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Unnoticed diversity in the *Riccia glauca-bifurca* group (Ricciaceae, Marchantiales): morphological differentiation and phylogeny of *R. gothica* and *R. pusilla* in Europe

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ABSTRACT

The genus *Riccia* is the most species-rich genus among complex thalloid liverworts in Europe. High intraspecific variability in presumed diagnostic thallus characters leads to problems in species delimitation. We started a morphological and phylogenetic study of 105 specimens assigned to *R. bifurca*, *R. glauca*, *R. gothica*, and an unknown entity morphologically related to these species. Selected characters were statistically analyzed and compared to molecular results obtained by sequencing three nuclear marker regions: the commonly used ITS2 and two single-copy markers, RPB1 and an anonymous locus, both specifically targeted for the first time in bryophytes. These data indicate, that *R. bifurca*, *R. glauca*, and *R. pusilla* (the unknown entity) display distinct lineages reflected in spore morphology. *Riccia gothica* forms a subclade within *R. pusilla*, thus we interpret the taxon as a subspecies of *R. pusilla*. Lectotypification of *R. pusilla* and *R. gothica* is carried out. Both subspecies are more widespread in Central Europe than previously assumed, but precise distribution is still unknown. We show that only spore characteristics are useful to distinguish the four taxa, which we present in detail. Similarity in spore morphology does not reflect phylogenetic relationships. Overinterpretation of thallus characteristics could lead to failure in determination of *Riccia*-species.

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Introduction

The taxonomy and phylogenetic relations in many bryophyte groups are still insufficiently resolved and obscured by phenotypic plasticity, convergent evolution and relatively few characters suitable for morphological discrimination at low taxonomical ranks (e.g. Yu et al. 2013; Kiebacher and Urmi 2023). Within the highly diverse genus *Riccia* L. (Marchantiales), many taxonomical problems exist, among others caused by a high variability in thallus characteristics within species (Müller 1916; Jovet-Ast 1986; Schuster 1992; Hugonnot and Chavoutier 2021). More stable seem to be the characteristics of the polar spores, which show a complex ornamentation differing between the proximal (inner part of tetrades) and the distal side (external part; Perold 1989; Damsholt and Hallingbäck 1986b; Jovet-Ast 1986; Pörtl et al. 2024). *Riccia* is globally the most species-rich genus within the thalloid liverworts and grows in various habitats (Perold 1989; Paton 1999; Singh 2014; Cargill et al. 2016). Moreover, the genus is ecologically important, for example, in terrestrial habitats *Riccia* species

function as pioneers and are involved in the formation of soil crusts (Volk 1984).

In our long-term project (Pörtl et al. 2020), we aim to solve taxonomic and phylogenetic problems in the genus for Europe. Until 2024, we have examined about 600 living collections of more or less all European *Riccia* species. Additionally, we studied herbaria specimens from various European collections. The most widespread *Riccia* species in Europe, especially in arable fields, include *R. glauca* L. and *R. bifurca* Hoffm. (Hodgettes and Lockhart 2020). Our findings after six years of research have shown us that based on thallus characteristics, those two species are in many cases not separable, but within spore characteristics they show clear and stable differences. In our study about the variability of spore characters (Pörtl et al. 2024), we found out, that characters with the highest diagnostic value are the number of areoles on the distal spore side diagonally, the ornamentation of the proximal spore side (areolate to not areolate), the consistency of the wing which surrounds the spores and the color of the spores in the mature state.

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In the course of our project, we came across two entities of specimens that share thallus characteristics with *R. glauca* and *R. bifurca*, but differ in spore ornamentation. One of the entities matched the morphological concept of *R. gothica* Damsh. & Hallingb, but the occurrence of this taxon in central Europe had been questioned. *Riccia gothica* was described from southern Scandinavia (Damsholt and Hallingbäck 1986a) as a small species with light brown, rarely dark brown, spores which are 60–80 µm in size. On the distal side 6–9 areolae are counted. The proximal faces also show areolae and the y-shaped ridge is not well-defined. The wing is characterized as narrow and irregularly crenulate. From Northern Europe, the species is only known from Sweden. It is restricted to coastal alvar vegetation, which is characterized by a very specific ecology and a wealth of endemic species (Tyler et al. 2018). Besides Sweden, the species is only reported from Germany and one single record exists from France (Meinunger and Schröder 2007; Frahm 2008). However, Hugonnot and Chavoutier (2021) considered the taxon to be problematic and did not accept *R. gothica* in the liverwort flora of France. According to Damsholt and Hallingbäck (1986a), *R. gothica* is most similar to *R. glauca*, while Meinunger and Schröder (2007) and Frahm (2008) state a similarity with *R. bifurca* based on the similarly light brown spores. The ornamentation of the spores of the other entity also showed similarities to those of *R. gothica*. However, it differs in the strikingly notched edge of the spore wing, more acute papillae at the walls of the areolae, leaving a more spiny impression, and the dark brown color. The three faces of the proximal side are usually well delimited by the y-marking. Considering thallus characteristics, we suspected this morphotype to be related to *R. gothica*, *R. bifurca*, or *R. glauca*. After searching for a Central European *Riccia* species that matched this spore type, we came across the protologue of *R. pusilla* (Warnstorf 1896), which is downgraded as a variety of *R. bifurca* [var. *pusilla* (Warnst.) K.Muell.] by Müller (1916) based on similarities in thallus characteristics. In his 2nd edition (Müller 1951–1958) even this variety is no longer mentioned and *R. pusilla* is completely included in the synonymy of *R. bifurca*. This concept has been followed by most modern *Riccia* monographs or bryophyte floras (e.g. Jovet-Ast 1986; Schuster 1992; Schumacker and Váňa 2005; Ros et al. 2007). Meylan (1924) classifies forms of *R. bifurca* with flat thallus margins as *R. pusilla*. However, we have noticed that the description of the spores in the protologue of Warnstorf (1896) does not match those of *R. bifurca* but has many similarities with our unknown entity.

Against this background, using an integrative approach combining a comprehensive morphological dataset with molecular data, we have addressed the following questions: (1) Do the Central European collections of *R. gothica* actually belong to the taxon described from Scandinavia? (2) How does *R. gothica* phylogenetically relate to *R. bifurca* and *R. glauca*? (3) What is the identity of *R. pusilla*? (4) Do similarities in spore ornamentation reflect the phylogeny?

We hypothesize that spore characteristics are more useful than thallus characteristics in resolving the taxa of this group proved by using molecular methods. Based on this, we postulate that species with greater similarities in their spore

characters are phylogenetically more closely related to each other. Thus, we assume that *R. bifurca* and *R. gothica* as well as *R. glauca* and *R. pusilla* are sister taxa, respectively.

Methods

Morphological studies on fresh material and herbarium specimens, dataset for statistical tests

We compiled a dataset comprising 105 specimens from ten European countries (Appendix 1) morphologically assigned to *Riccia gothica* (30), *R. bifurca* (33), *R. glauca* (32) and *R. pusilla* (12), whereby the latter includes the specimens of our unknown entity. The dataset also includes an isotype and a topotype of *R. gothica* (pg-596 and pg-875) as well as type material of *R. pusilla* from collections G and WU. In total, 80 samples are from recent collections from 2018 to 2023, where we were able to generate data of thallus characteristics from the living state. The remaining 25 specimens are from herbaria G, GJO, JE, W, WU, Z, as well as from private herbaria of the authors and other colleagues. From herbaria specimens we only generated spore data because it is difficult to recover accurate measurements from rehydrated thalli. The selection of spore characters is based on the findings of Pörtl et al. (2024). Within some specimens, single spore characters could not be levied, for example when the spores are strongly immature or kind of damaged. In Table 1 thallus and spore characters we used and tested for differentiation of the four taxa are listed. The table also shows how qualitative characters are classified in order allow statistical analyses. In total, six characters are used for delimitation of the taxa.

The ratio of height to width of three terminal thallus segment is measured by cross-sections within the upper third of the segment. The maximum width arises from measurements of at least ten terminal segments to compare the character between the four taxa. Per sample at least ten spores are analyzed and metric data are averaged. We provide the data

Table 1. Qualitative and quantitative characters used for differentiation of the taxa; classification of qualitative data is given.

	Character	Quantitative data	Qualitative data
Spore	no. of areolae diagonally distal	counting	
	integrity of areolae proximal		complete areolae (1), walls of areoles partly broken or forming maze (2), walls dissolved in vermiculate structures (3)
	consistency of spore wing		entire (1), fine papillose (2), crenulate (3), notched or wavy (4), deep and coarse notched (5)
Thallus	spore color		verbal description
	width terminal segment	measuring	
	ratio width : height terminal segment	measuring	

The number in the brackets represents the class used for the statistics.

sheet used for the analyses in [Appendix 3](#). Statistical analyses are performed with the software PAST (Hammer 2014). We used t-test for metric and Mann-Whitney-test for ordinal data. Spore color is obtained qualitatively by verbal description. For morphological descriptions of taxa, we also examined additional characteristics of thalli and spores such as diameter of the rosettes, number of divisions of thalli, shape and color of margins and apices of terminal segments, descriptions of the ventral scales and the spore size (including the wing). Habitus pictures are made with Olympus Tough TG-5 as well as Keyence VHX 7000. For microscopic measurements and light micrographs (LM) we used Olympus BX51 microscope equipped with a DP74 camera and Olympus cellSense software as well as Motic light microscope BA310 with Moticam 5.0MP camera and software supplied by the manufacturer. For stacking the software Helicon Focus (<http://www.heliconsoft.com/heliconsoft-products/helicon-focus/>) was used. Specimens of all recently collected *Riccia* samples are deposited in the GJO collections. From specimen bi-588 and pu-588, all material was used up for molecular analyses, but a new specimen from the same locality collected in 2024 is deposited in GJO (136623; 136625).

Sampling for molecular dataset

For phylogenetic inference we used a subset of the specimens examined morphologically comprising ten accessions of *R. bifurca*, six of *R. glauca*, eight of *R. gothica* (including the topotype, pg-875) and six of *R. pusilla*, representing our unknown entity ([Table 2](#) and [Appendix 1](#)). In addition, *Riccia ciliata* Hoffm. and *R. ciliifera* Link ex Lindenb. are included as outgroup. To obtain samples for sequencing about 15 thalli segments per specimen of the freshly collected material, were purified with distilled water. The samples were then dried in silica gel and stored in the freezer at -18°C . Four samples were prepared from herbaria specimens ([Table 2](#)).

Laboratory protocols

We extracted DNA from dried and crushed plant tissue using the NucleoSpin Plant II Mini kit (Machery-Nagel, Germany) following the manufacturer's protocol with the exception of an extended lysis step of 3 h. Our initial attempts to utilize classical chloroplast markers (psbA_trnK, atpB-rbcL, trnL-trnF) for phylogenetic inference revealed either no or very low inter-specific variability. Consequently, we focused on nuclear markers targeting the multi-copy ribosomal internal transcribed spacer 2 (ITS2) and two novel nuclear single-copy loci which are used here for the first time in bryophyte phylogenetics. These novel loci were developed screening genome skimming libraries of *Riccia glauca* and a Balkan specimen *Riccia* sp. and the *Marchantia polymorpha* reference genome (GCA_003032435.1). After first genome assembly in Spades v 3.15 (Prijbelski et al. 2020) genes were predicted using unannotated v 1.8.15 (Palmer and Stajich 2019) orthologs were identified using orthofinder (Emms and Kelly 2019) on translated sequences. Pairwise alignments of references and draft assemblies and primer development were carried out in

Geneious v. 11.1.5 (<https://www.geneious.com>) One locus is located in the gene coding the DNA-directed RNA polymerase II subunit (RPB1) and the other spans across a conserved intronic region within a putative protein coding gene (thereafter referred to as unidentified nuclear marker, UNM), which was confirmed to be single copy in the latest *Marchantia polymorpha* genome (GenBank Acc.No GCA_037833965.1), where it corresponds to protein BFI33365.1, located before the MpPAPK1.1 gene in chromosome AP031354.1. We performed PCR using reaction volume of 10 μl composed of 2 μl bovine serum albumin solution (4 mg/ml), 1.2 μl 2.5 μM solution of each forward and reverse primer, 5 μl DreamTaq Master Mix (ThermoFisher) and 0.6 μl DNA isolate. Primers and cycling programs are provided in [Table 3](#). For ITS2 we used two alternative forward primers and for UNM and RPB1 two alternative reverse primers. Cycling was preceded by five minutes initial denaturation at 95°C and followed by a final elongation step of ten minutes at 72°C . Amplicons were commercially sequenced by LGC Genomics (Germany) using amplification primers.

Phylogenetic analyses

We edited reads by visual inspection of chromatograms in Geneious v2024.0. One accession (pu-588) showed double peaks at three nucleotide positions in the first part of ITS2 and the second part was unreadable due to overlapping signals. We therefore excluded the sequence from the phylogenetic analyses but tentatively included it in the alignment to evaluate its affinities and if the ambiguities affect informative sites. We aligned edited sequences using the E-INS-i strategy in the online interface of MAfft v7 (Katoh and Standley 2013; Katoh et al. 2019) followed by manual edits. We scored indels using the simple coding strategy of Simmons and Ochoterena (2000) and performed phylogenetic analyses using Bayesian inference (BI) in MrBayes v3.2.6 (Ronquist et al. 2012) and maximum likelihood (ML) in RAxML v8.2.10 (Stamatakis 2014) using the GTR+G+I model for the nucleotide partition. In MrBayes we specified a gamma model for the indel partition and unlinked parameters across partitions. We set the number of generations to 1 M, the sample frequency to 100, the heating temperature to 0.1 and calculated a fifty percent majority rule consensus tree after discarding the first 25% of trees as burn-in. Convergence of the two runs was verified using Tracer v1.7.2 (Rambaut et al. 2018) to check that ESS values exceeded 200. In RAxML we used the thorough bootstrapping algorithm with the extended majority rule bootstrapping criterion to assess the support for the nodes of the best scoring tree out of 50 independent ML runs. Initial separate analyses of the markers revealed consistent topologies for the well supported clades between the two single-copy genes ([Appendix 2](#)) and we thus proceeded to concatenate these loci and run BI and ML analyses on this concatenated dataset with the same settings. We summarized the support of nodes from BI and ML analyses in TreeGraph 2 (Stöver and Müller 2010) displaying the BI topology.

Table 2. Samples used in the molecular dataset.

Species name	Provenance	ID <i>Riccia</i> -project	Voucher	ITS2	RPB1	UNM
<i>Riccia bifurca</i>	Sweden, Lekeberg	bi-606	GJO 111518	PQ777448	–	PQ871586
<i>Riccia bifurca</i>	Hungary, Vgrad Mountain, Visegrad	bi-263	GJO 95584	PQ777447	PQ784060	PQ871585
<i>Riccia bifurca</i>	Austria, Styria, Lannach	bi-557	GJO 111538	PQ777441	PQ784054	PQ871578
<i>Riccia bifurca</i>	Austria, Styria, Haselsdorf-Tobelbad	bi-430	GJO 97730	PQ777440	PQ784053	PQ871577
<i>Riccia bifurca</i>	Austria, Styria, Enzelsdorf	bi-588	NA, later collection from same locality GJO 1306623	PQ777442	PQ784055	PQ871579
<i>Riccia bifurca</i>	Austria, Styria, Gamlitz	bi-854	GJO 111553	PQ777444	PQ784057	PQ871581
<i>Riccia bifurca</i>	Austria, Lower Austria, Haidegg	bi-869	GJO 140146	–	–	PQ871584
<i>Riccia bifurca</i>	Austria, Styria, Vasoldsberg	bi-856	GJO 111557	PQ777445	PQ784058	PQ871582
<i>Riccia bifurca</i>	Austria, Styria, Pöllau near Hartberg	bi-656	GJO 111983	PQ777443	PQ784056	PQ871580
<i>Riccia bifurca</i>	Austria, Carinthia, Villach	bi-858	GJO 111641	PQ777446	PQ784059	PQ871583
<i>Riccia glauca</i>	Austria, Styria, Laßnitzhöhe	gl-961	GJO 140147	PQ777452	PQ784065	PQ871591
<i>Riccia glauca</i>	Spain, Canary Islands, La Palma, Los Sauces	gl-901	GJO 136623	PQ777455	PQ784068	PQ871593
<i>Riccia glauca</i>	Austria, Styria, Kirchberg a. d. Raab	gl-437	GJO 97720	–	PQ784063	PQ871589
<i>Riccia glauca</i>	Germany, Baden-Württemberg, Filderstadt	gl-654	GJO 112147	PQ777454	PQ784067	–
<i>Riccia glauca</i>	Austria, Styria, Gamlitz	gl-855	GJO 112186	PQ777451	PQ784064	PQ871590
<i>Riccia glauca</i>	Germany, Baden-Württemberg, Filderstadt	gl-630	GJO 112157	PQ777453	PQ784066	PQ871592
<i>Riccia pusilla</i> subsp. <i>gothica</i>	Switzerland, Reussebene	pg-960	GJO 140148	PQ777462	PQ784072	PQ871599
<i>Riccia pusilla</i> subsp. <i>gothica</i>	Switzerland, Aargau, Rottenschwiler Moos	pg-815	Z-134038	PQ777460	–	PQ871597
<i>Riccia pusilla</i> subsp. <i>gothica</i>	Slovenia, Gorenjaka, Škofja Loka	pg-615	GJO 111526	PQ777464	PQ784074	PQ871601
<i>Riccia pusilla</i> subsp. <i>gothica</i>	Austria, Styria, Bad Mitterndorf	pg-844	GJO 140149	PQ777459	PQ784071	PQ871596
<i>Riccia pusilla</i> subsp. <i>gothica</i>	Austria, Styria, Friesach	pg-465	GJO 100850; priv. herb. W. Obermayer 15218	PQ777457	PQ784070	PQ871595
<i>Riccia pusilla</i> subsp. <i>gothica</i>	Sweden, Öland, Södra Möckleby parish, Klinta alvar	pg-875	GJO 140150	PQ777463	PQ784073	PQ871600
<i>Riccia pusilla</i>	Austria, Styria, Enzelsdorf b. Hausmannstätten	pu-588	NA, later collection from same locality GJO 1306625	PQ777456	PQ784069	PQ871594
<i>Riccia pusilla</i> subsp. <i>gothica</i>	Switzerland, Aargau, Aristau, Oberschachen an der Reuss	pg-825	priv. herb. H. Hofmann 211377	PQ777461	–	PQ871598
<i>Riccia pusilla</i> subsp. <i>pusilla</i>	Austria, Styria, Jahrbach am Saßbach	pp-425	GJO 99688	PQ777465	PQ784075	PQ871602
<i>Riccia pusilla</i> subsp. <i>pusilla</i>	Austria, Styria, Enzelsdorf b. Hausmannstätten	pp-588	GJO 111541	PQ777458	PQ784076	PQ871603
<i>Riccia pusilla</i> subsp. <i>pusilla</i>	Switzerland, Luzern, Fischbach	pp-820	priv. herb. H. Hofmann 304954	PQ777468	–	PQ871606
<i>Riccia pusilla</i> subsp. <i>pusilla</i>	Austria, Carinthia, E Villach	pp-919	GJO 118673	PQ777467	PQ784078	PQ871605
<i>Riccia pusilla</i> subsp. <i>pusilla</i>	Austria, Styria, Enzelsdorf b. Hausmannstätten	pp-713	GJO 111573	PQ777466	PQ784077	PQ871604
<i>Riccia ciliifera</i> agg.	Spain, Canary Islands, Teneriffa, Punta del Hidalgo	ci-738	GJO 136579	PQ777450	PQ784062	PQ871588
<i>Riccia ciliata</i> (det. Kiebacher)	Italy, South Tyrol, Feldthurns, Tschiffnon	–	priv. herb. T. Kiebacher 4376	PQ777449	PQ784061	PQ871587

Results

Overall, our morphological and molecular analyses revealed that the Central European collections of *R. gothica* cannot be distinguished from the Scandinavian plants including the topotypic material. Furthermore, our results show that the unknown entity is morphologically and molecularly distinct, closely related to *R. gothica* and corresponds to historical material of *R. pusilla*. We consequently decided to recognize *R. gothica* as a subspecies of *R. pusilla*. Henceforth, we use this new combination.

Molecular results

The length of the ITS2, UNM and RPB1 alignments comprised 628, 783, and 1026 nucleotide positions, respectively, of which 187, 213, and 87 were variable and 80, 104, and 31 were parsimony informative. The topologies inferred from the two datasets (ITS2 and UNM+RPB1) were largely consistent except for the accessions of *R. glauca* (Figures 1 and 2). Within the target group, accessions of *Riccia bifurca* were resolved in a maximally supported basal clade separated by all other accessions by a long branch. *Riccia pusilla* + *R.*

glauca accessions formed a highly supported clade in both analyses (pp = 1 and bs = 94 in ITS2, 1/99 in UNM+RPB1) as well as the *R. pusilla* s.l. accessions within this clade (1/94 in both datasets). Accessions of *R. pusilla* were separated into two supported clades corresponding to the two spore morphologies assigned to the two subspecies *pusilla* and *gothica* (1/100 and 0.99/61 in ITS2, 1/84 and 0.78/73 in UNM+RPB1). Minor intraspecific differences (few SNPs and indels) in the ITS2 sequences of subsp. *gothica* resulted in the clustering of

the topotype (pg-875) in a poorly supported clade, along with three Swiss specimens, among the remaining Central European accessions. RPB1 and UNM sequences of the topotype were identical to Central European accessions. Accessions of *R. glauca* were resolved in a maximally supported clade sister to *R. pusilla* in ITS2 whereas the RPB1+UNM tree revealed a paraphyletic topology. Two of the accessions from Austria and Spain (gl-437, gl-901) appeared in a highly supported clade (1/99) sister to a clade (1/92) comprising the remaining *R. glauca* accessions and *R. pusilla*. Visual inspection of the ITS2 alignment, including the critical pu-588 accession, revealed that its sequence is identical to those of accessions of *R. pusilla* s.l. and that the three ambiguous sites correspond to the three SNPs distinguishing the two subspecies. The unreadable second part of the raw reads followed an insertion found in subsp. *gothica* accessions compared to those of subsp. *pusilla*.

Table 3. Primers and PCR cycling used for amplifying of the plastid *rps4-trnF* and the nuclear ITS region.

Locus	Primer name & sequence (5'-3')	Reference	PCR cycling
ITS2	F: ITS3 GCATCGATGAAGAACGCAGC	White et al. 1990	40×30sec 95°C
	F: TS3_Ric_F TGAAGAACGCAGCGAAATGCG	This study	40sec 57°C
	R: 25S-ITS4-R TCCTCCGCTTAGTGATATGCTTAAACTCAGC	Kiebacher et al. 2024	1 min 72°C
	F: UNM_1398_F TGAAGCAGGCTTTGGTTGAC	This study	40×30sec 95°C
	R: UNM_2138_R CTTCGGGCTKCGAACTTCGA	This study	40sec 57°C
UNM	UNM_2121_R CGAAGGATGCTGAAGTGTG	This study	1 min 72°C
	F: RPB1_7064_F AGTATAGGTATCGGAGATACTATTGC	This study	40×1 min 95°C 1 min
	R: RPB1_7764_R CAAAACCACGACTCTCGGG	This study	55°C
RPB1	R: RPB1_7752_R ACTCTCGGGACCGTAATCGTC	This study	1.5 min 72°C

Morphological description, ecology and distribution

Riccia pusilla* Warnst. subsp. *pusilla, Verhandlungen des Botanischen Vereins Berlin Brandenburg 37: 50, 1896 (Figures 3 and 4).

Lectotype (designated here): [Germany] Neuruppin, Thongruben bei der Irrenanstalt, Sept. 1894, leg. C. Warnstorff, WU 0157476.

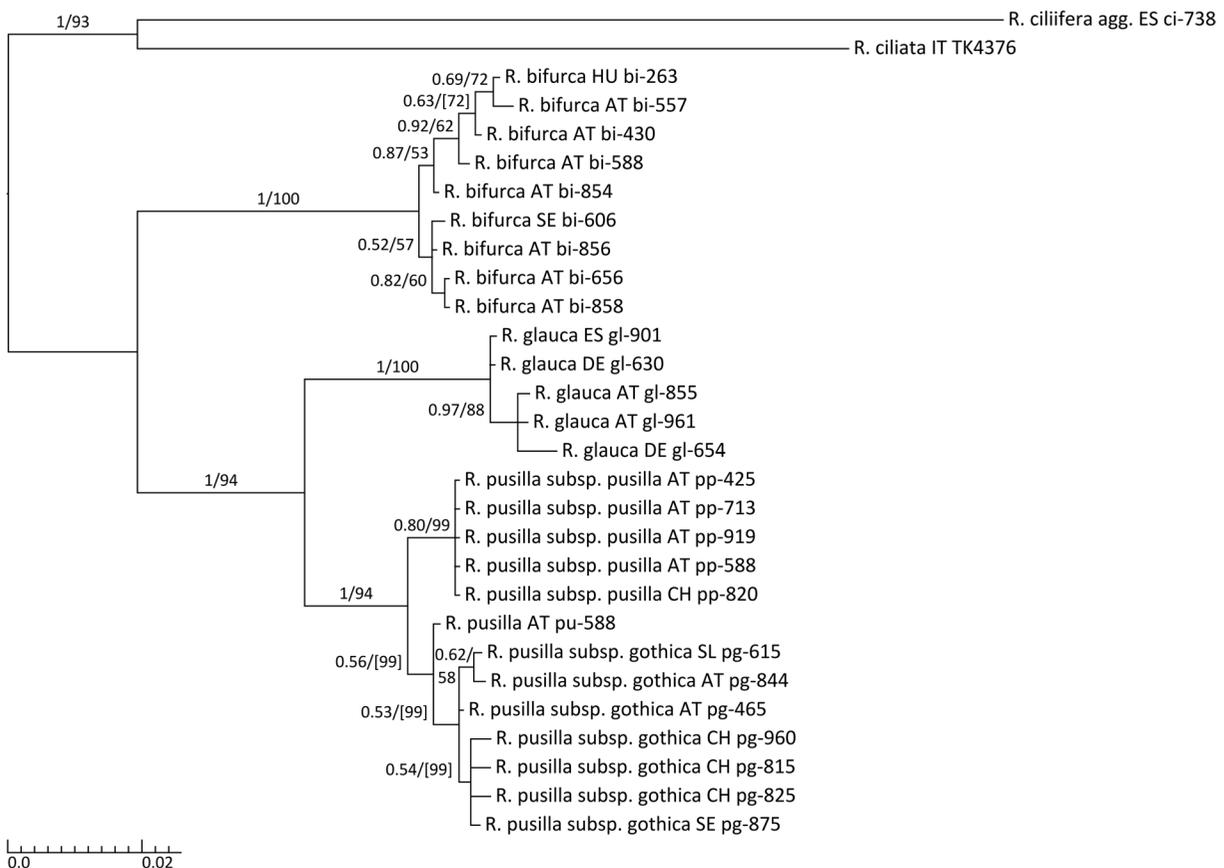


Figure 1. Bayesian inference (50% majority rule consensus tree) on the ITS2 dataset 1. Support values above branches are posterior probabilities followed by bootstrap support values ≥ 50 obtained from maximum likelihood analysis of the same dataset. Upper case letters after the epithets are country IDs in ISO 3166 format. GenBank accession numbers are provided in Table 2.

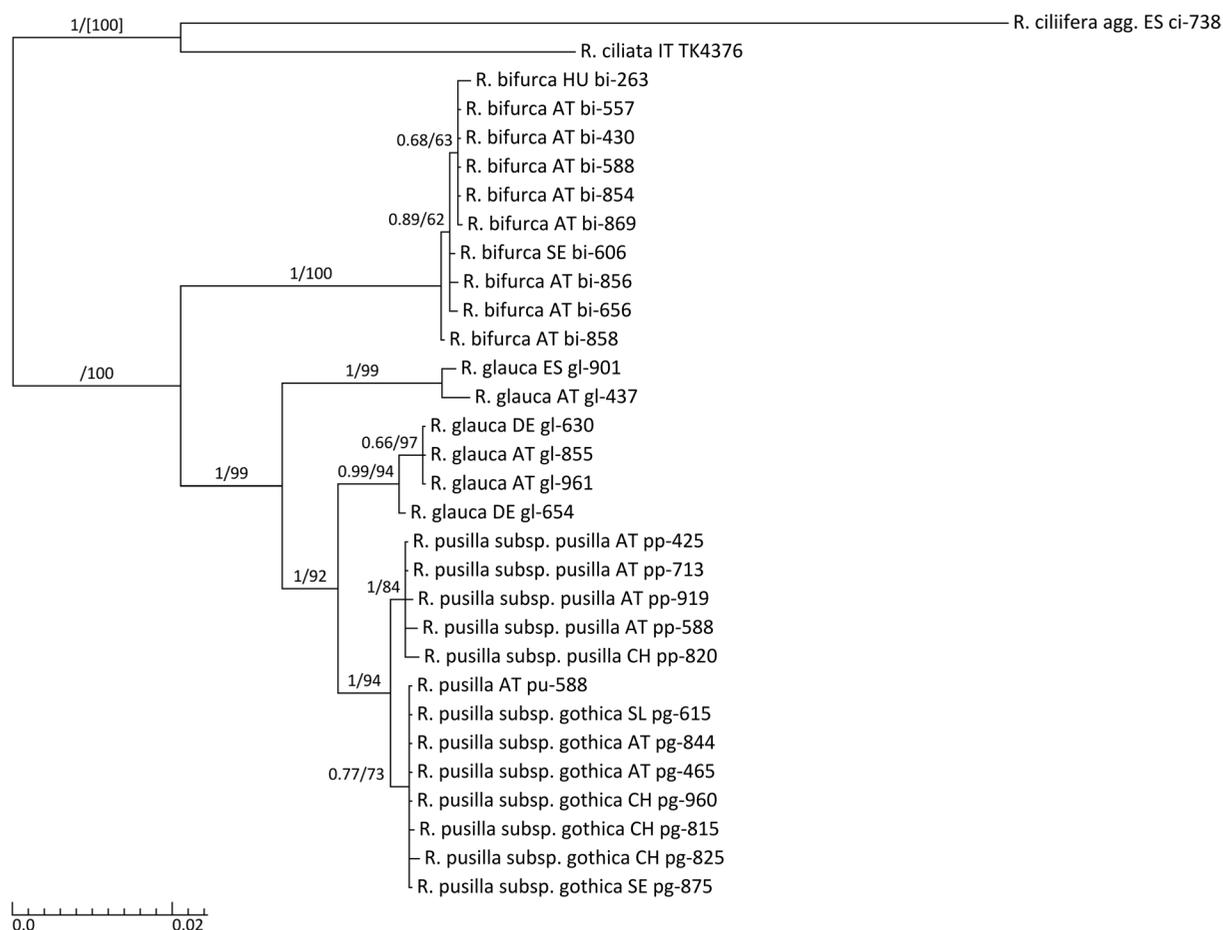


Figure 2. Bayesian inference (50% majority rule consensus tree) on the concatenated RPB1 and UNM data. Support values above branches are posterior probabilities followed by bootstrap support values ≥ 50 obtained from maximum likelihood analysis of the same dataset. Upper case letters after the epithets are country IDs in ISO 3166 format. GenBank accession numbers are provided in Table 2.

Note: The type material in WU and G examined is heterogeneous. The specimen in WU contains several broken rosettes of *R. pusilla* subsp. *pusilla* and subsp. *gothica* as well as *R. cavernosa*. Two fertile parts of rosettes that show spore characteristics as described in the protologue of *R. pusilla* by Warnstorf (1896) are selected as the lectotype and stored in a labelled envelope within the specimen. The two specimens in G designated as types could not be confirmed as types of *R. pusilla*. One specimen (Germania, Neuruppin bei der Irrenanstalt, 04.09.1894, leg. C. Warnstorf, G 00281881) comprises only *R. pusilla* subsp. *gothica*. The second specimen (Germania, Neuruppin, s. d., leg. C. Warnstorf, G 00281882) is assigned to *R. pusilla* subsp. *pusilla* but because the date is missing on the label it is uncertain if the material comes from the original collection. Both specimens bear the stamp 'Species Hepaticarum Stephani' and in Warnstorf's handwriting the names *Riccia pusilla* and *Riccia bifurca*. We have not examined syntypes deposited in MNHN and S.

Description

Plants in conspicuous small, complete rosettes, 1.0–2.0 cm in diameter (but mostly ≥ 1.5 cm), glaucous green to greyish green, with or without purple pigmented margins, without cilia; thalli 2–5 times dichotomously branched; terminal

segment 0.5–1.5 mm wide, with round to truncate apex; ratio height to width 1 to 3–5; flanks bulging, edges round to subacute; middle groove well developed, narrow at the apex, gradually widening towards the back; ventral scales inconspicuous, hyalin or a bit purple; sporophytes frequently. Spores dark to middle brown, 70–105 μm in diameter including the wing, distal surface diagonally with 8–12 areolae, proximal side with same sized or slightly smaller areolae, walls sometimes broken; papillae of walls high and truncate, giving an icky appearance; y-ridge well developed in the centre, broken polewards; wing narrow, roughly notched to wavy.

Ecology

Riccia pusilla subsp. *pusilla* occurs on moist to wet and base-rich soils. Former alluvial soils seem to be favorable, since the species is often found in damp to wet pioneer sites near larger watercourses. Also at the type locality Neuruppin there are larger rivers around. Specimens were collected from different habitats with a pioneer character, such as arable fields, dried out river and pond sludge, or clay pits, where Warnstorf originally collected it. It often occurs in even more moist and base rich habitats than other taxa of the complex and co-occur with *R. cavernosa*.

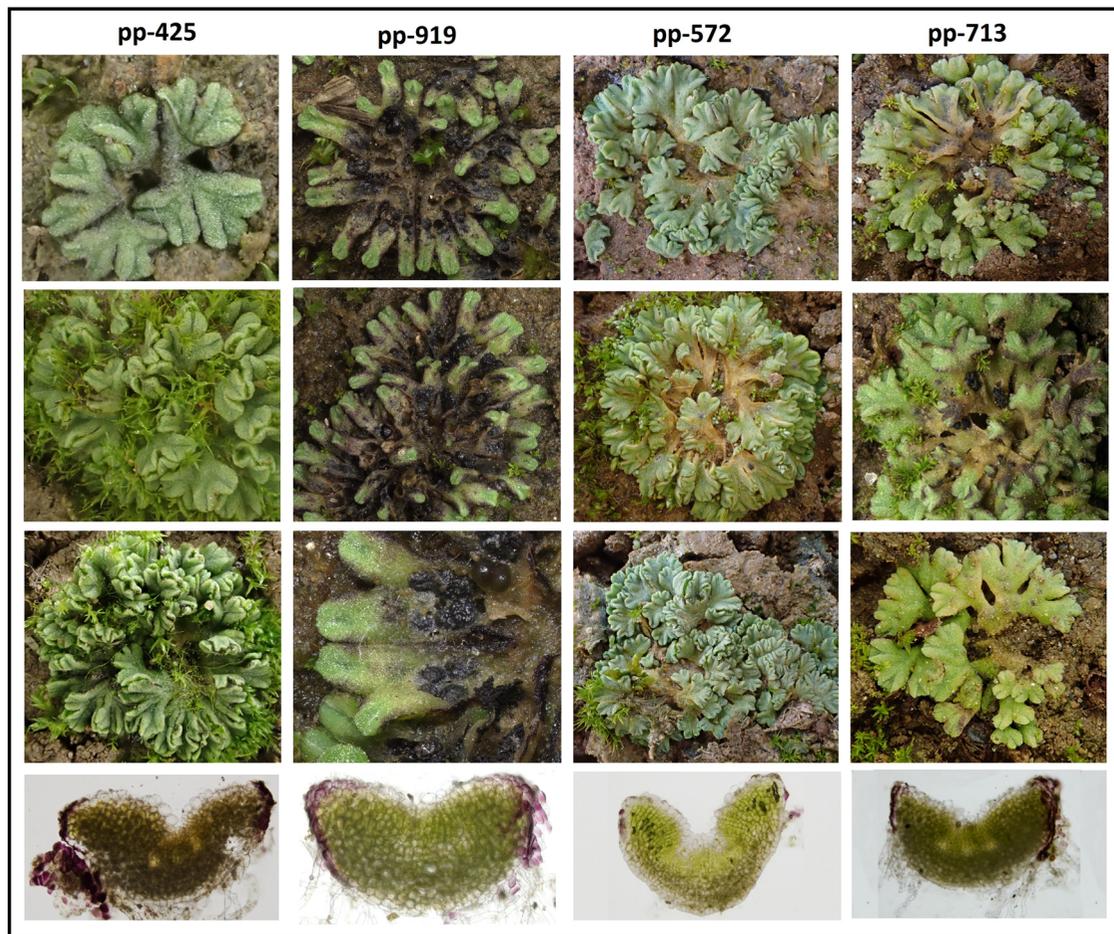


Figure 3. Habitus and cross-sections of terminal segments of four samples of *R. pusilla* subsp. *pusilla* from Austria. Voucher information is provided in Appendix 1.

Distribution

All of Warnstorff's historical collections originate from Germany. In addition, we can name the species for Austria, Slovenia and Switzerland. All collections are from the collin and lower montane altitudinal belt at elevations between 105 and 695 m.

Figure 5 shows distal and proximal sides of the spores from the lectotype. All capsules were still closed and spores were not fully mature. Thus, the spores do not show the typical dark brown color which we would expect. One the distal side 10–11 areolae could be counted, the proximal side shows closed, more or less same sized areolae. The spore wing is narrow and mostly irregularly notched.

***Riccia pusilla* subsp. *gothica* (Damsh. & Hallingb.) Pötl & C.Berg, comb. nov.** (Figures 6 and 7).

Basionym: *Riccia gothica* Damsh. & Hallingb., *Lindbergia* 12: 100, f. 1: 5–8. 1986[1987].

Lectotype (designated here): Sweden, Västergötland, Dala, Dala kalkbrott, Coordinates: latDD 58.25065; longDD 13.74965, 06.09.1985, leg. T. Hallingbäck and K. Damsholt, collector number 4248, UPS B-1109891. Isotype: Sweden, Västergötland, Dala, Dala kalkbrott, Coordinates: latDD 58.25065; longDD 13.74965, 06.09.1985, leg. T. Hallingbäck and K. Damsholt, collector number 4248, C s. n.

Note: in the protologue of *R. gothica* (Damsholt and Hallingbäck 1986a) two specimens, one in the collection C

and one in UPS, are cited. Thus, selection of a lectotype has been done here. We have not examined isotype material deposited in JE.

Description

Thalli as in subsp. *pusilla*. Spores light brown, translucent, 60–105 µm in full diameter, distal surface diagonally with 7–12 areolae, proximal side with same sized areolae, walls sometimes broken; papillae of walls mostly flat, conic to truncate; y-ridge badly developed, the three proximal facets badly separating; wing narrow or missing, mostly entire, sometimes notched.

Ecology

Riccia pusilla subsp. *gothica* occurs on moist, base rich to calcareous, open places in different habitats. In Central Europe it is mostly found on arable land, as well as on forest embankments, damp forest tracks, gravel banks and dried-out pond bottoms. In Sweden it has so far been found in the calcareous alvars.

Distribution

From Northern Europe *Riccia pusilla* subsp. *gothica* is known from Sweden. In Central Europe the taxon is known from Austria, Germany, Hungary, Slovenia and Switzerland. The subspecies is

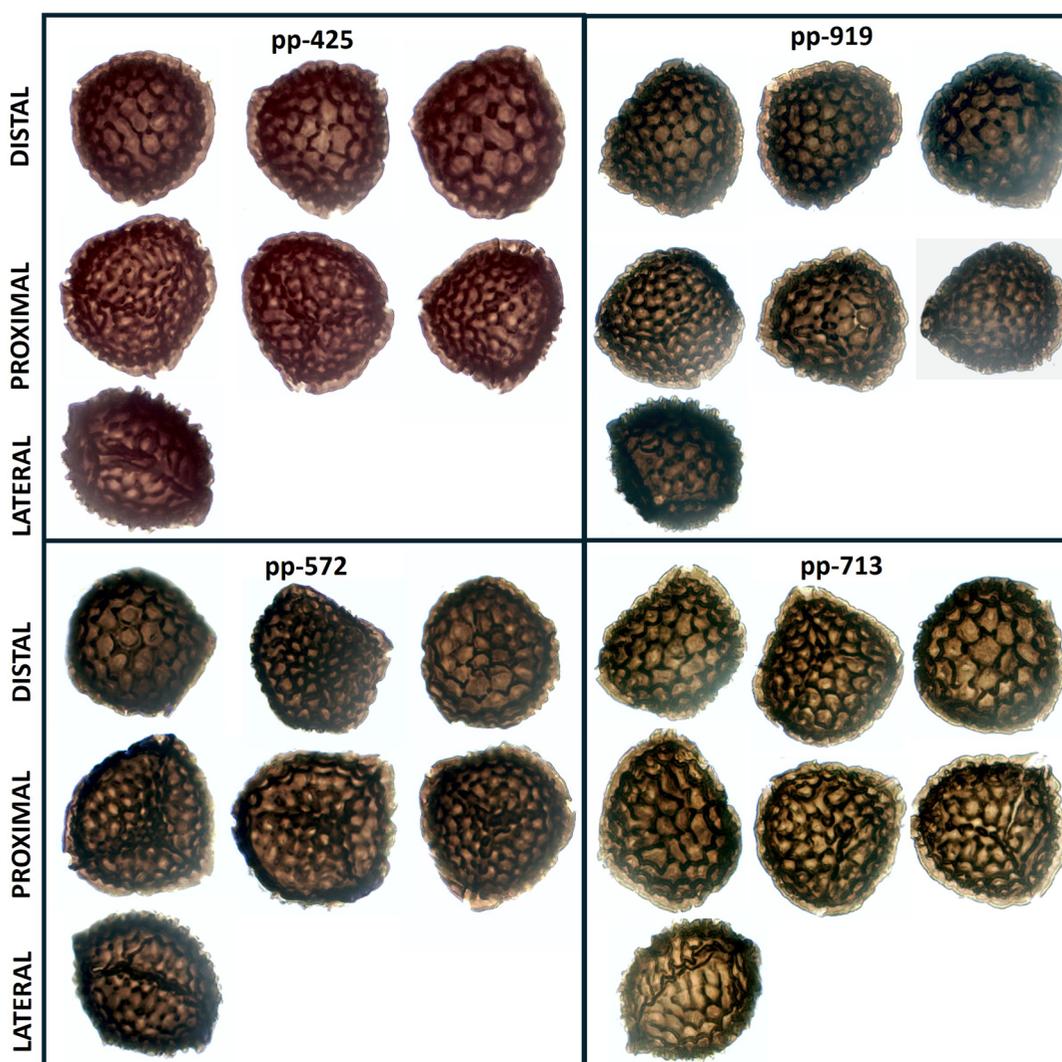


Figure 4. Distal, proximal and lateral sides of the spores of four specimens of *Riccia pusilla* subsp. *pusilla*. Voucher information is provided in Appendix 1.

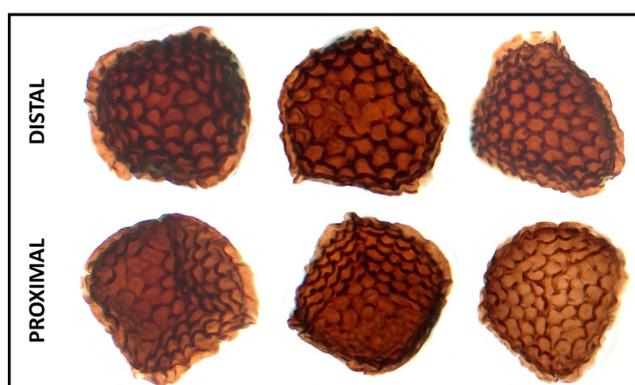


Figure 5. Distal and proximal sides of spores from the lectotype of *Riccia pusilla* subsp. *pusilla*; spores are not fully matured.

found in the colline to submontane altitudinal belt, with the highest known occurrence at 850m in Salztal, Austria.

Morphological differentiation of *Riccia pusilla* s. lat., *R. bifurca*, and *R. glauca*

Thallus characteristics are hardly differentiated within the four taxa. *Riccia bifurca* shows the broadest terminal

segments (Figures 8 and 9A). Between the three other taxa, no differences could be found. The ratio height to width of the terminal segments is approximately the same for all species and ranging from 2 to 5 (Figure 9B). Within spore characteristics the number of areolae on the distal spore side diagonally is 7 in *R. bifurca* and 9–10 in *R. glauca* and in both subspecies of *R. pusilla* (Figure 9A). Concerning the consistency of spore wings, *R. pusilla* subsp. *gothica* and *R. glauca* show a much lower value as the spores of those two taxa are mostly entire with smooth edges of the wing (Figure 9B). In contrast, *R. bifurca* and *R. pusilla* subsp. *pusilla* most often have strongly notched or wavy spore wings. Differences in proximal spore ornamentation between the species are reflected in the integrity of the areolae. *Riccia bifurca* received a significantly higher value in the boxplot (Figure 9C), as it is the only species tested here with vermiculate structures instead of complete areolae. Within all collections, *R. glauca* shows complete and considerably smaller areolae on the proximal side compared with those on the distal side. Within both subspecies of *R. pusilla*, specimens also show complete areole on proximal side, with areolae same sized or only slightly smaller than those on the distal side. In some samples single areolae are partly broken. The y-ridge at the proximal side varies from strong pronounced to somewhat broken

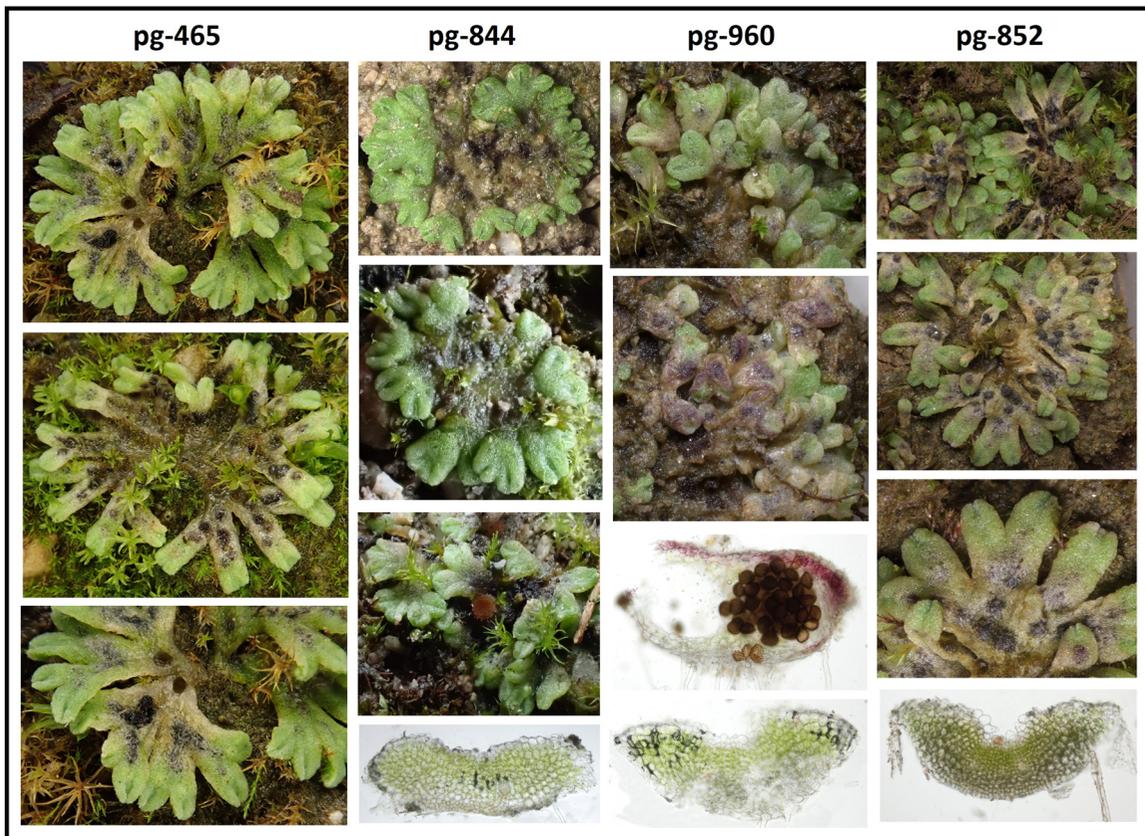


Figure 6. Habitus and cross-sections of four samples of *R. pusilla* subsp. *gothica* from Austria (pg-465, pg-844, pg-852) and Switzerland (pg-960). Voucher information is provided in [Appendix 1](#).

in all taxa. Within *R. pusilla* subsp. *gothica* the three facets often appear less distinct from each other, because the y-ridge is only badly recognizable, especially compared to the subsp. *pusilla*, where in all most cases a more or less strong y-ridge separates the facets. Within *R. pusilla* subsp. *pusilla* papillae of the walls of the areolae, especially on the distal side, are often higher and more acute, leaving a more spiky impression of the spores ([Figure 5](#)).

LM of distal and proximal spore side of the four taxa *R. pusilla* subsp. *gothica*, *R. pusilla* subsp. *pusilla*, *R. bifurca* and *R. glauca* are presented in [Figure 10](#). Concerning the color of the spores in mature state, *R. pusilla* subsp. *pusilla* and *R. glauca* have dark brown, opaque spores whereas *R. pusilla* subsp. *gothica* and *R. bifurca* have light brown, semi-translucent spores.

Spore morphology of type material of *R. pusilla*

Warnstorf (1896) described the spores of *R. pusilla* as blackish, opaque, showing hexagonal thickening bars (areolae) at all faces and the wing as narrow to irregularly notched and less translucent than in *R. bifurca*. Spores from the unknown entity show same ornamentation than those of the lectotype which in turn fits the description in the protologue (Warnstorf 1896). In all samples the wing is strongly notched, mostly narrow but in some cases broadly developed. Well matured spores, indicated by an open, dissolving capsule wall, are dark brown, while in still closed capsules immature spores are more light brown. The details in ornamentation do not differ within the state of maturity.

In recent collections the papillae on areolae walls are conspicuously acute and spiny, which could not be observed within the spores of historical specimens, including the lectotype. In all type material no spores could be found assigned to *R. bifurca*.

Diagnostic spore characters of *R. bifurca*, *R. glauca*, and *R. pusilla* s. lat

To reliably distinguish the four taxa treated here it is necessary to examine spore characteristics ([Figure 10](#)). *Riccia pusilla* subsp. *gothica* has light brown, translucent spores in mature state, similar to those of *R. bifurca*, but differs in having: (1) a higher number of areolae on the distal side (2) complete areolae with single broken areolae on the proximal spore side and (3) an entire, rarely notched spore wing in contrast to *R. bifurca*. The spores of the second subspecies *R. pusilla* subsp. *pusilla* differs in having (1) a dark brown, opaque color, (2) a roughly notched spore wing and (3) more acute papillae on areolae walls on both spore sides (best observed in lateral view, [Figure 4](#)). The spores of *R. glauca* have much smaller and denser areolae on the proximal side than those of the two subspecies of *R. pusilla*. In contrast, *R. bifurca* has vermiculate structures with isolated closed areolae on the proximal side. Diagnostic spore characteristics of those four taxa are presented in [Table 4](#) and used in the following determination key.

Key to differentiate *Riccia bifurca*, *R. glauca*, and *R. pusilla* s. lat. based on spore characteristics

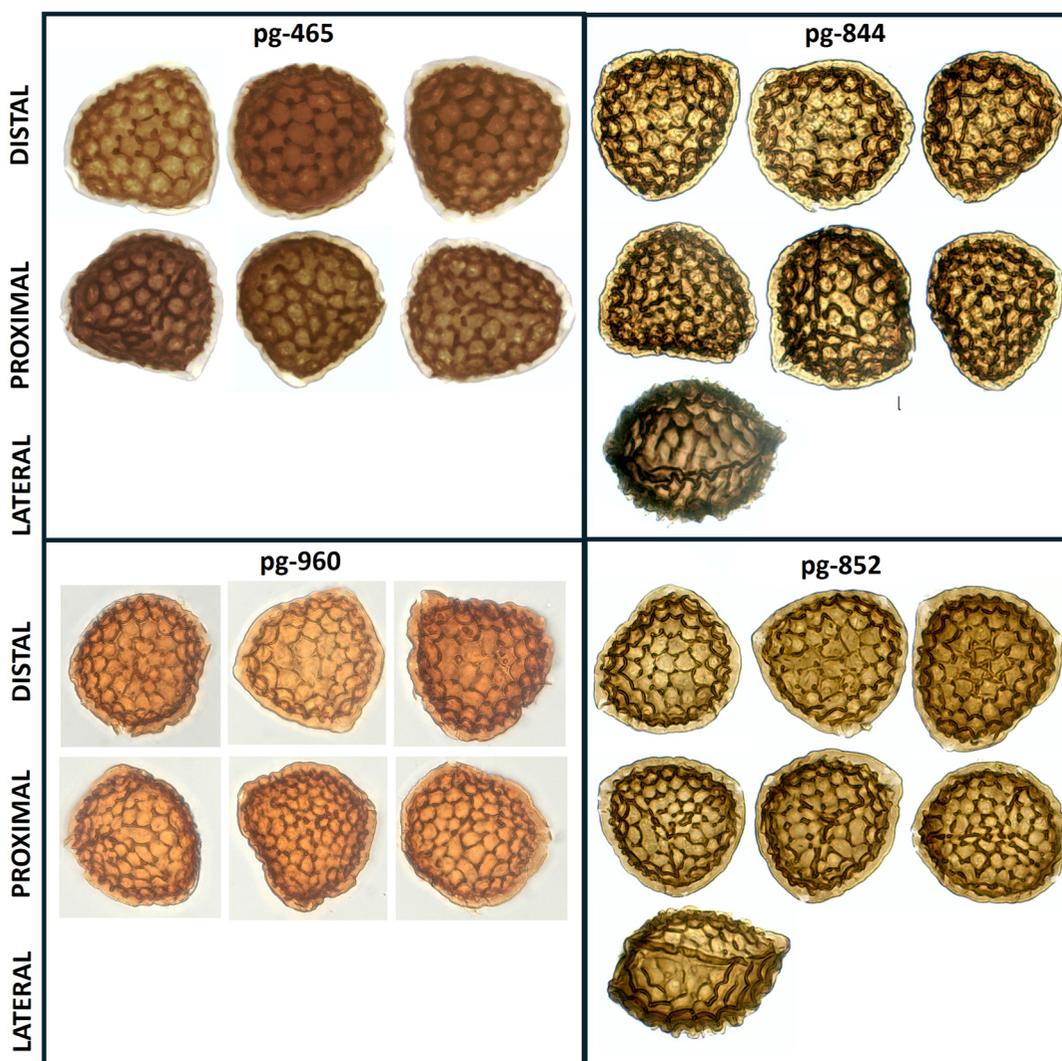


Figure 7. Distal, proximal and lateral sides of the spores of four samples of *R. pusilla* subsp. *gothica* from Austria (pg-465, pg-844, pg-852) and Switzerland (pg-960). Voucher information is provided in [Appendix 1](#).

- 1 proximal spore side with areolae resolved in vermiculate structures, mature spores light brown..... *R. bifurca*
 1* proximal spore side with complete areolae, only single walls broken, mature spores light to dark brown 2
 2 areolae on proximal side smaller and denser than on distal side, spore wing entire, spores dark brown *R. glauca*
 2* areolae on proximal side more or less as wide and dense as on distal side, spore wing entire or roughly notched, spores light or dark brown 3
 3 spore wing roughly notched, spores dark brown, papillae on areolae wall acute, plants growing in very moist, pH-neutral sites..... *R. pusilla* subsp. *pusilla*
 3* spore wing entire, spores light brown, translucent, papillae on areolae wall obtuse, plants growing in lime-influenced sites *R. pusilla* subsp. *gothica*

Discussion

Taxonomy and molecular patterns

Through the combination of morphological and phylogenetic methods we show that *R. bifurca*, *R. glauca*, *R. pusilla*

subsp. *pusilla* and *R. pusilla* subsp. *gothica* can be clearly separated. Our hypothesis that spore characteristics within these very similar looking taxa have a high taxonomic value can be confirmed by the genetic results. Within thallus characteristics a high variability is reflected in our data, whereby characteristics strongly overlap. Besides *R. bifurca* and *R. glauca*, also *R. pusilla* s.l. displays a distinct lineage in all three markers. Furthermore, the analyses consistently revealed two subclades within *R. pusilla* that are morphologically characterized by distinct spore ornamentation. The first subclade includes specimens from Central and Northern Europe which are assigned to *R. gothica* based on spore characteristics which indicates that they are the same taxa. The second subclade includes all specimens of our unknown entity which turned out to morphologically fit well to *R. pusilla*, a poorly known species described by Warnstorf (1896). However, one accession (pu-588), morphologically corresponding to *R. gothica*, showed ambiguous signals in the ITS2 chromatograms suggesting ITS variants corresponding to *R. gothica* and *R. pusilla* s.str. At the same time, we didn't observe ambiguities in the single copy locus UNM, while RPB1 was invariable for the two taxa ([Appendix 2](#)). This

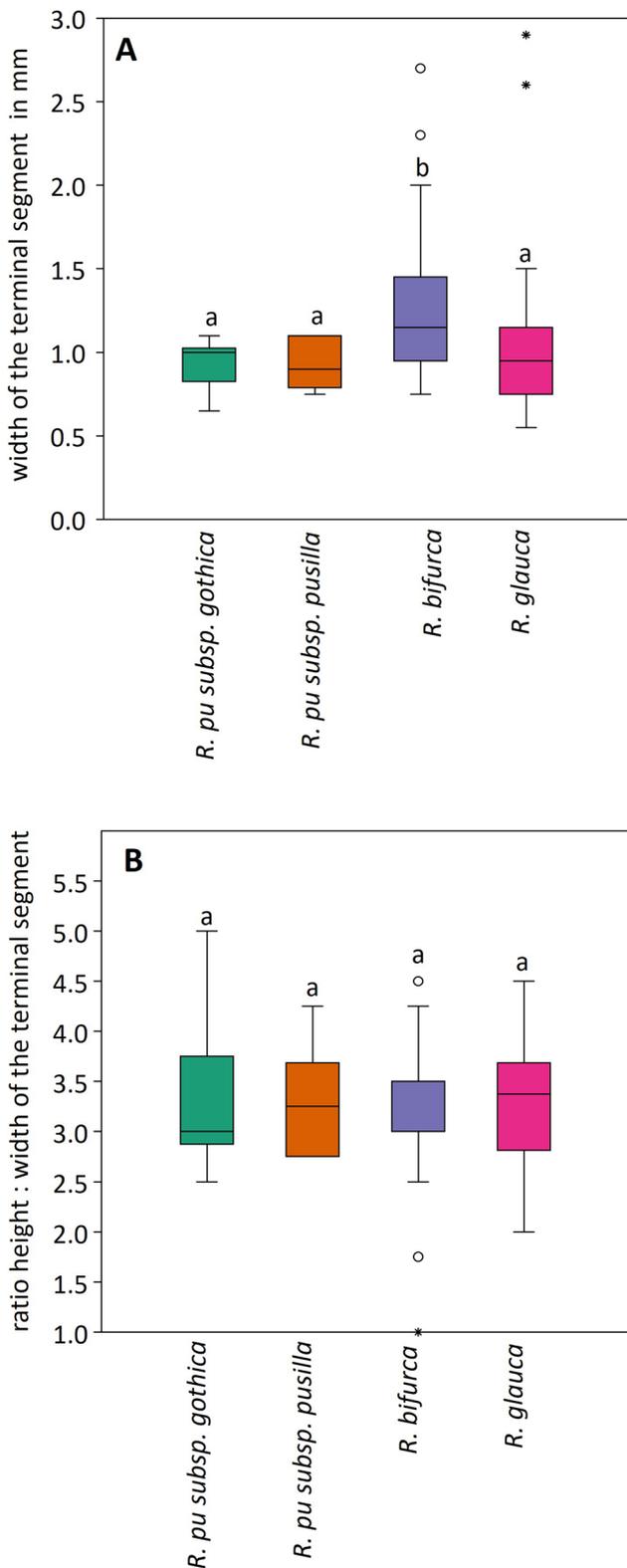


Figure 8. Thallus characteristics in *Riccia pusilla* subsp. *gothica* (n=9), *R. pusilla* subsp. *pusilla* (n=6), *R. bifurca* (n=27) and *R. glauca* (n=26), (A) width of the terminal segment in mm, (B) ratio height to width of the terminal segment. The letters a-c indicate groupings with values significantly different from each other ($p \leq 0.05$).

pattern could indicate a hybridization event between the two taxa resulting in a haploid gametophyte. Hybridization may have been facilitated by the occurrence of both taxa at the collection site of this specimen. This possible indication

of incomplete reproductive isolation has prompted us to propose the rank of subspecies for *R. gothica* and to not assign the critical accession (pu-588) to either subspecies (Figure 2, Table 2, and Appendix 2). From a morphological perspective, the clear differences between the two subspecies in spore ornamentation, would justify recognition of *R. gothica* at the species level. However, we can't exclude the possibility that the ambiguous signal in ITS in the pu-588 accession is due to a contamination with plant material of *R. pusilla* s.str. The absence of ambiguous signals in the single copy UNM locus could then be explained by the low amount of the *pusilla* component while the hundreds of copies of the rDNA operon in the genome may have provided a sufficient template to generate a detectable ITS2 amplicon. The variants detected in the pu-588 accession could also result from incomplete sorting of ancestral lineages, but this seems less likely because frequent sexual reproduction and short life cycles (one or even several per year), typical of our target species, usually facilitate rapid concerted evolution (e.g. Mano and Innan 2008).

The two loci, RPB1 and UNM, specifically targeted here for phylogenetic inference for the first time in bryophytes, proved to be suitable for such analyses at low taxonomic ranks in *Riccia* and are very likely a promising alternative or complement to commonly used markers in other groups as well. Especially, UNM showed a level of variability similar to that of the widely used ITS2 locus.

The lectotype material of *R. pusilla* comprises admixed the subspecies *gothica*, what we also observed within some other collections. These mixed populations raise the question of how these two taxa evolved. One explanation could be that they diverged in isolated refugia during the glacial oscillations (Hewitt 2004). The unfavorable cold and dry conditions likely led to small population sizes, which accelerated genetic drift and fostered the accumulation of genetic conflicts between the two entities (Escudero et al. 2019). These conflicts, in the current situation of mixed populations, may prevent re-homogenization. However, reproductive isolation may be incomplete, based on sample pu-588, which could represent a hybrid (see above). The potential of hybridization could be assessed by sequencing several gametophytes and sporophytes from mixed stands.

Further our results indicate, that morphological similarities between closely related *Riccia* species, do not necessarily reflected the phylogenetic relation. Our molecular data show that *R. pusilla* subsp. *gothica* is not closely related to *R. bifurca*, but closely to *R. glauca*. In contrast, characteristics of the spores, especially the proximal ornamentation and the light brown spore color, which is rare in the genus, would suggest a closer relationship of *R. bifurca* to *R. pusilla* subsp. *gothica* than to *R. glauca*. Such patterns seem to be rather common in bryophytes and can result from convergent evolution or stasis (e.g. Medina et al. 2018; Renner 2020; Kiebach and Urmi 2023).

Within *R. glauca* we detect paraphyletic clades in RPB1 and UNM. We proved again the collections and could not find morphological differences or geographic patterns, which could explain this grouping. The results could indicate cryptic speciation within these taxon.

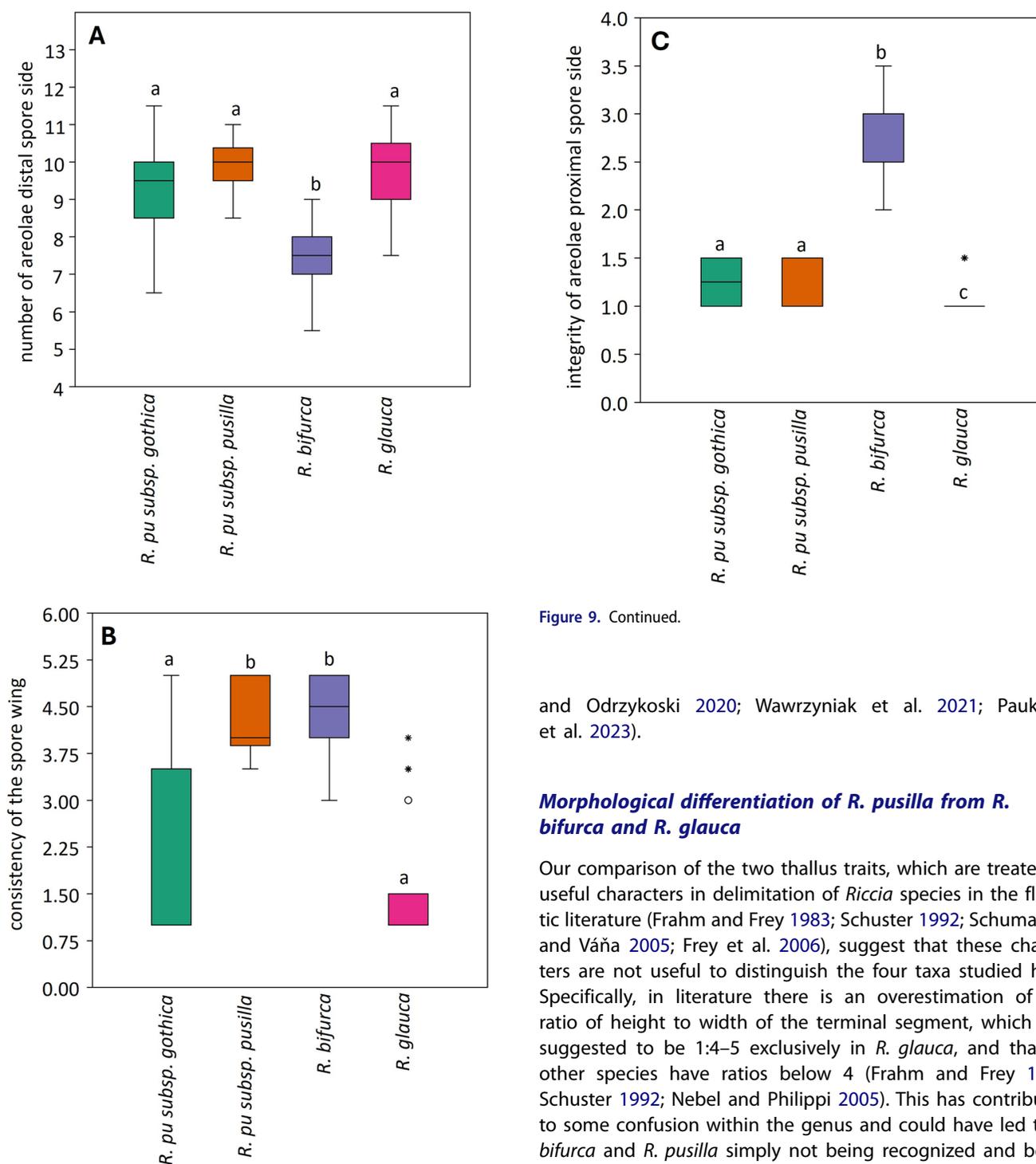


Figure 9. Continued.

and Odrzykoski 2020; Wawrzyniak et al. 2021; Pauksztó et al. 2023).

Morphological differentiation of *R. pusilla* from *R. bifurca* and *R. glauca*

Our comparison of the two thallus traits, which are treated as useful characters in delimitation of *Riccia* species in the floristic literature (Frahm and Frey 1983; Schuster 1992; Schumacker and Váňa 2005; Frey et al. 2006), suggest that these characters are not useful to distinguish the four taxa studied here. Specifically, in literature there is an overestimation of the ratio of height to width of the terminal segment, which was suggested to be 1:4–5 exclusively in *R. glauca*, and that all other species have ratios below 4 (Frahm and Frey 1983; Schuster 1992; Nebel and Philipp 2005). This has contributed to some confusion within the genus and could have led to *R. bifurca* and *R. pusilla* simply not being recognized and being mistaken for *R. glauca*. Purple pigmented parts of thalli, which should be typical for *R. pusilla* subsp. *gothica* (Damsholt and Hallingbäck 1986a), also occur within all other species presented here. For a reliable determination of these four taxa only spore characteristics are useful. A differentiation in the field or determination of sterile material is not possible. The combination of the spore color and the ornamentation of the distal and proximal side serve as diagnostic characteristics as reflected by the presented key. Care must be taken when interpreting the spore color of well-developed but not fully mature spores, which is indicated by a closed capsule (Berg and Pörtl 2020). The mature state of the sporophyte is reached, when the capsule wall is already open and starts to dissolve.

Figure 9. Spore characteristics in *Riccia pusilla* subsp. *gothica* ($n=30$), *R. pusilla* subsp. *pusilla* ($n=12$), *R. bifurca* ($n=33$) and *R. glauca* ($n=32$), (A) number of areolae diagonal on the distal side, (B) consistency of the spore wing, (C) integrity of areolae at the proximal side. The letters a-c indicate groupings with values significantly different from each other ($p \leq 0.05$).

Cargill et al. (2016) also found high genetic variability within some *Riccia* species. Further research considering a comprehensive set of specimens from different habitats and geographical regions is needed to clarify possible taxonomic diversity in the current concept of species like *R. glauca*. Also in other groups within the Marchantiales hints of cryptic speciation exist, e. g. *Aneura*, *Conocephalum* and *Pellia* (Akiyama

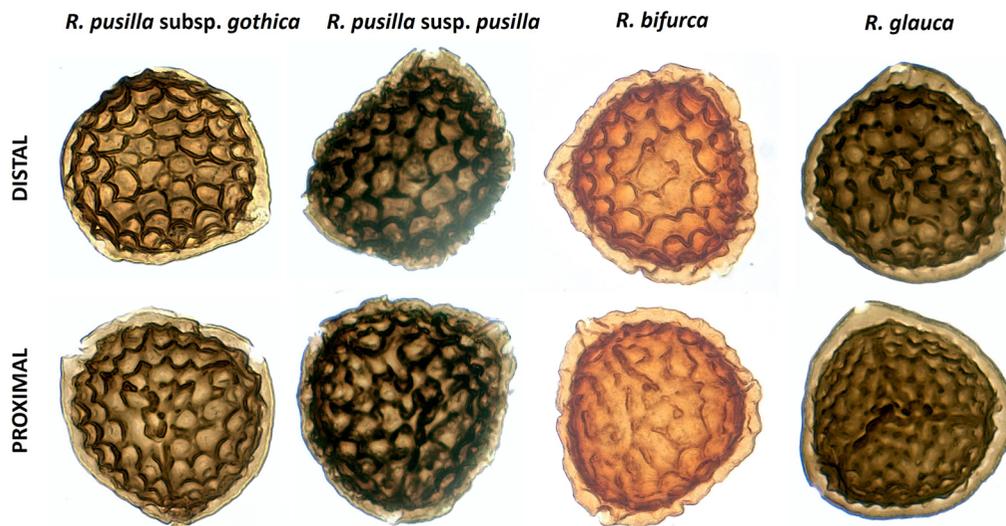


Figure 10. Spores images of *Riccia pusilla* subsp. *gothica* (pg-615), *R. pusilla* subsp. *pusilla* (pp-713), *R. bifurca* (bi-656) and *R. glauca* (gl-855).

Table 4. Overview of the diagnostic spore characters within *R. bifurca*, *R. glauca*, *R. pusilla* subsp. *Pusilla*, and *R. pusilla* subsp. *gothica*.

	<i>R. bifurca</i>	<i>R. glauca</i>	<i>R. pusilla</i> subsp. <i>pusilla</i>	<i>R. pusilla</i> subsp. <i>gothica</i>
Spore color	Light brown, translucent	Dark brown	Dark brown	Light brown, translucent
No areolae diagonally distal	6–8	9–10	8–12	7–12
Proximal ornamentation	Vermiculate, only single complete areolae	Complete areolae, smaller and much denser than distal	Complete areolae, same size or slightly smaller than distal, single walls sometimes broken	Complete areolae, same size as distal, walls sometimes broken
Wing width and consistency	Broad, wavy, strongly notched	Narrow, mostly entire, with smooth edges	Narrow, wavy, roughly notched	Narrow or missing, mostly entire, sometimes notched

Distribution and ecology

So far, *R. pusilla* subsp. *pusilla* is only known from Central Europe, its exact distribution is largely unknown. In this paper we present *R. pusilla* subsp. *gothica* for the first time for several countries in Central Europe, which confirms the idea of Meinunger and Schröder (2007) and Frahm (2008) that the taxon is much more wide spread in Central Europe than assumed (Hodgetts et al. 2020).

Nearly all finds of *R. pusilla* subsp. *pusilla* were made on former floodplains near large rivers, whereas subsp. *gothica* frequently also occurs in periodically dry environments such as arable fields. This suggests that the occurrence of *R. pusilla* s.str. is more strongly linked to constantly humid or wet sites. The taxon is regularly co-occurring with *R. cavernosa* as well as *Aphanorrhagma patens* (Hedw.) Lindb., which also prefer more constantly humid pioneer sites. *Riccia pusilla* subsp. *gothica* got not so high demands on humidity but prefers lime-influenced sites. In Central Europe, it occurs on different open, mostly anthropogenic, moist and calcified sides, like arable fields or embankments in forests. In Northern Europe, where the species is only known from Sweden, where it occurs on costal alvars, which are characterized by steppe-like, shallow alkaline soils over limestones. The actual distribution of both subspecies of *R. pusilla* will only become apparent in the future. *Riccia bifurca* and *R. glauca* mainly occur on arable fields, the latter is found less frequently in other pioneer habitats, which could indicate that the species prefer more nutrient rich sides than *R. bifurca*. Besides, we got the impression

that *R. bifurca* is also an overlooked species in Central Europe, since thallus characteristics are much more similar to those of *R. glauca* than modern literature suggest and spore characteristics are still too strongly neglected. The same applies to *R. pusilla* s. lat. Further revisions are planned, especially in the taxon *R. bifurca* and *R. glauca*, which easily could be confused with one of the two subspecies of *R. pusilla*.

Acknowledgment

We want to thank the curators of the collections G, JE, W, WU, and Z and all colleagues who kindly provided *Riccia* specimens to our project.

Authors' contributions

MP, TK and CB wrote the text, MP photographed and measured the freshly collected material, made LM, generated the dataset, carried out the statistical data analysis. TK choose the molecular method, managed the laboratory and interpreted the genetic results. CB and MP conceived the idea, made LM of spores and spore measurement. TH has taken over the sampling in Northern Europe and contributed his expertise on *R. gothica*, IT did preparatory work for sequencing, FM designed the primers for PCR. VF aligned the sequences and prepared the phylogenetic trees. MP, CB and TK collected samples in Central Europe. All authors revised the text and approved of its final form.

Disclosure statement

No potential conflict of interest was declared by the authors.

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