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Species delimitations in the *Cladonia cariosa* group (*Cladoniaceae*, Ascomycota)

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Abstract: Phenotypic characters, either morphological or chemical, have shown to be insufficient to delimit species boundaries in the genus *Cladonia*. The present study addresses the circumscription of species within the *Cladonia cariosa* group, examining a number of specimens of the currently accepted taxa *Cladonia cariosa*, *C. symphycarpa*, *C. acuminata*, *C. subcariosa* and *C. latiloba*. We employed maximum likelihood and Bayesian methods of phylogenetic reconstructions based on DNA sequences of ITS, *rpb2* and *ef1a* regions. Our results show that the *C. cariosa* group consists of at least four phylogenetic lineages. It is also shown that each of these lineages is chemically variable, which restricts the taxonomic value of the chemical differences within the group. However, anatomical differences, such as squamule surface and cortex structure, were found to correlate with the distinct lineages found in the phylogenetic analysis. This result confirms the taxonomic value of the cortical surface under SEM, as was found in other lichen groups.

Key words: chemistry, *Lecanorales*, lichens, taxonomy

Introduction

The circumscription of species in lichen-forming fungi has largely been based on morphological or chemical characters. Especially in groups of foliose and fruticose lichens, characters of the vegetative thallus have been widely used in the distinction of taxa. However, there is a growing body of evidence from DNA-based studies that morphological and chemical characters do not reflect the real number of species in lichenized fungi (Crespo & Lumbsch 2010). *Cladoniaceae* is a perfect example to illustrate the difficulties of species circumscriptions using phenotypic characters. Several phylogenetic studies have demonstrated a remarkable amount of phenotypic disparity in this family (Stenroos & DePriest 1998; Wedin *et al*. 2000; Stenroos *et al*. 2002*a*, *b*; Zhou *et al*. 2006; Lumbsch *et al*. 2010; Parnmen *et al*. 2010). In fact, it is well known among lichenologists that species recognition within the core genus of the family, *Cladonia*, is not an easy task (Thomson 1968; Ahti & Sohrabi 2006; Syrek & Kukwa 2008). Morphology-based species circumscriptions in *Cladonia* rely heavily on the secondary thallus, the so-called podetia, while the primary thallus is simpler and has been used to distinguish taxa only in a few instances (Ahti 2000). However, numerous species of *Cladonia* are morphologically variable (Ahti 2000; Kotelko & Piercey-Normore 2010), for example *C. furcata* (Huds.) Schrad. (Ahti & Hammer 2002), *C. ramulosa* (With.) J. R. Laundon (Ahti 2000; Burgaz & Ahti 2009) or *C. squamosa* Hoffm. (James 2009). The situation is complicated by chemical variability that is often not clearly associated with morphological differentiation.

Phylogenetic studies employing molecular data to address species circumscription in

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Cladonia have helped to elucidate evolutionary relationships, and to determine the taxonomic relevance of the phenotypic characters in species delimitation. For instance, in the *C. arbuscula* group, a number of taxa had been described on the basis of different morphologies. However, molecular data suggest that these morphotypes belong to one morphologically variable lineage (Piercey-Normore 2010). The chemical variability in the *C. chlorophaea* group could not be correlated with PCR-RFLP patterns found in studies which used ribosomal nuclear DNA (DePriest 1993, 1994). In the *C. pyxidata* and *C. pocillum* groups, neither species was found to be monophyletic, and the morphological variation was found to be determined by soil pH (Kotelko & Piercey-Normore 2010). In the cases of *C. subulata* (L.) F. H. Wigg. and *C. rei* Schaer., two morphologically and chemically variable species whose delimitation was disputed (Spier & Aptroot 2007), the presence of different secondary metabolites was shown to be correlated with different clades identified in phylogenetic studies using molecular data (Dolnik *et al*. 2010; Pino-Bodas *et al*. 2010).

This study focuses on the *Cladonia cariosa* group, defined by Culberson (1969), Harris (1975) and Culberson *et al*. (1993) as consisting of *C. brevis* (Sandst.) Sandst., *C. cariosa* (Ach.) Spreng., *C. polycarpia* G. Merr., *C. polycarpoides* Nyl., *C. sobolescens* Nyl. ex Vain., *C. subcariosa* Nyl., *C. subclavulifera* Asahina and *C. symphycarpa* (Flörke) Fr. These species are characterized by a persistent primary thallus and ascyphose podetia. Nevertheless *Cladonia brevis*, *C. polycarpia*, *C. polycarpoides*, *C. subcariosa*, *C. sobolescens* and *C. subclavulifera* differ in their secondary metabolites (Evans 1944; Culberson 1969; Park 1985; Huovinen *et al*. 1989; Culberson *et al*. 1993), but not in morphology, and so Ahti (2000) combined them in a single species, namely *C. subcariosa*. Consequently, the *Cladonia cariosa* group then consisted of *C. cariosa*, *C. subcariosa* and *C. symphycarpa*. *Cladonia subcariosa* was later shown to be distantly related to the *C. cariosa* group despite their morphological similarity (Kärkkäinen 2000). However, *C. cariosa* and *C. symphycarpa* constitute a monophyletic group along with *C. acuminata* (Ach.) Norrl. (Stenroos *et al*. 2002*a*). The latter differs morphologically from *C. cariosa* and *C. symphycarpa* in having sorediate podetia, unbranched or dichotomously branched near the tips (Ahti 2000). Hence, the *Cladonia cariosa* group currently includes *C. acuminata*, *C. cariosa* and *C. symphycarpa*, which are the focus of this study. These three taxa have production of the secondary metabolite atranorin and a calcareous substratum in common (Stenroos 2002*a*). The morphological characters used to distinguish *C. cariosa* from *C. symphycarpa* are subtle and variable and include squamule size and the amount of podetium fissures, which are more abundant in *C. cariosa* (Stenroos *et al*. 1992; Piercey-Normore 2003; Burgaz & Ahti 2009). Species identification is further complicated by the frequent lack of podetia in *C. symphycarpa* (Masselink & Sipman 1985; Carlin & Larsson 1994). The chemical variation has been widely employed to distinguish *C. cariosa* from *C. symphycarpa*, but it was found that they share several chemotypes (Harris 1975; Culberson *et al*. 1993; Piercey-Normore 2003; Bültmann & Lünterbusch 2008; Burgaz & Ahti 2009). *Cladonia cariosa* has seven chemotypes: atranorin only (the most common chemotype, including the type material); atranorin and fumarprotocetraric acid; atranorin and homosekikaic acid; atranorin and norstictic acid; atranorin and psoromic acid; atranorin and rangiformic acid or atranorin, fumarprotocetraric and rangiformic acids. Five chemotypes have been described for *C. symphycarpa*: atranorin only; atranorin and norstictic acid (the most common chemotype); atranorin, norstictic and stictic acids; atranorin and psoromic acid; and atranorin and fumarprotocetraric acid. The chemotype containing psoromic acid was described as *C. dahliana* (Kristinsson 1974), but some authors considered it as a synonym of *C. symphycarpa*, since they are morphologically indistinguishable (Ahti 1976; Randlane 1986; Ahti & Hammer 2002; Burgaz & Ahti 2009). The chemotype with stictic acid has been found only in Tierra de Fuego

(Stenroos & Ahti 1990; Stenroos *et al*. 1992). In specimens related to *C. symphycarpa* from Iceland and Andorra, bourgeanic acid was found (Culberson *et al*. 1993; Azuaga *et al*. 2001), but the identity of this material has not been confirmed. Given the high chemical variability of *C. cariosa* and *C. symphycarpa*, and the fact that several chemotypes are present in both species, the secondary metabolites cannot be used as discriminant characters to tell them apart. *Cladonia acuminata* consists of three chemotypes that are morphologically indistinguishable (Huovinen *et al*. 1989): atranorin and norstictic acid; atranorin only; and atranorin and psoromic acid. The chemotype with psoromic acid was described as C. *norrlinii* Vain. or *C. acuminata* var. *norrlinii* Lynge Ahti (2000) showed that the type material of *C. acuminata* var. *norrlinii* contains norstictic and not psoromic acid and consequently included this taxon in *C. acuminata*. In contrast, Harris (2009) described *C. acuminans* R. C. Harris as a different species from the psoromic acid chemotype, arguing that the distribution area of two chemical variants is different. Though both chemotypes coexist in North America, the one with psoromic acid is not present in Europe.

The aim of this study is to elucidate the species boundaries within the *C. cariosa* group and to examine whether the chemically variable taxa *C. acuminata*, *C. cariosa* and *C. symphycarpa* are correlated with phylogenetic lineages.

Materials and Methods

Taxon sampling

In this study we checked the identifications of 323 specimens of the following species: *Cladonia cariosa* (114 samples), *C. symphycarpa* (140 samples), *C. acuminata* (19 samples), *C. subcariosa* (49 samples) and *C. latiloba* Ahti & Marcelli (1 sample). The specimens are held in the herbaria B, BG, H, L, MACB, S and UPS, including the types of *C. cariosa* (H-ACH-1577) and *C. symphycarpa* (UPS). For the molecular study, specimens were chosen from different geographical origins, including most of the known chemical variability in the group (Table 1). The species were identified using morphological characters (squamule size and morphology of podetia), according to Ahti (2000), Ahti & Hammer (2002) and Burgaz & Ahti (2009). *Cladonia subcariosa* and *C. latiloba* were used here as outgroup, based on Kärkkäinen (2000) and on our data (Appendix 1). Though they do not belong to the *C. cariosa* group, *Cladonia cariosa* and *C. latiloba* are basal to it.

DNA extraction, PCR amplification and DNA sequencing

Before DNA extraction, the secondary metabolites were extracted by soaking the specimens in acetone for two hours, and the liquid was then used for thin-layer chromatography (TLC). The DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) was used to extract DNA, according to the manufacturer's instructions. The DNA was dissolved in 200 µl of buffer included in the kit. The three following loci were amplified: nuclear ITS rDNA using primer ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990), *rpb2* using two pairs of primers, RPB2-5F/RPB2-7R (Liu *et al*. 1999) and RPB2dRaq/RPB2rRaq (Pino-Bodas *et al*. 2010), and *ef1a* using CLEF-3F/CLEF-3R (Yahr *et al*. 2006). PCRs were carried out with Ready-to-Go-PCR Beads (GE Healthcare Life Sciences, UK). The volume of reaction was 25 µl for each tube, with 0·4 mM final concentration of primers. The volume of extracted DNA used for the PCR was 1 µl. The amplification programs were: 1) 94°C for 5 min; 5 cycles of 94°C for 30 s, 54°C for 30 s and 72 °C for 1 min; and 33 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 1 min; with a final extension of 72°C for 10 min (Martín & Winka 2000) for nuclear ITS rDNA, 2) initial denaturation at 94°C for 5 min; 40 cycles of 95°C for 1 min, 52°C for 30 s and 72°C for 2 min; with a final extension at 72°C for 10 min for *rpb2* region, and 3) initial denaturation at 94°C for 5 min; 35 cycles of 95°C for 1 min, 55°C for 30 s and 72°C for 1 min; with a final extension at 72°C for 10 min for *ef1a* region. PCR products were purified using the QIAquick gel extraction Kit (QIAGEN, Hilden, Germany). The purified DNA was dissolved in 40 µl of buffer included in the kit. The sequencing reactions were done at Secugen S. L. (CIB, Madrid, Spain) and Macrogen (South Korea) service (www.macrogen.com), with the same primers used for the PCR.

Sequence alignment and phylogenetic analysis

The alignments were made manually with SE-AL v2.0a11 (Rambaut 1996) for each locus separately. Eight ambiguous positions in the ITS rDNA matrix were removed, while the matrices of *ef1a* and *rpb2* did not contain ambiguous positions. Each region was analyzed by maximum parsimony (MP) and maximum likelihood (ML). MP analyses were made using PAUP version 4.0.b.10 (Swofford 2002), using heuristic searches with 1000 random taxon-addition replicates with TBR branch swapping and MulTrees option in effect, equally weighted characters and gaps treated as missing data. For the confidence analysis the bootstrap was applied, with 10 000 replicates, using the fast-step option. Congruence among the different topologies

TABLE 1. *List of taxa and specimens, locality, collection and GenBank accession numbers used in this study*

	Taxon	Locality and collection	ITS	*rpb2*	*ef1a*
Clade A	*C. cariosa* 1	Spain, Teruel, MACB 45292	JN621908	JN621940	JN621972
	C. cariosa 2	Spain, Lérida, MACB 94208	JN621909	JN621941	JN621973
	C. cariosa 3	USA, Michigan, S F53032	JN621912	JN621944	JN621976
	C. cariosa 4	Norway, Nord-Trondelag, BG L79658	JN621913	JN621945	JN621977
	C. cariosa 5	Finland, Uusimaa, H	JN621915	JN621947	JN621905
	C. cariosa 6	Finland, Tavastia Proper, H	JN621916	JN621948	JN621979
	C. cariosa 7	Russia, Karelia Republic, H	JN621917	JN621949	JN621980
	C. cariosa 8	Canada, Manitoba, H	JN621934	JN621950	JN621981
	C. cariosa 9	Spain, Barcelona, MACB 94207	JN621907	JN621939	JN621971
Clade B	*C. symphycarpa* 1	Spain, Burgos, MACB 93496	JN621918	JN621951	JN621982
	C. symphycarpa 2	Spain, Guadalajara, MACB 93559	JN621919	JN621952	JN621983
	C. symphycarpa 3	Sweden, Öland, S L50055	JN621923	JN621956	JN621988
	C. symphycarpa 4	USA, Michigan, S F53075	JN621924	JN621957	JN621989
	C. symphycarpa 5	Germany, Oldenburg, B 60 0122320	JN621925	JN621958	JN621990
	C. symphycarpa 6	Germany, Oldenburg, B 60 0125267	JN621926	JN621959	JN621984
	C. symphycarpa 7	Bosnia and Herzegovina, Sarajevo, MACB 101124	JN621931	JN621964	JN621995
	C. symphycarpa 8	Norway, Nordland, BG L784035	JN621914	JN621946	JN621978
Clade C	*C. acuminata* 1	USA, Alaska, H	JN621932	JN621965	JN621996
	C. acuminata 2	Canada, Manitoba, H	JN621933	JN621966	JN621997
	C. acuminata 3	Chile, Región XII Magallanes y Antartida, MACB 92017	JN621920	JN621953	JN621985
	C. acuminata 4	Spain, Palencia, MACB 92739	JN621922	JN621955	JN621987
	C. acuminata 5	Canada, Manitoba, H	JN621928	JN621961	JN621992
Clade D	*C. cariosa* s. lat. 1	Spain, Gerona, MACB 94205	FR695863	HQ340075	JN621904
	C. cariosa s. lat. 2	Portugal, Tras-os-Montes, MACB 93984	JN621906	JN621938	JN621970
	C. cariosa s. lat. 3	Spain, Ávila, MACB 93018	JN621910	JN621942	JN621974
	C. cariosa s. lat. 4	Spain, Granada, MACB 92995	JN621911	JN621943	JN621975
	C. symphycarpa s. lat. 1	Spain, Madrid, MACB 92737	JN621921	JN621954	JN621986
	C. symphycarpa s. lat. 2	Austria, Steiermark, UPS L135579	JN621927	JN621960	JN621991
	C. symphycarpa s. lat. 3	Ukranie, Donts'k Oblast, H	JN621930	JN621963	JN621994
	C. symphycarpa s. lat. 4.	Russia, Tuva Republic, H	JN621929	JN621962	JN621993
Outgroup	*C. subcariosa*	USA, New Jersey, H	JN621936	JN621968	JN621999
	C. subcariosa	USA, North Carolina, H	JN621935	JN621969	JN622000
	C. latiloba	Brazil, Santa Catalina, H	JN621937	JN621967	JN621998

inferred from the loci was tested following Lutzoni *et al.* (2004). Each clade with more than 75% bootstrap support in the single-gene analyses was scanned for conflict among loci. Since no incongruence was detected among loci, the datasets were combined. MrModeltest (Nylander 2004) was used for selecting the most appropriate nucleotide substitution model for each locus using the AIC criterion. The combined dataset was analyzed by ML and a Bayesian approach. The ML analysis was implemented using Tree-Puzzle 5.2 (Schmidt *et al.* 2002) assuming a GTR+I+G model. The Bayesian analysis was carried out using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). The combined dataset was partitioned into seven sections: ITS rDNA, and each of three codon positions of *ef1a* and *rpb2*, respectively. The model SYM+G was applied to the ITS part and each partition of *ef1a*, while the K80+G model was used for all partitions of *rpb2*. The posterior probabilities were approximated by sampling trees using Markov Chain Monte Carlo (MCMC). The posterior probabilities of each branch were calculated by counting the frequency of trees visited during MCMC analysis. Two simultaneous runs with 20 000 000 generations, each starting with a random tree and employing 4 simultaneous chains, were executed. Every 1000th tree was saved into a file. The first 1 000 000 generations (i.e. the first 1000 trees) were deleted as the 'burn in' of the chain. AWTY (Nylander *et al.* 2008) was used to determine when the chains reached the stationary stage. The 50% majority-rule consensus tree was calculated using the 'sumt' command of MrBayes.

The pairwise fixation index F_{ST} (Weir & Cockerham 1984) was calculated with DnaSP v. 5 (Librado & Rozas 2009) using the combined dataset. The F_{ST} was

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employed to estimate the differentiation among the clades following O'Brien *et al.* (2009) and Leavitt *et al.* (2011).

Morphology and chemistry

Microscopic measurements of the squamule thickness were carried out using hand-cut transverse sections. Three squamules of the primary thallus were measured for each specimen included in the molecular analyses (except when the material was scarce, in which case only one or two squamules were measured). The podetial squamules of *C. acuminata* were not studied. In addition, transverse sections of the squamules, cut with a Micron-ACP freezing microtome and stained with lactophenol blue solution, were made to study the cortex structure. The surfaces of the squamules were observed by Scanning Electron Microscopy (SEM) using a Hitachi S-3000N, and the specimens were prepared according to Hale (1973), vacuum-coated with gold-paladium and without critical-point drying. The statistical analyses of length, breadth, incision and thickness of the squamules, thickness of the cortex, the algal layer and the medulla were carried out with the STATGRAPHICS 5.1 software program. The Kolmogorov-Smirnov test was used to check normality, and Levene's test for equality of variances. All variables were normal and had homogeneous variances, hence we used one-way ANOVA to analyze the association of characters among the clades found in phylogenetic analyses, according to Wirth *et al.* (2008), Murillo *et al.* (2009) and Rivas-Plata *et al.* (2011). The probability level for significance was set at $P < 0{\cdot}05$. A Tukey HSD post-hoc test was performed to identify which differences among clades were significant.

The chemical composition was studied by TLC in 132 specimens following White & James (1985), using solvent systems A and B. Specimens studied by other researchers using TLC were not rechecked (unless they were used for the molecular study). Some old material not suitable for molecular studies was also not checked.

Results

Phylogenetic analyses

A total number of 97 new sequences was generated (Table 1) for this study (32 ITS rDNA, 32 *rpb2* and 33 *ef1α* sequences). The combined data matrix contained 2154 characters (628 in the ITS rDNA, 628 in the *ef1α* and 898 in the *rpb2* dataset), 1817 of which were constant, and 225 parsimony-informative (76 in the ITS rDNA, 54 in the *ef1α* and 95 in *rpb2* dataset). MP analysis generated 24 equally parsimonious trees, 490 steps long, with CI = 0·747 and RI = 0·893. ML analysis yielded a tree with a likelihood value of LnL = −6393·86, while the mean likelihood of the Bayesian tree sampling was LnL = −6108·97.

The phylogenetic reconstructions of the combined dataset using MP, ML and Bayesian analyses yielded trees with similar topologies. Figure 1 shows the tree of the Bayesian analysis. The specimens of the *C. cariosa* group form a strongly supported monophyletic group in all analyses. Clade A gathers only *C. cariosa* s.str. specimens, and clade B includes only *C. symphycarpa* specimens. *Cladonia acuminata* specimens (clade C) form a monophyletic group. A fourth clade (clade D) includes specimens that were identified as *C. cariosa* or *C. symphycarpa* on the basis of morphology and chemistry. The pairwise F_{ST} values showed genetic differentiation among the clades. The values varied from 0·59 to 0·79 (Table 2).

Morphological and chemical results

A re-examination of morphological characters revealed differences among the clades found in the phylogenetic analysis of the DNA sequence data. Clade A includes specimens with squamules significantly shorter than those in clades B and D and thinner than in clade B (Tables 3, 4). These squamules can be entire or have incisions that reach up to 30% of the entire squamule length. When observed using SEM, the surface of the squamules is smooth in some specimens, while in others it shows fissures and small cells that commonly do not exceed 10 μm in diameter (Fig. 2A). In transverse sections the cortex is smooth, and two layers can be distinguished within the upper cortex (Fig 2B). The outer cortex does not stain in lactophenol cotton-blue, indicating that this layer consists of dead mycobiont hyphal cells (i.e. an epinecral layer). Specimens in clade B usually have cracked squamules, which are significantly longer and thicker than those of clade A (Table 4). Their surface appears nearly smooth in SEM images, but with wide, shallow fissures (Fig. 2C). The cortex in clade B is thick and homogeneous and lacks an epinecral layer (Fig. 2D). Clade C includes specimens with squamules similar

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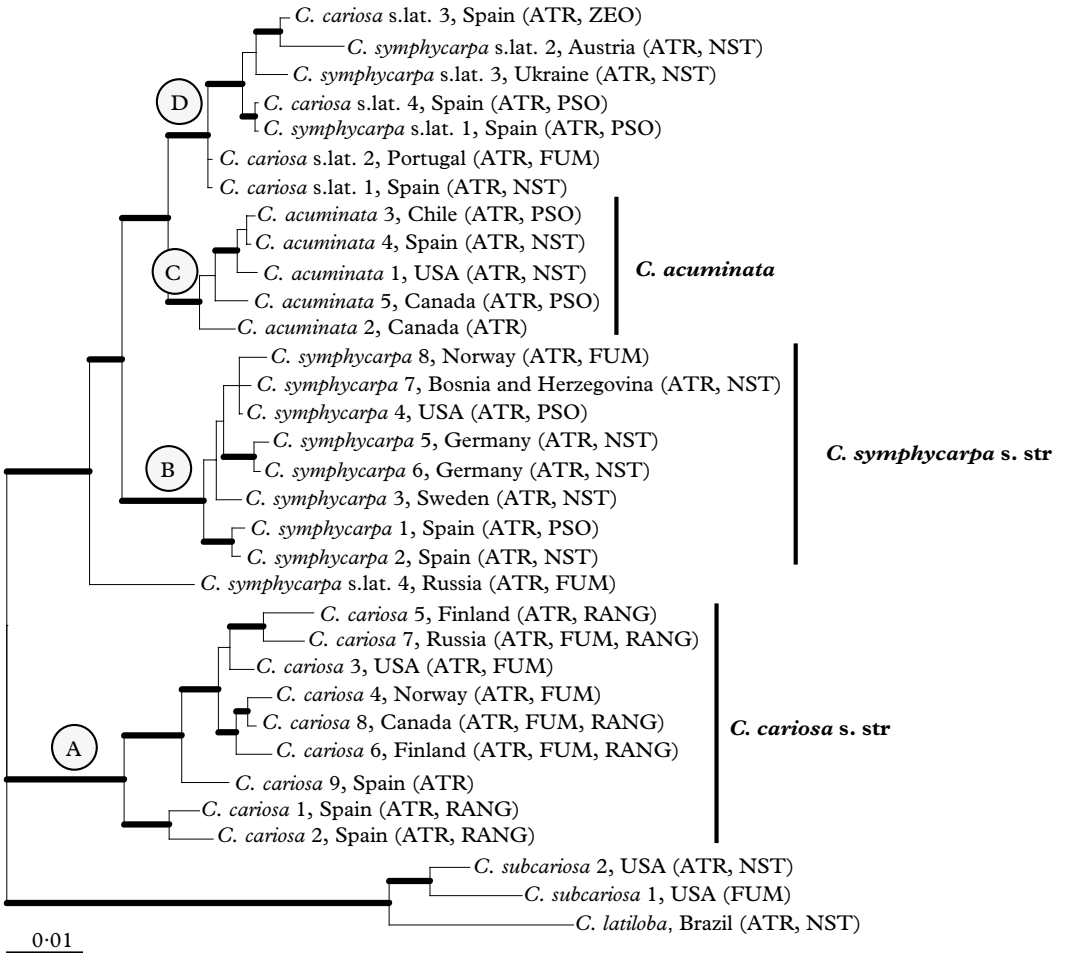

FIG. 1. Phylogeny of the *Cladonia cariosa* group based on a combined dataset (ITS rDNA, *rpb2* and *ef1a*). This is a 50% majority rule consensus tree of a Bayesian analysis. Branches supported with posterior probability ≥ 0·95 and bootstrap > 70 % are indicated in bold. ATR = atranorin, FUM = fumarprotocetraric acid, NST = norstictic acid, PSO = psoromic acid, RANG = rangiformic acid.

in size to those of clade B. The surface of the squamules is rough, with shallow cracks, showing an areolate-verrucose surface (Fig. 2E), and the cortex is homogeneous, without an epinecral layer (Fig. 2F). Squamules in clade D are similar in size to those in clades B and C. The squamule surfaces are strongly fissured, usually showing small cells about 5 μm in diameter, similar to those in clade A (Fig. 2G). Transverse sections of the cortex show a similar anatomy to those in clades B and C, lacking a well differentiated epinecral layer (Fig. 2H).

TABLE 2. *Pairwise F_{ST} values for combined dataset among clades*

	F_{ST}
Clade A-B	0·79823
Clade A-C	0·79425
Clade A-D	0·78799
Clade B-C	0·78610
Clade B-D	0·79260
Clade C-D	0·59728

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TABLE 3. *Variation (mean ± standard deviation) of morphological and anatomical characters of squamules in comparison with phylogenetic pattern*

	Clade A $n = 20$	Clade B $n = 24$	Clade C $n = 12$	Clade D $n = 20$	*P*
Length (mm)	2·51 ± 1·136	5·97 ± 2·397	5·04 ± 2·685	5·81 ± 1·419	0·002*
Width (mm)	1·19 ± 0·235	1·91 ± 0·653	2·22 ± 0·824	1·99 ± 0·727	0·03 *
Incision/length (mm)	0·12 ± 0·147	0·36 ± 0·201	0·23 ± 0·189	0·32 ± 0·259	0·06
Thickness (µm)	236·56 ± 65·771	340·09 ± 75·180	318·59 ± 64·452	275·84 ± 28·748	0·008*
Cortex (µm)	45·31 ± 10·134	62·93 ± 9·999	62·37 ± 9·265	60·10 ± 9·811	0·002*
Algal layer (µm)	29·71 ± 4·044	35·43 ± 6·348	37·22 ± 8·656	34·33 ± 4·210	0·091
Medulla (µm)	161·55 ± 58·114	241·73 ± 72·639	219 ± 57·968	182·63 ± 21·878	0·031*

* significant *P* values (< 0·05).

TABLE 4. *Tukey's multiple comparison test for significant results of the ANOVA analyses*

	Length	Width	Thickness	Cortex	Medulla
Clade A-B	$P < 0·05$	ns	$P < 0.05$	$P < 0·05$	$P < 0·05$
Clade A-C	ns	$P < 0·05$	ns	$P < 0·05$	ns
Clade A-D	$P < 0·05$	ns	ns	$P < 0·05$	ns
Clade B-C	ns	ns	ns	ns	ns
Clade B-D	ns	ns	ns	ns	ns
Clade C-D	ns	ns	ns	ns	ns

ns = not significant.

Table 5 summarizes the chemical results. The most frequent chemotype in *C. cariosa* is the one which contains atranorin alone, present in 18 specimens. In *C. symphycarpa* the most common is the chemotype with atranorin and norstictic acid. This chemotype is also the most frequent in *C. acuminata*. The chemotype of *C. cariosa* containing atranorin and homosekikaic acid, and the chemotypes of *C. symphycarpa* containing stictic acid and bourgeanic acid were not found among the specimens examined. The distribution of the different chemotypes in the clades, resulting from phylogenetic analyses, is shown in Figure 1 (which also shows the secondary metabolites found in the outgroup). Each clade included samples of several chemotypes.

Discussion

The diversity in this group falls into four strongly supported monophyletic lineages. The results of the F_{st} value estimations show low gene flow among these clades and support the separation of the distinct clades in the group. The use of F_{st} values to assess the gene flow and genetic differentiation, and to test species boundaries, follows Porter (1990). This author's method, known as Hybrid Zone Barrier, is especially useful in the recognition of species that have recently diverged (Shaffer & Thomson 2007). Subsequently, the F_{st} value has been applied to species delimitation in different organisms (Milankov *et al*. 2008; Korczynska *et al*. 2010; Mendonça *et al*. 2011), including lichenized fungi (O'Brien *et al*. 2009; Leavitt *et al*. 2011).

Following a genealogical concordance phylogenetic species recognition concept (Taylor *et al*. 2000), four species should be distinguished in the *C. cariosa* group instead of the three currently accepted. These four lineages are also supported by subtle morphological differences, such as squamule

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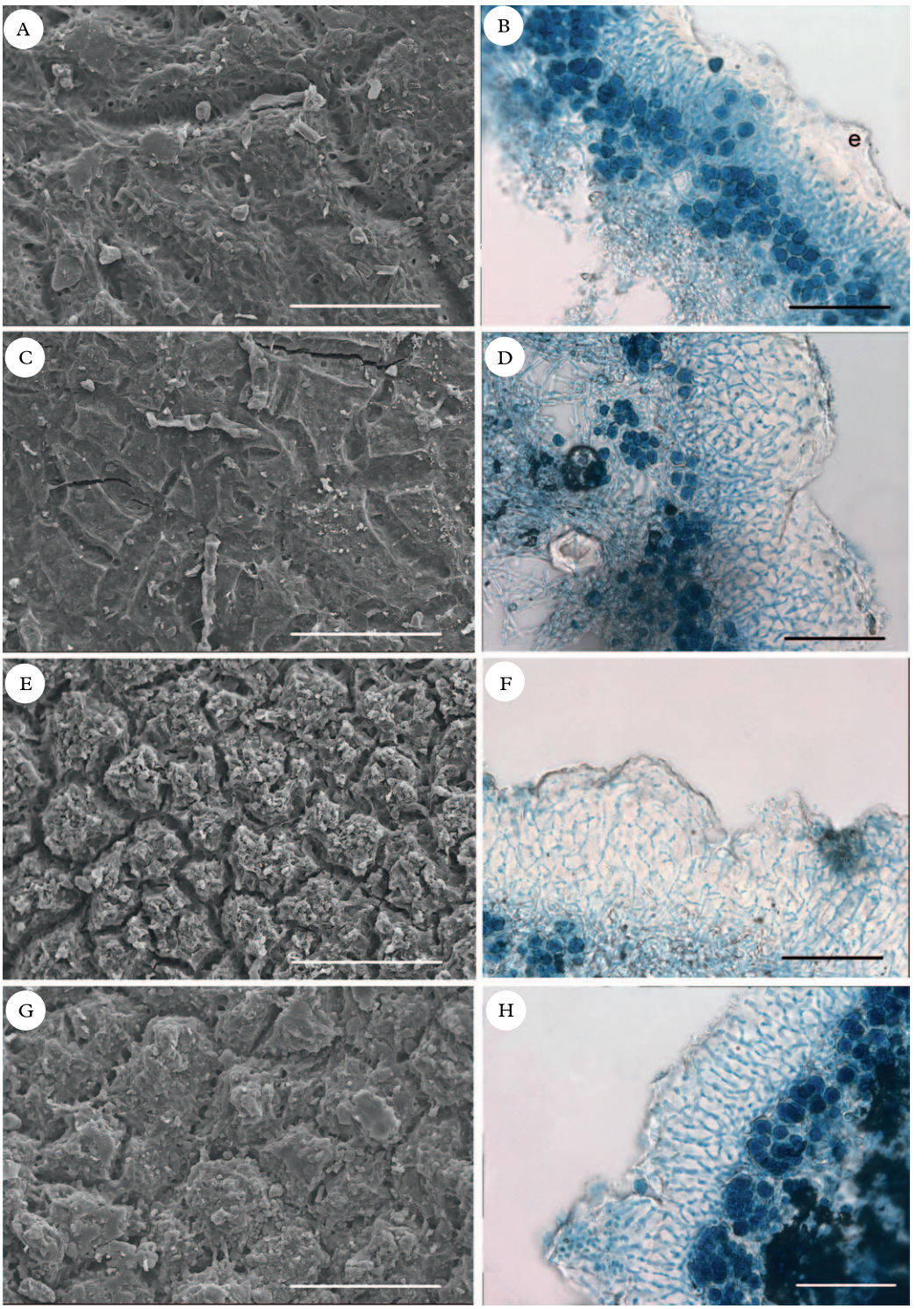

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size, thickness and surface of the squamules, and the cortex structure. These characters had previously been proposed by some authors to delimit the species within this group. The length, breadth and thickness of the primary squamules were used by Thomson (1983) and Ahti (2000) to distinguish *C. cariosa* from *C. symphycarpa*, and by Merrill (1909) to distinguish *C. subcariosa* from *C. symphycarpa*. Ahti (2000) and Ahti & Hammer (2002) noticed that squamule surfaces of *C. symphycarpa* were papillose-maculate when squamules reached maturity. Based on these preliminary observations, we were encouraged to study the squamule surfaces in more detail. The SEM observations show the existence of some micromorphological differences in the cortical surface of the different phylogenetic lineages that were previously unnoticed. The taxonomic value of the cortex surface was noted in *Alectoria* and *Cornicularia* (Hawksworth 1969) and in other *Parmeliaceae* (Hale 1973). In contrast to subtle morphological characters, our study clearly demonstrates that presence or absence of secondary metabolites is of limited taxonomic value in the *C. cariosa* group, as previously suggested (Bültmann & Lünterbusch 2008; Burgaz & Ahti 2009). A number of lineages include different chemotypes, and these chemotypes are largely shared among lineages. This is consistent with studies in some other groups of lichenized fungi, such as *Bryoria* in which the sections did not correlate with chemical characters (Myllys *et al.* 2011) or *Xanthoparmelia*, in which lineages consisted of different chemotypes (Leavitt *et al.* 2011).

Below, we attempt to clarify the identity of the clades found in our phylogenetic analyses, referring them to the current species and examining the appropriate type materials. Clade A contains specimens characterized by a small primary thallus (Table 2) and branched podetia, with many lengthwise fissures along them (Fig. 3A). As mentioned above, the chemistry of this clade is variable: atranorin only; atranorin and fumarprotocetraric acid; atranorin, fumarprotocetraric and rangiformic acids; and atranorin and rangiformic acid, but psoromic acid or norstictic acid are never present. The morphological characters of these specimens are similar to those of the *Cladonia cariosa* lectotype and we consider that this clade represents *Cladonia cariosa* s.str. Additional characters for this taxa have been found, such as the presence of a thick epinecral layer above the cortex in the primary thallus and a smooth or fissured surface (Fig. 2B).

The specimens within clade B have large squamules, often prostrate and cracked. The podetia (present only in two of the studied samples) are corticate, with areolate zones, and slightly fissured (Fig. 3D). The chemical variability of this clade includes: atranorin only; atranorin together with norstictic acid; atranorin and fumarprotocetraric acid; atranorin and psoromic acid. The morphology of these specimens is similar to the neotype of *C. symphycarpa*, and the four chemotypes described for *C. symphycarpa* (Huovinen *et al.* 1989) are present in this clade. We consider clade B as being *C. symphycarpa* s.str. Additional taxonomically useful anatomic characters have been found. The squamule surface is smooth in young parts, while in older zones (middle and inferior zone of the squamules) some wide, shallow fissures can be observed using SEM (Fig. 2C). The specimens containing psoromic acid do not form
a monophyletic clade, which is consistent with a taxonomic concept that includes *C. dahliana* as a chemotype within *C. symphycarpa*.

Clade C included all specimens identified based on morphology as *C. acuminata*, supporting that this species is monophyletic. The specimens have large squamules and sorediate, mostly unbranched podetia, that can be rarely dichotomously branched near

FIG. 2. *Cladonia cariosa* group, anatomy of the primary thallus in the different clades. A, C, E & G, SEM micrographs of squamule surfaces; B, D, F & H, transverse sections of squamules; A & B, clade A (e = epinecral layer); C & D, clade B; E & F, clade C; G & H, clade D. Scales: A, C, E & G = 100 μm; B, D, F & H = 50 μm. In colour online.

TABLE 5. *Chemical variation found in the* Cladonia *specimens examined*

ATR	FUM	NST	PSO	RANG	ZEO	*C. cariosa*	*C. symphycarpa*	*C. acuminata*	*C. subcariosa*	*C. latiloba*
+						18	3	1	—	—
+					+	3	—	—	—	—
+	+					10	3	—	—	—
+	+			+		3	—	—	—	—
+		+				9	38	7	1	1
+		+			+	—	1	—	—	—
+			+			3	10	2	—	—
+			+		+	1	3	—	—	—
+				+		2	—	—	—	—
		+				—	7	—	—	—
			+			—	4	1	—	—
+	+	+				—	1	—	—	—
	+					—	—	—	1	—

ATR = atranorin, FUM = fumarprotocetraric acid, NST = norstictic acid, PSO = psoromic acid, RANG = rangiformic acid and ZEO = zeorin.

the tips (Fig. 3C), as already described for this species (Ahti 2000; Ahti & Hammer 2002). The three chemotypes found for this taxon are present in our sampling. The three *C. acuminata* chemotypes form a single monophyletic group. This clade includes specimens originating from North America with the three chemotypes, along with a European sample which contains atranorin and norstictic acid. Consequently, we interpret *C. acuminans* as a synonym of *C. acuminata*. Additionally, *C. acuminata* differs from the other species in the areolate-verrucose cortical surface (Fig. 2E). A specimen from Spain that only had a primary thallus is included in this clade. The squamules of this sample are morphologically and anatomically similar to those of the other samples in the clade. This specimen extends the range in Europe of *C. acuminata*, the southern limit of which in Europe was in Tyrol (Nimis 1993).

Clade D includes specimens with a primary thallus consisting of large squamules, similar in size to those of *C. symphycarpa*, but with podetia (Fig. 3B) similar to those of *C. cariosa* (with many fissures). Chemically this clade is also variable. It includes the following chemotypes: atranorin and norstictic acid; atranorin and psoromic acid; atranorin and fumarprotocetraric acid; and atranorin and zeorin. The specimens in this clade are an intermediate morphotype between *C. cariosa* and *C. symphycarpa*. This putative species has squamule surfaces that are strongly fissured (Fig. 2G) and lacking an epinecral layer. Furthermore, while the other species occur on calcareous substrata, these samples are found on acid substrata at an altitude above 1000 m. No taxonomic conclusion is made here concerning this clade because Vainio (1887) described several taxa in the group and the type materials of these names need to be examined before taxonomic conclusions can be drawn.

One specimen (*Cladonia symphycarpa* sp.lat. 4) could not be assigned to any of the four major clades (Fig. 1) and may represent another species in the group. Additional studies including more samples of this group are needed to evaluate the taxonomic status

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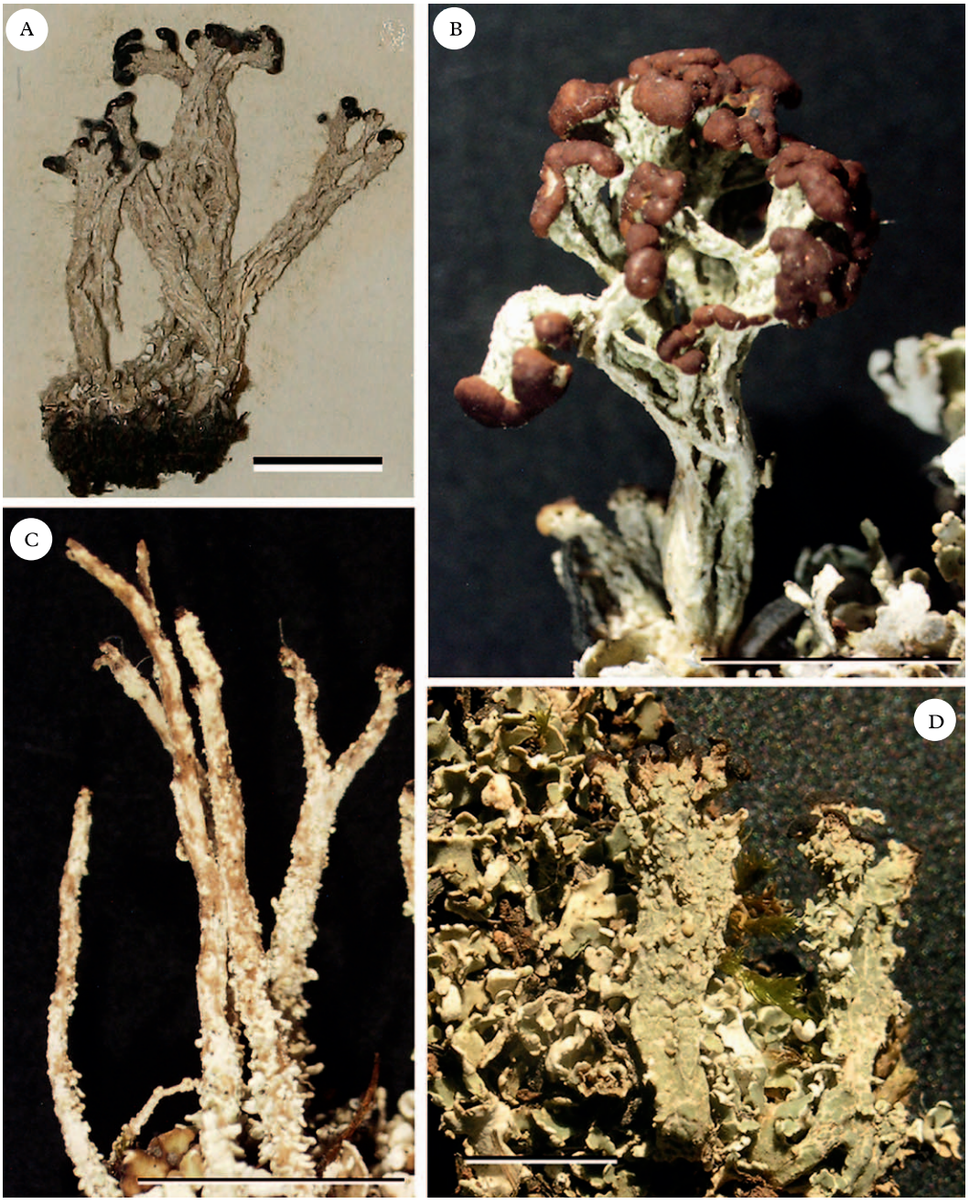

FIG. 3. Morphology of *Cladonia* species studied. A, *Cladonia cariosa* (lectotype); B, *C. cariosa* s. lat. (MACB 93018); C, *C. acuminata* (*T. Ahti* 63278); D, *C. symphycarpa* (MACB 101124). Scales = 5 mm. In colour online.

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of this specimen. In these future studies we will try to include more specimens and the chemotype of *C. cariosa*, containing atranorin and homosekikaic acid that was described from North America and Greenland (Harris 1975; Bültmann & Lünterbusch 2008).

This study suggests that the *C. cariosa* group contains a greater number of species than was traditionally recognized, and that there are subtle morphological differences among them.

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Appendix. The phylogenetic relationships of *Cladonia subcariosa* and *C. latiloba* with the *C. cariosa* group.

A matrix of ITS rDNA with 102 sequences from species included in the supergroup *Cladonia* (Stenroos *et al*. 2002) was constructed to infer phylogenetic relationships of *C. subcariosa* and *C. latiloba* with the *C. cariosa* group. Maximum Parsimony and Bayesian analyses were performed. The Bayesian analysis was carried out using the GTG+I+G model (this model was selected in MrModeltest as the best-fitting evolutionary model using the AIC criterion). The posterior probabilities of each branch were calculated by counting the frequencies of trees that were visited during the course of the MCMC analysis. Model parameters were estimated in each analysis for 10 000 000 generations sampled in 4 simultaneous chains, and every 1000th was saved into a file. The initial 1000 trees were discarded as burn-in. Using the "sumt" command of MrBayes, the 50% majority-rule consensus tree was calculated from 18 000 trees sampled after reaching likelihood convergence to calculate the posterior probabilities of the tree nodes.

The matrix contained 587 characters, 186 of them parsimony informative. MP analyses generated 1000 equally parsimonious trees, 795 step long, CI = 0·4579, RI = 0·7810 and RC = 0·3576. The Bayesian analysis yielded a tree with a likelihood value of Ln = −5435·17. *Cladonia subcariosa* and *C. latiloba* are not closely related to the *C. cariosa* group (Fig. A1). *Cladonia subcariosa* appears to be closely related to *C. caespiticia* with high support, while the relationship of *C. latiloba* is not resolved.

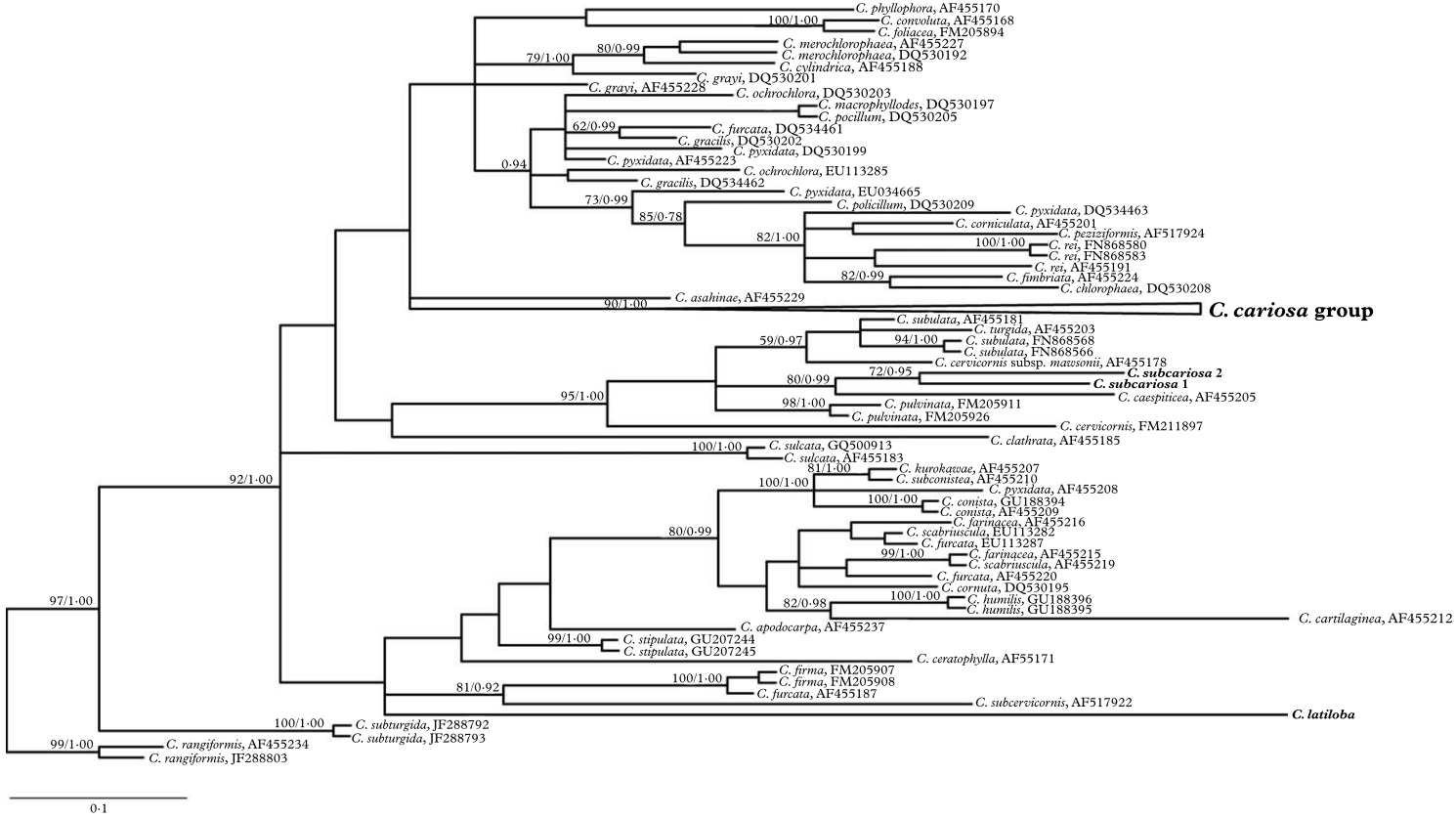

FIG. A1. Phylogenetic placement of *Cladonia subcariosa* and *C. latiloba* in the supergroup *Cladonia*. The 50% majority-rule consensus tree from a Bayesian analysis based on ITS rDNA. The boostrap values of MP analysis and posterior probability of Bayesian analysis are indicated on the branches.