Investigation into the Use of Molecular Methods to Distinguish between Species of *Caladenia* Subgenus *Calonema*

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Thesis abstract

The phylogeny of typical spider orchid (*Caladenia* subgenus *Calonema*) is investigated for the first time. The analyses were performed using 17 RAPD and 10 ISSR primers on 30 taxa representing the three spider orchid groups (the dilatata, patersonii and reticulata groups) yielding 135 RAPD and 63 ISSR polymorphic markers. The average number of polymorphic markers produced from 17 RAPD and 10 ISSR primers were 5.12 and 4.48, respectively. 76 RAPD markers and 38 ISSR markers were polymorphic within spider orchid species. The highest number of amplified DNA fragments were produced from OPE15 (8.77 fragments) and UBC 842 (6.71 fragments) while OPF04 (2.93 fragments) and UBC 825 (3.02 fragments) gave the smallest number of amplification products. The average Dice genetic similarity of pairs of individuals within a species ranged from 0.772 to 0.939 based on RAPD and from 0.770 to 0.976 for ISSR data.

The 117 spider orchid individuals analysed by RAPD, ISSR and combined data using cluster analysis were classified into three groups that correlate with those based on morphological analysis: reticulata, patersonii and dilatata. The phylogenetic analysis based on three data sets, presented relationships at the intraspecific level that were well supported by bootstrapping. However, the interspecific relationships within the groups remained unclear. Principal component analysis of RAPD and ISSR data also conducted indicating closed relationships between the reticulata and patersonii groups. High correlation (r=0.90) was detected between RAPD and ISSR markers by mean of Mantel test.

Phylogenetic relationships within the dilatata group were also investigated using ITS and the *trn*T (UGU) and *trn*L (UAA) 5'exon spacer sequences. The ITS and the *trn*T-L had the approximate sizes of 750 bp and 700 bp, respectively. The G+C content of ITS sequences varied from 41.67 to 42.12% which is higher than the G+C content of the *trn*T-L (25.80 to 26.26%). The aligned data matrix of the ITS region included a total of 671 sites including 544 constant characters, 102 parsimony uninformative characters and 25 parsimony informative characters. The aligned data matrix of the non-coding region of the *trn*T-L included a total of 677 sites that consisting of 617 constant characters, 49 parsimony uninformative characters and 11 parsimony informative characters.

The strict consensus tree inferred from both ITS and the *trn*T-L sequences and combined data had poor resolution of the dilatata grouping as indicated by unresolved polytomies that were supported with relatively low bootstrap value. The ITS phylogeny well resolved the separation of *C. venusta*, *C. cardiochila*, *C. tessellata* and *C. patersonii* from the dilatata spider orchid. The *trn*T-L phylogenetic relationships provided sufficient information to resolve the relationships between *C. flaccida*, *C. latifolia* and *L. menziesii* and typical spider orchids. The phylogenetic tree constructed from combined data tended to have the feature of ITS phylogenetic tree, as ITS sequences had more polymorphisms. The *trn*T-L sequence of *C. verrucosa* also showed specific sequences that might be useful in the identification of this species.

The use of molecular characters to assess the phylogenetic relationships among the species of *Caladenia* subgenus *Calonema* does not address the position of spider

orchids within their belonging group. This evidence indicates that taxonomic re-examination of the *Caladenia* subgenus *Calonema* is needed.

Student Declaration

"I, Chutima Kongjaroon, declare that the PhD thesis entitled Investigation into the Use of Molecular Methods to Distinguish between Species of *Caladenia* Subgenus *Calonema* is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my work".

Chutima Kongjaroon

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Chapter 1

Introduction

Orchids (Family *Orchidaceae*) are similar to many other monocotyledons in that they are herbaceous, have sheathing leaf bases, parallel venation, an inferior ovary, a single seed leaf and two sets of floral parts, arranged in threes: three sepals, situated under and between three inner petals (Backhouse and Jeanes, 1995). Plants within the Orchid family may be terrestrial; growing with their roots in soil, lithophytes: growing on rock, boulders and cliff faces, or epiphytes; growing on plant surfaces e.g. trunks and branches of trees. All species of the Orchid genus *Caladenia*, which is the focus of this thesis, are terrestrial (Cribb and Bailes, 1989; Backhouse and Jeanes, 1995; Jones and Jones, 2000).

Members of *Orchidaceae* possess a unique set of floral and vegetative features that distinguish them from other plant families (Backhouse and Jeanes 1995). There are five basic characteristics associated with orchid flowers including: zygomorphic arrangement of the petals and sepals (bilateral symmetry), a column (a structure located at the central of orchid flowers formed by the fusion of their reproductive parts (the stamens, and stigma), the rostellum (a little beak; this being the sharp apex of the stigma that separates the pollinia from the stigmatic surface), pollinia (an aggregation of the pollen into packets) and tiny seed that contains no endosperm. This last characteristic means that orchid seedlings are reliant on fungus to provide nutrient and ensure germination and early growth, a relationship that may persist into

adulthood (Cribb and Bailes, 1989; Backhouse and Jeanes, 1995). In addition, orchids commonly have a modified median petal, called the labellum, which is typically decorated with brightly coloured lobes, appendages, teeth, calli or shiny glands and can be hinged at the base. The labellum plays an important role in orchid pollination. Its main abilities are to attract and provide a landing platform for pollinators (Backhouse and Jeanes, 1995) or mimic the females of the insect pollinator species to effect pollination through pseudocopulation. Because of its important role in reproduction, the labellum is one structure that often varies between species and can be used for taxonomic identification.

1.1 Genus *Caladenia*

The genus *Caladenia* was described by Robert Brown in 1810 (Jones, 1988). The generic name is well chosen, being based on the Greek words meaning "beautiful gland" and refers to the ornamental calli that adorn the labellum of these orchids; calos = beautiful and adenos = gland. This genus is a large one, with over 243 named species (Hopper and Brown, 2004a). Most *Caladenia* species are endemic to Australia except for four species in New Zealand and one found in New Caledonia, Indonesia and Malaysia (Backhouse and Jeanes, 1995; Jones and Jones, 2000). There are presently, approximately 50 named *Caladenia* species in Victoria, plus several varieties, two subspecies and a single hybrid (Backhouse and Jeanes, 1995; Jeanes and Backhouse, 2000). There are an unknown number of un-named taxa (Jeanes and Backhouse, 2000).

1.2 Plant description

Caladenia species are terrestrial orchids that have a single green basal leaf which is generally long (10-35 cm), narrow (3-20 mm) and hairy. The hairs may be simple or

glandular (Jones, 1988; Backhouse and Jeanes, 1995). The two main Sections within the genus *Caladenia*, *Eucaladenia* (finger *Caladenia*) and *Calonema* (Spider *Caladenia*) can be distinguished from one another based on floral structure. The leaves of individual species within each of the two groups provide only very limited taxonomic information, varying mainly in terms of size and degree of hairiness.

As with most other terrestrial orchids, *Caladenia* produce underground storage organs called tuberoids, derived from root tissues, that are replaced annually. The tuberoid is the organ that survives over summer after the vegetative parts of the plant have withered, remaining dormant until autumn rains stimulate the growth of a new shoot. The pearly white, rounded, pea-sized tuberoids of *Caladenia* can be distinguished from those of other genera because they are wholly or partially enclosed in a shaggy, fibrous sheath which extends to the soil surface (Fig 1.1). The extent of the fibrous sheath has been highlighted as having some taxonomic significance at the level of genus or subgenus in recent treatments (Jones and Clements, 2002; Hopper and Brown, 2004a), but is highly variable between and within species. The orchid leaf primordium re-emerges through the sheath when nutrient reserves are sufficient for sexual reproduction, but are known to remain dormant in times of nutrient or water stress. The tuberoid is replaced annually on a short dropper (tuber stalk), so that a plant during the growing season will typically have a pair of tubers – one old tuber and one new (Fig 1.1). The new tuberoid is deeper in the soil than the previous one (Jones, 1988).

Patterns of vegetative reproduction in *Caladenia* vary. Most *Caladenia* produce only one replacement tuber, and reproduce almost exclusively by seed. A few species form

clumps by the production of multiple replacement tuberoids on droppers at the base of the parent plant. These species may form large colonies. The Victorian species, *C. latifolia,* produces lateral stolons and grows in large colonies sometimes with hundreds of stems potentially derived from the same genetic individual. A few species, such as *C. filifera*, increase more slowly by forming more than one dropper annually but not stolons. These plants grow in localized tufts (Jones, 1988). The only Victorian taxa of *Caladenia* section *Calonema* to do so are *C. filamentosa* var. *filamentosa* and *C. filamentosa* var. *tentaculata*. In general, tuberoids do not provide sufficient taxonomic information to distinguish individual species, so that the main identifying features for taxonomic purposes are the flower, flowering time, habitat and pollinators (if known).

The flowering period of *Caladenia* occurs mainly in spring. However, several species may flower as early as mid-winter, and a few during the summer period. Flowering of most species is enhanced significantly after fire in the previous summer or autumn. Fire removes competing vegetation and releases nutrients thereby accelerating growth. Plant dormancy is initiated by high temperature and dry soil in late spring (Backhouse and Jeanes, 1995).

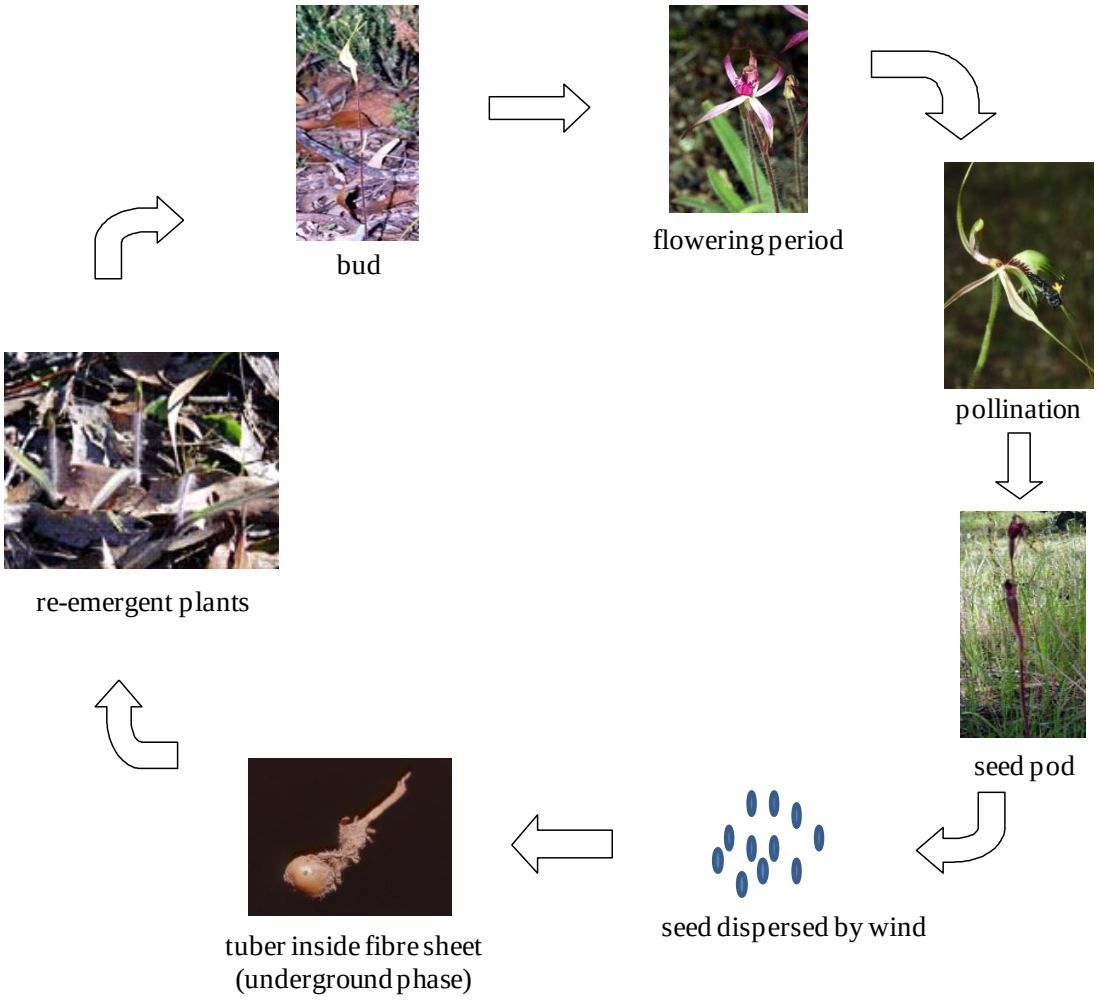


Figure 1.1 Spider orchid's life cycle.

1.2.1 Floral characteristic

Caladenia is a varied genus characterized by an erect inflorescence. The floral parts consist of three sepals and two petals that are similar in size and shape and longer than the modified third petal - the labellum (Fig 1.2). All species have a conspicuous labellum, hinged to the base of the column by a small claw. The labellum is usually three-lobed, and decorated with conspicuous calli that vary in shape, colour and arrangement. The margins of the lateral lobes are often fringed or deeply toothed, these being referred to as combs in some species (Jones, 1988; Backhouse and Jeanes, 1995). Typically, the shape of the labellum, the number, shape and arrangement of calli and the structure of the labellum margins provide features for taxonomic identification.

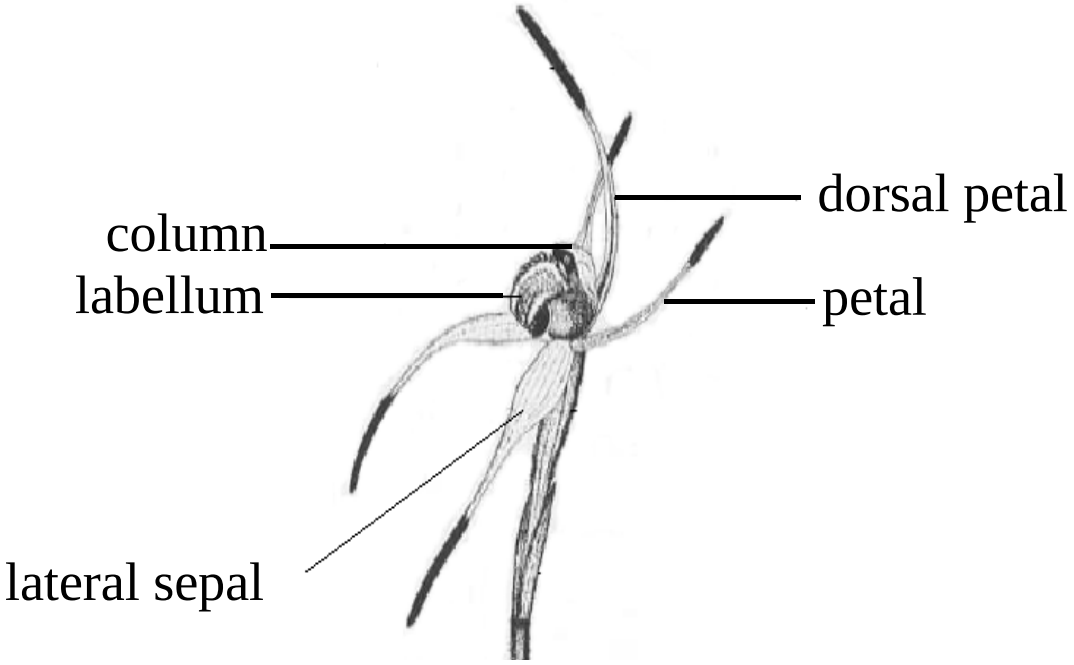


Figure 1.2 Floral form of a spider orchid (from Backhouse and Jeanes, 1995).

Caladenia prior to 2001, and as currently accepted by most Australian herbaria, consists of two sections: Section *Eucaladenia* comprises all those species considered to be "finger *Caladenia*", which are distinguished by relatively short, forward-projecting lateral petals and sepals with a short dorsal sepal that may be upright or hooded over the column. The spider orchids, which are the focus of this study, are located within Section *Calonema*. Spider orchids can be identified by their relatively large flowers with long tapering sepals and lateral petals collectively known as tepals since the petals and sepals cannot be easily distinguished from one another. These tepals typically have either numerous hair-like glands called osmophores or conspicuous club-like thickenings at their tips. In each case, the clubs or osmophores produce chemicals that act as sexual attractants for the plants' pollinators which are usually male thynnid wasps (Jones, 1988). The structure and colour of the osmophores and clubs assist with taxonomic identification. Another useful taxonomic feature is the flower's habit – i.e. whether the flower parts are stiffly spread, lax or deflexed (Backhouse and Jeanes, 1995).

1.3 Ecology

Caladenia species are distributed throughout Victoria and found in a wide variety of habitats including alpine meadow, open mallee scrublands, closed coastal scrublands, heathlands, heathy woodlands, woodlands and open forest (Fig 1.3) (Jones 1988; Backhouse and Jeanes, 1995). They are found generally in well-drained soils, including deep sands, sand and clay loams and dry skeletal soils (Backhouse and Jeanes, 1995).

Dry woodland

Coastal heath and heath woodland

Box-pine woodland

Mallee woodland

Figure 1.3 Variety of spider orchid's habitats (from Jeanes and Backhouse, 2000).

Some *Caladenia* species were named by their habitat or their distribution area, for example, *Caladenia actensis* refers to its origin at Australian Capital Territory (ACT) (Jones and Clements, 1999), *Caladenia pilotensis* refers to its distribution area at Mt. Pilot (Jones, 1991), *Caladenia australis* refers to its distribution area across southern Victoria ("*australis*" in Latin means southern; Carr, 1991), *Caladenia insularis* refers to the species occurrence on French Island ("*insula*" in Latin means an island; Carr, 1991) and *Caladenia montana* grows in mountainous areas; (Latin "*montanus*" means mountain; Carr, 1991). Some species names indicate their flowering time such as *Caladenia aestiva* - the Latin "*aestivus*" means summer, and *Caladenia brumalis* – the Latin "*brumalis*" means winter (Carr, 1991).

1.4 Taxonomy of genus *Caladenia*

Orchids belonging to this genus are classified into:

Subfamily: *Orchidoideae*

Tribe: *Diurideae*

Subtribe: *Caladeniineae*

1.4.1 Subtribe *Caladeniineae*

Most genera and species in the Orchid subtribe *Caladeniineae* are endemic to Australia. The subtribe consists of about 10-15 genera with 271 species. However, the actual number is currently under discussion due to differences between the published views of a number of groups of researchers, splitting the largest accepted genus, *Caladenia,* into genera of varying sizes. The other genera included within *Caladeniineae* are much smaller and have been variously classified by different authors. Segregate Genera that are generally accepted in recent times include *Elythranthera* (2 species), *Glossodia* (2 species), *Leptoceras* (1 species), *Eriochilus* (8

species) and *Adenochilus* (2 species) (Hopper and Brown, 2000; Kores *et al*., 2000; Hopper and Brown, 2004b).

According to Hopper and Brown (2004b) *Caladenia* now comprises about 243 species and 19 named hybrids after splitting off several newly proposed genera. Hopper and Brown informally proposed the genera *Praecoxanthus* (1 species) and *Cyanicula* (10 species) in their field guides (1988, 1991), and have since formally published these based on DNA sequencing (2000). These two genera are also accepted by Jones and Clements (2002) although they placed *C. deformis* in the monotypic genus *Pheladenia,* rather than *Cyanicula*. This placement is now accepted by Hopper and Brown (2004b). A further genus, *Ericksonella* (1 species), was described by Hopper and Brown (2004b) who disputed the name, but not the placement, by Jones and Clements (2002) of *C. saccharata* into *Glycorchis*. Other recently published genera are currently the subject of some dispute and are discussed further below.

Of the genera mentioned above, many are endemic to south-western Australia, including: *Ericksonella/Glycorchis*, *Elythranthera*, *Praecoxanthus*, 107 species of *Caladenia*, nine of the ten *Cyanicula* and six of the eight species of *Eriochilus.* Other taxa, including *Glossodia*, one species each of *Cyanicula* and *Adenochilus* and two of the eight species of *Eriochilus* are endemic to eastern states of the continent, however, *Adenochilus* is not found in Victoria. Within *Caladeni*a subgenus *Calonema*, 132 species are endemic across southern Australia, 56 are distributed in south-west with 76 in south-eastern Australia including New South Wales, South Australia, Victoria and Tasmania. A few species of *Caladenia* are also found on surrounding islands of

New Zealand, Chatham Islands, Auckland Islands, New Caledonia and Indonesia (Hopper and Brown, 2004b).

1.4.2 Genus *Caladenia* – early classification systems

The terrestrial Australian orchid genus *Caladenia* was erected by Robert Brown in 1810 (Brown, 1810, Hopper and Brown, 2004b). At this time the genus included only 13 species, including *C. flava* R. Br. The original descriptions for the various sections made no reference to the type species. Many of the plants were classified into section *Eucaladenia*. Brown also described the section *Leptoceras* with only two species including *C. menziesii* R. Br. and *C. macrophylla* R. Br.

The present circumscription of *Caladenia* as a monophyletic entity was done in January 1840 by Lindley (Hopper and Brown, 2004b). Lindley also described 14 new Western Australia species based on Drummond's collections of dried specimens (Hopper and Brown, 2001; Hopper and Brown, 2004b). Brown's section *Leptoceras* was elevated by Lindley to generic rank and he named two new sections in *Caladenia*; *Caladenia* section *Pentisia* (*C. gemmata*, *C. ixioides*) and *Caladenia* section *Calonema* (*C. filifera*, *C. denticulata*, *C. hirta*, *C. longicauda*, *C. discoidenia*). He also reclassified a rather heterogeneous group of species into section *Eucaladenia* including *C. alba*, and *C. carnea* (now in subgenus *Caladenia*), *C. angustata* and *C. congesta* (now in subgenus *Stegostyla*), *C. flava*, *C. latifolia*, *C. reptans*, *C. marginata*, *C. ochreata*, *C. elongate* and *C. mollis* (now in subgenus *Elevatae*), *C. caerulea* and *C. sericea* (now in *Cyanicula*) and *C. unguiculata* and *C. barbata* (now in *Pheladenia*). He also described *Glossodia emarginata* (now *Elythranthera emarginata*) with glossy pink petals and sepals (Hopper and Brown, 2004b).

Later in March 1840 Lindley classified 30 *Caladeni*a species from Eastern and Western Australia into three sections including 17 species in *Caladenia* section *Eucaladenia*, two species in *Caladenia* section *Pentisia* and 11 species in *Caladenia* section *Calonema*. However, according to recent molecular work, sections *Pentisia* and *Calonema* have been found to be heterogeneous classifications. Lindley also expanded *Leptoceras* to six species and *Glossodia* to four species (Hopper and Brown, 2004b).

The circumscription of *Caladenia*, rendering the genus polyphyletic, was expanded by including species of *Glossida*, *Cyrtostylis*, *Adenochilus*, *Chiloglottis*, *Rimacola* and *Lyperanthus* in 1871 by Reichenbach (Hopper and Brown, 2004b). He described four new *Caladenia* species from Western Australia including the distinctive *C. saccharata* and *C. barbarossa*. This last species was later named as the type species for the subgenus *Drakonorchis* (Hopper and Brown, 2000). This broadest concept of *Caladenia* was not accepted by Bentham (1873, cited by Hopper and Brown, 2004a). Bentham preferred Brown's concept that treated *Leptocerus* as a section of *Caladenia* rather than Lindley's distinct genus. Bentham also retained *Lyperanthus suaveolens* and *L. serratus* in *Caladenia* as Reichenbach did. Bentham also described a new *Caladenia* species, *C. aphylla* from Western Australia species and placed it in the monotypic genus *Praecoxanthus* (Hopper and Brown, 2000). In 1871 Reichenbach had some concern regarding the broad limitation of genus *Caladenia*. Bentham classified 27 *Caladenia* species into section rank including 13 species in *Caladenia* section *Eucaladenia*, 2 species in *Caladenia* section *Leptoceras*, 3 species in *Caladenia* section *Phlebochilus*, 7 species in *Caladenia* section *Calonema* and 2 species in *Caladenia* section *Pentisia*. *Glossodia* section *Elythranthera* was elevated

to generic status and Lindley's *Leptoceras fimbriata* was recognised as the new monotypic genus *Leporella*. However, the classification of the genus *Caladenia* remained polyphyletic (as we now understand) as other authors continued to include *Praecoxanthus*, and *Leptoceras* in *Caladenia* (Hopper and Brown, 2004b).

1.4.3 Genus *Caladenia* classification in 20th century

The genus *Caladenia* initially described by Robert Brown has remained in use in the broadest sense with the number of species increasing to more than 200 species. Over the past two decades, more species have been described in the genus, indicated by the "shift in species concepts from the polytypic morphology concept in wide use in Australia following Bentham to the application of evolutionary (Carr, 1986), phylogenetic, or taxonomic concepts" (Hopper and Brown, 2001). Hopper and Brown (2000) formally proposed that *Cyanicula* should be segregated as a genus distinct from *Caladenia*, along with the reinstatement of *Leptoceras* and the erection of genus *Praecoxanthus.* They also described five subgenera of *Caladenia* including two that were new: *C.* subgenus *Drakonorchis* (type species: *Caladenia barbarossa*) and *C. subgenus Elevata* (type species: *Caladenia flava*), along with *Caladenia* subgenus *Caladenia* (type species: *Caladenia carnea*), *Caladenia* subgenus *Calonema* (type species: *Caladenia patersonii*) and *Caladenia* subgenus *Phlebochila* (type species: *Caladenia cairnsiana*). Notably, they revised their earlier view of *Drakonorchis* as a separate genus after DNA studies (Hopper and Brown, 2000).

Szlachetko (2001a) divided *Caladenia* R. Br. into two sections; *Caladenia* and *Caladeniastrum* (*C. flava*). He also removed the section *Calonema,* consisting of the more than 70 species typically considered spider orchids from *Caladenia*. He further

segregated the former *Caladenia* Section *Calonema* into several genera including the monotypic *Jonesiopsis*, *Phlebochilus* (in which he placed a group of spider orchids with thick lips and short perianth segments) as well as *Calonema* with *Calonema filifera* as the type species. He used *Caladenia multiclavia* as the type species for *Jonesiopsis* but in his publication, it was actually printed as *Jonesyella multiclavia*, leading to subsequent claims by Jones and Clements that *Jonesiopsis* was invalid (Szlachetko, 2003). His new genera *Phlebochilus* and *Calonema* were subsequently found to be a heterogeneous mixture of taxa according to contemporary molecular phylogenetic analysis by other groups of researchers. These contemporary authors further complicated the taxonomic status of the Spider Orchids and seemed to depart significantly from traditional classification (Jones *et al*., 2001, Hopper and Brown, 2001). Later, Szlachetko's genus *Calonema* was changed to *Calonemorchis* since *Calonema* had already been used for a fungal genus in the Myxomycetes (Szlachetko, 2001b). Additionally, Szlachetko did not indicate types for *Calonema* and *Phlebochilus* which led later researchers to argue his names were invalid (Jones and Clements, 2002).

In September 2001, Hopper and Brown published their major work on the *Caladenia* Alliance. This work provided a detailed explanation of their species concepts, molecular biology, subgenera within *Caladenia* and descriptions of numerous new species that had been previously published informally in their field guides. Most relevant to this study on spider orchids are the two subgenera of *Caladenia* (Brown), *Calonema* and *Phlebochila*. As stated by Hopper and Brown (2001) the subgenus *Calonema* refers to spider orchids with long petals and sepals while the short-petalled and sepalled spider orchids belong to subgenus *Phlebochila*. A key feature of

Calonema, according to their description, is the four or more rows of calli on the labellum. The main difference between *Calonema* and *Phlebochila* is that *Phlebochila* has only two rows of labellum calli, or calli so crowded that individual rows cannot be identified.

Jones *et al*. (2001) proposed a new classification of *Caladenia* in the narrow concept that segregated *Caladenia* into several genera based on DNA sequence data obtained from ITS region using 70 species of *Caladeniinae*. The phylogenetic analysis of *Caladeniinae* separated *Leptoceras* and *Praecoxanthus* from other taxa and divided the rest of the *Caladeniinae* into three main groups. *Cyanicula deformis*, or *Caladenia deformis*, according to Hopper and Brown, was placed into the monotypic genus *Pheladenia* while *Glossodia* and *Elythranthera* were well separated from other entities. The remaining taxa of *Cyanicula* were related with *Caladenia saccharata* as a sister group. Then *Caladenia saccharata* was named as the monotypic genus *Glycorchis*.

The largest group containing species previously classified into *Caladenia* was separated into six new genera named *Petalochilus* (reinstated from *Caladenia* subgenus *Caladenia* and typified by *P. calciformis*), *Stegostyla* (a newly erected genus typified by *S. gracilis*), *Caladenia* ("*flava*" group typified by *C. flava*), *Calonema* (the "*filifera*" group of spider orchids along with the thick-lipped Western Australian species), *Drakonorchis* (elevated to generic rank, typified by *D. barbarossa*) and *Arachnorchis* (part of Section *Calonema* as typified by *C. patersonii* and commonly referred to as "spider orchids").

According to Jones *et al*. (2001), *Arachnorchis* is characterised by trichomes (hairs) of a multiseriate, eglandular nature with long white basal cell on the leaf and scape, elongated tepals with ends that are either tail-like (caudae) or have thickened clubs. Glandular structures called osmophores that are either globular or hemispherical are found on the tips of the tepals. Typically, a pair of yellow glands are found at the base of the column. *Calonema* similarly has trichomes on the leaf and scape. The flowers are usually large with long tapering or filamentous ends on the tepals. The shape of the osmophores is quite different, being cylindrical. The calli on the labellum are different in structure from *Arachnorchis*, being shiny and smooth.

Jones and Clements's (2001) genus *Arachnorchis* contains the same species as Hopper and Brown's (2000, 2001) subgenus *Calonema*, whereas Jones and Clements's genus *Jonesiopsi*s (originally published as *Calonema*, then *Calonemorchis*) includes the same species as Hopper and Brown's subgenus *Phlebochila.*

Jones and Clements (2002) then chose to accept *Calonemorchis* for the `*filifera*' group of spider orchids as proposed by Szatchetko in place of the previously incorrect *Calonema* because they claimed that neither *Phlebochilus* nor *Jonesiopsis* were validly published by Szlatchetko (2001a). Later, Jones and Clements (2003) accepted *Jonesiopsis* as validly published with *J. multiclavia* as the type after Kew Botanic Gardens accepted that the assignation of the name *Jonesyella* was an unintended mistake. Since Jones and Clements' molecular studies had placed *Caladenia multiclavia* to be in the same group as species such as *Caladenia filifera* and the thick-lipped Western Australian species they had to accept this as the first legally published

name for all the species in that group. However, they erected three subgenera; *Jonesiopsis* subgenus *Jonesiopsis* (containing *J. multiclavia* only), *Jonesiopsis* subgenus: *Phlebochilus* (containing the small, thick-lipped spider orchids with crowded flat calli) and *Jonesiopsis* subgenus *Calonema* (containing the filamentous or *`filifera'* type spider orchids). Afterwards, *Caladenia* subgenus *Elevatae* (*Caladenia* section *Caladeniastrum*) was elevated to generic rank and named *Caladeniastrum* by Szlachetko (2003).

Hopper and Brown (2004b) attempted to rectify the nomenclature confusion within Subtribe *Caladeniineae*. They accepted *Ericksonella* as a distinct genus but did not accept the name *Glycorchis* ascribed to it by Jones and Clements. As their circumscription (2000, 2001, 2003) they now propose that *Caladenia* consists of six subgenera; *Caladenia, Elevatae, Calonema, Phlebochilus, Drakonorchis and Stegostyla* (containing species currently placed by Jones and Clements in genus *Petalochilus*, *Caladenia*, *Arachnorchis*, *Jonesiopsis*, *Drakonorchis* and *Stegostyla*). They also provided recommended generic names to apply to their subgenera based on Szlachetko (2001a) and Jones *et al*. (2001) classification (Table 1.1). Hopper and Brown's large group classification referred to spider orchid that were originally placed into *Caladenia* subgenus *Calonema* Hopper and Brown (2004b). However, Jones et al. 2001 classified this same group into genus *Arachnorchis*. The comparison of spider orchid classification is shown in Table 1.2.

Table 1.1 Comparison of recently proposed *Caladenia* nomenclature

Caladenia nomenclature					
Hopper and Brown (subgenus)[1]	Type species	Jones and Clements (generic rank) [2]	Type species	Slatchetko (generic rank) [3]	Type species
C. subgenus *Caladenia*	*Caladenia carnea*	*Caladenia* and *Petalochilus*	*Caladenia flava*, *Petalochilus calyciformis*		
C. subgenus *Elevatae*	*Caladenia flava*	*Caladenia*	*Caladenia flava*	*C.* sect. *Caladeniastrum*,	*Caladenia flava*
C. subgenus *Calonema*	*Caladenia patersonii*	*Arachnorchis*	*Caladenia patersonii*	*Phlebochilus*	
C. subgenus *Phlebochilus*	*Caladenia cairnsiana*	*Calonema later Calonemorchis, then Jonesiopsis*	*Caladenia filifera*	*Calonema* later substitution by *Calonemorchis* , *Jonesiopsis*, *Phlebochilus*	not designated *Caladenia filifera* *Jonesiella multiclavia* not designated
C. subgenus *Drakonorchis*	*Caladenia barbarossa*	*Drakonorchis*	*Caladenia barbarossa*		
C. subgenus *Stegostyla*	*Caladenia gracilis*	*Stegostyla*	*Caladenia gracilis*		
		Petalochilus	*Petalochilus calciformis*		

[1] Hopper and Brown (2000), [2] Jones and Clements (2001, 2002, 2003) , [3] Szlatchetko (2001a,b)

Noted: It is understood that the preferred subgeneric nomenclature is that of Hopper and Brown (2004), although this is not mandatory. For simplicity, this interpretation will be used.

Caladenia is also closely related to *Cyanicula*, *Glossodia* and *Elythranthera*. *Caladenia* differs from the others in having new tubers produced on extended droppers, each tuber naked basally, with a few-layered tunica above, and the basal cell of hairs noticeably enlarged. In comparison with *Cyanicula*, *Caladenia* also differs in the absence of blue petals and sepals. *Elythranthera* and, to lesser extent *Glossodia* differ from *Caladenia* in having glossy flowers.

1.5 Spider orchids in Victoria

Victorian spider orchids are currently classified into genus *Caladenia* by Robert Brown, Section *Calonema* and include those species classified by Hopper and Brown as subgenus *Calonema*, or by Jones and Clements as genus *Arachnorchis* as well as two species from the *`filifera'* group that would belong to subgenus *Phlebochila* as described by Hopper and Brown (2001, 2004b) or genus *Jonesiopsis* (Jones and Clements, 2003). These latter two are *C. filamentosa* and *C. capillata (formerly C. filamentosa* var *filamentosa* and *C. filamentosa* var *tentaculata).* These differ from the other spider orchids in lacking a pair of yellow basal glands, having flat calli, and long filamentous tepals with differently shaped (long, thin thread-like tails) osmophores.

The remainder of non-Victorian 'spider orchids' can be divided into three main groups on morphological grounds (Jeanes and Backhouse 2000). However, in each group the actual number of taxa is not fully resolved, with the separation of morphologically similar taxa into separate species being questioned by some botanists, while others believe there are a number of, as yet, undescribed species.

The dilatata group

The dilatata group (Figure 1.4 and 1.7a) refers to spider orchids that have a labellum with a maroon apex and calli, and green marginal fringes or teeth. Members of the dilatata group considered to be found in Victoria are *C. amoena, C. toxochila, C. phaeoclavia, C. parva, C. verrucosa, C. dilatata, C. tentaculata, C. tensa* and *C. stricta*. There is still some debate about whether C. *phaeoclavia* and *C. parva* represent separate species as they apparently share the same pollinator. Recently, some authors have accepted *Caladenia dilatata* ssp *villosissima* (G. Carr pers com.) at species level and like other groups of spider orchids, the exact number of taxa is under discussion. The morphological differences of dilatata samples used in this study presents in Table 1.2.

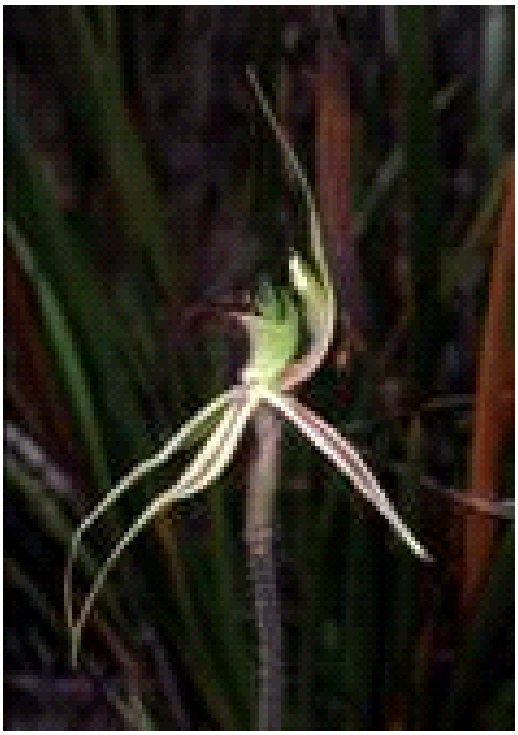


Figure 1.4 An example of floral form of dilatata group, photo by Wendy Probert.

Table 1.2 Characteristic comparisons within dilatata species (Backhouse and Jeanes 1995; Jones, 1991, 1999; Jeanes and Backhouse 2000)

FEATURE		*amoena*	*dilatata*	*parva*	*phaeoclavia*	*stricta*	*tensa*	*tentaculata*	*toxochila*	*verrucosa*	*villosissima*
	colour	green with red spot towards base	green with red dish basal spots	green with red spots towards base	green with red blotches towards base	green with red spots towards base	green with red spots towards base	green with red spots towards base	green with reddish basal spots	green with red blotches towards base	green with reddish spots
	shape	lanceolate	lanceolate	Lanceolate	narrowly lanceolate	lanceolate	lanceolate	lanceolate	lanceolate	narrow-lanceolate	lanceolate
	length (mm)	80	130	100	130	60	120	150	200	150	130
	width(mm)	10	15	10	10	10	15	20	10	10	15
	hairiness		hairy on both surfaces	hairy on upper and lower surfaces	hairy on upper and lower surfaces	slightly hairy on upper and lower surface	hairy on upper and lower surfaces	hairy on upon and lower surfaces	hairy on both surfaces	hairy on upper and lower surfaces	hairy on both surfaces
Flower stem	length(cm)	10	40	15	25	30	30	50	30	30	30
	colour	green to reddish	green or reddish	green	green or reddish	green	green or reddish	green or reddish	green to reddish	green or reddish	green or reddish
	shape	slender	slender	slender	slender	slender	slender	slender	slender	slender	slender
	hairiness	+[1]	+	+	+	+	+	+	+	+	+
Flower	number	1 (rarely 2)	1	1	1	1(rarely 2)	1 or 2	1 or 2	1	1	1-3
	size	20 mm across	to 50 across	to 30 across	to 40 across	to 40 mm across	50 mm across	100 mm across	40 mm. across	40 mm. across	to 40 mm. across
Perianth segment	colour segments	yellowish green	green	green	green	green	green	green	crystalline white	green	green
	stripe/streak	variable crimson	crimson	crimson	variable crimson	crimson	crimson	crimson	faint reddish	variable crimson	variable crimson
	length (mm)	25	50	30	40	30	40	80	25	40	40

		amoena	*dilatata*	*parva*	*phaeoclavia*	*stricta*	*tensa*	*tentaculata*	*toxochila*	*verrucosa*	*villosissima*
	shape	narrow, tapering abruptly	slender with filiform tips	long, slender with filiform tips	long, slender,	broadest just below middle, tapering abruptly to short filiform tips	broad at base tapering abrupty to slender, filiform tips	slender with filiform tips	abrupty tapering to long, slender filaments	generally shorter, slender, with filiform tips	long, slender,
	osmophore	yellow	yellowish brown	yellowish	brown		yellowish	yellowish	purplish brown	yellow	brown
Osmophores / clubs	location	sepals sometimes with indistinct osmophores	petals and sepals with osmophores forming distinct clubs	sepals with osmophores forming distinct clubs	sepals with osmophores forming short flat clubs	clubs and osmosphores absent	sepals with pale yellowish osmophores	sepals with osmophores forming long, narrow, indistinct clubs	forming short distinct clubs	sepals with osmophores as short, flat clubs	petals and sepals with osmophores forming distinct clubs
	length (mm)	3	20 sepals, 8 petals	7	10	30	40	15	3	10	
Segment habit	dorsal sepal	erect and incurved over column	erect to incurved over column	erect to incurved over column	erect to incurved	erect to incurved over column	erect to incurved over column	erect to incurved over column	erect, incurved	erect to incurved over column	erect to incurved over column
	lateral sepals	obliquely deflexed	obliquely deflexed, tips drooping, often crossed	strongly deflexed, often crossed	projecting stiffly forward, somewhat deflexed, parallel	stiffly spreading to obliquely deflexed	projecting stiffly forward, somewhat deflexed, often crossed	projecting forward, deflexed, often upcurved in distal half	widely spreading, deflexed, filaments drooping	projecting stiffy forward, somewhat deflexed, parallel or crossed	projecting stiffly forward, somewhat deflexed, parallel
	petals	spreading, obliquely deflexed, tips curved	dropping along side ovary	deflexed	spreading	stiffly spreading to obliquely deflexed	stiffly spreading	deflexed	widely spreading	stiffy spreading	stiffy spreading

		amoena	*dilatata*	*parva*	*phaeoclavia*	*stricta*	*tensa*	*tentaculata*	*toxochila*	*verrucosa*	*villosissima*
Labellum	colour	green	lamina white	lamina white	whitish lamina	Whitish lamina	whitish lamina	whitish lamina	pinkish	whitish lamina	white
	apex	maroon, recurved	maroon, recurved	maroon, recurved	marron, recurved	maroon, recurved	maroon, recurved	maroon, recurved	maroon, recurved	maroon, often with pale tips recurved	maroon, recurved
	margin tooth	short	short margins, blunt teeth	short, blunt, irregular teeth	distal margins with short blunt teeth	short, blunt teeth, distal margins irregularly toothed to crispate	basal margins with short, blunt teeth, apical margins crispate	short, blunt teeth	slender, curved teeth	short, blunt teeth	short, blunt teeth
	calli	reddish purple	maroon	maroon	maroon	maroon	maroon	maroon	pinkish	maroon	maroon
	shape	broadly V-shape	Large mobile tri-lobed	distinctly tri-lobed	tri-lobed	distinctly tri-lobed		large, mobile, distinctly tri-lobed		tri-lobed	tri-lobed
	lamina	+							not noticeably		
	mid-lobe	triangular, recurved at maroon apex, margin irregular	triangular, recurved at maroon apex	triangular, recurved at maroon apex	triangular, recurved at maroon apex	triangular	maroon apex	triangular, recurved at apex	triangular, recurved at apex	triangular, recurved at apex	triangular, recurved at apex
	club/calli (# row)	4	4	4	4	4	4	4	4	4	4

		amoena	*dilatata*	*parva*	*phaeoclavia*	*stricta*	*tensa*	*tentaculata*	*toxochila*	*verrucosa*	*villosissima*
	lateral lobe	obliquely erect with a few short teeth on distal margins	green erect, marginal teeth on distal half to 6 mm long	white lamina and greenish, obliquely erect, with a few short marginal teeth , to 3 mm long, on forward portion	green, erect	green to yellowish, erect	green, obliquely erect, divergent	green, erect, distal half with marginal teeth	green, erect, distal with marginal teeth	green, erect, distal with marginal teeth	green
	density	crowded	crowded	crowded	fairly crowded	crowed	moderately crowded	crowed	crowded	crowded	crowded
	shape	small-headed, laminar calli extending to mid-lobe	Stalked, clubbed laminar calli extending well on to mid-lobe	Stalked, clubbed laminar calli extending on the base of mid-lobe	clubbed laminar calli extending on to mid-lobe	stalked, clubbed laminar calli extending on the base of mid-lobe	stalked, clubbed laminar calli extending on to base of mid-lobe	stalked, clubbed laminar calli extending on the base of mid-lobe	stalked clubbed laminar calli extending nearly to apex	very congested, short, clubbed, warty-headed laminar calli extending on to mid-lobe	clubbed laminar calli extending on to mid-lobe
Column		bent sharly at about 90 degrees near middle									
Flowering period		Aug-Sept-Oct	Oct-Nov-Dec-Jan	Aug-Sept-Oct	Sept-Oct-Nov-Jan	Sept-Oct-Nov	Sept-Oct-Nov	Sept-Oct-Nov-Dec	Aug-Sept-Oct	Aug-Sept-Oct	Oct – Nov

[1] indicates the presenting of floral feature

The reticulata group

The reticulata group (Figure 1.5 and 1.7b) refers to those spider orchids that have sepals (and sometimes petals) tipped with distinct clubs and labellum margins fringed with short teeth. The reticulata group in Victoria consists of *C. aestiva, C. australis*, *C. brachyscapa* (believed extinct), *C. calcicola, C. clavigera, C. cruciformis, C. montana, C. sp. aff. fitzgeraldii*, *C. insularis*, *C. robinsonii*, *C. lowanensis, C. hastata*, *C. reticulata*, *C. pumila*, *C. valida*, and *C. xanthochila*. Plant descriptions of reticulate specimens used in this study showed in Table 1.3.

Figure 1.5 An example of floral form of the reticulata group, photo by Wendy Probert.

Table 1.3 Characteristic comparisons within reticulata species (Backhouse and an Jeanes 1995; Jones, 1991, 1999; Jeanes and Backhouse 2000)

FEATURE		*australis*	*calcicola*	*clavigera*	*cruciformis*	*hastata*	*insularis*	*lowanensis*	*reticulata*	*richardsiorum*	*robinsonii*
	colour	green	green with red spots near base	green with red spots at base	Dull green base blotched with red	green with a few red basal spots	green, reddish at base	green with red spots near base	green, base reddish	dull green, base irregularly blotched with red purple	green, base reddish
	shape	lanceolate	lanceolate	lanceolate	linear-lanceolate	lanceolate	lanceolate	lanceolate	lanceolate	linear-lanceolate	lanceolate
	length (mm)	80	120		50-110	120	85	120	120	160-220	100
	width(mm)	10	15		3.5-6	10	10	10	10	13-15	10
	hairyness	+, sparsely	+ densely on both side	+ with red spots at base	+,densely hirsute	+	+ sparely	+ densely on both surfaces	+, sparsely	+	+, sparsely
Flower stem	length(cm)	30	25	40	50-70	35	40	25	30	20-40	30
	colour	green or reddish	green or reddish	green to brown	Dark red to crimson	green or reddish	green or reddish	green	green or reddish		green or reddish
	shape	slender	slender	slender	wiry	slender	slender	tall, slender	tall, slender		tall, slender
	hairiness	+	+	+	+	+		+	+		+
Flower	#	1(occasionally 2)	1 or 2	1 or 2	1 really 2	1 to 3	1(occasionally 2)	1	1 to 3	1	1 rarely 2
	size	60 mm across	40 mm across	40 mm across	50-70 mm. Diam	80 mm across	60 mm across	40 mm across	50 mm across	40 mm across	40 mm across
	length (mm)	60	40	50		40	50	40	40		35

		australis	*calcicola*	*clavigera*	*cruciformis*	*hastata*	*insularis*	*lowanensis*	*reticulata*	*richardsiorum*	*robinsonii*
Perianth segment	colour segments	creamy yellow	glossy, greenish yellow		dark red to crimson or pinkish	creamy white with deep red to blackish	cream, pink or pale yellow	pale yellow to red	pale creamy yellow	yellowish-green	creamy yellow
	stripe/streak	red	faint to heavy red	faint to heavy red	dark red		red	faint to heavy red stripes on segments	basal red streaks and suffusions		red longitudinal streaks and suffusions
	shape	narrow	slender, tapering to filamentous points	slender, tapering to filamentous points	narrow	broad in basal third and abruptly tapering to narrow caudae		slender, tapering to filamentous points	broad at base, tapering to short filaments		broad at base, tapering to short filaments
	osmophore	dark red	yellow to red	black	Blackish red	black	red	deep red	dark red	prominent blackish	dark red
	location	sepaline	sepaline	ending in short, beady clubs	sepaline	tepaline	sepaline	sepaline	sepaline	sepaline	sepaline
	length (mm)	20	9			20	10	40	40	8-10	8
		forming distinct flattened club	forming distinct clubs			forming thickened club	forming distinct clubs petal sometimes shorter than clubs		forming distinct flattened club		distinct club
Segment habit	dorsal sepal	erect, incurved	erect	erect, incurved	erect, slightly recurred or recurved	erect or incurved	erect or incurved	erect to incurved	erect, incurved	linear to linear-oblong in proximal half then narrowed	erect, incurved

										to a linear	
		australis	*calcicola*	*clavigera*	*cruciformis*	*hastata*	*insularis*	*lowanensis*	*reticulata*	*richardsiorum*	*robinsonii*
	lateral sepals	spreading and decurved	stiffly spreading to deflexed	spreading, held stiffly or drooping	spreading stiffly in the shape of a cross	stiffly spreading	stiffly spreading	stiffly spreading or downcurved	spreading and slightly decurved	ovate-lanceolate in proximal half,slightly falcate divergent	spreading and slightly deflexed
	Petals	spreading and decurved	stiffly spreading to deflexed	spreading, held stiffly or drooping	spreading stiffly in the shape of a cross	stiffly spreading	stiffly spreading	stiffly spreading or downcurved	spreading and slightly decurved	lanceolate, tapered to a long-acuminate apex	spreading and slightly deflexed
Labellum	colour	lamina dark red, base yellowish with red streaks	glossy, shortly tooth in apical half							greenwish-cream, long acuminate apex	
	calli			purple		purple				dark reddish	
	shape	tri-lobed	obscurely tri-lobed	heart, indistinctly tri-lobed		not noticeably lobed	indistinctly in tri-lobed	tri-lobed	indistinctly tri-lobed	obscurely tri lobed	indistinctly tri-lobed
	lamina				ovate to ovate lanceolate in outline				yellow at base with red striations	broadly ovate-cordate in outline, curved throughout, erect in proximal half, distal half recurved	yellow at base with red striations

		australis	*calcicola*	*clavigera*	*cruciformis*	*hastata*	*insularis*	*lowanensis*	*reticulata*	*richardsiorum*	*robinsonii*
	mid-lobe	recurved, margins fringed with short, regular calli almost to tip	maroon, triangular, entire, recurved	reddish, recurved, marginal entire or shallowly toothed	broadly delate in outline when flattened, margins with straight or curved teeth	margins with fairly long, barbed teeth near base becoming small and irregular towards tip; apex greatly recurved	recurved, margins with short, regular fringes	triangular, recurved, basal margins with long calli, decreasing in size towards apex	recurved, margins with short calli nearly to apex	linear-ovate in outline when flat, margins with numerous, reddish, cream-tipped, obtuse teeth, decrescent towards apex	deep red, recurved, margins with short calli nearly to apex
	lateral lobe	erect, margins fringed calli to 2 mm long	greenish with maroon stripes, erect	green or yellowish, entire, horizontal or slightly rolled	margins splayed out		erect, marginal teeth short and regular	erect, distal half with long marginal calli	erect, fringed with short calli	Erect, proximal margins entire	obliquely erect, fringed with calli
	club/calli (#row)	4-6	4-6	4-6	4-6	4-6	6	4-6	4-6	6	4-6
	shape	widely spaced	crowed			slender, clubbed laminar	widely spaced		widely spaced		
Laminar calli		clubbed laminar extending to base of mid-lobe	flat-topped laminar calli, extending to based of mid-lobe	clubbed, extending to base of mid-lobe	Numerous, crowded, prominently stalked	clubbed laminar calli, extending to bend; margins	shiny clubbed extending to base of mid-lobe	glossy clubbed laminar extending to base of mid-lobe	clubbed laminar extending to base of mid-lobe, sometimes 2 rows extending nearly to apex	reddish with cream tips, stalked, crowded, decrescent towards apex	congested, clubbed, glossy extending to base of mid-lobe

		australis	*calcicola*	*clavigera*	*cruciformis*	*hastata*	*insularis*	*lowanensis*	*reticulata*	*richardsiorum*	*robinsonii*
Flowering period		Sept-Oct-Nov	Sept-Oct-Nov	Aug to Jan	Sept-Oct	Oct-Nov	Sept-Oct	Sept-Oct	Sept-Oct	late Sept-early Nov	Sept-Oct

[1] indicates the presenting of floral feature

The patersonii group

Those species in which the sepals end in long filamentous tips usually covered with glandular hairs, and labellum margins fringed with short to long teeth belong to the patersonii group (Fig 1.6 and 1.7c). The Victorian patersonii group includes: *C. audasii*, *C. flavovirens*, *C. fulva*, *C.*sp. aff. *colorata*, *C. concolor* Fitzg, *C.* sp. aff. *concolor*1, *C.* sp. aff. *concolor*2, *C. formosa*, *C. fragrantissima* subsp. *fragrantissima*, *C. fragrantissima* subsp. *orientalis*, *C. oenochila*, *C. pilotensis*, *C.* sp. aff. *oenochila*, *C. patersonii*, *C.* sp. aff. *patersonii*, C. *magnifica*, *C. venusta*, *C.* sp. aff. *venusta*1, *C.* sp. aff. *venusta*2, *C. rosella*, and *C. versicolor*, *C. thysanochila*. The characteristic comparison of patersonii samples used in this study indicated in Table 1.5.

Figure 1.6 An example of floral form of patersonii group, photo by Wendy Probert.

Table 1.4 Characteristic comparisons within patersonii species (Backhouse and Jeanes 1995, Jones, 1991, 1999, Jeanes and Backhouse 2000)

FEATURE		*Cardiochila*	*concolor*	*formosa*	*fragrantissima*	*oenochila*	*orientalis*	*patersonii*	*pilotensis*	*rosella*	*tessellata*	*venusta*
	colour	green with red spots	green with a few reddish basal spots	green with a few reddish basal spots	green with red basal spots	green with reddish basal spots	Green with reddish basal spots	green with reddish basal spots	dull green, base red to purple-blotched	green with reddish spots and blotches near base	green with basal red spots	green with reddish basal spots
	shape	lanceolate	lanceolate	lanceolate	lanceolate	lanceolate		lanceolate	lanceolate	lanceolate	lanceolate	lanceolate
	length (mm)	100	150	150	150	120	150	150	70-130	80	100	150
	Width (mm)	5	10	12	10	12	15	15	10-150	8	5	12
	hairiness	+ sparsely on both surfaces	+ sparsely	+ sparsely	+ on both surfaces	+ sparsely on both surfaces	+ hairy on both surfaces	+ sparsely on both surfaces	+	+	+ sparsely on both surfaces	+ sparsely on both surfaces
Flower stem	Length (cm)	30	30	50	40	30	200	30	250-320	17	30	60
	colour	green to reddish	dark green to greenish purple	dark green to greenish purple	green-brown	green-brown	green-brown	green-brown		green to reddish purple	green to reddish	green or reddish
	shape	slender and wiry	slender	slender	relatively stout	slender	relatively stout	slender	wiry	slender, erect	slender and wiry	slender
	hairiness		+	+	+	+	+	+	+	+		+
Flower	#	1 or 2	1 or 2	1 rarely2	1 to 3	1 or 2	1 or 2	1 or 2	1 or 2	1	1 or 2	1 or 2
	size	30 mm across some large forms to 50 mm across	80 mm across	60 mm across	80 mm across	80 mm across	100 mm across	80 mm across	70-100 mm diam	60 mm across	30 mm across	12 cm across
	perfume				strong					musk-scented		

		Cardiochila	*concolor*	*formosa*	*fragrantissima*	*oenochila*	*orientalis*	*patersonii*	*pilotensis*	*rosella*	*tessellata*	*venusta*
Perianth segment	colour segments	yellowish green and spotting	uniform deep purplish red	uniform pinkish red to blood-red	creamy white to pale yellowish green, often with reddish markings, rarely wholly red	pale greenish yellow inside, external covered with red glandular hairs		creamy white to pale yellowish	creamy white	pale to bright pink	yellowish green	white to pale yellow
	stripe/streak	faint to heavy red stripes						red at base	darker red		heavy maroon and suffusions on segments	
	colour of glandular hair tipped segment				dark reddish to black	red	dark reddish brown to black	reddish brown		dark red		brown
	length (mm)	25	50	80	80	60	120	80		50	25	
	shape	long, broadly lanceolate, tapering abruptly to fine points	long hairy, broad near base but tapering to filamentous, glandular tips	long, broad near base, abruptly tapering to long, filamentous, glandular tips	broad near base, abruptly tapering to long, slender filaments for most of their length	broad near base and abruptly tapering to slender filaments tips, all segments held rather stiffly	Slender filaments for most of their length	broad near base but tapering to slender filamentous tips, held rather stiffly		narrow, tapering to filamentous point	long, slender to broadly obovate, tapering to short fine points	broad near base but abruptly tapering to long, slender filaments for most of their length

		Cardiochila	*concolor*	*formosa*	*fragrantissima*	*oenochila*	*orientalis*	*patersonii*	*pilotensis*	*rosella*	*tessellata*	*venusta*
Segment habit	dorsal sepal	erect or incurved	erect and incurved	erect and incurved	erect and incurved	erect or incurved	erect, filament curving forward or backwards	erect and incurved	erect	stiffly erect or slightly incurved	erect and incurved	erect in basal half, filament often drooping forward or backwards
	lateral sepals	spreading and downcurved	widely spreading, obliquely deflexed to drooping	widely spreading, obliquely deflexed to drooping	widely spreading, obliquely deflexed, filaments drooping	widely spreading, deflexed	widely spreading, deflexed filaments drooping	widely spreading, obliquely deflexed, filaments drooping	divergent, obliquely deflexed to drooping	spreading, obliquely deflexed	deflexed, forward-pointing, parallel or crossed	widely spreading, deflexed, filaments drooping
	petals	spreading and downcurved	widely spreading, obliquely deflexed to drooping	widely spreading, obliquely deflexed to drooping	widely spreading, obliquely deflexed, filaments drooping	widely spreading, deflexed	widely spreading, deflexed filaments drooping	widely spreading, obliquely deflexed, filaments drooping	obliquely deflexed to drooping	spreading, obliquely deflexed	often strongly deflected against ovary	widely spreading, deflexed, filaments drooping
	sepaline	occasion short clubs (to 3 mm)										
Labellum	colour			paler pinkish red to blood-red		variably reddish to yellowish	reddish				greenish with maroon stripes	
	apex				recurved to coiled		recourved to coiled	recurved to coiled		strongly recurved		recurved to coiled
	calli				reddish	dark red		reddish, often pale-tipped	reddish	reddish, pale tipped	glossy purple	

		Cardiochila	*concolor*	*formosa*	*fragrantissima*	*oenochila*	*orientalis*	*patersonii*	*pilotensis*	*rosella*	*tessellata*	*venusta*
	margin tooth, colour	-	irregular teeth near recurved apex		reddish moderately long basal teeth becoming very short towards tip	dark red	very long basal teeth becoming very short towards tip	reddish, long, slender linear dimishing in size towards tip	very short, peg-like, purplish teeth		maroon	linear
	calli				reddish	dark red		reddish, often pale-tipped	reddish	reddish, pale tipped	glossy purple	
	shape	obscurely tri-lobed, broadly heart-shaped	obscurely tri-lobed	very obscurely tri-lobed	very obscurely tri-lobed	not noticeably tri-lobed		not noticeably tri-lobed		narrow, not noticeably tri-lobed	obscurely tri-lobed, broadly heart-shaped	obscurely tri-lobed
	mid-lobe	slightly deflexed, margin thickened, gladular, entire		margins almost entirely covered by short calli, decreasing in size towards tip of strongly recurved mid-lobe					ovate-deltate in outline when flattened, obtuse		maroon, slightly deflexed, margins thickened, glandular, entire	triangular with short, regular marginal teeth extending to tip

		Cardiochila	*concolor*	*formosa*	*fragrantissima*	*oenochila*	*orientalis*	*patersonii*	*pilotensis*	*rosella*	*tessellata*	*venusta*
	lateral lobe	erect, horizontal or slightly rolled under							erect		obliquely erect, entire or with a few short calli on distal margins	obliquely erect with numerous fairly long
	club/calli (# row)	2-4	4-6	4-6	4-6	4-6 (stalked)	4-6	4-6 (stalked)	4-6	4-6	4-6, often breaking up with 2 rows extending well on to mid-lobe	4-6 (stalked)
Laminar calli		narrow, dense central cluster of thick, short clubbed lamina calli extending to base of mid-lobe	short clubbed, extending on to base of mid-lobe	short clubbed, central rows extending well on to mid-lobe	short clubbed, extending to bend	extending to bend	extending to bend	clubbed, extending to bend	extending nearly to the labellum apex	elongate, finger liked, reduced ti irregular serrations at apex	broad central cluster of short, thick, clubbed, densely packed at base	reddish, pale tipped, clubbed laminar calli, middle rows extending on to mid-lobe
marginal calli			longest near base, decreasing to small			margins with slender linear teeth extending nearly to apex			linear, dark purplish, widely spreading, incurved			

		Cardiochila	*concolor*	*formosa*	*fragrantissima*	*oenochila*	*orientalis*	*patersonii*	*pilotensis*	*rosella*	*tessellata*	*venusta*
column									translucent green with red markings			
Flowering period		Aug-Sept-Oct-Nov	Sept-Oct	Sept-Oct	Sept-Oct-Nov	Aug-Sept-Oct	Aug-Sept-Oct-Nov	Sept-Oct	Sept-Oct	Jul-Aug-Sept-Oct	Sept-Oct-Nov	Sept-Oct-Nov

[1] indicates the presenting of floral feature

Figure 1.7 Floral forms of (a.) dilatata (b.) reticulata and (c.) patersonii.

There are species that do not fit obviously into the three main groups, for example, *Caladenia tessellata* and *Caladenia cardiochila*. These two are very similar, having relatively short tepals, and crowded calli (Figure 1.8).

Caladenia tessellata

Caladenia cardiochila

Figure 1.8 Floral forms of *Caladenia tessellata* and *Caladenia cardiochila*

1.6 Spider orchid classification status

It has been suggested that the Spider orchids be re-classified into a separate genus from *Caladenia* called *Arachnochis* (Jones *et al*., 2001). However, this classification is yet to receive widespread support since the defining characters and type species are contested. Furthermore, some species of spider orchids are extremely similar and difficult to distinguish from each other based on morphology alone. New approaches are needed to be explored in order to discriminate accurately between various species and/or to verify the taxonomic status of closely related species. Some of the most

threatened orchids in Victoria belong to *Caladenia*, with several being in section *Calonema*. It is thus very important that the taxonomy of these orchids be well understood so that conservation efforts are appropriately focused.

In order to precisely classify closely related taxa to species level, molecular data have been found to be powerful resources, sometimes better than traditional techniques such as morphological and physiological characters (Carvalho and Hauser, 1999). The evolutionary information of molecular data, either DNA or protein sequences, is often much more precise than that of morphological and physiological characters. Taxonomy based on molecular data should give a more accurate representation of relationships for closely related species classification. Thus, molecular data may provide a solution to taxonomic problems, as well as information about the possible evolutionary relationships of species within the genus. While it is generally assumed that molecular markers could well resolve spider orchid taxonomy better than morphological markers it is yet to be resolved which method or combination of methods would be most appropriate.

Chapter 2

Background

2.1 Plant taxonomy

Taxonomy is the science and practice of identifying and grouping individuals of organisms into units (taxa, typically species). This system arranges species into larger groups, such as genus, family, order, class and phylum, in order to indicate evolutionary relationships. This process produces a hierarchical classification based on the evolutionary distance of the various taxa. Although any classification system has imperfections, the ultimate aim is a procedure to organize living organism information into a system that is useful for humans (Judd *et al*., 2002; Tautz *et al*., 2003).

The species level of classification is traditionally used as the fundamental unit of biology when comparing genes, cells, and relatedness of organisms. However, the species concept can be crude and subjective when based solely on morphological and ecological relationships. There is a range of opinion and various, sometimes conflicting theories as to what constitutes a species. Some authors believe that the species concept is much more fluid and that 'species' exist at the higher level of organization and fluidity and are much more broadly defined and longer-lived than the human observing them (de Queiroz, 2007).

Charles Darwin used identifiable gaps in morphological characters to delimit species, a concept for which he coined the term the morphological species concept (Mallet, 2007). Other authors delimitate species using a system called the biological species concept. In the biological species concept, "Species are groups of actually interbreeding natural populations that are reproductively isolated from other such groups" (Mayr, 1970). When taxonomic criteria take a more minor rule, the conceptual issues of what is a species can be divided into a range of theories, collectively known as alternative species concept, (e.g. Ecological species, Evolutionary species, Phylogenetic species) (Mallet, 2007; de Queiroz, 2007). With the rise of ecology, the species concept, referred to as the ecological species, has been developed. The ecological species refers to the ecological niche occupied by the 'species' rather than a species concept based solely on interbreeding or morphological characters (Mallet, 2007). The evolutionary species concept is based on groups of unique organisms that share an evolutionary rate, tendencies and historical fate and can be traced back to a shared ancestor (de Queiroz, 2007).

A further refinement of the evolutionary species is the phylogenetic species concept which itself is divided into four categories depending on the properties of the taxa. The Hennigian species concept states that "species form when a single interbreeding population split into two lineages that do not exchange genetic material" (Mallet, 2007). De Queiroz (2007) stated that main property of his monophyletic species concept is an ancestor and all of its descendants can be commonly inferred from possession of shared, derived character states. The de Queiroz theory further states that groups of alleles of a given gene are descended from a common ancestral allele not shared with those of other species known as genealogical species concept (de

Queiroz, 2007). By using the diagnostic criterion formulated by de Queiroz, namely the form of fixed genetic differences at one or more inherited alleles, a more fundamental and statistically provable species concept can be formulated. This last segregate of the phylogenetic species concept, the Queirozian species concept, is defined as the proper basal cluster of organisms (species) that is able to be diagnosed and therefore distinct from other such clusters, and within which there is a parental pattern of ancestry and descent (Mallet, 2007).

There are two approaches to produce a Queirozian species classification: phylogenic and evolutionary history determination, and a classification on this history base, although both can be based on genetic relatedness (Judd *et al*., 2002). A phylogeny-based classification, whether based on genetics or morphology, attempts to arrange organisms into groups on the basis of their evolutionary history. Evidence from morphological, biochemical processes and gene sequence data suggests that all organisms on Earth are genetically related. Thus a phylogenetic tree can elucidate the similarities and differences among organisms and can be constructed by morphological, biochemical and/or genetic data (Buth and Murph, 1999; Judd *et al*., 2002; Kumar *et al*., 2009).

DNA makes up the genotype of the species, containing all the genetic information from which the species can be delimited and distinguished from other related but distinct species. Genetic information is encoded by sequences of nucleotides, forming a code that is nearly universal in all organisms and transmitted with some variation to coordinate development of the phenotype. The phenotype is the product of interactions between the genotype and the environment. Major changes in genetic

material are often reflected in phenotypes (Page and Holmes, 1998; Judd *et al*., 2002; Kumar *et al*., 2009). Alternatively, distinct genotypes may be indistinguishable using strictly morphological or phenotypic variation or lack thereof.

2.2 Plant genomes

Plants contain three different genomes: chloroplast, mitochondrial and nuclear. Each of these genomes differ in size. Chloroplast and mitochondria are primarily maternally inherited and have a circular, non-recombinant genome which makes them very useful tools for evolutionary studies (Palmer, 1987). Although the mitochondrial genome has been the most important source of molecular study in animals, it has not been as highly utilised in plants. Since there is a very low synonymous base substitution rate in plant mitochondrial DNA and a high rate of intramolecular recombination. These two problems create an extremely complex situation when large proportions of the genome are being examined (Palmer, 1992). In addition, gene order in the mitochondrion is variable and genes can be separated by large regions of non-coding DNA (Palmer, 1992).

In contrast, the chloroplast genome is conserved both within cells and within plant species. An important feature of the chloroplast genome is the presence of two long inverted repeat regions which encode the same genes separated by a single copy region and a large copy region. A potentially useful chloroplast character is that rearrangements are relatively rare so when these do occur they can be used to delimitate major plant groups (Page and Holmes, 1998; Petit *et al*., 1998; Judd *et al*., 2002). Because of the conservative nature of chloroplast DNA, it has been widely employed to assess plant phylogenetic relationships. For example, the genetic relationships among 225 moss species based on the cpDNA *rps*4 gene of cpDNA has

generated several novel systematic hypotheses due to major dissimilarities between taxa (Goffinet *et al*., 2001). The phylogenetic relationships among *Diurideae* (Orhidaceae) based on DNA sequence data from matK and *trn*L-F indicated that *Diurideae* are not monophyletic as currently delimited (Kores *et al*., 2001). The molecular phylogeny of *Genista* (Leguminosae) and related genera obtained from *trn*T-F of cpDNA also helped to clarify the position of taxa whose relationships were not well established (Pardo *et al*., 2004).

Nuclear genomes are biparentally inherited and have a large numbers of genes and copies. The order of genes in the nuclear genome is known to be stable, at least within species, and it may also be stable across groups of species (Judd *et al*., 2002; Xu, 2005). This characteristic is a valuable tool for plant classification and related areas in several disciplines such as plant identification (Benedetti *et al*., 2000; Lee at al., 2004), plant taxonomy (Sensi *et al*., 2003 and Roalson *et al*., 2004; Bekele *et al*., 2007; Oumar *et al*., 2008), plant genetic diversity (Missaoui *et al*., 2006) and plant evolution (Fehrer *et al*., 2007). Other DNA-based markers are currently being used and developed for further genome analysis studies include ribosomal DNA, low copy number genes and high copy number non-coding nuclear sequences (Judd *et al*., 2002).

2.3 Types of markers

Genetic markers have been used in many areas of biological study. Genetic markers usually do not directly affect biological processes; they are merely linked to the genes controlling a trait or biological process. Some genetic markers associated with major genes which are responsible for important characteristics and genes under selection. There are three types of genetic markers: visible markers or morphological markers,

which are phenotypic traits or characters; biochemical markers, which include allelic variants of proteins; and molecular markers, which reveal neutral sites of variation at the DNA sequence level (Kumar, 1999; Semagn *et al*., 2006). All genetic markers ultimately represent a difference in DNA sequences between individuals.

2.3.1 Morphological and anatomical and physiological characters

Morphological markers are characterized on morphological, physiological and pigmentation characters. Morphological characters have been used as a source of taxonomic evidence since the beginning of molecular-based plant taxonomy. Anatomical characters such as stem and leaf structure as well as embryo composition, spores, gametophytes, and gametangia, pollen structures and karyotype can be observed by light microscopy, transmission electron microscopy and/or scanning electron microscopy, depending on the detail of the object being studied. Secondary plant compounds known as biochemical characters also have been used for plant taxonomy. Examples of these are alkaloids, glucosinolates and flavonoids as well as indirect characters such as odors, tastes, and medicinal characters (Mallet, 1996; Kumar *et al*., 1999; Judd *et al*., 2002).

2.3.2 Molecular characters

The main protein character used as a marker is the isozyme. Isozymes refer to different structures of proteins with the same catalytic function (Judd *et al*., 2002). According to the enzyme classification commission, isozymes are accepted as species-specific variation of an enzyme system and can be visualized by electrophoresis and specific protein staining methods. However, isozyme variation may be a tissue type or developmental stage specific (Bank *et al*., 2001). Primary structures of protein or amino acid sequences have been used as taxonomic characters

instead of isozymes to construct phylogenetic trees. Isozyme analysis, however, is still a very useful as preliminary molecular tool to study genetic association among groups. For example, electrophoretic profiles obtained from six polymorphic isozymes provided 108 reliable molecular markers among four taxa in the genus *Chaenomeles*. Cluster analysis and multidimension scaling analysis of these taxa agreed with associations observed in a previous RAPD study (Garkava *et al*., 2000). Isozymes were also used to observe genetic relationships among American taxa of *Vigna savi* (Fabaceae) and resulted in several changes in Sections compared with morphology-based taxonomy (Jaaska, 2001). The isozyme banding patterns of *Petrocoptis A. braun* (*Caryophyllaceae*) seedlings did not closely agree with the taxonomic framework obtained from morphology characters, however, this data provided moderate support to the splitting of the genus into two groups based on morphology data and geographic distribution (Mayol *et al*., 2001). Peroxidase, superoxide, dismutase, glutamate dehydrogenase and malate dehydrogenase were found to be good molecular markers for characterization of *Boesenbergia* and related taxa with the dendrogram obtained showing a higher degree of relationships between *Boesenbergia* and *Scaphochlamys* than between *Boesenbergia* and *Kaempferia* (Vanijajiva *et al*., 2005).

DNA-based characters derived from DNA fingerprinting and DNA sequences have been found to be suitable molecular markers for investigating genetic variation. Molecular characters offer numerous advantages over morphological characters. They are stable and detectable in all tissues without being affected by the environment. Because of their inherited nature, they can often provide a clearer picture of relationships between organisms than those based on morphological and, to a lesser

extent, physiological characters. Molecular markers are much more abundant than morphological characters (Judd *et al*., 2002; Semagn *et al*., 2006; Kumar *et al*., 2009). Although, molecular technique has been developed to more precisely, quickly and cheaply assess genetic variation, there is no single molecular technique to solve the many questions in genome research. However, there are no molecular markers available to satisfy all requirements needed by researchers (Semagn *et al*., 2005; Kumar *et al*., 2009).

2.3.2.1 Type of DNA markers

A molecular marker or DNA marker is a small fragment of DNA sequence which can be classified into two groups: hybridization-based DNA markers and polymerase chain reaction (PCR)-based DNA markers. Restriction fragment length polymorphism (RFLP) is based on hybridization of a labeled probe to fragments of genomic DNA following digestion with restriction enzymes. The genomic DNA is digested with a restriction enzyme that cleaves the DNA at specific sites. Sequence mutations at the recognition site prevent digestion and produce differences in the length of the restriction fragments. The resultant fragments are separated by electrophoresis on an agarose or polyacrylamide gel and transferred by blotting onto a nylon membrane to allow hybridization with a probe. DNA polymorphisms can be detected by hybridization with a labeled probe such as a complementary DNA probe or ribosomal RNA probe (Tanksley *et al*., 1989).

PCR-based molecular markers are generated by amplification using short specific or arbitrary oligonucleotide primers (Williams *et al*., 1990). A mixture of oligonucleotide primer, Taq DNA polymerase, free deoxynucleotides and genomic

DNA are subjected to successive cycles of heating and cooling. The primers bind to complementary sites on the target DNA and Taq DNA polymerase amplifies the DNA sequence between the primers. DNA polymorphisms can be detected by differences in primer binding sites between individuals or the distance between them, which generate subsequent differences in banding patterns on agarose or polyacrylamide gel due to size differences in the amplified products. Random amplified polymorphic DNA (RAPD) markers are obtained from randomly generated primers, nine to ten nucleotides in length, with no prior knowledge of the target DNA sequence being necessary. Using RAPDs, several DNA fragments may be amplified by a single DNA primer in each of the DNA samples. Although most of these PCR products are generally common to all individuals assessed, some PCR fragments are amplified from one individual only (Williams *et al*., 1990). The presence or absence of PCR fragments is assumed to represent mutations in the primer-binding sites of genomic DNA (Wolfe and Liston, 1999).

One problem with the use of RAPD markers is reproducibility (Penner, 1996). RAPD markers may be affected by template concentration, primer annealing temperature, and magnesium chloride concentration (Penner, 1996). DNA amplification fingerprinting (DAF) is a molecular marker method that has been modified from the RAPD technique. DAF is performed with single short nucleotide (5-6 bases) primers of arbitrary sequence and amplification products are separated on polyacrylamide gel and visualized with silver staining. Due to the shorter target sequence and the high resolution electrophoresis, the DAF technique can potentially generate more scoreable and reproducible bands than the RAPD technique (Caetano-Anolles *et al*., 1991).

Another PCR-based molecular marker system is based on microsatellite markers. Microsatellites are also known as “simple sequence repeats (SSR)” (Tautz *et al*., 1986) “short tandem repeats (STRs)” (Winter and Kahl, 1995) or sequence tagged microsatellite site (STMS) (Huttel *et al*., 1999). They consist of head to tail tandem arrays of short DNA motifs that occur ubiquitously in eukaryotic genomes. Typically, they may be dinucleotides $(AC)_n$, $(CA)_n$, $(AT)_n$; trinucleotides $(TCT)_n$, $(TAA)_n$; or tetranucleotide $(TATG)_n$, where n is the number of repeating units within the microsatellite locus (Weising *et al*., 1998). PCR using primers designed for the sequences flanking these repeats can be used to generate polymorphisms due to frequent variation in the length of the repeat regions. Inter-simple sequence repeat (ISSR) marker is a subset of SSR, designed by anchoring of primers to a part of the SSR. The amplified products are the regions between closely spaced oppositely oriented SSRs (Wolfe *et al*., 1998). ISSR markers used to study genetic relationships of mulberry varieties grouped them according to their low, medium or high yielding attributes (Vijayan & Chatterjee, 2003). Amplified fragment length polymorphism (AFLP) analysis is a molecular marker method which combines the use of restriction enzymes with PCR amplification. The technique consists of three steps: (1) the digestion of genomic DNA with restriction enzymes and the ligation of DNA fragments with double stranded adapters; (2) selective amplification of sets of the restriction fragments with primers complementary to the adapters; and (3) DNA fragment analyses on a polyacrylamide gel (Vos *et al*., 1995).

Numerous phylogenetic studies have been employed by DNA profiles and DNA sequences. The study of genetic relationships in the genus *Houttuynia* Thunb., (Saururaceae) based on RAPD and ISSR markers classified 70 accessions into

groupings that correlated with chromosome numbers and geographic distribution (Wu *et al*., 2005). In some cases, DNA-based phylogeny has some advantages over morphological based studies. For example, a phylogenetic study of *Citrus* based on RAPDs, sequence-characterized amplified regions (SCARs), and cpDNA gave better resolution of phylogenetic relationships than morphological data. Furthermore, it provided means to develop specific markers to identify some *Citru*s groupings (Nicolosi *et al*., 2000). The use of random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR markers), ITS sequences and morphological characters (leaf volatile terpenoids) on 12 genotypes selected from *Juniperus* populations, varieties and species indicated that ISSR markers could detect individual differences, the ITS sequence data was useful for interspecific and intergeneric differences and the RAPD data for intermediate taxonomic levels. An analysis of eggplant germplasm (*Solanum melongena*) using morphological and AFLP data contributed to phylogenetic interpretations which showed that molecular data supported morphological conclusions regarding the similarities and differences among accessions (Furini and Wunder, 2004). Singh *et al*. (2004) found that phylogenetic relationships in *Ocimum* species based on RAPD markers separated five *Ocimum* species into two major clusters which agreed with a previous morphological study. This indicated that RAPD was a sensitive, precise and efficient tool for determining *Ocimum* species through genomic analysis. Kim *et al*. (2005) determined the phylogenetic relationship among *Pyrus pyrifolia* and *P. communis* cultivars using RAPD markers and found that 19 *P. pyrifolia* cultivars were divided into five groups, based on their parent and the close hybrid. RAPD markers also separated 19 *P. pyrifolia* cultivars from 5 *P. communis* cultivars. The advantages and disadvantages of each type of genetic marker are indicated in Table 2.2.

Table 2.1 Advantages and disadvantages of different molecular marker types

Type of molecular marker	Advantages	Disadvantages	References
Isozyme	• Quick and simple • Robust and reliable • Cost effective	• Large amounts of tissue required • Limited number of polymorphic bands • co-migration	Kumar *et al*., 2009
AFLP	• Multi-locus • High levels of polymorphism generated	• Dominant marker • Time-consuming • Laborious and expensive	Vos *et al*.,1995
ISSR	• Quick, simple and cheap • Multiple loci from a single primer • Small amounts of DNA required	• Dominant marker	Kojima *et al.*, 1998; Joshi *et al*., 2000
RAPD	• Quick, simple and cheap • Multiple loci from a single primer • Small amounts of DNA required	• Dominant marker • Problems with reproducibility	Penner, 1996; Welsh & McClelland, 1990; Williams et. al., 1990
RFLP	• Codominant marker • Robust and reliable	• Large amounts of DNA required • Limited polymorphism • Time-consuming	Beckmann & Soller,1986; Kochert, 1994; Tanksley *et al*., 1989
STMS, SSRs,STRs	• Codominant marker • Reproducible • Robust and reliable	• Time-consuming, • Laborious and expensive • Species-specific/genotype-specific • Expensive and may be subjected to intellectual property rights	McCouch *et al*., 1997; Powell *et al*., 1996

2.4 Uses of molecular markers in plant research

Molecular markers offer potential to advance plant research in several areas such as:

(1) determining the identity of plant genotypes (Lu and Myers , 2002; Tikunov *et al*., 2003; Terzopoulos *et al*., 2005; Anisimova *et al*., 2009: Minami *et al*., 2009);

(2) analysis of genetic variation (Herńandez-Verdugo *et al*., 2001; Arnau *et al*., 2002; Missaoui *et al*., 2006);

(3) plant genome analysis (Goertzen *et al*., 2003; Noir *et al*., 2004; Suda *et al*., 2005; Inoue-Nagata *et al*., 2007);

(4) plant improvement through breeding and genetic engineering (Moumeni *et al*., 2003; Vijayan and Chatterjee, 2003; Cammareri *et al*., 2004; Furini and Wunder, 2004; Gygax *et al*., 2004; Yue *et al*., 2006);

(5) plant ecological research (Hess *et al*., 2000; Fischer *et al*., 2004; Maunder *et al*., 2008; Ohtani *et al*., 2008; Chai *et al*., 2010)

(6) evolutionary biology (Martos *et al*., 2005; Vanin *et al*., 2006; Liu & Xue, 2007; Hao *et al*., 2009);

(7) management of plant genetic resources (Pradeepkumar *et al*., 2003; Furini and Wunder, 2004; Hayati *et al*., 2004; Dong-mei *et al*., 2007; Vashney *et al*., 2007) and

(8) evaluation of biodiversity and genotype distribution (Kapteyn *et al*., 2002; Galván *et al*., 2003; Toquica *et al*., 2003; Gemas *et al*., 2004: Souframanien and Gopalakrishana, 2004; Gontcharova & Gontcharov, 2009).

2.5 Constructing a phylogenetic tree with molecular markers

2.5.1 Phylogenetic trees

A phylogenetic tree is a graph composed of branches and nodes used to represent the historical or evolutionary relationships of groups of organisms, often at the species level. It consists of nodes, which represent taxonomic units (species, populations and individuals, both extant and their presumed ancestors) and branches that define the relationship between taxonomic units in terms of descent and ancestry. The nodes at the tips of the tree are constructed from observable features, such as protein sequences or morphological features and are called operational taxonomic units (OTUs) (Page and Holmes, 1998; Prevost and Wilkinson, 1999; Brocchiert, 2001)).

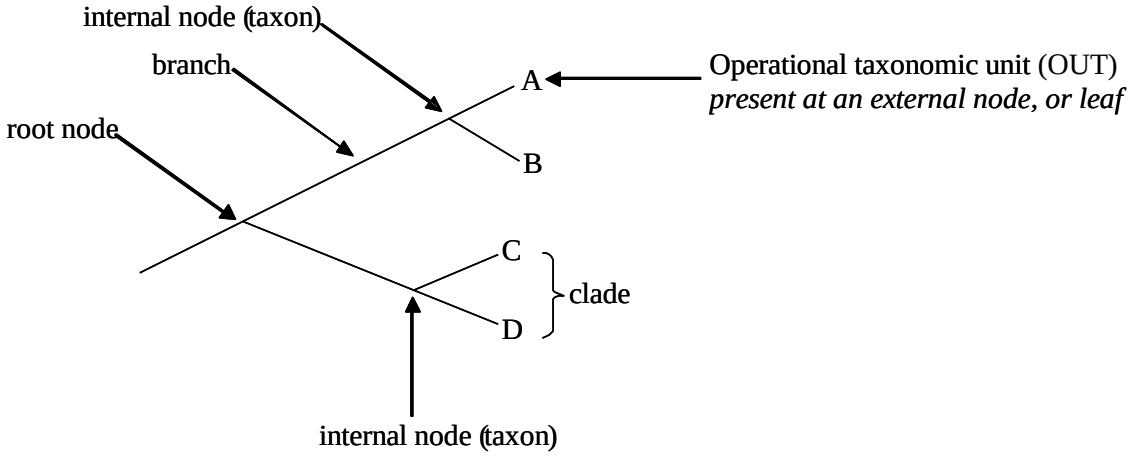


Figure 2.1 Diagram describing phylogenetic tree composition (Prevost and Wilkinson, 1999).

A node can be external, internal, or at the root of a tree. Internal nodes represent a common ancestor of two other operation taxonomic units that can represent many type of comparative taxa. An internal node is bifurcating if it has only two immediate

descendant lineages (branches). Bifurcating trees are also called binary or dichotomous. Any branch that divides, splits into two daughter branches. The splitting of lineages usually assumes that it is a binary process that results in the information of two species from a single ancestral species. This occurrence may not always happen, or a lack of suitable data may make it impossible to resolve the order in which species descended from a single common ancestor. In this case a tree that has a node with more than two immediate descendants will result, and is called multifurcating or a polytomy. Multifurcating trees are, by definition, non-binary. The branching pattern of a tree, defined by the relationships among the taxa in terms of ancestry, is called topology (Morrison, 1996; Prevost and Wilkinson, 1999; Brinkman and Leipe, 2001.).

Phylogenetic trees may be unscaled or scaled. In an unscaled tree or cladogram only the branching order of nodes is shown and the branch lengths are not proportional to the information represented at an external node (Morrison, 1996). A cladogram has the advantage of aligning the OTUs neatly in the vertical column. This may be especially useful if the tree has numerous of OTUs. Also this format allows the nodes to be placed along a time scale describing when the divergence event is estimated to have occurred (Page and Holmes, 1998; Brinkman and Leipe, 2001). Scaled trees called phylograms display both branching order and distance information. Distance is the number of amino acid or nucleic acid substitutions that have taken place along a branch. A phylogram has the helpful feature of conveying a clear visual idea of the relatedness of different characters within the tree (Morrison, 1996; Page and Holmes, 1998). A clade is a group of all the taxa that have been derived from a common ancestor and all of the descendants of a recent common ancestor. A clade is also called a monophyletic group. A tree is said to be additive if the distance between any

two OTUs is equal to the sum of the lengths of all the branches connecting them (Hall 1942; Morrison, 1996).

2.5.2 Methods for building phylogenetic trees

There are two significantly different methods for building phylogenetic trees within the field of taxonomy: phenetic and cladistic methods. The phenetic method or numerical classification constructs trees that express phenetic relationships as a phenogram based on overall similarity without regard to the evolutionary history of the character being considered and without trait selection. Phenetic methods build trees based on either homology, similarity due to common descent, or analogy, similarity due to independent origin by convergence and parallelism. This similarity can relate to molecular, phenotypic and/or anatomical characters (Sneath and Sokal, 1973; Morrison, 1996). On the other hand, cladistic methods or phylogenetic classifications aim to generate cladograms based on the similarity that reflects the evolutionary relationships between the objects under consideration. A phenogram may serve as an indicator of cladistic relationships but the phenetic and cladistic relationships will not necessarily be identical. Phenetic and cladistic relationships may become identical when a linear relationship between time of divergence and the degree of genetic or morphological divergence exists (Li, 1997).

2.5.3 Phylogenetic tree construction methods used in this study

The principal methods of making trees can be classified into two types: distance-based and character-based methods. Distance-based methods begin the construction of a tree by calculating the distances for all pairs of taxa and build a tree by considering the relationship among these distance values. The main distance-based methods are

the unweighted pair group method with arithmetic mean (UPGMA) and neighbor joining (NJ). In contrast, character-based methods analyze candidate trees based on relationships inferred directly from sequence alignments. These approaches are distinct from distance-based methods, since they do not involve an intermediary summary of the sequence data in the form of a distance matrix or resemblance matrix. Nonetheless they do depend on distance matrices. There are two main character-based methods: maximum parsimony (MP) and maximum likelihood (ML) (Nei and Kumar, 2000: Brocchievi, 2001).

2.5.3.1 Making trees using UPGMA distance-based method

The UPGMA is a simple tree-making algorithm that works by clustering the sequences data, DNA profile, morphology data and other evidence based on a distance matrix. The program first searches the pair of OTUs with the smallest distance between them. The branching pattern between them is defined as half of that distance resulting in placing a node at the midpoint. Then the two OTUs are grouped together into a cluster and the matrix is re-written with distances from the cluster to each of the remaining OTUs. As a result, the cluster serves as a substitute for two OTUs and the entire number in the matrix is now reduced by one. This process is repeated on the new matrix and is continued until the new matrix consists of a single entry OTU. Then, that set of matrices is used to construct the tree by starting at the root and moving out to the first two nodes represented by the last two clusters (Hall 1942; Sneath and Sokal, 1973; Morrison, 1996; Page and Holmes, 1998; Nei and Kumar, 2000).

2.5.3.2 Trees generated by maximum parsimony character-based methods

The assumption of Maximum parsimony (MP) is that taxa share a common characteristic because they inherited that characteristic from a common ancestor. Therefore, MP searches for the best tree defined as shortest branch lengths. The best tree requires the fewest number of changes to explain data in the alignment (Hall, 1942; Morrison, 1996). Under high rates of evolutionary change MP can perform extremely well to find the true evolutionary tree. However, MP can construct the wrong tree when more data is accumulated. This can be explained by a greater proportion of identical character states being shared by chance, between unrelated taxa rather than shared by a common ancestor between related taxa (Swafford and Sullivan, 2003).

The steps involved seeking the most parsimony explanations for observed data are

1. Identify informative sites. If a site is constant, then it is not informative. Parsimony-informative characters must have at least two states that occur in at least two OTUs
2. Invariant characters. These characters found at the same state in all OTUs, are useless and are ignored.
3. Construct trees. All possible trees are computed and the one with shortest branch length is chosen. An algorithm is used to determine the minimum number of steps for any given tree to be consistent with observed data.
4. Count the number of changes and select the shortest tree (or trees). The algorithm is used to evaluate a possible tree at each informative site, then add up the changes to calculate the minimum number of changes for particular tree. The most parsimonious trees are the tree or trees with lowest score (Hall, 1942; Kumar *et al*., 1993; Nei and Kumar, 2000).

In the program, PAUP, the tree-making criterion to parsimony can be determined by the user. It would be ideal to perform an exhaustive search of all possible trees to find the one with the shortest total branch lengths however; in practice this is impossible for more than 10-20 taxa, so it is necessary to perform a heuristic search. A heuristic search seeks an initial tree and then rearrangement (branch swapping) to improve the initial tree until the best tree is found. Both heuristic and exhaustive searches often result in the identification of several trees having the same minimal value for total branch length of the tree. Trees can be visualized as a phylogram or a cladogram (Hall, 1942).

2.5.4 Evaluation tree using randomizing tests and bootstrapping

The main criteria to assess the accuracy of phylogenetic trees are consistency, efficiency, and robustness. The most common approach to assess this is bootstrap analysis. Bootstrapping is not a technique to assess the accuracy of a tree but describes the robustness of the tree topology. The consistency of particular branching order finding by a tree-building algorithm was computed by using randomly-permuted version of the original data set (Brinkman and Leipe, 2001).

A multiple sequence alignment is used to generate a tree using some tree-building method. Nonparametric bootstrapping then makes an artificial data set of the same size as the original data set by randomly choosing columns from the multiple sequence alignment. This is usually performed with replacement, meaning the any individual character may be selected multiple times (or not at all). A tree is generated from the randomly selected data set and the proportion of each clade among all the bootstrap replicates is computed. A large number of bootstrap replicates, between 100 and 1000 are then generated by this process. The bootstrap trees are compared to the

original, implied tree, to determine the number of times the branching patterns are observed. The information from bootstrapping indicates the frequency with which each clade in the original tree is observed. The percentage of times that a given clade is supported in the original tree forms the basis on how often the bootstraps supported the original tree topology. Bootstrap values above 70% are generally considered to provide support for the clade designations (Navidi, 1995; Brinkman and Leipe, 2001).

Table 2.2 Examples of molecular based phylogeny studies in various plant groups.

Plant group	Sample size	Taxonomic level	Type of character	Type of markers	Tree building method	Software	Reference
Araceae, Sect. Monsteroideae, Lasioideae, Pothoideae	118 (43 taken from other study)	intergenera, infrafamily	DNA sequences	CpDNA *trn*L-*trn*F intergenic spacer	Parsimony	PAUP	(Tam *et al*.,2004)
Styrax (Styracaceae)	53 (1 outgroup)	52 accessions (39 species)	DNA sequences, restriction site	ITS, cpDNA (*trn*K, *rpo*C1, *rpo*C2 restriction sites)	Maximum parsimony	PAUP	(Fritsch, 2001)
Plantago (Plantaginaceae)	62 (3 outgroup)	57 species	DNA sequences	ITS, cpDNA *trn*L-*trn*F intergenic spacer	Maximum parsimony	PAUP	(Ronsted *et al*. ,2002)
Sesamum (Pedaliaceae)	75 accessions	Within accession	DNA profiling	ISSR	UPGMA	POPGENE	(Kim *et al*. ,2002)
Oryza (Poaceae)	28 accessions, 10 cultivars, 1 landrace 3 related genera	between accession, 21 species	DNA profiling	ISSR	UPGMA	NTSYS	(Joshi *et al*. ,2000)
Houttuynia (Saururaceae)	70 accessions	within and between accession	DNA profiling	RAPD, ISSR	UPGMA	NTSYS	(Wu *et al*., 2005)
Actinidia (Actinidiaceae)	31 species	within genus	DNA profiling	RAPD	UPGMA	NTSYS	(Huang *et al*. ,2002)
Cactaceae	11 species	11 species, 2 populations	DNA profiling	AFLP, cpSSR	UPGMA	TREECON	(Labra *et al*. ,2003)

AFLP: amplified fragment length polymorphism, ISSR: inter-simple sequence repeat, NJ: neighbor joining, UPGMA: unweighted pair-group method using arithmetric averages, PAUP: Phylogenetic analysis using parsimony, NTSYS: Numerical Taxonomy System

2.6 The used of molecular marker in orchid phylogenetic relationships

To date, molecular markers which reveal extensive polymorphism at DNA level has been widely used in various research disciplines in many levels of organism, including microorganisms, in the plant and animal kingdom. The use of various DNA markers have been applied for plant classification and identification study more often than the previously used traditional characters. Tsai *et al*. (2002) studied phenetic relationship and identification of subtribe Oncidiinae genotypes using RAPD markers. The results found that phenetic similarity among 24 accessions of subtribe Oncidiinae giving six major clusters and one individual not belonging to any of the six clusters. The dendrogram based RAPD also showed that genus *Miltonia* is separated from other genera among the Oncidiinae used in this study. In addition, the RAPD markers used in this study are an appropriate tool for Oncidiinae genotype identification.

In addition, RAPD markers have been used to assess genetic diversity in cultivated vanilla (Orchidaceae): *Vanilla planifolia*, and relationship with *V. tahitensis* and *V. pompona* by using RAPD (Beese *et al*., 2004). RAPD markers were proven to be species-specific markers and used successfully to analyse putative *V. planifolia* x *V. tahitensis* hybrid specimens. However, these markers failed to discriminate the different specimens from Central America. The UPGMA clustering indicated three main phenetic groups belonging to three *Vanilla* species assessed. Low levels of genetic diversity were detected in cultivated *V. planifolia* from Mexico which is in accordance with the vegetative mode of dispersion of vanilla plants. ISSR markers are also widely used for determining phylogenetic relationship. Wang *et al*. (2009a) used ISSR marker to understand phylogenetic relationships and identification of 31 *Dendrobium* species. The results found high levels of polymorphism (100%) among

278 ISSR loci generated from 17 ISSR primers at the generic level. The UPGMA of these species found six clusters indicating a polyphyletic genus with several well-supported lineages with similarity coefficients ranging from 0.532 to 0.730. This highly polymorphic and reliable amplification across *Dendrobium* indicated the utility of ISSR markers for species identification and genetic diversity within the genus. ISSR markers were also useful for assessing molecular diversity and relationships among *Cymbidium goeringii* cultivars (Wang *et al*., 2009b). The UPGMA and PCA grouped *C. goeringii* into two clusters roughly corresponding with geographical distribution.

The complicate technique of arbitrary marker, AFLP expects to generate more polymorphic markers to resolving genetic relationships among closely related species. This technique was applied in the investigation of phylogenetic relationships between *Diuris fragrantissima* and its closest relatives (Smith *et al*. 2007). High similarity obtained from AFLP markers was found between either population or species indicating a very close relationships between *D. fragrantissima*. The purple-flowered species were found to form individual phenetic clusters confirming taxonomic recognition of *D. fragrantissima*. AFLP markers were also applied to the endangered fen orchid (*Liparis loeselii*) by Pilon *et al* (2007) to conducted genetic diversity and ecological differentiation. The grouping indicated that *Liparis* populations clustered according to their habitat type rather than geographical location, with dune slack population from northern France clustering with dune slack population from Britain rather than with the northern France fen populations. However, the separation is not well resolved at the individual level since one genotype was found in both habitats.

Although genetic differentiation between populations was low, this result provided the occurrence of long distance gene flow, possibly across the English channel.

ITS sequences were used to determine genetic relationships in slipper orchids (*Cypripedioideae*, *Orchidaceae*; Cox *et al*., 1997), *Phalaenopsis* Blume (Orchidaceae; Tsi *et al*., 2006), *Goodyera* species (Orchidaceae, Cranichideae; Tsai *et al*., 2004), genera *Apostasia* and *Neuwiedia* subfamily Apostasioideae (Orchidaceae; Kocyan *et al*., 2004) and African subtribe Disinae (Orchidoideae, Orchidaceae; Bytebier *et al*., 2007). Phylogenetic relationships of the slipper orchids (*Cypripedioideae*, *Orchidaceae*) suggested the genera *Paphiopedilum*, *Phragmipedium*, *Mexipedium*, *Cypripedium* and *Selenipedium* are monophyletic. An ITS based cladogram placed *Mexipedium* sister to *Phragmipedium*. Even though the topology of the ITS tree was supported by low bootstrap value; the confidence in topology was defined by congruence with previous taxonomies, morphological, anatomical and cytological data (Cox *et al*., 1997). The accordance between molecular phylogeny and traditional classification was also found in phylogenetic relationships of the several *Goodyera* species (Shin *et al*., 2002) and African subtribe Disinae (Bytebier *et al*., 2007). The ITS phylogenetic analysis of several Korean *Goodyera* species reported by Shin *et al*. (2002) indicated that Korean *Goodyera* species (Tribe Cranichideae, Subtribe Goodyerinae) are monophylytic. It also supported previous morphological, geographical and RAPD analysis. In addition, the ITS analysis was in agreement with previous reports based on morphological characters that *G. maximowicziana* and *C. velutina* were more closely related to each other than to *G. macrantha*. The ITS sequence showed no differences between *G. schlechtendaliana* and *G. repens* even they share very similarity external morphology. Bytebier *et al* (2007) conducted

phylogenetic relationships within African subtribe Disinae (Orchidoideae, Orchidaceae) which include the large genus *Disa* and small genus *Schizodium* based on ITS, *trn*LF and *mat*K sequences. These results support a recircumscription of the genus *Disa* in the broadest possible sense while the generic status of *Schizodium* can no longer be supported as it is deeply embedded within the genus *Disa*. The ITS, *trn*LF and *mat*K sequences indicated that the genus *Aerides* (Epidendrodeae, Vandeae, Aeridinae) was monoplyletic. It consisted of three well-supported subclades which were only partly in accordance with previous reports based on floral character.

ITS sequences also provided valuable information to clarify the phylogeny of genus *Phalaenopsis* (Tsi *et al*., 2006). Molecular phylogeny of *Phalaenopsis* Blume (Orchidaceae) by Tsi *et al*. (2006) showed that ITS region of nuclear ribosomal DNA seemed to be sufficient for resolving infrageneric relationships in *Phalaenopsis* and showing monophyly of the genus. The ITS based phylogeny showed that genera *Doritis* and *Kingidium* can be treated as part of the genus *Phalaenopsis*. Within *Phalaenopsis*, subgenera *Aphyllae* and *Parishianae* were monophyletic and clustered with subgenus Proboscidioides, sections Esmeralda and Delliciosae of subgenus *Phalaenopsis*. Only section *Phalaenopsis*, subgenus *Phalaenopsis* is highly supported as monophyletic while moderate monophyly was found in section *Polychilos*, subgenus *Polychilos*. Furthermore, ITS sequences have proven to be a valuable tool for *Dendrobium* identification by developing species specific marker (Tsai *et al*., 2004). The ITS phylogeny of *Dendrobium* species (Orchidaceae) in Taiwan based on ITS resulted in four main clusters generated from 12 *Dendrobium* species. The ITS based cladogram supported *Epigeneium nakaharai* not being placed in the genus *Dendrobium*. The ITS tree also suggested that *D. furcatopedicellatum* and *D. somai*

could be placed in a different genus, *Grastidium*. However, the ITS phylogenetic relationship did not completely match the classification based on morphology characters (Tsai *et al*., 2004).

Apart from ITS, other regions of chloroplast genome have been used for phylogenetic construction in Orchidaceae. Bellusci *et al*. (2008) reported phylogenetic relationships in the genus *Serapias L*. based on non-coding regions of the chloroplast genome indicating the well-defined phylogenetic tree supported a division of taxa into two main clades, each including two minor groups which differed from those obtained using morphological characters. However, these two main clades reflected an early differentiation of flower size due to the shift from allo-to self-pollination. On the other hand, relationships within each minor group did not reflect floral size variation indicating that diversification resulted from genetic drift, local selection forces, and multiple independent transitions towards self-pollination and polyploidy.

The combination of ITS and plastid gene regions sequences has also found to be a useful tool for phylogenetic analysis. Kocyan *et al*., (2004) clearly resolved a phylogenetic tree of the genera *Apostasia* and *Neuwiedia* subfamily Apostasioideae (Orchidaceae) based on ITS, *trn*LF and *mat*K sequences. The result clearly showed that *Apostasioideae* formed a clade that was sister to Vanilloideae, Cypripediodeae, Orchidoideae and Epidendroideae. The cladograms of Orchidoideae in this study resulting in the same tree topology of previously reported cladograms based on morphology and molecular characters. Phylogenetic analysis indicated the Apostasioideae was sister to the remaining Orchidaceae. However, the genetic relationships among taxa within section *Apostasia* remained unresolved.

In conclusion, molecular markers from either fingerprinting or sequence analysis can provide sufficient information for phylogenetic proposes. However, the limitation of genetic variation within specimens and the choice of markers used have been potential factors for resolving molecular phylogeny.

2.7 Current status of molecular based classification in spider orchids

There are a few published reports of molecular phylogenies of orchid species in tribe *Diurideae*. Only one spider orchid species *Caladenia falcata*, was examined in the study of evolutionary relationships of the *Orchidaceae* based on chloroplast DNA sequences from *mat*K, and *trn*L-F. It was grouped into core Caladeniinae, which is sister to Acianthinae-Prasophyllinae. The core Caladeniinae consisted of *Eriochilus*, *Adenochilus*, *Leptoceras*, *Praecoxanthus* and the clade of *Caladenia*, *Cyanicula*, *Elythranthera* and *Glossodia* (Kores *et al*., 2001). *Caladenia longicauda* represented spider orchid species in the study of phylogenetics of *Diurideae* based on ITS region which found that Caladeniinae was sister to Diuridinae and Adenochilus. Caladeniinae included the genera of *Leptoceras*, *Praecoxanthus*, *Gossodia*, *Elythranthera*, *Cyanicula* and *Caladenia*. *Leptoceras* and *Praecoxanthus* were isolated from other taxa within Caladeniinae. The remaining taxa were separated into two groups, one containing *Cyanicula deformis*, *Glossodia*, and *Elythranthera* and the second group with the rest of *Cyanicula* and *Caladenia* (Clements *et al*., 2002). Using ITS, Jones *et al.* (2001) pointed out that *Caladenia cardiochila* and *Caladenia patersonii* were separated from *Caladenia tentaculata*. In Jones *et al*. (2001) spider orchids were classified into a new genus named *Arachnorchis*. To date, there have been no published phylogenetic studies based on molecular data among the spider orchid species complex.

Chapter 3

Genetic Relatedness among *Caladenia* Species Using RAPD Markers

3.1 Introduction

The principles of biological classification can be grouped into three basic approaches: traditional Linnean taxonomy, phenetic classification and phylogenetic or cladistic approaches. The Linnean system classifies the taxonomic groups based on a few key, usually morphological, traits. Phenetic classification groups the taxa based on shared characters and traits that can discriminate the taxa. Phenetic classifications are usually presented in the form of a dendrogram. The phylogenetic system classifies taxonomic units based on shared similarities as a consequence of common descent, and is presented in the form of a phylogenetic tree (Ganeshaiah *et al.,* 2000).

More recently, phylogenetic relationships among living organisms such as fungi, bacteria, animals and plants have been investigated using molecular as well as traditional morphological characters. In many cases, morphological parameters have generally not been sufficiently discriminative in species complexes and/or closely related species. Molecular markers are commonly used to characterize genetic relationships because they typically detect much higher levels of polymorphism. Furthermore, they can distinguish variation in alleles of genes both within and among populations as well as variation in the nucleotides, genes, chromosomes, or whole genomes of organisms. Amongst the various molecular marker-assisted techniques currently available, the Random Amplified Polymorphic DNA (RAPD) method has used to determine genetic relationships, genetic diversity and genetic variation within

living organisms because of its speed, low cost and requirement for only small amounts of DNA (Willams *et al.,* 1990). RAPD primers are not designed to recognize any specific DNA sequence and hence no previous knowledge of the target genome species is required. The method also requires only a single primer per reaction which binds at multiple and unknown positions in the genome resulting in a number of amplification fragments varying in size from DNA sample being compared. Amplified fragments can then separated by size on an agarose gel and stained with ethidium bromide to visualize the banding pattern for each individual. The molecular markers can then be identified by the presence or absence of the bands across all samples being studied. The similarity among samples can be then computed from the banding patterns in each sample to produce a similarity matrix for constructing a clustering pattern. RAPDs have been used to study genetic relationships or molecular phylogeny in *Actinidia* (Actinidiaceae; Huang *et al.,* 2002), *Cymbidium* (Orchidaceae; Choi *et al*., 2006), *Echinacea* (Asteraceae; Kapteyn *et al.,* 2002), *Gossypium* (Malvaceae; Khan *et al*., 2000), *Lansium* (Meliaceae; Song *et al*., 2000), *Lippia* (Verbenaceae; Viccini *et al*., 2004), *Osteospermum* (Asteraceae; Faccioli *et al*., 2000), *Paphiopedilum* and *Phragmipedium* (Orchidaceae; Chung *et al*., 2006), *Vanilla* (Orchidaceae; Verma *et al*., 2009) and numerous other genera. RAPD markers are dominant, that is an individual homozygous for a particular RAPD marker cannot be distinguished from a heterozygous individual bearing only one copy of that marker (Willams *et al*., 1990). The considerable ambiguity of RAPD marker bands has been discussed widely and has to be taken into account in any interpretation of data (Holzapfel *et al*., 2002). Moreover, problems with non-reproducible amplification fragments have been encountered with this technique. These problem can be improved by using only high quality DNA, by careful optimization of PCR conditions

(Weising *et al*., 1998) and repeating experiments several times to monitor reproducibility of bands.

The aim of this chapter was to investigate the genetic relationships among Victorian spider orchid species using RAPD markers and to evaluate the usefulness of RAPD marker as alternative character for spider orchid classification.

3.2 Material and methods

3.2.1 Plant materials

One hundred and seventeen spider orchid individual specimens from 30 taxa of *Caladenia* subgenus *Calonema* (Benth.) Hopper & A.P.Br., syn. *Arachnorchis (*D.L.Jones & M.A. Clem.) and three outgroup species (*Caladenia* sens. lat.) were collected from natural habitats in Victoria, South Australia and New South Wales (see Fig. 3.1 below).

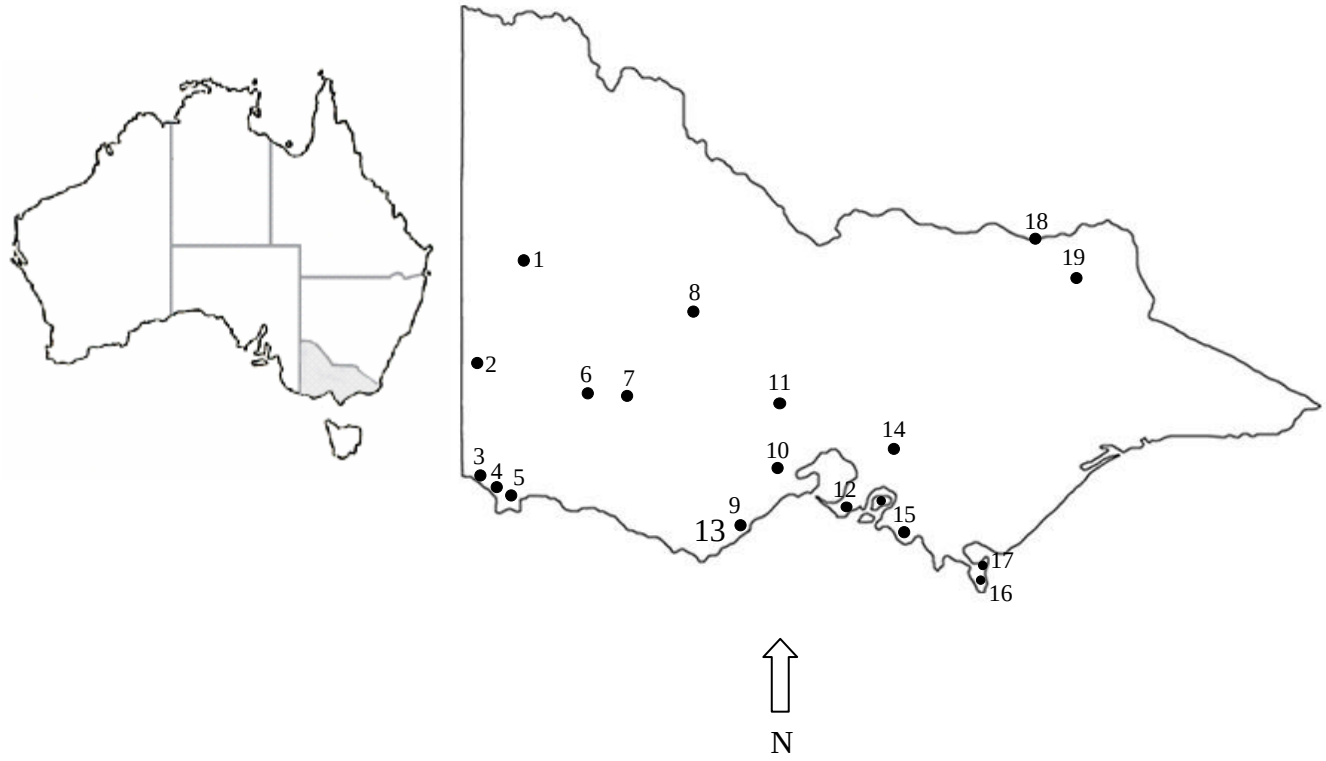


Figure 3.1 Maps of Australia and Victoria indicating sample locations.

1. Little Desert (Kiata, Diapur) (kia)
2. Langkoop, Mereek, Meerek
3.Nelson (Glenelg) (gle)
4. Swan Lake (swa)
5. Portland: Point Danger, Bats Ridge, Mt.Richmond (myr)
6. Grampians (Serra), Lake Fyans (graSR)
7. Stawell (sta)
8. Stuart Mill (stm)
9. Angelsea (ang)
10. Inverleigh (inv)
11. Castlemaine (Golden Pt), Chewton
12. Rosebud (ros)
13. French Island (fre)
14. Belgrave South (bel)
15. Wonthaggi (won)
16. Wilson's Promontary, Lilly Pilly Gully, Tidal Overlook, Derby Saddle (wilLP, wilTO, wilds)
17. Wilson's Promontary (wil)
18. Albury (alb)
19. Beechworth (Chiltern, Mt.Pilot)

Figures 3.2A-3.2D are photographs of flowers of the species included in this study. Figure 3.2A are outgroup species with Figures 3.2B-3.2D being spider orchids in the strict sense. Table 3.1 are the list of species and locations of samples used in this study.

C. latifolia

C. flaccida

L. menziesii

Figure 3.2A Photographs of the outgroup taxa *Caladenia* sens. str. and *Leporella* used in this study, photo by Gary Backhouse except *L. Menziesii* photo by Wendy Probert.

C. amoena

C. parva

C. phaeoclavia

C. stricta

C. tentaculata

C. villosissima

Figure 3.2B Photographs of the *Caladenia* subgenus *Calonema* taxa, dilatata alliance used in this study, photo by Gary Backhouse.

C. cardiochila

C. sp. aff. *colorata*

C.concolor

C. formosa

C. fragrantissima

C. oenochila

C. sp. aff. *patersonii*

C. pilotensis

C. rosella

Figure 3.2C Photographs of the *Caladenia* subgenus *Calonema* taxa, patersonii alliance used in this study, photo by Gary Backhouse.

C. tessellata *C. venusta* *C.* sp. aff. *venusta* (Stuart Mill)

Figure 3.2C (cont.)

C. australis *C. calcicola* *C. clavigera*

Figure 3.2D Photographs of the *Caladenia* subgenus *Calonema* taxa, reticulate alliance used in this study, photo by Gary Backhouse.

C. cruciformis

C. hastata

C. lowanensis

C. reticulata

C. richardsiorum

Figure 3.2 D (cont.)

Table 3.1 Accessions of *Caladenia spp.* used in this study.

species code	Species	Group	Location	Date[1]
C.latifolia	*C. latifolia*	outgroup	Cultivated, Roy. Bot. Gdns Melbourne	1/10/2002
J.flaccida	*C. flaccida*	outgroup	Knocker Tk, Omeo	10/10/2003
L.menziesii	*L. menziesii*	outgroup	Cultivated, Roy. Bot. Gdns Melbourne	1/10/2002
amo-RBG	*C. amoena*	dilatata	Cultivated, Roy. Bot. Gdns Melbourne	1/10/2002
par-lanLT	*C. parva*	dilatata	Longbottom track, Langkoop	1/10/2002
par-lanMSF1	*C. parva*	dilatata	Meerek State Forest	9/10/2002
par-lanMSF2	*C. parva*	dilatata	Meerek State Forest	9/10/2002
par-lanMSF3	*C. parva*	dilatata	Meerek State Forest	9/10/2002
pha-lanLT	*C. phaeoclavia*	dilatata	Longbottom track, Langkoop	1/10/2002
pha-lanMR1	*C. phaeoclavia*	dilatata	Mereek Rd, Langkoop	1/10/2002
pha-lanMR2	*C. phaeoclavia*	dilatata	Mereek Rd, Langkoop	1/10/2002
pha-lanMR3	*C. phaeoclavia*	dilatata	Mereek Rd, Langkoop	1/10/2002
str-idnGP1	*C. stricta*	dilatata	Little Desert NP (gravel pits)	12/10/2003
str-idnGP2	*C. stricta*	dilatata	Little Desert NP (gravel pits)	12/10/2003
str-idnGP3	*C. stricta*	dilatata	Little Desert NP (gravel pits)	12/10/2003
ten-graWP1	*C. tentaculata*	dilatata	Glenelg River Rd, Woolpooer, Grampians NP	21/10/2002
ten-graWP2	*C. tentaculata*	dilatata	Glenelg River Rd, Woolpooer, Grampians NP	21/10/2002
ten-graWP3	*C. tentaculata*	dilatata	Glenelg River Rd, Woolpooer, Grampians NP	21/10/2002
vil-lanLT	*C. villosissima*	dilatata	Longbottom track, Langkoop	1/10/2002
vil-lanMSF1	*C. villosissima*	dilatata	Meerek State Forest	9/10/2002
vil-lanMSF2	*C. villosissima*	dilatata	Meerek State Forest	9/10/2002
vil-lanMSF3	*C. villosissima*	dilatata	Meerek State Forest	9/10/2002
vil-lanMR1	*C. villosissima*	dilatata	Mereek Rd, Langkoop	1/10/2002
vil-lanMR2	*C. villosissima*	dilatata	Mereek Rd, Langkoop	1/10/2002
vil-lanMR3	*C. villosissima*	dilatata	Mereek Rd, Langkoop	1/10/2002
car-angHS1	*C. cardiochila*	patersonii	Anglesea (O'Donohues)	28/9/2003
car-angHS2	*C. cardiochila*	patersonii	Anglesea (O'Donohues)	28/9/2003
car-angHS3	*C. cardiochila*	patersonii	Anglesea (O'Donohues)	28/9/2003
colaff-gleGT1	*C. colorata (sp.aff.)*	patersonii	Gorge Track, Lower Glenelg NP	19/10/2002
colaff-gleGT2	*C. colorata (sp.aff.)*	patersonii	Gorge Track, Lower Glenelg NP	19/10/2002
colaff-gleGT3	*C. colorata (sp.aff.)*	patersonii	Gorge Track, Lower Glenelg NP	19/10/2002
con-chiCY1	*C. concolor*	patersonii	Cyanide Dam, Chiltern-Pilot NP	6/10/2003
con-chiCY2	*C. concolor*	patersonii	Cyanide Dam, Chiltern-Pilot NP	6/10/2003
con-chiCY3	*C. concolor*	patersonii	Cyanide Dam, Chiltern-Pilot NP	6/10/2003
con-alb1	*C. concolor*	patersonii	Albury NSW	1/10/2003
conaff-tya	*C. concolor (sp.aff.)*	patersonii	Murchison Hill, Tyaac	28/10/2003
for-lanLT1	*C. formosa*	patersonii	Longbottom track, Langkoop Mereek	30/9/2002
for-lanLT2	*C. formosa*	patersonii	Longbottom track, Langkoop Mereek	30/9/2002
for-lanLT3	*C. formosa*	patersonii	Longbottom track, Langkoop Mereek	30/9/2002
for-lanMSF1	*C. formosa*	patersonii	Meerek State Forest	9/10/2002
for-lanMSF2	*C. formosa*	patersonii	Meerek State Forest	9/10/2002
for-lanMSF3	*C. formosa*	patersonii	Meerek State Forest	9/10/2002
fra-awaDB1	*C. fragrantissima*	patersonii	Discovery Bay CP (Swan Lake)	20/10/2002
fra-awaDB2	*C. fragrantissima*	patersonii	Discovery Bay CP (Swan Lake)	20/10/2002
fra-awaDB3	*C. fragrantissima*	patersonii	Discovery Bay CP (Swan Lake)	20/10/2002
fra-mtr1	*C. fragrantissima*	patersonii	Mt. Richmond NP	18/10/2002
fra-mtr2	*C. fragrantissima*	patersonii	Mt. Richmond NP	18/10/2002
fra-mtr3	*C. fragrantissima*	patersonii	Mt. Richmond NP	18/10/2002
oen-belBW1	*C. oenochila*	patersonii	Belgrave South, Baluk Willam FFR	18/10/2002
oen-belBW2	*C. oenochila*	patersonii	Belgrave South, Baluk Willam FFR	18/10/2002
oen-belBW3	*C. oenochila*	patersonii	Belgrave South, Baluk Willam FFR	18/10/2002
oen-graSR1	*C. oenochila*	patersonii	Serra Rd, Grampians NP	1/10/2002

oen-graSR2	*C. oenochila*	patersonii	Serra Rd, Grampians NP	1/10/2002
oen-graSR3	*C. oenochila*	patersonii	Serra Rd, Grampians NP	1/10/2002
ori-won1	*C. orientalis*	patersonii	Wonthaggi Heathland Reserve	2/11/2002
ori-won2	*C. orientalis*	patersonii	Wonthaggi Heathland Reserve	3/11/2002
ori-won3	*C. orientalis*	patersonii	Wonthaggi Heathland Reserve	4/11/2002
pataff-inv1	*C. patersonii (sp.aff.)*	patersonii	Inverleigh Common FFR	28/09/2003
pataff-inv2	*C. patersonii (sp.aff.)*	patersonii	Inverleigh Common FFR	28/09/2003
pataff-inv3	*C. patersonii (sp.aff.)*	patersonii	Inverleigh Common FFR	28/09/2003
pil-beeMP1	*C. pilotensis*	patersonii	Mt. Pilot, Beechworth	4/10/2003
pil-beeMP2	*C. pilotensis*	patersonii	Mt. Pilot, Beechworth	4/10/2003
pil-beeMP3	*C. pilotensis*	patersonii	Mt. Pilot, Beechworth	4/10/2003
ros-RBG	*C. rosella*	patersonii	Cultivated, Roy. Bot. Gdns Melbourne	1/10/2002
tes-wilLP1	*C. tessellata*	patersonii	Lilly Pilly Gully (Wilsons Prom NP)	13/10/2002
tes-wilLP2	*C. tessellata*	patersonii	Lilly Pilly Gully (Wilsons Prom NP)	13/10/2002
tes-wilLP3	*C. tessellata*	patersonii	Lilly Pilly Gully (Wilsons Prom NP)	13/10/2002
ven-lanMSF1	*C. venusta*	patersonii	Meerek State Forest	9/10/2002
ven-lanMSF2	*C. venusta*	patersonii	Meerek State Forest	9/10/2002
ven-lanMSF3	*C. venusta*	patersonii	Meerek State Forest	9/10/2002
venfor-lanLT1	*C. venusta x C. formosa*	patersonii	Longbottom track, Langkoop Mereek	1/10/2002
venfor-lanLT2	*C. venusta x C. formosa*	patersonii	Longbottom track, Langkoop Mereek	1/10/2002
venfor-lanLT3	*C. venusta x C. formosa*	patersonii	Longbottom track, Langkoop Mereek	1/10/2002
venaff-stmFF1	*C. venusta (sp.aff.)*	patersonii	Stuart Mill FFR	30/09/2002
venaff-stmFF2	*C. venusta (sp.aff.)*	patersonii	Stuart Mill FFR	30/09/2002
venaff-stmFF3	*C. venusta (sp.aff.)*	patersonii	Stuart Mill FFR	30/09/2002
aus-wilLP1	*C. australis*	reticulata	Lilly Pilly Gully (Wilsons Prom NP)	13/10/2002
aus-wilLP2	*C. australis*	reticulata	Lilly Pilly Gully (Wilsons Prom NP)	13/10/2002
aus-wilLP3	*C. australis*	reticulata	Lilly Pilly Gully (Wilsons Prom NP)	13/10/2002
aus-wilTO1	*C. australis*	reticulata	Tidal Overlook TK Wilsons Prom NP	19/10/2001
aus-wilTO2	*C. australis*	reticulata	Tidal Overlook TK Wilsons Prom NP	19/10/2001
aus-wilTO3	*C. australis*	reticulata	Tidal Overlook TK Wilsons Prom NP	19/10/2001
cal-bat1	*C. calcicola*	reticulata	Bats Ridge, Portland	1/10/2001
cal-bat2	*C. calcicola*	reticulata	Bats Ridge, Portland	1/10/2001
cal-bat3	*C. calcicola*	reticulata	Bats Ridge, Portland	1/10/2001
cla-wilDS1	*C. clavigera*	reticulata	Derby Saddle, Wilsons Promontory NP	12/10/2002
cla-wilDS2	*C. clavigera*	reticulata	Derby Saddle, Wilsons Promontory NP	12/10/2002
cla-wilDS3	*C. clavigera*	reticulata	Derby Saddle, Wilsons Promontory NP	12/10/2002
cru-stmFF1	*C. cruciformis*	reticulata	Stuart Mill FFR (Little Dalynong FFR?)	30/09/2002
cru-stmFF2	*C. cruciformis*	reticulata	Stuart Mill FFR (Little Dalynong FFR?)	30/09/2002
cru-stmFF3	*C. cruciformis*	reticulata	Stuart Mill FFR (Little Dalynong FFR?)	30/09/2002
has-por1	*C. hastata*	reticulata	Portland	5/11/2002
has-por2	*C. hastata*	reticulata	Portland	5/11/2002
has-por3	*C. hastata*	reticulata	Portland	5/11/2002
ins-freMW1	*C. insularis*	reticulata	Mt. Wellington track, French Island	23/10/2002
ins-freMW2	*C. insularis*	reticulata	Mt. Wellington track, French Island	23/10/2002
ins-freMW3	*C. insularis*	reticulata	Mt. Wellington track, French Island	23/10/2002
ins-freMW4	*C. insularis*	reticulata	Mt. Wellington track, French Island	NA
ins-freMW5	*C. insularis*	reticulata	Mt. Wellington track, French Island	NA
ins-freMW6	*C. insularis*	reticulata	Mt. Wellington track, French Island	NA
ins-freQC1	*C. insularis*	reticulata	Quarry on Cemetery track, French Island	23/10/2002
ins-freQC2	*C. insularis*	reticulata	Quarry on Cemetery track, French Island	23/10/2002
ins-freQC3	*C. insularis*	reticulata	Quarry on Cemetery track, French Island	23/10/2002
ins-freQC4	*C. insularis*	reticulata	Quarry on Cemetery track, French Island	NA
ins-freQC5	*C. insularis*	reticulata	Quarry on Cemetery track, French Island	NA
low-kiaFR1	*C. lowanensis*	reticulata	Kiata Flora Reserve	21/11/2001
low-kiaFR2	*C. lowanensis*	reticulata	Kiata Flora Reserve	21/11/2001
low-kiaFR3	*C. lowanensis*	reticulata	Kiata Flora Reserve	21/11/2001

ret-staDL1	*C. reticulata*	reticulata	Deep Lead FFR, Stawell	11/10/2003
ret-staDL2	*C. reticulata*	reticulata	Deep Lead FFR, Stawell	NA
ret-staDL3	*C. reticulata*	reticulata	Deep Lead FFR, Stawell	NA
ric-norSA1	*C. richardsiorum*	reticulata	Nora Creina	26/10/2002
ric-norSA2	*C. richardsiorum*	reticulata	Nora Creina	26/10/2002
ric-norSA3	*C. richardsiorum*	reticulata	Nora Creina	26/10/2002
ric-souSA1	*C. richardsiorum*	reticulata	Southern SA	12/10/2002
ric-souSA2	*C. richardsiorum*	reticulata	Southern SA	12/10/2002
ric-souSA3	*C. richardsiorum*	reticulata	Southern SA	12/10/2002
rob-ros1	*C. robinsonii*	reticulata	Rosebud	NA
rob-ros2	*C. robinsonii*	reticulata	Rosebud	NA
rob-ros3	*C. robinsonii*	reticulata	Rosebud	NA

[1] indicates the sampling limited to this study

3.2.2 DNA extraction

Spider Orchid leaf samples were collected in the natural habitat by various spider orchid experts. One fourth of each leaf was cut and preserved by drying, using silica gel. Genomic DNA was extracted from leaf tissue using the DNeasy Plant Mini Kit from Qiagen (Clifton Hill/Australia). Plant tissue was ground under liquid nitrogen to a fine powder in a 1.5 ml microfuge tube. The cells were then lysed by adding 400 µl of lysis buffer AP1 before the sample thawed. This was then followed by the addition of 20 µl of 20 mg/ml RNase A and the mixture vortexed until all tissue clumps were dissipated. The mixture was incubated at 65^0C for 10 minutes and mixed 2-3 times by inverting the tube to lyse the cells. 130 µl of precipitate buffer AP2 was added to the lysate, mixed well before incubation on ice for 5 minutes to precipitate detergents, proteins and polysaccharides. The mixture was then centrifuged for 5 minutes at maximum speed to pellet viscous lysates and precipitates which can shear DNA during filtration. The supernatant was then applied to a Qiashredder spin column in a 2 ml collection tube and centrifuged for 2 min at maximum speed to remove cell debris and salt precipitates that are retained by the column. The flow-through fraction was transferred to a new tube without disturbing the pellet of cell-debris that may have passed through the Qiashredder column. A 0.5 volume of binding buffer AP3

containing 1 volume of 100% ethanol was added to the cleared lysate and mixed by pipetting. The mixture was then applied, in 650 µl aliquots, to a DNeasy spin column in a 2 ml collection tube and centrifuged at 12,000 rpm for 1 min to bind the DNA to the column. The DNeasy spin column was then placed in a new 2 ml collection tube, 500 µl of wash buffer used with subsequent flowthrough discarded. A further 500 µl of wash buffer AW was added to the column, the column centrifuged at 12,000 rpm for 2 minutes to dry column membrane. The DNeasy spin column was then transferred to a fresh microfuge tube and 50 µl of preheated elution buffer AE at 65^0C was added directly onto the column membrane. The column was incubated at room temperature for 5 min to increase the yield of DNA and then centrifuged at 12,000 rpm for 1 min to elute the DNA. This elution step was then repeated to produce a final eluate volume of 100 µl. The quality of extracted DNA was visually assessed by loading 1 µl of DNA stock on a 1.4% agarose gel (Promega Cooperation, USA) containing 0.3 ng/100ml ethidium bromide (BioRad, USA), and electrophoresed in TBE buffer (Appendix I). The gel was then visualized under UV illumination. The quantity of DNA was estimated by comparing with a Low DNA Mass Ladder (Fermentas, Australia). DNA of each individual was diluted to 10 ng/ml for PCR reactions.

3.2.3 Amplification of RAPD markers

PCR reactions were performed in 200 µl thin-wall tubes in a MJ research PTC-100 or PTC-200 thermocycler. The PCR reactions contained 40 ng of template DNA within a total volume of 25 µl. The reaction mixture contained: PCR buffer (20 mM Tris HCl pH 8.4, 50 mM KCl), 2.5 mM $MgCl_2$, 0.24 mM of each dNTP, 1 unit of Taq polymerase (Invitrogen, Australia) and 0.2 µM of primer (Operon Technologies,

synthesized by Invitrogen, Australia). The PCR amplification cycle included denaturation at 94^0C for 1 min followed by 35 cycles of 94^0C for 10 sec, 36^0C for 30 seconds and 72^0C for 1 min with a final extension at 72^0C for 7 min (Kongjaroon, 2002). Forty decamer primers of the Operon kits, OPE and OPF were tested for their capacity to produce stable and polymorphic RAPD patterns using five randomly selected DNA samples. Seventeen of these were selected for further experiment. Amplification products were then separated on 2% agarose containing 0.6 μg/ml of ethidium bromide in TBE buffer, and visualized under UV light.

3.2.4 Data analysis

Only reproducible bands from DNA profiles generated by RAPD-PCR were scored manually as "1" for presence, "0" for absence and "999" for unclear bands indicated as missing data. The intensity variation between bands of the same molecular weight across samples was not considered to be a polymorphism. The binary matrix generated was then analyzed using NTSYS-pc software (Numerical Taxonomy and Multivariate Analysis System) version 2.1 (Exeter Software Co., New York). The matrix was used to calculate the genetic similarity between individual pairs, by employing Dice similarity coefficient most widely used with RAPD data; using SIMQUAL (similarity for Qualitative Data). Dice coefficients are defined as $S_{ij} = 2n_{ij}/(n_i+n_j)$, where n_i and n_j are the number of fragments in individuals I and J, respectively, and $2n_{ij}$ is defined as the number of bands shared by both individuals, according to Nei and Li (1979).

The confidence limits of the dendrograms were estimated using the program WINBOOT (Yap and Nelson, 1996) using tab-delimited format in which 1000 replications were carried out. Ordination was used as an alternative method to

evaluate phenetic relationships in a non-hierarchical approach. A principal component analysis (Gower, 1966) was performed using the MDSCALE (Multidimensional scaling) to measure 'goodness of fit'; the distance in the configuration space to the monotone function of the original distances using a statistic called stress. Variances and the covariance coefficient were then calculated to produce a covariance matrix using the SIMINT (similarity for interval data) program. Then eigenvalues and eigenvectors for real symmetric matrices were calculated via EIGEN (Eigenvectors). Component coefficients were used to calculate a component score using the Proj program (Project) and position objects on a scatterplot using the first two Principal Components routine in the NTSYS-pc software.

Parsimony analysis was conducted using PAUP* 4.0 b10 (Swofford, 2001). A heuristic search was performed using the maximum parsimony optimality criterion. Starting trees for branch swapping were obtained by stepwise addition. Branch swapping utilized the tree-bisection-reconnection (TBR) algorithm with saving and swapping on all optimum trees (MulTrees on), and saving only the shortest trees or the shortest from each replicate. The resulting trees were used as starting trees in another round of TBR with the same parameters as the first and swapping on all trees. Support for tree topology was evaluated with 500 bootstrap replicates. A bootstrap analysis of 500 replicates was conducted using the same search criteria as the initial search. Tree was rooted using *C. latifolia*, *C. flaccida*, *L. menziesii* as outgroup taxa.

3.3 Results

3.3.1 RAPD marker profile

3.3.1.1 Number of monomorphic and polymorphic bands produced within spider orchid species and over the whole sample

A total of 135 reproducible, clearly scorable polymorphic bands were produced across the entire sample of spider orchid species and outgroup taxa of 120 individuals from 17 RAPD primers. A polymorphic band in this study has been taken to be a band that is missing in at least one individual (99%). An example of the DNA profile obtained from RAPD primers is shown in Figure 3.3. The approximate size of the largest fragment produced was 1.9 kb and the smallest easily detectable fragment produced was approximately 0.25 kb (Table 3.2). Of these, seven were monomorphic within the three outgroup species (*C. latifolia*, *C. flaccida* and *L. menziesii*). Forty-four monomorphic bands were found within spider orchid species. Some 117 bands were found to be polymorphic between both the spider orchid and outgroup species together while 76 were polymorphic within only the spider orchid species (Table 3.2). The total number of amplified fragments obtained over all 120 individual plants obtained from 135 markers was 10452. However, there was no marker specific to any particular spider orchid group and species observed in this study.

Table 3.2 Detailed RAPD analysis showing primers sequences, size of bands, number of monomorphic and polymorphic within either outgroup or ingroup.

Primer	Sequence (5'→3')	Approximate fragment size range (bp)	Number of markers				
			No. of bands monomorphic within outgroup taxa	No. of polymorphic bands within outgroup taxa	No. of monomorphic bands within spider orchid species	No. of polymorphic bands within spider orchid species	Total
OPE06	AAGACCCCTC	600-1400	0	7	4	4	10
OPE07	AGATGCAGCC	300-1200	0	7	5	2	7
OPE09	CTTCACCCGA	225-1300	2	5	3	4	8
OPE15	ACGCACAACC	250-1200	1	12	3	8	13
OPE16	GGTGACTGTG	550-1200	0	10	2	10	12
OPE18	GGACTGCAGA	400-1150	2	5	4	3	9
OPE19	ACGGCGTATG	650-1375	1	5	1	5	6
OPE20	AACGGTGACC	300-1150	0	7	3	4	8
OPF01	ACGGATCCTG	225-1200	1	10	2	5	11
OPF02	GAGGATCCTG	300-900	0	6	2	3	6
OPF03	CCTGATCACC	250-1375	0	10	4	5	10
OPF04	GGTGATCAGG	400-1150	0	5	1	4	5
OPF06	GGGAATTCGG	450-1375	1	6	2	5	7
OPF09	CCAAGCTTCC	800-1600	0	5	2	3	5
OPF13	GGCTGCAGAA	300-1600	0	6	2	5	8
OPF15	CCAGTACTCC	800-1500	1	3	1	4	5
OPF20	GGTCTAGAGG	700-1900	0	5	3	2	5
Total			9	114	44	76	135

3.3.1.2 Number of bands produced per primer and which primer produced the most and least polymorphism

An average of 5.12 DNA fragments was amplified per primer (Table 3.3). Of these, OPE15 primer amplified the most DNA fragments per primer of 8.77, whereas OPF04 amplified the fewest at 2.93 (Table 3.3). Polymorphism among outgroup samples ranged from 100% with primer OPE07, OPF02, OPF03, OPF04 and OPF20 to 55.56% with primer OPE18 (Table 3.3). Within the spider orchid species, the highest degree of polymorphism produced was 83.3% from OPE16 and OPE19 and the lowest was 28.5% from OPE07 (Table 3.3).

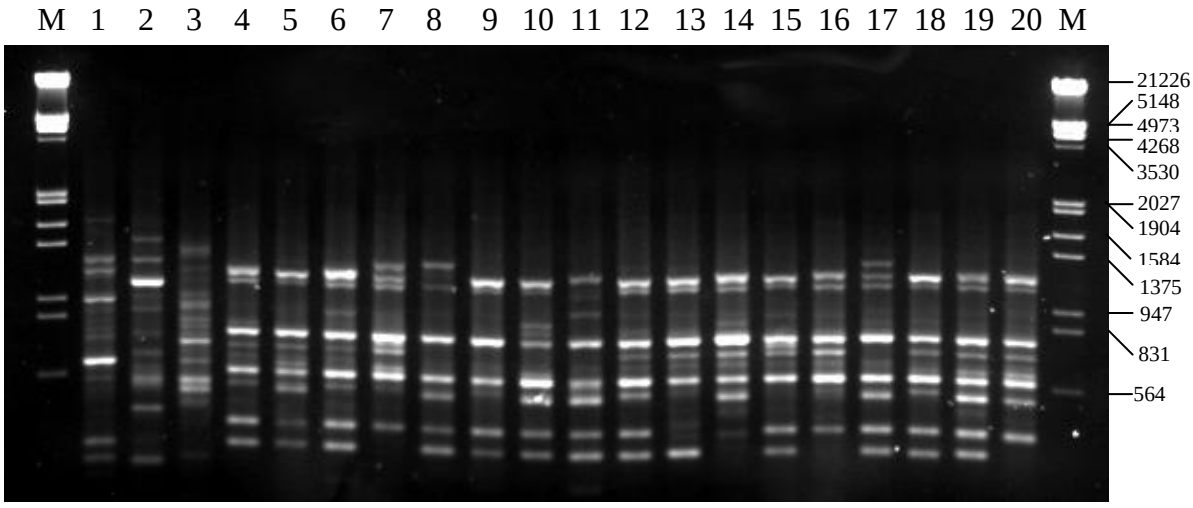


Figure 3.3 Amplification products generated from primer UBC 842 on 20 individual plants. Lanes M= λ DNA *EcoRI* and *Hind III* molecular weight marker, Lane 1=*C. flaccida*, Lane 2= *C. latifolia*, Lane 3= *L. menziesii*, Lanes 4-9=*C. australis*, Lanes 10-12=*C.calcicola*, Lanes 13-15= *C. clavigera*, Lanes 16-18= *C. cruciformis*, Lanes 19-20= *C. formosa*.

Table 3.3 The number and level of polymorphism revealed by RAPD primers.

Primer	Total No. of polymorphic fragments across all species tested	No. of polymorphic bands per primer (N_T)	Mean No of fragments per primer[a]	No. of polymorphic markers (N_P)		Polymorphism ($P= N_P/ N_T$ x 100)	
				Within outgroup samples	Within spider orchid species	Within outgroup	Within spider orchid species
OPE06	765	10	6.38	7	4	70.00	40.00
OPE07	692	7	5.77	7	2	100.00	28.57
OPE09	643	8	5.36	5	4	62.50	50.00
OPE15	1052	13	8.77	12	8	92.31	61.54
OPE16	900	12	7.5	10	10	83.33	83.33
OPE18	664	9	5.53	5	3	55.56	33.33
OPE19	514	6	4.28	5	5	83.33	83.33
OPE20	629	8	5.24	7	4	87.50	50.00
OPF01	478	11	3.98	10	5	90.90	45.45
OPF02	452	6	3.77	6	3	100.00	50.00
OPF03	657	10	5.48	10	5	100.00	50.00
OPF04	351	5	2.93	5	4	100.00	80.00
OPF06	574	7	4.78	6	5	85.71	71.42
OPF09	473	5	3.94	5	3	100.00	60.00
OPF13	650	8	5.42	6	5	75.00	62.50
OPF15	448	5	3.73	3	4	60.00	80.00
OPF20	510	5	4.25	5	2	100.00	40.00
Total	10452	135	5.12 (mean)	114	76	84.44 (mean)	56.30 (mean)

[a] total number of bands/number of species tested

3.3.1.3 Polymorphisms produced by primers within the spider orchid groups

Out of 135 polymorphic markers, the dilatata group produced the maximum number of polymorphic markers (69 bands, 51%), patersonii group produced 65 polymorphic bands (48%), while 60 polymorphic bands (44%) were found within the species of reticulata group (Table 3.4). OPE19 gave 83% (5 out of 6) of polymorphic markers produced in the reticulata group. Six out of seven (71.42%) polymorphisms produced by OPF06 were found in patersonii and dilatata groups. On the other hand, OPE07, OPE18 and OPF20 gave low numbers of polymorphic DNA fragments in all types of spider orchid species in this study (Table 3.4).

Table 3.4 The frequency of polymorphic products of RAPD primers in spider orchid group species.

Primer	Number of markers									Total
	reticulata group			patersonii group			dilatata group			
	Monomorphic		No. of Polymorphic bands (%)	Monomorphic		No. of Polymorphic bands (%)	Monomorphic		No. of Polymorphic bands (%)	
	(Absent in all)	(Present in all)		(Absent in all)	(Present in all)		(Absent in all)	(Present in all)		
E06	2	4	4 (40)	2	4	4 (40)	1	5	4 (40)	10
E07	0	6	1 (14)	0	5	2 (29)	0	4	3 (43)	7
E09	2	4	2 (25)	1	4	3 (38)	2	4	2 (25)	8
E15	3	6	4 (31)	3	6	4 (31)	2	2	7 (54)	13
E16	0	3	9 (75)	0	3	9 (75)	0	2	10 (83)	12
E18	3	4	2 (22)	3	5	1 (11)	2	4	3 (33)	9
E19	0	1	5 (83)	0	3	3 (50)	0	2	4 (67)	6
E20	1	3	4 (50)	1	3	4 (50)	2	5	1 (13)	8
F01	5	3	3 (27)	4	2	5 (45)	3	2	6 (54)	11
F02	2	2	2 (33)	1	2	3 (50)	1	2	3 (50)	6
F03	3	4	3 (30)	2	4	4 (40)	2	4	4 (40)	10
F04	0	1	4 (80)	0	1	4 (80)	0	2	3 (60)	5
F06	0	2	5 (71)	0	1	6 (86)	0	0	6 (86)	7
F09	0	3	2 (40)	0	3	2 (40)	0	2	3 (60)	5
F13	1	2	5 (62)	1	2	5 (63)	1	3	4 (50)	8
F15	0	2	3 (60)	0	1	4 (80)	0	1	4 (80)	5
F20	0	3	2 (40)	0	3	2 (40)	0	3	2 (40)	5
Total		53	60 (44)		52	65 (48)		47	69 (51)	135

3.3.2 Genetic variation within spider orchid species

A wide range of Dice coefficients of genetic similarity were observed among the spider orchid taxa and three outgroup species. The genetic similarity based on RAPD markers used in this study separated spider orchids from outgroups taxa. The lowest genetic similarity among outgroup species was 0.372 between *C. flaccida* and *L. menziesii* and the highest similarity is 0.435 between *C. flaccida* and *C. latifolia* (Appendix II). The mean genetic similarity (the average genetic similarity of pairs of individuals within a species) among spider orchid species ranged from 0.772 between *C. phaeoclavia* from Mereek Road, Langkoop and *C. concolor* from Albury, New South Wales to 0.939 between *C. oenochila* from Baluk Willam Flora and Fauna Reserve, Belgrave South and *C. fragrantissima* from Mt. Richmond National Park and between *C.* sp. aff. *concolor* from Murchison Hill, Tyaac and *C. cardiochila* from Anglesea (O'Donohues) (Table 3.5). In the dilatata group, the lowest mean genetic similarity value observed was 0.824 between *C. phaeoclavia* from Mereek Road, Langkoop and *C. villossisima* from Longbottom track, Langkoop. The highest mean value was 0.930 between *C. villossisima* from Meerek State Forest and *C. stricta* from Little Desert National Park (Table 3.5). The lowest mean genetic similarity value of 0.827 in the patersonii group was observed between *C.* sp. aff *venusta* from Stuart Mill Flora and Fauna Reserve and *C. rosella* from Royal Botanic Garden, Melbourne. The lowest mean genetic similarity (0.834) was observed between *C. calcicola* from Bats Ridge, Portland and *C. richardsiorum* from southern, South Australia while the highest mean genetic similarity (0.910) was between *C. calcicola* from Bats Ridge, Portland and *C. lowanensis* from Kiata Flora Reserve (Table 3.5).

Table 3.5 Average Dice coefficient of similarity values within spider orchid species. The range of values for each species pair is shown in parentheses next to the average values.

Group	Comparison pair[1]	Dice similarity coefficient values	
		Average minimum[2]	Average maximum[2]
Spider orchids	pha-lanMR/con-alb oen-belBW/fra-mtr Conaff-tya/car-angHS	0.776 (0.770-0.782)	 0.939 (0.933-0.944) 0.939 (0.932-0.949)
dilatata	pha-lanMR/vil-lanLT vil-lanMSF/str-ldnGP	0.824 (0.817-0.833)	 0.930 (0.923-0.938)
patersonii	Venaff-stmFF/ros-RBG oen-belBW/fra-mtr	0.827 (0.825-0.830)	 0.939 (0.933-0.944)
reticulata	cal-bat/ric-souSA cal-bat/low-kiaFR	0.842 (0.826-0.859)	 0.910 (0.899-0.920)

[1] Comparison pair selected from Dice similarity matrix based on their similarity coefficient value

[2] average minimum and average maximum values of genetic similarity of pairs of individuals within each species pair with range of Dice coefficient of similarity values listed in parentheses

Dice similarity coefficient of average mean genetic similarity observed within the species of spider orchids from different geographical regions was extremely high ranged from 0.831 to 0.969 (Table 3.6). The average mean genetic similarity in the dilatata group was detected in *C. parva*, *C. phaeoclavia* and *C. villosissima*

specimens. The mean genetic similarity between *C. parva* specimens collected from Longbottom track, Langkoop and Meerek State Forest was 0.924, with a minimum value of 0.921 and a maximum of 0.928 (Table 3.6). Specimens of *C. phaeoclavia* from Mereek Road, Langkoop and Longbottom track, Langkoop had the mean genetic similarity of 0.831, with a minimum value of 0.827 and a maximum of 0.832 (Table 3.6). The mean genetic similarity detected between *C. villosissima* specimens from Mereek Road, Langkoop and Longbottom track, Langkoop was 0.928, and Meerek State Forest was 0.930, with the same minimum value of 0.920 and a maximum of 0.937 (Table 3.6). Specimens of *C. villosissima* from Longbottom track, Langkoop and Meerek State Forest explained the mean genetic similarity of 0.899, with a minimum value of 0.894 and a maximum of 0.902 (Table 3.6)

The mean genetic similarity in the patersonii group was calculated for the same species and "sp. aff." taxa species, i.e. taxa that have very similar floral structure to a known species but are sufficiently different to be regarded by botanists as potentially representing a distinct taxon, e.g. *C. concolor*, *C.*sp. aff *concolor*, *C. formosa*, *C. fragrantissima*, *C. oenochila*, *C. venusta* and *C.*sp. aff *venusta*. Mean genetic similarities within the *C. concolor* taxa and the *C. fragrantissima* taxa were high. The mean genetic similarity between *C. concolor* from Albury, New South Wales and *C.* sp. aff. *concolor* from Murchison Hill, Tyaac was 0.962 (Table 3.6). The mean genetic similarity between *C. concolor* from Cyanide Dam, Chiltern-Pilot National Park and *C. concolor* from Albury, New South Wales was 0.969, with a minimum value of 0.967, and a maximum of 0.973 (Table 3.6). The mean genetic similarity between *C. concolor* from Cyanide Dam, Chiltern-Pilot National Park and *C. concolor* Albury, New South Wales was 0.969, ranging from 0.967 to 0.973 and

between *C. concolor* from Cyanide Dam, Chiltern-Pilot National Park and *C.* sp. aff. *concolor* from Murchison Hill, Tyaac was 0.960, ranging from 0.956 to 0.962 (Table 3.6). *C. formosa* from Longbottom track, Langkoop Mereek and *C. formosa* from Meerek State Forest had a mean genetic similarity of 0.937, with a minimum value of 0.908, and a maximum of 0.967 (Table 3.6*). C. fragrantissima* collected from Discovery Bay CP (Swan Lake) and Mt. Richmond National Park showed a genetic similarity value of 0.933, ranged from 0.925 to 0.938 (Table 3.6). A mean genetic similarity of 0.884 was detected between the specimens of *C. oenochila* collected from Belgrave South, Baluk Willam Flora and Fauna Reserve and Serra Road, Grampians National Park, ranging from 0.873 to 0.890. *C.* sp. aff. *venusta* from Stuart Mill Flora and Fauna Reserve and *C. venusta* from Meerek State Forest had a mean genetic similarity of 0.923, with a minimum value of 0.910, and a maximum of 0.947 (Table 3.6).

The mean genetic similarity between the same species within the reticulata group was observed from *C. australis*, *C. insularis* and *C. richardsiorum*, with the value of 0.942, 0. 888 and 0.912, respectively (Table 3.6). The minimum value of 0.921 and a maximum value of 0.959 were found between *C. australis* from Lilly Pilly Gully, Wilsons Promontory National Park and Tidal Overlook track, Wilsons Promontory National Park (Table 3.6). *C. insularis* from Quarry on Cemetery track, French Island and *C. insularis* from Mt. Wellington track, French Island had a minimum genetic similarity value of 0.871, and maximum value of 0.909 (Table 3.6). *Caladenia richardsiorum* collected from South Australia and from Nora Creina, South Australia had a minimum value of 0.902 and a maximum value of 0.925 (Table 3.6).

Table 3.6 Dice coefficient of similarity values between the same spider orchid species pairs from various locations. The range of values for each species pairs compared between species is shown in parentheses.

Group	Comparison pair	Dice similarity coefficient values[1] [2]
dilatata	par-lanLT/par-lanMSF	0.924 (0.921-0.928)
	pha-lanMR/pha-lanLT	0.831 (0.827-0.832)
	vil-lanMR/vil-lanLT	0.928 (0.920-0.937)
	vil-lanMR/vil-lanMSF	0.930 (0.920-0.937)
	vil-lanLT/vil-lanMSF	0.899 (0.894-0.902)
patersonii	con-alb/conaff-tya	0.962
	con-chiCY/con-alb	0.969 (0.967-0.973)
	con-chiCY/conaff-tya	0.960 (0.956-0.962)
	for-lanLT/for-lanMSF	0.937 (0.908-0.967)
	fra-swaDB/fra-mtr	0.933 (0.925-0.938)
	oen-belBW/oen-graSR	0.884 (0.873-0.890)
	venaff-stmFF/ven-lanMSF	0.923 (0.910-0.947)
reticulata	aus-wilLP/aus-wilTO	0.942 (0.921-0.959)
	ins-freQC/in-freMW	0.888 (0.871-0.909)
	ricsouSA/ric-norSA	0.912 (0.902-0.925)

[1] average Dice coefficient of similarity values

[2] minimum and maximum of Dice coefficient of similarity values are listed in parentheses

3.3.3 Cluster analysis

3.3.3.1 Cluster analysis obtained by Dice coefficients

The cluster analyses resulting from Dice coefficient will be explained together because of their similar dendrograms. Cluster analysis of 117 spider orchid

individuals and three outgroup taxa constructed by Dice coefficients in Figure 3.4 revealed three notable observations: 1) the outgroup species separated well from the spider orchids as expected (Figure 3.4). 2) different individuals within a species (as determined by experienced sample collectors) tended to be clustered closely with each other; and 3) the spider orchid grouping in this study supported the traditional grouping that the range of spider orchids under study can be classified into three groups based on their floral structure: reticulata group – the clubbed spider orchids; patersonii group - the glandular spider orchids; and dilatata group – the green-comb spider orchids (Jeanes and Backhouse, 2005). Thus, overall these molecular data supported results obtained from traditional methods of identification and classification of these plants.

Three main clusters comprising three groups of spider orchids were formed. The outgroup species, *C. flaccida*, *C. latifolia* and *L. menziesii* were found to be placed far apart from spider orchid species (Figure 3.4). The reticulata group are normally identified by sepals (and sometimes petals) tipped with distinct clubs that are formed by densely packed unicellular outgrowths (osmophores), and labellum margins fringed with short teeth (Jeanes and Backhouse, 2005). The forty- four specimens of these that were examined grouped into three clusters (I, II and III; Figure 3.4). Cluster I consisted of the species from Wilsons Promontory National Park including *C. australis* from Lilly Pilly Gully and Tidal Overlook Track, as well as *C. clavigera* from Derby Saddle, *C. calcicola* from Bats Ridge, Portland, *C. lowanensis* from Kiata Flora Reserve and *C. cruciformis* from Stuart Mill Flora and Fauna Reserve (Figure). Cluster II included the specimens of *C. insularis* from Mt. Wellington track, French Island, *C. insularis* from the Quarry on Cemetery track, French Island, *C. robinsonii*

from Rosebud and *C. reticulata* from Deep Lead Flora and Fauna Reserve, Stawell. Cluster III consisted of the spider orchids collected from Western Victoria and the border between Victoria and South Australia including *C. richardsiorum* and *C. hastata* (Figure 3.4).

The patersonii group is normally identified by sepals ending in long filamentous tips, usually covered with multicellular glandular hairs (not clubs) and labellum margins fringed with short to long teeth (Jeanes and Backhouse, 2000). The 51 specimens from this group examined clustered into four sub-groups, here named Clusters IV to VII. Cluster IV consisted of *C. formosa* from Longbottom Track, Langkoop, Mereek Rd and Meerek State Forest, a spider orchid hybrid taxon between *C. venusta* x *C. formosa* from Longbottom Track, Langkoop, Mereek, *C. fragrantissima* from Discovery Bay CP (Swan Lake) and *C. oenochila* from Baluk Willam Flora and Fauna Reserve, Belgrave South, which clustered with *C. fragrantissima* from Mt. Richmond National Park (Figure 3.4). However, the phylogenetic tree derived from the Dice coefficient indicated that one individual of *C. formosa* collected from Longbottom Track, Langkoop, Mereek clustered with the hybrid species with low bootstrap support (Figure 3.4). Cluster V comprised *C. rosella*, cultivated at Royal Botanic Garden Melbourne, *C. concolor* from Cyanide Dam, Chiltern-Pilot National Park, *C. concolor* from Albury, New South Wales, *C.* sp. aff. *concolor* from Murchison Hill, Tyaac, *C. cardiochila* from Anglesea, *C. orientalis* from Heathland Reserve, Wonthaggi and *C. pilotensis* from Mt. Pilot, Beechworth (Figure 3.4). Cluster VI included *C. oenochila* from Serra Road, Grampians National Park, *C.* sp. aff. *patersonii* from Inverleigh and *C. tessellata* from Lilly Pilly Gully, Wilsons Promontory National Park (Figure 3.4). The last cluster in the patersonii group was

the group of *C.* sp. aff. *venusta* from Stuart Mill Flora and Fauna Reserve, *C. venusta* from Mereek State Forest and *C.* sp. aff. *colorata* from George Track, Lower Glenelg National Park (Figure 3.4).

Members of the dilatata group have labella with a maroon apex and calli, and green marginal fringes or teeth. A total of 22 specimens from this group separated into two clusters (VIII and IX; Figure 3.4). Cluster VIII had *C. phaeoclavia* from Mereek Road, Langkoop, *C. tentaculata* from Glenelg River Road, Woolpooer, Grampians National Park. Cluster IX included *C. villosissima* from Mereek State Forest and Mereek Road, Langkoop, *C. stricta* from Little Desert National Park (gravel pits), *C. villosissima* from Longbottom track, Langkoop and *C. amoena*, cultivated at Royal Botanic Garden, Melbourne from plants collected at Wattle Glen, *C. parva* from Longbottom Track, Langkoop and Meerek State Forest and *C. phaeoclavia* from Longbottom Track, Langkoop (Figure 3.4).

Intraspecific similarities showed higher bootstrap values than the interspecific similarities for all taxa in this study. The Winboot program (Yap and Nelson, 1996) was used to perform UPGMA-based bootstrapping to test confidence in the groupings produced by this method. In general, the UPGMA clustering methods based on Dice and Jaccard coefficients indicated that the grouping of the same species clustered together with bootstrap value higher than 50% (Figure 3.4). However, all interspecific clustering were supported by bootstrap values lower than 50%.

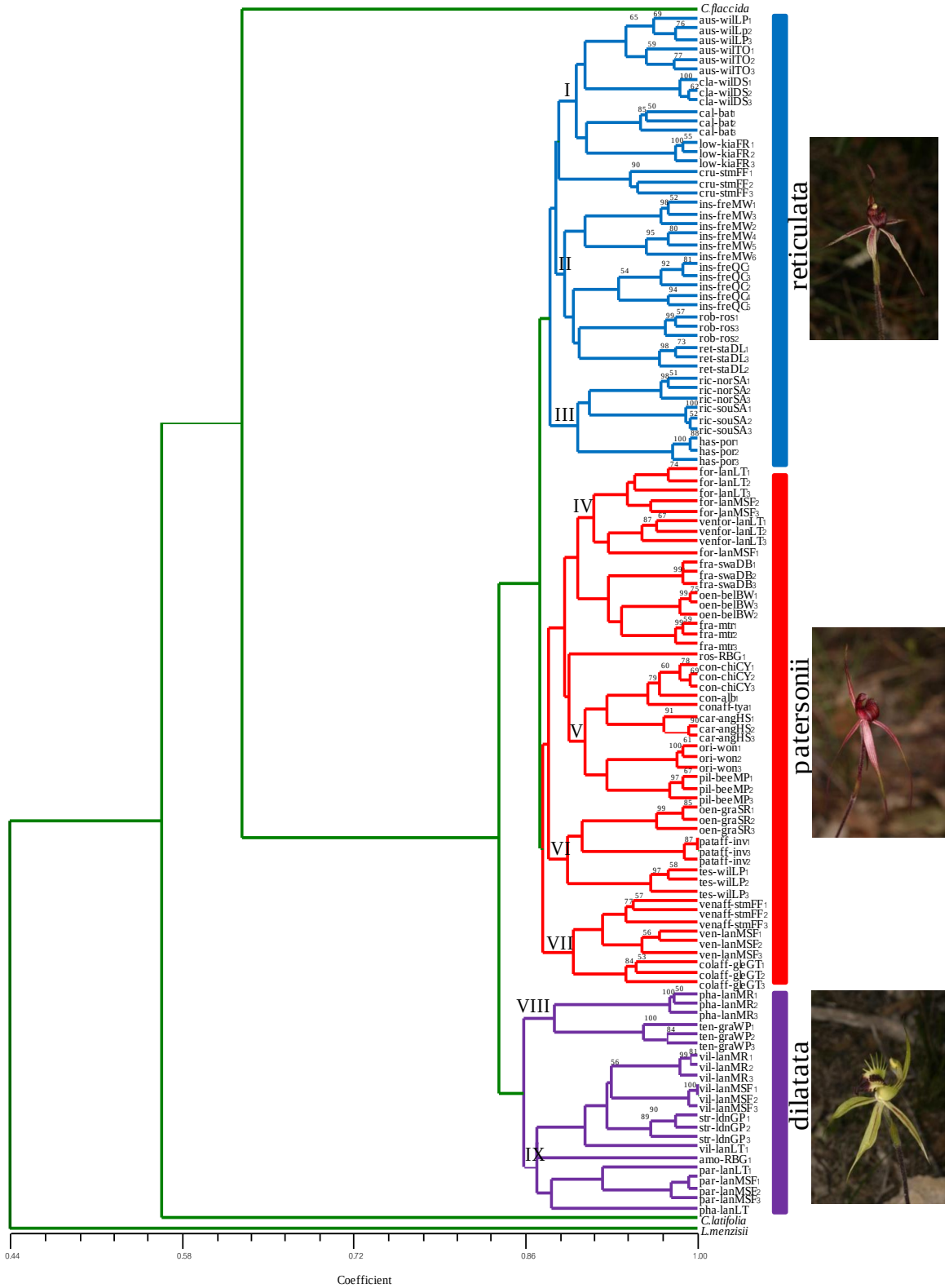


Figure 3.4 UPGMA Dendrogram showing genetic relationships of 117 spider orchid species and three outgroup taxa. The dendrogram was constructed using Dice similarity coefficient with bootstraps calculated above 50% are shown above the node.

3.3.3.2 Cluster analysis obtained by principal component analysis

The dendrograms obtained by UPGMA clustering method constructed indicated that spider orchid species have close genetic relatedness within the group and are distantly related to the outgroup taxa. In order to observe the genetic relatedness within spider orchids, outgroup taxa were removed from the analysis. Principal component analysis (PCA) was then performed in addition to cluster analysis only on data from the spider orchid species. The first and second principal component of the Dice genetic similarity matrices yielded 52% and 25% of the variance, respectively. A dispersion plot of all spider orchid individuals in the dimension defined by the two first components shows three main clusters of spider orchid groups indicating close genetic relationship between the reticulata and patersonii groups and more distant relationships with the dilatata group (Figure 3.5). The dilatata group separated in positive PCA 1 space while the patersonii group and one specimen from the reticulata group (*C. hastata*) seaparated in negative PCA 1 and positive PCA 2 space.

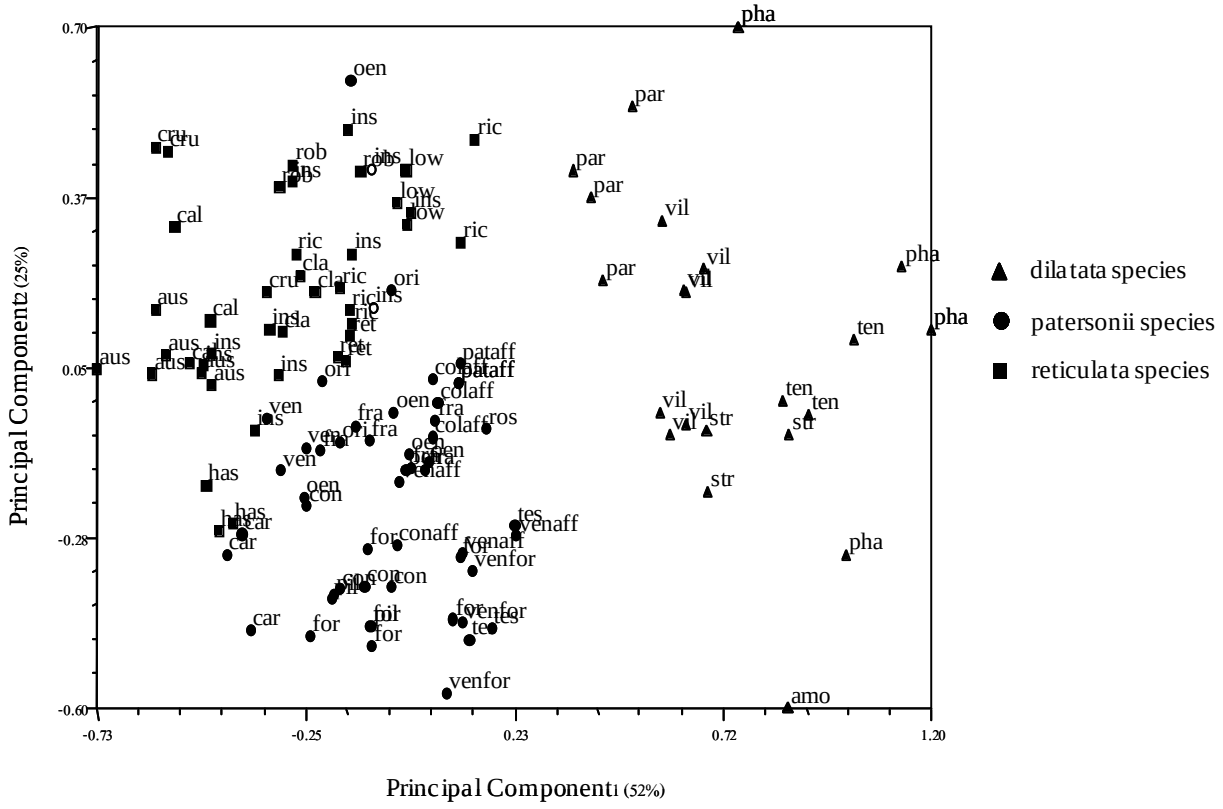


Figure 3.5 Principal component analysis plot of 117 *Caladenia spp.* individuals based on Dice similarity coefficients from 135 RAPD markers (stress1=0.20812; indicated as fair fit). The numbers in parentheses represent the percentage of variation accounted for by each principal component.

It is readily apparent that the dilatata group is well separated out from the other two spider orchid groups. In order to look at the genetic relatedness between the reticulata and patersonii groups, dilatata specimens were removed from the data set and re-analysed. The first and second principal component of the Dice genetic similarity matrices accounted for 41% and 31% of the variance, respectively (Figure 3.6). The majority of the reticulata group were distributed in negative PCA 1 and PCA 2 space. The patersonii group was well separated from the reticulata group with the exception of *C. venusta* which one specimen showed closely related to *C. insularis* and the other one indicated close relationship with *C. robinsonii*.

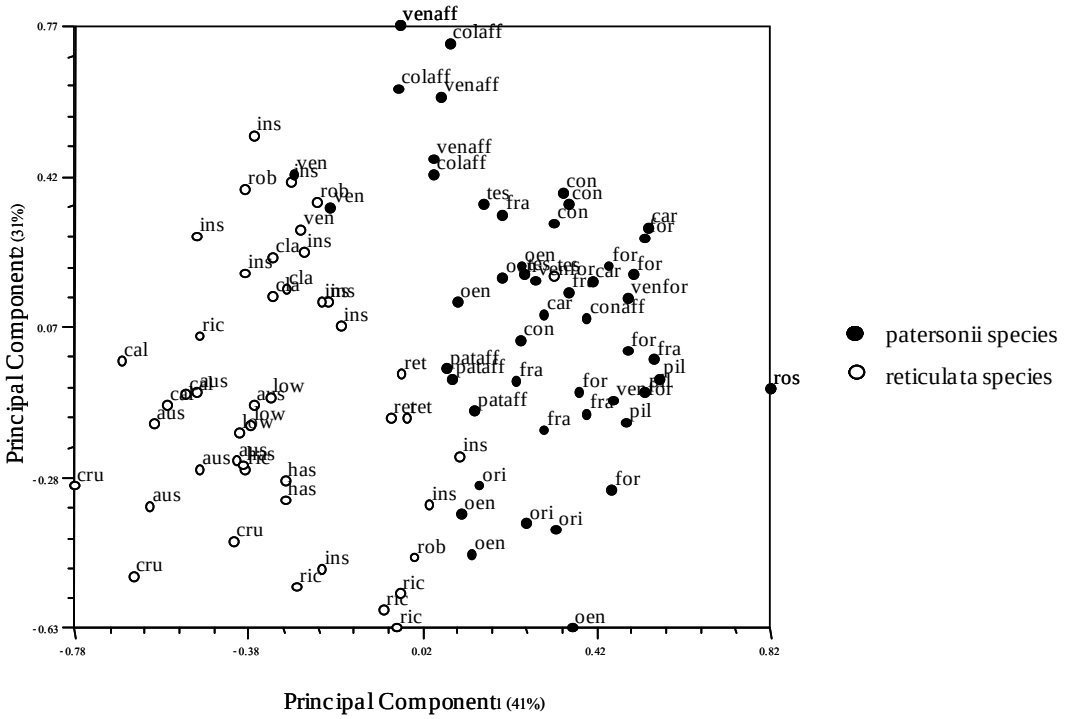


Figure 3.6 Principal component analysis plot of reticulata and patersonii individuals based on Dice similarity coefficients from 135 RAPD markers (stress1=0.24154; indicated as fair fit). The numbers in parentheses represent the percentage of variation accounted for by each principal component.

3.3.3.3 Parsimony analysis

The binary data obtained from RAPD marker was subjected to parsimony analysis. The cladogram indicated polytomies within the species of reticulata and patersonii. They were grouped into clade IV (Figure 3.7). The second clade included two species of Western distribution (*C. hastata* and *C. Richardsiorum*). The majority of representative of each species were group together with high bootstrap value support. The dilatata group was separated as their sister group.

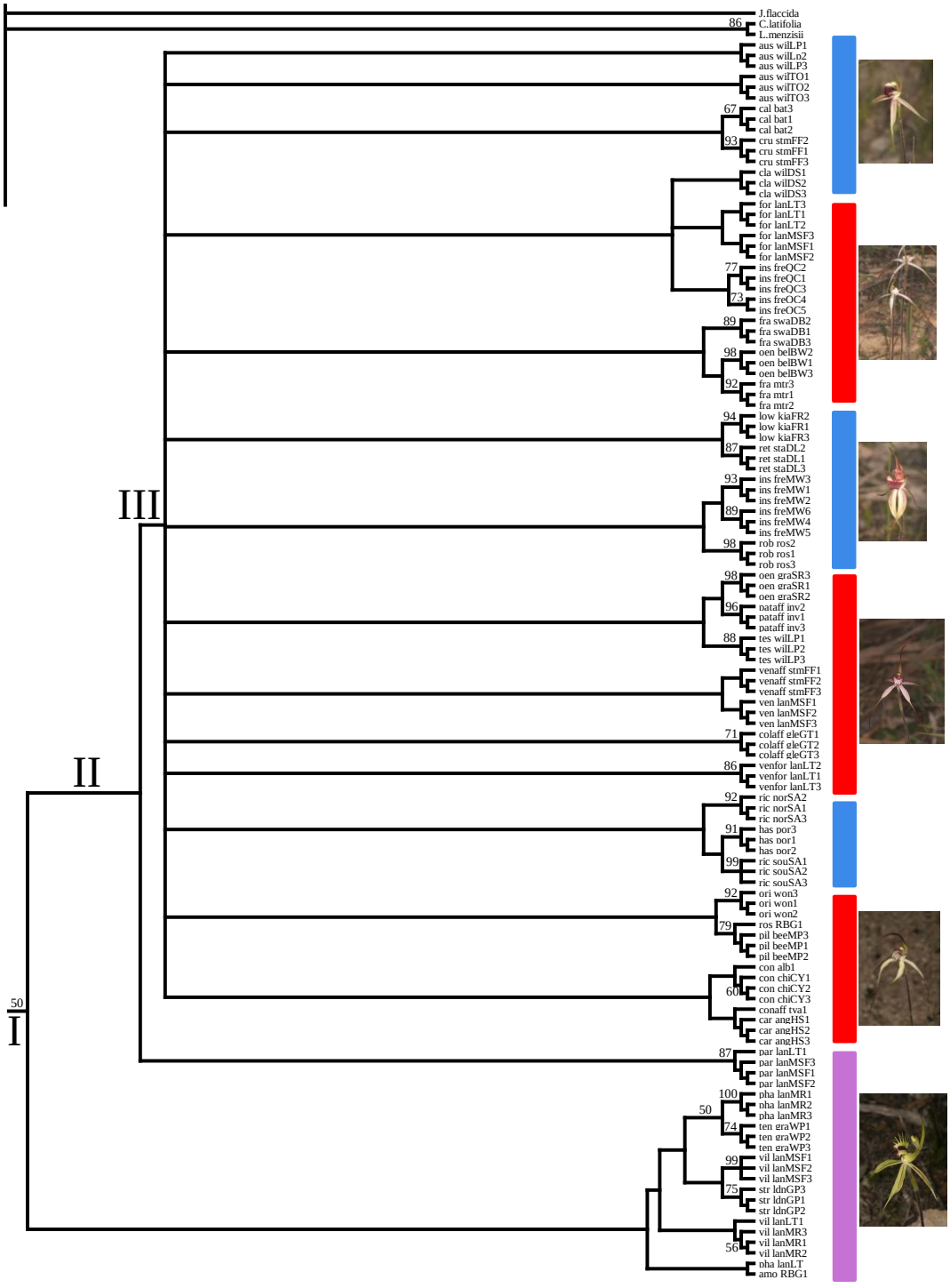


Figure 3.7 Single most parsimonious tree based on 135 RAPD markers (tree length, 850; CI=0. 1588, RI=0.6448). Bootstrap percentages greater than 50% are provided above the branches.

3.4 Discussion

3.4.1 Genetic variation among spider orchid species

This is the first report of the genetic relationships from a broad range of spider orchids in Victoria, Australia. The pair-wise estimates of genetic similarities at the molecular level obtained from RAPD markers revealed that most of spider orchid species showed a high degree of genetic similarity. Within 30 species of spider orchid, the similarity index ranged from 0.939 to 0.772. Furthermore, the mean genetic similarity values in each group of spider orchids were generally high, ranging from 0.824-0.930 in the dilatata group, 0.827-0.939 in the patersonii group and 0.834-0.910 in reticulata group. However, the similarity value obtained from RAPD markers in other studies on different plant groups were not as high as indicated here. For example, genetic similarity in *Aralia* ranged from 0.45 to 0.794 (Zhuravleve et al., 2003), 0.426 to 0.73 in *Huttuynia* (Wu et al., 2005), 0.59 to 0.92 in *Curcuma* (Nayak et al., 2006), 0.629 to 0.882 in *Paphiopedilum* (Chung et al., 2006), 0.72 to 0.55 in Prunus (Erturk et al., 2009), 0.110 to 0.97 in Zingiber (Bua-in et al., 2010) and 0.23 to 0.76 in *Phyllanthus* (Rout et al., 2010). The high genetic similarity was probably due to the fact that the RAPD primers used in this study yielded a low number of polymorphic bands in this sample of spider orchids. This probably reflects the selected RAPD primers that were chosen based on polymorphic bands produced between outgroup taxa and the randomly selected spider orchid DNA giving enough information to distinguish them. The selected strategy was based on the outgroup taxa chosen, *C. flaccida*, *C. latifolia* and *L. menziesii*. The outgroup species were previously included in the genus *Caladenia* but were not grouped with spider orchids, based on traditional morphological identification methods. As there was no molecular or genetic information available on the spider orchids, RAPD primers giving polymorphism

between members of different groups previously included within *Caladenia* were selected for screening the rest of spider orchids. Another explanation for having low polymorphism between spider orchids may be that there may be non-homologous bands of similar size present that mask differences in genetic sequences. In addition, RAPD markers are dominant and hence unable to distinguish between haplotypes, which is a disadvantage of this method, especially when comparisons are attempted to be made within and between closely related taxa (Rieseberg, 1996). The robustness of genetic similarity in this study was conducted by analysis of different similarity coefficients as well as parsimony analysis. Likewise, the robustness of the genetic relationships of Echinacea (Kapteyn et al., 2002) and flax species in the genus *Linum* L (Fu et al., 2002) was confirmed by different similarity coefficients.

3.4.2 Spider orchid relationships

It has been argued that a phylogenetic approach yields the most biologically meaningful framework for taxonomic decision-making (de Queizor and Gauthier, 1992). However, phylogenetic relationships among spider orchid species based on RAPD markers used in this study did not produce well resolved relationships. In addition, the RAPD-based phylogram had low bootstrap support. As a result, the confidence of the phylogenetic relationships was confirmed by the similarity groupings of Jaccard and Simple matching coefficients (Appendix, III). The robustness of genetic similarity conducted by different similarity coeffients was reported in genetic relationships of Echinacea species (Kapteyn et al., 2002). The separation of the outgroup taxa indicated that the RAPD markers used in this study proved a useful tool for clarifying genetic relatedness within subtribe Caladeniinae. These findings are in agreement with the study of Diurideae phylogeny by Kores *et*

al., 2001 based on *mat*K and *trn*L-F and that described by Clements et al. (2002) based on ITS analysis which pointed out that *Leptoceras menziesii* was genetically distant from *Caladenia*. Typical spider orchid species, *Caladenia* subgenus *Calonema*, were formed into three clusters, representing the reticulata, patersonii and dilatata groups. The three well-distinguished spider orchid groups were separated by RAPD markers used in this study. This evidence also found in cluster analysis based on RAPD marker that clearly identified genetic relationships in subtribe Oncidiinae and might provide genetic tools for indentifying Oncidiinae genotypes (Tsai *et al*., 2002).

In this study, RAPD markers divided fifteen recticulata species into three clusters. The group of *C. australis*, *C. calcicola*, *C. lowanensis* and *C. lowanensis* was separated from other recticulata based on their geographic distribution as well as the cluster of *C. hastata* and *C. richardsiorum*. Cluster analysis based on RAPD data has proven to support geographical distribution e.g. within *Vigna* (Massawe et al., 2003), *Salicornieae* (Murakeőzy et al., 2007) and *Vanilla* (Schluter et al., 2007). The rest of reticulata specimen was grouped together including *C. insularis*, *C. robinsonii* and *C. reticulata*. *Caladenia insularis* specimen from difference location did not join together. This indicated natural hybridization and/or speciation event among *C. insularis*. Since *C. insularis* from Mt. Wellington track is known to form a morphologically homogeneous population while the population from the Quarry on Cemetery track contains plants of varied appearance, these being considerably smaller and possibly of hybrid origin (Duncan, pers. Comm.). The ability of RAPD marker to detect speciation was also found in genetic relatedness in *Carthamu*s (Vilatersana et al., 2005), *Calamagrostis* (Saitou et al., 2007).

The position of *C. insularis* and *C. robinsonii* as identified by RAPD markers is not supported morphological characters. Some views of the taxonomic status of these two species suggest that these two above-mentioned species should be regarded as the same entity. However, RAPD profiles recognized *C. robinsonii* and *C. reticulata* as closely related species. This might due to the limited numbers of RAPD primer used in this study. Addition of more polymorphism marker such as AFLP might gain better resolution of phylogenetic analysis in the study of *Caladenia* species. Cluster analysis based on RAPD marker was previously reported to place closely related specimen in contrast with their morphological characters. This evidence found in beer and cooking banana cultivar (Pillay et al., 2001), *Cotoneaster tomentosus* and *C. socavianus* (Bartish et al., 2001) and the close relatedness of *Solanum scabrum* and *S. retroflexum* (Poczai et al., 2008).

Caladenia formosa and a taxon thought to be a hybrid between *C. venusta* x *C. formosa* are closely related as indicated by RAPD markers. This occurrence suggests that the presumed hybrid may have undergone backcrossing with *C. formosa*, or that the assumption of its hybrid status was incorrect and it represented extreme morphological variation within *C. formosa* sens. str. One individual of *C. formosa* from Langkoop Mereek was grouped with the presumed hybrid by the Dice method. This occurrence might support the idea of a backcrossing event between the hybrid and one of its parents. The ability of RAPD markers to detect hybrid species was also found in *Malus*. RAPD markers indicated that the position of a *Malus* hybrid was placed close to its parent on the dendrogram (Zhou and Li. 2000). According to RAPD based dendrogram, the hybrid of *Calamagrostis longiseta* var. *longe-aristata* also clustered with both parental clusters (Saitou et al., 2007).

The morphological differences in *C. fragrantissima* distributed in different location is that the one from Discovery bay CP (Swan Lake) has a creamy white flower whereas another from Mt. Richmond has a reddish flower. However, RAPD markers clustered the specimen of *C. fragrantissima* in the same clade. The explanation for this phenomenon is that the habitat fragmentation might play a role on species disruption and distribution patterns. Habitat fragmentation caused by human activity such as development of agriculture areas, rural development and/or climate change may force dispersing individuals to traverse a matrix habitat that separates suitable habitat fragments from each other (Ewers and Didham, 2006). *C. fragrantissima* occupying different environments could differ in their floral color. RAPD markers used in this study detected small differences but placed them in the same clade as sisters groups.

The grouping of *C. tessellata* is in contrast to the new classification system of Jeanes and Backhouse (2000). They propose that *C. cardiochila* and *C. tessellata* be placed into an informal group of their own (the 'cardiochila group'). Hopper and Brown (2000) also placed these two species in their *Caladenia* subgenus *Phlebochilus*, although they later conceded that the molecular evidence does not wholly support the morphological basis for their inclusion in the subgenus (Hopper and Brown, 2004a). Morphological characters of subgenus *Phlebochilus* include an unfringed, triangular or cordate labellum with calli sessile on or immersed in the upper surface, and tepals with no glandular hairs or clubs. However, the finding in this genetic study has positioned them apart from the other spider orchids. These findings both justify Hopper and Brown's caution and support Jones *et al*'s. (2001) placement of these two 'unique' species with the `true' spider orchids in the genus *Arachnorchis* or the equivalent subgenus *Calonema* as described by Hopper and Brown (2004a). The unique phenotype of *Cymbidium* was also detected by RAPD marker and the position

on RAPD based phenogram separated *C. lancifolium* and *C. aspidistrifolium* from other *Cymbidium* as distinct group (Choi et al., 2006).

The dilatata, or Green Comb spider orchid species have a cream or greenish perianth with variable crimson striping, and a cream to white labellum with green lateral lobes, a maroon tip and calli. RAPD marker classified closely related species between *C. phaeoclavia*, a brown-club spider orchid and *C. tentaculata*, a large-flowered plant with yellowish glandular tips. The grouping of different phynotypic species might occur by neutral hybridization which leading to speciation within dilatata group. This occurrence was also found in other orchids e.g. *Epidendrum* subgenus *Amphiglottium* (Pinheiro et al., 2009).

The position of Green Comb specimen on the RAPD based dendrogram did not support their similarity morphology might indicate long term hybridization within dilatata group species. The evidence to support the speciation hypothesis is the close relatedness and very similar floras structure of the species pairs *C. tentaculata* and *C. phaeoclavia*, *C. villosissima* and *C. stricta* and *C. phaeoclavia* and *C. parva*. Another point to be noted is that *C. phaeoclavia*, a medium-leafed species clustered with *C. parva* that was far removed geographically from the rest of *C. phaeoclavia* species in this study. Further molecular analysis of the congeneric *C. phaeoclavia*, *C. villosissima* and *C. parva* is required to address their genetic status. Another explanation for the findings in this study might be that these species really are just part of a single genetically and morphologically polymorphic species, a notion supported by studies of pollinator activity on these taxa in different areas. These pollinator studies showed that pollinators treated *C. parva* and *C. phaeoclavia* as the

same target species (C. Bower pers. comm.). Typically, pollinators of the spider Caladenias are species specific - a different species of Thynnid wasp being the obligate pollinator for each species of *Caladenia* (Bower, 1992; Dickson and Petit, 2006; Faast et al., 2009).

3.4.3 Biogeography

Although, RAPD-based phylogenetic analysis in this study did not resolve genetic relationships within the groups of spider orchid, some concordance between biogeographic and phylogenetic relationships was detected. In the reticulate group two species from Wilsons Promontory National Park, *C. australis* and *C. clavigera* were clustered together as indicated by their geography. The species from Western Victoria, *C. calcicola* was clustered with *C. lowanensis*. RAPD markers also grouped these species according to their distribution. The correlation between geographical distribution and RAPD markers was also reported within the sampled of *Houttuynia* (Wu et al., 2005), *Vanilla planifolia* specimens (Schluter et al., 2007) and *Catasetum* (Oliveira et al., 2010).

Even though unresolved taxonomic relationships within spider orchids remain, the genetic relationships obtained using RAPDs supplement the understanding of spider orchid taxonomy. This RAPD markers used in this present work gave low numbers of polymorphic markers to address the position of spider orchid species. The findings based on RAPD analysis mean that this technique can adequately delineate genetic relationships among these closely related spider orchid species, which were also compounded by the fact that limited polymorphic markers exist within the spider orchid genome, natural hybridization and/or introgression events often occur between

these. Thus RAPD markers used in this study are not suitable genetic markers to address the genetic relatedness among spider orchid. Further molecular work using more RAPD and/or AFLP markers need to be conducted in order to detect polymorphism within spider orchid genome.

In other genera of the family *Orchidaceae,* RAPD has been successfully used to delineate species level taxa. *Vanilla planifolia*, and it's relationship with *V. tahitensis* and *V. pompona* was clarified by using RAPD (Beese *et al*., 2004) RAPD markers were also used successfully as species-specific markers to analyse putative *V. planifolia* x *V. tahitensis* hybrid specimens. However, it failed to discriminate the other different specimens of Vanilla from Central America. The UPGMA clustering indicated three main phenetic groups belonging to three *Vanilla* species used. Low levels of genetic diversity were detected in cultivated *V. planifolia* specimens in Mexico which is in accordance with the vegetative mode of dispersion of vanilla plants (Schluter et al., 2007).

One possible explanation for unclear genetic relationships between spider orchid species in this study is that only 40 RAPD primers were used in the pre-screening step due to budgetary and time constraints. This limited number of primers may not have been enough to pick up the best primers in the study of genetic relationships in closely related species like spider orchids. Moreover, the focus for selection of plant species for use in the pre-screening step was genetic variation between spider orchids and outgroup taxa. As a result, the selected primers used in this study were based on those primers giving different banding patterns between spider orchids and outgroup taxa. As no other genetic information was available on spider orchids at this stage of the

study, it was not possible to be any more specific in the selection of spider orchid species for the preliminary study. In addition, the closeness of the outgroup taxa used in this study to the spider orchid groups was also in question and genetic distance between them had to be investigated first. For this reason the direction of this experiment was based on primers giving the optimum genetic variation between outgroup taxa and spider orchids. For further studies on spider orchid relationships based on RAPDs representatives from the members from each of the three groups, dilatata, reticulata and patersonii, should be included in the initial primer screening so that the primers selected are those that best separate these taxa.

Another point of view to be mentioned in this study is that RAPDs are known as dominant markers. As a result, the similarity at the sequence level of 'monomorphic' bands is questionable. In this study there may have been monomorphic fragments present of the same molecular weight but different nucleotide sequence across the entire spider orchid samples, which may have been the reason why 'genetic similarity' at the interspecific level was found, giving unresolved genetic relationships at that level. However, the study of Wu et al (1999) and Sales et al (2001) found that most co-migrating fragments are identical by lineage, at least at the intraspecific level. Even though co-migrating fragments in this study were not tested by sequence analysis, the dendrograms obtained from RAPDs data reflect the genetic similarity within species. However, the preliminary genetic relationships observation in this study placed the species belonging to each group into the right morphological grouping with some doubt about exact position within the group. The phylogeny of Jones *et al*. (2001) based on the internal transcribed spacer region of nuclear rDNA within *Caladenia* including three species of spider orchid also provided no resolution

of species level. Farrington *et al*. (2009) also found that the position within the group of subgenus *Calonema* was ambiguous based on combined data of *trn*L^{UAA} intron and *trn*Q-5'*rps* 16 intergenic spacer sequences. Further studies of genetic relationships among spider orchid species need to be carried out in order to answer questions about taxonomic status, i.e., of species within the groups. Understanding of the genetic relationships among spider orchids will be a good adjunct to conservation management that currently includes investigations into the pollination biology and the obligate mycobionts of these plants.

The position of spider orchid within their respective groups remained unclear. Therefore, further phylogenetic analysis was performed using ISSR marker to resolve genetic relationships of spider orchid at the interspecific level. The ISSR phylogeny will be presented in Chapter 4.

Chapter 4

Genetic Relatedness among *Caladenia* species

Using ISSR Markers

4.1 Introduction

Caladenia, a single hairy leaf orchid with long filamentous segments has a unique floral structure which can be recognized by spider like orchid. They can be classified into three groups based on their floral structure; reticulata or clubbed spider orchid, patersonii or glandular hairs spider orchid and dilatata or green comb (Jeanes and Backhouse, 2000). Due to their similarity floral structure, the identification based on their appearances is not suitable for taxonomic classification. Therefore, the molecular marker has been applied in this study.

Inter-simple sequence repeat (ISSR) markers are generated from single-primer PCR using designed primers from di-, trinucleotide repeat motifs such as $(GT)_n$, $(GA)_n$, and $(AGC)_n$ with a 5' or 3' anchoring sequence of one to three nucleotides (Gupta *et al.*, 1994; Fang and Roose, 1997; Godwin *et al.*, 1997; Wolfe *et al.*, 1998). Randomly anchoring sequences, used on a common microsatellite motif, produces unique banding profiles (Wolfe *et al.*, 1998). The amplified regions represent the nucleotide sequence between two SSR priming sites orientated on opposite DNA strands as shown in Figure 4.1 resulting in a multilocus marker system useful for fingerprinting, diversity analysis and genome mapping (Godwin *et al.*, 1997; Wolfe *et al.*, 1998). The tandem repeat units of 2-5 base pair motifs are abundant and distributed throughout

the eukaryotic genome. A large number of polymorphic bands can be generated from ISSR-PCR (Godwin *et al.,* 1997; Wolfe *et al.,* 1998). ISSR markers are inherited in a dominant or codominant Mendelian fashion (Gupta *et al.,* 1994). However, they are interpreted as dominant markers and scored diallelically with presence or absence of bands similar to RAPD data (Wolfe *et al.,* 1998). The absence of a band is interpreted as sequence divergence at the site of primer binding. This occurs by loss of a locus through the deletion of the site or chromosomal rearrangement (Wolfe and Liston, 1998). The ISSR technique is considered to be fast, simple, reproducible and capable of producing significant levels of polymorphism and is therefore appropriate for this study.

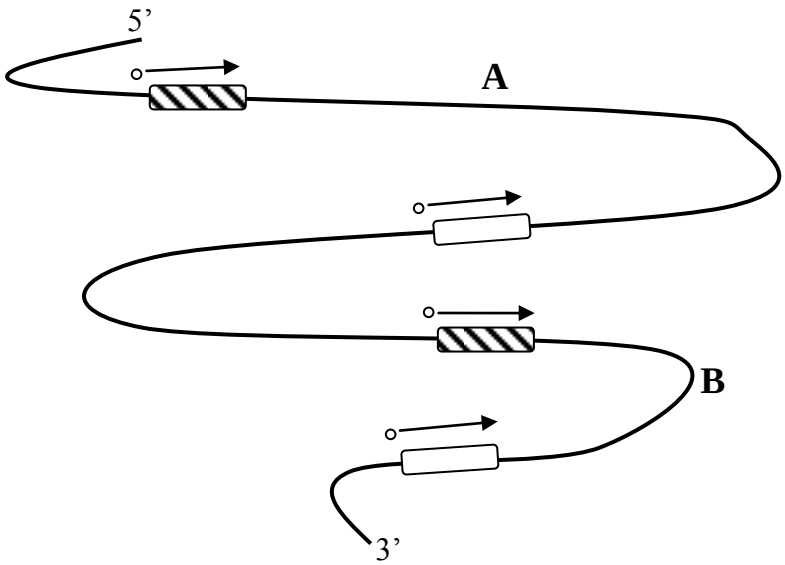


Figure 4.1 Diagram of ISSR primer annealing. A and B refer to inter-simple sequence repeat regions that are amplified if primer sequences anchored on the 5’ end of microsatellite regions are intact. Boxes with hash marks represent primer sequences orientated in the 5’ direction and clear boxes represent primer sequences on the complementary strand (from Wolfe *et al.,* 1998).

ISSR-PCR has been applied to several molecular approaches to examine various taxonomic levels including genus, species and variety. ISSR markers have also been used to identify and differentiate among accessions. For example, ISSR fingerprinting has been used for:

- cultivar identification in:
    - citrus (Fang and Roose, 1997),
    - strawberry (Arnau *et al.*, 2002) and
    - olive (Terzopoulos *et al.,* 2005),
- assessing hybridization in a natural population of *Penstemon* (Wolfe *et al.*, 1998)
- genetic diversity and relationships of
    - sweetpotato (Huang and Sun, 2000)
    - tea (Mondal, 2002)
    - lotus (Tian *et al*., 2008),
- genome mapping (Sankar and Moore, 2001; Collard, 2002; Kongjaroon, 2002)
- marker-assisted selection in chickpea (Millan *et al*., 2003)
- phylogenetic relationships of:
    - *Oryza* (Joshi *et al*. 2000)
    - *Cicer* (Iruela., 2002)
    - *Lycopersicon* (Tikunov, 2003)
    - *Dendrobium* (Wang *et al*., 2009a)
- population structure and genetic diversity of *Botrychium pumicola* (Francisco and Liston, 2001)
- genetic diversity in:
    - olives (Gemas *et al.,* 2004)

- *Vanilla* (Verma *et al*., 2009).

The aim of this chapter was to investigate the genetic relationships among Victorian spider orchid species in Victoria using ISSR markers and to evaluate the usefulness of ISSR marker as alternative character for spider orchid classification.

4.2 Material and methods

All samples used in this study were as described in Chapter 3.

4.2.1 Amplification of ISSR marker

The set of microsatellite UBC number 9 ISSR primers with different di-, tri-, and tetra-nucleotide repeats, were prescreened with some spider orchids which representative of each group and the outgroup taxa for optimizing PCR conditions. PCR amplification was carried out in a final volume of 25 µl in PCR buffer (20 mM Tris HCl pH 8.4, 50 mM KCl), 2.5 mM $MgCl_2$, 0.24 mM of each dNTP, 1 unit of Taq polymerase (Invitrogen, Australia) and 0.2 µM primer (obtained from the University of British Columbia, Canada). The PCR amplification cycle involved incubation at 94^0C for 5 min followed by 40 cycles of 94^0C for 1 min, 42-50^0C for 1 min (42^0C for UBC 842, 856, 864, 884, 45^0C for UBC 809, 825, 890, 48^0C for UBC 810, 848 and 50^0C for UBC 886) and 72^0C for 2 min with a final extension at 72^0C for 7 min. The PCR were repeated three times in order to examine the reproducible amplification product. Amplification products were then separated on 2% agarose containing 0.6 µg/ml of ethidium bromide in TBE buffer, and visualized under UV light.

4.2.2 Data analysis

Data analysis was carried out in the same manner as with Chapter 3. Cluster analysis was computed based on an original data of 68 reproducible polymorphic markers giving some misplacement of key species on the dendrogram. This was suspected to be due to the polymorphism of bands within the same species confounding the analysis. Thus, five markers, in particular, polymorphic bands within the same species were removed from the data set in order to determine whether at least the various species clustered into their expected groups and to get an idea of genetic relatedness between species. This is in similar way as previously reported by Huang, *et al*. (2002) that polymorphic markers within taxa were discarded from the analysis to avoid intrataxon variation that might confound the analysis of inter-taxa relationships. This would, of course, assume that the species as named by the collectors were correctly named. The grouping of these species based on RAPD analysis has confirmed that they were indeed the same species.

According to the higher cophenetic correlation values obtained by Dice genetic similarity coefficient in Chapter 3, the analysis based on ISSR markers was performed using Dice genetic similarity coefficient.

4.3 Results

4.3.1 ISSR marker profile

4.3.1.1 Number of monomorphic and polymorphic bands produced within spider orchid species

Of the 100 primers assessed, only nine dinucleotide and one trinucleotide motif ISSR primers gave clear and unambiguous banding. A total of 63 reproducible and clearly

scorable bands produced from the ten ISSR primers were assessed across the entire sample (117 spider orchid specimens and three outgroup taxa). Only polymorphic markers that were missing in at least one individual were selected as input data. An example of a DNA profile obtained from one of the ISSRs primer is shown in Figure 4.2. The approximate size of the largest DNA fragment produced was 1.4 kb and the smallest fragment produced was 0.25 kb (Table 4.1). The most informative primer was UBC 842, which consisted of a dinucleotide GA repeat unit anchored with YG, giving ten informative DNA fragments (Table 4.1). UBC 810 also has the GA repeat motif, but is anchored with AT and this produced eight polymorphic bands across all the samples in this study (Table 4.1). The dinucleotide AG motif anchored at the 5'-end with HBH, UBC 884 primer, also gave highly informative DNA fragments. The repeat units of CA anchored with RG (UBC 848) and dinucleotide AC motif anchored with YA (UBC 856) were also found to give a high number of amplification fragments. Among polymorphic loci, three loci were found to be monomorphic in the outgroup taxa and 8 loci were monomorphic within the sampled spider orchid species. Fifty-nine DNA fragments were observed to be polymorphic among spider orchid species and outgroup taxa together, whereas 57 DNA amplification fragments were polymorphic within spider orchid species. There was no specific marker obtained for spider orchid group identification and species identification.

Table 4.1 Detailed ISSR analysis showing primer sequences, band size, number of monomorphic and polymorphic within either outgroup or ingroup.

Primer	Sequence (5'→3')	Approximate fragment size range (bp)	Number of markers				
			No. of bands monomorphic within outgroup taxa	No. of polymorphic bands within outgroup taxa	No. of monomorphic bands within spider orchid species	No. of polymorphic bands within spider orchid species	Total
UBC 809	$(AG)_8G$	550-1000	0	4	1	4	5
UBC 810	$(GA)_8T$	300-900	3	4	0	8	8
UBC 825	$(AC)_8T$	750-1400	0	5	1	4	5
UBC 842	$(GA)_8YG$	250-1100	0	9	1	9	10
UBC 848	$(CA)_8RG$	480-1110	0	7	1	7	7
UBC 856	$(AC)_8YA$	450-1200	0	7	0	7	7
UBC 864	$(ATG)_6$	500-850	0	4	0	4	4
UBC 884	$HBH(AG)_7$	250-1000	0	7	2	7	9
UBC 886	$VDV(CT)_7$	500-800	0	3	1	3	4
UBC 890	$VHV(GT)_7$	700-1100	0	4	0	4	4
Total			3	54	8	57	63

[a] R stands for puRine, Y for pYrimidine, B for non-A, D for non-C, H for non-G, V for non-T residues

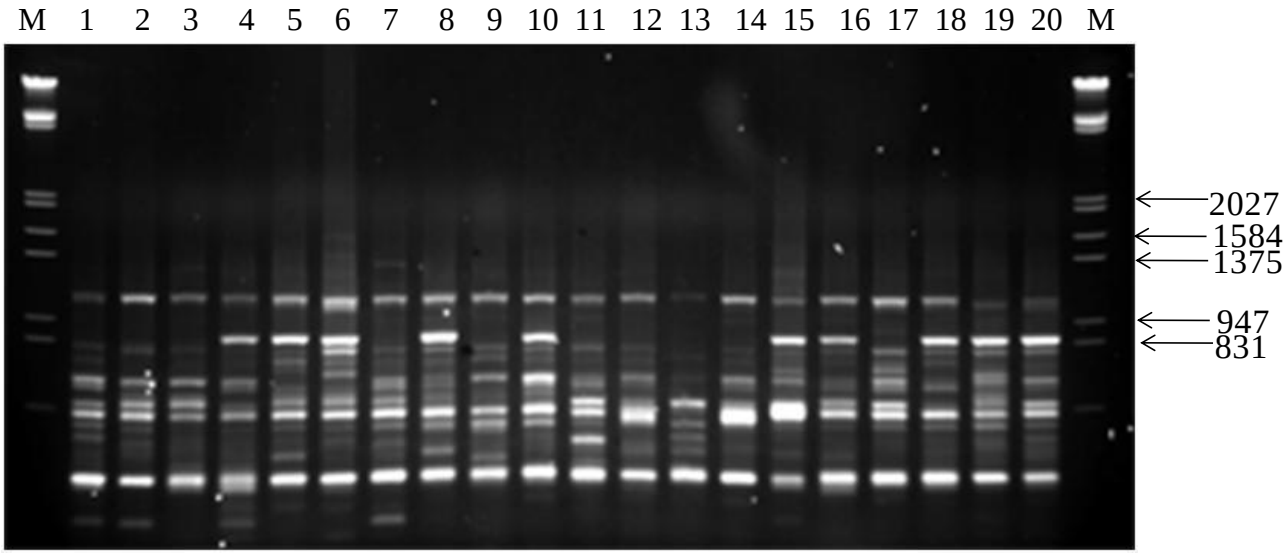


Figure 4.2 Amplification products generated from primer UBC 842 on 20 individual plants. Lanes M=λ DNA *EcoRI* and *Hind III* molecular weight marker, Lane 1=*C. formosa*, Lanes 2-4=*C. fragrantissima*, Lanes 5-7=*C. lowanensis*, Lanes 8-18=*C. insularis*, Lanes 19-20=*C. oenochila*.

4.3.1.2 Number of bands produced per primer and which primer produced the most and least polymorphism

The total number of amplified fragments across the entire samples obtained from the ten ISSR primers was 5375 with an average of 4.48 fragments per primer (Table 4.2). Of these, UBC 842 primer gave the highest average number of amplification products of 6.7 fragments, while UBC 825 (AC motif) produced an average of 3.0 DNA fragments. The nine polymorphic fragments out of ten amplification products produced from UBC 842 gave the highest number of polymorphisms within outgroup taxa. The polymorphism among outgroup taxa ranged from 50% produced from UBC 810, GA repeat unit anchored with T, to 100% amplified by UBC 825 (AC motif), UBC 848 (CA motif), UBC 856 (AC motif), UBC 864 (ATG motif) and UBC 890 (GT motif) (Table 4.2). All DNA fragments produced from UBC 810 (AG motif), UBC 848 (CA motif), UBC 856(AC motif), UBC 864 (ATG motif) and UBC 890 (GT motif) were polymorphic within spider orchid species.

Table 4.2 The number and level of polymorphism revealed by ISSR primers.

Primer	Total No. of polymorphic fragments across all species tested	No. of polymorphic bands per primer (N_T)	Average No of fragments per primer[a]	No. of polymorphic markers (N_P)		% Polymorphism ($P= N_P/ N_T$ x 100)	
				Within outgroup	Within spider orchid species	Within outgroup	Within spider orchid species
UBC 809	422	5	3.5	4	4	80	80
UBC 810	582	8	4.9	4	8	50	100
UBC 825	362	5	3.0	5	4	100	80
UBC 842	805	10	6.7	9	9	90	90
UBC 848	702	7	5.8	7	7	100	100
UBC 856	657	7	5.5	7	7	100	100
UBC 864	417	4	3.5	4	4	100	100
UBC 884	604	9	5.0	7	7	77.8	77.8
UBC 886	429	4	3.6	3	3	75	75
UBC 890	395	4	3.3	4	4	100	100

[a] total number of bands/number of species tested

4.3.1.3 Polymorphisms produced by primers within the spider orchid groups

Thirty-two polymorphic markers were found in the reticulata and patersonii groups (51% polymorphism) and 31 in dilatata groups (49% polymorphism) (Table 4.3). The highest number of polymorphic markers was produced from UBC 809 with 4 markers (80%) in the reticulata group, UBC 810 with 6 markers (75%) and UBC 890 with 3 markers (75%) in the patersonii group and UBC 825 with 4 markers (80%) in the dilatata group. UBC 856 produced only one polymorphic marker (14%) across all spider orchid group specimens.

Table 4.3 The frequency of polymorphic products of ISSR primers in spider orchid group species.

Primer	Number of markers									
	reticulata group			patersonii group			dilatata group			Total
	Monomorphic		Polymorphic (%)	Monomorphic		Polymorphic (%)	Monomorphic		Polymorphic (%)	
	(Absent in all)	(Present in all)		(Absent in all)	(Present in all)		(Absent in all)	(Present in all)		
UBC 809	0	1	4 (80)	1	1	3 (60)	0	2	3 (60)	5
UBC 810	1	2	5 (63)	1	1	6 (75)	1	2	5 (63)	8
UBC 825	0	2	3 (60)	1	2	2 (40)	0	1	4 (80)	5
UBC 842	0	6	4 (40)	1	4	5 (50)	0	4	6 (60)	10
UBC 848	0	3	4 (57)	0	3	4 (57)	0	3	4 (57)	7
UBC 856	1	5	1 (14)	1	5	1 (14)	1	5	1 (14)	7
UBC 864	0	2	2 (50)	0	2	2 (50)	1	2	1 (5)	4
UBC 884	1	3	5 (56)	1	3	5 (56)	2	3	4 (44)	9
UBC 886	0	3	1 (25)	0	3	1 (25)	0	3	1 (25)	4
UBC 890	0	1	3 (75)	0	1	3 (75)	0	2	2 (50)	4
Total		28	32 (51)		25	32 (51)		27	31 (49)	63

4.3.2 Genetic variation within spider orchid species.

The Dice coefficient was employed in the ISSR analysis to study relationships among the spider orchid species and three outgroup taxa. The genetic similarity among outgroup taxa, *C. latifolia* and *C. flaccida* was found to be 0.373, between *C. latifolia* and *L. menziesii* it was 0.321 and between *L. menziesii and C. flaccid* was 0.400. The mean genetic similarity among spider orchid species ranged from 0.770 between *C.* sp. aff. *concolor* from Murchison Hill, Tyaac and *C. tentaculata* from Grampians National Park and 0.976 between *C. parva* from Longbottom Track, Langkoop and *C. villossisima* from Meerek State Forest (Table 4.4). The genetic similarity observed in the dilatata group indicated that the lowest mean value was 0.800 between *C. phaeoclavia* and *C. parva* from Longbottom Track, Langkoop. The highest mean value was 0.976 between *C. parva* from Longbottom Track, Langkoop and *C. villossisima* from Meerek State Forest. The lowest mean genetic similarity value of 0.834 in patersonii group was obtained between *C.* sp. aff. *concolor* from Murchison Hill, Tyaac and *C. formosa* from Longbottom Track, Langkoop Meerek while the highest mean genetic similarity value of 0.961 was found between *C. venusta* from Meerek State Forest and *C.* sp. aff. *venusta* from Stuart Mill Flora and Fauna. In the reticulata group, the lowest mean genetic similarity (0.833) was observed between *C. lowanensis* from Kiata Flora Reserve and *C. australis* from Tidal Overlook Tk, Wilsons Promontory National Park, while the highest mean genetic similarity (0.939) was between *C. reticulata* from Deep Lead Flora and Fauna Reserve, Stawell and *C. hastata* from Portland.

Table 4.4 Average Dice similarity coefficient values between the same spider orchid species pairs from various locations.

Group	Comparison pair[1]	Dice similarity coefficient values	
		Average minimum[2]	Average maximum[2]
Spider orchids	conaff.-tya/ten-graWP	0.770 (0.764-0.773)	
	par-lanLT/vil-lanMR		0.976 (0.964-0.988)
dilatata	pha-lanLT/par-lanLT[3]	0.800	
	par-lanLT/vil-lanMR		0.976 (0.964-0.988)
patersonii	conaff.-tya/for-lanLT	0.834 (0.819-0.854)	
	ven-lanMSF/venaff.-stmFF		0.961 (0.857-0.969)
reticulata	low-kiaFR/aus-wilTO	0.833 (0.813-0.860)	
	rec-staDL/has-por		0.939 (0.917-0.957)

[1] comparison pair selected from Dice similarity matrix based on their similarity coefficient value

[2] average minimum and average maximum values of genetic similarity of pairs of individuals within each species pair with range of Dice's coefficient of similarity values are listed in parentheses

[3] one sample per species tested

The Dice similarity coefficient observed within the species of spider orchids from different geographical areas ranged from 0.834 to 0.976 (Table 4.5). The mean genetic similarity in the dilatata group was derived from *C. parva, C. phaeoclavia* and *C. villosissima*. The highest mean genetic similarity (0.914, range 0.905-0.929), was in the dilatata complex between *C. phaeoclavia* from Mereek Road, Langkoop and Longbottom Track. The lowest mean genetic similarity (0.834, range 0.822-0.844), was found in *C. parva* collected from Longbottom Track, Langkoop and Meerek State Forest.

Species in the patersonii group that have very similar floral structure but are regarded as distinct entities included *C. concolor, C.* sp. aff. *concolor, C. formosa, C. fragrantissima, C. oenochila, C. venusta* and *C.* sp. aff. *venusta*. The mean genetic similarity between *C. concolor* from Albury, New South Wales and *C.* sp. aff. *concolor* from Murchison Hill, Tyaac was 0.952 (Table 4.5). *Caladenia* sp. aff. *venusta* from Stuart Mill Flora and Fauna Reserve and *C. venusta* from Meerek State Forest had a highest mean genetic similarity of 0.961, (range 0.957-0.969). The lowest mean genetic similarity found between *C. concolor* from Cyanide, Chiltern-Pilot National Park and *C. concolor* from Albury, New South Wales and *C.* sp. aff. *concolor* from Murchison Hill, Tyaac was 0.911, ranged from 0.897 to 0.930 (Table 4.5).

The mean genetic similarity between the same species within the reticulata group was calculated from *C. australis, C. insularis* and *C. richardsiorum*. *C. australis* from Lilly Pilly Gully and Tidal Overlook Tk, Wilsons Promontory National Park. *Caladenia richardsiorum* collected from Southern, South Australia and Nora Creina,

South Australia had a highest mean genetic similarity value of 0.937 between individuals, range from 0.911 to 0.957. The genetic similarity between *C. insularis* specimens from Quarry on Cemetery Track, French Island and *C. insularis* from Mt. Wellington Track, French Island indicated the average value of 0.899, had a wider range of values with a minimum value of 0.864, and maximum value of 0.933.

Table 4.5 Dice coefficient of similarity values between the same spider orchid species pairs from various locations.

Group	Comparison pair	Dice similarity coefficient values[1 2]
dilatata	par-lanLT/par-lanMSF	0.834 (0.822-0.844)
	pha-lanMR/pha-lanLT	0.914 (0.905-0.929)
	vil-lanMR/vil-lanLT	0.976 (0.964-0.988)
	vil-lanMR/vil-lanMSF	0.873 (0.847-0.892)
	vil-lanLT/vil-lanMSF	0.856 (0.840-0.864)
patersonii	con-alb/conaff.-tya	0.952 (0.952-0.952)
	con-chiCY/con-alb	0.911 (0.897-0.930)
	con-chiCY/conaff.-tya	0.911 (0.897-0.930)
	for-lanLT/for-lanMSF	0.929 (0.902-0.955)
	fra-swaDB/fra-mtr	0.918 (0.889-0.936)
	oen-belBW/oen-graSR	0.936 (0.920-0. 957)
	venaff.-stmFF/ven-lanMSF	0.961 (0.957-0.969)
reticulata	aus-wilLP/aus-wilTO	0.903 (0.891-0.923)
	ins-freQC/in-freMW	0.899 (0.864-0.933)
	RicsouSA/ric-norSA	0.937 (0.911-0.957)

[1] average Dice coefficient of similarity values

[2] minimum and maximum of Dice coefficient of similarity values are listed in parentheses

4.3.3 Cluster analysis

4.3.3.1 Cluster analysis using the original data obtained by Dice coefficient

Cluster analyses constructed by Dice coefficient are shown in Figure 4.3. The resulting phylogenetic tree revealed three notable observations which were also found in the RAPD data analysis: 1). spider orchids fell into the same three groups as the ones based on their floral structure (reticulata: clubbed spider orchids; patersonii: glandular spider orchids; and dilatata: green comb spider orchids), 2). individuals of the same species from different geographical region tended to be clustered closely with each other and, 3). outgroup species separated well from spider orchids.

Forty-four samples from the reticulata group aggregated into five clusters (Figure 4.3). Cluster I consisted of the species of *C. australis* from Wilsons Promontory National Park including Lilly Pilly Gully and Tidal Overlook Tk (Figure 4.3). *Caladenia clavigera* (cluster II) from Derby Saddle was sister to *C. australis* (Figure 4.3). Cluster III included *C. lowanensis* from Kiata Flora Reserve which grouped together with the subcluster of *C. hastata* from Portland and *C. reticulata* from Deep Lead Flora and Fauna Reserve, Stawell. Three individuals of *C. insularis* from the Quarry on Cemetery Track, French Island were also included in this group (Figure 4.3). Cluster IV consisted of *C. calcicola* from Bats Ridge, Portland clustered with the subcluster of *C. cruciformis* from Stuart Mill Flora and Fauna Reserve and three individuals of the species of *C. insularis* from Mt. Wellington Track, French Island. Cluster V consisted of the remaining *C. insularis* from Mt. Wellington Track and Quarry on Cemetery Track, French Island and *C. robinsonii* from Rosebud that clustered together with the specimens of *C. richardsiorum* from Western Victoria and the border of Victoria and South Australia.

The second group was comprised of the 51 `patersonii complex' specimens including four sub-groups (cluster VI to cluster IX). Cluster VI contained *C. oenochila* from Belgrave South, Baluk Willam Flora and Fauna Reserve clustered together with the group of *C. orientalis* from Wonthaggi Heathland Reserve and *C.* sp. aff. *patersonii* from Inverleigh which joined together with the subcluster of *C.* sp. aff. *colorata* from George's Track, Lower Glenelg National Park which grouped with subcluster of *C.* sp. aff. *venusta* from Stuart Mill Flora and Fauna Reserve and *C. venusta* from Meerek State Forest (Figure 4.3). Cluster VII comprised *C. concolor* from Cyanide Dam, Chiltern-Pilot National Park, *C. oenochila* from Serra Road, Grampians National Park, *C. rosella*, cultivated at Royal Botanic Garden Melbourne from plant collected at Hurstbridge, *C. concolor* from Albury, New South Wales and *C.* sp. aff. *concolor* from Murchison Hill, Tyaac . Cluster VIII included *C. tessellata* from Lilly Pilly Gully, Wilsons Promontory National Park, *C. cardiochila* from Anglesea, and *C. pilotensis* from Mt. Pilot, Beechworth. Cluster IX comprised *C. formosa* from Longbottom Track, Langkoop Meerek clustered with the group of *C. venusta* x *C. formosa* hybrids from Longbottom Track, Langkoop Meerek and *C. formosa* from Meerek State Forest grouping with subcluster of *C. fragrantissima* from Discovery Bay CP (Swan Lake) and *C. fragrantissima* from Mt. Richmond National Park.

The members of the 'dilatata complex' separated into two clusters (Figure 4.3). Cluster X included *C. phaeoclavia* from Mereek Road, Langkoop and Longbottom Track, Langkoop and *C. tentaculata* from Glenelg River Road, Woolpooer, Grampians National Park joining with subcluster of *C. parva* from Longbottom Track, Langkoop and *C. villosissima* from Mereek Road, Langkoop (Figure 4.3). Cluster XI included *C. parva* from Meerek State Forest grouped with *C. stricta* from

Little Desert National Park (gravel pits) which clustered with the group of *C. villosissima* from Meerek State Forest and *C. amoena*, cultivated at Royal Botanic Garden from plants collected at Wattle Glen, Melbourne (Figure 4.3).

The confidence limits of UPGMA-based bootstrapping testing the groupings was performed. One thousand bootstrap replications were carried out. In general, the UPGMA clustering methods based on Dice coefficients supported intraspecific groupings as shown by bootstrap values of greater than 50% (Figure 4.3). However, all interspecific clusterings were supported by low bootstrap values (lower than 50%).

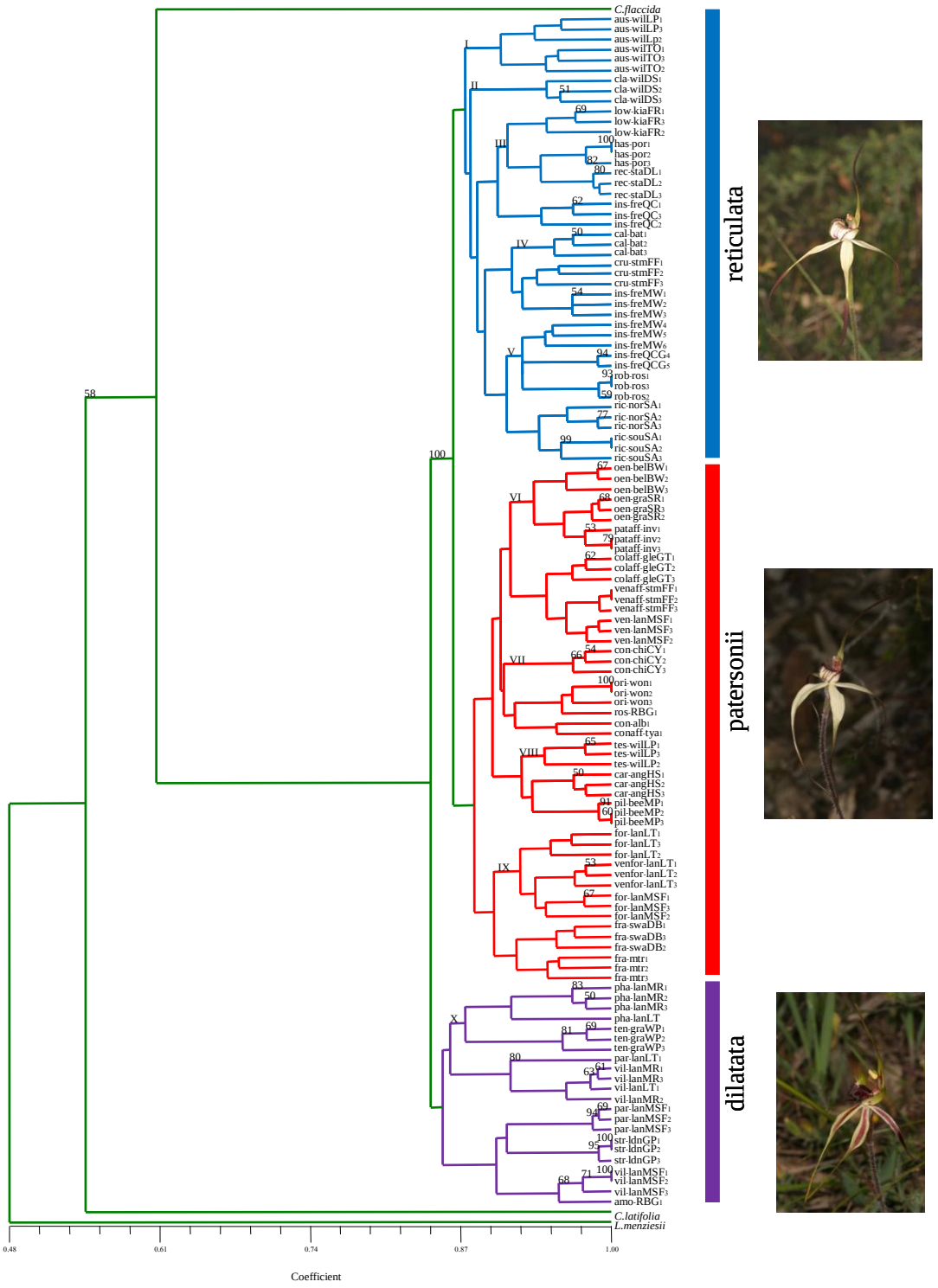


Figure 4.3 Dendrogram showing genetic relationships of 117 spider orchid species and three outgroup control species. The dendrogram was constructed using DICE coefficient of similarity and UPGMA clustering obtained from 63 ISSR markers. Bootstrap values above 50 are shown above the node.

As mentioned in Chapter 3, outgroup taxa were removed for principal component analysis in order to observe the relationships within spider orchids. The ISSR similarity matrix obtained by Dice coefficient without outgroup taxa was used as an input data for PCA analysis.

4.3.3.2 PCA

The PCA result was reflected in the scatterplot which clearly distinguished species of each group into three well-differentiated groups. The dilatata separated in negative PCA 1 and positive PCA 2. Majority of patersonii separated in negative PCA 1 and PCA 2 while most of reticulate group separated in negative PCA 1 and positive PCA 2. The first and second principal component of the genetic similarity matrix yielded equally 40% of the variance (Figure 4.4).

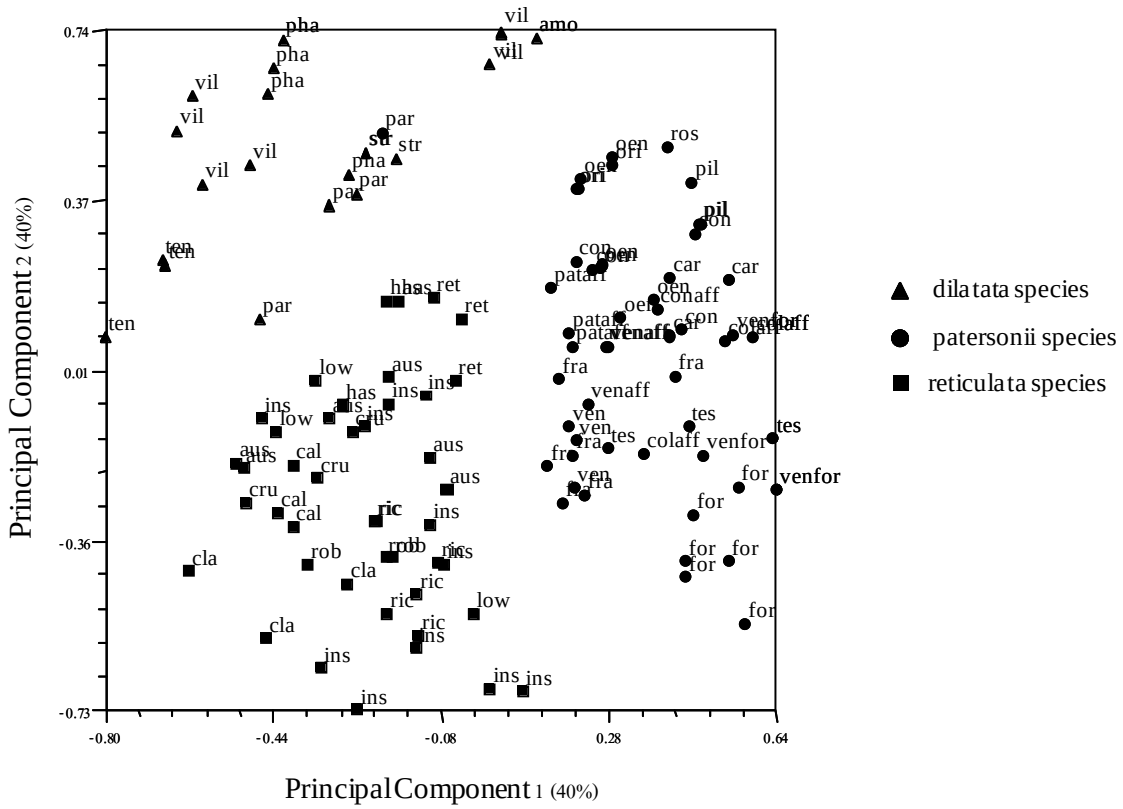


Figure 4.4 PCA plot of 117 spider orchid individuals individuals based on Dice coefficient obtained from 63 ISSR markers (stress1= 0.23086; indicated as fair fit). The numbers in parentheses represent the percentage of variation accounted for by each principal component

4.3.3.3 Parsimony analysis

Parsimony analysis obtained from 63 ISSR markers produced a single tree of length 531, and with CI = 0.1186 (Figure 4.5). Outgroup taxa was separated from spider orchid on clade I. Clade II consisted of the grouped of reticulata and dilatata species. Patersonii classified into clade III as their sister group.

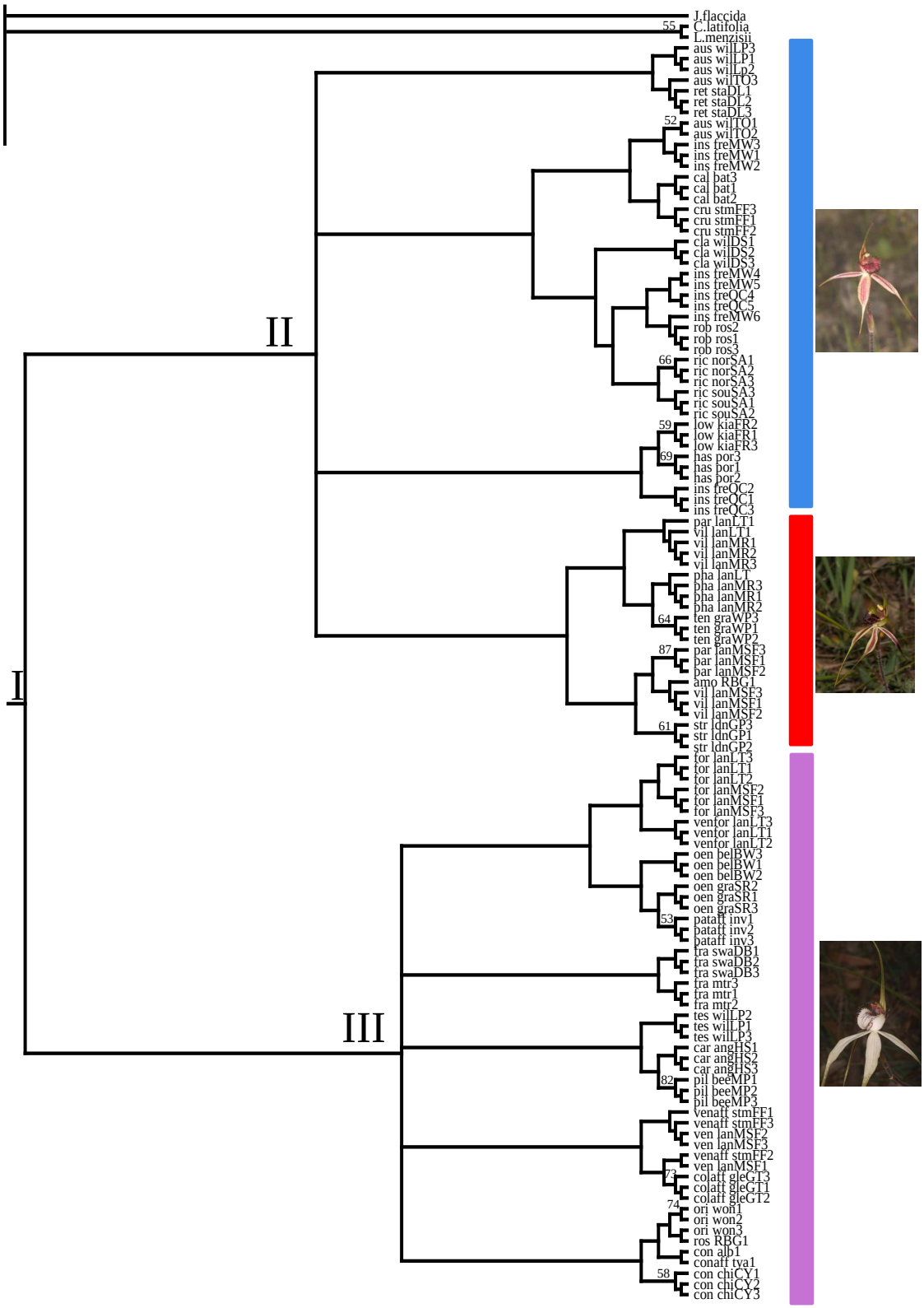


Figure 4.5 Single most parsimonious tree based on 63 ISSR markers (tree length, 531; CI=0.1186, RI=0.5791). Bootstrap percentages greater than 50% are provided above the branches.

4.4 Discussion

4.4.1 ISSR marker profile

Out of the 100 ISSR primers tested, only nine dinucleotide and one trinucleotide motif ISSR primers gave clear amplified DNA fingerprinting patterns under the PCR conditions optimized for this study. Poly GA motif ISSR primers were found to be the best primers and produced a high number of informative polymorphic loci generated from UBC 842 (10 markers) and UBC 810 (8 markers). Poly CA anchored at the 3'end with RG and poly AC anchored at the 3'end with YA also produced high number of informative bands. UBC 884 primer was the only one dinucleotide of AG motif which, anchored at 5'end with HBH, generated highly informative DNA fragments. ISSR genome scanning in *Caladenia* section *Calonema* suggested that mostly dinucleotide repeat units are distributed throughout *Caladenia* section *Calonema* genome. AG, GA, and AC dinucleotide motifs are found more frequently than CA, CT and GT motifs in *Caladenia* section *Calonema*. The CA and GT dinucleotide repeat units were also found in *Olea* genome (Terzopoulos *et al.,* 2005). Other dinucleotide motifs have been found abundance in plant genome such as the study by Vijayan and Chatterjee (2003) found that $(AG)_n$, $(TG)_n$ and $(AC)_n$ motifs were dispersed throughout the *Morus* genome. AG, GA and CT repeat units have also been reported in montane plant species from different families; Symplocaceae, Ericaceae and Theaceae (Deshpande *et al.,* 2001). Sarla *et al*. (2003) reported that AG and GA motifs were abundant in *Oryza nivara* accessions. As found here, the *Caladenia* section *Calonema* genome as well as the *Cicer* genome (as reported by Rajesh *et al.,* 2002) consisted of ATG trinucleotide motifs whereas other studies mentioned that both di-and trinucleotide motifs were very useful for amplification of polymorphic bands (Blair *et al.,* 1999, 2001, Mondal, 2002, Amel *et al.,* 2004; Chen *et al.,* 2005, Terzopoulos *et al.,* 2005). Bands were considered to be polymorphic if they were absent in one or more of the taxa tested, including the spider orchids and

outgroup taxa. The average number of bands amplified of 4.48 fragments per primer was particularly low in comparison with other studies based on ISSR-PCR; 20.6 fragments in *Lactoris* (Crawford *et al*., 2001), 10.8 fragments in *Camellia* (Mondal, 2002), 7.67 fragments (6.6 polymorphic bands per primer) in *Cicer* (Rajesh *et al.,* 2002) 9.9 fragments in *Isoëtes* (Chen *et al.,* 2005) and 16.3 frangments in *Dendrobium* (Wang *et al*., 2009). The ISSR patterns indicated the low ISSR primer binding sites in spider orchid species.

Unexpected DNA profiles were found within the same species since there were differences in banding patterns such as in *C. insularis* taxa as shown in Figure 4.2. This suggested that there was some genetic variation within the tandem repeat regions between individuals of the same species. This type of variation has also been found in some other species such as *C. fragrantissima*. The explanation of this variation is that neutral hybridization and/or speciation might play a role in the evolution of *Caladenia*. Furthermore, if the opportunity of repeat hybridization has occurred, the extensive gene flow may result in the extinction of one or both of the hybridization taxa via genetic assimilation (Ayres *et al*., 2004; Konishi and Takata, 2004 and Chapman and Burke, 2007). The occurrence of hybridization events could not affect the morphological characters since ISSR markers are not amplified from functional genes (Zietkiewicz *et al*., 1994).

4.4.2 Genetic variation among spider orchid species.

ISSR markers were employed in the analysis of genetic relatedness of spider orchid species including comparison to three outgroup taxa from related genera. *C. latifolia, C. flaccida* and *L. menziesii* had high degree of divergence that separated them from spider orchids. According to the genetic similarity based on short tandem unit analysis between outgroup taxa and spider orchids, *L. menziesii* and *C. formosa* from Longbottom Track, Langkoop Meerek had the lowest average genetic similarity, suggesting a furthest genetic distance

between them, while *L. menziesii* and *C. venusta* x *C. formosa* hybrids from Longbottom Track, Langkoop Meerek had the closest genetic background. Among the outgroup taxa *L. menziesii* was least divergent to the patersonii group, *C. formosa* and least diverged from its offspring the *C.venusta* x *C. formosa* hybrid. An explanation for this occurrence might be that the putative hybrid inherited those short nucleotide repeat units from *C. venusta* rather than *C. formosa*. It is possible that while the hybrid has a genetic background from *C. venusta*, morphologically it approaches more closely the *C. formosa* appearance. The greatest divergence among spider orchids was found between *C*. sp. aff. *concolor* from Murchison Hill, Tyaac and *C. tentaculata* from Serra Road, Grampians National Park. The closest spider orchid species were *C. parva* from Longbottom Track, Langkoop and *C. villosissima* from Meerek State Forest. This is because they belong to the dilatata ("green comb") group and have very similar floral structure.

Within the three spider orchid groups, the greatest divergence in the dilatata group was between *C. phaeoclavia* and *C. parva* from Longbottom Track, Langkoop. These specimens were collected from the same location and have very similar floral structures but are traditionally separated by flower size with *C. phaeoclavia* the larger of the two, although some orchidologists doubt the distinction between these two species (Jeans and Backhouse, 2000). This might imply genetic mutation or speciation among this group of spider orchids. The greatest divergence within the patersonii group was found between *C.* sp. aff. *concolor* from Murchison Hill, Tyaac and *C. formosa* from Longbottom Track, Langkoop Mereek. These also have a very similar appearance but *C. formosa* is larger-flowered with more numerous marginal labellum calli. The greatest divergence within the reticulata group occurred between *C. lowanensis* from Kiata Flora Reserve and *C. australis* from Tidal

Overlook Tk, Wilsons Promontory National Park, might also be explained by the presence of speciation even if they have some differences in their floral appearance.

Caladenia parva from Longbottom Track, Langkoop and *C. villossisima* from Meerek State Forest were most similar genetically in the dilatata group of species. *Caladenia venusta* from Meerek State Forest and *C.* sp. aff. *venusta* from Stuart Mill Flora and Fauna were genetically close in the patersonii group species. For these two pairs of taxa, their floral structures are very similar to one another. *Caladenia parva* and *C. villossisima* as well as *C. venusta* and *C.* sp. aff. *venusta* are distinguished largely by different flower size between the pairs. Therefore, less divergence should be expected between these than between species with distinctly different floral morphology. Furthermore, the close genetic background between them might indicate the similar occurrence of di-,trinucleotide tandem regions in their genomes. In contrast, in the reticulata group, some morphologically distinct species did not show expected differences in genetic distance based on ISSR analysis: *C. reticulata*, which has a creamy yellow flower with variable reddish streaks and has short red clubs on the tips of sepals and petals, from Deep Lead Flora and Fauna Reserve, Stawell was expected to be highly divergent from *C. hastata*, with a creamy white flower, labellum with purplish calli and marginal teeth, and with extensive dark red to almost black osmophores on the sepals and petals from Portland. However, less genetic divergence was seen between these two than other specimens of the group in this study. It is possible that this finding may be because of some degree of mutation based on di-, and trinucleotides repeat units in the species under investigation that gave a high degree of similarity based on ISSR DNA profile between *C. reticulata* and *C. hastata*. However, the recent studies in south-west Victoria indicate that *C. reticulata* and *C. hastata* are not close to each other based on their different pollinator species of thynnid wasp (Bower, pers. comm).

4.4.3 Spider orchid relationships

The UPGMA clustering method of ISSR data produced a similar dendrogram to the RAPD data with the exception of the position of spider orchid species within their respective groups. Firstly, the outgroup taxa, *C. flaccida*, *C. latifolia* and *L. menziesii*, were well separated from spider orchids. These results agree with the Diurideae phylogeny by Kores (2001) based on *mat*K and *trn*L-F that showed *L. menziesii* was distant from *C. latifolia* and the spider orchids. In addition, Clements *et al.* (2002) stated that *L. menziesii* was genetically distant from spider orchids based on ITS sequences. The molecular phylogeny of *Caladenia* based on ITS sequences by Dixon and Hopper (2009) also pointed out that *C. latifolia* was distant from spider orchid. This evidence indicated that ISSR used in this study provided potential markers for phylogenetic analysis in subtribe Caladeniinae.

The ISSR marker has been widely used to identify and determine relationships at the species and cultivar level (Raina *et al*., 2001; Pharmawati *et al*., 2004; Wang *et al*., 2009a; Wang *et al*., 2009b; Suranjana *et al*., 2010). The ISSR markers were detected in 30 *Caladenia*. A dendrogram was constructed to infer phylogenetic relationships among 30 *Caladenia* species using UPGMA analysis based on Jaccard's, Dice and simple matching similarity, and parsimony on ISSR data. Parsimony analysis also generated a tree that was in broad agreement with the two dendograms. The phylogenetic relationships obtained from ISSR marker are in good agreement with their floral structure as explained by Backhouse and Jeanns (2000); three main groups were recognized and most of the closely related species were grouped together. The first group was reticulata consisted of *C. australis*, *C. calcicola*, *C. clavigera*, *C. cruciformis*, *C. hastata*, *C. lowanensis*, *C. insularis*, *C. reticulata*, *C. richardsiorum and C. robinsonii*. They were grouped into five groups (I-V). Although the grouping did not reflect clear relationships among species under investigation, it indicated the

grouping of intraspecifics with the exception of *C. insularis*. As stated in Chapter 3 the variation in the appearance of *C. insularis* specimens used in this study could explain the differences reflected in this analysis. *Caladenia insularis* collected from Quarry on Cemetery Track and from Mt. Wellington Track, French Island did not cluster into the same clade. There is a significant difference in the size of the specimens from these locations – the *C. insularis* specimens from the Quarry (a dry site with a soil comprised of red gravel) are considerably smaller than those growing along Mt Wellington Track. This may indicate a potential speciation event in this population. Unlike the dendrogram based on ISSR marker classified 39 *Viola* species according to their morphological character and their distribution. This marker grouped the species from Europe in the same cluster as well as the species from Southern Europe placed in the same group. However, the majority of *Viola* species used in that study were grouped together based on their morphological characteristic (Yockteng *et al*., 2003). In addition, ISSR markers were used to obtained genetic relationships and distinguished genotype in 29 grapefruit (*Citrus papadisi* Macf.), five pummelo (*C. maxima* (Burm.) Merr.) and one *C. hassaku* Hort. Ex Tanaka accessions. The dendrogram based on UPGMA separated all pummelo accessions from grapefruit accessions. ISSR marker could be distinguished in all pummelo accessions while some grapefruit accessions remained unclear (Uzun *et al*., 2010). Even though the ISSR markers did not draw the taxonomy status of reticulata group of Spider Orchids, the interpretation could be complex if some *Caladenia* species have undergone a speciation event. In this case, the pattern of molecular markers in *C. insularis* could be affect by the speciation events. Further work on conspecies analysis should be conducted to address the genetic differentiation in *C. insularis*.

The cluster within the patersonii group supported a grouping that might be inferred based on their floral structure. For example, samples with large red flowers, *C. formosa*, *C. venusta* x

C. formosa and *C. formosa* grouped together with another group comprised of the large white-flowered species, *C. fragrantissima*. The group of flowers which are characterized by their reddish calli labellum with marginal teeth, the tips covered with reddish glandular hairs were grouped together. Because of their similarity on gross morphology, only a few morphological parameters were used as key characters in traditional plant classification. However, ISSR markers used in this study can be separated spider orchid species from each other. Genetic relationship obtained from ISSR markers supported by morphology. The ISSR based classification was also reported to support traditional classification in *Grevillea* (Pharmawati., 2004) and *Genista* (Hakki *et al*., 2010). ISSR markers also provided the relationship of small flowers lacking clubs and glandular hairs and with entire labellum margins *Caladenia tessellata* and *C. cardiochila.* Hopper and Brown (2004a) also placed small-flowered, thick-lipped species including *C. cardiochila* and *C. tessellata* into a separate group called *Caladenia* subgenus *Phlebochilus*, which supports the grouping found here.

The groupings within the dilatata group from the three different clustering methods gave the same subcluster within the group as with the previous methods of analysis. The cluster of *C. parva* and *C. villosissima* from different location were not placed in the same cluster. This might indicated the occurrence of hybridization of the species under investigation. Even though *C. phaeoclavia* has a floral appearance similar to *C. villosissima* and *C. parva*, it clustered with *C. tentaculata,* a big green-comb with yellowish glandular tips. Although this close relationship between the two species was not supported by morphology, ISSR markers indicated their close relationships. Another explanation for their high similarity is that they might have originated from hybridization event which is indicated by their potentially close relationships (Joshi *et al*., 2000). According to floral appearance, *C. villosissima*, *C.*

phaeoclavia and *C. parva* have close genetic background. However, ISSR markers did not place them together.

4.4.4 Biogeography

The geography grouping of spider orchid based on ISSR markers used in this study was also found in dilatata; *C. phaeoclavia*, a brown-clubbed spider orchid from Mereek Road, Langkoop was clustered with the one collected from Longbottom Track, Langkoop. Wang *et al*. (2009b) also found that ISSR based phylogram grouped *Cymbidium goeringii* into two clusters roughly correspondence to geographical region.

The ISSR technique is sensitive to low level of genetic variation, providing a very useful molecular tool for population analysis on a wide range of plant species, as well as for indentifying species, cultivars or population within the same species (Zietkiewicz *et al*., 1994; Raina *et al*., 2001; Wang *et al*., 2009a; Wang *et al*., 2009b). The findings in this study are still unclear due to the genetic background of the study plants. Phylogeny based ISSR carried out on *Dendrobium* species separated 31 *Dendrobium* species into six clusters. This high polymorphism was also detected and proved to be useful molecular markers for species identification and genetic diversity of the genus (Wang *et al*., 2009b). Furthermore, ISSR markers proved to be a useful molecular tool for identifying *Cymbidium* cultivars in the study of molecular diversity and relationship among *Cymbidium goeringii* cultivars (Wang *et al*., 2009b).

The good separation of the spider orchid groups revealed by ISSR data suggests that short tandem repeats which are dispersed throughout the genome of spider orchid are conserved sequences. The variation of unique sequences within the group can be used to classify spider

orchid into reticulata, patersonii and dilatata groups. However, separation of species within each group was not clear, possibly due to insufficient levels of ISSR length polymorphism found between different species using these particular combinations of primers. Other explanations could be introgression, hybridization and/or rapid unresolved speciation. In contrast to other studies, ISSR could classify almond accessions according to their original regions (Martin *et al*., 2003), resolved common bean cultivars into their gene pool (Galvan *et al*., 2003), grouped cultivated and wild of *Houttuynia* based on their chromosome numbers (Wu *et al*., 2005).

The ISSR technology is sensitive to low levels of genetic variation, providing a very useful molecular tool for studying population genetic on a wide range of plant species, as well as for identifying species, cultivar or population of the same species (Zietkiewicz *et al*., 1994; Ajibade *et al*., 2000; Raina *et al*., 2001 and Wang *et al*., 2009b) ISSR analysis is a PCR-based method with advantages of low cost and high-3fficiency as compared with other DNA genotyping technique.

The presence of the polymorphic bands within the same species could have affected analysis of genetic relatedness among the species examined. Possible explanations for variable bands within species could be (1) the sequences themselves have undergone mutation individually (2) the resolution of agarose gel electrophoresis. However, this study tested the reproducibility of ISSR markers by performing separate PCR runs and the amplification of markers obtained by primers used under the PCR condition used in this study was consistent. This indicates that the use of longer primers such as ISSR primer and higher annealing temperature-based PCR yield highly reproducible amplification products (Nagaoka and Ogihara, 1997; Moreno *et al*., 1998). As for the finding of monomorphic bands across species

or insufficient polymorphism, the use of polyacrylamide gel electrophoresis might help resolve this problem due to their ability to separate co-migration bands as they appear in agarose gels (Gupta *et al*., 1994 and Godwin *et al*., 1997). This would provide more polymorphic markers for analysis.

The low polymorphic ISSR markers did not clearly resolve genetic relationship among spider orchid species at the interspecific level. Therefore, RAPD and ISSR markers were combined to determine whether taxonomic relationship could be further clarified with more polymorphic markers. The investigation of genetic relatedness amongst *Caladenia* section *Calonema* species by combining the results RAPD and ISSR markers are presented in Chapter 5.

Chapter 5

Genetic Relatedness among *Caladenia* Species Using RAPD and ISSR Markers

5.1 Introduction

In previous chapters, it has been shown that while RAPDs and ISSRs can separate the three main subgroupings of spider orchids with strong bootstrap support, can identify genetic differences/similarities between different taxa (described and undescribed) and can distinguish between individuals within a species/taxon with strong bootstrap support, the exact phenetic relationships between individual taxa cannot be reliably shown. One of the reasons for this may be that the *Caladenia* taxa are too similar genetically, showing few polymorphisms, and/or that the markers used do not reflect to morphological characters. However, it may also be that too few suitable markers were employed – the primers chosen did not provide sufficient polymorphism to analyse the genetic relationships among spider orchid species. Combining the two data sets to increase the number of bands detected should increase the overall degree of polymorphism for genetic markers and may clarify the relationships between taxa.

DNA-based molecular markers including randomly amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR) and amplified fragment length polymorphisms (AFLP) have proven to be effective tools in the study of genetic relationships. This is due to the fact that they can be applied at low levels of genetic variability. Song *et al*. (2000) examined genetic relatedness among 85 *Lansium domesticum* Corr. accessions using ten RAPD primers, yielding 113 markers for

UPGMA cluster analysis. In this study Song was able to separate the 85 accessions into three main clusters that correlated with the thickness of fruit skin. Joshi *et al.* (2000) studied genetic diversity and phylogenetic relationships in *Oryza* using 30 ISSR primers to construct a UPGMA dendrogram. The ISSR-PCR clustered 42 *Oryza* genotypes according to their respective genomes. The bootstrap values supported phylogenetic inferences for groupings mostly at intraspecific levels. The UPGMA clustering pattern based on 115 ISSR markers was in agreement with the data obtained from studies on crossability, seed storage protein, isozyme, allozyme and RAPD marker analysis. Cluster analysis grouped three broad clusters of chickpea genotypes which showed intraspecific similarities supported by higher bootstrap values than the interspecific similarities (Rajesh *et al.*, 2002).

The combination of different marker type has also widely use in molecular genetics. For example, Huang and Sun (2000) determined genetic diversity and relationships of sweetpotato and its wild relatives in *Ipomoea* series *Batatas* (Convolvulaceae) by means of ISSR-PCR and restriction analysis of chloroplast DNA. Similar relationships at the interspecific level were obtained from both data sets. However, the higher polymorphism of ISSR markers gave a better separation of intraspecific accessions. Phylogenetic analysis in the genus *Cicer* and cultivated chickpea based on RAPD and ISSR markers (Iruela *et al.*, 2002). The resulting phylogenetic tree showed that the distribution pattern of variability between species was related to growth habit and geographical origin. Molecular profiles of Indian cashew varieties obtained from a combination of 58 RAPD and 38 ISSR markers was shown to be a molecular tool for the identification of 35 commercial cashew varieties (Archak *et al.*, 2003). The evaluation of genetic relationships in the genus *Houttuynia* based on RAPD and ISSR

markers showed that the grouping of *H. cordata* accessions based on ISSR data were clustered in relation to their chromosome number. While RAPD analysis grouping them based on geographic distribution (Wu *et al*., 2005).

A combination of RAPD and ISSR markers was also applied for phylogenetic relationships in *Vanilla* (Orchidaceae) showed that phylogenetic analysis based on RAPD was not exactly matched with the ISSR dendrogram. Specific grouping were obtained from each analysis with slight variation between two different markers. However, combined data analysis based on UPGMA clustering gave a high genetic interrelationship among species (Verma *et al*., 2009). Lu *et al*. (2009) investigated genetic diversity of broccoli and its related species and cultivar identification based on RAPD and ISSR markers. The results showed that RAPD and ISSR markers can be used to identify broccoli cultivar. The molecular analysis based on RAPD and ISSR marker combination revealed new evidence on genetic relationships among broccoli, cauliflower and their relatives. This finding also provided valuable information for taxonomic and phylogenetic investigations in *Brassica*.

Molecular markers generated by RAPD and ISSR target different regions of the genome, though in a random manner. A combination of RAPD and ISSR markers would give a better coverage of genome. Hence, the aim of this chapter was to evaluate the potential of RAPD and ISSR marker used in this study as a molecular tool for clarifying the genetic relationships among spider orchid species in Victoria.

The aim of this chapter was to investigate the genetic relationships among Victorian spider orchid species using combined data from RAPD and ISSR markers and to

evaluate the usefulness of these markers as alternative character for spider orchid classification.

5.2 Material and methods

5.2.1 Data analysis

The RAPD and ISSR data obtained as described in the previous chapters were combined. Results described in Chapters 3 and 4 indicated that the Dice coefficient similarity showed higher genetic similarity in either RAPD data or ISSR data than with Jaccard's and Simple matching coefficients. As a result, this analysis was undertaken using the Dice coefficient. Cophenetic matrices were derived from the dendrograms using the COPH (co-phenetic values) program, and the goodness-of-fit of the clustering to the matrix was calculated by comparing the original similarity matrices using MXCOMP program. The Mantel test (Mantel, 1967) was used to establish correspondence between the RAPD and ISSR matrices. This test provides a correlation index (*r*), which is a measure of the relatedness between the two matrices. Principal component analysis was also performed to display the relationships among spider orchids and within each particular group.

5.3 Results

5.3.1 Genetic variation within spider orchid species

Genetic similarity using pair-wise comparisons was calculated from the percentage of matched markers among spider orchid individuals, revealing narrow genetic diversity within spider orchid species (Figure 5.1). The close relationships of spider orchids resulted in the high degree of genetic similarity detected by RAPD and ISSR markers.

About 86% of the pair-wise comparisons among 117 spider orchid individuals showed a genetic similarity between 0.8-0.9, approximately 13% had a genetic similarity greater than 0.85 and about 1% exhibited a genetic similarity less than 0.80 (Figure 5.1).

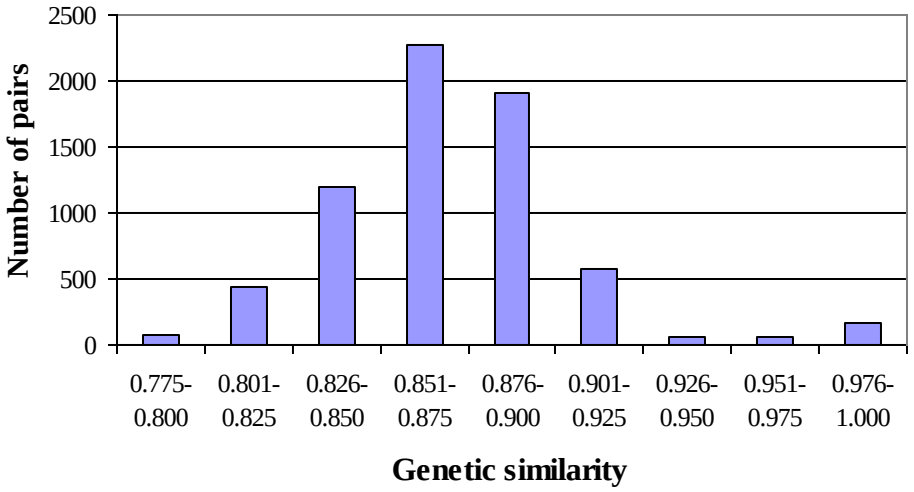


Figure 5.1 Pair-wise comparison of genetic similarity among spider orchid individuals.

The mean genetic similarity among individual specimens within each spider orchid species varied from 0.919 to 1. (one individual from *C. amoena*, *C.* sp. aff. *concolor C. rosella* Figure 5.2). *Caladenia insularis* originated from French Island and was collected from two different sites, Mt Wellington Track and Quarry on Cemetery Track had the lowest genetic similarity (Figure 5.2). *Caladenia australis* from two different locations of Wilsons Promontory National Park including Lilly Pilly Gully and Tidal Overlook Tk had a genetic similarity of 0.950 (Figure 5.2). *Caladenia richardsiorum* collected from Nora Creina South Australia and border of Victoria and South Australia exhibited a genetic similarity of 0.934 (Figure 5.2). *Caladenia*

concolor from Cyanide, Chiltern-Pilot National Park and from Albury, New South Wales gave a genetic similarity of 0.992 (Figure 5.2). A genetic similarity of 0.946 was found between *C. formosa* collected from Longbottom Track, Langkoop Mereek and Meerek State Forest while a value of 0.959 value was found between *C. fragrantissima* from Discovery Bay CP (Swan Lake) and Mt Richmond National Park. *C. oenochila* from Belgrave South, Baluk Willam Flora and from Fauna Reserve and Serra Road, Grampians National Park exhibited a genetic similarity of 0.954. *C. parva* from Longbottom Track, Langkoop and Meerek State Forest had a genetic similarity value of 0.959. *Caladenia phaeoclavia* from Mereek Road, Langkoop and Longbottom Track, Langkoop had a genetic similarity of 0.945. A genetic similarity of 0.938 was exhibited in the specimens of *C. villosissima* from Meerek State Forest and Mereek Road.

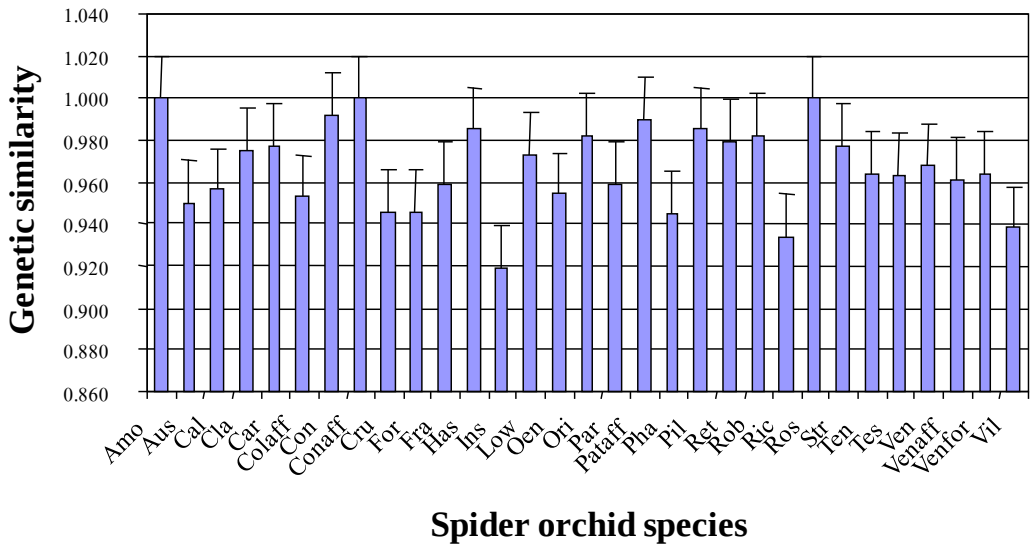


Figure 5.2 Average genetic similarity within each species (Amo: *C. amoena*, Aus: *C. australis*, Cal: *C. calcicola*, Car: *C. cardiochila*, Colaff: *C.* aff. *colorata*, Con: *C. concolor*, Conaff: *C.* aff *concolor*, Cru: *C. cruciformis*, For: *C. formosa*, Fra: *C. fragrantissima*, Has: *C. hastata*, Ins: *C. insularis*, Low: *C. lowanensis*, Oen: *C. oenochila*, Ori: *C. orientalis*, Par: *C. parva*, Pataff: *C.* aff *patersonii*, Pha: *C. phaeoclavia*, Pil: *C. pilotensis*, Ret: *C. reticulata*, Rob: *C. robinsonii*, Ric: *C. richardsiorum*, Ros: *C. rosella*, Ten: *C. tentaculata*, Tes: *C. tessellata*, Ven: *C. venusta*, Venaff: *C.* aff *venusta*, Venfor: *C. venusta* x *C. formosa* and Vil: *C. villosissima*

The mean genetic similarity within the dilatata, patersonii and reticulata groups varied from 0.907-0.912 with the similarity among specific pairs of each species ranging from 0.822 to 1.000 (Table 5.1). Although the value of mean genetic similarity of the entire sample did not show a big difference between each group, it could be used to classify spider orchids into three groups, groupings that are supported by traditional observations.

Table 5.1 Average genetic similarity within spider orchid groups

Item	dilatata	patersonii	reticulata	Overall
Average	0.907	0.912	0.909	0.869
Standard deviation	0.051	0.032	0.030	0.036
Min.	0.822	0.850	0.863	0.788
Max	1.000	1.000	0.985	1.000

The genetic similarity between spider orchid group species was determined from similarity coefficients between pairs of spider orchid among species groups: reticulata and patersonii groups, reticulata and dilatata groups and patersonii and dilatata groups (Figure 5.3). The genetic similarity among them was slightly different. Reticulata and patersonii groups share the highest genetic similarity among the three species groups, with an average of 0.869 (Figure 5.3). Reticulata and dilatata groups share the lowest genetic similarity with an average of 0.838 (Figure 5.3). The genetic similarity between patersonii and dilatata groups was 0.844 (Figure 5.3).

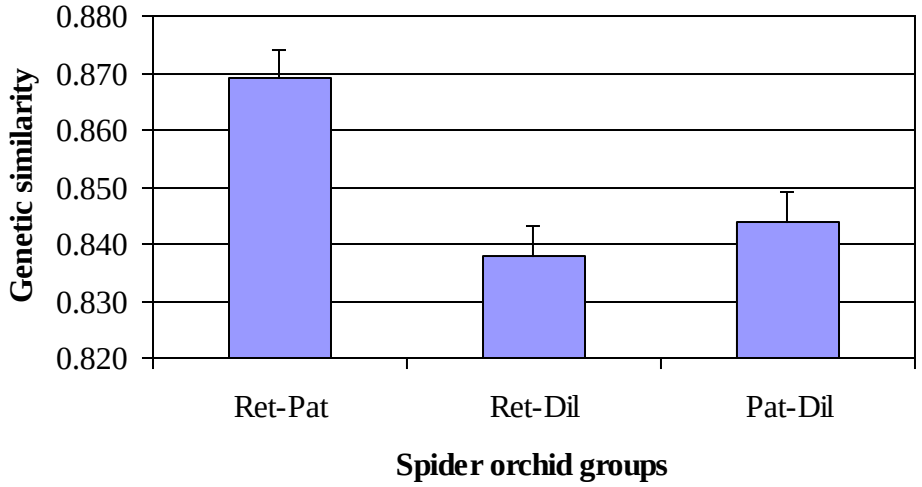


Figure 5.3 Average genetic similarity between spider orchid groups (Ret: reticulata group; Pat: patersonii group and Dil: dilatata group).

Table 5.2 Comparison of cophenetic correlation values obtained from combinations of three similarity coefficients and four clustering methods employed for analysis of combined data obtained from RAPD and ISSR

Clustering method	Similarity coefficients		
	DICE	Jaccard's	SM
UPGMA	0.975	0.964	0.965
WPGMA	0.973	0.963	0.960
Complete linkage	0.961	0.942	0.943
Single linkage	0.965	0.948	0.951

The dendrograms obtained by using the three different similarity coefficients; Dice, Jaccard's and SM and various different clustering methods; UPGMA, WPGMA, complete linkage and single linkage were investigated and the co-phenetic correlation

values produced by each coefficient was compared (Table 5.2). In general, high co-phenetic correlation values ranging from 0.942 to 0.975 were obtained where $r>0.9$ indicates a very good fit. The UPGMA method gave consistently higher co-phenetic correlation scores and Dice coefficient also gave consistently higher co-phenetic correlation values than either Jaccard's or SM coefficients. Therefore, the dendrogram illustrated in this Chapter was based on UPGMA clustering method and Dice similarity coefficient.

5.3.2 Cluster analysis obtained by Dice coefficients

Cluster analysis of 117 spider orchid individuals and three outgroup taxa constructed by Dice coefficient is shown in Figure 5.4. The bootstrap values seen at the nodes indicate that relationships are mostly reliable only at intra-specific levels. The UPGMA dendrogram based on the combined one that outgroup taxa; *Caladenia flaccida, Caladenia latifolia* and *Leptoceras menziesii* separated from typical spider orchid species (*Caladenia* section *Calonema*) by large genetic distance with strong bootstrap value support (*L. menziesii*, 88%; *C. latifolia*, 87%: *C. flaccida*, 100%) and three spider orchid groupings correlated with groupings allocated based on their floral structure which is supported by morphological classification.

The clubbed spider orchids (reticulata group) divided into three clusters. The first cluster consisted of *C. australis* from Lilly Pilly Gully and Tidal Overlook Tk from Wilsons Promontory National Park, *C. calcicola* from Bats Ridge, Portland, *C. clavigera* from Derby Saddle, Wilsons Promontory National Park and *C. lowanensis* from Kiata Flora Reserve (Figure 5.4). Cluster II comprised the sub-cluster of *C. insularis* from Mt Wellington Track, French Island three of which grouped with *C. robinsonii* from Rosebud before joining with the rest of the grouped species, and

another sub-cluster of *C. richardsiorum* collected from Nora Creina, South Australia and the border of Victoria and South Australia that grouped together with the sub-cluster of *C. insularis* from Quarry on Cemetery Track, French Island, *C. reticulata* from Deep Lead Flora and Fauna Reserve, Stawell and *C. hastata* from South Australia (Figure 5.4). Cluster III had only the species of *C. cruciformis* from Stuart Mill Flora and Fauna Reserve (Figure 5.4).

The second group of tailed spider orchids (patersonii group), was placed into clusters IV to VI. The first cluster of the group consisted of the sub-cluster of *C. formosa* from Longbottom Track, Langkoop Mereek and Meerek State Forest and the spider orchid hybrid between *C. venusta* x *C. formosa* from Longbottom Track, Langkoop Mereek, a sub-cluster of *C. fragrantissima* from Discovery Bay CP (Swan Lake) and Mt Richmond National Park and *C. oenochila* from Belgrave South, Baluk William Flora and Fauna Reserve (Figure 5.4). Cluster V included *C. orientalis* from Wonthaggi, Healthland Reserve, *C. cardiochila* from Anglesea, *C. pilotensis* from Mt Pilot, Beechworth, *C. concolor* from Cyanide, Chiltern-Pilot National Park, *C. concolor* from Albury, New South Wales, *C.* sp. aff. *concolor* from Murchison Hill, Tyaac and *C. rosella*, cultivated at Royal Botanic Garden Melbourne, (Figure 5.4). Cluster VI consisted of *C. oenochila* from Serra Road, Grampians National Park, *C.* sp. aff. *patersonii* from Inverleigh and *C. tessellata* from Lilly Pilly Gully, Wilsons Promontory National Park and the group of *C.* sp. aff. *venusta* from Stuart Mill Flora and Fauna Reserve, *C. venusta* from Meerek State Forest and *C.* sp. aff. *colorata* from Gorge Track, Lower Glenelg National Park (Figure 5.4).

The green comb (dilatata group) spider orchids were grouped into three clusters. Cluster VII had *C. phaeoclavia* from Mereek Road, Langkoop and *C. tentaculata* from Glenelg River Road, Woolpooer, Grampians National Park (Figure 5.4). Cluster VIII consisted of *C. parva* from Longbottom Track, Langkoop and Meerek State Forest, *C. villosissima* from Meerek State Forest and Mereek Road, Langkoop, *C. stricta* from Little Desert National Park (gravel pits), *C. villosissima* from Longbottom Track, Langkoop and *C. amoena*, cultivated at Royal Botanic Garden, Melbourne from parent plant from Wattle Glen (Figure 5.4). Cluster IX included *C. phaeoclavia* from Longbottom Track, Langkoop (Figure 5.4).

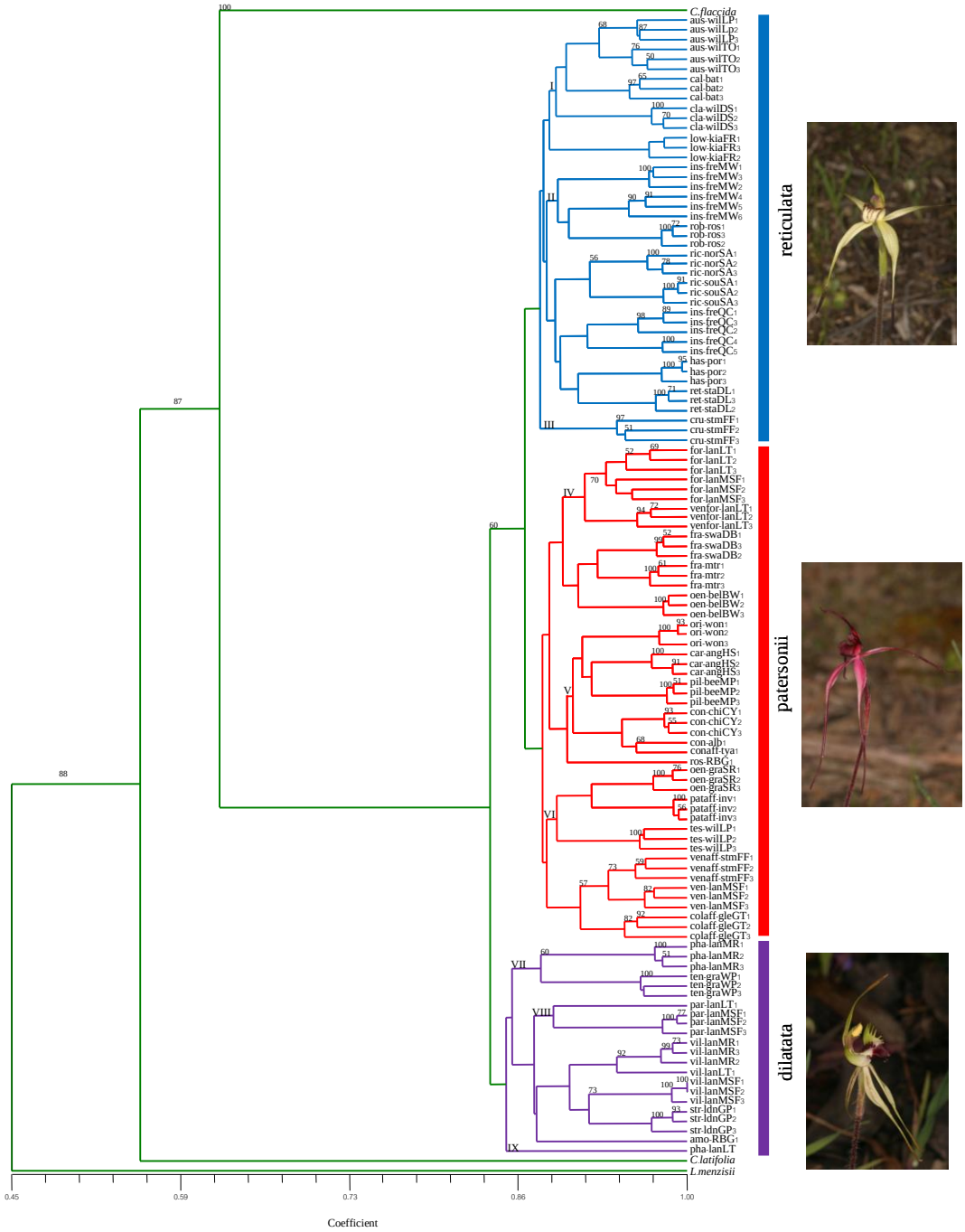


Figure 5.4 Dendrogram showing genetic relationships of 117 spider orchid species and three outgroup taxa. The dendrogram was constructed using DICE coefficient of similarity and UPGMA clustering. Bootstrap values above 50% are shown above the node.

5.3.3 Cluster analysis obtained by principal component analysis

5.3.3.1 Principal Component Analysis (PCA) of spider orchids

Association among the 117 spider orchid individuals revealed by principal component analysis is presented in Figure 5.5. The first and the second principal axes accounted, respectively, for 60% and 27% for total variation between individuals. As shown in the scatter plot, the PCA generated by 135 RAPD markers and 63 ISSR markers separated the three spider orchid groups with the dilatata group which separated in positive PCA 1 and PCA 2 showing further broad separation within the group. The reticulate separated in negative PCA 1 and positive PCA 2 while patersonii separated in negative PCA 1 and PCA 2. The reticulata and patersonii groups abut one another with some of the *C. insularis* specimens crossing over into the patersonii group.

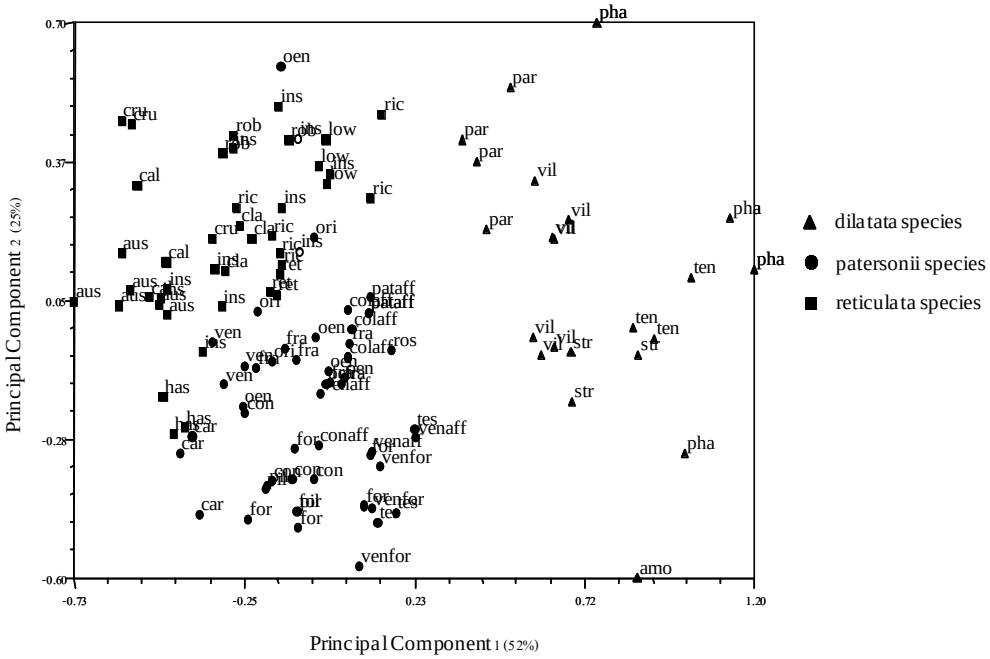


Figure 5.5 Principal components plot of 117 *Caladenia spp.* individuals based on Dice similarity coefficients from 135 RAPD markers and 63 ISSR markers (stress1= 0.18659; indicates as a good fit). The numbers in parentheses represent the percentage of variation accounted for by each principal component.

5.3.3.2 PCA of each spider orchid group

The first two components in the reticulata group explained 71% of the total variation. The scatterplot representation of the PCA clearly showed a broad spread of *C. insularis* whereby some of them had closer relationships to *C. robinsonii* rather than to their own species. The remainder of the group gave the similar groupings to that revealed by the UPGMA.

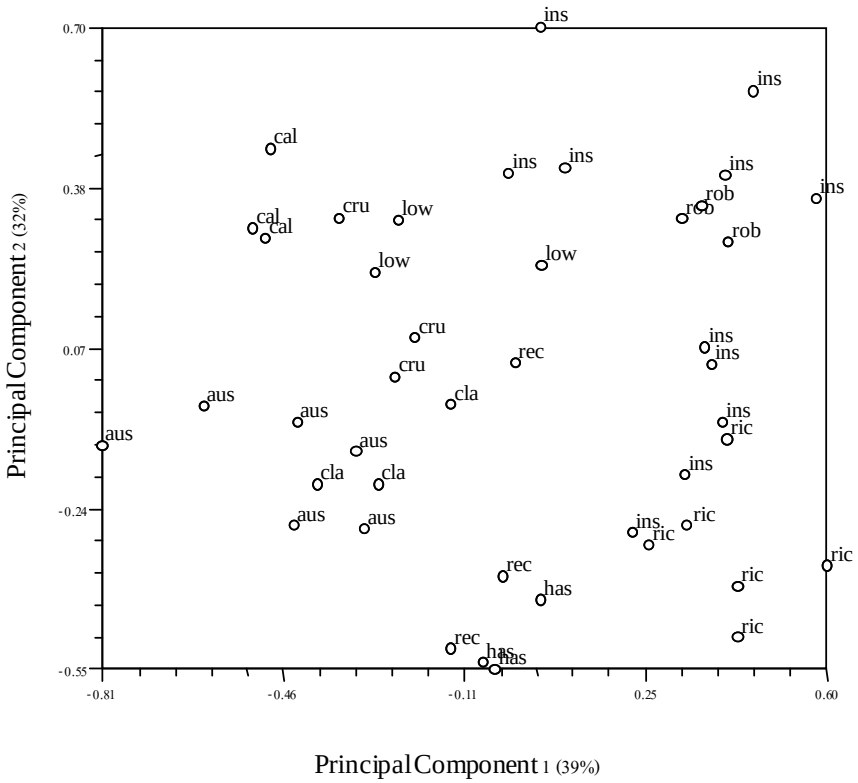


Figure 5.6 Principal components plot of 44 reticulata group individuals based on Dice similarity coefficients from 135 RAPD markers and 63 ISSR markers (stress1= 0.20107; indicated as fair fit). The numbers in parentheses represent the percentage of variation accounted for by each principal component.

The first two principal components of the patersonii group explained 44% and 31% of the variation, respectively. The PCA grouping was similar to UPGMA clustering method with the exception of the close grouping of *C. Oenochila* from different location.

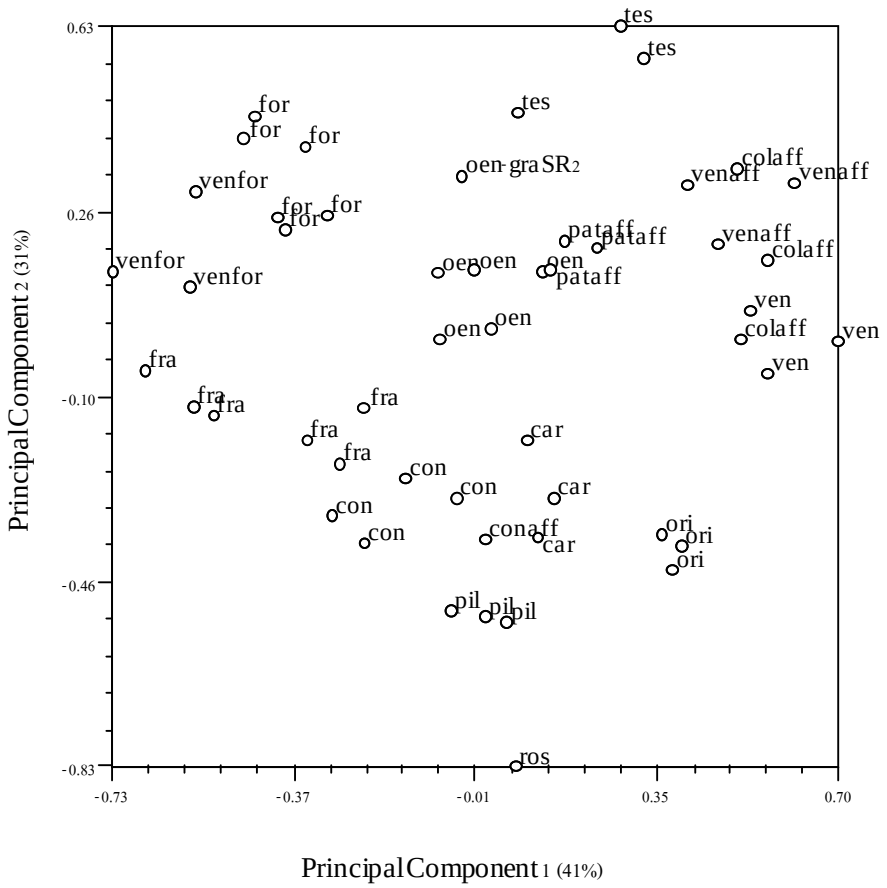


Figure 5.7 Principal components plot of 51 patersonii group individuals based on Dice similarity coefficients from 135 RAPD markers and 63 ISSR markers (stress1= 0.15252; indicated as good fit). The numbers in parentheses represent the percentage of variation accounted for by each principal component.

The first two components in the dilatata group explained 49% and 31% of the total variation, respectively. The PCA grouping also displayed similar grouping with dendrograms based on UPGMA cluster analysis. The only point to note was that *C. parva* collected from Longbottom Track, Langkoop (par-lanLT1) was closer to *C. stricta* and C. *villosissima* rather than *C. parva* from Mereek forest as indicted by the UPGMA. The group of *Caladenia villosissima* collected from Meerek State Forest and Mereek Road, Langkoop were distant apart.

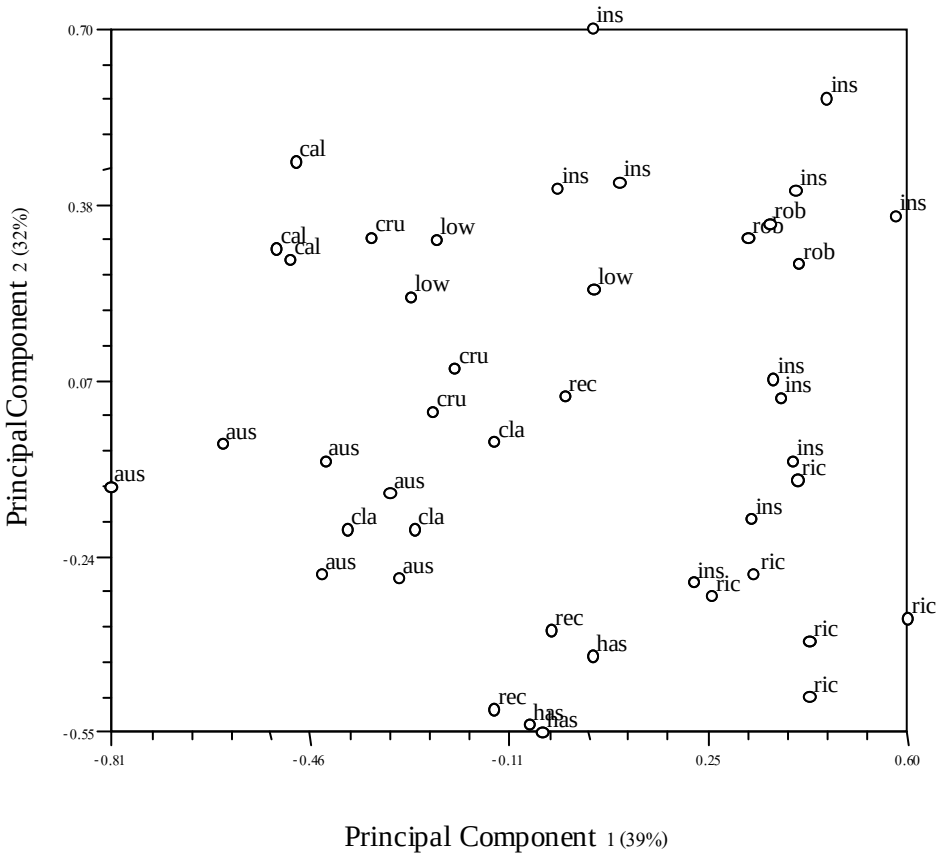


Figure 5.8 Principal components plot of 22 dilatata group individuals based on Dice similarity coefficients from 135 RAPD markers and 63 ISSR markers (stress1= 0.05893; indicated as excellent fit). The numbers in parentheses represent the percentage of variation accounted for by each principal component.

5.3.4 Comparison of RAPD and ISSR genetic similarities

The genetic similarity indices obtained through RAPD and ISSR analyses were compared using regression analysis performed by mean of a Mantel test. A high correlation (r=0.90) was observed between RAPD and ISSR markers, in term of their genetic similarity assessment among spider orchid species used this study.

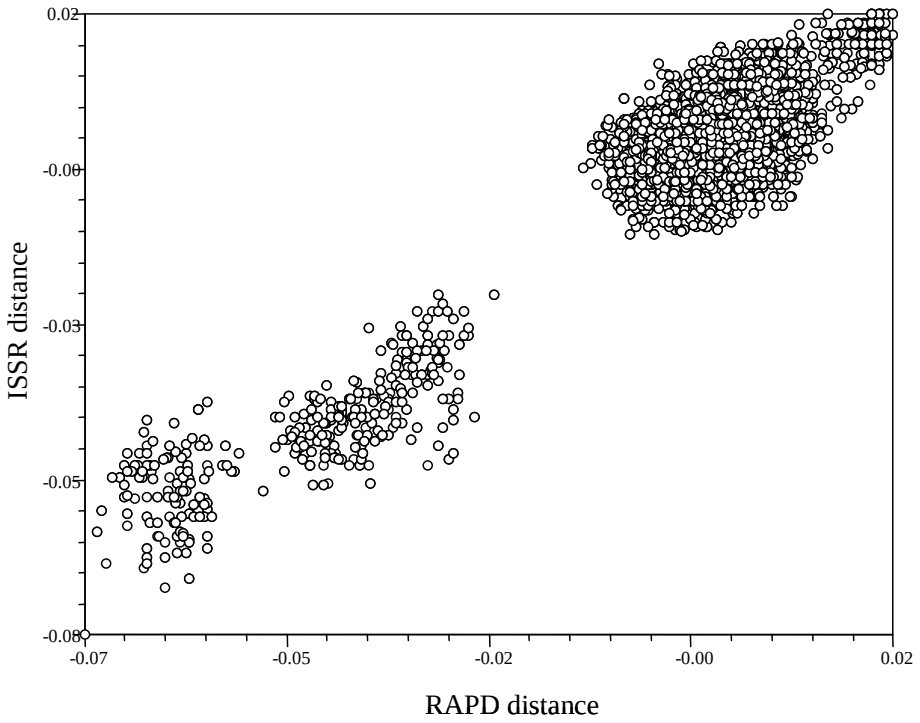


Figure 5.9 Comparison of genetic similarity estimates from RAPD and ISSR using Mantel test.

5.3.5 Parsimony analysis

The parsimony analysis of combined data produced a single tree of length 1381 with CI = 0.1434. The cladogram was similar to the result of using RAPD alone except it separated the group of *C. richardsiorum* and *C. hastata* as the sister group of the majority of reticulata and patersonii species. In addition, it also group *C. oenochila* and *C.* sp. aff. *patersonii* into clade III as their sister group (Figure 5.10).

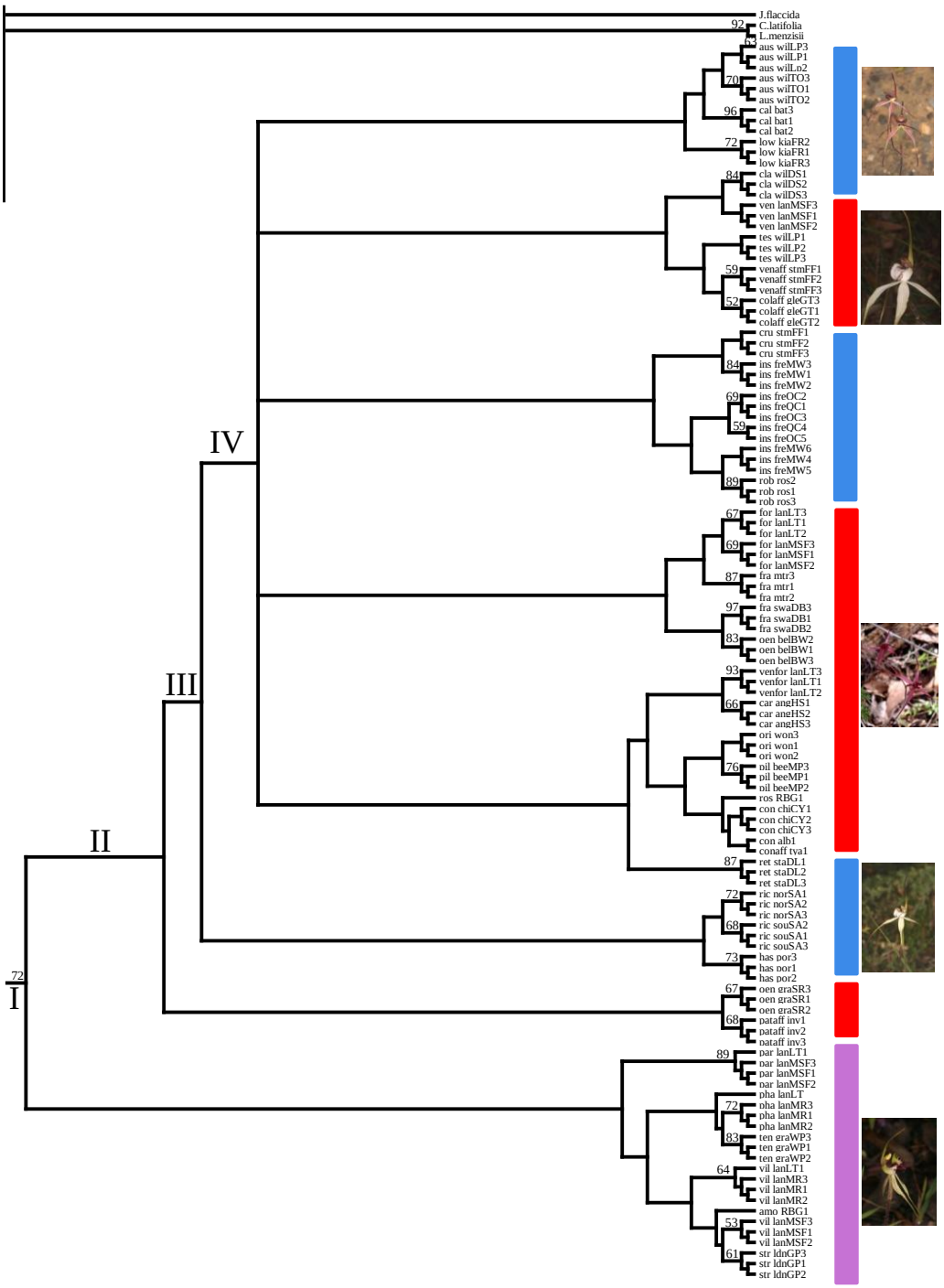


Figure 5.10 Single most parsimonious tree based on 135 RAPD and 63 ISSR markers (tree length, 1381; CI=0.1434. 1588, RI=0.8566). Bootstrap percentages greater than 50% are provided above the branches.

5.4 Discussion

5.4.1 Genetic differentiation within spider orchid species

The genetic differentiation among spider orchid species examined in this study was small as indicated by their high degree of genetic similarity. The majority of the pair-wise comparisons exhibited values of high genetic similarity between 0.8-0.9. High genetic similarities were also found in *Coffee arabic*a cultivars, which investigated mean genetic similarity within each cultivar varied from 0.880 to 0.969 using AFLP analysis (Steiger *et al.*, 2002). This was also the case in East African highland banana (*Musa spp.*), where a single group of closely related cultivars shared their similarity of 95.5 % based on RAPD analysis (Pillay *et al.*, 2001). These indicated that high genetic similarity obtained from DNA markers can highlight differences among specimens at the DNA level.

The low genetic differentiation within the species of spider orchid might be due to a recent diversification from a common ancestor, may be an artefact of sampling, or due to the limited number of samples available for use in this study. Many species of spider orchids are endangered species in Victoria and protected against intentional picking, uproot and destruction. As a result of the limited amount of material available, it was not possible to base sampling on appropriate numbers of plants per species at the time of conducting the DNA analysis. Nevertheless, with most species, three specimens of the same species were available to represent spider orchid species used in this study.

The mean genetic similarity within species collected from different locations ranged from 0.919 (*C. insularis*) to 0.992 (*C. concolor*). Eleven samples of *C. insularis*

collected from two different locations gave the lowest genetic similarity value suggesting that the species may have undergone mutation in some of the genetic regions examined, which are reflected by morphological differences between plants from the two locations that can be detected by an expert plant collector (M. Duncan personal communication). Alternatively, some of the *C. insularis* represents hybridisation between some other species. For example, *C. robinsonii* and a more widespread species such as *C. australis* which is found from Western Victoria, the Otways in Western Victoria through to Wilsons Promontory in the East, or *C. clavigera* which is widespread across Victoria. *Caladenia concolor* collected from two different sites, Cyanide Chiltern-Pilot National Park and Albury, New South Wales gave very high genetic similarity perhaps indicating a more continuous distribution area and hence gene flow in the past. Further experiments should be conducted to confirm this close genetic relationship either through morphology, pollination, genetic or molecular biology studies since there are presently large distances between the sites.

Three morphologically similar species of green comb spider orchid (dilatata group) which were variable in size, *C. parva*, *C. phaeoclavia* and *C. villosissima* (the latter having a very hairy leaf and stem), were collected from the same site. *Caladenia villosissima* had the lowest genetic similarity value of 0.938, perhaps due in part to the small sample size (seven plants) under investigation. The study of variation in plant size and genetic variation within these three species should be conducted to gain a better understanding of taxonomic status in these similar green comb spider orchids. It would be preferable to employ a larger sample size than that used for the current investigation.

High genetic similarity suggests that all members of *Caladenia* section *Calonema* have diverged only slightly from the ancestral taxon. A high degree of similarity can also be seen in their floral structures. They have a similar floral structure: long sepals and petals, some species ending with club or osmophores. They can be identified by the details of perianth segments such as overall shape, shape of calli on the labellum, shape of marginal fringes, the density and position of clubs on laminar calli and tepal tips. The similarity coefficient obtained from combined RAPD and ISSR markers was not as high as similarity coefficient value shown in this study. For example, Gupta *et al* (2008) conducted genetic diversity in *Jatropha curcas* genotypes showing similarity coefficient ranging from 0.41 to 0.89. The similarity coefficients of the rice bean landraces ranged from 0.559 to 0.777 (Muthusamy *et al*., 2008), from 0.20 to 0.80 in *Cucurbitaceae* (Sikdar *et al*., 2010) and from 0.56 to 0.81 in Iranian pistachio cultivars (Tagizad *et al*., 2010).

The six green comb species had the most genetic variation within the spider orchids in this study. This indicated the diverse range of genetic information obtained from RAPD and ISSR data within green comb species. They appear to possess more genetic differentiation than other spider orchid group species investigated in this study. The clade of dilatata demonstrated convergent evolution of their molecular markers based on the markers used in this study. The evaluation of genetic relationships in *Houttuynia* based on RAPD and ISSR markers indicated that the groups based on RAPD markers were more related with geographic distribution (Wu *et al*., 2005). The dendrogram based on RAPD and ISSR combined markers also indicated close relationships of *Cicer* (Iruela *et al*., 2002) and *Vigna* species (Abd El-Hady *et al*., 2010) from their geographic distribution.

The reticulata and patersonii groups have the highest genetic similarity between them in comparison with the paired comparisons between reticulata and dilatata groups, and patersonii and dilatata groups. The similarity of reticulata and patersonii can also be visualized by parsimony cladogram. The genetic similarity among spider orchid groups can be correlated to the similarity of their floral structure. The reticulata and patersonii generally have similar floral shape and colour with the exception of the tepal tips: which consist of tightly packed osmophores forming clubs in the reticulata group or glandular hairs (individual osmophores) in the patersonii group. Based on their floral features, there is indication of recent divergent evolution of their morphological characters. In contrast, the dilatata group have a unique floral structure with the green fringed labellum margin and maroon labellar apex and calli shared by all species. The high cophenetic correlation value obtained from three different similarity coefficients (Dice, Jaccard's and SM) and four clustering methods (UPGMA, WPGMA, complete linkage and single linkage) indicate the strength of RAPD and ISSR DNA profiles, which were a very good fit with clustering methods. The value of correlation coefficient between RAPD and ISSR markers was reported to be in good agreement found in *Amaranthaceae* and *Chenopodiaceae* (r = 0.83) (Ray and Roy, 2007), in rice bean (*Vigna umbellata*) landraces (r=0.673) (Muthusamy *et al*., 2008), in elite germplasms of *Cymbopogon winterianus* (r=0.965) Bhattacharya *et al*., 2010 and in Iranian pistachio cultivars (r = 0.83) (Tagizad *et al*., 2010). However, the moderate correlation was reported in *Houttuynia* (r=0.4078) (Wu *et al*., 2005) and *Cucurbitaceae* (r = 0.58) (Sikdar *et al*., 2010). The correlation between RAPD and ISSR based similarities in *Jatropha curcas* genotypes was low with value of 0.3318 (Gupta *et al*., 2008). However, the correlation of RAPD and ISSR was very

poor fit (r=0.1902) in the assessment of genetic diversity in *Melocanna baccifera* Roxb. (Lalhruaitluanga and Prasad, 2009).

5.4.2 Cluster analysis

The genetic relationships among spider orchid species revealed by cluster analysis of combined RAPD and ISSR binary data using UPGMA and Dice coefficient, as well as principal component analysis, led to the separation of spider orchids into three distinct groups as indicated by traditional classification based on their floral structure; clubbed spider orchids (reticulata group), tailed spider orchids (patersonii group), and green comb spider orchids (dilatata group). The genetic differentiation among spider orchids used in this study was small. Three outgroup taxa *Caladenia flaccida*, *Leptoceras menziesii* and *Caladenia latifolia* were well separated from the typical spider orchids (*Caladenia* subgenus *Calonema*) indicated by large distances at 0.623, 0.559 and 0.290 genetic similarity level with *C. flaccida*, *C. latifolia* and *L. menziesii*, respectively. Principal component analysis on the combined ISSR and RAPD data indicated that the three distinct groups of spider orchids could be further resolved into separate taxa as discussed below.

5.4.2.1 Reticulata group

The reticulata group or clubbed spider orchids which are identified by distinct club tips at sepals or sometime petals and short teeth margin labellum, grouped into three clusters mainly based on their floral structure. The greenish to yellowish flowered; *C. australis*, *C. calcicola*, *C. clavigera* and *C. lowanensis* clustered into clade I. Cluster II consisted of creamish to creamy yellow flowers; *C. insularis*, *C. robinsonii*, *C.*

richardsiorum, *C. reticulata C. hastata*. *Caladenia cruciformis* formed a separate cluster.

There are some points to note regarding much morphological variation in *C. insularis* collected from the Quarry on Cemetery Track, French Island as mentioned by the plant collector. This might be one possible reason why *C. insularis* from this site clustered with other species in this cluster rather than the rest of the species. This occurrence might indicate that the species has sympatric speciation at this site. Genetic similarity based on combined RAPD and ISSR data grouped the member of this cluster based on their morphology similarities mainly in their perianth segment colour rather than their geographical location. Their geographical distribution varies from a coastal or southerly location (*C. insularis*, *C. robinsonii, C. hastata*), a forest and low coastal South Australia (*C. richardsiorum*) whereas *C. reticulata* and *C. cruciformis* are inland and more central-western.

The PCA basically showed that the species are not clearly resolved. This was consistent with the fact that individuals of the same species from different locations cluster with other species, not one another. This suggested that hybridisation has occurred between some species, and/or speciation event has occurred in particular taxa. Another point to be considered is that a formerly widespread species has been fragmented by habitat loss.

5.4.2.2 Patersonii group

The patersonii group comprise the tailed spider orchids recognized by their sepals ending with long filamentous tips, variably covered with glandular hairs. This

grouping mainly was related to morphological character. The large-flowered consisted of *C. formosa*, *C. formosa x venusta*, *C. fragrantissima*, *C. oenochila* (South Belgrave) grouped together. The creamy to yellowish flowered plants; *C. orientalis*, *C. cardiochila*, *C. pilotensis* clustered with a group of red flowered; *C. concolor*, . sp. aff. *concolo*r and *C. Rosella*. The last cluster was a group of creamy white- to greenish yellow-flowered: *C. oenochila* (Serra Road, Grampians National Park), *C.* sp. aff. *patersonii* (Inverleigh), *C. tessellata*, *C.* sp. aff. v*enusta*, *C. venusta*, *C.* sp. aff. *colorata*.

Combined RAPD and ISSR markers grouped a smaller-flowered species with a heart-shaped labellum and tepals lacking clubs or glandular hair (*C. cardiochila* and *C. tessellata*) with typical tailed spider orchids. However, ISSR based dendrogram placed *C. cardiochila* and *C. tessellata* in the same group. This grouping is in agreement with the grouping of Hopper and Brown (2004b) which places *C. cardiochila* with Western Australian taxa with *C. tessellate* since both have a relatively broad labellum with entire margins, crowded calli, and short tepals into *Caladenia* subgenus *Phlebochilus* based on morphological characteristics, with a typical subgenus *Calonema* spider orchid. However, Jones and Clements (2001) had previously indicated that *C. tessellata* is closer to the *Arachnorchis* group which they suggested a separate genus for spider orchids.

PCA grouped *C. oenochila* from Serra Road, Grampians National Park and Belgrave South together while UPGMA cluster analysis placed them into different cluster. A possible explanation is that small sample size affects the PCA of particular group analysis. PCA also placed *C. cardiochila* and *C. tessellata* far apart although they

have similarity in their floral structure. This indicated that molecular study based on RAPD and ISSR used in this study did not support the morphological classification which Hopper and Brown (2004) and Jeanes and Backhouse (2000) separated them out from typical spider orchid.

Unlike the reticulata PCA, patersonii PCA resolved named species but not undescribed taxa closely related to described species. This might imply that the undescribed taxa are the same species as the formally described species. Because of their different geographical distributions and very similar floral appearance, the name of the described species most closely related to the undescribed species has been used to refer to this taxa. The further examination of these closely related species and the undescribed taxa need to be conducted using other source of genetic information to gain more evidence to determine taxonomic boundary.

5.4.2.3 Dilatata group

The dilatata group comprising the green comb spider orchids are recognized by a labellum having a maroon apex and calli and green marginal fringes or teeth. There were three main clusters consisted of the group of big and small flower; *C. phaeoclavia* (Mereek Road lankoop), *C. tentaculata* , the group of *C. parva*, *C. villosissima*, *C. stricta*, *C. Amoena* and a distinct clade of *C. phaeoclavia* (Longbottom Track, Langkoop).

The dendrogram placed small, *C. phaeoclavia* and large, *C. tentaculata* green comb spider orchids together. A possible explanation for this grouping is that the characters using in phenetic analysis obtained from DNA amplification fragments throughout

spider orchid genome can come from either coding or noncoding regions. Therefore, genetic similarity between these two species might arise from markers produced from non-coding region - fragments from regions which had no relation to floral structure. The only one grouping based on their distribution area found in the cluster of *C. parva* and *C. villosissima* from Langkoop.

C. phaeoclavia 'medium leaf', from Longbottom Track, Langkoop was treated as a distinct taxon in this analysis. This individual might have some genetic information different from others as indicated by medium leaf size that phenetic analysis based on combined RAPD and ISSR data placed as a distinct taxon. This might indicated the evidence of speciation within *C. phaeoclavia* species.

The PCA gave similar grouping, with the exception of *C. parva* collected from Longbottom Track, Langkoop being grouped closer to *C. stricta* and *C. villosissima* rather than to *C. parva* from Mereek State Forest as indicated by the UPGMA cluster analysis. This finding also might be the result of from small data size in an analysis. Further experiment using more specimen of each species from a range of locations should be conducted to confirm the relationships within green comb species.

The correlation coefficient determined by a Mantel test was 0.9 between RAPD and ISSR. A similarly high correlation of 0.95 between two data sets of AFLPs and ISSRs was also found in genetic relationships in *Cucurbita pepo*, (Paris *et al.*, 2003). The high correlation between these two data sets in this study indicated the strength of dendrograms obtained from either RAPD data or ISSR data. In addition, the complete concordance of the dendrogram produced using the different similarity coefficients

and clustering methods also suggested that the data were robust in terms of the relative similarity among and within taxa. However, the bootstrap values supported phylogenetic inference for the grouping mostly at intraspecific level, and the lack of strong bootstrap value at the interspecific level indicated weakly interspecific relationships within *Caladenia* subgenus *Calonema*. This indicated that further investigation of these relationships is needed. Because of the unresolved phylogenetic relatedness within the species groups among spider orchid species based on DNA profiling, the next chapter will describe the use of DNA sequencing technique to clarify the relationships among spider orchids.

Chapter 6

Genetic Relatedness within Dilatata Group Using ITS and cpDNA Regions

6.1 Introduction

The development and use of molecular data to resolve phylogenetic relationships at different taxonomic levels has become increasingly popular. The available molecular markers used for this purpose are obtained from either the nuclear genome or chloroplast genome in plants. Among the nuclear markers, the internal transcribed spacers (ITS) regions have been the most widely used in phylogenetic studies.

The internal transcribed spacers (ITS) are non-coding regions of DNA sequence that separate genes coding for the 28S, 5.8S, and 18S ribosomal subunits. Eukaryotic organisms have two internal transcribed spacers: ITS1 is embedded between the 18S gene and the 5.8S gene, and ITS2 is embedded between the 5.8S and the 28S gene. Ribosomal genes and spacers are found in tandem repeats that are thousands of copies long. These ribosomal RNA (rRNA) genes are highly conserved across taxa while the spacers between them may be species-specific. Therefore, the ITS region is widely used in taxonomy and molecular phylogenetics at the species level, and even within species (e.g. to identify geographic races). The conservation of the rRNA genes allows for the design of universal primers to amplify these regions by polymerase chain reaction (PCR) amplification in a wide range of taxa. The variation in the spacers has proven useful for distinguishing among a wide diversity of difficult-to-identify taxa including *Orhrys* species (Orchidaceae; Soliva *et al*., 2001), *Dendrobium*

species (Orchidaceae; Tsai *et al*., 2004), *Phalaenopsis* species (Orchidaceae; Tsai *et al*., 2006) and *Disa* species (Orchidaceae; Bytebier *et al*., 2007).

Chloroplast DNA analysis has become a useful tool for the study of plant molecular phylogeny (Chase and Albert, 1998). Chloroplasts are sub-cellular organelles with their own DNA which is different from the genomic DNA of a plant because it is inherited in a non-Mendelian fashion from one parent only. In plants, chloroplast DNA is mostly maternally inherited, does not undergo recombination and has highly conserved sequences (Taberlet *et al.*, 1991; Karp *et al.*, 1998). However, the non-coding regions of the chloroplast evolve at a higher rate than the more conserved coding regions and have therefore been employed for plant molecular phylogenetic investigation (Taberlet *et al*., 1991; Karp *et al*., 1998; Nickrent *et al*., 2004). Three regions flanking the chloroplast tRNA genes (*trn*T, *trn*L and *trn*F) have proven useful in addressing species-level questions and universal primers have been designed to amplify the conservative sequences (Taberlet *et al*., 1991). These Taberlet primers have been widely used in many species-level phylogenetic studies including Disa (Bellstedt *et al*., 2001), Medicago (Bena, 2001), Cytisus (Cubas *et al*., 2002), Hypochaeris (Samuel *et al*., 2003), Digitalis L. (Bräuchler *et al*., 2004) Fuchsia (Berry *et al*., 2004), Genista (Pardo *et al*., 2004), Arecuthobium (Nickrent *et al*., 2004).

The spider orchids within dilatata group were used in this study. These were chosen because of the subtle variation within their readily recognisable floral architectures. The aim of this chapter is to evaluate the level at which molecular data from ITS and *trn*T-L regions provide phylogenetic information to distinguish between the species in the dilatata group.

6.2 Materials and methods

6.2.1 Plant materials

Twenty-six spider orchid (*Caladenia* section *Calonema*) specimens, 22 from the dilatata group, three from the patersonii group, one from the reticulata group and three outgroup taxa from related genera were used in this study. Plants were collected from their natural habitats and in some cases from cultivated plants of known origin from the Royal Botanic Garden, Melbourne. The list of plant specimens is shown in Table 6.1.

Table 6.1 Details of *Caladenia spp.* used for sequencing analysis

species code	Species	Location
Outgroup		
C.latifolia	*C. latifolia*	Cultivated, Roy. Bot. Gdns, Melbourne
C.flaccida	*C. flaccida*	Knocker Track, Omeo
L.menziesii	*L. menziesii*	Cultivated, Roy. Bot. Gdns, Melbourne
car-angHS	*C. cardiochila*	Anglesea (O'Donohues)
tes-wilLP	*C. tessellata*	Lilly Pilly Gully (Wilsons Prom NP)
ven-lanMSF	*C. venusta*	Meerek State Forest
ret-staDL	*C. reticulata*	Deep Lead FFR, Stawell
Ingroup		
amo-RBG	*C. amoena*	Royal Botanic Garden from plants collected at Wattle Glen
par-lanLT	*C. parva*	Longbottom track, Langkoop
par-lanMSF	*C. parva*	Meerek State Forest
par-dod	*C. parva*	Dodd St St Andrews
par-too	*C. parva*	Peppermint Ridge Farm, Toorour
pha-lanLT	*C. phaeoclavia*	Longbottom track, Langkoop
pha-lanMR	*C. phaeoclavia*	Mereek Rd, Langkoop
pha-golEP	*C. phaeoclavia*	Golden point, expedition pass
pha-RBG	*C. phaeoclavia*	Cultivated, RBG Melbourne
str-idnGP	*C. stricta*	Little Desert NP (gravel pits)
str-diaRa	*C. stricta*	Diapur roadside
ten-graWP	*C. tentaculata*	Glenelg River Rd, Woolpooer, Grampians NP
ten-albGT	*C. tentaculata*	Albury NSW (Gap Trail)
tens-kiaFR	*C. tensa*	Kiata Flora reserve
tens-RBG	*C. tensa*	Cultivated, RBG Melbourne
tox-carSA	*C. toxochila*	Carapee Hills SA
tox-IdnKL	*C. toxochila*	Little Desert NP. Kiata Lowan Sanctuary
tox-mamSA	*C. toxochila*	Mambray Ck, SA
ver-mir	*C. verrucosa*	Miram Piram
vil-lanLT	*C. villosissima*	Longbottom track, Langkoop
vil-lanMSF	*C. villosissima*	Meerek State Forest
vil-lanMR	*C. villosissima*	Mereek Rd, Langkoop

6.2.2 Genomic DNA extraction

Genomic DNA extraction and assessment of DNA quality were carried out as described in chapter 3.2.

6.2.3 DNA amplification

6.2.3.1 ITS region amplification

Double-stranded DNA of complete ITS regions including ITS1, 5.8S and ITS2 was amplified from total genomic DNA using the ITS1 and ITS4 primers of White *et al*. (1990) synthesized by Invitrogen, Australia (ITS1, 5'-TCC GTA GGT GAA CCT GCG G-3'; ITS4, 5'-TCC TCC GCT TAT TGA TAT GC-3'). Amplification was carried out in 50-µl reaction volumes including 1 U Platinum®Taq DNA Polymerase (Invitrogen), 1X PCR buffer supplied by the manufacturer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 µM of each primer and 50 ng genomic DNA. Amplification was performed in a MJ research PTC-100 or PTC-200 thermocycler using the following conditions: denature at 95^0C for 5 min followed by 35 cycles of 95^0C for 1 min, 55^0C for 1 min and 72^0C for 1 min with a final extension at 72^0C for 7 min (modified from Lashermes *et al*., 1997). Amplified products were then separated on 1.4% agarose containing 0.6 µg/ml of ethidium bromide in TBE buffer, and visualized under UV light. Next PCR products were cut for purification.

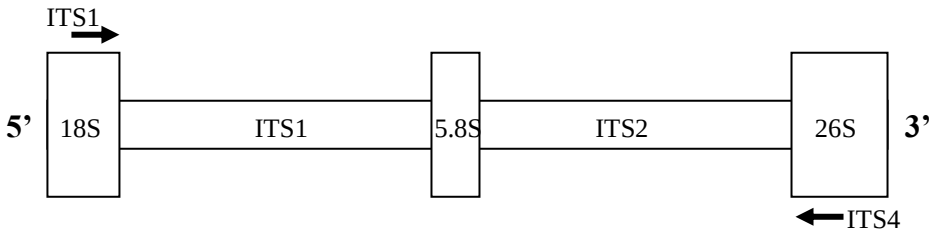


Figure 6.1 The three coding and two internal transcribed spacer regions of the nuclear ribosomal DNA repeat unit of a typical angiosperm (not drawn to scale). Arrows indicate approximate locations of the two primers used for PCR amplification of ITS region used in this study (Saar and Polans, 2000).

6.2.3.2 cpDNA regions amplification

Two cpDNA regions were amplified by polymerase chain reaction: the non-coding region of the *trn*T (UGU) and *trn*L (UAA) 5'exon spacer and *trn*L (UAA) 5'exon and *trn*F (GAA) intergenic spacer. The universal primers a, b, c and f of Taberlet *et al*. 1991 (Table 6.1 and Figure 6.2) were synthesized by Invitrogen, Australia. PCR reactions were conducted in 50-µl reaction volumes using Platinum®*Pfx* DNA Polymerase (Invitrogen). The PCR reaction was carried out using a modified protocol from the manufacturer. The amplification reactions contained 2X amplification buffer supplied by the manufacturer, 1.5 mM $MgSO_4$, 0.6 mM of each dNTP, 0.2 µM of each primer 0.75 U Platinum®*Pfx* DNA Polymerase (Invitrogen), and 100 ng genomic DNA. Both target regions required separate amplification parameters. The standard PCR condition was employed in the amplification of the *trn*T (UGU) and *trn*L (UAA) 5'exon spacer: denature at 94^0C for 5 min followed by 35 cycles of 94^0C for 1 min, 48^0C for 1 min and 72^0C for 3 min with a final extension at 72^0C for 5 min (Taberlet *et al*., 1991). The amplification reaction for *trn*L (UAA) 5'exon and *trn*F (GAA) intergenic spacer were performed with the following procedure: 1 cycle of 95^0C for 5 min and 35 cycles of 92^0C for 45 sec, 58^0C for 45 sec and 72^0C for 2 min with a final extension at 72^0C for 10 min (modified from Fujii *et al*., 2002). Amplification products were then separated on 1.4% agarose containing 0.6 µg/ml of ethidium bromide in TBE buffer, and visualized under UV light. The amplified target DNA regions were cut from the gel for the purification step.

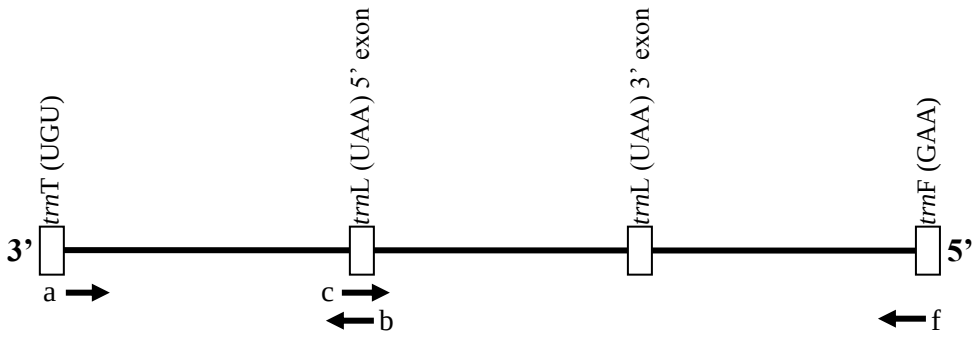


Figure 6.2 Approximate locations of *trnT-L* and *trn*LF primers used in this study (from Taberlet *et al*., 1991).

Table 6.2 Sequences of the *trnT-L* and *trn*LF primers used in this study (from Taberlet *et al*., 1991)

Name	5'-3' Sequence
a	CAT TAC AAA TGC GAT GCT CT
b	TCT ACC GAT TTC GCC ATA TC
c	CGA AAT CGG TAG ACG CTA CG
f	ATT TGA ACT GGT GAC ACG AG

6.2.4 DNA Template purification

Successful amplifications were purified using QIAquick Gel Extraction Kit (Qiagen, Australia), following the manufacturer's protocol. Purified PCR's were quantified by estimation using a low MassRuler^{TM} DNA Ladder (Fermentas Life Science, Australia) and then cycle sequenced using the same primers as were used for amplification.

6.2.5 Sequencing reaction

DNA sequencing was performed according to the method of Sanger *et al*. (1977) and was conducted using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v.3 (Applied Biosystems). Sequencing reactions were conducted in 20 µl final volumes containing 1 µl of the commercial BigDye solution, 1.5 mM $MgCl_2$, 37.5 mM Tris-HCl (pH 9.0), 3.2 pmoles of primer and ~20 ng of PCR product. Thermal cycling was undertaken in the MJ research PTC-100 or PTC-200 thermocycler according to the standard protocol (Applied Biosystems), consisting of initial denaturation at 96^0C for 1 minute followed by 25 cycles, each of 96^0C for 1 minute, 50^0C for 10 seconds and 60^0C for 4 minutes. The sequencing reactions were ethanol/EDTA-precipitated according to the supplied protocol (Applied Biosystems) and air-dried prior to being analysed on an ABI 373A automated sequencer at the Micromon DNA Sequencing Facility. Department of Microbiology, Monash University, Clayton, Australia. To ensure the accuracy of the sequences, both forward and reverse strands of PCR products were sequenced and regions of low-quality or ambiguous sequence were clarified through replicate sequencing reactions.

6.2.6 Sequencing analysis

Sequence editing and alignments were conducted using the BioEdit software package v 6.0.6 (www.mbio.ncsu.edu/Bioedit/bioedit.html; Hall, 1999). The quality of DNA sequence was assessed by visual analysis of the trace file using the Chromas software package v 2.31 (http://www.technelysium.com.au/chromas.html). Areas of ambiguity ('N' bases) could often be resolved by visual checking of the chromatogram; where this was not possible, replicate sequencing reactions were conducted, as described above.

6.2.7 Alignment of multiple sequences

Multiple sequence alignments of DNA were performed with the "ClustalX" version 1.83 program (Thompson *et al*., 1997). The multiple alignment parameters were a gap opening penalty of 10.0 and a gap extension penalty of 0.20.

6.2.8 Parsimony analysis

Parsimony analysis was conducted using PAUP* 4.0 b10 (Swofford, 2001). A heuristic search was performed using the maximum parsimony optimality criterion. Starting trees for branch swapping were obtained by stepwise addition. Branch swapping utilized the tree-bisection-reconnection (TBR) algorithm with saving and swapping on all optimum trees (MulTrees on), and saving only the shortest trees or the shortest from each replicate. The resulting trees were used as starting trees in another round of TBR with the same parameters as the first and swapping on all trees. Gaps were treated as missing data and branches with a minimum length of zero were collapsed. Support for tree topology was evaluated with 1000 bootstrap replicates. A bootstrap analysis of 1000 replicates was conducted using the same search criteria as the initial search. For both data sets, trees were rooted using *C. latifolia*, *C. flaccida*, *L. menziesii*, *C. cardiochila*, *C. tessellata*, *C. venusta* and *C. reticulata* as outgroup taxa.

6.3 Results

6.3.1 Sequence characteristic

According to RAPD and ISSR analysis, the grouping of specimens collected from the same location indicated that the obtaining phenograms did not well resolve the genetic

relationship within the group. As a result, the green comb group was chosen for sequence analysis and only one representative from each location was used. The total number of 29 individuals consisted of 22 specimens from the dilatata group used in previous chapters, some new specimens available at the time the experiment was conducted, and seven outgroup. The representatives *C. reticulata* from the reticulata group and, *C. venusta*, *C. cardiochila* and *C. tessellata* from patersonii group were used as outgroup.

6.3.1.1 ITS

The ITS region generated from ITS1 and ITS4 primers (White *et al*.,1990) gave a single strong amplified product of approximately 750 bp on agarose gel as shown in Figure 6.3. The boundaries of ITS1, ITS2 and adjacent coding region were determined by comparing the published sequences from GenBank (www.ncbi.nlm.nih.gov/Genebank/index.html). The ITS sequences were missing a portion of 13 to 18 bp from the 3' end of the ITS2 spacer due to poor sequencing of that region. Among 29 specimens, ITS sequences sizes ranged from 655 bp (*L. menziesii*) to 660 bp (all spider orchid specimens). The length of ITS1 of spider orchids was 249 bp, while ITS2 ranged from 416 bp to 660 bp. The 5.8S rDNA had a length of 162 bp across all samples. Mean base frequencies of spider orchid specimens were A=0.276, C=0.191, G=0.229 and T=0.305 which were not very different from the base frequencies of the three outgroup taxa (*C. latifolia*, *C. flaccida*, and *L menziesii*) which were A=0.264, C=0.192, G=0.239 and T= 0.308. The G+C content of spider orchids varied from 41.67 to 42.12%, again, similar to those in the three outgroup taxa which contained 42.64% G+C content in *C. latifolia*, 42.03% in *C. flaccida* and 44.58% in *L menziesii*. Multiple sequence alignment

required the addition of several gapped positions including 20 indel-characters (11 in ITS1, 2 in 5.8S and 7 in ITS2). The aligned data matrix of the ITS region included a total of 671 sites. Of these, 544 characters were constant, 102 characters were parsimony uninformative and 25 characters were parsimony informative.

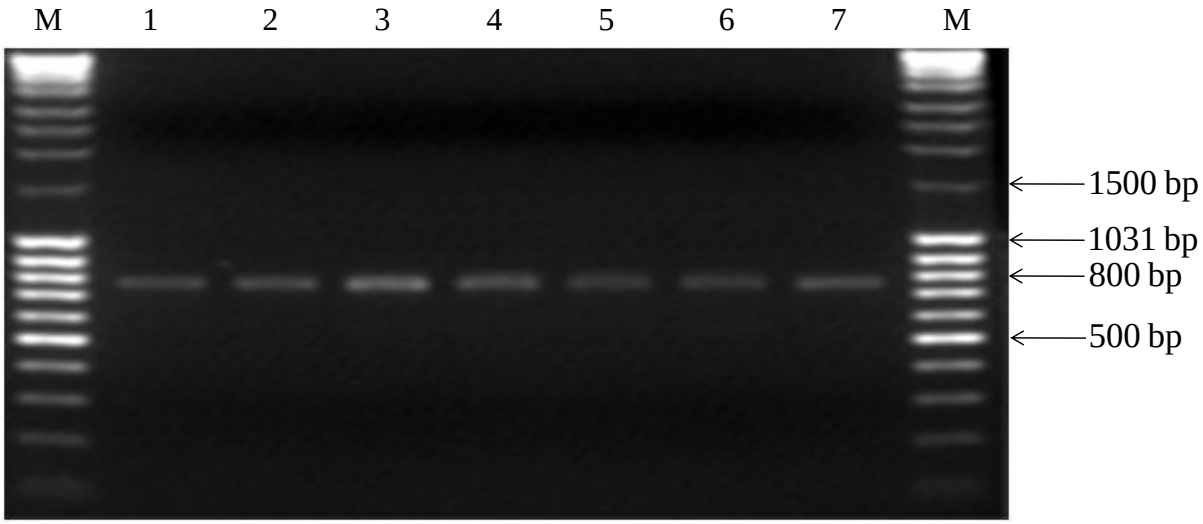


Figure 6.3 Amplification of ITS region generated from primer ITS1 and ITS4 on 7 individual plants. Lanes M=low DNA ladder molecular weight marker, Lane 1=*C. tentaculata*, Lane 2-3=*C. tensa*, Lane 4-6=*C. toxochila*, Lane 7=*C. verrucosa*.

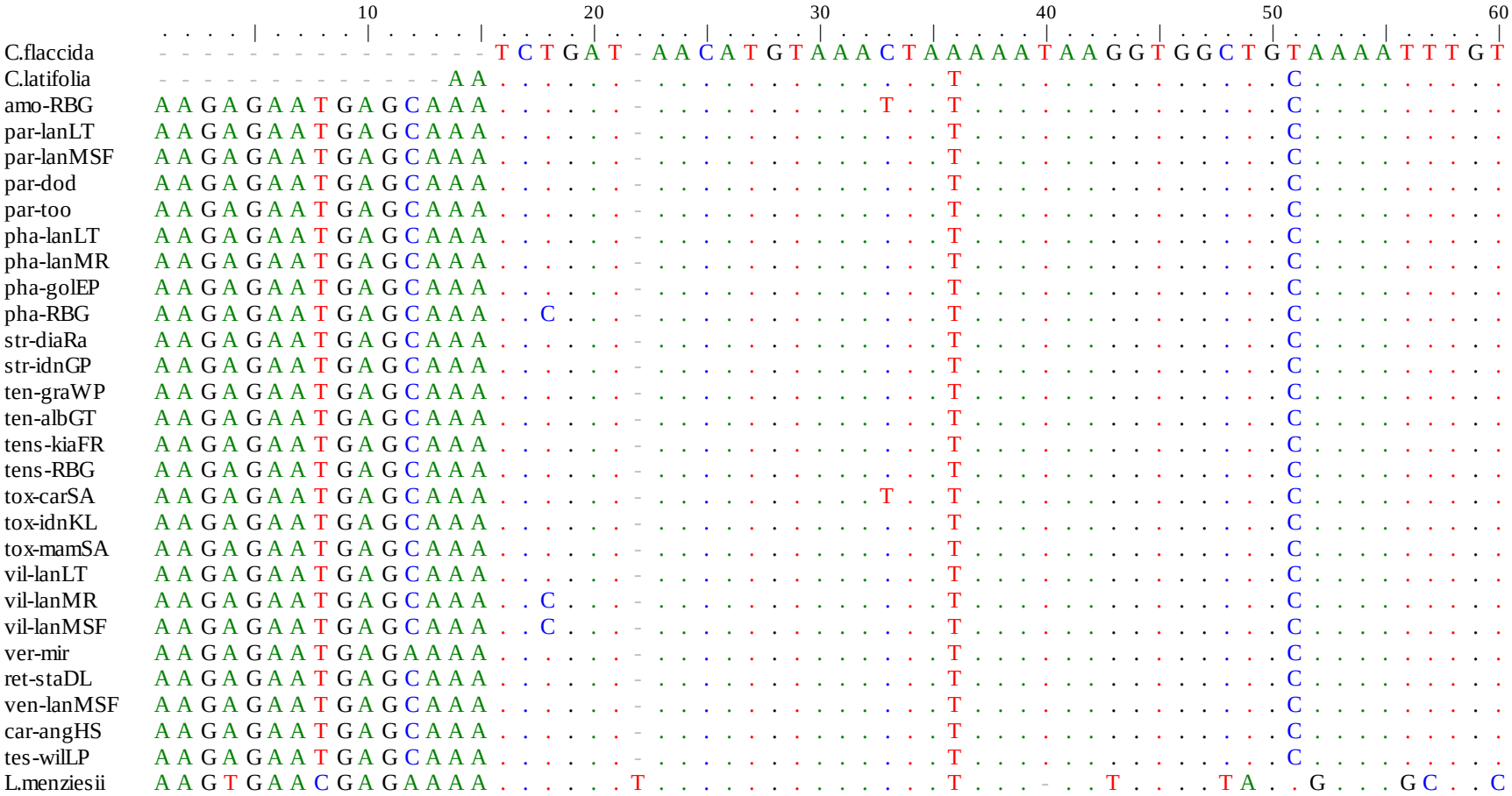


Figure 6.4 Sequences of internal transcribed spacer 1 (ITS1)-5.8 S rDNA-ITS2 fragment from 22 specimens from the dilatata group, three from the patersonii group and one from the reticulata group and three outgroup taxa from related genera. Sequences Identical with the top sequence are indicated by dots; and hyphens represent gaps.

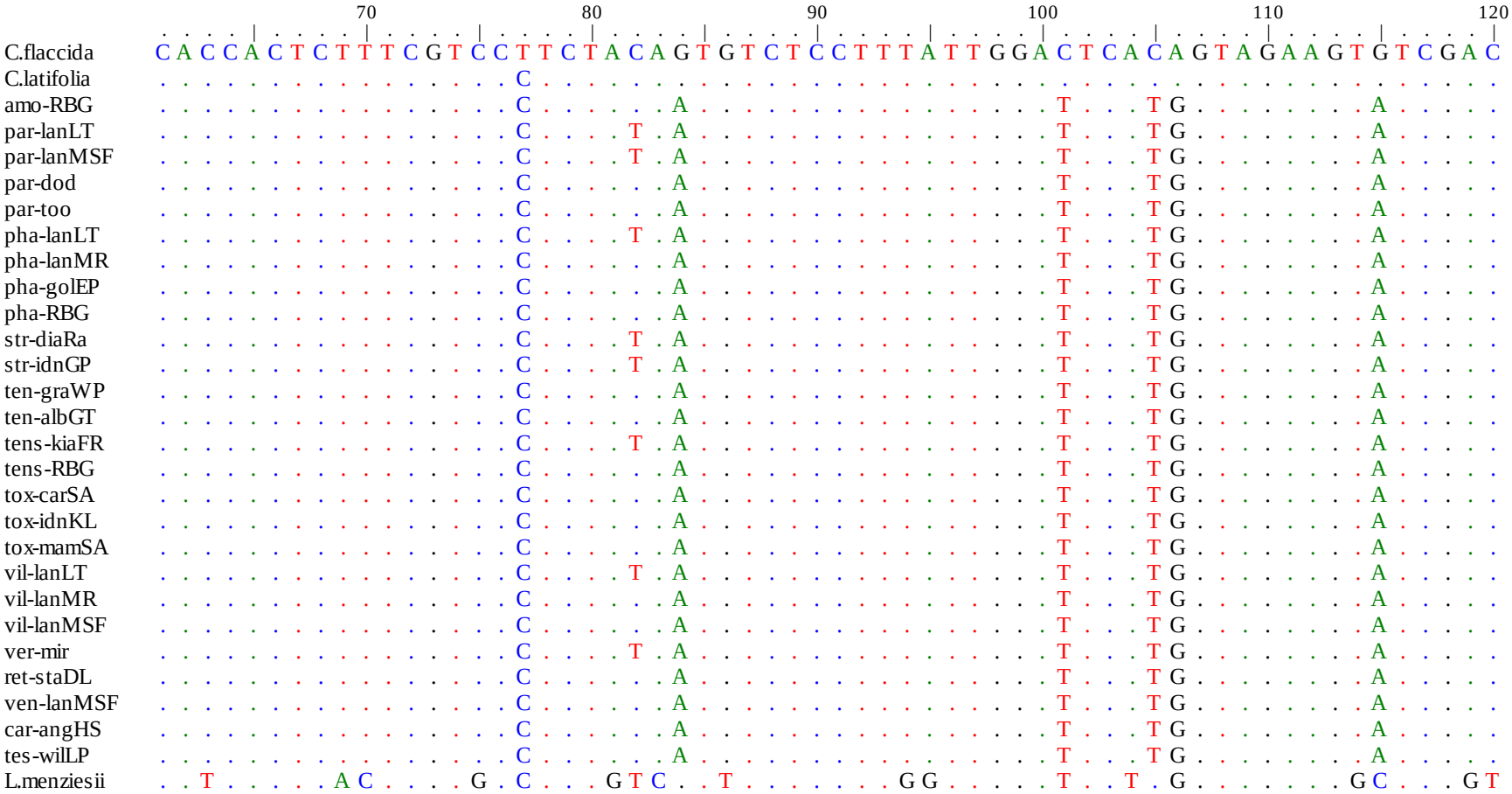


Figure 6.4 (continued)

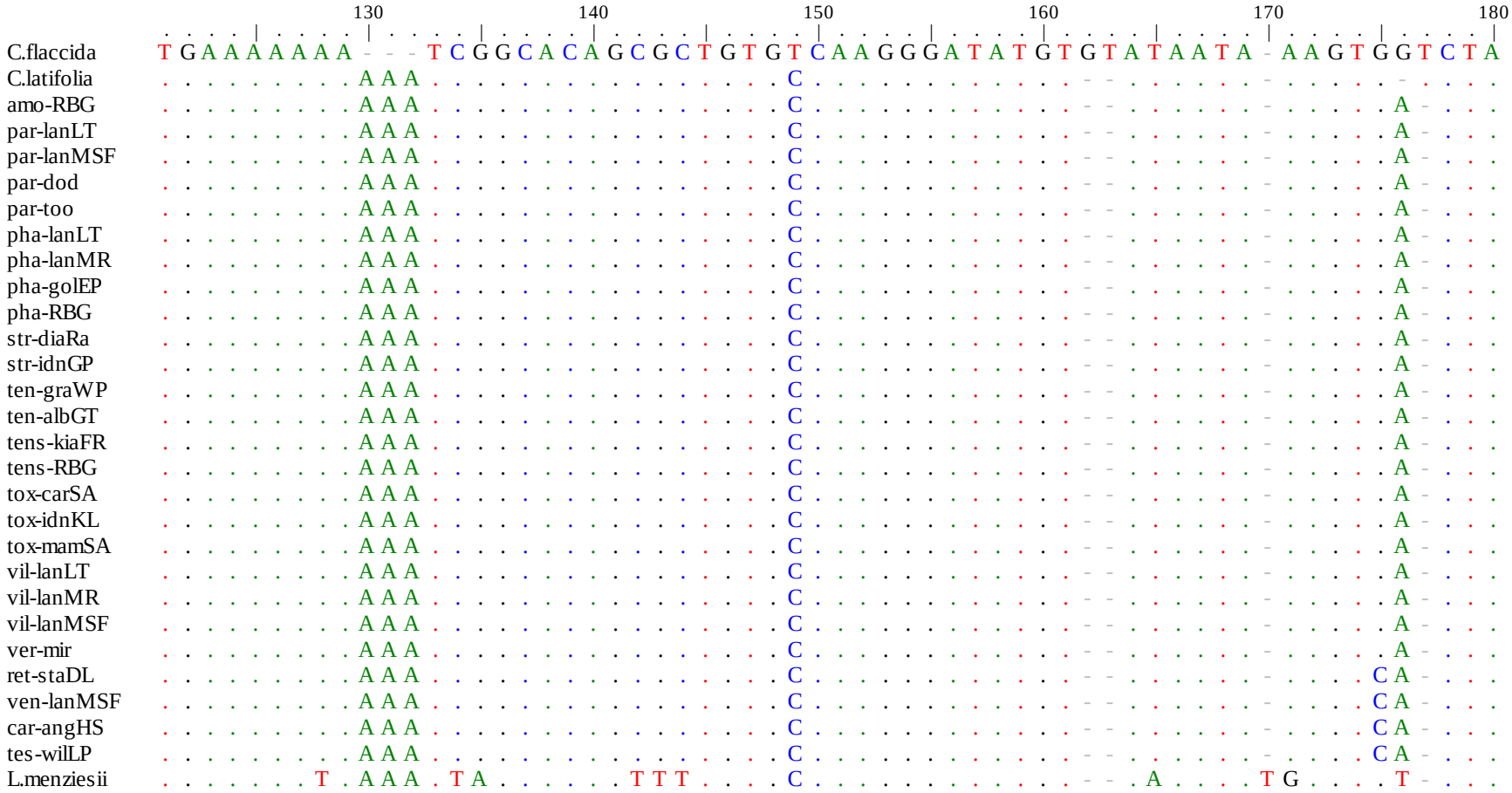


Figure 6.4 (continued)

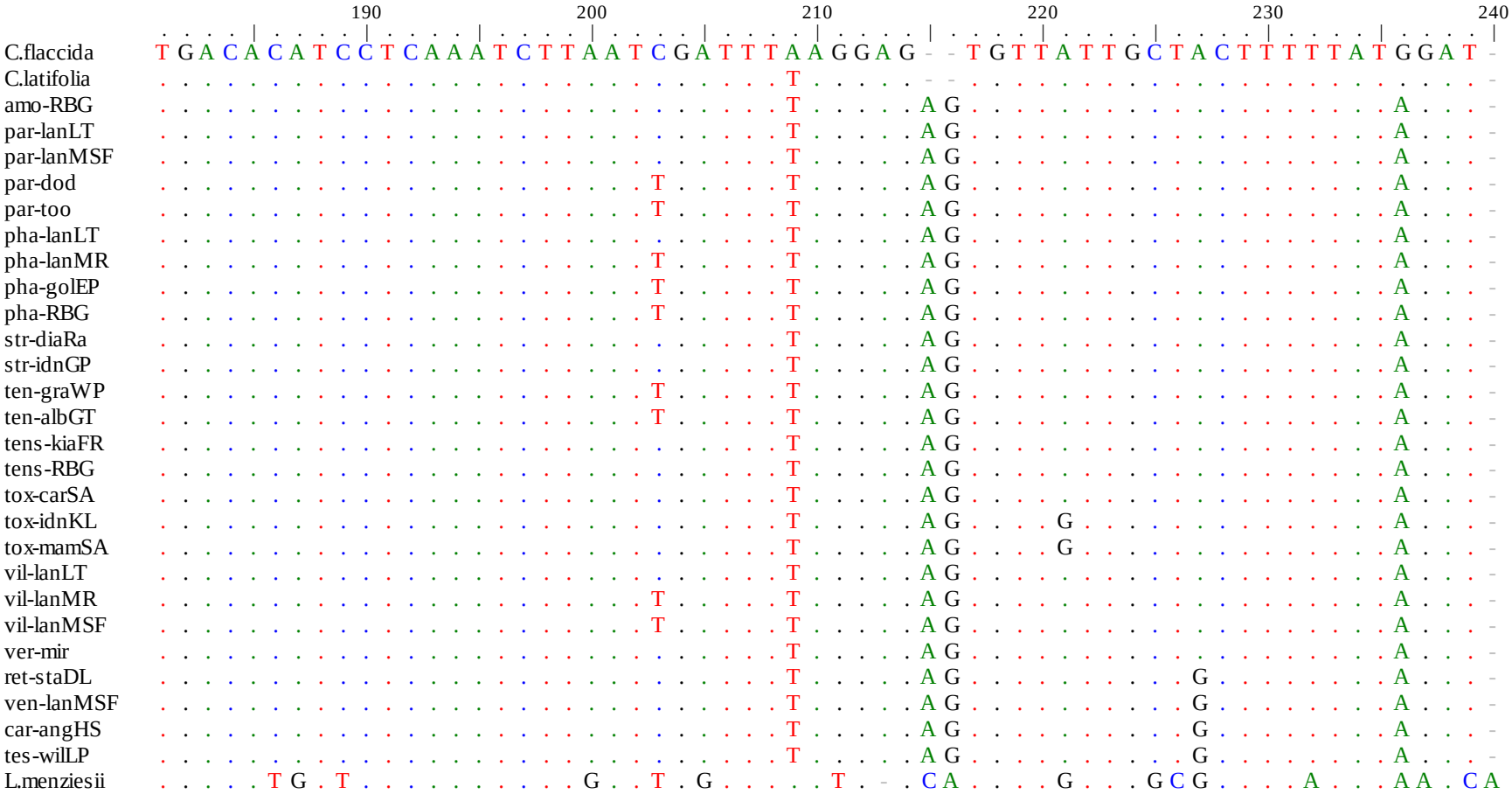


Figure 6.4 (continued)

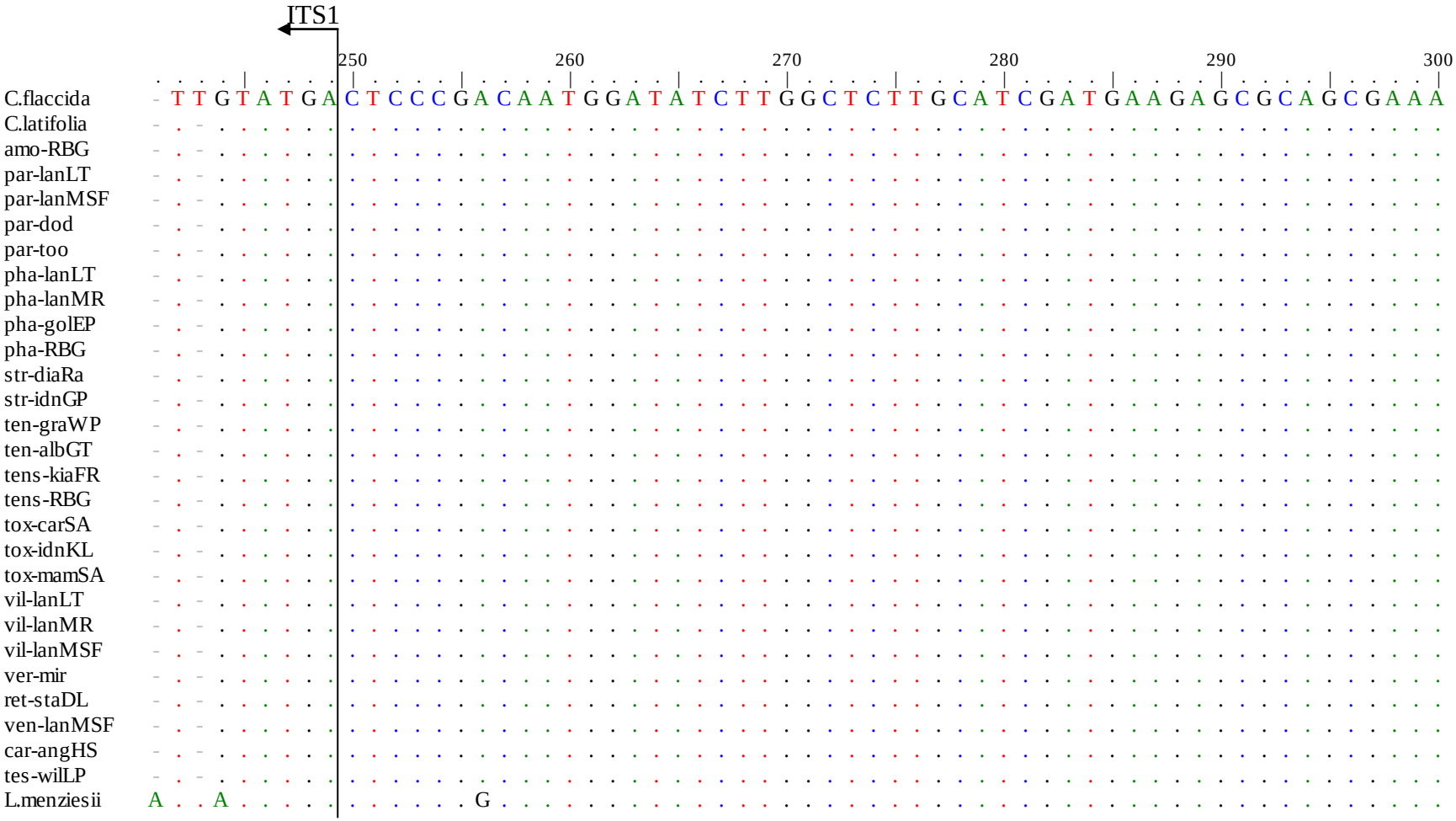


Figure 6.4 (continued)

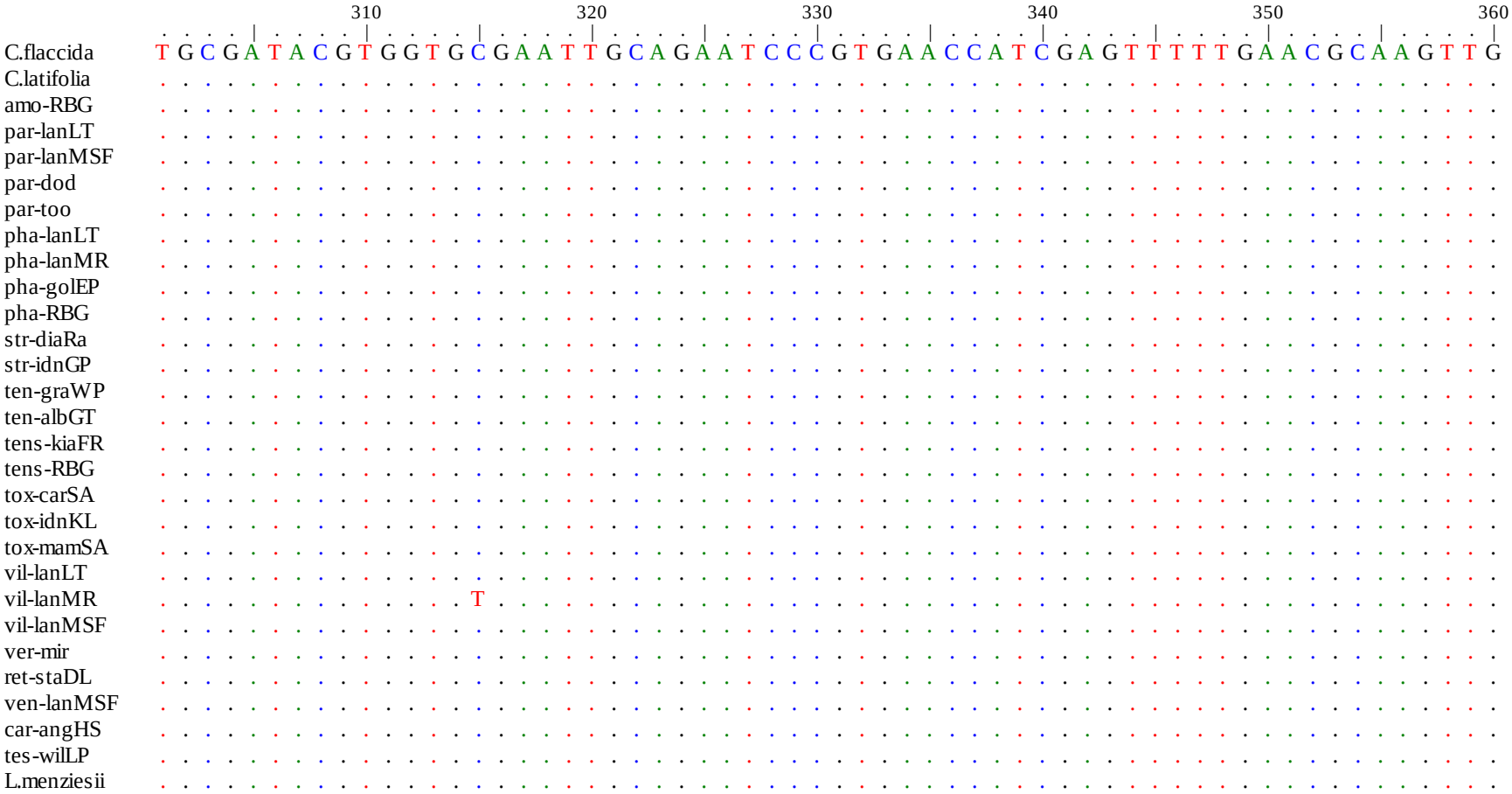


Figure 6.4 (cont.)

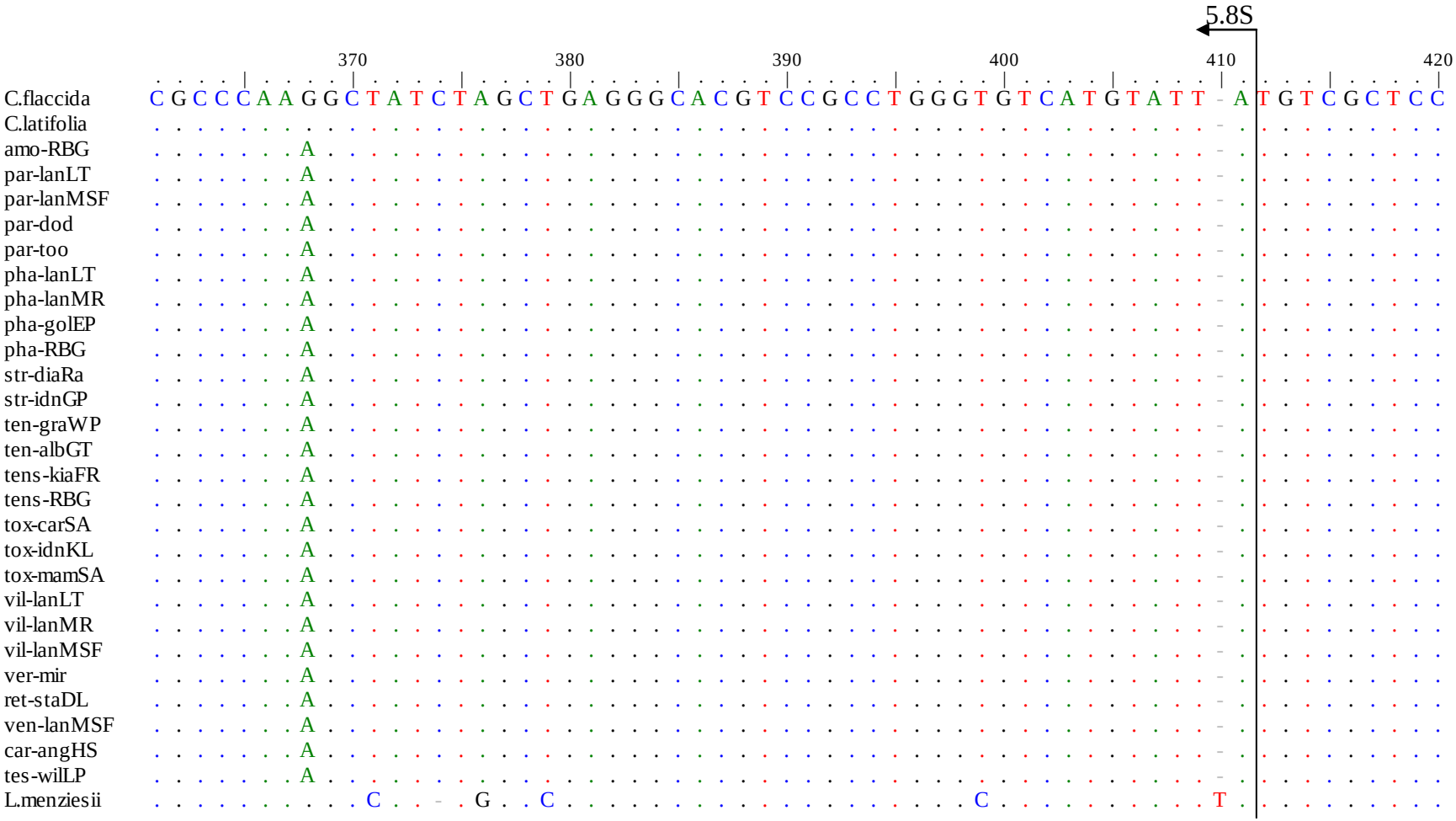


Figure 6.4 (cont.)

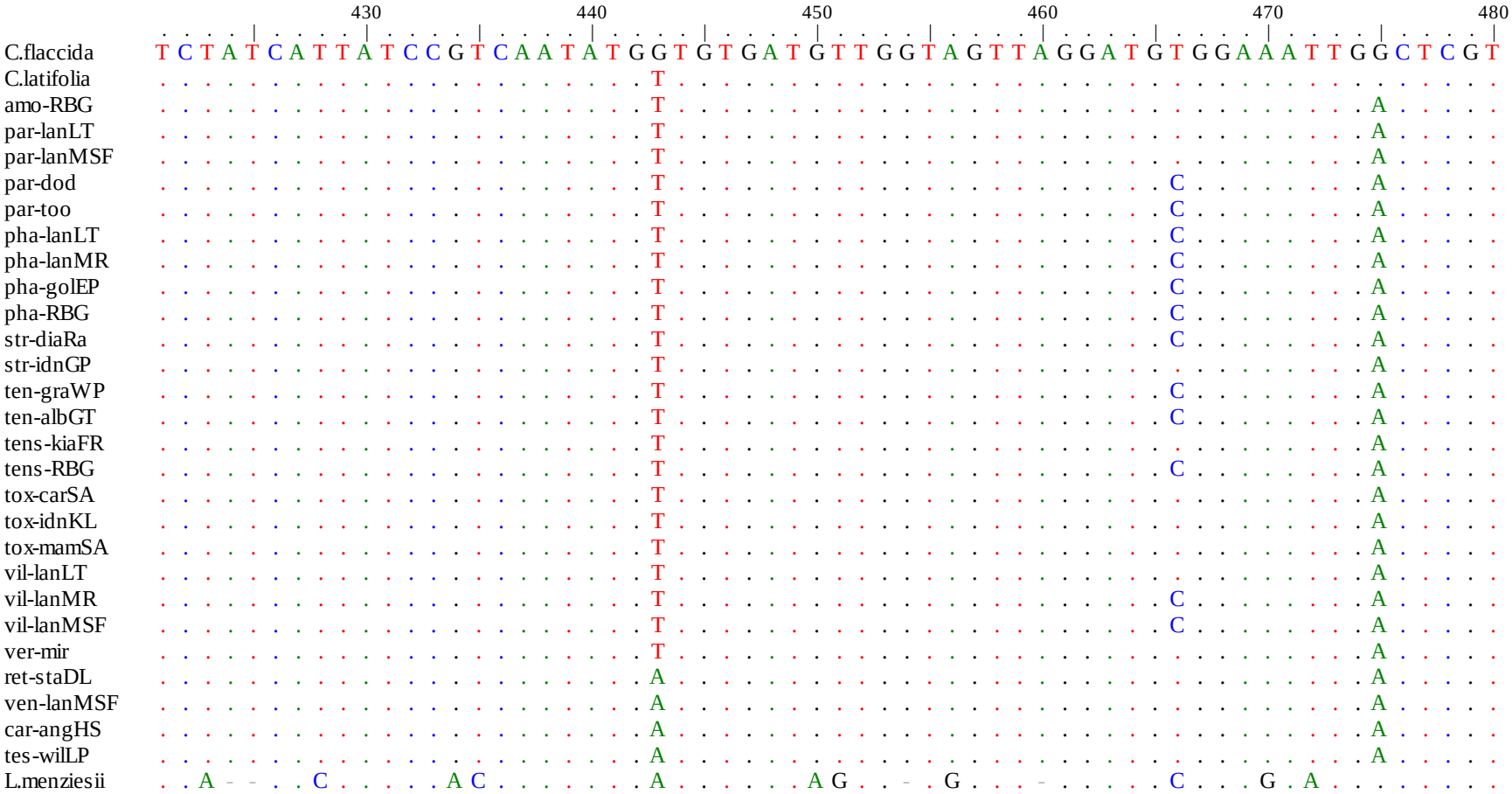


Figure 6.4 (cont.)

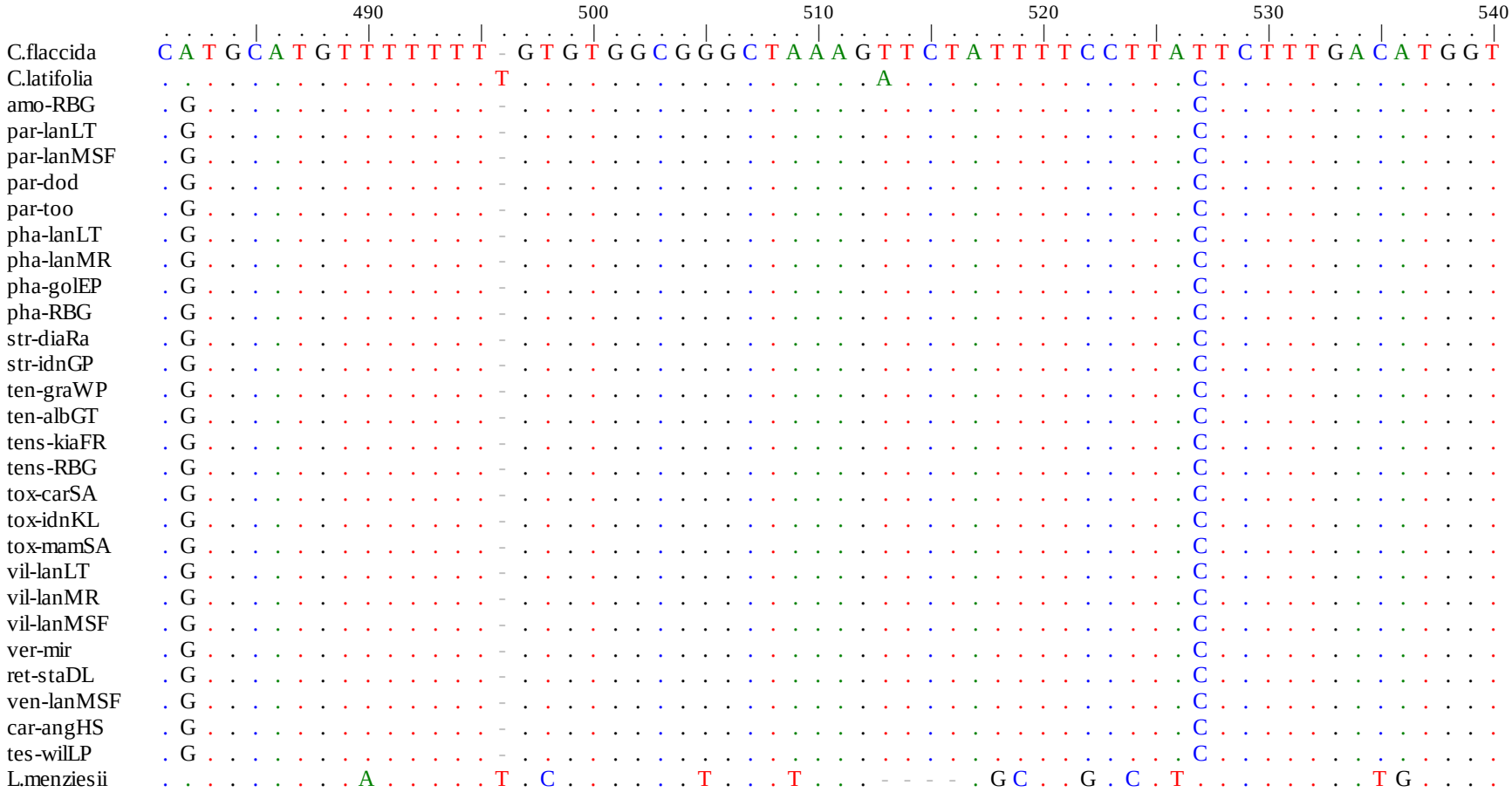


Figure 6.4 (continued)

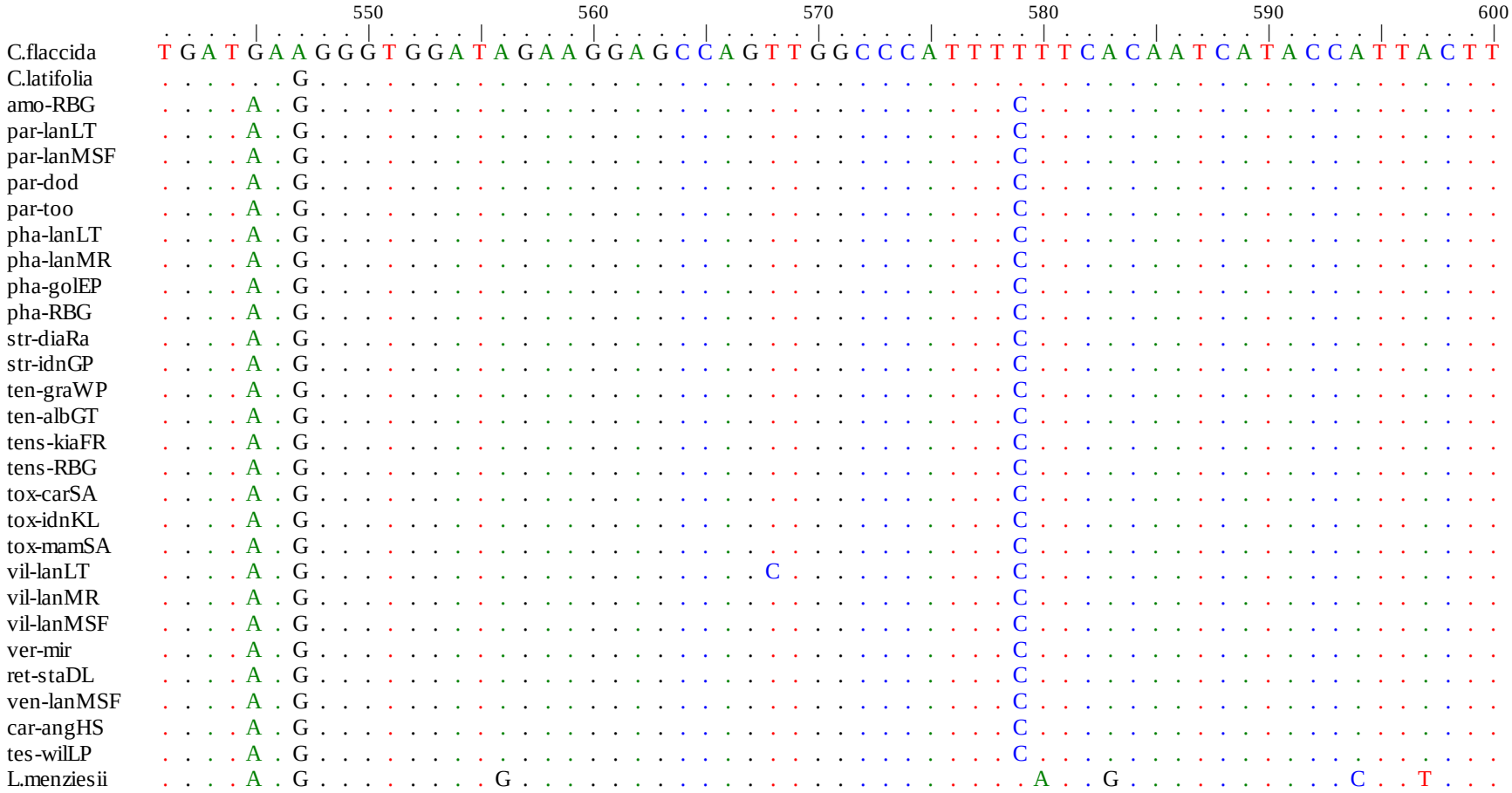


Figure 6.4 (cont.)

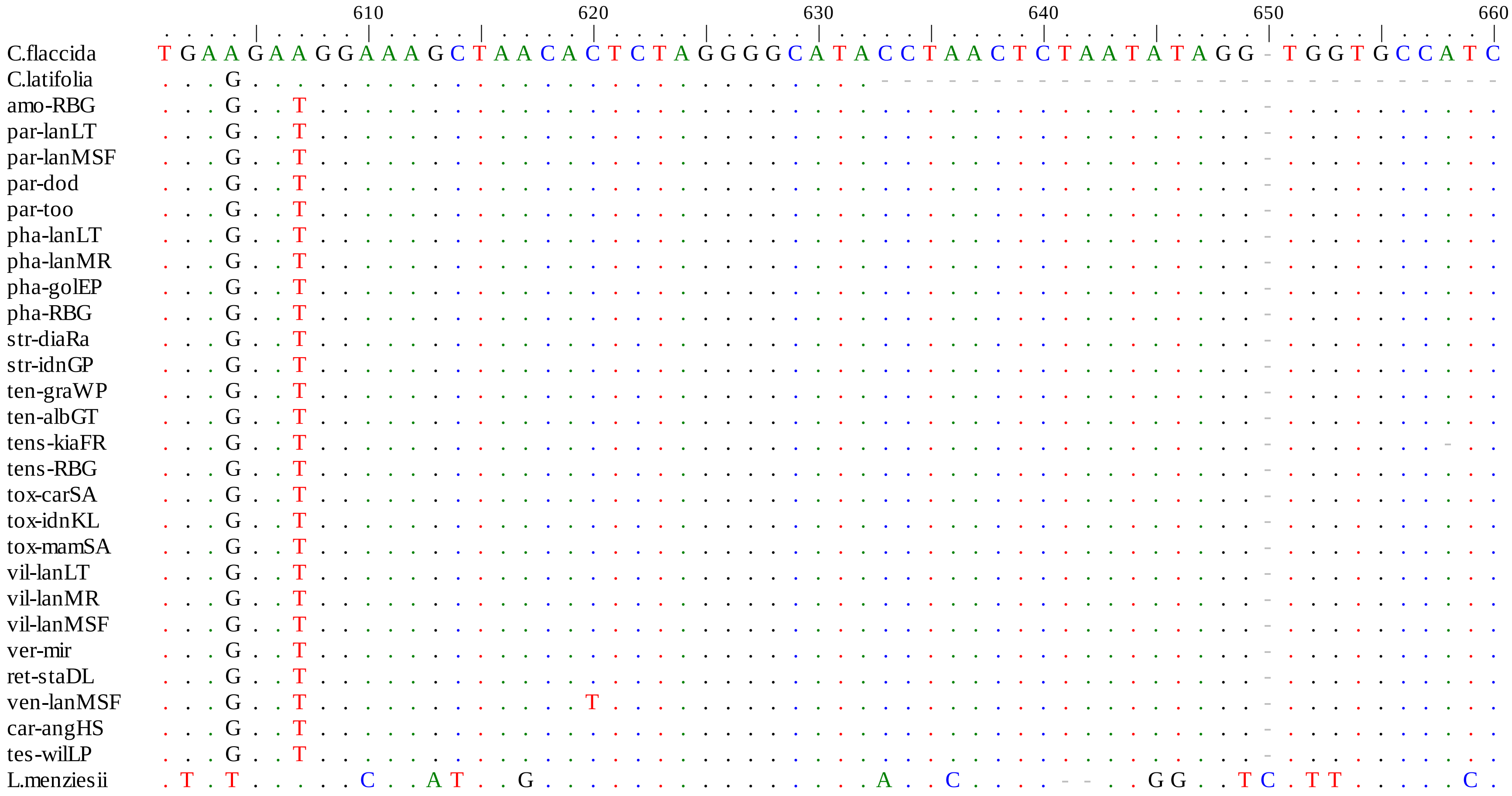


Figure 6.4 (cont.)

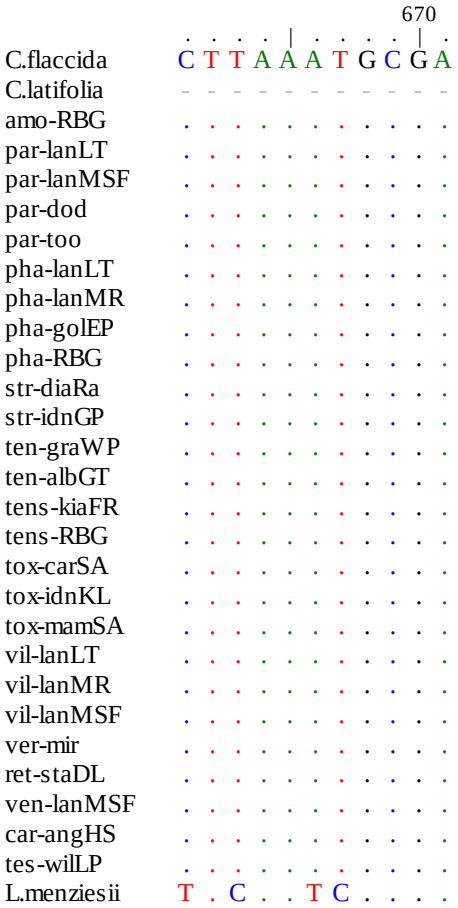


Figure 6.4 (cont.)

Transversion substitution in spider orchid ITS region found at position 12 (C→A) in *C. verrucosa*, 175 (G→C) and 444 (T→A) both in reticulata and patersonii outgroup (Table 6.3). The T→C transition occurred at position 18 in *C. phaeoclavia* RBG, and *C. villosissima* from Mereek Rd, Langkoop and Meerek State Forest (Table 6.3). While at position 467 found in *C. phaeoclavia* from Golden point, expedition pass, Longbottom track, Langkoop, Mereek Rd, Langkoop and RBG, *C. parva* from Dodd St St Andrews, *C. stricta* from Diapur roadside, *C. tentaculata* from Albury NSW (Gap Trail) and Glenelg River Rd, Woolpooer, Grampians NP, *C. tensa* Cultivated, RBG Melbourne and *C. villosissima* from Mereek Rd, Langkoop and Meerek State Forest(Table 6.3) . The last position of T→C mutation found in *C. villosissima* from Longbottom track, Langkoop at position 568 (Table 6.3). The C→T substitution found at position 33, 82, 203,305 and 602 mostly in the dialata group especially, *C. parva, C. phaeoclavia* and *C. villosissima* with the exception of position 602 found in *C. venusta* as indicated in Table 6.3**.** *Caladenia toxochila* from Little Desert NP. Kiata Lowan Sanctuary and Mambray Ck, SA had A→G transition at position 221 while *C. reticulate*, *C. cardiochila, C. tessellate* and *C. venusta* exhibited at position 227 (Table 6.3)

Table 6.3 Mutations in ITS within spider orchid species

Taxa[a]	Mutation	Sequence	Position[b]
ver-mir	Transversion	C-A	12
pha-RBG, vil-lanMR, vil-lanMSF	Transition	T-C	18
amo-RBG, tox-carSA	Transition	C-T	33
par-lanLT, par-lanMSF, ,str-diaRa, str-idnGP, tens-graWP, ver-mir, vil-lanLT	Transition	C-T	82
ven-lanMSF, car-angHS, ret-staDL, tes-wilLP	Transversion	G-C	175
par-dod, par-too, pha-golEP, pha-lanMR, pha-RBG, ten-albGT, ten-graWP, vil-lanMR, vil-lanMSF,	Transition	C-T	203
tox-idnKL, tox-mamSA	Transition	A-G	221
ret-staDL, car-angHS, tes-wilLP, ven-lanMSF	Transition	A-G	227
vil-lanMR	Transition	C-T	305
ret-staDL, car-angHS, tes-wilLP, ven-lanMSF	Transversion	T-A	444
pha-golEP, pha-lanLT, pha-lanMR, pha-RBG, par-dod, str-diaRa, ten-albGT, ten-graWP, tens-RBG, vil-lanMR, vil-lanMSF	Transition	T-C	467
vil-lanLT	Transition	T-C	568
ven-lanMSF	Transition	C-T	620

[a] species names are abbreviated as in Table 6.1

[b] nucleotide sites in aligned sequences are numbered consecutively from 5' to 3'

6.3.1.2 The non-coding region of the *trn*T (UGU) and *trn*L (UAA) 5'exon spacer

The *trn*T (UGU) and *trn*L (UAA) 5'exon spacer region was amplified between primers a (in the *trn*T) and b (in the *trn*L 5'exon) of Tablert *et al* (1991) as shown in Figure 6.2. The single strong amplified product band had an approximate size of 700 bp on agarose gel. However, sequencing indicated that the length of the non-coding region of the *trn*T (UGU) and *trn*L (UAA) 5'exon spacer region (excluding the outgroups) ranged between 593 to 606 bp. Mean base frequencies of spider orchids were A=0.391, C=0.128, G=0.133 and T=0.348 while the three outgroup taxa contained A=0.390, C=0.129, G=0.139 and G=0.341. The G+C content of ingroup taxa varied from 25.80 to 26.26% and outgroup taxa contained 27.29% in *C. latifolia*, 26.07% in *C. flaccida* and 27.23% in *L menziesii*. Multiple sequences alignment required the additional of a 16 indel-character. The aligned data matrix of the non-coding region of the *trn*T (UGU) and *trn*L (UAA) 5'exon spacer region included a total of 677 sites. Of these, 617 characters were constant, 49 characters were parsimony uninformative and 11 characters were parsimony informative.

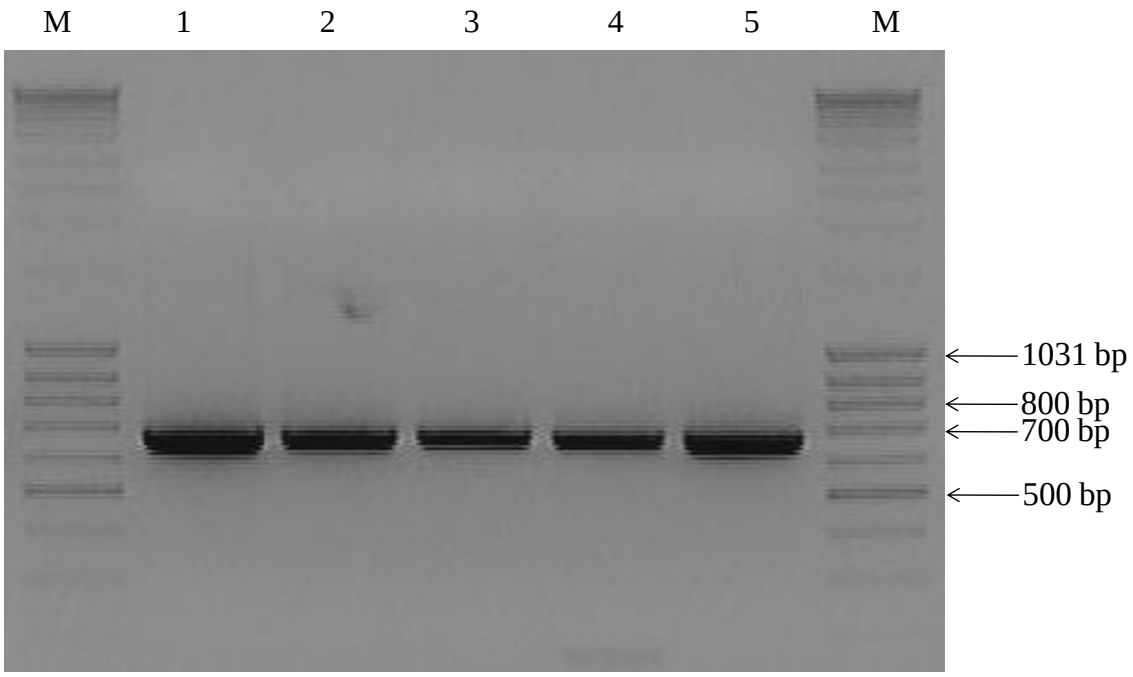


Figure 6.5 Amplification of the non-coding region of the *trn*T (UGU) and *trn*L (UAA) 5'exon spacer generated from primer "a" and "b" on 5 individual plants. Lanes M=low DNA ladder molecular weight marker, Lane 1=*C. parva*, Lane 2=*C. amoena*, Lane 3=*C. cardiochila*, Lane 4=*C. stricta*, Lane 5=*C .parva.*

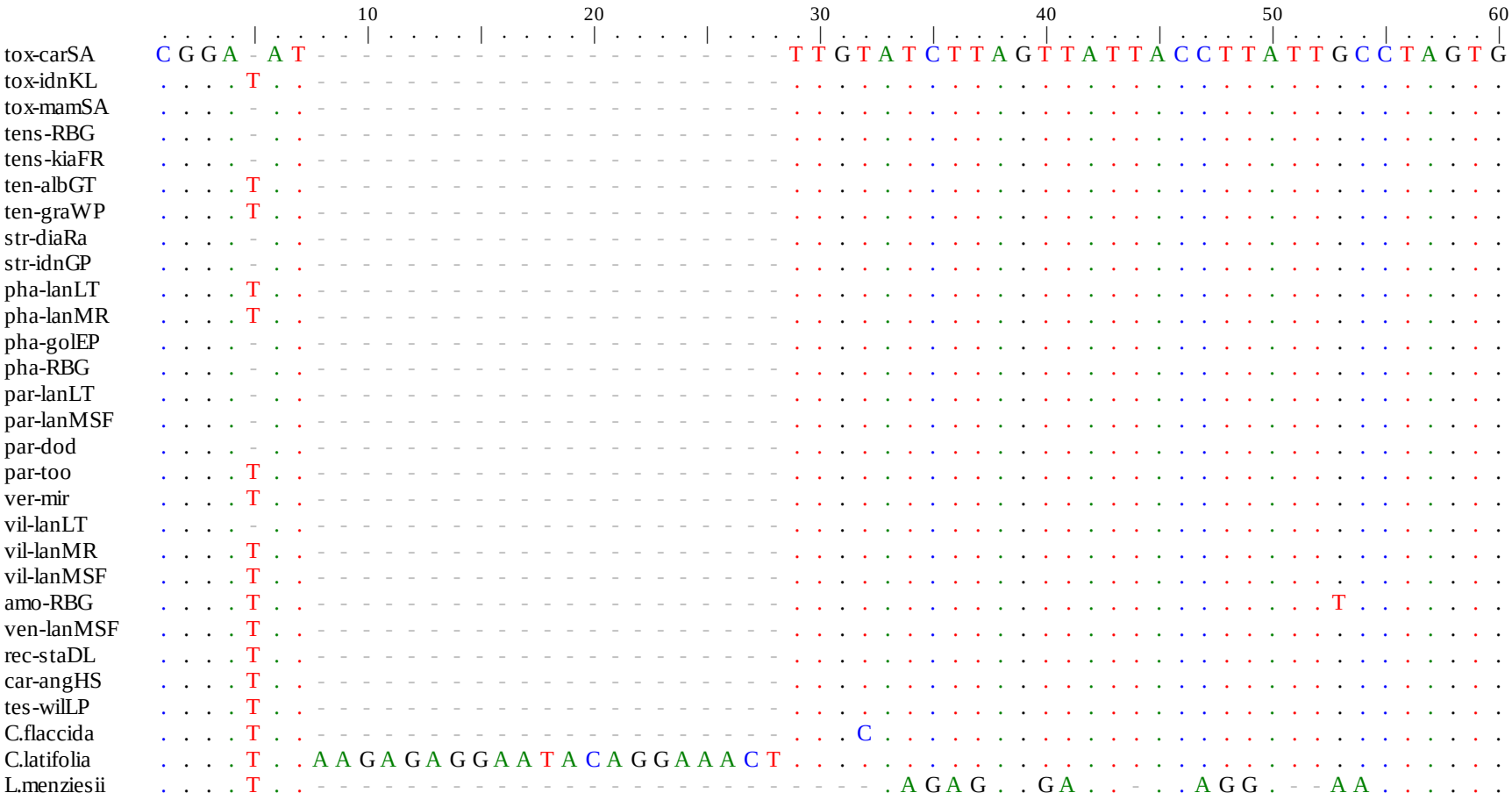


Figure 6.6 Sequences of the non-coding region of the *trn*T (UGU) and *trn*L (UAA) 5' exon spacer fragment from 22 specimens from the dilatata group, three from the patersonii group and one from the reticulata group and three outgroup taxa from related genera. Identities with the top sequence are indicated by dots; and hyphens represent gaps.

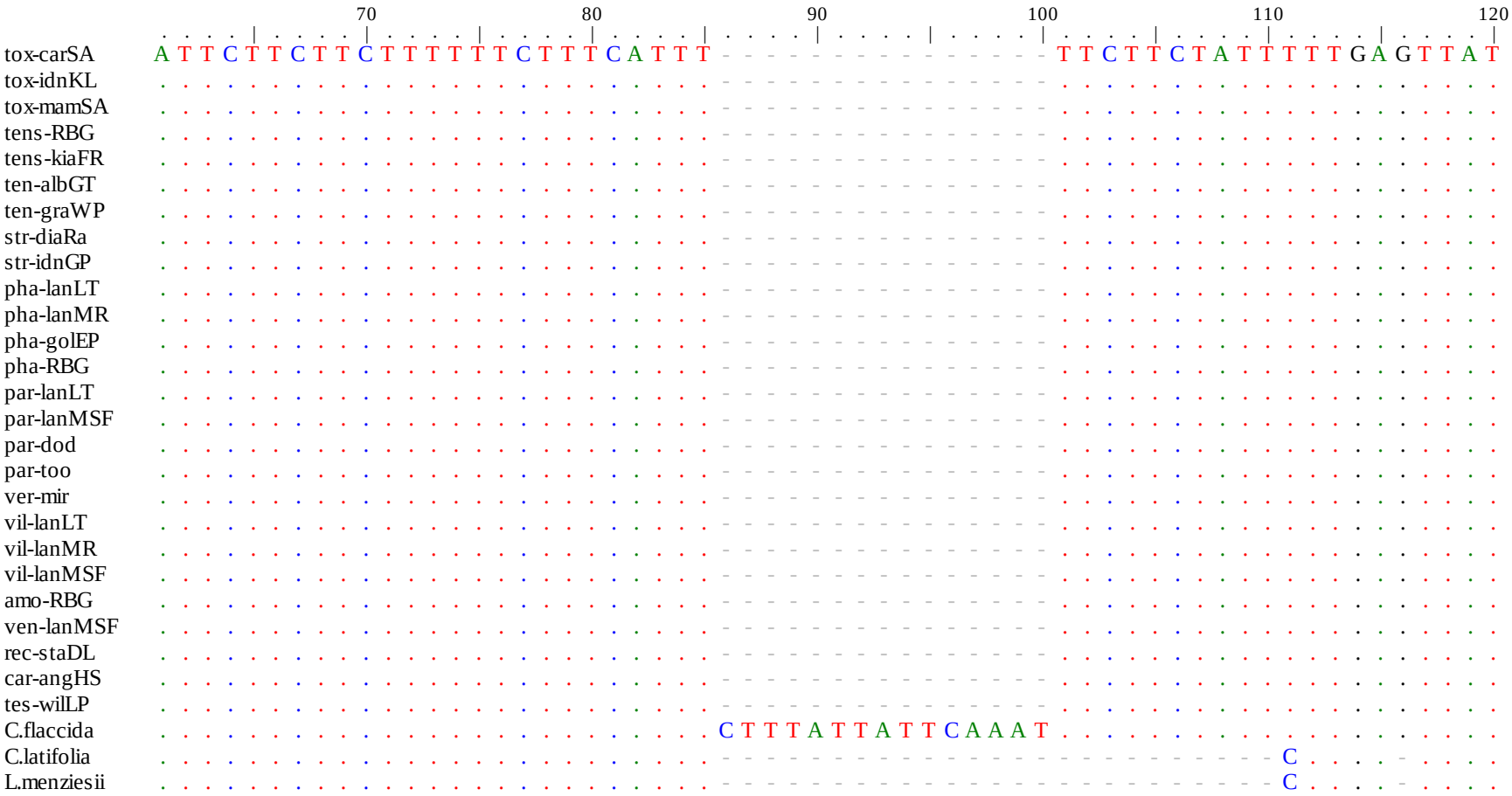


Figure 6.6 (cont.)

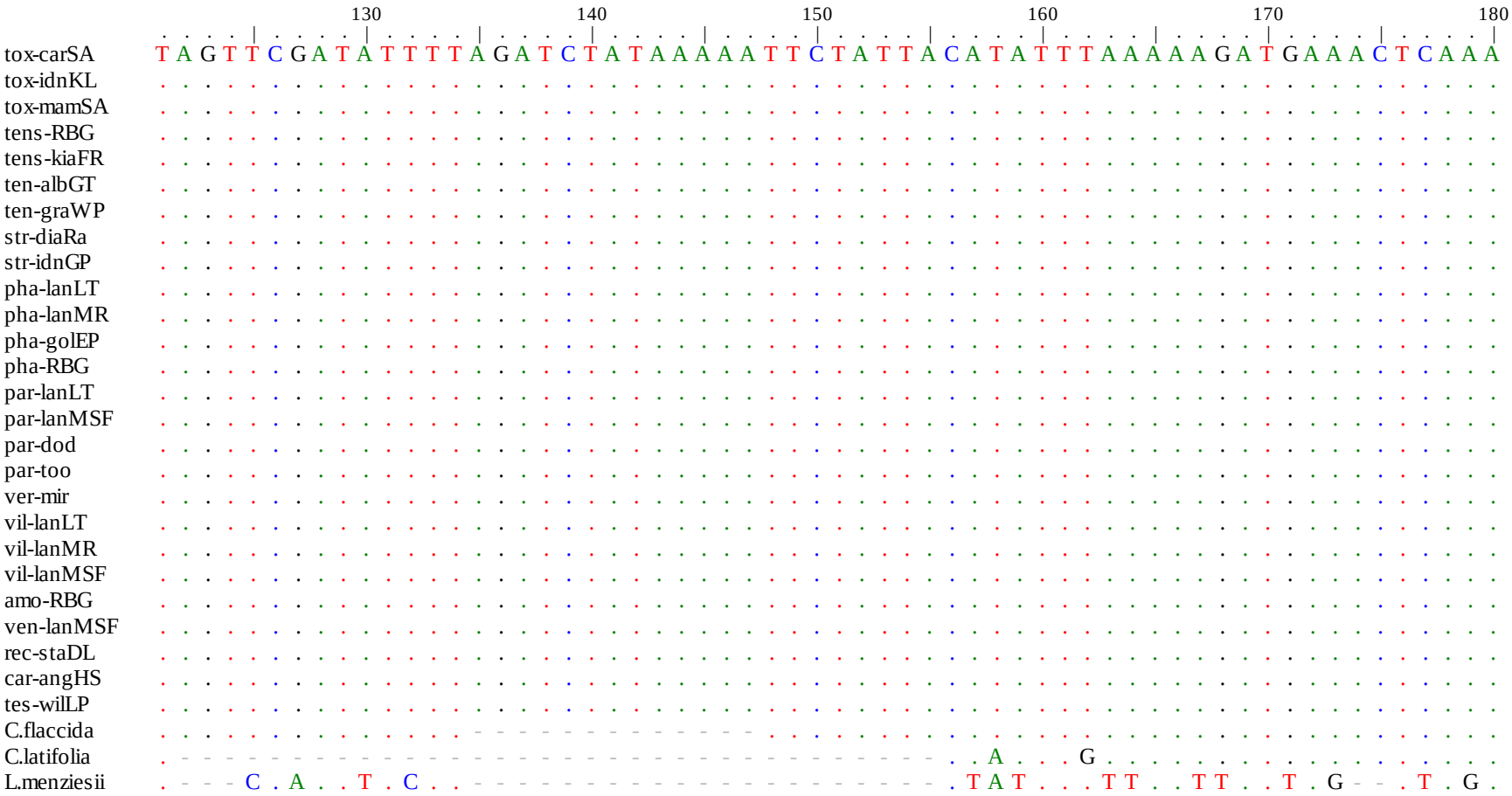
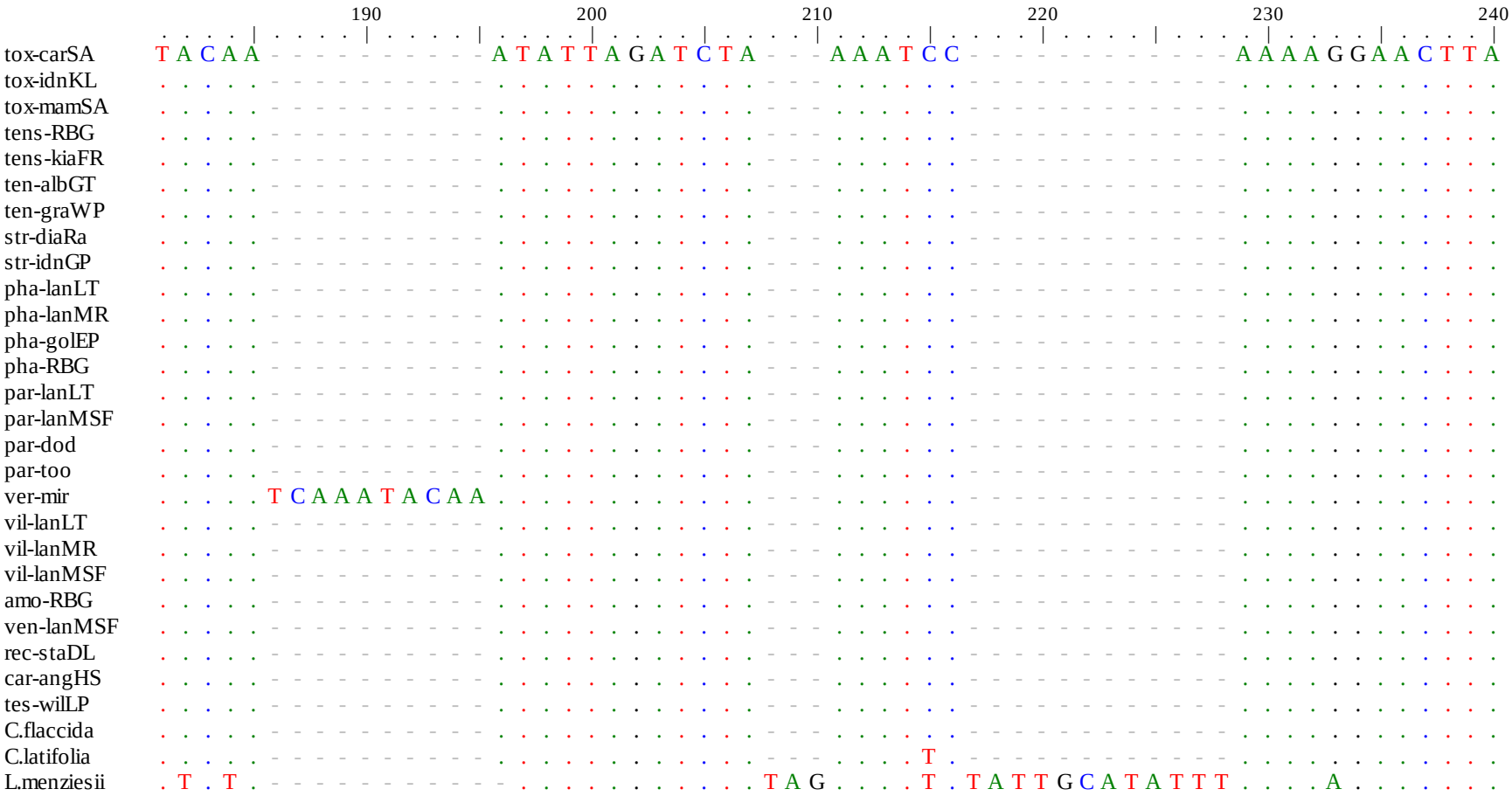


Figure 6.6 (cont.)



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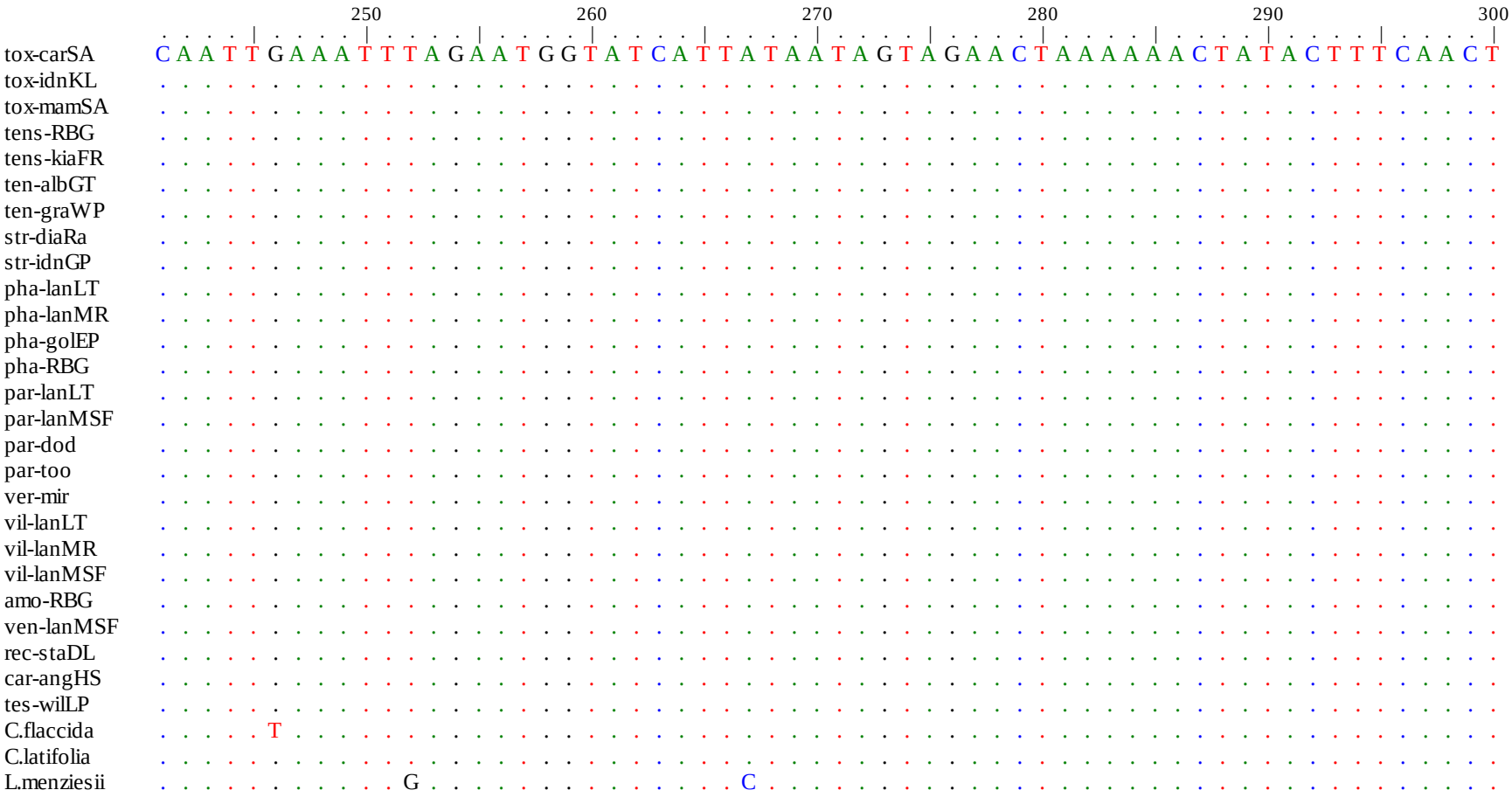

Figure 6.6 (cont.)

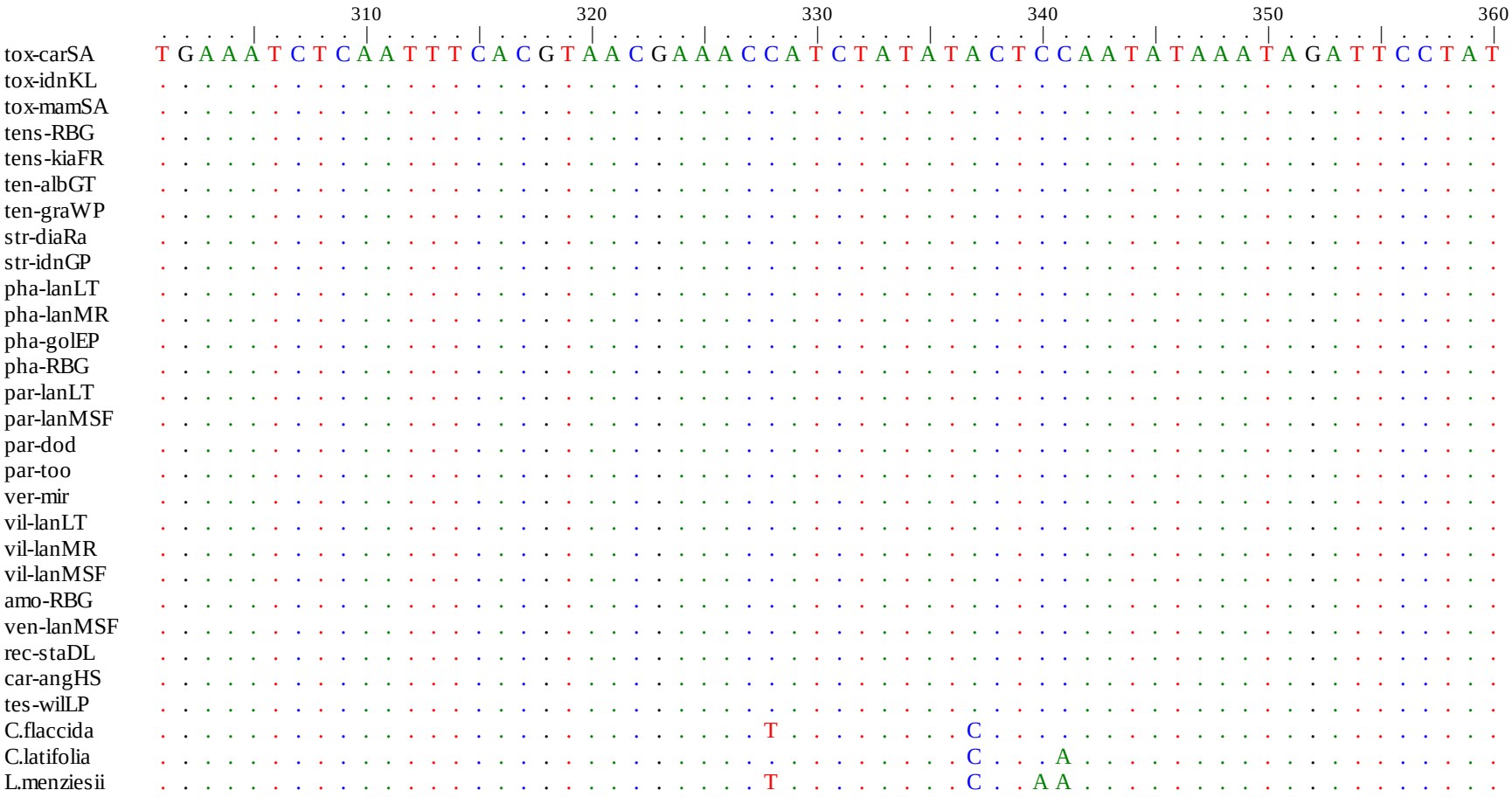

Figure 6.6 (cont.)

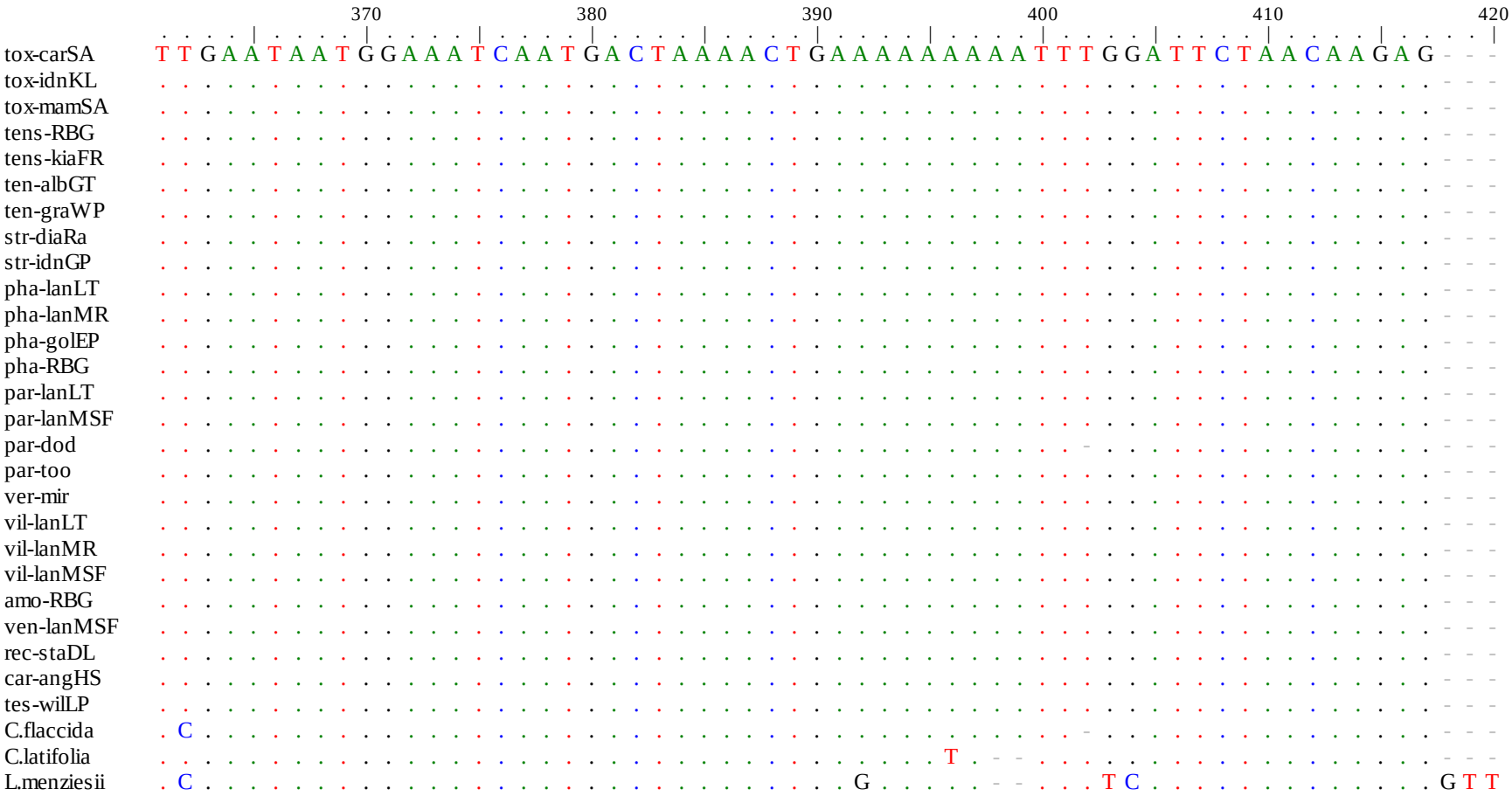

Figure 6.6 (cont.)

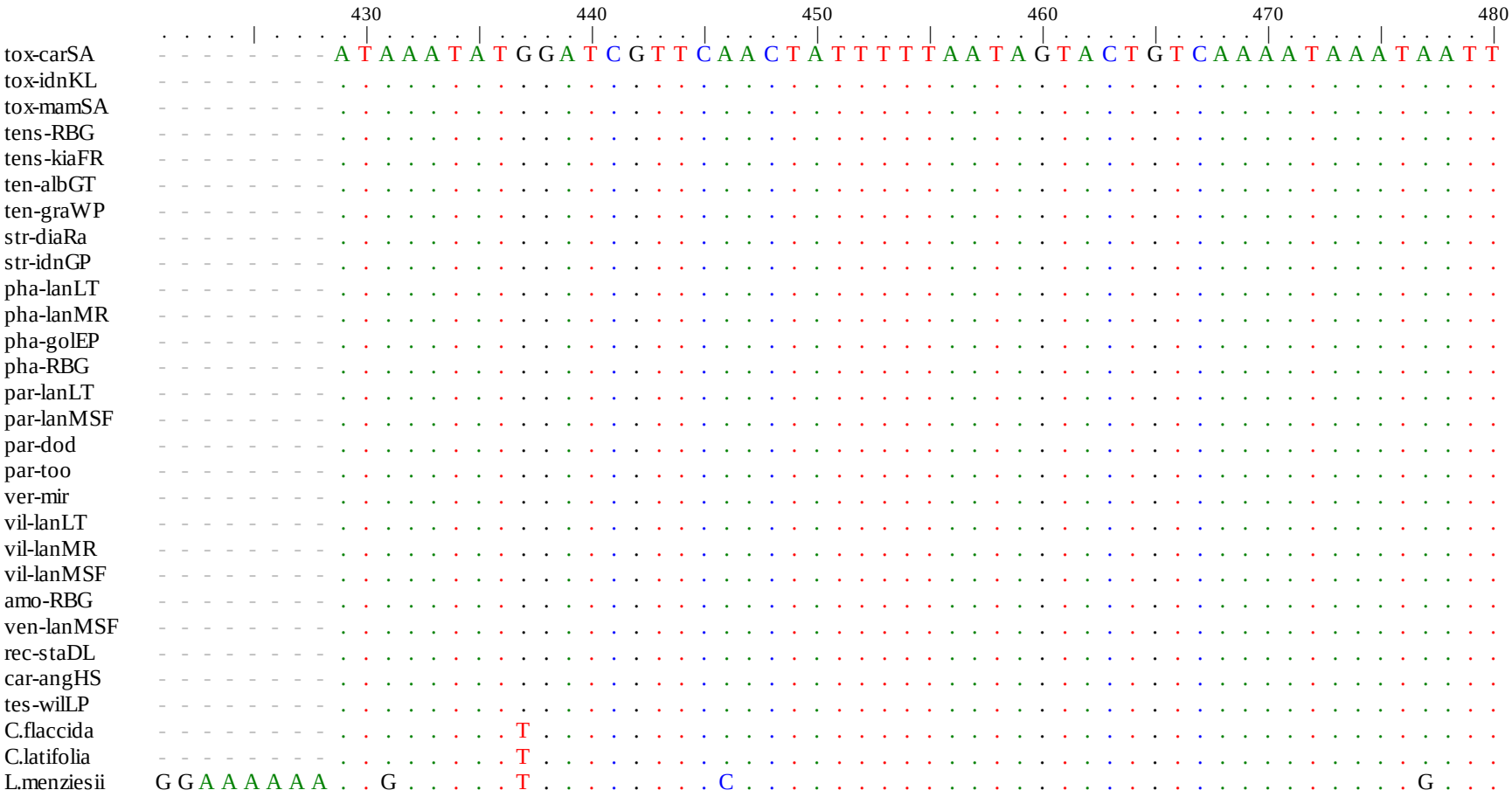

Figure 6.6 (cont.)

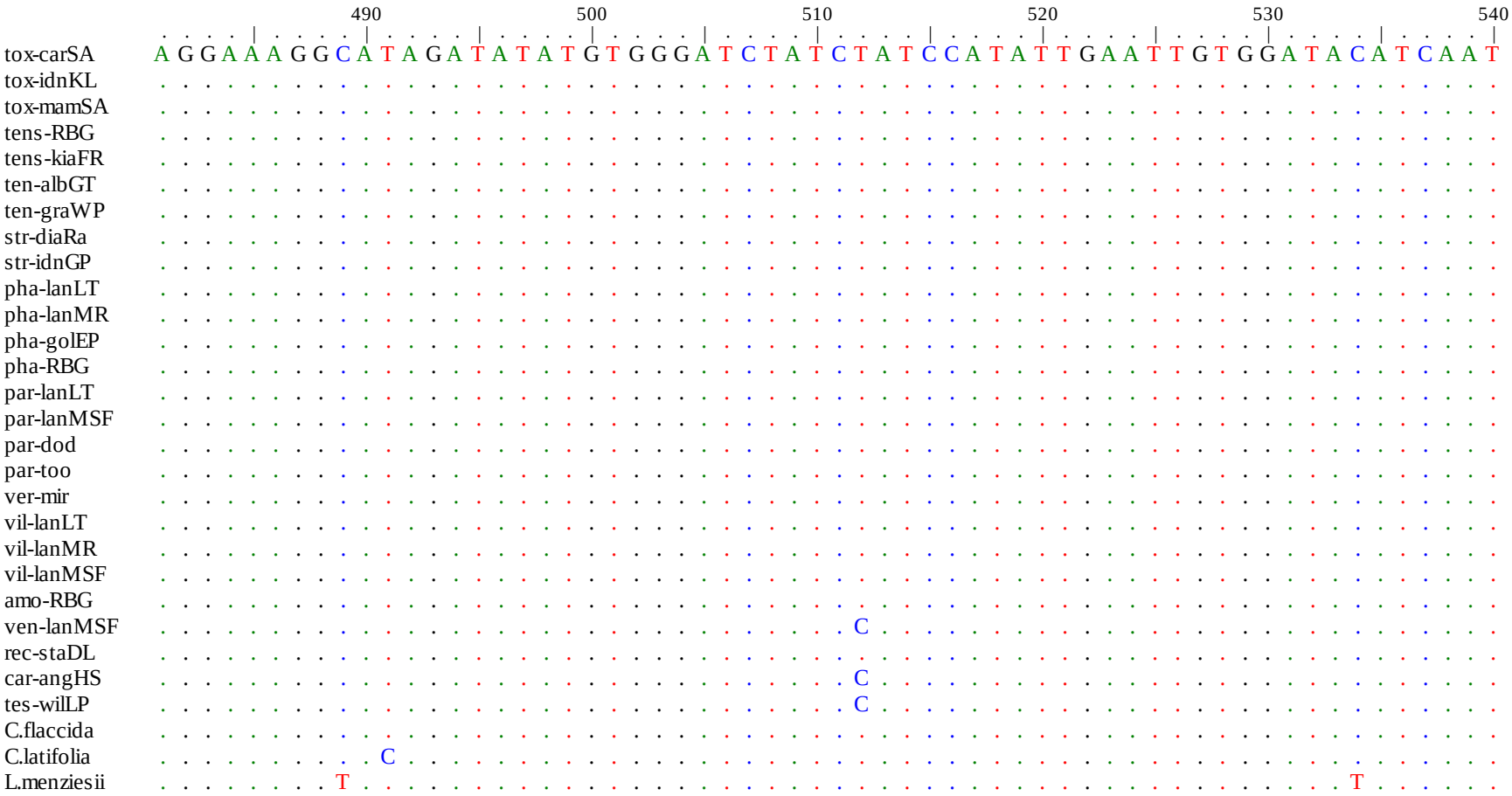

Figure 6.6 (cont.)

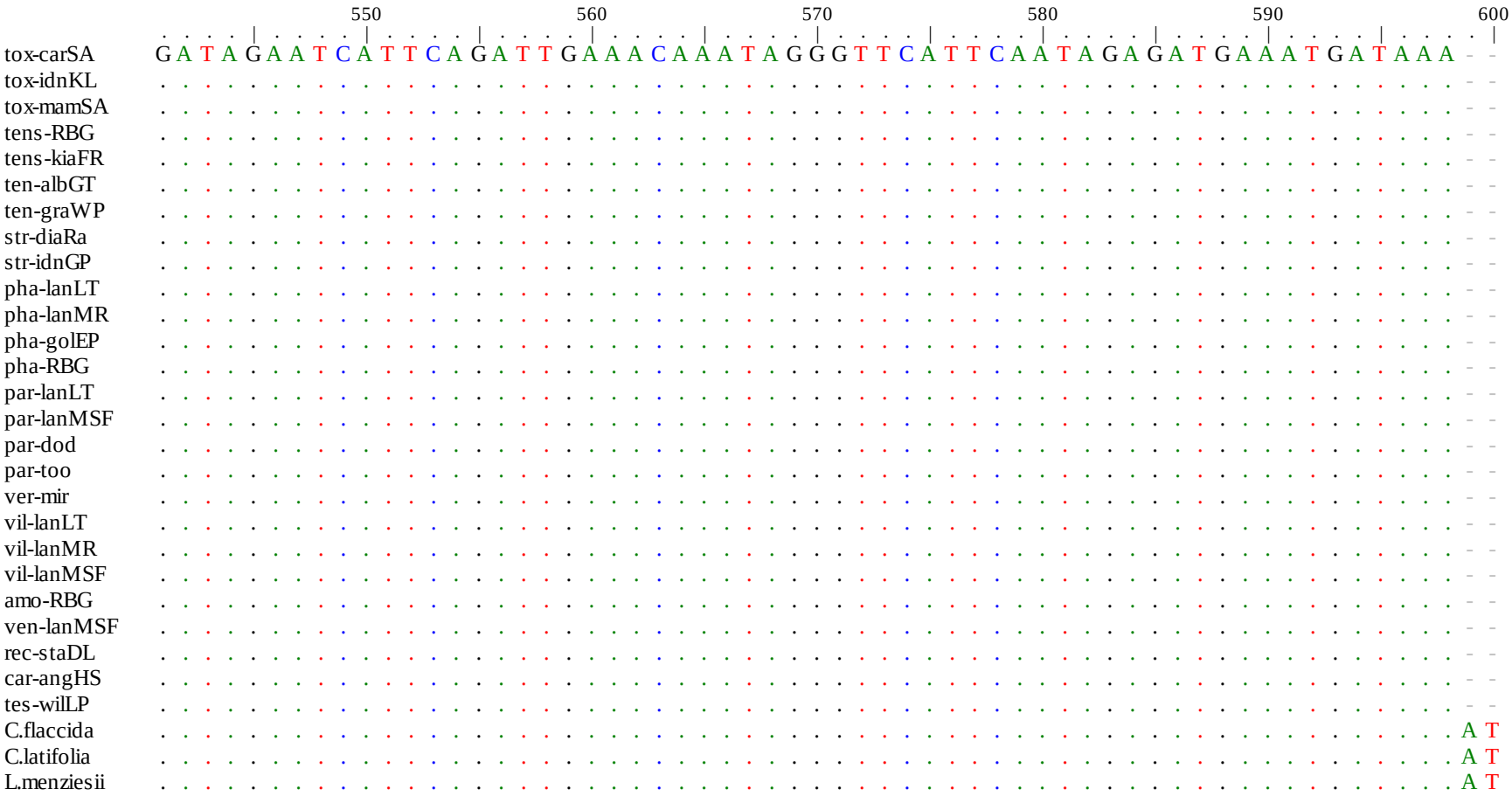

Figure 6.6 (cont.)

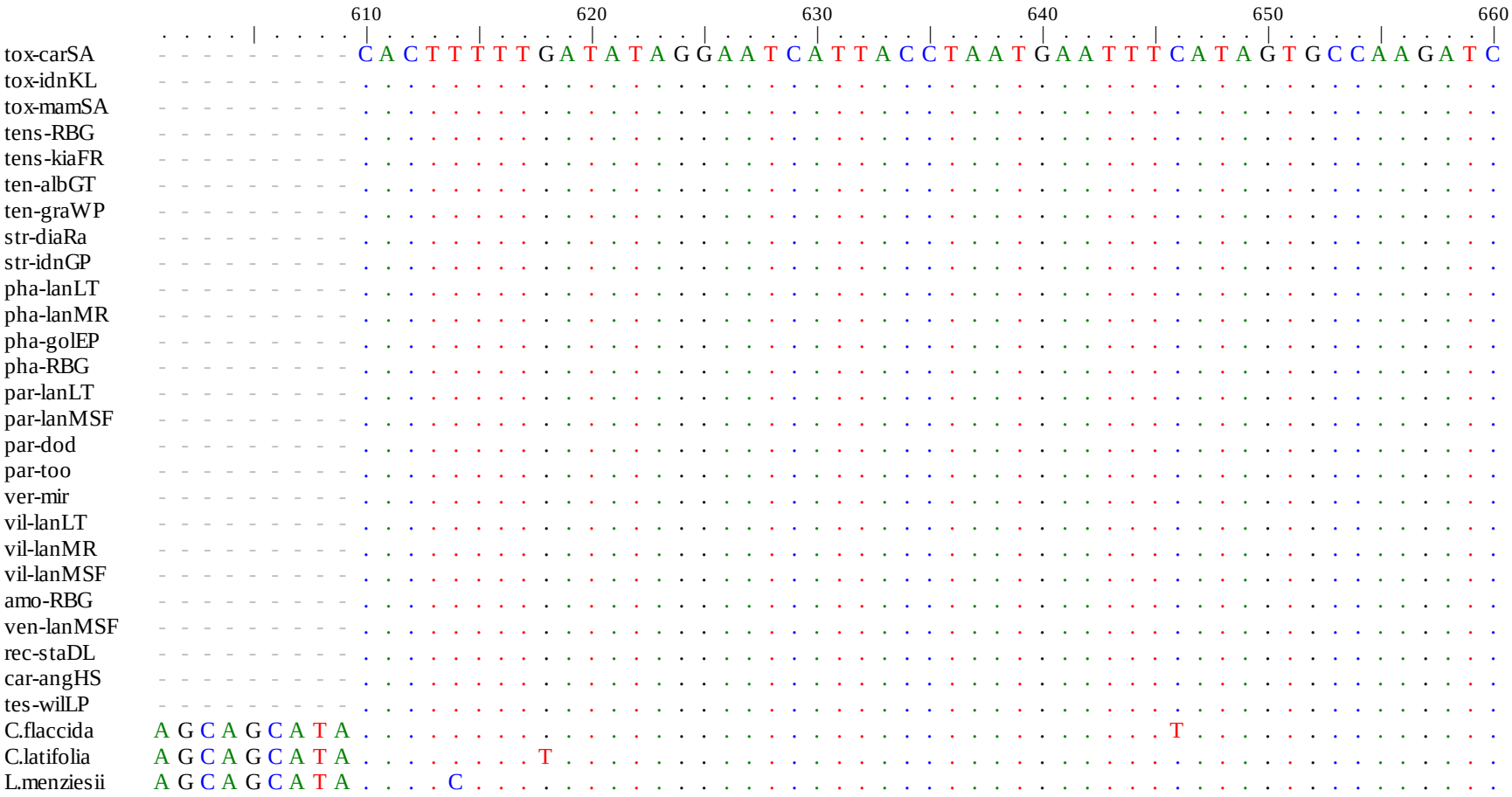

Figure 6.6 (cont.)

Mutation in *trn*T-L IGS within spider orchid species showed in Table 6.4. The G→T transversion was found in *C. amoena* at position 32. Another transversion between C →A at position 588 was found in *C. toxochila* collected from South Australia. The T→C transition occurred in the patersonii group used in this study. The G→A substitution was at position 586 found in *C. stricta* from Diapur and *C. toxochila* from Mambray Ck, South Australia and at position 589 in *C. parva* collected from Dodd Street St Andrews. A→G transition was at position 592 found in *C. recticulata* from Deep Lead FFR, Stawell. The insertion found in *C. verrucosa* while the deletion found in *C. parva* from Dodd Street St Andrews.

Table 6.4 Mutations in *trn*T-L IGS within spider orchid species

Taxa[a]	Mutation	Sequence	Position[b]
amo-RBG	Transversion	G-T	32
ver-mir	Insertion	TCAAATACAA	150-159
par-dod	Deletion	T	339
ven-lanMSF, car-angHS, tes-wilLP	Transition	T-C	440
str-diaRa, tox-mamSA	Transition	G-A	586
tox-carSA, tox-mamSA	Transversion	C-A	588
par-dod	Transition	G-A	589
ret-staDL	Transition	A-G	592

[a] species names are abbreviated as in Table 6.1

[b] nucleotide sites in aligned sequences are numbered consecutively from 5' to 3'

6.3.1.2 The non-coding region of the *trn*L (UAA) 5'exon and *trn*F (GAA) intergenic spacer

The *trn*L-F IGS region was amplified between primer c (in the *trn*L 5' exon) and f (in the *trn*F gene, Figure 6.7). The single strong amplified product band had an approximate size of 800 bp on agarose gel. However, there was some difficulty with reading the complete DNA sequences from target regions due to a high degree of homopolymer, poly "A" contained in the *trn*L-F region. This region was not included in the characteristics and parsimony analysis.

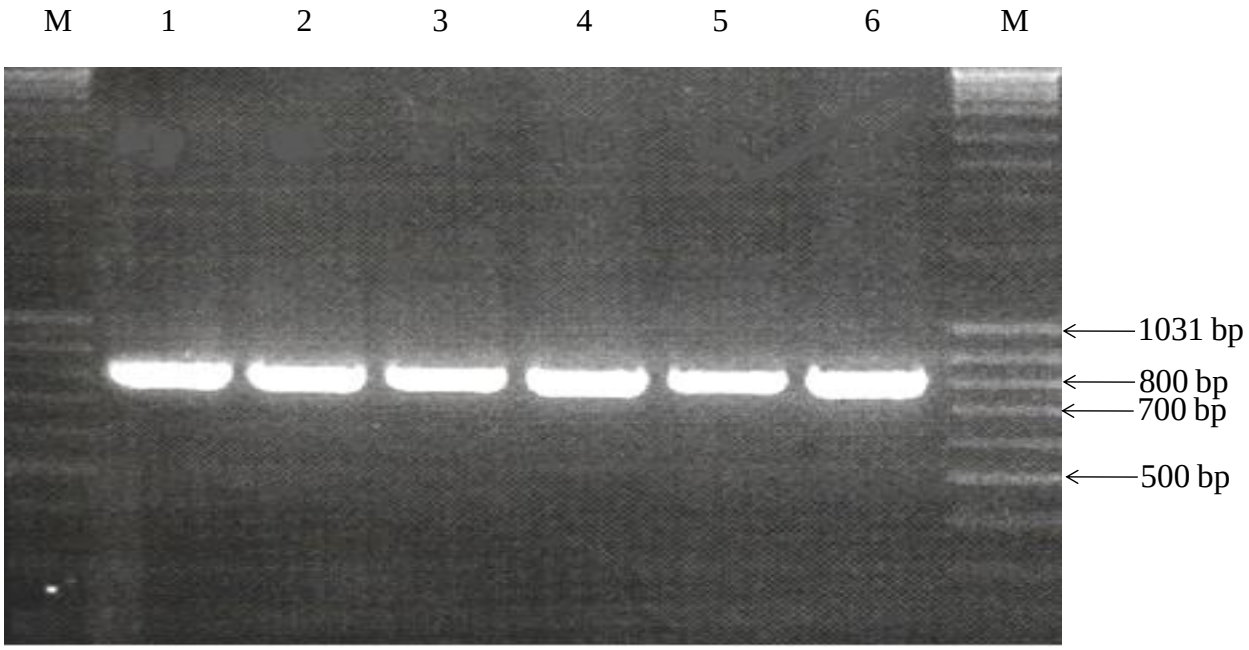

Figure 6.7 Amplification of the non-coding region of the *trn*L (UAA) 5'exon and *trn*F (GAA) intergenic spacer generated from primer "c" and "f" on 6 individual plants. Lanes M=low DNA ladder molecular weight marker, Lane 1=*C. verrucosa*, Lane 2=*C. parva*, Lane 3-4=*C. phaeoclavia*, Lane 5=*C. tentaculata*, Lane 6=*C. villosissima.*

6.3.2 Parsimony analysis

6.3.2.1 ITS analysis

The analysis of the ITS data set produced 142 parsimonious trees with a length of 148 steps, consistency index (CI) = 0.8851, homoplasy index (HI) = 0.1149, retention index (RI) = 0.7500 and rescaled consistency index (RC) = 0.6639. Figure 6.8 is the strict consensus of the most-parsimonious trees. The strict consensus tree specified *C. latifolia, C. flaccida,* and *L menziesii* as outgroups. The strict consensus tree had poor resolution of the dilatata grouping as indicated by the many unresolved polytomies and relatively low bootstrap values as shown in Figure 6.8. Strong support was shown for the relationships of the outgroup between *C. venusta* from Meerek State Forest and *C. cardiochila* from Anglesea (O'Donohues) (94% bootstrap) and the sister relationships of *C. verrucosa* from Miram Piram and the rest of the spider orchid specimens with the bootstrap value of 90%.

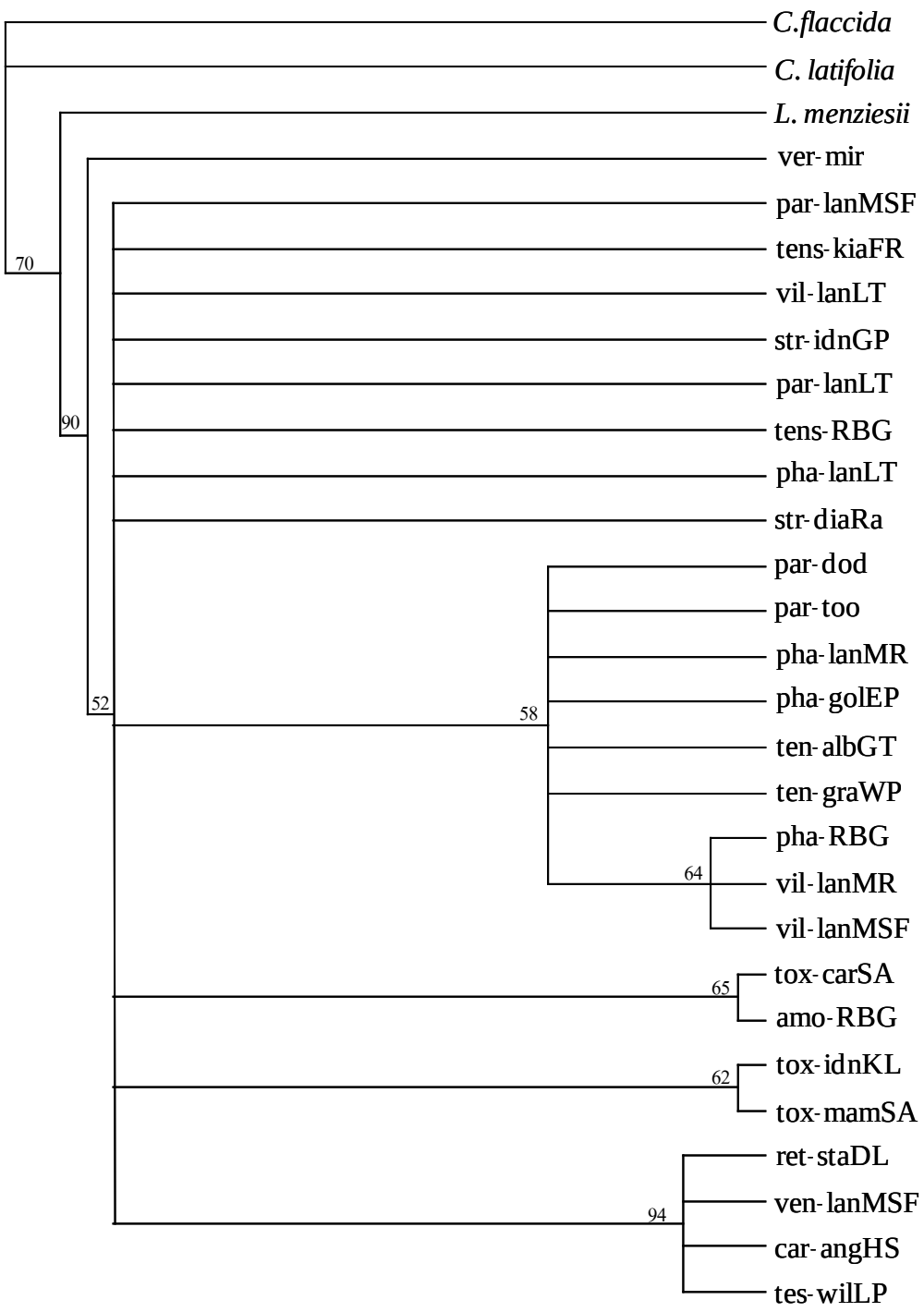

Figure 6.8 Strict consensus of 142 most parsimonious trees of 148 steps from the ITS sequence data most parsimony analysis (CI=0.8851, RI=0.7500, RC=0.6639, HI=0.1149) for 26 species of *Caladenia* section *Calonema* and three species of related genera. Bootstrap percentages greater than 50% are provided above the branches.

6.3.2.2 the *trn*T-L analysis

The analysis of the *trn*T (UGU) and *trn*L (UAA) 5'exon spacer region data set produced 72 parsimonious trees with a length of 64 steps, consistency index (CI) = 0.9531, homoplasy index (HI) = 0.0469, retention index (RI) = 0.7857 and rescaled consistency index (RC) = 0.7489. The strict consensus tree based on *trn*T-L intergenic spacer also had poor resolution indicated by unresolved polytomies as shown in Figure 6.9. However, the strict consensus tree separated the three outgroup taxa, *C. latifolia*, *C. flaccida*, and *L menziesii*, from the spider orchids as a sister group relationships of unresolved polytomies with bootstrap support at 90% (Figure 6.9). The consensus tree was also supported with relatively low bootstrap values. Strong support was shown for the relationships of outgroup taxa with one another with a bootstrap value of 90 between the relationships of *C. flaccida* and the monophyletic grouping of *C. latifolia* and *L. menziesii*.

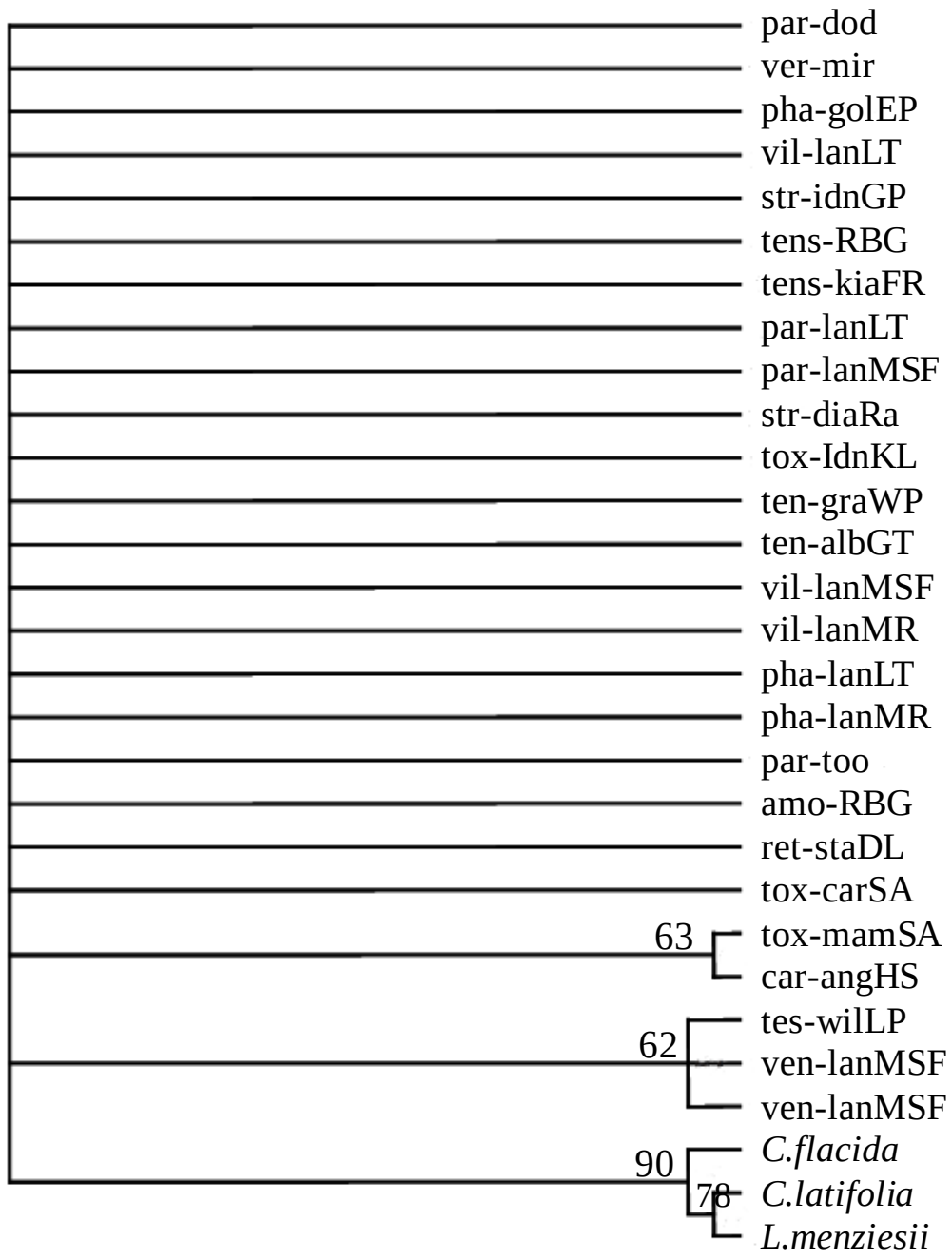

Figure 6.9 Strict consensus of 72 most parsimonious trees of 64 steps from the *trn*T-L sequence data most parsimony analysis (CI=0.9531, RI=0.7857, RC=0.7489, HI=0.0469) for 26 species of *Caladenia* section *Calonema* and three species of related genera. Bootstrap percentages greater than 50% are provided above the branches.

6.3.2.3 Combined data analysis

The combined dataset of ITS and the *trn*T-L (UAA) intergenic spacer region consisted of 1333 characters of which 36 were parsimony informative, 1151 characters were constant, and 146 characters were parsimony uninformative. The aligned matrix had 1333 positions including 39 indels. The analysis resulted in 72 parsimonious trees with a length of 216 steps, consistency index (CI) = 0.8704, homoplasy index (HI) = 0.1296, retention index (RI) = 0.6585 and rescaled consistency index (RC) = 0.5732. The strict consensus tree separated out the three outgroup taxa, *C. latifolia*, *C. flaccida*, and *L. menziesii*, at the base but their relationships were unresolved as shown in Figure 6.10. Low bootstrap values were also found. *Caladenia verrucosa* from Miram Piram was placed as sister to the unresolved polytomies with 100% bootstrap value. This unresolved polytomy consisted of *C. parva* from Meerek State Forest, *C. villosissima* from Longbottom track, Langkoop, *C. parva* from from Longbottom track, *C. stricta* from Little Desert NP (gravel pits), *C. tensa* from Kiata Flora reserve, *C. phaeoclavia* from Longbottom track, *C. stricta* from Diapur roadside, *C. toxochila* from Carapee Hills SA, *C. amoena* cultivated from Royal Botanic Garden, Melbourne, *C. toxochila* from Little Desert NP. Kiata Lowan, *C. toxochila* from Mambray Ck, and *C. tensa* cultivated from Royal Botanic Garden, Melbourne. Within the rest of the dilatata specimens: (1) *C. parva* from Dodd St St Andrews and Peppermint Ridge Farm, Toorour, *C. phaeoclavia* from Golden point, expedition pass and Mereek Rd, Langkoop, *C. tentaculata* from Albury NSW (Gap Trail) and Glenelg River Rd, Woolpooer, Grampians NP were unresolved; (2) the subclade of *C. phaeoclavia* pha-RBG, *C. villosissima* from Longbottom track and Meerek State Forest was supported by 62%

bootstrap value. The species of reticulata and patersonii spider orchids were clustered into the same subgroup at the basal unresolved polytomies.

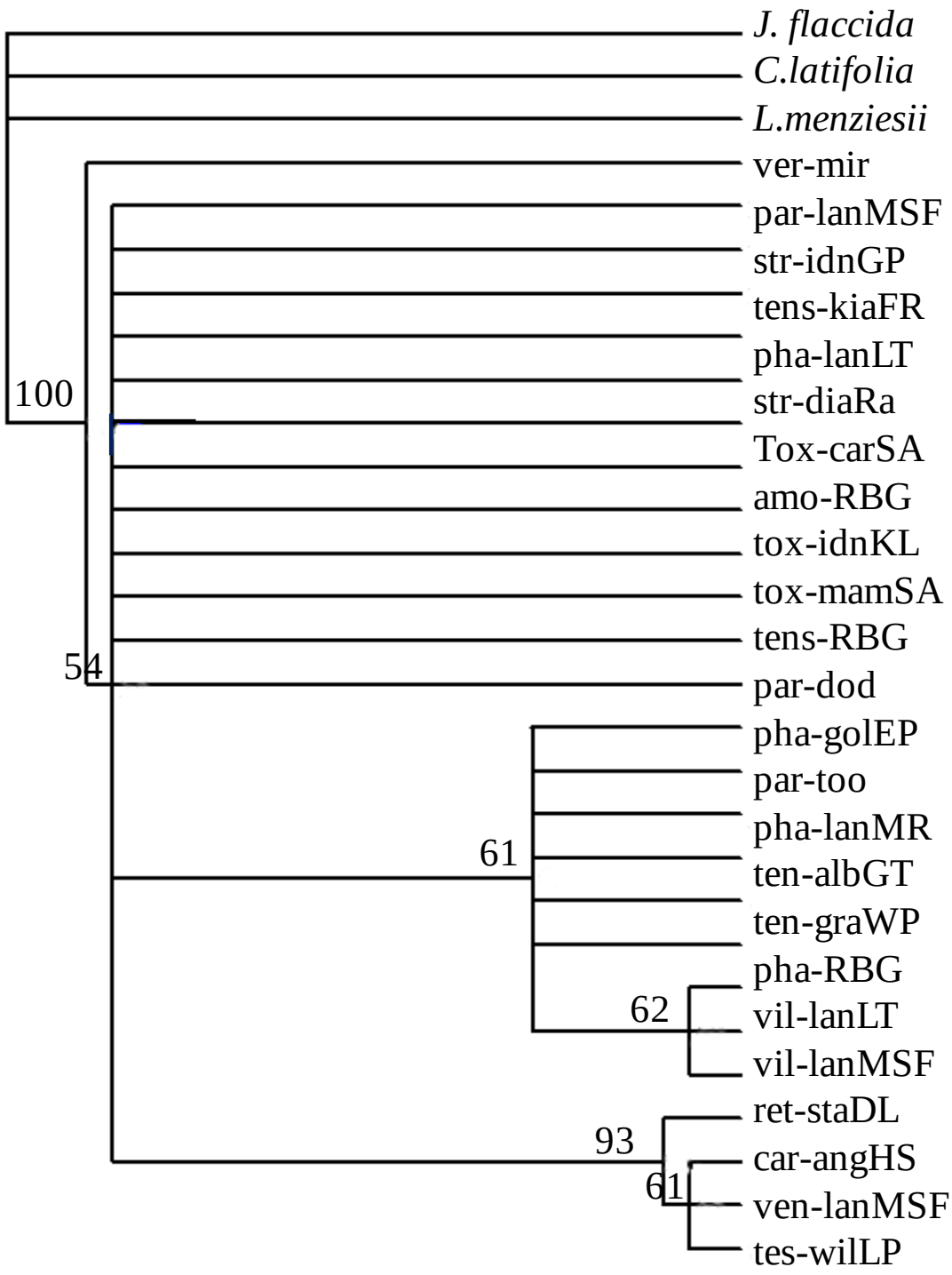

Figure 6.10 Strict consensus of 72 most parsimonious trees of 216 steps from the combined ITS and *trn*T-L sequences data most parsimony analysis (CI=0.8704, RI=0.6585, RC=0.5732, HI=0.1296) for 26 species of *Caladenia* section *Calonema* and three species of related genera. Bootstrap percentages greater than 50% are provided above the branches.

6.4 Discussion

6.4.1 Sequence characteristics

The spider orchid specimens collected from different locations across Victoria showed almost identical DNA sequences within species. ITS sequences had one base substitution for one to nine positions and *trn*T-L sequences also had a single base substitution for one to three positions across all the species tested. Overall, the ITS DNA sequences of examined specimens show highly conserved regions with only one of the outgroups, *L. menziesii*, indicating divergence from the other specimens. DNA sequences in the representatives of the reticulata and the patersonii group species had only two bases different as a transversion at position 175 between C and G and T→A transversion at position 444. Even though these bases transversion in ITS does not seem to be potential sequences for classification, it separated out these four samples of spider orchid from the dilatata group species. The lengths of ITS1 and ITS2 in spider orchid were 249 bp and 240 bp, respectively which are in general agreement with the reported lengths of ITS1 and ITS2 of less than 300bp in angiosperms (Baldwin *et al*., 1995). The range of ITS1 sequences varied from 187 to 298 bp in length and ITS2 from 187 to 252 bp (Baldwin *et al*., 1995; Downie and Katz-Downie, 1996; Padgett, 1997; Bena *et al*., 1998; Leskinen and Alstrom-Rapaport, 1999; Susanna *et al*., 1999; Hao *et al*., 2000; Bena, 2001; Sun *et al*., 2002; Martin *et al*., 2003a; Samuel *et al*., 2003; Martel *et al*., 2004; Tsai *et al*., 2006). Thus, the length of ITS2 in this study was at the high end of the range. The G+C content of *Caladenia* section *Calonema* was 41.67 to 42.12% which was lower than those of 62.03 to 66.10% reported in *Stylosanthes* (Stappen *et al*., 2002) and 53.72 % in Primuta (Kovtonyuk & Goncharov, 2009).

DNA sequences of the *trn*T-L intergenic spacer also indicated a highly conserved region within spider orchid species with the exception of base transition at the position of 440 in *C. venusta*, *C. cardiochila* and *C. tessellata* and the insertion of ten bases in *C. verrucosa* at the position 150-159. The three outgroup taxa from other genera expressed some regions differing from spider orchids; the position of 528-538 found in the three outgroup taxa from other genera; position 65-79 in *C. flaccida*, position, 7-28 in *C. latifolia* and a range of positions between 117-119, 126-136 and 325-335 in *L. menziesii*.

6.4.2 Phylogenetic relationships

6.4.2.1 Outgroup relationships of the dilatata group

The phylogenetic relationship of the dilatata group relative to the three outgroup taxa is well resolved using ITS and *trn*T-L sequences, although there was in unresolved polytomy within the spider orchids on *trn*T-L sequence analysis. However, *trn*T-L sequence parsimony analysis grouped *C. latifolia* with *L. menziesii* indicated by 78% bootstrap support. *Caladenia flaccida* was grouped as a sister to those taxa with high bootstrap support (90%) and the relationship of this species to *L. menziesii* has been reported by the study of Kores *et al*. (2001) based on *mat*K and *trn*L-F and Clements *et al.* (2002) based on ITS analysis. On the account of the divergent of their nuclear DNA and chloroplast DNA, their placement on the cladogram separated them far apart as well as their placement in this study. Relative to other spider orchids investigated in this study, the ITS sequencing of *C. reticulata* (reticulata group), *C. venusta*, *C. cardiochila* and *C. tessellata* (all from the patersonii group) supported a monophyly for the group even though relationships within outgroup taxa from reticulata and patersonii specimens indicated unresolved polytomies. This is due to

the highly conserved regions displayed in the ITS region of spider orchids. The relationship based on *trn*T-L sequence gave less resolution because of the highly conserved region within spider orchid species. The high bootstrap value supported divergent between ingroup taxa and outgroup taxa was reported in other orchid such as in *Disa*, *trn* L-F based sequence (Bellstedt *et al*., 2001); *Phalaenopsis* Blume, ITS based sequence (Tsai *et al*., 2006); *Disa*, ITS and two plastid (*trn*T-L and *matK*) based cladogram (Bytebier *et al*.,2007). However, in the study of Tasi *et al* (2006) some of their selected outgroup were supported by low bootstrap value.

6.4.2.2 Phylogenetic relationship within dilatata

The relationships of dilatata species obtained from ITS sequences were better resolved than that obtained from *trn*T-L sequences. Thus ITS parsimony analysis had a higher number of informative characters. However, parsimony informative characters in both ITS and *trn*T-L data were low as indicated by 3.7% and 1.6%, respectively. Other reported phylogenetic analyses using ITS and cpDNA based on *trn*L-F IGS at species level had higher numbers of parsimony informative characters such as in *Stylosanthes* (*Fabaceae*) with ITS sequencing being 14% (Stappen *et al*., 2002), 28.1 % (ITS) and 1.3% (*trn*L-F) in *Liparis* (*orchidaceae*, Tsutsumi *et al*., 2007) and in another case, ITS and *trn*L-F IGS being 6.4% and 3.8% parsimony informative characters, respectively (Hoggard *et al*., 2004). In the study of phylogeny of *Hypochaeris* (Asterceae, subfamily Cichorieae) there was a very high proportion of ITS parsimony informative characters (47.92%) and *trn*L-F IGS parsimony informative characters (9.7%) (Samuel *et al*., 2003). The ITS parsimony informative characters in this study was very low and indicated a highly conservative region within the ITS in spider orchid.

Parsimony informative characters in the study of *Cytisus* (Leguminosae) at generic and species levels were 16.9% in ITS, and 9.6% in *trn*L-F (Cupas *et al*., 2002). Asphodelaceae (Asparagales) at generic level had 14.9% parsimony informative characters in *trn*L-F (Chase *et al*., 2000), *Genista* (Leguminosae) at generic level had 46.3% parsimony informative characters with ITS and 27.8% with *trn*L-F (Pardo *et al*., 2004), Apostasiodeae (Orchidaceae) at subfamily and generic levels had 31.3% of ITS parsimony informative characters and 4.8% of *trn*L-F parsimony informative characters (Kocyan *et al*., 2004), Primulaceae at family level had 65.43 % of ITS parsimony informative characters (Matins *et al*., 2003a), Araliaceae at infrafamily level had 42.4% of ITS parsimony informative characters and 15.15% of *trn*L-F parsimony informative characters (Plunkett *et al*., 2004).

The combined data sets resulted in the tree similar to that based on ITS sequences due to the higher number of parsimony informative characters in ITS data. The sister position of *C. verrucosa* to the remaining spider orchids was most likely due to its insertion sequences of TCAAATACAA of *trn*T-L IGS between 150 to 159 base position. *Caladenia verrucosa* has the general morphology of the other dilatata group species but is distinguished in having four rows of very crowded, short clubbed, warty-headed maroon calli on the labellum lamina. Geographically it is largely isolated from the other members of the group, being confined to mallee communities in the north-west of the state. The separation by geographical distribution revealed by ITS was also found in *Phalaenopsis celebensis*. It is geographically separated from other species of the section Stauroglottis (Tsai *et al*., 2006).

Parsimony analysis obtained from ITS and combined data showed similar relationships within dilatata specimens, namely that the unresolved polytomies consisting of the sub-clade of reticulata and patersonii taxa, another sub-clade of *C. parva*, *C. phaeoclavia*, *C. tentaculata* and *C. villosissima*, and the rest of the unresolved dilatata taxa. A poorly resolved tree based on ITS and *trn*L-F is also found in *Ophrys* (Orchidaceae; Soliva *et al*., 2001). The dilatata species show remarkably little morphological variation, with a uniform and basic pattern: a maroon apex and calli with green marginal fringe or teeth on the labellum. Due to this unique morphology, it is difficult to make a clear statement about the relationships within dilatata group species based solely on morphological characteristics.

As shown by molecular data based on ITS and *trn*T-L sequences data, the grouping of dilatata remaines unresolved, due to the fact that only a few parsimony informative characters were found. However, the majority of similar species grouped together with the exception of the largest flowered species of the group, *C. tentaculata* and *C. tensa*. *Caladenia parva*, *C. phaeoclavia*, and *C. villosissima* are very similar in gross morphology and display the simplest architecture in the group: a green flower with variable crimson striping, the labellum white with green lateral lobes and a maroon apex. They are generally classified into different species by size and some differences on club color; *C. parva* is smallish tipped with yellowish clubs, *C. phaeoclavia* has 25-40 mm long tepals, with distinct yellowish to brownish clubs on the sepals, and *C. villosissima* has sepals to 40 mm long with brownish clubs and petals to 25 mm long. It is known that *C. parva* and *C. phaeoclavia* share the same pollinator, and recent studies in the South-West of Victoria in 2006 indicate that *C. villosissima* is also pollinated by the same species of thynnid wasp (C. Bower, pers. comm.). The identity

of these species has been problematic since their description (Jeanes and Backhouse 2000). On the account of RAPD and ISSR phylogeny, these species have undergone speciation and/or hybridization. Further study on genetic relationships within these species and genetic diversity among their population are desirable to reflect their genotypic relationships.

The very small-flowered dilatata species *C. toxochila* and *C. amoena*, were separated out from other dilatata specimens and they are not properly resolved from one another based on their ITS sequences data. Their separation from the remainder of the green-combs reflects their distinctive morphology within this group – flowers of smaller size than other species with unclubbed sepals and petals and very reduced fringes on the labellum margins. Surprisingly, however, *C. stricta*, another small species lacking clubs did not group with these sub-clades. It differs from the other two small-flowered species in having elongated labellar fringes. Further molecular markers such as the plastid gene ycf1 might be applied to clarify spider orchid relationships since this marker are highly variable across Orchidaceae and proved to be phylogenetically informative at the species level and (Neubig *et al*., 2009).

The remainder of the dilatata specimens also was still showed unresolved polytomies in combined data phylogeny tree. These consisted of: the small, clubbed flower, *C. parva, C. villosissima, C. phaeoclavia, C. stricta*; the small-flowered and clubless, *C. amoena*; a small-flower, *C. toxochila*; a large clubbed and stiffly tepaled flower, *C. tensa*. Even though there are some differences in floral architecture among dilatata species, the ITS and *trn*T-L parsimony analysis did not reflect these morphological differences. On the account of ITS and plastid DNA, some unresolved polytomies

were found in *Disa*. The relationship among species under investigation was supported by low bootstrap value (Bellstedt *et al*., 2001). Phylogenetic relationships of *Carex* based on ITS and ETS reported some unresolved polytomies among specimen (Roalson and Friar, 2004). The unresolved polytomies of *Alisma* based on ITS were also detected with low bootstrap value (Jacobson and Hedŕen, 2007). Hayashi *et al* (2001) also reported unresolved polytomies of *Clintonia* based on *rbcL* and *matK*.

The ITS and chloroplast trees are partly incongruent. Phylogenetic incongruence between different data sets can be caused by insufficient data and the contradiction to each other can result in between weakly supported clades. The incongruence phylogent analysis between nuclear and plastid data also found in other orchids; *Pleione* (Gravendeel *et al*., 2004) and *Satyrium* (Van der Niet *et al*., 2005). The weakly supported results do not necessarily reflect the evolutionary history of the taxa, however, they can be the result of homoplasy (Kellogg *et al*., 1996). As a result, homoplasty is usually not taken into account in a discussion on phylogeny. On the other hand, Farrington *et al* (2009) reported relationships of 32 chloroplast haplotypes from *trn*L^{UAA} intron and *trn*Q-5' *rps* 16 intergenic spacer showing that within the accession examined dilatata complex (*C. stricta*, *C. tensa* and *C. tentaculata*) were well resolved based on haplotype expression.

The use of two DNA fragments (one nuclear and one chloroplast) in the construction of a molecular phylogeny in *Caladenia* section *Calonema* has provided two independent sources of information with which to construct the molecular phylogeny. The ITS tree has been more informative than the *trn*T-L tree. However, neither ITS

nor the *trn*T-L region have been ideal as there are very few parsimony informative characters to investigate phylogenetic relationships among spider orchid species in this study. The ITS sequences was applied for phylogeny analysis in *Caladenia* and allied genera by Hopper, 2009. The ITS based phylogenetic relationship also did not address the relationship within spider orchids. It is not useful to make confident inferences about phylogenetic relationships at a species level in *Caladenia* section *Calonema* using ITS and *tr*nT-L sequences. However, the insertion sequence "TCAAATACAA" of *trn*T-L IGS at base position 150-159 in *C. verrucosa* might be a candidate for the development of a species-specific marker for identifying *C. verrucosa* in the dilatata complex, provided this is not found in other species not investigated in this study.

Chapter 7

General discussion

7.1 Choice of markers

In this study, RAPD and ISSR markers were used to investigate the genetic relationships amongst Victorian *Caladenia* subgenus *Calonema.* These methods were chosen because they are simple, quick, efficient and cost-effective techniques that generate markers without requiring existing genomic sequence information. Furthermore, these techniques are efficient at generations multiple loci for each individual in a single gel run. ITS and the plastid non-coding regions of the *trn*T (UGU) and *trn*L (UAA) 5'exon spacer sequences were also selected to examine the genetic relationship within *Caladenia* subgenus *Calonema* (dilatata group) species because these markers have been reported to address taxonomic and phylogenetic questions at lower taxonomic levels. The reason for the usefulness of these spacer regions is due to the more rapid evolution of these regions when compared to the coding sequences by the accumulation of indels. It is because of their rapid evolution that they provide more informative characters for taxonomic differentiation (Tam *et al*., 2004; Xu and Ban 2004). Due to the highly conserved coding regions across species and genera of chloroplast genome and ribosomal DNA, the pairs of universal primers can be designed to amplify non-coding regions flanked by coding regions (Taberlet *et al*., 1991; Bena *et al*., 1998).

7.2 Phylogenetic relationships within spider orchids as determined by RAPD and ISSR markers

The use of both the RAPD and ISSR methods produced the following results 1) the three outgroup species were well separated from the spider orchids as expected, with strong bootstrap support indicating the good choice of outgroup taxa; 2) individuals of the same species tended to be clustered closely with each other, generally with moderate to high bootstrap support with the exception of *C. insularis* from Mt Wellington Track, French Island; and 3) the spider orchid groupings in this study supported the traditional taxonomic groupings based on morphological differences of their floral structure: reticulata group – the clubbed spider orchids, patersonii group - the tailed spider orchids, and dilatata group – the green-comb spider orchids. However, bootstrap values for interspecific taxonomic groupings were generally at the lower end, and hence the binary data created from selected primers used in this study were not sufficient to reach any firm conclusions about the taxonomic position of spider orchid species within each respective group. However, the complete concordance of the dendrograms produced using different similarity coefficients and clustering methods, indicates that the data are robust in terms of the relative similarity among and within spider orchid species. This is the first report of molecular data based on DNA profiling that supports clear separation of the three groups of spider orchids.

Based on the results of RAPD, ISSR and combined data of molecular phylogenetic analysis of *Caladenia* subgenus *Calonema* in this study, species of the reticulata group, *C. robinsonii* and *C. insularis*, are particularly closely related. This is supported by morphological characters. They both have very similar floral structure

with the major morphological distinction between them being that *C. robinsonii* has slightly smaller marginal calli. Further studies of genetic diversity between these two species from various geographical distributions might help clarify the relationships between them.

The close relationships in the dilatata group, particularly between *C. tentaculata* and *C. phaeoclavia,* found by RAPD, ISSR and combined data are not supported by morphological data. This suggests that they might have high similarity of their genetic information especially the sequences of non-coding regions on their genome producing by the event of speciation. Further experiments based on the other regions of their genomes may help resolve this anomalous relationship. Unexpectedly, *C. phaeoclavia* and *C. parva* did not cluster into the same clade given their similar floral appearance. Current taxonomic separation is based on sepal size: *C. phaeoclavia* (25-40 mm long) is bigger than *C. parva* (20-30 mm long) but the data presented here indicated a significant degree of genetic differentiation between these species suggesting sympatric speciation. Future research using plant material from across the Victorian geographical distribution of these species needs to be conducted to clarify this genetic differentiation either within the same species or closely related species in *Caladenia* subgenus *Calonema*. Including *C. villosissima* into the investigation of *C phaeoclavia* and *parva* might also be interesting, as all three species have a similar appearance, and some taxonomists and orchidologists have suggested that the three are a single species.

7.3 Possible explanations for the low level of genetic differentiation found among spider orchid species

The apparent low level of genetic differentiation found among the species which resulted in an inability to clearly cluster the species within the three groups could result from a variety of factors. The most obvious explanation is that the species are in fact too closely related to be distinguishable by the molecular markers employed in this study.

The low number of polymorphic markers could be due to the primer prescreening that concentrated on indentifying polymorphism between spider orchids and the outgroup taxa that were believed to be well-separated out from the spider orchids, rather than primers based on genetic differences between type species from each group. The focus at that stage was to determine if spider orchids formed a distinct group compared with other orchids. In addition, no genetic information was available for any of the spider orchids, and the three groupings had not been genetically confirmed. It was therefore not possible prior to this study to reliably select type species from each group to assess which primers provide maximum polymorphism among them. To clarify relationships between these spider orchids other primers need to be tested, using primers that produce the most polymorphism within the spider orchid species.

In order to make informed decisions on suitable primers to examine phylogenetic relationships at lower taxonomic rank, especially among closely related species, primers that amplify regions spanning the whole genome of taxa of interest should be selected. Amplified fragments should be good representatives of the whole genome and useful for looking at genetic (dis) similarity among taxa used. The target regions

should also amplify regions where polymorphisms exist to gain proper genetic information to construct phylogenetic trees. Therefore, the various combinations of AFLP primers are needed to investigate the polymorphism among group representatives of the spider orchid genome. In addition, better resolution could be obtained, including separation of co-migrating bands and faint bands such as those found by separating the PCR products on polyacrylamide non-denaturing gel.

All in all, however, the results from this analysis have improved our understanding of relationships among the spider orchids by confirming that three morphological and genetic group exist within the spider orchids, as well as separating out species within the groups, albeit with low bootstrap support. The application of other genetic markers (e.g. AFLP) is likely to provide additional information to improve the resolution of intra & inter- specific relationships.

7.4 Phylogenetic relationships within the dilatata group from sequencing results (ITS and chloroplast DNA)

The use of ITS and *trn*T-L parsimony informative characters in the construction of molecular phylogeny separated the outgroup species from the spider orchids. In addition, representatives of the reticulata and the patersonii groups were separated out from the dilatata group. Within the dilatata group itself, however, there were unresolved polytomies. Thus, both ITS and the *trn*T-L sequences contained very few parsimony informative characters to investigate phylogenetic relationships within the dilatata group. However, an interesting and unique insertion sequence was found in the *trn*T-L IGS region in *C. verrucosa* relative to the other species examined in this study. If this proves not to be found in any other orchid species, it could well be used

as a specific marker to identify this species, using quicker and less expensive methods than sequencing by developing species-specific marker.

It is much more difficult to find morphological support for the phylogeny of the dilatata because all members in the group share a unique floral appearance. This study requires a more complete sampling and careful morphological investigation before any inferences can be made. The inclusion of more characters, both molecular data and morphological, will hopefully rectify the situation.

The issue of the group delimitation in *Caladeni*a subgenus *Calonema* is problematic, but not unsolvable. Additional informative characters from other loci are needed to study species delimitations and to elucidate the relationships among closely related species. Thus, further phylogenetic analysis among spider orchid species should be carried out using other targets of non-coding regions of chloroplast DNA. In addition, other alternative approaches such as the use of sequence data from low-copy nuclear genes (LCNG) and comparative anchor tagged sequences (CATS) may resolve phylogenetic relationships among closely related species (Hughes *et al*., 2006). Pre-testing of numerous parsimony informative characters obtained from representative spider orchid species would allow clarification of the position of spider orchids within the group. In addition, some species-specific DNA sequences may be found that could be used as a tool to identify particular species.

One must bear in mind that a sequence that readily separates out the distant outgroups, may not show enough variation to resolve the relationships within the ingroup, and a sequence that performs well within the group may not necessarily

distinguish the outgroup. Thus, in order to carefully infer phylogenetic relationships within closely related species, sequence variations need to be found both between the spider orchids and outgroup taxa as well as within the ingroup spider orchid species. These could then provide sufficient parsimony informative characters for distinguishing between the ingroup taxa.

7.5 Could the apparent lack of genetic variation reflect the true situation?

There is, of course, a simple (and the most obvious) explanation for the low genetic differentiation found within the spider orchids by any of the four methods used. It could actually reflect a true lack of genetic variation within *Caladenia* subgenus *Calonema* genome. The various phenotypes seen could be the result of environmental factors that alter gene expression, producing morphological variation across a geographical range within Victoria such as rainfall, soil nutrients, less efficient fungi, and more frost in drier areas. True lack of genetic variability or gene expression due to environmental conditions is unlikely to be the case for the entire *Caladenia* section. The three groups within *Caladenia* subgenus *Calonema* and most of the species within them were genetically separated as expected from morphological data, albeit with varying bootstrap support. However, for those species that were phenotypically very different yet clustered together in the genetic analysis, such as *C. tentaculata* and *C. phaeoclavia,* the explanation regarding genetic divergence may apply. This needs to be further investigated by conducting studies on population genetic divergence of these closely related species.

7.6 Conservation implications

Information on genetic variation has been used in conservation management efforts of endangered species. The decision about which populations and numbers of interesting species should be protected to conserve the greatest portion of the overall genetic diversity of the species is dependent on information pertaining to the genetic distinctness of individual populations (Sapir *et al*., 2003). Prior to population analysis, thorough phylogenetic analysis need to be done within a species of interest from various geographical distribution areas in order to estimate genetic divergence. However, the cluster analysis could include either genetic or morphological markers (Tryon, 1939 cited by Boettcher *et al*., 2010). This result should provide some large scale insights into the extent of dispersal (Hughes and Hollingsworth, 2008). A clear understanding of the degree of divergence in spider orchid species will be used in future prospecting and indentifying sites for in situ conservation.

As in the phylogeny presented in this study, *Caladenia* subgenus *Calonema* is shown as a natural monophyletic group. However, the positions of spider orchid species/taxa within the groups are still unclear. In order to elaborate an acceptable classification of *Caladenia* subgenus *Calonema*, it seems necessary to first establish clear relationships between taxa and to identify reliable morphological characters correlated with the clade recognized in the phylogeny. A phylogenetic analysis of more taxa sampled from different geographical distribution should allow the proposal of a comprehensive taxonomic treatment reflecting evolutionary relationships. Once the spider orchid relationships can be distinguished, the genetic diversity within spider orchid populations can be detected. Genetic differentiation among populations can be

calculated from the same data. Such information could play a very important role in the conservation program in terms of genetic resources.

If population-specific marker bands, i.e. alleles unique to a population, are found these need to receive extra focus if the broadest possible genetic range of the species is to be preserved. The results from population genetic studies may indicate whether there is fragmentation of the species of interest among subpopulations. It would be important to determine whether the extant populations could represent remnants of a formerly large population broken up through human activities or species that naturally develop in isolated populations. The re-connection of gene flow among fragments needs to be induced through *in-situ* and *ex-situ* conservation methods. In addition, re-vegetation corridors that allow habitats to link up and permit gene flow where the loss of a population through habitat conversion has taken place might be a suitable conservation strategy. Cross-pollinators also play an important role in maintaining distinct features in low genetic diversity populations of habitats that have produced fragmentation. On the other hand, high genetic diversity within fragmented populations requires cross-pollination to increase gene flow between populations isolated by habitat fragmentation.

Even though spider orchid relationships based on molecular markers used in this study did not address position of spider orchid species within the group. It supports three main groups of spider orchid as recognised by morphological character.

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Appendix I: Composition of buffers and solution used in this study.

Composition of 5X TBE (per litre)

54 g Tris base

27.5 g Boric acid

20 ml 0.5 M EDTA (pH 8.0)

Polyacrylamide gel

49 ml Diluent (Sequalgel)

14 ml Acrylamide concentrate (Sequalgel)

7 ml 10X TBE

70% Ammonium persulfate

70 μl TEMED (Sigma)

Gel loading buffers:

For agarose gels (6X)

0.25% (w/v) Bromophenol blue

0.25% (w/v) Xylene blue FF

40% (w/v) sucrose

For polyacrylamide gel

10 ml Formamide

0.25% (w/v) Bromophenol blue

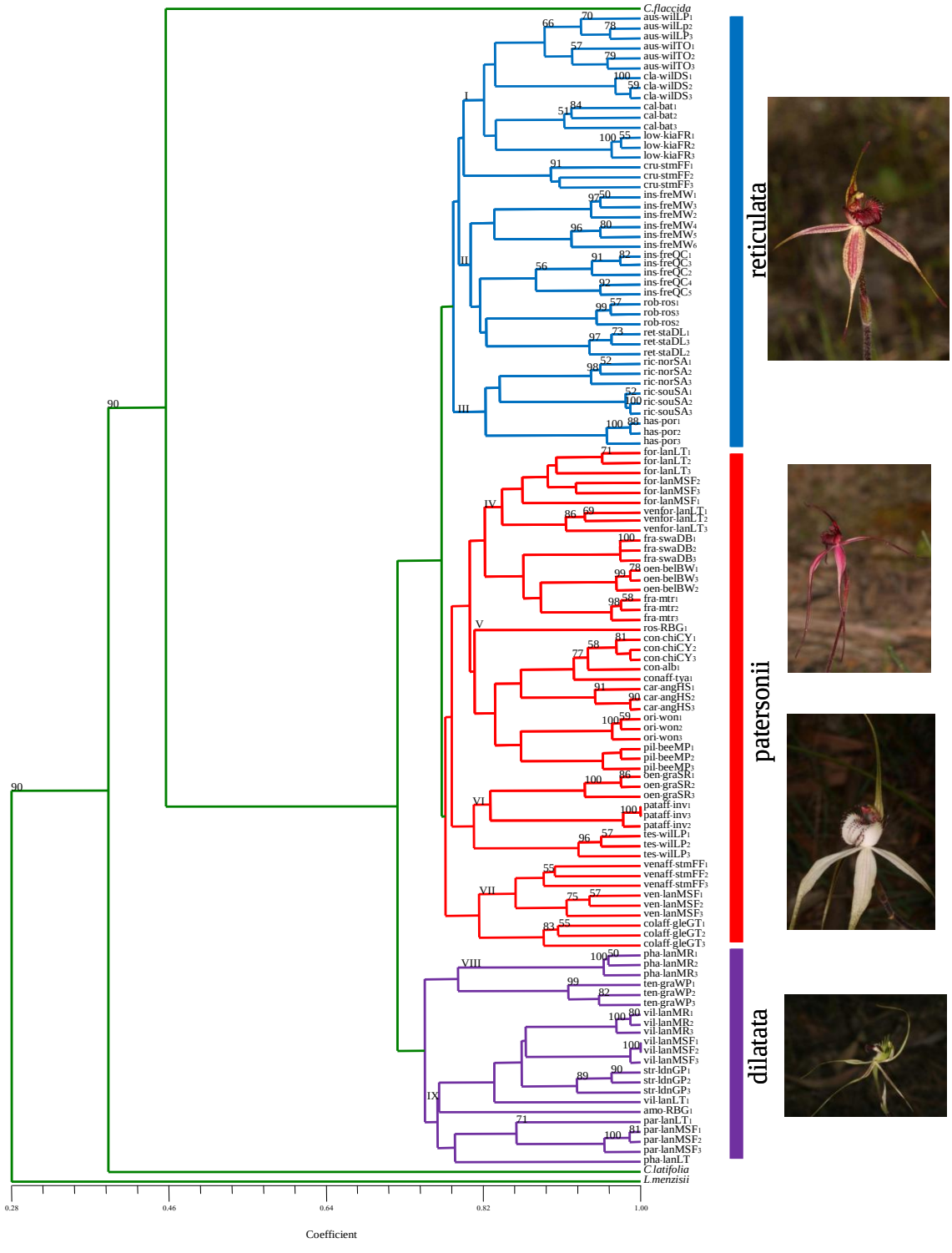
0.25% (w/v) Xylene blue FF

400 μl 0.5 M EDTA

Appendix II: File on CD

Appendix V: File on CD

Appendix III: UPGMA Dendrogram based on RAPD markers showing genetic relationships of 117 spider orchid species and three outgroup taxa. The dendrogram was constructed using Jaccard similarity coefficient with bootstraps calculated above 50% are shown above the node.



Appendix IV: UPGMA Dendrogram based on RAPD markers showing genetic relationships of 117 spider orchid species and three outgroup taxa. The dendrogram was constructed using Simple Matching similarity coefficient with bootstraps calculated above 50% are shown above the node.