The Lichenologist **48**(3): 209–227 (2016) doi:10.1017/S0024282915000572

Phylogenetic relationships among reindeer lichens of North America

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Abstract: *Cladonia* is one of the largest lichen-forming ascomycete genera. It was formerly divided into ten sections, three of which, *Crustaceae* (*Cladina*), *Tenues*, and *Impexae*, are called the reindeer lichens. While previous studies have elucidated the relationships between species and sections, they often examined only one or a few specimens of each species in the analysis. This study examined the monophyly of selected members of sections *Crustaceae*, *Tenues*, and *Impexae* and their relationships in the genus *Cladonia* using the internal transcribed spacer region of the nuclear ribosomal DNA (ITS rDNA) and the mitochondrial small subunit gene of the mitochondrial ribosomal DNA (mtSSU). The phylogenetic tree contained four clades, two representing species in section *Impexae*, one representing species that belong to sections *Crustaceae* and *Tenues*, and one clade with *C. arbuscula* and related species. Five of 22 species, *C. pycnoclada*, *C. stellaris*, *C. evansii*, *C. ciliata* and *C. subtenuis*, showed monophyly in the phylogenetic tree; some of these 5 species have been shown previously to be monophyletic. The thallus branching pattern was interpreted as an important heritable character using the mtSSU network. Three duplets of paraphyletic species were further examined using ITS rDNA haplotype networks and AMOVA analysis. The results for the species duplets showed some mixing of haplotypes but the AMOVA analysis provided support for species separation within the duplets. While the evidence supports distinct species, further study is needed to conclusively show separate species in these duplets.

Keywords: AMOVA, *Cladina*, haplotype network, ITS rDNA, monophyly, mtSSU, phylogeny

Accepted for publication 12 *December* 2015

Introduction

Cladonia is the largest genus in the lichen-forming fungal family *Cladoniaceae*, consisting of *c.* 459 accepted species (T. Ahti, August 2015, unpublished data). Based on morphology and secondary chemistry, Ahti (2000) divided the neotropical species of *Cladonia* into seven taxonomic sections and three sections were recognized in the segregate genus *Cladina* (Ahti 2000). The division was applicable to most species in the world. The group called *Cladina* (known as reindeer lichens) is most abundant in the coniferous belt of the Northern Hemisphere and in the *Nothofagus* regions in the Southern Hemisphere, but is also known in sandy areas of the south-eastern United States and elsewhere, as well as at high altitudes in many mountain ranges. While the lack of competitive ability of lichens with plants is well known, the reindeer lichens have adapted better than almost all other lichens to the terrestrial niches uninhabited by vascular plants and bryophytes. Some species, such as *Cladonia arbuscula*, *C. rangiferina*, *C. stygia* and *C. stellaris*, are important components of northern ecosystems where they provide vast areas of ground cover (Auclair & Rencz 1982; Shaver & Chapin 1991) and form a major component of the winter food for caribou and reindeer (Svihus & Holand 2000; den Herder *et al.* 2003). Knowledge of their species status would inform ecosystem management and maintenance of biodiversity.

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In recent times the genus *Cladina* has not been recognized by most authors because in phylogenetic studies it is nested within *Cladonia* and is not monophyletic (Stenroos *et al.* 2002, 2015; Guo & Kashiwadani 2004). The group of reindeer lichens constitute *Cladonia*, sections *Crustaceae* (= *Cladina* section *Cladina*), *Tenues*, and *Impexae*, which typically have highly branched ecorticate podetia and a soon disappearing crustose primary thallus. Section *Tenues* typically has anisotomic branching with dichotomous branches, section *Crustaceae* has anisotomic branching with two, three or four divisions to the branches, and section *Impexae* has isotomic or subisotomic branching with two, three, four, or five divisions to the branches. Phylogenetic relationships among all sections of *Cladonia* have been examined previously (Stenroos *et al.* 1997, 2002), but usually with only one to a few specimens of each species included in the analysis. While Stenroos *et al.* (2002) recommended studying a larger number of specimens for each species, knowledge is lacking of the number of monophyletic groups, the species composition of each monophyletic group, and their phylogenetic relationships. Earlier studies were focused on species identification (Ruoss 1987*a*, *b*; Ruoss & Ahti 1989) and recent studies show relationships among distant geographical collections of *Cladonia arbuscula* in the broad sense (Myllys *et al.* 2003; Piercey-Normore *et al.* 2010), but knowledge concerning phylogenetic relationships is insufficient. Monophyly and diagnosability are considered to be important criteria for species delimitation (e.g. Bacon *et al.* 2012). While monophyly may suggest low levels of intraspecies variation, the absence of monophyly may suggest ongoing speciation, incomplete lineage sorting, or interbreeding among species.

The goals of this study were: 1) to examine the monophyly of the sections *Crustaceae*, *Tenues* and *Impexae*; 2) to reconstruct the phylogenetic relationships among selected species of reindeer lichens in the genus *Cladonia*; and 3) to examine species delimitations.

Materials and Methods

Lichen specimens were collected from Canada or borrowed from herbaria, and additional sequences were obtained from the NCBI GenBank (Table 1). Sixty-two representative specimens are deposited in the University of Manitoba Herbarium (WIN) or the Botanical Museum, Finnish Museum of Natural History, Helsinki (H) (Table 1).

For the phylogenetic analysis, either spore cultures or pieces of dry thalli (10–20 mg) from the apical region of each lichen sample were selected and visually inspected for contaminating debris. The DNA was isolated using a modified CTAB (hexadecytrimethylammonium bromide) protocol (Grube *et al.* 1995). The polymerase chain reaction (PCR) of fungal DNA on the internal transcribed spacer 1 and 2 (ITS1 and ITS2) and the 5.8S of the nuclear ribosomal DNA (rDNA) was performed using the primers 1780F-5' (Piercey-Normore & DePriest 2001) and ITS2KL-3' (Lohtander *et al.* 1998), and on the mitochondrial small subunit (mtSSU) DNA using the primers mrSSU2 and mrSSU3R (Zoller *et al.* 1999). Where there were problems in amplifying across both ITS regions for some samples, the primers ITS1F, ITS2, ITS3 and ITS4 (White *et al.* 1990) were used to amplify the ITS region in two fragments. PCR reaction mixtures (20 µl) contained 20 ng of template DNA, 1 × PCR buffer (50 mM KCl, 20 mM Tris), 0·5 µM of each forward and reverse primer, 3·0 mM of $MgCl_2$ (2·0 mM $MgCl_2$ for mtSSU), 200 mM of each dNTP (Invitrogen Life Technologies, California, USA), and 0·1 U of Taq polymerase (Invitrogen Life Technologies, California, USA). Amplification was carried out in a Biometra® TGradient thermocycler (American Laboratory Trading Inc., Connecticut, USA). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min, and an extension at 72 °C for 1 min 30 s for all primers. For samples for which we had difficulties with PCR amplification, touchdown cycles were as follows: initial denaturation at 95 °C for 5 min; 1 cycle of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and an extension at 72 °C for 1 min 30 s. Annealing temperature of the following 3 cycles was dropped by 2 °C at each cycle (58, 56, 54) followed by 26 cycles with an annealing temperature of 52 °C.

Four to six identical 50 µl reaction volumes of PCR product were pooled for DNA sequencing and gel was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA) following the manufacturer's instructions. Cycle sequencing reaction volumes were 20 µl, containing 60–70 ng of purified DNA, BigDye V3.1 (Applied Biosystem, California, USA) and the same PCR primers that were used for sequencing. Reactions were cleaned using the ethanol/EDTA precipitation method (Applied Biosystem Handbook) according to the manufacturer's instructions. The dried product was dissolved in 20 µl formamide and loaded into a 96-well plate for sequencing on a 3130 Genetic Analyzer (Applied Biosystems, California, USA). The sequences were edited using Sequencher®

TABLE 1. *Collection location, collection numbers, and accession numbers for the* Cladonia *specimens used in this study. Specimens with collection numbers were used to generate sequences and those with references were obtained from GenBank.*

Section and species	Source: collection location, and collection number or reference	Accession no. for ITS or mtSSU
Genus *Cladonia*		
Section *Crustaceae*		
C. argentea	Guyana (Stenroos *et al.* 2002)	ITS: AF458305
C. arbuscula	Canada, Manitoba, *Athukorala* 9 (WIN)	mtSSU: KP001224
C. arbuscula	Canada, Manitoba, *Normore* 9461 (WIN)	ITS: KP001204 mtSSU: KP001225
C. arbuscula	Canada, Manitoba, *Normore* 5073 (WIN)	mtSSU: KP001229
C. arbuscula	Canada, Manitoba, *Athukorala* 7 (WIN)	ITS: KP001207
C. arbuscula	Finland (Myllys *et al.* 2003)	ITS: AY170789
C. arbuscula	Argentina, Tierra del Fuego (Myllys *et al.* 2003)	ITS: AY170787
C. arbuscula	USA, Georgia (Myllys *et al.* 2003)	ITS: AY170773
C. arbuscula	Finland (Myllys *et al.* 2003)	ITS: AY170771
C. arbuscula	Finland (Stenroos *et al.* 2002)	ITS: AF458293
C. arbuscula	USA, Alaska (Piercey-Normore *et al.* 2010)	ITS: GU169280
C. arbuscula	USA, Alaska (Piercey-Normore *et al.* 2010)	ITS: GU169285
C. arbuscula	USA, Alaska (Piercey-Normore *et al.* 2010)	ITS: GU169281
C. arbuscula	USA, Alaska (Piercey-Normore *et al.* 2010)	ITS: GU169284
C. arbuscula	Canada, British Columbia (Piercey-Normore *et al.* 2010)	ITS: GU169283
C. arbuscula	Finland (Piercey-Normore *et al.* 2010)	ITS: GU169223
C. conspicua	Canada, New Brunswick, *Ahti* 74398 & *Clayden* (H)	ITS: KT072714
C. dendroides	Guyana (Stenroos *et al.* 2002)	ITS: AF458295
C. densissima	Guyana (Stenroos *et al.* 2002)	ITS: AF458294
C. mitis	Canada, Manitoba, *Athukorala* 12 (WIN)	ITS: KP001209
C. mitis	Canada, Newfoundland, *Normore* 8804 (WIN)	ITS: KP001205 mtSSU: KP001223
C. mitis	Canada, Manitoba, *Normore* 1155 (WIN)	mtSSU: KP001228
C. mitis	Canada, Manitoba, *Normore* 9468 (WIN)	ITS: KP001206
C. mitis	Argentina, Tierra del Fuego (Myllys *et al.* 2003)	ITS: AY170764
C. mitis	Argentina, Tierra del Fuego (Myllys *et al.* 2003)	ITS: AY170759
C. mitis	Finland (Myllys *et al.* 2003)	ITS: AY170792
C. mitis	Argentina, Tierra del Fuego (Myllys *et al.* 2003)	ITS: AY170767
C. mitis	Argentina, Tierra del Fuego (Myllys *et al.* 2003)	ITS: AY170768
C. mitis	Argentina, Tierra del Fuego (Myllys *et al.* 2003)	ITS: AY170769
C. mitis	Canada, British Colombia (Piercey-Normore *et al.* 2010)	ITS: GU169274
C. mitis	Canada, Manitoba (Piercey-Normore *et al.* 2010)	ITS: GU169228
C. mitis	Greenland (Piercey-Normore *et al.* 2010)	ITS: GU169222
C. oricola	Canada, Nova Scotia, *Ahti* 71318 (H)	ITS: KT072715
C. rangiferina	Canada, Manitoba, *Ahti* 62933 (WIN)	mtSSU: KP001252
C. rangiferina	Canada, Manitoba, *Normore* 9469 (WIN)	ITS: KP001199
C. rangiferina	Canada, Manitoba, *Normore* 5278 (WIN)	mtSSU: KP001251
C. rangiferina	Canada, Manitoba, *Normore* 9462 (WIN)	ITS: KP001192
C. rangiferina	Canada, Ontario, *Normore* 6597 (WIN)	ITS: KP001194
C. rangiferina	Canada, Ontario, *Normore* 6767 (WIN)	ITS: KP001195
C. rangiferina	Canada, New Brunswick, *Normore* 6888 (WIN)	ITS: KP001191
C. rangiferina	Canada, Ontario, *Normore* 7202 (WIN)	ITS: KP001198
C. rangiferina	Canada, Ontario, *Normore* 7307 (WIN)	ITS: KP001193
C. rangiferina	Canada, Newfoundland, *Normore* 8810 (WIN)	ITS: KP001186
C. rangiferina	USA, Alaska, *Kotelko* 1043 (WIN)	ITS: KP001197
C. rangiferina	Canada, Manitoba, *Athukorala* 17 (WIN)	ITS: KP001202
C. rangiferina	Canada, Manitoba, *Athukorala* 22 (WIN)	ITS: KP001200
C. rangiferina	China (Han *et al.* unpublished data)	ITS: EU266113
C. rangiferina	USA, Washington (Smith *et al.* 2012)	ITS: JQ695918
C. rangiferina	USA, Washington (Smith *et al.* 2012)	ITS: JQ695920
C. rangiferina	Finland (Myllys *et al.* 2003)	ITS: AF458306
C. rangiferina	Sweden (Lumbsch *et al.* 2004)	mtSSU: AY300881

TABLE 1. *Continued*

Section and species	Source: collection location, and collection number or reference	Accession no. for ITS or mtSSU
C. rangiferina	India, *Sinha* 1643 (H)	ITS: KP001190
C. rangiferina subsp. *abbayesii*	Guyana (Stenroos *et al.* 2002)	ITS: AF458307
C. rotundata	Guyana (Stenroos *et al.* 2002)	ITS: AF457913
C. stygia	Canada, Newfoundland, *Normore* 7079 (WIN)	ITS: KP001196
C. stygia	Canada, Manitoba, *Normore* 9466 (WIN)	mtSSU: KP001249
C. stygia	Canada, Manitoba, *Normore* 9465 (WIN)	mtSSU: KP001248
C. stygia	Canada, Newfoundland, *Normore* 8811 (WIN)	ITS: KP001187 mtSSU: KP001250
C. stygia	Canada, Nova Scotia, *Normore* 6905 (WIN)	ITS: KP001188
C. stygia	Canada, Newfoundland, *Normore* 7111 (WIN)	ITS: KP001185
C. stygia	Canada, Manitoba, *Normore* 7674 (WIN)	ITS: KP001184
C. stygia	Canada, Yukon, *Kotelko* 1038 (WIN)	ITS: KP001182
C. stygia	USA, Alaska, *Kotelko* 1044 (WIN)	ITS: KP001183
C. stygia	USA, Alaska, *Kotelko* 1090A (WIN)	ITS: KP001189
C. stygia	Finland (Stenroos *et al.* 2002)	ITS: AF458308
C. stygia	Canada, Manitoba, *Athukorala* 24 (WIN)	ITS: KP001201
C. submitis	USA, New Jersey, *Lendemer* 1803 (H)	ITS: KP001218 mtSSU: KP001230
Section *Impexae*		
C. confusa	Bolivia, *Flakus* 4568 (H)	mtSSU: KP001234
C. confusa	Bolivia, *Flakus* 4645 (H)	mtSSU: KP001235
C. confusa	Brazil, Minas Gerais (Stenroos *et al.* 2002)	ITS: AF458296
C. confusa	Bolivia (Pino-Bodas *et al.* 2016)	ITS: KP941536
C. delavayi	Bhutan (Stenroos *et al.* 2002)	ITS: AF458304
C. evansii	USA, Georgia, *Lendemer* 21090 (H)	ITS: KP001203 mtSSU: KP001232
C. evansii	USA, Georgia, *Lendemer* 21623 (H)	mtSSU: KP001233
C. evansii	USA, Georgia, *Lendemer* 22296 (H)	mtSSU: KP001231
C. evansii	USA, Georgia (Stenroos *et al.* 2002)	ITS: AF458303
C. evansii	USA, Florida (Yahr *et al.* 2004)	ITS: AY753590
C. mediterranea	Spain, Balearic Islands (Pino-Bodas *et al.* 2016)	ITS: KP941524
C. mediterranea	Portugal, Algarve (Pino-Bodas *et al.* 2016)	ITS: KP941522
C. mediterranea	Portugal, Beira Litoral (Pino-Bodas *et al.* 2016)	ITS: KP941523
C. mediterranea	Portugal, Beira Litoral (Pino-Bodas *et al.* 2016)	ITS: KP941514
C. mediterranea	Portugal, Beira Litoral (Pino-Bodas *et al.* 2016)	ITS: KP941513
C. portentosa	Denmark, *Hansen* Lich. Danici Exs. 511 (H)	ITS: KP001216 mtSSU: KP001221
C. portentosa	Lithuania, *Ahti* 68573 (H)	ITS: KP001214 mtSSU: KP001222
C. portentosa	Spain, *Burgaz* s. n. 6 (H)	ITS: KP001215
C. portentosa	Spain, *Burgaz* s. n. 7 (H)	ITS: KP001213 mtSSU: KP001219
C. portentosa	Portugal, Beira Litoral (Pino-Bodas *et al.* 2016)	ITS: KP941508
C. portentosa	United Kingdom, Scotland (Pino-Bodas *et al.* 2016)	ITS: KP941530
C. portentosa	Spain, Burgos (Pino-Bodas *et al.* 2016)	ITS: KP941527
C. portentosa	Canary Islands (Pino-Bodas *et al.* 2016)	ITS: KP941503
C. portentosa	Canary Islands (Pino-Bodas *et al.* 2016)	ITS: KP941501
C. portentosa	Azores (Pino-Bodas *et al.* 2016)	ITS: KP941520
C. portentosa	Azores (Pino-Bodas *et al.* 2016)	ITS: KP941535
C. portentosa	Germany (Stenroos *et al.* 2002)	ITS: AF458302
C. portentosa subsp. *pacifica*	USA, Washington (Smith *et al.* 2012)	ITS: JQ695923
C. portentosa subsp. *pacifica*	USA, Washington (Smith *et al.* 2012)	ITS: JQ695922
C. portentosa subsp. *pacifica*	USA, Washington (Smith *et al.* 2012)	ITS: JQ695921
C. portentosa subsp. *pacifica*	USA, Alaska (Pino-Bodas *et al.* 2016)	ITS: KP941528
C. portentosa subsp. *pacifica*	USA, Alaska (Pino-Bodas *et al.* 2016)	ITS: KP941529
C. pycnoclada	Chile (Stenroos *et al.* 2002)	ITS: AF458298

TABLE 1. *Continued*

Section and species	Source: collection location, and collection number or reference	Accession no. for ITS or mtSSU
C. pycnoclada	Chile (Stenroos *et al.* 2002)	ITS: AF458299
C. pseudoevansii	USA, Alaska, *Talbot & Schofield* ADA127-X-01 (H)	mtSSU: KP001243
C. stellaris	Canada, Manitoba, *Normore* 9402 (WIN)	ITS: KP001210 mtSSU: KP001241
C. stellaris	Canada, Manitoba, *Normore* 9463 (WIN)	ITS: KP001211 mtSSU: KP001239
C. stellaris	Canada, Newfoundland, *Normore* 8808 (WIN)	ITS: KP001212 mtSSU: KP001238
C. stellaris	Canada, Manitoba, *Normore* 6496 (WIN)	mtSSU: KP001240
C. stellaris	Finland (Stenroos *et al.* 2002)	ITS: AF458301
C. stellaris	Canada, Manitoba (Piercey-Normore *et al.* 2010)	ITS: GU169230
C. stellaris var. *aberrans*	USA, Alaska (Piercey-Normore *et al.* 2010)	ITS: GU169229
C. terrae-novae	Canada, Newfoundland, *Normore* 8806 (WIN)	ITS: KP001180 mtSSU: KP001227
C. terrae-novae	Canada, Newfoundland, *Normore* 8809 (WIN)	ITS: KP001181 mtSSU: KP001226
C. terrae-novae	Canada, Newfoundland (Stenroos *et al.* 2002)	ITS: AF458300
Section *Tenues*		
C. ciliata	Spain, *Burgaz* s. n. 1 (H)	mtSSU: KP001247
C. ciliata	USA, Washington (Smith *et al.* 2012)	ITS: JQ695926
C. ciliata	USA, Washington (Smith *et al.* 2012)	ITS: JQ695924
C. ciliata	USA, Washington (Smith *et al.* 2012)	ITS: JQ695915
C. ciliata	Ireland (Stenroos *et al.* 2002)	ITS: AF488310
C. ciliata var. *tenuis*	Portugal, *Burgaz* s. n. 3 (H)	mtSSU: KP001244
C. ciliata var. *tenuis*	Spain, *Burgaz* s. n. 2 (H)	mtSSU: KP001245
C. ciliata var. *tenuis*	Spain, *Burgaz* s. n. 5 (H)	mtSSU: KP001246
C. ciliata var. *tenuis*	USA, Washington (Smith *et al.* 2012)	ITS: JQ695917
C. ciliata var. *tenuis*	Portugal (Stenroos *et al.* 2002)	ITS: AF488311
C. subtenuis	USA, Georgia, *Lendemer* 21941 (H)	ITS: KP001217 mtSSU: KP001237
C. subtenuis	USA, South Carolina, *Lendemer* 22118 (H)	mtSSU: KP001236
C. subtenuis	USA, Florida (Yahr *et al.* 2006)	ITS: DQ482684
C. subtenuis	USA, Florida (Yahr *et al.* 2006)	ITS: DQ482690
C. subtenuis	USA, South Carolina (Yahr *et al.* 2006)	ITS: DQ482710
C. subtenuis	USA, South Carolina (Yahr *et al.* 2006)	ITS: DQ482711
C. subtenuis	Canada, Nova Scotia (Stenroos *et al.* 2002)	ITS: AF457911
C. subtenuis	USA, Missouri (Yahr *et al.* 2006)	ITS: DQ482696
C. subtenuis	USA, Pennsylvania (Yahr *et al.* 2006)	ITS: DQ482706
Other sections of *Cladonia*		
C. amaurocraea	Finland (Stenroos *et al.* 2002)	ITS: AF455245
C. atlantica	USA, Massachusetts (Stenroos *et al.* 2002)	ITS: AF457884
C. bellidiflora	Finland (Stenroos *et al.* 2002)	ITS: AF453700
C. boryi	Canada, Newfoundland (Stenroos *et al.* 2002)	ITS: AF457907
C. caespiticia	Canada, Nova Scotia (Stenroos *et al.* 2002)	ITS: AF455205
C. cariosa	Finland (Stenroos *et al.* 2002)	ITS: AF455230
C. cenotea	Canada, Newfoundland (Stenroos *et al.* 2002)	ITS: AF457900
C. ceratophylla	Brazil, Minas Gerais (Stenroos *et al.* 2002)	ITS: AF455171
C. coccifera	Canada, Newfoundland (Stenroos *et al.* 2002)	ITS: AF454437
C. coccifera	Finland (Stenroos *et al.* 2002)	ITS: AF454436
C. cornuta	Chile, Prov. Magallanes (Stenroos *et al.* 2002)	ITS: AF455196
C. divaricata	Brasil, Minas Gerais (Stenroos *et al.* 2002)	ITS: AF457910
C. floerkeana	Finland (Stenroos *et al.* 2002)	ITS: AF453697
C. furcata	USA, Georgia (Stenroos *et al.* 2002)	ITS: AF455220
C. gracilis	Sweden (Stenroos *et al.* 2002)	ITS: AF455194

TABLE 1. *Continued*

Section and species	Source: collection location, and collection number or reference	Accession no. for ITS or mtSSU
C. lopezii	Brazil, Minas Gerais (Stenroos *et al.* 2002)	ITS: AF453279
C. merochlorophaea	Finland (Stenroos *et al.* 2002)	ITS: AF455227
C. metaminiata	Brazil, Minas Gerais (Stenroos *et al.* 2002)	ITS: AF453286
C. peziziformis	USA, North Carolina (Stenroos *et al.* 2002)	ITS: AF455182
C. pleurota	Canada, Nova Scotia (Stenroos *et al.* 2002)	ITS: AF454442
C. rangiformis	Faeroe Islands (Stenroos *et al.* 2002)	ITS: AF455234
C. ravenelii	USA, Georgia (Stenroos *et al.* 2002)	ITS: AF453688
C. rei	Canada, Nova Scotia (Stenroos *et al.* 2002)	ITS: AF455191
C. robbinsii	USA, North Carolina (Stenroos *et al.* 2002)	ITS: AF455167
C. squamosa	Sweden (Stenroos *et al.* 2002)	ITS: AF457886
C. strepsilis	Finland (Stenroos *et al.* 2002)	ITS: AF457880
C. subsubulata	Argentina, Tierra del Fuego (Stenroos *et al.* 2002)	ITS: AF457883
C. subulata	Finland (Stenroos *et al.* 2002)	ITS: AF455180
C. turgida	Finland (Stenroos *et al.* 2002)	ITS: AF455203
C. uncialis	USA, Washington (Stenroos *et al.* 2015)	ITS: KR019420
C. uncialis subsp. *biuncialis*	France (Stenroos *et al.* 2015)	ITS: KR019375
C. wainioi	Canada, Newfoundland, *Normore* 8805 (WIN)	ITS: KP001177
C. wainioi	Canada, Newfoundland, *Normore* 8807 (WIN)	ITS: KP001178
C. wainioi	Canada, Newfoundland (Stenroos *et al.* 2002)	ITS: AF455204

version 4.6 (Gene Codes Corporation, Michigan, USA). In addition, 57 accessioned DNA sequences were retrieved from NCBI GenBank and were included in the phylogenetic analysis. All sequences were automatically aligned using the ClustalX (Jeanmougin *et al.* 1998) program and manually edited.

To infer the relationships of the sections *Crustaceae*, *Tenues* and *Impexae* within *Cladonia* we constructed a matrix with 69 sequences (belonging to 51 species) of ITS rDNA. In this alignment, one sequence per major clade of the phylogeny presented by Stenroos *et al.* (2002) was included, along with one or two sequences per species of *Crustaceae*, *Tenues* and *Impexae*. The type species of each section were included. *Cladonia wainioi* was assigned as the outgroup taxon because of its basal position in the phylogenetic tree of the genus *Cladonia* (Stenroos *et al.* 2002). The ambiguous positions were identified and removed by Gblocks version 0.91b (Castresana 2000) using the less stringent options. Three separate ITS rDNA alignments were then constructed, one per monophyletic *Cladina* group identified in the previous analysis including multiple specimens per species. All nucleotide sequences generated in this study have been deposited in the NCBI GenBank (Table 1).

The alignments were subjected to maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses. Maximum parsimony analyses were run using PAUP* 4.0b10 (Swofford 2003), ML using RAxML 7.0.3 (Stamatakis 2006) and Bayesian analyses using MrBayes v3.2 (Ronquist & Huelsenbeck 2003; Ronquist *et al.* 2012). MP analyses were performed using tree bisection and reconnection branch swapping, heuristic searches with 1000 random addition replicates and bootstrap searches of 1000 resamplings (Felsenstein 1985) using the heuristic option. ML analyses were performed using the GTRGAMMA model and fast boostrap searches of 500 replicates. The best models of nucleotide substitution were estimated using jModelTest 2.1.1 (Darriba *et al.* 2012). These models were applied for the Bayesian analyses (Table 2). The Bayesian analysis used 10 000 000 generations with two parallel runs performed. In each run, four chains were used and initiated with a random tree. The convergence between runs was diagnosed by standard deviation of split frequencies <0·01. At the end of the runs, in all cases the split frequencies fell below 0·01. TRACER 1.5 (Rambaut & Drummond 2007) was used to plot the log-likehood scores of sample points against generation time. Afterwards, the first 2 500 000 generations were removed and the 50% majority-rule consensus tree was calculated. Bootstrap values greater than 70% and posterior probabilities greater than 0·95 are reported in the phylogenies.

Haplotype analysis was performed using 34 mtSSU sequences belonging to 14 species and one mtSSU sequence obtained from GenBank. A haplotype network analysis is recommended when polymorphism is low (Clement *et al.* 2000). The TCS program version 1.21 (Clement *et al.* 2000) was used to construct a parsimony network. The parsimony probability criterion (Templeton *et al.* 1992) with gaps coded as fifth character state and a 95% parsimony threshold for network relationships was used in the analysis. Chromatograms were examined to rule out miscalled bases. Haplotype analyses using the same procedure were also conducted on three pairs of species (*C. rangiferina*-*C. stygia*; *C. arbuscula*-*C. mitis*; and *C. portentosa*-*C. terrae-novae*) using the ITS rDNA alignments. An analysis of molecular variance (AMOVA) was conducted in GenAlEx ver. 6.5 (Peakall & Smouse 2012) with 999 permutations to determine the extent

TABLE 2. *Data from the alignments and analyses of the different clades of* Cladonia.

	Clades A & B	Clade C	Clade D
n	42	49	30
Aligned positions	589	603	577
PI positions	127	69	39
Model	SYM + G	SYM + Y + G	SYM + Y + G
CI	0·8327	0·6578	0·7357
RI	0·9281	0·7406	0·7874
Length of MP tree	269	263	140
N of MP trees	1000	1000	63
−Lnl (ML analysis)	2260·18	2390·08	1620·41
−Lnl (Bayesian analysis)	2310·44	2516·94	1700·01

n = number of sequences including the outgroup; PI = parsimony-informative; CI = consistency index; RI = retention index; MP = maximum parsimony; –Lnl = likelihood values; ML = maximum likelihood.

of shared polymorphism among species duplets. The same ITS rDNA alignments used for the haplotype networks were also used for AMOVA analysis. AMOVA populations were defined as species duplets and the species in each of the duplets were defined as subpopulations.

Results

Forty-four ITS rDNA sequences generated in this study and additional sequences from GenBank were used in the phylogenetic analyses. The alignment constructed to study the phylogenetic relationships of *Crustaceae*, *Tenues* and *Impexae* contained 537 unambiguously aligned sites, of which 190 were parsimony-informative. The ITS rDNA maximum parsimony analysis generated 1000 equally parsimonious trees with 847 changes. The consistency index (CI) and retention index (RI) were 0·4864 and 0·7540, respectively. The ML and Bayesian analyses (using GTRGAMMA model) produced a tree with a topology that was consistent with that of the MP tree and with likelihood values of −Lnl = 5167·301 and 5248·02, respectively. For the three other constructed phylogenies, Table 2 summarizes the data used in the analyses and models selected by jmodeltest.

The phylogeny shows a number of clades, among which four are notable: A, B, C, and D. Clades A and B represent section *Impexae*, are highly supported, and are basal to the taxa in the tree (Fig. 1). Clade C represents some species of section *Crustaceae* (*C. arbuscula*, *C. densissima*, *C. mitis* and *C. submitis*) with support values of 69% MP bootstrap (BS), 74% ML BS and 0·99 posterior probability (PP). Most of clade D represents species of sections *Crustaceae* and *Tenues* together, and is moderately supported (81% MP BS, 79% ML BS and 0·95 PP). Clades C and D form a polytomy with the *Unciales*.

The analysis of clades A and B with additional ITS rDNA sequences yielded some highly supported subclades (Fig. 2A). Clade A contains *C. stellaris*, which is sister to the only specimen of *C. delavayi*. However, *C. delavayi* (Himalayan) and *C. stellaris* are very different from one another in morphology and chemistry, as well as their ITS rDNA sequences. Clade B contains three well-supported subclades. Subclade B1 represents *C. portentosa* s. str. (including the recently synonymized species *C. azorica* and *C. macaronesica* according to Pino-Bodas *et al.* 2016) and *C. terrae-novae.* Neither *C. terrae-novae*, *C. portentosa* s. str., nor *C. portentosa* subsp. *pacifica* could be supported as monophyletic groups. *Cladonia evansii* alone forms subclade B2 (97% MP BS, 95% ML BS and 1·0 PP). The subclade B3 is represented by *C. pycnoclada*. *Cladonia confusa* is not monophyletic.

The analysis of clade C, representing *C. arbuscula*, *C. submitis*, *C. densissima* and *C. mitis*, shows two unsupported subclades, one with all the sequences of *C. mitis* and the other with most of the *C. arbuscula* sequences (Fig. 2B). It is remarkable that other

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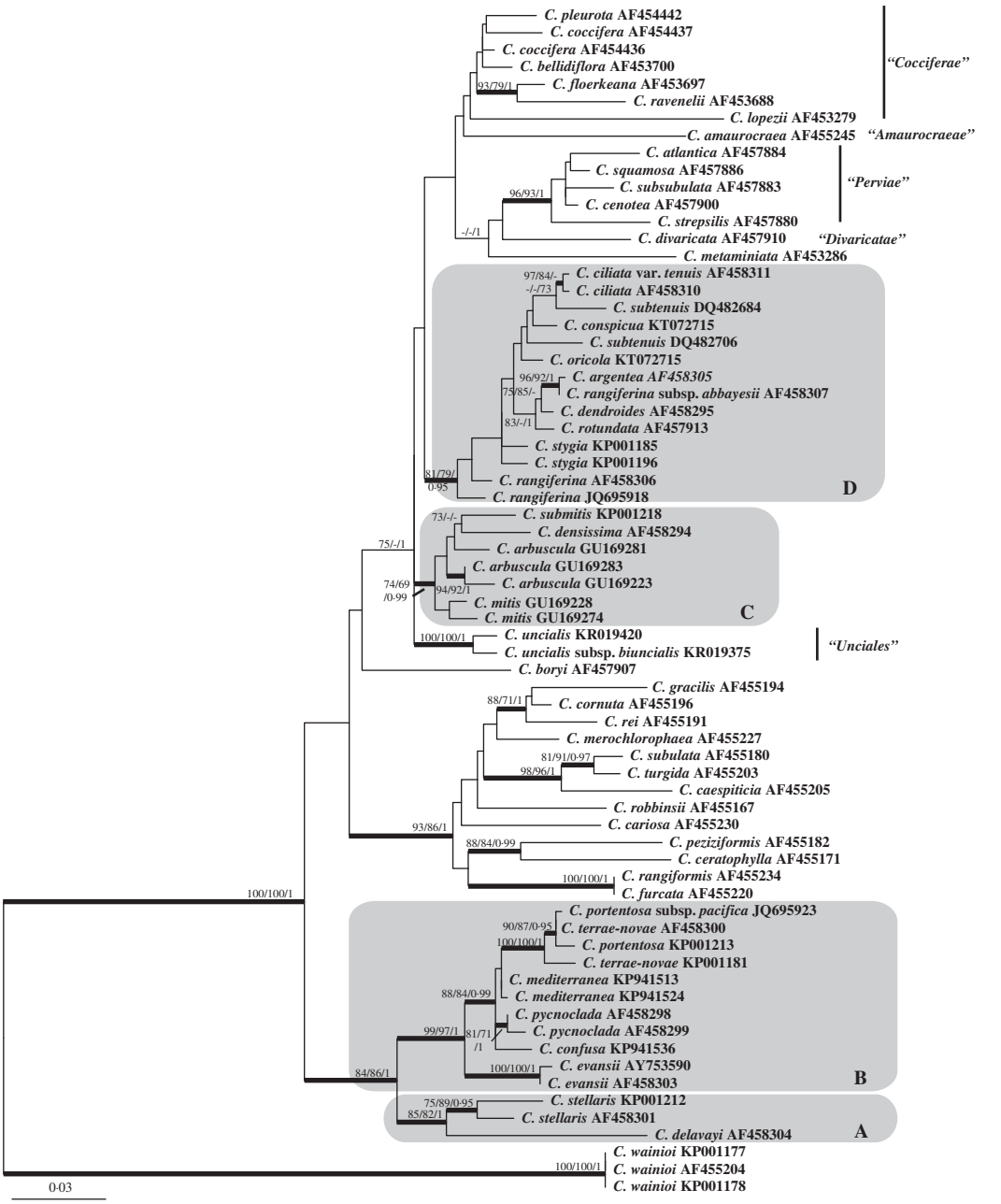

FIG. 1. Phylogenetic tree generated from ML analysis based on ITS rDNA sequences. It shows the placement of the sections *Crustaceae*, *Tenues*, and *Impexae* in the genus *Cladonia*. The taxonomic sections are represented as A, B, C, and D. The values on the branches are ≥70% bootstrap for MP and ML analyses and ≥0·95 posterior probability for Bayesian analysis. The thick branches represent branches supported in the three analyses.

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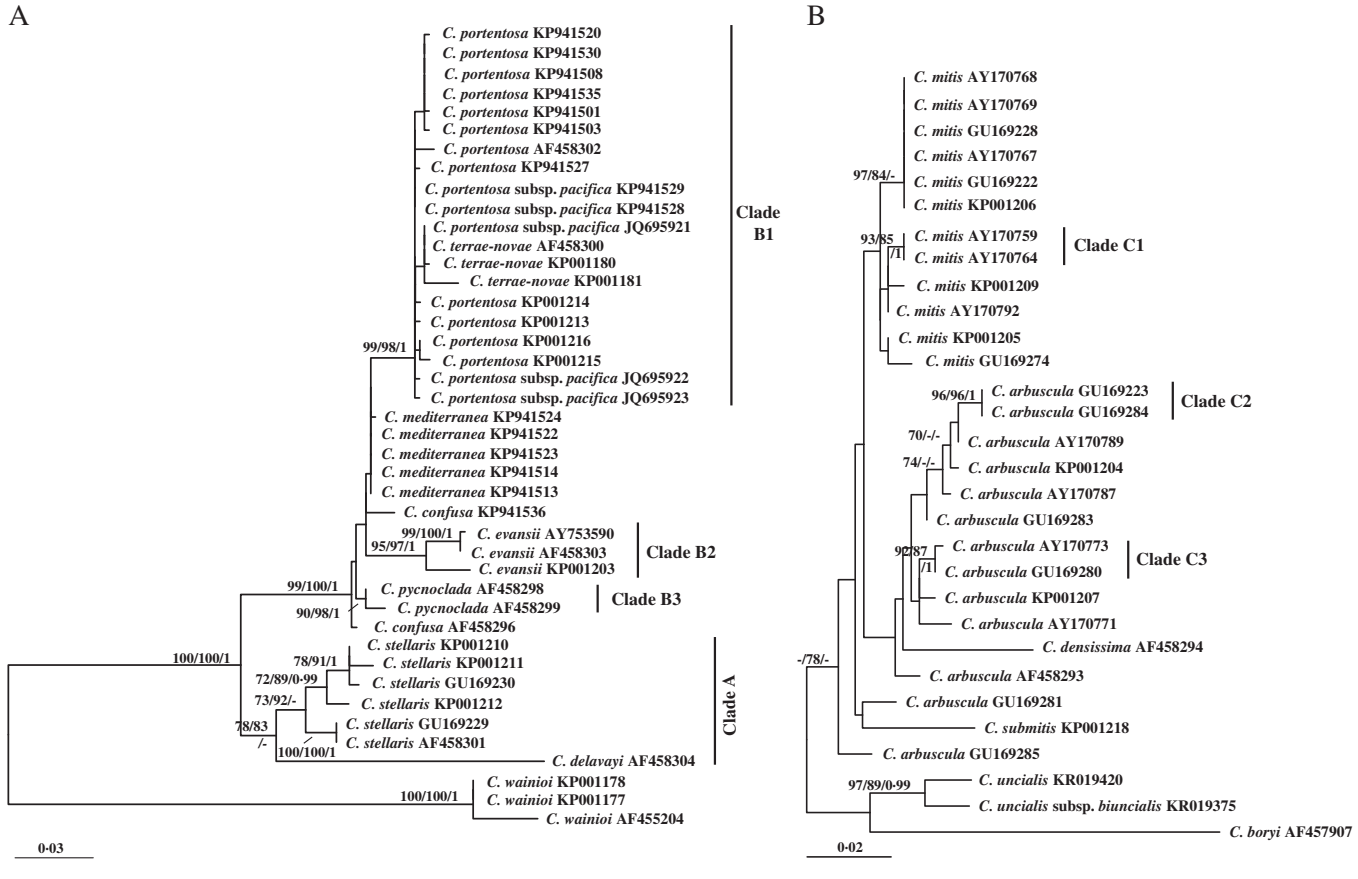

FIG. 2. Phylogenetic trees from ML analyses based on ITS rDNA sequences showing a wide sampling of sequences per species. A, analysis from clades A and B; B, analysis from clade C. Numbers on the branches indicate bootstrap support ≥70% in MP and ML analyses and posterior probabilities ≥0·95 from the Bayesian analysis. The subclades that are supported in the analyses are numbered in each tree.

chemically and morphologically distinct segregates, namely *C. submitis* and *C. densissima*, are also nested within *C. arbuscula*. The analysis thus suggests that even the rest of *C. arbuscula* may not be taxonomically uniform. One subclade containing two specimens of *C. mitis* (subclade C1) is supported. Two other subclades with specimens of *C. arbuscula* (subclades C2 and C3) are also supported.

The analysis of clade D with additional sequences shows several supported subclades (Fig. 3). Section *Tenues* is represented by two monophyletic groups within subclades D1 and D2; subclade D1 contains six sequences from *C. ciliata* (82% MP BS, 92% ML BS and 1·0 PP). However, there is no support for the separation of *C. ciliata* var. *tenuis* and var. *ciliata*. Subclade D2 contains eight sequences from *C. subtenuis* (only 54% MP BS, 84% ML BS and 1·0 PP). *Cladonia rangiferina* and *C. stygia* are not monophyletic. Subclade D3 consists of specimens representing *C. argentea*, *C. dendroides*, *C. rangiferina*, and *C. rotundata* (87% MP BS, 93% ML BS and 1·0 PP).

Thirty-four mtSSU sequences were generated in this study and one sequence was taken from GenBank. The alignment consisted of 477 positions with two single-nucleotide gaps at positions 31 and 223. The analysis was conducted using alignments with and without gaps and produced the same results. Additionally, missing bases were present near the beginning and end of 4 sequences (1 base in 2 sequences, 7 bases in 1 sequence, and 83 in 1 sequence). The mtSSU haplotype network produced 24 haplotypes from 33 sequences. The haplotype network is congruent with the ITS rDNA results, showing that the section *Crustaceae* is divided into two groups. The species included in the section *Tenues* are closely related to *C. rangiferina* and *C. stygia* (Fig. 4). The haplotypes of *C. arbuscula* and *C. mitis* were mixed but they appeared to be monophyletic in the ITS phylogeny, despite a lack of statistical support. One haplotype was shared between *C. rangiferina* and *C. stygia*. All other haplotypes are represented by a single species each.

Three duplet haplotype networks are shown that represent paraphyletic species groups from Fig. 2 and 3 (Fig. 5). The haplotype network of *C. portentosa-C. terrae-novae* was based on 560 aligned positions and produced 11 haplotypes from 14 sequences and no haplotypes were shared between species (Fig. 5A). The haplotype network of *C. arbuscula-C. mitis* was based on 564 aligned positions and produced 19 haplotypes from 25 sequences, with two of the haplotypes shared between species and incomplete clustering of the haplotypes within species (Fig. 5B). One loop (dotted line) in the network indicated homoplasy. Twenty-seven sequences of *C. rangiferina-C. stygia* were based on 472 aligned positions and produced 23 haplotypes with no haplotypes shared between the two species, but the *C. stygia* haplotypes were intermixed with those of *C. rangiferina* (Fig. 5C). Five loops (dotted lines) in the network indicated homoplasy.

The AMOVA analysis showed low to moderate levels of population differentiation between species duplets (Table 3) with low to moderate Phi values. The Phi statistic is a measure of allelic differentiation among subpopulations. A population which represents no allelic differentiation has a Phi value of 0 and a high level of differentiation when the Phi value is 1·0. In this study a population was defined as a species duplet and the subpopulations as the species. A null hypothesis of homogeneity of variance was tested for each of the three species duplets. Significant ($P<0·05$) genetic differences are shown for *C. rangiferina-C. stygia* and for *C. mitis-C. arbuscula*, suggesting a low level of homogeneity among the subpopulations (species), which implies that the species are differentiated from one another. The genetic differentiation for *C. portentosa-C. terrae-novae* was not significant, suggesting no differentiation between species of the species duplet. The partitioning of the total variance shows a higher level of variance within species than between species for all three duplets. Separate species are supported for each of *C. arbuscula*, *C. mitis*, *C. rangiferina* and *C. stygia*, but not for *C. portentosa* and *C. terrae-novae*.

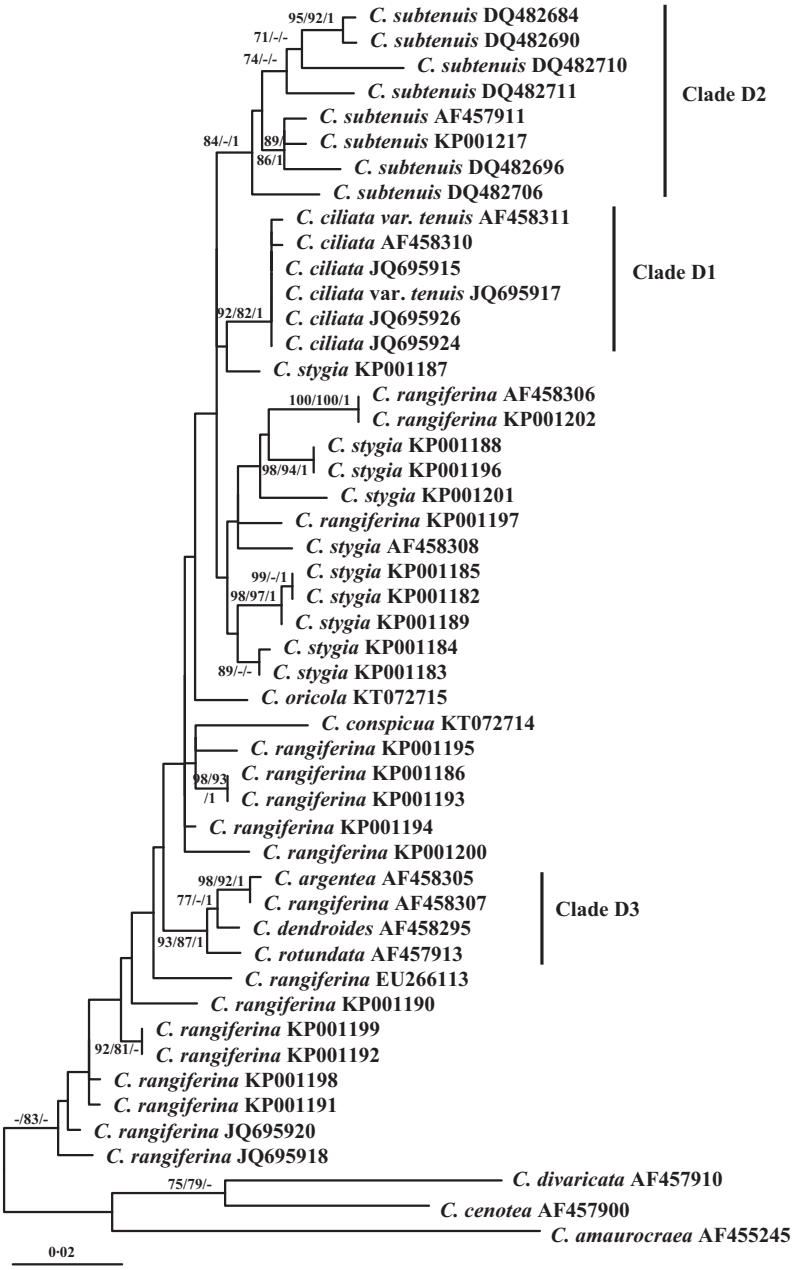

FIG. 3. Phylogenetic tree from ML analyses based on ITS rDNA sequences for clade D showing a wide sampling of sequences per species. Numbers on the branches indicate bootstrap support ≥70% in MP and ML analyses and posterior probabilities ≥0·95 from the Bayesian analysis. The subclades that are supported in the analyses are numbered.

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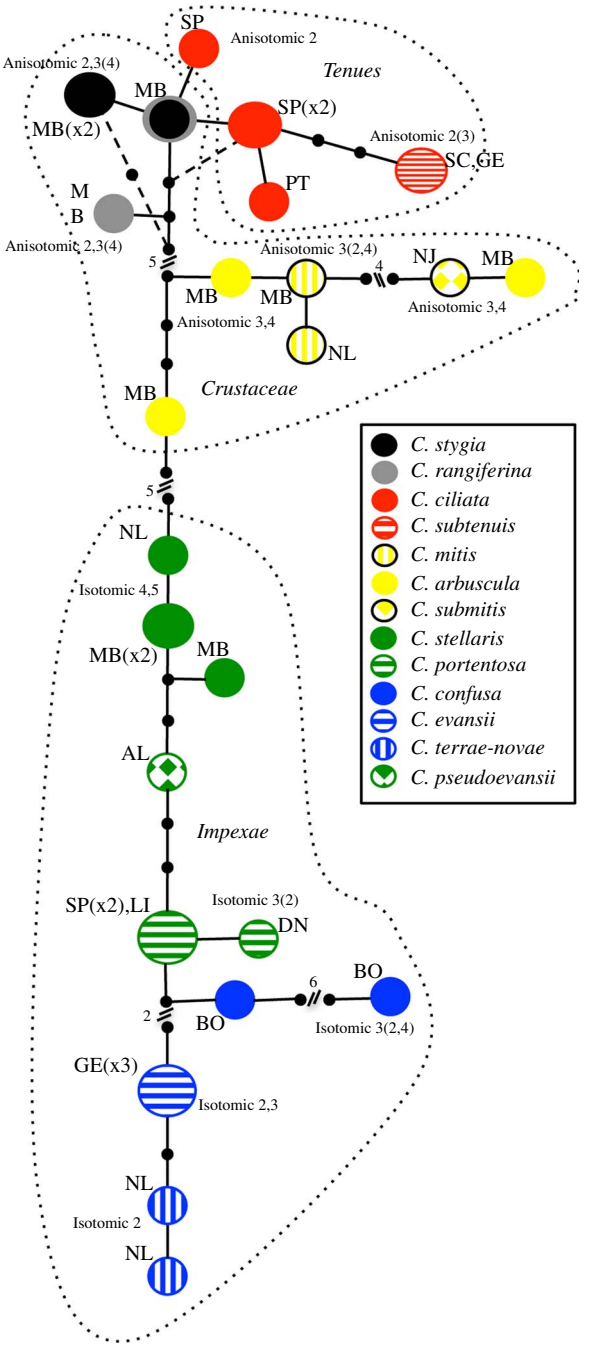

FIG. 4. Haplotype network of mtSSU sequences showing the relationship between *Cladonia* taxa (see legend). Thallus branching pattern is indicated beside each species with the number representing the branching, and the number in brackets representing the less common branching. The small solid black dots indicate distance between haplotypes and the size of the circles represents number of haplotypes (1 to 4 haplotypes). The numbers with double dashes on the lineages represent number of changes between the dots. MB = Manitoba; NL = Newfoundland and Labrador; SC = South Carolina; GE = Georgia; NJ = New Jersey; AL = Alaska; LI = Lithuania; DN = Denmark; BO = Bolivia; SP = Spain; PT = Portugal.

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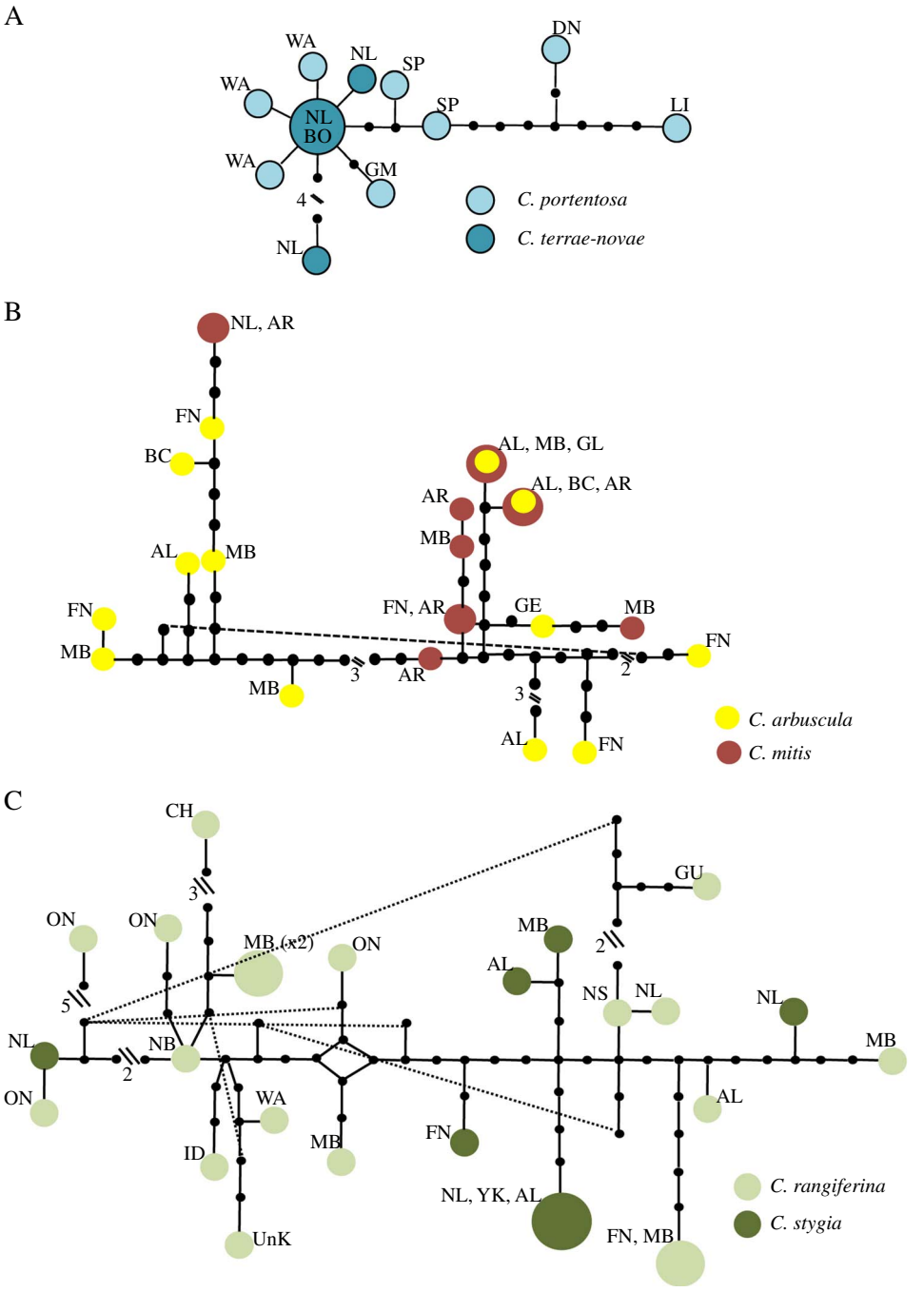

FIG. 5. Haplotype network of ITS rDNA sequences showing relationship between A) *C. portentosa* and *C. terrae-novae*, B) *C. arbuscula* and *C. mitis*, and C) *C. rangiferina* and *C. stygia* (see legends). The small solid black dots indicate distance between haplotypes and the size of the circles represents number of haplotypes (1 to 3 haplotypes). MB = Manitoba; NL = Newfoundland and Labrador; BC = British Columbia; NB = New Brunswick; NS = Nova Scotia; ON = Ontario; YK = Yukon; SC = South Carolina; GE = Georgia; NJ = New Jersey; WA = Washington; AL = Alaska; LI = Lithuania; DN = Denmark; BO = Bolivia; SP = Spain; PT = Portugal; GM = Germany; AR = Argentina; FN = Finland; GL = Greenland; CH = China; GU = Guyana; ID = India; UnK = unknown location.

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TABLE 3. *Results of AMOVA analyses between and within species for each species duplet;* C. rangiferina-C. stygia, C. arbuscula-C. mitis, *and* C. portentosa-C. terrae-novae.

Population (species comparison)	Subpopulation	d. f.	SS	MS	Observed variance	% of total variance	Phi statistic	*P*
C. rangiferina-C. stygia	Between species	1	9·195	9·195	0·414	8	0·078	0·034
	Within species	25	122·471	4·899	4·899	92		
C. arbuscula-C. mitis	Between species	1	23·955	23·955	1·459	23	0·228	0·001
	Within species	25	123·341	49·34	49·34	77		
C. portentosa-C. terrae-novae	Between species	1	4·042	4·042	0·216	7	0·070	0·188
	Within species	10	28·875	2·888	2·888	93		

d. f. = degrees of freedom; SS = sums of squares; MS = mean of squares; variance partitioning includes both observed and percent of total; Phi statistic = fixation index; *P*-value with significance inferred at 0·05.

Discussion

Phylogenetic relationships of the reindeer lichens

This is the most comprehensive study to date on the phylogeny of the reindeer lichens. It includes 22 species and several specimens for many of them, with an emphasis on *C. rangiferina-C. stygia*, *C. mitis-C. arbuscula* and also *C. portentosa-C. terrae-novae*. A number of previous phylogenetic studies proved that the former genus *Cladina* was not monophyletic (DePriest *et al.* 1999, 2000; Guo & Kashiwadani 2004; Stenroos *et al.* 2015); nevertheless, the species sampling was scarce and the composition of the different groups was not complete. Guo & Kashiwadani (2004) and Stenroos *et al.* (2015) concluded that the reindeer species form three independent groups, but relationships among them could not be determined. The results of the present study are consistent with these findings. While some morphological characteristics were not useful in elucidating the evolutionary relationships within certain subdivisions of *Cladonia* (Stenroos *et al.* 2002), thallus branching pattern showed a trend in species discrimination in this study. The anisotomic branching was common to both sections *Crustaceae* and *Tenues* and the isotomic branching was present in section *Impexae*.

The section *Impexae* is monophyletic and basal to the species in this phylogeny (Fig. 1). *Tenues*, along with most of the species of *Crustaceae*, forms a monophyletic group that is related to a clade consisting of species from the sections *Cocciferae* and *Perviae*, and the groups *Amaurocraeae* and *Divaricatae* (according to Stenroos *et al.* 2002). In the analyses by Stenroos *et al.* (2015) this group is related to *Cladonia uncialis*, though this relationship was not supported by most of the analyses here. Guo & Kashiwadani (2004) obtained a similar result. The third group is formed by *C. arbuscula*, *C. densissima*, *C. mitis*, and *C. submitis*. The phylogenetic relationships within this group of the genus *Cladonia*, however, are not statistically supported in our analyses. The phylogenetic analyses by Stenroos *et al.* (2015) showed this group to be related to the group *Divaricatae,* but this relationship was not supported. The study of additional loci will be necessary in order to more accurately understand the phylogenetic relationships among the different groups of reindeer lichens within the genus *Cladonia*.

Clades A and B: section *Impexae*

The section *Impexae sensu* Ahti (2000) is monophyletic, including *C. confusa*, *C. delavayi*, *C. evansii*, *C. mediterranea*, *C. portentosa*, *C. stellaris*, and C. *terrae-novae*. The characters used to define this section were the presence of dichotomous or trichotomous branching, hyaline slime in conidiomata (exception: *C. stellaris* has red slime), and the presence of perlatolic acid (Ahti 1984, 2000). Within section *Impexae*, *C. portentosa* (17 sequences) and *C. terrae-novae* (4 sequences) are morphologically similar to one another. They both have thallus

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branches that diverge in threes and sometimes twos, and both grow in a boggy habitat. They differ in that *C. terrae-novae* produces atranorin and perlatolic acid, while *C. portentosa* produces perlatolic acid alone (both contain usnic acid in addition although it may occasionally be absent; Ahti 1961; Orange 1993), and Ahti (1961) mentions minor differences in branching and surface structure. The two species are closely related but they are geographically separated from one another in North America where *C. portentosa* is distributed along the west coast (recognized as *C. portentosa* subsp. *pacifica*; Ahti 1961, 1984) and *C. terrae-novae* along the east coast. Allopatry may encourage divergence between these species but if the period of time has not been sufficient for complete divergence, they would show incomplete genetic divergence and an absence of monophyly. Monophyly of *C. portentosa* was reported by Smith *et al.* (2012) but they did not include *C. terrae-novae* in their analysis for comparison. While they could not be distinguished by the ITS rDNA phylogeny, the mtSSU haplotype network clearly separated the species, showing *C. confusa* and *C. evansii* to fall between them. The AMOVA analysis suggests that in addition to gene flow, another possible explanation is shared ancestral polymorphism where characters may be part of the reaction norm in both species but those characters do not exist in the current niches. The sample size for this species duplet was low, which may have biased the analysis (Fitzpatrick 2009). These conflicting results suggest that further study of these two species is still needed.

Cladonia stellaris is strongly supported as monophyletic and *C. delavayi* is basal to *C. stellaris*, which is consistent with Stenroos *et al.* (2002). *Cladonia stellaris* occupies a basal position in the *Impexae* (Fig. 1) and is a derived species in the evolution of the *Cladoniaceae* (Stenroos *et al.* 1997). *Cladonia stellaris* is more closely linked with other members of the *Impexae* in the mtSSU haplotype network than in the ITS rDNA phylogeny, which is consistent with the morphology. Choisy (1928) postulated that *C. stellaris* originated from the ancestors of *Cocciferae* and *Perviae*. While *C. delavayi* was originally thought to be a member of *Unciales* (des Abbayes 1958), it was proposed to move it to supergroup *Crustaceae* and group *Cladinae* because of this affiliation (Stenroos *et al.* 2002). Nevertheless, *C. delavayi* could be a new section of *Cladonia* on its own since both morphologically and chemically it is very different from the species included in section *Impexae*. *Cladonia delavayi* has thickly corticate podetia and, in addition to usnic acid, contains 4-O-methylcryptochlorophaeic and cryptochlorophaeic acids, characters not shared by any other species in *Impexae* or with *C. stellaris*. Molecular data would support this separation (Figs 1 & 2A).

Clade D: sections *Crustaceae* and *Tenues*

The characters used to separate *Crustaceae* from *Tenues* were the branching type and the colour of the slime in conidiomata (Ahti 1984, 2000). The species of section *Tenues* have dichotomous branching and conidiomata with red slime, while the species included in *Crustaceae* have predominantly tetrachotomous branching and hyaline slime. However, the phylogenetic results suggest that these characters may have evolved several times independently, they may represent ancestral characters with multiple losses or they may be silenced in some ecological niches.

The position of *C. rotundata*, *C. argentea*, and *C. dendroides*, nested within a clade of *C. rangiferina*, is consistent with Stenroos *et al.* (2002). *Cladonia rangiferina* and *C. stygia* were shown to be closely related in this study, which is also supported by Stenroos *et al.* (2002). The similar branching pattern may be explained by the non-monophyly of these two species, and the homoplasy in the mtSSU network. The two species differ by moist (*C. stygia*) and dry (*C. rangiferina*) habitats (Ruoss & Ahti 1989) but they may overlap in habitats with moderate levels of moisture. Additionally, the stereome in *C. stygia* is black and the slime in the conidiomata is red, whereas the stereome of *C. rangiferina* is grey or brown and the slime is colourless (Ahti & Hyvönen 1985; Ruoss & Ahti 1989). Haplotype networks and AMOVA analysis

imply frequent homoplasy and genetic differentiation between *C. rangiferina* and *C. stygia*. Therefore, they may not be reproductively isolated from each other and are in the early stages of speciation. The close physical proximity in geographical distribution may encourage interbreeding and obscure genetic divergence between these species.

The two species *C. ciliata* (six sequences) and *C. subtenuis* (eight sequences) are both monophyletic in the ITS rDNA phylogeny and are similar in their thallus branching pattern, but they show homoplasy with the *Crustaceae* in the mtSSU network. Yahr *et al.* (2006) reported a low level of population structure and inferred that recombination was occurring within *C. subtenuis*. Monophyly of *C. ciliata* was also reported by Smith *et al.* (2012); however, the two colour variants (chemotypes) *C. ciliata* var. *ciliata* (= f. *ciliata*) and *C. ciliata* var. *tenuis* (= f. *flavicans*) were not resolved, which supports the finding in this study. Moreover, the findings of this study support the synonymy of the sections *Crustaceae* (*Cladina*) and *Tenues* as recommended by Stenroos *et al.* (2002).

Clade C: *Cladonia arbuscula* and related species

Cladonia arbuscula and *C. mitis* (together with *C. submitis* and *C. densissima*) formed a highly supported clade in Fig. 1 but not in Fig. 2B which included a larger number of sequences. Possible explanations include the potential for paralogous ITS rDNA regions (Buckler *et al.* 1997), a failure of concerted evolution in the nuclear ribosomal repeats (Ambrose & Crease 2011), or divergence within *C. arbuscula* that may be detected using other genes or an increased number of specimens representing all the subspecies. The difficulty in separating the larger number of specimens of *C. arbuscula* and *C. mitis* was also consistent with Piercey-Normore *et al.* (2010) and with the mtSSU network (Fig. 4). However, Smith *et al.* (2012) showed both *C. mitis* and *C. arbuscula* to be monophyletic species although they had fewer specimens. An analysis with a larger number of specimens would have a higher probability of showing paraphyly than one with fewer specimens. Multiple gene phylogenies showed that *C. mitis* is supported as a monophyletic species when beta-tubulin, GAPDH, a group 1 intron in nuclear 18S rDNA, and the ITS rDNA were used in the phylogeny, but it was paraphyletic when the intron was omitted from the group of genes or when either gene was used alone in the analysis (Myllys *et al.* 2003). The reticulate nature of the haplotype analysis in this study, with one case of homoplasy, might suggest a lack of reproductive isolation between these two species. However, the AMOVA analysis indicates only a moderate level of genetic differentiation and a significant *P*-value, suggesting the species are genetically different from one another. The diagnostic characters overlap between the species, where *C. arbuscula* has denser branching of the apices with more browned and curved branch tips than *C. mitis*. *Cladonia mitis* produces usnic acid alone (or with rangiformic acid), whereas *C. arbuscula* produces both usnic acid and fumarprotocetraric acid, but this feature can be variable (Ruoss 1987*b*; Ruoss & Ahti 1989). The close evolutionary relationship between the two species and their physical proximity in similar habitats provides opportunities for gene flow and therefore interbreeding. Consequently, they may have had only a short history of reproductive isolation (Myllys *et al.* 2003), resulting in low resolution of *C. arbuscula* and *C. mitis* where sequence divergence (speciation) has lagged behind morphological evolution. Other evolutionary processes may also have influenced the patterns observed in this study, such as incomplete lineage sorting through speciation (Knowles & Carstens 2007). A coalescent-based approach using multiple loci will improve the resolution but may not remove the effects of incomplete lineage sorting, depending on the extent of speciation (Knowles & Carstens 2007).

Potential bias from ITS rDNA and sampling

The lack of monophyly observed in *C. arbuscula* and *C. mitis*, and similarly in

C. rangiferina and *C. stygia*, might suggest there is insufficient phylogenetic signal in the ITS rDNA region to resolve the morphological differences between these species. Among the several gene regions proposed for species discrimination in fungi (Taylor *et al.* 1999, 2000; Myllys *et al.* 2003), Pino-Bodas *et al.* (2013) concluded that the best combination for barcoding in *Cladonia* is *RPB2* and ITS rDNA. The ITS rDNA region was previously supported by Schoch *et al.* (2012) as a potential barcoding marker for fungi. While the ITS rDNA is widely used, species discrimination using ITS rDNA has been previously shown to be a challenge with some members of *Cladonia* (Fontaine *et al.* 2010; Kotelko & Piercey-Normore 2010; Kelly *et al.* 2011; Pino-Bodas *et al.* 2011). The greater intraspecific variation in the ITS rDNA observed with species of *Cladonia* such as *C. arbuscula*, *C. mitis*, *C. rangiferina* and *C. stygia*, might also suggest that evolutionary processes such as incomplete lineage sorting in a recent divergence obscures species delimitation (Knowles & Carstens 2007). Ribosomal DNA has been subject to interpretations of having divergent paralogs (Buckler *et al.* 1997) or failure of concerted evolution (Ambrose & Crease 2011) in a diversity of organisms. These explanations cannot be ignored in the interpretation of ribosomal DNA patterns.

In conclusion, the current study supported monophyly for five of 22 species in *Cladonia* sections *Crustaceae* and *Tenues*, some of which have also been shown to be monophyletic by other studies, but the 17 other species were not supported and *Impexae* was divided into two clades using a phylogenetic analysis of the ITS rDNA and a haplotype network of the mtSSU gene. The mtSSU network also illustrated a morphological trend of the thallus branching pattern in these lichens, where members of section *Impexae* have isotomic branching and *Tenues* and *Crustaceae* have anisotomic branching. Incomplete lineage sorting, recombination, gene flow, and recent divergence were considered as explanations for the reticulate nature of the haplotype networks of some species. These results emphasize the importance of examining the non-monophyletic species using multiple loci for a coalescence-based approach. In addition, further investigation of gene flow and recombination between and within the species duplets reported in this study might reveal more about the evolutionary status of these species.

The authors thank T. Booth (University of Manitoba) for providing light microscopic imaging facilities, R. Kotelko for providing field collections, and S. Toni for laboratory assistance. Funding was provided by the Natural Science and Engineering Research Council (NSERC) for a Canada Graduate Scholarship to SA and Discovery Grant to MPN. This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement No PIEF-GA-2013-625653 (RPB, SS).

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