




Standard Paper

Ramalina arsenii, an additional new species in the *R. pollinaria* group in Western Europe

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Abstract

Ramalina arsenii sp. nov. belongs to the *R. pollinaria* group and is easily recognized by its ITS barcode and several micro-morphological characters that are diagnostic in a European context: small size, less than 3 cm long; soralia developing on the underside of lobe apices; absence of excavate depressions on the lower side. Its ecological niche (i.e. rock outcrops and especially underhangs of slightly calcareous rocks, at low and mid altitudes) is also unique. *Ramalina arsenii* is frequent and locally abundant in France (Alps, Cantal) and Switzerland (western Alps), and is also known from Germany and the Spanish side of the Pyrenees.

Key words: evernic acid, ITS barcodes, lichen, *Ramalina carminae*, taxonomy

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Introduction

Enormous progress has recently been made in the evolutionary and taxonomic knowledge of the genus *Ramalina*, with the work of Pérez-Ortega *et al.* (2019), LaGreca *et al.* (2020) and Spjut *et al.* (2020). The preliminary evolutionary tree of Sérusiaux *et al.* (2010) has been broadly confirmed, and the generic boundaries within the fruticose *Ramalinaceae* are now well established (Spjut *et al.* 2020): three genera endemic to coastal deserts benefiting from regular fog along the Pacific coasts in the New World (*Niebla* and *Vermilacinia*) and the Atlantic coasts of SW Africa (*Namibialina* and a single *Vermilacinia* species shared with the New World), and a globally distributed genus with a much larger ecological amplitude (*Ramalina*).

Numerous works recently published for many well-established genera of macrolichens, based on easily accessible molecular data and being processed in an evolutionary context, has led to the recognition of more species than would be supported by data based only on morphological and chemical characters (production of secondary metabolites in the medulla). Examples can be found with *Cora* (Lücking *et al.* 2014), *Dendricosticta* (Simon *et al.* 2021), *Peltigera* (N. Magain *et al.*, unpublished data), *Usnea* (Truong & Clerc 2016) and *Vulpicidia* (Saag *et al.* 2014). However, problems may remain (Miadlikowska *et al.* 2018) and the merging of previously widely accepted species may also be supported by molecular and statistical evidence (*Bryoria*: Boluda *et al.* 2019).

Within the genus *Ramalina*, the delimitation of species as proposed by reference literature for geographic areas (Cannon *et al.*

(2021) for Great Britain and Ireland; Stenroos *et al.* (2016) for Finland) is either supported (or almost so) or requires major revision. An example of the first case can be found with LaGreca *et al.* (2020) who confirmed that only two species can be recognized within the *R. siliquosa* group, a chemically variable assemblage of saxicolous lichens in coastal areas of Western Europe, as previously demonstrated by Sheard (1978) based on morphological and chemical data. However, populations of the same species complex from East Asia are shown to belong to a distinct species. An example of the second case is the *R. bourgeana* clade in Macaronesia (Pérez-Ortega *et al.* 2019) which is expected to include many more species than the three recognized by Krog & Østhagen (1980), *R. bourgeana*, *R. cupularis* and *R. crispatula*.

A further example of a highly variable group that could be resolved into several species by assembling detailed morphological data and DNA-barcoding is the *Ramalina pollinaria* complex (Gasparyan *et al.* 2017). Indeed, three species are currently recognized: *R. labiosorediata* restricted to North America (Canada and USA) and two species found in Europe including the Caucasus region, *R. europaea* and *R. pollinaria*.

In the study of Gasparyan *et al.* (2017), one accession was left as '*R. aff. pollinaria* Switzerland GU827324' in an unresolved phylogenetic position. This material was included in Sérusiaux *et al.* (2010) as *R. pollinaria*, collected in Switzerland by P. van den Boom (#41227, hb. van den Boom, LG). It represents a further species in the *R. pollinaria* complex, known from France, Germany, Spain and Switzerland, that can be distinguished by its distinct ITS barcode and its ecological niche (underhangs of slightly calcareous rock outcrops at mid elevations in montane areas). As no epithet is available, it is described as new in this paper. In addition, ITS barcodes of our own records of *R. europaea* were assessed; thus the distribution of this species is expanded to further countries, including Belgium, Iran, Norway and Romania.

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Material and Methods

Specimens held in LG and the private herbarium of P. van den Boom were examined with a Wild M5A stereoscopic microscope (Heerbrugg, Switzerland). All four species dealt with in this paper and forming the so-called *pollinaria*-group have been studied (*c.* 60 specimens examined).

Genomic DNA was isolated following the protocol of Cubero *et al.* (1999). The nuclear internal transcribed spacer region (ITS) was amplified via polymerase chain reaction (PCR) for 18 specimens from available recent collections, using the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990).

PCR products that yielded high quality bands in agarose gel electrophoresis were purified using ExoSAP (Affymetrix, Santa Clara, California, USA). Amplicons were sequenced by Macrogen Inc. (Seoul, South Korea) using the amplification primers. Sequences were edited using Sequencher v. 4.9 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and subjected to MegaBLAST searches (Wheeler *et al.* 2007) in GenBank to detect potential contamination. Accessions of the three species recognized in the *R. pollinaria* group by Gasparyan *et al.* (2017) were assembled, together with our own collections of *R. europaea* and those assumed to represent a further species. Alignment was performed using MAFFT run in auto mode (Kato *et al.* 2009) and checked manually. All sequences newly generated for this study were deposited in GenBank (Table 1).

An initial ITS data matrix was generated with accessions retrieved from Gasparyan *et al.* (2017): three accessions of *Ramalina labiosorediata* including the type collection, four of *R. pollinaria* and two of *R. europaea* including the type collection. Our own accessions included 10 of *R. europaea* and eight of the putative new species. GenBank Accession numbers for all samples used in our analyses are included in Fig. 1A.

A maximum likelihood analysis (ML) was run with RAxML v. 8.0.0 (Stamatakis 2006) on this data matrix. Optimal tree and bootstrap searches were conducted with the rapid hill-climbing algorithm for 1000 replicates using the GTRCAT substitution model.

To analyze the genealogical relationships among the haplotypes, we constructed a haplotype network for all four species. This network was calculated using the program TCS v. 1.21 (Clement *et al.* 2000), which implements the statistical parsimony estimation as described by Templeton *et al.* (1992). The connection limit of the networks was fixed to 90% of the positions and gaps were treated as a fifth character state.

An additional ML analysis was performed with a more extensive sampling of *Ramalina* species, using the ITS subset of the data matrix build for the evolutionary tree of the genera *Namibialina* and *Ramalina* (fig. 9 of Spjut *et al.* (2020)). It includes three accessions of *R. labiosorediata*, four of *R. pollinaria*, eight of *R. europaea* and seven of the newly described *R. arsenii*. In total 130 accessions were included, with nine belonging to the genus *Namibialina*.

Thin-layer chromatography (TLC) was carried out following Orange *et al.* (2010) using solvents C and G for all new accessions of *R. europaea* and for *R. arsenii*.

Results

The ML and TCS analyses based on the first data matrix included 479 characters, of which 453 are constant, 3 are variable parsimony-uninformative and 23 parsimony-informative. All four expected taxa are retrieved with strong support (BS varying from 98 to 100); all other branches were not supported, except the branch supporting the four species, all producing evernic acid. All new accessions of both *R. europaea* and the newly

described *R. arsenii* produce evernic acid in their medulla. The evolutionary tree and the haplotype network are presented in Fig. 1A & B.

The ML and TCS analyses based on the second data matrix included 446 characters, of which 257 are constant, 49 variable parsimony-uninformative and 140 parsimony-informative. The branch comprising all four species here referred to the *pollinaria* group is not supported. This evolutionary tree is presented in Supplementary Material Fig. S1 (available online).

The haplotype of the single accession ‘aff. *pollinaria*’ from Switzerland (Gasparyan *et al.* 2017) is shared by other collections from the Alps (Switzerland and France), the ‘Massif Central’ (France) and the Pyrenees (Spain), and is also known at lower elevations in Germany. It is described as a new species that can be recognized by subtle morphological characters (see description and identification key). Furthermore, it is easily recognized by its ITS barcode, differing from the other three species by at least 7 substitutions in the haplotype network. We identify a 16 bp segment in ITS2 that provides a quick and accurate way for the recognition of all four species:

<i>R. europaea</i>	GGGT C CTTCGCCCGGG AGAT CCC
<i>R. labiosorediata</i>	GGGT C CTTCGCCCGGG ATAC CCC
<i>R. pollinaria</i>	GGGT T CTTCGCCCGGG AGAT CCC
<i>R. arsenii</i> sp. nov.	GGGT C CTTCGCCCGGG ATAT CCC

Ramalina arsenii is the only species with detectable variation in the ITS: one accession with a single substitution in ITS1, and an additional one plus an inversion in ITS2.

Taxonomy

Ramalina arsenii Sérusiaux, van den Boom & Magain sp. nov.

Mycobank No.: MB840281

In a European context, distinguished from other sorediate species producing evernic acid in their medulla by its ecological preference for slightly calcareous rocks, best developed in underhangs, rather small thalli (not exceeding 3 cm long), with soralia developing mainly on the underside of lobe apices.

Type: Switzerland, Valais canton, Saastal, near Saas-Grund, slightly calcareous rocks at edge of coniferous forest, 1680 m, 46°08.13'N, 007°56.19'E, August 2018, *E. Sérusiaux* s. n. (LG DNA 6389)(G—holotype; LG—isotype). GenBank Accession no.: MW892987.

(Fig. 2A–E)

Thallus saxicolous, fruticose, pale yellowish green to green; *branches* erect and ascending, or pendulous, especially for old individuals growing in dark underhangs, 1–2 cm long, rarely longer but specimens up to 3 cm long seen, starting as erect cushions of dichotomously divided and applanate lobes, with younger parts palmately or dichotomously branching; narrow longitudinal cortical pseudocyphellae seen on tiny branchlets, rarely opening with margins producing tiny verrucae; *soralia* first forming on dissolving, old lateral or terminal flattened branches, and eventually getting larger and expanding mainly on underside and/or at upper parts of lateral branches; in such cases, a soredioid underside may be extensive; smaller branches may not have soralia but produce delicate phylloid flattened lobes that can be a dominant feature of some thalli; old lobes may develop a helmet-shaped apex.

Table 1. Voucher information and GenBank Accession numbers for ITS sequences newly generated for *Ramalina arsenii* and *R. europaea* included in this study.

Species	Country, regional origin, year of collection, collection data, herbarium	GB Accessions
<i>Ramalina arsenii</i>	Switzerland, Valais, 2018, <i>E. Sérusiaux</i> DNA 6388, LG TYPE	MW892987
<i>R. arsenii</i>	Switzerland, Valais, 2018, <i>E. Sérusiaux</i> DNA 6389, LG	MW892988
<i>R. arsenii</i>	Germany, Rhineland-Palatinate, 2011, <i>E. Sérusiaux</i> DNA 2694, LG	MW892989
<i>R. arsenii</i>	France, Savoie, 2009, <i>E. Sérusiaux</i> DNA 827, LG	MW892990
<i>R. arsenii</i>	France, Cantal, 2020, <i>E. Sérusiaux</i> DNA 7475, LG	MW892991
<i>R. arsenii</i>	France, Cantal, 2020, <i>E. Sérusiaux</i> DNA 7477, LG	MW892992
<i>R. arsenii</i>	Spain, Catalonia, 2019, <i>P. van den Boom</i> DNA 7506, hb. van den Boom, LG	MW892993
<i>R. europaea</i>	Iran, Golestan, 2007, <i>H. Sipman</i> DNA 844, B	MW892977
<i>R. europaea</i>	Armenia, Syunik, 2011, <i>E. Sérusiaux</i> DNA 2670, LG	MW892978
<i>R. europaea</i>	Norway, Hedmark Ringsaker, 2008, <i>B. Anders</i> DNA 2776, O	MW892979
<i>R. europaea</i>	Romania, PN Piatra Craiului, 2015, <i>E. Sérusiaux</i> DNA 4352, LG	MW892980
<i>R. europaea</i>	Romania, PN Apuseni, 2015, <i>E. Sérusiaux</i> DNA 4354, LG	MW892981
<i>R. europaea</i>	Romania, PN Retezat, 2015, <i>E. Sérusiaux</i> DNA 4357, LG	MW892982
<i>R. europaea</i>	Romania, PN Retezat, 2015, <i>E. Sérusiaux</i> DNA 4361, LG	MW892983
<i>R. europaea</i>	Belgium, Namur, 2008, <i>E. Sérusiaux</i> DNA 539, LG	MW892984
<i>R. europaea</i>	France, Vaucluse, 2010, <i>M. Bertrand</i> DNA 2963, LG	MW892985
<i>R. europaea</i>	France, Savoie, 2009, <i>E. Sérusiaux</i> DNA 826, LG	MW892986

Apothecia very rare, found only once and only immature apothecia seen, with urceolate and pruinose discs < 0.2 mm diam.; *ascospores* not seen.

Chemistry. Usnic acid in the cortex and evernic acid in the medulla.

Etymology. This new species is named after our distinguished colleague Dr Arsen Gasparyan, who revised the taxonomy of the *Ramalina pollinaria* group (Gasparyan *et al.* 2017); he also published an interesting account of epiphytic lichens in Armenia (Gasparyan & Sipman 2016).

Selected specimens examined (TLC performed for all; DNA extracted and ITS sequence produced). *Ramalina arsenii*. **Switzerland:** same locality as type, *E. Sérusiaux* s. n. (LG DNA 6388; not included in the ML and TCS analyses). W of Chur, S of Flims Waldhaus, Safiental, N of Thalkirch, 46°39.33'N, 009°

17.47'E, 1745 m, vertical N-exposed rock, 2008, *P. van den Boom* 41227 (hb. van den Boom, LG DNA 501).—**Germany:** *Rhineland-Palatinate:* NW of Koblenz, southern shore of Laacher Zee, 50°24'32"N, 007°17'39"E, 275 m, slightly calcareous outcrop at edge of forest, 2011, *E. Sérusiaux* s. n. (LG DNA 2694).—**France:** *Dept. Cantal:* Vic-sur-Cère, 44°59.98'N, 002°38.80'E, 775 m, on rocks by river in deep shade, 2020, *E. Sérusiaux* s. n. (LG DNA 7477); *ibid.*, S of Mandailles-Saint-Julien, 45°03.77'N, 002°39.88'E, 1560 m, crevices in exposed outcrops, 2020, *E. Sérusiaux* s. n. (LG DNA 7475). *Dept. Savoie:* Haute-Maurienne, vallée de l'Arc en amont de Bessans, La Roche-du-Château, 45°20'48"N, 007°01'38"E, 1800 m, ophiolite outcrops by a river, 2009, *E. Sérusiaux* s. n. (LG DNA 827).—**Spain:** *Catalonia:* Val d'Aran, 42°50.14'N, 00°43.43'E, 915 m, under overhang in crevice, inside mixed forest, 2019, *P. & B. van den Boom* 58555 (hb. van den Boom, LG DNA 7506).

Ramalina europaea. **Armenia:** *Syunik Prov.:* E of Goris, Khndzoresk, 39°30'18"N, 046°25'54"E, 1300 m, volcanic breccia, 2011, *E. Sérusiaux* s. n. (LG DNA 2670).—**Belgium:** *Prov. Namur:* Ave-et-Auffe, along road to Han/s/Lesse, 50°07'01"N, 005°10'14"E, 235 m, underhang of calcareous outcrop, 2008, *E. Sérusiaux* s. n. (LG DNA 539).—**France:** *Dept. Savoie:* Haute-Maurienne, vallée de l'Arc en amont de Bessans, La Roche-du-Château, 45°20'48"N, 007°01'38"E, 1800 m, ophiolite outcrops by a river, 2009, *E. Sérusiaux* s. n. (LG DNA 826). *Dept. Vaucluse:* Fontaine de Vaucluse, bordure D24, 43°55'N, 005°08'E, 700 m, calcareous outcrop, facing north, 2010, *M. Bertrand* s. n. (hb. M. Bertrand, LG DNA 2963).—**Iran:** *Golestan:* Gorgan Distr., Tuskestan Forest, 36°44.98'N, 054°34.01'E, 650 m, disturbed deciduous forest, 2007, *H. Sipman* 55215B (B)(LG DNA 844).—**Norway:** *Hedmark:* Ringsaker, Brøttum sag, V for UTM WGS84: NN 8371 6447, 125 m, on tree, 2008, *Breili Anders* (O—L159241)(LG DNA 2776).—**Romania:** Parcul Natural Piatra Craiului, 45°32'24"N, 025°16'38"E, 1300 m, on *Quercus* trunk in open forest, 2015, *E. Sérusiaux* s. n. (LG DNA 4352). Parcul Natural Apuseni, Gàrda de Sus, 46°27.36'N, 022°49.42'E, 800 m, 2015, on *Tilia* trunk by river, *E. Sérusiaux* s. n. (LG DNA 4354). Parcul Natural Retezat, S of Hateg, 45°29.30'N, 022°55.28'E, 600 m, on trees at edge of forest, 2015, *E. Sérusiaux* s. n. (LG DNA 4357, 4361).

Discussion

In a European context and within the group comprising species producing soralia and evernic acid (*R. europaea* and *R. pollinaria*), *Ramalina arsenii* is easily recognized by its ecological niche (slightly calcareous rocks, especially in underhangs at low and mid elevations in Western Europe), its ITS barcode, its small size (less than 3 cm long), and its soralia developing mainly on the underside of lobe apices. This latter character makes it similar to *R. labiosorediata*, an endemic North American species that is usually epiphytic.

Ramalina carminae R. Arroyo & Sériñá (Arroyo *et al.* 2011; Nimis 2016) shares the same ecology as *R. arsenii*, growing in underhangs of rock outcrops in mountains. It is reported from Portugal, Spain and Sardinia in Italy. It is easily distinguished from *R. arsenii* by the more palmately branched lobe apices and the production of variolaric acid. Furthermore, it belongs to the *capitata-polymorpha* clade (Pérez-Ortega *et al.* 2019) which is part of the so-called 'early diverging clades' of the genus (Spjut *et al.* 2020), whereas the *fastigiata* group mainly includes only

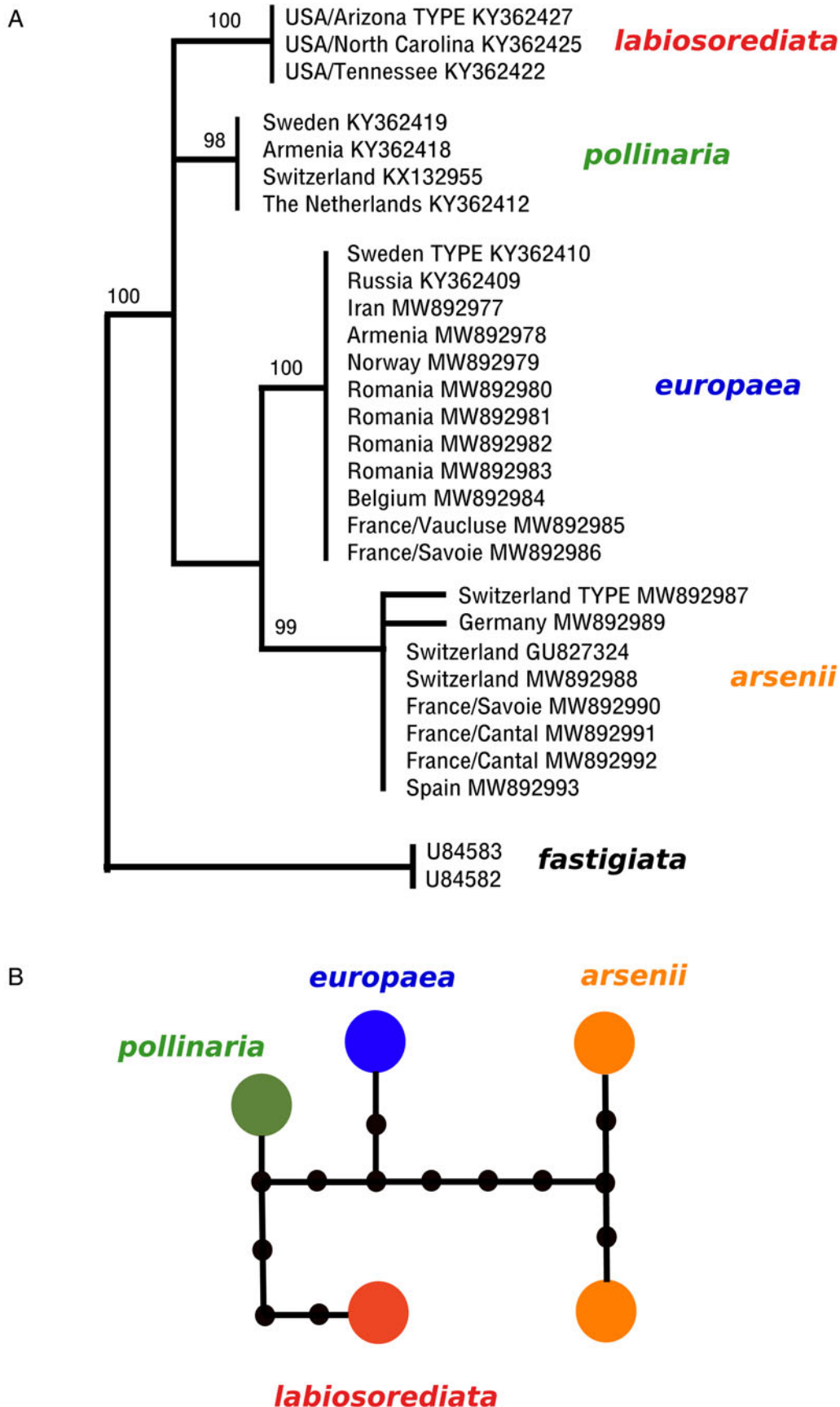


Fig. 1. A, evolutionary tree based on ITS sequences for the four species of the *Ramalina pollinaria* group, as circumscribed by Gasparyan *et al.* (2017) and optimized under maximum likelihood, with *R. fastigiata* as outgroup. GenBank Accession numbers are included and country of provenance provided. Numbers on branches indicate those with statistical support > 75%. B, haplotype network constructed with TCS v. 1.21 (Clement *et al.* 2000) with all four species of the *R. pollinaria* group; each bar between circles in the network represents a single mutation step, and each dot is an additional change. In colour online.

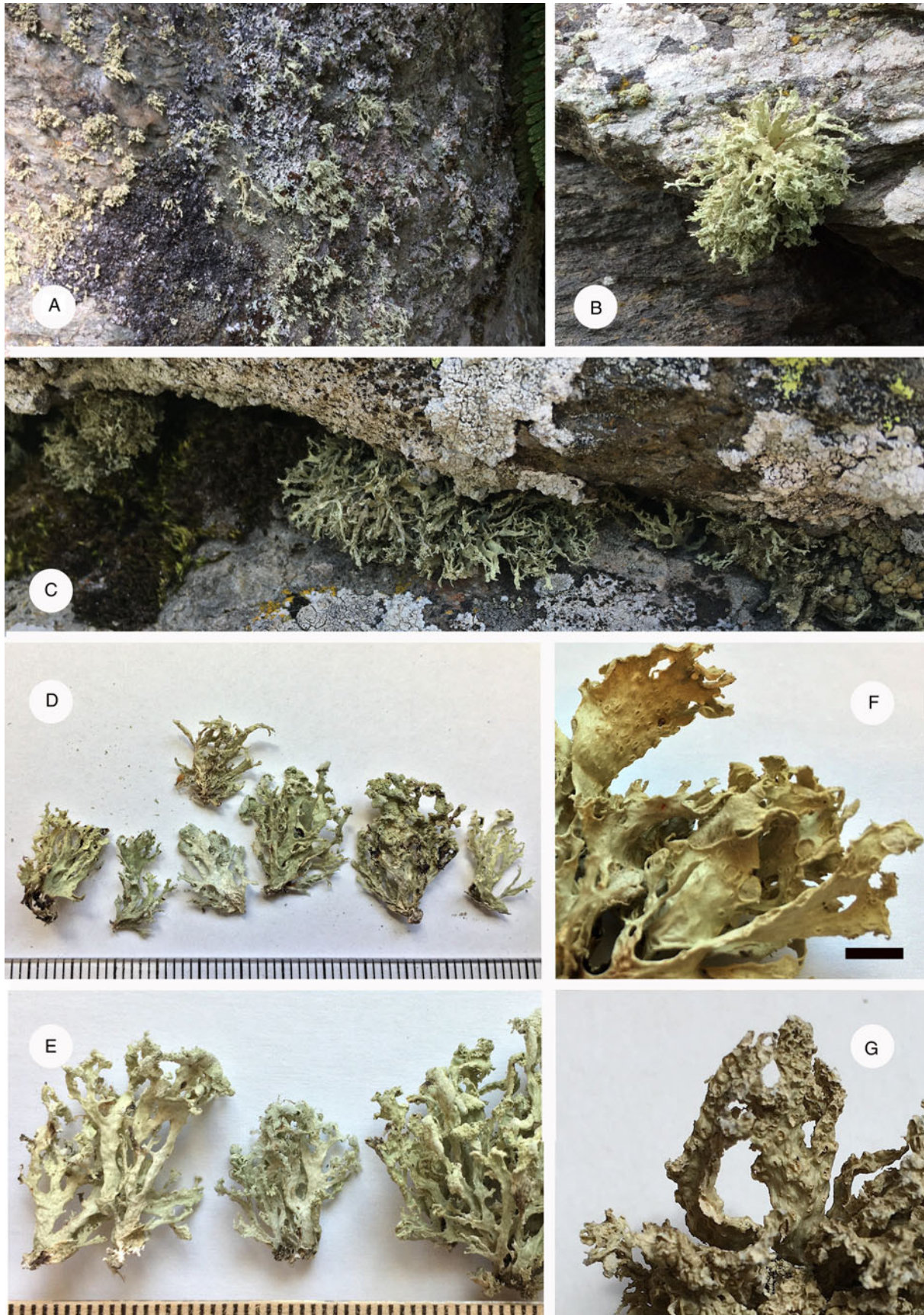


Fig. 2. *Ramalina arsenii* (A–E) and *R. europaea* (F & G). A & B, type locality of *R. arsenii*. A, general view of a sheltered outcrop where the species is abundant and easily detected. B, well-developed isolated thallus. C, sheltered underhangs in Cantal Dept., France, where the species thrives. D & E, individual lobes of the holotype collection (scales in mm). F, corticolous collection of *R. europaea* with typical ovoid excavate depressions on underside. G, the only saxicolous population found for *R. europaea*, occurring together with genuine *R. arsenii* (not seen here), with the same depressions, albeit much smaller than in epiphytic material. Scale: F = 4 mm. In colour online.

species producing evernic acid (*R. breviscula*, *R. carpatica*, *R. fastigiata* and all species of the *pollinaria* assemblage).

With the data available, *Ramalina arsenii* appears to be a rather widespread species, being found in the Western Alps (Switzerland and France), the 'Massif Central' (France) and the southern side of the Pyrenees (Spain), and is further known at lower elevations in Germany. Interestingly, it has been found once with *Ramalina europaea*, its sister species in our ML analysis (but without support), on the same rock outcrop in the French Alps (GenBank Accession MW892986 for *R. europaea* and MW892990 for *R. arsenii*). In such a context, the development, especially on the lower side, of ovoid excavate depressions is a


reliable diagnostic character for *R. europaea*. Such depressions are usually quite conspicuous in epiphytic material of *R. europaea* but are much smaller when saxicolous (Fig. 2F & G).

Phylogenetically closely related species thriving in the same locality and also in the same ecological microniche might not be a rare pattern in fungi (Taylor et al. 2014; Peay 2016; Truong et al. 2017). The genus *Ramalina* may provide an interesting model as a further similar case has been detected with the duo *Ramalina inaequalis* and *R. tingitana* (Spjut et al. 2020); indeed, these two species are easily recognized, even in the field, and are resolved as two distinct phylogenetic entities but share the same niche (e.g. rocky seashores in the western part of the Mediterranean Sea).

Key to the species of the *Ramalina pollinaria* group (expanded from Gasparyan et al. 2017)

- 1 Soralia mainly originating from the underside, irregularly lip-shaped and rather large (North America and Western Europe) 2
 - Soralia marginal to laminal, elongate to rounded, if terminal then starting out as small, punctiform structures on small, spine-like branchlets; Eurasia 3
- 2 (1) North America; mainly on trees but also reported from rock **R. labiosorediata**
 Western Europe; only on rock underhangs **R. arsenii**
- 3 (1) Soralia starting out terminally as small, punctiform structures on small, spine-like branchlets; old lobes may develop a helmet-shaped bulge at their apex, and develop ovoid excavate soralia, 0.1–0.2 mm across, rarely regular in saxicolous population; soredia granular, 50–70 µm diam.; on rocks and trees **R. europaea**
 Soralia from the beginning subterminal to laminal-marginal, irregularly elongate, not starting out on small branchlets and such branchlets absent or rare; soredia farinose, < 50 µm diam.; on rocks and trees **R. pollinaria**

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