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Phylogenetic position of a Pacific Northwest North American endemic cyanolichen, *Nephroma occultum* (Ascomycota, *Peltigerales*)

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Abstract: *Nephroma occultum* is a COSEWIC listed cyanolichen of ''Special Concern''. It is endemic to old-growth cedar-hemlock forests in western North America. This is the first study to place *N. occultum* into a phylogenetic framework using nucleotide sequence and secondary structure data. It also addresses the phylogenetic relationship between *N. occultum* and *N. arcticum*. Analysis of fungal Internal Transcribed Spacer (ITS) ribosomal DNA (rDNA) produced four major clades. The cyanobacterial transfer RNA^{Leu} intron (tRNA^{Leu}) from *N. occultum* was separated from that of *N. isidiosum* and fell between the Eurasian and North American epiphytic taxa. Examination of length and complexity of the folded secondary structures revealed different trends in the ITS1 and ITS2 rRNA regions. Even though *N. occultum* is endemic to North America, it seems more closely related to South American temperate rainforest species than to the sympatric *N. arcticum*. *Nephroma occultum* is alone among the studied species of *Peltigerales* in having an exceptionally long ITS1 region, and a different tRNA^{Leu} intron DNA sequence of the photobiont suggesting association with a unique genotype of *Nostoc*. It may be argued that the fitness of *N. occultum* may be influenced by the complex ITS1 RNA structure, a unique photobiont genotype undergoing a genetic bottleneck, no sexual reproduction to generate variation, and the inability to associate with different photobionts to adapt to changing habitats.

Key words: evolution, ITS rDNA, ITS secondary structure, *Nephroma occultum*, *N. arcticum*, phototype, phylogeny, tRNA^{Leu}

Introduction

The majority of lichens contain a green algal primary photobiont and only 10% have cyanobacteria as the primary photobiont (Friedl & Büdel 1996). About 3–4% of lichen-forming fungi associate primarily with a green alga but contain also cyanobacteria as secondary photobionts, compartmentalized in special structures (cephalodia). Furthermore some of these fungi have the ability to switch from green algal to a cyanobacterial primary photosynthetic partner, producing either similar (most *Peltigera* spp.) or distinct (*Sticta*/''*Dendriscocaulon*'') thalli. These associations can be attached or independent, and are referred to as morphotype pairs, photosymbiodemes (Honegger 1996), or phototype pairs (Jørgensen 1996). Although phototype pairs are thought to share the same fungal species, in the past they have been accorded different species epithets. In situations where the fungal partner of a phototype pair is the same species, only the older name is valid, a convention we follow here; but see White & James (1988).

Phenotypic characters such as morphology and secondary metabolites, and genotypic characters such as nucleotide sequences are commonly used to determine

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similarity and infer evolutionary relationships among species. Similar to other phenotypic characters, secondary structures of rRNA have also been used to infer similarity and evolutionary relationships (Lott *et al.* 1998; Piercey-Normore *et al.* 2004). Secondary structures of the spacer regions in ribosomal RNA have been reported to be involved in the ribosomal RNA processing for maturation of the ribosome (Good *et al.* 1997; Lalev & Nazar 1998; 1999; Lalev *et al.* 2000). The secondary structures retain a highly conserved configuration especially where proteins interact and cleavage occurs during rRNA maturation reflecting the critical nature of this process to survival of living organisms. The structure of the spacer regions thus provides an additional character to be used to examine similarity and infer evolutionary relationships.

The genus *Nephroma* belongs to the *Peltigerales* where it is thought to be basal to *Peltigera* and *Lobaria* (Miądlikowska & Lutzoni 2004) based on nuclear SSU and LSU ribosomal DNA. However, *Peltigera* was reported to be basal to *Nephroma* and *Lobaria* using mitochondrial SSU and nuclear LSU rDNA (Wiklund and Wedin 2003). The *Peltigerales* contain several examples of phototype pairs (see Tønsberg & Holtan-Hartwig 1983). Although resynthesis experiments provide the strongest support for occurrence of phototypes (Stenroos *et al.* 2003), nucleotide sequence comparisons can provide indirect evidence (Armaleo & Clerc 1991; Goffinet & Bayer 1997; Paulsrud *et al.* 1998). *Nephroma* is a genus of mostly epiphytic foliose lichens that form symbioses with either cyanobacterial partners alone (bipartite), or less often primarily with green algal partners and secondarily with cyanobacteria (tripartite). A current phylogenetic history of the genus *Nephroma* shows the intermixing of tripartite and bipartite species (Lohtander *et al.* 2002). The factors that drive the selection of photobionts are poorly known, but may be ecophysiological (photosynthetic efficiency), or biogeographical (availability of alternative photobiont). Two lichens that are morphologically distinct and ecologically differentiated, may in fact share the same fungus. One such case may be that of *N. occultum* Wetmore and *N. arcticum* (L.) Torss.

Nephroma occultum, a bipartite cyanobacterial species, is endemic to western North America, where it grows in old-growth cedar-hemlock forests and is designated as ''Special Concern'' by COSEWIC[1] (Goward 1996). It produces large numbers of vegetative propagules (isidia eroding to form soredia), which are thought to be dispersed by rain and animals. Apothecia have not been reported from this species suggesting a lack of sexual reproduction and recombination. To our knowledge, this species has not been placed into a phylogenetic framework. Wetmore (1980) suggested that the chemistry of *N. occultum* is similar to that of *N. arcticum* except for the presence of usnic acid in *N. occultum* and its rarity in *N. arcticum* (but see James & White 1987; White & James 1988). As a result he raised the question as to whether the two lichens have the same fungal species combining with the green alga, *Coccomyxa*, to form *N. arcticum* and the cyanobacterium, *Nostoc*, to form *N. occultum*. However, Tønsberg & Holtan-Hartwig (1983) reported a blue-green phototype of *N. arcticum* morphologically different from *N. occultum*. The objectives of this study were: (1) to determine the phylogenetic position of *N. occultum* relative to other species in the genus; (2) to investigate whether *N. occultum* is, if not the photomorph, the sister species to *N. arcticum*; and (3) to examine the phylogenetic position of the cyanobacterial partner associated with *N. occultum* and *N. arcticum*.

Materials and Methods

Lichen material

Samples used in this study include *N. occultum* (*Radies* C8 01, C28 01, BC, Canada); *N. isidiosum*

[1]The Committee on the Status of Endangered Wildlife in Canada (COSEWIC) is a coordinated effort among all levels of government, scientists, and all Canadians to conserve Canada's biodiversity. The national approach for the protection of species at risk is to prevent species in Canada from becoming extinct as a consequence of human activity.

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(Nyl.) Gyelnik (*Radies* H25 04, BC, Canada); *N. expallidum* (Nyl.) Nyl. (*Normore* 3041, MB, Canada), *N. antarcticum* (Jacq.) Nyl. (*Goffinet* 6550, Chile); *N. arcticum* (*Goward* 94-947a; *Goward* 04-22, BC, Canada). Samples were air-dried and vouchers have been deposited in the personal herbarium of D. Radies, University of British Columbia Herbarium (UBC), the University of Conneticut Herbarium (CONN), and the University of Manitoba Herbarium (WIN).

DNA extraction and PCR

Total DNA was extracted from a 1 cm thallus lobe using a CTAB (cetyltrimethylammonium bromide) extraction buffer and a protocol modified from Grube *et al.* (1995). The thallus was examined for discoloration or foreign particles before isolating DNA. DNA was resuspended in sterile dH_2O and amplified by the polymerase chain reaction (PCR). Amplification of the internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) of the fungal partner was from NSR1566-5′ (Gargas & DePriest 1996) and ITS4-3′ (White *et al.* 1990), and the transfer RNA^{Leu} intron ($tRNA^{Leu}$) from the cyanobacterial partner using TrnLF (5′-GGGGRTRTGGYGRAAT-3′) and TrnLR (5′-GGGGRYRGRGGGACTT-3′) (Paulsrud & Lindblad 1998). The primers for amplification of *N. antarcticum* and *N. arcticum* were BMBC-R, ITS4 (White *et al.* 1990) and a new primer ITSF (5′-CTTGGTCATTTAGAGGAASTAA-3′). Amplifications were performed in six 50 µl volumes and combined to produce 300 µl for DNA sequencing. Amplifications were performed in 1 × buffer (200 mM Tris-HCl (pH 8·4), 500 mM KCl), with 2·5 units Taq DNA polymerase (GibcoBRL, Burlington, ON, Canada), 200 µmol l^{-1} of each dNTP, 0·5 µmol l^{-1} of primer, and between 10 and 50 ng of DNA. Amplification conditions in Fisher Scientific Techne Genius were: initial template denaturing at 94°C for 5 min, then denaturing at 94°C for 1 min, annealing at 54°C for 1 min (50°C for *N. antarcticum* and *N. arcticum*), extension at 72°C for 2 min, for 33 cycles. PCR products were agarose gel purified by crushing frozen blocks of agarose, pipetting the buffer containing DNA into an Eppendorf tube, and subsequent precipitation with 0·2 volumes 5M NaCl and 2·5 volumes 100% ethanol. DNA was quantified on 1% agarose gel stained with ethidium bromide.

DNA sequencing

Double stranded PCR products were sequenced using BigDye Terminators Version 3.0 on a 377 and 377XL ABI DNA Sequencing Instrument (University Core DNA and Protein Services, University of Calgary, Calgary, Alberta) and following Goffinet *et al.* (2003) for *N. antarcticum* and *N. arcticum*. The DNA sequences were assembled into full-length sequences using Sequencher 4.1.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Both strands were sequenced from two PCR primers. Nucleotide sequences were aligned manually in Se-Al v 1.0 (Rambaut 2001) and imported into PAUP* 4.0b10 (Swofford 2003). Homologous regions in the alignment were evident except for 140 bases at the 5′ end of the ITS1. Comparison among secondary structures in this region yielded insufficient information on homology; therefore, separate analyses included and excluded these 140 bases producing the same phylogenetic history. All nucleotide sequences generated in this study have been deposited in NCBI GenBank and accession numbers are indicated on the phylogenies.

ITS secondary structure

The internal transcribed spacer regions and the 5.8S rDNA were defined based on the conserved sequence at the 3′ end of the 18S gene, the 5′ and 3′ ends of the 5.8S gene, and the 5′ end of the 26S gene (Hausner & Wang 2005). These regions were not present in GenBank sequences from the ITS1 of *N. australe*, *N. antarcticum* and the ITS2 of *N. antarcticum*. The stem loop structures were folded using the mfold web server (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi; Zuker 2003). If more than one fold was produced the final fold was based on comparisons with previously published ITS1 and ITS2 folds, maximization of the hydrogen bonding forming solid stems, and the largest negative delta g value (free energy).

Data analysis

The fungal ITS phylogeny of the genus *Nephroma* was inferred from 34 specimens, (including 7 new sequences), and rooted with *Lobaria retigera* (Bory) Trevis. based on Lohtander *et al.* (2002). Results from a BLAST search using *Nephroma* query sequences yielded results with species of *Lobaria* as the most similar sequences. The cyanobacterial $tRNA^{Leu}$ phylogeny was inferred from three new sequences of *Nephroma* and 24 accessioned DNA sequences retrieved from NCBI GenBank. The cyanobacterial $tRNA^{Leu}$ phylogeny was unrooted. GenBank sequences chosen for the $tRNA^{Leu}$ analysis were based on a BLAST (Altschul *et al.* 1997) search of the photobiont sequences from *N. isidiosum* and *N. occultum*. Photobiont sequences from the best matches (based on *E-score*) and several less similar matches were included in the analysis.

Phylogenetic and phenetic analyses were performed on four sets of data; (1) fungal ITS1 rDNA, (2) fungal ITS2 rDNA, (3) combined fungal ITS1, ITS2, and the 5.8S rDNA, and (4) cyanobacterial $tRNA^{Leu}$ intron. Phylogenetic determinations were based on Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses. MP trees were produced using the option tree bisection and reconnection (TBR) branch swapping. Heuristic searches were conducted using 1000 random addition replicates with a limit of 10 trees per search and bootstrap searches of 500 resamplings (Felsenstein 1985). Gaps were treated as missing data. ML trees were produced using default parameters including a substitution model of transition/transversion ratio set to 2, empirical base frequencies, and equal among-site rate

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TABLE 1. *Length (bp) and energy values (ΔG) for separate ITS regions of species in the genus* Nephroma *and in* Lobaria retigera

Clade	Species	ITS1		ITS2	
		Length (bp)	ΔG* (kcal mol^{-1})	Length (bp)	ΔG* (kcal mol^{-1})
A	*N. expallidum*	200–201	− 55·2	134	− 43·1
	N. laevigatum	196	—	136	—
	N. tangeriense	197	—	136	—
	N. sulcatum	201	—	138	—
	N. parile	197	− 56·1	135	− 40·4
	N. bellum	185†	—	136	—
B	*N. plumbeum*	197	—	140	—
	N. rufum	197	—	140	—
	N. helveticum	203	—	145	—
	N. tropicum	203	—	145	—
	N. cellulosum var *isidoferum*	205	—	145	—
	N. isidiosum	202	− 61·7	145	− 39·7
C	*N. australe*	191†	− 60·1	147	− 50·4
	N. occultum	291	− 83·7	150–152	− 44·8
	N. antarcticum	181†	− 53·2	145†	− 49·9
	N. cellulosum	204–205	− 66·7	147	− 49·0
	N. arcticum	183	− 62·0	142–143	− 44·5
D	*N. resupinatum*	173–175	− 60·1	149–150	− 46·4
Outgroup	*Lobaria retigera*	159	− 54·7	149	− 52·2

*Energy values were determined only for ITS RNA that was folded.
†Samples in which portions of the adjacent RNA coding regions were not available.

variation. Phenetic analyses were performed using the Neighbour Joining clustering algorithm and Kimura 2-parameter model (Kimura 1980).

Results

Genetic variation

Pairwise sequence similarity within species of *Nephroma* was variable for the genus but was greater than 95%. Sequence similarity was >99·6% for *N. expallidum*; >97·6% for *N. isidiosum*; >99·8% for *N. cellulosum* (Ach.) Ach.; 100% within each of *N. occultum* and *N. antarcticum*; >99·6% for *N. arcticum* (including the cyanobacterial phototype); and >95·5% for *N. resupinatum* (L.) Ach.

The DNA sequence length of the fungal ITS1 region ranged from 159 base pairs (bp) to 205 bp (46 bp range excluding *N. occultum* whose ITS is 291 bp long; Table 1). The length of the ITS1 region varied more than that of the ITS2 region, which ranged from 134 bp to 152 bp (18 bp range including *N. occultum*). The ITS1 of the outgroup taxon was the shortest, that of *N. resupinatum* (L.) Ach. longer, *N. arcticum* longer, and those of all other ingroup taxa lay between 191 bp to 205 bp except *N. occultum* (Table 1). The length of the ITS1 aligned matrix was 307 bp. The length of the ITS2 region in the outgroup taxon was 149 bp, it fluctuated in the ingroup taxa, and the shortest regions were in *N. expallidum* (Nyl.) Nyl. and *N. parile* (Ach.) Ach. (Table 1). The length of the ITS2 region in *N. occultum* was within the range of other taxa. The length of the ITS2 aligned matrix was 170 bp.

The length of the $tRNA^{Leu}$ intron in the cyanobacterial partner of *N. occultum* was 334 bp to 336 bp but ranged from 270 bp to 384 bp in the taxa in this study (Fig. 4). The $tRNA^{Leu}$ alignment was 467 bp long. Variation among the taxa included indels and single base substitutions. The same $tRNA^{Leu}$ intron (based on Paulsrud &

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TABLE 2. *Phylogenetic information for each of the ITS1, ITS2, and entire region (ITS1, ITS2, and 5.8S) for* Nephroma *and tRNA^{Leu} for the photobiont*

Information	ITS1	ITS2	ITS1, ITS2, and 5.8S	tRNA^{Leu}
Tree length (steps)	442	313	807	78
No. MP trees	233	16	245	88
CI	0·6584	0·5764	0·6270	0·8718
RI	0·8523	0·8203	0·8315	0·9107
No. characters	307	170	638	467
Var. uninformative	47	22	76	25
Var. informative	145	96	251	38

Lindblad 1998) was found in the cyanobacterial partners of each species, *N. occultum* and *N. arcticum*, but with seven single base substitutions separating them. A BLAST search indicated that photobionts of *N. occultum* were most similar (*E-value*=1e-179) to five GenBank accessions (AF509392–AF509396). All sequence matches were included in the analysis in addition to other less similar sequences.

The fungal ITS1 was more variable than ITS2 producing a larger number of MP trees and a longer tree length (Table 2). The tRNA^{Leu} region included fewer informative characters than the separate fungal ITS regions alone and their average Consistency (CI) and Retention (RI) indices were higher than that of the fungal nuclear loci. Pairwise nucleotide sequence similarities of the ITS1 among *Nephroma* species ranged from 39·4%–45·6% between *N. resupinatum* and *N. tropicum* (Müll.Arg.) Zahlbr. (the lowest), to 97·5% between *N. helveticum* Ach. and *N. tropicum* (the highest), and was greater than 92% within species. Pairwise nucleotide sequence similarities of the ITS2 among *Nephroma* species ranged from 45%–50% between *N. resupinatum* and *N. rufum* (Church. Bab.) P. James, to 98·7% between *N. cellulosum* var. *isidioferum* J. Murray and *N. isidiosum*, and was greater than 94% within species. Pairwise nucleotide sequence similarities of the cyanobacterial tRNA^{Leu} intron ranged from 93·3%–100% among all taxa.

Phylogenetic history

Phylogenetic inferences from the combined fungal ITS rDNA phylogeny for the genus *Nephroma* yielded four well-defined clades (A, B, C, and D; Fig. 1). Clade A with 94% bootstrap support consisted of *N. expallidum*, *N. laevigatum* Ach., *N. tangeriense* (Maheu & Gillet) Zahlbr., *N. sulcatum* P. James & F. J. White, and *N. parile*. *Nephroma expallidum*, collected from Manitoba, falls with other conspecific specimens (with 100% bootstrap support) in clade A. *Nephroma bellum* (Sprengel) Tuck. was sister to clade A. Clade B with 100% bootstrap support contained *N. plumbeum* (Mont.) Mont., *N. rufum*, *N. helveticum*, *N. tropicum*, *N. cellulosum* var. *isidioferum*, and *N. isidiosum*. *Nephroma isidiosum* was resolved as polyphyletic. Clade D with 100% bootstrap support contained only accessions of *N. resupinatum*. *Nephroma resupinatum* is the sister-group to a highly supported (i.e., 95% bootstrap support) lineage combining clades A, B, and C. The monophyly of clade C, which includes *N. australe* A. Rich., *N. cellulosum*, *N. antarcticum*, *N. occultum*, and *N. arcticum*, had low support (<50%). The cyanobacterial phototype of *N. arcticum* was monophyletic with the green algal phototypes (Fig. 1A). The relationships among the taxa in clade C were unresolved. Inferences under the maximum likelihood criterion yielded a single topology for this clade, identical to that of the MP tree, wherein *N. arcticum* is a sister species to the

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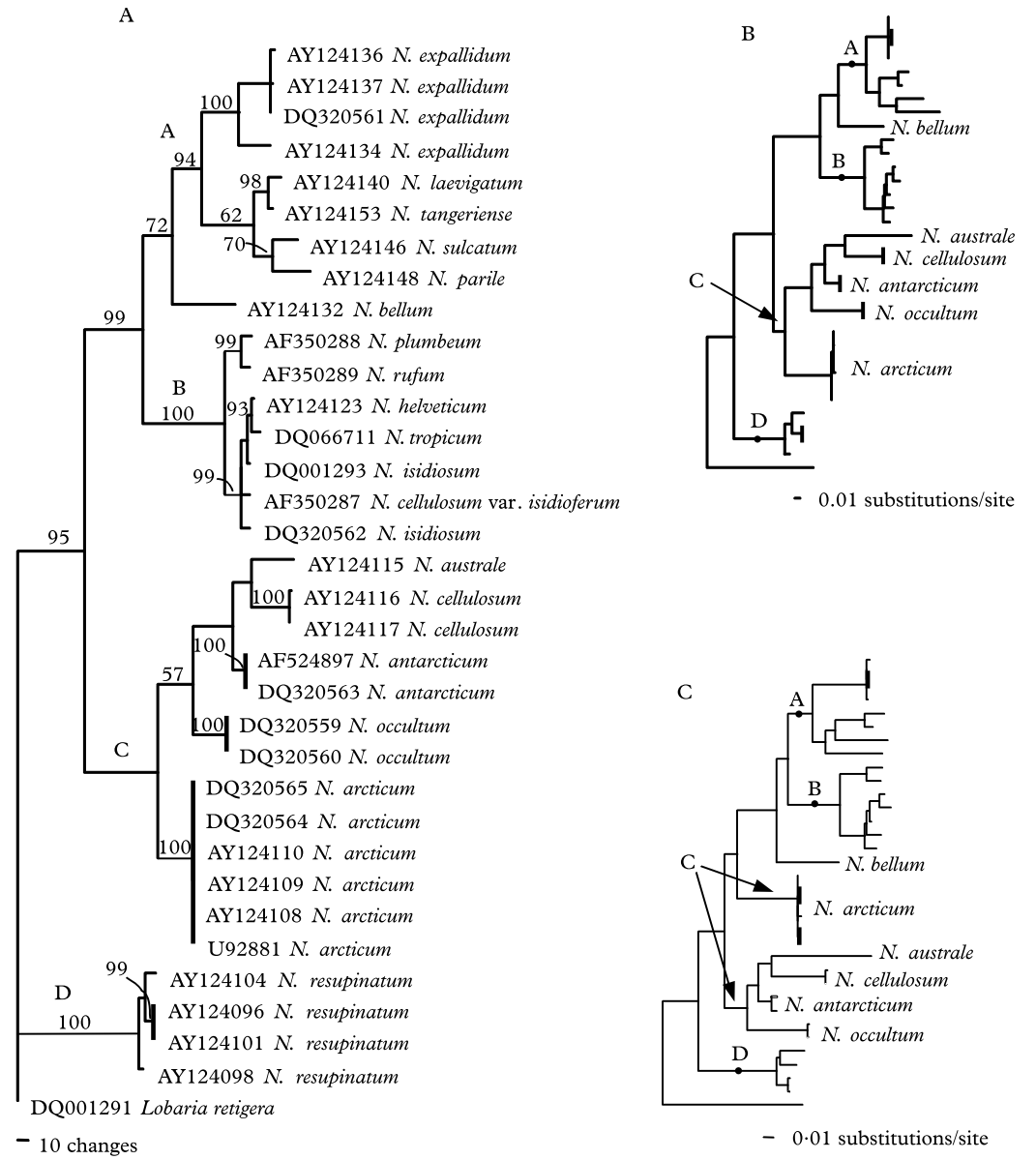

FIG. 1. Evolutionary history of species of *Nephroma* represented by two phylogenetic (MP and ML) and a phenetic (NJ) method based on combined ITS1, 5.8S, and ITS2 nucleotide sequences. A, one of 245 MP trees for 19 taxa; B, the ML tree (-Ln=4689·7868); C, the NJ tree. Bootstrap values of >50% are shown above the branches. *Lobaria retigera* was assigned the outgroup. The cyanobacterial phototype of *N. arcticum* is U92881.

grade containing *N. australe*, *N. cellulosum*, *N. antarcticum*, and *N. occultum*. By contrast the NJ analysis placed *N. arcticum* sister to clades A and B, and the lineage comprising *N. australe*, *N. cellulosum*, N. *antarcticum*, and *N. occultum* sister to this combined clade.

In the separate analyses of fungal ITS1 and ITS2, clades B and D consistently produced high bootstrap support (Figs 2 and 3).

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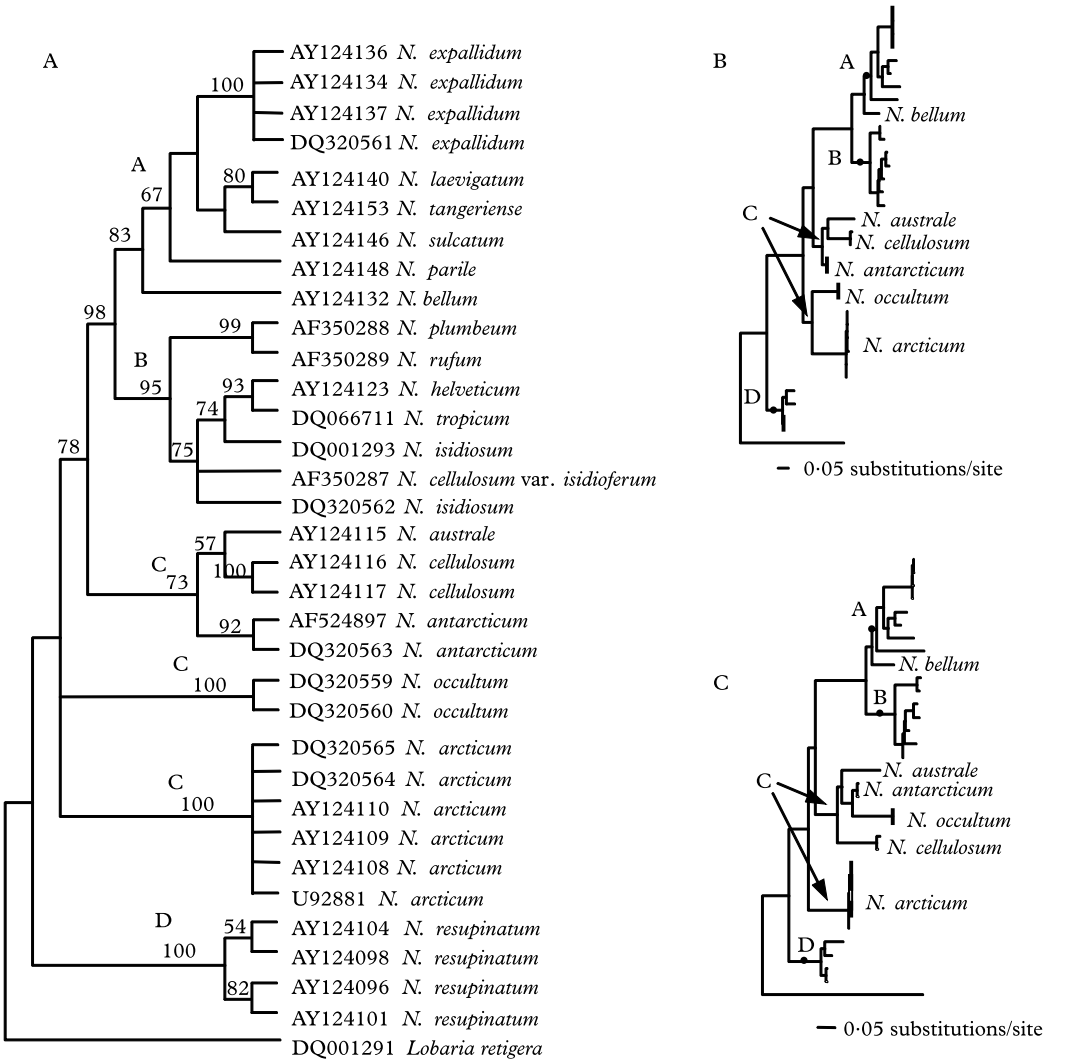

FIG. 2. Evolutionary history of species of *Nephroma* represented by two phylogenetic (MP and ML) and a phenetic (NJ) method based on ITS1 nucleotide sequences. A, strict consensus of 245 MP trees for 19 taxa; B, the ML tree (-Ln=2318·1876); C, the NJ tree. Bootstrap values of >50% are shown above the branches. *Lobaria retigera* was assigned the outgroup.

Nephroma isidiosum from Clade B was polyphyletic. Clade A contained the same members in both analyses but had low bootstrap support in the ITS1 analysis. Clade C was variable in topology between ITS1 and ITS2 regions as well as between ML, MP, and NJ analyses. The five species in clade C based on the combined analysis, were separated into three lineages forming a grade leading to clades A, B and *N. bellum*. Four of the five species within each of the three grades are monophyletic. The fifth species, *N. australe*, was represented by only a single specimen. The most likely tree (Fig. 2B) placed *N. occultum* as a sister species to *N. arcticum*, but the strict consensus of the most parsimonious ITS1 trees (Fig. 2A) showed that the two species were unresolved.

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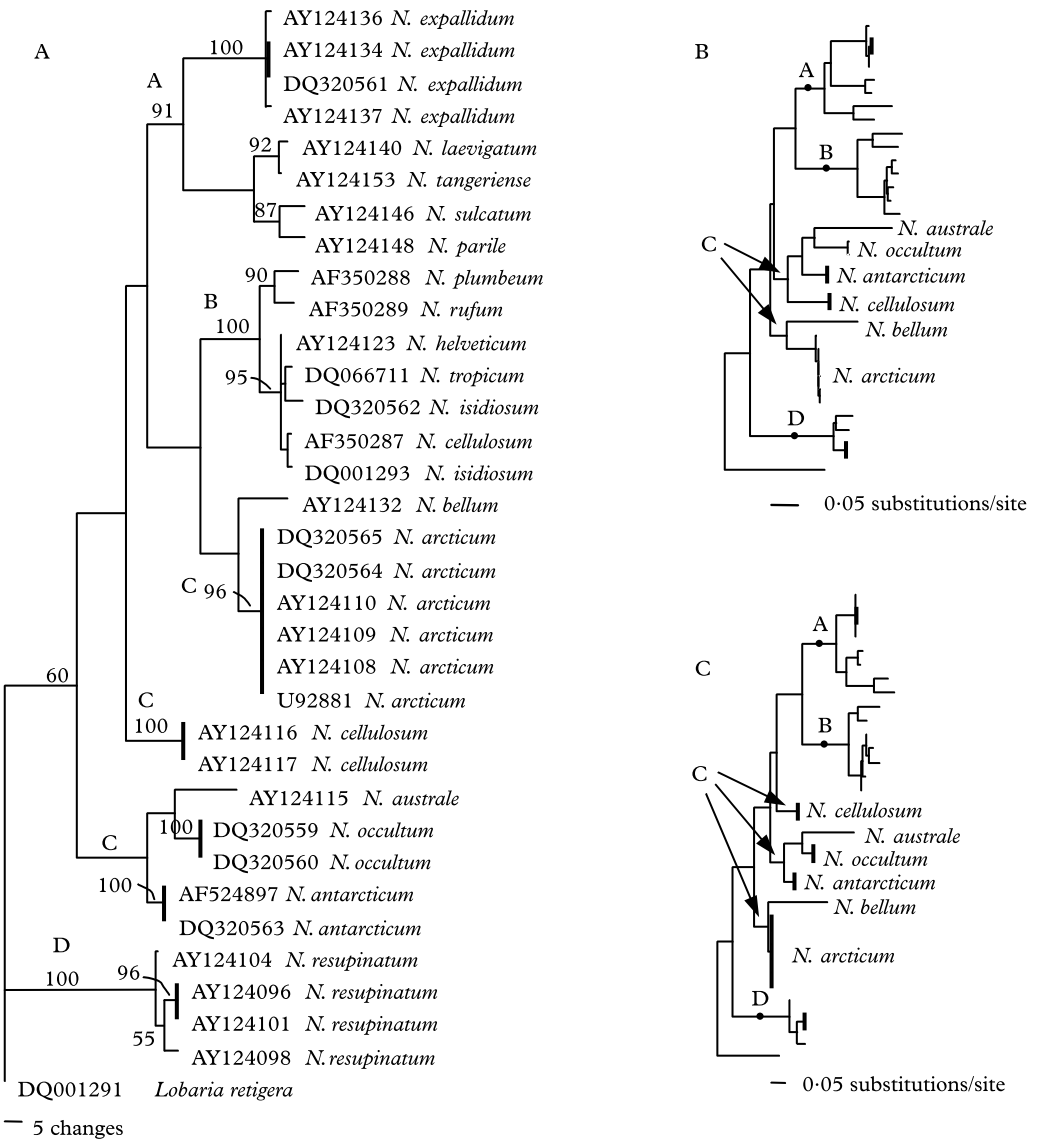

FIG. 3. Evolutionary history of species of *Nephroma* represented by two phylogenetic (MP and ML) and a phenetic (NJ) method based on ITS2 nucleotide sequences. A, one of 16 MP trees for 19 taxa; B, the ML tree (-Ln=1613·6292); C, the NJ tree. Bootstrap values of >50% are shown above the branches. *Lobaria retigera* was assigned the outgroup.

However, *N. arcticum* was basal to *N. australe*, *N. occultum*, *N. antarcticum*, and *N. cellulosum* in the NJ tree (Fig. 2C). Inferences from ITS 2 resolve members of "clade C" into a grade of three lineages, which are successively a clade composed of *N. antarcticum*, *N. australe* and *N. occultum*, followed by *N. cellulosum*, and at last a clade composed of *N. arcticum* and *N. bellum*. The most likely ITS2 tree placed *N. arcticum* basal to the grade containing *N. australe*, *N. occultum*, *N. antarcticum*, and *N. cellulosum* (Fig. 3B). However, the NJ analysis placed *N. arcticum* and *N. bellum* basal to *N. cellulosum*, *N.*

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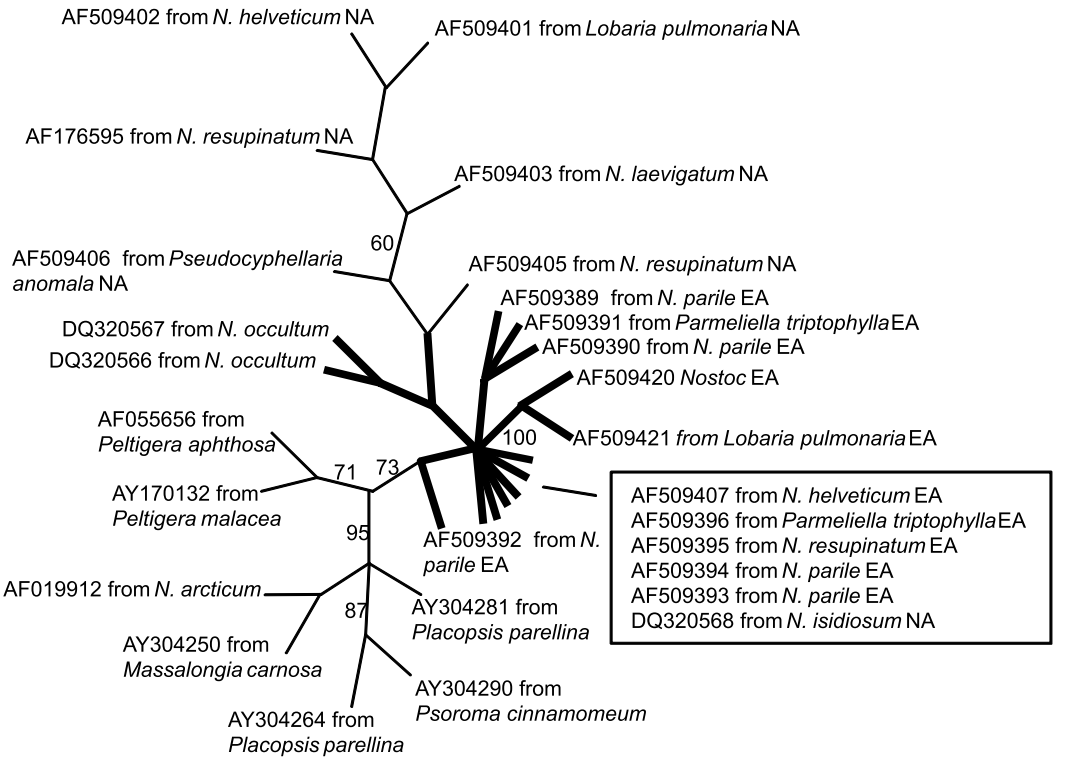

FIG. 4. Evolutionary history of cyanobacterial photobionts showing a 50% majority rule of 907 MP trees for 27 samples based on the $tRNA^{Leu}$ intron nucleotide sequences. Bootstrap values of >50% are shown above the branches. NA indicates a North American collection and EA indicates a Eurasian collection. Lineages in bold refer to the taxa that collapsed in the strict consensus tree.

australe, *N. occultum*, and *N. antarcticum* (Fig. 3C).

The cyanobacterial $tRNA^{Leu}$ phylogeny was less resolved than the fungal ITS phylogeny. The unrooted 50% majority rule consensus tree (Fig. 4) produced clades with low bootstrap support. In the strict consensus tree (data not shown) the taxa represented by thick black lineages (Fig. 4) formed a single unresolved clade. In the 50% majority rule consensus tree the photobionts of *N. occultum* were separated from the unresolved clade. The *N. occultum* photobiont was separated from the North American taxa. The *N. arcticum* photobiont was closely related to the terrestrial cyanobacterial guild (cyanobacteria from *Massalongia carnosa*, *N. arcticum*, *Peltigera aphthosa*, *P. malacea*, *Placopsis parellina*, and *Psoroma cinnamomeum*).

ITS secondary structures

Representative taxa from each clade were chosen and putative secondary structures of the ITS regions were folded (Fig. 5). All representatives of clade C were folded since *N. occultum* was located in clade C. The trend in the ITS1 region was toward an increase in length from the outgroup to the derived ingroup taxa where it remained approximately 200 bp long (except *N. occultum*). The foldings of ITS1 in the basal species in the tree were compact with the highest free energy value in the outgroup taxon (Table 1). The ITS1 structures contained a larger number of secondary and tertiary hairpins in the ingroup and the lowest free energy in *N. occultum*. Two regions that were conserved in nucleotide sequence were mapped onto the structures.

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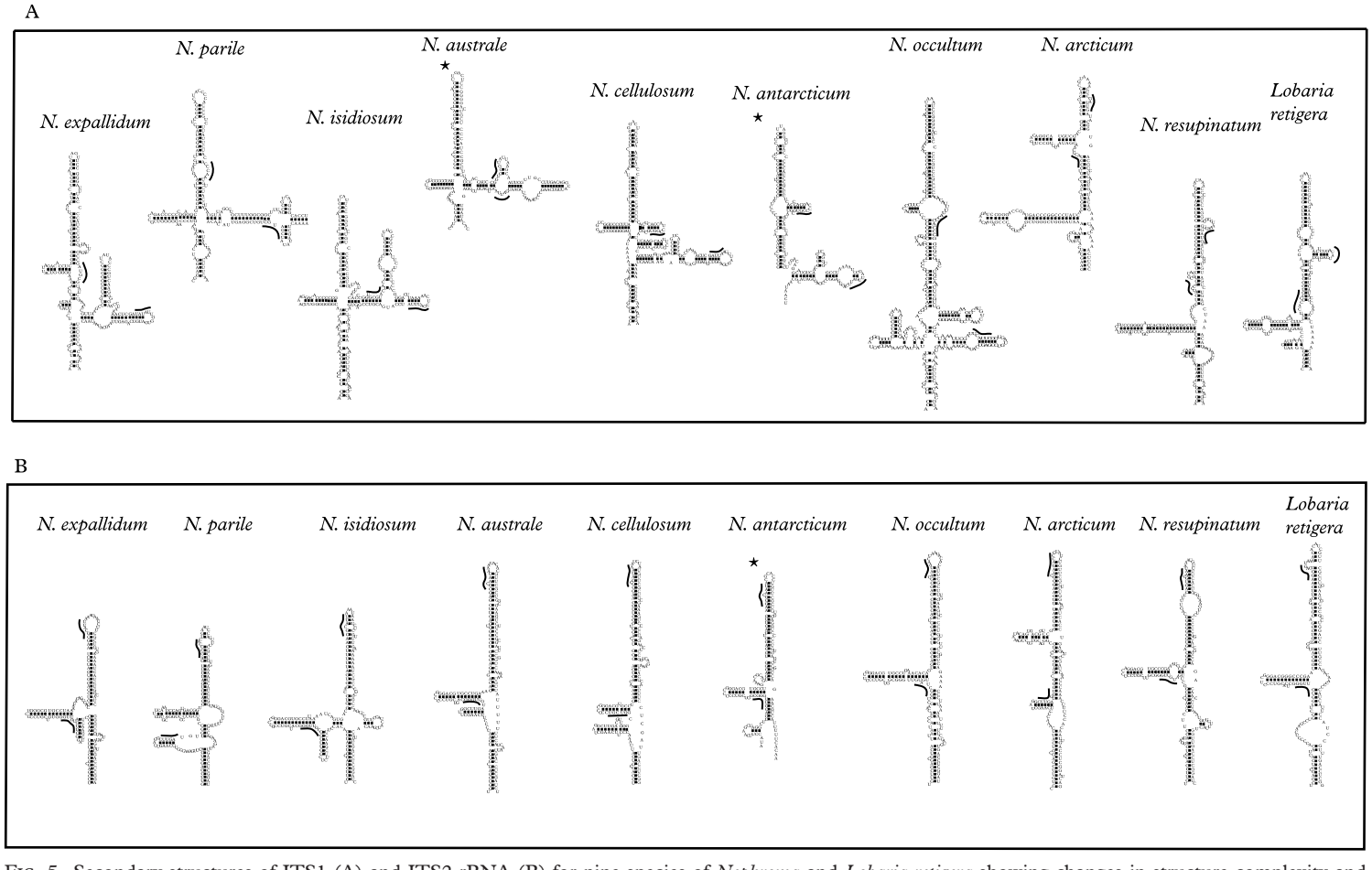

FIG. 5. Secondary structures of ITS1 (A) and ITS2 rRNA (B) for nine species of *Nephroma* and *Lobaria retigera* showing changes in structure complexity and conserved nucleotide sequences relative to position on fold. Species represent each of the major clades and all of clade C from Fig. 1 in the same order in which they are presented in Fig. 1 with the outgroup on the right side of the figure. The two solid lines adjacent to the structure represent nucleotide conserved regions. ⋆portions of adjacent RNA coding regions were not available.

The conserved regions shifted in the ITS1 structures from the left and upper right sides of the structures in the basal taxa to the right lower sides in the ingroup taxa. The ITS2 foldings were more conserved than the ITS1 foldings and a different trend was found. Length decreased slightly from basal to ingroup taxa (Table 1), but with the lowest free energy value in the outgroup. The ITS2 nucleotide conserved regions remained for the most part in the same positions on the structure. The upper left conserved region was highly consistent among all structures. The lower left region shifted between upper and lower right angles of a secondary stem loop in the structure. Minimum free-energy values (ΔG) of all ITS1 and ITS2 foldings were negative indicating that the secondary structures were thermodynamically stable.

Discussion

Phylogenetic history of *N. occultum*

Nephroma occultum shares with other members of clade C, a similar set of secondary compounds, characterized by the presence of usnic acid and hopane triterpenoids (T3) (White & James 1988). However, based on morphological features, the diagnostic surface reticulation in *N. occultum* is more similar to that in *N. papillosum* F. J. White and P. James, *N. microphyllum* Henssen, and *N. chubutense* Lamb (White & James 1988). *Nephroma occultum* belongs to the *N. microphyllum* species complex (White & James 1988). While all three species are restricted to Chile and Argentina *N. microphyllum* is more widespread than the other two species. The surface reticulation in *N. occultum* is different from the surface faveolation in *N. antarcticum* and *N. arcticum*, but the two features are combined in *N. cellulosum*. *Nephroma cellulosum*, *N. australe*, *N. antarcticum* and *N. occultum* were not included in the phylogeny in Lohtander *et al.* (2002). However, the placement of *N. resupinatum* basal to *N. arcticum* is consistent with the phylogenetic hypothesis proposed by Lohtander *et al.* (2002). The pattern was also consistent with Miądlikowska & Lutzoni (2004) even though only three species of *Nephroma* were included by these authors. Resolution of the phylogenetic position of *N. occultum* needs to be better addressed by comparison with South American species such as *N. papillosum, N. microphyllum*, and *N. chubutense.*

The hypothesis that *N. arcticum* and *N. occultum* are phototypes was not supported in this study. White & James (1988) arrived at a similar conclusion and suggested that a green phototype of *N. occultum* should be more similar to the green phototype of *N. papillosum*, a species restricted to Chile and Argentina, than to *N. arcticum*. The cyanobacterial phototype of *N. arcticum* (Tønsberg & Holtan-Hartwig 1983) is morphologically different from *N. occultum* (James & White 1987). The only evidence from this study to support the phototype hypothesis is that the nucleotide sequence alignment of the combined ITS region placed *N. occultum* in clade C with *N. cellulosum*, *N. australe*, *N. antarcticum*, and *N. arcticum* (Fig. 1A) and that *N. occultum* was a sister species to *N. arcticum* in the ITS1 ML phylogeny (Fig. 2C). However, stronger evidence to refute the hypothesis includes: (1) the dissimilarity of the ITS1 secondary structure between *N. occultum* and *N. arcticum* (Fig. 5); (2) the extensive ITS1 length discrepancy between *N. occultum* and *N. arcticum* (Table 1); (3) the separation of *N. arcticum* in all phylogenies; (4) the closer relationship of *N. occultum* to *N. cellulosum*, *N. antarcticum*, and *N. australe* than to *N. arcticum* in all analyses (Figs 1–3); and (5) the fungal ITS sequence of the joined blue-green and green morphotype of *N. arcticum* fell into the highly supported and invariable *N. arcticum* clade with other members of the species (Figs 1–3). Based on pairwise sequence similarities *N. occultum* was most similar to *N. antarcticum* in the ITS1 (89%) and the ITS2 (86%) nucleotide sequence. By contrast the fungal ITS1 regions of the phototype in *Lobaria amplissima* (Scop.) Forss. and *L. fendleri* (Tuck. ex Mont.) Lindau were identical (Stenroos *et al.* 2003). Although just two samples and a single locus are not sufficient to validate the hypothesis

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of a phototype-pair, neither are two samples and one locus sufficient to reject the hypothesis. If the difference in similarity is part of infraspecific variation, the hypothesis should also be examined by investigation of a larger number of South American species. The addition of South American species may provide internal support in the phylogeny. While a larger sample size is required to detect variability within the species, *N. occultum*, evidence from this study suggests that *N. occultum* and *N. arcticum* are different species and that *N. occultum* belongs to a South American clade.

Some species showed more sequence variation than other species. The ITS sequences of *N. arcticum* were more than 99% similar among six samples. Since 10 samples of *N. arcticum* produced almost identical ITS sequences in Lohtander *et al.* (2002), only two were used in their analysis. Intraspecific variation was evident in *N. isidiosum* between the sample collected in China and the one in British Columbia and may be explained by taxonomic heterogeneity. Both samples clustered in clade B with *N. cellulosum* var. *isidioferum*. *Nephroma cellulosum* var. *isidioferum* was treated as a distinct species (*N. lepidophyllum* Räsänen ex Gyelnik) based on the crowded phyllidia and a greyish cast in the centre of the thallus, but recently treated as a variety of *N. cellulosum* by White & James (1988). Based on the information presented in this study we support the previous taxonomic separation, and contend that *N. cellulosum* var. *isidioferum* be treated as a distinct species. A larger sample size is required to examine further this contention.

During this study a discrepancy was revealed regarding the nucleotide sequence of *N. isidiosum*. After a BLAST search was performed on the DNA sequence of *N. isidiosum*, significant matches were produced with other species of *Nephroma* and a sequence labelled as ''*Oropogon secalonicus*'' from Hur *et al.* (2005). However, the ''*Oropogon secalonicus*'' DNA sequence was identical to *N. isidiosum* from this study and the accession number (DQ001293) matched the name *N. isidiosum* in Hur *et al.* (2005). Therefore, we concluded that a mislabelling occurred during the original GenBank submission and that the sequence labelled as ''*Oropogon secalonicus*'' in GenBank was actually *N. isidiosum* as appears in Hur *et al.* (2005).

Decline of *N. occultum*

The relationship between *N. australe*, *N. cellulosum*, *N. antarcticum*, *N. occultum*, and *N. arcticum* in clade C (Fig. 1A) suggested that *N. occultum* was derived from ancestors of three tripartite (*N. australe*, *N. antarcticum*, *N. arcticum*) and one bipartite (*N. cellulosum*), epiphytic species of the temperate Southern Hemisphere. The tripartite condition may have originated once at the base of clade C and was subsequently lost twice, or it may have been gained three independent times. Both scenarios are equally parsimonious. *Nephroma australe* is broadly distributed in South America and in Australasia occupying shaded branches at forest margins (Green *et al.* 1997). Also widespread is *N. cellulosum*, an epiphyte found in shaded climax forests (Mark *et al.* 1963), and *N. antarcticum* distributed throughout temperate South America (White & James 1988). *Nephroma arcticum* diverged into a circumpolar northern species occupying subarctic, boreal and alpine regions (James and White 1987). Perhaps the four species (*N. australe*, *N. cellulosum*, *N. antarcticum*, *N. occultum*) were conspecific before the breakup of Pangea, and with subsequent vicariance events the species diverged retaining the adaptation to high humidity, low light, epiphytic habitats. These habitats survived in the Southern Hemisphere temperate zone but are declining in the Northern Hemisphere. The ancestor of *N. occultum* may have lost the ability to lichenize with green algal partners that form the tripartite lichens of its recent ancestors, and shifted, as *N. cellulosum*, to cyanobacteria as the primary photobiont. The reduction in habitat and the loss of the ability to lichenize with green algae may have played a role in the decline of *N. occultum* (Goward 1996).

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Since ecological factors play a role in development of different phototypes (Brodo & Richardson 1978; Ott 1988; Stocker-Wörgötter 1995), habitat conditions may partially govern the compatibility between photobiont and mycobiont. High humidity may cause cephalodia of a tripartite species to develop into cyanobacterial thalli (Ott 1988), or give the cyanobacterial phototype a photosynthetic advantage over the green algal phototype (Green *et al.* 1997). Rikkinen *et al.* (2002) reported that *Nostoc* from lichen associations separated into two clades representing those in terrestrial habitats and those in epiphytic habitats. The photobiont of *N. arcticum* was placed with the terrestrial guild. Although the cyanobacterial partner of *N. occultum* belongs to the epiphytic *Nephroma* guild, it did not contain $tRNA^{Leu}$ sequences identical to those in the phylogeny (Fig. 4) or any available in GenBank. Nucleotide sequence variation was reflected in six variable base substitutions in the photobionts of *N. occultum* in each of the variable regions 1 and 2 (as delimited by Paulsrud *et al.* 2000). High variation in tree topology was reflected in the strict consensus of 907 trees, but the 50% majority rule consensus tree separated the *N. occultum* photobionts from the Eurasian photobionts (Fig. 4) with low bootstrap support. The photobiont of *N. occultum* was more closely related to other epiphytic North American photobionts. Since the *N. occultum* photobiont is restricted to western North America, a bottleneck that limited genetic variation in the photobiont, may in part account for the endemism and the decline of the species. Analysis of a larger number of populations of *N. occultum* from western North America would shed light on the variation within the photobionts of *N. occultum* and the possibility of a genetic bottleneck in the photobiont.

Evolution of ITS secondary structures

Secondary structures of the ITS1 and ITS2 regions differed in complexity of the structure and level of sequence conservation among species of *Nephroma*. Even though both *N. arcticum* and *N. occultum* contained the distinct central extended hairpin of the ITS1 region (Fig. 5) required for cleavage and interactions during rRNA processing (Lalev & Nazar 1998), *N. occultum* had two additional secondary hairpins and one tertiary hairpin with several bulges that were not present in *N. arcticum*. The ITS1 structure of *N. arcticum* was more similar to those of the basal species, *N. resupinatum* and *Lobaria retigera*. Unlike the ITS1 region, the ITS2 region of *N. occultum* was very similar to that of all other species of *Nephroma* including the outgroup, *Lobaria retigera* (Fig. 5). The single centrally extended hairpin in the ITS2 is also thought to be important in protein interactions (Lalev & Nazar 1999) and plays a critical role in rRNA processing (Good *et al.* 1997). Cleavage and enzyme activity were affected by the relative position of both DNA sequence and structural elements in the ITS2 (Côté & Peculis 2001). Conservation of both DNA sequence and secondary structure in the ITS2 have been suggested to account for high sequence homology among species of *Candida* (Lott *et al.* 1998). DNA sequence homology and its relative position on the structure are highly conserved in the ITS2 across species of *Nephroma* (Fig. 5). However, the location of two homologous sequence regions in ITS1 shifted position on the secondary structure (Fig. 5) suggesting less selection for both DNA sequence and structure in the ITS1 than in the ITS2. In fact, Lalev & Nazar (1998) suggested that even though the ITS1 structure may be important for interactions during rRNA processing, it may not be critical to rRNA maturation and that it may only influence the overall efficiency of the maturation process. Less stringent selection on the ITS1 region would allow substitutions and indels to occur that would obscure sequence homology among species.

The ITS1 region of *N. occultum* is longer than that in any other species of *Nephroma* reported (Table 1), and is also longer than that of any *Peltigera* species yet examined (Goffinet & Bayer 1997; Goward & Goffinet 2000; Goffinet *et al.* 2003). Nevertheless it is within the range of ITS1 regions reported

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for ascomycetes (Hausner & Wang 2005). By contrast, the length of the ITS2 is similar to that of the other species of *Nephroma* (Table 2) and *Peltigera* (Goffinet & Bayer 1997). A different situation existed in *Candida* (Lott *et al*. 1998), where the ITS2 region varied widely in length ranging from 66 bp to 240 bp. The evolutionary trend toward shorter ITS lengths (Hausner & Wang 2005) was not observed in the ITS1 in this study. Members of *Nephroma* may be evolving toward longer ITS1 regions and toward a shorter ITS2 region. A minimum length of the ITS region would be the least number of nucleotides necessary to form a stable RNA structure. If this ''minimum length rule'' (Lott *et al*. 1998) is critical for optimal function of a ribosome, then it would follow that a maximum length rule might also exist. A maximum length rule is supported by the report that the ITS2 central helix cannot exceed a specific length in yeast (Côté & Peculis 2001). If the length became too long, secondary structures would become more complex, and the rRNA processing might become inefficient. Although the ITS1 length of *N. occultum* is within the range reported for ascomycetes (Hausner & Wang 2005) it may be approaching its limit for efficient rRNA processing (Good *et al*. 1997).

This is the first study to place *N. occultum*, a COSEWIC listed ''Special Concern'' species, into a phylogenetic framework using DNA sequence and secondary structure data. Even though *N. occultum* is endemic to North American western forests, it falls into a clade with South American temperate rainforest species. Its relationship with *N. arcticum*, *N. cellulosum*, *N. antarcticum*, and *N. australe* is likely to be more distant than its relationship to South American species in the *N. microphyllum* complex. *Nephroma occultum* is distinct among the studied species of *Peltigerales* in having an exceptionally long ITS1 region, which is reflected in the complex secondary structure of the ITS1. It is also distinct in the $tRNA^{Leu}$ intron of the photobiont suggesting association with a unique genotype of *Nostoc*. It may be argued that *N. occultum* contains a potentially inefficient ITS1, a unique photobiont genotype, no sexual reproduction to generate variation, and the inability to switch photobionts to adapt to different ecological habitats. With the continuing loss of old-growth forests along the humid west coast of North America, *N. occultum* is currently in a state of rapid decline (Goward 1996). These findings have major implications for the conservation biology of a species that may be ecologically and evolutionarily ill equipped to survive within its modern range in the Northern Hemisphere.

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