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REGULAR PAPER

Molecular phylogeny of the cosmopolitan aquatic plant genus *Limosella* (Scrophulariaceae) with a particular focus on the origin of the Australasian *L. curdieana*

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Abstract *Limosella* is a small aquatic genus of Scrophulariaceae of twelve species, of which one is distributed in northern circumpolar regions, two in southern circumpolar regions, two in the Americas, one endemic to Australia, and six in tropical or southern Africa or both. The Australasian *L. curdieana* has always been considered distinct but its close phylogenetic relationships have never been inferred. Here, we investigated the following alternative phylogenetic hypotheses based on comparative leaf morphology and habitat preferences or floral morphology: (1) *L. curdieana* is sister to the African *L. grandiflora*; or (2) it is closely related to a group of other African species and the northern circumpolar *L. aquatica*. We tested these hypotheses in a phylogenetic framework using DNA sequence data from four plastid DNA regions and the nuclear ITS region. These were analyzed using maximum parsimony and Bayesian inference. We obtained moderately resolved, partially conflicting phylogenies, supporting that accessions of *L. grandiflora* form the sister group to the rest of the genus and that *L. curdieana* groups with the African taxa, *L. africana* and *L. major*, and *L. aquatica*. Thus, the molecular evidence supports the second hypothesis. A biogeographic analysis suggests an out-of-southern Africa scenario and several dispersal events in the Southern Hemisphere. Past dispersal from southern Africa to Australasia is suggested, yet it cannot be excluded that a route via tropical Africa and temperate Asia has existed.

Keywords Aquatic plants · Biogeography · Dispersal · Lamiales · Phylogenetic inference

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Introduction

Limosella L. (mudworts) is a genus in Scrophulariaceae with twelve species (Cook 2004; Glück 1934) that are either aquatic or amphibious in wetlands. The genus is distributed in temperate and subtropical regions. Their habitat and small seeds may have facilitated the global distribution of the taxon by migrating birds (Darwin 1872). The worldwide but discontinuous distribution includes one species in northern circumpolar regions, two in southern circumpolar regions, two in Americas, one endemic to Australia, and six in tropical or southern Africa or both (Cook 2004; Glück 1934; Fig. 1). Given the species richness of the genus and the distribution patterns of the related genera, such as *Jamesbrittenia* Kuntze, *Lyperia* Benth., *Manulea* L., *Selago*

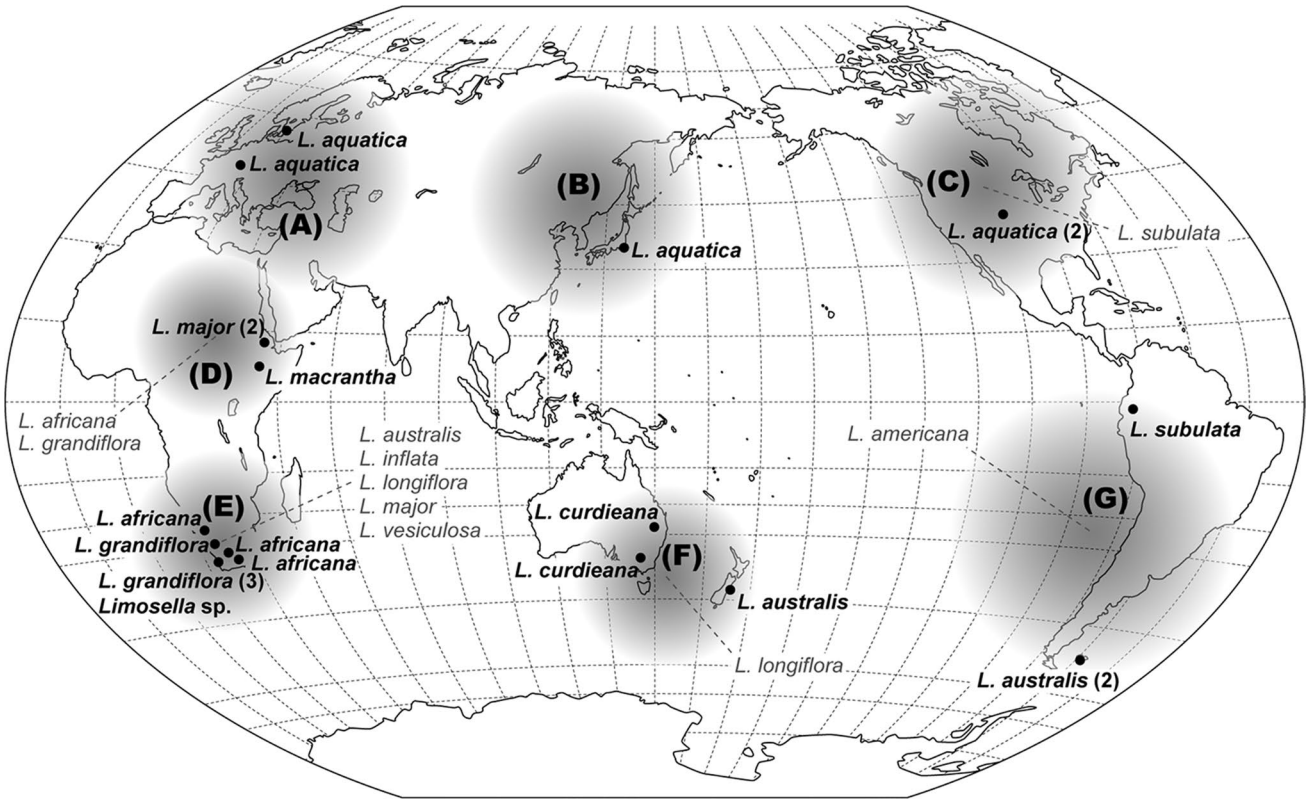

Fig. 1 Map of sampling localities of *Limosella* species. The main distribution areas of *Limosella* are roughly shaded with *circles* and letters referring to those used in Fig. 5. Species not included in this study are shown by area in *grey* font

L. (Kornhall and Bremer 2004; Oxelman et al. 2005), it is reasonable to postulate that *Limosella* originated in southern Africa (Kornhall and Bremer 2004).

While many of the species are either distributed in Africa or geographically close to Africa (*Limosella aquatica* L.), a few species are distributed in distant regions, i.e., *L. americana* Glück, *L. curdieana* F. Muell., and *L. subulata* E. Ives. Among these, *L. americana* described by Glück (1934) is sometimes recognized as a distinct taxon in Central and South America but was recently merged into *L. aquatica* (Brako and Zarucchi 1993; Cook 2004). *Limosella subulata* from North and South America has been recognized in some regional Floras (Brako and Zarucchi 1993; Crow and Hellquist 2000), but occasionally treated as a synonym of *L. australis* R.Br. (Cook 2004). *Limosella curdieana*, an Australasian endemic species, in contrast, is remarkable as it has never been synonymized, nor has its phylogenetic origin been inferred (Barker 1986, 1999; Harden 1992; Moore 1961).

Glück (1934) proposed an infrageneric classification of *Limosella* based on leaf morphology and habitat types, in which *L. curdieana* and *L. grandiflora* Benth (including its synonym, *L. capensis* Thunb.: Hilliard and Burtt 1986) are grouped together. On the basis of floral characters, however, *L. curdieana* appears to have affinities to the tropical African species, *L. africana* Glück and *L. major* Diels, as well as the circumpolar *L. aquatica* by having "petal lobes equal to or shorter than the sepals" (Figs. 2, 3; Barker 1986, 1999; Cook 2004; Godfrey and Wooten 1981; Gorshkova 1997; Harden 1992; Hong et al. 1998; Ivanina 2001; Moore 1961; Webb 1972; Yamazaki 1993).

We aim to test these two competing hypotheses for *Limosella curdieana* in Australasia and examine whether the species is phylogenetically related to an African species, *L. grandiflora*, or to a Northern circumpolar species and other African species, i.e., *L. africana* and *L. major*. For that purpose, we employed simultaneous molecular phylogenetic analyses of plastid DNA (subsequently referred to as ptDNA) and nuclear ITS (subsequently referred to as nrITS) DNA sequences based on our worldwide taxon sampling of *Limosella*.

Materials and methods

Taxon sampling

Samples of *Limosella* were collected in the field or obtained from herbaria (Table 1). We follow a broadened taxonomic concept of twelve species in the genus. Because the only

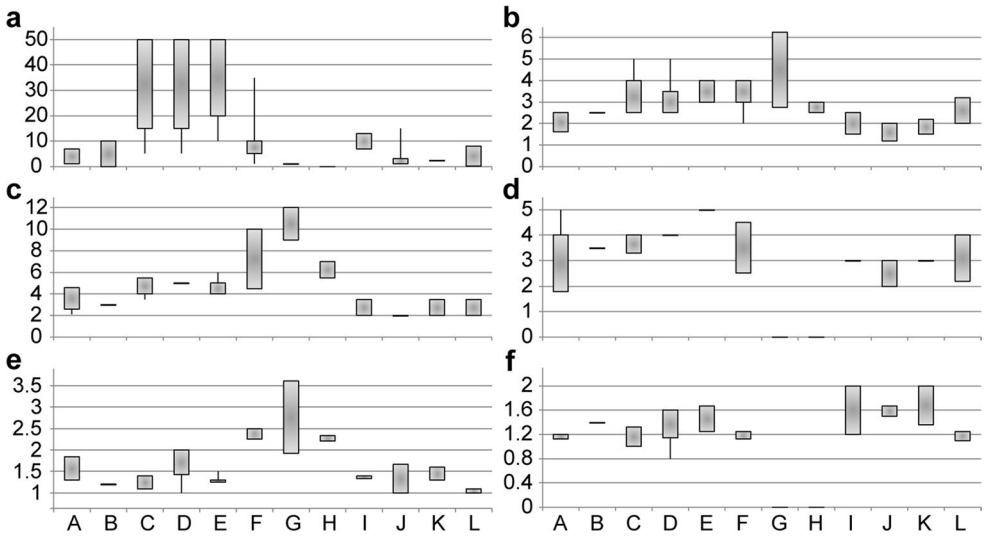

Fig. 2 Comparison of key morphological features of the petiolate-leaved *Limosella* species. Usual morphological variation is indicated with *boxes*; maximum and minimum values extracted from published taxon descriptions are indicated with *bars*. **a** Lengths of petioles; **b** length of sepals; **c** length of petals; **d** length of capsules; **e** the ratio of petal length to sepal length; **f** the ratio of capsule length to sepal length. A *vertical line* show traits (mm) in **a**–**d** and ratios in **e**, **f**. *A*: *L. africana* (Cook 2004); *B*: *L. africana* (Ghazanfar et al. 2008); *C*: *L. major* (Cook 2004); *D*: *L. major* (Philcox 1990); *E*: *L. major* (Ghazanfar et al. 2008); *F*: *L. grandiflora* (Cook 2004); *G*: *L. infata* (Cook 2004); *H*: *L. vesiculosa* (Cook 2004); *I*: *L. aquatica* (Hong et al. 1998); *J*: *L. aquatica* (Yamazaki 1993); *K*: *L. aquatica* (Gorshkova 1997); *L*: *L. curdieana* (Barker 1986, 1999; Harden 1992)

Fig. 3 *Limosella curdieana* in its natural habitat. Photos courtesy of the South Australian Seed Conservation Centre, Australia (http://www.saseedbank.com.au)

comprehensive revision made by Glück (1934) does not provide a key to the species, regional treatments were used for African (Cook 2004), Australasian (Barker 1986, 1999; Cook 2004; Harden 1992; Moore 1961), European (Webb 1972), North American (Crow and Hellquist 2000), and South American species (Cook 2004; Crow and Hellquist 2000). Yamazaki (1993) was consulted to confirm if our collection from Japan corresponded to the only species in the flora. Except for *L. grandiflora* with characteristic elongated stems (Cook 2004), positive identification of samples without flowers was problematic and these samples were treated as *Limosella* sp. Our taxon sampling, including four samples used in previous molecular phylogenetic studies (Kornhall and Bremer 2004; Oxelman et al. 2005), covers eight species: *L. africana* (3 specimens); *L. aquatica* (5; two from Europe, two from North America, and one from temperate Asia); *L. australis* (3; two from the Falklands and one from New Zealand); *L. curdieana* (2); *L. grandiflora* (4); *L. macrantha* R.E. Fr.(1); *L. major* (2); *L. subulata* (1); and *Limosella* sp. (1). The specimen UPS:BOT:V-120091, initially identified as *L. grandiflora*, was re-identified to be *L. africana*. In the present study we did not include specimens corresponding to *L. americana* in Central and South America, *L. inflata* Hilliard & B.L. Burtt and *L. vesiculosa* Hilliard & B.L. Burtt, both confined to small regions of South Africa, and *L. longiflora*, a widespread, close relative of *L. australis* (Cook 2004). Outgroup taxa were chosen following Kornhall and Bremer (2004) and Oxelman et al. (2005): species of *Lyperia* and *Jamesbrittenia*

Table 1 Specimen and voucher information for the taxa included in this study

Species	No.	Locality	Voucher	*ndhF*	*rbcL*	*rps16*	*trnL-trnF*	nrITS
Jamesbrittenia megadenia		South Africa	UPS:BOT:V-152759	AJ401404	n/a	n/a	AJ296511	AJ550584
Jamesbrittenia foliolosa		South Africa	Goldblatt P. & Porter L. 12488 (NBG)	n/a	AM235139	n/a	n/a	n/a
Lyperia antirrhinoides		South Africa	Hagstroem & Acock 1162 (S)	AJ401405	n/a	n/a	AJ296521	AJ616324
Lyperia tristis		South Africa	Manning J.C. 2854 (NBG)	n/a	AM235140	n/a	n/a	n/a
Limosella africana	α	South Africa: Eastern Cape; Elandsberg	UPS:BOT:V-120091	AJ550552	LC132983	LC133001[a]	AJ550525	AJ550587
Limosella africana	β	Namibia: Hunsberge; Nuobrivier	W. Giess & M. Muller 14313 (PRE)	n/a	LC132985	LC133003	LC133018	LC133035
Limosella africana	γ	South Africa: Central Cape; De Kom/Aarfontein	N.H. Helme 1541 (NBG)	n/a	LC132984	LC133002	LC133017	LC133034
Limosella aquatica	α	Hungary: South	YI1617 (TNS)	LC132973	LC132991	LC133009	LC133024	LC133041
Limosella aquatica	β	Sweden: Östergötland	UPS:BOT:V-155230	AJ550547	n/a	n/a	n/a	AJ550588
Limosella aquatica	γ	Japan: Saitama; Koshigaya	TD4036 (TNS)	LC132972	LC132990	LC133008	LC133023	LC133040
Limosella aquatica	δ	USA: Nebraska	NEB:295749	LC132974	LC132992	LC133010	LC133025	LC133042
Limosella aquatica	ε	USA: Nebraska	NEB:289070	LC132975	LC132993	LC133011	LC133026	LC133043
Limosella australis	α	UK: Falklands	K:14895	n/a	LC132986	LC133004	LC133019	LC133036
Limosella australis	β	UK: Falklands	K:39593	LC132969	LC132987	LC133005	LC133020	LC133037
Limosella australis	γ	New Zealand: Canterbury; Lake Forsyth	YI1769 (TNS)	LC132970	LC132988	LC133006	LC133021	LC133038
Limosella curdieana	α	Australia: Victoria; Gunbower Isl.	MEL:2371937	LC132976	LC132994	LC133012	LC133027	LC133044
Limosella curdieana	β	Australia: Queensland; South Glen	CANB:00703245	LC132977	LC132995	LC133013	LC133028	LC133045
Limosella grandiflora	α	South Africa: Northeast Cape; Springbok	A. Le Roux 2355 (PRE)	n/a	LC132981	LC132999	n/a	LC133032
Limosella grandiflora	β	South Africa: Western Cape; Riebeeckasteel	NBG:0272166-0	LC132967	LC132980	LC132998	LC133015	LC133031
Limosella grandiflora	γ	South Africa: Western Cape; Knolfontein	NBG:0230228-0	n/a	LC132978	LC132996	n/a	LC133029
Limosella grandiflora	δ	South Africa: Western Cape; Swartruggens	NBG:0249071-0	LC132966	LC132979	LC132997	LC133014	LC133030
Limosella macrantha		Ethiopia: Bale Prov.; Bale Mts. Natl. park	UPS:BOT:V-152745	AJ550553	n/a	n/a	AJ550526	AJ550586
Limosella major	α	Ethiopia: Begemdir Prov.; Simien	UPS:BOT:V-152744	AJ550548	n/a	n/a	n/a	AJ550585
Limosella major	β	Ethiopia: Arsi-Robe/Sertu	K:46167	LC158860	LC158859	LC158861	LC158862	LC158858
Limosella subulata		Ecuador: Napo; Río Chalupas	Lagaard 101769 (AAU)	LC132971	LC132989	LC133007	LC133022	LC133039
Limosella sp.		South Africa: Western Cape; Swartruggens	YI1991 (TNS)	LC132968	LC132982	LC133000	LC133016	LC133033

Sequences generated in the present study are underlined. Herbarium acronyms are in accordance with Index Herbariorum (http://sciweb.nybg.org/science2/IndexHerbariorum.asp)

[a] This replaces AJ609170

from the tribe Limoselleae Dumort., representing the sister genus to *Limosella* (*Lyperia*) and the basal genus of the tribe (*Jamesbrittenia*) for initial molecular phylogenetic analysis; those of *Barthlottia*, *Chenopodiopsis*, *Cromidon*, *Dischisma*, *Glumicalyx*, *Hebenstretia*, *Jamesbrittenia*, *Lyperia*, *Manulea*, *Melanospermum*, *Microdon*,

Phyllopodium, *Polycarena*, *Pseudoselago*, *Reyemia*, *Selago*, *Sutera*, *Tetraselago*, *Trieenea*, and *Zaluzianskya* for species tree analysis (Table 1; Online resource 1). *Glekia* is not included due to the lack of nrITS data.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from silica gel-dried leaf tissues or from herbarium specimens using the CTAB method described in Ito et al. (2010). Parts of the plastid DNA regions, *ndhF*, *rbcL*, *rps16* and *trnT-trnF,* and nrITS were PCR amplified with the following primers: ndhF-F2 (Oxelman et al. 1999) and ndhF-1955R.re (5′-CGATTATAKGACCAATTATATA) modified from Olmstead and Sweere's (1994) ndhF-1955R for *ndhF*; rbcL-F1F (Wolf et al. 1994) and rbcL-1379R (Little and Barrington 2003) for *rbcL*; rps16-1F and rps16-2R (Oxelman et al. 1997); "a" and "b" for *trnT-trnL* and "c" and "f" for *trnL-trnF* (Taberlet et al. 1991); ITS-4 and ITS-5 for nrITS (Baldwin 1992). PCR amplification was performed using TaKaRa Ex Taq polymerase (TaKaRa Bio, Shiga, Japan), and PCR cycling conditions were 94 °C for 60 s; then 30 cycles of 94 °C for 45 s, 52 °C for 30 s, 72 °C for 60 s; and finally 72 °C for 5 min. The PCR products were cleaned using ExoSAP-IT (GE Healthcare, Piscataway, NJ, USA) purification and amplified using ABI PRISM Big Dye Terminator ver. 3.1 (Applied Biosystems, Foster City, CA, USA) with the same primers as those used for the PCR amplifications. DNA sequencing was performed with an ABI PRISM 377 DNA sequencer (Applied Biosystems). Automatic base-calling was checked by eye using Genetyx-Win ver. 3 (Software Development Co., Tokyo, Japan). The sequences generated and their metadata were submitted to the DNA Data Bank of Japan (DDBJ), which is a GenBank data provider (Table 1).

Data analysis

We assembled two datasets of *Limosella*: (1) ptDNA (*ndhF*, *rbcL*, *rps16*, and *trnT-trnF*); and (2) nrITS. Sequences were aligned using Mafft ver. 7.058 (Katoh and Standley 2013) and then inspected manually. We used "leave gappy regions" option in Mafft to code gaps found in *ndhF*, *rps16*, *trnT-trnF*, and nrITS.

The incongruence length difference test (Farris et al. 1994) in PAUP* ver. 4.0b10 (Swofford 2002) was employed to test for phylogenetic congruence among the four ptDNA regions using a partition homogeneity test with 1,000 replicates. This test did not reveal significant heterogeneity among genes (P value >0.05), and all subsequent analyses were therefore performed with a combined data set of ptDNA.

Phylogenetic inference was performed using maximum parsimony (MP) in PAUP* (Swofford 2002) and Bayesian inference (BI; Yang and Rannala 1997). In the MP analysis, a heuristic search was performed with 100 random addition replicates involving tree-bisection-reconnection (TBR) branch swapping, with the MulTrees option in effect. The MaxTrees option was set at 100,000. Bootstrap analyses (Felsenstein 1985) were performed using 1,000 replicates with TBR branch swapping and simple addition of sequences. The MaxTrees option was set to 1,000. Gaps were treated as binary characters.

BI analyses were conducted with MrBayes ver. 3.2.2 (Ronquist and Huelsenbeck 2003; Ronquist et al. 2012) run on the CIPRES portal (Miller et al. 2010) after the best models had been determined in MrModeltest ver. 3.7 (Nylander 2002); these models were GTR + I + Γ and GTR + Γ for ptDNA and nrITS, respectively. For gap characters, the "datatype = standard" option of MrBayes was used and default prior settings were applied, i.e., "ratepr = variable". Analyses were run for 670,000 and 415,000 generations for ptDNA and nrITS, respectively, until the average standard deviation of split frequencies dropped below 0.01, sampling every 1,000 generations and discarding the first 25% as burn-in. The convergence and effective sampling sizes (ESS) of all parameters were checked in Tracer ver. 1.6 (Rambaut et al. 2014). All trees were visualized using FigTree ver. 1.3.1 (Rambaut 2009). Nodes are recognized as strongly, moderately, or weakly supported with: ≥95% bootstrap support (BS), ≥0.99 Bayesian posterior probabilities (PP); ≥70% BS, ≥ 0.95 PP; or <70% BS; <0.95 PP, respectively. The data matrices and the MP and BI trees are available at Treebase (TB2:S19401).

Species trees considering all samples were reconstructed for biogeographic analysis. *Limosella macrantha* was excluded due to incongruent positions between ptDNA and nrITS trees (see "Results"). A multispecies coalescent method (Heled and Drummond 2010) implemented in BEAST ver. 1.7.2 (Drummond et al. 2006; Drummond and Rambaut 2007) was performed. We ran *BEAST using the two data sets (ptDNA and nrITS) from 21 samples from seven ingroup taxa and assigning them to six terminal species, namely *L. aquatica*, *L. australis*, *L. curdieana*, *L. grandiflora*, *L. major*, plus *Limosella* sp.

We performed two independent runs of ten million generations of the MCMC chains, sampling every 1,000 generations. Convergence of the stationary distribution was checked by visual inspection of plotted posterior estimates using Tracer ver. 1.5 (Rambaut and Drummond 2007). After discarding the first 1,000 trees as burn-in, the samples were summarized in the maximum clade credibility tree using TreeAnnotator ver. 1.6.1 (Drummond and Rambaut

Table 2 Percentage of missing characters and gaps by DNA regions

	Missing characters	Total characters[a]	Percentage of missing characters (%)	Gap length	Total alignment length	Percentage of gaps (%)
rbcL	4,858	31,968	15.20	0	1,332	0.00
rps16	3,705	16,560	22.37	70	760	9.21
trnL	4,035	20,544	19.64	125	981	12.74
ndhF	21,833	46,872	46.58	12	3,937	0.30
nrITS	917	16,296	5.63	12	687	1.75

[a] Gaps are excluded

2007) with a posterior probability limit of 0.5 and summarizing mean node heights. The results were visualized using FigTree ver. 1.3.1 (Rambaut 2009).

Biogeographic analysis

Reconstruction of historical biogeography of *Limosella* was performed using RASP ver. 3.2 (Reconstruct Ancestral State in Phylogenies) (Yu et al. 2015). The Bayesian Binary Method (BBM; Ronquist and Huelsenbeck 2003) was selected because it: (1) accepts polytomies; (2) tends to suggest single distribution areas for ancestral nodes more often than others (Müller et al. 2015); and (3) is capable of providing unambiguous and informative results (Ito et al. 2016). BBM was conducted using the post burn-in species trees that resulted from the *BEAST analysis. The following seven biogeographic areas were defined: (a) Europe; (b) temperate Asia; (c) North America; (d) tropical Africa; (e) southern Africa; (f) Australasia; (g) South America (including Falklands). For the distribution of the species we used the area including the locality of accessions from the molecular phylogenetic analysis of the present study. Multiple ancestral states were allowed. The number of generations was set to 10 million and the first 10% of the samples were discarded as burn-in. All other parameters were kept at default settings.

Results

Molecular phylogeny

Sequences of the concatenated four ptDNA regions of *Limosella* consisting of 22 ingroup and two outgroup samples resulted in an alignment with a total length of 5,067 bp. In total 428 characters including 29 binary-coded indels were polymorphic, of which 214 were parsimony-informative. Percentage of missing characters and gaps were: 15.20 and 0.00% (*rbcL*); 22.37 and 9.21% (*rps16*); 19.64 and 12.74% (*trnL*); 46.58 and 0.30% (*ndhF*) (Table 2). Analysis of this data set yielded the imposed limit of 100,000 MP trees (tree length = 466 steps; consistency index = 0.96; retention index = 0.96). The strict-consensus MP tree and MrBayes BI 50% consensus trees showed no incongruent phylogenetic relationships. Therefore, the better resolved MrBayes tree is presented (Fig. 4a).

Limosella was divided into two strongly-supported groups: a clade of four accessions of *L. grandiflora* (group I; 100% BS; 1.0 PP) and the rest of the genus (100% BS; 1.0 PP). In the latter clade, *Limosella* sp. was placed as sister to the remaining accessions (89% BS; 1.0 PP). The other supported groups were (1) *L. australis* and *L. subulata* (group II; 88% BS; 1.0 PP) and (2) *L. aquatica*, *L. curdieana*, *L. macrantha*, and *L. major* (group III plus *L. macrantha*; <50% BS; 0.98 PP). Three accessions of *L. africana* were resolved in a polytomy with *L. australis*-*L. subulata* and *L. aquatica*-*L. curdieana*-*L. macrantha*-*L. major*.

The nrITS alignment consisting of 22 ingroup and two outgroup sequences had a total length of 691 bp. In total 121 characters including four binary-coded indels were polymorphic, of which 63 were parsimony-informative. Percentage of missing characters and gaps were: 5.63 and 1.75%, respectively (Table 2). Analysis of this data set yielded 22 MP trees (tree length = 166 steps; consistency index = 0.86; retention index = 0.89). The strict-consensus MP tree and MrBayes BI 50% consensus trees showed no incongruent phylogenetic relationships. Therefore, the better resolved MrBayes tree is presented (Fig. 4b).

A topology similar to that of ptDNA was recovered, with two strongly-supported clades: one comprising four accessions of *Limosella grandiflora* (group I; 87% BS; 1.0 PP) and another consisting of the remaining taxa (99% BS; 1.0 PP). In the clade of 17 accessions, *L. africana* UPS:BOT:V-120091 was placed as sister to the remaining accessions (68% BS; 0.99 PP). A clade of *L. macrantha* and *L. africana* β (87% BS; 0.98 PP) positioned sister to the remaining accessions (<50% BS; 1.0 PP). Group II, consisting of *L. australis* and *L. subulata* (88% BS; 0.99 PP) and group III (*L. aquatica*, *L. curdieana*, and *L. major*; 83% BS; 1.0 PP) formed a clade in the BI analysis (<50% BS; 0.97 PP), which was placed as sister to *L. africana* N.H. Helme 1541 (NBG).

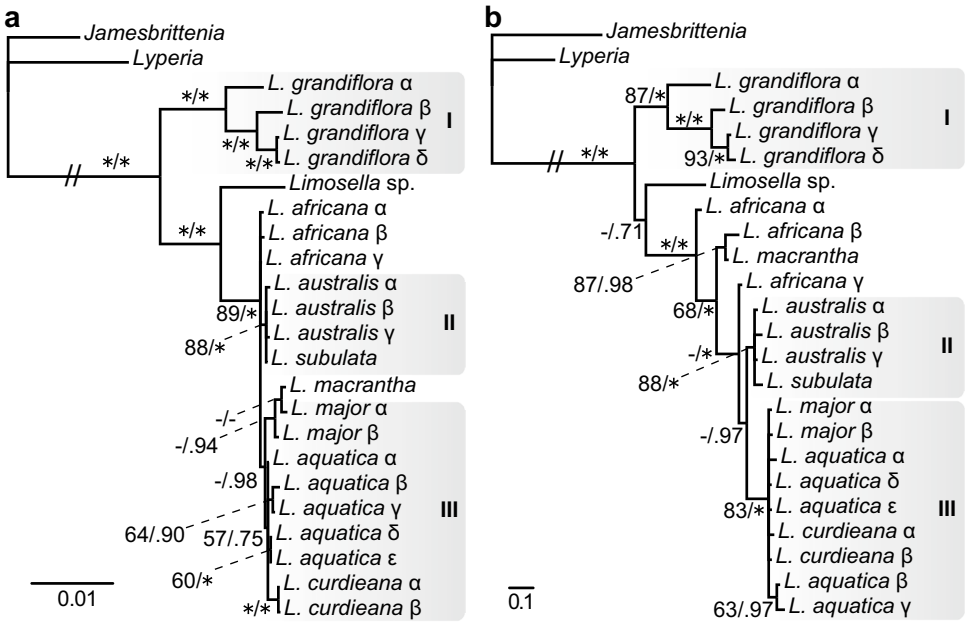

Fig. 4 MrBayes trees of *Limosella* based on **a** plastid DNA and **b** nuclear ITS. Branch lengths are proportional to molecular divergence among accessions. *Numbers* above or below the branches indicate bootstrap support (BS) calculated in maximum parsimony and Bayesian posterior probabilities (PP). BS < 50% and PP < 0.9 are indicated by *hyphens* while those of ≥95% and ≥0.99 are *asterisks*. Moderately- to strongly-supported groups are surrounded by round rectangles in background in gray and numbered. It should be noted, however, that group III is paraphyletic in ptDNA due to the inconsistent position of *L. macrantha* (see "Discussion")

The *BEAST species tree analysis retrieved two lineages: (a) BCDGHMPRSTZ (*Barthlottia*; *Chenopodiopsis*; *Cromidon*; *Dischisma*; *Glumicalyx*; *Hebenstretia*; *Manulea*; *Melanospermum*; *Microdon*; *Phyllopodium*; *Polycarena*; *Pseudoselago*; *Reyemia*; *Selago*; *Sutera*; *Tetraselago*; *Trieenea*; *Zaluzianskya*); (b) *Limosella* and *Lyperia* (Fig. 5).

Biogeographic reconstruction

The ancestral area of *Limosella* was inferred in southern Africa (1.0 PP); ambiguous results were obtained for the ancestral area of the most recent common ancestor (MRCA) of *L. aquatica*, *L. curdieana*, and *L. major* with highest probability for Australia (70.9 PP) (Fig. 5; Online resource 2).

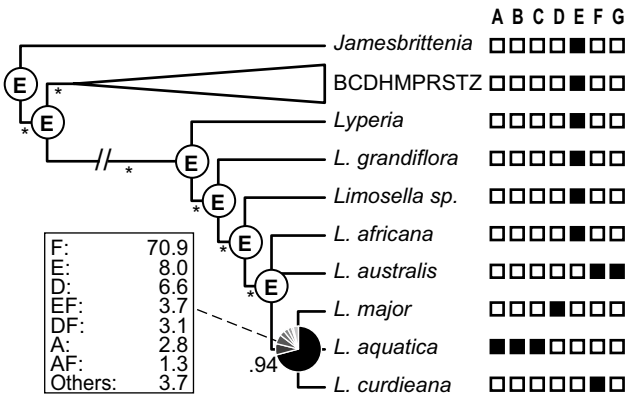

Fig. 5 Bayesian *BEAST species tree for *Limosella* based on plastid DNA and nuclear ITS data. The outgroup clade (BCDGHMPRSTZ: *Barthlottia*; *Chenopodiopsis*; *Cromidon*; *Dischisma*; *Glumicalyx*; *Hebenstretia*; *Manulea*; *Melanospermum*; *Microdon*; *Phyllopodium*; *Polycarena*; *Pseudoselago*; *Reyemia*; *Selago*; *Sutera*; *Tetraselago*; *Trieenea*; *Zaluzianskya*) has been collapsed. Values above or below the branches represent the Bayesian posterior probabilities (PP). PP ≥ 0.95 are indicated by *asterisks*. Clades without significant support (PP < 0.75) are collapsed. Ancestral areas inferred from Bayesian Binary MCMC (BBM) are shown on each node. Multiple posterior results are shown in a *box*. The area codes follow Fig. 1 (*A* Europe; *B* temperate Asia; *C* North America; *D* tropical Africa; *E* southern Africa; *F* Australasia; *G* Central and South America)

Discussion

Phylogeny of *Limosella* and the position of *L. curdieana*

The present study reconstructed the most detailed molecular phylogeny of the genus *Limosella* to date with a primary aim to test two competing hypotheses for ancestral relationships of the Australasian species, *L. curdieana*, i.e., whether the species is phylogenetically closely related to (1) the African species, *L. grandiflora*, or (2) a group composed of the two African species, *L. africana* and *L. major*, and the Northern circumpolar species, *L. aquatica*. The topologies recovered based on ptDNA and nrITS, respectively, are mostly congruent, except for a single accession of *L. macrantha* (see below). Both topologies resolve *L. grandiflora* as sister to the remaining species, including *L. africana*, *L. aquatica*, *L. curdieana*, and *L. major* (Fig. 4). The molecular evidence thus clearly rejected the hypothesis proposed by Glück (1934) and instead supported a relationship of *L. curdieana* with *L. africana*, *L. major* and *L. aquatica*.

Topological incongruence between ptDNA and nrITS

We detected a topological incongruence caused by our single accession of *Limosella macrantha*. In the ptDNA phylogeny this tropical African species (Ghazanfar et al. 2008; Glück 1934) is resolved in a clade including multiple accessions of the likewise tropical African species, *L. major*, whereas in the ITS phylogeny it is sister to southern African *L. africana*. Although morphological evidence suggests a close relationship between *L. macrantha* and *L. australis* (including specimens previously segregated as *L. subulata*) (Cook 2004; Glück 1934), the single accession of *L. macrantha* obtained from previous phylogenetic studies (Kornhall and Bremer 2004; Oxelman et al. 2005) was resolved as distantly related to *L. australis* (Fig. 4). Further taxon and data sampling will reveal whether this single specimen is part of an introgressive hybrid swarm or represents a hybrid. Alternatively, incomplete lineage sorting cannot be excluded as cause of the topological conflicts since this is common in closely related lineages such as *Limosella*.

Biogeography of *Limosella* and implications for dispersal

The present study supports a basal diversification of *Limosella* in southern Africa with moderate to strong support (Fig. 5), confirming a southern African origin of the genus as indicated by species richness of the ingroup and the distribution of sister genera. Thus, southern Africa seems to be a cradle for not only a number of drought-adapted taxa such as Crassulaceae (Mort et al. 2001), Scrophulariaceae (Oxelman et al. 2005), *Thesium* L. (Moore et al. 2010), Amaryllidoideae (Rønsted et al. 2012), and *Asparagus* L. (Norup et al. 2015) but also aquatics such as *Limosella*.

Bell et al. (2010) provided an estimated origin of Scrophulariaceae earlier than the mid-Paleogene, since tribes Myoporeae and Scrophularieae diversified ca. 51–53 mya. As *Limosella* is derived much later in the evolution of the family (Oxelman et al. 2005), vicariance due to plate tectonics is not an option to explain the discontinuous distribution worldwide and instead seed dispersal is more likely as is mentioned by Cook (2004): "the disseminules are small seeds, dispersed in mud and perhaps otherwise." The current distribution of *L. australis* (including *L. subulata*) in e.g., Ecuador, New Zealand, and the Falklands could be a case of "wind highways" existing in the Southern Hemisphere (Muñoz et al. 2004).

The biogeographic origin of *Limosella curdieana* remains uncertain, but a close relationship with *L. aquatica* from Europe, temperate Asia, and North America, and *L. major* from tropical Africa (Ethiopia) is moderately supported (Fig. 4). The biogeographic analysis suggests Australasia as the ancestral area for the MRCA of the three species and thus implies a dispersal route from southern Africa to Australasia, and then to temperate Asia and tropical Africa (Fig. 5). Alternatively, considering the facts that (1) migratory bird routes are well documented between Asia and Australasia (Boere and Stroud 2006), (2) case studies exist that reveal disjunct distributions between Asia and Australasia (*Lobelia* L.: Kokubugata et al. 2012; *Solenogyne* Cass.: Nakamura et al. 2012), and (3) young plant groups in Australia have predominantly migrated from Asia (Crisp and Cook 2013), dispersal to Australasia via temperate Asia may also explain the geographic isolation of *L. curdieana* in Australia.

Implications for taxonomy of *Limosella*

Our molecular phylogenetic results provide additional insights into the taxonomy of *Limosella*. The three specimens of *L. africana* show close relationships, especially in ptDNA analysis, but do not form a clade in either of the analyses. No significant morphological differences are observed among the specimens except leaf shape, i.e., linear-lanceolate leaves for *L. africana* α and ovate leaves for *L. africana* β and *L. africana* γ. Still, all three specimens could belong to *L. africana* var. *africana* Glück, even in the strict sense, because none of these have much-clustered, short-pedicellate flowers or fruits (Cook 2004; Glück 1934). The sterile *Limosella* sp. shows affinities to *L. africana* var. *africana* in vegetative morphology, i.e., ovate leaves. Additional taxon sampling in flower and fruit from the locality and/or nearby habitats will provide further insight into the affinities of this accession, e.g., whether it belongs to either of the varieties, or represents an undescribed taxon. Since non-monophyletic taxa may be the result of various processes such as incomplete lineage sorting, a proper investigation of the species status of *L. africana* would require sampling from multiple loci in a species tree context.

The single accession of *L. subulata* from Ecuador forms a clade with three accessions of *L. australis* from New Zealand and the Falklands. This result agrees with the taxonomic treatment by Cook (2004) who synonymized *L. subulata* with the widespread *L. australis*. Further studies based on increased taxon and data sampling focusing on the taxonomic status of these taxa are needed.

Conclusions

Within a phylogenetic framework we provided new insights into the evolutionary origin of *L. curdieana*, the only Australasian endemic species in the genus. Our molecular phylogenetic analyses based on ptDNA and nrITS data sets

revealed a basal diversification in southern Africa and close relationships between *L. curdieana* and northern circumpolar as well as a species from tropical Africa. The biogeographic analysis points to an out-of-southern Africa into the Northern Hemisphere and subsequent dispersal back into the Southern Hemisphere. Past dispersal from southern Africa to Australasia is suggested, however, we cannot exclude the existence of a route via tropical Africa and Asia.

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