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Assessing host specialization in symbiotic cyanobacteria associated with four closely related species of the lichen fungus *Peltigera*

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Heterocystous cyanobacteria form symbiotic associations with a wide range of plant and fungal hosts. We used a molecular phylogenetic approach to investigate the degree of host specialization of cyanobacteria associated with four closely related species of the lichenized fungus *Peltigera*, and to compare these strains with other symbiotic cyanobacteria. We conducted phylogenetic analyses on 16S, *rbc*LX, and *trn*L sequences from cyanobacteria associated with multiple specimens of each lichen species and from symbionts of other fungi and plants, as well as from free-living strains of *Nostoc* and related genera of cyanobacteria. The genus *Nostoc* comprises two divergent lineages, but symbiotic strains occur primarily within a single monophyletic lineage that also includes free-living representatives. Cyanobacteria from the same lichen species were often more closely related to strains from other species or to plant symbionts or free-living strains than to each other. These results indicate that host specialization is low for the genus *Nostoc*, and suggest that opportunities for coevolution with its partners may be rare.

Key words: cyanobacteria, host association, lichens, molecular phylogenetics, *Nostoc*, *Peltigera*, specialization, specificity, symbiosis

Introduction

Symbiotic associations are an important component of the ecology of many cyanobacterial lineages and include interactions with plants (Rai *et al*., 2000), fungi (Rai, 1990), animals (Wilkinson, 1992), and eukaryotic algae (Janson, 2002; Murakami *et al*., 2004). It has been proposed that tightly integrated ecological associations, if maintained faithfully over evolutionary timescales, can lead to coevolutionary patterns such as asymmetric evolutionary rates (Law & Lewis, 1983), gene-for-gene interactions (Flor, 1955) or cospeciation (Brooks, 1979). Because coevolution involves reciprocal evolutionary changes, it requires that each partner have a significant fitness effect on the other (Thompson, 1994). It has been suggested that, if a species limits the number of partners with which it interacts (specialization), this may increase the response of that species to selection imposed by those partners, facilitating coevolution (Whitlock, 1996; Kawecki, 1998).

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The cyanobacterial genus *Nostoc* presents an interesting case for studying host specialization because of the wide number of symbiotic associations formed by members of the genus. In addition to its role as a photosynthetic partner (photobiont) of a wide range of lichenized fungi (Tschermak-Woess, 1988), *Nostoc* also forms symbiotic associations with a number of different plants, including bryophytes (Adams, 2002), cycads (Costa & Lindblad, 2002), the flowering plant *Gunnera* (Bergman, 2002), and possibly the fern *Azolla* (Plazinski *et al*., 1990; but see Peters & Meeks, 1989; Baker *et al*., 2003), as well as the non-lichen fungus *Geosiphon pyriforme* (Kluge *et al*., 2002). Within lichens, *Nostoc* is thought to be the sole photobiont occurring in most members of the order Peltigerales *sensu* Miadlikowska & Lutzoni (2004) and some members of the Lichinales (Tschermak-Woess, 1988) and Arctomiaceae (Lumbsch *et al*., 2005). It also occurs as a secondary photobiont in many green algal lichens, including members of the Agyriaceae, Stereocaulaceae, and Peltigerales.

It is not currently known if the same strains of *Nostoc* are able to participate in these different

associations or if *Nostoc* comprises multiple lineages that are each specialized on a different host. For most lichens, the evolutionary advantages of symbiont specialization must be balanced by the requirement for re-establishing the association during each generation. While some lichen species have evolved specialized codispersal mechanisms such as soredia and isidia, which package together vegetative fungal hyphae and photobiont cells (Büdel & Scheidegger, 1996), many lichen fungi reproduce primarily through sexually produced ascospores. These ascospores disperse independently of the photobiont and must reassociate with the cyanobacteria or algae found in the immediate vicinity of the germinating ascospore. Specialization would reduce the number of potential photobiont partners that germinating spores are likely to encounter. Since the fungal partner is thought to be obligately symbiotic, whereas free-living *Nostoc* is ubiquitous in many terrestrial habitats (Potts, 2000), evolutionary pressure toward specialization may differ for the two partners.

Characterizing the degree of specialization in cyanolichens has been hampered by problems with cyanobacterial taxonomy, owing in large part to the lack of connection between studies based on the botanical and bacteriological traditions (Turner, 1997). The former emphasizes examination of freshly collected field material and dried herbarium material (Geitler, 1932; Komárek & Anagnostidis, 1989) while the latter is based on examination of axenically cultured reference strains, often with little information on the ecological distribution of the organisms (Rippka *et al*., 1979; Castenholz, 2001). For example, the genus *Nostoc* was traditionally characterized by the formation of large gelatinous colonies (Komárek & Anagnostidis, 1989). Since this character is not exhibited in culture, bacteriological treatments have defined *Nostoc* based on the production of motile hormogonia (Rippka *et al*., 1979). However, hormogonia are not always produced under standard cyanobacterial culture conditions and they have recently been reported in cultures of *Anabaena* (Svenning *et al*., 2005). Likewise, species were traditionally recognized based on differences in colony morphology. It has been shown that these characters cannot be used to diagnose monophyletic groups (Wright *et al*., 2001), but no bacteriological species concept has been proposed for the group. These problems are exacerbated in the case of symbiotic strains, because identification is usually based on microscopic observation of symbiotic tissues, where the morphology is often altered from the free-living state (Bergman & Hällbom, 1982). Even in cases where symbiotic cyanobacteria are isolated in pure culture, it is difficult to ensure that the strains obtained do not represent minor symbionts or epiphytes that grow faster than the primary symbiont in culture (Meeks, 1998; Meeks *et al*., 1988; Miao *et al*., 1997).

Molecular phylogenetic approaches can be used to overcome these difficulties by amplifying symbiont DNA directly from the host tissues and comparing them with axenically cultured reference strains that have been examined morphologically and physiologically. Sequence-based approaches can also discriminate among morphologically indistinguishable strains, allowing examination of fine-scale patterns of host-specialization among closely related hosts. We used multi-locus phylogenetics to determine the extent of host specialization in *Nostoc* by comparing the relatedness of strains associated with the same host and those associated with different hosts. This was carried out at nested taxonomic scales: closely related species within the genus *Peltigera* section *Peltigera sensu* Miadlikowska & Lutzoni (2000), *Peltigera* versus other cyanolichen genera, and plants and other fungi versus lichenized fungi. In order to determine the taxonomic breadth of these symbiotic strains, we have also developed a phylogenetic framework for heterocystous cyanobacteria by including sequences from well-studied reference strains representing six cyanobacterial genera. Specialization was examined for each partner by looking at the amount of genetic diversity among cyanobacterial strains associating with the same host taxon and by looking at the taxonomic diversity of hosts associated with each cyanobacterial lineage.

Materials and methods

Taxon sampling

Our taxon sampling is summarized in Table 1. We prepared two data sets: one to examine phylogenetic relationships among representative strains of different genera within the Nostocales (dataset 1), and one to examine patterns of host specialization for symbiotic strains (dataset 2). Dataset 1 included 16S and *rbc*LX sequences of seven *Nostoc* strains (five environmental isolates and one cycad symbiont, as well as DNA extracted directly from a colony of *Nostoc commune* collected by HEO), three strains each of *Anabaena*, *Aphanizomenon*, and *Nodularia*, and one strain of *Cylindrospermum*, plus one strain of *Fischerella* (Stigonematales), for a total of 18 strains. For examination of specialization (dataset 2), we included 16S, *rbc*LX, and *trn*L. This necessitated obtaining *trn*L sequences for all the above strains except the *Aphanizomenon* strains and two of the *Anabaena* strains, which were unavailable

Table 1. Cyanobacterial strains used in this study with host or substrate information, location, collector and voucher information, and Genbank/EMBL accession numbers

Taxon[a]	Host/Substrate[a]	Location	Voucher	16S accession	*rbc*LX accession	*trn*L accession	Dataset
Nostoc sp.	***Peltigera canina* 1 (L)**	USA	H. O'Brien 02031210-2 (DUKE)	DQ185225	DQ185282	DQ185167	2
Nostoc sp.	***Peltigera canina* 2 (L)**	USA	H. O'Brien 02060620 (DUKE)	DQ185230	DQ185287	DQ185172	2
Nostoc sp.	***Peltigera canina* 3 (L)**	Poland	K. Czyzewska 00925 (LOD-L)	DQ185239	DQ185296	DQ185181	2
Nostoc sp.	***Peltigera canina* 4 (L)**	Germany	H. O'Brien 01081204 (DUKE)	DQ185222	DQ185279	DQ185164	2
Nostoc sp.	***Peltigera canina* 5 (L)**	Unknown	D. Mollenhauer 1:1-064[b]	DQ185204	DQ185261	DQ185146	2
Nostoc sp.	***Peltigera didactyla* 1 (L)**	Germany	H. O'Brien 01072401 (DUKE)	DQ185215	DQ185272	DQ185157	2
Nostoc sp.	***Peltigera didactyla* 2 (L)**	Germany	H. O'Brien 01081202 (DUKE)	DQ185220	DQ185277	DQ185162	2
Nostoc sp.	***Peltigera didactyla* 3 (L)**	Poland	J. Miadlikowska 5233 (UGDA-L)	DQ185245	DQ185304	DQ185188	2
Nostoc sp.	***Peltigera didactyla* 4 (L)**	Iceland	D. Mollenhauer 1:1-066[b]	DQ185206	DQ185263	DQ185148	2
Nostoc sp.	***Peltigera didactyla* 5 (L)**	Unknown	D. Mollenhauer 1:1-067[b]	DQ185207	DQ185264	DQ185149	2
Nostoc sp.	***Peltigera membranacea* 1 (L)**	USA	H. O'Brien 02031211 (DUKE)	DQ185226	DQ185283	DQ185168	2
Nostoc sp.	***Peltigera membranacea* 2 (L)**	USA	H. O'Brien 02031212-2 (DUKE)	DQ185227	DQ185284	DQ185169	2
Nostoc sp.	***Peltigera membranacea* 3 (L)**	Canada	H. O'Brien 020708-0-9-1 (DUKE)	DQ185203	DQ185260	DQ185145	2
Nostoc sp.	***Peltigera membranacea* 4 (L)**	Canada	D. Bastian & M. Dignard 575 (QFA)	DQ185248	DQ185307	DQ185191	2
Nostoc sp.	***Peltigera membranacea* 5 (L)**	Russia	S. Tchabanenko & R. Rosentreter 24874 (OSU)	DQ185247	DQ185306	DQ185190	2
Nostoc sp.	***Peltigera rufescens* 1 (L)**	Germany	H. O'Brien 01073001 (DUKE)	DQ185217	DQ185274	DQ185159	2
Nostoc sp.	***Peltigera rufescens* 2 (L)**	Germany	H. O'Brien 01073008 (DUKE)	DQ185218	DQ185275	DQ185160	2
Nostoc sp.	***Peltigera rufescens* 3 (L)**	Germany	H. O'Brien 01081201 (DUKE)	DQ185219	DQ185276	DQ185161	2
Nostoc sp.	***Peltigera rufescens* 4 (L)**	Germany	H. O'Brien 01081203 (DUKE)	DQ185221	DQ185278	DQ185163	2
Nostoc sp.	***Peltigera rufescens* 5 (L)**	Poland	F. Lutzoni 99.07.18-24 (DUKE)	DQ185249	DQ185308	DQ185192	2
Nostoc sp.	***Peltigera rufescens* 6 (L)**	England	D. Mollenhauer 1:1-065[b]	DQ185205	DQ185262	DQ185147	2
Nostoc sp.	***Peltigera rufescens* 7 (L)**	Unknown	D. Mollenhauer 94.1[b]	DQ185214	DQ185271	DQ185156	2
Nostoc sp.	***Peltigera aphthosa* 1 (L)**	Switzerland	A. Zehnder SAG 39.87[bc]	DQ185252	DQ185311	DQ185195	2
Nostoc sp.	***Peltigera aphthosa* 2 (L)**	Switzerland	A. Zehnder SAG 41.87 [bc]	DQ185253	DQ185312	DQ185196	2
Nostoc sp.	***Peltigera degenii* (L)**	Canada	T. Ahti & A. Drozdowicz 45565 (dupl. DUKE)	DQ185244	DQ185303	DQ185187	2
Nostoc sp.	***Peltigera horizontalis* (L)**	USA	H. O'Brien 02031213-1 (DUKE)	DQ185228	DQ185285	DQ185170	2
Nostoc sp.	***Peltigera lepidophora* (L)**	Canada	C. Nuyt 10.083 (QFA)	DQ185246	DQ185305	DQ185189	2
Nostoc sp.	***Peltigera neopolydactyla* (L)**	USA	H. O'Brien 02031107 (DUKE)	DQ185224	DQ185281	DQ185166	2
Nostoc sp.	***Collema crispum* (L)**	Germany	H. O'Brien 01072402 (DUKE)	DQ185216	DQ185273	DQ185158	2
Nostoc sp.	***Leptogium gelatinosum* (L)**	USA	B. McCune 24548 (dupl. DUKE)	DQ185232	DQ185289	DQ185174	2
Nostoc sp.	***Lobaria amplissima* (L)**	Austria	R. Türk 19926 (GLU)	DQ185233	DQ185290	DQ185175	2
Nostoc sp.	***Lobaria hallii* (L)**	USA	J. Miadlikowska & B. McCune 08.11.00 (dupl. DUKE)	DQ185234	DQ185291	DQ185176	2
Nostoc sp.	***Massalongia carnosa* (L)**	USA	B. McCune 24704 (OSU)	DQ185235	DQ185292	DQ185177	2
Nostoc sp.	***Nephroma bellum* (L)**	Austria	E. Hansen 661 Exsic. (H)	DQ185236	DQ185293	DQ185178	2
Nostoc sp.	***Nephroma helveticum* (L)**	Canada	B. McCune 20046 (OSU)	DQ185237	DQ185294	DQ185179	2
Nostoc sp.	***Pannaria conoplea* (L)**	Austria	J. Hafellner 42749 (GZU)	DQ185238	DQ185295	DQ185180	2
Nostoc sp.	***Sticta beauvoisii* (L)**	USA	H. O'Brien 02031219-1[a] (DUKE)	DQ185229	DQ185286	DQ185171	2
Nostoc sp.	***Sticta fuliginosa* (L)**	USA	B. McCune 24547 (dupl. DUKE)	DQ185259	DQ185318	DQ185202	2
Nostoc sp.	***Anthoceros* sp. 1 (P)**	Italy	D. Mollenhauer 1:1-106b2[b]	DQ185209	DQ185266	DQ185151	2
Nostoc sp.	***Anthoceros* sp. 2 (P)**	Germany	D. Mollenhauer 1:1-150b[b]	DQ185213	DQ185270	DQ185155	2
Nostoc punctiforme	***Blasia pusilla* 1 (P)**	Germany	H. Pankow SAG 65.79[bc]	DQ185255	DQ185314	DQ185198	2

(*continued*)

Table 1. Continued

Taxon[a]	Host/Substrate[a]	Location	Voucher	16S accession	*rbc*LX accession	*Trn*L accession	Dataset
Nostoc sp.	***Blasia pusilla* 2 (P)**	Germany	D. Mollenhauer 1:1-115[b]	DQ185211	DQ185268	DQ185153	2
Nostoc sp.	***Blasia pusilla* 3 (P)**	Germany	D. Mollenhauer 1:1-125[b]	DQ185212	DQ185269	DQ185154	2
Nostoc sp.	***Cycas circinalis* (P)**	Brazil	M. C. Margheri SAG 29.90[bc]	DQ185250	DQ185309	DQ185193	2
Nostoc sp.	***Encephalartos natalensis* (P)**	Italy	D. Mollenhauer 1:1-108[b]	DQ185210	DQ185267	DQ185152	2
Nostoc punctiforme	***Macrozamia* sp. (P)**	Australia	R. Rippka ATCC 29133[d] (PCC 73102[e])	02000002[f]	02000040[f]	02000083[f]	1, 2
Nostoc sp.	***Stangeria paradoxa* (P)**	England	S. L. Trebon SAG 36.92[bc]	DQ185251	DQ185310	DQ185194	2
Nostoc punctiforme	***Geosiphon pyriforme* 1 (O)**	Germany	W. Koch SAG 69.79[bc]	DQ185257	DQ185316	DQ185200	2
Nostoc sp.	***Geosiphon pyriforme* 2 (O)**	Germany	D. Mollenhauer 1:1-088[b]	DQ185208	DQ185265	DQ185150	2
Nostoc punctiforme	***Gunnera manicata* (P)**	Germany	W. Koch SAG 68.79[bc]	DQ185256	DQ185315	DQ185199	2
***Nostoc commune* (F)**	Soil	USA	H. O'Brien 02011101 (DUKE)	DQ185223	DQ185280	DQ185165	1, 2
***Nostoc* sp. (F)**	Unknown	Unknown	Iowa St. Univ. PCC 7120[e]	003272[g]	003272[g]	003272[g]	1, 2
***Nostoc muscorum* (F)**	Soil	France	H. Jakob SAG 57.79[b]	DQ185254	DQ185313	DQ185197	1, 2
***Nostoc punctiforme* (F)**	Soil	France	M. Lefèvre SAG 71.79[b]	DQ185258	DQ185317	DQ185201	1, 2
***Nostoc* sp. (F)**	Soil	Indonesia	A. Watanabe PCC 6720[eh]	DQ185240	DQ185297	DQ185182	1, 2
***Nostoc* sp. (F)**	Soil	Senegal	P. A. Roger PCC 7423[eh]	DQ185242	DQ185301	DQ185185	1, 2
***Anabaena cf.* cylindrica (F)**	Fresh water	France	PMC 9705[i]	AJ293119[j]	AJ293165[j]	NA	1
***Anabaena variabilis* (F)**	Fresh water	USA	R. Tischer ATCC 29413[d] (PCC 7937[e])	01000001[f]	01000006[f]	01000021[f]	1, 2
***Anabaena macrospora* (F)**	Fresh water	France	PMC 9301[i]	AJ293115[j]	AJ293161[j]	NA	1
***Aphanizomenon flos-aquae* (F)**	Fresh water	Holland	PCC 7905[e]	AJ133154[k]	AJ293153[j]	NA	1
***Aphanizomenon flos-aquae* (F)**	Fresh water	France	PMC 9706[i]	AJ293129[j]	AJ293150[j]	NA	1
***Aphanizomenon flos-aquae* (F)**	Fresh water	France	PMC 9707[i]	AJ293130[j]	AJ293151[j]	NA	1
***Cylindrospermum stagnale* (F)**	Soil	Sweden	A. Neilson PCC 7417[eh]	AJ133163[k]	DQ185300	U83250[l]	1, 2
***Fischerella musicola* (F)**	Hot Spring	New Zealand	W. Koch PCC 7414[eh]	AB039003[m]	DQ185299	DQ185184	1, 2
***Nodularia spumigena* (F)**	Soil	Canada	R. N. Nordin PCC 73104[en]	DQ185241	DQ185298	DQ185183	1, 2
***Nodularia* sp. (F)**	Hot Spring	France	R. Pourriot PCC 7804[eo]	DQ185243	DQ185302	DQ185186	1, 2
***Nodularia harveyana* (F)**	Baltic Sea	Germany	M. Hübel 1983/300[o]	DQ185231	DQ185288	DQ185173	1, 2

[a]Name in bold indicates name used in text and figures. Letters refer to lifestyle (L–lichen photobiont, P–plant symbiont, O–other symbiont, F–free-living). [b]Strain provided by T. Friedl, Universität Göttingen, Germany. [c]Sammlung von Algenkulturen, Universität Göttingen, Germany. [d]American Type Culture Collection, USA.[e]Pasteur Culture Collection, Institut Pasteur, France. [f]All sequences obtained from the Department of Energy Joint Genome Institute (USA) shotgun genome sequence (Accession numbers begin with AAAY and AAEA for *N. punctiforme* PCC 73102 and *A. variabilis* ATCC 29413 respectively). [g]All sequences obtained from Kazusa DNA Research Institute (Japan) genome sequence (Accession numbers begin with NC_). [h]Genomic DNA provided by K. Pryer, Duke University, USA. [i]National Museum of Natural History, France. [j]Sequence obtained from Gugger *et al.* (2002). [k]Sequence obtained from Lyra *et al.* (2001). [l]Sequence obtained from Paquin *et al.* (1997). [m]Sequence obtained from Ishida *et al.* (2001). [n]Culture provided by John Waterbury, Woods Hole Oceanographic Institute, USA. [o]Genomic DNA from cultures provided by Jaana Lehtimäki, University of Helsinki, Finland.

for sequencing. We added photobiont sequences for five to seven specimens each of *Peltigera canina*, *P. didactyla*, *P. membranacea*, and *P. rufescens*. All four of these species are members of section *Peltigera sensu* Miadlikowska & Lutzoni (2000). We also included six other *Peltigera* photobionts, ten photobionts from seven other cyanolichen genera, ten plant symbionts, and two *Geosiphon pyriforme* symbionts (62 strains in total). Lichen specimens were collected approximately equally from North America and Europe, with a smaller portion from other parts of the world. *Peltigera canina* included specimens from North America and Europe and *P. membranacea* included specimens from North America, Europe, and Asia, while all specimens of *P. rufescens* and *P. didactyla* were from Europe.

Data collection

DNA was extracted both from cultures and directly from lichen specimens using the PUREGENE Ultrapure DNA extraction kit (Gentra Systems, Minneapolis, MN, USA). The 16S rDNA, *trn*L (a group I intron found in a conserved position of $tRNA^{Leu}$(UAA) gene across the cyanobacteria, including plant chloroplasts; Kuhsel *et al*., 1990) and *rbc*LX region (which includes the last 82 amino acids of the RUBISCO large subunit (*rbc*L), a putative chaperone gene (*rbc*X) and two intergenic spacers; Li & Tabita, 1997) were amplified using published primers (Wilmotte *et al*., 1993; Nübel *et al*., 1997; Paulsrud & Lindblad, 1998; Rudi *et al*., 1998; Turner *et al*., 1999). Each 25-µl PCR reaction consisted of: 25 µg BSA, 0.625 U *Taq* DNA polymerase (Abgene, Rochester, NY, USA), 1.5 mM $MgCl_2$, dNTPs (0.2 mM each), primers (0.5 µM each) and PCR buffer. Amplification of *rbc*LX followed Rudi *et al*. (1998), while the following thermal cycler profile was employed for *trn*L and 16S: an initial denaturation of 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, 52°C for 45 s, and 72°C for 3 min, with a final extension of 72°C for 10 min. Heterogeneous PCR products were cloned using the Topo-TA 5-minute PCR cloning kit (Invitrogen, Carlsbad, CA, USA) and cyanobacterial sequences were identified using BLAST (Altschul *et al*., 1997). PCR products were purified using Qiaquick PCR purification columns (Qiagen, Valencia, CA, USA) and sequenced using Big Dye chemistry with an ABI 3700 automated sequencer (PE Applied Biosystems, Foster City, CA, USA).

Data analysis

Sequences were assembled using Sequencher 4.2 (Gene Codes, Ann Arbor, MI, USA) and manually aligned using MacClade 4.0 (Maddison & Maddison, 2000). Sequences of 16S and *trn*L were aligned with the aid of the secondary structure information (Gutell, 1993; Paulsrud & Lindblad, 1998; Paulsrud *et al*., 1998; Costa *et al*., 2002). Sequences of *rbc*L and *rbc*X were aligned with reference to their amino acid translations. Ambiguously aligned regions were excluded from phylogenetic analyses. Gaps were treated as missing data. Parts of the *rbc*L-*rbc*X spacer (71 characters), *rbc*X–*rbc*S spacer (38 characters), and the 3′ end of the *rbc*X coding region (27 characters) could be aligned only for symbiotic strains and four of the free-living *Nostoc* strains. These characters were excluded for analysis of dataset 1. Dataset 2 was analyzed both with these sites excluded and with them included but with data from non-*Nostoc* taxa recoded as missing data. For *trn*L, the P6b stem-loop was excluded from the analyses. Class I (TDNGATT heptanucleotide repeats) and class II (NNTGAGT heptanucleotide repeats) loop types were identified by comparison of our alignment to that of Costa *et al*. (2002) and mapped onto the phylogeny. All sequences have been deposited in Genbank (see Table 1) and alignments have been deposited in Treebase (accession number: SN2461).

For each alignment (16S and *rbc*LX for dataset 1, 16S, *rbc*LX, and *trn*L for dataset 2), models of sequence evolution were fitted to the data using Modeltest 3.06 (Posada & Crandall, 1998). For *rbc*LX, the alignment was partitioned by codon position and by gene, with the *rbc*L–*rbc*X and *rbc*X–*rbc*S spacers pooled into a single partition, for a total of seven partitions. Models were selected for each of these seven partitions, as well as for the entire alignment. Separate maximum-likelihood phylogenies were obtained for datasets 1 and 2 for each locus using PAUP* 4.0b10 (Swofford, 2003), with 20 random addition replicates.

Branch support was assessed with maximum-likelihood bootstrapping, weighted maximum-parsimony bootstrapping, and Bayesian posterior probabilities. Maximum-likelihood bootstrapping was conducted on 1,000 replicates using the same model parameters and heuristic search options used for analyzing the original alignments. For parsimony bootstrapping, stepmatrices based on the inverse of the substitution rate matrix obtained from Modeltest were used to weight changes (Flores-Villela *et al*., 2000). To avoid violations of the triangle inequality, the smallest weight applied was 0.5 (Felsenstein, 2004). For the *rbc*LX alignments, separate stepmatrices were applied to each of the seven partitions. One thousand bootstrap replicates were conducted using 1,000 random addition replicates per bootstrap replicate, keeping a maximum of 1,000 most parsimonious trees per random addition replicate with PAUP*. Bayesian analyses were conducted using MrBayes 3.0b4 (Huelsenbeck & Ronquist, 2001). Model structure was based on the models specified by Modeltest. Separate models were applied to each partition of the *rbc*LX gene (seven partitions). Each analysis was run for 5 million generations with a sampling interval of 1,000 generations. Likelihood scores and model parameters were examined graphically to ensure that all runs reached stationarity and to determine the length of the burn-in interval. Posterior probabilities were derived from trees that were sampled after the burn-in interval of 1 million generations. Bootstrap values of 75% or higher and posterior probabilities of 95% or higher were considered significant.

In order to determine if our data could reject monophyly of the genus *Nostoc* we used the Shimodaira–Hasegawa (S–H) test (Shimodaira & Hasegawa, 1999). This involved reanalyzing the 16S

and *rbc*LX alignments for dataset 1 with all *Nostoc* strains (plus *Anabaena variabilis* ATCC 29413, see Results) constrained to be monophyletic using the likelihood settings as for the original analysis. We then tested if the likelihood scores for the constraint trees were significantly worse than the unconstrained phylogenies using the S–H test as implemented in PAUP*. We used resampling-estimated log likelihood bootstrapping with 1,000 replicates to create the null distribution.

Pairwise distances were calculated in PAUP* for each locus using the absolute number of differences and maximum-likelihood distances based on the models obtained from Modeltest for each locus. All characters were included for these calculations, though *trn*L comparisons were made separately for sequences with class I and II heptamer repeats in their P6b stem loops (see Discussion).

Results

Phylogenetic relationships among genera of heterocystous cyanobacteria

When analyzed separately, 16S and *rbc*LX sequences from taxonomic reference strains clustered into six groups (Fig. 1). Four of these groups received significant support by all three methods for both genes, while the other two groups contained a single taxon each. One cluster consisted of the three strains of *Nodularia*, one consisted of the three strains of *Aphanizomenon* plus two strains of *Anabaena,* two contained *Nostoc* strains and *Anabaena variabilis* ATCC 29413 (*Nostoc* I and *Nostoc* II), while the last two consisted of single strains of *Fischerella* and *Cylindrospermum*. One *Nostoc* cluster (*Nostoc* I in Fig. 1) contained three *Nostoc* reference strains (two soil isolates and one of unknown origin), as well as *Anabaena variabilis* from fresh water. The other *Nostoc* cluster (*Nostoc* II in Fig. 1) contained two soil isolates, a strain isolated from the cycad *Macrozamia*, and sequences obtained from a *Nostoc commune* colony. The sister groups of the *Nostoc*-containing clusters were different for the two genes, but there was no support for these relationships and monophyly of *Nostoc* could not be rejected for either gene ($p > 0.05$, S–H test). Within the *Anabaena*/*Aphanizomenon* cluster, the two genera were not distinct from each other, though support was low for relationships within this group.

Although relationships among these six groups differed between ML phylogenies inferred for the two genes, support was low for these relationships. There was well-supported conflict within the *Nostoc* I clade, with *Nostoc* PCC 7423 receiving strong support by all three methods for a different placement between the two genes (Fig. 1). There was also a discrepancy regarding the placement of *Anabaena* cf. *cylindrica* PMC 9705 within the *Anabaena*/*Aphanizomenon* cluster, though support was only moderately significant and only for Bayesian posterior probabilities.

Identity and diversity of symbiotic strains

When a broad sampling of symbiotic strains was added to the 16S alignment, a total of 50 unique genotypes were recovered out of a total of 62 strains (Table 2). All but one symbiotic strain grouped with the *Nostoc* II cluster, which also included two soil isolates and a field-collected colony of *Nostoc commune* (Fig. 2). One photobiont of the lichen *Peltigera didactyla* was placed as sister to *Nodularia* with high support from all three branch-support measures used. A symbiont of *Geosiphon pyriforme* was at the base of the *Nostoc* II clade, but the branch connecting it to the rest of the group was long and there was no parsimony bootstrap support for its inclusion in this cluster. Within the *Nostoc* II clade, support was low for most relationships, especially along the backbone. Most nodes that did receive support grouped taxa with very similar or identical sequences.

Similarly to the 16S results, 49 unique genotypes were recovered in the *rbc*LX tree (Table 2) and all symbiotic strains except the *Peltigera didactyla* 5 photobiont clustered within *Nostoc* II with high support (Fig. 3). Unlike the 16S tree, the strain isolated from *Geosiphon pyriforme* 2 was deeply nested within the *Nostoc* II clade. Rather than being sister to *Nodularia*, the *P. didactyla* 5 photobiont was sister to a clade that included *Nostoc* II and *Cylindrospermum*, though this arrangement was supported only by the Bayesian posterior probabilities. There was more support for nodes within *Nostoc* II than in the 16S tree, but *rbc*LX also failed to resolve backbone relationships within this clade with significant support. Similar results were obtained when the 3′ end of *rbc*X and the *rbc*X–*rbc*S spacer were excluded from the analysis (results not shown).

The *trn*L phylogeny, also resolved two distinct *Nostoc* clades, but there was no support for these groups, and *Cylindrospermum* and two *Nodularia* strains were nested within the *Nostoc* II clade while the other *Nodularia* strain was nested within *Nostoc* I (Fig. 4). The symbiont of *Geosiphon pyriforme* 2 was in an unresolved basal position, but the *Peltigera didactyla* 5 photobiont was nested within the *Nostoc* II clade. Support values were much lower with this locus, with only three tip clades receiving significant support. All *Nostoc* strains, except strains belonging to *Nostoc* I (PCC 7120, PCC 6720, PCC 7423) and the photobiont of *P. didactyla* 5, had P6b stem-loop sequences that

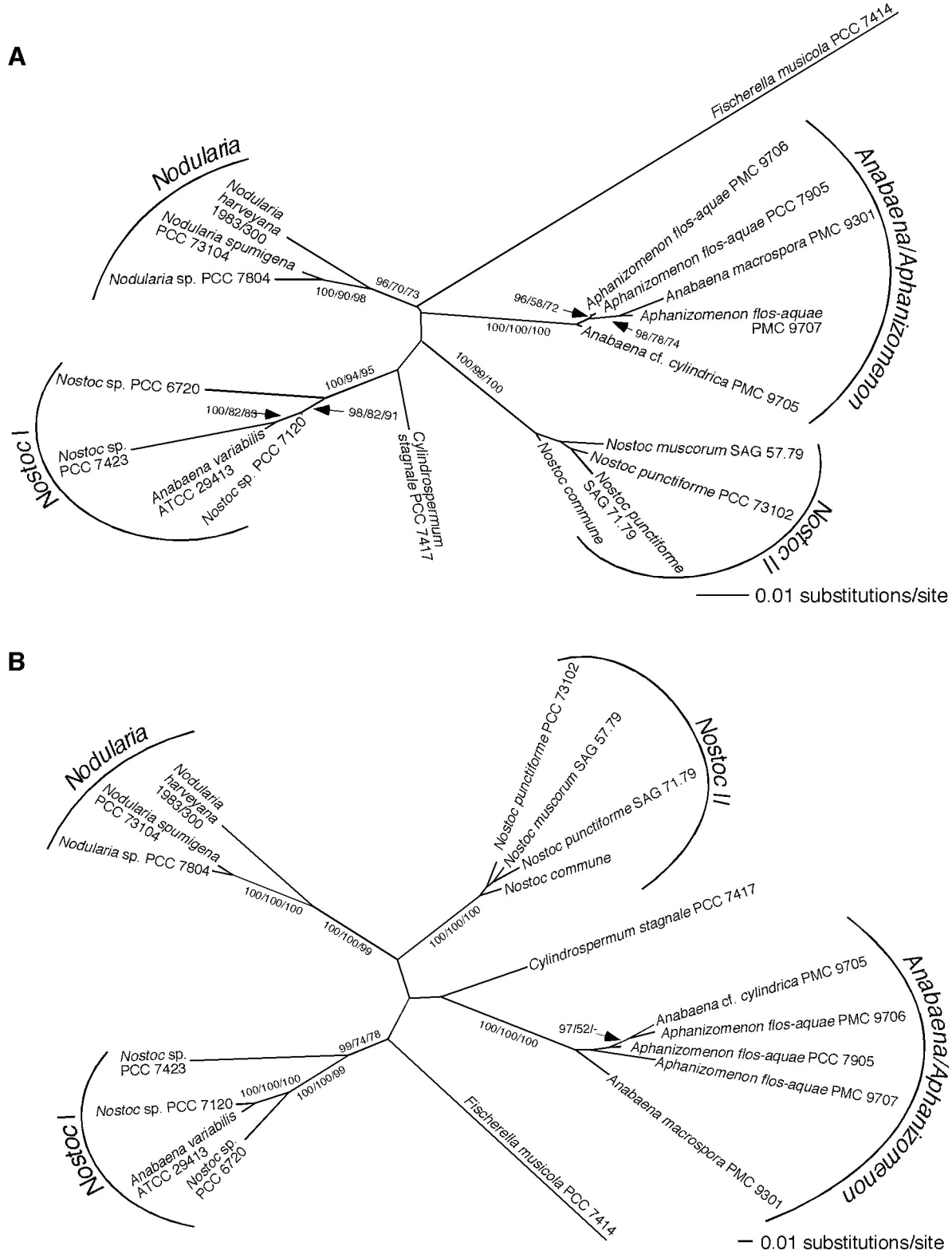

Fig. 1. Phylogenetic relationships among heterocystous cyanobacteria (Nostocales). Support values are indicated for nodes that received significant support from at least one method (Bayesian posterior probabilities ≥95%, maximum-likelihood bootstrap values ≥75%, and parsimony bootstrap values ≥75%; pp/ML-BS/MP-BS). *A* – Unrooted phylogram based on maximum-likelihood analyses of 16S rDNA sequences from reference strains (−lnL = 3940.06, topology obtained on 19 out of 20 random addition replicates). *B* – Unrooted phylogram based on maximum-likelihood analyses of *rbc*LX sequences from reference strains (−lnL = 4299.54, topology obtained on 9 out of 20 random addition replicates).

could be classified into one of two classes. Taxa that clustered together tended to have the same loop type, but clusters of sequences with the two types were distributed across the tree. When all characters were considered, *trn*L resolved nearly as many unique genotypes as the other loci (46 versus 49 and 50, Table 2), but much of this variation was in regions of the intron that had to be excluded

Table 2. Properties of loci used for phylogenetic analyses of dataset 2

Locus	Total length	No. excluded positions	No. variable included characters	No. unique genotypes[a]
16S	1,361–1,421	53	182	50
*rbc*LX	627–1,010	446	331	49
*trn*L	189–338	186	38	46

[a]Number of different sequence types recovered among the 62 strains sequenced for this study.

from phylogenetic analyses, resulting in several large unresolved clusters of taxa in the *trn*L phylogeny (Fig. 4).

In addition to the placement of the symbiont of *G. pyriforme* 2, there were two other cases of significantly supported conflicts between the 16S and *rbc*LX datasets. One involved the placement of *Nostoc* sp. PCC 7423 in the *Nostoc* I clade that was also observed in dataset 1. The other involved the relationship between the photobionts of *P. rufescens* 1 and *P. rufescens* 2, which had identical sequences in both 16S (Fig. 2) and *trn*L (Fig. 4), but had *rbc*LX sequences that differed by 20 substitutions (Fig. 5H) and that came out in different parts of the *rbc*LX tree, though their position was supported only by posterior probability values (Fig. 3). Other examples of strains that were identical at one locus but divergent at another included strains from *P. degenii* and *P. horizontalis* which had identical *trn*L sequences but differed at 26 positions in the 16S and by 36 positions in the *rbc*LX (Fig. 5E), and two cases where strains had identical 16S sequences and similar *rbc*LX sequences but had different *trn*L P6b classes (strains from *G. pyriforme* 1 versus *Macrozamia* sp., Fig. 5B; *Blasia pusila* 3 and *P. didactyla* 3 versus *Gunnera manicata*; Fig. 5F). In 11 pairwise comparisons, taxa with identical *trn*L sequences differed at both of the other loci while there were five and three comparisons, respectively, where only the *rbc*LX or the 16S was identical.

Host specialization

Symbionts of both plants and fungi were distributed throughout the *Nostoc* II clade for all three loci (Figs 2–4). Furthermore, each focal lichen species had photobionts that were also placed throughout the *Nostoc* II clade and, in the case of *P. didactyla*, sometimes outside of it (Figs 2, 3). Likewise, many lineages of closely related *Nostoc* strains were associated with many different hosts. There were a number of cases where *Nostoc* strains with identical sequences at all three loci were associated with different lichen species (*P. membranacea* and *P. horizontalis*, Fig. 5G; *P. canina*, *P. rufescens*, and *P. didactyla*, Fig. 5H) or with a lichen and a plant (*Collema crispum* and *Encephalartos natalensis*, Fig. 5D; *P. didactyla* and *Blasia pusilla*, Fig. 5F). The free-living strains belonging to *Nostoc* II were also distributed throughout the clade (Figs 2–4). Photobionts of the lichen genera *Nephroma*, *Pannaria*, and *Sticta* and the *Lobaria amplissima* photobiont clustered together with high support in both 16S and *rbc*LX analyses. They also formed a clade in the *trn*L tree, but without support.

Geographic structure

Within the *Nostoc* II clade, strains from specimens collected in North America and Europe were distributed throughout the tree, indicating that intercontinental dispersal has been frequent since the origin of the group (Figs 2–4). Symbiotic strains from South America and Oceania were also found in *Nostoc* II while free-living strains from Oceania and Africa were placed in *Nostoc* I. There were several cases of strains from different continents having identical genotypes at one or two loci, but strains sharing identical genotypes at all loci were always from the same continent (Fig. 5), suggesting that there is some population differentiation at intercontinental scales.

Discussion

The major phylogenetic relationships obtained in this study are in broad agreement with other phylogenies of heterocystous cyanobacteria. The intercalation of *Anabanea* and *Aphanizomenon* strains was also observed by Gugger *et al.* (2002) based on analyses of 16S and *rbc*LX sequences for a larger strain selection for these genera, although they did not sample the *Anabaena variabilis* strain that clustered in the *Nostoc* I clade in our analyses. Svenning *et al.* (2005) also reported that *Aphanizomenon* was nested within *Anabaena* based on analyses of 16S data. They also found *Nostoc*, *Nodularia*, and *Calothrix* strains clustered with *Anabaena*, though without support. Our study supports the finding of Svenning *et al.* (2005) that *Anabaena variabilis* clustered with *Nostoc* PCC 7120, although they found that this clade formed a sister group to two other *Nostoc* clades with high posterior probability. *Nostoc* clades I and II never shared a most recent common ancestor in our analyses, but we were unable to resolve the sister group placement of either *Nostoc* clade with significant support and we were unable to reject monophyly for *Nostoc*. A more complete sampling of other heterocystous cyanobacterial taxa and combined

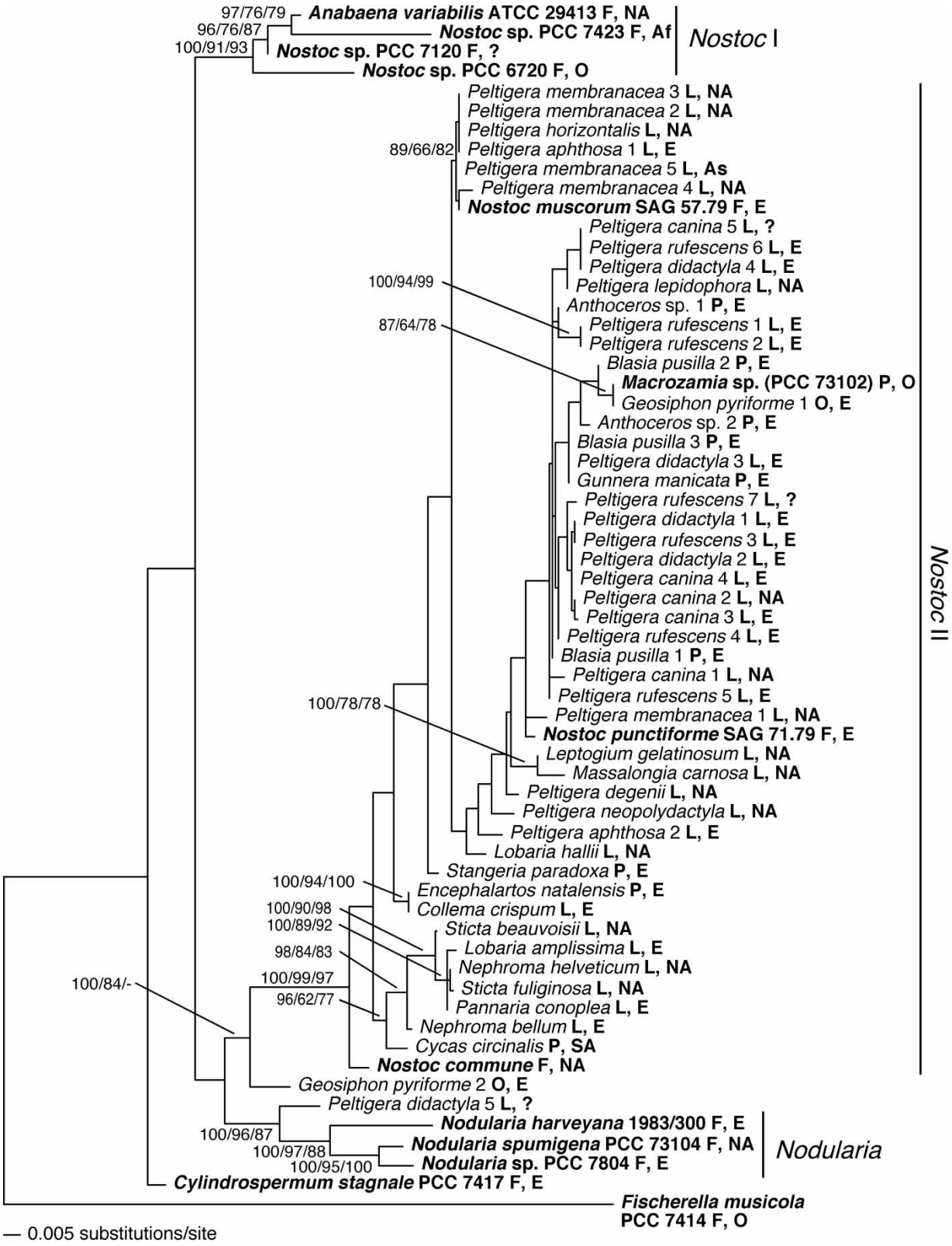

Fig. 2. Phylogenetic relationships among symbiotic and free-living cyanobacterial strains based on 16S rDNA. Maximum-likelihood phylogram rooted with *Fischerella musicola* PCC 7414 ($-\ln L = 5208.55$, topology obtained on 1 out of 20 random addition replicates). Names indicate host species for symbiotic strains and cyanobacterial names for free-living strains included as references. See Fig. 1 legend for explanation of node support values. Letters following name indicate lifestyle (L – lichen, P – plant, O – other symbiosis, F – free-living), followed by continent of origin (E – Europe, NA – North America, SA – South America, O – Oceania, As – Asia, Af – Africa, ? – unknown). Names in bold refer to strains included in both datasets 1 and 2.

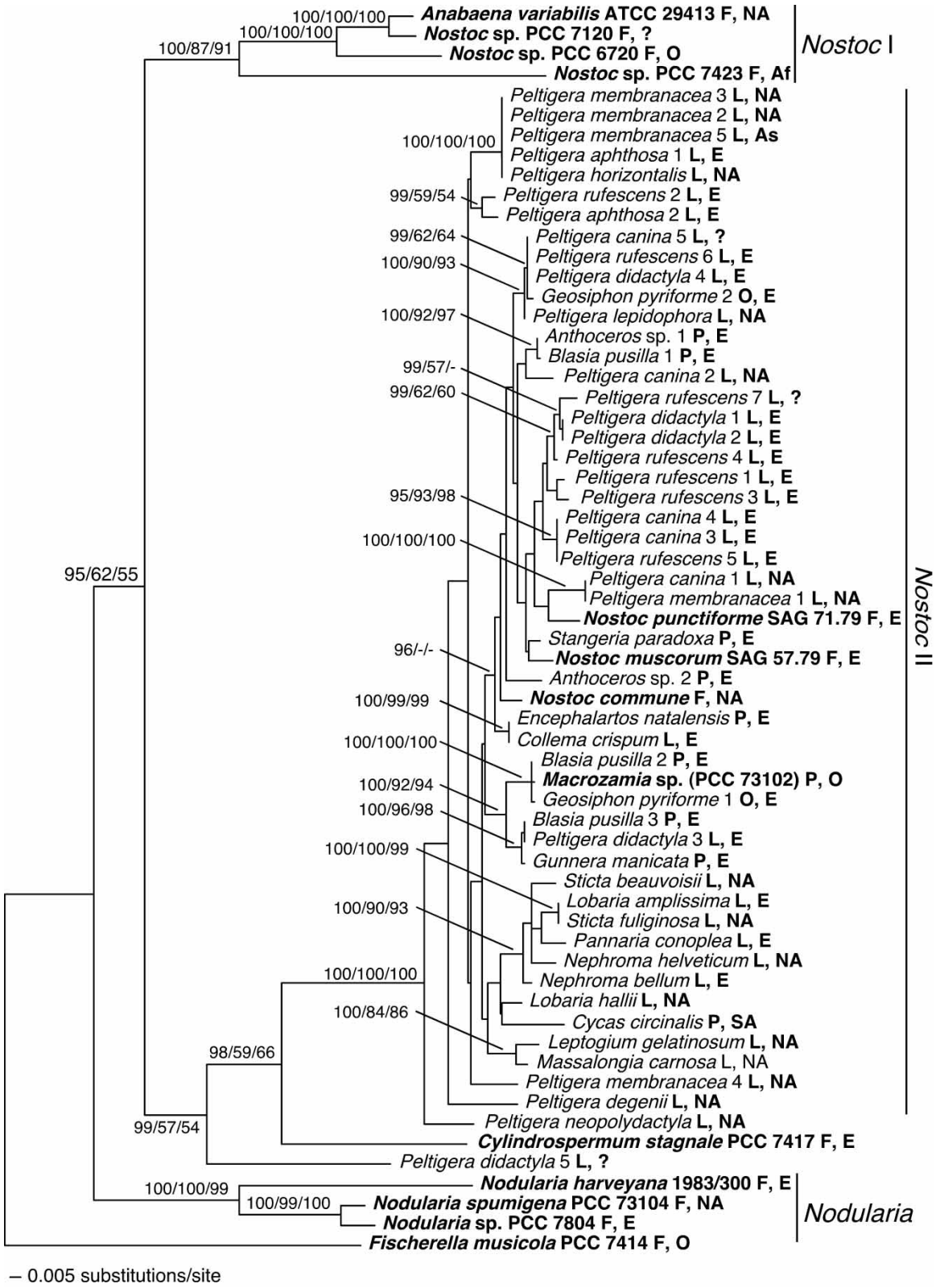

Fig. 3. Phylogenetic relationships among symbiotic and free-living cyanobacterial strains based on *rbc*LX. Maximum-likelihood phylogram rooted with *Fischerella musicola* PCC 7414 (–lnL = 5713.87, topology obtained on 5 out of 20 random addition replicates). See legend for Fig. 1 for explanation of node support values and for Fig. 2 for explanation of taxon names.

analyses of multiple loci will be needed to resolve confidently deep phylogenetic relationships within this group.

With the exception of one *Peltigera didactyla* photobiont, all symbiont strains were restricted to *Nostoc* II. Svenning *et al.* (2005) found that one bryophyte symbiont, two *Peltigera* photobionts, and five *Gunnera* symbionts, along with *Nostoc punctiforme* PCC73102, which was isolated from a cycad, all clustered together along with a number

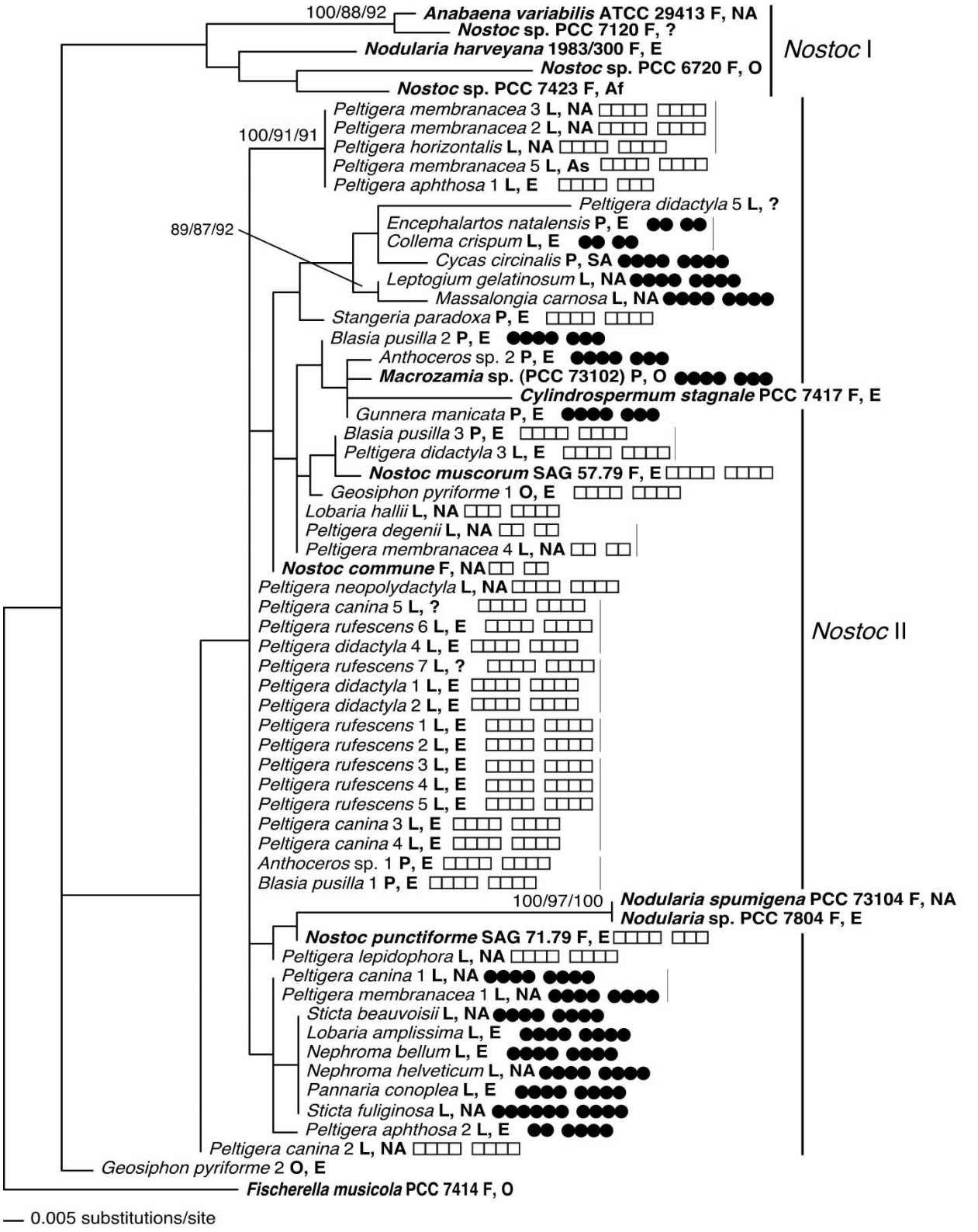

Fig. 4. Phylogenetic relationships among symbiotic and free-living cyanobacterial strains based on tRNA (Leu) intron. Maximum-likelihood phylogram rooted with *Fischerella musicola* PCC 7414 ($-\ln L = 709.61$, topology obtained on 9 out of 20 random addition replicates). See legend for Fig. 1 for explanation of node support values and for Fig. 2 for explanation of taxon names. Vertical lines after taxon names indicate strains with identical *trn*L intron sequences (other taxa on zero-length branches differ at positions that were excluded from phylogenetic analyses). Circles and squares indicate the sequence type of the P6b stem-loop (open square = class I − TDNGATT, filled circle = class II − NNTGAGT) and the number of heptamers on each side of the loop.

A

	Lo. amp	S. ful
Lo. amplissima	-	6
L, E	-	0
	-	+
S. fuliginosa	0.005	-
L, NA	0.000	-
	+	-

B

	Macro	Ge. Pyr1
Macrozamia sp	-	0
P, O	-	2
	-	x
Ge. Pyriforme 1	0.000	-
O, E	0.002	-
	x	-

C

	P. can1	P. mem1
P. canina 1	-	11
L, NA	-	0
	-	0
P. membranacea 1	0.009	-
L, NA	0.000	-
	0.000	-

D

	C. crisp	E. nat
C. crispum	-	0
L, E	-	0
	-	0
E. natalensis	0.000	-
P, E	0.000	-
	0.000	-

E

	P. deg	P. mem4
P. degenii	-	26
L, NA	-	36
	-	0
P. membranacea 4	0.022	-
L, NA	0.055	-
	0.000	-

F

	B. pus 3	P. did 3	Gu. man
B. pussila 3	-	0	0
P, E	-	0	2
	-	0	x
P. didactyla 3	0.000	-	0
L, E	0.000	-	2
	0.000	-	x
Gu. manicata	0.000	0.000	-
P, E	0.003	0.003	-
	x	x	-

G

	P. hor	P. mem2	P. mem3	P. aph1	P. mem5
P. horiontalis	-	0	0	0	1
L, NA	-	0	0	0	0
	-	0	0	+	1
P. membranacea 2	0.000	-	0	0	1
L, NA	0.000	-	0	0	0
	0.000	-	0	+	1
P. membranacea 3	0.000	0.000	-	0	1
L, NA	0.000	0.000	-	0	0
	0.000	0.000	-	+	1
P. aphthosa 1	0.000	0.000	0.000	-	1
L, E	0.000	0.000	0.000	-	0
	+	+	+	-	1
P. membranacea 5	0.001	0.001	0.001	0.001	-
L, As	0.000	0.000	0.000	0.000	-
	0.004	0.004	0.004	+	-

H

	P.can5	P.ruf6	P.did4	Anth1	B.pus1	P.ruf7	P.did1	P.did2	P.ruf1	P.ruf2	P.ruf3	P.ruf4	P.can4	P.can3	P.ruf5
P. canina 5	-	0	0	15	12	3	14	16	20	19	17	11	16	14	13
L, ?	-	0	0	13	14	1	20	20	13	18	17	18	15	15	15
	-	0	0	4	4	3	3	3	1	1	4	4	4	4	4
P. rufescens 6	0.000	-	0	15	12	3	14	16	20	19	17	11	16	14	13
L, E	0.000	-	0	13	14	1	20	20	13	18	17	18	15	15	15
	0.000	-	0	4	4	3	3	3	1	1	4	4	4	4	4
P. didactyla 4	0.000	0.000	-	15	12	3	14	16	20	19	17	11	16	14	13
L, E	0.000	0.000	-	13	14	1	20	20	13	18	17	18	15	15	15
	0.000	0.000	-	4	4	3	3	3	1	1	4	4	4	4	4
Anthoceros sp. 1	0.012	0.012	0.012	-	3	13	11	11	12	12	12	7	11	9	4
P, E	0.017	0.017	0.017	-	1	23	20	20	19	19	24	18	18	18	18
	0.016	0.016	0.016	-	0	3	3	3	3	3	4	4	4	4	4
B. pusilla 1	0.009	0.009	0.009	0.002	-	11	8	8	16	15	9	4	9	6	1
P, E	0.019	0.019	0.019	0.001	-	24	21	21	20	20	25	19	19	19	19
	0.016	0.016	0.016	0.000	-	3	3	3	3	3	4	4	4	4	4
P. rufescens 7	0.015	0.015	0.015	0.010	0.009	-	7	7	14	15	8	7	7	9	2
L, E	0.021	0.021	0.021	0.030	0.032	-	7	7	12	27	13	9	15	15	15
	0.012	0.012	0.012	0.012	0.012	-	0	0	2	2	1	1	1	1	1
P. didactyla 1	0.011	0.011	0.011	0.009	0.006	0.005	-	1	18	17	0	4	1	2	9
L, E	0.027	0.027	0.027	0.026	0.027	0.009	-	0	11	22	14	4	10	10	10
	0.012	0.012	0.012	0.012	0.012	0.000	-	0	2	2	1	1	1	1	1
P. didactyla 2	0.013	0.013	0.013	0.008	0.006	0.005	0.001	-	18	17	0	4	0	2	9
L, E	0.027	0.027	0.027	0.026	0.027	0.009	0.000	-	11	22	14	4	10	10	10
	0.012	0.012	0.012	0.012	0.012	0.000	0.000	-	2	2	1	1	1	1	1
P. rufescens 1	0.016	0.016	0.016	0.010	0.012	0.012	0.013	0.013	-	0	18	13	17	17	16
L, E	0.017	0.017	0.017	0.025	0.026	0.015	0.014	0.014	-	20	7	9	13	13	13
	0.004	0.004	0.004	0.012	0.012	0.008	0.008	0.008	-	0	3	3	3	3	3
P. rufescens 2	0.016	0.016	0.016	0.010	0.012	0.012	0.013	0.013	0.000	-	18	13	17	17	16
L, E	0.024	0.024	0.024	0.025	0.026	0.036	0.029	0.029	0.026	-	28	20	22	22	22
	0.004	0.004	0.004	0.012	0.012	0.008	0.008	0.008	0.000	-	3	3	3	3	3
P. rufescens 3	0.014	0.014	0.014	0.009	0.007	0.006	0.000	0.000	0.014	0.014	-	4	2	3	10
L, E	0.023	0.023	0.023	0.031	0.033	0.017	0.018	0.018	0.009	0.037	-	12	12	12	12
	0.004	0.004	0.004	0.016	0.016	0.004	0.004	0.004	0.011	0.011	-	0	0	0	0
P. rufescens 4	0.009	0.009	0.009	0.005	0.003	0.005	0.003	0.003	0.010	0.010	0.003	-	4	6	5
L, E	0.024	0.024	0.024	0.023	0.025	0.011	0.005	0.005	0.011	0.026	0.014	-	10	10	10
	0.016	0.016	0.016	0.016	0.016	0.004	0.004	0.004	0.011	0.011	0.000	-	0	0	0
P. canina 4	0.013	0.013	0.013	0.008	0.007	0.005	0.000	0.001	0.013	0.013	0.001	0.003	-	2	10
L, E	0.020	0.020	0.020	0.023	0.024	0.019	0.013	0.013	0.016	0.029	0.014	0.012	-	0	0
	0.016	0.016	0.016	0.016	0.016	0.004	0.004	0.004	0.011	0.011	0.000	0.000	-	0	0
P. canina 3	0.011	0.011	0.011	0.007	0.004	0.007	0.001	0.001	0.013	0.013	0.002	0.004	0.001	-	7
L, E	0.020	0.020	0.020	0.023	0.024	0.019	0.013	0.013	0.016	0.029	0.014	0.012	0.000	-	0
	0.016	0.016	0.016	0.016	0.016	0.004	0.004	0.004	0.011	0.011	0.000	0.000	0.000	-	0
P. rufescens 5	0.010	0.010	0.010	0.003	0.001	0.009	0.007	0.007	0.012	0.012	0.007	0.004	0.008	0.005	-
L, E	0.020	0.020	0.020	0.023	0.024	0.019	0.013	0.013	0.016	0.029	0.014	0.012	0.000	0.000	-
	0.016	0.016	0.016	0.016	0.016	0.004	0.004	0.004	0.011	0.011	0.000	0.000	0.000	0.000	-

Fig. 5. Pairwise distances for comparisons involving *Nostoc* II strains with identical sequences at one or more loci. Number of nucleotide substitutions above the diagonal and maximum-likelihood distances below. Within each cell, first row = 16S, second row = *rbc*LX, and third row = *trn*L. +, *trn*L sequences that differ in the number of heptanucleotide repeats in the P6b stem loop; X, *trn*L sequences that differ in heptamer repeat class. Grey boxes highlight taxa that have identical sequences at all three loci. All taxon names refer to the host species. See Fig. 2 legend for explanation of abbreviations. Letters above tables (*A–H*) designate different groups, with members that share identical genotypes with at least one other group member at one or more loci.

of free-living *Nostoc* isolates. They found that a sixth *Gunnera* symbiont and an isolate from the fern *Azolla* clustered with *Anabaena*, *Aphanizomenon*, and *Nodularia*. The symbiont of *Azolla* has usually been identified as *Anabaena azollae* (Peters & Meeks, 1989), so its grouping with *Anabaena* is to be expected. The findings of a lichen photobiont (*P. didactyla* 5) falling outside the *Nostoc* II clade in our study and of a *Gunnera* symbiont which does not cluster with other symbiotic strains by Svenning *et al.* (2005) suggest that both plants and fungi may be capable of associating with unusual partners under certain ecological conditions. These results, which are reminiscent of the isolation of *Calothrix* in a survey of bryophyte symbionts (West & Adams, 1997), deserve further study.

Our finding that each *Peltigera* species examined in detail associated with photobiont strains dispersed throughout the *Nostoc* II clade indicates that photobiont sharing is prevalent among lichen species. This result is further supported by the observation that *Nostoc* strains from each species are often more similar to strains from unrelated lichens or to plant symbionts than to strains associated with conspecific specimens. This is true both for sexual species with horizontal photobiont transmission (*P. canina*, *P. membranacea*, *P. rufescens*) and for species that produce specialized asexually derived codispersal propagules (*P. didactyla*). Sharing of photobiont genotypes among different host species has also been reported previously among members of the Nephromataceae (Rikkinen *et al.*, 2002; Lohtander *et al.*, 2003; Wirtz *et al.*, 2003), as well as within bryophytes (Costa *et al.*, 2001) and cycads (Costa *et al.*, 1999), but ours is the first study to include large enough sample sizes of both plant symbionts and lichen photobionts to infer that symbiont sharing among unrelated hosts is common. These results contradict earlier studies, based on smaller datasets, which suggested that species-level host specialization was prevalent in cyanolichens and that host species was a better predictor of symbiont genotype than geography (Paulsrud *et al.*, 1998, 2000). Wirtz *et al.* (2003) suggested that the lack of host specialization observed in Antarctic cyanolichens might be due to selection pressure for generalism in harsh environments, but our data indicate that low host specialization can be found also in temperate cyanolichens. Given the differences in photobiont specificity documented even among closely related species of green algal lichens (Yahr *et al.*, 2004), it would be advisable to conduct careful studies on a case-by-case basis for a broad sampling of lichen species before drawing general conclusions about host specialization.

A possible explanation for conflicting reports about the degree of cyanobiont specialization in lichens is that earlier studies were often based on comparisons of small numbers of distantly related host taxa. Photobionts of most members of the lichen families Nephromataceae and Lobariaceae appear to be specialized to a basal lineage within the *Nostoc* II clade (Rikkinen *et al.*, 2002; Lohtander *et al.*, 2003). This pattern was also observed with our data. Rikkinen *et al.* (2002) attributed this photobiont specialization to ecological differences in the hosts (epiphytic versus terricolous), but the observed pattern could also be due to genetically determined host specialization that evolved after the Nephromataceae and Lobariaceae diverged from the Peltigeraceae. These possibilities could be distinguished by sampling terricolous members of the Nephromataceae and Lobariaceae and epiphytic members of the Peltigeraceae, or by sampling more members of other families of cyanolichens that occur in both terricolous and epiphytic habitats, such as the Collemataceae.

There are numerous examples of apparent topological incongruence between the phylogenies inferred from the different loci included in this analysis. Such incongruence is often interpreted as evidence that the different genes have different evolutionary histories, indicating inter-locus recombination, or horizontal gene transfer, among diverged strains (Syvanen, 1994; Rudi *et al.*, 1998). Because the stochastic nature of the evolutionary process can cause phylogenetic methods to indicate incorrect relationships (Koonin *et al.*, 2001), however, conflicting topologies that do not receive significant branch support are likely to be the result of phylogenetic artifacts rather than horizontal gene transfer. Even when conflicts do receive significant support, caution should be taken in inferring horizontal transfer unless multiple independent support measures are significant. In particular, Bayesian posterior probabilities can be misleading in cases where internodes are short because current implementations of Bayesian phylogenetic inference are unable to accommodate polytomies in relationships among taxa (Alfaro *et al.*, 2001; Reeb *et al.*, 2004; Lewis *et al.*, 2005). We did have some examples where incongruent relationships received significant support from more than one of the support measures used, which suggests that some exchange of genetic material between different *Nostoc* strains is occurring in natural populations, but most topological conflicts involved unsupported nodes for one or both loci.

An alternative approach to identifying inter-locus recombination is to compare the pairwise distances for each locus. There are several cases

where taxa in our dataset have identical sequences at one locus but are highly diverged at another (Fig. 5). Since substitution rates for the three loci are roughly comparable (compare the scale bars in Figs 2–4), it is extremely unlikely that one locus would accumulate a large number of substitutions while another locus remained unchanged since the taxa diverged, indicating that the loci must have different evolutionary histories. This case is particularly strong in examples where taxa have identical 16S or *rbc*LX sequences but different *trn*L intron types. In the absence of recombination, it would require coordinated changes at the same position of each heptamer, converging on the same intron type in different lineages to explain our data. In these examples, however, we are unable to distinguish inter-locus recombination between *trn*L and the other loci from intra-locus recombination within *trn*L between the P6b stem-loop and the rest of the intron. It has been shown that these two stem-loop types have a punctate distribution on phylogenies inferred from the remainder of the *trn*L locus (Oksanen *et al*., 2004). The two stem-loop types are also spread across our *trn*L phylogeny (Fig. 4), but the lack of branch support means we cannot rule out the monophyly of the stem-loop types.

While our data indicate that genetic material is being exchanged among strains within both *Nostoc* I and *Nostoc* II, the same major clusters were recovered in analyses of both 16S and *rbc*LX, suggesting that horizontal gene transfer between members of different clades may be uncommon. Recombination has also been observed in populations of *Nodularia* from the Baltic Sea (Barker *et al*., 2000). It has been suggested that the observation of genealogical concordance among groups but not within may provide an operational proxy for the application of the biological species concept (Dykhuizen & Green, 1991; Dettman *et al*., 2003). According to this criterion, *Nostoc* clade II would comprise a single, globally distributed species. This view is also supported by the absence of major discontinuities in the genetic distances between clusters within the *Nostoc* II clade in the 16S and *rbc*LX phylogenies (Acinas *et al*., 2004). If *Nostoc* II does represent a single species, it would mean that it is capable of forming symbiotic associations with a wide range of plant and fungal partners. It would also imply that all these plant and fungal hosts are specialized on a single symbiont species. High host diversity and low symbiont diversity would be consistent with the prediction of Law & Lewis (1983) that selective forces within a mutualistic environment favor reduced diversity. Alternatively, this difference might reflect divergent selective forces in the non-symbiotic phase of their life cycles due to the ability of *Nostoc* to occur free-living in nature. However, *Nostoc* II contains high levels of genetic diversity: for example, the level of *rbc*L sequence divergence between strains within this clade (up to 6%) is almost twice as high as has been found across the Cycads, which comprise an entire order of land plants (Rai *et al*., 2003). Careful physiological studies will be needed to determine how much of this genetic diversity contributes to functional phenotypic diversity.

While lichen-forming fungi are highly specialized at higher taxonomic scales (i.e. orders of fungi are often restricted to a single photobiont genus), green algal photobiont genera commonly associate with unrelated lineages of fungi (Rambold *et al*., 1998; Piercey-Normore & DePriest, 2001) and sharing of photobiont genotypes among related species is common (Kroken & Taylor, 2000; Helms *et al*., 2001; Yahr *et al*., 2004). Our findings that, with the exception of the Nephromataceae/Lobariaceae photobiont lineage, host specialization is low for the genus *Nostoc* indicate that patterns are similar for cyanolichens and suggest that opportunities for coevolution may be low in these symbioses. The tightly integrated and organized symbiotic tissues found in lichens, cycads, and *Gunnera* are highly evolved structures, but they are primarily created by the host and may have evolved without requiring any reciprocal evolutionary changes in the generalist symbionts. The broad range of *Nostoc* strains compatible with each *Peltigera* species we examined, along with the ubiquitous presence of free-living *Nostoc* in terrestrial habitats, even under xeric conditions, may also help to explain the paradox of how such obligately symbiotic organisms without specialized mechanisms for codispersal of both partners have been able to be ecologically dominant in many habitats and to spread throughout all major land masses (Martínez *et al*., 2003).

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