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ORIGINAL PAPER

Molecular identification and phylogeny of an aquatic moss species in Antarctic lakes

Kengo Kato · Tomotsugu Arikawa · Satoshi Imura · Hiroshi Kanda

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Abstract Due to the morphological variability, the identification of moss species can be difficult when the plant grows in submerged environments. The taxonomic status of an aquatic moss found in lakes of the Sôya Coast region, East Antarctica, had been controversial, and then, it was investigated by molecular phylogenetic and haplotype network analysis of two chloroplast regions (*rps*4 and *trn*L-F) and/or the nuclear ribosomal ITS region. Based on the results of the analyses, the moss was assigned to the genus *Leptobryum* and determined to be conspecific with *Leptobryum wilsonii* (Mitt.) Broth. described from South America. Almost no genetic variation was observed between all samples from Antarctic lakes and some samples of *L. wilsonii* from Chile. Molecular and geohistorical evidence suggests that immigration of *L. wilsonii* into Antarctic lakes took place during the Holocene via long-distance dispersal from South America. This study gives a clear example of the widespread assumption that most of the Antarctic moss species are post-glacial immigrants.

Keywords Aquatic moss · *Leptobryum wilsonii* · Long-distance dispersal · Molecular phylogeny · Moss pillar · Moss taxonomy

K. Kato (✉) · S. Imura · H. Kanda
Department of Polar Science, The Graduate University for Advanced Studies (SOKENDAI), 10-3 Midori-cho, Tachikawa, Tokyo 190-8518, Japan
e-mail: kkato@nipr.ac.jp

T. Arikawa
Tottori Prefectural Museum, 2-124 Higashi-machi, Tottori 680-0011, Japan

S. Imura · H. Kanda
National Institute of Polar Research, 10-3 Midori-cho, Tachikawa, Tokyo 190-8518, Japan

Introduction

In various lakes of the Sôya Coast region, Dronning Maud Land, East Antarctica (Fig. 1), two aquatic moss species are currently recognized as components of the benthic vegetation (e.g., Imura et al. 2003). As reported by Kanda and Iwatsuki (1989), one is an aquatic form of *Bryum pseudotriquetrum* (Hedw.) P. Gaertn., B. Mey. & Scherb., a common species in Antarctic terrestrial environments. The identification of the other species is still controversial. The unidentified moss is sterile and is represented by non-characteristic filiform plants possessing oblong-lanceolate and unbordered leaves with short and weak costa and characteristic pale brown rhizoidal tubers (asexual propagules) (Fig. 2a–d). This moss is also the main component of "moss pillar," a unique column or pillar-shaped vegetational structure consisting of two aquatic mosses, algae, and microorganisms (e.g., Nakai et al. 2012a, b), which has been found only at the bottom of some of the lakes in this region (Fig. 2e; Imura et al. 1999, 2003).

As summarized in Table 1, the moss has a long and complex taxonomic history. Nakanishi (1977) reported a moss with rhizoidal tubers from the bottom of several lakes in the Sôya Coast region and noted its external similarity to *Bryum korotkevicziae* Sav. & Smirn. and the variety *hollerbachii* Sav. & Smirn. that described from lakes in the Bunger Hills (66°18′ S, 100°45′ E), East Antarctica (Savich-Lyubitskaya and Smirnova 1959, 1960). Because the plants were sterile, Ochi (1979) treated the moss as a *Bryum* sp. and Imura and Kanda (1986) described its smooth rhizoids and spherical tubers but did likewise. Kanda and Iwatsuki (1989) considered it to be a *Dicranella* sp. The species was identified as *Leptobryum pyriforme* (Hedw.) Wilson by Imura et al. (1992) based on its rhizoidal tubers and synoicous inflorescences under culture

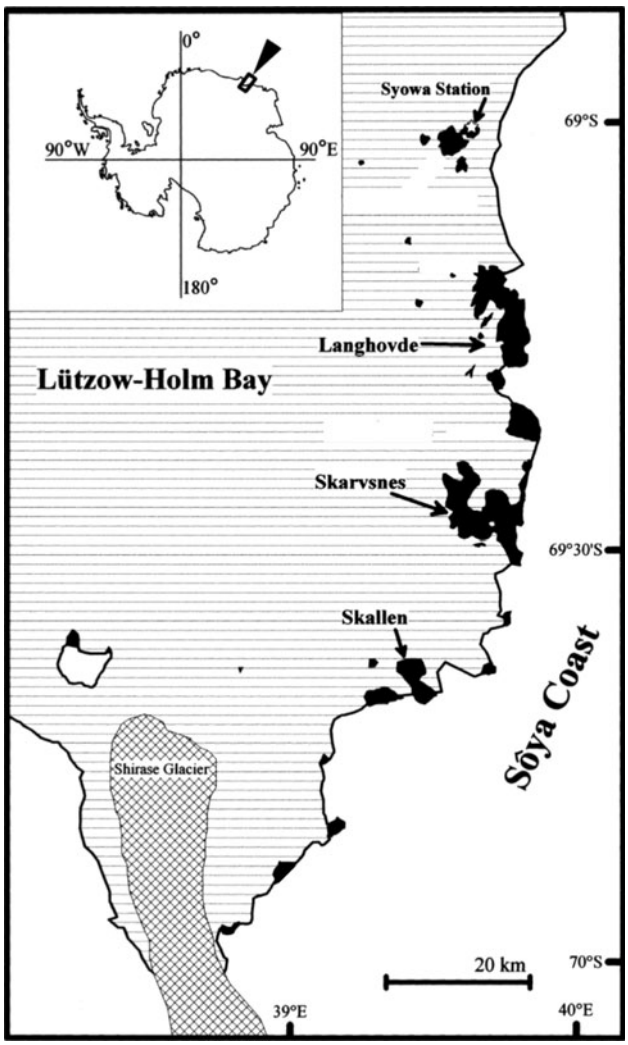


Fig. 1 Location of the Sôya Coast region and the three ice-free areas, Langhovde, Skallen, and Skarvsnes

conditions, but later Imura et al. (1999) treated it merely as a *Leptobryum* sp. In a comprehensive review of the genus *Leptobryum*, Arts (2001) recognized only two valid species, *L. pyriforme* and *L. wilsonii*. Using the illustration by Kanda and Iwatsuki (1989) as a reference, he considered the aquatic moss from Sôya Coast region to be *L. wilsonii* based on leaf characters. This species designation was questioned by Imura et al. (2003), however, because of the synoicous inflorescence—considered to be characteristic of *L. pyriforme* rather than *L. wilsonii*—that had been previously observed on cultured plants (Imura et al. 1992). The most recent taxonomic interpretation of this moss is that of Ochyra et al. (2008). These authors argued that sexuality (i.e., monoecy or dioecy) is not always a reliable diagnostic character for these species, especially under culture conditions, and recognized it as *L. wilsonii*. At the same time, based on type specimens of *L. wilsonii* from South America, they proposed the new combination *Pohlia wilsonii* (Mitt.) Ochyra, as they considered that there was a taxonomic affinity between *L. wilsonii* and *Pohlia* section *Cacodon* comprising the propaguliferous species in the genus (e.g., Shaw 1984). Morphological similarities between *L. wilsonii* and some species of *Pohlia* have also been noted by other authors (Shaw 1985 and Arts 1995). This species was actually even described previously as *Pohlia integra* (Cardot) A.J. Shaw (Shaw 1982). Recent molecular phylogenetic studies, however, suggest that *Leptobryum* and *Pohlia* are only distantly related (e.g., Cox et al. 2000; Goffinet et al. 2001; Guerra et al. 2011).

Apart from classification difficulties with respect to *L. wilsonii* versus *Pohlia*, much of the taxonomic confusion surrounding the aquatic moss from the Sôya Coast region is due to the sterile condition and phenotypic plasticity of the plant. It is well known that morphological characters of mosses often vary when the plants are submerged (e.g., Lodge 1959; Priddle 1979). In this study, we performed a molecular phylogenetic analysis to determine the taxonomic status of this puzzling moss. In addition, we conducted a haplotype network analysis to uncover detailed phylogenetic relationships and genetic variation within related taxa. Using the results of these analyses and geohistorical considerations, we also examined the origin of this species and its immigration history into Antarctic lakes.

Materials and methods

Plant materials

In the Sôya Coast region, an aquatic moss with rhizoidal tubers has been found in 26 lakes in three ice-free areas: Langhovde (one lake), Skallen (one lake), and Skarvsnes (24 lakes) (Imura et al. 2003 as *Leptobryum* sp.). In this study, 14 samples of this moss that collected from 11 lakes in the three ice-free areas were used for DNA sequencing as shown in Table 2. After collection, 12 of the samples were cultured under laboratory conditions to obtain high-quality DNA. The other two samples were kept frozen until DNA extraction.

Because the aquatic moss was thought to belong to *Leptobryum* (e.g., Arts 2001 as *L. wilsonii*; Imura et al. 1992 as *L. pyriforme*) or *Pohlia* (Ochyra et al. 2008 as *P. wilsonii*), we also included 10 samples of *L. pyriforme* from various regions of the world and four samples of *L. wilsonii* from South America (Table 2) to test their phylogenetic relationships to this species. The 10 samples of *L. pyriforme* were selected from a set of 49 samples used in a preliminary global phylogeographic study of *L. pyriforme* based on *rps*4, *trn*L-F, and ITS regions (Kato and Imura, unpublished data), and were chosen to represent the

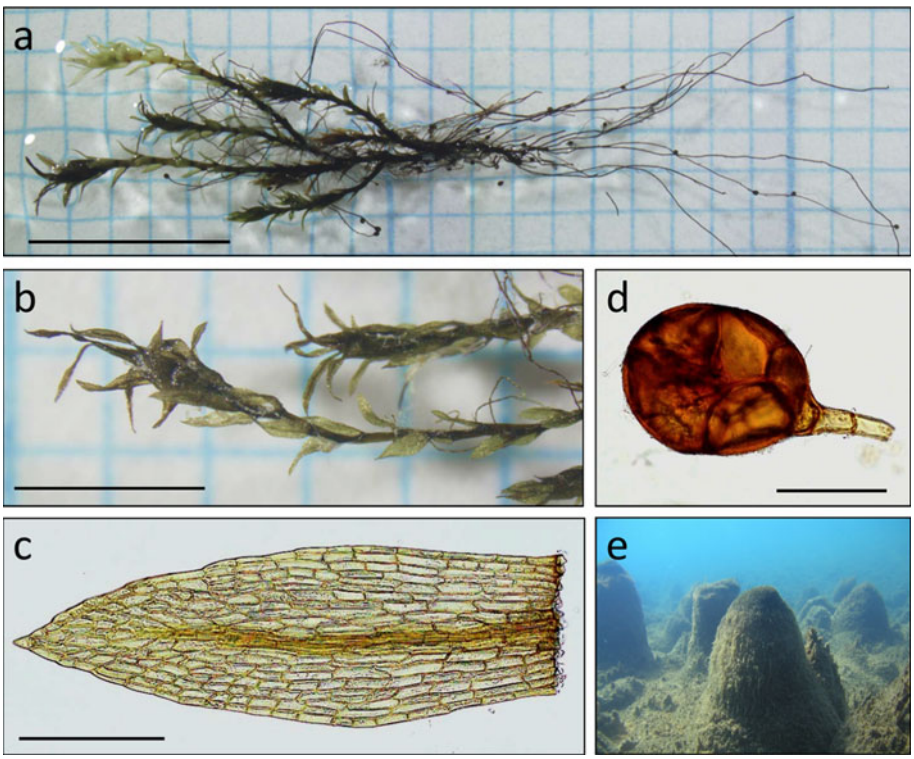


Fig. 2 Photographs of the aquatic moss and the moss pillars. (**a**) Habit, wet; (**b**) upper stem portion, dry; (**c**) stem leaf; (**d**) rhizoidal tuber; (**e**) moss pillars found in lake Hotoke-ike. *Scale bars* **a** 5.0 mm; **b** 2.0 mm; **c** 200 μm; **d** 100 μm

Table 1 Taxonomic history of the aquatic moss found in lakes of the Sôya Coast region

Reference	Taxonomic treatment of the aquatic moss (subject or content of the study)
Nakanishi (1977)	*Bryum* cf. *korotkevicziae* or *Bryum* cf. *korotkevicziae* var. *Hollerbachii* (the first report of the moss in lakes of the Sôya Coast region)
Ochi (1979)	*Bryum* sp. (a taxonomic review of the genus *Bryum* in Antarctica)
Imura and Kanda (1986)	*Bryum* sp. (the description of rhizoidal tubers of the moss)
Kanda and Iwatsuki (1989)	*Dicranella* sp. (a taxonomic study of two aquatic moss species in the Sôya Coast region)
Imura et al. (1992)	*Leptobryum pyriforme* (the description of cultured plants of the moss)
Imura et al. (1999)	*Leptobryum* sp. (the first report and description of moss pillars)
Arts (2001)	*Leptobryum wilsonii* (a taxonomic review of the genus *Leptobryum*)
Imura et al. (2003)	*Leptobryum* sp. (a distribution survey of aquatic mosses in the Sôya Coast region)
Ochyra et al. (2008)	*Pohlia wilsonii* (the latest comprehensive taxonomic monograph of Antarctic mosses)

maximum intraspecific variation currently known for this species. Sequence data for *Pohlia* species were obtained from DNA databases (DDBJ/EMBL/GenBank) as shown in Table 3.

DNA extraction, PCR, and DNA sequencing

Total DNA was extracted using a modified version of the standard CTAB method (Murray and Thompson 1980). Nucleotide sequences of two chloroplast DNA (cpDNA) regions—the ribosomal protein S4 gene (*rps*4) and the *trnL* (UAA) 5′ exon–*trnF* (GAA) exon region (*trn*L-F)—and the internal transcribed spacer region of nuclear ribosomal DNA (ITS: ITS1-5.8S rDNA-ITS2) were amplified for each sample by polymerase chain reaction (PCR). PCR was performed using 0.5 units of *Takara Ex Taq* (Takara Bio, Shiga, Japan) or 0.4 units of *Kod FX Neo* (Toyobo, Osaka, Japan) in 20-μl reaction volumes according to each manufacturer's instructions. Reaction conditions for PCRs using *Takara Ex Taq* consisted of 4 min of initial denaturation at 94 °C, followed by 30–35 cycles of denaturation (94 °C; 30 s), annealing (52 °C for cpDNA regions, 55 °C for ITS region; 30 s), and extension (72 °C; 60 s), ending with a final extension step (72 °C; 7 min). Samples for which PCR using *Takara Ex Taq* was unsuccessful were amplified using *Kod FX Neo*

Table 2 Specimens sequenced in this study, including location, voucher (herbarium), sample ID, and GenBank accession number for *rps*4, *trn*L-F, and ITS

Taxon	Location	Voucher specimen (Herbarium)	Sample ID	GenBank accession number		
				*rps*4	*trn*L-F	ITS
L. pyriforme	Belgium. Gent	20120531-0001 (NIPR)	BEL	AB795407	AB795617	AB795589
L. pyriforme	Canada. Nunavut	Allen 19666 (DUKE)	CAN	AB795408	AB795618	AB795590
L. pyriforme	China. Hebei	Sulayman 10354 (HIRO)	CHN	AB795409	AB795619	AB795591
L. pyriforme	Mexico. Concepción del Oro	Cardenas 1155 (DUKE)	MEX	AB795410	AB795620	AB795592
L. pyriforme	South Africa. Grahamstown	Vanderpoorten 214 (DUKE)	ZAF	AB795413	AB795623	AB795595
L. pyriforme	USA. Alaska Fairbanks	19910315-0117 (NIPR)	AK1	AB795404	AB795614	AB795586
L. pyriforme	USA. Alaska Shumagin islands	Schofield 106046 (DUKE)	AK2	AB795405	AB795615	AB79S587
L. pyriforme	USA. Alaska Chirikof island	Schofield 117938 (DUKE)	AK3	AB795406	AB795616	AB795588
L. pyriforme	USA. Missouri	Anderson 26021 (DUKE)	USA	AB795411	AB795621	AB795593
L. pyriforme	West Antarctica, Deception island	R.I.L. Smith 3644a (AAS)	WANT	AB795412	AB795622	AB795594
L. wilsonii	Bolivia. La Paz	Lewis 87-1222 d-6(DUKE)	BOL	AB795418	AB795628	AB79S600
L. wilsonii	Chile. Potosí	Moreno 12908 (DUKE)	CHL1	AB795419	AB795629	AB795601
L. wilsonii	Chile. Biobío Province	Goffinet 5573 (DUKE)	CHL2	AB795420	AB795630	AB795602
L. wilsonii	Chile. Biobío Province	Goffinet 5577 (DUKE)	CHL3	AB795421	AB795631	AB795603
Aquatic moss	Langhovde, Lake Akebi-lke	20120531-0038 (NIPR)	AMI	AB795417	AB795627	AB795599
Aquatic moss	Skallen, Lake Koke-Numa	20120531-0036 (NIPR)	AM2	AB795425	AB795635	AB795607
Aquatic moss	Skarvsnes, Lake A-7-lke	20120531-0019* (NIPR)	AM3	AB795414	AB795624	AB795596
Aquatic moss	Skarvsnes, Lake Ageha-lke	20120531-0014* (NIPR)	AM4	AB795415	AB795625	AB795597
Aquatic moss	Skarvsnes, Lake Ageha-lke	20120531-0020* (NIPR)	AM5	AB795416	AB795626	AB795598
Aquatic moss	Skarvsnes, Lake Hotoke-lke	No specimen registered*	AM6	AB795422	AB795632	AB795604
Aquatic moss	Skarvsnes, Lake Hotoke-lke	No specimen registered*	AM7	AB795423	AB795633	AB795605
Aquatic moss	Skarvsnes, Lake Jizo-lke	20120531-0009* (NIPR)	AM8	AB795424	AB795634	AB795606
Aquatic moss	Skarvsnes, Lake Kuwai-lke	20120531-0008* (NIPR)	AM9	AB795426	AB795636	AB795608
Aquatic moss	Skarvsnes, Lake Naga-lke	20120531-0048* (NIPR)	AM10	AB795427	AB795637	AB795609
Aquatic moss	Skarvsnes, Lake Namazu-lke	No specimen registered*	AM11	AB795428	AB795638	AB795610
Aquatic moss	Skarvsnes, Lake Nise-hyoutan-lke	20120531-0021* (NIPR)	AM12	AB795429	AB795639	AB795611
Aquatic moss	Skarvsnes, Lake Nise-hyoutan-lke	20120531-0040* (NIPR)	AM13	AB795430	AB795640	AB795612
Aquatic moss	Skarvsnes, Lake Shimo-tenpyo-lke	20120531-0047* (NIPR)	AM 14	AB795431	AB795641	AB795613

Voucher specimens marked with asterisks were used for culturing

under the following reaction conditions: initial denaturation at 94 °C (2 min), followed by 30–35 cycles of denaturation (98 °C; 10 s), annealing (58 °C; 30 s), and extension (68 °C; 60 s). Primers used for amplification of the two cpDNA regions were *rps*5 (Nadot et al. 1995) and trnas (Souza-Chies et al. 1997) for *rps*4, and trnC and trnF (Taberlet et al. 1991) for *trn*L-F. From the cultured material, the entire ITS region was amplified using external primers ITS1 and ITS4 (White et al. 1990). From the uncultured material, the ITS region was amplified in two parts (i.e., ITS1 and ITS2 regions) using external primers ITSBF (5′-CATTAAACCTTATCATTTAGAGG AAGGAG-3′) or ITS1 for the forward primer, and ITS4 for the reverse primer, in combination with internal primers ITSC bryo and ITSD bryo (Sabovljevic et al. 2005) (We developed ITSBF to avoid fungal contamination problems.). Primers used for DNA sequencing were identical to those used for PCR amplification. Amplified fragments were purified with ExoSAP-IT (GE Healthcare, Waukesha, WI, USA), and DNA sequencing in both directions was accomplished using a BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The resulting sequences for each DNA region were assembled and edited using DNA Baser version 3 (Heracle Software, Lilienthal, Germany).

Detailed information of samples sequenced in this study, including voucher, sample ID, and GenBank accession number, is listed in Table 2. These samples are represented as their sample IDs throughout this paper (e.g., 14 samples of the aquatic moss from lakes of the Sôya Coast region are represented as AM1–AM14).

Table 3 Voucher information for the 61 samples downloaded from DNA databases, listed as follows: genus, species, voucher specimen (herbarium), and GenBank accession numbers for *rps*4 and *trn*L-F

Genus	Species	Voucher specimen (Herbarium)	GenBank accession number	
			*rps*4	*trn*L-F
Acidodontium	*Acidodontium heteroneuron* (Spruce ex Mitt.) Broth.	Churchill 13550 (NY)	AF521673	AY150341
Amblyodon	*Amblyodon dealbatus* (Hedwig) Bruch & W.P. Schimp.	Schofield 89289 (DUKE)	AY499653	AY501425
Anacolia	*Anocolio menziesii* (Turner) Paris	Miller 8103 (MO)	AF491029	AF497135
Anomobryum	*Anomobryum julaceum* (Schrad. ex P. Gaertn., B. Mey. & Scherb.) Schimp.	Cox 112 (RNG)	AF023786	AF023739
Aplodon	*Aplodon wormskioldii* (Hornem.) R. Br.	Nimis (NY)	AY039047	AY039072
Aulacomnium	*Aulacomnium androgynum* (Hedw.) Schwagr.	J. R. Shevock 16833 (UC)	AY857766	AY857795
Bartramia	*Bartramia stricta* Brid.	Longton 4871 (RNG)	AF023799	AF023756
Brachymenium	*Brachymenium nepalense* Hook.	Long 23614(E)	AY078338	AY078311
Brochymitrion	*Brachymitrion jamesonii* Taylor	Litt 117 (NY)	AY499627	AY501399
Bryum	*Bryum argenteum* Hedw.	Hedderson 10385 (RNG)	AY078318	AY078291
Cyrtomnium	*Cyrtomnium hymenophyllum* (Bruch & Schimp.) Holmen	Hedderson 4779 (RNG)	AF023792	AF023764
Dicranum	*Dicranum scoparium* Hedw.	Rumsey 18/2/99 s.n. (Pers. Herb.)	AF234158	AF234159
Epipterygium	*Epipterygium tozeri* (Grev.) Lindb.	Cano, MUB 21892 (MUB)	JF277306	JF277340
Funaria	*Funaria hygrometrica* Hedw.	Price 2258 (G)	AJ845203	AJ847853
Haplodontium	*Haplodontium reticulatum* (Hook.) Broth.	Cox 1306/00 (DUKE)	AF521692	AY150360
Hedwigia	*Hedwigia ciliata* (Hedw.) P. Beauv.	Hedderson 11771 (RNG)	AJ251309	AF233587
Imbribryum	*Imbribryum alpinum* (Huds. ex With.) N. Pedersen	Hedderson 11428 (RNG)	AF023783	AF023738
Leiomitrium	*Leiomitrium plicatum* (P. Beauv.) Mitt.	Goffinet 823 (Pers. Herb.)	AY618359	AY636029
Leptobryum	*L. wilsonii* (Mitt.) Broth.	Gotfinet 5608 (DUKE)	AF306992	AY501424
Leptobryum	*L. wilsonii* (Mitt.) Broth.	Cano & Jimenez, MUB 28078 (MUB)	JF277301	JF277335
Leptostomum	*Leptostomum macrocarpum* (Hedw.) Bach. Pyl.	Fletcher s.n. (RNG)	AF023790	AF023744
Leptostomum	*Leptostomum inclinans* R. Br.	Streimann, 15467 (RNG)	AY078313	AY078287
Leucolepis	*Leucolepis acanthoneuro* (Schwägr.) Lindb.	R. R. Hales 4883 (UC)	AY857789	AY857821
Meesia	*Meesia muelleri* C. Müll. & Hampe	Streimann 53400 (H)	AY499648	AY501420
Meesia	*Meesia triquetra* (H. Richter) Ångström	Schofield 99251A (DUKE)	AF306995	AY501419
Meesia	*Meesia uliginosa* Hedw.	Schofield 93204 (DUKE)	AF306994	AY501418
Mielichhoferia	*Mielichhoferia bryoides* (Harv.) Wijk & Margad.	Hedderson 11713 (RNG)	AF023794	AF023765
Mielichhoferia	*Mielichhoferia elongata* (Hoppe & Hornsch.) Wijk & Margad.	Shaw sn (RNG)	AF023793	AF023766
Mnium	*Mnium hornum* Hedw.	Guerra et al. MUB 28763 (MUB)	JF277309	JF277343
Neomeesia	*Neomeesia paludella* (Besch.) Deguchi	Goffinet 5862 (DUKE)	AF306993	AY501421
Orthbodontium	*Orthodontium lineare* Schwägr.	Hedderson s.n. (RNG)	AF023800	AF023768
Orthbotrichbum	*Orthbotrichbum affine* Schrad. ex Brid.	Vitt Exs. 43 (DUKE)	AY618365	AY636021
Paludella	*Paludella squarrosa* (Hedw.) Brid.	Vitt 34205 (DUKE)	AF306996	AY501422
Pentastichella	*Pentastichella pentasticha* (Mont.) Müll. Hal. exTher.	Goffinet 5489 (DUKE)	AY618373	AY636009
Philonotis	*Philonotis fontana* (Hedw.) Brid.	Virtanen 2056 (H)	AF491031	AF497121
Phyllodrepanium	*Phyllodrepanium falcifolium* (Schwägr.) Crosby	Buck 32969 (NY)	AF143074	AF161167
Plagiobryum	*Plagiobryum uliqinosum* (Brid.) N. Pedersen	Hakeliers.n. (S)	AF521690	AY150358
Plagiomnium	*Plagiomnium affine* (Blandow ex Funck) T.J. Kop.	Cano, MUB 28651 (MUB)	JF277324	JF277358
Pohlia	*Pohlia andalusica* (Höhn.) Broth.	Guerra, MUB 21460 (MUB)	JF277302	JF277336
Pohlia	*Pohlia annotina* (Hedw.) Lindb.	Guerra, MUB 22534 (MUB)	JF277303	JF277337
Pohlia	*Pohlia camptotrachela* (Renauld & Cardot) Broth.	Guerra et al., MUB 23665 (MUB)	JF277305	JF277339
Pohlia	*Pohlia chilensis* (Mont.) A.J. Shaw	Cano, MUB 18036 (MUB)	JF277317	JF277351

Table 3 continued

Genus	Species	Voucher specimen (Herbarium)	GenBank accession number	
			*rps*4	*trn*L-F
Pohlia	*Pohlia cruda* (Hedw.) Lindb.	Gallego et al., MUB 17925 (MUB)	JF277325	JF2773S9
Pohlia	*Pohlia elongata* Hedw.	Guerra, MUB 27402 (MUB)	JF277314	JF277348
Pohlia	*Pohlia longicolla* (Hedw.) Lindb.	Brugués, MUB 22225 (MUB)	JF277310	JF277344
Pohlia	*Pohlia melanodon* (Brid.) A.J. Shaw	Guerra et al., MUB 28437 (MUB)	JF277308	JF277342
Pohlia	*Pohlia nutans* (Hedw.) Lindb.	Cano, MUB 27489 (MUB)	JF277318	JF277352
Pohlia	*Pohlia wahlenbergii* (F.Weber & D.Mohr) A.L. Andrews	Muñoz, MUB 15768 (MUB)	JF277307	JF277341
Ptychostomum	*Ptychostomum pallescens* (Schleich. ex Schwägr.) J.R. Spence	Hedderson 10487 (RNG)	AY078333	AY078306
Rhacocarpus	*Rhacocarpus purpurascens* (Brid.) Paris	D. H. Norris 77393 (UC)	AY857792	AY857823
Rhizogonium	*Rhizogonium novae-hollandiae* (Brid.) Brid.	Streimann 36688 (RNG)	AF023827	AF023752
Rhodobryum	*Rhodobryum spathulatum* (Hornsch.) Pócs	Redfearn 35546 (DUKE)	AF521695	AY150363
Rosulabryum	*Rosulabryum capillare* (Hedw.) J.R. Spence	Hedenäs B11066(S)	AF521682	AY150350
Schizymenium	*Schizymenium campylocarpum* (Arn. & Hook.) A.J. Shaw	Cano & Jimenez, MUB 28187 (MUB)	JF277313	JF277347
Splachnum	*Splachnum ampullaceum* Hedw.	Schofield 99074 (DUKE)	AY039044	AY039069
Tayloria	*Tayloria splachnoides* (Schleich. ex Schwägr.) Hook.	Hakelien 19.8.1992(H)	AY039062	AY039087
Tayloria	*Tayloria froelichiana* (Hedw.) Mitt., ex Broth.	Long 20882 (DUKE)	AY039059	AY039084
Tetraplodon	*Tetraplodon mnioides* (Sw. ex Hedw.) Bruch & Schimp.	Shaw 9082 (DUKE)	AY499644	AY501416
Timmia	*Timmia austriaca* Hedw.	Schofield 98363 (DUKE)	AF223035	AF229892
Trachycystis	*Trachycystis microphylla* (Dozy & Molk.) Lindb.	CB84(SHNU)	FJ572592	FJ572445
Voitia	*Voitia nivalis* Hornsch.	Long 26833 (DUKE)	AY039051	AY039076

Sequence alignment and phylogenetic analysis

A molecular phylogenetic analysis was performed based on cpDNA (*rps*4 and *trn*L-F) regions. In addition to the 28 samples sequenced in this study, sequence data for 61 samples were obtained from DNA databases (DDBJ/EMBL/GenBank) and incorporated into the analysis. These additional samples included two accessions of *L. wilsonii* (Goffinet 5608 and MUB 28078), 10 species of *Pohlia*, and 49 samples representing species from 43 other genera (Table 3).

Sequences from each region were pre-aligned using the MUSCLE algorithm (Edgar 2004) as implemented in MEGA 5.05 (Tamura et al. 2011), followed by manual refinement. Incomplete data at the beginning and end of sequences as well as sites characterized by ambiguous alignment, the presence of insertion/deletions (indels), and mixed bases were excluded from further analysis. Samples with completely identical sequences in the final aligned matrix were treated as a single operational taxonomic unit (OTU).

Phylogenetic relationships were assessed using maximum likelihood (ML), maximum parsimony (MP), and neighbor-joining (NJ) methods as implemented in MEGA 5.05. Selection of nucleotide substitution models for ML was also performed using MEGA 5.05. Based on the lowest Akaike information criterion (AIC) value, GTR+G+I was selected as the best-fit model for constructing the ML tree. The ML tree was inferred from a close-neighbor-interchange heuristic search with an automatically generated initial tree. The MP tree was obtained using a close-neighbor-interchange algorithm set at search level 3, in which the initial trees were obtained using 10 random addition replicates. For the NJ tree, the maximum composite likelihood method was used to calculate evolutionary distances. In each method, branch support was assessed using bootstrap (1,000 replicates). Because all three methods (ML, MP, and NJ) produced largely congruent tree topologies, only the ML tree is presented. Bootstrap support values (BS) greater than 50 both on the MP and NJ trees were overlaid to assess the robustness of each branch of the ML phylogram.

Sequence alignment and haplotype network analysis

The results of sequence alignment and phylogenetic analysis suggested that the aquatic moss belongs to the genus *Leptobryum* and is conspecific with *L. wilsonii* (cf. Fig. 3).

Some informative *trn*L-F sites were excluded during sequence alignment with phylogenetically distant species; however, phylogenetic resolution within *Leptobryum* was reduced. Consequently, only samples of *Leptobryum* species and the aquatic moss were used in subsequent analyses. Phylogenetic relationships and genetic variations among these samples were evaluated by constructing haplotype networks based on both cpDNA sequence data and a combined data set of sequences of cpDNA and the rapidly evolving nuclear ITS region. For the haplotype network based on cpDNA sequence data, we analyzed the 28 samples sequenced in this study (Table 2) and two samples of *L. wilsonii* from DNA databases (Goffinet 5608 and MUB 28078 in Table 3). For the haplotype network based on the cpDNA-ITS combined sequence data, only the 28 samples sequenced in this study were analyzed.

Alignment of each genomic region was carried out as for the phylogenetic analysis, except that indels were included. Haplotype network analyses were performed using statistical parsimony as implemented in TCS (Clement et al. 2000), with the parsimony connection limit set to 95 % and indels were treated as fifth character states. Genetic variation among samples, represented as a percentage, was calculated based on the number of variable sites and alignment length. The calculation was performed using a pairwise sequence-identity matrix program implemented in BioEdit (Hall 1999), followed by manual confirmation.

Results

Sequence alignment and phylogenetic analysis

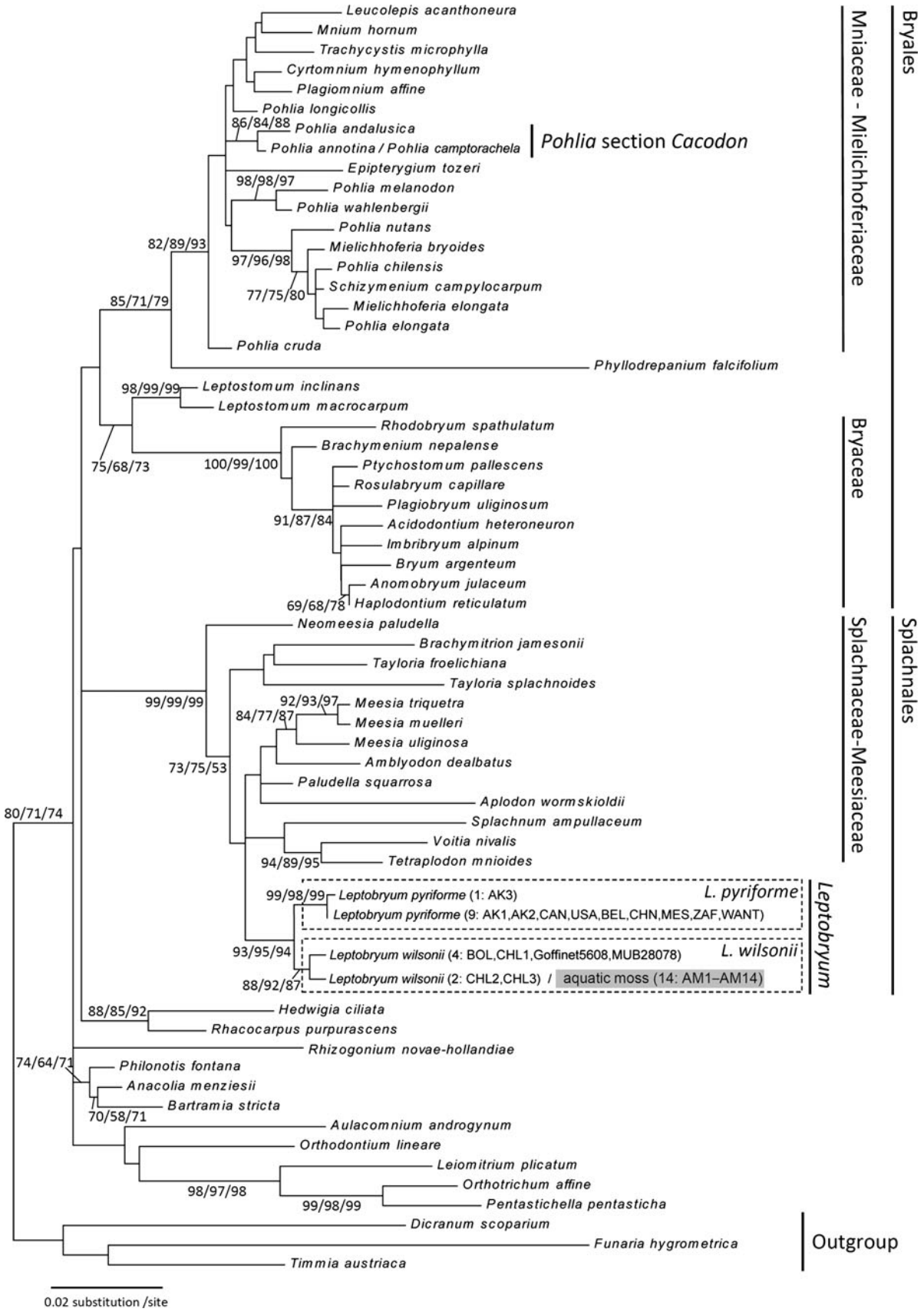
The final aligned cpDNA matrix for 86 samples contained 786 characters (518 *rps*4 and 268 *trn*L-F), of which 486 were constant, 143 were autapomorphic, and 157 were parsimony informative. Because two samples of *L. wilsonii* (CHL2 and CHL3) and 14 samples of the aquatic moss (AM1–AM14) had completely identical sequences, they were treated as one OTU. Of the remaining samples, four samples of *L. wilsonii* (BOL, CHL1, Goffinet 5608, and MUB 28078), nine samples of *L. pyriforme*, and two samples of *Pohlia* (*P. annotina*, and *P. camptotrachela*) were treated as each one OTU. Consequently, a total of 62 OTUs were used in the analysis. The ML tree (ln-likelihood = −5198.97) showing phylogenetic relationships among these samples is presented in Fig. 3.

In the ML tree, the OTU comprising two samples of *L. wilsonii* (CHL2 and CHL3) and 14 samples of the aquatic moss (AM1–AM14) is to be part of a well-supported clade (BS = 93 [ML]/95 [MP]/94 [NJ]) that also includes the remaining 14 *Leptobryum* samples (Fig. 3). This *Leptobryum* clade, which consists of two well-supported subclades corresponding to each species of the genus, *L. pyriforme* and *L. wilsonii*, is closely allied to samples of Meesiaceae and Splachnaceae, and together with them forms a well-supported larger clade (BS = 99/99/99) corresponding to Splachnales. On the other hand, 10 samples of *Pohlia* are included in a moderately supported clade (BS = 82/89/93) corresponding to the Mniaceae-Mielichhoferiaceae lineage reported by Guerra et al. (2011). Within this clade, samples of *Pohlia* are not monophyletic, although the three samples of *Pohlia* sect. *Cacodon* (*P. andalusica*, *P. annotina*, and *P. camptotrachela*) constitute a moderately supported clade (BS = 86/84/88).

Haplotype network analysis

The final matrix (including gaps) of cpDNA and ITS sequence data for the samples contained 1719 characters (570 *rps*4, 431 *trn*L-F, and 718 ITS). Statistical parsimony networks based on cpDNA and cpDNA-ITS combined sequence data are shown in Fig. 4. In the network based on cpDNA sequences (Fig. 4a), two samples of *L. wilsonii* (CHL2 and CHL3) and 14 samples of the aquatic moss (AM1–AM14) are assigned to one haplotype because they have completely identical sequences. Four samples of *L. wilsonii* (BOL, CHL1, Goffinet 5608, and MUB 28078) are assigned to two haplotypes, and 10 samples of *L. pyriforme* are to six haplotypes.

In the network based on cpDNA-ITS combined data (Fig. 4b), four unconnected subnetworks were obtained under the 95 % connection limit (= 18). Subnetworks-1 and subnetworks-2 contain the five haplotypes associated with samples of *L. wilsonii* and the aquatic moss; subnetworks-3 and subnetworks-4 comprise eight haplotypes representing *L. pyriforme*. Within subnetwork-1 (Fig. 4b), the three haplotypes are closely related, being separated from one another only by variation in the ITS region. Thirteen samples of the aquatic moss (AM2–AM14) are assigned to one of the haplotypes, which is separated from another haplotype, consisting of a single sample of the aquatic moss (AM1), by one indel. The other haplotype, consisting of two samples of *L. wilsonii* (CHL2 and CHL3), is separated from the two aquatic moss haplotypes (AM1 and AM2–AM14) by one base substitution and three or four indels. Subnetwork-2 contains two haplotypes, each corresponding to a single sample of *L. wilsonii* (BOL and CHL1), which are separated by five base substitutions and 12 indels. The most distant haplotypes of *L. wilsonii* and the aquatic moss, CHL2-CHL3 and BOL, vary by seven cpDNA sites (base substitutions) and 62 ITS sites (21 base substitutions and 41 indels), which correspond to *L. wilsonii* intraspecific variation of 4.1 % (69 variable sites out of 1681 total).Within *L. pyriforme*, haplotypes,


Leucolepis acanthoneura
Mnium hornum
Trachycystis microphylla
Cyrtomnium hymenophyllum
Plagiomnium affine
Pohlia longicollis
86/84/88
Pohlia andalusica
Pohlia annotina / Pohlia camptorachela
Pohlia section Cacodon
Epipterygium tozeri
98/98/97
Pohlia melanodon
Pohlia wahlenbergii
Pohlia nutans
82/89/93
Mielichhoferia bryoides
97/96/98
Pohlia chilensis
77/75/80
Schizymenium campylocarpum
85/71/79
Mielichhoferia elongata
Pohlia elongata
Pohlia cruda
Phyllodrepanium falcifolium
98/99/99
Leptostomum inclinans
Leptostomum macrocarpum
Rhodobryum spathulatum
Brachymenium nepalense
75/68/73
100/99/100
Ptychostomum pallescens
Rosulabryum capillare
Plagiobryum uliginosum
91/87/84
Acidodontium heteroneuron
Imbribryum alpinum
Bryum argenteum
Anomobryum julaceum
69/68/78
Haplodontium reticulatum
Neomeesia paludella
Brachymitrion jamesonii
Tayloria froelichiana
Tayloria splachnoides
99/99/99
92/93/97
Meesia triquetra
84/77/87
Meesia muelleri
Meesia uliginosa
73/75/53
Amblyodon dealbatus
Paludella squarrosa
Aplodon wormskioldii
80/71/74
Splachnum ampullaceum
Voitia nivalis
94/89/95
Tetraplodon mnioides
99/98/99
Leptobryum pyriforme (1: AK3)
L. pyriforme
Leptobryum pyriforme (9: AK1,AK2,CAN,USA,BEL,CHN,MES,ZAF,WANT)
93/95/94
Leptobryum wilsonii (4: BOL,CHL1,Goffinet5608,MUB28078)
L. wilsonii
88/92/87
Leptobryum wilsonii (2: CHL2,CHL3) / aquatic moss (14: AM1–AM14)
Leptobryum
88/85/92
Hedwigia ciliata
Rhacocarpus purpurascens
Rhizogonium novae-hollandiae
74/64/71
Philonotis fontana
Anacolia menziesii
Bartramia stricta
70/58/71
Aulacomnium androgynum
Orthodontium lineare
Leiomitrium plicatum
98/97/98
Orthotrichum affine
99/98/99
Pentastichella pentasticha
Dicranum scoparium
Funaria hygrometrica
Timmia austriaca
Outgroup
Mniaceae - Mielichhoferiaceae
Bryaceae
Splachnaceae-Meesiaceae
Splachnales
Bryales
0.02 substitution /site

◀ **Fig. 3** Maximum likelihood tree (ln-likelihood = −5198.97) based on cpDNA regions, with the phylogenetic position of the aquatic moss highlighted in gray. Number of samples and sample IDs are listed in *parentheses*. *Numbers* near branches are bootstrap support values above 50 for branches recovered using maximum likelihood, maximum parsimony, and neighbor-joining methods

consisting of one sample each, AK3 and CHN or USA, are the most distantly separated. They are separated by variations occurring in three cpDNA sites (base substitutions) and 56 ITS sites (19 base substitutions and 37 indels), corresponding to *L. pyriforme* intraspecific variation of 3.6 % (59 variable sites out of 1630 total). The closest haplotypes between *L. wilsonii* (including the aquatic moss) and *L. pyriforme*, AM1 and BEL-ZAF, or MEX-WANT are separated by changes in 13 cpDNA sites (12 base substitutions and one indel) and 119 ITS sites (29 base substitutions and 90 indels). This is equivalent to 7.8 % interspecific variation (132 variable sites out of 1688 total).

Discussion

In this study, we found that two samples of *L. wilsonii* from Chile (CHL2 and CHL3) and 14 samples of the aquatic moss from Antarctic lakes (AM1–AM14) had completely identical cpDNA sequences (Fig. 4a) and almost identical ITS sequences (Fig. 4b; subnetwork-1). The phylogenetic analysis accordingly placed the aquatic moss in the *L. wilsonii* subclade of the genus *Leptobryum* (Fig. 3). The aquatic moss is clearly conspecific with *L. wilsonii* as previously suggested by morphological examination (e.g., Arts 2001). In contrast, the new combination *P. wilsonii* proposed by Ochyra et al. (2008) is unacceptable because the molecular phylogenetic evidence indicates that *Pohlia* is distantly separated from *Leptobryum* at the order level (Fig. 3).

Members of the genus *Leptobryum* are defined by their shiny pear-shaped inflated capsules, long-pointed perichaetial leaves, and abundant characteristic rhizoidal tubers (e.g. Arts 2001; Brotherus 1924). In the taxonomic revision of Arts (2001), *L. pottiaceum* Dusén, *L. escomelii* Thér., *L. stellatum* (Herzog) Broth., and *P. integra* (Cardot) A.J. Shaw were reduced into synonymy under *L. wilsonii*; as a consequence, only two species—*L. pyriforme* and *L. wilsonii*—were recognized in the genus. Arts (2001) also recognized no intraspecific taxa in any of the two *Leptobryum* species. In the haplotype network analysis in our study, samples of *L. wilsonii* (including the aquatic moss) were separated into two subnetworks based on cpDNA-ITS combined sequences (Fig. 4b). These results suggest the possibility that each subnetwork may correspond to an independent taxon in the genus. We therefore attempted to evaluate the intraspecific variation in *L. wilsonii* by comparing it with intraspecific variation in *L. pyriforme* and interspecific variation between *L. pyriforme and L. wilsonii*. The *L. wilsonii* intraspecific variation, calculated as 4.1 %, was slightly higher than that observed within *L. pyriforme* (3.6 %) but lower than between the two species (7.8 %). It is thus inconclusive to recognize the two subnetworks of *L. wilsonii* as independent infrageneric taxa based on the current molecular data; however, the monospecificity of *L. pyriforme* also seems to be uncertain, as shown by the existence of unconnected networks in the species. To achieve a more precise taxonomic circumscription of *L. wilsonii* and also *L. pyriforme*, further molecular and morphological examinations with numerous samples, including type specimens of described taxa, may be needed.

Leptobryum pyriforme is widely known as a cosmopolitan weedy species and a pioneer of bare or disturbed land (e.g., Bradbury 2006). This species is even reported from maritime Antarctica: from Deception Island (63°00′ S, 60°40′ W) (specimen WANT in this study) (Lewis Smith 1984) and from Galindez Island (65°14′ S, 64°14′ W) (Ochyra and Tyshchenko 2006). In contrast, the distribution of *L. wilsonii* is more restricted. Outside Antarctica, occurrences of this species are concentrated in several South American countries (i.e., Argentina, Bolivia, Chile, Ecuador, Peru, and Uruguay), with only one known collection from Lesotho, Southern Africa (Arts 1995 as *Pohlia integra*), and Mexico, North America (Shaw 1982 as *P. integra*). In these regions, most specimens of *L. wilsonii* have been collected from moist and wet habitats as river-bank, ditch-side, seepage slope or wet rock in high elevation areas above 1,300 m up to 4,600 m (Arts 1995; Churchill et al. 2000; Arts 2001). Since this species seems to prefer wet condition but has never been collected from totally submerged environments, it is typically regarded as terrestrial moss species. In Antarctica, however, *L. wilsonii* has never been reported from terrestrial environments, having been found only in lakes of the Sôya Coast region and probably of the Schirmacher Oasis (70°45′ S, 11°38′ E). In the latter region, located over 1,000 km west of the Sôya Coast region, a scant collection of *Leptobryum* has been reported once from Lake Zub (Tewari and Pant 1996). Their voucher specimen was not available during our study, but judging from their short description and illustrations of its leaves and tubers, this moss appears to be conspecific with the aquatic moss from the Sôya Coast region, namely *L. wilsonii*. Populations of *L. wilsonii* in these Antarctic lakes are geographically separated, and their habitat type also varies from non-Antarctic populations.

In Antarctica, as well as *L. wilsonii*, occurrences of mosses in submerged environments have been well known. At present, totally 12 species, which correspond to ca. 11 % of Antarctic moss flora, are listed as aquatic mosses

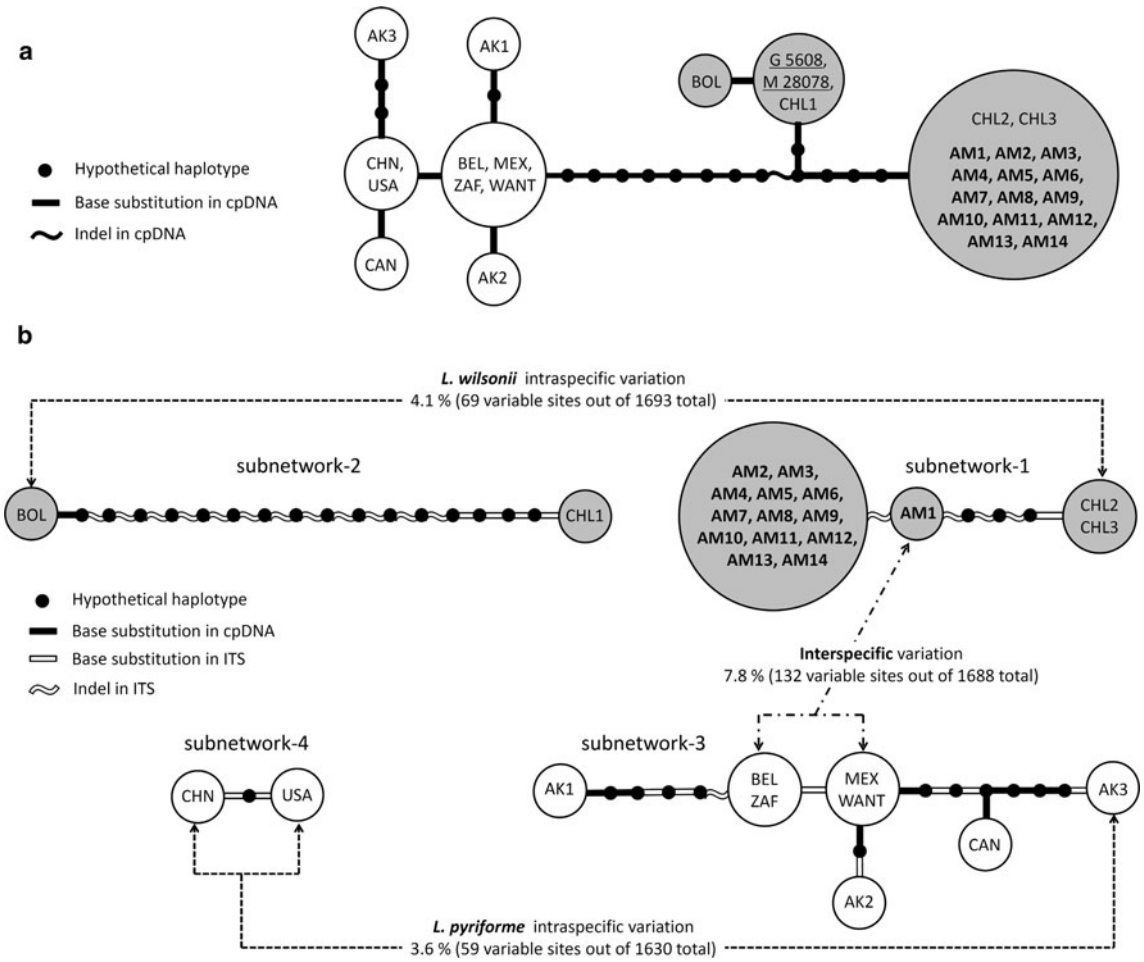


Fig. 4 Statistical parsimony networks for studied samples of two *Leptobryum* species and the aquatic moss. (**a**) The network for 30 samples based on cpDNA sequences. Samples from DNA databases are underlined and abbreviated; G 5608 corresponds to Goffinet 5608 and M 28078 to MUB 28078. (**b**) The network for 28 samples based on cpDNA-ITS combined sequences. *Circle* size is proportional to the number of samples assigned to an identical haplotype. Haplotypes with *gray* backgrounds consist of samples of *L. wilsonii* and the aquatic moss. Haplotypes with *white* backgrounds consist of samples of *L. pyriforme*. A *filled circle* represents a hypothetical haplotype. The *straight* and *wavy line* connecting each haplotype represents a single base substitution and a single indel between them. The *colors* of *straight* and *wavy lines* indicate whether the variations occurred in cpDNA regions (*black*) or the ITS region (*white*). Sample IDs of the aquatic moss are indicated in *bold* type. Bidirectional *arrows* with comments show the genetic variation between designated haplotypes

in Antarctica, although they also exist as terrestrial mosses elsewhere in the world (reviewed in Li et al. 2009). Nine of them have been observed both in Antarctic lakes and on their surrounding lands, and therefore, it seems to be reasonable to presume that these aquatic mosses have been derived from terrestrial populations of same species as suggested by Priddle (1979). The other three species [*Drepanocladus longifolius* (Mitt.) Paris, *Plagiothecium orthocarpum* Mitt. and *L. wilsonii*] have been known to be exclusively submerged in Antarctica. In comparison with the latter two species, populations of *D. longifolius* which have been found in some lakes in the northern Antarctic Peninsula region are geographically close to non-Antarctic (e.g., subantarctic and southern South American) populations (Ochyra et al. 2008; Li et al. 2009). In contrast, a population of *P. orthocarpum*, which has been collected once from Lake Glubokoye in the Schirmacher Oasis (Savich-Lyubitskaya and Smirnova 1964 as *P. simonovii* Sav. & Smirn.), is geographically distant to non-Antarctic (e.g., subantarctic South Georgia and Kerguelen islands) populations (Ochyra et al. 2008), as well as *L. wilsonii*. Thus, these two species could be regarded as highly isolated in Antarctic lakes, and therefore, the immigration processes of them are the topic of high interest.

Almost no genetic variation was observed between samples of *L. wilsonii* from Chile (CHL2 and CHL3) and those from Antarctic lakes (AM1–AM14) (Fig. 4b; subnetwork-1). In bryophyte taxa, such high sequence similarity among disjunct populations has been explained by either relictualism combined with slow evolutionary rates

(e.g., stenoevolution sensu Frey et al. 1999, 2010) or recent long-distance dispersal (e.g., Shaw et al. 2003; Vanderpoorten et al. 2008). In the case of this study, the latter theory is more applicable to the distribution of *L. wilsonii* in Antarctic lakes, as the former theory (i.e., the persistence of this species in Antarctica before the last glacial period) must be rejected based on several lines of evidence. First, extant lakes of the Sôya Coast region are believed to have been formed during the Holocene (Iwasa et al. 2000; Seto et al. 2002; Matsumoto et al. 2006, 2010). For example, the oldest record of lake sediment cores so far reported in this region is 7,030 ± 59 years before present (BP) in Lake Skallen-Oike, which was inferred by radiocarbon dating (Matsumoto et al. 2010). The aquatic moss communities in this region are also thought to have been established relatively recently. The radiocarbon age of the oldest layer containing moss debris has been confirmed by Seto et al. (2002) as 3,200 years BP in Lake Akebi-ike and by Matsumoto et al. (2006) as 1,110 years BP in Lake Namazu-ike. Second, *L. wilsonii* has never been reported from terrestrial habitats in Antarctica, only from lakes. If *L. wilsonii* had survived in some Antarctic refugia during the last glacial period prior to lake formation in the Sôya Coast region, it might be expected to persist in present terrestrial habitats rather than lakes. These considerations, taken together, suggest that the immigration of *L. wilsonii* into Antarctic lakes took place during the Holocene by means of long-distance dispersal from other continents, presumably South America where this species has its current maximum occurrence. Based on their very low rates of endemism, most of Antarctic moss species have typically been assumed to be post-glacial immigrants from other continents (e.g., Peat et al. 2007; Convey et al. 2008; Ochyra et al. 2008). The immigration process of *L. wilsonii* into Antarctic lakes suggested by the molecular and geohistorical evidence in this study is to be an example supporting this widespread assumption.

A remaining important subject is the process of range expansion, which shapes the present distribution of *L. wilsonii* in Antarctic lakes. This process, which presumably corresponds to the frequency of dispersal events into Antarctic lakes from other continents and dispersal events among Antarctic lakes, may be detectable by analysis of genetic diversity and population genetic structure of this species. In this study, samples from Antarctic lakes had almost identical sequences. This suggests that Antarctic populations of this species are genetically nearly homogeneous, but this is not conclusive because of the small sample size in this study (14 samples) and the presumed slow evolutionary rate of the Antarctic plants due to their asexual reproduction. Population genetic analysis based on hypervariable DNA markers (e.g., microsatellites) along with exhaustive sampling of Antarctic and additional non-Antarctic samples of *L. wilsonii* would be needed to clarify this question.

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