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Reticulate evolution in diploid and tetraploid species of *Polystachya* (Orchidaceae) as shown by plastid DNA sequences and low-copy nuclear genes

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• *Background and Aims* Here evidence for reticulation in the pantropical orchid genus *Polystachya* is presented, using gene trees from five nuclear and plastid DNA data sets, first among only diploid samples (homoploid hybridization) and then with the inclusion of cloned tetraploid sequences (allopolyploids). Two groups of tetraploids are compared with respect to their origins and phylogenetic relationships.
• *Methods* Sequences from plastid regions, three low-copy nuclear genes and ITS nuclear ribosomal DNA were analysed for 56 diploid and 17 tetraploid accessions using maximum parsimony and Bayesian inference. Reticulation was inferred from incongruence between gene trees using supernetwork and consensus network analyses and from cloning and sequencing duplicated loci in tetraploids.
• *Key Results* Diploid trees from individual loci showed considerable incongruity but little reticulation signal when support from more than one gene tree was required to infer reticulation. This was coupled with generally low support in the individual gene trees. Sequencing the duplicated gene copies in tetraploids showed clearer evidence of hybrid evolution, including multiple origins of one group of tetraploids included in the study.
• *Conclusions* A combination of cloning duplicate gene copies in allotetraploids and consensus network comparison of gene trees allowed a phylogenetic framework for reticulation in *Polystachya* to be built. There was little evidence for homoploid hybridization, but our knowledge of the origins and relationships of three groups of allotetraploids are greatly improved by this study. One group showed evidence of multiple long-distance dispersals to achieve a pantropical distribution; another showed no evidence of multiple origins or long-distance dispersal but had greater morphological variation, consistent with hybridization between more distantly related parents.

Key words: Allopolyploidy, consensus network, filtered supernetwork, low-copy nuclear genes, Orchidaceae, phylogenetic analysis, *Polystachya*, reticulate evolution.

INTRODUCTION

The significance and extent of natural hybridization in angiosperm evolution has been widely recognized (Paun *et al.*, 2007; Wissemann, 2007), with an estimated 25 % of vascular plants forming hybrids with other species (Mallet, 2005) and perhaps 11 % of plant species having arisen as a result of hybridization (Ellstrand *et al.*, 1996). Outcomes of hybridization are complex and not predictable from case to case. Changes in ploidy are common, and confirmed examples in the literature of allopolyploid speciation are more common than those of homoploid hybridization, which is possibly due to easier detection and confirmation of allopolyploids in the wild compared with homoploids (Hegarty and Hiscock, 2008). Polyploidy is a common product of hybridization (Soltis and Soltis, 2000; Sang *et al.*, 2004), usually following the union of a pair of unreduced gametes from the two parent species, although other mechanisms can also result in polyploid offspring. As well as an immediate and mostly effective barrier to introgression with their parent species due to the difference in chromosome number (even though triploid bridges still make this possible in some cases; Husband, 2004), allopolyploids express novel combinations of genes relative to both parents and often exhibit genomic and epigenetic instability and immediate plasticity in gene expression and regulatory networks (Osborn *et al.*, 2003; Baack and Rieseberg, 2007; Chen, 2007; Leitch and Leitch, 2008). This can have an effect on colonization and dispersal abilities and allow them to occupy environmental niches unavailable to the parent species (Soltis and Soltis, 2000; Otto, 2007; Hegarty and Hiscock, 2008). Homoploid hybrids can also exhibit extreme large-scale genomic changes, such as increases in genome size due to increased retrotransposon activity (Baack and Rieseberg, 2007).

In addition to hybridization, gene flow between species via introgression is a common event, with the genomes of many species apparently permeable to alleles from related species (Baack and Rieseberg, 2007; Lexer *et al.*, 2009). The phenomena of hybridization and introgression can confound efforts to reconstruct the phylogeny of such groups. Often, only data from the plastid genome is used in phylogeny reconstruction, and the uniparental nature of plastid DNA masks reticulation. When both plastid and biparentally inherited nuclear DNA have been used in a study they have often given conflicting phylogenetic signals (e.g. Rieseberg *et al.*, 1996; Schilling and Panero, 1996; Oh and Potter, 2003; Kelly *et al.*, 2010),

but relatively few studies have compared plastid DNA sequences with more than one nuclear locus. Incongruence between nuclear loci or between nuclear and organellar DNA can be interpreted as a sign of interspecific hybridization, but it also can arise as a result of stochastic or population-level events causing individual gene trees to differ from the underlying species tree (McBreen and Lockhart, 2006; Holland *et al.*, 2008). The methods used to analyse multiple loci and interpret incongruence in phylogenetic results are still under development (Linder and Rieseberg, 2004; McBreen and Lockhart, 2006).

Previous work on *Polystachya* (Polystachyinae; Vandeae; Orchidaceae) suggested that the genus might be well suited to the study of reticulate evolution due to variation in ploidy including some tetraploid species groups (Rupp, 2008; Russell *et al.*, 2010). The genus comprises approx. 240 species distributed pantropically, with centres of diversity in Africa and smaller species numbers in the Indian Ocean islands, southern Asia and the Neotropics. Species radiations have occurred in the Neotropics and Madagascar, and these include polyploid clades with $2n = 4x = 80$ chromosomes (Russell *et al.*, 2010). One group of polyploid species with morphological and genetic similarity to the pantropical species, *Polystachya concreta*, has dispersed throughout the tropics relatively recently; another group represented, for example, by *P. rosea* and *P. clareae* has remained endemic to Madagascar and the Malagasy Islands. Some species from these two groups are illustrated in Fig. 1.

Previous studies on the genus have used plastid DNA sequences. Although these data were useful in constructing a well-supported phylogenetic hypothesis, as they have been in many other studies of plant evolution, the maternal inheritance of plastid DNA prevented any conclusions about the incidence of reticulate evolution. In this study, the analysis is extended to biparentally inherited nuclear DNA. The aim is to compare the results of plastid DNA analysis with those from several nuclear genes using supernetwork and consensus network analyses to gauge the extent to which hybridization has been important in *Polystachya* evolution. Reticulation amongst diploids is investigated using incongruence between gene trees as a potential hybridization signal. This strategy is then extended to tetraploid accessions for which homoeologous gene copies from low-copy nuclear genes can be cloned and sequenced to establish their origin. Two major groups of tetraploids are compared in terms of their morphological and biogeographical traits, but there are others in *Polystachya* for which sampling of species and individuals does not permit an effective study.

MATERIALS AND METHODS

Material for DNA extraction came from the collections of the Botanical Garden of the University of Vienna, the collection of Isobyl la Croix in Ross-shire, Scotland, and field collections made by the authors. DNA samples were also obtained from the DNA Bank of the Royal Botanic Gardens, Kew (http://data.kew.org/dnabank/homepage.html). See the Appendix for accession details and GenBank accessions. See Russell *et al.* (2010) for details of material preservation, DNA extraction and ploidy of *Polystachya* species. Much ploidy information was obtained using genome size measurements from Rupp (2008) and Rupp *et al.* (2010).

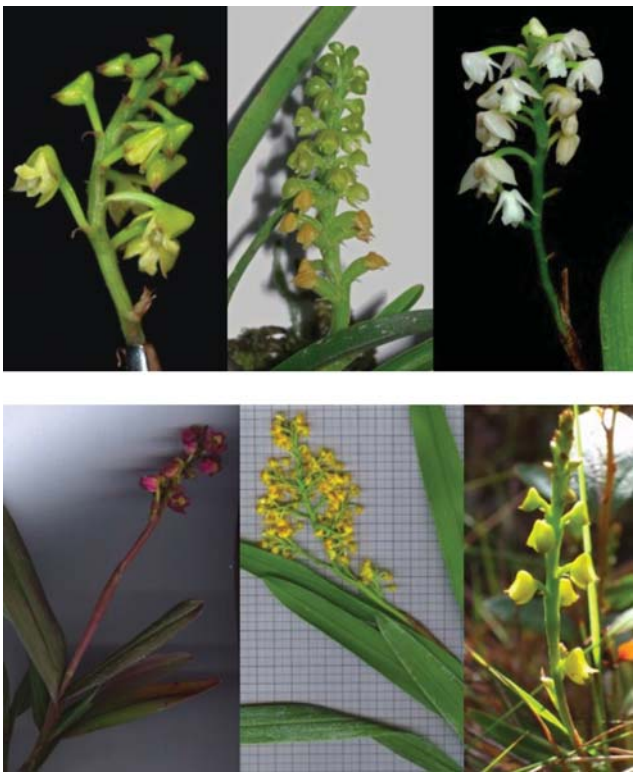

FIG. 1. Top row: three examples of *Polystachya* from the pantropical tetraploid group (photos: R. Hromniak, University of Vienna Botanical Garden; left to right: *P. concreta* from Laos, *P. masayensis* from Costa Rica and *P. concreta* from Réunion). Bottom row: three examples of plants from the Malagasy endemic tetraploid group (photos: A. Sieder, University of Vienna Botanical Garden; left to right: *P. tsaratananae*, *P. clareae* and *P. monophylla*).

A number of nuclear genes known to be low- or single-copy in angiosperms were screened and the following loci selected, based on their ease of amplification and sequencing: *PgiC* between exons 11 and 15; *PhyC* exon 1; and *Rpb2* intron 23. *PgiC* codes for phosphoglucose isomerase, an essential glytolytic enzyme. It has been used in phylogenetic studies in Dipterocarpaceae (Kamiya *et al.*, 2005), *Stephanomeria* (Compositae; Ford *et al.*, 2006) and *Clarkia* (Onagraceae), in which it is present in two copies (Thomas *et al.*, 1993; Ford and Gottlieb, 2003). *PhyC* is a member of the phytochrome family of genes, which code for photoreceptive proteins in plants and regulate a wide range of flowering and developmental pathways. It has been used in a number of phylogenetic studies in Phyllanthaceae (Samuel *et al.*, 2005), Poaceae (Mathews and Sharrock, 1996) and across monocots (M. Kinney, University of Missouri, *et al.*, unpub. res.) and other angiosperms (Saarela *et al.*, 2007). *Rpb2* codes for the second largest subunit of the RNA polymerase enzyme and has been used in phylogenetic studies in *Chamaedorea* (Arecaceae; Thomas *et al.*, 2006), *Hordeum* (Poaceae; Sun *et al.*, 2009), and across angiosperm families (Oxelman *et al.*, 2004). DNA samples were initially amplified using universal primers: for *PgiC* and *Rpb2*, primers were taken from the literature (Rongal *et al.*, 2005; Ford *et al.*, 2006). For

TABLE 1. *Nuclear low-copy and ITS primers used in this study*

PgiC AA11F	TTYGCNTTYTGGGAYTGGGT	Universal primer	Ford *et al.* (2006)
PgiC AA16R	CCYTTNCCRTTRCTYTCCAT	Universal primer	Ford *et al.* (2006)
PgiC Pol E12F1	GTTGGTGTGCTTCCKTTGTCTC	*Polystachya*-specific	This study
PgiC Pol E12F2	CTCTCCAATATGGATTTCCAATC	*Polystachya*-specific	This study
PgiC Pol E15R	AAGTGCTTGAGARTATGGTAATATAGC	*Polystachya*-specific	This study
PgiC Pol I12F1	AGTAATTTAAGAGTCAGTGGTGATCG	*Polystachya*-specific internal sequencing primer	This study
RPB2-INT-23F	CAACTTATTGAGTGCATCATGG	Universal primer	Rønçal *et al.* (2005)
RPB2-INT-23R	CCACGCATCTGATATCCAC	Universal primer	Rønçal *et al.* (2005)
RPB2-POL-23F1	CTCCATTCACTGATGTTACGG	*Polystachya*-specific	This study
RPB2-POL-23F2	GGAGATGCTACTCCATTCACTG	*Polystachya*-specific	This study
RPB2-POL-23R	GAACAGTGGTCARCCTCCAAG	*Polystachya*-specific	This study
phyc503f	TCVGGGAAGCCSTTYTAYGC	Monocot-specific	This study
phyc1705r	GRATWGCATCCATYTCAACATC	Monocot-specific	This study
phyc515f-OR	AAGCCSTTYTAYGCAATTCTACACCG	Orchid-specific	This study
phyc1699r-OR	ATWGCATCCATYTCAACATCKTCCCA	Orchid-specific	This study
phyc524f-OR	GCAATTCTACACCGTATCAATGA	Orchid-specific internal sequencing primer	This study
phyc1690r-OR	TCAACATCKTCCCATGGAAGGCT	Orchid-specific internal sequencing primer	This study
phyc974f-OR	GCTCCTCATGGMTGTCATGCTCA	Orchid-specific internal sequencing primer	This study
phyc1145r	CCTGMARCARGAACTCACAAGCATATC	Monocot-specific internal sequencing primer	This study
ITS 18s F	ACCGATTGAATGGTCCGGTGAAGTGTTCG	Universal primer	Gruenstaeudl *et al.* (2009)
ITS 26s R	CTGAGGACGCTTCTCCAGACTACAATTCG	Universal primer	Gruenstaeudl *et al.* (2009)
ITS 5·8S F	ACTCTCGGCAACGGATATCTCGGCTC	Universal internal sequencing primer	Gruenstaeudl *et al.* (2009)
ITS 5·8S R	ATGCGTGACGCCCAGGCAGACGTG	Universal internal sequencing primer	Gruenstaeudl *et al.* (2009)

PhyC, universal monocot primers were designed from GenBank sequences. When a clean single PCR band was obtained using universal primers, the product was cloned using the pGEM-T Easy system (Invitrogen) following the manufacturer's instructions to assess copy number and amount of within-sample variation (e.g. between different alleles at the same locus). The resulting sequences were aligned and *Polystachya*-specific primers were designed from conserved areas using Primer3 (Rozen and Skaletsky, 1999). Primer details are given in Table 1.

Plastid DNA sequences came from the *rps16* intron, the *rps16–trnK* spacer and the *trnK* intron, including *matK*, and were already available from a previous study (Russell *et al.*, 2010). The ITS region (ITS1-5·8S–ITS2 nuclear ribosomal DNA) was also sequenced as an additional source of data. The high copy number of ITS sequences in the nuclear genome makes the region relatively easy to amplify and sequence, and it is commonly used in plant phylogenetics. Results from ITS are often contrasted with plastid sequences to show possible reticulation (Hodkinson *et al.*, 2002; Schwarzbach and Rieseberg, 2002; Chase *et al.*, 2003; van den Berg *et al.*, 2009). Although some properties of nrDNA (multiple copy number, concerted evolution, and frequent occurrence of pseudogenes) sometimes makes its use in phylogenetics problematic, especially in the study of hybrids (van den Hof *et al.*, 2008; Alvarez and Wendel, 2003; Feliner and Rossello, 2007), it was felt that in the context of a multi-gene study, involving several plastid and nuclear gene regions, ITS sequences could provide useful additional information in this study.

There are fewer *Polystachya* species included in this study than in the Russell *et al.* (2010) paper; samples were excluded because some nuclear genes could not be amplified, directly sequenced or, in the case of tetraploids, successfully cloned, either because of deficiencies in the PCR protocols or because the DNA samples contained too little intact nuclear DNA. Taxon sampling of *Polystachya* tetraploids includes five groups found in the Russell *et al.* (2010) study, two of which comprise multiple accessions. Nine accessions belong to a pantropical group with affinities to *P. concreta*; five accessions belong to a group endemic to the Malagasy islands; three other accessions from mainland Africa occur separately with diploid sister species.

DNA amplification and sequencing

After initial cloning to design primers and develop PCR protocols for sequencing low-copy nuclear genes, initial analyses suggested the *PgiC*, *PhyC* and *Rpb2* genes were effectively single-copy in diploids and that sequences in different individuals could be treated as orthologous. For diploid accessions, these genes were then sequenced directly from PCR products, whereas PCR products from tetraploids were cloned to amplify homoeologous gene copies separately if more than one was present. Plastid and ITS sequences were obtained directly from PCR products.

In this study, 20-μL PCR reactions used, with 18·0 μL ABGene ReddyMix PCR Master Mix, 0·5 μL of each primer at 20 μM, and 1·0 μL template DNA. Thermocycling was performed with initial denaturation at 80 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, annealing for 30 s and 72 °C for 2 min, and a final extension of 72 °C for 5 min. Annealing temperature was usually 55 °C for *PgiC* and 58 °C for *PhyC*, *Rpb2* and ITS. PCR products for direct sequencing were cleaned with a mixture of 1 unit CIAP (calf intestinal alkaline phosphatase; Fermentas) and 10 units exonuclease I (Fermentas) to degrade single-stranded DNA fragments and dNTPs in the PCR product (Werle *et al.*, 1994). The mixture was incubated at 37 °C for 45 min, then denatured at 80 °C for 15 min. *PgiC*, *PhyC* and *Rpb2* PCR products from tetraploid accessions were gel-purified and cloned using the pGEM-T Easy cloning system (Invitrogen) following the manufacturer's instructions. Colonies were fixed in TE buffer, and subsequent amplification and sequencing were performed

using vector primers M13F, M13R, SP6 and T7. Five to fifteen colonies were sequenced per accession – an attempt was made to always sequence the higher number, but some samples had a high sequencing failure rate even from clones.

Cycle sequencing was carried out in 10-μL reactions with 1·0 μL ABI BigDye Terminators kit, 1·0 μL sequencing primer at 3·2 μM, and 8·0 μL cleaned-up PCR product, cycling with 30 cycles of 96 °C for 10s, 50 °C for 5s, and 60 °C for 4 min. Sequencing was performed on a 48 capillary sequencer, Applied Biosystems (ABI) 3730 DNA Analyzer, following the manufacturer's protocols.

Analysis of diploids

Sequences were edited with FinchTV (Geospiza Inc.) and assembled with either AutoAssembler 1.4.0 (ABI) or LaserGene 7·1 SeqMan (DNASTAR Inc.). They were aligned initially with MUSCLE (Edgar, 2004), and these alignments were adjusted by eye in MacClade (Maddison and Maddison, 2005) following the guidelines of Kelchner (2000). Non-alignable end sequences and gap-rich sequences (>50 % missing data) were excluded from the analyses. Characteristics of sequence alignments are presented in Table 2.

Individual gene trees were constructed using maximum parsimony and Bayesian analyses. Parsimony analyses were conducted in PAUP*4·10b (Swofford, 2003) using a two stage heuristic search strategy with tree bisection and reconnection branch swapping, saving a maximum of 10 000 trees. Bootstrap percentages (BP) were calculated using 1000 heuristic search replicates, saving ten trees per replicate with tree bisection and reconnection branch swapping. Bayesian trees were made in MrBayes 2·1 (Huelsenbeck and Ronquist, 2001) using the facilities of the Computational Biology Service Unit at Cornell University (http://cbsuapps.tc.cornell.edu) and the University of Oslo Bioportal (https://www.bioportal.uio.no). Best-fitting nucleotide substitution models were determined beforehand using MrModeltest 2·3 (Nylander, 2004) following the Akaike information criterion in each case. Two independent sets of four metropolis-coupled Markov chain Monte Carlo runs were executed for five million generations, sampling every 500 generations, with chains heated to 0·2, a burnin of 25 % and default priors. Nucleotide models were: *PgiC*, GTR + G; *PhyC*, GTR + I + G; *Rpb2*, HKY + G; plastid DNA, GTR + I + G; ITS, GTR + G. The program Tracer (Rambaut and Drummond, 2007) was used to check the runs had reached stationarity, and effective sample size of all the parameters was high (>100).

To illustrate incongruities between the individual gene trees, a filtered supernetwork (Splitstree 4·10; Huson and Bryant, 2006) was constructed from the five 50 % bootstrap consensus trees from parsimony analysis, filtering the splits to show only those present in a minimum of two input trees.

Consensus networks were constructed in Dendroscope 2·3 (Huson *et al.*, 2007) using the galled network algorithm (in which each inferred reticulation is independent of all the others; Linder and Rieseberg, 2004) with a 20 % threshold for network construction. Input trees were 50 % bootstrap consensus trees. Since five gene trees were analysed, a 20–39 % threshold effectively excluded incongruent clades unique to a single gene tree without support from any of the other trees. A threshold setting of 40–59 % would have excluded incongruent clades found in one or two gene trees, further reducing the possibility of a false positive reticulation signal but resulting in much reduced overall resolution in the consensus network. Constructing supernetworks and consensus networks without filtering in this way would generate a reticulation for each of the incongruities between the input trees, but incongruity alone does not necessarily signify reticulate evolution. It can also be due to processes such as deep coalescence, gene duplication, recombination or character homoplasy within

TABLE 2. *Characteristics of the five loci used to construct individual gene trees, and parsimony scores of the equally most-parsimonious (e.m.p.) trees after analysis in PAUP**

Locus	No. of characters included	Potentially parsimony-informative characters	No. of e.m.p. trees found	Length of e.m.p. trees	Consistency index	Retention index
Plastid DNA						
Diploids only	4422	346	72	1072	0·75	0·81
Diploids and tetraploids	4419	377	432	1177	0·74	0·83
ITS						
Diploids only	815	162	>10 000	499	0·61	0·82
Diploids and tetraploids	815	172	>10 000	562	0·60	0·84
PgiC						
Diploids only	1035	146	4014	490	0·86	0·88
Diploids and tetraploids	1033	176	>10 000	535	0·84	0·91
PhyC						
Diploids only	1183	81	1661	253	0·82	0·89
Diploids and tetraploids	1183	93	8222	313	0·84	0·93
Rpb2						
Diploids only	833	156	>10 000	513	0·87	0·90
Diploids and tetraploids	833	184	>10 000	570	0·85	0·91

individual genes (Linder and Rieseberg, 2004). Hybridization is hypothesized to cause more large-scale genomic changes affecting many genes, and so incongruence due to hybridization should be detectable from consistent differences in the phylogenetic signal from different genes. Filtering the clades used to construct phylogenetic networks allows only the more consistent differences in phylogenetic signal to be presented (McBreen and Lockhart, 2006; Holland *et al.*, 2008).

Analysis of tetraploids

Submatrices of cloned DNA sequences from each sample were aligned in MacClade, and chimeric sequences, those cloned only a single time, were removed. *In vitro* recombination of DNA sequences is a problem when cloning products of PCR reactions in which multiple alleles or paralogous gene copies have been amplified (Cronn *et al.*, 2002; Anthony *et al.*, 2007; Kelly *et al.*, 2010). Since alignments of cloned PCR products generated by this study were small (5–15 sequences with 800–1200 sites from each cloned sample), it was most expedient to screen the sequences by eye for chimeric clones. Unrooted neighbor-joining trees of the clones from a sample were made, and these were used to find the most distant sequences, termed type 1 and type 2. This information was used as an aid to screening the submatrix of cloned sequences by eye in MacClade for evidence of recombination. Recombinant sequences were identified as those that shared characteristic mutations (single nucleotide polymorphisms or indels) with both type 1 and type 2 sequences at different points along their length (Salmon *et al.*, 2010). The recombination detection programs included in the RDP3 package (Martin *et al.*, 2005) were usually unable to detect chimeric sequences that were obvious to the eye, and when they did detect recombination, they gave wrong results both for identities of the parental sequences and positions of recombination breakpoints. Anthony *et al.* (2007) found similar problems when using these programs to detect chimeric sequences, and this might be because the programs require higher levels of sequence divergence to be effective (Posada and Crandall, 2001). Occasional single nucleotide polymorphisms and small indels in the sequences were expected as a result of the cloning procedure (Speksnijder *et al.*, 2001) or DNA damage prior to amplification (Lindahl, 1993) and not taken as evidence of either *in vitro* recombination or sequence paralogy. To counteract the effect of mutations introduced in the course of cloning, consensus sequences were made for each of the parental sequences identified for each set of clones (i.e. from a given accession) to be used in subsequent analyses.

Sequences from tetraploids were aligned with directly sequenced diploid species and analysed using parsimony and MrBayes in the same way as for the diploids-only data matrices. See Table 2 for characteristics of the alignments. After constructing individual gene trees, each sequence from the tetraploid samples was assigned to a particular sequence type based on its similarity to diploid accessions, so that the taxon labels could be made consistent between gene trees. The 50 % bootstrap consensus trees were then used as input trees to construct a galled consensus network in Dendroscope. Differences in taxon sampling between the individual gene trees, due to difficulties in amplifying and cloning the available material and the occurrence of only one of the parental sequence types for some loci, required correction using the Z-closure algorithm (Huson *et al.*, 2004). This is built into the network construction methods in Dendroscope and uses the phylogenetic information shared between the input trees to overcome gaps in the taxon sampling of individual gene trees. Holland *et al.* (2008) found that the effects of potential false splits introduced by the Z-closure algorithm are offset by count-based filtering of the splits during network construction. It was found that, using Z-closure, a 20–39 % threshold for network construction in Dendroscope resulted in some clades that only appeared in one of the input trees being used to calculate reticulations in the consensus network, contrary to the present purpose of filtering the incongruent clades. Therefore a more stringent 40 % threshold was used to construct a consensus network from the combined diploid and tetraploid data to avoid poorly supported reticulations at the cost of overall resolution. Tetraploid samples for which different sequence types appeared in separate clades were manually reconnected using hybridization nodes in enewick format (Cardona *et al.*, 2008) and redrawn in Dendroscope.

RESULTS

Analysis of diploids

Individual gene trees for the three low-copy nuclear genes, the combined plastid DNA and ITS sequences are presented (Fig. 2) in the form of 50 % bootstrap consensus trees with the species names coloured according to their phylogenetic position in the plastid trees of Russell *et al.* (2010). Table 2 provides tree scores from maximum parsimony analysis of each data set in PAUP*. Each of the tree topologies is unique, although many clades are shared by more than one tree. Majority rule consensus trees from Bayesian analysis were congruent with parsimony trees but with higher resolution than the parsimony strict consensus trees. Since strict consensus trees for the nuclear genes contained clades that received low bootstrap and posterior probability support and Bayesian posterior probabilities are often unrealistically high (Simmons *et al.*, 2004), the option was taken to present the bootstrap consensus trees here.

The topology of the plastid tree (Fig. 2A) agrees well with the more complete plastid trees presented in Russell *et al.* (2010). The previous plastid study identified five main clades, I–V, which are also found in the plastid tree in this study with high bootstrap percentages and posterior probabilities. However, not all of them are present in all of the gene trees. Plastid DNA analysis found a number of species-poor, early diverging clades sister to the larger clade containing clades I–V; in the nuclear gene trees these relationships were unresolved. Clades II, III, IV and V are not present (i.e. do not appear as monophyletic groups of accessions) in the *PgiC* tree; clades I and II are not present in *PhyC*; clades I, II and III are not present in *Rpb2*. However, although the trees show many differences, many of these are not strongly supported by bootstrap percentages and posterior probabilities of clades, especially in the nuclear gene trees.

Differences between the trees are represented graphically by a filtered supernetwork (Fig. 3). This is an implicit reticulate

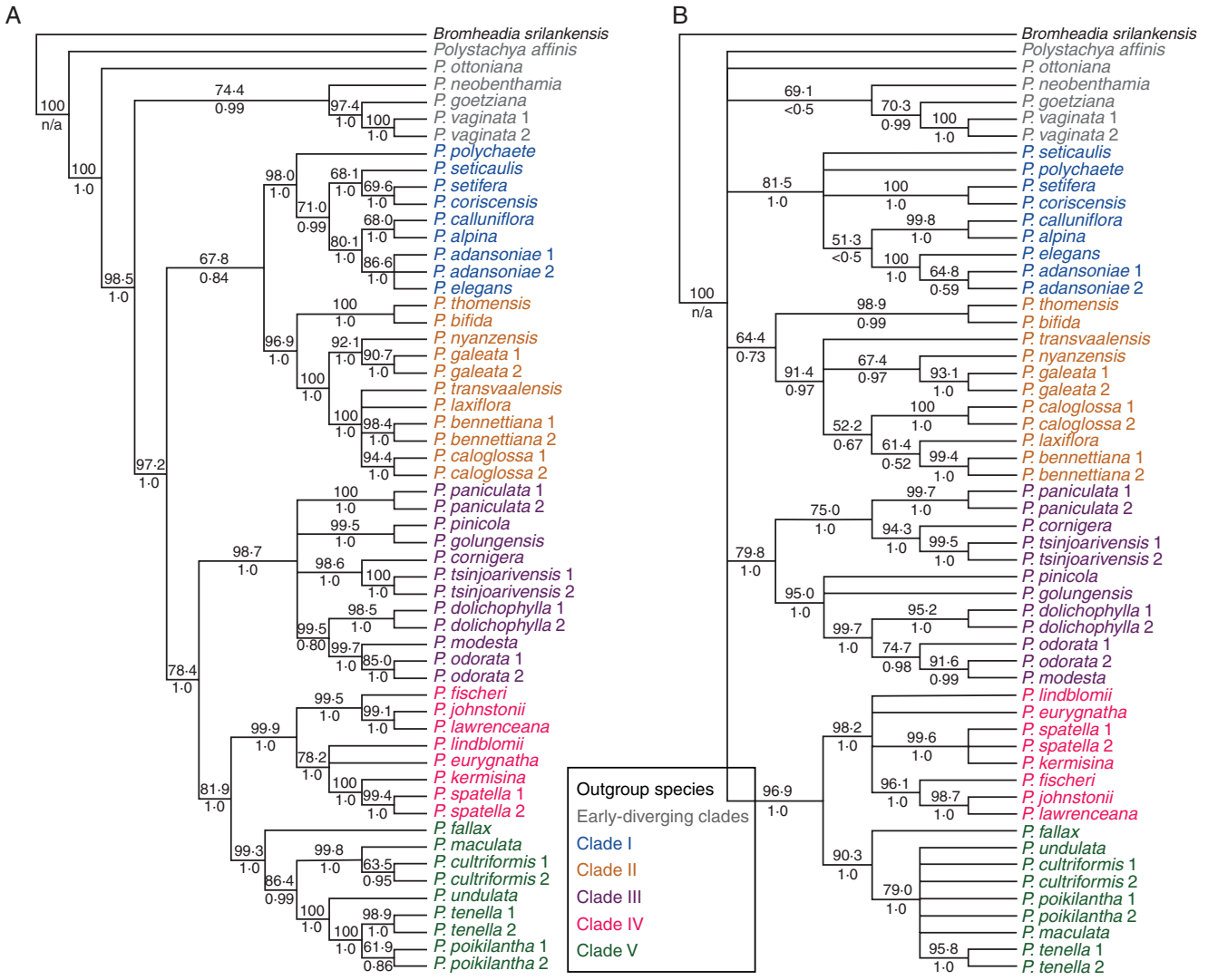

FIG. 2. Fifty per cent bootstrap consensus trees from maximum parsimony analysis of diploid samples, using five loci: plastid DNA (A), ITS (B), *PgiC* (C), *PhyC* (D) and *Rpb2* (E). Numbers above branches are bootstrap percentages; numbers below branches are Bayesian posterior probabilities. Species' names are coloured according to their correspondence to the main clades identified by plastid DNA analysis: clades I–V and species-poor, early diverging clades (Russell *et al.*, 2010).

network: cycles in the network represent conflicting phylogenetic signals rather than explicit phylogenetic hypotheses. Areas of incongruence according to Fig. 3 are at the base of the tree, the bases of clades I and III, the *P. bennettiana/P. transvaalensis* group in clade II and throughout clade V except for *P. fallax*. Despite the fact that the input trees were incongruent, the five main clades still group together in the supernetwork. A supernetwork (not shown) obtained without filtering the input trees shows a greater degree of conflict between splits at the core of the network, but the main clades are still recovered.

The consensus galled network (Fig. 4), in which cycles explicitly represent alternative phylogenetic inferences between the trees, collapses relationships between the main clades to a four-way polytomy at the core of the tree (the 'backbone' if the tree was rooted). The accessions involved in reticulations are *P. spatella* 1 (but not 2), *P. poikilantha* 1 and *P. poikilantha* 2. As with the filtered supernetwork, the five main clades expected from plastid DNA analysis are all recovered, and the outer branches are generally well resolved.

Analysis of tetraploids

Overall, 60 out of 232 cloned sequences appeared chimeric (25·8 %) from tetraploid accessions from which two homoeologous sequences were recovered, but chimeric sequences were not distributed evenly among the three nuclear genes. The following percentages of clones were chimeric: *PhyC*, 36·9 %; *PgiC*, 22·1 %; *Rpb2*, 9·6 %. When cloned tetraploid sequences were included with diploid sequences in parsimony analysis, again none of the individual gene trees was congruent with any other (Fig. 5). As with the diploids-only data, Bayesian analysis agreed with the parsimony trees but with greater resolution overall. Due to the occurrence of clades in the strict

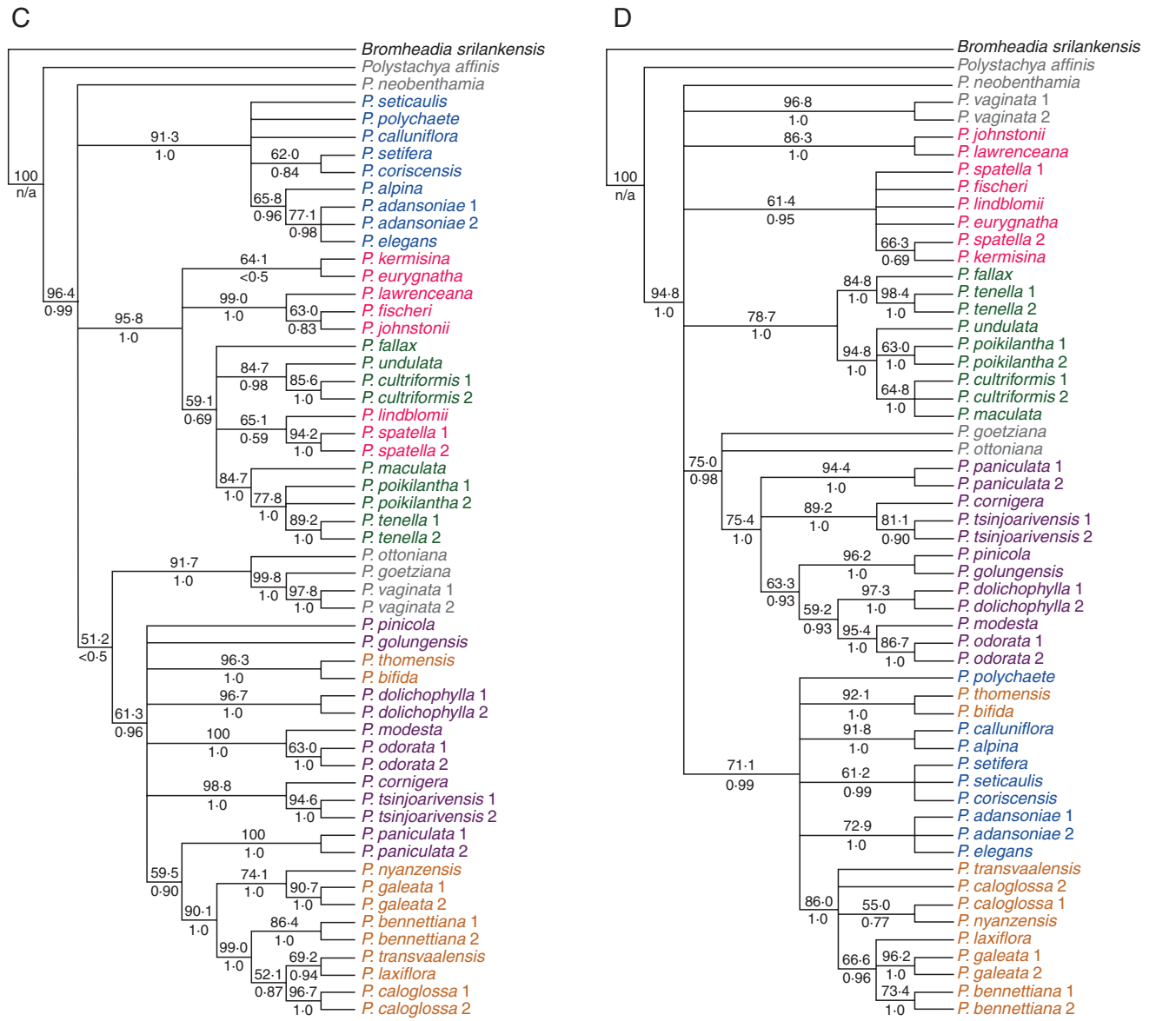

FIG. 2. Continued.

consensus parsimony trees that received no bootstrap or posterior probability support, the bootstrap consensus trees are presented here, with maximum parsimony tree scores presented in Table 2.

With cloning, two distinct sequence types were found for almost all members of the pantropical tetraploid group for all three nuclear low-copy genes; only a single *PhyC* sequence type (Fig. 5D) was recovered in the sample *P. concreta* 1 (Madagascar), but it is unclear whether this is due to the loss of one copy of *PhyC* in some populations or PCR bias: 12 clones were sequenced, which should have easily recovered a product that was half of the PCR product. By contrast, from members of the Malagasy endemic clade only a single copy of *PgiC* (Fig. 5C) and *Rpb2* (Fig. 5E) could be recovered, but two copies of *PhyC*. The two *PhyC* copies had sequences similar to those of *P. odorata* and *P. cultriformis*; the *PgiC* and *Rpb2* sequences were all similar to *P. odorata*, whereas the plastid sequences and ITS were all similar to *P. cultriformis*. Since the network construction methods using the Z-closure algorithm do not require all of the parental sequences to be present in all of the samples, there was enough phylogenetic information in the five data sets to resolve the relationships of these species with confidence in spite of missing data or copy number reduction in *PgiC* and *Rpb2*. In constructing the consensus networks, an estimate had to be made of the parental haplotype of each gene copy in the tetraploids so that the terminal taxa of the individual trees could be correlated to each other. In the case of the pantropical tetraploids, each sequence could be said to have similarity to that of either *P. modesta* or *P. golungensis* (both diploids). These two species were not inferred to be the exact parental species, but rather diploid representatives of the two sequence types found in the tetraploids for each gene. Similarly, in the case of the Malagasy tetraploids, each sequence was similar to either *P. cultriformis* or

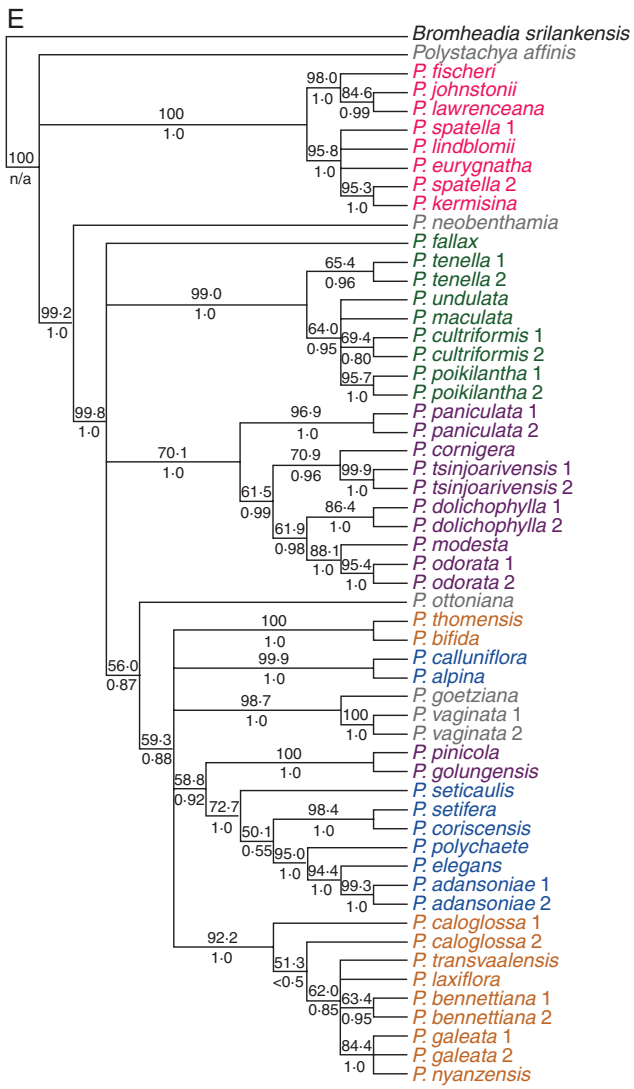

FIG. 2. Continued.

P. modesta, and in the case of *P. piersii* each sequence was similar to either *P. cultriformis* or *P. fischeri*.

The consensus galled supernetwork (40 % threshold with five input gene trees using the Z-closure method to correct for differences in taxon sampling) including the tetraploid sequences is shown in Fig. 6. Relationships among the diploid species are similar to the results from analysing diploids alone, but the higher threshold for network construction has resulted in lower resolution overall. *Polystachya fischeri* is the one diploid species involved in a reticulation in Fig. 6; it was not involved in any reticulations in Fig. 4.

The parental sequences of pantropical tetraploids from Indian Ocean islands and Asia (eastern group) are closely related and unresolved. Parental sequences from the Neotropical members of the group are also closely related to each other, but they form a clade distinct from the eastern group. Among the eastern group, it was possible to differentiate between specimens that belonged to the same clade after network analysis but for which the plastid sequences corresponded to different reticulation edges. Branches corresponding to the plastid DNA sequences of the tetraploids are coloured green in Fig. 6, and each reticulate branch is labelled to indicate whether it is represented in the plastid, ITS or low-copy nuclear gene trees.

The Malagasy endemic group also comprises allotetraploids, with one parent from clade III and the other from clade V. The genetic divergence between the parents is greater in the Malagasy tetraploids than in the pantropical group, and the genetic variation within the group is higher. However, the species appear to have originated from the same pair of parents (one from section *Cultriformes* and one from section *Polystachya*), despite the relatively high morphological variation compared with the pantropical clade.

Polystachya piersii from Kenya is revealed as an allotetraploid arising from distantly related parent species, one in clade IV and the other in clade V. Its clade V parent is more closely related to the accession *P. cultriformis* 1 (also from Kenya) than *P. cultriformis* 2 (from Madagascar). It was not possible to distinguish different parental sequences from another two tetraploid accessions, *P. bella* and *P. pubescens*;

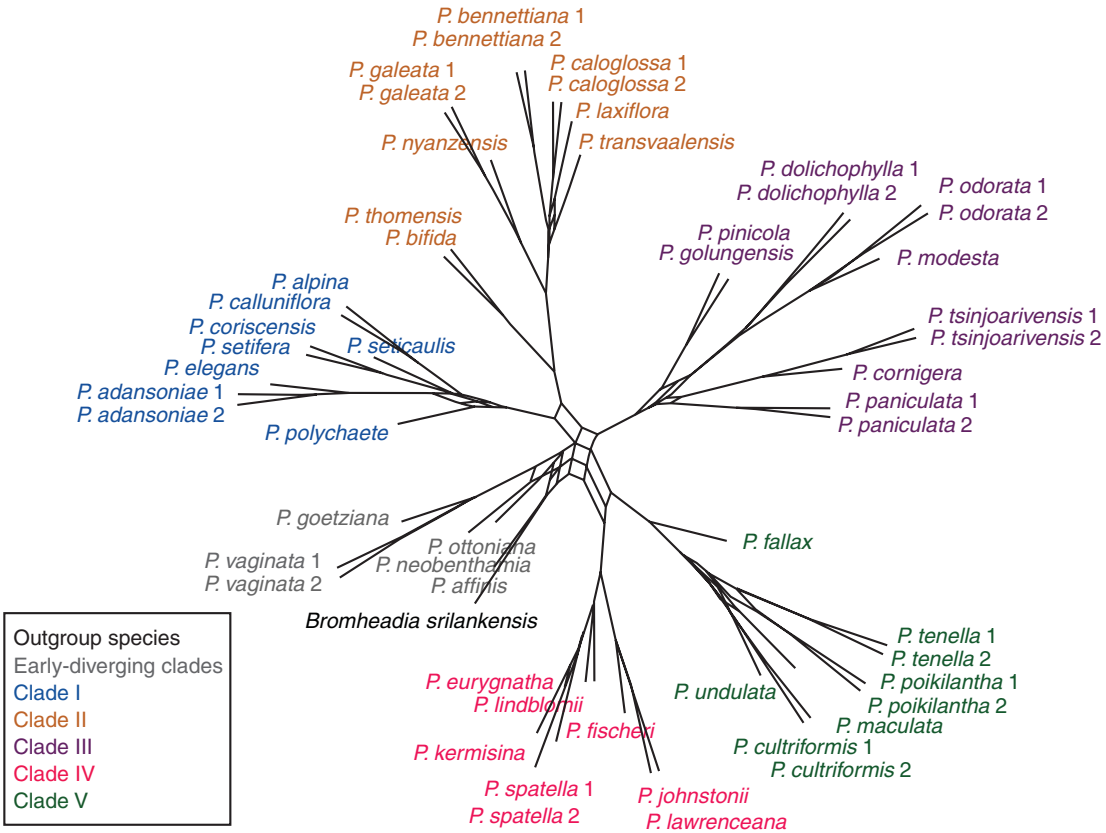

FIG. 3. Filtered supernetwork using the five 50 % bootstrap consensus gene trees from parsimony analysis as input trees, with MinNumberTrees set at 2. Species' names are coloured according to their correspondence to the main clades identified by plastid DNA analysis: clades I–V and species-poor, early diverging clades (Russell *et al.*, 2010).

they appear in the consensus network in an unresolved position at the base of clade V and sister to *P. fischeri*, respectively.

DISCUSSION

The results presented here provide a significant modification to our understanding of *Polystachya* phylogenetics and illustrate the utility of low-copy nuclear genes in resolving reticulate relationships in angiosperms. Although the number of species included in this study is lower than in a previous phylogenetic study of *Polystachya* using plastid data alone (Russell *et al.*, 2010), the inclusion in this study of DNA sequences from multiple loci provides a qualitative test of the accuracy of the plastid DNA results and allows information on hybridization and reticulation to be added to our hypothesis of *Polystachya* evolution. The importance of using multiple gene trees instead of inferring reticulations from comparison between, say plastid DNA and a single nuclear locus, is highlighted by Linder and Rieseberg (2004). This is because stochastic and population-level events can lead to misleading results in individual gene trees. Although in discarding incongruities that are unique to single gene trees some evidence for reticulation in the genus has inevitably been discarded, the reticulations retained are more likely to have accompanied large-scale genomic changes affecting multiple genes. Those affecting only one gene tree could be due to introgression or lineage sorting and thus do not affect large portions of the genomes of these taxa.

Despite apparently high levels of incongruence between diploid gene trees, supernetwork and consensus network analysis revealed the incongruence to occur mainly at deeper phylogenetic levels. The main clades identified by Russell *et al.* (2010) using only plastid sequences, with greater taxon sampling, are not found in all the gene trees produced for this study, but are recovered by the filtered supernetwork and consensus network methods. Relationships between the main clades are not resolved, and support for deeper-level phylogenetic structure in any of the individual trees is not reproduced by any of the other trees except for the position of *P. affinis* as sister to the remainder of the genus. This is similar to the findings of Murphy *et al.* (2008) for Braconidae (Hymenoptera) using a filtered supernetwork approach.

The results question the relationship of *P. pokilantha* as a sister species to *P. tenella* as found by plastid, ITS and *PgiC* trees and the monophyly of *P. spatella* with respect to *P. kermisina* as found by plastid DNA data. This could be interpreted as possible homoploid hybridization between ancestors of these species, but phenomena other than hybridization could account for differences observed in the trees, especially given the low bootstrap and posterior probability support for many of the incongruent clades. Heterogeneous rates of sequence divergence between and within genes could be confounding the tree-building algorithms or the

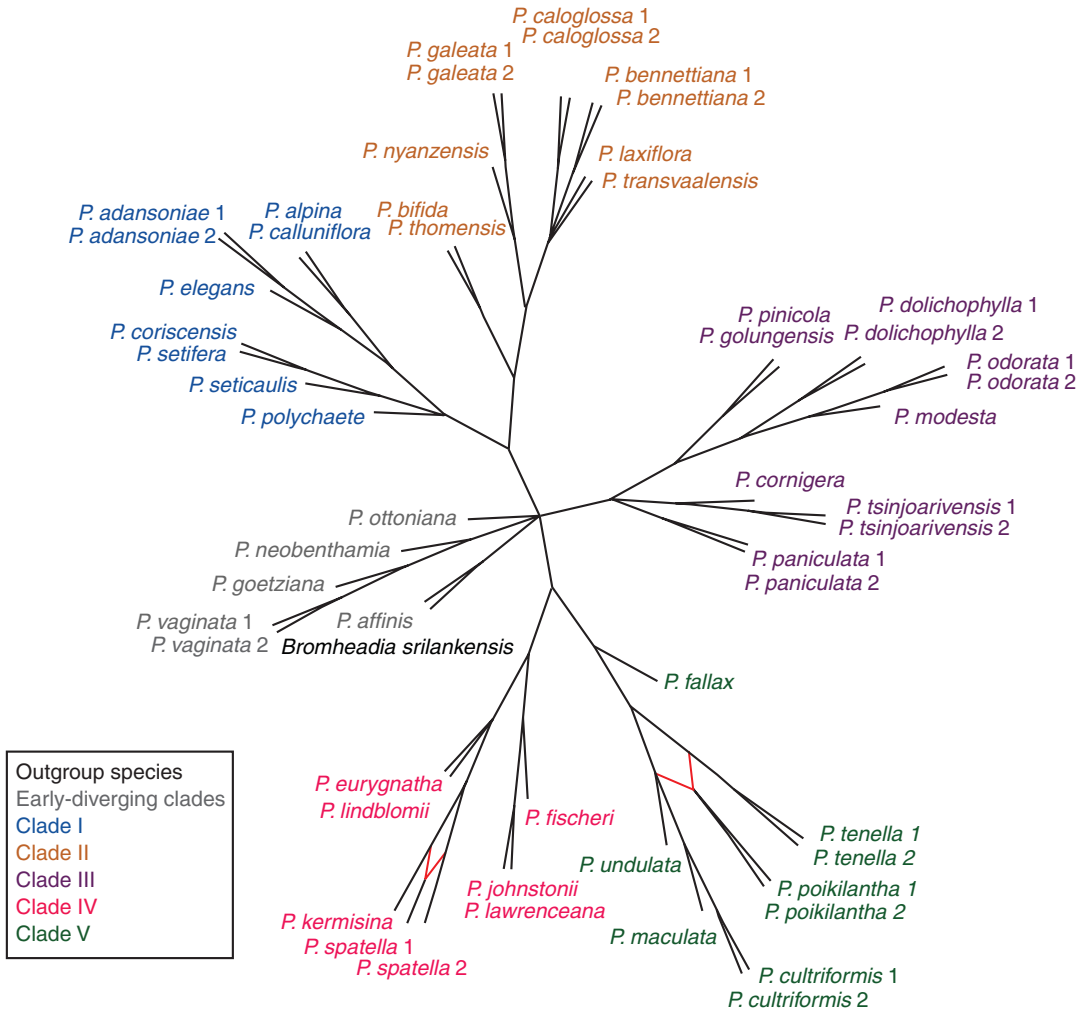

FIG. 4. Unrooted consensus galled network (20 % threshold for network construction) summarizing incongruities between the five individual gene trees of diploid species using 50 % bootstrap consensus trees as input. Branch lengths are not to scale; only the topology is shown. Red lines represent reticulations. Species names are coloured according to their correspondence to the main clades identified by plastid DNA analysis: clades I–V and species-poor, early diverging clades (Russell *et al.*, 2010).

differences could simply result from sampling error (not enough variation to obtain a clear answer). Reticulation events inferred between diploid species were not found to be consistent between analyses. When the taxon sampling was changed to include cloned tetraploids and the analysis changed to include Z-closure and a higher threshold for network construction, the above-mentioned reticulations were not recovered but instead *P. fischeri* was represented as involved in a reticulation, sister to both *P. pubescens* and *P. piersii*. The fact that these are both polyploid accessions and not present in all of the input gene trees makes it likely that this reticulation is the result of a lack of information in the input trees (Holland *et al.*, 2008), especially for the clade IV parent of *P. piersii*, which is only present in the plastid and *Rpb2* data. Homoploid hybrids are likely to lose one parental copy fairly soon after their formation, and thus homoploid hybridization is best detected by looking for linkage disequilibrium, for which large numbers of loci are needed (Chase *et al.*, 2010). Homoploid hybridization in angiosperms is clearly difficult to detect (Hegarty and Hiscock, 2008), and more than three loci would be needed to document this robustly.

More direct evidence of hybrid origins comes from cloning and sequencing the duplicated nuclear genes present in tetraploids (e.g. Petersen and Seberg, 2009). Polyploidy is present in at least eight *Polystachya* clades (Russell *et al.*, 2010), but nuclear loci were often difficult to amplify and sequence. In this study, the cloning efforts were focused on five groups of polyploids including two groups comprising multiple accessions and three comprising single accessions. The proportion of recombinant sequences among tetraploid clones (25·9 %) was higher than would be expected if these were natural recombinants (e.g. 2·4 % among homoeologous expressed sequence tags in *Gossypium*; Salmon *et al.*, 2010), supporting the present interpretation of these sequences as chimeric and the result of PCR-mediated recombination. Identifying the chimeric and parental sequences by eye based on single nucleotide polymorphisms and indels characteristic to each homoeologous sequence was possible with matrices with fewer cloned sequences produced in this study.

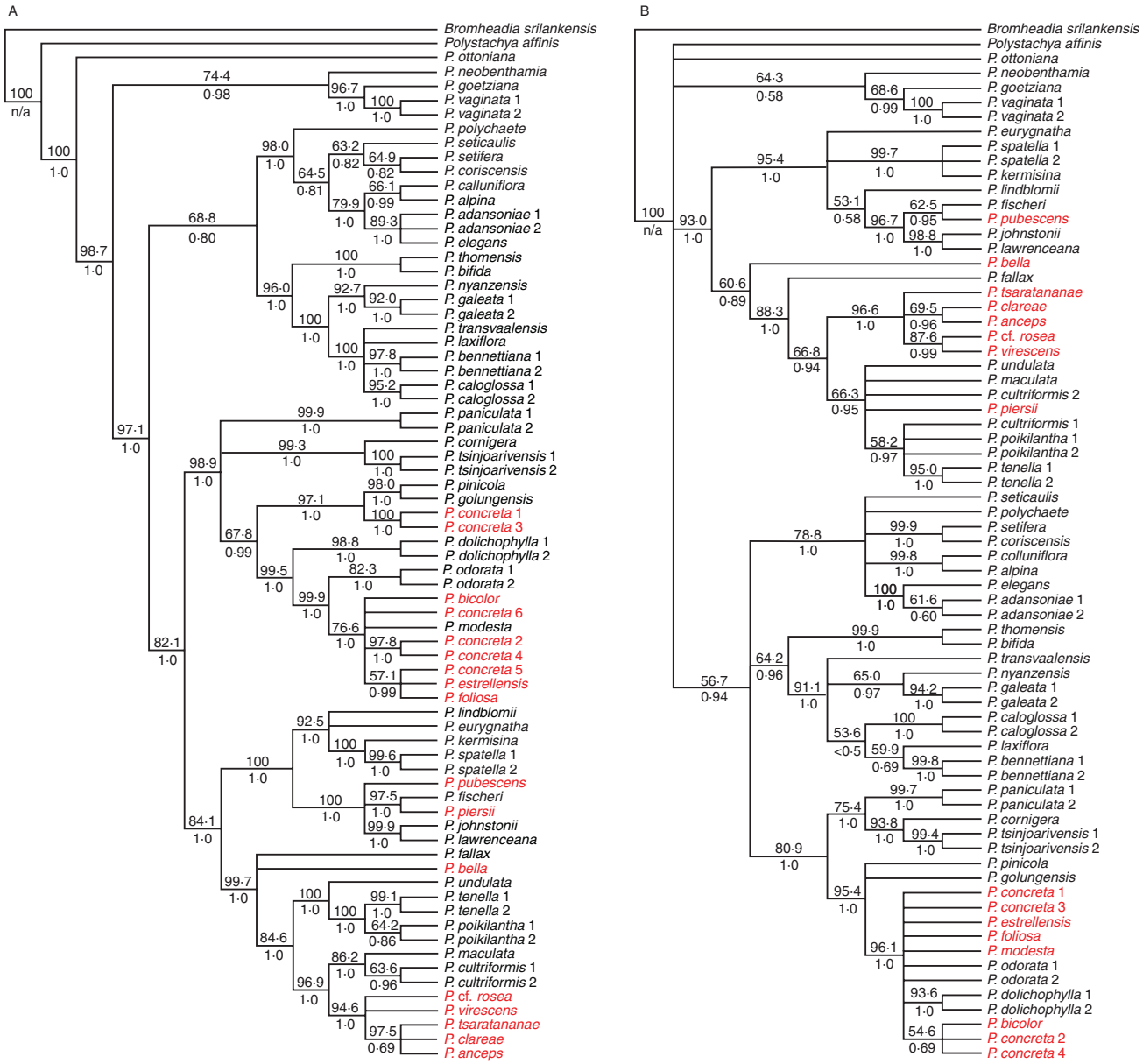

FIG. 5. Fifty per cent bootstrap consensus trees from maximum parsimony analysis of diploid and tetraploid samples, using five loci: A, plastid DNA; B, ITS; C, *PgiC*; D, *PhyC*; E, *Rpb2*. Numbers above branches are bootstrap percentages; numbers below branches are Bayesian posterior probabilities. Tetraploid accessions are shown in red.

For matrices with more sequences, an automated detection technique would be desirable, such as that used by Salmon *et al.* (2010).

The pantropical group, including *P. concreta*, mostly appears in the plastid trees (Russell *et al.*, 2010) in a clade within which there is no resolution due to low levels of divergence; this is sister to *P. dolichopylla*, along with *P. odorata* and *P. modesta*, also with low levels of sequence variation between samples as far apart as Laos, Madagascar and Brazil. A second, smaller group of *P. concreta* samples occurs in a separate clade sister to *P. golungensis*. Analysis of low-copy nuclear genes gives greater resolution for this group and reveals it to comprise allotetraploid species. The two clades of *P. concreta* found by plastid DNA are hybrids between the same parent species; in Fig. 5 they are drawn as separate groups because the accessions within the two parents contributing their plastid genome are different in each group, providing evidence of independent origins of some populations. The Neotropical tetraploids of the *P. concreta* group have different origins from those in Asia and the Indian Ocean, so we can deduce at least three independent origins of the pantropical *Polystachya* tetraploid group, all of which have dispersed rapidly and recently from the centre of *Polystachya* distribution in Africa (there may potentially be more than three independent origins of the pantropical group accessions in this study, but we are unable to infer more than three from these data). From the diploid taxa included in this study, *P. modesta* is

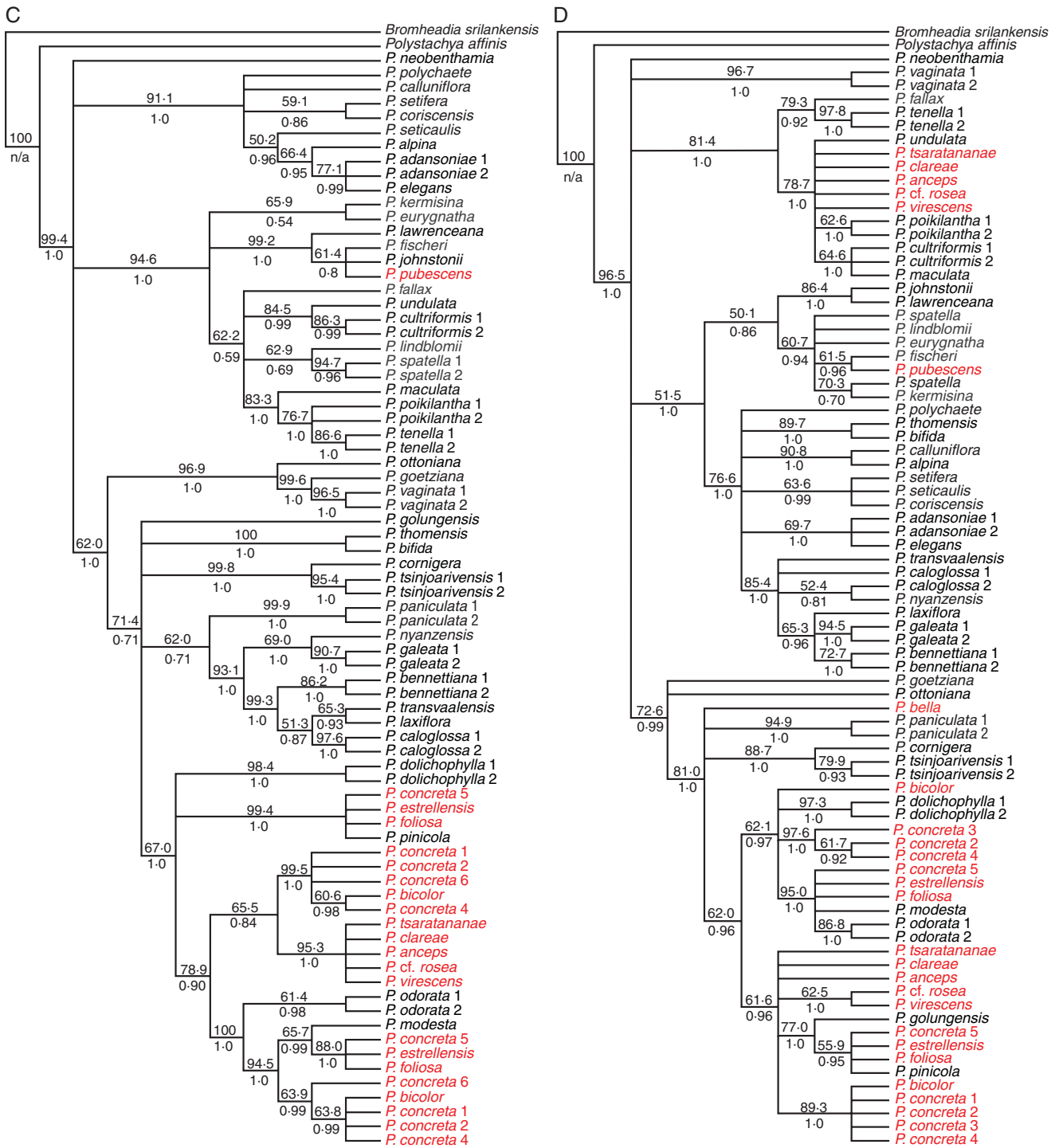

FIG. 5. Continued.

morphologically the most similar to pantropical tetraploid accessions and could be one of the parent species. Some Neotropical species including *P. foliosa* bear similarity to *P. golungensis* in flower size, shape and colour, and could be considered intermediate in morphology between, for example, *P. golungensis* and *P. modesta* or *P. odorata*. From their nuclear DNA sequences, the eastern group of pantropical tetraploids could share one parent species with the Malagasy tetraploids. These hypotheses of parental species are speculative; confident identification of the parent species would require broader taxon sampling and detailed morphological analysis.

Increased dispersal capability is commonly found in allopolyploids (Chase *et al.*, 2003; Hegarty and Hiscock, 2008), and

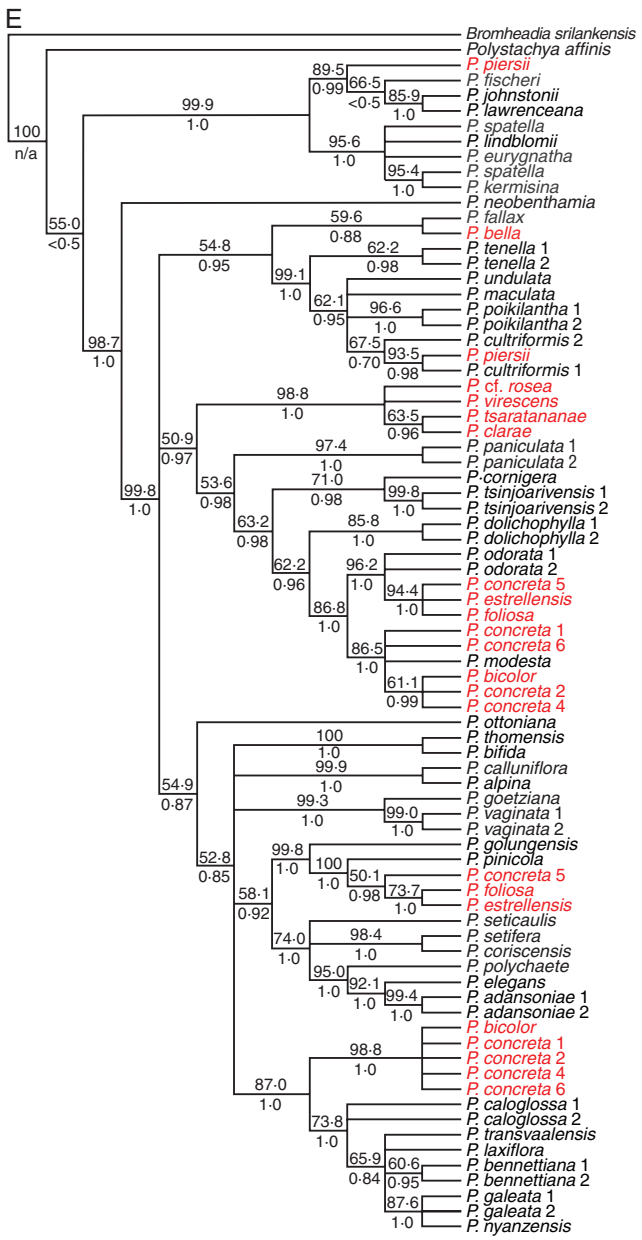

FIG. 5. Continued.

in *Polystachya* the capability for long-distance dispersal has arisen repeatedly among a certain set of hybrid offspring from relatively closely related parents. The presence of the Neotropical diploid *P. pinicola* as a sister to the Neotropical tetraploids (Fig. 6) suggests that dispersal of diploids might have been followed by allotetraploidy. Dierschke *et al.* (2009) found evidence for bicontinental hybrid origins of New Zealand *Lepidium* (Brassicaceae); the present results suggest a similar scenario is possible for Neotropical *Polystachya*, although greater taxon sampling would be required to confirm this. The wide distribution of *Polystachya* is unusual in Orchidaceae; only ten other genera have a comparable pantropical distribution (Dressler, 1993). Although orchid seeds appear adapted for wind dispersal due to their small size and internal air spaces, most seeds do not travel more than a few metres from their parent plant (Carey, 1998; Murren and Ellison, 1998). However, there are several recorded occurrences of long-distance dispersal in orchids (Arditti and Ghani, 2000), and it is not surprising given the large numbers of seeds produced by each capsule that over the course of time some of them are transported much further than most. Reasons for the apparently greater dispersal capacity of the pantropical tetraploids compared with the rest of the genus are unknown but could include a greater ability to be transported long distances and/or greater ability for seeds to germinate and establish populations in new areas. The particular adaptations that have allowed this would be worth further investigation.

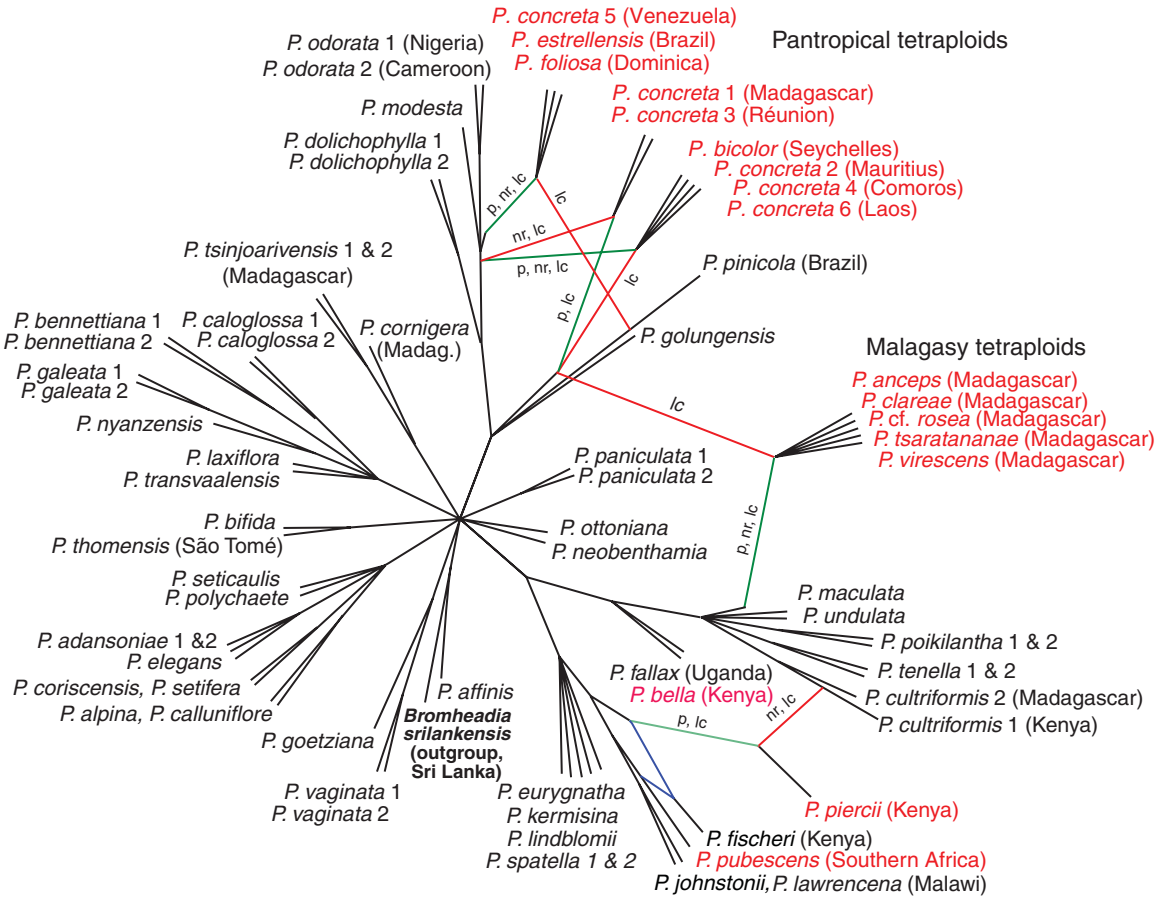

FIG. 6. Unrooted consensus galled network (40 % threshold for network construction) summarizing incongruities and allopolyploids in the five individual gene trees of diploid and tetraploid accessions, using 50 % bootstrap consensus trees as input. Branch lengths are not to scale; only the topology is shown. Reticulations inferred solely from incongruence between the gene trees are coloured blue. Red- and green-coloured branches represent reticulations involving cloned tetraploids, for which the parental sequences were manually reconnected as hybridization nodes. Green branches represent relationships according to the plastid DNA tree; red branches are only present in nuclear DNA trees; these branches are also annotated if they are represented in the plastid (p), ITS (nr) or nuclear low-copy (lc) gene trees. Species names of the cloned tetraploid samples are coloured red. Accessions are from mainland tropical Africa unless otherwise indicated.

The Malagasy tetraploids are also shown to be hybrids, and with parental species both genetically and morphologically more distant from each other than is the case with the pantropical group. Plastid DNA from all five accessions (representing five species) indicated a maternal parent from section *Cultriformes*, suggesting a single ancestral hybrid species from which the clade has subsequently diversified, although wider sampling would be necessary to rule out multiple independent origins. The fact that only one copy each of *Rpb2* and *PgiC* could be sequenced in these species is probably due to preferential PCR amplification, but Feldman *et al.* (1997) found that low-copy DNA sequences can be eliminated from allopolyploid genomes rapidly in a non-random manner.

In contrast to the pantropical group, the Malagasy allotetraploids have not shown any remarkable long-distance dispersal capability, with the species remaining endemic to Madagascar and the Comoros (located approx. 340 km from Madagascar). The morphological variation within the group, however, is greater than within the pantropical species, which is consistent with hybridization between genetically more distant parents and subsequent speciation (Paun *et al.*, 2009). Conversely, lower morphological variation among members of the pantropical group is consistent with hybrid origins from more closely related parents (Hegarty and Hiscock, 2005).

Members of the Malagasy allotetraploids have floral morphology (texture and shape of perianth segments; shape of inflorescence) consistent with members of section *Cultriformes* from clade V. The vegetative morphology (with multiple leaves on each shoot and an ovoid basal pseudobulb obscured by leaf bases) is more similar to members of section *Polystachya* in clade III (which includes the pantropical group). In the previous study based on plastid DNA sequences (Russell *et al.*, 2010), the apparent transition from the single-leaved habit of section *Cultriformes* to a section *Polystachya*-type habit with foliose shoots in the Malagasy species was interpreted as a loss of the single-leaved character. The results of this study allow that conclusion to be modified and suggest that if the clade originated with hybrids that were morphologically intermediate between the parents, then the single-leafed character was probably not 'lost' in the Malagasy group as a result of selection but was not among the characters inherited by members of the clade when it

originated. Diploid *P. cultriformis* (clade V) is extant in Madagascar, and this species or one of its ancestors is likely to be one of the parent species; from clade III the only members of section *Polystachya*, for which ploidy data are available, are tetraploids, but it is possible that diploid members of this group also occur there. Morphology of the Malagasy tetraploids is consistent with hybrid origins between *P. cultriformis* and a *P. concreta* relative. Field observations of Madagascan *Polystachya* have led workers to believe that hybridization is a common and ongoing process (G. Fischer, University of Salzburg, and A. Sieder, University of Vienna Botanical Garden, pers. comms.), and further investigations into the role of hybridization in evolutionary processes on the island would be rewarding. This would require increased taxon sampling, more detailed morphological and geographical studies, and data from more genes and population markers.

Allotetraploid *P. piersii* appears to be a hybrid between *P. cultriformis* and a relative of the *P. fischeri*/*P. johnstonii*/*P. lawrenceana* group from clade IV. Kenyan *P. cultriformis* is more closely related to *P. piersii* than to Madagascan *P. cultriformis*. Its morphology also appears intermediate between the two groups. *Polystachya piersii* has similar floral morphology to *P. cultriformis*, but its habit and vegetative morphology are more similar to clade IV members of section *Affines*. It is still not possible to say whether another two accessions, *P. bella* and *P. pubescens*, are allo- or autotetraploid; although we were unable to distinguish two parental sequences from any of the genes sequenced from either accession this could be because they lack hybrid origins or because, if hybrids, their parental species are too closely related for consistent sequence differences to be discerned or homoeologous gene copies from one of the parents have been lost or failed to amplify.

As well as the five groups represented by tetraploid accessions in this study, polyploidy occurs in several other groups in *Polystachya*; here the focus has been on taxa for which nuclear DNA could be amplified and cloned. At least two species occur as both diploids and tetraploids so, although this study has focused on hybrid clades, autopolyploidy might also be an important process in the genus. Further study on other tetraploid groups would contribute to our understanding of the significance of polyploidy in the evolution and biogeography of the genus in the African mainland.

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APPENDIX

Accession list: species name; country of origin when known; herbarium voucher when present; accession number of the living collection of the Royal Botanic Gardens, Kew or the HBV (University of Vienna Botanical Garden) where applicable; accession number for the DNA bank of the Jodrell Laboratory, Royal Botanic Gardens, Kew, where applicable; GenBank accession numbers for DNA sequences.

Accession	Country	Herbarium voucher	Living collection (Kew/HBV)	Kew DNA bank	GenBank accession numbers				
					Plastid	ITS	*PgiC*	*PhyC*	*Rpb2*
Bromheadia srilankensis Kruiz. & de Vogel	Sri Lanka	Chase 15746 (K)	NA	15746	GQ145086	HM018544	HM018560	HM018513	HM018526
Polystachya adansoniae Rchb.f. 1	Nigeria	*Bytebier 429/94/469* (EA)	NA	17957	GQ145088	GU556632	GU556782	GU556701	GU556852
Polystachya adansoniae 2	Cameroon	*A. Russell 92* (YA)	NA	NA	GQ145089	HM018545	HM018561	HM018514	HM018527
Polystachya affinis Lindl.	Nigeria	*Chase 21165* (K)	Kew 1981-4996	21165	GQ145090	GU556633	GU556783	GU556702	GU556853
Polystachya alpina Lindl.	Cameroon	*A. Russell 67* (YA)	NA	NA	GQ145092	GU556634	GU556784	GU556703	GU556854
Polystachya anceps Ridl.	Madagascar	*Fischer & Sieder FS4068* (WU)	NA	NA	GQ145094	GU556692	HM018562	GU556755-GU556756	NA
Polystachya bella Summerh.	Kenya	*Beatrice 783* (EA)	NA	17950	GQ145095	HM018546	NA	HM018515	HM018528
Polystachya bennettiana Rchb.f. 1	Kenya	*Beatrice 338/94/418* (EA)	NA	17958	GQ145096	HM018547	HM018563	HM018516	HM018529
Polystachya bennettiana 2	Unknown	*Mughambi & Odhiambo 81/01* (EA)	NA	19186	GQ145097	HM176598	HM018564	HM018517	HM018530
Polystachya bicolor Rolfe (=*P. concreta* (Jacq.) Garay & H.R.Sweet)	Seychelles	*A. Russell Kew-2003-406* (WU)	Kew 2003-406	25884	GQ145120	GU556686	GU556836-GU556837	GU556760-GU556761	GU556907-GU556908
Polystachya bifida Lindl.	São Tomé	NA	Kew 2001-3989	25885	GQ145100	GU556636	GU556787	GU556706	GU556857
Polystachya calluniflora Kraenzl.	Cameroon	*A. Russell 63* (YA)	NA	NA	GQ145104	GU556638	GU556788	GU556708	GU556859
Polystachya caloglossa Rchb.f. 1	Cameroon	*A. Russell 41* (YA)	NA	NA	GQ145105	HM018548	HM018565	HM018518	HM018531
Polystachya caloglossa 2	Cameroon	*A. Russell 104* (YA)	NA	NA	GQ145106	GU556639	GU556789	GU556709	GU556860
Polystachya clareae Hermans	Madagascar	*Fischer & Sieder s.n.* 27/1/2007 (WU)	NA	NA	GQ145109	GU556684	GU556833	GU556757-GU556758	GU556904
Polystachya concreta (Jacq.) Garay & H.R.Sweet 1	Madagascar	*Chase 17854* (K)	Kew 1997-4474	17854	GQ145110	GU556685	GU556834-GU556835	GU556759	GU556905-GU556906
Polystachya concreta 2	Mauritius	NA	HBV ORCH07278	NA	GQ145118	GU556687	GU556840-GU556841	GU556764-GU556765	GU556913-GU556914
Polystachya concreta 3	Réunion	NA	HBV 'Reunion 1'	NA	GQ145117	GU556688	NA	GU556766-GU556767	GU556914-GU556915
Polystachya concreta 4	Comoros	Photograph	HBV ORCH07417	NA	GQ145119	GU556698	GU556842-GU556843	GU556768-GU556769	GU556915-GU556916
Polystachya concreta 5	Venezuela	NA	HBV ORCH06361	NA	GU556925	NA	GU556846-GU556847	GU556770-GU556771	GU556917-GU556918
Polystachya concreta 6	Laos	*A. Russell ORCH06415* (WU)	HBV ORCH06415	NA	GU556926	NA	GU556848-GU556849	NA	GU556919-GU556920

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APPENDIX *Continued*

Accession	Country	Herbarium voucher	Living collection (Kew/HBV)	Kew DNA bank	GenBank accession numbers				
					Plastid	ITS	*PgiC*	*PhyC*	*Rpb2*
Polystachya coriscensis Rchb.f.	Unknown	*A. Russell ORCH07314* (WU)	HBV ORCH07314	NA	GQ145122	GU556641	GU556791	GU556711	GU556862
Polystachya cornigera Schltr.	Madagascar	*Fischer & Sieder FS3208* (WU)	NA	NA	GQ145123	GU556642	GU556792	GU556740	HM018532
Polystachya cultriformis (Thouars) Lindl. ex Spreng. 1	Unknown	*Mugambi & Odhiambo 054/98/ 1607* (EA)	NA	19182	GQ145124	GU556643	GU556793	GU556713	GU556863
Polystachya cultriformis 2	Madagascar	*Fischer & Sieder FS1045* (WU)	HBV FS1045	NA	GQ145125	GU556644	GU556794	GU556714	GU556864
Polystachya dolichophylla Schltr. 1	Cameroon	*Chase 25886* (K)	Kew 1989-1745	25886	GQ145128	GU556646	GU556796	GU556716	GU556865
Polystachya dolichophylla 2	Unknown	Photograph	HBV ORCH03072	NA	GU556927	GU556647	GU556797	GU556712	GU556866
Polystachya elegans Rchb.f.	Cameroon	*A. Russell 74* (YA)		NA	GQ145129	GU556648	GU556798	GU556718	GU556867
Polystachya estrellensis Rchb.f. (=*P. concreta* (Jacq.) Garay & H.R.Sweet)	Brazil	*A. Russell ORCH06604* (WU)	HBV ORCH06604	NA	GQ145114	GU556693	GU556838-GU556839	GU556762-GU556763	GU556909-GU556910
Polystachya eurygnatha Summerh.	Unknown	Photograph	NA	NA	GQ145131	GU556649	GU556799	GU556719	GU556868
Polystachya fallax Kraenzl.	Uganda	*Chase 17922* (K)	Kew 2001-4022	17922	GQ145132	GU556650	HM018566	GU556720	GU556869
Polystachya fischeri Rchb.f. ex Kraenzl.	Kenya	*Pearce 616/94/607* (EA)	NA	17964	GQ145133	GU556651	GU556800	GU556721	GU556870
Polystachya foliosa (Hook.) Rchb.f.	Dominican Republic	NA	Kew 2001-3986	25887	GQ145135	GU556690	HM018567-HM018568	GU556772-GU556773	GU556921-GU556922
Polystachya galeata (Sw.) Rchb.f. 1	Unknown	*Chase O-1496* (K)	Kew 1972-1958	O-1496	GQ145139	GU556652	GU556801	GU556722	GU556871
Polystachya galeata 2	Unknown	'*C283*' (K)	NA	9041	GU556928	GU556653	GU556802	GU556723	GU556872
Polystachya goetziana Kraenzl.	Kenya	*Bytebier 1772* (EA)	NA	17955	GQ145141	GU556654	GU556803	GU556724	GU556873
Polystachya golungensis Rchb.f.	Unknown	*A. Russell ORCH05170* (WU)	HBV ORCH05170	NA	GQ145143	GU556655	GU556804	GU556725	GU556874
Polystachya johnstonii Rolfe	Unknown	Photograph	HBV ORCH06241	NA	GQ145149	GU556657	GU556806	GU556727	GU556876
Polystachya kermisina Kraenzl.	Rwanda	Photograph	HBV ORCH07240	NA	GQ145150	GU556658	GU556807	GU556728	GU556877
Polystachya lawrenceana Kraenzl.	Malawi	Photograph	NA	NA	GQ145152	HM018549	HM018569	HM018519	HM018533
Polystachya laxiflora Lindl.	Unknown	*A. Russell ORCH07315* (WU)	HBV ORCH07315	NA	GQ145153	GU556659	GU556808	GU556729	GU556878
Polystachya lindblomii Schltr.	Kenya	*Bytebier 1142/98/ 1695* (EA)	NA	17967	GQ145154	GU556660	GU556809	GU556730	GU556879
Polystachya maculata P.J.Cribb	Burundi	Photograph	HBV ORCH07263	NA	GQ145156	GU556696	GU556810	GU556731	GU556880

Polystachya modesta Rchb.f.	Unknown	NA	HBV ORCH05165	NA	GQ145159	GU556662	GU556812	GU556733	GU556882
Polystachya neobenthamia Schltr.	Unknown	Photograph	HBV ORCH07214	NA	GQ145087	GU556663	GU556813	GU556734	GU556883
Polystachya nyanzensis Rendle	Unknown	*A. Russell ORCH06425* (WU)	HBV ORCH06425	NA	GQ145163	HM018550	HM018570	HM018520	HM018534
Polystachya odorata Lindl. 1	Nigeria	*Chase 17857* (K)	Kew 1970-2771	17857	GQ145164	GU556664	GU556814	GU556735	GU556884
Polystachya odorata 2	Cameroon	*A. Russell 42* (YA)	NA	NA	GQ145165	GU556665	GU556815	GU556736	GU556885
Polystachya ottoniana Rchb.f.	Unknown	NA	Kew 2005-964	25888	GQ145168	GU556666	GU556816	GU556737	GU556886
Polystachya paniculata (Sw.) Rolfe 1	Ethiopia	NA	Kew 1984-4977	25889	GQ145170	GU556667	GU556818	GU556739	GU556888
Polystachya paniculata 2	Unknown	Photograph	HBV O99B26-1	NA	HM018557-HM018559	HM018551	HM018571	HM018521	HM018535
Polystachya piersii P.J.Cribb	Kenya	*Beatrice 101/95/ 1186* (EA)	NA	17948	GQ145172	HM018552	NA	NA	HM018536-HM018537
Polystachya pinicola Barb.Rodr.	Brazil	NA	HBV ORCH06606	NA	GQ145174	GU556668	GU556819	GU556717	GU556889
Polystachya poikilantha Kraenzl. 1	Kenya	*Bytebier 956/97/524* (EA)	NA	19261	GQ145176	GU556669	GU556820	GU556741	GU556890
Polystachya poikilantha 2 (var. *leucorhoda* (Kraenzl.) P.J.Cribb & Podz.)	Unknown	Photograph	HBV ORCH06272	NA	GQ145177	HM018553	HM018572	HM018522	HM018538
Polystachya polychaete Kraenzl.	Kenya	NA	Kew 2001-3987	25890	GQ145178	GU556670	GU556821	GU556742	GU556891
Polystachya pubescens Rchb.f.	Unknown	*Kurzweil 1849* (K)	NA	O-700	GQ145179	HM018554	HM018573	HM018523	NA
Polystachya cf. *rosea* Ridl.	Madagascar	*Fischer & Sieder FS796* (WU)	HBV FS796	NA	GQ145185	GU556689	GU556850	GU556774-GU556775	GU556923
Polystachya seticaulis Rendle	Congo	*Chase 17924* (K)	Kew 2001-3981	17924	GQ145186	GU556671	GU556822	GU556743	GU556892
Polystachya setifera Lindl.	Unknown	*Chase O-1493* (K)	Kew 1983-2403	O-1493	GQ145187	GU556672	HM018574	GU556744	GU556893
Polystachya spatella Kraenzl. 1	Kenya	*Bytebier 949* (EA)	NA	17951	GQ145188	GU556673	GU556823	GU556745	GU556894
Polystachya spatella 2	Kenya	*Khayota 381* (EA)	NA	19263	GQ145189	HM018555	HM018575	HM018524	HM018539
Polystachya tenella Summerh. 1	Kenya	*Bytebier 955/97/ 1524* (EA)	NA	17952	GQ145193	GU556674	GU556824	GU556746	GU556895
Polystachya tenella 2	Kenya	*Bytebier 955/97/ 1523* (EA)	NA	19262	GQ145194	GU556675	GU556825	GU556747	GU556896
Polystachya thomensis Summerh.	São Tomé	*Chase 17858* (K)	Kew 2001-3989	17858	GQ145196	GU556677	GU556827	GU556748	GU556898
Polystachya transvaalensis Schltr.	Kenya	*Bytebier 951/97/ 1519* (EA)	NA	17969	GQ145197	GU556678	GU556828	GU556749	GU556899
Polystachya tsaratananae H.Perrier	Madagascar	*Chase 17861* (K)	Kew 2001-2413	17861	GQ145199	GU556691	GU556851	GU556776-GU556777	HM018540
Polystachya tsinjoarivensis H.Perrier 1	Madagascar	*Fischer & Sieder FS3209* (WU)	NA	NA	GQ145201	HM018556	HM018576	HM018525	HM018541

Continued

Appendix *Continued*

Accession	Country	Herbarium voucher	Living collection (Kew/HBV)	Kew DNA bank	GenBank accession numbers				
					Plastid	ITS	*PgiC*	*PhyC*	*Rpb2*
Polystachya tsinjoarivensis 2	Madagascar	Photograph	HBV FS4182	NA	GQ145202	GU556679	GU556829	GU556750	HM018542
Polystachya undulata P.J.Cribb & Podz.	Unknown	*Chase 17862* (K)	Kew 2001-3975	17862	GQ145203	GU556680	GU556830	GU556751	GU556900
Polystachya vaginata Summerh. 1	Kenya	*Bytebier 566/95/1140* (EA)	NA	17949	GQ145204	GU556681	GU556831	GU556752	GU556901
Polystachya vaginata 2	Kenya	*Bytebier 452/97/1587* (EA)	NA	19265	GQ145205	GU556682	GU556832	GU556753	GU556902
Polystachya virescens Ridl.	Madagascar	*Fischer & Sieder FS1002* (WU)	HBV FS1002	NA	GQ145206	GU556697	HM018577	GU556778-GU556779	HM018543