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PHYLOGENETIC ANALYSIS OF *PAPAVER* L.

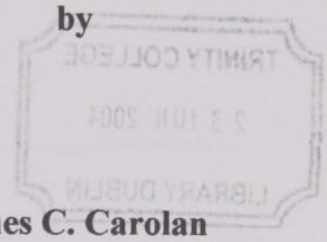
PHYLOGENETIC ANALYSIS OF *PAPAVER* L.

Thesis presented to the University of Dublin, Trinity College

for the

Degree of Doctor of Philosophy (Ph.D.)

by



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2003

based on research carried out under the joint supervision

of

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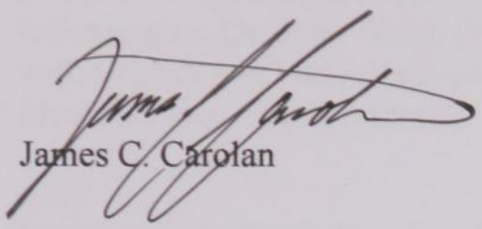
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James C. Carolan

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ABSTRACT

The phylogenetic relationships of *Papaver* L. were investigated using morphological, molecular and phytochemical characters. Such information is of great utility because *Papaver* species produce highly valuable secondary metabolites, including morphine thebaine and codeine. Two molecular regions have been implemented in this analysis, the internal transcribed spacer region (ITS) of 18S-26S nuclear ribosomal DNA and the *trn*L intron and the *trn*F intergenic spacer region of plastid origin. The resulting phylogenetic trees based on parsimony analysis demonstrate that the genus is not monophyletic unless a number of closely related genera (such as *Roemeria* and *Stylomecon*) and *Meconopsis cambrica* are included in *Papaver*. Three distinct clades are resolved: Clade 1 consists of section *Argemonidium* and *Roemeria heterophylla*; Clade 2 consists of section *Meconella* and Clade 3 consists of all remaining sections of *Papaver* including *Meconopsis cambrica* and *Stylomecon*. A number of diagnostic indels and characteristic sets of nucleotide sequences support these groupings.

The results of the molecular analysis and results from the mapping of morphological characters onto a combined molecular tree suggest that a number of morphological characters such as valvate dehiscence of capsule, dark and light coloured filaments have arisen through convergent evolution. The distribution and evolution of isoquinoline type alkaloids was evaluated in the same way. In some cases the synthesis of similar alkaloid types in certain sections of *Papaver* was shown to be a result of evolutionary relationship and common descent. The results of this thesis also demonstrate that particular alkaloid types are found only in certain groups of sections which will broaden basic knowledge of these groups and aid their utilisation as sources of important secondary metabolites.

Currently recognised sections were reviewed and a revised classification recognising six subgenera, eight sections and four subsections for *Papaver* is proposed. Sections *Argemonidium* and *Meconella* are elevated to the rank of subgenus (*Papaver* subgenus *Argemonidium* and subgenus *Meconella*). Section *Horrida* (South Africa) is elevated to the rank of subgenus, as is section *Californicum* (North West America). *Meconopsis cambrica* and *Stylomecon heterophylla* are recognised as *Papaver cambrica* and *Papaver lemonii*, respectively. *Papaver cambrica* is placed within *Papaver* at the subgenus level (*Papaver* subgenus *Cambrica*) in recognition of

differences in capsule characteristics between this species and other representatives of *Papaver*. *Papaver lemonii* is transferred to subgenus *Californicum* and recognised within a new section (section *Lemonii*). The sixth subgenus (subgenus *Papaver*) comprises sections *Meconidium, Oxytona, Papaver, Pilosa, Pseudopilosa and Rhoeadium* acknowledging the close affinity between these sections from a molecular point of view. Section *Rhoeadium* is recognised here as comprising four subsections. Based on the groupings within the molecular phylograms and a number of morphological and phytochemical considerations *Papaver glaucum* has been transferred to a subsection of section *Rhoeadium* (subsection *Glauca*). A division between *Papaver dubium* type species and *P. rhoeas/P. arenarium* type species is accommodated by the transfer of *P. dubium* type species to new section *Rhoeadium* subsection *Dubia.* Species within the *P. rhoeas/P. arenarium* group are transferred to section *Rhoeadium* subsection *Rhoeadium. Papaver macrostomum* which was separated from the genetically similar *P. rhoeas* group into a new section (*Carinatae* Kadereit) based solely on differences in capsule characteristics is transferred here to a subsection of section *Rhoeadium* (subsection *Carinatae*).

Amplified fragment length polymorphism (AFLP) DNA markers in conjunction with Neighbor Joining and PCO analysis were employed to assess genetic variation among species of *Papaver* section *Oxytona*. This section is important from a phytochemically point of view as its members accumulate economically important morphinane alkaloids without the accumulation of codeine and morphine (the key constituents of opium as a source of narcotic abuse). Individual alkaloids are species-specific in this section and it is necessary for the utilisation of these species as sources of valuable alkaloids to be able to identify accessions correctly. AFLP fingerprinting was also used to assess the genetic fidelity between *in vitro* cell lines of *Papaver bracteatum* and mature plants derived from the propagation of their callus cultures (results published in Carolan *et al.*, 2002). The results from this analysis demonstrated that the hexaploid (*Papaver pseudo-orientale*) was more similar to the diploid *P. bracteatum* than to the tetraploid *P. orientale.* AFLP fingerprinting identified hybrids between the species of this section and was able to differentiate between species of *Oxytona* from a range of cultivars.

Genomic *in situ* hybridisation (GISH) and fluorescent *in situ* hybridisation (FISH) were also used to assess the evolution of species within *Papaver* section *Oxytona*. Using total genomic DNA from *Papaver bracteatum* as a probe on chromosomes of *P. pseudo-orientale* demonstrated the presence of two sets of *P. bracteatum* genomes confirming its status as a diploid progenitor to the hexaploid species. The 18S-5.8S-26S repeat was hybridised to chromosomes of all species of *Oxytona* with *Papaver bracteatum*, *P. orientale* and *P. pseudo-orientale* having six, thirteen and eighteen loci for this particular repeat, respectively.

Combining the groupings from the AFLP analysis and the limited *in situ* hybridisation data gives support to the hypothesis that the tetraploid *Papaver orientale* arose from *P. bracteatum* and another diploid Alpine species (as yet unidentified). The hexaploid *P. pseudo-orientale* is a polyploid arising from *P. bracteatum* and *P. orientale* progenitors.

CHAPTER 1. Introduction

1.1 Taxonomy

Papaver L., consists of c. 80 annual, biennial or perennial herbs with many varieties and forms mostly native to central and southern Europe or temperate and subtropical regions of the Old World (Kadereit, 1988a). One species, *Papaver aculeatum,* Thunb. is indigenous to South Africa and one species *Papaver californicum* A. Gray is indigenous to Western North America. The plants are mostly hispid (sometimes glabrous) and produce white, rarely coloured, latex. The leaves are usually basal rosulate, petiolate, pinnatifid or lobed. The leaf margins are entire or toothed, scalloped, or incised. Inflorescences are cymiform, with solitary flowers (sometimes many) borne by long scapes or peduncles. The flowers are mostly red (rarely violet), yellow or white. There are usually two to three sepals that are caducous and distinct and four (sometimes six) petals crumpled in bud, with those of the outer whorl generally larger then the inner whorl. The stamens are numerous comprising white, violet or purple/black filiform filaments (rarely clavate) and purple/black or yellow anthers. The ovary has few or several united carpels but is sometimes incompletely multilocular by placental intrusion. The styles are absent and stigmas range in number from 3-22 radiate on sessile, more or less lobed discs, crowning the ovary. The capsules are obovoid, club-shaped, obconical to oblong-cylindrical, erect, pored or short-valved immediately beneath a persistent or sometimes deciduous stigmatic disc.

Papaver was traditionally classified within the order Papaverales, comprising the families Papaveraceae, Capparaceae, Cruciferae and Resedaceae (Fedde, 1936; Engler, 1964). However chemotaxonomic and anatomical traits such as the presence of laticifers and the production of benzylisoquinoline alkaloids, which are characteristic to Papaveraceae and not to Capparaceae, highlighted the distinction between the two families (Hutchinson, 1959). Resulting classifications associated Papaveraceae (including the Fumariaceae) within the order Ranunculales (Gottlieb *et al.* 1993; Kubitzky, 1993). Within Papaveraceae three (Fedde, 1936), seven

(Takhtajan, 1959) or four (Kadereit, 1993a) subfamilies are recognised; the later classification is followed here.

Kadereit's classification (Table 1.1; 1993a) recognises four subfamilies, Papaveroideae, Chelidonioideae, Eschscholzioideae and Platystemonoideae with the c.260 species of Papaveraceae divided into 23 genera. The subfamily Papaveroideae consists of nine genera forming two taxonomic groupings. The first group consists of the genera *Arctomecon*, *Argemone*, *Canbya* and *Romneya* but this grouping is viewed to be artificial and not a monophyletic group (Kadereit, 1993a). The genera of the second group consisting of *Papaver*, *Meconopsis*, *Stylomecon* and *Roemeria* form a monophyletic grouping sharing diagnostic phytochemical and anatomical characters such as semicampyltropous ovules and a seed coat with a fine layer of crystals, making them distinct from other genera of the subfamily. Additionally meconic acid is found only within species of these four genera (Cordel, 1981). The relationships that exist between these four genera of Papaveroideae form a primary focus of the systematic evaluation presented in this thesis.

Table 1.1 Subdivision of the Papaveraceae (Kadereit, 1993; after Mihalik, 1998).

Family	**Papaveraceae**			
Subfamily	Chelidonioideae	Eschcholzioideae	Platystemonoideae	Papaveroideae
Genus	*Chelidonium* (Tourn.)	*Eschscholzia* Cham.	*Platystemon* Benth.	*Meconopsis* Vig.
	Hylomecon Maxim.			*Papaver* L.
		Hunnemannia A.Juss	*Hesperomecon* Greene	*Roemeria* Medic.
	Stylophorum Nutt.			*Stylomecon* Benth.
		Dendromecon Benth.	*Meconella* Nutt.	*Arctomecon* Tor. et Frem.
	Eomecon Hance			
	Sanguinaria L.			
	Macleaya R. Br.			*Argemone* L.
	Bocconia (Plum.) L.			*Canbya* Parry
	Glaucium (Tourn.) Adans.			*Romneya* Harv.
	Dicranostigma Hook. et Thoms.			

Bernhardi (1833) divided *Papaver* L. into four sections: *Lasiotrachyphylla, Oxytona, Rhoeades and Mecones*. Elkan (1839) introduced the sections *Milthantha* and *Horrida*, and Prantl & Kunding (1889) included the section *Pilosa*. Fedde (1909) introduced the section *Argemonorhoedes* and *Orthorhoeades* while excluding the section *Carinatae*. Gunther (1975) divided the section *Pilosa*, and combined *Papaver* with the genus *Roemeria* in a new section. Preininger *et al.* (1981) divided the section *Mecones* into the sections *Papaver* and the section *Mecones* based on chemotaxonomic distinction. Infrageneric taxonomic treatments are given in Table 1.2.

Table 1.2 Historical surveys of the sections of *Papaver*. Note that the genus has become progressively more divided with time until the most recent treatment of Kadereit (1993) who recognises 11 sections.

Bernhardi (1833)	*Lasiotrachphylla* Bernh.; *Milthantha* Bernh.; *Oxytona* Bernh.; *Rhoeades* Bernh
Elkan (1839)	*Horrida* Elk.; *Macrantha* Elk.; *Pyramistigmata* Elk.; *Rhoeades* Bernh.; *Scapiflora* Reich.
Prantl (1889)	*Horrida* Elk.; *Lasiotrachphylla* Bernh.; *Macrantha* Elk.; *Milthantha* Elk.; *Pilosa* Prantl; *Rhoeades* Bernh.
Fedde (1909)	*Argemonorhoeades* Fedde; *Carinatae* Fedde; *Horrida* Elk.; *Macrantha* Elk.; *Mecones* Bernh.; *Milthantha* Elk.; *Orthorhoeades* Fedde; *Pilosa* Prantl; *Scapiflora* Reich.
Flora Europaea (1964)	*Argemonorhoeades* Fedde; *Carinatae* Fedde; *Macrantha* Elk.; *Mecones* Bernh.; *Orthorhoeades*; *Papaver* L.; *Pilosa* Prantl.; *Scapiflora* Reich.;
Gunther (1975)	*Argemonidium* Spach.; *Carinatae* Fedde; *Horrida* Elk.; *Lasiotrachyphylla* Bernh.; *Mecones* Bernh.; *Milthantha* Elk.; *Oxytona* Bernh.; *Papaver* L.; *Pilosa* Prantl; *Rhoeades* Bernh.; *Roemeria* M.
Preininger (1986)	*Argemonidium* Spach.; *Carinatae* Fedde; Glauca J. Nov & V. Prein.; *Horrida* Elk.; *Macrantha* Elk.; *Meconella* Spach; *Mecones* Bernh.; *Milthantha* Elk.; *Papaver* L.; *Pilosa* Prantl; *Pseudo-pilosa* Gunther; *Rhoeadium* Bernh.; *Roemeria* M.
Kadereit (1993)	*Argemonidium* Spach.; *Carinatae* Fedde; *Californicum* Kadereit; *Horrida* Elk.; *Macrantha* Elk.; *Meconidium* Bernh.; *Meconella* Spach; *Papaver* L.; *Pilosa* Prantl; *Pseudopilosa* Gunther; *Rhoeadium* Bernh.;

1.2 Sectional characteristics of *Papaver*

1.2.1 Section *Meconella* Spach.

Section *Meconella* (Hanlet, 1969; Randel, 1974, 1977) contains c. 30 perennial species (Table 1.3; Figure 1.1) characterised by scapose growth habit with

distributions predominantly including arctic-alpine habitats. The main distribution for this section is Central, Inner and East Asia, Siberia, Scandinavia through Greenland and North Canada. Additionally species can be found in mountainous regions of Europe and the Rocky Mountains, U.S.A. (Randel, 1974). Representatives of this section are furthermore characterised by their bristly cylindrical or spherical capsules, which open valvately (rectangular pores are formed from the retraction of the carpel subapically and continuing along the placentae giving rise to distinct valves). The stigmatic discs, which are usually flat, have deep incisions between the stigmatic rays giving rise in some cases to stigmatic discs comprising only of stigmatic tissue (Kadereit, 1988a). The pinnatisect leaves, which are often simple or dissected, form rosettes from which a single hispid or glabrous floral axis with a solitary flower is produced. The species in this section have numerous light coloured stamens borne on light coloured filiform anthers. The petals are wedge-shaped and range in colour from yellow, orange to white. It has been noted that some species of the section (e.g. *Papaver alpinum* L.) show wide infraspecific variability and variable levels of ploidy, which can make correct taxonomic identification difficult (Kadereit, 1988a).

1.2.2 Section *Meconidium* Bernh.

Section *Meconidium* (Cullen, 1965; Kadereit, 1988a; Kadereit, 1993b) comprising four biennial species occupy a continuous geographical range limited to South and East Turkey, the Caucasus Mountains, North Iraq and North West Iran, (Table 1.4; Figure 1.2). Basal leaves are pinnatifid to pinnatisect having few to many teeth and form a rosette from a thick main root in the first year, with the flowering stem emerging in the second year.

Cauline leaves on the axis are usually small, sometimes even reduced to scales or absent. When present, the sessile cauline leaves are pinnatifid to serrate (Kadereit, 1993b). As with section *Meconella,* species of section *Meconidium* shed their seed through valves. The two-valved buds can be glabrous or densely setose and more usually globose in shape. The capsules bear the characteristic sessile stigmatic disc. The broadly ellipsoid to ovoid capsules can be bristly or glabrous, are sometimes ribbed, and bear a conical stigmatic disc comprising three-five stigmatic rays.

Flowers are usually in racemes or panicles, are rarely solitary, and the petals (usually four) range in colour from orange to brown. Stamens are usually light in colouration with orange anthers and white/yellow filiform filaments. Taxonomic difficulties have arisen with previous classifications of members of this section due to fact that the rosette leaves tend to die off before anthesis begins resulting in herbarium samples with cauline leaves and flowers only (Kadereit, 1993b).

Table 1.3 Species and subspecies of *Papaver* section *Meconella.*

Species	**Subspecies**
Papaver alboroseum Hult.	
P. alpinum L.	*alpinum* L.
	corona-sancti-stephani Zap.
	degenii Urum et Jav.
	ernesti-mayeri Mgf.
	kerneri (Hay.) Fedde
	rhaeticum Mgf.
	sendtneri Kern.
	tataricum Nyar.
P. angrenicum Pazij.	
P. canescens Tolm.	
P. croceum Ledeb.	*chinese* Randel
	croceum Ledeb.
	longiscapum Randel
P. czeckanowskii Tolm.	
P. indigirkense Jurc.	
P. laestadianum Nordh.	
P. lapponicum Tolm.	
P. leucotrichum Tolm.	
P. macounii Greene.	
P. microcarpum DC.	
P. minutifolium Tolm.	
P. miyabeanum Tatew.	
P. nivale Tolm.	
P. nudicaule L.	*amurense* Busch
	baicalense Tolm.
	leiocarpum Turz.
	nudicaule L.
	rubro-aurantiacum (DC) Fedde
	xanthopetalum (Trautv.) Fedde
P. pulvinatum Tolm.	
P. pygmaeum Rydb.	
P. radicatum Rottb.	*dahlianum* (Nordh.) Randel
	ovatilobum Tolm.
	polare Tolm.
	radicatum Rottb.
P. relictum (Lundstr.) Nordh.	
P. tianschanicum Popov	
P. walpolei Pors.	

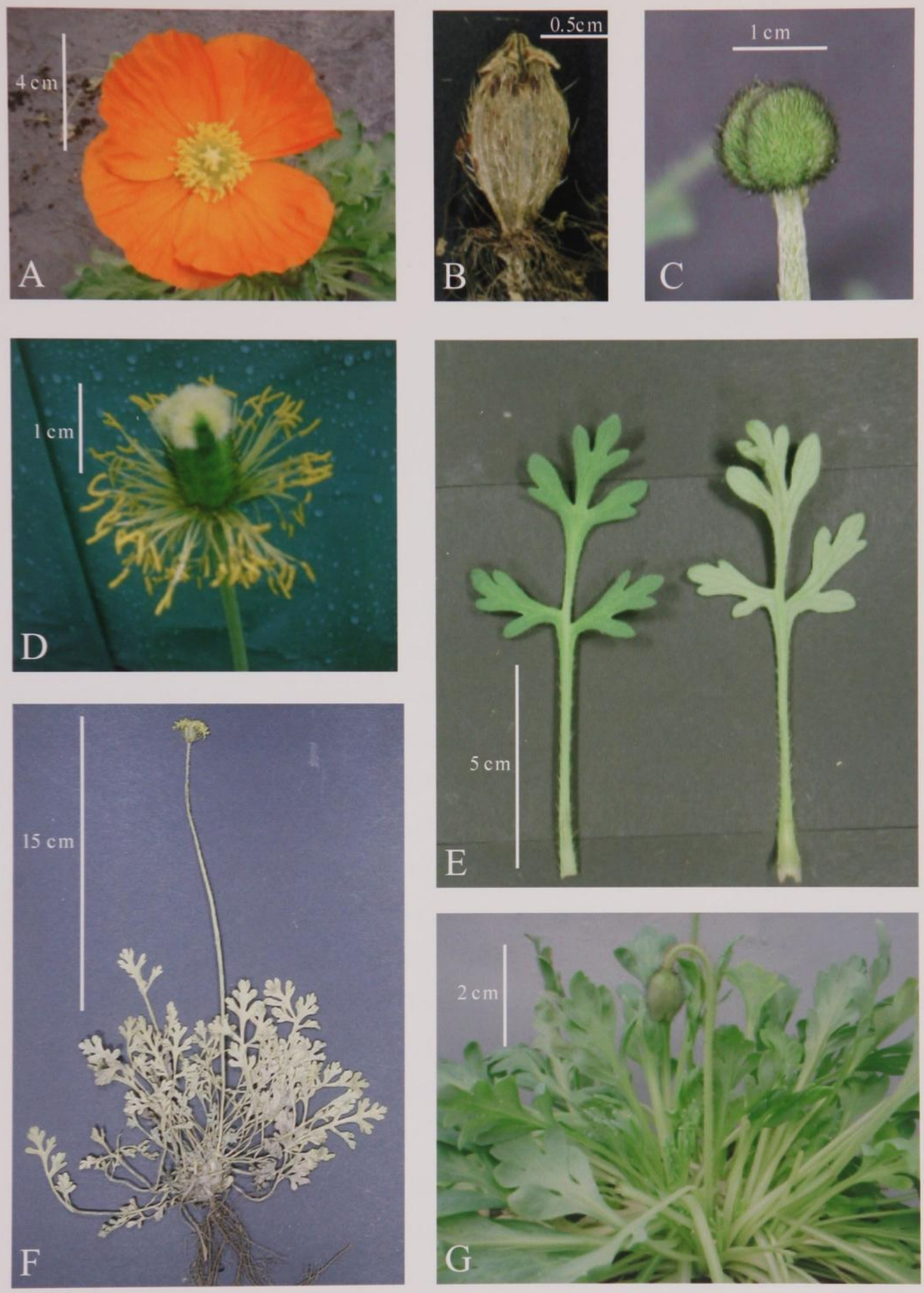


Figure 1.1. Characteristic features of *Papaver* sect. *Meconella*: A, light coloured petals (usually yellow, but orange here); B, bristly capsule; C, two-valved bud; D, light coloured anthers and filiform filaments; E, pinnatisect leaves; F, solitary flowering axis; G, scapose habit. Figures A-F: *Papaver croceum*.

Table 1.4 Species and subspecies of *Papaver* section *Meconidium*.

Species	Subspecies
Papaver armeniacum L.	*armeniacum* *microstigmum* Boiss *pilgerianum* Fedde
P. curviscapum Nab.	
P. libanoticum Boiss	*libanoticum* Kadereit *polychaetum* Schott & Kotschy ex Boiss.
P. persicum Lindl.	*microcarpum* Boiss *persicum* Lindl. *tauricolum* Boiss

1.2.3 Section *Californicum* Kadereit

The only native annual *Papaver* species of North America, *P. californicum* A.Gray (Figure 1.3), is the sole member of this section. Similar to the previous two sections, dehiscence of the capsules is through valves. Fedde (1909) placed it within the section *Rhoeadium* but Kadereit (1988b) separates this species from *Rhoeadium* due its possession of characters more similar to sections *Meconella* and *Meconidium*. It has a slender, ribbed, glabrous capsule which is ellipsoid to obovoid in shape bearing a pyramidical stigmatic disc consisting of four to eight stigmatic rays. Basal leaves are most often glabrous and pinnatisect. The cauline leaves are somewhat reduced and sessile and rarely found on the upper half of the flowering stem. The flowers are usually in many-flowered racemose inflorescences. The glabrous, globose buds usually bear four obovate, yellow petals, surrounding yellow anthers borne on light coloured filiform, rarely clavate filaments (Kadereit, 1988b).

Morphological similarities such as leaf shape and other vegetative characters (Ernst, 1962) exist between this species and *Stylomecon heterophylla* (Benth.) G. Taylor the only representative of the genus *Stylomecon* G. Taylor. The similarity between the two species (before floral and capsule characters are produced) and the similar geographical distributions (*S. heterophylla* is found predominantly in North West America) led to the inclusion of *S. heterophylla* in *Papaver* as *Papaver lemonii* Greene (Greene, 1888), before Taylor transferred the later into a new genus. The main

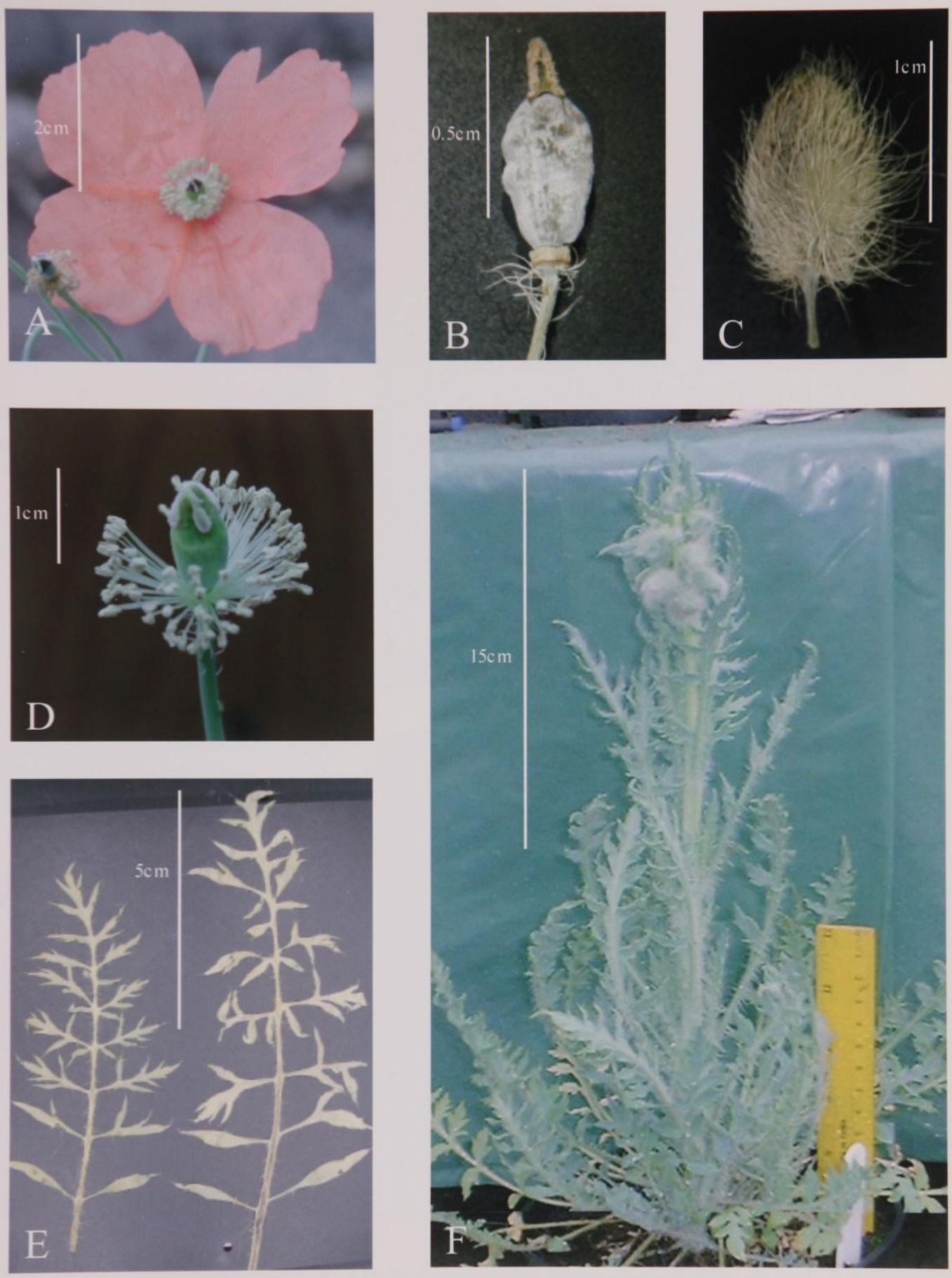


Figure 1.2. Characteristic features of *Papaver* sect. *Meconidium*: A, light coloured petals (usually pale orange); B, ellipsoid to ovoid capsule bearing a cylindrical stigmatic disc; C, two-valved setose bud; D, light coloured anthers; E, pinnatisect leaves; F, rosette forming plant with many flowered inflorescence. Figures A-F: *Papaver armeniacum* ssp. *armeniacum*

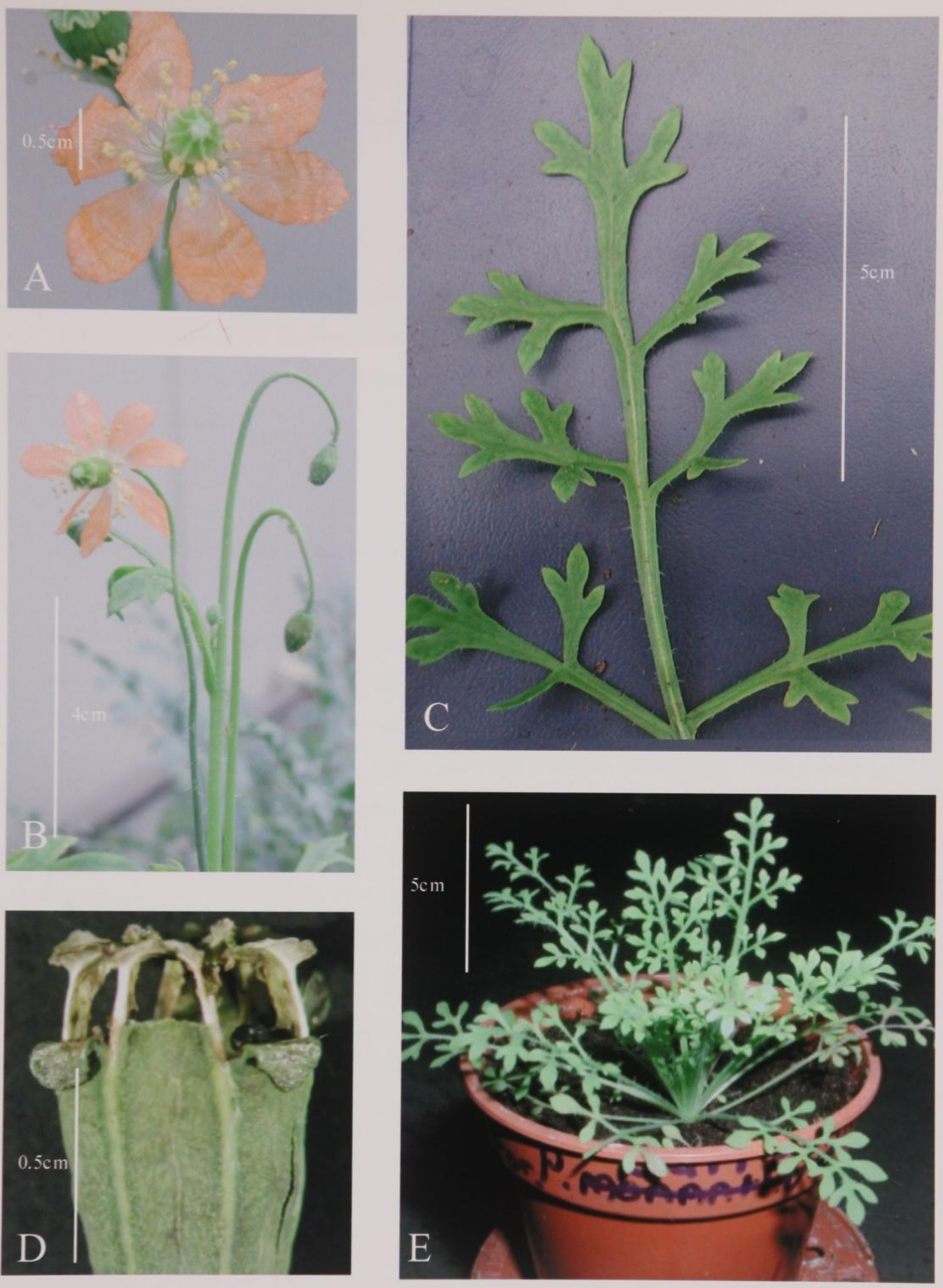


Figure 1.3. Characteristic features of monotypic *Papaver* sect. *Californicum*: A, light coloured petals (usually yellow) and light coloured anthers; B, many-flowered racemose inflorescences; C, pinnatisect leaves; D, ribbed, glabrous obovoid capsule (showing distinct valves); E, rosette forming plant. Figures A-F: *Papaver californicum.*

differences between *S. heterophylla* and *P. californicum* are primarily those of the capsule, which in *S. heterophylla* are relatively narrow and long, bearing a style on top of a flat ovary apex.

Results from the analysis of chloroplast DNA led Kadereit *et al.* (1997) to regard the possession by *Meconopsis discigera* Prain. of a style on top of a flat ovary apex to be a plesiomorphy. They highlight the fact that this particular character "could be interpreted as a reminiscence of the origin of *P. californicum/S. heterophylla* from within *Meconopsis*".

1.2.4 Section *Argemonidium* Spach. (= *Argemonorhoeades* Fedde)

Kadereit (1986a) reviewed this section on geographical and morphological grounds and concluded that it contains four annual half-rosette species (Table 1.5; Figure 1.4). *Papaver apulum* Ten., *P. argemone* L. and *P. pavonium* Fisch. et May. are closely related and occur allopatrically from around the Adriatic Sea through Turkey-Iran to the Himalayas. The fourth species *P. hybridum*, occupies a wide range from the Macronesian Islands towards the Himalayas (Kadereit, 1986a, 1988a). This section is considered distinct from the other sections, as these species possess stigmatic discs, which are a continuation of the capsule, forming a solid plug known as the 'apical plug' (Markgraf, 1958; Kadereit, 1986a). In addition representatives of this section show a distinctive growth form because after the formation of basal rosette leaves one very long internode is formed followed by much shorter internodes (Kadereit, 1988a). This growth form is not found in any other species of *Papaver*. In contrast to the preceeding sections representatives of section *Argemonidium* shed their seed through pores and not valves. The cylindrical to obovoid capsules are extremely bristly, having four to eight stigmatic rays. Species of this section produce a basal rosette of pinnatisect leaves with few and very long internodes followed by shorter internodes. The branches are straight and setose with the upper leaves being sessile. The clavate (rarely filiform) filaments are violet-black and pollen colour can differ from species to species and may be pink, purple or blue (Landolt, 1967; Kadereit, 1986a).

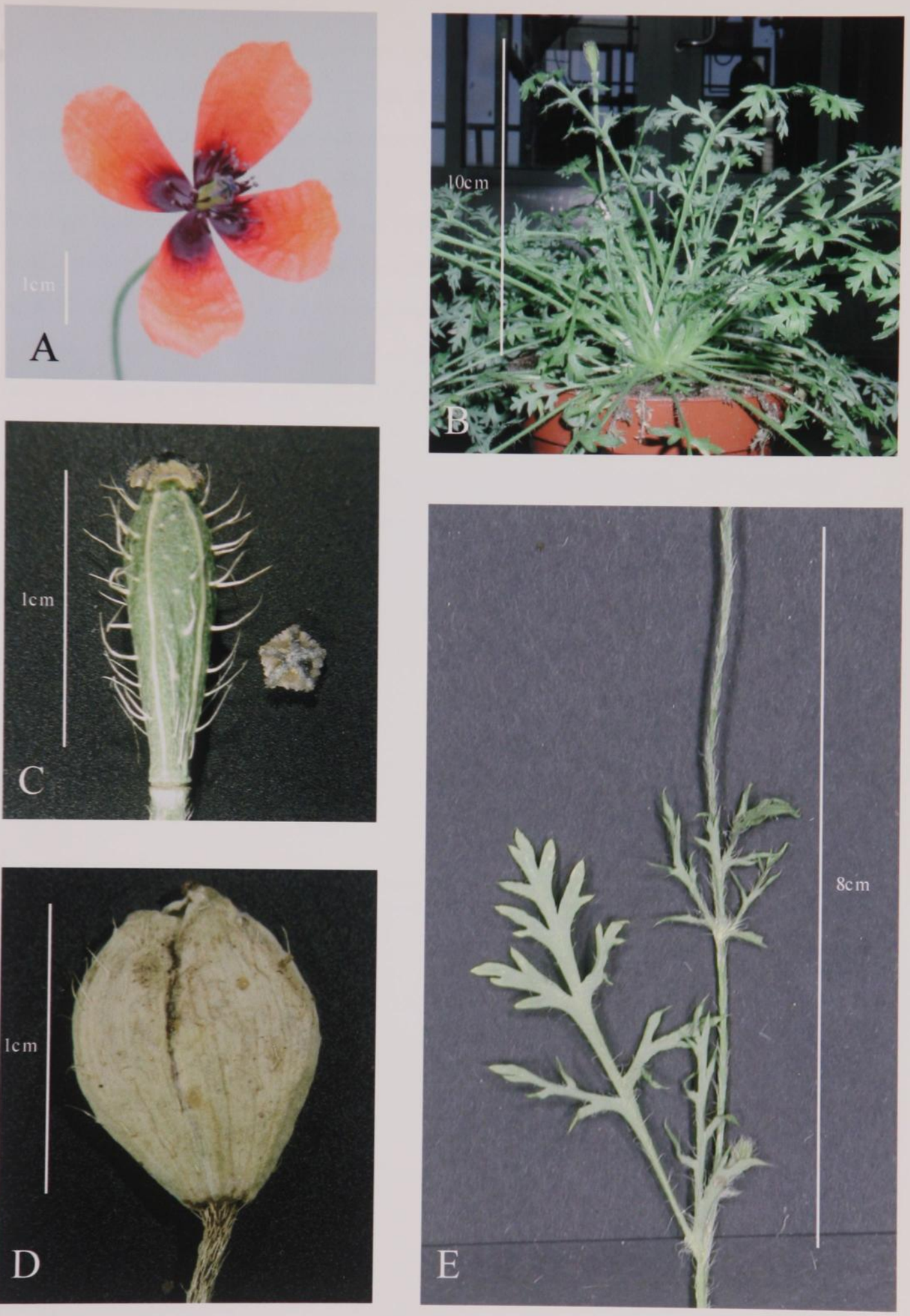


Figure 1.4. Characteristic features of *Papaver* sect. *Argemonidium*: A, dark coloured petals (usually red) and dark coloured anthers with clavate filaments; B, rosette forming plant; C, bristly capsules; D, obovoid bud; E, pinnatisect leaves. Figure A-E: *Papaver argemone* ssp. *argemone*.

Argemonidium not only has many characters that make it distinct from the other sections but also shares characters with species of different genera, specifically the genus *Roemeria* Medik. *Roemeria* comprises three annual species and has been linked to section *Argemonidium* by Gunther (1975) who combined the two as a section within *Papaver*. Morales Torres *et al.* (1988) also placed the species of *Argemonidium* into *Roemeria*. Shared characters include polyporate instead of three-colpate pollen grains, which are typical of *Papaver* (Ernst, 1962) and long internodes from the basal rosette. Additionally most species of *Argemonidium* have filaments, which are clavate, again a character not typical of *Papaver* but found in *Roemeria refracta* D.C. (Kadereit, 1997). A restriction site analysis of chloroplast DNA (Kadereit & Sytsma, 1992) and a RFLP analysis of the chloroplast *trnK* region (Kadereit, 1997) provided more evidence for the close relationship existing between *Argemonidium* and *Roemeria,* giving support to the author's suggestion that *Papaver* sect. *Argemonidium* and *Roemeria* 'might well have originated from within *Meconopsis* from an ancestor related to that of *Papaver* sect. *Meconella*' (Kadereit, 1997).

Table 1.5 Species and subspecies *Papaver* section *Argemonidium.*

Species	Subspecies
Papaver apulum Ten.	
P. argemone L.	*argemone*
	davisii Kadereit
	meiklei Kadereit
	minus (Boiv.) Kadereit
	nigrotinctum (Fedde) Kadereit
P. hybridum L.	
P. pavonium Fisch. et Mey.	*pavonium* Kadereit
	ocellatum (Woron) Kadereit

1.2.5 Section *Pilosa* Prantl

Section *Pilosa* contains one perennial half-rosette subscapose species and five subspecies found predominantly in West Turkey (Table 1.6; Figure 1.5). Kadereit (1996) recognises only one species, *Papaver pilosum,* classifying the other members



Figure 1.5. Herbarium specimen of *Papaver pilosum* ssp. *pilosum*: diagnostic characters include light coloured petals (usually orange or yellow), dark coloured anthers, obovoid glabrous capsules bearing a flat stigmatic disc, light coloured filiform anthers, obovoid bud and pinnatifid leaves (photographed at the Royal Botanic Gardens, Edinburgh, Scotland).

of this section as subspecies of this one. The species of section *Pilosa* shed their seed through pores and not valves. The basal leaves are petiolate, long-lanceolate, dentate and in some cases dissected, and can be sparsely to densely covered with appressed hairs. The axis bears sessile leaves with convulate vernation (Gunther, 1975). The obovoid to ellipsoid capsules are more often glabrous but can be setose, bearing a flat to pyramidical stigmatic disc with five-eight stigmatic rays. The buds, which are ovoid to globose with a glabrous to half appressed indumentum, are borne on short to long pedicels. The few to many flowers can be pale orange or salmon-coloured. The filiform filaments and anthers are yellow.

Table 1.6 Species and subspecies *Papaver* section *Pilosa.*

Species	**Subspecies**
Papaver pilosum Sibth. et Smith	
	glabrisepalum Kadereit
	pilosum Wendt ex Kadereit
	spasipilosum Wendt
	spicatum Wendt ex Kadereit
	strictum Wendt ex Kadereit

1.2.6 Section *Pseudopilosa* Pop. et Gunther

This section contains three to five perennial species of subscapose to scapose habit found predominantly from South West Asia to North Africa and Southern Spain (Table 1.7; Figure 1.6). The section was initially classified as part of section *Pilosa* (Popov, 1937), but these were subsequently separated based on morphological and phytochemical characteristics (Gunther, 1975). Wendt (1976) and Kadereit (1996) recognise three species and five subspecies. Section *Pseudopilosa* can be differentiated from section *Pilosa* by its revolute leaf vernation instead of convulate leaf vernation. Capsules are obconical to narrowly obovoid, sometimes ribbed bearing a more or less flat stigmatic disc with four-ten stigmatic rays. Leaves decrease in size towards the apex and are oblanceolate to lanceolate, coarsely serrate to pinnatifid, and can have a densely setose indumentum on upper and lower surface. As with section *Pilosa,* species of section *Pseudopilosa* have pale orange to salmon- coloured petals

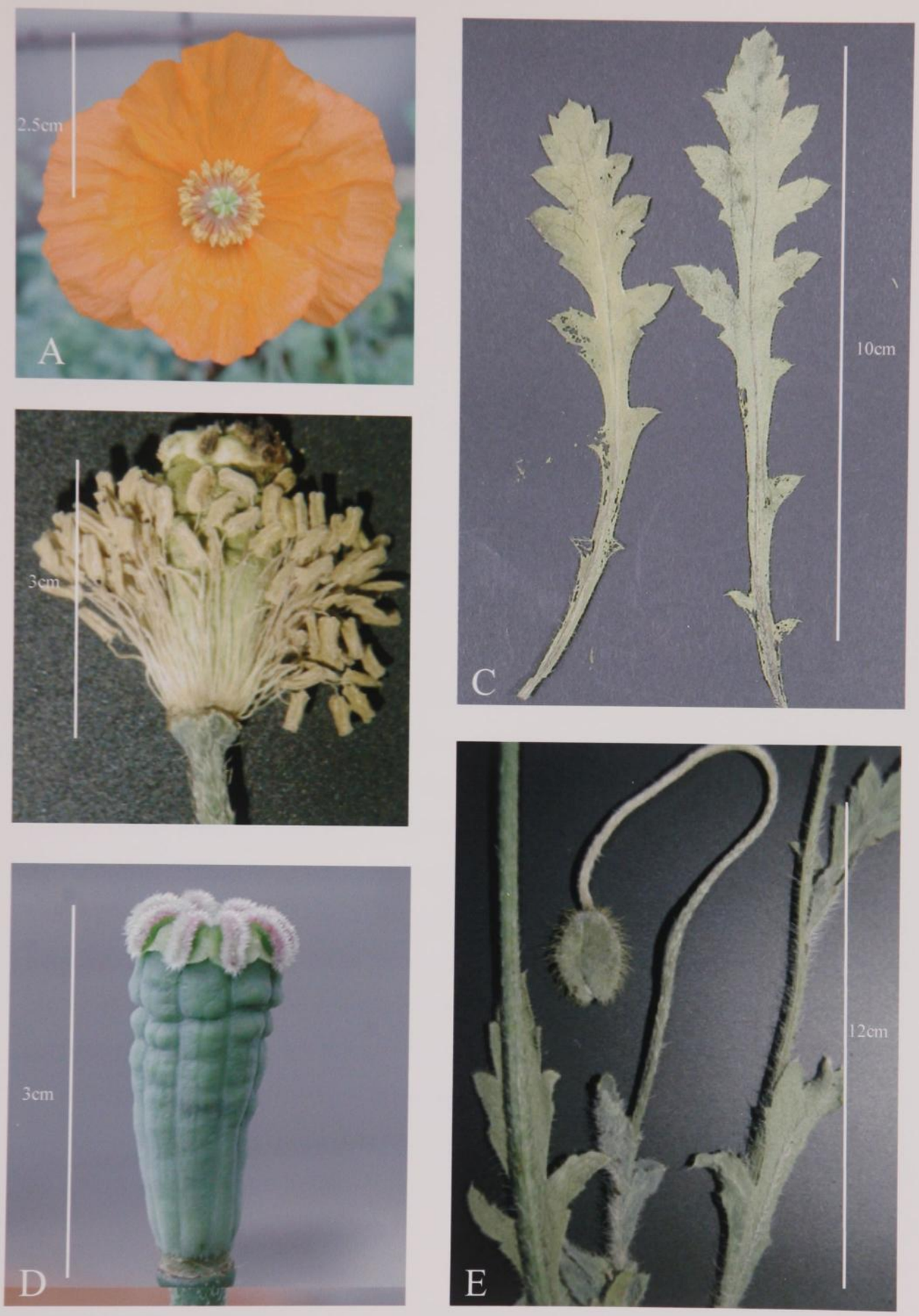


Figure 1.6. Characteristic features of *Papaver* sect. *Pseudopilosa*: A, light coloured petals (usually orange); B, light coloured filiform anthers; C, pinnatifid leaves; D, obovoid glabrous capsules, bearing a flat stigmatic disc; E, obovoid densely setose two-valved bud and numerous flowers. Figures A-E: *Papaver atlanticum* ssp. *atlanticum*.

and yellow filiform filaments.

Table 1.7 Species and subspecies of *Papaver* section *Pseudopilosa.*

Species	Subspecies
Papaver atlanticum Ball et Cross.	*atlanticum* Ball et Cross.
	mesatlanticum Maire.
P. lateritium Koch.	*lateritium* Koch.
	monanthum Trautv.
P. rupifragum Boiss et Reut.	

1.2.7 Section *Horrida* Elkan

This monotypic section includes *Papaver aculeatum* Thunb. (Figure 1.7) and is probably endemic to Eastern South Africa. *Papaver aculeatum* is found in Australia but Kadereit (1998c) views this as a result of synanthropic spread. The habit of this species is usually annual, but in some circumstances it may be biennial (Gunther, 1975). *Papaver aculeatum* is characterised by having oval to narrowly elliptical incised leaves with an indumentum of relatively long bristles. The lower leaves narrow towards their base while the upper leaves are sessile with auriculate-amplexicaulous base. All green parts of plant are covered with patent bristles. The poricidal capsule is obovoid and glabrous with a flat stigmatic disc consisting of five-eleven stigmatic rays. The flowers consist of four obovate, orange petals, usually in racemes or panicles and are rarely solitary. The filaments are filiform and both filaments and anthers are yellow in colour (Kadereit, 1988c).

1.2.8 Section *Carinatae* Fedde

Section *Carinatae* is also monotypic and contains the annual species, *Papaver macrostomum* Boiss et Huet. (Figure 1.8) with three varieties (Kadereit, 1987) found growing in Iran, Iraq and Turkey. The flowers are mostly in many-flowered racemes with four flabelliform to obovate, dark red to crimson petals, with or without black basal marking often with a white apical margin. The capsules are mostly glabrous, ovoid or cylindrical with six-eight stigmatic rays, with the flat stigmatic disc

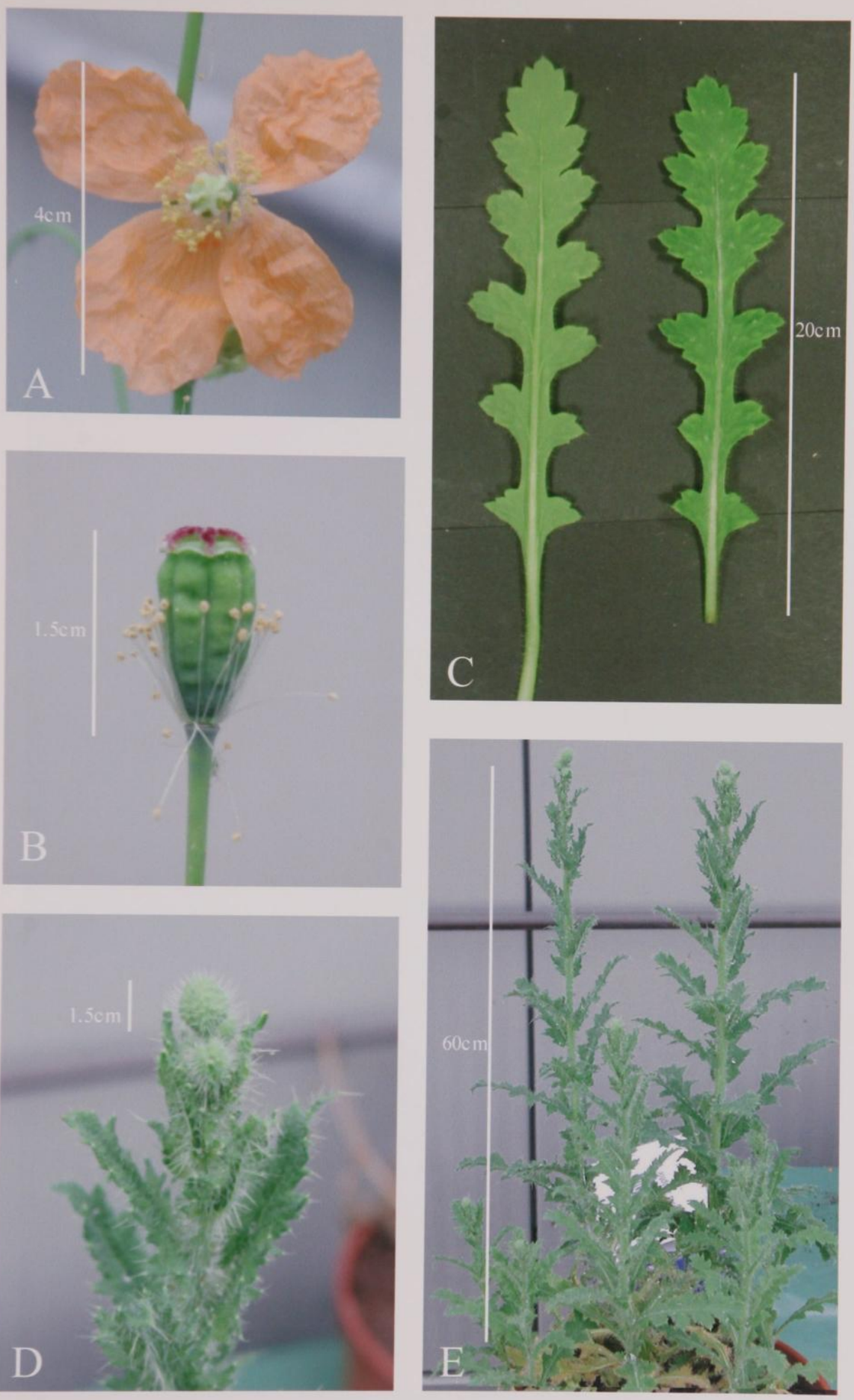


Figure 1.7. Characteristic features of *Papaver* sect. *Horrida*: A, light coloured petals (usually orange); B, narrow glabrous capsules, bearing a flat stigmatic disc with light coloured filiform anthers; C, pinnatifid leaves; D, flowers in racemes, obovoid bud and plant covered in bristles; E, erect annual herb. Figures A-E: *Papaver aculeatum*.



Figure 1.8. Herbarium specimen of *Papaver macrostomum*: diagnostic characters include deciduous stigmatic disc which becomes detached from capsule at maturity and dark coloured filaments and anthers (photographed at the Royal Botanic Gardens, Edinburgh, Scotland).

becoming detached from the capsule at maturity. Sparse or dense patent hairs cover the leaves and sepals. The lower leaves are petiolated and are larger than the sessile upper leaves and are usually pinnatisect. The four petals, which are broader than long are usually dark crimson to red in colour with or without a purple spot often with a white apical margin. The filaments are filiform and black.

1.2.9 Section *Rhoeadium* Spach.

Papaver section *Rhoeadium* consists of seventeen (Table 1.8; Figure 1.9) predominantly annual species (biennials are known to exist under special circumstances, Gunther, 1975; Markgraf, 1986). The centre of origin for this section is South West Asia and the Aegean area. However some species can be found in Central or Western Mediterranean, the Balkans or the West Himalayas (Kadereit, 1989). Morphologically these species are characterised by poricidal capsules which are glabrous and globose to subglobose or ellipsoid bearing a flat stigmatic disc (rarely pyramidical) comprising of five-eighteen stigmatic rays. The stem may be simple or branched to different degrees. The leaves, which are pinnatifid or pinnatisect with dentate segments, are sessile and in some species are covered by spiny hairs. Petal colour is crimson to red (sometimes white or pale violet) and often with a quadrangular black spot (sometimes with a white apical margin). Filaments are filiform and black in colour. Anthers are predominantly dark in colour but yellow and violet anthers have been encountered (Kadereit, 1989).

Kadereit (1989) recognises three groups within *Rhoeadium*, based on geographical and morphological observations. The first group contains species with longer than broader capsules such as *Papaver dubium* L. and contains only tetraploid (2n=28) and hexaploid (2n=42) species. The second group contains diploid species (2n=14) including *P. arenarium* Marsch. et Bich. and *P. commutatum* Fisch et Mey. and is diverse in its morphology. The third group, although showing a greater amount of morphological uniformity than the *P. arenarium* group, consists of diploid species including *P. rhoeas* L. Kadereit (1989) regards the latter two groups to be more closely related to each other than to the first group. Previous molecular analysis (Kadereit & Sytsma, 1992) based on a restriction site analysis of chloroplast DNA,

suggests that the section may not in fact be monophyletic. This analysis did not include a sufficient amount of taxa to clearly resolve phylogenetic relationships within the section and other sections of *Papaver*. However a separation between the *P. dubium* group and the *P. rhoeas* group was observed with the latter group showing a similarity to members of the section *Papaver* (Tourn.) L.

Table 1.8 Species and subspecies of *Papaver* section *Rhoeadium*.

Species	Subspecies
Papaver arachnoideum Kadereit	
P. arenarium Marsch. et Bich.	
P. carmeli Feinbrun	
P. chelidoniifolium Boiss et Buhse	
P. clavatum Boiss et Hausskn.	
P. commutatum Fisch et Mey.	*commutatum*
P. dubium L.	*euxinum* Kadereit *dubium* Kadereit *erosum* Kadereit *glabrum* Kadereit *laevigatum* Kadereit *lecoquii* Syme
P. guerlekense Stapf	
P. humile Fedde	
P. pinnatifidum Moris	
P. purpureomarginatum Kadereit	
P. rechingeri Kadereit	
P. rhoeas L.	
P. stylatum Boiss et Bal. ex. Bios	
P. tenuifolium Boiss et Hohem.	
P. umbonatum Boiss	

1.2.10 Section *Oxytona* (Bernh.) = *Macrantha* Elk.

The three perennial species (Table 1.9; Figure 1.10) found predominantly in the Caucasus Mountains, East Turkey and North West Iran (Goldblatt, 1974), are characterised by their scapose habit and a rosette with long thick and bristly pinnately dissected leaves, a thick main root with fleshy lateral branches, and a long almost always unbranched flower axis (Troll, 1964). The glabrous poricidal capsule, which is obovoid to globose, bears a flat to pyramidical stigmatic disc comprising seven-twenty stigmatic rays. The four-six petals range in colour from crimson-red to orange and a basal spot may be present or absent. Filaments are filiform (sometimes clavate)



Figure 1.9. Characteristic features of *Papaver* sect. *Rhoeadium*: A, dark coloured petals (usually red, sometimes violet) and dark (sometimes light) coloured anthers with filiform filaments; B, poricidal, glabrous capsules, bearing a flat stigmatic disc; C, obovoid, setose bud; D, erect annual herb with flowers in racemes; E, pinnatifid leaves. Figures A, C-E: *Papaver rhoeas*, Figure B: *Papaver dubium* ssp. *dubium*.

and black-dark violet in colour. The taxonomy of this section is discussed in greater depth in chapter 6.

Table 1.9 Species of *Papaver* section *Oxytona.*

Species
Papaver bracteatum Lindl.
P. orientale L.
P. pseudo-orientale (Fedde) Medv.

1.2.11 Section *Papaver* (Tourn.) L.

Kadereit (1986b) recognisies four annual species (Table 1.10; Figure 1.11), distributed from the Western Mediterranean and South West Turkey to Cyprus, Iran, Afghanistan and Pakistan. The glabrous poricidal or indehiscent capsule which can be obovoid, elliptical or ovoid to globose, bears a flat stigmatic disc with five-twenty-two stigmatic rays which can be overlapping or non-overlapping. Leaves are obovate, oblong pinnatipartite to undivided. The lower leaves are usually petiolated and the upper leaves take various forms from dissected to undivided, sessile, or strongly auriculate-amplexicaulous (Kadereit, 1986b). Plants may be glabrous or with different setae covering various organs. Flower buds are ovoid to oblong. The petals are rotund or reniform, of various colours (from white to dark violet) and sizes, and basal marks may exist. The filaments are filiform or clavate and are whitish to pale purple to dark violet bearing oblong yellow anthers (Kadereit, 1986b).

As with section *Rhoeadium,* the monophyly of section *Papaver* is questionable in light of molecular phylogenetic analysis. The restriction site analysis of chloroplast DNA (Kadereit and Sytsma, 1992) demonstrated that members of this section such as *Papaver glaucum* and *P. gracile* share a close affinity to *P. rhoeas* and *P. dubium. Papaver somniferum* resolved separately to these species. Many morphological and geographical similarities exist between the two sections (as outlined by Kadereit, 1988a). However the author viewed these similarities (including molecular



Figure 1.10. Characteristic features of *Papaver* sect. *Oxytona*: A, orange or red coloured petals with or without a dark basal spot and dark coloured anthers and filaments; B, poricidal, glabrous capsules, bearing a pyramidical stigmatic disc; C, pinnately dissected leaves; D erect perennial herb with solitary flowers. Figures A&D: *Papaver pseudo-orientale*; Figures B&C: *Papaver bracteatum.*

similarities) as an indication of the close affinity between the two sections rather than a taxonomic separation within the sections.

Table 1.10 Species and subspecies of *Papaver* section *Papaver*.

Species	Subspecies
Papaver decaisnii Hochst. Steud. ex Elkan	
P. glaucum Boiss et Hausskn	
P. gracile Boiss	
P. somniferum L.	*setigerum* (DC.) Corb.
	somniferum Kadereit

1.3 Sectional affinities within *Papaver*

Morphological (Kadereit *et al.*, 1994) and molecular (Kadereit & Sytsma, 1992; Kadereit *et al.*, 1997) phylogenetic trees demonstrate that *Papaver sensu lato* is not a monophyletic group. Species of *Papaver* are considered distinct morphologically primarily by the possession of a stigmatic disc with sessile stigmata, but uncertainty has arisen regarding the homology of this structure between various groups of species within *Papaver* (Soltis & Soltis, 1995; Kadereit *et al.*, 1997). However certain sections of *Papaver* share characteristic morphological traits with other genera of Papaveroideae. Based on molecular characters, Kadereit and Sytsma (1992) and Kadereit *et al.* (1997) view these shared characters between the four genera of Papaveroideae a result of recent shared ancestry, with *Roemeria* and *Stylomecon* having close affinities to *Papaver* section *Argemonidium* and *Papaver* section *Californicum* respectively. It was also shown that *Meconopsis cambrica* L., the only Western European species of this genus with an otherwise primarily Himalayan distribution, grouped within *Papaver* and separate from the other members of *Meconopsis*. This affinity was interpreted as representing the paraphyletic nature of *Papaver* and indicates that certain sections of *Papaver* have arisen from within *Meconopsis* either directly or from an ancestor of the European *Meconopsis cambrica*. It was anticipated that the molecular characterisation of *Papaver* and these closely related genera undertaken in this project would give further insight into the

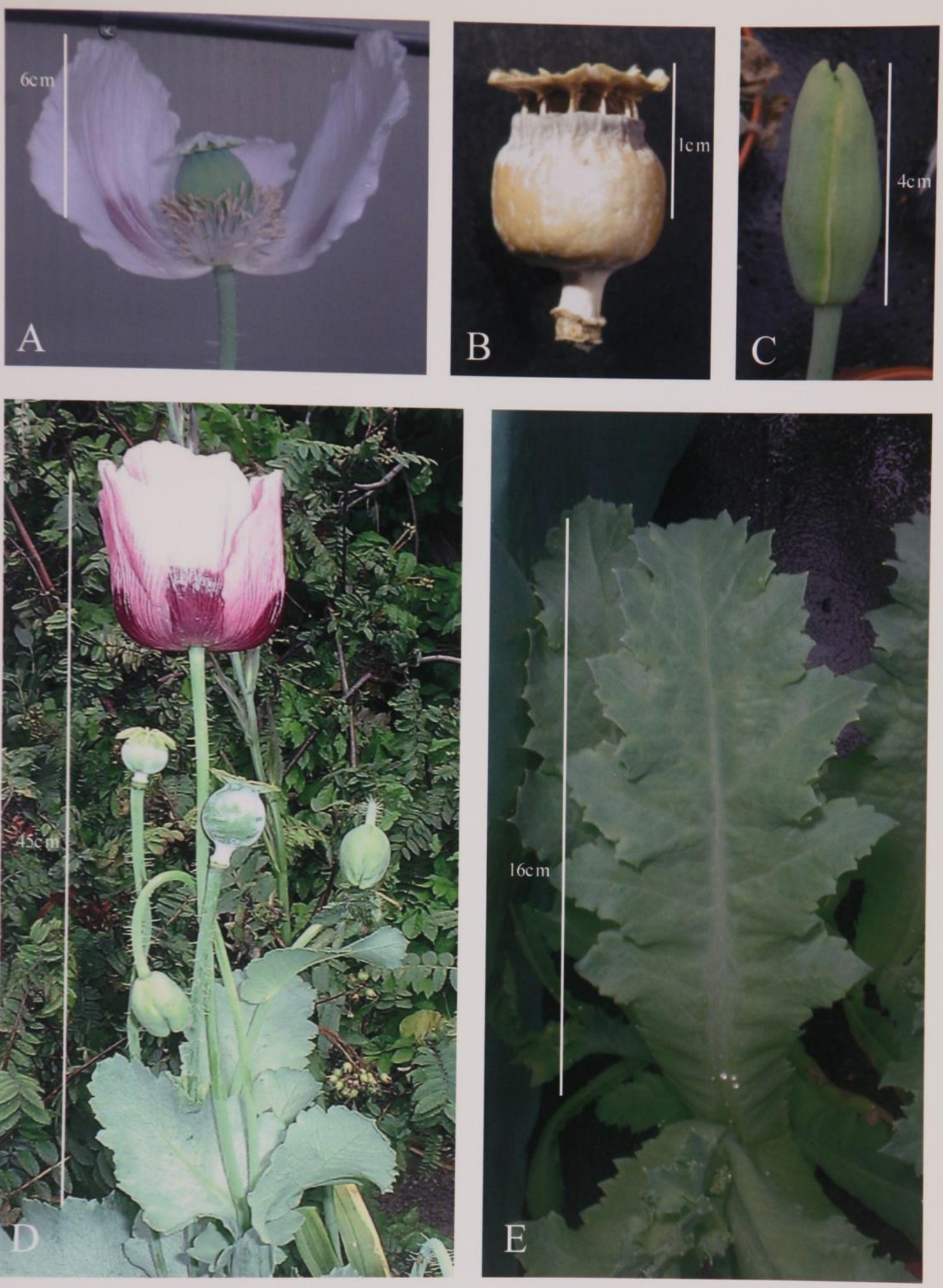


Figure 1.11. Characteristic features of *Papaver* sect. *Papaver*: A, light coloured anthers and filiform filaments; B, poricidal, glabrous ovoid or obovoid capsules, bearing a pyramidical stigmatic disc; C, glabrous, obovoid two-valved bud; D, erect annual herb with flowers in racemes; E, lanceolate to dissected auriculate-amplexicaulous leaves. Figures A, C-E: *Papaver somniferum*; B: *Papaver glaucum*.

relationships that exist within Papaveroideae. The latest revision of *Papaver* (Table 1.11) made by Kadereit who (1988a) recognises eleven sections consisting of c. 80 species and this classification will be followed henceforth. Four groups within *Papaver* were recognised based on diagnostic morphological characters coupled with geographical distributions.

Table 1.11 Division of the sections of *Papaver* into groups determined by specific diagnostic morphological characters (Kadereit, 1988).

Meconella	*Argemonidium*	*Pilosa*	*Carinatae*
Meconidium		*Pseudopilosa*	*Rhoeadium*
Californicum		*Horrida*	*Oxytona*
			Papaver

The first group consists of sections *Californicum, Meconella* and *Meconidium* and is characterised by having light coloured filiform filaments, light coloured anthers and valvate dehiscence of seed. Group two consists of *Argemonidium* alone and is characterised by having dark coloured clavate filaments, dark coloured anthers and poricidal dehiscence of seed. The third group comprises sections *Horrida, Pilosa and Pseudopilosa* and with light coloured filiform filaments, light coloured anthers and poricidal dehiscence of seed. Finally group four comprises sections *Carinatae, Oxytona, Papaver* and *Rhoeadium,* and is characterised by having, dark coloured (sometimes light) filiform (sometimes clavate) filaments, dark coloured anthers and poricidal dehiscence of seed.

1.4 Aims of this thesis

Without further molecular investigation it is difficult to ascertain whether or not any of characters used to define taxonomic groups have evolved through convergent morphological evolution. It was the primary aim of this project not only to investigate the phylogenetic relationships that exist within *Papaver,* its species and sections, but also to ascertain if the currently used sectional groupings are based on characters that indicate recent shared ancestry or whether these groupings are artificial

and without any phylogenetic significance. The systematic affinities of the sections of *Papaver* were examined using a number of different types of character, including molecular, morphological and phytochemical.

In addition, molecular techniques will be employed in an attempt to differentiate between the species of section *Oxytona* and assess the relationship and origin of these species. This section warrants special attention due to the significance of the morphinane alkaloids which are produced by its species. Difficulties have arisen in the identification and differentiation of these species without diagnostic floral characters which are produced in the second year of growth. Thus a molecular fingerprinting technique was assessed for suitability to provide a means of identifying and differentiating these species without the reliance on morphological characters. It was anticipated that this would aid not only allow a better understanding of the relationships that exist between these species but also aid future phytochemical and *in-vitro* studies which require the correct identification of material before use.

The specific objectives of this thesis including the methods employed to suit these objectives include:

- To assess systematic relationships between and within sections of *Papaver* using nucleotide sequence polymorphisms (Chapter 2). The two molecular regions, which have been implemented in this thesis, are the internal transcribed spacer region (ITS) of 18S-26S nuclear ribosomal DNA and the combined *trnL* intron and the *trnF* intergenic spacer region of plastid origin. Combining sequences from different genomes (nuclear, chloroplast and mitochondrial) is common in modern molecular phylogenetics and has resulted in greater understanding of relationship within a wide range of plant groups (e.g. Taberlet *et al.*, 1991; Sun *et al.*, 1994; Baldwin *et al.*, 1995; Kelchner *et al.*, 1997; Hodkinson *et al.*, 2002a). In addition the utilisation of these particular gene regions and their subsequent combination has not been applied to *Papaver* in previous molecular studies. Previous molecular analysis suffered from lack of species sampling. For example the study of Kadereit & Sytsma, (1992) did not include representatives from all sections of *Papaver* and the study of Kadereit *et al.* (1997) although including representatives from all sections predominantly included single accessions to represent a species

and single species to represent polytypic sections. In this thesis, where possible, all species were represented by more than one accession and the polytypic sections were represented by a number of different species. It was anticipated that these two regions would be suitable to assess relationship at the sectional and generic level in *Papaver* as they had proven useful at this level in other plants (e.g. Wendel *et* al., 1995; Gielly and Taberlet, 1996; Gielly *et al.*, 1996; Wendel & Doyle, 1998; Hodkinson *et al.*, 2002). The higher degree of species sampling undertaken in this thesis compared to other studies has also allowed a more thorough examination of sectional relationships within *Papaver* to be made.

- To assess the significance of morphological traits as systematic characters (Chapter 3) and to better understand their origin (character evolution). In an attempt to ascertain the suitability of species diagnostic morphological characters for phylogenetic reconstruction, a morphological phylogenetic analysis of *Papaver* was undertaken. Before the molecular studies of Kadereit & Sytsma (1992), detailing possible systematic affinities of sections within *Papaver,* all previous classifications and phylogenetic estimations were based on morphological (and sometimes phytochemical) similarities. However, it is becoming increasingly obvious that the use of morphological characters (morphometric data) as the basis for phylogenetic analysis is strengthened when combined with molecular evidence (see review by Soltis & Soltis, 1995). In addition, convergent morphological characters (shared/similar characters that have arisen from separate ancestral lineages), which potentially result in inaccurate classifications, can be identified when morphological similarities are assessed in light of groupings based on molecular similarities. The characters that have traditionally been used to identify the sections of *Papaver* were examined cladistically, and the groupings found by this analysis were compared to those found in the molecular analysis. Thus characters that were similar but not because of shared ancestry may be identified. Additionally a number of important morphological characters that have been used to estimate groupings of sections within *Papaver* (c.f. Table 1.11) were mapped onto a molecular tree to assess their suitability to define such groups.

- To assess the significance of phytochemical traits (alkaloids) as systematic characters (Chapter 4) and their origin by estimating whether sections that produce similar alkaloid types do so by virtue of similar descent. Similarities or differences in phytochemical spectra can be extremely informative for the classification of plant species and have been used to aid classifications of *Papaver* on a number of occasions (Gunther, 1975; Preininger *et al.*, 1981; Tetenyi, 1993; reviewed by Waterman, 1998). The economic importance of the morphinane alkaloids thebaine, codeine and morphine produced by opium poppy (*Papaver somniferum,* section *Papaver*) has given rise to a vast array of literature detailing the types of alkaloids a wide range of poppy species predominantly produce. For all accessions included in the molecular analysis a list of reported alkaloids obtained from the literature was made. Instead of using individual alkaloids as characters for a certain species which is detrimental for a number of reasons (discussed in Chapter 4), alkaloids were grouped based on the alkaloid type (based on chemical structure) and the biosynthetic route (biogenetic groups) responsible for their production. Groupings of sections within *Papaver* as indicated by the analysis of these groups were compared to groups obtained in the molecular analysis to assess firstly whether they can be used as strict cladistic characters and secondly to estimate the amount of phylogenetic significance (signal) they represent. Certain biogenetic groups of alkaloids were mapped onto a cladogram obtained from the molecular analysis in an attempt to ascertain whether convergence between sections that produce similar alkaloid types has occurred.

- To propose a revised classification (if required) for *Papaver* and its sections based on interpretations of groupings obtained from the analyses conducted in Chapters, 2, 3 & 4).

- To conduct a detailed phylogenetic analysis of *Papaver* L. section *Oxytona* (Chapter 6). The aims of this section of the thesis are further divided and it was first intended to estimate the relationships that exist between the species of the section based on cytological, phytochemical and molecular characters

(Chapter 6) and secondly to utilise molecular techniques to differentiate between the species to aid in the utilisation of these species as sources of economically important phytochemicals (Chapter 6 &7). ITS and *trnL-F* nucleotide sequences generally do not provide sufficient polymorphisms to estimate relationships at the species level (Soltis & Soltis, 1998). To examine relationship at this taxonomic level other molecular techniques better suited to this task were employed. The estimation of relationship within *Oxytona* and investigation of the origin of these species was based on a number of techniques to produce morphological, phytochemical and cytological characters. The genetic fingerprinting technique AFLP (discussed in Chapter 6) was also employed to differentiate between the species of the section and infer the phylogenetic relationship that exists between the species. To assess the origin of the tetraploid and hexaploid species of this section, *in situ* hybridisation technologies were applied which have been used successfully to ascertain the parental progenitors of tetraploid and hexaploid species (e.g. Bennett *et al.*, 1992; Moscone *et al.* 1996; Hodkinson *et al.*, 2002; Lim *et al.*, 1998; Lim *et al.*, 2000).

- To apply genetic fingerprinting methods to assess of genetic variability of *in vitro* cultured *Papaver bracteatum* (section *Oxytona*). This chapter (Chapter 7) involved the utilisation of AFLP fingerprinting as a means of differentiating and identifying material used to initiate *in vitro* cultures. Considering that many of the alkaloids produced by species of *Papaver* are species-specific it is critical that material is identified correctly. Additionally hybrids have the potential to effectively reduce the yields of valuable alkaloids and can alter the expected phytochemical spectra of a certain accession (Milo *et al.*, 1990; Levy & Milo, 1991) and their identification is thus essential. The difficulties of differentiating *Oxytona* material, makes genetic fingerprinting an essential prerequisite for experimentation involving these species. *In vitro* technologies could potentially be used to mass-produce certain chemotypes of individual species and it is essential to track the genetic stability of such accessions (and thus ensure genetic uniformity) through the tissue culture and regeneration processes. AFLP fingerprinting was used here for such purposes and to attempt to explain previously ambiguous results (Hook *et al.*, 1998).

CHAPTER 2. Molecular phylogenetic analysis of *Papaver* L.

2.1 Introduction

Phylogenetics in its simplest definition is the study of evolutionary history. The term phylogeny, in its etymology, means simply the 'origin or birth of tribes' (Sporne, 1974). Phylogenetic taxonomy is a subdiscipline of phylogenetics that classifies organisms on the basis of their degree of evolutionary relatedness. Determining the relationship between organisms can be achieved by the examination of a variety of characters such as morphological, molecular, phytochemical and chromosomal as long as these characters have a heritable genetic basis. One of the governing principles when choosing characters to determine relationship between organisms is that the characters are homologous i.e. shares a resemblance in structure and evolutionary origin (Doyle & Davis, 1998). In the case of using DNA or RNA to infer relationships between organisms, the characters used range from single nucleotides within a sequenced genomic region to fragments of DNA ranging in size, with fragments of the same size deemed homologous (either through experimentation or assumptions). For detailed reviews of homology see Donoghue (1992), Hillis (1994) and Doyle & Davis (1998).

It is essential to differentiate between homologous and homoplasious characters in phylogenetics. Two similar characters or character states may be assumed to be homologous and apomorphic i.e. derived through common descent, when in fact the similarity may have been derived independently. Homoplasy may be the result of parallelism, which is a similarity of non-homologous characters due to chance occurrence or similar selection under similar conditions; or character reversal, which is the reversal of a shared derived character (synapomorphy) back to a similar condition to the ancestral state. Additionally, when studying DNA evidence the distinction between paralogy and orthology must be understood. Paralogy is homology that arises via gene duplication, whereas orthology is homology that arises via speciation (Fitch, 1970; Hillis, 1994; Doyle & Davis, 1998).

For the phylogenetic appraisal of *Papaver* nucleotide sequencing was implemented. The aim here was to produce phylogenetic trees that provide a graphical representation of shared ancestry between all individuals of the genus available for study. Sequences from all available taxa were compared to each other, and to distantly related taxa, in an attempt to identify which characters are primitive/ancestral (plesiomorphy) or derived (apomorphy), relative to homologous characters in related taxa (Henning, 1966). As stated, a synapomorphy is a derived character, which is shared between two or more individual taxa, and it is with synapomorphic characters that sequence phylogenetic trees based on parsimony methods are made.

With nucleotide sequence analysis systematists are dealing with the smallest units of evolutionary change i.e. the nucleotide, and the variation that allows phylogenetic inferal is provided for by mutations (mutations defined here as all types of molecular change). Sequences accumulate changes over time through mutation, which are classified according to the change caused in the nucleotide sequence (Li & Graur, 1991; Li, 1997). Although mutations occur rarely, the most common mutation is the point mutation or substitution, which is a change of one nucleotide for another. A transition is a replacement of a purine (adenine and guanine) with a purine or a pyrimidine (thiamine and cytosine) with a pyrimidine. A replacement of a purine with a pyrimidine or vice versa is termed a transversion and is generally less common than a transition (Swofford *et al.*, 1996). Deletions/insertions ('indels') result from the removal/addition of one or more nucleotides from a particular sequence and are extremely informative with respect to phylogenetic reconstruction. Other mutations encountered in nucleotide sequencing are inversions, which describe a rotation of 180° by a DNA duplex, and duplications which are the copying and insertion of a nucleotide sequence.

A significant problem when attempting to produce phylogenetic trees from variations in nucleotide sequences is that the number of substitutions observed is generally less than the actual number of substitutions that have occurred during evolutionary history. Mutations can occur which reflect the incorrect number of substitutions and include: multiple substitutions (e.g. A to C to T) at a site in one sequence and coincidental

substitutions (e.g. C to G in one sequence, C to A at the same site in other sequence), both of which give the impression that only one substitution occurred (Swofford *et al.*, 1996). Additionally mutations can be undetected altogether (concealed), caused by parallel substitutions (e.g. T to A in one sequence, T to A at the same site in other sequence), convergent substitutions (e.g. A to C to T in one sequence, A to T at the same site in other sequence) and back substitutions (e.g. C to T to C) at a site in one sequence. As evolutionary time progresses since the divergence of studied individuals these concealed substitutions become increasingly more significant to phylogenetic construction (Li, 1997).

2.1.1 Genetic regions implemented in the systematic study of *Papaver*

Selection of a suitable DNA sequence for phylogenetic inferal at this particular taxonomic level within *Papaver* (i.e. between species and closely related genera) is dependent on a number of factors (Soltis & Soltis, 1998). Firstly all regions chosen contain regions that are non-coding, i.e. regions with intergenic spacers or introns. Non-coding DNA experiences relatively less evolutionary constraint in comparison to coding regions of the genome, resulting in a greater level of sequence divergence. Thus, at the generic level choosing a protein-coding sequence, such as the plastid ribulose 1,5, biphosphate carboxylase large subunit (*rbcL*), which is important to the overall photosynthetic metabolism of an organism, may result in data matrices with insufficient genetic divergence to infer evolutionary relationships between closely related taxa, as it is imperative that this region remains relatively unchanged through successive generations. Additionally the relatively expensive nature of most molecular techniques used to aid systematic studies requires choosing regions of the genome that can be amplified and sequenced with relative ease.

The two molecular regions, which have been implemented in this analysis, are the internal transcribed spacer region (ITS) of 18S-26S nuclear ribosomal DNA (Figure 2.1) and the *trnL* intron and the *trnF* intergenic spacer region (Figure 2.2) of plastid origin. The presence of conserved coding regions flanking the non-coding regions allows for successful amplification and sequencing using universal primers applicable to a wide range of plant groups (Taberlet *et al.*, 1991; Sun *et al.*, 1994; Baldwin *et al.*, 1995; Kelchner *et al.*, 1997; Hodkinson *et al.*, 2002a). In addition, all regions are

relatively small in size (i.e. *trnL- trnF* ~500- 900bps; and ITS ~700bps), which also promotes successful amplification and sequencing (Taberlet *et al.*, 1991; Baldwin *et al.*, 1995; Kelchner *et al.*, 1997).

2.1.2 18S-26S internal transcribed spacer regions (ITS-1, ITS-2) of nuclear ribosomal DNA

Nuclear Ribosomal DNA (nrDNA) codes for cytoplasmic ribosomal RNAs. The entire nrDNA repeat is present in up to many thousands of copies arranged in tandem repeats at one or more chromasomal loci, with the majority being located around the nuclear organising region (NOR) (Appels & Baum, 1992; Soltis and Soltis, 1999). This high copy number promotes the detection, amplification, cloning and sequencing of nrDNA. Nuclear DNA is bi-parentally inherited, thus providing evidence of evolutionary history for both the maternal and paternal lineages. In eukaryotes, the rDNA cistron encodes 18S, 5.8S and 26S rRNAs, separated by two internal transcribed spacers (ITS-1 and ITS-2), and flanked by the 5' and 3' external transcribed spacers (5'-ETS and 3'-ETS; Figure 2.1). Each repeat is separated from the next by an intergenic spacer (IGS). The 18S forms the ribosomal small subunit (SSU) and the 5.8S and the 26S form most of the large subunit (LSU; Hershkovitz *et al.*, 1999). From a functional perspective the ITS region is part of the transcriptional unit of nrDNA but the spacer segments of the transcriptional unit are not incorporated into mature ribosomes. Instead, ITS-1 and ITS-2 of the nrDNA transcript appear to function, at least in part, in the maturation of nrRNAs (Baldwin *et al.*, 1995). The ITS-1 and ITS-2 regions are under less evolutionary constraint than the genes they adjoin, but do appear to be under some evolutionary pressure (van der Sande *et al.*, 1992). Combining ITS-1 and ITS-2 often results in sufficient variation between organisms to allow the construction of accurate phylogenetic trees at the inter-specific and intra-generic level (Wendel *et* al., 1995; Wendel & Doyle, 1998; Hodkinson *et al.*, 2002; Simpson *et al.*, 2003)

Phylogenetic reconstruction using the ITS-1 and ITS-2 regions recognise that this gene family undergoes rapid concerted evolution, via unequal crossing over and gene conversion (Cronn *et al.* 1996; Hodkinson *et al.*, 2002). This promotes intragenomic

uniformity of repeat-units and in general, promotes accurate reconstruction of species relationships from those sequences (Baldwin *et al.*, 1995). However problems with using the ITS-1 and ITS-2 regions can arise if concerted evolution fails to homogenise ITS paralogues through a succession of speciation events. The probability of unknowingly sampling sequences with different evolutionary histories and thus producing mis-representative phylogenetic trees is a real danger (Baldwin *et al.*, 1995). If infragenomic ITS polymorphism is present cloning and sequencing of all paralogues must carried out in order to separate the orthologous sequence (Baldwin *et al.*, 1995; Hodkinson *et al.*, 2002).

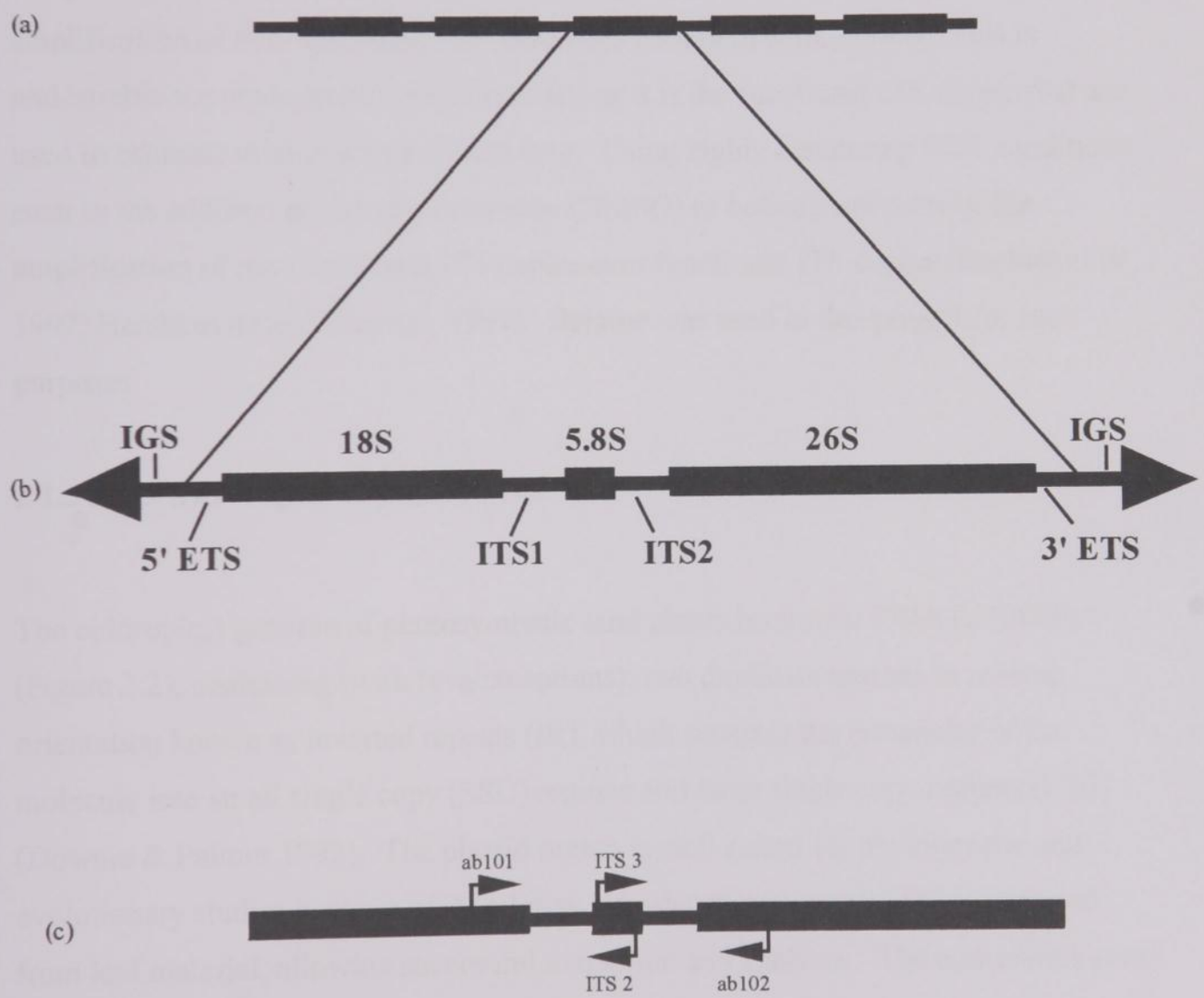


Figure 2.1 (a) Typical structure of angiosperm ribosomal array. (b) Coding regions of the 18S small subunit (SSU), 5.8S, and 26S large subunit (LSU) are shown as heavy rectangles. IGS= intergenic spacer, ETS= external transcribed spacer, ITS= internal transcribed spacer. (c) Position of annealing and direction of synthesis for the primers used in this study.

In addition, successful amplification and subsequent sequencing can be hindered by the formation of secondary structures. The GC content of angiosperm nrDNA is usually higher than 50% and sometimes exceeds 75% (Hershkovitz and Zimmer, 1996, 1997). This high GC content means that for ITS-1 and ITS-2 rRNA transcripts and single strand templates there is the potential for the formation of folds into helices (double stranded stems with terminal loops) or more complex structures (Conn & Draper, 1998) via Watson-Crick intrastrand base-pairing (Baldwin *et al.*, 1995). These structures can potentially block polymerisation steps during amplification and sequencing and also effect strand mobility in the sequencing gel. The relatively lower secondary structure stability of non-functional ITS single strand copies in comparison to functional ITS single strand copies, can result in the preferential amplification of non-functional ITS sequences (Baldwin *et al.*, 1995). This is undesirable for phylogenetic reconstruction as it is the functional ITS copies that are used to estimate relationship between taxa. Using highly denaturing PCR conditions such as the addition of dimethylsulfoxide (DMSO) or betaine can prevent the amplification of non-functional ITS copies over functional ITS copies (Buckler *et al.*, 1997; Hershkovitz and Zimmer, 1997). Betaine was used in this project for such purposes.

2.1.3 *trnL- trnF* region of plastid DNA

The chloroplast genome of photosynthetic land plants is circular DNA (c.120kb) (Figure 2.2), containing (with few exceptions), two duplicate regions in reverse orientation known as inverted repeats (IR), which separate the remainder of the molecule into small single copy (SSC) regions and large single copy regions (LSC) (Downie & Palmer 1992). The plastid region is well suited for phylogenetic and evolutionary studies because of its relative abundance within total DNA extracted from leaf material, allowing successful extraction and analysis. The conservative rate of nucleotide substitution within the plastid genome and relatively slow rate of plastid protein coding gene evolution (on average fivefold slower than plant nuclear genes; Wolfe *et al.*, 1987) make plastid DNA regions appropriately suited to resolve plant phylogenetic relationships at higher taxonomic levels (e.g. Downie & Palmer, 1992; Bayer & Starr, 1998). Noncoding regions of the plastid genome evolve more rapidly

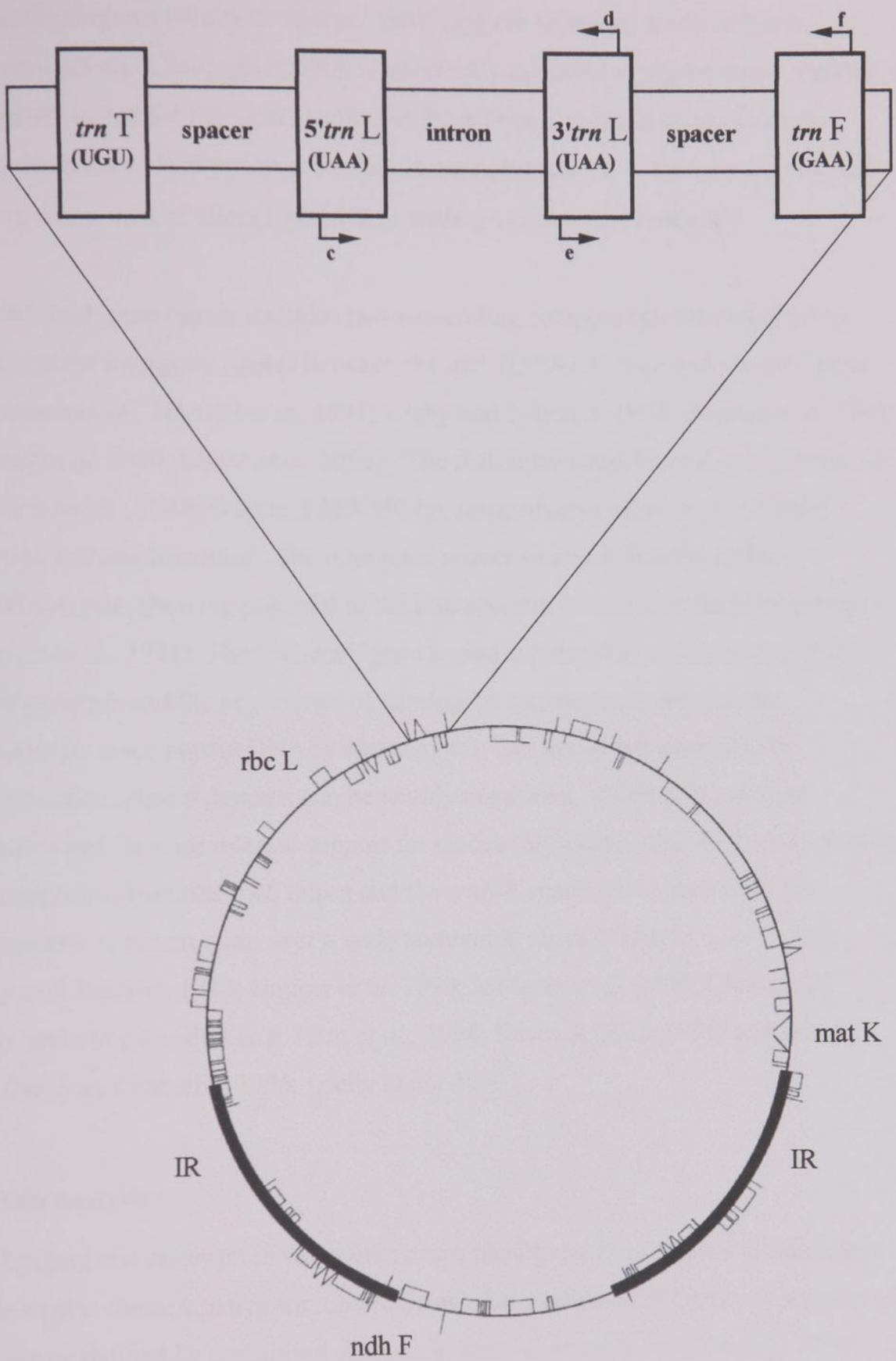


Figure 2.2 Structure of plastid DNA of *Nicotiana tabacum* L. (Solonaceae). Presented are positions of the *trnT* exon (UGU), the *trnL-T* intergenic spacer, the *trnL* intron, the *trnL* 3' and 5' exons (UAA) the *trnL-F* intergenic spacer and the *trnF* exon (GAA). The relative position of annealing and direction and f primers (Taberlet *et al.*, 1991) used in this study. After Bayer and Starr, (1998).

than coding regions (Wolfe & Sharpe, 1988) and are therefore useful at lower taxonomic levels. Chloroplast DNA is maternally inherited in angiosperms, making it particularly suited for the identification of the maternal parental progenitors of polyploid species. Hodkinson *et al.* (2002) used plastid *trnL-F* sequences to identify the maternal parent of allopolyploid *Miscanthus* × *giganteus* (Poaceae).

The *trnL-trnF* gene region includes two non-coding components: the *trn*L (UAA) intron and the intergenic spacer between the *trnL* (UAA) 3' exon and the *trnF* gene (hereafter *trnL-F*; Taberlet *et al.* 1991; Gielly and Taberlet, 1994; Hopper *et al.* 1999; Molvray *et al.* 1999; Chase *et al.* 2000). The *trn*L intron and the *trnL-trnF* spacer are relatively small (350-600 bp and 120-350 bp, respectively), allowing successful detection and amplification. The intergenic spacer generally has the higher substitution rate, showing potential at the interspecific level in certain plant groups (Taberlet *et al.*, 1991). The *trnL-trnF* gene region is present as a single copy in the plastid genome; and the occurrence of paralogous sequences is not an issue. Furthermore, since plastid DNA is inherited as a unit and is not subjected to recombination, plastid datasets can be readily combined, which may enhance resolution and increase internal support for clades (Soltis & Soltis, 2000). Nucleotide polymorphisms from the *trnL* intron and the *trnL-F* spacer have been used for phylogenetic reconstruction over a wide taxonomic range (Taberlet *et al.*, 1991; Gielly and Taberlet, 1994; Hopper *et al.* 1999; Molvray *et al.* 1999; Chase *et al.* 2000), including families (e.g. Ham *et al.*, 1994; Bayer & Starr, 1998) and genera (e.g. Gielly and Taberlet, 1996; Gielly *et al.*, 1996).

2.2 Data analysis

The fundamental assumption when inferring a phylogeny from a set of characters is that ancestral characteristics are inherited, and that evolutionary history of a group of taxa can be defined by examining changes in these characteristics (Hennig, 1950, 1966). Evolutionary relationships between groups of organisms are illustrated by means of a phylogenetic tree, which are graphs composed of nodes and branches. The terminal nodes represent the taxonomic units (taxa) and the branches define the relationships among the units in terms of descent and ancestry.

2.2.1 Maximum parsimony

The phylogeny of *Papaver* in this thesis was inferred from the character matrix under the principle of maximum parsimony. This was achieved by generating a large number of hypothetical trees using a search algorithm and then selecting the 'best fit' trees according to specific optimality criteria (Swofford *et al.*, 1996). The most widely used optimality criterion, parsimony, searches for minimum length trees (maximum parsimony trees) that minimize the amount of evolutionary change (transformations from one character state to another) needed to explain the amount of dis/similarity existing within a group of taxa based on the matrix of characters. Determining the most parsimonious trees depends largely on how characters are treated within the optimality criteria. Trees obtained from parsimony analyses can be rooted or unrooted phylograms. Choosing the shortest trees also serves to maximise congruence between characters. Unrooted phylograms are trees in which the earliest point in time or common ancestor is not identified (Swofford *et al.*1996).
Evolutionary trees can be rooted using an outgroup, which is usually a relative of the main group of taxa (ingroup) under study (e.g. Henning, 1950, 1966; Hillis, 1987). An assumption made when using outgroup rooting is that the members of the ingroup are monophyletic i.e. more closely related to each other than to the outgroup (Judd *et al.*, 1999). Inclusion of an outgroup allows the polarity or directionality of characters to be identified and assessed. Examining changes in characters from primitive (plesiomorphic) states to derived (apomorphic) states allows the 'directionality' of a character to be obtained. This method identifies primitive characters states by comparing the character states within a monophyletic group (ingroup) to a sample taxon from a sister group (outgroup), thereby identifying the character states which are ancestral (common in both) and those that are derived (present in ingroup only), (Judd *et al.*, 1999). By determining the directionality of character states it is possible to impose constraints upon permissible character changes. This is achieved in the choice of parsimony method, which includes, Wagner, Fitch, Dollo and Camin-Sokal parsimony. Wagner parsimony (Kluge and Farris, 1969; Farris, 1970) treats binary or unordered multistate characters and permits free irreversibility; Fitch parsimony, the method most frequently used for DNA sequence data, including this thesis (Fitch, 1971), imposes no constraints on character changes permitting transformation into any other state. Dollo parsimony (Farris, 1977) permits each derived character to

originate only once and Camin-Sokal parsimony (Camin-Sokal, 1965) prohibits reversals from a derived state to a relatively more ancestral condition.

The physical production and assessment of phylogenetic trees from a matrix of characters is based on two distinct procedures (i) a search for the most parsimonious tree or sets of trees (optimal tree(s)) and (ii) the testing of the most parsimonious trees to assess the reliability of branching patterns and characters within the trees (support).

2.2.2 Maximum likelihood

An additional form of optimality-criteria search method is maximum likelihood (ML; Cavalli-Sforza & Edwards, 1967; Felsenstein, 1981; Felsenstein, 2004). In short the principle of likelihood suggests that the explanation that makes the observed outcome the most likely (i.e. the most probable) is the explanation preferred (Page & Holmes, 1998). Maximum likelihood analyses involve the use of a model-based approach in an attempt to find the tree that makes the observed data more probable. Phylogenies are inferred by locating those trees that yield the highest likelihood (Swofford *et al.*, 1996), under a given model, for generating the observed data. For sequence data these models include the branch length, a base substitution model and in some cases a Gamma distribution. The substitution model attempts to account for the variation of homologous nucleotides by specifying the probabilities of a given nucleotide substitution and base frequencies. These models differ in the assumptions on the specific substitution parameters (Swofford and Begle, 1993).

One of the first models developed and considered the simplest model of nucleotide substitution is the Jukes-Cantor (JC) model (1969), which assumes that the four bases have equal frequencies and that transversions and transitions occur at the same rate. Kimura's 2 parameter (K2P) model (Kimura, 1980) is an extension of the JC model and is based on the assumption that transitions and transversion occur at different rates i.e. for a given nucleotide there are three possible changes, one of which is a transition and the other two possible change being transversions (Swofford and Begle, 1993). Felsenstein (1981) extended the JC model further by developing a model (F81) to take into consideration that the frequency of certain nucleotide substitutions is dependant on the variation in base composition, i.e. if some bases are more frequent in a given sequence then substitutions involving that nucleotide are more likely. The

F81 model assumes unequal frequencies for the four nucleotides and that transversions and transitions are equally likely. The Hasegawa, Kishino, Yano (1985) model (HKY85) basically combines the K2P and F81 models by allowing unequal base frequencies, and allowing transitions and transversions to occur at different rates. Gamma distribution allows the analysis to accommodate rate heterogeneity across sites.

2.2.3 Searching for optimal trees

There are a number of methods used to search for optimal trees including exact and heuristic methods. Exact searches (exhaustive, branch and bound searches) are based on algorithms that generate all possible trees (exact search) or guarantee to find the shortest tree by generating only optimal trees (branch and bound, Swofford and Begle, 1993). Optimal trees are retained in both methods. Heuristic methods do not guarantee that the optimal solution is found but can, if conducted appropriately, search the total tree space efficiently and either find the optimal tree(s) or get close to the optimal tree(s). Heuristic methods work by creating an initial tree and continually rearranging the tree until the optimal tree is produced. Each remaining taxon is successively added to the taxon tree with the addition sequence being predetermined using a number of methods. These methods include the addition of taxa in the same order as they are found in the data matrix ('as is') to random addition of taxa based on certain permutations (Swofford and Begle, 1993). A more detailed method i.e. 'closest' evaluates all remaining taxa in the dataset by adding them to every branch in the taxon-branch combination, retaining the taxon tree that requires the smallest increase in tree length for the next step. The 'simple' method predetermines the addition sequence before stepwise addition by determining the distance between taxa and a reference taxon. This distance is known as an advancement index (Farris 1970) and the taxa are added in order of increasing 'advancement' (Swofford and Begle, 1993). Tree searches often involve several (e.g. 1000) repetitions of random addition sequence so that the sequences are not trapped on a suboptimal 'island' of potential trees. An additional heuristic option is 'branch-swapping' which involves the dismantling and rebuilding of trees in an attempt to produce a tree with a smaller number of steps according to one of several methods: including nearest-neighbour interchange (NNI), subtree pruning (SPR) and tree bisection and reconnection (TBR).

NNI swapping involves the interchanging of subtrees connected by any internal branch of the main tree. Trees containing the smallest number of steps are kept. In SPR, subtrees are 'pruned' from the tree and subsequently 'grafted' to a different location on the tree. TBR involves the bisection of a tree along a branch yielding two subtrees, which are subsequently reconnected by joining a different pair of braches (Swofford and Begle, 1993).

2.2.4 Estimating support of clades

Once the most parsimonious or likeliest tree or set of trees has been obtained there are a number of methods for testing the reliability of branching patterns and characters within a given tree. These methods (reviewed by Swofford *et al.*, 1996) include Bootstrap (Felsenstein, 1985) and Jacknife (see Mueller & Ayala, 1982) resampling, and the Bremer support method (Bremer, 1994). Bootstrap analysis was chosen as the preferred method to place support values on the trees produced in this thesis. "Bootstraping" involves the resampling of characters from within the data, with replacement, to create a series of bootstrap samples (new data sets) the same size as the original data (Felsenstein, 1985). Each of these is then subjected to a heuristic search to produce a set of bootstrap trees. Finally, a majority rule consensus tree, which involves the comparison of the topologies of the most parsimonious trees (retaining only branches which appear in a minimum number of trees) is produced. Each group (an inferred monophyletic group) within the consensus tree is assigned a percentage dependant on how many times it appears in the set of bootstrap trees, thus allowing the ability to assign support values to a hypothesised relationship. For example a group of taxa that group together in 90% of the bootstrap trees will be assigned a value of 90% on the particular branch that separates this group from the rest of the taxa. A bootstrap value of 95% can be considered as a good support for a clade. However lower values can also indicate support (Salamin *et al.*, 2003).

Additional methods assess the reliability of characters within a particular tree. This is achieved by examining the behaviour ("fit") of a specific character on a particular tree topology using descriptive statistical indices. The consistency index (CI; Kluge & Farris, 1969), the retention index (RI) and the rescaled consistency index (RC; Farris, 1989) give a statistical indication to the reliability of a character with respect to other

characters in the data matrix. A low index score for a character reflects that a character is adding to tree length without increasing support for any of the branches thus allowing the probable homology/homoplasy of a character to be measured. When they are summed over all characters in the matrix they can give an indication of the homoplasy in the whole dataset.

2.3 Materials and methods

2.3.1 Outgroup selection

Outgroup taxa were selected on the basis of the chloroplast DNA restriction site analysis of Kadereit and Sytsma (1992) and the morphological works of Kadereit (1993). Although attempts were made to use groups from outside the Papaveroideae to polarise trees, alignments were ambiguous and could not be used for phylogenetic reconstruction. These included *Chelidonium majus* L. and *Macleaya cordata* Willd. (Papaveraceae subfamily Chelidonioideae) The nine genera of the Papaveroideae have been separated into two groups (Kadereit, 1993; discussed in Chapter 1, table 1.1). The first group (including *Papaver*) is viewed to be monophyletic thus accessions obtained from these genera have been included in the ingroup analysis. The second group consists of *Arctomecon*, *Argemone*, *Canbya* and *Romneya.* It is important when selecting an outgroup that this particular species is not part of the ingroup and *Argemone mexicana* Linn. and *Eschscholtzia californica* Cham. (Papaveraceae subfamily Eschcholzioideae) were thus chosen as the outgroup taxa.

2.3.2 Plant material

The majority of the material used in this analysis was cultivated from seed obtained from various botanic gardens and commercial sources and grown to maturity either at the National Botanic Garden, Glasnevin, Ireland, or in the glasshouse of the School of Phramacognosy, Trinity College Dublin, Ireland. Voucher specimens were kept for each accession used and were stored in the Herbarium of the Department of Botany, Trinity College Dublin, Ireland. Each accession was given a unique identification number and an equivalent DNA bank reference number and subsequently stored at the

School of Botany DNA Bank. Sequences will be posted in Genbank the National Centre for Biotechnology Information (NCBI) database.

2.3.3 DNA extraction

DNA extraction, purification, quantification, amplification, cycle sequencing and a portion of sequencing on the ABI Prism automated sequencer was carried out by the author at the molecular systematics laboratory, in the Department of Botany, TCD. Some sequences reactions were run on the sequencer at the Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, England. Total genomic DNA was extracted from 0.05-0.1g of fresh or silica dried leaf material using the hexadecyltrimethyl ammonium bromide (CTAB) based extraction buffer modified from Doyle & Doyle (1987; reagent concentrations, protocol and materials are detailed in Appendix1.1, Hodkinson, unpublished). The crude DNA extract was then purified using the Concert™ Rapid PCR Purification System (Life Technologies, 1999; Appendix 1.2), a cleaning method that removes phenolics, small RNA molecules, polysaccharides and other impurities. Each DNA sample was then labelled and quantified using a spectrophotometer (Ependorf, Biophotometer) and then stored at -20°C or -70°C. Total genomic DNA and PCR products were visualised by running samples on an agarose gel (protocol, concentrations and materials are detailed in Appendix1.3).

2.3.4 PCR amplification

The polymerase chain reaction ('PCR', Mullis & Faloona, 1987), a method for amplifying DNA sequences, was used to amplify the targeted *trnL-trnF* and ITS sequences from the total DNA template using a GeneAmp® PCR System 9700 thermal cycler. For amplification and sequencing of the ITS region the forward and reverse primers here called "ab101" and "ab102" of Sun *et al.* (1994) were used. The *trnL* intron and the *trnL-trnF* spacer were amplified using primers "c" and "f" of Taberlet *et al.* (1991). Difficulties were encountered when attempting to amplify certain sequence regions for certain accessions, this included all DNA extracted from herbarium specimens. Their successful amplification and subsequent sequencing was however achieved using internal primers "ITS-2" and "ITS-3" for ITS (Baldwin *et al.*, 1995) and primers "d" and "e" for the *trnL* intron and the *trnL-trnF* spacer

Table 2.1 Accessions used for molecular analysis. NBG: National Botanic Gardens, Glasnevin, Ireland; TCD BG: Trinity College Dublin, Botanic Gardens; SoP: School of Pharmacognosy, Department of Pharmacy, Trinity College Dublin. MBG: University of Mainz Botanic Garden, Germany; RBGE Royal Botanic Gardens Edinburgh, Scotland; UCBG: University of Copenhagen Botanic Gardens, Denmark. CPG: Chelsea Physics Garden, London, England. Cult: Cultivated material from seed. Herb: DNA extracted from herbarium material. CS: Commercial source. DNA: material provided as DNA.

Genus and species	Section	Accession number	Source, Origin & Material Type	Accession Information (Botanic Gardens)
Papaver aculeatum	*Horrida*	J-131	MBG, Origin unknown, Cult.	No accession information
Papaver aculeatum	*Horrida*	J-151	NBG, Losetto, South Africa, Cult.	2000.0659
Papaver aculeatum	*Horrida*	J-158	NBG, Origin unknown, Cult.	No accession information
Papaver albiflorum spp. *albiflorum*	*Rhoeadium*	J-100	NBG, Origin unknown, Cult.	2000.0600
Papaver alpinum spp. *rheaticum*	*Meconella*	J-150	MBG, Origin unknown, DNA	No accession information
Papaver alpinum spp. *alpinum*	*Meconella*	J-102	NBG, Bergomense, Italy, Cult.	2000.0568
Papaver anomalum	*Meconella*	J-106	NBG, Origin unknown, Cult.	2000.1321
Papaver anonalum 'album'	*Meconella*	J-078	NBG, CS, Cult.	2000.1806 (T&M seed suppliers)
Papaver apulum	*Argemonidium*	J-084	NBG, Czech Republic, Cult.	2000.0601
Papaver argemone	*Argemonidium*	J-153	NBG, Origin unknown, Cult.	No accession information
Papaver argemone	*Argemonidium*	J-165	UCBG, Origin unknown, Cult.	2000.1717, E4823-0025
Papaver armeniacum ssp. *armeniacum*	*Meconidium*	J-154	NBG, Origin unknown, Cult.	Prof. K
Papaver armeniacum ssp. *armeniacum*	*Meconidium*	J-095	NBG, Czech republic, Cult.	2000.0604
Papaver armeniacum ssp. *armeniacum*	*Meconidium*	J-087	NBG, Turkey, Cult.	2000.0651, IPK 507 182
Papaver armeniacum ssp. *armeniacum*	*Meconidium*	J-082	NBG, Czech Republic, Cult.	2000.0795, Brno 450
Papaver armeniacum ssp. *armeniacum*	*Meconidium*	J-103	NBG, Czech Republic, Cult.	2000.0793, Brno 446
Papaver armeniacum ssp. *armeniacum*	*Meconidium*	J-109	NBG, Czech Republic, Cult.	2000.0608, Czech 101
Papaver armeniacum ssp. *armeniacum*	*Meconidium*	J-115	CPG, Origin unknown, Cult.	No accession information
Papaver atlanticum	*Pseudopilosa*	J-092	NBG, Czech Republic, Cult.	2000.0603.
Papaver atlanticum	*Pseudopilosa*	J-005	TCD BG, Origin unknown Cult.	No accession information
Papaver atlanticum	*Pseudopilosa*	J-163	MBG, Origin unknown, Cult.	No accession information
Papaver atlanticum	*Pseudopilosa*	J-156	MBG, Origin unknown, Cult.	No accession information
Papaver atlanticum	*Pseudopilosa*	J-178	RBGE, Origin unknown, Herb.	19820128A
Papaver bracteatum	*Oxytona*	J-022	SOPG, Iran, Cult.	ARYA II, PI.381605
Papaver bracteatum	*Oxytona*	J-028	SOPG, Iran, Cult.	ARYA II, PI.381605
Papaver bracteatum	*Oxytona*	J-031	SOPG, Iran, Cult.	ARYA II, PI.381605
Papaver bracteatum	*Oxytona*	J-073	SOPG, Origin Unknown, Cult.	No accession information
Papaver bracteatum var. goliath	*Oxytona*	J-123	NBG, Origin unknown, Cult.	2000.14445
Papaver bracteatum	*Oxytona*	J-157	MBG, Origin unknown, Cult.	No accession information
Papaver californicum	*Californicum*	J-170	MBG, California, U.S.A., Cult.	No accession information
Papaver croceum	*Meconella*	J-015	NBG, Origin Unknown, Cult.	1999.00340
Papaver croceum	*Meconella*	J-104	SOPG, CS, Cult.	T&M seed suppliers

Table 2.1 *continued*

Genus and species	Section	Accession number	Material Type	Accession Information (Botanic Gardens)
Papaver croceum	*Meconella*	J-105	NBG, Origin Unknown, Cult.	2000.0653
Papaver commutatum	*Rhoeadium*	J-101	NBG, Origin Unknown, Cult.	2000.0605
Papaver commutatum	*Rhoeadium*	J-161	UCBG, Origin Unknown, Cult.	2000.1703, S0000-0020
Papaver dubium ssp. *dubium*	*Rhoeadium*	J-024	Dublin, Ireland	Wild material
Papaver dubium ssp. *dubium*	*Rhoeadium*	J-162	UCBG, Origin Unknown, Cult.	2000.1704
Papaver dubium ssp. *dubium*	*Rhoeadium*	J-164	NBG, Origin Unknown, Cult.	2000.1706
Papaver dubium ssp. lecoquii	*Rhoeadium*	J-174	NBG, Origin Unknown, Cult.	2000.0606
Papaver glaucum	*Papaver*	J-089	NBG, Czech Republic, Cult.	2000.0609, Czech 102
Papaver glaucum	*Papaver*	J-177	MBG, Czech Republic, Cult.	No accession information
Papaver glaucum	*Papaver*	J-189	SOPG, Origin unknown, CS	949D Chiltern Seeds
Papaver hybridum	*Argemonidium*	J-167	UCBG, Origin Unknown, Cult.	1999.1723, E4823-0005
Papaver lateritium	*Pseudopilosa*	J-116	CPG, Origin unknown, Cult.	No accession information
Papaver lateritium	*Pseudopilosa*	J-181	RBGE, Origin Unknown, Herb.	19520333A
Papaver litwinowii	*Rhoeadium*	J-168	NBG, Czech Republic, Cult.	2000.0610
Papaver miyabeanum	*Meconella*	J-019	NBG, Origin unknown, Cult.	1999.0339
Papaver miyabeanum	*Meconella*	J-186	SOPG, CS, Cult.	951Y Chiltern Seeds
Papaver macrostomum	*Carinatae*	J-159	RBGE, Iran, Herb.	Herbarium no.34182
Papaver macrostomum	*Carinatae*	J-160	RBGE, Iran, Herb.	Herbarium no.34139
Papaver nudicaule ssp. *nudicaule*	*Meconella*	J-086	NBG, Origin unknown, Cult.	2000.0598. Kiel 698
Papaver orientale	*Oxytona*	J-035	SOPG, Turkey, Cult.	No accession information
Papaver orientale	*Oxytona*	J-135	NBG, Origin unknown, Cult.	No accession information
Papaver orientale	*Oxytona*	J-155	MBG, Origin unknown, Cult.	No accession information
Papaver pavonium ssp. *pavonium*	*Argemonidium*	J-138	MBG, Origin unknown, Cult.	No accession information
Papaver pilosum ssp. *strictum*	*Pilosa*	J-081	NBG, Turkey, Cult.	2000.0768
Papaver pilosum ssp. *pilosum*	*Pilosa*	J-182	MBG, Origin unknown, Cult.	No accession information
Papaver pilosum ssp. *pilosum*	*Pilosa*	J-193	SOPG, CS, Cult.	Chiltern Seeds
Papaver pilosum ssp. *spicatum*	*Pilosa*	J-180	RBGE, Origin undetermined, Herb.	19772119A
Papaver pseudo-orientale	*Oxytona*	J-014	TCD BG, Origin unknown Cult.	No accession information
Papaver pseudo-orientale	*Oxytona*	J-039	NBG, Turkey, Cult.	1999.0341
Papaver pseudo-orientale var. salmon queen	*Oxytona*	J-040	SOPG, Origin unknown, Cult.	No accession information
Papaver pseudo-orientale	*Oxytona*	J-076	NBG, Turkey, Cult.	2000.0794. Brno 449
Papaver pseudo-orientale	*Oxytona*	J-080	NBG, Turkey, Cult.	2000.0770
Papaver pseudo-orientale	*Oxytona*	J-093	NBG, Turkey, Cult.	2000.0632. Jena 4009
Papaver pseudo-orientale	*Oxytona*	J-137	SOPG, Origin unknown, Cult.	No accession information
Papaver pseudo-orientale	*Oxytona*	J-139	NBG, Turkey, Cult.	2000.0794

Table 2.1 *continued*

Genus and species	Section	Accession number	Material Type	Accession Information (Botanic Gardens)
Papaver radicatum spp subglabosum	*Meconella*	J-094	NBG, Origin unknown, Cult.	2000.0769
Papaver rhoeas var. cedric morris	*Rhoeadium*	J-090	NBG, CS, Cult.	T&M seeds
Papaver rhoeas	*Rhoeadium*	J-147	MBG, Origin unknown, DNA	No accession information
Papaver rhoeas	*Rhoeadium*	J-175	UCBG, Denmark, Cult.	2000.1708
Papaver rupifragum	*Pseudopilosa*	J-017	NBG, Origin unknown, Cult.	1999.0342
Papaver rupifragum	*Pseudopilosa*	J-099	NBG, Origin unknown, Cult.	2000.0615
Papaver rupifragum	*Pseudopilosa*	J-196	SOPG, CS, Cult.	951C Chiltern Seeds
Papaver somniferum ssp. *setigerum*	*Papaver*	J-016	TCD BG, Origin unknown Cult.	No accession information
Papaver somniferum ssp. *somniferum*	*Papaver*	J-068	NBG, Origin unknown, Cult.	No accession information
Papaver somniferum ssp. *somniferum*	*Papaver*	J-126	SOPG, Slovenia, Cult.	69/1 genebank med plant
Papaver somniferum ssp. *somniferum*	*Papaver*	J-142	SOPG, Hungary, Cult.	69/5 1 Lekduna
Papaver somniferum ssp. *proliferum*	*Papaver*	J-166	MBG, Origin unknown, Cult.	No accession information
Papaver somniferum var. *lacinatum*	*Papaver*	J-176	SOPG, CS, Cult.	Chiltern Seeds
Papaver somniferum	*Papaver*	J-130	MBG, Origin unknown, Cult.	No accession information
Papaver somniferum	*Papaver*	J-021	SOPG, Turkey, Cult.	No accession information
Argemone mexicana		J-191	SOPG, U.S.A., Cult	No accession information
Eschscholzia californica		J-012	TCD BG, Origin unknown Cult.	No accession information
Meconopsis betonicifolia		J-032	TCD BG, Origin unknown Cult.	1998.0451
Meconopsis cambrica		J-001	TCD BG, Origin unknown Cult.	No accession information
Meconopsis cambrica		J-048	NBG, Origin unknown, Cult.	1992.0611
Roemeria refracta		J-171	TCD, Origin unknown, Herb	No accession information
Romneya coulterii		J-009	TCD BG, Origin unknown Cult.	No accession information
Stylomecon heterophylla		J-183	SOPG, U.S.A., Cult	No accession information

(Taberlet *et al.*, 1991). Sequences of all primers used are shown in Table 2.2. The PCR reaction mix was placed in the thermal cycler and subjected to a series of cycles with varying temperature treatments (thermal cycling). Successive cycles promote the denature and renature of DNA and in turn the exponential amplification of the target DNA (Mullis & Faloona, 1987). Denaturation was generally at 94°C. Annealing of primers to complementary regions of the template DNA was at 50-55°C and was followed by extension at 72°C, where a transcribed copy of the target sequence was generated between the primers in the 5'-3' direction. Extension was catalysed by *Taq* polymerase (©Promega Inc.), which is the recombinant and modified form of the *Thermus aquaticus* YT11 DNA polymerase gene expressed in *Escherischi coli*.

Table 2.2 Primer sequences used in this study.

Primer	**Nucleotide sequence**	**Source**
ab101	5'-ACGAATTCATGGTCCGGTGAAGTGTTC-3'	Sun *et al.*, 1994
ab102	5'-TAGAATTCCCCGGTTCGCTCGCCGTTA-3'	Sun *et al.*, 1994
ITS-2	5'-GCTGCGTTCTTCATCGATGC-3'	Baldwin *et al.*, 1995
ITS-3	5'-GCATCGATGAAGAACGCAGC-3'	Baldwin *et al.*, 1995
trnL-F primer 'c'	5'-CGAAATCGGTAGACGCTACG-3'	Taberlet *et al.*, 1991
trnL-F primer 'f'	5'-ATTTGAACTGGTGACACGAG-3'	Taberlet *et al.*, 1991
trnL-F primer 'd'	5'-GGGGATAGAGGGACTTGAAC-3'	Taberlet *et al.*, 1991
trnL-F primer 'e'	5'-GGTTCAAGTCCCTCTACTCC-3'	Taberlet *et al.*, 1991

2.3.5 Optimisation of PCR conditions

Successful PCRs rely on the optimisation of PCR reaction mix concentrations, PCR thermal cycling temperatures, reagent concentrations and cycling temperatures. Optimal reagent concentrations and temperatures, found by experimentation (Carolan – see below) are given in table 2.3. Difficulties were encountered when attempting to amplify the target regions from ITS. Initially many bands were produced when only one was expected and this was most likely due to the low specificity of the primers, resulting in the amplification of non-target sequences. Annealing temperatures were

increased by 1°C increments from 50°C until only single bands were obtained (55°C). Successful amplification and sequencing reactions were obtained when annealing temperatures were gradually decreased from 56°C to 54°C to 52°C over the first 3 cycles. The initial high annealing temperature allowed only target sequences to be amplified in the first cycle and the subsequent drops in annealing temperate allowed for more productive amplification of the target DNA from the first few cycles. The remaining cycles used an annealing temperature of 52°C. PCR reaction mix components and thermal cycling protocol for the ITS region are given as Table 2.3 (reagent concentrations, protocol and materials are detailed in Appendix 1.4). Little difficulty was encountered when attempting to amplify the *trnL-F* region and the reaction mix components and thermal cycling protocol for the *trnL-F* region are given as Table 2.4).

2.3.6 Nucleotide sequencing

Cycle sequence reactions (Sanger *et al.*, 1977) were carried out on the amplified PCR products, using fluorescently-labelled dye terminators and carried out on an MJ Research PTC-200 DNA Engine. The BigDye terminators (ABI PRISM ® BigDye Terminators) are dideoxynucleotide triphosphates (ddNTPs) linked to an energy transfer dye composed of a fluorescein donor and one of four d-rhodamine-based acceptors (Rosenblum *et al.*, 1997). The thermal cycle protocol and sequencing reaction mix are detailed in Table 2.5 and a complete protocol including materials and reagent components and concentrations is detailed in Appendix 1.5. Products obtained from the cycle sequence reaction were purified using an ethanol precipitation technique (reagent concentrations, protocol and materials given in Appendix 1.6). The cycle sequence products were combined with 50µl of 100% ethanol and 2µl of sodium acetate in a 0.5ml tube and were subjected to a number of multiple centrifugation and drying steps to pellet the products. Purification removes unincorporated dye-labelled terminators and other unwanted reaction components. If not removed prior to

Table 2.3. PCR reaction mix components and thermal cycling protocol for the ITS region.
*Concentrations are outlined in Appendix 1.4

PCR Reaction Mix

	ITS Mix	
Component	**Volume (µl)**	**Concentration (or amount)**
Sterile ultrapure H_2O	29.25	
Promega Buffer (10x)	5.0	1x
DNTPs (10mM)	1.0	0.2mM
Forward primer 100ng/µl (e.g. ab101)	0.5	50ng
Reverse primer 100ng/µl (e.g. ab102)	0.5	50ng
$MgCl_2$ 25mM	3.0	1.5mM
Taq polymerase (5 units/µl)	0.25	1.25 units
Template DNA	0.5	100-300ng
Betaine	10.0	
Total volume	50.0	

PCR Thermal Cycling Protocol

	Cycle Phase	Temperature (°C)	Duration (minutes)
Cycle 1	1. Denaturing	97	1
	2. Annealing	56	1
	3. Extension	72	3
Cycle 2	1. Denaturing	97	1
	2. Annealing	55	1
	3. Extension	72	3
Cycle 3	1. Denaturing	97	1
	2. Annealing	54	1
	3. Extension	72	3
Standard cycle (28 Cycles)	1. Denaturing	97	1
	2. Annealing	52	1
	3. Extension	72	3
Final cycle	1. Denaturing	97	1
	2. Annealing	52	1
	3. Extension	72	3
	4. Soak	4	∞ (hold)

Table 2.4. PCR reaction mix components and thermal cycling protocol for the *trnL/trnF* region. *Concentrations are outlined in Appendix 1.4

PCR Reaction Mix

***trnL-trnF* Mix**

Component	Volume (µl)	Concentration (or amount)
Sterile ultrapure H_2O	39.25	
Promega Buffer (10x)*	5.0	1x
DNTPs (10mM)	1.0	0.2mM
Forward primer 100ng/µl (e.g. primer c)	0.5	50ng
Reverse primer 100ng/µl (e.g. primer f)	0.5	50ng
$MgCl_2$ 25mM	3.0	1.5mM
Taq polymerase (5 units/µl)	0.25	1.25 units
Template DNA	0.5	100-300ng

PCR Thermal Cycling Protocol

	Cycle Phase	Temperature (°C)	Duration (minutes)
Standard cycle	1. Denaturing	97	1
(32 Cycles)	2. Annealing	52	1
	3. Extension	72	3
Final cycle	1. Denaturing	97	1
	2. Annealing	52	1
	3. Extension	72	3
	4. Soak	4	∞(hold)

analysis, certain sequencing reaction components interfere with data collection. Pellets were suspended in 25µl template suppressant reagent (TSR, Applied Biosystems) and heated at 95°C for 6 minutes to denature the dsDNA. Samples were subsequently placed on ice, spun down and loaded onto the automated sequencer of Botany, T.C.D.). Forward and reverse strands were sequenced in separate reactions. When a completed sequencing reaction mix is run through a sequencing capillary a continuous stream of progressively longer (by one base) fragments pass through the polymer (Rosenblum *et al.*, 1997). The sequencer separates the labelled sequence

extension products using the polymer that is a molecular sieve matrix and identifies the labelled terminal nucleotide by its specific emission wavelength excited by a laser beam, producing a sequential banding pattern, which is read as a single copy of the original target DNA template.

Sequencing Analysis Software™ v3.7 was used to automatically process the raw sample files and to assign bases to matching sequence peaks. Forward and reverse strands were aligned and corrected using Sequencher™ version 3.1 (©Gene Codes Corporation, 1998) and a contiguous sequence for the target DNA region obtained. Consensus sequences for all accessions were imported into PAUP version 4.0b 10(Swofford, 2003) where sequences were aligned by inserting gaps manually within

Table 2.5. Sequencing reaction mix components and thermal cycling protocol. *Concentrations are outlined in Appendix 1.5

Cycle Sequence Reaction Mix

Component	Volume (µl)
Sterile ultrapure H_2O	1.8
Sequencing buffer *	3.5
Primer 5ng/µl	0.7
Applied Biosystems Dye Terminator mix	1.0
Template DNA (c. 500ng/ µl)	2.0
Total	10.0

Thermal Cycling Protocol

	Cycle Phase	Temperature (°C)	Duration (minutes or seconds)
Standard cycle	1. Denaturing	97	0:10
(24 Cycles)	2. Annealing	52	0:05
	3. Extension	72	4:00
Final cycle	1. Denaturing	97	0:10
	2. Annealing	52	0:05
	3. Extension	72	4:00
	4. Soak	4	∞ (hold)

the data matrix. The resulting matrix was then subjected to maximum parsimony (MP) using PAUP to provide phylogenetic trees (as outlined in section 2.4.2 and 2.4.6).

The handling of gaps and indels has produced much debate and they are generally totally discarded or totally included from the resulting analysis. Many authors have proposed methods for handling indels (Swofford, 1993; Baldwin *et al.*, 1995; Hibbett *et al.*, 1995; Kjer, 1995; Flores-Villela *et al.*, 2000; Kjer *et al.*, 2001). By meticulously editing the electropherograms base by base a number of incorrectly called bases were identified and corrected. Where alignment was questionable every polymorphic nucleotide within the final aligned matrix was checked against the original electropherogram to ensure that they were correct. Any sequence with large amounts of heterogeneity or that were of poor quality were repeated or omitted from the final analysis. Regions of the sequence alignment that contained a substantial number of alignment gaps were omitted from the analyses, as the positional homology within these regions is generally uncertain (Swofford *et al.*, 1996).

2.4 Results

2.4.1 The ITS dataset

The length of ITS-1, ITS-2 and the 5.8S gene were confirmed using a comparative alignment of *Papaver rhoeas* ITS-1, ITS-2 and the 5.8s gene regions obtained from GenBank and the NCBI website (Schwarzbach & Kadereit, 1999; accession AF098920). The final aligned matrix had a total length of 668 characters (not including outgroups and accessions outside *Papaver*; Table 2.6). Of the total matrix, 307 characters were variable and 208 were considered phylogenetically informative (Table 2.6). Little variation was encountered within the 5.8s region, with only 9 of the 163 nucleotides (representing 3.2%) being variable and parsimony informative. The ITS-1 and ITS-2 non-coding regions ranged in length from 249 to 261bp and 243 to 254bp respectively. A considerable proportion of both regions were variable. Of the 150 variable sites within ITS-1 (representing 54.6% of the ITS-1 region), 111 were parsimony informative. The 131 variable sites found within ITS-2 (representing 52.4% of the ITS-2 region) consisted of 88 parsimony informative characters. The

G+C content of both ITS-1 and ITS-2 ranged from 50.5 to 60.2% and 55.3 to 63.8% respectively. Independent analysis of the ITS-1 and ITS-2 regions yielded broadly congruent cladograms (results not shown) and for the purposes of this study both regions were combined for parsimony analysis.

Table 2.6. Sequence characteristics of the 5.8s gene region and the ITS-1 and ITS-2 non-coding regions for *Papaver* accessions sequenced in this study.

	ITS-1	5.8s	ITS-2	Combined ITS-1, 5.8S and ITS-2 regions
Length range (bp)	249-261	162-163	243-254	654-678
Aligned length (bp)	275	163	250	688
G + C content range (%)	50.5-60.2	51.5-56.5	55.3-63.8	52.4-60.2
Number of variable sites	150 (54.6%)	26 (16.0%)	131 (52.4%)	307
Number of potentially informative sites	111 (40.4%)	9 (3.2%)	88 (35.2%)	208
Number of constant sites	125 (45.4%)	137 (84.0%)	119 (47.6%)	381
Number of autapomorphic sites	39 (14.2%)	17 (10.4%)	43 (17.2%)	99
Number of indels	14	1	21	36
Indel size range (bp)	1-6	1	1-6	1-6

2.4.2 Parsimony analysis of ITS data set

Maximum parsimony analyses of the final matrix were performed using the heuristic search algorithms of PAUP with 1000 replicates of random addition sequence (holding 25 trees at each replication) and with tree bisection reconnection (TBR) branch swapping on multiple trees (multrees on). Gaps were treated as missing data. With respect to the ITS analysis a region of the matrix with questionable alignment corresponding to positions 311-324 base pairs was omitted from the analysis due to the high number of alignment gaps within this region.

Parsimony analysis of the matrix (including outgroups) generated 453 equally most parsimonious trees of 972 steps with a consistency index (CI) of 0.548 and a retention index (RI) of 0.799. Clade support was examined using 1000 bootstrap replicates (Felsenstein 1985) of a heuristic search with 100 replicates of TBR branch swapping on multiple trees. One randomly selected tree is shown here as a cladogram (Figure 2.3). All branches were present in the strict consensus tree. To aid in the

visualisation of clades within the trees and to better illustrate branch lengths, they were also displayed as a phylograms (Figure 2.4). Cladograms convey only branching information, while phylograms draw branch lengths proportional to the number of inferred changes according to the current reconstruction (Swofford & Begle, 1993).

In total 74 accessions representing 39 species were used with all sections of *Papaver* represented. Additional phylogenetic information was obtained by the inclusion of a number of representatives from outside the genus but from within Papaveroideae. These were included on the basis that a number of sections from within *Papaver* share significant morphological and phytochemical characteristics with members of different genera (Kadereit *et al.*, 1997).

Three distinct clades are found within the ITS tree. Clade 1 (Figure 2.4) consists of section *Argemonidium*, which resolves sister to all the other sections of *Papaver* in all equally most parsimonious trees. The grouping of *Argemonidium* has 100% bootstrap support. The single representative of *Roemeria* (*Roemeria refracta*), groups with the species of *Argemonidium* and all representatives within this clade are characterised by having a diagnostic nucleotide sequence (at position 82-90) of TCTTTTTG, instead of –CCCCTTG, which is found in all other species of the genus including the representatives of *Meconopsis* and *Stylomecon heterophylla*. Clade 2 comprises section *Meconella* and is well supported (94% bootstrap support). In addition all representatives of this section possess a three base-pairs deletion at position 245-248 of the final aligned matrix with respect to the other sections and species of *Papaver*. *Meconopsis betonicifolia* sister to clade 3 in all equally parsimonious trees, but with less than 50% bootstrap support. Clade 3 (76% bootstrap support) comprises the remaining sections of *Papaver* including *Meconopsis cambrica* and *Stylomecon heterophylla*. A well-supported group (94%) within clade 3, comprising of sections *Meconidium, Oxytona, Papaver, Pilosa, Pseudopilosa Rhoeadium, Carinatae* and the species *Meconopsis cambrica* is present within all equally parsimonious trees indicating that these six sections including *Meconopsis cambrica* are a monophyletic group within *Papaver*. Sections *Papaver* and *Rhoeadium* do not resolve as monophyletic when examined alone, as indicated by the grouping of *Papaver glaucum* (section *Papaver*) with representatives of section *Rhoeadium* (including *Carinatae*, 61% bootstrap support) and the grouping of *P. litwinowii* with the

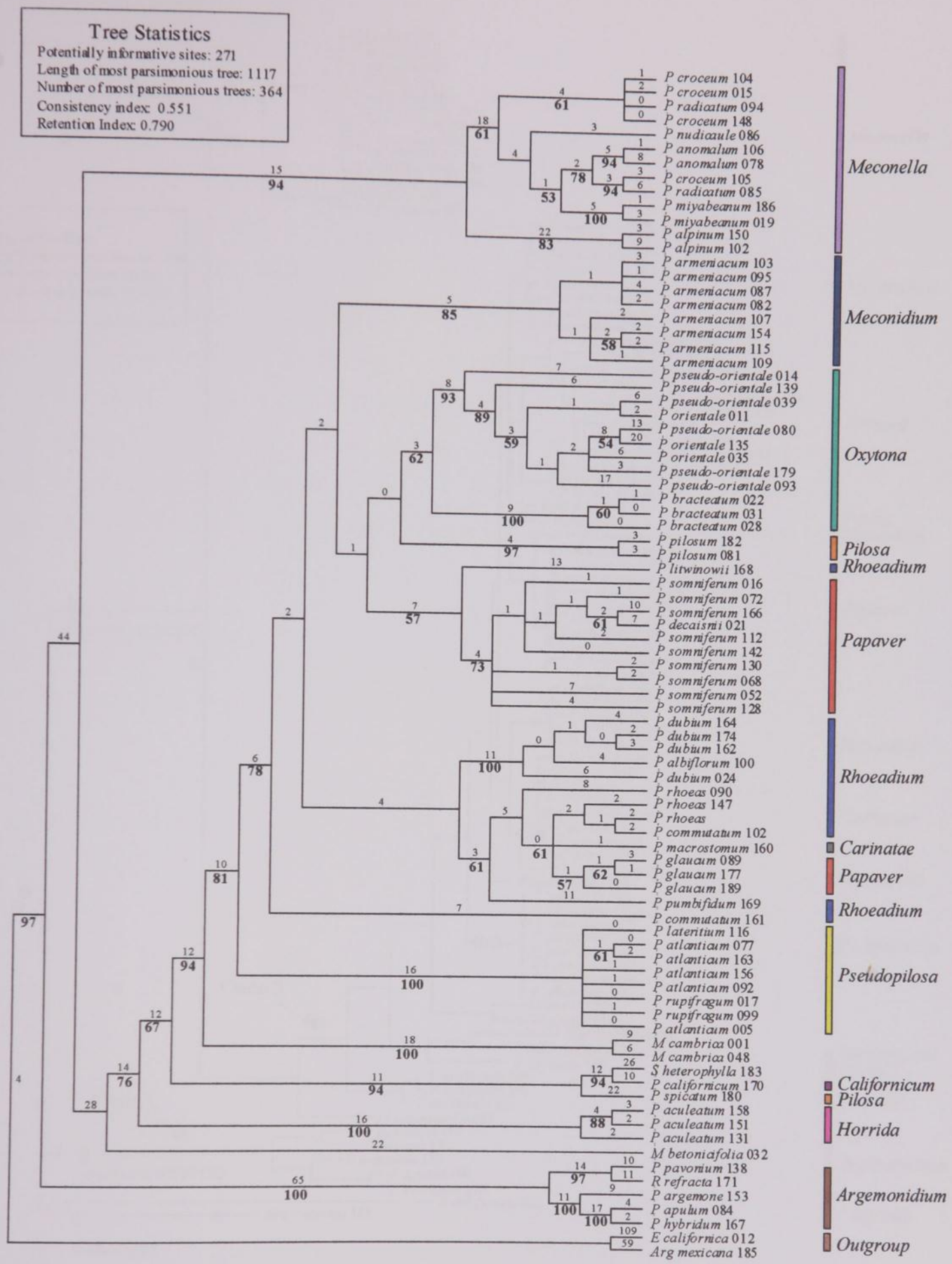


Figure 2.3 1 of 364 equally most parsimonious trees (shown as a cladogram) generated from the ITS data-set using maximum parsimony with heuristic search options. Support for each node is represented by bootstrap values (%) below the branch (shown only when >50%). Numbers above branches indicate the numbers of character changes along each lineage. All branches appeared in the strict consensus tree.

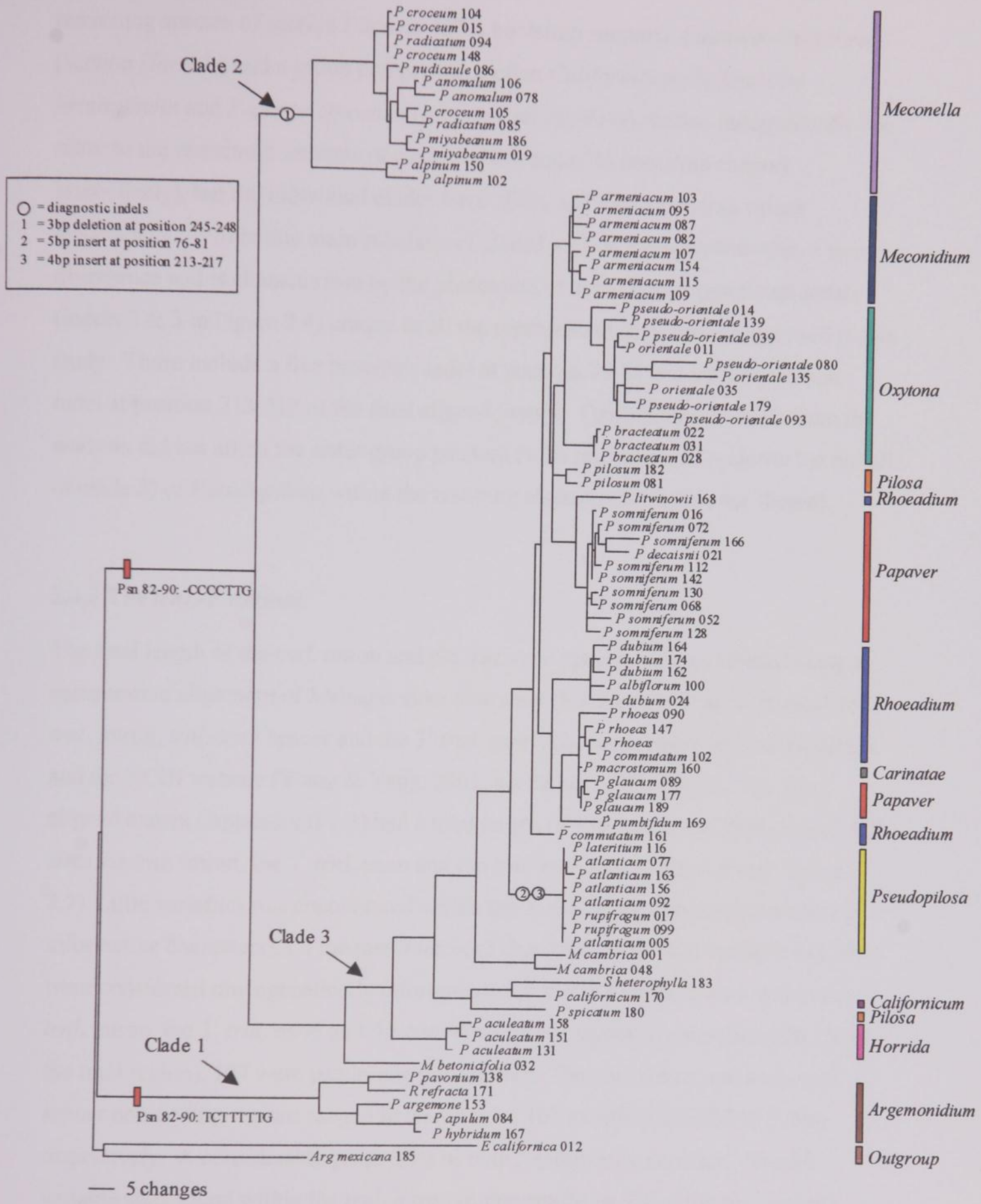


Figure 2.4 1 of 364 equally most parsimonious trees (shown as a phylogram) generated from the ITS data-set using maximum parsimony with heuristic search options (same tree as in Figure 2.3). Three well resolved clades separate within the phylogram (discussed in text) indicating the suitability of ITS regions for phylogenetic inference within *Papaver*. Diagnostic indels (circles) and some substantial sequence differences (red bars) are shown.

remaining species of section *Papaver* (57% bootstrap support). *Papaver aculeatum* (section *Horrida*) and a group comprising section *Californicum*, *Stylomecon heterophylla* and *Papaver spicatum* (section *Pseudopilosa*) resolve independently but sister to the remaining sections of clade 3 (76% and 67% bootstrap support respectively), but the individual clades have 100% and 98% bootstrap values respectively. Within this main subclade of clade3 section *Pseudopilosa* shows most divergence and is characterised by the possession of a number of significant indels (indels 2 & 3 in Figure 2.4) unique to all the representatives of this section used in this study. These include a five base-pair indel at position 76-81 and a four base-pair indel at position 213-217 of the final aligned matrix. Omitting these indels from the analysis did not affect the sister group position (with respect to the majority but not all of clade 3) of *Pseudopilosa* within the resulting cladograms (results not shown).

2.4.3 The *trnL-F* dataset

The total length of the *trnL* intron and the *trnL-trnF* spacer, were confirmed using a comparative alignment of *Menispermum dauricum* (Menispermaceae; Ranunculales), *trnL* intron, *trnL-trnF* spacer and the 3' *trnL* exon sequences obtained from GenBank and the NCBI website (Wang & Yang, 2001; accession AF335293.1). The final aligned matrix (Appendix II 1.3) had a total length of 936 characters (509, 50 and 377 sites for *trnL* intron, the 3' *trnL* exon and the *trnL-trnF* spacer respectively; Table 2.7). Little variation was encountered within the 3' *trnL* exon region (no parsimony informative characters). Of the total matrix of characters, 307 were variable and 208 were considered phylogenetically informative. Of the 188 variable sites within the *trnL* intron, the 3' *trnL* exon and the *trnL-trnF* spacer regions (representing 20.1% of the total region), 107 were parsimony informative. The *trnL* intron and *trnL-trnF* spacer non-coding regions ranged in length from 467 to 499bp and 353 to 375bp respectively. A considerable proportion of both regions were constant. The 86 variable sites found within the *trnL* intron (representing 16.9% of the *trnL* intron) consisted of 45 parsimony informative characters and the *trnL-trnF* spacer contained 100 variable characters (representing 26.5%) of which 63 were parsimony informative. The G+C content of both the *trnL* intron and *trnL-trnF* spacer ranged from 32 to 36.5% and 33.6 to 41.4% respectively. Independent analysis of the *trnL* intron and the *trnL-trnF* spacer regions yielded congruent cladograms (results not

shown) and for the purposes of this study both regions were combined for parsimony analysis. Furthermore these are part of the non-recombining plastid genome and so should have the same phylogenetic history and are frequently combined for phylogenetic reconstruction (e.g. Hopper *et al.*, 1999; Chase *et al.*, 2000; Hodkinson *et al.*, 2002).

Table 2.7. Sequence characteristics of the *trnL* intron, *trnL-trnF* spacer, and *trn L- trnL/trnF* combined non-coding regions sequenced in this study

	***trnL* intron**	***trnL* exon**	***trnL-trnF* spacer**	**Combined (*trn L* intron + *trnL/trnF* spacer)**
Length range (bp)	467-499	50	353-375	870-924
Aligned length (bp)	509	50	377	936
G + C content (%)	32-36.5	44-48	33.6-41.4	49.2
Number of variable sites	86 (16.9%)	2 (4%)	100 (26.5%)	188
Number of potentially informative sites	45 (8.8%)	0 (0%)	63 (16.7%)	107
Number of constant sites	423 (83.1%)	48 (96%)	277 (73.3%)	748
Number of autapomorphic sites	41 (8.1%)	2 (4%)	37 (9.8%)	80
Number of indels	13	0	9	22
Indel size range (bp)	1-28	0	1-9	1-28

2.4.4 Parsimony analysis of *trnL-F* data set

Maximum parsimony analyses of the final matrix were performed using the heuristic search algorithms of PAUP, for 1000 random addition sequence replicates, retaining 25 trees per replicate to reduce time spent swapping on large numbers of trees with bisection reconnection (TBR) branch swapping on all trees (multrees on). Gaps were treated as missing data. A region of the matrix with questionable alignment corresponding to positions 606-642 base pairs was omitted from the analysis due to the high number of alignment gaps within this region. Parsimony analysis of the matrix (including outgroup) generated 489 equally parsimonious trees of 407 steps with a consistency index (CI) of 0.713 and a retention index (RI) of 0.891. Clade

support was examined using 1000 bootstrap replicates of a heuristic search with 100 replicates of TBR branch swapping on multiple trees. One randomly selected tree is shown as a cladogram (Figure 2.5) and a phylogram (Figure 2.6).

As with the ITS analysis, the separation of sections *Meconella* (Clade 1, 86% bootstrap support) and *Argemonidium* (Clade 2, 80% bootstrap support) from the main group containing the remaining sections of *Papaver* is evident from the cladograms produced from the *trnL-F* region. Both sections are well supported with 100% bootstrap values. *Meconopsis betonicifolia* forms a well-supported group (86% bootstrap values) with representatives of section *Meconella.* Members of section *Meconella* possessed a characteristic 4 base-pair insert at position 248, which is also present in the outlying sections, such as *Californicum, Horrida* and *Meconopsis betonicifolia*. This insert was also present in *Papaver spicatum* (accession 180; section *Pilosa*). An additional characteristic only found within the members of *Meconella* was a TTTTTA sequence at positions 747-752 base pairs. All other members of clade 3 had the nucleotide sequence TATATA at this particular region. The separation of section *Argemonidium* is well characterised by a number of indels specific to these species including a 5 base-pair deletion at position 255-260 and a 2bp insertion at position 709-711. The polymorphism described at positions 747-752 is also evident within *Argemonidium* with these species (including *Stylomecon heterophylla*) having the nucleotide sequence GCGATA at this particular locus (Figure 2.6). *Papaver apulum* and *P. hybridum* share a 10 base pair deletion at position 642-652, which is not found in *P. pavonium.*

The remainder of the sections form a poorly supported clade (clade 3; 68% bootstrap support). Sections *Horrida*, *Californicum* and *Meconopsis cambrica* resolve independently but sister to the main group with similar topologies as found in the ITS trees. Within clade 3 a polytomy exists comprising sections *Carinatae, Meconidium, Oxytona, Papaver, Pilosa, Pseudo-pilosa* and *Rhoeadium* (71% bootstrap support). Section *Oxytona* groups with section *Meconidium,* with section *Pilosa* resolving as sister to these (in all parsimonious trees but with no bootstrap support). Section *Pseudopilosa* is a well defined monophyletic group (96% bootstrap support). This

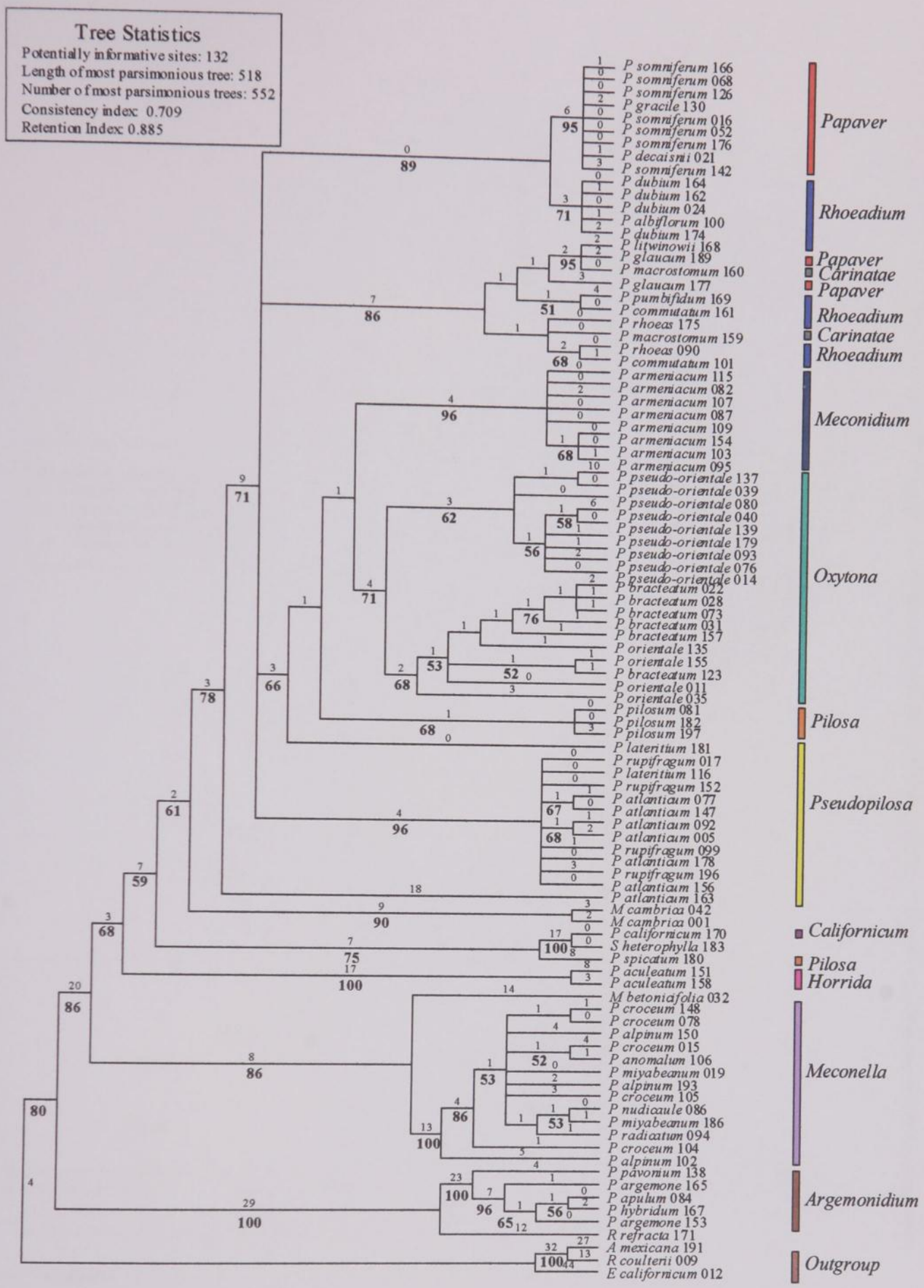


Figure 2.5 1 of 552 equally most parsimonious trees (shown as a cladogram) generated from the *trnL-F* data-set using maximum parsimony with heuristic search options. Support for each node is represented by bootstrap values (%) below the branch (shown only when >50%). Numbers above branches indicate the numbers of character changes along each lineage. All branches appeared in the strict consensus tree.

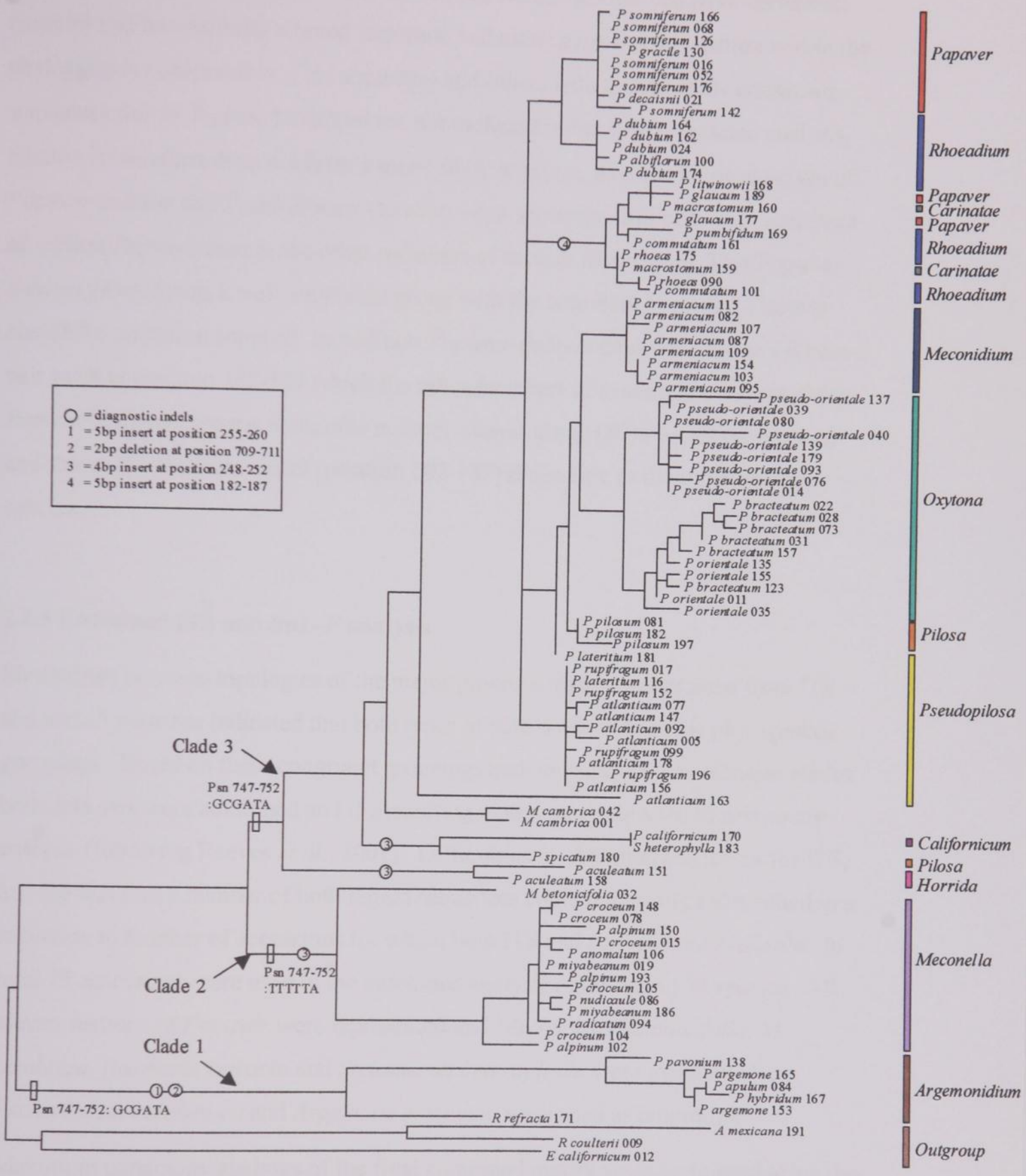


Figure 2.6 1 of 552 equally parsimonious trees (shown as a phylogram) generated from the *trnL-F* data-set in using maximum parsimony with heuristic search options (same tree as in Figure 2.5). Three well resolved clades separate within the phylogram (discussed in text) indicating the suitability of *trnL-F* regions for phylogenetic inference within *Papaver*. Diagnostic indels and some substantial sequence differences are shown.

however excludes accession 181 (*P. lateritium*) which was derived from herbarium material and the resulting aligned sequence had many gaps and its position within the phylogram is questionable. This accession and others with questionable consensus sequences due to degraded material are not included in the final combined analysis. Section *Rhoeadium* does not form a monophyletic group, with the representatives of *Papaver dubium* and *P. albiflorum* showing more sequence similarity with members of section *Papaver* than to the other members of section *Rhoeadium.* This *Papaver dubium* group forms a well-supported group with the members of section *Papaver* also (89% bootstrap support). In addition *Papaver dubium* does not possess a 6 base-pair indel at position 182-187 which the other members of section *Rhoeadium* share. *Papaver glaucum* groups within the main *P. rhoeas* group (86% bootstrap support) and shares the particular indel (position 182-187) diagnostic to these *Rhoeadium* species.

2.4.5 Combined ITS and *trnL-F* analysis

Similarities between topologies of the major groups within trees obtained from ITS and *trnL-F* matrices indicated that both types of data produced similar phylogenetic groupings. Based on these congruent groupings and bootstrap values of major clades both data sets were combined and the resulting matrix was subjected to parsimony analysis (following Reeves *et al.*, 2001). Difficulties in obtaining sequences for ITS, and the fact that a number of both region sequences could not be aligned resulted in a reduction in number of accessions for which both ITS and *trnL-F* were available. In total 75 accessions were used in the combined analysis representing 34 species. All eleven sections of *Papaver* were represented and *Meconopsis betonicifolia, M. cambrica, Roemeria refracta* and *Stylomecon heterophylla* were included also. *Eschscholzia californica* and *Argemone mexicana* were used as outgroups.

Maximum parsimony analyses of the final combined matrix were performed using the heuristic search algorithms of PAUP, for 1000 random addition sequence replicates, retaining 25 trees per replicate to reduce time spent swapping on large numbers of trees with tree bisection reconnection (TBR) branch swapping on all trees (multrees on). Gaps were treated as missing data. Parsimony analysis of the matrix (including outgroup) generated 488 equally most parsimonious trees of 1503 steps with a

consistency index (CI) of 0.601 and a retention index (RI) of 0.800. Clade support was examined using 1000 bootstrap replicates (Felsenstein 1985) of a heuristic search with 100 replicates of TBR branch swapping on multiple trees. One randomly selected tree is shown here as a cladogram (Figure 2.7).

The combination of the ITS and *trnL-F* data sets resulted in increased bootstrap supports for the majority of the groupings from the individual analyses. As with the previous analyses three clades are resolved. Clade 1 comprises section *Argemonidium* (including *Roemeria refracta*; 100% bootstrap support). The separation of *Argemonidium* from the remaining sections of *Papaver* is also well supported having 85% bootstrap support. Clade 2 comprises section *Meconella* (including *Meconopsis betonicifolia*) and bootstrap support for this grouping increased to 85%. This particular section is monophyletic within the genus as demonstrated by the 100% bootstrap value obtained in the combined analysis. Clade 3 (100% bootstrap support comprises the remaining sections of the genus including *Meconopsis cambrica* and *Stylomecon heterophylla* and support for this clade increased to 100% in comparison to 68 and 76% for this group in the ITS and *trnL-F* analysis respectively. Section *Horrida* is sister to the rest of clade 3 (66% bootstrap support). The single representative of section *Californicum* shares a close affinity with *Stylomecon heterophylla* (bootstrap support of 100%) and groups with *Papaver spicatum* (section *Pilosa*, 92% bootstrap support). DNA for this particular accession was supplied from a degraded herbarium specimen and until this grouping is confirmed using fresh material the positioning of this species should be ignored. Support for the positioning of *Meconopsis cambrica* and more importantly the separation from the other representative of *Meconopsis* also increased to 96% in comparison to the single gene trees. Within clade 3 the main group of sections including *Carinatae, Meconidium, Oxytona, Papaver, Pilosa, Pseudopilosa* and *Rhoeadium* is evident and well supported (87% bootstrap support). Of these, section *Pseudopilosa* shows most divergence and is monophyletic within *Papaver* with a 100% bootstrap value.

Sections *Meconidium* (100% bootstrap support), *Oxytona* (93% bootstrap support) and *Pilosa* (100% bootstrap support) form a weakly resolved clade (63% bootstrap support) within clade 3. The separation within section *Rhoeadium* is also evident with the division of the *Papaver dubium* group from the other representatives of *Rhoeadium*. An affinity exists between *Papaver dubium* and *Papaver albiflorum* with

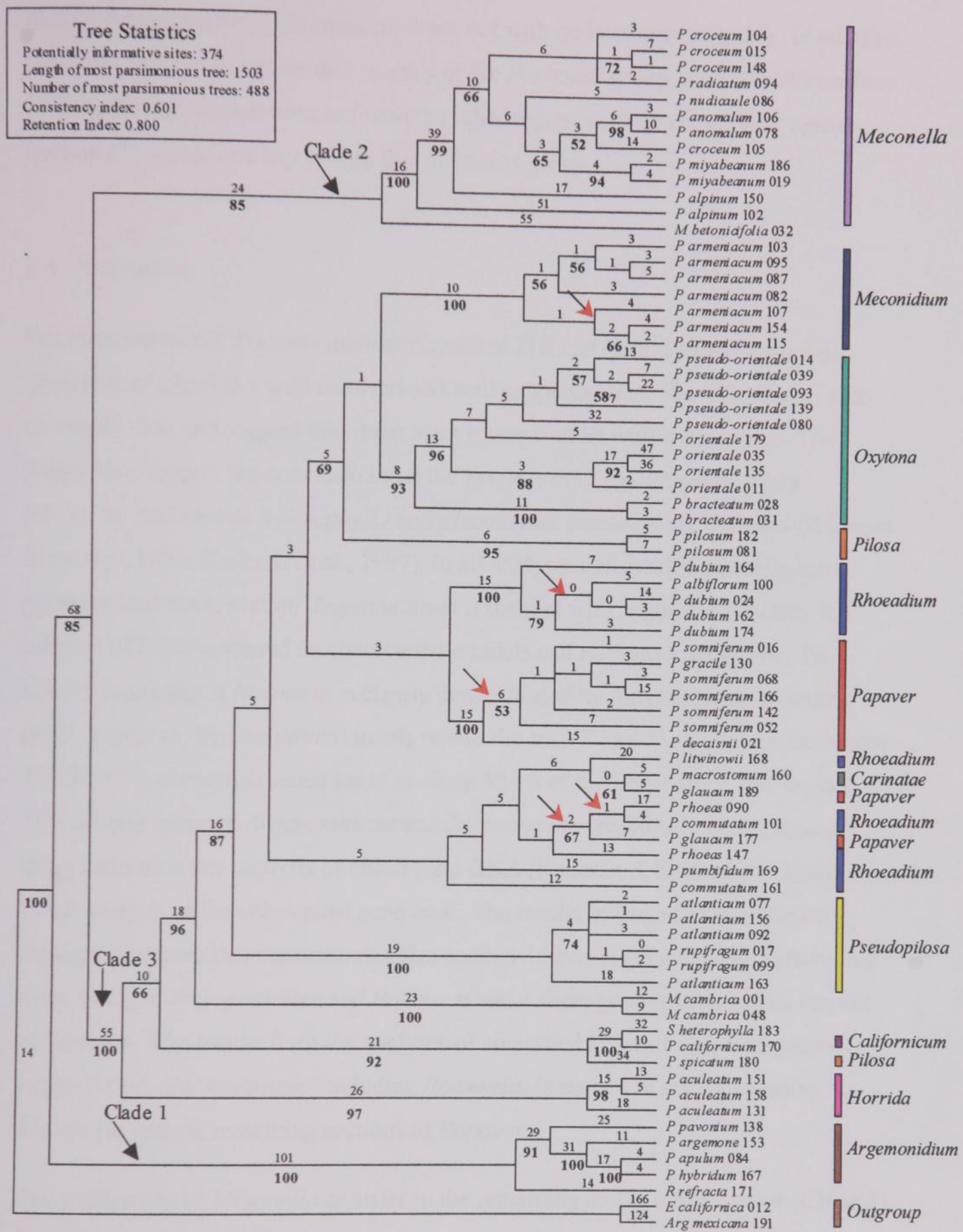


Figure 2.7 1 of 488 equally most parsimonious trees (shown as a cladogram) generated from the combined ITS and *trnL-F* data-sets using maximum parsimony with heuristic search options. Support for each node is represented by bootstrap values (%) below the branch (shown only when >50%). Numbers above branches indicate the numbers of character changes along each lineage. Branches that did not appear in the strict consensus tree are indicated by a red arrow head.

section *Papaver* (in all parsimonious trees but with no bootstrap support). In addition *Papaver glaucum* groups within species of the *P. rhoeas* group of section *Rhoeadium* (67% bootstrap support) and not with the other representatives of section *Papaver*. Section *Carinatae* resolves within this *P. rhoeas* group also.

2.4 Discussion

The combination of *Papaver* nuclear ribosomal ITS and plastid *trnL-F* nucleotide sequences resulted in a well resolved and well supported tree. The ITS, *trnL-F* and combined data sets suggest that three main lineages exist within the genus. The results also support the conclusion that the genus is only monophyletic when *Roemeria, Stylomecon heterophylla* and *Meconopsis cambrica* are included (Kadereit & Sytsma, 1992; Kadereit *et al.*, 1997). In all analyses and resulting equally most parsimonious trees, section *Argemonidium* is distinct with respect to the other ten sections of *Papaver* based on characteristic indels and nucleotide sequence. Its affinity to the genus *Roemeria* is clearly demonstrated by the possession of unique (with respect to *Argemonidium*) indels within the *trnL-F* region and the characteristic TCTTTTTG nucleotide sequence at position 82-90 of the ribosomal ITS-1 region. This affinity between *Argemonidium* and *Roemeria* was previously demonstrated using restriction site analysis of chloroplast DNA (Kadereit & Sytsma, 1992) and RFLP analysis of the chloroplast gene *trnK*. The results of these analyses yielded phylogenetic trees that demonstrated that section *Meconella* occupied a position as a sister group to *Argemonidium* and *Roemeria* and a sister group position with respect to *Papaver*. The results from the analyses of combined ITS and *trnL-F* sequences suggests that *Argemonidium* (including *Roemeria*) is sister to a group including *Meconella* and the remaining sections of *Papaver*.

The positioning of *Meconella* as sister to the remaining sections of *Papaver* (Clade 3) is well supported in the combined analysis (85% bootstrap support). The grouping of the Asian representative of *Meconopsis* (*Meconopsis betonicifolia*) with *Meconella* and not sister to all sections of *Papaver* (including *Argemonidium*) as demonstrated by previous molecular analyses (Kadereit *et al.*, 1997) is unexpected. However its position is justified not only by nucleotide similarities, such as the TTTTTTA nucleotide sequence at position 747-752 of the *trnL-F* region (found only in

Meconopsis betonicifolia and *Meconella*) but also by the possession of a characteristic 4 base-pair indel at position 248-252 of the same gene region shared by *Meconopsis betonicifolia* and representatives of section *Meconella, Californicum* and *Horrida*. Assuming that section *Meconella* represents a sister group to Clade 3, coupled with the outlying positioning of *Californicum* and *Horrida* within Clade 3, it may be postulated that these three sections represent relatively ancient lineages within *Papaver*, one that had a close affinity to Asian species of *Meconopsis*. Such phylogenetic interpretations are further expanded in Chapter 5 in light of morphological and phytochemical analysis.

Clade 3 comprises the majority of the sections of *Papaver* and receives 100% bootstrap support in the combined analysis. The independent sister group positions of *Californicum* and *Horrida* are congruent with previous molecular analysis (Kadereit & Sytsma, 1992; Kadereit *et al.*, 1997). The affinity between *Papaver californicum* and *Stylomecon heterophylla* is well supported (100% bootstrap support in the combined analysis). The results from the combined analysis and previous molecular analysis (Kadereit *et al.*, 1997), in combination with morphological and biogeographical similarities supports the view of Kadereit *et al.* (1997) that *Stylomecon* arose from within *Papaver*. DNA from *Papaver spicatum* (accession 180) was obtained from herbarium material and the resultant sequence had many gaps in the final aligned matrix. Due to this its position within the phylogenetics trees (and trees used in later chapters) should be ignored.

The European representative of *Meconopsis, Meconopsis cambrica,* occupies a well-supported (96% in the combined analyses) sister group position to the remaining sections of *Papaver* (within Clade 3). This position and its separation from Asian *Meconopsis* have been previously demonstrated from a molecular point of view (Kadereit *et al.* 1997) and our results further support the view that two distinct lineages within *Meconopsis* s.l. exist. However it would be inappropriate to draw further conclusions about the infrageneric relationships of *Meconopsis* unless further representatives of this genus were added to the analysis.

Within clade 3 lies a well supported (87% bootstrap support in the combined analysis) group of sections including *Carinatae, Meconidium, Oxytona, Papaver, Pilosa, Pseudopilosa* and *Rhoeadium* (hereafter described as the core sections of *Papaver*)

which based on molecular evidence seem to represent the only monophyletic group of sections within *Papaver*. Section *Pseudopilosa* shows the most divergence having a number of diagnostic indels (discussed previously). The separation of *Pseudopilosa* from *Pilosa* based on morphological and phytochemical differences (Popov, 1937; Gunther, 1975; Kadereit, 1996) is well supported by the results of the combined analysis. Sections *Meconidium*, *Oxytona* and *Pilosa* group in all equally parsimonious trees although the bootstrap support for this grouping is low (69% in the combined analysis).

Within the group comprising *Carinatae, Meconidium, Oxytona, Papaver, Pilosa* and *Rhoeadium*, the inter-sectional relations are poorly defined. Differences between the *trnL-F* and ITS trees are most probably due to the different rates of evolution (different mutation rates) experienced by both regions. If we assume that this group of sections represent the more derived sections within *Papaver*, the polytomy encountered in the trees obtained from the maternally inherited *trnL-F* region may be explained by the lack of polymorphisms to distinguish relationships between the sections (insufficient time for polymorphisms to occur). In addition a relatively small amount of incongruence between the *trnL-F* and ITS data sets occurs regarding sections *Rhoeadium* and *Papaver*. Within section *Rhoeadium* three distinct groups of species are recognised (Kadereit, 1988). Representatives for two of these groups (*Papaver rhoeas* group and the *P. dubium* group) were included in this study. It is obvious under their current taxonomic circumscription that neither section is monophyletic. From the *trnL-F* trees the *Papaver dubium* group is clearly allied to representatives of section *Papaver* (89% bootstrap support in the *trnL*-F analysis) and shares greater sequence similarity to this section than to the *P. rhoeas* group. However in the trees obtained from the biparentally inherited ITS region the *Papaver dubium* group is weakly allied to the *P. rhoeas* group (bootstrap support <50%). Incongruence between data sets may arise for a number of reasons including homoplasy in the data, chance events of lineage sorting at speciation, different levels of nucleotide polymorphisms and/or introgressive hybridisation (e.g. Moore, 1995; Doyle, 1997; Klein *et al.*, 1998). However, it has been demonstrated that incongruent groupings from nuclear and plastid genealogies can provide additional phylogenetic information, through the detection of introgression, hybridisation and allopolyploidy events (e.g. Moore, 1995; Hodkinson *et al.*, 2002). With respect to sections *Papaver*

and *Rhoeadium* the incongruent trees suggest that introgressive hybridisation may have occurred. The positioning of *Papaver dubium* with section *Papaver* suggests that these species have shared a common ancestral lineage. The other species of *Rhoeadium* all share a diagnostic 5 base-pair insertion within the ITS region which is not found in any other section of *Papaver* except in *P. glaucum* (section *Papaver*) which continually groups within the *P. rhoeas* clade in the ITS, *trnL-F* and combined analysis. This again demonstrates that these two sections are not monophyletic. *Papaver macrostomum* (section *Carinatae*) groups consistently with *P. glaucum* within the *P. rhoeas* group in all analyses. The amount of sequence similarity and the possession of the 5 base-pair indel common to this group tends to suggest that the separation of this species from the obviously related *P. rhoeas* into a new section due to the possession of a deciduous stigmatic disc is not supported. This interrelationship between these two sections has been demonstrated on a molecular level previously (Kadereit & Sytsma, 1992) with *Papaver glaucum* associating within a group consisting of *P. rhoeas* and *P. macrostomum* (section *Carinatae*) with *P. dubium* resolving outside and sister to this group. However the results from the analyses performed in this study are better resolved and suited for the investigation of phylogenetic relationships within *Papaver* especially at the sectional level. The phylogenetic and taxonomic implications of the groupings of species and sections obtained from the molecular analysis of this chapter are discussed and expanded further in Chapter 5 in light of morphological, phytochemical and biogeographical considerations.

CHAPTER 3. Morphological phylogenetic analysis of *Papaver* L.

3.1 Introduction

In an attempt to ascertain the suitability of morphological characters for phylogenetic reconstruction, a morphological phylogenetic analysis of *Papaver* was undertaken. The use of morphological characters (morphometric data) as the basis for phylogenetic analysis is the subject of much debate. Problems arise with the interpretation of characters and their evaluation as un/informative characters (Kitching *et al.*, 1998; Bateman, 2001; Weins, 2001). The phylogenetic value of a character may be assessed by ascertaining whether a specific character is qualitative/quantitative and/or discrete/continuous, with discrete and qualitative characters often being preferred (Thiele, 1993; Kitching *et al.*, 1998). Within *Papaver* there is a wide spectrum of morphological variation between the various sections. Kadereit (1996) assumes that extinction has been wide-spread within the genus, thus giving rise to the amount of morphological variation that exists between extant species.

A plethora of characters may be obtained to identify species according to their original monographs and to aid differentiation between closely related species and subspecies. However when applying the characters of one species to another in an attempt to postulate relationship it is essential that these characters are homologous with respect to those species. Homologous characters are those that are similar in appearance and are shared due to common descent (see review by Donoghue, 1999). Identification of apomorphies (shared derived characters) is critical for correct phylogenetic appraisal when using shared characters between organisms. For example valvate dehiscence of seed from three of the eleven sections of *Papaver* may be indicative of shared ancestry and thus be a plesiomorphous character, or one of these particular sections may have evolved this character separately but in parallel and thus the character is synapomorphic. Whether these characters are actually suitable for phylogenetic inferal may be ascertained by molecular systematic evaluation.

Ascertaining whether characters are shared or derived was achieved in this chapter by the comparison of cladograms derived from morphological characters with those derived from molecular characters and the subsequent mapping of morphological characters onto a combined molecular tree to provide insight into diagnostic morphological character evolution. This method of comparison of DNA based phylogenies to morphological characters has provided novel interpretations of morphological character evolution and their significance as phylogenetic characters (see review by Soltis & Soltis, 1995).

3.2 Methods

Twelve unordered characters based on the examination of voucher and herbarium specimens created from cultivated and collected material (Chapter 2, Table 2.1) were scored and the resulting character state matrix is presented in Appendix 2.1. Many characters from prior taxonomic treatments were adopted for this analysis. Difficulties in applying these characters to species from outside the genus occurred due to the fact that the homology of these characters cannot be demonstrated with any amount of certainty. An example of this concerns mode of dehiscence of seed. Kadereit (1988) points out that valvate dehiscence of seed may be considered a basic diagnostic character of Papaveraceae, but questions the comparison of valvate dehiscence in capsules with a stigmatic disc to such dehiscence without a disc. Thus, morphological characters alone were used only to estimate intersectional relationships within *Papaver* with all representatives from other genera omitted from the analysis. However *Argemone mexicana* was scored for these characters and used only to root the resulting cladograms.

3.2.1 Morphological characters used for cladistic analysis

1. Dehiscence of capsule: valvate (0), poricidal (1). *Papaver* sects. *Californicum, Meconella* and *Meconidium* are characterised by dehiscence of seed through valves. All other sections of *Papaver* shed their seed through pores.

2. Filament colour: light (0), dark (1). Filaments of *Californicum, Horrida, Meconella, Meconidium, Pilosa,* and *Pseudo-pilosa* possess light-coloured filaments, whereas representatives of *Argemonidium, Carinatae* and *Oxytona* have dark coloured filaments. Sections *Papaver* and *Rhoeadium* include species of both light and dark coloured anthers and they have been scored accordingly.
3. Filaments: filiform (0), clavate (1). Representatives of *Californicum, Carinatae, Horrida, Meconella, Meconidium, Pilosa, Pseudopilosa* and *Rhoeadium* possess filiform filaments. *Argemonidium* has clavate filaments and species of section *Papaver* and *Oxytona* can have filiform or clavate filaments.
4. Capsules: with bristles (0), without bristles (1). The majority of sections within *Papaver* possess glabrous capsules. Sections with bristly capsules include *Argemonidium* and *Meconella.*
5. Stigmatic disc: a continuation of the capsule (0), separate to the capsule (1). *Papaver* section *Argemonidium* is distinct from all other sections of the genus as its stigmatic disc is a continuation of the capsule (apical plug).
6. Florescence: solitary (0), more than one (1). Sections *Californicum, Carinatae, Horrida, Meconidium, Papaver, Pilosa* and *Rhoeadium* produce more than one flower, whereas, *Argemonidium, Meconella, Oxytona* and *Pseudopilosa* produce solitary flowers.
7. Habit: annual (0), biennial (1), perennial (2).
8. Leaf vernation: convolute (0), revolute (1). The majority of sections within *Papaver* produce leaves with revolute vernation (the leaf has a distinct roll upwards). However *Papaver* section *Pilosa* has convolute leaf vernation (where the leaf rolls backwards).
9. Leaves: pinnatisect (0), pinnatifid/pinnatipartite (1), not divided/lanceolate (2). There are three distinct types of leaf shape within *Papaver.* The first is shared between sections *Argemonidium, Californicum, Meconella* and *Meconidium,* where leaves are extremely dissected (pinnatisect) and bipinnate. The majority of the remaining sections produce leaves, with pinnatipartite or pinnatifid division of lobes. Lanceolate leaves are common to sections *Pilosa, Pseudopilosa* species such as *Papaver somniferum* (section *Papaver*) and *P. orientale* (section *Oxytona*)
10. Stigmatic rays: <10 (0), >10 (1). This character may be regarded as the most variable of the characters chosen for the cladistic analysis. Populations of the same species can produce capsules with varying number of stigmatic rays.

However, within a species the variation is generally not greater than ten. It cannot be assumed that two species that produce the same number of stigmatic rays are related. It is the author's opinion based on observation that species of the same section tend to produce similar number of stigmatic rays and that the species of *Papaver* either have less or more than ten stigmas.

11. Anther colour: light (0), dark (1). Many of the sections of *Papaver* demonstrate homogeneity with regards to anther colour. However, some sections e.g. *Papaver* and *Rhoeadium*, contains accession which produce both light and dark coloured anthers and these have been scored accordingly.
12. Pollen polyporate (0), tricolpate (1). This character was first described by Ernst (1962) to differentiate between section *Argemonidium* and the other sections of *Papaver* and was subsequently used in the morphological evaluation of *Papaver* and related genera by Kadereit *et al.* (1997). Representatives of section *Argemonidium* have polyporate pollen in contrast to the tricolpate pollen grains of the rest of *Papaver*.

3.2.2 Phylogenetic analysis

Accessions used in the molecular phylogenetic analysis were scored for the above characters based on measurements and observations made on voucher material. The matrix of character states for morphological analysis (Appendix 2.1) was imported into PAUP™ v4.0b10 (Swofford, 2003) where it was analysed separately and in combination with the combined molecular data set (ITS and *trnL-F*). Resulting matrixes were subjected to maximum parsimony methods (discussed in chapter 2) to provide phylogenetic trees. Maximum parsimony analyses of the resulting matrices were performed using the heuristic search algorithms of PAUP with 1000 replicates of random addition sequence (holding 25 trees at each replication) and with tree bisection reconnection (TBR) branch swapping on multiple trees (multrees on). In an attempt to visualise the evolution of some of the major diagnostic characters for *Papaver*, these characters were mapped onto the combined molecular tree (Chapter 2, Figure 2.7). Detailing the CI and RI values for individual characters is useful in determining whether or not those characters are homologous or homoplasious with respect to a group of taxa. It is generally accepted that characters with low CI and RI

values indicate that the character is liable to be homoplasious and that the trait that this character describes may have evolved more than once.

3.3 Results

3.3.1 Cladistic analysis of morphological characters

The morphological matrix had 12 characters all of which were parsimony informative. Parsimony analysis of the morphological characters yielded 193 equally most parsimonious trees (Figure 3.1) of 28 steps with a consistency index (CI) of 0.536 and a retention index (RI) of 0.933. CI and RI values for individual morphological characters are shown in Table 3.1. Within the tree a separation between section *Argemonidium* and the other sections of *Papaver* exists, with *Argemonidium* resolving as a sister group to these sections. Based on the characters chosen, all species of section *Argemonidium* form a moderately well supported group (74% bootstrap support). *Papaver californicum* resolves sister to the remaining sections of *Papaver* (excluding section *Argemonidium*) within a clade receiving 76% bootstrap support. Sections *Meconella* and *Meconidium* form a polytomy with a group comprising sections *Carinatae*, *Horrida*, *Oxytona, Papaver, Pilosa* and *Pseudopilosa*. These groups receive no bootstrap support but are present in all equally most parsimonious trees. Within this main group sections *Carinatae, Pilosa* and *Pseudopilosa* form a polytomy with a group comprising sections *Oxytona, Papaver* and *Rhoeadium*, with sections *Oxytona* and *Papaver* resolving as sister to each other (<50% bootstrap support but in all equally most parsimonious trees). Incidentally *Papaver croceum* (accession 148; section *Meconella*) resolves within this polytomy and differs from the other *P. croceum* accessions in that it has glabrous rather than bristly capsules.

The combined molecular and morphological analysis produced 702 trees with a length of 1693, CI=0.581 and RI=0.796 and varied negligibly from the combined molecular tree, due to small number of parsimonious characters in comparison to the relatively large number obtained for the molecular analysis. The topology of the combined molecular/morphological trees (not shown) is exactly the same as that of the trees generated from molecular characters alone.

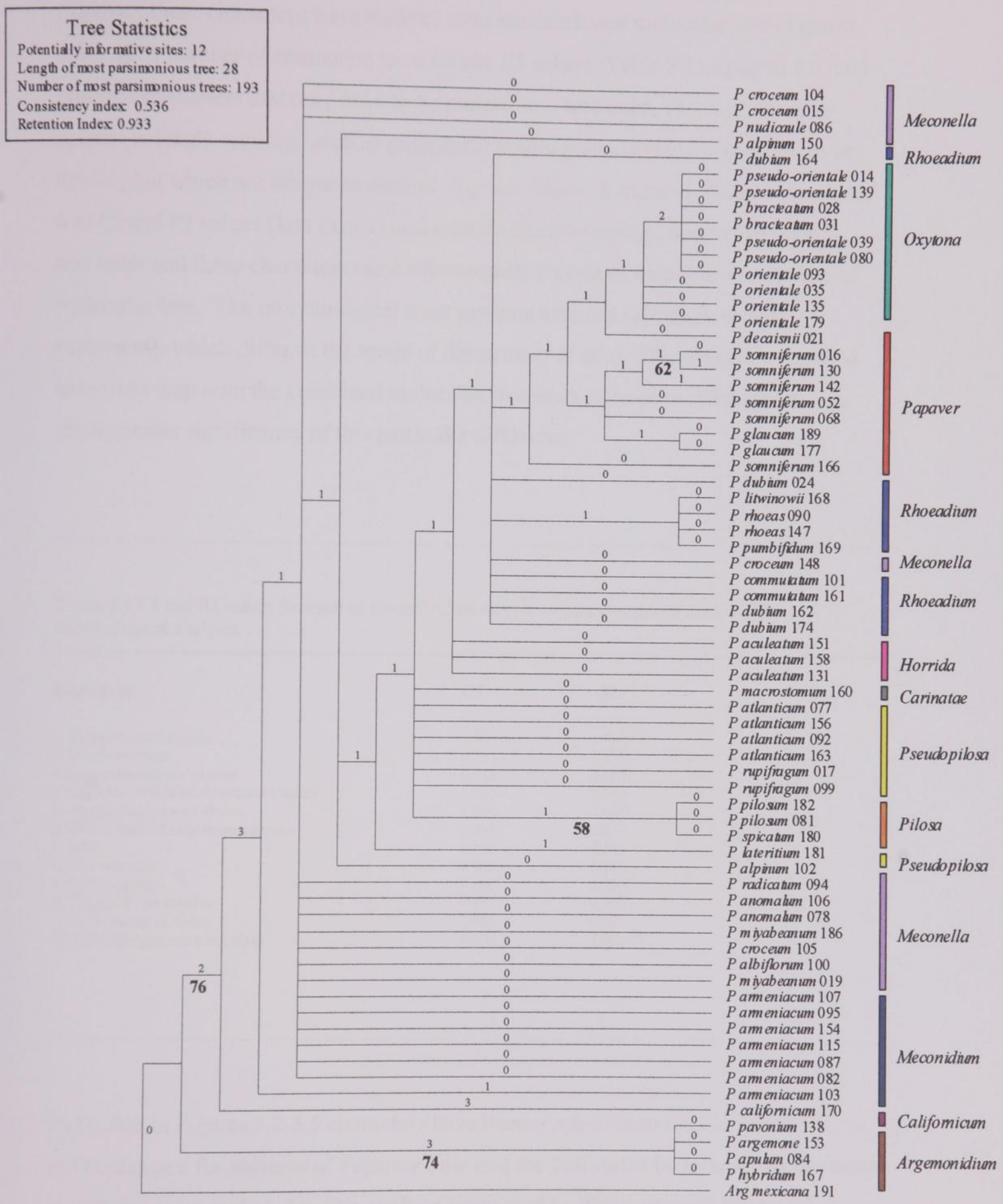


Figure 3.1. One of 193 equally most parsimonious trees from a morphological analysis of *Papaver* using 12 characters. Branch lengths are above branches and bootstrap values are below. Tree statistics are also shown.

In an attempt to visualise the evolution of some of the major diagnostic characters for *Papaver*, these characters were mapped onto the combined molecular tree (Figures 3.2-3.4). A number of characters have CI and RI values (Table 3.1) equal to 1.0, and describe characters that only have to be plotted on a tree once, varying only with respect to single sections, such as polyporate pollen grains and the possession of an apical plug which are unique to section *Argemonidium*. A number of characters have low CI and RI values (less than 1) and include filament colour, number of flowers, and habit and these characters were subsequently chosen to map onto the combined molecular tree. The morphological trees separate sections (although weakly supported), which differ in the mode of dehiscence of seed. This character was also chosen to map onto the combined molecular tree in an attempt to estimate the phylogenetic significance of this particular difference.

Table 3.1 CI and RI values for each of the individual morphological characters used in the morphological analyses

Character	CI	RI
1. Dehiscence of capsule	1.000	1.000
2. Filament colour	0.333	0.933
3. Filaments filiform/ clavate	0.333	0.667
4. Capsules with bristles/ without bristles	1.000	1.000
5. Apical plug present/ absent	1.000	1.000
6. Florescence solitary/ more than one	0.333	0.867
7. Habit	0.400	0.889
8. Leaf vernation	1.000	1.000
9. Leaf dissection	0.500	0.939
10. Stigmatic ray number	0.667	0.917
11. Colouration of anther	0.667	0.917
12. Pollen polyporate/ tricopolate	1.000	1.000

Note, that in Figures 3.2-3.5 characters have been mapped onto the combined molecular tree for sections of *Papaver* only and the trees must be interpreted ignoring the other genera included in this analysis (indicated by blank spaces). Figure 3.2 details the dehiscence of seed characters mapped onto the combined molecular tree. A distinct separation exists between sections *Californicum, Meconella* and *Meconidium*. Figure 3.3 details filament characters mapped onto a combined

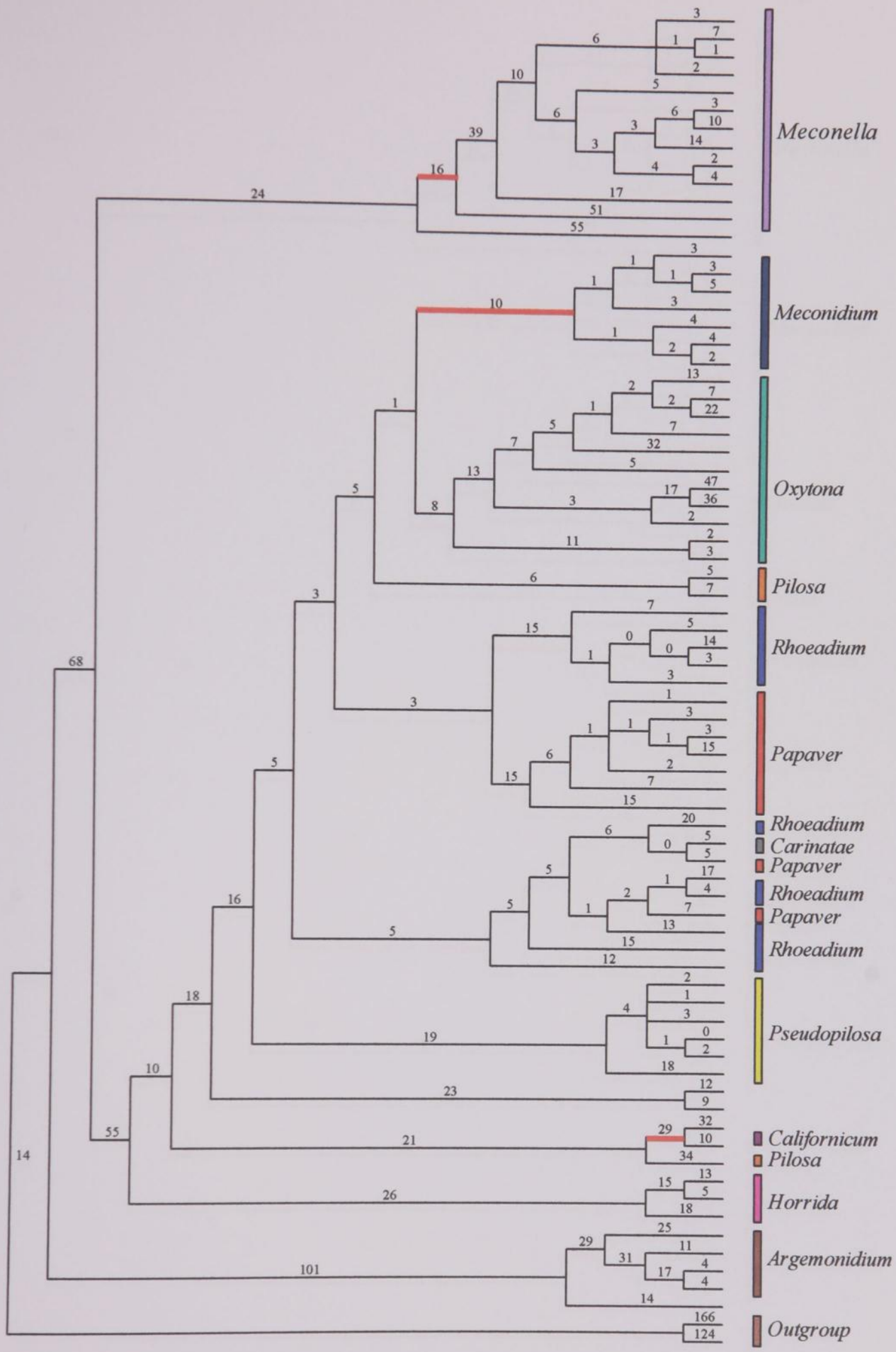


Figure 3.2 Dehiscence of seed characters mapped onto the combined molecular tree. Red lines represent character state changes and describe sections of *Papaver* whose members shed their seed through valves.

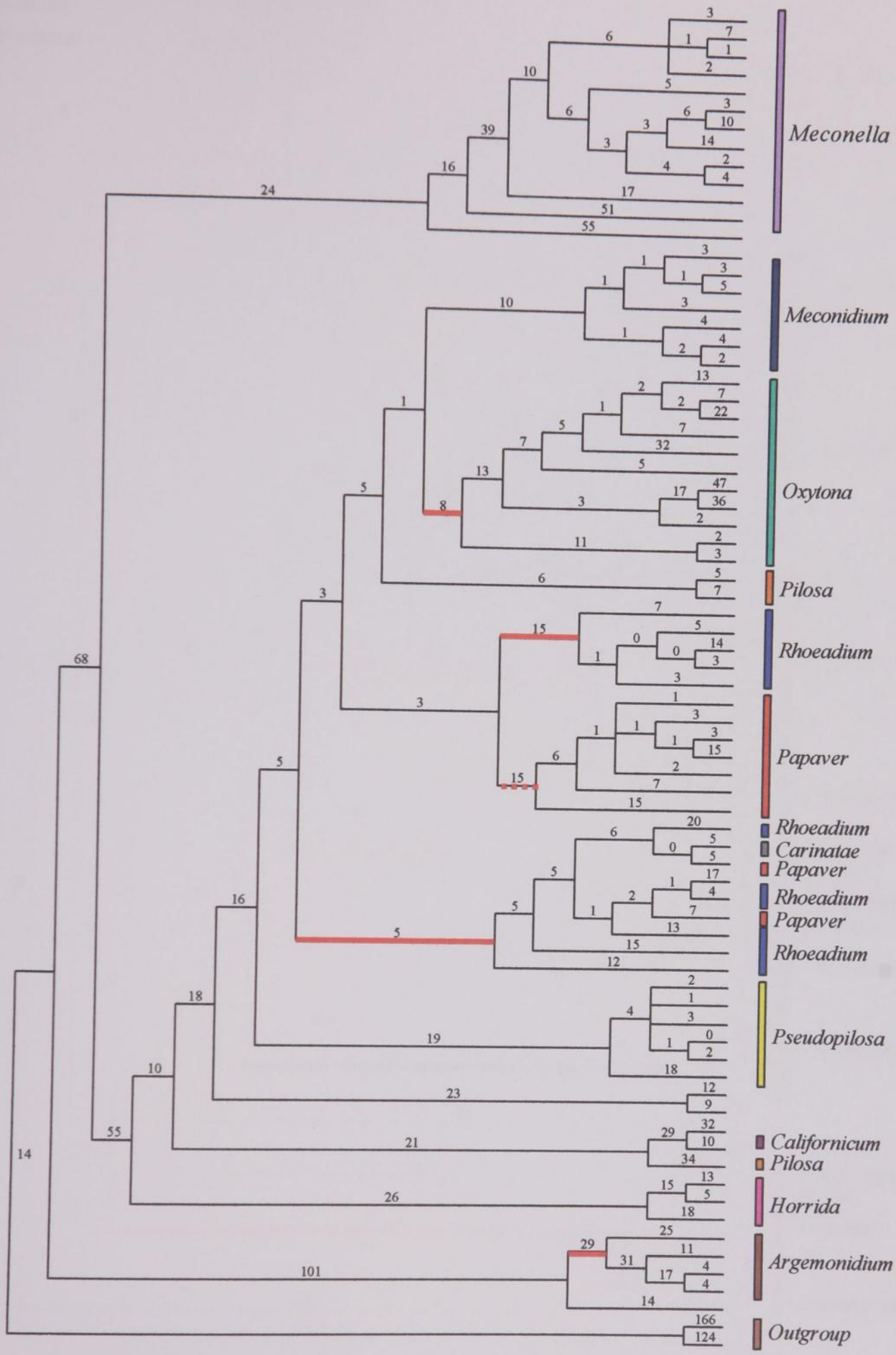


Figure 3.3 Filament characters mapped onto the combined molecular tree. Red lines represent character state changes and describe sections of *Papaver* whose members possess dark coloured filaments. Dashed red line indicates a group with dark or light coloured filaments.

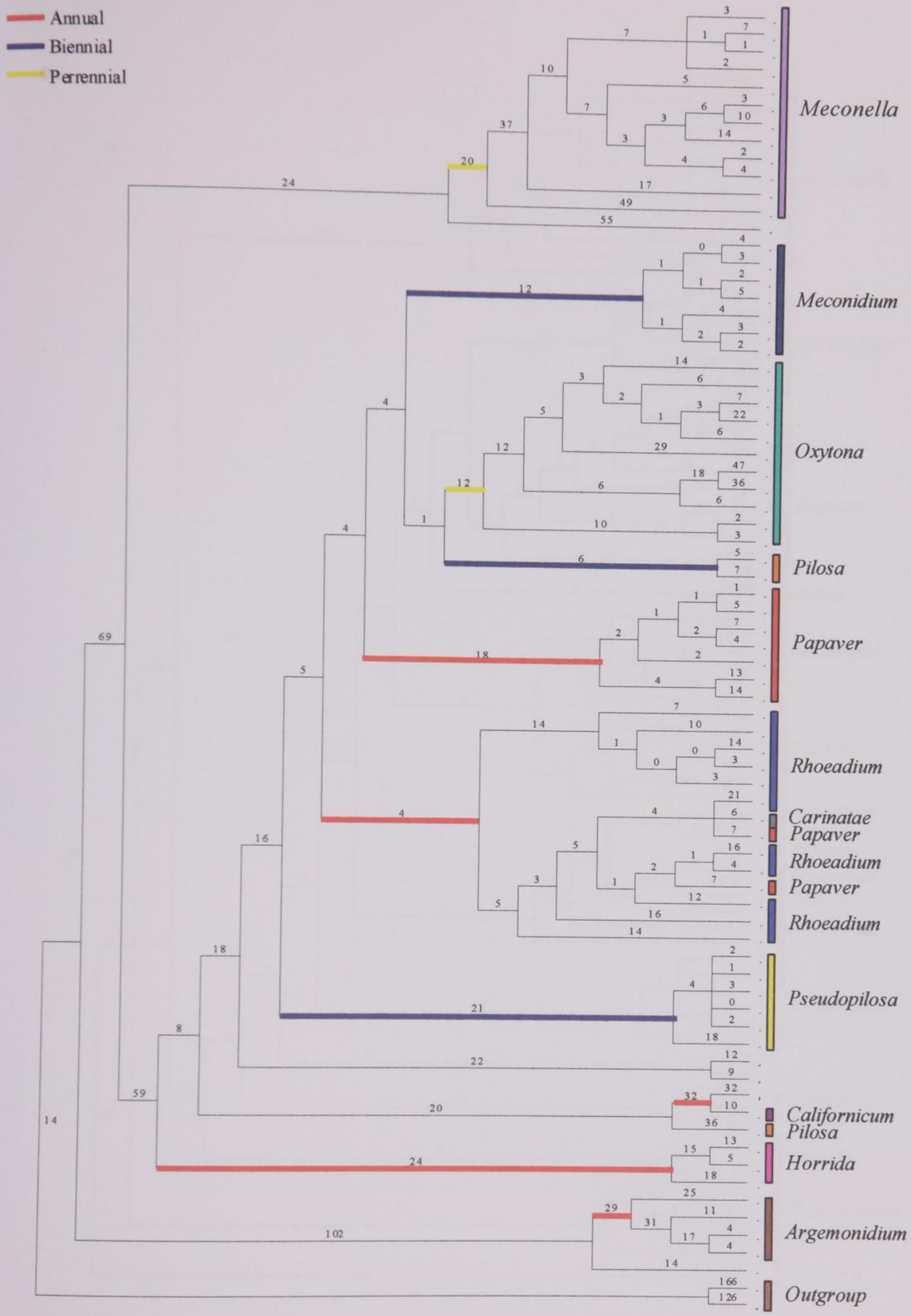


Figure 3.4 Habit characters mapped onto the combined molecular tree. Different coloured bars represent character state changes.

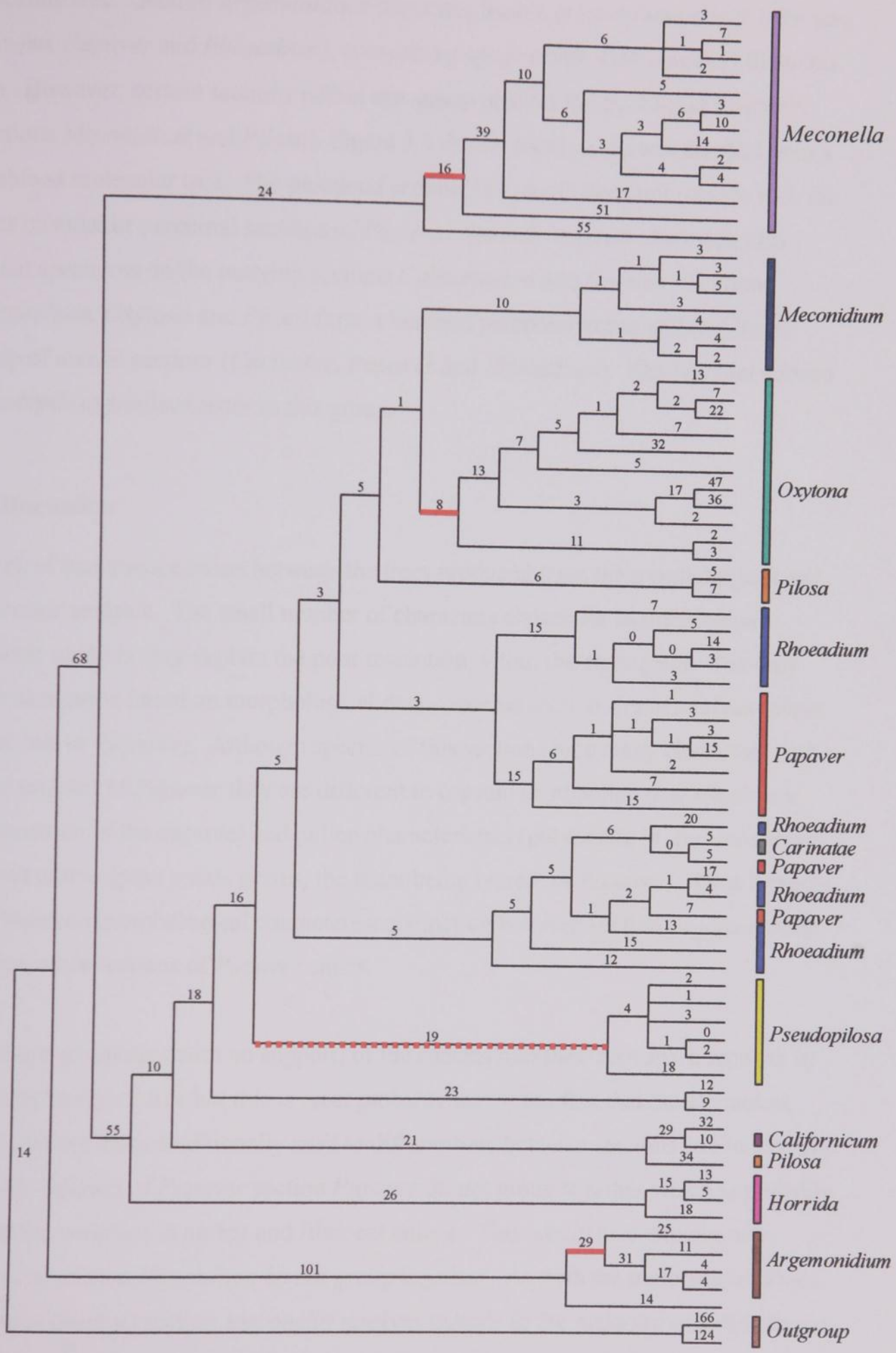


Figure 3.5 Differences in flower number mapped onto the combined molecular tree. Red lines represent character state changes and describe sections of *Papaver* whose members produce single flowers. Dashed red line indicates sections that produce single or numerous flowers.

molecular tree. Section *Argemonidium* separates from a group of sections (*Carinatae, Oxytona, Papaver* and *Rhoeadium*), comprising species with dark coloured filaments also. However, certain sections within this group possess light coloured filaments (sections *Meconidium* and *Pilosa*). Figure 3.4 details habit characters mapped onto a combined molecular tree. The perennial section *Meconella* does not resolve with the other biennial or perennial sections of *Papaver*. Section *Argemonidium* contains annual species as do the outlying sections *Californicum* and *Horrida*. Sections *Meconidium, Oxytona* and *Pilosa* form a biennial/perennial group within a larger group of annual sections (*Carinatae, Papaver* and *Rhoeadium*). The biennial section *Pseudopilosa* resolves sister to this group.

3.4 Discussion

A lack of congruence exists between the trees produced from the morphological and molecular analysis. The small number of characters chosen for morphological cladistic analysis may explain the poor resolution within the cladogram. The only clear separation based on morphological data concerns section *Argemonidium* (sister to the rest of *Papaver*). Although species of this section share many characters with other sections of *Papaver* they are different in capsule (a stigmatic disc which is a continuation of the capsule) and pollen characteristics (polyporate in *Argemonidium* instead of tricolpate pollen grains, the latter being typical of *Papaver*). Thus it seems that based on morphological characters a distinction between section *Argemonidium* and the other sections of *Papaver* exists.

There are groupings (with no support) of the species into their respective sections in the morphological tree but this is most probably due to the fact that the characters examined are those traditionally used to differentiate between sections and to identify species. Species of *Papaver* section *Papaver* do not group together which is probably due to the variation in anther and filament colour. This would also explain why species of section *Rhoeadium* do not group together. As with the molecular analysis (previous chapter) section *Meconella* resolves outside to the main group consisting of the majority of the sections of *Papaver* in this case forming a polytomy with representatives of section *Meconidium* and sister to a group comprising sections *Carinatae, Horrida, Oxytona, Papaver, Pilosa* and *Pseudopilosa* (with no bootstrap

support but groups are present in all equally most parsimonious trees). The polytomy of *Meconella* and *Meconidium* indicates that based on the characters used in the morphological analysis these two sections are very similar, a view in accordance with the groupings of Kadereit (1988a). Within the group comprising *Carinatae*, *Horrida*, *Oxytona, Papaver, Pilosa* and *Pseudopilosa*, sections *Oxytona* and *Papaver* form a group that has no bootstrap support but is present in all equally parsimonious trees indicating a close affinity between these sections based on the characters chosen for analysis. Section *Rhoeadium* resolved as sister to these sections and again shares a number of characters with sections *Oxytona* and *Papaver*. Section *Horrida* resolved as sister to *Oxytona, Papaver* and *Rhoeadium* a result incongruent with the molecular analysis. This positioning can be explained by the number of similar characters such as poricidal dehiscence of capsule. Section *Pseudopilosa* resolved as sister to *Horrida, Oxytona, Papaver* and *Rhoeadium*. Discounting section *Horrida* this positioning of *Pseudopilosa* is congruent with that found in the molecular analysis.

It is evident that this restricted set of morphological characters is not suited for estimating phylogeny at this taxonomic level although some phylogenetic signal is present within the matrix. The CI and RI values for the morphological analysis were 0.54 and 0.94 respectively, an indication of the high levels of homoplasy within the morphological analysis. Although the groupings within the morphological analysis are similar to those of Kadereit (1988a) based on morphological similarities (c.f. Chapter 1, Table 1.11), the high levels of homoplasy indicate that convergent morphological evolution may have occurred within *Papaver*.

Mapping potentially homoplasious characters onto the tree obtained from the combined molecular analysis allowed a close examination of character evolution to be made. In the morphological analysis most groupings had little phylogenetic significance or support and phenomena such as divergence or convergence could not be detected readily. Once these characters were mapped onto the trees such phenomena could be visualised and the phylogenetic significance of the individual character could be assessed.

Figure 3.2 details the position of sections with valvate dehiscence of seed. With regards to the outlying position of sections *Meconella* and *Californicum* with respect

to the other sections of *Papaver* suggests that this character is primitive. However this character is also found in section *Meconidium* which resolves within a strongly supported within a group consisting of sections *Carinatae, Oxytona, Papaver, Pilosa, Pseudopilosa* and *Rhoeadium* (core *Papaver* sections). Its presence here suggests that this character is a synapomorphy for the section *Meconidium*. Thus the results of this analysis suggest that valvate dehiscence has evolved independently a number of times within *Papaver*.

Additionally the dark coloured filaments (Figure 3.3) seem to have evolved more than once. Molecular and morphological data separate section *Argemonidium* from the other dark coloured filament sections (*Carinatae, Oxytona, Papaver* and *Rhoeadium*) and indicates that this character is synapomorphic with respect to these sections. With regards to the monophyletic group consisting of sections *Meconidium, Oxytona, Pilosa, Papaver, Rhoeadium* and *Carinatae* dark coloured filaments are found in sections *Rhoeadium*, *Carinatae* and *Papaver*. Section *Oxytona* also consists of members with dark coloured filaments and its positioning with sections *Meconidium* and *Pilosa* (species with light coloured filaments) may be explained in two ways. Firstly, the species of section *Oxytona* evolved this character separately and independently to the other dark coloured filament sections from an ancestor of sections *Pilosa* or *Meconidium* that may have had light coloured anthers. Alternatively, the topology of the sections with species having dark coloured filaments may be interpreted as having evolved from a common ancestor, but this trait was subsequently lost in sections *Pilosa* and *Meconidium*.

Figure 3.4 details sections with respect to their habit. There is very little phylogenetic significance with these characters as demonstrated by the lack of grouping of sections with similar habit. For example, *Meconella* is sister to the core group of *Papaver* and contains perennial species and shares this character with section *Oxytona* (which is obviously distantly related). The biennial sections *Meconidium* and *Pilosa* are independently resolved sister groups to section *Oxytona* and this character may be ancestral for the *Meconidium, Oxytona* and *Pilosa* group. The perennial characteristic of *Oxytona* may have evolved from a biennial ancestor. The annual habit is typically considered to have evolved from perennial ancestors in angiosperms with the annual species generally considered to be more advanced (see review by Stebbins, 1974;

Crawford *et al.*, 1992). Within *Papaver* annual habit is the more common giving the species of these sections distinct advantages in terms of reproducing and highlights why many species of *Papaver* are extremely successful ruderals. The biennial and perennial species are native to arctic alpine habitats where annual habit may be considered energy expensive. A far more suitable strategy would be to produce flowers in summer, cross pollinate, then die back and grow through vegetative propagation in the spring, a strategy adopted by species of section *Oxytona* for example. Regarding the annual habit as the norm within *Papaver* the results of this analysis suggest that a divergence to the perennial or biennial habit annual species from an annual ancestor has occurred many times.

The evolution of single flowers was also investigated (Figure 3.5). With regards to sections *Argemonidium* and *Meconella* their sister group positions with respect to the rest of *Papaver* may indicate that the production of single flowers is a plesiomorphic character for *Papaver*. Section *Oxytona* also consists of species with single flowers but its distance from the pre-mentioned sections allows the interpretation that this character is apomorphic and that convergence has occurred with respect to this character. Section *Pseudopilosa* comprises species that have single to many flowers but the material examined by the author all had single flowers and were scored accordingly. Based on the separation from the other single flowered species, members of this section may demonstrate a transition from the production of single to many flowers, as is common to the majority of sections within the core *Papaver* group.

It is evident from the combined analysis of morphological and molecular characters that convergent morphological evolution has occurred on numerous occasions within *Papaver*. This highlights the difficulties in relying on morphological characters alone for the inferal of phylogenetic relationships and that many of the groupings of sections within *Papaver* (Kadereit, 1988a) are artificial. Without in-depth molecular phylogenetic analysis of *Papaver* the identification of convergence could not have been possible and indicates the benefits from a phylogenetic point of view of examining molecular and morphological datasets in combination. The taxonomic and phylogenetic significance of these results are discussed in greater detail in Chapter 5.

CHAPTER 4. Chemotaxonomic evaluation of *Papaver* L.

4.1 Introduction

Similarities or differences in phytochemical spectra can be extremely informative for the classification of plant species. Phytochemical data when used alone or in combination with morphological and molecular characters can result in the production of comprehensive phylogenetic trees (Singh, 1999). With the advent of more precise analytical techniques, the wide range of chemical structures and compounds produced by plants have become apparent and they have provided taxonomists with additional information on which to base classifications. Natural chemical constituents are divided into micromolecules (molecular weight less than 1000) and macromolecules (molecular weight greater than 1000). Furthermore micromolecules can be subdivided into primary (compounds involved in vital metabolic pathways such as aconitic acid, citric acid and protein amino acids) and secondary metabolites (compounds which are the by-products of metabolism such as alkaloids, flavonoids, non-proteinic amino acids and terpenoids), both of which exhibit a wide variation in chemical diversity, distribution and structure (Herbert, 1989; Singh, 1999). With regards to using phytochemicals for the systematic study of a group of related species, it is often secondary metabolites that provide the most informative characters (Gershenzon & Mabry, 1983; Harborne & Turner, 1984). Primary metabolites would be analogous to coding regions of DNA that often offer little phylogenetic information because their function is essential and thus variation (with respect to systematic evaluation) would be reduced. Secondary metabolites have been used to substantiate botanical classifications across many different taxonomic ranks from the order (e.g. Cronquist, 1977, 1988; Dahlgren, 1980; Dahlgren *et al.*, 1981; Gottlieb *et al.*, 1993) to the genus (e.g. Gunther, 1975; Preininger, 1986; Middleton, 1992; Aziz *et al.*, 1993; Matsuura *et al.*, 1994). In addition secondary metabolites have been demonstrated to show inter-specific and even infra-specific variation to be used for study at lower taxonomic levels (see reviews by Gershenzon & Mabry, 1983; Harborne & Turner, 1984).

With respect to *Papaver* L., alkaloids often represent the secondary metabolite most suitable for systematic evaluation. Alkaloids are organic nitrogen-containing bases

(usually with a heterocyclic ring). They have diverse structures but can be classified within a few biogenetic groups primarily because they are formed from a relatively small number of amino acids and the skeleton of these amino acids are retained largely intact in the derived alkaloids (Herbert, 1989). The economic and medicinal importance of many of these alkaloids has stimulated extensive studies into the presence/absence of particular phytochemicals across a wide range of poppy species (see reviews by Tetenyi, 1993; Mihalik, 1998; Waterman, 1998). In addition the elucidation of enzymatic pathways responsible for the synthesis of certain alkaloidal types has increased the significance of alkaloids as taxonomic characters and has led to more detailed chemotaxonomic evaluation (Gunther, 1975; Preininger *et al.*, 1981; Tetenyi, 1993; reviewed by Waterman, 1998).

The alkaloids of *Papaver* are those based on the benzylisoquinoline nucleus (Figure 4.1), which form a vast and complex group of alkaloids displaying a large array of structural types (Cordell, 1981). These alkaloids can be subdivided into several constitutional types (biogenetic groups; Figure 4.2), which are directly or indirectly derived from reticuline the key intermediate in the biosynthesis of the benzyltetrahydroisoquinoline (BTIQ) alkaloids. The 1-benzyltetrahydroisoquinoline skeleton contains a tetrahydroisoquinoline (TIQ) segment and a benzyl moiety (Cordell, 1981; Preininger *et al.*, 1981).

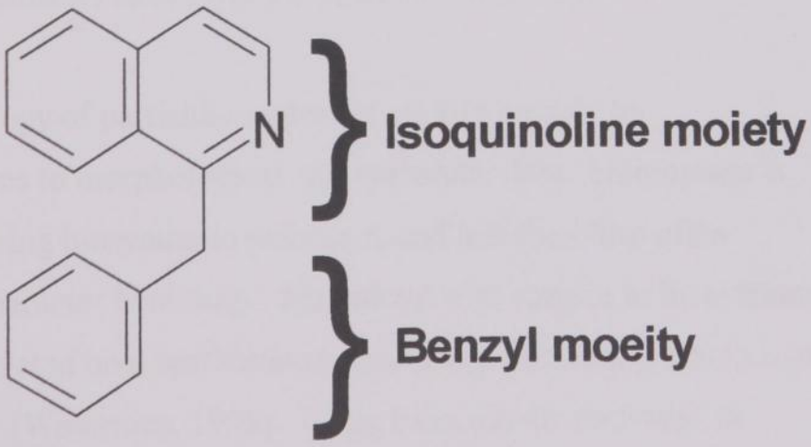


Figure 4.1 The benzylisoquinoline nucleus (BIQ), which provides the molecular skeleton for a variety of alkaloids including reticuline, the biosynthetic precursor to many of the alkaloid types, synthesised by *Papaver* species.

Each taxonomic section of *Papaver* has characteristic biosynthetically linked groups of compounds, which can be used to trace relationships between the species. In *Papaver* twelve alkaloid groups (tetrahydroisoquinolines, benzylisoquinolines, aporphines, promorphines, protoberberines, phthalideisoquinolines, secophthalideisoquinolines, rhoeadines, benzophenanthradines, rhoeadines, papaverrubines) have been detected containing almost 200 compounds (Preininger, 1986). With the aid of molecular analysis it should be possible to demonstrate whether a shared biosynthetic pathway (BP) between two plants indicates a close relationship or not.

Interpreting the systematic value of phytochemical distributions between species depends primarily on the understanding of the biosynthetic mechanisms through which those compounds are formed (Waterman, 1998). The use of single biosynthetic products as cladistic characters may be detrimental for many reasons. The most important of these is the lack of equivalence between presence and true absence. The reported presence of a compound can be accepted as evidence for its presence, lack of such a report cannot necessarily be assumed to indicate true absence (Saunders & Brown, 1999). Many of the alkaloids produced by a particular species may have been converted into other alkaloids and may not, as yet, have been detected even though the particular pathway that produced it is present. It has been suggested that such a problem can be avoided by the analysis of the occurrence of groups of compounds that are biosynthetically linked (biogenetic groups) rather than the occurrence of specific phytochemicals (Saunders & Brown, 1999; Barkman, 2001).

The need to confirm homology of particular pathways applies equally to phytochemical data as it does to morphological and molecular data. Homoplasy is common within groups sharing biosynthetic pathways, and it is therefore often difficult to ascertain true character homology. Homology with respect to biosynthetic pathways would describe related taxa synthesising chemically different products using the same synthetic pathway (Waterman, 1998). Using biosynthetic pathways or biogenetic groups to characterise individual plant taxa allows the identification of problems that effect the interpretation of alkaloid distribution, including parallelism (also known as convergence; the occurrence of alkaloids with similar structures in seemingly unrelated parts of the plant kingdom), and divergence (the situation where

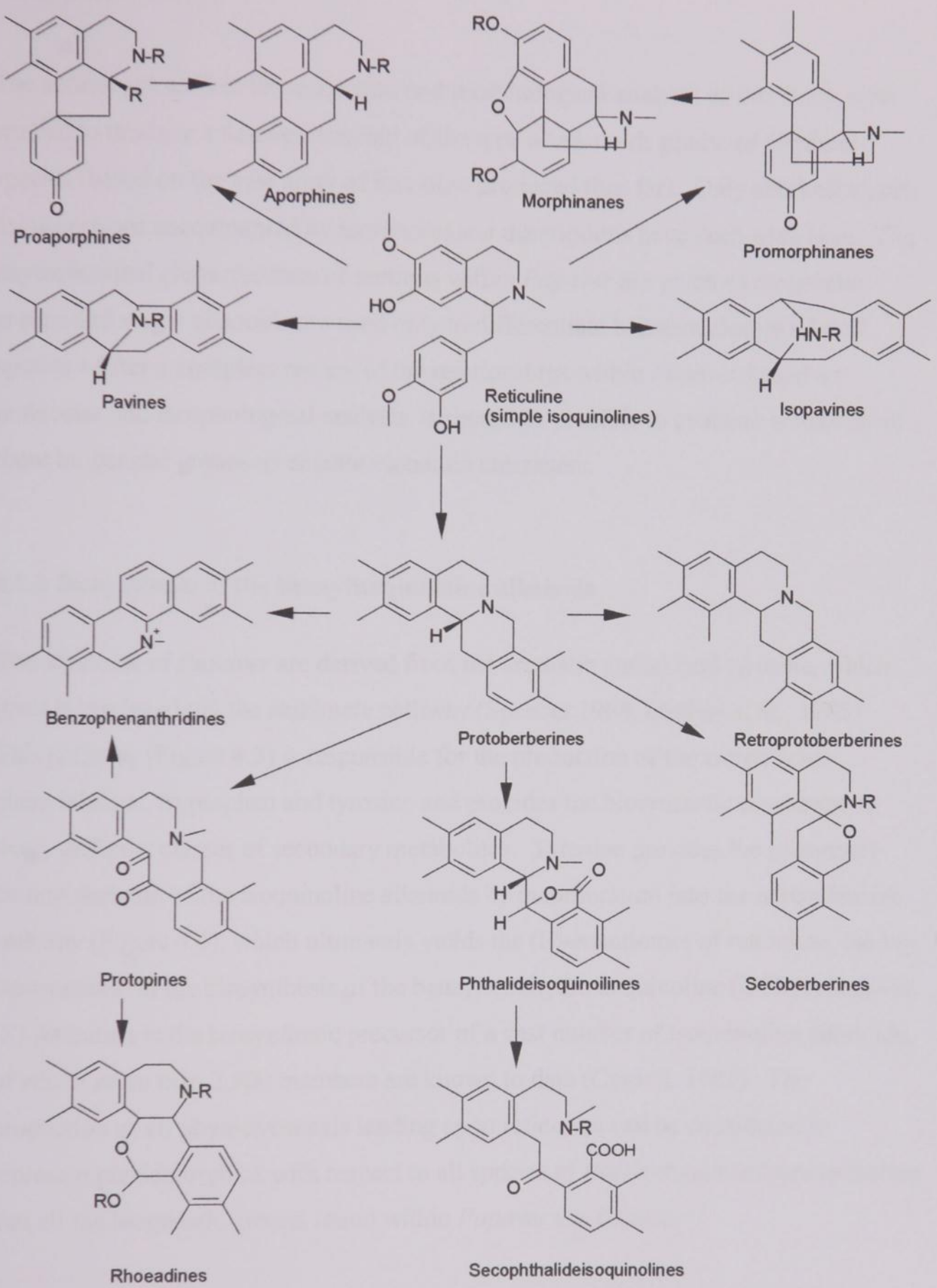


Figure 4.2 Simplified biosynthetic relationships between the isoquinoline alkaloids found in *Papaver* species derived from reticuline (modified after Preininger, 1986).

related taxa produce alkaloids with different biosynthetic origins (Hegnauer, 1966; Waterman, 1998).

The accessions used in the molecular and morphological analysis of this study were studied to produce a detailed account of the type of alkaloids produced for these species (based on the vast array of literature produced thus far). Only alkaloid reports for accessions accompanied by morphological descriptions have been used here. The phytochemical characteristics of sections within *Papaver* are given as biogenetic groups and single alkaloids are used only to differentiate between closely related species. After a complete review of the relationships within *Papaver* based on molecular and morphological analysis, it should be possible to evaluate suitability of these biogenetic groups as chemotaxonomic characters.

4.1.2 Biosynthesis of the benzylisoquinoline alkaloids

The alkaloids of *Papaver* are derived from the aromatic amino acid tyrosine, which itself is produced via the shikimate pathway (Spencer 1968; Mothes *et al.*, 1985). This pathway (Figure 4.3) is responsible for the production of the amino acids phenylalanine, tryptophan and tyrosine and provides the biosynthetic precursors to many different classes of secondary metabolites. Tyrosine provides the precursory carbon skeleton of the isoquinoline alkaloids by incorporation into the norcoclaurine pathway (Figure 4.4), which ultimately yields the (S)-enantiomer of reticuline, the key intermediate in the biosynthesis of the benzyltetrahydroisoquinoline (BTIQ) alkaloids. (S)-reticuline is the biosynthetic precursor of a vast number of isoquinoline alkaloids, of which more than 2,500 members are known to date (Cordell, 1981). The production of all phytochemicals leading up to reticuline can be considered to represent plesiomorphies with respect to all species of *Papaver*, as it is from reticuline that all the biogenetic groups found within *Papaver* are derived.

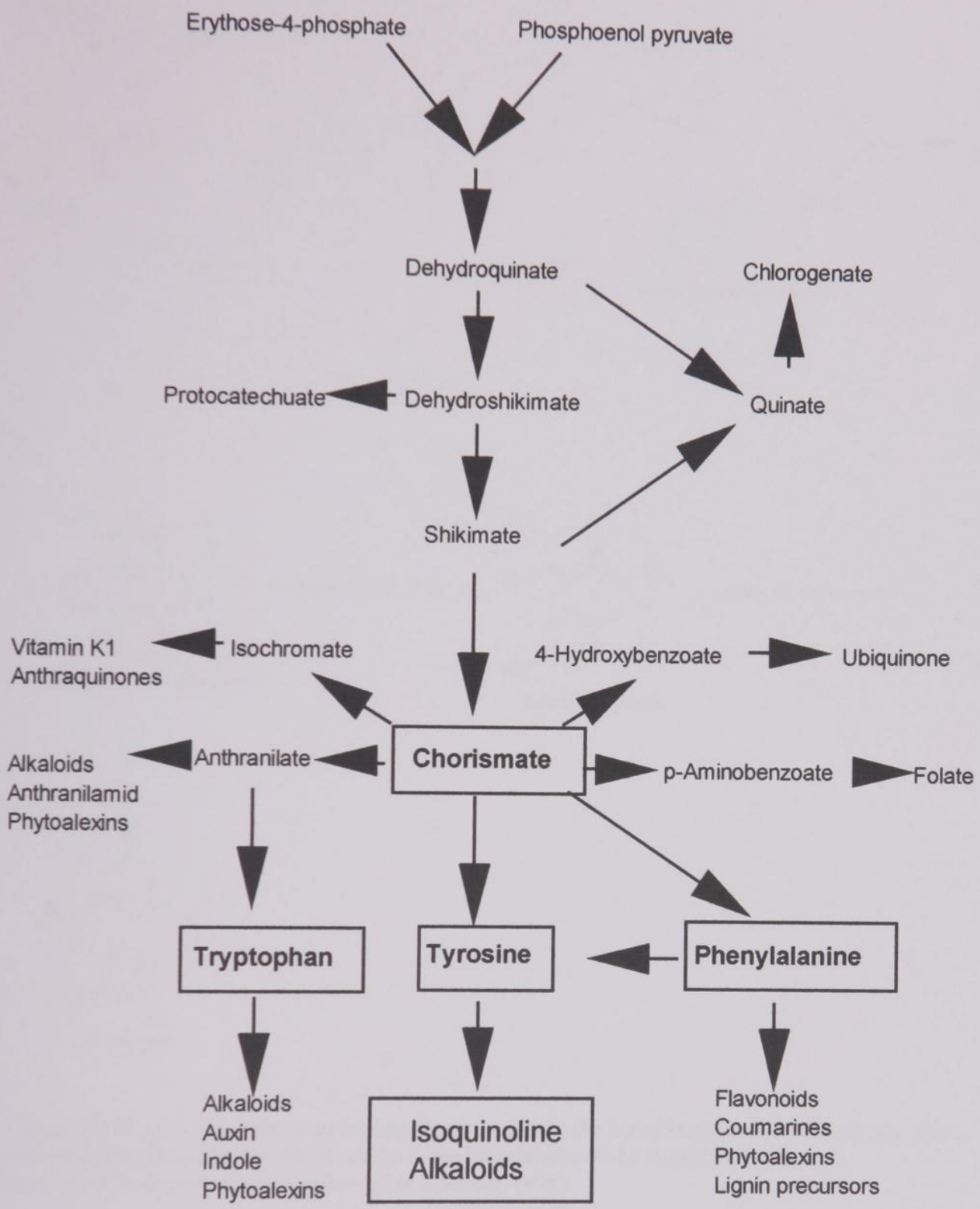


Figure 4.3 A schematic diagram showing different classes of secondary metabolites that arise from aromatic amino acids and from intermediates of the shikimate pathway (after Psenak, 1998).

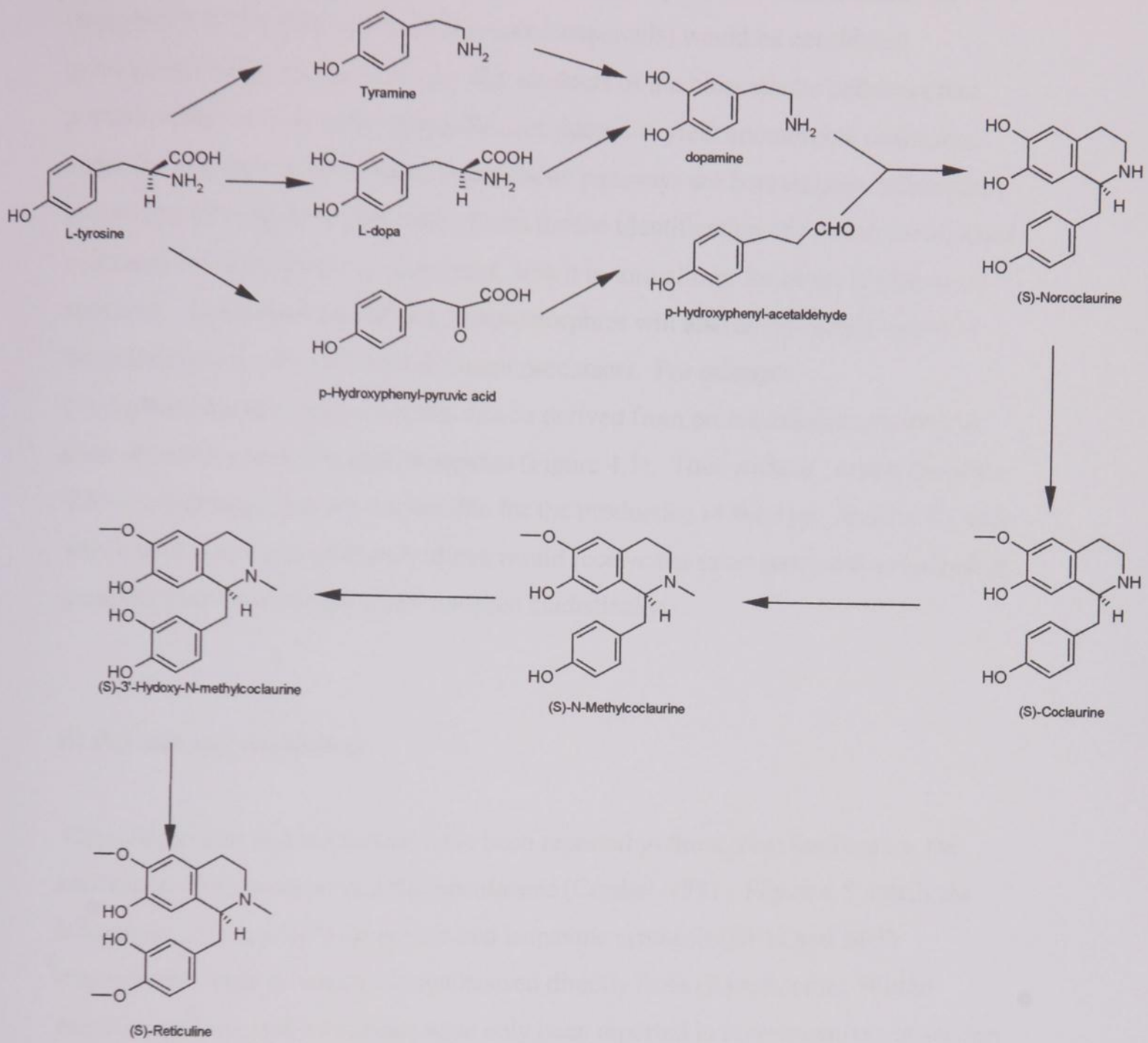


Figure 4.4 Biosynthetic sequences leading from tyrosine to the benzylisoquinoline alkaloid precursors. The norcoclaurine pathway, which results in the biosynthesis of the important precursory benzylisoquinoline alkaloid reticuline (after Kutchan, 1998).

4.1.3 Description of the various biogenetic groups used as cladistic characters

In order to utilise phytochemicals as phylogenetic characters it is important to trace through the biosynthetic pathways (BP) responsible for their production back to their simplest constituent (Waterman, 1998; Barkman, 2001). Once this is detailed is it possible to differentiate between the sections of *Papaver* and identify at which point

along the pathway differentiation occurs. Up to these points of differentiation all components of the pathway (enzymes and compounds) would be considered primitive/ancestral (plesiomorphy). The products of the biosynthetic pathways that produce different biogenetic groups are considered to yield apomorphic characters, under the assumption that shared biosynthetic pathways are homologous. Subsequent knowledge of biogenetic pathways allows for the identification of convergent alkaloid evolution and homoplasious characters, which in turn allows for better phylogenetic appraisal. In addition identifying synapomorphies will also aid in identification of biogenetic groups derived from different precursors. For example benzophenanthridine type alkaloids can be derived from protoberberines directly or from protoberberines through protopines (Figure 4.1). Thus without knowledge of the different pathways that are responsible for the production of this type of alkaloid, taxa which produced benzophenanthridines would receive the same score and a misleading grouping may be achieved when analysed cladistically.

(i) Pavines and isopavines

The c.20 pavines and isopavines have been reported in three plant families i.e. the Lauraceae, Papaveraceae and Ranunculaceae (Cordell, 1981). Figure 4.5 details the biogenetic pathway (BP) for pavine and isopavine synthesis (BP1a and BP1b respectively), both of which are synthesised directly from (R)-reticuline. Within *Papaver,* pavines and isopavines have only been reported in representatives of section *Meconella* (Preininger, 1986; Maturova *et al.*, 1966; Randel, 1974). Characteristic alkaloids of the group include amurensine, amurensinine, amurine, nudaurine, alpinigine, alpinine, papaverrubine and muramine (Biot & Flentje, 1960; Maturova *et al.*, 1966; Randel, 1974). The isopavines amurensine and amurensinine have been detected in almost all species of this section and can be considered diagnostic chemotaxonomic characters (Preininger, 1986).

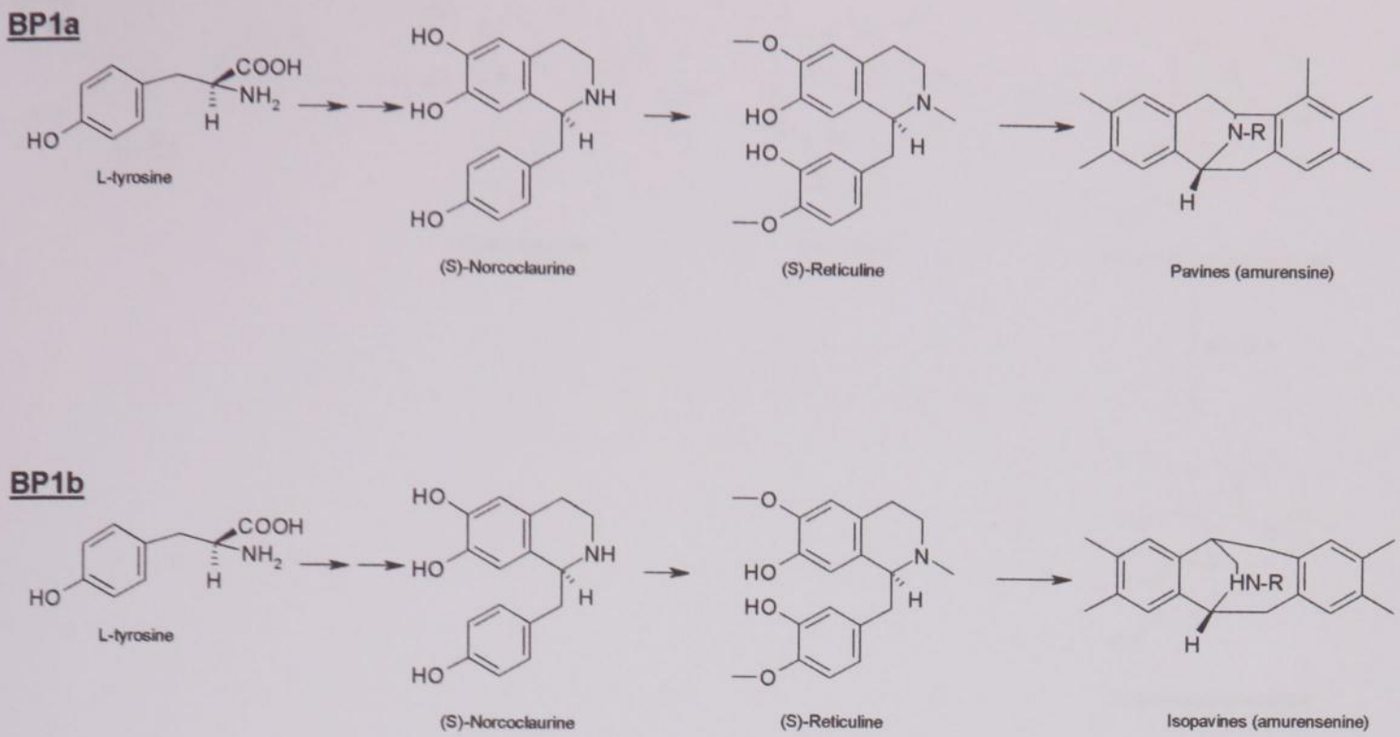


Figure 4.6 Biogenetic pathways responsible for the synthesis of pavine (BP1a) and isopavine alkaloids (BP1b).

(ii) Proaporphines and aporphines

The c.30 naturally occurring proaporphine alkaloids discovered to date are distributed in the plant families Euphorbiaceae, Lauraceae, Menispermaceae, Nymphaceae and Papaveraceae (Cordell, 1981). They are directly derived from (S)-reticuline by the production of a cyclohexadienone system incorporated into the tetrahydroisoquinoline system. Proaporphines may be of two types: (i) isoquinolines with an unreduced dienone system such as pronuciferine and (ii) those with reduced or partially reduced dienone systems such as orioline (Cordell, 1981). Figure 4.6 details the biogenetic pathway for proaporphine synthesis (BP2a). Aporphine and aporphinoid alkaloids constitute a large (over 100 compounds) naturally occurring subgroup of the benzylisoquinolines (Cordell, 1981). Distribution of these tetracyclic bases have been reported in a wide range of plant families including the Annonaceae, Berberidaceae, Fumariaceae, Lauraceae, Magnoliaceae, Papaveraceae, Piperaceae and Rutaceae (Preininger, 1981 and Rios *et al.*, 2000). The basic skeleton of aporphine alkaloids differs from bezylisoquinoline alkaloids (such as reticuline and N-methyl-cocluarine) by having a single bond between the two aromatic rings (Herbert, 1989). It has been demonstrated with respect to *Papaver somniferum* that a number of pathways exist for

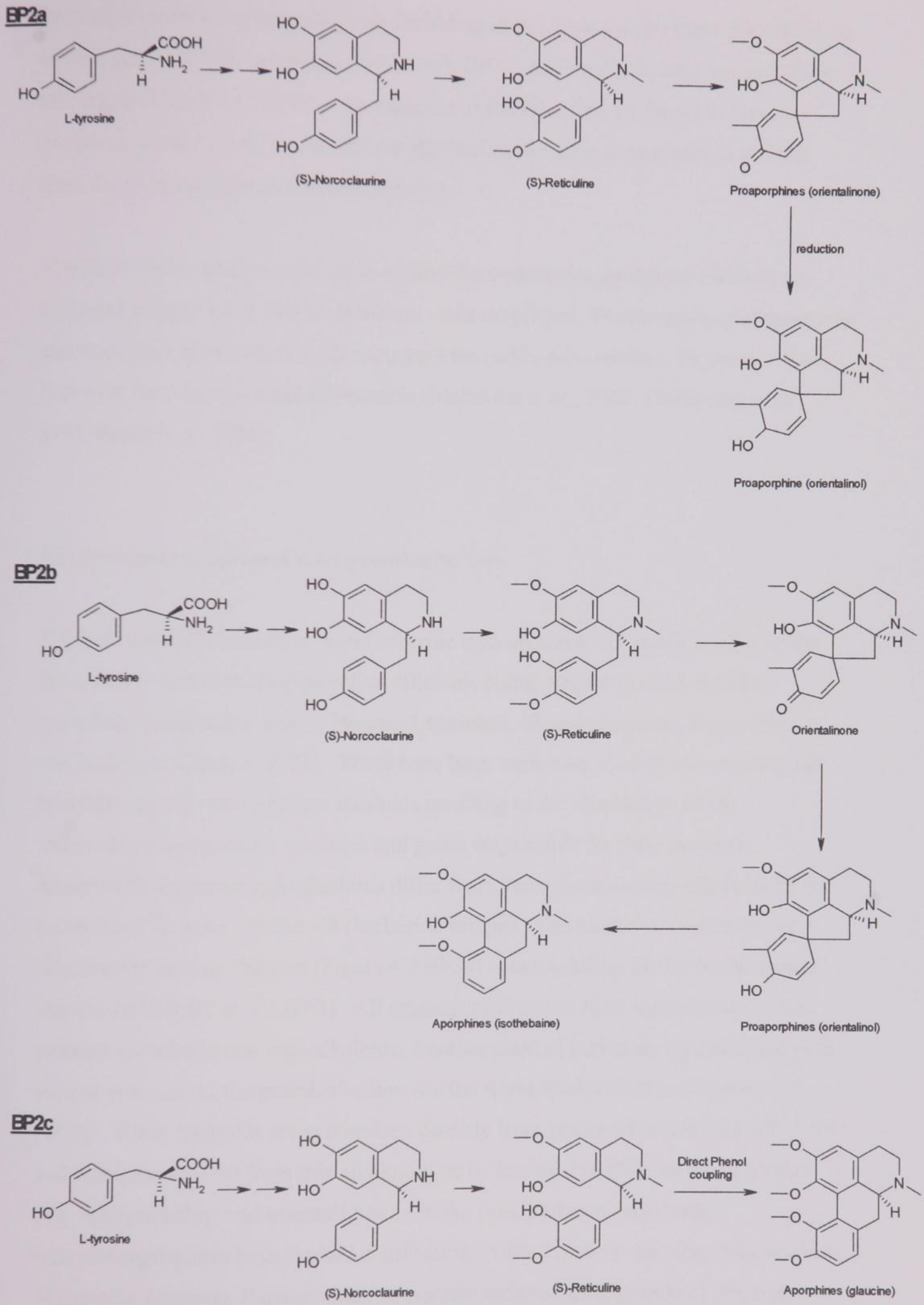


Figure 4.6 Biogenetic pathways responsible for the synthesis of proaporphine (BP2a) and aporphine alkaloids (BP2b and BP2c).

the formation of aporphine alkaloids including direct biosynthesis from (S)-reticuline as with glaucine and isoboldine (Figure 4.6, BP2c) and synthesis from proaporphine precursors (Figure 4.6, BP2b). An example of the latter can be found in *Papaver pseudo-orientale* where the aporphine alkaloid isothebaine is synthesised directly from the proaporphine alkaloid orientaline.

Where possible, species reported to synthesise a particular aporphine alkaloid are separated depending on the biosynthetic route employed. Proaporphine and aporphine alkaloids have been reported for sections *Meconella, Meconidium, Oxytona, Pilosa, Papaver, Pseudopilosa* and *Rhoeadium* (Maturova *et al.*, 1966; Phillipson, *et al.*, 1981; Preininger. 1986)

(iii) Protoberberines and retroprotoberberines

The c.40 naturally occurring protoberberine type alkaloids are considered to be the most widely distributed isoquinoline alkaloids being present in many families including Annonaceae, Berberidaceae, Lauraceae, Menispermaceae, Papaveraceae and Rutaceae (Cordell, 1981). There have been numerous studies investigating the biosynthesis of protoberberine alkaloids resulting in the elucidation of the intermediate compounds, enzymes and genes responsible for their synthesis. Structurally berberine type alkaloids differ from benzylisoquinoline alkaloids by the presence of an extra bond at C8 (berberine bridge). The oxidative cyclisation of reticuline to protoberberines (Figure 4.7 BP3a) is catalysed by the berberine bridge enzyme (Battersby *et al.*, 1963). All sections of *Papaver* have representatives that produce protoberberine type alkaloids. Another class of berberine type alkaloid with similar structure to the protoberberines are the retroprotoberberines (Figure 4.7 BP3b). These alkaloids are synthesised directly from protoberberines (Cordell, 1981) and are differentiated from this alkaloid type by having 2 methylene dioxy groups (e.g. mecambridine and orientalidine). Unlike protoberberine alkaloids, retroprotoberberines have limited distribution within *Papaver*. Sections *Meconidium, Meconella, Oxytona, Papaver* and *Rhoeadium* accumulate alkaloids of this type (Maturova *et al.*, 1966; Phillipson, *et al.*, 1981; Preininger. 1986).

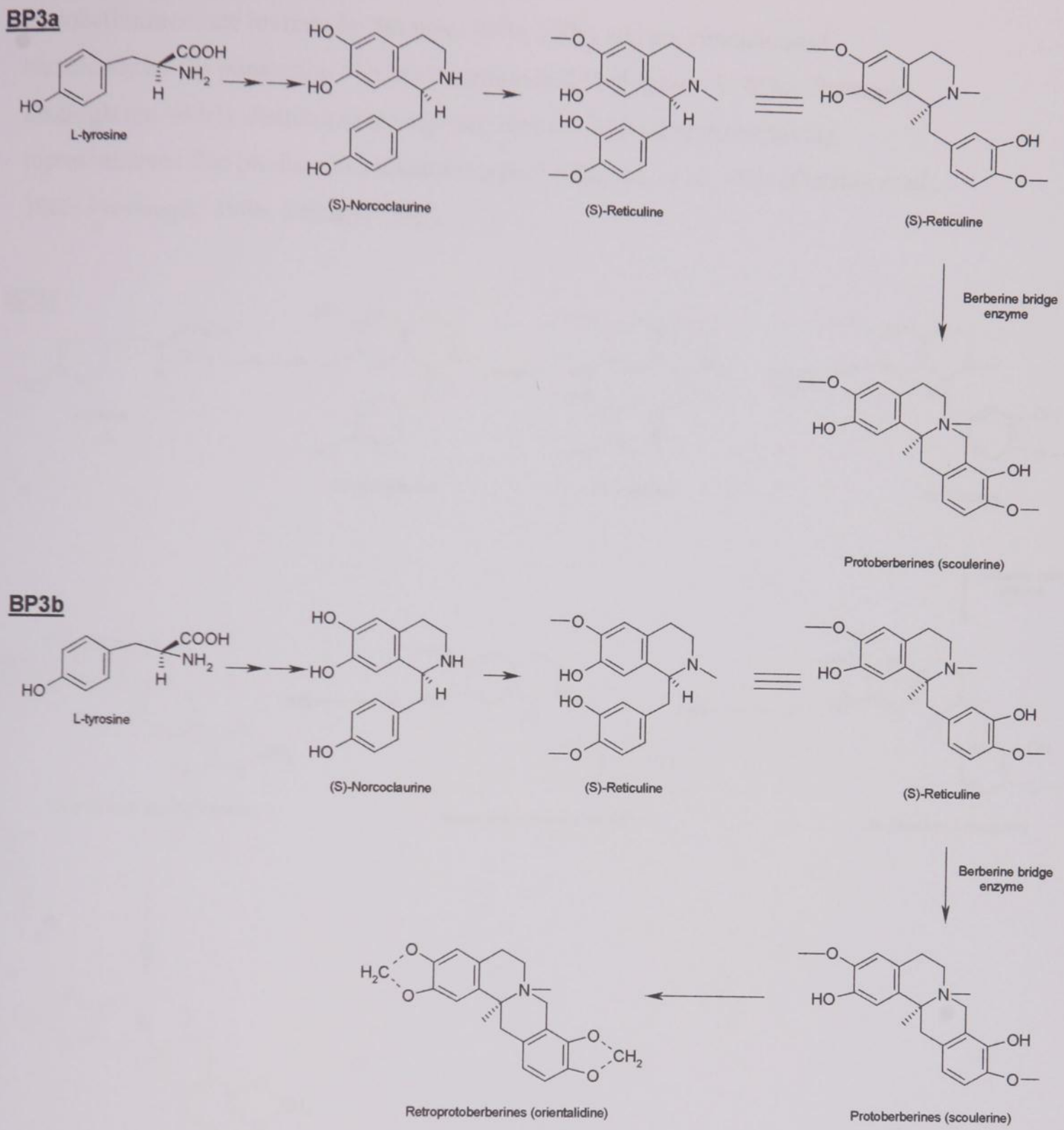


Figure 4.7 Biogenetic pathways responsible for the synthesis of protoberberine (BP3a) and retroprotoberberine alkaloids (BP3b).

(iv) Protopines

The c.20 naturally occurring protopine type alkaloids are distributed in the plant families Berberidaceae, Fumariaceae, Papaveraceae, Ranunculaceae and Rutaceae (see review by Onda & Takahashi, 1988). Protopines are synthesised directly from

protoberberines (see reviews by Santavy, 1970; 1979) and are characterised chemically by the possession of a ketone group at C14 (Figure 4.8 BP3c). Protopine alkaloids are widely distributed throughout *Papaver* with all sections having representatives that produce this alkaloids type (Phillipson, *et al.*, 1981; Oztekin *et al.*, 1985; Preininger, 1986; Tetenyi, 1993).

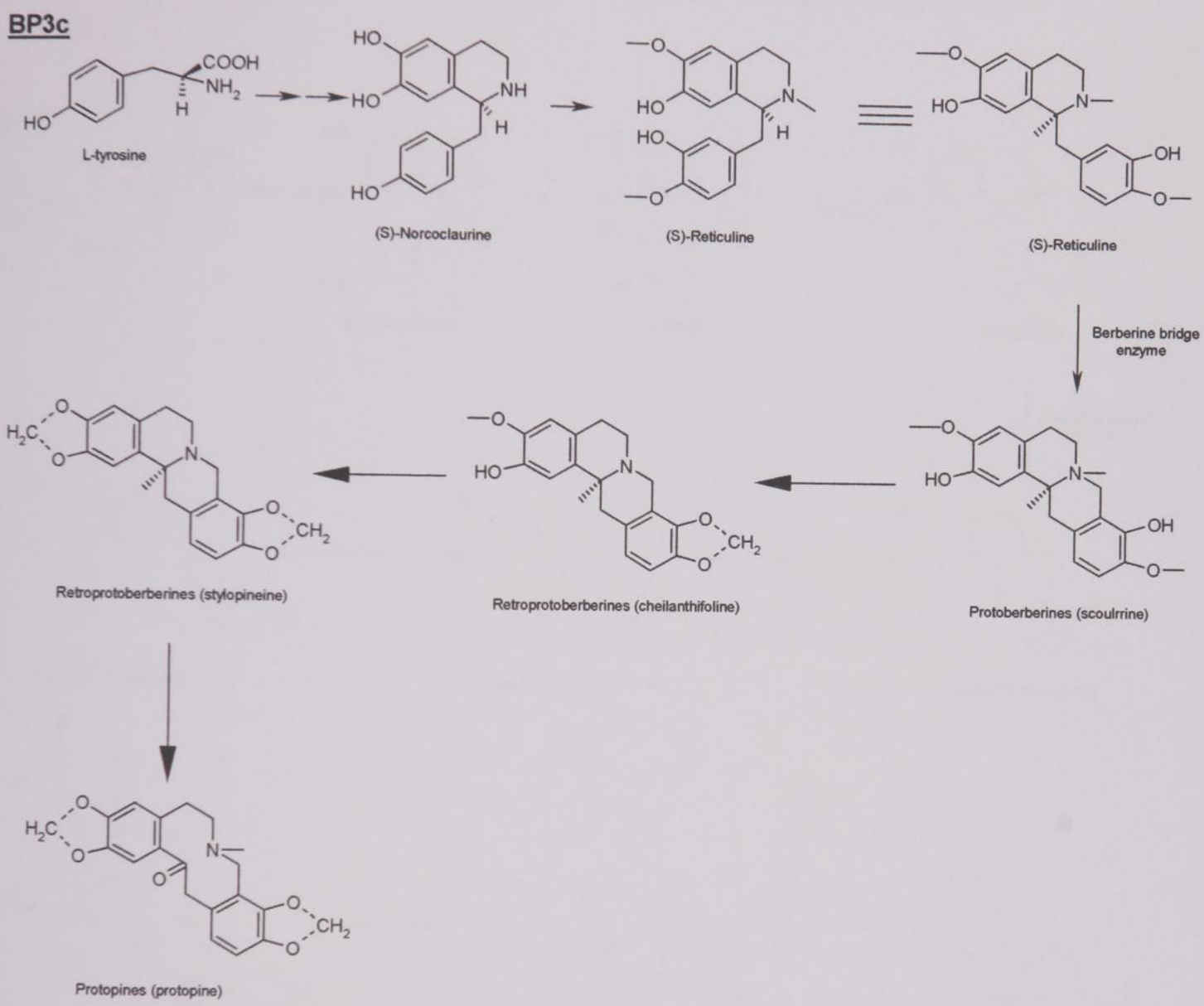


Figure 4.8 Biogenetic pathways responsible for the synthesis of protopine alkaloids (BP3c).

(v) Benzophenanthridines

Benzophenanthridine alkaloids have been reported in the Capifoliaceae, Fumariaceae, Meliaceae, Papaveraceae and the Rutaceae. Sixteen genera synthesise these alkaloids including *Argemone, Eschscholtzia, Meconopsis, Papaver*, and *Stylomecon* (see

review by Simanek, 1985). Benzophenanthridine alkaloids are synthesised from protoberberines through protopines by the cleavage of the N-C-6 bond and the formation of a new bond between C-6 and C-13 (Simanek, 1985). Figure 4.9 details the production of benzophenanthridine alkaloids (BP3d). Benzophenanthradines have been reported for all sections of *Papaver* except sections *Californicum, Pseudopilosa* and *Horrida* (Maturova, *et al.*, 1966; Oztekin *et al.*, 1985; Preininger, 1986; Tetenyi, 1993).

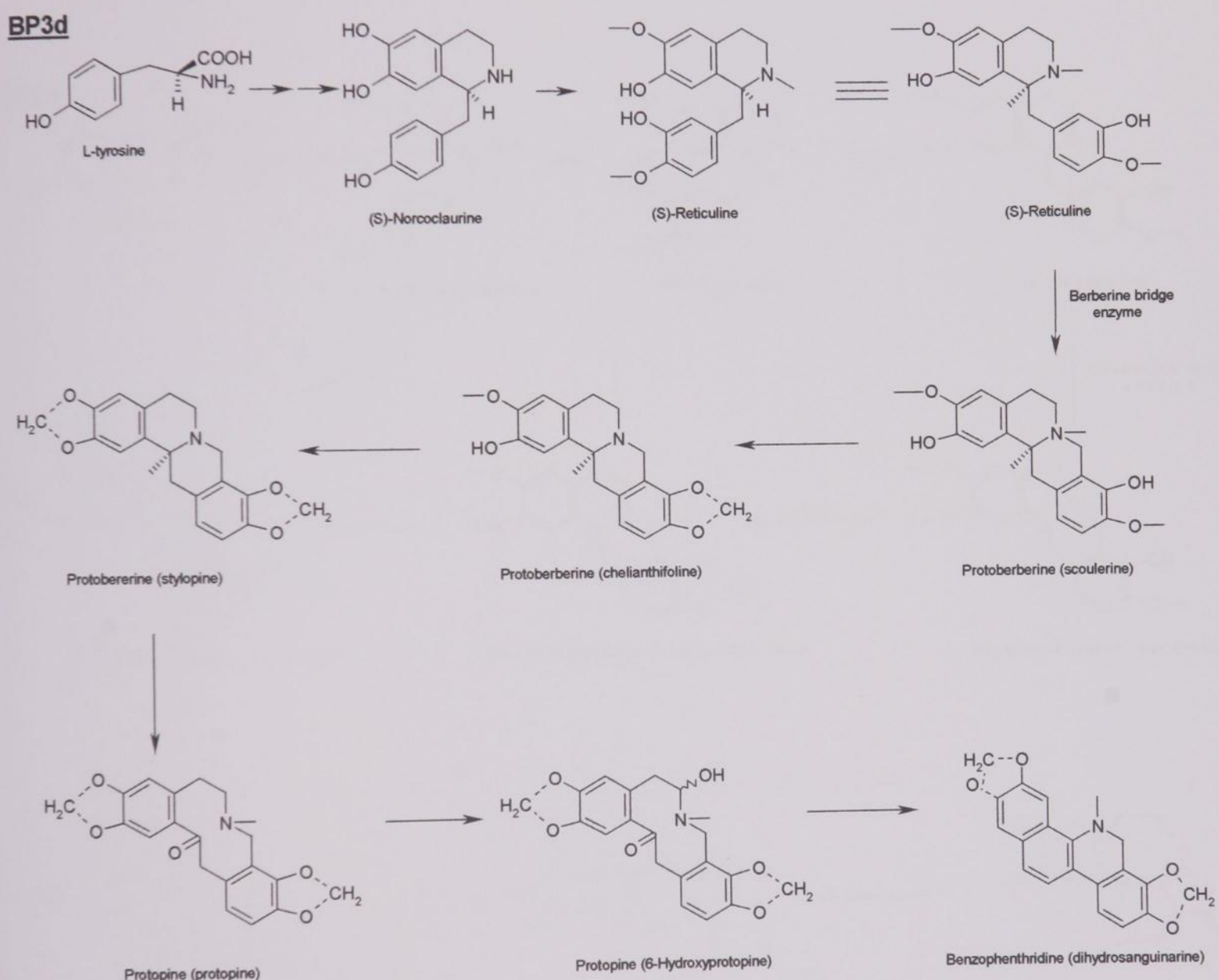


Figure 4.9 Biogenetic pathways responsible for the synthesis of benzophenanthridine alkaloids (BP3d).

(vi) Rhoeadines

In addition to benzophenanthridine alkaloids, protopines (from protoberberines) provide the molecular skeleton for the production of rhoeadine type alkaloids

including papaverrubines (Ronsch, 1986; Figure 4.10 BP3e). The 25 naturally occurring rhoeadine alkaloids discovered to date are found only within members of *Meconopsis, Papaver* and *Bocconia frutescens* L. (Cordell, 1981; Ronsch, 1986). Rhoeadine alkaloids such as rhoeadine and alpinigenine are characterised by the possession of a seven-membered N-heterocyclic ring. Rhoeadine alkaloids are widely distributed throughout *Papaver* and all sections of the genus produce this alkaloid type (Santavy, 1979; Cordell, 1981; Preininger, 1986; Tetenyi, 1993).

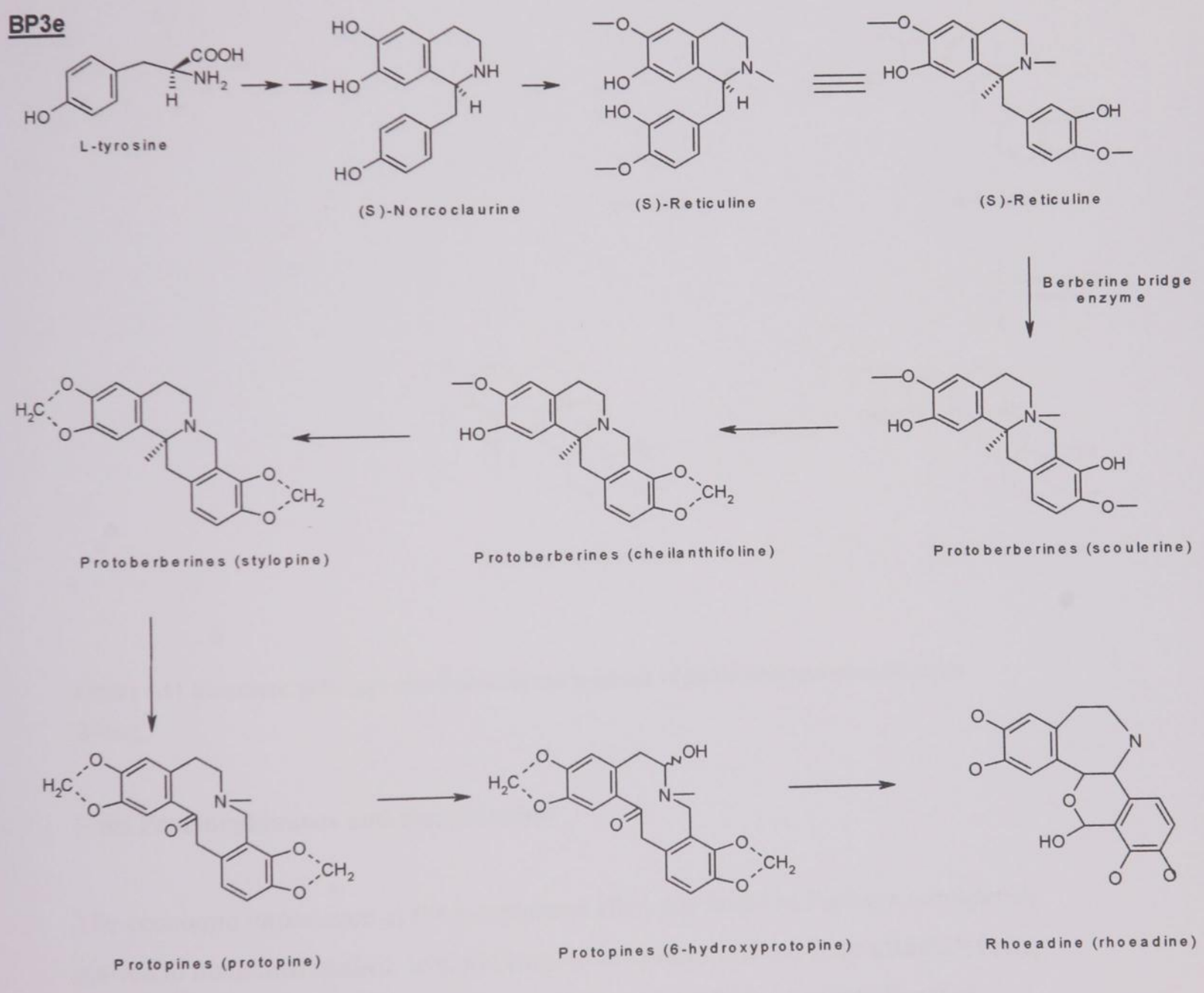


Figure 4.10 Biogenetic pathways responsible for the synthesis of rhoeadine alkaloids (BP3e).

(vii) Phthalideisoquinolines

Phthalideisoquinolines are another group of alkaloids derived from protoberberines by oxidative modification (Figure 4.11, BP3f) and are found in plant families such as the Berberidaceae and Papaveraceae (Cordell, 1981). Phthalideisoquinolines, which have a limited distribution within the genus, have been reported in sections *Meconidium, Pilosa, Rhoeadium* and *Papaver* (Phillipson *et al.*, 1981; Sariyar *et al.*, 1983; Preininger, 1986; Tetenyi, 1993).

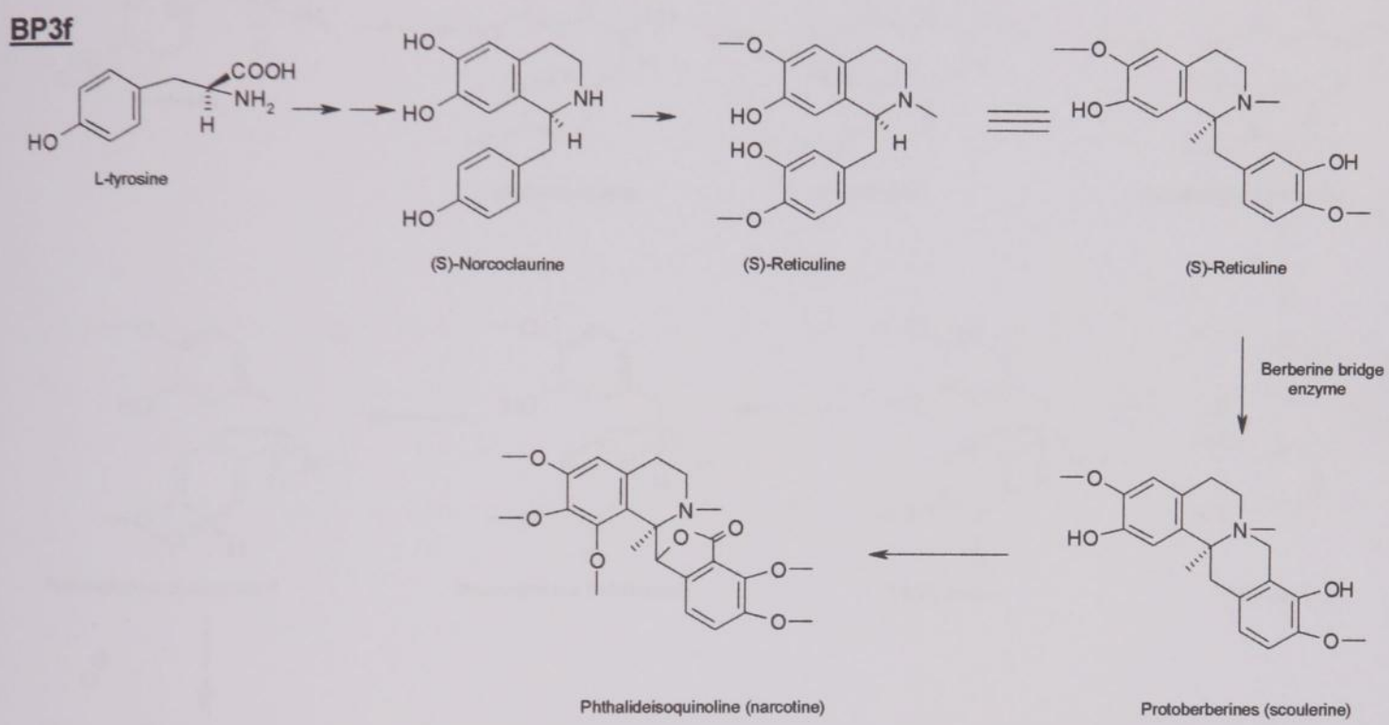


Figure 4.11 Biogenetic pathways responsible for the synthesis of phthalideisoquinoline alkaloids (BP3e).

(viii) Promorphinanes and morphinanes

The economic importance of the morphinane alkaloids found in *Papaver somniferum* has led to numerous studies, with the enzymatic synthesis of the these alkaloids being almost completely elucidated (see review by Kutchan, 1998). As with the other biogenetic groups of *Papaver*, reticuline provides the molecular skeleton for promorphinane and morphinane type alkaloid synthesis. One critical difference however is that the (R)-isomer of reticuline is the key intermediate compound.

(S)-reticuline is converted to (R)-reticuline through the 1,2-dehydroreticulinium ion (Figure 4.12, BP4a). Morphinanes have limited distribution within *Papaver* and are found only in section, *Meconidium, Oxytona, Papaver, Pilosa* and *Rhoeadium.* The precursors of morphinanes the promorphinanes have thus far been reported in sections *Argemonidium, Meconella, Meconidium, Oxytona, Papaver, Pilosa* and *Rhoeadium* (Santavy, 1979; Oztekin *et al.*, 1985; Preininger, 1986 Tetenyi, 1993).

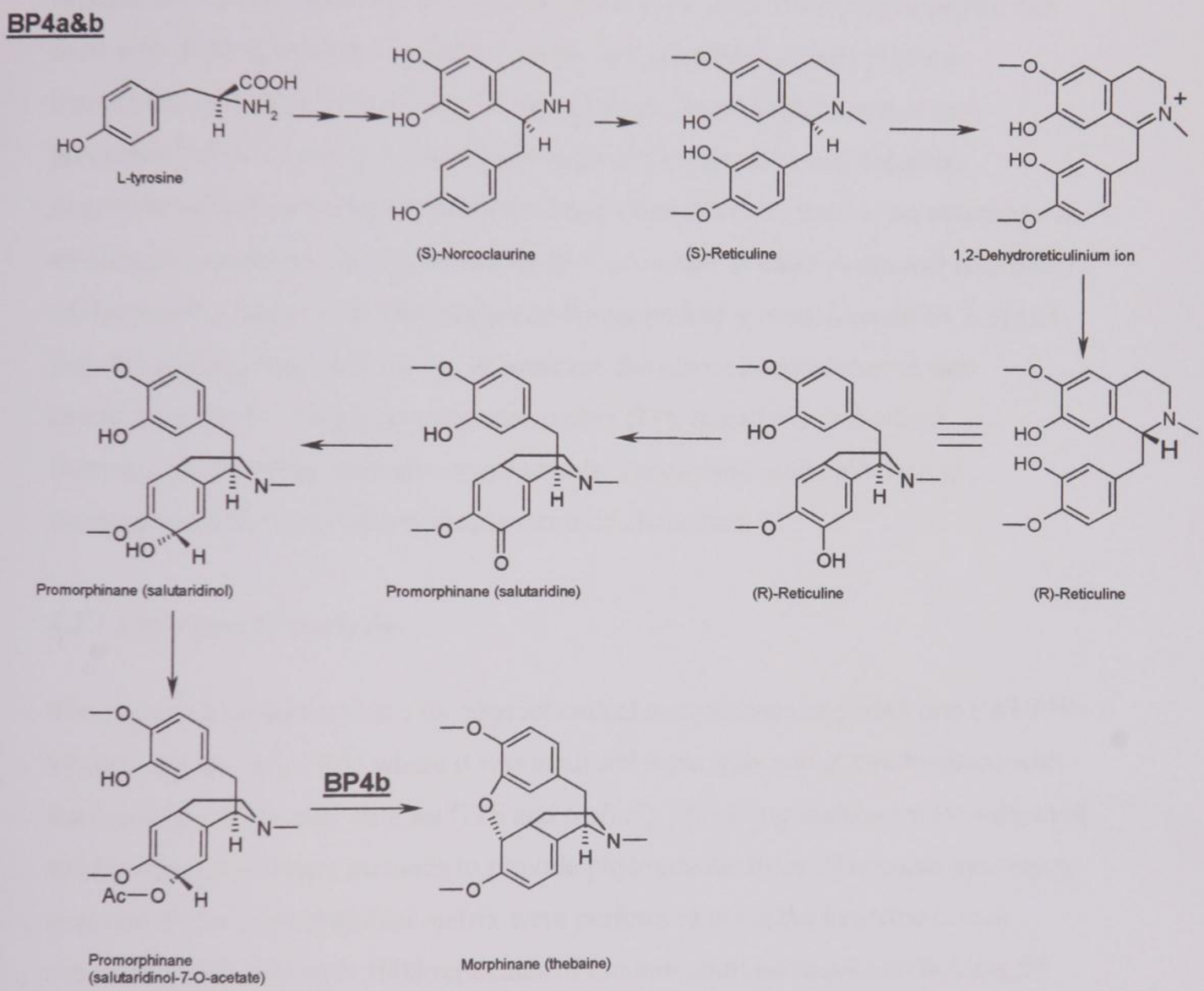


Figure 4.12 Biogenetic pathways responsible for the synthesis of promorphinane (BP4a) and morphinane (BP4b) alkaloids.

alkaloid (BP4b; Kutchan, 1998). Morphinane alkaloids have limited distribution within *Papaver*, with only five of the seven sections that produce promorphinanes, reportedly producing morphinane alkaloids, such as *Meconidium, Oxytona, Papaver,*

Pilosa and *Rhoeadium* (Santavy, 1979; Oztekin *et al.*, 1985; Preininger, 1986 Tetenyi, 1993).

4.2 Methods

Thirteen unordered characters based on individual biosynthetic pathways (described in Section 4.1.3) were scored for each accession used in the molecular analysis. For each individual accession a literature search was conducted and a list of the biosynthetic pathways responsible for the alkaloids found in that species was produced (Table 4.1), which formed the basis of a presence/absence matrix. *Argemone mexicana* was also scored for these characters and used as an outgroup. In an attempt to ascertain the taxonomic level most suited to these characters parsimony analysis of the matrix was first conducted for individual species (Appendix 2.2) and then for each section (Table 4.1). In addition, the phytochemical matrix was combined with the combined molecular matrix (ITS & *trnL-F*). Individual biosynthetic pathways were also mapped onto a combined molecular tree to investigate further their potential as systematic characters.

4.2.1 Phylogenetic analysis

The matrix of character states for phytochemical analysis was imported into PAUP™ v4.0b10 (Swofford, 2003) where it was analysed separately and in combination with the combined molecular data set (ITS and *trnL-F*). Resulting matrixes were subjected to maximum parsimony methods to provide phylogenetic trees. Maximum parsimony analyses of the phytochemical matrix were performed using the heuristic search algorithms of PAUP with 1000 replicates of random addition sequence (holding 25 trees at each replication) and with tree bisection reconnection (TBR) branch swapping on multiple trees (multrees on), permitting ten trees to be held at each step with TBR branch swapping. Clade support was examined using 1000 bootstrap replicates of a heuristic search with 100 replicates of TBR branch swapping on multiple trees. In an attempt to visualise the evolution of some of the major diagnostic characters for *Papaver*, these characters were mapped onto the combined molecular tree (Chapter 2, Figure 2.7). Detailing the CI and RI values for individual characters is useful in

determining whether or not those characters are homologous or homoplasious with respect to a group of taxa.

4.3 Results

4.3.1 Cladistic analysis of phytochemical characters

The phytochemical matrix had 12 characters all of which were parsimony informative. The heuristic search yielded 126 equally most parsimonious trees (Figure 4.13) of 19 steps with a consistency index (CI) of 0.579 and a retention index (RI) of 0.945. CI and RI values for individual phytochemical characters are shown in Table 4.2. The trees obtained from the analysis of phytochemical characters for individual species are poorly resolved and consist of a large polytomy. Within this polytomy three distinct groups are present. The first consists of the species of section *Meconella* (<50% bootstrap support but present in all equally parsimonious trees) demonstrating that these species may be homogenous with respect to the types of alkaloids they produce. The second group comprises some species of sections *Meconidium, Oxytona* and *Papaver* (<50% bootstrap support but present in all equally parsimonious trees). The third group comprises sections *Argemonidium, Carinatae, Horrida, Pseudopilosa* and *Rhoeadium,* along with the other remaining species of sections *Oxytona* and *Papaver* (<50% bootstrap support but present in all equally parsimonious trees). The positioning of species of sections *Oxytona* and *Papaver* in the second and third group suggests that species within these sections are heterogeneous for alkaloid production. *Papaver dubium, P. litwinowii, P. pumbifidum* (all section *Rhoeadium*), *P. californicum* (section *Californicum*), *P. pilosa* and *P. spicatum* (section *Pilosa*) are unresolved.

The biogenetic groups for individual species were grouped into their respective taxonomic sections (Table 4.1) and the resulting matrix was subjected to parsimony analysis using a heuristic search. The analysis yielded 40 equally most parsimonious trees of 17 steps (one of which is shown as figure 4.14) with CI and RI values of 0.706 and 0.808 respectively. Within the tree a polytomy is resolved consisting of section *Argemonidium,* a group comprising the monotypic sections of *Papaver* (i.e.

sections *Carinatae, Californicum* and *Horrida*) and a group comprising sections *Meconella, Meconidium, Oxytona, Papaver, Pilosa, Pseudopilosa* and *Rhoeadium* (<50% bootstrap support but present in all equally parsimonious trees). Within this group section *Pseudopilosa* resolves sister to the other sections (<50% bootstrap support but present in all equally parsimonious trees). Section *Meconella* is sister to a group comprising *Meconidium, Oxytona, Papaver, Pilosa* and *Rhoeadium* (64% bootstrap support), and within this group a group comprising sections *Oxytona* and *Pilosa* are sister to sections *Meconidium, Papaver* and *Rhoeadium* (62% bootstrap support).

The combined molecular and morphological analysis produced 702 trees (not shown), with a length of 1693, CI=0.581 and RI=0.796. Adding phytochemical data to the combined ITS and *trnL-F* matrix produced no difference in the topology of the resulting trees. This is due to the small number (11) of informative characters provided by the phytochemical data as apposed to the 374 informative characters provided by the combined molecular data. The topology of the combined molecular/phytochemical trees is exactly the same as that of the trees generated from molecular characters alone.

A number of characters have CI and RI values (Table 4.2) equal to 1.0 and describe characters, which are diagnostic to single sections such as pavine, isopavine, aporphine and proaporphine biosynthesis. A number of characters have low CI and this may be interpreted as indicating that the characters are homoplasious and indicative of convergent evolution. Certain alkaloid types such as rhoeadines and protopines are found in all sections of the genus and this character may be considered diagnostic with respect to the genus. The low CI values for phthalideisoquinoline, promorphinane and morphinane production indicates homoplasy with respect to these characters. These characters are phylogenetically informative and were chosen to be mapped onto the combined molecular tree. In addition the accumulation of retroprotoberberines seems to be significant as this type of alkaloids is found only in four of the eleven sections. This character was also chosen to map onto the combined molecular tree. The phytochemical biogenetic groups mapped onto the molecular tree are shown in Figures 4.15-4.20. Figure 4.15 details the distribution of pavine and

isopavine alkaloids within *Papaver*, which are found within species of section *Meconella* only. Figure 4.16 details sections of *Papaver* that do not accumulate aporphine and proaporphine alkaloids and include sections *Argemonidium, Californicum* and *Horrida.* Figure 4.17 details the distribution of retroprotoberberine alkaloids within *Papaver.* A group comprising sections *Carinatae, Meconidium, Oxytona, Papaver* and *Rhoeadium* produce this alkaloid type. Section *Pilosa,* which is a member of this group, does not produce this alkaloid type. Figure 4.18 details the distribution of phthalideisoquinoline alkaloids within *Papaver.* A group comprising sections *Carinatae, Meconidium, Papaver, Pilosa* and *Rhoeadium* produce this alkaloid type. Section *Oxytona*, a member of this group does not produce this alkaloid type. Figure 4.19 details the distribution of morphinane alkaloids within *Papaver.* A group comprising sections *Carinatae, Meconidium, Oxytona, Papaver, Pilosa* and *Rhoeadium* produce this alkaloid type.

4.4 Discussion

Figure 4.13 shows one of the 126 trees obtained from the cladistic analysis of phytochemical characters. The small number of characters chosen for cladistic analysis may explain the poor resolution within the cladogram. From a phylogenetic and taxonomic point of view the tree is not well resolved or supported. Many of the groups within the tree have very little bootstrap support and many of the sections are heterogeneous for alkaloid production. Those sections which are monophyletic (as demonstrated by the molecular analysis; Chapter 2) do not group together and include *Meconidium, Papaver, Oxytona* and *Rhoeadium.* This again not only demonstrates that many of the species within these sections are heterogeneous for alkaloid production but that the resolution of alkaloid biosynthetic pathways as cladistic characters are not particularly suited for examining relationships at the species level.

To investigate this further all species were grouped into their respective sections and a parsimony analysis was carried out at the sectional level. The groupings within these particular trees (one of which is shown as Figure 4.14) are consistent with those from the molecular analysis. One significant group within the phytochemical tree for

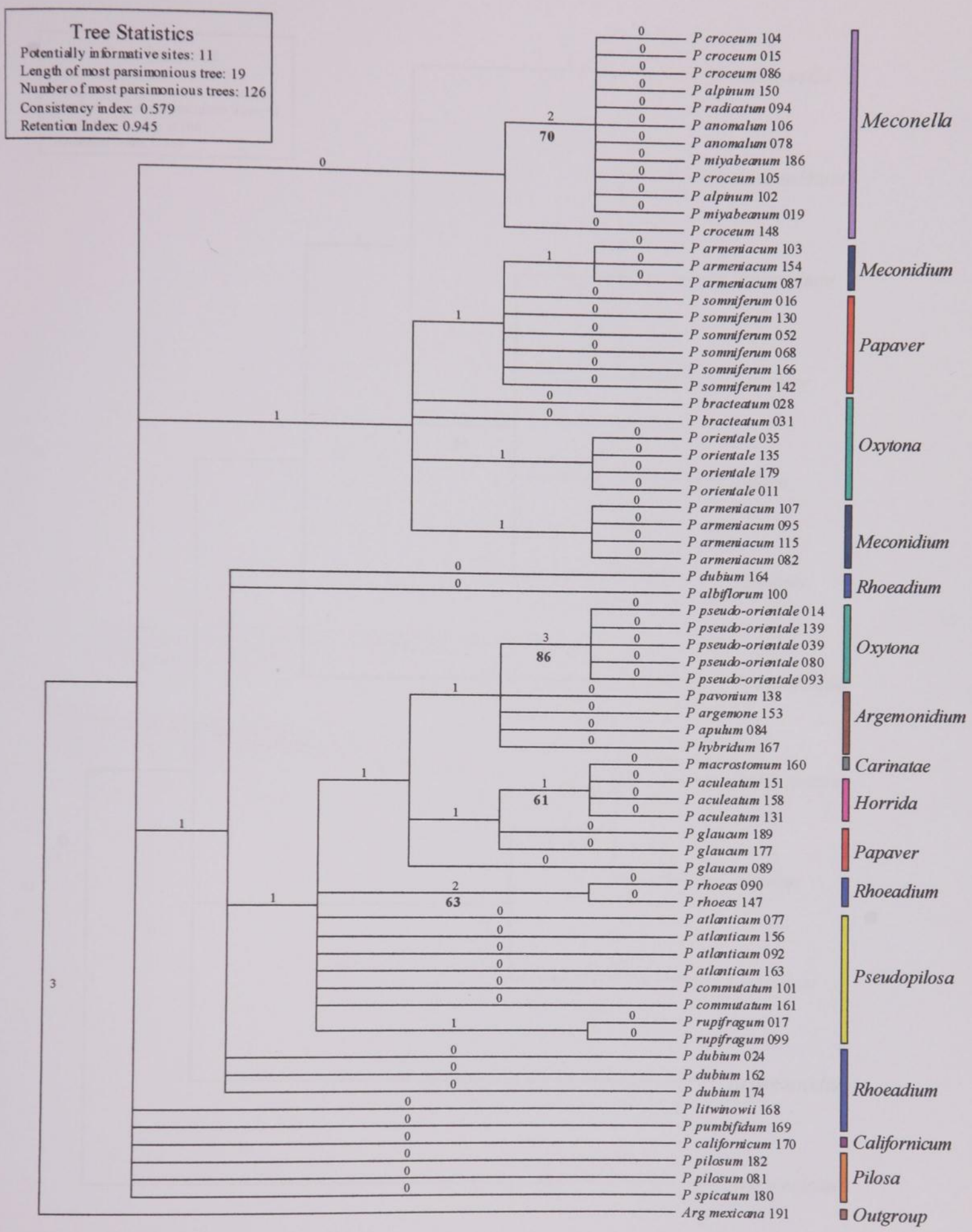


Figure 4.13 One of 126 equally most parsimonious trees from a phytochemical analysis of *Papaver* using 13 characters. Branch lengths are above branches and bootstrap values are below. Tree statistics are also shown.

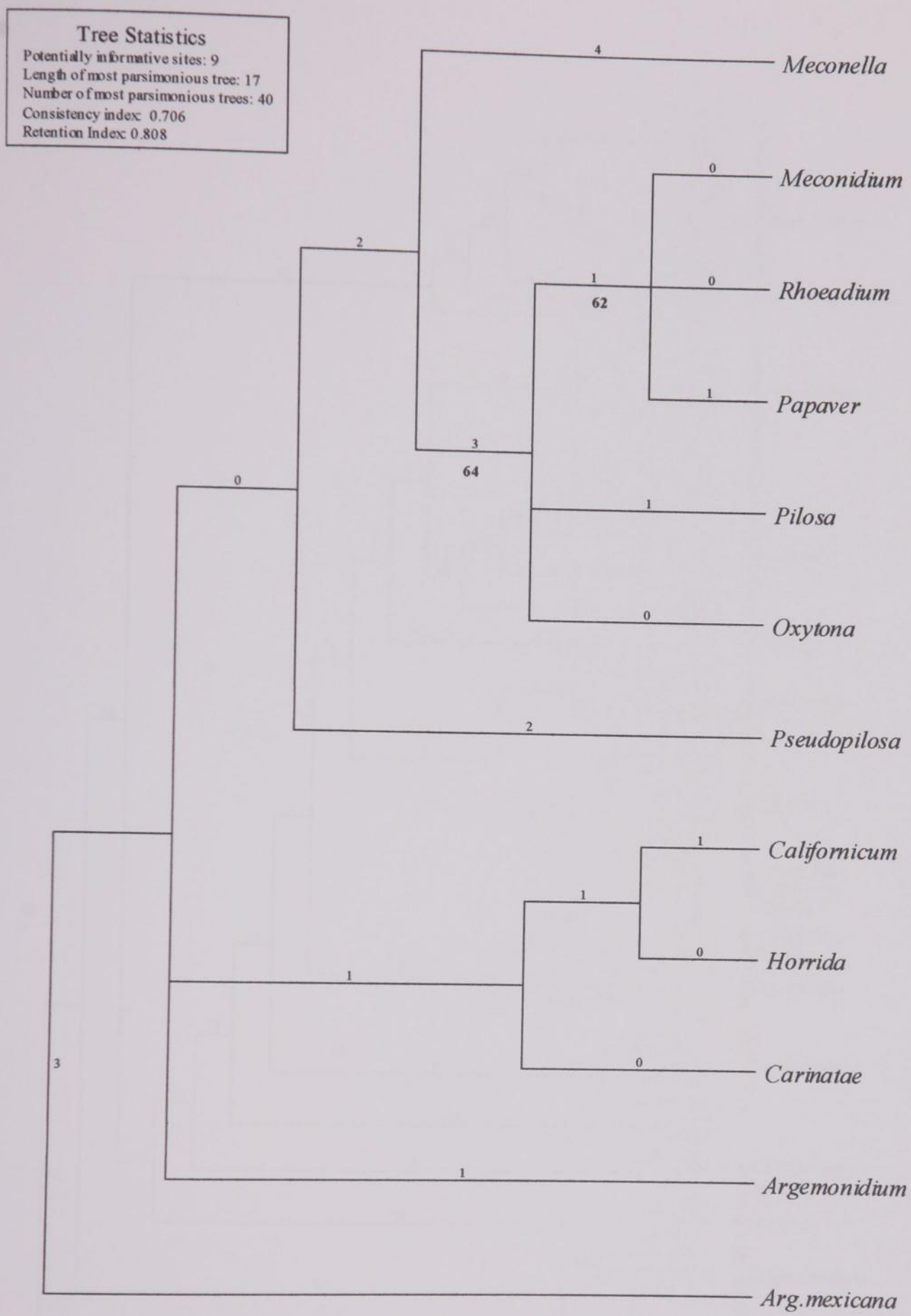


Figure 4.14 One of 40 equally parsimonious trees from a phytochemical analysis of the sections of *Papaver* using 13 characters. Branch lengths are above branches and bootstrap values are below. Tree statistics are also shown.

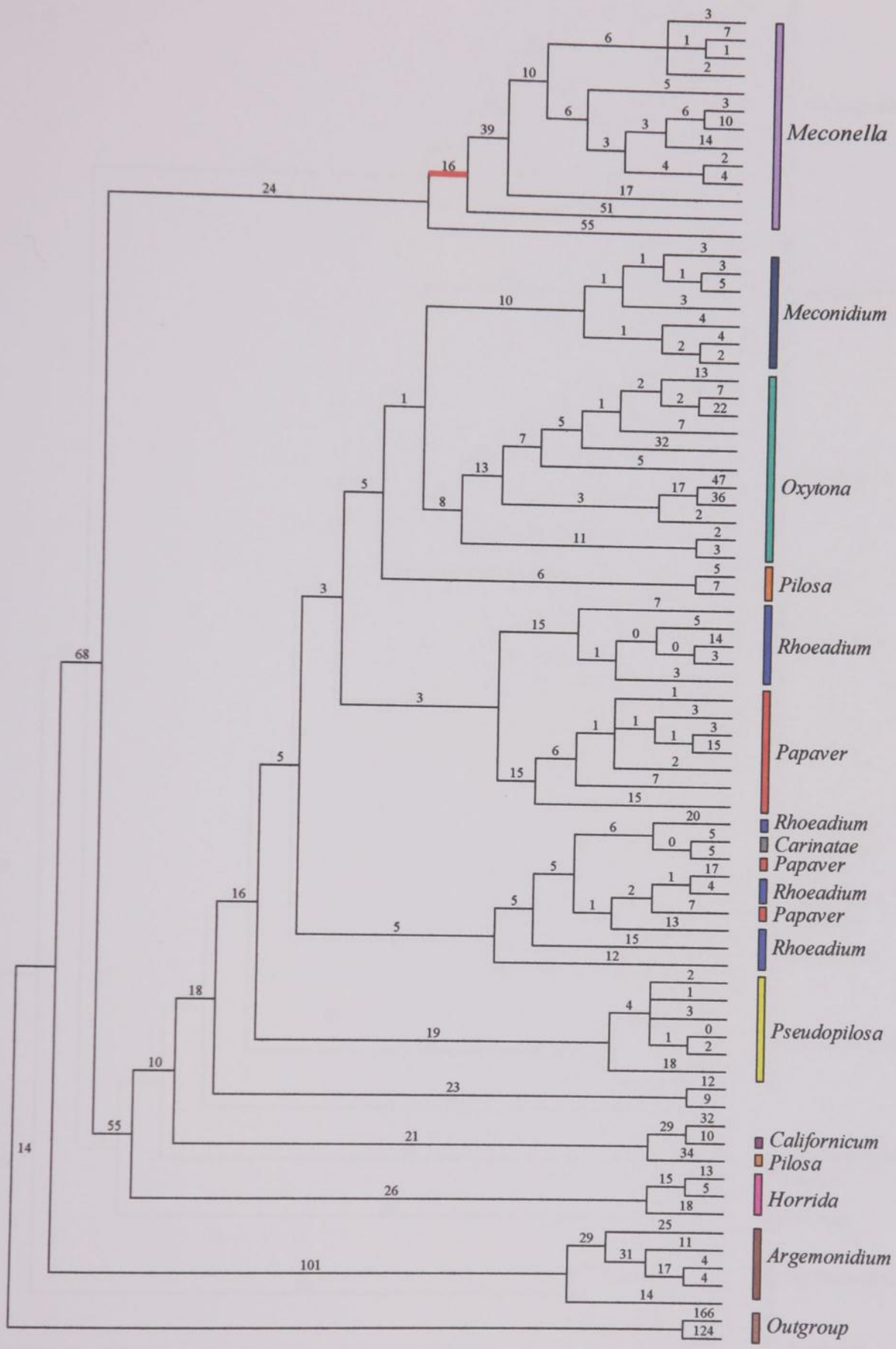


Figure 4.15 Pavine and isopavine biosynthesis mapped onto a combined molecular tree. Red lines represent character state changes and describe sections of *Papaver* whose members produce pavine and isopavine alkaloids.

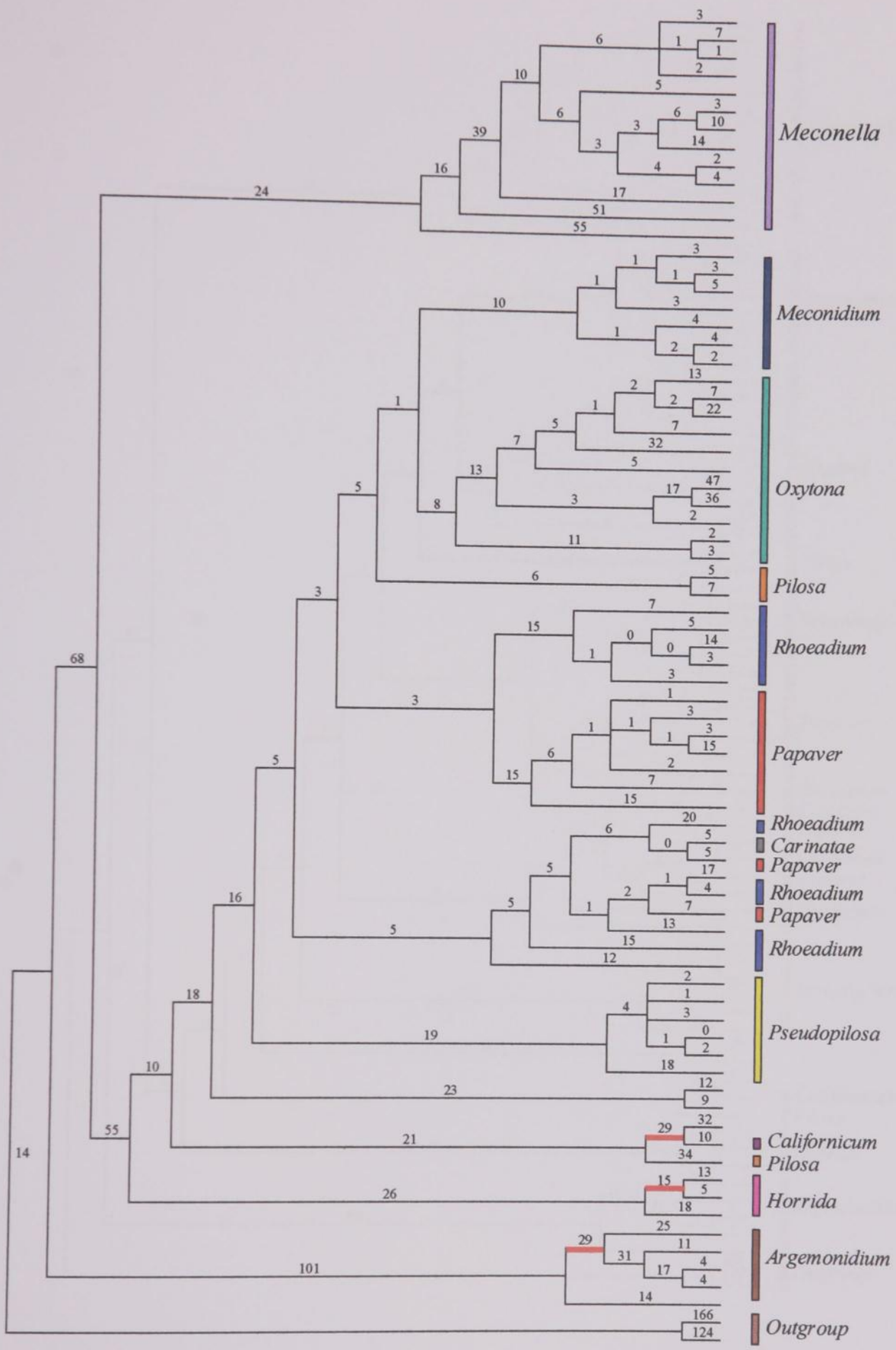


Figure 4.16 Aporphine and proaporphine biosynthesis mapped onto a combined molecular tree. Red lines represent character state changes and describe sections of *Papaver* whose members do not produce aporphine and proaporphine alkaloids.

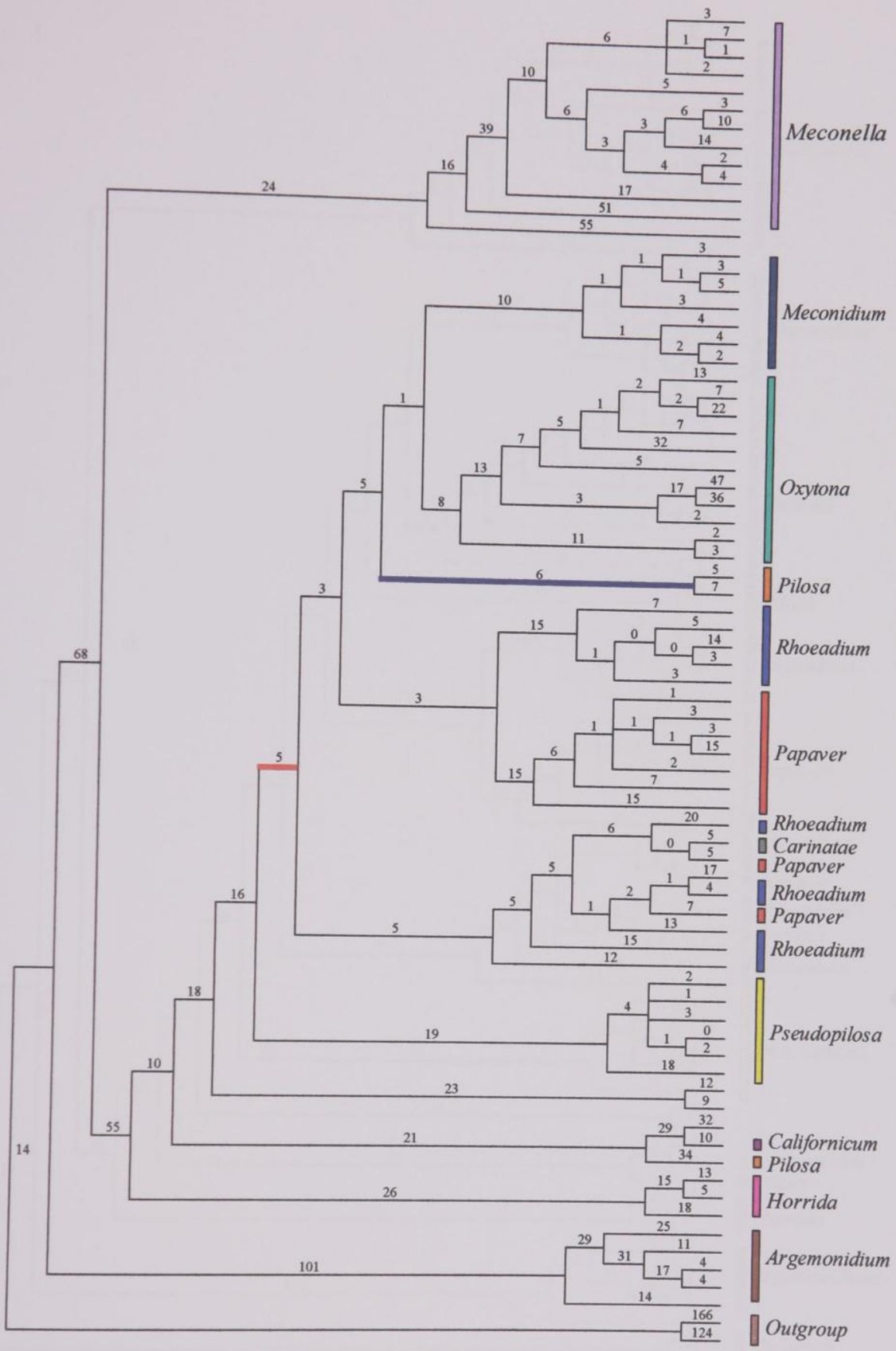


Figure 4.17 Retroprotoberberine biosynthesis mapped onto a combined molecular tree. Red lines represent character state changes and describe sections of *Papaver* whose members produce retroprotoberberine alkaloids. The blue line indicates sections whose members do not produce retroprotoberberine alkaloids.

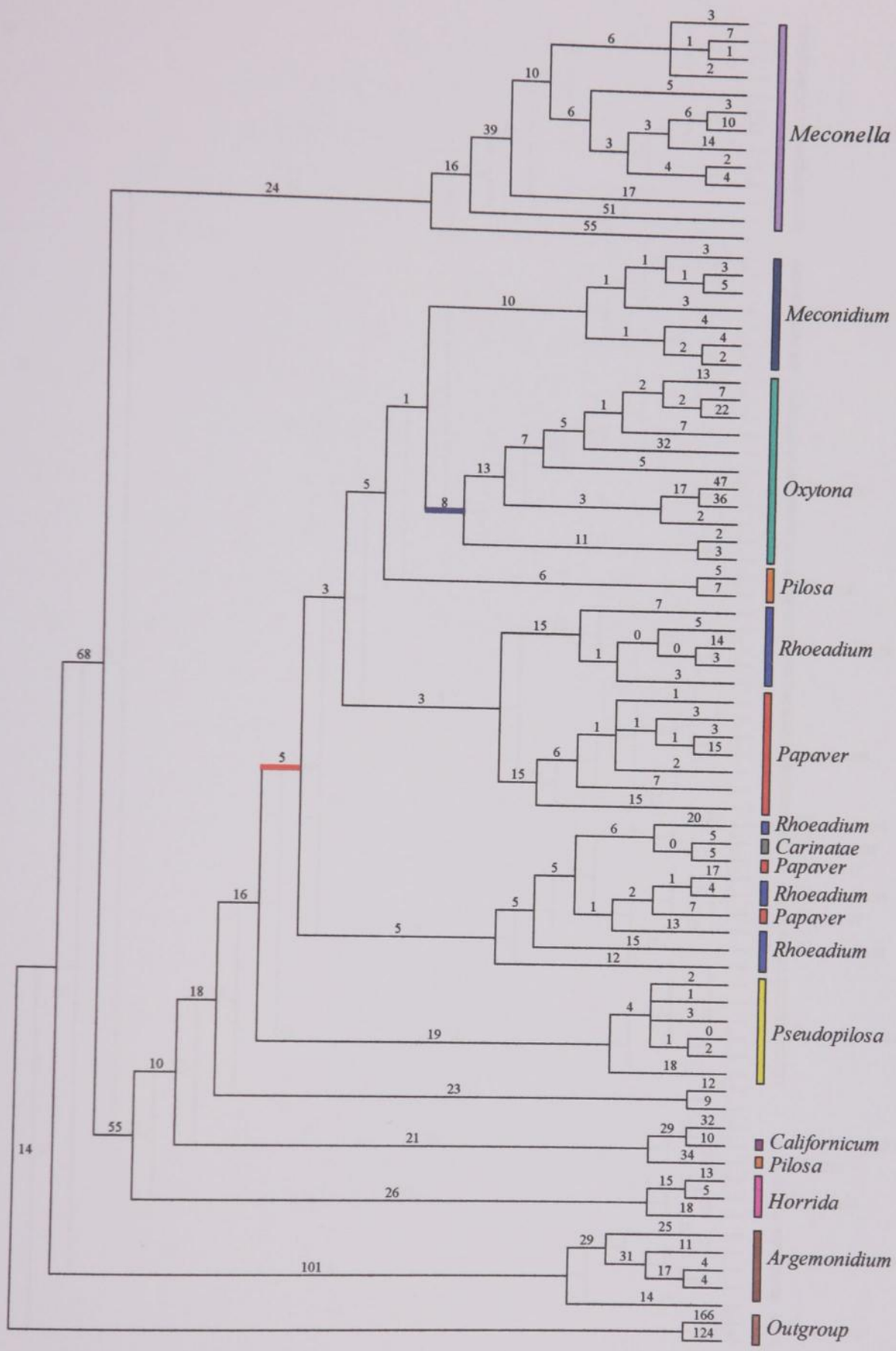


Figure 4.18 Phthalideisoquinoline biosynthesis mapped onto a combined molecular tree. Red lines represent character state changes and describe sections of *Papaver* whose members produce phthalideisoquinoline alkaloids. The blue line indicates sections whose members do not produce phthalideisoquinoline alkaloids.

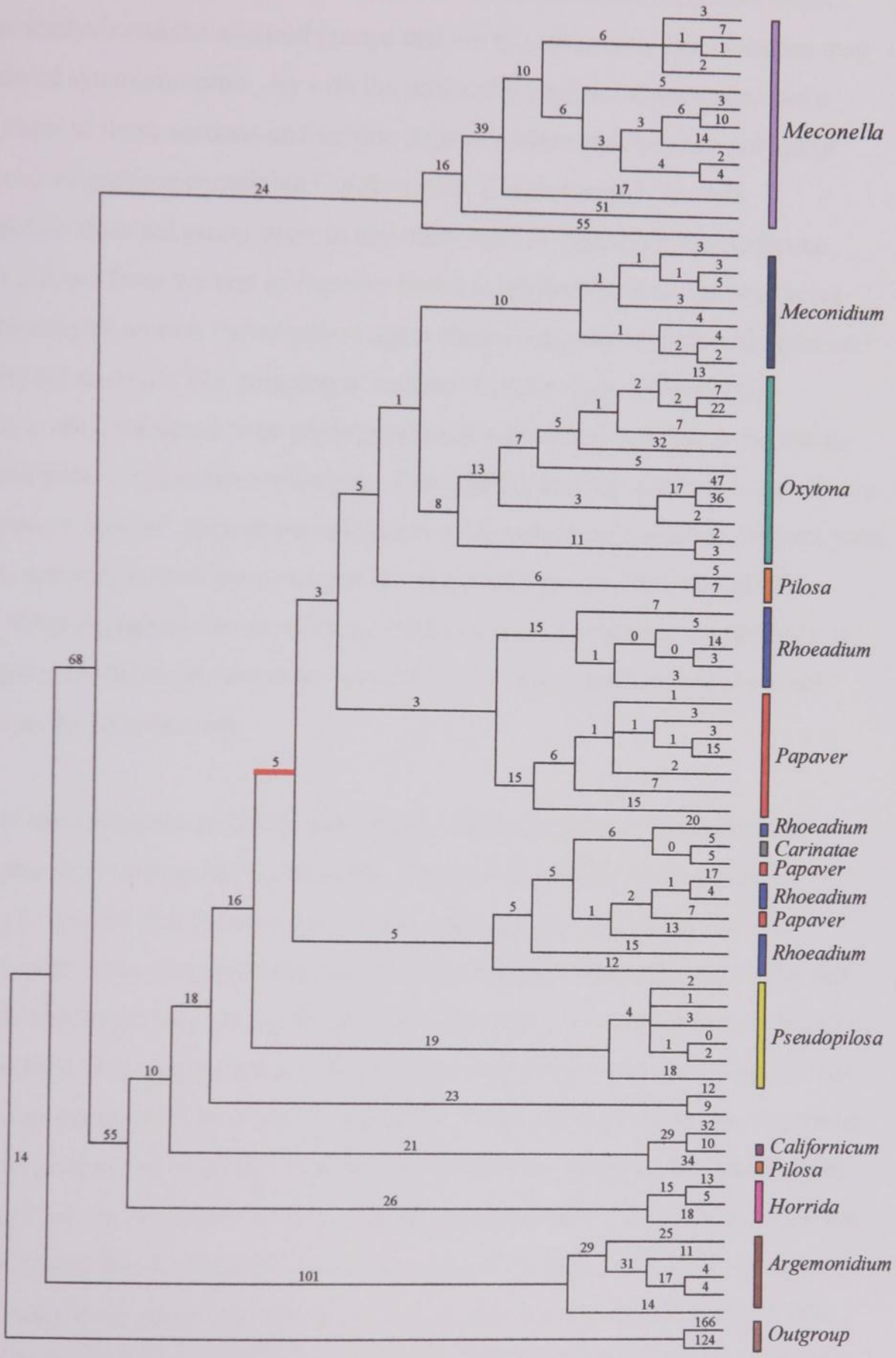


Figure 4.19 Morphinane biosynthesis mapped onto a combined molecular tree. Red lines represent character state changes and describe sections of *Papaver* whose members produce morphinane alkaloids.

individual sections, comprises sections *Meconidium, Oxytona, Papaver, Pilosa* and *Rhoeadium* (64% bootstrap support). These five sections resolved together in the molecular analysis and the alkaloid groups that are in common to these sections may be considered synapomorphic. As with the molecular analysis section *Meconella* resolves sister to these sections and section *Argemonidium* resolves in a polytomy with a group of sections containing *Californicum, Carinatae* and *Horrida. Argemonidium* does not group sister to any other section indicating this particular section is distinct from the rest of *Papaver* based on phytochemical characteristics. The positioning of section *Pseudopilosa* again shows congruence with molecular and morphological analysis. The grouping of sections *Californicum, Horrida* and *Carinatae* is not considered to be phylogenetically significant as it has no bootstrap support and these sections are monotypic. Considering that this analysis is based on a combination of species' phytochemical spectra to represent each section, sections with more than one species will have more of the biogenetic groups represented in the analysis. With regards to the use of biogenetic pathways as cladistic characters it is evident that such characters are more suited for estimating phylogeny at sectional level than to the species level.

Potentially homoplasious and other informative phytochemical characters were mapped onto their corresponding branches. Figure 4.15 details those sections, consisting of species that produce pavine and isopavine type alkaloids. Section *Meconella* is the only section of *Papaver* that accumulates these alkaloids. The fact that pavine and isopavines are not found in the core *Papaver* sections but are found in *Meconella* their sister group and in the outgroup (*Argemone mexicana*) suggests that pavine and isopavine synthesis may be a primitive character lost to the other sections. Conversely, pavine and isopavine biosynthesis could have evolved independently in these groups but this would be a less parsimonious possibility. If we consider pavine and isopavines in terms of molecular complexity, we see that these alkaloids are formed directly from reticuline with often one or two steps (section 4.1.3-i). As the species of *Papaver* diverge and evolve, the biosynthetic pathways and the alkaloid types that are characteristic to those species seem to become more complex.

Figure 4.16 shows those sections of *Papaver* which do not accumulate proaporphine and aporphine type alkaloids. These types of alkaloids are not produced by species of

sections *Argemonidium, Californicum* and *Horrida.* The absence of this alkaloid type in these sections may be interpreted a number of ways. If it is assumed that it is a result of shared ancestry it is evident that all three sections occupy independent sister group positions within the cladograms generated from the molecular analysis. However section *Californicum* is found in North America and *Horrida* is found in South Africa thus it is implausible that these species lack aporphines and proaporphines because of shared ancestry. In addition based on morphological and molecular analysis section *Argemonidium* is distinct from *Papaver* and it is difficult to ascertain whether any characters (morphological or phytochemical) are suggestive of relationship. Alternatively it seems that these sections have simply lost the ability to accumulate these particular alkaloid groups and this pattern is due to convergence.

The distribution of retroprotoberberines (Figure 4.17) is interesting as they are found only within a group of sections that is consistently found within the molecular cladograms. This group comprises sections *Meconidium, Oxytona, Papaver* and *Rhoeadium* and thus the production of retroprotoberberines is an apomorphic character. As detailed in section 4.1.2 (iii) of this chapter retroprotoberberines are formed from protoberberine precursors. This particular group (including section *Pilosa*) is resolved in the analysis of biosynthetic pathways for individual sections (Figure 4.14). In addition, protoberberines are involved in the separate biosynthetic pathway responsible for the production of protopines, benzophenanthridines, rhoeadines and phthalideisoquinolines. This latter pathway is common to the majority of the sections of *Papaver* and thus may be considered symplesiomorphic and these pathways could be considered primitive from a phylogenetic point of view. The biosynthetic pathway responsible for the conversion of retroprotoberberines from protoberberines however is common to the more advanced (interpreted from molecular phylogenies) sections and reflects congruence with respect to these sections between molecular and phytochemical characteristics. Section *Pilosa*, which belongs to this group of sections based on the molecular analysis, does not accumulate retroprotoberberines. This absence probably reflects that the species of *Pilosa* or the progenitors of section *Pilosa* simply lost the ability to employ the particular biosynthetic pathway.

Figure 4.18 details the distribution of phthalideisoquinoline alkaloids within *Papaver*. As with retroprotoberberines, phthalideisoquinoline alkaloids are derived from protoberberines and again are characteristic to the group of sections containing *Meconidium, Papaver, Pilosa* and *Rhoeadium* (section *Oxytona* seems to have lost its ability to accumulate these alkaloids). Although this alkaloid type is characteristic to many genera within the Berberidaceae and Papaveraceae (Cordell, 1981), it is difficult to ascertain from the results of this particular study whether their presence in these genera is due to convergence. Convergence and evolutionary reversal is common within biosynthetic pathways, and it is difficult to ascertain true character homology (Saunders, 1999). However with regards to *Papaver,* it seems that the presence of retroprotoberberines and phthalideisoquinolines represents a divergence from the more common pathway involving protoberberine (described above) and is common to the more advanced sections of *Papaver*.

The group comprising *Meconidium, Oxytona, Papaver, Pilosa* and *Rhoeadium* is again characterised by morphinane alkaloid production (Figure 4.19). Morphinanes are not found in any other genus throughout the plant kingdom and thus their accumulation within this group of sections is considered to be an apomorphic character. There have been no reversals within this group for morphinane production.

In conclusion, alkaloid biosynthetic pathways when used alone as cladistic characters do give some indication of relationship especially at the level of the sectional rank. Trees based on biosynthetic pathways for individual sections (rather than individual species) within *Papaver* are congruent with molecular phylogenetic trees. Congruence between molecular and phytochemical trees exist with respect to the topology of major clades especially that of sections *Meconidium, Oxytona, Papaver, Pilosa* and *Rhoeadium,* indicating a strong phylogenetic relationship between these sections. This indicates that the biosynthetic pathways have value as systematic characters. The data demonstrate that the possession of similar biosynthetic pathways and thus the production of similar alkaloidal spectra are due to shared ancestry when groupings from molecular trees are considered. They also allow an assessment of whether certain pathways are primitive or advanced. It is evident with respect to *Papaver* that an understanding of the pathways involved in alkaloid biosynthesis allows the identification of homoplasious characters by determining at which point

along certain pathways differentiation occurs between different species. Additionally this is demonstrated by examining common biosynthetic pathways found in taxonomically diverse species. The taxonomic and phylogenetic significance of these results are discussed in greater detail in Chapter 5.

CHAPTER 5. Taxonomic implications of molecular, morphological and phytochemical analysis

5.1 Introduction

The results of Chapters 2, 3 & 4 have given fresh insight into the systematic relationships within *Papaver* and allowed the importance of traditionally used traits based on morphology, phytochemistry and biogeography to be evaluated. In many cases support is given to previous taxonomic classifications and views on groupings within the genus based on shared characteristics. However it has been demonstrated that a number of these characteristics have evolved in parallel (due to convergent evolution) and the identification of homoplasious characters allows for a revised classification of the genus and a better understanding of the evolution of many of the sections within *Papaver*.

5.1.1 Implications for section *Argemonidium*

The four species of this annual section (all included in this study) are well differentiated in capsule and petal characteristics (Kadereit, 1986) but are clearly related as demonstrated by the groupings within the molecular phylogenetic trees. From a morphological perspective the characters that have traditionally been used to define this section and its species (Fedde, 1909; Ernst, 1962; Cullen, 1965; Kadereit, 1986) such as the possession of an apical plug, long internodes from the basal rosette, polyporate pollen grains and bristly capsules can be considered to be homologous characters with respect to these species and indicative of shared ancestry. Within *Argemonidium, Papaver apulum* and *P. hybridum* are very similar in both ITS and *trnL-F* sequences indicating a close relationship. These two species differ from *Papaver argemone* and *P. pavonium* in seed morphology (number and shape of cells of seed coat) and the absence of subapical processes on their sepals (Kadereit, 1986). However, *Papaver apulum* shares considerable similarities in phytochemical spectra (Vent, 1972) and pigment distribution (Acheson *et al.*, 1962) with *P. argemone*, which in turn has an obvious affinity with *P. pavonium*, demonstrated by similarities in seed shape and structure. *Papaver pavonium* ssp. *pavonium* is the only

representative of *Argemonidium* that displays filiform rather than clavate filaments and this similarity to the other sections of *Papaver* has been viewed to reflect the sister group nature of the species with respect to the other members of *Argemonidium* (Kadereit *et al.*, 1997). The sister group position of *Papaver pavonium* is also demonstrated by the results of the analyses undertaken in this project. Previous reports on chromosome numbers within this section have shown that a number of these species show a dysploid reduction of the haploid chromosome number from seven to six (discussed further by Kadereit, 1986). *Papaver apulum* is diploid having 2n=12 (Sugiura, 1936; McNaughton, 1960), *P. pavonium* (diploid) has 2n=12 (Suguira, 1931; 1936) and 2n=14 (Podlech & Dieterle, 1969) and *P. argemone* ssp. *argemone* is hexaploid having either 2n=40 (Koopmans, 1954) or 2n=42 (Suguira, 1936; 1940; McNaughton, 1960). *Papaver hybridum* is diploid with 2n=14 with no records of dysploid reduction for this species in the literature. The possession of reduced and unreduced chromosome numbers for *Papaver argemone* and *P. pavonium* has been viewed to reflect a parallel nature of dysploid reduction and not necessarily an indication of shared ancestry directly (Suguira, 1936; Podlech & Dieterle, 1969). However, the extremely close relationships that exist between species within the section, as demonstrated by the results of this analysis, suggest that the disposition for reduction in chromosome numbers may be a heritable, homologous and apomorphic.

Interspecific relationships within *Argemonidium* have not been fully investigated here, but the results of the combined ITS and *trnL-F* matrix show that well resolved topologies can be produced and supported. The aim of this research was to examine the inter-sectional relationships and affinities within *Papaver,* with these gene regions being extremely well suited to this task. Completion of an evaluation of the relationships existing within section *Argemonidium* can only occur when all species (including all subspecies) are included in a phylogenetic analysis with more than one accession for every species being sampled. To complete this, it is suggested that any future molecular work is accompanied by a cytological investigation.

Regarding the position of *Argemonidium* within *Papaver* it is obvious that the species of this section are clearly distinct based on evidence from the molecular, morphological and phytochemical analyses undertaken in this project. The close

relationship between the species of *Argemonidium* and their distinction from the rest of *Papaver* is demonstrated by the highly supported groupings within the ITS, *trnL-F* and combined analyses, but also by a number of diagnostic indels and polymorphic sets of nucleotides found within these regions. The five base pair indel and the two base pair indel at positions 255-260 and 709-711 respectively of the *trnL-F* region, coupled with the GCGATA and the TCTTTTG sequences found at positions 747-752 of the *trnL-F* and 82-90 of the ITS regions respectively, are indicative not only of the strong relationship that exists within *Argemonidium,* but also of the separation (on the molecular level) of this section from the other sections of the *Papaver.* These diagnostic molecular differences can be considered to be highly useful molecular markers for the identification of species of *Argemonidium* and the differentiation of these species from other *Papaver* species.

The molecular separation of *Argemonidium* on the molecular level is mirrored in the results from the parsimony analysis of molecular and phytochemical characters. Although insufficient resolution was obtained from cladograms based on these characters alone to appraise phylogenetic relationships of *Papaver*, one clear trend was observed, i.e. section *Argemonidium* is distinct from both a morphological and phytochemical point of view from the other sections of *Papaver.* Morphologically these species are distinct in their possession of an apical plug, the unusual growth form produced after the formation of the basal rosette of leaves (a long internode followed by followed by shorter internodes), polyporate pollen grains and clavate filaments (filiform only for *Papaver pavonium* ssp. *pavonium*). All other sections of *Papaver* comprise species with 3-colpate pollen grains and a distinct stigmatic disc which is not a continuation of the capsule. Although clavate filaments are present in sections *Oxytona* and *Papaver,* the results of the molecular analysis demonstrate that *Argemonidium* and these sections are not monophyletic and that the similarity in filament structure has arisen due to convergent evolution. Section *Argemonidium* is also characterised by having dark coloured filaments and anthers, but again the presence of such filaments and anthers in other sections of *Papaver* (such as *Carinatae, Oxytona, Papaver* and *Rhoeadium*) is a result of convergence. In addition the tendency for reduction in chromosome number (described above) has thus far only been demonstrated in section *Argemonidium* and in no other section of the *Papaver.* The results of these analyses support the groupings of Kadereit (1988a), of the

distinction of *Argemonidium* into a group on its own within *Papaver* (c.f. Chapter 1, Table 1.11).

Phytochemically, representatives of *Argemonidium* produce a range of alkaloid types, which are generally found in all sections of *Papaver* including a number of other genera within Papaveraceae. These include benzophenanthridine, protoberberine, protopine and rhoeadine alkaloid types (Santavy, 1979; Tetenyi, 1993). Significantly those alkaloid types which are considered advanced such as retroprotoberberines, phthalideisoquinolines and morphinanes are absent from species of *Argemonidium*, supporting its sister group placement within *Papaver*.

From a taxonomic point of view the most significant relationship involving *Argemonidium* is with members of the genus *Roemeria.* This affinity has been suggested by previous authors based on morphological observations (Gunther, 1975; Morales Torres *et al*., 1988) and demonstrated using molecular techniques (Kadereit & Sytsma, 1992; Kadereit *et al.*, 1997). The results of the analyses undertaken in this study support these views. The diagnostic and unique indels and nucleotide sequences described above for *Argemonidium* are found also in the representative of *Roemeria* (*R. refracta*) used in the molecular analyses. This affinity is also demonstrated by morphological characteristics. For example many of the characters that separate *Argemonidium* from *Papaver* are also shared with *Roemeria* including polyporate pollen grains, long internodes from the basal rosette (shared with *R. hybrida*) and clavate dark coloured filaments (shared with *R. refracta*). In addition dysploid reduction in the chromosome number has been reported for the tetraploid *Roemeria hybrida* where accessions have been demonstrated to have either 2n=22 or 2n=24 (Safonova, 1991). The well-supported grouping of *Argemonidium* and *Roemeria* from the ITS, *trnL-F* and combined analysis confirms that these characters are indicative of shared ancestry and relationship.

Regarding the actual evolutionary relationships within the *Argemonidium-Roemeria* group and suggestions for the taxonomic classification of these species, difficulties arise due to incongruence between the ITS and *trnL-F* phylogenetic trees and also between these results and results from the previous molecular analyses of Kadereit & Sytsma (1992) and Kadereit *et al.* (1997). The cladogram produced from the

biparentally inherited ITS analysis clearly shows a similarity between *Papaver pavonium* ssp. *pavonium* and *Roemeria refracta,* with the latter species being sister to this species and resolving within section *Argemonidium.* This placement was also demonstrated by Kadereit & Sytsma (1992) and Kadereit *et al.* (1997). Based on the molecular similarity between *Papaver pavonium* ssp. *pavonium* and *Roemeria refracta,* and the fact that *Papaver pavonium* shares a similar geographical distribution with *Roemeria refracta* and *Roemeria hybrida* Kadereit & Sytsma (1992) and Kadereit *et al.* (1997) postulated that *Roemeria* had arisen from within section *Argemonidium* and most probably directly from *Papaver pavonium* or an ancestor of this species. However from the analyses of the maternally inherited *trnL-F* region, section *Argemonidium* and *Roemeria* are sister groups indicating that *Roemeria* can be considered distinct from section *Argemonidium* but not distinct from *Papaver.*

Incongruence with uniparentally and biparentally inherited regions is sometimes attributed to different parental lineages in allopolyploid formation from hybrids. However since the two species in question are both diploid (Podlech & Dieterle, 1969; Safonova, 1991) polyploidy cannot be used to explain the different positions found within both cladograms. Thus conclusions about the direction of evolution in light of these results depends largely on which tree is preferred. What is certain from the results of this analyses is that section *Argemonidium* and *Roemeria* are more closely related to each other than to any other section of *Papaver,* and based on the ITS and *trnL-F* regions form a well-supported monophyletic group. Future classifications and systematic studies must ensure that the affinity between section *Argemonidium* and *Roemeria* is highlighted and investigated.

Regarding the taxonomic classification of *Argemonidium*, I am of same opinion as George Bentham as outlined in his letter to Asa Gray dated February 1, 1861. ' Where a large group is natural and well defined I do not like lopping off a few species here and there on account of some remarkable exceptional character, for it is that which carried to excess has produced the present lamentable chaos in our botanical system' (quote taken from Stevens, 1997). However the 'remarkable exceptional character' with respect to *Argemonidium* can be applied to a considerable quantity of molecular and morphological characteristics (described above), suggesting that the removal of *Argemonidium* from *Papaver* may be justified and could allow a clearer definition of

Papaver. It is becoming increasingly evident that the stigmatic disc in the various subgroups of *Papaver* is not structurally homologous (Kadereit *et al.*, 1997 and results from this study). Representatives of *Argemonidium* possess a stigmatic disc which is a continuation of the capsule and the results from these analyses suggest that this structure is not homologous with the stigmatic disc displayed by the majority of sections within *Papaver*. This character has traditionally been used to define the genus taxonomically and it is becoming evident that either paraphyletic groups have been classified within *Papaver*, or that obviously related groups have been separated from the genus based only on differences in capsule.

Based on the molecular regions used in this study and morphological characters these two groups of species are clearly related but differ significantly in capsule characteristics (*Roemeria* possesses linear valvate capsules without a stigmatic disc). If so much variation in this capsule is demonstrated in obviously related groups of species then the significance of this character as a means of uniting certain species (without molecular phylogenetic information) becomes less certain. Other examples of seemingly closely related species differing in capsule characteristics are outline below with respect to other sections within *Papaver*. Based on this and the molecular and morphological distinction of *Argemonidium*, it is proposed that this section should be given a higher taxonomic rank within *Papaver*. Whether *Argemonidium* is distinguished from *Papaver* into a separate genus with *Roemeria* (as the results of this analysis are congruent with) would depend on further molecular and cytological investigation and on the opinion on how best to 'chop up' a phylogenetic tree. Based on this I take a conservative view of the results of this analysis and suggest that *Argemonidium* be retained within *Papaver* and elevated to the level of subgenus i.e. *Papaver* subgenus *Argemonidium*. This classification is outlined in view of other significant groupings within *Papaver* and in light of molecular, phytochemical and morphological considerations at the end of this chapter.

5.1.2 Implications for section *Meconella*

The scapose, perennial species of section *Meconella* (represented in this study by *Papaver alpinum, P. anomalum, P. croceum, P. miyabeanum* and *P. radicatum*) form a monophyletic group based on the ITS and *trnL-F* analysis (receiving 100%

bootstrap support in the combined molecular analysis). In both the ITS and *trnL-F* analyses the species of this section form well supported groups and the separation of this section from *Papaver* (excluding section *Argemonidium*) is also supported. Comparing the two gene regions, it is evident that the ITS region contains a higher number of polymorphic sites and yields a better-resolved and supported grouping. From a morphological perspective the characters that have traditionally been used to define this section and its species (Hanlet, 1969; Randel, 1974; Kadereit, 1988a) such as bristly, valvate capsules, simple or dissected pinnatisect leaves, light coloured anthers borne on light coloured filiform filaments and yellow, orange or white wedge-shaped petals, can be considered homologous characters with respect to these species and indicative of shared ancestry.

From a morphological point of view a separation within the section exists corresponding to the amount of leaf dissection displayed by different species. Of the species available for this study *Papaver alpinum, P. miyabeanum* and *P. radicatum* had finely dissected leaves, whereas *Papaver apulum, P. croceum* and *P. nudicaule* had broad leaf lobes. This distinction has previously been noted by Kadereit (1990) and Kadereit & Sytsma (1992) specifically describing *Papaver alpinum.* The authors of these studies regarded finely dissected leaves to be a primitive character and suggest that these species may be closely related to section *Argemonidium*, which consists of species with similar leaf characteristics. Although *Papaver alpinum* separates from and is sister to the other *Meconella* species in the combined analysis, the other representatives with finely dissected leaves show more sequence similarity with the species with broad leaf lobes. This may be interpreted as indicating the more advanced broad lobes have originated from a lineage of species with dissected leaves and the results of this analysis support the views of Kadereit, (1990) and Kadereit & Sytsma (1992) that finely dissected leaves are a plesiomorphic character. The combination of dissected and broad leaf species within the main group of *Meconella* species in the trees produced from the molecular analysis implies that the origin of the broader leaf within this section occurred on more than one occasion or that a reversal back to the more primitive state has occurred in some species. However as demonstrated by the separation of *Papaver croceum* (accession 105) from the other *P. croceum* representatives (although they are clearly similar from a morphological point of view) the suitability of using ITS and *trnL-F* to examine interspecific relationships

within *Meconella* is questioned. Insufficient taxon sampling renders any attempt to postulate the relationships existing within the section hypothetical at best. For example the section is widely distributed across Central, Inner and East Asia, Siberia, Scandinavia through Greenland and North Canada, with representatives found also in mountainous regions of Europe and the Rocky Mountains, U.S.A. (Randel, 1974; Kadereit, 1988a). The species of this section included in this study represent limited pockets within this distribution. Additionally only five of the thirty species of this section were available for molecular analysis and many of these exist at variable levels of ploidy (Randel, 1974; Kadereit, 1988a). To obtain a further insight into the phylogenetic relationships within *Meconella* it would be necessary to include all species (if possible) and accompany molecular data with a cytological investigation.

It is clear that nucleotide sequence data taken from ITS and *trnL-F* regions permit a better understanding into the phylogenetic position of this section within *Papaver*. In the trees obtained from both regions and the combined datasets, section *Meconella* resolves sister to the remaining sections of *Papaver* (excluding *Argemonidium*). This specific positioning of section *Meconella* is incongruent with topologies obtained from previous molecular analysis of *Papaver* based on a restriction site analysis of chloroplast DNA (Kadereit & Sytsma, 1992) and an RFLP analysis of the chloroplast gene *trnK* (Kadereit *et al.*, 1997). The results from these published analyses suggest that *Meconella* is sister to all sections of *Papaver* (Kadereit & Sytsma, 1992) and that sections *Meconella* and *Argemonidium* resolve as sister groups to each other (Kadereit *et al.*, 1997). However the branch support for the sister grouping of these two sections (in the latter study) is low (<50% bootstrap support) indicating that this grouping may not be phylogenetically robust. There is a clear separation of sections *Argemonidium* and *Meconella* based on the results reported here, which is well supported in the ITS, *trnL-F* and combined cladogram. Additionally the close affinity of the species of *Meconella* to each other, and their separation from the other sections of *Papaver* is demonstrated by the highly supported groupings within the molecular cladograms and also by a number of diagnostic indels and polymorphic sets of nucleotides found within the regions analysed. These include a TTTTTA sequence found at positions 747-752 of the *trnL-F*, a four base pair indel at positions 248-252 of the *trnL-F* region and a three base pair indel at positions 82-90 of the ITS region. The distinction of section *Meconella* is also demonstrated by phytochemical

characteristics such as the accumulation of a number of pavine and isopavine type alkaloids which are uncommon to the other sections of the genus (Maturova *et al.*, 1966; Randel, 1974).

Based on the topology of the major clades within the molecular trees, it seems that section *Meconella* has arisen from a basal node in the tree and that the species of this section are derived from a separate and relatively older lineage with respect to the other sections of the genus (excluding *Argemonidium*). In addition the Asian representative of *Meconopsis* (*Meconopsis betonicifolia*) included in this analysis resolves as a sister to section *Meconella* and shares the diagnostic indel and nucleotide sequences of *Meconella* as described above.

Regarding the taxonomic placement of section *Meconella* within *Papaver*, it is evident that a significant amount of distinction exists between the two. Although species of section *Meconella* possess a sessile stigmatic disc similar to the stigmatic discs typical of *Papaver*, it has been noted by previous authors (Randel, 1977; Kadereit, *et al.*, 1997) that the stigmatic disc is dissimilar to the type displayed by other sections of *Papaver* (excluding *Argemonidium*). The stigmatic disc of *Meconella* consists in some cases of stigmatic tissue only or there are deep incisions between the stigmatic rays. In addition certain species of *Meconella* have polyporate instead of tricolpate pollen, a characteristic also found in some species of *Meconopsis* and *Papaver* section *Argemonidium*. No species of *Meconella* with polyporate pollen were included in this study. This symplesiomorphic character again suggests a close affinity between section *Meconella* and *Meconopsis*. If the current circumscription of *Papaver* is followed and section *Meconella* is retained within the genus, a strict interpretation of the trees produced in this analysis would imply that *Meconopsis betonicifolia* is also recognised as a member of *Papaver*. To retain *Meconopsis* as a genus would require a separation of *Argemonidium, Californicum, Horrida* and *Meconella* from *Papaver* into different genera or within *Meconopsis*. However one of the limitations of this project was the lack of inclusion of more representatives of *Meconopsis*, without which the origins of the 'fringe' sections of *Papaver* cannot be determined with sufficient confidence. Due to this I am reluctant to suggest an elevation of *Meconella* to the rank of genus and suggest that its distinction from the

core section of *Papaver* be acknowledged at the level of subgenus, i.e. *Papaver* subgenus *Meconella.*

5.1.3 Implications for sections *Californicum* and *Horrida*

Papaver sections *Californicum* and *Horrida* are distributed outside the main range of modern poppies. *Papaver aculeatum* (section *Horrida*) is native to South Africa and is characterised by having an indumentum of relatively long bristles, poricidal capsule, light coloured filiform filaments and anthers. All green parts of plant are covered with patent bristles (Kadereit, 1988c). *Papaver californicum* (section *Californicum*) is characterised by having slender, ribbed, glabrous capsule, a many-flowered racemose inflorescence, light coloured anthers and filaments and valvate dehiscence of capsule and is native to the west coast of North America (Kadereit, 1988b). In the ITS, *trnL-F* and combined cladograms both sections resolve from basal nodes deep within the main group of *Papaver* sections (Clade 3, 100% bootstrap support) and are sister to a 'core' group of *Papaver* sections. *Papaver aculeatum* shares morphological and cytological characteristics with sections *Pilosa* and *Papaver.* Similarities between *Papaver aculeatum* and *P. somniferum* (section *Papaver*) include auriculate-amplexicaulous leaves and a chromosome base number of n=11, both characteristics found only in these two species. However both characters must be viewed not to be homologous and are indicative of convergent evolution in light of the results of the molecular analysis. Similarities between *Horrida* and *Pilosa* include racemose inflorescence, light coloured filiform filaments and the possession of long capsules with flat stigmatic discs (Kadereit, 1988c). *Papaver aculeatum* possesses a chemical composition that resembles that of *P. pilosum* species, both containing salutaridine and aculeatine (Maturova *et al.*, 1966). However, these characteristics are found in a number of other sections and as concluded from the morphological analysis (Chapter 3), phytochemical analysis (Chapter 4) and indicated by the separation of these two sections in the molecular analysis, may be the result of convergent evolution.

Papaver californicum shares characteristics with section *Meconidium* including valvate dehiscence of capsule and light coloured filiform filaments. However the geographic separation and the separation demonstrated on a molecular level questions

whether these characters are truly symplesiomorphic. Phytochemically *P. californicum* is characterised by the presence of rhoeadine and protopine alkaloids, and muramine and bezylisoquinoline laticerine (Santavy 1979), the later being characteristic of section *Pilosa* (Takao *et al.*, 1976) but again these similarities may be the result of convergence rather than relationship.

The results from the molecular analysis support the view of Kadereit *et al.*, (1997) that *Stylomecon heterophylla* arose from within *Papaver* and should not be considered as a genus. *Stylomecon heterophylla* and *Papaver californicum* share a similar distribution with both being native to California and found growing in similar habitats (Kadereit, 1988b). Morphological similarities between these species include leaf shape and other vegetative characters (Ernst, 1962). The two species are differentiated by differences in capsule, with *Stylomecon heterophylla* possessing a distinct style, which is similar to that displayed by some representatives of *Meconopsis*. In the ITS, *trnL-F* and combined analysis both species form a well-supported group (100% bootstrap support in the combines trees) and very little difference in nucleotide sequences between the two was observed. *Stylomecon heterophylla* possesses the four base pair indel diagnostic to sections *Meconella, Californicum* and *Horrida* (including *Meconopsis betonicifolia*) at position 248-252 in the *trnL-F* region. This affinity between the two species has been demonstrated on a molecular level previously (Kadereit *et al.*, 1997). The lack of polymorphic molecular characters between the two species suggests that the divergence of *Stylomecon heterophylla* from *Papaver californicum* (or its ancestor) occurred relatively recently.

Regarding the taxonomic placement of sections *Californicum* and *Horrida*, the results of the molecular analysis can be interpreted in a number of ways. Both sections resolve as sister to the core group of *Papaver* comprising *Carinatae, Papaver, Pilosa, Pseudopilosa, Oxytona, Meconidium* and *Rhoeadium* in a well-supported clade (100% bootstrap support). However sections *Californicum* and *Horrida* possess the characteristic 4 base-pair indel at position 248-252 in the *trnL-F* region, shared with *Meconopsis betonicifolia* and representatives of section *Meconella* and further suggests that *Californicum* and *Horrida* are separate from the main core of *Papaver*. The disjunctive geographic distributions of *Californicum* (North America) and

Horrida (South Africa) indicate a wider distribution of *Papaver* at some point during the evolutionary history of the genus with extinction occurring within North America and Central Africa leaving these two sections isolated (Randel, 1974 & Kadereit *et al.*, 1997). Taking into account the outlying positions of section *Californicum* and *Horrida* in the molecular trees and their present day distribution these two sections may have been derived from a relatively ancient lineages within *Papaver*. A distinction from the core species of *Papaver* is therefore suggested. In addition a separation between *Californicum* and *Horrida* and the core sections of *Papaver* on a phytochemical level exists. Those alkaloids considered advanced and characteristic to the core group sections within *Papaver*, such as retroprotoberberines (present in sections *Meconidium, Oxytona, Papaver* and *Rhoeadium*), phthalideisoquinolines (present in sections *Meconidium, Papaver, Pilosa* and *Rhoeadium*) and morphinanes have not been reported for sections *Californicum* and *Horrida*. Thus their outlying positions within the molecular trees, their disjunct distributions, the similarities in *trnL-F* diagnostic indels with section *Meconella* and the lack of accumulation of 'advanced' alkaloids, suggests that sections *Californicum* and *Horrida* should be separated from the core sections of *Papaver*. In recognition of this and the distinct distribution of these two sections, I propose that sections *Californicum* and *Horrida* be elevated to the rank of subgenus retaining them within *Papaver*, i.e. *Papaver* subgenus *Californicum* and *Papaver* subgenus *Horrida* respectively. The separation of *Stylomecon heterophylla* from *Papaver* is not justified based solely on differences in capsule characteristics. Based on the clear affinity between this species and *Papaver californicum* as indicated by similarities of morphological characteristics, habit, geographical distribution and nucleotide sequences within the ITS and *trnL-F* gene regions I suggest that it should be placed within subgenus *Californicum* as a section and recognised as *Papaver lemonii* Greene. Thus *Papaver* subgenus *Californicum* comprises two sections, i.e., section *Californicum* (*Papaver californicum*) and section *Lemonii* (*Papaver lemonii*), two closely related sections which are separated by differences in capsule characteristics.

5.1.4 Implications for *Meconopsis cambrica*

The results of the molecular analyses reported here highlight a separation of the European representatives of *Meconopsis* (*Meconopsis cambrica*) from the single

representative of Asian *Meconopsis* with the former occupying a well-supported (96% bootstrap support in the combined analysis) sister group position to the remaining sections of *Papaver* (excluding *Argemonidium, Californicum, Horrida* and *Meconella*). This separation was previously demonstrated from a molecular point of view (Kadereit *et al.* 1997) and our results further support the view that two distinct lineages within *Meconopsis* exist and that *Meconopsis* in its current circumscription is neither monophyletic nor distinct from *Papaver*. The separation of the two representatives of *Meconopsis* included in this study is also supported by a difference in diagnostic nucleotide sequence at position 747-752 of the *trnL-F* region. *Meconopsis cambrica* has the sequence TATATA which is found in all other species examined (excluding those from *Argemonidium, Californicum, Horrida* and *Meconella*) and *M. betonicifolia* has TTTTTA which is characteristic of section *Meconella*. Determining the direction of the evolution of plants recognised as within *Papaver* is difficult. The results of this study may be interpreted in two ways. Firstly that *Meconopsis cambrica* arose in parallel to the Asian representatives of *Meconopsis* or secondly that *Papa*ver (more specifically sections *Carinatae, Papaver, Pilosa, Pseudopilosa, Oxytona, Meconidium* and *Rhoeadium*) arose from within a lineage represented today by *Meconopsis cambrica*. Both views were also proposed by Kadereit *et al.* (1997) when interpreting the results of their RFLP analysis of the chloroplast gene *trnK,* with those authors favouring the later view based on geographical, phytochemical and morphological considerations. Thus it would seem that the taxonomic classification of this species (and incidentally *Papaver* itself) is subject to interpretation. If *Meconopsis cambrica* is recognised as *Meconopsis* and not *Papaver* then based on the results of this analysis *Papaver* as a genus comprises sections *Carinatae, Meconidium, Oxytona, Papaver, Pilosa, Pseudopilosa* and *Rhoeadium* only. This would require the separation of section *Argemonidium, Californicum, Horrida* and *Meconella* from the genus either into new genera in their own right or into *Meconopsis*. However, if these sections are to be retained within *Papaver* either as sections or subgenera as suggested here it would be necessary to include *Meconopsis cambrica* within *Papaver* as a monotypic section and the recognition of *Meconopsis cambrica* back to *Papaver cambrica* L is required. A new subgenus could also be recognised, i.e. *Papaver* subgenus *Cambrica.*

5.1.5 Implications for sections *Carinatae, Meconidium, Oxytona, Papaver, Pilosa, Pseudopilosa* and *Rhoeadium*

Within Clade 3 of the combined analysis a well-supported group comprising sections *Carinatae, Meconidium, Oxytona, Papaver, Pilosa, Pseudopilosa* and *Rhoeadium* is resolved (87% bootstrap support). This group of sections is deemed by Kadereit *et al.*, (1997) to represent the typical representatives of the genus *Papaver*. In addition this group of sections (excluding *Pseudopilosa*) is homogenous in phytochemical distribution as demonstrated by the phytochemical analysis (Chapter 4). The accumulation of retroprotoberberines (present in sections *Meconidium, Oxytona, Papaver* and *Rhoeadium*), phthalideisoquinolines (present in sections *Meconidium, Papaver, Pilosa* and *Rhoeadium*) and morphinanes (present in sections *Meconidium, Oxytona, Papaver, Pilosa* and *Rhoeadium*) within this group only, suggests that these alkaloid types are synapomorphic. The fact that certain sections within this group do not accumulate some of these diagnostic alkaloid types (e.g. section *Oxytona* does not accumulate phthalideisoquinolines) represents a lack of employment of the biosynthetic pathway responsible for their production and is not indicative of a separate ancestry.

Section *Pseudopilosa* (represented in the combined molecular analysis by *Papaver atlanticum* and *P. rupifragum*) form a well supported group in the combined analysis (100% bootstrap support) indicating that the characters traditionally used to define this section such as revolute leaf vernation, poricidal dehiscence of capsule and light coloured filiform filaments are homologous with respect to these species. The species of this section are of subscapose to scapose habit found predominantly from South West Asia to North Africa and Southern Spain. Little sequence divergence has occurred between the species of this section indicating that they have diverged relatively recently. Section *Pseudopilosa* is distinct to the other sections of this main group of sections from a molecular and phytochemical point of view. None of the representatives of *Pseudopilosa* accumulate any of the alkaloid types diagnostic to this core group (sections *Carinatae, Meconidium, Oxytona, Papaver, Pilosa* and *Rhoeadium*) and this section may have arisen before the diversification within the core group occurred. In addition representatives of *Pseudopilosa* are characterised by

having a five and four base pair indel at positions 76-81 and 213-217 of the ITS region respectively, both of which are not found in any other species of *Papaver*.

Section *Pilosa*, comprising a single perennial subscapose species and a number of subspecies (Kadereit, 1996) found predominantly in West Turkey, is characterised morphologically by convolute leaf vernation, poricidal dehiscence of capsule, few to many flowers and light coloured filiform filaments. The separation of *Pilosa* from *Pseudopilosa* based on morphological and phytochemical differences (Popov, 1937; Gunther, 1975; Kadereit, 1996) is supported by the results of the combined analysis. Within the cladograms generated from the molecular analysis this section resolves as a sister group to sections *Oxytona* and *Meconidium*. Section *Oxytona* comprises three perennial species (all of which are represented in this study) found predominantly in the Caucasus Mountains, East Turkey and North West Iran (Goldblatt, 1974), and are characterised by their scapose habit, poricidal dehiscence of capsule and dark coloured filaments and anthers. Section *Meconidium,* comprising four biennial species (represented by *Papaver armeniacum*) occupy a continuous geographical range limited to South and East Turkey, the Caucasus Mountains, North Iraq and North West Iran and are characterised morphologically by the possession of bristly (which can be bristly within species) capsules, poricidal dehiscence of seed and light coloured filaments and anthers. Within the molecular phylograms, sections *Meconidium, Oxytona* and *Pilosa* continually resolve together but clearly the sections are heterogeneous with respect to morphology and the identification of synapomorphies that define this group is difficult. The grouping of sections *Oxytona* and *Pilosa* is entirely not unexpected. Species of both sections inhabit similar geographical distributions including the Caucasus Mountains, East Turkey and North West Iran (sections *Oxytona*) and West Turkey (section *Pilosa*). However species of section *Oxytona* possess dark coloured filaments, a character deemed advanced (Kadereit *et al.*, 1997). Section *Meconidium* shares light coloured filaments with section *Pilosa* but differs in the mode of dehiscence of seed, which is valvate for the former and poricidal for the later. Thus it would seem that the lineage responsible for the origin of this particular group probably contained species with the ancestral characters such as light coloured filaments and valvate dehiscence of capsule.

The remaining sections of this 'core' *Papaver* group are *Carinatae*, *Papaver* and *Rhoeadium*. The results of the molecular analysis clearly question whether these sections are monophyletic in their present taxonomic circumscription. *Papaver* section *Rhoeadium* consists of seventeen predominantly annual species (Gunther, 1975; Kadereit, 1989) and is represented in this study by *Papaver albiflorum, P. commutatum, P. dubium, P. litwinowii and P. rhoeas.* The centre of origin for this section is South West Asia and the Aegean area with some species found in Central or the Western Mediterranean, the Balkans or Western Himalayas (Kadereit, 1989). Characteristic morphological traits include poricidal capsules and dark (sometimes light) coloured filaments. However, the section is extremely diverse in morphological characteristics. Kadereit (1989) recognises three groups within section *Rhoeadium*, based on geographical and morphological observations. The first group contains species with longer than broader capsules such as *Papaver dubium* and contains only tetraploid (2n=28) and hexaploid (2n=42) species. The second group contains diploid species (2n=14) including *Papaver arenarium* and *P. commutatum* and is diverse in its morphology. The third group, although showing a greater amount of morphological uniformity than the *P. arenarium* group, again consists of diploid species including *P. rhoeas.* The similarity of the *P. rhoeas* group and the *P. arenarium* group (the later being represented by *P. commutatum*) alluded to by Kadereit (1989) is supported by the results of this study. In addition the representatives of both groups possess a diagnostic five base pair indel at position 182-187 of the *trnL-F* region. The possession of this indel coupled with the relatively well-supported grouping of the species of the *P. rhoeas* and *P. arenarium* group suggests that although separating these groups satisfies classifications based on morphological and geographical considerations, these two groups are each distinctly monophyletic and the morphological characters exhibited by these two groups are homologous.

A clear separation exists between the *Papaver rhoeas/P. arenarium* groups and the *P. dubium* group. From the *trnL-F* trees the *Papaver dubium* group is clearly allied to representatives of section *Papaver* (89% bootstrap support in the *trnL*-F analysis) and shares greater sequence similarity to this section than to the *P. rhoeas* group. However in the trees obtained from the biparentally inherited ITS region the *Papaver dubium* group is weakly allied to the *P. rhoeas* group (bootstrap support <50%).

Considering that *Papaver dubium* is tetraploid it may be the case that it has arisen as a polyploid from a cross between representatives or ancestors of sections *Papaver* and *Rhoeadium*.

The single representative of section *Carinatae*, *Papaver macrostomum,* consistently grouped within this *P. rhoeas* group and shares its diagnostic indel. *Papaver macrostomum,* with a distribution including Iran, Iraq and Turkey, possesses all the morphological characteristics displayed by species of section *Rhoeadium* as demonstrated by the results of the morphological investigation, but has been separated into a new section based on the possession of a deciduous stigmatic disc (Fedde, 1909; Kadereit, 1987). This characteristic is unusual and represents a divergence from the poricidal and valvate dehiscence displayed by the rest of *Papaver.* However it is becoming increasingly obvious that capsule characteristics may not represent the once thought absolute diagnostic character upon which to base phylogenetic estimations. No evidence for the separation of *Papaver macrostomum* from *Papaver* section *Rhoeadium* is found from the analysis of the ITS and *trnL-F* regions reported here. Based on this the author suggests the placement of *Papaver macrostomum* into *Papaver* section *Rhoeadium* and into a new subsection i.e. subsection *Carinatae* in recognition of the diversity in capsule characteristics.

The four annual representatives of *Papaver* section *Papaver* (represented in this study by *Papaver glaucum* and *P. somniferum*) from the Western Mediterranean and South West Turkey to Cyprus, Iran, Afghanistan and Pakistan do not form a monophyletic group based on the molecular analysis of ITS and *trnL-F* regions. Species of this section are characterised by the possession of strongly auriculate-amplexicaulous leaves, poricidal dehiscence of capsule and dark (sometimes light) coloured filaments. A separation exists between *Papaver glaucum* and the other species of the section, with this species showing more similarity to the *Papaver rhoeas* clade. This division within section *Papaver* has previously been demonstrated on a molecular level by the restriction site analysis of chloroplast DNA (Kadereit and Sytsma, 1992). The results of their analysis also demonstrated that members of this section such as *Papaver glaucum* and *P. gracile* share a close affinity to *P. rhoeas* and *P. dubium.* Many morphological and geographical similarities exist between the two sections (as outlined by Kadereit, 1988a). However the author viewed these similarities

(including molecular similarities) as an indication of the close affinity between the two sections rather than a taxonomic separation within the sections. Phytochemically *Papaver glaucum* differs from *P. somniferum* in that it does not accumulate morphinane alkaloids but produces similar alkaloids to those found in *P. rhoeas* (Preininger *et al.*, 1981; Preininger, 1986). *Papaver gracile, P. glaucum* and *P. decaisnii*, like the majority of *Papaver,* have a base chromosome number of seven. *Papaver somniferum* has a base chromosome number of eleven (Hammer & Fritsch, 1977). These differences in chromosome numbers and alkaloid spectra led Novak & Preininger (1980) and Preininger *et al.* (1981) to separate these three species into a new section *Glauca.* Reckin (1973) transferred these species to section *Rhoeadium.* In addition the possession of the diagnostic five base pair indel at position 182-187 of the *trnL-F* region characteristic to the *Papaver rhoeas* group by *P. glaucum* questions further the classification of *Papaver glaucum* within section *Papaver*. Although the results of this analysis clearly indicate a separation within section *Papaver,* it would be necessary to increase taxon sampling for sections *Rhoeadium* and *Papaver* to investigate further the relationships between these sections. Including taxa from different locations and of different phenotypes would also allow a better understanding of the phylogenetic significance of these groupings to be obtained.

The results of this study suggest that these two closely related sections arose from similar ancestors. This could be the same lineage that gave rise to section *Pseudopilosa,* and later on sections *Meconidium*, *Oxytona* and *Pilosa,* with the divergence of sections *Rhoeadium* and *Papaver* probably having occurred somewhere in between these two events. Regarding the taxonomic classification of these sections, it is evident that all seven sections are recognised as being derived from the same lineage and that these sections represent the more advanced sections of *Papaver*. Although a number of 'primitive' morphological characters are displayed by certain sections of this group, it has been proposed that the possession of primitive traits is a result of reversal from a more advanced state and that certain characters have evolved more than once (c.f. Chapter 3). To accommodate the separation of these sections from the prementioned subgenera, a new subgenus, i.e. *Papaver* subgenus *Papaver* is recognised. Within this subgenus six sections are recognised: sections *Meconidium, Oxytona, Papaver, Pilosa, Pseudopilosa* and *Rhoeadium.* The affinity between sections *Papaver* and *Rhoeadium* is recognised formally by the expansion of section

Rhoeadium into the following subsections: *Rhoeadium* (comprises the species of the *P. rhoeas* and *P. arenarium* groups of Kadereit, (1989), *Dubia* comprises the species of the *P. dubium* group (Kadereit, 1989), *Carinatae* (comprises *P. macrostomum*) and *Glauca,* comprises *P. glaucum* (Preininger *et al.,* 1981). Of these four subsections *Glauca* is the most contentious. The molecular analysis clearly demonstrates that *Papaver glaucum* is embedded in a group with *P. rhoeas,* but due to similarities with *P. somniferum* and evidence that it was a progenitor to *P. somniferum* (Kadereit, 1986c) this species is separated into this subsection, and represents an intermediate group between sections *Papaver* and *Rhoeadium.* Whether *P. decaisnii* and *P. gracile* are transferred to this subsection with *P. glaucum* will only be determined by future molecular analysis including these species.

5.2 Proposed classification based on molecular, morphological and phytochemical considerations.

One of the primary aims of this project was to determine whether the groups of sections proposed by Kadereit (1988a) based on morphological similarities could be supported by results obtained from molecular analysis. It is clear that his recognition of four groups (i.e. Group 1, *Californicum, Meconella* and *Meconidium*; Group 2, *Argemonidium*; Group 3; *Horrida, Pilosa* and *Pseudopilosa*; Group 4, *Carinatae, Oxytona, Papaver* and *Rhoeadium*) within the genus makes sense from a morphological point of view. However, the results from the molecular analyses conducted here demonstrate that certain characters, that have been traditionally viewed to be homologous and indicative of shared ancestry, have arisen more than once and that reversal from advanced back to primitive states has occurred for some characters. In recognition of the number of obviously paraphyletic groups within the phylogenetic trees produced in this analysis, a revision of the taxonomic classification of *Papaver* is required. The main difficulty in this has been how to define the taxonomic 'boundaries' of *Papaver.* I have based this revised classification on the monophyletic groups observed within the molecular phylograms with reference to morphology and phytochemistry. Until more representatives of *Meconopsis* and *Roemeria* are included in a molecular analysis it is prudent to retain sections *Argemonidium* and *Meconella* within *Papaver* as subgenera. The remaining sections

of *Papaver* including *Stylomecon heterophylla* and *Meconopsis cambrica* are monophyletic when these particular species are recognised as *Papaver lemonii* and *Papaver cambrica* respectively. The degree of division within the main group of *Papaver* (sections *Californicum, Cambrica, Carinatae, Horrida, Meconidium, Oxytona, Papaver, Pilosa, Pseudopilosa* and *Rhoeadium*) is subject to interpretation and opinion. One interpretation may be to combine these sections into a single subgenus of *Papaver*. However, taking morphological, phytochemical and geographical characteristics into consideration, and recognising that the sister relationship of the outlying sections (*Californicum*, *Horrida* and *Cambrica*) to the core group of *Papaver* sections probably represents different evolutionary histories, separating these groups into subgenera seems the most suitable option (table 5.1).

Table 5.1 Proposed taxonomic classification of sections within *Papaver*.

Subgenus	Section	Subsections
Argemonidium		
Californicum	*Californicum, Lemonii*	
Cambrica		
Horrida		
Meconella		
Papaver	*Meconidium*	
	Oxytona	
	Papaver	
	Pilosa	
	Pseudopilosa	
	Rhoeadium	*Carinatae, Dubia, Glauca, Rhoeadium*

CHAPTER 6. Phylogenetic analysis of *Papaver* L section *Oxytona* Bernh. = (*Macrantha* Elk.).

6.1 Introduction

Papaver section *Oxytona* Bernh., last revised by Goldblatt (1974), consists of three perennial species, in a polyploid series including the diploid *Papaver bracteatum* Lindl. (2n=14), the tetraploid *P. orientale* L. (2n=28), and the hexaploid *P. pseudo-orientale* Fedde (2n=42) with distributions including North Iran (Alborz Mountains), the Caucasus Mountains, East Turkey and North West Iran. The three species share many morphological characters and their position within this section is justified by studies including the phylogenetic analysis of DNA sequences from the nuclear ITS-1, ITS-2 and the 5.8S gene and the plastid *trn*L- *trn*F region (this thesis, Chapter 2). The section is distinct morphologically by the possession of dark coloured anthers, filiform filaments, poricidal dehiscence of seed and an ovoid capsule bearing a flat to pyramidical stigmatic disc. Goldblatt (1974) concluded that from all the morphological characters available for the section, that the differences in bud, petal and mature capsule characteristics and the position and insertion of leaves, may be considered as diagnostic taxonomic characters.

A brief summary of diagnostic morphological characters for section *Oxytona* is given in Table 6.1. The uppermost leaf is found on the upper third of the stalk in *Papaver bracteatum* and *P. pseudo-orientale*, while in *P. orientale* it is situated around the mid-line and often as three leaves grouped together. Bracts are found only in *P. bracteatum* and in some individuals of *P. pseudo-orientale*, with those found in *P. bracteatum* being larger and more numerous (3-8). The flower buds of *P. bracteatum* and *P. pseudo-orientale* are erect throughout their growth but the calyx bristles of *P. pseudo-orientale* are more sparsely spread compared to the based bristles of *P. bracteatum*. The bud of *P. orientale* is pendulous during development and straitens just before the flower opens. The petals of *P. bracteatum* are a crimson-red colour with square or long dark coloured blotches from the base to mid-line. *Papaver pseudo-orientale* and *P. orientale* have orange red-petals, but *P. pseudo-orientale*

usually has a dark rectangular blotch above the base, which can be absent in some individuals. The petals of *Papaver orientale* are free of the rectangular blotch.

Table 6.1 Diagnostic morphological characters used to define the species of section Oxytona. Many of the character states can overlap and cause confusion when identifying plants to the level of species (after Goldblatt, 1974)

Character	*Papaver bracteatum*	*Papaver orientale*	*Papaver pseudo-orientale*
Petal characteristics	Deep red, base black streak	Orange no markings	Orange/red black centre
Ploidy	Diploid 2n=2x=14	Tetraploid 2n=4x=28	Hexaploid 2n=6x=42
Flowers	Bractate	Ebractate	Bractate/ebractate
Petals	6 rarely 4	4 rarely 6	4 or 6
Petal colour	Dark red, dark markings running to base	Pale orange	Orange, black rectangular markings near base
Leaves	Dentate-bidentate	Serrate	Deeply pinnatasect-subcompound
Leaf margins	5-7	Dentate	Irregularly dentate 5-6
Floral bracts	3-8	Absent	Absent or present1-4
Bud (during development)	Erect, oval, oblong before opening	Pendulous	Erect/oval
Calyx	3-valved	2-valved	2-3 valved
Bristles	Thick/adpressed	Slender/subpatent	Slender/subpatent
Anthers	Linear/dark purple	Oblong/yellow or pale violet	Linear/pale violet
Mean pollen diameter (μm)	25.5	27.4	28.8
Ovary	Ovoid	Ovoid	Ovoid
Ovary disc	Flat/concave or conical	Slightly convex	Slightly convex
Stigmatic rays	12-24	8-15	9-19
Capsule	≈ 3.0 cm wide	≈ 2.0 cm wide	≈ 2.5 cm wide

With the characters of Table 6.1 the differentiation of these species is straight forward. However these perennial species do not produce many of these diagnostic characters until the second, often third year of growth, which hinders their identification and ultimately the identification of hybrids. Until these morphological characters are scorable the only aerial character available to differentiate between these species is leaf shape and amount of leaf dissection. There is some variation in the leaves in the group, those of *P. orientale* are different in being less dissected and somewhat narrower, but this distinction is not consistent (Goldblatt, 1974). In

addition this character is considerably variable and often the amount of variation within a species makes the differentiation between them difficult if not impossible. Goldblatt (1974) reported that the morphological ranges of variation of *P. bracteatum, P. orientale* and *P. pseudo-orientale*, when nearing their extremes can often overlap, especially with *P. orientale* and *P. pseudo-orientale*. Interspecific hybridisation does occur (Goldblatt, 1974; Milo *et al.*, 1990; Pyysalo *et al.*, 1988; Levy & Milo, 1991) and due to phenotypic similarities, the mis-identification of plant material is common.

The identification of species within this section is therefore problematic and is exacerbated by the abundance of ornamental cultivars and garden varieties of "oriental poppies" that have been developed. Since their introduction into Europe by Tournefort during the eighteenth century (Goldblatt, 1974), the 'oriental poppy' has been extensively cultivated and many new varieties and cultivars have been bred. Many of these cultivars are strikingly different from the original 'true' species but a number do however resemble these species making the differentiation between them more complicated. Considering the difficulties in obtaining material from their natural habits, most studies involving these species depend on the exchange of seed between Botanic Gardens. It is the author's experience that of the 30 accessions described as *Papaver orientale* under cultivation at the National Botanic Gardens, Glasnevin in Dublin only one accession was truly this species with the rest being *Papaver pseudo-orientale* or cultivars of this particular species. Considering the time it takes to cultivate these species from seed to maturity it is essential that cultivars are identified and eliminated from further study. A number of the more common cultivars of the 'oriental poppy' were screened genetically to ascertain the amount of genotypic variation that exists between cultivated and wild taxa of *Papaver bracteatum, P. orientale* and *P. pseudo-orientale.*

The aims of this part of the project are:

(i) to estimate the relationships that exist between the species of section *Oxytona* based on cytological, phytochemical and molecular characters.

(ii) to utilise molecular techniques to differentiate between the species to aid the utilisation of these species as sources of economically important phytochemicals.

6.1.2 Phytochemical characteristics of section *Oxytona*

As with most other species of *Papaver*, species of section *Oxytona* produce phytochemical spectra that are species-specific with considerable variation of alkaloid amounts existing for each species within the section (Phillipson, 1983, see review by Tetenyi, 1986). *Papaver orientale* predominantly produces the morphinane alkaloid oripavine and mecambridine has also been reported (Phillipson *et al.*, 1981). The proaporphine isothebaine is the dominant alkaloid produced by *Papaver pseudo-orientale* (Theuns *et al.*, 1986), with macranthaline, orientalidine and salutaridine reported as minor constituents (Sariyar & Baytop, 1980). *Papaver bracteatum* accumulates the morphinane alkaloid thebaine as its dominant alkaloid. Several chemotypes for *P. bracteatum* have been reported (Fairbairn & Hakim, 1973; Meshulam & Lavie, 1980; Bohm, 1981) including races that produce thebaine only, thebaine and alpinigenine, thebaine (dominant alkaloid) with trace amounts of orientalidine and thebaine (dominant) with trace amounts of orientalidine and isothebaine (Cheng, 1972). The biosynthetic pathways responsible for the characteristic phytochemicals produced by members of section *Oxytona* are detailed in Figure 6.1.

Certain varieties of *Papaver bracteatum* have been shown to accumulate up to 98% thebaine of their total alkaloid production (Neubauer & Mothes, 1963). Thebaine is the natural precursor to codeine and morphine in *Papaver somniferum* and is used as a starting material for the production of opiate analgesics, including codeine, oxymorphone and oxycodone (McNicholas & Martin, 1984). Species of *Oxytona* do not however possess the enzymatic pathways necessary to convert thebaine and oripavine into codeine and morphine. During the 1970's the United Nations called for extensive studies into the utilization of *Papaver bracteatum* as a source of thebaine and the possible replacement of *P. somniferum* as the major source of morphinane alkaloids (UN report, 1976). The world wide social problem that has arisen from the illicit trading of opium and more specifically the ease of conversion of morphine into its semi-synthetic derivative heroin has highlighted the dangers of the large scale cultivation of this particular species. Despite the addictive nature of morphine, its powerful analgesic properties, from a medical point of view, have made it one of the most important plant products discovered to date.

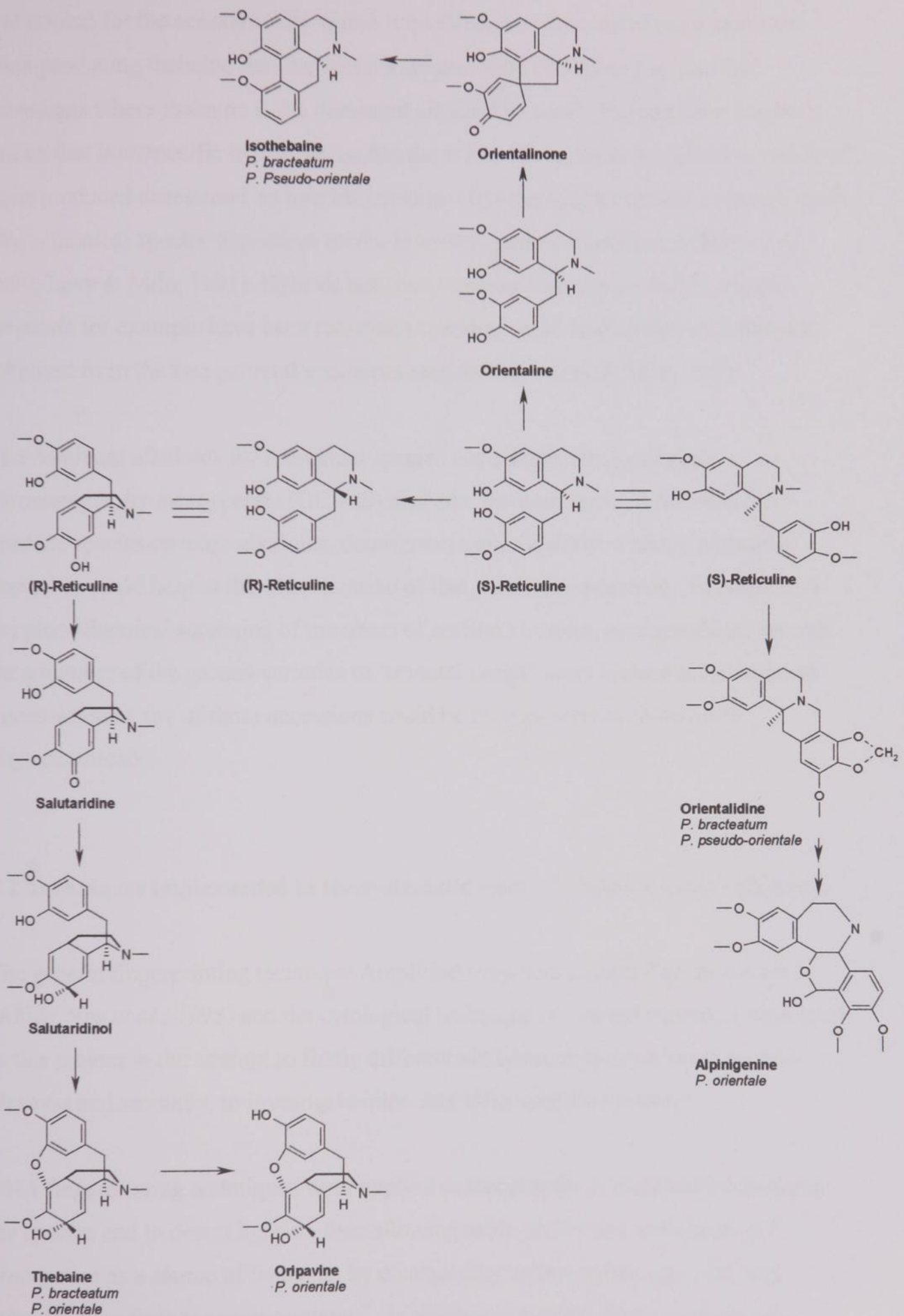


Figure 6.1 Biosynthesis of the major alkaloids of section *Oxytona* (modified after Nyman & Bruhn, 1979).

It is critical for the economically viable use of *Papaver bracteatum* as a means of mass-producing thebaine that the species are correctly identified but also that accessions where thebaine is the dominant alkaloid be used. In addition it has been shown that interspecific hybridisation has the effect of reducing the effective yields of mass produced accessions, as hybrids between *Oxytona* species produce characteristic phytochemical spectra dependent on the inherited genetic information (Milo *et al.*, 1990; Levy & Milo, 1991). Hybrids between *Papaver bracteatum* and *P. pseudo-orientale* for example have been reported to produce both isothebaine and thebaine inherited from the two parental genotypes respectively (Levy & Milo, 1991).

The dominant alkaloids for individual species were determined using gas chromatography mass spectra (GC/MS) analysis. Because some of the alkaloids produce species-specific alkaloids, determination of alkaloids within a particular accession could help in the identification of that particular accession. In addition to the phytochemical screening of members of section *Oxytona*, phytochemical spectra for a number of the garden varieties of 'oriental poppy' were carried out in order to assess whether any of these accessions could be used as sources for valuable phytochemicals.

6.2 Techniques implemented in the systematic study of *Papaver* section *Oxytona.*

The genetic fingerprinting technique Amplified Fragment Length Polymorphism (AFLP; Vos *et al.*, 1995) and the cytological technique *in situ* hybridisation were used in this project in the attempt to firstly differentiate between the species of section *Oxytona* and secondly, to investigate inter- and infra-specific variation.

DNA fingerprinting techniques were applied in this chapter to differentiate between the species and to detect hybrids, thus allowing more productive utilisation of *P. bracteatum* as a source of thebaine, by disregarding misidentified material (and hybrids) from further experimentation. In addition the different chemotypes of *Papaver bracteatum* were screened in the attempt to differentiate between them and to find molecular markers that can be used to assess whether certain accessions will produce thebaine only as its dominant alkaloid, thus offering the potential to gain

elevated yields of thebaine of mass produced accessions by omitting accessions with heterogeneous phytochemical spectra from further experimentation.

6.2.1 Amplified Fragment Length Polymorphisms (AFLP)

AFLP is a molecular technique for generating DNA fingerprints, based on the selective amplification of restriction fragments from a total digest of genomic DNA (Vos *et al.*, 1995). The AFLP process produces 'fingerprint' patterns of different fragment lengths and the differences in fragment lengths can be attributed to base changes in the restriction site or the primer extension site, or to indels in the body of the DNA fragment. The resulting complex fingerprints are reproducible between laboratories and provide a wide number of informative markers derived from many loci dispersed throughout the genome (Ridout & Donini, 1999). The technique is suited to differentiating between closely related species, varieties and cultivars, including near-isogenic lines. They are also suited for subsequent phylogenetic interpretation of any relationship that exists between such taxonomic ranks. However, when applying the technique to species, the assumption that shared fragments (markers) are homologous diminishes as the species become more distantly related. Thus the technique cannot be used successfully above certain taxonomic ranks depending on the study group (Karp *et al.*, 1996). AFLP has been used to detect genetic variation in a variety of plants (Mackill *et al.*, 1996; Paul *et al.*, 1997; Waugh *et al.*, 1997; Hodkinson *et al.*, 2000; Carolan *et al.*, 2002).

AFLP fingerprinting involves three steps. Firstly the restriction enzymes *EcoRI* and *MseI* are used to digest total genomic DNA. Double-stranded oligonucleotide primers specific to these restriction sites are then ligated to the ends of the fragments. Secondly a preselective PCR amplification of a subset of all fragments in the total digest is carried out. This involves using preselective primers, which have a selective nucleotide that will recognise the subset of the restriction fragments. This step achieves a 16-fold reduction in the number of fragments amplified in the subsequent steps. The third part of the AFLP reaction process involves a selective amplification of a subset of the fragments generated in the preselective amplification step. The primers used (in this Chapter) have a *Mse*I or an *EcoR*I recognition sequence. The *EcoR*I primers are labelled with a fluorescent dye, which ultimately yields

fluorescently labelled amplified fragments (Applied Biosystems, AFLP Plant Mapping Kit literature). The selective amplification step results in a 256-fold reduction in the number of fragments generated in the initial restriction of total genomic DNA, which results in a manageable number of fragments to score. Labelled fragments are then resolved by gel electrophoresis. Homology is assumed between fragments of the same size and these fragments are scored as present/absent and the resulting matrix is subjected to distance, clustering and ordination analyses.

6.2.2 Data analysis

A wide variety of methods for the estimation of relatedness are associated with AFLP data. The most common of these and those used in this project are described below.

6.2.2.1 Distance and clustering methods

Estimation of phylogeny from AFLP markers has traditionally been based on the utilization of algorithms that produce single trees based on 'distance' methods. Distance methods are based on predefined formulae, which estimate the difference between taxa within a given matrix (overall distance) and convert the initial matrix into a 'pairwise distance matrix'. A common algorithm for creating a pairwise distance matrix is that of Nei and Li (1979) which measures overall similarity between taxa (Li & Graur, 1991) and computes evolutionary distances for all pairs of taxa using the formula: 2a/2a+2b+2c (a= the number of markers in common, b= the number of markers in one phenotype and c= the number of markers in the other phenotype, i.e. a matching coefficient). The production of trees from the resulting distance values between taxa involves the use of 'clustering' algorithms. Many clustering methods construct a tree by linking the least distant (more similar) pairs of taxa, followed by successively more distant taxa. These trees are base solely on the relative numbers of similarities and differences between a set of characters.

6.2.2.2 UPGMA and NJ

The two distance methods commonly employed are UPGMA (Unweighted Pair Group Method using Arithmetic Averages; Sneath & Sokal, 1973) and NJ (Neighbor

Joining; Saitou & Nei, 1987). These methods ultimately produce a single tree, which compared to parsimony methods for example, which involve the generation and comparison of large number of trees, UPGMA and NJ are computationally and temporally rapid for small numbers of taxa. Once characters within a matrix are converted to distances, valuable phylogenetic information is lost by not allowing the specific character(s) that contributes to branch length to be identified. By producing single trees these methods are limited by not allowing competing hypotheses for tree production to be tested and often the topology of a tree generated by clustering methods is determined by the order in which taxa are added to the growing tree (Page & Holmes, 1998). UPGMA is further limited by assuming the rates of change are equal between all taxa on a tree (i.e. characters are ultrametric). NJ has a distinct advantage over UPGMA, as it does not assume that all characters are ultrametric. This method involves the modification of the distance matrix to adjust for the differences in the rate of evolution of each taxon. The least distant pairs of nodes are linked and their common ancestral node is added to the tree while their terminal nodes are pruned from the tree. This continues until two nodes remain (Saitou & Nei, 1987). Internal support for groups within the tree can be calculated using bootstrap replicates (Felsenstein, 1985).

6.2.2.3 Similarity algorithms and ordination techniques

In addition to cluster analysis and the resulting production of trees based on distances calculated between accessions, a number of ordination techniques can be used to process AFLP data. Ordination techniques determine the placement of taxa (based on a variety of distance or similarity measures and algorithms) in multi-dimensional space and are represented usually by a scatter diagram (Singh, 1999) in two or three dimensions. For the ordination method used here principle coordinate analysis (PCO) similarity between taxa is calculated using a similarity coefficient (e.g. Dice distances, 1945), which produce a value between taxa based on certain criteria such as number of polymorphic bands shared between two accessions and the number of bands common to one accession and not the other. PCO is well suited for binary data (0,1) such as AFLP marker matrices. Generally, a dissimilarity matrix (distance matrix) is created by subtracting the similarity coefficient between two accessions from 1, and using this value to create a new matrix. Scatter-plots based on these values are

generated using the ordination technique. Eigenvalues are calculated from the similarity matrix and plotted in n-dimensional space with n= the number of characters. The technique is based on the assumption that if a line represents a character, all accessions could be placed along the line according to the value for that particular character. PCO then determines a line through the multi-dimensional cloud of points that account for the greatest amount of variation (the first principle axis, x-axis). A second axis (second principle axis, y-axis) is then produced perpendicular to the first and accounts for the next greatest amount of variation (Singh, 1999). Sometimes the first few axis account for the majority of the variation between accessions and only two dimensions need to be plotted. Scatter-plots are interpreted by assuming that accessions, which are positioned close to each other, share greater similarity than those that are relatively more distant.

6.3 *In situ* hybridisation (ISH)

The combination of molecular and cytological data is commonplace with many phylogenetic studies. The information obtained from a simple chromosome count can be invaluable to the correct interpretation of phylogenetic groupings obtained from molecular data. Further characterisation of the chromosomes can be achieved with techniques based on molecular *in situ* hybridisation (Gall & Pardue, 1969; John *et al.*, 1969). *In situ* hybridisation involves the localisation of DNA or RNA directly to biological material (in this case chromosomes). The DNA probe is labelled in such a way to allow visualization and the determination of the probes locus. This in turn allows the characterisation of chromosomes through the production of physical maps, which can be compared between species in an attempt to ascertain phylogenetic relationship. In cases where the probe is labelled with a fluorescent dye the technique is known as fluorescent *in situ* hybridisation (FISH). Resurgence in cytological investigations has occurred over the last decade as a result of the advent of fluorescent technologies and the resulting combination of both technologies has resulted in the resolution of phylogenetic relationship between distantly related and closely related plant species. Many authors have reviewed the application of *in situ* hybridisation (Schwarzacher *et al.*, 1989; Leitch *et al.*, 1990; Leitch *et al*, 1991; Leitch & Bennett, 1997). In addition *in situ* hybridisation can be applied across different taxonomic ranges with the choice of probe ultimately deciding the resolution of the data for

taxonomic investigations (Stace & Bailey, 1999). For example Adams *et al.* (2000) were able to further elucidate phylogenetic relationship existing within the genus *Aloe* L. using fluorescent *in situ* hybridisation, to characterise the distribution of 5s and 18S-5.8S-26S rDNA repeats in the chromosomes of a number of *Aloe* species. When combined with nucleotide sequence data from the same regions they achieved further understanding into the relationships that exist within the species of that particular genus.

In situ hybridisation is well suited for the elucidation of relationship between polyploid species and more specifically resolving phylogenetic issues involving polyploid species and species that exist as polyploid series (e.g. diploid, tetraploid and hexaploid) as found within *Papaver* section *Oxytona*. Polyploids are species with cell nuclei containing more than two genomes i.e. three or more sets of chromosomes. Diploid species contain two sets of chromosomes. Within the angiosperms polyploidy is common with Grant (1981) estimating that 47% to 52% and Leitch & Bennett (1997) estimating that up to 90% of all angiosperm species are polyploid, respectively. There are two basic types of polyploid species, autopolyploids, which contain more than two genetically identical genomes, and allopolyploids, which combine genomes from more than one ancestral species (Leitch & Bennett, 1997). Characterisng chromosomes with *in situ* methods offer the potential to discover the parental progenitors to polyploid species (for examples see Bennett *et al.*, 1992; Moscone *et al.* 1996; Hodkinson *et al.*, 2002; Lim *et al.*, 1998; Lim *et al.*, 2000). These technologies have been successful in elucidating the parental genome donors of a number of species, including the tobacco plant *Nicotiana tobacum* where it was demonstrated that both *N. tomentosiformis* and *N. sylvestris* or their ancestors both contributed their genomes (T and S-genomes respectively) to the polyploid hybrid (Parokonny & Kenton, 1995; Moscone *et al.* 1996; Lim *et al.*, 2000).

Goldblatt (1974) suggests that the tetraploid *Papaver orientale* arose from an autopolyploidy event of a hybrid species. Milo *et al.*, (1988) produced a number of artificial hybrids between *Papaver* section *Oxytona* species to examine the chromosome behaviour at meiosis. These results were combined with isozyme and chloroplast DNA restriction fragment data. They demonstrated that genetic information from *Papaver bracteatum* was transferred to the resulting interspecific

hybrids, highlighting a close affinity between the species of the section. *Papaver orientale* and *P. pseudo-orientale* possessed similar restriction endonuclease fragments and are more closely related to each other than to their diploid progenitor. From this they postulated that the origin of *Papaver pseudo-orientale* occurred recently and that *P. orientale* was the maternal donor contributing both sets of chromosomes and its cytoplasm. It was anticipated that *in-situ* hybridisation technologies will provide further evidence for the autoployploidy nature of section *Oxytona* by demonstrating the presence of the *Papaver bracteatum* genome in *P. orientale* and both *P. bracteatum* and *P. orientale* genomic material within the hexaploid *P. pseudo-orientale*.

6.4 Materials and Methods

6.4.1 Plant material and DNA extraction

The majority of the material used in this analysis (Table 6.2) was cultivated from seed obtained from various Botanic Gardens and commercial sources and grown to maturity either at the National Botanic Garden, Glasnevin, Ireland or in the glasshouse of the Department of Phramacognosy, Trinity College Dublin, Ireland. Voucher specimens were kept for each accession used and were stored in the Herbarium of the Department of Botany, Trinity College Dublin, Ireland (TCD). Each accession was given a unique identification number and an equivalent DNA bank reference number and subsequently stored at the Department of Botany DNA bank. The methods for DNA extraction have been outlined previously in Chapter 2 section 2.3.3.

Table 6.2 Samples used for AFLP, morphological, phytochemical and cytological analysis. NBG: National Botanic Gardens, Glasnevin, Ireland; TCD BG: Trinity College Dublin, Botanic Gardens; SoPG: Gardens of the School of Pharmacy, Trinity College Dublin. cv: cultivar

Genus and species	Accession number	Source, Origin & Material Type	Accession Information (Botanic Gardens)
Papaver bracteatum	J-020	SOPG, Iran, Cult.	ARYA II, PI.381605
Papaver bracteatum	J-028	SOPG, Iran, Cult.	ARYA II, PI.381605
Papaver bracteatum	J-029	SOPG, Iran, Cult.	ARYA II, PI.381605
Papaver bracteatum	J-030	SOPG, Iran, Cult.	ARYA II, PI.381605
Papaver bracteatum	J-031	SOPG, Iran, Cult.	ARYA II, PI.381605
Papaver bracteatum	J-157	MBG, Origin unknown, Cult.	No accession information
Papaver orientale	J-008	TCD BG, Origin unknown, Cult.	No accession information
Papaver orientale	J-035	SOPG, Turkey, Cult.	No accession information
Papaver orientale	J-135	NBG, Origin unknown, Cult.	No accession information
Papaver pseudo-orientale	J-018	NBG, Origin unknown, Cult.	1999.0341
Papaver pseudo-orientale	J-034	SOPG, Origin unknown, Cult.	No accession information
Papaver pseudo-orientale	J-037	SOPG, Origin unknown, Cult.	No accession information
Papaver pseudo-orientale	J-054	NBG, Origin Unknown, Cult.	No accession information
Papaver pseudo-orientale	J-055	NBG, Origin Unknown, Cult.	No accession information
Papaver pseudo-orientale	J-062	NBG, Origin Unknown, Cult.	No accession information
Papaver pseudo-orientale	J-108	TCD BG, Iran, Cult.	2000.1888. Halle 3626
Papaver pseudo-orientale	J-139	NBG, Turkey, Cult.	2000.0794
Cultivars and unidentified garden varieties			
Papaver orientale 'Allegro'	J-122	TCD BG, CS, Cult.	No accession information
Papaver orientale 'unknown'	J-137	SOPG, Turkey, Cult.	No accession information
Papaver orientale 'Picote'	J-121	NBG, CS, Cult.	2000.1441
Papaver orientale 'Goliath'	J-123	NBG, CS, Cult.	2000.1445
Papaver orientale 'Black & White'	J-132	NBG, CS, Cult.	2000.1448
Papaver orientale 'Pattys Plum'	J-140	NBG, CS, Cult.	2000.1437
Papaver orientale 'Salmon Queen'	J-040	SOPG, Origin unknown, Cult.	No accession information
Papaver orientale 'unknown'	J-070	NBG, Origin Unknown, Cult.	No accession information
Papaver orientale 'unknown'	J-133	NBG, Origin Unknown, Cult.	No accession information
Papaver pseudo-orientale	J-091	NBG, Origin Unknown, Cult.	No accession information
Papaver pseudo-orientale	J-134	TCD BG, Origin unknown Cult.	No accession information
Papaver pseudo-orientale	J-096	NBG, Turkey, Cult.	2000.0794
Papaver pseudo-orientale	J-093	NBG, Turkey, Cult.	2000.0632. Jena 4009
Outgroup			
Papaver somniferum	J-016	TCD BG, Origin unknown Cult.	No accession information

6.4.2 Morphological analysis

A variety of morphological characters were scored for each accession and subjected to a cladistic analysis. The majority of characters were based on diagnostic characters detailed by Goldblatt (1974) and include:

1. Petals: with (0) or without spots (1). *Papaver orientale* is always devoid of spots, but spots are most always present in *P. bracteatum*. *Papaver pseudo-orientale* can be with or without spots.
2. Petal colour: crimson/red (0), orange (1) or other (2). *Papaver bracteatum* always has crimson flowers, while those of *P. orientale* and *P. pseudo-orientale* are orange. The garden cultivars can have a range of petal colours including white, pink, purple and red.
3. Petals: bractate (0), ebractate (1). Bracts are always present for *Papaver* bracteatum, absent for *P. orientale* and can be present or absent for *P. pseudo-orientale*.
4. Leaves: pinnatisect (0), lanceolate (1). The leaves of *Papaver bracteatum* are pinnatisect and deeply divided, whereas the leaves of *P. orientale* are less divided and almost lanceolate.
5. Calyx: 2-valved (0), 3-valved (1).
6. Buds during development: erect (0), pendulous (1). The cauline stem of *Papaver orientale* is different from the other two specs, as it possesses a stem that is pendulous before flowering.
7. Capsule: ovoid (0), obovoid (1). The capsules of *Papaver bracteatum* and *P. pseudo-orientale* are ovoid in shape. The capsule of *Papaver orientale* is more slender and obovoid in shape.
8. Stigmatic disc: flat (0), pyramidical (1). The stigmatic discs of *Papaver bracteatum* and *P. orientale* are generally pyramidical, while the stigmatic disc of *P. pseudo-orientale* is flat.
9. Setae: adpressed (0), subpatent (1). Most of the material examined had setae which were slender and adpressed. However some of the taxa examined had subpatent setae.
10. Capsule width: <2cm (0), >2cm (1). Although the width of capsules varied within the same plant, accessions for *Papaver orientale* had capsules no wider than 2cm.

11. Stigmatic rays: <10 (0), >10 (1). *Papaver orientale* generally has no more than 10 stigmatic rays.

Accessions were scored according to the characters above (Table 6.3). The resulting matrix was subjected to maximum parsimony analyses, performed using the heuristic search algorithms of PAUP with 1000 replicates of random addition sequence (holding 25 trees at each replication) and with tree bisection reconnection (TBR) branch swapping on multiple trees (multrees on). Clade support was examined using 1000 bootstrap replicates of a heuristic search with 100 replicates of TBR branch swapping on multiple trees

6.4.3 Phytochemical analysis

The stigmatic discs from mature poppy capsules were removed and the capsules were hollowed out. Alkaloid extraction was based on a general chloroform extraction procedure which is detailed in Appendix 1.7 (including materials and reagent concentrations). Analysis of residues was carried out using mass spectrophotometry, which is a method of analysis based upon the formation of ions, which are deflected, by a magnetic or electrostatic field. The pattern that the ions make is characteristic to the analyte. Samples are introduced to a transfer line where they pass through a direct-coupled capillary column. These ions then pass through to the ion trap, which consists of a central ring-shaped electrode and two end cap electrodes. The compound flows into the ion trap where electrons produced by the filament are periodically allowed into the trap, controlled by an electronic gate. These electrons cause the compound to be ionised in the mass spectrophotometer by causing an electron to be removed from somewhere on the molecule (Electron Impact/Ionisation; EI), thus destabilising its molecular structure forming molecular and fragment ions. Each fragment will have a specific mass-to-charge (m/z) ratio. These fragment ions are subsequently stored and satbilised in the ion trap cavity. The central electrode produces an oscillating radio-frequency (RF) electric field, which causes the molecular and fragment ions to move in stable trajectories (the ions effectively become "trapped"). Application of a RF field at the end cap electrodes causes some

Table 6.3 Character matrix of morphological characters for use for phylogenetic analysis of *Papaver* section *Oxytona*. For a description of each character and character state see text.

Genus and species	Accession number	MC1	MC2	MC3	MC4	MC5	MC6	MC7	MC8	MC9	MC10	MC11
Papaver bracteatum	J-020	0	0	0	0	1	0	0	1	1	1	1
Papaver bracteatum	J-028	0	0	0	0	1	0	0	1	1	1	1
Papaver bracteatum	J-029	0	0	0	0	1	0	0	1	1	1	1
Papaver bracteatum	J-030	0	0	0	0	1	0	0	1	1	1	1
Papaver bracteatum	J-031	0	0	0	0	1	0	0	1	1	1	1
Papaver bracteatum	J-157	0	0	0	0	1	0	0	1	1	1	1
Papaver orientale	J-008	1	1	1	1	0	1	1	1	0	0	0
Papaver orientale	J-035	1	1	1	1	0	1	1	1	0	0	0
Papaver orientale	J-135	1	1	1	1	0	1	1	1	0	0	0
Papaver orientale 'Salmon Queen'	J-040	0	2	0	0	0	0	0	0	0	1	0
Papaver orientale	J-070	1	2	0	0	1	0	1	0	0	1	1
Papaver pseudo-orientale 'Picote'	J-121	0	1	0	0	1	0	0	0	0	1	1
Papaver pseudo-orientale 'Allegro'	J-122	0	1	0	0	1	0	0	0	0	0	1
Papaver orientale 'Goliath'	J-123	0	0	0	0	1	0	0	0	0	1	0
Papaver orientale 'Black & White'	J-132	0	2	1	1	1	0	0	0	0	1	1
Papaver orientale	J-133	0	2	1	1	1	0	0	0	0	1	0
Papaver orientale	J-137	0	1	0	0	1	0	0	0	0	1	1
Papaver orientale 'Patty's Plum'	J-140	0	2	1	0	1	0	0	0	0	1	1
Papaver pseudo-orientale	J-018	0	1	0	1	1	0	0	0	0	1	1
Papaver pseudo-orientale	J-034	0	1	0	0	0	0	0	0	0	1	0
Papaver pseudo-orientale	J-037	0	1	0	0	0	0	0	0	0	1	0
Papaver pseudo-orientale	J-054	1	1	1	1	0	1	1	1	0	0	0
Papaver pseudo-orientale	J-055	0	1	0	1	0	1	1	1	0	0	0

Table 6.3 (continued)

Genus and species	Accession number	MC1	MC2	MC3	MC4	MC5	MC6	MC7	MC8	MC9	MC10	MC11
Papaver pseudo-orientale	J-062	0	1	0	0	1	0	0	0	0	1	1
Papaver pseudo-orientale	J-091	0	1	0	0	1	0	1	0	1	1	0
Papaver pseudo-orientale	J-093	0	1	0	1	1	0	0	0	0	1	1
Papaver pseudo-orientale	J-096	0	1	0	1	1	0	0	0	0	1	1
Papaver pseudo-orientale	J-108	0	1	1	0	0	0	1	1	0	1	1
Papaver pseudo-orientale	J-134	0	1	0	0	1	0	0	0	0	1	1
Papaver pseudo-orientale	J-139	0	1	0	0	0	0	0	0	0	1	0
Papaver somniferum	J-016	1	2	1	0	0	1	0	0	0	1	1

ion trajectories to become unstable and such ions are dumped from the trap into holes in the end caps where they are detected. Ions with a low m/z ratio are ejected with RF voltages of lower amplitudes. The RF voltage is sequentially increased thus causing ion fragments with greater m/z values to be subjected. Once ejected the stream of ion fragments are detected by an electron multiplyer and converted to digital signal, which is processed by specific computer software where a mass spectrum is produced. The mass spectrum is essentially a graphical representation of the ion intensities versus the mass-to-charge ratio. From these mass spectra the typical phytochemical spectra for a number of accessions were obtained.

For gas chromatographic mass spectrum (GC/MS) analysis, samples were injected in splitless mode (all the injected sample went on to the column) onto a GC capillary column (30 m x 0.25 mm i.d., Va 35MS) coupled to a Saturn 2000 MSD (EI 70eV). The injector temperature and the GC-MS transfer line were held at 260°C and the column programmed from 100 °C, held for three minutes, rising to 300°C at 15°C per minute. Helium was used as the carrier gas at a flow rate of 1 ml/min (helium helps to focus the trajectory of the ions which yields sharper mass peaks as they are scanned out). Mass spectra were analysed using the Saturn® GC/MS Workstation software v.5.2. Only dominant alkaloids were reported and reference samples of thebaine and isothebaine were also analysed to determine reference m/z values (molecular weights) and retention times.

6.4.4 Cytological analysis

6.4.4.1 Root pre-treatment and fixation

A number of accessions were grown from seed or transplanted from living collections (Table 6.3) and grown under glasshouse conditions. When root tips were actively growing they were collected and placed in a saturated solution of Gammaxene (2 grams in 200ml boiling water: reagent concentrations, protocol and materials are given as Appendix 1.8). Generally root tips were collected early in the morning when metaphase activity was at its highest. Gammaxene serves to suspend cell activity thus keeping many of the cells at the metaphase stage of development. Root-tips were

suspended in gammaxene for 4 hours and then transferred to freshly prepared Carnoy's solution (1:3 Glacial Acetic Acid :Absolute alcohol) which fixed the cells.

6.4.4.2 Chromosome counts

Root tips were hydrolysed in prewarmed 1N HCl at 60 ºC for 8 minutes, transferred into 5 ml of Feulgen solution (Feulgen solution stains exposed aldehyde groups following hydrolysis) and placed in the dark for 30 minutes. Stained root tips were then placed onto the corner of a glass microscope slide and a drop of 45% (v/v) glacial acetic acid was placed on the tip of the root. Using a hypodermic needle the tip was macerated. A glass coverslip was then placed over the material. Slides with suitable numbers of cells at metaphase were sealed with standard nail polish (to stop the slide from drying out) and viewed with a light microscope at a magnification of ×1000. Materials are given as Appendix 1.8.

6.4.5 AFLP analysis

AFLP reactions were carried out using the commercially available kits of Applied Biosystems AFLP™ Plant Mapping Kit. Reagent concentrations and materials are detailed further in Appendix 1.10

6.4.5.1 Restriction/Ligation reaction

The total DNA for each accession was quantified using a spectrophotometer (Amersham Pharmacia, Ultraspec 1000) and the quantity of DNA used in the AFLP process for each accession was standardised. The restriction enzyme master mix (Enzyme Master Mix; Table 6.4) was prepared in a sterile tube for the appropriate number of DNA samples. The total volume was brought up to (n)0.5µl, (where n=the number of samples) with ultra-pure deionised water, vortexed for 5 seconds, centrifuged to spin down contents and stored on ice. The *Mse*I and *EcoR*I adaptor pairs were annealed by heating to 95˚C for 5 minutes and allowed to cool over 10 minutes. A second master mix, the Restriction/Ligation Mater Mix; Table 6.5) was then prepared including the annealed adaptor pairs. In a labelled 0.2ml PCR tube,

2.25µl of the enzyme master mix was added to 0.5µl of the restriction/ligation master mix. 2.75µl of template DNA (100ng/µl) was added. The contents were vortexed, spun down and incubated at 37°C for 120 minutes in a thermal cycler. 94.5µl of $TE_{0.1}$ buffer was added to each restriction/ligation sample to dilute them.

Table 6.4 Restriction/Ligation enzyme master mix components and incubation protocol for AFLP. Reagent components are detailed in Appendix 1.9.

Restriction Enzyme Master Mix

	Volume
Component	1 reaction
10× Promega T4 DNA Ligase Buffer	0.05µl
0.5M NaCl	0.05 µl
BSA (1mg/ml)	0.025 µl
MseI	0.5 units
EcoRI	0.5 units
Promega T4 DNA Ligase	0.5 units
Sterile ultrapure H_2O	To make a volume of 0.5 µl
Total volume	0.5 µl

Restriction/Ligation Enzyme Master Mix

	Volume
Component	1 reaction
Promega T4 Ligase buffer	0.5 µl
0.5M NaCl	0.5 µl
BSA (1mg/ml)	0.25µl
*Mse*I adaptor	0.5 µl
*EcoR*I adaptor	0.5 µl
Enzyme Master Mix	0.5 µl
Template DNA	100 ng
Sterile ultrapure H_2O	To make a volume of 5.5 µl
Total volume	0.5 µl

Incubation	95°C for 120 minutes

6.4.5.2 Preselective amplification

The preselective amplification was carried out by adding 2.0µl of the dilute restriction/ligation DNA, 7.5µl AFLP core mix (which consists of buffer, nucleotides and AmpliTaq® DNA polymerase) and 0.5µl of the AFLP preselective primer pairs. The samples were then vortexed and spun down, placed in a thermal cycler and subjected to the temperature control parameters outlined in Table 6.5. 5.0µl of the preselective amplification product was diluted in 95.0µl $TE_{0.1}$ buffer. The remaining 0.5µl was run on a 1.2% agarose gel to ensure the restriction/ligation steps were successful.

Table 6.5. Preselective Amplification Reaction Mix components and thermal cycling parameters. Reagent components are detailed in Appendix 1.9.

Preselective Amplification Reaction Mix

Component	Volume (µl)
AFLP preselective primers	0.5
AFLP core Mix	7.5
Diluted restriction/ligation product	2.0
Total volume	10.0

Thermal Cycling Protocol

	Cycle Phase	Temperature (°C)	Duration
Cycle 1	1. Extension	72	2 min
Standard cycle	1. Denaturing	97	1 sec
(20 cycles)	2. Annealing	56	30 sec
	3. Extension	72	2 min
Final cycle	1 Extension	60	30 min
	2 Soak	4	∞ (hold)

6.4.5.3 Selective amplification

The final step of the AFLP process involves a selective amplification of a subset of the fragments generated in the previous two steps. This was achieved by combining 1.5µl of diluted preselective amplification reaction product, 7.5µl AFLP core mix, 0.5µl of *Mse*I primer and 0.5µl of labelled *EcoR*I primer. The *EcoR*I primer is complimentary to the I adaptor sequence and is labelled with a fluorescent dye (either FAM (blue), JOE (green) or NED (yellow) to allow detection of fragments. The *Mse*I primer is also complimentary to the *Mse*I adaptor sequence but is unlabeled. The resulting mixture was vortexed, spun down and loaded onto a thermal cycler and subjected to the temperature parameters outlined in Table 6.6. Preparation and loading of samples for electrophoresis is detailed in Appendix 1.9.

Table 6.6. Selective Amplification Reaction Mix components and thermal cycling parameters.

Selective Amplification Reaction Mix

	Volume
	Volume (µl)
Component	
*EcoR*I labelled primer (1µM)	0.5
*Mse*I primer (5µM)	0.5
AFLP core Mix	7.5
Diluted preselective PCR product	1.5
Total volume	10.0

Selective Primer Pair Combinations

TAMRA (yellow)	*EcoR*I-ACC
	*Mse*I-CAT
JOE (green)	*EcoR*I-AGG
	*Mse*I-CAG

Table 6.6 (continued)

Thermal Cycling Protocol			
	Cycle Phase	Temperature (°C)	Duration
Cycle 1	1. Denaturing	94	2 min
	2. Annealing	65	30 sec
	3. Extension	72	2 min
Cycle 2-11	1. Denaturing	94	1 sec
	2. Annealing	64 decreasing by 1 over each subsequent cycle until 56	2 min
	3. Extension	72	2 min
Standard cycle	1. Denaturing	94	1 sec
(22 cycles)	2. Annealing	56	30 sec
	3. Extension	72	2 min
Final cycle	1 Extension	60	30 min
	2 Soak	4	∞ (hold)

6.4.6 Data analysis

AFLP profiles were scored using the Genotyper 1.1 software of Applied Biosystems. DNA fragments ranging between 50-500 base pairs in size from the AFLP analysis were scored as presence/absence characters and analysed with Neighbor-Joining (NJ) using Nei & Li distances (Nei & Li, 1985) with the phylogenetic analysis program PAUP 4.0bv10 (Swofford, 2003). Internal support for groupings was assessed using the bootstrap procedure with 1000 replicates (Felsenstein, 1985). Principal co-ordinates analysis (PCO) was performed with Le Progiciel R v4.0d (Casgrain, 1999) using Dice similarity (Dice, 1945) which is equivalent to Nei & Li distances (NJ).

6.4.7 *In situ* hybridisation

In situ hybridisation experiments were carried out in the Department of Biological Studies, Queen Mary's University, Westfield, London.

6.4.7.1 Enzyme digestion and slide preparation

Fixed root tips about 1cm long were washed in 1×enzyme buffer for 5 minutes to remove any fixative. This was repeated 3 times. Root tips were incubated at 37°C in pre-warmed digestion enzyme (3% (w/v) drilase, 0.3% (w/v) cellulase R10 and 0.3% (w/v) pectolase Y23, ICN Biomedical Co.) in 1× enzyme buffer solution for 25 minutes in a 1.5ml microcentrifuge tube tube. This step served to soften the cells and digest the cytoplasm. The enzyme solution and the root tips were poured gently into a Petri dish filled with 1×enzyme buffer. The partially digested root tips were left at room temp for at least 2 hours to equilibrate. Under a dissecting microscope the root cap was teased off with a needle. The meristem tip was excised and gently pipetted onto a chromic acid washed slide. The excess enzyme buffer was carefully soaked up with the corner of a filter paper and a drop of 60% acetic acid was added. The protoplasts were dispersed with a thin needle in the acid and gently covered with a round coverslip (Lim *et al.* 1998; Leitch *et al.* 2000). Putting gradual pressure on the centre of the coverslip squashed the cells. Excess acid was soaked up with a piece of filter paper. Slides were examined using phase contrast microscopy and slides with a high number of cells in metaphase and where chromosomes which where distinct and non-overlapping were kept and marked. Adequate slides were placed in liquid nitrogen for 4 seconds and the coverslip was removed with a razor blade. Slides were kept at 4°C until required. A list of materials including stock concentrations is detailed in Appendix 1.10a.

6.4.7.2 Probe Generation

The probe chosen for hybridisation to root tip metaphase chromosome spreads was the (pTa71), which contains a 9 kb *Eco*RI fragment of 18S-5.8S-26S rDNA and the non transcribed spacer from wheat (Gerlach & Bedbrook, 1979). The labelling of DNA involves the incorporation of labelled nucleotides that are conjugated to biotin, digoxigenin or fluorochromes (Leitch *et al.,* 1994*)* and the FISH protocol described here is based on protocols previously described by Lim *et al.* (1998) and Leitch *et al.* (2000). The two labels used here were biotin-11-dUTP (Sigma-Aldrich) and digoxigenin-11-dUTP (Roche Diagnostics). Biotin has a strong affinity to avidin and strepatavidin which when conjugated to a fluorochrome are used to detect the probe

hybridisation sites. Hybridisation sites containing digoxigenin are visualized by using antibodies for digoxigenin conjugated to fluorochromes. Probes where labelled by nick translation (Leitch *et al.*, 1994), which involves the employment of two enzymes, DnaseI and *E. coli* DNA polymerase, to incorporate labelled nucleotides into both strands of the DNA probe by nicking the DNA and substituting nucleotides. The nick translation reaction mix is detailed in Tables 6.7 and 6.8 (a list of materials including stock concentrations are detailed in Appendix 1.11b). The outlined reagents were combined in a 1.5 ml microcentirfuge tube, mixed and centrifuged briefly. The reagents were then placed in a thermal-cycler and incubated at 15°C for 90 minutes. Adding 5µl EDTA stopped the reaction. The DNA probes were purified using the ethanol precipitation method and resuspended in 10µl 1×TE buffer (described in Protocol 1.6).

Table 6.7. DNA labelling with digoxigenin-11-dUTP by nick translation. Reagent components are detailed in Appendix 1.10b.

Probe label mix

Component	**Volume (µl)**
Nick translation buffer (10×)	5.0
Dithiothreitol (DTT; 100mM)	1.0
Unlabelled nucleotide mix (0.5mM)	5.0
Digoxigenin-11-dUTP (0.65mM)	1.0
Pta71 DNA (200 ng/µl)	5.0
Sterile ultrapure H_2O	28.0
DNA Polymerase I/DnaseI solution	5.0
Total volume	50.0
Incubation	15°C for 90 minutes

In addition to using the pTa71 probe, genome *in-situ* hybridisation was attempted on the tetraploid and hexaploid species using total genomic DNA probes generated for the diploid species. The labelling of the DNA was by nick translation and the reagent

composition was the same as for the pTa71 probe. It was intended to use the diploid *Papaver bracteatum* DNA to hybridise to the tetraploid *P. orientale* and labelled DNA from *P. bracteatum* and *P. orientale* to hybridise to the hexaploid *P. pseudo-orientale* in an attempt to investigate the origins of the tetraploid and hexaploid species.

Table 6.8. DNA labelling with biotin-11-dUTP by nick translation. Reagent components are detailed in Appendix 1.10b.

Probe label mix

Component	**Volume (µl)**
Nick translation buffer (10×)	5.0
Dithiothreitol (DTT; 100mM)	1.0
Unlabelled nucleotide mix (0.5mM)	5.0
biotin-11-dUTP (0.65mM)	2.5
pTa71 DNA (200 ng/µl)	5.0
Sterile ultrapure H_2O	26.5
DNA Polymerase I/Dnase I solution	5.0
Total volume	50.0
Incubation	15°C for 90 minutes

Probe incorporation was checked by loading approximately 1µl of the resuspended probe-labelling product onto a 5cm×5cm piece of Hybond N^+ membrane (Amersham Pharmacia Biotech). The membrane was transferred to a series of buffers (detailed in Appendix 1.11c), and the probe was detected by adding 1ml NBT/BCIP detection reagent (Life Technologies) and processed in the dark. A compact, dark spot indicated successful probe incorporation.

6.4.7.3 Material preparation for *in-situ* hybridisation

The preparation of slides with high quality chromosome spreads involves a number of steps before the probe can be hybridised to the chromosomes (a list of materials and

stock concentrations are detailed in Appendix 1.11d). Slides were placed in an oven at 37°C overnight. 100µl of 0.2µg/ml DNase A was added to the slide and covered with a plastic coverslip. The DNaseA treatment served to remove cellular RNA to avoid the probe from binding to it. The slides were incubated at 37°C for 1 hour. The slides were then washed in 2×SSC three times for 5 minutes each. The slides were placed in 0.01N HCl for two minutes and 100µl of 1.0µg/µl pepsin was added, covered with a plastic coverslip and incubated for 10 minutes at 37°C. Pepsin is thought to make the chromosomes more accessible to the probe (Leitch *et al.*, 1994). The reaction with pepsin was stopped by placing the slide in water for 2 minutes and subsequently washed in 2×SSC twice for 5 minutes each. The slides were fixed using freshly prepared 4% (w/v) paraformaldehyde for 10 minutes, which helps to maintain the chromosome structure enabling a higher denaturation temperature (Leitch *et al.*1994). The slides were once again washed in 2×SSC three times for 5 minutes each. Slides were dehydrated by placing them in coplin jars containing an ethanol series of 70%, 90% and 100% for 5 minutes each (starting with the lowest). The slides were drained of ethanol and air-dried.

6.4.7.4 Hybridising the probe to the chromosomes

The hybridisation mix was set up as detailed in Table 6.9 and details on materials, reagent components and stock concentrations can be found in Appendix 1.11e. The hybridisation mix was denatured at 75°C for 10 minutes, vortexed and placed on ice. The slides were denatured by placing them in coplin jar containing 70% formamide solution at 70°C for 2 minutes. The slides were then placed in an ice-cold ethanol series (70%, 90% and 100%) for 3 minutes each and allowed to air dry. 40µl of the denatured hybridisation solution was added to each slide, covered with a plastic coverslip and incubated at 37°C overnight in a humid chamber.

After the *in situ* hybridisation reaction the slide was washed stringently to ensure that weakly bound and non-specifically bound probe was 'stripped' away. After incubation the slides were placed in a Coplin jar containing 2×SSC at 42°C until the coverslip became detached from the slides. The slides were then transferred to another coplin jar containing 'stringent wash' (20% formamide in 0.1×SSC) twice for

5 minutes each at 42°C. The stringent wash solution contains salts and formamide, which result in the destabilization of weakly bound probes (Leitch *et al.*, 1994).

Table 6.9. DNA:DNA *in situ* hybridisation reaction mix. Reagent components are detailed in Appendix 1.10e.

Hybridisation reaction mix

Component	Volume (µl)	Final Concentration
Formamide	20.0	50%
20× SSC	4.0	2×
Probe DNA-dig (100ng/slide)	1.0	2.5ng/µl
Probe DNA-bio (100ng/slide)	1.0	2.5ng/µl
Blocking DNA	2.0	250ng/µl
10% (w/v) SDS	0.5	
50% (w/v) dextran sulphate	8.0	1.25%
Sterile ultrapure H_2O	3.5	10.%
Total volume	40.0	

Slides were washed in 2×SSC 3 times at 42°C for 3 minutes each and then transferred to another Coplin jar containing 4×SSC/Tween for 5 minutes. Slides were drained (never allowing them to dry off) and 200µl of BSA-DNA block was added to each, covered in a plastic coverslip and incubated at 37°C for 5 minutes. The coverslips were removed from the slides and excess solution was drained off. 50µl of detection reagent (anti-digoxigenin FITC and avidin Cy3 fluorochrome conjugates for digoxigenin and biotin probe detection respectively) was then added to each slide, covered with a plastic coverslip and incubated at 37°C for 1 hour. Slides were then washed in 4×SSC/Tween 3 times at 37°C for 5 minutes. Slides were drained of solution and 100µl of DAPI was added to counterstain the chromosomes. The slides were covered with a plastic coverslip and incubated at 37°C for 10 minutes. After incubation the slides were rinsed briefly in 4×SSC/Tween, drained and 2 drops of antifade solution was added (reduces the fading of fluorescence during slide scanning

and photography). A transparent coverslip was placed over the material on the slide and the excess antifade was removed with filter paper.

Visualisation of chromosome spreads with hybridised probes were examined using Leitz Aristoplan or Leica DMRA2 epifluorescent microscopes fitted with an Orca ER camera and Open Lab software® (Improvision). All images were processed using Adobe Photoshop® V. 5.0 and treated for colour contrast and brightness uniformly.

6.5 Results

6.5.1 Parsimony analysis of morphological characters

The morphological matrix had 11 characters all of which were parsimony informative. The heuristic search yielded 33 equally most parsimonious trees (Figure 4.13) of 19 steps with a consistency index (CI) of 0.364 and a retention index (RI) of 0.807. All *Papaver bracteatum* accessions resolved together in a moderately supported group (bootstrap value of 71%). Those accessions (accessions 035 & 135) identified as *Papaver orientale* as defined by the morphological characters described by Goldblatt (1974) resolved together (with bootstrap support < 50%) but within a larger group consisting of a number of cultivars and those accessions identified as *P. pseudo-orientale. Papaver orientale* 'Goliath' (accession 123) groups with the *Papaver orientale* accessions. Resolving as sister to the main *Papaver bracteatum, P. orientale* and *P. pseudo-orientale* group is a group of accessions (accessions 093, 108, 137, 096 and 133), which had dubious identity, either displaying characteristics of both *P. orientale* and *P. pseudo-orientale.* A number of cultivars resolved outside these main groups and generally possessed petals which were different colours to typical red and orange displayed by *Oxytona* species.

6.5.2 Phytochemical analysis

The retention times and mass fragmentation spectra for a number of accessions used in the morphological and molecular analysis are given as Table 6.10. Although the molecular weight of the expected dominant alkaloids produced by *Papaver*



Figure 6.2 One of 33 equally most parsimonious trees from a morphological analysis of *Papaver* section *Oxytona* using 13 characters. Branch lengths are above branches and bootstrap values are below. Tree statistics are also shown. Support for each node is represented by bootstrap values (%) below the branch (shown only when >50%). Numbers above branches are branch length indices. Black arrows indicate branches that collapse in the strict consensus trees and red arrows indicate branches of zero branch length

bracteatum and *P. pseudo-orientale* (i.e. thebaine and isothebaine) is 311 these two alkaloids can be differentiated as the have diagnostic retention times. All accessions of *Papaver bracteatum* (accessions 020, 031, 028 and 029) produced thebaine as the dominant alkaloid (RT 20.51mins; *m/z* 311). Oripavine was identified by its molecular weight (297) and by the fact that this alkaloid (RT 19.57mins; *m/z* 297) was present in those accessions identified as 'true' *Papaver orientale* (accessions 35 and 135) based on the morphological description of Goldblatt (1974). The molecular weight is consistent with previous GC/MS reports (Theuns *et al.*, 1987). Isothebaine (RT 21.80mins; *m/z* 311) was the dominant alkaloid produced by all other accessions analysed including those accessions identified *Papaver pseudo-orientale*, all cultivars of *Papaver orientale* and those accessions with questionable identities.

Table 6.10 Selected ions, retention times, and ion ratios of thebaine, isothebaine and a number of selected accessions.

Compound/Accession	Accession number	Retention Time (min)	Monitored Ions	Ion Ratio Percent (Abundance)	Alkaloid identity
Thebaine		20.51	311,296	100,75	
Isothebaine		21.80	311,294	100,50	
Papaver bracteatum	J-020	20.51	311,296	100,75	Thebaine
Papaver bracteatum	J-031	20.51	311,296	100,75	Thebaine
Papaver bracteatum	J-028	20.51	311,296	100,75	Thebaine
Papaver bracteatum	J-029	20.51	311,296	100,75	Thebaine
Papaver orientale	J-035	19.57	297,283	100,25	Oripavine
Papaver orientale	J-040	21.80	311,294	100,50	Isothebaine
Papaver orientale	J-070	21.80	311,294	100,50	Isothebaine
Papaver orientale	J-135	19.57	297,283	100,25	Oripavine
Papaver orientale	J-121	21.80	311,294	100,50	Isothebaine
Papaver orientale	J-123	20.51	311,296	100,75	Thebaine
Papaver orientale	J-137	21.80	311,294	100,50	Isothebaine
Papaver orientale	J-140	20.51	311,296	100,75	Thebaine
Papaver pseudo-orientale	J-034	21.80	311,294	100,50	Isothebaine
Papaver pseudo-orientale	J-054	21.80	311,294	100,50	Isothebaine
Papaver pseudo-orientale	J-055	21.80	311,294	100,50	Isothebaine
Papaver pseudo-orientale	J-062	21.80	311,294	100,50	Isothebaine
Papaver pseudo-orientale	J-093	21.80	311,294	100,50	Isothebaine
Papaver pseudo-orientale	J-096	21.80	311,294	100,50	Isothebaine
Papaver pseudo-orientale	J-134	21.80	311,294	100,50	Isothebaine

6.5.3 Cytological analysis

The diploid chromosome number for a number of accessions was recorded and given in Table 6.11. No deviation from n=7 was experienced for any of these accessions. It

was not possible to obtain chromosome counts for all accessions used in the morphological and molecular analysis as a number of these accessions were obtained as leaf material with accompanying vouchers. In addition it was not possible to transplant some accessions from the living collections of the Botanic Gardens from which material was obtained. A number of photographs when developed but were poorly focused and the majority of chromosome counts were made directly from the microscope by focusing up and down through the slide. All the accessions for *Papaver bracteatum* had 2n=14. Those accessions which were acquired as "*Papaver orientale*" and that had 42 chromosomes were *Papaver pseudo-orientale*. Accessions of *Papaver orientale* were therefore identified by having 2n=28. All accessions supplied as *Papaver pseudo-orientale* and that had 2n=42 were correctly identified.

Table 6.11 Chromosome numbers and ploidy for a number of accessions representing section *Oxytona*.

Genus and species	Accession number	Chromosome number	Ploidy/ possible origin
Papaver bracteatum	J-020	14	Diploid
Papaver bracteatum	J-031	14	Diploid
Papaver bracteatum	J-028	14	Diploid
Papaver bracteatum	J-029	14	Diploid
Papaver bracteatum	J-155	14	Diploid
Papaver orientale	J-008	28	Tetraploid
Papaver orientale	J-035	28	Tetraploid
Papaver orientale	J-135	28	Tetraploid
Papaver orientale	J-137	35	*P. orientale* × *P. pseudo-orientale* hybrid
Papaver orientale	J-070	42	Hexaploid
Papaver orientale	J-040	42	Hexaploid
Papaver pseudo-orientale	J-034	28	*P. bracteatum* × *P. pseudo-orientale* hybrid
Papaver pseudo-orientale	J-037	28	*P. bracteatum* × *P. pseudo-orientale* hybrid
Papaver pseudo-orientale	J-054	42	Hexaploid
Papaver pseudo-orientale	J-055	42	Hexaploid
Papaver pseudo-orientale	J-062	42	Hexaploid
Papaver pseudo-orientale	J-091	35	*P. orientale* × *P. pseudo-orientale* hybrid
Papaver pseudo-orientale	J-096	35	*P. orientale* × *P. pseudo-orientale* hybrid
Papaver pseudo-orientale	J-108	35	*P. orientale* × *P. pseudo-orientale* hybrid

A number of accessions (accessions 034 and 37), which displayed morphological characteristics of *Papaver pseudo-orientale*, had in a reduced number of chromosomes and were determined to be probable hybrids of this species and *P. bracteatum* having 28 chromosomes (7 from *P. bracteatum* and 21 from *P. pseudo-orientale*). A number of cultivars, 'Salmon Queen' (accession 040) and 'Patty's Plum' (accession 140) were hexaploid having 2n=42. Some accessions which have been described previously as being very difficult to identify (accession 091, 096 and

137), displaying morphological traits of both *Papaver pseudo-orientale* and *P. orientale* were identified as probable hybrids between the two species possessing 35 chromosomes.

6.5.4 AFLP analysis

A total of 226 markers ranging in size from 50-288bps (fragment sizes) were generated from the two primer pair combinations used in the analysis. Nei and Li distances are given as Appendix 2.3. The Neighbour Joining tree generated from the AFLP markers is given as Figure 6.3. The tree generated from NJ analysis was in congruence with the UPGMA tree (not shown). Two major groups were resolved, the first (clade 1; 98% bootstrap support) consists of *Papaver bracteatum, P. pseudo-orientale* (possessing 2n=42) and a number of cultivars and the second (clade 2; 77% bootstrap support) consists of *P. orientale* and the remaining cultivars and those accessions which displayed morphological characters of both *P. orientale* and *P. pseudo-orientale*. All accessions of *Papaver bracteatum* grouped together and this group was well supported (97% bootstrap support). The hybrids identified from the cytological analysis that arose between *Papaver pseudo-orientale and P. bracteatum* grouped within clade 1 (accessions 034 and 37), and the accessions that arose from crosses between *P. orientale* and *P. pseudo-orientale* grouped within clade 2 (accessions 91, 96, 108 and 137).

Figure 6.4 shows the principle coordinate analysis (PCO) of the AFLP data, which is essentially in congruence with the NJ analysis. Dice distances and the corresponding eigenvalues and eigenvectors generated from the PCO analysis are given as Appendices 2.4 and 2.5 respectively. The first two axes cumulatively accounted for 44.8% of the data variance (representing 34.1 and 10.7 respectively). Three main groups are evident. The first group contains those accessions identified as *Papaver bracteatum* and those accessions which were hybrids of *P. bracteatum* × *P. pseudo-orientale*. Group two consists of *Papaver pseudo-orientale*. The third group comprises *Papaver pseudo-orientale,* many of the cultivars and those accessions, which from the cytological analysis were identified as hybrids between *P. pseudo-orientale* and *P. orientale*.

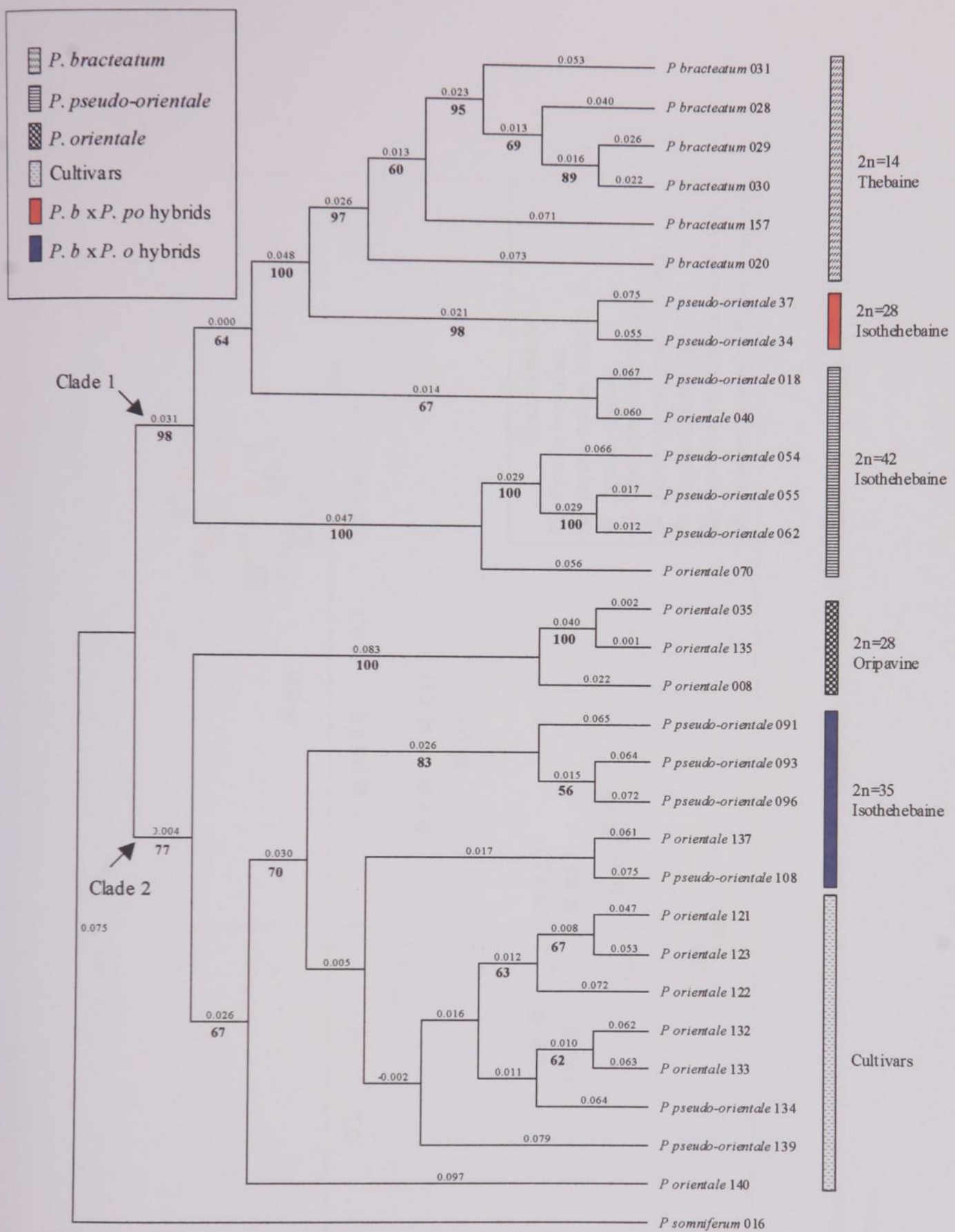


Figure 6.3 Neighbour Joining tree based on AFLP data using Nei & Li's distance measure, rooted on *Papaver somniferum*. Support for each node is represented by bootstrap values (%) below the branch (shown only when >50%). Numbers above branches are branch lengths. Characters from the cytological and phytochemical analysis are also shown. *P.b* × *P. po*: *P. bracteatum* × *P. psuedo-orientale* hybrid; *P.b* × *P.o*: *P. bracteatum* × *P. orientale* hybrid.

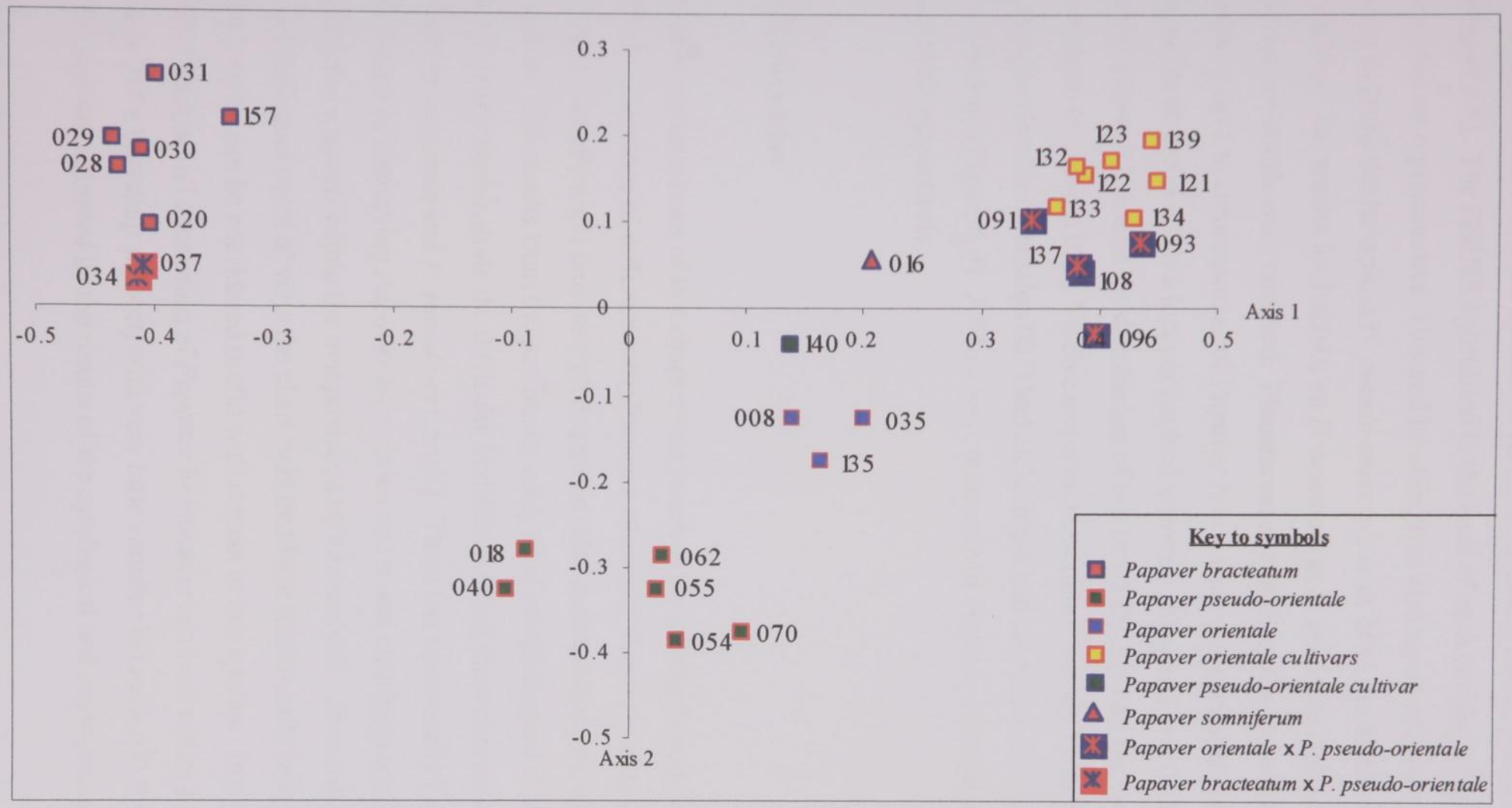


Figure 6.4 Principal co-ordinates analysis (PCO) of AFLP data. The first two axes cumulatively accounted for 44.8% of the data variance (34.1and 10.7 respectively). The key to the symbols is also shown.

6.5.5 *In situ* hybridisation

Labelled total genomic DNA taken from *Papaver bracteatum* (PbDNA) was hybridised to chromosome spreads of itself, *P. orientale* and *P. pseudo-orientale* (Figure 6.6). The PbDNA hybridised to the ends of each chromosome in the *P. bracteatum* representative. This end labelling was also noted when the PbDNA was used to probe the hexaploid *P. pseudo-orientale* with 28 of the 42 chromosomes being labelled. No results for PbDNA on *P. orientale* or *P. orientale* DNA on itself and *P. pseudo-orientale* were obtained. Fluorescent *in situ* hybridisation with the rDNA probe pTa71 to chromosomes of *Papaver bracteatum* (accession 031) showed six sites (three 18S-5.8S-26S loci per haploid genome). Each site was telomeric (Figure 6.7). Figure 6.8 details the distribution of the 18S-5.8S-26S loci for *Papaver orientale* (accession 035). Twelve major and one minor loci are identified. *Papaver pseudo-orientale* (accession 062) had eight major and six minor loci for 18S-5.8S-26S distribution (Figure 6.9). All loci were telomeric in *Papaver orientale* and *P. pseudo-orientale* respectively.

6.6 Discussion

One of the intentions of this chapter was to assess the genetic fingerprinting technique AFLP as a means of differentiating between species of *Papaver* section *Oxytona* and to give further insight into their phylogenetic relationship that exists within this section. The results from the parsimony analysis of morphological characters (Figure 6.2), clearly demonstrate the difficulty in differentiating these species (especially *Papaver orientale* and *P. pseudo-orientale*). There has traditionally been little difficulty in identifying *Papaver bracteatum* and it was clearly grouped (90% bootstrap support) within the tree produced by this analysis. Thus from a morphological point of view the characters that have traditionally been used to define this species can be considered useful with respect to this species. In the molecular AFLP analysis all accessions of *Papaver bracteatum* resolved within a well-supported clade (97% bootstrap support) with very little variance between AFLP fingerprints. This was corroborated by the results of the cytological and phytochemical analysis as

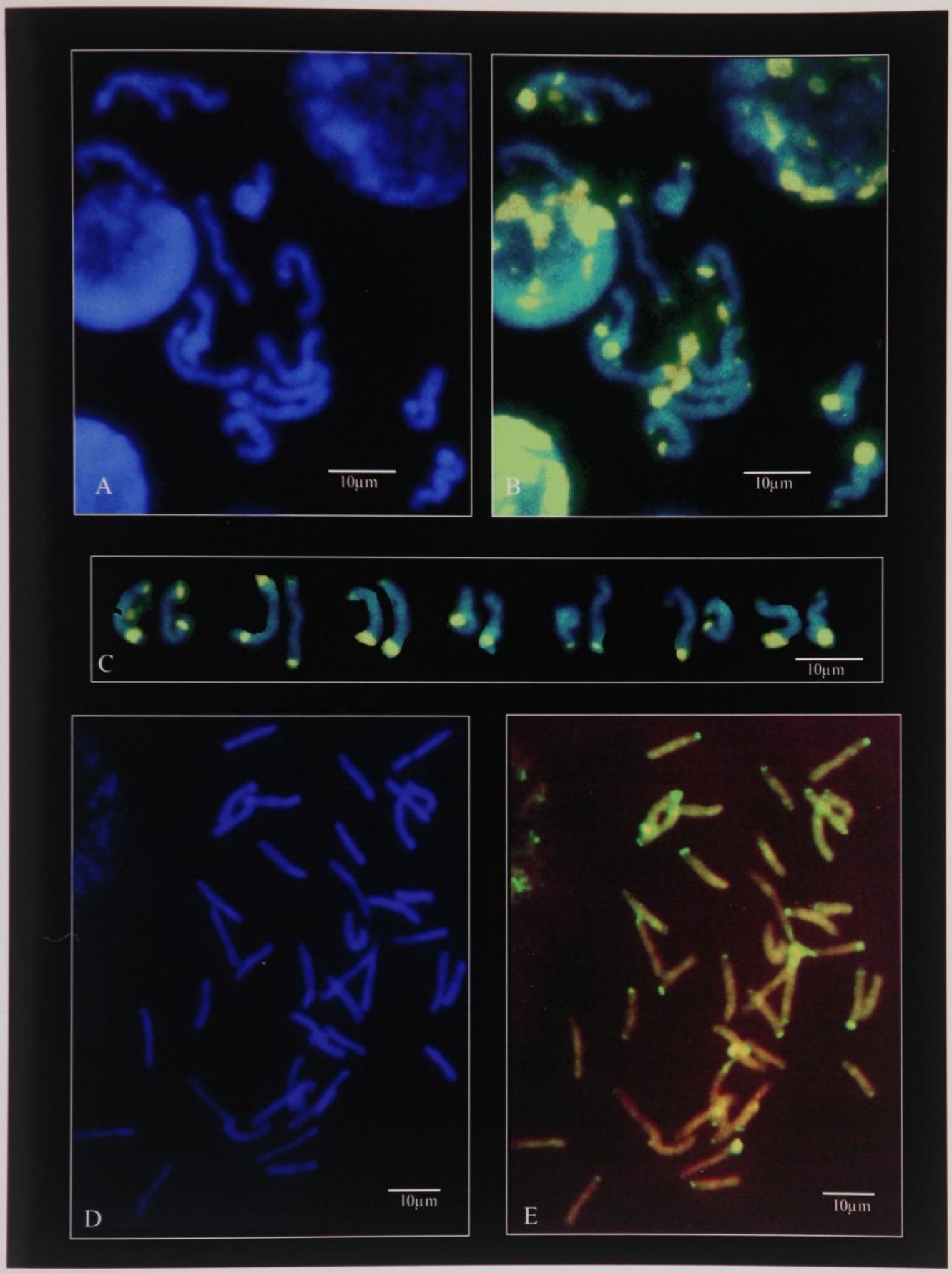


Figure 6.6 (A-C): Chromosomes of *Papaver bracteatum*; (D&E): Chromosomes of *P. pseudo-orientale.* GISH performed by probing with biotinylated total genomic DNA from *Papaver bracteatum* on itself (B&C) and on *P. pseudo-orientale* (E). Hybridised areas fluoresce bright yellow; A&D: Metaphase chromosomes stained with DAPI; C: Karyogram of *Papaver bracteatum.*

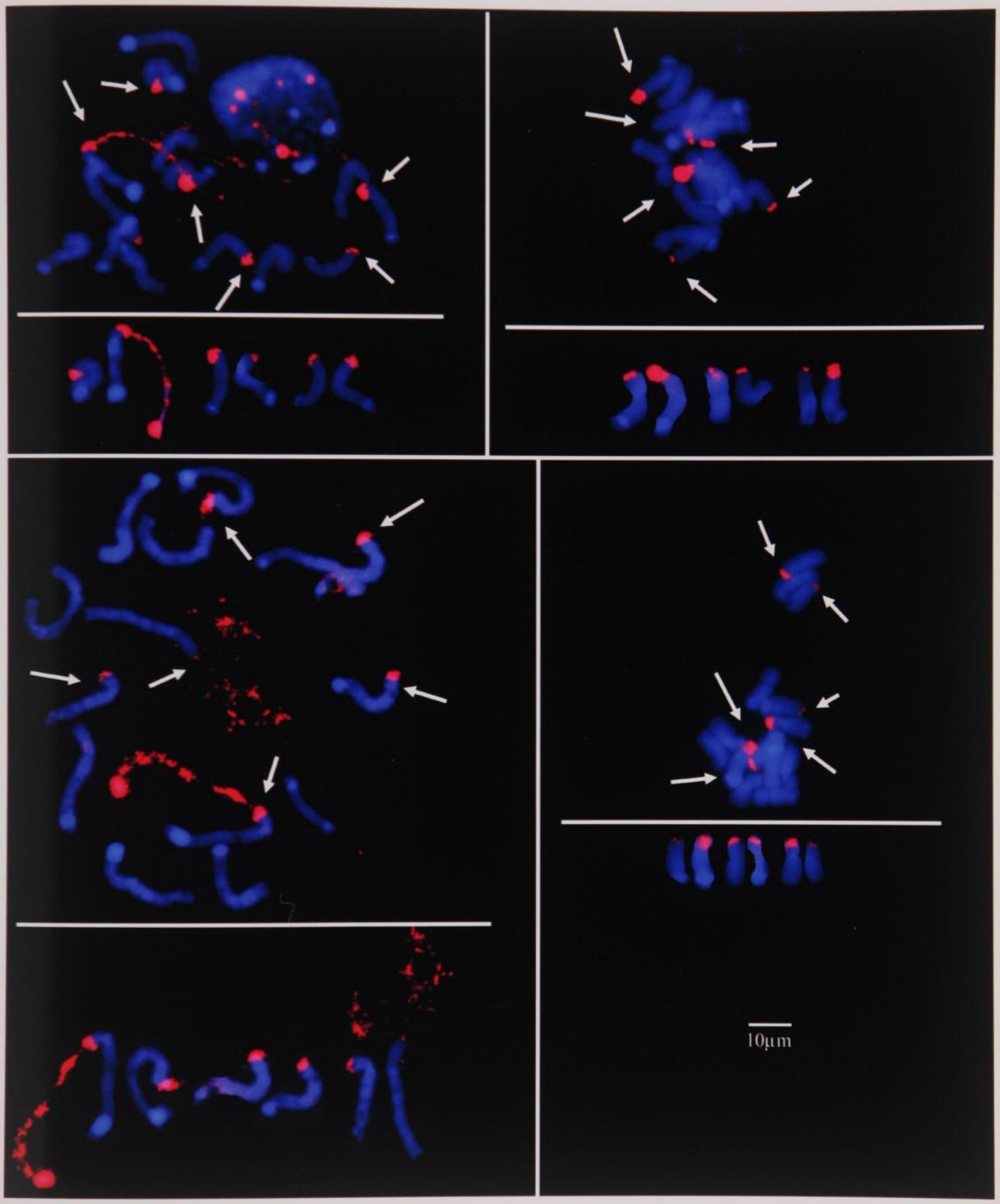


Figure 6.7 Chromosomes of *Papaver bracteatum* stained with DAPI, showing three major loci for probe hybridisation, corresponding to 18S-5.8S-26S rDNA repeat distribution.

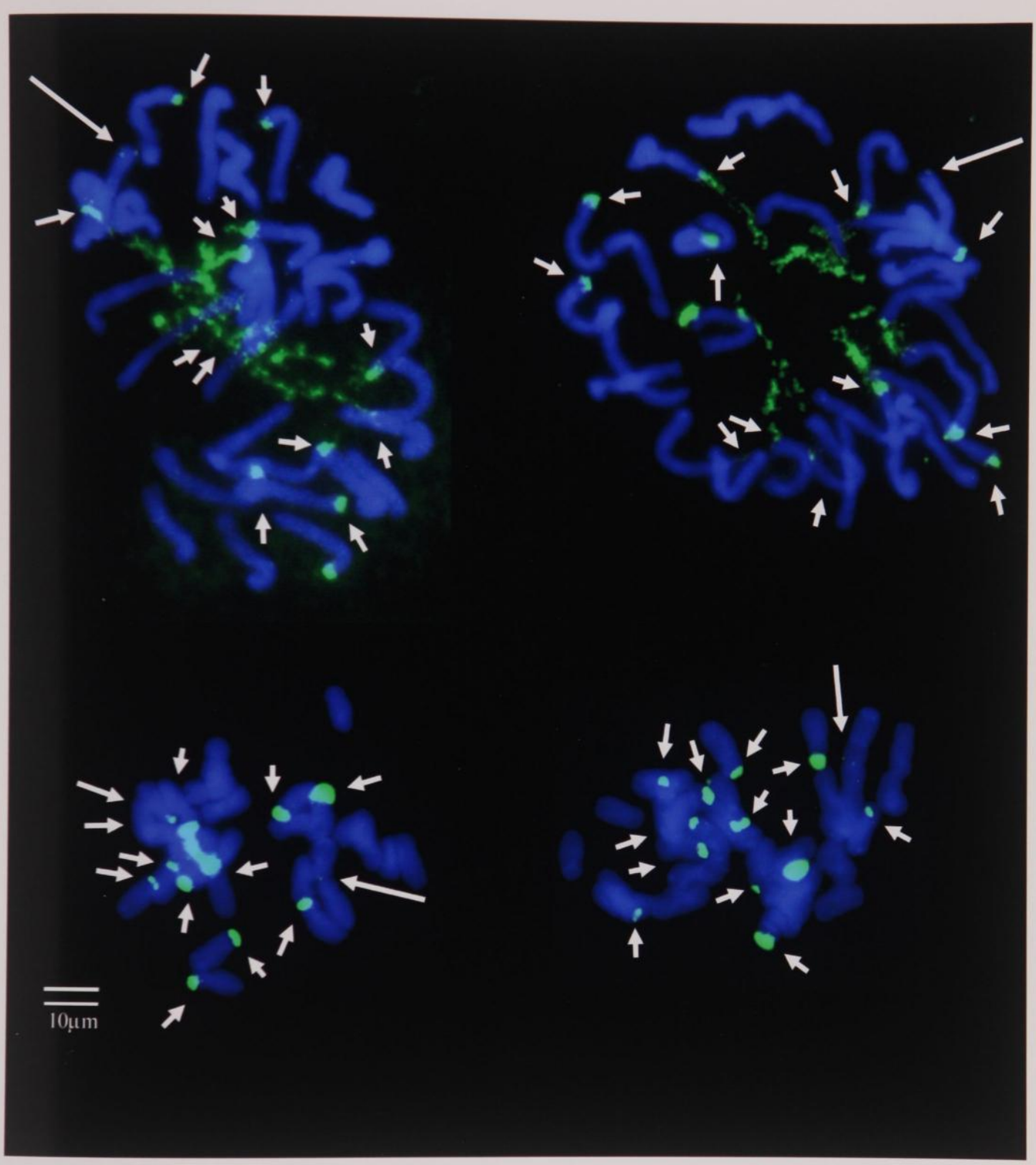


Figure 6.8 Chromosomes of *Papaver orientale* stained with DAPI, showing twelve major loci (indicated by small arrows) and one minor locus (indicated by larger arrows) for probe hybridisation, corresponding to 18S-5.8S-26S rDNA repeat distribution.

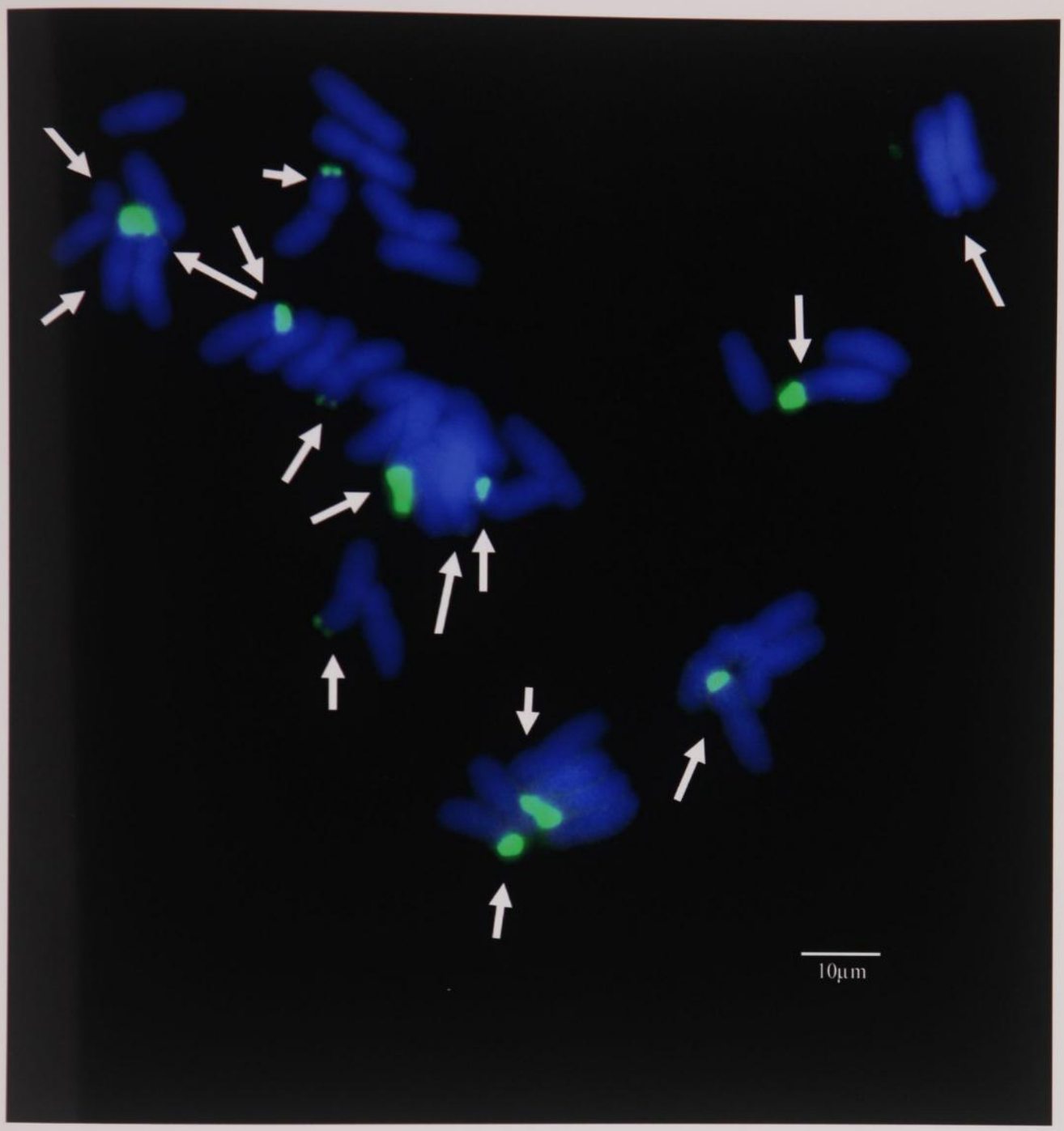


Figure 6.9 Chromosomes of *Papaver pseudo-orientale* stained with DAPI, showing eight major (indicated my small arrows) and six minor (indicated by larger arrows) loci for probe hybridisation, corresponding to 18S-5.8S-26S rDNA repeat distribution. This particular photograph shows 40 chromosomes only. Two chromosomes were lost during slide preparation but the number of loci was consistent with other cells consisting of 42 chromosomes (not shown).

all *Papaver bracteatum* accessions analysed, had 2n=14 and produced thebaine as the dominant alkaloid. The three accessions identified as *Papaver orientale* (accessions 008, 035 and 135) in accordance to Goldblatt's description (1974, c.f. Table 6.1) resolved together in a well-supported group (100% bootstrap support) within the tree generated from the AFLP analysis. Based on morphological characters these three accessions also grouped together but the distinction from *Papaver pseudo-orientale* was not well defined. However the identity of these particular accessions was confirmed by the cytological and phytochemical analysis, as all had 2n=28 and produced oripavine as their dominant alkaloid.

The embedding of *Papaver orientale* within the group comprising *P. pseudo-orientale* from the morphological analysis highlights the similarity between these species and more specifically the overlap that exists between a number of morphological characters displayed by the accessions used in this analysis. Based on morphological characters, a group of accessions (accessions 093, 108, 137, 096 and 133) separate from the main *Papaver bracteatum, P. orientale* and *P. pseudo-orientale* group. These accessions proved difficult to identify even with floral characteristics, with many showing characteristics of both *Papaver orientale* and *P. pseudo-orientale*. For example accessions 093 and 096 possessed characters such as pendulous buds before flowering, pyramidical stigmatic disc, four orange petals, low number of stigmatic rays and extremely fine adpressed setae. These characters are displayed by *Papaver orientale* but are also found in *P. pseudo-orientale* (Goldblatt, 1974; except pyramidical stigmatic disc). The fact that these particular accessions also had petals with purple spots (a character never displayed by *Papaver orientale*) would suggest that these accessions were *P. pseudo-orientale* based on morphology alone. Within the tree generated from the AFLP analysis these particular accessions show genetic dissimilarity to the other *Papaver pseudo-orientale* accessions, but instead group more closely to *Papaver orientale.* The results suggest the presence of *Papaver orientale* genomic material within these particular accessions. Chromosome counts demonstrate that a number of accessions (accessions 096, 137 and 108) were hybrids formed between *Papaver orientale* and *P. pseudo-orientale* having 35 chromosomes. Thus it would seem that where a particular accession displays morphological characters not typical of those used to define a particular species of *Oxytona,*



Figure 6.9 A&B, Typical *Papaver pseudo-orientale* (accessions 062 and 054 respectively); C, *P. orientale* × *P. pseudo-orientale* hybrid (accession 108); D, *P. orientale* × *P. pseudo-orientale* hybrid (accession 096); E, *Papaver pseudo-orientale* (accession 134), most likely a *P. orientale* × *P. pseudo-orientale* hybrid; F, *Papaver pseudo-orientale* (accession 133), most likely a *P. orientale* × *P. pseudo-orientale* hybrid. Although similarities in petal characteristics exist between all accessions certain subtle differences are found such as the combination of spots with pyramidal stigmatic disc which suggests that many of theses accessions are hybrids. *Papaver pseudo-orientale* generally has flat stigmatic discs.

divergence in morphology could be explained by hybridisation and/or introgressive hybridisation. Figure 6.9 details the floral characteristics of *Papaver pseudo-orientale* and those accessions with problematic identities. This is highlighted by the positioning of accession 091,within the morphological tree with *Papaver pseudo-orientale* (accession 062). In the tree produced by the AFLP analysis it is evident that these accessions are distinct with accession 091 grouping within the *Papaver orientale* group (clade 2) along with the other accessions of questionable identity (accessions 093, 108, 137, 096 and 133). This accession was also believed to be of hybrid origin having 35 chromosomes and groups with another hybrid, accession 096 within a well-supported clade (83% bootstrap support). In addition the two other *Papaver orientale* × *P. pseudo-orientale* hybrids identified from the cytological analysis (accessions 108 and 137) resolve, together within clade 2.

Those accessions identified as *Papaver pseudo-orientale* by having 2n=42 chromosomes and morphological characters consistent with the description of Goldblatt, 1974) resolve with *Papaver bracteatum* within a well-supported group (98% bootstrap support) in the AFLP analysis. However two of these accessions (i.e. accessions 037 and 034) are sister to the *Papaver bracteatum* group (100% bootstrap support) and do not group with the other *P. pseudo-orientale* accessions. This unexpected position (as these accessions possessed morphological characters typical of *Papaver pseudo-orientale*) was explained by the cytological investigation as these accessions had 28 chromosomes, which could be interpreted as representing a hybridisation between *Papaver bracteatum* and *P. pseudo-orientale* (receiving 7 and 21 chromosomes from *P. bracteatum* and *P. pseudo-orientale* respectively). Accessions 018 and 040 also demonstrate such a pattern within the AFLP analysis, which again may indicate that they are in fact of hybrid origin. However one of these, accession 040, was available for a chromosome count and it possessed 42 chromosomes indicating that it is *Papaver pseudo-orientale*. Thus the separation of this group from the other *Papaver pseudo-orientale* accessions may represent a degree of genetic diversification (or evidence of hybridisation/introgression).

Regarding the number of cultivars included in this study, it is evident that there are two types. The first group shows more genetic similarity to *Papaver orientale* than to *P. pseudo-orientale.* However many of these cultivars usually possess spots on their

petal a characteristic of the latter species. The positioning of these cultivars within clade 2 and that fact that those accessions, which were identified as hybrids of *Papaver orientale* × *P. pseudo-orientale,* resolve as sister to this group would suggest that these cultivars are of hybrid origin. Unfortunately only two of these were available for chromosome count with one accession (137) having 35 chromosomes confirming its hybrid status. Cross breeding is common when attempting to develop new garden and crop varieties (Jones *et al.,* 1997; Lyrene *et al.,* 2003; Ramanna & Jacobsen, 2003). However determining the identity of cultivars with genetic methods is made difficult by the fact that often cultivars are bred with other cultivars and the exact historical records of these crosses are seldom kept. The results of this investigation however suggests that many of the cultivars supplied as *Papaver orientale* (or oriental poppies) are hybrids and the genetic similarity between *P. orientale* and these cultivars can be interpreted as indicating that this species was the starting material for crosses and manipulation in the attempt to discover accessions with aesthetically pleasing phenotypes. A number of cultivars resolve within clade 1 and include accessions 070 ('Black & White') and 040 ('Salmon Queen'). This genetic similarity to *Papaver pseudo-orientale* was confirmed by the fact that these accessions were hexaploid having 2n=42. The position within clade 1 and their separation from the other cultivars examined in this analysis suggests that these particular cultivars have been developed from *P. pseudo-orientale* and not *P. orientale.*

The results from the alkaloid analysis are generally consistent with the groupings of the AFLP analysis. All accessions of *Papaver bracteatum* that grouped together in the AFLP analysis produced thebaine as the dominant alkaloid, a result consistent with previous reports (e.g. Fairbairn & Helliwell, 1977; Theuns *et al.,* 1987; Hook *et al.,* 1988). Accessions identified as *Papaver pseudo-orientale* based on morphological characters, hexaploid status and resolving within clade 1 of the AFLP analysis produced isothebaine as the dominant alkaloid. Those accessions identified as *Papaver orientale* were the only accessions to produce oripavine as the dominant alkaloid. The hybrids that arose from *Papaver bracteatum* × *P. pseudo-orientale* (accessions 034 and 037) produced isothebaine as the dominant alkaloid a result consistent with previous reports (Milo *et al.,* 1990; Levy & Milo, 1991). Our results however contrast with Levy & Milo (1990), as no traces of thebaine were recorded for

the hybrid accession. The hybrids that arose from *Papaver orientale* × *P. pseudo-orientale* produced isothebaine as the dominant alkaloid, confirming their status as hybrids. These displayed the phytochemical characteristics of *Papaver pseudo-orientale* and the genetic and morphological characteristics of *P. orientale*. *Papaver pseudo-orientale* 'Salmon Queen' produced thebaine as its dominant alkaloid, a result congruent with previous phytochemical analyses (Corrigan & Martin, 1981). This cultivar had 42 chromosomes and can be considered to be a variety of *Papaver pseudo-orientale*, the alkaloid profile most probably reflects that it is a chemotype of this species. Chemotypes of *Papaver pseudo-orientale* which produce thebaine as their dominant alkaloid rather than isothebaine have previously been reported (Lalezari & Shafiee, 1978). Cultivar 'Goliath' also produced thebaine also as its dominant alkaloid and coupled with the 'Salmon Queen' cultivar represent those cultivars analysed in this study most worthy of further investigation.

The results of the genetic fingerprinting analysis indicate that AFLP is well suited to differentiate between the species of *Oxytona*. Without flowering characteristics these species are extremely difficult to identify. It is evident from the results of this research that AFLP fingerprinting can be employed to aid in the identification of material without reliance on these characters, on the condition that correctly identified reference material is used also. In addition AFLP fingerprinting has the potential to identify hybrid accessions. Many of the hybrids identified in this analysis were not obvious from a morphological point of view, but slight differences in morphology coupled with anomalous positioning within the AFLP trees highlighted the uncertain identity of these particular accessions. Hybrids were confirmed using simple chromosome counts, thus highlighting not only the necessity but also the advantage of using a multi-discipline approach to species identification.

An objective of the research described in this chapter was to use the results of the *in situ* hybridisation investigation and the results of the AFLP analysis to further investigate the phylogenetic relationship that exists between the species of *Papaver* section *Oxytona*. The only successful *in situ* hybridisation using genomic DNA (GISH) of one species as a probe on the chromosomes of another was obtained using *Papaver bracteatum* total genomic DNA on chromosomes of itself and the hexaploid *P. pseudo-orientale* (c.f. Figure 6.6). The results demonstrate that the total genomic

probe labelled the ends of all 14 chromosomes of *P. bracteatum* and the ends of 28 of the 42 chromosomes of *P. pseudo-orientale*. Probing of chromosomes with total genomic DNA of a potential parental species usually results in the labelling of the complete chromosome or not. The results of this analysis show hybridisation of the probe to the ends of each chromosome (satellite repeat regions).

Where individual chromosomes have shown hybridisation in the satellite repeat areas (end labelling) phenomena such as recombination and translocation have been suggested to explain the apparent lack of hybridisation along the whole chromosome (Stace & Bailey, 1999). However such explanations could not possibly account for this pattern of hybridisation along all chromosomes. A similar hybridisation pattern was reported by Stace & Bailey (1999), who in their work on *Festuca rubra* (Poaceae), found that when the total genomic DNA of a potential progenitor was used as a probe on the complete set of this species chromosomes only the terminal segments of a set of chromosomes hybridised to the probe. They explained this by suggesting that it is the highly repetitive DNA that forms most of the species-specific DNA (see Anamthawat-Jonsson & Heslop-Harrison, 1995) and when DNA from a closely related species is used as blocking DNA the more conserved (between species) central regions of the chromosome are inhibited from hybridising with the probe. This might explain the situation in *Papaver pseudo-orientale* as DNA from the closely related *P. orientale* was used as blocking DNA. However this explanation is rejected as the same banding pattern was obtained when total genomic DNA form *Papaver bracteatum* was used as a probe on itself, with no blocking DNA being used. Stace & Bailey (1999), when interpreting similar results conclude that 'it must be a physical and/or chemical difference between the DNA of euchromatin and heterochromatin that is causing the difference in labelling between those two regions'. To confirm this, however, would require a significant amount of further experimentation.

To examine the results of the GISH analysis with respect to hypotheses explaining the origins of the tetraploid and hexaploid species of *Papaver* section *Oxytona* the assumption that the chromosomes in both *Papaver bracteatum* and *P. pseudo-orientale* with the terminal hybridisation of *P. bracteatum* DNA are homologous has

to be made. It is hypothesised (Goldblatt, 1974, Nyman & Bruhn, 1979) that *Papaver orientale* is an allotetraploid descendant of a hybrid of *P. bracteatum* and another diploid alpine species (as yet unidentified). It is also postulated that *Papaver pseudo-orientale* arose from a hybrid of *P. orientale* × *P. bracteatum.* Therefore *P. orientale* would contain a single genome of *P. bracteatum*, and *P. pseudo-orientale* would contain two *P. bracteatum* genome compliments. The results of the GISH analysis demonstrate this with 28 chromosomes of *Papaver pseudo-orientale* possibly being homologous with the 14 chromosomes of *P. bracteatum* and the unlabelled 14 chromosomes possibly being those of the other diploid ancestor. The identification of two sets of *Papaver bracteatum* genomes within the hexaploid species can be explained as follows: *P. pseudo-orientale* is a polyploid of *P. bracteatum* (diploid progenitor) and a tetraploid species. Subsequently the tetraploid species is an allopolyploid with *P. bracteatum* being the diploid progenitor also. This tetraploid progenitor to *Papaver pseudo-orientale* may be *P. orientale* as suggested by Goldblatt (1974) but the results presented here do not conclusively demonstrate this. To identify the progenitors of *Papaver pseudo-orientale* and to determine the origin of the tetraploid *Papaver orientale* requires further experimentation. This would include using total genomic DNA from *Papaver orientale* on the chromosome compliment of *P. pseudo-orientale*. The origin of *Papaver orientale* may be deduced by using total genomic DNA from *P. bracteatum* as a probe. Additionally some of the alpine diploid species that share a geographic distribution with *P. bracteatum* (such as *Papaver lateritium* ssp. *monanthum*) could be included in future studies in an attempt to identify the other progenitor of the tetraploid species. It would also be necessary to attempt using total genomic DNA from *P. bracteatum* as a probe on other diploid species within *Papaver* to investigate whether the terminal hybridisation patterns (c.f. Figure 6.6C) are common across a wide range of *Papaver* species. If it were the case that this satellite repeat pattern was present in chromosomes of *P. bracteatum* only, then the status of *P. bracteatum* as a progenitor of *P. pseudo-orientale* would be confirmed.

The results of the AFLP analysis are also broadly congruent with the views of Goldblatt (1974) that *Papaver bracteatum* and a species from outside the section were the progenitors of *P. orientale.* This is demonstrated by the separate grouping of *Papaver orientale* from *P. bracteatum* in the AFLP tree and PCO analysis indicating a

genetic dissimilarity between the two species. This genetic dissimilarity probably represents the presence of genomic material of the other diploid species. As demonstrated from the GISH analysis, two *Papaver bracteatum* genome compliments are present in *Papaver pseudo-orientale*. This is congruent with the AFLP and PCO analyses as those accessions identified as *Papaver pseudo-orientale* grouped more closely to *P. bracteatum* than to *P. orientale*. The results of the GISH analysis when combined with the AFLP analysis support the views that *P. orientale* arose from *P. bracteatum* and another diploid species and that *P. pseudo-orientale* arose from *P. bracteatum* and *P. orientale* progenitors.

Mapping of the 18S-5.8S-26S rDNA repeats in *Papaver bracteatum* identified three loci per haploid chromosome compliment. *Papaver orientale* had six major and one minor locus for the same rDNA repeat. If it was the case that *Papaver orientale* was an autopolyploid with two *P. bracteatum* genomes it would contain only twelve loci. The extra minor locus suggests that another diploid species with three major and one minor loci was the progenitor of this species with *P. bracteatum*, a conclusion also drawn from the AFLP analysis. If *Papaver pseudo-orientale* was an allopolyploid arising from *P. bracteatum* and *P. orientale* the expected number of loci for 18S-5.8S-26S rDNA repeats would be the sum of both species compliment (19 loci per diploid compliment). However the results of this analysis suggest that *P. pseudo-orientale* has 14 loci (eight major and six minor). Three possible explanations could explain this apparent reduction in the number of 18S-5.8S-26S loci:

i) During the process of interspecific hybridisation and subsequent polyploidy changes in rDNA locus number and location has occurred. This may also have involved uniparental ribosomal gene deletion. Such uniparental ribosomal gene deletion has previously been reported in a number of allopolyploid species, but thus far only in monocot species such as *Zingeria* (Kotseruba *et al.*, 2003), *Milium* (Bennett & Thomas, 1991), *Avena* (Fominaya *et al.*, 1988) and *Scilla* (Vaughan *et al.*, 1993). To the authors knowledge no reports for uniparental ribosomal gene deletion in a member of dicot family exist.

ii) For successful *in situ* hybridisation and probe detection it is necessary for a sufficient number of copies of the repeat rDNA to be present at any given locus. If however this number falls below the particular threshold, which is generally accepted to be >10kb (Jiang & Gill, 1994), detection of the locus may not occur.

iii) The previous two explanations for the reduction in the number of 18S-5.8S-26S loci for *Papaver pseudo-orientale* are made under the assumption that *P. bracteatum* and *P. orientale* are the progenitors of this particular species. It may be the case that different species are the progenitors of *Papaver pseudo-orientale*. However, the results from the sequencing of ITS1, 5.8s and ITS-2 regions (c.f. Chapter 2), AFLP analysis, morphological analysis and phytochemical analysis all demonstrate that a strong relationship exists between species of *Oxytona* and that *Papaver pseudo-orientale* did arise from *P. bracteatum* and *P. orientale* progenitors.

As a method for attempting to ascertain the origins of the species of *Oxytona* it seems that mapping of the 18S-5.8S-26S loci is not suitable. It is extremely difficult to ascertain whether a similar number of loci in two species is a direct indication of relationship. It would be necessary to evaluate a number of other diploid *Papaver* species for 18S-5.8S-26S distribution, in order to ascertain whether those loci for *Papaver bracteatum* where diagnostic and characteristic to that species only. Thus any similarities in the number of loci in *P. orientale* would be indicative of direct relationship. Thus it would seem that mapping the 18S-5.8S-26S loci does not possess the resolution necessary to conclusively demonstrate the relationships at the sectional level for *Papaver* section *Oxytona.*

In situ hybridisation is an extremely expensive technique to undertake, and the results of this analysis are those that were possible to achieve under the limited funding assigned to this part of my research. Although the results are limited and inconclusive it is the author's opinion that *in situ* hybridisation techniques offer the best opportunity to finally and conclusively demonstrate the origins of the representatives of *Papaver* section *Oxytona.*

Chapter 7 Using AFLP markers for species differentiation and assessment of genetic variability of *in vitro* cultured *Papaver bracteatum* (section *Oxytona*)

7.1 Introduction

A considerable amount of research concerning the production of isoquinoline alkaloids by *in vitro* cultured material derived from *Papaver bracteatum* has been conducted over recent decades (see review by Szoke, 1998 for examples). There has been little substantial success in producing good yields of thebaine from callus or suspension cultures derived from *Papaver bracteatum* or *P. somniferum* cells. The production of morphinane alkaloids is known to be associated with cell differentiation and morphogenetic processes (Kutchan *et al.*, 1983; Constabel, 1985; Kamo & Mahlberg, 1988). Morphinane alkaloid synthesis is dependent on the presence of specialised cells known as laticifers (Kutchan *et al.*, 1983; Rush *et al.*, 1985), which are absent in undifferentiated poppy cultures. The detection of such alkaloids often coincides with the development of laticifers in differentiated plant material that develop in a similar way to young seedlings.

The majority of reported alkaloids from callus and suspension *Papaver bracteatum* cultures are the benzophenanthridine type including sanguinarine, dihydrosanguinarine and oxysanguinarine, which are produced in minor quantities in intact plants of the genus *Papaver* but are found as the dominant constituents of undifferentiated callus and suspension cultures (Cline & Coscia, 1988). The majority of research involving the *in vitro* production of isoquinoline alkaloids concerns the production of these alkaloids under different cultivation factors (Table 7.1). Very little attention, however, has been given to the variation in alkaloid production due to differences in genotype. Genetic variation among micropropagated plants is well documented (see review by Rani & Raini 2000). Somaclonal variation, whether it is at the molecular, morphological or the phytochemical level, must be detected and minimised if true-to-type plants are to be produced. The use of molecular techniques has allowed rapid detection of such variation and helped elucidate the factors and mechanisms that cause the loss of genetic uniformity.

Table 7.1. Media and culture conditions reported for the growth of *P. bracteatum* cell cultures CM: coconut milk; MS: Murashige and Skoog; 2,4-D: 2,4-dichlorophenoxyacetic acid; NAA: napthalene acetic acid; IAA: indole acetic acid: Kin: kinetin (after Hook *et al.* 1988).

Medium	Auxin mg/l	Cytokinen mg/l	Other supplement	Culture type	Other conditions	Alkaloid present	Reference
MS (no glycine)	2,4-D(1)	Kin(0,1)	CM(7%)	Callus	26-hr dark	Sanguinarine nor-sanguinarine dihydrosanguinarine oxysanguinarine chelirubine	Ikutsu *et al.*, (1974)
MS	2,4-D(1)	Kin(0,1)	Polypeptone CM	Suspension	26-hr dark 3% sucrose	Magnoflorine	Kamimura & Akutsu (1976)
MS	NAA(1)	Kin(0,1)	CM(10%)	Callus suspension	3% sucrose	Thebaine protopine stylopine	Kamimura & Nishikawa (1976)
MS	2,4-D(1)	Kin(0,1)		Callus	26-hr dark 3% sucrose	Thebaine	Kamimura *et al.*, 1976)
MS	2,4-D(0.1)			Suspension	24°C 24-hr light	Thebaine	Shafiee *et al.*, (1976)
MS	2,4-D(1)	Kin(0-0.1)	Various	Callus		Sanguinarine orientalidine	Lockwood, (1981)
MS	IAA(1)			Root suspension	15-hr light	Thebaine	Zito & Staba, (1982)
MS	2,4-D(1)	Kin(0,1)	Vit.C(10mg/l)	Suspension	12-hr light	Sanguinarine orientalidine	Lockwood, (1981)
MS	2,4-D(0.22) NAA(0.186)			Suspension	Continuous dim light	Sanguinarine orientalidine	Hook *et al.*,(1988)
MS	2,4-D(0.22) NAA(0.186)		Copper	Suspension	18-hr light	Sanguinarine orientalidine	Lecky *et al.*, (1992)

Molecular techniques such as restriction fragment length polymorphisms (RFLPs) and the various polymerase chain reaction (PCR) techniques such as random amplified polymorphic DNA (RAPD; Williams *et al.*, 1990) and AFLP have been used extensively to generate markers to assess genetic diversity between parent material, clones and micropropagated plantlets. RAPD fingerprints have been successfully employed for the assessment of genetic fidelity of tissue culture-derived plants including pine (Isabel *et al.*, 1993), wheat (Brown *et al.*, 1993), sugarcane (Taylor *et al.*, 1995), garlic (Al-Zahim *et al.*, 1999) and peach (Hashmi *et al.*, 1997). RFLPs have been successfully used to detect fluctuations of DNA methylation between regenerated plantlets and the original *in vitro* material. Changes in DNA methylation effects the expression of genes ultimately leading to the loss of functional proteins (Matzke & Matzke, 1991) and the stability of DNA methylation is considered to be a primary influence on the maintenance of genetic uniformity between micropropagated plants and the material from which they are derived. RFLP analysis using methylation-sensitive restriction endonucleases (Brown, 1989) has been employed successfully to a number of different species including maize (Brown & Lorz, 1986; Brown, 1989; Brown *et al.*, 1991; Kaepler & Phillips, 1993) and rice (Muller *et al.*, 1990). Although RAPD fingerprints were thought to circumvent the technical problems (degree of expertise, cost and use of radio-isotopes) encountered when using RFLP (Rani & Raini, 2000), the technique itself has been fraught with difficulties especially with reproducibility of results. AFLP fingerprints however are highly reproducible and generally produce a large number of polymorphic markers (Ridout & Donini, 1999; Hodkinson *et al.*, 2000) and it has been demonstrated that AFLP fingerprinting can be applied to the study of genetic variation in poppies (cf. chapter 6). They have the potential to identify ambiguities between parent material and regenerated plantlets and to detect somaclonal variation in *Papaver in vitro* clones and propagated plants (this chapter and Carolan *et al.*, 2002, see Appendix 3).

Considerable amounts of research have been conducted on *Papaver bracteatum* as a source of producing isoquinoline alkaloids and the potential of mass-producing high thebaine yielding strains of this species through tissue culture and micropropagation by Ingrid Hook and co-workers at the Department of Pharmacognosy, School of Pharmacy, Trinity College, Dublin. The mass propagation of high alkaloid producing cell lines demonstrates one of the beneficial uses of *in vitro* technologies to the

successful utilization of *Papaver* species. *Papaver bracteatum* plants are almost self-incompatible which makes the creation of phytochemically pure lines through selfing very difficult to achieve. Day *et al* (1986) reported successful regeneration of micropropagated plants from embryogenic callus culture with little phytochemical variation occurring between micropropagated offspring and parent plant material. The effects of somaclonal variation, however, have the potential to diminish effectiveness of tissue culture as a means of producing significant quantities of genetically and phytochemically uniform accessions. The ability of AFLP fingerprinting to differentiate between closely related species and to assess genetic variance between closely related accessions (as demonstrated by the results of the previous chapter) warranted an investigation into whether AFLP fingerprinting was capable of identifying the genetic constitution of *in vitro* generated plants and assessing the stability and quality of repeatedly sub-cultured cell lines or genetic variance between cell cultures. The collection of basic phytochemical (dominant alkaloid) and cytological data (chromosome counts) were to aid the interpretation of results.

Several cell cultures were developed by Hook *et al.* (1988) from seeds of *P. bracteatum* originally supplied as ARYA II. Plants developed from these seeds exhibited morphological and phytochemical characteristics typical of *P. bracteatum* as described by Goldblatt (1974). On repeated sub-culture one cell line exhibited organogenesis, and subsequently differentiated into *in vitro* plantlets, some of which were grown on to maturity under garden conditions. The resulting plants did not resemble the parent, exhibiting morphological characters similar but not identical to those of *P. pseudo-orientale*. This therefore brought into question the identity of the original seed used to initiate the cultures or indicated that the cell culture process was not stable and had induced morphological change possibly via somaclonal variation.

The aims of this part of the project include using molecular fingerprinting methods to identify plant material (to the level of species) used to initiate callus culture and assess the amount of genotypic variation occurring between cultured material and parent stock.

7.2 Materials and Methods

7.2.1 Plant material and DNA extraction

Plant material was obtained from a number of sources listed in Table 7.2, and included four species: *Papaver orientale*, *P. bracteatum*, *P. pseudo-orientale* and *P. somniferum*. Capsules were collected from mature plants from May to July 2000, four weeks after flowering and dried in a fan-assisted oven at <35 °C. Not all accessions were available for alkaloid analysis and chromosome counts. The methods for DNA extraction have been outlined previously in Chapter 2, section 2.3.3.

Table 7.2 *Papaver* material used in study. NBG: National Botanic Gardens, Glasnevin, Ireland; TCD BG: Trinity College Dublin, Botanic Gardens; Foxrock Park, School of Pharmacognosy, Trinity College Dublin.

Accession	Source/information	Accession code
P. bracteatum	ARYA II P.I 381605	22
P. bracteatum	ARYA II P.I 381605	30
P. bracteatum	ARYA II P.I 381605	31
In vitro hybrid	Culture initiated from seed of 2nd generation ARYA II	43
Micropropagated hybrid	Micropropagated through tissue culture of accession 43	34
P. pseudo-orientale	Foxrock park	36
P. pseudo-orientale	Foxrock park (cv. Salmon Queen)	40
P. pseudo-orientale	Foxrock park	37
P. pseudo-orientale	NBG 000341	18
P. pseudo-orientale	TCD BG	02
P. orientale	Foxrock park	35

7.2.2 *In vitro* culture of *Papaver bracteatum*

Callus cultures were established in 1984 (Hook *et al.*, 1988) from surface sterilized seeds plated onto an agar-solidified MS salt medium (Murashige & Skoog, 1962) containing 2,4-D (0.22 mg/l), NAA (0.186 mg/l), glycine (2 mg/l), nicotinic acid (0.5 mg/l), pyridoxine HCl (0.5 mg/l), mesoinositol (200 mg/l), thiamine HCl (0.5 mg/l),

and sucrose (30 g/l). Subculturing and media preparation were carried out by the author on these continually subcultured cell lines for the duration of this project. Cultures grew as beige-coloured aggregates and were subcultured every four to six weeks onto fresh medium. Subsequently aggregates showing organogenesis were transferred to hormone-free MS agar where shoot and root regeneration occurred. Regenerated *in vitro* plantlets were also subcultured onto fresh medium every 4-6 weeks, and maintained to the present day. For alkaloid analysis *in vitro* grown plantlets were harvested after six weeks growth and dried as above. Roots formed and these shoots provided the material for cytological analysis.

7.2.3 Cytological analysis

Seeds from identified accessions were germinated under constant light at 25 ºC on wet filter paper in plastic Petri dishes. Root tips were taken when growth reached 1.5 cm. Chromosome counts were also made on young rapidly growing roots of *in vitro* cultured plantlets. The methods for cytological analysis have been outlined previously in Chapter 6, section 6.3.4.

7.2.4 AFLP analysis

AFLP reactions used the AFLP Plant Mapping Kit supplied by Applied Biosystems, following their standard protocols and the methods for AFLP analysis have been outlined previously in Chapter 6 section 6.4.5. Two different primers were used here for the selective amplification step and were *Mse*CTC/*Eco*ACG and *Mse*CAG/*Eco*AGC. Data analysis was outlined previously in Chapter 6, section 6.4.6 and included NJ and PCO.

7.2.5 Alkaloid analysis

The methods for the extraction of alkaloids and their subsequent identification by GC/MS were outlined previously in Chapter 6, section 6.3.3. In addition to GC/MS, alkaloid spectrum was examined using thin layer chromatography (TLC). The

resultant residue was dissolved in 0.5 ml methanol and run on silica gel plates (Silica gel 60 F_{254}; Merck) developed in acetone: toluene: ethanol: conc. ammonia (40:40:12:2.5). Visualization of alkaloids was with UV light and Dragendorff's reagent. Reference compounds were thebaine (2.0 mg/ml), isothebaine (1.4 mg/ml), oripavine (0.3 mg/ml) and salutaridine (2.5 mg/ml).

7.3 Results

7.3.1 AFLP analysis

AFLP generated 254 polymorphic markers, of which 146 were shared between individuals. A NJ tree from the AFLP analysis that groups accessions according to genetic similarity is given in Figure 7.1. There are three major divisions, representing the three species of section *Oxytona* respectively (*P. orientale*, *P. bracteatum* and *P. pseudo-orientale*). The *in vitro* grown plant and the micropropagated plant grouped with the *P. pseudo-orientale* accessions. The results of the PCO analysis are illustrated in Figure 7.2. The first two axes cumulatively accounted for 60.4% of the data variance (representing 38.3 and 22.1 respectively). The *in vitro* grown plant and the micropropagated plant did not group with the *P. bracteatum* accessions, but again formed a distinct group with the *P. pseudo-orientale.*

7.3.2 Alkaloid analysis

Alkaloid profiles for accessions where capsules were available are given in Table 7.3. Reference standards of thebaine, isothebaine, oripavine and salutaridine had R_f values of 0.54, 0.57, 0.42 and 0.46 respectively. The *in vitro* grown plant and the plant grown from the micropropagated plantlet produced isothebaine (R_f 0.57) as the dominant alkaloid with several unidentified alkaloids present as minor constituents. For reference, capsules from *P. bracteatum* (accessions 22, 30 and 31) and *P. pseudo-orientale* (accessions 36 and 40) produced thebaine (R_f values of 0.54) and isothebaine (R_f value of 0.57) as dominant alkaloids respectively with *P. orientale* (accession 35) producing oripavine (R_f value of 0.42) as its dominant alkaloid.

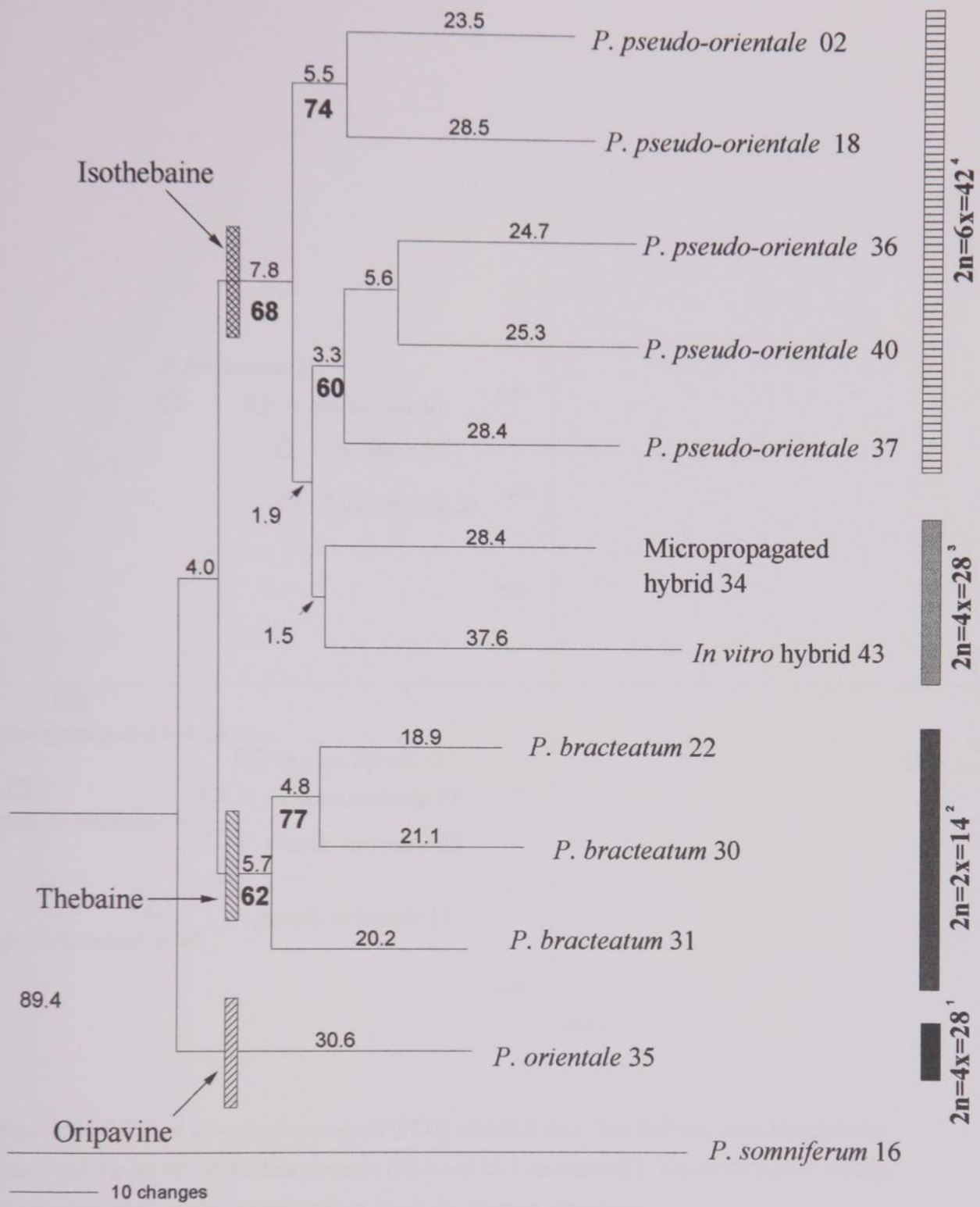


Figure 7.1 Neighbour Joining tree based on AFLP data. *P. b x P. po* : *Papaver bracteatum* × *Papaver pseudo-orientale* hybrid. Three groups representing the three species of *Oxytona* are apparent. The *in vitro* plant and the regenerated plant resolve within the *P. pseudo-orientale* group. [1] Based on chromosome count for accession 36; [2] Based on chromosome count for accessions 34 and 43; [3] Based on chromosome count for accessions 22 and 31; [4] Based on chromosome count for accession 35. Values above branches are genetic distances and values below are bootstrap percentages.

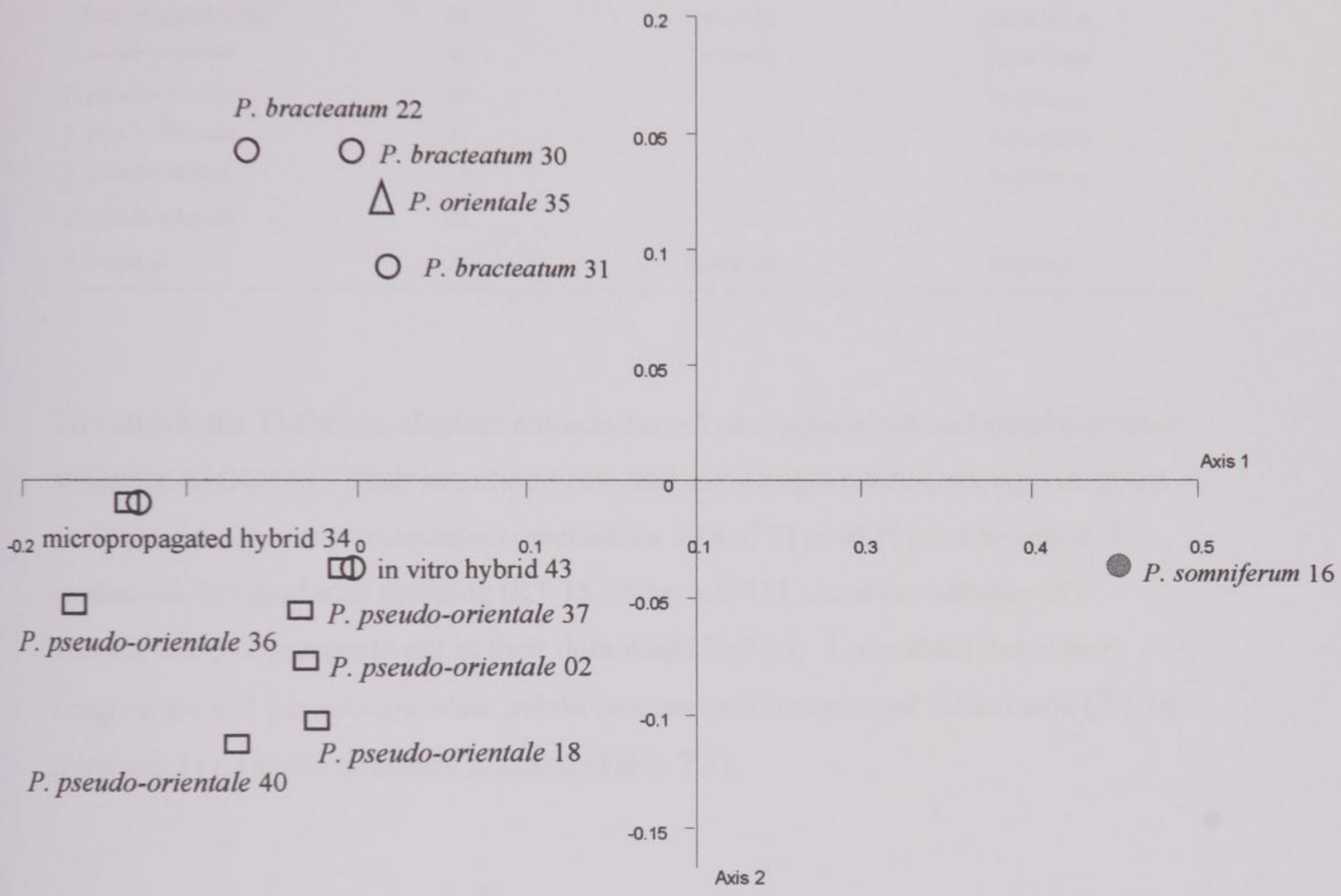


Figure 7.2 Principal co-ordinates analysis (PCO) of AFLP data. The first two axes cumulatively accounted for 60.4% of the data variance (38.3 and 22.1 respectively). The *in vitro* plant and the regenerated plant group separately from the *P. bracteatum* accessions.

Table 7.3 Dominant alkaloids (assessed by thin layer chromatography and gas chromatographic mass spectra analysis) and chromosome numbers counted for a selection of accessions.

Accession	Accession code	Chromosome numbers	Dominant alkaloid
P. bracteatum	22	-	-
P. bracteatum	30	2n=2x=14	Thebaine
P. bracteatum	31	2n=2x=14	Thebaine
In vitro hybrid	43	2n=4x=28	Isothebaine
Micropropagated hybrid	34	2n=4x=28	Isothebaine
P. pseudo-orientale	36	2n=6x=42	Isothebaine
P. pseudo-orientale	40	-	Isothebaine
P. pseudo-orientale	37	-	Isothebaine
P. pseudo-orientale	18	-	Isothebaine
P. pseudo-orientale	02	-	-
P. orientale	35	2n=2x=28	Oripavine

To validate the TLC data, alkaloid extracts for selected accessions and standards were subjected to GC/MS. Their retention times and mass fragmentation spectra are given as Table 7.4. *Papaver bracteatum* (accessions 30 and 31) and *P. pseudo-orientale* (accession 40) produced thebaine (RT 15.2mins *m/z* 311,), and isothebaine (RT 16mins *m/z* 311,) respectively as their dominant alkaloids. The extract from the *P. bracteatum* × *P. pseudo-orientale* hybrid (accession 43) contained isothebaine (RT 16 mins *m/z* 311,) as the dominant alkaloid (Table 7.3).

Table 7.4 Selected ions, retention times, and ion ratios of thebaine, isothebaine and four selected accessions.

Compound/Accession	Retention Time (min)	Monitored Ions	Ion Ratio Percent (Abundance)
Thebaine	15.2	311,296	100,75
Isothebaine	16	311,294	100,50
Accession 30	15.2	311,296	100,25
Accession 31	15.2	311,296	100,25
Accession 40	16	311,294	100,60
Accession 43	16	311,294	100,60

7.3.3 Cytological analysis

Cells from root tips of *in vitro* cultured *P. bracteatum* contained 28 chromosomes as detected in metaphase of mitosis. Chromosome counts for available accessions are given in Table 7.3. Members of the *P. bracteatum* AFLP group were diploid with 2n=14, while members of the *P. pseudo-orientale* group were hexaploid with 2n=42 (excluding the hybrid). *Papaver orientale* had 28 chromosomes and is tetraploid (2n=28).

7.4 Discussion

The NJ tree generated from the AFLP markers showed three major groups, which correspond to the three species, *P. bracteatum*, *P. orientale* and *P. pseudo-orientale*. *Papaver bracteatum* accessions resolved independently from a group which contains the *P. pseudo-orientale* accessions. *Papaver orientale* resolved as a sister group to the other two species. The results of the PCO analysis are congruent with these conclusions. Accessions labelled as *P. orientale* shared morphological characters with *P. pseudo-orientale,* having orange petals with large dark spots on each. These two accessions (02 and 18) grouped with other *P. pseudo-orientale* plants. AFLP generated markers are therefore highly efficient when used in conjunction with morphological, phytochemical and cytological characters to identify plant material.

Regenerated plantlets grown on from the *P. bracteatum* cultures should group with *P. bracteatum* if they have remained genetically stable before and during tissue culture. However, this *in vitro* derived plantlet did not associate with *P. bracteatum* and instead grouped more closely with *P. pseudo-orientale*. These AFLP groupings show that genomic material from *P. pseudo-orientale* is present within the *in vitro* grown plant and the micropropagated accession, indicating that a hybridisation event may have occurred between *P. pseudo-orientale* and the maternal *P. bracteatum*, from which seed was obtained to initiate the *in vitro* plants. The *in vitro* plant and the micropropagated accession group together but are not identical which suggests that somaclonal variation may have occurred during their culture. AFLP generated

fingerprints would therefore have potential to detect loss of genetic uniformity due to somaclonal variation in poppies.

The results from the alkaloid analysis (Table 7.3) support the conclusion that the *in vitro* grown plant is a hybrid. If the *in vitro* grown plant bred true from *P. bracteatum*, thebaine would be the expected dominant alkaloid (Hook *et al.*, 1988). Phytochemical spectrum and alkaloid quantity is influenced by parent genotype and ploidy. For example, hybrids between *Oxytona* species produce characteristic phytochemical spectra dependant on the inherited genetic information (Milo *et al.*, 1990; Levy & Milo, 1991). The wide spectrum of alkaloids found in the differentiated *in vitro* plant (including isothebaine) demonstrates the presence of *P. pseudo-orientale* genomic material and its resulting biosynthetic pathways. Hybrids between *P. bracteatum* and *P. pseudo-orientale* have been reported to produce both isothebaine and thebaine inherited from the two parental genotypes respectively (Levy & Milo, 1990). Our results contrast with Levy and Milo (1990), as no traces of thebaine were recorded for the hybrid accession. The alkaloid spectrum found within each species is congruent with the groupings of the AFLP analysis with three groups all producing diagnostic dominant alkaloids.

To confirm that the material used to initiate the *in vitro* plant arose from hybrid seed, chromosomes were counted and compared to counts for other studied accessions. *Papaver bracteatum, P. orientale* and *P. pseudo-orientale* had 14, 28 and 42 chromosomes respectively. The *in vitro* plant had 28 chromosomes and is therefore a tetraploid. Evidence has been provided to show that the cultured plant is likely to be a hybrid with genomes from both *P. bracteatum* and another species (probably *P. pseudo-orientale*). The hybrid clusters closer to *P. pseudo-orientale* than *P. bracteatum* (in both NJ and PCO analyses), which indicates that it might have inherited more chromosomes from *P. pseudo-orientale* than *P. bracteatum*. In this case, one explanation is that it inherited seven chromosomes from *P. bracteatum* and twenty-one from *P. pseudo-orientale* (Figure 7.3).

Goldblatt (1974) cites examples of *Papaver* (section *Oxytona*) hybrids with somewhat intermediate characteristics, and stresses that some of the diagnostic characters when used alone could lead to mis-identifications. Hybrids are not always morphologically

intermediate between their parents (Riesberg, 1995) and great care needs to be taken when evaluating their characters. They are often just as likely to exhibit the characteristics of one or other of the parental species. The regenerated plant

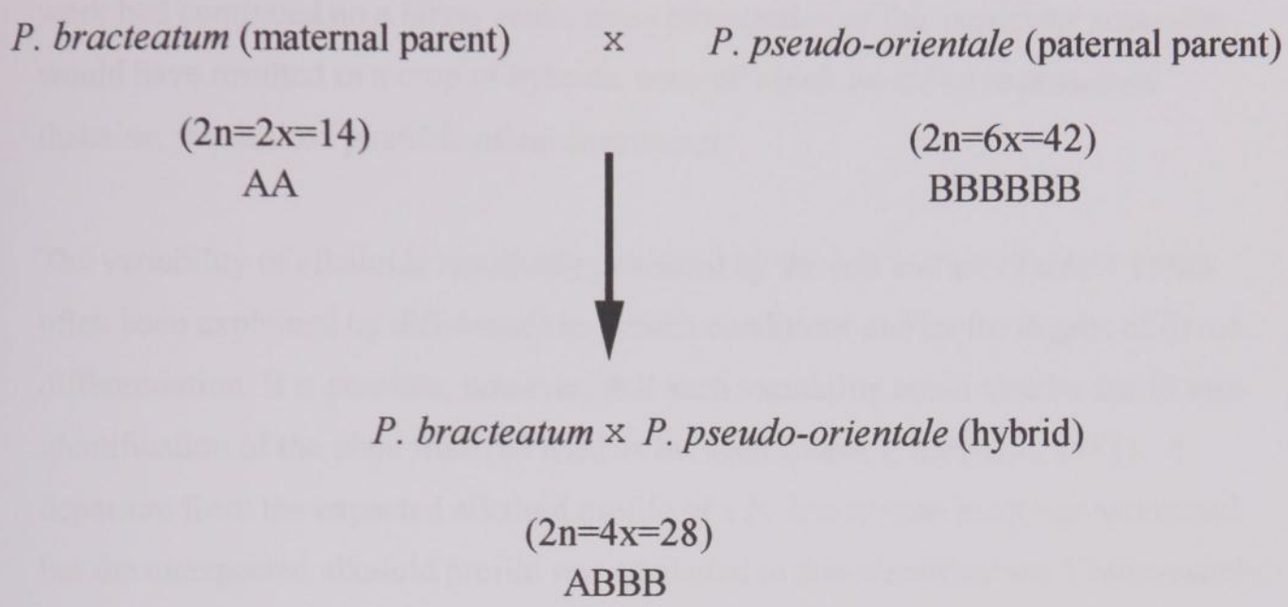


Figure 7.3 Probable genomic combination for a hybrid arising from *P. bracteatum* × *P. pseudo-orientale*. A hybrid would inherit seven chromosomes from *P. bracteatum* and twenty-one from *P. pseudo-orientale*.

resembled *P. pseudo-orientale* more closely in its gross morphology than *P. bracteatum*, having six orange petals with faded central purple spots. Single bracts were present but not found on all flowering stems. The 2-valved calyx was borne on a pendulous flowering stem becoming erect when mature. Uncharacteristic fine setae were subpatent on the lower portion of the flowering stem, becoming adpressed further up. The capsule bore a concave stigmatic disc comprising 14 stigmatic rays. There was, therefore, little indication from morphological data that *P. bracteatum* was a parent to this particular accession. The original *P. bracteatum* plant had, in fact, been garden grown in the presence of *P. pseudo-orientale* and a hybridisation event was possible. Molecular data supplied by AFLP in combination with chromosome

counts confirm that such an event did occur. This is therefore an example of a situation where a hybrid is not morphologically intermediate between its parents as it inherited more characters from one of them.

In our research, the rapid identification of the hybrid cell line allowed us to review the various sources of plant material used to initiate callus and suspension cultures. If this work had continued on a larger scale, mass propagation of this particular accession would have resulted in a crop of hybrids, none of which would have possessed thebaine, the desired phytochemical constituent.

The variability of alkaloids reportedly produced by the cell culture (Table 7.1) has often been explained by differences in growth conditions and by the degree of tissue differentiation. It is possible, however, that such variability could also be due to mis-identification of the plant material used as the seed source (Phillipson, 1981). A departure from the expected alkaloid profile of a *P. bracteatum* plant was witnessed but the unexpected alkaloid profile was attributed to mis-identification. Unfortunately, other studies that report high variability in alkaloid profiles often fail to provide evidence for correct identification of their material. It has also been demonstrated that AFLP markers can be used efficiently to characterise *in vitro* grown plants, original parent material and other genotypes of the same or closely related species.

The most fundamental requirement for the use of any plant species in the pharmaceutical industry is its correct identification. Poppy species belonging to section *Oxytona* are not sufficiently distinct to base identification solely on morphological characters. Regenerated *Papaver* plantlets from tissue culture could be grown on to maturity and identified using morphological characters and chromosome numbers but a growing period of two years is required to produce flowers. AFLPs combined with cytogenetic analysis offer a powerful way to rapidly differentiate species and identify potential hybrids. AFLP clearly has great potential as a way of monitoring the genetic stability of *in vitro* plants particularly those that possess superior phytochemical traits and that are multiplied by tissue culture methods.

The DNA fingerprinting technique AFLP combined with standard cytogenetic techniques offer the possibility to improve the utilisation of *Papaver bracteatum* as a pharmaceutical resource. The markers can be used for marker-aided selection and for the study of quantitative trait loci (Mackill *et al.*, 1996; Paul *et al.*, 1997; Waugh *et al.*, 1997). In addition the results of this section of the thesis demonstrate that AFLPs have a wider utility in the field of plant systematics for the study of hybridisation and introgression in poppies.

CHAPTER 8

8.1 Summary

The phylogenetic relationships between sections of the genus *Papaver* were evaluated using parsimony analysis of biparentally inherited nuclear rDNA ITS and maternally inherited plastid *trnL-F* sequences in combination with morphological and phytochemical characters (Chapters 2, 3 & 4). Trees based on individual gene regions were broadly congruent and combining the data improved resolution and support in the trees. The results demonstrate that *Papaver* is not monophyletic unless the genera *Roemeria, Stylomecon* and the species *Meconopsis cambrica* are included, a result previously demonstrated by Kadereit *et al.* (1997).

Within the genus, three distinct groups are recognised. The first comprises *Papaver* section *Argemonidium* (including *Roemeria*). A number of unique indels and diagnostic sets of nucleotide sequences support not only the clear affinity between section *Argemonidium* to *Roemeria*, but also the separation of this group from the other sections of *Papaver*. The second group comprises section *Meconella.* In addition the close affinity between *Papaver californicum* and *Stylomecon heterophylla* is demonstrated. The third group comprises sections *Carinatae, Californicum, Horrida, Meconidium, Oxytona, Papaver, Pilosa, Pseudopilosa* and *Rhoeadium.* This group also includes *Stylomecon heterophylla* and *Meconopsis cambrica* and their recognition as *Papaver lemonii* and *Papaver cambrica* is recommended.

Within this core group of *Papaver* an affinity between sections *Meconidium, Oxytona* and *Pilosa* was demonstrated. A close relationship also exists between sections *Papaver* and *Rhoeadium* and neither is deemed monophyletic in their current taxonomic circumscription. A distinct grouping within section *Rhoeadium* corresponding to a *Papaver rhoeas* group and a *P. dubium* group exists. These groups were recognised previously by Kadereit (1989). *Papaver glaucum* (section *Papaver*) is more closely related to members of section *Rhoeadium* (specifically the *P. rhoeas* group) than to *Papaver somniferum* (section *Papaver*). The sole representative of

section *Carinatae, Papaver macrostomum,* is placed into the closely related section *Rhoeadium* based on molecular and morphological similarities.

The morphological and phytochemical characters used in this thesis, although essential for species identification, were not suited for cladistic analysis when used alone. A more productive strategy concerning their use was to subsequently map these characters onto a tree obtained from the combined molecular analysis (Chapters 3 & 4). This yielded novel interpretations of the relationships that exist within *Papaver.* A number of characters that have been traditionally used to suggest groupings within the genus were demonstrated to have arisen through convergent evolution and include valvate dehiscence of capsule and light coloured filaments. The distribution and evolution of isoquinoline type alkaloids was evaluated in the same way. In some cases the synthesis of similar alkaloid types in certain sections of *Papaver* was shown to be a result of relationship and common descent. The results of this thesis demonstrate that certain alkaloid types are found only in certain groups of sections, which will broaden basic knowledge of these groups and aid further their utilisation as sources of important secondary metabolites.

Based on the results of the molecular, morphological and phytochemical analysis undertaken in this thesis, and in light of geographic distributions, a new classification of *Papaver* is proposed. Effort has been made to maintain sections that have traditionally been described within *Papaver. Papaver* is recognised here as consisting of six subgenera, eight sections and four subsections.

Sections *Argemonidium* and *Meconella* are elevated to the rank of subgenus (*Papaver* subgenus *Argemonidium* and subgenus *Meconella*). Section *Horrida* (South Africa) is elevated to the rank of subgenus, as is section *Californicum* (North West America). *Meconopsis cambrica* and *Stylomecon heterophylla* are recognised as *Papaver cambrica* L. and *Papaver lemonii* Greene, respectively. *Papaver cambrica* is placed within *Papaver* at the subgenus level (*Papaver* subgenus *Cambrica*) in recognition of differences in capsule characteristics between this species and other representatives of *Papaver. Papaver lemonii* is transferred to subgenus *Californicum* and recognised within a new section (section *Lemonii*). The sixth subgenus (subgenus *Papaver*) comprises sections *Meconidium, Oxytona, Papaver, Pilosa, Pseudopilosa* and *Rhoeadium* acknowledging the close affinity between these sections from a molecular

point of view. Section *Rhoeadium* is recognised here as comprising four subsections. Based on the groupings within the molecular phylograms, and a number of morphological and phytochemical considerations, *Papaver glaucum* has been transferred to a subsection of section *Rhoeadium* (subsection *Glauca* Preininger). A division between *Papaver dubium*-type species and *P. rhoeas/P. arenarium* type species is accommodated by the transfer of *P. dubium* type species to new section *Rhoeadium* subsection *Dubia.* Species within the *P. rhoeas/P. arenarium* group are transferred to section *Rhoeadium* subsection *Rhoeadium. Papaver macrostomum* which was separated from the genetically similar *P. rhoeas* group into a new section (*Carinatae* Kadereit) based solely on differences in capsule characteristics is transferred here to a subsection of section *Rhoeadium* (subsection *Carinatae*).

The genetic fingerprinting technique AFLP was shown to be extremely well suited to address problems with species differentiation and identification within section *Oxytona* (Chapters 6 & 7). This section is important from a phytochemical point of view as its members accumulate economically important morphinane alkaloids without the accumulation of codeine and morphine (the key constituents of opium as a source of narcotic abuse). A fundamental requirement for using any plant species as a source of medicinally important compounds is that correct identification occurs. Individual alkaloids are species-specific in this section, thus highlighting the need to be able to differentiate between these species. AFLP fingerprinting was suited to this task. The resulting NJ trees and PCO analysis helped assess the inter-relationships of its species and investigate the polyploid origin of *Papaver orientale* (tetraploid) and *P. pseudo-orientale* (hexaploid). AFLP fingerprinting identified hybrids between the species of this section and was able to differentiate between species of *Oxytona* from a range of cultivars. AFLP fingerprinting was also used to assess the genetic fidelity between *in vitro* cell lines of *Papaver bracteatum* and mature plants derived from the propagation of their callus cultures (results published in Carolan *et al.* (2002).

The origin of the tetraploid *Papaver orientale* and the hexaploid *P. pseudo-orientale* could not be resolved conclusively based on the results of the *in situ* hybridisation experiments (Chapter 6). The results of the GISH and FISH analysis are limited but demonstrate that *P. bracteatum* is a diploid progenitor of *P. pseudo-orientale.* Genome *in situ* hybridisation, using the total genomic DNA of *Papaver bracteatum* as a probe on chromosomes of *P. pseudo-orientale* revealed that two sets of the *P.*

bracteatum genome are present within *P. pseudo-orientale.* Comparing the loci for the distribution of the 18S-5.8S-26S (the pTa71 probe) repeat suggests that the tetraploid *Papaver orientale* may have arisen from an autopolyploidy event involving a hybrid of *Papaver bracteatum* and another diploid species. If *Papaver pseudo-orientale* arose from *P. bracteatum* and *P. orientale* the subsequent number of loci for 18S-5.8S-26S distribution in *P. pseudo-orientale* suggests that it has in fact lost some of these loci. Combining the groupings from the AFLP analysis and the limited *in situ* hybridisation data, support is given to the view that the tetraploid *Papaver orientale* arose from *P. bracteatum* and another diploid alpine species (as yet unidentified) and the hexaploid *P. pseudo-orientale* is a polyploid arising from *P. bracteatum* and *P. orientale* progenitors.

8.2 Future work

Future work and suggested additions to the work carried out in this thesis have been discussed in the respective chapters. Increasing the number of taxa sampled would be a priority. This would include not only including more species of *Papaver,* but also the inclusion of representatives of the species often placed in genera *Meconopsis* and *Roemeria.* Without these the phylogenetic relationships of the subgenera of *Papaver* may not be fully understood. Obtaining chromosome counts for all individuals used in the molecular analysis would aid in the interpretation of these results. In addition other gene regions could be sequenced and combined (if suitable) with the ITS and *trnL-F* matrices.

Regarding the systematics of *Papaver* section *Oxytona* it is obvious that AFLP fingerprinting is suited to the identification and differentiation of *Papaver bracteatum, P. orientale* and *P. pseudo-orientale.* This technique could be applied to other sections and subsections, thus increasing the understanding of the interspecific relationships that exist within these taxonomic groups.

Although AFLP fingerprinting can show relationships, I feel that *in situ* hybridisation has the potential to conclusively demonstrate the origin of the tetraploid *Papaver orientale* and the hexaploid *P. pseudo-orientale.* Future work here would include using total genomic DNA from *Papaver bracteatum* as a probe on the chromosomes of *P. orientale* and to use total genomic DNA of *P. orientale* as a probe on

chromosomes of *P. pseudo-orientale* in an attempt to demonstrate whether these species are the progenitors (a view commonly held with researchers working on this subject). In addition, comparative mapping and determination of the chromosomal loci for other gene regions would assist in this matter.

It is anticipated that at least four peer-reviewed papers will be produced from this work. One has already been published (Carolan *et al.*, 2002 Appendix 3). A paper is in preparation including the molecular phylogenetic assessment of *Papaver* and interpretation of morphological and phytochemical characters. A paper is also in preparation that will include the work on *Papaver* section *Oxytona* based on the AFLP, FISH and GISH analyses. A final paper is planned to cover taxonomic implications based on the results of this thesis, formally proposing a new system of classification for *Papaver* L.

References

Adams, S.P., Leitch, I.J., Bennett, M.D., Chase, M.W. & Leitch, A.R., (2000) Ribosomal DNA evolution and phylogeny in *Aloe* (Asphodelaceae). Am. J. Bot., 87: 1578-1583

Al-Zahim, M.A., Ford-Lloyd, B.V. & Newbury, H. J., (1999) Detection of somaclonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. Plant Cell Rep. 18: 473-477

Anamthawat-Jonsson, K, & Heslop-Harrison, J.S., (1995) Establishing the relationships between closely related species using total genomic DNA as a probe. In: Methods of Molecular Biology (Clapp, J.P. ed.) Humana Press Inc. U.S.A., pp. 209-225

Appels, R. & Baum, B., (1992) Evolution of the NOR and 5S DNA loci in the Triticeae. In: Molecular Systematics of Plants, (Soltis, P.S., Soltis, D.E. & Doyle, J.J. eds.) Chapman & Hall, New York, pp., 92-116

Aziz, K., Husain, S.Z. & Jahan, N. (1993) Chemotaxonomic studies of the genus *Medicago* L. (Papilionoideae) from Pakistan. Pakistan J. Bot., 25: 111-117

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Cambell, C.S. & Donoghue, M.J. (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Missouri Bot. Gard., 82: 247-277

Barkman, T.J., (2001) Character coding of secondary chemical variation for use in phylogenetic analyses. Biochem. Syst. & Evol., 29: 1-20

Bateman, R.M., (2001) Evolution and classification of European orchids: Insights from molecular and morphological characters. Journal Europäischer Orchideen, 33: 33–119.

Battersby, A. R., Francis, R. J., Hirst, M. & Staunton, J. (1963) Biosynthesis of the Berberine Bridge. Proc. Chem. Soc., 268-278

Bayer, R.J. and Starr, J.R. (1998) Tribal phylogeny of the Asteraceae based on two non-coding chloroplast sequences, the *trnL* intron and *trnl/trnF* intergenic spacer. Ann. Mo. Bot. Gard ,85: 242-256

Bennett, S.T. & Thomas, S.M., (1991) Karyological analysis and genome size in *Milium* (Gramineae) with special reference to polyploidy and chromosomal evolution. Genome, 34: 868-878

Bennett, S.T., Kenton, A.Y. & Bennett, M.D. (1992) Genomic *in situ* hybridisation reveals the allopolyploid nature of *Milium montianum* (Graminae). Chromosoma, 101: 420-424

Bernhardi, J.J., (1833) Uber den character und die verwandtschaft der Papaveraceen und Fumariaceen. Linneae 8:401-447

Biot, H.G. & Flentje, H., (1960) Nudeaurin, Muramin and Amurensin, drei neue Papaver-Alkaloide. Naturwissenschafen. 47, 180-186

Bohm, H., (1981) *Papaver bracteatum* Lindl. Results and problems of the research on a potential medicinal plant. Pharmazie, 36: 660-667

Bremer, K., (1994) Branch support and tree stability. Cladistics, 10: 295-304

Brown, P.T.H., (1989) DNA methylation in plants and its role in tissue culture. Genome, 31:717-729

Brown, P.T.H. & Lorz, H., (1986) Molecular changes and possible origin of somaclonal variation. In: Somaclonal variation and crop improvement (Semal, J., ed.) Dordrecht: Martinus Nijhoff, 138-159

Brown, P.T.H., Lange, F.D., Kranz, E. & Lorz, H. (1993) Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. Mol. Gen. Genet., 237: 311-317

Buckler, E.S., Ippoliti, A. & Holtsford, T.P (1997) The evolution of ribosomal DNA: divergent paralogues and phylogenetic implications, Genetics, 145:821-832

Camin, J.H. & Sokal, R.R., (1965) A method for deducing branching sequences in phylogeny. Evol., 19: 311-326

Carolan, J.C., Hook, I.L.I., Walsh, J.J. & Hodkinson, T.R., (2002) Using AFLP markers for species differentiation and assessment of genetic variability of *in vitro* cultured *Papaver bracteatum* (section *Oxytona*) In Vitro Cell. Dev. Biol.-Plant, 38: 300-307

Casgrain, P., (1999) Le Progiciel Rv4.0d1. Development release.

Cavalli-Sforza, L.L. & Edwards, A.W.F., (1967) Phylogenetic analysis: Models and estimation procedures. Evol., 32:550-570

Chase, M.W., de Bruijn, A.Y, Reeves, G., Cox, A.V., Rudall, P.J., Johnson, M.A.T. and Eguiarte, L.E., 2000. Phylogenetics of Asphodelaceae (Asparagales): an analysis of plastid *rbcL* and *trnL-F* DNA sequences. Ann. Bot. (London), 86: 935-956

Cheng, P. C., (1972) Cultivation and analysis of *Papaver bracteatum* Lindl., M. Sc. Thesis, Univ. Mississippi.

Cline, C.D. & Coscia, C.J., (1988) Stimulation of sanguinarine production by combined fungal elicitation and hormonal deprivation in cell suspension cultures of *Papaver bracteatum.* Plant Physiol., 86:161-165

Conn, G.L. & Draper, D.E., (1998) RNA structure. Curr. Opin. Struct. Biol., 8: 278-285

Constabel F., (1985) Morphinan alkaloids from plant cell cultures. In: The chemistry of the isoquinoline alkaloids (Phillipson J.D., Roberts M.F. & Zenk M.H., eds.). Springer, Berlin Heidelberg, New York, pp. 357-264

Cordell G.A., (1981) In: Introduction to Alkaloids. (Cordell, G.A. ed.). John Wiley and Sons, New York, Chichester, Bribane, Toronto, pp. 490-512

Corrigan, D. & Martin, E., (1981) The thebaine content of ornamental poppies belonging to the *Papaver* section *Oxytona*. Plant. Med., 42: 45-49

Cronn, R.C., Zhao, X.P., Paterson, A.H. and Wendel, J.F., (1996) Polymorphism and concerted evolution in a tamdemly repeated gene family: 5S ribosomal DNA in diploid and allopolyploid cottons. J. Molec. Evol., 42: 685-705

Cronquist, A., (1977) On the taxonomic significance of secondary metabolites in Angiosperms. Plant Syst. Evol., Suppl., 1:179-189

Cronquist, A., (1988) The Evolution and Classification of Flowering Plants. (2nd ed). New York Botanical Garden, Bronx, New York.

Cullen, J., (1968) In: Flora of Turkey and the East Aegean Islands" (Davis, P.H., ed.), Vol.I. Edinburgh, 213-247

Dahlgren, R.M.T., (1980) A revised system of classification of angiosperms. Bot. J. Linn. Soc., 100: 197-203.

Dahlgren, R.M.T., Rosendal-Jensen, S. & Nielsen, B.J., (1981) A revised classification of the angiosperms with comment on the correlation between chemical and other characters, In: Phytochemistry and Angiosperm Phylogeny (Young, D.A. & Seigler, D.S., eds.). Praeger, New and Unwin, London, U.K. pp. 144-149

Day, K.B., Draper, J. & Smith, H., (1986) Plant regeneration and thebaine content of plants derived from callus culture of *Papaver bracteatum*. Plant Cell Rep., 5: 471-474

Dice, L.R., (1945) Measures of the amount of ecological association between species. Ecology, 76: 295-302

Donoghue, M.J., (1992) Homology, In: Keywords in evolutionary biology (Fox Keller, E, & Lloyd, E.A., eds.), Harvard University Press Cambridge, Massachusetts, London, England, pp. 170-179

Downie, S.R., & Palmer, J.D., (1992) Use of chloroplast DNA rearrangements in reconstructing plant phylogeny. In: Molecular systematics of plants II (Solstis, D.E., Solstis P.S. & Doyle, J.J. eds.), Kluwer Academic Publishers, London., pp. 14-35

Doyle, J.J., (1997) Trees within trees: Genes and species, molecules and morphology. Syst. Biol., 54: 537-553

Doyle, J.J. and Doyle J.L., (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. Bot. Soc. Amer., 19: 11-15

Doyle,J.J. & Davis, J.I., (1998) Homology in molecular phylogenetics: a parsimony perspective In: Molecular systematics of plants II (Solstis, D.E., Solstis P.S. & Doyle, J.J. eds.), Kluwer Academic Publishers, London, pp.101-131

Elkan L., (1839) "Tentamen monographie generis *Papaver*" Regimentii Borussorum, Koningsberg.

Engler, A., (1964) Engler's syllabus der Pflanzenfamilien. Zwolfte Aufgbe von Hans Melchior Band II. Gebruder Borntraeger, Berlin, pp.178-181

Ernst, W.R., (1962) A comparative morphology of the Papaveraceae. Ph.D. Thesis (unpbl.), Stanford University, Stanford, California.

Fairbairn, J. W. & Williamson E.M., (1973) Meconic acid as a chemotaxonomic marker in the *Papaveraceae,* Phytochemistry, 17: 2087-2089

Fairbairn, J. W. & Hakim F., (1973) *Papaver bracteatum* Lindl. A new source of plant opiates. J. Pharm. Pharmacol. 25: 353-358

Fairbairn, J. W. & Helliwell, K. (1977) *Papaver bracteatum* Lindl. Thebaine content in relation to plant development. J. Pharm. Pharmacol. 29: 65-69

Farris, J. S., (1970) Methods for computing Wagner trees. Syst. Zool., 19: 83-92

Farris, J. S., (1977) Phylogenetic analysis under Dollo's Law. Syst. Zool., 26: 77-88

Farris, J. S., (1989) The retention index and the rescaled consistency index. Cladistics, 5:417-419

Fedde F., (1909) in "Das Pflanzenreich" (A. Engler, ed.). Englemann, Leipzig 40: p. 228

Fedde, F., (1936) *Papaveraceae*. In *Die naturlichen Pflanzenfamilien* 2. Aufl. (Engler, A. & Prantl eds.)

Feher, J. (1932) Kleistogamia uj esetei Pipacs fajokon. Bot. Kozlem XXX, 192-194

Felsenstein, J., (1981) Evolutionary trees from DNA sequences: A maximum likelihood approach. J. Mol. Evol., 17: 368-376

Felsenstein, J. (1985) Confidence limits on Phylogenies: An approach using the bootstrap. Evolution, 39: 783-791

Fitch, W.M., (1970) Distinguishing homologous from analogous proteins. Syst. Zool., 19: 99-113

Fitch, W. M., (1971) Toward defining the course of evolution: minimal change for a specific tree topology. Syst. Zool., 20: 406-416

Flores-Villela, O., Kjer, K. M., Benabib, M. & J. W. Sites, J.W., (2000) Multiple data sets, congruence and hypothesis testing for the phylogeny of basal groups of the lizard genus *Sceloporus* (Squamata, Phrynosomatidae). Syst. Biol., 49: 713–739

Fominaya, A., Vega, C. & Ferrer, E., (1988) Giemsa C-banded karyotypes of *Avena* species. Genome, 30: 627–632

Gall, J.G., & Pardue, M.L. (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc. Nat. Acad. Sci., 63: 378-382

Gielly, L.P. & Taberlet, P. (1996) A phylogeny of the European gentians inferred from chloroplast *trn*/L(UAA) intron sequences. Bot. J. Linn. Soc., 120:57-75

Gielly, L.P., Yuan, Y.M., Kupfer, P. & Taberlet, P. (1994) Phylogenetic use of noncoding regions in the genus *Gentiana* L.: Chloroplast *trn*/L(UAA) intron versus nuclear ribosomal internal transcribed spacer sequences. Molec. Phylogenetic. Evol., 5: 460-466

Gerardy, R. & Zenk, M.H., (1993) Formation of salutaridine from (R)- reticuline by a membrane bound cytochrome P-450 enzyme from *Papaver somniferum*. Phytochemistry, 32: 79-86

Gershenzon, J. & Mabry, T.J., (1983) Secondary metabolites and the higher classification of angiosperms. Nordic. J. Bot., 3:5-34

Goldblatt, P., (1974) Biosystematic studies in *Papaver* section *Oxytona*. Ann. Mo. Bot. Gard., 61: 264-296

Gottlieb, O.R., Kaplan, M.A.C & Zocher, D.H.T (1993) A chemosystematic overview of *Magnoliidae, Ranunculidae, Cariophyllidae* and *Hamamelidae.* In: The families and genera of vascular plants Vol.II (Kubitzki, K., Rohwer, J.G. & Bittrich, V., eds.), Springer-Verlag, Berlin

Grant, V.D., (1981) Plant speciation. Columbia University Press. New York. pp. 72-94

Greene, E.L., (1888) New or noteworthy species, II. Pittonia, 1: 168

Grossheim, A.A., (1950) Flora Caucasica Moscow & Leningrad, IV: 93-94.

Gunther, K.F., (1975) Beitrage zur Morphologie und verbreitung der *Papaveraceae* 2. Teil: Die Wuchsformen der *Papaverae, Escoscholzieae* und *Platystemonoideae.* Flora, 164: 393-436

Ham, R.C.H.J. van, Hart, H., Mes, T. & Sandbrink, J.M. (1994) Molecular evolution of noncoding-regions of the chloroplast genome in the Crassulaceae and related species. Curr. Genet., 25: 558-566

Hanlet, P., (1969) Revision der mongolischen taxa von *Papaver* L. sect. *Scapiflora* Rchb. Sowie studien zur systematic und evolution dieser section. Habilitationsschrift, Martin Luther Universitat, Halle-Wittenberg, pp 63-70

Hashmi, G., Huettel, R., Meyer, R., Krusberg, L. & Hammerschlag, P., (1997) RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. Plant Cell Rep., 6: 624-627

Harborne, J. B., (1967) Comparative Biochemistry of the Flavonoids, Academic Press, pp. 149-152

Harborne, J. B. & Turner. B.L., (1984) Plant Chemosystematics, Academic Press, London, pp. 143-155

Hasegawa, M., Kishino, H. & Yano, T., (1985) Dating the human-ape split by a molecular clock of mitochondrial DNA. J. Molec. Evol., 22: 160-174.

Hegnauer, R., (1966) In: Comparitive Phytochemistry (Swain, T. ed.), Academic Press, London, U.K, pp. 49-62

Henning, W., (1950) Grundzuge einer theorie der phylogenetischen systematic. Deutscher Zentralverlag, Berlin, pp. 201-222

Henning, W., (1966) Phylogenetic Systematics. Urbana, University of Illinois Press

Herbert, R.B., (1989) Alkaloids In: The Biosynthesis of Secondary Metabolites (2nd ed.).Chapman and Hall, London, U.K. pp. 120-181

Hershkovitz, M.A. & Zimmer, E.A., (1996) Conversion patterns in angiosperm ITS2 sequences. Nuc. Acid. Res., 24: 2857-2867

Hershkovitz, M.A. & Zimmer, E.A., (1997) On the evolutionary origins of the cacti. Taxon, 46: 217-232

Hershkovitz, M.A., Zimmer, E.A. & Hahn, W.J. (1999) Ribosomal DNA sequences and angiosperm systematics. In: Molecular systematics and plant evolution (P.M. Hollingsworth, R. M. Bateman & R. J. Gornall, eds.), Taylor & Francis, London., pp. 268-326

Hibbet, D. S., Fukumasa-Nakai, Y., Tsuneda, A. &Donoghue, M.J., (1995) Phylogenetic diversity in shiitake inferred from nuclear ribosomal DNA. Mycologia, 87: 618–638

Hillis, D.M., (1987) Molecular versus morphological approaches to systematics. Ann. Rev. Ecol. Syst., 18: 23-42

Hillis, D.M., (1994) Homology in molecular biology,. In: Homology. The hierarchial basis of computational biology, (Hall, B.K., ed.), Acedemic Press, London, pp. 339-368

Hodkinson, T.R, Renvoize, S.A & Chase, M.W., (1997) Systematics of *Miscanthus*. Aspects Appl. Biol., 49:189-198

Hodkinson, T.R., Renvoize, S.A., Ni Chonghaile, G., Stapleton, M.A., Chase, M.W., (2000) A comparison of ITS nuclear rDNA sequence data and AFLP markers for phylogenetic studies in *Phyllostachys* (Bambusoideae, Poaceae). J. Plant Res., 113: 259-269

Hodkinson, T.R., Chase, M.W., Takahashi, C., Leitch, I.J., Bennett, M.D. & Renvoize, S.A., (2002) The use of DNA sequencing (ITS and *trnL-F*), AFLP and fluorescent *in-situ* hybridisation to study allopolyploid *Miscanthus* (Poaceae). Amer. J. Bot., 89: 279-286

Hook I., Sheridan H. & Wilson G., (1988) Alkaloids of the cell cultures derived from strains of *Papaver bracteatum*. Phytochem., 27: 2137-2141

Hopper, S.D., Fay, M.F., Rossetto, M., and Chase, M.W., (1999) A molecular phylogenetic analysis of the bloodroot and kangaroo paw family, Haemodoraceae: taxonomic, biogeographic and conservation implications. Bot. J. Linn. Soc., 131: 285-299.

Hutchinson, J., (1959) Families of flowering plants. 2nd ed. Oxford University Press, London, New York.

Isabel, N., Bovin, R., Levasseur, C., Charest, P. M., Bousquet, J. & Tremblay, F. M., (1996) Occurrence of somaclonal variation among somatic embryo derived white spruce (*Picea glauca*, Pinaceae). Amer. J. Bot., 83:1121-1130

Ikustu, A., Syono, K. & Furuya, T., (1974) Alkaloids of callus tissues and redifferentiated plantlets in the Papaveraceae. Phytochemistry, 13: 2175-2141

Jiang, J.M. & Gill, B.S. (1993) A zebra chromosome arising from multiple translocations involving non-homologous chromosomes. Chromosoma, 102: 612-618

John, H.A., Birnstiel, M.L. & Jones, K.W., (1969) RNA-DNA hybrids at the cytological level. Nature, 223: 582-587

Jones, M.P., Dingkuhn, M., Alukosnm, G.K. & Semon M. (1997) Interspecific *Oryza sativa* L. X *O. glaberrima* Steud. Progenies in upland rice improvement Euphytica, 94: 237-246

Jukes, T.H., & Cantor C.R., (1969) Evolution of protein molecules. In: Mammalian protein metabolism. (Munro, H.N., ed.).Academic Press, New York. pp. 21-132

Judd, W.S., Campbell, C.S., Kellogg, E.A. and Stevens, P.F., (1999) In Plant Systematics: A phylogenetical approach. Sinauer Associates, Sunderland MA, USA pp. 149-152

Kadereit, J.W., (1986a) A revision of *Papaver* section *Argemonidium.* Notes Roy. Bot. Gard. Edinburgh, 44: 25-43

Kadereit, J.W., (1986b) A revision of *Papaver* L. sect *Papaver* (Papaveraceae). Bot. Jahrb. Syst., 108: 1-16

Kadereit, J.W., (1986c) *Papaver somniferum* L. (Papaveraceae): A triploid hybrid? Bot. Jahrb. Syst., 106: 221-244

Kadereit, J.W., (1987) A revision of *Papaver* sect *Carinatae* (Papaveraceae). Nord. J. Bot., 7: 501-504

Kadereit, J.W., (1988a) Sectional affinities and geographical distribution in the genus *Papaver* L. (*Papaveraceae*). Beitr. Biol. Pflanzen, 63: 139-156.

Kadereit, J.W., (1988b) *Papaver* L. sect *Californicum* Kadereit, a new section of the genus *Papaver*. Rodora, 90: 7-13

Kadereit, J.W., (1988c) The affinities of the south-hemispherical *Papaver aculeatum* Thunb. (Papaveraceae). Bot. Jahrb. Syst., 109: 335-341

Kadereit, J.W., (1989) A revision of *Papaver* section *Rhoeadium* Spach. Notes Roy. Bot. Gard. Edinburgh, 45: 225-286

Kadereit, J.W., (1990) Some suggestions on the geographical origin of the Central, West and North European synantropic species of *Papaver* L. Bot. J. Linn. Soc.,103: 221-231

Kadereit, J.W., & Sytsma, K.J., (1992) Disassembling *Papaver*: a restriction site analysis of chloroplast DNA. Nord. J. Bot., 12: 205-217

Kadereit, J.W., (1993a) *Papaveraceae.* In: The families and genera of vascular plants Vol.II (Kubitzki, K., Rohweer, J.G. & Bittrich, V., eds.), Springer-Verlag, Berlin, pp. 20-33

Kadereit, J.W., (1993b) A revision of *Papaver* sect. *Meconidium.* Edinb. J. Bot., 50: 125-148

Kadereit, J.W., Blattner, F.R., Jork, K.B. & Schwarzbach, A.E., (1994) Phylogenetic analysis of the *Papaveraceae* s.l. (incl. *Fumariaceae, Hypecoaceae* and *Pteridophyllum*) based on morphological characters. Bot. Jahrb. Syst., 116: 361-390

Kadereit, J.W., (1996) A revision of *Papaver* L. sects *Pilosa* Prantl and *Pseudopilosa* M. Popov ex Gunther (Papaveraceae). Edinb. J. Bot., 53: 285-309

Kluge, A.G. & Farris, J.S..(1969) Quantitative phyletics and the evolution of anurans. Syst. Zool., 18: 1-32

Kotseruba, V., Gernand, D., Meister, A. & Houben, A., (2003) Uniparental loss of ribosomal DNA in the allotetraploid grass *Zingeria trichopoda* ($2n = 8$). Genome: 156-163

Kubitzki, K., (1993) In: Kubitzki, K., Rohwer, J.G. & Bittrich, V. (eds.) The families and genera of vascular plants Vol.II. Springer-Verlag, Berlin, pp.1-12

Kutchan, T.M., (1998) In: The Alkaloids (Cordell, G.A. ed.). Molecular Genetics of Plant Alkaloid Biosynthesis. Academic Press, New York, 50: 275-316

Kutchan, T.M., Ayabe S. Krueger R.J., Coscia E.M. & Coscia C.J., (1983) Cytodifferentiation and alkaloid accumulation in cultured cells of *Papaver bracteatum*. Plant Cell Rep., 2: 281-284

Lalezari, I., Nasseri P. & Asgharian R., (1973) Isolation of alpinigeneine from *Papaver bracteatum*, J. Pharm. Sci. 62: 1718

Lalezari, I. & Shafiee, A., (1978) Alkaloids of the *Papaver* genus *P. orientale* and *P. pseudo-orientale*. Pazhoohandeh (Tehran), 13: 50-56

Landolt, E., (1967) *Papaver alpulum* Ten. Am Alpensudfuss. Bauhinia, 3:265-269

Lecky, R., Hook, I. & Sherridan, H., (1992) Enhancement of dihydrosanguinarine production in suspension cultures of *Papaver bracteatum*, I. Medium modifications J. Nat. Prod., 55: 1513-1517

Leitch, A.R., Mosgoller, W., Schwarzacher, T., Bennett, M.D. & Heslop-Harrison, J.S. (1990) Genomic *in situ* hybridization of section nuclei shows chromosome domains in grass hybrids. J. Cell Sci., 95: 335-341

Leitch, A.R., Schwarzacher, T., Mosgoller, W., Bennett, M.D. & Heslop-Harrison, J.S., (1991) Parental genomes are separated throughout the cell cycle in a plant hybrid. Chromosma, 101: 206-213

Leitch, A.R., Schwarzacher, T., Jackson, D. & Leitch, I, J. (1994) *In situ* hybridization: A practical guide, BIOS Scientific Publishers

Leitch, I.J. & Bennett, M.D., (1997) Polyploidy in angiosperms. Trends in Plant Sci. Reviews. 2, 12: 470-476

Levy, A. & Milo J., (1991) Inheritance of morphological and chemical characters in interspecific hybrids between *Papaver bracteatum* and *Papaver pseudo-orientale*. Theor. Appl. Genet., 81: 537-540

Li, W.H & Graur, D., (1991) Fundamentals of molecular evolution. Sinauer Associates, Sunderland MA, USA.

Li, W.H..(1997) Molecular evolution. Sinauer Associates, Sunderland MA, USA.

Lim,K.Y, Leitch, I.J & Leitch, A.R., (1998) Genomic characterisation and the detection of raspberry chromatin in polyploid Rubus. Theoret. Appl. Genet., 97:1027-1033

Lim, K.Y, Kovarik, A., Matyasek, R., Bezdek, M., Lichtenstein, C.P, Leitch A.R., (2000) Gene conversion of ribosomal DNA in *Nicotiana tabacum* is associated with undermethylated, active gene units. Chromosoma, 109: 161-172

Ljungdahl, H., (1922) Zur Zytologie der Gattung Papaver. Svensk. Bot.. Tidsk., 16: 103-116

Lockwood, G.B., (1984) Alkaloids of cell suspensions derived from four *Papaver* species and the effect of temperature stress. Z Pflanzenphysiol, 114: 361-363

Lockwood, G.B., (1981) Orientalidine and isothebaine from cell cultures of *Papaver bracteatum.* Phytochemistry, 20:1463-1464

Lyrene, P.M., Vorsa N. & Ballington J.R., (2003) Polyploidy and sexual polyploidization in the genus *Vaccinium.* Euphytica., 133: 27-36

Mackill, D.J.; Zhang, Z.; Redona, E.D. & Colowit, P.M., (1996) Level of polymorphism and genetic mapping of AFLP markers in rice. Genome, 39:969-977

Markgraf, F., (1986) Familie *Papaveraceae.* In: Illustrietrte Flora von Mittelurropaa Band IV. Teil 1. (3rd ed.) (Hegi, ed.)

Massicot, J.,(1961) Biosynthesis of alkaloids in Poppy seedlings, Ann. Pharmaceut, France, 19(1) p.44

Matsuura, N., M. Iunuma, & Tanaka, T., (1994) Phylogenetic analysis in genus *Euchresta* based on secondary metabolites. Biochem. Syst. & Ecol., 22: 621-629

Maturova, M., Pavlaskova, D. & Santavy, M., (1966) Isolierung der Alkaloide aus einger arten der Gattung Papaver. Planta Med., 37: 22-41

Matzke, M.A. & Matzke, A.J.M., (1991) Differential inactivation and methylation of a trans gene in plants by two suppressor loci containing homologous sequences. Plant. Mol. Biol., 16: 821-830

McNicholas, L.F. & Martin, R., (1984) New and experimental therapeutic role for naloxone and related opiate antagonists, Drugs, 27: 81-93

Medvedev, J. S., (1918) On a new poppy, *Papaver pseudo-orientale*. Isv. Kavkazsk. Mus. (Ann. Caucas. Dus.), 11:204-207

Meshulam, H. & Lavie, D. (1980) The alkaloid constituents of *Papaver bracteatum* Arya II. Phytochemistry, 19: 2633-2635

Middleton, D.J., (1992). Chemotaxonomic studies in the genus *Gaultheria* and related genera. Bot. J. Lin. Soc. 110: 313-324

Mihalik, E., (1998) Taxonomy. In: Poppy, The genus *Papaver* (Bernath, J., ed.) Hardwood Academic Publishers. The Netherlands, pp. 7-45

Milo J., Levy A., Palevitch D. & Ladizinsky G., (1990) Genetic evidence for the conversion of the morphinian alkaloid thebaine to oripavine in interspecific hybrids between *Papaver bracteatum* and *Papaver orientale*. Heredity 64: 367-370

Molvray, M., Kores, P.J. & Chase, M.W., (1999) Phylogenetic relationships within *Korthalsella* (Viscaceae) based on nuclear ITS and plastid *trnL-F* sequence data. Amer. J. Bot., 86: 249-260

Moore, W.S., (1995) Inferring phylogenies from mtDNA variation: Mitochondrial-gene trees versus nuclear gene trees. Evolution, 49: 718-726

Morale Torres, C., Mendoza Castellon, R. & Romero Garcia, A.T., (1988) La pisicion sistematica de *Papaver argemone* L.: interes evolutivo del orden *Papaverales* : 1. Lagascalia, 15:181-189

Moscone, E.A., Matzke, M.A. & Matzke, A.J.M., (1996) The use of combined FISH/GISH in conjunction with DAPI counterstaining to identify chromosomes containing transgene inserts in amphidiploid tobacco. Chromosoma, 105: 231–236

Mothes, K., Schutte, H.R. & Luckner, M., (1985) *Biochemistry of Alkaloids*, VEB Deuther Verlag der Wissenschaften, Berlin pp. 325-332

Mueller, L.D. & Ayala, F.J., (1982) Estimation and interpretation of genetic distance in empirical studies. Genet. Res., 40:127-137

Muller, E., Brown, P.T.H. & Hartke, S., (1990) DNA variation in tissue cultured-derived rice plants. Theor. Appl. Genet., 80: 673-679

Mullis, K. B. & Faloona, F. A., (1987) Specific synthesis of DNA *in vitro* via a polymerase catalyzed chain reaction. Methods Enzymol., 155: 335-350

Murashigee, T. & Skoog, F.A., (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant, 15:473-497

Nei, M. & Li, W. H., (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA, 76: 5269-5273

Nemeckova, A., Santavy, F. & Waltera D., (1967) Coll. Czech. Chem. Commun., 35, 1733

Preininger V., (1986) Chemotaxonomy of Papaveraceae and Fumariaceae. In: The Alkaloids, Acedemic Press, London, U.K. and New York U.S.A., 29: 2-98

Psenak, M., (1998) Biosynthesis of morphinane alkaloids. In: Poppy, The genus *Papaver* (Bernath, J., ed.). Hardwood Academic Publishers. The Netherlands, pp. 159-181

Pyysalo, H., Widen, C.J., Salemink, C.A., Lewing, E., Rousi, A. & Ojala, A., (1988) Interspecific hybridization in Papaver: 2. Alkaloid contents of Papaver somniferum and species of the section Oxytona and their interspecific hybrids. Ann. Bot. Fenn., 25: 1-10

Qui, Y.L., Lee, J., Bernasconi-Quadroni, F., Soltis, D.E., Soltis, P.S., Zanis, M., Zimmer, E.A., Chen, Z., Savolainene, V. & Chase, M., (1999) The earliest angionsprms: evidence from mitochondrial, plastid and nuclear genomes, Nature 402: 404-407

Ramanna, M.S. & Jacobsen E., (2003) Relevance of sexual polyploidization for crop improvement – A review. Euphytica, 133:3-8

Randel, U., (1974) Beitrage zur Kenntnis der Sippenstruktuir der Gattung *Papaver* L. Sectio *Scapiflora* Reihenb. Im vergleich mit *P. alpinum* L. (*Papaveraceae*). Feddes Repert., 86:19-37

Randel, U., (1977) Uber die grondlandischen Vertreter der section *Lasiotrachyphylla* Bernh. (Papaveraceae). Feddes Repert., 88: 421-450

Rani, V. & Raini, S. N., (2000) Genetic fidelity of organized meristem-derived micropropagated plants: A critical reappraisal. In Vitro Cell. Dev. Biol., 36: 319-330

Reckin, J., (1973) A contribution to the cytology of *Papaver gracile* Auch. Including proposals for a revision of the section *Mecones*. Caryologia, 26: 245-251

Reeves, G., Chase, M.W, Goldblatt, P., Rudall, P., Fay, M.F., Cox, A.V., Lejeune, B. and Sousa-Chies, T., (2001) Molecular systematics of Iridaceae. Evidence from four plastid DNA regions. Amer. J. Bot., 88: 2074-2087

Ridout, C.J. & Donini, P., (1999) Use of AFLP in cereals research. Trend. Plant Sci., 4: 76-79

Riesberg, L.H., (1995) The role of hybridization in evolution: Old wine in new skins. Amer. J. Bot., 82:944-953

Rios, J.L., Manez, S., Giner, R.M. & Recio, M.C., (2000) Biological Aspects of Aporphinoid Alkaloids. In The Alkaloids, (G.A. Cordell, ed.), Academic Press, New York, 53: 57-117

Ronsch H., (1986) Rhoeadine alkaloids. In: The Alkaloids (Brossi, A. ed.). Academic Press, New York, 28: 1-93

Rosenblum, B.B., Oaks, F., Menchen, S. & Johnson, B., (1997) Improved single-strand DNA sizing accuracy in capillary electrophoresis. Nuc. Acid. Res., 25: 3925-3929

Rush, M.D., Kutchan, T.M. & Coscia, C.J., (1987) Correlation of appearance of morphinan alkaloids and laticifer cells in germinating *Papaver bracteatum* seedlings. Plant Cell Reports, 4:237-240

Sande C.A.F.M. van der, Kwa, M., Nues, R.W., Heerikhuizen, H.J., van, Raue H.A. & Planta, R.J., (1992) Functional analysis of the internal transcribed spacer 2 of Saccharomyces cerevisiae ribosomal DNA. J.Mol. Biol., 223: 899-910

Saitou, N. & Nei, M., (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. and Evol., 4: 406-425.

Salamin, N., Chase, M.W., Hodkinson, T.R. & Savolainen, V. (2003) Assessing internal support with large phylogenetic DNA matrices. Mol. Phyl. Evol., 27: 528-539

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A., 74:5463-5467

Sansome, F. & Philip J., (1939) Recent Advances in Plant Genetics (2nd ed). London pp. 19-30

Santavy, F., (1970), Papaveraceae Alkaloids. In: The Alkaloids (Manske, R.H.F. ed.). Academic Press, New York, 12: 333-454

Santavy, F., (1979), Papaveraceae Alkaloids. In: The Alkaloids (Manske, R.H.F. & Rodrigo, R.G.A. eds.). Academic Press, New York, 17: 385-544

Sariyar, G. & Baytop, T., (1980) Alkaloids from *Papaver pseudo-orientale* (*P. lasiotrix*) of Turkish origin. Planta Med., 46, 378-380

Sariyar, G., Baytop, T. & Phillipson, J.D., (1983) A new protopine alkaloid from Turkish *Papaver curviscapum* 1-methoxyallocryptopine. Planta Med., 46: 378-380

Saunders, R. & Brown, G.D., (1999) Biosynthetic pathways and transformation series in the cladistic analysis of chemical data. Systematics Association, Second Biennial Conference, Book of Abstracts. p. 42.

Schwarzbach, A.E. & Kadereit, J.W., (1999) Phylogeny of prickly poppies, Argemone (Papaveraceae), and the evolution of morphological and alkaloid characters based on ITS nrDNA sequence variation, Plant Syst. Evol. 218: 257-279

Schwarzacher, T., Leitch, A.R., Bennet, M.D. & Heslop-Harrison, J.S. (1989) *In situ* localization of parental genomes in a wide hybrid. Ann. Bot., 64: 315-324

Shafiee, A., Lalezari, I. & Yassa, N., (1976) Thebaine in tissue culture of *Papaver bracteatum* Lindl, Population Arya II. Lloydia, 39: 380-381

Simanek, V., (1985) Benzophenanthridine Alkaloids. In: The Alkaloids(Brossi, A. ed.). Academic Press, New York, 26: 185-240.

Simpson, D.A., Furness, C.A., Hodkinson, T.R., Muasya, A.M. & Chase, M.W., (2003) Phylogenetic relationships in Cyperaceae subfamily Mapanioideae inferred from pollen and plastid DNA sequence data. Am. J. Bot., 90: 1071-1086

Singh, G., (1999) in 'Plant Systematics'. Science Publishers, Inc. New Hampshire, U.S.A.

Sneath, P.H.A. & Sokal, R.R., (1973) Numerical Taxonomy. W.H. Freeman and Company, San Fransisco.

Soltis, P. S. & Soltis, D.E., (1995) Plant molecular systematics: Inferences of phylogeny and evolutionary processes. Evol. Biol., 28: 139-194

Soltis, P. S. & Soltis, D.E., (1998) Choosing an approach and an appropriate gene for phylogenetic analysis. In: Molecular Systematics of Plants II. (Soltis, D.E., Soltis P.S. & Doyle, J.J. eds) Kluwer Academic Publishers, London, pp. 1-42

Soltis, P.S., Soltis, D.E. & Chase, M.W., (1999) Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. Nature, 402: 402-404

Soltis, P. S. & Soltis, D.E., (2000) Contributions of plant molecular systematics to studies of molecular evolution. Plant. Mol. Biol., 42: 45-75

Spencer, I.D., (1968) The biosynthesis of alkaloids and other nitrogenous secondary metabolites. In: Comprehensive biochemistry, (Florkin, N. & Stotz, E.H., eds.), Elsevier, North Holland & New York, pp. 300-330

Sporne, K.R., (1974) Morphology of Angiosperms. Hutchinson Univ. Library, London, pp. 65-72

Stace, C.A. & Bailey, J.P., (1999) In: Molecular systematics and plant evolution (P.M. Hollingsworth, R. M. Bateman & R. J. Gornall, eds). Taylor & Francis, London, pp.199-210.

Stevens, P.F., (1997). What kind of classification should practising taxonomist used to be saved. Plant diversity in Malesia III - In: Proceedings of the third international Flora Malesiana symposium (J. Dransfield, M.J.E. Coode & D.A. Simpson, eds.), pp. 231–246.

Sun, Y., Skinner, D.Z., Liang, G.H. & Hulbert, S.H., (1994) Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. Theor. Appl. Genet., 89: 26-32.

Swofford, D.L. & Begle D.P., (1993) PAUP Phylogenetic Analysis Using Parsimony. Version 3.1 User's Manual. Illinois Natural History Survey., pp. 1-57

Swofford, D. L., (2003) PAUP*: Phylogenetic analysis using parsimony (* and other methods), version 4.0b 10. Sinauer Associates, Sunderland, Massachusetts.

Swofford, D.L., Olsen, G.J., Waddell, P.J. & Hillis, D.M., (1996) Phylogenetic inference. In: Molecular Systematics (2nd edn) (Hillis, D.M., Moritz, C. & Mable, B.K., eds.), Sinauer Associates, Sunderland MA, USA., pp.407-514

Szoke, E., (1999) *In vitro* biosynthesis of poppy alkaloids. In: Poppy, The genus *Papaver* (Bernath, J., ed.). Hardwood Academic Publishers. The Netherlands, pp. 189-207

Taberlet, P.L., Gielly, G., Pautou & Bouvet, J., (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Pl. Molec. Biol., 17: 1105-1109

Takhtajan, P., (1959) *Die evolution der Angiospermen.* Fischer, Jena. pp. 149-152

Tam, W.H.J., Constabel, F. & Kurz, W.G.W., (1980) Codeine from cell suspension cultures of *Papaver somniferum.* Phytochemistry, 19: 486-487

Taylor, P. W. J., Geijskes, J. R., Ko, H. L., Fraser, T. A., Henry, R. J. & Birch, R. J., (1995) Sensitivity of random amplified polymorphic DNA to detect genetic variation in sugarcane during tissue culture. Theor. Appl. Genet., 90:1169-1173

Tetenyi, P., (1993) Chemotaxonomy of the genus *Papaver.* Acta. Hort., 344:154-165

Tetenyi, P., (1997) Opium poppy (*Papaver somniferum*): Botany and horticulture. Horticult. Rev., 19: 373-410

Theuns, H.G., Janssen, R.H. & Salemink, C.A., (1987) The alkaloids of the Papaver *section Oxytona* Berhn. Herbs, Spices and Medicinal Plants, 2:57-110

Thiele, K., (1993) The Holy Grail of the Perfect Character: the Cladistic Treatment of Morphometric Data, Cladistics, 9: 275-304

Trease, G.E. & Evens W.C., (1983) Pharmacognosy-12th Edition. Bailliere Tindall, London pp. 435-452

Troll, W., (1964) *Die Inflorescezen.* Gustav Fischer Verlag, Jena.

United Nations Document (1976) Scientific Research on *Papaver bracteatum*, Report of the Fourth Working Group. ST/SOA/SER.J/23: p.6

Vaughan, H.E., Jamilena, M., Ruiz, R.C., Parker, J.S., & Garrido,R.M.A., (1993) Loss of nucleolar-organizer regions during polyploid evolution in *Scilla autumnalis.* Heredity, 71: 574-580

Vos, P.R., Hodges, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Kuiper, M. & Zabeau, M., (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res., 23: 4407-4410

Waterman, P.G., (1998) Alkaloid Chemosystematics. In: The Alkaloids Academic Press, London, U.K. and New York U.S.A., 50: 537-565

Waugh, R., Bonar, N., Baird, E., Thomas, B., Graner, A., Hayes, P. & Powell, W., (1997) Homology of AFLP products in three mapping populations of barley. Mol. Gen. Genet., 255:311-321

Wiens, J., (2001) Character analysis in morphological phylogenetics: Problems and solutions. Syst. Biol., 50: 689-699.

Wendel, J.F., Schnabel, A. & Seelanan, T., (1995) An unusual ribosomal DNA sequence from *Gossypium gossypioides* reveals ancient, cryptic, intergenomic introgression. Mol. Phyl. Evol., 4: 298-313

Wendel, J.F. & Doyle J.J., (1998) Phylogenetic incongruence: Window into genome history and molecular evolution. In: Molecular Systematics of Plants II. (Soltis, D.E., Soltis P.S. & Doyle, J.J. eds) Kluwer Academic Publishers, London, pp. 265-296

Wendt, N., (1976) Beitrage zur sippenstrukturforschung an der gattung *Papaver* L., sektion *Pilosa* Prantl, sektion *Pseudopilosa* Popov ex Gunther und section *Oxytona* Bernh. Ph.D. Dissertation. Berlin

Williams, J. G. K., Kubelik, A. R., Livak, J., Rafalski, J. A. & Tingey, S. V., (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535

Wolfe, K.H., Li, W-H & Sharp, W.H., (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast and nuclear DNAs. Proc. Nat. Aced. Sci. U.S.A., 84: 9054-9058

Wolfe, K.H. & Sharp, W.H., (1988)
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Yasui, K., (1921) On the behavior of chromosomes in the meotic phase of some artificially raised *Papaver* hybrids. Bot. Mag., Tokyo, 35:154-167

Yasui, K., (1936) Cytogenetic studies in artificially raised interspecific hybrids of *Papaver* IV. Interspecific hybrids of *P. orientale* and *P. bracteatum*. Cytologia, 7: 535-542

Yasui, K., (1937a) Cytogenetic studies in artificially raised interspecific hybrids of *Papaver* VI. The trigenomic hybrids of *Papaver*. Cytologia. Jubilee, 1101-1112

Yasui, K., (1937b) Cytogenetic studies in artificially raised hybrids of *Papaver* VII. *P. somniferum* X *P. bracteatum*. Cytologia, 8: 331-334

Zenk, M.H., (1985) Enzymology of benzylisoquinoline alkaloid formation. In: The Chemistry and Biology of Isoquinoline alkaloids (Phillipson, J.D., Roberts, M.F. & Zenk, M.H. eds.). Springer-Verlag, Berlin, pp. 240-256

Zito, S.W. & Staba, E.J., (1982) Thebaine from root cultures of *Papaver bracteatum.* Planta Med., 45:53-54

Appendix 1

1.1. Isolation of total genomic DNA using CTAB

Materials

2× CTAB Buffer (100mM Tris-HCl pH 8.0, 1.4M NaCl, 20mM EDTA, 2%CTAB w/v)

2-mercaptoethanol

CI (24:1 chloroform: isoamyl alcohol)

Wash buffer (70% ethanol)

TE Buffer (10mM Tris HCl pH8.0, 1mM EDTA)

EDTA= Ethylenediaminetetraacetate

CTAB= Hexadecyltrimethylammonium bromide

Equipment

10ml capped centrifuge tubes (labelled)

Pipettes (Gilson, P200, P5000)

Pipette tips

Transfer pipettes

Weighing boats

Pestles

Mortars

10ml capped tubes (labelled)

Grant SUB14 / Memmert W200 water bath

Harrier 15/ 80 centrifuge

Fume hood

Protocol:

1. Pre-warm a pestle, mortar and 10ml of 2×CTAB extraction buffer combined with 40µl of 2-mercaptoethanol to 65°C.
2. Weigh between 0.05-0.1g of leaf material, cut up into small pieces in the pestle.

3. Grind the leaf into a fine powder and add a small amount of pre-warmed extraction buffer.
4. When the leaf material is almost ground add the remainder of the extraction buffer.
5. Place the slurry back into a labelled 12ml centrifuge tube, sealed and incubate at 65°C for approximately 10 minutes with occasional mixing.
6. Add 10ml of Cl solution to each tube and mix gently.
7. Place in a water bath at 65°C for 10 minutes.
8. Release pressure from tube and place on a shaker in the horizontal position for approximately 30 minutes.
9. Weigh tubes to aid balancing the cetrifuge and centrifuge at 4500g for 10 minutes
10. Remove the upper layer (aqueous phase containing the DNA) with a transfer pipette and place in a labelled 12ml capped tubes.
11. Add equal volumes of ice-cold isopropanol to each DNA sample.
12. Store all samples at -20°C for at least 24 hours to further precipitate the DNA.
13. Following precipitation, centrifuge the samples at 2500g for 10 minutes to pellet the DNA.
14. Pour off the supernatant and add 3ml of wash buffer to each tube, and mix gently.
15. Centrifuge the samples once more at 2500 rpm for 5 minutes to pellet the DNA.
16. Decant off the supernatant and place the tubes upside down for 5 minutes on a paper towel to let any excess wash buffer drain away.
17. Turn tube the right way up and allow to air dry in a fume hood for approximately 25-30 minutes to remove all traces of ethanol.
18. Suspend the pellet in 0.5ml 1×TE and transfer to a labelled 1.5ml microcentrifuge tube.
19. Store extracted DNA at -80°C until required.

1.2 Spin column cleaning of total genomic DNA using the Concert Rapid PCR Purification System

Materials

H1 binding solution (conc. guanidine hydrochloride, EDTA, Tris-HCl and isopropanol)

H2 Wash Buffer (NaCl, EDTA and Tris – HCl) including 70% ethanol.

TE Buffer (10mM Tris – HCl (pH 8.0) and 0.1mM EDTA)

Equipment

1ml spin cartridges

2ml wash tubes

1.5ml recovery tubes

Pipettes (P20, P200 and P1000)

Pipette tips (P200 and P1000)

Whirlmixer (Fison Scientific Equipment)

Heating block (Grant QBT2)

Microcentrifuge (IEC Micromax)

Protocol

1. Preheat an aliquot of TE Buffer (50µl per sample cleaned) to 65°C.
2. Add four times the volume of H1 solution to each DNA sample and mix thoroughly.
3. Transfer each DNA sample to the spin cartridge.
4. Place the spin cartridges into the wash tubes and centrifuge at 13000g for one minute.
5. Discard the flow through and place the spin cartridges once more into a wash tube.
6. Add 700µl of H2 solution to each spin cartridge and centrifuge at 13000g for one minute.
7. Discard the flow-through once more and centrifuge for a third time at 13000g for one minute

8. Place the spin cartridges into labelled 1.5ml recovery tubes and add 50µl of TE buffer directly to the centre of the spin cartridge. Incubate each sample at room temperature for one minute and centrifuge at 13000g for two minutes.
9. Store the DNA solution in the recovery tube at –20°C until required.

1.3. Gel electrophoresis to visualise total genomic DNA

Materials

TBE 10 x (0.89M Tris, 0.89M Boric acid, 0.1M EDTA pH 8.3)

80ml 1.2% agarose gel (1.2g of agarose (GIBCO BRL), 100ml of 1xTBE, 2µl (10mg/ml of ethidium bromide)

Loading Buffer (sucrose and bromophenol blue)

0.5 µg/µl of 1Kb ladder (GIBCO BRL)

Equipment

Parafilm

20µl pipette (P20)

200µl pipette tips

Gel rig (Horizon 11.14, Life Technologies, GIBCO BRL Horizontal Electrophoresis Appartus)

Power pack (EC105, E-C Apparatus Corporation)

UVP Dual-intensity transilluminator (Kodak)

Kodak LPU120 Digital Camera

ID Image analysis software (Kodak Digital Science™)

Protocol

1. Prepare a 1.2% agarose gel and allow cooling to 50°C before adding 2µl ethidium bromide (10 µg/µl) .
2. Pour mixture into an electrophoresis tray and allow to set.

3. Load 5µl of total genomic DNA or PCR product, each with 1µl of loading buffer, into separate wells.
4. Mix 1µl of 1kb ladder, 1µl loading buffer and 3µl ultrapure water as a reference to estimate DNA range or sequence size.
5. Run the gel in 1×TBE at 112V for 30- 45 minutes.
6. Place the agarose gel above a UV transilluminator and photograph with digital camera.

1.4. ITS PCR amplification-reaction mixtures

Materials

Sterile ultrapure H_2O

10× thermophilic buffer (Promega) DETAILS

DNTPs each at 10mM

Forward primer 100ng/µl (ab101 or ITS-3)

Reverse primer 100ng/µl (ab102 or ITS-2)

Forward primer 100ng/µl (*trn*L-F 'c' or 'e')

Reverse primer 100ng/µl (*trn*L-F 'f' or 'd')

$MgCl_2$ 25mM

Taq polymerase (5 units/µl)

Betaine (carboxymethyl)trimethylammonium inner salt

Equipment

0.2ml PCR tubes

Pipettes (P2, P200)

Pipette tips

Microcentrifuge (IEC Micromax)

Thermal cycler (GeneAmp® PCR System 9700)

Protocol

1. Prepare a Master Mix as outlined in Chapter 2, Table 2.3 and 2.4 for ITS and *trnL-F* respectively
2. Place 0.5µl of template DNA into a labelled 0.2ml PCR-tube.
3. Add 49.5µl of the Master-Mix to the DNA, vortex briefly and spin down the contents of the PCR tubes.
4. Load the tubes (ensuring that there are no bubbles) into the thermal cycler.
5. Subject the mix to the reaction conditions outlined in Chapter 2, Table 2.3 and 2.4 for ITS and *trnL-F* respectively
6. Purify PCR products using the spin-column cleaning (protocol 1.2). Run 5µl of the PCR product on a 1.2% agarose gel to see if amplification was successful.
7. Store at 4°C.

1.5. Cycle sequencing of PCR products using Applied Biosystems Big Dye Terminator Cycle Sequencing Kit

Materials

Sterile ultrapure H_2O

Sequencing buffer (5mM $MgCl_2$, 200mM Tris HCl buffer at pH9.0)

Primer 5ng/µl

Dye Terminator/Taq/dNTP 'Pink' mix (Applied Biosytems; contains dNTPs, Amplitaq DNA polymerase, $MgCl_2$, Tris-HCl buffer at pH 9.0)

Template DNA (c. 500ng/ µl)

Equipment

0.2ml PCR tubes

Pipettes (P2, P210)

Pipette tips

Microcentrifuge (IEC Micromax)

Thermal cycler (GeneAmp® PCR System 9700)

Protocol

1. Place the reaction components as outlined in Chapter 2, Table 2.5 into a labelled 0.2ml PCR-tube.
2. Vortex briefly and spin down the contents of the PCR tubes.
3. Load the tubes (ensuring that there are no bubbles) into the thermal cycler.
4. Subject the cycle sequence mix to the reaction conditions outlined in Chapter 2, Table 2.5.
5. Store samples at 4°C wrapped in foil to prevent degradation of fluorescent dyes by light.

1.6. Purification of sequenced DNA samples using ethanol precipitation

Materials

100% Ethanol (EtOH)

Sodium Acetate (NaAc)

70% Ethanol (EtOH)

Equipment

1.5ml microcentrifuge tubes or 0.5ml centrifuge tubes (labelled)

Pipettes (P2, P20, P200. P1000)

Pipette tips

Paper towels

Microcentrifuge (IEC Micromax)

Thermal cycler (GeneAmp® PCR System 9700)

Protocol

1. Transfer each sample into a 1.5ml or 0.5ml centrifuge tube.
2. Add 2µl of NaAc and 50µl of 100% EtOH to each tube.

3. Incubate the samples at room temperature for ten minutes and then place on ice (or in the -20°c freezer) for a further ten minutes.
4. Centrifuge each sample at 13000g for 25 minutes.
5. Carefully discarded the supernatant and add 300µl of 70% EtOH to each tube.
6. Centrifuge each sample at 13000g for 15 minutes.
7. Discard the supernatant and repeat steps 5 to 7.
8. Air-dry the DNA pellet overnight and store in foil at -20°C.

1.7. Alkaloid extraction

Materials

Acetic acid (NaOAc)

10% NH_4OH (dilute ammonia).

Anhydrous sodium sulphate ($Na_2 SO_4$)

Chloroform ($CHCl_3$).

Methanol (CH_3OH)

Equipment

250ml conical flask

Bucchnar flask (250ml)

Round bottom flask (250ml)

Separating funnel (250ml)

Blender (Krupps)

Rotary table

Rotary Evaporator

Protocol

1. Hollow out then crush mature poppy capsules and grind to a powder in a blender
2. Place 0.5g of this material into a 250ml conical flask.

3. Add 50 ml of acetic acid (NaOAc) and place the mixture on a rotary table for 1 hour.
4. Filter the mixture under vacuum and allow to dry. Scrape off the material remaining on the filter paper into the 250ml conical flask and extracted for a second time with 5% acetic acid. Again place on a rotary table for 1 hour.
5. After filtering the second mixture under vacuum and combining both extracts into a 250ml conical flask adjust pH to 9.0 by adding 10% NH_4OH (dilute ammonia).
6. Pour solution into a 250ml separating funnel and add 50ml of Chloroform ($CHCl_3$).
7. Shake the mixture gently opening the valve to release gas build up.
8. Filter the bottom layer (chloroform layer) through anhydrous sodium sulphate (Na_2SO_4) and collect in a 250ml round bottom flask.
9. Repeat the chloroform extraction step twice more, combining them.
10. Evaporate the filtrate to dryness on a rotary vacuum, and store in a fridge until needed.
11. Dissolve the dried extract with 0.5ml Methanol (MeOH; CH_3OH).

1.8. Root pre-treatment, fixation and chromosome counts.

Materials

Gammaxene ®, a saturated solution of hexachlorocyclohexane (Sigma) in water,

Fixative (3 parts ethanol: 1 part glacial acetic acid)

45% (v/v) glacial acetic acid

1N HCl

Feulgen solution

Equipment

Microscope slides and coverslips

Water bath

Light microscope

10ml vials

Protocol

1. Ensuring that plants have been well watered 24 hrs before collection, collect fresh fast growing roots early in the morning.
2. Place roots in 10ml of a saturated solution of gammaxene (2g in 100ml of boiling water, allowing to cool) for 4 hrs at room temperature in the dark.
3. Remove roots from this solution and place in 10ml of fixative (3 parts ethanol: 1 part glacial acetic acid, freshly prepared). Store in fridge for at least 24 hours
4. Preheat an aliquot of 1N HCl (10ml per sample) to 60 ºC .
5. Place fixed roots into 1N HCl (at 60 ºC) for 8 minutess.
6. Transfer roots into 5ml Feulgens solutions for 30 minutes and store in darkness.
7. Remove the stained part of the root tip to a microscope slide and macerate with the end of a hypodermic needle.
8. Place a small drop of 45% (v/v) glacial acetic acid onto root material.
9. Take a coverslip and gentle place it on the material applying gentle pressure.
10. Seal the edges of the coverslip with a varnish.
11. Examine the root material for cells in metaphase and obtain a chromosome count.

Materials

Sterile ultrapure H_2O

AFLP Applied Biosystems Kit (Kit components detailed in Chapter 6, Tables 6.4, 6.5, 6.6)

10× Promega T4 DNA Ligase Buffer comprises: 50mM Tris-HCl (pH 7.8, 10mM)

$MgCl_2$, 10mM dithiothreitol, 1mM ATP, 25µg bovine serum albinum.

1 x $TE_{0.1}$ buffer (20mM Tris-HCl, 0.1 mM EDTA, pH8.0)

GeneScan-500 Rox size standards (Applied biosystems)

Dye Primer Matrix Standard Kit.

Equipment

0.2ml PCR tubes

Pipettes (P2, P20, P200)

Pipette tips

Microcentrifuge (IEC Micromax)

Thermal cycler (GeneAmp® PCR System 9700)

Protocol

Restriction/Ligation reaction, preselective amplification and selective amplification procedures are described in Chapter 6 section 6.4.5.

Protocol for loading and electrophoresis of samples

1. Prepare the loading buffer mix, by combining the following in a 2 ml tube as:

   180µl formamide

   70µl blue loading dye supplied with size standard

   50µl Gs-500 ROX-labelled size standard.

2. Add 2.5 µl of the loading buffer mix to a 0.2ml PCR tubes.
3. Add the sample to the tube: 0.75µl for FAM-labelled products, 0.80µl for JOE-labelled products and 1.5µl for TAMRA- labelled products.
4. Heat samples to 90°C for 3 minutes, spin down and place on ice.
5. Load samples onto automated sequencer.

Appendices 1.10a-e based on protocols of Lietch *et al.* (1994)

1.10a. Enzyme digestion and slide preparation.

Materials

10× enzyme buffer (40mM citric acid and 60mM sodium citrate (pH 4.8). Dilute 1 part 10× enzyme buffer with 9 parts water to give 1× enzyme buffer.

Digestion enzyme solution: 0.3% (w/v) drilase, 0.3% (w/v) cellulase R10 and 0.3% (w/v) pectolase Y23 (ICN Biomedical Co.) in 1× enzyme buffer.

60% glacial acetic acid in water.

Liquid Nitrogen.

Equipment

1.5ml microcentrifuge tubes

Water bath

Petri dishes

Dissecting microscope

Stereomicroscope

Microscope slides

Round coverslip

Filter paper

Protocol

Enzyme digestion and slide preparation protocol described in Chapter 6 section 6.4.7.1.

1.10b. Probe Generation

Materials

Sterile ultrapure H_2O

10× Nick translation buffer (0.5M Tris-HCl, pH 7.8; 0.05M $MgCl_2$; 0.5 mg/ml bovine serum albumin).

Unlabelled nucleotide mix (0.5mM dCTP, dATP and dGTP in 100ml Tris-HCl, pH 7.5).

DNA for labelling (0.5-1.0 µg/ml).

Digoxigenin-11-dUTP labelled nucleotide mix (0.35mM digoxigenin-11-dUTP, 0.65mM dTTP

0.4 mM biotin-11-dUTP (Sigma)

100 mM ditiothreitol

DNA Polymerase I/Dnase I solution (0.4 units/ µl; Gibco BRL)

0.3M EDTA, pH 8.0)

70% ethanol

1× TE Buffer (10mM tris HCL pH8.0, 1mM EDTA)

Equipment

1.5ml microcentrifuge tubes

Pipettes (P2, P20, P200. P1000)

Pipette tips

Microcentrifuge (IEC Micromax)

Thermal cycler (GeneAmp® PCR System 9700)

Protocol

The probe generation protocol has previously been described in Chapter 6 section 6.4.7.2 .

1.10c. Checking Probe incorporation

Materials

Hybond N^+ membrane

Buffer-1 (0.1 M Tris-HCl, pH 7.5 and 0.15 M NaCl).

Buffer-2 (0.5% (w/v). Blocking reagent (Roche Molecular Biochemicals) in buffer-1), *dissolve by heating at 50 to 70°C for at least 1hr.*

1:500 dilution of anti-biotin conjugated to alkaline phosphate (Vector Laboraties) in buffer-1 or a 1:5000 dilution of anti-digoxigenin conjugated to alkaline phosphatase (Roche Molecular Biochemicals).

Buffer-3 (0.1 M Tris-HCl, pH 9.5, 0.1 M Nacl, 0.05 M $MgCl_2$), NBT/BCIP mixture (Roche Molecular Biochemicals).

Equipment

Petri dishes

Parafilm

Water bath

Water proof container

Protocol

1. Cut a small square of Hybond N^+ membrane and soak in buffer-1 (in a petri dish) for 5 minutess. Blot dry.
2. Load 0.1µg DNA onto membrane and leave to dry for 5 minutess
3. Place the membrane in the Petri dish and add buffer-1 for 1 min., drain and add buffer-2 and incubate at room temperature for 15 minutes.
4. Drain the membrane and add either anti-biotin/alkaline phosphatase or anti digoxigenin/alkaline as appropriate
5. Cover the membrane with parafilm and incubate at 37°C for 30 min.
6. Wash the membrane in excess buffer-1, 3 times for 5 minutes each.
7. Transfer the membrane to buffer-3 for 2 minutes.
8. Add NBT/BCIP mixture, apply fresh cover and incubate in the dark for 10 minutes. Allow blue-black colour to develop. Wash the membrane in water and air dry.
9. A good clear dot indicates strongly labelled probes.

1.10d. Material preparation for *in-situ* hybridisation

Materials

2× SSC (0.3M NaCl, 0.03M sodium citrate)

100 µg/ml of RNase A in 10mM Tris-HCl (pH 7.5), 15mM NaCl.

0.01M HCl

1 µg/ml pepsin (Sigma) in 0.01M HCl.

Fixative (4% (w/v) paraformaldehyde)

Ethanol series, 70%, 90% and 100%.

Equipment

Coplin Jars

Water Bath

Thermometer

Humid chamber (made by placing 2× SSC soaked paper towels in a box that can be incubated in a water bath at 37°C.

Protocol

The protocol for material preparation for *in-situ* hybridisation has previously been described in Chapter 6 section 6.4.7.3.

1.10e. Hybridising the probe to the chromosomes

Materials

Sterile ultrapure H_2O

Formamide

20× SSC (3M NaCl, 0.3M sodium citrate). Dilute to 2× SSC with water.

Probe DNA-dig.

Probe DNA-bio.

Blocking DNA

10% (w/v) SDS. Sodiumdodecyl sulphate in water

Formamide denaturing solution (70% formamide in 2× SSC)

50% (w/v) dextran sulphate

Ethanol series (70%, 90% and 100%)

Stringent wash (20% formamide in 0.1×SSC)

Tween 20 (polyoxyethylenesorbitan monolaurate)

2× SSC/Tween (4 part 20× SSC, 16 parts H_2O and add 0.2% Tween 20)

BSA (5%9w.v) BSA in 4× SSC Tween 20

Cy3-Avidin (Amersham Pharmacia; 10 μg/ml)

Antidigigoxigenin FITC (Roche Biochemicals; 10 μg/ml)

McIlvaine's buffer (*0.1M* citric acid, *0.2M* NA_2HPO_4)

DAPI (10 μg/ml)

Antifade (Vector Laboratories)

Equipment

Coplin Jars

Water Bath

Thermometer

Humid chamber (made by placing 2× SSC soaked paper towels in a box that can be incubated in a water bath at 37°C.

Protocol

The protocol for DNA:DNA hybridisation, post hybridisation washes and probe detection and visualisation are described in Chapter 6, section 6.4.7.4.

	MC1	MC2	MC3	MC4	MC5	MC6	MC7	MC8	MC9	MC10	MC11	MC12
Papaver bracteatum J-123	1	1	0	1	1	0	2	1	1	1	1	1
Papaver bracteatum J-157	1	1	0	1	1	0	2	1	1	1	1	1
Papaver californicum J-170	0	0	0	0	1	1	0	1	0	1	0	0
Papaver commutatum J-101	1	1	0	1	1	1	0	1	1	1	0	1
Papaver commutatum J-161	1	1	0	1	1	1	0	1	1	1	0	1
Papaver confine J-174	1	1	0	1	1	1	0	1	1	0	0	0
Papaver croceum J-148	0	0	0	0	1	1	2	1	0	0	0	0
Papaver croceum J-104	0	0	0	0	1	1	2	1	0	0	0	0
Papaver croceum J-015	0	0	0	0	1	1	2	1	0	0	0	0
Papaver decaisnii J-021	1	1	0	1	1	0	0	1	1	1	1	0
Papaver dubium J-024	1	1	0	1	1	1	0	1	1	0	0	0
Papaver dubium J-162	1	1	0	1	1	1	0	1	1	0	0	0
Papaver dubium J-164	1	1	0	1	1	1	0	1	1	0	0	0
Papaver glaucum J-089	1	1	0	1	1	1	0	1	1	0	1	1
Papaver glaucum J-131	1	1	0	1	1	1	0	1	1	0	1	1
Papaver hybridum J-167	0	1	0	0	0	0	0	1	0	0	0	1
Papaver lateritium J-011	1	0	0	1	1	1	2	1	1	0	0	0
Papaver lateritium J-116	1	0	0	1	1	1	2	1	1	0	0	0
Papaver lateritium J-116	1	0	0	1	1	1	2	1	1	0	0	0
Papaver lateritium J-181	1	0	0	1	1	1	2	1	1	1	0	0
Papaver litwinowii	1	1	0	1	1	1	0	1	1	1	0	0
Papaver macrostomum J-159	1	1	0	1	1	1	2	1	1	1	0	0
Papaver miyabeanum J-186	0	0	0	0	1	1	2	1	0	0	0	0
Papaver miyabeanum J-019	0	0	0	0	1	1	2	1	0	0	0	0
Papaver nudicaule ssp. *nudicaule* J-086	0	0	0	0	1	1	2	1	0	0	0	0
Papaver orientale J-014	1	1	0	1	1	0	2	1	1	1	1	1
Papaver orientale J-093	1	1	0	1	1	0	2	1	1	1	1	1
Papaver orientale J-135	1	1	0	1	1	0	2	1	1	0	1	1

	MC1	MC2	MC3	MC4	MC5	MC6	MC7	MC8	MC9	MC10	MC11	MC12
Papaver orientale J-179	1	1	0	1	1	0	2	1	1	0	1	1
Papaver pavonium J-138	1	1	0	0	0	0	0	1	0	0	0	1
Papaver pilosum J-081	1	0	0	1	1	1	2	0	1	0	0	0
Papaver pilosum J-182	1	0	0	1	1	1	2	0	1	0	0	0
Papaver pilosum J-184	1	0	0	1	1	1	2	0	1	0	0	0
Papaver pseudo-orientale J-039	1	1	0	1	1	0	2	1	1	1	1	1
Papaver pseudo-orientale J-040	1	1	0	1	1	0	2	1	1	1	1	1
Papaver pseudo-orientale J-137	1	1	0	1	1	0	2	1	1	1	1	1
Papaver pseudo-orientale J-139	1	1	0	1	1	0	2	1	1	1	1	1
Papaver pumbifidum	1	1	1	1	1	1	0	1	1	1	0	0
Papaver radicatum spp *subglabosum* J-094	0	0	0	0	1	1	2	1	0	0	0	0
Papaver radicatum ssp. *ovatilobum* J-085	0	0	0	0	1	1	2	1	0	0	0	0
Papaver rhoeas (cedric morris) J-090	1	1	1	1	1	1	0	1	1	1	0	1
Papaver rhoeas J-175	1	1	1	1	1	1	0	1	1	1	0	1
Papaver rupifragum J-017	1	0	0	1	1	1	2	1	1	0	0	0
Papaver rupifragum J-099	1	0	0	1	1	1	2	1	1	0	0	0
Papaver somniferum J-016	1	0	1	1	1	1	0	1	1	1	1	0
Papaver somniferum J-052	1	1	0	1	1	1	0	1	1	1	1	1
Papaver somniferum J-068	1	1	0	1	1	1	0	1	1	1	1	1
Papaver somniferum J-126	1	0	1	1	1	1	0	1	1	1	1	0
Papaver somniferum J-142	1	0	0	1	1	1	0	1	1	1	1	0
Papaver somniferum J-166	1	1	0	1	1	1	0	1	1	1	0	0
Papaver somniferum J-176	1	0	0	1	1	1	0	1	1	1	1	0
Papaver spicatum J-180	1	0	0	1	1	1	2	0	1	0	0	0
Argemone mexicana J-191	0	1	1	0	1	0	0	1	1	0	0	0

2.2 Binary matrix of biogenetic pathways (BP) responsible for the biosynthesis of alkaloids characteristic to each species used in the molecular analysis. For a description of each group see text. No information on alkaloid production is indicated by ?

Genus and species		BP1a	BP1b	BP2a	BP2b	BP2c	BP3a	BP3b	BP3c	BP3d	BP3e	BP3f	BP4a	BP4b	Source
Papaver aculeatum	J-131	0	0	0	0	0	0	0	1	0	1	0	0	0	Maturova *et al.*, 1966; Phillipson, *et al.*, 1981; Preininger. 1986
Papaver aculeatum	J-151	0	0	0	0	0	0	0	1	0	1	0	0	0	Maturova *et al.*, 1966; Phillipson, *et al.*, 1981; Preininger. 1986
Papaver aculeatum	J-158	0	0	0	0	0	0	0	1	0	1	0	0	0	Maturova *et al.*, 1966; Phillipson, *et al.*, 1981; Preininger. 1986
Papaver albiflorum	J-100	0	0	0	1	0	1	0	1	0	1	0	0	1	Preininger, 1986
Papaver alpinum spp. *rheaticum*	J-150	1	1	1	1	0	1	0	1	1	1	0	1	0	Phillipson, *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Papaver alpinum spp. *alpinum*	J-102	1	1	1	1	0	1	0	1	1	1	0	1	0	Phillipson, *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Papaver anonalum	J-078	1	1	1	1	0	1	0	1	1	1	0	1	0	Preininger *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Papaver anomalum	J-106	1	1	1	1	0	1	0	1	1	1	0	1	0	Preininger *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Papaver apulum	J-084	0	0	0	0	0	1	0	0	0	1	0	0	0	Preininger, 1986; Tetenyi, 1993
Papaver apulum	J-084	0	0	0	0	0	1	0	0	0	1	0	0	0	Preininger, 1986; Tetenyi, 1993
Papaver argemone	J-153	0	0	0	0	0	1	0	0	0	1	0	0	0	Preininger, 1986; Tetenyi, 1993
Papaver armeniacum ssp. *armeniacum*	J-082	0	0	1	1	0	0	0	1	1	1	0	1	1	Phillipson, *et al.*, 1981; Preininger, 1986; Sariyar, 1983
Papaver armeniacum ssp. *armeniacum*	J-087	0	0	1	1	0	0	0	1	1	1	0	1	1	Phillipson, *et al.*, 1981; Sariyar, 1983; Preininger, 1986
Papaver armeniacum ssp. *armeniacum*	J-103	0	0	1	1	0	0	0	1	1	1	0	1	1	Phillipson, *et al.*, 1981; Sariyar, 1983; Preininger, 1986

Continued

Genus and species		BP1a	BP1b	BP2a	BP2b	BP2c	BP3a	BP3b	BP3c	BP3d	BP3e	BP3f	BP4a	BP4b	Source
Papaver armeniacum ssp. *armeniacum*	J-154	0	0	1	1	0	0	0	1	1	1	0	1	1	Phillipson, *et al.*, 1981; Preininger, 1981; Sariyar, 1983
Papaver armeniacum ssp. *microstigmum*	J-095	0	0	1	1	0	0	0	1	1	1	0	1	1	Phillipson, *et al.*, 1981; Sariyar, 1983; Preininger, 1986
Papaver armeniacum ssp.*microstigmum*	J-115	0	0	1	1	0	0	0	1	1	1	0	1	1	Phillipson, *et al.*, 1981; Sariyar, 1983; Preininger, 1986
Papaver atlanticum	J-156	0	0	0	1	0	1	0	1	1	1	0	0	0	Preininger, 1986; Tetenyi, 1993
Papaver atlanticum	J-178	0	0	0	1	0	1	0	1	1	1	0	0	0	Preininger, 1986; Tetenyi, 1993
Papaver bracteatum	J-028	0	0	1	1	0	1	0	1	1	1	0	1	1	Preininger, 1986; Tetenyi, 1986; Hubert *et al.*, (1994)
Papaver bracteatum	J-031	0	0	1	1	0	1	0	1	1	1	0	1	1	Preininger, 1986; Tetenyi, 1986; Hubert *et al.*, (1994)
Papaver californicum	J-170	0	0	0	0	0	1	0	1	0	1	0	0	0	Preininger, 1986; Tetenyi, 1986;
Papaver commutatum	J-101	0	0	0	1	0	1	0	1	1	1	0	0	0	Preininger, 1986
Papaver commutatum	J-161	0	0	0	1	0	1	0	1	1	1	0	0	0	Preininger, 1986
Papaver croceum	J-015	1	1	1	1	0	1	0	1	1	1	0	1	0	Preininger *et al.*, 1981; Preininger, 1986;Tetenyi, 1993
Papaver croceum	J-086	1	1	1	1	0	1	0	1	1	1	0	1	0	Preininger *et al.*, 1981; Preininger, 1986;Tetenyi, 1993
Papaver croceum	J-104	1	1	1	1	0	1	0	1	1	1	0	1	0	Preininger *et al.*, 1981; Preininger, 1986;Tetenyi, 1993
Papaver croceum	J-104	1	1	1	1	0	1	0	1	1	1	0	1	0	Preininger *et al.*, 1981; Preininger, 1986;Tetenyi, 1993
Papaver croceum	J-148	1	1	1	1	0	1	0	1	1	1	0	1	0	Preininger *et al.*, 1981; Preininger, 1986;Tetenyi, 1993
Papaver dubium ssp. *dubium*	J-024	0	0	1	1	0	1	0	1	1	1	0	0	0	Preininger *et al.*, 1981; Preininger, 1986;Tetenyi, 1993
Papaver dubium ssp. *dubium*	J-162	0	0	1	1	0	1	0	1	1	1	0	0	0	Preininger *et al.*, 1981; Preininger, 1986;Tetenyi, 1993
Papaver dubium ssp. *lecoquii*	J-164	0	0	1	1	0	1	0	1	1	1	0	0	0	Preininger *et al.*, 1981; Preininger, 1986;Tetenyi, 1993
Papaver decaisnii	J-021	0	0	0	0	0	1	0	1	1	1	0	1	1	Preininger *et al.*, 1981 Preininger, 1986;Tetenyi, 1993

Continued

Genus and species		BP1a	BP1b	BP2a	BP2b	BP2c	BP3a	BP3b	BP3c	BP3d	BP3e	BP3f	BP4a	BP4b	Source
Papaver glaucum	J-177	0	0	0	0	0	1	0	1	0	1	0	0	0	Preininger *et al.*, 1981 Preininger, 1986;Tetenyi, 1993
Papaver glaucum	J-189	0	0	0	0	0	1	0	1	0	1	0	0	0	Preininger *et al.*, 1981 Preininger, 1986;Tetenyi, 1993
Papaver gracile	J-130	0	0	1	1	0	1	0	1	1	1	1	1	0	Preininger *et al.*, 1981 Preininger, 1986;Tetenyi, 1993
Papaver hybridum	J-167	0	0	0	0	0	1	0	0	1	1	0	1	0	Preininger, 1986; Tetenyi, 1993
Papaver macrostomum	J-160	0	0	0	1	0	1	0	1	1	1	0	0	0	Preininger *et al.*, 1981; Preininger, 1986;Tetenyi, 1993
Papaver miyabeanum	J-019	1	1	1	1	0	1	0	1	1	1	0	1	0	Phillipson, *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Papaver miyabeanum	J-186	1	1	1	1	0	1	0	1	1	1	0	1	0	Phillipson, *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Papaver orientale	J-011	0	0	1	1	0	1	0	1	0	1	0	1	1	Preininger, 1986; Tetenyi, 1986; Hubert *et al.*, (1994)
Papaver orientale	J-035	0	0	1	1	0	1	0	1	0	1	0	1	1	Preininger, 1986; Tetenyi, 1986; Hubert *et al.*, (1994)
Papaver orientale	J-135	0	0	1	1	0	1	0	1	0	1	0	1	1	Preininger, 1986; Tetenyi, 1986; Hubert *et al.*, (1994)
Papaver orientale	J-179	0	0	1	1	0	1	0	1	0	1	0	1	1	Preininger, 1986; Tetenyi, 1986; Hubert *et al.*, (1994)
Papaver pavonium	J-138	0	0	0	0	0	1	0	1	1	1	0	1	0	Preininger *et al.*, 1981; Preininger, 1986;Tetenyi, 1993
Papaver pilosum	J-081	0	0	1	1	0	1	0	1	1	1	0	1	0	Oztekin *et al.*, 1985; Preininger, 1986;Tetenyi, 1993
Papaver pilosum	J-182	0	0	1	1	0	1	0	1	1	1	1	1	1	Phillipson, *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Papaver pseudo-orientale	J-014	0	0	0	0	1	1	0	0	1	1	0	1	1	Preininger, 1986; Tetenyi, 1986; Hubert *et al.*, (1994)
Papaver pseudo-orientale	J-039	0	0	0	0	1	1	0	0	1	1	0	1	1	Preininger, 1986; Tetenyi, 1986; Hubert *et al.*, (1994)
Papaver pseudo-orientale	J-080	0	0	0	0	1	1	0	0	1	1	0	1	1	Preininger, 1986; Tetenyi, 1986; Hubert *et al.*, (1994)
Papaver pseudo-orientale	J-093	0	0	0	0	1	1	0	0	1	1	0	1	1	Preininger, 1986; Tetenyi, 1986; Hubert *et al.*, (1994)

Continued

Genus and species		BP1a	BP1b	BP2a	BP2b	BP2c	BP3a	BP3b	BP3c	BP3d	BP3e	BP3f	BP4a	BP4b	Source
Papaver pseudo-orientale	J-139	0	0	0	0	1	1	0	0	1	1	0	1	1	Preininger, 1986; Tetenyi, 1986; Hubert *et al.*, (1994)
Papaver pumbifidum	J-169	?	?	?	?	?	?	?	?	?	?	?	?	?	
Papaver radicatum spp *subglabosum*	J-094	1	1	1	1	0	1	0	1	1	1	0	1	0	Phillipson, *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Papaver rhoeas	J-090	0	0	0	1	0	1	0	1	1	1	1	0	1	Preininger, 1986; Kalav & Sariyar, 1989; Tetenyi, 1993
Papaver rhoeas	J-147	0	0	0	1	0	1	0	1	1	1	1	0	1	Preininger, 1986; Kalav & Sariyar, 1989; Tetenyi, 1993
Papaver rupifragum	J-017	0	0	0	1	0	1	0	1	0	1	0	0	0	Preininger *et al.*, 1981; Preininger, 1986;Tetenyi, 1993
Papaver somniferum	J-016	0	0	1	1	0	1	0	1	1	1	1	1	1	Phillipson, *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Papaver somniferum	J-052	0	0	1	1	0	1	0	1	1	1	1	1	1	Phillipson, *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Papaver somniferum	J-068	0	0	1	1	0	1	0	1	1	1	1	1	1	Phillipson, *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Papaver somniferum	J-142	0	0	1	1	0	1	0	1	1	1	1	1	1	Phillipson, *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Papaver somniferum	J-166	0	0	1	1	0	1	0	1	1	1	1	1	1	Phillipson, *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Papaver spicatum	J-180	0	0	1	1	0	1	0	1	1	1	1	1	1	Phillipson, *et al.*, 1981; Preininger. 1986; Tetenyi, 1993
Argemone mexicana	J-191	1	0	0	0	1	1	0	1	1	0	0	0	0	Phillipson, *et al.*, 1981; Preininger. 1986

2.3 Nei & Li genetic distances for accessions used in Neighbour-Joining analysis.

	031	035	037	008	054	055	062	070	018
Papaver bracteatum J-031	1								
Papaver orientale J-035	0.7308	1							
Papaver pseudo-orientale J-037	0.6792	0.8772	1						
Papaver orientale J-008	0.359	0.5116	0.4545	1					
Papaver pseudo-orientale 054	0.2	0.2963	0.3636	0.3902	1				
Papaver pseudo-orientale 055	0.2264	0.386	0.4483	0.4091	0.8727	1			
Papaver pseudo-orientale 062	0.2642	0.386	0.3793	0.4091	0.8	0.8276	1		
Papaver orientale J-070	0.1667	0.2692	0.3019	0.4103	0.84	0.7925	0.8302	1	
Papaver pseudo-orientale J-018	0.2632	0.2857	0.2326	0.2759	0.45	0.3721	0.4186	0.4211	1
Papaver pseudo-orientale J-091	0.2	0.1111	0.1455	0.2927	0.3462	0.4364	0.5091	0.48	0.2
Papaver pseudo-orientale J-093	0.16	0.1111	0.1455	0.2927	0.4615	0.4727	0.4727	0.48	0.25
Papaver pseudo-orientale J-096	0.2222	0.1724	0.1356	0.2667	0.2857	0.3729	0.4068	0.4074	0.2273
Papaver somniferum J-016	0.0952	0.1739	0.1702	0.2424	0.1818	0.1702	0.2128	0.2381	0.125
Papaver orientale J-137	0.1667	0.1538	0.1132	0.2564	0.4	0.3396	0.4528	0.4167	0.3158
Papaver pseudo-orientale J-108	0.1633	0.1132	0.0741	0.15	0.2353	0.2593	0.3333	0.3673	0.1538
Papaver orientale J-121	0.125	0.0769	0.1132	0.1538	0.32	0.3019	0.3019	0.2917	0.1579
Papaver bracteatum J-020	0.8163	0.6792	0.6296	0.25	0.3137	0.3333	0.3704	0.2857	0.359
Papaver orientale J-122	0.0851	0.1176	0.1923	0.1579	0.2857	0.3077	0.2308	0.2553	0.1622
Papaver orientale J-123	0.1277	0.0784	0.0769	0.1579	0.2857	0.2692	0.2692	0.2553	0.1622
Papaver orientale J-132	0.12	0.0741	0.1091	0.1951	0.3846	0.4	0.4	0.36	0.15
Papaver orientale J-133	0.1852	0.1379	0.1695	0.1778	0.3929	0.3729	0.4407	0.3704	0.2273
Papaver pseudo-orientale J-134	0.1455	0.1356	0.1333	0.2609	0.3509	0.4	0.4333	0.3636	0.1778
Papaver orientale J-135	0.7059	0.9818	0.8571	0.4762	0.3019	0.3929	0.3929	0.2745	0.2927
Papaver pseudo-orientale J-139	0.1633	0.1509	0.1481	0.2	0.2745	0.3333	0.3704	0.4082	0.1538
Papaver bracteatum J-028	0.902	0.7636	0.7143	0.381	0.2642	0.25	0.2857	0.1961	0.2927
Papaver bracteatum J-029	0.9388	0.7925	0.7037	0.4	0.2353	0.2593	0.2963	0.2041	0.2564
Papaver bracteatum J-030	0.92	0.7778	0.6909	0.3902	0.2692	0.2909	0.3273	0.24	0.25
Papaver orientale J-140	0.3137	0.3636	0.3571	0.5238	0.4906	0.5	0.6429	0.549	0.2439
Papaver pseudo- orientale J-040	0.2727	0.3333	0.3673	0.3429	0.5652	0.4898	0.5306	0.5455	0.5294
Papaver bracteatum J-155	0.8	0.6441	0.6	0.3043	0.2456	0.2667	0.3667	0.2545	0.2667
Papaver pseudo-orientale J-034	0.72	0.9259	0.8364	0.439	0.3077	0.3273	0.3636	0.28	0.35

	091	093	096	016	137	108	121	020	122
Papaver bracteatum J-031									
Papaver orientale J-035									
Papaver pseudo-orientale J-037									
Papaver orientale J-008									
Papaver pseudo-orientale 054									
Papaver pseudo-orientale 055									
Papaver pseudo-orientale 062									
Papaver orientale J-070									
Papaver pseudo-orientale J-018									
Papaver pseudo-orientale J-091	1								
Papaver pseudo-orientale J-093	0.8462	1							
Papaver pseudo-orientale J-096	0.7857	0.6786	1						
Papaver somniferum J-016	0.3182	0.3182	0.3333	1					
Papaver orientale J-137	0.76	0.72	0.6667	0.381	1				
Papaver pseudo-orientale J-108	0.6275	0.549	0.6182	0.3721	0.7347	1			
Papaver orientale J-121	0.6	0.64	0.6296	0.2381	0.5833	0.6122	1		
Papaver bracteatum J-020	0.1569	0.1176	0.1818	0.1395	0.1224	0.12	0.0816	1	
Papaver orientale J-122	0.5306	0.5714	0.4528	0.2927	0.5957	0.625	0.6809	0.125	1
Papaver orientale J-123	0.5714	0.6122	0.6038	0.3415	0.6383	0.625	0.8085	0.125	0.6957
Papaver orientale J-132	0.6154	0.6538	0.6071	0.3182	0.64	0.6275	0.76	0.1176	0.6939
Papaver orientale J-133	0.6071	0.6429	0.5667	0.3333	0.6296	0.6182	0.6296	0.1455	0.566
Papaver pseudo-orientale J-134	0.7368	0.7018	0.7213	0.3673	0.7273	0.7143	0.7273	0.1071	0.5926
Papaver orientale J-135	0.1132	0.1132	0.1754	0.1778	0.1569	0.1154	0.0784	0.6923	0.12
Papaver pseudo-orientale J-139	0.7451	0.6275	0.7273	0.3256	0.6939	0.76	0.6939	0.16	0.5833
Papaver bracteatum J-028	0.1509	0.1509	0.1754	0.1333	0.1569	0.1154	0.0784	0.8077	0.08
Papaver bracteatum J-029	0.1569	0.1176	0.1818	0.1395	0.1224	0.12	0.0816	0.84	0.0833
Papaver bracteatum J-030	0.1923	0.1538	0.2143	0.1364	0.16	0.1176	0.08	0.8235	0.0816
Papaver orientale J-140	0.6792	0.6038	0.5614	0.3111	0.549	0.4615	0.5098	0.3077	0.4
Papaver pseudo- orientale J-040	0.2609	0.3043	0.32	0.2105	0.2727	0.2222	0.1818	0.4	0.186
Papaver bracteatum J-155	0.2456	0.1754	0.2623	0.0408	0.2182	0.1786	0.1455	0.75	0.1481
Papaver pseudo-orientale J-034	0.0769	0.1154	0.1786	0.1818	0.16	0.1176	0.08	0.6667	0.1224

	123	132	133	134	135	139	028	029	030
Papaver bracteatum J-031									
Papaver orientale J-035									
Papaver pseudo-orientale J-037									
Papaver orientale J-008									
Papaver pseudo-orientale 054									
Papaver pseudo-orientale 055									
Papaver pseudo-orientale 062									
Papaver orientale J-070									
Papaver pseudo-orientale J-018									
Papaver pseudo-orientale J-091									
Papaver pseudo-orientale J-093									
Papaver pseudo-orientale J-096									
Papaver somniferum J-016									
Papaver orientale J-137									
Papaver pseudo-orientale J-108									
Papaver orientale J-121									
Papaver bracteatum J-020									
Papaver orientale J-122									
Papaver orientale J-123	1								
Papaver orientale J-132	0.7755	1							
Papaver orientale J-133	0.6415	0.75	1						
Papaver pseudo-orientale J-134	0.7037	0.7368	0.7541	1					
Papaver orientale J-135	0.08	0.0755	0.1404	0.1379	1				
Papaver pseudo-orientale J-139	0.625	0.5882	0.6182	0.75	0.1538	1			
Papaver bracteatum J-028	0.08	0.0755	0.1754	0.1034	0.7407	0.1538	1		
Papaver bracteatum J-029	0.0833	0.0784	0.1455	0.1071	0.7692	0.16	0.9615	1	
Papaver bracteatum J-030	0.0816	0.1154	0.1786	0.1404	0.7547	0.1961	0.9434	0.9804	1
Papaver orientale J-140	0.48	0.4528	0.4561	0.5517	0.3704	0.5385	0.3333	0.3462	0.3396
Papaver pseudo- orientale J-040	0.1395	0.1739	0.16	0.1961	0.3404	0.2222	0.383	0.3111	0.3043
Papaver bracteatum J-155	0.1852	0.2105	0.2295	0.1935	0.6207	0.2143	0.7586	0.75	0.7719
Papaver pseudo-orientale J-034	0.0816	0.0769	0.1429	0.1053	0.9057	0.1176	0.717	0.7451	0.7308

	140	040	155	034
Papaver bracteatum J-031				
Papaver orientale J-035				
Papaver pseudo-orientale J-037				
Papaver orientale J-008				
Papaver pseudo-orientale 054				
Papaver pseudo-orientale 055				
Papaver pseudo-orientale 062				
Papaver orientale J- 070				
Papaver pseudo-orientale J-018				
Papaver pseudo-orientale J-091				
Papaver pseudo-orientale J-093				
Papaver pseudo-orientale J-096				
Papaver somniferum J-016				
Papaver orientale J-137				
Papaver pseudo-orientale J-108				
Papaver orientale J-121				
Papaver bracteatum J-020				
Papaver orientale J-122				
Papaver orientale J-123				
Papaver orientale J-132				
Papaver orientale J-133				
Papaver pseudo-orientale J-134				
Papaver orientale J-135				
Papaver pseudo-orientale J-139				
Papaver bracteatum J-028				
Papaver bracteatum J-029				
Papaver bracteatum J-030				
Papaver orientale J-140	1			
Papaver pseudo- orientale J-040	0.4681	1		
Papaver bracteatum J-155	0.3103	0.3529	1	
Papaver pseudo-orientale J-034	0.3396	0.3478	0.6316	1

2.4 Eigenvalues generated from principle coordinate analysis using AFLP data

Number	Eigenvalues	% of variance	cumulative %
1	4.35049	34.07067	34.07%
2	1.27151	10.73291	44.80%
3	0.54899	5.25645	50.06%
4	0.46925	4.652	54.71%
5	0.42515	4.31776	59.03%
6	0.37082	3.90597	62.94%
7	0.24408	2.9453	65.88%
8	0.21964	2.76009	68.64%
9	0.18783	2.51892	71.16%
10	0.16766	2.36605	73.53%
11	0.11856	1.99392	75.52%
12	0.09905	1.84606	77.37%
13	0.07913	1.69505	79.06%
14	0.07415	1.65727	80.72%
15	0.06249	1.5689	82.29%
16	0.05696	1.52697	83.81%
17	0.04027	1.4005	85.21%
18	0.0288	1.31354	86.53%
19	0.02386	1.27614	87.80%
20	0.00768	1.15349	88.96%
21	0.0052	1.13464	90.09%
22	0	1.08265	91.18%
23	-0.00166	1.04252	92.22%
24	-0.00696	1.00701	93.22%
25	-0.01164	0.99681	94.22%
26	-0.01299	0.92588	95.15%
27	-0.02235	0.82959	95.98%
28	-0.03505	0.75754	96.73%
29	-0.04456	0.71672	97.45%
30	-0.04994	0.61568	98.07%
31	-0.06327	0.55925	98.63%
32	-0.07072	0.48255	99.11%
33	-0.08083	0.44249	99.55%
34	-0.08612	0.25806	99.81%
35	-0.11045	0.19064	100.00%
36	-0.11935	0	100.00%
37	-0.1445	0	100.00%

Appendix 2.5 Eigenvectors generated from principle coordinate analysis using AFLP data

	Axis 1	Axis 2	Axis 3	Axis 4
Papaver bracteatum J-031	-0.3977	0.2656	-0.0599	0.0043
Papaver orientale J-035	-0.4352	0.0798	0.0844	-0.0379
Papaver pseudo-orientale J-037	-0.4149	0.0336	0.0376	-0.0555
Papaver orientale J-008	-0.1425	-0.1082	0.3039	-0.1821
Papaver pseudo-orientale 054	0.0407	-0.3861	-0.1042	-0.0228
Papaver pseudo-orientale 055	0.0242	-0.3271	-0.1056	-0.1316
Papaver pseudo-orientale 062	0.0294	-0.2885	-0.0938	-0.1382
Papaver orientale J- 070	0.0966	-0.3765	-0.0438	-0.0824
Papaver pseudo-orientale J-018	-0.0863	-0.281	-0.1052	0.3421
Papaver pseudo-orientale J-091	0.3835	0.0448	-0.0204	-0.1523
Papaver pseudo-orientale J-093	0.4021	-0.0284	0.0102	-0.0757
Papaver pseudo-orientale J-096	0.3433	0.1058	0.0002	-0.049
Papaver somniferum J-016	0.2065	0.0584	0.453	0.243
Papaver orientale J-137	0.3811	0.0512	0.0157	0.0269
Papaver pseudo-orientale J-108	0.411	0.1718	-0.015	0.0827
Papaver orientale J-121	0.4498	0.1484	-0.094	0.0175
Papaver bracteatum J-020	-0.4017	0.0937	-0.1149	0.1243
Papaver orientale J-122	0.389	0.1527	-0.0484	0.1396
Papaver orientale J-123	0.4442	0.1951	-0.0468	0.0973
Papaver orientale J-132	0.4342	0.077	-0.1077	0.0144
Papaver orientale J-133	0.3631	0.1171	-0.1201	0.0107
Papaver pseudo-orientale J-134	0.4294	0.103	-0.0135	-0.0594
Papaver orientale J-135	-0.4271	0.0697	0.0792	-0.0242
Papaver pseudo-orientale J-139	0.3803	0.1653	-0.0242	-0.0711
Papaver bracteatum J-028	-0.4303	0.1622	0.0021	0.0434
Papaver bracteatum J-029	-0.4351	0.1927	0.0142	-0.0034
Papaver bracteatum J-030	-0.4111	0.1808	-0.0187	-0.0565
Papaver orientale J-140	0.1393	-0.0409	0.1048	-0.1933
Papaver pseudo- orientale J-040	-0.1034	-0.3262	0.0062	0.1575
Papaver bracteatum J-155	-0.3349	0.2167	-0.2059	-0.03
Papaver pseudo-orientale J-034	-0.4355	0.0646	0.0718	0.0559

Appendix 3

Peer reviewed publications

Using AFLP markers for species differentiation and assessment of genetic variability of *in vitro* cultured *Papaver bracteatum* (section *Oxytona*) *In Vitro* Cell. Dev. Biol. Plant 38:300-307