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Genetic variation and phylogenetic relationships of a pantropical species group in *Polystachya* (Orchidaceae)

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Amplified fragment length polymorphism (AFLP) markers were used to investigate the relationships among *Polystachya* accessions from a group of closely related pantropical tetraploids. Before starting with the fingerprinting analyses, the polyploid accessions were first included in a phylogenetic analysis using low-copy nuclear DNA data to establish their relationships, which confirmed that they belonged to a species group of closely related allotetraploids. Neo- and Palaeotropical polyploid accessions formed two hybrid clades with apparently independent origins. Sampling for the AFLP analyses included single accessions from much of the range of the genus and populations from Costa Rica (CR) and Sri Lanka (SL) to compare population structure and genetic diversity in these two areas in more detail. A splits graph of the complete AFLP data showed three major clusters corresponding to three sources of population sampling (*P. concreta*, SL; *P. foliosa*, CR; *P. masayensis*, CR), with individual accessions from Africa and Indian Ocean islands showing a closer relationship to *P. concreta* from SL than to the two CR species. Individual accessions from the Neotropics occurred in more isolated positions in the splits network, with little resolution. Some *P. foliosa* accessions clustered with *P. masayensis*, suggesting some hybridization between the two species, and this was confirmed by Bayesian structure analysis. However, the splits network, structure and analyses of molecular variance indicated a generally high level of genetic divergence between the two CR species, despite their recent hybrid origin, occurrence in largely the same localities and occasional hybridization. *Polystachya foliosa* from CR had a higher degree of population-level genetic structure ($\Phi_{ST} = 0.291$) than *P. masayensis* from CR ($\Phi_{ST} = 0.161$) and *P. concreta* from SL ($\Phi_{ST} = 0.138$), possibly because of its occurrence within a larger and more environmentally diverse continuous range than the other two species. Genetic divergence between Neo- and Palaeotropical members of the pantropical tetraploid group of *Polystachya* and the nonmonophyly of *P. concreta* suggested that *P. concreta s.l.* should be split and the use of this epithet should be confined to the Neotropics (the type is from Martinique). Other names should be used in Africa and the Asian tropics. *Botanical Journal of the Linnean Society*, 2011, **165**, 235–250.

ADDITIONAL KEYWORDS: AFLP – biogeography – Costa Rica – epiphytic orchid – low-copy nuclear genes – phylogenetic analysis – *Polystachya concreta* – *Polystachya foliosa* – *Polystachya masayensis* – population structure – splits network – Sri Lanka.

INTRODUCTION

Polystachya Hook. is a large tropical orchid genus (*c.* 250 species) occurring in Africa, southern Asia and the Americas. The centre of diversity is in Africa, but one group of closely related and taxonomically problematic species is found throughout the tropics. Although DNA sequence analysis has been informative in studies of the genus as a whole, relationships between members of this pantropical species group

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[including *P. concreta* (Jacq.) Garay & Sweet, *P. foliosa* (Hook.) Rchb.f., *P. estrellensis* Rchb.f., *P. bicolor* Rolfe and other species] have not been well resolved because of low levels of sequence divergence between the species in both plastid and nuclear genes (Russell *et al.*, 2010a, b). Morphologically, species boundaries between widely dispersed populations have also been difficult to determine. Chromosome counts and genome size measurements (Rupp *et al.*, 2010; Russell *et al.*, 2010b) have shown that members of the pantropical group occurring outside Africa are allotetraploid, and comparisons of DNA sequences from low-copy nuclear genes have indicated that plants found in the eastern half of the distribution originated separately from those in the west. These factors indicate recent origins coupled with a high capability for long-distance dispersal. Other studies have shown comparable properties in allopolyploids from other families (e.g. in *Castilleja* Mutis ex L.f., Orobanchaceae: Tank & Olmstead, 2009; *Santalum* L., Santalaceae: Harbaugh, 2008), but the tendency for rapid long-distance dispersal is not commonly seen in orchid genera, in spite of their dust-like, wind-dispersed seeds. There are 11 orchid genera with a pantropical distribution comparable with that of *Polystachya*, although several more have trans-Atlantic or trans-Pacific distributions (Dressler, 1993).

The pantropical *Polystachya* species group is taxonomically complicated. Much of the variation seen among populations throughout the tropics is taxonomically combined under the name *Polystachya concreta* (Jacq.) Garay & Sweet. The name is associated with a large number of synonyms (Garay & Sweet, 1974), some of which are morphologically diverse, and although populations from different areas tend to appear subtly different (based on characters such as flower size and colour, inflorescence shape, overall size of plants, and number and shape of leaves), these morphological differences are difficult to define consistently.

Although we know from previous work (Russell *et al.*, 2010a, b) that *P. foliosa* belongs to a group of Neotropical allotetraploids with close affinities to *P. concreta*, the relationships of *P. masayensis* Rchb.f. have not yet been established using DNA sequences. For this study, we obtained nuclear DNA sequences of the low-copy nuclear genes *PhyC* and *Rpb2* from Costa Rican *P. foliosa* and *P. masayensis* and Sri Lankan *P. concreta*. *PhyC* is a member of the phytochrome gene family, important in photoregulatory pathways; *Rpb2* codes for a component of RNA polymerase. Both genes have been used widely in plant phylogenetics (e.g. Mathews & Sharrock, 1996; Oxelman *et al.*, 2004; Samuel *et al.*, 2005; Thomas *et al.*, 2006; Sun, Pourkheirandish & Komatsuda, 2009). They were used in a previous study (Russell *et al.*, 2010a) to determine the phylogenetic relationships among *Polystachya* tetraploids. The Costa Rican and Sri Lankan accessions can be aligned with existing sequences to confirm that they belong to the same species groups as the *P. concreta* and *P. foliosa* accessions from that study. Plastid data for this species group have been useful in demonstrating the close relatedness of members of this group, but as maternally inherited sequences, they can give incomplete results in the case of allopolyploid species. For example, although most members of the pantropical tetraploid *Polystachya* clade have similar plastid sequences, some accessions from Madagascar and La Réunion have received their plastid genomes from a different parent and appear to be unrelated to conspecific accessions from other populations; the cloning and sequencing of low-copy nuclear genes have revealed that this is a result of the hybrid origins of the group, and both parental sequences can be found in their nuclear genomes (Russell *et al.*, 2010a, b).

Compared with previous evolutionary studies of the genus, the present study focuses on the pantropical *Polystachya* group in more detail, including population-level sampling in Sri Lanka and Costa Rica. A *P. concreta*-like entity grows natively in Sri Lanka and is the only species there. Twenty-one species are currently recognized in the Neotropics (Govaerts *et al.*, 2009, *World Checklist of Monocotyledons*), three of which occur in Costa Rica. One of these, *P. lineata* Rchb.f., is rarely recorded. The two more common species are *P. foliosa* and *P. masayensis* (Fig. 1), often found growing together. Costa Rica is an ideal place to collect because *Polystachya* populations can be found readily, and the presence of only two common species simplifies the study design and analysis whilst providing the necessary material to answer the questions posed.

As an alternative to DNA sequencing, we chose to use amplified fragment length polymorphism (AFLP) markers to estimate the genetic distances and probable dispersal histories among accessions of *P. concreta*-like plants from Sri Lanka, *P. foliosa* and *P. masayensis* from Costa Rica, and isolated specimens referred to *P. concreta* and *P. foliosa* from other Indian Ocean islands, Africa and South America. AFLP analysis typically generates a large number of markers for genetic fingerprinting and is appropriate for population-level studies and closely related species with little sequence divergence between accessions (Meudt & Clarke, 2007), including evolutionary studies of polyploid groups (Hedrén *et al.*, 2001; Guo *et al.*, 2006; Albach, 2007). The specific aims of the study were as follows: (i) to determine the phylogenetic relationships of Costa Rican and Sri Lankan *Polystachya* accessions; (ii) to investigate the phylogenetic

Figure 1. Inflorescences of *Polystachya* species native to Costa Rica: A, *P. foliosa*; B, *P. masayensis*. Photographs © Lankester Botanical Gardens.

relationships among populations of the pantropical *Polystachya* group from around the world; and (iii) to assess the extent to which *P. foliosa* and *P. masayensis* are genetically distinct in Costa Rica.

MATERIAL AND METHODS

Sampling of *Polystachya* was carried out at 17 sites in Costa Rica from March to April 2008 and four sites in Sri Lanka in April 2009. Leaf material from 1–24 plants in each population was preserved in silica gel for DNA extraction (Chase & Hills, 1991). Voucher specimens were collected from all populations and are held at JBL and PDA herbaria (Jardín Botánico Lankester, Costa Rica, and Royal Botanic Gardens, Sri Lanka). In addition, 14 DNA samples from isolated *P. concreta*-like individuals from around the world were included. See Table 1 (Costa Rican and Sri Lankan populations) and Appendix (individual accessions from other areas) for accession details.

DNA extraction was performed using a cetyltrimethylammonium bromide (CTAB) protocol (Russell *et al*., 2010b) modified from those of Doyle & Doyle (1987), Li *et al*. (2007) and Tel-Zur *et al*. (1999). Primers for polymerase chain reaction (PCR) were those used by Russell *et al*. (2010a): *Rpb2*-Pol-23F1 (CTCCATTCACTGATGTTACGG); *Rpb2*-Pol-23R (GA ACAGTGGTCARCCTCCAAG); *PhyC*e1F2-or (AAGC CSTTYTAYGCAATTCTACACCG); and *PhyC*e1R2-or (ATWGCATCCATYTCAACATCKTCCCA). PCR was carried out in 20-μL reactions using 18.0 μL ABGene ReddyMix PCR Master Mix, 0.5 μL of each primer at 20 μM and 1.0 μL of template DNA. The PCR programme included an initial denaturation at 80 °C for 5 min, 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 2 min, followed by a final extension of 72 °C for 5 min. Products were gel-purified and cloned using the pGEM-T Easy cloning system (Promega), following the manufacturer's protocol. TE minipreps were made from successful transformants, and these were used as template DNA for amplification and cycle sequencing employing SP6 and T7 vector primers. Cycle sequencing was carried out in 10-μL reactions with 1.0 μL of ABI

Table 1. Populations from Costa Rica and Sri Lanka with collection numbers, location, number of individuals (*N*) and within-population marker variation expressed as fragment polymorphism and Nei's gene diversity index (H_S). The values were calculated arithmetically for populations with three or more individuals using AFLPdat (H_S) and by Bayesian analysis of dominant population genetic data using Hickory v1.1 (h_s)

Population	Location	*N*	No of variable bands	% of variable bands	H_S (AFLPdat)	$h_s \pm$ SD (Hickory)
P. concreta (Sri Lanka)						
Samuel SL-H	7°16′N 80°38'E	10	99	65	0.22	0.30 ± 0.01
Samuel SL-L	7°17′N 80°46′E	5	81	53	0.25	0.30 ± 0.01
Samuel SL-P	6°47′N 79°55′E	4	53	34	0.19	0.28 ± 0.01
Samuel SL-RBG	7°16′N 80°36′E	3	57	36	0.24	0.30 ± 0.01
P. foliosa (Costa Rica)						
Bogarín 4146	9°48′N 83°50′W	1	–	–	–	–
Bogarín 4167	9°48′N 83°42′W	1	–	–	–	–
Bogarín 4199	9°49′N 83°33′W	13	108	69	0.23	0.27 ± 0.01
Bogarín 4200	9°49′N 83°32′W	2	36	23	–	–
Bogarín 4218	9°50′N 83°53′W	10	90	57	0.19	0.24 ± 0.01
Bogarín 4821	9°47′N 83°46′W	1	–	–	–	–
Bogarín 5036	9°58′N 84°38′W	4	73	47	0.26	0.26 ± 0.01
Bogarín 5066	10°20′N 84°0′W	11	86	56	0.20	0.25 ± 0.01
Pupulin 6097	10°45′N 85°5′W	1	–	–	–	–
Pupulin & Castelfranco s.n.	9°31′N 84°5′W	1	–	–	–	–
Russell 116	9°53′N 83°39′W	7	94	59	0.28	0.30 ± 0.01
Russell 119	10°3′N 83°37′W	2	17	11	–	–
Russell 120	9°50′N 83°51′W	12	79	50	0.17	0.23 ± 0.01
Russell 121	8°42′N 83°12′W	8	83	53	0.21	0.25 ± 0.01
Russell 122	8°38′N 83°11′W	20	64	41	0.14	0.20 ± 0.01
P. masayensis (Costa Rica)						
Bogarín 4168	9°48′N 83°42′W	10	71	45	0.17	0.26 ± 0.01
Bogarín 4254	10°3′N 83°37′W	2	44	28	–	–
Bogarín 4255	9°53′N 83°39′W	17	108	69	0.22	0.30 ± 0.01
Karremans 2224	9°52′N 83°48′W	1	–	–	–	–
Russell 111	9°48′N 83°50′W	1	–	–	–	–

BigDye Terminator kit, 1.0 μL of sequencing primer at 3.2 μM and 8.0 μL of PCR product cleaned using 1 unit of calf intestinal alkaline phosphatase (Fermentas) and 10 units of exonuclease I (Fermentas) (Werle *et al*., 1994). The thermocycling programme included 30 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Sequencing was performed on a 48-capillary sequencer, Applied Biosystems (ABI) 3730 DNA Analyser, following the manufacturer's protocols.

Sequences were assembled with LaserGene 7.1 SeqMan (DNASTAR Inc.), and alignments of the cloned sequences from each accession were made. As described in Russell *et al*. (2010a), chimeric sequences were identified by eye and excluded, and the two putatively parental sequence types from the *P. foliosa* and *P. masayensis* accessions were identified and aligned with pre-existing DNA sequences (Russell *et al*., 2010a). Nonalignable and gap-rich (> 50% missing data) regions were excluded from the analysis. The matrix contained 1984 included characters, 272 of which were potentially parsimony informative. Parsimony analysis on the combined *PhyC/Rpb2* matrix was performed in PAUP* (Swofford, 2003) following a two-stage heuristic search strategy (1000 replicates, saving the ten shortest trees per replicate, followed by branch swapping on the resulting saved trees) with tree bisection–reconnection (TBR) branch swapping and MaxTrees set to 10 000. Bootstrap percentages (BPs) were calculated using 1000 random resampling replicates, saving ten trees per replicate.

AFLP data were collected using a standard laboratory protocol (Vos *et al*., 1995), with *Eco*RI and *Mse*I restriction enzymes and ligation to adapters. Sixteen primer combinations were tested on a few samples, and the three that gave the best signal distribution and clearest traces were then applied to the remainder of the samples (EcoRI-ACT/MseI-CTA,

EcoRI-ACC/MseI-CTA, EcoRI-AGG/MseI-CAG). Preselective and selective PCRs were performed consecutively, and the amplified fragments were run on a 48-capillary sequencer. Fragments were size-calibrated and scored using GeneMarker software (SoftGenetics). Some samples were duplicated between runs as internal controls, and these duplicated samples were used to create a panel of reproducible markers that could then be applied to the remainder of the samples using automatic scoring with manual checking. The final matrix contained 158 polymorphic markers between 100 and 450 bp in size, coded as present or absent for 161 accessions.

Network analysis was performed on the entire dataset using Splitstree (Huson & Bryant, 2006) employing standard Nei–Li distances. Standard genetic diversity indices for AFLP data were calculated for the Costa Rican and Sri Lankan population samples. Nei's measure of average gene diversity per locus H_S (Nei, 1973), which is identical to the measure of average differences within populations (ADW; McCain, Groth & Roelfs, 1992), was calculated in AFLPdat (Ehrich, 2006) using the formula $H_S = n/(n-1)\{1 - [\text{freq}(1)^2 + \text{freq}(0)^2]\}$ averaged across all markers. Analysis of molecular variance (AMOVA) (Excoffier, Smouse & Quattro, 1992) was performed in FAMD v1.2 (Schlüter & Harris, 2006) based on standard Jaccard similarities; this generates the measure Φ_{ST}, the proportion of genotypic variance resulting from among-population differences. The three species for which accessions were grouped at the population level were subjected separately to two-level AMOVA. The Costa Rican populations could be grouped by species and populations, and so were also subjected to three-level AMOVA. We also used Hickory v1.1 (Holsinger, Lewis & Day, 2002) as an alternative approach to calculate h_s and $\theta^{(II)}$, measures of within-population genetic diversity and among-population genetic variation. Hickory uses a Bayesian method to infer numerous population genetic statistics from dominant data, without assuming that the populations are in Hardy–Weinberg equilibrium.

We also used Structure 2.2 (Pritchard, Stephens & Donnelly, 2000) to analyse the genetic make-up of Costa Rican individuals and populations from both *P. foliosa* and *P. masayensis*. The data were entered as tetraploid dominant genotypes, and Bayesian analysis was performed applying an admixture model, a burn-in of 10 000 generations and a subsequent run length of 100 000 generations, testing values of k (assumed number of genetic populations) between 1 and 16 with three replicates per k value. R-script Structure-2.2-sum (Ehrich, 2008) was used to determine the most appropriate value of k for the data, as the modal value of the ΔK parameter.

RESULTS

Parsimony analysis of the combined *PhyC*/*Rpb2* DNA data found the maximum 10 000 shortest trees, with a length of 801 steps, consistency index (CI) of 0.79 and retention index (RI) of 0.89. The strict consensus is shown in Figure 2. Tetraploid accessions had two copies of both genes and, as a result, appear in two clades; these have been redrawn on the tree as reticulations. Both clades arose from a parent sister to *P. odorata* Lindl. and/or *P. modesta* Rchb.f., but the clade containing Indian Ocean island *P. concreta* and *P. bicolor* accessions had another parent sister to a *P. golungensis* Rchb.f./*P. pinicola* Barb.Rodr. clade, whereas the clade comprising Neotropical *P. concreta*, *P. estrellensis*, *P. foliosa* and *P. masayensis* had a parent sister to *P. pinicola*. The two Costa Rican species belong to an allotetraploid clade including other Neotropical *P. foliosa* and *P. concreta* accessions. The accessions of *P. concreta* from Sri Lanka belong to another allotetraploid clade comprising Palaeotropical members of the group.

A splits graph of the complete data matrix is presented in Figure 3. Our sample of *P. concreta* from Cameroon groups with *P. estrellensis* from Brazil and *P. foliosa* from Dominica. Single specimens of *P. concreta* from Madagascar, Indian Ocean islands and Laos cluster together, as do the Sri Lankan *P. concreta* accessions. From the Costa Rican populations that make up the main focus of this study, *P. masayensis* accessions cluster together, and some *P. foliosa* accessions also cluster with *P. masayensis* instead of the main *P. foliosa* group. Single accessions of *P. concreta* and *P. foliosa* from Venezuela appear to be relatively isolated genetically, although one of them groups with two Costa Rican *P. foliosa* accessions.

Collection numbers for Sri Lankan and Costa Rican populations are presented in Table 1, with co-ordinates of collecting localities, AFLP marker polymorphism and two estimates of Nei's gene diversity index H_S/h_s. The AFLPdat figure (H_S) is obtained directly from fragment presence and absence; the Hickory figure (h_s) is inferred from Bayesian analysis, taking into account the fact that the matrix comprises dominant data and without assuming Hardy–Weinberg equilibrium. It is an average of estimates sampled from a Monte Carlo Markov chain, with standard deviation. All populations except *Bogarín 5036* have h_s higher than H_S, but estimates are correlated. H_S varies from 0.14 (*Russell 122*; *P. foliosa*) to 0.28 (*Russell 116*; *P. foliosa*), whereas h_s varies from 0.20 (*Russell 122*; *P. foliosa*) to 0.30 (several populations from all three species).

The results from AMOVA of the Costa Rican and Sri Lankan accessions are presented in Table 2 with cor-

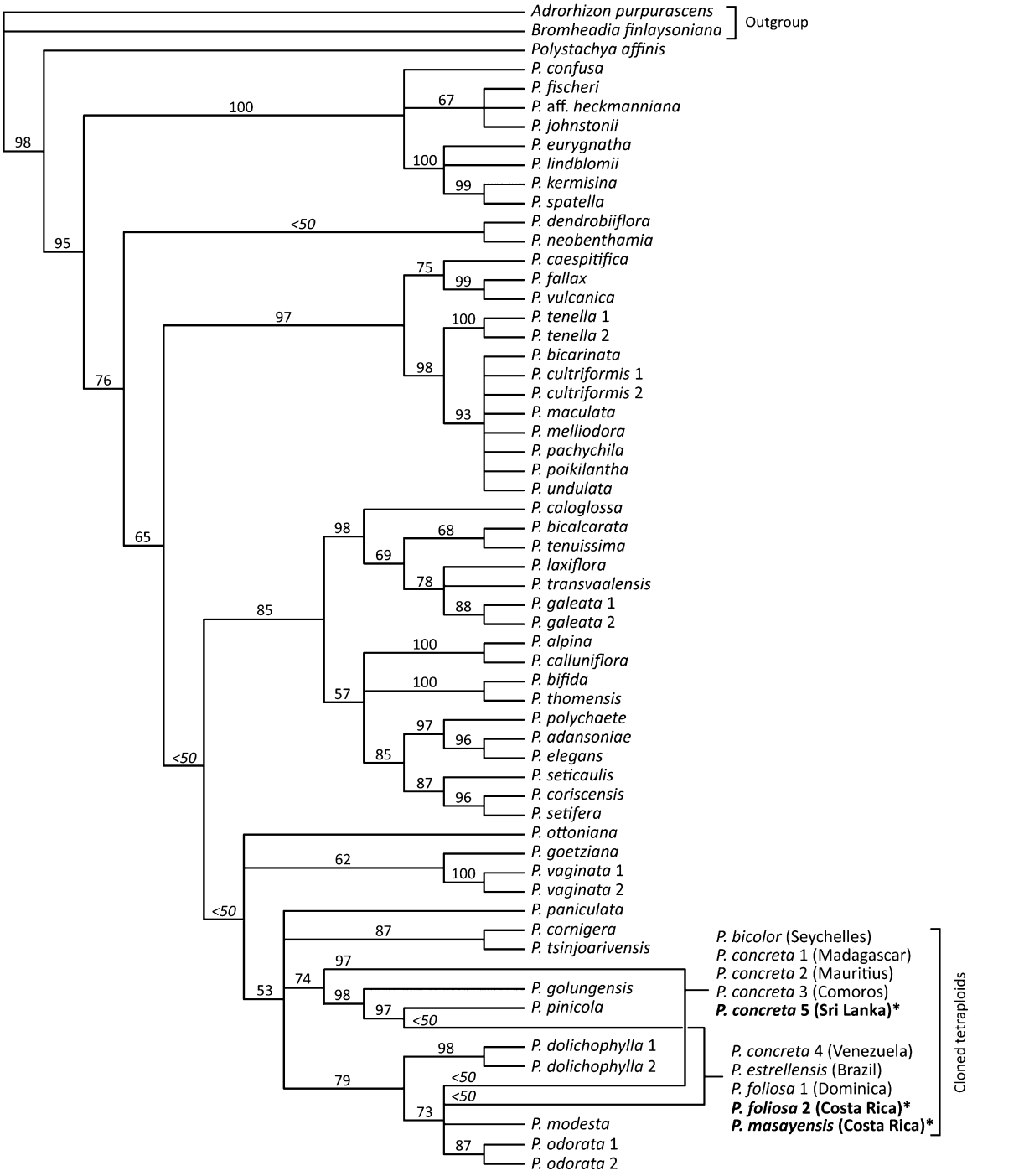

Figure 2. Strict consensus tree from maximum parsimony analysis of a combined *PhyC*/*Rpb2* DNA matrix. Numbers above the branches are bootstrap percentages. Tetraploid sequences, including those from the two Costa Rican species (marked with an asterisk), were obtained by cloning polymerase chain reaction products and aligning two parental sequence types separately in the matrix. As a result, the tetraploid accessions occur twice, with the parental sequences appearing in different clades, and have been redrawn here to show their putative hybrid origins. The remaining species included here are diploids.

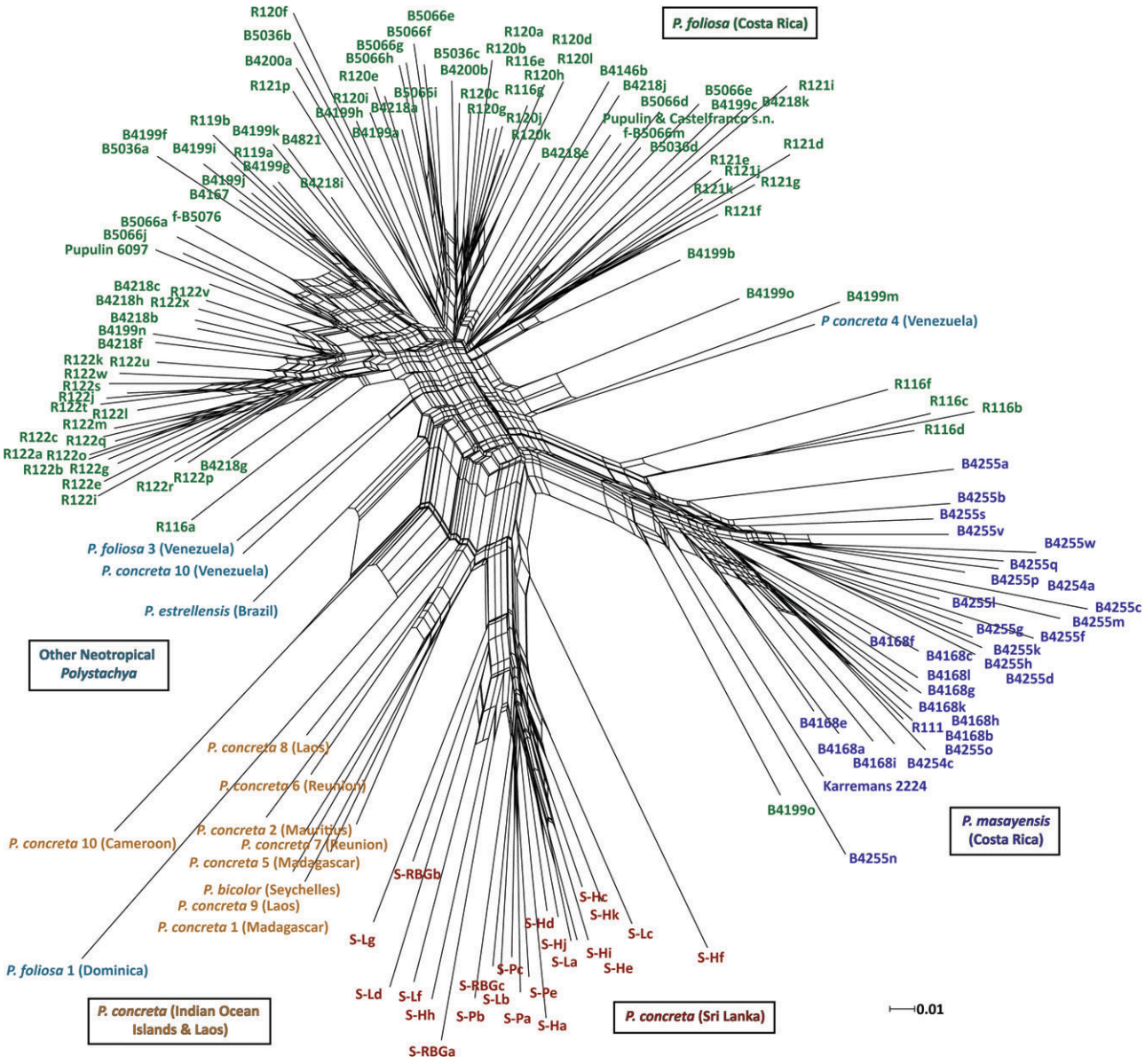

Figure 3. Splits network from neighbour-net analysis of the complete amplified fragment length polymorphism (AFLP) dataset in Splitstree, based on standard Nei–Li distance data. Red, Sri Lankan *Polystachya concreta*; yellow, African and Indian Ocean samples; light blue, Neotropical samples excluding Costa Rica; dark blue, Costa Rican *P. masayensis*; green, Costa Rican *P. foliosa*.

responding Φ_{ST} values representing the proportion of genetic variance attributable to among-population variation. This is highest for *P. foliosa*, at 0.291, and lowest for Sri Lankan *P. concreta*, at 0.138. A separate, three-level AMOVA for the combined Costa Rican data shows that the greatest contribution (48.9%) to variance in the Costa Rican samples at this level is from genetic differences between the two species. An alternative measure of among-population variation is provided by $\theta^{(II)}$ from Hickory v1.1. This, like h_s above, is calculated using a Bayesian algorithm explicitly assuming dominant data. It assumes that the included populations are a random sample of all possible populations, incorporating stochasticity as a source of variation. Again, $\theta^{(II)}$ estimates suggest that *P. foliosa* has the highest proportion of among-population variation, $\theta^{(II)} = 0.20$, compared with 0.10 for *P. masayensis* and 0.13 for Sri Lankan *P. concreta*.

Structure-2.2-sum shows a bimodal distribution of ΔK, with the highest peak at $k = 2$ and a secondary peak at $k = 6$ (Fig. 4). Normally, the modal value indicates where average likelihood values for structure

Table 2. Analysis of molecular variance (AMOVA) using FAMD v1.2 and Bayesian analysis of dominant population genetic data using Hickory v1.1 for Sri Lankan and Costa Rican accessions. Φ_{ST}, as calculated by FAMD, and $\theta^{(II)}$, as calculated by Hickory, are the resulting measures of genetic differentiation among populations

	AMOVA				Hickory
Species	df	Variance components	% variation	Φ_{ST}	$\theta^{(II)}$ ± SD
P. concreta (Sri Lanka)				0.138	0.13 ± 0.02
Among populations	3	0.008	13.8		
Within populations	18	0.052	86.2		
P. foliosa (Costa Rica)				0.291	0.20 ± 0.01
Among populations	14	0.014	29.1		
Within populations	79	0.033	70.9		
P. masayensis (Costa Rica)				0.161	0.10 ± 0.02
Among populations	4	0.007	16.1		
Within populations	26	0.036	83.9		
All Costa Rican populations (*P. foliosa* and *P. masayensis*)				0.626	
Among species	1	0.044	48.9		
Among populations, within species	18	0.012	13.7		
Within populations	105	0.034	37.4		

runs, plotted for increasing values of k, stop increasing sharply as k increases and start to plateau. A bimodal distribution of ΔK for Costa Rican population data with the highest peak at $k = 2$ could be a result of high genetic variability between the two species, with additional genetic structure apparent below the species level, as indicated by the second peak. Therefore, Structure-2.2-sum results are presented in Figure 4 for $k = 2$ and $k = 6$. In both cases, *P. masayensis* populations share the same genetic population with some admixture from the *P. foliosa* genetic group(s), whereas *P. foliosa* shows a similarly small amount of admixture from the *P. masayensis* genetic group, mostly in the population *Russell 116*. The extra genetic populations assumed in the $k = 6$ run are admixed among the *P. foliosa* populations and do not strictly conform to the populations as they occur in the field; however, *Russell 120*, *121* and *122* are three populations with larger numbers of sampled individuals ($n \geq 8$) that also correspond to three genetic groups with little admixture from other groups. From Figure 5, the genetic groups from the $k = 6$ model also do not appear to correlate with the geographical locations of collecting sites; two of the most genetically distinct *P. foliosa* populations occur close together, 7.6 km apart in the south of Costa Rica.

DISCUSSION

DNA sequence analysis of two low-copy nuclear genes (Fig. 2) allowed us to confirm that Costa Rican *P. masayensis* and *P. foliosa* are closely allied species with recent hybrid origins and belong to the same species group as other Neotropical *Polystachya* accessions (Russell *et al*., 2010a, b). Similarly, Sri Lankan *P. concreta* is closely related to other Palaeotropical members of the pantropical tetraploid species group. The additional accessions included in this analysis did not allow greater resolution of the phylogenetic relationships of this group, relative to previous work using plastid and low-copy DNA sequences, because of the low levels of sequence divergence between them.

More detailed phylogenetic information came from AFLP data. On the global scale, accessions from the pantropical *Polystachya* species group cluster geographically and by species (Fig. 3). Sri Lankan *P. concreta* populations show the closest relationships with other Indian Ocean island accessions. Single accessions from Brazil, Venezuela and Dominica appear in the network in isolated positions; they appear to be more closely related to the Costa Rican populations than to other accessions from the Palaeotopics, except for one accession of *P. foliosa* from Dominica and one of *P. estrellensis* from Brazil. Figure 3 shows a degree of genetic separation between *P. foliosa* and *P. masayensis* in Costa Rica, comparable with the separation seen between these two species and accessions from Sri Lanka, Madagascar and the Indian Ocean islands, with the exception of five *P. foliosa* individuals that group close to *P. masayensis*. Although we know from DNA sequence data that the two species are closely related, this suggests that they are fully separated species and, as expected from related species growing in close proximity, produce occasional hybrids.

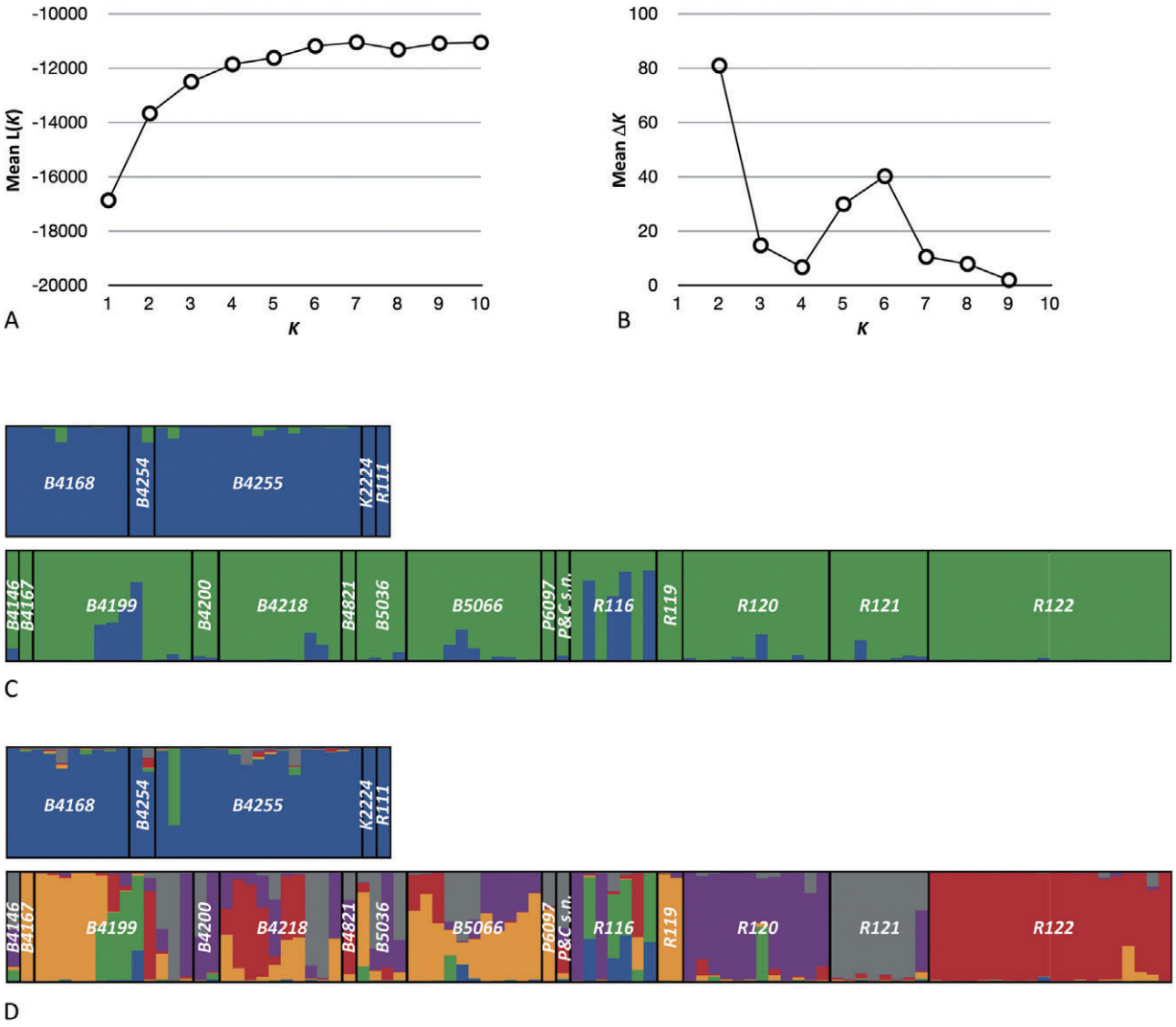

Figure 4. Structure v2.2 analysis of Costa Rica amplified fragment length polymorphism (AFLP) accessions: A, mean log likelihood values of Structure 2.2 runs for different values of k; B, ΔK values as calculated by Structure 2.2-sum; C, results of admixture analysis when $k = 2$, with accessions represented as columns grouped by population; D, results of admixture analysis when $k = 6$. Colours represent different genetic groups.

Indices of population genetic variation also support this result. Three-level AMOVA on the Costa Rican populations (Table 2) shows between-species variance to be higher than both among-population/within-species and within-population variance, indicating a high level of genetic separation between the two species. Two-level AMOVA of the species for which multiple samples were gathered per population (*P. concreta* from Sri Lanka; *P. foliosa* and *P. masayensis* from Costa Rica) indicates that *P. foliosa* has higher among-population variation than the other two species; this is corroborated by a higher $\theta^{(II)}$ value from Bayesian analysis in Hickory. In all three species, within-population variance accounted for most of the variation (70.9–86.2%). The low levels of among-population variation shown here are consistent with other studies of epiphytic orchids (Ackerman & Ward, 1999; Murren, 2003; Trapnell, Hamrick & Nason, 2004; Ávila-Díaz & Oyama, 2007). Epiphytes have generally been found to have low population structure, because of the transient nature of the epiphytic habitat, high capacity for the wind dispersal of seeds (Murren & Ellison, 1998) and high pollinator mobility (Trapnell & Hamrick, 2005). The higher level of among-population variation in *P. foliosa* could be caused by biological differences, such as

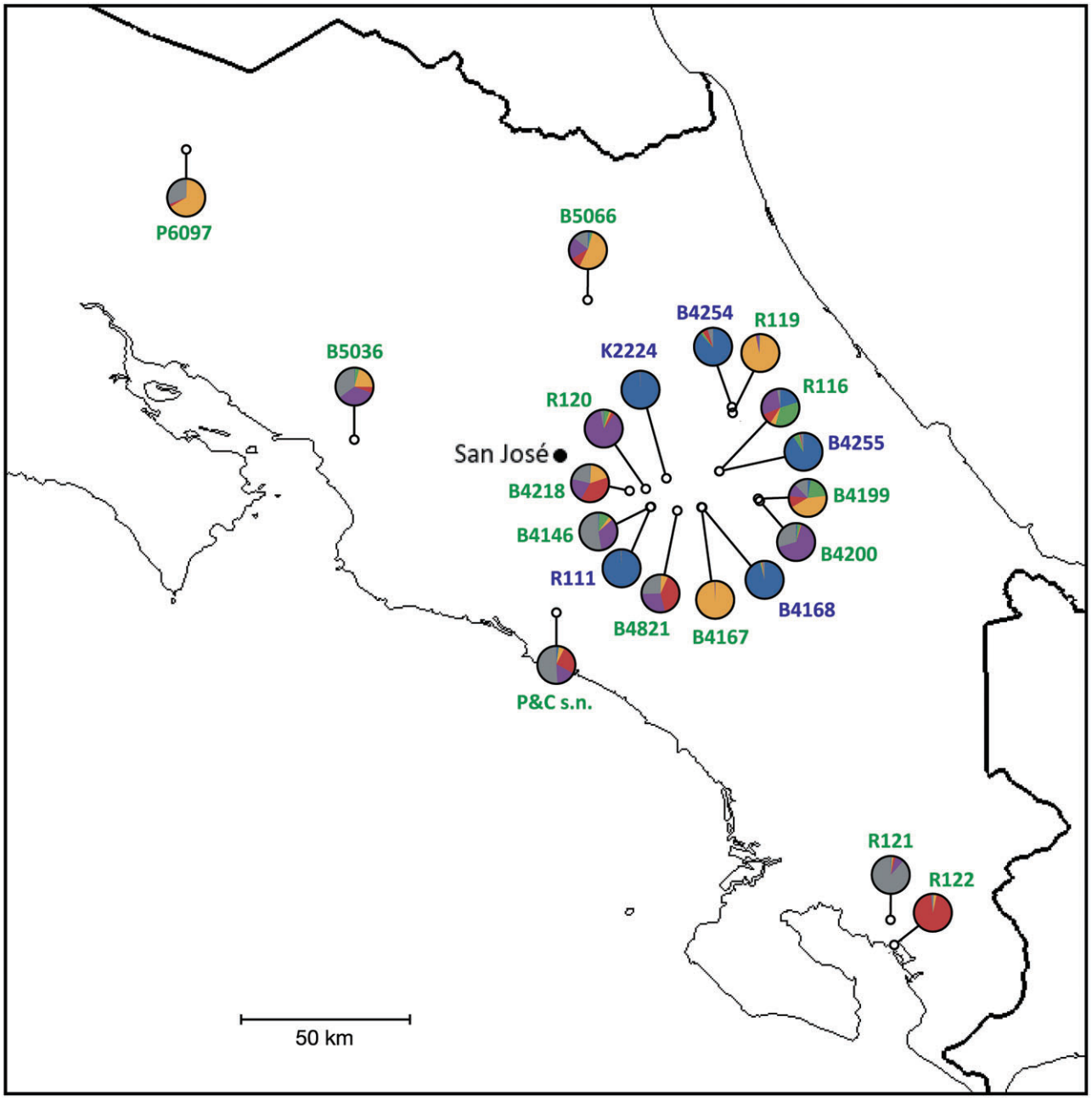

Figure 5. Map of collecting sites in Costa Rica. Small open circles are locations of *Polystachya* populations. Pie charts show genetic make-up of each population from Structure 2.2 analysis ($k = 6$). Blue population names are *P. masayensis* populations; green names are *P. foliosa* populations.

different breeding systems: if there were greater levels of self-pollination or apomixis in *P. masayensis* and Sri Lankan *P. concreta*, this might reduce the measures of population variability relative to *P. foliosa*. Pansarin & Amaral (2006) found that *P. estrellensis* in south-eastern Brazil reproduces primarily by selfing, whereas *P. concreta* in the same part of Brazil is primarily outcrossing with a range of Apidae and Halactidae pollinators. However, there are no consistent differences in within-population genetic diversity (Table 1) between the three species to provide evidence for major differences in breeding system (although some populations have lower diversity indices than others). More populations would need to be collected and field observations of pollination systems made to draw firm conclusions. Other

reasons could be that *P. foliosa* is more widespread and more variable in its habitat preferences than is *P. masayensis*, factors which could contribute to greater population structure in the former (Wallace, 2004; Ávila-Díaz & Oyama, 2007), and population variation in *P. concreta* in Sri Lanka could be reduced because of its occurrence on an island; island population groups would generally be expected to have lower population diversity than mainland equivalents (Frankham, 1997).

From DNA data, *P. foliosa* and *P. masayensis* diverged only recently and grow in the same locations and same habitats: *P. foliosa* is more common and less restricted in its distribution, but all *P. masayensis* populations occur in close proximity to *P. foliosa* populations, except for the single specimen *Karremans 2224*. One might therefore expect introgression to be common between the populations. Structure 2.2 analysis (Figs 4, 5) agreed with neighbour-network analysis (Fig. 3) in finding evidence of admixture in two populations of *P. foliosa* growing at the same locality as *P. masayensis* populations, especially under the $k = 2$ model, but other populations showed little introgression. There was a small amount of admixture between the genetic groups of the two species in all populations, but a tendency for highly admixed individuals to mostly belong to *P. foliosa* populations. The results show a high degree of separation between the two species, and this is also seen in the three-level AMOVA results, discussed above.

This is a comparable situation to *Dactylorhiza* (Orchidaceae) in Europe, another group in which allotetraploid complexes with multiple independent origins are known to occur (Hedrén, 1996; Hedrén *et al*., 2001; Pillon *et al*., 2007). In *Dactylorhiza*, co-occurring species (diploid or tetraploid) tend to inhabit different microhabitats when they co-occur (Ståhlberg, 2009) and, although hybrid zones exist, the parental species retain their genetic identity. In this case, occasional introgression has probably been an important factor allowing rapid colonization of new habitats in northern Europe following the last glacial maximum (Hedrén, 2003).

Applying the $k = 6$ model instead of $k = 2$ to Costa Rican populations served to add more structure to the *P. foliosa* populations only (Fig. 4); the *P. masayensis* populations still showed no genetic structure. This supports the conclusion from AMOVA that *P. foliosa* has higher among-population diversity than does *P. masayensis*. Although most *P. foliosa* populations comprising more than three individuals had a mixed genetic makeup from five of the six genetic populations identified by Structure 2.2, three populations were relatively homogeneous. In some populations, the presence of individuals from apparently different genetic groups could indicate a tendency for suitable habitats to be occupied when they appear by opportunistic individuals from different populations, possibly after seed dispersal from considerable distances (Murren & Ellison, 1998). The presence of multiple population founders would therefore lead to greater within-population genetic diversity compared with populations that have arisen from a single individual, as is more likely to be the case in the three homogeneous populations. The lack of geographical correlation with genetic structure evident from Figure 5 is consistent with a high dispersal capability, and it would be interesting to see at what spatial scales population genetic structure in this species starts to correlate with geographical distance.

Considering the Eastern Hemisphere accessions, the clustering together of Indian Ocean island *P. concreta* accessions (Fig. 3), excluding those from Sri Lanka, may suggest a colonization of Sri Lanka by direct long-distance dispersal rather than by a 'Lemurian Stepping Stones' (Schatz, 1996) route between Africa and Asia. It is clear from DNA sequence data that dispersal out of Africa/Madagascar occurred relatively recently. Considering the large distances between island groups in the Indian Ocean, it probably would have been a stochastic process driven by occasional wind transfer of seed (Arditti & Ghani, 2000). There were probably multiple dispersals to different island groups or to Asia, rather than an identifiable dispersal route between Africa and Asia; a similar situation has occurred in *Exacum* L. (Gentianaceae; Yuan *et al*., 2005). However, greater sampling of Malagasy and African accessions would be needed to confirm specific hypotheses for dispersal routes across the Indian Ocean.

Figure 3 shows that Neotropical accessions of *P. concreta* are genetically closer to other Neotropical species (*P. foliosa*, *P. masayensis*) than to Palaeotropical *P. concreta*. Although broader sampling from the full distribution of this species would be needed to strengthen our conclusions, this study agrees with our findings from nuclear DNA sequences (Fig. 2 and Russell *et al*., 2010a) that Neo- and Palaeotropical tetraploids originated independently from hybrids between different sets of parents. The Garay & Sweet (1974) definition of *P. concreta* incorporated considerable morphological variation between populations in different parts of the world and was too broad. The results from this study strengthen the view that the species should be revisited, and the morphological and genetic diversity clearly present should be taxonomically recognized; correct species definitions have wider benefits for conservation and future biological work.

If, as shown by the present study and Russell *et al*. (2010a), Neo- and Palaeotropical *P. concreta* have separate origins and lack sufficient genetic similarity

Table 3. Suggested application of epithets for members of the pantropical allotetraploid *Polystachya* species group

Present name	Provenance	Suggested name	Type
P. foliosa (Hook.) Rchb.f.	Neotropics	*P. foliosa* (Hook.) Rchb.f.	Guiana. C.S. Parker *s.n.* (K)
P. masayensis Rchb.f.	Neotropics	*P. masayensis* Rchb.f.	Nicaragua. Oersted *s.n.* (W)
P. concreta (Jacq.) Garay & Sweet	Neotropics	*P. concreta* (Jacq.) Garay & Sweet	Martinique. Description in Jacquin, 1760. *Enumeratio systematica plantarum*, p. 30
P. concreta (Jacq.) Garay & Sweet	Palaeotropics	*P. mauritiana* Spreng.	Mascarene Islands. Description in *Systema vegetabilium* **3:** 742 (1826)
P. bicolor Rolfe	Seychelles	*P. bicolor* Rolfe	Seychelles. *Thomasset 58* (K)
P. estrellensis Rchb.f.	Brazil	*P. estrellensis* Rchb.f.	Brazil. *Beyrich s.n.* ii.1823

to be considered conspecific, the name *P. concreta* (Jacq.) Garay & Sweet should be applied to Neotropical accessions (Jacquin's original collections and description were from Martinique; Garay & Sweet, 1974), whereas, in the Palaeotropics, the name *P. mauritiana* Spreng. can be applied as the earliest valid name based on Palaeotropical specimens (Table 3). The taxonomy of other species included in this study would be unaffected. Here, we have used the names *P. estrellensis* Rchb.f and *P. bicolor* Rolfe for some accessions; these are sometimes considered as synonyms of *P. foliosa* Rchb.f., *P. mauritiana* Spreng. and *P. concreta* (Jacq.) Garay & Sweet. The synonym *P. tessellata* Lindl. is often applied to *P. concreta* on mainland Africa. Although the present study confirms the genetic dissimilarity between Neo- and Palaeotropical *Polystachya* tetraploids found by Russell *et al*. (2010a), we would need more widespread sampling to draw broader conclusions about the status of these taxa.

From Figure 3, some single accessions of *P. foliosa* appear to be genetically closer to *P. concreta* accessions than to the main group of Costa Rican *P. foliosa* populations. This could be because they are too genetically dissimilar from other accessions in the study for the neighbour-net algorithm to group them accurately from the AFLP data. However, small differences in flower shape, size and insertion on the rachis are apparently not an indication of shared descent over larger areas, even though they can be used to discern taxa within smaller regions (e.g. between *P. concreta* and *P. estrellensis* in south-eastern Brazil; Pansarin & Amaral, 2006). This is a taxonomically difficult group, reflected in the large number of synonyms and competing species delimitations used historically and currently (Russell, 2007), and substantial taxonomic work will need to be carried out to establish species definitions that can be consistently applied to the entire pantropical tetraploid group.

Further work should involve wider and finer scale sampling from the study areas to enable genetic structure to be correlated with geographical and ecological differences among populations. Additional knowledge of the biology of these plants would be useful in interpreting genetic structure results, for example observations of the breeding systems of the species involved using controlled pollination experiments. A greater number of primer pairs to detect more AFLP markers would also enable more detailed genetic information to be obtained, and might increase the resolution of phylogenetic analyses, including members of this species group from throughout its range.

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APPENDIX

Voucher information and GenBank accession numbers for samples in the phylogenetic analysis and samples from outside Sri Lanka and Costa Rica included in the AFLP study. HBV = Botanical Garden of the Uiversity of Vienna.

Species name	Provenance	Voucher	*PhyC*	*Rpb2*
Adrorhizon purpurascens Hook.f.	Sri Lanka	*Chase 15745* (K)	GU556699	NA
Bromheadia finlaysoniana (Lindl.) Miq.	Brunei	*Duangjai 039* (BRUN, K)	GU556700	NA
Polystachya adansoniae Rchb.f.	Nigeria	*Bytebier 429/94/469* (EA)	GU556701	GU556852
Polystachya affinis Lindl.	Nigeria	*Chase 21165* (K)	GU556702	GU556853
Polystachya alpina Lindl.	Cameroon	*A. Russell 67* (YA)	GU556703	GU556854
Polystachya bicalcarata Kraenzl.	Cameroon	*A. Russell 81* (YA)	GU556704	GU556855
Polystachya bicarinata Rendle	Kenya	*Bytebier 621/95/1226* (EA)	GU556705	GU556856
Polystachya bicolor Rolfe	Seychelles	*A. Russell Kew2003-406* (WU)	GU556760-GU556761	GU556907-GU556908
Polystachya bifida Lindl.	Sao Tome	Kew living collection: 2001-3989	GU556706	GU556857
Polystachya caespitifica Lindl.		*A. Russell ORCH06423* (WU)	GU556707	GU556856
Polystachya calluniflora Kraenzl.	Cameroon	*A. Russell 63* (YA)	GU556708	GU556859
Polystachya caloglossa Rchb.f.	Cameroon	*A. Russell 104* (YA)	GU556709	GU556860
Polystachya concreta (Jacq.) Garay & H.R.Sweet 1	Madagascar	*Chase 17854* (K)	GU556759	GU556905-GU556906
Polystachya concreta (Jacq.) Garay & H.R.Sweet 2	Mauritius	HBV living collection ORCH07278	GU556764-GU556765	GU556913-GU556914
Polystachya concreta (Jacq.) Garay & H.R.Sweet 3	Comoros	HBV living collection ORCH07417 – photo voucher	GU556768-GU556769	GU556915-GU556916
Polystachya concreta (Jacq.) Garay & H.R.Sweet 4	Venezuela	HBV living collection ORCH06361	GU556770-GU556771	GU556917-GU556918
Polystachya concreta (Jacq.) Garay & H.R.Sweet 5	Sri Lanka	*Samuel et al. SW/SL/P4* (PDA)	NA	HQ704885-HQ704886
Polystachya concreta (Jacq.) Garay & H.R.Sweet 6	Reunion	HBV living collection ORCH06417	NA	NA
Polystachya concreta (Jacq.) Garay & H.R.Sweet 7	Reunion	HBV living collection "C&S–Reunion 1"	GU556766-GU556767	GU556914-GU556915
Polystachya concreta (Jacq.) Garay & H.R.Sweet 8	Laos	HBV living collection ORCH07344 – photo voucher	NA	GU556911-GU556912
Polystachya concreta (Jacq.) Garay & H.R.Sweet 9	Laos	*A. Russell ORCH06415* (WU)	NA	GU556919-GU556920
Polystachya concreta (Jacq.) Garay & H.R.Sweet 10	Venezuela	*A. Russell ORCH08068* (WU)	NA	NA
Polystachya confusa Rolfe	Kenya	*Bytebier et al 122* (EA)	GU556710	GU556861
Polystachya coriscensis Rchb.f.		*A. Russell ORCH07314* (WU)	GU556711	GU556862
Polystachya cornigera Schltr.	Madagascar	*Fischer & Sieder FS3208* (WU)	GU556740	HM018532
Polystachya cultriformis (Thouars) Lindl. ex Spreng. 1	Kenya	*Mugambi & Odhiambo 054/98/1607* (EA)	GU556713	GU556863
Polystachya cultriformis (Thouars) Lindl. ex Spreng. 2	Madagascar	*Fischer & Sieder FS1045* (WU)	GU556714	GU556864
Polystachya dendrobiiflora Rchb.f.		*Mugambi & Odhiambo 064/98/1622* (EA)	GU556715	NA
Polystachya dolichophylla Schltr. 1	Cameroon	*Chase 25886* (K)	GU556716	GU556865
Polystachya dolichophylla Schltr. 2		HBV living collection ORCH03072 – photo voucher	GU556712	GU556866
Polystachya elegans Rchb.f.	Cameroon	*A. Russell 74* (YA)	GU556718	GU556867
Polystachya estrellensis Rchb.f.	Brazil	*A. Russell ORCH06604* (WU)	GU556762-GU556763	GU556909-GU556910
Polystachya eurygnatha Summerh.		Photo voucher—contact author	GU556719	GU556868
Polystachya fallax Kraenzl.	Uganda	*Chase 17922* (K)	GU556720	GU556869

APPENDIX *Continued*

Species name	Provenance	Voucher	*PhyC*	*Rpb2*
Polystachya fischeri Rchb.f. ex Kraenzl.	Kenya	*Pearce 616/94/607* (EA)	GU556721	GU556870
Polystachya foliosa (Hook.) Rchb.f. 1	Dominica	Kew living collection 2001-3986	GU556772-GU556773	GU556921-GU556922
Polystachya foliosa (Hook.) Rchb.f. 2	Costa Rica	*F. Pupulin & D. Castelfranco s.n.* (JBL)	HQ704879-HQ704880	HQ704885-HQ704886
Polystachya foliosa (Hook.) Rchb.f. 3	Venezuela	HBV living collection ORCH07028	NA	NA
Polystachya galeata (Sw.) Rchb.f. 1		*Chase O-1496* (K)	GU556722	GU556871
Polystachya galeata (Sw.) Rchb.f. 2		*C283* – spirit (K)	GU556723	GU556872
Polystachya goetziana Kraenzl.	Kenya	*Bytebier 1772* (EA)	GU556724	GU556873
Polystachya golungensis Rchb.f.		*A. Russell ORCH05170* (WU)	GU556725	GU556874
Polystachya aff. *heckmanniana* Kraenzl.	Malawi	Photo voucher—contact author	GU556726	GU556875
Polystachya johnstonii Rolfe		HBV living collection ORCH06241 – photo voucher	GU556727	GU556876
Polystachya kermisina Kraenzl.	Rwanda	HBV living collection ORCH07240 – photo voucher	GU556728	GU556877
Polystachya laxiflora Lindl.		*A. Russell ORCH07315* (WU)	GU556729	GU556878
Polystachya lindblomii Schltr.	Kenya	*Bytebier 1142/98/1695* (EA)	GU556730	GU556879
Polystachya maculata P.J.Cribb	Burundi	photo voucher—contact author	GU556731	GU556880
Polystachya masayensis Rchb.f.	Costa Rica	*Karremans 906* (JBL)	HQ704881	HQ704884
Polystachya melliodora P.J.Cribb	Tanzania	*Chase 17923* (K)	GU556732	GU556881
Polystachya modesta Rchb.f.		HBV living collection ORCH05165	GU556733	GU556882
Polystachya neobenthamia Schltr.		HBV living collection ORCH07214 – photo voucher	GU556734	GU556883
Polystachya odorata Lindl. 1	Nigeria	*Chase 17857* (K)	GU556735	GU556884
Polystachya odorata Lindl. 2	Cameroon	*A. Russell 42* (YA)	GU556736	GU556885
Polystachya ottoniana Rchb.f.		Kew living collection 2005-964	GU556737	GU556886
Polystachya pachychila Summerh.		*A. Russell ORCH07310* (WU)	GU556738	GU556887
Polystachya paniculata (Sw.) Rolfe	Ethiopia	Kew living collection 1984-4977	GU556739	GU556888
Polystachya pinicola Barb.Rodr.	Brazil	*A. Russell ORCH06603* (WU)	GU556717	GU556889
Polystachya poikilantha Kraenzl.	Kenya	*Bytebier 956/97/524* (EA)	GU556741	GU556890
Polystachya polychaete Kraenzl.	"Congo"	Kew living collection 2001-3987	GU556742	GU556891
Polystachya seticaulis Rendle	"Congo"	*Chase 17924* (K)	GU556743	GU556892
Polystachya setifera Lindl.		*Chase O-1493* (K)	GU556744	GU556893
Polystachya spatella Kraenzl.	Kenya	*Bytebier 949* (EA)	GU556745	GU556894
Polystachya tenella Summerh. 1	Kenya	*Bytebier 955/97/1523* (EA)	GU556746	GU556895
Polystachya tenella Summerh. 2	Kenya	*Bytebier 955/97/1524* (EA)	GU556747	GU556896
Polystachya tenuissima Kraenzl.	Kenya	*Bytebier 428/94/468* (EA)	NA	GU556897
Polystachya thomensis Summerh.	Sao Tome	*Chase 17858* (K)	GU556748	GU556898
Polystachya transvaalensis Schltr.	Kenya	*Bytebier 951/97/1519* (EA)	GU556749	GU556899
Polystachya tsinjoarivensis H.Perrier	Madagascar	HBV living collection FS4182 – photo voucher	GU556750	HM018542
Polystachya undulata P.J.Cribb & Podz.		*Chase 17862* (K)	GU556751	GU556900
Polystachya vaginata Summerh. 1	Kenya	*Bytebier 566/95/1140* (EA)	GU556752	GU556901
Polystachya vaginata Summerh. 2	Kenya	*Bytebier 452/97/1587* (EA)	GU556753	GU556902
Polystachya vulcanica Kraenzl.	Kenya	*Bytebier 954/97/1522* (EA)	GU556754	GU556903