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Delimitation and diversity of the genus *Badula* Juss. (Primulaceae)- rare endemic trees of the Mascarene archipelago

Thesis submitted for the degree of Doctor of Philosophy

February 2010

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Declaration

I, Ruth Emily Bone, declare that this thesis has not been submitted as an exercise for a degree at this or any other university. This thesis includes the unpublished work of others, in the form of contributed DNA sequences. Wherever included, the contributor is acknowledged beneath each chapter heading. All data analyses and writing are my own work. The Library may lend or copy this thesis upon request.

Summary

The western Indian Ocean Mascarene archipelago is a trio of geologically young volcanic islands that have never been connected to a continent or continental fragments. The islands are biologically diverse, and 72 % of the 885 native angiosperm species are endemic. *Badula* Juss. (Primulaceae) is one of the few genera currently treated as a Mascarene endemic with a distribution across all three of the Mascarene islands. Consequently it provides an ideal opportunity to investigate patterns of colonization in the region. However the endemic status of *Badula* is questionable because no clear distinction can be made between this group and *Oncostemum* Juss., endemic to Madagascar and the Comoro Islands. Furthermore, varying levels of morphological differentiation are evidenced within *Badula*, meaning that the delimitation of some species is also unclear, a problem exacerbated by the extreme rarity of several Mauritius and Rodrigues species and the consequent paucity of material available for description. The main aims of this research were: 1) to determine the generic delimitation of *Badula* relative to the allied genus *Oncostemum*, and assess the status of *Badula* as a Mascarene endemic; 2) to infer patterns of colonization by *Badula* among the islands; 3) to investigate whether the species delimitations of the current classification of *Badula* are supported by molecular data; 4) to determine whether plant species considered so rare that they are termed 'the living dead' have low genetic diversity.

I sequenced nuclear (ITS) and plastid (*trnS-trnG, trnG, accD, psbA-trnH*) regions of the genome for multiple accessions of all species of *Badula* and ~14 *Oncostemum* taxa, and carried out phylogenetic analyses (Bayesian and parsimony methods) of partitioned and combined data. I used AFLP markers to further elucidate inter-specific relationships within *Badula* (alone, and in combination with DNA sequences). I generated parsimony and neighbour-joining (NJ) trees from these data and distance matrices for analysis by principle coordinates analysis (PCoA). To estimate genetic diversity measures, I genotyped every individual of the Critically Endangered *Badula* species of Mauritius with AFLP markers. I calculated genetic diversity (phenotypic diversity; H_{PH}) and made multiple comparisons of these 'species-level' genetic diversity estimates. I used the analysis of molecular variance (AMOVA) technique to estimate levels of genetic differentiation among and within these rare species, and further characterized intra-specific diversity by NJ and PCoA methods. Because DNA sequences of the CR species from Rodrigues revealed considerable intra-

specific variation, I complemented the AFLP data with additional information from a survey of plastid genome regions (*rpoB-trnC, 5'trnL-trnT, rpl16*) to identify haplotypes.

Badula was shown to be monophyletic in relation to *Oncostemum* (with strong support; 100 BP, 1.0 PP), although nested within an *Oncostemum* clade. Further work is needed to elucidate the relationship of *Badula* to the *Oncostemum* clades, and the taxonomic rank of the *Badula* clade may change as a consequence of further studies. Nevertheless, this work has found strong evidence for the distinction of *Badula* as an evolutionarily unique unit, endemic to the Mascarene islands. In general, the analysis methods revealed geographical groupings among *Badula* species, by island. The results indicate the existence of an isolated single-species clade that represents Rodrigues (*B. balfouriana*), a group of morphologically and ecologically varied, and clearly distinguished species in Mauritius, and the contrasting clade of La Réunion taxa, where the descendants of a common ancestor have diversified into a variety of apparently narrowly endemic species and other morphologically intermediate and more widespread forms, that may suggest incipient speciation on this geologically young island. The Rodrigues and La Réunion clades both received moderate to strong support, but the monophyly of the Mauritius taxa was ambiguous, or had low support, and further studies are required. Within the geographical groups, thirteen *Badula* taxa were resolved as taxon-specific clades and support the current classification (Coode, 1981). Genetic diversity levels varied among the CR species from Mauritius, with *B. crassa* showing significantly lower genetic diversity (H_{PH} 0.091) than *B. ovalifolia* (H_{PH} 0.159), *B. reticulata* (H_{PH} 0.143) and a more widespread congener, *B. insularis* (H_{PH} 0.164). *B. balfouriana* from Rodrigues was shown to have the highest genetic diversity of all CR species surveyed (H_{PH} 0.292) and five haplotypes were identified among the seven wild plants (four samples from *ex situ* collections were identified as a single haplotype). These high levels were interpreted as evidence of relictual diversity among samples of these relatively long-lived, and presumably once more widespread perennial species, and were also restricted to those species that continue to show signs of reproductive success (fruit set and/ or seedling recruitment). Without intervention, genetic diversity is expected to decline over subsequent generations.

Badula represents one of the most diverse plant groups in the Mascarene archipelago and is an ideal candidate for further studies of angiosperm speciation and diversity in these islands, but without concerted efforts to protect what remains, many of these species face irrecoverable decline.

Acknowledgements

I have been very fortunate to meet so many of the people responsible for the discovery, protection and restoration of the flora and fauna of the Mascarene islands. Among them are many friends and colleagues, of whom I have asked countless favours during the course of this research work. In particular I would like to thank Claudia Baider and Vincent Florens for supporting me in all aspects of this work. For assistance in the field during prospecting and collecting work I would also like to thank Kersley Pynee of the Mauritius Herbarium; Gabriele D'Argent and Jean-Claude Sevathian of the Mauritius Wildlife Foundation; Mario Allett and staff of the Petrin office of the National Parks and Conservation Service; Richard Payandee; Frederic Robert and his mountaineering team at Incentive Partners Ltd. (Mauritius) and Yan de Maroussem of Yanature for assistance in reaching the summit of Mt. Le Morne; Dominique Strasberg who provided assistance in the field in La Réunion and who, with Christophe Thébaud and Joeri Strijk, contributed leaf and DNA samples and sequences for some of the La Réunion taxa. I benefited hugely from a visit to the California Academy of Sciences (CAS) in 2007 and would especially like to thank Peter Fritsch for his encouragement and support throughout this work, for his contribution of *Oncostemum* samples and ITS sequences and for giving me the opportunity to work in the Centre for Comparative Genomics at CAS. Thanks to Boni Cruz and Brian Simison, also of CAS, for laboratory training and support. Thanks to Gerry Douglas at TEAGASC, Kinsealy Co. Dublin, for micropropagation efforts and use of facilities, and Peter Krogstrup of Copenhagen botanical garden for provision of material from cultivated collections. Thanks also to Lori Lach & Christopher Kaiser for helpful discussions on insect-plant interactions.

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and the Labour, Victoire and Martin families in Dublin and Mauritius who made me feel so welcome in both places. Most of all, thank you to Gina.

In December 2003 I arrived in Mauritius to take up the post of Horticulture Co-ordinator for the Mauritian Wildlife Foundation. I was based at the nursery on Ile aux Aigrettes, a low corallitic islet in the southeast lagoon that bears one of the last coastal forests in Mauritius. During the first meeting with my new colleagues I was given a list of 160 Critically Endangered indigenous plant species, mostly endemic to Mauritius and mostly known from fewer than twenty-five individuals. In extreme cases, like the coral tree *Chassalia boryana* (Rubiaceae) and the endemic palm *Hyophorbe amaricaulis* (Arecaceae), a single plant has been found. Over the two years that I lived and worked in Mauritius I became aware of how little is known about the flora, and what scant information is available to the people who strive to protect it. I hope this work proves to be a useful contribution to conservation, ecological restoration and documentation of diversity in the Mascarene islands.

11th of October 2009, Dublin

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All photographs are the author's own apart from those listed below:
In Figure 1.2 the image of *Hyophorbe lagenicaulis* was taken by Dave Stepninsky, and the *Phelsuma* image is from the Round Island warden archive. In Figure 4.1 the image of *Badula ovalifolia* fruits is from the archive of Claudia Baider and Vincent Florens.

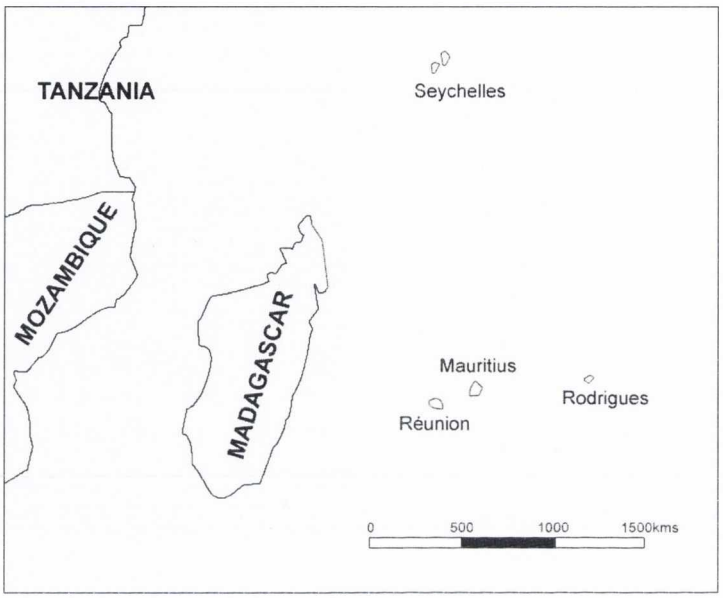
1. General introduction

1.1 The Mascarene archipelago

The Mascarene archipelago comprises three volcanic islands (La Réunion, Mauritius and Rodrigues) in the western Indian Ocean, just north of the Tropic of Capricorn and 700 to 1,400 km east of Madagascar (Fig. 1.1). The islands have never been connected to land of continental origin and are thought to have emerged at least 8 million years ago (Myr). Potassium-argon methods have been used to date Mauritius to 8 – 10 Myr (McDougall & Chamalaun 1969), La Réunion to 2.1 Myr (McDougall 1971) and Rodrigues to c. 1.5 Myr (McDougall *et al.* 1965). However, in recent years the estimated age for Rodrigues has been disputed and it has been suggested that it is the oldest of the three islands, at 10 Myr or more (Giorgi & Borchellini, 1998, in Cheke & Hume 2008). This issue remains unresolved for the present time. However, the proposal that Rodrigues is the oldest of the archipelago is intuitively more likely than it being the youngest due to the highly eroded topology of the island in comparison to the others (Cheke & Hume 2008). For example, La Réunion (2.1 Myr) bears the highest point in the Indian Ocean (Piton des Neiges at 3,070 m above sea level; Micheneau *et al.* 2008). In addition, La Réunion has no reef and the southeast coast of the island, Grand Brûlé, is charred and blackened from the most recent eruption of Piton de la Fournaise. In contrast, the highest point on Rodrigues is Mont Limon at 398 m asl, the island has an extensive reef and there are no historical records of volcanic activity. Cheke and Hume (2008) draw parallels with the Hawaiian islands[1] and liken "the large, high and still volcanically active island of Hawaii" to La Réunion, the "older and progressively more eroded high islands (Maui, Oahu, Kauai)" to Mauritius and the "low islands with broad lagoons and residual basaltic hills (Niihau, Necker)" to Rodrigues. In addition, the contrasting forms of the three Mascarene islands exemplify the pattern of emergence, development, erosion and submergence that characterizes the life-span of oceanic islands (Whittaker & Fernández-Palacios 2007).

[1] However here the similarity ends because, unlike the Hawaiian archipelago, the Mascarene islands were not all formed by the same volcanic 'hotspot': Mauritius and La Réunion have a common origin, not shared by the more isolated island of Rodrigues (Cheke & Hume, 2008).

Fig. 1.1. Location of the Mascarene archipelago



1.2 Biogeography

While the topography of some Hawaiian islands may reflect those of the Mascarenes, comparisons of colonization and species diversification patterns among the biota of these archipelagos have been hampered by a lack of data for the Mascarenes, which have received relatively little research interest compared to other island groups (Cuenca *et al.* 2008; Micheneau *et al.* 2008). Yet the flora and fauna of the archipelago show high levels of endemism and the islands form part of the Madagascar and Indian Ocean Islands Biodiversity Hotspot (Myers *et al.* 2000). The studies of *Badula* Juss. (Primulaceae) presented here are among the first to deal with one of the sixteen plant genera endemic to the archipelago[2] (see Table 1.1).

[2] The studies of Le Pechon *et al.* (2009), which includes sampling of *Trochetia* D.C. (Malvaceae), and Cuenca *et al.* (2008) which includes all species of *Hyophorbe* Gaertn. (Arecaceae), are among the few available.

Table 1.1. Endemic plant genera of the Mascarene islands

A total of 35 genera are endemic to the Mascarene islands. Genera are listed below for the respective islands, but if present on more than one island they are listed under 'Mascarenes'. Data are compiled from (Mabberley 2008) and C. Baider & V. Florens, pers. comm. *Badula* Juss. is highlighted in bold.

Island(s)	Family	Endemic genus	No. of species
La Réunion	Asclepiadaceae	*Trichosandra* Decne.	1
	Asteraceae	*Eriotrix* Cass.	2
	Asteraceae	*Faujasia* Cass.	5
	Campanulaceae	*Berenice* Tul.	1
	Escalloniaceae	*Forgesia* Comm. ex Juss.	1
	Malvaceae	*Ruizia* Cav.	1
	Orchidaceae	*Bonniera* Cordem.	2
Mauritius	Arecaceae	*Tectiphiala* H.E.Moore	1
	Asteraceae	*Cylindrocline* Cass.	2
	Campanulaceae	*Nesocodon* Thulin	1
	Lythraceae	*Tetrataxis* Hook.f.	1
	Malvaceae	*Astiria* Lindl.	1
	Sapindaceae	*Hornea* Baker	1
	Saxifragaceae	*Roussea* Sm.	1
Rodrigues	Asteraceae	*Sarcanthemum* Cass.	1
	Rubiaceae	*Doricera* Verdc.	1
	Rubiaceae	*Ramosmania* Tirveng. & Verdc.	2
	Rubiaceae	*Scyphochlamys* Balf. f.	1
	Turneraceae	*Mathurina* Balf. f.	1
Mascarenes	Arecaceae	*Acanthophoenix* H. Wendl.	1
	Arecaceae	*Dictyosperma* H. Wendl. & Drude	1
	Arecaceae	*Hyophorbe* Gaertn.	5
	Arecaceae	*Latania* Comm. ex Juss.	3
	Asteraceae	*Faujasiopsis* C. Jeffrey	3
	Asteraceae	*Monarrhenus* Cass.	2
	Asteraceae	*Parafaujasia* C. Jeffrey	2
	Campanulaceae	*Heterochaenia* A. DC.	3
	Euphorbiaceae	*Cordemoya* Baill.	1
	Malvaceae	*Trochetia* DC.	6
	Monimiaceae	*Monimia* Thou.	3
	Orchidaceae	*Arnottia* A. Rich.	2
	Primulaceae	***Badula* Juss.**	**14**
	Psiloxylaceae	*Psiloxylon* Thou. ex Tul.	1
	Rubiaceae	*Fernelia* Comm. ex Lam.	3
	Rubiaceae	*Myonima* Comm. ex Juss.	6

Cadet (1977) recognised the influence of the neighbouring floras of Madagascar and the African continent on the Mascarenes, and suggested that 70 to 80% of the flora of La Réunion originated from there. More recent studies on the Araliaceae (Plunkett *et al.* 2004), Malvaceae (Le Pechon *et al.* 2009), Orchidaceae (Micheneau *et al.* 2008) and Rubiaceae (Malcomber 2002) have added further examples of a predominantly Malagasy origin, while it has been suggested that the ancestor of the Mascarene endemic family Psiloxylaceae (Myrtales) arrived from Africa following long distance dispersal (Sytsma *et al.* 2004). A smaller proportion of genera have affinities with floras further afield, such as members of the Escalloniaceae (Stevens 2001 onwards) and, most remarkable of all, the endemic bottle-palm genus *Hyophorbe* (Fig. 1.2), whose closest relatives occur in Central and South America (Cuenca *et al.* 2008; Dransfield 1996).

Among the Mascarene islands, phylogenetic reconstructions have revealed varied explanations for patterns of diversification within species-rich groups. For example, among Mascarene angraecoid orchids (which have wind-born, dust-like seeds) evidence has been found for repeated dispersal events from Madagascar, rather than radiations on the islands (Micheneau *et al.* 2008). Similarly, within the Dombeyoideae at least three colonisation events from Madagascar to the Mascarenes were inferred by phylogenetic analyses of morphological data (Le Pechon *et al.* 2009). In contrast, within the same group there is some evidence to suggest that the gecko-pollinated endemic genus *Trochetia* had a Mauritius origin, and subsequently colonized the other islands (Le Pechon *et al.* 2009). Colonization from Mauritius to La Réunion was likewise inferred by Richardson *et al.* (2003) for the genus *Phylica* L.

Among the fauna, a single colonization of the Mascarene islands followed by intra-island speciation is evident for many of the endemic reptile species (Austin & Arnold 2001; Austin *et al.* 2004). For the genus *Phelsuma* Gray (the day geckos, Fig. 1.2) Austin *et al.* (2004) were unable to determine whether Rodrigues or Mauritius was colonized first, but found evidence to suggest that La Réunion was the last island to be reached, while the *Cylindraspis* Fitzinger giant tortoises first invaded Mauritius, and subsequently Rodrigues (Austin & Arnold 2001). Sadly the Mascarenes have been so transformed by human activity that many of these endemic lineages are threatened by extinction.

Fig. 1.2. Endemic species of the Mascarene islands

Clockwise from left: The bottle palm *Hyophorbe lagenicaulis* L.Bailey (with the author collecting seeds)- although common in cultivation, the only putative wild populations of this Mauritian endemic occur on the offshore islet Round Island. An endemic day gecko of the genus *Phelsuma* from Mauritius (Round Island). *Trochetia boutoniana* F.Friedmann, Mauritius.



1.3 Decline and extinction

With no permanent human population until the 16th century, the archipelago developed a remarkable flora and fauna where some of the bizarre traits peculiar to island species, such as gigantism and flightlessness among birds, were commonplace. More than twenty-eight endemic bird species have become extinct since human settlement (Cheke 1987), among them the famous dodo of Mauritius (*Raphus cucullatus* L.). In addition, numerous species of reptiles have been lost, including at least one species of giant skink (*Leiolopsima mauritiana* Günther) (Austin & Arnold 2006) and the entire giant tortoise genus *Cylindraspis* was extinct by 1830, despite 16th century reports of its abundance (Austin & Arnold 2001; Cheke 1987).

Of the three Mascarene islands, Mauritius has experienced the most complex colonial history, having been settled but later abandoned by the Dutch (c. 1638 and 1710, respectively), colonized by the French (1715) and seized by the British in the Battle of Grand Port (1810), before finally gaining independence in 1968, while retaining control of Rodrigues. A consequence of this tumultuous history, combined with the favourable topology and rich resources of the island, has been the rapid and widespread depletion of its native vegetation, as ebony and other timber was extracted and land was cleared for sugar cane cultivation (see Fig. 1.3). Some colonists realised the negative affects of this devastation and in 1761 the French East India Company made an unsuccessful request to the Governor of Mauritius to cease deforestation (Baker 1877). The subsequent British administration substantially increased sugar production and by 1860 10 % of global sugar exports came from Mauritius (Baker 1877). In 1877 the so-called Guardian of Woods and Forests wrote in his annual report: "naturalists complain that the indigenous vegetation is disappearing and being replaced by foreign plants which they know nought of… the sooner the greater part of the indigenous trees which are left disappear, the better it will be for all concerned" (Vaughan & Wiehe 1937). In the same year, Baker (1877) described the flora as "a mere wreck of what it was a hundred years ago."

Destruction of native forests of Mauritius continued well into the 1960s and '70s, largely as a consequence of the *Travail pour tous* programme which funded deforestation to make way for tea and pine plantations (Cheke & Hume 2008; Gabriel D'Argent, pers. comm.). By 1993 only 5 % of the island supported native vegetation, with 63 % in the southwest (Safford 1997). Today less than 2 % is considered rich in

native species and no pristine areas remain (Atkinson & Sevathian 2005). Introduction of alien species, such as macaque monkeys (*Macaca fascicularis* Raffles), wild pigs (*Sus scrofa* L.) and a host of invasive plant species, has further contributed to the demise of native vegetation and thwarts regeneration efforts. Consequently, 160 of the 670 plant species native to Mauritius are Critically Endangered (Atkinson & Sevathian 2005) under IUCN criteria.

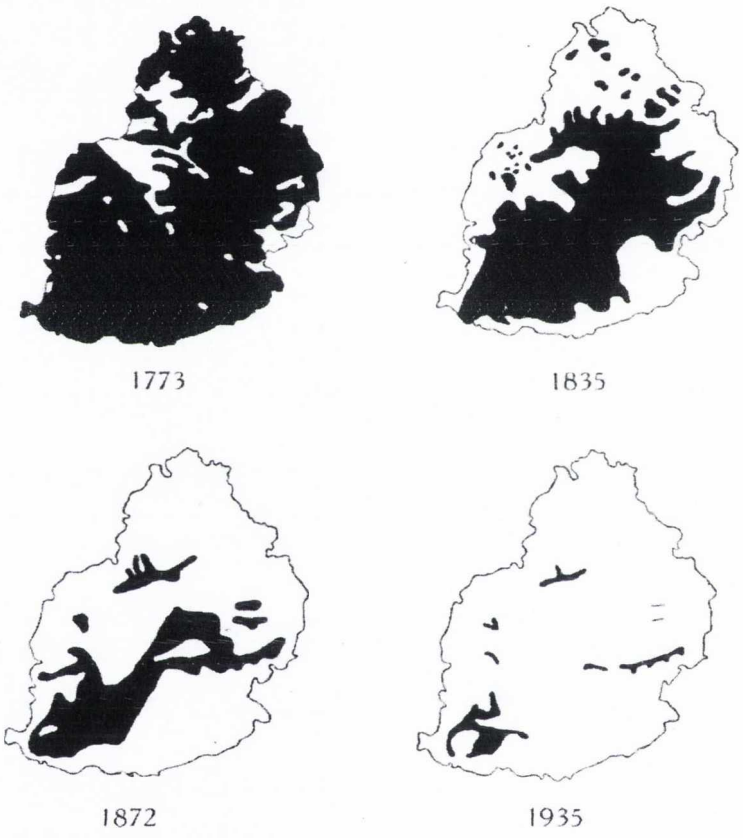


Fig. 1.3. Maps illustrating the decline of native vegetation in Mauritius
Shaded areas show the extent of native vegetation in 1773 and its decline up to 1935. Today the shaded southwest area (shown for 1935) is largely included in the Black River Gorges National Park (established in 1993). Maps reproduced from Vaughan and Wiehe's seminal study on the plant communities of Mauritius (Vaughan & Wiehe 1937).

Despite this bleak history, increased exploration has lead to many recent discoveries by both national and international research teams. For example, the ant genus *Discothyrea* Roger was recorded in Mauritius for the first time in 2005 and a new endemic species (*D. berlita* Fisher) was described, confined only to a remnant patch of native forest on Le Pouce mountain (Fisher 2005). Examples from the flora include a new genus of lichens (Timdal 2002), new species of pteridophytes (e.g. Janssen & Rakotondrainibe 2006) and angiosperms (e.g. Klackenberg 2005) and re-discovery of species once thought extinct, such as *Badula ovalifolia* A.DC. (Florens *et al.* 2008), *Crinum mauritianum* Lodd. (K. Pynee, pers. comm.) and *Trochetia parviflora* Bojer (V. Florens & J. C. Sevathian, pers. comm.). Both the Forest and Reserves Act (1983) and the establishment of the Black River Gorges National Park (6,754 ha) a decade later, have facilitated conservation of the remaining native vegetation (Strahm 1996a). Following the work of Strahm (1993) fenced plots (Conservation Management Areas; CMAs) were created to protect rare plants from feral animals and competition from alien plants (removed bi-annually by weeding teams). Reintroduction programmes and *inter-situ* methods[3] (sensu Burney & Burney 2007) have been in place for several years using nursery-grown material derived from the few surviving individuals of rare endemic species, such as *Diospyros* L. on offshore Round Island (Gasson *et al.* 1998). In addition, several long-term reintroduction programmes have been undertaken for rare birds, and the Mauritius Kestrel (*Falco punctatus* Temminck) is one of three endemic bird species deemed likely to have become extinct had it not been for the intervention of conservation groups (Butchart *et al.* 2006).

Cheke and Hume (2008) describe 18th century Rodrigues as an island that "regressed from desert-island paradise to ecological ruin". Like Mauritius, Rodrigues was plundered for its resources. In particular the giant tortoises were valued for their meat and were traded among the islands and further afield. By 1733 a permanent 'tortoise outpost' had been established on the island, and an account from 1736 describes how every ship bound for France held 400 tortoises (North-Coombes, 1994 in Cheke & Hume 2008). The island bears fewer inaccessible tracts of land than Mauritius and La Réunion. Consequently, virtually all hills and valleys were cleared to extract

[3] *Inter-situ* being the use of palaeo-ecological data to infer the past ranges of species and guide reintroduction plans.

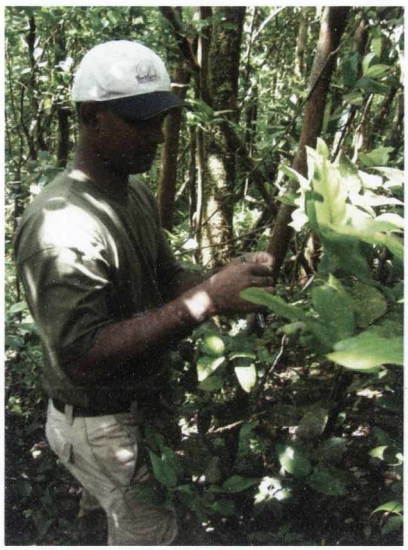
timber or to make way for agriculture (Fig. 1.4) and by the early 19th century only small relicts of the original vegetation remained (Balfour 1877, 1879b).

Rodrigues does not suffer the industrial scale sugar cane cultivation common to Mauritius or the same level of urbanisation and coastal hotel development, and today the landscapes of Rodrigues are dominated by subsistence agriculture. Thanks to the stewardship of local conservation practitioners (from the Mauritian Wildlife Foundation Rodrigues and the Forestry Service) native plants survive and vegetation restoration projects have been underway for several years (R. E. Bone, pers. obs.).

The vegetation of La Réunion has fared better than that of the other islands, largely due to the rugged topology, but also due to the different economic pressures on the island, which is a French Overseas Department and consequently part of the European Union and the Euro-zone. A large proportion of the vegetation of La Réunion has nevertheless been destroyed or degraded and felling of indigenous trees from the primary forests continues, particularly for the highly prized timber of *Acacia heterophylla* Willd. (Fabaceae). However, compared to the native vegetation cover of Mauritius (< 2%; Atkinson & Sevathian 2005) and Rodrigues (< 1%; Strahm 1996b), La Réunion has retained a relatively large amount (30%; Strasberg *et al.* 2005). At higher elevations, four of the nineteen habitat types identified by Strasberg *et al.* (2005) have retained more than 80 % of their original extent. For example the 'subalpine shrublands' cover 100 % of their original distribution (Strasberg *et al.* 2005), and extensive areas of the *Domebeya* dominated 'coloured tree forest' can be found (Fig. 1.4; Le Pechon *et al.* 2009). In 2007, a National Park was created and covers over 40 % of the island's area (D. Strasberg, pers. comm.).

Fig. 1.4. Examples of vegetation in Mauritius, Rodrigues and La Réunion

Top left: Botanist Kersley Pynee of the Mauritius Herbarium collecting leaf samples of *Memecylon cordatum* Lam. in mesic forest on the flanks of Mt. Cocotte, Mauritius. Top right: Agricultural land in southern Rodrigues. Below: The 'coloured tree forest' of upland La Réunion, dominated by *Dombeya spp.* (pictured in flower, right of centre, and continuing into the background).







1.4 *Badula*

The genus *Badula* Juss. has traditionally been treated as a member of the Myrsinaceae, however this family was included in the Primulaceae in the most recent update of the classification of angiosperm orders and families (The Angiosperm Phylogeny Group 2009). Prior to that, the Myrsinaceae was re-circumbscribed by Ståhl and Anderberg (2004), however no sub-divisions of the family were provided in this treatment. Consequently, the sub-family Myrsinoideae sensu Mez (1902) is used here, to define the group of genera most closely allied to *Badula*.

The Myrsinoideae is dominated by woody tropical genera, including *Amblyanthus* A. DC. of north-east India, *Cybianthus* Mart. from the neotropics, *Hymenandra* A. DC. of south-east Asia and *Oncostemum* Juss. of Madagascar and the Comoro Islands (Mez 1902; Ståhl & Anderberg 2004). Within this group, generic delimitations are considered "unclear" (Ståhl 1996; Ståhl & Anderberg 2004) and among the published phylogenies dealing with the Myrsinaceae/ Primulaceae (e.g. Anderberg *et al.* 2002; Anderberg & Ståhl 1995; Anderberg *et al.* 1998; Källersjö *et al.* 2000; Yesson *et al.* 2009), very few Myrsinoideae genera have been included, leaving phylogenetic relationships unresolved.

Badula is a genus of shrubs and small trees that predominantly occurs in the understorey of upland mesic forests of the Mascarene islands. It is one of two Myrsinoideae genera native to the archipelago (the other being *Embelia* Burm.f.; three species) and it is one of the sixteen genera considered endemic to the islands (see Table 1.1). With fourteen species, it is also the most species-rich. The 1997 IUCN Red List (Walter & Gillett 1998) includes virtually all of them, and nine are listed as Endangered or a more severe category. Several of the Mauritian *Badula* species were also included on a rare plant list drawn up by the Mauritian Wildlife Foundation in recent years (R. E. Bone, pers. obs.), and were estimated to number fewer than ten individuals. The only species known from Rodrigues, *Badula balfouriana* (O.Kuntze) Mez, has met a similar fate by becoming a Red List species.

When I first wrote the research proposal for the work presented here, some mystery surrounded the identity of a *Badula* plant that grew in a small clearing surrounded by *goyave de chine* (*Psidium cattleianum* Sabine) on the upland plateau of Mauritius, which has since been identified as *Badula reticulata* A.DC. by J. Bosser at the Paris Herbarium (C. Baider, pers. comm.). Another cluster of plants had been found

that resembled *Badula* in every way except by the unusual pyramidal fruit, and these plants are now recognised as the previously presumed extinct *Badula ovalifolia,* the only *Badula* species known with this fruit type (the fruits were not previously described; Florens *et al.* 2008). In the most recent account of *Badula,* Coode (1981) could not find reliable characters to separate *Badula* from the Malagasy genus *Oncostemum* Juss. Unable to investigate this further, Coode (1981) stressed that the generic delimitation of *Badula* was in need of revision. Consequently, the endemic status of *Badula* is in question.

Ståhl and Anderberg (2004) cite Coode's assertion that there is confusion over the distinction between this genus and *Oncostemum* (Coode 1981). In addition, within the genus, species delimitation is unclear for some of the La Réunion taxa and several *Badula* species were described from few herbarium specimens, often without field observations to complement them, due to extreme rarity or even presumed extinction (Coode 1981). Furthermore, no data were available regarding the genetic diversity of the rare species of *Badula*, indeed the work of Besse *et al.* (2003) on native *Psiadia* Jacq. (Asteraceae) and that of Dulloo (1998) on *Coffea* L. (Rubiaceae) are the only two studies I have been able to find that provide genetic diversity measures for *any* native plants species in Mauritius.

Soltis and Gitzendanner (1999) argue that phylogenetic information should be used to identify evolutionarily distinct lineages and "also should be considered in setting priorities for conservation." They state that "in conjunction with biogeography [it can be used to identify] areas that harbour many actively speciating groups" and that phylogeny should inform species concepts. Finally they call for "the integration of systematics in the establishment of conservation priorities and the development of strategies to preserve Earth's biota." In summing up the aims of the Global Strategy for Plant Conservation, Lowry II and Smith (2003) similarly call for an end to the "gulf" that separates botanists and conservationists, and recognise that "in practice, [to achieve the aims of the strategy] means someone must provide reliable information about what species these are, where they occur and what threats they face."

The occurrence of *Badula* on all three of the Mascarene islands, and the presence of more than one species on two of the islands, provides an ideal opportunity to test hypotheses of colonization among angiosperms in the region. In addition, these species show varied levels of morphological differentiation, and show different within-island distributions and levels of rarity. Consequently they present opportunities for

assessment of endemism and studies of speciation and genetic diversity. The studies I present here have the following aims:

1. To determine the generic delimitation of *Badula* and establish whether this genus can continue to be considered endemic to the Mascarene islands (Chapter 2):

    Nuclear (ITS) and plastid (*trnS-trnG, trnG*) markers are used to determine whether molecular data support the taxonomic delimitation of *Badula* and *Oncostemum*. In addition, this work comprises the first phylogeny of any speciose genus of woody tropical Primulaceae and therefore represents a significant contribution to the understanding of evolutionary relationships for this large family.

2. To infer patterns of colonization by *Badula* among the islands (Chapters 2 and 3):

    A variety of molecular markers, including AFLP and DNA sequences (ITS, *trnS-trnG, trnG, accD, psbA-trnH*) are used to investigate the biogeography of *Badula* in the Mascarene islands. In particular, this work aims to determine whether few or multiple colonization events may have caused species-richness in *Badula*, whether there is evidence for allopatric or sympatric speciation and whether speciation maybe on-going. This work contributes to the growing body of research on the origins and speciation patterns within the flora of the Mascarene islands.

3. To investigate whether the species concepts of Coode (1981) are supported by molecular data (Chapter 3):

    Using the molecular markers described for point 2, this study provides valuable baseline data to inform future taxonomic treatments of *Badula.* In addition the results aid conservation assessment for those taxa where species delimitations are currently unclear, by facilitating identification of evolutionarily significant units.

4. To determine whether plant species considered so rare that they are termed the 'living-dead' (sensu Janzen 1986) have low genetic diversity (Chapters 4 and 5):

This work investigates whether the Critically Endangered species of Mauritius and Rodrigues are genetically depauperate, (where available, comparisons were made to a more widespread congener) to inform conservation strategies. In addition, ecological traits were used to make inferences about the capacity of these species for survival. Finally, the only *ex situ* collections of a Critically Endangered *Badula* species (*B. balfouriana* of Rodrigues) were investigated to determine whether the collections are of conservation value.

2. Phylogenetics of the Mascarene endemic genus *Badula* Juss. and its Malagasy ally, *Oncostemum* Juss. (Primulaceae)

Submitted as: Ruth E. Bone, Joeri S. Strijk, Peter W. Fritsch, Trevor R. Hodkinson, Dominique Strasberg, Christophe Thébaud, Steve Waldren. Phylogenetics of the Mascarene endemic genus *Badula* Juss. relative to its Malagasy ally, *Oncostemum* Juss. (Primulaceae). *Botanical Journal of the Linnean Society* (under review).

2.1 Abstract

Sequences from nuclear and chloroplast DNA were used for phylogenetic reconstruction of the Mascarene endemic genus *Badula* Juss. (Primulaceae) and the allied genus *Oncostemum* Juss., a more species-rich and morphologically diverse genus endemic to Madagascar and the Comoro islands. The delimitation of these genera rests on stamen characters that are known to vary within both groups. I elucidated the phylogenetic relationships between these genera and investigated inter-specific relationships within *Badula*. The results indicate that *Badula* is monophyletic and nested within *Oncostemum*, rendering the latter paraphyletic under its current circumscription. The non-monophyly of *Oncostemum*, however, had low statistical support. In addition, non-concordance was found between Perrier de la Bâthie's infra-generic classification of *Oncostemum* and the clades recovered in these analyses. Within *Badula*, there is evidence for the monophyly of several species, consistent with the most recent classification of the genus- despite it being written when few specimens were available in some cases due to extreme rarity. Furthermore, the taxa from Rodrigues and La Réunion formed separate clades. Monophyly of taxa from Mauritius, however, was ambiguous.

2.2 Introduction

The isolated volcanic archipelago of the Mascarene Islands (La Réunion, Mauritius and Rodrigues) lies c. 2,000 km east of the African coast and supports a flora of high diversity and endemism, with 73 % of angiosperms and 22 % of pteridophytes listed as endemic to the island chain (Strahm 1996b). The Mascarenes are part of the Madagascar and Indian Ocean Islands Biodiversity Hotspot (sensu Myers *et al.* 2000) and have been categorized as an IUCN Centre of Plant Diversity (Strahm 1994).

Despite international recognition of its rich biodiversity, the literature shows a paucity of studies on the origins and mode of speciation of the flora, compared to those of other oceanic island groups (Micheneau *et al.* 2008). This trend has improved in recent years, with increased conservation and systematics research activity throughout the Mascarenes that has included phylogenetic and biogeographic studies of endemic groups in several plant families, such as Araliaceae (Plunkett *et al.* 2004); Malvaceae (Le Pechon *et al.* 2009); Orchidaceae (Micheneau *et al.* 2008); Pandanaceae (Callmander 2003); and Rubiaceae (Malcomber 2002). Many Mascarene plants, however, may become extinct before they can be adequately documented, with 39 % of the 670 species indigenous to Mauritius suffering extreme rarity, a level of threat superseded only by the flora of the remote Atlantic island St Helena (Walter & Gillett 1998).

Badula is the most species-rich genus endemic to the Mascarenes, and provides one of the most spectacular examples of morphological diversity in the Mascarene flora. Furthermore, the genus warrants attention from conservation agencies owing to the restricted distribution ranges and very small population sizes of several taxa, which survive in fragmented upland habitats where many are considered on the brink of extinction. Yet, like several other pairs of woody tropical Primulaceae genera [e.g. *Amblyanthus* A.DC. and *Amblyanthopsis* Mez; *Ctenardisia* Ducke and *Yunckeria* Lundell; *Rapanea* Aubl. and *Myrsine* L. (Ståhl & Anderberg 2004)] the delimitation of *Badula* and its Malagasy ally *Oncostemum* is not clear (Coode 1981).

The name *Badula* was first proposed by Jussieu (1789) based on specimens from Commerson's La Réunion herbarium and characterized by the presence of axillary racemose-paniculate inflorescences, a short corolla tube that divides into five corolla lobes, sub-sessile anthers and a short style that bears a capitate stigma. De Candolle (1834) argued that stigma-form distinguished *Badula* from *Ardisia* Sw., and inflorescence-form distinguished it from *Myrsine* L., and thus described seven species from La Réunion and Mauritius. Subsequently de Candolle (1841; 1844) described many new species across a broad distribution extending from Central and South America to the Philippines, but all were later treated as synonyms in other Primulaceae genera (e.g. *Ardisia, Embelia* Burm.f., and *Oncostemum*).

Jussieu (1830) proposed the genus *Oncostemum* and distinguished it from *Badula* by the presence of stamens fused to form a tube surrounding the style, versus distinct anthers inserted simply onto the corolla. The taxonomic treatment by Mez

(1902) modified this to include various degrees of filament and/or anther fusion in *Oncostemum*. This definition was later reinforced by Perrier de la Bâthie (1953) in his treatment of *Oncostemum* for the *Flore de Madagascar et des Comores*, where he described the genus as having stamens united in a tube, always by the filaments, and often by the anthers. Perrier de la Bâthie (1953) treated *Oncostemum* as endemic to Madagascar, the Comoros and Mauritius, and in addition recognized fourteen *Badula* species: eleven endemic to the Mascarenes and three to Madagascar (Perrier de la Bâthie 1952, 1953). In the most recent treatment of *Badula*, for the *Flore des Mascareignes*, Coode (1981) emphasized the lack of clear distinction between *Badula* and *Oncostemum* due to the variation in floral characters and determined that, although it is possible that they are distinct, the delimitation of the genera is in need of review. For the purposes of the *Flore des Mascareignes*, Coode chose to treat all *Badula* and *Oncostemum* occurring in the Mascarenes as *Badula*, and circumscribed this genus as a Mascarene endemic comprising 14 species (Coode 1976, 1981). Consequently, a new combination was published for *O. platyphyllum* (A.DC.) Mez, becoming *Badula platyphylla* (A.DC.) Coode (Coode 1976) and *O. reticulatum* (A.DC.) Mez was listed as a synonym of *Badula reticulata* A.DC. (Coode 1981). These Mauritian endemics were described as distinct from other Mascarene *Badula* by the unique characters of long, pendulous inflorescences (50 mm – 300 mm) and filaments fused into a ring, but bearing distinct anthers (Coode 1976, 1981).

Despite the lack of clear morphological distinction from *Oncostemum*, several characters are shared by the *Badula* species of the Mascarenes, including the presence of thick stems that are often densely covered with leaf scars, leaves that are clustered towards the tips of branches and often red-tinged at the base and along the petioles, caducous and ciliate bracteoles (subtending the pedicels), and densely spotted or lined corolla lobes (Coode 1981). At the infra-generic level, Coode (1981) also noted difficulty in distinguishing certain *Badula* species from one another, particularly on La Réunion where he recorded intermediate forms between *B. barthesia* (Lam.) A.DC., *B. borbonica* A.DC., and *B. grammisticta* (Cordem.) Coode (Coode 1981), an observation corroborated by other workers (D. Strasberg, pers. obs.). Some characters essential to the identification of *Badula* species are cryptic (such as leaf markings) or absent (such as habit) on herbarium specimens and identification is often dependent on detailed notes by collectors or additional observations of fresh material (Coode 1981; R. E. Bone, pers. obs.). During preparation of the account for *Flore des Mascareignes*, two

species (*B. crassa* A.DC. and *B. platyphylla*) were noted as having not been seen in the wild for several years and were considered very likely extinct (Coode 1981). *Badula ovalifolia* A.DC. was also listed as extinct in the 1997 IUCN *Red List of Threatened Plants* (Walter & Gillett 1998), and the island of origin for this species was uncertain due to specimens in different herbaria bearing different annotation, despite being regarded as duplicates made from the same collection (Coode 1981; Florens *et al.* 2008). In addition the habit, fruit and flower morphology of some species were unknown due to the paucity of historic collections and rarity of plants. Fortunately, exploration and collection by local botanists in Mauritius has intensified in recent years and has lead to the re-discovery of species previously considered extinct, as exemplified by the recent establishment of *Badula ovalifolia* as an extant Mauritian endemic (Florens *et al.* 2008). *Badula crassa* remains the only species of dubious origin. Like *Badula ovalifolia* and *B. insularis* A.DC., the type specimen of *B. crassa* is labelled '*in Borbonia* [La Réunion] *aut Mauritio*' (de Candolle 1834). Only two other specimens were known when the Flora was published: one labelled Mauritius and one labelled La Réunion (Coode 1981). Specimens initially thought to resemble *Badula crassa* were collected in La Réunion during preparation of the Flora but were subsequently assigned to the new specific epithet of *B. fragilis* Bosser & Coode (Coode 1979). In addition, soon after publication of the Flora, three populations of *Badula crassa* were discovered in Mauritius (two of which remain, constituting a total of five plants). In the absence of a positive record of *Badula crassa* in La Réunion in the past 150 years, I regard this species as a Mauritian endemic (although arguably due to extinction in La Réunion), and consequently recognize all *Badula* species as single-island endemics.

The classification of *Oncostemum* (Perrier de la Bâthie 1953) also needs attention. It relies heavily on characters from the inflorescence (or infructescence) to group species. For example, sepal morphology is used to split the genus into two large groups, within which several sub-groups are defined based on inflorescence form and flower number. And, although *Oncostemum* exhibits a striking diversity of habit and leaf-form, it is only within the sub-groups that vegetative characters are used to distinguish species, and I therefore hypothesize that this classification does not represent natural groups.

In this chapter I present the first phylogenetic study of *Badula* and *Oncostemum*, using a data set of molecular sequences from nuclear and chloroplast

DNA. I aim to elucidate the relationship between these two genera, assess their classification, and investigate inter-specific relationships of *Badula*.

2.3 Materials and Methods

Taxon sampling

Leaf material of *Badula* and *Oncostemum* was collected from wild populations in Madagascar and the Mascarenes, and dried in silica gel for DNA extraction (Chase & Hills 1991). Multiple samples of each of the fifteen *Badula* taxa recognised by Coode (1981) were included (Table 2.1). The three *Badula* species of Madagascar (Perrier de la Bâthie 1952) have not been treated in any classification since 1953 (Perrier de la Bâthie) and are known only from the type specimens. Sampling has therefore not included these species. The primary focus of my research was *Badula*. *Oncostemum* is species-rich (c. 100 species) and this study could not encompass sampling of *Oncostemum* in its entirety. However, every attempt has been made to sample across the morphological diversity seen in the genus (in the field and among herbarium specimens). Identifications were provided by P. W. Fritsch (California Academy of Sciences), and all mounted material was checked against Perrier de la Bâthie's descriptions a second time (R. E. Bone, pers. obs.). However, in many cases, characters essential to the identification of *Oncostemum* species, such as anther form, were missing in the vouchers associated with samples, preventing unambiguous identification. These identifications are indicated as *'affinis'*.

Over collection of individual plants in the genus *Badula* is associated with reduction in population size in at least one Critically Endangered *Badula* species (Florens *et al.* 2008). Collections made for this study were therefore carried out cautiously, and herbarium vouchers were made for at least one individual of each taxon, with photographs and existing geo-referenced vouchers then used as reference for further population level sampling. Samples collected in this way are indicated with '*cf.*' and a reference specimen number (*i.e.* an existing specimen of the same taxon from the same locality). In cases of extreme rarity (fewer than fifteen individuals known), detailed photographs were taken of plant parts and habit, and mounted and labelled *in lieu* of herbarium vouchers. These photographic specimens were deposited in a herbarium (Trinity College Dublin).

Outgroup selection

A detailed phylogenetic framework is lacking for the woody tropical Primulaceae and generic delimitation in these groups is reported as vague and unclear (Ståhl 1996; Ståhl & Anderberg 2004). However, phylogenetic analysis of DNA sequences from the *rbcL* gene has provided some evidence for the placement of *Ardisia* as sister to *Oncostemum* (Källersjö *et al.* 2000). In addition, analysis of GenBank® published ITS sequences for several woody Primulaceae genera (*Ardisia, Monoporus* A. DC., *Rapanea* and *Stylogyne* A. DC.) has revealed moderate support for the position of *Ardisia* as sister to a monophyletic *Oncostemum-Badula* group (R. E. Bone, unpubl. data). I therefore used *Ardisia* as the outgroup in these analyses.

Selection of markers

Samples representative of the morphological diversity of the ingroup genera were sequenced during a pilot study of the internal transcribed spacer of nuclear ribosomal DNA (ITS) and several of the plastid markers described in Shaw *et al.* (2005). Based on the results of this study, the plastid *trnS-trnG* intergenic spacer and the *trnG* intron were selected for this work. Two other plastid markers, *accD* and *psbA-trnH*, both of which have been utilized in DNA barcoding studies (Chase *et al.* 2005) were also sequenced.

Molecular methods

DNA extractions were carried out with DNeasy® Plant Mini kits (Qiagen Inc., Valencia, CA 91355, USA) following the manufacturer's protocol, or the CTAB method of Doyle and Doyle (1987) as modified by Hodkinson *et al.* (2007), with purification through JETquick spin columns (GENOMED GmbH, Löhne, Germany) following the manufacturer's protocol.

DNA regions were amplified with either a Bio-Rad MyCycler (Bio-Rad Laboratories, Hercules, CA 94547, USA) or a Gene Amp 9700 thermocycler (Applied Biosystems, Warrington, Cheshire, UK). The ITS region, including the 5.8S gene, was amplified with the primers ITS 5 (modified from White *et al.* 1990) and ITS 25 R (Nickrent *et al.* 1994). The *trnS-trnG* intergenic spacer and the *trnG* intron were co-amplified with the primers specified in Shaw *et al.* (2005) with one exception: *5'trnG2S* was modified to 5'- TCG AAT CGG TCA TTG AGA GGT -3' after Lu (unpublished) for use as an internal primer during cycle sequencing. The plastid coding

region *accD* was amplified and sequenced with the primers and protocols listed on the DNA Barcoding website of the Royal Botanical Gardens Kew (http://www.kew.org/barcoding/protocols.html). We used primer set 1F and 4R for amplification and sequencing. For successful amplification, a total of 38 cycles proved sufficient. The plastid intergenic spacer region, *psbA-trnH*, was amplified and sequenced with the primers described in Sang, Crawford and Stuessy (1997).

The amplification mixture contained 2 - 4 μL of template DNA (approximately 50 - 100ng/μL), 10 μL of buffer (5x; Promega, Southampton, UK), 1 μL of dNTPs (10 mM; Invitrogen, Paisley, UK), 3 μL of $MgCl_2$ (25 mM), 0.5 μL of each primer (25 μM), 0.25 μL *Taq* polymerase (5 U/ μL), and ultra-pure water to bring the total reaction volume to 50 μL. The PCR amplification parameters for ITS were: initial denaturation of 96° C for 5 minutes, followed by 40 cycles (each being: 94° C for 30 seconds, 46° C for 30 seconds, and 72° C for 1 minute) with a final extension of 72° C for 7 minutes. For *trnS-trnG* and *trnG*, the protocol given in Shaw *et al.* (2005) was followed, with the annealing temperature raised from 66° C to 68° C if double banded products were amplified. PCR reactions for *psbA-trnH* consisted of an initial denature step of 5 minutes at 94° C, followed by 28 cycles of 1 minute denature at 94° C, 1 minute annealing at 50° C, and 2 minutes extension at 72° C, with a final 10 minute extension at 72° C. Amplified products were purified with either JETquick spin columns or the enzymes exonuclease I (Exo) and Shrimp Alkaline Phosphotase (SAP) (USB Corporation, Ohio, USA) following the manufacturers' protocols.

Sequencing was carried out with Big Dye Terminator v. 1.1 or v 3.1 cycle sequencing kits (Applied Biosystems) following the manufacturer's protocols. Cycle sequencing reactions were cleaned with ethanol precipitation and then run on Applied Biosystems automated capillary sequencers (AB310, AB3100 or AB3130xl).

Phylogenetic analyses

Sequences were edited and assembled with Sequencher v. 4.7 (Gene Codes Corp., MI, USA) or Autoassembler v. 2.0 (Applied Biosystems). Each base was checked for agreement between the forward and reverse strands, and contiguous sequences were then aligned manually in Se-Al v. 2.0a11 (Rambaut 2002) by following the guidelines in Kelchner (2000).

A first round of analyses was carried out to address the relationship between *Badula* and *Oncostemum* using ITS and the combined *trnS-trnG* and *trnG* data sets

(hereafter referred to as *trnSGG*) with sampling across all accessions (Table 2.1). Preliminary analyses were carried out separately for the nuclear and chloroplast data sets because of the different modes of inheritance of nuclear and plastid genomes (biparental and uniparental, respectively) and therefore the potential for these markers to infer different evolutionary histories (Wendel & Doyle 1998). Because no incongruence with bootstrap support (BP) exceeding 50%, or posterior probability (PP) exceeding 0.66, was found in the resulting phylogenetic trees, subsequent analyses were performed on the combined ITS and *trnSGG* data, hereafter referred to as Matrix 1.

Upon establishing the monophyly of *Badula*, a second round of analyses was undertaken with a sub-sample of the accessions (Table 2.1). By adding two plastid molecular markers (*accD* and *psbA-trnH*) to the existing data set and retaining two *Oncostemum* samples as outgroups, I aimed to improve phylogenetic resolution at the inter-specific level in *Badula*. Separate analyses were conducted for nuclear and chloroplast data sets, again to check for congruence of topologies, before a final analysis was completed with a combined ITS and cpDNA (*trnSGG*, *accD* and *psbA-trnH*) data set, hereafter referred to as Matrix 2.

Maximum parsimony (MP) and Bayesian inference (BI) analyses were carried out for Matrices 1 and 2 and the separate nuclear and cpDNA matrices. For MP analyses, potentially parsimony-informative insertion-deletion mutations (indels) of any length were coded separately (representative of the most parsimonious number of mutational events) as presence/absence (binary) characters and added to the matrix, as suggested by Kelchner (2000). Only gaps of equal size across all terminals were scored. In the *trnSGG* region, the length of two long mononucleotide repeat regions (made up of poly-Ts followed by poly-As of approximately 130 bp in length) at 411 bp could not be ascertained with confidence due to the high rate of mutation at these sites; they were therefore excluded from all analyses. However, a 19 bp insertion, between the two mononucleotide repeats, was recovered in all accessions of *Badula balfouriana* (see Table 2.2) and was scored as described above.

MP analyses were conducted using the heuristic search options in PAUP* 4.0b10 (Swofford 2002). One-thousand replicates of random-sequence addition were employed, with 35 trees saved per replicate to increase efficiency of searches in tree space and reduce time spent in exhaustive 'hill-climbing' on sub-optimal islands of trees (Swofford *et al.* 1996), and with the tree bisection-reconnection (TBR) option in

effect. Statistical support for clades was estimated by using bootstrap analyses (Felsenstein 1985) with 1,000 replicates of simple sequence addition and TBR branch swapping (see Salamin *et al.* 2003).

For Bayesian analyses, the matrices were partitioned (each partition corresponding to a separate DNA region) to allow selection and implementation of different models of DNA substitution. These were determined in Modeltest (Posada & Crandall 1998) and the Akaike Information Criterion (AIC)[4] was used to select models of 'best-fit' for each partition, which were initiated in MrBayes v. 3.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) by setting the appropriate nucleotide substitution-rate matrix, the proportion of invariable sites and the rate variation across sites, while the parameter values were free to vary. In combined Matrix 1 the models selected were the general-time-reversible (GTR) model (Tavaré 1986) with a proportion of invariable sites *(I)* and a γ-distribution *(G)* of rate variation across sites (GTR+*I*+*G*) for the *trnSGG* partition, and the TrN model (Tamura & Nei 1993) + *G* for *ITS*. Because the latter cannot be implemented in MrBayes, the model with next best AIC score, HKY (Hasegawa *et al.* 1985) + *G*, was used. In combined Matrix 2, the model used for *ITS* was the same as that for combined Matrix 1; for *trnSGG*, the F81 (Felsenstein 1981) + *I* model was used; for *psbA-trnH*, the K81 (Kimura 1981) model with bases occurring in unequal frequencies (uf) + *I* + *G* best fit the data, but could not be implemented in MrBayes and the next-best model (F81+*I*+*G*) was selected; and for *accD*, the transitional model TIM (Posada 2005) was selected, but could not be implemented in MrBayes and the next-best model (GTR) was selected.

Two concurrent runs were initiated in MrBayes, each for 5,000,000 generations of four randomly started Monte Carlo Markov Chains using the default setting of 1 hot and 3 cold chains. Sampling was carried out every 100 generations. In each analysis, stationarity of log-likelihood values was reached after 50,000 or 100,000 generations and the plot of -lnL values (calculated in MrBayes with *sump*) indicated that convergence between the two runs had been achieved. 5,000 or 10,000 samples, respectively, were therefore discarded as 'burn-in' and posterior probabilities were calculated from the remaining samples.

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[4] The AIC for model *i* is calculated as $AIC_i = -2 \ln L_i + 2 K_i$ where K_i is the number of free parameters in the *i*th model and L_i is the maximum likelihood value of the data under the *i*th sample. Hierarchical likelihood ratio tests can also be used but make comparsions of two models at a time following a stepwise procedure. The AIC compares multiple models simultaneously, and is therefore more likely to find the best model. The model with the minimum AIC should be selected (Posada & Crandall 1998).

Table 2.1. Taxa, voucher and locality data for all accessions sequenced (DNA sequences will be submitted to GenBank)

Letters in parentheses indicate locations of voucher specimens, following *Index Herbariorum* acronyms (Thiers continuously updated). Where listed, tDNA numbers correspond to the DNA Bank from Laboratoire Evolution et Diversité Biologique, Unité Mixte de Recherches 5174 CNRS-Université de Toulouse, F31062, Toulouse, Cedex 9. France. Island names are abbreviated as follows: M for Mauritius, Mad for Madagascar, Re for La Réunion, and Rod for Rodrigues. All latitude and longitude data conform to the WGS-84 reference co-ordinate system.

Taxon	Voucher/ Herbarium	Locality		
		Place name	Latitude	Longitude
Ardisia sp.	R. Bone 45/ MAU	Mt. Calbasse (M)	S 20° 10' 22"	E 57° 35' 06"
Ardisia elliptica (Thunb.)	R. Bone 26/ MAU	Gaulette Serrai (M)	S 20° 12' 15"	E 57° 38' 17"
Oncostemum Juss. Group I (H. Perr.)				
Oncostemum aff. ankifiense Mez	P. Fritsch 1504/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 15' 50"	E 47° 25' 15"
Oncostemum aff. ankifiense Mez	P. Fritsch 1696/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 15' 50"	E 47° 25' 07"
Oncostemum aff. forsythii Mez	F. Almeda 7913/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 15' 36"	E 47° 25' 12"
Oncostemum aff. forsythii Mez	P. Fritsch 1553/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 15' 00"	E 47° 27' 00"
Oncostemum aff. forsythii Mez	P. Fritsch 1640/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 15' 00"	E 47° 25' 00"
Oncostemum aff. neriifolium Baker	P. Fritsch 1727/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 13' 00"	E 47° 24' 00"
Oncostemum aff. pachybotrys Mez	P. Fritsch 1488/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 15' 48"	E 47° 24' 50"
Oncostemum Juss. Group II (H. Perr.)				
Oncostemum aff. acuminatum Mez	G. de Nevers 11611/ CAS	Details inaccessible (among unmounted material CAS)		
Oncostemum aff. denticulatum H. Perr.	P. Fritsch 1601/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 15' ?"	E 47° 27' ?"
Oncostemum aff. denticulatum H. Perr.	P. Fritsch 1610/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 16' 14"	E 47° 25' 31"
Oncostemum aff. elephantipes H. Perr.	G. de Nevers 11557/ CAS	Details inaccessible (among unmounted material CAS)		
Oncostemum aff. gracile Mez	P. Fritsch 1736/ CAS	Vatovavy, Fianarantsoa (Mad)	S 21° 24' 00"	E 47° 56' 00"

Table 2.1 continued

Taxon	Voucher/ Herbarium	Locality		
		Place name	Latitude	Longitude
Oncostemum aff. nervosum Baker	P. Fritsch 1505/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 15' 50"	E 47° 25' 15"
Oncostemum aff. ovato-acuminatum H.Perr.	P. Fritsch 1641/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 15' 00"	E 47° 25' 00"
Oncostemum aff. ovato-acuminatum H.Perr.	P. Fritsch 1669/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 15' 35"	E 47° 25' 40"
Oncostemum aff. palmiformae H.Perr.	P. Fritsch 1697/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 15' 48"	E 47° 25' 18"
Oncostemum aff. seyrigii H.Perr.	P. Fritsch 1742/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 16' 05"	E 47° 25' 39"
Oncostemum Juss. indet.				
Oncostemum sp.	P. Fritsch 1628/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 17' 00"	E 47° 25' 00"
Oncostemum sp.	F. Almeda 8088/ CAS	Ranamofana, Fianarantsoa (Mad)	S 21° 15' 36"	E 47° 25' 12"
Oncostemum sp.	*s. n.* (tDNA 97)	Marojejy (Mad)	*No record*	
Oncostemum sp.	Strijk 687/ MO	Andasibe-Matidea (Mad)	S 18° 56' 06"	E 48° 25' 30"
Oncostemum sp.	Strijk 728/ MO	Andasibe-Matidea (Mad)	S 18° 47' 36"	E 48° 26' 00"
Badula balfouriana (O. Kuntze) Mez	R. Bone 51/ TCD	Grande Montagne (Rod)	S 19° 42' 32"	E 63° 27' 47"
Badula balfouriana (O. Kuntze) Mez	R. Bone 52/ TCD	Grande Montagne (Rod)	S 19° 42' 32"	E 63° 27' 46"
Badula balfouriana (O. Kuntze) Mez	R. Bone 54/ TCD	Nr Malabar (Rod)	S 19° 42' 35"	E 63° 26' 29"
Badula balfouriana (O. Kuntze) Mez	R. Bone 55/ TCD	Mt. Cimètiere (Rod)	S 19° 42' 34"	E 63° 25' 31"
Badula balfouriana (O. Kuntze) Mez	R. Bone 56/ TCD	Grande Montagne (Rod)	S 19° 42' 19"	E 63° 28' 03"
Badula barthesia (Lam.) A. DC.	*cf.* D. Strasberg 333/REU (tDNA1)	Grande Montagne (Re)	*No record*	
Badula barthesia (Lam.) A. DC.	*s. n.* (tDNA 26)	La Roche Marveilleuse, Cilaos (Re)	S 21° 09' 32"	E 55° 29' 17"
Badula barthesia (Lam.) A. DC.	*cf.* Cadet 3303/ REU (tDNA 134)	Morne des Patates, St Denis (Re)	S 20° 56' 13"	E 55° 27' 40"
Badula barthesia (Lam.) A. DC.	*cf.* Cadet 5574/ REU (tDNA 163)	Foret a Mare Joseph (Re)	S 21° 07' 25"	E 55° 29' 05"

Table 2.1 continued

Taxon	Voucher/ Herbarium	Locality		
		Place name	Latitude	Longitude
Badula barthesia (Lam.) A. DC.	*s. n.* (tDNA 265)	Les Radiers (Re)	S 21° 10' 51"	E 55° 48' 21"
Badula barthesia (Lam.) A. DC.	D. Strasberg 71/ REU	Makes (Re)	*No record*	
Badula barthesia (Lam.) A. DC.	LR 569/ REU	Basse vallée (Re)	*No record*	
Badula borbonica A. DC. var. *borbonica*	*cf.* LR571/ REU (tDNA 37)	Basse vallée (Re)	*No record*	
Badula borbonica A. DC. var. *borbonica*	*cf.* LR509/ REU (tDNA 77)	Bras Laurent, Ste Suzanne (Re)	*No record*	
Badula borbonica A. DC. var. *borbonica*	*s.n.* (tDNA 113)	Plaines des Lianes (Re)	S 21° 01' 38"	E 55° 36' 35"
Badula borbonica A. DC. var. *borbonica*	D. Strasberg 164/ REU	Makes, fin route de la scierie (Re)	*No record*	
Badula borbonica A. DC. var. *borbonica*	D. Strasberg 194/ REU	Grand Etang (Re)	*No record*	
B. borbonica var. *macrophylla* (Cordem.) Coode	LR 497/ REU	Piton des Songes (Re)	*No record*	
B. borbonica var. *macrophylla* (Cordem.) Coode	D. Strasberg 99/ REU	Piton de Bébour (Re)	*No record*	
B. borbonica var. *macrophylla* (Cordem.) Coode	J. Dupont, *s. n.* (12/04/2008)	Plaine des Palmistes (Re)	S 21° 16' ?"	E 55° ?' ?"
Badula crassa A. DC.	R. Bone 22/ TCD	Nr. Gross Roche, Yemen (M)	S 20° 21' 23"	E 57° 25' 58"
Badula crassa A. DC.	R. Bone 24/ TCD	Yemen (M)	S 20° 21' 23"	E 57° 25' 58"
Badula crassa A. DC.	R. Bone 58/ TCD	Mt. Le Morne (M)	S 20° 26' 59"	E 57° 19' 22"
Badula decumbens (Cordem.) Coode	*s.n.* (tDNA 86)	Entre-deux (Re)	S 21° 16' 05"	E 55° 27' 31"
Badula decumbens (Cordem.) Coode	LR471/ REU	Col Bellevue (Re)	*No record*	
Badula decumbens (Cordem.) Coode	LR 472/ REU	Col Bellevue (Re)	*No record*	
Badula fragilis Bosser & Coode	*s. n.* (tDNA 12)	Sentier de la Plaine des Cafres (Re)	S 21° 13' 28"	E 55° 34' 13"
Badula fragilis Bosser & Coode	*cf.* R. Bone 69/ REU (tDNA 46)	Footpath, Coteau Monique, Bébour (Re)	*No record*	
Badula fragilis Bosser & Coode	*cf.* Cadet 4078/ REU (tDNA 98)	Bébour (Re)	*No record*	

Table 2.1 continued

Taxon	Voucher/ Herbarium	Locality		
		Place name	Latitude	Longitude
Badula fragilis Bosser & Coode	*cf.* R. Bone 70/ REU (tDNA 212)	Belouve 2 (Re)	S 21° 04' 39"	E 55° 32' 40"
Badula grammisticta (Cordem.) Coode	*s. n.* (tDNA 63)	Rivière de l'Est (Re)	*No record*	
Badula grammisticta (Cordem.) Coode	*cf.* Cadet 6606/ REU (tDNA 69)	Plateau Duvernay, Bébour (Re)	*No record*	
Badula grammisticta (Cordem.) Coode	*cf.* Cadet 6606/ REU (tDNA 101)	Bébour (Re)	*No record*	
Badula grammisticta (Cordem.) Coode	*s. n.* (tDNA 119)	Plaine des Lianes (Re)	S 21° 01' 40"	E 55° 36' 30"
Badula grammisticta (Cordem.) Coode	D. Strasberg 55/ REU	Bras Laurent (Re)	*No record*	
Badula insularis A. DC.	R. Bone 4/ MAU	Petrin (M)	S 20° 24' 19"	E 57° 28' 27"
Badula insularis A. DC.	R. Bone 11/ MAU	Alexandra Falls (M)	S 20° 25' 56"	E 57° 27' 22"
Badula insularis A. DC.	R. Bone 39/ MAU	Mt. Le Pouce (M)	S 20° 12' 02"	E 57° 31' 31"
Badula multiflora A. DC.	R. Bone 13/ MAU	Piton de la Riviere Noire (M)	S 20° 24' 35"	E 57° 24' 32"
Badula multiflora A. DC.	R. Bone 17/ MAU	Piton de la Riviere Noire (M)	S 20° 25' 18"	E 57° 25' 23"
Badula multiflora A. DC.	R. Bone 19/ MAU	Piton de la Riviere Noire (M)	*No record*	
Badula nitida (Coode) Coode	*cf.* LR514/ REU (tDNA 75)	Bras Laurent, Ste Suzanne (Re)	*No record*	
Badula cf. nitida (Coode) Coode	D. Strasberg 48/ REU	Bras Laurent (Re)	*No record*	
Badula cf. nitida (Coode) Coode	D. Strasberg 50/ REU	Bras Laurent (Re)	*No record*	
Badula nitida (Coode) Coode	LR 514/ REU	Bras Laurent (Re)	S 21° 58' 33"	E 55° 33' 29"
Badula nitida (Coode) Coode	LR 515/ REU	Bras Laurent (Re)	S 21° 58' 33"	E 55° 33' 29"
Badula ovalifolia A. DC.	R. Bone 28/ TCD	Maccabé (M)	S 20° 24' 00"	E 57° 27' 06"
Badula ovalifolia A. DC.	R. Bone 29/ TCD	Maccabé (M)	S 20° 24' 00"	E 57° 27' 06"
Badula ovalifolia A. DC.	R. Bone 31/ TCD	Brise Fer, Picadilly Circus (M)	S 20° 22' 30"	E 57° 26' 44"

Table 2.1 continued

Taxon	Voucher/ Herbarium	Locality		
		Place name	Latitude	Longitude
Badula platyphylla (A. DC.) Coode	R. Bone 2/ TCD	Petrin CMA (M)	S 20° 24' 29"	E 57° 28' 11"
Badula platyphylla (A. DC.) Coode	R. Bone 3/ MAU	Petrin CMA (M)	S 20° 24' 28"	E 57° 28' 10"
Badula platyphylla (A. DC.) Coode	R. Bone 38/ TCD	Laing, outside Petrin CMA (M)	S 20° 24' 19"	E 57° 28' 00"
Badula reticulata A. DC.	R. Bone 9/ TCD	Nr. Petrin-Macchabé track (M)	S 20° 24' 09"	E 57° 27' 48"
Badula reticulata A. DC.	R. Bone 10/ TCD	Nr. Petrin-Macchabé track (M)	S 20° 24' 09"	E 57° 27' 48"
Badula reticulata A. DC.	R. Bone 21/ TCD	Nr. Petrin-Macchabé track (M)	S 20° 24' 10"	E 57° 27' 48"
Badula sieberi A. DC.	R. Bone 8/ MAU	Pigeon Wood (M)	S 20° 26' 39"	E 57° 28' 59"
Badula sieberi A. DC.	R. Bone 12/ MAU	Piton de la Riviere Noire (M)	*No record*	
Badula sieberi A. DC.	R. Bone 44/ MAU	Mt. Le Pouce (M)	S 20° 11' 55"	E 57° 31' 45"
Badula sieberi A. DC.	R. Bone 49/ MAU	Mt. Calbasse (M)	S 20° 10' 51"	E 57° 35' 04"

2.4 Results

Amplification and sequencing

Seventy-six accessions were included in Matrix 1. Two accessions failed to amplify for *trnSGG* (*Oncostemum sp.* 97 and *B. barthesia* 163) and were therefore scored as missing data. The aligned ITS partition of the matrix consisted of 687 nucleotide positions and included 116 variable characters, 74 of which were potentially parsimony-informative. The *trnSGG* partition consisted of 1,426 nucleotide positions (after exclusion of the two long mononucleotide repeat regions), 67 of which were variable and 23 of which were potentially parsimony-informative. In addition, two unique insertions were found in the *trnSGG* region. The first (19 bp in length) was revealed to be diagnostic for samples representative of *Badula balfouriana* and the second (25 bp) was diagnostic for *B. crassa* (Table 2.2).

Forty-nine accessions were included in Matrix 2 (47 *Badula* and 2 *Oncostemum*). The aligned *trnSGG* partition consisted of 1,415 nucleotide positions, 21 of which were variable and 13 of which were potentially parsimony-informative. The aligned ITS partition consisted of 672 nucleotide positions, 40 of which were variable and 28 of which were potentially parsimony-informative. The *accD* partition consisted of 276 nucleotide positions, of which 21 were variable and 16 were potentially parsimony-informative. The *psbA-trnH* partition consisted of 525 nucleotide positions, 19 of which were variable and 13 of which were potentially parsimony-informative.

Table 2.2. Diagnostic insertions in the *trnSGG* region

Position of the insertion refers to the aligned matrix for this region

Position	Insertion sequence	Taxon
420	ACT TTA ACT ACT TTA TTT T	*Badula balfouriana*
1199	GAT TTG AAT TGG TAG AGA AGA AAT T	*Badula crassa*

Phylogenetic analyses

Matrix 1 (*Badula* and *Oncostemum*)

The MP analysis recovered 22,813 equally most parsimonious trees of 233 steps (total length), with a consistency index (CI) of 0.84 and a retention index (RI) of 0.93. One of these trees is illustrated in Figure 2.1, with arrows indicating nodes that collapse in the strict consensus tree (Fig. 2.2). Bayesian analysis produced an almost identical topology (PP values shown on the MP tree, Fig. 2.1). *Badula* and *Oncostemum* form a monophyletic group (100 BP, 1.0 PP) relative to *Ardisia. Oncostemum* is paraphyletic and forms two main clades in all analyses (Clades A and B in Fig. 2.1), with low to moderate support (Clade A 63 BP, 0.83 PP; Clade B 64 BP, 0.99 PP) that are successively sister to a strongly supported *Badula* clade (Clade C, 100 BP, 1.0 PP). However, the non-monophyly of *Oncostemum* was only weakly supported (<50 BP; 0.67 PP). Clade A contains *Oncostemum* aff. *ankifiense, O. pachybotrys* and an unidentified *Oncostemum* (97). The position of *O.* aff. *elephantipes* was unresolved within Clade B, where it forms a trichotomy with two further clades. The first (60 BP, 0.91 PP) contains *O.* aff. *denticulatum, O.* aff. *seyrigii and O.* aff. *neriifolium.* The second (66 BP, 1.0 PP) contains *O.* aff. *ovato-acuminatum*, two unidentified *Oncostemum* accessions (PF1628 and FA8088), *O.* aff. *forsythii, O.* aff. *gracile, O.* aff. *acuminatum* and *O.* aff. *nervosum.*

Badula balfouriana, the only *Badula* species known from Rodrigues, forms a distinct and well supported clade (96 BP, 0.85 PP) sister to a core group of all other *Badula* species from La Réunion and Mauritius (63 BP, 0.90 PP). Within the core *Badula* clade, a La Réunion group is weakly supported (54 BP, 0.85 PP) and forms a trichotomy with two lineages from Mauritius. One of these comprises *Badula multiflora* (90 BP, 1.00 PP), whereas all other Mauritian taxa group in a larger clade. The latter clade, however, has low support (< 50 BP, < 0.8 PP), and collapses to form a polytomy with the La Réunion and *B. multiflora* clades in the strict consensus tree (Fig. 2.2).

Some sub-groupings within the Mauritian taxa are resolved, including a *Badula platyphylla* + *B. reticulata* clade (97 BP, 1.00 PP) and a *B. ovalifolia* clade (88 BP, 1.0 PP). The analyses also revealed intra-specific variation within some Critically Endangered species, such as *B. balfouriana* and *B. ovalifolia*, each of which are known from less than ten remaining individuals in the wild.

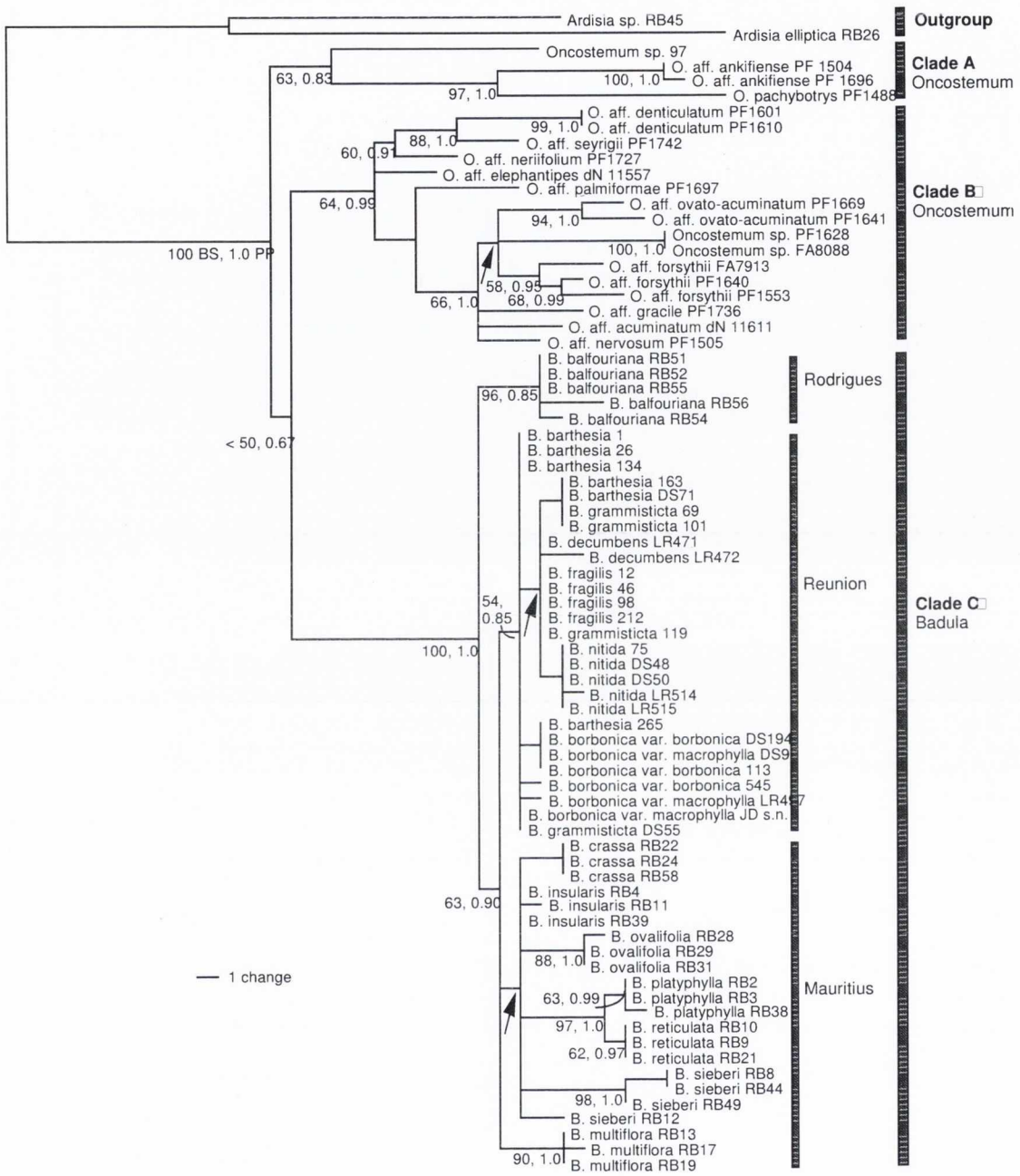


Fig. 2.1 Phylogram showing 1 of 22,813 equally most parsimonious trees from the combined analysis of Matrix 1 Length 233, CI= 0.84, RI= 0.93. Branch lengths (ACCTRAN optimisation) are indicated by the scale bar. Bootstrap percentages (BP), followed by posterior probability values (PP) from a Bayesian analysis, are shown below branches (within the major clades, BP < 50 and PP < 0.8 are not shown). Arrows indicate nodes that collapse in the strict consensus (Fig. 2.2).

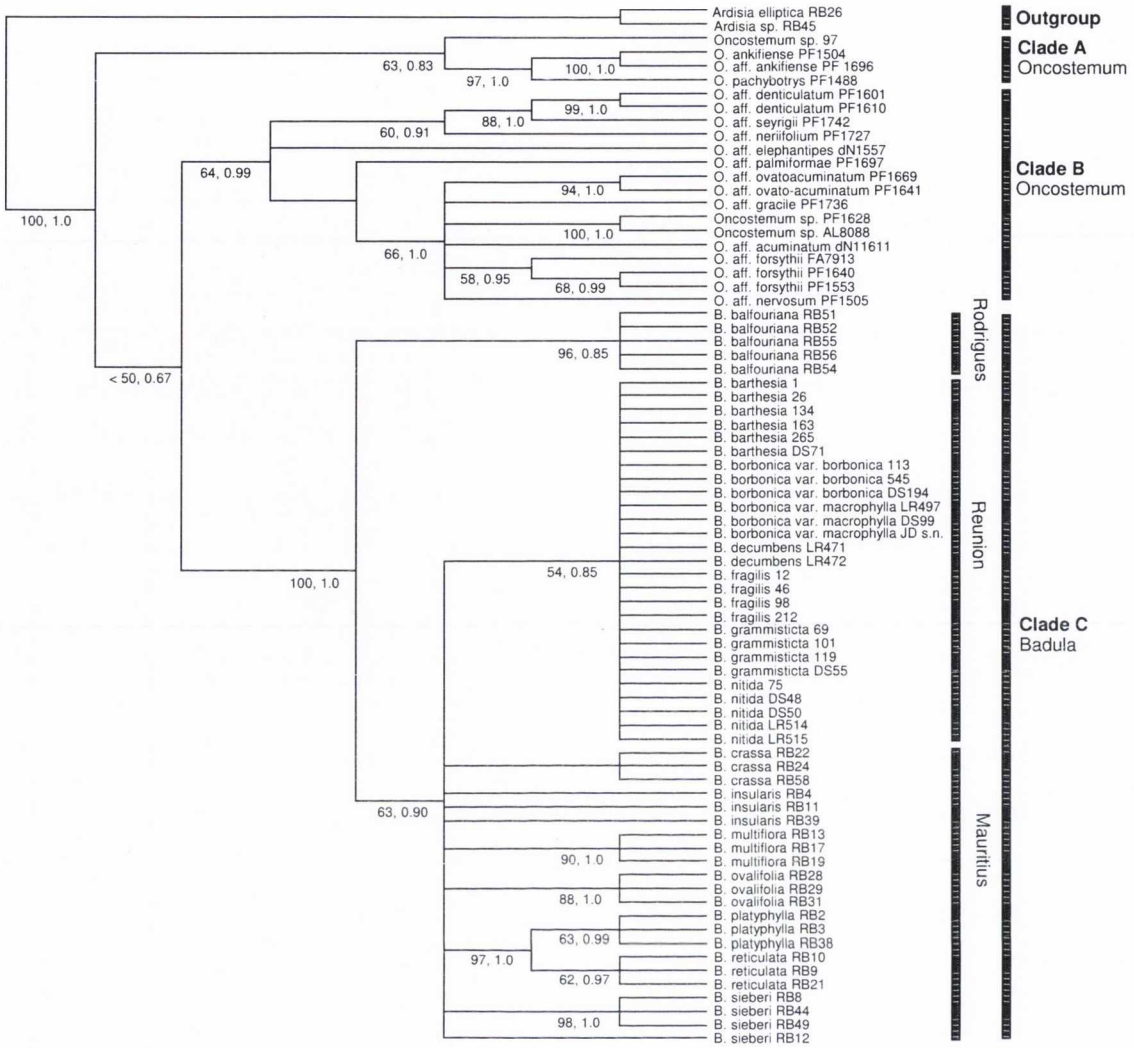


Fig. 2.2 The strict consensus tree from the combined analysis of Matrix 1

The strict consensus of 22,813 equally most parsimonious trees. Bootstrap percentages (BP), followed by posterior probability values (PP) from a Bayesian analysis, are shown below branches (within the major clades, BP < 50 and PP < 0.8 are not shown).

Matrix 2 (*Badula*)

The ITS and cpDNA phylogenetic trees were congruent and the final Bayesian and MP analyses were therefore based on the combined data (ITS, *trnSGG, accD* and *psbA-trnH*). The MP analysis recovered 37,520 equally most parsimonious trees of 125 steps (total length), with CI = 0.84 and RI= 0.93; Table 2.3). The same first-diverging clade of *B. balfouriana* was recovered in this analysis, and the support values were raised to 100 BP and 1.0 PP (Table 2.3). Likewise, support for the La Réunion clade is higher in this analysis (73 BP, 1.0 PP), and within it strong support was found for the *Badula nitida* clade (99 BP, 1.0 PP). However, no other La Réunion species-specific groups are retained in the strict consensus.

The same topology of Mauritian taxa was recovered in the strict consensus of this analysis as that of Matrix 1 (Fig. 2.1), but with increased support for most species-specific clades, including the *Badula ovalifolia* clade (which increased to 97 BP, 1.0 PP) and the *B. reticulata* + *B. platyphylla* clade (which increased to 99 BP, 1.0 PP). Increased branch lengths were observed in some cases, revealing further intra-specific variation. The position of *B. sieberi* accession RB12 is unresolved in all analyses (from Matrices 1 and 2).

Table 2.3. Results of MP and BI analyses of Matrix 2, combined and partitioned (nuclear/ cpDNA), compared to Matrix 1.

Within *Badula,* the same geographical and taxonomic clades shown in Fig. 2.1 were recovered by these analyses. Clades present in the strict consensus trees for each analysis are indicated with a tick.

	Combined Matrix 1 *trnSGG* & ITS		Combined Matrix 2 *cpDNA* & ITS		cpDNA partition from Matrix 2		Nuclear ITS DNA partition from Matrix 2	
Geographical groups	Strict cons.	BP, PP	Strict cons.	BP, PP	Strict cons.	BP, PP	Strict cons.	BP, PP
Rodrigues	✓	96, 0.85	✓	100, 1.0	✓	99, 1.0	✓	60, 0.9
Réunion	✓	54, 0.85	✓	73, 1.0	x	<50,0.88	✓	61, 0.89
Mauritius	x	0	x	0	x	0	x	0
Taxon groups								
Badula	✓	100, 1.0	✓	100, 1.0	✓	96, 1.0	✓	100, 1.0
B. balfouriana	✓	96, 0.85	✓	100, 1.0	✓	99, 1.0	✓	60, 0.9
B. nitida	✓	<50, 0.77	✓	99, 1.0	✓	97, 1.0	x	0
B. crassa	✓	85, 0	✓	82, 0	✓	81, 0	x	0
B. ovalifolia	✓	88, 1.0	✓	97, 1.0	✓	93, 1.0	✓	76, 1.0
B. platyphylla, B. reticulata	✓	97, 1.0	✓	99, 1.0	✓	87, 1.0	✓	92, 1.0
B. platyphylla	✓	63, 0.99	✓	63, 1.0	x	0	✓	60, 1.0
B. reticulata	✓	62, 0.97	✓	61, 0.96	✓	63, 0.99	x	0
B. sieberi excluding RB12	✓	98, 1.0	✓	96, 1.0	x	0	✓	98, 1.0
B. multiflora	✓	90, 1.0	✓	91, 1.0	✓	53, 0.99	✓	75, 0.81

2.5 Discussion

Delimitation of Badula *and* Oncostemum

The results suggest that *Badula* is nested within *Oncostemum* and thus it is possible that *Oncostemum* is paraphyletic relative to *Badula.* This relationship was only found in the strict consensus tree, however, and received low bootstrap and posterior probability support. It is therefore more conservative to treat the relationship as a trichotomy comprising *Badula* and the two *Oncostemum* clades.

The generic delimitation of *Badula* and *Oncostemum* based on stamen fusion, originally proposed by Jussieu and later modified by Mez to include fusion of the filaments, has long been considered inadequate and in the molecular analyses presented in this chapter accessions with similar stamen types did not group together. For example, Clade B (Fig. 2.1) includes *O.* aff. *ovato-acuminatum*, with distinct anthers, and *O.* aff. *denticulatum* and *Oncostemum sp.* PF1628 and FA8088, all with anthers fused into a tube. In Clade A, *O. pachybotrys* PF1488 also has anthers fused into a tube. In Clade C, two species named *Oncostemum* in the classification of Mez (1902; based on the presence of filaments fused into a ring, but bearing free anthers) were subsequently treated as *Badula* (*B. platyphylla* and *B. reticulata*; Coode, 1976) and these results indicate that these species are indeed nested within the *Badula* group. The definition of androecial characters could once again be modified to describe *Badula* as always having distinct anthers, with filaments free or fused, but this character alone cannot be used to delimit the genera due to the variation of these characters within *Oncostemum.*

Badula

Badula forms a robust endemic Mascarene clade in these analyses (100 BP, 1.0 PP). *Badula balfouriana*, the only species known from Rodrigues, forms a strongly supported monophyletic group in these analyses, sister to all other *Badula* species. Further geographical grouping of taxa occurs within this core *Badula* clade, where a moderately supported La Réunion clade forms a trichotomy with the two clades from Mauritius. One of these comprises *Badula multiflora*, and the position of this clade is ambiguous in relation to other Mauritian species. In the strict consensus tree (Fig. 2.2) the larger Mauritius clade collapses and, with the exception of *B. platyphylla* and *B. reticulata*, relationships among the Mauritian species are unresolved. Thus there is little

evidence to suggest that the Mauritian species evolved from a single ancestral lineage, and additional data are required to further examine whether *Badula* diversity on Mauritius has single or multiple origins.

Within the geographical groups, several moderately to strongly supported species-specific clades agree with the current classification of *Badula*. For example, the specific epithet *Badula fragilis* was published with some reluctance because information was lacking at the time regarding the morphologically similar *B. crassa,* then considered extinct (Coode 1979). These results confirm the distinctness of these two species, with the four accessions of *B. fragilis* all falling within the La Réunion clade, separate from the three accessions of *B. crassa.*

As noted by Coode, some of the La Réunion taxa are difficult to distinguish from one another, and intermediate forms have been recorded between *B. barthesia, B. borbonica* and *B. grammisticta*. The phylogeny recovered two mixed clades (*B. barthesia* + *B. grammisticta*; and *B. barthesia* + *B. borbonica* var. *borbonica* + *B. borbonica* var. *macrophylla*), both poorly supported (<50 BP, 0.8 PP), and other accessions of these taxa were unresolved within the La Réunion clade. This lack of differentiation for the markers studied may corroborate the existence of intermediate morphological forms and incipient, or very recent, speciation among populations occurring on this geologically young island of approximately 2 to 3 Myr (McDougall 1971).

Oncostemum

These results indicate the non-monophyly of groups used in the current classification of *Oncostemum*. For example, although Clade A (Fig. 2.1) contained representatives exclusively from Group I of *Oncostemum sensu* Perrier de la Bâthie (1953) (*Oncostemum* aff. *ankifiense* and *O. pachybotrys*), other species from Perrier de la Bâthie's Group I were nested within Clade B (*O.* aff. *neriifolium* and *O.* aff. *forsythii*), supporting my opinion that this classification is not based on natural groups.

Oncostemum is species-rich (c. 100 species) and, although every attempt has been made to sample across the morphological diversity seen in the genus, the primary focus of my research was *Badula* and could not encompass comprehensive sampling of *Oncostemum*. Since Perrier de la Bâthie's 1953 publication in *Flore de Madagascar et des Comores*, collecting effort in the region has increased immensely and several herbaria hold numerous unidentified *Oncostemum* specimens. Further work is needed to

identify morphological traits that can be used to distinguish the clades resolved here (Fig. 2.1) and, until *Oncostemum* can be investigated in more depth both taxonomically and phylogenetically, I recommend that nomenclatural changes are not made to it.

Concluding remarks

I have found evidence for the existence of a lineage endemic to the Mascarene Islands, whether or not this group, with more evidence, is eventually assimilated into a larger genus that also includes Malagasy lineages. This indicates that the Mascarenes were colonized only once by the common ancestor of *Badula* (*sensu* Coode, 1981), with subsequent diversification following establishment. Moreover, this clade is composed of species endemic to single islands suggesting that dispersal between the Mascarene islands has occurred just a few times with intra-island speciation playing a predominant role in the generation of current *Badula* species diversity. The existence of many *Badula* species is precarious and they should be amongst the highest conservation priorities. Promising evidence of intra-specific variation was found within the severely threatened species *Badula balfouriana, B. ovalifolia* and *B. platyphylla*, and should be further investigated due to the implications for conservation strategies.

3. Species origins and delimitations in the genus *Badula* Juss. (Primulaceae): evidence for extensive within-island radiations in the Mascarene archipelago

To be submitted as: Ruth E. Bone, Trevor R. Hodkinson, Christophe Thébaud and Steve Waldren. Species origins and delimitations in the genus *Badula* Juss. (Primulaceae): evidence for extensive within-island radiations in the Mascarene archipelago

3.1 Abstract

I aimed to elucidate infra-generic relationships in the genus *Badula* (Primulaceae), one of the most species-rich endemic genera of the Mascarene archipelago, and part of the Madagascar and Indian Ocean Islands Biodiversity Hotspot. Due to the rarity of plants, paucity of herbarium specimens and intermediate morphological forms among some taxa, difficulties in species delimitation were encountered during the last taxonomic treatment of the group. Using DNA sequences and AFLP markers (alone and in combination), I recovered statistically robust clades for thirteen of the fifteen known *Badula* taxa using parsimony and distance methods. Some accessions from La Réunion were not resolved in taxon-specific clades however. This finding confirms the existence of morphologically intermediate forms, which may be a consequence of very recent or incipient speciation, as inferred by the very short branch lengths recovered from these analyses for taxa on this geologically young island (< 3 Myr). Taxa from Rodrigues and La Réunion each formed separate, well supported clades, indicating that an ancestral *Badula* species colonized each of these islands only once. However, contrasting relationships were resolved among the species from Mauritius. In particular, the position of the Mauritian species *Badula crassa* differed between the distance and parsimony methods, and in the NJ and PCoA analyses this species was resolved as outlying all other *Badula* species from Mauritius. The morphological and ecological affinities of this species and the Rodrigues endemic *Badula balfouriana* are described and lend credence to their position as sister-species in the NJ tree. However bootstrap analysis did not support either of the topologies and further evidence is needed to clarify the origins of Mauritian *Badula.*

3.2 Introduction

Recognition of species that are unique (endemic) to archipelagos or individual islands is critical to determine conservation priorities in threatened island floras. It relies upon extensive biological inventories, including in-depth systematic investigations, and identification of cryptic species in lineages that lack substantial morphological differentiation across their range (e.g. Hebert *et al.* 2004; Okuyama & Kato 2009). While there is currently much effort to fill these knowledge gaps, there are still islands in which many plant groups have not been thoroughly investigated and for which the geographical origin of colonizing lineages and the patterns of species diversification have not been studied. The Mascarene archipelago of the Western Indian Ocean is such an island group. Despite international recognition of its rich biodiversity, the literature shows a paucity of studies on the origins and evolution of the Mascarene flora compared to those of other oceanic island groups, such as the Hawaiian archipelago, Galapagos Islands and Canary Islands (Micheneau *et al.* 2008). Furthermore, while several phylogenetic studies deal with endemic representatives of more widespread genera, such as *Angraecum* Bory (Micheneau *et al.* 2008), *Gaetnera* Lam. (Malcomber 2002), *Phylica* L. (Richardson *et al.* 2001) and *Polyscias* J. R. Forst. & G. Forst. (Plunkett *et al.* 2004), the study of *Badula* Juss. (Primulaceae) presented in the preceding chapter (Chapter 2) is among the first to deal with one of the sixteen plant genera recognised as endemic throughout the archipelago (see Table 1.1; C. Baider and V. Florens, unpubl.). This genus is possibly the most species-rich of the Mascarenes and provides one of the most spectacular examples of morphological diversity in the flora, ranging from narrow monocaulous shrubs and trees with leaves in apical clusters (e.g. *B. borbonica* and *B. decumbens*), to spindly divaricate forms (*B. grammisticta* and *B. platyphylla*) or stout many-branched trees that can reach the canopy of upland forests (e.g. *B. fragilis* and *B. insularis*). The fifteen *Badula* taxa are each considered endemic to a single island (*cf.* Chapter 2 for a discussion of species provenance). Fourteen taxa are distributed equally between the two larger Mascarene islands of La Réunion (2,500 km²) and Mauritius (1,800 km²), and one species (*Badula balfouriana*) is found on Rodrigues (110 km²), the eastern-most and putatively the oldest of the Mascarene islands. As such, *Badula* represents an ideal group for the study of patterns of colonisation and diversification in the Mascarene flora.

The last treatment of *Badula* (Coode 1981) cited several problems with species delimitation in the genus and mentioned the existence of intermediate forms for species

from La Réunion. Furthermore, little material was available for description due to the rarity of some *Badula* species, which lead Coode (1979) to comment that: "to add another specific epithet... before the existing ones are really understood is perhaps unfortunate." Three species were considered extinct as the Flora went to press and, although these species have since been re-discovered, they suffer extreme rarity and are known from fewer than ten wild plants. Phylogenetic analyses of DNA sequences from one nuclear (*ITS*) and four chloroplast regions (*trnS-trnG, trnG, psbA-trnH* and *accD*) found strong evidence for the monophyly of *Badula* in relation to the allied genus *Oncostemum* Juss. from Madagascar (Chapter 2). The phylogenetic reconstruction also recovered two island clades and some species-specific clades. However, several accessions, in particular those from La Réunion, showed a lack of sequence divergence and their phylogenetic positions were unresolved. Amplified fragment length polymorphisms (AFLP; Vos *et al.* 1995) have successfully resolved inter-specific relationships among closely related taxa such as *Ophrys* L. (Devey *et al.* 2008), *Phylica* L. (Richardson *et al.* 2003) and *Quercus* L. (Kelleher *et al.* 2005) and are considered appropriate markers for systematics studies of closely related taxa, when DNA sequences may show low levels of variation (Hodkinson *et al.* 2000).

The Mascarenes comprise three main islands of volcanic origin, 2,000 km from the nearest continent (Africa) and 800 km from the nearest island of continental origin (Madagascar; Fig. 1.1). Rodrigues, to the east, was long considered the youngest Mascarene island based on Potassium-Argon dating of McDougall *et al.* (1965). However, more recent estimates place it as the oldest of the archipelago at 10 Myr (Giorgi & Borchellini, 1998 in Cheke & Hume 2008), congruent with the eroded topology of the island (which reaches only 393m at the highest point of Mt Limone) and the earlier observations of Darwin (1842), who noted the extensive reef. Mauritius is an estimated 8-10 Myr (McDougall & Chamalaun 1969) while Réunion, to the west, is the youngest island (< 3Myr; McDougall 1971) and remains volcanically active.

Among the studies available for more widespread genera, colonization and speciation patterns of varying complexity have been revealed. Annual cyclonic storms, generally moving from east to west, have been cited as a causal factor for organism dispersal in the region, for example Dijkstra (2007) cites westward storms as the most likely explanation of the distribution of *Hemicordulia* Tau dragonflies and other winged animals in the region. Colonisation of La Réunion from Mauritius has been reported in the plant genus *Phylica* (Richardson *et al.* 2003), however eastward migration of

organisms in the Mascarenes is also recorded. For example, Micheneau *et al.* (2008) found evidence for the Malagasy origin of angraecoid orchids of the Mascarenes, and suggested that the dust-like seeds may have reached the islands due to irregularities in cyclone patterns that cause secondary storms in an easterly direction.

Here I aim to elucidate the phylogeny for *Badula* and the delimitations of its species, using AFLP and new DNA sequence data, and examine the role of geography in explaining the diversification of *Badula* in the Mascarenes.

3.3 Materials and Methods

Sampling

Leaf samples were collected from wild plants and dried in silica gel following the method of Chase and Hills (1991). Collecting was carried out cautiously due to the rarity of many *Badula* species (see Chapter 2, section 2.3). Every *Badula* taxon is represented by between one and five accessions (see Fig. 3.1 for collecting localities). A total of 39 accessions were genotyped in this study (Table 3.1). DNA sequences were available for 29 of the accessions (sequenced in a previous study; Chapter 2) and I therefore sequenced the ten remaining accessions to provide DNA sequences for every AFLP genotyped individual.

DNA extraction

DNA extractions were carried out with DNeasy® Plant Mini kits (Qiagen Inc., Valencia, CA 91355, USA) by following the manufacturer's protocol, or the CTAB method of Doyle and Doyle (1987) as modified by Hodkinson *et al.* (2007), with purification through JETquick spin columns (GENOMED GmbH, Löhne, Germany) following the manufacturer's protocol.

Molecular markers

AFLP reactions were carried out using the Applied Biosystems AFLP Plant Mapping Kit for regular size genomes following the manufacturer's protocol, except that all reaction volumes were halved. All digestion and amplification reactions were carried out on a Gene Amp 9700 thermocycler (Applied Biosystems). Total DNA was digested using two restriction enzymes (*Eco*RI and *Mse*I) and specific oligonucleotide adaptors were ligated to restriction sites on these fragments, which were then used for

two successive PCR reactions (pre-selective and selective). The pre-selective PCR used primers provided in the kit and amplified a sub-set of the fragments, reducing them approximately 16-fold. These PCR products then under-went a second, selective PCR, which reduced the number of fragments further still (approximately 256-fold). One primer in each primer pair incorporated a fluorescent dye (always *Eco*RI) to facilitate visualization of amplified fragments. Twelve different primer combinations were tested in the selective PCR using a sub-set of the digested DNA samples. Four pairs were selected for final analysis based on the quality of the fingerprints they produced. These were: (FAM dye-labelled) *Eco*RI-ACT + *Mse*I-CAG, (FAM) *Eco*RI-ACT + *Mse*I-CAA; (FAM) *Eco*RI-ACA + *Mse*I- CAG, and (NED) *Eco*RI-AGC + *Mse*I-CTG. The samples were run on an AB3130xl Applied Biosystems automated capillary sequencer and were analysed using Genemapper v. 4.0 (Applied Biosystems).

For ten accessions (Table 3.1), DNA regions were amplified and sequenced for the *trnS-trnG* spacer, the *trnG* intron and ITS following the methods described in Chapter 2.

Fig. 3.1 Collecting localities for samples included in the study

The co-ordinates pf the collection localities are given in Table 3.1, with a list of sample numbers. Collection localities are indicated by filled black circles and the highest peaks on each island are indicated by a black triangle, with the height above sea level in parentheses. The maps are not representative of the arrangement of the islands in the archipelago (refer to Fig. 1.1).

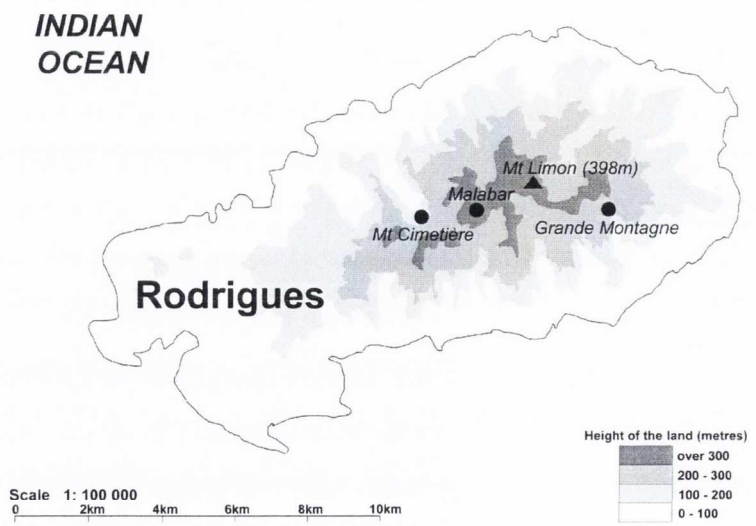


Fig. 3.1 Continued

Collection localities are indicated by filled black circles and the highest peaks on each island are indicated by a black triangle, with the height above sea level in parentheses.

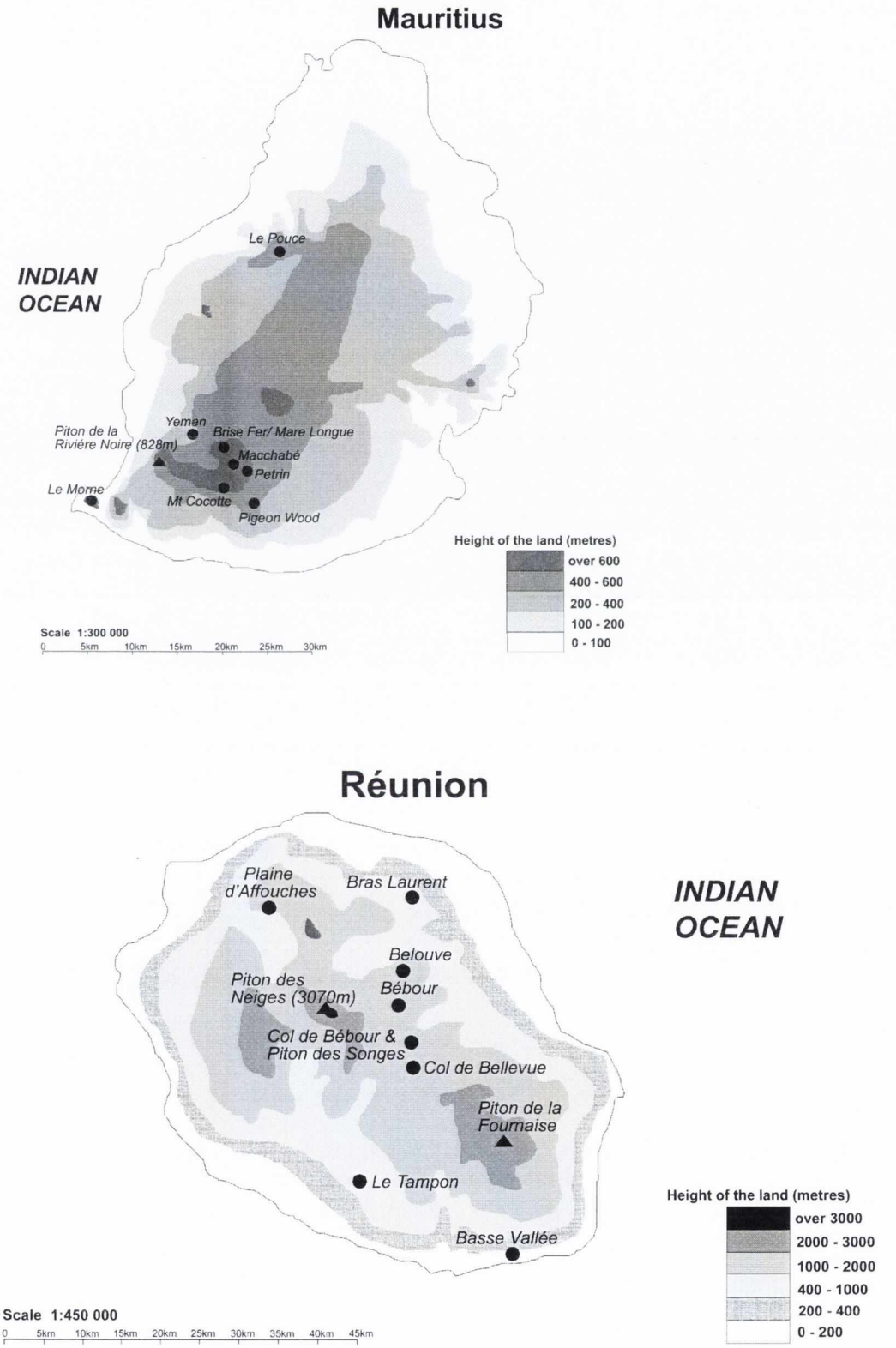


Table 3.1 Taxa, voucher and locality data for all sequenced and genotyped accessions (DNA sequences will be submitted to GenBank)

Herbarium acronyms follow *Index Herbariorum* (Thiers continuously updated) and indicate locations of voucher specimens. Where listed, DNA Bank numbers correspond to the DNA Bank, Laboratoire Evolution et Diversité Biologique, Unité Mixte de Recherches 5174 CNRS-Université de Toulouse, F31062, Toulouse, Cedex 9. France. Collecting locality abbreviations are M for Mauritius, Re for La Réunion and Rod for Rodrigues (see Figure 3.1 for their distribution). *Accession numbers marked with an asterisk indicate those sequenced in a previous study (Chapter 2).

		Locality		
Taxon	Voucher/ Herbarium	Place name	Latitude	Longitude
Badula balfouriana (O. Kuntze) Mez	R. Bone 51/ TCD *	Grande Montagne (Rod)	S 19° 42' 32"	E 63° 27' 47"
Badula balfouriana (O. Kuntze) Mez	R. Bone 52/ TCD*	Grande Montagne (Rod)	S 19° 42' 32"	E 63° 27' 46"
Badula balfouriana (O. Kuntze) Mez	R. Bone 54/ TCD *	Nr Malabar (Rod)	S 19° 42' 35"	E 63° 26' 29"
Badula balfouriana (O. Kuntze) Mez	R. Bone 55/ TCD *	Mt. Cimètiere (Rod)	S 19° 42' 34"	E 63° 25' 31"
Badula balfouriana (O. Kuntze) Mez	R. Bone 56/ TCD*	Grande Montagne (Rod)	S 19° 42' 19"	E 63° 28' 03"
Badula barthesia (Lam.) A. DC.	D. Strasberg & B. Warren 314/ REU	Plaine d'Affouches (Re)	*No record*	
Badula barthesia (Lam.) A. DC.	D. Strasberg & B. Warren 333/ REU	Plaine d'Affouches (Re)	*No record*	
Badula barthesia (Lam.) A. DC.	LR 569/ REU	Basse vallée (Re)	*No record*	
B. aff. *borbonica* A. DC. var. *borbonica*	R. Bone 68/ REU	Nr. Le Tampon (Re)	c. S 21° 15' ?	E 55° 36' ?
B. borbonica var. *macrophylla* (Cordem.) Coode	LR 497/ REU*	Piton des Songes (Re)	*No record*	
B. borbonica var. *macrophylla* (Cordem.) Coode	D. Strasberg 99/ REU*	Piton de Bébour (Re)	*No record*	
Badula crassa A. DC.	R. Bone 22/ TCD *	Nr. Gross Roche, Yemen (M)	S 20° 21' 23"	E 57° 25' 58"
Badula crassa A. DC.	R. Bone 24/ TCD*	Yemen (M)	S 20° 21' 23"	E 57° 25' 58"
Badula crassa A. DC.	R. Bone 58/ TCD *	Mt. Le Morne (M)	S 20° 26' 59"	E 57° 19' 22"
Badula decumbens (Cordem.) Coode	LR471/ REU*	Col Bellevue (Re)	*No record*	
Badula decumbens (Cordem.) Coode	LR 472/ REU*	Col Bellevue (Re)	*No record*	

Table 3. 1 continued

Taxon	Voucher/ Herbarium	Locality		
		Place name	Latitude	Longitude
Badula fragilis Bosser & Coode	R. Bone 69/ REU	Coteau Monique, Bébour (Re)	S 21° 04' 38"	E 55° 32' 40"
Badula fragilis Bosser & Coode	*cf.* Cadet 4078/ REU (tDNA 98)*	Bébour (Re)	*No record*	
Badula fragilis Bosser & Coode	*cf.* R. Bone 70/ REU (tDNA 212)*	Belouve 2 (Re)	S 21° 04' 39"	E 55° 32' 40"
Badula grammisticta (Cordem.) Coode	*cf.* Cadet 6606/ REU (tDNA 69)	Bébour (Re)	*No record*	
Badula grammisticta (Cordem.) Coode	*cf.* Cadet 6606/ REU (tDNA 101)	Plateau Duvernay, Bébour (Re)	*No record*	
Badula insularis A. DC.	R. Bone 4/ MAU*	Petrin (M)	S 20° 24' 19"	E 57° 28' 27"
Badula insularis A. DC.	R. Bone 39/ MAU*	Mt. Le Pouce (M)	S 20° 12' 02"	E 57° 31' 31"
Badula insularis A. DC.	R. Bone 65/ MAU	Cocotte (M)	S 20° 26' 24"	E 57° 28' 22"
Badula multiflora A. DC.	R. Bone 13/ MAU*	Piton de la Riviere Noire (M)	S 20° 24' 35"	E 57° 24' 32"
Badula multiflora A. DC.	R. Bone 17/ MAU*	Piton de la Riviere Noire (M)	S 20° 25' 18"	E 57° 25' 23"
Badula multiflora A. DC.	R. Bone 19/ MAU*	Piton de la Riviere Noire (M)	*No record*	
Badula cf. nitida (Coode) Coode	D. Strasberg 48/ REU*	Bras Laurent (Re)	*No record*	
Badula nitida (Coode) Coode	LR 514/ REU*	Bras Laurent (Re)	S 21° 58' 33"	E 55° 33' 29"
Badula nitida (Coode) Coode	LR 515/ REU*	Bras Laurent (Re)	S 21° 58' 33"	E 55° 33' 29"
Badula ovalifolia A. DC.	R. Bone 30/ TCD	Maccabé (M)	S 20° 24' 00"	E 57° 27' 06"
Badula ovalifolia A. DC.	R. Bone 31/ TCD*	Brise Fer, Picadilly Circus (M)	S 20° 22' 30"	E 57° 26' 44"
Badula platyphylla (A. DC.) Coode	R. Bone 2/ TCD *	Petrin CMA (M)	S 20° 24' 29"	E 57° 28' 11"
Badula platyphylla (A. DC.) Coode	R. Bone 3/ MAU*	Petrin CMA (M)	S 20° 24' 28"	E 57° 28' 10"
Badula platyphylla (A. DC.) Coode	R. Bone 38/ TCD*	Laing, outside Petrin CMA (M)	S 20° 24' 19"	E 57° 28' 00"
Badula reticulata A. DC.	R. Bone 9/ TCD*	Nr. Petrin-Macchabé track (M)	S 20° 24' 09"	E 57° 27' 48"

Table 3. 1 continued

Taxon	Voucher/ Herbarium	Locality		
		Place name	Latitude	Longitude
Badula reticulata A. DC.	R. Bone 21/ TCD*	Nr. Petrin-Macchabé track (M)	S 20° 24' 10"	E 57° 27' 48"
Badula reticulata A. DC.	R. Bone 72/ TCD	Mare Longue (M)	S 20° 23' 55"	E 57° 27' 28"
Badula sieberi A. DC.	R. Bone 8/ MAU*	Pigeon Wood (M)	S 20° 26' 39"	E 57° 28' 59"

Data analysis

AFLP markers

DNA fragments ranging in size from 50 bp to 500 bp were visualized as electropherograms using Genemapper v.4.0 (Applied Biosystems). Fragments were scored manually as presence (1) or absence (0) characters. All samples were Weak, ambiguous peaks were removed from the matrix before analysis. Mantel tests were performed (for 999 permutations) and measured by the Mantel correlation coefficient calculated in GenAlEx (Smouse & Long 1992; Smouse *et al.* 1986) to test for congruence between datasets generated by the four different primer pairs. Subsequent analyses included all AFLP data as one combined matrix.

Phylogenetic analyses

All phylogenetic analyses were conducted in PAUP* 4.0b10 (Swofford 2002). Maximum parsimony (MP) was performed using the heuristic search option with one thousand replicates of random-sequence addition and the tree bisection-reconnection (TBR) option in effect. Thirty-five trees were saved per replicate for branch swapping to increase efficiency of searches in tree space and reduce time spent in exhaustive 'hill-climbing' on sub-optimal islands of trees (Swofford *et al.* 1996). In addition, the AFLP dataset was analysed using the neighbour-joining (NJ) method (cf. Swofford *et al.* 1996) computed with Nei-Li distances (Nei & Li 1979) which weights data in favour of shared bands.

Statistical support for clades recovered in all phylogenetic analyses was estimated with the bootstrap procedure (Felsenstein 1985) with 1,000 replicates of simple sequence addition and TBR branch swapping, or 1,000 NJ replicates. Final phylogenetic analyses were carried out on a combined matrix of the AFLP and sequence data following the procedures described above.

Where phylogenetic reconstructions are presented as rooted trees, *Badula balfouriana* was selected as the outgroup based on the work presented in Chapter 2 (which found strong statistical support for the sister relationship of *Badula balfouriana* to all other *Badula* species, in relation to the allied genus *Oncostemum*). In order to determine whether the position of *Badula balfouriana* was affected by the increased number of potentially parsimony informative characters (from AFLPs) and the accessions sampled (that differed from Chapter 2) a phylogenetic analysis was executed with a combined dataset that included DNA sequences from an *Oncostemum* sample.

The resulting phylogeny, rooted with *Oncostemum*, showed the same sister relationship of *B. balfouriana* (data not shown), congruent with earlier findings (Chapter 2). The *Oncostemum* sequences were removed from the matrix for all subsequent analyses.

Principle Coordinates Analysis

Principle Coordinates Analysis (PCoA) is commonly used to identify patterns of variation in AFLP derived genetic-fingerprint data (e.g. Devey *et al.* 2008; Gill 2007; Hodkinson *et al.* 2002; Kelleher *et al.* 2005; Richardson *et al.* 2003). The binary AFLP data are first of all converted to a pairwise distance matrix. The PCoA then converts these values to Eigenvectors and plots them in multi-dimensional space, allowing the major axes of variation in the data to be identified (Gill 2007; Peakall & Smouse 2005). Each successive axis explains proportionally less of the variation in the data.

Because of the existence of many shared absences (0s) in my data-set, I used the Sørensen-Dice distance measure (Dice 1945; Sørensen 1948), which weights data in favour of presence of shared bands and is equivalent to Nei-Li distance (Cole & Kuchenreuther 2001). The distance matrix was calculated in Le Progiciel R v.4.0d (Casgrain 1999) using the Simil package, followed by PCoA ordination computed in GenAlEx (Peakall & Smouse 2006).

3.4 Results

AFLP data

The selected primer pairs produced a large number of AFLP markers for analysis, with a total of 744 bands, of which 448 were parsimony informative. The primers pairs and corresponding results were as follows: *Eco*RI-ACT + *Mse*I-CAG produced 194 bands including 107 parsimony informative characters (PIC); *Eco*RI-ACT + *Mse*I-CAA, 138 bands with 110 PIC; *Eco*RI-ACA + *Mse*I-CAG, 256 with 154 PIC; *Eco*RI-AGC + *Mse*I-CTG, 156 with 77 PIC. All Mantel tests revealed significant positive correlations between all primer pairs (Mantel correlation coefficient *Rxy* 0.4 to 0.6, $P = 0.001$).

Phylogenetic analyses of the AFLP data

The two phylogenetic methods (NJ and MP; Fig. 3.2 and Fig. 3.3 respectively) gave similar results and, where the tree topologies were in agreement, there was strong bootstrap support for clades recovered by both methods. In the NJ tree, where taxa are represented by more than one accession, most taxa form monophyletic groups. Exceptions are accessions of the La Réunion taxa *Badula barthesia* (DS333 & DS314), *B.* aff. *barthesia* (LR569) and *B.* aff. *borbonica* var. *borbonica* (RB68), whose positions were unresolved among the clade of La Réunion taxa. All taxa except *Badula crassa* are resolved as part of distinct geographical clades, by island. *Badula crassa* (from Mauritius) was placed as sister to *B. balfouriana* (from Rodrigues). When rooted (with *B. balfouriana*) this meant that *B. crassa* was placed as sister to all other *Badula* species of the ingroup. The MP analysis recovered two trees of length 1973. The consistency (CI) and retention indices (RI) were low (0.31 and 0.45, respectively), but this was not unexpected given that the analysis was based on binary rather than nucleotide data. Again, most taxa (when represented by more than one accession) formed monophyletic groups, and several have moderate to strong support in both analyses. In this analysis, the only exception was *Badula reticulata* of Mauritius, which is shown to be paraphyletic with *Badula platyphylla* nested within it (although this relationship is weakly support; < 50 BP). The La Réunion accessions unresolved by the NJ analysis, were here resolved in two distinct clades (*B. barthesia* DS333 and DS314 together, and *B.* aff. *borbonica* var. *borbonica* RB68 with *B.* aff. *barthesia* LR569), although neither clade was supported by bootstrap analysis (<50 BP). In addition, the three

geographically defined clades seen in the NJ tree were again resolved here and grouped taxa from each of the Mascarene islands. The Rodrigues and La Réunion clades both had strong support (100 and >90 bootstrap percentage, BP, respectively), but the Mauritius clade, although present in both the MP and NJ trees, had no bootstrap support in either analysis and different topologies were recovered for the Mauritius clade by each method.

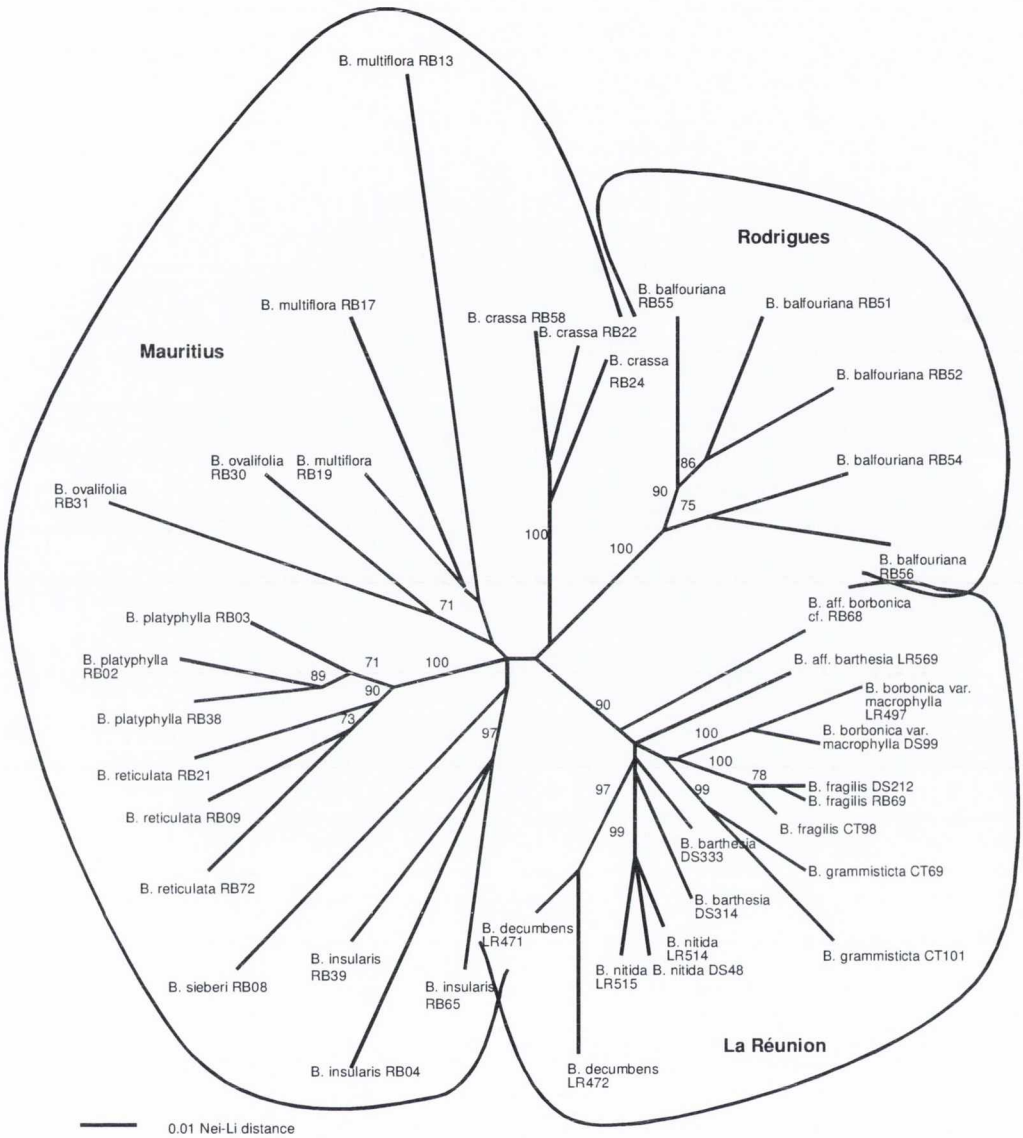


Fig. 3.2 NJ tree from analysis of AFLP data, presented as an unrooted phylogram

Numbers above branches are bootstrap (BP) values after 1,000 NJ replicates (shown where >50 BP). Branch lengths indicate genetic distance. All taxa except *Badula crassa* fall into geographical clades, by island (indicated by the curved outline & labelled by island names). *B. crassa*, however, forms a weakly supported clade with *B. balfouriana* from Rodrigues (<50 BP).

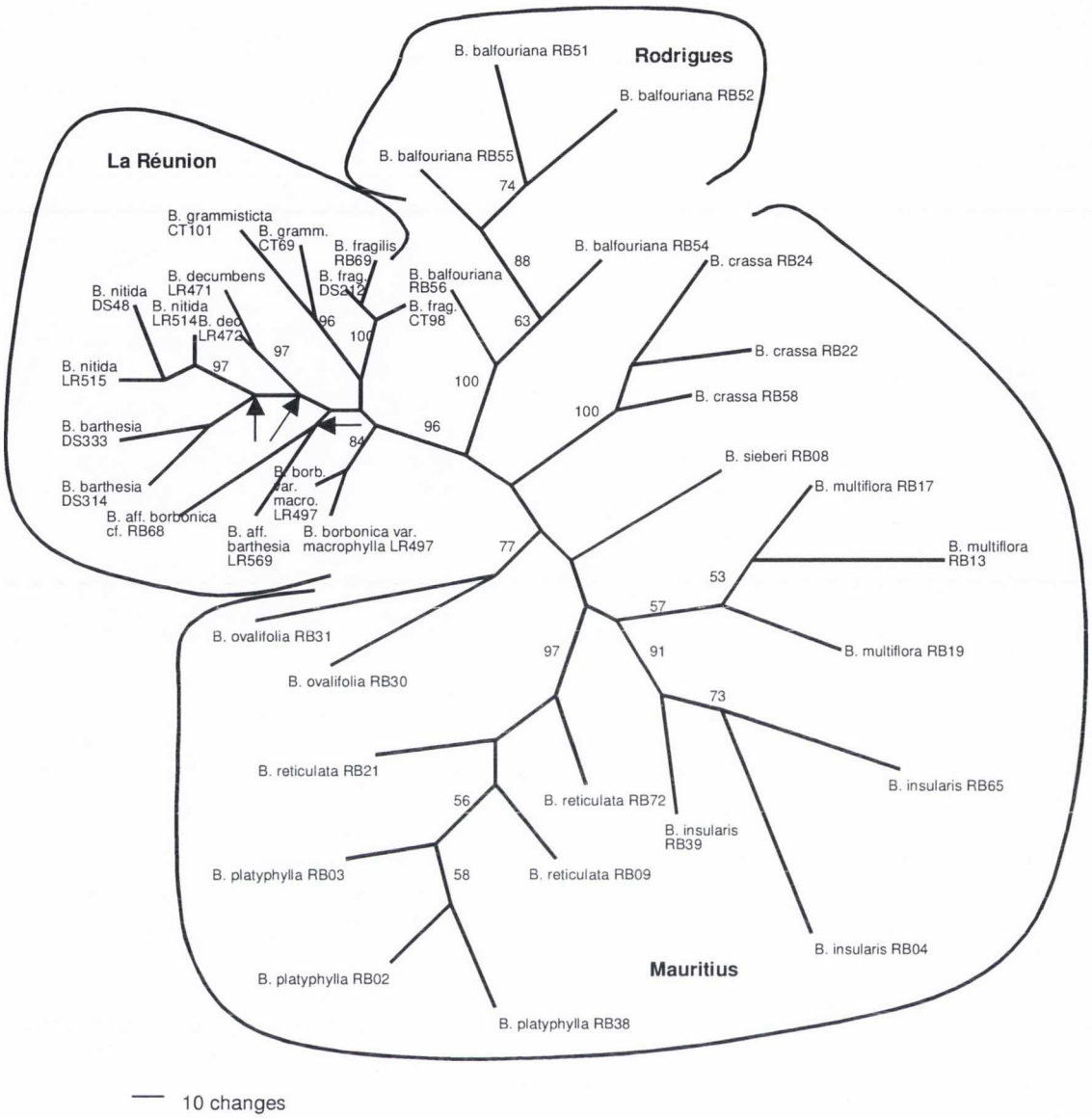
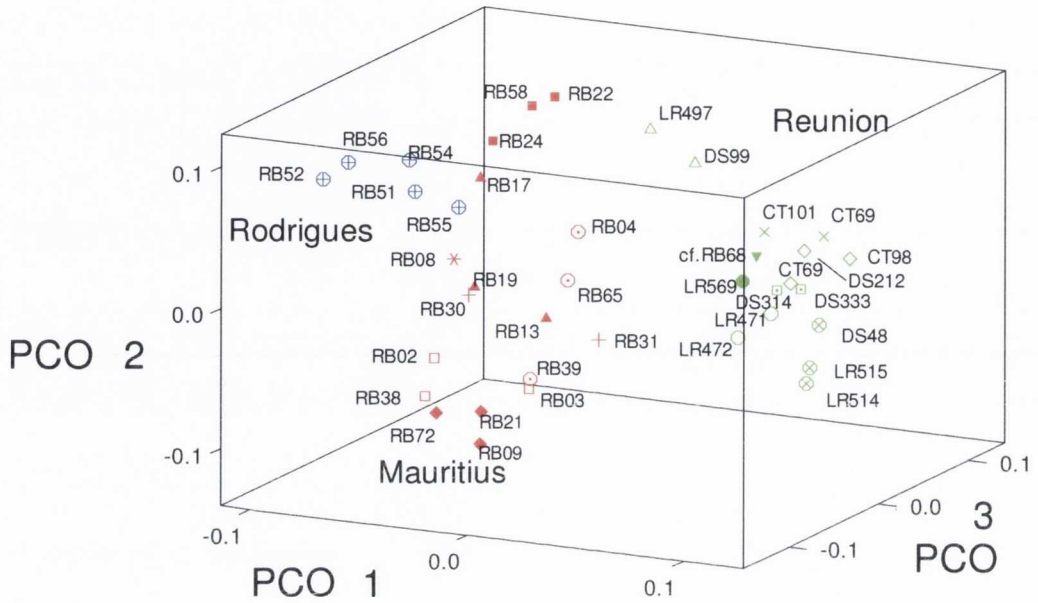


Fig. 3.3. One of the two maximally parsimonious trees for the AFLP data presented as an unrooted phylogram. Tree length 1973, CI 0.31, RI 0.45. Values above branches are bootstrap values (shown where >50 BP) after 1,000 simple sequence addition replicates. Branch lengths indicate the number of character state changes. Arrows indicate branches that collapse in the strict consensus tree. All taxa fall into geographical clades, by island (highlighted by the curved line, and labelled by island name). *Badula crassa* is resolved as part of the Mauritius clade, and outlying other Mauritius taxa. Where used, abbreviations are as follows: B. dec. is *B. decumbens* LR472; B. frag. DS212 and CT98 are accessions of *B. fragilis*; B. gramm. CT69 is *B. grammisticta* CT69.

The results from the PCoA are illustrated in Figure 3.4. The first two axes account for 48.85 % of the variance in the data, with 27.69 % for axis 1 and 21.17 % for axis 2. The third axis contributed 15.65 % of the data variance, resulting in a cumulative value of 64.5 % for the first three axes. The taxa are separated broadly into three geographical groups, representative of taxa from the three islands. In addition some taxonomic groupings can be seen, for example among La Réunion taxa, the three accessions of *Badula nitida* (LR514, LR515 and DS48) are clustered together, and the two accessions of *B. borbonica* var. *macrophylla* (DS99 and LR497) appear somewhat distant from other the rest of La Réunion group. Among taxa from Mauritius, the three accessions of *B. reticulata* (RB09, RB21 and RB72) are grouped, and the *B. platyphylla* samples are placed nearby. *Badula crassa* (Mauritius) clusters with *B. balfouriana* (Rodrigues) on PCO 1 and PCO2 but is separated from it by the variation in the third axis. A single *B. multiflora* accession (RB17) is also grouped with these taxa.

Fig. 3.4 Plot of the Principle Coordinates Analysis for the AFLP data

The plot of the first, second and third axes explain 27.69, 21.17 % and 15.65% of the variance in the dataset, respectively. Taxa highlighted in red are all from Mauritius, those from La Réunion are in green and those from Rodrigues are in blue. See Legend for symbols corresponding to each taxon. Accession numbers follow table 3.1. All accessions appear to be broadly segregated by geographic origin.



Legend

Rodrigues taxa	La Réunion taxa	Mauritius taxa
⊗ *B. balfouriana*	⊡ *B. barthesia*	■ *B. crassa*
	● *B. aff. barthesia*	⊙ *B. insularis*
	▼ *B. aff. borbonica var. borbonica*	▲ *B. multiflora*
	△ *B. borbonica var. macrophylla*	+ *B. ovalifolia*
	○ *B. decumbens*	□ *B. platyphylla*
	◇ *B. fragilis*	◆ *B. reticulata*
	× *B. grammisticta*	✳ *B. sieberi*
	⊗ *B. nitida*	

DNA sequence data

Sequences of the ITS region and the co-amplified *trnS-trnG* spacer and *trnG* intron (hereafter referred to as *trnSGG*), were obtained for nine of the ten accessions listed in Table 3.1 (novel to this work) and added to sequences of 29 accessions from a previous study (to be made available in GenBank®; cf. Chapter 2). *Badula ovalifolia* RB30 was not successfully amplified for the ITS region, and *B. insularis* RB65 was not amplified for *trnSGG*. These samples were therefore scored as missing data in the

aligned matrices. In the ITS matrix, 672 characters were included of which 9 were potentially parsimony informative, and for the *trnSGG* matrix a total of 1,408 characters were included, 9 of which were also potentially parsimony informative. Preliminary analyses were carried out separately for the nuclear and chloroplast data sets due to the potential for different evolutionary histories to be inferred with markers from the two genomes (Wendel & Doyle 1998).

The analyses recovered contrasting topologies (see Appendix 1). The ITS data showed some resolution of the spine of the tree, with 88 % bootstrap support for the clade of ingroup taxa (all species except the outgroup *B. balfouriana*). Within this clade a trichotomy was resolved comprising two weakly supported (≤ 56 BP) Mauritius clades and a polytomous La Réunion clade with no boostrap support (<50 BP). The smaller of the Mauritius clades (with 53 BP) included all *Badula multiflora* accessions and *B. sieberi* RB08. *Badula ovalifolia* RB30 was also placed here, however this accession was scored as missing data for the ITS matrix and its position should be interpreted as unresolved in this analysis. All other Mauritius accessions were included in the larger clade (with 56 BP). In the latter group, five *Badula reticulata* and *B. platyphylla* accessions formed the only moderately supported clade of this analysis (85 BP).

By contrast, the *trnSGG* data produced a tree with a polytomous spine, but grouped several accessions into taxon-specific clades unresolved by the ITS phylogeny (*B. crassa, B. grammisticta, B. multiflora, B. nitida, B. ovalifolia* and *B. reticulata*; Appendix 1). However, only the *B. crassa* clade received over 80 BP support. Because no incongruence with moderate or strong bootstrap support was found (60 BP >) subsequent analysis proceeded with combined data.

The combined data-set comprised a total of 2,080 characters (of which 18 were potentially parsimony informative) and recovered 512 maximally parsimonious trees, of length 36, with a CI of 0.92 and RI of 0.96. The strict consensus tree from this analysis (Fig. 3.5), rooted with *Badula balfouriana*, recovered the same clades outlined above, but statistical support was improved for all taxon-specific clades. As above, the only grouping of different taxa occurred for *B. reticulata* and *B. platyphylla*, and all six accessions formed a clade with increased support (87 BP), within which further differentiation between the species was seen. In addition, a La Réunion clade was recovered by the combined analysis, but despite being retained in the strict consensus of all 512 trees, it had no bootstrap support (<50 BP). The Mauritius clade collapsed to a

polytomy in the strict consensus, and similarly had no bootstrap support, whereas the *B. balfouriana/* Rodrigues clade was robust (95 BP; retained in the un-rooted strict consensus, not shown). Apart from *Badula balfouriana*, few other taxon-specific groups were resolved by the DNA sequence data however. Among those retained in the strict consensus tree are *B. multiflora* (73 BP), *B. grammisticta* (64 BP) and *B. nitida* (63 BP), which are not strongly supported by bootstrap values.

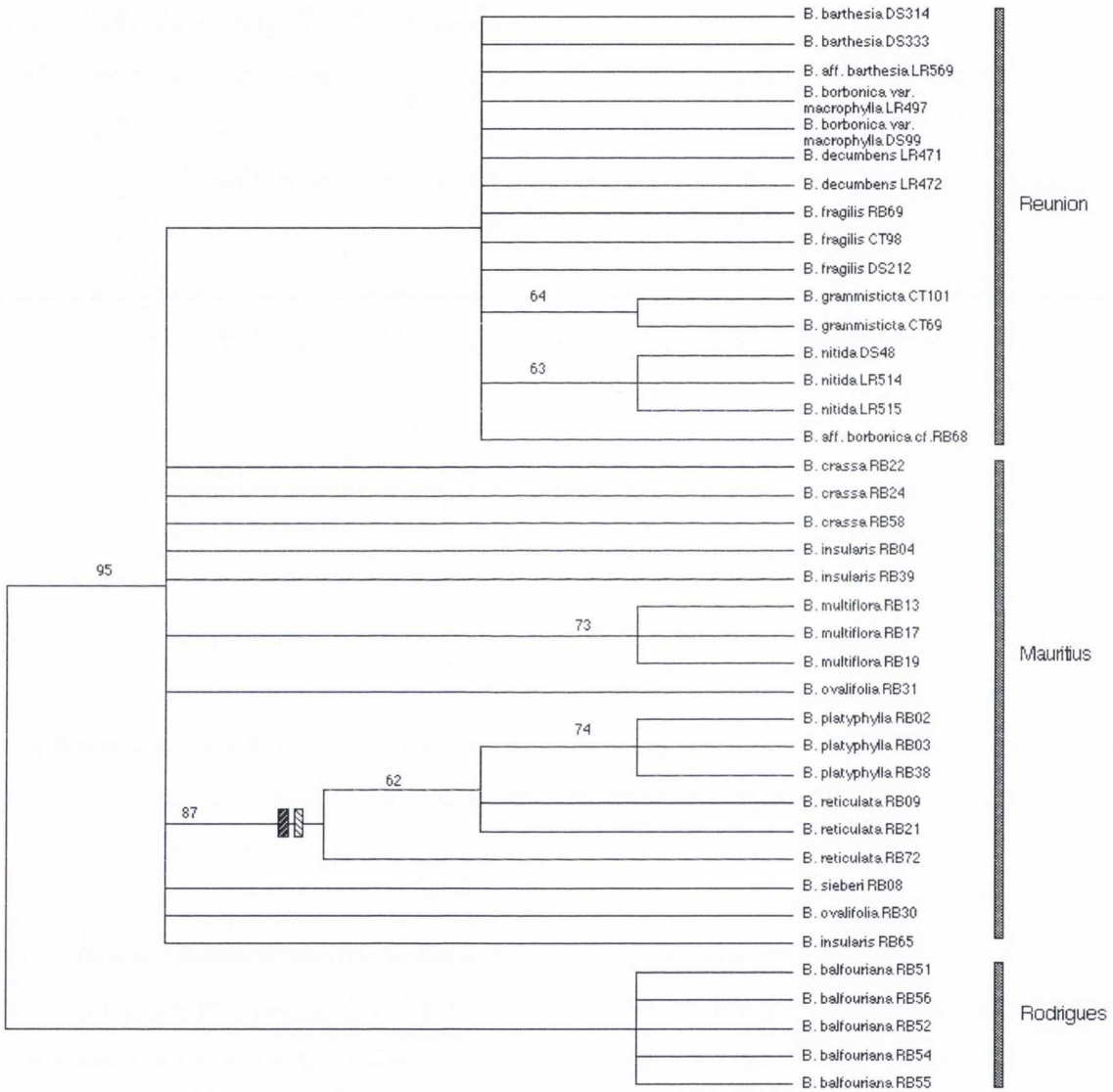


Fig. 3.5 The strict consensus tree for the combined ITS and *trnSGG* data set.

The MP analysis recovered 512 maximally parsimonious trees, of length 36, with a CI of 0.92 and RI of 0.96. Values above branches are bootstrap values after 1,000 simple sequence addition replicates (shown when >50 BP). Vertical bars indicate the geographical groups of taxa. Shaded bars at the base of the *Badula platyphylla/ B. reticulata* clade represent morphological synapomorphies referred to in the text: these species share the unique character of fused filaments (Coode, 1981) and long (50 – 300 mm), pendulous inflorescences (R. E. Bone, pers. obs.; Coode, 1976; 1981).

Phylogenetic analysis of the combined AFLP and DNA sequence data

Topologies generated by maximum parsimony analysis of each dataset revealed agreement on composition of all clades with greater than 50 BP, and hence that the different markers complemented one another in elucidation of phylogenetic relationships. Final parsimony analysis therefore proceeded with a combined AFLP and sequence data that included 2,824 characters of which 466 were potentially parsimony informative. Figure 3.6 shows one of the two most parsimonious trees (length 2,010 steps, CI 0.32 and RI 0.46) recovered by the MP analysis, as an unrooted phylogram. The topology is generally in agreement with those of previous analyses of the AFLP data but with increased statistical support for some clades (e.g. support for the *Badula multiflora* clade increased from 57 to 76 BP).

Geographical groups

The maximum parsimony analyses of all datasets each show geographical groupings of species by island. In the strict consensus tree (Fig. 3.7) of the combined AFLP and sequence data, three island clades are retained, and statistical support was strong for the Rodrigues and La Réunion clades (100 and 98 BP respectively). The Mauritius clade had no statistical support however (< 50 BP), congruent with earlier findings (Chapter 2). The NJ and PCoA results broadly agree with these results and taxa are clustered into three groups of accessions from the three islands. However, in the NJ tree, all *Badula crassa* accessions (from Mauritius) cluster with *B. balfouriana* of Rodrigues.

Taxonomic groups

More than one sample representative of a taxon was included for thirteen of the fifteen *Badula* taxa. In these cases moderate to strongly supported taxon-specific clades were recovered in the combined analysis for all but one species (*B. barthesia*, <50 BP). In addition, the lone sample of *B.* aff. *borbonica* var. *borbonica* (RB68) grouped with *B.* aff. *barthesia* (LR569), which morphologically resembles an intermediate form of *Badula barthesia* and *B. grammisticta* (D. Strasberg, pers. comm.), but this clade had no statistical support (<50 BP).

The most robust taxon-specific clades (100 BP) in the combined analysis were those that comprised *Badula fragilis* (three accessions), *Badula crassa* (three accessions) and *Badula balfouriana* (five accessions). Differentiation within species

was also resolved in these three species. In the *Badula fragilis* clade, accession CT68 was resolved as sister to a moderately supported clade of RB69 and DS212 (78 BP and retained in the strict consensus tree). Among the *Badula crassa* accessions, RB58 was placed as sister to RB22 and RB24; a relationship retained in the strict consensus tree but not supported by bootstrap analysis (< 50 BP). In the *Badula balfouriana* clade sample RB56 out-lies the other accessions, which are successively sister to one another, with bootstrap support for some relationships.

Other well supported taxon-specific clades were recovered for the La Réunion species *Badula grammisticta* (98 BP), *B. decumbens* (96 BP) and *B. nitida* (99 BP). Within the latter clade, *B. nitida* LR514 was sister to the remaining two accessions (LR515 and DS48) but this relationship had no support (<50 BP). The two accessions of *B. borbonica* var. *macrophylla* also form a clade (86 BP). The Mauritian species *Badula insularis, B. multiflora* and *B. ovalifolia* all formed moderate to well supported clades (92, 76 and 83 BP respectively).

Relationships between taxa were retained in the strict consensus tree from the combined data analysis, but were weakly supported. For example, among the La Réunion taxa, *Badula borbonica* var. *macrophylla* was resolved as sister to all other La Réunion taxa, a position also supported by the PCoA of the AFLP data (Fig. 3.4). All other La Réunion taxa formed two further clades: one comprising *Badula fragilis* and *B. grammisticta* as sister species, and the second being a polytomy of four taxa (of which *B. nitida* and *B. decumbens* were each grouped into well supported clades). However these supra-specific clades were not supported by bootstrap values (none exceed 50 BP). Among the Mauritius taxa, the strict consensus tree recovered *Badula crassa* as sister to a large clade of all Mauritian *Badula*. Within this large clade, *Badula ovalifolia* was the most phylogenetically outlying species, then *Badula sieberi*, which in turn was sister to the remaining Mauritian species of *B. insularis* + *B. multiflora* and a *B. platyphylla, B. reticulata* clade. In the latter clade, strong support (99 BP) was revealed for the outlying position of *B. reticulata*, but there was a lower bootstrap value for the relationship of this species to accessions of *B. platyphylla* (71 BP). The *B. platyphylla, B. reticulata* clade was the only one that grouped Mauritian species with significant bootstrap support.

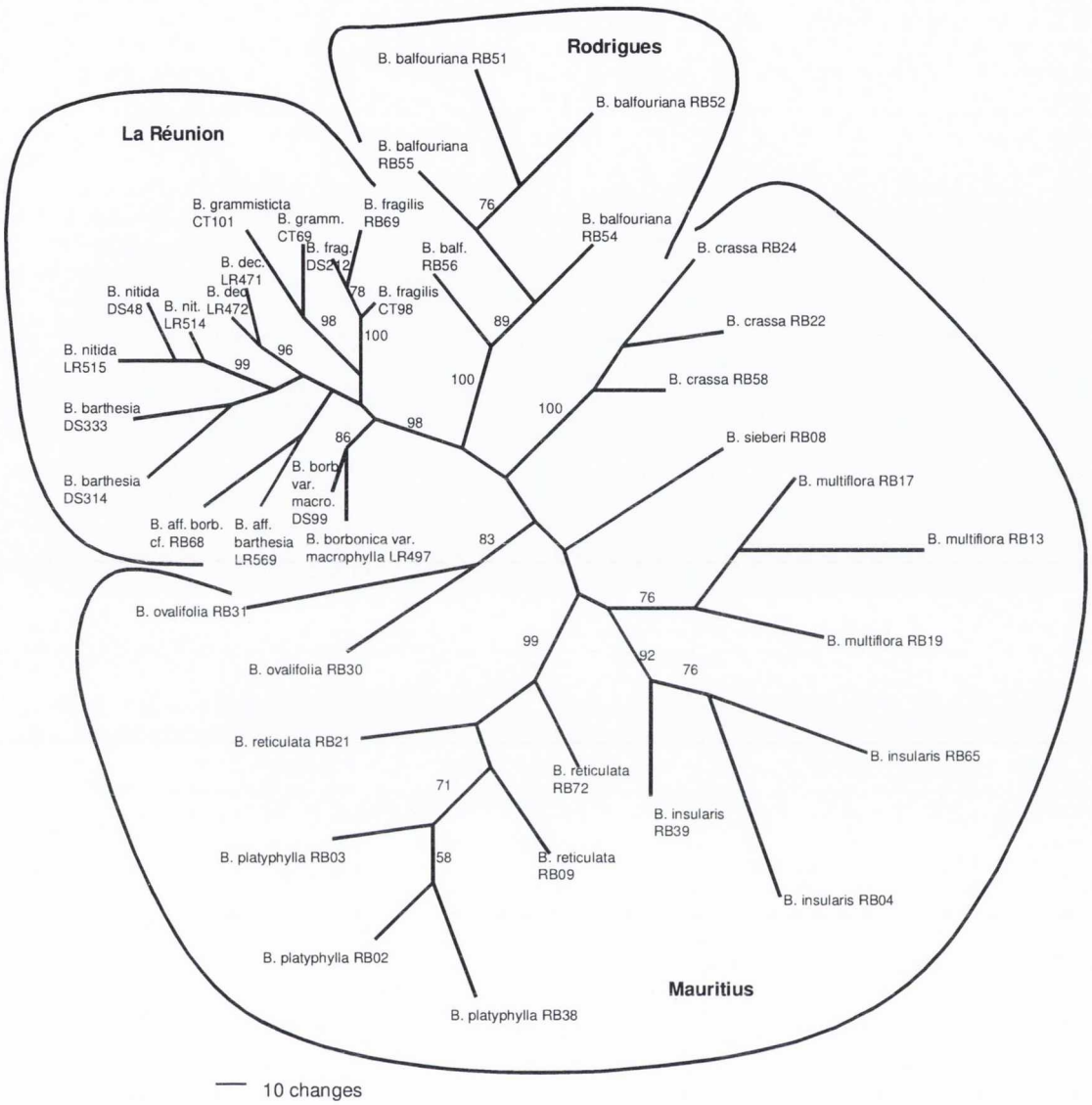


Fig. 3.6. One of the two most parsimonious trees of the combined AFLP and DNA sequence data, presented as an unrooted phylogram.

Length 2,010 steps, CI 0.32 and RI 0.46. Values above branches are bootstrap percentage values (not shown if < 60 BP). Where used, abbreviations are as follows: B. balf. is *B. balfouriana*; B. aff. borb. cf. RB68 is *B. aff. borbonica* var. *borbonica* cf. RB68; B. dec. is *B. decumbens*; B. frag. is *B. fragilis*; B. gram. is *B. grammisticta;* B. nit. is *B. nitida.*

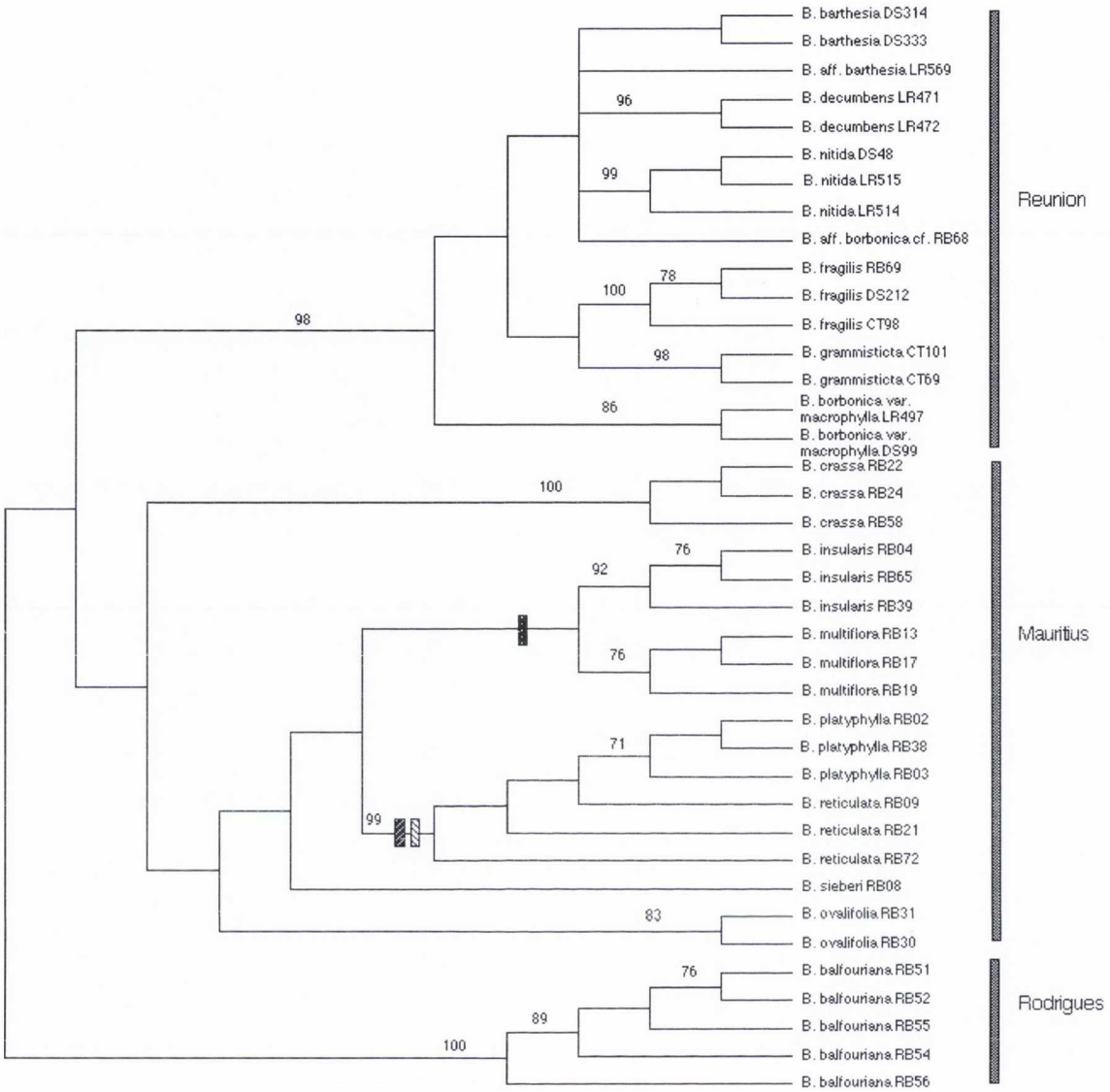


Fig. 3.7. Strict consensus of the two maximally parsimonious trees recovered from the combined AFLP and sequence data

Two most parsimonious trees were recovered by the analysis (one is shown is Fig. 3.6). Values above branches represent bootstrap percentages (BP) shown if > 60 BP. Vertical grey bars indicate the geographical groups of taxa. Taxa from Rodrigues and La Réunion are resolved in strongly supported clades (100 BP and 98 BP respectively). Taxa from Mauritius also form a clade, suggesting a common origin, however there is no bootstrap support (<50 BP) for this clade. Within geographical groups, several well supported taxon-specific clades are resolved. Shaded bars at the base of clades represent morphological synapomorphies referred to in the text: among Mauritian taxa, black dots in the leaf lamina are found only in *B. multiflora* and *B. insularis* (R. E. Bone, pers. obs.); *B. platyphylla* and *B. reticulata* are united by the unique character of fused filaments (Coode, 1981) and long, pendulous inflorescences (R. E. Bone, pers. obs.; Coode, 1976; 1981).

3.5 Discussion

Taxonomic groupings

The combined analysis of AFLP and DNA sequence data has recovered clades consistent with the current classification of *Badula* (Coode 1981). Virtually all accessions were resolved in taxon-specific clades, and the majority of these have moderate to strong support. For example, in agreement with the results of Chapter 2, there is clear distinction of *Badula crassa* and *Badula fragilis* into separate clades. In Figure 3.7, *Badula nitida* (raised to species rank by Coode from *B. barthesia* Bojer var. *nitida* Coode) is shown to be monophyletic and is resolved as sister to *B. barthesia*, although there is no statistical support for this relationship. In addition, *Badula* aff. *borbonica* var. *borbonica* (cf. RB68) does not group with *B. borbonica* var. *macrophylla* but falls into a separate clade. Further sampling of *B. borbonica* var. *borbonica* is needed however, to resolve relationships of these varieties and verify the identity of this sample. Among Mauritian taxa the sister relationship of *B. multiflora* and *B. insularis* is supported by a synapomorphy of black markings within the leaf lamina (R. E. Bone, pers. obs.). Likewise, several synapomorphies unite *B. reticulata* and *B. platyphylla*, including the long pendulous inflorescences and fusion of the filaments (Coode 1981).

Biogeography

The phylogenetic reconstructions presented here have identified a clear pattern of geographical discrimination within *Badula,* with those taxa from Rodrigues and La Réunion separated into two distinct groups with strong support (100 BP and 90-98 BP respectively, in both NJ and MP analyses), congruent with the results of Chapter 2. It is therefore inferred that the *Badula* taxa of Rodrigues and La Réunion are derived from a single ancestral species unique to each island and that, following establishment, the *Badula* lineages of each island diversified independently.

The relationships among Mauritian taxa are less well defined however. Previous workers reported a similarly ambiguous Mauritian clade due to the unresolved position of *Badula multiflora* (Chapter 2). Here the position of a different Mauritian species, *Badula crassa,* is ambiguous and varies among data analysis methods. In the MP tree (Fig. 3.3) and PCoA (Fig. 3.4) of AFLP data and the MP tree of the combined AFLP and sequence data (Fig. 3.7) *Badula crassa* is present in a clade that contains all

Mauritian taxa, whereas the NJ tree places this species as sister to *B. balfouriana* of Rodrigues and out-lying a clade of all *Badula* species from Mauritius and La Réunion. The morphological affinity of *Badula crassa* and *B. balfouriana* (Coode 1981) supports the genetic similarity of these species inferred by the NJ analysis. In addition, the dry forest habitat of both species (otherwise unknown in *Badula*) represents an ecological synapomorphy for them.

If the contrasting phylogenetic reconstructions are rooted using *Badula balfouriana* (following Chapter 2) some different inferences about the colonization patterns of *Badula* can be made. For example the rooted MP strict consensus tree (Fig. 3.7) infers that the common ancestor of the genus *Badula* gave rise to *B. balfouriana* on Rodrigues and two separate ancestors of the Mauritius and La Réunion taxa. Within the Mauritius clade, a common ancestor then gave rise to *B. crassa* and the remaining *Badula* species on the island. In contrast, if the NJ tree (Fig. 3.2) was rooted *B. balfouriana* (not shown), *B. crassa* would be resolved in an outlying position sister to a clade comprising all other Mauritian and La Réunion *Badula* species. Given the current status of *B. crassa* as a Mauritian endemic, this would suggest that a sister species to *B. crassa* evolved in Mauritius and gave rise to the two separate *Badula* lineages of Mauritius and La Réunion.

The sister relationship of *Badula crassa* and *B. balfouriana* in the NJ tree has no bootstrap support however (<50 BP), and these species are grouped by very short branch lengths (Fig. 3.2). The alternative phylogenetic reconstruction, presented by the MP analysis, similarly recovered no bootstrap support for the sister relationship of *B. crassa* to the other Mauritian species (<50 BP), despite the clade being retained in the strict consensus tree (Fig. 3.7). Statistical support was also not recovered for other groupings in the spine of the Mauritius clade in the strict consensus tree (with the single exception of *B. platyphylla* and *B. reticulata*, 99 BP). Given the absence of statistical support for the Mauritius clade, patterns of diversification cannot yet be determined. There is a possibility that multiple colonizations have lead to the establishment of more than one lineage on this island (consistent with the monophyly of *Badula,* but the lack of a common ancestor for the Mauritius taxa), a pattern also recovered in Chapter 2 where the position of *Badula multiflora* was resolved as ambiguous. The similarity of habitats occupied by *Badula balfouriana* and *B. crassa* may be indicative of niche conservatism within the Mascarene flora (sensu Wiens & Graham 2005) rather than the direct relatedness of these species, and the morphological similarity may be a

consequence of convergence. Further evidence is required to determine the origins of the Mauritius *Badula* species and the affinity of *Badula crassa* and *B. balfouriana*.

Despite these limitations, some inferences regarding the relative time-scale of colonisation events can be drawn from comparison of the estimated ages of the three islands and the differing branch lengths (revealed by both character state changes and Nei-Li genetic distance) among individuals and among species of *Badula*. For example, branch lengths among taxa on the youngest and western-most island (La Réunion, < 3 Myr) are much shorter than those from the older islands (Mauritius, c. 8 Myr and eastern-most Rodrigues c. 10+ Myr) as exemplified by the greater character state divergence *within* some Mauritian species (e.g. *Badula insularis*) than *between* several of the La Réunion taxa (e.g. *B. fragilis* and *B. borbonica* var. *macrophylla*). This suggests that rapid and recent speciation has occurred on La Réunion relative to the establishment of species on Mauritius and Rodrigues. Furthermore, examination of the Soil Map of Mauritius (Anon. 1962) reveals that *B. crassa* is the only *Badula* species that occurs on (and is apparently limited to) Magenta type soils derived from Older Volcanic Series parent rock restricted to the southwest of Mauritius (Parish & Feillafé 1965), while *B. balfouriana* is the only *Badula* species that currently occurs on Rodrigues, thought to be the oldest of the Mascarene islands (Cheke & Hume 2008). These two species are indicated as putative outliers in the *Badula* phylogeny (*B. balfouriana* in Chapter 2 and, with less certainty, *B. crassa* in this chapter). This again provides some support for the hypothesis that ancestral *Badula* became established on Rodrigues and Mauritius some time before it reached La Réunion.

Diversification and micro-endemism

The monophyly of the La Réunion *Badula* taxa infers that they have evolved on this island following a single successful colonisation event by an ancestral species. Subsequent speciation may have then arisen in a number of ways, for example by micro-allopatry, parapatry or sympatry or as a consequence of hybridisation. Intermediate forms between some of the more widespread taxa (*B. borbonica* varieties, *B. barthesia* and *B. grammisticta*) are reported where their distributions overlap (Coode, 1981; D. Strasberg pers. comm., 2008), suggesting that they are not reproductively isolated, and that hybridisation may occur. In addition, the phylogenetic position of two accessions (RB68 and LR569) from La Réunion were not clearly resolved by these analyses. On the other hand, species such as *B. decumbens* and *B. fragilis* appear to be micro-endemics that grow only in specific locations where, despite their close proximity

to other more 'promiscuous' species, intermediate forms are unknown (R. E. Bone, pers. obs.) and my results corroborate their distinctness.

On the upland plateau of Mauritius, the endemic species *Badula reticulata, B. multiflora*, and *B. insularis* grow in the immediate vicinity of one another, spaced only a few metres apart (e.g. at Brise Fer; R. E. Bone pers. obs., 2008) yet no intermediate forms are known. In other cases, ecological barriers appear to segregate species. For example *Badula crassa,* which appears to have been historically restricted in range to the dry west and southwest coast of the island. *Badula reticulata* and *Badula platyphylla* also occur in different habitats. Both species occur on the upland plateau in the southwest of Mauritius, and in some cases they are separated by a distance of less than 2 km, however, *Badula reticulata* appears to be restricted in distribution to the remaining fragments of upland forest in Mauritius, where it grows in humic soils beneath a dense Sapotaceae dominated canopy, while *B. platyphylla* grows in the neighbouring heath-like vegetation on water-logged laterite gravel and thin soils. These species are morphologically and genetically similar and were resolved as sister taxa in previous work (Chapter 2), and the NJ analysis of the AFLP data supports this relationship. However the MP analyses (of the AFLP data alone, and in combination with DNA sequence data) gave conflicting results and inferred that *Badula platyphylla* is nested within *B. reticulata*.

Concluding remarks

The phylogenetic reconstructions of *Badula* recovered several robust taxon-specific clades that were not evident in previous analyses based on DNA sequence data alone. With the addition of AFLP data, the number of potentially parsimony informative characters was raised from 18 (from both the *trnSGG* and ITS datasets) to 466. As such, my results confirm the utility of AFLP markers in species delimitation among taxa where a lack of sequence divergence (and in some cases morphological characters) distinguish them. These results indicate that dispersal between the Mascarene islands has occurred just a few times with intra-island speciation playing a predominant role in the generation of current *Badula* species diversity. While the results support the species delimitations of the current classification of *Badula*, not all accessions sampled from La Réunion were resolved in taxon-specific clades, and on-going speciation is inferred within *Badula* on this geologically young island. *Badula sensu* Coode (1981) represents a remarkable example of evolutionary radiation in island plants. Studies of species

biology and ecology are required to determine the drivers of diversification in *Badula* and how species distinctions are maintained.

4. Effects of extreme rarity on species-level genetic diversity in four critically endangered island endemics (genus *Badula*, Primulaceae)

To be submitted as: Ruth E. Bone, Claudia Baider, Trevor R. Hodkinson and Steve Waldren. Effects of extreme rarity on species-level genetic diversity in four critically endangered island endemics (genus *Badula,* Primulaceae).

"There are many breeding populations on managed lands that do not so much survive on new resources as persist on scraps of the old" (Janzen 1986)

4.1 Abstract

I used AFLP markers to estimate genetic diversity (*H*) from every known individual (above seedling size) of four Critically Endangered *Badula* Juss. species from the Indian Ocean island of Mauritius, where 45 % of angiosperm species are endemic, and 39.2 % of the flora is threatened. I gathered ecological data relevant to the life history traits of each of the study species, examined the effects of extreme rarity on their genetic diversity and drew inferences for the likely survival of these species from these ecological and genetic data. I found significantly lower genetic diversity in *Badula crassa* (H_{PH} 0.091), compared to two other species (*B. reticulata* H_{PH} 0.143 and *B. ovalifolia* H_{PH} 0.159) and the more widespread congener (*B. insularis* H_{PH} 0.164). In addition, *B. platyphylla* (H_{PH} 0.116) was shown to have lower genetic diversity than the latter two species. On gathering all available ecological data, I inferred that low genetic diversity in *B. crassa* and *B. platyphylla* may have been caused by reproductive isolation, whereas *B. ovalifolia* and *B. reticulata* show signs of continued reproductive success, and consequently may have retained genetic diversity levels similar to that seen in the more widespread congener. However, the presumed longevity of these small trees may mean that the relatively high genetic diversity levels detected are due to relict samples of previously more extensive populations. Subsequent generations are therefore likely to show an extreme bottleneck effect and, without the immediate intervention of conservation practitioners, these endemic species face irrecoverable decline.

4.2 Introduction

Endemic species are prone to endangerment and extinction (Frankham 1997) and narrowly endemic species, such as those unique to small islands, are often

considered especially susceptible to extinction associated with habitat fragmentation and degradation (e.g. Maunder *et al.* 2002; Turner 1996). Since the early 17th century, island extinctions of bird, mammal and flowering plant species have been estimated to account for 81 %, 60 % and 36 % of all extinctions in these taxa, respectively (Primack 1998). Within island floras, examples of extreme rarity lead Cronk (1997) to observe that species "often exist in isolated fragments [where] community structure has broken down and all that may remain are a few plants of one species on a cliff." It is perhaps intuitive that these small, isolated plant populations would suffer depleted genetic diversity, due to genetic processes such as inhibited gene flow, increased genetic drift and inbreeding (Rossetto *et al.* 2004; Young *et al.* 1996). In cases of extreme rarity, it has been suggested that remnant individuals represent the 'living dead' of degraded ecosystems (Janzen 1986). However, varying levels of genetic diversity are associated with the life history traits of higher plants (Hamrick & Godt 1996). Nybom and Bartish (2000) reported highest genetic diversity within populations of widespread perennial species that were long-lived, out-crossing, late succession colonisers with highly vagile seeds (wind and/ or water dispersed). In fragmented landscapes these traits have been used to explain retention of genetic diversity in species expected to suffer genetic bottlenecks. For example, higher than expected genetic diversity has been found in adult populations of the long-lived gymnosperm *Araucaria nemorosa* Laub. in New Caledonia (Kettle *et al.* 2007). Likewise, in the fast growing rainforest tree *Elaeocarpus grandis* F.Muell., life history traits (such as longevity, out-crossing and efficient seed dispersal) were associated with a negligible difference in detected heterozygosity between undisturbed and fragmented populations (Rossetto *et al.* 2004).

While these studies provide genetic diversity measures for species affected by habitat fragmentation, few address cases of rarity as extreme as those described by Janzen (1986) and Cronk (1997). In Mauritius, 160 of the 670 native plant species are classified as Critically Endangered (CR) under IUCN criteria (Atkinson & Sevathian 2005) and in several cases fewer than 25 individuals are known to exist in the wild (Atkinson *et al.* Unpublished; Strahm 1996b). Conservation practitioners in the tropics are typically faced with a lack of baseline data that, if available, could be used to assess the conservation status of species. In Mauritius, the studies of *Psiadia* by Besse *et al.* (2003) and *Coffea*, by Dulloo (1998) are the only two I am aware of that address genetic diversity of native plant species. However, it is imperative that data quantifying existing diversity levels are made available to facilitate long-term conservation strategies.

The study system

The western Indian Ocean island of Mauritius has experienced dramatic habitat loss. Soon after the first permanent human settlement of the island at the end of the 16th century, native forests were cleared for timber extraction and creation of sugar cane plantations. By 1830 Mauritius produced 10 % of global sugar exports (Baker 1877) and the native trees, described in 1598 as "so close to each other that one can hardly walk in the forest," had been reduced to upland fragments (Brouard 1963). Today less than 2 % of forested lands are rich in native species (J. Mauremootoo, pers. comm.; Atkinson & Sevathian 2005). The introduction of macaque monkeys (*Macaca fascicularis*), wild pigs (*Sus scrofa*), rats (*Rattus rattus* L.) and a host of invasive plant species has further contributed to the demise of native vegetation in Mauritius and thwarts regeneration efforts (Fisher 2005; Florens *et al.* 2008; e.g. Lorence & Sussman 1986; Safford 1997).

Badula Juss. (Primulaceae) is one of sixteen genera endemic to the Mascarene archipelago (C. Baider and V. Florens, unpubl.; Coode 1981) and is among the few Mascarene endemic genera that have been the subject of phylogenetic (Chapter 2) and biogeographic studies (Chapter 3). The taxonomic status of *Badula* has been called into question because morphological distinction from the allied Malagasy genus *Oncostemum* Juss. is unclear (e.g. Chapter 2; Coode 1981; Ståhl & Anderberg 2004). However, in the phylogenetic reconstructions presented in Chapter 2, strong evidence was found for the monophyly of *Badula*, and as such it can be considered an evolutionarily distinct unit representative of a lineage unique to the Mascarene islands. *Badula* comprises 14 species of perennial trees and shrubs (Coode 1981), all of which are considered single island endemics (e.g. Chapter 2; Florens *et al.* 2008). Recent speciation has been inferred from a lack of DNA sequence divergence among *Badula* species (Chapter 2). In addition, data from a combination of molecular markers, and the diversity of morphological and ecological forms, have been interpreted as evidence of radiation into new habitats following colonisation of the islands (Chapter 3). The five species included in the present study, namely *Badula crassa* A.DC., *B. insularis* A.DC., *B. ovalifolia* A.DC., *B. platyphylla* (A.DC.) Coode and *B. reticulata* A.DC., are all endemic to the Mascarene island of Mauritius. No morphologically intermediate forms are known and phylogenetic studies support their status as distinct taxa (Chapters 2 and 3). However, *B. platyphylla* and *B. reticulata* share several morphological affinities, including fused filaments and long pendulous inflorescences (Coode 1981), and *B.*

platyphylla has been treated as a subspecies of *B. reticulata* in past taxonomic accounts (de Candolle 1844). Molecular data support the morphological similarity of these species, and they have been resolved as sister taxa in previous studies (Chapter 2).

In this paper I draw together all available ecological data (both published and unpublished) to provide a summary of life history traits and ecological attributes of the study species. I then use molecular markers to estimate the genetic diversity of each species, by sampling all known individuals above seedling size. Finally, I use the research findings to infer the potential for species recovery and suggest practical conservation measures.

Fig. 4.1. The study species

Clockwise from top left: *Badula crassa* at Yemen, with stem held by botanist Gabriel D'Argent. *Badula crassa* on Mt. Le Morne. The more widespread congener, *Badula insularis*, growing at Mt. Cocotte (location of one of the few extensive populations of this species). Pendulous inflorescences of *Badula reticulata*. Divaricate branching habit of *Badula platyphylla*. The pyramidal fruits of *Badula ovalifolia* (unique among *Badula spp.*).



4.3 Materials and methods

Data generation

Sampling

Leaf samples were collected from wild plants and dried in silica gel following the method of Chase and Hills (1991). A total of 30 individuals were included, representing all known plants (above seedling size) of the rare species, *Badula crassa, B. ovalifolia, B. platyphylla* and *B. reticulata*, and a sample from a closely related and more widespread species, *B. insularis* (included for comparative purposes; sensu Gitzendanner & Soltis 2000). For *Badula insularis,* a sample based on mean sample size for the rare species was used, comprised of individuals from four localities throughout the species' range. Due to the rarity of plants, detailed photographic voucher specimens were made where necessary, rather than taking full herbarium specimens (see Table 4.1 and Fig. 4.2 for accessions numbers and sampling distribution, respectively).

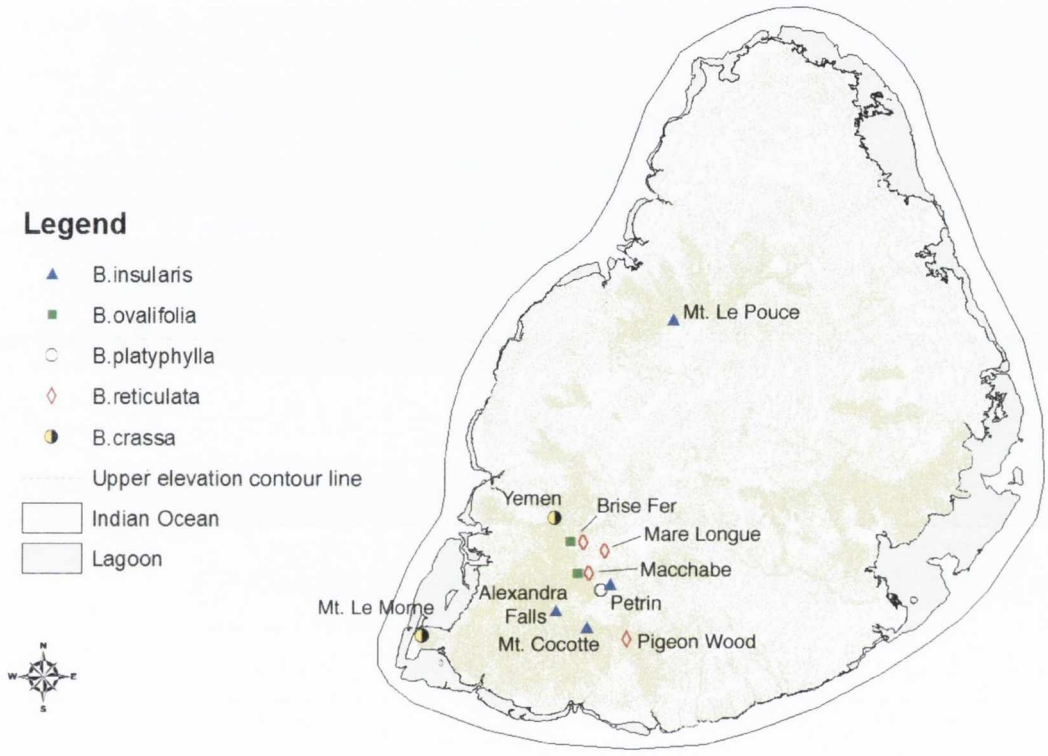
Table 4.1. Taxa and voucher numbers of all genotyped accessions

Herbarium acronyms follow *Index Herbariorum* (Thiers continuously updated) and indicate locations of voucher specimens. Detailed photographs were taken of plant parts and habit, and mounted and labelled and deposited in a herbarium. The use of *cf.* indicates full voucher specimens from the same population as the sample. Refer to Tables 2.1 and 3.1 for co-ordinates of collecting localities, and Fig. 4.2 for a map of their distribution. Sub-adults trees are indicated by asterisks.

Taxon	Sample no. used in text	Voucher/ Herbarium	Locality
Badula crassa A. DC.	RB22	R. Bone 22/ TCD	Yemen
Badula crassa A. DC.	RB23	R. Bone 23/ TCD	Yemen
Badula crassa A. DC.	RB24	R. Bone 24/ TCD	Yemen
Badula crassa A. DC.	RB57	R. Bone 57/ TCD	Mt. Le Morne
Badula crassa A. DC.	RB58	R. Bone 58/ TCD	Mt. Le Morne
Badula insularis A. DC.	RB04	R. Bone 04/ MAU	Petrin
Badula insularis A. DC.	RB11	R. Bone 11/ MAU	Alexandra Falls
Badula insularis A. DC.	RB39	R. Bone 39/ MAU	Mt. Le Pouce
Badula insularis A. DC.	T03	*cf.* R. Bone 65/ MAU	Mt. Cocotte
Badula insularis A. DC.	T17	*cf.* R. Bone 65/ MAU	Mt. Cocotte
Badula insularis A. DC.	T20	*cf.* R. Bone 65/ MAU	Mt. Cocotte
Badula ovalifolia A. DC.	RB28	R. Bone 28/ TCD	Macchabé
Badula ovalifolia A. DC.	RB29	R. Bone 29/ TCD	Macchabé
Badula ovalifolia A. DC.	RB30	R. Bone 30/ TCD	Macchabé
Badula ovalifolia A. DC.	RB31	R. Bone 31/ TCD	Brise Fer*
Badula ovalifolia A. DC.	RB32	R. Bone 32/ TCD	Brise Fer
Badula platyphylla (A. DC.) Coode	RB01	R. Bone 01/ TCD	Petrin CMA
Badula platyphylla (A. DC.) Coode	RB02	R. Bone 02/ TCD	Petrin CMA
Badula platyphylla (A. DC.) Coode	RB03	R. Bone 03/ MAU	Petrin CMA
Badula platyphylla (A. DC.) Coode	RB38	R. Bone 38/ TCD	Petrin, outside CMA
Badula platyphylla (A. DC.) Coode	RB78	R. Bone 78/ TCD	Petrin CMA
Badula platyphylla (A. DC.) Coode	RB79	*cf.* R. Bone 03/ MAU	Petrin CMA
Badula reticulata A. DC.	RB07	R. Bone 07/ TCD	Pigeon Wood
Badula reticulata A. DC.	RB09	R. Bone 09/ TCD	Macchabé
Badula reticulata A. DC.	RB10	R. Bone 10/ TCD	Macchabé
Badula reticulata A. DC.	RB21	R. Bone 21/ TCD	Macchabé
Badula reticulata A. DC.	RB71	R. Bone 71/ TCD	Mare Longue*
Badula reticulata A. DC.	RB72	R. Bone 72/ TCD	Mare Longue
Badula reticulata A. DC.	RB74	R. Bone 74/ TCD	Mare Longue
Badula reticulata A. DC.	RB75	R. Bone 75/ TCD	Brise Fer

Fig. 4.2. Map of Mauritius showing the distribution of collections made

Dark green shading indicates upland areas of Mauritius (> 300 m above seal level). Virtually all collections came from the remaining fragments of mesic forest on the southwest upland plateau. Exceptions are all localities of *Badula crassa* (which comprise the dryland areas of Mt. Le Morne, and Yemen, in the rain shadow of the western edge of the plateau) and *Badula insularis* on Mt. Le Pouce. See Fig. 1.1 for the location of Mauritius.



Marker selection

Phylogenetic studies of *Badula* using highly variable regions of the nuclear and chloroplast genomes (ITS, *trnS-trnG, trnG, accD, psbA-trnH*) have revealed low or absent intra-specific variation within the study species (Chapter 2), and to my knowledge no nuclear or organelle microsatellite markers have been developed for *Badula* or closely allied genera (*Ardisia* is the closest relative with GenBank® published microsatellite sequences). Amplified Fragment Length Polymorphisms (AFLP; Vos *et al.* 1995) yield reproducible banding patterns (or genetic fingerprints) by selective amplification of fragments from a digest of total DNA, using short universal primer pairs (Ridout & Donini 1999). AFLPs facilitate screening for polymorphism without prior knowledge of the target genome, making them ideal markers for studies of tropical tree species where typically little information is available (e.g. Kremer *et al.* 2005).

DNA extraction and genotyping

DNA extractions were carried out withDNeasy® Plant Mini kits (Qiagen Inc., Valencia, CA 91355, USA) by following the manufacturer's protocol, or the CTAB method of Doyle and Doyle (1987) as modified by Hodkinson *et al.* (2007), with purification through JETquick spin columns (GENOMED GmbH, Löhne, Germany) following the manufacturer's protocol.

AFLP reactions were carried out using the Applied Biosystems AFLP Plant Mapping Kit for regular size genomes (Applied Biosystems, Warrington, Cheshire, UK) following the manufacturer's protocol, except that all reaction volumes were halved. All digestion and amplification reactions were carried out on a GeneAmp 9700 thermocycler (Applied Biosystems).

Total DNA was digested using two restriction enzymes (*Eco*RI and *Mse*I) and specific oligonucleotide adaptors were ligated to restriction sites on these fragments, which were then used for two successive PCR reactions (pre-selective and selective). The pre-selective PCR used primers provided in the kit and amplified a sub-set of the fragments, reducing them approximately 16-fold. These PCR products then underwent a second, selective PCR, which reduced the number of fragments further still (approximately 256-fold). One primer in each primer pair incorporated a fluorescent dye to allow automated visualization of amplified fragments. Twelve different primer combinations were tested in the selective PCR using a subset of the digested DNA samples. Four pairs were selected for final analysis based on the quality of the fingerprints they produced. These were: (FAM dye-labelled) *Eco*RI-ACT + *Mse*I-CAG, (FAM) *Eco*RI-ACT + *Mse*I-CAA, (FAM) *Eco*RI-ACA + *Mse*I- CAG, (NED) *Eco*RI-AGC + *Mse*I-CTG. The samples were run on an Applied Biosystems automated capillary sequencer, AB3130xl, and were analysed using Genemapper v 4.0 (Applied Biosystems). The methods followed those described in Chapter 3.

Data analysis

AFLP data scoring

DNA fragments ranging in size from 50 bp to 500 bp were visualized as electropherograms using Genemapper v. 4.0 (Applied Biosystems). Fragments were scored manually as presence (1) or absence (0) characters. All fragments were scored blind (i.e. labelled with arbitrary numbers to prevent inadvertent bias during data scoring). Weak or ambiguous peaks were removed from the matrix before analysis.

Similarly, loci scored as missing data for some accessions were removed from the matrix (across all accessions). Mantel tests were performed (for 999 permutations) in GenAlEx (Peakall & Smouse 2006) to test for congruence between datasets generated by the four different primer pairs. Subsequent analyses included all AFLP data as a combined matrix.

Species differentiation

The extreme rarity of the study species (and consequently the small samples sizes) meant that these data could not be partitioned into populations within a species, but rather each species was treated as a single 'population' for analysis purposes. Genetic differentiation within and among species was estimated by the Analysis of Molecular Variance procedure (AMOVA; Excoffier *et al.* 1992) computed in GenAlEx (Peakall & Smouse 2006). AMOVA requires a Euclidean metric (Excoffier *et al.* 1992; Huff *et al.* 1993; Peakall *et al.* 1995). I therefore used the genetic distance option in GenAlEx (Huff *et al.* 1993; Peakall & Smouse 2006) to calculate an appropriate distance matrix[5]. For these binary data, a *Phi* statistic (*Φpt)* was computed, analogous to the fixation index *Fst* (Peakall & Smouse 2006), with statistical significance calculated after 999 AMOVA permutations. For additional pair-wise comparison of species groups I used Nei's genetic distance (Nei 1978; Nei 1992) and ordination by principle coordinates analysis (PCoA), both computed in GenAlEx (Peakall & Smouse 2006).

Genetic diversity measures

With AFLP markers, the number of alleles amplified at each locus is unknown. For the purposes of data analysis, all loci were therefore assumed to be bi-allelic and the distinction between genotypes was estimated from the relative frequencies of the observed band (interpreted as the marker allele) and the frequency of the visually absent band, which was interpreted as the 'null' allele (Lynch & Milligan 1994). Many statistical measures of genetic diversity (*H*) rely on assumptions of Hardy-Weinberg Equilibrium, including random mating by out-crossing (e. g. Lynch & Milligan 1994). In this study however, because of the rarity of plants, mixed mating and out-crossing are unlikely or may not occur at all (i.e. plants may self-pollinate rather than out-cross, or produce seeds by apomixis). In such cases, Kremer *et al.* (2005) recommend that

[5] Based on the Euclidean metric $E = \{\varepsilon^2_{xy}\} = n\,[1 - 2n_{xy}/\,2n]$ where n is the total number of polymorphic sites. This value corresponds to the number of band differences between individuals (Huff *et al.* 1993).

genetic diversity is estimated by calculation of phenotypic diversity (H_{PH}) (sensu Mariette *et al.* 2002a; Mariette *et al.* 2002b).

I calculated H_{PH} in a Microsoft Excel™ spreadsheet, using the formula given by Kremer *et al.* (2005). For comparison across all species I used the Kruskal-Wallis Test, computed in Minitab™ v.13.30 (Minitab 2000), which compared medians of ranked H_{PH} values. A one-way ANOVA was also computed and gave the same result. I therefore made multiple comparisons (Tukey's Honestly Significant Difference, HSD, calculated in Minitab™) to identify which species showed significant differences in H_{PH} values. Other diversity measures calculated for each species included the number of amplified bands, the number of polymorphic bands, and the number of private bands (present only in one species but not present in all individuals of that species).

Patterns of intra-specific variation

Following the comparative analyses among species, genetic differentiation among the individuals within each species was assessed by ordination with PCoA, and with dendrograms. For the ordination, I used the Sørensen-Dice distance measure (Dice 1945; Sørensen 1948), which weights data in favour of the presence of shared bands. Distance matrices were calculated in Le Progiciel R v.4.0d (Casgrain 1999) using the Simil package, followed by ordination computed in GenAlEx (Peakall & Smouse 2006). Dendrograms were produced with the neighbour-joining (NJ) method (cf. Swofford *et al.* 1996) and were computed with Nei-Li distances (Nei & Li 1979) in PAUP* 4.0b10 (Swofford 2002), which are synonymous with Sørensen-Dice distances (Cole & Kuchenreuther 2001). Statistical support for clades was estimated with the bootstrap procedure (Felsenstein 1985) with 1,000 NJ replicates, undertaken in PAUP* 4.0b10 (Swofford 2002). Within each species, allele frequencies were calculated for each individual (see Appendix 2; following Freeland 2005) to determine whether allele frequency appeared to be affecting the positions of accessions resolved by these analyses.

Compilation of ecological data

For each of the study species, all available ecological data were compiled from a review of the literature, historical records from herbarium specimens [G-DC, K and MAU; acronyms follow *Index Herbariorum* (Thiers continuously updated)], unpublished data from field workers in Mauritius, and personal observations (R. E. Bone and C. Baider) made during field work in 2007 and 2008. Soil and rainfall data

follow Parish and Feillafé (1965), while vegetation types are those summarized by Lorence (1978). Associated plant genera are listed from personal observation and from herbarium records. The census estimates are based on personal observations in the field, communication with botanical field workers in Mauritius and herbarium records. Insect observations were made between September 2003 and March 2004 by Kaiser-Bunbury (pers. comm., 2006; Kaiser-Bunbury *et al.* in press).

4.4 Results

AFLP markers

The four primer pairs yielded amplified bands for a total of 591 loci, with varied number of loci amplified for each species (see page 87). Among the four primer pairs, the number of bands amplified were as follows: (FAM dye-labelled) *Eco*RI-ACT + *Mse*I-CAG produced 162 bands, (FAM) *Eco*RI-ACT + *Mse*I-CAA produced 105, (FAM) *Eco*RI-ACA + *Mse*I- CAG produced 198, (NED) *Eco*RI-AGC + *Mse*I-CTG produced 126. Mantel tests among all possible dataset pairs revealed significant positive correlation (Mantel correlation coefficient *Rxy* 0.43 to 0.61, $P = 0.001$). The data were therefore combined for further analysis.

Species differentiation

The AMOVA indicated that a far greater proportion of the variation was found within (79 %) rather than among species (21 %) (Table 4.2). The latter value was reflected in the 0.21 Φ_{PT} statistic, and suggests that these species show a moderate level of genetic differentiation (Freeland 2005). However, differentiation among species was not equal when pair-wise comparisons of Φ_{PT} values were made (which range from the low value of 0.06 for the differentiation between *Badula reticulata* and *B. platyphylla*, to the highest value of 0.37 for the differentiation between *B. platyphylla* and *B. crassa*; see Table 4.3. The differentiation between all five species was statistically significant ($P < 0.05$). However, the Φ_{PT} value for differentiation of *Badula reticulata* from *B. platyphylla* was borderline ($P = 0.043$) at the 95 % significance level.

Table 4.2 Results for the AMOVA for the combined AFLP data set

ΦPT is analogous to the fixation index, Fst

Source	*df*	SS	MS	Est. Var.	% Variation	Stat.	Value	*P*
Among *spp.*	4	500.125	125.031	12.896	21	*ΦPT*	0.21	0.001
Within *spp.*	25	1207.575	48.303	48.303	79			
Total	29	1707.700		61.199	100			

Table 4.3 Pairwise comparisons of species *ΦPT* (PhiPT) values

Values are *ΦPT* values, followed (in parentheses) by the probability values from the AMOVA based on 999 permutations. All probability values are statistically significant ($P < 0.05$) but the value for the differentiation of *Badula reticulata* and *B. platyphylla* is borderline (in bold, *P* 0.043).

	B. crassa	***B. insularis***	***B. ovalifolia***	***B. platyphylla***	***B. reticulata***
B. cras.	**				
B. ins.	0.274 (0.006)	**			
B. oval.	0.273 (0.009)	0.125 (0.017)	**		
B. plat.	0.370 (0.003)	0.181 (0.001)	0.151 (0.005)	**	
B. ret.	0.325 (0.001)	0.196 (0.002)	0.183 (0.002)	0.060 (**0.043**)	**

The PCoA based on Nei's genetic distance (GD; Fig. 4.3), revealed a similar pattern of low level genetic differentiation between *Badula platyphylla* and *B. reticulata* (GD 0.012), followed by a higher value of 0.02 GD between *B. insularis* and *B. ovalifolia*. These two species pairs differed from each other by between 0.022 and 0.03 GD. The most genetically distant of all of the study species is *Badula crassa* (GD 0.059 to 0.072 from all other species). Axis 1 explained 57.02 % of the variation, axis 2 explained 23.73 % (cumulative total of 80.75 %) and axis 3 (not shown) explained 12.89 %.

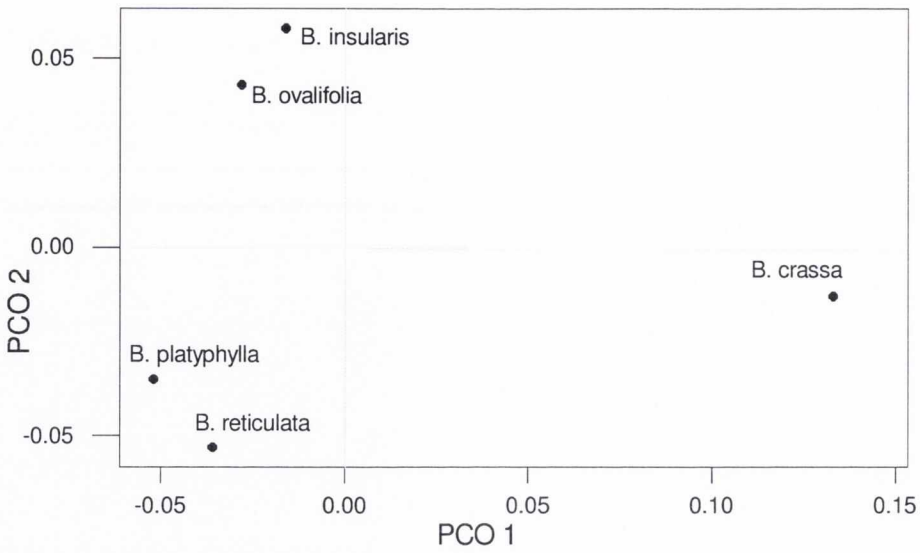


Fig. 4.3 Genetic differentiation between species based on Nei's genetic distance

Ordination of the Principle Coordinates Analysis results: Axes 1 and 2 explain 57.02 % and 23.73 % of the variation, respectively (a cumulative total of 80.75 %).

Genetic diversity

Summary statistics and diversity measures are given in Table 4.4. In *Badula crassa* and *B. platyphylla,* 187 and 194 bands (i.e. marker alleles) were amplified respectively; approximately 100 fewer than for *Badula reticulata*. In *B. crassa,* 23.35 % of loci were polymorphic, which is the lowest value among these species, and half that of *B. reticulata* (46.87 %). *Badula platyphylla* showed a higher level of polymorphism than *B. crassa* but, at 30.46 %, this was approximately 10 to 15 % lower than the other three species. The number of private bands varied similarly among species (Table 4.4). *Badula platyphylla* and *B. crassa* showed the lowest numbers of 20 and 34, respectively. This represented 10 % of the total number of bands amplified for *B. platyphylla*, and 18 % for *B. crassa*. Far more private bands were amplified for *Badula reticulata* (68, or 23 % of the total number of bands amplified). However the highest proportions of private bands were found within *Badula ovalifolia* (68 bands, representing 27 % of the total number amplified) and *B. insularis* (83 bands, representing 29 % of the total).

Table 4.4 Genetic diversity of rare *Badula* species and a widespread congener (*B. insularis*).
Estimated H_{PH} (± standard error) calculated in Excel following Kremer *et al.* (2005). N indicates the number of plants sampled for each species. Number of private bands indicates those unique to the species, but not present in every individual.

Species	N	No. of bands	No. private bands	% poly-morphic loci	H_{PH}
B. crassa	5	187	34	23.35	0.091 ± 0.01
B. insularis	6	288	82	47.21	0.164 ± 0.01
B. ovalifolia	5	249	68	41.62	0.159 ± 0.01
B. platyphylla	6	194	20	30.46	0.116 ± 0.01
B. reticulata	8	291	68	46.87	0.143 ± 0.01

The Kruskall-Wallis test indicated that there was a statistically significant difference among the means of ranked H_{PH} values (H = 70.62, $P = < 0.001$ when adjusted for ties between ranks). In addition a one-way ANOVA was calculated and gave the same statistically significant result ($P = < 0.001$). Tukey's HSD was therefore calculated and the results are summarized in Figure 4.4. *Badula crassa* was shown to have the lowest genetic diversity (H_{PH} 0.091). The difference between this H_{PH} value and those for all species, except *B. platyphylla* (H_{PH} 0.116), was significant. *B. platyphylla* showed a similar level of genetic diversity to *B. reticulata* (H_{PH} 0.143), but the difference between the genetic diversity of *B. platyphylla* and the remaining two species was statistically significant. In contrast, the two Critically Endangered species *Badula reticulata* (H_{PH} 0.143) and *B. ovalifolia* (H_{PH} 0.159), showed similar high genetic diversity levels to the widespread congener *B. insularis* (H_{PH} 0.164) and the differences in genetic diversity of these three species were not statistically significant.

Fig. 4.4 Multiple comparisons of the H_{PH} genetic diversity values
Species are arranged in ascending order (left to right) of H_{PH} values (from Table 4.4), which are given below the species names. Any H_{PH} values connected by a horizontal line cannot be separated statistically. Where a line does not connect values, the difference is statistically significant.

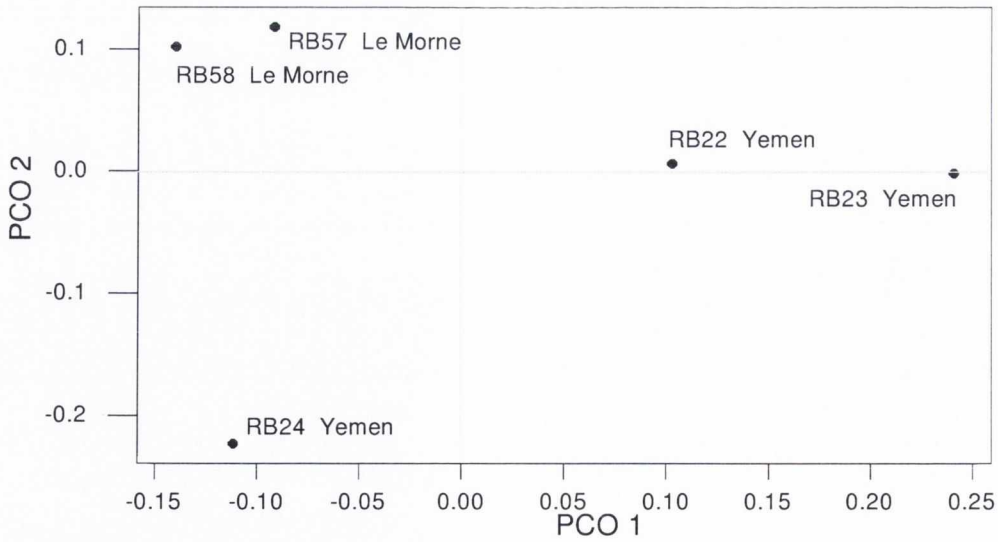
B. crassa	*B. platyphylla*	*B. reticulata*	*B. ovalifolia*	*B. insularis*
0.091	0.116	0.143	0.159	0.164

Structure of diversity within each species

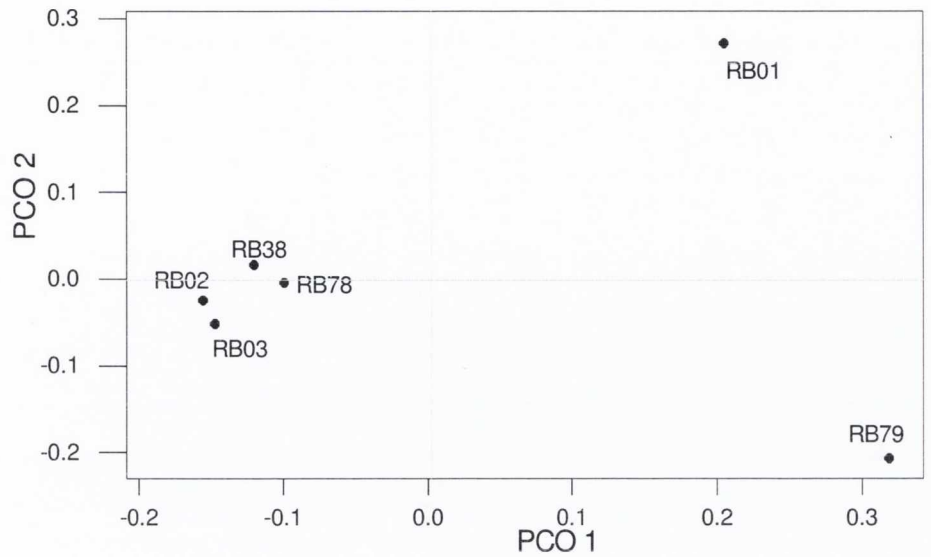
The results of the PCoA (Fig. 4.5) and NJ tree-building analyses (Fig. 4.6) both illustrate intra-specific variation found in the five study species and can be used to identify genetically distant individuals within each species. In addition, some distinct intra-specific groups were recovered by the NJ trees (in two cases with 100 % bootstrap support; BP). Within each species, allele frequencies were calculated for each individual (Appendix 2) and, in some cases, accessions revealed much lower allele frequencies than for the majority of individuals. All fingerprint profiles (trace files) were checked manually for quality and no clear discrepancies were found. These values therefore appear to represent genuine differences in allele frequency among individuals. However there is a possibility that AFLP artefacts, such as scoring error, may be contributing to the polymorphism values measured here (see section 6.5 for a discussion of limitations of the data). The outlying positions of the samples of *Badula insularis* (RB11), *B. ovalifolia* (RB28) and *B. platyphylla* (RB01 and RB78) should therefore be interpreted with caution until these values can be verified.

Fig. 4.5 Principle coordinates plots for the first and second axes for each species, based on ordination of Sørenson-Dice distances

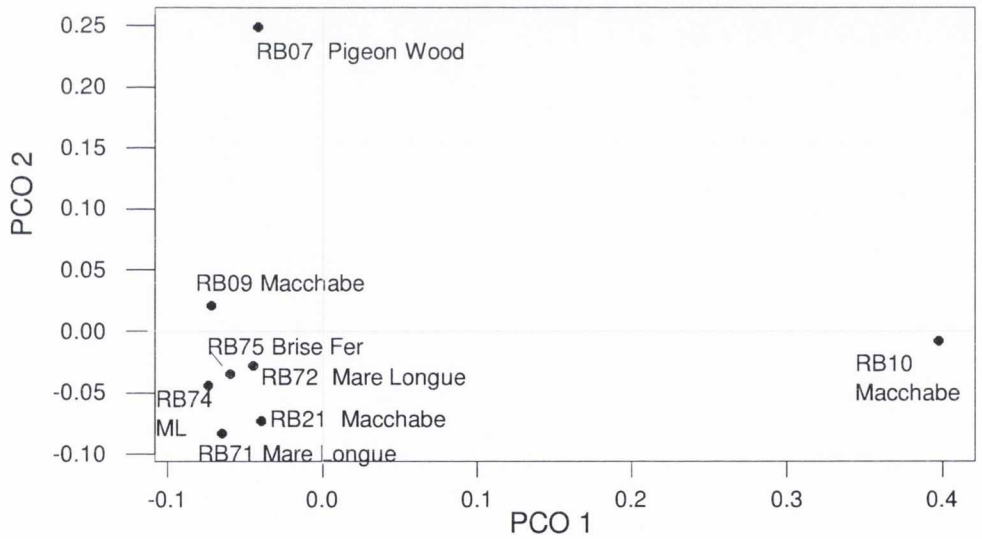
Plots for each species are arranged in ascending order of genetic diversity values (H_{PH})



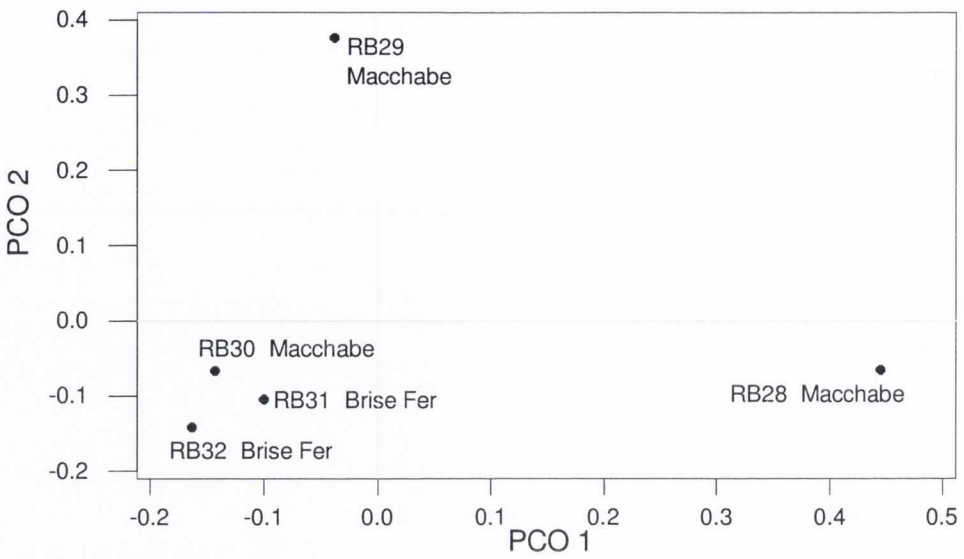
4.5.1 *B. crassa*: Axes 1 and 2 explain 38.43 % and 25.99 % of the variance respectively (a cumulative total of 64.42 %)



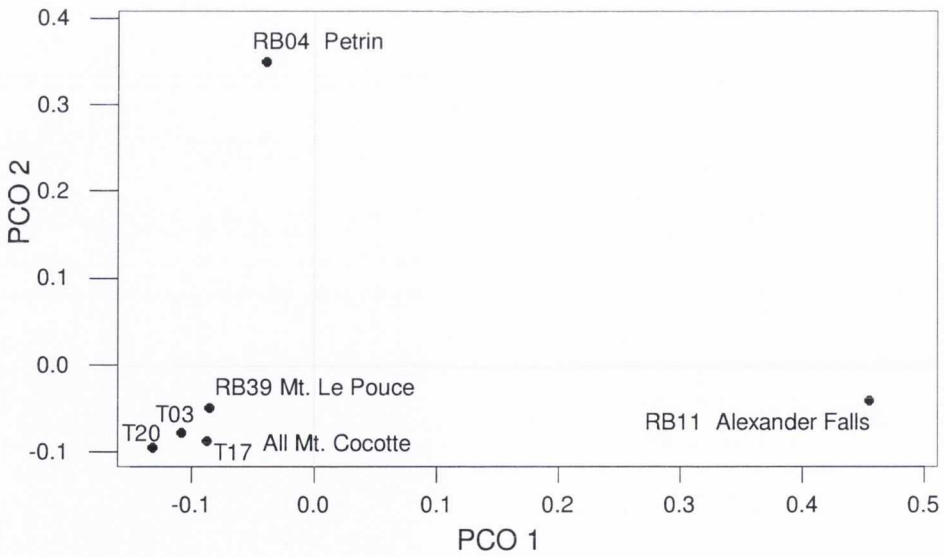
4.5.2 *B. platyphylla*: Axes 1 and 2 explain 39.93 % and 22.42 % of the variance respectively (a cumulative total of 61.75 %). All samples are from Petrin, currently the only recorded locality for this species.



4.5.3 *B. reticulata*: Axes 1 and 2 explain 35.71 % and 15.37 % of the variance respectively (a cumulative total of 51.08 %). RB74 ML is from Mare Longue



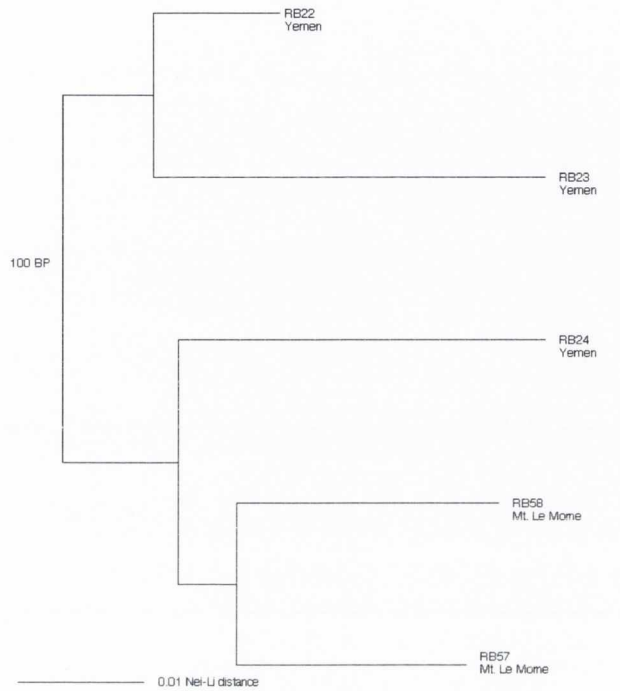
4.5.4 *B. ovalifolia:* Axes 1 and 2 explain 39.76 % and 27.98 % of the variance respectively (a cumulative total of 67.74 %)



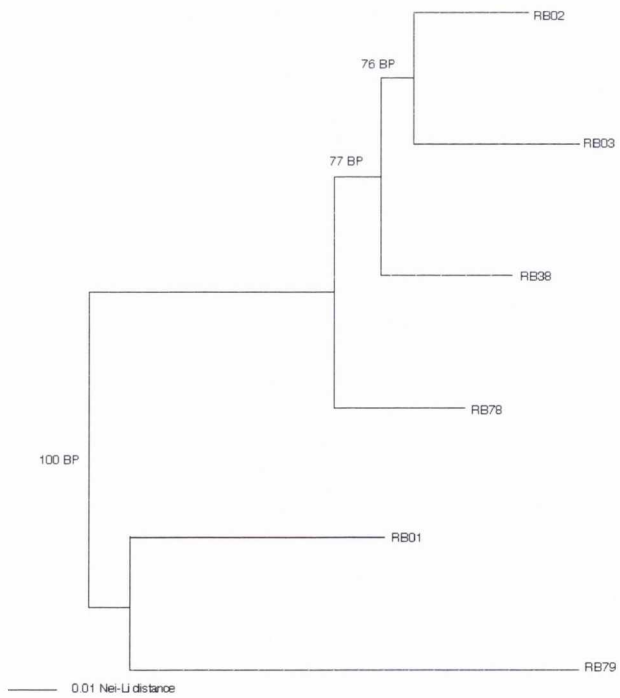
4.5.5 *B. insularis*: Axes 1 and 2 explain 33.96 % and 19.86 % of the variance respectively (a cumulative total of 53.82 %). Accessions T03, T17 and T20 were sampled across the broad area of Mt. Cocotte, the most extensive population of this species known.

Fig. 4.6 NJ trees for each species, showing genetic distance of individuals

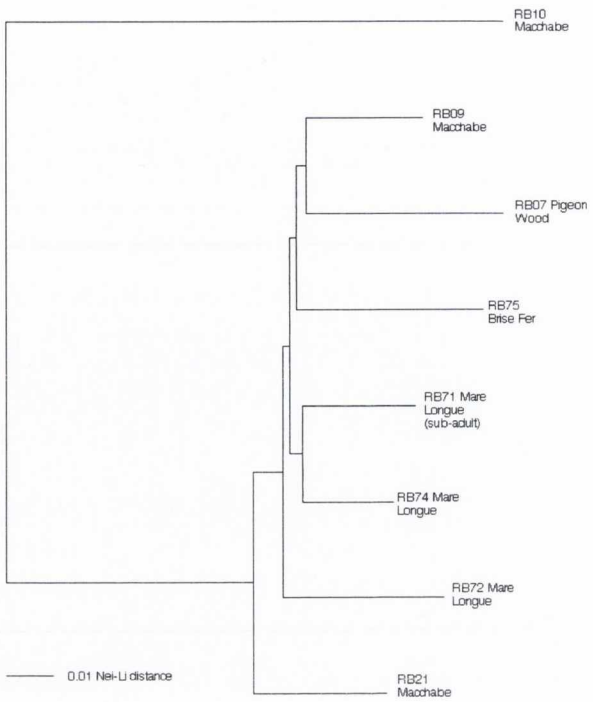
Based on Nei-Li genetic distance (equivalent to Sørenson-Dice). NJ bootstrap values are shown where BP >50. Trees for each species are presented in ascending order of genetic diversity (H_{PH} values)



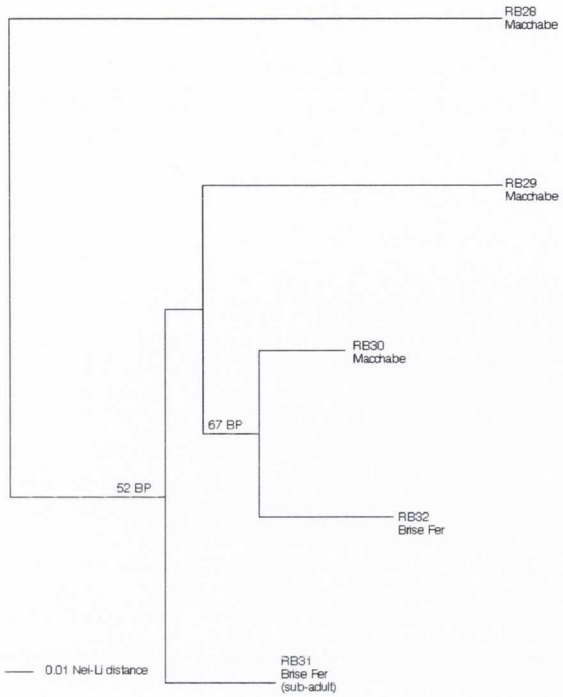
4.6.1 *Badula crassa*



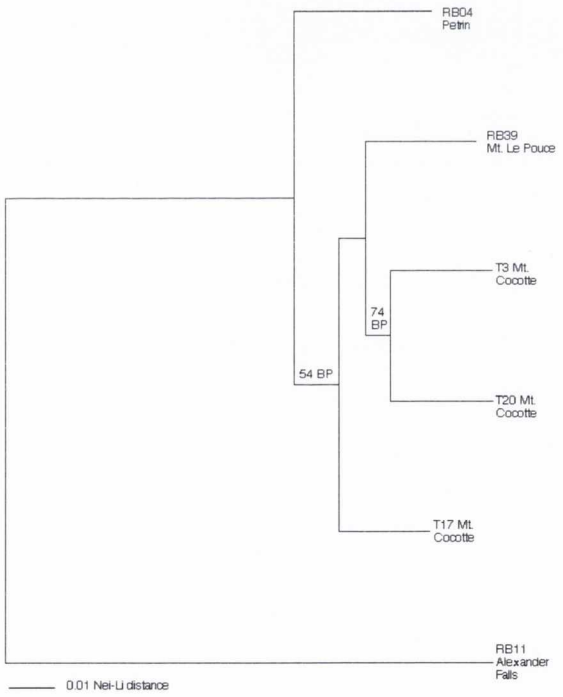
4.6.2 *Badula platyphylla*



4.6.3 *Badula reticulata*



4.6.4 *Badula ovalifolia*



4.6.5 *Badula insularis*

Ecological attributes of the study species

The survey of available data indicated that, with the exception of *Badula insularis*, the study species are restricted in distribution to southwest Mauritius where they occupy a variety of late succession habitats (Table 4.5). These range from the thin, waterlogged laterite soils of the heathlands (*B. platyphylla*) and the humic latosols of the lower montane wet forests (*B. ovalifolia* and *B. reticulata*), to the relictual patches of dry evergreen forest that survive on the Magenta type soils of the rain-shadowed southwest coast (*B. crassa*). Rainfall in these areas varies from a high of 5,000 mm in the super-humid region to a low of 1,000 mm on the southwest coast.

The census estimates for the Critically Endangered species are extreme, with each species currently known from fewer than nine individuals (Table 4.6). While these estimates may change (following extensive searches in the vicinity of known plants) it is unlikely that these species will ever be found in such abundance that they can be considered anything other than Critically Endangered. In addition, despite being more widespread, *B. insularis* cannot be regarded as common and is restricted to upland remnants of heath and forest.

Available information documenting potential for recovery among these species is summarized in Table 4.6. Little information about the reproductive biology of *Badula* was available. However all species are known to have simple, bisexual, apparently unspecialised flowers that could be pollinated by generalists (R. E. Bone, pers. obs.; Coode 1981). Studies of plant-pollinator communities in Mauritius by Kaiser (2006) and Kaiser-Bunbury *et al.* (in press) found a prevalence of generalized pollination systems dominated by flies, and several fly species were among insect visitors Kaiser recorded on *Badula* (*B. platyphylla* and *B. insularis*; Table 4.6). The European honey bee (*Apis mellifera* L.) has been cited as a functional substitute for extirpated native pollinators in Mauritius (Kaiser-Bunbury & Müller 2009), and was among recorded insect visitors to *B. insularis*, but not *B. platyphylla* (Kaiser-Bunbury *et al.* in press). Little is known of the potential pollinator role of other insect visitors recorded by Kaiser, and he has suggested that some visitors recorded on flowers of *B. platyphylla* may act as nectar or pollen robbers. In addition he notes that competition among pollinators is very high and dominated by invasive invertebrates, which may have caused displacement of native pollinators (C. N. Kaiser-Bunbury, pers. comm.). It

is not known whether *Badula* can self-pollinate[6], however self-compatibility has been recorded in other Primulaceae genera with bisexual flowers, e.g. *Ardisia* (Pascarella 1997).

The fleshy fruits of *Badula* are single-seeded and ripen to a deep reddish colour (R. E. Bone, pers. obs.; Coode 1981) possibly making them attractive to birds (common to other Myrsinaceae genera such as Ardisia; Amsberry 2008). Seeds of various *Badula* species have been germinated successfully in cultivation (including *B. reticulata*; A. Hardoar pers. comm.) and seedlings of *B. ovalifolia* are known in the wild (Florens *et al.* 2008). While *B. ovalifolia* and *B. reticulata* both flower and fruit regularly, fruiting in *B. crassa* and *B. platyphylla* has not been recorded in more than fifteen years (see Table 4.5). Furthermore, the lack of vigour in the remaining plants is an immediate threat to survival of the latter two species, in particular this has meant that vegetative propagation efforts to date have failed (R. E. Bone, pers. obs; G. Douglas, pers. comm.). Consequently, the existing genetic diversity within each of these species has not yet been secured in *ex situ* collections. Other threats include predation of unripe fruits by rats and non-native monkeys, disturbance of seedlings by feral pigs and damage by wood boring insects (R. E. Bone, pers. obs. 2004; Florens *et al.* 2008). Furthermore, encroachment by alien plants in upland mesic forest is extreme: Florens *et al.* (2008) recorded that the alien species *Ligustrum robustum* (Roxb.) Blume (Oleaceae) and *Psidium cattleianum* (Myrtaceae) constitute more than 80 % of woody stems over 1.3 m in height. Consequently, competition for resources is high among plants in this environment and possibly prevents recruitment of *Badula* seedlings, while it certainly impedes exploration of the forest to find more individuals for conservation.

[6] Experiments carried out in 2008 to test for autogamous self-pollination of *Badula insularis* resulted in aborted flower buds in all monitored inflorescences (R. E. Bone and C. Baider, unpublished data).

Table 4.5 Summary of habitat characteristics for the five study species

Species	Distribution	Alt. (above sea level)	Soil type	Rainfall per annum	Vegetation type	Associated plant genera
B. crassa	Dryland areas of south-west Mauritius (live plants known only at Yemen & Mt. Le Morne)	200 to 500m	Magenta type soils (occur only in Tourelle du Tamarin. Yemen & Le Morne) & boulder- strewn terrain (Yemen)	1,000 to 1,600 mm	Dry Evergreen Forest	Dominated by natives incl.: *Cyphostemma; Erythroxylon; Eugenia; Euodia; Fernelia; Lomatophyllum; Monimiastrum; Premna; Psiadia*
B. insularis	Occurs across upland areas including the super-humid region of the SW plateau & the Moka Mt. Range (Mt. Le Pouce & Mt. Calbasse).	400 to 790m	Generally occurring on humic ferruginous latosols, also on the laterite & humic gleys common to Heath Formations	2,000 to 5,000 mm	Various: Heath Formations (incl. *Erica/ Phylica* heath), Lower Montane Wet Forest (LMWF) & LMWF 'high forest'	On heaths: *Helicrysum; Gaetnera; Ochna; Phillipia; Phylica.* At Mt. Le Pouce: *Badula (B. sieberi); Erythrospermum; Roussea.* In LMWFs: *Badula (B. multiflora & B. sieberi); Gaetnera; Sideroxylon.* In LMWFs exotics dominate (e.g. *Ligustrum; Psidium)*
B. ovalifolia	Restricted to the super-humid region of the upland plateau (currently known only at Brise Fer & Macchabé)	620 to 670m	Humic ferruginous latosols	4,000 to 5,000 mm	Lower Montane Wet Forest 'high forest'	Dominated by exotics incl.: *Ligustrum; Psidium* Natives incl: *Chassalia; Diospyros; Eugenia; Nuxia; Syzygium*
B. platyphylla	Recorded only on the SW plateau (Petrin)	c. 675m	Laterite and low humic gleys with a permanently high water table	3,500 to 4,500 mm	Heath Formations: Currently known only at the Petrin *Erica/ Phylica* heath	Dominated by natives incl.: *Antirhea; Gaetnera; Helicrysum; Lycopodium; Ochna; Phillipia; Phylica; Tambourissa*
B. reticulata	Known in the super-humid region of the upland plateau (from Macchabé to Brise Fer & towards Mare Longue), & in the Savanne Range (at Pigeon Wood)	630 to 690m	Humic ferruginous latosols	4,000 to 5,000 mm	Lower Montane Wet Forest & LMWF 'high forest'	Dominated by exotic species incl.: *Ardisia; Ligustrum; Psidium; Ravenala;* Native spp. incl: *Badula (B. insularis, B.multiflora & B. sieberi); Chassalia; Diospyros; Eugenia; Nuxia; Syzygium*

Table 4.6 Factors affecting regenerative potential of the five study species

N is the number of documented wild plants. For the Critically Endangered (CR) species where this number is marked '!' it is considered an accurate estimate of the number plants extant today, and the discovery of more individuals is considered unlikely, however where census estimates are contained by quotation marks, these figures are conservative, and it is suggested that more individuals may be found following concentrated search efforts in the vicinity of known plants.

Species	N	Condition of plants	Insect visitors to flowers	Last known fruiting	Signs of seedling recruitment
***B. crassa* (CR)**	5!	Yemen plants decrepit (few branches, few leaves & hollowed/ cankered stems). Le Morne plants in good health (stout trunks bearing many foliose branches)[7]	No records	Nov. 1992[8]	None
B. insularis	> 1000	Generally good. Some plants suffer from extensive insect damage to leaves	Coleoptera (*Chaetocnema sp.*); Diptera (*Chrysomya megacephala, Eristalinus flaeolus, Musca domestica, Ornidia obesa, Phorinia sp., Stomorhina lunata, Trirhithrum* aff. *nigerrimum*); Hymenoptera (*Apis mellifera*); Hymenoptera, Formicidae (*Technomyrmex albipes*); Lepidoptera (*Zizeeria knysa*)	2009 (Fruiting regularly, in abundance)	No seedlings found but young plants seen at Mt. Cocotte in 2008
***B. ovalifolia* (CR)**	"5"	All adult plants have insect damaged stems, but re-sprout. 2 adult trees have died since 1997	No records	2009	1 sub-adult tree & 1 seedling in the wild.
***B. platyphylla* (CR)**	6![9]	All known plants are mature & bear little new material	Coleoptera (*Ahasverus advena*, 2 indet. Mordellidae spp., *Chaetocnema sp., Nitidulidae sp.*); Hymenoptera, Formicidae (*Technomyrmex albipes* & 2 *Brachmyrmex spp.*); Diptera (*Stomorhina lumata* & *Villa unifasciata*)	No records	No fruits or seedlings found. Flowering last recorded in 2003-2004[9]
***B. reticulata* (CR)**	"8"	Adult plants appear healthy & vigorous. The only known sapling was accidentally cut back during weeding,	No record	2009	Flowers regularly. 1 sapling found in 2008

[7] In 2007, a search for a population of *Badula crassa* at Tourelle du Tamarin, last searched in the 1990s, recovered no living plants, and one dead (R. E. Bone and J.C. Sevathian pers. obs.).
[8] Herbarium specimen record (number MAU 22130);
[9] Kaiser-Bunbury (in press) recorded 10 plants at Petrin in 2003-2004, but only 6 could be found during more recent prospecting work.

4.5 Discussion

Species differentiation

Previous phylogenetic studies based on DNA sequences of *Badula* have revealed little sequence divergence in the genus (Chapter 2). My results identified moderate differentiation among the five species in the study ($\Phi_{PT} = 0.21$) and all were resolved as genetically distinct from one another (at a 95 % significance level). However, the *Phi* statistics (Φ_{PT}) and PCoA revealed varied levels of differentiation between the species. In particular *B. reticulata* and *B. platyphylla* were found to be genetically similar to one another, and *B. crassa* was placed as the most genetically distinct among these species. These findings are congruent with previous phylogenetic analyses which placed *B. reticulata* and *B. platyphylla* as sister taxa (Chapter 2) and found some evidence for the distinction of *B. crassa* in an outlying position from the Mauritian species (Chapter 3). The AMOVA attributed the majority of genetic variation in the dataset to intra-specific variation (79 %).

Varying levels of genetic diversity were revealed in these analyses (Table 4.4), and in some cases the differences between species were statistically significant (Fig. 4.4). The numbers of bands amplified for each species was also revealed as being markedly different among species. In particular the species with the lowest genetic diversity (*Badula crassa* and *B. platyphylla*) had the lowest numbers of bands (187 and 194, respectively) and the lowest proportion of private bands (18 % and 10 %, respectively, of the total number amplified). There is some evidence to suggest that the number of fragments derived from AFLP analysis is dependant roughly on the size of the genome (Meudt & Clarke 2007). Therefore, in addition to the conservation implications of these contrasting results (discussed below), these data may also have implications for species differentiation by indicating possible ploidy differences among species.

Genetic diversity and ecological attributes: implications for species recovery

Badula crassa was shown to have significantly lower genetic diversity (H_{PH} 0.091) than three of the five species sampled. Furthermore, accessions were clustered into two strongly supported (100 BP) distinct groups by the NJ tree indicating intra-specific divergence. Interestingly, one specimen from Yemen (RB24) was resolved as more similar to the specimens on Mt. Le Morne (RB57 and RB58), than to the other

plants at Yemen (RB22 and RB23), which grow adjacent to one another. Although specimen RB24 is located in Yemen, it is some distance apart from the other two specimens, and geographic isolation may explain this clustering, and may have lead to deleterious genetic processes, such as an absence of gene flow among past populations, and increased inbreeding within them. Unfortunately, no live plants were recovered during the search of Tourelle du Tamarin in 2007 for a previously recorded population of *B. crassa* there (see legend to Table 4.6 and Fig. 4.7). Had it been possible, inclusion of samples from this locality may have further elucidated patterns of genetic differentiation among *B. crassa* plants. Fruiting has not been recorded in this species since 1992 and flowering is unknown in recent years (although arguably it may have occurred un-witnessed at Mt. Le Morne, which is rarely visited due to inaccessibility). In light of the ecological information available for *B. crassa*, it is likely that this species is no longer able to regenerate in the wild. Urgent remedial action is required to prevent extinction of this micro-endemic, which is restricted to xeric conditions and Magenta type soils (Parish & Feillafé 1965) of the southwest of Mauritius. This species has long populated the priority lists of conservation practitioners in Mauritius, and was named in the 'top thirty threatened plants' by Strahm (1996b). However, the scope for successful vegetative propagation of this species is bleak due to the recalcitrant nature of surviving plant material, and thus practitioners wishing to save this species from extinction face a difficult task.

Badula platyphylla also has low genetic diversity values (H_{PH} 0.116) when compared to the more widespread congener and the CR species *Badula ovalifolia*. Like *B. crassa*, strongly supported clusters were recovered by bootstrap analysis of the NJ tree for *B. platyphylla,* however the two outlying samples were shown to have far lower allele frequencies than other accessions (Appendix 2) and this result should be interpreted with caution until these values can be verified. Nevertheless, the scope for recovery of *B. platyphylla* is more promising than for *B. crassa* however, since this species continues to bear flowers and insect visitors have been recorded, however no fruit set has been found and some insect visitors are possibly pollen robbers (C. N. Kaiser-Bunbury, pers. comm.). Human intervention is therefore essential, and in particular experiments with hand-pollination should be made in an attempt to produce fruit-set.

The two other CR species surveyed, *B. reticulata* (H_{PH} 0.143) and *B. ovalifolia* (H_{PH} 0.159), were shown to have genetic diversity that was not significantly different

from that of the more widespread congener *B. insularis* (H_{PH} 0.164). The outlying positions of accessions RB28 (*Badula ovalifolia*, Fig. 4.6.4) and RB11 (*B. insularis*, Fig. 4.6.5) may be caused by the low allele frequency of these samples (Appendix 2) and should be interpreted with caution. However, evidence of partitioning of genetic diversity into groups was also found among other accessions of these three species. Relationships were not robust when tested by NJ bootstrap analyses ($\leq$ 74 BP in all cases), suggesting that genetic isolation of individuals may be less established than in *Badula crassa* and that at least some moderate amount of gene flow was facilitated in previous generations of each of these species. Both *B. ovalifolia* and *B. reticulata* are known to flower and fruit regularly, and more individuals may yet be found in the forested areas in the vicinity of the known plants. Indeed some accessions of *B. reticulata*, sampled here, were found during weed clearance in 2007 and 2008. In addition, sub-adult plants of both species and seedlings of *B. ovalifolia* have been found in the wild in the last two years. These species may have the capacity to regenerate in the wild given adequate protection from fruit predators, seedling disturbance and invasion by alien weeds. However, there are currently no data available on insect visitors or the potential of these species to self-pollinate or produce seeds by apomixis, and therefore it is not known whether gene flow continues to occur among individuals. Without intervention, these species are likely to suffer the same fate as *B. crassa* and *B. platyphylla*, i.e. experience a steady depletion of genetic diversity, increased genetic isolation of individuals, and a decline in reproductive success.

Badula insularis- *at risk?*

A limitation of this study has been the lack of a truly widespread congener in Mauritius, for comparative purposes. *Badula insularis* is the most widespread *Badula* species in Mauritius, but it can by no means be considered common. For the purposes of this study a species-level sample was used that included sampling from four separate localities (see Table 4.1 and Fig. 4.2). Sample size was confined to the mean sample size of the four Critically Endangered species, which in turn were each limited to all known adult and sub-adult trees. A diversity of insect visitors has been recorded for *B. insularis* and fruit set is reported as heavy, for example at Petrin in 2004 (C. N. Kaiser-Bunbury, pers. comm.) and at Mt. Cocotte in 2008 (R. E. Bone, pers. obs.). Consequently, this species does not appear to be at risk of extinction. However, in 2008 inflorescences on eighteen plants were monitored for fruit-set within the single large

population of *B. insularis* at Mt. Cocotte, and all flowers aborted before producing fruit (R. E. Bone and C. Baider, unpublished). The same sample of the population was genotyped (using the methods followed in this study) and a genetic diversity measure of H_{PH} 0.114 was calculated (N = 18; R. E. Bone, unpublished data); a value similar to that of *B. platyphylla* reported here (H_{PH} 0.116). I therefore recommend that more detailed studies of *B. insularis* are carried out to investigate population-level genetic diversity of this species. The species-level sampling from a range of localities of *B. insularis* used in this study (and its relatively high H_{PH} value) may obscure low level genetic diversity within populations of this species, and partitioning of genetic diversity among populations due to an absence of gene flow.

Conservation recommendations

Without the immediate intervention of practitioners, the four Critically Endangered endemic species face irrecoverable decline. Species recovery plans should be implemented to address the immediate threats posed by habitat degradation, but should also incorporate breeding programmes among all possible pairs of individuals to minimize inbreeding effects and maximize genetic diversity of seed set, and the subsequent generations. I therefore make the following recommendations:

- Cautious and gradual removal of alien weed species is necessary in the areas inhabited by *Badula* species in Mauritius, to reduce competition for resources between *Badula* species and native plants, allow *Badula* seedling recruitment and facilitate exploration for more individuals of the critically endangered species.
- Close monitoring of all remaining trees of *B. crassa* and *B. platyphylla* should be carried out to assess whether flowering continues to occur.
- Out-crossing by hand-pollination is required in the four critically endangered *Badula* species to minimize kinship (i.e. cross-pollination should be carried out between individuals that have been shown to be genetically most distant from one another) and thus maximize genetic diversity of seed set. Pending further investigation, this may also prove necessary among populations of *B. insularis.*
- Developing fruits should be given adequate protection from fruit-predators.
- Accurate records of the parentage of seedlings raised in cultivation will be essential to prevent inadvertent loss of genetic diversity among nursery-stock.

Where possible, genetic diversity of nursery raised material should be assessed prior to out-planting to detect negative consequences of *ex situ* cultivation (e.g. Kettle *et al.* 2008).

- In the case of *B. crassa*, if no further evidence of flowering can be found, drastic action will be necessary. The use of formative pruning techniques should be considered, to stimulate growth of meristematic tissue and provide material for micro- or macro-propagation.

Concluding remarks

Contrary to my suspicions that these species may represent 'the living dead' and therefore show depauperate levels of genetic diversity, the results suggest that when compared to a more widespread species, considerable genetic diversity has been retained in some of these critically endangered island endemics, despite their extreme rarity. However the relatively high H_{PH} values of *B. reticulata* and *B. ovalifolia* may be partly explained by the longevity of plants and they may comprise 'relicts' of historic genetic diversity levels within previously inter-connected populations. For those species showing evidence of continued reproductive success, subsequent generations are likely to show an extreme bottleneck effect. In the short-term, immediate action is required to secure the retained genetic diversity levels of the current generations of these species. In addition, long-term amelioration of the local habitat is essential to prevent their extinction.

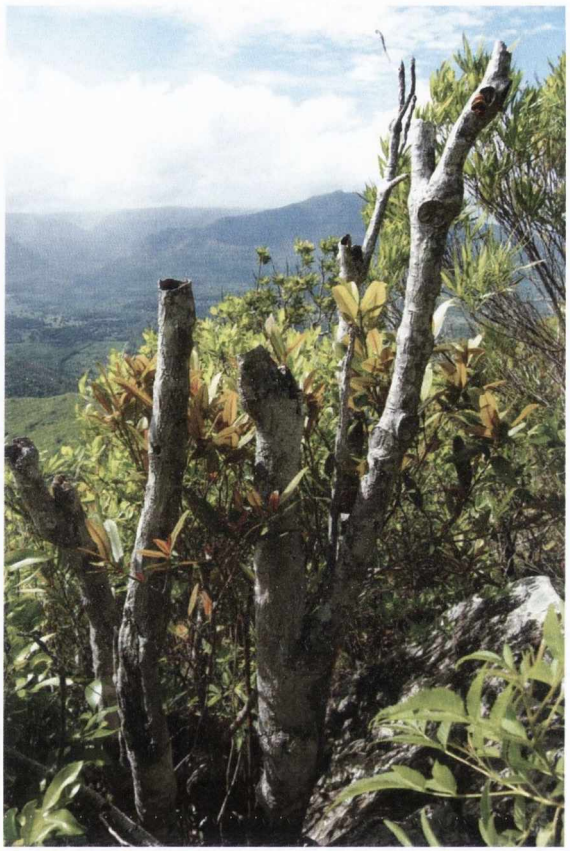


Fig. 4.7. A dead plant at Tourelle du Tamarin, thought to be *Badula crassa*

The upland plateau of Mauritius and the National Park form the horizon. Living plants of *Badula crassa* were last recorded here in 1992, leaving only two localities of living plants.

5. Genetic diversity of a Critically Endangered Rodrigues island endemic, *Badula balfouriana* (Primulaceae), as revealed by plastid DNA and AFLP markers

To be submitted as: Ruth E. Bone, Trevor R. Hodkinson and Steve Waldren. Genetic diversity of a wild Critically Endangered Rodrigues island endemic *Badula balfouriana* (Primulaceae) as revealed by plastid DNA and AFLP markers

5.1 Abstract

Quantification of genetic diversity levels within critically endangered species is invaluable for the development of sampling strategies for reintroduction and other conservation purposes. In cases of extreme rarity, common to island floras, genetic diversity measures may comprise total genetic diversity of a given species, rather than comparisons among and within con-specific populations. *Badula balfouriana* (O.Kuntze) Mez (Primulaceae) is a small tree endemic to the western Indian Ocean island of Rodrigues, where it is known from fewer then ten wild plants. I aimed to estimate the 'relictual' genetic diversity of this species using both plastid DNA and AFLP markers, and also include results from a survey of samples held in European *ex situ* collections. Evidence of high genetic diversity was found among wild plants with plastid haploid diversity ($H = 0.272$) and AFLP genetic diversity (phenotypic diversity) methods ($H_{PH} = 0.292$), and these measures were especially high relative to values that have been reported for other *Badula* species. The survey of plastid DNA regions recovered five haplotypes among the wild plants, however the *ex situ* specimens were found to be genetically uniform using these markers ($H = 0$). AFLP markers revealed that every wild plant was genetically unique, and I therefore recommend that AFLP markers are used to further investigate all *ex situ* collections. In addition, efforts should be made to protect existing genetic diversity of *B. balfouriana* by clonal propagation of all wild plants.

5.2 Introduction

Badula balfouriana is a small tree endemic to the western Indian Ocean island of Rodrigues, the most remote and putatively the oldest island of the volcanic Mascarene archipelago (Giorgi & Borchellini, 1998 in Cheke & Hume 2008). The vegetation of Rodrigues was modified and degraded long before it was scientifically

described (Strahm 1996a). As a member of the Transit of Venus Expedition, Balfour (1879a) noted that Rodrigues had "an exceedingly fragmentary flora" and had suffered deforestation, erosion and invasion by exotic species to a similar extent to St. Helena. In 1874 he recorded 108 seed plants as probable introduced species (Balfour 1879b) and noted the rarity of native plants, commenting that "in several cases the plant from which I have gathered my specimen was the only individual of the species which I have seen on the island" (Balfour 1877).

The flora of Rodrigues is currently estimated to comprise 145 indigenous species (Frodin 2001) including five endemic genera (four of which are monotypic, see Table 1.1; C. Baider and V. Florens, unpubl.) and other regional endemics including the Mascarene endemic genus *Badula.* In Rodrigues, *Badula* is represented by a single species, *B. balfouriana*, which was among the collections made by Balfour. Mez (1902) considered this species as very similar to *B. crassa* A.DC. from Mauritius, but *B. balfouriana* can be distinguished by the variable form of its leaves (which appear to be heterophyllous, bearing narrowly linear juvenile leaves and wide adult leaves) and the large size of the fruits (Coode 1981). In the field, distinctive purple coloration can often be seen on the undersides of juvenile leaves (along the margins and/ or mid-rib; Fig. 5.1), and adult leaves do not show the black dot-dash markings common to those of *B. crassa* (R. E. Bone, pers. obs.). Recent molecular phylogenetic studies have also found evidence of the distinctness of *Badula balfouriana* from other species (Chapters 2 and 3).

Fig. 5.1. Juvenile leaves of *Badula balfouriana*



Today eight wild plants of *Badula balfouriana* are known (R. Payandee pers. comm.) and only seven of these could be found during field work in 2007 (R. E. Bone pers. obs.). Strahm (1996b) listed the species as among the highest of conservation priorities and in the 'top ten' threatened plants of Rodrigues and it is Critically Endangered (CR) according to IUCN criteria. Consequently, *ex situ* collections were established, primarily from cuttings of a plant at Mt. Cimètiere taken by Jean-Yves Lesouëf of the Conservatoire Botanique National de Brest, France (Kristensen *et al.* 2005). Material from this plant was sent to Copenhagen University Botanical Garden, supplemented with seed collections made by Karen Thingsgaard in 1985 and 1987 on Grande Montagne, and later a micropropagation protocol was established (Kristensen *et al.* 2005). To my knowledge, *B. balfouriana* is the only *Badula* species to have been cultivated using *in vitro* methods[10]. Explants from the Copenhagen *in vitro* collection have also been duplicated at the TEAGASC Kinsealy Research Centre, Co. Dublin.

Development of an appropriate propagule sampling strategy is an essential first step towards establishment of plant nursery stock suitable for reintroduction purposes (Touchell *et al.* 1997). Material secured in *ex situ* collections for subsequent out-planting must be genetically diverse if inadvertent genetic bottlenecks are to be avoided within restored and reintroduced populations. Examples of the negative consequences of inappropriate sampling include a reintroduced population of the endangered Hawaiian endemic Mauna Kea silversword (*Argyroxiphium sandwicense* DC. subsp. *sandwicense*), where Robichaux *et al.* (1997) found evidence of a 73 % reduction in detectable RAPD polymorphism associated with establishment using seeds from only two maternal plants, and subsequent recruitment within the same population. Similarly, Kettle *et al.* (2008) detected an extreme genetic bottleneck using molecular markers within nursery collections of seed-raised plants in the endangered New Caledonian conifer *Araucaria nemorosa.* If left unchecked this can lead to total genetic uniformity, as was reported for *Lysimachia minoricensis* Rodrig., a Balearic endemic herb that survives only in botanical gardens (Calero *et al.* 1999). Data quantifying existing diversity levels are therefore of great value in the development of long-term conservation strategies. In cases of extreme rarity, common to the floras of island archipelagos such as the Mascarene islands, genetic diversity measures may comprise

[10] Attempts to establish other *Badula* species (following the protocol of Kristensen *et al.*, 2005) were made by R. E. Bone, G. Douglas and S. Egan (TEAGASC) in 2007 and 2008, but were unsuccessful due to a lack of active meristematic tissues and inadequate sterilization of material (collected in the upland mesic forests of Mauritius). Contamination was cited by Kristensen *et al.* (2005) as a major problem and significantly impeded success of micropropagation efforts.

total genetic diversity of a given species, rather than comparison among con-specific populations (e.g. Chapter 4).

Here I used a combination of AFLP markers and sequences from a number of plastid (chloroplast) DNA regions to investigate genetic variation among the wild individuals of *Badula balfouriana*. In addition I use plastid DNA markers to carry out a survey of *B. balfouriana* specimens held in *ex situ* collections (two samples from mature plants and two samples derived from explants established *in vitro*). I aimed to:

- Investigate previous reports (Chapter 2) of intra-specific variation in wild *Badula balfouriana* using AFLP and DNA sequence based markers
- Use the AFLP data to calculate genetic diversity measures of the wild plants for comparison to CR *Badula* species of Mauritius
- Use plastid markers to identify wild haplotypes and calculate a haploid genetic diversity measure
- Compare plastid DNA haplotype diversity of the wild and *ex situ* material
- Establish if any unique plastid haplotypes can be identified in the *ex situ* collections

5.3 Materials and Methods

Sampling and DNA extraction

Samples were collected from wild plants, from pot grown plants held in the living collections of the University of Copenhagen Botanical Garden (B. G.) or from *in vitro* collections (held at Kinsealy, County Dublin, but donated by Copenhagen B. G.). In the case of wild plants, specimens were recorded using detailed photographs of plant parts and habit, which were mounted, labelled and deposited in the Trinity College Dublin (TCD) herbarium *in lieu* of full specimen vouchers. For cultivated plants, living specimens held at the University of Copenhagen Botanical Garden serve as vouchers for accession numbers P2004 (full accession number P2004-5074) and S1985 (full accession S1985-0650). Accession P2004-5074 was donated to Copenhagen B. G. by the Conservatoire Botanique National de Brest, France. According to Kristensen *et al.* (2005) this accession was originally collected by Jean-Yves Lesouëf at Mt. Cimètiere and accession S1985-0650 was collected by Karen Thingsgaard at Grande Montagne.

The samples held at Kinsealy, Co. Dublin, (Accessions Kins. 1 and Kins. 2) were donated by Copenhagen B. G. but the original accessions number are unknown. Three accessions from the Grande Montagne reserve have been numbered by the Mauritian Wildlife Foundation for monitoring purposes, as follows: Accession RB50 is MWF founder 002, RB51 is MWF founder 003, RB52 is MWF founder 004.

Samples derived from wild collected and pot grown plants were dried in silica gel following the method of Chase and Hills (1991). In the case of *in vitro* collections, entire explants were removed from the growing medium, rinsed with distilled water, and stored in 2 ml micro-centrifuge tubes submerged in a 1:1 acetone to pure ethanol solution (see Fernandez-Manjarres *et al.* 2006). After 24 hours, excess solution was poured off and the explants were placed on coverless Petri dishes and left to dry in a fume hood for 48 hours. (See Fig. 5.2 for collecting localities; accession details are given on page 101).

All DNA extractions were carried out with DNeasy® Plant Mini kits (Qiagen Inc., Valencia, CA 91355, USA) by following the manufacturer's protocol, or the CTAB method of Doyle and Doyle (1987) as modified by Hodkinson *et al.* (2007), with purification through JETquick spin columns (GENOMED GmbH, Löhne, Germany) following the manufacturer's protocol.

Identification of polymorphic DNA regions

A previous study (Chapter 2) reported intra-specific variation in the *trnS-trnG* spacer, which included five samples of *Badula balfouriana.* Here, *trnS-trnG* and four other plastid DNA regions (*rpoB-trnC, 5'trnL-trnT, rps16* and *rpl16*) were sequenced to search for polymorphism. See Table 5.1.

DNA amplification and sequencing

DNA regions were amplified with a Gene Amp 9700 thermocycler (Applied Biosystems, Warrington, Cheshire, UK). The polymerase chain reaction (PCR) primers for *trnS-trnG* and *trnG* (co-amplified), *rpoB-trnC* and *5'trnL-trnT* followed Shaw *et al.* (2005), with the exception of the *5'trnG2S* primer (used as an internal primer for the *trnS-trnG* spacer) which was modified to 5'- TCG AAT CGG TCA TTG AGA GGT -3' after Lu (unpublished). The amplification parameters for these primers also followed Shaw *et al.* (2005) and were optimised as follows: For *trnS-trnG* and *trnG* the annealing temperature was raised from 66° C to 68° C if double banded products were amplified;

for *rpoB-trnC,* 30 amplification cycles were used and an annealing temperature of 48° C; for *5'trnL-trnT* an annealing temperature of 55° C was optimal. The *rpl16* and *rps16* regions were amplified using the primers given by Jordan *et al.* (1996) and Oxelman *et al.* (1997) respectively. In both cases, the amplification parameters given in Oxelman *et al.* (1997) were followed, with 30 amplification cycles and an annealing temperature of 52° C. All PCR reactions contained 2 - 4 μL of template DNA (approximately 50 - 100ng/μL), 10 μL of buffer (5x; Promega, Southampton, UK), 1 μL of dNTPs (10 mM; Invitrogen Ltd., Paisley, UK), 3 μL of Mg Cl_2 (25 mM), 0.5 μL of each primer (25 μM), 0.25 μL *Taq* polymerase (5 U/ μL; Promega), and ultra-pure water to bring the total reaction volume to 50 μL.

Amplified products were purified with JETquick spin columns (GENOMED) following the manufacturers' protocols. Sequencing was carried out on an Applied Biosystems automated capillary sequencer (AB3130xl) with a Big Dye Terminator v 3.1 cycle sequencing kit (Applied Biosystems) by following the manufacturer's protocols, and cleaned with ethanol precipitation. Sequences were edited and assembled with Autoassembler v. 2.0 (Applied Biosystems). Each base was checked for agreement between the forward and reverse strands, and contiguous sequences were then aligned manually in PAUP* 4.0b10 (Swofford 2002). Polymorphisms in the plastid DNA matrix were scored using binary characters to represent the most parsimonious number of single mutational events at a given locus following the guidelines of Kelchner (2000). Multi-state characters were not found and the presence or absence of a character state change was scored using binary characters (0, 1). The resulting binary data matrix was used for all subsequent analysis, including generation of the distance matrix and parsimony analysis.

AFLP markers

The AFLP and haplotype studies were carried out at different times. *Ex situ* material was not available when the genotyping work was carried out. Consequently only samples of wild plants were screened for variation using AFLP markers. This method has been successfully used to detect genetic diversity in other rare *Badula* species (Chapter 4). See Chapter 4 for details of the AFLP methods.

DNA fragments ranging in size from 50 bp to 500 bp were visualized as electropherograms using Genemapper v.4.0 (Applied Biosystems, Warrington, Cheshire, UK). Fragments were scored manually as presence (1) or absence (0)

characters. All fragments were scored blind (i.e. labelled with arbitrary numbers to prevent inadvertent bias during data scoring). Weak, ambiguous peaks were removed from the matrix before analysis, and any loci with missing data were removed. Mantel tests were performed (for 999 permutations) and measured by the Mantel correlation coefficient calculated in GenAlEx (Smouse & Long 1992; Smouse *et al.* 1986) to test for congruence between datasets generated by the four different primer pairs. Subsequent analyses included all AFLP data as a combined matrix.

Relationships among accessions

Distance matrices were calculated from the binary plastid DNA and AFLP datasets with different distance measures using Le Progiciel R v.4.0d (Casgrain 1999). Because plastid DNA is haploid, only one allele is found at each locus, per individual. I therefore used a Euclidean distance measure[11]. In contrast, AFLP markers may be derived from anywhere in the plant genome, including the nucleus. Consequently, the number of alleles amplified at a given locus is unknown and it is difficult to determine whether the markers are homozygous or heterozygous. For the purposes of data analysis loci are assumed to be bi-allelic. The presence of a band is interpreted as the 'marker' allele, and the absence of the band is interpreted as the 'null' allele (Lynch & Milligan 1994). In addition, it is unknown whether band absence is due to a lack of amplification or because it is truly absent. It is therefore desirable to weight the data in favour of shared bands. Here I have used the Sørensen-Dice distance measure (Dice 1945; Sørensen 1948), calculated in Le Progiciel R v.4.0d (Casgrain 1999) using the Simil package.

The distance matrices generated from both haplotype and AFLP data were then converted into EigenVectors for principle coordinates analysis (PCoA) computed in GenAlEx (Peakall & Smouse 2006). Mantel tests (conducted in GenAlEx) were used to determine whether genetic and geographic distances were significantly correlated (using GenAlEx). In addition, a pairwise matrix of total character distance was calculated in PAUP* 4.0b10 (Swofford 2002) to facilitate direct comparison of the total distance between all possible pairs of samples.

The AFLP matrix was subjected to an exhaustive parsimony analysis executed in PAUP* 4.0b10 (Swofford 2002), followed by bootstrap analysis (10,000 replicates)

[11] Pairwise calculations were made following $\sqrt{\sum(p_j - q_j)^2}$ where p_j represents the jth locus of one sample and q_j represents the jth locus of the opposing sample

to determine support for clades (Felsenstein 1985). Connection lengths between haplotypes were calculated in Arlequin v. 3.1 (Excoffier *et al.* 2005) based on modifications to the algorithm supplied by Rohlf (1973, in Excoffier *et al.* 2005) and visualized using a Minimum Spanning Tree (MST).

Genetic diversity

For the DNA sequence data, haploid genetic diversity was calculated in GenAlEx (Peakall & Smouse 2006) by the formula $h = 1 - \sum p_i^2$ where p_i is the frequency of the i-th (or marker) allele. This value was then averaged across all loci to give mean haploid diversity for the species (H).

To estimate genetic diversity (H) from the dominant AFLP markers, I used the H_{PH} (phenotypic diversity sensu Mariette *et al.* 2002a; Mariette *et al.* 2002b) calculated using the formula given by Kremer *et al.* (2005). This method does not rely on assumptions of Hardy-Weinberg Equilibrium, which are likely to be inappropriate in this study where out-crossing is limited due to the rarity of plants, or may not occur at all (i.e. plants may self-pollinate rather than out-cross, or produce seeds by apomixis). In addition, use of this measure allowed comparison with previous studies of Critically Endangered *Badula species* (Chapter 4).

5.4 Results

Detection of polymorphism in plastid DNA

The polymorphisms found were transversions (purine - pyrimidine substitutions), insertions or deletions (indels). Six variable loci were identified across the eleven samples: the seven remaining wild plants and material from four plants in cultivation (Table 5.1). The *rpl16* region was the most variable sequence surveyed. However *rps16* was invariant across all accessions and was excluded from further analysis.

Table 5.1. Primers used to amplify plastid DNA regions where polymorphisms were detected and descriptions of the six polymorphic loci

Locus	Primer	Reference	Position of polymorphism†	Description
1	*trnS-trnG*	Shaw *et al.* (2005)	449	Indel (29 bp)
2	*rpoB-trnC*	Shaw *et al.* (2005)*	1012	Transversion (T / A)
3	*5'trnL-trnT*	Taberlet *et al.* (1991)	436	Indel (1bp)
4	*rpl16*	Jordan *et al.* (1996)	310	Transversion (C / G)
5	*rpl16*	Jordan *et al.* (1996)	683	Transversion (G / T)
6	*rpl16*	Jordan *et al.* (1996)	721	Indel (14 bp)

* Modified from Ohsako and Ohnishi (2000)

† Position in base pairs in aligned matrix of each plastid region

Plastid haplotypes recovered and their distribution

Five haplotypes were identified (Table 5.2). Haplotype A was the most common among the *ex situ* collections, where all four accessions had this haplotype). However, among wild plants this haplotype is rare and represented by only one plant (RB50). Haplotype B was the next most common and was represented by three wild plants from three localities. The three remaining haplotypes (C, D and E) are represented by single wild plants: two from Grande Montagne Reserve and one from the valley floor near Malabar.

Fig. 5.2. Map of Rodrigues showing collecting localities of samples

The accession numbers collected at each locality are given in Table 5.2. Mt. Limon is the highest point of Rodrigues. Collecting localities are indiciated with solid black circles. (See Figure 1.1 for the location of Rodrigues).

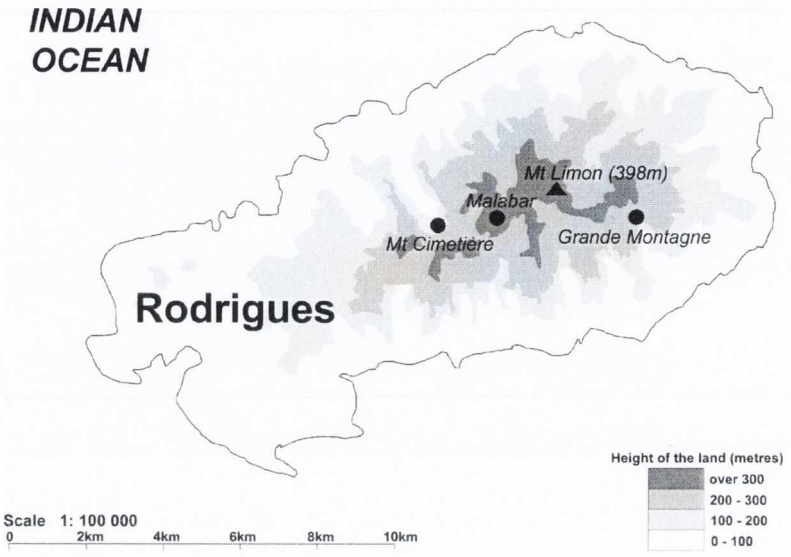


Table 5. 2. Accession details and haplotypes of *Badula balfouriana*, their distribution and the genetic diversity (*H*) of wild and cultivated plants

Allelic composition refers to the presence of an allele form (1) that differs from the normal allele (0) at a given locus. 'Normal' corresponds to the most common form. Six variable loci were identified across the eleven samples. The six loci are listed in Table 5.1 and the primer used to amplify each locus is given below. *H* = mean haploid diversity (calculated in GenAlEx) given as the mean for the wild vs. cultivated plants. Sample numbers correspond to accession numbers for voucher specimens.

Sample	Haplotype	Allelic composition at polymorphic loci						Presence of ≥ 1 unique alleles	Source
Wild plants									$H = 0.272 \pm 0.027$
RB50	A	0	0	0	0	0	0	No	Grande Montagne
RB51	B	0	0	0	0	1	0	No	Grande Montagne
RB53	B	0	0	0	0	1	0	No	Malabar roadside
RB55	B	0	0	0	0	1	0	No	Mt. Cimètiere
RB52	C	0	1	0	1	1	0	Yes	Grande Montagne
RB54	D	0	0	1	0	0	0	Yes	Nr. Malabar
RB56	E	1	0	0	0	1	1	Yes	Grande Montagne
Cultivated									$H = 0$
P2004	A	0	0	0	0	0	0	No	Mt. Cimètiere
S1985	A	0	0	0	0	0	0	No	Grande Montagne
Kins. 1	A	0	0	0	0	0	0	No	Kinsealy (*in vitro*)
Kins. 2	A	0	0	0	0	0	0	No	Kinsealy (*in vitro*)
Primer/ amplified region		*trnS-_trnG*	*rpoB-trnC*	*5'trnL-trnT*	*rpl16*	*rpl16*	*rpl16*		

AFLP data

The selected primer pairs produced a large number of AFLP markers for analysis, with a total of 230 bands (marker alleles) from seven accessions, of which 80.43 % were polymorphic across accessions.

Table 5.3. Polymorphism and genetic diversity of the wild plants detected by AFLP data

N is the number of accessions

No. of marker alleles	N	% Polymorphism	$H_{PH} \pm$ S.E.
230	7	80.43	0.292 ± 0.01

Genetic diversity of **Badula balfouriana**

Plastid genetic diversity *(H)* of 0.272 was measured from 'haploid diversity' calculated from the sequence data of the wild plants. In addition a similar estimate of *H* was calculated for the wild plants from the AFLP data (H_{PH}) as 0.292. However, among the *ex situ* collections *H* was 0 (all samples were Haplotype A). H_{PH} was not calculated for the *ex situ* collections (AFLP data are not available for these accessions).

Relationships among accessions

The haplotype and AFLP distance matrices used for PCoA are shown in Tables 5.4 and 5.5, respectively. Due to the different evolutionary history of the markers (AFLPs being predominantly derived from the nuclear genome, whereas the haplotype markers are from the chloroplast genome), correlation between the two datasets is not expected. The results are nevertheless in agreement for the placement of some accessions. For example both analyses reveal that RB54 (Haplotype D) is distinct from RB51 and RB55 (both Haplotype B), and RB52 (Haplotype C).

Table 5.4. Euclidean distance matrix calculated from the haplotype data

Euclidean distances were calculated for all pairwise comparisons of the six loci described in Table 5.2. These distances were used for ordination by PCoA, see Fig. 5.3.

Accession	RB50	RB51	RB52	RB53	RB54	RB55	RB56
RB50	0						
RB51	1	0					
RB52	1.73	1.41	0				
RB53	1	0	1.41	0			
RB54	1	1.41	2	1.41	0		
RB55	1	0	1.41	0	1.41	0	
RB56	1.73	1.41	2	1.41	2	1.41	0

Table 5.5. Sørenson-Dice pairwise distance matrix of the AFLP data

Used to generate the PCoA, Fig. 5.4.

Accession	RB50	RB51	RB52	RB53	RB54	RB55	RB56
RB50	0						
RB51	0.3145	0					
RB52	0.3036	0.2776	0				
RB53	0.2863	0.2763	0.2812	0			
RB54	0.2961	0.3574	0.3468	0.314	0		
RB55	0.3247	0.3279	0.3171	0.3083	0.3448	0	
RB56	0.3067	0.4191	0.3667	0.3504	0.3186	0.3929	0

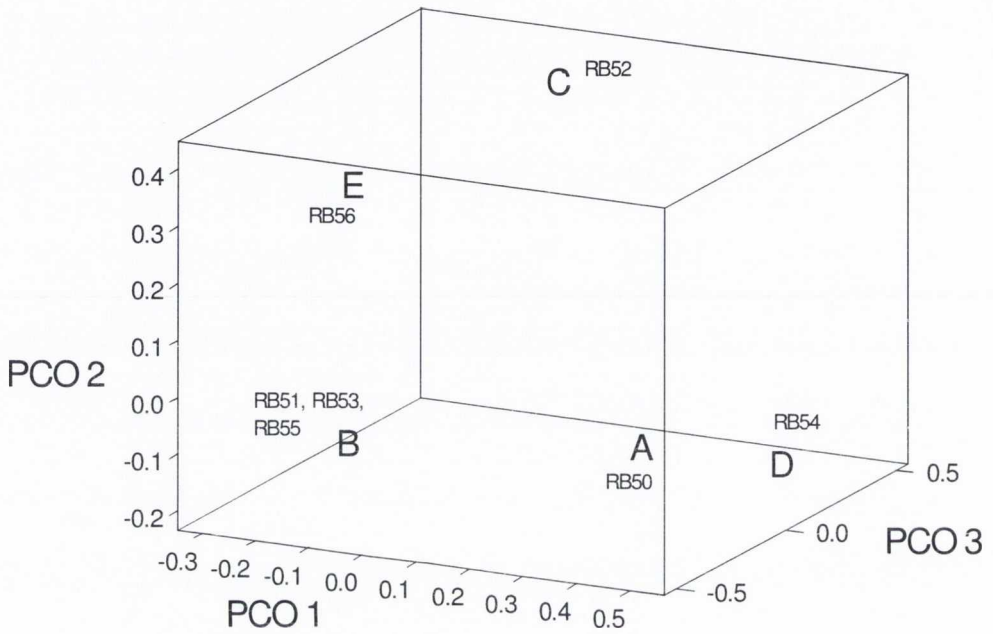
Table 5.6. Total character pairwise distance matrix of the AFLP data

Included to facilitate pairwise comparison of samples based on the total number of characters that differ between them.

Accession	RB50	RB51	RB52	RB53	RB54	RB55	RB56
RB50	0						
RB51	78	0					
RB52	75	73	0				
RB53	69	71	72	0			
RB54	69	89	86	76	0		
RB55	75	81	78	74	80	0	
RB56	69	101	88	82	72	88	0

In the PCoA of plastid data (Fig. 5.3) axis 1 explains 31.72 % of the variance in the data. Four samples cluster on this axis (RB51, 52, 53, 55 and 56; Haplotypes B, C and E) but are distinguished from RB50 (Haplotype A) and the more distant sample RB54 (Haplotype D). Axis 2 (which explains 29.2 % of the variance) distinguishes RB52 (Haplotype C) and RB56 (Haplotype E) from the cluster of Haplotype B samples, however the difference between samples RB56 and RB52 is only shown on axis 3, which explains 26.73 % of the variation (meaning that all three axes explain a cumulative total of 87.64 % of the variance in the data).

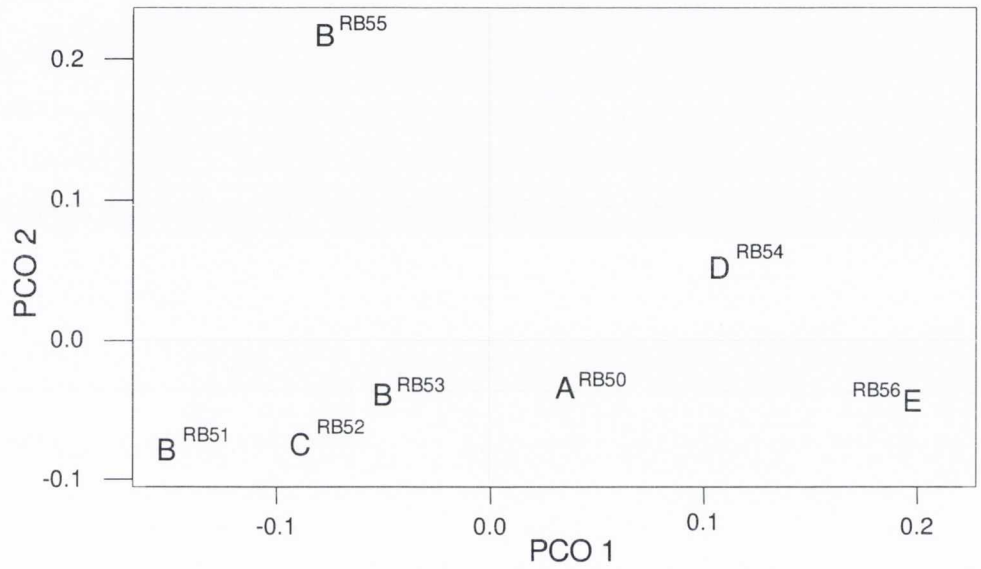
Fig. 5.3. Plot of Principle Coordinates Analysis of the distance matrix derived from plastid DNA
Only the wild plants are included here, to allow comparison with the AFLP dataset (all *ex situ* plant material matches Haplotype A). Ordination is based on a PCoA of the Euclidean distance matrix derived from plastid DNA (Table 5.4). Data points are identified by the haplotype code given in Table 5.2. Labels at each point give the accession number. Axis 1 explains 31.72 %, axis 2 explains 29.2 % and axis 3 explains 26.73 % of the variance (a cumulative total of 87.64 %).



In the PCoA of AFLP data (Fig. 5.4) axis 1 explains 24.53 % of the variance in this dataset, and shows samples RB51, RB53 and RB55 (all representative of Haplotype B) and sample RB52 (the unique representative of Haplotype C) clustered together. Sample RB56 (Haplotype E) is revealed as the most genetically distinct sample. Axis 2 is less informative (17.49 %) but reveals RB55 (Haplotype B) as distinct from the other samples identified as this Haplotype by the plastid DNA. In addition, a second distance matrix was calculated for the AFLP data to show the total character difference between all possible pairs of samples (Table 5.6). This matrix is in agreement with the Sørenson-Dice matrix (Table 5.5), and both distance measures reveal that the greatest distance in the AFLP dataset lies between samples RB51 (Haplotype B) and RB56 (Haplotype E). High values were calculated between several other pairs of samples identified as different haplotypes, but also between samples identified as the same haplotype by the plastid DNA survey. For example, accessions RB51 and RB55 (both haplotype B) differ by a total character difference of 81 (or Sørenson-Dice distance 0.3279; Table 5.5),

which is a greater distance than that between Haplotypes A and E (a difference of 0.3067 in Table 5.5, or 69 in Table 5.6), or Haplotypes D and E (0.3186 in Table 5.5, or 72 in Table 5.6).

Fig. 5.4. Plot of Principle Coordinates Analysis of the distance matrix derived from AFLP data
Ordination is based on PCoA of the Sørenson-Dice distance matrix from AFLP markers (Table 5.5). Data points are identified by the haplotype code (identified by the plastid DNA) given in Table 5.2. Axis 1 explains 24.53 % and axis 2 explains 17.49 % of the variance in the data (a cumulative total of 42.01 %).



The positions of accessions RB52 (Haplotype C) and RB56 (Haplotype E) relative to each other appear to vary the most amongst the two PCoA plots, with the plastid data identifying them as less genetically distant from one another than the AFLP data. But the most significant difference between the two datasets is the position of samples RB51, 53 and 55 in the AFLP PCoA, where they are revealed to be genetically distinct, in contrast to the plastid data that identified them all as the same haplotype (B).

Geographical isolation cannot consistently be used to explain genetic distance among accessions. For example, the three samples identified as Haplotype B by the plastid data are each from three different localities on the island (Table 5.2 and Fig. 5.2). Mantel tests did not recover a significant positive correlation between geographic distance and genetic distance from the haplotype data (Mantel Test correlation coefficient *Rxy* 0.212, $P = 0.270$) or the AFLP data (*Rxy* -0.083, $P = 0.379$).

The parsimony analysis of the AFLP data (Fig. 5.5) recovered two most parsimonious trees. A total of 230 characters were included in the analysis and 109 of these were parsimony informative. Several character state changes (annotated above

branches in Fig. 5.5) resolved every sample included in the survey as genetically unique. Congruent with the distance measures, samples RB56 and RB51 are among the samples most genetically distant from one another. In addition, bootstrap analysis revealed that the positions of RB54 and RB56 have some support (66 BP). A more robust value of 72 BP was recovered for the grouping of RB51, RB53 and RB55 (all Haplotype B) with RB52 (Haplotype C), which is generally congruent with the analysis of the haplotype data. Furthermore, in the strict consensus tree, two clades collapse and the topology resembles the cladogram for the plastid data. However the two datasets do not agree on the position of sample RB56 (Haplotype E).

For the plastid data, the Minimum Spanning Tree (MST) placed RB50 (Haplotype A) and RB54 (Haplotype D) in close proximity to one another, congruent with their position in the PCoA (Fig. 5.3). The greatest differences are between Haplotypes C and D and Haplotypes D and E, positioned at the terminals of each of the MST branches (Fig. 5.6), again in agreement with the position of these samples in the PCoA of haplotype data (Fig. 5.3).

Fig. 5.5. Unrooted cladogram recovered by parsimony analysis of AFLP

945 trees were evaluated and two equally most parsimonious trees were recovered, of length 296. One of these is presented below. Arrows indicate branches that collapse in the strict consensus tree. Of the 230 characters included, 109 were parsimony informative. Branch lengths (number of character state changes between accessions) are shown above branches. Numbers annotated with 'BP' indicate bootstrap support after 10,000 replicates. Letters at branch terminals refer to the haplotype codes given in Table 5.2.

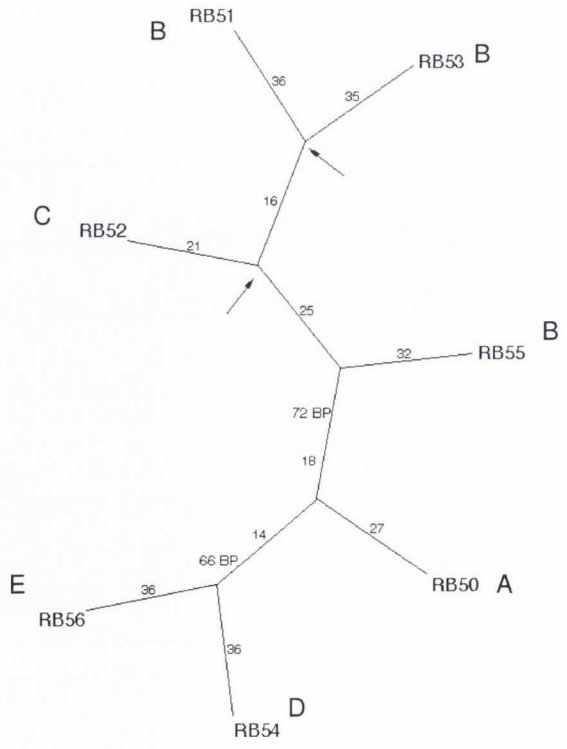
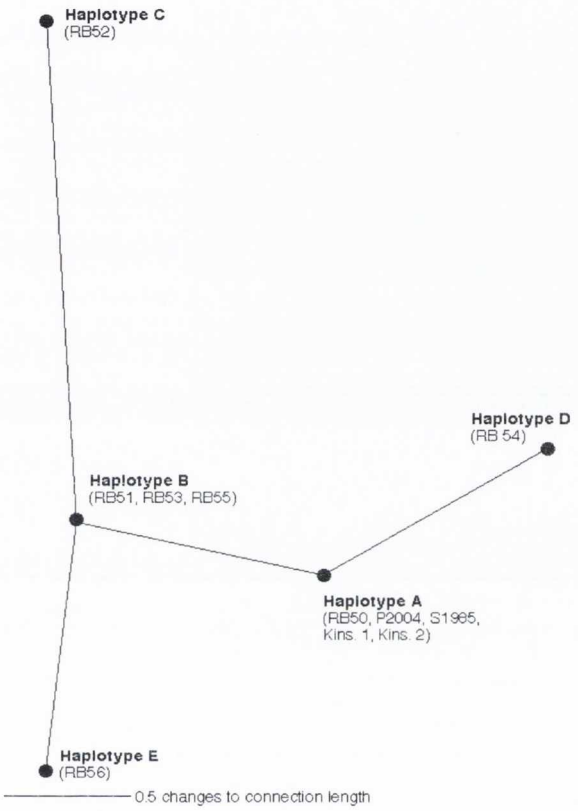


Fig. 5.6 Minimum Spanning Tree of the haplotype data

Letters at branch terminals refer to the haplotype codes given in Table 5.2. All accessions of cultivated plants were identified as Haplotype A (see Table 5.2). Haplotypes A and B are positioned here as being somewhat intermediate between the three most genetically distinct Haplotypes (C, D and E).



5.5 Discussion

Comparison of AFLP and plastid DNA markers

The AFLP technique yields fragments from a digest of total genomic DNA, including nuclear DNA, which can infer different evolutionary histories from those inferred from the maternally inherited plastid genome as a consequence of processes such as hybridisation, introgression and lineage sorting (Wendel & Doyle 1998). The markers may also show a different facility in detection of polymorphism due to varying rates of evolution among the sampled areas of the genome. The differences between the two types of markers may therefore be the cause of incongruence between the two data sets, as indicated by the Mantel test results.

The AFLP data identified all wild samples as being genetically unique. The plastid DNA markers, however, identified five haplotypes across the seven wild plants. AFLP data are not currently available for the *ex situ* collections, and my findings (based on plastid DNA only) identified that all four samples match Haplotype A (of accession number RB50). One sample in cultivation at Copenhagen (P2004-5074) is thought to be derived from collections made at Mt. Cimètiere by Jean-Yves Lesouëf (see Table 5.2; Kristensen *et al.* 2005), a locality where only one wild sample is known today, which I have identified as a different haplotype (accession RB55, Haplotype B). It therefore seems likely that unique genetic diversity, perhaps extinct among wild plants, is represented among the cultivated plants, despite the uniformity reported here using plastid markers. I recommend that AFLP data are generated for these samples, and other specimens held in botanical garden collections.

Genetic distinctness of individuals

In terms of allelic composition (Table 5.2), Haplotypes A, B, and D all differ from one another by just one allele in the selected regions of the chloroplast genome (see Table 5.1). By contrast, Haplotypes C and E each differ from Haplotypes A and B, and from one another, by two unique alleles. The greatest differences, however, are shown by pairwise comparisons of Haplotypes C and D and Haplotypes D and E (Table 5.4), also illustrated by the outlying positions of these haplotypes in the Minimum Spanning Tree (Fig. 5.6).

The positions of samples differed among datasets. For example, accession RB56 (Haplotype E) was resolved as genetically distant from other samples by PCoA of both

the AFLP and plastid DNA distance matrices, but its position varied among the datasets. In addition, sample RB52 (Haplotype C) was placed with the three Haplotype B samples in parsimony analyses of the AFLP dataset, but the distance matrices derived from these datasets were not in agreement, and the plastid data consistently placed sample RB52 as more distant from the Haplotype B samples, than the AFLP data. The two methods have nevertheless identified several samples of this species as genetically distinct. In particular the AFLP data has identified every surviving plant as being a unique genotype.

Geographic isolation

No clear pattern of correlation was evidenced by comparison of genetic and geographic distances. Table 5.2 shows that three specimens all with Haplotype B are from three different localities, and suggest that isolation by distance is not of great significance. Given the age of the trees (in some cases known to survive at maturity for at least 24 years), it seems more likely that genetic isolation of individuals is a consequence of discontinuity of a once more widespread population(s), and the sampling reflects only what remains.

Genetic diversity of the species

These data provide valuable information regarding the genetic diversity of remnant trees of *Badula balfouriana*, one of the rarest Rodrigues endemic species. Genetic diversity levels in *B. balfouriana* far exceed the H_{PH} values calculated in a previous study of Critically Endangered *Badula* species from Mauritius, that ranged from 0.091 ± 0.01 (S.E.) to 0.159 ± 0.01 (Chapter 4). Moreover, the value presented here also exceeds the H_{PH} 0.164 ± 0.01 calculated for the more widespread congener of that study, *B. insularis*. *B. balfouriana* plants are capable of living in excess of 24 years (Kristensen *et al.* 2005) and it is likely that the plants listed by Strahm (1996) are among those sampled here. It may be the case that longevity of plants has allowed this species to retain historic genetic diversity levels, i.e. presumably this species once comprised more extensive inter-breeding and inter-connected populations (see Chapter 4).

In the *Badula* phylogeny of Chapter 2, that used the *trnS-trnG* spacer, and during preliminary investigation of the *5'trnL-trnT* and *rpoB-trnC* spacers (R. E. Bone, unpublished), *B. balfouriana* was the only *Badula* species that showed evidence of intra-specific variation. In addition, substantial sequence divergence distinguishes *B.*

balfouriana from other *Badula* species and it has been resolved as outlying all other *Badula* species, including a single clade of all known La Réunion taxa (Chapter 2). This information, coupled with the estimated age of Rodrigues as the oldest of the Mascarene islands (c.10 Myr), suggest that *B. balfouriana* is at least as old as the common ancestor of the La Réunion taxa. The long establishment of *Badula balfouriana* may help to explain the higher than expected genetic diversity levels reported here because it could have accumulated a greater number of mutations than other, more recently evolved species.

Recommendations

The DNA sequence data revealed that the available material from *ex situ* collections was genetically uniform (all samples were Haplotype A). These specimens nevertheless represent a valuable conservation collection since only one wild plant is known to survive with the same haplotype identity (sample RB50 from Grande Montagne). In addition, accession P2004-5074 (held at Copenhagen botanical garden) may be a clonal representative of a wild plant from Mt. Cimètiere. Only one plant, with a different genetic composition, was found at this locality during field work undertaken for this study (accession RB55, Haplotype B). Therefore the *ex situ* collections may hold unique genetic material from plants that have not survived in the wild. More extensive sampling of *ex situ* collections should be made (e.g. to include samples in cultivation at the Conservatoire Botanique National de Brest, France, and any that may occur within local *ex situ* collections on Rodrigues) to determine whether any other haplotypes are represented in current collections. These results indicate that AFLP markers have the potential to detect genetic polymorphism in *B. balfourina* not revealed by the plastid markers. I therefore also recommend that AFLPs are used to further characterize *ex situ* collections.

Conservation strategies for *Badula balfouriana* should include vegetative propagation of all individuals (by either macro- or micropropagation methods) to ensure that all existing genetic diversity is secured *ex situ* (sensu Atkinson *et al.* Unpublished). It is also imperative that the wild plants are protected *in situ.* Three specimens (accessions RB50, RB51, and RB53) grow in the Grande Montagne reserve, which is under active conservation management. However, some plants grow at the periphery of this reserve where they are at risk of neglect and encroachment by invasive alien weeds such as *Furcraea* Vent. and *Litsea glutinosa* (Lour.)Robinson. In addition, one of the

largest specimens, accession RB56, grows just outside the boundary of the reserve. Plants at the other localities on the island, Mt Cimètiere and near Malabar, are particularly at risk. During field work in 2007, the locality of accession RB54 was being used for charcoal burning. This plant is a unique haplotype (D) that is among the most genetically distinct of the remaining plants and which, in terms of the plastid data, represents one fifth of the remaining genetic diversity of *Badula balfouriana*. Accession RB53 grows in a dense stand of *Syzigium jambos* (L.) Alston along the roadside and bordering a housing area, where it is vulnerable to clearance. The only known wild plant of *Ramosmania rodriguesii* Tirveng. grows a few metres away, partially protected by a cage, but threatened by over-collection due to the beneficial properties associated with very rare plants in Rodrigues. Urgent action is needed to protect the remaining wild *B. balfouriana* plants from clearance. However, it is a task that local conservation practitioners will have to undertake with considerable skill, to prevent this species from suffering the same fate as other rare plant species in Rodrigues, by becoming the target of collectors.

6. General discussion

6.1 Summary

The genus *Badula* is distributed across the Mascarene archipelago. Several species occur on two of these islands, where they exhibit varying levels of morphological differentiation and rarity. In order to improve understanding of the systematic relationships of this genus I aimed to determine the generic delimitation of *Badula* relative to the morphologically similar Malagasy genus *Oncostemum*, assess the endemic status of *Badula,* determine whether evidence gleaned from molecular data supported the species delimitations of Coode (1981) and infer biogeographic patterns among *Badula* species across the Mascarene archipelago. In addition, I aimed to gain a deeper understanding of the conservation requirements of this genus by investigating the genetic diversity of Critically Endangered *Badula* species.

In order to determine the generic delimitation of *Badula*, I used a combination of nuclear and plastid markers of all species of *Badula* and several samples of *Oncostemum* (14 taxa) selected to be representative of the broad variety of morphological forms of this genus. I then carried out phylogenetic analyses (using Bayesian and Maximum Parsimony methods) to infer phylogenetic relationships among these genera. In addition, I aimed to investigate infra-generic relationships for *Badula* with these data. To further elucidate these relationships, I used AFLP markers in combination with DNA sequences and both Maximum Parsimony and distance methods. I also used AFLP markers to investigate the genetic diversity of four Critically Endangered *Badula* species endemic to Mauritius, known from fewer than nine wild plants, and made comparisons to the most widespread *Badula* species in Mauritius, *B. insularis*. *Badula balfouriana* is the lone *Badula* species on the most remote, and putatively the oldest of the Mascarene islands, Rodrigues. Unlike other *Badula* species, *B. balfouriana* showed evidence of intra-specific variation among DNA sequences of individuals sequenced for phylogenetic analysis. I therefore sampled further regions of the plastid genome of this species and was able to identify five haplotypes among the seven wild plants, and a single haplotype among *ex situ* collections held at Copenhagen Botanical Garden and TEAGASC, Kinsealy, Co. Dublin. AFLP markers were used to further characterize genetic diversity of the wild plants and identified each as being genetically distinct. Here I provide a synthesis of the results presented in the thesis, and make suggestions for further work.

6.2 Generic delimitation of *Badula*- a Mascarene endemic?

At the commencement of the research presented here, evidence drawn from the literature inferred that *Badula* and the Malagasy genus *Oncostemum* should possibly be considered as one genus, there being no reliable morphological characters by which to distinguish them (the original androecial characters used by Jussieu are known to vary among species of both genera; R. E. Bone, pers. obs.; Coode, 1981). The endemic status of *Badula* was thus called into question, with implications for the conservation assessment of the genus. In an effort to resolve this issue, phylogenetic reconstructions for the two genera were undertaken using several genetic markers (Chapter 2).

Oncostemum is speciose and morphologically diverse. Moreover, its current classification (Perrier de la Bâthie 1953) is in need of revision since it does not appear to be based on natural groups (a hypothesis supported by the results presented in Chapter 2). The classification is also difficult to use due to a reliance on characters of the inflorescence (which are not always available). Consequently, identifications are either lacking, or are ambiguous. The size of the genus (c. 100 *spp.*), the numerous unidentified specimens held in herbaria, internationally, and the lack of previous phylogenetic studies of *Oncostemum,* made sampling a challenging task. However, thanks to a collaboration with Peter Fritsch at the California Academy of Sciences (CAS) I was able to include *Oncostemum* samples in this study. I surveyed the voucher specimens held at CAS and made a selection of samples across the range of morphological diversity of this genus (names used here are based on determinations made by P. Fritsch or myself). Additional samples were kindly contributed by Joeri Strijk (see Table 2.1). The phylogenetic reconstructions therefore include 22 *Oncostemum* samples representing 14 taxa and which show clear morphological differentiation. It was hoped that this strategy would prove adequate for the purposes of this study- i.e. to determine whether molecular data support the distinction of *Badula* and *Oncostemum*.

The results provide some evidence (although weakly supported by bootstrap analysis) for the paraphyly of *Oncostemum,* with *Badula* nested within an *Oncostemum* clade (Clade A, Fig. 2.1), and as such they cannot be used to directly address the question of whether *Badula* can continue to be considered an endemic genus. The position of *Badula* in the phylogenetic reconstruction has implications for the classification of both genera, with the possibility of *Badula* (this name having priority)

being re-circumscribed as one genus, with a Madagascan and Indian Ocean Islands distribution. Further work is needed before any nomenclatural changes are made. This work should include thorough sampling of *Oncostemum* from a broad geographical distribution, a task which was beyond the scope of this study. In addition, a survey of the morphological characters of *Oncostemum* should be conducted, to determine whether a physical distinction can be made between this genus and *Badula*. This work would also inform the classification of *Oncostemum,* an important component of the Malagasy flora, and one in need of taxonomic revision. Again, assessment of the morphology of *Oncostemum* was beyond the scope of this study.

Generic delimitation is problematic among several Primulaceae genera, particularly those from the tropics (Chapter 2) and no phylogeny is currently available that encompasses comprehensive sampling of tropical woody Primulaceae genera. In this study, the position of *Badula* and *Oncostemum* was assessed in relation to other genera by using ITS sequences available from GenBank®. Parsimony analyses revealed an outlying position for accessions of *Ardisia, Monoporus, Rapanea* and *Stylogyne* in relation to *Badula* and *Oncostemum*. Extensive sampling and phylogenetic reconstruction of Primulaceae genera are required, to provide a phylogenetic framework not only for *Badula* and *Oncostemum*, but also for other Primulaceae genera for which extensive data are lacking (e.g. the genus *Monoporus*).

Despite the remaining question over the taxonomic rank of the Mascarene *Badula*, and thus the status of the genus as a Mascarene endemic, the results presented in Chapter 2 have nevertheless identified *Badula* as a monophyletic group. This clade represents a lineage unique to the Mascarene islands and it should remain a high priority for conservation.

6.3 Species delimitations

Within the genus *Badula*, species delimitation has been hampered by a lack of specimens, in particular for species from Rodrigues and Mauritius where the floras are among the World's most threatened (Walter & Gillett 1998) and habitat loss and degradation is so severe that under 2 % of the land area is rich in native species (Atkinson & Sevathian 2005). During scientific exploration of Rodrigues in the early 19th Century, type specimens were often collected from plants already considered rare

(Balfour 1877) and, in 1960s Mauritius, the last known population of *B. platyphylla* at that time was destroyed as native vegetation was cleared to create pine plantations (Coode 1981). Almost all *Badula* species were included in the 1997 Red Data List (Walter & Gillett 1998) and nine were listed as Threatened or a more severe category, including two species that were presumed to be extinct at that time (*B. ovalifolia* and *B. platyphylla*). In La Réunion, native vegetation is more extensive and less degraded. Some *Badula* species are nevertheless considered rare, but distributions and numbers appear to be naturally localized (e.g. *B. decumbens;* R. E. Bone, pers. obs.) rather than limited by the encroachment of invasive alien plants or through clearance of vegetation. Other La Réunion taxa, such as *B. barthesia* and *B. borbonica* var. *borbonica*, are more widespread and can be found in many areas of upland forest. However, in these cases species delimitations are unclear not because material is lacking, but due to the presence of intermediate forms (Coode 1981; D. Strasberg, pers. comm.). Elucidation of inter-specific relationships within *Badula* was therefore one aim of the work undertaken for this thesis.

The phylogenetic reconstructions of *Badula* presented in Chapters 2 and 3 include at least one accession of each *Badula* species and the taxa below species rank (*B. borbonica* var. *borbonica* and *B. borbonica* var. *macrophylla).* The dataset created for Chapter 2 included sequences from one nuclear (ITS) and four plastid DNA regions [*trnS-trnG* and *trnG (*i.e. *trnSGG), accD, psbA-trnH*] for all *Badula* taxa. Some robust taxon-specific clades were resolved, primarily for the Rodriguan species (*B. balfouriana*) and some species from Mauritius (e.g. *B. multiflora, B. ovalifolia* and *B. sieberi*). In addition, unique polymorphisms in one of the plastid regions proved to be diagnostic for *B. balfouriana* and *B. crassa* (Table 2.2).

However, relationships among taxa from La Réunion were generally unresolved and showed little sequence variation. In Chapter 3, AFLP markers were used to try to further elucidate inter-specific relationships. In combination with data from the ITS and *trnSGG* regions, clear genetic differentiation among almost all taxa was seen, and was generally consistent across analysis methods, for example among the La Réunion taxa individuals of *B. decumbens, B. grammisticta* and *B. fragilis* all formed species specific groups. Exceptions were the morphologically intermediate samples *B.* aff. *borbonica* var. *borbonica* (RB68) and *B.* aff. *barthesia* LR569, which were resolved by some, but not all analyses, as sister taxa but with no bootstrap support. Similarly, *B. barthesia* DS333 and DS314 also formed a group in some analyses (NJ only), but again had no

support. Among the taxa from Mauritius, all except *B. platyphylla* formed robust groups in all analyses. This species was resolved as sister to *B. reticulata* in earlier work (Chapter 2), and by the NJ tree of Chapter 3, but Maximum Parsimony analyses placed *B. platyphylla* as nested within *B. reticulata*, although this relationship was weakly supported (56 BP). In addition, the distance methods used in Chapter 4 resolved the five study species from Mauritius as distinct (see Fig. 4.3), and while the differentiation between *B. platyphylla* and *B. reticulata* was statistically significant ($P = 0.043$), the other species appeared to be more clearly differentiated (P from 0.001 to 0.017).

6.4 Origins

Badula represents a lineage unique to the Mascarene islands and, under its current circumscription, comprises an endemic genus that is the most speciose of the archipelago (see Table 1.1). Few studies are available that have documented patterns of speciation in the flora of Mascarenes, particularly by comparison to floras of other island groups (Micheneau *et al.* 2008). Several studies that compare widespread and endemic angiosperm genera are currently underway, as part the EC funded HOTSPOTS project lead by Vincent Savolainen, RBG Kew. Among them are studies of *Psiadia* (Asteraceae) and members of the Monimiaceae (Strijk *et al.*, in progress) that will mark a significant contribution to knowledge of speciation patterns in the Mascarene region. Here I have presented phylogenetic reconstructions with the primary aim of elucidating the generic and infra-generic relationships of *Badula.* However, this work also allows some inferences to be made about the patterns of diversification seen among *Badula* taxa.

In the analyses of Chapter 2, virtually all taxa formed clades that corresponded to their geographic distribution. In particular, two robust clades for the taxa from Rodrigues and La Réunion provided strong evidence for single colonization events of these islands by ancestral *Badula* species. Among the species from Mauritius however, differing results were produced in the molecular phylogenies and no clear pattern emerged for the origins of *Badula* on this island. For example, in Chapter 2 the position of *B. multiflora* proved ambiguous, but with the addition of AFLP data (Chapter 3) *B. crassa* was resolved in different positions that varied with the methods used. Further evidence for the distinctness of *B. crassa* was found in the study of Critically

Endangered *Badula* species (Chapter 4), where it was identified as the most genetically distinct from other Mauritian species (*B. insularis, B. ovalifolia, B. platyphylla* and *B. reticulata).* Further investigation of the relationship between *B. balfouriana* and *B. crassa* may provide clues to the process of colonization and adaptation by *Badula* among, and within, the islands. *B. crassa* shares several morphological and ecological affinities with *B. balfouriana*, which supports the genetic similarity of these species inferred by the distance methods (Chapter 3). For example, they are the only *Badula* species adapted to xeric conditions and *B. crassa* is the only *Badula* species that occurs on older volcanic 'Magenta type' soils of Mauritius (sensu Parish & Feillafé 1965), while *B. balfouriana* is restricted to what is putatively the oldest island of the archipelago, Rodrigues. The southwest promontory of Mt. Le Morne, is one of the two remnant localities of *B. crassa,* and stands apart from the central highland plateau of Mauritius where it forms the wind-swept coastline in the path of cyclonic storms in the region. A scenario of colonisation of Mauritius by an ancestor of *B. balfouriana* and *B. crassa* can thus be envisaged, with subsequent dispersal on Mauritius to wet forests further inland, followed by radiation into new habitats (dispersal from wet to dry habitats has been proposed for species of Mascarene ebony; Venkatasamy *et al.* 2006). Where today the upland plateau of Mauritius harbours areas of relatively homogenous (and degraded) mesic forest, there would have once been a diversity of altitudinally stratified niches and a more rugged topology, resembling contemporary La Réunion. Speciation in this varied environment is far more plausible than in the isolated patches of forest found today, where surviving species may only persist due to the restriction of their range. Thus, the uplands of Mauritius may be not only be a refuge for the remaining genetic diversity of several Critically Endangered species, but may also be a refuge for the descendants of previously far more extensively distributed upland species, whose habitats have since eroded away.

Whether an ancestral *Badula* species colonized Mauritius or Rodrigues first cannot be determined yet, and subsequent colonisations of Mauritius from the ancestral source of *Badula* cannot be ruled out, and would be consistent with the ambiguity of the Mauritius clades presented in this work. Nevertheless, a more recent colonization of La Réunion does seem probable, based on the evidence of short branch lengths within the La Réunion clade and a lack of clear morphological differentiation among taxa on the island. However, more evidence is needed to determine the origins of the Mauritius clade before a hypothesis of colonization among these islands is developed further.

Moreover, the origins of *Badula* from further afield will remain obscure until a more detailed phylogeny for allied genera becomes available.

6.5 Genetic diversity

The rarity of some *Badula* species is so extreme that they could be relegated to the 'the living dead' described by Janzen (1986). Estimations of genetic diversity (Chapters 4 and 5) were therefore expected to reveal these species as being genetically depauperate. However, contrasting levels of genetic diversity were calculated from AFLP data for the four Critically Endangered *Badula* species of Mauritius, and for *B. ovalifolia* and *B. reticulata* these values did not differ significantly from a more widespread congener included in the study (*B. insularis*). Furthermore, AFLP and plastid DNA data indicated that *B. balfouriana*, the only *Badula* species known from Rodrigues, has higher genetic diversity than other *Badula* species surveyed, including *B. insularis*.

In the literature, varied levels of genetic diversity are reported among rare and endangered plants, and various explanations have been offered as to why these may differ. For example, in the Wollemi pine (*Wollemia nobilis* Jones, Hill & Allen, Araucariaceae) no genetic diversity was detected (by AFLP or SSR markers) among a sample taken across the remnant population of ~100 plants (Peakall *et al.* 2003). The authors considered both technical issues (the facility of the markers to detect polymorphism) and biological factors (genetic drift, reports of clonality in the species and naturally low level genetic diversity for the Araucariaceae) when drawing conclusions about the reasons for such low levels of detected genetic diversity (Peakall *et al.* 2003). Within the rare Epacridaceae species, *Leucopogon obtectus* Benth., ~90 % of loci were recorded as polymorphic among the >400 remaining individuals, distributed across several populations, and little differentiation was seen among them (Zawko *et al.* 2001). A presumed reliance on out-crossing, facilitating gene flow between populations, was among the suggested explanations for the mode in which this species has maintained comparatively high genetic diversity.

In the case of *Badula* species included here (Chapters 4 and 5), plants are thought to have persisted for several years in a state of rarity. During Balfour's visit to Rodrigues as part of the Transit of Venus Expedition, many native plants were already

considered rare, and the flora decimated (Balfour 1877, 1879a). For example, in the 19th Century only two or three trees of *Zanthoxylum paniculatum* Balf.f. could be found (Balfour 1879a), and today two mature trees are known and occur at the same type-specimen locality (Bone 2005). Kristensen *et al.* (2005) mention the persistence of specimens of *B. balfouriana* for over 24 years, and some of the trees recorded in Strahm's 'top twenty' list of rare plants in Rodrigues (Strahm 1996b) are likely to be within the sample used in the study presented here (Chapter 5). In Mauritius, *B. crassa* has persisted in low numbers for decades (R. E. Bone, pers. obs.; herbarium data) and was thought to be extinct when the treatment of *Badula* for the *Flore des Mascareignes* was published by Coode 1981. In 2007, no live plants were found at a known historic locality of this species (Tourelle du Tamarin, Fig. 4.7), and three of the five remaining plants are in a decrepit state and appear to be reproductively dormant. The putative longevity of these geographically, and apparently reproductively, isolated plants was given as a possible explanation for the low levels of genetic diversity revealed in this species, which was the lowest value among the *Badula* species surveyed. However, *B. ovalifolia* and *B. reticulata* showed levels that were not significantly lower than the value calculated for the more widespread congener, and consequently a less bleak prognosis was described for *B. ovalifolia* and *B. reticulata,* which both show signs of natural regeneration (through continued flowering, fruit production and apparently successful seedling recruitment, albeit hampered by the detrimental affects of feral animals and invasive plant species). The situation for the endemic *Badula* species of Mauritius is nevertheless so severe that even the most widespread *Badula* species on the island, *B. insularis*, is probably at risk. Further studies are needed to determine whether this species is in decline.

Of all the *Badula* species for which genetic diversity was surveyed, *B. balfouriana* showed by far the highest level, based on both AFLP and plastid DNA markers. In comparison to other *Badula* species surveyed, considerable intra-specific variation was also revealed in the phylogenetic reconstructions (Chapter 2) and it was identified as the most genetically distinct *Badula* species in the Mascarene islands. However this species (and the Critically Endangered species from Mauritius) survive in such low numbers that inbreeding may become inevitable and cause the genetic homogeneity of subsequent generations. Conservation actions should include artificial pollination to minimize inbreeding effects (by crossing all most genetically distant pairs of plants) and raising and out-planting offspring as part of carefully managed species

recovery programmes. *Badula balfouriana* is currently the only Critically Endangered *Badula* species secured in botanical gardens. The four samples contributed from *ex situ* collections were all identified as a single haplotype (A). However, records indicate that one plant held at Copenhagen Botanical Garden (accession P2004-5074) is derived from collections made by Jean-Yves Lesouëf at Mt. Cimètiere. The haplotype survey identified this plant as Haplotype A, yet the only known living plant at this locality today was identified as Haplotype B. This *ex situ* plant may therefore represent genetic diversity now extinct in the wild. AFLP markers facilitated better resolution of genetic differentiation in this species, where applied (wild plants only) and AFLP markers must now be used to further characterize this garden material.

Limitations of the genetic diversity measures

The genotyping work included in Chapters 4 and 5 has not been repeated to test for reproducibility. Some indication of the reliability of the results can be drawn from the high accuracy and reproducibility of AFLP markers (Vos *et al.* 1995), which have the facility to distinguish genomic fragments that differ by as little as one base pair (Krauss 2000), and thus aid assessment of homology. In addition, four primer pairs were used for the samples, and each yielded over 100 bands (marker alleles), for example the data set used in Chapter 4 comprised a total of 591 markers. These data sets were assessed for congruence by subjecting them to Mantel tests (between all possible pairs of data), and in each case a significant correlation between data sets was revealed (indicated by Mantel test correlation coefficients of *Rxy* 0.43 to 0.61, $P < 0.05$). In addition, the samples used in Chapter 5 were also sequenced, and the resulting genetic diversity measure ($H = 0.272 \pm 0.027$) was similar to the value calculated from the AFLP data ($H_{PH} = 0.292 \pm 0.01$). While these data serve as an indication of the reliability of the values stated in Chapters 4 and 5, tests for reproducibility are required. It is therefore recommended that new DNA extractions are prepared, for at least a sub-set of the samples, so that they may be genotyped a second time using the same primer pairs. In addition, sub-sets of the data should be re-scored to allow an estimation of scoring error. Together these measures would allow assessment of the reproducibility of all markers and verification of the high genetic diversity levels recorded for these Critically Endangered island endemics.

6.6 Conclusion

This work has found strong evidence for the distinction of *Badula* as an evolutionarily unique unit, and has established that this lineage is endemic to the Mascarene islands. Evidence has also been found in support of the current infra-generic classification of *Badula* (Coode 1981). This clade comprises fourteen species, all of which are confirmed as endemic to single islands of the archipelago. Here they occupy a diverse array of habitats, from the dry, low-altitude forests of Rodrigues and southwest Mauritius, to upland heaths and mesic forests of Mauritius and La Réunion. The results indicate the existence of an isolated single-species clade that represents Rodrigues (*B. balfouriana*), a group of morphologically and ecologically varied, and clearly distinguished species in Mauritius, and the contrasting clade of La Réunion taxa, where the descendants of a common ancestor have diversified into a variety of apparently narrowly endemic species (such as the glaucous-leaved *B. decumbens*) and other morphologically intermediate forms, that may suggest incipient speciation on this geologically young island. As such, *Badula* represents one of the most diverse plant groups in the Mascarene islands and is an ideal candidate for further studies of angiosperm speciation in these islands. More work is needed to determine the timing of speciation events (currently under-way, with DNA sequence data contributed from this research; Strijk *et al.*, in progress) to allow elucidation of the pattern of colonization by ancestors of *Badula* among the islands. In particular, the mode of colonization of Mauritius remains obscure and it is not known whether few or many events led to the diversity of species found on the island today. In addition, further studies, including detailed morphological and cytological data, are required to further investigate the intermediate forms of *Badula* found in some areas of La Réunion. Of utmost importance, however, is the protection of these threatened species from extinction. The Critically Endangered species of Mauritius and Rodrigues need intensive conservation management, and in La Réunion, continued protection of habitats is imperative to safeguard the evolutionary potential of this diverse group.

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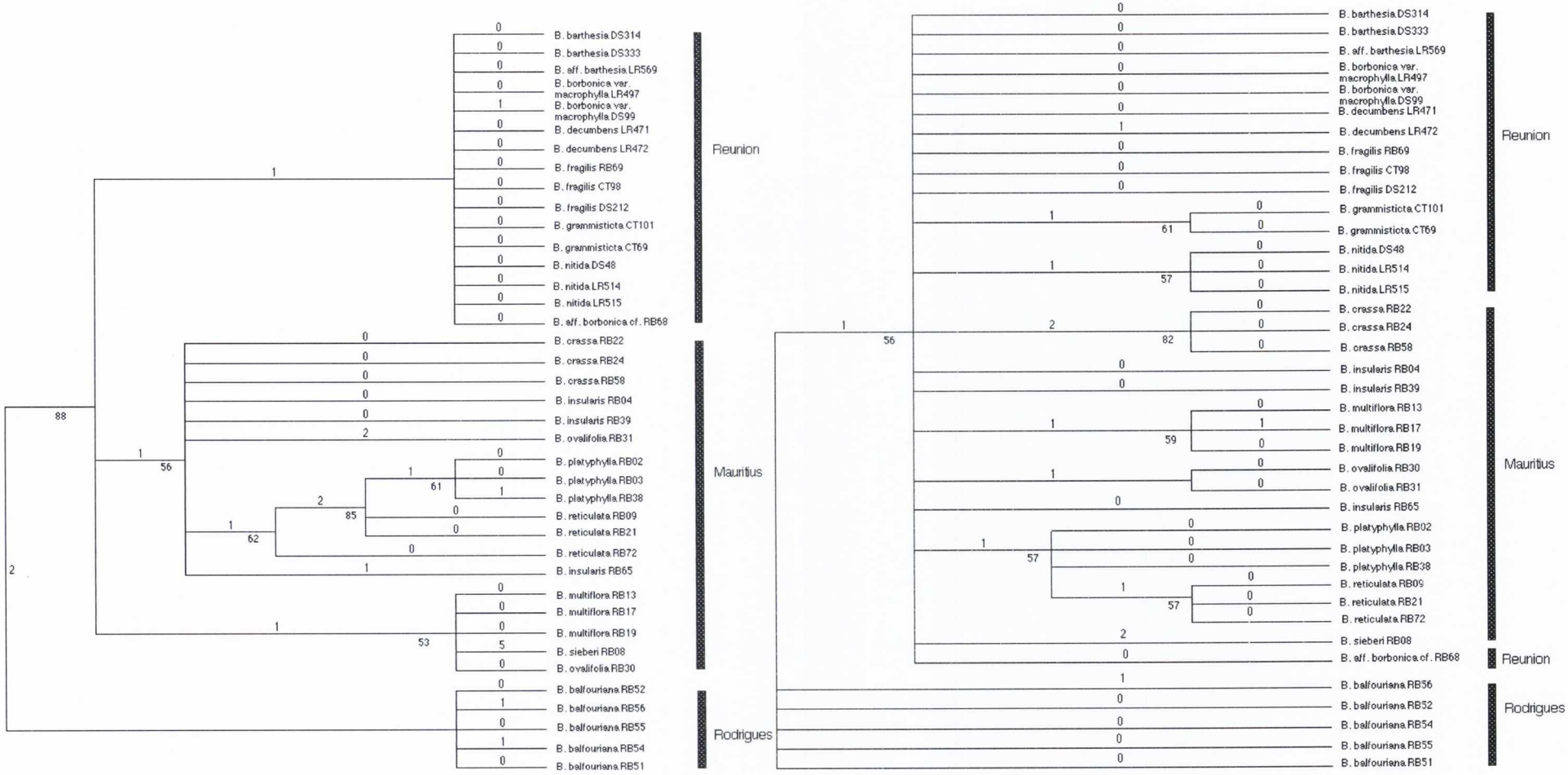
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Left: Rooted cladogram showing 1 of 3,768 MP trees from the ITS data
The tree was rooted using *B. balfouriana* (see Chapter 3). Tree length is 21. Numbers above branches indicate branch length. Numbers below branches indicate bootstrap support for clades (where 50>). Vertical bars indicate the island origins of taxa.

Right: Rooted cladogram showing 1 of 21 MP trees from the *trnSGG* data
The tree was rooted using *B. balfouriana* (see Chapter 3). Tree length is 14. Numbers above branches indicate branch length. Numbers below branches indicate bootstrap support for clades (where 50>). Vertical bars indicate the island origins of taxa.

Allele frequency per individual (or heterozygosity sensu Freeland, 2005*) was calculated and is reported below for all accessions (Chapter 4). Samples highlighted in bold show low allele frequencies in comparison to other samples (see Chapter 4).

Badula crassa

Acc. #	RB22	RB24	RB58	RB23	RB57
*H**	0.18	0.19	0.20	0.18	0.17

B. insularis

Acc. #	RB04	**RB11**	RB39	T03	T17	T20
*H**	0.22	**0.08**	0.20	0.23	0.19	0.21

B. ovalifolia

Acc. #	**RB28**	RB29	RB31	RB30	RB32
*H**	**0.07**	0.17	0.21	0.21	0.21

B. platyphylla

Acc. #	RB02	RB03	RB38	**RB01**	**RB79**	RB78
*H**	0.20	0.21	0.18	**0.08**	**0.06**	0.17

B. reticulata

Acc. #	RB10	RB09	RB21	RB71	RB72	RB74	RB75	RB07
*H**	0.15	0.20	0.21	0.20	0.17	0.20	0.20	0.16

* Sum of all alleles per individual divided by the total number of loci (see Freeland 2005, p. 21).