The Lichenologist **42**(3): 323–338 (2010) © British Lichen Society, 2010
doi:10.1017/S0024282909990569

Convergent evolution in *Cladonia gracilis* and allies

Kyle M. FONTAINE, Teuvo AHTI and Michele D. PIERCEY-NORMORE

Abstract: Members of the *Cladonia gracilis* group of lichen fungi are common terrestrial lichens where morphological features are more similar between members of the *C. gracilis* species complex and allied species outside the complex than they are between subspecies within the complex. The objectives of this study were to examine whether the *Cladonia gracilis* species complex is monophyletic, to determine whether morphological similarity is supported by genetic variation, and to examine the utility of the polyketide synthase (PKS) gene for phylogenetic studies among closely related species. Two loci, the ketosynthase region of the PKS gene and the internal transcribed spacer (ITS) region of nuclear ribosomal DNA, were sequenced and analysed by Maximum Parsimony, Bayesian and haplotype network analyses. Functional differences were also inferred through ITS2 RNA secondary structures and non-synonymous changes in translated PKS amino acid sequences. The monophyly of the *C. gracilis* complex is supported by 71% bootstrap in the ITS phylogeny, and 92% bootstrap with greater than 95% posterior probability in the PKS phylogeny. Morphological similarity is not always supported by genetic similarity. The PKS gene is less variable than the ITS but the PKS supports species hypotheses that are reflected in the ITS2 RNA model. We conclude that monophyly of the *C. gracilis* complex can be supported if *C. cornuta*, *C. coniocraea* and *C. ochrochlora* are included in the complex. In addition, *C. maxima*, *C. phyllophora* and *C. subchordalis* are supported as monophyletic species outside the *C. gracilis* complex. *Cladonia maxima* may form a separate species and variation among podetial morphology may be explained by convergent evolution.

Key Words: *Cladonia maxima*, *Cladonia phyllophora*, compensatory base changes, ITS rDNA sequences, non-synonymous substitutions, polyketide synthase gene

Introduction

The genus *Cladonia* P. Browne is a large and variable genus of lichenized ascomycetes that are mainly terrestrial on soil, decaying wood or at the base of trees. The genus, comprising more than 400 species distributed world-wide, has been divided into seven taxonomic sections (Ahti 2000) based on morphological characters. Previous phylogenetic studies using morphological characters showed low resolution among the morphological forms within the genus (Hyvönen *et al*. 1995; Stenroos *et al*. 1997). Recent phylogenetic work based on nucleotide characters shows more resolution (Piercey-Normore & DePriest 2001; Stenroos *et al*. 2002). The extensive work by Stenroos *et al*. (2002; see also Guo & Kashiwadani 2004) included representative members of all sections of the genus *Cladonia*. *Cladonia* section *Cladonia* contains a number of species complexes with different levels of morphological and chemical variability. Within section *Cladonia*, the *Cladonia gracilis* species complex and its allies are highly variable in morphology but have a low level of variation in secondary compounds. Ahti (1980) included seven species in the group but up to 23 species may belong here. More recently, Stenroos *et al*. (2002) and Guo & Kashiwadani (2004) treated it provisionally as "subgroup *Graciles*".

Morphological features are more similar between some members of the *Cladonia gracilis* species complex and species outside the complex, than they are between subspecies within the complex. For example, *C. gracilis* subsp. *turbinata* and *C. phyllophora* have similar podetial morphology often

K. M. Fontaine and M. D. Piercey-Normore: Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba, Canada, R3T 2N2. Email: pierceyn@cc.umanitoba.ca
T. Ahti: Botanical Museum, P. O. Box 7, FI-00014, Helsinki University, Finland.

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

containing broad shallow scyphi and large brown apothecia. However, *C. gracilis* subsp. *turbinata* and *C. gracilis* subsp. *elongata* are very different in appearance where *C. gracilis* subsp. *elongata* has very narrow scyphi or none at all. Overlapping morphological features of *C. gracilis* subsp. *turbinata* and *C. phyllophora* may suggest that these species intergrade in nature (Ahti 1980) making them difficult to distinguish. *Cladonia gracilis* subsp. *elongata* is superficially similar to *C. maxima*, an ally of the *C. gracilis* group. Both species contain a smooth podetial cortex, and they may have blunt podetia or they may produce small cups. The difference is that *C. maxima* has a yellowing podetial base and *C. gracilis* subsp. *elongata* has a blackening podetial base (Ahti 1980).

A more distantly related species, *C. multiformis*, is placed in a different taxonomic section (*Ascyphiferae*) than *C. gracilis* (section *Cladonia*, Ahti 2000). One of the diagnostic features of *C. multiformis* is the perforated shallow cup, but at least two morphological forms of *C. multiformis* may exist; one with shallow cups and one without cups containing funnelled branches in a cetrarioid growth form (see photograph in Hinds & Hinds 2007). Both growth forms may exist on the same podetium or they may be found separately. The use of different growth forms in classification is presumably artificial (Stenroos & DePriest 1998) and may be influenced by environmental conditions or reflect developmental stages in the lichen.

Similarly, the production of secondary compounds in lichens is thought to be influenced by environmental and developmental conditions (Hawksworth 1976; Leuckert *et al*. 1990; Culberson & Armaleo 1992; Stocker-Wörgötter 2001). The taxonomic value of lichen secondary compounds is not well understood as they are known to vary qualitatively and quantitatively within species and populations (Culberson *et al*. 1988) and are thought to undergo convergent evolution (Culberson 1986; Gowan 1989). Most species and subspecies in the *C. gracilis* group and its allies contain a single secondary compound, fumarprotocetraric acid, and some taxa secondarily produce atranorin (Ahti 1980). These secondary compounds are polyketides, which are produced by a multidomain polyketide synthase (PKS) encoded by a series of genes and arranged in clusters on the chromosome (Keller & Hohn 1997; Schümann & Hertwech 2006 and references therein; Shwab & Keller 2008). The ketostynthase (KS) region of the PKS gene has previously produced phylogenetic resolution between species and genera in studies such as those within the *Parmeliaceae* (Opanowicz *et al*. 2005), *Lecanora* and related genera (Grube & Blaha 2003) and *Pertusaria* and related genera (Schmitt *et al*. 2005). Another phylogenetic marker, the ITS rDNA, is a standard among species level phylogenies (DePriest 2004), and is often used to reflect evolution of the species. The ITS2 rRNA secondary structure is also becoming an effective tool for inferring phylogenetic histories (Coleman 2003; Müller *et al*. 2007).

The objectives of this study were 1) to determine whether the *C. gracilis* species complex forms a monophyletic group based on nucleotide sequences, 2) to determine whether morphological similarity between *C. phyllophora* and *C. gracilis* subsp. *turbinata* and between *C. maxima* and *C. gracilis* subsp. *elongata* is supported by genetic similarity, and 3) to evaluate the utility of a PKS gene for phylogenetic studies among closely related species.

Methods

Taxon sampling

All seven members of the *C. gracilis* group according to the taxonomic revision by Ahti (1980) were included in this study. Three of the seven allies were available only from GenBank accessions. Three members of the *C. gracilis* species complex and one member from each of the *C. cornuta* and *C. ecmocyna* complexes were included. Other species, such as *Cladonia ochrochlora, C. coniocraea, C. phyllophora* (section *Cladonia*) and *C. multiformis* (section *Ascyphiferae*), were also included (Table 1). These taxa were chosen because they present morphological difficulties with members of the *C. gracilis* group. The assigned outgroup taxa (for the ITS rDNA only) were *Cladia aggregata* and *Pycnothelia papillaria* as members of the family *Cladoniaceae* but outside the

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

genus *Cladonia*. The annotation for the PKS gene translation is based on *C. foliacea* (EF363873).

Thin layer chromatography and isolation of genomic DNA

Secondary metabolites were extracted from podetial tissue 1·5–2 centimetres in length. Podetia were examined under a dissecting microscope and contaminating debris was removed. Vouchers are deposited in the cryptogamic division of the University of Manitoba Herbarium (WIN). A TLC protocol was modified from Culberson (1972) using solvent A (toluene 180ml: dioxane 45ml: glacial acetic acid 5ml) and solvent C (toluene 200ml: glacial acetic acid 20ml). The CTAB (hexadecytrimethylammonium bromide) DNA extraction protocol was modified from Grube *et al*. (1995) and performed on the same piece of thallus tissue that was used for TLC. This ensured that the podetium that produced the polyketide was the same podetium producing the DNA sequence in the analysis.

DNA amplification and sequencing

The fungal ITS gene (ITS1, ITS2 and the 5.8S) of the nuclear ribosomal DNA (nrDNA) and a portion of the ketosynthase (KS) domain of the polyketide synthase (PKS) gene were amplified by the Polymerase Chain Reaction (PCR). The DNA was amplified in 50 µl PCR reactions consisting of 10–50 ng of DNA, PCR buffer [50 mM KCl; 100 mM Tris-HCl (pH 8·3)], 2·0 mM $MgCl_2$, 200 µM of each dNTP, 0·5 µM of each primer, and 2 units of Taq DNA polymerase (Invitrogen, Burlington, ON, Canada). The ITS1, 5.8S, and ITS2 rDNA were amplified using the fungal specific primer 1780F-5′ (5′- CTGCGGAAGGATC ATTAATGAG-3′) (Piercey-Normore & DePriest 2001) and universal primer ITS4-3′ (5′-TCCTCCG CTTATTGATATGC-3′) (White *et al*. 1990). All amplifications of the ITS gene were performed on a Techne Genius thermal cycler (Fisher Sci, Nepean, ON, Canada) using a touchdown PCR cycle with initial denaturation of the DNA at 94°C for 5 min; then 36 cycles with denaturation at 94°C for 1 minute, annealing at 60°C for two cycles (×2), 58°C (×2), 56°C (×2), 54°C (×30) for 1 min and extension at 72°C for 1·5 min; followed by 4°C hold for infinity. Initial amplification of a portion of the KS domain was accomplished using the LC1 (5′-GATCCIAGITTT TTTAATATG-3′) and LC2C (5′-GTICCIGTICC GTGCATTTC-3′) (Bingle *et al*. 1999) degenerate primer pair. Because these primers often amplified multiple products, a set of non-degenerate nested primers were designed to amplify a single product. The new primers were PKS1-F (5′-TACGAAGCCC TAGAAATGGC-3′) and PKS2-R (5′-ACGTTTG GCAGTTTCCTGTC-3′). The amplification conditions were an initial denaturation of DNA at 94°C for 1 min; then 30 cycles with denaturation set at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min; followed by 4°C hold for infinity.

Eight identical 50 µl reactions were pooled to produce 400 µl of PCR product for sequencing. To purify, the DNA was precipitated by adding 0·2 volumes of 5M NaCl and 2·5 volumes of 100% ethanol, centrifuged at 13 000 rpm, and the pellet washed with 300 µl of cold 80% ethanol. The pellet was resuspended in 20 µl sterile distilled water, which was loaded on an agarose gel for electrophoresis and excision of the band. The gel blocks were frozen overnight at −20°C, crushed between a folded piece of parafilm, and the liquid from the thawing gel mass was pipetted into a 1·5 ml centrifuge tube. The DNA was precipitated again and resuspended in 20 µl sterile distilled water. To quantify the DNA, intensities of electrophoresced bands were compared with that of a known quantity using a 1 kb plus DNA ladder (Invitrogen, Burlington, ON, Canada). Results were visualized using an AlphaImager 2200 transilluminator (Alpha Innotech, Fisher Scientific, Nepean, ON, Canada).

For DNA sequencing, cycle sequencing reactions were performed using a Biometra T-Gradient thermal cycler (Fisher Sci, Nepean, ON, Canada) using BigDye ver. 3.1 and sequenced on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to manufacturer's directions. Sequences were edited using Sequencher v. 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA) and manually aligned using Se-Al v. 1.0 (Rambaut 2001). DNA sequencing was done using PCR primers, but when the ITS rDNA showed ambiguous base calling, the sample was sequenced again using primers that anneal to the 5.8S region ITS2-3′ (5′-GCTGCGTTCTTCATCGATGC-3′) and ITS3-5′ (5′-GCATCGATGAAGAACGCAGC-3′) (White *et al*. 1990).

Data analyses

Phylogenetic analysis using Maximum Parsimony (MP) was performed in PAUP 4.0 (Swofford 2003) using a heuristic search with 1000 replicates of random addition of taxa, tree-bisection-reconnection (TBR) branch swapping, MulTrees off, and saving no more than 1000 trees in each replicate. Bootstrap values were determined by using 1000 bootstrap replicates (Felsenstein 1985) with simple addition of taxa and TBR branch swapping. Bootstrap values greater than 70 are recorded on the phylogeny. Bayesian analysis, in MrBayes v3.1.2 (Huelsenbeck *et al*. 2001; Ronquist & Huelsenbeck 2003), was performed using 2 400 000 generations for the ITS rDNA and 1 000 000 generations for the PKS analysis. A six-parameter GTR (General Time Reversible) model was applied with a gamma shaped parameter and proportion of invariable sites uniformly distributed. This was the best model estimated with Modeltest 3.7 (Posada & Crandall 1998) that was available in MrBayes. Both runs converged on similar likelihood values and were terminated when the standard deviation of split frequencies fell below 0·01. Posterior probability values greater than 95 are reported on the phylogeny. The Kishino-Hasagawa test (Kishino and Hasagawa 1989) was performed on the ITS and PKS data sets.

Haplotype networks were performed in TCS (Clement *et al*. 2000) using the criteria that all gaps were treated as missing data and the probability of parsimony

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

TABLE 1. *List of taxa, collection source, and accession numbers (Acc.) for the DNA sequences deposited in NCBI GenBank.*

Taxon name	Source	ITS Acc.	PKS Acc.
Cladonia coniocraea (Flörke) Spreng.	Han *et al.* unpub. (2007); China	EU034663	–
C. coniocraea (Flörke) Spreng.	Canada, Newfoundland, 2007, *Normore* 7937 (FU)*	FJ536347	FJ536383
C. coniocraea (Flörke) Spreng.	Canada, Newfoundland, 2007, *Normore* 7991 (FU)	FJ536348	FJ536384
C. cornuta (L.) Willd.	Canada, Newfoundland, 2007, *Normore* 7936 (FU)	FJ536349	FJ536386
C. cornuta (L.) Willd.	Canada, Manitoba, 2006, *Normore* 6065 (FU)	FJ536350	FJ536385
C. cornuta (L.) Willd.	Canada, Newfoundland, 2006, *Normore* 7046 (FU)	FJ536351	FJ536387
C. cornuta (L.) Willd.	Canada, Saskatchewan, 2008, *Normore* 8232 (FU)	FJ536352	FJ536388
C. cornuta (L.) Willd.	Stenroos *et al.* (2002); Chile	AF455197	–
C. cornuta (L.) Willd.	Stenroos *et al.* (2002); Estonia	AF455196	–
C. ecmocyna Leight.	Stenroos *et al.* (2002); Greenland	AF455199	–
C. gracilis subsp. *elongata* (Jacq.) Vain.	Canada, Newfoundland, 2007, *Normore* 7770 (AT)	FJ536353	FJ536389
C. gracilis subsp. *elongata* (Jacq.) Vain.	Canada, Newfoundland, 2007, *Normore* 7771 (AT)	FJ536354	FJ536390
C. gracilis subsp. *elongata* (Jacq.) Vain.	Canada, Newfoundland, 2007, *Normore* 7992 (AT, FU)	FJ536355	FJ536392
C. gracilis subsp. *elongata* (Jacq.) Vain.	Stenroos *et al.* (2002); Chile	AF455198	–
C. gracilis subsp. *elongata* (Jacq.) Vain.	Canada, Newfoundland, 2007, *Normore* 7933 (FU)	FJ536356	FJ536391
C. gracilis subsp. *elongata* (Jacq.) Vain.	Canada, Newfoundland, 2007, *Normore* 7835c (FU)	FJ536357	FJ536393
C. gracilis subsp. *elongata* (Jacq.) Vain.	Canada, Newfoundland, 2007, *Normore* 7988 (FU, AT)	FJ536358	FJ536394
C. gracilis subsp. *elongata* (Jacq.) Vain.	Canada, Yukon, 2006, *Kotelko* 1021 (FU)	FJ536359	FJ536411
C. gracilis (L.) Willd. subsp. *gracilis*	Stenroos *et al.* (2002); Sweden	AF455194	–
C. gracilis subsp. *turbinata* (Ach.) Ahti	Canada, Manitoba, 2006, *Normore* 5478 (AT)	FJ536360	–
C. gracilis subsp. *turbinata* (Ach.) Ahti	Canada, Ontario, 2006, *Normore* 6677 (FU)	FJ536361	FJ536414
C. gracilis subsp. *turbinata* (Ach.) Ahti	Canada, Manitoba, 2007, *Normore* 8005 (FU)	FJ536362	FJ536415
C. gracilis subsp. *turbinata* (Ach.) Ahti	Canada, Manitoba, 2007, *Normore* 8007 (FU)	FJ536363	FJ536412
C. gracilis subsp. *turbinata* (Ach.) Ahti	Canada, Yukon, 2006, *Kotelko* 860 (FU)	FJ536364	FJ536417
C. gracilis subsp. *turbinata* (Ach.) Ahti	Canada, Manitoba, 2006, *Fontaine* 38 (FU)	–	FJ536413
C. gracilis subsp. *turbinata* (Ach.) Ahti	Canada, Manitoba, 2007, *Fontaine* 105 (FU)	FJ536365	FJ536416
C. gracilis subsp. *turbinata* (Ach.) Ahti	Stenroos *et al.* (2002); Finland	AF455193	–
C. gracilis subsp. *turbinata* (Ach.) Ahti	Canada, British Columbia, 2008, *Normore* 8096 (FU)	FJ536366	FJ536410
C. gracilis (L.) Willd.	Hur unpub. (2006); China	DQ394380	–
C. gracilis (L.) Willd.	Lim & Hur unpub. (2005); Canada	DQ219313	–
C. gracilis (L.) Willd.	Hur unpub. (2007); China	DQ534462	–
C. gracilis (L.) Willd.	Beiggi & Piercey-Normore (2007); Canada	DQ530202	–
C. macroceras (Delise) Hav.	Hur *et al.* (2005); China (probably *C. macroptera*)	DQ001277	–
C. macroceras (Delise) Hav.	Stenroos *et al.* (2002); Iceland	AF455200	–
C. maxima (Asahina) Ahti	Canada, Newfoundland, 2007, *Normore* 7934 (FU)	FJ536367	FJ536398
C. maxima (Asahina) Ahti	Canada, Newfoundland, 2007, *Normore* 7938 (FU)	FJ536368	FJ536399
C. maxima (Asahina) Ahti	Canada, Newfoundland, 2007, *Normore* 7835b (FU)	FJ536369	FJ536395

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

TABLE 1. *Continued*

Taxon name	Source	ITS Acc.	PKS Acc.
C. maxima (Asahina) Ahti	Canada, Newfoundland, 2007, *Normore* 7861 (FU)	FJ536370	FJ536396
C. maxima (Asahina) Ahti	Canada, Newfoundland, 2007, *Normore* 7935 (FU)	FJ536371	FJ536397
C. maxima (Asahina) Ahti	Stenroos *et al.* (2002); Canada	AF455195	–
C. multiformis G. Merr.	Canada, Ontario, 2006, *Normore* 7294 (FU)	FJ536372	FJ536403
C. multiformis G. Merr.	Canada, Manitoba, 2007, *Normore* 8010a (FU)	FJ536373	FJ536404
C. multiformis G. Merr.	Canada, Manitoba, 2007, *Normore* 8010b (FU)	FJ536374	FJ536405
C. multiformis G. Merr.	Canada, Manitoba, 2006, *Normore* 5706 (FU)	FJ536375	FJ536400
C. multiformis G. Merr.	Canada, Manitoba, 2006, *Normore* 5918a (FU)	FJ536376	FJ536401
C. multiformis G. Merr.	Canada, Manitoba, 2006, *Normore* 5918b (FU)	FJ536377	FJ536402
C. multiformis G. Merr.	Canada, Manitoba, 2006, *Kotelko* 792 (FU)	FJ536378	FJ536406
C. multiformis G. Merr.	Canada, Manitoba, 2006, *Normore* 6122 (FU)	FJ536379	–
C. ochrochlora Flörke	Stenroos *et al.* (2002); Sweden	AF455192	–
C. ochrochlora Flörke	Ahti *et al.* (2008); Thailand	EU113284	–
C. ochrochlora Flörke	Ahti *et al.* (2008); Thailand	EU113285	–
C. phyllophora Hoffm.	Canada, Manitoba, 2006, *Normore* 5485 (FU)	FJ536380	FJ536407
C. phyllophora Hoffm.	Canada, Manitoba, 2007, *Normore* 8006 (FU)	FJ536381	FJ536408
C. phyllophora Hoffm.	Canada, Newfoundland, 2007, *Normore* 8018, (FU)	FJ536382	FJ536409
C. phyllophora Hoffm.	Stenroos *et al.* (2002); Finland	AF455170	–
C. phyllophora Hoffm.	Beiggi & Piercey-Normore (2007); Canada	DQ530196	–
C. squamosissima (Müll. Arg.) Ahti.	Han *et al.* unpub. (2007); China	EU266112	–
C. subchordalis A. Evans	Stenroos *et al.* (2002); Chile	AF455175	–
C. subchordalis A. Evans	Stenroos *et al.* (2002); Chile	AF455174	–
C. subchordalis A. Evans	Stenroos *et al.* (2002); Chile	AF455173	–

*FU = fumarprotocetraric acid, AT = atranorin

was set at 90%. One data set was used for the ITS rDNA which excluded three taxa; RK1021, MN5485, and MN6065, because the sequences contained large numbers of ambiguous positions. All taxa were included in the PKS data set.

RNA folding of the ITS2 was done using the mfold web server (Zuker 2003; http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi). If more than one fold was produced by the mfold program, the final fold was based on comparisons with previously published ITS2 folds (http://its2.bioapps.biozentrum.uni-wuerzburg.de/cgi-bin/index.pl?about), maximization of the hydrogen bonding forming solid stems, and the largest negative delta g value (free energy). The ITS2 was folded and used in the phylogenetic comparisons because more information is available for ITS2 models (Coleman 2003; Müller *et al.* 2007; http://its2.bioapps.biozentrum.uni-wuerzburg.de/cgi-bin/index.pl?about) than for ITS1.

Results

The ITS rDNA phylogeny was made up of 36 sequences from this study and 25 sequences from GenBank (Fig. 1), and produced 269 MP trees with a length of 356 steps. The alignment consisted of 622 characters with 115 of them being phylogenetically informative. The CI was 0·7444 and the RI 0·8613. *Cladonia multiformis* (section *Ascyphiferae*) is the most distantly placed species from members of section *Cladonia* and forms a highly supported clade. Within section *Cladonia*, *C. subchordalis* is more basal than *C. phyllophora* and the *C. gracilis* group, which form terminal sister clades. These form monophyletic clades with high levels of support. The *C. gracilis* complex forms a derived clade with bootstrap support of 71% and includes *C. coniocraea*, *C. ochrochlora* and *C. cornuta*. Members of *Cladonia maxima* form a group within the *C. gracilis* clade.

The model for the ITS2 RNA secondary structure for *C. gracilis* and allies contains three helices around an open single stranded region (not shown). Helix I in the model shows a single substitution in the terminal bulge that varies among the five taxa (Fig. 2). Helix II is more variable than the other helices. Helix II in *C. gracilis* subsp. *turbinata* and *C. gracilis* subsp. *elongata* is almost identical except for substitutions within the species (open boxes). The difference between *C. gracilis* subsp. *turbinata* and *C. phyllophora* is a Compensatory Base Change (CBC) in the lower stem of helix II, a full base pair insertion in the upper stem, and two single base insertions on the 5′ side of the stem in *C. phyllophora*. The difference between *C. gracilis* subsp. *elongata* and *C. maxima* is two CBCs and the loss of a bond in the terminal bulge. Two double CBCs and two single CBCs separate *C. multiformis* and *C. maxima*. *Cladonia multiformis* and *C. phyllophora* are separated by one double CBC, three single CBCs, and the loss of a bond at the terminal bulge. *Cladonia multiformis* differs from the other species by having three single CBCs and two double CBCs. Helix III is less variable than the other helices and is identical in *C. gracilis* subsp. *elongata* and *C. gracilis* subsp. *turbinata*. *Cladonia maxima* contains a substitution and loss of a bond in the lower stem creating a bulge, and one CBC in the upper stem. The helix III in *C. phyllophora* is identical to that of *C. gracilis* subsp. *elongata* and *C. gracilis* subsp. *turbinata*.

The midpoint rooted phylogeny based on the KS region of the PKS gene contains 35 samples from this study (Fig. 3A), and produced 12 MP trees with length of 104 steps. The alignment consists of 407 characters with 61 of these being phylogenetically informative. The CI is 0·8161 and the RI is 0·9521. The clade representing the *C. gracilis* species complex is highly supported with 92% bootstrap and greater than 95% posterior probability. *Cladonia multiformis* is not monophyletic as one member (5918b) falls outside the clade. *Cladonia maxima* and *C. phyllophora* are each monophyletic with bootstrap support of 98% and a posterior probability greater than 95%, respectively. *Cladonia maxima* falls outside the *C. gracilis* clade with high bootstrap support at 92%.

After the 50bp insertion in the coding region of the KS domain was removed, the region was translated to an amino acid sequence. Four non-synonymous changes were present in the translated coding region. These non-synonymous changes separated *C. multiformis* and *C. phyllophora* from all other species (Fig. 3B).

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

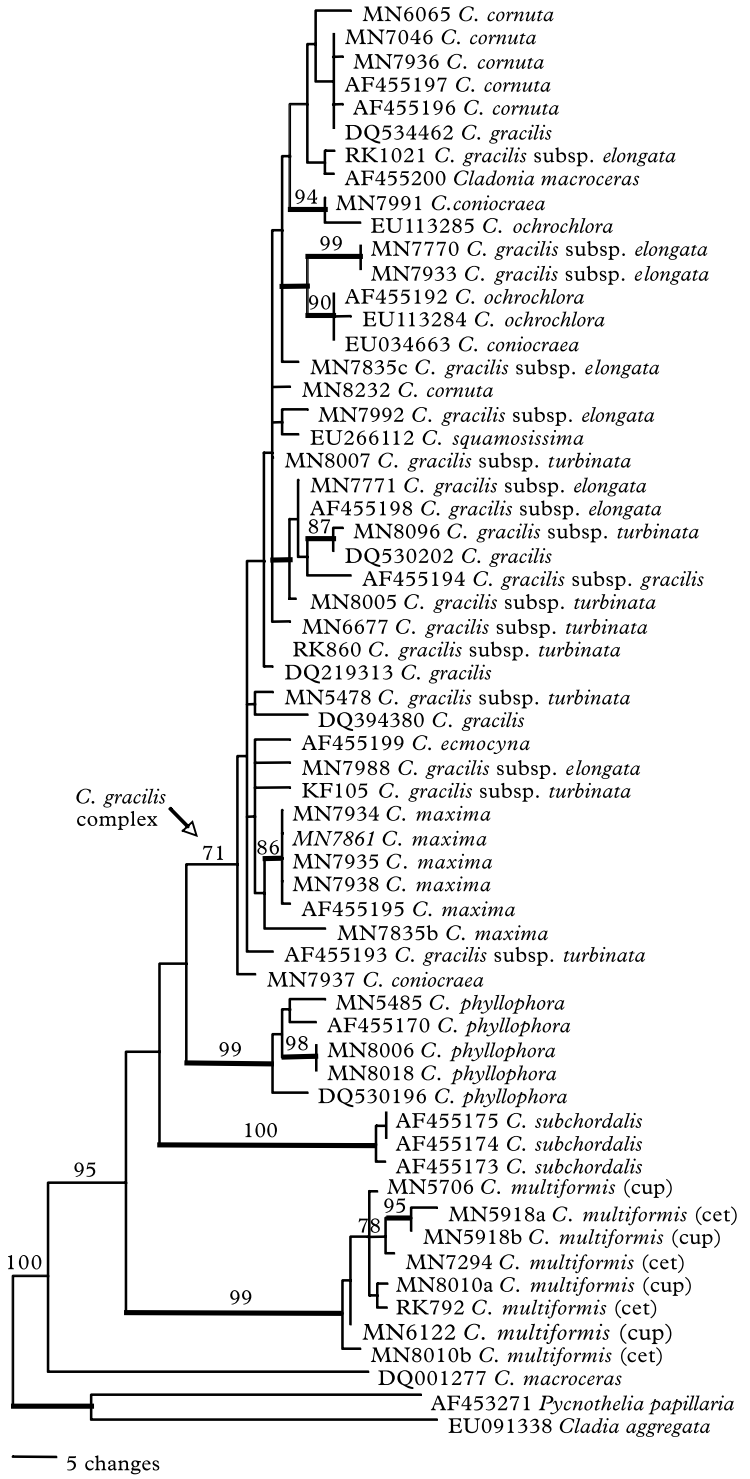


FIG. 1. Phylogenetic hypothesis of *Cladonia gracilis* species complex and allies. One of 269 MP trees based on ITS rDNA sequences. Numbers above the branches are bootstrap values greater than 70%, and thick lines are lineages supported by more than 95% posterior probability. "Cet" refers to the cetrarioid and "cup" refers to the cup-forming growth forms of *C. multiformis*. The outgroup was designated as *Pycnothelia papillaria* and *Cladia aggregata*.

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

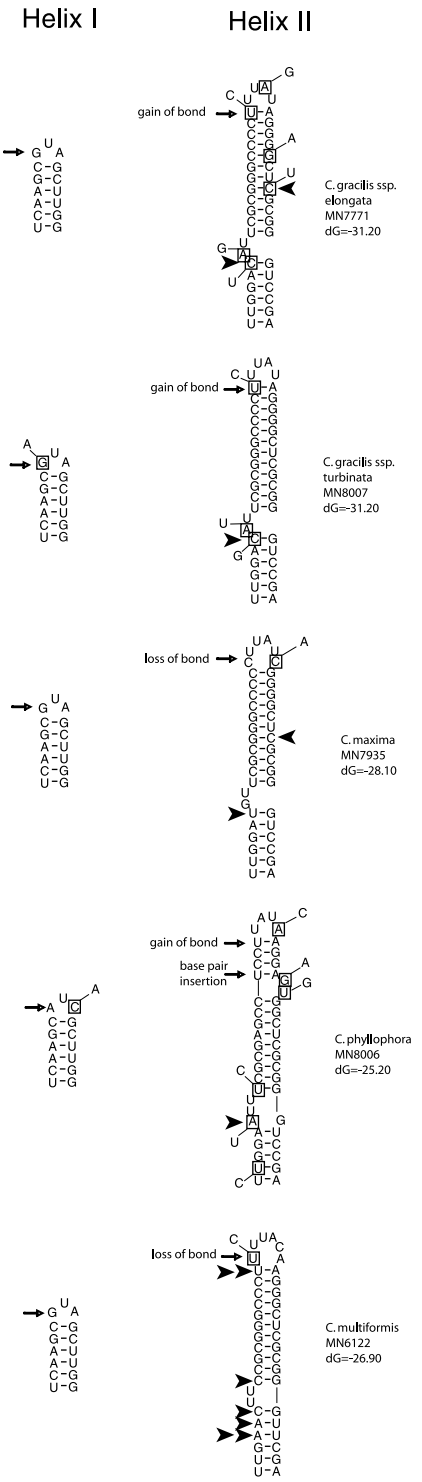


FIG. 2. Helices I and II of the secondary structure model of ITS2 rRNA representing five species of *Cladonia gracilis* and allies in this study. Changes are indicated on the structures as solid arrow heads representing CBC between species; open boxes connected to a substitution represent substitutions within species only; solid arrows indicate other changes as indicated. Species name, collection number of the folded sequence, and the dG of the fold are placed to the right of the folds.

An incongruence test was performed on the PKS and the ITS data sets to determine whether the data sets produced topologies that were significantly different from one another. The Kishino-Hasagawa (KH) test showed that six of the ten trees from the PKS data produced an evolutionary history that was not significantly different from that of the ITS, but the remaining four PKS trees differed from the ITS topology in the placement of *C. maxima* and *C. phyllophora*. Substitutions in six positions of the PKS alignment were responsible for *C. phyllophora* being placed outside *C. maxima*. However, homoplastic substitutions in two positions placed *C. maxima* outside *C. phyllophora* in the four significantly different PKS trees.

The ITS haplotype network produced 27 haplotypes (represented by collection numbers) from 33 taxa (three taxa were removed from the analysis because of large amounts of missing data) and the maximum number of connection steps at 90% probability was 15 steps (Fig. 4). The ITS network showed a taxonomic pattern similar to that of the PKS but the ITS network contained secondary connections within the network. The haplotype network based on the PKS nucleotide sequence produced 20 haplotypes (represented by collection numbers) in 35 taxa and the maximum number of connection steps at 90% probability was 12 steps (Fig. 5). The networks showed no geographic pattern but they are in agreement with the general taxonomic pattern shown in the phylogenetic trees. Both networks place *C. multiformis* (Cmul) in an ancestral position and *C. maxima* (Cmax) in a derived position. The networks differ in the placement of *C. phyllophora* (Cphy), which is ancestral in the ITS and derived in the PKS network.

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

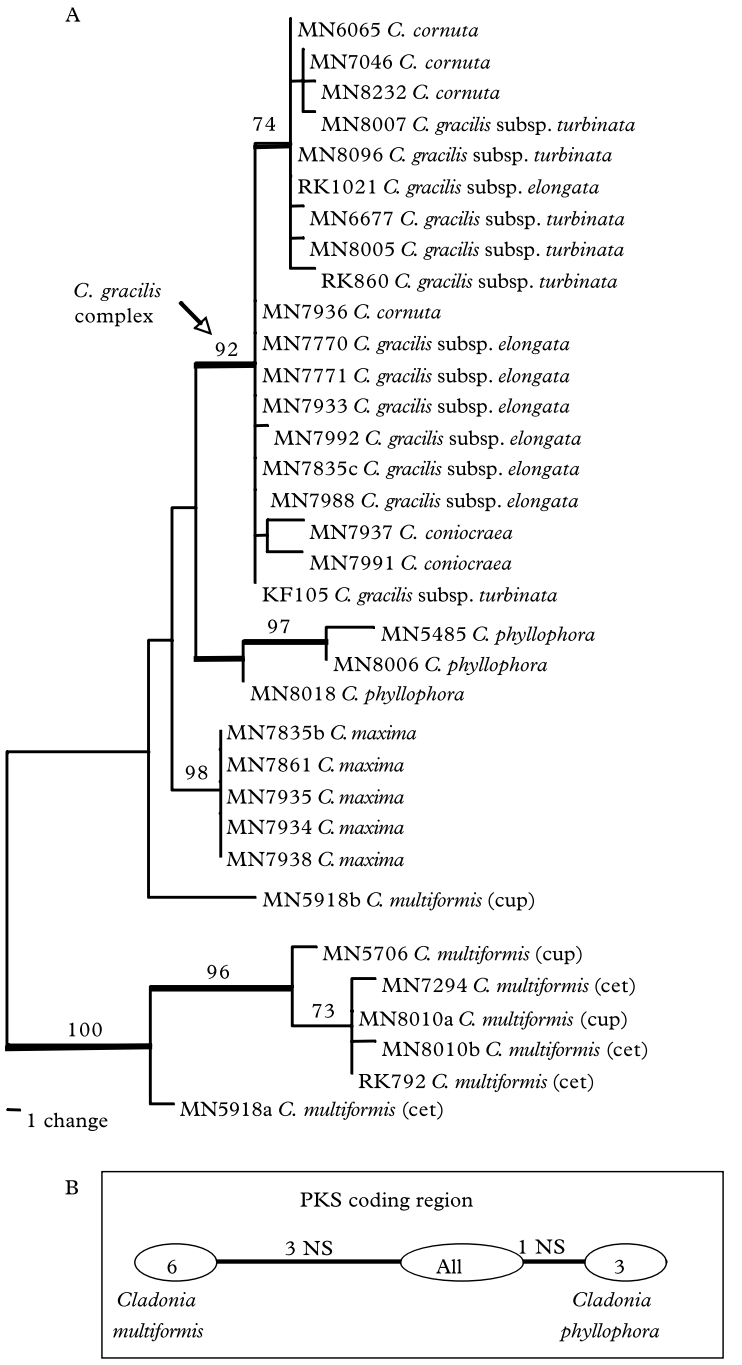


FIG. 3. A, One of 12 most parsimonious trees showing the phylogenetic hypothesis for the *C. gracilis* complex and allies based on a portion of the KS region in the PKS gene. Numbers above the branches are bootstrap values greater than 70%, and thick lines are lineages supported by more than 95% posterior probability. "Cet" refers to the cetrarioid and "cup" refers to the cup-forming growth forms of *C. multiformis*. The tree is midpoint rooted; B, hand-drawn haplotype network of the amino acid sequence showing non-synonymous changes between species.

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

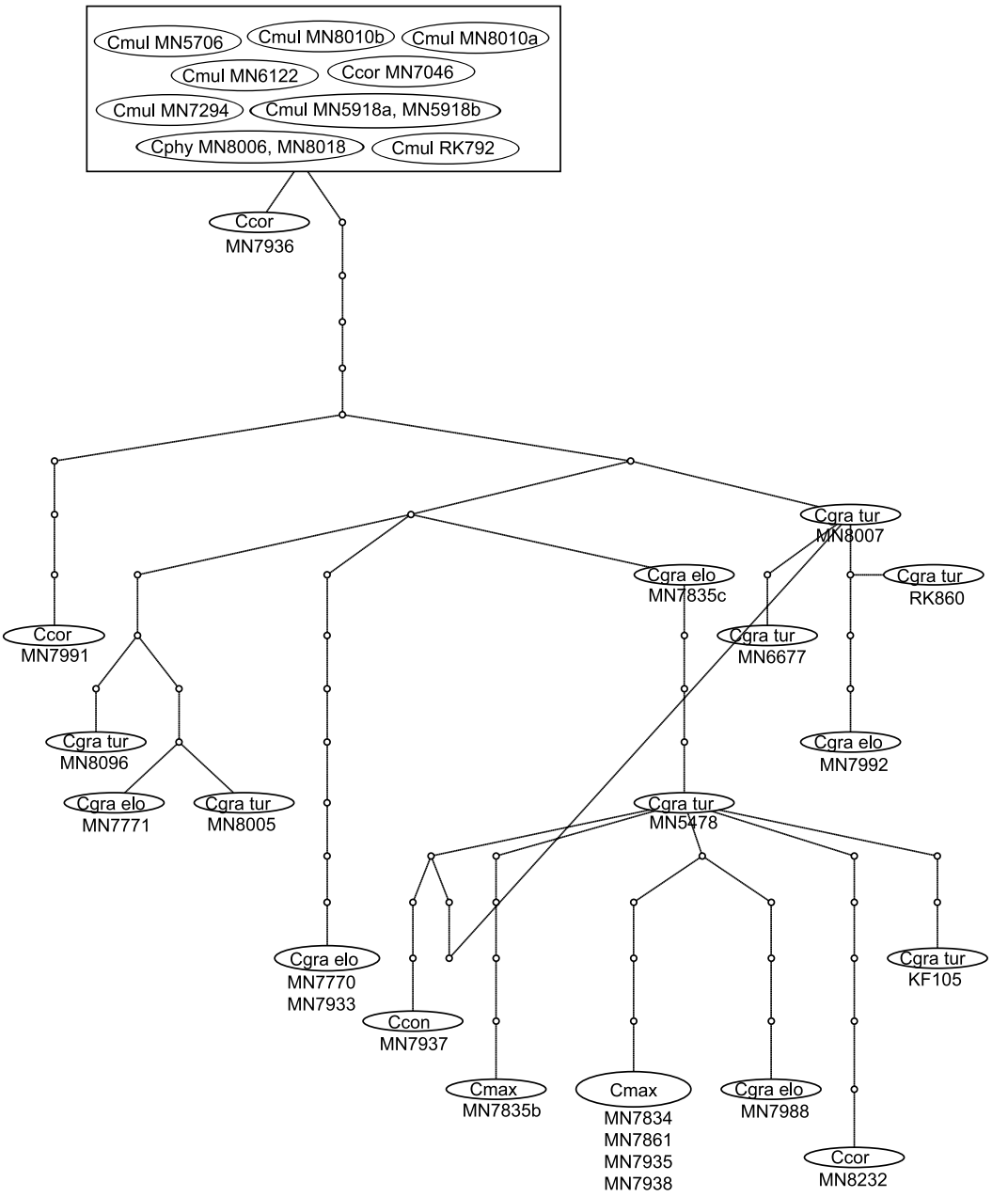


FIG. 4. Haplotype networks of *Cladonia gracilis* complex and allies based on nucleotide sequences of ITS rDNA (except Cgra tur RK1021, Cphy MN5485, and Ccor MN6065). Sample names for derived haplotypes are enclosed in oval spaces and those for ancestral haplotypes are enclosed in boxes. Sample names are represented by the first letter of the genus and the first three letters of the species epithet. The number of changes between haplotypes is represented by lines.

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

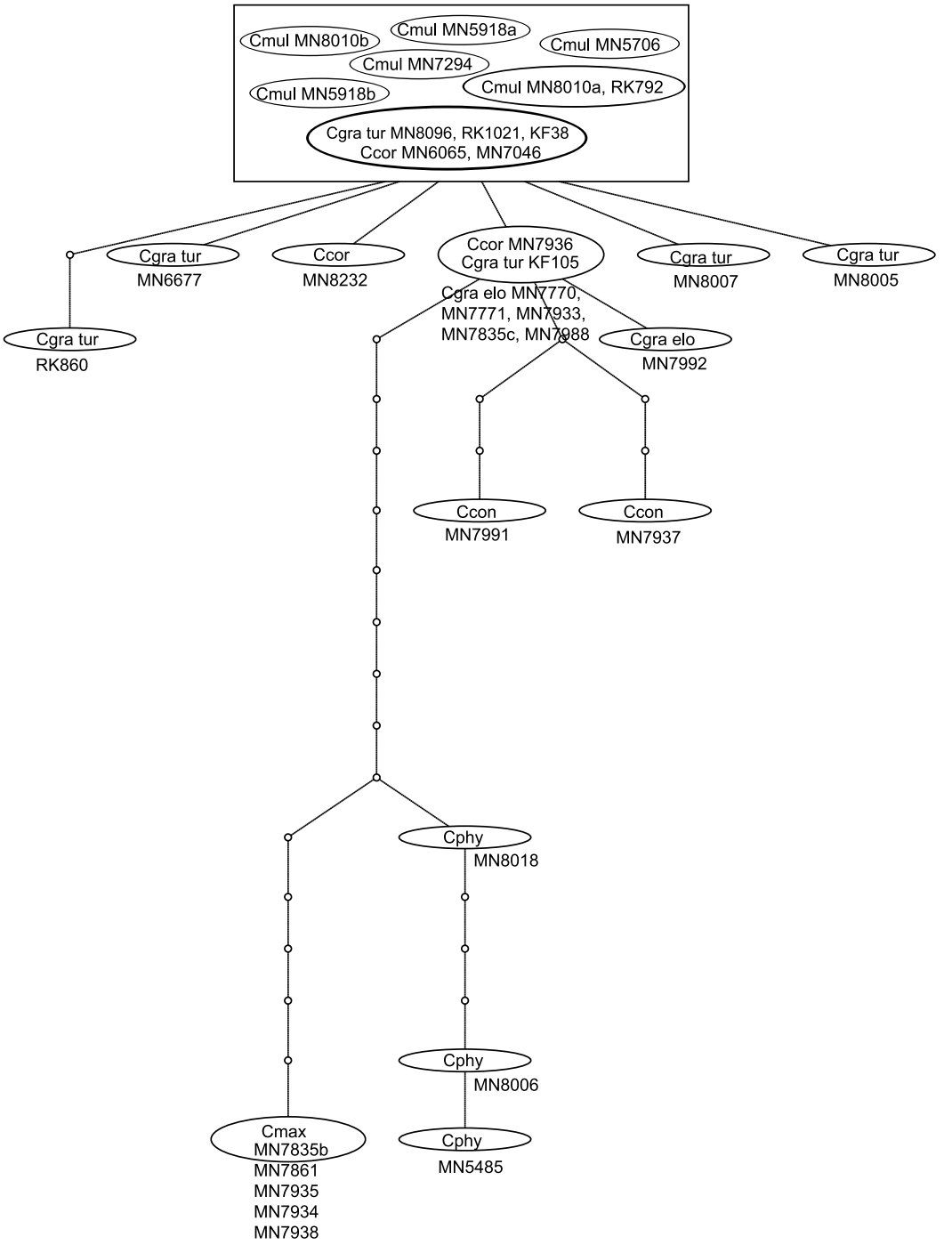


FIG. 5. Haplotype networks of *Cladonia gracilis* complex and allies based on nucleotide sequences of KS region of the PKS gene. See legend for Fig. 4 for further explanation.

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

All samples used in this study produced fumarprotocetraric acid and a small number (five samples) produced atranorin. These samples did not cluster together in either tree and were unresolved in the PKS tree.

Discussion

Speciation of *C. gracilis* and allies

Monophyly of the *Cladonia gracilis* complex can be supported by phylogenetic analyses for both ITS and PKS data sets if allied species (*C. coniocraea* and *C. ochrochlora*) are included in the complex. *Cladonia ochrochlora* was previously confirmed as a member of the complex by Stenroos *et al.* (2002). *Cladonia coniocraea* is now also a confirmed member of the complex using both ITS and PKS loci. These two species are difficult to distinguish using morphological characters. Other species previously included in the *C. gracilis* complex by Ahti (1980) are *Cladonia macroceras*, *C. maxima*, *C. squamosissima*, *C. subchordalis*, *C. cornuta*, *C. ecmocyna* and *C. gracilis*. The inclusion of five of these species is supported by this study. *Cladonia subchordalis* clearly falls outside the clade labelled as *C. gracilis* complex. The presence of three CBCs, a seven-base insert and a shift in the base pairing of the lower stem of helix II (data not shown) support *C. subchordalis* forming a separate biological species Additional support for monophyly of the *C. gracilis* clade is the absence of CBCs in helices of the ITS2 secondary structure from members within the complex, suggesting that these members may interbreed (Coleman 2003; Müller *et al.* 2007). Alternatively, recent speciation may be occurring among taxa within the *C. gracilis* clade resulting in low support for separation of morphological species.

The ancestral haplotype was hypothesized to be *C. multiformis* and most members of the *C. gracilis* complex are derived based on the frequency of haplotypes. Haplotype frequencies are thought to correlate with age under a neutral coalescent theory (Castelloe & Templeton 1994) where the highest frequency of haplotypes are considered the most ancestral forms. This is in agreement with the phylogenetic hypothesis where members of the *C. gracilis* complex show low levels of resolution suggesting a recent speciation. The distance between *C. multiformis* and *C. phyllophora* with the *C. gracilis* complex was also supported by the PKS amino acid sequence of the coding region, but with *C. multiformis* more distant than *C. phyllophora*.

The haplotype network for the ITS region produced a genealogical pattern corresponding loosely with a taxonomic rather than a geographic pattern. A geographic pattern might be more evident from a uniparentally inherited plastid rather than from nuclear loci where recombination occurs. The distinct geographic pattern produced by mitochondrial loci in ungulates was not supported by nuclear loci (Schaschl *et al.* 2003), which was interpreted to result from sexual reproduction or balanced polymorphism. The retention of multiple alleles (as in balanced polymorphism) might be an argument in this study in favour of convergence of cup forms from ancestral lineages that do not express the cup-forming phenotype. The alleles required for cup formation must be retained within the ancestral lineage to be expressed under specific environmental conditions or during developmental stages. In addition, the lack of correspondence between geographic origin and pattern in the haplotype network may also be caused by small sample size, since small samples may be prone to sampling artifacts. Sexual reproduction and recombination may contribute to the absence of a geographic variation since the morphological species in this study have not been shown to be reproductively isolated.

Evolution of cup morphology

Despite the morphological similarities between *C. maxima* and *C. gracilis* subsp. *elongata*, and between *C. phyllophora* and *C. gracilis* subsp. *turbinata*, both allies, *C. maxima* and *C. phyllophora* form phylogenetic species separate from the morphologically similar members of the *C. gracilis* complex in this study. This is supported by a number of CBCs and other changes in the

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

ITS2 secondary structures (Fig. 2) suggesting these species are reproductively isolated from the *C. gracilis* complex (Coleman 2003; Müller *et al.* 2007). The evolution of cup-forming morphologies in *C. phyllophora* and *C. maxima*, distinct from the cup-formers in the *C. gracilis* complex, suggests that evolution of the cup form is converging and that it may serve as an important function for these species. Polyphyly among cup-forming species was also present in studies by Stenroos *et al.* (2002) and Beiggi & Piercey-Normore (2007). Other examples of convergent evolution include growth forms in the *Cladoniaceae* (Stenroos & DePriest 1998), ascomatal type in higher fungi (Grube & Hawksworth 2007; Hawksworth & LaGreca 2007), and the mazaediate "pin lichens" from the *Caliciales* (Tibell 1998). One function suggested for the cup in sorediate lichens is dispersal of propagules by rainwater. This splash cup mechanism of dispersal is known to occur in plants (Brodie 1951; Nakanishi 2004), and it has also been described for sorediate dispersal in *Cladonia chlorophaea* (Bailey 1966). However, none of the cup forming species in this study produce propagules, eliminating sorediate dispersal as a possible function. Another function for a non-sorediate cup may be to retain water near the developing apothecia. This would prolong development time for asci and spores when the surrounding air dries, perhaps allowing asci to reach maturity, since the amount of precipitation is thought to influence spore production (Verseghy 1965). A slower rate of thallus dehydration by water retention may also induce ascal release of spores aiding spore dispersal. In most of these species the cup is closed allowing water to accumulate near the developing apothecia on the cup margin. However, the cup in *C. multiformis* is perforated allowing water to escape into the hollow podetium thus perhaps capturing a larger amount of water for an even longer retention and slower rate of dehydration. The two morphological forms of *Cladonia multiformis* in this study are a cetrarioid form with funnelled extensions from the cup margin, and a cup-forming morphology without the extensions. One hypothesis is that the apothecia-bearing cups are the 'mature' podetia and the funnel shaped cetrarioid forms are immature forms. The funnel shaped cetrarioid forms that grow from the cup margins may direct water toward the cups for prolonged development of the apothecia. More ecological research is required to study the function and correlation of cup forms with specific habitat parameters.

Two morphological forms of *C. multiformis* from the same herbarium packet were represented by MN8010a and MN8010b; and from the same individual podetium by MN5918a and MN5918b. The ITS variation between 8010a and 8010b (seven differences) suggests they were different individuals growing side by side in a non-clonal fashion. This may reflect a different growth strategy by *C. multifomis* than by *C. subtenuis* where clonal growth was hypothesized in a single patch of *C. subtenuis* (Beard & DePriest 1996). The ITS sequences of samples MN5918a and MN5918b were identical (no differences) suggesting the single podetium is made of a single fungal genotype. The non-zero branch lengths in Fig. 1 are the result of problems associated with large amounts of missing data (Kearney & Clark 2003; Wiens 2006), but the ITS haplotype network placed the two samples as the same haplotype (Fig. 3A).

The utility of the PKS gene as a phylogenetic marker

The PKS gene is less variable than the ITS with approximately half the number of phylogenetically informative characters and higher CI and RI indices, but it provided strong support for the clades inferred to be reproductively isolated by CBC in the ITS2 secondary structure. The disagreement between the PKS and ITS trees indicated by the KH test is predominantly at the position of the clade containing five members of *Cladonia maxima*. Low resolution in one set of trees, as with the ITS in this study, may account for the incongruence. Other explanations may also account for this apparent discrepancy. 1) Fungal chimera may exist where two or more fungal strains make up a

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

single podetium. Armaleo & Clerc (1991) demonstrated fungal chimera in photosymbiodemes. Alternatively, a single podetium may consist of more than one fungal strain. 2) Recombination and sexual reproduction may occur among these species suggesting they may not be separate biological species. Culberson *et al.* (1988) found that *C. merochlorophaea* and *C. grayi* were interbreeding but were reproductively isolated from *C. cryptochlorophaea*, implying that morphologically similar chemospecies may be either interbreeding or reproductively isolated. 3) Paralogous genes for each of the ITS rDNA and the PKS may be possible. Multiple copies of the ITS rDNA were suspected in individual *Symbiodinium* genomes (Reimer *et al.* 2006). Different ITS copies may exist with incomplete concerted evolution. Although paralogs of PKS genes have been reported; three copies for some members of the *Parmeliaceae* (Opanowicz *et al.* 2005) and as many as 14 copies suggested for *Cladonia grayi* (D. Armaleo, pers. comm.), the nucleotide sequences of paralogs show larger differences than are present in this study.

In conclusion, this study has corroborated previously untested hypotheses. Monophyly of the *C. gracilis* species complex is supported with the inclusion of *Cladonia coniocraea* and *C. ochrochlora*. When additional taxa are examined, the *C. gracilis* complex may contain a larger number of species and subspecies than is currently hypothesized. Convergent evolution of cup morphology may explain differences between morphological and genetic variation in the phylogenetic trees. The ecological significance of the cup morphology needs to be examined further. The phylogenetic position of *C. maxima* may be argued based on the ITS, but it may form a species separate from the *C. gracilis* species complex. *Cladonia phyllophora* and *C. multiformis* form monophyletic groups with support for morphological and biological species. The KS region is less variable than that of the ITS rDNA, but it verified clades of species and was in agreement with changes in the ITS2 RNA model, supporting the use of the PKS gene as a phylogenetic marker for this group of lichens.

We thank Rhonda Kotelko for providing samples, H. Hernandez (Manitoba Conservation) for kindly providing collecting permit number 20429, and Parks Canada for permit number WAP-2005-498. Funding was provided by Natural Sciences and Engineering Research Council of Canada (NSERC).

References

Ahti, T. (1980) Taxonomic revision of *Cladonia gracilis* and its allies. *Annales Botanica Fennici* **17:** 195–243.

Ahti, T. (2000) Cladoniaceae, *Flora Neotropica Monograph 78*. New York: The New York Botanical Garden.

Ahti, T., Parnmen, S. & Mongkolsuk, P. (2008) Three new species of *Cladonia* from Thailand. *Sauteria* **15:** 15–19.

Armaleo, D. & Clerc, P. (1991) Lichen chimeras: DNA analysis suggests that one fungus forms two morphotypes. *Experimental Mycology* **15:** 1–10.

Bailey, R. H. (1966) Studies on the dispersal of lichen soredia. *Journal of the Linnean Society, Botany* **59:** 479–490.

Beard, K. H. & DePriest, P. T. (1996) Genetic variation within and among mats of the reindeer lichen, *Cladina subtenuis*. *Lichenologist* **28:** 171–182.

Beiggi, S. & Piercey-Normore, M. D. (2007) Evolution of ITS ribosomal RNA secondary structures in fungal and algal symbionts of selected species of *Cladonia* sect. *Cladonia* (Cladoniaceae, Ascomycotina). *Journal of Molecular Evolution* **64:** 528–542.

Bingle, L. E. H., Simpson, T. J. & Lazarus, C. M. (1999) Ketosynthase domain probes identify two subclasses of fungal polyketide synthase genes. *Fungal Genetics and Biology* **26:** 209–223.

Brodie, H. J. (1951) The splash-cup dispersal mechanism in plants. *Canadian Journal of Botany* **29:** 224–234.

Castolloe, J. & Templeton, A. R. (1994) Root probabilities for intraspecific gene trees under neutral coalescent theory. *Molecular Phylogenetics and Evolution* **3:** 102–113.

Clement, M., Posada, D. & Crandall, K. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9:** 1657–1660.

Coleman, A. W. (2003) ITS2 is a double-edged tool for eukaryote evolutionary comparisons. *Trends in Genetics* **19:** 370–375.

Culberson, C. F. (1972) Improved conditions and new data for the identification of lichen products by a standardized thin-layer chromatographic method. *Journal of Chromatography* **72:** 113–125.

Culberson, C. F. (1986) Biogenetic relationships of the lichen substances in the framework of systematics. *Bryologist* **89:** 91–98.

Culberson, C. F. & Armaleo, D. (1992) Induction of a complete secondary-product pathway in a cultured lichen fungus. *Experimental Mycology* **16:** 52–63.

Culberson, C. F., Culberson, W. L. & Johnson, A. (1988) Gene flow in lichens. *American Journal of Botany* **75:** 1135–1139.

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

DePriest, P. T. (2004) Early molecular investigations of lichen-forming symbionts: 1986–2001. *Annual Review of Microbiology* **58:** 273–301.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783–791.

Gowan, S. P. (1989) A character analysis of the secondary products of the Porpidiaceae (lichenized Ascomycotina). *Systematic Botany* **14:** 77–90.

Grube, M. & Blaha, J. (2003) On the phylogeny of some polyketide synthase genes in the lichenized genus *Lecanora*. *Mycological Research* **107:** 1419–1426.

Grube, M. & Hawksworth, D. L. (2007) Trouble with lichen: the re-evaluation and re-interpretation of thallus form and fruit body types in the molecular era. *Mycological Research* **111:** 1116–1132.

Grube, M., DePriest, P. T., Gargas, A. & Hafellner, J. (1995) DNA isolation from lichen ascomata. *Mycological Research* **99:** 1321–1324.

Guo, S. & Kashiwadani, H. (2004) Recent study on the phylogeny of the genus *Cladonia* (s. lat.) with the emphasis on the integrative biology. *National Science Museum Monographs, Tokyo* **24:** 207–225.

Hawksworth, D. L. (1976) Lichen chemotaxonomy. In *Lichenology: Progress and Problems* (D. H. Brown, D. L. Hawksworth & R. H. Bailey, eds): 139–184. London: Academic Press.

Hawksworth, D. L. & LaGreca, S. (2007) New bottles for old wine: fruit body types, phylogeny, and classification. *Mycological Research* **111:** 999–1000.

Hinds, J. W. & Hinds, P. L. (2007) *The Macrolichens of New England*. New York: New York Botanical Garden Press.

Huelsenbeck, J. P., Ronquist, F., Nielsen, R. & Bollback, J. P. (2001) Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* **294:** 2310–2314.

Hur, J. S., Wang, L. S., Oh, S. O., Kim, G. H., Lim, K. M., Jung, J. S. & Koh, Y. J. (2005) Highland macrolichen flora of northwestern Yunnan, China. *The Journal of Microbiology* **43:** 228–236.

Hyvönen, J., Ahti, T., Stenroos, S. & Gowan, S. P. (1995) Genus *Cladina* and the section Unciales of the genus *Cladonia* (Cladoniaceae, lichenized Ascomycotina), a preliminary phylogenetic analysis. *Journal of the Hattori Botanical Laboratory* **78:** 243–253.

Kearney, M. & Clark, J. M. (2003) Problems due to missing data in phylogenetic analyses including fossils: a critical review. *Journal of Vertebrate Paleontology* **23:** 263–274.

Keller, N. P. & Hohn, T. M. (1997) Metabolic pathway gene clusters in filamentous fungi. *Fungal Genetics and Biology* **21:** 17–29.

Kishino, H. & Hasagawa, M. (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *Journal of Molecular Evolution*. **29:** 170–179.

Leuckert, C., Ahmadjian, V., Culberson, C. F. & Johnson, A. (1990) Xanthones and depsidones of the lichen *Lecanora dispersa* in nature and of its mycobiont in culture. *Mycologia* **82:** 370–378.

Müller, T., Philippi, N., Dandekar, T., Schultz, J. & Wolf, M. (2007) Distinguishing species. *RNA* **13:** 1469–1472.

Nakanishi, H. (2004) Splash seed dispersal by raindrops. *Ecological Research* **17:** 663–671.

Opanowicz, M., Blaha, J. & Grube, M. (2005) Detection of paralogous polyketide synthase genes in *Parmeliaceae* by specific primers. *Lichenologist* **38:** 47–54.

Piercey-Normore, M. D. & DePriest, P. T. (2001) Algal switching among lichen symbioses. *American Journal of Botany* **88:** 1490–1498.

Posada, D. & Crandall, K. A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

Rambaut, A. (2001) *Se-Al: Sequence Alignment Editor V2.0*. Oxford: University of Oxford.

Reimer, J. D., Takishita, K., Ono, S., Maruyama, T. & Tsukahara, J. (2006) Latitudinal and intracolony ITS-rDNA sequence variation in the symbiotic dinoflagellate genus *Symbiodinium* (Dinophyceae) in *Zoanthus sansibaricus* (Anthozoa: Hexacorallia). *Phycological Research* **54:** 122–132.

Ronquist, F. & Huelsenbeck, J. P. (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574.

Schaschl, H., Kaulfus, D., Hammer, S. & Suchentrunk, F. (2003) Spatial patterns of mitochondrial and nuclear gene pools in chamois (*Rupicarpra* r. *rupicapra*) from the Eastern Alps. *Heredity* **91:** 125–135.

Schmitt, I., Martin, M. P., Kautz, S. & Lumbsch, H. T. (2005) Diversity of non-reducing polyketide synthase genes in the Pertusariales (lichenized Ascomycota): a phylogenetic perspective. *Phytochemistry* **66:** 1241–1253.

Schümann, J. & Hertwech, C. (2006) Advances in cloning, functional analysis and heterologous expression of fungal polyketide synthase genes. *Journal of Biotechnology* **124:** 690–703.

Shwab, E. K. & Keller, N. P. (2008) Regulation of secondary metabolite production in filamentous ascomycetes. *Mycological Research* **112:** 225–230.

Stenroos, S. K. & DePriest, P. T. (1998) SSU DNA phylogeny of cladoniiform lichens. *American Journal of Botany* **85:** 1548–1559.

Stenroos, S., Ahti, T. & Hyvönen, J. (1997) Phylogenetic analysis of the genera *Cladonia* and *Cladina* (Cladoniaceae, lichenized Ascomycota). *Plant Systematics and Evolution* **207:** 43–58.

Stenroos, S., Hyvönen, J., Myllys, L., Thell, A. & Ahti, T. (2002) Phylogeny of the genus *Cladonia* s. lat. (Cladoniaceae, Ascomycetes) inferred from molecular, morphological, and chemical data. *Cladistics* **18:** 237–278.

Stocker-Wörgötter, E. (2001) Experimental studies of the lichen symbiosis: DNA-analyses, differentiation and secondary chemistry of selected mycobionts, artificial resynthesis of two- and tripartite symbioses. *Symbiosis* **30:** 207–227.

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press

Swofford, D. L. (2003) *PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods), Version 4.0*., Sunderland, MA: Sinauer Associates.

Tibell, L. (1998) Crustose mazaediate lichens and the Mycocaliciaceae in temperate South America. *Bibliotheca Lichenologica* **71:** 1–107.

Verseghy, K. (1965) Effect of dry periods on the spore production of lichens. *Acta Biologica Hungarica* **16:** 85–104.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide to Methods and Applications*. (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315–322. New York: Academic Press, Inc.

Wiens, J. J. (2006) Missing data and the design of phylogenetic analyses. *Journal of Biomedical Informatics* **39:** 34–42.

Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* **31:** 3406–3415.

Accepted for publication 25 *September* 2009

https://doi.org/10.1017/S0024282909990569 Published online by Cambridge University Press