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Genetic diversity and species delimitation of the zeorin-containing red-fruited *Cladonia* species (lichenized Ascomycota) assessed with ITS rDNA and *β*-tubulin data

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Abstract: Zeorin-containing red-fruited *Cladonia* species, the so-called *C. coccifera* group, are widespread terrestrial lichens which share most of their secondary substances but differ morphologically. The main objective of this study was to explore whether the current delimitation of these species is supported by molecular data. A total of 52 European and North American specimens of *C. coccifera*, *C. deformis*, *C. diversa*, and *C. pleurota* were examined. The internal transcribed spacer regions of the nuclear ribosomal DNA and the *β*-tubulin gene loci were sequenced for phylogenetic analyses. Traditional morphological species circumscriptions in zeorin-containing members of the *C. coccifera* group are not supported by molecular data. *Cladonia coccifera*, *C. deformis*, and *C. pleurota* were recovered as polyphyletic in both gene topologies; *C. diversa* formed a lineage in the ITS phylogeny but this was not statistically supported. We detected chemical patterns of the presence/absence of porphyrilic and/or isousnic acid which may help to characterize two lineages. Our results also show incongruence between the two molecular markers studied. Therefore, we focused on possible explanations of this phenomenon. Five major evolutionary mechanisms can potentially result in phylogenetic discordance between genes: presence of pseudogenes, horizontal gene transfer, gene paralogy, incomplete lineage sorting, and hybridization. These mechanisms are briefly discussed. We consider incomplete lineage sorting and/or hybridization to best explain the incongruence.

Key words: bootscanning, *Cladoniaceae*, Cocciferae, Lecanoromycetes, lichens, taxonomy

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Introduction

The genus *Cladonia* P. Browne represents one of the largest genera of lichen-forming fungi, with more than 400 described species (Ahti 2000). *Cladonia* species are often major contributors to overall biomass in the ground-layer vegetation in arctic and alpine tundra, in lichen woodlands, on rock outcrops, on heaths, and on peatlands (Lechowicz & Adams 1974). Several *Cladonia* species, commonly termed reindeer lichens, serve as a winter food source for animals. Other species are found in habitats where higher plants are not competitors, such as wood or burned habitats. These lichens usually develop two distinct kinds of thallus morphology: a horizontal primary thallus (foliose or crustose, largely absent in reindeer lichens) and a vertical secondary thallus called a podetium (fruticose, bearing the hymenia). These thalli are among the most complex and aesthetic in lichens and, not surprisingly, there is a tremendous variation in morphological details, which provides many characters for classification. As frequently found with lichens, the interpretation of phenotypic variation of the thallus has been controversial (Stenroos & DePriest 1998; Stenroos *et al.* 2002; Divakar *et al.* 2006; Grube & Hawksworth 2007).

The traditional species circumscription of *Cladonia* is based on morphological and chemical characters. However, several recent molecular studies have revealed a lack of

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correlation between morphological and molecular data, and many traditionally delimited species are problematic or even artificial in light of these data (Myllys *et al.* 2003; Kotelko & Piercey-Normore 2010; Piercey-Normore *et al.* 2010; Pino-Bodas *et al.* 2012*a*, *b*). The incongruence between morphological and genetic data is usually attributed either to significant intraspecific variation of the species as a response to environmental conditions, or to genetic recombination (e.g., Fontaine *et al.* 2010; Kotelko & Piercey-Normore 2010).

Zeorin-containing red-fruited *Cladonia* species are conspicuous lichens, two of which were distinguished by Linnaeus (1753). Similar to most other *Cladonia* species, members of this aggregate usually grow in habitats with a low rate of competition from vascular plants (e.g., on sandy or rocky soils, on thin soil over rock, on bark, or on rotten wood). Currently, the aggregate of zeorin-containing red-fruited and scyphose (cup-forming) *Cladonia* species consists of five species worldwide, of which four are known from Europe and North America [*C. coccifera* (L.) Willd., *C. deformis* (L.) Hoffm., *C. diversa* Asperges ex S. Stenroos, and *C. pleurota* (Flörke) Schaer]. The fifth species, *C. sinensis* S. Stenroos & J. B. Chen (Stenroos *et al.* 1994), has a limited distribution in South-East Asia, and was not included in the analysis.

This group of species is characterized by similar chemical patterns (presence of usnic acid derivates and zeorin, occasionally accompanied by porphyrilic acid), and species within this group are delimited morphologically. The size, shape and location of the vegetative propagules on the podetia are traditionally considered as the most important diagnostic characters separating species (e.g., Asperges 1983; Stenroos 1989). The shape and width of the podetium is another relevant morphological feature commonly used to distinguish the species belonging to this group (e. g., Asperges 1983; Osyczka 2011; Ahti & Stenroos 2012).

Cladonia coccifera (Fig. 1A & B) is an esorediate species with gradually expanded cups. The surface of the podetium is areolate corticate, covered by bullate and scaly plates. This species had often been confused with *C. diversa*, *C. pleurota* or *C. borealis* (Stenroos 1989; Osyczka 2011). *Cladonia deformis* (Fig. 1C & D) is easy to recognize when well developed. Podetia are usually tall, relatively narrow and farinose sorediate. However, it might also be short-podetiate and then difficult to distinguish from *C. pleurota* (Osyczka 2011). *Cladonia pleurota* (Fig. 1G & H) is morphologically very variable (Stenroos 1989). Young individuals are usually completely granulose sorediate, but when fertile the surface may turn almost totally corticate and is partly covered by granules or verruculae (Stenroos 1989). *Cladonia diversa* (Fig. 1E & F), described by Asperges (1983), is the most controversial species. The podetia of this species are usually slender and microsquamulose-granulose. Because of its obvious morphological similarity to *C. coccifera* (esorediate podetia covered by irregular plates and/or granules), the natural status of this species was disputed by Stenroos (1989). However, recently Ahti & Stenroos (2012) became "more convinced that it is an acceptable taxon".

Until now, no comprehensive attempt has been made to assess phenotypically circumscribed red-fruited *Cladonia* species within a molecular phylogenetic context. However, some species belonging to this aggregate were included in previous studies which focused on the generic phylogeny of *Cladonia* (Stenroos *et al.* 2002), a study of lichen diversity in some Antarctic regions (Lee *et al.* 2008), or a DNA-barcoding study of taxonomically diverse lichens in the UK (Kelly *et al.* 2011). Stenroos *et al.* (2002) examined four species belonging to this aggregate (from 1 to 4 specimens per species) and the possible polyphyly of *C. coccifera*.

Many phylogenetic surveys using sequence data inferred the evolution of *Cladonia* at higher taxonomical levels. Myllys *et al.* (2003) investigated the genetic diversity of two closely related putative species, *C. arbuscula* and *C. mitis*. The analysis involved four markers: ITS rDNA, a group I intron in SSU rDNA at position 1516 (according to *Escherichia coli* numbering), two introns in β-tubulin gene, and a single intron in the GAPDH

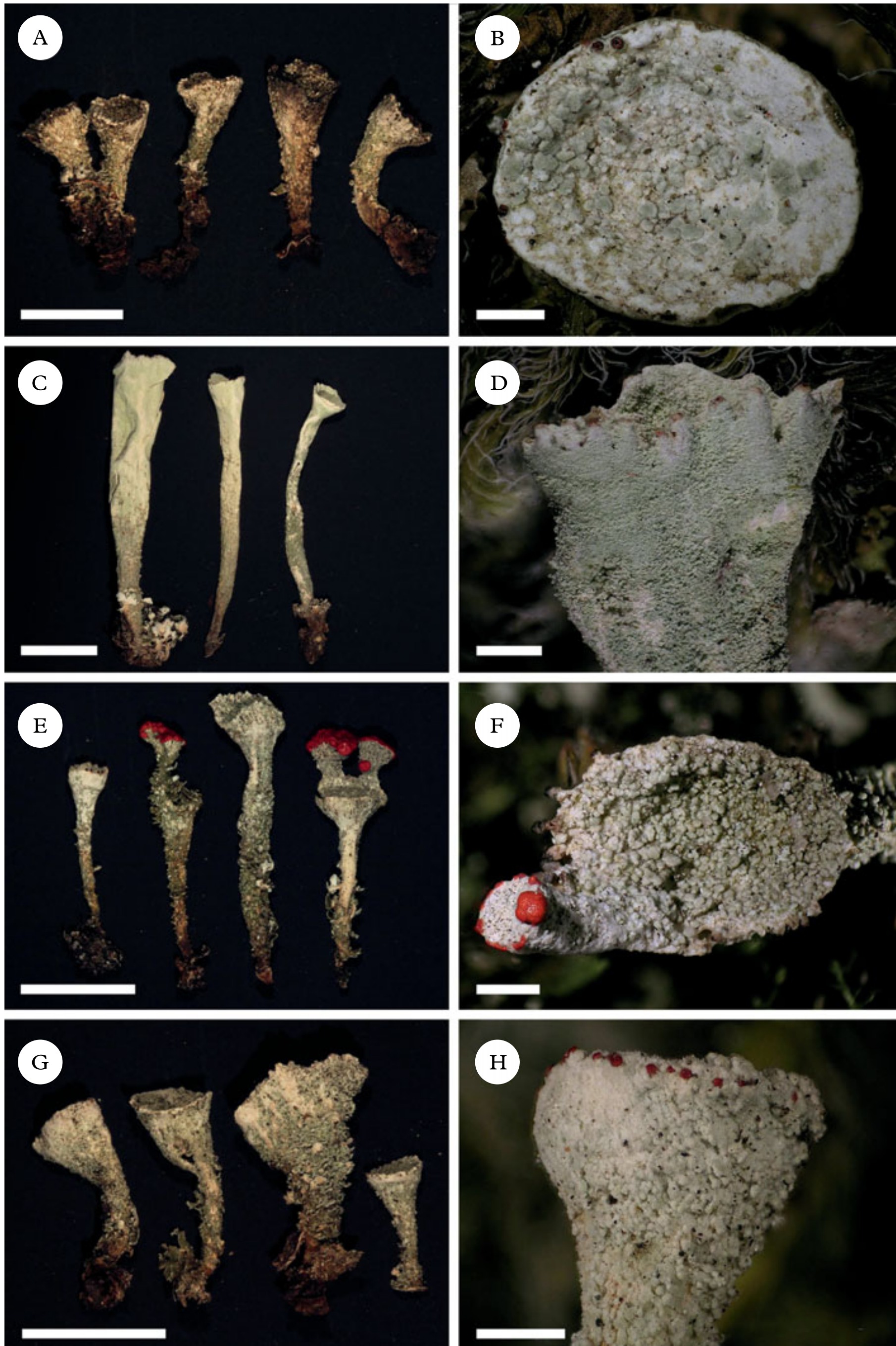

Fig. 1. Morphology of *Cladonia* species studied. A & B, *Cladonia coccifera* (CL179); C & D, *C. deformis* (CL176); E & F, *C. diversa* (CL173, topotype); G & H, *C. pleurota* (CL136). Scales: A, C, E & G = 5 mm; B, D, F & H = 1 mm. In colour online.

gene. Surprisingly, a significant conflict between the four gene regions was detected. According to their conclusions, Myllys *et al.* (2003) regarded either incomplete lineage sorting or recombination as the most likely reason for the incongruences among the markers. Recently, Fontaine *et al.* (2010) studied the *C. gracilis* complex by using ITS rDNA and polyketide synthase (PKS) genes and obtained similar results, but they suggest paralogy in both genes as an alternative explanation of the incongruence identified between individual gene trees.

In the present study, inferences from ITS rDNA and an intron-containing portion of the β-tubulin gene were used to explore the genetic diversity of currently recognized zeorin-containing red-fruited *Cladonia* species. We examined numerous collections of all the four currently accepted zeorin-containing red-fruited *Cladonia* species known from the European continent and North America. In addition, we address possible explanations for the incongruence between individual gene trees detected in this study, similar to other multilocus studies of *Cladonia* (Myllys *et al.* 2003; Fontaine *et al.* 2010).

Materials and Methods

Species sampling and determination

The material for this study was either collected by the authors or obtained from the following herbaria: BG, CBFS, GZU, NY, PL, PRA, PRC, and PRM. A total of 52 samples were collected, largely in Europe (44 specimens); eight collections were made in North America (Table 1). All the specimens were examined by the first author and revised by S. Stenroos and T. Ahti. Patterns in secondary metabolite variation were identified by thin-layer chromatography (TLC) on Merck silica gel 60 F254 pre-coated glass plates in solvent systems A, B and C, according to Orange *et al.* (2001). *Cladonia crispata* and *C. squamosa* were used as an outgroup, based on the study of Stenroos *et al.* (2002).

DNA extraction, PCR, and DNA sequencing

Fine ground lichen material was used for total genomic DNA extraction with the CTAB protocol (Cubero *et al.* 1999) or the Invisorb Spin Plant Mini Kit (Invitek). The fungal nuclear ITS region and an intron-containing portion of the β-tubulin gene were amplified with the following primers: ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990), and Bt3-LM and Bt10-LM (Myllys *et al.* 2001). In most cases, PCR reactions were prepared for a 30 µl final volume containing 4·05 µl double-distilled water, 3 µl 10 × *Taq* polymerase reaction buffer (10 mM Tris; pH 8·3), 1·8 µl $MgCl_2$ (25 mM), 3 µl of 2·5 mM dNTPs, 0·15 µl *Taq* DNA polymerase, 1·5 µl of each of the 10 mM primers. Amplifications consisted of an initial 2 min denaturation at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 54°C (ITS)/51°C (β-tubulin), 1 min at 72°C, and a final extension of 7 min at 72°C.

The PCR products were quantified on a 1% agarose gel stained with ethidium bromide and cleaned either with QIAquick PCR Purification Kit (Qiagen) or JetQuick PCR Product Purification Kit (Genomed), according to the manufacturer's protocols.

Sequencing of PCR products was performed with an Applied Biosystems (New York, USA) automated sequencer (ABI 3730xl) at Macrogen Corp. in Seoul, Korea. The PCR primers were also used for sequencing.

Sequence alignment and model selection

Sequences were initially aligned using CLUSTAL X 1.83 (Thompson *et al.* 1997) and MUSCLE (Edgar 2004). ITS sequences (comprising ITS1, 5.8S, and ITS2 regions) were aligned on the basis of their rRNA secondary structure information (see below) with MEGA 4 (Kumar *et al.* 2008). For subsequent phylogenetic analyses, the alignments were minimalized to contain the unique sequences only. Alignments can be downloaded at http://botany.natur.cuni.cz/algo/align/03_Cladonia_ITS.nex (ITS) http://botany.natur.cuni.cz/algo/align/03_Cladonia_BT.nex (β-tubulin).

For both ITS and β-tubulin datasets, suitable partitioning strategy and partition-specific substitution models were selected in a multi-step process (Verbruggen *et al.* 2010). Initially, guide trees were obtained by carrying out a second-level maximum likelihood (ML) search on the unpartitioned dataset with an HKY + Γ_8 model with TreeFinder (Jobb *et al.* 2004) by using the Bayesian information criterion (BIC). Then, the datasets were divided by five (ITS) and six (β-tubulin), respectively, different partitioning strategies. For each partition present in these partitioning strategies, 12 different nucleotide substitution models were evaluated (F81, HKY, GTR, and their combinations with Γ, I, and Γ +I). Subsequently, Bayesian information criterion (BIC) calculations were performed for all potential partitioning strategies, assuming the guide tree and evaluated models for each partition. For both datasets, three partitioning strategies with the best fit to the data (lowest BIC scores) were retained for further analysis. In the next step, the best models of sequence evolution were selected for individual partitions by using the BIC. Finally, the partitioning strategies were re-evaluated using the selected models for particular partitions. This BIC-based model selection procedure selected the following models. For the ITS rDNA dataset, the strategy with 3 partitions was selected: i) ITS1 region (HKY + Γ_8), ii) 5.8S rDNA (HKY), and iii) ITS2 region (HKY + Γ_8). In the case of the β-tubulin dataset, the strategy with two partitions was selected as the best: (i) first and second codon positions of exon (HKY), and (ii) third codon position of exon and intron region (HKY).

Table 1. *List of lichen taxa used in this study with collection information and GenBank accession numbers.*

Taxon name	DNA extraction No.	Collection No. (herbarium)	Locality	GenBank No.	
				ITS	β-tubulin
Cladonia deformis	C8	*Peksa* 918 (PL)	Czech Republic, Chvaletice	HE611205	HE611257
	CL102	*Steinová* 110 (PRC)	Czech Republic, Brdy, Žd'ár	HE611184	HE611236
	CL175	*Steinová* 330 (PRC)	Finland, Suomossalmi	HE611190	HE611242
	CL176	*Steinová* 336 (PRC)	Finland, Varkaus	HE611186	HE611238
C. diversa	CL54	*Bouda* 777*	Czech Republic, NP Českosaské Švýcarsko, Babylon	HE611164	HE611216
	CL106	*Steinová* 400 (PRC)	Portugal, Beira Alta, Parque Natural de Serra da Estrela	HE611165	HE611217
	CL130	*Vondrák* 6242 (CBFS)	Denmark, Bornholm, Jomfrugården	HE611166	HE611218
	CL172	*Steinová* 351 (PRC)	Belgium, Kalmthout, Van Ganzenven	HE611167	HE611219
	CL173	*Steinová* 352 (PRC)	Belgium, Kalmthout, Van Ganzenven – topotype	HE611168	HE611220
	CL174	*Steinová* 353 (PRC)	Netherlands, Grenspak De Zoom-Kalmthoutse Heide	HE611169	HE611221
C. coccifera	CL3	*Peksa* 84 (PL)	Czech Republic, Lužické hory, Studenec	HE611154	HE611206
	CL31	*Hafellner* 66608 (GZU)	Austria, Stubalpe, Größenberg	HE611155	HE611207
	CL32	*Hafellner* 66785 (GZU)	Austria, Stubalpe, Ofnerkogel	HE611156	HE611208
	CL39	*Hafellner* 66214 (GZU)	Austria, Stubalpe, Lichtengraben	HE611157	HE611209
	CL52	*Bouda* 778*	Czech Republic, Novohradské hory, Kraví hora	HE611158	HE611210
	CL60	*Peksa* 359 (PL)	Czech Republic, Lužické hory, Studenec	HE611159	HE611211
	CL90	*Steinová* 43 (PRC)	Czech Republic, Krkonoše, Velká kotelní jáma	HE611160	HE611212
	CL93	*Steinová* 81 (PRC)	Czech Republic, Českosaské Švýcarsko, Křepelčí důl	HE611161	HE611213
	CL105	*Steinová* 401 (PRC)	Spain, Somosierra, arroyo de la Peña del Chorro	HE611162	HE611214
	CL141	*Steinová* 242 (PRC)	Austria, NP Nockberge, Erlacher Bockhütte	HE611163	HE611215
	CL120	*Beeching* 3100 (NY)	USA, Missouri, Iron Co., Pilot Knob National Wildlife Refuge	HE611170	HE611222
	CL178	*Steinová* 332 (PRC)	Norway, NP Rondane, Einsethøe	HE611171	HE611223
	CL179	*Steinová* 334 (PRC)	Finland, Heinola, Pirttijärvi lake	HE611172	HE61122
C. pleurota	B18	*Peksa* 820 (PL)	Slovakia, Vel̆ká Fatra, Harmanec	HE611191	HE611243
	C6	*Peksa* 588 (PL)	Czech Republic, Chvaletice	HE611181	HE611233
	CL26	*Palice* 11305 (PRA)	Czech Republic, Dolní Loučky, Pásník	HE611193	HE611245
	CL36	*Hafellner* 65635 (GZU)	Austria, Stubalpe, Lahnhofen	HE611194	HE611246
	CL43	*Peksa* 562 (PL)	Czech Republic, Brdy, Hřebenec	HE611182	HE611234
	CL44	*Peksa* 564 (PL)	Czech Republic, Brdy, Hřebenec	HE611183	HE611235
	CL45	*Peksa* 563 (PL)	Czech Republic, Brdy, Hřebenec	HE611195	HE611247

Table 1. *Continued*

Taxon name	DNA extraction No.	Collection No. (herbarium)	Locality	GenBank No.	
				ITS	β-tubulin
C. pleurota	CL64	*Vondrák* 3631 (CBFS)	Romania, Retezat Mountains, Cheile Butii	HE611187	HE611239
	CL67	*Vondrák* 2868 (CBFS)	Czech Republic, Křivoklátksko, Na Andělu	HE611173	HE611225
	CL73	*Peksa* 574 (PL)	Czech Republic, Chvaletice	HE61117	HE611226
	CL77	*Steinová* 22 (PRC)	Austria, Zirbitzkogel, Linderhütte	HE611192	HE611244
	CL81	*Lendemer* 7139 (NY)	USA, New Jersey, Burlington Co., Rutgerds Pinelands Field Station	HE611175	HE611227
	CL84	*Steinová* 84 (PRC)	Czech Republic, Českosaské Švýcarsko, Křepelčí důl	HE611201	HE611253
	CL85	*Steinová* 103 (PRC)	Czech Republic, Brdy, Žd'ár	HE611196	HE611248
	CL98	*Steinová* 45 (PRC)	Czech Republic, Krkonoše, Kotel	HE611188	HE61124
	CL99	*Steinová* 99 (PRC)	Czech Republic, Brdy, Žd'ár	HE611202	HE611254
	CL100	*Steinová* 65 (PRC)	Czech Republic, Slavkovský Les, Křížky	HE611176	HE611228
	CL101	*Steinová* 108 (PRC)	Czech Republic, Brdy, Žd'ár	HE611203	HE611255
	CL104	*Steinová* 126 (PRC)	Czech Republic, Brdy, Hřebenec	HE611185	HE611237
	CL107	*Harris* 51548 (NY)	USA, Connecticut, Fairfield Co., Redding, Highstead Arboretum	HE611177	HE611229
	CL109	*Lendemer* 720 (NY)	USA, Missouri, Iron Co. Pilot Knob National Wildlife Refuge	HE611178	HE611230
	CL111	*Harris* 52433 (NY)	USA, Missouri, Iron Co. Pilot Knob National Wildlife Refuge	HE611179	HE611231
	CL113	*Lendemer* 10223 (NY)	Canada, Island of Newfoundland, Big Otter Pond	HE611197	HE611249
	CL115	*Lendemer* 10384 (NY)	Canada, Island of Newfoundland, Burry Heights Center	HE611198	HE611250
	CL117	*Lendemer* 10563 (NY)	Canada, Island of Newfoundland, Ha-Ha Mountain	HE611199	HE611251
	CL128	*Steinová* 164 (PRC)	Czech Republic, Sedlčansko, Drbákov-Albertovy skály	HE611180	HE611232
	CL136	*Steinová* 215 (PRC)	Finland, Helsinki, Rastila	HE611200	HE611252
	CL148	*Steinová* 241 (PRC)	Austria, Gurktaler Alpen, Nassbodensee	HE611189	HE611241
	CL150	*Steinová* 187 (PRC)	Finland, Vantaa, Fagersta	HE611204	HE611256

* private herbarium

Molecular data and phylogenetic analyses

Possible substitution saturation of both markers studied that would imply a low reliability of phylograms (Lopez *et al.* 1999; Muschner *et al.* 2003) was assessed by two different approaches. Firstly, we plotted the uncorrected distances against the corrected distances, determined with the respective model of sequence evolution estimated by the BIC-based model selection as described above (HKY + Γ_8 for ITS rDNA and HKY for the β-tubulin dataset). Secondly, the phylogenetic signal present in the data partitions was estimated by ML mapping (Strimmer & von Haeseler 1997) using the Tree-puzzle 5.2 program (Schmidt *et al.* 2002).

The phylogenetic trees were inferred with Bayesian inference (BI), Maximum Likelihood (ML) and Maximum Parsimony (MP). A Bayesian analysis was implemented using MrBayes version 3.1 (Ronquist & Huelsenbeck 2003). Two parallel MCMC runs were carried out for 2 million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF value between simultaneous runs was 0·004683 in ITS and 0·002003 in the β-tubulin. ML and MP phylograms were obtained using Garli version 2.0, and PAUP version 4.0b10 (Swofford 2002), respectively. The same programs were used for bootstrap analyses. ML analyses consisted of rapid heuristic searches (100 pseudo-replicates) by using automatic termination (the genthreshfortopoterm command set to 100 000). The weighted parsimony (wMP) bootstrapping (1000 replications) was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences (the number limited to 10 000 for each replicate), and gap characters treated as a fifth character state. The weight to the characters was assigned using the rescaled consistency index on a scale of 0 to 1000. New weights were based on the mean of the fit values for each character over all of the trees in memory.

The secondary structures of ITS sequences were constructed in order to detect presumed sexual barriers between studied species. The incompatibility for sexual reproduction between species can be ascertained by the presence of compensatory base changes (CBSs; so-called CBS approach) (Coleman 2000; Müller *et al.* 2007). The secondary structures of ITS sequences were constructed using the Mfold computer program version 2.3, (Walter *et al.* 1994; Zuker 2003), with the folding temperature set to 25°C. The structures were compared with published ITS secondary structures of *Cladonia* species (Beiggi & Piercey-Normore 2007). Common secondary structures were created by using RnaViz (version 2; De Rijk *et al.* 2003) and used to identify compensatory base changes (CBCs) and hemi-CBCs.

Analyses of hybridization

Two different attempts were used to detect hybridization events in the diversification of *Cladonia* species. Firstly, incongruence between the ITS and β-tubulin-derived trees was examined using NeighborNet analysis as implemented by the program Splits Tree 4 (Huson & Bryant 2006). This method provides a visualization of the extent to which a collection of gene trees suggests contradictory taxon relationships. If a collection of gene trees has congruent topologies, consensus networks will be tree-like, and where the relationships are incongruent, the graphs will be net-like (McBreen & Lockhart 2006). To explain the incongruent relationships displayed by a network analysis in terms of reticulation events, a consensus network was constructed.

Secondly, the evidence of hybridization was evaluated by the bootscanning method (Salminen *et al.* 1995) on the concatenated sequence dataset. We used two different programs to run bootscanning analyses: 1) the alignment was analyzed by SimPlot version 3.5.1 (Lole *et al.* 1999), using the bootscan option and default settings; 2) several different algorithms (RDP, GENECONV, Chimaera, MaxChi, BootScan, SiScan and 3Seq) were run to determine the presence of recombination using the Recombination Detection Program, Rdp3, v. 3.22 (Martin *et al.* 2010). In the case of positive recombination detection, bootstrap support curves were visualized to locate hybrid sequences, and to reveal potential parent sequences present in the alignment.

Results

Secondary chemistry

All samples studied contained zeorin and usnic acid as major lichen substances. We detected four chemotypes which differ by the presence/absence of accessory substances isousnic and porphyrilic acids. Isousnic acid (chemotype 1) was present in 19 specimens, porphyrilic acid in 12 specimens (chemotype 2), both were present (chemotype 3) in 10 specimens and neither of the two mentioned (chemotype 4) were found in 11 specimens (Table 2).

Analysis of the molecular data

Amplification products of the ITS1, 5.8S, and ITS2 regions of the ribosomal rRNA gene were *c.* 600 bp long, while those of the two exon and one intron regions of the β-tubulin genes were *c.* 700 bp in length. Although the number of nucleotides analyzed differed accordingly (ITS: 546 bp, β-tubulin: 674 bp), the datasets were comparable in the amount of phylogenetic signal. Although the ITS dataset contained more variable sites (ITS: 44 sites; β-tubulin: 31 sites), the number of parsimony-informative characters was the same for both loci (31). No ambiguous positions that could bias the inference of phylogeny were detected.

Testing the data partitions for substitution saturation (distribution of the uncorrected vs corrected distances) revealed a practically linear correlation, indicating no saturation in both ITS and β-tubulin data (see Appendix 1). Similarly, the results of likelihood mapping demonstrated a strong phylogenetic

TABLE 2. *Number of* Cladonia *specimens studied of each chemotype*

	chemotype 1 ZEO, USN, ISO	chemotype 2 ZEO, USN, POR	chemotype 3 ZEO, USN, ISO, POR	chemotype 4 ZEO, USN
Cladonia coccifera	1	10	0	2
C. deformis	1	1	0	1
C. diversa	0	1	0	5
C. pleurota	17	0	10	3
Total	19	12	10	11

ISO = isousnic acid; POR = porphyrilic acid; USN = usnic acid; ZEO = zeorin

signal detected in both ITS and β-tubulin loci (89·2% and 94·1% of the fully resolved quartets, respectively).

Phylogenetic analyses

The Bayesian, MP and ML analyses yielded trees with similar topology. Figure 2 shows the phylogram obtained from the Bayesian analysis. It revealed three well-supported (#1, #2, and #4) and one moderately supported (#3) lineages. Lineage #1 comprised 24 identical sequences belonging to *C. deformis* and *C. pleurota*. *Cladonia pleurota* strains also formed lineage #2. In contrast, lineage #4 contained sequences belonging to both *C. coccifera* and *C. diversa*, and lineage #3 comprised three *C. coccifera* strains.

Compared to the β-tubulin gene tree, ITS phylogenetic analysis inferred a clearly different topology. Three of the four well-resolved lineages of β-tubulin phylogeny were not resolved, but separated into different and distantly related clades (Fig. 2). Lineage #1 was separated into four lineages (#1a, #1b, #1c, and #1d). Whereas lineages #1a, #1c, and #1d contained both *C. pleurota* and *C. deformis* specimens, lineage #1b comprised only the *C. pleurota* strains. Lineage #4 from the β-tubulin gene tree formed lineages #4a and #4b in the ITS phylogram. Although receiving low support, they were obviously unrelated. Clade #4a was composed of all the analyzed *C. diversa* strains, whereas lineage #4b contained sequences belonging to *C. coccifera*. Finally, lineage #3 was split into two lineages: unsupported lineage #3a and lineage #3b containing only one sequence. Lineage #2 was the only one that was inferred with high statistical support by both ITS and β-tubulin phylogenetic analyses.

Since ITS and β-tubulin phylogenies were obviously not congruent, concatenated analysis was not performed.

Hybridization tests

A visual comparison of ITS and β-tubulin phylograms indicated a discrepancy in relationships among some taxa in both markers. For example, *C. diversa* formed a highly supported monophyletic clade together with some *C. coccifera* strains in the β-tubulin tree (lineage #4), but it created a separated lineage #4a in the ITS phylogram (which was, however, not statistically supported). A consensus network constructed from the trees obtained from the Bayesian analysis of the β-tubulin gene and ITS suggested contradictory taxon relationships. This network (Fig. 3) explains the conflict between source-tree topologies as a consequence of the hybridization event. Based on the investigation of concatenated datasets, the Phi test did find statistically significant evidence for recombination ($P = 1{\cdot}1 \times 10^{-7}$).

The presence of the recombination event was also examined by two tests. Rdp3 analysis detected two hybridization events within both ITS and β-tubulin loci, which led to two hybrid lineages: #4a (all *C. diversa* strains) and #4b (some *C. coccifera* strains) (Fig. 3). The recombination was detected by three different tests implemented in

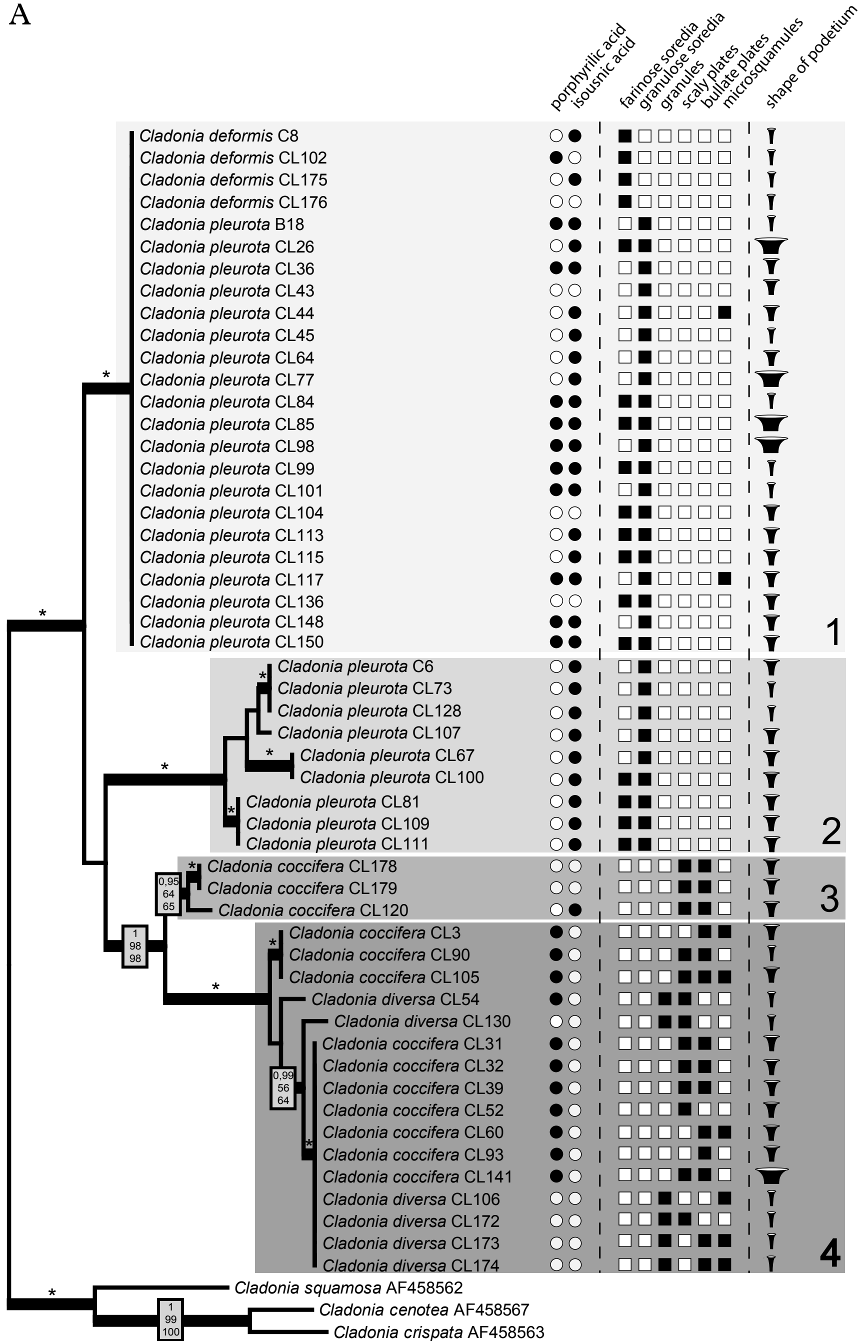

FIG. 2. Comparison of Bayesian topologies based on the β-tubulin (A) and ITS rDNA (B) datasets, together with observed chemical and morphological characters. For the analyses, an HKY + Γ_8 model for ITS1 and ITS2 regions, and HKY model for 5.8S rDNA, three codon positions and exon of β-tubulin gene was used. Values at the nodes indicate statistical support estimated by three methods: MrBayes posterior node probability (top), maximum likelihood bootstrap (in the middle) and maximum parsimony bootstrap (bottom). Thick branches represent nodes receiving PP support $\geq$0·90; asterisks (*) indicate statistical support 1/100/100. Only values receiving PP support $\geq$0·90 are shown. Species affiliation to four β-tubulin clades (including the corresponding sub-clades on the ITS rDNA tree) is indicated. Scale bar: estimated number of substitutions per site. [narrow podetium symbol], displays narrow, slender podetia; [cup symbol], moderately broad cups; [broad cup symbol], extremely broad podetia.

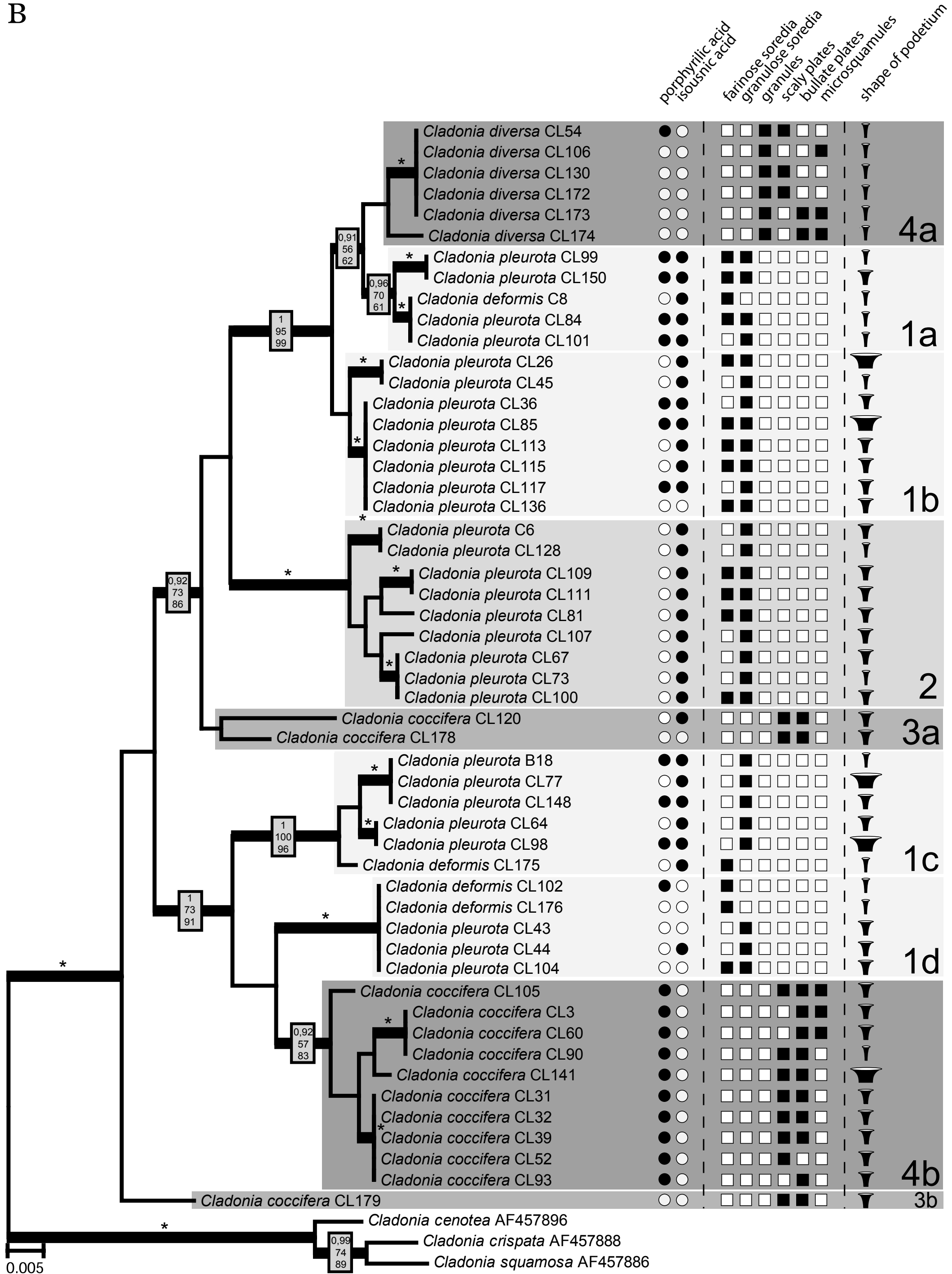

FIG. 2. *Continued*

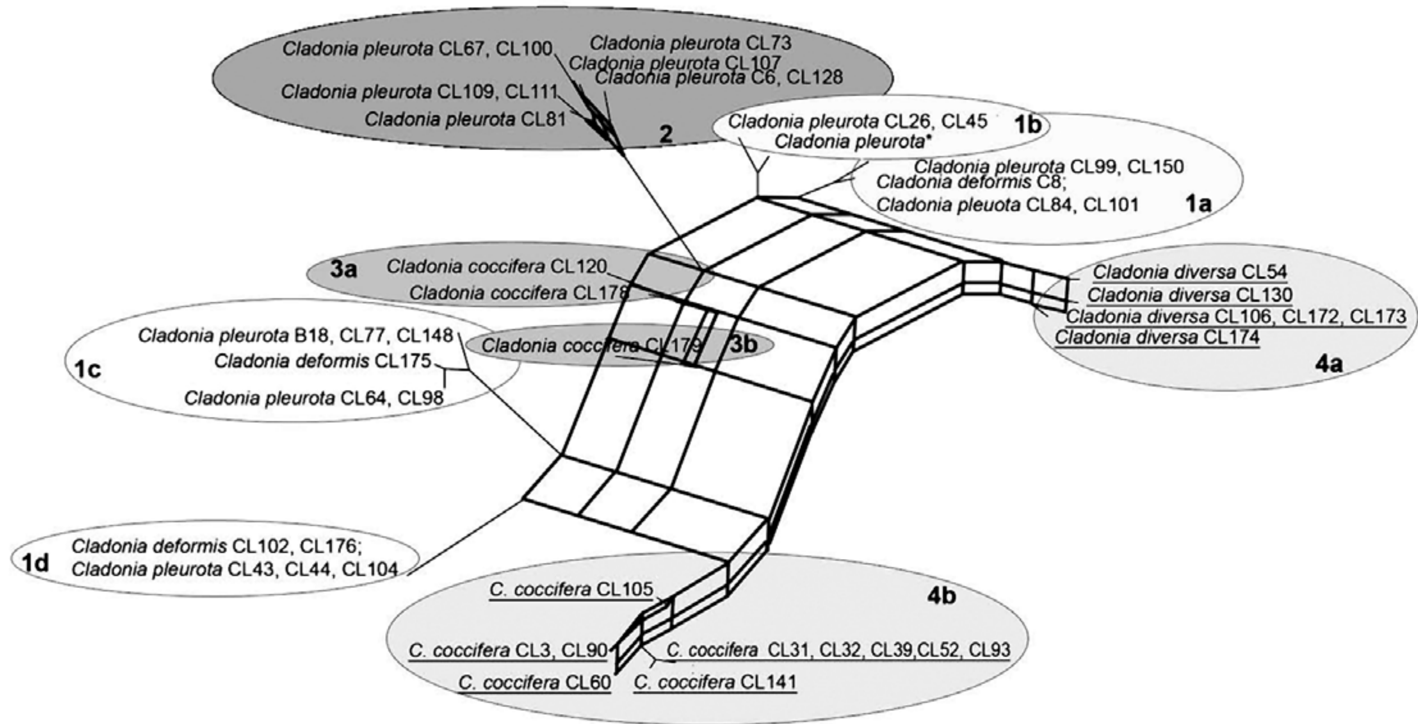

Fig. 3. Consensus network inferred from Bayesian trees from ITS and β-tubulin data. Thick lines represent hybridization events. Taxa involved in hybridization events are underlined. Scale bar shows the estimated number of substitutions per site.

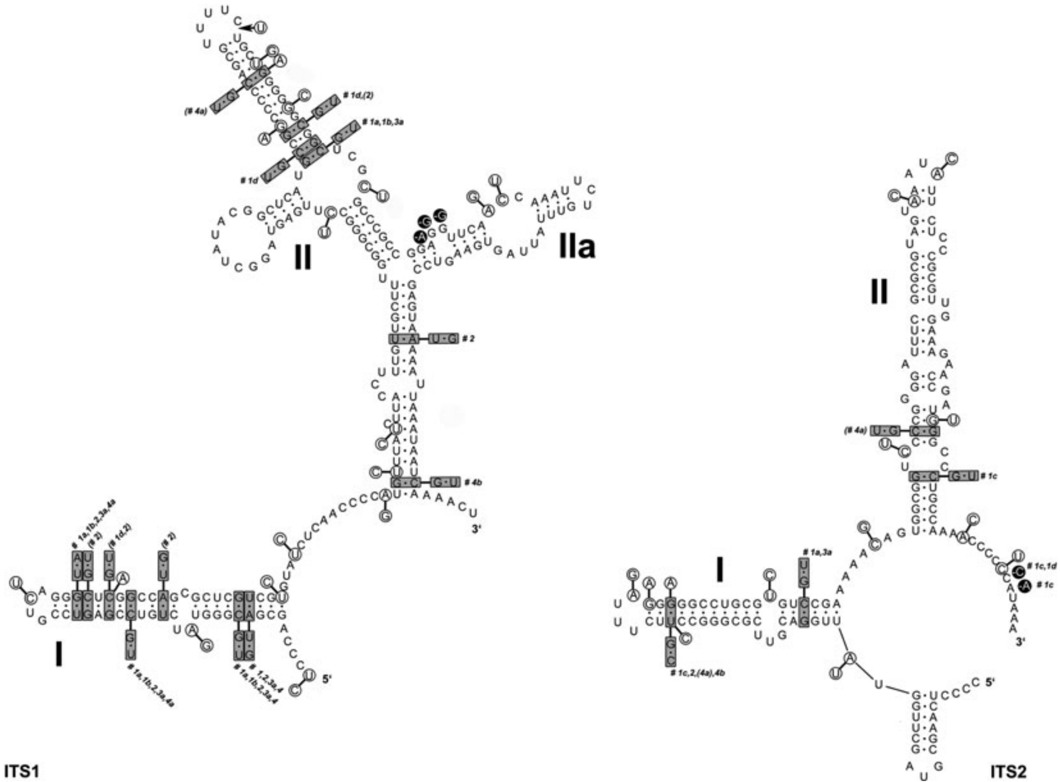

FIG. 4. Secondary structure models of ITS1 and ITS2 transcripts of *Cladonia coccifera* (DNA extraction number CL93) derived from comparison of 9 zeorin-containing red-fruited *Cladonia* lineages. Base changes between the different *Cladonia* genotypes are indicated: the base pairs marked in grey boxes indicate hemi-CBCs; single base changes are marked in circles. The numbers next to the boxes (#1a–4b) specify the *Cladonia* clades in which the base changes occurred (see Figs 2 & 3).

Rdp3: MaxChi ($P = 0{\cdot}0084$), Chimaera ($P = 0{\cdot}0030$) and 3Seq ($P = 0{\cdot}0006$). These hybrid lineages formed highly supported monophyletic clade #4 in the β-tubulin tree. Based on the bootscanning analysis conducted by the SimPlot program, we discovered the probable ancestral lineages of both hybrids. In fact, only one parent lineage was unequivocally inferred for both hybrids. In the case of *C. diversa* (#4a), lineage #1a was identified as ancestral, whereas in the second case (#4b), the ancestral lineage was found to be lineage #1c. The second ancestral lineage in both cases of hybridization was unknown; however, SimPlot identified a hybrid as the ancestor of the other one, and vice versa. From this, we concluded that both of the hybrid lineages had a common, as yet unknown, parent lineage.

ITS1 and ITS2 secondary structure

A common organization of ITS1 and ITS2 secondary structures was found in all *Cladonia* strains. The ITS1 secondary structure comprised two main paired regions (helices I and II), with two additional lateral helices (IIa and IIb) on helix II. Helix I was more divergent than helix II. The ITS2 structure was more conserved than that of ITS1. It contained three paired regions (helices I, II, and III). Helix I was identical in all the specimens examined, whereas helices II and III showed a small degree of divergence. The ITS secondary structures were compared with the lineages inferred in the ITS phylogram (Fig. 4) to check the occurrence of compensatory base changes (CBCs, nucleotide changes at both sides of the paired bases)

and hemi-CBCs (change on only one side of the nucleotide pair, but still preserving pairing), according to Coleman (2000, 2003). We revealed no CBC and 161 hemi-CBCs in all the lineages. The number of hemi-CBCs varied from zero (#3b) to six (#2) between the different lineages. Altogether, 15 hemi-CBC sites were identified in both the ITS regions. ITS1 contained 11 hemi-CBCs, of which seven were located in helix I. Four hemi-CBCs identified in ITS2 were situated in helices II and III. The highest number of hemi-CBCs (seven) was determined between lineages #1d–1a, #1d–4a, #1c–1a, #1c–2, and #1c–4a. In contrast, no hemi-CBC was identified between lineages #1a–4a.

Discussion

Phenotypic and genetic variability of red-fruited *Cladonia* species

The four species considered in this paper are chemically very similar but differ morphologically. The most obvious differences characterizing these species are the size and shape of the podetium, the character of the podetium surface, and the character and size of the vegetative propagules. However, three of these species were shown to be polyphyletic. Only *Cladonia diversa* formed a monophyletic group in the ITS phylogeny, although it was not supported statistically. Traditionally, this species was regarded as a member of the *C. coccifera* group. Stenroos (1989) doubted the status of *C. diversa* and found that its total variation is still obscure. Recently, Ahti & Stenroos (2012) accepted the species as a valid taxon. All the specimens studied have slender, narrow scyphi and their surfaces are covered by microsquamules, irregular plates and granules (Fig. 2). Furthermore, we can also confirm its preference for sandy substrata (out of six specimens, four were collected from sandy dunes, one from a sandstone), as previously reported by Asperges (1985), Christensen & Johnsen (2001), Hasse (2005), and Osyczka (2009). Also, chemical patterns are consistent with other sources (e.g. Osyczka 2011; Ahti & Stenroos 2012). James (2009) proposed porphyrilic acid to be a stabile compound of this species, but according to our results this cannot be confirmed (porphyrilic acid was detected in only one specimen of *C. diversa*).

The specimens morphologically identified as *C. coccifera* were distributed in three lineages (#3a, #3b, #4b). Two of these (#3a and #3b) were phylogenetically distant from lineage #4b. Ten specimens representing lineage #4b showed wide morphological variability (Fig. 2), particularly in the features of the vegetative propagules and the shape of podetia. The surface of podetia in some specimens was largely covered with scaly and bullate plates, whereas microsquamules dominated on podetia of other specimens studied. The shape also varied from narrow to very broad cups. In contrast, lineages #3a and #3b contained specimens that were morphologically more uniform and consistent with the traditional delimitation (however, only three *C. coccifera* specimens were inferred in these lineages). According to our results, clade #4b differed chemically from clades #3a and #3b. Whereas specimens involved in the lineage #4b are characterized by the presence of porphyrilic acid and the absence of isousnic acid, the three specimens from the other two lineages lacked porphyrilic acid. Interestingly, whereas these specimens were collected in Norway and Missouri, the specimens from the lineage #4b were sampled from Central Europe and Spain. This suggests a possible geographical pattern in the distribution (and also chemical variation) of these two lineages, which should be studied more carefully with wider sampling.

Although *C. deformis* is regarded as a distinct species, the specimens were found in three lineages: #1a, #1c, as well as #1d together with *C. pleurota*. These two species are traditionally distinguished by the size and shape of the podetium, and by the size of the soredia. *Cladonia deformis* usually forms elongated farinose-sorediate podetia, whereas *C. pleurota* is characterized by shorter and more coarsely sorediate podetia. The four studied specimens of *C. deformis* did not show any uniform chemical pattern. In one case (specimen Cl102 from the Czech Republic)

we detected porphyrilic acid, which has not been reported for this species previously.

On the basis of the findings of Stenroos *et al.* (2002), *C. pleurota* appeared to be a monophyletic species. However, our results with a more detailed sampling of the species disprove the monophyly of this taxon. Specimens of *C. pleurota* as currently understood are spread across five lineages (#1a, #1b, #1c, #1d, and #2). Although the specimens were studied thoroughly, we did not detect any morphological criteria that would properly characterize any of these lineages (Fig. 2). The podetial surface of all specimens used for the analysis was covered with granulose soredia, sometimes accompanied by farinose soredia in varying amounts. The shape of podetia exhibited considerable variation (from very narrow to short and extremely broad cups) that, however, does not correspond with the genetic diversity of the material studied. Discordance between the high morphological variation and sequence data has recently been discussed by Pérez-Ortega *et al.* (2012), with respect to vagrant forms in *Cetraria aculeata*. However, the induction of growth variation as suggested for vagrant vegetative offspring cannot apply in *Cladonia*. It seems that, on the contrary, there are at least some chemical patterns or tendencies which may help us to define some clades. Lineage #2 comprises nine samples with identical chemical characteristics (chemotype 1: presence of isousnic acid and absence of porphyrilic acid). Porphyrilic acid appears to be a constant substance in samples of clade #1c, but with only six specimens studied we refrain from drawing taxonomic conclusions.

Discordance among gene-tree genealogies

In our study, we analyzed two commonly used and well-established molecular markers for Ascomycota: ITS rDNA and an intron-containing portion of the β-tubulin gene. The strong conflict between the ITS and β-tubulin topologies seems to be a recurrent phenomenon already found by other authors investigating *Cladonia* phylogenies (Myllys *et al.* 2003). Moreover, it could in fact represent a more widespread phenomenon than generally anticipated, as it has also been found in other lichen groups (e. g., Ertz *et al.* 2009). Phylogenetic incongruences among genes can occur for many reasons, including the presence of pseudogenes, gene paralogy, horizontal gene transfer, incomplete lineage sorting (ILS), and hybridization. Alternatively, the presence of hyphae from more species growing together in the same podetium could be another possible explanation for the conflict (Kotelko & Piercey-Normore 2010). We can clearly rule this out in our dataset, as we have unambiguous signals with the ITS primers.

Pseudogenes are dysfunctional relatives of known genes that have lost their protein-coding ability or are otherwise no longer expressed in the cell (Vanin 1985). Their base compositions are different from those of functional genes, and they evolve very rapidly (Buckler *et al.* 1997). Pseudogenous clones are characterized by occasional deletions in genes and spacers, by increased non-synonymous mutations in the otherwise almost identical rRNA-coding regions (Grimm & Denk 2008; Harpke & Peterson 2008), or by low predicted secondary structure stability in ribosomal genes or spacers (Buckler *et al.* 1997). However, the almost identical 5.8S rRNA sequences (only one substitution was detected), absence of long-branching artefacts in the phylograms, and the absence of deletions suggest the non-pseudogenous nature of any of the ITS sequences analyzed. In addition, conserved sequences of 3′ and 5′ ends also suggest that they are not pseudogenes.

Horizontal gene transfer (HGT) is the transfer of genes across species. This mechanism is well known mainly in bacteria, but it also occurs in the evolution of Eukaryota (Keeling & Palmer 2008; Khaldi *et al.* 2008; Marcet-Houben & Gabaldon 2010). However, an HGT event is an unlikely source for the conflict between tree topologies in our dataset because it would imply a recent HGT event between two eukarya lineages, which is rare (Won & Renner 2003) and generally involves the transfer of introns (Rot *et al.* 2006).

Gene paralogy occurs if a gene in an organism is duplicated to occupy two different

positions in the same genome and can also be responsible for the conflict between two phylogenies. Although paralogs of the β-tubulin gene are known from different groups of fungi (e.g., Begerow *et al.* 2004; Corradi *et al.* 2004; Msiska & Morton 2009), they have not been reported from lichenized Ascomycota. Moreover, in our case, intragenomic variation should be found in β-tubulin sequences to explain the incongruence in our dataset by potential gene paralogy. However, we did not find an ambiguous signal in the β-tubulin sequences.

Incomplete lineage sorting, also called deep coalescence, is a phenomenon that can cause conflicting gene and species trees. ILS represents the incomplete random sorting of alleles at many loci independently due to short intervals between divergence events (Blanco-Pastor *et al.* 2012). ILS has been reported in many different groups of organisms, more likely in those species which have a large population size and a short time between divergences (e.g., Morando *et al.* 2004; Jakob & Blattner 2006; Pollard *et al.* 2006). This process is difficult to distinguish from interspecific hybridization and both may even occur simultaneously (Seehausen 2004; Meng & Kubatko 2009). Although several methods distinguishing these two evolutionary processes have been recently proposed (e.g., Holland *et al.* 2008; Bloomquist & Suchard 2010), many independent loci are needed for their implementation and it is difficult to uncover multiple reticulation events (Blanco-Pastor *et al.* 2012).

Since we are not able to distinguish ILS and hybridization, we assume both could be responsible for the incongruence in our dataset. Here we propose the phylogenetic consequences of these two scenarios.

The presence of ILS would indicate that zeorin-containing *Cladonias* spp. probably diverged relatively recently (Leache & Fujita 2010). The ITS and β-tubulin phylogenies would represent gene trees, which would not correspond with the species tree. To be able to describe the phylogenetic relationships within this group, even in the presence of incomplete lineage sorting, it will be necessary to study more loci (e.g., Knowles & Carstens 2007), which will definitely be within reach with the ongoing *Cladonia* genome project hosted at Duke University.

Interspecific hybridization is regarded as one of the major factors responsible for conflicts among different loci (e.g., Taylor *et al.* 2000; Fehrer *et al.* 2007; Ertz *et al.* 2009). Similar incongruences have been detected in other lineages of *Cladonia* (Myllys *et al.* 2003; Fontaine *et al.* 2010; Kotelko & Piercey-Normore 2010). We assume hybridization should be considered as an important mechanism, possibly influencing the evolution of the lichen genus *Cladonia*, resulting in reticulate evolution that may contribute to the species diversification. Although hybridization is not yet known in lichens, it has been proved to occur in most fungal phyla (e.g., Brasier *et al.* 1998, 1999; Xu *et al.* 2000; Craven *et al.* 2001*a*, *b*).

If the effect of hybridization is considered, only one parent lineage could be identified in both hybridization events. The second ancestral lineage is unknown, which could have two alternative explanations: 1) the parent lineage is extinct and could therefore not be detected; 2) we did not sample and analyze the parent lineage. To better understand the species concept and delimitation in the group of zeorin-containing red-fruited *Cladonia* lichens, it will be important to address the question of frequency of hybridization events more carefully in the future. The suggested hybridization could represent either an exceptional ancient event or a common ongoing process. Sexual compatibility/incompatibility between two organisms can be detected by a comparison of the secondary structure of the ITS (so-called CBS approach). The presence of compensatory base changes (CBSs) indicates incompatibility for sexual reproduction between species (Coleman 2003; Müller *et al.* 2007). In our case, the absence of CBCs reveals that there are presumably no reproduction barriers among the species studied, and hence, we can conclude that the second alternative is more feasible.

Species circumscriptions in *Cladonia*

Similarly to other recent studies focusing on *Cladonia* (Fontaine *et al.* 2010; Kotelko & Piercey-Normore 2010; Pino-Bodas *et al.*

2010, 2012*a*), our investigations clearly revealed the incongruence between the phylogenetically inferred lineages and traditional, morphologically and/or chemically delimited species. In fact, we were not able to find any phenotypic feature to unambiguously define the lineages in most cases (except *Cladonia diversa*, and chemical patterns in the lineages #2 and #4b).

In general, there are two alternatives for interpreting this incongruence. The phylogenetic units could be either regarded as populations of a morphologically variable species or accepted as 'cryptic' or incipient species.

The question of how to treat the 'cryptic' species within the traditionally defined nominal species has been discussed by many authors, advocating two different attitudes. Some authors (e.g., Kotelko & Piercey-Normore 2010) have suggested a more conservative attitude in maintaining the traditional delimitation of the species, even when they are not supported by molecular data. They have argued for the possible implications of these species for ecophysiological studies, and more generally, for the detection and preservation of rare or unusual species. Conversely, other authors (Grube & Kroken 2000) have proposed applying the phylogenetic species concept and thus defining the well-supported phylogenetic lineages as cryptic species. They mentioned that morphological characterization of the species is often facilitated after finding cryptic lineages with molecular data, as it may then become apparent which characters are significant. They also claimed that the knowledge of cryptic species is useful in investigations at fine scales of taxonomic resolution, such as for interpretations of ecophysiological differences and microhabitat preferences (Grube & Kroken 2000).

Considering the findings in this study, we adopt the second opinion, understanding the well-supported phylogenetic lineages as separate species, but without describing them formally for the time being. One lineage indeed consisted of morphologically well-characterized specimens of *C. diversa*, traditionally recognized as a species. Moreover, *C. coccifera* samples belonging to clade #4b shared identical chemical characteristics (chemotype 2; presence of porphyrilic acid and absence of isousnic acid) and appeared to differ chemically from the other *C. coccifera* strains (lineages #3a and #3b). It is therefore very likely that the other lineages, even if morphologically indistinguishable, also represent separate species. Zeorin-containing red-fruited *Cladonia* species have wide morphological and ecological amplitudes, and thus, the correlation between phylogenetically separated lineages and different phenotypic characters should be studied more comprehensively in the future.

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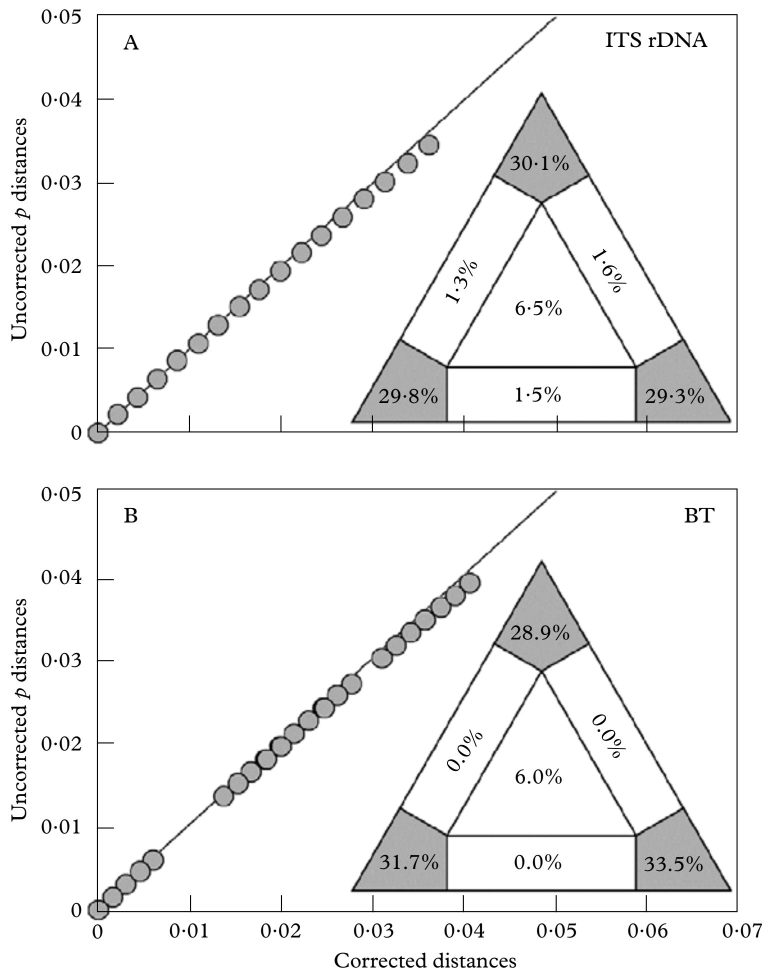
Appendix 1. Analysis of substitution saturation.



The graphs visualize the saturation of the ITS rDNA and β-tubulin datasets by plotting ML-corrected distances against uncorrected p-distances. Corrected distances are calculated using models estimated by PAUP/Modeltest for each specific data partition. A, analysis of ITS rDNA sequences; B, analysis of β-tubulin sequences. The triangles in the lower right of the graphs illustrate likelihood mapping results. The values in the panels indicate proportion of fully resolved (corners), partially resolved (along the sides), and fully unresolved quartets (in the centre).