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Standard Paper

A molecular phylogenetic evaluation of the *Ramalina siliquosa* complex, with notes on species circumscription and relationships within *Ramalina*

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Abstract

Lichens of the *Ramalina siliquosa* complex dominate seashore cliffs in Europe and South-East Asia, but their taxonomy has been vigorously debated for over a century. On many cliffs, they exhibit a bewildering zonation of chemotypes that resembles the classic zonation of organisms that occupy the littoral zone below. Do the chemotypes represent separate species, or infraspecific variation? To better understand the systematics of this group, sequences from four genetic loci (ITS, IGS, *RPB1* and *RPB2*) were obtained for 59 samples from Denmark, France, Iceland, Norway, UK, Japan and Korea, including all major chemotypes. Maximum likelihood analysis of these sequences, together with sequences from 36 other *Ramalina* species, reveals that the complex comprises two distinct phylogenetic lineages, each including multiple chemotypes. These two putative species-level lineages correspond to the currently accepted taxa *R. cuspidata* and *R. siliquosa*. There is no evidence that these two taxa are phylogenetic sister species. Consequently, the explanation of this chemotype complex as an example of 'sibling speciation' is rejected. Specimens traditionally called '*R. siliquosa*' from South-East Asia form a third clade, identified here as *R. semicuspidata*, with an additional, divaricatic acid chemotype. Other results include a robustly supported clade of *Ramalina* species that produce medullary depsides and depsidones; this clade includes another well-supported clade of south-eastern United States coastal plain and tropical *Ramalina* species. By contrast, large, strap-shaped *Ramalina* species that lack medullary depsides and depsidones occur in separate lineages. In addition, close relationships between the following groups of species are indicated: *R. farinacea* with *R. subfarinacea*; *R. fraxinea* with *R. leptocarpha*, *R. menziesii* and *R. subleptocarpha*; *R. sinensis* with *R. unifolia*. Furthermore, a new, variolaric acid-only chemotype is reported for *R. farinacea*, and a new, acid-deficient chemotype is reported for a more broadly circumscribed *R. culbersoniorum*.

Key words: Lecanoromycetes, lichens, phylogeny, *Ramalinaceae*, systematics, taxonomy

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Introduction

Second only to being a classic example of symbiosis, lichenized fungi are well known as producers of unique secondary metabolites (see Rankovíc (2015) and references therein). For the systematist, these compounds take on special significance as taxonomic characters because of their high congruence with morphological variation and their ease of identification, even in old museum specimens (reviews: Culberson 1969*a*; Brodo 1986; Rogers 1989). Some morphological species, however, comprise multiple, morphologically indistinguishable chemical races, or chemotypes (Culberson 1969*a*; Culberson & Culberson 1970; Culberson 1986). Traditionally, in cases where such chemotypes have their own distinct ecologies or geographical ranges, they are interpreted as sibling species, or as subspecies (review: Lumbsch 1998). Sometimes, rather than the presence or absence of individual compounds, the presence or absence of chemosyndromes (Culberson & Culberson 1977) has been used to delimit species (review: Elix & Stocker-Wörgötter 2008). In most cases, however, the interpretation of taxonomic rank based on chemotype has traditionally been assigned subjectively, without rigorous evaluation using experimental or quantitative methods.

Phylogenetic analyses of DNA sequence data have provided a more detailed picture of the evolution of chemical variation in

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lichenized fungi, including the polyketide synthase (PKS) genes that control production of the secondary metabolites themselves (e.g. Muggia *et al.* 2008). DePriest (1994, 1995) was the first to use DNA to evaluate the taxonomic status of chemotypes: using RFLP patterns from small subunit ribosomal DNA (SSU), she demonstrated that, in the Southern Appalachians, only one of the chemotypes of the well-studied *Cladonia chlorophaea* chemotype complex (*Cladonia grayi* G. Merr. ex Sandst.) possessed unique patterns that merited recognition at the species level. More recently, analyses of nuclear ribosomal internal transcribed spacer region (ITS) sequences suggest that two other chemotypes, *C. merochlorophaea* Asahina and *C. novochlorophaea* (Sipman) Brodo & Ahti, may also be monophyletic (Dolnik *et al.* 2010). In the chemically rich *Ramalina americana* chemotype complex, LaGreca (1999) used ITS sequences to divide the complex into two species: one, *R. americana* Hale, is largely acid-deficient and occurs in the northern half of the geographical range of the complex; the other, *R. culbersoniorum* LaGreca, encompasses five chemotypes and occupies the southern half. In another, well-studied chemotype complex in *Ramalina*, the *R. farinacea* complex, Stocker-Wörgötter *et al.* (2004) found no evidence from ITS sequences for elevating any of the chemotypes to species status. During his work on *Lepraria*, Lendemer (2012) discovered that a chemically variable species he described as *L. normandinoides* Lendemer & R. C. Harris actually comprises two sympatric sibling species, based on ITS sequence data. The original species was re-circumscribed to include the protocetraric acid and acid-deficient chemotypes, while a segregate species (called *L. oxybapha* Lendemer) was erected for the fumarprotocetraric chemotype. A phylogenetic investigation of the well-studied, chemically heterogeneous *Parmotrema perforatum* complex (Widhelm *et al.* 2016) concluded that individuals producing an orcinol-type depsidone (alectoronic acid) comprise one phylogenetic lineage, while those producing ß-orcinol depsidones comprise two separate lineages. More recently, a molecular phylogenetic study of the *Usnea cornuta* complex (Gerlach *et al.* 2019) found a strong correlation between nine robustly supported lineages and their chemistry; most of them were characterized by only one chemotype. Furthermore, in a ground-breaking investigation of chemical variation in lichens, Spribille *et al.* (2016) provided evidence that the presence of *Cyphobasidium* yeasts in the cortex might influence the production of secondary metabolites by demonstrating that the frequency of such yeasts is significantly different in two chemospecies of *Bryoria*: *B. tortuosa* (G. Merr.) Brodo & D. Hawksw. (with more vulpinic acid and more yeast) and *B. fremontii* (Tuck.) Brodo & D. Hawksw. (with less vulpinic acid and less yeast). How the occurrence of yeast on the surfaces of lichens might influence the chemistry of those lichens, however, remains obscure.

One of the most fascinating, and best-documented, examples of chemotypes exhibiting different ecologies is the *Ramalina siliquosa* complex. These lichens grow in dense mats on maritime, granitic cliffs in western Europe, Iceland and South-East Asia (Lynge 1940; Kashiwadani 1992; Smith *et al.* 2009; Moon 2013; Nimis 2016; Stenroos *et al.* 2016), where they form the major component of vegetation from the high-tide zone up to where vascular plants become dominant (Fig. 1). The *Ramalina siliquosa* complex consists of seven morphologically similar chemotypes, all with different but broadly overlapping geographical distributions (Culberson *et al.* 1977; Hamada 1985; Kashiwadani 1992). Chemical variation in this complex was first discovered by Nylander (1870), who used spot tests to differentiate two chemotypes. Later, Zopf (1906) showed that the chemotypes of this complex exhibit different ecologies: on a maritime cliff in Sweden, he observed that one chemotype (salazinic acid) grows towards the bottom of the cliff, closer to the sea, than another (protocetraric acid). Expanding on Zopf's observation, Culberson & Culberson (1967) discovered that on a maritime cliff at Holyhead, Wales, chemotypes of this complex display a bewildering zonation: one chemotype (hypoprotocetraric acid) is found only at the very top; another (stictic acid) occurs exclusively at the very bottom, nearest the water's edge; in between, an additional three chemotypes (salazinic, acid-deficient and norstictic) are arranged in distinct bands according to elevation. A similar pattern was discovered on a cliff in westernmost Portugal (Culberson 1969*b*) except that, unlike the Welsh locality, the protocetraric chemotype grows at the very top of the cliff; at Holyhead, this chemotype is found only on boulders and stone walls in more sheltered, inland localities. In fact, throughout Europe, the protocetaric and hypoprotocetraric chemotypes are the only ones that occur at substantial distances inland; the others are restricted to the coast. On the Danish island of Bornholm in the Baltic Sea (Søchting 1976), the pattern found was identical to that found at the Swedish locality (the zonation of which is further characterized by Culberson *et al.* 1977). On cliffs in north-west Spain and north-west France, Alvarez *et al.* (2001) and Parrot *et al.* (2013), respectively, reported no less than seven chemotypes but did not discuss their zonation. Of the seven known chemotypes, the one containing 4-*O*-demethylbarbatic acid is the rarest, its only localities being a cliff in north-west Spain (Culberson *et al.* 1977) and cliffs in north-west France (Parrot *et al.* 2013). In all these seashore environments, biological zonation is the rule; every species of invertebrate and alga below the high-tide level has its own place in the total community (Lewis 1964; Moore & Seed 1986). In other words, in Europe, the individual chemotypes of these lichens behave, ecologically, like species of other organisms on these cliffs. This compelled Culberson (1986) to assert that the chemotypes of the *R. siliquosa* complex are closely related sympatric species, declaring them 'the best example of sibling species marked phenotypically by natural-product chemistry'.

In South-East Asia, the chemistry of these lichens is not as thoroughly documented as it is in Europe, but three of the European chemotypes (salazinic, protocetraric and acid-deficient) have been verified in Japan (Culberson 1970; Hamada 1985; Kashiwadani 1992). Unlike in Europe, zonation of chemotypes does not occur in Japan or Korea; individuals of all chemotypes appear to grow side by side (H. Kashiwadani & K. Moon, personal observation), although the concentration of salazinic acid in the salazinic chemotype has been shown to be highly correlated with temperature (Hamada 1981).

The morphology of the *Ramalina siliquosa* complex is quite variable, but not useful in a reliable way for diagnosing chemistry. In Europe, it has been shown that individuals at the bottom of cliffs (i.e. norstictic, stictic and acid-deficient chemotypes) tend to have melanized bases and pycnidia, and are often unbranched, or, when branched, the branches are primarily from the apex of the lobes; those at the top (i.e. salazinic, hypoprotocetraric and protocetraric chemotypes) tend to be non-melanized and branch primarily from the base of the thallus (Culberson 1967; Søchting 1976; Sheard 1978*b*). Interestingly, these two broad morphotypes correlate precisely with the two distinct biogenetic pathways proposed for their secondary products (Culberson *et al.* 1977; Sheard 1978*a*). This correlation has been used to justify a two-species taxonomy for the complex in Europe (i.e. *R. cuspidata* Nyl. and

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Fig. 1. Lichens of the *Ramalina siliquosa* chemotype complex covering a maritime cliff on the Isle of Skye, Inner Hebrides, Scotland, UK.

R. siliquosa (Huds.) A. L. Small), which has been followed in recent floras (e.g. Smith *et al.* 2009; Stenroos *et al.* 2016). In South-East Asia, two morphotypes have also been noted, similar to those in Europe (Kashiwadani 1992). Unlike in Europe, however, no correlation of morphology with chemotype, or heights on cliffs, has been observed (H. Kashiwadani & K. Moon, personal observation).

There have been two previous genetic studies of the *Ramalina siliquosa* complex. One study (Mattsson & Kärnefelt 1986), utilizing a phenetic analysis of isozymes, showed that on the cliff in Sweden where Zopf (1906) first observed the zonation of the chemotypes, three groups could be discerned. One of these groups comprised individuals of the norstictic, stictic and acid-deficient chemotypes; the other two contained individuals of the salazinic, protocetraric and acid-deficient chemotypes. A parsimony analysis of this same data set (Mattsson 1990), however, indicated that each of the chemotypes yielded a different isozyme pattern, and thus should be accepted as distinct taxonomic entities. The other genetic study of the complex, by Culberson *et al.* (1993), utilized chemical analyses of spore progeny from thalli collected on the cliff in Wales that was the site of their very first study of the complex (Culberson & Culberson 1967).

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Of over 300 spores analyzed, 96% matched the maternal thallus, indicating a very high level of reproductive isolation for the chemotypes. Of the six chemotypes detected on that cliff, only the acid-deficient and norstictic chemotypes appeared to interbreed with each other.

There have been multiple phylogenetic studies of the genus *Ramalina* (Marsh 1996; LaGreca 1997; LaGreca & Lumbsch 2001; Joneson 2003; Stocker-Wörgötter *et al.* 2004; Sérusiaux *et al.* 2010; Timsina *et al.* 2012; Pérez-Vargas & Pérez-Ortega 2014; Gasparyan *et al.* 2017), and of species complexes within *Ramalina* (Groner & LaGreca 1997; LaGreca 1999; Ohmura *et al.* 2008; Hayward *et al.* 2014; Gumboski *et al.* 2018). Most of these studies utilized the ribosomal ITS region alone, although some combined ITS with another locus, such as *ß*-tubulin, the mitochondrial small subunit (mtSSU), or the IGS, SSU or large subunit (LSU) regions of ribosomal DNA. Each of these studies revealed one or more strongly supported, monophyletic species; however, most of the deeper, internal branches in these published phylogenies lack statistical support. To paraphrase Timsina *et al.* (2012), it appears that molecular data generated thus far for *Ramalina* are best used for species diagnosis rather than phylogenetic reconstruction.

Despite being the subject of various investigations for 150 years, the systematics of the *Ramalina siliquosa* complex remain poorly understood. How many species does the complex actually comprise? Do the Asian populations represent separate species? The current paper addresses these questions by reconstructing a phylogeny for the *R. siliquosa* complex using nucleotide sequences of four loci from 59 individuals representing six chemotypes across the geographical range of the complex. These results are discussed in the context of a preliminary phylogeny of the genus *Ramalina*, including 45 additional samples representing 36 taxa. The application of the 'sibling species' concept to these and other lichens is also explored.

Material and Methods

Taxon sampling, secondary product identification, and morphological examination

We obtained sequence data from 59 individuals of the *Ramalina siliquosa* chemotype complex from throughout its geographical range. These samples represent all of the major chemotypes and morphotypes and include 20 samples from South-East Asia (Supplementary Material Table S1, available online). A total of 24 additional samples representing 21 additional species of *Ramalina* and one species of *Niebla*, mostly from North America and Europe, were also sequenced. The nomenclatural authorship of all species included in our phylogenetic analyses is provided in Supplementary Material Table S1. All of the common species of *Ramalina* occurring in North America (Esslinger 2019) were sampled. For one of these species, *R. culbersoniorum*, three additional specimens from portions of its geographical range not included by LaGreca (1999), including two additional chemotypes not sequenced in that study, were sequenced in order to further understand the circumscription of this chemically diverse species. In addition, 19 ribosomal DNA internal transcribed spacer region (ITS) sequences and two ribosomal intergenic spacer region (IGS) sequences from 15 *Ramalina* species were retrieved from GenBank to contribute to the analyses (see Supplementary Material Table S1). *Niebla homalea* was selected as the outgroup because previous studies (Sérusiaux *et al.* 2010; Miadlikowska *et al.* 2014; Gasparyan *et al.* 2017) strongly suggest that the segregate genus *Niebla* is both monophyletic and closely related to *Ramalina*.

Secondary metabolites of all specimens were determined by TLC, using solvents A, B′ and C (Culberson & Ammann 1979; Culberson & Johnson 1982); spots were visualized using 10% sulphuric acid sprayed over the plates, followed by heating at 110 °C for *c.* 5–15 min. In addition, we closely examined the morphology of 159 Japanese and Korean specimens of the *Ramalina siliquosa* complex, deposited in the herbarium of the National Institute of Biological Resources, Korea (NIBR) and herbarium TNS.

DNA isolation, PCR amplification and sequencing

For specimens extracted before 2014, total genomic DNA was extracted using the DTAB/CTAB method of Armaleo & Clerc (1995). For specimens extracted more recently, either the Prepease DNA Isolation Kit (USB, Cleveland, OH, USA; product discontinued) or the DNeasy Plant Mini Kit (Qiagen, Germany) were used, following the plant leaf extraction protocol. The ITS locus (ITS1 + 5.8S + ITS 2; *c.* 500 bp total) was amplified and sequenced for all samples except four (see Supplementary Material Table S1). Fragments were also amplified from the intergenic spacer region (IGS) of ribosomal DNA (*c.* 400 bp) and two low-copy, protein-coding markers: the largest subunit of the RNA polymerase II gene (*RPB1*; *c.* 830 bp) and the first part of the second-largest subunit of the RNA polymerase II gene (*RPB2*; *c.* 800 bp). Sequencing success rates among these three loci were more variable than for ITS (Supplementary Material Table S1). All primers used to amplify and sequence loci used in this study are given in Table 1. For the most part, PCR amplifications prior to 2014 were performed in 50 µl reactions following the method described in LaGreca (1999); more recent amplifications were conducted in 25 µl reactions using Ready-To-Go PCR Beads (GE Healthcare, Foster City, CA, USA) following the manufacturer's instructions. The PCR amplifications on the South-East Asian *R. siliquosa* and *R. sinensis* samples were performed in 20 µl volumes using AccuPower PCR tubes (Bioneer, Republic of Korea) containing 2 µl of extracted DNA solution, 1 µl each of 10 pmol/µl of each primer (Table 1), and 16 µl of deionized sterile water. PCR products were quantified on 1% agarose gels and stained with ethidium bromide or Dyne LoadingSTAR (DYNE BIO, Republic of Korea). Complementary strands were sequenced from cleaned PCR products using the same primers as for amplifications. Sequencing reactions were performed using BigDye v.3.1 or ABI PRISM 3730XL (Applied Biosystems Inc., Foster City, CA, USA) and run on an ABI automated sequencer according to recommended protocols (Applied Biosystems Inc.).

Sequence alignment and analysis

Contigs were assembled and edited using Sequencher v.4.10 (Gene Codes Inc., Ann Arbor, MI, USA). Two of the loci sequenced, ITS and IGS, include a number of difficult to align regions, resulting in ambiguous alignments. To address this, we tested two alignment strategies. The first was a traditional alignment using MAFFT v.7 (Katoh & Standley 2013). For the protein-coding loci (*RPB1* and *RPB2*), we used the G-INS-i alignment algorithm and '1PAM/K = 2' scoring matrix, with an offset value of 0.9, and the remaining parameters were set to default values. For the ribosomal ITS and IGS loci, we used the same

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Table 1. Information for the primers used in this study, including literature references.

Primer	Locus	Primer sequence (5′ to 3′)	Reference
BMB-CR	ITS rDNA	GTACACACCGCCCGTCG	Lane *et al.* (1985)
SLG-1	ITS rDNA	TTGCGCAACCTGCGGAAGGAT	Groner & LaGreca (1997)
ITS-1F	ITS rDNA	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns (1993)
ITS-4a	ITS rDNA	TCCTCCGCTTATTGATATGC	White *et al.* (1990)
IGS-12a	IGS rDNA	AGTCTGTGGATTAGTGGCCG	Carbone & Kohn (1999)
NS1R	IGS rDNA	GAGACAAGCATATGACTAC	Carbone & Kohn (1999)
gRPB1-a	*RPB1*	GAKTGTCCKGGWCATTTTGG	Stiller & Hall (1997)
fRPB1-c	*RPB1*	CNGCDATNTCRTTRTCCATRTA	Matheny *et al.* (2002)
RPB2-6F	*RPB2*	ATGGGYAARCAAGCYATGGG	Liu *et al.* (1999)
fRPB2-7cr	*RPB2*	CCCATRGCTTGYTTRCCCAT	Liu *et al.* (1999)

parameters with the exception of an offset value set to 0.0 rather than 0.9. The resulting alignment was manually adjusted as necessary. The second approach utilized the GUIDANCE2 server (Sela *et al.* 2015; http://guidance.tau.ac.il/ver2/) to remove regions aligned with low confidence (i.e. ambiguous regions) from the data set. In the GUIDANCE2 alignment, the multiple sequences alignment algorithm was set to MAFFT, implementing the 'globalpair' pairwise alignment method. Following the alignment step, GUIDANCE scores were calculated and residues with a GUIDANCE score < 0.90 were masked.

Alignments for each of the four loci (ITS, IGS, *RPB1* and *RPB2*) were analyzed separately with a maximum likelihood (ML) criterion using RAxML v.8.2.10 (Stamatakis 2014) as implemented on the CIPRES Science Gateway v.3.3 (Miller *et al.* 2010), using *Niebla homalea* as the outgroup. For each of the four single-locus trees, the ML analyses for the MAFFT versus GUIDANCE alignments did not reveal any conflicts; so, going forward, we used the more conservative GUIDANCE alignments. The single-locus topologies based on these GUIDANCE alignments were all congruent, so a concatenated data set was analyzed. Initial models of DNA sequence evolution for each marker were selected with jModelTest v.0.1 (Posada 2008), using the AIC criterion. For all four markers, the GTRCAT option (a General Time Reversible model of nucleotide substitution under a Gamma model of rate heterogeneity) provided the best fit for our data. Additional exploratory analyses of alternative substitution models and partition strategies yielded topologies and nodal support values similar to GTRCAT, so GTRCAT was used for the concatenated data set for all final ML analyses. Additionally, for all ML analyses, the extended majority-rule consensus tree criterion was used. Branch support was estimated using 1000 pseudoreplicates and a non-parametric bootstrap approach.

A Bayesian phylogenetic hypothesis was also inferred from the concatenated GUIDANCE alignment using BEAST v.1.8.3 (Drummond & Rambaut 2007; Heled & Drummond 2010), also using *Niebla homalea* as the outgroup. The Yule-Process was implemented as the tree prior (branching model). The data matrix was partitioned by individual loci, implementing the GTR+G+I substitution model for each partition based on the jModelTest results performed prior to the ML analyses. Trees were estimated under both a strict molecular clock and an uncorrelated relaxed lognormal molecular clock (Drummond *et al.* 2006). For both strict and relaxed lognormal estimates, two independent MCMC runs of 15 million generations were performed, sampling every 1000 steps. Chain mixing and convergence were inspected using the program Tracer v.1.6 (Rambaut & Drummond 2003), considering ESS values > 200 as good indicators. After excluding the first 25% of sampled trees as burn-in, trees from the two independent runs were combined using the program LogCombiner v.1.8.3 (Rambaut & Drummond 2003), and the final MCMC tree was estimated from the combined posterior distribution of trees using TreeAnnotator v.1.8.3 (Rambaut & Drummond 2009). The exclusion criterion of 25% was used because it was well above the point of convergence that was identified by inspecting the average standard deviation of split frequencies value.

Results

A total of 255 new sequences were generated for this study and aligned with 21 sequences downloaded from GenBank. The final, concatenated, four-locus alignment is available online as Supplementary Material File 1, and also from the Dryad Digital Repository: https://doi.org/10.5061/dryad.08kprr4zd. Our combined data set contained 2507 aligned positions, of which 886 were variable within the ingroup. Of these variable characters, 169 occurred in the ITS region, 184 in the IGS, 233 in the *RPB2* and 300 in the *RPB1*. The total proportion of gaps and indeterminable characters in the alignment was 52.77%. The ML tree (Fig. 2) had a final ML optimization likelihood value of −14969.549914. Clades with thickened lines are supported by Maximum Likelihood Bootstrap (MLBS) values ≥75%; those denoted by capital letters are discussed in the text. The following taxa and clades have branch lengths much longer than other branches in the ML tree: *Ramalina denticulata*, *R. leiodea*, *R. ovalis*, *R. pacifica*, *R. sayreana*, and clade M (= the branch containing *R. celastri* and *R. ovalis*). The Bayesian phylogeny (see Supplementary Material Fig. S1, available online) exhibited no significant (MLBS values ≥75%) conflicts with the ML tree but was less resolved.

At the outset of this study, the ITS region was amplified using the primers BMB-CR and SLG-1 (Table 1), which anneal further towards the 5′ end of the rDNA SSU than does the primer ITS-1F, our preferred 5′ primer in recent years. Use of BMB-CR and SLG-1 caused PCR amplification of multiple PCR products, including both algal rDNA (*c.* 1250 bp) and fungal rDNA; in addition, in certain samples, two different sizes of

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Fig. 2. Phylogenetic relationships within the genus *Ramalina* based on a maximum likelihood analysis of concatenated ITS, IGS, *RPB1* and *RPB2* sequences. Bootstrap values ≥ 75% are given above the internodes; these branches are depicted with thickened lines. Clades marked with capital letters are discussed in the text. Clades highlighted in grey are samples from the ingroup, the *R. siliquosa* complex. For chemotype abbreviations of samples from the *R. siliquosa* complex, see Table 2. Chemotype numbers for the *R. americana* and *R. culbersoniorum* samples follow the numbering systems of Culberson *et al.* (1990) and LaGreca (1999). The dashed line indicates the branch connecting the two parts of the tree, which was divided because of space considerations. Scale = nucleotide substitution rate.

fragments of fungal rDNA were amplified (*c.* 750 bp vs *c.* 900–1000 bp). Depending on the sample, sequencing of the larger fragment revealed the presence of Group I introns at either position 1512 or 1516 of the 18S rDNA (using the system of Gargas *et al.* (1995)). *Ramalina celastri*, *R. complanata*, *R. montagnei*, *R. paludosa* and *R. willeyi* contained the 1512 intron, while *R. americana*, *R. culbersoniorum*, *R. roesleri* and *R. sinensis* contained the 1516 intron. None of the samples possessed introns at both positions. A BLAST search (Altschul *et al.* 1997) using the position 1512 intron of *R. paludosa* as a query yielded multiple similar rDNA SSU sequences, the most similar (93% similarity) from *Anthracothecium nanum* (Zahlbr.) R. C. Harris (GenBank # KT232207), followed by *R. complanata* (92%; GenBank # FJ356152). Another BLAST search using the position 1516 intron from a *R. culbersoniorum* sample as a query yielded many similar rDNA SSU sequences, all from other *Ramalina* species; the most similar (95%) was from *R. complanata* (GenBank # HQ650720). We have

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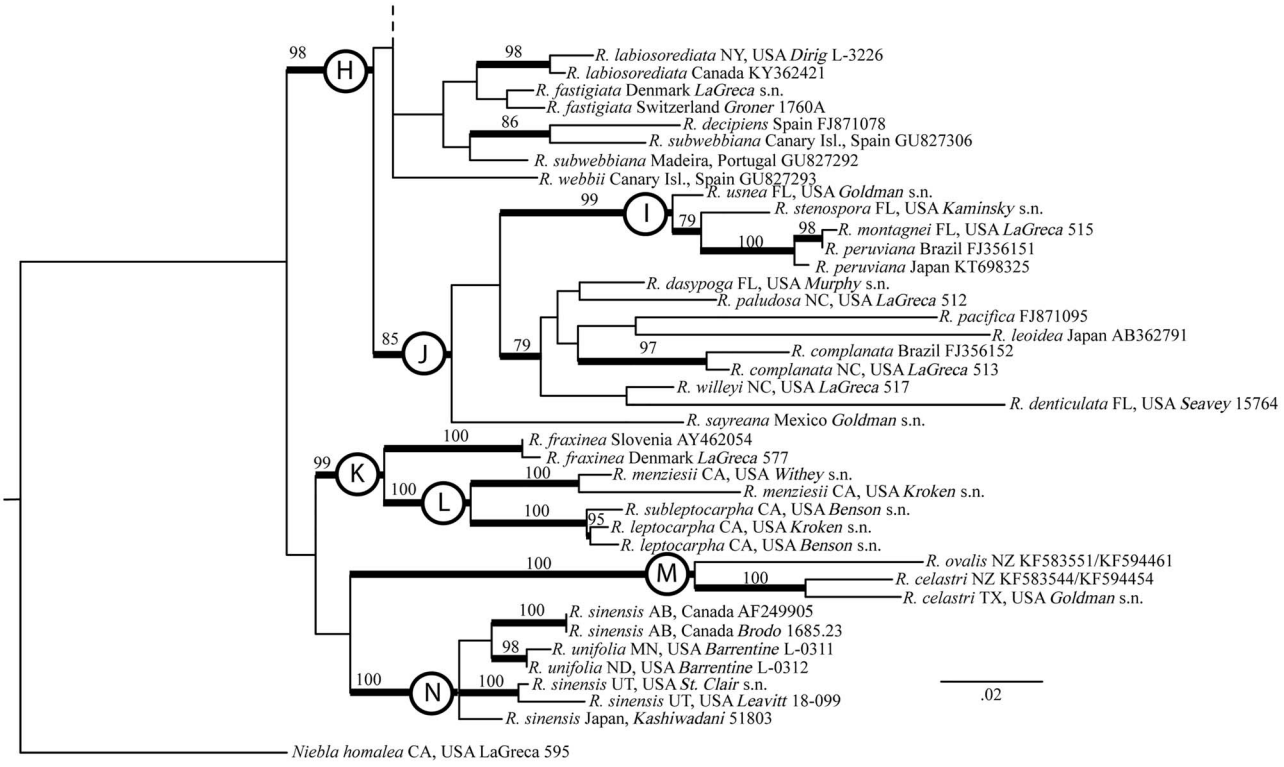

Fig. 2. (Continued)

deposited the two intron sequences used in our BLAST searches in GenBank (position 1512: GenBank # MN906756, 1516: GenBank # MN906757). Group I introns from both of these positions are commonly found in various lineages of lichenized fungi (e.g. DePriest & Been 1992; Bhattacharya *et al.* 2000; Gutiérrez *et al.* 2007). Additional details of all the introns found in the present study, including their inferred folded secondary structures and alignment among different taxa, are given in LaGreca (1997).

The ML tree (Fig. 2) shows the 59 ingroup samples forming three well-supported clades (A, C and G), none of which are direct phylogenetic sisters. Most of the backbone branches of the reconstructed tree have low support (MLBS < 75%), except for one strongly supported clade (H) comprising all *Ramalina* species included in this study that produce medullary depsides and depsidones. By contrast, a number of strongly supported (MLBS > 75%), more derived groups (clades B, D, E, F and I–N) can be discerned. One of these is a clade (B) pairing *R. roesleri* with one of the clades of ingroup samples (C) as sister species. Another strongly supported clade comprises all south-eastern USA coastal plain and tropical *Ramalina* species (clade J). This includes a well-supported clade comprising *R. montagnei*, *R. peruviana*, *R. stenospora* and *R. usnea* (clade I). All geographically and chemically disparate specimens of *Ramalina culbersoniorum* form a strongly supported clade with other specimens of that species (E). Specimens of *R. farinacea* and *R. subfarinacea* group with strong support in clade F. Clade K includes *R. fraxinea* as sister to a clade (L) comprising *R. menziesii* and a fertile/sorediate species pair, *R. leptocarpha* and *R. subleptocarpha*. Finally, there is a robust clade pairing *R. celastri* with *R. ovalis* (M), and another strongly supported clade (N) comprising multiple specimens of *R. sinensis* together with two specimens of *R. unifolia*. Neither K, M nor N are robustly placed within the global phylogeny presented.

Discussion

The Ramalina siliquosa *complex*

Our ML analysis (Fig. 2) provides strong evidence that the European members of the *Ramalina siliquosa* complex comprise two distinct evolutionary lineages ('phylogenetic species' *sensu* Mishler (1996)), supporting a two-species classification (Table 2): *R. siliquosa* (Huds.) A. L. Sm. s. str. (clade C; 96% MLBS), including the hypoprotocetraric, protocetraric and salazinic chemotypes and *R. cuspidata* Nyl. (clade G; 99% MLBS), including the norstictic and stictic chemotypes. These are the oldest available names for these two taxa (for thorough taxonomic reviews see Laundon 1966; Sheard & James 1976; Sheard 1978*a*). The grouping of our samples into these two clades supports the two hypothetical biogenetic pathways proposed for their secondary products by previous workers (Culberson *et al.* 1977; Sheard 1978*a*). Interestingly, acid-deficient samples fall into both lineages. This result agrees with Sheard (1978*a*), who observed that some individuals of the acid-deficient chemotype possess the typical morphology (i.e. terete branches with pigmented bases and pycnidia) of *R. cuspidata*, but others key out to *R. siliquosa* s. str. (i.e. flatter branches with non-pigmented bases and pycnidia). This also makes sense given that lichen secondary products can feasibly be caused by regulatory repression of the involved PKS pathways. For example, differential silencing of the atranorin versus norstictic acid PKS pathways in the cortex versus the medulla of *Parmotrema hypotropum* (Nyl.) Hale has been proposed (Armaleo *et al.* 2008), and large differences in

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Table 2. Two competing taxonomies for the *Ramalina siliquosa* chemotype complex.

Chemotype	Abbreviation	Chemical species *sensu* Culberson (1967) and Culberson *et al.* (1993)	Morphological species *sensu* Sheard & James (1976), Sheard (1978*a*), Smith *et al.* (2009) and Stenroos *et al.* (2016)
stictic	ST	*R. curnowii* Cromb. ex Nyl.	*R. cuspidata*
norstictic	NST	*R. stenoclada* W. L. Culb.	*R. cuspidata*
acid-deficient	AD	*R. atlantica* W. L. Culb.	[*R. cuspidata* or *R. siliquosa*]
salazinic	SAL	*R. crassa* (Del. ex Nyl.) Mot.	*R. siliquosa*
protocetraric	PR	*R. siliquosa*	*R. siliquosa*
hypoprotocetraric	HYPO	*R. druidarum* W. L. Culb.	*R. siliquosa*
4-*O*-demethylbarbatic	NBAR	*R. zopfii* W. L. Culb. *et al.*	not treated
divaricatic	DIV	not treated	not treated

PKS gene expression have been demonstrated between two single-spore isolates from the same lichen, *Cladonia grayi* (Armaleo *et al.* 2011).

The occurrence of the *Ramalina siliquosa* complex in South-East Asia has been well known since Nylander's time (Nylander 1890), with three chemotypes (acid-deficient, protocetraric and salazinic) that mirror those found in Europe (Culberson 1970). One additional chemotype, producing divaricatic acid as its major medullary product, is reported here as new; it differs from the others in producing an orcinol-type *para*-depside (protocetraric and salazinic acids are both ß-orcinol depsidones). Unlike in Europe, the chemotypes in South-East Asia do not display zonation on cliffs (H. Kashiwadani & K. Moon, personal observation). South-East Asian material has been identified until now as *R. siliquosa* but is slightly different morphologically. Based on our ML analysis of 20 *R. siliquosa* specimens from Japan and Korea (Fig. 2, clade A; 96% MLBS), the South-East Asian populations belong to a separate species which we originally thought was new to science. A review of the literature, however, revealed a little-known variety of *R. scopulorum* (Ach.) Ach., var. *semicuspidata* Räsänen (Räsänen 1940), which was elevated to species level by Sheard (1978*a*) in a short footnote (*op. cit.*, p. 936). Due to its obscurity, as well as an incomplete description and an absence of photographs in the original protologue, we provide a thorough account of this species in the 'Taxonomic Conclusions' section at the end of this paper.

Additional records of the *Ramalina siliquosa* complex from North America, South America and Africa exist in both the literature and herbaria (Howe 1913; Fink 1935; Calvelo & Liberatore 2002; Gumboski *et al.* 2018; CNALH 2019). Most of these records are clearly misidentifications but others warrant further inspection; they may represent separate species, like *R. semicuspidata*. Furthermore, in Europe, the *R. siliquosa* complex occurs to the south in Portugal, Spain and Italy (Culberson *et al.* 1977; Nimis 2016), countries not included in the present study. Future investigations of this species complex in southern Europe might reveal other species, and provide answers to other questions, such as the taxonomic status of the rare 4-*O*-demethylbarbatic chemotype ('*Ramalina zopfii*', Table 2).

Relationships among other Ramalina *species*

In order to both identify a sister group for the *Ramalina siliquosa* complex and provide a preliminary phylogeny for the genus, 45 *Ramalina* samples representing 36 other taxa, mainly from North America and Europe, were added to our four-locus data set. As in previous studies (e.g. Pérez-Vargas & Pérez-Ortega 2014; Gasparyan *et al.* 2017; references therein), the relationships among many of the *Ramalina* taxa included in our broader phylogeny remain unresolved (Fig. 2). This lack of resolution indicates that the evolutionary history of the genus may be too complex to be adequately captured by a dichotomously branching phylogeny based on only a few loci. A number of clades, however, were reconstructed with MLBS values ≥ 75%. For example, clade J (85% MLBS) includes all south-eastern United States coastal plain and tropical species. Although there are no obvious morphological characters uniting these species, all of them (except perhaps *R. sayreana*) occur on the coastal plain of the south-eastern United States or in humid, tropical habitats. There is little meaningful resolution within this clade, except for one monophyletic group (clade I; 99% MLBS) that includes only species with fusiform spores. This supports Howe's (1912) proposed section *Fusisporae*, comprising all species of *Ramalina* with fusiform spores.

Clade E (98% MLBS) indicates that a broader circumscription is needed for *Ramalina culbersoniorum*, a chemically rich species segregated from *R. americana* (which, unlike *R. culbersoniorum*, is almost always acid-deficient) on the basis of an ITS phylogeny (LaGreca 1999). Subsequent phylogenetic studies of *Ramalina* (Stocker-Wörgötter *et al.* 2004; Timsina *et al.* 2012; Pérez-Vargas & Pérez-Ortega 2014; Gasparyan *et al.* 2017) have all indicated that *R. culbersoniorum* is a robustly supported, monophyletic species. The present study expands the sampling by LaGreca (1999) of *R. culbersoniorum* with additional loci, one specimen from Florida and one each from two Midwestern states, Missouri and Arkansas. The Florida specimen (*LaGreca* 558, DUKE) represents the divaricatic/sekikaic chemotype (chemotype '8' *sensu* Culberson *et al.* 1990), which is the most southern chemotype and the only chemotype known at the time that was not sequenced by LaGreca (1999). It clearly falls with the other *R. culbersoniorum* samples in our tree. The Missouri specimen (*Ladd* 19804, FH) is a lecanoric/evernic individual (chemotype '5' *sensu* Culberson *et al.* 1990) that also falls within *R. culbersoniorum*. The Arkansas specimen (*Ladd* 21904, NY) is acid-deficient, and on that basis we expected it to be placed within *R. americana*; however, this sample also groups with *R. culbersoniorum*. Based on these results, we confirm that *R. culbersoniorum* occurs in the Midwest; however, we must also expand the circumscription of *R. culbersoniorum* to include an acid-deficient chemotype.

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Clade F (81% MLBS) indicates that *Ramalina farinacea* and *R. subfarinacea* are closely related, corroborating ideas put forward by numerous lichenologists (Culberson 1966; Hawksworth 1968; Krog & James 1977). In our analysis, samples of *R. subfarinacea* form a monophyletic group within a paraphyletic *R. farinacea*, the latter group including samples from both North America and Europe. More intensive sampling of this widespread species complex will be required to properly distinguish *R. subfarinacea* from *R. farinacea*. Notably, one of the *R. farinacea* samples sequenced in our study (*LaGreca* 514, DUKE) contains variolaric acid only. Variolaric acid is found in some chemotypes of *R. farinacea* in Europe (Zedda 2002), where it always co-occurs with other medullary substances (usually protocetraric acid). However, no other medullary substances could be detected in the variolaric acid-containing specimen we included in our analysis. This specimen, from Newfoundland, Canada, represents the first record of variolaric acid in a North American specimen of *R. farinacea* (*cf.* Bowler & Rundel 1978), and also represents a new chemotype for this species.

Clade H (98% MLBS) is remarkable because it contains all of the *Ramalina* species in our data set that produce depsides and depsidones in the medulla. This supports the suggestion of Stocker-Wörgötter *et al.* (2004) and Timsina *et al.* (2012) that *Ramalina* species containing medullary products are derived. By contrast, all *Ramalina* species that lack these medullary products are found in clades K, L, M and N. Furthermore, interestingly, compared to all but one of the species (*R. usnea*) in Clade H, the species in these four clades (K, L, M and N) all produce large, horsey, strap-shaped thalli. It has been shown that environmental stress limits the growth of lichen thalli but it also seems to induce the production of secondary metabolites; in such situations, accumulated carbohydrates may be shifted to other pathways to produce secondary metabolites that are not essential for growth (Culberson & Armaleo 1992; Stocker-Wörgötter 2001). In other words, slower mycelial growth resulting from inadequate nutrients may be linked to the production of secondary metabolites (Bu'Lock 1961; Fox & Howlett 2008), which is related to the carbon-nutrient balance hypothesis (Bryant *et al.* 1983). This was the explanation put forward by Timsina *et al.* (2013) to explain the negative relationship they observed between culture diameter and the amounts of secondary metabolites produced by cultures of *R. dilacerata*, and by Hyvärinen *et al.* (2002) to explain nutrient content in *Cladonia stellaris* (Opiz) Pouzar & Vězda relative to herbivory. In other words, if cell growth and secondary metabolism are indeed competing processes (Bu'Lock 1961), then the inability of the species in clades K, L, M and N to produce medullary depsides and depsidones might allow those species to spend more energy on growth, resulting in larger thalli.

Clade L (100% MLBS) includes three species endemic to the west coast of North America: *Ramalina leptocarpha*, *R. menziesii* and *R. subleptocarpha*. *Ramalina menziesii*, a pendulous *Ramalina* with holes in its thallus, is sister to a clade containing the other two species, both of which are strap-shaped and without holes. *Ramalina leptocarpha* and *R. subleptocarpha* are a classic lichen 'species pair' (Rundel & Bowler 1976; Tehler 1982), the former being exclusively sexual and the latter reproducing only by soredia. Species pairs have been a popular subject for molecular phylogenetic studies of lichens at the species level (e.g. Lohtander *et al.* 1998; Myllys *et al.* 2001; Buschbom & Mueller 2006), with most concluding they are merely populations of the same species (but see Widhelm *et al.* (2016) and Grewe *et al.* (2018)). More extensive sampling will be needed to adequately address whether this is the case here. Clade M (100% MLBS) supports the results of Hayward *et al.* (2014) that *R. ovalis* is a distinct species from the morphologically similar but more broad-ranging *R. celastri*, which is sister to it. Equally interesting, however, is how the New Zealand specimen of *R. celastri* pairs with the Texas specimen of *R. celastri* in a well-supported clade (100% MLBS). This indicates that *R. celastri* may be a nearly cosmopolitan lichen species, being reported from North America, South America, Africa and Australasia; recent studies (e.g. Leavitt *et al.* 2015, 2018) have demonstrated that widespread lichen species such as this might be more common than previously thought. Clade N (100% MLBS) pairs *R. sinensis*, a widespread, strap-shaped *Ramalina* known from South-East Asia and western and northern North America, with *R. unifolia*, a North American endemic species (Thomson 1990) known from Minnesota, Wisconsin and the Dakotas. In our analysis, *R. sinensis* is paraphyletic to a strongly supported (98% MLBS), monophyletic *R. unifolia*. The two species are strikingly similar, each bearing wide, flat lobes with broad, ecorticate areas on the lower surface. Additional sampling is required to assess the delimitation of *R. unifolia* from *R. sinensis*.

The 'sibling species' concept in lichens

Whereas cryptic species are defined as morphologically identical (or nearly identical) species (reviews: Mayr 1970; Futuyma & Kirkpatrick 2017; Struck *et al.* 2017), sibling species can be thought of as a special subset of cryptic species that are each other's closest relatives (Steyskal 1972; Bickford *et al.* 2007). The term 'sibling species' is widely used among zoologists and entomologists (e.g. Rohland *et al.* 2010; Lee & Lin 2012) but the concept has also been used by botanists (e.g. Grant 1981; Prata *et al.* 2018). Culberson (1986) argued for sympatric sibling speciation in a 'sharply telescoped environment' as the evolutionary mechanism producing the seven European sibling chemospecies he recognized in the *Ramalina siliquosa* complex (Table 2). The present study has demonstrated, however, that only two species exist in Europe (*R. cuspidata* and *R. siliquosa*) and, furthermore, they are not sibling species. Neither the majority-rule (Fig. 2) nor the single-gene trees (not shown) support a sister relationship for *R. cuspidata* and *R. siliquosa* s. str. In fact, the majority-rule tree indicates that the epiphytic, fistulose species *R. roesleri* is sister to *R. siliquosa* (80% MLBS) and that this pair (clade B; 80% MLBS), in turn, is sister to two other fistulose species, *R. almquistii* and *R. dilacerata* (clade D; 75% MLBS). Although cryptic, the species that comprise the *R. siliquosa* complex are not sibling species.

Many similar studies have recently demonstrated cryptic species in lichens (e.g. Singh *et al.* 2015; Del-Prado *et al.* 2016) but, as in the case of the *R. siliquosa* complex, these species-level lineages are not always sibling species. For example, in a study of the widespread lichen *Parmelina quercina* (Willd.) Hale, a single, nominal species was revealed to be four separate species, each with a distinct biogeographical distribution, three of which were closely related (Argüello *et al.* 2007). However, the fourth species within the *P. quercina* complex, *P. elixia* Argüello & A. Crespo, was subsequently found to belong to a distinct, distantly related evolutionary lineage, *Austroparmelia* (Crespo *et al.* 2010). Similar patterns of cryptic, species-level lineages not forming sibling species include examples in the brown parmelioids (Leavitt *et al.* 2016), *Xanthoparmelia* (Hodkinson & Lendemer 2011), *Parmelia* (Divakar *et al.* 2015), *Porina* (Baloch & Grube 2009) and others.

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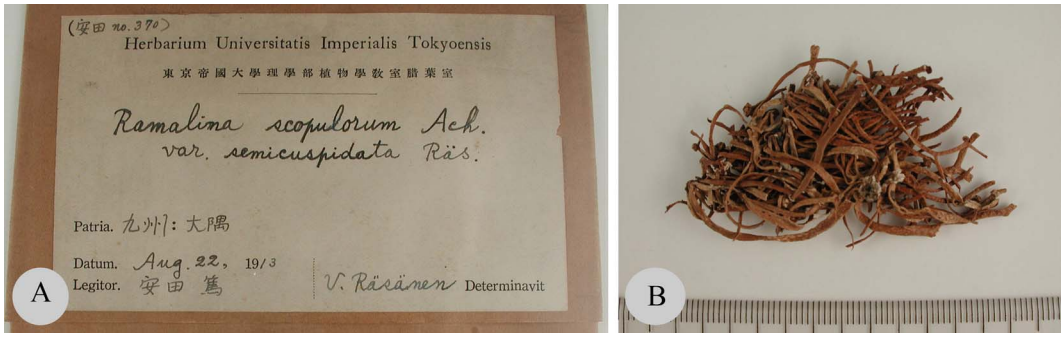

Fig. 3. Isotype of *Ramalina semicuspidata* (*A. Yasuda* 370, TNS). A, specimen label; B, intermixed thalli. Scale = mm. In colour online.

The range of evolutionary processes that have been associated with diversification of morphologically cryptic species are numerous, from morphological stasis (Nevo 2001) to convergence (Grube & Kantvilas 2006) to novel symbiotic interactions (Schneider *et al.* 2016). In the *Xanthoparmelia pulla* group, rare intercontinental dispersal, followed by diversification, resulted in multiple cryptic species (de Paz *et al.* 2012). Similarly, in the morphologically variable *Leptogium furfuraceum-L. pseudofurfuraceum* complex, transoceanic dispersal produced four geographically disjunct phylogenetic lineages (Otálora *et al.* 2010). In these examples, there is strong evidence for allopatric sibling speciation, driven by geographical isolation. A similar scenario might explain why the South-East Asian species revealed in the present study, *Ramalina semicuspidata*, is morphologically similar to the European *R. siliquosa* complex; however, phylogenetic evidence for an intercontinental dispersal event is lacking (Fig. 2). By contrast, sympatric speciation, the process by which sibling species co-occur in the same habitats, is reportedly much less common than allopatric speciation (Futuyma & Kirkpatrick 2017). This is because morphologically similar, co-occurring sister species cannot coexist over time: one either gets outcompeted or adapts to a different ecological niche (Zeigler 2014).

Therefore, contrary to ideas put forward by Culberson (1986), the *Ramalina siliquosa* complex is not an example of sympatric sibling speciation but rather an example of parallel, or perhaps convergent, evolution (a possibility hinted at by Culberson *et al.* (1993)). Another supposed example of sympatric sibling speciation in lichens discussed by Culberson (1986) was also recently debunked: the *Parmotrema perforatum* complex. In two classic papers about this complex, Culberson (1973) and Culberson & Culberson (1973) proposed six sympatric sibling species, each characterized by different combinations of chemistry and reproductive mode (sexual vs asexual). Using a combination of phylogenetic analysis and multi-species coalescent species delimitation methods, Widhelm *et al.* (2016) found that although all their apotheciate samples sorted into three separate, well-supported clades, the relationships among these clades did not correlate with the similarity of their secondary chemistries. Furthermore, no correlation was found between their reconstructed phylogeny and the reproductive mode of their samples. Although a test of monophyly for the *P. perforatum* complex awaits a more comprehensive analysis at the genus level, Widhelm *et al.* (2016) reduced the complex from six putative sibling species to four and, furthermore, demonstrated that the Culbersons' traditional sibling species and chemospecies are not supported by phylogenetic analyses.

If the chemotypes of *Ramalina cuspidata* and *R. siliquosa* are not sibling species, then what is responsible for their remarkable vertical zonation? In the sublittoral and littoral zones below where these lichens grow, multiple ecological factors influence patterns of zonation, including tides, wave action, type of rock, steepness of the topography, salinity and herbivory/predation (e.g. Underwood & Jernakoff 1984; Farrell 1991; Sarver & Foltz 1993; Chu *et al.* 2000). On rocky coasts, a steep salt fall gradient may exist, as was demonstrated in Portugal using *R. canariensis* J. Steiner as a biomonitor (Figueira *et al.* 1999*a*, *b*). Perhaps the chemotypes that occur lower down these maritime cliffs have a higher tolerance for salt than those above them; moderate salt tolerance was, in fact, demonstrated in one isolate of the *R. siliquosa* complex by Yamamoto *et al.* (2001), who grew fungal cultures on media of varying concentrations of NaCl. An alternative, or perhaps complementary, explanation is that different algal photobionts of these lichen fungi possess different levels of salt tolerance, much like the photobionts of some *Lepraria* spp. possess different levels of tolerance to rain exposure (Peksa & Škaloud 2011). In other words, the algae may actually be driving the chemotype zonation. Yet another factor that could be causing the zonation of chemotypes on these cliffs is mite herbivory. Studies of one cliff in Bornholm revealed that *R. siliquosa* is grazed more heavily by orbatid mites than *R. cuspidata* (Gjelstrup & Søchting 1979). Future investigations of these lichens, pairing ecological sampling methods with modern, phylogenomic approaches, might uncover the mechanisms underlying their zonation.

Taxonomic Conclusions

Ramalina semicuspidata (Räsänen) Sheard

Canadian Journal of Botany **56**, 936 (1978).—*Ramalina scopulorum* Ach. var. *semicuspidata* Räsänen, *Journal of Japanese Botany* **16**, 87 (1940); type: Japan, Kyushu, Prov. Ohsumi, 22 August 1913, *Yasuda* 370 (TUR—holotype; TNS—isotype) [TLC: usnic and salazinic acids].

Diagnosis. Morphologically close to *Ramalina cuspidata* and *R. siliquosa*, differing mainly by the presence of pseudocyphellae and the rare production of scattered soredia which are initiated by isidia-like protuberances.

(Figs 3 & 4)

Thallus saxicolous, erect or rarely subpendulous, caespitose, 1.5–3 (–8) cm long, growing from a common holdfast. *Surface* pale yellow-green (reddish brown in herbaria), holdfast unpigmented to rarely blackened. *Branches* solid, simple or sparingly branched,

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Fig. 4. Habit of *Ramalina semicuspidata* in Japan (*Kashiwadani* 51488, TNS).

1–3(–5) mm wide, dorsiventral or terete, dorsiventral branches flattened or more or less slightly canaliculate, matt or subnitid, surface smooth or uneven, irregularly ridged by protruded pycnidia, rarely foveolate. *Pseudocyphellae* ellipsoid or orbicular, often with a slit or tiny cracks near the centre, laminal or marginal, sparse or rarely very conspicuous, especially towards the base. *Soredia* rare; when present, initiated as isidiate protuberances, and not arranged in soralia but instead scattered marginally or subterminally on the main branches. *Thallus* 300–800(–1000) µm thick; cortex indistinct, *c.* 10 µm thick; chondroid tissue continuous or dissected by pseudocyphellae, often penetrating into the medulla, clearly to moderately cracked, 50–180(–300) µm thick.

Apothecia common, subterminal, submarginal or laminal (lateral on terete branches); *disc* flat, becoming convex with age; thalline exciple entire, pseudocyphellate; *hymenium* 60–65 µm high; *hypothecium* 30–40 µm thick; *proper exciple* 50–100 µm thick; chondroid tissue of thalloid exciple conspicuous, often connected with exciple; *ascospores* hyaline, broadly ellipsoid, 2-celled, with or without additional septa, 10–12 × 4–5 µm.

Pycnidia common, unpigmented.

Secondary chemistry. Four chemotypes (races) are known: 1) usnic and salazinic acids; 2) usnic and divaricatic acids (previously unreported for this taxon); 3) usnic and protocetraric acids; 4) usnic acid only (acid-deficient).

Ecology and distribution. This lichen grows on non-calcareous, maritime rocks in Japan and Korea.

The protocetraric chemotype of *Ramalina semicuspidata* is rare, being known from only a handful of Japanese samples (Culberson 1970; Hamada 1985; Kashiwadani 1992). Unfortunately, no fresh material of the protocetraric chemotype was available for DNA extraction but the known specimens are morphologically indistinguishable from all other individuals included in this study, so they are provisionally included within *R. semicuspidata*. The divaricatic acid race (reported here as new), the salazinic acid race, and the acid-deficient races, by contrast, are commonly found in both Japan and Korea.

Selected specimens examined. Race 1, usnic and salazinic acids. **Japan:** *Hokkaido*: Prov. Nemuro, Cape Nosappu, *Kurokawa* 65711, *Lich. Rar. Crit. Exs.* no. 679 (TNS, US). *Honshu*: Prov. Izu (Shizuoka Pref.), Kamo-gun, Minamiizu-cho, Irozaki Harbour, *Kashiwadani* 51488 (TNS); Shimoda-city, Cape Tsumekizaki, *Kashiwadani* 51491 & 51493 (CPU, NIBR, TNS); *ibid.*, *Tanaka* s. n. (hb. *Kashiwadani* 51690, NIBR, TNS); Kamo-gun, Hamazaki-mura (Shimoda-city), Suzaki, *Kurokawa* s. n., *Lich. Jap. Exs.* no. 290 (TNS, US); Prov. Shima (Mie Pref.), Shima-gun. Daiwô-zaki, *Murai* s. n., *Lich. Jap. Exs.* no. 145 (TNS, US); Shimoda-city, Suzaki, *Lich. Min. Cogn. Exs.* no. 321, *Shibuichi* 8382 & *Yoshida* (DUKE, FH, TNS, US); Prov. Kii (Wakayama Pref.), Cape Kajino-zaki, Nishi-Muro-gun, *Watari* s. n. (DUKE). *Kyushu*: Prov. Bungo (Ohita Pref.), Ohita-city, Saganoseki-cho, Sekizaki, *Umezu* 4-1 (hb. *Kashiwadani* 51505, NIBR, TNS); Kita-amabe-gun, Saganoseki-cho, Sekizaki, *Matsumoto* & *Iwashina* s. n., *Lich. Min. Cogn. Exs.* no. 43 (TNS, US); Prov. Higo (Kumamoto Pref.), Amakusa-gun, *c.* 1.4 km NNE of Cape Shikizaki, Tomioka, *Moon* 16034 & *Kashiwadani* (NIBR); *ibid.*, *Kashiwadani* 51830, *Takeshita* & *Moon* (NIBR, TNS); Prov. Tsushima (Nagasaki Pref.), Tsushima-city, Kamitsushima-machi, south end of Mogihama swimming beach, *Kashiwadani* 51626 & *Moon* (NIBR, TNS). *Shikoku*: Prov. Awa (Kagawa Pref.), Shodo-gun, Shodo-shima Island, *Lich. Min. Cogn. Exs.* no. 170, *Moon* 3516 (DUKE, FH, TNS, US).—**Korea:** *Incheon*: Jabong-do

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Island, *Moon* 13197 (CPU, NIBR, TNS); Mo-do Island, Modo Port, *Moon* 15287 & *Kashiwadani* (NIBR, TNS). *Prov. Gangwon-do*: Yangyang-gun, Hajodae, *Kashiwadani* 51529 (NIBR, TNS). *Prov. Gyungsangbuk-do*: Pohang-shi, Nam-gu, near the Daedongbae Elementary branch school, *Moon* 13430 & *Kashiwadani* (NIBR). *Prov. Jeollanam-do*: Goheung-gun, Mondol beach, *Moon* 13712 (NIBR, TNS). *Prov. Jeju*: Jeju-shi, Yongduam Rock, *Moon* 15676 & *Kashiwadani* (NIBR, TNS).

Race 2, usnic and divaricatic acids. **Japan:** *Kyushu*: Prov. Tsushima (Nagasaki Pref.), Tsushima-city, Kamitsushima-machi, Ajiro, *Kashiwadani* 51634 & *Moon* (NIBR, TNS); *ibid*., *Kashiwadani* 51638 & *Moon* (NIBR, TNS).—**Korea:** *Incheon*: Shi-do Island, Sugi Beach, *Moon* 15291 & *Kashiwadani* (NIBR, TNS); Jabong-do Island, *Moon* 13195 (NIBR, TNS). *Prov. Gangwon-do*: Yangyang-gun, Sol Beach, *Kashiwadani* 51538 (NIBR, TNS). *Prov. Gyungsangnam-do*: Tongyoung-shi, Sanyang-eup, Shinjeon-ri, *Moon* 13377 (NIBR). *Prov. Jeju*: Jeju-shi, Yongduam Rock, *Moon* 15675 & *Kashiwadani* (NIBR, TNS); Seogwipo-shi, Cape Seopjikoji, *Moon* 15589, *Kashiwadani* & *Ahn* (NIBR, TNS).

Race 3, usnic and protocetraric acids. **Japan:** *Honshu*: Prov. Rikuzen (Miyagi Pref.), Oga-gun, Senjojiki, *Sasaki* 8224 (TNS); Prov. Shimofusa (Chiba Pref.), Unakami-gun, Cape Inobu, *Imazeki* s. n. (TNS); Prov. Izu (Shizuoka Pref.), Kamo-gun, N of Yahatano, *Kurokawa* 70981 & 70983 (TNS); S of Itho, Yahatano, Hasgudate, *Sasaki* s. n. (DUKE, TNS) (Kashiwadani 1992).

Race 4, usnic acid only. **Japan:** *Honshu*: Prov. Sagami (Shizuoka Pref.), Mitsuiwa, Cape Manazuru, *Hisauchi* 7 (TNS); Prov. Izu (Shizuoka Pref.), Kamo-gun, Tsumezaki, *Shibuichi* 4461 (TNS); *ibid*., *Kurokawa* 701009 (TNS); Prov. Noto (Ishikawa Pref.), Wajima-city, Aramiko-jima, *Satomi* s. n. (TNS) (Kashiwadani 1992); Prov. Owari (Aichi Pref.), Chita Peninsula, Cape Hazu, *Takahashi* 327 (TNS). *Kyushu*: Prov. Hizen (Nagasaki Pref.), *Faurie* s. n. (KYO, TNS).—**Korea:** *Incheon*: Jabong-do Island, *Moon* 13198 (NIBR, TNS); Jabong-do Island, around Meolgot, *Moon* 13489 & *Kashiwadani* (NIBR). *Prov. Gangwon-do*: Yangyang-gun, Hajodae, *Kashiwadani* 51532 (NIBR, TNS). *Prov. Gyungsangbuk-do*: Gyungju-shi, Gampo-up, Ohryu Beach, *Moon* 13460 (CPU, NIBR). *Prov. Jeju*: Jeju-shi, U-do Island, Dolkanee, *Moon* 15597, *Kashiwadani* & *Ahn* (NIBR, TNS); Pukcheju-gun (= Jeju-shi), Hado-ri, *Lich. Min. Cogn. Exs.* no. 216, *Kashiwadani* 43860 & *Moon* (DUKE, FH, TNS, US). *Prov. Jeollabuk-do*: Gunsan-si, Munyeo-do Island, *Moon* 13191 (NIBR). *Prov. Jeollanam-do*: Goheung-gun, Geogeum-do Island, Shinchon-ri, *Moon* 13914 (CPU, NIBR); Jindo-gun, Setbae shelter, *Moon* 15236 & *Ahn* (NIBR).

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