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Systematics of the Woody Bamboos
(Tribe Bambuseae)

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Thesis submitted for the Degree of Doctor of Philosophy
University of Dublin
December 2001
Systematics of the Woody Bamboos (Tribe Bambuseae)

Declaration

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Gráinne Ní Chonghaile

II
Bamboo

Unchanged on our planet for two hundred million years
Continuity between past and present
Imbued with a mysterious grace
You rise to the sky pushed by the force of life
You are the harmony of opposites
Serenity, whispers of wisdom, for those who contemplate you
In the hollow of your stems you are filled with the breath of the earth
Generous to man, a helpful friend
Green and vigorous in the heart of winter, you link us to eternity
To overcome the madness and excitement at the end of the millennium
Study bamboo and rediscover harmony!

By Muriel Crouzet 2000
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May the Force be with you all..................Always
Abstract

In this thesis the monophyly of the tribe Bambuseae and the inter-relationships of its constituents were assessed using comparative morphological character analysis and sequence analysis of four non-coding DNA regions, three of plastid origin (trnL intron, trnL-trnF spacer and the rpl16 intron) and one of nuclear origin (internal transcribed spacer (ITS) of rDNA).

In the morphological analysis, the temperate woody bamboos were resolved as a monophyletic group. However due to an overall lack of defining characters, internal resolution with support was lacking. The molecular study provided support for a monophyletic woody bamboo lineage and temperate clade. However, support for a monophyletic tropical lineage and, furthermore, Palaeotropical and Neotropical sub-clades was lacking. In all datasets analysed the internal branches of the tropical bamboo lineages were well supported while those of the temperate clade were weakly supported. It is hypothesised that this feature may be attributed to one or a combination of factors: over-taxonomy, rapid radiation, sequence rate heterogeneity and/or recent radiation of the temperate bamboo lineage. All datasets proved informative at resolving evolutionary relations at the intergeneric level in the tropical bamboos. However, only one molecular dataset, the ITS dataset, proved informative at resolving relationships at the intergeneric level in the temperate clade.

Comparative sequence analysis, using trnL-trnF and ITS sequences and AFLP analysis, were used to characterise the putative hybrid, Hibanobambusa tranquillans and its parents, in an attempt to elucidate its origin. The results questioned the hypothesis proposed by Kashiwagi, whereby, Phyllostachys nigra var. henonis and Sasa are suggested to be the parents for the putative hybrid. Furthermore, ITS sequence data provided evidence which questions whether H. tranquillans is an intergeneric hybrid. Several bamboo species
(Shibataea kumsaca, Pseudosasa owartarii and Sinobambusa tootsik) which share similarities in their morphology with *H. tranquillans*, were found to group closely with this species. Although this result could support either Shibataea, Pseudosasa or Sinobambusa as potential parents of the hybrid, it also could suggest an alternative conclusion: that *H. tranquillans* is not a hybrid at all but is simply closely related to these species as a result of non-reticulate evolution.
Chapter 1

Introduction

1.1 Woody Bamboos: A General Review

The woody bamboos are members of the grass family Poaceae. They collectively form the tribe Bambuseae and along with their herbaceous allies constitute the sub-family Bambusoideae (GPWG, 2001). To date there are in excess of one thousand species documented, constituting forty-nine to ninety genera, depending on the type of classification system used (Clayton & Renvoize, 1986; Stapleton et al., 1995).

Woody bamboos are commonly known as forest grasses due to their tree-like habit and ecological affinities with forest ecosystems. In brief, there are a number of morphological and anatomical characters that set woody bamboos apart from other grasses: their woody, usually hollow culms; complex rhizome and branch systems; pseudopetiolate leaf blades; prominent sheathing organs; external ligules; trimerous floral arrangement and arm and fusoid cells within the leaf blades. In addition, many woody taxa exhibit cyclical, gregarious, monocarpic flowering behaviour, many having unusually long flowering intervals which range from fifteen years to over one hundred years in duration (McClure, 1966; Dransfield & Widjaja, 1995; Judziewicz et al., 1999). This phenomenon has attracted much attention and several hypotheses have been proposed to explain it. These include: predator satiation, gap creation and energy partitioning (McClure, 1966; Janzen, 1976; Clark, 1997). Whether this phenomenon is in direct response to one or all of the aforementioned ecological pressures, or has evolved in response to an alternative influence, has yet to be verified (Clark, 1997).
From a geographic perspective, woody bamboos are indigenous to all continents with the exception of Europe and the Antarctic. Their greatest density and diversity is in South-East Asia and South America, occurring in tropical, subtropical and temperate regions. Bamboos inhabit a wide spectrum of habitats from lowland to mountain forests in both dry and humid tropics, on wasteland, swamps and dry or regularly flooded riverbanks (Dransfield & Widjaja, 1995).

Intensive investigations into the morphology, anatomy, physiology, biology, ecology and taxonomy of these grasses have only been initiated in the latter half of the last century. Due to the recent origin of these topics, much has yet to be discovered and understood. Inventories of natural bamboo populations are incomplete in many countries; yet, those that have been undertaken reveal a high proportion of undescribed taxa, indicating that much of the biodiversity remains undocumented (Stapleton & Rao, 1991). Indeed, many woody bamboo taxa have only been recognised and made known to science within the last generation, particularly those of the New World (Judziewicz et al., 1999; Ohmberger, 1999). As a result, the taxonomy of the Bambuseae is in a perpetual state of change. At the present time the taxonomic limits of sub-tribes and genera within the Bambuseae and their inter-relationships remain uncertain. Morphological and anatomical studies are ongoing and terminology has yet to be devised for certain aspects of the bamboo plant (Stapleton, 1997; Judziewicz et al., 1999). With regards to the physiology, biology and ecology of the woody bamboos, much emphasis had initially been placed on those species of economic importance in a bid to optimise biomass, growth etc. However, with a greater emphasis now being placed on the conservation of biodiversity, it is anticipated that those plants of lesser economic importance will soon be subject to further investigations (Li et al., 1998; Li et al., 1999).

1.2 Taxonomic Treatments Of The Bambuseae

There has been a number of classification systems constructed in the last twenty years for the Bambuseae (Keng, 1982; Watson et al., 1985; Clayton & Renvoize, 1986; Soderstrom
& Ellis, 1987; Stapleton et al., 1994; Wang, 1996; Yi, 1998; Ohrnberger, 2000). These classifications were constructed using a combination of morphological, anatomical, cytological and physiological characters. Comparison of these classifications shows considerable variation regarding the constituents and sub-divisions defined within the tribe. The question as to whether any of these classification systems represent natural or artificial groupings has yet to be comprehensively assessed. This particular study aims to evaluate two of these classifications: one constructed by Clayton & Renvoize (1986) and the other based on a consensus list developed by Clark et al. (1995) (see tables 1.1, 1.2).

‘Genera Graminum, Grasses of the World’ is an impressive comprehensive review of the family Gramineae published by Clayton and Renvoize in 1986. A three level classification system was adopted recognising sub-families, tribes and sub-tribes. All groupings are established on the basis of morphological, anatomical, cytological and physiological characters. According to Clayton and Renvoize (1986) forty-nine genera constitute the Bambuseae, each of which are assigned to one of three sub-tribes, Arundinariinae, Bambusiinae and Melocanninae. The sub-tribes are defined on the basis of presence and type of ovary appendage and also inflorescence structure. The Arundinariinae were deemed most primitive, having a simple ovary appendage and inflorescence. The sub-tribe includes a mixture of temperate and tropical woody bamboo species. Those included in the sub-tribe Bambusiinae are also of temperate or tropical origin. These woody bamboos possess a more complex inflorescence (usually compound or iterauctant) with a broadly conical and usually hairy ovary appendage. The sub-tribe Melocanninae constitute tropical woody bamboo genera, characterised by the possession of a long stiff tapering ovary appendage and iterauctant inflorescence (Clayton & Renvoize, 1986).

<table>
<thead>
<tr>
<th>Arundinariinae</th>
<th>Bambusiinae</th>
<th>Melocanninae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sinarundinaria Nakai</td>
<td>Ráceobambos Holttum</td>
<td>Schizostachyum Reichenb.</td>
</tr>
<tr>
<td>Colanthella McClure &amp; Smith</td>
<td>Nasus Juss</td>
<td>Oxylenanthera Munro</td>
</tr>
<tr>
<td>Thamnoedanus Munro</td>
<td>Pseudoeois Camus</td>
<td>Melocanna Trin.</td>
</tr>
<tr>
<td>Aulonemia Goudot</td>
<td>Heckel Camus</td>
<td>Ochtandra Thw.</td>
</tr>
</tbody>
</table>
Table 1.1. Classification of the Bambuseae according to Clayton & Renvoize (1986)

<table>
<thead>
<tr>
<th>Formerly Recognised Genera</th>
<th>New Genera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olmea Soderstrom</td>
<td>Decaryochloa Camus</td>
</tr>
<tr>
<td>Myriocladus Swallen</td>
<td>Paedia Franch</td>
</tr>
<tr>
<td>Glaziophyton Franch</td>
<td>Grestania Balansa</td>
</tr>
<tr>
<td>Chusquea Kunt</td>
<td>Semiarundinaria Nakai</td>
</tr>
<tr>
<td>Neurolepis Meisser</td>
<td>Phyllostachys Sieb. &amp; Zucc.</td>
</tr>
<tr>
<td>Arundinaria Mehanx</td>
<td>Shibataea Nakai</td>
</tr>
<tr>
<td>Hitchcockella Camus</td>
<td>Arthrostachys Ruprecht</td>
</tr>
<tr>
<td>Pernierbamibus Camus</td>
<td>Apocléada McClure</td>
</tr>
<tr>
<td>Guadualia Camus</td>
<td>Elstrochus McClure</td>
</tr>
<tr>
<td>Sasa Mak. &amp; Shib.</td>
<td>Athrostachys Bentham</td>
</tr>
<tr>
<td>Pseudosasa Nakai</td>
<td>Atractantha McClure</td>
</tr>
<tr>
<td>Indocalamus Nakai</td>
<td>Rhpidocladum McClure</td>
</tr>
<tr>
<td>Acalytopora Chu &amp; Chao</td>
<td>Actinocladum Soderstrom</td>
</tr>
<tr>
<td>Indosasa McClure</td>
<td>Merostachys Spreng</td>
</tr>
<tr>
<td>Sinobambusa Nakai</td>
<td>Bambusa Schreb.</td>
</tr>
<tr>
<td>Chimonobambusa Makino</td>
<td>Oreobambusa Schum.</td>
</tr>
<tr>
<td></td>
<td>Throstachys Gamble</td>
</tr>
<tr>
<td></td>
<td>Gigantochloa Munro</td>
</tr>
<tr>
<td></td>
<td>Dendrocalamus Nees</td>
</tr>
<tr>
<td></td>
<td>Melocalamus Bentham</td>
</tr>
<tr>
<td></td>
<td>Dinobambus Hise</td>
</tr>
</tbody>
</table>

The classification system documented in the publication ‘Bamboos of the World’ by Ohrnberger (2000) is based on a consensus list compiled by several bamboo taxonomists (Clark et al., 1994). The identification of genera and their assignment to various sub-tribes is based upon the investigations of Soderstrom et al. (1987) and also those studies carried out by the respective authors (Clark, 1995). The consensus assigns all formerly recognised genera to one of nine sub-tribes, which represent the temperate genera, the Palaeotropical genera and the Neotropical genera. Originally, the consensus was looked upon as a temporary measure, providing a foundation upon which subsequent studies could be based. However since its construction, this consensus has been used as a classification system. This classification/consensus list has since been modified with the inclusion of an
additional sub-tribe: Thamnocalaminae. To date, 90 genera have been recognised in this system (Table 1.2) (Clark, 1995).

<table>
<thead>
<tr>
<th>Sub-tribe</th>
<th>Genera included in the respective sub-tribes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperate sub-tribes:</td>
<td></td>
</tr>
<tr>
<td>Palaeotropical sub-tribes:</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.2. Classification of the Bambuseae according to Clark (1995)

<table>
<thead>
<tr>
<th>Subtribe</th>
<th>Genera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hickeliinae</td>
<td>Decaryochloa Camus, Grendania Balans, Hickelia Camus, Hickcochella Camus, Nastus Jussieu, Parietarialex Camus, Temburongia Dransfield &amp; Wong.</td>
</tr>
<tr>
<td>Neotropical sub-tribes:</td>
<td></td>
</tr>
<tr>
<td>Chusqueinae</td>
<td>Chusquea Kunth, Neurolepis Mezner.</td>
</tr>
<tr>
<td>Guadunae</td>
<td>Criciuma Soderstrom &amp; Londono, Bremocladon Soderstrom &amp; Londono, Guaduna Kunth, Olmeca Soderstrom, Oltea (McClure &amp; Smith) Calderon &amp; Soderstrom.</td>
</tr>
</tbody>
</table>
1.3 Woody Bamboos, People, Flora and Fauna

Woody bamboos are a prominent feature of the landscape in many parts of the world particularly in Central and South America and Asia. In western Amazonia, as one example, hundreds of thousands of square kilometres are dominated by jungles of Guadua, called pacales or tabocais, which are visible from satellites (Judziewicz et al., 1999). Due to their dominance, it is not of any great wonder that a wide range of associations have developed between bamboo and other organisms, including man.

Woody bamboos play a significant role as a natural resource in South-East Asia and Central and South America, both from a cultural and economic point of view. In many communities, past and present, bamboos have been used in the construction of homes and fencing, as a fuel, as a food source and in the production of domestic utensils, weapons, musical instruments, snare traps, fishing harpoons and even rafts (Dransfield & Widjaja, 1995; Judziewicz et al., 1999). Indeed, many industries rely on bamboo as a raw material including the construction, paper and food industries. The bamboo’s commercial and horticultural viability has promoted a major escalation in demand, extending beyond the boundaries of their native countries to the world market (Dransfield & Widjaja, 1995).

Bamboos have also been reported to have medicinal properties and have been used in the treatment of various ailments such as kidney infections, fevers and aching limbs. In addition bamboos have also become incorporated into myths and legends down through the centuries. The tribes of the middle Orinoco River (Venezuela) consider guaduales to be sacred forests inhabited by an evil spirit “Marmar”. The Jivaroan tribes of the upper Amazon of Peru and Ecuador sowed forests of Guadua near their dwellings in homage to the earth-mother god “Nunkut”. Even today there is a folk belief in southern Columbia and Ecuador that a person who encounters a flowering Guadua at midnight on a Friday is destined to become rich (Judziewicz et al., 1999)!
From an ecological and conservation standpoint, bamboos are highly important in many ecosystems. Bamboos play a considerable role in soil conservation. Their dense network of rhizomes and roots often contribute to the prevention of gully and sheet erosion more effectively than many tree species. In essence they provide a useful and low cost means of slope stabilisation (Stapleton, 1994; Judziewicz et al., 1999). Indeed, various woody bamboo species have played vital roles in soil redevelopment projects in Asia. Bamboo plantations have proven effective in the accumulation of organic matter, plant nutrients and soil stabilisation in areas which have been severely depleted of nutrients and soil as a result of coal mining (Singh & Singh, 1998).

Bamboo dominated habitats have been reported to support vast communities of unusual invertebrates, amphibians, birds and other animals. Investigations have revealed aquatic insects and amphibians unique to the miniature pond environments within culm internodes (Judziewicz et al., 1999). In a study of *Chusquea culeou* Desvaux a variety of parasites, aphids, thrips, mites and caterpillars were observed living in and around young buds (Widmer, 1998). A large number of bird species and small rodents feed or supplement their diets with the fruits, rhizome buds and emerging shoots of woody bamboos and also use the vegetative structures of the bamboo plant in nest building (Pearson et al., 1994; Judziewicz et al., 1999; Meserve et al., 1999). Larger animals known to be closely associated with the bamboo plant include the endangered giant pandas (*Ailuropoda melanoleuca*) and red pandas (*Ailurus fulgens*) of China. These animals evolved to fill a highly specialised niche as bamboo feeders. In the wild, they feed almost exclusively on bamboo leaves, shoots and fruits (Wei, 1999). In Madagascar there are three sympatric bamboo lemur-species (genus *Hapalemur*). These too are year round bamboo specialists. It is estimated that 88% of their diet consists of bamboo and they rely primarily on the species *Cathariostachys madagascariensis* that comprises 72-95% of their diets. Based on the updated IUCN Red List Criteria, two species, *H. simus* and *H. aureus*, have been reported to be most at risk of extinction by the end of the this century as a result of a decline in their habitats (Tan, 1999).
1.4 Systematics

Its Origin And Definition

Taxonomy and classification are two activities integral to the study of systematics, one of the most ancient practices in biology (Lewin, 1996; Li, 1997). Collectively they involve the identification of groups of organisms (taxonomy) and the subsequent placement of organisms in a logically organised hierarchical scheme of relationships (classification). Systematics has its origin in Greek science whereby classifications were constructed wholly on the basis of intuitive comparative morphology and not common ancestry (Lewin, 1996). In fact, the concept of evolution did not become known or widely accepted to science until the publication of Charles Darwin's 'On the Origin of Species' in 1859. Following the advent of Darwinism scientists strove to incorporate evolutionary relationships into classification systems; such biological classifications had to be hierarchial as, in theory, evolution produced a tree-like, diverging, hierarchical pattern of similarities among living things (Lewin, 1996).

Central to recognising evolutionary relationships among groups of organisms is the ability to distinguish between two types of physical similarity: homology and analogy. Homology is a type of similarity that arises as a result of common ancestry. Analogy is a superficial likeness that is the result of convergence rather than common ancestry. Although the distinction between both types is relatively easy to express in words, the distinction between both is frequently difficult to detect in practice (Lewin, 1996).

Two schools were established in the 1960s, phenetism and cladism, both aimed at breaking through this methodological impasse. Although both shared a common goal, philosophically they were extremely different, prompting an active debate over which was the more correct (Wiley et al., 1991; Lewin, 1996).
The pheneticists do not explicitly seek genealogies, but seek to group species on the basis of overall similarity. The pheneticists aim to avoid evolutionary uncertainty by assuming overall similarity represents a reasonable guide to evolutionary relationships, thereby avoiding the need to distinguish between homology and analogy (Wiley et al., 1991; Foley et al., 1996; Lewin, 1996). Subjectivity is minimised in the methodology as a large number of characters are compared by statistical techniques, with each character carrying equal weight regardless of its evolutionary meaning. Phenetics, or numerical taxonomy as it is also known, employs purely algorithmic cluster methods such as UPGMA (Unweighted Pair Group Method with Arimethic Mean) to construct phenograms or ordination methods to group individuals in a multidimensional space. Such methods use distance measures as an indication of similarity or dissimilarity between pairs of taxa. The result is a multivariate cluster statistic of pairs of species, ultimately yielding a hierarchy of clusters that represent a description of relationships (Lewin, 1996).

It is generally accepted that phenetics is less suitable for elucidating evolutionary relations than cladism as it lacks a well-founded evolutionary criterion upon which its methods should be based. Its inability to distinguish homologous characters from homoplasious ones only contributes to a distorted topology of evolutionary relations. It has also become evident that different phenetical methods result in incongruent hierarchies as a consequence of different algorithms producing varying measures of character similarity (i.e. different distance matrices can produce different tree topologies). In addition, some methods, such as UPGMA, perform on the assumption that the rate of evolutionary divergence is constant among different lineages, however, in cases where such an assumption does not hold, an erroneous topology is most likely to arise (Foley et al., 1996; Lewin, 1996; Li, 1997).

Cladism, most coherently formulated by Willi Hennig (a German entomologist) aims to reconstruct the phylogenetic relations between organisms, and then employs these relations in the construction of classification systems. Phylogenetic relations are established wholly on the basis of a specific type of character termed shared derived homology
(synapomorphy). A shared derived homology is defined as a character unique to a particular group of species and their most recent common ancestor. Synapomorphies define monophyletic groups (Li, 1997). In theory cladistic studies should result in classifications whereby species are grouped solely according to their most recent common ancestor. Furthermore, these classifications should be monophyletic for all such characters. Even those synapomorphies not formally used to define the classification should fall into the same groups because all evolutionary changes occur in the same phylogenetic tree.

Unlike phenetical methods, cladistic methods of phylogenetic inference are not purely algorithmic methods. Cladistic methods seek to accomplish their goal by defining an evolutionary criterion for comparing and evaluating alternative phylogenetic trees. Such methods entail two logical steps. The first step is to define an optimality criterion for evaluating phylogenetic trees and the second is to implement specific algorithms that will evaluate the alternative trees and find the more favourable tree(s) under the conditions specified in the criterion. There are two principal phylogenetic inference methods used in cladistics and these are known as Parsimony and Maximum Likelihood (Swofford et al., 1996). Parsimony methods operate on the criterion that trees that require the minimal amount of evolutionary steps to explain a given set of data are the more accurate phylogenetic hypotheses (Swofford et al., 1996). Maximum Likelihood methods evaluate an evolutionary hypothesis in terms of the probability that a proposed model of the evolutionary process and the hypothesised history would give rise to the observed data. It is conjectured that a history with a higher probability of giving rise to the observed data is preferable to one with a lower probability (Swofford et al., 1996).

Optimality criterion methods are often the more favoured means of phylogenetic inference as they have deep philosophical justification which phenetics lacks. They are objective in that they seek not to construct evolutionary groups but rather to discover them through the analysis of synapomorphic characters. Moreover, cladism is the only school of taxonomy that explicitly seeks to discover monophyletic groups (natural evolutionary groups).
Dobzhansky once stated that 'nothing in biology makes sense except in the light of evolution' (Soltis & Soltis, 1995). Soltis and Soltis (1995) elaborated that 'everything makes a lot more sense in the light of phylogeny'. The development of robust phylogenetic hypotheses provides a foundation for studies of both patterns and processes of evolution (Soltis & Soltis, 2000).

Since its inception, the discipline systematics has in its own way evolved. Today it aims to catalogue biodiversity and classify it according to evolutionary relationships, rather than intuition, based upon a combination of biology and statistical techniques (Li, 1997). The development of a phylogenetic perspective is an ongoing process. With further investigations underway there is no doubt that the present views on phylogeny will continue to change.

**Molecular Systematics**

Molecular systematics strives to discover and elucidate evolutionary relations through the analysis of genetic data using a combination of biotechnological and statistical techniques.

Today systematists can choose from a wide range of data sources for phylogenetic information. In addition to macro-, micro-morphological and anatomical characters, systematists are now equipped with the biological and technological competence to implement biochemical (secondary metabolites such as flavonoids and terpenoids) and genetic material (such as DNA, RNA and amino acids) in research (Minelli, 1993; Gielis, 1995). Genetic data or molecular data have proven to be highly useful for phylogenetic inference (Gielis, 1995). When compared to other types of data, genetic data holds a number of exclusive attributes that favour its use in systematics:

1. Genetic data provide genotypes, which are the most detailed anatomy of any organism.
2. Unlike morphological data or otherwise, genetic data tends not to be so easily modified by the environment. Environmental factors such as climate, nutrition, geographical position etc cannot modify genetic material.

3. Genetic data sets tend to be much larger than morphological or biochemical data sets, offering many characters for phylogenetic analysis.

4. Given that there has been a lot research in genome mapping and understanding the processes of heredity and evolution at work in these genomes, the homology of various genetic regions can be more easily assessed with such knowledge at hand.

5. Molecular data, be it a complete genome, gene or region of DNA or amino acid sequence can be selected for analysis based on its rate of evolution permitting the application of molecular markers appropriate for the taxonomic level of enquiry.

6. Molecular data is highly suitable to algorithmic methods of analysis (Soltis & Soltis, 1995).

In general molecular science has a relatively recent history. Much of genetics is founded on the publication of an Augustinian monk, Gregor Mendel, in 1866. He proposed that ‘heritable factors’ were responsible for the physical characters of organisms and that differences in these ‘factors’, that are today termed chromosomes, gave rise to biodiversity (Campbell, 1999). However it was not until the 1950s that the content of these ‘factors’ (chromosomes) were defined by James Watson and Francis Crick (1953). The content of Mendel’s ‘factors’ became known as DNA (deoxyribo-nucleic acids), its structure: a double helix, and its function: to transfer biological information between generations (Minelli, 1993). Further genetic research followed (and continues to this day) and many advances were made including the development of the neutral theory by Misoto Kimura; his work has been instrumental in the comprehension of the evolutionary process at the molecular level; and the synthesis of the first amino acid sequence by Fred Sanger and colleagues (1954), which paved the way for the synthesis of the first DNA sequence (Minelli, 1993; Ridley, 1996). Scientific innovations such as these gave rise to the establishment of a wide spectrum of molecular fields including functional genomics, and molecular systematics.
Prior to the early 1980s there was limited use made of genetic data in systematic research. Genetic data was still a new resource and hence in the early stages of investigation. In addition, there were few suitable analytical molecular techniques available because biotechnology and information technology were very much underdeveloped at that time. Initially molecular systematic investigations were achieved using protein and DNA assay techniques such as protein immunology, DNA-DNA hybridisation, restriction fragment length polymorphism (RFLP), and DNA sequencing (Appendix I) (Avise, 1989). These techniques were labour intensive, time consuming and costly to run as certain experiments (i.e. sequencing) involved in vivo techniques. However during the mid 1980s an in vitro amplification technique, Polymerase Chain Reaction (PCR) (Appendix I) was developed by Kary Banks Mullis (Soltis & Soltis, 2000). The introduction of PCR proved to be revolutionary; the protocol was less complex and time consuming, highly specific, and financially more feasible. In addition, PCR facilitated the automation of other techniques (such as DNA sequencing), and also the development of novel PCR based fingerprint methods such as Randomly Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) (Appendix I) which are useful for the detection, characterisation and evaluation of biodiversity. PCR and PCR-based techniques facilitated molecular taxonomic research at many levels which otherwise would not have been so easily achieved using former methods (Karp et al., 1996).

Although databases and analytical programs have been developed since the 1960s, the rapid rate at which they are produced today only began in the late 1980s following the explosive growth in biotechnology and information technology. As the amount of biological data increased so too did the demand for new and improved computer-based analytical programs. In response to this a new scientific field was established – bioinformatics. Bioinformatics fuses biology with mathematics and computer science. It provides the necessary technological facilities required to exploit biological data. Bioinformatics covers a wide range of topics from retrieving and aligning sequences, to predicting structure and function of gene products (Boguski, 1998). Several phylogenetic programs have been
developed to help calculate trees from molecular data. The best-known software for phylogenetics are PAUP (Phylogenetic analysis using parsimony) Phylip (Phylogenetic Inference Package), GCG (Genetics Computer Group) Wisconsin Package and MacClade (Lake & Moore, 1998; Appendix I). The internet has become an important component of bioinformatics. Courtesy of the world wide web (www), there are numerous sites available that provide databases (such as Genbank), database retrieval systems (such as Sequence retrieval system (SRS), Entrez and DBGET) (Appendix I), analytical programs and also literature (Lake & Moore, 1998).

Since its inception the pace at which molecular systematics has developed has greatly accelerated. Nucleotide sequences have now replaced proteins as the main source of data particularly since the invention of PCR. There have also been major advances in evolutionary theory and the development of sophisticated methods for recovering evolutionary information (Page et al., 1998).

The success of molecular systematics is reflected in the dramatic rise in the number of scientific papers produced in this field (Page et al., 1998). In years to come it is anticipated that the use of molecular data in conjunction with morphological data and improved analytical tools will significantly enhance the current understanding of organismal evolution and refine current classifications so that they convey a more accurate representation of phylogenies. Molecular systematics is coming of age!

**Problematic Issues in Systematics**

Despite the fact that the theory upon which systematics is based is well founded there are several problematic issues which the practicing systematist should be aware of prior to engaging in research.

The value of different types of data in phylogenetic research has been a heated subject of discussion for many years. The advent of molecular systematics has led to arguments about
the relative value of molecular versus morphological data. Some claim that molecular characters are relatively weak while others claim that morphological characters are likely to be misleading or uninformative. It needs to be recognised that all types of data have distinct advantages and disadvantages. As a result certain sources of data be they morphological, genomic or otherwise may be more suitable for addressing certain phylogenetic questions while others may not be so informative. Therefore the real concern for the systematist is awareness of the applicability and limitations of the data chosen for systematic analysis (Hillis & Moritz, 1998).

There is a growing awareness that reliance on a single data set may often result in insufficient phylogenetic resolution or misleading inference. Accordingly it is increasingly widespread practice to apply multiple data sets to a common group of taxa (Wendel & Doyle, 2000). In general, studies that incorporate multiple data sets will provide much better descriptions and interpretations of biological diversity than those that focus on just one approach (Hillis & Moritz, 1998).

One of the consequences of analysing multiple data sets is that the phylogenies inferred may differ from each other in one or more details. This phylogenetic incongruence is not a rare occurrence, to the contrary, it is almost the rule rather than the exception, being evident in varying degrees (Wendel & Doyle, 1998). Given the prevalence of phylogenetic incongruence, the question naturally arises as to whether two or more independent data sets should only be analysed separately or whether they should be combined in a global analysis. Despite considerable discussion in the past and present regarding this issue no clear consensus has emerged as to the most appropriate course of action (Wendel & Doyle, 1998).

Not all incongruence is equal in magnitude and the topological differences that exist between competing phylogenies may have a number of causes (summarised in Table 1.1 and see appendix) some of which are artefactual. Consequently efforts are being made to
measure incongruence and evaluate whether it is real and hence biologically significant or spurious due to insufficient character evidence, excessive homoplasy or some other cause (Wendel & Doyle, 1998).

‘Phylogenetic incongruence is usually regarded as an undesirable result, which represents an impediment to achieving phylogenetic understanding’ (Wendel & Doyle, 2000). However discordances such as these can be insightful, reflecting one or more evolutionary processes (such as convergent evolution, hybridisation, introgression, lineage sorting, paralogy conflation etc) that would have otherwise gone undetected in the absence of phylogenetic incongruence (Wendel & Doyle, 2000).

<table>
<thead>
<tr>
<th>Technical causes</th>
<th>Biological Processes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insufficient data</td>
<td>Convergent evolution</td>
</tr>
<tr>
<td>Gene choice</td>
<td>Rapid diversification</td>
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<tr>
<td>Sequecing error</td>
<td>Hybridisation/introgression</td>
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<td>Taxon sampling</td>
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<td>Horizontal transfer</td>
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<td>Intragenic recombination</td>
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<td></td>
<td>Orthology/paralogy conflation</td>
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<td></td>
<td>Rate heterogeneity among taxa/molecular sites</td>
</tr>
</tbody>
</table>

Table 1.2. Phenomena that may give rise to phylogenetic incongruence amongst independent data sets representing a common group of taxa.

It should be noted that phylogenetic discordance may be attributed to one or more or a combination of biological and technical factors and in many instances it is very difficult to pinpoint its exact origin (Wendel & Doyle, 1998).

Arguments continue over the efficiency, consistency and robustness of competing methods used in phylogenetic studies (Hillis & Moritz, 1998). Regardless of where one stands on this issue, it is very clear that no method of phylogenetic inference is completely resistant
to systematic error\(^1\). Systematic error is defined as a deviation between a parameter of a population and an estimate of that parameter due to incorrect assumptions in the estimation method. In other words, systematic error occurs when the evolutionary process that gave rise to the data set violates the assumptions of the phylogenetic method used in the analysis. Systematic error generates a bias within the evaluation of alternative phylogenies favouring particular branching patterns and decreasing the support for others. Consequently, systematic error may overwhelm the legitimate support for the correct tree and lead the researcher to an incorrect conclusion (Hillis & Moritz, 1998).

Realistically, systematic error should be expected in any given systematic study; one can never be overly confident that no violation of assumptions has occurred. However measures can be taken to reduce the affect of systematic error in an analysis. For instance phylogenetic assumptions can be assessed using a ‘test of model fit statistic’. In addition unreliable data that contributes to systematic error can be eliminated from the analysis (Hillis & Moritz, 1998).

### 1.5 Bamboo Systematics: Past And Present

Bamboos have been documented since the early seventeenth century. In a publication entitled ‘Pinax’ by Caspar Bauhin (1623) bamboos were listed as ‘Arundos abor’ that translates as woody or treelike reed. Bamboos were first formerly taxonomised in 1753 in a publication entitled ‘Species Plantarum’ by Carolus Linneaus. Linneaus denoted the name ‘Arundos bambos’ to the bamboos documented at that period. However this simplified system of nomenclature became gradually more complex in structure when it was realised that the myriad of bamboo species included under ‘Arundos’ represented a number of different genera. This resulted in the allocation of species to separate genera.

\(^1\) One may argue that systematic error is irrelevant in parsimony analysis since it has been claimed that parsimony does not make assumptions on evolutionary change. However, parsimony does make assumptions regarding evolutionary change; the difficulty lies in stating what exactly these assumptions are. At a minimum, acceptance of an optimal tree under parsimony criterion requires one to assume that conditions that can cause parsimony to estimate an incorrect tree are unlikely to occur (Hillis & Moritz, 1998).\(^1\)
By the early nineteenth century bamboos were recognised as one of the major subfamilies within the grasses (the Bambusoideae). This was followed by the first system of classification for bamboos. A German botanist, Nees von Esenbeck (1835), in his treatment of the Brazilian bamboos recognised three groups—two of which were woody, Bambuseae and Arundinareae, and one of which was herbaceous, Streptochaeteae (Soderstrom, 1985). Further taxonomic studies followed (including the work of Ruprecht (1839) and the monographs of Munro (1868) and Gamble (1978)) and from these investigations new species and genera were defined and classifications were modified accordingly (Soderstrom, 1980).

Although there was a general consensus amongst taxonomists that woody bamboos had herbaceous allies (although there were several exceptions such as Prat, 1960) the definition of these herbaceous relatives was highly controversial (Renvoize & Clayton, 1986; Clark et al., 1995).

Throughout the last century independent authorities gave rise to a series of classification systems that varied markedly in the number of genera and divisions assigned to the Bambusoideae, particularly the number of herbaceous taxa included in the subfamily (Prat, 1960; Watson et al.; 1985; Clayton & Renvoize, 1986; Soderstrom & Ellis, 1987; Kellogg & Campbell, 1987; Tzvelev, 1989). In general, the woody bamboos were included in a broadly defined subfamily, Bambusoideae sensu lato, which also contained a heterogeneous collection of herbaceous taxa whose taxonomic relationships with each other and the Bambuseae were uncertain (Renvoize & Clayton, 1986; Clark et al., 1995).

Despite the inconsistencies evident between independent classifications, most taxonomists believed that the Bambusoideae represented the most ancestral extant grass taxa since these grasses exhibited, amongst other characteristics, primitive floral morphologies. Yet certain members of the bambusoid clade possessed morphological characters that were considered advanced such as the woody culms and extensive lateral branching systems of the
Bambuseae. However such features were for the most part overlooked as grass taxonomy at that time seemed to have a bias toward floral characteristics (Clark et al., 1995).

The problematic circumstance of bamboo taxonomy was highlighted in 1993 following the detailed investigation of the Bambusoideae s.l. by Kellogg and Watson. They concluded that the classifications constructed by Soderstrom and Ellis (1987) and other authors did not reflect monophyletic groupings. Furthermore they stated that the polyphyletic nature of these groupings were attributed directly to the implementation of symplesiomorphic morphological and anatomical characters in taxonomic studies.

The results from this assessment highlighted several critical issues; if future bamboo classifications were to reflect the phylogenetic relations of true bamboos the definition of a bamboo required clarification and the criteria upon which the classifications were built needed to be re-evaluated.

The advent of molecular systematics invigorated bamboo taxonomy and offered hope that stable classification systems may be established. The implementation of plastid and nuclear sequence data in assessing the monophyly of groups within the Bambusoideae has progressed at speed with many researchers contributing to the growing knowledge (Kellogg and Watson, 1993; Davis & Soreng, 1995; Clark et al., 1995; Zhang et al., 1995; Duvall & Morton, 1996; Hsiao et al., 1999; Zhang et al., 2000; GPWG, 2000, 2001). These studies supported a robust monophyletic Bambusoideae *senso stricto* constituting two or three cladistically monophyletic lineages; the Bambuseae and the herbaceous Olyreae and Buergsiochloideae (Zhang et al., 1995; Duvall & Morton, 1996; Hsiao et al., 1999; GPWG, 2000, 2001). In addition the molecular investigations conducted by Clark et al. (1995) resolved for the first time *Anomochloa, Streptochaeta* and *Pharus*, as the basal most taxa to all grasses, a hypothesis that had been suggested in the past (Soderstrom, 1981) but never verified until then. Subsequently, *Streptochaetaceae* and *Anomochloaceae*, and *Pharoaceae* were subscribed to separate subfamilies, Anomochloooideae and Pharoideae respectively (Clark
& Judziewicz, 1996). In recent times the Grass Phylogeny Working Group (2000, 2001) have confirmed that Anomochlooideae and Pharoideae are the earliest diverging lineages of the grasses with Puelioideae as sister to the core grasses.

Studies of the Bambusoideae s.str. have defined three major divisions, which correspond to the herbaceous bamboo lineage, the temperate woody bamboo lineage and the tropical woody bamboo lineage. Furthermore, the tropical woody bamboos sub-divide into New World and Old World clades. Unfortunately, resolution within these clades has been weakly supported (Clark et al., 1995; Zhang et al., 1995; Zhang & Clark, 2000). Subsequent molecular systematic investigations have been carried out on the Bambuseae however such studies have been restricted to answering phylogenetic questions relevant to one or several genera (Friar et al., 1991, 1994; Watanabe, 1994; Hodkinson et al., 2000; Loh et al., 2000).

Despite the aforementioned discoveries, the systematics of the woody bamboos remains shrouded in controversy. Although previous molecular investigations have resolved a cladistically monophyletic woody bamboo clade it must be noted that these investigations did not contain a comprehensive representation of the members constituting the Bambuseae. In other words these studies did not specifically serve to assess the monophyly of the tribe as documented in any of the classifications proposed in previous years (Keng, 1982; Watson et al., 1985; Clayton & Renvoize, 1986; Soderstrom & Ellis, 1987; Stapleton et al., 1994; Wang, 1996; Yi, 1998; Ohrnberger, 2000). Furthermore, most of the genetic data employed in these studies did not provide enough character variation to resolve relationships at the intergeneric level.

Without a comprehensive assessment of the monophyly of the Bambuseae and its sub groupings as defined in the more recent classifications (i.e. Clayton & Renvoize, 1986, Orhnberger, 2000), the problematic nature associated with the systematics of this group will not improve.
In years to come, it is anticipated that further research into the morphology and genetics of these plants will lead to the discovery of reliable characters, which can be implemented in systematic investigations with confidence. In turn, it is hoped that such investigations will enhance the current understanding of bamboo evolution and refine the current classification of the Bambusoideae and its constituents, including the alignment and definition of tribes and genera (Clark, 1995).
1.6. Methods for Phylogenetic Inference

Maximum likelihood is a model-based approach to phylogenetic inference; it evaluates specific evolutionary hypotheses using explicit models of evolutionary change (Lewis, 2000). Phylogenetic inferences are made through the evaluation of the probability that the chosen model generated the observed data; phylogenies are then inferred by finding those trees that yield the highest likelihood's (Swofford et al., 1996).

The models employed in likelihood methods are base substitution models that serve to account for the conversion of one nucleotide sequence to another. A number of such models have been developed differing in the assumptions made regarding specific substitutional parameters such as the substitution rate and the base frequency parameter (Swofford et al., 1993). The Jukes/ Cantor model (1969) assumes that the frequency for all four bases are the same and that all substitutions (transversions and transitions) occur at the same rate. The Kimura 2- Parameter model (1980) differs in that it assumes that transitions and transversions do not occur at the same rate. The Hasegawa/ Kishino/ Yana model (1985) differs from the former models in that it assumes unequal base frequencies in addition to unequal transition and transversion rates of substitution (Lewis, 2000).

Depending on the model chosen, all parameters may be fully defined (as in the Jukes/ Cantor model) or some may need to be estimated from the data prior to analysis (as in the Kimura 2- Parameter model and the Hasegawa/ Kishino/ Yana model) (Swofford et al., 1993). The flexibility of those models that do not fix all substitution parameters enables systematists to tailor their analyses to the particular details of the group or genes understudy (Lewis, 2000).

"The best evolutionary model is one that captures only those features of reality that are necessary to answer the question at hand, more detailed models simply cloud the picture" (Lewis, 2000). In other words the models used for statistical inference, should include only
enough parameters to capture the most important aspects of the process being modelled. One effect of adding too many parameters in the context of phylogenetic inference is that the distinction between alternative tree topologies becomes increasingly small. So how does one choose the best model? The answer is data exploration and hypotheses testing of the models used. It is only through such measures that the factors most important to that particular group or gene can be discovered. Failure to account for factors, such as site to site rate heterogeneity and non-independence among sites, in an analysis will result in the occurrence of phenomena such as long branch attraction and base compositional attraction which ultimately gives rise to erroneous phylogenetic hypotheses (Lewis, 2000).

Parsimony methods do not employ explicit substitution models to evaluate competing phylogenetic hypotheses. This approach infers phylogeny through the application of a basic principle: that the tree topology that requires the smallest number of evolutionary changes to explain the differences observed among the individuals under study is the more accurate phylogenetic hypothesis (Li, 1997). Although the assumptions made regarding the evolutionary process using the parsimony criterion is not clear, at the very least it can be said that parsimony assumes that different sites or characters are independent and that a bifurcating tree adequately depicts the underlying phylogeny.

There are a number of phylogenetic inference approaches available that are based on the parsimony principle, differing from each other with regards to the constraints or lack of them placed on the means through which characters transform from one state to another. Fitch and Wagner parsimony are the simplest methods imposing no (Fitch) or minimal (Wagner) constraints on permissible character state changes. Wagner parsimony assumes that intervening states are measured on an interval scale; thus it is appropriate for binary, ordered multistate and continuous character datasets. The Wagner method assumes that any transformation from character state to another also implies a transformation through any intervening states, as determined by the ordering relationship. Therefore if a character with state ‘A’ changes to state ‘D’, the character must transform from ‘A’ to ‘B’ to ‘C’ and
finally to ‘D’. Fitch parsimony differs from the former method in that any state can transform to another, i.e. ‘A’ can transform directly into state ‘D’ without transforming into the intermediate states of ‘B’ and ‘C’. Fitch parsimony is thus suitable for datasets that have unordered multistate characters. Both methods permit free reversibility; that is, change of character states in either direction is assumed to be equally probable (i.e. the probability of ‘A’ changing to ‘D’ is equal to the probability of ‘D’ changing to ‘A’) (Swofford et al., 1998).

Much of the phylogenetic information available has resulted from the application of parsimony methods to discrete character sets. One reason for this is that an analysis using the maximum likelihood approach is a lot more time consuming. The larger the dataset and the more parameters included in a substitution model means a considerably higher expenditure of computing effort in the calculation of likelihood’s. Moreover systematists tend to lack confidence in the capabilities of such methods the principal reason being that likelihood methods make too many assumptions regarding the evolutionary process (Swofford et al., 1998; Lewis, 2000).

Unfortunately debates concerned with identifying the best optimality criterion for phylogenetic inference are futile as there is no way of determining with certainty the effectiveness of any criterion at recovering the true phylogeny (Lewis, 2000). The best that can be done is to make informed decisions regarding the way phylogenetic investigations are carried out; this can only be achieved through research into the group under study, the characters used and the inference methods currently available.

In this thesis Fitch parsimony was employed in the sequence analysis of the Bambuseae. Maximum likelihood methods were not employed as the computation time required to analyse the datasets was too great. In addition to parsimony, a distance method was also employed in the research, principally as an indicator of the robustness of groupings obtained in the parsimony analyses. The distance method employed was the Neighbour
Joining (NJ) distance method. The NJ method is an additive tree method. Like all distance methods, the evolutionary distances are computed for all pairs of taxa, and a phylogenetic tree is constructed by using an algorithm, in this case NJ, based on some functional relationships among the distance values (Li, 1997).
1.7. Research Objectives

The objectives of the current research entitled ‘Systematics of the woody bamboos (Tribe Bambuseae)’ aims to evaluate the tribe Bambuseae through comparative morphological and DNA sequence analysis. The main objectives of this research are given below:

1. Assess the monophyly of the tribe Bambuseae through morphological analysis and sequence analysis of DNA regions of both nuclear and plastid origin.

2. Assess the sub-tribal and intergeneric relations of its constituents.

3. Evaluate two recently constructed classification systems proposed for the tribe (Clayton & Renvoize, 1986; Clark et al., 1995).

4. Assess the utility of the morphological characters and DNA regions employed for taxonomic investigations at this level.

In addition to the above, this thesis aims to characterise the putative intergeneric hybrid Hibanobambusa tranquillans Koidzumi and its parents using comparative sequence analysis of both plastid and nuclear DNA and also AFLP analysis.
Chapter 2

Morphological Analysis of the Temperate Bamboos

2.1 Introduction: Bamboo morphology

The Bamboo Seedling

After fruit formation, seed dispersal and perhaps a period of dormancy, the life of a new bamboo plant begins through seed germination and subsequent growth. Essentially the Bambusoid seedling consists of a short coleoptile, which is generally not elevated above the germinating caryopsis. The first one to several foliage leaves are very small, often represented merely by a point. The first expanded blade is usually broad and held horizontally away from the seedling (McClure, 1966; Soderstrom, 1980; Judziewicz et al., 1999).

The Mature Bamboo Plant

Subsequent growth and cell differentiation transforms the juvenile plant into the adult form consisting of a woody culm with an extensive rhizome and lateral culm branching system.

Rhizome Systems

The bamboo rhizome is a segmented subterranean stem which functions to support aerial culms, store food reserves and also allow vegetative reproduction. Rhizomes evolved throughout the grass family but are best developed in the Bambuseae (Judziewicz et al., 1999).
Like all other regions of the bamboo plant, the rhizome is made up of repeating units/segments. Each unit/segment consists of a node, an internode, a sheath, a bud and one or more roots (McClure, 1966; Stapleton, 1994a, b; Judziewicz et al., 1999). Internally, the rhizome exhibits an organisation similar to that of a stem, except that an endodermal-like layer may be present around the vascular tissue as in roots (Judziewicz et al., 1999). A single rhizome may be comprised of many segments, but there are essentially two recognisable portions, the rhizome neck and the rhizome proper. Typically the rhizome neck consists of a number of short segments that lack buds or roots and it is narrower than the rhizome from which it arose. The neck functions in the physical placement of the rhizome proper both vertically and horizontally. The rhizome proper includes several to many segments, each of which bears a bud and a few to many roots. Each node of the rhizome regardless of its position bears a scale-like structure called the rhizome sheath which acts as a protective shroud around the node and internode during early development (McClure, 1966; Stapleton, 1994a, b; Judziewicz et al., 1999).

Two general patterns of branching, sympodial and monopodial branching, are recognised in bamboos. In sympodial branching each successive axis becomes dominant in turn as it develops, whereas in monopodial branching only one axis is dominant and secondary axes are derived from it. The terms sympodial and monopodial branching are not only restricted to the rhizome form but are also used to describe the branching pattern in other parts of the plant such as in the culm branches and the inflorescences. Another branching form found to occur in the bamboo rhizome is amphipodial branching. In this form both sympodial and monopodial branching occur (McClure, 1966).

Morphologically two broadly defined rhizome types, pachymorph and leptomorph, are recognised which are closely correlated with branching pattern (McClure, 1966; Stapleton, 1994a, b; Judziewicz et al., 1999). The pachymorph rhizome has a short thick rhizome proper. Its maximum diameter is usually somewhat greater than that of the culms that arise from it. Pachymorph rhizomes always have sympodial branching, such that each rhizome
apex turns upward and gives rise to an aerial culm (determinate growth). The transition from rhizome proper to aerial culm is usually abrupt. The rhizome segments are wider than they are long, asymmetrical and solid. A single bud is evident in each segment. It is subcircular in outline, asymmetrically dome-shaped and its apex is divergent from the parent (Judziewicz et al., 1999). Adventitious roots may be restricted to the nodal line but usually are found throughout each segment, being concentrated on the ventral surface. The neck of pachymorph rhizomes may be short or highly elongated, sometimes extending several metres (Judziewicz et al., 1999).

Leptomorph rhizomes differ from the pachymorph type in that they are comprised of a short neck and a long slender rhizome proper with a maximum diameter no greater and usually somewhat less than the culms originating from it. Generally they exhibit monopodial branching, however, this is not always the case. The dominant axis usually grows indeterminately, but, in some cases it turns upward to form a culm, although a transitional zone intervenes (the metamorph II axis of McClure, 1966). The segments are longer than they are wide, relatively symmetrical and usually hollow. A single bud per node is evident which tends to be somewhat triangular in outline and more or less boat-shaped, and the apex points parallel to the parent axis. The roots tend to be restricted to a ring just above the nodal line.

At present, morphological studies of the bamboo rhizome generally recognise and differentiate between four to five rhizome categories:

1. Short necked pachymorph rhizomes
2. Long-necked pachymorph rhizomes
3. Leptomorph rhizomes with single culms
4. Leptomorph rhizomes with tillering culms
5. Amphimorph rhizomes
These differences encountered in rhizome morphology and branching patterns are, most probably, ecological adaptations. The formation of clumps may help bamboos to occupy gaps and out-compete other plants for space and light, whereas diffuse spacing of culms may represent a strategy for searching out other gaps in a forest ecosystem. It is also possible that under certain substrate and environmental conditions, rhizomes may become altered from their original form. For example, long necked pachymorph bamboo species may produce shorter necked rhizomes (McClure, 1966; Judziewicz et al., 1999).

Culm

The aerial vegetative axes of a bamboo plant are called the culms. They consist of an alternating sequence of nodes and internodes as in the rhizome (Figure 2.1). In fact, the rhizomes, culms and branches of a bamboo plant form an interconnected system of segmented vegetative axes (Judziewicz et al., 1999). The repeating units that make up a culm consist of a node, an internode, a bud and a culm sheath (one or more root primordia may also be present). Differentiation, development and maturation along a given axis occur from the base towards the apex, thus the most fully developed and taxonomically characteristic leaves, buds and branch complements are found at the mid culm nodes.

The nodal regions bear the culm sheaths, the buds found in the axil of the culm sheath and the subsequent branch complement. Externally, the lowermost boundary of the node in bamboos is the nodal line or sheath scar. The uppermost boundary of the node is called the supra-nodal ridge; the intervening zone is known as the nodal zone and this is where the bud is inserted. If root primordia are present they too are found in this region. Internally, the node is a solid, horizontal plate of anastomosing vascular tissue known as the nodal diaphragm.

The internodes are the intervening regions between the successive nodes on the culm. The internodes may be completely terete, flattened or grooved above the bud/branch complement. Internally, the internodes are often hollow with a well-defined lumen. Wall
Figure 2.1. Culm of *Phyllostachys pubescens* illustrating the alternate pattern of nodes and internodes (Starosta, 1996).
Systematics of the Woody Bamboos (Tribe Bambuseae)

thickness varies from very thin with a large lumen to very thick with a small lumen. Exceptions to this trend are the completely solid internodes that are characteristic of the genus Chusquea and certain species of other genera such as Merostachys ternata Nees, Guadua amplexifolia Presl. and Guadua glomerata Munro. The surface of the internode varies greatly among taxa in texture and indument, ranging from completely smooth surfaces to grooved or pubescent surfaces. Surfaces may also be roughened to a lesser or greater extent by warty excrescences or sharp trichomes. The internodes are usually green when young but may change colour with maturity. A waxy, glaucous bloom is often present on young internodes and heavier white floury deposits may also be observed.

The culm sheath is found arising at each node enveloping the developing internodes (Figure 2.2). Its presence prevents mechanical abrasion and pest damage during the developing stages of the young internodes. A culm sheath consists of an expanded sheath, a reduced or at least modified blade and an inner ligule. Auricles and or oral setae may be present and a band of tissue known as the girdle may be present connecting the base of the sheath to the nodal line. The girdle is composed of a tough but elastic tissue. It is not unusual for the girdle to be scabrous or pubescent, especially in species with a climbing habit such as Dinochloa spp. Indeed, the girdle may be a different colour from the sheath as in Merostachys spp. The sheath blade is highly variable in size, position and morphology. It may be narrow, broad, smaller, equal or larger in length than the sheath. It also varies in shape (from linear to lanceolate), in symmetry, indument and stature. Culm sheath’s characteristics may be diagnostic for certain genera and are critical for the identification of species. The best-developed and most representative samples of these organs are also found in the midculm regions of the bamboo plant (McClure, 1966; Judziewicz et al., 1999).

Bud Complement

In woody bamboos single buds or multiple buds are observed in the nodal region of the culms. The bud complement is centred in the axil of the culm sheath borne at each node.
Figure 2.2. Culm sheath of *Bambusa vulgaris* 'Vittata' (Starosta, 1996)
Along the vegetative axis the bud complements are arranged in a distichous fashion (McClure, 1966; Judziewicz et al., 1999). The shape of the bud, presence or absence of the promotory and hairs, the morphology of the protective prophylls and the relative size and arrangement of the buds can also be useful in woody bamboo taxonomy (Stapleton, 1993; Judziewicz et al., 1999).

The bud prophyll is the first leaf of the axis that is produced by the growth of a bud. The prophyll encloses and protects the delicate branch tip until growth is initiated. The prophyll is adaxial in position and keeled; the margins clasp the bud while the wings are closely appressed to the main axis. The wings of the culm buds can be inconspicuous or quite prominent and they are often ciliate and pubescent. In Chusquea subgenus Rettbergia as well as other species of Chusquea in which the central bud is dome-shaped, the prophyll lacks wings entirely. The wings tend to be much less developed in the rhizome buds and are absent in the dome-shaped buds, which are found exclusively on pachymorph rhizomes (Judziewicz et al., 1999).

The bud complement may be elevated on an unsegmented swelling called the promontory. Its origin and function are uncertain but it has been suggested that the promontory along with a persistent, rough girdle may be an adaptation for the climbing or vining habit encountered in some woody bamboo taxa such as Alvimia and Athroostachys (Judziewicz et al., 1999).

**Branch Complement**

The complex branching of the Bambuseae presumably is adaptive for light competition, for which the rapid extension of photosynthetic surfaces and optimal positioning of leaves are important factors. The overall pattern of branching is monopodial; that is, the main culm is the main axis that gives rise to lateral branches. Branching may be described as either intravaginal, extravaginal or infravaginal. Intravaginal branching is a condition resulting from the branches growing more or less appressed to the culm, emerging through the mouth
Systematics of the Woody Bamboos (Tribe Bambuseae)

of the sheath without rupturing it. Extravaginal branching occurs when branch development is divergent to the culm axis and the branches emerge through the sheath by rupturing the sheath at its base. Infravaginal branching is a modification of the latter in which the girdle is well developed and the branches emerge horizontally and downward by rupturing the girdle (McClure, 1966; Stapleton, 1994a, b; Judziewicz et al., 1999).

Variation in branch complements among woody bamboo species is remarkable, and the number, arrangement and pattern of branching provides invaluable clues to the identity and inter-relationships of bamboos (Stapleton, 1993) (Figure 2.3). The concepts and terminology regarding branching have not been fully developed and further investigations are warranted before a consistent interpretation of branching patterns can be developed that can be applied to all bamboos (Stapleton, 1997). The number of branches varies from one to many depending on the species. Perhaps the most common arrangement is the branch complement in which the primary branch grows from a single bud and remains strongly dominant, with a few to many smaller lateral branches arising from buds at the basal nodes of the primary branch. Another variant is the development of several sub-equal branches. In some such cases, subsequent rebranching above the branch base occurs i.e. Apoclada. The arrangement of the branches ranges from linear to crescent-shaped to verticillate (Judziewicz et al., 1999).

Thorns are modified branches and in the Bambuseae thorns arise from branches of various orders that have become dwarfed, hardened, sharp-pointed and usually curved. Guadua is the only New World genus known to bear thorns; Bambusa, an Old World tropical bamboo, is another genus where such structures are evident in some species.

Foliage Leaves

Bamboo leaves consist of two basic parts, the sheath and the leaf blade. The foliage leaves, whose principal function is photosynthesis, have relatively small sheaths and well-
Figure 2.3. Branch complement of *Chimonobambusa quadranularis* (Starosta, 1996)
developed leaf blades. In addition to the leaf sheath and the leaf blade, an inner ligule is always present on the adaxial side. The leaves are distichously arranged (Figure 2.4). Along any given axis leaves at the basal most nodes tend to be less well developed. On a leafy branch, the lowermost leaves are bract-like and are often called cataphylls (Judziewicz et al., 1999).

Sheaths are often green and presumably photosynthetic but probably more importantly function to support the leaf blades. The ridges or lines observed on the outer surface of the sheaths are the external manifestations of the vascular strands that run length-wise and eventually extend into the blade providing the vascular connection to the culm. The group of vascular strands that form the midrib of the blade may be prominent along the back of the blade and form a projecting keel. The inner surface of the sheath is typically smooth and shiny. The sheaths may be persistent or deciduous. Like the culm sheath, the sheath's summit bears both an inner ligule. Auricles, cilia and oral setae can also be present depending on the taxon (Judziewicz et al., 1999) (Figure 2.5).

The bamboo leaf blades vary in length from several centimetres to several metres i.e. Neurolepis nobilis Munro. Woody bamboos possess a constriction at the base of the blade called a pseudopetiole. This structure can also vary in length from several millimetres to fifty centimetres i.e. Neurolepis nobilis. Variation in indument, texture and the presence or absence of tessellation can be observed in different genera and species. Foliage dimorphism, as manifested in size differences between early growth and latter growth, is known to occur in some species (Judziewicz et al., 1999).

**Spikelets and Synflorescences**

In general, a grass spikelet consists of a series of overlapping, distichous bracts, some of which bear flowers in their axes. Usually the lowermost bracts of the spikelet are empty bearing neither floral axes nor buds; these are traditionally known as glumes. In woody bamboos the number of glumes is highly variable. There may be one to several or they may
Figure 2.4. Distichously arranged leaves of *Pleioblastus viridistriatus* ‘Auricoma’ (Starosta, 1996)

Figure 2.5. Auricles with radiating oral setae on the leaf sheath of *Phyllostachys bambusoides* ‘Violascens’ (Starosta, 1996)
be completely absent. Above the glumes there are one to several florets. In bamboos, one or more reduced florets are produced at the apex of the spike as growth ceases. These florets are often poorly developed or have abortive reproductive structures; such florets are known as rudimentary florets. The segmented axis of the spikelet is called the rachis and the pedicel upon which the spikelet is borne is called the peduncle.

Each spikelet represents a compact inflorescence consisting of a bracteate, sessile flowered spike. An aggregation of these spikelets is termed a synflorescence (Judziewicz et al., 1999).

In many bamboos and, indeed, in all other grasses, the synflorescence develops in one period of growth and all spikelets produced reach maturity in that same period – such synflorescences are termed semelauctant synflorescences. However, in some bamboos, synflorescences are produced in which the spikelets are at different stages of development. These arise through growth of buds in bracts immediately below the base of the spikelets; such synflorescences are called iterauctant synflorescences. Subtending bracts, prophylls and gemmiparous bracts are found in iterauctant synflorescences but are usually vestigial or absent in semelauctant synflorescences (Judziewicz et al., 1999).

The spikelet terminal to the principal axis of the synflorescence is called the main florescence and the lateral branches are known as paraclades.

Within a multi-flowered spikelet maturation usually occurs from the base towards the apex. It has also been observed that this process of spikelet maturation can begin in the central region of a spike and proceed towards both ends. Within a synflorescence the process of spikelet maturation is determinate; beginning with the terminal spikelet and proceeding downwards. Bamboo synflorescences may be categorised as one of the following (Judziewicz et al., 1999):
1. Spicate synflorescences: the peduncle of each spikelet is reduced or completely absent. The main axis is straight or zigzag.
2. Racemose synflorescences: each spikelet is borne on a well-defined peduncle along the main axis but there is no branching.
3. Paniculate synflorescence: each spikelet is pedunculate and there is more than one order of branching.
4. Capitate synflorescences: the arrangement is similar to that found in paniculate synflorescences except the internodes are shorter, giving a dense globose appearance.

Flowers

The flowers are composed of lodicules, stamens and a pistil (Figure 2.6). A palea is present at the base of the floral axis, which terminates in the pistil. The floral structures are whorled. The flowers are always bisexual in the Bambuseae, although it is possible to find staminate flowers in the higher order pseudospikelets in those species with highly branched pseudospikelets.

The lodicules are small flaps of tissue found at the base of the flower. At anthesis, the lodicules swell up and force the lemma and palea enclosing the delicate flower to open so that the stamens and stigmas can be exserted. In most grasses there are two lodicules but in bamboos there are often three. The lodicules of the woody bamboos are long, pointed and heavily vascularised and also may be fringed with short micro hairs (Judziewicz et al., 1999).

The stamens, usually two, three or six, are arranged in one or two whorls. The female structure consists of a solitary pistil whose swollen base contains one ovule. The apex of the ovary narrows and tends to quickly branch into a short style or styles, which bear the hispid to plumose stigmas that receive the pollen grains. In woody bamboos there are usually two to three stigmas (Judziewicz et al., 1999).
Figure 2.6. The suspended stamens of *Phyllostachys fimbriligula* (Starosta, 1996).
Relatively little is known about pollination and other aspects of bamboo reproductive biology due to the long flowering intervals that are encountered in these grasses. However, it appears that wind pollination is the principal means found amongst the woody genera. The stamens are usually exserted first. After the pollen has been shed the stigmas appear, a behaviour that facilitates out crossing (Judziewicz et al., 1999).

Caryopsis

The caryopsis of most woody bamboos is dry, narrow and cylindrical. The caryopsis usually has a long hileum and a small embryo. The pericarp is usually thin and reddish or reddish-brown in colour. In some species (i.e. Actinocladum verticillatum and some species of Merostachys) an achene-like or nucoid caryopsis is produced. Even more striking are the fleshy, olive or plum sized bacoid fruits of Alvimia, Olmeca and some species of Guadua and Melocanna (Judziewicz et al., 1999).
2.2 Morphological Classification Systems

The act of classifying objects or organisms stretches far back into the history of mankind (Lewin, 1996). From a botanical perspective, prior to the age of molecular science and biotechnology, classifications were based wholly upon morphological and anatomical characters. The first classification systems were primarily based on macro morphological characters, chiefly the structure of the inflorescences and also the floral structures. However, as time and research progressed publications on micro-morphological characters of anatomical and cytological origin began to appear. Such characters were included in classifications in a bid to improve and refine systems, so that they may represent natural evolutionary groups (Tzvelev, 1989).

In the past there have been number of classifications devised for the Bambuseae (Keng, 1982; Watson et al., 1985; Clayton & Renvoize, 1986; Soderstrom & Ellis, 1987; Stapleton et al., 1994; Wang, 1996; Yi, 1998; Ohrnberger, 2000). These systems have been established using a variety of character types including morphological, anatomical, cytological and physiological characters. However, comparison of these classifications reveals marked variation regarding the number of genera and divisions defined. The incongruencies encountered are attributed to several factors:

1. Traditionally, grass classification systems placed greatest emphasis on the inflorescence and floral structures of plants in defining species, genera and other taxonomic divisions. However in the Bambuseae, such characters are a limited resource due to the unusually long flowering intervals exhibited by most woody bamboos. Indeed in some species, flowering specimens have never been collected (McClure, 1996). Consequently systematists have found themselves in a compromised position whereby examples of these characters were not available. As a result alternative sources such as taxonomic descriptions and/or dried herbarium specimens had to be used. It has been found that taxonomic descriptions can be inaccurate and herbarium specimens may be badly preserved or of questionable
origin. The use of such material in the construction of classification systems would without any doubt lead to errors (personal observation).

2. Woody bamboos exhibit a considerable degree of morphological plasticity. In the past taxonomists have been inconsistent with regards to the region of the plant sampled during investigations. Consequently this gave rise to multiple taxonomic treatments for certain woody bamboo taxa (personal observation). It has been recently advised that morphological descriptions should be restricted to the mid-culm nodes and internodes; such regions represent the most fully developed and taxonomically characteristic features of the bamboo plant (Judsievicz et al., 1999).

3. The taxonomy of woody bamboos lacks character standardisation. As a result various features of the bamboo plant have received different interpretations regarding their taxonomic significance in classification systems (Stapleton & Rao, 1996). As of yet the interpretation and terminology given to the structure of the synflorescence of woody bamboos is under constant debate (Judsievicz et al., 1999; personal observations). Since woody bamboos exhibit no marked distinction in their morphology between vegetative and floral zones, morphologists find it difficult to define the limits of these zones.

4. In the past, most bamboo classifications have been based on a limited number of defining characters. In addition some of these datasets usually included either vegetative or reproductive characters. Few classifications were based on datasets that included both vegetative and reproductive characters. For example Tzvelev (1989) based his treatment of the woody bamboos on anatomical characteristics of the leaf blade, while Clayton and Renvoize (1986) defined taxonomic divisions on the basis of ovary appendage and also inflorescence structure. Furthermore, some of these systems were geographically limited to specific regions or countries (Wang, 1996; Yi, 1997).

In recent years there has been great initiative amongst bamboo taxonomists to enhance the understanding of bamboo structure and form, to discover new characters and evaluate the

### 2.3 The Current Morphological Analysis

The current morphological study was a collaborative effort involving Dr. C.M.A. Stapleton (bamboo taxonomist at RBG, Kew) and the author. The objectives of the study are summarised as follows:

1. Investigate the inter-relationships of the temperate woody bamboos using both vegetative and floral characters.
2. Assess the utility of morphological characters in systematic studies.
3. Compare the resulting bamboo groups with those defined in the classification systems defined by Clayton & Renvoize (1986) and Clark (1995).

Both Dr. Stapleton and the author carried out data collection at the Royal Botanic Gardens, Kew. Character selection and phylogenetic analysis of the complete character matrix was exclusively carried out by the author.

### 2.4 Materials and Methods

Morphological examinations were carried out using both living and herbarium specimens. All examinations were carried out on mature plants and, where relevant, at the mid-culm region of each plant. Taxonomic descriptions were also employed in scoring characters for the respective taxa. The initial character dataset consisted of forty characters. However due to limitations attributed by character availability and homoplasy the number of characters used in the actual analysis was reduced to 24. The data set included both vegetative and floral characters. These are summarised in Table 2.1.
Systematics of the Woody Bamboos (Tribe Bambuseae)

<table>
<thead>
<tr>
<th>Character Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Rhizomes: leptomorph = 0; pachymorph = 1</td>
<td></td>
</tr>
<tr>
<td>2. Clump form: unicaespitose = 0; culms single, spreading = 1, pluricaespitose = 2</td>
<td></td>
</tr>
<tr>
<td>3. Thorn: present = 0; absent = 1</td>
<td></td>
</tr>
<tr>
<td>4. Stimulation on internodes: absent = 0; present = 1</td>
<td></td>
</tr>
<tr>
<td>5. Prophylle: 2-keeled = 0; 1-keeled = 1; absent = 2</td>
<td></td>
</tr>
<tr>
<td>6. Margins: open at front = 0; fused at front = 1</td>
<td></td>
</tr>
<tr>
<td>7. Promontory: absent = 0; present = 1</td>
<td></td>
</tr>
<tr>
<td>8. Number of initial mid-culm branches: 1 = 0; 2 = 1; 3 = 2; 4 \leq 10 = 3; 11 \leq 20 = 4; 21 &lt; 5</td>
<td></td>
</tr>
<tr>
<td>9. Branches: subequal = 0; not subequal = 1</td>
<td></td>
</tr>
<tr>
<td>10. Basal nodes: not compressed = 0; 1-5 nodes compressed = 1; 6&lt; nodes compressed = 2</td>
<td></td>
</tr>
<tr>
<td>11. Lateral branch replications: absent = 0; present = 1</td>
<td></td>
</tr>
<tr>
<td>12. Culm sheaths: deciduous = 0; persistent = 1</td>
<td></td>
</tr>
<tr>
<td>13. Foliage leaves: non-tessellate = 0; tessellate = 1</td>
<td></td>
</tr>
<tr>
<td>14. Synflorescence: paniculate = 0; racemose = 1; single spikelet = 2</td>
<td></td>
</tr>
<tr>
<td>15. Synflorescence: not unilateral = 0; unilateral = 1</td>
<td></td>
</tr>
<tr>
<td>16. Synflorescence: bractete = 0; ebractete = 1</td>
<td></td>
</tr>
<tr>
<td>17. Fasciulation: absent = 0; present = 1</td>
<td></td>
</tr>
<tr>
<td>18. Ultimate branches of synflorescence with prophyl at point of branching: present = 0; absent = 1</td>
<td></td>
</tr>
<tr>
<td>19. Lateral spikelets: sessile = 0; on a promontory = 1</td>
<td></td>
</tr>
<tr>
<td>20. Florets: more than 1 = 0; 1 = 1</td>
<td></td>
</tr>
<tr>
<td>21. Basal florets: much larger than higher ones = 0; of similar size = 1</td>
<td></td>
</tr>
<tr>
<td>22. Number of stamens: 6 = 0; 3 = 1</td>
<td></td>
</tr>
<tr>
<td>23. Number of stigmas: 3 = 0; 2 = 1; 1 = 2</td>
<td></td>
</tr>
<tr>
<td>24. Culms: hollow = 0; solid = 1</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1. The list of characters included in the analysis.

**Phylogenetic Analysis**

The morphological dataset was initially compiled using Excel version 9.0. Characters were mainly scored on the basis of their presence or absence and most characters had but two states. All characters were scored using integers (1-5). On completion, the dataset was
imported into PAUP version 4.0b8 (PPC). The matrix was then subjected to both parsimony and distance methods.

2.5 Results

The morphological data set constituted 29 bambusoid taxa. All resulting trees were rooted using both species of Chusquea, C. foliosa and C. coronalis, as the outgroup species. Optimal trees were obtained using two phylogenetic inference methods, the Fitch Parsimony method and the Neighbor Joining (NJ) distance method. The parsimony analysis employed a heuristic search (1000 replicates). Starting trees were obtained using random stepwise addition (1000 replicates). The branch swapping algorithm used was nearest neighbor interchange (NNI). Bootstrap values (BS) were also obtained via a heuristic search (1000 replicates). The search parameters employed in estimating bootstrap support were identical to those employed in the parsimony analysis.

The Neighboring Joining distance method (NJ) employed the mean character difference distance measure. Branch support for the resulting tree was also estimated using bootstrap analysis. Bootstrap support was estimated using the same parameters as those employed in the NJ analysis.

Topologies obtained following parsimony search

Following parsimony and bootstrap analyses, two hundred equally parsimonious trees were retained, each consisting of 63 evolutionary steps. Consistency and retention indices were 0.4 and 0.7 respectively (Figure 2.1). The resulting trees were well resolved, however, the internal branches within the trees were, for the most part, without bootstrap support (Figures 2.2).

The main characteristic of the phlogenetic trees obtained in the parsimony analysis was strong support for the monophyly of the temperate clade (94% BS). The internal branches
Systematics of the Woody Bamboos (Tribe Bambuseae)

of the trees are unsupported with the exception of one, the *Chimnobambusa quadrangularis-C. marmorea* branch (73% BS).

**Topologies obtained following Neighbor Joining analysis**

As in the parsimony analysis, the NJ tree supported a monophyletic temperate bamboo clade (78% BS) (Figure 2.3). Within the temperate clade several assemblages were supported, these included a *Hibonobambusa-Semiarundinaria-Shibataea-Phyllostachys-Sinobambusa-Chimonobambusa* group (61% BS), within which a *Chimonobambusa quadrangularis-C. marmorea* group (92% BS) was defined, an *Arundinaria tecta-Bashania fangiana* group (65% BS), a *Borinda-Fargesia* group and an *Ampelocalamus-Himalayacalamus-Drepanostachyum* group (66% BS).
Figure 2.1. One of 100 parsimonious trees obtained upon analysis of the morphological dataset. Integers represent the number of characters supporting each branch.
Systematics of the Woody Bamboos (Tribe Bambuseae)

Figure 2.2. Bootstrap consensus tree obtained following a parsimony search. Bold integers represent the bootstrap support values for the respective branches.
Minimum evolution: 52.2

Figure 2.3. Bootstrap consensus tree obtained following a NJ search. Bold integers represent the bootstrap values for the respective branches.
<table>
<thead>
<tr>
<th>Character Code</th>
<th>Consistency Index value</th>
<th>Retention Index value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.667</td>
<td>0.889</td>
</tr>
<tr>
<td>2</td>
<td>0.286</td>
<td>0.643</td>
</tr>
<tr>
<td>3</td>
<td>1.000</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>0.200</td>
<td>0.556</td>
</tr>
<tr>
<td>5</td>
<td>0.400</td>
<td>0.250</td>
</tr>
<tr>
<td>6</td>
<td>0.143</td>
<td>0.333</td>
</tr>
<tr>
<td>7</td>
<td>0.250</td>
<td>0.667</td>
</tr>
<tr>
<td>8</td>
<td>0.556</td>
<td>0.667</td>
</tr>
<tr>
<td>9</td>
<td>0.143</td>
<td>0.250</td>
</tr>
<tr>
<td>10</td>
<td>0.667</td>
<td>0.889</td>
</tr>
<tr>
<td>11</td>
<td>0.250</td>
<td>0.750</td>
</tr>
<tr>
<td>12</td>
<td>0.167</td>
<td>0.445</td>
</tr>
<tr>
<td>13</td>
<td>0.500</td>
<td>0.800</td>
</tr>
<tr>
<td>14</td>
<td>0.300</td>
<td>0.562</td>
</tr>
<tr>
<td>15</td>
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<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>17</td>
<td>0.333</td>
<td>0.667</td>
</tr>
<tr>
<td>18</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>19</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>20</td>
<td>0.500</td>
<td>0.500</td>
</tr>
<tr>
<td>21</td>
<td>0.500</td>
<td>0.667</td>
</tr>
<tr>
<td>22</td>
<td>0.0250</td>
<td>0.0</td>
</tr>
<tr>
<td>23</td>
<td>0.200</td>
<td>0.429</td>
</tr>
<tr>
<td>24</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Table 2.2. CI and RI values for each character employed in the analysis.
2.6 Discussion

The lack of resolution with support in the phylogenetic trees is attributed mainly to an insufficient number of defining characters. The CI and RI values for most of the characters employed in the analysis are low which is indicative of character homoplasy (Table 2.2). Consequentially, the phylogenetic significance of these assemblages is questionable.

Resolution with strong bootstrap support for a temperate woody bamboo group is in agreement with the findings of Clark et al. (1995), Zhang et al. (1995) and Zhang & Clark (2000). Only one internal branch was supported in both the parsimony and NJ trees, that being the *C. quadrangularis-C. marmorea* branch (Figures 2.2, 2.3). This particular assemblage does bear significance since the species *C. quadrangularis* has been in the past recognised as a separate genus, *Oreocalamus*. However, further evidence is required from alternative data to verify these results.

In contrast with the parsimony results, those obtained following NJ analysis show increased support for the internal branches of the temperate woody bamboo clade. Although bootstrap support for these sub-clades is weak (Figure 2.3) (with the exception of the *C. quadrangularis-C. marmorea* assemblage), some of these sub-clades do bear significance from a morphological and ecological perspective. The largest group recognised in the NJ tree is the *Hibanobambusa-Semiarundinaria-Shibataea-Phyllostachys-Sinobambusa-Chimonobambusa* assemblage. These taxa share similarities in their floral characteristics such as fully bracteate synflorescences, prophylls at the point of branching in the synflorescences and sessile lateral spikelets. The *Ampelocalamus-Himalayacalamus-Drepanostachyum* assemblage reflects an ecological trait exclusive to these particular bamboos; these bamboos occupy subtropical to lower temperate habitats as indicated by the absence of tessellation in their leaf venation.

It was originally intended to include a much wider selection of characters in the investigation. The initial list contained 40 characters, including features of the leaves,
sheaths and glumes of these grasses, however the utility of these characters in the analysis proved problematic. First, a detailed morphological analysis of the bamboo synflorescence and floral structures was not possible due to inadequate specimens. Only two living specimens were in flower at the time of data collection, those being *Phyllostachys flexuosa* and *Fargesia nitida*, and herbarium specimens for most of the other species included in the analysis were lacking, in poor condition or of questionable identity (that is, many herbarium specimens consisted of insufficient material to verify their taxonomy). Second, it was concluded that variation in features associated with the leaves and sheaths were mainly due to ecological responses or were only useful at the inter-specific level of taxonomy. For instance characteristics, such as, whether a culm sheath blade is erect or reflexed appears to be a purely ecological response. Since bamboos grow in a telescopic manner, it makes sense for the sheath blade to be apressed to the culm during internode elongation to prevent damage. However with maturation, the blade becomes reflexed in a bid to maximise the surface area for light absorption. The presence of appendages such as auricles, fimbriae and ligules at the base of sheath blades and foliage leaves have proven to be highly variable within temperate genera, thus such characters were deemed unsuitable for systematic investigations at the inter-generic level.

It must be emphasised that this investigation is preliminary and further studies are necessary to evaluate the potential of morphological characters in phylogenetic studies. Despite the difficulties encountered, the investigation has highlighted the potential of bamboo branching characteristics at the inter-generic taxonomic level. Preliminary analyses of the mid-culm buds of a range of temperate bamboo genera revealed patterns that seemed to be exclusive to one or several temperate genera. This discovery supports the findings of Stapleton (1991 & 1997). However, due to limited time and resources a detailed investigation into the interpretation and evolution of these patterns was not possible. It is recommended that further investigations should be carried out to evaluate more accurately the utility of such characters in resolving evolutionary relations.
Chapter 3

Molecular phylogenetic analyses of the tribe Bambuseae

3.1 Molecular data and DNA sequencing

Molecular data

The explosion of data derived from studies of DNA has revolutionised the practice of plant systematics. Molecular approaches are now an integral part of most plant systematic studies. Molecular investigations have illuminated, in some cases resolved well, relationships within many groups of plants (Olmstead et al., 1994). Molecular data employed in systematics can be of mitochondrial, plastid or nuclear origin. The potential of each genome in phylogenetic studies depends on their nucleotide mutational/substitutional rate. DNA that is structurally conserved generally has a relatively low rate of evolution and is therefore best applied to studies at the higher taxonomic levels and vice versa. In general, mitochondrial DNA tends to be the slowest evolving genome followed by plastid DNA and finally nuclear DNA (Wolfe et al., 1987; Soltis et al., 2000).

A genome is composed of both coding and non-coding DNA. Due to diminished functional constraints, non-coding DNA tends to be more susceptible to rapid evolution than coding DNA. As a result coding and noncoding regions within a genome exhibit substitutional rate heterogeneity. The evolutionary rate of change for a particular DNA region may also vary in different lineages, meaning that a region of DNA which is informative at a particular taxonomic level for one group of organisms may not be of similar significance for another (Soltis et al., 1998).
Molecular phylogenetic inferences are often based on mutations that arise in the genomes of organisms, giving rise to nucleotide sequence variation. These mutations alter the content and/or the length of gene regions. Mutations may affect a single or several nucleotides. Mutations may be termed synonymous or non-synonymous: synonymous mutations are those that do not affect gene expression, whereas non-synonymous mutations are those, which alter gene expression. Furthermore, mutations may be classified according to the type of change they incur on the DNA molecule. These are summarised in the following text:

1. Substitution mutations refer to the replacement of a nucleotide(s) by another. Substitution mutations may be classified as transitions or transversions. Transitions are substitutions that occur within purines or pyrimidines, whereas, transversions are substitutions that occur between purines and pyrimidines.

2. Gene conversion refers to the non-reciprocal transfer of genetic data from one chromatid to another.

3. Crossing-over, also termed reciprocal recombination, refers to the mutual exchange of genetic information between linked loci.

4. Insertions and deletions refer to the addition or removal of one or more nucleotides from a sequence.

5. Inversions refer to the rotation by 180° of a segment of a DNA duplex (Lewin, 1996; Li, 1997).

Plant molecular systematics has relied primarily on the plastid genome for suitable characters. Plastids are intracellular organelles that contain their own genetic systems and a number of chloroplast components are encoded in their genomes. Plastids, responsible for photosynthesis and other biochemical tasks, are semiautonomous endosymbionts derived from previously free-living cyanobacteria (Palmer, 1990; Palmer et al., 2000). Most plastid genomes of higher plants consist of circular molecules about 120-160 kb in length (Shimada et al., 1991). These molecules are characterised by a large, ca 25 kb, inverted repeat which divides the remainder of the genome into one large and one small single copy
Sequence data has shown that the nucleotide substitution rate of plastid genes is conservative relative to plant nuclear genes. As a result, plastid DNA has mainly been employed in phylogenetic studies at higher taxonomic levels (Morton et al., 1993). Plastid DNA has several advantages that favour its use in systematic studies:

1. The chloroplast genome is relatively small. As a result it is straightforward to examine the whole genome using restriction site analysis, or portions of it using DNA sequencing.

2. Most genes are essentially single copy, therefore sequence heterogeneity is not an issue.

3. Despite its conservative nature, different portions of the plastid genome evolve at different rates. As a result a wide range of possibilities exist for resolving relationships using data from the plastid genome.

4. Plastid DNA is inherited uniparentally, usually maternally in Angiosperms, and is not subject to recombination. Therefore, plastid DNA data can be readily combined which may increase internal support for clades (Soltis et al., 2000).

The main disadvantage encountered when using chloroplast sequences in phylogenetics, particularly at lower taxonomic levels, is the occurrence of introgression which if undetected may bias estimates of phylogeny (Chapter 4 of thesis; Soltis et al., 2000).

Sequences from the nuclear genome (nrDNA) have not been utilised to the same degree as plastid DNA. However in recent years the number of published systematic papers in which nrDNA datasets have been used, has rapidly increased (Soltis et al., 2000). Nuclear DNA is the largest genome encoding a diverse selection of genes. Despite its large size, most
attempts to infer phylogeny with nuclear gene sequences have involved nuclear ribosomal DNA (rDNA). There are thousands of copies of rDNA cistrons in most plant nuclear genomes. These are arranged in tandem repeats distributed at one to several chromosomal loci, in particular the nucleolar organising region (NOR) (Liston et al., 1996). As with most nuclear genes, rDNA is bi-parentally inherited. The cistron is composed of three genes, the 18S, 5.8S and 26S, and each gene is separated by an internal transcribed spacer (ITS). Each repeat unit is separated from the other by an intergenic spacer (IGS). The approximate lengths of the three coding regions and the ITS regions are relatively uniform throughout the angiosperms (18S gene ca. 1800 bp, 5.8S gene ca 160 bp, the 26S gene ca 3,300 bp and the ITS region ca 500 bp). In contrast, the length of the IGS may vary considerably from 8-15 kb amongst different genera (Baldwin et al., 1995; Soltis et al., 2000). The attributes that favour the use of nuclear ribosomal DNA in systematics may be summarised as follows:

1. The high copy number of the rDNA gene family promotes the detection, amplification, cloning and sequencing of the region.

2. Nuclear ribosomal DNA may be employed at a variety of taxonomic levels. The gene family is composed of conserved regions (18S, 5.8S and 26S genes) that can be used to infer phylogeny at higher taxonomic levels. In addition, rDNA is composed of more rapidly evolving segments (ITS, IGS) that may be useful at the generic and specific level.

3. From the standpoint of phylogeny reconstruction, this gene family undergoes rapid concerted evolution, via unequal crossing-over and gene conversion. This property promotes intragenomic uniformity of repeat units and in general, promotes accurate reconstruction of species relationships from those sequences (Baldwin et al., 1995).

The most significant problem encountered when using sequence data from rDNA is the occurrence of sequence heterogeneity. Sequence heterogeneity arises when concerted evolution fails to homogenise all repeat units of a gene family. Concerted evolution is defined as those mechanisms whereby sequence variation amongst repeat types of a gene
family is eliminated, via gene conversion and or unequal crossing-over, giving rise to homogenous repeat types (Chapter 4). Duplications can also cause error because incorporation of sequences that are paralogous, rather than orthologous, into phylogenetic analyses, can give rise to inaccurate speciation hypotheses (Baldwin et al., 1995; Soltis et al., 2000).

Unlike animal mitochondrial DNA (mtDNA), the mtDNA in plants has been little used in phylogenetic studies. The lack of emphasis has been attributed to several characteristics that make this genome very difficult to analyse:

1. Plant mtDNA is very large and highly variable in size.
2. Foreign DNA sequences of unknown origin, may be present in plant mtDNA.
3. Large duplications are frequently created or lost.
4. Recombination of repeats can occur at a high frequency creating a very complex genome structure.
5. Plant mtDNA can rearrange very quickly, the result being that closely related species may not hold the same order of mitochondrial genes.
6. The rate of nucleotide substitution is very low compared to plastid or nuclear DNA (Olmstead et al., 1994; Soltis et al., 2000).

**DNA sequencing**

Initially, most phylogeny reconstructions were based on restriction site mapping of the entire plastid genome because DNA sequencing was too time-consuming and costly a process to employ in systematic studies. However, the advent of PCR technology and automated DNA sequencing rapidly placed DNA sequencing to the fore as a major source of comparative molecular data (Minelli, 1993; Olmstead et al., 1994). Sequencing has a number of advantages that favour its use over restriction site analysis in systematic studies:

1. Sequencing requires a minute amount of template DNA. Badly degraded DNA can often be sequenced thereby enabling the use of herbarium specimens and even
fossils as sources of DNA, whereas restriction site analysis requires relatively high molecular weight DNA.

2. DNA sequencing examines each base pair individually, thereby minimising the multiple hit problem inherent in restriction site analysis. In addition, insertions and deletions that may be too small and thus beyond the sensitivity of restriction site analysis, can be identified using sequencing analysis.

3. DNA sequencing has a wider application in phylogenetic studies. The problem of upper and lower limits of resolution inherent in restriction site analysis is much less severe for sequence data (Olmstead et al., 1994).

When choosing sequence data for systematic analysis several criteria should be met:
1. It is important that the rate of sequence divergence is appropriate to the phylogenetic questions being addressed.
2. The sequence data should be of sufficient length to provide enough phylogenetically informative characters.
3. The sequences must be readily aligned. Sequence alignment is essential for the correct assessment of character homology.
4. Sequences must be orthologous if speciation events are to be accurately recorded (Olmstead et al., 1994).

3.2 Genetic regions employed in the systematic study of the woody bamboos (Bambuseae)

This thesis is concerned with the assessment of evolutionary relationships at the inter-generic level. It was decided that DNA segments with relatively high substitution rates were the most appropriate molecular character sets to employ in the investigation. Genetic regions were chosen on the basis of their performance in addressing similar phylogenetic questions in previous studies. Four non-coding regions were used in this study, three (trnL intron, trnL-trnF intergenic spacer and rpl16 intron) of plastid origin and one (ITS) of nuclear origin.
The *trnL-trnF* gene region, *ca* ~900 bp, lies in the large single copy region of the plastid genome. It includes two non-coding components: the *trnL* (UAA) intron and the intergenic spacer between the *trnL* (UAA) 3' exon and the *trnF* gene (Taberlet *et al.*, 1991). In previous systematic studies, it has been shown that the *trnL-trnF* non-coding regions are useful in resolving phylogenetic relations between closely related taxa. Comprehensive assessment of *trnL-trnF* has demonstrated that the intergenic spacer generally has a higher substitutional rate than the intron, showing potential in taxonomic studies at the infrageneric level in many groups (Soltis *et al.*, 2000). The lower substitutional rate of the *trnL* intron tends to limit its application to the inter-generic and higher taxonomic levels (Taberlet *et al.*, 1995; Soltis *et al.*, 2000). However in a phylogenetic analysis of the genus *Gentiana* L. (*Gentianaceae*), the *trnL* intron proved informative at the intra-generic level (Gielly *et al.*, 1994; Gielly *et al.*, 1996). The *trnL-trnV* gene region, to our knowledge, has never been used in phylogenetic studies of the tribe Bambuseae.

The *rpl16* intron, *ca* 1400 bp, is a component of the single copy gene encoding for the ribosomal protein L16 (Brosius *et al.*, 1976; Posno *et al.*, 1986; Jordon *et al.*, 1996). Previous studies have revealed the potential of this region in resolving phylogenies at the interspecific level (Jordon *et al.*, 1996). Recently, *rpl16* has been used in a phylogenetic study of the Neotropical representatives of the Bambuseae. The analysis emphasised its usefulness at the intergeneric and interspecific levels (Kelchner & Clark, 1997).

ITS-1 and ITS-2 are the internal transcribed spacers of 18S–26S nuclear ribosomal DNA. This region is approximately 600 bp in length and includes the 5.8S gene. The ITS region is part of the transcriptional unit of rDNA, but, the spacer segments are not incorporated into the mature ribosomes. Instead, the ITS-1 and ITS-2 regions of the rDNA transcript appear to function, at least in part, in the maturation of nrRNAs (Baldwin *et al.*, 1995). Previous studies have shown ITS-1 and ITS-2 to be informative at both the inter-generic and intra-generic level (Hsiao *et al.*, 1994, 1995, 1999; Oxelman *et al.*, 1995; Sun *et al.*, 1995; Downie *et al.*, 1996; Liston *et al.*, 1996; Baldwin *et al.*, 1998; Gernandt *et al.*, 1999).
In a study assessing the phylogenetic utility of these spacer regions in monocots, the resulting tree topologies were in general agreement with those based on molecular data from ribosomal RNA and plastid DNA (Hsiao et al., 1994). Furthermore, phylogenetic investigations that employed rDNA, such as those carried out on the Apioidae and *Picea*, highlight that ITS datasets are useful for phylogenetic inference among closely related species (Smith et al., 1994; Downie et al., 1996). The internal transcribed spacers of rDNA have been sequenced for several woody bamboo taxa (Hsiao et al., 1994) but as yet a comprehensive analysis of the tribe, using rDNA, has never been undertaken.

### 3.3 Materials and Methods

One hundred and twelve bamboo taxa were used in this investigation, six of which were Olyreae species and the remainder were representative of the tribe Bambuseae. Information regarding the names and sources of the respective specimens are provided in Appendix III.

**Isolation of genomic DNA**

Total DNA was extracted from 0.05-0.1 grams of silica gel dried leaf material using a modified hexadecyltrimethyl ammonium bromide (CTAB) method from Doyle & Doyle (1990) (Appendix II: protocol 1.0). The crude DNA extract was then purified using the Concert Rapid PCR Purification System or the CsCl gradient purification technique (Appendix II: protocols 1.1, 1.2, 1.5). Each DNA sample was then quantified using gel electrophoresis and stored at -20°C or -70°C (Appendix II: protocol 1.3).

**Amplification of target DNA regions**

Both nuclear and plastid DNA regions were amplified using the Polymerase Chain Reaction (PCR) (Table 3.1, Appendix II: protocol 1.4). The reactions were carried out using a Perkin Elmer PE 480 thermocycler. The amplified products were quantified using
gel electrophoresis and then purified using the Concert Rapid PCR Purification System (Gibco BRL) before being stored at -20°C (Appendix II; protocols 1.3, 1.5).

**Sequencing of amplified PCR products**

Cycle sequence reactions were carried out on the amplified PCR DNA products using the Big Dye Terminator sequence mix produced by Applied Biosystems™. All cycle sequence reactions were carried out using a MJ Research Peltier Thermocycler PTC200 (Appendix II; protocol 1.6). The sequenced samples were then purified (Appendix II: protocol 1.7). Subsequently, the cycle sequenced samples were denatured (Appendix II: protocol 1.8) and run on either the Applied Biosystems™ 377 genetic Analyser (at the Royal Botanic Gardens, Kew, Richmond, Surrey, U.K.) or on the 310 Genetic Analyser (at the Department of Botany, Trinity College, Dublin) using the ABI Prism™ 310 module SEQ POP6 (1.0-mL) E or the ABI Prism™ 310 module SEQ POP6 RAPID (1.0-mL) E run files. The sequences were then processed using ABI Prism™ DNA Sequencing Analysis Software, version 2.1.1.

**DNA Analysis**

Successfully sequenced DNA samples were edited and assembled using Gene Codes Sequencher package version 3.1. The sequences were then imported into PAUP version 4.0b8 (PPC) and aligned by eye. The resulting matrix was then subjected to parsimony and distance methods.
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### 3.4 Results

#### 3.41 Phylogenetic Analysis of the Bambuseae based on sequence comparison of the trnL-trnF noncoding regions

The trnL-trnF data set constitutes 83 bambusoid taxa, 80 of which are representative of the tribe Bambuseae and the remainder are members of the tribe Olyreae. The 3 olyroid taxa were used as outgroup species in the analyses. Previous molecular investigations using the ndhF plastid gene region (Clark et al., 1995) and also the recent findings of the Grass Phylogeny Working Group (2001) have resolved the herbaceous bamboos as sister to the woody bamboos. The trnL-trnF data set contained 1199 bases, 991 of these characters were included in all analyses, 785 characters were constant, 93 were variable but uninformative and 113 were parsimony informative. Sequence variation in this dataset was mainly attributed to substitutions (mainly point substitutions), insertions and deletions (indels) and, to a much lesser degree, inversions. Greatest variation was found within the

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**Table 3.1: Primers used in PCR and Cycle Sequence Reactions**

<table>
<thead>
<tr>
<th>Genetic Origin</th>
<th>Target Region</th>
<th>Primer Base Sequence</th>
</tr>
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</table>
| cpDNA          | trnL-F (Taberlet et al., 1991) | Forward primer: CGAAATCGGTAGACGCTACG  
Reverse primer: ATGAACTG GTGACACGAG |
| mtDNA          | ITS-1, ITS-2 (Sun et al., 1994) | Forward primer AB101: ACGAATTCATGGTCCCGTGAAGTGT  
Reverse primer AB102: AGAATTCCC CGGTCCGTCCCGGT |
| cpDNA          | rpl16 (Jordan et al., 1996) | Forward primer F21: GCATGCTTAGCTGACTGCTG  
Reverse primer R1661: CGTACCCATTTTTCACCACGAG |

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spacer region located at, approximately, 600 bp-1100 bp. Four large deletions were encountered in the spacer. These deletions were characteristic of the tropical bamboos and were scored manually using 01 characters, on the basis of their presence or absence in all sequences.

Optimal trees were obtained using two phylogenetic inference methods, the Fitch parsimony method and the Neighbor Joining (NJ) distance method. The parsimony analysis employed a heuristic search (1000 replicates). Starting trees were obtained using random stepwise addition (1000 replicates). The branch swapping algorithm used was nearest neighbor interchange. Bootstrap values (BS) were also obtained via a heuristic search (1000 replicates). The search parameters employed in estimating bootstrap support were identical to those employed in the parsimony analysis.

The Neighbor Joining distance method (NJ) employed the Hasegawa/Kishino/Yana (HKY85) distance measure. All base substitutions were given equal weights and the gamma shape parameter was estimated prior to analysis using maximum likelihood. Branch support for the resulting tree was also estimated using bootstrap analysis. Bootstrap support was estimated using the same parameters as those employed in the NJ analysis.

**Topologies obtained following parsimony search**

Following parsimony and bootstrap analyses, two hundred equally parsimonious trees were retained (Figure 3.1), each consisting of 310 steps. Consistency and retention indices were 0.8 and 0.9 respectively. The resulting trees supported a robust (100% BS) monophyletic tribe Bambuseae. All trees were well resolved, however, the internal branches within the trees were, for the most part, poorly supported (Figure 3.2).

In this analysis, the temperate woody bamboos were strongly supported (100% BS) as a monophyletic lineage. However, internal groupings within the clade were highly unstable, as illustrated by the lack of bootstrap support (Figure 3.2).
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The tropical woody bamboos were resolved as paraphyletic. The Palaeotropical species *Dendrocalamus giganteus* was positioned outside the core tropical bamboo group and sister to the temperate clade. Support for its placement was weak (54% BS) and was mainly attributed to point mutations. Removal of this species from the analysis resulted in a robustly supported monophyletic tropical clade (88% BS; tree not shown).

With the exception of *D. giganteus*, the remaining tropical woody bamboos were placed into two lineages corresponding to the Palaeotropical and Neotropical bamboos. However, bootstrap values for these branches were low (55% and 66% respectively). Within the core tropical bamboo group, four supported sub-clades were identified, the *Neurolepis-Chusquea* lineage (82% BS), the *Aulonemia-Rhipidocladum-Otatea* lineage (63% BS), the *Bambusa-Dendrocalamus-Gigantochloa-Neomicrocalamus-Oreobambos-Racemobambos* lineage (62% BS) and the *Melocanna-Pseudostachyum-Schizostachyum-Thyrsostachys* lineage (99% BS). In these sub-clades, further sub-divisions were defined: the *Aulonemia-Rhipidocladum* lineage (98% BS), the *Chusquea* lineage (74% BS), within which, *C. sp* and *C. coronalis* formed a weakly supported monophyletic group (52% BS); the *Bambusa* lineage (57% BS), within which, a *Bambusa multiplex* group (91% BS) was defined; the *Schizostachyum-Thyrsostachys* lineage (65% BS), within which a *Schizostachyum* lineage (88% BS).

**Tree obtained using Neighbor Joining**

The tree (Figure 3.3) obtained following an analysis using NJ was congruent with the parsimony trees. A monophyletic tribe Bambuseae was resolved consisting of a paraphyletic tropical clade and a robustly supported monophyletic temperate clade. Within the temperate clade resolution with support was weak; the highest bootstrap values attained was 59% for the *Arundinaria* and the *Borinda-Chimonobambusa-Fargesia-Yushania-Indocalamus* groups. The phylogenetic position of *D. giganteus* was well supported (83% BS). Within the core tropical bamboos, the sub-clades identified resembled those of the parsimony trees, however support for several of these sub-clades differed from those values.
obtained on parsimony analysis, such as the *Chusquea* sp.-*C. coronalis* lineage (85% BS), *Bambusa*-Dendrocalamus-Gigantochloa-Neomicrocalamus-Oreobambos-Racemobambos lineage (85% BS) and the *Melocanna*-Pseudostachyum-Schizostachyum-Thyrsostachys lineage (51% BS). Several supported branches were exclusive to the NJ tree; these included the branches supporting the *Bambusa*-Dendrocalamus-Gigantochloa-Racemobambos-Oreobambos sub-clade (65% BS), the *Bambusa*-Gigantochloa-Racemobambos sub-clade (61% BS), the *Bambusa*-Racemobambos sub-clade (50% BS), the *Pseudostachyum*-Schizostachyum-Thyrsostachys sub-clade (56% BS), the *Schizostachyum*-Thyrsostachys sub-clade (78% BS) and the Thyrsostachys siamensis-Schizostachyum caudatum-*S. zollingeri* sub-clade (56% BS).

**Discussion**

The resolution of a monophyletic tribe Bambuseae in all analyses is a result that supports previous molecular investigations (Clark *et al.*, 1995; Duvall *et al.*, 1996; Hsiao *et al.*, 1999; Zhang *et al.*, 1995; Zhang & Clark, 2000; Kelchner & Clark, 1997; GPWG, 2000, 2001). The phylogenetic significance of the information obtained from the *trnL*-trnF dataset varies greatly between the temperate and the tropical woody bamboos. Apart from the high bootstrap value (100% BS, 96% BS) obtained for the monophyly of the temperate bamboo lineage in all analyses, which is also in agreement with the results obtained in Chapter 2 of this thesis and previous molecular studies (Clark *et al.*, 1995; Zhang *et al.*, 1995; Zhang & Clark, 2000), there is little support for the internal branches (Figure 3.2, 3.3). Those branches that are supported have low bootstrap values and the majority of these assemblages, with the exception of a further weakly supported polytomy, consist of only two taxa which are mainly members of the same genus: *Shibataea chinensis* and *S. kumasaca*, Himalayacalamus falconeri and *H. hookerianus*; *Arundinaria gigantea* and *A. tecta*. Resolution with poor support was directly attributed to the low number of synapomorphic characters, usually one character, supporting each branch (Figure 3.1). Given the overall scarcity of characters in this clade, the long branch of *Yushania alpina* (8 autapomorphic characters) is interesting. Being one of only two African representatives...
(the other being *Thamnocalamus tessellatus*) of the temperate clade included in this study its position is noteworthy. It would appear to be distinct from Asian members of the genus, to which it bears the closest morphological similarity, and also from North American and Asian members of *Arundinaria*, the genus in which it was first described.

In contrast, the inferred relationships of the tropical woody bamboos are, for the most part, well supported. The Neotropical taxa are divided into two distinct lineages. The first lineage to be discussed includes *Neurolepis elata*, *Chusquea delicatula*, *C. coronalis* and *C. sp.* (82% BS in Figure 3.2, 92% BS in Figure 3.3). The monophyly of the genus *Chusquea* and the position of *Neurolepis* as a sister genus to *Chusquea* is supported by previous molecular findings. In those investigations by Clark *et al.* (1995), Zhang *et al.* (1996) and Kelchner & Clark (1997), molecular data supported a close sister relationship between *Chusquea* and *Neurolepis*. The second lineage containing *Otatea acuminata*, *Aulonemia longiaristata* and *Rhipidocladum harmonicum* is supported by a bootstrap value of 63% in the parsimony trees only (Figure 3.2). Within this lineage, *Otatea acuminata* is resolved as a sister species to a robust *Aulonemia* and *Rhipidocladum* group (98% BS). These findings are in agreement with previous molecular studies carried out by Zhang *et al.* (1996) and Kelchner & Clark (1997).

The Palaeotropical woody bamboos are also divided into two lineages in all analyses. The *Melocanna-Pseudostachyum-Schizostachyum-Thrysostachys* sub-clade was resolved with 99% BS in the parsimony trees (Figure 3.2). Within this sub-clade, *Thrysostachys siamensis* (traditionally regarded as closely related to *Bambusa*, *Gigantochloa* etc. on the basis of its short ovary appendage) was found to be sister to the *Schizostachyum* species (this particular branch was weakly supported). The second lineage was not as robust as the former with regards to bootstrap support. All representatives of the *Bambusa* genus formed a weakly supported monophyletic sub-clade, with two cultivars of *B. multiplex* in a further sub-clade supported by a bootstrap of 91%. In the NJ tree (Figure 3.3), the same sub-clades were defined, although bootstrap values assigned to these branches were in some cases
conflicting with those of the parsimony trees. Those uniquely supported branches bear little significance in the analysis as their bootstrap values were low and were not found in the parsimony tree.

The phylogenetic position of *D. giganteus* is interesting. Despite the fact that its position is weakly supported in the parsimony analysis, it should be noted that sequence comparison of this species reveals synapomorphies shared with the temperate or tropical bamboos. Furthermore, exclusion of the four manually scored base deletions from the parsimony analysis, resulted in an increased bootstrap support for the placement of *D. giganteus* as a sister taxon to the temperate clade, which is similar to the findings of the NJ analysis. In addition, following AFLP analysis of, amongst other genera, *Dendrocalamus* by Loh *et al.* (2000), *D. giganteus* was found to be distantly related to other members of its genus and also the genus *Bambusa* (a genus which is regarded, on the basis of similar sheath, leaf, branch and floral morphology, to be closely related to *Dendrocalamus*). The position of this species provides reason to question whether the tropical woody bamboos do represent a monophyletic clade as this investigation finds little support for this theory. It is also unclear whether two discrete sub-clades, corresponding to the palaeo- and neotropical bamboo lineages represents an accurate reflection of the true phylogeny of these grasses because bootstrap values are also found to be low (as in Clark *et al.* (1995) and Zhang *et al.* (1996)) and internal branches within these clades have higher bootstrap support.

It is apparent from the analyses undertaken that the distribution of steps within this dataset is mainly concentrated along the branches leading to the tropical woody bamboos (Figure 3.1). The skewed distribution of these characters may be attributed to one, or a combination, of the following factors:

1. The tropical and temperate bamboos may exhibit rate heterogeneity regarding the evolution of the *trnL-trnF* noncoding gene region, with the temperate bamboos evolving at a much slower rate.
The temperate bamboos may have experienced rapid radiation. As a result, insufficient accumulation of genetic variation within existing lineages comprising the temperate clade reduces resolution and support for sub-clades. In other words, the species may have undergone rapid morphological radiation/speciation but there has been insufficient time for molecular variation to accumulate.

The temperate bamboos may be over taxonomised, as a result, the species and genera currently defined may have been elevated to ranks they do not justify. As mentioned previously (Chapter 1, 2), woody bamboo taxonomy is highly controversial, as independent authorities fail to come to a consensus regarding their nomenclature and hierarchical treatment.

It is impossible to draw conclusions from the trnL-trnF data as to the cause of the poor resolution within the temperate clade. Further investigations are required in order to ascertain whether the distribution pattern of characters, and correspondingly the bootstrap support in this analysis is a trend within the tribe Bambuseae or a unique artefact attributed to the evolutionary mechanisms at work within this particular DNA region.

Regarding the taxonomic treatment of the tribe Bambuseae, it is apparent from this study and previous investigations (Clark et al., 1995; Zhang et al, 1996; Zhang & Clark, 2000; Kelchner & Clark, 1997) that the recognition of sub-tribes containing constituents of both temperate and tropical origin (as in the classification constructed by Clayton and Renvoize (1986)) are not an accurate reflection of the currently supported phylogenetic hypothesis for the woody bamboos. The results from this research does provide support the treatment of the tropical bamboos as suggested in Clark (1995) with the exception of Racemobambos hepburnii, Neomicrocalamus andropogonifolius and Thyrsostachys siamensis. The two larger palaeotropical sub-clades of the trnL-trnF trees represent the sub-tribes Bambusinae and Melocanninae. The recognition of the sub-tribe Racemobambosinae to which N. andropogonifolius and R. hepburnii, are constituents, was not supported. In addition, T. siamensis, which is treated in Clark (1995) as a member of the sub-tribe Bambusinae, is
according to the trnL-trnF data associated with those species that represent the palaeotropical sub-tribe Melocanninae. Molecular support for an intermediate position for this species between these two sub-tribes was found in cpDNA data (Watanabe et al., 1994). Furthermore, AFLP data has resolved this species as a distant relative to Bambusa, Gigantochloa and Dendrocalamus (Loh et al., 1999). Amongst the Neotropical bamboos further groupings reflect the taxonomic treatment described in Clark (1995). The Glaziophyton-Rhipidocladum lineage and the Chusquea-Neurolepis lineage reflect those constituents of the sub-tribes Arthrostylidiinae and Chusqueinae respectively.

Given the fact that sampling of the tropical bamboos in this study was limited and also, representation of most genera consisted of a single species, suggestions of an alternative taxonomic treatment for these genera would be premature at this stage.
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Tree Length: 310
CI: 0.8
RI: 0.9

Figure 3.1. One of 200 equally parsimonious trees obtained following comparative sequence analysis of the \textit{trnL-trnF} sequence data. Bold integers represent the number of steps supporting each branch.
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Temperate woody bamboos:  
Palaeotropical woody bamboos:  
Neotropical woody bamboos:  
Herbaceous bamboos:  

Figure 3.2. Bootstrap consensus tree. Bold integers represent the bootstrap support values obtained for the respective branches following an analysis using identical search parameters as those employed in the parsimony analysis.
Figure 3.3. NJ tree obtained following an analysis of the trnL-trnF dataset using the HKY85 distance measure. Bold integers represent the bootstrap support values for the respective branches.
3.42 Phylogenetic analysis of the Bambuseae using the rpl16 sequence dataset

The rpl16 data set constitutes 31 bamboo taxa, 28 of which are woody bamboo species and the remainder are herbaceous bamboos (Olyreae), Pariana radiciflora, Sucrea maculata and Beugersiochloa bambusoides. As in the trnL-trnF analysis, all resulting trees were rooted using the olyroid grasses as outgroup species. The limited sampling within this dataset is attributed to unsuccessful amplification and sequencing of this region for a large number of bamboo species. The factor(s) attributing to this difficulty were never fully identified, although it is suspected that microsatellite regions were interfering with sequencing. The rpl16 sequences were on average 900-1262 base pairs in length before alignment, 940 of these bases were included in the analyses. 827 characters were found to be constant, 99 were variable but uninformative and 69 were deemed parsimony informative. The rpl16 intron consisted of large portions of DNA that were structurally conserved. These regions were interrupted by indels, many of which were distinct to a particular species/ genus or groups of species/ genera. Therefore, those indels deemed phylogenetically informative were scored on the basis of their presence or absence, and included in the analysis. Optimal phylogenetic trees were obtained in the parsimony analysis via a heuristic search (1000 replicates) using random stepwise addition, and the NNI branch-swapping algorithm. Bootstrap values were also obtained using the heuristic search option. 1000 replicates were carried out using identical search parameters to those employed in the parsimony analysis. The Neighbor Joining analysis employed the HKY85 distance measure and the gamma shape parameter was estimated prior to analysis using maximum likelihood. Bootstrap support was estimated using identical parameters to those used in the NJ analysis.

Results obtained from the parsimony analysis

Two hundred equally parsimonious trees were obtained, consisting of 206 steps and consistency and retention indices of 0.8 and 0.9 respectively (Figures 3.4). All trees were
well resolved and the monophyly of the tribe Bambuseae was strongly supported (100% BS) (Figure 3.5). The temperate woody bamboos were also strongly supported as a monophyletic lineage (98% BS). However, internal branch support within the temperate clade was poor; only three branches had bootstrap support: the *Drepanostachyum falcatum-Indocalamus tessellatus* f. *hamadae* lineage (57% BS), the *Pleioblastus simonii*-P. *linearis-Pseudosasa japonica*-Arundinaria *gigantea* lineage (62% BS) and the *Pseudosasa japonica*-Arundinaria *gigantea* lineage (90% BS) (Figure 3.5).

The tropical woody bamboos were not resolved as a monophyletic lineage (Figure 3.5). However, the Palaeotropical bamboos (represented by the genera *Schizostachyum*, *Sinocalamus*, *Dendrocalamus* and *Bambusa*) were resolved as a monophyletic group (81% BS) within which several sub-clades were defined: the *Sinocalamus-Dendrocalamus-Bambusa* sub-clade (64% BS), and the *D. hamiltonii*-D. *membranaceus* sub-clade (94% BS). The Neotropical bamboos were not resolved as a monophyletic group. Instead the genera representing the Neotropical bamboos (*Chusquea*, *Glaziophyton*, *Otatea*, *Rhipidocladum* and *Neurolepis*) were positioned on one of three separate branches that corresponded to the *Chusquea* lineage (100% BS), within which a *C. culeou* and *C. vulcanalis* lineage was supported (83% BS), the *Glaziophyton-Otatea-Rhipidocladum* lineage (81% BS) and the *Neurolepis aperta* branch (unsupported).

**Neighbouring Joining Analysis**

The Neighbor Joining tree (Figure 3.6) gave a similar topology as the parsimony trees supporting a monophyletic woody bamboo lineage (100% BS), a temperate woody bamboo clade (90% BS) and Palaeotropical sub-clade (90% BS). As in the parsimony bootstrap consensus tree, internal branches of the temperate clade were poorly supported. Five branches were given bootstrap values these being the *Indocalamus-Phyllostachys-Oligostachyum-Drepanostachyum-Ampelocalamus* (55% BS) branch, the *Phyllostachys-Drepanostachyum-Ampelocalamus* branch (51% BS), the *Pleioblastus simonii*-P. *linearis*-
Pseudosasa japonica-A. gigantea branch (50% BS), the Pleioblastus linearis-Pseudosasa japonica-Arundinaria gigantea branch (72% BS) and the Pseudosasa japonica-Arundinaria gigantea branch (90% BS).

As in the previous analysis the monophyly of the tropical bamboos was not supported. In addition, the Neotropical bamboos were also resolved on three separate branches that corresponded to the Chusquea lineage (100% BS), within which the C. culeou-C. vulcanalis branch was supported (82% BS), the Glaziophyton-Otatea-Rhipidocladum lineage (69% BS), within which the Glaziophyton-Otatea branch was supported (57% BS) and the Neurolepis aperta branch (unsupported). The topology within the Palaeotropical bamboo sub-clade was as follows; Schizostachyum luzonicum was resolved as sister to a Sinocalamus-Dendrocalamus-Bambusa sub-clade (65% BS). Within this sub-clade both Dendrocalamus species formed a monophyletic group (96% BS).

Discussion

As in the morphological trees, the trnL-trnF trees and previous molecular investigations (Clark et al., 1995; Duvall et al., 1996; Hsiao et al., 1999; Zhang, 1996; Zhang & Clark, 2000; Kelchner & Clark, 1997; GPWG, 2000, 2001), the monophyly of the woody bamboos was well supported in this analysis (Figures 3.5, 3.6). In addition, the hypothesis regarding the monophyly of the temperate bamboos was reinforced with further evidence from the analysis of the rpl16 data set. However, beyond this hierarchical status, resolution within the temperate clade was generally weakly supported with the exception of the Arundinaria gigantea-Pseudosasa japonica lineage (Figures 3.5, 3.6). The association between Pseudosasa japonica and Arundinaria gigantea repeats the findings of Zhang (1996) from ndhF sequence data. As the type species of Arundinaria and the sole North American representative of the temperate clade, this association is of bio-geographical and taxonomic interest. Firstly it suggests that a Bering Straits land bridge was indeed crossed by ancestors of Arundinaria gigantea, when this lineage expanded from Asia into North
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America. Secondly it suggests that Asian species sometimes considered members of the genus *Arundinaria*, on the basis of morphological similarity to *Arundinaria gigantea*, may be less closely related to *A. gigantea* than even morphologically distinct species such as *Pseudosasa japonica*.

The general lack of definition within the temperate group may be attributed to poor sampling, but it is more likely to be attributed to low levels of sequence divergence. Sequence variation between these taxa was generally very low as exemplified by the short branch lengths consisting mainly of 1-2 base changes (Figure 3.4), even for those genera that are regarded as morphologically distinct (i.e. *Phyllostachys*). In an analysis by Hodkinson *et al.* (2000) using both ITS sequence data and AFLP data, the genus *Phyllostachys* was resolved as a robust monophyletic lineage, but in this particular analysis of the *rpl16* intron, both *Phyllostachys* species, *P. edulis* and *P. nigra* var. *henonis*, were not supported as a monophyletic group. The general lack of evolutionary signal in this dataset for the temperate bamboo taxa may be suggestive of over-taxonomy, rapid radiation or rate heterogeneity, as discussed in the previous section, 3.31.

Within the tropical bamboos, further similarities were found between the topologies of the *rpl16* and the *trnL-trnF* trees such as the recognition of separate Neotropical and Palaeotropical lineages (Figures 3.5, 3.6).

As in the *trnL-trnF* trees the taxonomic treatment of the woody bamboos by Clayton and Renvoize (1986) was not supported. Instead the results supported the treatment of the woody bamboos as suggested in Clark (1995) in that temperate and tropical woody species should be placed in separate groups. Representatives of the Bambusinae sub-tribe (*Bambusa, Dendrocalamus* and *Sinocalamus*) were well supported as a natural evolutionary group while *Schizostachyum luzonicum*, a putative member of the Melocanninae sub-tribe, was positioned sister to this group, however, its position was weakly supported (Figure 3.5, 3.6). Bootstrap support for the *Chusquea-Neurolepis* lineage was lacking in the parsimony
and NJ trees, contradicting the findings of the *trnL*- *trnF* study and also those of Clark *et al.* (1995), Zhang (1996) and Kelchner & Clark (1997) in which a well supported *Chusquea*- *Neurolepis* lineage was resolved.
Figure 3.4. One of 200 equally parsimonious trees obtained following analysis of the rpl16 dataset. Integers indicate the number of steps supporting each branch.
Figure 3.5. Bootstrap consensus tree. Bold integers represent the bootstrap support values obtained for the respective branches following an analysis using identical search parameters as those employed in the parsimony analysis.
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Minimum evolution: 0.21

- *Arundinaria gigantea*
- *Pseudosasa japonica*
- *Pleioblastus linearis*
- *Pleioblastus simonii*
- *Sasa palmata f. nebulosa*
- *Ampelocalamus scandens*
- *Drepanostachyum falcatum*
- *Phyllostachys nigra var. henonis*
- *Indocalamus tessellatus*
- *Oligostachyum oedogonatum*
- *Phyllostachys edulis*
- *Indocalamus tessellatus f. hamadae*
- *Thamnoocalamus spathiflorus var. aristatus*
- *Sasa veitchii var. veitchii f. minor*
- *Bambusa multiplex var. gracilis*
- *Sinocalamus oldhamii*
- *Bambusa vulgaris*
- *Dendrocalamus membranaceus*
- *Dendrocalamus hamiltonii*
- *Bambusa longisquamiculata*
- *Schizostachyum luzonicum*
- *Chusquea delicatula*
- *Chusquea euleou*
- *Chusquea vulcanolis*
- *Neurolepis aperta*
- *Glaziophyton mirabile*
- *Ottelia acuminata*
- *Rhipidocladum pittieri*
- *Parana racicifolia*
- *Beugersiochloe bambusoides*
- *Suctea maculata*

- 0.005 substitutions/site

Temperate woody bamboos:

Palaeotropical woody bamboos:

Neotropical woody bamboos:

Herbaceous bamboos:

Figure 3.6. Bootstrap consensus tree. Bold integers represent the bootstrap support values obtained for the respective branches following an analysis using identical search parameters as those employed in the NJ analysis.
3.43 Phylogenetic analysis based on ITS1-5.8S-ITS2 sequences

The ITS1-5.8S-ITS2 (ITS) dataset constituted 56 taxa, all of which were woody bamboo species. Unlike the previous analyses, species from the tribe Olyreae were not used as outgroup taxa in the following analyses due to unsuccessful sequencing and the unavailability of these particular bamboos (with the exception of *Lithacne humilis*) from the GenBank database. *Lithacne humilis* was initially used as an outgroup species however as a result of sequence alignment problems it was concluded that the DNA sample was unsuitable. As a result all trees resulting from the analyses were rooted using four Neotropical bamboos, *Chusquea delicatula*, *C. foliosa*, *Neurolepis elata* and *Alvimia gracilis*, as outgroup species. The sequences obtained for the respective taxa were on average 600-800bp in length prior to alignment. In the study, 829 characters were included, 540 were constant, 143 were variable but uninformative and 144 were regarded parsimony informative. Genetic variation between the sequences was greatest in the spacer regions. This variation was mainly attributed to point mutations, mainly base substitutions and to a lesser degree, insertions and deletions. All insertions/deletions were treated as missing data. Highly variable sites were excluded from the analyses as base alignment within these regions was not satisfactory.

Similar inference methods were employed in this particular study, as used in the phylogenetic analyses of the *trnL-trnF* and *rpl16* datasets. Optimal parsimony trees were obtained using a heuristic search (1000 replicates, via random stepwise addition and the nearest neighbor interchange branch swapping algorithm). During stepwise addition 50 trees were retained at each step. The resulting topologies were evaluated using bootstrap analysis. The bootstrap analysis (1000 replicates) employed identical parameters to the parsimony analysis. The NJ analysis used the HKY85 distance measure. As in the *trnL-trnF* and *rpl16* studies, the gamma shape distribution was estimated prior to analysis using maximum likelihood. Branch support in the resulting tree was evaluated using bootstrap analysis (1000 replicates).
Topologies obtained using parsimony

Two hundred optimal trees (Figure 3.7, 3.8) obtained following parsimony consisting of 459 steps. The Cl and RI values were 0.7 and 0.8 respectively. All parsimonious trees were well resolved, however, as in the topologies obtained following the analyses of the trnL-trnF and rpl16 datasets, many of the internal branches were without significant support. The temperate clade was supported as a monophyletic lineage (85% BS). Within the clade greatest support was found along the branches representing the Drepanostahyum-Himalayacalamus lineage (78% BS), the Himalayacalamus lineage (81% BS), the Phyllostachys lineages (100%, 73% BS), the Thamnocalamus lineages (69%, 97% BS), the Bashania lineage (75% BS), the Pseudosasa-Sinobambusa lineage (76% BS) and also the Yushania lineage (84%, BS).

The Palaeotropical woody bamboos were not supported as a monophyletic group. Three Palaeotropical species, Neomicrocalamus andropogonifolius, Schizostachyum caudatum and S. zollingerii formed a weakly supported group (62% BS). Within, both species of Schizostachyum formed a strongly supported monophyletic lineage (100% BS). The position of Racemobambos hepburnii was unsupported.

Resulting tree obtained NJ analysis

The tree topology (Figure 3.9) obtained following NJ analysis also resolved a monophyletic temperate clade (100% BS). In addition, many of the internal lineages supported in the NJ tree were broadly congruent with those of the parsimony trees. However, this distance method did recognise other lineages (such as the Fargesia murielae-F. nitida lineage) that were unsupported in the parsimony trees. However, support for these branches were predominantly low with the exception of the large sub-clade representing the Acidosasa-Pleioblastus-Pseudosasa-Sinobambusa-Arundinaria-Hibanobambusa-Sasa-Shibataea lineage (74% BS).
The Palaeotropical woody bamboos were not resolved as a monophyletic lineage. However, *Racemobambos hepburnii*, *Schizostachyum caudatum* and *S. zollingerii* were grouped together in a weakly supported sub-clade (64% BS). Within this sub-clade both species of *Schizostachyum* were supported as a robust lineage (100% BS). In the NJ bootstrap analysis the position of *Neomicrocalamus andropogonifolius* was unsupported.

**Discussion**

The resolution of a monophyletic temperate woody bamboo lineage (Figures 3.8,3.9) supports previous findings (morphological, trnL-trnF and rpl16 results, Clark *et al.*, 1995; Zhang, 1996, Zhang & Clark, 2000; Duvall *et al.*, 1996; Hsiao *et al.*; 1999; Kelchner & Clark, 1997; GPWG, 2001). Unlike previous investigations, the ITS dataset provides resolution, with strong bootstrap support (in both the parsimony and NJ trees), for several, mainly peripheral, branches within the temperate clade. However, the overall lack of bootstrap support for the majority of the temperate species remains the most prominent feature of the clade. Those groups that are well supported, demonstrate, to a limited degree, phylogenetic relations between temperate bamboo species. For instance *Drepanostachyum falcatum*, *Himalayacalamus cupreus* and *H. falconeri* are strongly supported as a monophyletic group in both parsimony and NJ trees. Morphological characteristics such as the possession of a large number of bud initials, simple open semelauctant inflorescences with strong fasciculation and delicate glumes, support this group (personal observations). The robustly supported *Phyllostachys* group is a distinct feature within the topology of the temperate clade (in all analyses). The long-branch length supporting this group is not found in any of the other supported lineages (figure 3.7). The *Phyllostachys* group supports previous findings (Hodkinson *et al.*, 2000), whereby members of this genus have been resolved as a monophyletic group. Although these lineages indicate that these species are more closely related to each other than with other taxa included in the clade, beyond that there is little information provided which reflects a supported hierarchical pattern of evolution. All species, including those within
the supported sub-groups, are embedded in a large polytomy, the relationships between the majority of these taxa are still unsupported.

From a taxonomic standpoint, the monophyly of the temperate clade distinct from the Palaeotropical bamboos contradicts the taxonomic treatment of the Bambuseae by Clayton & Renvoize (1986). Furthermore, the improved bootstrap support within the temperate clade may be interpreted as an indication of the polyphyletic nature of the genera defined in this classification. For instance, in this classification *Drepanostachyum falcatum*, *Himalayacalamus falconeri* and *H. cupreus* are treated as members of *Sinarundinaria* and *Thamnocalamus*, respectively, while the genera *Drepanostachyum* and *Himalayacalamus* are not recognised (these two genera incidentally are defined in the classification system published in Clark (1995)). In the ITS analysis, high bootstrap support was recorded for a close sister relationship between these three species (Figure 3.8,3.9). This assemblage may suggest that the constituents of *Sinarundinaria* and *Thamnocalamus* do not constitute discrete monophyletic lineages. However, it must be emphasised that this data does not provide conclusive evidence in support of the taxonomic treatment of these species as defined in the system published in Clark (1995). As mentioned, the majority of the branches supported in both the parsimony and NJ trees are the peripheral branches rather than the internal ones. Thus, there is no evidence provided for the relationships existing between these supported groups and other species included in the analysis.

It is reasonable to state that a poorly resolved temperate clade is not a feature confined to plastid DNA. Therefore, the hypotheses suggested in section 3.31 to explain the lack of structure in the temperate clade remain undisputed possibilities. The reason as to why only several taxa form well-supported groups in the ITS dataset is puzzling. If the temperate bamboos have only recently diverged, perhaps those groups that are defined, such as *Phyllostachys*, represent the older members of this lineage. In addition, those taxa that are unsupported in the ITS trees may also reflect over-taxonomy. Alternatively, given the fact that base substitutional rates are high in the ITS region, it is plausible that a certain portion
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of these random base changes represent phylogenetic noise (Wenzel & Siddal, 1999). If the phylogenetic noise is significant within this dataset, this phenomenon may influence the topology within the temperate clade. Therefore, without similar results from other investigations, the phylogenetic relationships of the temperate woody bamboos remains under speculation.

Unlike the trnL-trnF trees, the Palaeotropical woody bamboo species, *Racemobambos hepburnii*, *Schizostachyum caudatum*, *S. zollingerii* and *Neomicrocalamus hepbrunii* were not supported as a monophyletic lineage. This may be attributed to poor sampling of the Palaeotropical bamboos or to the presence of phylogenetic noise in the ITS dataset.

The number of species included in the ITS dataset is not comparable in size with the trnL-trnF dataset. Decreased sampling of the woody bamboos in this dataset was attributed to sequence heterogeneity of repeat ITS units. The lack of concerted evolution in the nrDNA seems to be a feature of many woody bamboos. The occurrence of this phenomenon is worthy of further investigations. Two factors that may attribute to sequence heterogeneity are suggested:

1. Organisms with relatively long generation periods usually exhibit slow evolutionary rates. Therefore, the time required for homogenisation of bi-parentally inherited repeat units, such as ITS, will inevitably take longer in these organisms than in those with shorter generation times. Most woody bamboos exhibit long cyclical flowering intervals that supports the theory that woody bamboos may have slow evolving genomes.

2. Sequence variation in bi-parentally inherited repeat types is also indicative of hybridisation. Given the gross morphological and genetic similarities encountered, in the temperate bamboos, such an event cannot be overlooked.
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Minimum evolution: 0.32

Figure 3.9. Bootstrap consensus tree obtained following a NJ search using the HKY85 distance measure. Bold numbers represent the bootstrap values for the respective branches. Those taxonomic names in brackets represent the treatment of the respective taxa in the classification system constructed by Clayton & Renvoize (1986).
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Figure 3.8. Bootstrap consensus tree obtained following analysis of the ITS sequence data using identical parameters as those employed in the parsimony search. Bold integers represent the bootstrap values. Those taxonomic names in brackets represent the treatment of the respective taxa in the classification system constructed by Clayton & Renvoize (1986).
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Tree length: 459
CI: 0.7
RI: 0.8

Acidosasa purpurea
Ampelocalamus patellaris
Ampelocalamus scandens
Ampelocalamus micanthogynus (Dendrocalamus)
Dendrocalamus falcatus (Sinarundinaria)
Himalayocalamus cuogens (Thamnocalamus)
Himalayocalamus falconeri (Thamnocalamus)
Phyllostachys bissetii
Phyllostachys dulcis
Phyllostachys flexuosa
Phyllostachys bambus

Bashania fargesii (Arundinaria)
Bashania fargesii (Arundinaria)
Bashania qingchengshanensis (Arundinaria)
Indocalamus solidus
Indocalamus tessellatus f. hamadake
Indocalamus tessellatus
Fargesia murielae (Sinarundinaria)
Fargesia nitida (Sinarundinaria)
Yushania changii (Sinarundinaria)
Yushania mazzancini (Sinarundinaria)
Fargesia utilis (Sinarundinaria)
Yushania maculata (Sinarundinaria)
Yushania bolian (Sinarundinaria)
Yushania yunnanensis (Sinarundinaria)
Fargesia dracocephala (Sinarundinaria)
Chimonobambusa marmorea
Chimonobambusa tanoidissima
Chimonobambusa quadrangularis
Chimonobambusa szechuenensis
Gaulterogynhanna mengkohkensa (Indocalamus)
Yushania alpina (Arundinaria)
Thamnocalamus spatuliflorus subsp. crassistachys
Thamnocalamus spatuliflorus subsp. aristatus
Thamnocalamus spatuliflorus
Arundinaria teita
Hibakobambusa tranquillans (Semiarmundinaria)
Pleioblastus pygmaeus Distichus (Arundinaria)
Pseudosasa amabilis (Arundinaria)
Sinobambusa taoisk
Sasa palmata f. nebulosa
Sasa verticillata var. verticillata f. minor
Arundinaria gigantea
Shibataea kumasaka
Pseuosasa ovatarii
Pleioblastus oleosis (Arundinaria)
Chusquea delicatula
Chusquea floribunda
Neurolepus elata
Neocimicocalamus andropogonfolius
Schizostachyum caudatum
Schizostachyum zollingeri
Racemobambos hephburnii
Alpinia gracilis

Temperate woody bamboos:
Palaeotropical woody bamboos:
Outgroup species:

5 changes

Figure 3.7. One of 200 equally parsimonious trees obtained following sequence analysis of the ITS dataset. Integers represent the number of steps supporting each branch. Those taxonomic names in brackets represent the treatment of the respective taxa in Clayton & Renvoize (1986).
3.44 Combined analysis of the ITS1-5.8S-ITS2, the *trnL-trnF* and *rpl16* datasets

It has been demonstrated that evolutionary signals are often additive across different DNA sequence matrices and phylogenetic noise is averaged (Wenzel *et al.*, 1999). That is, if two datasets of similar size with s support and n% noise are combined in a phylogenetic analysis, their combination will produce 2s support but still only n% noise. In a study by Barret *et al.* (1991) it was revealed that a signal too weak to determine a tree in either of two datasets can emerge to dominate in a combined analysis. With this in mind, the ITS, the *trnL-trnF* and the *rpl16* datasets were combined. Twelve taxa common to all datasets were included in the analysis and were subjected to identical analytical treatments (parsimony and NJ analyses) as in the previous studies. Those genera of tropical origin, *Chusquea delicatula*, *Neurolepis* species and *Schizostachyum* species were used as outgroup taxa in the analyses. Congruency tests for assessing similarity or conflict between tree topologies generated from different data sets were not used in this study. Tests such as the incongruence length difference (ICD) tests, have been used by various workers (Hvelsenbeck *et al.*, 1996) as an indication of whether data from independent datasets should be combined. This is a contentious issue. However, a growing number of studies have shown that measures of incongruence should not be used as indicators of datasets combinability (Yoder *et al.*, 2001) because these tests can be completely misleading.

The author believes that an approach that looks at one node at a time and considers the relative support (be it bootstrap or another measure of support) of the groupings is the best way to assess congruence between datasets, such an approach is advocated by Weins (1998) and Reeves *et al.* (2001).

Since all three datasets did not include the same species for each genus, the sequences of different members of the same genus were combined. As a result the names applied to the some of the sequences included in the analysis include the genus name only.
Resulting parsimony trees and NJ tree

The combined dataset included 2559 characters in total, 2301 were constant, 174 were variable but uninformative and 84 characters were parsimony informative. Ten equally parsimonious trees were obtained, each consisting of 323 steps. The CI and RI values were 0.8 and 0.7 respectively (Figure 3.10).

The topologies obtained from the parsimony (Figure 3.11) analyses resolved a monophyletic temperate clade (100% BS). Bootstrap support was obtained for an *Indocalamus* lineage (64% BS), a *Thamnocalamus-Arundinaria-Pleioblastus-Sasa* lineage (54% BS) and an *Arundinaria-Pleioblastus-Sasa* lineage (65% BS).

As in the parsimony analysis, a monophyletic temperate clade was resolved (100% BS) in the NJ tree (Figure 3.12). Similarly an *Indocalamus* lineage (91% BS) and an *Arundinaria-Pleioblastus-Sasa* lineage (81% BS) were defined. Within the *Arundinaria-Pleioblastus-Sasa* lineage, *Pleioblastus* and *Sasa* formed a monophyletic group (72% BS). Furthermore an *Ampelocalamus-Drepanostachyum-Phyllostachys* lineage was defined, supported by a bootstrap value of 68%. Within this lineage *Drepanostachyum falcatum* and *Phyllostachys* formed a monophyletic lineage (61% BS).

Discussion

The phylogeny inferred from the combined ITS-*trnL-trnF-rpl16* dataset supports the previous results obtained in this research, in that a robust monophyletic temperate clade is defined in both the parsimony and Neighbor Joining analyses. However, due to the low number of taxa included in the data set it is not possible to assess the potential of using combined data sets in this phylogenetic investigation as the dataset is not comparable in size with the previous datasets. Furthermore, the supported groups defined in the analyses are weakly supported or not at all in the parsimony tree and thus cannot be given much
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weight. Increased sampling is necessary if the potential of the ITS-trnL-trnF-rpl16 dataset is to be accurately assessed.
Figure 3.10. One of 10 equally parsimonious trees obtained following comparative sequence analysis of the combined trnL-trnF-ITS-rpl16 dataset. Integers represent the branch lengths.
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Figure 3.11. Bootstrap consensus tree obtained following analysis of the combined trnL-trnF-ITS-rpl16 dataset using identical search parameters as those employed in the parsimony analysis. Bold numbers represent the bootstrap support values for the respective branches.
Minimum evolution: 0.12

- *Ampelocalamus scandens*
  - *Drepanostachyum falcatum*
    - *Phyllostachys*
      - *Indocalamus tessellatus*
        - *Indocalamus tessellatus f. bancrodeae*
  - *Arundinaria gigantea*
    - *Pleioblastus*
      - *Sasa palmata f. nebulosa*
        - *Thamnocalamus spathiflorus subsp. aristatus*
  - *Clusia gigantea*
    - *Neurolepis*
      - *Schizostachyum*

0.005 substitutions/site

Temperate woody bamboos: 🔴
Outgroup species: 🔴

Figure 3.12. NJ tree obtained using the HKY85 distance measure. Bold values represent the bootstrap support percentages for the respective branches.
Chapter 4

Hibanobambusa tranquillans (Koidzumi) Maruyama & H Okamura, a putative intergeneric hybrid

4.1 Research objectives

The objective of the following research was to characterise the putative hybrid, *Hibanobambusa tranquillans*, and its parents, in an attempt to elucidate its origin and depending on the results obtained, trace the direction of any hybridisation. The investigation employed comparative sequence analysis of the plastid trnL-trnF non-coding gene regions and the nuclear ribosomal ITS spacer regions. It was anticipated that the bi-parentally inherited ITS sequences may identify (possibly via sequence heterogeneity) the parents of this putative hybrid. The implementation of plastid DNA in the analysis aimed to identify the maternal parent species. Amplified Fragment Length Polymorphism (AFLP) was also employed in the study. This method is a multi-locus fingerprinting technique (Appendix I, II) and has been found highly suitable at resolving phylogenetic relationships at the lower taxonomic levels in the woody bamboos. The potential of this technique was highlighted in the systematic study of the genus *Phyllostachys* (Hodkinson et al., 2001), of the genera constituting the sub-tribe Bambusiinae (Loh et al., 2000) and also in the study of allopolyploidy in *Miscanthus* (Poaceae, not a woody bamboo genus) (Hodkinson et al., in press). It was anticipated that the resulting data obtained from the AFLP analysis would provide further evidence in support of, or in conflict with the hypothesis that suggests *H. tranquillans* is an inter-generic hybrid.
4.2 Hybrid species, harsh beginnings

From a taxonomic perspective a hybrid can be defined as a taxon that arises from a cross between parents of different species. In molecular terms a hybrid is often interpreted as any individual, which arises from a cross that involves parents of differing genotypes.

Hybridisation occurs when the reproductive barriers between species fail and gene flow is permitted giving rise to a progeny whose genotype is a combination of both species (Judd et al., 1999). The survival and subsequent success of hybrid progenies relies wholly on their ability to increase and maintain viable populations. In other words, hybrids must be capable of reproduction, even if this is non-sexual. Hybrids can be sterile, usually because the chromosome pairs, which consist of one chromosome contributed by each parent, do not segregate regularly at meiosis. In such cases hybrids are likely to go extinct. However they may alternatively undergo polyploidisation which would render them interfertile among themselves and hence to speciate (Ridley, 1996). Hybrids may also be interfertile with one or both parental species, thus enabling them to interbreed producing new offspring (introgression). At some point the population may become reproductively isolated from the parental species and evolve into a new hybrid species.

Initially when hybrids arise within a plant population their numbers are quite few and thus if the plant hybrid is fertile but infertile with both parents, the probability of finding a suitable mate (i.e. a hybrid of the same genotype) would be low. In order to cross this transition stage, where it is rare, the hybrid would have to employ alternative reproductive means other than sexual cross-fertilization, such as self-fertilization, parthenogenesis or asexual reproduction, to increase its abundance and its chances of survival (Arnold, 1992; Ridley, 1996).

Another potential problem is ecological competition. When a hybrid arises, it often occupies the same ecological niche as the parental species and is likely to have similar
ecological needs as those of its competitors. In a bid to increase the chance of survival, the hybrid would have to adapt to ecological niches different to that of its parents. Otherwise competition with the established parent populations particularly at the initial stage of its existence may drive the hybrid to extinction (Ridley, 1996).

The phenomenon of plant hybridisation has received much attention and data on naturally occurring plant hybrids has been collected and compiled into comprehensive listings of putative and verified examples of inter-specific and inter-generic hybrids (Stace, 1984; Rieseberg et al., 1993). Phylogenetic data, in the form of molecular data have also revealed instances of reticulate evolution within plant groups. Discordances between rDNA and cpDNA phylogenies have often proven to be reflective of past hybridisation and introgressive hybridisation events (Rieseberg, 1991; Rieseberg et al., 1991; Arnold, 1992). These findings support earlier conclusions that reticulate evolution is a widespread occurrence in the plant kingdom (Stebbins, 1959; Arnold, 1992).

Reviews have also been made regarding the evolutionary significance of plant hybridisation, including its role in the origin of allopolyploids, the origin of adaptations, the origin of ecotypes and species, increased genetic diversity and the breakdown or reinforcement of isolating barriers (Stebbins, 1959; Potts et al., 1988; Potts et al., 1990; Schemske et al., 1990; Abbot, 1992; Soltis et al., 1993; Judd et al., 1999). Aside from its evolutionary significance, hybridisation events also impact a number of other types of investigations including conservation. Hybridisation may lead to increased diversity in some instances or to possible extinction of populations or species in others via introgression (Judd et al., 1999).

The study of plant hybridisation has been misled by several generalisations made regarding character expression in hybrids including (1) hybrids are morphologically intermediate; (2) hybrids are uniformly less fit than the parental species; (3) character coherence is a diagnostic feature of introgressive populations; and (4) the inclusion of hybrids in cladistic
trees will lead to erroneous topologies (Rieseberg et al., 1993). Critical assessments have suggested that these assumptions are incorrect or at least not universal to all known hybrids. Firstly, hybrids are a mosaic of parental, intermediate and extreme characters. Secondly, studies have revealed that hybrids may be more fit, less fit, of intermediate fitness or of equivalent fitness to both parents. Thirdly, studies have also shown that character correlations in hybrid populations appear to be the exception rather than the rule. And finally breakdown in cladistic structure or the occurrence of major topological changes in phylogenetic trees will arise only if the hybrid is of distantly related parentage (McDade, 1990; McDade, 1992a; Rieseberg et al., 1993; Rieseberg, 1995; Judd et al., 1999).

4.3 Molecular analyses and its contribution to the study of hybridisation

Molecular investigations into hybridisation have been aided by the many technological advances, such as DNA sequencing, RFLP, AFLP, ISH (in situ hybridisation; including GISH, genomic in situ hybridisation), which have provided scientists with an almost unlimited number of molecular markers. These molecular markers have greatly enhanced the ability to detect hybridisation and quantify the degree of introgression, as well as to more precisely identify different genealogical classes of hybrids (Rieseberg, 1995; Stace et al., 1999). In addition, molecular studies have enabled the generation of detailed linkage maps for hybridising species, providing information regarding the genomic location and linear order of the markers. These maps have been used to assess the genomic contribution of parental taxa to suspected hybrid species or introgressant populations (Stace et al., 1999). They also have potential in identifying changes in gene order and by inference, structural changes in the genomes of the hybrids (Rieseberg, 1995). The results arising from these molecular studies can be used to infer the genetic processes involved in hybridisation and provide data for the generation of hypotheses regarding such issues as the generation of novel or extreme characters, i.e. hybrizymes, in hybrids (Rieseberg & Noyes, 1993; Rieseberg, 1995; Rieseberg & Ellstrand, 1998).
It must be emphasised that many molecular investigations are not without their limitations. Firstly, the potential of phylogenetic optimality criteria in detecting hybridisation events is somewhat controversial. It has been shown that analytical techniques such as parsimony can be ineffective in detecting hybrids since parsimony is based upon a hierarchical pattern of evolution and does not account for the potential occurrence of reticulate evolution (McDade, 1990). Secondly, if the hybrids under study are between closely related species there may be little detectable signal to allow for the diagnosis of hybrids in phylogenetic trees (McDade, 1990; Rieseberg, 1995). Thirdly, the phylogenetic position occupied by the hybrids in phylogenetic trees cannot be predicted accurately. Rather, the position of hybrids in phylogenetic trees appears to be largely dependent on the type of data used in the analysis and whether the hybrids express a high proportion of primitive vs. derived parental characters; and if derived characters are expressed, from which parent they have been inherited (Rieseberg et al., 1993).

When using molecular markers in assessing hybridisation events, investigators should take several important issues into consideration. First of all, it is important that the investigator is aware of the mode of inheritance of the particular DNA region under study. Depending on whether it is of biparental co-dominant inheritance or of uniparental dominant inheritance, the resulting data will provide varying information regarding the hybridisation event. Secondly, the applicability and limitations of the molecular and analytical techniques employed in the study must be taken into consideration and where possible appropriate measures should be undertaken to minimise error. Thirdly, the investigator should be aware of the potential occurrence of intragenic recombination, concerted evolution, and non-Mendelian inheritance patterns in hybrids which can result in data that are difficult to interpret or even misleading (Rieseberg et al., 1993; Wendel et al., 1995a,b, 2000).

Hybrids that are genetically intermediate are often resolved basally with their parents in a
phylogenetic tree. However, most hybrids are not genetically intermediate because of the occurrence of evolutionary processes such as introgression, intragenic recombination and concerted evolution. The consequence of these processes can give rise to hybrid genotypes within which evidence of its origin can be concealed. That is to say, the genetic contribution of one parent to the genotype of the hybrid may be altered in various gene regions as a result of one or a combination of the aforementioned processes. Phylogenetic analysis of those DNA regions affected by one or more of the above would give rise to a biased result in which the hybrid would be positioned closely to that parent with which it shares greatest similarity, thereby giving an impression that its relationship with the parent species is a result of non-reticulate evolution (Wendel et al., 1998). Introgression refers to backcrosses between a hybrid and one or both parents. If a hybrid were interfertile with only one parent, parent 'A', backcrossing with parent 'A' would result in the hybrid sharing more genetic similarity with that particular parent. The degree of similarity between both the hybrid and parent 'A' would be dependent upon the extent to which backcrossing occurred. If the occurrence of introgression were significant in the history of the hybrid, phylogenetic analysis of gene regions involved in the introgressive events would give rise to a topology in which the hybrid would be positioned closest to parent 'A'. Intragenic recombination leads to the evolution of composite molecules that possess characteristics of both parental alleles. If intragenic recombination occurs the position of the hybrid relative to the position of its parents in a phylogenetic tree would depend on several factors (Wendel et al., 1998):

1. The percentage of genetic material contributed by each parent in the gene region used in the analysis.
2. The amount of sequence divergence between the two parental alleles.
3. The relative antiquity of the recombination event.

Intragenic recombination has been recorded in a number of phylogenetic studies such as the study of Gossypium, in which the species Gossypium gossypioides was found to be a hybrid species that resulted from recombination between two highly divergent ITS sequences that
were brought together in a common nucleus as a result of interspecific hybridisation (Wendel et al., 1995b). Another example was found on the analysis of Alcohol dehydrogenase (Adh) sequence data of maize where recombination was documented among the alleles at Adh-1 and Adh-2 (Gaut et al., 1993c). As mentioned in Chapter 3, concerted evolution is important when inferring phylogenies from molecular data that is of biparental co-dominant inheritance and occurs as multiple copies in a genome. But in the detection of hybrids and their parents such a phenomenon can be problematic. Sequence heterogeneity can be interpreted as an indicator of past hybridisation events. However, such indicators can be removed from a gene family through concerted evolution. If the DNA sequence undergoes a capture event, the repeat type of one parent would be homogenised throughout all repeat units of the hybrid, thereby removing all trace of the second parent from that genetic region (Wendel et al., 1998).

Given that certain evolutionary processes can give misleading information regarding the phylogeny of an organism it is advisable to include several molecular regions in an analysis. In this way, if one of the previously described phenomena occurred in the biological history of one genetic region, comparison of the resulting topology with that obtained from other datasets would highlight its presence via homoplasy.

4.4 The taxonomic status of Hibanobambusa tranquillans

Natural hybridisation is well known in most grasses, in the Grass Hybrid Database approximately 4,000 hybrids have been recorded (Freeling, 2001). These include hybrids of interspecific origin, such as Spartina anglica (Raybould et al., 1991; Chapman 1996) and also hybrids of inter-generic origin such as Poa labradorica (Darbyshire et al., 1992). However the occurrence of natural hybrid species in the woody bamboos has rarely been documented (Clark et al., 1989). The long flowering interval exhibited by most woody bamboo taxa is one factor influencing this statistic; the scarcity of flowering amongst this group of grasses reflects at least in part the lack of opportunity for this phenomenon to arise.
(Clark et al., 1989). In the past, several putative hybrids have been reported, however the acquisition of conclusive evidence of hybridisation for these species has never been obtained and thus for most, their taxonomy remains shrouded in controversy (McClure, 1973; Clark et al., 1989; Okamura et al., 1991).

*Hibanobambusa tranquillans* is native to Mt. Hiba, Shimane Prefecture, Southern Honshu, Japan. There it was originally found growing amongst stands of *Sasa veitchii* f. *tyugokensis* by Dr. Hantaro Uchida in 1932 (Okamura et al., 1991). Since its discovery this species was placed in several genera including *Sinoarundinaria*, *Semiarundinaria* and *Phyllostachys* (Ohmberger, 2000). It was not until 1971 that this species was finally classified as a new genus, *Hibanobambusa* (Okamura, 1991). Currently *Hibanobambusa* is regarded as a monotypic genus, containing the species *H. tranquillans*. It is hypothesized that *H. tranquillans* first appeared towards the end of the 19th century when *P. nigra* var. *henonis* flowered simultaneously with *S. veitchii* f. *tyugokensis* resulting in successful cross fertilization (Okamura et al., 1991; Ohnberger, 2000). It is also assumed that *S. veitchii* f. *tyugokensis* is the maternal parent and *P. nigra* var. *henonis* is the paternal parent species (Okamura et al., 1991).

Suspicions regarding the origin of *H. tranquillans* arose in 1979 when Marugama et al. suggested that *H. tranquillans* was the result of an inter-generic hybridisation event between species of *Sasa* and *Semiarundinaria*. Kashiwagi, who maintained that the parents of the putative hybrid were *Sasa veitchii* f. *tyugokensis* and *Phyllostachys nigra* var. *henonis*, disputed this parentage. The justification for this hypothesis was supposed morphological intermediacy. It is claimed that *H. tranquillans* shares traits with both of these putative parents (Okamura et al., 1991), the genus exhibiting similar branching and inflorescence structures to those of *Phyllostachys*, while the number of flowers per spikelet and also the number of stamens are closer to those of *Sasa* (Okamura et al., 1991).

Apart from morphological intermediacy, there is little evidence to support or refute
hypotheses regarding the origin of *H. tranquillans*. While the original locality from which the specimens were documented seems to be a natural one, there is no documentation that any of the putative parents now occur in the vicinity (Clark *et al.*, 1989). The only molecular investigation carried out on *H. tranquillans* and its putative parents, using RAPD, proved to be inconclusive although *H. tranquillans* was found to be more closely related to *Sasa* than *Phyllostachys* or *Semiarundinaria* (Flannery, 1999).

### 4.5 Morphology of *H. tranquillans*

This native of Japan has leptomorph rhizomes with culms reaching a height of 2-6m tillering to give a pluriscaespitose arrangement. The culms are smooth, glabrous, hollow and sulcate. The internodes are short (4.5-11cm in length), bearing one-three branches in the mid-culm and upper nodes. The leaves are lanceolate to oblong in shape, acuminate, tessellate, glabrous, coriaceous, with scabrous margins. The ligule is truncated and the auricles have well developed radiating oral setae. The culm sheaths are deciduous, tessellate with soft white hairs. The sheath blade is reduced and linear in shape. The sheaths bear a ligule and fimbriate auricles. The inflorescence is bracteate with fasciculation towards its base (similar to *Phyllostachys*). Each spikelet is multi-flowered containing 2-3 flowers, each enclosing 6 stamens, reminiscent of the genus *Sasa* (*Phyllostachys* has 3 stamens) (Koidzumi, 1941,1942; Okamura *et al.*, 1991).

While the inflorescence is similar to that of *Phyllostachys*, bracteate inflorescences such as these are also found in several other bamboo genera, including *Semiarundinaria*, *Sinobambusa* and *Shibataea*. In addition, significant differences are encountered within the vegetative branching of this species when compared to *Phyllostachys*. The branch complement contains compressed nodes, a basal branch node with no lateral branch or bud, and fused prophyll margins. These three characters, which are never found in *Phyllostachys*, are found in *Sasa*, *Indocalamus* and some members of *Pseudosasa*, three genera in which relatively large leaf blades are found (Table 4.1).
### Table 4.1. Character comparison between *H. tranquillans*, putative parents and other morphologically similar taxa.

<table>
<thead>
<tr>
<th>Character</th>
<th><em>Phyllostachys</em></th>
<th><em>Hibano bambusa tranquillans</em></th>
<th><em>Sasa</em></th>
<th><em>Shibataea</em></th>
<th><em>Sinobambusa</em></th>
<th><em>Pseudosasa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Suture above lateral</td>
<td>present</td>
<td>absent</td>
<td>absent</td>
<td>present</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>branch initials</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of branch initials</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Basal nodes compressed</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Replication of</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>laterals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bracteate synflorescence</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Lateral spikelets sessile</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>?</td>
</tr>
<tr>
<td>Number of stamens</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 4.1. Character comparison between *H. tranquillans*, putative parents and other morphologically similar taxa.
4.6 Materials and Methods

Molecular investigations were carried out on twelve taxa (Table 4.2). Two cultivars of *Hibabombussa* were used in the study, *H. tranquillans* and *H. tranquillans* ‘Shiroshima’. In addition to the putative parent species, *Sasa, Semiarundinaria* and *Phyllostachys*, several other taxa, which share morphological characteristics with *H. tranquillans*, were also included in the analysis. *Sasa veitchii* f. *tyugokensis* was not available, as a result *S. veitchii* var. *veitchii* f. *minor* and/or *S. palmata* f. *nebulosa* were employed in the investigations. In the ITS sequence dataset, *Phyllostachys nigra* var. *henonis* was not included due to sequencing difficulties, as a result *Phyllostachys flexuosa* was employed as a representative of *Phyllostachys*. Furthermore, failure to sequence the trnL-trnF region for *Sasa veitchii* var. *veitchii* f. *minor* resulted in the inclusion of *Sasa palmata* f. *nebulosa* in the dataset.

| *Hibabombussa tranquillans* (Koidzumi) Maruyama & H Okamura |  |
| *H. tranquillans* ‘Shiroshima’ Koidzumi |  |
| *Indocalamus tessellatus* (Munro) Keng |  |
| *Phyllostachys nigra* var. *henonis* (Mittord) Muroi |  |
| *P. flexuosa* (Carrière) A & C Rivièrè |  |
| *Pseudosasa japonica* (Siebold ex Makino) Makino |  |
| *Sasa veitchii* var. *veitchii* f. *minor* Makino |  |
| *S. palmata* f. *nebulosa* (Makino) S Suzuki |  |
| *Semiarundinaria fastuosa* f. *viridis* Makino |  |
| *S. yamadorii* Muroi |  |
| *Shibataea chinensis* Nakai |  |
| *Shibataea kumasae* (Steudel) Makino ex Nakai |  |

Table 4.2. Taxa employed in the investigation
Isolation of genomic DNA

Total DNA was extracted from 0.05-0.1 grams of silica gel dried woody bamboo leaf material using a modified hexadecyltrimethyl ammonium bromide (CTAB) method from Doyle & Doyle (1990) (Appendix II: protocol 1.0). The crude DNA extract was then purified using the Concert Rapid PCR Purification System or the CsCl gradient purification technique (Appendix II: protocols 1.1, 1.2, 1.5). Each DNA sample was then quantified using gel electrophoresis and stored at -20°C or -80°C (Appendix II: protocol 1.3).

Amplification of target DNA regions

Both nuclear and plastid DNA regions were amplified using the Polymerase Chain Reaction (PCR) (Table 4.3 and Appendix II: protocol 1.4). The reactions were carried out using a Perkin Elmer PE 480 thermocycler. The amplified products were quantified using gel electrophoresis and then purified using the Concert Rapid PCR Purification System before being stored at -20°C (Appendix II: protocols 1.3, 1.5).

Sequencing of amplified PCR products

Cycle sequence reactions were carried out on the amplified DNA products using the Big Dye Terminator sequence mix of Applied Biosystems™. All cycle sequence reactions were carried out using a MJ Research Peltier Thermocycler PTC200 (Appendix II: protocol 1.6). The sequenced samples were then purified (Appendix II: protocol 1.7). Subsequently, the cycle sequenced samples were denatured (Appendix II: protocol 1.8) and run on an Applied Biosystems™ 310 Genetic Analyser using the ABI Prism™ 310 module SEQ POP6(1.0-mL)E or the ABI Prism™ 310 module SEQ POP6 RAPID (1.0-mL)E run files at the Department of Botany, Trinity College, Dublin or run on the ABI 377 DNA sequencer at the Royal Botanic Gardens in Kew, Surrey, U.K. The sequences were then analysed using ABI Prism™ DNA Sequencing Analysis Software, version 2.1.1.
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Data analysis

Successfully sequenced DNA samples were edited and assembled using Genecodes-Sequencher package version 3.1. The sequences were then imported into PAUP version 4.0b8 (PPC) (Swofford, 1999) and aligned by eye. The resulting matrix was then assessed by eye for shared characteristics and also subjected to maximum parsimony and Neighbor Joining analysis.

The most parsimonious trees were obtained using an exhaustive or a heuristic search retaining only those trees with a minimal score. The resulting topologies were then assessed for bootstrap support, using branch and bound, or a heuristic search. Optimal trees from the \textit{trnL-trnF} dataset were obtained using a heuristic search (1000 replicates) with random stepwise addition and the branch swapping algorithm Tree Bisection and Reconnection (TBR). Bootstrap values for the respective branches were estimated following 100 bootstrap replicates using identical search parameters as those employed in obtaining the optimal trees. In the phylogenetic analysis of the ITS1-5.8S-ITS2 sequences, the resulting trees were obtained using an exhaustive search. Bootstrap analysis was achieved using the branch and bound search method.

<table>
<thead>
<tr>
<th>Genetic Origin</th>
<th>Target Region</th>
<th>Primer Base Sequence</th>
</tr>
</thead>
</table>
| cpDNA          | \textit{trn L-trnF} (Taberlet et al., 1991) | Forward primer: CGAAATCGGTAGACGCTACG  
Reverse primer: AT1GAACCTGG1GACACGAG |
| nrDNA          | ITS1, ITS2 (Sun et al., 1994) | Forward primer: AB101: ACGAATTCATGGGTCCCGTGGAAGTGTC  
Reverse primer: AB102: AGAAATCCCCCGTGTCGCTCGCGT |

Table 4.3. Primers used in PCR and Cycle Sequence Reactions
Amplified Fragment Length Polymorphism (AFLP)

AFLP (Amplified Fragment Length Polymorphism) reactions were carried out using the Perkin Elmer Applied Biosystems AFLP™ Plant Mapping kit. The kit was composed of three modules (Appendix II: protocol 1.9).

1. Ligation and Preselective Amplification module containing EcoR1 and Mse1 adaptor pairs, preselective primers and preselective amplification mix.
2. Core Mix module which composed of a mix of buffer, nucleotides and AmpliTaq® DNA polymerase.
3. The Selective Amplification module, which contained a set of primers complementary to the EcoR1 adaptor sequence and labelled at the 5’ end with a fluorescent dye (either FAM (blue), JOE (green) or TAMRA (yellow) and a set of primers complementary to the Mse1 adaptor sequence (Table 4.4).

<table>
<thead>
<tr>
<th>Fluorescent Label</th>
<th>Primers Complementary to Adaptor Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM (blue)</td>
<td>labe</td>
</tr>
<tr>
<td>JOE (green)</td>
<td>labelled EcoR1-ACG and Mse1-CTC</td>
</tr>
<tr>
<td>NED (yellow)</td>
<td>labelled EcoR1-AAC and Mse1-CIG</td>
</tr>
</tbody>
</table>

Table 4.4. Primers used in the Selective Amplification Module.

The AFLP fingerprints were then assessed using the Neighbor Joining (NJ) distance method and also Principal Coordinate analysis (PCO). The NJ analysis employed the Nei & Li distance method. Bootstrap values were estimated using identical parameters to those employed in the original NJ search. Principal Coordinate analysis was carried out using Le Progiciel package version 4.0. The PCO analysis employed the Sørensen Dice similarity measure \((2a/ (2a+b+c))\) in calculating the genetic variance between the respective taxa. These values were then used for the PCO analysis and imported into Excel version 9.0 and plotted on a scatter plot. Dice distances approximate Nei and Li distances for restriction sites.
4.7 Results

Two equally parsimonious trees (Figure 4.1) were obtained on analysis of the *trnL-trnF* dataset. Bootstrap analysis of the sequences provided moderate support for the *Indocalamus-Shibataea* lineage (71% BS). The positions of all other woody bamboo species were unsupported in the bootstrap analysis.

Phylogenetic analysis of the ITS sequences (Figure 4.2) resolved a strongly supported *Phyllostachys-Indocalamus* lineage (100% BS). In addition, a *Hibanobambusa-Shibataea-Pseudosasa-Sinobambusa* sub-clade was identified (77% BS) within which, the *Pseudosasa-Shibataea* and *Hibabobambusa-Sinobambusa* branches were supported by values of 75% and 56%, respectively.
Figure 4.2. An unrooted cladogram of one of six equally parsimonious trees obtained following an exhaustive search of the ITS1-5.8S-ITS2 dataset. Bold integers represent the bootstrap support values. Those species highlighted in bold are the putative hybrids and parents.

Neighbor Joining analysis (Figure 4.3) of the AFLP dataset resolved a well-supported Hibanobambusa lineage (100% BS). The positions of Sasa veitchii var. veitchii f. minor and Phyllostachys nigra var. henonis, relative to the Hibanobambusa lineage were unsupported. However, Phyllostachys nigra var. henonis did have the greatest branch length (0.404). Manual comparison of the AFLP data, revealed a higher number of characters shared by the putative hybrids and Sasa veitchii var. veitchii f. minor rather than Phyllostachys (Table 4.5).
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Figure 4.3. Unrooted cladogram illustrating the topology obtained following Neighbor Joining analysis of the AFLP data set. Bold integers represent the bootstrap support values for the respective branches. Those integers that are not in bold represent the branch lengths. Those species highlighted in bold are the putative hybrids and parents.

<table>
<thead>
<tr>
<th>Number of characters shared by <em>H. tranquillans</em>, <em>H. tranquillans</em> 'Shiroshima' and <em>Sasa veitchii var. veitchii f. minor</em></th>
<th>137</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of characters shared by <em>H. tranquillans</em>, <em>H. tranquillans</em> 'Shiroshima' and <em>Phyllostachys nigra var. henonis</em></td>
<td>64</td>
</tr>
<tr>
<td>Number of characters shared by <em>H. tranquillans</em> and <em>H. tranquillans</em> 'Shiroshima'</td>
<td>59</td>
</tr>
</tbody>
</table>

Table 4.5. Number of shared characters found on comparison of the AFLP data for the respective taxa.
Figure 4.4 Scatter plot illustrating the degree of genetic divergence obtained following PCO analysis of the AFLP dataset. The first axis accounts for 71.35% of the total variance and the second 27.63%.

Principle coordinate analysis of the AFLP data provided similar results to the NJ tree, whereby, *P. nigra* var. *henonis* (based on percentage variance) was most divergent from *H. tranquillans* and *H. tranquillans* 'Shiroshima' (Figure 4.4). The first plot on the axis 1 contains the highest percentage of variance (71.35%) and although both species of *H. tranquillans* are intermediate between both putative parent species, these two species show greatest similarity with *Sasa veitchii* var. *veitchii* f. *minor* along this axis (Figure 4.4, Table 4.6). The Y-axis exhibits only 27.63% of the total genetic variance. Along this axis, *Hibanobambusa* is most similar to *Phyllostachys*.
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Table 4.6. Distance values (Di=1-Si) illustrating the amount of genetic variance between the respective taxa.

<table>
<thead>
<tr>
<th></th>
<th>Phyllostachys nigra var. henonis</th>
<th>Hibanobambusa tranquillans</th>
<th>H. tranquillans 'Shiroshima'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phyllostachys nigra var. henonis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. tranquillans</em></td>
<td>0.525</td>
<td></td>
<td></td>
</tr>
<tr>
<td>'Shiroshima'</td>
<td>0.5315</td>
<td>0.0773</td>
<td></td>
</tr>
<tr>
<td><em>Sasa veitchii</em> var. veitchii f. minor</td>
<td>0.59</td>
<td>0.3504</td>
<td>0.3505</td>
</tr>
</tbody>
</table>
4.8 Discussion

As in the investigations carried out by Flannery (1999 unpublished), *H. tranquillans* and *H. tranquillans* ‘Shiroshima’ have been shown to be closely allied and are therefore interpreted as members of the same species. The Neighbor Joining analysis carried out on the AFLP dataset resolved both as a monophyletic group with 100% bootstrap support. The Dice similarity statistics of the data estimated the percentage of genetic variance between the two accessions as 0.0773 (Table 4.6). In both axis 1 and 2 of the PCO scatter plot both accessions clustered tightly together. The two *Hibanobambusa* accessions were intermediate between *Sasa* and *Phyllostachys* on the first axis of the PCO (which accounted for 71.35% of the data variance) but were more much more closely allied with *Sasa*. AFLP markers are distributed throughout the genome and are therefore well suited to the study of hybrids. If the two parental genomes of an F1 hybrid are similar in size, AFLP markers should be inherited approximately equally from each parental species (such that the hybrid should be intermediate between the two parents in the PCO). In the case of *Hibanobambusa*, a putative recent hybrid, little time would have occurred for it to reproduce and therefore the hybrid could be expected to show intermediacy in AFLP profiles. This is, indeed, the case but a much closer association is shown to *Sasa* than to *Phyllostachys*. It is possible that introgression in the direction of *Sasa* has occurred, that AFLP markers have shown asymmetrical inheritance, or that *Phyllostachys* is not a parent of *Hibanobambusa* (see discussion below).

The ITS parsimony analysis grouped both accessions of *Hibanobambusa* in the same clade, however, due to a weak evolutionary signal, the data failed to recognise both accessions as a distinct lineage. In the *trnL*-trnF sequence dataset the *Hibanobambusa* lineage was not defined, as a result of weak evolutionary signal. For the remainder of the discussion, both the species and cultivar shall be collectively referred to as *H. tranquillans*.

The results presented question the hypothesis proposed by Kashiwagi, whereby
Phyllostachys nigra var. henonis and Sasa are suggested to be the parents for the putative hybrid, H. tranquillans. In most datasets analysed, Phyllostachys was the most divergent genus, not showing any significant genetic affinities with H. tranquillans (Figures 4.2, 4.3 and 4.4). In the ITS phylogenetic trees, Phyllostachys nigra var. henonis possessed the largest number of autapomorphies (47) and was separated from the sub-group containing H. tranquillans by a bootstrap value of 100%. The results obtained following analysis of the AFLP dataset using NJ and PCO revealed a similar pattern whereby P. nigra var. henonis was not supported as a close relative to H. tranquillans (Figures 4.3, 4.4).

The trnL-trnF sequence data did not support the hypothesis regarding Sasa as the maternal parent for the putative hybrid species. It was anticipated that the maternally inherited trnL-trnF sequences would reveal an association between H. tranquillans and the ovum donor, as a result of chloroplast capture. But given the high degree of homogeneity exhibited in the sequences for all taxa (with the exception of Indocalamus tessellatus and Shibataea kumasaca), the lack of support for such a group neither proves nor disproves the suggested parentage and hybrid status. Manual sequence comparison of the trnL-trnF sequences revealed only one synapomorphy shared exclusively between Sasa and Hibanoabambusa. Despite the lack of bootstrap support, the plastid dataset does suggest that Indocalamus and Shibatae are the most divergent species (71% BS) in the dataset.

Not only does the ITS sequence data (Figure 4.2) strongly suggest (100% BS) that P. flexuosa and I. tessellatus are distantly related to H. tranquillans, it also indicates a close relationship between Sasa, Hibanoabambusa, Shibatae, Pseudosasa and Sinobambusa. Moreover, the parsimony trees provided bootstrap support for a Hibanoabambusa, Shibatae, Pseudosasa and Sinobambusa lineage (77% BS) and also for a Sinobambusa tootsik-H. tranquillans lineage (56% BS). The Hibanoabambusa, Shibatae, Pseudosasa and Sinobambusa lineage cannot be attributed immediately to phylogenetic noise since morphological studies have revealed a number of characteristics common to these species (Table 4.1). Although this finding could support either Shibatae, Pseudosasa or
Sinobambusa as potential parents of a hybrid *H. tranquillans*, the ITS data also suggests an alternative conclusion: that *H. tranquillans* is not a hybrid at all but is simply closely related to species such as *Sinobambusa* as a result of non-reticulate evolution. Another feature worth noting in the ITS dataset is that the ITS sequences of *H. tranquillans* do not exhibit sequence heterogeneity, which is normally indicative of a recent hybridisation event. In this case, this characteristic of the rDNA sequences may be indicative of two possibilities:

1. *H. tranquillans* is not a hybrid.
2. The hybridisation event that gave rise to *H. tranquillans* is an old one. Concerted evolution homogenised the tandem repeat units of this particular rDNA region to that which is most similar to *Sinobambusa tootsik*. Such old hybridisation events may be common in the bamboos, and therefore would not warrant contemporary hybrid taxon status.

Since *Semiarundinaria* was not included in the ITS dataset as a result of sequencing difficulties attributed to sequence heterogeneity, the potential of this genus as a parent to the putative hybrid has not been fully assessed. Once more, the exclusion of the generic representatives, *Shibataea, Pseudosasa, Sinobambusa* and also *Semiarundinaria*, from the AFLP analysis has resulted in failure to thoroughly assess the relationship of these species with the putative hybrid.
Chapter 5

Concluding thoughts

5.1 The systematics of the Bambuseae

Comparative analysis of morphological characters proved uninformative for resolving phylogenetic relationships in the temperate woody bamboos. This was attributed to an insufficient number of defining characters in the dataset and also a high incidence of homoplasy as indicated by the low CI and RI values. Despite the problematic circumstance of the morphological analysis the research did bring to attention the potential use of branching patterns at the inter-generic taxonomic level. It must be emphasised that the investigation was preliminary and warrants further investigations.

It is apparent that the results obtained following molecular analysis of the woody bamboos support their treatment of the woody bamboos as a monophyletic lineage, in which the temperate and tropical woody bamboos form distinct lineages (Chapter 3: section 3.4). Resolution of this geographic division within the Bambuseae conflicts with the treatment of the woody bamboos in the classification system established by Clayton & Renvoize (1986). The results of this sequence analysis and also previous molecular studies (Clark et al., 1995; Zhang, 1996; Zhang & Clark, 2000) highlight that the sub-tribes defined under this particular system are unnatural. The classification published by Clark (1995) places the temperate and tropical bamboos in separate sub-tribes. However, these sub-tribes could not be fully assessed in this analysis as a consequence of poor genetic variation in the temperate clade and also inadequate sampling of the tropical woody bamboos. Those groups that are well supported in at least two of the data sets analysed include the *Chusquea* lineage (trnL-trnF, rpl16), and also the *Schizostachyum* lineage (trnL-trnF, ITS). Sequence analysis of the ITS data did provide insight into the phylogenetic relationships between the temperate bamboos, however on the whole it was not possible to infer a
hierarchical pattern due to the predominance of unsupported resolution for the internal branches of this clade.

Several interesting findings were made in this research. The position of *D. giganteus* in the *trnL-trnF* parsimony and NJ trees contradicts previous molecular investigations (Clark et al., 1995; Zhang, 1996; Zhang & Clark, 2000; Kelchner & Clark, 1997) in that the tropical woody bamboos and, furthermore, the Palaeotropical bamboos did not represent a monophyletic clade (Chapter 3: sub-section 3.41). However, due to sequencing difficulties this species was not included in the ITS, *rpl16* and combined datasets, thus, it was not possible to investigate the phylogenetic significance of this result any further. Little support was also obtained for the monophyly of the Neotropical bamboo lineage (Chapter 3: sub-sections 3.41, 3.42) which is congruent with the findings of Clark et al. (1995) and Zhang (1996), Zhang & Clark (2000). Yet, it must be emphasised that sampling of the tropical bamboos was limited and thus verification of these findings warrants further sampling.

Although the phylogenetic relations between the temperate taxa remain under speculation, this research has unveiled an unusual molecular pattern in the temperate bamboos. Much less genetic variation was detected in the temperate clade than in the tropical bamboos. This meant that little internal structure was found within the temperate clade while the tropical sub-clades were well supported (Chapter 3: sub-sections 3.41, 3.42, 3.43). The cause(s) of this phenomenon can only be hypothesised. In Chapter 2 the problematic circumstance of morphological studies of the woody bamboos was discussed. Poor resolution within the temperate bamboos may reflect to a degree over-taxonomy whereby the species and genera currently defined may have been elevated to ranks they do not justify. Rapid radiation is another potential contributor to the poor resolution in the temperate woody bamboos. Such an event has been proposed in several plant groups and is expressed in phylogenetic studies as poorly resolved clades (Schwarzbach et al., 1995; Francisco Ortega et al., 1996; Gielly et al., 1996; Jorgensen et al., 1999; Vijverberg et al., 1999).
1999; Chan et al., 2001). Similarly the skewed distribution of characters in all molecular datasets, in which the distribution of steps are concentrated along the branches of the tropical species, may suggest evolutionary rate heterogeneity. Slowly evolving DNA can explain the low genetic variance encountered in the temperate bamboos. Another theory worthy of consideration is that the temperate woody bamboos may represent the most recently diverged lineage. Given that long generation periods are exhibited by both temperate and tropical bamboos, it is quite possible that the rate of evolutionary change may not differ significantly between these lineages. It has been proposed that the woody bamboos originated in the tropics of the Southern Hemisphere and radiation of a temperate lineage occurred later in the evolutionary history of these grasses (Judziewicz et al., 1999).

If the temperate lineage is younger than the tropical woody bamboos, the lack of genetic variation may be the result of inadequate time for the accumulation of sufficient variation in the slowly evolving genomes of the temperate bamboos. Since the above stated hypotheses are all expressed in a similar manner in phylogenetic trees identification of the exact cause(s) is beyond the scope of this research.

The molecular regions used in this dataset varied with regards to their potential in evaluating the phylogenetic relations existing between the constituents of the tribe Bambuseae. All regions were informative at the inter-generic level for the tropical woody bamboos, despite limited sampling particularly in the ITS and rpl16 datasets. The plastid regions, particularly the trnL-trnF spacer and the rpl16 intron, provided distinct molecular markers, mainly in the form of base deletions that are characteristic of the tropical bamboos. As mentioned in the previous section, resolution within the temperate clade was poor in all datasets. However, ITS did exhibit the greatest amount of internal support within this clade and thus is worthy of further investigations at this level. Both the rpl16 and ITS regions were problematic from a practical perspective, microsatellites in the rpl16 intron and sequence heterogeneity in the ITS sequences posed as major obstacles in establishing a molecular dataset for these regions. Cloning is a solution for future molecular studies of the Bambuseae using ITS, however resolution for the problematic
nature of the \textit{rp/16} intron cannot be suggested in this thesis.

This thesis has revealed that much is to be understood about the Bambuseae with regards to their phylogeny. It is hoped that the results obtained can be used as a foundation upon which future studies can be based. It is recommended that further investigations should implement other molecular regions of both nuclear and plastid origin, and the potential of combined analyses should be explored further with increased sampling of the temperate and tropical bamboos. Combined analyses have been shown to be useful in a number of plant groups (Reeves \textit{et al.}, 2001; APG, 1998) including grasses (GPWG, 2001). It will be increasingly possible to perform combined analyses on woody bamboos in the future because DNA sequence data accumulating at a steady rate in GenBank.

Large multi-gene matrices are often favoured for phylogenetic reconstruction but they are not always available. In such situations it is possible that a super-tree approach discussed by Salamin \textit{et al.} (2002) may enable separate analyses be combined to provide new hypotheses and insights into bamboo evolution. Super-trees have been shown by Salamin \textit{et al.} (2002) to be useful for phylogenetic analysis of the grass family. They combined 55 published phylogenies to build one of the most comprehensive phylogenetic trees published for the grass family including 403 taxa. Such trees are desirable for studying macro-evolutionary processes and for classification purposes.

Alternative techniques to DNA sequencing should also be investigated. As demonstrated by Hodkinson \textit{et al.} (2000) and Loh \textit{et al.} (2001) AFLP is useful at deciphering taxonomic relations between closely related species and genera.

It is anticipated that given the speed at which molecular technology is improving and genetic information is accumulating, molecular systematists may soon be equipped with the necessary tools and data to accurately explain the historical relationships between bamboos and indeed other mysterious plant groups.
5.2 Molecular analysis of *Hibanolambusa tranquillans* and it's putative parents

As indicated from the analyses in Chapter 4 of this thesis, confirmation of a hybridisation event is not always straightforward using molecular data. Sequence variance was found to be very low in the *trnL-trnF* and ITS data sets for most of the species analysed, with the exception of *Phyllostachys* in the ITS analysis (Chapter 4: Figure 4.2). This indicates that most species included in the analysis are closely related to each other, particularly *Sasa*, *Hibanolambusa*, *Sinobambusa* and *Pseudosasa*, or are the result of a relatively recent divergence. The implementation of phylogenetic methods in the study of hybridisation may be inadequate for these genera, as there may be little detectable signal to allow for the detection of a hybridisation event. Furthermore, it must be noted that both the plastid and nuclear datasets represent a very small portion of a complete genome. As exemplified in the study of *Gossypium gossypioides*, several independent datasets were required to elucidate the phylogeny of this inter-specific hybrid (Wendel *et al.*, 1995b). Alternatively, the results obtained may be indicative of a hierarchical evolutionary trend, within which *H. tranquillans* is more closely related to *Sinobambusa tootsik* as a result of shared derived characters common to these species.

The results from the AFLP analysis show that the putative hybrid is intermediate between the two putative parents on the first axis of the PCO, but groups more closely with *Sasa* (Chapter 4, Figure 4.4). In all analyses undertaken a high genetic distance between *Phyllostachys* and *Hibanolambusa*, was recorded (Chapter 4: section 4.), which suggests that *Phyllostachys* may not be involved in the origins of *Hibanolambusa*.

To conclude it is recommended that further studies be undertaken spanning the molecular, morphological, and cytological fields. Sampling should include, in addition to *Hibanolambusa*, *Sasa*, *Phyllostachys* and *Semiarundinaria*, members of *Shibataea*, *Pseudosasa* and *Sinobambusa*. From the combined results of the AFLP and DNA sequence
Systematics of the Woody Bamboos (Tribe Bambuseae)

analysis presented here it is difficult to implement *Phyllostachys* in the origin of *Hibanobambusa*. The status of *H. tranquillans* as an inter-generic hybrid is therefore questionable.
Appendix I

Glossary

AFLP, Amplified Fragment Length Polymorphism

A highly sensitive method for detecting high numbers of DNA polymorphism. Following restriction enzyme digestion of DNA a subset of DNA fragments is selected for PCR amplification and subsequently run out on a sequencing gel and polymorphisms are detected using laser (Schlindwein, 1995).

DBGET

An integrated database retrieval system developed by the Institute for Chemical Research, Kyoto University and the Human Genome Centre of the University of Tokyo, that is available through the Genome Net. DBGET provides access to about 20 databases (Lewitter, 1998).

DNA-DNA hybridisation

This method is based on the double stranded nature of the DNA molecule, and the fact that paired nucleotides on the two complementary strands are held together by hydrogen bonds. The protocol involves hybridising single-stranded DNA from two species and measuring the thermal stability of the heteroduplex. This is achieved by gradually raising the temperature, and monitoring the course of molecular dissociation to single strands. The thermal stability of the heteroduplex is compared with the thermal stability of the
homoduplex of both species. The thermal stability of any duplex depends largely on the similarity of nucleotide sequences in the two strands as only complementary bases form hydrogen bonds. The measure of difference in thermal stability between homoduplexes and heteroduplexes provides a quantitative estimate of the genetic divergence between the two species (Avise, 1994).

**Entrez**

An integrated database retrieval system developed by the National Centre for Bioinformatics (NCBI). It is an entry point for exploring distinct but integrated databases. The Entrez system provides access to nucleotide and protein sequence databases, molecular 3-D structures database, genomes and maps database and also literature (Lewitter, 1998).

**GCG Wisconsin Package**

The Wisconsin package is a computer package containing over 130 programs, which cover a wide range of biological disciplines. Based on published algorithms from fields of mathematical and computational biology the package includes: sequence comparison, database searching and retrieval, DNA/ RNA secondary structure prediction, evolutionary analysis, fragment assembly, gene finding and pattern recognition, mapping and protein analysis and translation (Genetics Computer Group, 2000).

**MacClade**

A computer program for phylogenetic analysis. Its analytical strength is in studies of character evolution. It also provides tools for entering and editing data and phylogenies,
and for producing tree diagrams and charts (Maddisson & Maddisson, 2000).

**PAUP, Phylogenetic Analysis Using Parsimony**

Software package for the inference of evolutionary trees. This package is available for use on Macintosh, Windows, Unix/VMS or Dos-based format. The package can be used for the analysis of molecular, morphological and behavioural data to infer evolutionary biology. In addition to parsimony, PAUP also contains maximum likelihood and distance methods (Swofford, 2000).

**PCR, Polymerase Chain Reaction**

A method developed by Kary Banks Mullis (1983) for amplifying a DNA base sequence. The technique is an automated procedure, which involves amplifying a DNA base sequence using heat stable polymerase, two 20-base primers and other reagents. The reaction mix is placed in a thermocycler and is subjected to a series of cycles with varying temperature treatments. Successive cycles promote denature and renature of DNA, and in turn, the exponential amplification of the target DNA (Schlindwein, 1995, Hillis *et al.*, 1991).

**Phylip, Phylogenetic Inference Package**

A computer package containing programs for inferring phylogenies. Methods available in the package include parsimony, distance matrix and likelihood methods. Phylip can be used for the analysis of molecular sequences, gene frequencies, restriction sites, distance matrices and discrete 0/1 characters (Felsenstein, 2000).
RAPD, Randomly Amplified Polymorphic DNA

A widely used technique for amplifying anonymous sequences of DNA using PCR with arbitrary primers. The discrete DNA products that result are separated on an agarose gel in the presence of Ethidium Bromide and visualised under ultraviolet light. The polymorphisms are detected as the presence or absence of bands and result presumably from sequence differences in the primer binding sites (Schlindwein, 1995; Karp et al., 1996).

RFLP, Restriction Fragment Length Polymorphism

A non-PCR based technique for detecting polymorphism. The technique involves the digestion of DNA using restriction enzymes that recognise (typically) 4-6 base pair motifs and cleave the DNA at specific sites. The resulting fragments are separated by gel electrophoresis and transferred to a filter by blotting, to which radioactive, or non-radioactive probes are then hybridised. Specific probe-enzyme combinations give highly reproducible patterns for a given individual but variation between individuals arises when mutations alter restriction sites, or the fragment lengths between them (Karp et al., 1996).

Sequencing

A PCR based technique that decodes the sequence in which nucleotide bases occur in a region of DNA. Such a technique is largely used in addressing phylogenetic questions (Schlindwein, 1995).

Sequencing Retrieval System (SRS)

An integrated database retrieval system developed by the European Bioinformatics Institute (EBI). It is a homogeneous interface to over 80 biological databases. SRS includes
sequence and sequence related databases and also, databases concerned with metabolic pathways, transcription factors, protein 3-D structure, genome mapping, mutations and locus specific mutations (Lewitter, 1998).
Appendix II

Molecular Protocols

Protocol 1.0

Isolation of total genomic DNA using CTAB

<table>
<thead>
<tr>
<th>Materials</th>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2xCTAB Buffer (100mM Tris-HCl pH 8., 0.1M NaCl, 20mM EDTA, 2% CTAB)</td>
<td>10ml capped centrifuge tubes (labelled)</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>Pipettes (P200, P5000)</td>
</tr>
<tr>
<td>24:1 chloroform:isoamyl alcohol</td>
<td>Pipette tips</td>
</tr>
<tr>
<td>Wash buffer (70% ethanol)</td>
<td>Transfer pipettes</td>
</tr>
<tr>
<td>EDTA = Ethylenediaminetetraacetate</td>
<td>Weighing boats</td>
</tr>
<tr>
<td>CTAB = Hexadeyltrimethyl Ammonium Bromide</td>
<td>Petri dishes</td>
</tr>
<tr>
<td></td>
<td>Mortars</td>
</tr>
<tr>
<td></td>
<td>10ml capped tubes (labelled)</td>
</tr>
<tr>
<td></td>
<td>Grant SUB14/ Memmert W200 water bath</td>
</tr>
<tr>
<td></td>
<td>Harrier 15/80 centrifuge</td>
</tr>
<tr>
<td></td>
<td>Fume hood</td>
</tr>
</tbody>
</table>

Procedure

1. 10ml of 2xCTAB extraction buffer and 40ul of 2-mercaptoethanol were placed in labelled 12ml capped centrifuge tube (fume hood).

2. The tubes and pestles and mortars were then preheated at 65°C in a water bath.

3. Each extraction required 0.05-0.1g of leaf material, cut up into small pieces.

4. Extractions were carried out in a fume hood. Using initially a small amount of extraction buffer the leaf material was ground up using a pre-heated pestle and
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mortar.

5. When the leaf material was almost ground to a pulp the remainder of the extraction buffer was added.

6. The slurry was then placed back into the 12ml centrifuge tube, sealed and incubated at 65°C for approximately 10 minutes with occasional mixing.

7. 5ml of CI was then added to each tube and mixed gently.

8. The lid was released briefly to release any gas that had accumulated and then tightened once more.

9. The tubes were placed on a shaker in a horizontal position for approximately 30 minutes.

10. All samples were then centrifuged at 4500 rpm for 10 minutes.

11. The samples were then carefully removed ensuring not to disturb the separation.

12. The aqueous (upper) phase containing the DNA was removed using a transfer pipette and placed in 12ml capped tubes.

13. Equal volumes of isopropanol were added to each DNA sample.

14. The tubes were sealed and inverted to precipitate the DNA.

15. All samples were stored at -20°C for at least a week to further precipitate the DNA.

16. Following precipitation the samples were centrifuged at 2500rpm for 10 minutes to pellet the DNA.

17. The supernatant was then poured off and 3ml of wash buffer was added to each tube, and mixed gently.

18. The samples were centrifuged once more at 2500rpm for 5 minutes to pellet the DNA.

19. The supernatant was decanted off and the tubes were placed upside down for 5
minutes on a paper towel to let any excess wash buffer drain away.

20. The tubes were then turned right way up and allowed to air dry in a fume hood for approximately 25-30 minutes to remove all traces of ethanol.

21. At this stage the DNA pellets were either subjected to Caesium Chloride (CsCl) gradient purification or were suspended in 1ml of TE buffer.

22. The eluted DNA samples were then transferred to 1.5ml micro centrifuge tubes and stored in a -20°C or -80°C freezer.
Protocol 1.1

Purification of tDNA using CsCl equilibrium gradient centrifugation

(continued from step 20 of protocol 1.0)

<table>
<thead>
<tr>
<th>Materials</th>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>tDNA</td>
<td>Beckman Centrifuge tubes (15x51mm)</td>
</tr>
<tr>
<td>CsCl, Ethidium Bromide solution</td>
<td>Transfer pipettes</td>
</tr>
<tr>
<td>Butanol/SSC</td>
<td>3ml capped tubes</td>
</tr>
<tr>
<td></td>
<td>1ml syringes</td>
</tr>
<tr>
<td></td>
<td>Edmund Buhler Kl-2 Shaker</td>
</tr>
<tr>
<td></td>
<td>Ultra centrifuge</td>
</tr>
<tr>
<td></td>
<td>UVP Dual-Intensity transilluminator</td>
</tr>
</tbody>
</table>

Protocol

1. Having pelleted the DNA, 3ml of CsCl EtBr solution was added to each tube.

2. The tubes were capped, covered with foil and placed in a vertical position on a shaker for 24 hours.

3. The mixture was then transferred using transfer pipettes to labelled Beckman centrifuge tubes, which were subsequently heat-sealed (care was taken that all tubes were free of air bubbles and of equal weight).

4. All samples were then centrifuged at 35000 rpm for 24 hours.

5. Following centrifugation the resulting layers of DNA suspended in each CsCl EtBr solution were carefully removed under UV illumination using a 1ml syringe and needle. Each recovered sample was placed in a clean-capped 3ml tube.

6. One and a half times the volume of butanol/ SSC was added to each tube. The tubes were sealed and inverted several times to remove the ethidium bromide from the DNA.
7. The DNA, the lower aqueous phase, was then removed using transfer pipettes and placed in labelled dialysis tubing. The DNA material was then separated from CsCl through dialysis (see protocol 1.2).

8. Following dialysis, each sample was then transferred to 1.5ml eppendorf tubes, quantified using gel electrophoresis (see protocol 1.3) and stored in a -20° or -80° freezer.
Protocol 1.2

Dialysis of CsCl gradient samples

<table>
<thead>
<tr>
<th>Materials</th>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultra pure water</td>
<td>Sterile dialysis tubing</td>
</tr>
<tr>
<td>Four litres of TE solution (50ml of 80xTE in 4l of ultra pure water)</td>
<td>500ml Beaker</td>
</tr>
<tr>
<td></td>
<td>2l Beakers</td>
</tr>
<tr>
<td></td>
<td>Clamps (labelled)</td>
</tr>
<tr>
<td></td>
<td>Transfer pipettes</td>
</tr>
<tr>
<td></td>
<td>AGB 1000 Hotplate &amp; Stirrer</td>
</tr>
<tr>
<td></td>
<td>Magnetic fly</td>
</tr>
<tr>
<td></td>
<td>1.5 ml eppendorf tubes (labelled)</td>
</tr>
</tbody>
</table>

Procedure

1. Dialysis tubing was rinsed in ultra pure water before use.

2. The dialysis tubing was then cut in to lengths of approximately 6-7cm and placed in a 500ml beaker of ultra pure water.

3. The DNA-CsCl solution was transferred to the dialysis tubing and sealed at both ends using clamps.

4. The samples were suspended in 2 litres of ultra pure water and mixed slowly for four hours.

5. The samples were then transferred to a second beaker containing 2 litres of TE solution. The samples were suspended in the solution for a period of four hours, subjected to gentle mixing.
6. Step 5 was repeated and then the DNA samples were transferred, using transfer pipettes, to eppendorf tubes and stored at -20°C or -80°C.
Systematics of the Woody Bamboos (Tribe Bambuseae)

Protocol 1.3

Estimation of DNA fragment size, quantity and quality using agarose gel electrophoresis

### Materials

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>80ml of 1.2% agarose gel (1.2g of agarose, gel strength of a 2% w/v gel), 100ml of 1xTBE, 2uL of Ethidium Bromide</td>
<td>Parafilm</td>
</tr>
<tr>
<td>Loading Dye (Bromophenol blue and Sucrose)</td>
<td>20μl pipette (P20)</td>
</tr>
<tr>
<td>0.5μg of 1Kb ladder</td>
<td>Pipette tips</td>
</tr>
</tbody>
</table>

### Equipment

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel rack (Horizontal III, Life Technologies, Gibco BRL, Horizontal Electrophoresis Apparatus)</td>
<td>Power pack (EC105, E-C Apparatus Corporation)</td>
</tr>
<tr>
<td>UVP Dual-intensity transilluminator</td>
<td></td>
</tr>
</tbody>
</table>

### Procedure

1. 8 μl of tDNA or 5 μl of amplified PCR products were mixed with 0.5μl of loading dye.

2. The mixture was then pipetted into a well on an agarose gel (the first and the last well of each lane were left empty).

3. Once all samples were loaded onto the gel, 3 μl of 1Kb ladder, mixed with 2μl of loading dye, was placed into the first and last well of each lane on the gel.

4. Each sample was then subjected to 118V for 30-45 minutes.

5. The agarose gel was then placed above a UV light box and the DNA samples were visualised and quantified through the comparison of the intensity of florescence of the highweight band in each sample to the florescence of the different bands of the 1Kb ladder. The fragment length of the band of the 1Kb ladder with a similar florescence to that of the sample being quantified was then used to calculate
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(in ng per µl) the quantity of DNA in each sample.

Formula used for estimating the concentration of each DNA sample:

\[
\text{Size of fragment/ total size of 1kb ladder x 100}
\]
Protocol 1.4

Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Materials</th>
<th>Volume (µl)</th>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>tDNA</td>
<td>1.0-2.5</td>
<td>1.5 ml and 0.5ml eppendorf tubes (labelled)</td>
</tr>
<tr>
<td>Mineral oil</td>
<td></td>
<td>Pipettes (P2, P10, P20, P200, P1000)</td>
</tr>
<tr>
<td><strong>Amplification reagents:</strong></td>
<td></td>
<td>Pipette tips</td>
</tr>
<tr>
<td>Promega 25 mM MgCl₂</td>
<td>6.0-8.0</td>
<td>Scotsman AF-10 Ice Maker</td>
</tr>
<tr>
<td>Promega Thermophilic Poly. 10x Buffer Primers (conc. 100ng/µl)</td>
<td>10.0</td>
<td>Whirlmixer (Fison Scientific Equipment)</td>
</tr>
<tr>
<td>dNTPs (conc. 100µg/µl)</td>
<td>1.0</td>
<td>Micro centrifuge (IEC Micromax)</td>
</tr>
<tr>
<td>Betaine (for ITS amplification only)</td>
<td>2.0</td>
<td>Thermocycler: PE-480</td>
</tr>
<tr>
<td>Sigma Taq polymerase</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Ultra pure water</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Procedure

1. All tDNA samples and reagents were thawed and placed on ice.
2. An aliquot from each tDNA sample was placed in a separate 0.5ml tube.
3. A master mix was then made in a 1.5ml tube adding all reagents according to the sequence stated above.
4. The master mix was then vortexed and aliquotted out into each 0.5ml tube. The total reaction volume was 100 µl per sample.
5. Each sample was then centrifuged briefly and one to two drops of mineral oil was then added to each 0.5ml tube.
6. The samples were then placed into a thermocycler and subjected to the following temperature treatments:
a. Denaturing at 97°C for 1 minute.

b. Annealing at 50-52°C for 1 minute.

c. Strand extension at 72°C for 3 minutes.

d. Steps a-c were repeated for thirty cycles.

e. Final strand extension at 72°C for 7 minutes.

f. Incubation at 4°C indefinitely.

7. The PCR products were then quantified using gel electrophoresis and subsequently purified (see protocols 1.3 and 1.5). The purified PCR products were then refrigerated at –20°C.
Protocol 1.5

Concert Rapid PCR Purification System

<table>
<thead>
<tr>
<th>Materials</th>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1 binding Solution (conc. Guanidine hydrochloride, EDTA, Tris - HCl and isopropanol)</td>
<td>1ul spin cartridges</td>
</tr>
<tr>
<td>H2 Wash Buffer (NaCl, EDTA and Tris - HCl)</td>
<td>2ul wash tubes</td>
</tr>
<tr>
<td>TE Buffer (10mM Tris - HCl (pH 8.0) and 0.1mM EDTA)</td>
<td>1.5ul recovery tubes</td>
</tr>
<tr>
<td></td>
<td>Pipettes (P20, P200 and P1000)</td>
</tr>
<tr>
<td></td>
<td>Pipette tips</td>
</tr>
<tr>
<td></td>
<td>Whirlmixer (Fison Scientific Equipment)</td>
</tr>
<tr>
<td></td>
<td>Heating block (Grant QBT2)</td>
</tr>
<tr>
<td></td>
<td>Microcentrifuge (IEC Micromax)</td>
</tr>
</tbody>
</table>

Procedure

1. An aliquot of TE Buffer (50µl per sample cleaned) was preheated to 65° to 70°C.
2. Four times the volume of H1 was added to each DNA sample and mixed thoroughly.
3. Each DNA sample was then transferred to the spin cartridges.
4. The spin cartridges were placed into the wash tubes and were then centrifuged at 13000rpm for one minute.
5. The flow through was discarded and the spin cartridges placed once more into the wash tubes. 700µl of H2 was added to each spin cartridge and then centrifuged at 13000rpm for one minute.
6. The flow through was once more discarded and the spin cartridges replaced. Each sample was then centrifuged for a third time at 13000rpm for one minute.
7. The spin cartridges were then placed into the recovery tubes and 50μl of TE buffer was added directly to the centre of the spin cartridge.

8. Each was then incubated at room temperature for one minute and then centrifuged at 13000rpm for two minutes. The DNA solution in the recovery tube was then stored at -20 or -80°C.
Protocol 1.6

Big Dye Terminator Cycle Sequence Reactions

<table>
<thead>
<tr>
<th>Materials</th>
<th>Volume (μl)</th>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified PCR Products</td>
<td>1.5-3.5</td>
<td>Big Dye™ Sequencing Mix 1.0</td>
</tr>
<tr>
<td>Sequencing reagents:</td>
<td></td>
<td>0.2μl Eppendorf tubes (labelled)</td>
</tr>
<tr>
<td>Primers (conc.5ng/μl)</td>
<td>0.7</td>
<td>P10, P2 pipettes</td>
</tr>
<tr>
<td>Sequencing Buffer</td>
<td>3.5</td>
<td>Pipette tips</td>
</tr>
<tr>
<td>Ultra Pure Water</td>
<td></td>
<td>Scotsman AE 10 Ice Maker</td>
</tr>
<tr>
<td>Big Dye™ Sequencing Mix</td>
<td>1.0</td>
<td>Microcentrifuge: Scot Lab (MSE) Micro Centaur</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thermocyclers, MJ Research Peltier</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thermocycler PTC 200</td>
</tr>
</tbody>
</table>

Procedure

1. Both the PCR products and sequencing reagents were thawed and kept on ice.
2. All reagents were added to each tube in the sequence stated above.
3. Each sample was made up to 10μl using ultra pure water.
4. Each reaction sample was then spun down and placed in a thermocycler. The conditions used in the reaction were as follows:
   a. Denaturation at 96°C for 10 secs.
   b. Annealing at 50°C for 1 sec.
   c. Sequence extension at 60°C for 4 minutes.
   d. Steps a-c were repeated 24 times.
   e. Samples were then incubated at 4°C indefinitely.
5. The samples were subsequently purified (see protocol 1.6) and stored at a temperature of -20°C.
**Protocol 1.7**

**Purification of Cycle Sequenced DNA Samples**

<table>
<thead>
<tr>
<th>Materials</th>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle sequenced DNA</td>
<td>1.5ml eppendorf tubes or 0.5ml centrifuge tubes (labelled)</td>
</tr>
<tr>
<td>100% Ethanol (EtOH)</td>
<td>Pipettes (P2, P20, P200, P1000)</td>
</tr>
<tr>
<td>Sodium Acetate (NaAc)</td>
<td>Pipette tips</td>
</tr>
<tr>
<td>70% Ethanol (EtOH)</td>
<td>Paper towels</td>
</tr>
<tr>
<td></td>
<td>Microcentrifuge (JEC Micromax)</td>
</tr>
</tbody>
</table>

**Procedure**

1. Each sample was transferred into a 1.5ml or 0.5ml centrifuge tube.
2. To each tube 2μl of NaAc and 50ul of 100% EtOH was added.
3. The samples were incubated at room temperature for ten minutes and then placed on ice or in a -20°C freezer for a further ten minutes.
4. Each sample was then centrifuged at 13000rpm for 25 minutes.
5. The supernatant was carefully discarded and 300μl of 70% EtOH was then added to each tube.
6. Each sample was centrifuged at 13000rpm for 15 minutes.
7. The supernatant was carefully discarded and steps 5 to 7 were repeated.
8. The pelleted DNA was air-dried overnight under paper towels and then stored at -20°C.
Protocol 1.8

Denaturing of Samples prior to Sequencing using the ABI Prism™ 310 Genetic Analyser

Procedure

1. 25μl of TSR was added to each sample DNA pellet.
2. The samples were then vortexed slightly and heated for two minutes at 95°C.
3. The DNA samples were then chilled on ice, vortexed once more and then centrifuged briefly.

---

1 This procedure was not used for those samples sequenced on the ABI 377 DNA Genetic Analyser at the Royal Botanic Gardens, Kew.
Protocol 1.9

**Amplified Fragment Length Polymorphism (AFLP)**

AFLP (Amplified Fragment Length Polymorphism) reactions were carried out using the Applied Biosystems AFLP™ Plant Mapping kit. The kit was composed of three modules.

1. Ligation and Preselective Amplification module containing EcoRI and MseI adaptor pairs, preselective primers and preselective amplification mix.
2. Core Mix module which composed of a mix of buffer, nucleotides and AmpliTaq® DNA polymerase.
3. The Selective Amplification module that contained a set of primers complementary to the EcoRI adaptor sequence and labelled at the 5' end with a fluorescent dye (either FAM (blue), JOE (green) or TAMRA (yellow) and a set of primers complementary to the MseI adaptor sequence.

The AFLP protocol was a modified version of that suggested by the manufacturer as follows.

1. The adaptor pairs were annealed by heating to 95°C for five minutes and allowed to cool over a ten-minute period. The tube was then centrifuged at maximum speed (13000 rpm) for 10 sec.
2. An enzyme master mix was prepared to contain the following for each restriction ligation reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x Promega T4 DNA Ligase buffer</td>
<td>0.05 µl</td>
</tr>
<tr>
<td>0.5M NaCl</td>
<td>0.05 µl</td>
</tr>
<tr>
<td>BSA (at 1mg/ml)</td>
<td>0.025 µl</td>
</tr>
<tr>
<td>MseI</td>
<td>0.5 Units</td>
</tr>
<tr>
<td>EcoRI</td>
<td>2.5 Units</td>
</tr>
<tr>
<td>Promega T4 DNA Ligase</td>
<td>0.5 Units</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>To make a volume of 0.5 µl</td>
</tr>
</tbody>
</table>
3. Restriction-ligation reactions were prepared to contain the following for each reaction:

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promega T4 Ligase buffer</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>0.5M NaCl</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>BSA (at 1 mg/ml)</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>Mael Adaptor</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>EcoRI Adaptor</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Enzyme master mix (from step 2 above)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>DNA template</td>
<td>100 ng</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>To make up to a reaction volume of 5.5 µl</td>
</tr>
</tbody>
</table>

4. The restriction-ligation reactions were carried out at 37 °C for 2.5 hours in a PTC 100 MJ Research Thermal Cycler (the heated lid option was used).

5. A total of 94.5 µl of TE were added to the reactions and the reactions were stored on ice or at -20°C until used.

6. A preselective amplification was carried out using the preselective primers. The reactions contained:

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFLP Preselective Primers</td>
<td>8.0 µl</td>
</tr>
<tr>
<td>Core Mix</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>Diluted restriction-ligation product (from step 5 above)</td>
<td>2.0 µl</td>
</tr>
</tbody>
</table>

The reaction specifications for the PCR amplification were as follows:

<table>
<thead>
<tr>
<th>Denaturing</th>
<th>Annealing</th>
<th>Extension</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94 °C for 1 sec</td>
<td>56 °C for 30 sec</td>
<td>72 °C for 2 min</td>
<td>1</td>
</tr>
<tr>
<td>60 °C for 30 min</td>
<td>60 °C for 30 min</td>
<td>60 °C for 30 min</td>
<td>20</td>
</tr>
</tbody>
</table>

A 4 °C soak ended the reaction. All ramp times were set at one degree per second.
7. A total of 5 μl of the PCR product were diluted in 95μl TE₀.₁ and the reactions were stored on ice or at 2-6°C until used.

8. A selective amplification reaction was performed for each primer pair with each reaction containing:

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI labelled primer</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Msel primer</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Core Mix</td>
<td>7.5 μl</td>
</tr>
<tr>
<td>Diluted PCR product</td>
<td>1.5 μl</td>
</tr>
</tbody>
</table>

The primers used in the final analysis were:

- FAM (blue) labelled EcoRI-ACA and Msel-CAC
- JOE (green) labelled EcoRI-ACG and Msel-CTC
- NED (yellow) labelled EcoRI-AAC and Msel-CTG

The reaction specifications for the selective amplification PCR are much more strict than those for the preselective amplification. The conditions are set out below:
The three PCR products for each sample were combined as follows: 0.6μl of FAM product, 0.8μl JOE product, 1.3μl NED product, 0.5μl ROX 500S size standard and 24μl of formamide. This was heated to 95°C for five minutes and then stored on ice until ready. The samples were then run on an ABI PRISM® 310 Genetic Analyser with GeneScan data collection software.
Appendix III

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Tribe</th>
<th>Species name</th>
<th>Accession No.</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Genbank</td>
</tr>
<tr>
<td>21Trevor</td>
<td>Bambuseae</td>
<td><em>Alvimia gracilis</em> Soderstrom &amp; Londêno</td>
<td>4388 A.M.de Carvalho, Brazil,CEPEC</td>
<td>Brazil</td>
</tr>
<tr>
<td>163B</td>
<td>Bambuseae</td>
<td><em>Ampelocalamus mianringensis</em> (Li &amp; Liang) Li &amp; Stapleton</td>
<td>1050/C.Stapleton</td>
<td>Yunnan</td>
</tr>
<tr>
<td>18B</td>
<td>Bambuseae</td>
<td><em>Ampelocalamus patellaris</em> (Gamble) Stapleton</td>
<td>Stapleton 873</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Genbank</td>
</tr>
<tr>
<td>94B</td>
<td>Bambuseae</td>
<td><em>Arundinaria gigantea</em> (Walter) Muhlenberg</td>
<td>MWC 1995</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>143B</td>
<td>Bambuseae</td>
<td><em>Arundinaria tecta</em> (Walter) McClure</td>
<td>1994-35</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>78B</td>
<td>Bambuseae</td>
<td><em>Aulonemia longiartistata</em> Clark &amp; Londono</td>
<td>1389 L.C.Clarke, P.Asimbaya 1380, Ecuador,QCA</td>
<td>Ecuador</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Genbank</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Genbank</td>
</tr>
<tr>
<td>30B</td>
<td>Bambuseae</td>
<td><em>Bambusa multiplex</em> 'Alphonose Karr' (Loureiro) Raeuschel ex Schultes &amp; Schultes</td>
<td>s.n.</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>58B</td>
<td>Bambuseae</td>
<td><em>Bambusa textilis</em> McClure</td>
<td>1132 Stapleton</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>135B</td>
<td>Bambuseae</td>
<td><em>Bambusa vulgaris</em> Schrader ex Wendland</td>
<td>1973-21090</td>
<td>U.S.</td>
</tr>
<tr>
<td>144B</td>
<td>Bambuseae</td>
<td><em>Bashania fangiana</em> Camus</td>
<td>1995-3993</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>142B</td>
<td>Bambuseae</td>
<td><em>Bashania fargesi</em> (Camus) Keng &amp; Yi</td>
<td>1991-1512</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>162B</td>
<td>Bambuseae</td>
<td><em>Bashania qingchengshanensis</em> Keng &amp; Yi</td>
<td>1995-3867</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>Code</td>
<td>Tribe</td>
<td>Species Description</td>
<td>Year</td>
<td>Location</td>
</tr>
<tr>
<td>------</td>
<td>-----------</td>
<td>----------------------------------------------------------</td>
<td>--------</td>
<td>----------------</td>
</tr>
<tr>
<td>512B</td>
<td>Bambuseae</td>
<td>Borinda emeryi Stapleton</td>
<td>1992-0401A</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>157B</td>
<td>Bambuseae</td>
<td>Borinda macelureana (Bor) Stapleton</td>
<td>1992</td>
<td>Rushford 5051</td>
</tr>
<tr>
<td>146B</td>
<td>Bambuseae</td>
<td>Borinda perlonga</td>
<td>1995-4215</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>145B</td>
<td>Bambuseae</td>
<td>Borinda sp.</td>
<td>1996-1754</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>3B</td>
<td>Bambuseae</td>
<td>Chimonobambusa marmorea (Mitford) Makino</td>
<td>1973-20180</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>56B</td>
<td>Bambuseae</td>
<td>Chimonobambusa macrophylla f. intermedia Wen &amp; Ohmb.</td>
<td>1124</td>
<td>Stapleton</td>
</tr>
<tr>
<td>6B</td>
<td>Bambuseae</td>
<td>Chimonobambusa quadrangularis (Fenzl) Makino</td>
<td>1988-3398</td>
<td>U.S.</td>
</tr>
<tr>
<td>57B</td>
<td>Bambuseae</td>
<td>Chimonobambusa szechuanensis (Rendle) Keng</td>
<td>1128</td>
<td>Stapleton</td>
</tr>
<tr>
<td>67B</td>
<td>Bambuseae</td>
<td>Chimonobambusa takedissinoda Hseuh &amp; Yi Ex Ohmb.</td>
<td>1989-315</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>73B</td>
<td>Bambuseae</td>
<td>Chusquea coronalis Soderstrom &amp; Calderón</td>
<td>1129</td>
<td>Stapleton</td>
</tr>
<tr>
<td>26B</td>
<td>Bambuseae</td>
<td>Chusquea culeou Desvaux</td>
<td>1130</td>
<td>Stapleton</td>
</tr>
<tr>
<td>19B</td>
<td>Bambuseae</td>
<td>Chusquea delicatula Hitchcock</td>
<td>1985</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>71B</td>
<td>Bambuseae</td>
<td>Chusquea foliosa Clark</td>
<td>1138</td>
<td>Stapleton</td>
</tr>
<tr>
<td>53B</td>
<td>Bambuseae</td>
<td>Chusquea sp.</td>
<td>1133</td>
<td>Stapleton</td>
</tr>
<tr>
<td></td>
<td>Bambuseae</td>
<td>Chusquea vulcanalis</td>
<td>1136</td>
<td>Stapleton</td>
</tr>
<tr>
<td>165B</td>
<td>Bambuseae</td>
<td>Dendrocalamus giganteus Munro</td>
<td>452</td>
<td>Stapleton (1987)</td>
</tr>
<tr>
<td>147B</td>
<td>Bambuseae</td>
<td>Dendrocalamus membranaceus Munro</td>
<td>1992-3549</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>12B</td>
<td>Bambuseae</td>
<td>Drepanostachyum falcatum (Nees) Keng</td>
<td>1996-1426TOWER</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>63B</td>
<td>Bambuseae</td>
<td>Fargesia dracocephala Yi</td>
<td>1989-1914</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>14Tevor</td>
<td>Bambuseae</td>
<td>Fargesia fungosa Yi</td>
<td>3257</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>61B</td>
<td>Bambuseae</td>
<td>Fargesia murielae (Gamble) Yi</td>
<td>1973-20162</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>154B</td>
<td>Bambuseae</td>
<td>Fargesia nitida (Mitford) Keng</td>
<td>s.n.</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>Code</td>
<td>Tribe</td>
<td>Species Description</td>
<td>Accession</td>
<td>GenbankLink</td>
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<tr>
<td>------</td>
<td>-----------</td>
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</tr>
<tr>
<td>50B</td>
<td>Bambuseae</td>
<td>Fargesia utilis Yi</td>
<td>1995-3861</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>140B</td>
<td>Bambuseae</td>
<td>Gaoligongshania megalothyrsa (Handel-Mazzetti) Li, Hseuh &amp; Xia</td>
<td>U.K.</td>
<td></td>
</tr>
<tr>
<td>140B</td>
<td>Bambuseae</td>
<td>Gigantochloa verticillata (Steudel) Widjaja</td>
<td>1973-12238</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>149B</td>
<td>Bambuseae</td>
<td>Hibanoahambusa tranquilans (Koidzumi) maruyama &amp; Okamura s.n.</td>
<td>RBG, Kew</td>
<td></td>
</tr>
<tr>
<td>150B</td>
<td>Bambuseae</td>
<td>Hibanoahambusa tranquilans 'Shiroshima' (Koidzumi) Maruyama &amp; Okamura s.n.</td>
<td>RBG, Kew</td>
<td></td>
</tr>
<tr>
<td>14B</td>
<td>Bambuseae</td>
<td>Himalayacalamus cupreus Stapleton</td>
<td>1144 Stapleton</td>
<td>RBG, Kew</td>
</tr>
<tr>
<td>10B</td>
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