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ITS sequences of nuclear ribosomal DNA support the generic placement and the disjunct range of *Plagiochila (Adelanthus) carringtonii*

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Abstract – According to phylogenetic analyses of nrDNA ITS1 and ITS2 sequences (including the 5.8S unit) *Plagiochila carringtonii* and *P. carringtonii* subsp. *lobuchensis* form a monophyletic lineage. *Plagiochila carringtonii* is sister to *P. sandei*, type species of *P.* sect. *Cucullatae*, and is placed in a clade with *P. asplenioides* and *P. porelloides* of sect. *Plagiochila*. A placement of *Plagiochila* (*Adelanthus*) *carringtonii* in another genus is clearly rejected by Kishino-Hasegawa-tests. Infraspecific ITS variation in *Plagiochila* can be high and potentially provides a tool for the analysis of dispersal events.

Jungermanniales / Plagiochilaceae / *Plagiochila* sect. *Carringtoniae* / nuclear ribosomal DNA / internal transcribed spacer

INTRODUCTION

The European *Adelanthus carringtonii* Balfour was placed in *Nardia*, *Jungermannia*, *Odontoschisma*, and *Jamesoniella* until Grolle (1964) detected perianths in a related Himalayan subspecies [*Plagiochila carringtonii* (Balfour) Grolle subsp. *lobuchensis* Grolle]. Chemically, *P. carringtonii* belongs to the most common *Plagiochila* chemotype (2,3-secoaromadendrane type, Rycroft *et al.*, 1999). Inoue (1965) set up a monotypic sect. *Carringtoniae.* Grolle (pers. comm. 1999) contemplated the idea that *P. carringtonii* should be placed in its own genus and initiated an analysis of the phylogenetic relationships of *Plagiochila carringtonii*, based on nucleotide sequence variations in the internal transcribed spacer regions of nuclear ribosomal DNA. The investigation expands a first study of ITS sequence variations of members of sect. *Arrectae* Carl, *Cucullatae* Schiffn., *Contiguae* Carl, and *Glaucescentes* Carl (Heinrichs *et al.*, in press).

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MATERIALS AND METHODS

Material of species to be sequenced (Table 1) was carefully cleaned before drying. Upper parts of shoots were selected from fresh herbarium specimens (*Gradstein 9970*) or were preserved over silica gel prior to extraction of genomic DNA using PUREGENE Genomic DNA Isolation Kit (Gentra Systems).

The PCR primers P1 (5'-TGT ACA CAC AAT GCA GCA AAC CAG CG-3') and P2 (5'-CGG GTA ATC TTG CCT GAT CTG AG-3') (Heinrichs *et al.*, in press) were used to amplify the internal transcribed spacer (ITS) of the ribosomal DNA, which encompasses the 5.8S gene and both the ITS1 and ITS2 regions. Amplification by the polymerase chain reaction (PCR, Saiki et al. 1988) was performed in a total volume of 50 µl containing 2U Taq-DNA-polymerase (Promega, Heidelberg, Germany), 5 µl Taq polymerase reaction buffer (Promega), 4 µl 25 mM $MgCl_2$, 4 µl dNTP-Mix (2,0 mM each, MBI-Fermentas, St. Leon-Rot, Germany), 1 µl of each of the two primers (50 pmol each) and 1 µl of the genomic DNA (100-500 ng). The reactions were performed in 32 cycles under the following conditions: 40 s denaturation at 94C, 30 s annealing at 54C and 40 s elongation at 72C - using hot-start-PCR with 10 min of denaturation at 94°C before adding the Taq at 80°C. Finally there were 10 min of elongation at 72°C.

PCR products were cloned into the pCR4-Topo Vector (Invitrogen Life Technologies, Karlsruhe, Germany) following the TOPO TA Cloning Kit manufacturer's protocol and transformed into TOP10 Chemically Competent *Escherichia coli* by the heat shock method (Sambrok *et al.*, 1989). Sequencing was done on an LI-COR DNA Sequencer Long Reader 4200 using the thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, England).

Phylogenetic trees were inferred using distance, maximum parsimony (MP), and maximum likelihood (ML) criteria using PAUP* version 4.0b8 (Swofford, 1998).

Manual alignment of 17 ITS1-, 5.8S and ITS2 sequences (Table 1) representing one outgroup (*Herbertus*) and 14 ingroup taxa (13 species and one subspecies of *Plagiochila*) lead to a data set including 814 putatively homologous sites. To decide on the evolutionary model, which fits the data best, the program Modeltest 3.04 (Posada & Crandall, 1998) was used, which employs two statistics: the likelihood ratio test (LRT) and the Akaike information criterion (AIC, Akaike, 1974). Based on the results of the tests, the model selected by the hierarchial LRT was the HKY85 model (Hasegawa *et al.*, 1985) with gamma shape parameter (G) for among site variation calculated from the data set (HKY85+G). The ML method (with the HKY85+G model) was used for phylogenetic analyses.

The confidence of branching was assessed using 100 bootstrap resamplings in ML-analysis (using the HKY85+G model) and 1000 bootstrap resamplings in distance (neighbour-joining method using the HKY85+G model) and unweighted MP (with 10 addition-sequence replicates) of the data set (Felsenstein, 1985).

A user-defined tree with *P. carringtonii* as basis of the ingroup was generated by modifying the treefile of the 'best tree' (Fig. 1) using TreeVIEW (version 1.6.2, Page, 1996). To compare the user-defined topology with the 'best tree', the sequence data file was loaded into PAUP* and used for

Tab. 1. Geographic origins, voucher numbers, and GenBank accession numbers of the investigated taxa. Vouchers are deposited at GOET, duplicates of those marked with # were distributed in 'Bryophyta Exsiccata Generis Plagiochilae' (BEGP, Heinrichs & Anton 2001). Asterisks indicate species of which sequences were taken from GenBank).

Taxon	*Origin*	*Voucher*	*Accession number*
**Herbertus subdentatus* (Steph.) Fulford	Bolivia, La Paz	Groth s.n.	AJ413177
P. asplenioides (L.) Dumort.	Germany, Lower Saxony, Göttingen	# Heinrichs & Groth 4339, BEGP 124	AJ414629
**P. bifaria* (Sw.) Lindenb.	Tenerife	Drehwald 3922	AJ413173
**P. buchtiniana* Steph.	Bolivia	Groth s.n.	AJ413306
P. carringtonii (Balf.) Grolle subsp. *carringtonii*	Scotland	Rycroft 00041	AJ414630
P. carringtonii subsp. *lobuchensis* Grolle	Bhutan	Long 28857	AJ414631
**P. deflexirama* Taylor	Costa Rica	# Heinrichs et al. 4163, BEGP 14	AJ413310
**P. diversifolia* Lindenb. & Gottsche	Ecuador	Holz EC-01-17	AJ413308
**P. longispina* Lindenb. & Gottsche	Costa Rica	# Heinrichs et al. 4148, BEGP 4	AJ413307
P. porelloides (Torrey ex Nees) Lindenb.	U.S.A., West Virginia	Holz - 01-117	AJ414632
P. porelloides	Germany, Lower Saxony, Göttingen	# Heinrichs & Groth 4340, BEGP 125	AJ414633
**P. punctata* (Taylor) Taylor	Scotland	Rycroft 01013	AJ413174
**P. sandei* Dozy ex Sande Lac.	Philippines	Schwarz 5732	AJ413176
P. sandei	Indonesia	# Gradstein 9970, BEGP 107	AJ414634
**P. spinulosa* (Dicks.) Dumort.	Scotland	Rycroft 01012	AJ413175
**P. tocarema* Gottsche	Costa Rica	Heinrichs et al. CR199	AJ413309
**P. virginica* Evans	Tenerife	Rycroft 01068	AJ413311

Kishino-Hasegawa-tests (KH, Kishino and Hasegawa, 1989); comparisons [KH tests using bootstrap (1000 replicates) with full optimization, one-tailed test] were based on ML (model of evolution selected by Modeltest) and MP criteria (Table 2).

Tab. 2. Comparisons of the maximum likelihood tree in Figure 1 with a user-defined tree by Kishino-Hasegawa-tests (using bootstrap [with 1000 bootstrap replicates] with full optimization, one-tailed test) using maximum likelihood (ML) and maximum parsimony (MP) methods.

	ML (HKY+G) [a]		*MP*	
Tree topology	*Diff-lnL* [b]	*P* [c]	*Diff. length* [d]	*P*
1. (Best tree; Fig.1)	(3694.8)	–	(518)	–
2. Sect. *Carringtoniae* as basis of the ingroup	50.3 ± 13.9	0.0002*	30	< 0.0001*

[a] Maximum likelihood (ML) using the model after Hasegawa *et al.* (1985) with estimated proportion of invariable sites and gamma shape (HKY+G)
[b] Difference in -log-likelihood between the best tree (Fig. 1) and the user-defined tree.
[c] Probability of getting a more extreme T-value under the null hypothesis of no difference between the two trees (one-tailed test)
[d] Difference in tree length between the best tree (Fig. 1) and the user-defined tree.
[e] -log-likelihood of the best tree (Fig. 1).
[f] Length of the optimal tree in the maximum parsimony analysis.

* User defined tree significantly worse than the best tree at $P < 0.05$.

RESULTS

When aligned with the sequence of *P. carringtonii* subsp. *carringtonii* (769 bp) the subsp. *lobuchensis* displays a 25 bp loss in ITS1 and differences in 16 further positions. Similar differences are found between the two *P. porelloides* and *P. sandei* specimens investigated: the *P. porelloides* sequences differ in 19 positions, the *P. sandei* sequences in 25 positions.

The molecular investigation (Fig. 1) produced a monophyletic lineage for *P. carringtonii* and *P. carringtonii* subsp. *lobuchensis* and confirms the disjunct range of the species (Grolle, 1964; Paton, 1999). *Plagiochila carringtonii* is sister to *P. sandei*, type species of *P.* sect. *Cucullatae* (Grolle, 1976), and is placed in a clade with *P. asplenioides* and *P. porelloides*. The generic placement of *P. carringtonii* in *Plagiochila* is strongly supported by the molecular data and confirms the results of Grolle (1964), Lewis (1970) and Rycroft *et al.* (1999). A placement of *Plagiochila carringtonii* in another genus is clearly rejected by KH tests (Table 2).

Identification of members of the *Plagiochila asplenioides* complex is notoriously difficult (e.g. Grolle, 1967; Schuster, 1980) and largely based on plant size. Variation of ITS sequences is congruent with that of morphological characters and the 'morphological species' are supported by molecular differentiation: a specimen of *P. porelloides* from Göttingen, Lower Saxony, Germany shares a clade with a *P. porelloides* specimen from West Virginia, U.S.A., both being sister to a *P. asplenioides* specimen from Göttingen.

DISCUSSION

Plagiochila carringtonii stands out by its laterally appressed, suborbicular leaves with edentate or 1-4 toothed margin, leaf cell walls with large, nodulose trigones, granular oil bodies, simple androecia with dorsally overlapping male

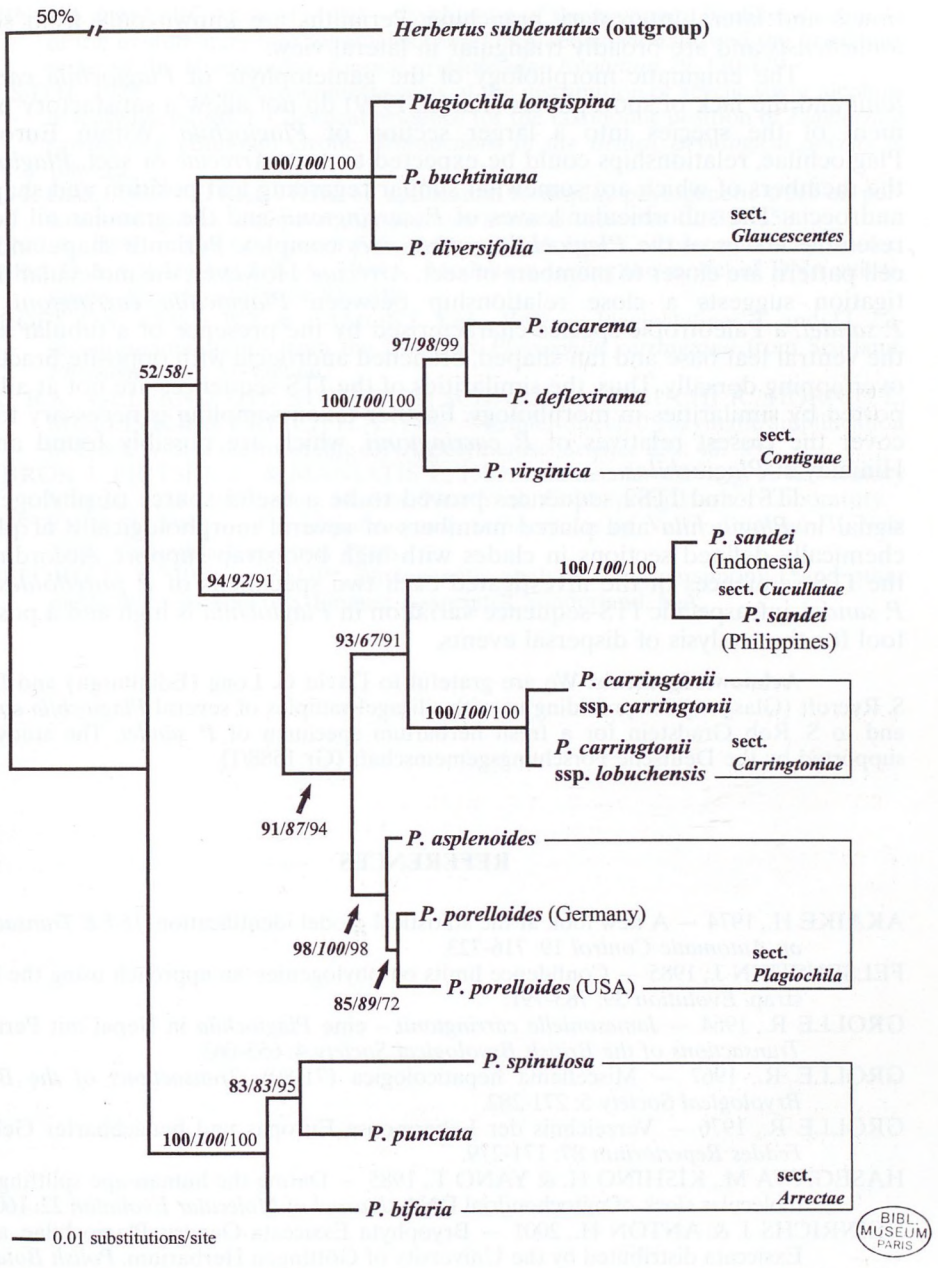

Fig. 1. Molecular phylogeny of *Plagiochila* species based on ITS1-, 5.8S-, and ITS2-nrDNA sequence comparisons using 814 aligned positions. The rooted tree resulted from a maximum likelihood analysis of 17 sequences (including the outgroup sequence), using the HKY85 (HKY85 + G; Hasegawa *et al.*, 1985) model with estimated gamma shape (G = 0.725) and transition/transversion ratio (Ti/Tv = 1.724), calculated as the best model by Modeltest 3.04 (Posada & Crandall, 1998); bootstrap percentage values (> 50%) were determined for maximum likelihood (using HKY85 + G; bold), neighbour-joining (HKY85 + G; bold italics) and unweighted maximum parsimony (not bold) methods.

bracts and lateral-intercalary branching. Perianths are known only from subsp. *lobuchensis* and are broadly triangular in lateral view.

The enigmatic morphology of the gametophyte of *Plagiochila carringtonii* and the lack of sporophytes (Paton, 1999) do not allow a satisfactory placement of the species into a larger section of *Plagiochila.* Within European Plagiochilae, relationships could be expected to sect. *Arrectae* or sect. *Plagiochila* the members of which are somewhat similar regarding leaf position and shape of androecia. The suborbicular leaves of *P. carringtonii* and the granular oil bodies resemble leaves of the *Plagiochila asplenioides* complex. Perianth shape and leaf cell pattern are closer to members of sect. *Arrectae.* However, the molecular investigation suggests a close relationship between *Plagiochila carringtonii* and *P. sandei*, a Paleotropic species characterised by the presence of a tubular sac at the ventral leaf base and fan-shaped, branched androecia with opposite bracts not overlapping dorsally. Thus, the similarities of the ITS sequences are not at all supported by similarities in morphology. Further taxon sampling is necessary to discover the closest relatives of *P. carringtonii*, which are possibly found among Himalayan *Plagiochilae.*

ITS1 and ITS2 sequences proved to be a useful source of phylogenetic signal in *Plagiochila* and placed members of several morphologically or phytochemically defined sections in clades with high bootstrap support. According to the ITS sequences of the investigated each two specimens of *P. porelloides* and *P. sandei*, infraspecific ITS-sequence variation in *Plagiochila* is high and a possible tool for the analysis of dispersal events.

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