

Integrative taxonomy substantiates the presence of three *Radula* species in Austria: *Radula complanata*, *R. lindenbergiana*, and *R. visianica* (Porellales, Jungermanniopsida)

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Abstract – A chloroplast DNA phylogeny of *Radula* substantiates the presence of three *Radula* species in Austria, *R. complanata*, *R. lindenbergiana* and *R. visianica*, all members of subg. *Radula*. *Radula visianica* has been observed at a few localities in the Austrian Alps, and is resolved in a sister relationship with a clade comprising *R. complanata*, *R. jonesii*, *R. lindenbergiana* and *R. quadrata*. *Radula visianica* resembles tiny alpine forms of *R. lindenbergiana* but differs in its more narrowly ovate leaf lobes and slightly more elongate leaf lobules. Despite extensive morphological overlap, molecular evidence unambiguously supports separate species status.

Holarctic / liverwort / molecular phylogeny / Radulaceae

INTRODUCTION

Integrative taxonomy aims to integrate evidence from multiple sources to derive robust hypotheses about relationships (Dayrat, 2005; Schlick-Steiner *et al.*, 2010). Combining phylogeny with morphology enables more reflective assessments of true bryophyte diversity than may be achieved from studies focusing solely on morphology (Szweykowski *et al.*, 2005; Bakalin & Vilnet, 2014; Heinrichs *et al.*, 2015). Integrative studies have sometimes questioned the accuracy and utility of the traditional concept of broadly defined bryophyte species with wide, often intercontinental ranges, by repeatedly identifying morphologically similar but genetically clearly distinct local taxa (Heinrichs *et al.*, 2010; Ramaya *et al.*, 2010; Hedenäs *et al.*, 2014).

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A prime example in this regard is the subcosmopolitan leafy liverwort genus *Radula*. This genus includes some 250 species (Yamada, 1986; Söderström *et al.*, 2016) and is notorious for its rather uniform and limited morphology, seen, e.g., in the complete absence of underleaves, predominantly entire-margined leaves as well as exclusively lateral branches, mostly of the terminal “*Radula* type”. Integration of molecular data in taxonomic studies of *Radula* was the basis for a greatly revised supraspecific classification (Devos *et al.*, 2011b). This revealed the presence of morphologically similar species in several independent lineages (Renner, 2015), and that some species had been assigned to the wrong subgenus on the basis of morphology alone. This situation hampers morphology-based reconstructions of relationships and species circumscriptions. Perhaps unsurprisingly, given the uncomplicated plant morphology and scarcity of qualitative characters, integrative studies of *Radula* species have demonstrated numerous incongruences between morphology-based classifications and molecular topologies, and resulted in comprehensive revisions of species circumscriptions and hypotheses on range formation (Patiño *et al.*, 2013, 2017; Renner *et al.*, 2013a, 2013b; Renner, 2014).

Only two species of *Radula* are considered to occur in Austria, the generitype *R. complanata* and *R. lindenberiana*, a morphologically closely related but dioicous rather than parioicous species (Paton, 1999; Devos *et al.*, 2011a). Recently, a tiny *Radula* was observed by H. Köckinger (*in lit.*) at several localities in the Austrian Alps. This plant resembled some alpine forms of *R. lindenberiana* but seemed to differ by its sometimes long drawn out leaf lobule apex and more narrowly ovate leaf lobe. Subsequent literature studies and revision of type material pointed to similarities with *R. visianica*, an apparently extinct species that was described by Massalongo (1904) based on specimens collected in Northern Italy.

Here we present the results of a study of the alpine *Radula* based on morphological and chloroplast DNA sequence evidence. We substantiate the existence of a third *Radula* species in Austria and identify it as *R. visianica*.

MATERIAL AND METHODS

Morphological investigation

Several accessions of a small-sized *Radula* morphotype were collected in the Austrian Alps by H. Köckinger (see Results). These plants did not fully match the morphology of the two common Austrian *Radula* species *R. complanata* and *R. lindenberiana* but resembled the presumed extinct *Radula visianica*, previously known only from two collections from Northern Italy. Isotype material of *R. visianica* was borrowed from the herbaria FH and S, and compared with the Austrian specimens. A loan request to VER, where the holotype is housed, remained unanswered.

Taxa studied in molecular analyses, DNA extraction, PCR amplification and sequencing

Gametophytical plant tissue was isolated from dried herbarium specimens of *Radula complanata* (one accession), *R. lindenberiana* (three accessions) and putative *R. visianica* (three accessions) (Table 1). Total genomic DNA was isolated

Table 1. Taxa used in the present study, including information about the origin of the studied material, voucher information, as well as GenBank accession numbers. New sequences in bold face

<i>Taxon</i>	<i>Voucher</i>	<i>Accession Number</i>
<i>Radula appressa</i> Mitt.	Réunion, Arts R U18/16 (BR)	KF187293
<i>Radula appressa</i>	Madagascar, Pócs 90113/AH (EGR)	HM992465
<i>Radula appressa</i>	Malawi, Hodgetts M2222a	KF187214
<i>Radula australis</i> Austin	U.S.A. (I), Shaw s.n. (DUKE)	KF187286
<i>Radula australis</i>	U.S.A. (II), N.N. 6091 (DUKE)	KF187274
<i>Radula australis</i>	U.S.A. (III), Shaw 6089 (DUKE)	HM992477
<i>Radula carringtonii</i> J.B.Jack	Madeira (I), DV s.n.	KF187264
<i>Radula carringtonii</i>	Madeira (II), DV s.n.	KF187262
<i>Radula carringtonii</i>	Madeira (III), Schäfer-Verwimp & Verwimp 25638 (hb SV)	KF187166
<i>Radula carringtonii</i>	Tenerife, Devos ND061007_2B	KF187235
<i>Radula complanata</i> (L.) Dumort.	Alaska, Shaw F960/7 (DUKE)	KF187282
<i>Radula complanata</i>	China, Whittimore 4532 (H3196609)	KF187157
<i>Radula complanata</i>	Germany (I), Schäfer-Verwimp 35477 (M)	KY271816
<i>Radula complanata</i>	Germany (II), Vanderpoorten 3403 (LG)	KF187207
<i>Radula complanata</i>	Slovakia, Schäfer-Verwimp & Verwimp 21315 (hb SV)	KF187169
<i>Radula evelynae</i> K.Yamada	Comoros, Pócs et al. 9288/R (EGR)	HM992468
<i>Radula fendleri</i> Gottsche	Ecuador, Schäfer-Verwimp & Preussing 23250 (hb SV)	HM992424
<i>Radula formosa</i> (C.F.W.Meissn. ex Spreng.) Nees	Fiji Isls. (I), Pócs s.n. (EGR)	HM992471
<i>Radula formosa</i>	Fiji Isls. (II), N.n. 03279/A	KF187225
<i>Radula grandis</i> Steph.	New Zealand (I), Glenny CHR571846 (CHR)	HM992457
<i>Radula grandis</i>	New Zealand (II), Renner AK286379 (AK)	KF187193
<i>Radula hicksiae</i> K.Yamada	Australia, Curnow & Streimann 3689 (CBG)	HM992443
<i>Radula iwatsukii</i> K.Yamada	Malaysia, Schaefer-Verwimp & Verwimp 18757/A (hb SV)	HM992426
<i>Radula japonica</i> Gottsche	Japan, Higuchi 1198 (BR)	HM992481
<i>Radula javanica</i> Gottsche	Bolivia, Churchill et al. 22187 (MO)	HM992448
<i>Radula javanica</i>	Fiji Isls. (1), Renner NSW889523 (NSW)	KF440506
<i>Radula javanica</i>	Fiji Isls. (2), Renner NSW889520 (NSW)	NSW889520
<i>Radula jonesii</i> Bouman, Dirkse & K.Yamada	Tenerife I, Devos ND061007_7	KF187251
<i>Radula jonesii</i>	Tenerife II, Devos ND061007_7	KF187253
<i>Radula jonesii</i>	Tenerife III, Devos ND061007_5	KF187248
<i>Radula lindenbergiana</i> Gottsche ex C.Hartm.	Austria (I), Styria, 1950 m, Köckinger 15003 (M)	KY271815

<i>Taxon</i>	<i>Voucher</i>	<i>Accession Number</i>
<i>Radula lindenbergiana</i>	Austria (II), Styria, 1650 m, Köckinger 15004 (M)	KY271814
<i>Radula lindenbergiana</i>	Austria (III), Styria, 1400 m, Köckinger 15005 (M)	KY271813
<i>Radula lindenbergiana</i>	Caucasus, Konstantinova k525/5-07 (KBAI)	GU737752
<i>Radula lindenbergiana</i>	Azores, Vanderpoorten <i>et al.</i> 11/15(LG)	GU737765
<i>Radula lindenbergiana</i>	Gran Canaria, Vanderpoorten GC12 (LG)	GU737770
<i>Radula lindenbergiana</i>	Turkey, Papp, B 48190/h (BP)	GU737760
<i>Radula macrostachya</i> Lindenb. & Gottsche	Costa Rica, Gradstein & Dauphin DB12894 (GOET)	HM992404
<i>Radula macrostachya</i>	Suriname, Muñoz 98-21 (DB12900)	KF187167
<i>Radula madagascariensis</i> Gottsche	Australia (I), Renner <i>et al.</i> NSW896938 (NSW)	KF440520
<i>Radula madagascariensis</i>	Australia (II), Kilgour s.n. (NSW)	KF440483
<i>Radula madagascariensis</i>	Madagascar, Szabo 9614/DV (EGR)	HM992466
<i>Radula marojezica</i> E.W.Jones	Madagascar, Pócs 90103/AE (EGR)	HM992467
<i>Radula multiflora</i> Gottsche <i>ex</i> Schiffn.	French Polynesia, Wood NY9604 (NY)	HM992453
<i>Radula neotropica</i> Castle	Honduras, Allen NY11935 (NY)	HM992452
<i>Radula obconica</i> Sull.	U.S.A. (1), Shaw 4874 (DUKE)	HM992446
<i>Radula obconica</i>	U.S.A. (2), Shaw 5829 (DUKE)	KF187289
<i>Radula physoloba</i> Mont.	New Zealand (I), Schäfer-Verwimp & Verwimp 14303 (hb SV)	KF187183
<i>Radula physoloba</i>	New Zealand (II), Schäfer-Verwimp & Verwimp 13776 (hb SV)	KF187172
<i>Radula polyclada</i> A.Evans	Alaska, Shaw F956 (DUKE)	HM992472
<i>Radula prolifera</i> Arnell	Alaska (I), Schofield 115792 (DUKE)	HM992445
<i>Radula prolifera</i>	Alaska (II), Schofield 109112	KF187203
<i>Radula quadrata</i> Gottsche	Kenya, Pócs <i>et al.</i> 9230/S (EGR)	HM992462
<i>Radula queenslandica</i> K.Yamada	Australia, Curnow 3846 (CBG)	HM992441
<i>Radula reflexa</i> Nees & Mont.	Fiji Islands, Pócs s.n. (EGR)	HM992460
<i>Radula varilobula</i> Castle	Dominica, Hill NY21274 (NY)	HM992454
<i>Radula visianica</i> C.Massal.	Austria (I), Köckinger 14993 (M)	KY271818
<i>Radula visianica</i>	Austria (II), Köckinger 14995 (M)	KY271819
<i>Radula visianica</i>	Austria (III), Köckinger 14979 (M)	KY271817
<i>Radula voluta</i> Taylor	Tanzania, Pócs <i>et al.</i> 88123/B (EGR)	KF187215
<i>Radula wichurae</i> Steph.	Madeira, Schäfer-Verwimp & Verwimp 26018 (hb SV)	HM992419

using the Invisorb Spin Plant Mini Kit (Stratec Molecular GmbH, Berlin, Germany) and the chloroplast DNA marker *trnL-trnF* was chosen for subsequent phylogenetic analyses. This marker has already been used in several molecular studies of *Radula* (Devos *et al.*, 2011a; Patiño *et al.*, 2013; Renner *et al.*, 2013a, 2013b; Renner, 2014) and the corresponding sequences are available from Genbank (<http://www.ncbi.nlm.nih.gov/genbank/>). Amplification of the *trnL-trnF* region was carried out with 0.4 μ L MyTaq DNA Polymerase (Bioline Reagents Ltd., UK), 11 μ L reaction buffer, 1 μ L of upstream primer (10 μ M), 1 μ L of downstream primer (10 μ M), and 1 μ L of template DNA. This mix was filled up to a total volume of 50 μ L with double-distilled water. The primer pair *trnL*lejF and *trnL/trnF*-R was employed and the PCR program as described in Gradstein *et al.* (2006). Bidirectional sequences were generated by an ABI 3730 capillary sequencer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing primers were the same as used for the PCR. Newly generated sequences were assembled and edited with PhyDE v.0.9971 (<http://www.phyde.de/index.html>). First, the newly generated sequences were compared with GenBank sequences using the BLASTN program (Altschul *et al.*, 1990). The BLAST searches indicated an affiliation of all sequences to *Radula* subg. *Radula* (data not shown). Based on the phylogenies presented by Devos *et al.* (2011a, b), accessions of *Radula* subg. *Radula* and its sister lineage *R.* subg. *Amentuloradula* were selected to form the ingroup. Two representatives of *Radula* subg. *Volutoradula* were chosen as outgroup.

Phylogenetic analyses

All sequences were aligned manually in Bioedit version 7.0.5.2 (Hall, 1999) and lacking parts of sequences marked as missing. Maximum likelihood (ML) analyses were conducted with RAxML version 8 (Stamatakis, 2006, 2014) with the extended majority rule bootstrapping criterion (Pattengale *et al.*, 2010). jModelTest version 2 (Darriba *et al.*, 2012) was used to determine the appropriate DNA substitution model, rate of invariable sites and gamma rate heterogeneity according to the Akaike information criterion (AIC; Akaike, 1973). The analysis resulted in a TPM1uf+G model. This model was not available in RAxML. Hence, the best-fitting overparameterized model, GTR+G, was employed following the suggestion of Posada (2008). ML bootstrap values (BV) of each node were visualized using FigTree 1.4 (<http://beast.bio.ed.ac.uk/figtree>). Bootstrap percentage values (BPV) $\geq 70\%$ were regarded as good support (Hillis & Bull, 1993).

RESULTS

Molecular investigation

The ingroup splits into two main clades corresponding to *Radula* subg. *Amentuloradula* (BPV = 100) and *R.* subg. *Radula* (BPV = 80). Three accessions identified as *Radula visianica* form a monophyletic lineage (BPV = 99) within one of the robust subclades (BPV = 95) of *Radula* subg. *Radula*. They are placed sister to a clade (BPV = 84) with five accessions of *R. complanata*, two accessions of *R. jonesii*, seven accessions of *R. lindenberghiana* (including three Austrian accessions with small-sized, alpine forms), and a single accession of *R. quadrata*. *Radula*

complanata, *R. jonesii* and *R. lindenberghiana* are polyphyletic on the basis of specimen determinations; a third accession identified as *R. jonesii* clusters with *R. obconica* in another subclade of *R.* subg. *Radula*. Several *Radula* species with multiple accessions are monophyletic (e.g., *R. australis*, *R. carringtonii*, *R. grandis*); others are para- or polyphyletic (e.g., *R. javanica*, *R. madagascariensis*, *R. prolifera*).

***Radula visianica* specimens examined**

Radula visianica C.Massal., *Annali di botanica* 1(4): 2. 1904. Type: Italy, “prov. di Padova”, al mt. Sengiari sopra Torreglia, non lungi dal luogo dove trovavasi la villa che un giorno possedeva il defunto professore R. de Visiani; 23 febbraio 1878, coll. C. Massalongo [holotype, VER (not seen), isotypes FH!, S!]
 Further specimens examined: **Austria. Carinthia:** Karawanken Mts.: Uschowa SE of Bad Eisenkappel, 1200 m, 2015, *H. Köckinger 14995* [M, priv. herb. Váňa]; **Styria:** Eisenerzer Alpen: Wildfeld, Hóchststein, 1680 m, 2015, *H. Köckinger 14993* [M]; Hóchststein N of Aflenzer Bürgeralm, 1700 m, 2014, *H. Köckinger 14979* [M]. For a description and figures see Massalongo (1904), Müller (1951-1958) and Castle (1964).

DISCUSSION

The molecular investigation provides convincing evidence for the presence of a third *Radula* species in the Austrian Alps (Fig. 1). *Radula visianica* is related to the other two Austrian *Radula* species – *R. complanata* and *R. lindenberghiana* – but clearly separated from the *R. complanata-lindenberghiana*-clade. Presence of a Kenyan accession of *R. quadrata* in the *R. complanata-lindenberghiana*-clade further substantiates the specific status of *R. visianica*.

Radula visianica shows a remarkably wide morphological variations which may in part be related to habitat differences. The two investigated isotypes each consist of several isolated shoots. The FH-material was separated from the holotype by Victor Schiffner. It is more scio-hygromorphic (cf. Massalongo, 1904: 298, Figs 1-3 and Castle, 1964: 196, Fig. 5) than the shoots present in the Karl Müller herbarium in S (cf. Müller, 1951-1958: 1198, Fig. 464). Possibly, the type material originated from several patches that may have grown under slightly different conditions. Leaves of the shoots in FH have a narrowly ovate outline and are more patent than those of the S isotype with more triangular and more distinctly forward pointing leaves. Corresponding to the narrower lobes, the lobules of the FH material are also narrower (and smaller in relation to the dorsal lobes) than those of the S-isotype. Only the FH isotype includes a few shoots with discoid gemmae on some distal lobe margins. Gemmae are extremely rare in the Austrian high-altitude plants. A remarkable feature shared by both isotypes and the Austrian collections are the distinctly spreading and almost flat dorsal lobes, also observed by Castle (1964). The Austrian material originates from higher altitudes than the type material and predominantly consists of smaller phenotypes (shoots about 0.3-1.0 mm wide, those of the type material up to 1.5 mm wide). The leaves in the largest shoots of the Austrian specimens have sickle-shaped, rather flat lobules and in these features agree with those depicted by Müller (1951-1958; Fig. 464 b, c) and Massalongo (1904, Fig. 1); however, the small-sized phenotypes may differ considerably

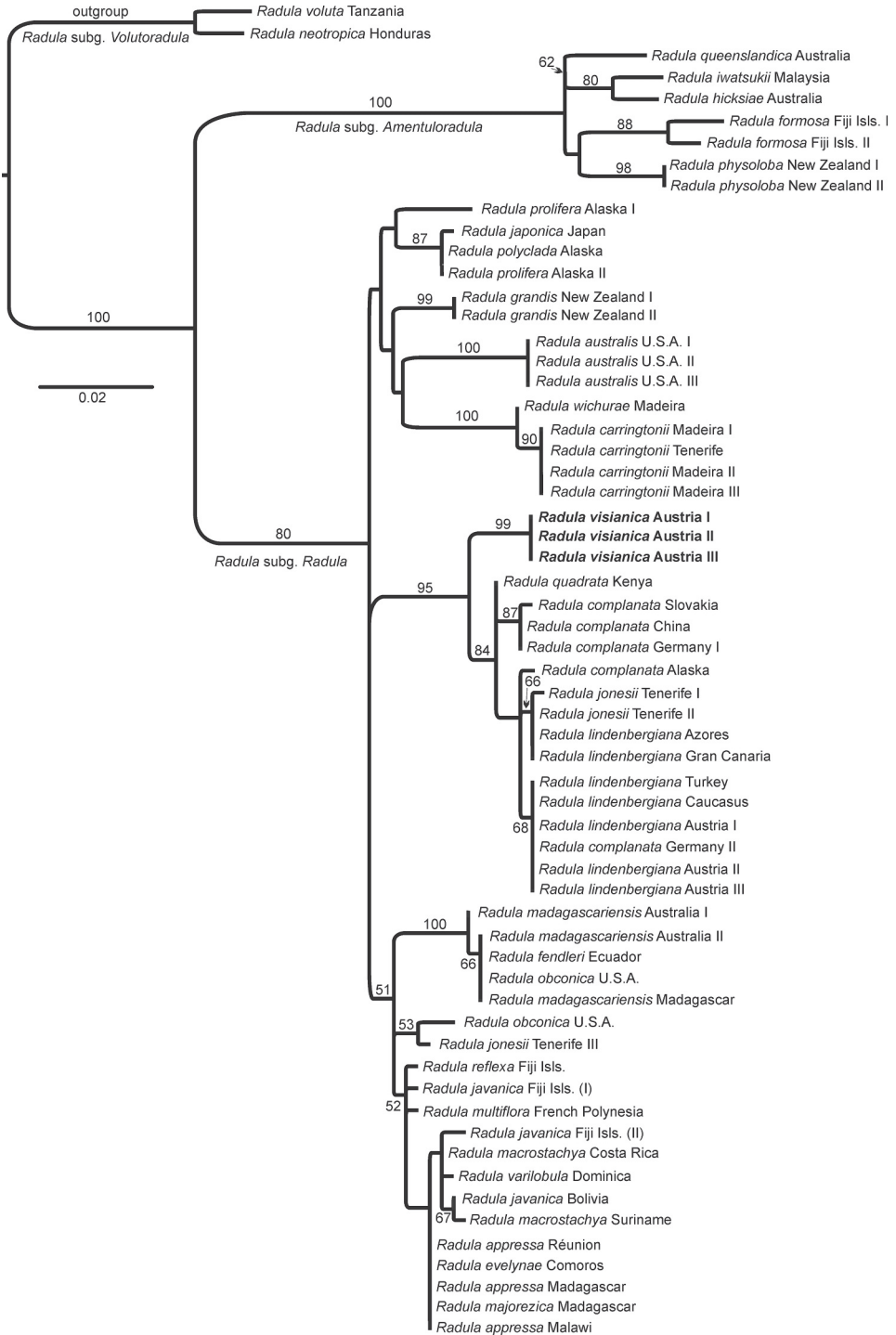
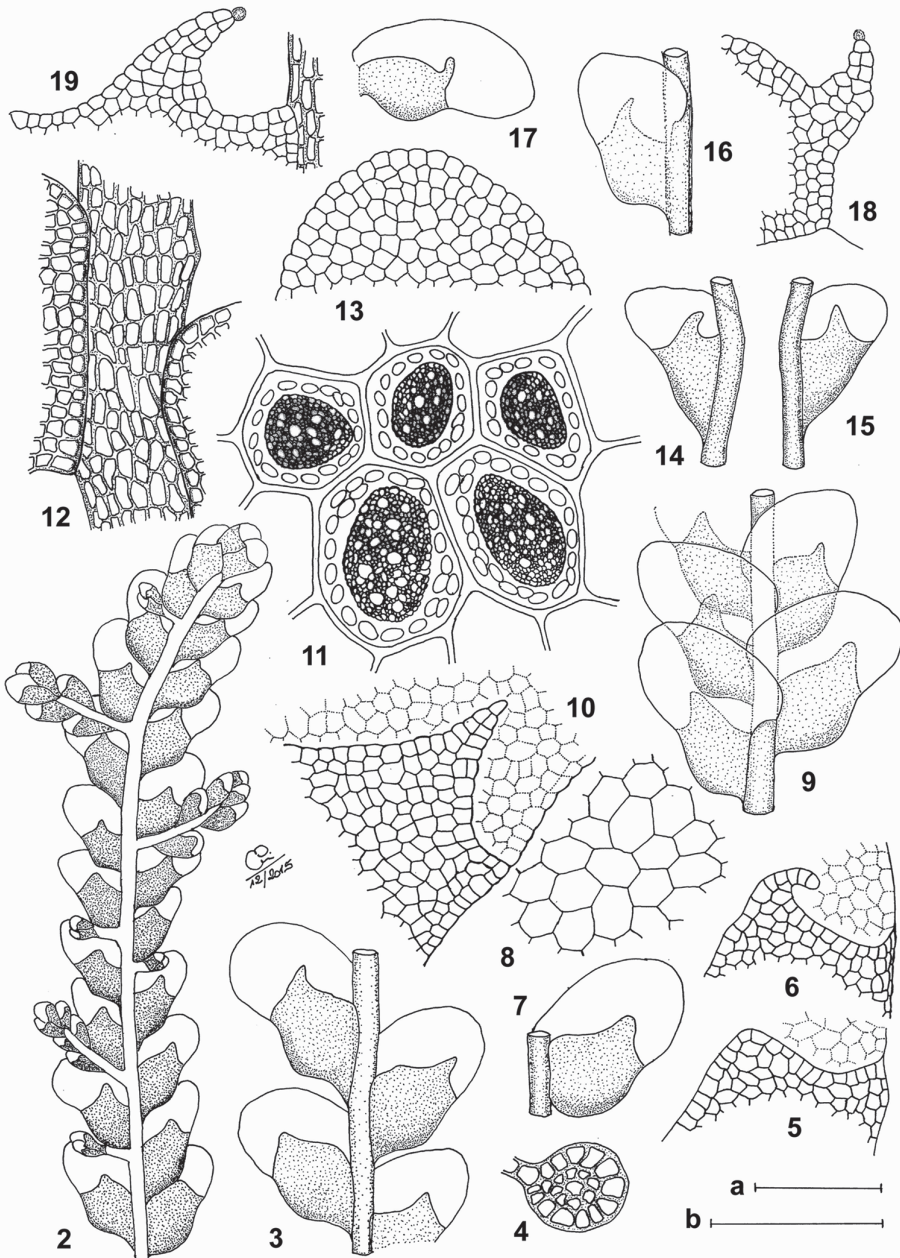


Fig. 1. A most likely phylogram resulting from maximum likelihood analysis of aligned *trnL-trnF* sequences of *Radula*. Bootstrap percentage values > 50 are indicated at branches.



Figs 2-19. *Radula visianica* C. Massal. 2. upper portion of shoot in ventral view. 3. portion of plant from median shoot sector in ventral view. 4. cross section of stem. 5-6. upper sectors of leaf lobules. 7. leaf in ventral view. 8. basal leaf cells. 9. shoot sector in dorsal view. 10. upper sector of leaf lobule. 11. leaf cells with oil bodies and chloroplasts. 12. portion of ventral stem surface. 13. leaf lobe apex. 14-17. leaves with elongated lobule apex. 18-19. apices of leaf lobules [2, 3, 5, 6, 9-12 from *HK 14995*; 4, 7, 8, 13 from *HK 14979*; 14-19 from *HK 14979*; scales: a = 730 μm for 2; 100 μm for 4, 5, 6, 10, 12, 13, 18, 19; 50 μm for 8; b = 500 μm for 3, 7, 9, 14, 15, 16, 17; 20 μm for 11].

(Figs 2-19). Here, the basal portion of the lobule may be inflated and the keel with the lobe may be strongly curved. The lobules of such forms are not much longer than wide and the apex is often bluntly acute, blunt or even rounded (Figs 2, 3, 7, 9, 13). Such forms may also include distinctly saccate lobules with the uppermost sector of the keel forming a straight line. However, even the most tiny phenotypes include at least single leaves with finely extended, often curved lobule apices (Fig. 6) terminating in an uniseriate tip, sometimes ending in a slime papilla (Figs 18, 19). The minute alpine forms of *R. visianica* (Figs 2-19) differ in size (and shape of leaf lobe) from “typical” *R. complanata* and *R. lindenberghiana*; however, some alpine forms of *R. lindenberghiana* approach the size of the *R. visianica* DNA vouchers and also tend to produce some slightly elongated lobule apices. We included three such phenotypes in our molecular investigation and resolved them in a clade with *R. lindenberghiana* accessions from the Caucasus and Turkey. Our findings provide some evidence that plant size is not a good character for separating *Radula* species. Similarly to the situation in *R. lindenberghiana*, *R. visianica* may be able to produce larger gametophytes, especially when it grows in less harsh environments than in the Austrian Limestone Alps. The somewhat larger size of the Northern Italian type material of *R. visianica* indicates a similar size variation than substantiated for *R. lindenberghiana*; however, the *R. visianica* type material is not available for sequencing.

All Austrian specimens of *R. visianica* were collected at altitudes above 1000 m and grew on rock, not on bark. Due to their small size, they resemble *Lejeunea cavifolia* rather than a species of *Radula*, and may thus be easily overlooked. A detailed description of the ecology and habitat of *R. visianica* was published by Köckinger, 2016.

Perspectives

Currently it is unclear if *Radula visianica* is a local endemic or a more widespread species that has not yet been recognized as such. Our study adds to growing evidence that species diversity of *Radula* remains poorly known, despite much morphology-based taxonomic work (e.g., Castle, 1936, 1967; Yamada, 1979). Evident problems with the current species classification, knowledge, and standards of identification are reflected in the poly- or paraphyly of several binomials. Only an extension of the sampling and critical revision of published DNA vouchers will allow to solve the evident taxonomic problems and to arrive at reliable estimates of global species diversity of *Radula*, and of liverworts generally (Renner *et al.*, 2013a, b; Bechteler *et al.*, 2017).

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