

NrITS sequences and morphology indicate a synonymy of the Patagonian *Plagiochila rufescens* Steph. and the Central American *Plagiochila bicuspidata* Gottsche

Henk GROTH, Florian A. HARTMANN,
Rosemary WILSON & Jochen HEINRICHS*

Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Abteilung Systematische
Botanik, Untere Karspüle 2, D-37073 Göttingen, Germany

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Abstract – A maximum likelihood analysis is performed of 20 nrITS sequences of representatives of *Plagiochila* sect. *Arrectae* and *P.* sect. *Rutilantes*. *Plagiochila rufescens* from Chile and *P. bicuspidata* from Costa Rica form a well supported monophyletic lineage within the *P.* sect. *Rutilantes*. A sequence of *P. exigua* (sect. *Rutilantes*) from the British Isles composes with *P. caduciloba*, *P. cuneata* and *P. steyermarkii* a clade that is clearly separated from the *P. bicuspidata* clade.

As a result of the molecular investigation, *P. rufescens* is excluded from the synonymy of *P. exigua* and placed in the synonymy of *P. bicuspidata*. Several years old herbarium specimens of *P. bicuspidata* from Costa Rica and Chile still exhibit a distinct odour of peppermint which is unknown from European *P. exigua*. Only vigorous plants of *P. bicuspidata* can be separated from *P. exigua* by morphological characters.

Jungermanniales / Plagiochilaceae / *Plagiochila* sect. *Rutilantes* / *Plagiochila bicuspidata* / *Plagiochila exigua* / *Plagiochila rufescens* / nuclear ribosomal DNA / internal transcribed spacer

INTRODUCTION

Species of *Plagiochila* are notorious for extensive variation in gametophytic characters, in particular leaf shape and dentition. However, identification keys usually focus on leaves and so species concepts are largely based on leaf characters. A taxonomically difficult group in this regard is the *P.* sect. *Rutilantes* Carl that was originally set up for a small group of Neotropical Plagiochilae (Carl, 1931). Groth *et al.* (2002) demonstrated that members of *P.* sect. *Caducilobae* Inoue (Inoue, 1975), which include some of the tiniest species of *Plagiochila* with frequent vegetative reproduction by caducous or fragmenting leaves such as *P. exigua* (Taylor) Taylor and *P. bicuspidata* Gottsche, belong to the *Rutilantes*. As a result, the formerly purely neotropical section covers nearly the complete geographic range of *Plagiochila*. Leaves of the former *Caducilobae* are small, variable in shape and are often beset with only a few teeth. In many specimens, most

* Correspondence and reprints: jheinri@gwdg.de

stems are completely denuded. The taxonomic significance of these reduced forms is extremely difficult.

Inoue (1980) established a wide species concept for *Plagiochila exigua* [as *P. corniculata* (Dum.) Dum.] and extended the range of this originally holarctic species (e.g., Schuster, 1959) to Africa and South America. He proposed a synonymy of *P. exigua* and the Patagonian *P. rufescens* Steph., a taxon with hitherto uncertain sectional placement (Carl, 1931: 142). However, acceptance of a wider phenotypic variation and of wide ranges holds the danger of creating non-monophyletic units. Hässel de Menendez (1983) kept both taxa separate and doubted the occurrence of *P. exigua* [as *P. corniculata*] in Southern South America. Recently (courtesy of Uwe Drehwald, Göttingen) fresh material of Chilean *P. rufescens* became available for study. The moistened plants exhibited a distinct odour of peppermint, which is well known from *P. rutilans* and its varieties (Heinrichs *et al.*, 2001; Heinrichs *et al.*, 2002a; Rycroft & Cole, 2001; Rycroft, 2003) as well as from further neotropical representatives of the sect. *Rutilantes* (*P. bicuspidata*, *P. cuneata* Lindenb. & Gottsche, and *P. loriloba* Herzog ex Carl; Groth *et al.*, 2002) but not from *P. exigua* (e.g., Rycroft *et al.*, 1998; Anton, 2001).

The observation of the peppermint odour led to a closer investigation of *P. rufescens* and its allies.

MATERIALS AND METHODS

DNA extraction – Upper parts of few shoots were isolated from *Drehwald 3201* and *Drehwald & Drehwald 4729* (GOET) and extracted with Invisorb Spin Plant Mini Kit (Invitex).

PCR-amplification – The 5'- primer Hep2f (5'-GAG TCA TCA GCT CGC GTT GAC -3') and the 3' primer HepCr (TCT CCA GAC TAC AAT TCG CAC A) were used to amplify the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA containing ITS1, ITS2 and the 5.8S gene. Polymerase chain reaction (PCR; Saiki *et al.*, 1988) was performed in a total volume of 50 µl, containing one unit Taq-DNA-polymerase (SilverStar, EuroGenTech), 5 µl Taq polymerase reaction buffer (EuroGenTech), 2 µl MgCl₂ (50 mM), 1 µl dNTP-mix (10 mM, MBI Fermentas), 2 µl dimethylsulfoxid, 1 µl of forward and reverse primer each (10 mM), and 1 µl template. PCR was carried out using the following program: 120s 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 in one step (10 min 72°C). Sequencing was carried out on an ABI 3100 capillary sequencer using the BigDye™ Terminator Cycle Sequencing v2.0 kit (PE Biosystems).

Phylogenetic analyses – 20 nrITS sequences of representatives of *Plagiochila* sects. *Arrectae* Carl, *Fuscoluteae* Carl and *Rutilantes* Carl from previous studies (Groth *et al.*, 2002; Heinrichs, 2002; Heinrichs *et al.*, 2002a) as well as new nrITS sequences of *Plagiochila gymnocalycina* and *P. rufescens* (= *P. bicuspidata*, see below) were used for the phylogenetic analyses (Table 1). The lacking ITS2 or partial ITS2 sequences of *P. bicuspidata* and of *P. caduciloba* were coded as “missing data”.

The ITS1 and ITS2 sequences were aligned manually in BioEdit version 5.0.9 (Hall, 1999), resulting in a dataset including 604 putatively homologous sites

Table 1. Geographic origins, voucher numbers, and GenBank/EMBL accession numbers of the investigated taxa. If not indicated otherwise, vouchers are deposited at GOET; duplicates of those marked with an asterisk (*) were distributed in “Bryophyta Exsiccata Generis Plagiochilae” (BEGP; Heinrichs & Anton, 2001).

<i>Taxon</i>	<i>Origin</i>	<i>Voucher</i>	<i>Accession number</i>
<i>P. aerea</i> Taylor	Costa Rica	Heinrichs et al. 4321, B.E.G.P. 95	AJ422028
<i>P. bicuspidata</i> Gottsche	Costa Rica	Holz s.n.	AF539457
<i>P. bicuspidata</i> Gottsche	Chile	Drehwald 3201	AY390532
<i>P. bidens</i> Gottsche	Brazil	Gradstein 5378 (G)	AF539458
<i>P. bifaria</i> (Sw.) Lindenb.	Tenerife	Drehwald 3922	AJ413173
<i>P. caduciloba</i> H.L.Blomq.	U.S.A.	Schuster 39376a	AF539459
<i>P. cuneata</i> Lindenb. & Gottsche	Bolivia	Heinrichs et al. 4093	AF539460
<i>P. deflexa</i> Mont. & Gottsche	Costa Rica	*Heinrichs & al. 4160, B.E.G.P. 10	AJ416083
<i>P. exigua</i> (Taylor) Taylor	Ireland	Hakelier s.n.	AF539461
<i>P. fuscolutea</i> Taylor	Costa Rica	*Heinrichs & al. 4400, B.E.G.P. 148	AJ416086
<i>P. gymnocalycina</i> (Lehm. & Lindenb.) Mont.	Bolivia	*Drehwald & Drehwald 4729, B.E.G.P. 153	AY390531
<i>P. loriloba</i> Herzog ex Carl	Costa Rica	Holz CR-00-466	AF539464
<i>P. patzschkei</i> Steph.	Ecuador	Holz EC-01-389	AJ422018
<i>P. punctata</i> (Taylor) Taylor	British Isles	Rycroft 01013	AJ413174
<i>P. retrorsa</i> Gottsche	Costa Rica	Heinrichs & al. 4154	AJ422021
<i>P. rutilans</i> Lindenb. var. <i>moritziana</i> (Hampe) J.Heinrichs	Ecuador	*Holz EC-01-404, B.E.G.P. 131	AJ416080
<i>P. rutilans</i> Lindenb. var. <i>rutilans</i>	Bolivia	Groth 101	AJ416081
<i>P. rutilans</i> Lindenb. var. <i>standleyi</i> (Carl) J. Heinrichs & D.S. Rycroft	Ecuador	*Holz EC-01-404, B.E.G.P. 131	AJ416079
<i>P. spinulosa</i> (Dicks.) Dumort.	British Isles	Rycroft 01012	AJ413175
<i>P. stricta</i> Lindenb.	Ecuador	Holz EC-01-478	AJ416647
<i>P. trichostoma</i> Gottsche	Costa Rica	*Heinrichs et al. 4324, B.E.G.P. 56	AJ416028

(alignment available from JH). Previous analyses (e.g., Groth *et al.*, 2002; Heinrichs, 2002) demonstrated the sister-group relationship of *Plagiochila* sects. *Arrectae* and *Rutilantes*. Hence, *P. aerea* Taylor and *P. fuscolutea* Taylor (*P. sect. Fuscoluteae*) were used as outgroups in the phylogenetic analyses.

Phylogenetic trees were inferred using maximum likelihood (ML) criteria as implemented in PAUP* version 4.0b10 (Swofford, 2003). To decide on the evolutionary model which best fits the data, the program Modeltest 3.06 (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 hierarchical 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). A maximum likelihood analysis with the HKY85+G model was implemented as heuristic search with 10 random addition sequence replicates, branch swapping algorithm TBR and MULTREES

on. The confidence of branching was assessed using 100 bootstrap resamplings in ML-analysis (using the HKY85+G model, heuristic search with 10 addition sequence replicates),

RESULTS

Molecular investigation - The ingroup sequences are separated into two well supported clades assignable to *Plagiochila* sect. *Rutilantes* and *P.* sect. *Arrectae* (Fig. 1). The *P.* sect. *Rutilantes* clade is subdivided into three subclades. *Plagiochila rutilans* and its two varieties form a subclade with *P. deflexa* Mont. & Gottsche; the topology has no bootstrap support. *Plagiochila trichostoma* Gottsche is placed as sister to *P. gymnocalycina* (Lehm. & Lindenb.) Mont. (bootstrap support of 92). The former *Caducilobae* (*P. bicuspidata*, *P. caduciloba* H.L.Blomq., *P. cuneata* Lindenb. & Gottsche, *P. exigua*, *P. loriloba* Herzog ex Carl, *P. steyermarkii* H.Rob.) form a third, well supported subclade (bootstrap support of 95).

P. rufescens is placed sister to *P. bicuspidata* (bootstrap support of 100) and clearly separated from *P. exigua*. The topology throws doubt on a conspecificity of *P. exigua* and *P. rufescens* and points at a possible synonymy of *P. rufescens* with *P. bicuspidata*.

Morphology - Mature shoots of *P. rufescens* have leaves that are in all slightly longer than those of typical *P. exigua* and dorsally at least shortly decurrent (Fig. 2, A-C, E; leaves of *P. exigua* usually not decurrent). These plants fully agree with Central American *P. bicuspidata*. Weak shoots of *P. rufescens* and of *P. bicuspidata*, especially those which are frequently provided with caducous leaves, are morphologically inseparable from European *P. exigua*. However, 6 years old moistened material of *P. rufescens* from Chile and 3 years old moistened material of *P. bicuspidata* from Costa Rica exhibit a distinct odour of peppermint that has never been reported from holarctic *P. exigua* (e.g., Schuster, 1959; Rycroft *et al.*, 1998; Anton, 2001).

As a result of the molecular and the morphological investigations, *P. rufescens* is excluded from the synonymy of *P. exigua* (Inoue, 1980) and placed in the synonymy of *P. bicuspidata*:

Plagiochila bicuspidata Gottsche, *Mexik. Leverm.*: 43. 1863; *P. cuneata* Lindenb. & Gottsche var. *bicuspidata* (Gottsche) Herzog, *Hedwigia* 74: 87. 1934. **Type**: Mexico, Oaxaca, "Sempoaltepec, 6/42, *Liebmann 581b* (10283) p.p." (lectotype, designated by Grolle & Heinrichs 1999: 523. C [c.per.] !).

= *Plagiochila rufescens* Steph., *Bih. Kongl. Svenska Vetensk.-Akad.* 26 (III, 6): 32. **Type**: Chile, 1897, *Dusén 270* (holotype, G 20878 [male] !).

= *Plagiochila cuneata* Lindenb. & Gottsche f. *defolians* Herzog, *Rev. Bryol. Lichénol.* 23: 32, *nom. inval.* [Art. 32.1(c)]. Original material: Chile, Fray Jorge, *Schwabe 196 p.p.* (JE [ster.] !)

Gametophyte – **Plants** delicate, 5-50 mm long and 0.5-2.6 mm broad, in thin patches or mats or growing isolated among other bryophytes and lichens, with short creeping stoloniform shoots giving rise to ascending or prostrate aerial shoots, fresh with distinct odour of peppermint. **Stems** brown, upper parts often

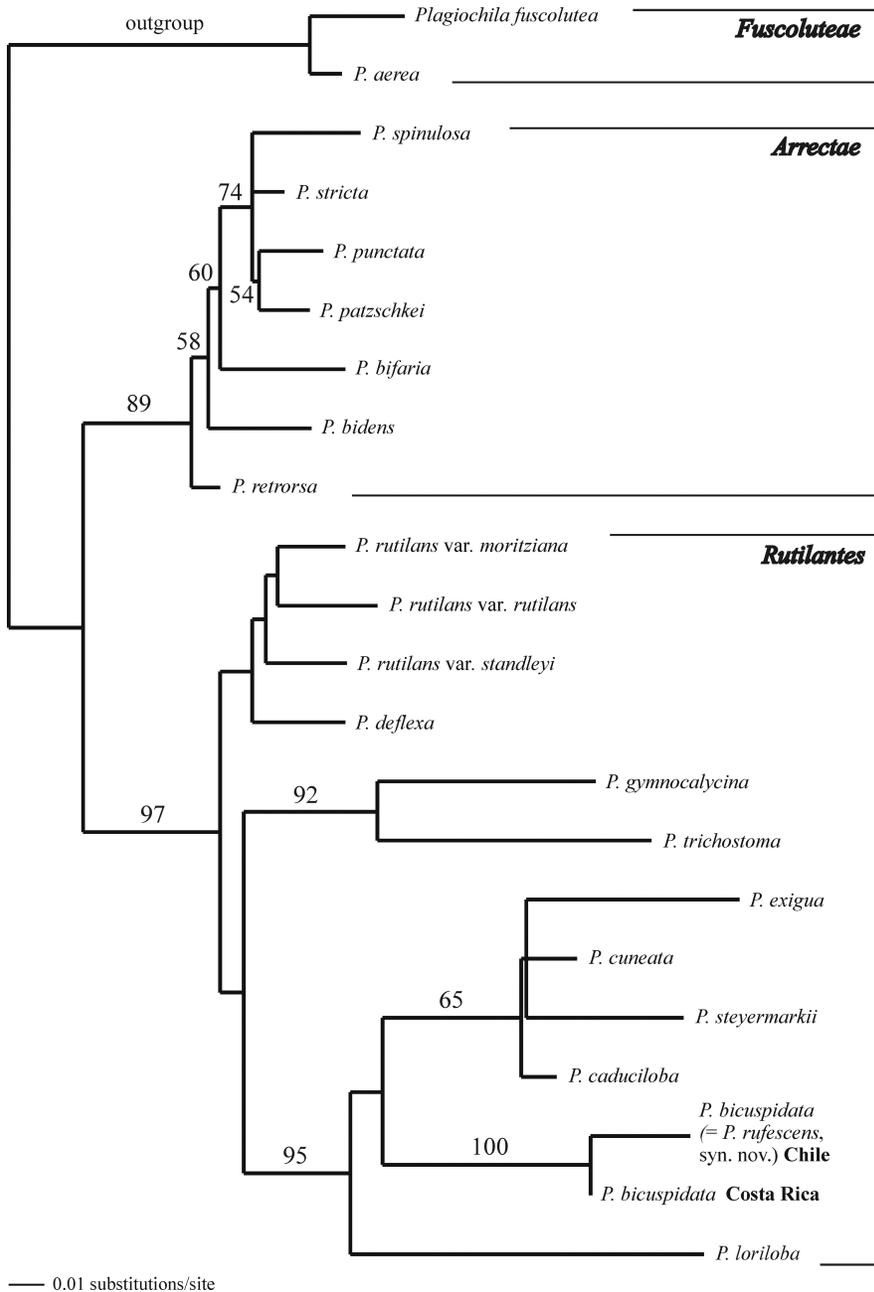


Fig. 1. Molecular phylogeny of *Plagiochila* species based on nrITS1 and ITS2 DNA sequence comparisons using 604 aligned positions. The rooted tree resulted from a maximum likelihood analysis based on the HKY+G model with estimated gamma shape ($G = 0.555$, and $Ti/Tv = 1.796$). Bootstrap percentage values ($> 50\%$) were determined for maximum likelihood (using HKY85+G) method.



Fig. 2. *Plagiochila bicuspidata* Gottsche: **A**, **B**, parts of shoots, dorsal view; **C**, part of shoot with lateral-intercalary branch, ventral view; **D**, top of female plant with sporophyte, dorsal view; **E**, top of male plant with androecium, ventral view [A from lectotype of *P. bicuspidata* (C); B, C from *Drehwald 3201*, Chile (GOET); D from *Heinrichs et al. AHH 150*, Costa Rica (GOET), E from holotype of *P. rufescens* (G)].

pale brown, pale yellow or pale green, dorsoventrally flattened, widely exposed both dorsally and ventrally, near base *ca* 70-130 × 90-160 μm thick, in cross section *ca* 6-10 cells across, the cortical cells in (1-)2(-3) layers, *ca* 7-14 × 10-22 μm, moderately to distinctly thick-walled (in weak shoots often only slightly thick-walled), medullary cells *ca* 14-25 × 18-35 μm, thin-walled or slightly thick-walled, trigones lacking or small, triangular. **Branches** lacking or sparse, of the lateral intercalary type. Short dorsal and ventral sectors of aerial shoots occasionally with rhizoids.

Leaves alternating, remote (below perianths occasionally imbricate), wide spreading or weakly bent to the ventral side, occasionally transversely inserted, at *ca.* 45-90° with stem, oblong-elliptical, oblong, occasionally slightly obovate, *ca* 0.3-1.1(-1.5) mm long and 0.2-0.5(-0.6) mm broad, *ca* 1.2-3.0(-4.2) times as long as wide, dorsally hardly to moderately decurrent, ventrally hardly to shortly decurrent, 0.15-0.30(-0.50) bilobed (some leaves occasionally with three lobes), the lobes broadly to elongate triangular, straight or curved, often with a short, blunt apical cell, ventral margin with 0-2 teeth, dorsal margin with 0-1 tooth, teeth triangular, with an elongate or short, acute or blunt terminal cell.

Leaf areolation regular to irregular, cells in leaf center mostly +/- isodiametric with scattered elongate cells or cells broader than long, *ca* 15-32 × 16-43 μm, 0.7-1.6(-2.2) times as wide as long, cells towards leaf base in all slightly more elongate, especially in distinctly elongate leaves. **Trigones** (small to) medium sized to large, (triangular to) subnodulose to nodulose, rarely confluent, intermediate thickenings rarely present on long cell walls; cuticle smooth. Oil bodies small, globose to ellipsoideal, homogeneous, colourless, *ca* 9-20 per cell. Underleaves small, vestigial, built by 1-4 short cilia often terminated by slime papillae.

Androecia simple, becoming intercalary, bracts in 4-11(-15) pairs, imbricate to remote, not or only weakly overlapping dorsally, inflated basally, basal part mostly composed of hyaline, thin-walled cells, apical part obliquely spreading, dentition similar to that of leaves. Antheridia usually 1 per bract.

Gynoecea terminal, subgynoeceal innovations frequent in absence of fertilization, 1-2(-3) in number; bracts larger than the subtending leaves and with up to 5 teeth on ventral margin, often inserted in some distance to the perianth; **perianth** elongate elliptical in dorsal view, cylindrical in lateral view, unwinged but dorsally occasionally with a low ridge, mouth moderately arched upwards, with elongate triangular to ciliate teeth, occasionally in addition with some long, slender lobes.

Sporophyte – Capsule exceeding the perianth 1.2-2.1 mm, +/- globose; valves straight, not twisted, 0.25-0.35 mm broad and 0.8-1.0 mm long; epidermal cells in surface view slightly broader than long to elongate, with 1-3 thickenings on most walls; innermost layer composed of +/- isodiametric to elongate, partly transversally directed cells with 1-4 nodulose thickenings on most walls; valves in cross section 40-60 μm thick, 3-4 stratose, epidermal cells slightly larger than inner ones, 20-30 μm thick. **Spores** globose, baculate, *ca* 13-20 μm in diameter. Elaters 5-9 μm in diameter, with one spiral band, central portion occasionally with two spiral bands.

Selected specimens examined: MEXICO. MEXICO: 27 km N of Mexico-City, near Passo Puerto de la Cruces, 3000 m, 1975, *Düll 2/333* (JE). COSTA RICA. CARTAGO: P. N. Volcán Irazu, 3300 m, 1999, *Holz CR 99-354* (GOET); SAN JOSÉ: Cordillera Talamanca, Páramo Buena Vista, 3200 m, 1999, *Heinrichs et al. AHH 150* (GOET). VENEZUELA. MERIDA: WSW of Santo Domingo, 3200 m, 1982, *Liesner 13836* (U). CHILE. AISÉN:

Carretera Austral, La Junta, 1997, *Drehwald 3201* (GOET); **COQUIMBO**: Loma Frai Jorge, 670 m, 1917, *Skottsberg & Skottsberg 57* (JE).

Plagiochila bicuspidata grows scattered in mountainous regions of Central America and Venezuela. In addition, the species occurs in Southern South America where it is locally abundant (Drehwald, pers. com. 2003). Most likely *P. bicuspidata* is more widespread in South America but a search in the extensive *Plagiochila* indet. collections of U and GOET remained unsuccessful. Specimens from Colombia proved to be wrongly identified.

Beside *P. exigua*, *Plagiochila bicuspidata* is mostly confused with *P. papillifolia* Steph. and *P. cuneata* Lindenb. & Gottsche. *P. papillifolia* can easily be separated by its papillose leaves (Heinrichs *et al.*, 2002b; leaves of *P. bicuspidata* smooth). Mature leaves of *P. cuneata* Lindenb. & Gottsche are provided with 2-3 elongate, often fragmenting apical lobes (leaves of *P. bicuspidata* usually with only 2 lobes; lobes usually not fragmenting).

DISCUSSION

Molecular methods become increasingly important for testing the monophyly of morphologically defined species. Several case studies have demonstrated the polyphyly of morphologically outlined moss species (e.g., *Fontinalis antipyretica* Hedw. (Shaw & Allen, 2000); *Hygroamblystegium tenax* (Hedw.) C. Jens. (Vanderpoorten *et al.*, 2001)) and pointed out the necessity to test present species concepts. However, several studies in *Plagiochila* confirm the ranges of species that were circumscribed by morphological characters (e.g., *P. bifaria* (Sw.) Lindenb. (Heinrichs, 2002); *P. carringtonii* (Balf.) Grolle (Renker *et al.*, 2002); *P. stricta* Lindenb. (Rycroft *et al.*, 2002); overview in Groth *et al.*, 2003).

Plagiochila exigua is the first species of this numerous genus whose synonymy and range is corrected as an outcome of a molecular study. Rycroft *et al.* (1998) pointed out that Peruvian *P. exigua* (Asakawa & Inoue, 1987) shows considerable differences to plants from the British Isles, throwing doubt on an occurrence of the species in tropical America. Unfortunately no sequencable material of *Plagiochila exigua* was available from outside Europe. Hence, an advanced discussion of the range of this morphologically strongly reduced species is regarded to be premature at the moment.

Although *P. exigua* is excluded from the bryophyte flora of Southern South America, the occurrence of *P. sect. Rutilantes* in the region is confirmed by the sequence of Chilean *P. bicuspidata*.

The three subclades of the *Rutilantes* lineage do not correspond with a certain morphology or chemistry. *Plagiochila gymnocalycina* (illustrated in Heinrichs *et al.*, 1998), which clusters with the type of the former *P. sect. Permista* Carl, *P. trichostoma* (Heinrichs *et al.*, 2002a) is often provided with caducous leaves and resembles members of the former *P. sect. Caducilobae*. Central American *P. deflexa*, which is placed as sister to *P. rutilans* is chemically nearly inseparable from European *P. exigua* (Anton *et al.*, 2001), which differs strongly in size, leaf shape and leaf dentition. Hence, no further subdivision of the *P. section Rutilantes* is undertaken.

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