ORIGINAL RESEARCH PAPER

Multilocus DNA analysis supports *Didymodon gelidus* (Musci, Pottiaceae) as a distinct endemic of the austral polar region

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Abstract

The taxonomic position of the Antarctic subendemic species *Didymodon gelidus* Cardot is controversial, notably because of its notorious sterile condition. Considering the overall appearance and the reddish coloration of the plants, the leaf areolation, reaction of the leaf lamina with KOH, and the presence of multicellular axillary gemmae, this species was considered to be conspecific with the Holarctic *D. brachyphyllus* (Sull.) R. H. Zander. As a result, the latter was established as a bipolar species. Recent detailed morphological and anatomical studies have revealed a number of features which enable recognition of *D. gelidus* and *D. brachyphyllus*, including the shape of leaves, leaf apices and basal leaf cells, as well as costal anatomy. Here, within a larger-scale project focused on the evolution and biogeographical connections of Antarctic endemic mosses, we analyzed the genetic relationships of *D. gelidus* and *D. brachyphyllus* to confront the morphology-based conclusions. We selected five geographically distinct collections per species and applied a multilocus DNA analysis based on nuclear (ITS) and plastid (*atpIH*, *trnLF*, *trnG*, *rps4*) sequences to assess the genetic differentiation of these two taxa. We also placed their lineages in a wider phylogenetic context using an extended sampling of *Didymodon* taxa and select other representatives of Pottiaceae. Our results showed a clear genetic differentiation of the Southern Hemisphere (*D. gelidus*) and Northern Hemisphere (*D. brachyphyllus*) plants. Moreover, the phylogenetic analysis showed that *D. gelidus* formed a strongly supported clade on its own which was distantly related to *D. brachyphyllus*. Accordingly, the two taxa do not represent geographical vicariants. *Didymodon gelidus* must be considered a distinct endemic species of the austral polar region, having its optimal occurrence in the Antarctic and weakly penetrating northward to South Georgia and Îles Kerguelen in the Subantarctic. The relatively isolated phylogenetic position of *D. gelidus* likely suggests its old age.

Keywords

Antarctica; biodiversity; Bryophyta; *Didymodon gelidus*; Îles Kerguelen; molecular analyses; South Georgia; Subantarctica; taxonomy

Introduction

Mosses (Bryophyta) are land plants that exist in a wide variety of habitats, but they are often considered to be plants of harsh environments. They are especially well adapted to low temperatures and therefore they are predominant constituents of terrestrial vegetation in the polar regions as well as altimontane elevations in the alpine zone of the

mountains throughout the world. Like all land plants, mosses have life cycles with an alteration of generations but, in contrast to vascular plants, the haploid gametophytes are the dominant generation with prominent and, for the most part, long-lived plants. The diploid sporophytes are produced on the gametophytes and are nutritionally dependent on them. Sometimes they are produced only occasionally and in some species they are entirely unknown; this is especially true for the climatically most extreme habitats.

Lack of known sporophyte production has serious taxonomic implications because these structures possess some morphological and anatomical features which are often essential for establishing taxonomic affinities and relationships of the species in question. This refers especially to the sporangium (a terminal spore producing capsule), which, in the vast majority of moss species, opens by an apical operculum covering a capsule mouth which is most often surrounded by single of double rings of peristome teeth. They present great diversity in morphological structure and exhibit many conservative characters, but generally show consistency within particular groups of mosses [1–3]. Within large genera, some remarkable diversity of sporophytes is observed and hence a lack of fertile material makes imperfect any taxonomic analyses based exclusively on gametophyte characters, since moss gametophytes often exhibit a convergent similarity in distantly related groups. Currently, the widespread use and common application of molecular analyses has become a standard tool in taxonomic studies and they have opened up new possibilities for determining the relationships and affinities of problematic taxa. In the case of notoriously sterile species, this is practically the only chance for solving the problem of their taxonomic position [4–7].

The moss flora of Antarctica consists of 115 species and two varieties of mosses [8–14]. For almost half, some 55 species and two varieties, sporophytes are unknown in the Antarctic, and in five species they have never been detected. These are Antarctic endemics including *Andreaea depressinervis* Cardot, *Coscinodon lawianus* (J. H. Willis) Ochyra, *Syntrichia sarconeurum* Ochyra & R. H. Zander, *Didymodon gelidus* Cardot, and *Bryoerythrophyllum antarcticum* (L. I. Savicz & Smirnova) P. Sollman [8,14]. The first four of these are taxonomically isolated species of unclear affinities and some attempts to establish their relationships on the basis of an assessment of gametophyte characters have given contradictory results depending on the characters which have been stressed in such analyses. The best and most illustrative example of this is the story of *Didymodon gelidus*.

Taxonomic history of *Didymodon gelidus*

Didymodon gelidus (Fig. 1, Fig. 2) was collected for the first time on January 6, 1843 by J. D. Hooker in West Antarctica, on the small Cockburn Island on the northeast coast of the Antarctic Peninsula, during the British Antarctic voyage of 1839–1843 under the command of Captain James Clark Ross. This specimen, now preserved in the Hooker herbarium in BM, was determined as *Tortula gracilis* Hook. & Grev. and under this name it was published and illustrated by Wilson and Hooker [15] in *Flora antarctica*. *Tortula gracilis* has been considered identical to *Didymodon acutus* (Brid.) K. Saito [16] or *Didymodon rigidulus* Hedw. var. *gracilis* (Hook. & Grev.) R. H. Zander [17], but Ochyra et al. [18] demonstrated that the plants from Cockburn Island correctly belong to *D. gelidus*.

This species was collected again in 1902 in southern Victoria Land in East Antarctica by the British South Polar Expedition of 1901–1904 from the ship Discovery under the command of Captain Robert F. Scott. In the report from this expedition [19], it was described as a new species, *Didymodon gelidus* Cardot. The species was subsequently recorded from the volcanic Deception Island in the South Shetland Islands, West Antarctica [20] and on Edward VII Land, East Antarctica, by the U. S. Antarctic Expedition of 1933–1935. The latter collection was described by Bartram [21] as a new taxon, *Barbula byrdii* E. B. Bartram. The species was subsequently rediscovered in 1980 on Deception Island and the specimens were distributed in *Bryophyta Antarctica Exsiccata* as No. 31 and 56 [19,22]. Finally, *D. gelidus* was found on King George Island, the largest island of the South Shetland Islands archipelago, and described and illustrated in the moss flora of this island [23], and Seppelt and Green [24] described and illustrated this species in the moss flora of southern Victoria Land.

Fig. 1 *Didymodon gelidus* in a locality in the maritime Antarctic (King George Island, South Shetland Islands). (**A**) individual tuft habit, (**B**) tuft detail, and (**C**) population in its habitat: crevices of moist, overhanging rocks. Photo: M. Ronikier.
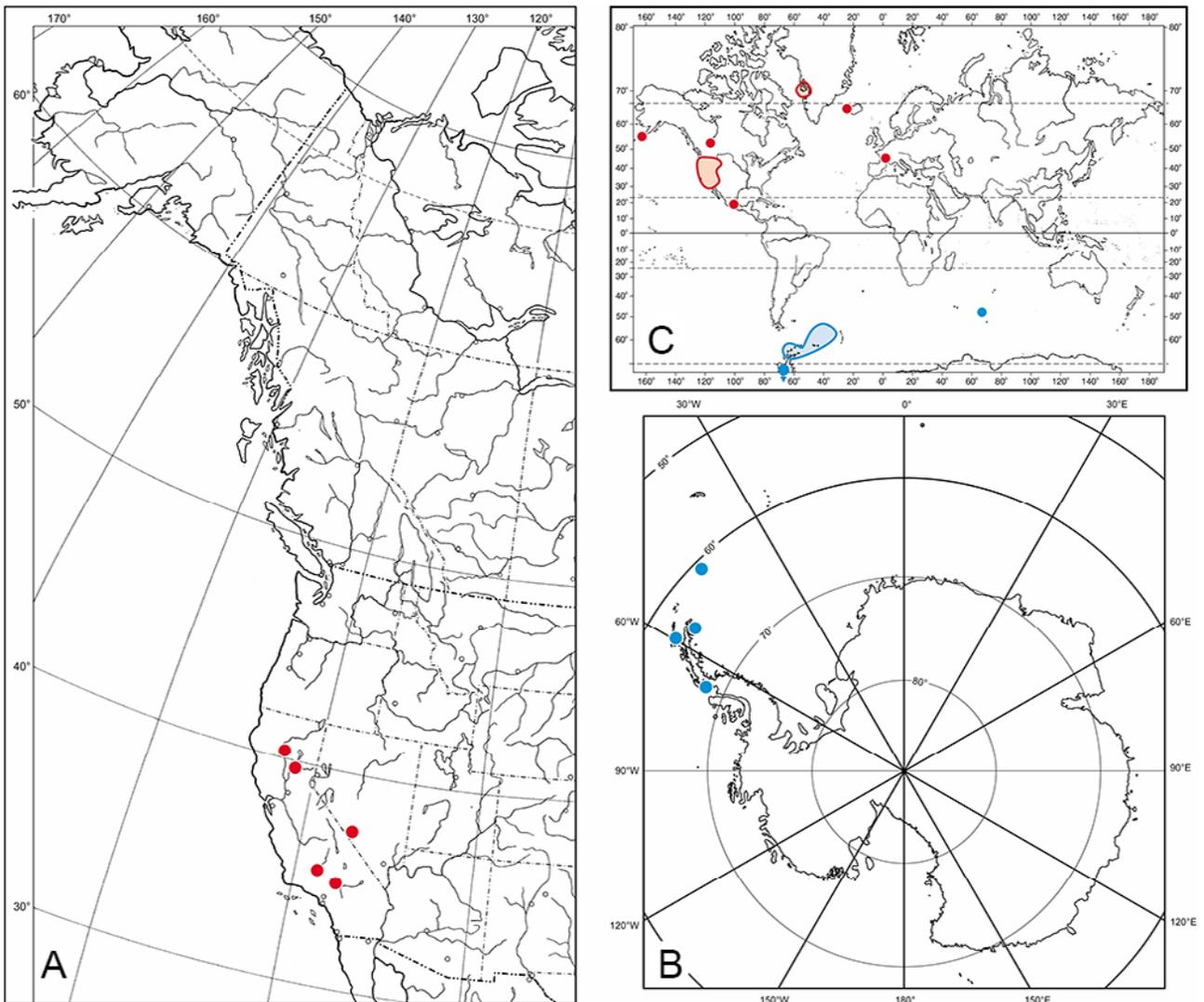

Fig. 2 Geographical distribution of the studied specimens of *Didymodon brachyphyllus* (**A**) and *D. gelidus* (**B**) and general distribution of these taxa (**C**) with *D. brachyphyllus* in red and *D. gelidus* in blue (occurrence on Alexander Island beyond the map is indicated by the arrow; localities in Victoria Land and Marie Byrd Land are not shown).

The barbuloid mosses collected in the Antarctic have not been taxonomically assessed for over 8 decades and they have been treated as Antarctic endemic species [23–25]. Zander [26] was the first to transfer *Barbula byrdii* to *Bryoerythrophyllum* P. C. Chen as *B. byrdii* (E. B. Bartram) R. H. Zander but without documenting arguments in support of this taxonomic conclusion. A detailed taxonomic evaluation of both *Barbula byrdii* and *Didymodon gelidus* was provided nearly a decade later [27,28]. These studies showed that the two Antarctic species are indistinguishable and at the same time their conspecificity with the Holarctic *Didymodon brachyphyllus* (Sull.) R. H. Zander, a species known mainly from western North America from Alaska to Mexico and West Greenland, was proposed. These two species, *D. gelidus* and *D. brachyphyllus*, are separated by the dramatic bipolar disjunction but share overall similarity in appearance of the plants, including their reddish coloration, shape and papillosity of distal and median laminal cells, the reaction of the leaf lamina with KOH, and the presence of multicellular axillary gemmae.

Finally, on the basis of recent detailed anatomical and morphological studies, Jiménez and Ochyra [29] presented a completely opposite taxonomic concept of these species of *Didymodon* Hedw. and supported *D. gelidus* and *D. brachyphyllus* as distinct species. The latter species has ovate to ovate-triangular leaves in contrast to ovate- to oblong-lanceolate leaves in *D. gelidus*. The leaf apices are shortly apiculate by one or more conical cells in *D. brachyphyllus*, whereas the leaf apices in *D. gelidus* are obtuse or widely acute and usually cucullate. The basal laminal cells are quadrate, oblate, or shortly rectangular with distinctly thickened transverse walls, especially towards the margins, whilst the basal cells in *D. gelidus* are shortly rectangular to rectangular, lax, and evenly thin-walled. Finally, the costa is elliptical in transverse section in *D. brachyphyllus*, without an adaxial coastal pad of cells, with a single row of guide cells and a small adaxial stereid band. In contrast, the costa in *D. gelidus* is terete in transverse section, with two–three rows of enlarged guide cells, without an adaxial stereid band, and with an adaxial costal pad of papillose cells.

All the aforementioned characters clearly separate the northern plants known as *Didymodon brachyphyllus* and the southern plants designated as *D. gelidus*. Yet, to be entirely sure whether these morphological and anatomical differences are sufficient to discriminate the northern and southern plants, we decided to check whether these differences extend also to the genetic level. We sequenced several DNA regions in selected samples of the two species to assess the degree of differentiation. We also placed the lineages of our focus taxa in a wider phylogenetic context based on available data to verify whether they represent a pair of geographically vicariant sister taxa or their relationships are more distant than expected based on gametophyte morphology analysis.

Material and methods

Plant material

For the sequencing analysis of *Didymodon gelidus* and *D. brachyphyllus*, we selected five collections of each taxon derived from geographically distinct localities in the main parts of the species' ranges (Tab. 1, Fig. 2). For *Didymodon gelidus*, we investigated accessions from the following regions: the South Orkney Islands (Signy Island) and the South Shetland Islands (Deception Island), the west coast of the Antarctic Peninsula (Fallières Coast, Léonie Island) in the maritime Antarctic, and the northeast coast of the Antarctic Peninsula (Vega Island in the James Ross Island group). For *D. brachyphyllus*, we studied several samples from California (Butte, Riverside, and Kern counties) and Nevada (Nye County) in the western part of North America, the main part of its distribution area.

All specimens used in the study were thoroughly verified based on their anatomical and morphological features using light microscopy. The analysis of ITS ribotypes and plastid haplotypes was specifically designed to test the taxonomic concept about the level of genetic divergence of the two allopatric species proposed by Jiménez and Ochyra [29]. Additionally, for three DNA regions (see below) we extended the taxon sampling for a phylogenetic overview of relationships of our target clades by including further

Tab. 1 Taxa and samples included in the analysis of *Didymodon gelidus* and *D. brachyphyllus* genetic differentiation (accessions analyzed in this study) with the voucher's reference and GenBank accession number for each particular molecular region, as well as the geographic origin of the specimens.

Voucher herbarium reference	GenBank accession No.					Geographical origin
	ITS	*atpHI*	*trnLF*	*trnG*	*rps4*	
Didymodon brachyphyllus						
J. R. Shevock 27819		MK307917	MK307947	MK307925	MK307938	USA, California, Butte County
J. R. Shevock 24064	MK307933	MK307919	MK307949	MK307927	MK307940	USA, California, Riverside County
J. R. Shevock 24003		MK307920	MK307950		MK307941	USA, California, Kern County
M. Lüth 8355		MK307921	MK307951			USA, California, Tehama County
J. R. Shevock 23706	MK307932	MK307918	MK307948	MK307926	MK307939	USA, Nevada, Nye County
Didymodon gelidus						
R. I. L. Smith 08062B	MK307937		MK307955	MK307930	MK307945	South Orkney Islands, Signy Island
R. I. L. Smith 11919A	MK307934	MK307922	MK307952	MK307928	MK307942	South Shetland Islands, Deception Island
A. Morton (No. Smith 05820B)				MK307931	MK307946	South Shetland Islands, Deception Island
R. I. L. Smith 08940	MK307935	MK307923	MK307953	MK307929	MK307943	Western side of the Antarctic Peninsula, Fallieres Coast, Leonie Island
R. I. L. Smith 07842	MK307936	MK307924	MK307954		MK307944	Eastern side of the Antarctic Peninsula, Vega Island

sequences of selected *Didymodon* species and several closely related representatives of the Pottiaceae family based on GenBank resources (see supplementary material, Tab. S1, for details). The taxon sampling was dependent on available sequences and although not entirely the same across the DNA regions studied, it provided a sufficient background for analyses.

DNA isolation, amplification, and sequencing

DNA isolation, amplification (PCR), and sequencing followed protocols as described by Saługa et al. [30] using primers also cited in that paper. The following genomic regions were sequenced in *Didymodon gelidus* and *D. brachyphyllus* for the present study: nuclear ribosomal internal transcribed spacer region (ITS) and, from the plastid genome, *atpI–atpH* intergenic spacer (*atpIH*), *trnL*(UAA)5′exon–*trnF*(GAA) region (*trnLF*), tRNA^Gly (UCC) gene intron (*trnG*), and ribosomal small protein 4 (*rps4*).

Sequence alignments and data analysis

Our original DNA sequences of each region were edited and aligned using Geneious v10.1.3 (Biomatters, USA) using Geneious alignment with default settings. In the case of plastid DNA alignments for haplotype network analysis, the four regions were concatenated and analyzed together assuming linked inheritance.

Alignments with external sequence data were assembled for the nuclear ITS region and combined *trnLF*/*trnG* plastid regions, for which relatively comprehensive data for *Didymodon* spp. and related representatives of Pottiaceae were available in GenBank resources (https://www.ncbi.nlm.nih.gov/genbank/). These extended cpDNA alignments were prepared in accordance with the above, while the extended ITS alignment was

aligned using Muscle option. All gaps were excluded from the ITS sequences before phylogenetic inference. As outgroups, we used *Trichostomum crispulum* Bruch (accession number: KT380402) and *T. brachydontium* Bruch (accession number: KT380387) for ITS, and *T. brachydontium* (accession numbers: KT380308, KT380234) for the plastid data. All alignments were trimmed with BioEdit v7.2.5 [31] and subjected for further analyses as described below. The original sequences from this study are available in the GenBank database (see Tab. 1 for accession numbers).

Diversity of haplotypes was studied in *Didymodon gelidus* and *D. brachyphyllus* using sequences generated in this study. The program Popart v4.8.4 (http://popart.otago.ac.nz) was used to calculate the number and network relationships of haplotypes (i.e., unique sequences) in both ITS and combined plastid data set using the statistical parsimony-based TCS method with default settings described by Templeton et al. [32]. Taking into account that missing data may have a profound effect on species network reconstruction, we excluded from the analysis specimens with the most incomplete data (sequences of several regions lacking): one *Didymodon gelidus* accession from the Deception Island [South Shetland Islands (A. Morton, No. Lewis Smith 05820B)] and one accession of *D. brachyphyllus* from the USA [California, Tehama County (M. Lüth 8355)].

Phylogenetic placement of *Didymodon gelidus* and *D. brachyphyllus* clades was analyzed based on extended ITS, and concatenated plastid *trnLF* and *trnG* data sets using two methods: maximum likelihood (ML) and Bayesian inference (BI). Maximum likelihood trees were prepared with RAxML v8.2.11 implemented in the Geneious, from 1,000 replicates, using rapid bootstrapping option, and GTR GAMMA model of evolution, and default settings. Posterior probabilities values were calculated with MrBayes. Prior to Bayesian inference, jModelTest was used to select the best evolutionary model, GTR+I+G model for ITS, HKY+I+G model for *trnLF*, and TIM1+G model for *trnG*. For each individual DNA region, we applied the following settings: MCMC chain length was set to 1.0×10^7 and stored on tree every 1,000 generations. Twenty-five percent of the trees were discarded as burn-in, and a 50% majority-rule consensus tree was constructed. Convergence and effective sample size was monitored with Tracer. All trees generated from these analyses were imported and visualized into FigTree. We assigned examined specimens to a clade if they formed a distinct group with >80% support values in the maximum likelihood (bootstrap support) and Bayesian analysis (posterior probabilities).

Results

Divergence of haplotypes between *D. gelidus* and *D. brachyphyllus*

Thirty-nine nucleotide sequences of one nuclear and four plastid regions were generated in this study for *Didymodon gelidus* and *D. brachyphyllus* (Tab. 1). The alignments for the respective regions had the following length: 781 bp – ITS, 340 bp – *atpIH*, 337 bp – *trnLF*, 561 bp – *trnG*, and 687 bp – *rps4*.

Within the *Didymodon gelidus* and *D. brachyphyllus* sequences, the total nucleotide diversity was π = 0.030 and 0.015 for the ITS and plastid data, respectively. North American specimens (morphologically assigned to *D. brachyphyllus*) were separated from the Antarctic plants (morphologically assigned to *D. gelidus*) by 15 segregating sites in ITS and 18 sites in the plastid regions. Accordingly, haplotypes pertaining to the two allopatric groups were highly isolated in the networks (Fig. 3). No intraspecific variation was detected in the Antarctic plants, which were all characterized by a single haplotype. Intraspecific variation was detected in the North American samples, where two relatively distinct haplotypes were detected in the ITS (2r and 3r; Fig. 2) data and three closely related haplotypes were found in the cpDNA data (2cp–4cp).

Phylogenetic analysis

Trees generated using ML and BI gave generally similar topologies. In both nuclear and plastid phylogenetic trees, *D. gelidus* and *D. brachyphyllus* formed separate and
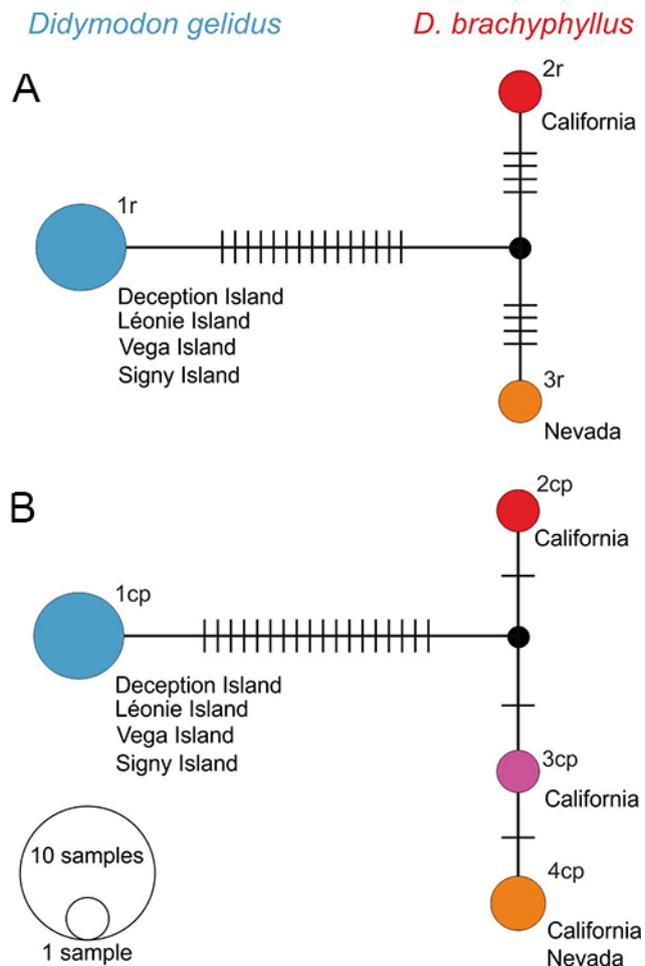
Didymodon gelidus   *D. brachyphyllus*

Fig. 3 Statistical parsimony haplotype networks showing structuring of (**A**) nuclear ITS (ribotypes 1r–3r) and (**B**) concatenated plastid DNA (haplotypes 1cp–4cp) sequences in *Didymodon gelidus* and *D. brachyphyllus* samples. The circle size reflects the haplotype frequency; bars and black dot indicate haplotypes not detected in the data set.

phylogenetically distant clades (Fig. 4). In all cases, the austral polar *D. gelidus* formed a fully supported and isolated clade on its own. The phylogenetic situation of *D. brachyphyllus* was less straightforward because it formed a clade closely related to *D. vinealis* and *D. nicholsonii*, among others, but these connections received a lower support and differed to some extent among data sets as regards the within-clade topology.

Discussion

Molecular data together with morphological assessment often provide an optimal background for conclusive evidence for the taxonomic delimitation (e.g., [30,33, 34]). Our multilocus genetic analysis based on both nuclear and plastid loci revealed a strong divergence between *Didymodon gelidus* and *D. brachyphyllus*, two species long considered conspecific and representing a widely distributed bipolar taxon (Fig. 2). No genetic data have been available for *Didymodon gelidus* thus far and our data present for the first time the approximate location of this austral taxon in the phylogeny of the genus. *Didymodon brachyphyllus* was represented in published phylogenies by a single accession only [35] [as *D. lamyanus* (Schimp.) Thér., later synonymized with *D. brachyphyllus* [16]], which was placed within a clade comprising the species circumscribed in sect. *Vineales* (Steere) R. H. Zander, in agreement with our present results. The available taxon sampling for the ITS and cpDNA data sets in our study differs (ITS data being available for a higher number of taxa), which makes problematic a comparative analysis of specific relationships of *D. gelidus*. Broadly, in both data sets it appears related (albeit not closely) to *D. rigidulus* Hedw., while in the ITS phylogeny, it is a sister clade to a group with weakly supported internal relationships, including *D. acutus*, *D. glaucus* Ryan, *D. cordatus* Jur.,

and *D. icmadophilus* (Müll. Hal.) K. Saito. This clade was revealed very distant from the clade representing species of "sect. *Vineales*" (containing *D. brachyphyllus* – as *D. lamyanus*) also in the phylogeny of *Didymodon* published by Werner et al. [35], in accordance with our results.

The clear molecular delimitation and divergent position of *Didymodon gelidus* corroborates the recent fine-scale analysis of morphological and anatomical features which displayed several features differentiating the Southern and Northern Hemisphere populations [29]. Moreover, they are not geographically vicariant sister species but are distant in the phylogeny of the genus *Didymodon*. This conclusion confirms the difficulties inherent for the interpretation of taxonomic relationships of taxa known exclusively in the sterile condition and importance of genetic data support for appropriate species delimitation and circumscription of their relationships.

The unequivocal reinstatement of *Didymodon gelidus* as a species in its own right has interesting and far-reaching implications. Based on our phylogenetic data together with demonstration of clear morphological features [29], the species is firmly established as an Antarctic subendemic having the main center of its occurrence in the continent which is the core of the ancient Gondwana. Also, its isolated occurrence on South Georgia and in Îles Kerguelen supports the hypothesis that the species evolved in Gondwana because these two subantarctic islands are also continental remnants of this supercontinent. Although our data were not time-calibrated and this aspect cannot be directly discussed, it might be hypothesized that the species could have even evolved
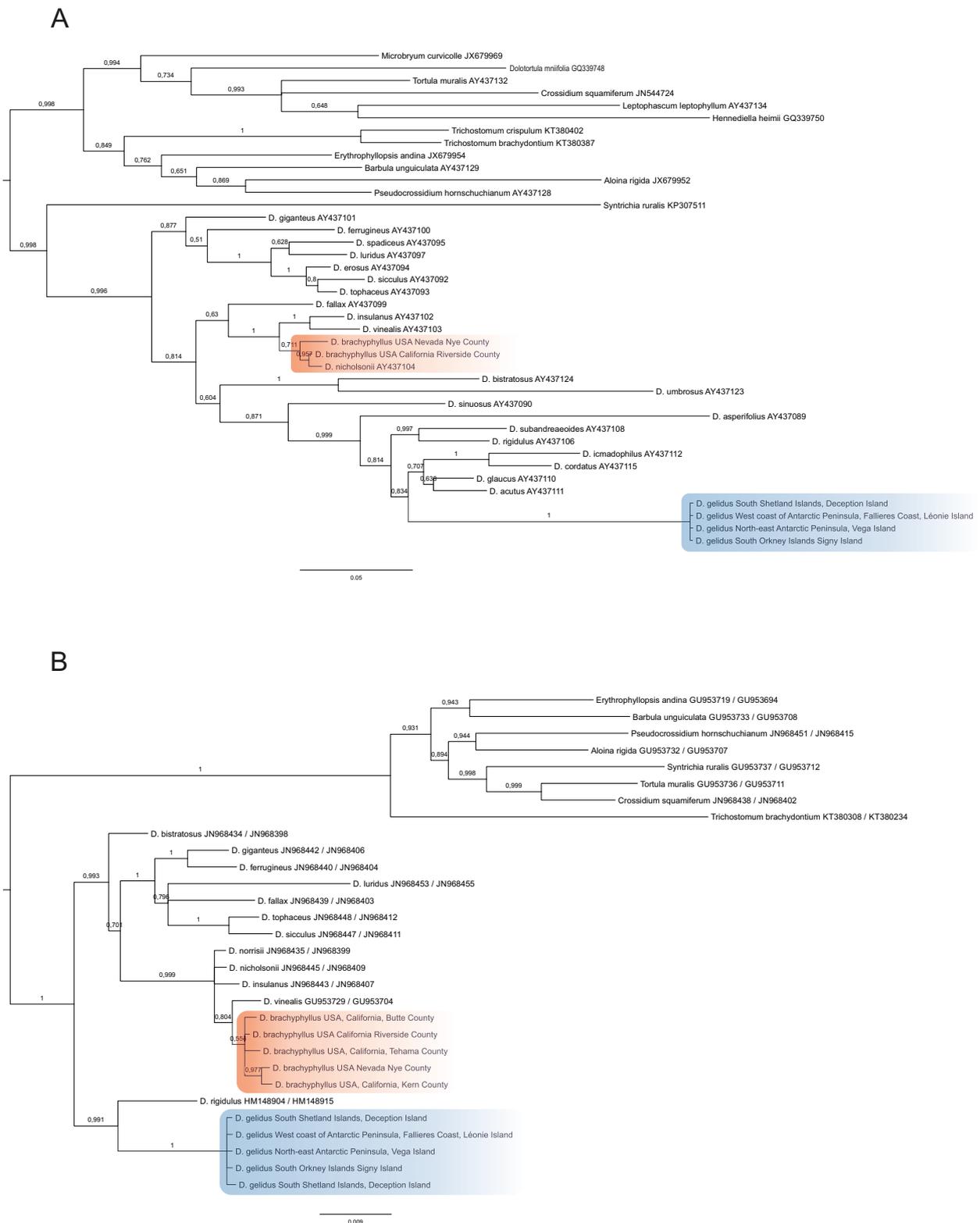
Fig. 4 Bayesian inference (BI) trees displaying the phylogenetic position of *Didymodon gelidus* and *D. brachyphyllus* within the genus *Didymodon*, based on (**A**) nuclear ribosomal internal transcribed spacer (ITS1-5.8S-ITS2) and (**B**) concatenated noncoding cpDNA markers (*trnLF* and *trnG*). Branch support values given are Bayesian posterior probabilities. Clades of *D. gelidus* and *D. brachyphyllus* are marked in blue and red, respectively. Background taxa are provided with respective GenBank accession numbers. Maximum likelihood trees had a similar topology and support of branches (see Fig. S1).

early in Gondwana, from where it reached Îles Kerguelen and South Georgia. The early divergence would be supported by the taxonomical isolation of *D. gelidus*.

The studied Antarctic populations of *Didymodon gelidus* were genetically coherent and displayed no intraspecific diversity, although inference of their phylogeography will require a more complete sampling (in particular including Îles Kerguelen and South Georgian material), which was beyond the scope of this study. The future range-wide phylogeographic survey and phylogenetic analyses including several austral species, especially *D. cardotii* (Dusén) R. H. Zander, *Didymodon fuscus* (Müll. Hal.) J. A. Jiménez & M. J. Cano, and *D. torquatus* (Taylor) Catches. should throw more light on the origin of *D. gelidus* and relationships with austral species of this genus and other pottialean mosses. In any case, this species, along with *Syntrichia sarconeurum* (Saługa M., Ochyra R., Ronikier M., unpublished data), provide a strong argument that some Antarctic species are actually palaeoendemics which could perhaps have survived in the Antarctic from the period preceding the break-up of Gondwana.

Acknowledgments

Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3609/0:

Tab. S1   List of additional DNA sequences retrieved from GenBank resources and used for the phylogenetic analysis of *Didymodon gelidus* and *D. brachyphyllus*.

Fig. S1   Maximum likelihood (ML) trees displaying the phylogenetic position of *Didymodon gelidus* and *D. brachyphyllus* within the genus *Didymodon*.

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