

Molecular and morphological evidence for an intercontinental range of the liverwort *Lejeunea pulchriflora* (Marchantiophyta: Lejeuneaceae)

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Abstract Examination of type specimens and additional vouchers has revealed that the African *Taxilejeunea pulchriflora* and the tropical Asian *Lejeunea propagulifera* and *Lejeunea tamaspocsii* show considerable morphological overlap; however, morphological similarity does not necessarily indicate genetic similarity. Thus, sequences of two chloroplast regions (trnL-trnF, rbcL) and the nuclear ribosomal ITS region were generated from accessions of these taxa from Tanzania, Ethiopia, Thailand, Malaysia, and the Fiji Islands and integrated into an existing *Lejeunea* dataset. Phylogenetic analyses of the three-marker dataset resolved the three taxa in a robust monophyletic lineage and indicated relationships to Neotropical and Holarctic species. Based on the morphological similarities and the low molecular variation of the investigated accessions, we consider the three taxa as conspecific and propose two synonyms and a new combination, *Lejeunea pulchriflora*. *Lejeunea pulchriflora* is characterized by its asymmetrical leaf lobes, small, distant underleaves, toothed perianth

keels, and autoicy and ranges from tropical Africa to the Pacific region.

Keywords Integrative taxonomy · Paleotropics · Porellales · *Taxilejeunea* · *Trachylejeunea*

Introduction

Lejeuneaceae include a diverse array of epiphytes that grow on the bark of trunks, branches, and twigs but also on living leaves (Wilson et al. 2007b). They are especially common in humid tropical lowland forests, where up to 70 % of the liverworts present belong to this family (Cornelissen and Ter Steege 1989; Zartman 2003; Sonnleitner et al. 2009). The Lejeuneaceae crown group evolved largely in the age of the angiosperms (Feldberg et al. 2014), and it has been suggested that the rapid adaptation to new niches in broad-leaved, angiosperm-dominated forests led to enormous morphological homoplasy (Schuster 2001). Morphology-based phylogenetic data matrices of Lejeuneaceae indeed contain a high degree of homoplasy and are of limited usability for the classification of this family into genera and species (Gradstein et al. 2003). The problems in structuring the huge diversity of Lejeuneaceae is seen in deviating estimates of species diversity, with recent estimates ranging from 750 (Wilson et al. 2007a) to 1700 species (He and Zhu 2011). They are also evident when comparing the conflicting genus concepts of different authors (Spruce 1884–1885; Mizutani 1961; Schuster 1963). While some 100 genera have been proposed to accommodate species of Lejeuneaceae (Ahonen et al. 2003), only 68 were accepted by Gradstein (2013) based on a screening of the literature and application of the monophyly principle. Although this classification was published only recently, several genus concepts have already been changed, and several new supraspecific

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taxa have been proposed (e.g., Wang et al. 2014; Schäfer-Verwimp et al. 2014; Wei et al. 2014).

With some 300 species, *Lejeunea* Lib. is one of the largest genera of the Lejeuneaceae (Lee 2013) and probably the best example to illustrate the difficulties in establishing a reliable classification at the species level and above (Heinrichs et al. 2013). Currently, a broad genus concept of *Lejeunea* is used by most authors and some 25 genera are accepted as its synonyms (e.g., Grolle 1983; Reiner-Drehwald 2005a; Gradstein and Reiner-Drehwald 2007; Pócs 2011; Heinrichs et al. 2012; Ye et al. 2013). Several authors also included *Microlejeunea* Steph. and *Otigoniolejeunea* (Spruce) Schiffn. in *Lejeunea* (e.g., Mizutani 1961; Schuster 1980; Reiner-Drehwald and Ilkiu-Borges 2007), but recent molecular work supported their status as independent genera (Dong et al. 2013; Czumay et al. 2013; Wei et al. 2014). *Lejeunea* can be separated from these and other related genera by a lack of ocelli (Dong et al. 2013), but otherwise, the genus is known for its heterogeneous morphology and incongruences of morphology-based classifications and molecular phylogenies (Heinrichs et al. 2013). The classification of *Lejeunea* into the subgenera *Lejeunea* and *Crossotolejeunea* Spruce thus does not reflect morphological disparities yet addresses the two main clades found in molecular phylogenies. Species once treated as synonyms of a single *Lejeunea* binomial may in fact nest in these two main clades (Renner et al. 2011), as do elements initially assigned to a single supraspecific element. An extreme example in this regard is *Taxilejeunea* (Spruce) Schiffn. whose species are distributed over nearly all clades of *Lejeunea* (Heinrichs et al. 2013).

The high degree of morphological homoplasy in *Lejeunea* necessitates the inclusion of molecular data in reconstructions of relationships and the circumscription of species. These data allow to balance between different species concepts and to explore the genetic variation of individuals sharing a similar morphology (Shaw 2001; Heinrichs et al. 2009).

In the framework of a revision of Asian and African *Lejeunea* species, we extended the molecular dataset of Heinrichs et al. (2013) and revised several species with narrow distribution ranges. Based on the outcome of our study, we transfer the African *Taxilejeunea pulchriflora* Pearson to *Lejeunea* and propose its conspecificity with two Asian binomials.

Material and methods

Morphological investigation

Herbarium specimens of *Lejeunea* (incl. *Taxilejeunea*) were loaned from the Eszterházy Károly College herbarium (EGR), the Conservatoire et Jardin botaniques de la Ville de Genève

(G), the Göttingen University herbarium (GOET), the Herbarium Haussknecht (JE), the Swedish Museum of Natural History (S), and the Herbarium of the University Kebangsaan Malaysia (UKMB). Plant material was examined by light microscopy. Drawings of type specimens and DNA vouchers were produced using a Leica DM1000 microscope equipped with a drawing tube. Figure 1g is redrawn from Fig. 3a (*Taxilejeunea pulchriflora*) of Jones (1976).

DNA extraction, PCR amplification, and sequencing

Plant tissue was isolated from dried herbarium specimens initially identified as *Lejeunea propagulifera* Gradst., *Lejeunea tamaspocsii* G.E. Lee, and *Taxilejeunea pulchriflora* (Appendix S1). Total genomic DNA was isolated using the Invisorb Spin Plant Mini Kit (Strattec Molecular GmbH, Berlin, Germany) prior to amplification. The *rbcL* gene was amplified with the PCR protocol in Gradstein et al. (2006), but the solution was filled up with double-distilled water to a total volume of 50 μ L. When products were not obtained in the initial PCR with the primers *rbcL*-1Pl-F and M1390-R, a nested PCR was conducted by running the same program with 1 μ L of the previous PCR and the primer pair *rbcL*-210-F and *rbcL*-1200-R. Amplification of the *trnL*-F region was carried out with 0.4 μ L MyTaq DNA Polymerase (Bioline Reagents Ltd., UK), 11 μ L reaction buffer, 1 μ L of upstream primer (10 μ M), 1 μ L of downstream primer (10 μ M), and 1 μ L of template DNA. This mix was filled up to a total volume of 50 μ L with double-distilled water. The primer pair *trnL*leJF and *trnL*/*trnF*-R was employed and the PCR program as described in Gradstein et al. (2006). For the nrITS1-5.8S-ITS2 region, the PCR primers and program of Hartmann et al. (2006) were used, with the same amount of reagents as described above for the *trnL*-F region. The nrITS1 and ITS2 regions were amplified separately. If this method failed, the nrITS1-5.8S-ITS2 region was amplified and a semi-nested PCR was carried out following the same protocol, yet amplifying the nrITS1 and ITS2 regions separately. The primer pair for the first PCR was Bryo18SF and Bryo26SR. For the semi-nested PCR, the primers Bryo18SF, Bryo5.8SR, Bryo5.8SF, and Bryo26SR were used (Hartmann et al. 2006).

Bidirectional sequences were generated by an ABI 3730 48 capillary sequencing machine using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing primers were the same as used for the PCR. Newly generated

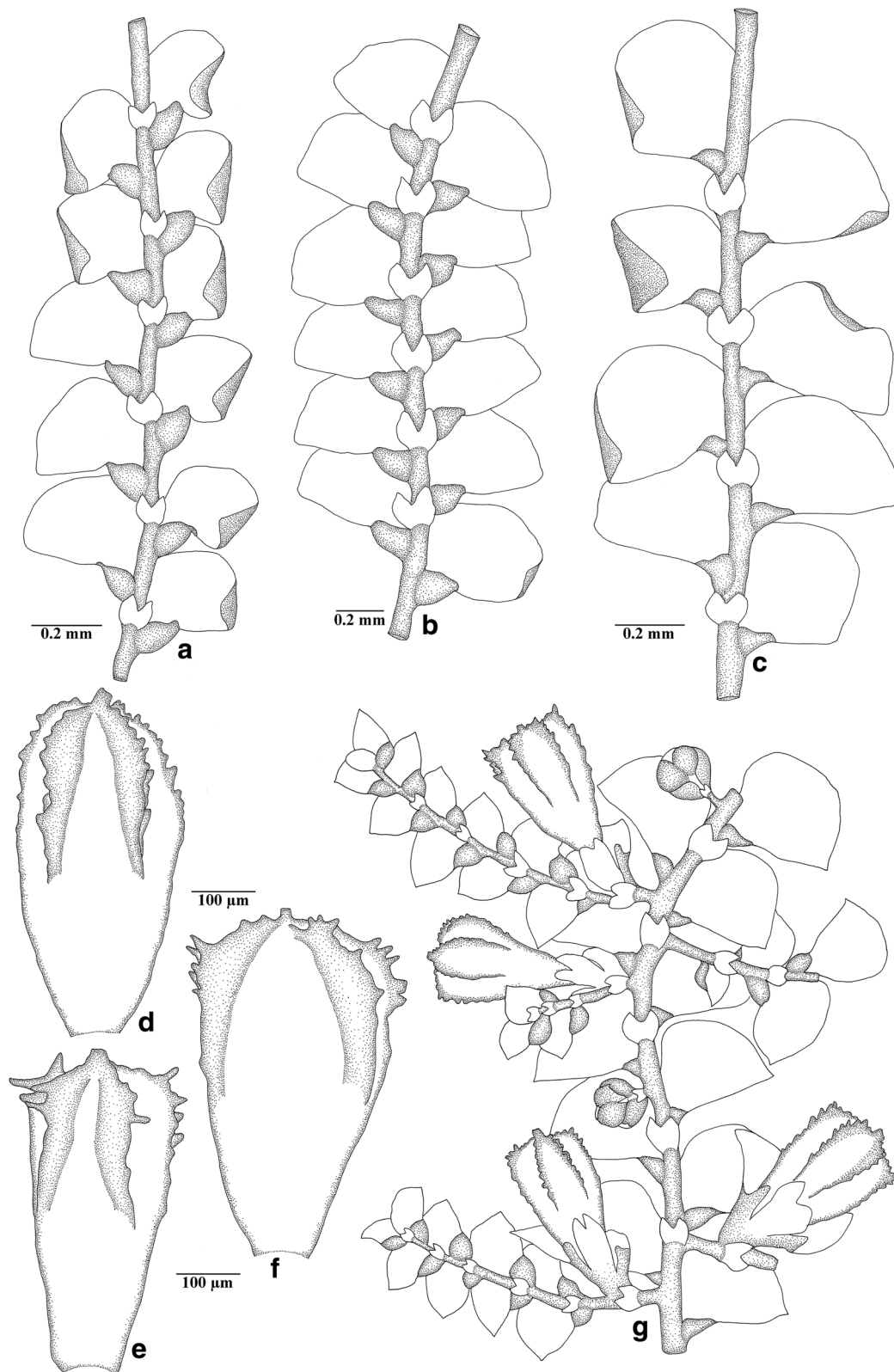


Fig. 1 *Lejeunea pulchriflora*. **a, b, c, g** Portions of gametophytes in ventral view. **d, e, f** Perianths in lateral view, each with the two ventral keels exposed [**a, e** from holotype of *Trachylejeunea schiffneri*; **b, d** from

holotype of *Lejeunea tamaspocsii*; **c, f** from holotype of *Taxilejeunea pulchriflora*; **g** redrawn from Jones (1976, Fig. 3, as *Taxilejeunea pulchriflora*)]

sequences were assembled and edited with PhyDE v.0.9971 (<http://www.phyde.de/index.html>).

Phylogenetic analyses

The new sequences were integrated in the *Lejeunea* alignment of Heinrichs et al. (2013) using BioEdit version 5.0.9 (Hall 1999), and a sequence subset was chosen for phylogenetic analyses. Taxa used in the molecular study, including GenBank accession numbers and voucher details, are listed in Appendix S1. Missing sequence stretches were coded as unknown and ambiguously aligned regions were excluded from the dataset.

Maximum likelihood (ML) analyses were conducted with RAxML version 8 (Stamatakis 2006, 2014). jModelTest 2 (Darriba et al. 2012) was employed to determine the appropriate DNA substitution model, rate of invariable sites, and gamma rate heterogeneity according to the Akaike information criterion (AIC; Akaike 1973) for the chloroplast data and the nuclear data. The analyses resulted in a GTR+G+I model for the chloroplast data and a TIM1+G+I model for the nuclear data. Since RAxML does not allow to implement the TIM1 model, the GTR+G+I model was used for both partitions. Trees were generated by selecting ten independent runs and the multiparametric bootstrap option autoMRE resulting in 300 bootstrap replicates. ML bootstrap values (BVs) of each node were visualized using FigTree 1.4 (<http://beast.bio.ed.ac.uk/figtree>). Compatibility of the chloroplast and nuclear regions was explored by comparison of the trees obtained from independent maximum likelihood (ML) analyses of each region. The trees were compared by eye to identify conflicting nodes with bootstrap values higher than 70 % (Mason-Gamer and Kellogg 1996). The trees gave no evidence of incongruence. Hence, the datasets were combined and a ML analysis was conducted with a chloroplast and a nuclear partition.

Results

Morphology

Morphological comparison of 19 specimens assigned to either *Taxilejeunea pulchriflora*, *L. tamaspocsii*, or *L. propagulifera* revealed that they have asymmetrical leaf lobes, small, distant, bifid underleaves, and triangularly obovate, beaked perianths with one dorsal, two ventral, and two lateral keels (Fig. 1). The perianth keels are beset with two rows of one to four cells long appendices. Density, length, and number of the appendices are variable and does not follow a geographical pattern (Fig. 1d–g).

Molecular phylogeny

Lejeunea splits into two well-supported main lineages (Fig. 2) of which one corresponds to subg. *Crossotolejeunea* (BV 78) and the other to subg. *Lejeunea* (BV 100). Species with toothed or ciliate perianth keels are diffusely distributed and occur in both main lineages. Accessions originally assigned to *L. propagulifera* (Fiji Isls.), *L. tamaspocsii* (Malaysia, Thailand), and *Taxilejeunea pulchriflora* (Ethiopia, Tanzania) nest in subg. *Lejeunea*, in a clade including both species with smooth perianth keels [*Lejeunea lamacerina* (Steph.) Schiffn., *Lejeunea laeta* (Lehm. & Lindenb.) Lehm. & Lindenb.] and species with ciliate/dentate perianth keels [*Lejeunea cristulata* (Steph.) M.E. Reiner & Goda, *Lejeunea grossitexata* (Steph.) M.E. Reiner & Goda, *Lejeunea paucidentata* (Steph.) Grolle, *Lejeunea sporadica* Besch. & Spruce]. They form a polytomous clade with a BV of 100; the Asian accessions nest in a subclade with a BV of 67. Branches within the crown group of this clade are short and comparable to the variation seen in *Lejeunea grossitexata*. Accessions of *L. lamacerina* form a monophyletic lineage splitting into a North American and a European/Macaronesian clade.

Based on the molecular phylogeny and the morphological investigation, we propose to treat *L. propagulifera* and *L. tamaspocsii* as synonyms of *Taxilejeunea pulchriflora*:

Lejeunea pulchriflora (Pearson) G.E. Lee, Bechteler, Pócs, Schäf-Verw. & Heinrichs, *comb. nov.*

≡ *Taxilejeunea pulchriflora* Pearson, Ark. Bot. 19: 15 (1924). TYPE: Africa, Kenya, Eastern Mount Elgon, Kaimosi, 6 August 1920, *G. Lindblom s.n.* (holotype: S-PA!).

= *Crossotolejeunea kilimandjarica* S. Arnell, Bull. Res. Coun. Israel Sect.D. Bot. 10: 322 (1961). TYPE: Africa, Kilimandjaro, Lower slopes near Kibo Hotel, on shaded earth face, 1500 m alt., *E. Esterhuysen 27263* (holotype: S-PA, not seen), *syn. fide Jones (1976)*.

= *Trachylejeunea schiffneri* Herzog, Svensk Botanisk Tidskrift 42: 239 (1948) ≡ *Crossotolejeunea schiffneri* (Herzog) R.M. Schust., Beih. Nova Hedwigia 9: 120 (1963) ≡ *Stenolejeunea schiffneri* (Herzog) Pócs, Annales Botanici Fennici 32: 267 (1995) ≡ *Lejeunea propagulifera* Gradst., Phytotaxa 9: 54 (2010). TYPE: Java, Salak, Tjapus, ad saxa, ca. 800 m alt., *V. Schiffner no. 3278* (holotype: JE!), *syn. nov.*

= *Lejeunea tamaspocsii* G.E. Lee, Polish Botanical Journal 58: 69 (2013). TYPE: Peninsular Malaysia, Pahang, Cameron Highlands, Goh Tong Jaya, area around the waterfall, 845 m alt., 3 March 2011, *G.E. Lee 2194* (holotype: UKMB!), *syn. nov.*

Plants autoicous, 0.6–1.0 mm wide, irregularly branched. Stems 70–100 µm in diameter, about four to six cells high in cross section, epidermal cells 7, 25–40 µm wide, medullary cells 4–7, 13–18 µm in diameter. Leaf lobes distant to contiguous, 0.5–0.6 × 0.4–0.5 mm,

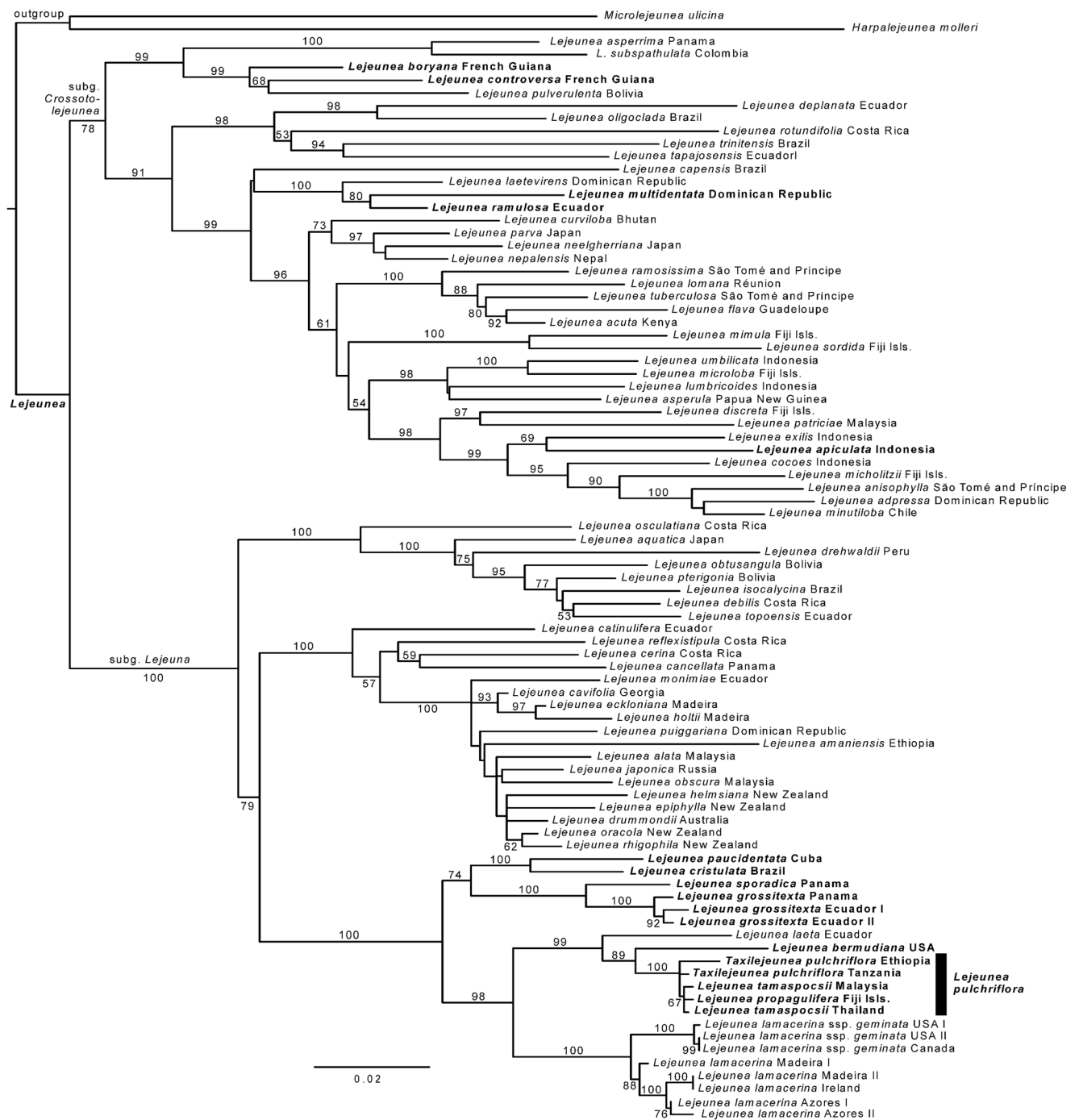


Fig. 2 Maximum likelihood phylogeny of *Lejeunea* based on a combined nrITS-trnL-F-rcbL alignment. Species with ciliate or toothed perianth keels in bold face. Accessions of *Lejeunea pulchriflora* from

Ethiopia, Tanzania, Malaysia, Thailand, and the Fiji Islands form a monophyletic lineage with a BV of 100 (black bar)

ovate, asymmetrical with strongly arched dorsal margin and straight to slightly curved ventral margin; leaf apex narrowly rounded, sometimes varying from obtuse (main stems) to acuminate (branches); leaf margin entire to crenulate due to slightly projecting marginal cells. *Leaf cells* rather isodiametric; cell walls hyaline with well-developed trigones and occasionally with intermediate thickenings;

cuticle rough by numerous minute papillae; oil bodies three to eight per cell, compound, longly oval $3 \times 10\text{--}12 \mu\text{m}$. *Leaf lobules* sometimes reduced, $0.15\text{--}0.20 \times 0.12\text{--}0.15 \text{ mm}$, ovate, keel curved, free margin flat to slightly incurved; the hyaline papilla on the proximal side of the apical tooth. *Underleaves* $0.1 \times 0.1\text{--}0.15 \text{ mm}$, up to two times wider than the stem, distant, suborbicular to

wider than long, bilobed, lobes to 1/2 of underleaf length, about four cells wide, sinus narrow to broad, widely V-shaped; underleaf margin entire to crenulate, base straight. *Androecia* intercalary on main shoots or on short lateral branches; male bracts in 2–3(-5) pairs; male bracteoles 1; antheridia not seen. *Gynoecia* on main shoots or on lateral branches, with one innovation; female bracts and bracteoles oblong to obovate; perianths 0.6–0.8×0.3 mm, triangularly obovate, with five keels, the keels more or less toothed or cristate, teeth one to four cells long, one to two cells broad at base; beak two to three cells long. Vegetative propagation by means of ribbon-like regenerants from leaf margin.

Discussion

A species in a nomenclatural context corresponds to a hypothesis on the relationships of individuals (Renner et al. 2013a). Morphologically circumscribed species have usually been considered to include closely related individuals, ideally only those that also match the biospecies concept, i.e., a reproductively isolated unit within the categories space and time (Willmann 1985). Several recent studies on bryophytes demonstrated that morphological disparity does not necessarily correspond to molecular disparity (Shaw 2000; Shaw et al. 2008; Wachowiak et al. 2007; Feldberg et al. 2009; Fuselier et al. 2009; Heinrichs et al. 2010, 2011; Kreier et al. 2010) and that morphologically similar accessions may nest in different main lineages of molecular phylogenies (Renner 2015; Heinrichs et al. 2015). This holds especially true for *Lejeunea*, where accessions originally assigned to *Lejeunea tumida* Mitt. belong to four species that nest in different clades (Renner et al. 2011). All these studies provided evidence for a higher number of morphologically cryptic or near cryptic bryophyte species and questioned the hypothesis of generally larger distribution ranges of bryophytes compared to angiosperms (Shaw 2001). The possible incongruence of morphological and molecular data necessitates the introduction of an integrative taxonomy that considers different lines of evidence (Dayrat 2005).

Lejeunea tamasocsii was described as a tropical Asian species that was distinguished from other local species by its asymmetrical leaf lobes, small, distant underleaves, toothed or cristate perianth keels with two rows of one to four cells long appendices, and its autoicy (Lee and Gradstein 2013). In the context of revisionary studies of Paleotropical Lejeuneaceae, we noted that the same suite of characters was assigned to the African *Taxilejeunea pulchriflora* (Pearson 1924). The taxonomic status of *Taxilejeunea* has been controversial since the 1960s when Mizutani (1963, 1966, 1970) transferred most Asian species of *Taxilejeunea* to *Lejeunea*, while the genus

Taxilejeunea is still accepted in tropical America (Gradstein and Costa 2003) and Africa (Wigginton 2004), although it was noted that the generic separation is not distinct (Reiner-Drehwald 2005b). The main diagnostic character of *Taxilejeunea* is repeatedly innovating female branches; however, Wilson et al. (2007a) and Heinrichs et al. (2013) demonstrated that species with such structures nest in several independent lineages of *Lejeunea*. Molecular phylogenies thus support inclusion of *Taxilejeunea* in *Lejeunea*, but as a consequence of the rapidly changing genus concepts in Lejeuneaceae, it must be considered that not all *Lejeunea* elements have already been transferred to this genus or even been recognized as such. Accordingly, *Taxilejeunea pulchriflora* still needs to be transferred to *Lejeunea* and the new combination is formalized in this study. The difficulties in establishing a reliable classification of Lejeuneaceae are also seen in a second binomial that we regard as conspecific with *L. pulchriflora*. *Trachylejeunea schiffneri* Herzog was established based on type material from Java (Herzog 1948) and alternatively treated as a member of *Crossotolejeunea* (Schuster 1963) and *Stenolejeunea* R.M. Schust. (Pócs et al. 1995), before Söderström et al. (2010) reduced *Stenolejeunea* to a synonym of *Lejeunea*. These authors introduced the binomen *L. propagulifera* for *Trachylejeunea schiffneri* because the heterotypic binomen *Lejeunea schiffneri* Steph. disabled a new combination. The Asian *L. propagulifera*, however, is morphologically similar to the African *L. pulchriflora*, as was already pointed out by Jones (1976) based on Herzog's protologue. We consider *L. propagulifera* to belong to *L. pulchriflora* because (1) it lacks any morphological disparities besides the occasional production of propagules (Pócs et al. 1995), (2) it is nested in a robust monophyletic lineage with Ethiopian and Tanzanian accessions of *L. pulchriflora*, and (3) the short crown group branches within this clade provide evidence for low sequence variation. Accessions of *L. tamasocsii* are also nested in this lineage, and we consider them to belong to *L. pulchriflora* (Fig. 2).

According to our revision, *L. pulchriflora* (= *L. propagulifera* and *L. tamasocsii*, syn. nov.) is disjunctly distributed from tropical Africa to the Pacific region (Fig. 3). Paleotropical ranges are not rare in bryophytes (Pócs 1976, 1992); however, only a few have been tested using molecular phylogenetic evidence.

In the present example, morphology is congruent with molecular variation, yet morphology alone does not allow to identify the species that are most closely related to *L. pulchriflora*. Prior to the introduction of molecular data, the variation in female involucre has been regarded as a valuable tool to classify *Lejeunea* into subgenera and sections or to delimit certain elements as satellite genera (Schiffner 1893). The toothed perianth keels of *L. pulchriflora* are not uncommon in *Lejeunea* and have previously been reported from members of *Crossotolejeunea*, a genus now included in *Lejeunea*

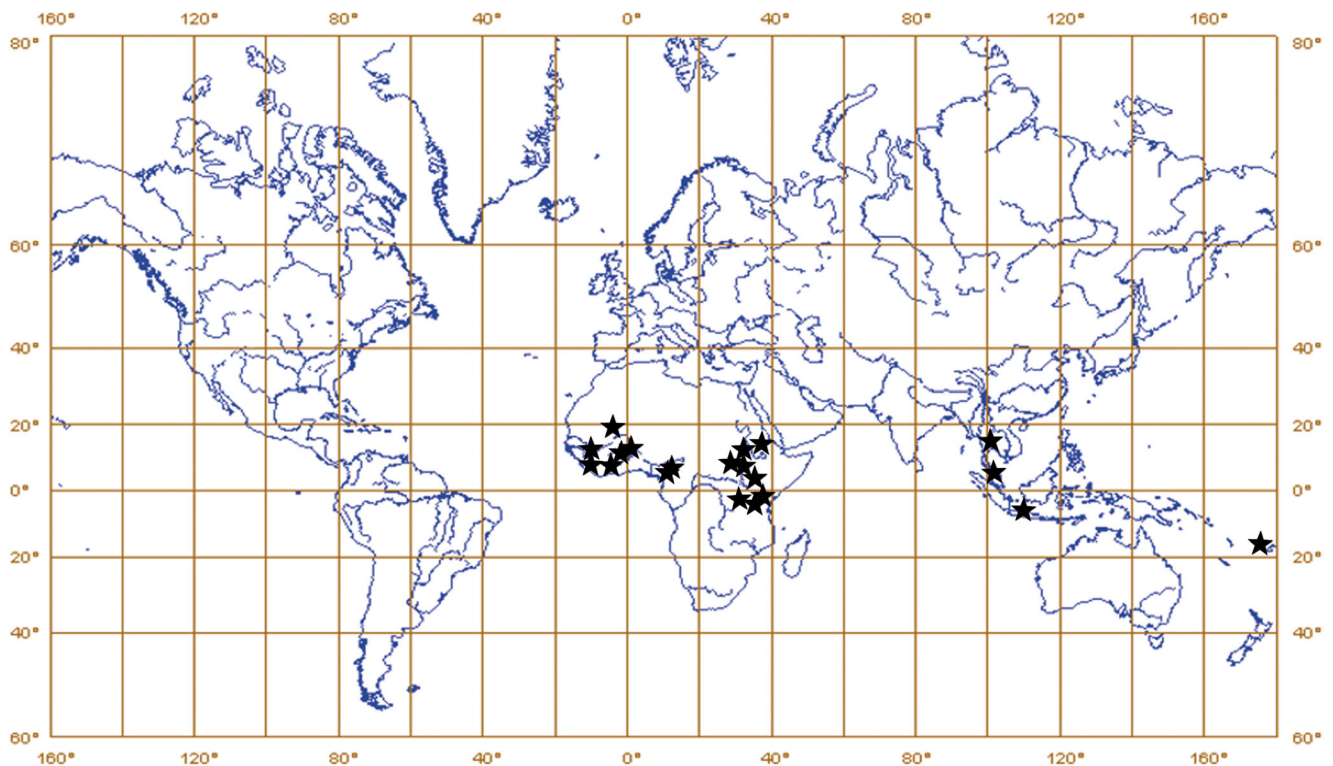


Fig. 3 Map showing the distribution of *Lejeunea pulchriflora* across the Paleotropics. Records based on own revisionary studies and Wigginton (2009)

(Reiner-Drehwald and Goda 2000). *Crossotolejeunea* species nest in both main *Lejeunea* clades (Heinrichs et al. 2013), as is also seen in the present study. The type species of *Crossotolejeunea*, *Lejeunea boryana* Mont., nests in the “subg. *Crossotolejeunea*-clade” (Fig. 2), whereas other former *Crossotolejeunea* elements belong to subg. *Lejeunea* (*Lejeunea bermudiana*, *L. cristulata*, *L. grossitexta*, *L. paucidentata*, *L. sporadica*). *Lejeunea pulchriflora* is nested in this largely neotropical clade; however, the closely related holarctic species *L. lamacerina* has smooth or at best weakly crenulate perianth keels (Schuster 1980).

Although the five accessions of *L. pulchriflora* nest in a polytomy, the Asian accessions are separated from the African accessions with a BV of 67 (Fig. 2). Our data do not show if the investigated populations still maintain the potential to interbreed successfully, but the short crown group branches indicate a recent separation, with similar variation seen in many bryophyte species. It is also clear that both morphological and phylogenetic species delimitations rely on a certain degree of subjectivity (Lang et al. 2015). Unfortunately, liverwort material suitable for molecular investigation is not easy to obtain from many regions of the Paleotropics, and a more detailed investigation is hampered by the low availability of DNA vouchers. Many morphologically circumscribed species of *Lejeunea* are highly diverse at the molecular level (Heinrichs et al. 2013), as is seen in the example *L. lamacerina* with a deep split into an American and a Macaronesian/European

clade (Fig. 2). Especially, such taxa are in urgent need of integrative studies with a dense sampling and inclusion of accessions from throughout their range (Fuselier et al. 2009; Hedenäs 2010; Heinrichs et al. 2011; Renner et al. 2013b). These studies would allow to introduce a stable classification of liverworts and a reliable identification of accessions for barcoding (Hollingsworth et al. 2009). Such studies would also improve our knowledge on true species numbers which are crucial for the reconstruction of diversification rates, biogeographical patterns, and understanding of endemism (Feldberg et al. 2014; Patiño et al. 2014; Villarreal et al. 2015).

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