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Molecular data favours a monogeneric *Peltulaceae* (Lichinomycetes)

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Abstract: The family *Peltulaceae* is currently composed of the three genera *Peltula*, *Phyllopeltula* and *Neoheppia*. The last two genera, both with two species, are distinguished from *Peltula* only by a small number of morphological characters. The morphology of the genus *Peltula* varies from peltate-umbilicate thalli to squamulose-semifruticose or squamulose-compound types, as well as subfoliose-compound and crustose types. All types have an upper epinecral layer and possess medullary cavities of various sizes; a lower cortex is normally present but is usually not developed in the subfoliose and crustose types. The genera *Neoheppia* and *Phyllopeltula* differ from the common *Peltula* morphology by crustose-areolate and subfoliose-compound thalli, respectively. Both *Neoheppia* and *Phyllopeltula* are additionally characterized by the absence of medullary cavities and lower cortices. To investigate the phylogenetic validity of *Phyllopeltula* and *Neoheppia*, we sequenced six loci from representatives of these two genera together with 37 species from *Peltula*. Despite the relatively high amount of conflict among loci, the results clearly indicate that both *Phyllopeltula* and *Neoheppia* are not monophyletic, and are nested within the genus *Peltula.* Consequently, we subsumed species of these two genera within the genus *Peltula.*

Key words: *Neoheppia, Peltula*, phylogeny, *Phylopeltula*, taxonomy

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Introduction

The lichen family *Peltulaceae* is exclusively associated with unicellular cyanobacterial photobionts, mostly of the genus *Chroococcidiopsis*, and presently comprises the three genera *Peltula*, *Phyllopeltula* and *Neoheppia* with a total of *c.* 47 species (Wetmore 1970; Bubrick & Galun 1984; Büdel 1987*a*, *b*, 1995; Egea 1989; Büdel & Elix 1997; Kalb 2001; Donner 2013; Marques *et al.* 2013; Makryi 2016). However, the pantropical species *Phyllopeltula corticola* (Büdel & R. Sant.) Kalb was recently found to form chimeras with unicellular green algae incorporated into the thallus (Aptroot & Schumm 2010). Members of the *Peltulaceae* occur worldwide in arid and semi-arid climatic regions. However, under humid conditions they can exist in microclimatic arid islands (Büdel *et al.* 2000; Becker 2002) as far north or east as Sweden or Baikal Siberia. The majority of species belong to the genus *Peltula*. Species from this genus have squamulose-compound (Figs 1P & U, 2A & R) and squamulose-semifruticose thalli (Fig. 2E, H–K), or exhibit singular peltate thalli with an umbilicus or central hyphal strand (Fig. 1A–I). All squamules of compound thalli are minute and usually grow tightly together (Büdel 1987*a*); they are not gelatinous (homoiomerous) but usually stratified (heteromerous) with an upper epinecral layer, a photobiont layer followed by a medulla with numerous air spaces of various sizes and typically a lower cortex (Fig. 2O & Q). An upper cortex is mostly absent but the epinecral layer serves as a

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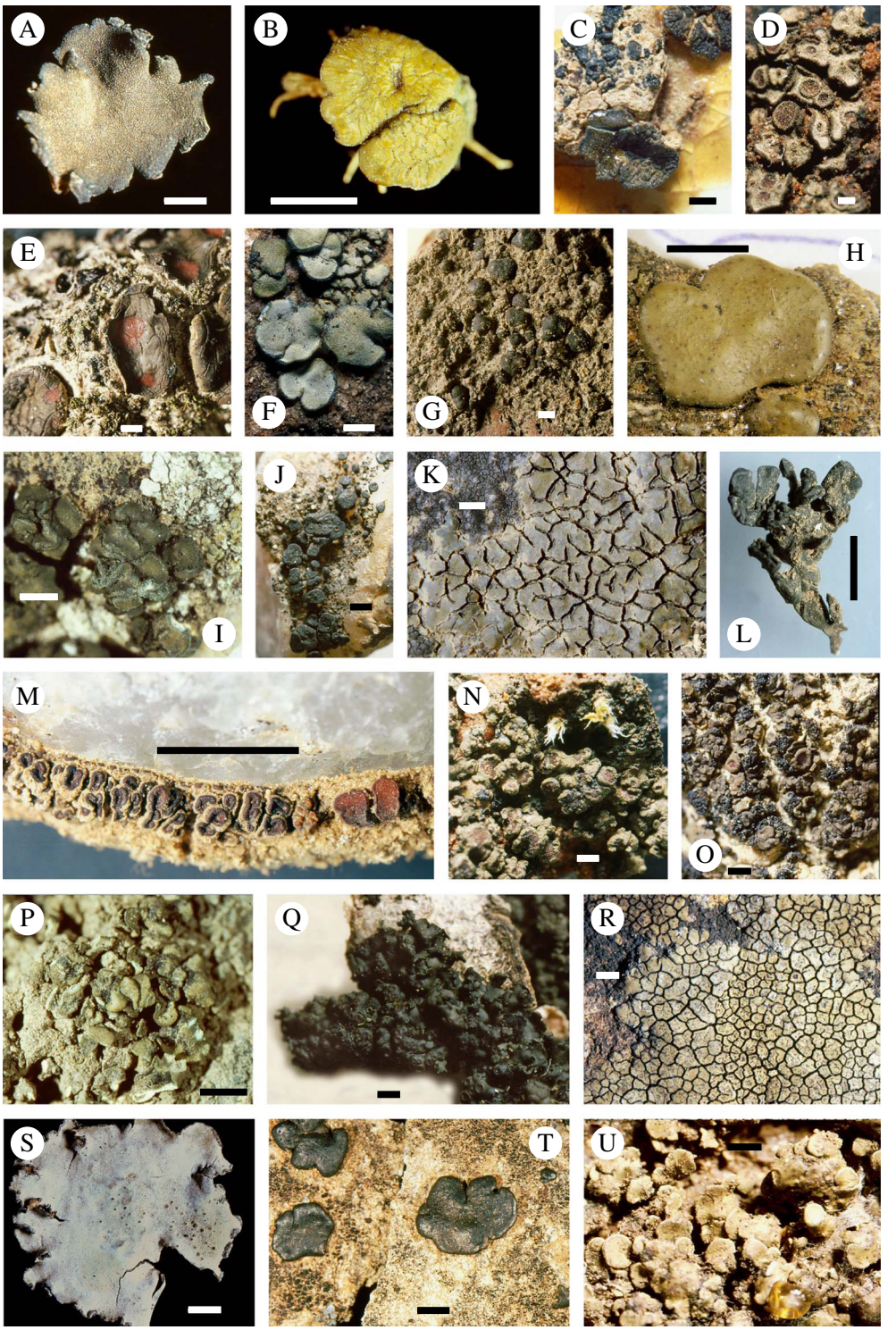


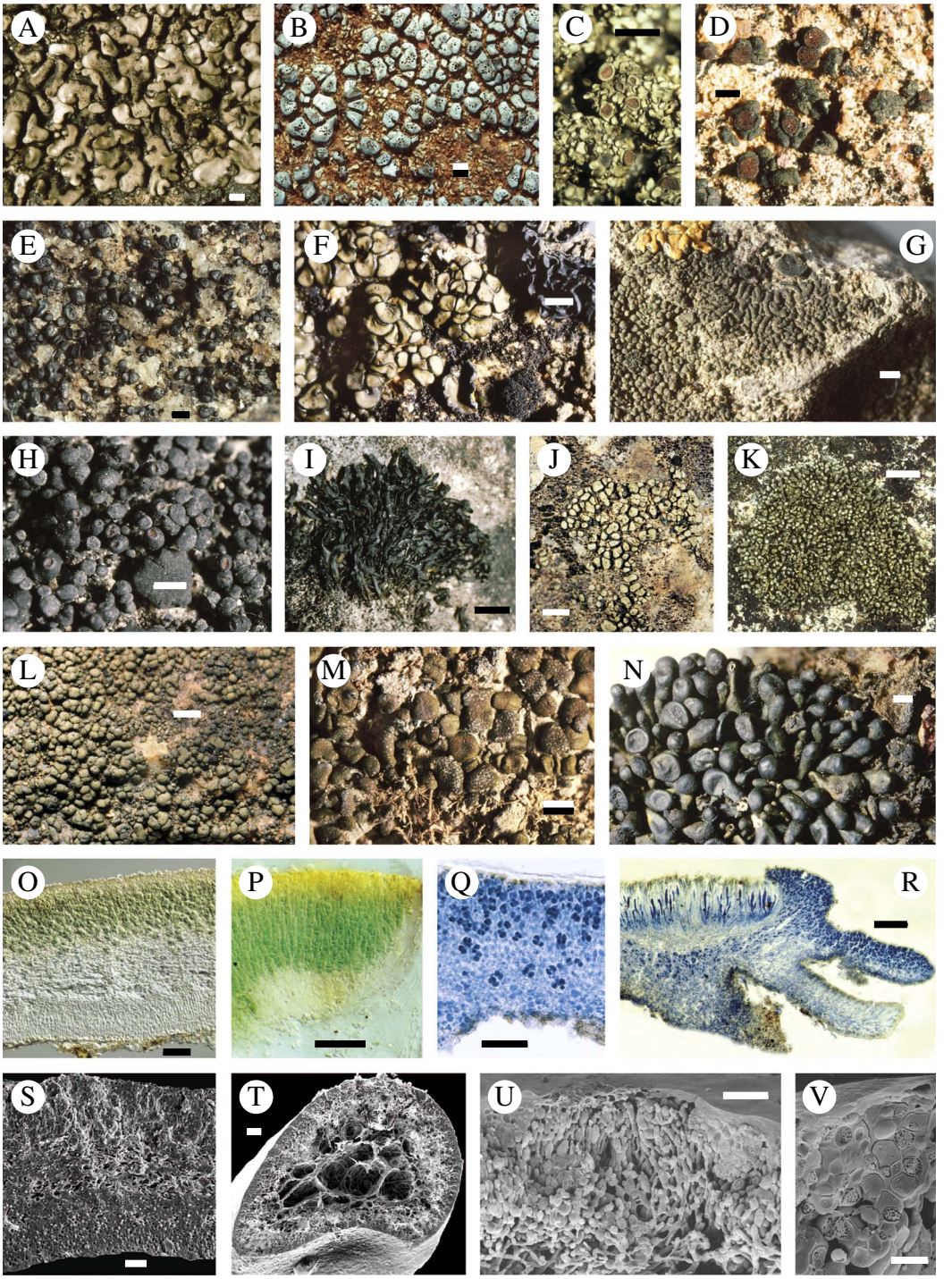
protective layer for the photobiont and is often yellowish to brown pigmented (Büdel 1990; Büdel & Lange 1994). Soredia and isidia are rare but not unknown in a small number of species. Apothecia occur frequently in the *Peltulaceae*, with the exception of *P. lingulata* (Vain.) Swinscow & Krog and *P. crispatula* (Nyl.) Egea. Additionally, lichen substances are lacking in the *Peltulaceae* with the exception of *P. langei* Büdel & Elix, from which a yellow pigment similar to Myeloconon C could be extracted (Büdel & Elix 1997).

The remaining two genera, *Neoheppia* (Zahlbruckner 1909; Büdel 1995) and *Phyllopeltula*, comprise only two species each. *Neoheppia cataractae* Büdel & Sérus. (Fig. 1K) and *N. brasiliensis* Zahlbr. (Fig. 1R) differ from the remaining *Peltulaceae* by lacking a lower cortex (Fig. 2P) with their thalli being secondarily cracked, giving the impression of compound thalli (Fig. 1K & R). *Phyllopeltula steppae* Kalb and *P. corticola* (Büdel & R. Sant.) Kalb (Fig. 1Q) develop compound thalli with subfoliose squamules and, in contrast to rock- or soil-inhabiting *Peltula* and *Neoheppia* species, colonize bark (Büdel 1987*a*; Kalb 2001). Their thalli do not show internal air spaces in cross-section (Fig. 2P & Q).

Reproductive structures are uniform across all members of the family: a polysporous ascus with a gelatinous sheath and simple, single-celled, colourless spores. Pycnidia, conidiophores and pycnospores are also uniform among the species. The separation of the three genera necessarily relies on morphological characters only: thallus morphology and anatomy, substratum attachment and habitat, characters that have been shown to be of varying taxonomic use in cyanolichens (Wedin *et al*. 2009; Otálora *et al*. 2013, 2014) and many other lichen groups (Printzen 2010; Ekman *et al*. 2014). For example, the lichen genera *Coenogonium* and *Dimerella* have identical apothecia but strikingly different thallus morphologies (filamentous vs. crustose, with intermediate forms), although molecular phylogenetic studies have shown that they are congeneric (Kauff & Lutzoni 2002). Within the *Peltulaceae*, the limited number of growth forms (such as fruticose vs. subfruticose or crustose-squamulose) are often hard to distinguish, and intermediate forms exist, which further complicate the use of such characters.

The few morphological characters, their presence/absence, and the many modifications and intermediate forms make it difficult to characterize putative monophyletic lineages based on morphological data, and to date there is no comprehensive phylogenetic hypothesis for the *Peltulaceae*. Egea (1989) made a first attempt to find assemblies of putatively closely related species. He identified two major evolutionary lineages, mainly according to substratum fixation: one lineage containing species attached to the substratum with an umbilicus (e.g. *P. euploca* (Ach.) Poelt or *P. africana* (Jatta) Swinscow & Krog; Fig. 1A & F), the other lineage comprising species forming rhizohyphae (e.g. *P. patellata* (Bagl.) Swinscow & Krog or *P. psammophila* (Nyl.) Egea; Fig. 1C & D). Subfruticose thalli were considered as the most advanced traits for the *Peltulaceae*. Büdel (1987*a*) described two groups of closely related species: the so-called 'form group' comprising *P. congregata* (Nyl.) Swinscow & Krog, *P. capensis* (Brusse) Büdel, *P. cylindrica* Wetmore and *P. tortuosa* (Nees) Wetmore (Figs 1L, 2A & K), three of which develop squamulose-semifruticose thalli, and the 'critical group' (*P. impressa* (Vain.) Swinscow & Krog, *P. marginata* Büdel, *P. placodizans* (Zahlbr.) Wetmore,

FIG. 1. *Peltula* morphology. A, *P. africana*, *Bu* 14304b, South Africa; B, *P. radicata*, type species of the genus, isotype PC, North Africa; C, *P. psammophila*, lectotype, H-Nyl 30904, Algeria; D, *P. patellata*, *Bu* 18058a, Central Australia; E, *P. richardsii*, *Schultz*, Sonoran Desert, Arizona, USA; F, *P. euploca*, Central Australia; G, *P. omphaliza*, *Vainio* 11551; H, *P. sonorensis*, holotype ASU, Arizona, USA; I, *P. bolanderi*, *Bu* 14119b, South Africa; J, *P. boletiformis*, *Bu* 14117, South Africa; K, *P. cataractae*, topotype, Zaire BR 1330; L, *P. capensis*, isotype, LD, South Africa; M, *P. inversa*, *Bu* 15058, Namibia; N, *P. imbricata*, holotype MEL, Australia; O, *P. hassei*, lectotype FH, California; P, *P. michoacanensis*, FH, isotype, Mexico; Q, *P. corticola*, holotype UPS, *Santesson* 20745, Kenya; R, *P. brasiliensis*, holotype W, Brazil; S, *P. farinosa*, holotype M, *Bu* 14327b, South Africa; T, *P. coriacea*, *Bu* 14095a, South Africa; U, *P. rodriguesii*, isolectotype BM 2333, Island of Rodriguez. Scales: A, B, E, N, P, S & T = 2 mm; C, D, G, I–K, O, Q, R & U = 1 mm; F & L = 5 mm; H = 3 mm; M = 10 mm.



P. umbilicata (Vain.) Swinsc. & Krog and *P. zahlbruckneri* (Hasse) Wetmore; Fig. 2B, F, G, J & L), comprising species with squamulose-compound thalli.

Thus, the question arises whether the subdivision of *Peltulaceae* into three genera, based on thallus morphology and anatomical variation, reflects the evolutionary history of this family. Are there morphological characters that can be used to delimit natural groups of related taxa in the *Peltulaceae*? Neither the monophyly of any of the above-mentioned lineages, nor the monophyly of the three genera *Peltula*, *Phyllopeltula* and *Neoheppia*, have yet been explicitly verified with molecular data. Although the reproductive features of the *Peltulaceae* are too uniform and unsuitable for phylogenetic analysis, the usefulness of the remaining morphological characters (thallus anatomy, thallus growth form, substratum) currently used for genus and species delimitation has yet to be shown.

The aim of this study is to test the current morphology-based genus delimitation within *Peltulaceae* and the monophyly of *Phyllopeltula* and *Neoheppia* using a molecular phylogenetic analysis based on six gene loci.

Materials and Methods

Taxon sampling

Thirty-seven of the 47 species classified within *Peltulaceae* were included in this study (Table 1). No specimens were available for *P. applanata* (Zahlbr.) J. C. Wei, *P. minuta* (H.Magn.) Golubk., *P. tenuis* Büdel & Henssen and *P. zabolotnoji* (Elenk.) Golubk., and the material for *P. koflerae* Henssen & Büdel and *P. langei* was too poor for a molecular approach. The new species *P. lobata* J. Marques *et al.*, *P. pannarioides* Makryi and *P. rosulata* Makryi were not available at the time of the analyses (Marques *et al.* 2013; Makryi 2016). The monophyly of the *Peltulaceae* has been shown previously (Schultz *et al.* 2001). Three species of *Lichinella* and *Trichoglossum hirsutum* (Pers.) Boud. were chosen as outgroup. Six genetic loci (nuclear ribosomal small subunit RNA gene (nrSSU), nrLSU, nuclear ribosomal internal transcribed spacer region (ITS), mitochondrial ribosomal small subunit RNA gene (mtSSU), and the protein coding genes RNA polymerase II second largest subunit (*RPB2*) and beta-tubulin (β-tub) were analyzed. Voucher data and GenBank Accession numbers are given in Table 2.

DNA isolation and sequencing

DNA isolation followed the protocol of Hofstetter *et al.* (2002). Polymerase chain reactions (PCR) were performed to amplify parts of six loci. The reaction was prepared to a final volume of 25 µl containing 4 µl dNTPs, 2·5 µl buffer, 1 µl BSA (bovine serum albumin, 10 mg ml^{-1}, New England Biolabs, #B9001S), 1·25 µl of each primer (10 µM), 0·3 µl TAQ DNA polymerase (Abgene, Rochester, NY, USA), 10 µl DNA extraction solution at different dilution stages, and PCR buffer N (Abgene, #AB-0288). Half of the reactions were prepared using 12·5 µl PCR mastermix (Promega, Mannheim, Germany, #M7505), 0·625 µl BSA (20 mg/ml, Fermentas, St.-Leon-Rot, Germany, #B14), 2·5 µl of each primer (10 µM), 1·875 µl nuclease-free water (Promega), and 5 µl template DNA at different dilution stages. Several combinations of forward/reverse primers were used for amplification: nsu131/NS24, nsu131/nsu1088, nsu1088R/NS24 (nrSSU), LR0R/LR7, LR0R/LR5, LR5R/LR7 (nrLSU), mrSSU1/mrSSU3R, mrSSU1/mrSSU2R (mtSSU), ITS1/LR5 (ITS), *RPB2*-7F/*RPB2*-11aR, *RPB2*-7F/*RPB2*-11bR, f*RPB2*-7cF/f*RPB2*-11aR (*RPB2*/7–11), Bt3-LM/Bt10-LM, T1/T22, T1/Bt2b, Bt2a/T22 (β-tub) (lutzonilab.org/primer-sequences). All amplification primers were also used for sequencing (at a concentration of 5 µM) plus a

FIG. 2. *Peltula* morphology and anatomy. A, *P. congregata*, Ivory Coast; B, *P. umbilicata*, South Africa; C, *P. auriculata*, holotype M, *Gröger* 18, Venezuela; D, *P. deserticola*, *Bu* 18073, Australia; E, *P. santessonii*, holotype UPS, *Santesson* 23620; F, *P. zahlbruckneri*, MB *Nash* 8772, USA; G, *P. placodizans*, lectotype W, *Blumer* 112, Arizona, USA; H, *P. clavata*, *Bu* 18047, Australia; I, *P. lingulata*, Ivory Coast; J, *P. marginata*, isotype *Bu* 14017a, South Africa; K, *P. tortuosa*, Venezuela; L, *P. impressa*, MEL *Brownlie* 37806, Australia; M, *P. impressula*, *Golubkova* 1036, Mongolia; N, *P. kofleri*, holotype LD, *Kofler* 31010, South Africa; O, *P. euploca*, cross-section of peltate thallus with upper epinecral layer, cyanobiont layer, medulla and lower cortex; P, *P. cataractae*, topotype, cross-section of crustose thallus with cyanobiont arranged in vertical parallel rows; Q, *P. corticola*, cross-section of leaf-like thallus with compact cyanobiont layer and medulla without cavities; R, *P. obscurans* var. *hassei*, cross-section of crustose-leafy thallus; S, *P. farinosa*, cross fracture of peltate thallus with numerous cavities in cyanobiont layer and medulla, LT-SEM; T, *P. tortuosa*, cross fracture of subfruticose thallus with large medullary cave, LT-SEM; U, *P. coriacea*, cross fracture of upper thallus layer with pycnidium, LT-SEM; V, *P. coriacea*, cross fracture of upper cyanobiont and epinecral layer, LT-SEM. Scales: A, B, D, E, H, L & N = 1 mm; C & I = 5 mm; F & G = 3 mm; J = 2 mm; K = 10 mm; M = 4 mm; O & S–U = 20 µm; P & R = 100 µm; Q = 50 mm; V = 5 µm. LT-SEM = Low temperature scanning electron micrograph.

TABLE 1. *Growth forms of the lichen genus* Peltula *and their morphological and anatomical characteristics.*

Species	Growth form	Anatomy	Substratum	Growth form-type	Habitus figures
P. farinosa *P. euploca* *P. omphaliza* *P. sonorensis* *P. bolanderi* *P. psammophila* *P. radicata* *P. patellata* *P. richardsii* *P. africana* *P. obscuratula*	Peltate-umbilicate, often singular thalli, rarely compound thalli, attached by an umbilicus or central strand of hyphae	Main type with upper epinecral layer, cyanobiont layer, medulla with cavities and lower cortex (Fig. 2O & S)	Mostly inclined rock surfaces, rarely soil	1	1A–I, S 2O
P. cylindrica *P. santessonii* *P. zahlbruckneri* *P. placodizans* *P. clavata* *P. lingulata* *P. marginata* *P. tortuosa* *P. capensis* *P. boletiformis*	Squamulose-semifruticose	Large medullary cavities (Fig. 2T)	Rock, temporarily inundated	2	1J, L 2E–K, T
P. congregata *P. umbilicata* *P. coriacea* *P. rodriguesii* *P. impressa* *P. obscurans* var. *deserticola* *P. crispatula* *P. obscurans* var. *hassei* *P. michoacanensis*	Squamulose-compound, rarely singular	Main type (see type 1), (Fig. 2 R, U, V)	Rock flats, seepage rocks after rain, rarely soil	3	1P, T, U 2A, B, D, L, R, U, V
P. corticola *P. steppae* *P. obscurans* var. *hassei* *P. auriculata* *P. imbricata* *P. leptophylla*	Subfoliose, compound	No medullary cavities, no lower cortex (Fig. 2Q)	Rock and tree bark	4	1N, O, Q 2C, Q, R
P. brasiliensis *P. cataractae*	Crustose-areolate	No medullary cavities, no lower cortex, deeply penetrating cyanobiont layer (Fig. 2P)	Rock, sometimes inundated	5	1K, R 2P
P. inversa	Crustose	Inverse thallus anatomy	Hypolithic on the lower site of quartz rocks	6	1M

variety of other primers: nssu634, nssu897R, SR6, SR7, SR7R, SR10, SR10R, SR11, SR11R, NS6 (nrSSU), LR2, LR2R, LR3, LR3R, LR6, LR6R (nrLSU), ITS4 (ITS), T2, T10, T12, T222, Bt698F, Bt705R (β-tub) (lutzonilab.org/primer-sequences). The primers Bt698F (5′-TCC GTT CGG TCA ACT CTT C-3′) and Bt705R (5′-TCA ACT TTT CCG TCC CGA-3′) were newly designed for this study. PCR was performed as follows: one initial cycle of 3 min at 95 °C, followed by 34 cycles of 1 min at 95 °C, 45 s at a variable annealing temperature depending on the targeted region, 2 min at 72 °C (1 min 30 s for *RPB2*/7–11 and β-tub). After completion of the 34 cycles, samples were held for 10 min at 72 °C. Gel electrophoresis was conducted on a TAE 1% agarose gel. PCR products were stained either with SYBRGreen I (Cambrex, East Rutherford, NJ, USA, #50513) or GelStar (Biozym, Hess. Oldendorf, Germany). A quantity of 0·5 µl $MgCl_2$ (25 mM) was added to some reactions, in combination with a raised number of cycles. All ribosomal RNA genes (nrSSU, nrLSU, mtSSU) and the ITS region were annealed at 50 °C, *RPB2*/7–11 at 52 °C, and β-tub primers Bt3-LM and Bt10-LM at 60 °C or 52 °C. PCR reactions showing multiple bands or a weak signal on the gel were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA and Karlsruhe, Germany, #K4500). Cloning PCR reactions were prepared to a volume of 25 µl as for the standard PCR, replacing the template DNA solution with the same amount of nuclease-free water. At least four clone colonies were picked and added to the PCR

TABLE 2. *Voucher information and GenBank Accession numbers for the species of* Peltulaceae *used in this study. Accession numbers are newly generated for this study with the exception of those in italics.*

Species	Origin & collection year	Hb. No.	GenBank Accession number					
			nrSSU	nrLSU	mtSSU	ITS	*RPB2*/7–11	β-tub
Peltulaceae								
Neoheppia brasiliensis	South Africa, 1983	hb. B.B. 14083a	MF766298	MF766421	MF766339	MF766380	MF804874	MF776090
N. cataractae	Dem. Rp. Congo, 1947	hb. B.B. 1329	MF766299	MF766422	MF766340	MF766381	MF804875	MF776091
Peltula africana	South Africa, 1990	hb. B.B. 14304b	MF766261	MF766384	MF766302	MF766343	MF804877	MF776053
P. auriculata ISOTYPE	Venezuela, 1992	hb. B.B. 24902	MF766262	MF766385	MF766303	MF766344	MF804860	MF776054
P. bolanderi	Mexico, 1993	hb. B.B. 20196e	MF766263	MF766386	MF766304	MF766345	MF804891	MF776055
P. boletiformis	South Africa, 2003	hb. B.B. 14911a-1	MF766264	MF766387	MF766305	MF766346	MF804892	MF776056
P. capensis	South Africa, 1994	hb. B.B. 14382b 2	MF766265	MF766388	MF766306	MF766347	MF804893	MF776057
P. clavata	Australia, 1987	hb. DUKE 164 (18047a)	MF766266	MF766389	MF766307	MF766348	MF804861	MF776058
P. congregata	South Africa, 2003	hb. B.B. 14909b-1	MF766267	MF766390	MF766308	MF766349	MF804896	MF776059
P. coriacea	South Africa, 2003	hb. B.B. 14500a-1	MF766268	MF766391	MF766309	MF766350	MF804878	MF776060
P. crispatula	Morocco, 1987	hb. B.B. 21001a	MF766269	MF766392	MF766310	MF766351	MF804862	MF776061
P. cylindrica	South Africa, 2003	hb. B.B. 14920a-1	MF766270	MF766393	MF766311	MF766352	MF804894	MF776062
P. euploca	Mexico, 1993	hb. B.B. 20162a	MF766271	MF766394	MF766312	MF766353	MF804879	MF776063
P. euploca ssp. *sorediosa*	South Africa, 2003	hb. B.B. 14921c-1	MF766272	MF766395	MF766313	MF766354	MF804880	MF776064
P. farinosa	Mexico, 1993	hb. B.B. 20119a	MF766273	MF766396	MF766314	MF766355	MF804898	MF776065
P. imbricata	Australia, 1987	hb. B.B. 18060a	MF766274	MF766397	MF766315	MF766356	MF804899	MF776066
P. impressa	Mexico, 1993	hb. B.B. 20140f	MF766275	MF766398	MF766316	MF766357	MF804863	MF776067
P. inversa	Namibia, 2001	hb. Pretoria 15058	MF766276	MF766399	MF766317	MF766358	MF804881	MF776068
P. leptophylla	Mexico, 1993	hb. B.B. 20128a	MF766277	MF766400	MF766318	MF766359	MF804864	MF776069
P. lingulata	South Africa, 1994	hb. B.B. 14452a	MF766278	MF766401	MF766319	MF766360	MF804882	MF776070
P. marginata	South Africa, 2003	hb. B.B. 14920d-1	MF766279	MF766402	MF766320	MF766361	MF804883	MF776071
P. michoacanensis	Mexico, 1993	hb. B.B. 20140l	MF766280	MF766403	MF766321	MF766362	MF804900	MF776072
P. obsc. var. *deserticola*	South Africa, 2003	hb. B.B. 14900b-1	MF766281	MF766404	MF766322	MF766363	MF804865	MF776073
P. obsc. var. *deserticola*	South Africa, 2003	hb. B.B. 14902d-1 2	MF766282	MF766405	MF766323	MF766364	MF804866	MF776074
P. obsc. var. *hassei*	South Africa, 1994	hb. B.B. 14354a	MF766283	MF766406	MF766324	MF766365	MF804895	MF776075
P. obscuratula 1	Morocco, 1987	hb. B.B. ex hb. Murcia	MF766284	MF766407	MF766325	MF766366	MF804867	MF776076
P. omphaliza	Mexico, 1993	hb. B.B. 20148b	MF766285	MF766408	MF766326	MF766367	MF804884	MF776077
P. patellata	Mexico, 2003	hb. M.S. 16254b	MF766286	MF766409	MF766327	MF766368	MF804868	MF776078
P. placodizans	Mexico, 1993	hb. B.B. 20112a	MF766287	MF766410	MF766328	MF766369	MF804885	MF776079
P. psammophila	Canary Islands, 1985	BM 761074	MF766288	MF766411	MF766329	MF766370	MF804869	MF776080
P. radicata	Yemen, 2002	hb. M.S. 14241a	MF766289	MF766412	MF766330	MF766371	MF804870	MF776081
P. richardsii	Mexico, 1993	hb. B.B. 20194a	MF766290	MF766413	MF766331	MF766372	MF804871	MF776082
P. rodriguesii	Namibia, 1990	hb. B.B. 15901	MF766291	MF766414	MF766332	MF766373	MF804872	MF776083
P. santessonii	South Africa, 2003	hb. B.B. 14912b-1	MF766292	MF766415	MF766333	MF766374	MF804886	MF776084
P. sonorensis	Mexico, 1993	hb. B.B. 20196d	MF766293	MF766416	MF766334	MF766375	MF804887	MF776085
P. tortuosa	Venezuela, 1996	hb. B.B. 24039b	MF766294	MF766417	MF766335	MF766376	MF804888	MF776086

TABLE 2 (*continued*).

Species	Origin & collection year	Hb. No.	GenBank Accession number					
			nrSSU	nrLSU	mtSSU	ITS	*RPB2*/7–11	β-tub
P. umbilicata	South Africa, 2003	hb. B.B. 14901a-1	*DQ782887*	*DQ832334*	*DQ922954*	*DQ832333*	*DQ832335*	MF776087
P. zahlbruckneri	Mexico, 1993	hb. B.B. 20157a	MF766295	MF766418	MF766336	MF766377	MF804889	MF776088
Phyllopeltula corticola	Yemen, 2002	hb. M.S. 14201	MF766296	MF766419	MF766337	MF766378	MF804873	MF776089
P. steppae HOLOTYPE	Venezuela, 1989	hb. K.K. 23948	MF766297	MF766420	MF766338	MF766379	MF804890	–
Lichinaceae								
Lichinella cribellifera	USA, AZ, 2003	hb. M.S. 16290a	–	–	MF766341	–	MF804876	MF776092
L. iodopulchra	USA, NM, 2003	hb. M.S. 16319a	MF766300	*DQ782916*	–	MF766382	*DQ832328*	–
L. nigritella	USA, AZ, 2003	hb. M.S. 16228d	MF766301	–	MF766342	MF766383	MF804897	MF776093
Outgroup (*Geoglossaceae*)								
Trichoglossum hirsutum	–	–	*AY544697*	*AY544653*	*AY544758*	*AY789314*	*AY641087*	*AY536845*

hb. B.B.: B. Büdel, Kaiserslautern, Germany; hb. M.S.: M. Schultz, Hamburg, Germany; hb. K.K.: K. Kalb, Neumarkt, Germany; hb. DUKE: Duke University, Durham, NC, USA.

reaction. PCR from cloning plasmids was performed as follows: one cycle of 10 min at 94 °C, followed by 34 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min 30 s at 72 °C. The Montage single-column kit (Millipore, Billerica, MA, USA and Schwalbach, Germany, #UFC7PC) was used for PCR product cleaning. Sequencing reactions were prepared to a total volume of 10 µl, containing 1 µl of Big Dye (Big Dye Terminator Cycle Sequencing Kit, Abi Prism, Perkin-Elmer, Applied Biosystems), 2 µl of 5 µM primer, 3 µl sequencing buffer (5 ml 1M $MgCl_2$, 200 µl 1M Tris at pH 9, 795 µl sterile double-distilled H_2O), and 4 µl purified PCR product as template DNA. Sequencing PCR was performed as follows: one cycle of 2 min at 96 °C, followed by 24 cycles of 30 s at 96 °C, 15 s at 50 °C and 4 min at 60 °C. All samples were sequenced on an ABI automated sequencer (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA).

Data set assembly

Sequence fragments were assembled using Sequencher 4.2 and 4.5 (Gene Codes, Ann Arbor, MI, USA). Previous to this study, sequences of only two species of *Peltula* were available from GenBank (www.ncbi.nlm.nih.gov/blast). BLAST searches (Altschul *et al.* 1997) resulted in ambiguous results and the identity of newly sequenced *Peltulaceae* sequences could not be verified prior to phylogenetic analyses. Ribosomal sequences were aligned using SAM 3.5 (Karplus *et al.* 1998) with a model obtained by training with an automatic ClustalW alignment from BioEdit 7.04.1 (Hall 1999). The single locus alignments were manually adjusted, and ambiguous regions (Lutzoni *et al.* 2000) and introns were excluded from phylogenetic analyses.

Test for topological incongruence

To test for topological incongruence among loci, we used the program compat3 (available at lutzonilab.org/downloadable-programs). For each individual locus of the combined data set, 500 bootstrap replicates (Felsenstein 1985) were generated with RAxML 7.2.7 (Stamatakis 2006; Stamatakis *et al.* 2008) and all pairwise comparisons between the six loci were performed. A conflict between two loci was assumed when a clade was supported as monophyletic with a bootstrap frequency ≥ 75% in one tree, but supported, using the same bootstrap threshold, as non-monophyletic in another (Mason-Gamer & Kellogg 1996). By comparing the significant topological differences, individual sequences causing conflicts were identified. Conflicting gene sequences were removed from alignments and the test was repeated until no further conflicts could be detected. Constraint-based filters (Ihlen & Ekman 2002) were used to check for congruence between different data sets. Constraints were defined for the whole topology as well as for single relationships in a phylogenetic tree. Congruence between data partitions was assumed if a constraint was present in at least 5% of the trees of a BMCMC sample.

Phylogenetic analyses

Phylogenetic searches were carried out by implementing maximum likelihood (ML) and Bayesian analyses. All analyses were run on the computer cluster of the Nano + Bio Center (University of Kaiserslautern, Germany).

For the ML analyses, the combined data set was split into eight partitions: mtSSU, nrLSU, nrSSU, ITS (excluding unalignable spacer regions), *RPB2* 1st, *RPB2* 2nd, *RPB2* 3rd codon position, β-tub 1st, β-tub 2nd, and β-tub 3rd codon position. ML analyses were carried out using RAxML 7.2.7 and 7.2.8 (Stamatakis 2006), implementing a GTR model of nucleotide substitution (Rodriguez *et al.* 1990) with a gamma shaped distribution and a proportion of invariable sites, and searching for the most likely tree with 500 heuristic replicates. Bootstrap frequencies (Felsenstein 1985; Stamatakis *et al.* 2008) were estimated with 500 replicates.

For the Bayesian analyses, the combined data sets were partitioned as described above. Three independent runs with 10 000 000 generations and four independent chains each were started with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), sampling every 500th tree. The burn-in fraction of sampled trees was estimated both by eye with ln-likelihood plots and using AWTY (Nylander *et al.* 2008) to ensure that the runs reached stationarity.

Only nodes that received posterior probabilities (PP) ≥ 0·95 and ML-bootstrap support values (ML-BS) ≥70% were interpreted as strongly supported.

Results

Morphology

Ascomatal features as well as ascoma ontogeny are largely uniform within the *Peltulaceae* and cannot be used to separate species (Büdel 1987*a*, *b*; Egea 1989). On the basis of thallus morphology and vegetative thallus structure, six different growth types are distinguished. Two types share a common thallus anatomy while the remaining groups possess differing anatomical features (Table 1, Figs 1 & 2).

Phylogenetic analyses

The concatenated alignments included a total of 10 212 characters. The alignment of the nrSSU contained a total of eight introns, and the nrLSU contained only one intron. After excluding hypervariable regions and introns, the final data set length for phylogenetic analyses was 5583 characters (nrLSU: 1321, nrSSU: 1635, ITS: 238, mtSSU: 703, *RPB2*: 876, β-tub: 810).

The pairwise topological comparisons showed large amounts of conflict among clades supported with bootstrap ≥75%, especially between the mtSSU and the other loci (Fig. 3). After removing one sequence from the nrLSU alignment, one nrSSU sequence, three β-tub sequences and nine mtSSU sequences, all topological conflicts were resolved.

When analyzed in combination, both the original data set and the conflict-free data set produced very similar tree topologies, where only a few single taxa or small monophyletic groups of two or three taxa changed position. However, none of these alternative phylogenetic relationships were well supported by bootstrap frequencies or posterior probabilities. Alternatively, a complete removal of the mtSSU alignment did not alter the topology but resulted in minor changes of branch support. Therefore, we decided to use the conflict-free data set including the mtSSU for further discussion of the results.

The results of the combined phylogenetic analyses, after the removal of conflicting sequences, are shown in Fig. 4. The *Peltulaceae* form a well-supported (90% ML-BS, 1·0 PP) monophyletic clade. The three outgroup species of the genus *Lichinella* also form a monophyletic clade with high support (100% ML-BS, 1·0 PP).

Growth form versus phylogeny

Both *Phyllopeltula* and *Neoheppia*, two genera that were separated from *Peltula* due to their crustose or foliose growth (Büdel 1987*a*, *b*, 1995; Kalb 2001), are non-monophyletic (Fig. 4) and the representative species of these two genera are interspersed among *Peltula* species. *Phyllopeltula corticola* shares a most recent common ancestor with *Peltula leptophylla* (80% ML-BS, 1·0 PP), whereas *Phyllopeltula steppae* is sister to a clade comprising *Phyllopeltula corticola*, *Neoheppia brasiliensis* and five other *Peltula* taxa (60% ML-BS, 0·98 PP). *Neoheppia brasiliensis* is monophyletic with *Peltula farinosa* (80% ML-BS, 1·0 PP), and *N. cataractae* is a sister taxon to *Peltula boletiformis* (90% ML-BS, 1·0 PP). The polyphyly of *Phyllopeltula* and *Neoheppia* is consistent with the results of the single-gene analyses (see Supplementary Material Figs S1–S6, available online) and is

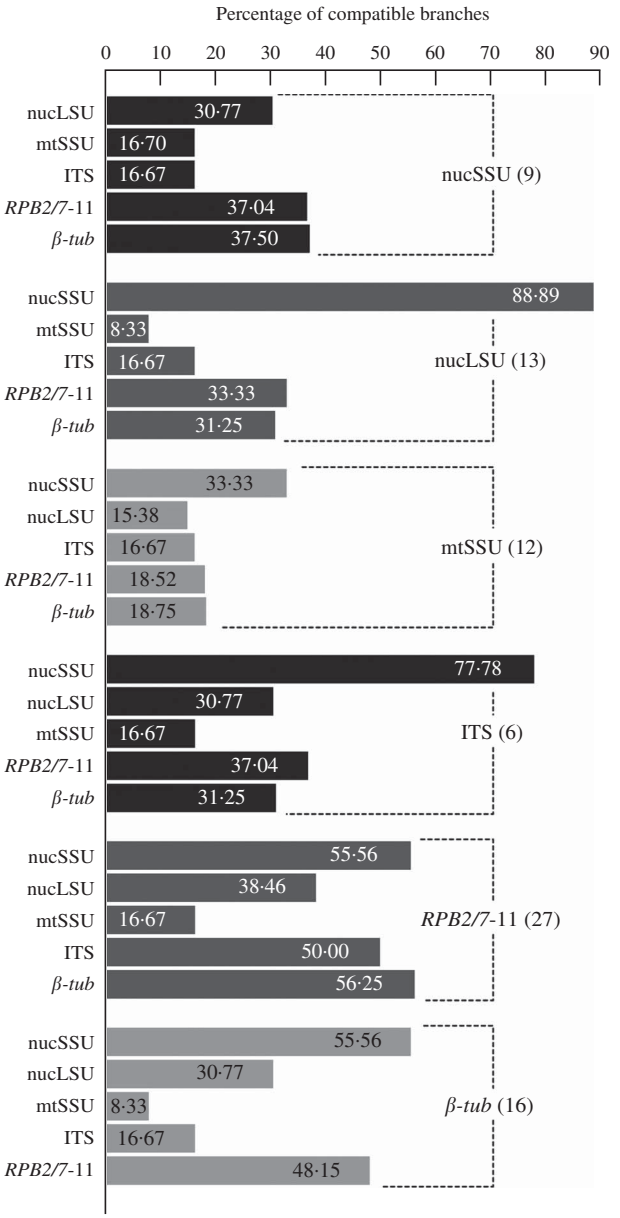


FIG. 3. Pairwise topological comparisons of the six gene loci used in this study of *Peltulaceae* using constraint-based filtering. Bars represent the percentage of compatible branches. The presence of all supported branches (i.e. constraints) of each gene (single bars) was tested in the BMCMC tree sample of each other gene (bars grouped by dashed line). The number of constraints tested for each gene is given in brackets after the name of the gene.

well supported in both ML and Bayesian phylogenetic trees.

The different types of growth form in *Peltula* (peltate, squamulose, subfruticose and subfoliose) are scattered across the tree, underlining that growth form as a character appears to be unsuitable to track phylogenetic relationships within the *Peltulaceae*. The two

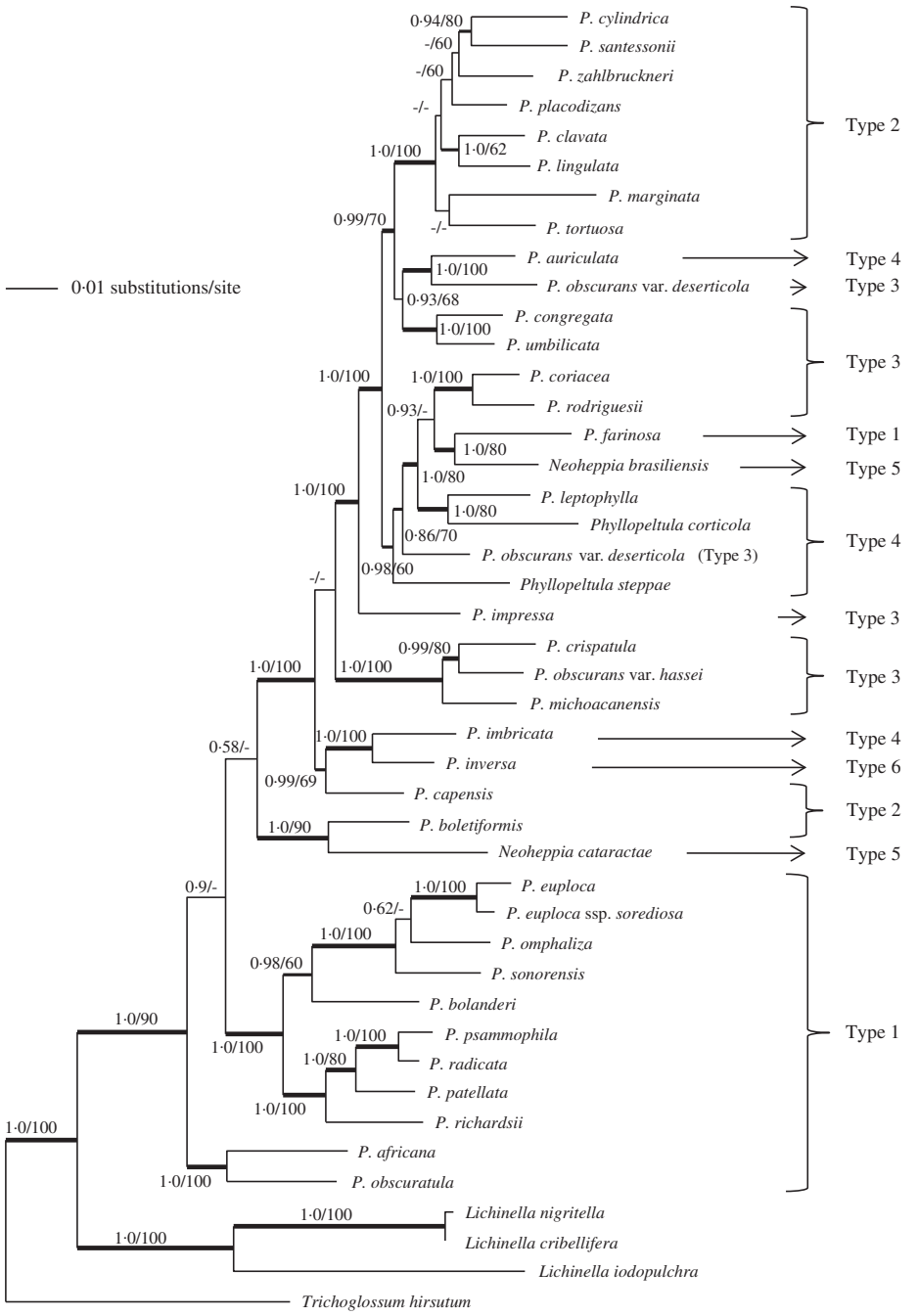


FIG. 4. Best phylogenetic tree for the family *Peltulaceae* based on the six-locus data set calculated with RAxML, using a GTR + I + G model (likelihood value = −34 009·803617). Thicker branches indicate PP ≥ 0·95 (before slash) or ML-BS ≥ 70% (after slash) support. If one of the values is absent (denoted by a dash), the respective branch was not present in the associated analysis. Growth types based on thallus morphology and vegetative thallus structure are indicated by 'Type 1–6'. For explanation, see Table 1. Genus is *Peltula* unless otherwise indicated.

smaller clades that resulted from the first split within the genus *Peltula*, both supported with 100% ML-BS and PP, exhibit peltate growth (type 1, Table 1); however, other peltate taxa are present across the rest of the tree (Fig. 4).

The same is true for the few remaining characters that are usually used to delimit species groups within the genus, such as the epinecral layer or anatomy of the medulla and presence/absence of the lower cortex. No other morphological and anatomical characters, individually or in combination, show any discernible pattern for the characterization of any of the clades present in the tree. None of the presumed evolutionary lineages described by Büdel (1987*a*) or Egea (1989) is in accordance with the results presented in this paper.

Peltula obscurans var. *hassei* and *P. obscurans* var. *deserticola* are not part of a monophyletic species. The two representatives of *Peltula obscurans* var. *deserticola* are not monophyletic either. While one is a well-supported sister to *P. auriculata* (100% ML-BS, 1·0 PP), the other forms a paraphyletic group with *Phyllopeltula steppae* (Fig. 4). *Peltula obscurans* var. *hassei* is sister with *P. crispatula* (80% ML-BS, 0·99 PP) in a well-supported clade with *P. michoacanensis*, with high support (100% ML-BS, 1·0 PP).

New Combinations and Synonyms

Peltula brasiliensis (Zahlbr.) Büdel, Kauff & Bachran comb. nov.
MycoBank No.: MB 821278

Basionym: *Neoheppia brasiliensis* Zahlbr., *Denkschr. kaiserl. Akad. Wiss. Wien math.-naturw. Kl.* **83:** 144 (1909); type: Brazil, São Paulo, Salto Grande do Rio Paranapanema, 1901, *V. Schiffner* 20702 (W—holotype, not seen; SI—isotype).

(Fig. 1R)

Peltula cataractae (Büdel & Sérusiaux) Büdel, Kauff & Bachran comb. nov.
MycoBank No.: MB 821279

Basionym: *Neoheppia cataractae* Büdel & Sérusiaux, *Mycotaxon* **54:** 140 (1995); type: Zaïre, Tshopo Waterfalls near Kisangani, 1947, *J. Léonard* 1329 (BR—holotype).

(Fig. 1K)

Peltula corticola Büdel & R. Sant.
MycoBank No.: MB 130538

Biblioth. Lichenol. **23:** 79 (1987); type: Kenya, *Santesson* 20745 (UPS—holotype).—*Phyllopeltula corticola* (Büdel & R. Sant.) Kalb, *Biblioth. Lichenol.* **78:** 158 (2001).

(Fig. 1Q)

Peltula hassei (Zahlbr.) Büdel, Kauff & Bachran comb. nov.
MycoBank No.: MB 821280

Basionym: *Heppia hassei* Zahlbr., *Beih. Bot. Centralbl.* **13:** 157 (1902); type: California, Palm Springs, *H. E. Hasse* (W—lectotype).
Peltula obscurans var. *hassei* (Zahlbr.) Wetmore, *Ann. Missouri. Bot. Gard.* **57:** 191 (1971).

(Fig. 1O)

Peltula leptophylla (Vain.) Büdel & M. Schultz comb. nov.
MycoBank No.: MB 822531

Basionym: *Heppia leptophylla* Vain., *Acta Soc. Flora Fauna Fenn.* 7(1)**:** 216 (1890); type: "supra rupem graniticam litoralem, Rio de Janeiro, n. 135b, leg. Edw. Vainio, 1885"; *Lich. Bras. Exs.* 1891 (TUR-VAIN 12474—isotype!).

Peltula steppae (Kalb) Büdel, Kauff & Bachran comb. nov.
MycoBank No.: MB 821277

Basionym: *Phyllopeltula steppae* Kalb, *Biblioth. Lichenol.* **78:** 159 (2001); type: Venezuela, Lara, Distr. Torres, *c.* 35 km E of Barquisimeto, 20 Aug. 1989, *Kalb* (M—holotype).

Discussion

Peltula, Phyllopeltula and *Neoheppia*

The single-locus tree topologies are not identical when compared to each other or to the combined analysis (Supplementary Material Figs S1–S9, available online). However, they all concur that *Phyllopeltula* and *Neoheppia* are not monophyletic. Since reproductive features cannot be used in species delimitation (see Introduction), the prior generic concept is exclusively based on morphological characters. *Phyllopeltula* and *Neoheppia* were segregated from *Peltula* based on the morphology of their cortex and the substratum, features that are

likely to adapt quickly to environmental factors. Phenotypic plasticity in lichens is not necessarily reflected in a corresponding genetic variability (Purvis 1997). In the *Peltulaceae*, the creation of an epinecral layer is dependent on the light intensity of the existing habitat (Büdel 1990; Büdel & Lange 1994). Growth types of *Peltula* (peltate, squamulose and subfruticose) are connected to characteristic habitats with different water availabilities.

The results from the combined molecular phylogenetic analyses, confirmed by the analyses of the single-locus data sets, clearly highlight that the separation of *Neoheppia* and *Phyllopeltula* from *Peltula* into distinct genera does not reflect the evolutionary history of this family. Consequently, the two genera *Phyllopeltula* and *Neoheppia* are subsumed here within *Peltula*.

The genus *Phyllopeltula* was originally established by Kalb (2001) based on *Peltula corticola*, together with *Phyllopeltula steppae* as a new species. Thus, *Phyllopeltula corticola* becomes a synonym of *Peltula corticola*, whereas *Peltula steppae* is proposed as a new combination for *Phyllopeltula steppae*. The genus *Neoheppia*, originally erected by Zahlbruckner (1909), is a synonym of *Peltula*. Consequently, *Peltula brasiliensis* and *P. cataractae* are proposed as new combinations for *Neoheppia brasiliensis* and *N. cataractae*, respectively.

The two varieties *Peltula obscurans* var. *deserticola* (Zahlbr.) Wetmore and *P. obscurans* var. *hassei* (Zahlbr.) Wetmore were established due to their differences in thallus morphology by Wetmore (1970), based on the two species *Heppia deserticola* Zahlbr. and *H. hassei* Zahlbr. Both species were treated as synonyms of *P. obscurans* and ranked as varieties of this species by the author. As neither are supported as varieties of *P. obscurans*, and cluster independently with other species of *Peltula* in this study, *P. obscurans* var. *hassei* is resurrected here as a species while *P. obscurans* var. *deserticola* is not a monophyletic entity and needs further investigation.

Species delimitation in *Peltula*

The anatomical uniformity of structures associated with sexual reproduction in the *Peltulaceae* leaves only morphological and anatomical characters of vegetative thalli for delimiting species within the genus *Peltula*. Main characters, among others, are the different growth forms (e.g. peltate, subfruticose, squamulose), attachment to the substratum (rhizines, umbilicus), presence or absence of apothecia, isidia, and characters associated with thallus medulla and cortex. The different *Peltula* species are currently distinguished by unique combinations of these features. It is thus not unexpected that the few attempts to establish phylogenetic lineages within the genus (or family) based on morphology have remained controversial (Büdel 1987*a*, *b*; Egea 1989). Our molecular analyses presented here confirm the difficulties with identifying characters that could play a role in characterizing presumably monophyletic evolutionary lineages. Morphological characters exhibit lability and, consequently, finding unique character states or combinations of characters for any of the clades has not been possible (results not shown). Despite the apparent lack of alternative characters, it is still not known whether phenotypic features can be diagnostic at the species level within the genus *Peltula*. The taxon sampling of this study is insufficient to answer this question. Printzen (2010) emphasized that the phylogenetic usefulness of specific characters varies among families and orders. However, certain taxa do have specific traits. For example, some *Peltula* species are characterized by shield-like (peltate) thalli, either with an umbilicus or a strand of hyphae (rhizines), occurring as many individuals (compound) or as single thalli (e.g. *P. euploca*, *P. bolanderi*, *P. radicata*, *P. patellata*, *P. africana*; Fig. 1A, B, D, F & I) and the squamulose to semifruticose group is characterized by large medullary cavities in the thalli that can occur in temporarily inundated habitats (e.g. *P. tortuosa*, *P. zahlbruckneri*, *P. clavata*, *P. lingulata*; Fig. 2F, H, I & K). It seems that there is a considerable amount of intraspecific variation in the thallus morphology of several species. This needs to be investigated systematically with a considerably enlarged

taxon sampling. Considering the poor taxonomic value of morphological and anatomical features of vegetative thalli in the family *Peltulaceae*, scientists should refrain from describing new species until a clearer connection between genetically and morphologically defined "species" is established.

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Supplementary Material

For supplementary material accompanying this paper visit https://doi.org/10.1017/S0024282918000105

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