The LINNEAN SOCIETY of London BOTANICAL Journal of the Linnean Society

Botanical Journal of the Linnean Society, 2012, **170**, 176–196. With 5 figures

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

ARAYA CHOCHAI[1,2]*, ILIA J. LEITCH FLS[1], MARTIN J. INGROUILLE[2] and MICHAEL F. FAY FLS[1]

[1]*Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3DS, UK*
[2]*Department of Biological Sciences, Birkbeck, University of London, Malet Street, London WC1E 7HX, UK*

Received 24 February 2012; revised 30 May 2012; accepted for publication 24 June 2012

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. *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,

*Corresponding author. E-mail: a.chochai@kew.org

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* (in part)	*Chromatoneura Tessellata*		*Barbata*	*Sigmatopetalum*	Section *Barbata*
	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 *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

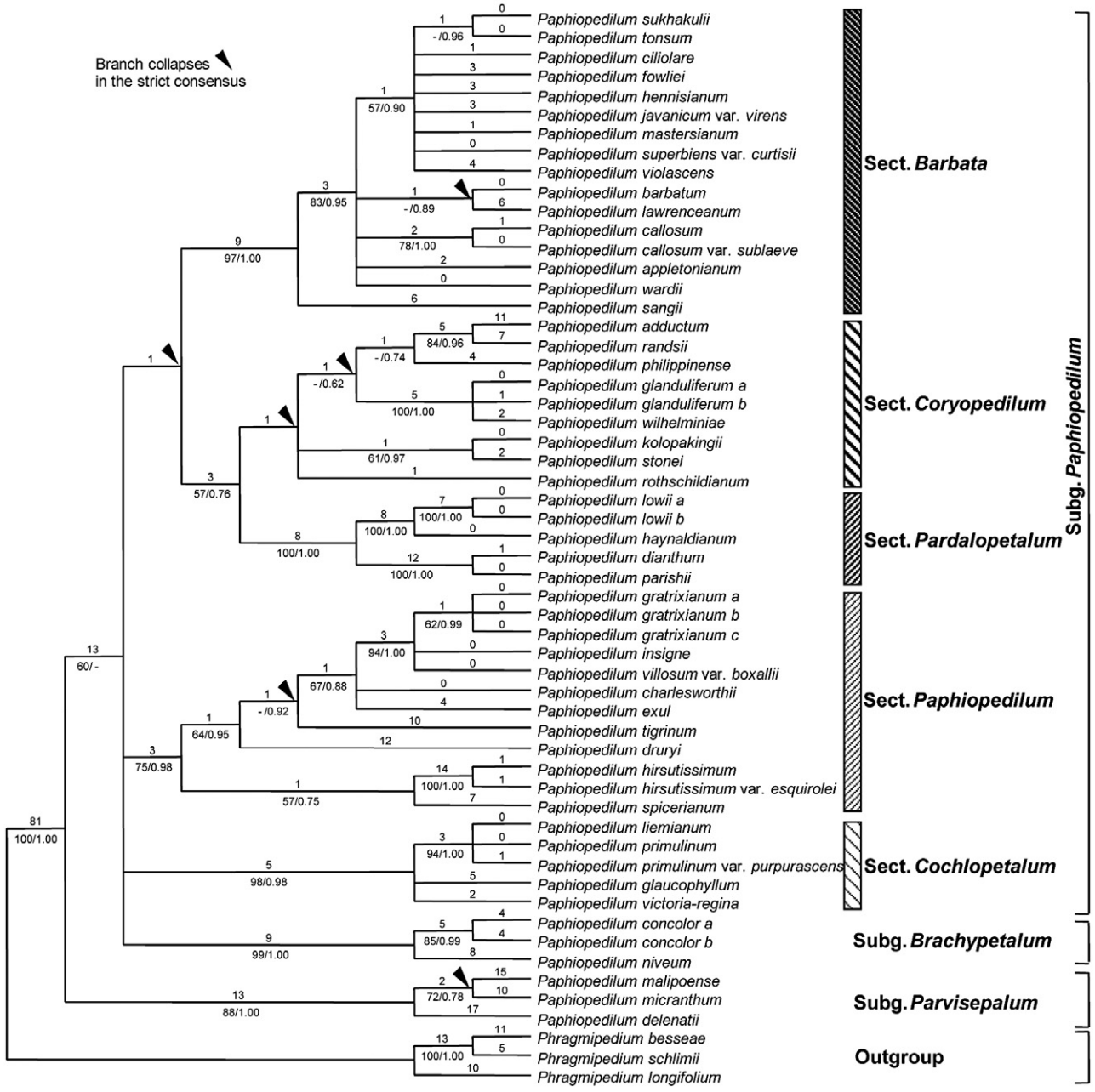

Figure 1. One of 35 most-parsimonious trees from the analysis of the internal transcribed spacer (ITS) region for *Paphiopedilum*. Tree length = 425, consistency index = 0.82, retention index = 0.90. Numbers above branches are branch lengths and numbers below branches are bootstrap percentages ≥ 50 and 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).

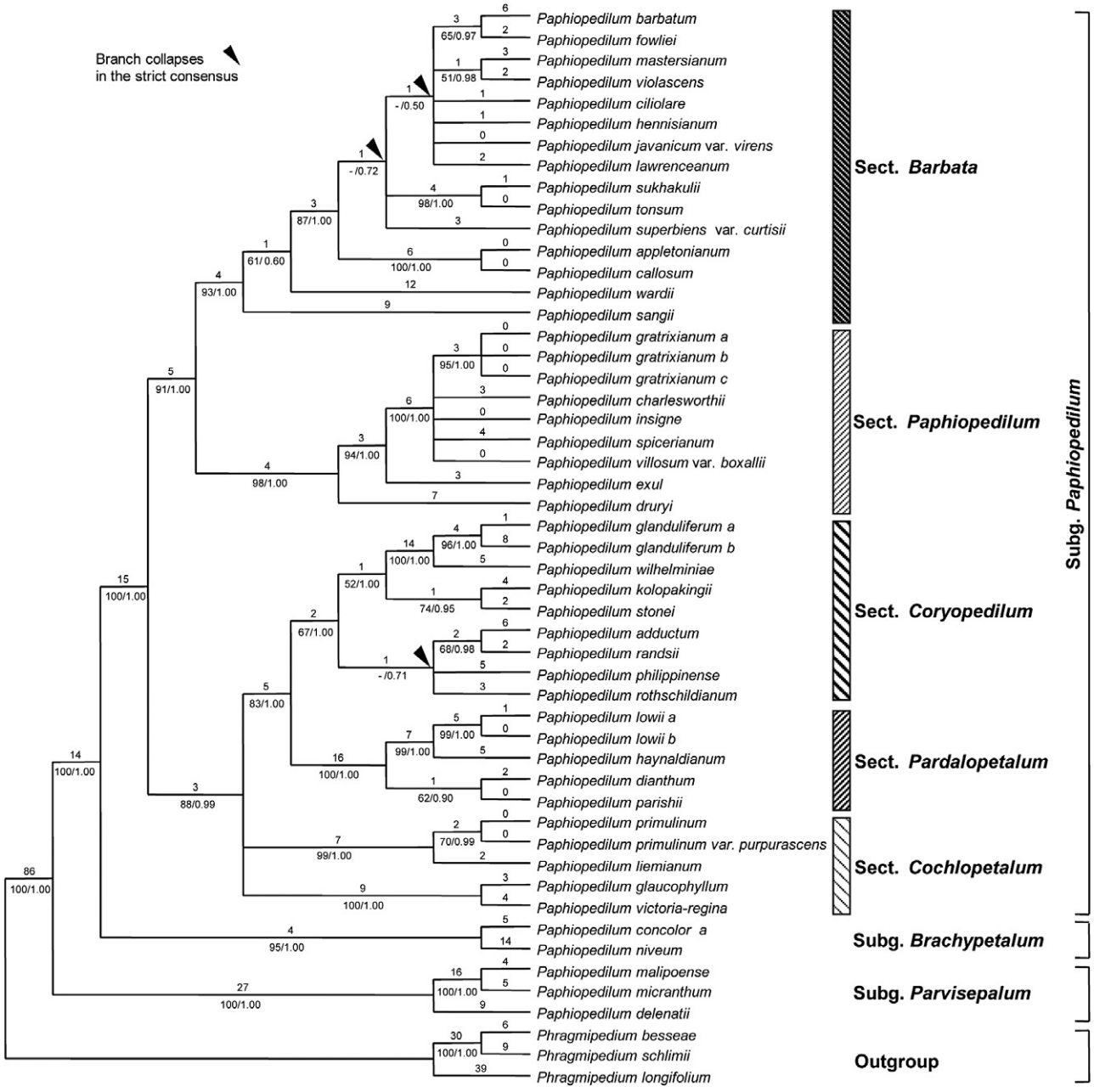

Figure 2. One of 20 most-parsimonious trees from the analysis of plastid (partial *matK*, *ycf1*, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*) regions for *Paphiopedilum*. Tree length = 520, consistency index = 0.84, retention index = 0.92. Numbers above branches are branch lengths and numbers below branches 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

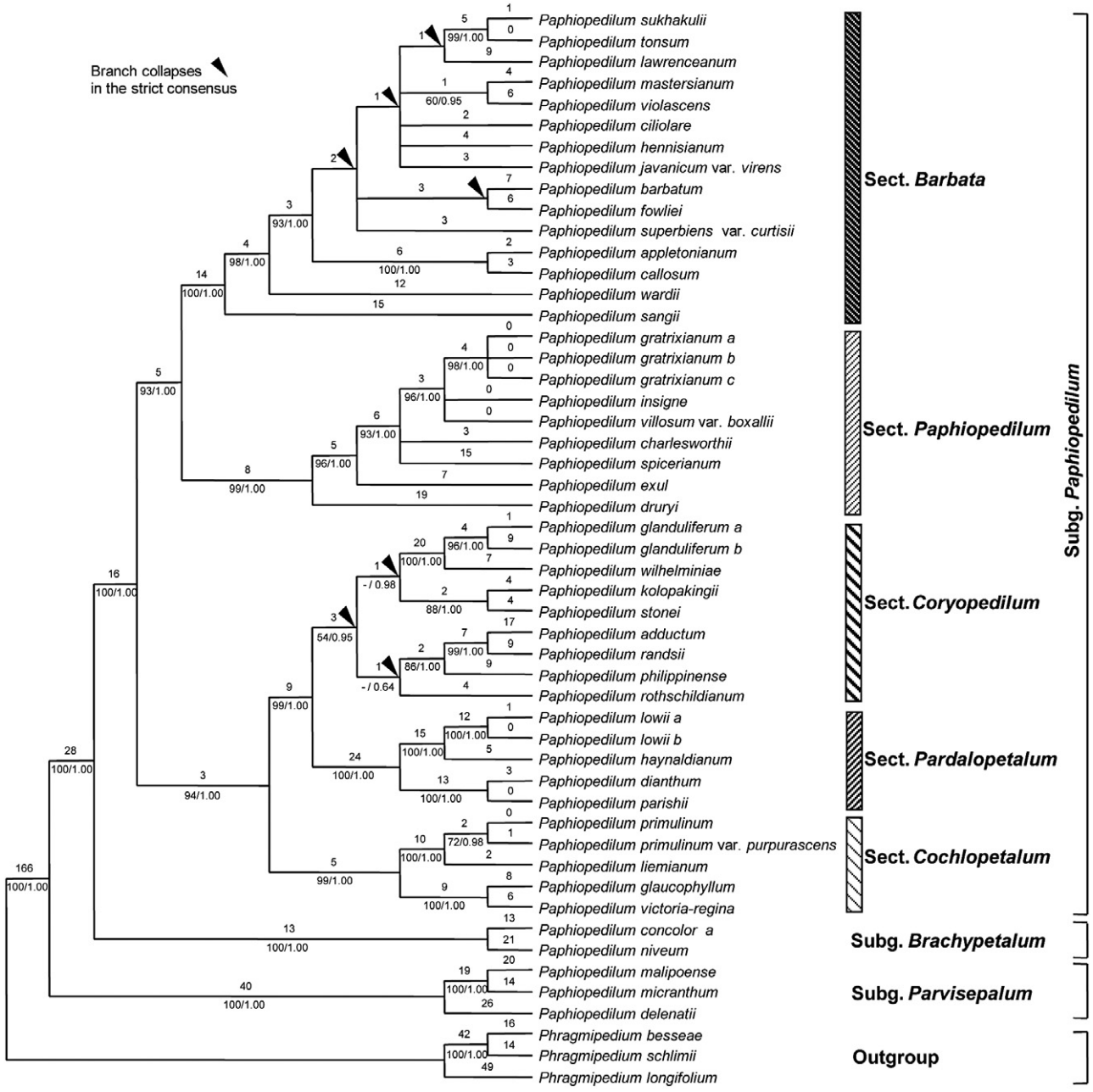

Figure 3. One of 120 most-parsimonious trees from the combined analysis of internal transcribed spacer (ITS) and plastid (partial *matK*, *ycf1*, *psaA-ycf3ex3* and *trnF*(GAA)-*ndhJ*) regions for *Paphiopedilum*. Tree length = 920, consistency index = 0.83, retention index = 0.91. Numbers above branches 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).

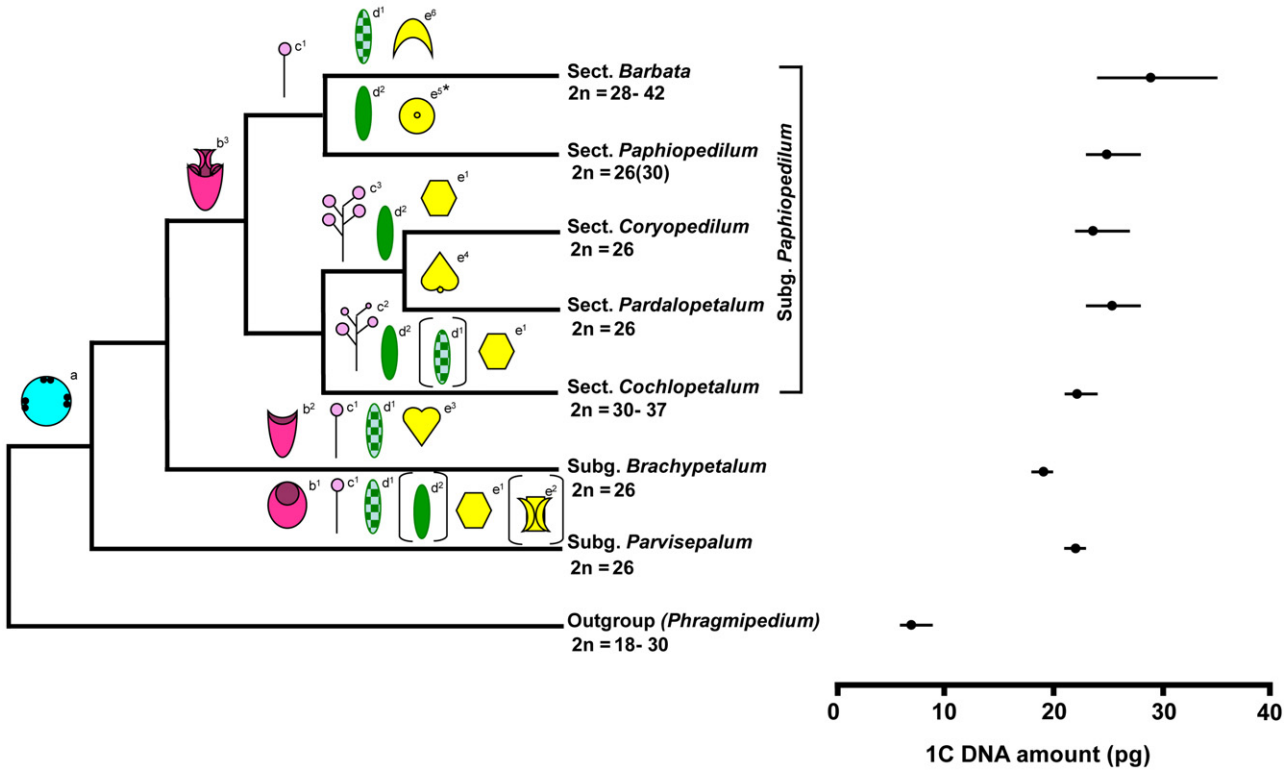

Figure 4. Morphological characters, chromosome numbers and genome size ranges (mean value indicated by a circle) mapped onto a phylogenetic framework from the combined DNA sequence data. a, unilocular ovary with parietal placentation; b^1, inflated lip; b^2, ovoid shaped lip; b^3, lip with only incurved 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

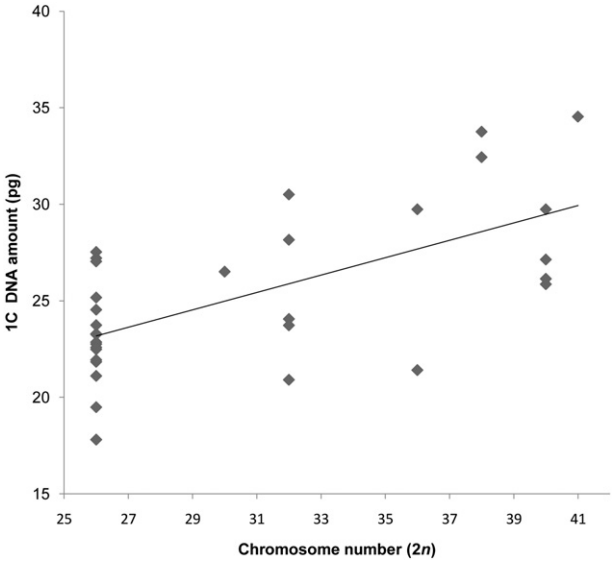

Figure 5. The relationship between genome size and chromosome number for 32 *Paphiopedilum* spp. Pearson's correlation coefficient $r = 0.632$, $P < 0.001$.

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).

ACKNOWLEDGEMENTS

We thank Dr J. Clarkson, Dr D. Devey, Dr L. Lledó, R. Cowan, L. Csiba and E. Kapinos (Jodrell Laboratory, RBG, Kew) for their technical support and advice, and thanks especially to J. Joseph, M. Sanchez, Dr H. Saslis-Lagudakis, Dr J. Pellicer, Dr L. Kelly, Dr R. Kynast, Dr I. Kahandawala and Dr M. Zarrei for their advice, support and valuable help. Thanks also to C. Ryan and B. Kompalli (The Tropical Nursery RBG, Kew) for giving access to living plant collections and to anonymous reviewers for their valuable comments on this manuscript.

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