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Reinstatement of *Plagiochila* sect. *Abietinae* (Plagiochilaceae, Jungermanniopsida)

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Abstract – The classification of the speciose leafy liverwort genus *Plagiochila* into species and sections is still in a preliminary state. Based on phylogenetic analyses of a dataset comprising nuclear ITS and chloroplast *rps*4 and *rbc*L sequences, we reject an earlier proposed synonymy of the Asian-Australasian *Plagiochila* sect. *Abietinae* and the pantropical *P.* sect. *Vagae*. A monophyletic sect. *Abietinae* is recognised, containing a clade with five accessions of *P. abietina* sister to a single accession of *P. hampeana*. This *Abietinae* clade nests in a larger *Plagiochila* clade which also retrieves monophyletic sects. *Fruticosae*, *Trabeculatae*, *Peculiares*, *Plagiochila* and *Poeltiae*. *Plagiochila* sect. *Vagae* is placed sister to *P*. sect. *Africanae*. Two *P. abietina* accessions from Australia form a sister relationship with a clade comprising three *P. abietina* accessions from the Fiji Islands. Based on the example of *P. patula*, we discuss the benefits of including type or topotype specimens in revisionary studies considering molecular and morphological evidence.

Barcoding / integrative taxonomy / Jungermanniales / liverwort / Lophocoleineae / *Plagiochila* sect. *Vagae*

INTRODUCTION

Plagiochila (Dumort.) Dumort., with some 700 currently accepted species (Söderström *et al.*, 2016), is among the largest genera of liverworts. Its members share dioicy, perianths with two keels of which the dorsal is usually slightly longer than the ventral, alternating or rarely subopposite foliation, and nearly exclusively lateral branching (Heinrichs, 2002). Members of *Plagiochila* are conspicuous and abundant elements of tropical rainforests but are also diverse in subtropical and temperate regions of the world. Numerous attempts to classify *Plagiochila* into supraspecific entities have been published. Early classification systems relied on a

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few characters of the gametophyte including leaf shape, leaf cell pattern, branching mode, and the position of the perianths (e.g., Lindenberg, 1839-1843; Schiffner, 1900; Dugas, 1929; Carl, 1931). Molecular phylogenies of *Plagiochila* identified extensive morphological homoplasy and led to revisions of the morphology-based classifications (e.g., Heinrichs, 2002; Groth *et al.*, 2003, 2004; Groth, 2006; Patzak *et al.*, 2016); however, the taxon sampling of these studies is still too sparse to introduce a comprehensive sectional classification.

The pantropical *Plagiochila* sect. *Vagae* Lindenb. is possibly the largest section of *Plagiochila* and may include some 100 species (Söderström *et al.*, 2016). In its original circumscription, *P.* sect. *Vagae* was a heterogeneous assemblage of taxa (Lindenberg, 1839-1843). However, designation of the lectotype *P. patula* (Sw.) Lindenb. led to a narrower concept of the section to species with (1) frequent terminal branching, which contributes to pseudo-dichotomous or pinnate shoot system architecture, (2) asexual reproduction by pluricellular propagules or plantlets from leaf surfaces, (3) perianths cylindrical to campanulate to obdeltoid and often winged, and (4) capsules with rather delicate valves with thickenings in all layers (Heinrichs *et al.*, 2002). Especially the leaf-borne cladia (Fig. 8) are a striking character of *Vagae* and may be regarded as a synapomorphy of the section. However, the molecular study of Groth (2006) resolved an accession of *P. abietina* (Nees) Mont. & Nees within *Vagae*. This species is the type of *P.* sect. *Abietinae* Schiffn.; hence Söderström *et al.* (2015) lowered *P.* sect. *Abietinae* to a synonym of *P.* sect. *Vagae*. Members of *Abietinae* have pinnate gametophytes with numerous terminal branches, postically secund leaves with an often distinct vitta, and lack vegetative distribution by propagules (Schiffner, 1900; Inoue, 1984). The synonymy of *Abietinae* and *Vagae* thus blurs the morphological circumscription of *Vagae* and we tested this synonymy by sequencing further accessions of former *Abietinae* species.

MATERIALS AND METHODS

Taxon sampling, DNA extraction, PCR amplification and sequencing

To investigate the relationship between *P.* sects. *Vagae* and *Abietinae,* six specimens from the former *P.* sect. *Abietinae* [one *P. hampeana* Gottsche and five *P. abietina* (Nees) Nees & Mont., Inoue (1984)] were obtained and a marker set of nuclear (nr) ITS, and chloroplast (cp) *rps*4 and *rbc*L sequences was compiled. A further six specimens of *P. patula* were obtained on account of this species being the type of *P.* sect. *Vagae* (Heinrichs *et al.*, 2002). DNA-extraction, PCR amplification and sequencing was carried out as described in Patzak *et al.* (2016), with the exception of *P. hampeana* and *P. patula* nrITS amplification, for which forward primer Hep4F (5' – CGT TGT GAG AAG TTC ATT AAA CC – 3') and reverse primer HepDR (5' – CCG CYT AGT GAT ATG CTT AAA CTC – 3') (Feldberg *et al.*, in press) were used. Polymerase chain reactions were set up as 50 µL reactions containing 2.5 units MyTaq DNA polymerase (Bioline), 1 µL of each primer (10 µM; final concentration of 0.2 µM), 10 µL of 5x MyTaq Reaction Buffer (Bioline, containing 5 mM dNTPs and 15 mM $MgCl_2$), 36.75 µL H_2O, and 1 µL of DNA. The PCR protocol was as follows: 120 s initial denaturation at 92°C, followed by 30 cycles of 60 s denaturation at 92°C, 50 s annealing at 51°C, and 90 s elongation at 72°C. Final elongation was carried out for 10 min at 72°C.

First, newly generated *Abietinae* seqences were compared with GenBank sequences using the BLASTN program (Altschul *et al.*, 1990). BLASTN searches indicated a close relationship of *Abietinae* and *Vagae* but a more distant homology to members of section *Vagae*. On this basis sampling was expanded to include taxa from *P.* sect. *Vagae* and several other closely related sections (sects. *Africanae* Heinrichs, *Cucullatae* Carl, *Peculiares* Schiffn., *Poeltiae* Inoue, *Plagiochila, Fruticosae* Inoue and *Trabeculatae* S.Hatt. ex Inoue, as well as the species *P. andina* Steph, which has not yet been assigned to any section) and selected sequences were downloaded from Genbank, bringing the number of species sampled to 52 in total (61 accessions, Table 1). Trees were rooted on *P. turgida* Herzog (sect. *Hylacoetes* Carl) and *P. adianthoides* Lindenb. (sect. *Adianthoideae* Lindenb.) which fall in a sister clade to remaining taxa as determined by Patzak *et al.* (2016).

Phylogenetic analyses

Sequences for each marker were aligned separately in BIOEDIT V.7.2.5 (Hall, 1999) using CLUSTALW (Thompson *et al.*, 1994), and then manually adjusted by eye. Regions lacking sequence data were marked as missing. Maximum likelihood (ML) analyses were carried out using RAXML V 8.0 (Stamakatis, 2014) as implemented on the CIPRES portal V 2.2 (Miller *et al.*, 2010) with the extended majority rule bootstrapping criterion. The Akaike information criterion (Akaike, 1973) implemented in JMODELTEST 2 was used to determine the most appropriate nuclear substitution models to use in phylogenetic analyses (Darriba *et al.*, 2012). The best fits were TIM2 + I + G for nrITS and GTR + I + G for both *rps*4 and *rbc*L. Since the TIM2 model is not available in RAXML the GTR + I + G model was used for nrITS as well, as suggested by Posada (2008). The three ML trees resulting from separate analyses of each locus were compared by eye. As there were no incongruences between the different markers [i.e. no conflicts with bootstrap values (BV) greater than 70%], the three alignments were concatenated (Mason-Gamer & Kellogg, 1996) and a combined ML analysis was conducted. The resulting tree was computed from 10 independent runs and autoMRE based multiparametric bootstrapping resulting in 360 bootstrap iterations. Clades with bootstrap values of 70% or more were considered to be well supported (Hillis & Bull, 1993).

Bayesian inference was carried out using MrBayes V 3.2.6 (Ronquist & Huelsenbeck, 2003; Ronquist *et al.*, 2011) on the CIPRES Science Gateway (Miller *et al.*, 2010), using the same partitions and models as in ML. Two MCMC analyses with four chains each were conducted for 20×10^6 generations with default priors. Parameter values and trees were sampled every 1000 generations. Tracer V 1.6 was used to check if the runs had converged and the initial 10% trees were discarded as burn in (http://tree.bio.ed.ac.uk/software/tracer/) and the maximum clade credibility topology was constructed from the remainder of trees using TreeAnnotator V 1.8.2 (both part of the BEAST package, Drummond *et al.*, 2012) with posterior probabilities along each node. Clades with posterior probabilities (BP) ≥ 0.95 were considered well supported (Larget & Simon, 1999).

Table 1. Taxa used in the present study, including information about the origin of the studied material, voucher information, as well as GenBank accession numbers. New sequences in bold face

Taxon	*Origin*	*Voucher*	*nrITS*	*rps4*	*rbcL*
P. abietina	Australia	Renner *et al.* 6977 (NSW848775)	**KX090153**	**KX090171**	**KX090165**
P. abietina	Australia	Renner *et al.* 7274 (NSW848776)	**KX090154**	**KX090172**	**KX090166**
P. abietina	Fiji	Renner *et al.* 5464 (NSW890131)	**KX090150**	–	**KX090162**
P. abietina	Fiji	Renner *et al.* 5486 (NSW890173)	**KX090151**	**KX090170**	**KX090163**
P. abietina	Fiji	Renner *et al.* 5778 (NSW895657)	**KX090152**	–	**KX090164**
P. adianthoides	Costa Rica	Heinrichs *et al.* 4314 (GOET)	AJ422027	AY438204	DQ194108
P. andina	Bolivia	Heinrichs & Müller 4046 (GOET)	DQ194028	DQ193974	DQ194111
P. arbuscula	Japan	Yamaguchi 18280 (HIRO)	AY550131	AY547692	DQ194112
P. asplenioides	Italy	Schäfer-Verwimp & Verwimp 35859 (M)	KT992544	KT992688	KT992617
P. austinii	USA	Risk 10849 (Duke)	AJ748130	AY608099	DQ439699
P. bantamensis	Japan	Yamaguchi 16890 (HIRO)	AY275160	AY547695	DQ194084 & DQ194070
P. barteri	Malawi	O’Shea M7062a (GOET)	AJ866749	AJ866764	–
P. carringtonii	Bhutan	Long 28857 (GOET)	AJ414631	AY438209	DQ194121
P. carringtonii	Great Britain	Rycroft 00041 (GOET)	AJ414630	–	–
P. colorans	Rwanda	Buchbender & Fischer 1115 (GOET)	AJ866751	AJ866765	DQ194123
P. corrugata	Brazil	Lüth 3490 (GOET)	AJ744788	–	–
P. deflexirama	Costa Rica	Heinrichs 11 (GOET)	AY550135	AY547698	DQ194128
P. disticha	Ecuador	Holz 436 E/5-01 (GOET)	AJ422014	AY438214	DQ194130
P. divergens	Kenya	Solga s.n. (GOET)	DQ194027	DQ193981	DQ194102
P. ericicola	Tanzania	Pócs *et al.* 87172/S (GOET)	AJ866748	–	–
P. fastigiata	Mexico	Gradstein 8274 (GOET)	AJ744790	DQ193989	DQ194139
P. flexuosa	Japan	Kurita 147 (HIRO)	AY550138	AY547703	DQ194140
P. frondescens	Indonesia	Schäfer-Verwimp & Verwimp 20704 (GOET)	AY438237	AY438219	DQ194141
P. fruticosa	India	Long 23002 (GOET)	AY438235	AY438217	–
P. fusifera	Seychelles	Pócs 9342/B (GOET)	AJ866746	AJ866760	DQ194145
P. hampeana	Indonesia	Gradstein 11036 (GOET)	**KX090149**	–	**KX090161**
P. hakkodensis	Japan	Yamaguchi 12271 (HIRO)	AY275164	AY547705	DQ194149
P. heterostipa	Malawi	O’Shea M7070a (GOET)	AJ866735	AJ866757	DQ194080 & DQ194066
P. incerta	Madagascar	Pócs 9447/L (GOET)	AJ866737	AJ866761	DQ194101
P. integerrima	Malawi	O’Shea M7552a (GOET)	AY275166	AY547707	DQ194095
P. javanica	Indonesia	Gradstein 10209 (GOET)	AJ744791	DQ193998	DQ194154

Taxon	*Origin*	*Voucher*	*nrITS*	*rps4*	*rbcL*
P. korthalsiana	Indonesia	Gradstein 10258 (GOET)	DQ194049	DQ194000	DQ194156
P. laetevirens	Ecuador	Sauer MS86 (GOET)	AJ744792	–	–
P. magna	Japan	Kurita 258 (HIRO)	AY275167	AY438221	DQ194162
P. montagnei	French Guiana	Holz FG0049 (GOET)	AJ744793	–	–
P. orbicularis	Japan	Kurita 132 (HIRO)	AY275168	AY438222	DQ194167
P. ovalifolia	Japan	Ohnishi 5723 (HIRO)	AY275169	AY547711	–
P. patula	Jamaica	Schäfer-Verwimp 34919 (M)	**KX090155**	–	–
P. patula	Jamaica	Schäfer-Verwimp 34940 (M)	**KX090156**	**KX090173**	**KX090167**
P. patula	USA	Düll 2331-1 (hb Schäfer-Verwimp)	**KX090157**	**KX090174**	–
P. patula	Ecuador	Schäfer-Verwimp & Verwimp 33171 (hb S-V)	**KX090158**	**KX090175**	**KX090168**
P. patula	Dominican Republic	Schäfer-Verwimp & Verwimp 26997 (hb S-V)	**KX090159**	**KX090176**	**KX090169**
P. patula	Domincan Republic	Schäfer-Verwimp & Verwimp 26938 (hb S-V)	**KX090160**	**KX090177**	–
P. patula	Ecuador	Sauer MSE063 (GOET)	AJ744794	–	–
P. peculiaris	Bhutan	Long 28832 (GOET)	AY550141	AY547716	DQ194176
P. poeltii	India	Long 22802 (GOET)	AY550142	AY547717	DQ194177
P. porelloides	Madeira	189207 (LISU)	DQ159994	–	–
P. pulcherrima	Japan	Ohnishi 5771 (HIRO)	AY438239	AY438223	DQ194179
P. raddiana	Ecuador	Holz 045 E/5-01 (GOET)	AJ422020	AY438225	DQ194181
P. renitens	Malaysia	Schäfer-Verwimp & Verwimp 18736/A (GOET)	AY569441	AY569440	DQ194184
P. sandei	Indonesia	Gradstein 9970 (GOET)	AJ414634	AY438228	DQ194191
P. sciophila	Japan	Ohnishi 5400 (HIRO)	AY275171	AY547724	DQ194193
P. semidecurrens	Nepal	Long 21348 (GOET)	AY275172	AY438227	DQ194194
P. squamulosa	Kenya	Chuah 0310/AB (GOET)	AJ744796	AJ866758	DQ194098
P. streimannii	Indonesia	Gradstein 10309 (GOET)	AJ866744	AJ866763	DQ194197
P. strictifolia	Malawi	Hodgetts M2490a (GOET)	AJ866734	AJ866759	–
P. subplana	French Guiana	Holz FG 32 (GOET)	AY275174	AY438224	DQ194199
P. subtropica	Nepal	Long 17359 (GOET)	AY550145	AY547726	DQ194200
P. tamariscina	Ecuador	Sauer MS165 (GOET)	AJ744799	–	–
P. teysmannii	Indonesia	Gradstein 10308 (GOET)	AJ866745	AJ866762	DQ194203
P. trabeculata	Japan	Kurita 257 (HIRO)	AY550146	AY547727	DQ194204
P. turgida	Ecuador	Holz 070 E/5-01 (GOET)	AJ422024	AY547729	DQ194206
P. virginica	Tenerife	Rycroft 01068 (GOET)	AJ413311	–	–

RESULTS

The nrITS alignment consisted of 757 base pairs (bp), *rps*4: 573 bp and *rbc*l: 1219 bp. Maximum likelihood and Bayesian analyses of this data resulted in largely similar topologies; hence only the ML topology is depicted, with likelihood bootstrap and Bayesian posterior probabilities indicated on branches (Fig. 1). The backbone of the phylogeny is largely unresolved whereas the sectional clades achieve BVs from 84 to 100 % and BPs of 0.99 or 1.00. *Plagiochila* sect. *Vagae* is placed sister to *P*. sect. *Africanae* (BV 79, BP 1.00). *Plagiochila* sect. *Abietinae* (*P. abietina*, *P. hampeana*) is resolved outside *P*. sect. *Vagae* and has a sister relationship with *P*. sect. *Poeltiae* (BV 60, BP 0.78). A clade with members of *P*. sects. *Abietinae*, *Fruticosae, Peculiares*, *Plagiochila*, *Poeltiae* and *Trabeculatae* receives a BV of 59 and a BP of 1.00. *Plagiochila hampeana* is placed sister to a clade with five accessions of *P. abietina* (BV 100, BP 1.00). The latter splits into an Australian clade (BV 99, BP 1.00) and a clade with accessions from the Fiji Islands (BV 100, BP 1.00). Six accessions of *P. patula* form a monophyletic lineage (BV 90, BP 1.00) within a derived clade of *P*. sect. *Vagae*, with one accession from Ecuador placed sister to a clade with accessions from the Dominican Republic, Jamaica, and U.S.A. (BV 100, BP 1.00).

DISCUSSION

Our results indicate that the Asian-Australasian *P*. sect. *Abietinae* (Figs 2-7) is not an element of the pantropical *P*. sect. *Vagae* as proposed by Söderström *et al.* (2015) based on the results of Groth (2006), and thus needs to be reinstated. The *P. abietina* sequence data of Groth (2006) may have been the result from a lab contamination or misidentified material. Carl (1931) considered *Abietinae* one of the morphologically best circumscribed sections of *Plagiochila* and pointed to the pinnate habit of the shoots and the postically secund leaves with spinose marginal teeth, a basal vitta and distinct, bulging trigones (Figs 2-7). These characters resemble members of *P*. sects. *Arrectae* Carl, *Durae* Carl and *Peculiares* rather than *Vagae*; however, members of the former three sections have intercalary branches (Heinrichs, 2002; Groth, 2006). *Abietinae* and *Vagae* share the frequent presence of terminal branches, yet perianths of *Abietinae* are unwinged and propagules are lacking; *Vagae* leaves are usually neither unilaterally inserted, spinosely dentated nor provided with a vitta. So & Grolle (2001) revised *P*. sect. *Abietinae* and included species with propagules (*P. exinnovata* Steph., *P. norfolkiensis* Steph.), however, these species hardly belong to *Abietinae* and may be members of *Vagae*. The same may hold true for the putative *Abietinae* members *P. cymata* Inoue & Grolle and *P. ulata* Inoue & Grolle (So & Grolle, 2001).

Members of *Abietinae* are covered with variously shaped paraphyllia (Figs 3, 7; Inoue, 1984) but these occur also in some members of sect. *Vagae* (e.g., *P. streimannii* Inoue), sect. *Fruticosae* Inoue (*P. pulcherrima* Horik.), subsect. *Caulimammillosae* Grolle & M.L.So (e.g., *P. aspericaulis* Grolle & M.L.So) (So, 2001), sect. *Fuscoluteae* Carl (e.g. *P. paraphyllina* Herzog, Heinrichs, 2002) and others. The character states “presence/absence of paraphyllia” and “presence/absence of terminal branches” demonstrate extensive morphological homoplasy which hampers a comprehensive sectional classification of *Plagiochila* based on morphological evidence (Söderström *et al.*, 2016). The introduction of geographically

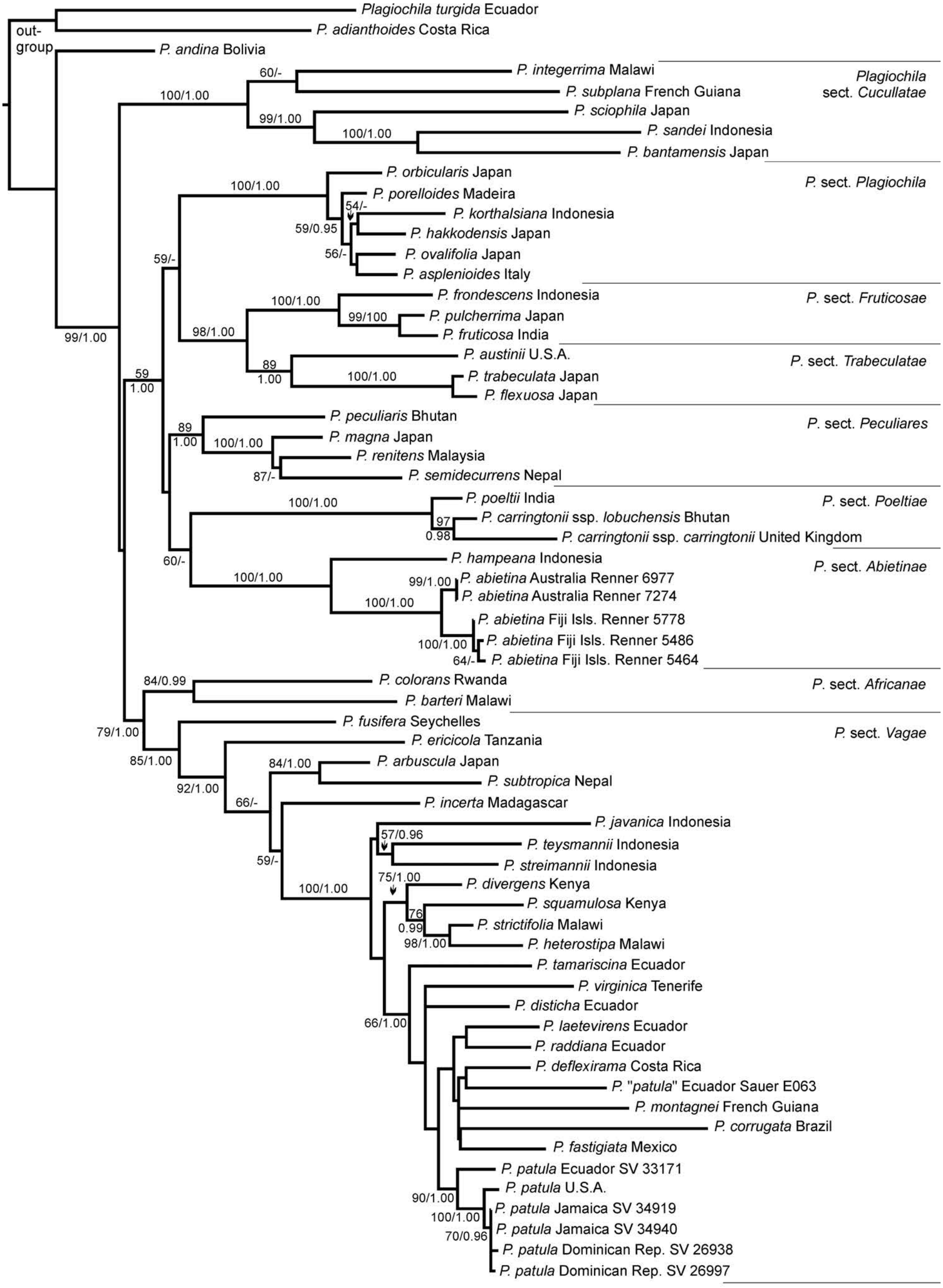
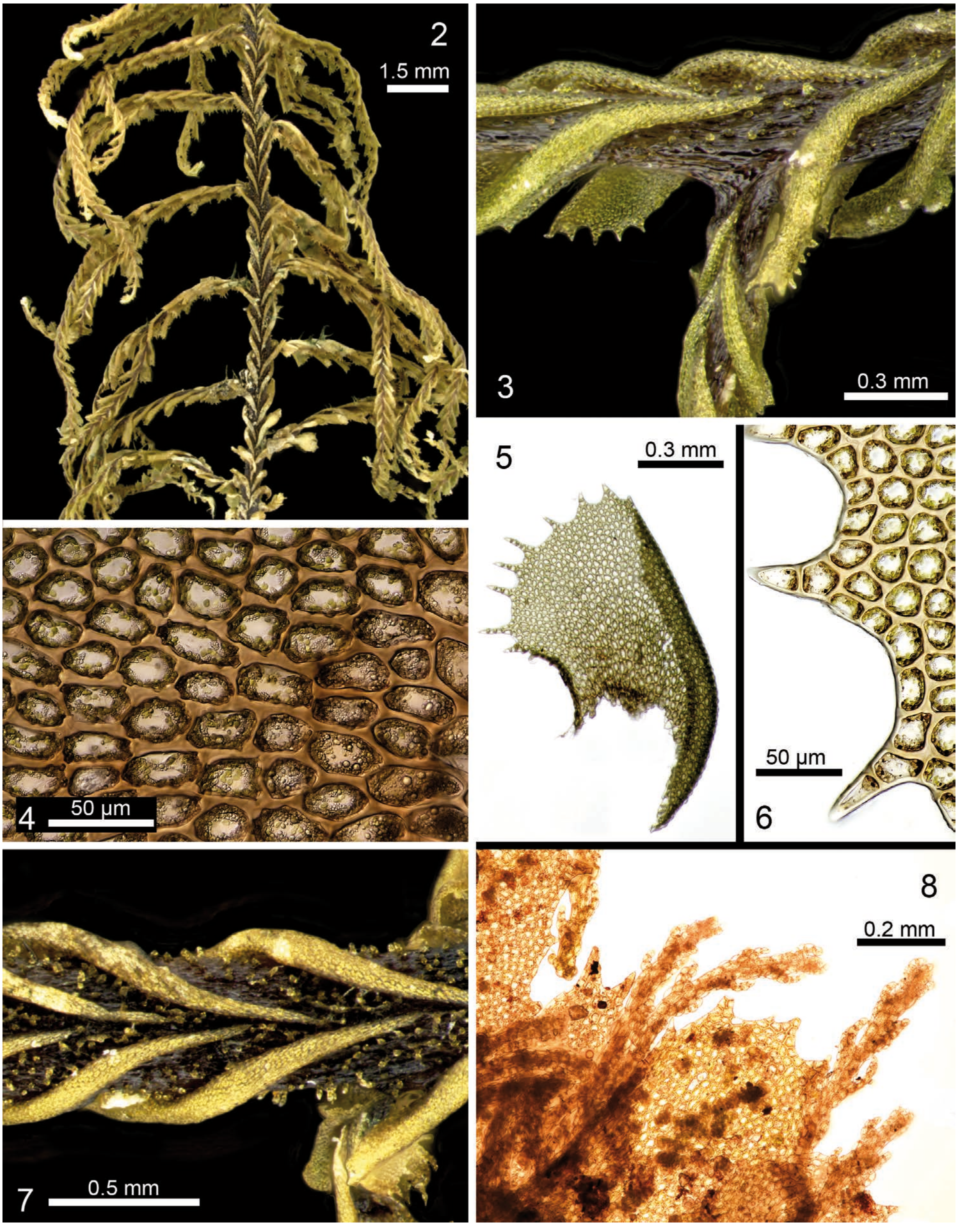


Fig. 1. Maximum likelihood phylogeny of *Plagiochila* sect. *Vagae* and allies based on a concatenated nrITS, cp *rps*4 and *rbc*L sequence alignment. Bootstrap percentage values ≥ 50 and Bayesian posterior probabilities ≥ 0.95 are indicated on branches.



Figs 2-8. **2-7.** *Plagiochila* (sect. *Abietinae*) *abietina* (Australia, Renner 6977). **2.** Part of shoot in dorsal view**. 3**. Part of shoot with a lateral-terminal branch in dorsolateral view. **4.** Elongate lamina cells at leaf base. **5.** Leaf. **6.** Part of dorsal leaf margin. **7.** Part of shoot in dorsal view with numerous spine-like paraphyllia. **8.** *Plagiochila* (sect. *Vagae*) *patula* (Dominican Republic, Schäfer-Verwimp & Verwimp 26997): upper parts of leafs in ventral view with numerous leaf-borne propagules and plantlets.

separated sections (Carl, 1931) was also not confirmed by molecular data (Groth *et al.*, 2003) and is a further obstacle to a morphology-based supraspecific classification.

Not only is the supraspecific classification of *Plagiochila* in a very preliminary state but so is the circumscription of species. Including multiple accessions of *P. abietina* in our phylogenetic analyses (Fig. 1) provides evidence for two geographically separated clades. Luxuriant phenotypes of *P. patula* from the West Indies are placed sister to a minute sized phenotype from Florida, USA, whereas another luxuriant phenotype from Ecuador is placed sister to the West Indian-North American clade. Another Ecuadorian specimen that was earlier identified as *P. patula* is placed in some distance to the above accessions and may belong to another species. It may be that West Indian representatives are not conspecific with morphologically similar Andean plants, however, a comprehensive geographical sampling is necessary to arrive at more reliable hypotheses. A first step towards a better understanding of *Plagiochila* species is the inclusion of type or at least topotype material in molecular investigations. In the case of *P. patula* we were able to include two accessions from Jamaica, from where Swartz described this species in the 18th century (Heinrichs *et al.*, 1998). We consider these sequences suitable for barcoding purposes (Will *et al.*, 2005); however, the exact morphological variation and geographical range of *P. patula* still needs to be reconstructed by an integrative study. Our ongoing work on Australasian, African and Neotropical *Plagiochila* provides evidence for frequent incongruence of current morphological species concepts and sequence data. A deeper understanding of the global diversity of *Plagiochila* thus needs inclusion of several thousand accessions which should be studied both morphologically and molecularly (Patzak *et al.*, 2016).

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