Phylogenetics, genome size evolution and population genetics of slipper orchids in the subfamily cypripedioideae (orchidaceae)

Phylogenetics, genome size evolution and population genetics of slipper orchids in the subfamily Cypripedioideae (Orchidaceae)

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Declaration

I hereby confirm that this thesis is my own work and the material from other sources used in this work has been appropriately and fully acknowledged.

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Abstract

Slipper orchids (subfamily Cypripedioideae) comprise five genera; *Paphiopedilum*, *Cypripedium*, *Phragmipedium*, *Selenipedium*, and *Mexipedium*. Phylogenetic relationships of the genus *Paphiopedilum,* were studied using nuclear ribosomal ITS and plastid sequence data. The results confirm that *Paphiopedilum* is monophyletic and support the division of the genus into three subgenera *Parvisepalum*, *Brachypetalum* and *Paphiopedilum*. Four sections of subgenus *Paphiopedilum* (*Pardalopetalum*, *Cochlopetalum*, *Paphiopedilum* and *Barbata*) are recovered with strong support for monophyly, concurring with a recent infrageneric treatment. Section *Coryopedilum* is also recovered with low bootstrap but high posterior probability values. Relationships in *Barbata* remain unresolved, with short branch lengths and narrow geographical distributions suggesting it may have undergone rapid radiation. Genome sizes were measured for seven taxa in *Paphiopedilum* and chromosome and genome size data mapped onto the phylogenetic framework, showing no clear trend in increase in chromosome number in the genus. The diploid chromosome number of $2n = 26$ in subgenera *Parvisepalum* and *Brachypetalum* suggests it is the ancestral condition, with higher chromosome numbers in *Cochlopetalum* and *Barbata* pointing to centric fission possibly having occurred independently in these sections. Although species in *Barbata* have larger genome sizes than other sections, any trend of genome size evolution remains unclear in the genus. Eight primer pairs for plastid microsatellites were designed from consensus sequences generated from different genera, most of them shown to be applicable across the subfamily. High levels of variation in allele size were observed at interspecific levels but at intraspecific level, low levels were observed in *Cypripedium calceolus*. The application of plastid microsatellites for population genetic analyses in *C. calceolus* was limited because few of them are polymorphic and low numbers of alleles were detected. Results were generally congruent with a previous study. Within the limits of this data, the plastid haplotype distribution of *C. calceolus* in western and northern Europe could indicate possible recolonisation routes from three main refugia, following glaciations. Size variation has also been detected in other species in some markers but sampling was sparse.

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Chapter 1: General Introduction

1.1 The Family Orchidaceae

The family Orchidaceae, which is placed within the order Asparagales of the monocotyledons (Chase *et al.*, 2000), is one of the largest families of angiosperms, consisting of c. 779 genera (Mabberley, 2008) and c. 26,567 species (WCSP, 2013). It is a cosmopolitan family, found in most environments in the world, ranging from northern Sweden and Alaska to Tierra del Fuego and Macquarie Island, and is only absent from the most extreme desert habitats (Dressler, 1981). In habit, orchids can be found as terrestrials, epiphytes or lithophytes. The majority of orchids in temperate regions are terrestrial, occurring in grassland and woodland habitats, whereas most orchids in the tropics are epiphytes on forest or woodland trees, or lithophytes on rocks or cliffs (Pridgeon *et al.*, 1999). Orchids are diverse in size. Although many species of *Bulbophyllum* Thouars have sometimes thought to have been the smallest orchids, the central American orchid *Platystele jungermannioides* (Schltr.) Garay is probably the smallest, being only a few millimetres in size (Dressler, 1981). The longest orchid plants are the vines of *Vanilla* Plum. ex. Mill. often growing to several metres but the largest orchids overall are thought to be *Grammatophyllum speciosum* Blume and *G. papuanum* J.J.Sm. the stems of which can grow to up to 5 metres and form massive clumps (Dressler, 1981).

Orchid classifications based on morphological characters, especially anther features, have been proposed several times in the past. The most recent widely accepted classification based on morphological characters is that of Dressler (1993), in which he recognised five subfamilies, namely Apostasioideae Rchb.f., Cypripedioideae Lindl., Orchidoideae, Spiranthoideae Dressler and Epidendroideae Lindl. Although the system of Dressler (1993) reflected some phylogenetic concepts, a more comprehensive, cladistic analysis based on morphological characters was that of Freudenstein and Rasmussen (1999).

During the last 20 years, molecular techniques, using DNA sequences have become more important for evaluations of those previous classifications and for proposing new classifications for Orchidaceae reflecting evolutionary relationships. Several molecular phylogenetic studies, including those of Chase *et al.* (1994), Neyland and Urbatsch

(1995; 1996), Cox *et al.* (1997) and Cameron *et al.* (1999), have been undertaken and have contributed to the revising of orchid classification. Chase *et al.* (2003) proposed a subfamilial orchid classification based on DNA sequence data, in which they recongnised five subfamilies in Orchidaceae: Apostasioideae, Vanilloideae Szlach., Cypripedioideae, Orchidoideae and Epidendroideae. In their phylogenetic tree, Apostasioideae is sister to the rest of the family; the second branching clade is Vanilloideae, followed by Cypripedioideae, which is sister to the clade of Orchidoideae and Epidendroideae. In this new classification, subfamily Spiranthoideae was not recognised and spiranthoid orchids were placed in subfamily Orchidoideae. In addition, *Vanilla* and its relative genera were recognised as subfamily Vanilloideae.

1.2 Subfamily Cypripedioideae

Slipper orchids are mainly terrestrial orchids, although they are also found in other forms, such as lithophytes and epiphytes. The floral characteristics of the slipper orchids are highly distinctive, the flowers being formed of a slipper-shaped lip, two fertile stamens, a shield-like staminode and united lateral sepals or a synsepal (Cox *et al.*, 1997). Taxonomically, they belong to subfamily Cypripedioideae. This subfamily contains five genera; *Paphiopedilum* Pfitzer, *Phragmipedium* Rolfe, *Selenipedium* Rchb.f., *Cypripedium* L. and *Mexipedium* V.A.Albert & M.W.Chase.

Slipper orchids, which consist of approximately 150–170 species (Cox *et al.*, 1997), are widely distributed throughout temperate regions in Eurasia and North America and tropical regions in Central and South America and Asia (Pridgeon *et al.*, 1999). *Cypripedium* has a mostly Northern Hemisphere distribution, ranging from Northern America to Europe and temperate Asia. *Paphiopedilum* is distributed in tropical Asia. *Phragmipedium* and *Selenipedium* inhabit tropical Central and South America and a monotypic genus, *Mexipedium*, is restricted to Southern Mexico only (Pridgeon *et al.*, 1999).

The five genera of slipper orchids cannot be distinguished by any unique morphological characters but a combination of morphological characters, including leaf type, number of locules, type of placentation and sepal aestivation, along with their geographical distribution, circumscribes each genus (Atwood, 1984; Cox *et al.*, 1997). Tropical Asiatic *Paphiopedilum* has conduplicate leaves, unilocular ovaries with parietal placentation and imbricate sepal aestivation. Northern Hemisphere *Cypripedium* has plicate leaves, perforate sepal aestivation and unilocular ovaries with parietal placentation. Tropical South American *Selenipedium* also has plicate leaves and

perforate sepal aestivation, as well as trilocular ovaries with axile placentation. Two other genera have conduplicate leaves apart from *Paphiopedilum*: Central and South American *Phragmipedium* has valvate sepal aestivation and a trilocular ovary with axile placentation and *Mexipedium*, restricted to Mexico, has unilocular ovaries with parietal placentation and valvate sepal aestivation (Atwood, 1984; Albert and Chase, 1992; Cox *et al.*, 1997).

Phylogenetic studies of subfamily Cypripedioideae were undertaken by Albert (1994), using morphological characters and *rbcL* sequence data. The results supported the monophyly of *Cypripedium, Paphiopedilum* and *Phragmipedium*, with *Selenipedium* sister to the rest of the family and the clade of *Mexipedium* and *Phragmipedium* sister to *Paphiopedilum* but sampling was sparse. A more comprehensive study was carried out by Cox *et al.* (1997), using the nuclear ITS region, the phylogenetic relationships among genera being congruent with those of Albert (1994) and they also provided support for the robustness of those relationships. Recently, Guo *et al.* (2012) used sequence data obtained from six plastid and two low copy nuclear regions for a phylogenetic study of subfamily Cypripedioideae, their results disagreeing with previous studies by finding *Cypripedium* to be sister to the rest of the subfamily and forming the first branching clade but being congruent with the results from phylogenetic studies at higher level (Freudenstein *et al.*, 2004; Cameron, 2006; Gorniak *et al.*, 2010).

1.3 Conservation of the slipper orchid subfamily Cypripedioideae

Slipper orchids have distinctive flowers, hence their common name, as the lip is sac-shaped and looks like an old fashioned lady's slipper. These highly unusual and beautiful flowers are extremely desirable to collectors, which has led to a serious problem with over-collecting impacting on wild populations. Along with degradation of their habitats, this has caused a serious decline in many species, with several becoming severely threatened in the wild. *Paphiopedilum* spp. and *Phragmipedium* spp. are listed in Appendix I of the Convention on International Trade of Endangered Species (CITES) and the remaining taxa in subfamily Cypripedioideae are included under Orchidaceae on Appendix II (CITES, 2012).

Cypripedium calceolus L., even though it is one of the most widespread species in the Northern Hemisphere (Cribb, 1997), is rare in many countries in Europe because of over-collecting and habitat destruction (Roberts, 2003). This species is designated as 'Near Threatened' on the IUCN European red List Appendix 2 (Bilz *et al.*, 2011), and is

also listed in the Convention on the Conservation of European Wildlife and Natural Habitats (the Bern Convention), Appendix I, Strictly Protected Flora Species (Council of Europe, 1979) and on European Council Directive 92/43/EEC on the Conservation of Natural Habitats and of Wild Fauna and Flora Habitats Directive, Annex II and IV (Council of Europe, 1992). *Cypripedium calceolus* is one of the rarest orchids in Britain, its numbers having declined dramatically, the cause of which is mainly over-collecting by both botanists and gardeners (Ramsay and Stewart, 1998), with only one original clump and some reintroduced plants surviving in the wild in the north of England (Fay and Cowan, 2001). *Cypripedium calceolus* has been the focus of various conservation activities in the UK, including site management, hand pollination, guarding round the clock, *ex situ* seed germination and DNA fingerprinting techniques, because of its extreme rarity (Fay and Cowan, 2001). A conservation programme in England for the re-establishment of this slipper orchid has been carried out using *in vitro* seed germination in the laboratory, with plants being reintroduced to natural sites and ensuring subsequent seed set by hand pollination (Ramsay and Stewart, 1998).

1.4 Genus *Paphiopedilum*

The genus *Paphiopedilum*, comprising *c.* 72 species (Averyanov *et al.*, 2003), is the largest of the five genera in subfamily Cypripedioideae, the name, referring to the shape of the lip of the flowers, being derived from the Greek word meaning 'the slipper of Aphrodite' (Cribb, 1998). This genus is formed of terrestrial, lithophytic or epiphytic orchids, with short rhizomes in most species but with elongated rhizomes found in some species, such as *P. armeniacum* S.C.Chen & F.Y.Liu and *P. micranthum* Tang & F.T.Wang (Cribb, 1998). *Paphiopedilum* leaves are coriaceous, conduplicate, and spreading or suberect, their shape varying from ligulate to elliptic-oblong, which can be useful features for discriminating at subgeneric and specific levels (Cribb, 1998). Leaf shapes vary from linear-ligulate, found in sections *Paphiopedilum* K.Karas. & K.Saito and *Coryopedilum* Pfitzer, to elliptic-oblong in subgenus *Brachypetalum* (Hallier f.) Pfitzer (Cribb, 1998). There are two distinctive patterns of the upper leaf surface; uniformly green or tessellated; this character is taxonomically useful in some cases (Cribb, 1998). In all of the species in sections *Paphiopedilum* and *Coryopedilum*, the upper surfaces of the leaves are uniformly green, whereas species in subgenera *Parvisepalum* K.Karas. & K.Saito (mostly), *Brachypetalum* and section *Barbata* (Kraenzl.) V.A.Albert & Börge Pett. have mottled upper leaf surfaces (Cribb, 1998).

Most species of *Paphiopedilum* have erect or arching inflorescences, although relatively horizontal peduncles are found in some species that grow on sheer, rocky cliffs, such as *P. helenae* Aver., *P. dianthum* Tang & F.T.Wang, and *P. henryanum* Braem, and in the epiphytic species, *P. villosum* (Lindl.) Stein (Averyanov *et al.*, 2003). The number of flowers borne on inflorescences can be taxonomically useful, species in sections *Coryopedilum*, *Pardalopetalum* (Hallier f.) Pfitzer and *Cochlopetalum* Hallier f. ex K.Karas. & K.Saito bear three or more flowers, while the others produce mostly a single flower but two-flowered inflorescences are also found rarely (Cribb, 1998). The shape of the lateral petals of *Paphiopedilum* are the most variable and elaborate, compared with other genera in the subfamily (Atwood, 1984), and can be another useful feature for classification; elliptic to subcircular shapes are found in subgenera *Parvisepalum* and *Brachypetalum*, linear, spirally twisted petals in section *Cochlopetalum* and tapering petals in section *Coryopedilum* (Cribb, 1998). The slipper shaped lip of *Paphiopedilum* is the third petal, which has been highly modified to form a deep sac that functions as an insect trap. There are three sepals, of which the two lateral sepals are united to form a synsepal. Characteristic shapes and patterns of the dorsal sepal can often be used to distinguish species (Cribb, 1998). Ovaries of species in *Paphiopedilum* are inferior and unilocular with parietal placentation. Two fertile stamens are attached to the stigma, forming a short column. The other stamen, attached to the end of the column, is an infertile, shield-like in shape staminode. The shape of the staminode in different species of *Paphiopedilum* is variable; cordate, obcordate, lunate and reniform shapes are most commonly found, and it is considered a useful character for distinguishing species within the genus; for example, lunate staminodes are commonly found in section *Barbata*, whereas obcordate staminodes occur commonly in section *Pardalopetalum* (Cribb, 1998). However, the staminode may not be a useful character for identification when closely related species are compared, such as among *P. barbatum* (Lindl.) Pfitzer, *P. callosum* (Rchb.f.) Stein [= *P. crossii* (E.Morren) Braem & Senghas] and *P. lawrenceanum* (Rchb.f.) Pfitzer (Braem and Chiron, 2003).

1.4.1 Taxonomic history of the genus *Paphiopedilum*

The genus *Paphiopedilum* was first described by Pfitzer (1886), although the first botanist who recognised the tropical slipper orchids as distinct from the temperate genus *Cypripedium* was Rafinesque (1838), who named two genera; *Cordula* Raf. and *Stimegas* Raf., based on *Cypripedium insigne* Wall. ex Lindl. and *C. venustum* Wall. ex Sims respectively (Cribb, 1998). The name *Paphiopedilum* of Pfitzer was accepted by

many botanists, but Rafinesque's slipper orchid names were abandoned (Atwood, 1984; Cribb, 1998; Braem and Chiron, 2003). In 1959, in accordance with the International Code of Botanical Nomenclature, the name *Paphiopedilum* was conserved over the slipper orchid names of Rafinesque (Rickett and Stafleu, 1959).

Infrageneric classifications of the genus have been proposed by various authors (Pfitzer, 1894; Hallier, 1896; Pfitzer, 1903; Brieger, 1971; Karasawa and Saito, 1982; Atwood, 1984; Cribb, 1987; Braem, 1988; Cox *et al.*, 1997; Braem *et al.*, 1998; Cribb, 1998; Braem and Chiron, 2003; Averyanov *et al.*, 2003). An overview of previous infrageneric classifications is shown in Table 1.1.

Table 1.1. An overview of infrageneric classifications of genus *Paphiopedilum* (modified from Cribb 1998).

Pfitzer (1894)	Hallier (1896)	Pfitzer (1903)	Brieger (1971)	Karasawa & Saito (1982)	Atwood (1984)
Coelopedilum group	*Coelopedilum* group				
a. *Eremantha Tessellata* (in part)	*Aphanoneura Brachypetalum*	*Brachypetalum*	*Brachypetalum*	*Brachypetalum*	*Brachypetalum*
				Parvispepalum	
b. *Polyantha*	*Chromatoneura Viridia Polyantha*	*Anotopedilum*	*Polyantha*	*Polyantha*	*Paphiopedilum*
	XI *Streptopetalum* (in part)	Section *Coryopedilum*	Section *Streptopetalum*	Section *Mastigopetalum*	Section *Coryopedilum*
	XII *Mastigopetalum*	Section *Gonatopedilum*	Section *Mastigopetalum*		
		Section *Prenipedilum*			
		Otopedilum			
	XI *Streptopetalum* (in part)	Section *Mystropetalum*	Section *Polyantha*	Section *Mystropetalum*	Section *Pardalopetalum*
	X *Pardalopetalum*	Section *Pardalopetalum*		Section *Polyantha*	
	XIII *Cochlopetalum*	Section *Cochlopetalum*	Section *Cochlopetalum*	*Cochlopetalum*	Section *Cochlopetalum*
a. *Eremantha Viridia*	*Chromatoneura Viridia Eremantha*		*Paphiopedilum*	*Paphiopedilum*	Section *Paphiopedilum*
	VIII *Stictopetalum*	Section *Stictopetalum*	Section *Stictopetalum*	Section *Stictopetalum*	
	IX *Neuropetalum*	Section *Neuropetalum*	Section *Paphiopedilum*	Section *Paphiopedilum*	
	V *Thiopetalum*	Section *Thiopetalum*		Section *Thiopetalum*	
	VII *Cymatopetalum*	Section *Cymatopetalum*			
	VI *Ceratopetalum*	Section *Ceratopetalum*		Section *Ceratopetalum*	
a. *Eremantha Tessellata*	*Chromatoneura Tessellata*		*Barbata*	*Sigmatopetalum*	Section *Barbata*
(in part)	II *Sigmatopetalum*	Section *Spathopetalum*	Section *Sigmatopetalum*	Section *Spathopetalum*	
	IV *Drepanopetalum*	Section *Blepharopetalum*	Section *Blepharopetalum*	Section *Sigmatopetalum*	
				Section *Blepharopetalum*	
				Section *Punctatum*	
				Section *Planipetalum*	
	III *Clinopetalum*	Section *Phacopetalum*	Section *Barbata*	Section *Barbata*	

Table1.1. Continued

Cribb (1987)	Braem (1988), Braem *et al*. (1998) and Braem & Chiron (2003)	Cox *et al.* (1997)	Cribb (1998)	Averyanov *et al.* (2003)
Brachypetalum	*Brachypetalum*	*Brachypetalum*	*Brachypetalum*	*Brachypetalum*
Section *Brachypetalum*				
Section *Parvisepalum*	*Parvispepalum*	*Parvisepalum*	*Parvisepalum*	*Parvisepalum*
				Section *Parvisepalum*
				Section *Emersonianum*
Paphiopedilum	*Polyantha*	*Paphiopedilum*	*Paphiopedilum*	*Paphiopedilum*
Section *Coryopedilum*	Section *Mastigopetalum*		Section *Coryopedilum*	Section *Coryopetalum*
Section *Pardalopetalum*	Section *Mystropetalum*	Section *Pardalopetalum*	Section *Pardalopetalum*	Section *Pardalopetalum*
	Section *Polyantha*			
Section *Cochlopetalum*	*Cochlopetalum*	Section *Cochlopetalum*	Section *Cochlopetalum*	Section *Cochlopetalum*
Section *Paphiopedilum*	*Paphiopedilum*	Section *Paphiopedilum*	Section *Paphiopedilum*	Section *Paphiopedilum*
	Section *Stictopetalum*			
	Section *Paphiopedilum*			
	Section *Thiopetalum*			
	Section *Ceratopetalum*			
Section *Barbata*	*Sigmatopetalum*	Section *Barbata*	Section *Barbata*	Section *Barbata*
	Section *Spathopetalum*			
	Section *Sigmatopetalum*			
	Section *Blepharopetalum*			
	Section *Punctatum*			
	Section *Planipetalum*			
	Section *Barbata*			

Pfitzer (1894) divided his genus *Paphiopedilum* into two main groups, including additional tropical species that been transferred by Stein (1892) (Cribb, 1998). He placed all the species that have a unilocular ovary in a group, *Coelopedilum* (= genus *Paphiopedilum s.s.*), which he further divided into two subgroups (*Eremantha* and *Polyantha*). He also placed all trilocular ovary species in the *Phragmopedilum* group (= genus *Phragmipedium s.s.*).

Hallier (1896) followed Pfitzer (1894) by dividing *Paphiopedilum* into two main groups but his infrageneric classification within the *Coelopedilum* group was more complicated than that of Pfitzer. Because the treatments of Pfitzer and Hallier did not state the ranks for their groups, those unranked infrageneric names do not have priority. However, some of those names have been used for ranks by subsequent authors (Cribb, 1998).

Pfitzer (1903) revised his system in Engler's Das Pflanzenreich, largely following Hallier's work. He distinguished 47 species of the genus *Paphiopedilum* and divided them into three subgenera [*Brachypetalum* (Hallier f.) Pfitzer, *Anotopedilum* Pfitzer and *Otopedilum* Pfitzer] and 14 sections, based on vegetative, floral and inflorescence morphology, excluding species in the *Phragmopedilum* group, for which he accepted the generic concept of Rolfe (1896), transferring those species into genus *Phragmopedilum* (Pfitzer) Rolfe emend. (= genus *Phragmipedium sensu* Rolfe.).

Several years later, Brieger (1971), largely following the treatment of Pfitzer (1903), proposed a revision of the infrageneric treatment for the genus. He recognised 68 species in four subgenera [*Brachypetalum* (Hallier f.) Pfitzer, *Polyantha* (Pfitzer) Brieger, *Paphiopedilum* and *Barbata* (Kraenzl.) Brieger] and nine sections, based on vegetative, floral and inflorescence morphology.

Karasawa and Saito (1982), in their revision of the genus *Paphiopedilum*, recognised a new subgenus, *Parvisepalum* K.Karas. & K.Saito, for *P. delenatii* Guillaumin and the newly discovered species *P. micranthum* and *P. armeniacum*. Based on morphological and chromosome data, they divided genus *Paphiopedilum* into six subgenera [*Brachypetalum*, *Parvisepalum* K.Karas. & K.Saito, *Polyantha* (Pfitzer) Brieger, *Cochlopetalum* (Hallier f. ex Pfitzer) K.Karas. & K.Saito, *Paphiopedilum* K.Karas. & K.Saito and *Sigmatopetalum* Hallier f. ex K.Karas. & K.Saito] and 13 sections. In addition, they assessed 17 morphological and chromosome characters for evolutionary trends, from primitive to derived conditions and proposed phyletic relationships within the genus. Based on these analyses, Karasawa and Saito identified two lines of evolution in the genus. Subgenus *Brachypetalum* was considered to be derived from

subgenus *Parvisepalum*, which possesses the most primitive character conditions. In contrast, the most derived characters are found in subgenus *Cochlopetalum*, derived from subgenus *Polyantha* and subgenus *Sigmatopetalum*, derived from subgenus *Paphiopedilum*.

Atwood (1984) studied the phylogenetic relationships within subfamily Cypripedioideae based on cladistic analysis of morphological and chromosome data, using the Wagner groundplan-divergence method. In his study, he considered previous infrageneric classifications of other authors, mainly that of Pfitzer (1903) and Brieger (1971) but he did not include the work of Karasawa and Saito (1982). Phylogenetic relationships within *Paphiopedilum*, elucidated from a cladogram produced from his results, showed an evolutionary trend from section *Coryopedilum* Pfitzer (= subgenus *Polyantha* section *Mastigopetalum* of Karasawa and Saito) toward section *Barbata* (= subgenus *Sigmatopetalum* of Karasawa and Saito) and he suggested the former as the most primitive group and the latter as the most derived group. However, this cladogram showed unclear relationships between *Brachypetalum* and the remaining groups in the genus. From his results, he suggested dividing the genus into two subgenera [*Brachypetalum* (Hallier f.) Pfitzer and *Paphiopedilum*] and five sections. The five sections [*Coryopedilum*, *Pardalopetalum* (Hallier f.) Pfitzer, *Cochlopetalum* (Hallier f.) Garay, *Paphiopedilum* and *Barbata* (Kraenzl.) Atwood] of the largest subgenus, *Paphiopedilum*, of Atwood are broadly similar to the four subgenera of Karasawa and Saito (1982). In his study, *P. delenatii* was included but not the related species *P. micranthum* and *P. armeniacum* that were newly discovered and had been placed by Karasawa and Saito in their new subgenus *Parvisepalum*. In contrast, Atwood placed *P. delenatii* in subgenus *Brachypetalum* and did not consider subdividing another infrageneric rank for this species.

In his monograph, Cribb (1987) divided the genus into two subgenera [*Brachypetalum* Hallier f. (Pfitzer) and *Paphiopedilum*], following Atwood (1984) in the infrageneric treatment of the genus. However, he followed Karasawa and Saito (1982) in recognition of subgenus *Parvisepalum* but reduced it to sectional level within subgenus *Brachypetalum*, in which he also included two newly discovered species; *P. malipoense* S.C.Chen & Z.H.Tsi and *P. emersonii* Koop. & P.J.Cribb. Subgenus *Brachypetalum* was further divided into two sections, *Parvisepalum* (K.Karas. & K.Saito) P.J.Cribb and *Brachypetalum* [= *Concoloria* (Kraenzl.) V.A.Albert & Börge Pett.]. Subgenus *Paphiopedilum* was divided into five sections, as in Atwood (1984),

because he considered, from the phylogenetic results of Atwood, that this subgenus was monophyletic and thus other groups should be considered to be at sectional level.

In a monograph of the genus *Paphiopedilum*, Braem (1988) accepted the treatment of Karasawa and Saito (1982), although he further subdivided several subsections in his treatment. The infrageneric treatments in the following editions of his work (Braem *et al.*, 1998; Braem and Chiron, 2003) are as in the first monograph.

Albert and Pettersson (1994) proposed combining all genera with conduplicate leaves (*Paphiopedilum*, *Phragmipedium* and *Mexipedium*) under *Paphiopedilum*, based on a cladistic analysis of subfamily Cypripedioideae but their infrageneric treatment was not accepted by other taxonomists.

Cox *et al.* (1997) used nuclear ribosomal DNA internal transcribed spacer (ITS) sequence data for a molecular phylogenetic study of subfamily Cypripedioideae. According to their molecular data for the genus *Paphiopedilum* they tentatively proposed elevating section *Parvisepalum* (K.Karas. & K.Saito) P.J.Cribb and section *Concoloria* (Kraenzl.) V.A.Albert & Börge Pett. [= section *Brachypetalum sensu* Cribb (1987)] of subgenus *Brachypetalum* to subgenera *Parvisepalum* and *Brachypetalum*, and suggested sections *Coryopedilum* and *Pardalopetalum* be combined. Also, they suggested simplification of the subsectional treatment of Braem (1988).

In the second edition of his monograph, the infrageneric classification of Cribb (1998), which was based mainly on morphological characters and chromosome data, also followed the molecular study of Cox *et al.* (1997). *Paphiopedilum* was subdivided into three subgenera in his classification: *Parvisepalum* K.Karas. & K.Saito, *Brachypetalum* (Hallier f.) Pfitzer and *Paphiopedilum* K.Karas. & K.Saito. As in his previous treatment, there remained five sections in subgenus *Paphiopedilum* [*Coryopedilum* Pfitzer, *Pardalopetalum* Hallier f. & Pfitzer, *Cochlopetalum* Hallier f. ex Pfitzer, *Paphiopedilum* K.Karas. & K.Saito and *Barbata* (Kraenzl.) V.A.Albert & Börge Pett.]. Representative species of subgenera and sections in this classification are shown in Figure 1.1.

Figure 1.1. Representative *Paphiopedilum* species in subgenera and sections of infrageneric classification of Cribb (1998). A) subgenus *Parvisepalum*: *P. delenatii*, B) subgenus *Brachypetalum*: *P. concolor*, C–G) subgenus *Paphiopedilum*, C) section *Cochlopetalum*: *P. primulinum*, D) section *Pardalopetalum*: *P. haynaldianum*, E) section *Coryopedilum*: *P. philippinense*, F) section *Barbata*: *P. sukhakulii*, G) section *Paphiopedilum*: *P. gratrixianum* (Photographs: A. Chochai).

Averyanov *et al.* (2003) largely followed the infrageneric classification of Cribb (1998) but subgenus *Parvisepalum* was divided into two sections: *Parvisepalum* Aver. & P.J.Cribb and *Emersonianum* Aver. & P.J.Cribb. Section *Parvisepalum* was made up of species with tessellated leaves, whereas two species, differentiated mainly by having plain green leaves, *P. hangianum* Perner & O.Gruss and *P. emersonii*, formed the newly recognised section *Emersonianum*.

1.4.2 Ecology and distribution of *Paphiopedilum*

Paphiopedilum has a distribution from India and southern China through Southeast Asia and the Malesian islands to the Solomon Islands (Cribb, 1998) (Figure 1.2). Species in subgenus *Parvisepalum* inhabit mostly southern China and northern Vietnam. Also found in mainland Asia is subgenus *Brachypetalum*, which has a distribution through south China, southeast Myanmar, Thailand, northern Malaysia, Vietnam, Laos, and Cambodia (Averyanov *et al.*, 2003). The most isolated distribution is that of a species belonging to section *Paphiopedilum*, *P. druryi* (Bedd.) Stein, which is endemic to southern India, more than 2000 km away from the nearest other species (Cribb, 1998). Other species in section *Paphiopedilum* are distributed from India to southern China, Thailand, Vietnam, Laos, and Cambodia, with most species being endemic to relatively localised areas, although two species, *P. hirsutissimum* (Lindl. ex Hook.) Stein and *P. villosum*, have the widest distributions which are also coincident with the distribution range of the section (Averyanov *et al.*, 2003). Two sections are restricted to islands, *Cochlopetalum* being endemic to Java and Sumatra and *Coryopedilum* endemic to the Philippines, Borneo, Sulawesi and New Guinea (Cribb, 1998). Section *Pardalopetalum* is more widespread, ranging from mainland southeast Asia, the Malay Archipelago, eastward to Sulawesi and Luzon in the Philippines (Cribb, 1998). The largest section, *Barbata*, has a distribution nearly the same as that of the genus, with four species; *P. appletonianum* (Gower) Rolfe, *P. callosum*, *P. bullenianum* (Rchb.f.) Pfitzer and *P. javanicum* (Reinw. ex Lindl.) Pfitzer, being the most widely distributed, whereas most of the endemic species in this section are found in Sumatra, Borneo and the Philippines (Averyanov *et al.*, 2003).

Paphiopedilum species are mainly terrestrials, most often growing in shade on the forest floor in evergreen or seasonally deciduous mountain forests, although five species are epiphytic; *P. parishii* (Rchb.f.) Stein, *P. lowii* (Lindl.) Stein, *P. villosum* are truly epiphytic, and *P. hirsutissimum* and *P. glanduliferum* (Blume) Stein are facultative

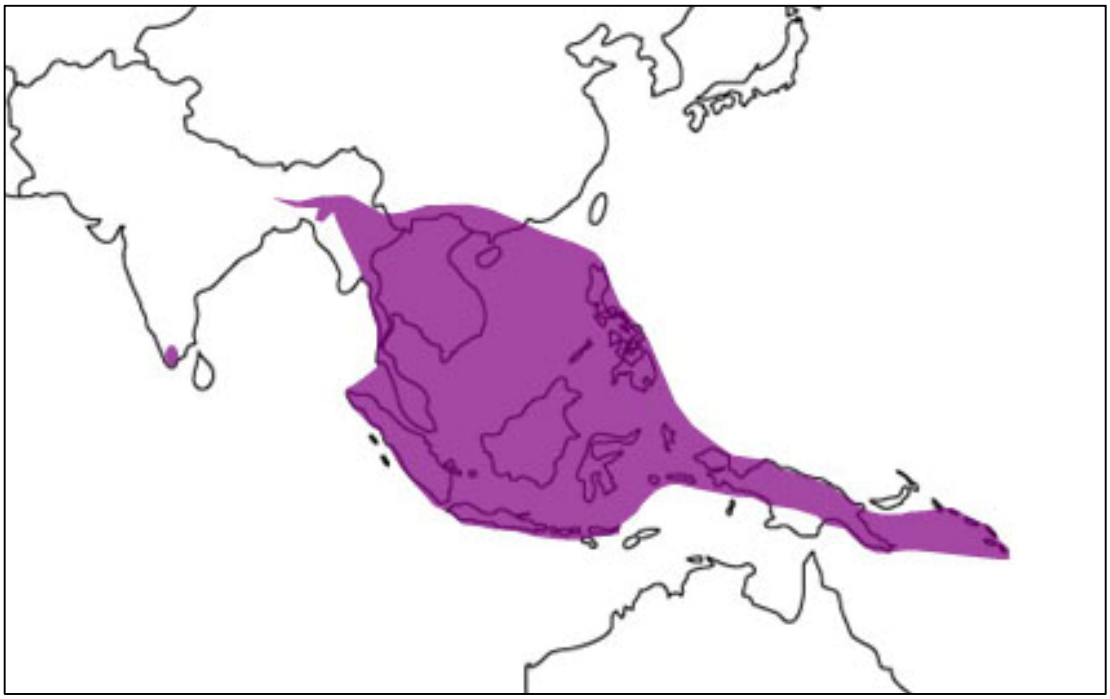

Figure 1.2. Distribution map of the genus *Paphiopedilum* in tropical Asia. (adapted from Cribb, 1998). Outline map: © Bruce Jones Design Inc. 2009.

epiphytes (Cribb, 1998). Several species are reported to be lithophytic on limestone rocks or cliffs, such as *P. concolor* (Bateman) Pfitzer and *P. hirsutissimum* (Lindl. ex Hook.) Stein var. *esquirolei* (Schltr.) K.Karas. & K.Saito (Fowlie, 1989; Fowlie, 1990b; Fowlie, 1990a), although species growing in those rocky habitats are not always truly lithophytic but can grow on litter accumulated in cracks in the rocks (Cribb, 1998).

Relatively few studies have been conducted into pollination in *Paphiopedilum* species but those studies that there have been, have, in most cases, reported that hoverflies are the pollinators (Atwood, 1985; Bänziger, 1994; 1996; 2002; Bänziger *et al.*, 2012; Shi *et al.*, 2007; 2009). However, bees have been observed as pollinators in *P. micranthum* (subgenus *Parvisepalum*) (Bänziger *et al.*, 2008) and a recent study has reported that bees are the pollinators in two closely related species, *P. thaianum* Iamwir. and *P. niveum* (Rchb.f.) Stein, of subgenus *Brachypetalum* (Bänziger *et al.*, 2012). Pollination by deceit has been reported as a mechanism in *Paphiopedilum*; both brood site and food deception (Atwood, 1985; Bänziger, 1994; Bänziger, 1996; Bänziger, 2002). A pollination study into *P. rothschildianum* (Rchb.f.) Stein by Atwood (1985) showed that hoverflies were deceived by flowers mimicking brood sites, from which a spicy or peppery scent was emitted to attract female hoverflies to the staminode in order to lay their eggs, during the process of which, they would slip into the lip. Because the only way to exit the pouch-like lip is by passing the stigma and

anthers behind the staminode, they deposit pollen on the stigma or pick up pollen from the anthers. Other species effect pollination by food deceit, such as *P. villosum*, in which the flowers produce a urine-like scent, that, along with colour contrast, possibly attracts hoverflies from a long distance, while closer to the flower, they are lured by the glittering staminode appearing like droplets of honeydew or moisture (Bänziger, 1996). Food deception by mimicking *Rhododendron* spp. flowers that supply a nectar reward was suggested as a mechanism for *P. micranthum* (Averyanov *et al.*, 2003). Although cross pollination by insects is predominant in species of *Paphiopedilum*, it has recently been found that an autonomous self pollination mechanism exists in *P. parishii*, by which the anthers transform into liquid, moving directly onto the stigma (Chen *et al.*, 2012).

1.5 *Cypripedium calceolus*

One of the most well known slipper orchids in Europe is the lady's slipper orchid, *Cypripedium calceolus* which was first described by Linnaeus in his *Species Plantarum* in 1753, the species being the type of the genus *Cypripedium*. This terrestrial species is long-lived, with the rhizome and buds perennating during the winter (Kull, 1999). Inflorescences can bear one, or mostly two (rarely three) flowers with maroon (rarely green) sepals and petals, a yellow, spotted within with red, slipper-like lip and a yellow, spotted with red, staminode (Cribb, 1997). *Cypripedium calceolus* inhabits deciduous and coniferous woodland, and is also found in open scrub and alpine meadows, usually growing in shade, occasionally in full sunlight (Kull, 1999). It is a well known calcicole species in Europe, usually growing on alkaline soils, although on rare occasions it has been found on neutral soil (Pridgeon *et al.*, 1999). This species reproduces both sexually, being pollinated by bees (Antonelli *et al.*, 2009), and asexually, by new ramets, which are produced from the horizontal rhizomes (Brzosko *et al.*, 2002). *Cypripedium calceolus* has a wide distribution, from northern and western Europe, including Britain, Scandinavia, France, northeastern Spain, Germany and northern Italy, eastward to Siberia and China and possibly as far as Japan, also previously occurring in Greece where it is now extinct (Cribb, 1997).

1.6 Phylogenetics and genome size evolution

Plant taxonomy is the study involved with the identification, classification and nomenclature of plants. Classification is the grouping of taxa into a unit of any rank according to certain criteria. Following the concept of evolution from Darwin, it is widely

accepted by taxonomists that classifications should reflect the evolutionary relationships, or phylogeny, of taxa. This concept was implemented in an approach called 'phylogenetic systematics' by Hennig in 1950 (Kitching *et al.*, 1998), or cladistics, as it was termed later by Mayr in 1969 (Stace, 1989). Cladistics refers to the hierarchical grouping of taxa in terms of sister-group relationships which can be represented in branching diagrams called cladograms (Kitching *et al.*, 1998). Advances in molecular techniques since that time, including polymerase chain reaction or PCR and DNA sequencing, have provided effective techniques and become routine for the reconstruction of phylogenetic relationships. The molecular phylogenetic study based on *rbcL* sequence by Chase *et al.* (1993) was a landmark in the use of molecular data for higher level plant classification and provided a basis for the APG (Angiosperm Phylogeny Group) classification of flowering plants, established in 1998 (APG I) and updated in 2003 (APG II) and 2009 (APG III), which proposed a modern classification based mainly on molecular data and revising previous classification systems (APG, 1998; APG, 2003; APG, 2009).

A phylogenetic framework is not only important for improving classification systems by reflecting the evolutionary relationships between taxa but is also crucial for understanding biological evolutionary processes, including vegetative and reproductive morphology, cytology and genome size. As genomes exist in all organisms, they are one of the most important biological characters, with the potential to reveal evolutionary trends, including that of genome size. The first study of genome size in plants was the important work on *Zea mays* and *Tradescantia* spp. by Swift in 1950 in which he coined the term 'C-value' for the amount of DNA content, which refers to 'the DNA content of unreplicated haploid chromosome compliment' (Bennett and Smith, 1976). Later, in the 1960s, variation in genome size of more than 50-fold was reported in angiosperms (McLeish and Sunderland, 1961), an interspecific variation of 40-fold between species of family Ranunculaceae (Rothfels *et al.*, 1966) and 5-fold within the genus *Vicia* L. (Martin and Shanks, 1966), which provided evidence for a wide variation of genome size within plant families and genera, independently of ploidy level (Bennett and Leitch, 2005). Recently, a high range of variability has been found across the eukaryotes, estimated to span a range of more than 40,000 fold (Leitch *et al.*, 2009). This massive variation of genome size raises many questions that may be addressed by viewing genome size data within a phylogenetic context (Leitch *et al.*, 2005).

This was done in a large scale study to determine evolutionary trends of genome size changes in angiosperms, first by Leitch *et al.* (1998) and extended by Soltis *et al.*

(2003), which found that the ancestral genome size of angiosperms is small (i.e. < 1.4 pg). Small genomes are found in all the major clades of angiosperms, very large genomes (i.e. > 14 pg) having evolved only in some derived families in the monocots and Santalales, suggesting they evolved independently and on separate occasions, although both increases and decreases may have happened. Several other studies of genome size evolution at familial and generic levels (e.g. Wendel *et al.*, 2002; Johnston *et al.*, 2005; Price *et al.*, 2005) have been carried out within a phylogenetic framework and showed that both increase and decrease in genome size have occurred in angiosperms divergence (Leitch *et al.*, 2005). More genome size data was included in a study that was extended to cover all the land plants and the same pattern of increases and decreases in the evolution of genome size, with a very small (i.e. ≤ 1.4 pg) or intermediate (i.e. > 3.5 to < 14.0 pg) ancestral C-value occurring throughout land plants (Leitch *et al.*, 2005).

Genome size is thought to have an influence on life form and life history strategies (Bennett, 1972; Chase *et al.*, 2005; Leitch *et al.*, 2009). Also, genome size has been shown to be correlated with guard cell size (Beaulieu *et al.*, 2008), rate of diversification (Knight *et al.*, 2005) and rarity (Vinogradov, 2003). The observation made by Vinogradov (2003) that genome size corresponds directly with rarity, with species having large genomes more likely to be rare than those with smaller genome sizes and at greater risk of extinction. Most slipper orchids have large genome sizes in comparison with most other flowering plants (Cox *et al.*, 1998; Leitch *et al.*, 2009), many of them are also under threat and occur only in small populations (Cribb, 1998; Nicolè *et al.*, 2005) . This is exacerbated by over-collection of plants from the wild and habitat destruction, meaning that the development of effective conservation strategies is even more crucial.

1.7 Population genetics and plant conservation

Population genetics has great value in determining genetic variation in endangered species, helping inform the decision making process in determining the appropriate methods for their conservation (Fay and Krauss, 2003). Genetic diversity, which is key to survival in changing environments (Frankham, 2005) and ensuring reproductive fitness (Reed and Frankham, 2003), can be affected by various factors, including genetic drift, population bottlenecks, natural selection and method of reproduction (Freeland *et al.*, 2011). Species with small populations are more likely to be affected by genetic drift and inbreeding, these factors potentially altering their patterns of

genetic diversity and fitness (Ellstrand and Elam, 1993). In addition, population sizes of endangered species might be small and could have been affected by bottlenecks or founder effects, and thus the effect of inbreeding depression could be of significant importance for conservation strategies (Hedrick and Kalinowski, 2000). Population structure is not only the consequence of present events but also past events, thus, it is crucial to be able to understand how those ancient events have contributed to the migration and the formation of the genetic components of present day species and populations (Hewitt, 1999). The geographical distribution of plant species in the present day is thought to be largely influenced by climatic changes in the Quaternary period and understanding and reconstructing these changes comes under the heading 'phylogeography', in which advances in molecular techniques have helped reveal the locations of glacial refugia and routes of postglacial recolonisation (Comes and Kadereit, 1998).

Levels of genetic diversity can be assessed using various DNA-based techniques, including DNA sequencing, AFLP [amplified fragment length polymorphisms, Vos *et al.* (1995)], nuclear and plastid microsatellites (reviewed in Fay and Krauss, 2003). DNA-based techniques have been utilised for orchid conservation, such as AFLP in *Pseudorchis albida* (L.) Á.Löve & D.Löve (Duffy *et al.*, 2011), *Phragmipedium longifolium* (Warsz. & Rchb.f.) Rolfe (Muñoz *et al.*, 2010); nuclear microsatellites in *Gymnadenia conopsea* (L.) R.Br. (Gustafsson, 2000), *Serapias vomeracea* Briq. (Pellegrino *et al.*, 2006) and plastid microsatellites in *Cypripedium calceolus* (Fay and Cowan, 2001; Fay *et al.*, 2009) and *Cephalanthera* spp. (Micheneau *et al.*, 2010). However, developing molecular markers, including AFLP and nuclear microsatellites, has been reported to be problematic in species that have large genome sizes (i.e. 1C-value > 15 pg) because of problems with PCR amplification (Garner, 2002; Fay *et al.*, 2005; Kahandawala, 2009). Thus, genome size is a factor in deciding which techniques are applicable for detecting genetic diversity in populations, which is essential for determining which conservation strategies should be implemented (Leitch *et al.*, 2009). The measurement of genetic diversity using molecular techniques, along with other methods, can facilitate the determination of priorities and help identify the most appropriate conservation management processes, as well as being the most cost effective strategy (Fay and Krauss, 2003).

1.8 Thesis structure

This PhD research comprises five chapters. Chapter two is a molecular phylogenetic study of the genus *Paphiopedilum*, using DNA sequencing techniques to obtain DNA sequence data from nuclear and plastid regions in order to address generic, subgeneric and sectional circumscription and to investigate the phylogenetic relationships within the genus. Chapter three is an investigation of evolutionary trends in genome size and chromosome number, by mapping chromosome number and genome size data for genus *Paphiopedilum* from the literature and some new genome size measurements from this study onto the phylogenetic framework from chapter two. Chapter two and chapter three were combined into a paper published under the title 'Molecular Phylogenetics of *Paphiopedilum* (Cypripedioideae; Orchidaceae) based on nuclear ribosomal ITS and plastid sequences' (Chochai *et al.*, 2012, Appendix). Chapter four concerns the development of plastid microsatellites for slipper orchids in the subfamily Cypripedioideae and the testing of those markers on a range of slipper orchids, with a focus on *Cypripedium calceolus*, to determine their cross applicability. Also, the utility of plastid microsatellites were assessed for a population genetics study of *Cypripedium calceolus* to examine levels of genetic variation. The last chapter is a general discussion, in which the results from the previous chapters were consolidated.

Chapter 2: Molecular phylogenetics of *Paphiopedilum* based on nuclear ribosomal ITS and plastid sequences

2.1 Introduction

The first comprehensive study of the molecular phylogenetics of subfamily Cypripedioideae was that of Cox *et al.* (1997), using nuclear ribosomal DNA internal transcribed spacer (ITS) sequence data. The circumscriptions of sections in *Paphiopedilum* were, in general, congruent with the previous infrageneric classification of Cribb (1987). However, the result did not support the division of the genus into two subgenera, *Brachypetalum* and *Paphiopedilum*, because subgenus *Brachypetalum* was found to be paraphyletic to subgenus *Paphiopedilum*. Section *Concoloria* [= section *Brachypetalum sensu* Cribb (1987)] of subgenus *Brachypetalum* was nested in a clade of subgenus *Paphiopedilum*. In addition, section *Coryopedilum* was weakly supported as being paraphyletic to the monophyletic section, *Pardalopetalum*. Cox *et al.* (1997) tentatively proposed elevating section *Parvisepalum* and section *Concoloria* of subgenus *Brachypetalum* to subgenera *Parvisepalum* and *Brachypetalum*, and suggested combining sections *Coryopedilum* and *Pardalopetalum* in their infrageneric treatment. Also, they suggested simplification of the subsectional treatment of Braem (1988), because groupings of only a few species are less useful in understanding the relationships among the groups. Although the ITS results of Cox *et al.* (1997) suggested that the infrageneric classification of Cribb (1987) was mainly well defined, it did not provide support for monophyly of the largest subgenus, *Paphiopedilum*. In addition, the phylogenetic relationships between sections in subgenus *Paphiopedilum* remained unclear, because the resulting tree did not have sufficient bootstrap support for those clades.

The infrageneric classification of Cribb (1998) in the second edition of his monograph, mainly based on morphological characters and chromosome data, also followed the molecular study of Cox *et al.* (1997). Cribb subdivided *Paphiopedilum* into three subgenera in his classification: *Parvisepalum*; *Brachypetalum*; and *Paphiopedilum*. Five sections of subgenus *Paphiopedilum* (*Coryopedilum*, *Pardalopetalum*, *Cochlopetalum*, *Paphiopedilum* and *Barbata*) remained, as in his previous treatment.

Averyanov *et al.* (2003) followed the outline of the infrageneric classification of Cribb

(1998), but they further divided subgenus *Parvisepalum* into two sections: *Parvisepalum* and *Emersonianum*. The new section *Emersonianum* was recognised to include *P. hangianum* and *P. emersonii*, which were differentiated mainly by these species having plain green leaves, whereas species of section *Parvisepalum* have tessellated leaves.

In a recently published paper (Guo *et al.*, 2012), six plastid DNA regions and two low-copy nuclear genes were used to study phylogenetics and biogeography in subfamily Cypripedioideae. As in earlier studies, *Paphiopedilum* was shown to be monophyletic, and was strongly supported as sister to *Phragmipedium*/*Mexipedium*. Sampling of *Paphiopedilum* spp., however, was rather sparse (eight species only) and the focus was on relationships between, rather than within, the genera.

The analysis of nuclear DNA regions alone, such as ITS, as in the study of Cox *et al.* (1997), may be inadequate for obtaining the necessary resolution of phylogenetic relationships at lower levels, although they may evolve rapidly (e.g. Álvarez and Wendel, 2003). Sequence data from other loci, such as plastid DNA, can be useful for investigating the relationships between closely related species. Although generally evolving relatively slowly, various regions of the plastid genome have undergone more rapid evolution, potentially providing more variation for studying closely related taxa (e.g. Shaw *et al.*, 2005; Shaw *et al.*, 2007). These data can also be utilised to test phylogenetic relationships independently and can be combined with data from other loci. Furthermore, unlike nuclear loci, plastid loci are uniparentally inherited (maternally in the case of slipper orchids, as for most flowering plants; Corriveau and Coleman, 1988), thus avoiding the potential problem of multiple copies, as found in the nuclear genome.

In this study, five DNA regions from nuclear and plastid genomes were selected for phylogenetic reconstruction. ITS region, comprising the internal transcribed spacers, ITS1 and ITS2 that evolve rapidly and the conserved region, ribosomal 5.8S rRNA gene, is a popular region for use in phylogenetic studies, mostly at specific level, because it evolves more rapidly than plastid DNA and although multiple copies are present in the plant genome, they have generally been homogenised by concerted evolution (Baldwin *et al.*, 1995; Álvarez and Wendel, 2003). *matK*, one of the most rapidly evolving protein coding genes in the plastid genome, coding for the enzyme maturase kinase which is involved in splicing type II introns from RNA transcript, is approximately 1500 base pairs and is located in the large single copy region in the

plastid genome (Wolfe, 1991; Wolfe *et al.*, 1992). *ycf1*, found in the plastid genome, is hypothetically a protein-coding region for chloroplast open reading frame 1 and has been found to be more variable than *matK* but less variable than ITS in Orchidaceae (Neubig *et al.*, 2009). Noncoding plastid regions have been useful for phylogenetic studies, mostly at lower levels, because they evolve faster than coding regions, due to the fact that they are thought to be under less functional constraint (Gielly and Taberlet, 1994). A new set of universal primers for sequencing noncoding regions located in the large single copy region of the plastid genome were developed by Ebert and Peakall (2009) and these can be utilised for phylogenetic studies and to help in searching for plastid microsatellites for use in population genetic studies. Two pairs of primers were selected to generate *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ* regions for this study.

2.1.1 Aims of this study

The aims of this study were to collect DNA sequence data from nuclear (ITS) and plastid [partial *matK*, *ycf1*, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*] loci to address generic, subgeneric and sectional circumscription and to investigate phylogenetic relationships within the genus.

2.2 Materials and methods

2.2.1 Plant materials

Most DNA samples were obtained from the DNA Bank at the Jodrell Laboratory (RBG Kew). In addition, some leaf material was obtained for DNA extraction from the living plant collection at the Tropical Nursery (RBG Kew). Because samples for two species, *P. hangianum* and *P. emersonii*, of subgenus *Parvisepalum* section *Emersonianum* in the treatment of Averyanov *et al.* (2003), were not available, the question of the monophyly of this group was not addressed. The taxon sampling used in this study was based on the infrageneric treatment of Cribb (1998) for sampling subgenera *Parvisepalum*, *Brachypetalum* and *Paphiopedilum* (sections *Coryopedilum, Pardalopetalum*, *Cochlopetalum*, *Paphiopedilum* and *Barbata*). The morphological terms used also follow Cribb (1998). Outgroup taxa were sampled from *Phragmipedium*, the sister genus of *Paphiopedilum* Cox *et al.* (1997). All species of *Paphiopedilum* and the outgroups used in this study, with voucher information, are listed in Table 2.1.

Table 2.1. Materials used for molecular phylogenetics in this study.

Taxa	Voucher/source	GenBank accession numbers				
		ITS	***matK***	***ycf1***	***psaA-ycf3ex3***	***trnF*(GAA)-*ndhJ***
Subgenus *Parvisepalum*						
Paphiopedilum delenatii Guillaumin	Chochai 39746 (K)	JQ929314	JQ929368	JQ929521	JQ929419	JQ929470
Paphiopedilum malipoense S.C.Chen & Z.H.Tsi	Z6	JQ929336	JQ929388	JQ929541	JQ929439	JQ929490
Paphiopedilum micranthum Tang & F.T.Wang	M.W. Chase O-629 (K)	JQ929338	JQ929390	JQ929543	JQ929441	JQ929492
Subgenus *Brachypetalum*						
Paphiopedilum concolor (Bateman) Pfitzer (a)	Z17	JQ929312	JQ929367	JQ929520	JQ929418	JQ929469
Paphiopedilum concolor (Bateman) Pfitzer (b)	Yang Ping, Guizhem. Luo s.n.	JQ929313	–	–	–	–
Paphiopedilum niveum (Rchb.f.) Stein	36862*, Kew 1990-996** (no voucher)	JQ929339	JQ929391	JQ929544	JQ929442	JQ929493
Subgenus *Paphiopedilum*						
Section *Paphiopedilum*						
Paphiopedilum hirsutissimum (Lindl. ex Hook.) Stein	Chochai 36808 (K)	JQ929327	–	–	–	–
Paphiopedilum hirsutissimum (Lindl. ex Hook.) Stein var. *esquirolei* (Schltr.) K.Karas. & K.Saito	M.W. Chase O-642 (K)	JQ929328	–	–	–	–
Paphiopedilum charlesworthii (Rolfe) Pfitzer	M.W. Chase O-632 (K)	JQ929310	JQ929365	JQ929518	JQ929416	JQ929467
Paphiopedilum insigne (Wall. ex Lindl.) Pfitzer	Chochai 36821 (K)	JQ929329	JQ929381	JQ929534	JQ929432	JQ929483
Paphiopedilum exul (Ridl.) Rolfe	36804*, Kew 1977-2853** (no voucher)	JQ929317	JQ929371	JQ929524	JQ929422	JQ929473
Paphiopedilum gratrixianum (Mast.) Rolfe (a)	Chochai 36809 (K)	JQ929322	JQ929376	JQ929529	JQ929427	JQ929478
Paphiopedilum gratrixianum (Mast.) Rolfe (b)	Chochai 40235 (K)	JQ929323	JQ929377	JQ929530	JQ929428	JQ929479
Paphiopedilum gratrixianum (Mast.) Rolfe (c)	Chochai 40236 (K)	JQ929324	JQ929378	JQ929531	JQ929429	JQ929480
Paphiopedilum villosum (Lindl.) Stein var. *boxallii* (Rchb.f.) Pfitzer	Chochai 36822 (K)	JQ929354	JQ929405	JQ929558	JQ929456	JQ929507
Paphiopedilum tigrinum Koop. & N.Haseg.	ex Paul Phillips-Rathcliffe	JQ929351	–	–	–	–
Paphiopedilum druryi (Bedd.) Stein	Chochai 36811 (K)	JQ929316	JQ929370	JQ929523	JQ929421	JQ929472
Paphiopedilum spicerianum (Rchb.f.) Pfitzer	M.W. Chase O-643 (K)	JQ929347	JQ929399	JQ929552	JQ929450	JQ929501

Table 2.1. Continued

Taxa	Voucher/source	GenBank accession numbers				
		ITS	***matK***	***ycf1***	***psaA-ycf3ex3***	***trnF*(GAA)-*ndhJ***
Section *Barbata*						
Paphiopedilum appletonianum (Gower) Rolfe	M.W. Chase 5897 (K)	JQ929306	JQ929362	JQ929515	JQ929413	JQ929464
Paphiopedilum sangii Braem	O-822* (no voucher)	JQ929346	JQ929398	JQ929551	JQ929449	JQ929500
Paphiopedilum mastersianum (Rchb.f.) Stein	M.W. Chase 5900 (K)	JQ929337	JQ929389	JQ929542	JQ929440	JQ929491
Paphiopedilum violascens Schltr.	O-825* (no voucher)	JQ929355	JQ929406	JQ929559	JQ929457	JQ929508
Paphiopedilum tonsum (Rchb.f.) Stein	M.W. Chase 5902 (K)	JQ929352	JQ929403	JQ929556	JQ929454	JQ929505
Paphiopedilum barbatum (Lindl.) Pfitzer	M.W. Chase 5898 (K)	JQ929307	JQ929363	JQ929516	JQ929414	JQ929465
Paphiopedilum callosum (Rchb.f.) Stein	Z4	JQ929308	JQ929364	JQ929517	JQ929415	JQ929466
Paphiopedilum callosum (Rchb.f.) Stein var. *sublaeve* (Rchb.f.) P.J.Cribb	Z32	JQ929309	–	–	–	–
Paphiopedilum hennisianum (M.W.Wood) Fowlie	Z30	JQ929326	JQ929380	JQ929533	JQ929431	JQ929482
Paphiopedilum fowliei Birk	M.W. Chase O-644 (K)	JQ929318	JQ929372	JQ929525	JQ929423	JQ929474
Paphiopedilum javanicum (Reinw. ex Lindl.) Pfitzer var. *virens* (Rchb.f) Stein	M.W. Chase O-635 (K)	JQ929330	JQ929382	JQ929535	JQ929433	JQ929484
Paphiopedilum lawrenceanum (Rchb.f.) Pfitzer	Chochai 36824 (K)	JQ929332	JQ929384	JQ929537	JQ929435	JQ929486
Paphiopedilum ciliolare (Rchb.f.) Stein	Z25	JQ929311	JQ929366	JQ929519	JQ929417	JQ929468
Paphiopedilum superbiens (Rchb.f.) Stein var. *curtisii* Braem	Z5	JQ929350	JQ929402	JQ929555	JQ929453	JQ929504
Paphiopedilum sukhakulii Schoser & Senghas	M.W. Chase 5901 (K)	JQ929349	JQ929401	JQ929554	JQ929452	JQ929503
Paphiopedilum wardii Summerh.	M.W. Chase 5903 (K)	JQ929356	JQ929407	JQ929560	JQ929458	JQ929509
Section *Pardalopetalum*						
Paphiopedilum dianthum Tang & F.T.Wang	Z23	JQ929315	JQ929369	JQ929522	JQ929420	JQ929471
Paphiopedilum parishii (Rchb.f.) Stein	Z3	JQ929340	JQ929392	JQ929545	JQ929443	JQ929494
Paphiopedilum lowii (Lindl.) Stein (a)	Z22	JQ929334	JQ929386	JQ929539	JQ929437	JQ929488
Paphiopedilum lowii (Lindl.) Stein (b)	Chochai 36810 (K)	JQ929335	JQ929387	JQ929540	JQ929438	JQ929489
Paphiopedilum haynaldianum (Rchb.f.) Stein	M.W. Chase O-175 (K)	JQ929325	JQ929379	JQ929532	JQ929430	JQ929481

Table 2.1. Continued

Taxa	Voucher/source	GenBank accession numbers				
		ITS	***matK***	***ycf1***	***psaA-ycf3ex3***	***trnF*(GAA)-*ndhJ***
Section *Cochlopetalum*						
Paphiopedilum glaucophyllum J.J.Sm.	Z21	JQ929321	JQ929375	JQ929528	JQ929426	JQ929477
Paphiopedilum liemianum (Fowlie) K.Karas. & K.Saito	36858*, Kew 1990-8000** (no voucher)	JQ929333	JQ929385	JQ929538	JQ929436	JQ929487
Paphiopedilum primulinum M.W.Wood & P.Taylor	Chochai 36827 (K)	JQ929342	JQ929394	JQ929547	JQ929445	JQ929496
Paphiopedilum primulinum M.W.Wood & P.Taylor var. *purpurascens* (M.W.Wood) P.J.Cribb	36860*, Kew 2001-3172** (no voucher)	JQ929343	JQ929395	JQ929548	JQ929446	JQ929497
Paphiopedilum victoria-regina (Sander) M.W.Wood	M.W. Chase O-630 (K)	JQ929353	JQ929404	JQ929557	JQ929455	JQ929506
Section *Coryopedilum*						
Paphiopedilum philippinense (Rchb.f.) Stein	Chochai 36807 (K)	JQ929341	JQ929393	JQ929546	JQ929444	JQ929495
Paphiopedilum randsii Fowlie	M.W. Chase O-636 (K)	JQ929344	JQ929396	JQ929549	JQ929447	JQ929498
Paphiopedilum kolopakingii Fowlie	Z18	JQ929331	JQ929383	JQ929536	JQ929434	JQ929485
Paphiopedilum stonei (Hook.) Stein	Z7	JQ929348	JQ929400	JQ929553	JQ929451	JQ929502
Paphiopedilum adductum Asher	36820*, Kew 1992-3661** (no voucher)	JQ929305	JQ929361	JQ929514	JQ929412	JQ929463
Paphiopedilum glanduliferum (Blume) Stein (a)	M.W. Chase O-716 (K)	JQ929319	JQ929373	JQ929526	JQ929424	JQ929475
Paphiopedilum glanduliferum (Blume) Stein (b)	M.W. Chase O-717 (K)	JQ929320	JQ929374	JQ929527	JQ929425	JQ929476
Paphiopedilum wilhelminiae L.O.Williams	36825*, Kew 2005-2702** (no voucher)	JQ929357	JQ929408	JQ929561	JQ929459	JQ929510
Paphiopedilum rothschildianum (Rchb.f.) Stein	Chochai 36806 (K)	JQ929345	JQ929397	JQ929550	JQ929448	JQ929499
Outgroup						
Phragmipedium besseae Dodson & J.Kuhn	Z16a	JQ929358	JQ929409	JQ929562	JQ929460	JQ929511
Phragmipedium schlimii (Linden ex Rchb.f.) Rolfe	M.W. Chase O-183 (VA)	JQ929360	JQ929411	JQ929564	JQ929462	JQ929513
Phragmipedium longifolium (Warsz. & Rchb.f.) Rolfe	Z9	JQ929359	JQ929410	JQ929563	JQ929461	JQ929512

*Kew DNA bank number, **Kew living collection number.

2.2.2 DNA extraction

For additional DNA samples, genomic DNA was extracted from fresh plant material, following the modified 2 × cetyl trimethylammonium bromide (CTAB) method of (Doyle and Doyle, 1987). DNA samples were purified by either caesium chloride/ethidium bromide density gradients or DNA purification columns (NucleoSpin Extract II Columns; Macherey-Nagel, GmbH & Co. KG, Germany) according to the manufacturer's protocols.

2.2.3 Amplification

For the nuclear ribosomal spacers ITS1 and ITS2 and the 5.8S ribosomal gene, primers 17SE (ACG AAT TCA TGG TCC GGT GAA GTG TTC G) and 26SE (TAG AAT TCC CCG GTT CGC TCG CCG TTA C) of Sun *et al.* (1994) and ITS4 (TCC TCC GCT TAT TGA TAT GC) and ITS5 (GGA AGT AAA AGT CGT AAC AAG G) of White *et al.* (1990) were used for amplification and sequencing. Amplification of ITS region was carried out in 25 µl volumes containing 22.5 µl ReddyMix PCR Mastermix (1.5mM $MgCl_2$, ABGene, Epsom, Surrey, UK), 0.5 µl bovine serum albumin (0.04%), 0.5 µl each of forward and reverse primers (100 ng/ µl) and template DNA approximately 50–100 ng. The PCR profile was as follows: 2 minutes of initial denaturation at 94^{o}C, followed by 30 cycles of 94^{o}C denaturation for 1 minute, 48^{o}C annealing for 1 minute and 72^{o}C extension for 1.5 minutes and final elongation 72^{o}C for 4 minutes.

Partial *matK*, approximately 800 bp in length, was amplified using primers 390F (CGA TCT ATT CAT TCA ATA TTTC) and 1326R (TCT AGC ACA CGA AAG TCG AAGT) of Sun *et al.* (2001). Amplification was carried out in 40 µl volumes containing 8 µl 5x buffer, 2.4 µl $MgCl_2$, 4 µl bovine serum albumin (0.04%), 2 µl each of forward and reverse primers (100 ng/ µl), 0.8 µl dNTPs, 1.6 µl DMSO (dimethyl sulphoxide 4%) and template DNA approximately 50–100 ng, then the volume was adjusted by adding sterile, deionised water. An alternative PCR condition was used with 25 µl volumes containing 22.5 µl ReddyMix PCR Mastermix (2.5mM $MgCl_2$, ABGene, Epsom, Surrey, UK) 0.5 µl bovine serum albumin (0.04%), 0.5 µl of each primer (100 ng/ µl) and template DNA approximately 50–100 ng. The PCR programme consisted of 3 minutes of initial denaturation at 94^{o}C, followed by 26 cycles of 94^{o}C denaturation for 30 seconds, 51^{o}C annealing for 40 seconds and 72^{o}C extension for 1 minute and final elongation 72^{o}C for 7 minutes.

A portion, approximately 1500 bp, from the 3′ end of *ycf1* was amplified using primers 3720F (TAC GTA TGT AAT GAA CGA ATG G) and 5500R (GCT GTT ATT GGC ATC AAA CCA ATA GCG) and two additional internal primers; IntF (GAT CTG GAC CAA TGC ACA TAT T) and IntR (TTT GAT TGG GAT GAT CCA AGG) were also used for sequencing (Neubig *et al.*, 2009). The PCR profile for *ycf1* region, using a 'touchdown', consisted of 3 minutes of initial denaturation at 94^{o}C, followed by 8 cycles of 94^{o}C denaturation for 30 seconds, 60-51^{o}C annealing (reducing 1^{o}C per cycle) for 1 minute and 72^{o}C extension for 3 minutes, followed by 30 cycles of 94^{o}C denaturation for 30 seconds, 50^{o}C annealing for 1 minute and 72^{o}C extension for 3 minutes and final elongation 72^{o}C for 3 minutes (Neubig *et al.*, 2009).

The non-coding plastid regions, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*, were amplified using the primer pairs: ANU_cp051-L (GTT CCG GCG AAC GAA TAAT) and ANU_cp052-R (GTC GGA TCA AGC TGC TGAG), ANU_cp061-L (CCT CGT GTC ACC AGT TCA AA) and ANU_cp062-R (TGG ATA GGC TGG CCC TTAC) of Ebert and Peakall (2009), respectively. Amplification was carried out in 25 µl volumes containing 22.5 µl ReddyMix PCR Mastermix (2.5mM $MgCl_2$, ABGene, Epsom, Surrey, UK) 0.5 µl bovine serum albumin (0.04%), 0.5 µl of each primer (100 ng/ µl) and template DNA approximately 50–100 ng. The PCR profile for both non-coding regions consisted of 3 minutes of initial denaturation at 94^{o}C, followed by 28 cycles of 94^{o}C denaturation for 1 minute, 48^{o}C annealing for 1 minute and 72^{o}C extension for 1 minute followed by final elongation 72^{o}C for 7 minutes.

All amplified PCR products were visualised by 1% agrose gel electrophoresis, then purified using NucleoSpin Extract II columns according to the manufacturer's protocols. The cycle sequence reactions were carried out in 10 µl volumes containing 1.75 µl of 5× sequence buffer, 0.5 µl of Big Dye Terminator kit (Applied Biosystems, Inc., Warrington, Cheshire, UK), 0.75 µl of each primer (10 ng/ µl) and approximately 50 ng/ µl cleaned PCR products, then the volume was adjusted by adding sterile, deionised water. The cycle sequencing profile was composed of 26 cycles of 96^{o}C denaturation for 10 seconds, 50^{o}C annealing for 5 seconds and 60^{o}C extension for 4 minutes. The cycle sequencing products were cleaned by ethanol precipitation and then run on an ABI 3730 automated sequencer. Raw sequences were edited and assembled using Sequencher 4.1 software (Gene Codes Inc., Ann Arbor, MI, USA). The resulting sequences were then aligned manually. All sequences were deposited in GenBank.

2.2.4 Parsimony analysis

Sequence data were analysed independently and in combination, using the maximum parsimony criterion in PAUP* version 4.0b10 for Macintosh (Swofford, 2002). All characters were treated as unordered and equally weighted (Fitch, 1971). Parsimony analyses were conducted using a heuristic search strategy, with 1000 replicates of random taxon addition, tree–bisection–reconnection (TBR) branch swapping with MulTrees in effect, gaps treated as missing data and saving no more than ten trees per replicate. Support for groups was evaluated using 1000 replicates of bootstrap (Felsenstein, 1985), with simple addition and TBR swapping, saving ten trees per replicate. Groups were retained when bootstrap percentages (BP) ≥ 50.

2.2.5 Bayesian analysis

The best-fit models for nucleotide substitution for the data matrix of each region were determined by the Akaike information criterion test (Akaike, 1974) as implemented in MrModeltest version 2.2 (Nylander, 2004). The general time reversible model of substitution with gamma distribution (GTR + G) was selected for ITS, partial *matK* and *psaA-ycf3ex3* data and the general time reversible model of substitution with gamma distribution and invariable sites (GTR + I + G) was selected for *ycf1* and *trnF*(GAA)-*ndhJ* data. All analyses were carried out using the parallel version of MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) through the University of Oslo Bioportal (http://www.bioportal.uio.no). Two runs of four Monte Carlo Markov chains (MCMC; Yang and Rannala, 1997) were performed for 10 000 000 generations and a tree was sampled every 1000 generations. Each parameter estimation obtained from the results of two runs was checked in Tracer version 1.5 (http://tree.bio.ed.ac.uk/software/tracer) to ascertain whether they had obtained proper effective sample size and to verify that stationary state had been reached. Trees from the first 10% of generations were discarded as burn-in. The remaining trees were combined to build a 50% majority-rule consensus tree in PAUP* version 4.0b10.

2.3 Results

2.3.1 Alignment of data sets

The ITS data matrix of 56 taxa, three of which were the outgroup, comprised 778 characters, of which 196 were potentially parsimony informative (25.2%). Analysis of ITS sequences yielded 35 equally most-parsimonious trees of 425 steps, consistency

index (CI) = 0.82, retention index (RI) = 0.90. One of the most-parsimonious trees was chosen randomly. Tree topology, bootstrap percentages (BP), branches that collapse in the strict consensus tree obtained from maximum parsimony analysis and Bayesian posterior probability values (PP) are indicated in Figure 2.1. In the ITS tree, the genus *Paphiopedilum* is monophyletic, with strong support (100 BP, 1.00 PP). Subgenus *Parvisepalum* is the first branching clade with 88 BP and 1.00 PP support for monophyly. The support for monophyly of subgenus *Brachypetalum* was 99 BP and 1.00 PP. Subgenus *Paphiopedilum* forms a polytomy with subgenus *Brachypetalum* (60 BP, – PP). Sections *Barbata*, *Pardalopetalum* and *Cochlopetalum* were well supported with 97 BP, 1.00 PP, 100 BP, 1.00 PP and 98 BP, 0.98 PP, respectively. Section *Paphiopedilum* had moderate bootstrap support (75 BP) but high PP values (0.98). There was no support for section *Coryopedilum*, and it did not form a clade in the strict consensus tree. In subgenus *Paphiopedilum*, the relationships within some sections were still not well supported.

The plastid data matrix [partial *matK*, *ycf1*, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*], including 51 taxa (it was not possible to obtain sequences for five taxa that were included in the ITS matrix), three of which were the outgroup, comprised 4353 characters, of which 281 were potentially parsimony informative (6.5%). Analysis of a combined plastid region matrix yielded 20 equally most-parsimonious trees of 520 steps, CI = 0.84, RI = 0.92. One of the most-parsimonious trees was randomly chosen, and the tree topology, bootstrap percentages, branches that collapse in the strict consensus tree obtained from maximum parsimony analysis and Bayesian posterior probability values are indicated in Figure 2.2. The tree of the combined plastid regions was more resolved than the ITS tree. The genus *Paphiopedilum* is monophyletic, with strong support (100 BP, 1.00 PP). The division of the genus into three subgenera is also well supported (100 BP, 1.00 PP for all). Support for the monophyly of *Paphiopedilum* subgenera *Parvisepalum*, *Brachypetalum* and *Paphiopedilum* is 100 BP, 1.00 PP, 95 BP, 1.00 PP and 100 BP, 1.00 PP, respectively. In subgenus *Paphiopedilum*, sections *Barbata*, *Paphiopedilum* and *Pardalopetalum* are well supported with 93 BP, 1.00 PP, 98 BP, 1.00 PP and 100 BP, 1.00 PP, respectively. Section *Coryopedilum* has weak bootstrap support (67 BP) but high PP support (1.00). Section *Cochlopetalum* forms two clades in a polytomy, with the clade formed by sections *Coryopedilum* and *Pardalopetalum*. In subgenus *Paphiopedilum*, the relationships within some sections are still not well supported.

The combined data matrix included 51 taxa (but excluded those for which only ITS data

was available), of which three were outgroups and comprised 4884 characters, of which 463 were potentially parsimony informative (9.5%). Analysis of the combined data matrix yielded 120 equally most-parsimonious trees of 920 steps, CI = 0.83, RI = 0.91. One of the most-parsimonious trees was randomly chosen. Tree topology, bootstrap percentages, branches that collapse in the strict consensus tree obtained from maximum parsimony analysis and Bayesian posterior probability values are indicated in Figure 2.3. The genus *Paphiopedilum* is monophyletic, with strong support (100 BP, 1.00 PP). The division of the genus into three subgenera is well supported (100 BP, 1.00 PP for all). The monophyly of *Paphiopedilum* subgenera *Parvisepalum*, *Brachypetalum* and *Paphiopedilum* is well supported, with BP 100, 1.00 PP for each node. In subgenus *Paphiopedilum*, sections *Barbata*, *Paphiopedilum*, *Pardalopetalum* and *Cochlopetalum* have strong support with 100 BP, 1.00 PP, 99 BP, 1.00 PP, 100 BP, 1.00 PP and 99 BP, 1.00, respectively. Only section *Coryopedilum* has weak bootstrap support (54 BP) and it collapses to form a polytomy with section *Pardalopetalum* in the strict consensus; however, it has a high PP value (0.95). In subgenus *Paphiopedilum*, the relationships within some sections are still not well supported.

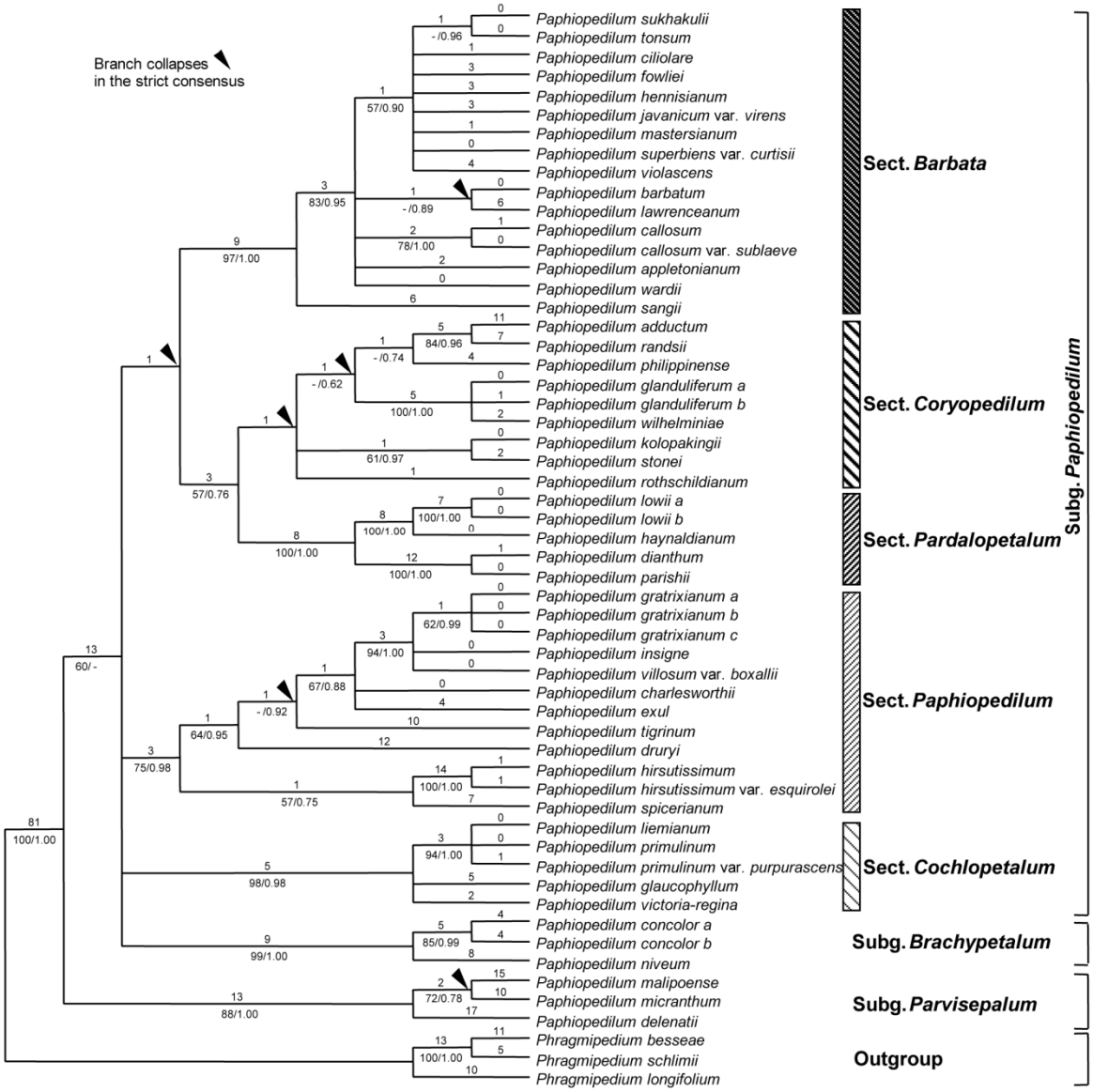

Figure 2.1. One of 35 most-parsimonious trees from the analysis of the internal transcribed spacer (ITS) region for *Paphiopedilum.* Tree length = 425, consistency index = 0.82, retention index = 0.90. Numbers above branches are branch lengths and numbers below branches are bootstrap percentages ≥ 50 and posterior probability values ≥ 0.50. Arrows indicate clades that collapse in the strict consensus tree obtained from maximum parsimony analysis. The infrageneric treatment follows Cribb (1998).

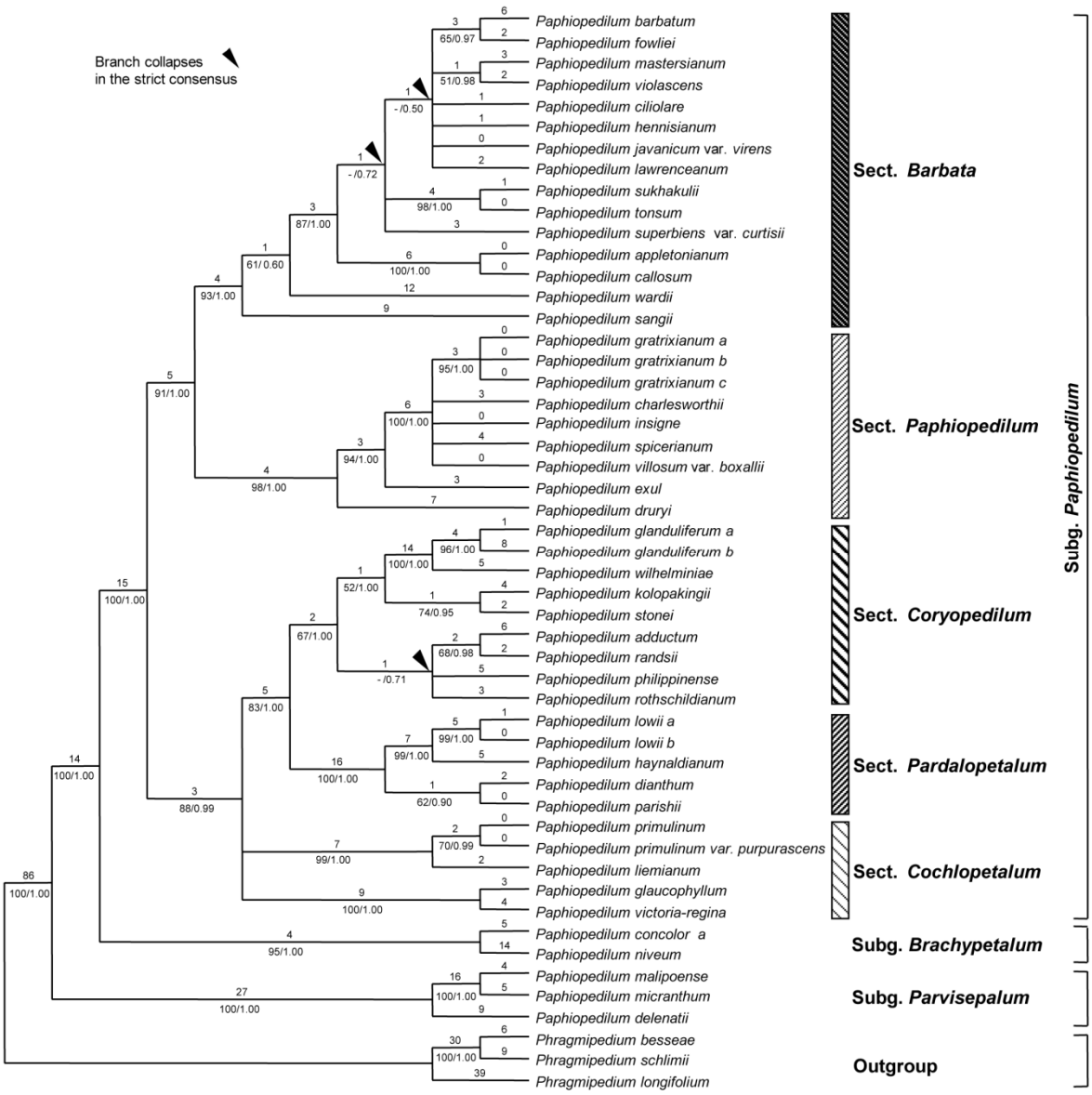

Figure 2.2. One of 20 most-parsimonious trees from the analysis of plastid [partial *matK*, *ycf1*, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*] regions for *Paphiopedilum*. Tree length = 520, consistency index = 0.84, retention index = 0.92. Numbers above branches are branch lengths and numbers below branches are bootstrap percentages ≥ 50 and posterior probability values ≥ 0.50. Arrows indicate clades that collapse in the strict consensus tree obtained from maximum parsimony analysis. The infrageneric treatment follows Cribb (1998).

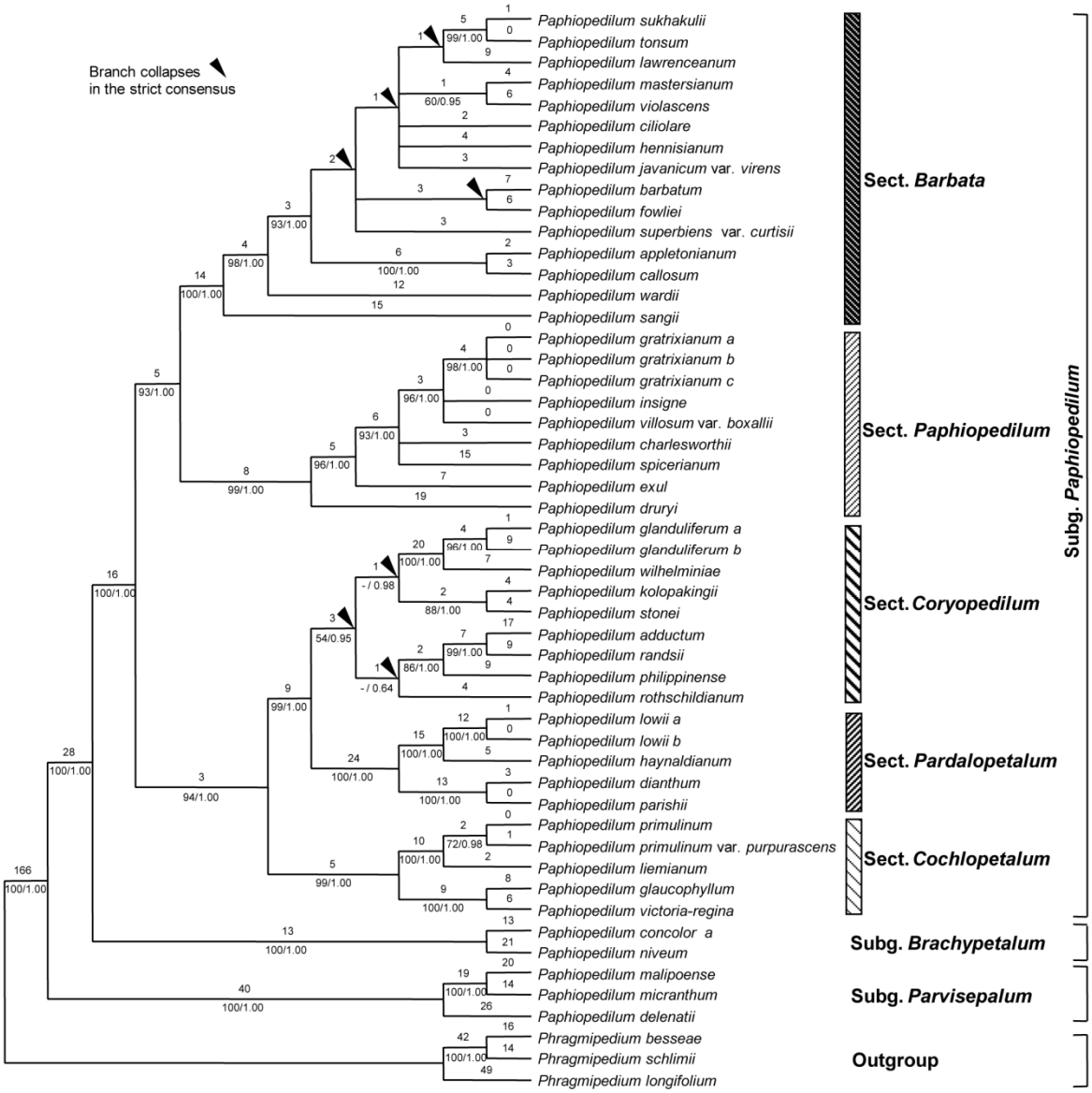

Figure 2.3. One of 120 most-parsimonious trees from the combined analysis of internal transcribed spacer (ITS) and plastid [partial *matK*, *ycf1*, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*] regions for *Paphiopedilum.* Tree length = 920, consistency index = 0.83, retention index = 0.91. Numbers above branches are branch lengths and numbers below branches are bootstrap percentages ≥ 50 and posterior probability values ≥ 0.50. Arrows indicate clades that collapse in the strict consensus tree obtained from maximum parsimony analysis. The infrageneric treatment follows Cribb (1998).

2.4 Discussion

2.4.1 Congruence of ITS and plastid data

The results from two separate matrices of ITS and plastid data showed no conflict between strongly supported branches (> 75 BP, > 0.90 PP) when compared node by node. Groupings within the genus in both ITS and plastid trees are generally as described in the treatment of Cribb (1998), but the relationships along the backbone are less resolved in the ITS tree. The results in the plastid trees had better bootstrap support, but the resulting trees from separate analyses of each individual plastid region [partial *matK*, *ycf1*, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*] lacked resolution because of low levels of divergence (data not shown). The combined data set produced more resolved trees, mostly with strong bootstrap support. In general, the increase in clade support in the combined tree (Figure 2.3) indicates congruence between the ITS and plastid data. The only place where there was lower clade support when the plastid and nuclear data sets were combined was in section *Coryopedilum,* suggesting some possible conflict between data sets in this part of the phylogenetic tree. However, the branches concerned receive only low bootstrap support.

2.4.2 Phylogenetic relationships in the genus *Paphiopedilum*

Overall, the results from all analyses showed general congruence with the previous infrageneric treatment of Cribb (1998), and confirm that *Paphiopedilum* is monophyletic, which is congruent with the results of previous studies (Albert, 1994; Cox *et al.*, 1997).

2.4.2.1 Subgenus *Parvisepalum*

Subgenus *Parvisepalum*, characterised by tessellated leaves [except two species, *P. hangianum* and *P. emersonii*, which have plain green leaves; Averyanov *et al.* (2003)], a single-flowered inflorescence, a flower with an inflated lip and a convex (mostly) or conduplicate staminode (Cribb, 1998) (Figure 2.4), was found to be the first branching clade with strong support in this study (Figures 2.2, 2.3). This confirms the results of Cox *et al.* (1997) and the suggestion of Chen and Tsi (1984) that *P. malipoense* and its closely related species are the 'basal group' (i.e. early diverging) of the genus. Chen and Tsi (1984) suggested that *Paphiopedilum* and *Cypripedium* were related via this species (subgenus *Parvisepalum*) by considering the similarity of the flower characters.

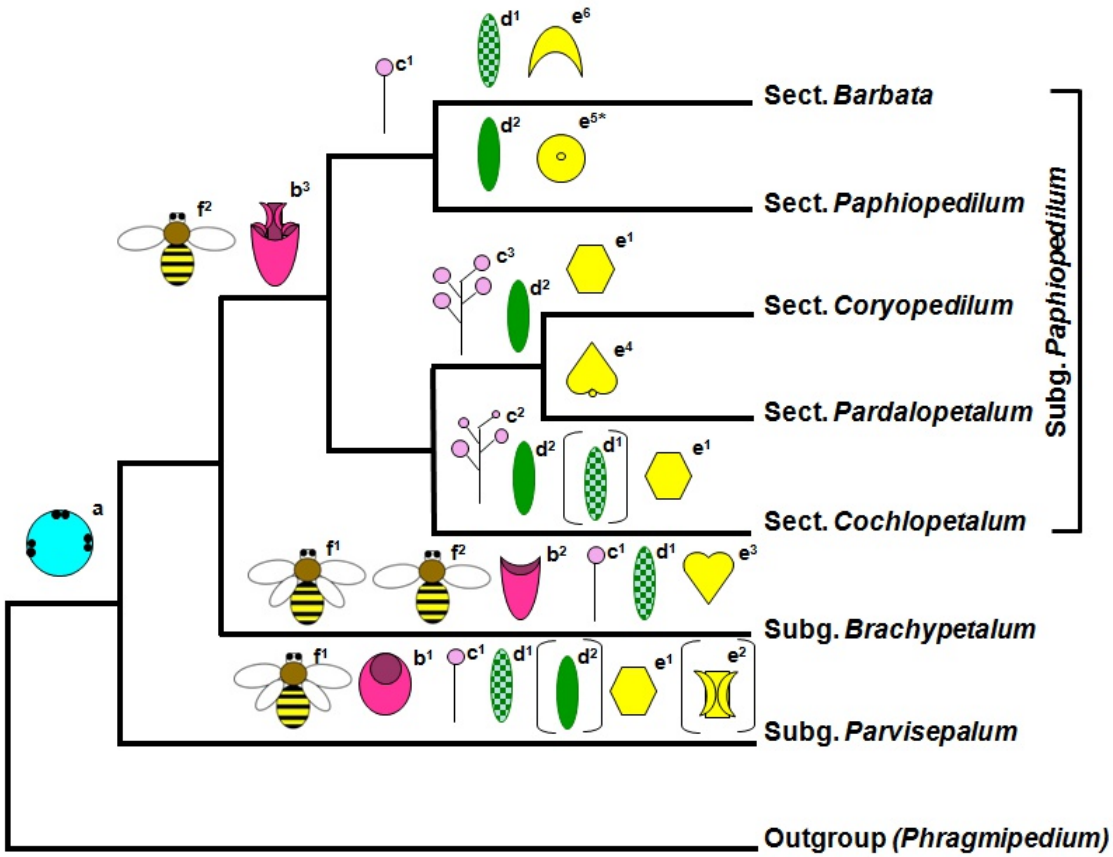

Figure 2.4. Morphological characters and pollinators mapped onto a phylogenetic framework from combined DNA sequence data. a, unilocular ovary with parietal placentation; b^1, inflated lip; b^2, ovoid shaped lip; b^3, lip with only incurved side lobes; c^1, (mostly) single-flowered inflorescence; c^2, multi-flowered with successively opening; c^3, multi-flowered with simultaneously opening; d^1, tessellated leaves; d^2, plain green leaves; e^1, convex staminode; e^2, conduplicate staminode; e^3, staminode with uni- or tridentate apex; e^4, obcordate staminode with basal protuberance; e^5, staminode with an umbo (* indicates more shape variations in the section); e^6, (mostly) lunate shape staminode); f^1, bee pollinator; f^2, hoverfly pollinator.

However, Cribb (1987) stated that the similarities between the flowers of *Paphiopedilum* and the other genera, for example *P. armeniacum* and *C. irapeanum* La Llave & Lex. or *P. delenatii* and *Phragmipedium schlimii* (Linden ex Rchb.f.) Rolfe, are the result of similar pollination syndromes, with bees as pollinators. Research has shown that most species are pollinated by hoverflies (Atwood, 1985; Bänziger, 1994; 1996; 2002; Bänziger *et al.*, 2012; Shi *et al.*, 2007; 2009). However, *P. micranthum* (subgenus *Parvisepalum*) was observed to be pollinated by bees (Bänziger *et al.*, 2008) and it has recently been reported that bees are the pollinators for two species in subgenus *Brachypetalum* (Bänziger *et al.*, 2012) (Figure 2.4). The results from the studies of Albert (1994) and Cox *et al.* (1997) pointed to *Paphiopedilum* differing extensively from both *Cypripedium* and *Phragmipedium*, not only in morphological characters but also in molecular characters. In this study, the results from the combined data of five DNA regions also showed that there are high levels of molecular divergence between *Paphiopedilum* and *Phragmipedium*.

2.4.2.2 Subgenus *Brachypetalum*

Subgenus *Brachypetalum*, characterised by tessellated leaves, one- or two- (rarely three-) flowered inflorescences, flowers white or yellow in colour, an involute margined ovoid shaped lip and a staminode that is uni- or tridentate at its apex (Cribb, 1998) (Figure 2.4), is a monophyletic group, with high support values from both BP and PP in all analyses. From plastid and combined data (Figures 2.2, 2.3), subgenus *Brachypetalum* is strongly supported as sister to subgenus *Paphiopedilum*. This result supports the recognition of subgenus *Parvisepalum* by Karasawa and Saito (1982), which was found to differ morphologically from the remaining species in subgenus *Brachypetalum*, and the elevation of section *Parvisepalum sensu* Cribb (1987) to subgeneric level in the second edition of his monograph by Cribb (1998), a change suggested by the ITS result of Cox *et al.* (1997). Although both *Parvisepalum* (most species) and *Brachypetalum* have tessellated leaves and a sporophytic chromosome number of 26, their flowers are clearly different (Figures 2.4, Table 3.2). Approximately seven species of subgenus *Parvisepalum* are distributed mostly in southern China and Vietnam, whereas the four species of *Brachypetalum* have a wider distribution in mainland south-east Asia (Cribb, 1998).

2.4.2.3 Subgenus *Paphiopedilum*

There is conflict between the classical infrageneric classifications concerning the division of subgenus *Paphiopedilum* into several sections or several subgenera in the most recent monographs of the genus. In the monographs of Braem (Braem, 1988; Braem *et al.*, 1998; Braem and Chiron, 2003), following the work of Karasawa and Saito (1982), subgenus *Paphiopedilum sensu* Cribb is divided into four subgenera (*Paphiopedilum*, *Sigmatopetalum*, *Polyantha* and *Cochlopetalum*). This disagrees with the treatment of Cribb in his monographs (Cribb, 1987; Cribb, 1998), in which he placed plants with different leaf colour (plain green vs. tessellated), number of flowers in the inflorescences [one, or rarely two or three, flowers vs. multiple flowers], number of chromosomes (constant 2*n* = 26 vs. variable) and pattern of blooming (simultaneous vs. successive), in one subgenus (Braem and Chiron, 2003). However, Cribb considered subgenus *Paphiopedilum* to be monophyletic, based on the cladistic study of Atwood (1984) and he treated other groups at sectional levels in this subgenus. Braem (in Braem and Chiron, 2003) also argued that the ITS tree from Cox *et al.* (1997) did not disagree with his subgeneric treatment. That is because there is no support for the robustness of the clade of subgenus *Paphiopedilum sensu* Cribb, as mentioned previously.

The results from this study show that subgenus *Paphiopedilum sensu* Cribb, which consists of species in which only the side lobes of the lip are incurved (Cribb, 1998) (Figure 2.4), is clearly monophyletic, with strong support from the plastid and combined data analyses (Figures 2.2, 2.3), and the subgenus is split into two main lineages. The first lineage includes three sections of multi-flowered species (*Coryopedilum*, *Pardalopetalum* and *Cochlopetalum*) and the second lineage includes two sections of mostly single-flowered species (*Paphiopedilum* and *Barbata*) (Figures 2.2–2.4). These are all sections as defined in the treatment of Cribb (1998). These lineages are different from the results of Cox *et al.* (1997), in which multi-flowered and (mostly) single-flowered sections are placed in the same clades. In the current study, multi-flowered inflorescences occur only in sections *Coryopedilum*, *Pardalopetalum* and *Cochlopetalum*, and thus this character appears to be a synapomorphy for this clade.

The tessellated leaf character found in the early diverging subgenera *Parvisepalum* (except two species) and *Brachypetalum*, is absent in most clades of subgenus *Paphiopedilum* (Figure 2.4). Reversions of this character are found in all species of section *Barbata* and in two species of section *Cochlopetalum* and it appears to occur

independently. Tessellated leaves are thought to play a role as camouflage as an anti-herbivory mechanism in understorey herbaceous plants growing in sun-flecked light conditions (Givnish, 1990), but there is no obvious evidence for the value of this adaptation *in Paphiopedilum*. Most species, including those with plain green and tessellated leaves, grow in similar, shady forest floor habitats, although a few plain green leaved species have been found in open sunny situations and some tessellated leaved species are found in deep shade (Cribb, 1998).

All sections in subgenus *Paphiopedilum* are strongly supported (both BP and PP) in the analyses of combined data, except section *Coryopedilum*, which has weak BP support (54 BP) for monophyly, collapsing in the strict consensus tree from parsimony analysis to form a polytomy with section *Pardalopetalum*. However, in the tree obtained from Bayesian analysis, *Coryopedilum* has 0.95 PP clade support (Figure 2.3). Previously, the results from ITS data of Cox *et al.* (1997) showed section *Coryopedilum* (no BP support, jackknife > 0.63 at some nodes) to be paraphyletic to a monophyletic section *Pardalopetalum sensu* Cribb (1987), and they tentatively proposed a combination of these sections. However, Cribb (1998), in the second edition of his monograph, did not accept these molecular results, because he noted that these sections are probably sister groups, based on morphological characters. The sections share plain green leaves, multi-flowered inflorescences that open simultaneously and a chromosome number of 2*n* = 26 (Figure 2.4, Table 3.2). Considering floral morphology, they can be clearly distinguished, with *Coryopedilum* having long tapering petals, a porrect lip and a convex staminode, whereas *Pardalopetalum* has distinctive dorsal petals that are reflexed at the base and an obcordate staminode with a basal protuberance and tridentate apex (Cribb, 1998). The *c*. 11 species of section *Coryopedilum* are found in the Malesian islands, and most are endemic to single islands. In contrast, section *Pardalopetalum* is more widespread, the four species being distributed through mainland south-east Asia, and the Malay Archipelago to Sulawesi and the Philippines (Cribb, 1998). In this study (Figures 2.1–2.3), these sections are sister groups, with 57 BP and 0.76 PP from ITS data, 83 BP and 1.00 PP from the plastid data and 99 BP and 1.00 PP from the combined data. There is no support for monophyly from the ITS data for *Coryopedilum*. Although bootstrap support from plastid data and combined data is low (67 BP and 54 BP respectively), support from Bayesian analysis is high, with 1.00 PP from plastid data and 0.95 PP from the combined data. However, *Coryopedilum* collapsed in the strict consensus trees of parsimony analyses of ITS data and combined data. In contrast, section *Pardalopetalum* has strong support, with 100 BP and 1.00 PP in all analyses. Results from this study therefore suggest that

section *Coryopedilum*, although clearly differing from section *Pardalopetalum* morphologically, shows insufficient levels of molecular divergence to support the monophyly of this section. Including more variable regions, such as low-copy nuclear regions, would possibly help in obtaining a clearer pattern. The low level of molecular divergence in *Coryopedilum* could possibly be explained by its selfing mode of reproduction, resulting from geitonogamy, and an absence of centric fission events (see Chapter 3). Species with multi-flowered inflorescences that open simultaneously, as found in sections *Coryopedilum* and *Pardalopetalum*, are more susceptible to geitonogamy or pollination among flowers on the same individual plant (Kliber and Eckert, 2004). This self-pollination by geitonogamy is thought to be disadvantageous, because it produces inbred offspring and requires pollinators to visit, as in outcrossing (Eckert, 2000). Although the floral features of orchids favour outcrossing, most orchids are self-compatible, which could facilitate reproduction in widely separated plants where outcrossing is not possible (Dressler, 1981). Because most species in section *Coryopedilum* are endemic to single Malesian islands (Cribb, 1998), they occur in small populations that are more likely to be geitonogamous than those of species in section *Pardalopetalum,* which are distributed more widely.

The *Cochlopetalum* clade is recovered in trees from ITS data (98 BP and 0.98 PP) and combined data (99 PP and 1.00 PP), but not in the plastid tree. In the combined tree, section *Cochlopetalum* is sister to a clade formed by sections *Coryopedilum* and *Pardalopetalum* (94 BP and 1.00 PP). Section *Cochlopetalum* is similar to its sister group in having multi-flowered inflorescences, but it differs in its flowers, which open successively, and in the variation in chromosome numbers ($2n$ = 30–37) (Figure 2.4, Table 3.2). In addition, linear, spirally twisted petals are a distinctive character for the section, including approximately five species that are endemic to Java and Sumatra (Cribb, 1998). These three sections, which share plain green leaves [except *P. victoria-regina* (Sander) M.W.Wood and *P. victoria-mariae* (Sander ex Mast.) Rolfe of section *Cochlopetalum*, which have faintly tessellated leaves; Cribb, 1998] and multi-flowered inflorescences, are together, sister to a clade consisting of sections *Paphiopedilum* plus *Barbata*, with strong support (100 BP and 1.00 PP from both plastid and combined data). The clade of sections *Paphiopedilum* and *Barbata* is characterised by single-flowered (rarely two-flowered) inflorescences (Figure 2.4). Both sections are monophyletic, with strong support: 98 BP and 1.00 PP from plastid data and 99 BP and 1.00 PP from combined data for *Paphiopedilum*; 93 BP and 1.00 PP from plastid data; and 100 BP and 1.00 PP from combined data for *Barbata* (Figures 2.2, 2.3). Section *Paphiopedilum* differs from section *Barbata* in having green leaves and chromosome

numbers in most species of 2*n* = 26, except in *P. druryi* and *P. spicerianum* (Rchb.f.) Pfitzer (2*n* = 30), whereas the tessellated-leaved section, *Barbata* shows considerable variation in chromosome number (2*n* = 28–42) (Figure 2.4, Table 3.2). Many species in section *Paphiopedilum* are characterised by a staminode with an umbo in the middle, whereas most species in section *Barbata* have a lunate staminode (Cribb, 1998) (Figure 2.4).

Phylogenetic relationships in section *Barbata* are unresolved, with many internal branches collapsing to a polytomy in the strict consensus tree for the parsimony analysis and 50% majority tree from Bayesian analyses (Figures 2.1–2.3). Atwood (1984) suggested that section *Barbata* was the most derived group, and this section was derived from section *Paphiopedilum,* based on his Wagner groundplan-divergence cladogram. However, that suggestion cannot be inferred from this current phylogenetic study, because it can only be inferred that both sections share a most recent common ancestor. The short branch lengths in section *Barbata* shown on the combined tree in this study and the narrow geographical distribution on Malesian islands of most species in this section might suggest a recent rapid radiation in the section (Cox *et al.*, 1997). Although numerous molecular characters from five DNA regions both from nuclear and plastid loci were included in this study, the relationships in this section remain unresolved. To obtain better resolution in this section, the use of more variable regions such as low-copy nuclear sequences could be helpful.

Chapter 3: Genome size and chromosome number evolution within the genus *Paphiopedilum*

3.1 Introduction

The term genome size has been used somewhat ambiguously and with various meanings. The term 'C-value', which was coined by Swift (1950), is often used to refer to genome size, in which 'C' stands for 'Constant' (Bennett and Smith, 1976). In this study, the term genome size refers to 1C-value, the meaning of which, as defined by Greilhuber *et al.* (2005) is 'DNA content of one non-replicated holoploid genome with the chromosome number *n*'.

Genome size in angiosperms varies *c*. 2400-fold, from that of the carnivorous plant *Genlisea margaretae* Hutch. (Lentibulariaceae), 1C-value of only 0.065 pg, to that of the monocot *Paris japonica* (Franch. & Sav.) Franch. (Melanthiaceae), the largest known genome of 1C = 152.23 pg (Greilhuber *et al.*, 2006; Pellicer *et al.*, 2010; Bennett and Leitch, 2011). Most angiosperms have a small genome size; based on an analysis of > 6000 species, the modal and median of 1C values are only 0.6 and 2.9 pg (Bennett and Leitch, 2010). Species with very large genome sizes [i.e. 1C ≥ 35 pg, Kelly and Leitch (2011)] are found mainly in monocots, including Orchidaceae. Among angiosperms, based on available data, Orchidaceae have the greatest variation in genome size, ranging 168-fold from 1C = 0.33 pg in *Oncidium maduroi* Dressler to 55.4 pg in *Pogonia ophioglossoides* (L.) Ker Gawl. (Leitch *et al.*, 2009).

Many species of subfamily Cypripedioideae have large genome sizes, ranging > 10-fold, from 1C = 4.1 pg in *Cypripedium molle* Lindl. to 43.1 pg in *C. fargesii* Franch. and *Cypripedium* is the most variable genus in the subfamily (Kahandawala, 2009; Leitch *et al.*, 2009). *Paphiopedilum* spp. also have large genome sizes, ranging nearly two-fold, from 1C = 17.80 pg in *P. godefroyae* (God.-Leb.) Stein, to 34.53 pg in *P. wardii* Summerh., whereas *Phragmipedium* spp. have smaller genomes and a narrower range, varying 1.5-fold, from 1C = 6.1 to 9.18 pg (Cox *et al.*, 1998).

A considerable amount of chromosome data is available for *Paphiopedilum* (e.g. Karasawa, 1978; 1979; 1982; 1986; Karasawa and Aoyama, 1980; 1988; Karasawa and Tanaka, 1980; 1981; Karasawa and Saito, 1982; Karasawa *et al.*, 1997; Cox *et al.*,

1998). The diploid chromosome number in the genus varies from 2*n* = 26 to 42 (Table 3.2). All species so far analysed in subgenera *Parvisepalum* and *Brachypetalum* have a chromosome number of 2*n* = 26 and many species in subgenus *Paphiopedilum* also have 2*n* = 26. In section *Paphiopedilum*, most species have 2*n* = 26, except for two species which have 2*n* = 30. Chromosome numbers in section *Cochlopetalum* range from 30 to 37 and section *Barbata* is the most variable, with chromosome numbers ranging from 2*n* = 28 to 42. Despite the variation in chromosome number, the total number of chromosome arms ('nombre fondamental' or n.f., Matthey, 1949) appears to be conserved in most species of the genus (n.f. = 52), which might suggest karyotype evolution via Robertsonian change, either producing telocentric chromosomes by centric fission or producing metacentric chromosomes by centric fusion (Robertson, 1916). The first report to postulate Robertsonian change as a cause of total arm number retention in *Paphiopedilum* was that of Duncan and Macleod (1949). Cox *et al.* (1998) studied the evolution of genome size and karyotype in Cypripedioideae by mapping chromosome number and genome size data onto a phylogenetic tree based on ITS data (Cox *et al.*, 1997). The results for *Paphiopedilum* showed evolutionary trends of an increase in the number of chromosomes and telocentric chromosomes and a decrease in metacentric chromosomes, suggesting the predominant direction of karyotype evolution was via centric fission, leading to higher chromosome numbers. It also showed an increase in genome size. However, the phylogenetic tree used for their study did not provide support for phylogenetic relationships between sections of *Paphiopedilum*, as mentioned previously and these hypotheses need to be reassessed in a phylogenetic framework with better resolution and support.

Genome size can be measured by different methods, but flow cytometry and Feulgen microdensitometry are those most commonly used. Both methods have their advantages and drawbacks (Greilhuber, 2005). Feulgen microdensitometry is a time consuming procedure because root materials have to be collected and prepared for chromosome staining. Although flow cytometry is more popular because it is a quicker procedure and is more convenient (Doležel *et al.*, 2007), it has been reported that inhibitors such as anthocyanin adversely affect florescence DNA staining (Bennett *et al.*, 2008). Many species of *Paphiopedilum*; most species in subgenera *Parvisepalum* and *Brachypetalum* and some species of sections *Paphiopedilum*, *Cochlopetalum* and *Barbata*, have purple marks on the underside of their leaves (Cribb, 1998) indicating the presence of anthocyanin, such as is found in *P. venustum* Pfitzer (Rutter and Willmer, 1979), thus using leaf materials for measuring DNA content by flow cytometry for those species is perhaps not practical. Feulgen microdensitometry has been used

previously for DNA content measurement of species in subfamily Cypripedioideae (Cox *et al.*, 1998; Kahandawala, 2009). In this study, Feulgen microdensitometry was used to measure DNA content using the Feulgen reaction for nuclei staining (Feulgen and Rossenbeck, 1924), which is the reaction of leucofuchsin acid (Schiff's reagent) with free aldehyde groups of hydrolysed DNA that turns to a purple stain. The density of stained DNA is then measured using a microdensitometer (Bennett and Smith, 1976).

3.1.1 Aims of this study

The aims of this study were to obtain additional genome size data for *Paphiopedilum* and the more robust phylogenetic trees from Chapter two were used as a framework to analyse evolutionary trends in genome size and chromosome number in the genus.

3.2 Materials and methods

3.2.1 Chromosome number and genome size data

Chromosome numbers for *Paphiopedilum* and *Phragmipedium* were taken from literature (Karasawa, 1979; 1980; 1982; 1986; Karasawa and Aoyama, 1980; 1988; Karasawa *et al.*, 1997; Cox *et al.*, 1998; Bennett and Leitch, 2010; Lan and Albert, 2011). Most genome size data were obtained from literature (Narayan *et al.*, 1989; Cox *et al.*, 1998; Bennett and Leitch, 2010). Seven species were measured for nuclear DNA content by Feulgen microdensitometry, according to Greilhuber and Temsch (2001) and Greilhuber (2005). Root materials used in this study were obtained from the living plant collection at the Tropical Nursery (RBG Kew). Root tips were all fixed with a fresh mixture of 3:1 (v/v) ethanol: glacial acetic acid and maintained at 4^{o}C for 24 hours before being transferred to 70% ethanol for storage at -20^{o}C until proceeding further. The fixed root tips were rinsed in sterile deionised water, then hydrolysed in 5M HCL at 20^{o}C for 60 minutes, before rinsing again in sterile deionised water and staining with pararosaniline solution (Sigma, Poole, UK) for 90 minutes in dark conditions. They were then washed three times in SO_2 water (100 ml H_2O, 5 ml 10% sodium metabisulphite) for 10 minutes, following which, the root tips were transferred to sterile deionised water and stored for up to 24 hours at 4^{o}C in the dark before squashing in 45% (v/v) acetic acid for the preparation of slides. Nuclei from ten mid-prophase cells (4C) per slide were measured and three slides analysed in total using a Vickers M85a microdensitometer and each nucleus was read three times. *Allium cepa* L. 'Ailsa Craig' (1C = 16.79 pg; Bennett and Smith, 1976) was used as the calibration standard. The

4C-value of each sample was calculated against the 4C-value of the standard in picograms and converted to give the 1C-value.

3.3 Results

Genome size data obtained from this study (seven taxa) and from the literature (25 taxa) are listed in Table 3.1. In Figure 3.1, genome size range (1C-value), mean value and chromosome number for each section within the genus are mapped onto the combined tree.

Table 3.1. Sources of genome size data used in this study (Chromosome number data are taken from Karasawa, 1979; 1980; 1982; 1986; Karasawa, Aoyama and Kamimura, 1997; Cox *et al*., 1998; Bennett and Leitch 2010; Lan and Albert, 2011).

Taxa	Voucher/source	Chromosome number (2*n*)	1C-value (pg)
Subgenus *Parvisepalum*			
Paphiopedilum armeniacum S.C.Chen & F.Y.Liu	Bennett & Leitch, 2010	26	21.10
Paphiopedilum delenatii Guillaumin	Cox *et al*., 1998	26	21.83
Paphiopedilum micranthum Tang & F.T.Wang	Cox *et al*., 1998	26	22.75
Subgenus *Brachypetalum*			
Paphiopedilum concolor (Bateman) Pfitzer	Cox *et al*., 1998	26	19.48
Paphiopedilum godefroyae (God.-Leb.) Stein	Cox *et al.,* 1998	26	17.80
Subgenus *Paphiopedilum*			
Section *Paphiopedilum*			
Paphiopedilum insigne (Wall. ex Lindl.) Pfitzer	Kew 2001-2843	26	27.52 (0.59)*
Paphiopedilum gratrixianum (Mast.) Rolfe	Kew 1979-975	26	25.16 (0.46)*
Paphiopedilum druryi (Bedd.) Stein	Kew 1982-1398	30	26.50 (0.47)*
Paphiopedilum villosum (Lindl.) Stein	Narayan *et al*., 1989	26	22.48
Section *Barbata*			
Paphiopedilum appletonianum (Gower) Rolfe	Cox *et al*., 1998	38	32.43
Paphiopedilum mastersianum (Rchb.f.) Stein	Cox *et al*., 1998	36	29.73
Paphiopedilum tonsum (Rchb.f.) Stein	Cox *et al*., 1998	32	28.15
Paphiopedilum barbatum (Lindl.) Pfitzer	Cox *et al*., 1998	38	33.75
Paphiopedilum bullenianum (Rchb.f.) Pfitzer var. *celebesense* (Fowlie & Birk) P.J.Cribb	Bennett & Leitch, 2010	40	25.85
Paphiopedilum callosum (Rchb.f.) Stein	Cox *et al*., 1998	32	24.05
Paphiopedilum lawrenceanum (Rchb.f.) Pfitzer	Bennett & Leitch, 2010	40	26.13
Paphiopedilum ciliolare (Rchb.f.) Stein	Bennett & Leitch, 2010	32	30.50
Paphiopedilum purpuratum (Lindl.) Stein	Bennett & Leitch, 2010	40	27.13
Paphiopedilum sukhakulii Schoser & Senghas	Cox *et al*., 1998	40	29.73

Table 3.1. Continued

Taxa	Voucher/source	Chromosome number (2*n*)	1C-value (pg)
Paphiopedilum wardii Summerh.	Cox *et al.*, 1998	41	34.53
Section *Pardalopetalum*			
Paphiopedilum parishii (Rchb.f.) Stein	Kew 1986-1038	26	27.20 (0.68)*
Paphiopedilum lowii (Lindl.) Stein	Bennett & Leitch, 2010	26	24.53
Paphiopedilum haynaldianum (Rchb.f.) Stein	Bennett & Leitch, 2010	26	22.85
Section *Cochlopetalum*			
Paphiopedilum liemianum (Fowlie) K.Karas. & K.Saito	Kew 1990-8000	32	23.72 (0.48)*
Paphiopedilum primulinum M.W.Wood & P.Taylor	Cox *et al.*, 1998	32	20.90
Paphiopedilum victoria-mariae (Sander ex Mast.) Rolfe	Cox *et al.*, 1998	36	21.40
Section *Coryopedilum*			
Paphiopedilum philippinense (Rchb.f.) Stein	Cox *et al.*, 1998	26	23.25
Paphiopedilum kolopakingii Fowlie	Kew 1983-5478	26	21.93 (0.86)*
Paphiopedilum stonei (Hook.) Stein	Kew 1998-2185	26	23.28 (0.46)*
Paphiopedilum adductum Asher	Bennett & Leitch, 2010	26	27.03
Paphiopedilum glanduliferum (Blume) Stein	*Cox et al.*, 1998	26	23.73
Paphiopedilum rothschildianum (Rchb.f.) Stein	Cox *et al.*, 1998	26	22.58
Outgroup			
Phragmipedium besseae Dodson & J.Kuhn	Cox *et al.*, 1998	24	7.08
Phragmipedium longifolium (Warsz. & Rchb.f.) Rolfe	Cox *et al.*, 1998	20, 21, 22, 23	6.10
Phragmipedium caudatum (Lindl.) Rolfe	Cox *et al.*, 1998	28	9.18
Phragmipedium lindleyanum (R.H.Schomb. ex Lindl.) Rolfe	Cox *et al.*, 1998	22	8.03
Phragmipedium pearcei (Rchb.f.) Rauh & Senghas	Cox *et al.*, 1998	20, 21, 22	6.33

*Standard deviations of 1C-value measured in this study shown in parentheses (pg)

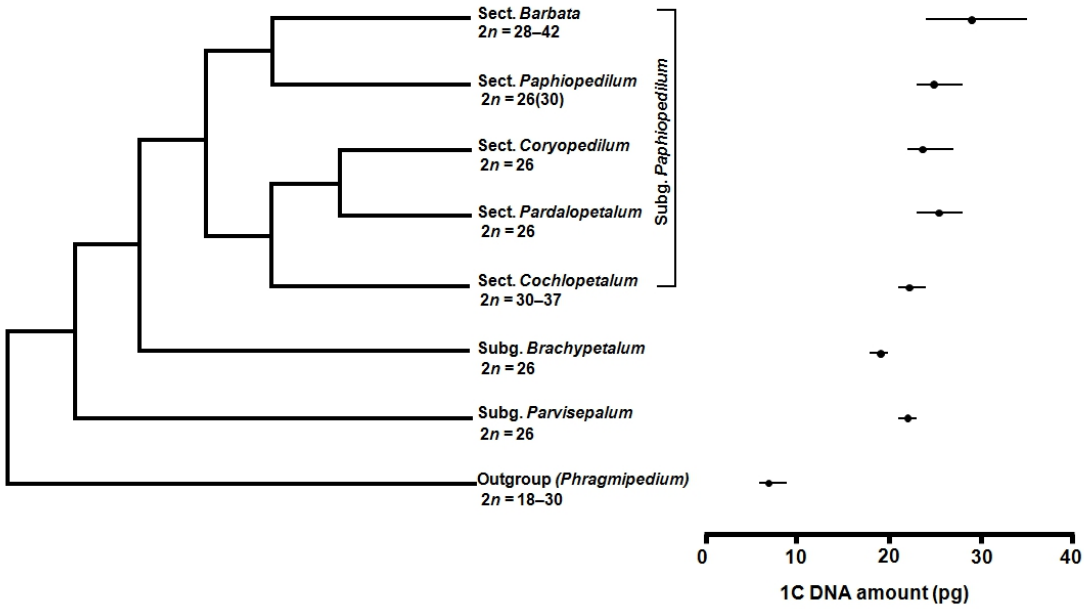

Figure 3.1. Chromosome numbers and genome size ranges (mean value indicated by a circle) mapped onto a phylogenetic framework from the combined DNA sequence data.

3.4 Discussion

3.4.1 Genome size and chromosome number evolution in the genus *Paphiopedilum*

Mapping chromosome number data onto the phylogenetic framework from the combined sequence data does not show clearly if there is a trend towards an increase in chromosome number as proposed by Cox *et al.* (1997; 1998) (Figure 3.1). There are two major lineages in subgenus *Paphiopedilum*, the first lineage composed of three sections (*Coryopedilum*, *Pardalopetalum* and *Cochlopetalum*). All species in the first two sections of this clade have a chromosome number of 2*n* = 26, whereas species of section *Cochlopetalum* have chromosome numbers that vary from 2*n* = 30 to 2*n* = 37. Similarly, in the second lineage, species of section *Paphiopedilum* have a chromosome number of 26 (except two species, *P. druryi* and *P. spicerianum*, with 2*n* = 30), whereas variable chromosome numbers, between 2*n* = 28 and 42, are found in the sister section *Barbata.* Although the topology of sections in subgenus *Paphiopedilum* in this phylogenetic framework is different from the study of Cox *et al.* (1997; 1998), the patterns are similar, in that sections with variable chromosome numbers are paired with sections with a constant chromosome number.

However, it has been shown from both phylogenetic frameworks that the first branching subgenus, *Parvisepalum*, and subgenus *Brachypetalum*, which is sister to subgenus *Paphiopedilum*, have a chromosome number of 2*n* = 26, with all metacentric chromosomes, and this could indicate that 2*n* = 26 is the ancestral condition for the genus, as suggested previously, because this number is found in most species of the genus (e.g. Karasawa, 1979). Also, the higher chromosome number and the presence of telocentric chromosomes could indicate a more derived condition, given the phylogenetic position of species with higher chromosome numbers. These results suggest that centric fission has contributed to the karyotype changes observed in the genus and superimposing the data onto the phylogenetic tree, indicate that centric fission has occurred independently in sections *Barbata* and *Cochlopetalum* (Figure 3.1).

There have been other studies that support a hypothesis of centric fission, for example, that of Karasawa and Tanaka (1980) who studied C-banding patterns of *P. callosum* (2*n* = 32) and found them to be similar to *P. insigne* (Wall. ex Lindl.) Pfitzer [=*P. insigne* (Wall. ex Lindl.) Pfitzer var. *sanderae* (Rchb.f.) Pfitzer, 2*n* = 26]. They postulated centric fission as a cause of karyotype changes.

Jones (1998), in a review of Robertsonian change in karyotype evolution, supported the hypothesis of centric fission in *Paphiopedilum*. He suggested that the small population sizes and inbreeding in *Paphiopedilum* could contribute to explaining the karyotype variation observed. Indeed, all species of section *Cochlopetalum* and most species of section *Barbata* that have a high chromosome number are endemic to the Malesian islands, and it has been suggested that centric fission may be under selection, as it has the potential to increase genetic recombination, enabling adaptation to the environments on islands (Cox *et al.*, 1998; Leitch *et al.*, 2009). However, this is clearly not always the case, as species of section *Coryopedilum*, most of which are also restricted to individual Malesian islands (Cribb, 1998), all have a chromosome number of 2*n* = 26. Although Cox (in Pridgeon *et al.*, 1999) suggested that the higher chromosome number of 2*n* = 30 in *P. druryi* (section *Paphiopedilum*) might be correlated with its narrow endemicity (in southern India), clearly other factors are involved in driving centric fission. This is because the only other species in section *Paphiopedilum* with 2*n* = 30 is *P. spicerianum*, which has a wider distribution. It is found in north-east India, north-west Burma and south-west China (Cribb, 1998).

The range in genome size, as represented by 32 species (44% of the genus), is from

1C = 17.80 pg in *P. godefroyae* to 1C = 34.53 pg in *P. wardii* (1.9-fold range; see Tables 3.1, 3.2 and Figure 3.1). The lowest genome sizes are found in species belonging to subgenus *Brachypetalum* (mean 1C = 18.64 pg) and the highest genome sizes are found in section *Barbata* (mean 1C = 29.27 pg). Mapping the genome size range of *Paphiopedilum* spp. onto the phylogenetic framework obtained in this study shows that there is no clear trend of genome size increase in the genus (Figure 3.1). The greatest range and largest genomes were found in section *Barbata*, which is also characterised by being the most variable in terms of chromosome number ($2n$ = 28–42). However, section *Cochlopetalum*, which is also variable in chromosome number ($2n$ = 30–37), has a similar range of genome size to other sections and subgenera characterised by $2n$ = 26 (Table 3.2).

Table 3.2. Range of chromosome number, number of chromosome arms (n.f.) and genome size data [minimum (min.), maximum (max.) and mean of 1C-value in picograms (pg)], number of species with 1C-value and representation in percentage. Chromosome number data are taken from Karasawa (1979, 1980, 1982, 1986), Karasawa and Aoyama (1980, 1988), Karasawa, Aoyama and Kamimura (1997), Cox *et al*. (1998), Bennett and Leitch (2010) and Lan and Albert (2011); sources of genome size data are listed in Table 3.1.

Taxa	Chromosome number (2*n*)	n.f.	Min. 1C-value (pg)	Max. 1C-value (pg)	Mean 1C-value (pg)	No. species with 1C-value	Representation (%)
Subgenus *Parvisepalum*	26	52	21.10	22.75	21.89	3	43
Subgenus *Brachypetalum*	26	52	17.80	19.48	18.64	2	50
Subgenus *Paphiopedilum*							
Section *Cochlopetalum*	30–37	48–50	20.90	23.72	22.01	3	60
Section *Pardalopetalum*	26	52	22.85	27.20	24.86	3	75
Section *Coryopedilum*	26	52	21.93	27.03	23.63	6	55
Section *Paphiopedilum*	26(30)*	52	22.48	27.52	25.42	4	29
Section *Barbata*	28–42	52–56	24.05	34.53	29.27	11	41
Phragmipedium (outgroup)	18–30	34–39	6.10	9.18	7.34	5	33

**P. druryi* and *P. spicerianum* 2*n* = 30

When plotting chromosome number against genome size data (Figure 3.2), a weak but significant relationship was found (Pearson's correlation coefficient r = 0.632, P < 0.001), suggesting that, as chromosomes undergo fission, it is often accompanied by an increase in genome size. The source of additional DNA in the genome is unclear, but is likely to comprise a diverse array of different types of repetitive DNA, including retrotransposons (Bennetzen, 2005).

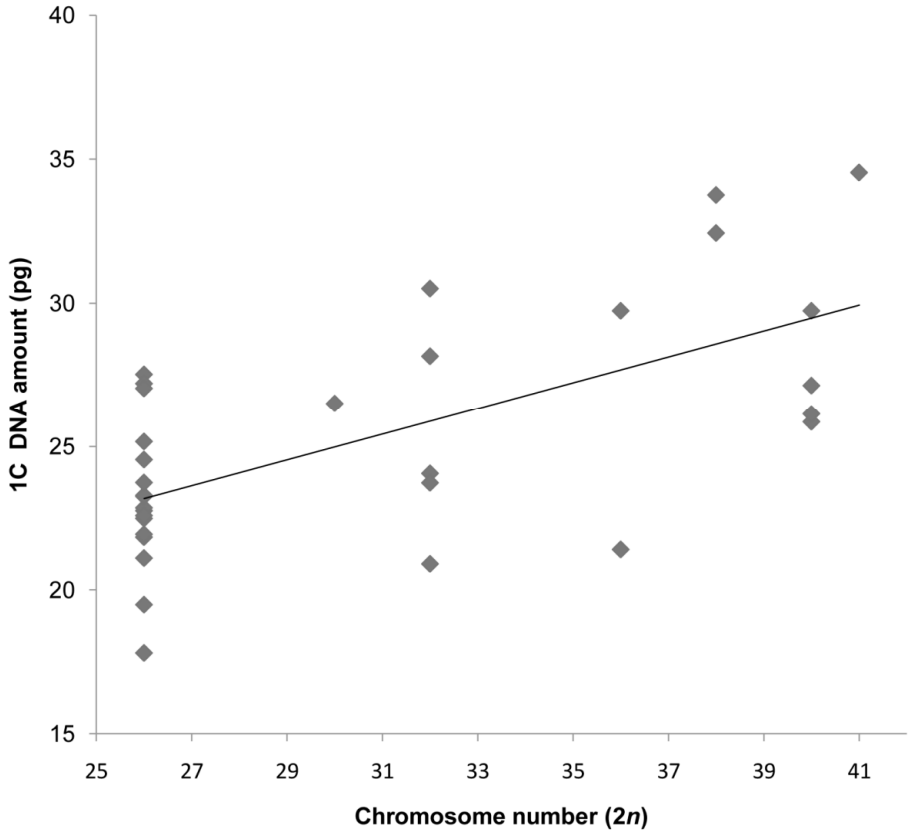

Figure 3.2. The relationship between genome size and chromosome number for 32 *Paphiopedilum* spp. Pearson's correlation coefficient r = 0.632, P < 0.001.

The relationship between chromosome number and genome size in *Paphiopedilum* differs from that of closely related genera. *Phragmipedium* has a variable chromosome number (2*n* = 18–30), but a smaller mean genome size and a narrower range (1.5-fold, 1C = 6.10 to 9.18 pg) (Cox *et al.*, 1998). *Cypripedium* is the most variable genus in subfamily Cypripedioideae in terms of genome size, with values ranging 10.5-fold (1C = 4.1 to 43.1 pg), but the chromosome number in most species is constant (2*n* = 20) (Leitch *et al.*, 2009; Kahandawala, 2009).

Lan and Albert (2011) studied the evolution of ribosomal DNA in *Paphiopedilum* using fluorescence *in situ* hybridisation and assessed the data according to the phylogenetic

framework of Cox *et al.* (1997). Although the results show variation of rDNA multiplication in *Paphiopedilum*, they found no evidence for a clear relationship between the increase in number of chromosomal locations of rDNA and the increase in chromosome number and genome size. Using the more robust phylogenetic framework from the current study, the multiplication of 25S rDNA loci observed by Lan & Albert occurred twice independently in *Paphiopedilum*, once in subgenus *Parvisepalum* and once in the clade formed by sections *Coryopedilum* and *Pardalopetalum* of subgenus *Paphiopedilum*. The multiplication event of 5S rDNA loci happened only in subgenus *Paphiopedilum*, whereas the early diverging subgenera *Parvisepalum* and *Brachypetalum* retained the ancestral number of two major sites, as also found in the outgroups *Phragmipedium* and *Mexipedium*.

Genome size is thought to have an influence on life form, habit and ecology. Annual plants are characterised by small genomes, whereas perennials have a larger range of genome sizes, and species with large genomes are all obligate perennials (Bennett, 1972). Leitch *et al.* (2009) found that epiphytic orchids have small genomes (mean 1C = 3.0 pg, range 0.33–8.5 pg), whereas terrestrial species have a much wider range (mean 1C = 18.3 pg, range 2.9–55.4 pg). This might be caused by selection for small guard cell sizes, because species with small guard cells are shown to respond more rapidly to water stress than those with larger cells (Aasamaa *et al.*, 2001; Hetherington and Woodward, 2003). As guard cell size has been shown to be correlated with genome size (Beaulieu *et al.*, 2008), this means that if small guard cells were selected, a consequence would be that small genomes would be selected as well (Leitch *et al.*, 2009). Most *Paphiopedilum* spp. are terrestrials, with only five being epiphytic: *P. parishii*, *P. lowii*, *P. villosum*, *P. hirsutissimum* and *P. glanduliferum*, the last two species being facultative epiphytes (Cribb, 1998). Nevertheless, in contrast to the observations of Leitch *et al.* (2009) that orchids with large genome sizes appear to be restricted to a terrestrial habit, the genome size of these epiphytic *Paphiopedilum* species is large (mean 1C = 24.49 pg, range 22.48–27.20 pg) and similar to those found in terrestrial species (mean 1C = 25.40 pg, range 17.80–34.53 pg). These observations suggest that water stress is unlikely to be a strong selective pressure on cell size in this case, perhaps because the high rainfall in habitats where *Paphiopedilum* spp. are found is seasonal. In addition, other features, such as thick leathery leaves, could also be strategies that enable their survival in the dry season (Cribb, 1998).

Vinogradov (2003) observed that species with large genomes are likely to be rarer than those with smaller genomes and that species with large genomes may be more likely to be at risk of extinction. This was tested by Knight *et al.* (2005), who hypothesised that constraints on adaptation in species with large genome sizes led to lack of diversity and speciation and the vulnerability of populations to environmental change, possibly leading to extinction. Most slipper orchids, including *Paphiopedilum*, have large genome sizes compared with angiosperms as a whole (Cox *et al.*, 1998; Leitch *et al.*, 2009). *Paphiopedilum* spp. are endangered because of the over-collecting of wild plants and degradation of their habitats. This, together with consequences of having large genome sizes, makes conservation of *Paphiopedilum* in the wild all the more important.

Chapter 4: Development of plastid microsatellites for slipper orchids (subfamily Cypripedioideae)

4.1 Introduction

4.1.1 Plastid microsatellites

The plastid genome is a circular haploid chromosome found in plastids such as chloroplasts. It is 120–160 kbp in size in most land plants (Green, 2011) with several thousand copies being found in each plant cell (Bendich, 1987). Generally, the plastid genome is transmitted through one parent, being maternally inherited in orchids and most angiosperms, but paternally inherited in gymnosperms (Corriveau and Coleman, 1988). The mitochondrial genome is also a circular haploid, uniparentally inherited chromosome but it is transmitted only maternally in plants (Ennos *et al.*, 1999). In contrast to those two organellar genomes, the nuclear genome is made up of linear, diploid or polyploid chromosomes in higher plants, which are biparentally inherited (Ennos *et al.*, 1999). Recombination, which happens among genes of homologous chromosomes in the nuclear genome, is extremely rare or absent in plastid and mitochondrial genomes, although intrachromosomal recombination is found in the mitochondrial genome in plants (Atlan and Couvet, 1993; Ennos *et al.*, 1999).

Microsatellites, or simple sequence repeats (SSRs), are repetitive tandem sequences, typically of one to six bases, found in prokaryotes and eukaryotes, where they are located in non-coding and coding regions in the nuclear, mitochondrial and plastid genomes (reviewed by Zane *et al.*, 2002; Ennos *et al.*, 1999; Provan *et al.*, 1999c; Li *et al.*, 2002). Microsatellites differ from minisatellites, which are repeat units of more than 10 bp, up to 100 bp (Richard and Pâques, 2000). Replication-slippage could be involved in microsatellite length variation where the addition or deletion of repeat units occurs during DNA replication (Levinson and Gutman, 1987).

Plastid microsatellites are repetitive tandem sequences, usually with more than 10 repeat units for mononucleotide sequences, in which poly-A or poly-T are found more commonly, than the combination of those bases (Fay *et al.*, 2009). However, some dinucleotide and trinucleotide plastid microsatellites have been reported in some plant species (Deng *et al.*, 2007; Provan *et al.*, 2001; Powell *et al.*, 1995b; Sebastiani *et al.*, 2004).

Plastid microsatellites are distributed across the plastid genome, and over 200 mononucleotide repeats have been reported in complete plastid sequences of six plant species (Powell *et al.*, 1995b). Similar repeat motifs are also found in nuclear microsatellites but are mostly dinucleotide repeats, A-T is the most common in plants (Powell *et al.*, 1996). Most microsatellites found in the mitochondrial genome of rice and other cereal plants are dinucleotide repeats (Rajendrakumar *et al.*, 2007; 2008). More recent research into 16 mitochondrial genomes of plants, reported the mononucleotides, poly-A or poly-T, as being more common than other types of repeats (Kuntal and Sharma, 2011).

4.1.2 Applications of plastid microsatellite markers

The uniparental mode of inheritance and the non-recombinant nature of the plastid genome allows an understanding of pollen flow contribute to genetic structure of population. By measuring genetic differentiation between plastid markers, (usually maternally inherited and transmitted via seeds in angiosperms) and nuclear markers that are biparentally inherited and in which gene flow is transmitted by both pollen and seeds, the difference between the two measurements reveals the level of pollen flow relative to seed flow, a greater difference indicating more pollen flow. (Ennos, 1994; Ennos *et al.*, 1999). Although variation in the number of microsatellites repeated in plastid DNA is lower than in nuclear DNA, polymorphism of mononucleotide microsatellites from the plastid genome has been reported as an alternative tool to detect intraspecific variations (Powell *et al.*, 1995a; Powell *et al.*, 1995b). Plastid microsatellites are suitable markers for revealing genetic diversity patterns, such as ancient bottlenecks, founder effect and genetic drift because the haploid nature of plastid genome leads to an effective population size half that of the diploid nuclear genome, heteroplasmy being scarce (Birky *et al.*, 1989; Provan *et al.*, 2001).

A study using plastid microsatellite markers supported those of a previous study using restriction fragment length polymorphism (RFLP) that there is a lack of variation in the plastid genome of *Pinus torreyana* Parry ex Carrière, indicating a bottleneck (Provan *et al.*, 1999b). Similarly, low levels of genetic diversity revealed by plastid microsatellites in most Mediterranean populations of *Pinus halepensis* Mill. show population bottlenecks arising from founder events (Morgante *et al.*, 1997). Also, low levels of plastid microsatellite variation have been found in *Abies nebrodensis* (Lojac.) Mattei in comparison to three other *Abies* species studied, which, it was suggested, was a result of a genetic bottleneck (Parducci *et al.*, 2001). In addition, plastid microsatellites have

been used for studying phylogeography in various plants, such as *Picea abies* (L.) H.Karst. in which the distribution of plastid haplotypes helped reveal the geographical patterns of populations relating to recolonisation events during the post-glacial period (Vendramin *et al.*, 2000). Also, eight gene pools associated with glacial refugia and recolonisation routes have been identified for *Pinus pinaster* Aiton from plastid microsatellite data, allowing a detailed reconstruction of the phylogeography of this species (Bucci *et al.*, 2007).

In angiosperms, plastid markers, including microsatellites, have been used for a phylogeographic study of *Carpinus betulus* L. and *C. orientalis* Mill. (Grivet and Petit, 2003), the results showing distinctive haplotypes, indicating no gene flow between them and it has been suggested, similar Italian and Balkan refugia. Results from plastid microsatellites supported previous research using nuclear markers that *Phaseolus vulgaris* L. originated in Mesoamerica and indicated central Mexico as a cradle of its diversity. Also, a putative ancentral plastid genome was identified in a group of accessions distributed between Northern Mexico and Peru (Desiderio *et al.*, 2013). Plastid microsatellites obtained from DNA extracted from herbarium specimens helped reveal five distinct haplotypes of submerged plants, *Zannichellia* spp. in specific localities across Europe (Triest *et al.*, 2007). A study using plastid DNA, including microsatellites, revealed a recent divergence between *Coffea arabica* L. and *C. eugenioides* S.Moore and suggested the maternal parent of *C. arabica* was an ancestor or close relative of *C. eugenioides* and not *C. eugenioides* itself, as previously thought. Also, the lack of variability in plastid microsatellites and SNP within *C. arabica* was suggested as being a result of the recent origin of this species by an allopolyploidisation event or by a severe population bottleneck event (Tesfaye *et al.*, 2007).

In Orchidaceae, plastid repeat sequences, such as minisatellites, have been used in a study of *Anacamptis palustris* (Jacq.) R.M.Bateman, Pridgeon & M.W.Chase to reveal a historic bottleneck in small populations of this species by measuring observed and expected gene diversity and analysis of haplotype number and haplotype frequency distribution (Cozzolino *et al.*, 2003b). Additionally, the distribution of plastid haplotypes from a combined data set of plastid minisatellites and microsatellites helped reconstruct phylogeographic history by identifying four main evolutionary lineages (Cozzolino *et al.*, 2003a). A study based on plastid haplotype data combined with nuclear DNA data found that the British and Irish *Dactylorhiza majalis s.l.* complex has at least four genetically distinct groupings and that some subspecies that had previously been

considered distinct, based on anthocyanin content, could be included in these groups. Also, results from plastid haplotype data revealed the origins of allopolyploidy and that *D. majalis* ssp. *traunsteinerioides* (Pugsley) R.M.Bateman & Denholm and *D. majalis* ssp. *praetermissa* (Druce) D.M.Moore & Soó originated from the Continental *D. majalis s.l.* complex (Hedrén *et al.*, 2011). A combination of amplified fragment length polymorphisms (AFLP), DNA sequence and plastid microsatellite data has helped to reveal genetic relationships among populations of the North American orchids, *Cleistes divaricata* (L.) Ames and *C. bifaria* (Fernald) Catling & K.B.Gregg in which it was found that *C. bifaria* populations were split into two groups, one of which was grouped with *C. divaricata* (Smith *et al.*, 2004). Plastid microsatellites have also been used to measure levels of genetic variation in populations of three widespread *Cephalanthera* species, intraspecific variation being detected in two species, the exception being *C. damasonium* Druce, a possible cause of which was suggested to be its breeding system (Micheneau *et al.*, 2010).

In subfamily Cypripedioideae, Fay and Cowan (2001) developed plastid microsatellite markers from the *trnL-F* region for detecting genetic variation in *Cypripedium calceolus*. Two loci containing microsatellites were screened (orch1, cyp2), but only one was found to be polymorphic (cyp2). Although the sampling from Eurasia was sparse, this marker detected five alleles. These results had implications for developing a conservation strategy, as the UK samples showed a different allele for two plants that might not be native, in which case, the authors suggested, those plants should be excluded from a reintroduction programme. A more extensive study, using plastid microsatellite markers for *C. calceolus*, was carried out by Fay *et al.* (2009). Using a combination of 14 polymorphic regions of plastid microsatellites (rps16-1, rps16-2, cyp2, *accD-psaI*) and indels (10 indels in the *accD-psaI* intergenic spacer), 23 haplotypes were recovered; some were widespread, supporting the assumption of at least occasional long-distance seed dispersal in orchids. Results from analysis of molecular variance (AMOVA) showed a pattern of genetic variation in which most of the variation was from within, rather than among populations of *C. calceolus*.

4.1.3 Development of plastid microsatellite markers

The highly conserved nature of sequences in the plastid genome could possibly help facilitate primer design for plastid microsatellites, to enable cross amplification in closely and even in distantly related species (Powell *et al.*, 1995a; Powell *et al.*, 1995b). This is unlike nuclear microsatellites, in which the higher rate of mutation leads

to the lack of conserved flanking areas for designing primers for divergent taxa (Zane *et al.*, 2002).

The development of plastid microsatellite markers is different from that of nuclear microsatellites. Because of the short length of plastid microsatellites and the need for genome specificity, primers cannot be developed by screening DNA libraries but instead require sequence data from closely related species. From these, polymorphic nucleotide repeats can be obtained and primers designed from completed plastid genome data (Provan *et al.*, 2001). In addition, the nature of plastid microsatellites, with short nucleotide lengths and a smaller range of alleles, allows allele size to be confirmed with sequence data, unlike nuclear microsatellites, which are longer and often have more alleles (Provan *et al.*, 2001).

The development of universal primers for plastid microsatellites has been reported for eudicots (Weising and Gardner, 1999). Also, the use of universal primers has been reported successfully in many families of monocots and eudicots (Chung and Staub, 2003), including the grass (Provan *et al.*, 2004) and the legume families (Angioi *et al.*, 2009). Plastid microsatellites have been successfully developed in individual orchid species, such as *Dactylorhiza incarnata* (L.) Soó (Hedrén, 2009), *Epidendrum* spp. (Pinheiro *et al.*, 2009), *Cephalanthera* spp. (Micheneau *et al.*, 2010) and *Dendrobium officinale* Kimura & Migo (Xu *et al.*, 2011). In some of these cases, specific primers proved to be cross-amplifiable between species.

4.1.4 Advantages and limitations

Mutation rates have been found to be lower in plastid than in nuclear genomes, inferred from the synonymous substitution rate when comparing sequences from both genomes. Indeed the rate of mutation in the plastid genome is less than half that in the nuclear genome (Wolfe *et al.*, 1987). This leads to a problem in obtaining enough variation from plastid microsatellites for the study of closely related species (Provan *et al.*, 2001). Consequently, nuclear microsatellites are advantageous and more popular markers. However, a lower mutation rate helps in the development of primers from highly conserved flanking areas. One obvious disadvantage of uniparentally inherited markers is that they provide only one side of parental history, usually the maternal side. However, the lack of recombination in plastid markers helps to reveal a clear species history for the study of phylogeography (Echt *et al.*, 1998). Ziegenhagen *et al.* (1998) studied the paternity of silver fir (*Abies alba* Mill.) trees using two plastid microsatellite loci, each of five alleles. In a small population near to two adult trees, six out of twenty

four young trees had plastid microsatellite haplotypes that differed from the adult trees, suggesting gene flow via pollen into the population, thus indicating the potential utility of plastid microsatellites for paternity analysis.

Plastid microsatellites have some advantages over other fingerprinting methods, such as AFLPs (Vos *et al.*, 1995), because only a single short locus is amplified, which allows them to be used with low DNA quality and quantity, such as DNA samples extracted from herbarium specimens (Fay and Cowan, 2001). Also, the AFLP method has been reported to be problematic with species that have large genome sizes (i.e. 1C value > 15 pg), including *Cypripedium calceolus* (Fay *et al.*, 2005). Problems with the amplification of nuclear microsatellites have also been reported for species with large genomes (Garner, 2002; Kahandawala, 2009). Species in subfamily Cypripedioideae have a large range of genome sizes, ranging from 4.1 to 43.1 pg with mean DNA content 1C = 25.8 pg. Because many species have large genome sizes (Cox *et al.*, 1998; Leitch *et al.*, 2009). Plastid microsatellites are thus more applicable than AFLPs and nuclear microsatellite protocols.

4.1.5 Conservation of the slipper orchid subfamily Cypripedioideae

Because of their beautiful and peculiar flowers, slipper orchids are desirable to many plant collectors. This has resulted in over-collecting from the wild, which, together with deforestation of their habitats, means many slipper orchids today are endangered. *Paphiopedilum* spp. and *Phragmipedium* spp. are listed in Appendix I of the Convention on International Trade of Endangered Species (CITES). The rest of the taxa in subfamily Cypripedioideae are included in Orchidaceae on Appendix II (CITES, 2012).

Cypripedium calceolus, despite being one of the most widespread species in the Northern Hemisphere (Cribb, 1997), is rare in many countries in Europe, due to over-collecting and habitat destruction (Roberts, 2003). In Britain, only one original clump and some reintroduced plants survive in the wild in the north of England (Fay and Cowan, 2001). This drastic decline is mainly due to the over-collecting of wild plants by both botanists and gardeners (Ramsay and Stewart, 1998). This species is designated as ‘Near Threatened’ on the IUCN European red List Appendix 2 (Bilz *et al.*, 2011), and is also listed on the Convention on the Conservation of European Wildlife and Natural Habitats (the Bern Convention), Appendix I, Strictly Protected Flora Species (Council of Europe, 1979) and on European Council Directive 92/43/EEC on the Conservation of

Natural Habitats and of Wild Fauna and Flora Habitats Directive, Annex II and IV (Council of Europe, 1992).

Many slipper orchids are endangered and exist in small populations (Cribb, 1998), for example, *C. calceolus*, although its distribution range is wide, often occurs in small populations, which are isolated and decreasing in size (Nicolè *et al.*, 2005). This may lead to a loss of genetic diversity, which is a key for adapting to changing environments (Frankham, 2005) and maintaining reproductive fitness (Reed and Frankham, 2003). Thus, monitoring genetic variation in those endangered species helps in choosing appropriate methods for their conservation. Genetic variation within and between populations is important for selecting plants for reintroduction and reinforcement (Fay and Krauss, 2003).

4.1.6 Aims of this study

The aim of this study was to develop a set of plastid microsatellite markers for species in subfamily Cypripedioideae. Primers were tested on a range of accessions in the subfamily to determine their cross applicability. Plastid microsatellite markers were examined to determine if they were variable in size length (base pairs). In addition, plastid microsatellites were assessed for their utility in a population genetics study of *Cypripedium calceolus* to examine levels of genetic variation. Obtaining polymorphic plastid microsatellites is useful for studying genetic diversity for conservation purposes.

4.2 Materials and methods

4.2.1 DNA samples and sequence data

All DNA samples used in this study were from the DNA bank, the Jodrell Laboratory, RBG Kew. The DNA samples were used for developing plastid microsatellite markers for genera in subfamily Cypripedioideae (*Cypripedium*, *Paphiopedilum* and *Phragmipedium*) and other subfamilies in Orchidaceae (*Epidendrum* L., *Vanilla* and *Orchis* L.). Plastid genome data for *Phragmipedium longifolium* were obtained from Dr. W. Mark Whitten (Florida Museum of Natural History, USA). Taxa used for designing plastid microsatellite primers are listed on Table 4.1. DNA samples for testing the applicability of those markers for the subfamily comprised 248 accessions in total [*Cypripedium*, 177 accessions (includes *C. calceolus* 137 accessions); *Paphiopedilum*, 66 accessions and *Phragmipedium* five accessions]. There were also nine accessions of DNA samples of *Vanilla* spp. for testing with microsatellite markers.

4.2.2 Search for plastid microsatellites and primer design

Plastid microsatellites (n > 9), including poly-A, poly-T and a combination of both bases (n > 5), were searched for in the plastid genome of *Phragmipedium longifolium* (Figure 4.1). The locations of selected microsatellites and flanking areas were identified in plastid loci by blasting with nucleotide data on GenBank (http://blast.ncbi.nlm.nih.gov/; Figures 4.2, 4.3).

Table 4.1. List of taxa used in the sequence alignments for designing plastid microsatellite primers. Plastid genome data of *Phragmipedium longifolium* obtained from Dr. W. Mark Whitten were also included in the alignments.

Region	Taxa	Voucher/source
trnL-F1 and *trnL-F 2*	*Vanilla planifolia* Andrews	AY557223***
	Mexipedium xerophyticum (Soto Arenas, Salazar & Hágsater) V.A.Albert & M.W.Chase	FR851215***
	Selenipedium aequinoctiale Garay	JF825973***
	Cypripedium molle Lindl.	FR851216***
	Paphiopedilum wardii Summerh.	EF156246***
	Phragmipedium longifolium (Warsz. & Rchb.f.) Rolfe	EF156250***
ycf1 3'	*Cypripedium calceolus* L.	MFF19106 (K)
	Paphiopedilum wardii Summerh.	M.W. Chase 5903 (K)
	Paphiopedilum violascens Schltr.	O-825* (no voucher)
	Phragmipedium besseae Dodson & J.Kuhn	Z16a
	Phragmipedium longifolium (Warsz. & Rchb.f.) Rolfe	Z9
trnF(GAA)-*ndhJ*	*Cypripedium calceolus* L.	MFF19106 (K)
	Paphiopedilum stonei (Hook.) Stein	Z7
	Paphiopedilum superbiens (Rchb.f.) Stein var. *curtisii* Braem	Z5
	Phragmipedium longifolium (Warsz. & Rchb.f.) Rolfe	Z9
psbD-trnT	*Cypripedium flavum* P.F.Hunt & Summerh.	5725* (no voucher)
	Paphiopedilum adductum Asher	36820*, Kew 1992-3661** (no voucher)
	Paphiopedilum glanduliferum (Blume) Stein (a)	M.W. Chase O-716 (K)
	Phragmipedium longifolium (Warsz. & Rchb.f.) Rolfe	Z9
trnC(GAA)-*petN*	*Paphiopedilum parishii* (Rchb.f.) Stein	Z3
clpPex3-clpPex2	*Cypripedium formosanum* Hayata	M.W. Chase 16308 (K)
	Paphiopedilum parishii (Rchb.f.) Stein	Z3
trnT(UGU)-*L*(UAA)	*Paphiopedilum parishii* (Rchb.f.) Stein	Z3

*Kew DNA bank number, **Kew living collection number, *** GenBank accession number of downloaded sequences

651 TAATTTTATTTATTAATATATATTAATATATTAATATAAATCAAAGTATTTA
652 TAAAATATTCTAAATATTCTATCTAAGAAAAGTATTTATGTATTGTATTTA
653 TGCGTTAAAGTATAAGGTAATAATGAGAAAGTAGAATTATCATTTCTTTTC
654 TTCAATTTTAACTTATTGCTTCGTTCAGTGTTTAGATGAGTTATGCCTATCG
655 CTATCGCACAATAAGCAAAAAAAGGATTTAAATATTGTTTCTTTATAAGCT
656 TTATAAGAAATAAGAATAAATAAGAATTCGGTTCAGGTTACGAATCCCCC
657 GAATCTTGATCAGTCAAAGTCAACAAAGCAAGATCTTGAAACACATTTTC
658 TTTTCTATTTTAGAAAAATATGAATCCATTTCCTAGATAAATCAAACTTAT
659 TGATTCATTATGATATGCAGTAGACTCATAATTATAATGGAAGATGAAGA
660 TGGCTAATTCATGAATTGAAGAAAACGGGCCCTTTTAACTCAGCGGTAGA
661 GTAACGCCATGGTAAGGCGTAAGTCATCGGTTCAAATCCGATAAAGGGCT
662 TTTTATACTCAACTAAAATCTGAGTCTTTGTTTTTCGGCGTAAAATAGAAA
663 TCTTTTTTATATTTGTAAAAAAAAAAGAAAACGACCACACCATTATGATTA
664 AAATGAGTTCTGATTATTCTGAGTTCTAGTTCGAAGATTGAACAATTAATC
665 ATTATTATAATGATTAATTGCTCTGATTAGGAGGAGTCGTGACATAGTAAT
666 CCTCTTCATGTCTCATTATTCCATTTTTTGTCCTGAGACATAAATATCCAAC
667 CCTTCTTATTATGAATCGATAAACAAACGTATTCCTACTTCGACATCGTTC
668 CTATAACCCAACGTCAAATTATAAATAAAGTAAAAGAAAGTAAGTGGACC
669 TGACCCATTGAATCATTACTATATCACATCATATGATTTAAGAAAAGATCT
670 TGAATGTATATATATATCAGTCAATTGCTATTCTGATATTTAAATTCGATA
671 TAAATTCAGTGGATTTTTTATTTCGTTAGACCATTGGACCACACAAGGCAA
672 AAATTTTTCGATATTTTTTATTTAATCCTCTTGTTACTGGATGCTCCATAGA
673 ACTAAATCGCTATTCTTTTACTTTCCACTACATATAAAATACAATTTTAAA
674 TGCGGATTTAAAATATTTTTCCTTGATTTCAGAATCAGATATTGATTTGTTG
675 TTACACCGATGGAATAGAGAATAAATGCACGAAGGGGGCTTTATTTTTTA
676 GTTTACCATTATTTAATATTGATAGGGAAAAATAGGGAATCCGTTTGGTTT
677 GACCCCCTCAAAGATATACTCTGGAATATAAGTCATAGGATAATTCAGGG
678 TTCCAATGGTCATGTTATTTTTCTTTTATTTTATATAGTACATAGTCAGTAC

Figure 4.1. Mononucleotide microsatellite and flanking areas in the *Phragmipedium longifolium* plastid genome obtained from Dr. W. Mark Whitten.

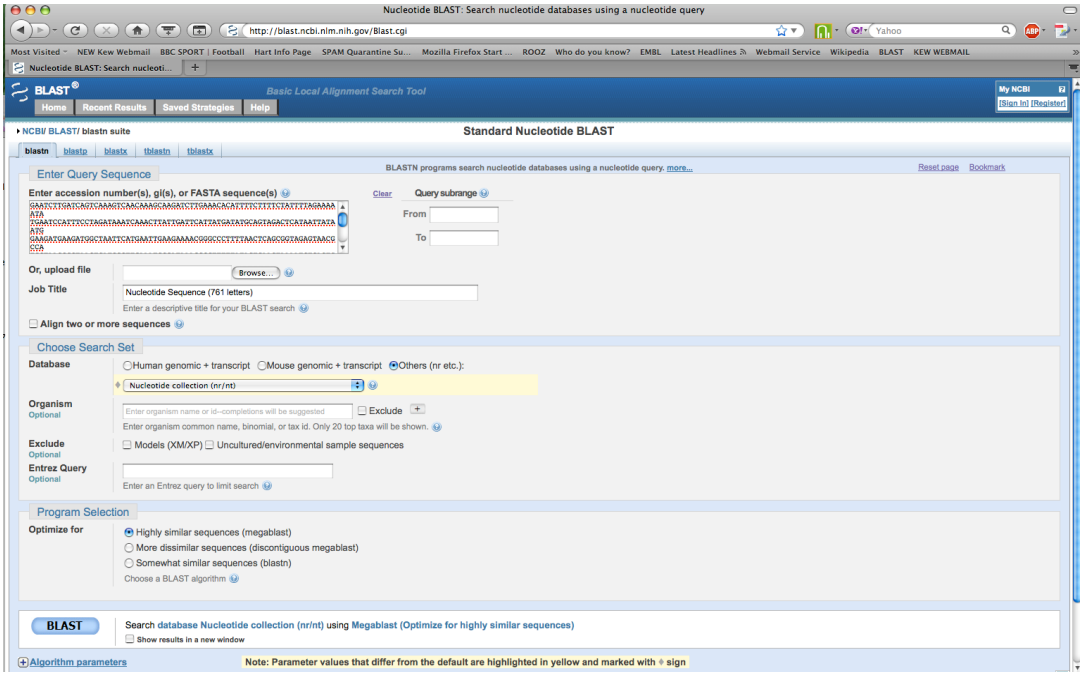

Figure 4.2. Searching location of microsatellite against nucleotide database on GenBank using Blast search engine.

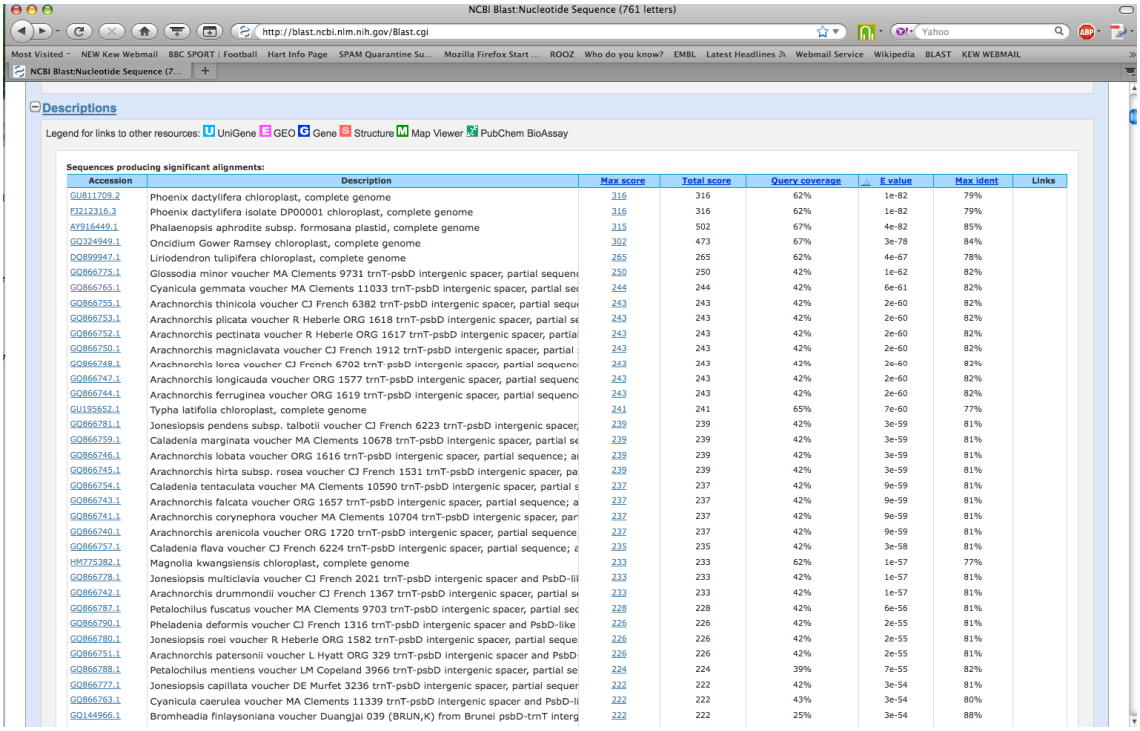

Figure 4.3. Results from Nucleotide Blast search showing location of the selected microsatellite on the plastid genome.

Eighteen plastid regions identified in the plastid genome of *Phragmipedium longifolium* were selected for generating sequence data using universal primers available at the Jodrell Laboratory, six regions of which, *rps16-trnK* and *clpPex1-psbB* (Ebert and Peakall, 2009); *rpl14-rps8-infA-rps36*, *ndhA, psbB-psbT*, and *trnQ*(UGG)-*rps16* (Shaw *et al.*, 2007) were not able to produce PCR products or sequences. Twelve plastid regions; *ycf1* 3'and *ycf1* 5' (Neubig *et al.*, 2009); *psaA-ycf3ex3*, *trnF(GAA)-ndhJ, atpI-rps2*, *trnC*(GCA)-*petN* and *clpP*ex3-*clpP*ex2 (Ebert and Peakall, 2009); *psbD-trnT, petL-psbE* and *trnL-rpl32* (Shaw *et al.*, 2007); *rpl16* (Jordan *et al.*, 1996); *trnT*(UGU)-*L*(UAA) (Taberlet *et al.*, 1991) were amplified successfully. In addition, sequences for *matK*, *atpB-rbcL* and *trnL-F* were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/nuccore/). Universal primers from publications, used for amplifying plastid regions, are listed in Table 4.2.

Table 4.2. List of universal primers from publications used for amplifying plastid regions.

Region	Primer	Sequences (5' to 3')	Reference
trnL-F	N/A	N/A	N/A
ycf1 3'	3720F 5500R IntF	TACGTATGTAATGAACGAATGG GCTGTTATTGGCATCAAACCAATAGCG GATCTGGACCAATGCACATATT	Neubig *et al.* (2009)
ycf1 5'	1F 1200R	ATGATTTTTAAATCTTTTCTACTAG TTGTGACATTTCATTGCGTAAAGCCTT	Neubig *et al.* (2009)
*psaA-ycf3*ex3	ANU_cp051-L ANU_cp052-R	GTTCCGGCGAACGAATAAT GTCGGATCAAGCTGCTGAG	Ebert and Peakall (2009)
trnF(GAA)-*ndhJ*	ANU_cp061-L ANU_cp062-R	CCTCGTGTCACCAGTTCAAA TGGATAGGCTGGCCCTTAC	Ebert and Peakall (2009)
psbD-trnT	psbD trnT(GGU)-R	CTCCGTARCCAGTCATCCATA CCCTTTTAACTCAGTGGTAG	Shaw *et al.* (2007)
petL-psbE	petL psbE	AGTAGAAAACCGAAATAACTAGTTA TATCGAATACTGGTAATAATATCAGC	Shaw *et al.* (2007)
atpB-rbcL	N/A	N/A	N/A
atpI-rps2	ANU_cp029-L ANU_cp030-R	TTGGAAACCTCCTATTTGC ATTTGTGAGGGCCGTTCT	Ebert and Peakall (2009)
trnC (GCA)-*petN*	ANU_cp037-L ANU_cp038-R	CAGGGGACTGCAAATCCTT TACCATTAAAGCAGCCCAAG	Ebert and Peakall (2009)
matK	N/A	N/A	N/A
*clpP*ex3-*clpP*ex2	ANU_cp089-L ANU_cp090-R	ACTAGCAGGTTGGTGAATCAT AACGTCTAGCATTCCCTCAC	Ebert and Peakall (2009)
rpl16	F71 R1661	GCTATGCTTAGTGTGTGACTCGTTG CGTACCCATATTTTTCCACCACGAC	Jordan *et al.* (1996)
trnL-rpl32	trnL(UAG) rpL32-F	CTGCTTCCTAAGAGCAGCGT CAGTTCCAAAAAAACGTACTTC	Shaw *et al.* (2007)
trnT(UGU)-*L*(UAA)	a b	CATTACAAATGCGATGCTCT TCTACCGATTTCGCCATATC	Taberlet *et al.* (1991)

N/A, sequences were downloaded from GenBank.

In most cases, amplification of all plastid regions was carried out in 25 µl volumes, containing 22.5 µl ReddyMix PCR Mastermix (2.5mM $MgCl_2$, ABGene, Epsom, Surrey, UK). 0.5 µl bovine serum albumin (0.04%), 0.5 µl of each primer (100 ng/ µl) and template DNA approximately 50–100 ng. Alternative PCR protocol was carried out in 19 µl volumes containing 0.4 µl of dNTP (100mM), 0.4 µl of Taq polymerase (5 u/ µl), 1.2 µl of $MgCl_2$ (25mM), 4 µl of 5× GoTaq Flexi Buffer, 2 µl bovine serum albumin (0.04%), 1 µl of each primer (100 ng/ µl) and template DNA approximately 50–100 ng. All PCR reactions were performed on ABI GeneAmp PCR System (Applied Biosystems).

The PCR profile for most plastid regions consisted of 3 minutes of initial denaturation at 94°C, followed by 28 cycles of 94°C denaturation for 1 minute, 48°C annealing for 1 minute and 72°C extension for 1 minute and final elongation 72°C for 7 minutes. An alternative PCR programme, 'touchdown plastid', consisted of 3 minutes of initial denaturation at 94°C, followed by 12 cycles of 94°C denaturation for 30 seconds, 66°C annealing (reducing 1.5°C per cycle) for 30 seconds and 72°C extension for 45 seconds then followed by 30 cycles of 94°C denaturation for 30 seconds, 47°C annealing for 30 seconds and 72°C extension for 45 seconds, and final elongation at 72°C for 30 seconds.

The PCR profile for *ycf1* region (both 3' and 5'), using a 'touchdown', consisted of 3 minutes of initial denaturation at 94°C, followed by 8 cycles of 94°C denaturation for 30 seconds, 60–51°C annealing (reducing 1°C per cycle) for 1 minute and 72°C extension for 3 minutes, followed by 30 cycles of 94°C denaturation for 30 seconds, 50°C annealing for 1 minute and 72°C extension for 3 minutes, and final elongation, 72°C for 3 minutes (Neubig *et al.*, 2009).

The PCR profile for *rpl16* region consisted of 2 minutes of initial denaturation at 94°C, followed by 30 cycles of 94°C denaturation for 1 minute, 52°C annealing for 1 minute and 72°C extension for 3 minutes, and final elongation, 72°C for 7 minutes (Davis *et al.*, 2007).

The PCR profile for *trnL-rpl32* regions consisted of 5 minutes of initial denaturation at 95°C, followed by 30 cycles of 95°C denaturation for 1 minute, 53°C annealing for 1 minute and 72°C extension for 2 minutes, and final elongation at 72°C for 10 minutes (Riggins, 2008).

The PCR profile for *trnT-L* regions consisted of 3 minutes of initial denaturation at 94°C, followed by 30 cycles of 94°C denaturation for 1 minute, 50°C annealing for 1 minute and 72°C extension for 3 minutes, and final elongation, 72°C for 7 minutes (Taberlet *et al.*, 1991).

All amplified PCR products were visualised by 1% agrose gel electrophoresis, then purified using NucleoSpin Extract II columns according to the manufacturer's protocols. The cycle sequence reactions were carried out in 10 µl volumes containing 1.75 µl of 5× sequence buffer, 0.5 µl of Big Dye Terminator kit (Applied Biosystems, Inc., Warrington, Cheshire, UK), 0.75 µl of each primer (10 ng/ µl) and approximately 50 ng/ µl cleaned PCR products, then the volume was adjusted by adding sterile, deionised water. The cycle sequencing profile was composed of 26 cycles of 96°C denaturation for 10 seconds, 50°C annealing for 5 seconds and 60°C extension for 4 minutes. The cycle sequencing products were cleaned by ethanol precipitation and then run on an ABI 3730 automated sequencer. Raw sequences were edited and assembled using Sequencher 4.1 software (Gene Codes, Inc., Ann Arbor, Michigan USA). The resulting sequences for each region were aligned using Muscle alignment (http://www.ebi.ac.uk/Tools/msa/muscle/), then manually aligned in PAUP* version 4.0b10 for Macintosh (Swofford, 2002).

Forward and reverse primers for selected plastid microsatellites of each region were designed manually from relatively conserved flanking areas (Figure 4.4). Primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany). All forward primers of each marker were labelled with fluorescent dye for detecting size variation in microsatellites.

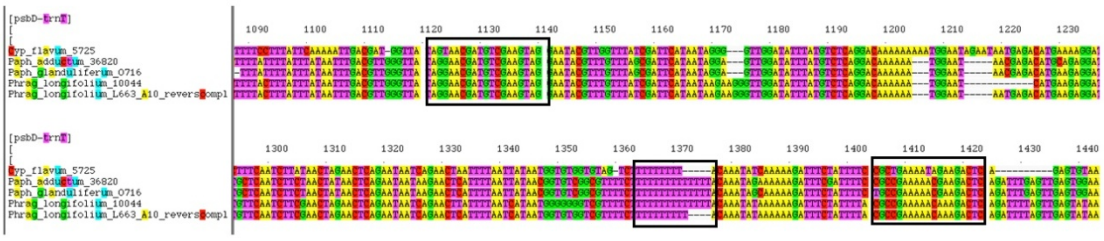

Figure 4.4. Partial sequence of *psbD-trnT* region showing microsatellite and flanking areas for designing forward and reverse primers.

4.2.3 Plastid microsatellite amplification and genotyping

PCR amplifications were carried out in 10 µl volumes containing 9 µl ReddyMix PCR Mastermix (2.5mM $MgCl_2$, ABGene, Epsom, Surrey, UK) 0.4 µl bovine serum albumin (0.04%), 0.1 µl of each primer (100 ng/ µl) and template DNA approximately 50–100 ng. The PCR profile for plastid microsatellites consisted of 3 minutes of initial denaturation at 94°C, followed by 28 cycles of 94°C denaturation for 1 minute, 48°C annealing for 1 minute and 72°C extension for 1 minute, and final elongation, 72°C for 7 minutes. The PCR products were visualised on 1% agarose gel by electrophoresis. The PCR products were diluted with Milli-Q water and multiplexing of PCR products was carried out by diluting PCR products of two or three markers that differ in size and labelling colours for loading in a single lane. Then, the diluted products were run on an ABI 3730 automated sequencer with 10.0 µl of Hi-Di formamide (Applied Biosystems) and 0.15 µl of an internal size standard (GeneScan-500 Rox, Applied Biosystems). Sizes of microsatellites (bp) were determined by Genemapper ver. 4.0 (Applied Biosystems).

4.2.4 Data analysis

Statistical analysis was carried out using GenAlEx 6.5 b3 (Peakall and Smouse, 2006; Peakall and Smouse, 2012). Unbiased haploid diversity (*uh*) was calculated: $uh = (n/n\text{-}1)*(1-\sum pi^2)$, where *pi* = frequency of *i*th allele, *n* = sample size. Linkage disequilibrium (LD) for a haploid disequilibrium test was calculated for the *Paphiopedilum* dataset only: *Ve/Vo* (index of linkage disequilibrium), where *Ve* = expected variance of *K* (the number of loci for which two individuals differ), *Vo* = observed variance. For the *Cypripedium calceolus* population analysis, because of sparse sampling, for the purposes of statistical analysis, one sample from Italy was combined with samples from Austria as 'Eastern Alps', a sample from the Black Forest, Germany, was combined with samples from Switzerland as 'Switzerland/Germany' and a sample from Muhu Island, Estonia was combined with samples from Gotland Island, Sweden as 'Baltic Islands'. Haplotypes were defined from the different combinations of alleles. Distribution of genetic diversity within and among populations, using AMOVA (Excoffier *et al.*, 1992) were estimated using Phi_{PT} parameter (F_{ST} analogue) in GenAlEx 6.5 b3 (Peakall and Smouse, 2006; Peakall and Smouse, 2012). Levels of significance were obtained by the permutation test (9999 permutations). Pairwise Phi_{PT} values between each population were also estimated by GenAlEx 6.5 b3: Phi_{PT} = AP / (WP + AP) = AP / TOT, where AP= estimated variance among populations, WP =

estimated variance within a population, TOT = total estimated variance (Peakall and Smouse, 2006; Peakall and Smouse, 2012). The programme NETWORK 4.6.1.0 (http://www.fluxus-engineering.com) was used to construct a median-joining (MJ) network (Bandelt *et al.*, 1999) based on the plastid microsatellite haplotypes. MJ uses maximum-parsimony criteria to reconstruct intraspecific phylogenetic relationships. For UK samples only, haplotype network analysis of a combination of two polymorphic markers, ACpsbD of the present study and cyp2 of Fay and Cowan (2001) was also carried out.

4.3 Results

4.3.1 Size variation of plastid microsatellites on DNA sequence data

Alignments of DNA sequence data for 15 regions were prepared, to identify size variation in plastid microsatellites. Eight loci, containing potential size variable microsatellites (i.e. n > 9 mononucleotide repeats) from seven plastid regions were selected for primer design. For the *trnL-F* region, for which sequences were downloaded from GenBank, two potential size variable microsatellites were identified. The first *trnL-F* locus contained a poly-T repeat, including T_{17} in *Cypripedium*, T_{10} in *Paphiopedilum*, T_{11} in *Phragmipedium* and T_{10} in *Vanilla*. The second, *trnL-F* locus, also a Poly-T repeat, was found in *Phragmipedium* (T_{9-10}) and *Mexipedium* (T_{10}). For the *ycf1* 3' region, *Orchis* and *Vanilla* samples were not successfully amplified and sequenced but a potentially length variable microsatellite was found in *Cypripedium* (A_{10})*, Paphiopedilum* (A_{13}) and *Phragmipedium* (A_{10}). For the *trnF*(GAA)-*ndhJ* region, a potentially length variable microsatellite was found in *Paphiopedilum* ($T_{12-14}CATAT_{12-14}$) and *Phragmipedium* ($T_{7-9}CATAT_{10}$). For the *psbD-trnT* region, *Vanilla* and *Orchis* samples were not successfully amplified and sequenced but a potentially length variable microsatellite was identified in *Paphiopedilum* (T_{14}) and *Phragmipedium* (T_{10-14}). For the *trnC*(GCA)-*petN* region, *Cypripedium*, *Vanilla* and *Orchis* samples were not successfully amplified and sequenced, but a potentially length variable microsatellite, $T_{11,}$ was found in *Paphiopedilum* and *Phragmipedium*. For the *clpP*ex3-*clpP*ex2 region, only the *Orchis* sample was not successfully amplified and sequenced, but a potentially length variable microsatellite was found only in *Paphiopedilum* (T_{13}). For the *trnT*(UGU)-*L*(UAA) region**,** *Cypripedium* and *Orchis* samples were not successfully amplified and sequenced, but a potentially length variable microsatellite was found only in *Paphiopedilum* (A_{13}). See Table 4.3 for characteristics of the eight plastid microsatellite primers.

Table 4.3. Characteristics of eight plastid microsatellite primer pairs designed in this study. Degenerate positions are K (= T or G), Y (= C or T), R (= G or A), M (= C or A), W (= A or T).

Region	Primer	Sequences (5' to 3')	repeat motif						Size (bp)	Tm (°C)
			Cypripedium	***Paphiopedilum***	***Phragmipedium***	***Mexipedium***	***Selenipedium***	***Vanilla***		
trnL-F (1)	ACtrnL-1F	TCCCTCTATCCCCAAGAAAA	T_{17}	T_{10}	T_{11}	T_8	*repeat absent	T_{10}	139–164	55.3
	ACtrnL-1R	KATTYGGATCYRTTTGTGAA								51.2
trnL-F (2)	ACtrnL-2F	TTCACAAAYGGATCCGAATA	T_4	T_5	T_{9-10}	T_{10}	*repeat absent	*repeat absent	165–207	52.2
	ACtrnL-2R	CYCCTCAATTTCTTAGATCT								52.2
ycf1 3'	ACycf1-F	GCTGTTGAAGAAGATTAYGC	A_{10}	A_{13}	A_{10}	**	**	N/A	145–151	54.2
	ACycf1-R	GATCMGCCCATCTTAATTGA								54.2
trnF(GAA)-*ndhJ*	ACtrnF-F	ACACAGTACAAATCAACCCC	*T_6CATAT$_3$	T_{12-14}CATAT$_{12-14}$	T_{7-9}CATAT$_{10}$	**	**	*	138–151	55.3
	ACtrnF-R	TGAMCTTTGTAYCGCGCACA								57.3
psbD-trnT	ACpsbD-F	TAGKAACGATGTCGAAGTAG	T_9	T_{14}	T_{10-14}	**	**	N/A	256–290	54.2
	ACpsbD-R	GAGTCTTYKWTTTTCRGCR								52.4
trnC (GCA)-*petN*	ACtrnC-F	CTCTGAGTGCTCAGAAATGAA	N/A	T_{11}	T_{11}	**	**	N/A	131–206	55.9
	ACtrnC-R	ACGAATCCACTCGTAAAAAG								53.2
*clpP*ex3-*clpP*ex2	ACclpP-F	CTTGTCTGTGGTTTAGCAATC	T_7	T_{13}	T_8	**	**	*T_7	127–134	55.9
	ACclpP-R	TTCCACACCAGAAGTCTCTTT								55.9
trnT(UGU)-*L*(UAA)	ACtrnT-F	TATCATTATACACAAGAGGACG	N/A	A_{13}	A_6	**	**	*A_7	106–110	54.7
	ACtrnT-R	ATATCTATGCCTTKACTCCC								54.2

N/A, amplification failed; Tm, melting temperature; * , excluded for designing primers, * * , no DNA samples (*Mexipedium* and *Selenipedium* sequence data were downloaded from GenBank).

4.3.2 Primer design

Eight primer pairs were designed from seven plastid regions (out of 15 regions), *trnL-F*, *ycf1* 3', *trnF*(GAA*)-ndhJ*, *psbD-trnT*, *trnC*(GCA*)-petN*, *clpP*ex3-*clpP*ex2 and *trnT*(UGU)-*L*(UAA). Details of designed primers are listed in Table 4.3. Two primer pairs were developed for two microsatellites in the *trnL-F* region: ACtrnL-1, developed from consensus sequences of species of *Cypripedium*, *Paphiopedilum*, *Phragmipedium*, *Mexipedium* and *Vanilla*, and ACtrnL-2 developed from the same species excluding *Vanilla* spp. because the flanking areas for this locus were highly variable in comparison with the rest of the sequences. Primer pairs for microsatellites of *ycf*1, *psbD-trnT* and *clpP*ex3-*clpP*ex2 regions (ACycf1, ACpsbD, ACclpP, respectively) were developed from consensus sequences of species of *Cypripedium*, *Paphiopedilum* and *Phragmipedium*. Primer pairs for microsatellites of *trnF*(GAA*)-ndhJ*, *trnC*(GCA*)-petN* and *trnT*(UGU)-*L*(UAA) regions (ACtrnF, ACtrnC and ACtrnT, respectively) were developed from consensus sequences of species of *Paphiopedilum* and *Phragmipedium*.

4.3.3 Primer test and cross applicability in subfamily Cypripedioideae

Six primer pairs designed in this study, ACtrnL-2, ACycf1, ACpsbD, ACclpP, ACtrnF, and ACtrnT, were tested with 248 accessions of subfamily Cypripedioideae [*Cypripedium* (177), *Paphiopedilum* (66) and *Phragmipedium* (5)]. Two primer pairs, ACtrnC and ACtrnL-1, were tested with 111 accessions, excluding accessions of *Cypripedium calceolus* (137), because ACtrnC was preliminarily tested with negative results. ACtrnL-1 is the same locus as cyp2 (Fay and Cowan, 2001; Fay *et al.*, 2009) that was previously tested with this species. ACtrnL-1 was also tested with nine accessions of *Vanilla* spp. Also, two primer pairs (rps16-1 and rps16-2), previously developed for *C. calceolus* (Fay *et al.*, 2009), were tested with 111 accessions of Cypripedioideae but excluding all accessions from *C. calceolus* (137). The results for the primer test can be found in Tables 4.4–4.6. Five markers, ACtrnL-1, ACtrnL-2, ACclpP, ACycf1, rps16-1, were successfully amplified in all accessions of *Paphiopedilum* spp. (66). Nine markers were successfully amplified in all accessions of *Phragmipedium* spp. (5), except for one accession for ACtrnF. ACtrnC amplified well in *Paphiopedilum* and *Phragmipedium* spp. but was least successfully amplified in *Cypripedium* spp., (only 17.5%, excluding *C. calceolus* accessions).

Table 4.4. Allele size of plastid microsatellites in taxa of genera *Paphiopedilum*, *Cypripedium*, *Phragmipedium* and *Vanilla*.

Taxa	Section	accession no.	ACpsbD	ACtrnL-1	ACtrnL-2	ACtrnC	ACtrnF	ACclpP	ACtrnT	ACycf1	rps16-1	rps16-2
Paphiopedilum bullenianum	*Barbata*	39145	285	140	171	260	145	127	109	141	140	294
Paphiopedilum bullenianum	*Barbata*	39146	285	140	171	260	145	127	109	141	140	294
Paphiopedilum callosum	*Barbata*	10039	286	141	171	377	144	127	107	141	141	257
Paphiopedilum callosum var. *sublaeve*	*Barbata*	10054	285	139	171	531	143	127	107	141	141	257
Paphiopedilum callosum var. *sublaeve* (syn. = *P. warnerianum*)	*Barbata*	39144	286	141	171	478	143	127	108	141	141	257
Paphiopedilum superbiens var. *curtisii*	*Barbata*	10040	285	141	171	290	151	127	109	141	139	241
Paphiopedilum appletonianum	*Barbata*	5897	286	140	171	430	143	127	108	141	141	241
Paphiopedilum barbatum	*Barbata*	39143	287	141	171	375	143	127	109	141	140	241
Paphiopedilum barbatum	*Barbata*	39147	286	144	171	323	143	127	110	141	140	240
Paphiopedilum barbatum	*Barbata*	39148	286	144	171	323	143	127	110	141	140	240
Paphiopedilum barbatum	*Barbata*	39149	285	141	171	500	143	127	108	141	141	257
Paphiopedilum barbatum	*Barbata*	5898	287	141	171	376	143	127	109	141	140	241
Paphiopedilum ciliolare	*Barbata*	10052	285	141	160	320	149	127	109	141	140	240
Paphiopedilum fowliei	*Barbata*	O-644	285	145	171	323	147	127	106	141	140	240
Paphiopedilum hennisianum	*Barbata*	10053	285	141	171	—	131	127	108	141	140	228
Paphiopedilum javanicum var. *virens*	*Barbata*	O-635	285	140	171	310	148	127	109	141	140	246
Paphiopedilum lawrenceanum	*Barbata*	36824	291	140	171	200	151	127	110	141	140	241
Paphiopedilum mastersianum	*Barbata*	5900	285	141	171	397	121	127	109	141	140	241
Paphiopedilum sangii	*Barbata*	O-822	286	140	171	320	150	125	110	150	141	251
Paphiopedilum sukhakulii	*Barbata*	5901	285	141	171	266	144	127	109	141	140	247
Paphiopedilum tonsum	*Barbata*	5902	285	141	171	266	144	127	109	141	140	247
Paphiopedilum tonsum var. *braemii*	*Barbata*	O-824	291	141	171	210	150	127	110	141	140	242
Paphiopedilum violascens	*Barbata*	O-825	285	141	171	383	133	127	108	141	140	240
Paphiopedilum wardii	*Barbata*	5903	287	140	164	352	148	127	109	141	140	309
Paphiopedilum concolor	*Brachypetalum*	10048	290	141	206	285	141	126	108	141	141	338
Paphiopedilum concolor	*Brachypetalum*	O-1273	289	140	206	315	139	126	108	141	138	347
Paphiopedilum niveum	*Brachypetalum*	36862	289	139	206	297	141	126	110	141	140	322
Paphiopedilum liemianum	*Cochlopetalum*	36858	288	140	206	273	140	127	109	141	140	261
Paphiopedilum liemianum	*Cochlopetalum*	O-631	288	140	206	273	140	127	109	141	140	261
Paphiopedilum primulinum	*Cochlopetalum*	36827	288	140	206	294	141	127	113	141	140	272
Paphiopedilum primulinum var. *purpurascens*	*Cochlopetalum*	36860	288	140	206	294	141	127	113	141	140	272

Table 4.4. Continued

Taxa	Section	accession no.	ACpsbD	ACtrnL-1	ACtrnL-2	ACtrnC	ACtrnF	ACclpP	ACtrnT	ACycf1	rps16-1	rps16-2
Paphiopedilum glaucophyllum	*Cochlopetalum*	10049	291	142	206	292	143	127	116	141	140	237
Paphiopedilum victoria-regina	*Cochlopetalum*	O-630	292	140	206	337	143	127	117	141	141	237
Paphiopedilum philippinense	*Coryopedilum*	36807	290	141	206	—	158	130	117	141	141	241
Paphiopedilum randsii	*Coryopedilum*	O-636	297	139	206	391	145	137	—	141	154	241
Paphiopedilum rothschildianum	*Coryopedilum*	36806	286	140	206	430	141	138	117	141	142	217
Paphiopedilum wilhelminiae	*Coryopedilum*	36825	290	138	206	—	145	142	—	141	138	—
Paphiopedilum adductum	*Coryopedilum*	36820	289	139	206	385	141	133	—	141	154	255
Paphiopedilum glanduliferum	*Coryopedilum*	O-716	291	138	206	373	152	131	117	141	136	236
Paphiopedilum glanduliferum	*Coryopedilum*	O-717	291	139	206	415	141	132	118	141	137	353
Paphiopedilum kolopakingii	*Coryopedilum*	10089	—	140	206	470	158	132	116	141	141	232
Paphiopedilum stonei	*Coryopedilum*	10042	291	139	206	412	150	133	116	141	146	282
Paphiopedilum charlesworthii	*Paphiopedilum*	O-632	288	138	171	281	143	127	113	141	140	251
Paphiopedilum druryi	*Paphiopedilum*	36811	285	140	206	273	143	127	104	140	140	267
Paphiopedilum exul	*Paphiopedilum*	36804	307	138	206	345	148	127	109	141	140	258
Paphiopedilum gratrixianum	*Paphiopedilum*	36809	288	138	171	268	144	129	113	140	140	251
Paphiopedilum gratrixianum	*Paphiopedilum*	40235	288	138	171	268	145	129	113	140	140	251
Paphiopedilum gratrixianum	*Paphiopedilum*	40236	288	138	171	268	144	129	113	140	140	251
Paphiopedilum gratrixianum	*Paphiopedilum*	40237	288	138	171	268	144	129	113	140	140	251
Paphiopedilum hirsutissimum	*Paphiopedilum*	36808	287	142	208	279	141	127	111	200	140	225
Paphiopedilum hirsutissimum var. *esquirolei*	*Paphiopedilum*	O-642	287	141	208	272	142	129	113	200	140	225
Paphiopedilum insigne	*Paphiopedilum*	36821	289	138	171	306	147	127	113	141	140	252
Paphiopedilum spicerianum	*Paphiopedilum*	O-643	288	138	171	354	145	127	113	141	140	259
Paphiopedilum tigrinum	*Paphiopedilum*	O-713	289	140	171	73	—	127	111	140	140	262
Paphiopedilum villosum var. *boxallii*	*Paphiopedilum*	36822	289	138	171	306	147	127	113	140	140	252
Paphiopedilum dianthum	*Pardalopetalum*	10051	289	139	199	206	139	130	106	141	139	266
Paphiopedilum dianthum	*Pardalopetalum*	O-641	289	139	199	206	139	130	106	141	139	266
Paphiopedilum dianthum	*Pardalopetalum*	5899	289	139	199	206	139	130	106	141	139	266
Paphiopedilum haynaldianum	*Pardalopetalum*	O-175	284	144	199	137	130	129	103	141	137	245
Paphiopedilum lowii	*Pardalopetalum*	10050	289	140	199	193	133	128	104	141	137	245
Paphiopedilum lowii	*Pardalopetalum*	36810	291	140	199	193	133	128	104	141	137	245
Paphiopedilum parishii	*Pardalopetalum*	10038	287	139	199	208	138	131	106	141	140	265

Table 4.4. Continued

Taxa	Section	accession no.	ACpsbD	ACtrnL-1	ACtrnL-2	ACtrnC	ACtrnF	ACclpP	ACtrnT	ACycf1	rps16-1	rps16-2
Paphiopedilum delenatii	*Parvisepalum*	36826	288	142	206	246	139	127	102	141	140	237
Paphiopedilum delenatii	*Parvisepalum*	39746	288	142	206	245	139	127	102	141	140	237
Paphiopedilum malipoense	*Parvisepalum*	10041	286	139	206	268	140	129	102	141	140	237
Paphiopedilum micranthum	*Parvisepalum*	O-629	288	142	206	242	138	129	109	141	141	235
Cypripedium acaule	*Acaulia*	141.19	—	147	211	—	140	125	96	148	137	458
Cypripedium plectrochilum	*Arietinum*	35014	444	158	—	—	123	125	97	144	141	—
Cypripedium plectrochilum	*Arietinum*	141.20	438	157	206	—	123	125	97	—	142	—
Cypripedium guttatum	*Bifolia*	O-958	329	146	200	595	140	125	98	148	137	400
Cypripedium guttatum	*Bifolia*	141.05	329	145	200	—	140	125	98	148	137	400
Cypripedium guttatum	*Bifolia*	141.21	329	145	200	595	140	125	98	148	137	400
Cypripedium calceolus (137 accesions)	*Cypripedium*	see Table 4.5	437–441	*	206	*	140–141	121	97–98	148	*	*
Cypripedium cordigerum	*Cypripedium*	O-952	—	189	202	—	141	121	102	148	152	279
Cypripedium farreri	*Cypripedium*	141.03	—	180	206	—	141	121	100	148	139	274
Cypripedium fasciolatum	*Cypripedium*	5723	—	182	206	—	141	121	99	148	139	274
Cypripedium fasciolatum	*Cypripedium*	O-1270	—	179	213	—	141	121	98	148	138	279
Cypripedium fasciolatum	*Cypripedium*	141.11	—	180	206	—	141	121	100	148	139	274
Cypripedium henryi	*Cypripedium*	141.09	439	190	206	—	141	121	99	148	146	284
Cypripedium himalaicum	*Cypripedium*	141.10	—	153	206	—	141	121	98	148	138	280
Cypripedium macranthos	*Cypripedium*	141.06	—	188	211	—	141	121	—	148	144	284
Cypripedium macranthos (syn. = *C. rebunense*)	*Cypripedium*	O-957	—	188	211	—	141	121	—	148	144	284
Cypripedium montanum	*Cypripedium*	141.22	—	190	206	—	141	121	97	148	139	293
Cypripedium parviflorum var. *pubescens*	*Cypripedium*	19107	432	199	206	—	140	121	99	148	149	282
Cypripedium parviflorum	*Cypripedium*	18786	433	199	206	—	140	121	99	148	149	281
Cypripedium segawai	*Cypripedium*	141.02	—	196	206	—	141	121	99	148	—	—
Cypripedium tibeticum	*Cypripedium*	5724	438	136	—	—	138	125	98	148	135	—
Cypripedium tibeticum	*Cypripedium*	141.18	—	178	206	—	141	121	98	148	138	280
Cypripedium yunnanense	*Cypripedium*	141.07	—	190	206	—	141	121	97	148	139	293
Cypripedium fasciculatum	*Enantiopedilum*	O-1269	332	180	208	—	99	124	—	141	137	326
Cypripedium formosanum	*Flabellinervia*	16308	312	137	200	364	140	125	99	148	137	278
Cypripedium formosanum	*Flabellinervia*	*141.*16	—	136	200	—	140	125	99	148	137	278
Cypripedium formosanum	*Flabellinervia*	O-956	312	137	200	364	140	125	99	148	137	278

Table 4.4. Continued

Taxa	Section	accession no.	ACpsbD	ACtrnL-1	ACtrnL-2	ACtrnC	ACtrnF	ACclpP	ACtrnT	ACycf1	rps16-1	rps16-2
Cypripedium japonicum	*Flabellinervia*	141.17	312	136	200	354	140	125	99	148	137	278
Cypripedium californicum	*Irapeana*	141.01	332	165	200	—	133	126	95	148	138	294
Cypripedium flavum	*Obtusipelata*	5725	257	143	200	—	140	125	125	148	142	299
Cypripedium flavum	*Obtusipelata*	141.15	257	143	200	—	140	125	98	148	141	299
Cypripedium passerinum	*Obtusipelata*	10045	255	144	200	409	140	131	95	148	142	349
Cypripedium reginae	*Obtusipelata*	141.14	255	145	200	—	140	131	96	148	142	298
Cypripedium subtropicum	*Subtropica*	O-888	—	—	—	—	—	—	—	—	—	275
Cypripedium wardii	*Subtropica*	O-887	—	—	—	—	—	—	—	—	—	—
Cypripedium wardii (unverified)	*Subtropica*	141.12	—	166	206	—	152	125	98	148	136	—
Cypripedium bardolphianum	*Trigonopedia*	5722	438	136	—	—	138	125	98	148	135	—
Cypripedium lichiangense	*Trigonopedia*	O-953	—	146	—	—	169	125	99	148	136	279
Cypripedium lichiangense	*Trigonopedia*	141.08	383	140	—	580	147	125	98	148	136	—
Cypripedium margaritaceum	*Trigonopedia*	7911	—	146	—	—	169	125	99	148	136	—
Cypripedium hotei		10046	469	187	209	—	141	121	101	148	140	288
Phragmipedium besseae	*Micropetalum*	10047	284	156	191	128	—	131	103	141	136	299
Phragmipedium caudatum	*Phragmipedium*	O-176	293	146	207	128	135	132	103	148	136	561
Phragmipedium lindenii	*Phragmipedium*	10043	293	145	207	127	135	132	103	148	136	562
Phragmipedium longifolium	*Lorifolia*	10044	286	134	206	117	136	133	103	148	136	402
Phragmipedium vittatum	*Lorifolia*	O-1293	290	141	210	129	141	132	103	148	136	522
Vanilla annamica		O1274	*	166	*	*	*	*	*	*	*	*
Vanilla cf. barbellata		O-591	*	—	*	*	*	*	*	*	*	*
Vanilla havilandii		8098	*	—	*	*	*	*	*	*	*	*
Vanilla imperialis		O-587	*	177	*	*	*	*	*	*	*	*
Vanilla imperialis		8099	*	177	*	*	*	*	*	*	*	*
Vanilla kinabaluensis		O-788	*	—	*	*	*	*	*	*	*	*
Vanilla planifolia		O-170	*	161	*	*	*	*	*	*	*	*
Vanilla planifolia		O-582	*	163	*	*	*	*	*	*	*	*
Vanilla roscheri		O-540	*	—	*	*	*	*	*	*	*	*

—, no amplified products; *, samples excluded for primer test

Table 4.5. Allele size of plastid microsatellites in taxa of *Cypripedium calceolus*.

Locality	accession no.	ACpsbD	ACtrnF	ACtrnL-2	ACclpP	ACtrnT	ACycf1
Austria (Central Tyrol; Coll: 1881)	12734	—	—	—	—	—	—
Austria (South East Tyrol; Coll: 1878)	12735	—	—	—	—	—	—
Austria (A)	12737	437	141	206	121	97	148
Austria (B)	12738	439	141	206	121	97	148
Austria (C)	12739	437	141	206	121	97	148
Austria (D)	12740	437	141	206	121	97	148
Austria (E)	12741	—	141	206	121	97	148
Austria (F)	12742	439	141	206	121	97	148
China (Manchuria)	A03	—	141	—	—	—	—
Denmark (Skindbjerg)	106.01	439	141	206	121	97	148
Denmark (Skindbjerg)	106.02	439	141	206	121	97	148
Denmark (Skindbjerg)	106.03	439	141	206	121	97	148
Denmark (Skindbjerg)	106.04	439	141	206	121	97	148
Denmark (Bjergeskov)	106.05	439	141	206	121	97	148
Denmark (Bjergeskov)	106.06	439	141	206	121	97	148
Denmark (Bjergeskov)	106.07	439	141	206	121	97	148
Denmark (Bjergeskov)	106.08	439	141	206	121	97	148
Denmark (Skindbjerg)	109.01	439	141	206	121	97	148
Denmark (Skindbjerg)	109.02	439	141	206	121	97	148
Denmark (Skindbjerg)	109.03	439	141	206	121	97	148
Denmark (Skindbjerg)	109.04	439	141	206	121	97	148
Denmark (Bjergeskov)	109.05	439	141	206	121	97	148
Denmark (Bjergeskov)	109.06	439	141	206	121	97	148
Denmark (Bjergeskov)	109.07	439	141	206	121	97	148
Denmark (Bjergeskov)	109.08	439	141	206	121	97	148

Table 4.5. Continued

Locality	accession no.	ACpsbD	ACtrnF	ACtrnL-2	ACclpP	ACtrnT	ACycf1
Denmark (Skindbjerg)	110.01	439	141	206	121	97	148
Denmark (Skindbjerg)	110.02	439	141	206	121	97	148
Denmark (Skindbjerg)	110.03	439	141	206	121	97	148
Denmark (Skindbjerg)	110.04	439	141	206	121	97	148
Denmark (Bjergeskov)	110.05	439	141	206	121	97	148
Denmark (Bjergeskov)	110.06	439	141	206	121	97	148
Denmark (Bjergeskov)	110.07	439	141	206	121	97	148
Denmark (Bjergeskov)	110.08	439	141	206	121	97	148
Denmark (Skindbjerg)	111.01	439	141	206	121	97	148
Denmark (Skindbjerg)	111.02	439	141	206	121	97	148
Denmark (Skindbjerg)	111.03	439	141	206	121	97	148
Denmark (Skindbjerg)	111.04	439	141	206	121	97	148
Denmark (Skindbjerg)	111.05	439	141	206	121	97	148
Denmark (Skindbjerg)	111.06	439	141	206	121	97	148
Denmark (Skindbjerg)	111.07	439	141	206	121	97	148
Denmark (Skindbjerg)	111.08	439	141	206	121	97	148
Denmark (Skindbjerg)	111.09	439	141	206	121	97	148
Denmark (Skindbjerg)	111.10	439	141	206	121	97	148
Estonia	9484	441	141	206	121	97	148
France (Les Nonieres)	O-1115	439	141	206	121	97	148
France (Col du Prayet)	O-1116	440	141	206	121	97	148
France	O-871	439	141	206	121	97	148
Germany (Black Forest, Lembach)	16399	439	141	206	121	97	148
Germany	O-882	—	141	206	121	98	148

Table 4.5. Continued

Locality	accession no.	ACpsbD	ACtrnF	ACtrnL-2	ACclpP	ACtrnT	ACycf1
Italy (Dolomites)	O-754	439	141	206	121	97	148
Poland (Wigry National Park)	12470	—	141	206	121	97	148
Poland (Wigry National Park)	12471	—	141	206	121	97	148
Poland (Roztocze National Park)	12472	—	141	206	121	97	148
Poland (Roztocze National Park)	12473	—	141	206	121	97	148
Poland (Roztocze National Park)	12474	—	141	206	121	97	148
Poland (Roztocze National Park)	12475	—	141	206	121	97	148
Poland (Roztocze National Park)	12476	—	141	206	121	97	148
Poland (Roztocze National Park)	12477	—	141	206	121	97	148
Russia (Aginskoje; Coll: 1948)	12733	—	—	—	—	—	—
Russia (Nr. Leningrad)	A06	—	141	206	121	97	148
Russia (Chitinskaya; 1962)	A02	—	140	—	121	97	148
Russia (Amur Oblast; 1957)	A04	—	140	—	121	97	148
Spain (Pyrenees)	1P	440	141	206	121	97	148
Spain (Pyrenees)	2P	439	141	206	121	97	148
Spain (Pyrenees)	3P	439	141	206	121	97	148
Spain (Pyrenees)	4P	439	141	206	121	97	148
Spain (Pyrenees)	1SG	439	141	206	121	97	148
Spain (Pyrenees)	2SG	439	141	206	121	97	148
Spain (Pyrenees)	3SG	439	141	206	121	97	148
Spain (Pyrenees)	4SG	439	141	206	121	97	148
Spain (Pyrenees)	5SG	439	141	206	121	97	148
Sweden	A05	—	141	206	121	97	148
Sweden (Kallgatburg, North Gotland)	O-1380	439	141	206	121	97	148

Table 4.5. Continued

Locality	accession no.	ACpsbD	ACtrnF	ACtrnL-2	ACclpP	ACtrnT	ACycf1
Sweden (Gotland)	O-1387	439	141	206	121	97	148
Sweden	1.1_1	439	141	206	121	97	148
Sweden	1.2_2	439	141	206	121	97	148
Sweden	1.3_3	439	141	206	121	97	148
Sweden	1.4_4	439	141	206	121	97	148
Sweden	1.5_5	439	141	206	121	97	148
Sweden	10.1_4	439	141	206	121	97	148
Sweden	10.2_5	439	141	206	121	97	148
Sweden	10.3_6	439	141	206	121	97	148
Sweden	11.1_7	439	141	206	121	97	148
Sweden	11.2_8	—	141	206	121	97	148
Sweden	11.3_9	—	141	206	121	97	148
Sweden	11.4_10	—	141	206	121	97	148
Sweden	2.1_6	439	141	206	121	97	148
Sweden	2.2_7	439	141	206	121	97	148
Sweden	2.3_8	439	141	206	121	97	148
Sweden	3.1_9	439	141	206	121	97	148
Sweden	3.2_10	439	141	206	121	97	148
Sweden	3.3_11	439	141	206	121	97	148
Sweden	4.1_12	439	141	206	121	97	148
Sweden	4.2_13	439	141	206	121	97	148
Sweden	4.3_14	439	141	206	121	97	148
Sweden	4.4_15	439	141	206	121	97	148
Sweden	5.1_16	439	141	206	121	98	148

Table 4.5. Continued

Locality	accession no.	ACpsbD	ACtrnF	ACtrnL-2	ACclpP	ACtrnT	ACycf1
Sweden	5.2_1	439	141	206	121	98	148
Sweden	6.1_5	439	141	206	121	97	148
Sweden	5.3_2	439	141	206	121	98	148
Sweden	5.4_3	439	141	206	121	98	148
Sweden	5.5_4	439	141	206	121	98	148
Sweden	6.2_6	439	141	206	121	97	148
Sweden	6.3_7	439	141	206	121	97	148
Sweden	6.4_8	439	141	206	121	97	148
Sweden	7.1_9	440	141	206	121	97	148
Sweden	7.2_10	439	141	206	121	97	148
Sweden	7.3_11	439	141	206	121	97	148
Sweden	7.4_12	439	141	206	121	97	148
Sweden	8.1_13	439	141	206	121	97	148
Sweden	8.2_14	439	141	206	121	97	148
Sweden	8.3_15	439	141	206	121	97	148
Sweden	9.1_16	439	141	206	121	97	148
Sweden	9.2_1	440	141	206	121	97	148
Sweden	9.3_2	440	141	206	121	97	148
Sweden	9.4_3	439	141	206	121	97	148
Switzerland (Creux du Vent n1)	12435	440	141	206	121	97	148
Switzerland (Creux du Vent n2)	12436	437	141	206	121	97	148
Switzerland (Creux du Vent n3)	12437	—	141	206	121	97	148
Switzerland (Orvin Sonvilier)	12438	439	141	206	121	97	148
Switzerland (Pavillion La Neuveville)	12439	439	141	206	121	97	148

Table 4.5. Continued

Locality	accession no.	ACpsbD	ACtrnF	ACtrnL-2	ACclpP	ACtrnT	ACycf1
Switzerland (Kandosteg Oeschinen)	12440	439	141	206	121	97	148
Switzerland (Gasterntal)	12441	439	141	206	121	97	148
Switzerland (Eggiwil)	12442	—	141	206	121	97	148
Switzerland (ex Zurich BG)	O-714	439	141	206	121	97	148
Switzerland	A01	439	141	206	121	97	148
UK (UK1)	O-1078	439	141	206	121	97	148
UK (UK3)	O-1080	439	141	206	121	97	148
UK (UK4)	O-1081	440	141	206	121	97	148
UK (UK5)	O-1082	439	141	206	121	97	148
UK (UK6)	O-1083	440	141	206	121	97	148
UK (UK7)	O-1084	439	141	206	121	97	148
UK (UK8)	O-1085	439	141	206	121	97	148
Yugoslavia (Sveti Ana; Coll: 1938)	12736	—	—	—	—	—	—
Unknown (RBG Kew, LivColl. s.n)	38939	439	141	206	121	97	148
Unknown (RBG Kew, Tropical Nursery)	19106	440	141	206	121	97	148
Unknown (RBG, Kew)	141.04	439	—	206	121	98	148

—, no amplified products.

Table 4.6. Summary of size variation, number of alleles and success of amplification of plastid microsatellites and unbiased haploid diversity (*uh*).

Locus	Taxa	No. samples	No. amplified products	Amplification %	Size range	No. alleles	*uh*
ACpsbD	*Paphiopedilum*	66	65	98.48	284–307	11	0.865
	Cypripedium (inc. *C. calceolus*)	177	134	75.71	255–469	15	0.438
	Cypripedium calceolus	137	113	82.48	437–441	4	0.227
	Phragmipedium	5	5	100	284–293	4	0.900
ACtrnL-1	*Paphiopedilum*	66	66	100	138–145	7	0.807
	Cypripedium (exc. *C. calceolus*)	40	38	95	136–199	23	0.969
	Phragmipedium	5	5	100	134–156	5	1.000
	Vanillla	9	5	55.56	161–177	4	0.900
ACtrnL-2	*Paphiopedilum*	66	66	100	160–208	6	0.644
	Cypripedium (inc. *C. calceolus*)	177	162	91.53	200–213	7	0.216
	Cypripedium calceolus	137	130	94.89	206	1	0.000
	Phragmipedium	5	5	100	191–210	4	0.900
ACtrnC	*Paphiopedilum*	66	63	95.45	73–531	46	0.987
	Cypripedium (exc. *C. calceolus*)	40	7	17.5	354–595	5	0.905
	Phragmipedium	5	5	100	117–129	4	0.900
ACtrnF	*Paphiopedilum*	66	65	98.48	121–158	19	0.925
	Cypripedium (inc. *C. calceolus*)	177	170	96.05	99–169	9	0.275
	Cypripedium calceolus	137	132	96.35	140–141	2	0.030
	Phragmipedium	5	4	80	135–141	3	0.833
ACclpP	*Paphiopedilum*	66	66	100	125–142	12	0.635
	Cypripedium (inc. *C. calceolus*)	177	170	96.05	121–131	5	0.232
	Cypripedium calceolus	137	132	96.35	121	1	0.000
	Phragmipedium	5	5	100	131–133	3	0.700
ACtrnT	*Paphiopedilum*	66	63	95.45	102–118	13	0.886
	Cypripedium (inc. *C. calceolus*)	177	167	94.35	95–125	9	0.389
	Cypripedium calceolus	137	132	96.35	97–98	2	0.101
	Phragmipedium	5	5	100	103	1	0.000
ACycf1	*Paphiopedilum*	66	66	100	140–200	4	0.272
	Cypripedium (inc. *C. calceolus*)	177	169	95.48	141–148	3	0.024
	Cypripedium calceolus	137	132	96.35	148	1	0.000
	Phragmipedium	5	5	100	141–148	2	0.400
rps16-1	*Paphiopedilum*	66	66	100	136–154	9	0.604
	Cypripedium (exc. *C. calceolus*)	40	37	92.5	135–152	12	0.898
	Phragmipedium	5	5	100	136	1	0.000
rps16-2	*Paphiopedilum*	66	65	98.48	217–353	32	0.961
	Cypripedium (exc. *C. calceolus*)	40	31	77.5	274–458	17	0.955
	Phragmipedium	5	5	100	299–562	5	1.000

4.3.4 Polymorphism of plastid microsatellites in Cypripedioideae

All plastid microsatellite markers are variable in size (length in base pairs, bp) (Tables 4.4–4.6). There is size variation in each genus, for all markers, except ACtrnT and rps16-1 in *Phragmipedium* spp. There were four to 46 alleles detected among markers in *Paphiopedilum*, ACtrnC being the most polymorphic marker, producing 46 alleles from 63 amplified products (*uh* = 0.987), ranging between 73 and 531 bp and ACycf1 was the least polymorphic marker with only four alleles detected from 66 amplified products (*uh* = 0.272) ranging from 140 to 200 bp. There were three to 23 alleles

detected among all markers in *Cypripedium*, ACtrnL-1 being the most polymorphic, with 23 alleles detected from 38 amplified products (*uh* = 0.969). Considering six markers tested for all accessions of the genus *Cypripedium*, including 137 accessions of *C. calceolus*, ACpsbD was the most polymorphic marker, detecting 15 alleles from 134 amplified products (*uh* = 0.438), with a range of 255–469 bp and ACycf1 was the least polymorphic marker, producing only three alleles from 169 amplified products (*uh* = 0.024) ranging between 141–148 bp. In *Phragmipedium*, there were one to five alleles. ACtrnL-1 and rps16-2 were the most polymorphic markers, detecting five alleles from five amplified products (*uh* = 1.000) ranging between 134–156 and 299–562 bp. There was only one allele detected by ACtrnT (103) and rps16-1 (136) markers for this genus (*uh* = 0.000). In *C. calceolus* accessions alone, there were one to four alleles detected. However, three primers detected only one allele from 130 (ACtrnL-2) or 132 (ACclpP and ACycf1) amplified products (*uh* = 0.000). There were only three polymorphic markers out of six markers tested on *C. calceolus*, ACpsbD detecting four alleles out of 113 amplified products ranging from 437 to 441 bp (*uh* = 0.227), ACtrnT detected two alleles from 132 amplified products (*uh* = 0.101) and ACtrnF detected two alleles from 132 amplified products (*uh* = 0.030).

In other species apart from *C. calceolus*, although the sample size at intraspecific level was low, with only two to five individuals represented for each taxon, most primers were found to be polymorphic, except for ACycf1 only (Table 4.4, summary Table 4.7). Intraspecific polymorphisms were found in *Paphiopedilum callosum* (Rchb.f.) Stein var. *sublaeve* (Rchb.f.) P.J.Cribb (ACpsbD, ACtrnL-1, ACtrnC, ACtrnT), *P. barbatum* (ACpsbD, ACtrnL-1, ACtrnC, ACtrnT, rps16-1, rps16-2), *P. concolor* (ACpsbD, ACtrnL-1, ACtrnC, ACtrnF, rps16-1, rps16-2), *P. glanduliferum* (ACtrnL-1, ACtrnC, ACtrnF, ACclpP, ACtrnT, rps16-1, rps16-2), *P. gratrixianum* (Mast.) Rolfe (ACtrnF), *P. lowii* (ACpsbD), *P. delenatii* (ACtrnC), *Cypripedium plectrochilum* Franch. (ACpsbD, ACtrnL-1, rps16-1), *C. guttatum* Sw. (ACtrnL-1), *C. fasciolatum* Franch. (ACtrnL-1, ACtrnL-2, ACtrnT, rps16-1, rps16-2), *C. tibeticum* King ex Rolfe (ACtrnL-1, ACtrnF, ACclpP, rps16-1), *C. formosanum* Hayata (ACtrnL-1), *C. flavum* P.F.Hunt & Summerh. (ACtrnT, rps16-1), *C. lichiangense* S.C.Chen & P.J.Cribb (ACtrnL-1, ACtrnF, ACtrnT) and *Vanilla planifolia* Andrews (ACtrnL-1) (Table 4.7).

Table 4.7. Summary of allele size of amplified PCR products detected by polymorphic plastid microsatellite markers for taxa with multiple accessions of *Paphiopedilum*, *Cypripedium* and *Vanilla*.

Taxa	ACpsbD	ACtrnL-1	ACtrnL-2	ACtrnC	ACtrnF	ACclpP	ACtrnT	rps16-1	rps16-2
P. callosum var. *sublaeve*	285, 286 (n = 2)	139, 141 (n = 2)	*	478, 531 (n = 2)	*	*	107, 108 (n = 2)	*	*
P. barbatum	285, 286, 287 (n = 5)	141, 144 (n = 5)	*	323, 375, 376, 500 (n = 5)	*	*	108, 109, 110 (n = 5)	140, 141 (n = 5)	240, 241, 257 (n = 5)
P. concolor	289, 290 (n = 2)	140, 141 (n = 2)	*	285, 315 (n = 2)	139, 141 (n = 2)	*	*	138, 141 (n = 2)	338, 347 (n = 2)
P. glanduliferum	*	138, 139 (n = 2)	*	373, 415 (n = 2)	141, 152 (n = 2)	131, 132 (n = 2)	117, 118 (n = 2)	136, 137 (n = 2)	236, 353 (n = 2)
P. gratrixianum	*	*	*	*	144, 145 (n = 4)	*	*	*	*
P. lowii	289, 291 (n = 2)	*	*	*	*	*	*	*	*
P. delenatii	*	*	*	245, 246 (n = 2)	*	*	*	*	*
C. plectrochilum	438, 444 (n = 2)	157, 158 (n = 2)	*	*	*	*	*	141, 142 (n = 2)	*
C. guttatum	*	145, 146 (n = 3)	*	*	*	*	*	*	*
C. fasciolatum	*	179, 180, 182 (n = 3)	206, 213 (n = 3)	*	*	*	98, 99, 100 (n = 3)	138, 139 (n = 3)	274, 279 (n = 3)
C. tibeticum	*	136, 178 (n = 2)	*	*	138, 141 (n = 2)	121, 125 (n = 2)	*	135, 138 (n = 2)	*
C. formosanum	*	136, 137 (n = 3)	*	*	*	*	*	*	*
C. flavum	*	*	*	*	*	*	98, 125 (n = 2)	141, 142 (n = 2)	*
C. lichiangense	*	140, 146 (n = 2)	*	*	147, 169 (n = 2)	*	98, 99 (n = 2)	*	*
C. calceolus	437, 439, 440, 441 (n = 113)	**	*	**	140, 141 (n = 132)	*	97, 98 (n = 132)	**	**
V. planifolia	**	161, 163 (n = 2)	**	**	**	**	**	**	**

n, number of amplified PCR products; ACycf1 was monomorphic in all amplified accessions; * , monomorphic markers or no amplified products; ** , primers were not tested.

4.3.5 Population genetic study of *Cypripedium calceolus*

4.3.5.1 Haplotypes in *Cypripedium calceolus*

There were only three polymorphic microsatellites found in *C. calceolus* and only two samples from eastern Russia had a unique allele for ACtrnF (140). Excluding all missing data and unknown samples, five haplotypes were obtained from a combination of two polymorphic markers, ACpsbD and ACtrnT, from 110 samples from eight populations of *C. calceolus* in Europe; Eastern Alps, Denmark, France, Spain, Baltic Islands, Sweden, Switzerland/Germany and UK (Figure 4.5). The most common haplotype, H2 (alleles 439, 97), was found in all populations. Haplotype, H4 (alleles 440, 97) was found in five populations. Haplotype, H1 (alleles 437, 97) was found in two populations. There are two unique haplotypes; H3 (alleles 439, 98), found in Sweden and H5 (alleles 441, 97), found in the Baltic Islands (only Muhu, Estonia).

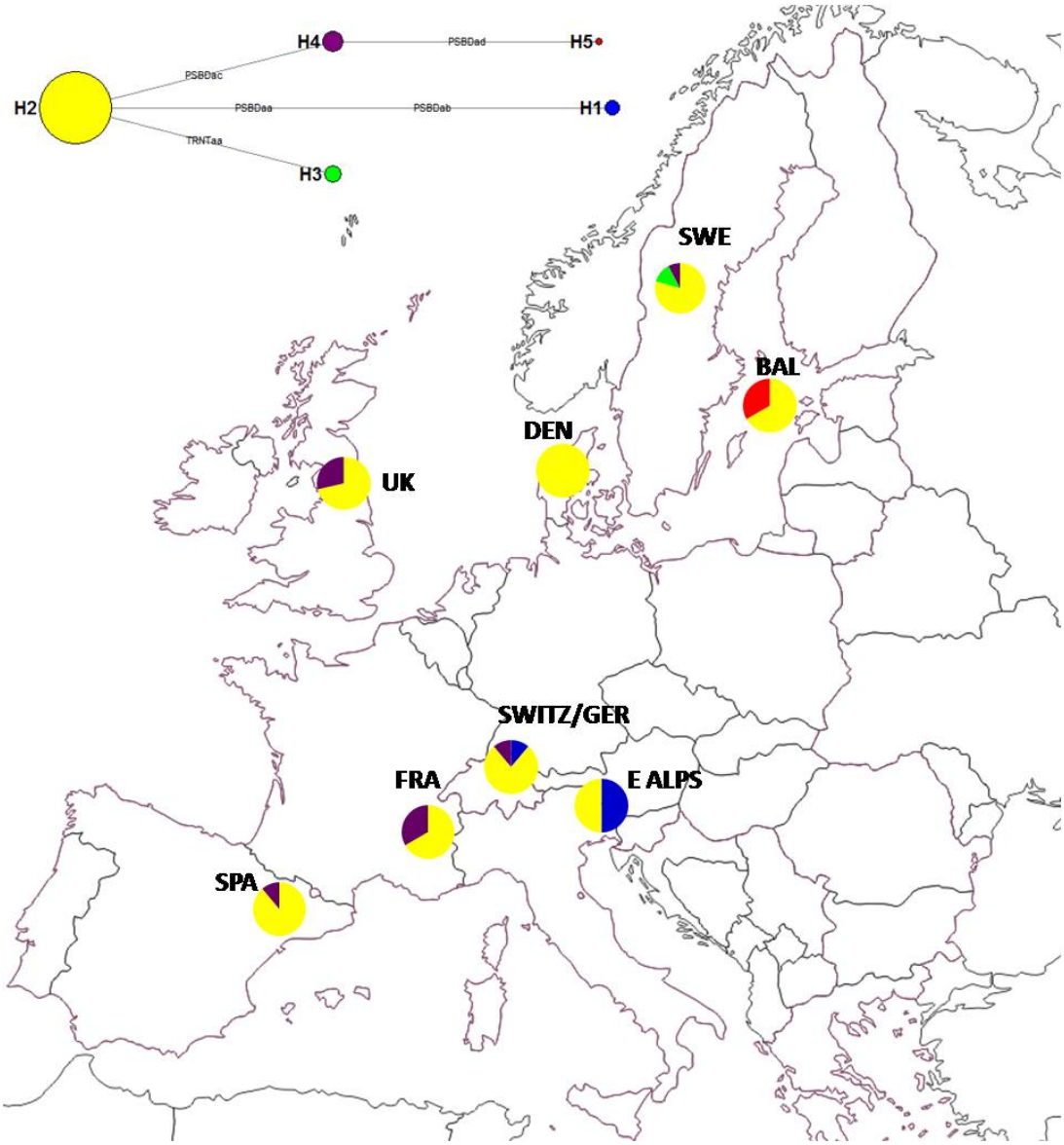

Figure 4.5. Median-joining network for plastid haplotypes (inset) of *Cypripedium calceolus* and haplotype distribution in western and northern Europe. Colours show frequencies of haplotypes within each population. UK, United Kingdom; SPA, Spain; FRA, France; SWITZ/GER, Switzerland/Germany; E ALPS, Eastern Alps, DEN, Denmark; BAL, Baltic Islands; SWE, Sweden. Outline map: © Daniel Dalet / D-maps.com.

4.3.5.2 Genetic diversity in *Cypripedium calceolus*

Fifty percent of polymorphic microsatellite loci were found in most populations. The highest was in Sweden (100%), whereas that of Denmark had no polymorphism (0%). The highest values for total number of alleles and number of haplotypes were found in Sweden and Switzerland/Germany. Two unique alleles and haplotypes were observed in two populations, in Sweden (98, H3) and the Baltic Islands (441, H5; only Estonia). The mean value of unbiased haploid diversity (*uh*) is 0.214, the lowest value (0.000) was for Denmark and the highest value (0.333) was for France and the Baltic Islands (see Table 4.8).

4.3.5.3 Genetic structure of *Cypripedium calceolus* populations

The results from the analysis of molecular variance (AMOVA) across all populations, showed that 28% of variation is due to difference among populations, whereas 72% of variation is attributed to difference within populations (Phi_{PT} = 0.278, P < 0.01) (Table 4.9). Most pairwise Phi_{PT} values between populations were insignificant (P > 0.05) (Table 4.10). However, Phi_{PT} values with P < 0.001 were found between Denmark and Spain (0.177); Phi_{PT} values with P < 0.01 were found between the Eastern Alps and Denmark (0.754), and between the Eastern Alps and Sweden (0.647); and Phi_{PT} values with P < 0.05 were found in the Eastern Alps and Spain (0.506), between the Eastern Alps and the UK (0.517), between France and Spain (0.000), between Denmark and the UK (0.507), and between Denmark and Sweden (0.077).

Table 4.8. Genetic diversity for plastid microsatellites of *Cypripedium calceolus* populations.

Population	Sample size	Polymorphism loci (%)	Number of total alleles	Number of haplotypes	Number of unique haplotypes	Unbiased haploid diversity (*uh*)
Eastern Alps	6	50.00	3	2	0	0.300
Denmark	34	0.00	2	1	0	0.000
France	3	50.00	3	2	0	0.333
Spain	9	50.00	3	2	0	0.111
Baltic Islands	3	50.00	3	2	1	0.333
Sweden	39	100.00	4	3	1	0.188
Switzerland/Germany	9	50.00	4	3	0	0.208
UK	7	50.00	3	2	0	0.238
Mean		50.00	1.563			0.214

Table 4.9. Analysis of molecular variance (AMOVA) within and among *Cypripedium calceolus* populations.

Source of variation	df	SS	MS	Est. Var.	Variation (%)	Phi_{PT} Statistic	*P* value
Among populations	7	9.068	1.295	0.089	28%	0.278	<0.01
Within populations	102	23.668	0.232	0.232	72%		

Levels of significance was based on 9999 permutations.

Table 4.10. Genetic differentiation between pairs of *Cypripedium calceolus* populations [Phi_{PT} value (below diagonal) and statistical significance; *P* values based on 9999 permutations (above diagonal). NS = not significant].

Population	1	2	3	4	5	6	7	8
Eastern Alps (1)	–	<0.010	NS	<0.050	NS	<0.010	NS	<0.050
Denmark (2)	0.754	–	NS	<0.001	NS	<0.050	NS	<0.050
France (3)	0.406	0.732	–	<0.050	NS	NS	NS	NS
Spain (4)	0.506	0.177	0.000	–	NS	NS	NS	NS
Baltic Islands (5)	0.466	0.732	0.000	0.175	–	NS	NS	NS
Sweden (6)	0.647	0.077	0.030	0.000	0.361	–	NS	NS
Switzerland/Germany (7)	0.250	0.000	0.000	0.000	0.151	0.030	–	NS
UK (8)	0.517	0.507	0.000	0.000	0.000	0.066	0.045	–

4.3.5.4 Haplotypes in *Cypripedium calceolus* UK plants

Analysis of the haplotype network of the UK samples of *C. calceolus* alone was also carried out, using a combination of two polymorphic loci of plastid microsatellites [ACpsbD from this study and cyp2 from Fay and Cowan (2001)] and four haplotypes were recovered (Figure 4.6). Haplotype H1 (alleles 439, 198) was the most widespread, being found in UK1, UK3, UK7 and UK8. The other three haplotypes, H2 (alleles 439, 199), H3 (alleles 440, 197) and H4 (alleles 440, 198) were found in UK5, UK4 and UK6, respectively.

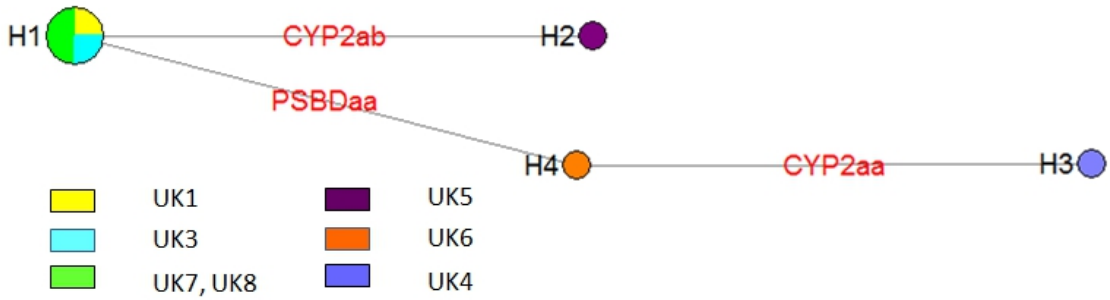

Figure 4.6. Median-joining network for plastid haplotype in UK samples of *Cypripedium calceolus*. The analysis includes two polymorphic microsatellite markers, ACpsbD (this study) and cyp2 (Fay and Cowan, 2001). Colours represent different localities in the UK.

4.4 Discussion

4.4.1 Limitations in the development of plastid microsatellite markers for the slipper orchid subfamily Cypripedioideae

There was difficulty in amplifying plastid regions containing microsatellites using universal primers from the literature for each genus, probably due to non-coding regions being highly variable for different divergent species, causing mismatches between primers and template DNA. Designing internal primers would probably help to improve the success of amplification. Also, there was a problem with stutter sequence, which is a PCR artefact caused by sequences containing poly-A or poly-T mononucleotide repeats affecting *Taq* polymerase in PCR reactions (Devey *et al.*, 2009), thus, nucleotide bases were unable to be determined in those sequences. These problems in obtaining sequences for use in designing primers resulted in some

limitation in obtaining microsatellite markers of some plastid regions. Also, because of the high variation in non-coding sequences of plastids between genera, in other words, lack of a conserved flanking area for designing primers, degenerate primers had been designed from consensus sequences and this could also reduce the amplified products. In addition, potential variable size microsatellites identified for each plastid region, based on complete sequence data of *Phragmipedium longifolium*, may not guarantee variable size microsatellites at the same loci in other related genera, thus diminishing the number of potential size variable microsatellite markers.

4.4.2 Primer test and cross applicability in the subfamily Cypripedioideae

Most new microsatellite markers were successfully amplified across a range of taxa in subfamily Cypripedioideae, the percentage of success varying from 75.71% to 100%. Two markers (rps16-1 and rps16-2), developed for *Cypripedium calceolus* in previous research (Fay *et al.*, 2009), were successfully cross amplified in most taxa in the subfamily (77.5–100%) (Table 4.6). A high rate of amplification shows that most markers are reproducible and transferable and this indicates that there is a sufficient level of conserved flanking areas of most markers in this subfamily. However, an exception is ACtrnC, for although being well amplified in *Paphiopedilum* spp. (95.45%) and *Phragmipedium* spp. (100%), there was only 17.5% amplification for *Cypripedium* spp. (excluding all accessions of *C. calceolus*) (Table 4.6). In general, levels of success in amplification depend on specificity between primer binding sites and DNA templates. A higher rate of amplification is a result of highly conserved flanking areas of priming sites.

The sequence data for non-coding plastid regions shows that they are highly variable among genera in subfamily Cypripedioideae, meaning that they lack conserved flanking areas for designing universal primers. However, degenerate primers were designed to resolve this problem but this could lower the amplification efficiency. Also, for those regions for which it was possible to obtain sequences but not for all the genera desired, the microsatellite primers had to be designed without all sequence samples for each genus. Thus, those primers may not be specific to the DNA templates of excluded taxa, leading to lower success of microsatellite amplification, such as in the case of ACtrnC for *Cypripedium*. Only ACtrnL-1 was tested for *Vanilla* spp. with only 55.56% success (Table 4.6). This could be because the primer binding site is variable in *Vanilla* spp., and degenerate primers from a consensus of different *Vanilla* spp. may need to be designed.

For *C. calceolus*, the DNA samples, accessions 12733–12736, were not able to be amplified by any markers. These four DNA samples were extracted from herbarium specimens obtained from different countries: Austria (collected in 1878, 1881), Russia (Aginskoje; 1948) and Yugoslavia (1938). This negative result is probably due to the problem of low quality DNA. However, some DNA samples extracted from herbarium specimens were successfully amplified in most markers [Russia (nr. Leningrad; 1937, Amur Oblast; 1957, Chitinskaya; 1962)].

In this study, the same PCR profile and conditions for amplification of microsatellite markers were used for simplicity. Modification of the PCR profile and conditions would help to increase the success of PCR amplification.

4.4.3 Polymorphism of plastid microsatellites in Cypripedioideae

Size variation in PCR products was observed within and/or among species, as detected from plastid microsatellite markers in this study (Tables 4.4–4.7). All ten markers were found to be polymorphic within each genus, the exception being *Phragmipedium spp*. in which two markers, (ACtrnT and rps16-1) were found to be monomorphic. In general, polymorphism at interspecific level among ten markers was high in *Paphiopedilum* (*uh* = 0.272–0.987). The level of polymorphism among six markers (ACtrnL-2, ACtrnF, ACycf1, ACtrnT, ACpsbD, ACclpP) was low (*uh* = 0.024–0.438) when considering all accessions of *Cypripedium* spp., because most accessions were of *C. calceolus* (137 of 177), in which this low level of polymorphism reflects low numbers of alleles for each marker in this species. For four markers (ACtrnL-1, ACtrnC, rps16-1, rps16-2), accessions of *C. calceolus* were excluded, the level of polymorphism of *Cypripedium* spp. was found to be high (*uh* = 0.898–0.969). Although only five species of *Phragmipedium* were studied here, most markers were found to be polymorphic (*uh* = 0.400–1.000), and there were only two monomorphic microsatellites (see Table 4.6).

At intraspecific level in this study, only three of six markers tested in *C. calceolus* were polymorphic. Polymorphism in these three markers was low (two or four alleles, *uh* = 0.030–0.227) (Table 4.6). A low level of polymorphism in plastid microsatellites has been reported in a previous study of this species, in which, between two and four alleles were found among four microsatellite markers, whereas, the more informative size data were from insertion/deletion fragments obtaining by sequencing (Fay *et al.*, 2009). These plastid markers for *C. calceolus* also showed lower levels of genetic variation in terms of number of alleles compared with nuclear microsatellites (nine and

eight alleles from two scorable polymorphic markers, IK8 and IK9) (Kahandawala, 2009; Pedersen *et al.*, 2012). Also, low numbers of alleles from plastid markers have been reported in other orchid studies, as in *Dendrobium officinale*, (two to four alleles, expected heterozygosity or H_e = 0.133–0.703] (Xu *et al.*, 2011), *Epidendrum* spp. (two or three alleles, H_e = 0.105–0.666) (Pinheiro *et al.*, 2009). However, the combination of those markers was suggested to be useful for discriminating among populations in the taxa studied.

In this study, only one allele was detected from ACtrnL-2 (206), ACclpP (121), ACycf1 (148) for *Cypripedium calceolus*. Two alleles were detected from ACtrnT (97, 98) and ACtrnF (140, 141). However, the level of polymorphism detected in those two primers was low (*uh* = 0.101 for ACtrnT and *uh* = 0.030 for ACtrnF), and only one most frequent allele was found. Allele 98 of ACtrnT was only found in seven accessions from Sweden (5), Germany (1) and unknown (1). The most frequent allele detected from ACtrnF was 141 bp long, the other, 140 bp long, being found only in two samples from eastern Russia (Chitinskaya and Amur Oblast). The other Russian sample, from the western part (nr. Leningrad), had the 141 bp allele. Although ACpsbD was the most polymorphic marker (*uh* = 0.227), varying between 437–441 bp, the most frequent allele, out of a total of four alleles, was 439. The other alleles of lengths, 437, 440 and 441 bp were found only in some samples. However, it was not possible to obtain the ACpsbD marker in many accessions, especially samples from Poland, due to difficulty in amplification.

At the intraspecific level, most alleles detected by each marker were found to be only a few base pairs different. However, a high level of size variation was found in ACtrnC and rps16-2 (Table 4.7). A greater variation in allele size was found between species (Table 4.4, 4.6). Highly different size length in some markers suggested that variations could involve insertion/deletion (indels) events, rather than repeat variation and this could be investigated by the sequencing of amplified products. In addition, some plastid markers showed an allele size range greater than expected, suggesting non-specific PCR amplification could have occurred. Further work in sequencing those regions needs to be carried out to test homology of those plastid microsatellites. As allele size is measured from PCR product that is composed of microsatellite and flanking regions, the variation in flanking regions could be a source of size homoplasy (Hale *et al.*, 2004). It has been suggested that those fragments resulting from insertion/deletion events could be useful for understanding the evolution of the plastid genome (Chung and Staub, 2003).

Caution should be exercised in using plastid microsatellite regions with high mutation rates for phylogenetic study at interspecific levels (e.g. Doyle *et al.*, 1998), because any variation in length could be size homoplasy, in which the same allele size may not be identical by descent (Estoup *et al.*, 1995). However, it has been suggested that because of the lower rates of mutation of plastid microsatellites compared with nuclear microsatellites, the effect of size homoplasy could be overcome by including more loci, enabling inference of phylogenetic relationships among closely related species (Provan *et al.*, 2001).

The utility of plastid microsatellites for phylogenetic study at interspecific levels for closely related species has been reported in taxa such as *Hordeum* spp. (Provan *et al.*, 1999a), *Aegilops* spp. and *Triticum* spp. (Ishii *et al.*, 2001), *Oryza* spp. (Provan *et al.*, 1997), *Vitis* spp. (Arroyo-García *et al.*, 2002), *Phaseolus* spp. (Angioi *et al.*, 2009) and *Solanum* spp. (Bryan *et al.*, 1999), but caution has to be exercised in making comparisons between cultivated taxa and wild taxa. However, various causes of size homoplasy have been observed from comparison of sequences of *Clusia* spp., namely, base substitutions within microsatellites, base substitutions in the flanking regions, indels in the flanking regions, multiple microsatellites within a fragment, and forward/reverse mutations of repeat length (Hale *et al.*, 2004).

Variability among plastid microsatellite markers is considered to be related, due to the non-recombinant nature of the plastid genome. This means the association between them can be investigated in order to infer if the origins of alleles are recurrent, as there is a negative correlation between linkage disequilibrium and homoplasy (Provan *et al.*, 2001). In this study, there was found to be high linkage disequilibrium between ten plastid microsatellite loci in *Paphiopedilum* (*Ve/Vo* = 2.855, $P < 0.01$), which is similar to the result from a study of *Phaseolus* spp. (Angioi *et al.*, 2009). Thus, it is probable that homoplasy would not be affected in a further analysis of relationships among closely related species of *Paphiopedilum* spp. at interspecific level. However, the results in this study differ from those of the study of *Clusia* spp., in which no link was found between microsatellite loci, suggesting forward/backward size homoplasy (Hale *et al.*, 2004).

A positive correlation between the number of repeat motifs and number of alleles was observed in a genetic study of common wheat and related species (Ishii *et al.*, 2001). The number of repeat motifs observed from *Cypripedium* sequence data was low, the number varying between n = 4–10, except in the case of *trnL-F* (1) region (n = 17)

(Table 4.3). Designed primers were excluded from testing with *C. calceolus* as discussed previously. This is probably the reason for the low number of alleles in each marker for *C. calceolus*. The higher number of repeat motifs of some regions found in *Paphiopedilum* and *Phragmipedium* sequence data (Table 4.3) would suggest a greater number of alleles for those designed primers, although this could not be concluded here, due to the low number of samples of the taxa in this study. However, positive correlations between length of microsatellite and allele numbers have not been observed from developed microsatellites based on DNA sequences of *Nicotiana tabacum* L. (Weising and Gardner, 1999).

4.4.4 Utility of plastid microsatellites for study of *Cypripedium calceolus* populations

4.4.4.1 Haplotype diversity in *Cypripedium calceolus*

In general, the number of haplotypes was low (five haplotypes) in this study because they were obtained from only two polymorphic microsatellite markers (Figure 4.5). For ACtrnT marker, most samples had the same allele (97 bp), except for five samples (98 bp) from Sweden and one sample from Germany, in which case it was excluded from all analyses because it was not amplified for ACpsbD. As for ACpsbD marker, although it was found to be quite variable (four alleles), it was not amplified in many samples of *C. calceolus*.

In a previous study of the genetic diversity of *C. calceolus* (Fay *et al.*, 2009), 23 haplotypes were recovered from 14 plastid length variable loci but only four loci were from plastid microsatellites, thus, most variability was from insertion/deletion sequences.

4.4.4.2 Genetic diversity in *Cypripedium calceolus*

In general, unbiased haploid diversity (*uh* = 0.000–0.333, mean = 0.214) (Table 4.8) for each population in Europe is found to be relatively lower than the gene diversity results of Fay *et al.* (2009) from 14 polymorphic loci from plastid DNA (0.000–1.000, mean = 0.675), probably because only two polymorphic plastid microsatellites have been included in this study. Denmark is the only population in this study that has no genetic diversity, which is congruent with the results from studies of plastid DNA markers (Fay *et al.*, 2009; Kahandawala, 2009). The study of Danish populations using nuclear microsatellites also revealed no variation (Kahandawala, 2009; Pedersen *et al.*, 2012)

and it was suggested that the lack of variability could be because of a genetic bottleneck or prolonged inbreeding in Danish populations.

Compared with the results from allozyme studies for *C. calceolus* populations, high genetic diversity was obtained by using three polymorphic markers for seven populations in Estonia (observed heterozygosity or H_o= 0.400–0.530), except for one population, in which low genetic diversity was observed (H_o = 0.160), in which case it was suggested the founder effect was the cause (Kull and Paaver, 1997). Lower levels of genetic diversity were obtained from a population study in two regions (NE Poland and SE France) using five polymorphic isozyme loci [H_o= 0.155 and 0.137, mean = 0.145, H_e = 0.164 and 0.140, mean = 0.150 (Brzosko *et al.*, 2009)]. In addition, similar levels of genetic variation were observed in three Polish populations using five polymorphic isozyme loci [H_o = 0.156, H_e = 0.184 (Brzosko *et al.*, 2002)] and further work was carried out by them on 32 Polish populations, using six polymorphic isozyme loci [H_o= 0.143, H_e = 0.149 (Brzosko *et al.*, 2011)]; however, these levels of variation were considered relatively high when compared with other endangered or rare taxa.

4.4.4.3 Genetic structure of *Cypripedium calceolus* populations

Most genetic variability for *C. calceolus* in this study was due to variation within populations (72%, Table 4.9.), which is similar to the results from Fay *et al.* (2009) who obtained slightly higher variation within *C. calceolus* populations (82.5%); however, the F_{ST} analogue or Phi_{PT} value was slightly higher in this study (0.278, $P < 0.01$) than the F_{ST} of the previous study (0.175, $P < 0.001$). Compared to studies of allozyme polymorphism, most genetic diversity was also due to variation within populations as found in the population study of *C. calceolus* in Poland [98.4%, F_{ST} = 0.016, $P < 0.05$ (Brzosko *et al.*, 2002); 79.9%, F_{ST} = 0.201, $P < 0.001$ (Brzosko *et al.*, 2011)] and that from NE Poland and SE France [92.45%, F_{ST} = 0.075, $P < 0.001$ (Brzosko *et al.*, 2009). Most pairwise Phi_{PT} values between populations in this study were low and insignificant. Although this could be because of the low level of polymorphic markers and small sample size, it could possibly indicate that there is genetic similarity between that pair of populations. However, high Phi_{PT} values found between populations in the Eastern Alps and other populations indicated the differentiation of this population from the others (Table 4.10).

4.4.4.4 Post-glacial recolonisation of *Cypripedium calceolus*

A dramatic decrease in infrageneric variation could have been due to the effects of glaciation, as is thought to be the case in many plants and animals in temperate regions (Hewitt, 1996). During the last ice age, when ice advanced southwards in Europe, populations migrated south to potential refugia, where there were warmer areas. These were the Iberian, Italian and Balkan peninsulas, which are separated east to west by sea. Later, as the ice retreated, some species with the potential for long distance dispersal would have rapidly expanded from their refugia to recolonise areas and establish populations, so that by the time other individuals arrived they would contribute only little of their genetic material, as a result of the population bottleneck, which would lead to a reduction in the average number of alleles per locus and a decrease in heterozygosity (Nei *et al.*, 1975; Hewitt, 1996).

The widespread common haplotype found in all populations, known as haplotype H2 in this study (Figure 4.5), has been suggested as being an indicator for such an ancient population bottleneck (Echt *et al.*, 1998). The results from a more extensive study of *C. calceolus* have also shown some widespread haplotypes, suggesting a potential for supporting the wind dispersal pattern of light-weight orchid seeds (Fay *et al.*, 2009). Seeds of *Cypripedium calceolus*, although said to be large for a terrestrial orchid in temperate regions, are still tiny, being approximately 1.2 x 0.3 mm, which could be dispersed by wind (Kull, 1999). However, the preliminary results from a field study on populations of *C. calceolus*, *Epipactis helleborine* (L.) Crantz and *Goodyera repens* R.Br. found that seeds of those orchids are more usually found nearby their mother plants, indicating mostly short distance dispersal (Brzosko *et al.*, 2009). Several more studies have suggested mostly short distance seed dispersal in orchids, such as in *Spiranthes spiralis* (L.) Chevall. (Machon *et al.*, 2003), *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase, *Dactylorhiza majalis* (Rchb.) P.F.Hunt & Summerh. and *Pseudorchis albida* (L.) Á.Löve & D.Löve (Jersáková and Malinová, 2007), *Orchis purpurea* Huds. (Jacquemyn *et al.*, 2007) and *Cypripedium macranthos* Sw. (Chung *et al.*, 2009).

Haplotype H2 is widespread, suggesting that it could have come originally from any of three main post glacial refugia, Iberian, Italian or Balkan. Haplotype H4 is also widely distributed but to a lesser extent and is found in four populations in western and northern Europe, meaning that it could have recolonised in a north-easterly direction from the Iberian refugium. East-west mountain ranges such as the Alps, are said to

obstruct migration to the northern regions, resulting in Italian populations being isolated from the others (Taberlet *et al.*, 1998). Haplotype H1, which is the only haplotype that is differentiated by more than one mutational step, is restricted to central Europe (Switzerland/Germany and Eastern Alps), thus, it could have come originally from the Italian refugium. Two unique haplotypes, H3 and H5, were found only in northern Europe; in Sweden and the Baltic Islands (Estonia only), respectively. These haplotypes perhaps originated from the Balkan refugium, although H5 is closely related to H4. However, due to the absence of samples from eastern Europe and the Balkan region, the recolonisation routes cannot be established (see Figure 4.5).

4.4.4.5 *Cypripedium calceolus* UK plants

From the analysis of the haplotype network of the UK samples alone, using a combination of two polymorphic loci of plastid microsatellites (ACpsbD from this study and cyp2 from Fay and Cowan (2001), four haplotypes were recovered, compared with the haplotype network from Fay *et al.* (2009), in which five haplotypes were recovered within the UK samples, from a combination of 14 polymorphic markers, including cyp2. Results from this study show that the most widespread haplotype, H1, is found in samples that are known to originate from the UK, whereas those samples, UK5 and UK6, from putatively introduced plants have different haplotypes from them (Figure 4.6). These results support the results of Fay *et al.* (2009) (H17 and H2 in their results, respectively). Although UK4 is known to be of wild origin, the haplotype is different from the rest of the UK origin samples confirming the findings of Fay *et al.* (2009), and is derived from haplotype H4 (UK6), however, the plant grows on a different soil type (magnesian limestone). In the study of Fay *et al.* (2009), this plant shared the same haplotype (H10 in their study) with those of France, Switzerland and Spain. The genetic variation in the plants of UK origin was also confirmed from nuclear microsatellite data (Kahandawala, 2009). On the basis of the results of Fay *et al.* (2009), it was decided to exclude from the Natural England conservation programme seedlings from those putatively introduced plants originating from self pollination and from cross pollination with those and other plants. The present study also supports that result.

Chapter 5: General discussion

5.1 Phylogenetic study of *Paphiopedilum*

The phylogenetic study of the genus *Paphiopedilum* in this thesis is presently the most comprehensive, in that it uses five sets of DNA sequence data, from both nuclear (ITS) and plastid regions [partial *matK*, *ycf1*, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*]. The tree results from separate ITS and plastid data were generally congruent, as there were no conflicts shown between strongly supported clades (Figures 2.1, 2.2). Infrageneric groups were recovered from both ITS and plastid trees, as recognised by Cribb (1998). However, the relationships elucidated from a combined plastid tree were resolved to a higher degree than the ITS tree, although individual trees obtained from each plastid region showed a lack of resolution. Mostly, there was an increase in the robustness of clades of the tree from a combination of ITS and plastid data, indicating congruence between the data. The robust phylogenetic tree resulting from the combined DNA sequence data was used for assessing existing infrageneric classifications (Figure 2.3), as discussed in Chapter two and provided a phylogenetic framework for elucidating evolutionary trends of genome size and chromosome number, as discussed in Chapter three (Figure 3.1).

Paphiopedilum was confirmed to be a monophyletic group in this study, agreeing with the results of previous studies by Cox *et al.* (1997) and Albert (1994). In general, the phylogenetic relationships within the genus elucidated by molecular data in this study, were congruent with the infrageneric classification of Cribb (1998), in which the genus was divided into three subgenera, *Parvisepalum*, *Brachypetalum* and *Paphiopedilum*. The phylogenetic relationships among the subgenera and sections within the genus were reconstructed from combined sequence data, mostly with strong clade support, unlike the phylogenetic relationships elucidated from ITS data alone in a previous study by Cox *et al.* (1997), which lacked support for clade robustness. The first branching clade of the genus was the monophyletic subgenus *Parvisepalum*, as found in the results of Cox *et al.* (1997). This result confirms that of Karasawa and Saito (1982), who recognised subgenus *Parvisepalum*, which they considered to be morphologically distinct from the other species in subgenus *Brachypetalum*. This also agrees with the treatment of Cribb (1998), who, based on the ITS results of Cox *et al.* (1997), elevated section *Parvisepalum* to a subgenus in the second edition of his monograph. Species in this subgenus were also considered by Chen and Tsi (1984), from their floral

characters, to be the 'basal group' (i.e. earliest diverging) of the genus, due to their flowers being similar to those of *Cypripedium*. Cribb (1987) suggested related pollination syndromes as a cause for the floral similarities, such as an inflated lip with involute margins, in species in subgenus *Parvisepalum* and *Cypripedium*. Although hoverflies have been reported as pollinators from several studies over many years for *Paphiopedilum* (Atwood, 1985; Bänziger, 1994; 1996; 2002; Bänziger *et al.*, 2012; Shi *et al.*, 2007; 2009), *P. micranthum*, which belongs to subgenus *Parvisepalum*, that is, the earliest diverged group, was observed to be pollinated by bees (Bänziger *et al.*, 2008). It has also recently been discovered that bees are the pollinators in two closely related species of subgenus *Brachypetalum* (*P. thaianum* and *P. niveum*) (Bänziger *et al.*, 2012). As subgenus *Brachypetalum* was found to be the second most early diverging group and a sister group to the largest subgenus, *Paphiopedilum*, both Cox *et al.* (1997) and this study, it has been suggested that they form a link between *Cypripedium* and *Paphiopedilum* (Bänziger *et al.*, 2012) (Figure 2.4).

In bee pollinated *Paphiopedilum* species, only species in subgenus *Parvisepalum* have flowers that are similar to bee pollinated *Cypripedium* species. The flowers of the two bee pollinated species in subgenus *Brachypetalum* are more typically *Paphiopedilum*-like in form, but have a scent similar to the flowers of species in *Parvisepalum.* This has been suggested as a possible reason for bee attraction, as scent has been found to be important in many bee pollinated flowers (Bänziger *et al.*, 2012). Different kinds of scent have been detected in the flowers of *Paphiopedilum* species in subgenus *Brachypetalum*, depending on the pollinators they are associated with, for example, Bänziger *et al.* (2012) noted a pleasant, lemongrass-like scent in flowers of *P. thaianum* which is pollinated by bees, whereas those of *P. godefroyae*, that are pollinated by hoverflies, smell unpleasantly like rancid butter and human perspiration, similar to another hoverfly pollinated species, *P. bellatulum* (Rchb.f.) Stein (Bänziger, 2002). In hoverfly pollinated species of *Paphiopedilum*, many floral characters, including aphid-like decoys (glandular hairs, hairy black warts), honeydew or moisture mimicking (glittering staminode), a bright yellow staminode and scent have been considered to be attractions for hoverflies by deception, as they are seeking pollen, nectar or honeydew for food, or brood sites for their aphid-eating larvae (Atwood, 1985; Bänziger, 1994; Bänziger, 1996; Bänziger, 2002; Bänziger *et al.*, 2012; Shi *et al.*, 2009).

In the earlier diverging genus in subfamily Cypripedioideae, *Cypripedium* (Cox *et al.*, 1997; Guo *et al.*, 2012), the shift from bee, in early diverging groups, to fly pollination in

later diverging groups has also been observed (Bänziger *et al.*, 2012). Recently, Li *et al.* (2012) observed *Cypripedium sichuanense* Perner and *C. micranthum* Franch. were pollinated by dung flies and fruit flies respectively, in which flowers attract flies by mimicking the scents and colours of decaying flesh, including a rotting smell, green or purple-brown colours that look and smell like rotten meat and a trap-like lip. Two other species closely related to *Cypripedium sichuanense* and *C. micranthum* have previously been observed to be fly pollinated; *C. fargesii* Franch. (Ren *et al.*, 2011) and *C. lentiginosum* P.J.Cribb & S.C.Chen (Liu *et al.*, 2008) (Li *et al.*, 2012).

The results of this study confirmed the largest subgenus, *Paphiopedilum*, to be a monophyletic group, with strong support from the combined data. This agrees with the infrageneric treatment of this group as a subgenus by Cribb (1998), rather than its division into several subgenera, as in the treatment of Braem (Braem, 1988; Braem *et al.*, 1998; Braem and Chiron, 2003). In addition, five sections recognised by Cribb (1998) (*Pardalopetalum*, *Coryopedilum, Cochlopetalum*, *Paphiopedilum* and *Barbata*) were all recovered in this study, mostly with strong support for grouping, with the exception of section *Coryopedilum*. There are two main lineages within the subgenus *Paphiopedilum*. The first lineage consists of three sections: *Coryopedilum*, *Pardalopetalum* and *Cochlopetalum*, all with species which have multi-flowered inflorescences and the second lineage consists of two sections: *Paphiopedilum* and *Barbata* of mostly single-flowered species (Figure 2.4).

Within the multi-flowered species clade, two sections: *Coryopedilum* and *Pardalopetalum*, consisting of species in which the flowers open simultaneously, were grouped together, sister to section *Cochlopetalum* that has species with flowers which open successively. Section *Pardalopetalum* had strong support for monophyly, whereas section *Coryopedilum* obtained a low bootstrap value (54 BP) for clade support and collapsed into a polytomy with section *Pardalopetalum* in a strict consensus tree, although it obtained 0.95 PP value from Bayesian analysis (Figure 2.3). Cox *et al.* (1997) tentatively proposed it should be combined with section *Pardalopetalum*, due to them being paraphyletic in their ITS tree but Cribb (1998) disagreed, suggesting the distinctive flower characters of both sections showed them probably to be sister groups. The low level of molecular divergence in *Coryopedilum* could possibly be related to its selfing mode of reproduction, resulting from geitonogamy, and an absence of centric fission events. Species in *Coryopedilum* and *Pardalopetalum* are prone to geitonogamy or pollination among flowers of the same plant (Kliber and Eckert, 2004), as they both have multi-flowered inflorescences that

open simultaneously. The floral mechanisms of orchids facilitate outcrossing, though most are also self-compatible, probably to ensure reproduction in isolated plants that are unable to cross pollinate (Dressler, 1981). The species of section *Coryopedilum* are narrow endemics in the Malesian islands with small populations (Cribb, 1998), making them more susceptible to geitonogamy than species in *Pardalopetalum* which have wider distributions. The low level of molecular divergence in *Coryopedilum* may provide evidence of relatively recent speciation in this section as it spread through Malesia. The adoption of geitonogamy in this clade provides support for Baker's Law, the shift from outbreeding self-incompatibility to self-compatibility and selfing exhibited by many island colonists (Cheptou, 2012).

Paphiopedilum parishii and *P*. *dianthum,* suggested to be sister species in the findings of this molecular study, are thought to have different pollination strategies. Hoverflies are the pollinators of *P. dianthum* but although *P. parishii* has been reported to be pollinated by hoverflies (Bänziger, 2002), it has recently been found by Chen *et al.* (2012) to have an autonomous self pollination mechanism which functions by means of the transformation of the anther from solid to liquid, with this liquid moving directly onto the stigma. In other respects the floral characters (such as a pouched lip and aphid-like spots) indicate adaptation to cross pollination by hoverflies. It has been suggested that this self pollination mechanism is an adaptation to situations where pollinators are limited (Chen *et al.*, 2012).

Taxa with similar characters have been a cause of doubt as to whether they should be given species, subspecies or variety status. Those similar species pairs of *Paphiopedilum* that have several distinguishing characters and distinct distribution are considered to be discrete species by authors including Cribb (1998). In section *Barbata*, many different species (27) have been recognised by authors such as Cribb (1998) and Averyanov *et al.* (2003). However, results from this present study show this is debatable, as the phylogenetic relationships within this largest section are unresolved, with many internal branches collapsing to polytomy. The short branch lengths in section *Barbata* shown on the combined tree in this study and the narrow geographical distribution on islands in Malesia of most species might suggest a recent rapid radiation in the section (Cox *et al.*, 1997). Although numerous molecular characters from five DNA regions, both from nuclear and plastid loci, have been included in this study, the relationships in this section remain unresolved.

The geological development of Malesia is extremely complex but, to simplify, it was formed by the addition of successively more land on the eastward side of the Sunda shelf of western Malesia, comprising modern Thailand, Indo-China, Peninsula Malaysia and Sumatra, caused by tectonic movements (Hall, 2009). The narrowing of the Makassar Strait and uplift of islands in the region known as Wallacea, as the Indo-Australian plate moved northwest to collide with the Eurasian plate, was the culmination of this process and the formation of islands in this region has been postulated as occurring during the early Miocene to early Pliocene (Hall, 2009). These events coincide in the geological time scale with the period that species in section *Barbata* probably diversified.

Based on the molecular clock hypothesis of Zuckercandl and Pauling (1965), that sequences have evolved at a relatively constant rate over time, the divergence of lineages can be estimated. In practice, it has been found in many cases that genes display 'non-clock behavior', because the evolutionary rate depends on various factors, such as metabolic rates and generation times, however, 'relaxed molecular clock' approaches have been developed to allow for variation in the rates of nucleotide substitution (Lemey *et al.*, 2009). Molecular dating methods are continually developing, helping to elucidate clear time scales of diversification (Sanderson, 1997; 2002; Drummond and Rambaut, 2007). Obtaining an accurate time scale is necessary for understanding the underlying mechanisms for the diversification of species in section *Barbata* in order to reveal patterns of diversification. Good sampling, that represents the range of their geographical distribution, would need to be included for a phylogeographic study. However, there is a limitation on collecting samples of these endangered orchids, as they are protected by CITES for international trading and by local or national legislation in many cases, thus, it is also important to be able to clarify the origin of any samples obtained for research, as well as being aware that revealing their location in the wild would probably lead to those species becoming threatened by collecting, as has been the case with some species of slipper orchids soon after they were first described (Pillon and Chase, 2007).

From the molecular dating of subfamily Cypripedioideae (Guo *et al.*, 2012), it has been shown that subgenus *Paphiopedilum* underwent divergence during the late Miocene. This molecular clock analysis was based on a combination of six plastid sequences. However no slipper orchid fossils are available, so the analyses using *matK* and *rbcL* sequences had to be performed firstly at familial level on taxa within Orchidaceae, with fossil calibration carried out using pollinaria preserved in Dominican amber for the

Goodyerinae (15–20 Mya) (Ramirez *et al.*, 2007) and macrofossils of *Dendrobium* (20–23 Mya) and *Earina* (20–23 Mya) (Conran *et al.*, 2009). The age of the root of the tree was determined by using the oldest monocot fossil for the maximum age (110–120 Mya) and the oldest known Asparagales (93–105 Mya) for the minimum age (Friis *et al.*, 2004; Ramirez *et al.*, 2007). Sampling within the subgenus *Paphiopedilum* was sparse, with only one species representing each section in their study; thus, the chronology of the diversification of section *Barbata* could not be determined precisely but it could be assumed to have occurred in the late Miocene or later.

The geological complexity and dynamism of Malesia is thought to be a possible cause of diversification, as this region is also one of the most species-rich areas in the world (Roos *et al.*, 2004). The effects of Malesian geology have been hypothesised as being drivers for diversification in a massive, diverse genus, *Begonia* L., in which c. 500 out of 1500 species are found in Malesia (Thomas *et al.*, 2012). The route of dispersal in *Begonia* has been suggested as being from west to east in the Malesian islands, because of the successive emergence of islands in the eastern part of the region (Thomas *et al.*, 2012); this could be a similar scenario to that in *Paphiopedilum* section *Barbata*, as the seeds of orchids are minute and have the potential for long distance dispersal by wind (Dressler, 1981). Although several studies have shown that most orchid seeds fall nearby their mother plants, including those of the slipper orchids; *Cypripedium calceolus* (Brzosko *et al.*, 2009) and *C. macranthos* (Chung *et al.*, 2009), Dressler (1981) pointed out that on a geological time scale there is much more chance that some seeds carried by the wind could have germinated a long distance from their mother plants.

Atwood (1984) previously suggested that the genus *Paphiopedilum* could have migrated southward from mainland southeast Asia to the Malay Peninsula first, then to the western part of the Malesian islands, before expanding to the Philippines and New Guinea. He also suggested that the shallow sea level in the western part of the Malesian islands would have led to the exposure of large areas of dry land available for colonisation following even small changes in sea level and these could have been major routes for the distribution of genus *Paphiopedilum* to the east, although the persistent Luzon Strait deep water channel would have remained a barrier to migration. The distribution of plants and animals over land bridges connecting mainland Southeast Asia and the western Malesian islands, when sea levels were lower during the late Pleistocene has been postulated (Voris, 2000). The occurrence of land bridges at that time has been suggested as routes for the migration and subsequent

diversification of treeshrew species (Roberts *et al.*, 2011) and the carnivorous plant genus *Nepenthes* L. (Meimberg *et al.*, 2001). Though most species in section *Barbata* are narrow endemics, *P. javanicum* and its variety have disjunct patterns of distribution as they are found in North Borneo, Sumatra, Java, Bali and Flores but are absent from Lombok and Sumbawa (Cribb, 1998). This species could have migrated by land bridges in western Malesia and the absence of this species from most parts of Wallacea may be explained by there being no land bridges in the Wallacea region during the Pleistocene (Voris, 2000).

The Middle/Late Miocene was a period of rapid diversification of the South American orchid genus *Hoffmannseggella* H.G.Jones, according to a study by Antonelli *et al.* (2010) and they also postulated a link between rapid evolutionary radiation and climate change. This was supported by Gustafsson *et al.* (2010), who estimated that species diversified in the late Miocene, in which case the link between rapid radiation and climatic fluctuations was strengthened. This coincides with the time in which *Paphiopedilum* section *Barbata* can be estimated to have undergone diversification. However, as *Hoffmannseggella* is restricted to high altitudes, a cooling climate, following the climatic optimum in the Mid Miocene, allowed their diversification by allowing expansion over a greater range (Antonelli *et al.*, 2010). In the case of species in *Paphiopedilum* section *Barbata*, their habitats were affected by various geographical and climatic changes in Malesia, including global cooling and the expansion of ice sheets leading to fluctuating sea levels, tectonic movements and island formation and changes in global ocean currents, including the Indonesian Throughflow between the Pacific and Indian Oceans (Hall, 2009; Shevenell *et al.*, 2008). Any or all of the factors may have played a role in the expansion of section *Barbata* and more evidence needs to be found from further research. Recently, a study of oxygen and carbon isotopes in planktic and benthic foraminifera and bulk carbonate samples from sediments in Central Java has shown that sea temperatures peaked in the mid Miocene before decreasing in the late Miocene, coinciding with the closing of the Indonesian Throughflow and climatic cooling on a global scale (Akmaluddin *et al.*, 2010).

Hybridisation is also thought to have played an important role as a mechanism underlying speciation. It has been estimated that over a quarter of all plant species, especially those most recently diverged, can form interspecific hybrids with other species (Mallet, 2005). Occurrences of natural hybridisation have been reported in many orchid genera, such as several species of *Paphiopedilum*, which are suspected to have originated from natural hybridisation (Cribb, 1998). For example, *P.* x

expansum has been considered to be a natural hybrid between *P. hennisianum* (M.W.Wood) Fowlie and *P. philippinense*, as a single plant was observed by Atwood (1989) among populations of putative parent species on the island of Cebu. Cribb (1998) considered *P. x hermanii* a natural hybrid between *P. hirsutissimum* var. *esquirolei* and *P. barbigerum* Tang & F.T.Wang based on Cox's molecular result (in Cribb, 1998). *P. x hermanii* was suggested by Averyanov *et al.* (2003) to be a more or less stabilised natural hybrid between *P. hirsutissimum* var. *esquirolei* and *P. helenae* (closely allied to *P. barbigerum*), based on the observation of Averyanov and Hiep that *P. x hermanii* occurs in between populations of those putative parent species.

Artificial hybridisation in *Paphiopedilum* has been utilised extensively for horticulture and hybrids can occur even between more distantly related species. For example, the hybrid 'Kevin Porter' is a cross between species in subgenera *Brachypetalum* and *Parvisepalum* [*P. bellatulum* x *P. micranthum*] (Cribb, 1998). This suggests that there is sufficient genetic similarity between species, that when different species occur in the same area, as sympatric species, hybridisation is possible. The evidence for hybridisation can usually be inferred when comparing tree topologies obtained from independent data sets that show incongruencies (Sang and Zhang, 1999) but tree topologies between ITS and plastid trees obtained in this study seem to be congruent, thus, hybridisation could not be inferred in this case. This congruence between ITS and plastid data is also found in another deceptive orchid genus, *Ophrys* L. (Soliva *et al.*, 2001). ITS sequences in *Paphiopedilum* could possibly have undergone concerted evolution, as this region was suggested to be prone to concerted evolution for homogeneity through unequal crossing over and gene conversion (Baldwin *et al.*, 1995; Álvarez and Wendel, 2003). Low copy nuclear genes are suggested to be more suitable markers because they are less likely to have undergone concerted evolution (Sang, 2002), therefore, these regions would help to reveal more evidence of hybridisation within the genus as a driver for evolution of *Paphiopedilum*.

Low copy nuclear genes are alternative markers for improving the robustness of clades that are unresolved from data of nuclear ITS and plastid regions, such as the results in sections *Barbata* and *Coryopedilum* in this study. They have the potential to reveal more phylogenetic information because the rate of nucleotide substitution is higher than that of plastid DNA (Wolfe *et al.*, 1987). However, they are not recommended to be used primarily, unless the resolution of phylogenetic relationships elucidated from nuclear ribosomal and plastid sequences is insufficient, due to the fact that it could be

difficult to distinguish between orthology and paralogy and they usually demand more work for cloning and require higher quality DNA (Sang, 2002).

5.2 Genome size and chromosome number evolution in *Paphiopedilum*

Obtaining a robust phylogenetic framework from the analyses discussed in Chapter two has helped enable an assessment of the evolutionary trends of genome size and chromosome number for the genus *Paphiopedilum*. A trend towards an increase in chromosome number, as proposed by Cox *et al.* (1997; 1998), was not shown clearly in this study, due to subgenus *Paphiopedilum* being divided into two main lineages, both of which are composed of sections comprising species with a consistent chromosome number of 2*n* = 26 paired with sections that have variable chromosome numbers (Figure 3.1). Because the first and second branching clades (*Parvisepalum* and *Brachypetalum*) of the phylogenetic framework were sections of species with a chromosome number of 2*n* = 26 with all metacentric chromosomes, as congruent with the results from the ITS tree of Cox *et al.* (1997), this could indicate the ancestral condition for the genus *Paphiopedilum*. This was also suggested previously by most species in the genus having a chromosome number of 2*n* = 26 (e.g. Karasawa, 1979). The phylogenetic position of species with large chromosome numbers and the presence of telocentric chromosomes indicates that these are a more derived condition. These karyotype changes in the genus were probably caused by centric fission, independently in sections *Barbata* and *Cochlopetalum*, as shown by the superimposition of the data onto the phylogenetic tree.

Robertsonian change; centric fission or fusion, is found relatively scarcely in higher plants compared to animals, but is widely scattered taxonomically and is found, for example, in the cycad genus *Zamia* L. (Jones, 1998). Recently, Olson and Gorelick (2011) re-assessed published chromosome data of *Zamia* spp. by mapping them onto a phylogenetic framework, the results showing that the earliest diverging species have low chromosome numbers which are mostly metacentric and submetacentric, thus, a trend of an increase in chromosome number was inferred in *Zamia*, suggesting Robertsonian centric fission was involved.

Only seven taxa in *Paphiopedilum* were measured for DNA content, due to the difficulty in obtaining root materials for the genome size study. However, the genome size data gathered for this study and from previous studies cover approximately 44% of taxa in the genus (Table 3.1). Genome sizes of *Paphiopedilum* spp. range from 1C = 17.80 pg in *P. godefroyae* in section *Brachypetalum*, to 34.53 pg in *P. wardii* in section *Barbata*,

representing a nearly two-fold increase. Mapping the genome size range of *Paphiopedilum* spp. onto the phylogenetic tree shows that there is no clear trend of genome size increase in the genus (Figure 3.1). The smallest genome sizes are found in species belonging to section *Brachypetalum*, which is the second branching group in the phylogenetic framework (mean 1C = 18.64 pg) and the highest genome sizes are found in section *Barbata* of the subgenus *Paphiopedilum* (mean 1C = 29.27 pg). *Barbata* was thought to be the most derived group (Atwood, 1984), although it could not be inferred from the results of this study. The greatest range and largest genomes were found in section *Barbata*, which is also characterised by being the most variable in terms of chromosome number (2*n* = 28–42). However, section *Cochlopetalum*, which is also variable in chromosome number (2*n* = 30–37), has a similar range of genome size to other sections and subgenera characterised by 2*n* = 26. This result was different from the previous research by Cox *et al.* (1998) which used a different phylogenetic framework and showed a trend of increasing genome size for *Paphiopedilum*.

The result differs from studies of closely related genera. Genome size data was mapped onto the phylogenetic framework of *Cypripedium*, in which it was shown that the ancestral condition was likely to be a small genome size, suggesting a trend of an increase in genome size for the genus (Kahandawala, 2009). For the genus *Phragmipedium*, in which genome sizes are small, there is a trend towards an increase in genome size, as observed by Cox *et al.* (1998) but only relatively few samples were included in their study. There have been several reports of small genome size as an ancestral condition, as in angiosperm families (Leitch *et al.*, 1998), Liliaceae (Peruzzi *et al.*, 2009; Leitch *et al.*, 2007) and Brassicaceae (Lysak *et al.*, 2009). Because genome size data was not available for all taxa sampled on the phylogenetic trees in this study, many gaps were present when considering genome size data for individual taxa. Including additional DNA sequence data in the phylogenetic study for those taxa with genome size data available and including more genome size data for the genus would help to understand the changes in genome size within each clade. In addition, better resolution of internal clades would be required for understanding genome size evolution among taxa, such as for section *Barbata*, in which the internal clades were particularly unresolved.

When chromosome numbers were plotted against genome size data, a weak but significant relationship was found (Figure 3.2), suggesting that as chromosomes undergo centric fission, it is often associated with an increase in genome size. The

precise origin of additional DNA in the genome is unclear, but it is likely to comprise a diverse array of different types of repetitive DNA, including retrotransposons (Bennetzen, 2005). One probable source of the additional DNA is telomeric sequences, which are required to stabilise the chromosome ends after centric fission (Leitch *et al.*, 2009). Another source of repetitive DNA, found to be highly abundant in the centromere, is centromeric satellite DNA, which has been reported in many plant species (Jiang *et al.*, 2003). In addition, plant retrotransposons, which are usually transcriptionally inactive, can be activated under stress, such as wounding, pathogen attack and tissue culture (Wessler, 1996; Grandbastien, 1998). Also, it has been reported in *Arabidopsis* spp. and Brassicaceae that heat stress could activate retrotranspons (Ito *et al.*, 2011; Ito *et al.*, 2013). Some plant retrotransposon families have been reported as being located specifically in centromeres and have been termed centromeric retrotransposons (Jiang *et al.*, 2003). Thus, it is possible that fission of the centromere may trigger the amplification of centromere-specific retrotransposons, also contributing to genome size increases, The origin of satellite repeats is unclear, but it has been postulated in some studies that centromeric tandem repeats can be derived from centromeric retrotransposons, due to sequence similarity between them, as has been observed in wheat and its relatives (Cheng and Murata, 2003) and maize (Sharma *et al.*, 2013).

However, these mechanisms could differ from those of closely related genera of *Paphiopedilum* because the relationship between chromosome number and genome size is different among them. *Phragmipedium*, which with *Mexipedium* is sister to *Paphiopedilum*, has a variable chromosome number (2*n* = 18–30) but a smaller mean genome size and a narrower range (1.5-fold, 1C = 6.10 to 9.18 pg) (Cox *et al.*, 1998). *Cypripedium* is the most variable genus in subfamily Cypripedioideae in terms of genome size, with values ranging 10.5-fold (1C = 4.1 to 43.1 pg) but the chromosome number in most species is constant (2*n* = 20) (Kahandawala, 2009; Leitch *et al.*, 2009). Because there was no centric fission in *Cypripedium*, such telomeric sequences and centromeric DNA that could have arisen as a consequence of centric fission, as may be the case in *Paphiopedilum*, are unlikely to be the source of additional DNA but the greater likelihood is that it is due to retrotransposons interspersed throughout the genome, as was suggested by Kahandawala (2009).

Physical chromosome mapping techniques, such as fluorescence *in situ* hybridisation (FISH), would be useful methods for the investigation of the physical organisation of repetitive DNA in the genome, in which they can be characterised and localised on

chromosomes, thus allowing their role in genome evolution to be fully understood (Jiang and Gill, 2006). Recent times have seen the advance of next generation sequencing technologies, which have been used for studying repetitive sequences, including retrotransposons in *Nicotiana* spp. (Renny-Byfield *et al.*, 2011) and *Silene latifolia* Poir. (Macas *et al.*, 2011). Kelly and Leitch (2011) pointed out that although these techniques have been used mostly for plants with smaller genome sizes (i.e. < 3500 Mb), they have the potential to allow a rapid advance of the study of genome evolution in plants with large genome sizes, such as *Fritillaria* spp.

5.3 Development of plastid microsatellites for Cypripedioideae

Monitoring the genetic diversity of endangered species is crucial for their conservation. Many molecular markers are available for this purpose, each with their own advantages and limitations. To determine which strategies should be used in each case, genome size should to be taken into consideration (Leitch *et al.*, 2009). The development of molecular markers such as AFLP and nuclear microsatellites for species with large genome sizes (i.e. 1C-value > 15 pg) has been reported as being difficult due to problems with PCR amplification (Fay *et al.*, 2005; Garner, 2002). This includes species in subfamily Cypripedioideae, of which *Cypripedium calceolus* (Fay *et al.*, 2005; Fay *et al.*, 2009; Kahandawala, 2009) has a 1C value of 32.4 pg (Bennett *et al.*, 2000). The negative effects on the amplification of nuclear microsatellites for species with large genome sizes were pointed out by Garner (2002), in which there is a smaller proportion of target DNA in relation to non-target DNA and an increase in non-specific binding of primers, therefore, the amount of primer available for PCR amplification is reduced. In the case of AFLP, samples with large genome sizes are found to be not amplified adequately for band scoring (Fay *et al.*, 2005). It has been reported that species with large genome sizes are more likely to be at risk of extinction (Vinogradov, 2003) and there are constraints on speciation associated with large genome size (Knight *et al.*, 2005) as discussed previously in Chapter 3. Inbreeding depression has also been suggested as a possible reason for an increased extinction rate for island populations, especially endemic species (Frankham, 1998), as is the case with many species of *Paphiopedilum*. This makes monitoring genetic diversity in order to determine effective conservation strategies all the more important but assessing genetic diversity has its limitations in plants with large genome sizes (Leitch *et al.*, 2009).

Plastid microsatellites are more applicable than other markers as they require only a single short locus to be amplified, which allows DNA of low quality and quantity to be used (Fay and Cowan, 2001). In this study, plastid microsatellites were developed for species across the subfamily Cypripedioideae, as most of these species are endangered and are rare in the wild (Cribb, 1998; Nicolè *et al.*, 2005). Eight primer pairs for plastid microsatellites were designed from flanking regions of consensus sequences generated from different genera. The results of PCR amplification from testing those newly designed primers with samples from widely ranging taxa in the subfamily have shown most of them to be applicable across the subfamily Cypripedioideae (Table 4.6), suggesting there are more or less conserved flanking areas between species in different genera, allowing successful amplification from degenerate primer pairs.

At the interspecific level, high levels of variation in allele size were observed among species in each genus, in terms of the number of alleles, size range and unbiased haploid diversity (Table 4.6). Highly variable allele size suggests indels could be the source of variation in those markers. Also, some plastid markers showed an allele size range greater than expected, which suggests non-specific PCR amplification could have occurred. To test the homology of those plastid microsatellite markers, further work in sequencing those regions needs to be carried out. At intraspecific level, low levels of variation in size length were observed in *C. calceolus* samples (Tables 4.6–4.7). Size variation has also been detected in other species in some markers but the number of samples included is too small for them to be evaluated for their utility for population studies, because of the limitation in obtaining samples for these endangered and rare species (Table 4.7).

The number of repeat motifs observed from sequences used for designing primers tends to be correlated with the number of alleles detected from plastid microsatellites in this study, as has also been observed in wheat and related species by Ishii *et al.* (2001). This correlation might be the reason for the lower number of alleles detected in *C. calceolus*, as low repeat motifs were found in most *Cypripedium* sequences. The greater number of repeat motifs in *Paphiopedilum* and *Phragmipedium* sequences might suggest a higher number of alleles would be detected from plastid microsatellites but that could not be evaluated here as only a small number of samples were tested (see Table 4.3).

The application of plastid microsatellite markers for population genetic analyses in *C. calceolus* was limited in this study because few of them are polymorphic and low numbers of alleles were detected. The results from statistical analyses in general, show congruence with the previous study by Fay *et al.* (2009), as most genetic variation was found within populations rather than among them but unbiased haploid diversity was found to be lower than their gene diversity, due to lower numbers of polymorphic markers in this study (Table 4.8–4.9). Although the number of haplotypes was lower than in the previous study, there was a similarity, in that one haplotype was found to be widely distributed (Figure 4.5), as was suggested previously by Fay *et al.* (2009) due to the potential for wind dispersal in orchids because of their light weight seeds. The distribution of plastid haplotypes of *C. calceolus* in western and northern Europe in this study could indicate possible recolonisation routes of this species from three main refugia following glaciation. However, more haplotype data, from a larger number of polymorphic plastid markers and a wider range of samples from eastern Europe and eastern Asia need to be included in further research in order to establish those routes more accurately.

The implications for population studies of using plastid microsatellite markers for the conservation of *C. calceolus* in the UK has been addressed previously with regard to decision making for the selection of plants to be reintroduced to the wild (Fay *et al.*, 2009). The results from the haplotype data of the UK plants in this study emphasise the findings that those UK plants are genetically variable and the haplotype of putatively introduced plants differs from the native ones (Figure 4.6). Also, the difference in haplotype of native plants occurs in different ecological settings and should be taken into consideration when reintroducing plants to the wild. The reintroduction of plants from different ecological habitats could potentially cause problems for the survival of the plants in those habitats, as genetically different plants might be under selective pressure for adaptation to specific environments and so not be adapted to the new habitat. Also, it would result in the replacement of genetically different plants, rather than conserving the original genotype (Schuiteman and de Vogel, 2003).

Recently, next generation sequencing methods have been reported, identifying advantages for their application in the development of nuclear microsatellite markers, as they are quicker and cheaper than conventional development, which involves cloning methods for microsatellite isolation (Zalapa *et al.*, 2012). This application has been reported on for orchid species, including *Cypripedium kentuckiense* C.F.Reed and *Pogonia ophioglossoides* (Pandey and Sharma, 2012), thus it could also be

applicable for *Cypripedium calceolus* and other slipper orchids. Plastid microsatellites remain an alternative marker of choice when species samples consist of small quantities or low quality DNA (Fay and Cowan, 2001). They are also good markers for revealing historical lineages without being obscured by recombination, as can be the case with nuclear markers (Echt *et al.*, 1998). In addition, because they represent half the effective population size, compared to nuclear genomes, they are considered to be good indicators for revealing ancient bottlenecks, founder effects and genetic drift (Provan *et al.*, 2001).

In summary, the molecular phylogenetic study of the genus *Paphiopedilum*, using DNA sequencing techniques to obtain DNA sequence data from nuclear and plastid regions helped to address generic, subgeneric and sectional delineation and helped to elucidate the phylogenetic relationships within the genus. However, further work of including more sequence data from variable regions needs to be undertaken for those clades which remain unresolved. Also, this research provided a phylogenetic framework for addressing the question of the direction of evolutionary processes for other biological characters, such as that addressed here for genome size and chromosome number evolution in *Paphiopedilum*. Species in this genus and other slipper orchids have large genome sizes and there is a correlation between inflated genomes and an increased rate of extinction. This, together with over-collecting and habitat destruction, makes the development of conservation strategies for slipper orchids all the more important. Here, it is shown that plastid molecular markers would be better alternative markers over other approaches that have problems caused directly by the negative effects of plants having large genome sizes. These newly developed plastid markers would help in the assessment of levels of genetic diversity, although low polymorphisms were detected at intraspecific level in *Cypripedium calceolus*. New technologies, such as next generation sequencing, would help in further work for developing nuclear microsatellites, which would provide more variation than plastid markers. However, the uniparental nature of of plastid markers will remain useful to address questions of phylogeography when more samples and more markers become available.

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Appendix

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Molecular phylogenetics of *Paphiopedilum* (Cypripedioideae; Orchidaceae) based on nuclear ribosomal ITS and plastid sequences

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Phylogenetic relationships in the genus *Paphiopedilum* were studied using nuclear ribosomal internal transcribed spacer (ITS) and plastid sequence data. The results confirm that the genus *Paphiopedilum* is monophyletic, and the division of the genus into three subgenera *Parvisepalum*, *Brachypetalum* and *Paphiopedilum* is well supported. Four sections of subgenus *Paphiopedilum* (*Pardalopetalum*, *Cochlopetalum*, *Paphiopedilum* and *Barbata*) are recovered as in a recent infrageneric treatment, with strong support. Section *Coryopedilum* is also recovered, with low bootstrap but high posterior probability values for support of monophyly. Relationships in section *Barbata* remain unresolved, and short branch lengths and the narrow geographical distribution of many species in the section suggest that it possibly underwent rapid radiation. Mapping chromosome and genome size data (including some new genome size measurements) onto the phylogenetic framework shows that there is no clear trend in increase in chromosome number in the genus. However, the diploid chromosome number of $2n = 26$ in subgenera *Parvisepalum* and *Brachypetalum* suggests that this is the ancestral condition, and higher chromosome numbers in sections *Cochlopetalum* and *Barbata* suggest that centric fission has possibly occurred in parallel in these sections. The trend for genome size evolution is also unclear, although species in section *Barbata* have larger genome sizes than those in other sections. © 2012 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2012, **170**, 176–196.

ADDITIONAL KEYWORDS: centric fission – chromosome number – evolution – genome size – infrageneric classification.

INTRODUCTION

The genus *Paphiopedilum* Pfitzer comprises *c.* 72 species (Averyanov *et al.*, 2003), distributed from India and southern China through south-east Asia and the Malesian islands to the Solomon Islands (Cribb, 1998). Most species are terrestrial, but some are epiphytic or lithophytic (Cribb, 1998). This genus is the largest of the five genera of slipper orchids in subfamily Cypripedioideae (Orchidaceae). The other genera are *Phragmipedium* Rolfe, *Selenipedium* Rchb.f., *Cypripedium* L. and *Mexipedium* V.A.Albert & M.W.Chase. Floral characteristics of the slipper orchids are a slipper-shaped lip, two fertile stamens, a shield-like staminode and united lateral sepals or a synsepal (Cox *et al.*, 1997). There is no unique morphological character to distinguish the slipper orchid genera from each other, but they can be distinguished by a combination of morphological characters, including leaf type, number of locules, type of placentation and geographical distribution (Cox *et al.*, 1997). The characteristics of *Paphiopedilum* are conduplicate leaves, imbricate sepal aestivation and a unilocular ovary with parietal placentation. *Paphiopedilum* can be distinguished from the Northern Hemisphere *Cypripedium* and the tropical American *Selenipedium* by those genera having plicate leaves and perforate sepal aestivation. In addition, *Selenipedium* is distinguished by a trilocular ovary with axile placentation. Among the conduplicate leaved genera,

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Paphiopedilum can be distinguished from the central to southern American *Phragmipedium* by that genus having valvate sepal aestivation, a trilocular ovary and axile placentation and from the monotypic *Mexipidium*, which is restricted to Mexico, by *Mexipedium* having valvate sepal aestivation (Atwood, 1984; Albert & Chase, 1992; Cox *et al.*, 1997).

The beautiful and often bizarre flowers of slipper orchids are not only attractive to insects but also to plant collectors, which have made them popular ornamental plants and has led to over-collection of plants from the wild, and this, along with the destruction of their habitat, means that many species are endangered or even facing extinction. The Convention on International Trade of Endangered Species (CITES) lists *Paphiopedilum* on Appendix I (CITES, 2012).

Paphiopedilum was first described by Pfitzer in 1886. Subsequently, infrageneric classifications of the genus have been proposed by various authors (Pfitzer, 1894, 1903; Hallier, 1896; Brieger, 1971; Karasawa & Saito, 1982; Atwood, 1984; Cribb, 1987, 1998; Braem, 1988; Cox *et al.*, 1997; Braem, Baker & Baker, 1998; Averyanov *et al.*, 2003; Braem & Chiron, 2003). An overview of previous infrageneric classifications is shown in Table 1.

The first comprehensive study of the molecular phylogenetics of subfamily Cypripedioideae was that of Cox *et al.* (1997), using nuclear ribosomal DNA internal transcribed spacer (ITS) sequence data. The circumscriptions of sections in *Paphiopedilum* were, in general, congruent with the previous infrageneric classification of Cribb (1987). However, the result did not support the division of the genus into two subgenera, *Brachypetalum* (Hallier f.) Pfitzer and *Paphiopedilum* K.Karas. & K.Saito, because subgenus *Brachypetalum* was found to be paraphyletic to subgenus *Paphiopedilum*. Section *Concoloria* (Kraenzl.) V.A.Albert & Börge Pett. (=section *Brachypetalum sensu* Cribb, 1987) of subgenus *Brachypetalum* was nested in a clade of subgenus *Paphiopedilum*. In addition, section *Coryopedilum* Pfitzer was weakly supported as paraphyletic to the monophyletic section, *Pardalopetalum* Hallier f. & Pfitzer. Cox *et al.* (1997) tentatively proposed elevating section *Parvisepalum* (K.Karas. & K.Saito) P.J.Cribb and section *Concoloria* of subgenus *Brachypetalum* to subgenera *Parvisepalum* K.Karas. & K.Saito and *Brachypetalum*, and suggested combining sections *Coryopedilum* and *Pardalopetalum* in their infrageneric treatment. Also, they suggested simplification of the subsectional treatment of Braem (1988), because groupings of only a few species are less useful in understanding the relationships among the groups. Although the ITS results of Cox *et al.* (1997) suggested that the infrageneric classification of Cribb (1987) was mainly well defined, it did not provide support for monophyly of the largest subgenus, *Paphiopedilum*. In addition, the phylogenetic relationships between sections in subgenus *Paphiopedilum* remained unclear, because the resulting tree did not have sufficient bootstrap support for those clades.

The infrageneric classification of Cribb (1998) in his second edition of the monograph, mainly based on morphological characters and chromosome data, also followed the molecular study of Cox *et al.* (1997). Cribb subdivided *Paphiopedilum* into three subgenera in his classification: *Parvisepalum*; *Brachypetalum*; and *Paphiopedilum*. Five sections of subgenus *Paphiopedilum* (*Coryopedilum*, *Pardalopetalum*, *Cochlopetalum* Hallier f. ex Pfitzer, *Paphiopedilum* and *Barbata* (Kraenzl.) V.A.Albert & Börge Pett.) remained, as in his previous treatment.

Averyanov *et al.* (2003) followed the outline of the infrageneric classification of Cribb (1998), but they further divided subgenus *Parvisepalum* into two sections: *Parvisepalum* and *Emersonianum* Aver. & P.J.Cribb. The new section *Emersonianum* was recognized to include *P. hangianum* Perner & O.Gruss and *P. emersonii* Koop. & P.J.Cribb, which were differentiated mainly by these species having plain green leaves, whereas species of section *Parvisepalum* have tessellated leaves.

The analysis of nuclear DNA regions alone, such as ITS, as in the study of Cox *et al.* (1997), may be inadequate for obtaining the necessary resolution of phylogenetic relationships at lower levels, although they may evolve rapidly (e.g. Álvarez & Wendel, 2003). Sequence data from other loci, such as plastid DNA, can be useful for investigating the relationships between closely related species. Although generally evolving relatively slowly, various regions of the plastid genome have undergone more rapid evolution, potentially providing more variation for studying closely related taxa (e.g. Shaw *et al.*, 2005, 2007). These data can also be utilized to test phylogenetic relationships independently and can be combined with data from other loci. Furthermore, unlike nuclear loci, plastid loci are uniparentally inherited (maternally in the case of slipper orchids, as for most flowering plants; Corriveau & Coleman, 1988), thus avoiding the potential problem of paralogous copies found in the nuclear genome.

In a recently published paper (Guo *et al.*, 2012), six plastid DNA regions and two low-copy nuclear genes were used to study phylogenetics and biogeography in subfamily Cypripedioideae. As in earlier studies, *Paphiopedilum* was shown to be monophyletic, and it was strongly supported as sister to *Phragmipedium*/*Mexipedium*. Sampling of *Paphiopedilum* spp., however, was rather sparse (eight species only) and the focus was on relationships between, rather than within, the genera.

Table 1. An overview of infrageneric classifications of genus *Paphiopedilum* (modified from Cribb 1998).

Pfitzer (1894)	Hallier (1896)	Pfitzer (1903)	Brieger (1971)	Karasawa & Saito (1982)	Atwood (1984)
Coelopedilum **group**	*Coelopedilum* **group**				
a. *Eremantha Tessellata* (**in part**)	*Aphanoneura Brachypetalum*	*Brachypetalum*	*Brachypetalum*	*Brachypetalum*	*Brachypetalum*
				Parvisepalum	
b. *Polyantha*	*Chromatoneura Viridia Polyantha*	*Anotopedilum*	*Polyantha*	*Polyantha*	*Paphiopedilum*
	XI *Streptopetalum* (**in part**)	**Section** *Coryopedilum*	**Section** *Streptopetalum*	**Section** *Mastigopetalum*	**Section** *Coryopedilum*
	XII *Mastigopetalum*	**Section** *Gonatopedilum*	**Section** *Mastigopetalum*		
		Section *Prenipedilum*			
		Otopedilum			
	XI *Streptopetalum* (**in part**)	**Section** *Mystropetalum*	**Section** *Polyantha*	**Section** *Mystropetalum*	**Section** *Pardalopetalum*
	X *Pardalopetalum*	**Section** *Pardalopetalum*		**Section** *Polyantha*	
	XIII *Cochlopetalum*	**Section** *Cochlopetalum*	**Section** *Cochlopetalum*	*Cochlopetalum*	**Section** *Cochlopetalum*
a. *Eremantha Viridia*	*Chromatoneura Viridia Eremantha*		*Paphiopedilum*	*Paphiopedilum*	**Section** *Paphiopedilum*
	VIII *Stictopetalum*	**Section** *Stictopetalum*	**Section** *Stictopetalum*	**Section** *Stictopetalum*	
	IX *Neuropetalum*	**Section** *Neuropetalum*	**Section** *Paphiopedilum*	**Section** *Paphiopedilum*	
	V *Thiopetalum*	**Section** *Thiopetalum*		**Section** *Thiopetalum*	
	VII *Cymatopetalum*	**Section** *Cymatopetalum*			
	VI *Ceratopetalum*	**Section** *Ceratopetalum*		**Section** *Ceratopetalum*	
a. *Eremantha Tessellata*	*Chromatoneura Tessellata*		*Barbata*	*Sigmatopetalum*	**Section** *Barbata*
(**in part**)	II *Sigmatopetalum*	**Section** *Spathopetalum*	**Section** *Sigmatopetalum*	**Section** *Spathopetalum*	
	IV *Drepanopetalum*	**Section** *Blepharopetalum*	**Section** *Blepharopetalum*	**Section** *Sigmatopetalum*	
				Section *Blepharopetalum*	
				Section *Punctatum*	
				Section *Planipetalum*	
	III *Clinopetalum*	**Section** *Phacopetalum*	**Section** *Barbata*	**Section** *Barbata*	

Cribb (1987)	Braem (1988), Braem *et al.* (1998) and Braem & Chiron (2003)	Cox *et al.* (1997)	Cribb (1998)	Averyanov *et al.* (2003)
Brachypetalum	*Brachypetalum*	*Brachypetalum*	*Brachypetalum*	*Brachypetalum*
Section *Brachypetalum*				
Section *Parvisepalum*	*Parvisepalum*	*Parvisepalum*	*Parvisepalum*	*Parvisepalum*
				Section *Parvisepalum*
				Section *Emersonianum*
Paphiopedilum	*Polyantha*	*Paphiopedilum*	*Paphiopedilum*	*Paphiopedilum*
Section *Coryopedilum*	**Section** *Mastigopetalum*	**Section** *Coryopedilum*	**Section** *Coryopedilum*	**Section** *Coryopetalum*
Section *Pardalopetalum*	**Section** *Mystropetalum*	**Section** *Pardalopetalum*	**Section** *Pardalopetalum*	**Section** *Pardalopetalum*
	Section *Polyantha*			
Section *Cochlopetalum*	*Cochlopetalum*	**Section** *Cochlopetalum*	**Section** *Cochlopetalum*	**Section** *Cochlopetalum*
Section *Paphiopedilum*	*Paphiopedilum*	**Section** *Paphiopedilum*	**Section** *Paphiopedilum*	**Section** *Paphiopedilum*
	Section *Stictopetalum*			
	Section *Paphiopedilum*			
	Section *Thiopetalum*			
	Section *Ceratopetalum*			
Section *Barbata*	*Sigmatopetalum*	**Section** *Barbata*	**Section** *Barbata*	**Section** *Barbata*
	Section *Spathopetalum*			
	Section *Sigmatopetalum*			
	Section *Blepharopetalum*			
	Section *Punctatum*			
	Section *Planipetalum*			
	Section *Barbata*			

Genome size in angiosperms varies *c.* 2400-fold, from that of the carnivorous plant *Genlisea margaretae* Hutch. (Lentibulariaceae), 1C-value of only 0.0648 pg, to that of the monocot *Paris japonica* (Franch. & Sav.) Franch. (Melanthiaceae), the largest known genome of 1C = 152.23 pg (Greilhuber *et al.*, 2006; Pellicer, Fay & Leitch, 2010; Bennett & Leitch, 2011). Most angiosperms have a small genome size; based on an analysis of > 6000 species, the modal and median of 1C values are only 0.6 and 2.9 pg (Bennett & Leitch, 2010). Species with very large genome sizes (i.e. 1C ≥ 35 pg, Kelly & Leitch, 2011) are found mainly in monocots, including Orchidaceae. Among angiosperms, based on available data, Orchidaceae have the greatest variation in genome size, ranging 168-fold from 1C = 0.33 pg in *Oncidium maduroi* Dressler to 55.4 pg in *Pogonia ophioglossoides* (L.) Ker Gawl. (Leitch *et al.*, 2009).

Many species of subfamily Cypripedioideae have large genome sizes ranging > 10-fold from 1C = 4.1 pg in *Cypripedium molle* Lindl. to 43.1 pg in *C. fargesii* Franch., and *Cypripedium* is the most variable genus in the subfamily (Kahandawala, 2009; Leitch *et al.*, 2009). *Paphiopedilum* spp. also have large genome sizes, ranging nearly two-fold, from 1C = 17.80 pg in *P. godefroyae* (God.-Leb.) Stein to 34.53 pg in *P. wardii* Summerh., whereas *Phragmipedium* spp. have smaller genomes and a narrower range, varying 1.5-fold, from 1C = 6.1 to 9.18 pg (Cox *et al.*, 1998).

A considerable amount of chromosome data is available for *Paphiopedilum* (e.g. Karasawa, 1978, 1979, 1982, 1986; Karasawa & Aoyama, 1980, 1988; Karasawa & Tanaka, 1980, 1981; Karasawa & Saito, 1982; Karasawa, Aoyama & Kamimura, 1997; Cox *et al.*, 1998). The diploid chromosome number in the genus varies from $2n = 26$ to 42. All species so far analysed in the subgenera *Parvisepalum* and *Brachypetalum* have a chromosome number of $2n = 26$, and many species in subgenus *Paphiopedilum* also have $2n = 26$. In section *Paphiopedilum*, most species have $2n = 26$, except for two species, which have $2n = 30$. Chromosome numbers in section *Cochlopetalum* range from 30 to 37, and section *Barbata* is the most variable, with chromosome numbers ranging from $2n = 28$ to 42. Despite the variation in chromosome number, the total number of chromosome arms ('nombre fondamental' or n.f., Matthey, 1949) appears to be conserved in most species of the genus (n.f. = 52), which might suggest karyotype evolution via Robertsonian change, either producing telocentric chromosomes by centric fission or producing metacentric chromosomes by centric fusion (Robertson, 1916). The first report to postulate Robertsonian change as a cause of total arm number retention in *Paphiopedilum* was that of Duncan & MacLeod (1949). Cox *et al.* (1998) studied the evolution of genome size and karyotype in Cypripedioideae by mapping chromosome number and genome size data onto a phylogenetic tree based on ITS data (Cox *et al.*, 1997). The results for *Paphiopedilum* showed evolutionary trends of an increase in the number of chromosomes and telocentric chromosomes and a decrease in metacentric chromosomes, suggesting the predominant direction of karyotype evolution was via centric fission, leading to higher chromosome numbers. It also showed an increase in genome size. However, the phylogenetic tree used for their study did not provide support for phylogenetic relationships between sections of *Paphiopedilum*, as mentioned previously, and these hypotheses need to be reassessed in a phylogenetic framework with better resolution and support.

The aims of this study were to collect DNA sequence data from nuclear (ITS) and plastid (partial *matK*, *ycf1*, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*) loci to address generic, subgeneric and sectional circumscription and to investigate phylogenetic relationships within the genus. In addition, the more robust phylogenetic trees were used as a framework to analyse evolutionary trends in genome size and chromosome number in the genus.

MATERIAL AND METHODS

Plant material

Most DNA samples were obtained from the DNA Bank at the Jodrell Laboratory (RBG, Kew). In addition, some leaf material was obtained for DNA extraction from the living plant collection at the Tropical Nursery, (RBG, Kew). As samples for two species, *P. hangianum* and *P. emersonii*, of subgenus *Parvisepalum* section *Emersonianum* in the treatment of Averyanov *et al.* (2003), were not available, we were not able to address the question on the monophyly of this group. The taxon sampling used in this study was based on the infrageneric treatment of Cribb (1998) for sampling subgenera *Parvisepalum*, *Brachypetalum* and *Paphiopedilum* (sections *Coryopedilum*, *Pardalopetalum*, *Cochlopetalum*, *Paphiopedilum* and *Barbata*). The morphological terms used also follow Cribb (1998). Outgroup taxa were sampled from *Phragmipedium*, the sister genus of *Paphiopedilum* (Cox *et al.*, 1997). All species of *Paphiopedilum* and the outgroups used in this study, with voucher information, are listed in Table 2.

MOLECULAR STUDY

DNA extraction

For additional DNA samples, genomic DNA was extracted from fresh plant material, following the

Table 2. Materials used for molecular phylogenetics in this study

Taxa	Voucher/source	GenBank accession numbers				
		ITS	*matK*	*ycf1*	*psaA-ycf3ex3*	*trnF*(GAA)-*ndhJ*
Subgenus *Parvisepalum*						
Paphiopedilum delenatii Guillaumin	Chochai 39746 (K)	JQ929314	JQ929368	JQ929521	JQ929419	JQ929470
Paphiopedilum malipoense S.C.Chen & Z.H.Tsi	Z6	JQ929336	JQ929388	JQ929541	JQ929439	JQ929490
Paphiopedilum micranthum Tang & F.T.Wang	M.W. Chase O-629 (K)	JQ929338	JQ929390	JQ929543	JQ929441	JQ929492
Subgenus *Brachypetalum*						
Paphiopedilum concolor (Bateman) Pfitzer (a)	Z17	JQ929312	JQ929367	JQ929520	JQ929418	JQ929469
Paphiopedilum concolor (Bateman) Pfitzer (b)	Yang Ping, Guizhem. Luo *s.n.*	JQ929313	–	–	–	–
Paphiopedilum niveum (Rchb.f.) Stein	36862*, Kew 1990–996† (no voucher)	JQ929339	JQ929391	JQ929544	JQ929442	JQ929493
Subgenus *Paphiopedilum*						
Section *Paphiopedilum*						
Paphiopedilum hirsutissimum (Lindl. ex Hook.) Stein	Chochai 36808 (K)	JQ929327	–	–	–	–
Paphiopedilum hirsutissimum (Lindl. ex Hook.) Stein var. *esquirolei* (Schltr.) K.Karas. & K.Saito	M.W. Chase O-642 (K)	JQ929328	–	–	–	–
Paphiopedilum charlesworthii (Rolfe) Pfitzer	M.W. Chase O-632 (K)	JQ929310	JQ929365	JQ929518	JQ929416	JQ929467
Paphiopedilum insigne (Wall. ex Lindl.) Pfitzer	Chochai 36821 (K)	JQ929329	JQ929381	JQ929534	JQ929432	JQ929483
Paphiopedilum exul (Ridl.) Rolfe	36804*, Kew 1977–2853† (no voucher)	JQ929317	JQ929371	JQ929524	JQ929422	JQ929473
Paphiopedilum gratrixianum (Mast.) Rolfe (a)	Chochai 36809 (K)	JQ929322	JQ929376	JQ929529	JQ929427	JQ929478
Paphiopedilum gratrixianum (Mast.) Rolfe (b)	Chochai 40235 (K)	JQ929323	JQ929377	JQ929530	JQ929428	JQ929479
Paphiopedilum gratrixianum (Mast.) Rolfe (c)	Chochai 40236 (K)	JQ929324	JQ929378	JQ929531	JQ929429	JQ929480
Paphiopedilum villosum (Lindl.) Stein var. *boxallii* (Rchb.f.) Pfitzer	Chochai 36822 (K)	JQ929354	JQ929405	JQ929558	JQ929456	JQ929507
Paphiopedilum tigrinum Koop. & N.Haseg.	ex Paul Phillips-Rathcliffe	JQ929351	–	–	–	–

Table 2. *Continued*

Taxa	Voucher/source	GenBank accession numbers				
		ITS	*matK*	*ycf1*	*psaA-ycf3ex3*	*trnF*(GAA)-*ndhJ*
Paphiopedilum druryi (Bedd.) Stein	Chochai 36811 (K)	JQ929316	JQ929370	JQ929523	JQ929421	JQ929472
Paphiopedilum spicerianum (Rchb.f.) Pfitzer	M.W. Chase O-643 (K)	JQ929347	JQ929399	JQ929552	JQ929450	JQ929501
Section *Barbata*						
Paphiopedilum appletonianum (Gower) Rolfe	M.W. Chase 5897 (K)	JQ929306	JQ929362	JQ929515	JQ929413	JQ929464
Paphiopedilum sangii Braem	O-822* (no voucher)	JQ929346	JQ929398	JQ929551	JQ929449	JQ929500
Paphiopedilum mastersianum (Rchb.f.) Stein	M.W. Chase 5900 (K)	JQ929337	JQ929389	JQ929542	JQ929440	JQ929491
Paphiopedilum violascens Schltr.	O-825* (no voucher)	JQ929355	JQ929406	JQ929559	JQ929457	JQ929508
Paphiopedilum tonsum (Rchb.f.) Stein	M.W. Chase 5902 (K)	JQ929352	JQ929403	JQ929556	JQ929454	JQ929505
Paphiopedilum barbatum (Lindl.) Pfitzer	M.W. Chase 5898 (K)	JQ929307	JQ929363	JQ929516	JQ929414	JQ929465
Paphiopedilum callosum (Rchb.f.) Stein	Z4	JQ929308	JQ929364	JQ929517	JQ929415	JQ929466
Paphiopedilum callosum (Rchb.f.) Stein var. *sublaeve* (Rchb.f.) P.J.Cribb	Z32	JQ929309	–	–	–	–
Paphiopedilum hennisianum (M.W.Wood) Fowlie	Z30	JQ929326	JQ929380	JQ929533	JQ929431	JQ929482
Paphiopedilum fowliei Birk	M.W. Chase O-644 (K)	JQ929318	JQ929372	JQ929525	JQ929423	JQ929474
Paphiopedilum javanicum (Reinw. ex Lindl.) Pfitzer var. *virens* (Rchb.f) Stein	M.W. Chase O-635 (K)	JQ929330	JQ929382	JQ929535	JQ929433	JQ929484
Paphiopedilum lawrenceanum (Rchb.f.) Pfitzer	Chochai 36824 (K)	JQ929332	JQ929384	JQ929537	JQ929435	JQ929486
Paphiopedilum ciliolare (Rchb.f.) Stein	Z25	JQ929311	JQ929366	JQ929519	JQ929417	JQ929468
Paphiopedilum superbiens (Rchb.f.) Stein var. *curtisii* Braem	Z5	JQ929350	JQ929402	JQ929555	JQ929453	JQ929504
Paphiopedilum sukhakulii Schoser & Senghas	M.W. Chase 5901 (K)	JQ929349	JQ929401	JQ929554	JQ929452	JQ929503
Paphiopedilum wardii Summerh.	M.W. Chase 5903 (K)	JQ929356	JQ929407	JQ929560	JQ929458	JQ929509
Section *Pardalopetalum*						
Paphiopedilum dianthum Tang & F.T.Wang	Z23	JQ929315	JQ929369	JQ929522	JQ929420	JQ929471
Paphiopedilum parishii (Rchb.f.) Stein	Z3	JQ929340	JQ929392	JQ929545	JQ929443	JQ929494
Paphiopedilum lowii (Lindl.) Stein (a)	Z22	JQ929334	JQ929386	JQ929539	JQ929437	JQ929488
Paphiopedilum lowii (Lindl.) Stein (b)	Chochai 36810 (K)	JQ929335	JQ929387	JQ929540	JQ929438	JQ929489
Paphiopedilum haynaldianum (Rchb.f.) Stein	M.W. Chase O-175 (K)	JQ929325	JQ929379	JQ929532	JQ929430	JQ929481

Table 2. *Continued*

Taxa	Voucher/source	GenBank accession numbers				
		ITS	*matK*	*ycf1*	*psaA-ycf3ex3*	*trnF*(GAA)-*ndhJ*
Section *Cochlopetalum*						
Paphiopedilum glaucophyllum J.J.Sm.	Z21	JQ929321	JQ929375	JQ929528	JQ929426	JQ929477
Paphiopedilum liemianum (Fowlie) K.Karas. & K.Saito	36858*, Kew 1990–8000† (no voucher)	JQ929333	JQ929385	JQ929538	JQ929436	JQ929487
Paphiopedilum primulinum M.W.Wood & P.Taylor	Chochai 36827 (K)	JQ929342	JQ929394	JQ929547	JQ929445	JQ929496
Paphiopedilum primulinum M.W.Wood & P.Taylor var. *purpurascens* (M.W.Wood) P.J.Cribb	36860*, Kew 2001–3172† (no voucher)	JQ929343	JQ929395	JQ929548	JQ929446	JQ929497
Paphiopedilum victoria-regina (Sander) M.W.Wood	M.W. Chase O-630 (K)	JQ929353	JQ929404	JQ929557	JQ929455	JQ929506
Section *Coryopedilum*						
Paphiopedilum philippinense (Rchb.f.) Stein	Chochai 36807 (K)	JQ929341	JQ929393	JQ929546	JQ929444	JQ929495
Paphiopedilum randsii Fowlie	M.W. Chase O-636 (K)	JQ929344	JQ929396	JQ929549	JQ929447	JQ929498
Paphiopedilum kolopakingii Fowlie	Z18	JQ929331	JQ929383	JQ929536	JQ929434	JQ929485
Paphiopedilum stonei (Hook.) Stein	Z7	JQ929348	JQ929400	JQ929553	JQ929451	JQ929502
Paphiopedilum adductum Asher	36820*, Kew 1992–3661† (no voucher)	JQ929305	JQ929361	JQ929514	JQ929412	JQ929463
Paphiopedilum glanduliferum (Blume) Stein (a)	M.W. Chase O-716 (K)	JQ929319	JQ929373	JQ929526	JQ929424	JQ929475
Paphiopedilum glanduliferum (Blume) Stein (b)	M.W. Chase O-717 (K)	JQ929320	JQ929374	JQ929527	JQ929425	JQ929476
Paphiopedilum wilhelminiae L.O.Williams	36825*, Kew 2005–2702† (no voucher)	JQ929357	JQ929408	JQ929561	JQ929459	JQ929510
Paphiopedilum rothschildianum (Rchb.f.) Stein	Chochai 36806 (K)	JQ929345	JQ929397	JQ929550	JQ929448	JQ929499
Outgroup						
Phragmipedium besseae Dodson & J.Kuhn	Z16a	JQ929358	JQ929409	JQ929562	JQ929460	JQ929511
Phragmipedium schlimii (Linden ex Rchb.f.) Rolfe	M.W. Chase O-183 (VA)	JQ929360	JQ929411	JQ929564	JQ929462	JQ929513
Phragmipedium longifolium (Warsz. & Rchb.f.) Rolfe	Z9	JQ929359	JQ929410	JQ929563	JQ929461	JQ929512

*Kew DNA bank number.
†Kew living collection number.

modified 2 × cetyl trimethylammonium bromide (CTAB) method of Doyle & Doyle (1987). DNA samples were purified by either caesium chloride/ethidium bromide density gradients or DNA purification columns (NucleoSpin Extract II Columns; Macherey-Nagel, GmbH & Co. KG, Germany) according to the manufacturer's protocols.

Amplification

The nuclear ribosomal spacers, ITS1 and ITS2, and the 5.8S ribosomal gene were amplified using the primers of Sun *et al.* (1994) and White *et al.* (1990). Partial *matK*, approximately 800 bp in length, was amplified using the primers of Sun, McLewin & Fay (2001). An approximately 1500-bp portion from the 3′ end of *ycf1* was amplified using the primers of Neubig *et al.* (2009). The non-coding plastid regions, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*, were amplified using the primers of Ebert & Peakall (2009).

All amplified PCR samples were purified using NucleoSpin Extract II columns according to the manufacturer's protocols. The PCR product was then sequenced using a Big Dye Terminator kit (Applied Biosystems Inc., Warrington, UK). The cycle sequencing products were cleaned by ethanol precipitation and then run on an ABI 3730 automated sequencer. Raw sequences were edited and assembled using Sequencher 4.1 software (Gene Codes Inc., Ann Arbor, MI, USA). The resulting sequences were then aligned manually. All sequences were deposited in GenBank.

Parsimony analysis

Sequence data were analysed independently and in combination, using the maximum parsimony criterion in PAUP* version 4.0b10 for Macintosh (Swofford, 2002). All characters were treated as unordered and equally weighted (Fitch, 1971). Parsimony analyses were conducted using a heuristic search strategy, with 1000 replicates of random taxon addition, tree–bisection–reconnection (TBR) branch swapping with MulTrees in effect, gaps treated as missing data and saving no more than ten trees per replicate. Support for groups was evaluated using 1000 replicates of bootstrap (Felsenstein, 1985), with simple addition and TBR swapping, saving ten trees per replicate. Groups were retained when bootstrap percentages (BP) ≥ 50.

Bayesian analysis

The best-fit models for nucleotide substitution for the data matrix of each region were determined by the Akaike information criterion test (Akaike, 1974) as implemented in MrModeltest version 2.2 (Nylander, 2004). The general time reversible model of substitution with gamma distribution (GTR + G) was selected for ITS, partial *matK* and *psaA-ycf3ex3* data and the general time reversible model of substitution with gamma distribution and invariable sites (GTR + I + G) was selected for *ycf1* and *trnF*(GAA)-*ndhJ* data.

All analyses were carried out using the parallel version of MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003) through the University of Oslo Bioportal (http://www.bioportal.uio.no). Two runs of four Monte Carlo Markov chains (MCMC; Yang & Rannala, 1997) were performed for 10 000 000 generations and a tree was sampled every 1000 generations. Each parameter estimation obtained from the results of two runs was checked in Tracer version 1.5 (http://tree.bio.ed.ac.uk/software/tracer) to ascertain whether they had obtained proper effective sample size and to verify that stationary state had been reached. Trees from the first 10% of generations were discarded as burn-in. The remaining trees were combined to build a 50% majority-rule consensus tree in PAUP* version 4.0b10.

Chromosome number and genome size data

Chromosome numbers for *Paphiopedilum* and *Phragmipedium* were taken from the literature (Karasawa, 1979, 1980, 1982, 1986; Karasawa & Aoyama, 1980, 1988; Karasawa *et al.*, 1997; Cox *et al.*, 1998; Bennett & Leitch, 2010; Lan & Albert, 2011). Most genome size data were taken from the literature (Narayan, Parida & Vij, 1989; Cox *et al.*, 1998; Bennett & Leitch, 2010). Seven species were measured for nuclear DNA content by Feulgen microdensitometry according to Greilhuber & Temsch (2001) and Greilhuber (2005). Ten nuclei of mid-prophase cells (4C) were measured per slide and three slides were analysed in total using a Vickers M85a microdensitometer and each nucleus was read three times. *Allium cepa* L. 'Ailsa Craig' (1C = 16.75 pg; Bennett & Smith, 1976) was used as the calibration standard. The 4C-value of each sample was calculated against the 4C-value of the standard in picograms and converted to give the 1C-value.

RESULTS

Alignment of data sets

The ITS data matrix of 56 taxa, three of which were the outgroup, comprised 778 characters, of which 196 were potentially parsimony informative (25.2%). Analysis of ITS sequences yielded 35 equally most-parsimonious trees of 425 steps, consistency index (CI) = 0.82, retention index (RI) = 0.90. One of the most-parsimonious trees was chosen randomly. Tree

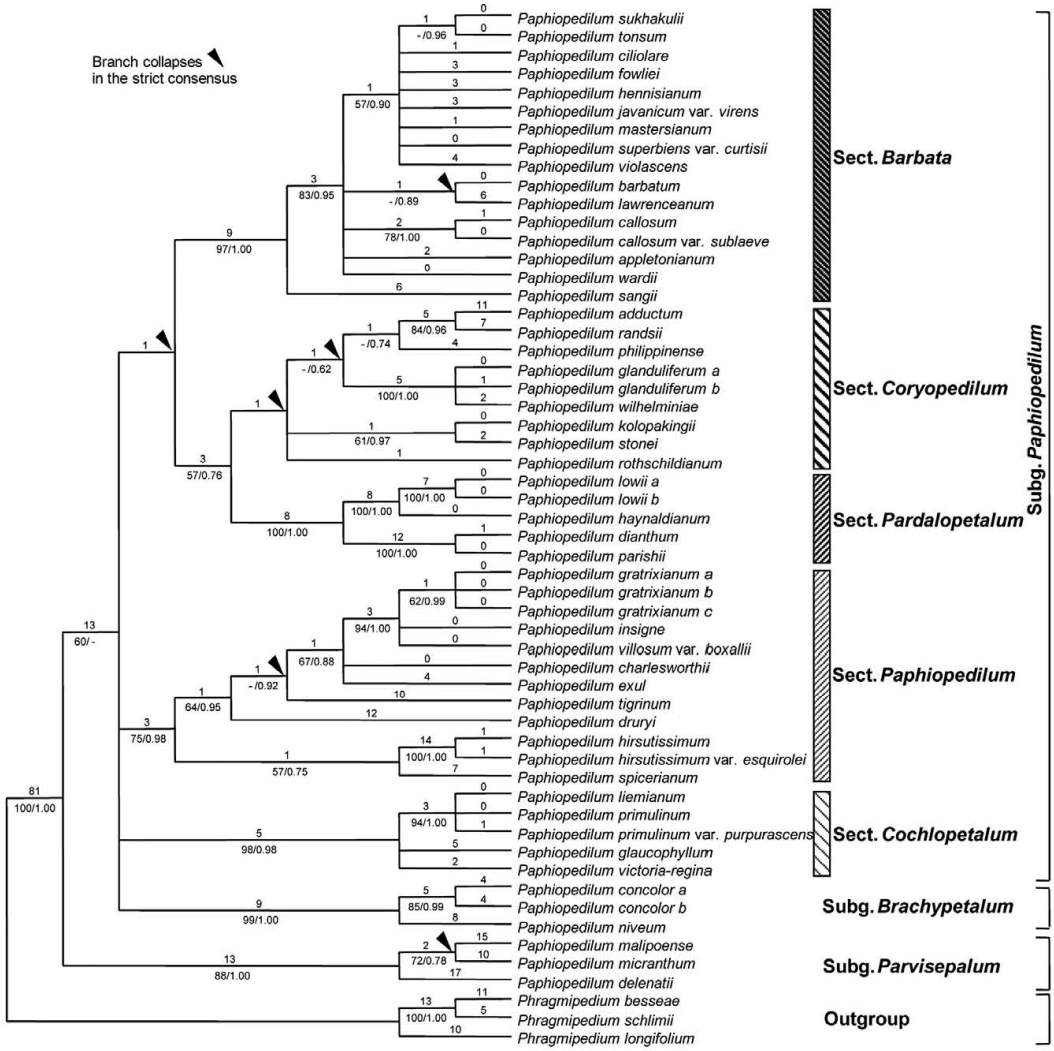

Figure 1. One of 35 most-parsimonious trees from the analysis of the internal transcribed spacer (ITS) region for *Paphiopedilum*. Tree length = 425, consistency index = 0.82, retention index = 0.90. Numbers above branches are branch lengths and numbers below branches are bootstrap percentages ≥ 50 and posterior probability values ≥ 0.50. Arrows indicate clades that collapse in the strict consensus tree obtained from maximum parsimony analysis. The infrageneric treatment follows Cribb (1998).

topology, bootstrap percentages (BP), branches that collapse in the strict consensus tree obtained from maximum parsimony analysis and Bayesian posterior probability values (PP) are indicated in Fig. 1. In the ITS tree, the genus *Paphiopedilum* is monophyletic, with strong support (100 BP, 1.00 PP). Subgenus *Parvisepalum* is the first branching clade with 88 BP and 1.00 PP support for monophyly. The support for monophyly of subgenus *Brachypetalum* was 99 BP and 1.00 PP. Subgenus *Paphiopedilum* forms a polytomy with subgenus *Brachypetalum* (60 BP, – PP). Sections *Barbata*, *Pardalopetalum* and *Cochlopetalum* were well supported with 97 BP, 1.00 PP, 100 BP, 1.00 PP and 98 BP, 0.98 PP,

respectively. Section *Paphiopedilum* had moderate bootstrap support (75 BP) but high PP values (0.98). There was no support for section *Coryopedilum*, and it did not form a clade in the strict consensus tree. In subgenus *Paphiopedilum*, the relationships within some sections were still not well supported.

The plastid data matrix [partial *matK*, *ycf1*, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*], including 51 taxa (it was not possible to obtain sequences for five taxa that were included in the ITS matrix), three of which were the outgroup, comprised 4353 characters, of which 281 were potentially parsimony informative (6.5%). Analysis of a combined plastid region matrix yielded 20 equally most-parsimonious trees of 520 steps, CI = 0.84, RI = 0.92. One of the most-parsimonious trees was randomly chosen, and the tree topology, bootstrap percentages, branches that collapse in the strict consensus tree obtained from maximum parsimony analysis and Bayesian posterior probability values are indicated in Fig. 2. The tree of the combined plastid regions was more resolved than the ITS tree. The genus *Paphiopedilum* is monophyletic, with strong support (100 BP, 1.00 PP). The division of the genus into three subgenera is also well supported (100 BP, 1.00 PP for all). Support for the monophyly of *Paphiopedilum* subgenera *Parvisepalum*, *Brachypetalum* and *Paphiopedilum* is 100 BP, 1.00 PP, 95 BP, 1.00 PP and 100 BP, 1.00 PP, respectively. In subgenus *Paphiopedilum*, sections *Barbata*, *Paphiopedilum* and *Pardalopetalum* are well supported with 93 BP, 1.00 PP, 98 BP, 1.00 PP and 100 BP, 1.00 PP, respectively. Section *Coryopedilum* has weak bootstrap support (67 BP) but high PP support (1.00). Section *Cochlopetalum* forms two clades in a polytomy, with the clade formed by sections *Coryopedilum* and *Pardalopetalum*. In subgenus *Paphiopedilum*, the relationships within some sections are still not well supported.

The combined data matrix included 51 taxa (but excluded those for which only ITS data was available), of which three were outgroups, and comprised 4884 characters, of which 463 were potentially parsimony informative (9.5%). Analysis of the combined data matrix yielded 120 equally most-parsimonious trees of 920 steps, CI = 0.83, RI = 0.91. One of the most-parsimonious trees was randomly chosen. Tree topology, bootstrap percentages, branches that collapse in the strict consensus tree obtained from maximum parsimony analysis and Bayesian posterior probability values are indicated in Fig. 3. The genus *Paphiopedilum* is monophyletic, with strong support (100 BP, 1.00 PP). The division of the genus into three subgenera is well supported (100 BP, 1.00 PP for all). The monophyly of *Paphiopedilum* subgenera *Parvisepalum*, *Brachypetalum* and *Paphiopedilum* is well supported, with BP 100, 1.00 PP for each node. In subgenus *Paphiopedilum*, sections *Barbata*, *Paphiopedilum*, *Pardalopetalum* and *Cochlopetalum* have strong support with 100 BP, 1.00 PP, 99 BP, 1.00 PP, 100 BP, 1.00 PP and 99 BP, 1.00, respectively. Only section *Coryopedilum* has weak bootstrap support (54 BP) and it collapses to form a polytomy with section *Pardalopetalum* in the strict consensus; however, it has a high PP value (0.95). In subgenus *Paphiopedilum*, the relationships within some sections are still not well supported.

Genome size evolution

Genome size data obtained from this study (seven taxa) and from the literature (25 taxa) are listed in Table 3. In Fig. 4, genome size range (1C-value), mean value and chromosome number for each section within the genus are mapped onto the combined tree.

DISCUSSION

Congruence of ITS and plastid data

The results from two separate matrices of ITS and plastid data showed no conflict between strongly supported branches (> 75 BP, > 0.90 PP) when compared node by node. Groupings in the genus in both ITS and plastid trees are generally as described in the treatment of Cribb (1998), but the relationships along the backbone are less resolved in the ITS tree. The results in the plastid trees had better bootstrap support, but the resulting trees from separate analyses of each individual plastid region [partial *matK*, *ycf1*, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*] lacked resolution because of low levels of divergence (data not shown). The combined data set produced more resolved trees, mostly with strong bootstrap support. In general the increase in clade support in the combined tree (Fig. 3) indicates congruence between the ITS and plastid data. The only place where there was lower clade support when the plastid and nuclear data sets were combined was in section *Coryopedilum*, suggesting some possible conflict between data sets in this part of the phylogenetic tree. However, the branches concerned receive only low bootstrap support.

Phylogenetic relationships in the genus *Paphiopedilum*

Overall, the results from all analyses showed general congruence with the previous infrageneric treatment of Cribb (1998), and confirm that the genus *Paphiopedilum* is monophyletic, which is congruent with the results of previous studies (Albert, 1994; Cox *et al.*, 1997).

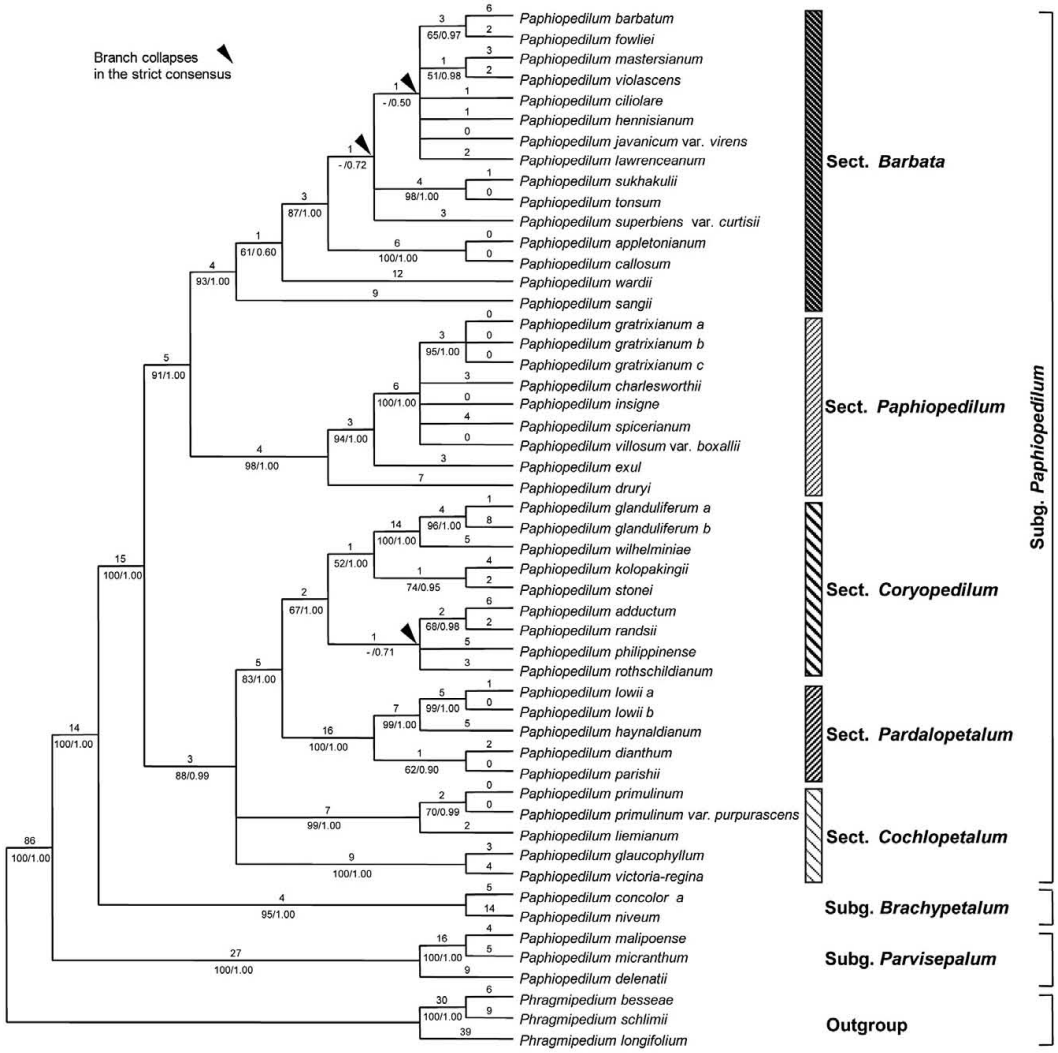

Figure 2. One of 20 most-parsimonious trees from the analysis of plastid (partial *matK*, *ycf1*, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*) regions for *Paphiopedilum*. Tree length = 520, consistency index = 0.84, retention index = 0.92. Numbers above branches are branch lengths and numbers below branches are bootstrap percentages ≥ 50 and posterior probability values ≥ 0.50. Arrows indicate clades that collapse in the strict consensus tree obtained from maximum parsimony analysis. The infrageneric treatment follows Cribb (1998).

Subgenus *Parvisepalum*

Subgenus *Parvisepalum*, characterized by tessellated leaves (except two species, *P. hangianum* and *P. emersonii*, which have plain green leaves; Averyanov *et al.*, 2003), a single-flowered inflorescence, a flower with an inflated lip and a convex (mostly) or conduplicate staminode (Cribb, 1998) (Fig. 4), was found to be the first branching clade with strong support in this study (Figs 2, 3). This confirms the results of Cox *et al.* (1997) and the suggestion of Chen & Tsi (1984) that *P. malipoense* S.C.Chen & Z.H.Tsi and its closely related species are the 'basal group' (i.e. early diverging) of the genus. Chen & Tsi (1984) suggested that

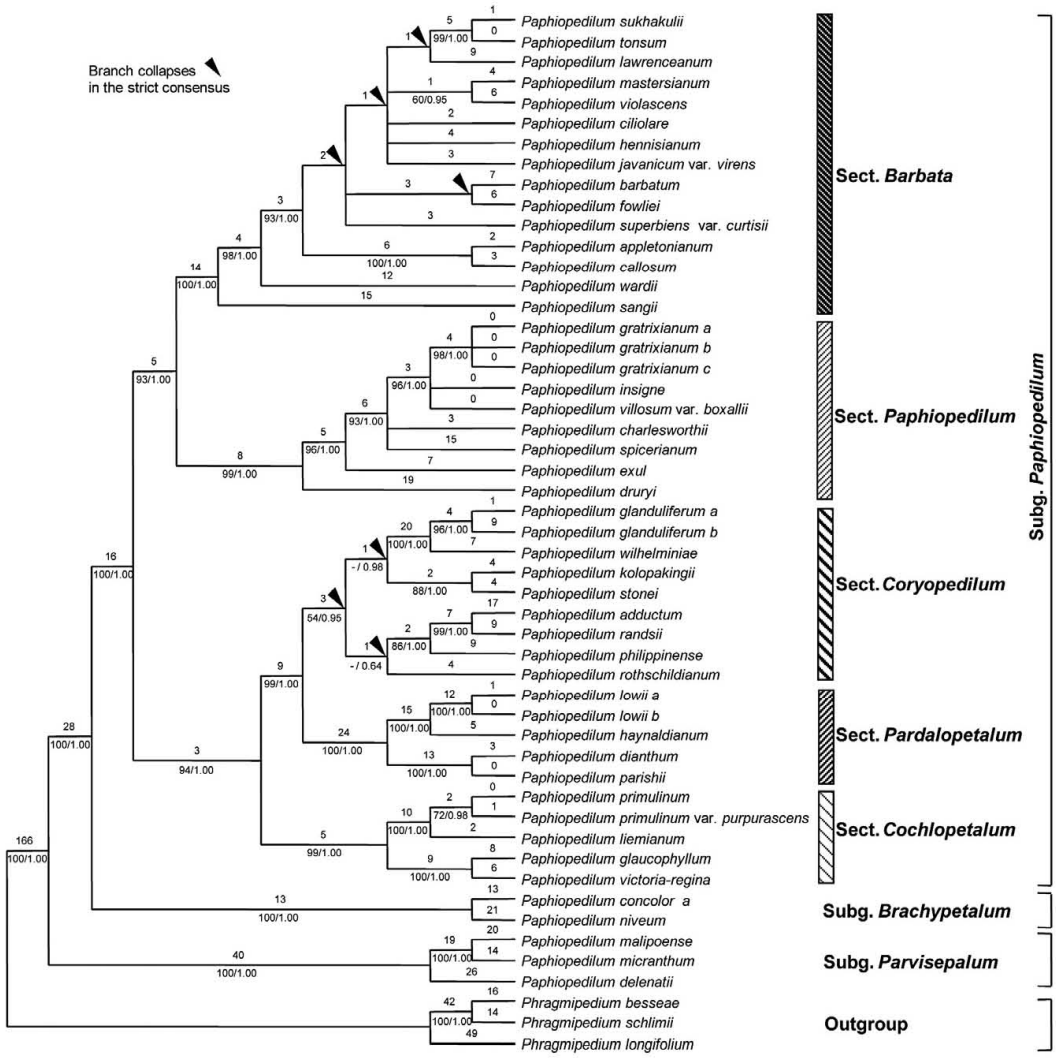

Figure 3. One of 120 most-parsimonious trees from the combined analysis of internal transcribed spacer (ITS) and plastid (partial *matK*, *ycf1*, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*) regions for *Paphiopedilum*. Tree length = 920, consistency index = 0.83, retention index = 0.91. Numbers above branches are branch lengths and numbers below branches are bootstrap percentages ≥ 50 and posterior probability values ≥ 0.50. Arrows indicate clades that collapse in the strict consensus tree obtained from maximum parsimony analysis. The infrageneric treatment follows Cribb (1998).

Paphiopedilum and *Cypripedium* were related via this species (subgenus *Parvisepalum*) by considering the similarity of the flower characters. However, Cribb (1987) stated that the similarities between the flowers of *Paphiopedilum* and the other genera, for example *P. armeniacum* S.C.Chen & F.Y.Liu and *C. irapeanum* La Llave & Lex. or *P. delenatii* Guillaumin and *Phragmipedium schlimii* (Linden ex Rchb.f.) Rolfe, are the result of similar pollination syndromes with bees as pollinators. Research into seven species in subgenus *Paphiopedilum* and one species in subgenus *Brachypetalum* showed that all of them are pollinated by hoverflies (Atwood, 1985; Bänziger, 1994, 1996, 2002; Shi *et al.*, 2007, 2009), but there is

Table 3. Sources of genome size data used in this study (chromosome number data are taken from Karasawa *et al.*, 1997; Karasawa, 1979, 1980, 1982, 1986; Cox *et al.*, 1998; Bennett & Leitch, 2010; Lan & Albert, 2011)

Taxa	Voucher/source	Chromosome number ($2n$)	1C-value (pg)
Subgenus *Parvisepalum*			
Paphiopedilum armeniacum S.C.Chen & F.Y.Liu	Bennett & Leitch, 2010	26	21.10
Paphiopedilum delenatii Guillaumin	Cox *et al.*, 1998	26	21.83
Paphiopedilum micranthum Tang & F.T.Wang	Cox *et al.*, 1998	26	22.75
Subgenus *Brachypetalum*			
Paphiopedilum concolor (Bateman) Pfitzer	Cox *et al.*, 1998	26	19.48
Paphiopedilum godefroyae (God.-Leb.) Stein	Cox *et al.*, 1998	26	17.80
Subgenus *Paphiopedilum*			
Section *Paphiopedilum*			
Paphiopedilum insigne (Wall. ex Lindl.) Pfitzer	Kew 2001–2843	26	27.52 (0.59)*
Paphiopedilum gratrixianum (Mast.) Rolfe	Kew 1979–975	26	25.16 (0.46)*
Paphiopedilum druryi (Bedd.) Stein	Kew 1982–1398	30	26.50 (0.47)*
Paphiopedilum villosum (Lindl.) Stein	Narayan *et al.*, 1989	26	22.48
Section *Barbata*			
Paphiopedilum appletonianum (Gower) Rolfe	Cox *et al.*, 1998	38	32.43
Paphiopedilum mastersianum (Rchb.f.) Stein	Cox *et al.*, 1998	36	29.73
Paphiopedilum tonsum (Rchb.f.) Stein	Cox *et al.*, 1998	32	28.15
Paphiopedilum barbatum (Lindl.) Pfitzer	Cox *et al.*, 1998	38	33.75
Paphiopedilum bullenianum (Rchb.f.) Pfitzer var. *celebesense* (Fowlie & Birk) P.J.Cribb	Bennett & Leitch, 2010	40	25.85
Paphiopedilum callosum (Rchb.f.) Stein	Cox *et al.*, 1998	32	24.05
Paphiopedilum lawrenceanum (Rchb.f.) Pfitzer	Bennett & Leitch, 2010	40	26.13
Paphiopedilum ciliolare (Rchb.f.) Stein	Bennett & Leitch, 2010	32	30.50
Paphiopedilum purpuratum (Lindl.) Stein	Bennett & Leitch, 2010	40	27.13
Paphiopedilum sukhakulii Schoser & Senghas	Cox *et al.*, 1998	40	29.73
Paphiopedilum wardii Summerh.	Cox *et al.*, 1998	41	34.53
Section *Pardalopetalum*			
Paphiopedilum parishii (Rchb.f.) Stein	Kew 1986–1038	26	27.20 (0.68)*
Paphiopedilum lowii (Lindl.) Stein	Bennett & Leitch, 2010	26	24.53
Paphiopedilum haynaldianum (Rchb.f.) Stein	Bennett & Leitch, 2010	26	22.85
Section *Cochlopetalum*			
Paphiopedilum liemianum (Fowlie) K.Karas. & K.Saito	Kew 1990–8000	32	23.72 (0.48)*
Paphiopedilum primulinum M.W.Wood & P.Taylor	Cox *et al.*, 1998	32	20.90
Paphiopedilum victoria-mariae (Sander ex Mast.) Rolfe	Cox *et al.*, 1998	36	21.40
Section *Coryopedilum*			
Paphiopedilum philippinense (Rchb.f.) Stein	Cox *et al.*, 1998	26	23.25
Paphiopedilum kolopakingii Fowlie	Kew 1983–5478	26	21.93 (0.86)*
Paphiopedilum stonei (Hook.) Stein	Kew 1998–2185	26	23.28 (0.46)*
Paphiopedilum adductum Asher	Bennett & Leitch, 2010	26	27.03
Paphiopedilum glanduliferum (Blume) Stein	Cox *et al.*, 1998	26	23.73
Paphiopedilum rothschildianum (Rchb.f.) Stein	Cox *et al.*, 1998	26	22.58
Outgroup			
Phragmipedium besseae Dodson & J.Kuhn	Cox *et al.*, 1998	24	7.08
Phragmipedium longifolium (Warsz. & Rchb.f.) Rolfe	Cox *et al.*, 1998	20, 21, 22, 23	6.10
Phragmipedium caudatum (Lindl.) Rolfe	Cox *et al.*, 1998	28	9.18
Phragmipedium lindleyanum (R.H.Schomb. ex Lindl.) Rolfe	Cox *et al.*, 1998	22	8.03
Phragmipedium pearcei (Rchb.f.) Rauh & Senghas	Cox *et al.*, 1998	20, 21, 22	6.33

*Standard deviations of 1C-values measured in this study are shown in parentheses (pg).

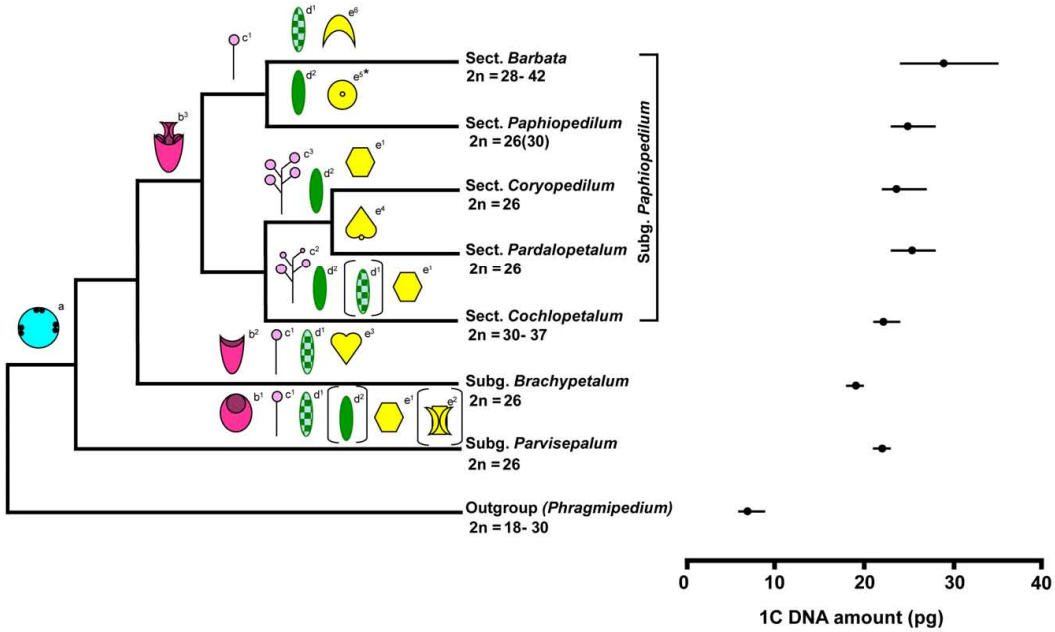

Figure 4. Morphological characters, chromosome numbers and genome size ranges (mean value indicated by a circle) mapped onto a phylogenetic framework from the combined DNA sequence data. a, unilocular ovary with parietal placentation; b^1, inflated lip; b^2, ovoid shaped lip; b^3, lip with only incurved side lobes; c^1, (mostly) single-flowered inflorescence; c^2, multi-flowered with successive opening; c^3, multi-flowered with simultaneous opening; d^1, tessellated leaves; d^2, plain green leaves; e^1, convex staminode; e^2 , conduplicate staminode; e^3, staminode with uni- or tridentate apex; e^4, obcordate staminode with basal protuberance; e^5, staminode with an umbo (* indicates more shape variations in the section); e^6, (mostly) lunate shape staminode.

no such research for species in subgenus *Parvisepalum*. The results from the studies of Albert (1994) and Cox *et al.* (1997) pointed to *Paphiopedilum* differing extensively from both *Cypripedium* and *Phragmipedium*, not only in morphological characters but also in molecular characters. In this study, the results from the combined data of five DNA regions also showed that there are high levels of molecular divergence between *Paphiopedilum* and *Phragmipedium*.

Subgenus *Brachypetalum*

Subgenus *Brachypetalum*, characterized by tessellated leaves, one- or two- (rarely three-) flowered inflorescences, flowers white or yellow in colour, an involute margined ovoid shaped lip and a staminode that is uni- or tridentate at its apex (Cribb, 1998) (Fig. 4), is a monophyletic group, with high support values from both BP and PP in all analyses. From plastid and combined data (Figs 2, 3), subgenus *Brachypetalum* is strongly supported as sister to subgenus *Paphiopedilum*. This result supports the recognition of subgenus *Parvisepalum* by Karasawa & Saito (1982), which was found to differ morphologically from the remaining species in subgenus *Brachypetalum*, and the elevation of section *Parvisepalum sensu* Cribb (1987) to subgeneric level in the second edition of his monograph (Cribb, 1998), a change suggested by the ITS result of Cox *et al.* (1997). Although both *Parvisepalum* (most species) and *Brachypetalum* have tessellated leaves and a sporophytic chromosome number of 26, their flowers are clearly different (Fig. 4). Approximately seven species of subgenus *Parvisepalum* are distributed mostly in southern China and Vietnam, whereas the four species of *Brachypetalum* have a wider distribution in mainland south-east Asia (Cribb, 1998).

Subgenus *Paphiopedilum*

There is conflict between the classical infrageneric classifications concerning the division of subgenus *Paphiopedilum* into several sections or several subgenera in the most recent monographs of the genus.

In the monographs of Braem (Braem, 1988; Braem *et al.*, 1998; Braem & Chiron, 2003), following the work of Karasawa & Saito (1982), subgenus *Paphiopedilum sensu* Cribb is divided into four subgenera (*Paphiopedilum*, *Sigmatopetalum* Hallier f. ex K.Karas. & K.Saito, *Polyantha* (Pfitzer) Brieger and *Cochlopetalum* (Hallier f. ex Pfitzer) K.Karas. & K.Saito). This disagrees with the treatment of Cribb in his monographs (Cribb, 1987, 1998), in which he placed plants with different leaf colour (plain green vs. tessellated), number of flowers in the inflorescence [one or rarely two (three) flowers vs. multiple flowers], number of chromosomes (constant $2n = 26$ vs. variable) and pattern of blooming (simultaneous vs. successive), in one subgenus (Braem & Chiron, 2003). However, Cribb considered subgenus *Paphiopedilum* to be monophyletic based on the cladistic study of Atwood (1984) and he treated other groups at sectional levels in this subgenus. Braem (in Braem & Chiron, 2003) also argued that the ITS tree from Cox *et al.* (1997) did not disagree with his subgeneric treatment. That is because there is no support for the robustness of the clade of subgenus *Paphiopedilum sensu* Cribb, as mentioned previously.

The results from this study show that subgenus *Paphiopedilum sensu* Cribb which consists of species in which only the side lobes of the lip are incurved (Cribb, 1998) (Fig. 4), is clearly monophyletic, with strong support from the plastid and combined data analyses (Figs 2, 3), and the subgenus is split into two main lineages. The first lineage includes three sections of multi-flowered species (*Coryopedilum*, *Pardalopetalum* and *Cochlopetalum*) and the second lineage includes two sections of mostly single-flowered species (*Paphiopedilum* and *Barbata*) (Figs 2–4). These are all sections as defined in the treatment of Cribb (1998). These lineages are different from the results of Cox *et al.* (1997), in which multi-flowered and (mostly) single-flowered sections are placed in the same clades. In the current study, multi-flowered inflorescences occur only in sections *Coryopedilum*, *Pardalopetalum* and *Cochlopetalum*, and thus this character appears to be a synapomorphy for this clade.

The tessellated leaf character found in the early diverging subgenera *Parvisepalum* (except two species) and *Brachypetalum*, is absent in most clades of subgenus *Paphiopedilum* (Fig. 4). Reversions of this character are found in all species of section *Barbata* and in two species of section *Cochlopetalum*, and it appears to occur independently. Tessellated leaves are thought to play a role as camouflage for anti-herbivore defence in understorey herbaceous plants growing in sun-flecked light conditions (Givnish, 1990), but there is no obvious evidence for the value of this adaptation in *Paphiopedilum*. Most species, including those with plain green and tessellated leaves, grow in similar shady forest-floor habitats, although a few plain green leaved species have been found in open sunny situations and some tessellated leaved species are found in deep shade (Cribb, 1998).

All sections in subgenus *Paphiopedilum* are strongly supported (both BP and PP) in the analyses of combined data, except section *Coryopedilum*, which has weak BP support (54 BP) for monophyly, collapsing in the strict consensus tree of parsimony analysis to form a polytomy with section *Pardalopetalum*. However, in the tree obtained from Bayesian analysis, *Coryopedilum* has 0.95 PP clade support (Fig. 3). Previously, the results from ITS data of Cox *et al.* (1997) showed section *Coryopedilum* (no BP support, jackknife > 0.63 at some nodes) to be paraphyletic to a monophyletic section *Pardalopetalum sensu* Cribb (1987), and they tentatively proposed a combination of these sections. However, Cribb (1998), in the second edition of his monograph, did not accept these molecular results, because he noted that these sections are probably sister groups based on morphological characters. The sections share plain green leaves, multi-flowered inflorescences that open simultaneously and a chromosome number of $2n = 26$ (Fig. 4). Considering floral morphology, they can be clearly distinguished, with *Coryopedilum* having long tapering petals, a porrect lip and a convex staminode, whereas *Pardalopetalum* has distinctive dorsal petals that are reflexed at the base and an obcordate staminode with a basal protuberance and tridentate apex (Cribb, 1998). The *c.* 11 species of section *Coryopedilum* are found in the Malesian islands, and most are endemic to single islands. In contrast, section *Pardalopetalum* is more widespread, the four species being distributed through mainland south-east Asia, and the Malay Archipelago to Sulawesi and the Philippines (Cribb, 1998). In this study (Figs 1–3), these sections are sister groups, with 57 BP and 0.76 PP from ITS data, 83 BP and 1.00 PP from the plastid data and 99 BP and 1.00 PP from the combined data. There is no support for monophyly from the ITS data for *Coryopedilum*. Although, bootstrap support from plastid data and combined data is low (67 BP and 54 BP, respectively), support from Bayesian analysis is high, with 1.00 PP from plastid data and 0.95 PP from the combined data. However, *Coryopedilum* collapsed in the strict consensus trees of parsimony analyses of ITS data and combined data. In contrast, section *Pardalopetalum* has strong support, with 100 BP and 1.00 PP in all analyses. Results from this study therefore suggest that section *Coryopedilum*, although clearly differing from section *Pardalopetalum* morphologically, shows insufficient levels of molecular divergence to support monophyly of this section.

Including more variable regions such as low-copy nuclear regions would possibly help in obtaining a clearer pattern. The low level of molecular divergence in *Coryopedilum* could possibly be explained by its selfing mode of reproduction, resulting from geitonogamy, and an absence of centric fission events (see below). Species with multi-flowered inflorescences that open simultaneously, as found in sections *Coryopedilum* and *Pardalopetalum*, are more susceptible to geitonogamy or pollination among flowers on the same individual plant (Kliber & Eckert, 2004). This self-pollination by geitonogamy is thought to be disadvantageous, because it produces inbred offspring and requires pollinators to visit, as in outcrossing pollination (Eckert, 2000). Although the floral features of orchids favour outcrossing, most orchids are self-compatible, which could facilitate reproduction in widely separated plants where outcrossing is not possible (Dressler, 1981). Because most species in section *Coryopedilum* are endemic to single Malesian islands (Cribb, 1998), they occur in small populations that are more likely to be geitonogamous than those of species in section *Pardalopetalum,* which are distributed more widely.

The *Cochlopetalum* clade is recovered in trees from ITS data (98 BP and 0.98 PP) and combined data (99 PP and 1.00 PP), but not in the plastid tree. In the combined tree, section *Cochlopetalum* is sister to a clade formed by sections *Coryopedilum* and *Pardalopetalum* (94 BP and 1.00 PP). Section *Cochlopetalum* is similar to its sister group in having multi-flowered inflorescences, but it differs in its flowers, which open successively, and in the variation in chromosome numbers ($2n = 30$–37) (Fig. 4). In addition, linear, spirally twisted petals are a distinctive character for the section, including approximately five species that are endemic to Java and Sumatra (Cribb, 1998). These three sections, which share plain green leaves [except *P. victoria-regina* (Sander) M.W.Wood and *P. victoria-mariae* (Sander ex Mast.) Rolfe of section *Cochlopetalum*, which have faintly tessellated leaves; Cribb, 1998] and multi-flowered inflorescences, are together sister to a clade consisting of sections *Paphiopedilum* plus *Barbata* with strong support (100 BP and 1.00 PP from both plastid and combined data). The clade of sections *Paphiopedilum* and *Barbata* is characterized by single-flowered (rarely two-flowered) inflorescences (Fig. 4). Both sections are monophyletic with strong support: 98 BP and 1.00 PP from plastid data, and 99 BP and 1.00 PP from combined data for *Paphiopedilum*; 93 BP and 1.00 PP from plastid data; and 100 BP and 1.00 PP from a combined data for *Barbata* (Figs 2, 3). Section *Paphiopedilum* differs from section *Barbata* in having green leaves and chromosome numbers in most species of $2n = 26$ except in *P. druryi* (Bedd.) Stein and *P. spicerianum* (Rchb.f.) Pfitzer ($2n = 30$), whereas the tessellated-leaved section, *Barbata* shows considerable variation in chromosome number ($2n = 28$–42). Many species in section *Paphiopedilum* are characterized by a staminode with an umbo in the middle, whereas most species in section *Barbata* have a lunate staminode (Cribb, 1998) (Fig. 4).

Phylogenetic relationships in section *Barbata* are unresolved, with many internal branches collapsing to a polytomy in the strict consensus tree for the parsimony analysis and 50% majority tree from Bayesian analyses (Figs 1–3). Atwood (1984) suggested that section *Barbata* was the most derived group, and this section was derived from section *Paphiopedilum* based on his Wagner groundplan-divergence cladogram. However, that suggestion cannot be inferred from this current phylogenetic study, because it can only be inferred that both sections share a most recent common ancestor. The short branch lengths in section *Barbata* shown on the combined tree in this study and the narrow geographical distribution on Malesian islands of most species in this section might suggest a recent rapid radiation in the section (Cox *et al.*, 1997). Although we included numerous molecular characters from five DNA regions both from nuclear and plastid loci in this study, the relationships in this section remain unresolved. To obtain better resolution in this section, the use of more variable regions such as low-copy nuclear sequences could be helpful.

Genome size and chromosome evolution in the genus *Paphiopedilum*

Mapping chromosome number data onto the phylogenetic framework from the combined data does not show clearly if there is a trend towards an increase in chromosome number, as proposed by Cox *et al.* (1997, 1998) (Fig. 4). There are two major lineages in subgenus *Paphiopedilum*, the first lineage composed of three sections (*Coryopedilum*, *Pardalopetalum* and *Cochlopetalum*). All species in the first two sections of this clade have a chromosome number of $2n = 26$, whereas species of section *Cochlopetalum* have chromosome numbers that vary from $2n = 30$ to $2n = 37$. Similarly, in the second lineage, species of section *Paphiopedilum* have a chromosome number of 26 (except two species, *P. druryi* and *P. spicerianum*, with $2n = 30$), whereas variable chromosome numbers, between $2n = 28$ and 42, are found in the sister section *Barbata*. Although the topology of sections in subgenus *Paphiopedilum* in this phylogenetic framework is different from the study of Cox *et al.* (1997, 1998), the patterns are similar, in that sections with variable chromosome numbers are paired with sections with a constant chromosome number.

However, it has been shown from both phylogenetic frameworks that the first branching subgenus, *Parvisepalum*, and subgenus *Brachypetalum*, which is sister to subgenus *Paphiopedilum*, have a chromosome number of $2n = 26$, with all metacentric chromosomes, and this could indicate that $2n = 26$ is the ancestral condition for the genus, as suggested previously, because this number is found in most species of the genus (e.g. Karasawa, 1979). Also, the higher chromosome number and the presence of telocentric chromosomes could indicate a more derived condition given the phylogenetic position of species with higher chromosome numbers. These results suggest that centric fission has contributed to the karyotype changes observed in the genus and, superimposing the data onto the phylogenetic tree, indicate that centric fission has occurred independently in sections *Barbata* and *Cochlopetalum* (Fig. 4).

There have been other studies that support a hypothesis of centric fission, for example Karasawa & Tanaka (1980), who studied C-banding patterns of *P. callosum* (Rchb.f.) Stein ($2n = 32$) and found them to be similar to *P. insigne* (Wall. ex Lindl.) Pfitzer [=*P. insigne* (Wall. ex Lindl.) Pfitzer var. *sanderae* (Rchb.f.) Pfitzer, $2n = 26$]. They postulated centric fission as a cause of karyotype changes.

Jones (1998), in a review of Robertsonian change in karyotype evolution, supported the hypothesis of centric fission in *Paphiopedilum*. He suggested that the small population sizes and inbreeding in *Paphiopedilum* could contribute to explaining the karyotype variation observed. Indeed, all species of section *Cochlopetalum* and most species of section *Barbata* that have a high chromosome number are endemic to the Malesian islands, and it has been suggested that centric fission may be under selection as it has the potential to increase genetic recombination, enabling adaptation to the environments on islands (Cox *et al.*, 1998; Leitch *et al.*, 2009). However, this is clearly not always the case, as species of section *Coryopedilum*, most of which are also restricted to individual Malesian islands (Cribb, 1998), all have a chromosome number of $2n = 26$. Although Cox (in Pridgeon *et al.*, 1999) suggested that the higher chromosome number of $2n = 30$ in *P. druryi* (section *Paphiopedilum*) might be correlated with its narrow endemicity (in southern India), clearly, other factors are involved in driving centric fission. This is because the only other species in section *Paphiopedilum* with $2n = 30$ is *P. spicerianum*, which has a wider distribution. It is found in north-east India, north-west Burma and south-west China (Cribb, 1998).

The range in genome size, as represented by 32 species (44% of the genus), is from 1C = 17.80 pg in *P. godefroyae* to 1C = 34.53 pg in *P. wardii* (1.9-fold range; see Tables 3–4 and Fig. 4). The lowest genome sizes are found in species belonging to section *Brachypetalum* (mean 1C = 18.64 pg) and the highest genome sizes are found in section *Barbata* (mean 1C = 29.27 pg). Mapping the genome size range of *Paphiopedilum* spp. onto the phylogenetic framework obtained in this study shows that there is no clear trend of genome size increase in the genus (Fig. 4). The greatest range and largest genomes were found in section *Barbata*, which is also characterized by being the most variable in terms of chromosome number ($2n = 28$–42). However, section *Cochlopetalum*, which also is variable in chromosome number ($2n = 30$–37), has a similar range of genome size to other sections and subgenera characterized by $2n = 26$ (Table 4).

When plotting chromosome number against genome size data (Fig. 5), a weak but significant relationship was found (Pearson's correlation coefficient $r = 0.632$, $P < 0.001$), suggesting that, as chromosomes undergo fission, this is often accompanied by an increase in genome size. The source of additional DNA in the genome is unclear, but is likely to comprise a diverse array of different types of repetitive DNA, including retrotransposons (Bennetzen, 2005).

The relationship between chromosome number and genome size in *Paphiopedilum* differs from that of closely related genera. *Phragmipedium* has a variable chromosome number ($2n = 18$–30), but a smaller mean genome size and a narrower range (1.5-fold, 1C = 6.10 to 9.18 pg) (Cox *et al.*, 1998). *Cypripedium* is the most variable genus in subfamily Cypripedioideae in terms of genome size, with values ranging

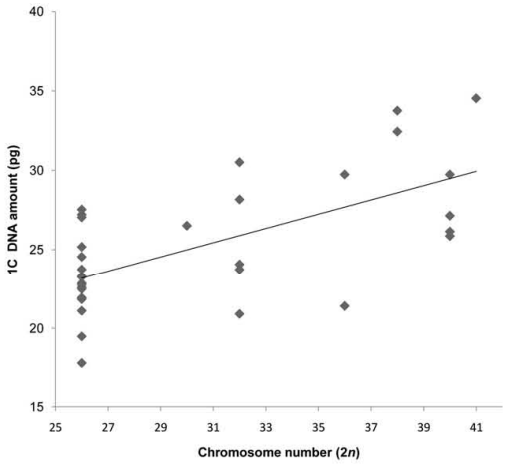

Figure 5. The relationship between genome size and chromosome number for 32 *Paphiopedilum* spp. Pearson's correlation coefficient $r = 0.632$, $P < 0.001$.

Table 4. Range of chromosome number, number of chromosome arms (n.f.) and genome size data [minimum (min.), maximum (max.) and mean of 1C-value in picograms (pg)], number of species with 1C-value and representation in percentage. Chromosome number data are taken from Karasawa (1979, 1980, 1982, 1986), Karasawa & Aoyama (1980, 1988), Karasawa *et al.* (1997), Cox *et al.* (1998), Bennett & Leitch (2010) and Lan & Albert (2011); sources of genome size data are listed in Table 3

Taxa	Chromosome number ($2n$)	n.f.	Min. 1C-value (pg)	Max. 1C-value (pg)	Mean 1C-value (pg)	No. species with 1C-value	Representation (%)
Subgenus *Parvisepalum*	26	52	21.10	22.75	21.89	3	43
Subgenus *Brachypetalum*	26	52	17.80	19.48	18.64	2	50
Subgenus *Paphiopedilum*							
Section *Cochlopetalum*	30-37	48–50	20.90	23.72	22.01	3	60
Section *Pardalopetalum*	26	52	22.85	27.20	24.86	3	75
Section *Coryopedilum*	26	52	21.93	27.03	23.63	6	55
Section *Paphiopedilum*	26 (30)*	52	22.48	27.52	25.42	4	29
Section *Barbata*	28–42	52–56	24.05	34.53	29.27	11	41
Phragmipedium (outgroup)	18–30	34–39	6.10	9.18	7.34	5	33

**P. druryi* and *P. spicerianum* $2n = 30$.

10.5-fold (1C = 4.1 to 43.1 pg), but the chromosome number in most species is constant ($2n = 20$) (Leitch *et al.*, 2009).

Lan & Albert (2011) studied the evolution of ribosomal DNA in *Paphiopedilum* using fluorescence *in situ* hybridization and assessed the data according to the phylogenetic framework of Cox *et al.* (1997). Although the results show variation of rDNA multiplication in *Paphiopedilum*, they found no evidence for a clear relationship between the increase in number of chromosomal locations of rDNA and the increase in chromosome number and genome size. Using the more robust phylogenetic framework from the current study, the multiplication of 25S rDNA loci observed by Lan & Albert occurred twice independently in *Paphiopedilum*, once in subgenus *Parvisepalum* and once in the clade formed by sections *Coryopedilum* and *Pardalopetalum* of subgenus *Paphiopedilum*. The multiplication event of 5S rDNA loci happened only in subgenus *Paphiopedilum*, whereas the early diverging subgenera *Parvisepalum* and *Brachypetalum* retained the ancestral number of two major sites, as also found in the outgroups *Phragmipedium* and *Mexipedium*.

Genome size is thought to have an influence on life form, habit and ecology. Annual plants are characterized by small genomes, whereas perennials have a larger range of genome sizes, and species with large genomes are all obligate perennials (Bennett, 1972). Leitch *et al.* (2009) found that epiphytic orchids have small genomes (mean 1C = 3.0 pg, range 0.33–8.5 pg), whereas terrestrial species have a much wider range (mean 1C = 18.3 pg, range 2.9–55.4 pg). This might be caused by selection for small guard cell sizes, because species with small guard cells are shown to respond more rapidly to water stress than those with larger cells (Aasamaa, Sober & Rahi, 2001; Hetherington & Woodward, 2003). As guard cell size has been shown to be correlated with genome size, then selection for small guard cells would result in selection for a small genome (Beaulieu *et al.*, 2008). Most *Paphiopedilum* spp. are terrestrials, with only five being epiphytic; *P. parishii* (Rchb.f.) Stein, *P. lowii* (Lindl.) Stein, *P. villosum* (Lindl.) Stein, *P. hirsutissimum* (Lindl. ex Hook.) Stein and *P. glanduliferum* (Blume) Stein, the last two species being facultative epiphytes (Cribb, 1998). Nevertheless, in contrast to the observations of Leitch *et al.* (2009), the genome size of these epiphytic species is large (mean 1C = 24.49 pg, range 22.48–27.20 pg), similar to those found in the terrestrial species (mean 1C = 25.40 pg, range 17.80–34.53 pg). These observations suggest that water stress is unlikely to be a strong selective pressure on cell size in this case, perhaps because the high rainfall in habitats where *Paphiopedilum* spp. are found is seasonal. In addition, other features, such as thick

leathery leaves, could also be strategies that enable their survival in the dry season (Cribb, 1998).

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