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THE USE OF NEXT GENERATION SEQUENCING TO SOLVE PHYLOGENETIC INCONGRUENCES IN ORCHIDACEAE JUSS.

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ABSTRACT

Background. Current evidence suggests that for more robust estimates of species tree and divergence times, several unlinked genes are required. However, most phylogenetic trees for non-model organisms are based on single sequences or just a few regions, using traditional sequencing methods. Techniques for massive parallel sequencing or Next Generation Sequencing are an alternative to traditional methods that allow access to hundreds of DNA regions. Here we use this approach to resolve the phylogenetic incongruence found in *Polystachya* Hook. (Orchidaceae), a genus that stands out due to several interesting aspects, including cytological (polyploid and diploid species), evolutionary (reticulate evolution) and biogeographical (species widely distributed in the tropics and high endemism in Brazil). The genus has a notoriously complicated taxonomy, with several sections that are widely used but probably not monophyletic.

Methods. We generated the complete chloroplast genome of 48 individuals from one clade within the genus. The method consisted in construction of genomic libraries, hybridisation to RNA probes designed from available sequences of a related species, and subsequent sequencing of the product. We also tested how well a smaller sample of the chloroplast genome would perform in phylogenetic inference in two ways: by duplicating a fast region and analysing multiple copies of this dataset, and by sampling without replacement from all non-coding regions in our alignment. We further examined the phylogenetic implications of non-coding sequences that appear to have undergone hairpin inversions (reverse complemented sequences associated with small loops).

Results. We retrieved 131,214 bp, including coding and non-coding regions of the chloroplast genome. The phylogeny was able to fully resolve the relationships among all species in the targeted clade with high support values. The first divergent species are represented by African accessions and the most recent ones are among Neotropical species.

Discussion. Our results indicate that using the entire chloroplast is a better option than screening highly variable markers, especially when the expected tree is likely to contain many

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short branches. The phylogeny inferred is consistent with the proposed origin of the genus, showing a probable origin in Africa, with later dispersal into the Neotropics, as evidenced by a clade containing all Neotropical individuals. The multiple positions of *Polystachya concreta* (Jacq.) Garay & Sweet in the phylogeny are explained by allotetraploidy. *Polystachya estrellensis* Rchb.f. can be considered a genetically distinct species from *P. concreta* and *P. foliosa* (Lindl.) Rchb.f., but the delimitation of *P. concreta* remains uncertain. Our study shows that next generation sequencing provides a powerful tool for inferring relationships at low taxonomic levels, even in taxonomically challenging groups with short branches and intricate morphology.

KEYWORDS: Orchids; next-generation sequencing; phylogenetics; hybridisation; complete genome; chloroplast; *Polystachya*.

INTRODUCTION

Orchidaceae is considered the largest family of Flowering Plants, with over 25,000 species (Dressler, 1990). The family probably dates back to the Late Cretaceous, as indicated by fossil-calibrated molecular phylogenies (Gustafsson, Verola & Antonelli, 2010; Ramirez et al., 2007, 2011). *Polystachya* Hook. is an orchid genus containing 240 species, with most species found in Africa (Dressler, 1993). Thirteen species are reported from the Neotropical region (Mytnik-Ejsmont, 2011), but this number may increase when considering the endemic species from Brazil that were not accounted for in Mytnik-Ejsmont (2011) or were considered synonymous (Barros et al., 2010).

Recent studies have shown a number of peculiar cytological, evolutionary and biogeographic aspects of *Polystachya*. The genus has diploid and polyploid species, the latter recently formed in the Neotropics and Madagascar (Rupp et al., 2010; Russell et al., 2010b). Unlike most genera of Orchidaceae, *Polystachya* has a wide geographical distribution range (Pridgeon et al., 2005; Fig. 1), having species that are Pantropical or have a transatlantic distribution. On the other hand, the Neotropics presents a high level of endemism. Brazil, as an example, has 12 species of which 10 are endemic (Barros et al., 2010). In addition, there is evidence of reticulate evolution in the genus and hybridisation with independent origins (Russell et al., 2010a).

The monophyly of the genus has been reported in the latest studies (Russell et al., 2010b; Mytnik-Ejsmont, 2011), which contrasts starkly with the low level of monophyly observed in the taxonomic sections described within the genus. Those 15 sections (Kraenzlin, 1926; Summerhayes, 1942, 1947 *apud* Russell et al., 2011b; Brenan, 1954; Cribb, 1978) are based on morphological characters and have been useful for field identification and inventories, but do not find support as natural groupings in the molecular studies currently available (Russell et al., 2010b; Mytnik-Ejsmont, 2011). According to those molecular studies, all sections are polyphyletic or paraphyletic, except Isochiloides (Russell et al., 2010b).

Section Polystachya has been described as comprising 32 species worldwide and is the only section with species of pantropical distribution (Mytnik-Ejsmont, 2011). However, according to molecular analyses, some species of this section appear to be more related to species of other sections (Russell et al., 2010b; Mytnik-Ejsmont, 2011). These studies highlight the need for new infrageneric divisions based on robust molecular evidence. Russell et al. (2010b), using chloroplast markers, defined five different clades that could be used as the basis for a revised classification of new sections within the genus. Clade III (sensu Russel et al., 2010b) includes species from five different sections (Polystachya, Eurychilae, Caulescentes, Superpositae, Polychaete) and is divided into distinct subclades of morphologically diverse plants. These species are Pantropical (such as *P. concreta*), Neotropical (such as *P. foliosa*), Malagasy endemics (such as *P. henrici*) or African (such as *P. odorata*). The relationships among Clade III species remain unresolved because specimens of *P. concreta*, *P. foliosa*, *P. henrici*, and *P. modesta* form a large polytomy, due to low levels of divergence between sequences. In addition, several distinct subclades are unresolved at the base. The molecular phylogenetic studies produced so far have included about 35% of the recognized species within the genus, and used only a small number of nuclear (PgiC between exons 11 and 15, PhyC exon 1, Rpb2 intron 23 and ITS in Russell et al., 2010a; only ITS in Mytnik-Ejsmont, 2011) and chloroplast markers (rps16 intron, rps16 exon 2, rps16-trnK spacer, trnK intron excluding matK, matK and psbD-trnT spacer in Russell et al., 2010b; rps16-trnK, rps16 and rpl32-trnL in Mytnik-Ejsmont, 2011).

New methods of DNA sequencing as well as the development of more powerful algorithms are propelling the replacement of trees generated from one or a few genes to those constructed from hundreds of them (Edwards, 2009). The improvement of massively parallel

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sequencing techniques – or Next Generation Sequencing (NGS) – has increased the amount of data available for biological research, whether the fully annotated reference genomes of species under study have been sequenced or not (Bräutigam & Gowik, 2010). However, despite its obvious potential, NGS technology is underused in most studies of plant systematics (Cronn et al., 2012; Carstens et al., 2013; Eaton & Ree, 2013), probably as a result of a prevailing focus on non-model organisms (which require *de novo* genomic sequencing and its inherent challenges), the need to sample many individuals per species and the absence of well-established protocols (McCormack, 2013). One method that increases the efficiency of NGS for non-model species compared to other genomic partitioning strategies is sequence capture (or hybridisation-based enrichment), which is based on the prior selection of loci of interest (Lemmon & Lemmon, 2013). The main benefit of this technique is that the number of specific sequences obtained can be very high, which makes it an advantageous method compared to PCR-based approaches if the objective is to sequence several individuals and multiple loci. Furthermore, sequence capture when combined with NGS platforms, such as Illumina, also reduces the costs of the process (Lemmon, Emme & Lemmon, 2012).

The necessity of a molecular phylogenetic framework for (and a morphological taxonomic revision of) *Polystachya* is clear. It requires a well-resolved phylogenetic hypothesis in order to clarify the relationships between species and also to redefine new infrageneric sections. In this paper, we explore the use of complete chloroplast genomes, obtained by sequence capture and massively parallel sequencing, to solve the phylogenetic inconsistencies found within Clade III of *Polystachya* (sensu Russell et al., 2010b). We also explore whether sequencing the entire chloroplast using NGS was worthwhile, compared to PCR and Sanger sequencing of a few fast-evolving loci. We hope that the results generated here can be extended to the rest of the genus and thus result in new interpretations of the evolutionary and biogeographic history of the group.

MATERIALS & METHODS

Sampling and DNA Extraction

We sampled 19 species and 48 individuals (Table 1, Fig. 1), of which 15 were collected in different locations in continental Brazil and three were collected on Trindade Island in the South Atlantic. The DNA of the 15 Brazilian samples was extracted from 10 mg

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of tissue dried with silica gel and using the DNeasy Plant Mini Kit (Qiagen). DNA samples for the remaining 33 individuals were provided by the University of Vienna and the DNA bank of the Royal Botanic Gardens, Kew. To this sample of individuals, selected because they cluster in Clade III (as defined in Russell et al., 2010b), we added *Polystachya tessellata* Lindl., supposedly synonymous of *P. concreta*. We also included multiple samples of *P. concreta*, because previous studies have reported a lack of monophyly for this species and several synonymous species. Permits to collect were provided by the Ministério do Meio Ambiente (MMA), Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) and Sistema de Autorização e Informação em Biodiversidade (SISBIO), with registration number 29478-1.

Polystachya bicolor Rolfe and *P. melanantha* Schltr. were chosen as outgroups. All studies conducted to date resolve *P. melanantha* as an outgroup with respect to the Clade III species. *Polystachya bicolor* has already been treated as a synonym of *P. rosea* (Mytnik-Ejsmont, 2011), with an uncertain position in the phylogenetic trees generated thus far, being sometimes closely related to *P. concreta* and other associated species (Mytnik-Ejsmont, 2011) and sometimes closely related to species from other clades (Russell et al., 2010b; Mytnik-Ejsmont, 2011).

Probe Design for DNA Capture

We used complete chloroplast genome of *Phalaenopsis aphrodite* subsp. *formosana* (NC_007499.1) (Chang et al., 2006) as the reference for the design of capture probes, because there is no completely sequenced chloroplast genome of a *Polystachya*. According to molecular analyses, *Polystachya* and *Phalaenopsis* belong to different sub-tribes but are closely related within the Vandeae tribe (Van Den Berg et al., 2005; Górniak, Paun & Chase, 2010; Freudenstein & Chase, 2015). The use of a quite distantly related species is possible by the DNA capture kit (MyBaits), which is able to support differences larger than 5% between probe sequences and target sequences (e.g., Li et al., 2013). The complete sequence of the chloroplast genome of *Phalaenopsis aphrodite* subsp. *formosana* was divided into blocks with 360 bp (reference blocks) and every other block was skipped. Additionally, fragments with a base repeated more than seven times on a row were avoided. Finally, the reference blocks

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totalled 63,720 bp and were brought together into a single FASTA file and sent to MYcroarray (Ann Arbor, Michigan, USA) to produce the probes.

Data Generation

Sonication and genomic library preparation

Extracted DNA was randomly fragmented by sonication using a Covaris S220 instrument (Covaris, Woburn, Massachusetts, USA), in order to evenly cover the full genome. Adapters were incorporated into the fragmented DNA using NEXTflexTM DNA Sequencing Kit and NEXflexTM Barcodes kit (BIOO Scientific, Austin, Texas, USA). Uniquely indexed adapters were used for each sample. We selected fragments between 300 and 400 bp using AgencourtAMPure XP magnetic beads kit (Beckman Coulter). The genomic library was amplified following the program: 98ºC for 2 minutes; 14 cycles (98ºC for 30 seconds; 65ºC for 30 seconds; 72ºC for 60 seconds); 72ºC for 4 minutes. The products were purified using a QIAquick PCR Purification Kit (Qiagen). The genomic DNA concentrations before and after sonication and the amplification of the library were measured in a NanoDrop 2000c instrument (Thermo Fisher Scientific, Waltham, MA, USA) (Table 1) to ensure that the final concentration exceeded 400ng/ µL.

Enrichment and sequencing

Before the enrichment, equimolar amounts (400ng/µL) of each amplified library were pooled into six reactions, each one containing eight indexed samples. The enrichment method involves the selection of genomic regions and capture of DNA samples before sequencing (Mamanova et al., 2010). The enrichment was performed with MYBaits target enrichment system (MYcroarray, Ann Arbor, Michigan), following the manufacturer's instructions. The probes were recovered using Dynabeads® MyOneTM Streptavidin C1 (Invitrogen Dynal AS, Oslo, Norway).

To increase DNA concentration, 14 cycles of PCR were performed for each hybridisation reaction using Herculase II Fusion DNA Polymerase (Agilent, Waldbronn, Germany) and the following program: 98ºC for 30 seconds; 14 cycles (98ºC for 20 seconds; 60ºC for 30 seconds; 72ºC for 60 seconds), 72ºC for 5 minutes. Sequencing was performed on

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the Illumina MiSeq platform (San Diego, California, USA) by the Genomics Core Facility (University of Gothenburg, Sweden).

Sequence editing

Illumina reads were processed using tools in the CLC assembly cell (CLC Bio, Aarhus, Denmark). Firstly, the Illumina adapter sequences were removed and low-quality sequences were excluded. Then the reads were mapped onto the reference sequence used for probe design and converted in FASTA format. Consensus sequences out of the mapped reads were obtained using the SAMTools software. Alignment of the species sequences was performed using the auto strategy in MAFFT - Multiple Sequence Alignment Software Version 7 (Katoh & Standley, 2013) and later manually refined using Geneious Pro (Biomatters). In the last step we aligned the sequenced samples with the *Phalaenopsis aphrodite* subsp. *formosana* (NC_007499.1) chloroplast genome to obtain the sequenced region annotation.

Alignment and Phylogenetic Analysis

Hairpin inversions

Micro-structural features of chloroplast non-coding sequences can have a profound influence on the multiple sequence alignment, and hence also the phylogeny. Hairpins (short stem-loop structures in single stranded DNA or RNA), for example, can create sites that allow small inversions to occur at a high enough frequency that homoplasious inversions can be observed among sequences from closely related species (Kelchner & Wendel, 1996). Sometimes the inverted sequence is not so short and can disrupt phylogenetic analysis, leading to strongly supported but spurious groupings (Joly et al., 2010). Non-coding sequences, such as group II chloroplast introns, contain many such stem-loop structures (Kelchner, 2002).

We examined the non-coding sequences in our alignment for inverted (reverse complemented) sequences and tested for their effect on the phylogenetic inference. This was done by excluding all but one character of the inversion (to down-weight the inversion to a single event) and rerunning the analysis. The selected character to represent the inversion was arbitrarily chosen. This was done to avoid recoding the inversions as indel characters and

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creating a new, small partition (with only eight characters) that would have required many additional parameters, in comparison to our approach.

Site exclusions

The alignment process can sometimes be confronted with small regions that are difficult to align, probably most often due to overlapping indel events. We identified several such regions and excluded them using the nexus block commands from the Bayesian analysis. Poor alignment excluded sites: 54459-54465, 55220-55224, 55484-55489, 67162-67167, 67426-67431, 68186-68192, 118838-118843, 119266-119364, 119493-119726, 120012-120019, 120867-120872, 121295-121393, 121522-121755, 122041-122048. Inverted loop-associated excluded sites: 74668-74669, 88120-88128, 90474-90481, 94193-94205, 94696-94699, 97241-97245, 104712-104716, 106631-106635.

The aligned sequences were partitioned based on the chloroplast annotation of 116 functional genes, seven pseudogenes and two partitions that concatenated untranslated regions – one partition containing introns with secondary structure and the other concatenating all intergenic sequences. The files that contained the introns were submitted to trimAl v1.2 (Capella-Gutiérrez, Silla-Martínez & Gabaldón, 2009) to test different gap deletion settings.

Faster region assessment

We used the sequences from one sample on GenBank (FS1045) of *Polystachya cultriformis* and examined two published markers, psbD-trnT (870 length aligned to our samples) and matK (1521 length aligned to our samples). We compared these sequences pairwise to one of our samples, *P. estrelensis*8, to check which was the faster-evolving region. We then ran a MrBayes v.3.2.4 (Ronqvist et al., 2012) analysis on the faster region, as a representative of a fast part of the chloroplast genome (fast cpDNA hereafter), with one copy of our dataset trimmed to this region alone plus the GenBank sequence. We then ran successive analyses, using additional copies of the dataset interleaved in the same file, to explore the increase in support with an increase of characters evolving under the same model. This was to discover how much of the fast cpDNA data would be needed to achieve high support on most nodes in the phylogeny (i.e., among species but not necessarily within species).

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Random sample from all non-coding regions

A single region copied many times proved ineffective in recovering most nodes with support (see Results). We therefore explored using random samples of characters without replacement from among all of the non-coding regions in our dataset to test how much data from faster regions would yield supported trees across most nodes. We expected this approach to be less subject to the limitations caused by the stochastic nature of mutations coupled with the limited size of any one region. By sampling across many regions, even those few characters that have changed on short branches might be sampled occasionally. In contrast, a single region, by chance, may simply not contain any characters changing on a specific short branch.

We sampled without replacement 4%, 8% and 16% of the non-coding data using delete-fraction jackknifing in the seqboot program v3.69 (from http://evolution.genetics.washington.edu/phylip.html), excluding the poorly aligned parts and with down-weighting of the inverted loops (by excluding all but one character of each loop), in 20 replicates each. The approximate average (and range) of PP was taken across the 20 replicates to get an indication of the likely support for clades that a non-coding dataset of these sizes would generate. These values were plotted on the whole alignment analysis to compare to the support received when using the whole dataset. Given that the largest dataset we used here (hereafter the "16% dataset", or ~9.2kb) failed to recover support for all nodes found in the whole genome analysis (see Results), we did not end up analysing the smaller replicates.

Phylogenetic analysis

MrBayes v.3.2.4 analyses were used for phylogenetic inference. These analyses were run for five million generations (two million for the random sample replicates), using a mixed substitution model (plus gamma and invariant sites) to account for among-site rate variation. Priors on branch lengths were set to unconstrained: exponential (100) to minimise the chance of inferring incorrectly long branches (Marshall, 2010), otherwise with default settings. The paired runs were checked for convergence and high effective samples sizes in the MrBayes output and Tracer v.1.6 (Rambaut et al., 2014), respectively. Burn-in generations were removed by discarding 10% of the samples of parameters and trees while summarising in TreeAnnotator v.1.8 (Rambaut and Drummond, 2010) to ascertain clade posterior

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probabilities. Analyses using the character partitions were also done, returning nearly identical results to the analysis described above, so they are not reported further.

RESULTS

Our NGS approach allowed the capture of coding and non-coding regions throughout the chloroplast genome. We recovered approximately 132kb, after the exclusion of gaps, representing 116 genes, seven pseudogenes, as well as regions with intergenic sequences and introns with secondary structure. Compared to the reference annotation, seven genes contained frameshifts that are usually associated with pseudogenisation and corresponded to previously reported pseudogenised genes in orchids (Luo et al., 2014).

We excluded eight of the 48 samples due to the low quality of the sequencing results (Table1). These eight samples showed lower DNA concentrations after the genomic library construction assembly, which may be the cause of low quality sequencing. The remaining 40 samples were submitted to the EMBL/ENA database under accession numbers ERS2203551-ERS2203590. The coding regions have 48,308 polymorphic sites (38,4%). Introns with secondary structure and regions with intergenic sequences have 21,264 (16,9%) and 56,226 (44,7%) polymorphic sites, respectively. The alignment of the concatenated data showed an unbalanced (but fairly typical) mean nucleotide composition of A=29,9%, C=19,9%, G=19,4% e T=30,8%.

Analysis Results

Hairpin inversions

In the non-coding part of alignment, we found evidence for eight putative small inversions (Table 2), based on the presence of inverted repeated motifs that could form stems at least four bp long. Stems of this length or longer are part of models of group II structures (Michel, Umesono & Ozeki, 1989; Toor, Hausner & Zimmerly, 2001; Kelchner, 2002) and are consistent with sequence patterns observed by one of us in the rpL16 intron sequences in other taxa (Pfeil et al., 2002).

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Faster region assessment

The pairwise identity between *Polystachya concreta*5 and *P. concreta*8 (whose common ancestor is relatively old and near the crown of *Polystachya*) for psbD-trnT was 98.7%. The pairwise identity for these samples for matK was 99.2%. PsbD-trnT was therefore used as the representative fast cpDNA region. The analysis with a single copy of this dataset (870 bp) yielded a MCC tree (Fig. 2 upper) with only five nodes with high support (>0.95 PP). Increasing the number of copies did not result in much improvement. The analysis using 16 copies of psbD-trnT (~14 kb) produced a MCC tree (Fig. 2 lower) containing only eight highly supported nodes.

Random sample from all non-coding regions

The pairwise identity between *Polystachya concreta*5 and *P. concreta*8 was 98.7% across 58 236 bp of non-coding region contained in our alignment. This compares to 98.8% identity between the same samples across the coding regions in our alignment.

The replicate datasets that sampled 16% of the original non-coding alignment (excluding poorly aligned parts and down-weighting the inverted loops) failed to return all nodes found in the whole genome analysis. Of 13 selected nodes found in the tree from the whole genome (four subtended by relatively long branches, four by medium length branches, and five by short branches), only five were found with high support across most or all replicates (i.e., at least 16 of 20 replicates had ≥0.95 PP). Three of the selected nodes instead had five or fewer replicates with high support (≥0.95 PP), but only one or no replicates that contained highly supported contradictory nodes (thus the support for the expected node was ≤0.05). Finally, five of the nodes had generally poor support among replicates (i.e., five or fewer replicates had ≥0.95 PP along with six or more nodes with ≤0.05 PP). The 16% datasets (~9.2 kb) recovered from seven to 21 highly supported nodes among replicates (mean=14.8), with more nodes recovered in 19 of 20 replicates than was the case with the larger repeated psbD-trnT dataset (~14 kb and eight supported nodes). This character sampling strategy was probably more reflective of the underlying support for various nodes than using repeated copies of a single small dataset.

The mutually exclusive foliosa1/concreta2 versus foliosa1/foliosa2 clades (see below) were also examined in the 16% datasets. In the first case (foliosa1/concreta2), just four replicates contained this clade with high or moderate support (≥0.90 PP). The contradictory

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second grouping (foliosa1/foliosa2) was found with a similar level of support (≥0.90 PP) in only two replicates. The fact that both groupings could be recovered, with high support, in at least some replicates suggests that the original dataset contains the signal of both clades. A NeighborNet analysis (Fig. 3 inset) confirmed that a mixture of patterns exists in the original dataset involving foliosa1, foliosa2, concreta1 and concreta2.

Phylogenetic analyses

Analyses with and without the inverted loops (the latter by down-weighting to a single character) returned almost identical trees. The results of only the latter analysis is presented in this section. The tree we recovered was able to resolve the phylogenetic relationships among the groups of the large clade selected for this study, with high support values on almost every node (Fig. 3). The tree was characterized by a large clade with relatively short branches containing only sequences from the Neotropics, with a grade of a few small clades and single sequences containing the remaining sequences (Fig. 3). The large clade contained 21 sequences from Brazil, Dominica and Venezuela, whereas the grade included 19 sequences from tropical central and eastern Africa, as well as Madagascar and the nearby islands (Fig. 3).

The grade recovered include a few geographically identifiable clades (Fig. 3). One of these, attaching fairly deeply within the crown, consists of four Malagasy sequences (*Polystachya humbertii*1, *P. humbertii*2, *P. oreocharis* and *P. tsinjoarivensis*2) that are sister to a Kenyan sequence (*P. eurychila*). Another clade comprises a Kenyan sequence (*P. golungensis*) and one from Reunion (*P. concreta*8). A third clade contains a pair of central African sequences, one from Cameroon (*P. odorata*2) and one from Nigeria (*P. odorata*1). A fourth clade contains sequences from central Africa (*P. concreta*5 from Cameroon), Madagascar (*P. tesselata*1), the Comoros (*P. concreta*9) and two sequences without certain provenance. Finally, a fifth pair of sequences were from samples collected from Mauritius (*P. concreta*7) and Madagascar (*P. tesselata*2). Lineages containing only a single sequence in this grade included samples from Kenya (*P. melanantha* and *P. steudneri*) and Cameroon (*P. dolichophylla*).

Sequences from the widely sampled and widely distributed *P. concreta* did not form a monophyletic group and occurred on different branches of the tree, separated by several well supported nodes (Fig. 3). Similarly, the two *P. tessellata* sequences from Madagascar did not

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form a clade. *Polystachya estrellensis* sequences form a clade with *P. concreta* sequences collected in Brazil. Although the sequences of *P. estrellensis* are thus paraphyletic, whether the taxon itself is paraphyletic cannot be established for certain here. The identification of this *P. concreta* sample could be wrong, given that the identification of these species is confused in Brazil and sometimes they are considered synonymous (see also Discussion).

With versus without loops

The down weighting of the inversions we identified (by excluding all but one character per inversion) resulted in a similar, but not identical, phylogenetic inference. The differences among the maximum clade credibility (MCC) trees involved *P. foliosa1*, *P. foliosa2*, *P. concreta1*, *P. concreta2*, *P. concreta3* and *P. concreta4*. The analysis with the inversions included returned this tree (Fig. 3 inset and Fig. 4 inset):

((concreta3,concreta4):1,((foliosa1,foliosa2):0.98,(concreta1,concreta2):0.98):0.97)

with clade posterior probabilities (PP) listed after each node. In contrast, the inference resulting from down-weighted inversions returned this tree (Fig. 3 main panel and Fig. 4 main panel):

((foliosa1,concreta2):1,((concreta3,concreta4):1,(foliosa2,concreta1):0.76):1)

There are several supported differences between these trees, with at least one corresponding to the way the inverted loops are weighted. When the entire loops are analysed, *P. foliosa1* and *P. foliosa2* are supported as sisters, with these two sequences appearing to share two loop inversions (if this topology is correct; Fig. 4 inset). However, down-weighting the inversions produces a tree consistent instead with two independent inversions (Fig. 4 main panel).

DISCUSSION

Chloroplast genome sequence provides a robust phylogeny

In this work we used the nearly complete chloroplast sequences of 40 *Polystachya* samples to infer a robust plastid phylogeny. The dataset significantly increased the phylogenetic resolution within the genus. Thus, our results suggest that increasing the number of molecular markers has the potential to solve not only the relationships among species, but also to identify new *Polystachya* clades and define new sections. The delimitation of new

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425 sections will, however, depend upon the inclusion of more taxa than was done by this study –
426 in other words a higher coverage of the genus. Below we highlight some of the clades
427 recovered, their morphological and/or geographical characterisation, and a comparison with
428 previous studies.

429 ***Polystachya bicolor/rosea* position contradicts Russell's Clade III**

430 We selected two species as outgroups, *P. bicolor* (=*P. rosea*) and *P. melanantha*, in
431 which *P. bicolor/rosea* appears in a clade together with samples of *P. concreta* (from
432 Cameroon and from the Comoros), *P. tesselata* (= *P. concreta*) and *P. modesta*. Not
433 surprisingly, the clade that includes *P. bicolor/rosea* is deeply nested within the ingroup, thus
434 contradicting the monophyletic Clade III presented by Russell et al. (2010 b).
435 In prior studies, *Polystachya bicolor/rosea* has an uncertain position in the
436 phylogenetic trees. In an analysis using plastid markers and Bayesian inference, this species
437 appears in a large polytomy with *P. concreta* and other related species (Mytnik-Ejsmont,
438 2011), or related to species of other clades (Russell et al., 2010b) depending on the marker
439 used. A phylogeny using nuclear data (ITS sequences) highlighted the lack of monophyly of
440 this species (Mytnik-Ejsmont, 2011), which may be connected to the difficulty in identifying
441 it. *Polystachya bicolor/rosea* is often mistaken for *P. concreta*, since differentiation between
442 these is made by subtle differences in the shapes of leaves, and the size and colour of the
443 flowers. Unlike *P. concreta*, which has a pantropical distribution, *P. bicolor/rosea* is
444 restricted to Madagascar, Comoros and the Seychelles (Mytnik-Ejsmont, 2011).

445 **Brazilian sequences form a clade**

446 The monophyletic nature of the group formed by the Brazilian accessions, contrasting
447 with the paraphyletic group made up of African accessions, is consistent with the hypothesis
448 that Africa is the area of highest species diversity for this genus (Dressler, 1993) and probably
449 the centre of origin with a subsequent (i.e., more recent) dispersal into the Neotropics (Russell
450 et al., 2010 a; Russell et al., 2010b).

451 **Hybrid origins of some taxa suggested**

452 The hybrid origin of *P. concreta* is a possible explanation for this species being found
453 in different positions in the tree (Russell et al., 2010b). *Polystachya concreta* individuals that
454 have dispersed out of Africa are tetraploid, whereas plants found in continental Africa can be

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diploid or tetraploid. The sister taxa of African *P. concreta* are diploid (Russell et al., 2010b), indicating that tetraploidy is a derived state in *P. concreta*. Allotetraploidy in *P. concreta* has been confirmed by analysis of low copy nuclear genes (Russell et al., 2010a).

Interspecific hybridisation events, as in *P. concreta*, are considered a source of chloroplast genome exchange via introgression. Chloroplast genome exchange among species is sometimes suggested as an explanation for the inconsistencies between phylogenetic trees based on nuclear and plastid markers in, e.g., *Populus* (Salicaceae) (Smith & Sytsma, 1990; Tsitrone, Kirkpatrick & Levin, 2003), *Nothofagus* (Nothofagaceae) and Crassulaceae (Mort et al., 2002; Acosta & Premoli, 2010). In *Nothofagus*, chloroplast capture results in the association of chloroplast genomes with geographic locations, rather than taxonomic relationships (Acosta & Premoli 2010). Relationships based on geographic location could be explored as a possible explanation for the proximity of *P. concreta* (accesses from Brazil) with *P. estrellensis* (also from Brazil) and not with non-Brazilian accessions of *P. concreta*. In this case a study of nuclear markers of these taxa would be needed.

Neotropical species

Relationships in the group that includes *P. concreta*, *P. foliosa*, *P. estrellensis* and other species are not well resolved due to the low sequence divergence levels between species found in both plastid and nuclear genes (Russell et al., 2010a; Russell et al., 2010b; Russell et al., 2011; Mytnik-Ejsmont, 2011). Generally, the morphological variation observed in this group is identified as *P. concreta*. Although *P. estrellensis* is considered a valid species on the official plant list of Brazil (Barros et al., 2010), there is no consensus on synonymy with *P. concreta*. This can be seen in the herbarium identifications that sometimes consider them as two distinct species, but sometimes as the same species. The same occurs with *P. foliosa*, a name which would only be correctly applied to plants from the Amazon basin, the Guyana Shield and the West Indies (Peraza-Flores, Fernández-Concha & Romero-González, 2011). This circumscription is not accepted by Mytnik-Ejsmont (2011), who considers *P. estrellensis* and *P. foliosa* to be synonymous.

Genetic dissimilarity between African and Neotropical tetraploids was reported by Russell et al. (2010) and Russell et al. (2011), but the delimitation *P. estrellensis*, *P. concreta* and *P. foliosa* remained uncertain. According to our results, under a molecular perspective, *P. estrellensis* should be considered distinct from *P. concreta*. Moreover, our results do not

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corroborate the placement in synonymy of *P. estrellensis* and *P. foliosa* as proposed by Mytnik-Ejsmont (2011). In our tree *P. foliosa* forms a highly supported group with some *P. concreta* sequences (from samples collected in Brazil). Finally, although our results indicate a possible separation of Brazilian and African *P. concreta*, the delimitation of this species remains uncertain, considering that there is no generic taxonomic revision that has rigorously analysed the morphological variation in this species. Moreover, considering the reticulated evolution by Russell et al. (2010a), further investigation with nuclear markers would be necessary.

Taken together, our analysis suggests that *P. estrellensis* can be considered a distinct species from *P. concreta* and *P. foliosa*, and that Brazilian and African *P. concreta* should probably be treated as different species. Evidence of hybridisation influencing the evolution of *P. concreta* (Russell et al., 2010a; Russell et al., 2010b) highlights how importance it will be to also consider bi-parentally inherited nuclear DNA when inferring of phylogenetic relationships between this species and other species of the genus. The placement in synonymy of *P. estrellensis* and *P. foliosa* proposed by Mytnik-Ejsmont (2011) was not confirmed by this study. In our results, *P. foliosa* forms a highly supported clade including Brazilian samples of *P. concreta*.

Implications for Data Requirements

The entire chloroplast more useful than a fast subset

Using a relatively large number of chloroplast sequences we were able to resolve the polytomy involving the Neotropical species. But, if on one hand this dataset is promising in the formulation of more robust phylogenetic hypotheses, on the other hand, the complete chloroplast genome sequencing may be costly for the systematic projects that consider genera with many species (Särkinen & George, 2013), such as *Polystachya*, which has about 250 species. This was the main motivation for testing how well a smaller sample of the chloroplast genome would perform in phylogenetic inference. This was done in two ways: by duplicating a fast region and analysing multiple copies of this dataset, and by sampling without replacement from all non-coding regions in our alignment.

We found that sampling without replacement up to ~9 kb of non-coding sequence (16% of our alignment) was not sufficient to return a robust inference across all nodes. This

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517 was in contrast to the analysis of the entire chloroplast and showed that in the case of these
518 samples of *Polystachya*, more data were needed to resolve their relationships. The cost of
519 primers, amplification and Sanger sequencing of only three or four regions begins to exceed
520 that of gene capture of the entire chloroplast. It is therefore more cost effective and produces a
521 more robust result to undertake the collection of the entire chloroplast genome. That said, our
522 16% sample did resolve some nodes with high support, and other nodes obtained moderate to
523 high support from a few of the replicates. This suggests that these data are on the way to
524 resolving most nodes, but a gradual increase in resolving power occurs as characters are
525 added.
526 Duplicating a single fast region even 16 times, in this case psbD-trnT copies totalling
527 ~14 kb, failed to achieve a robustly resolved phylogeny. The results for the psbD-trnT
528 duplicated analysis was poorer even than that of sampling fewer but more representative
529 characters across the non-coding region (above). It appears that a small sample size (only 870
530 bp of independent sequence sites) is a serious source of stochastic error in this case. Sampling
531 one versus 16 copies of the same dataset only slightly increased the number of resolved nodes
532 (but still falling short of the number of nodes usually resolved with support by the smaller
533 16% sample), confirming the limitations of the original dataset.

534 **Homoplastic hairpin inversions affect phylogenetic analysis**

535 One issue raised here that is rarely taken into account in analyses of whole
536 chloroplasts is that sequence patterns at the small scale, namely hairpin inversions of loops,
537 can still have an effect on phylogenetic inference, despite using very large data sets. Our
538 results indicate that at least some of the differences between the trees inferred using entire
539 loops versus down-weighted loops were driven by these hairpin loop inversions. This kind of
540 phylogenetic effect has been observed in other cases, although with smaller data sets (e.g.,
541 Kim and Lee, 2005; Joly et al., 2010). If loops invert in a single molecular event (as is
542 currently believed: Kelchner and Wendel, 1996; Kim and Lee 2005), such as an intra-
543 molecular recombination, then there is no good reason to use each character state difference
544 found between sequences in the entire loop in an analysis. This simply inflates the
545 phylogenetic impact of a single event, treating it instead as many independent events
546 (corresponding to the number of character state differences in the inversion), as also noted by

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Kim and Lee (2005). As shown here, a larger data set simply does not give licence to ignore known analytical pitfalls.

Together, these findings show that sampling the entire chloroplast, analysed carefully, is a better option than sampling a few (even a dozen or more) fast regions. This is true, at least in *Polystachya*, but a similar result has also found by other studies, such as Parks, Cronn. & Liston (2009) for *Pinus*. Based on cost alone, it seems there is no benefit to be gained by screening the chloroplast for faster markers when there are many short branches in the particular tree, as there are here. Whole chloroplast analyses are likely to be a better way forward than sampling individual chloroplast markers in addressing many phylogenetic questions. If gene capture is used, as it was here, it is also very easy to add probes to unlinked nuclear regions, further increasing the power of this approach as a general solution to the issue of data sampling.

CONCLUSIONS

Our results show that significantly increasing the number of nucleotides can be an effective option in the phylogenetic inference of taxonomic challenging taxa, such as the orchid genus *Polystachya*. We generated complete chloroplast sequences of 48 *Polystachya* specimens using a combination of Illumina NGS sequencing and a sequence capture, which solved a notorious polytomy for Neotropical species. Our tests on how well a smaller sample of the chloroplast genome would perform in phylogenetic inference shows that the whole chloroplast is a better option than selecting just a few highly variable markers. Full plastid genomes appear particularly powerful when the expected tree is likely to contain many short branches, but nonetheless need to be analysed with care.

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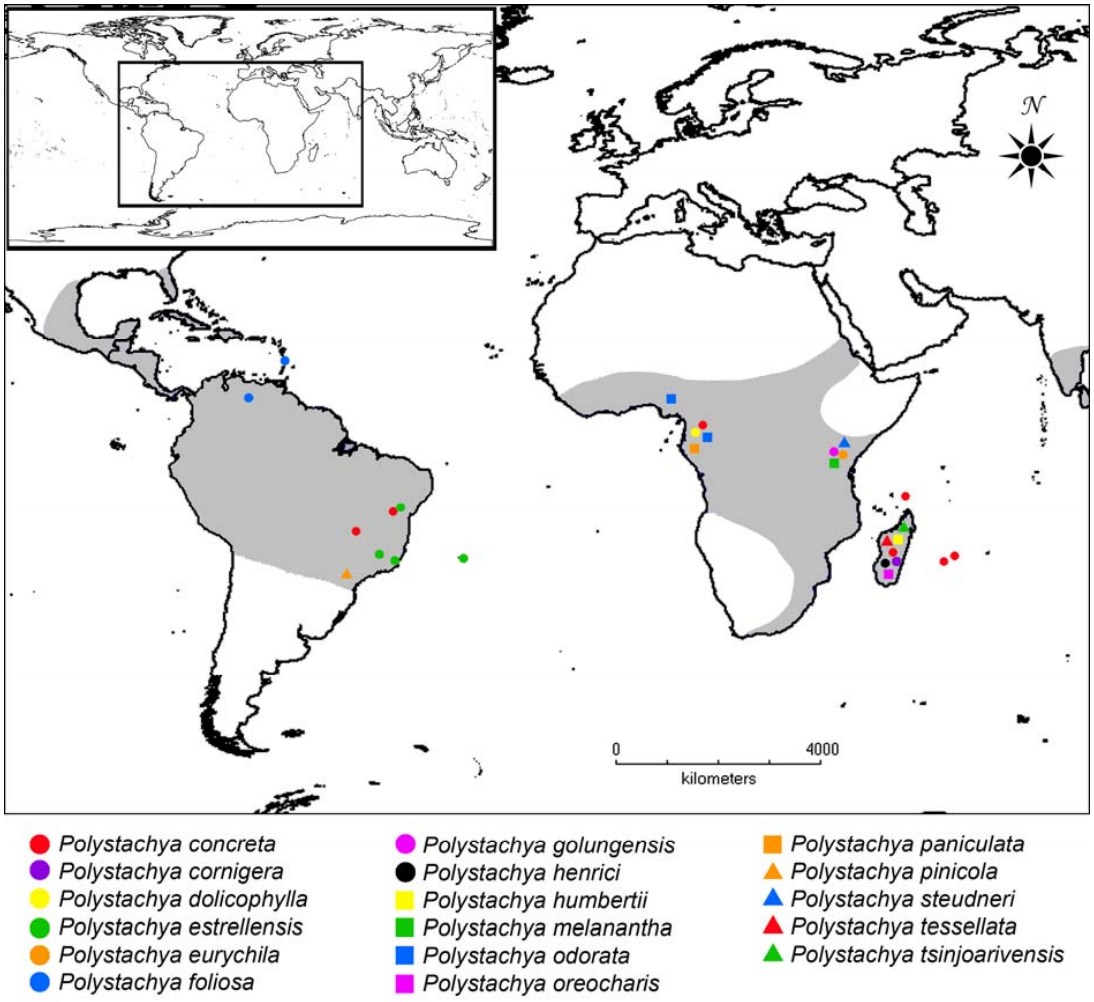
728 ## FIGURES

729



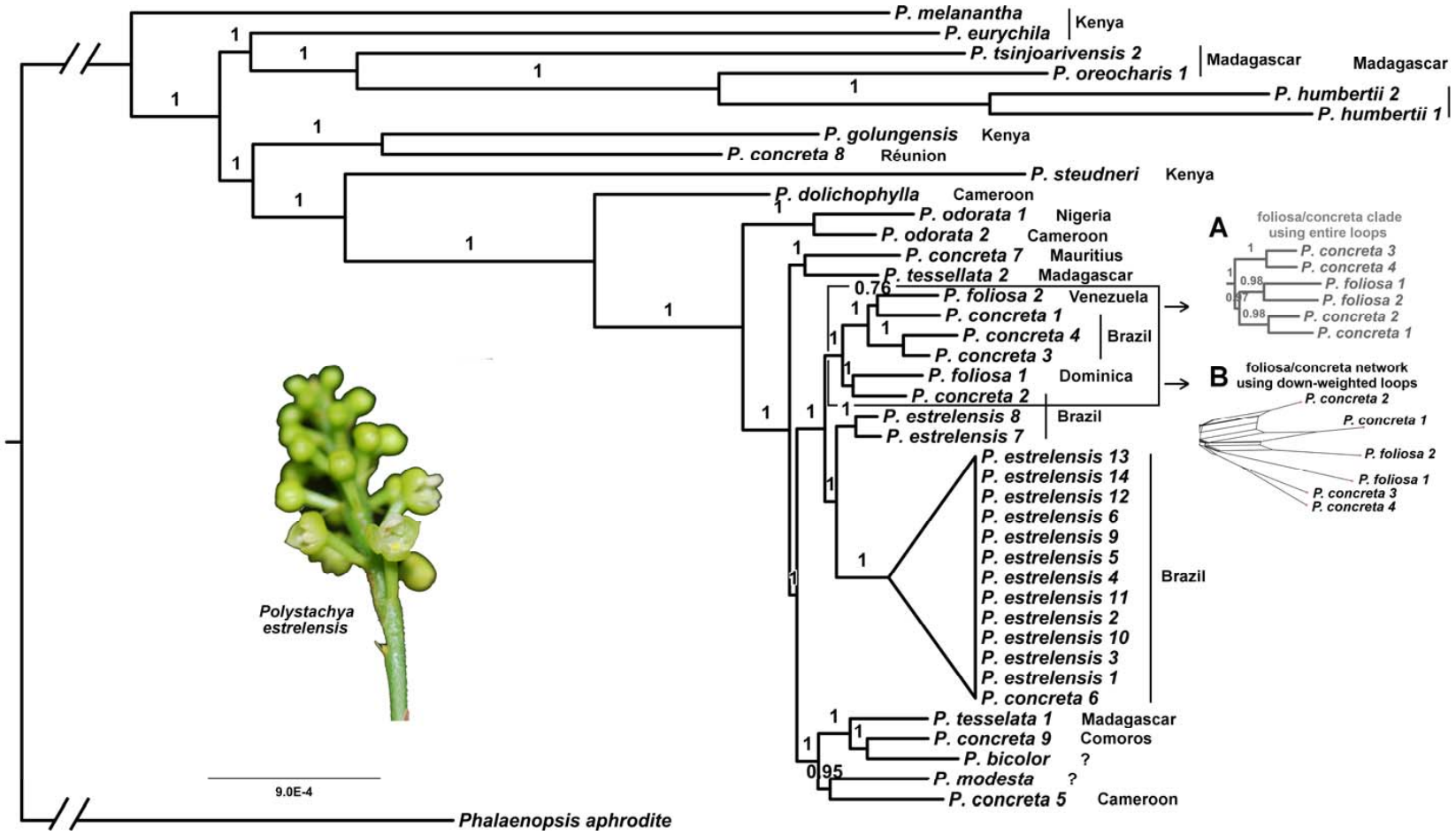
730

731 **Figure 1 – Distribution of *Polystachya* and the location of samples used in this study.**

732 Grey shading shows the distribution of the genus. Coloured symbols show the location of samples

733 used here, with the species determination of each sample as per Table 1.

Figure 2 – Phylogeny of the psbD-trnT region estimated using Bayesian analysis and rooted using *Phalaenopsis aphrodite*. Posterior probabilities are show above branches. Scale bar is in substitutions per site. The two branches leading to the root have been foreshortened to reduce space and are thus not to scale. *2A*: Phylogeny based on a single copy of psbD-trnT. *2B*: Phylogeny based on 16 identical copies of the psbD-trnT data set.

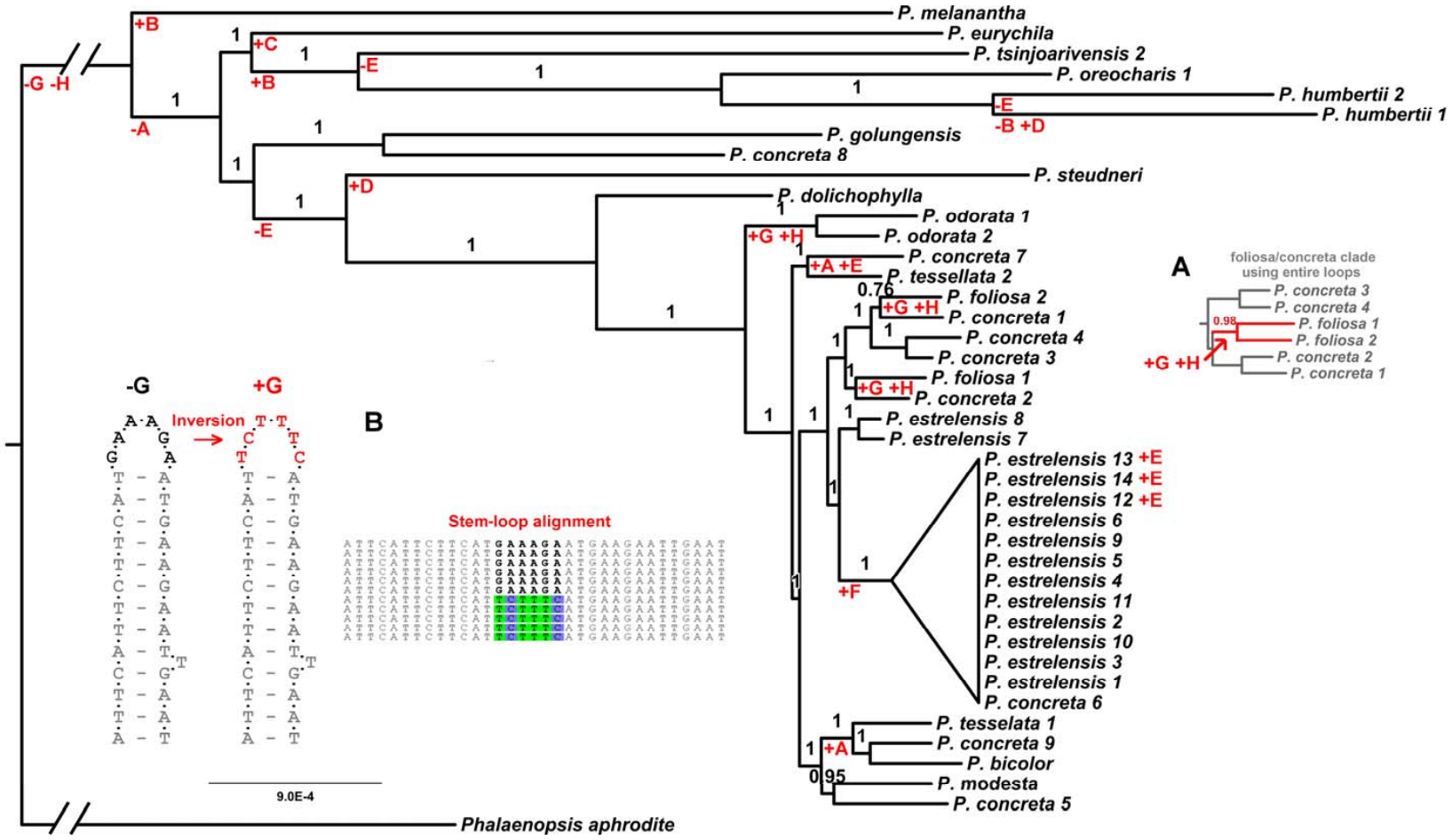


740

741 **Figure 3 – Plastid phylogeny from Bayesian analysis rooted using *Phalaenopsis aphrodite*.** Posterior probabilities are show above branches.

742 The *Polystachya estrelensis* group has been collapsed to reduce detail. Scale bar is in substitutions per site. The two branches leading to the root have

743 been foreshortened to reduce space and are thus not to scale. The two insets are not at the same scale as the main figure. *Main Figure*: phylogeny based
744 on the data set with poorly aligned regions excluded and loops down-weighted. *3A* (in grey): phylogeny of the foliosa/concreta group based on the full
745 inclusion of loops. *3B* (in black): NeighborNet network of the foliosa/concreta group based on the down-weighted loops. *Polystachya estrelensis* photo
746 credit: N. Lopes de Abreu.
747



748

749 **Figure 4 – Parsimonious gains and losses of non-coding loop inversions in *Polystachya* relative to the outgroup sequence, *Phalaenopsis***

750 ***aphrodite*, mapped on to the plastid phylogeny.** The letter codes designate loops as per Table 2. *Main Figure*: phylogeny estimated using down-

751 weighted loops (from the main panel, Fig. 1). Where equally parsimonious interpretations were possible, accelerated transformation has been used. *4A*:
752 part of the phylogeny estimated using entire loops for the foliosa/concreta group. The mapping of gains of two loop inversions shared by foliosa1 and
753 foliosa2 on this topology is in constrast to the mapping on the topology using down-weighted loops (main figure). *4B (from left to right)*: a diagramatic
754 representation of the stem-loop structure with the majority form of the loop sequence (in black); the stem-loop structure with the proposed inversion of
755 the loop sequence (in red); the consequence on the alignment before down-weighting of the loop sequence (loop seqeunce in bold – majority form;
756 loop sequence with back colours – inverted form).

757

TABLES

Table 1: Species and sample information of the accessions used in the study. Species analysed; location of the collection and voucher; DNA concentration and purity before and after genomic library assembly.

SPECIES	LOCATION	VOUCHER	ENA REFERENCE	BEFORE GENOMIC LIBRARY ASSEMBLY			AFTER GENOMIC LIBRARY ASSEMBLY	
				Concentration (ng/uL)	Purity 260/280	Volume	Concentration (ng/uL)	Purity 260/280
P. bicolor (=P.rosea)	-	Kew 25884		20.1	1.51	50	31.7	1.93
P. concreta (1)	*Brazil, Distrito Federal*	N.L.Abreu 254		39.2	1.92	75	17.2	1.92
P. concreta (2)	*Brazil, Distrito Federal*	N.L.Abreu 254		33.6	1.96	75	31.9	1.9
P. concreta (3)	*Brazil, Bahia State*	N.L.Abreu 251		18	1.78	70	29.9	1.97
P. concreta (4)	*Brazil, Bahia State*	N.L.Abreu 251		8.5	1.52	130	38.3	1.9
P. concreta (5)	*Cameroon*	A. Russell 40 (YA)		35	1.82	8	15	1.75
P. concreta (6)	*Brazil*	HBV ORCH 066004		21.9	1.53	6	9.8	1.9
P. concreta (7)	*Mauritius*	HBV ORCH 07278		26	1.78	13	32.4	1.85
P. concreta (8)	*Réunion*	HBV “Chase & Samuel 1”		34	1.82	13	30	1.85
P. concreta (9)	*Comoros*	HBV ORCH 07417		23.7	1.83	13	30.4	1.86
**P. concreta (10)*	*Madagascar*	Fischer&Sieder FS3210 (WU)		21	1.81	13	9.4	1.63
**P. concreta (11)*	*Madagascar*	Kew 17854		70	1.65	67	9.6	1.6
**P. cornigera*	*Madagascar*	Fischer&Sieder FS3208 (WU)		22.2	1.76	11	7.7	1.58
P. dolichophylla	*Cameroon*	Kew 25886		108.9	1.05	55	30.3	1.89
P. estrellensis (1)	*Brazil, Minas Gerais State*	N.L.Abreu 255		29.8	1.81	65	33.7	1.9
P. estrellensis (2)	*Brazil, Minas Gerais State*	N.L.Abreu 255		17.8	2.06	90	26	1.54
P. estrellensis (3)	*Brazil, Minas Gerais State*	N.L.Abreu 255		11.9	1.82	75	35	1.86
P. estrellensis (4)	*Brazil, Espírito Santo State*	N.L.Abreu 253		12.8	1.77	65	18.7	1.84
P. estrellensis (5)	*Brazil, Espírito Santo State*	N.L.Abreu 253		22.4	1.87	70	21	1.96

P. estrellensis (6)	*Brazil, Espírito Santo State*	N.L.Abreu 253		28.2	1.79	70	26.3	1.96
P. estrellensis (7)	*Brazil, Bahia State*	N.L.Abreu 252		25.4	1.91	70	29.3	1.98
P. estrellensis (8)	*Brazil, Bahia State*	N.L.Abreu 252		14.6	1.79	70	22.4	2.02
P. estrellensis (9)	*Brazil, Bahia State*	N.L.Abreu 256		27.8	1.61	90	20.4	2.05
P. estrellensis (10)	*Brazil, Bahia State*	N.L.Abreu 256		23.8	1.81	75	23.1	1.93
P. estrellensis (11)	*Brazil, Bahia State*	N.L.Abreu 256		22.3	1.68	90	28	1.97
P. estrellensis (12)	*Brazil, Trindade's Island*	Nilber		10.1	1.64	130	30	1.88
P. estrellensis (13)	*Brazil, Trindade's Island*	Nilber		12.5	1.64	130	30	1.83
P. estrellensis (14)	*Brazil, Trindade's Island*	Nilber		10.3	1.48	130	34.7	1.84
P. eurychila	*Kenya*	Kew 17963		207.6	0.94	85	13.7	1.98
P. foliosa (1)	*Dominica*	Kew 25887		14.7	1.58	56	31.8	1.96
P. foliosa (2)	*Venezuela*	HBV ORCH 07082		30.2	1.73	8	18.6	1.79
P. golungensis	*Kenya*	Kew 17966		104.7	1.14	115	26.1	1.88
**P. henrici*	*Madagascar*	Kew 17856		22	1.52	56	7.7	2.04
P. humbertii (1)	*Madagascar*	Fischer&Sieder FS2079 (WU)		116.3	1.82	13	29.4	1.77
P. humbertii (2)	*Madagascar*	Fischer&Sieder FS3017 (WU)		35.1	1.93	11	34.2	1.89
P. melanantha	*Kenya*	Kew 17954		207.2	0.94	50	12.2	1.8
P. modesta	-	HBV ORCH 05165		56.8	1.35	13	31.7	1.93
P. odorata (1)	*Nigeria*	Kew 17857		33.7	1.56	58	46.8	1.8
P. odorata (2)	*Cameroon*	A. Russell 42 (YA)		36.9	1.91	13	26.2	1.91
P. oreocharis (1)	*Madagascar*	Fischer&Sieder FS2082 (WU)		58	1.74	13	30.2	1.87
**P. oreocharis (2)*	*Madagascar*	Fischer&Sieder FS3152 (WU)		20.2	1.82	13	10.5	1.8
**P. paniculata (2)*	*Cameroon*	L. Pearce 27 (YA)		56.1	1.4	8	8.1	1.98
**P. pinicola*	*Barzil*	HBV ORCH 06606		31	1.11	7	9.2	1.98
P. steudneri	*Kenya*	Kew 17956		10.6	1.45	58	33.5	1.87
P. tessellata (1) (=P.concreta)	*Madagascar*	Kew 17859		176.2	1.1	58	32.7	1.89
P. tessellata (2)	*Madagascar*	Kew 17860		216.1	1.1	49	33.3	1.93

(=P.concreta)								
**P. tsinjoarivensis (1)*	*Madagascar*	Fischer&Sieder FS3209 (WU)		19.3	1.83	13	9.3	1.79
P. tsinjoarivensis (2)	*Madagascar*	HBV FS4182		22.2	1.75	13	17.2	1.64

761 *Species excluded due to low quality sequencing.
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763

764 **Table 2: Alignment co-ordinates and sequences of putative stem-loop structures that appear to have undergone inversion in *Polystachya*.** The
765 loops that appear to have undergone inversions are underlined. Bases in red would be treated as phylogenetically informative if the alignment doesn't
766 take into account the inversion. Bases not involved in stem formation are in subscript.
767

Loop	Alignment co-ordinates	Sequence (majority)	Sequence (minority)	Substitutions before/after inversion reverted	Sequences (minority)
A	74,660-74,678	TCTATCTA-GAA-TAGATAGA	TCTATCTA-TTC-TAGATAGA	3/0	bicolor, concreta7, concreta9, tesselata1, melanantha, Phalaenopsis
B	88,106-88,142	GGCCCAATCTTT$_C$-TTTTTTTGAGGA-AAAGATTGGGCC	GGCCCAATCTTT$_C$-TCCTCAAAAAAA-AAAGATTGGGCC	8/0	humbertii2, oreocharis1, tsinjoarivensis2, melanantha
C	90,456-90,496	AGTAAGAACTCAGCG-GGGTAAGGCCT-CGCTGAGTTCTTACT	AGTAAGAACTCAGCG-AGGCCTTACCC-CGCTGAGTTCTTACT*	7/0	eurychila
D	94,175-94,222	ATTGAAGTAATGAGCCC-CAAGATGAATATGA-GGGCTCATTACTTCAAT	ATTGAAGTAATGAGCCC-TCATATTCATCTTG-GGGCTCATTACTTCAAT	8/0	humbertii1, steudneri
E	94,679-94,717	GTATCTAAGGAAGATCC-AAAGG-GGATCTTCCTTAGATAC	GTATCTAAGGAAGATCC-CTTCT-GGATCTTCCTTAGATAC	5/2	estrelensis12, estrelensis13, estrelensis14, eurychila, concreta7, concreta8, golungensis, oreocharis1, melanantha, humbertii1, Phalaenopsis
F	97,230-97,258	AACGTCCAGTG-CCAAAGT-CACTGAATGGG	^CCCATTCAGTG-ACTTTGG-CACTGAATGGG	6/0	All 13 sequences of the estrelensis clade (cartooned in Figs. 2 and 4)
G	104,698-104,730	ATTC$_A$ATTCTTC-ATTCTTTCAA-GAAGAATGAAT	ATTC$_A$ATTCTTC-ATGAAAGAAA-GAAGAATGAAT	6/0	foliosa1, foliosa2, odorata1, odorata2, Phalaenopsis
H	106,618-106,650	ATTCATTCTTCAT-GAAAGA-ATGAAGAAT$_T$GAAT	ATTCATTCTTCAT-TCTTTC-ATGAAGAAT$_T$GAAT	6/0	foliosa1, foliosa2, odorata1, odorata2, Phalaenopsis

768 *Occurs in only one sequence. ^Only the sequences with the inversion appear to form the stem in this case.