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Evolution of the tRNA^{Leu} (UAA) Intron and Congruence of Genetic Markers in Lichen-Symbiotic Nostoc

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Abstract

The group I intron interrupting the tRNA^Leu UAA gene (trnL) is present in most cyanobacterial genomes as well as in the plastids of many eukaryotic algae and all green plants. In lichen symbiotic Nostoc, the P6b stem-loop of trnL intron always involves one of two different repeat motifs, either Class I or Class II, both with unresolved evolutionary histories. Here we attempt to resolve the complex evolution of the two different trnL P6b region types. Our analysis indicates that the Class II repeat motif most likely appeared first and that independent and unidirectional shifts to the Class I motif have since taken place repeatedly. In addition, we compare our results with those obtained with other genetic markers and find strong evidence of recombination in the 16S rRNA gene, a marker widely used in phylogenetic studies on Bacteria. The congruence of the different genetic markers is successfully evaluated with the recently published software Saguar, which has not previously been utilized in comparable studies.

Introduction

Cyanobacteria form symbioses with many different types of organisms, and in terrestrial ecosystems Nostoc is the most common genus of symbiotic cyanobacteria. Symbiotic genotypes of Nostoc establish well-defined symbioses with a plethora of lichen-forming fungi [1,2], some thalloid bryophytes [3,4], all cycads [5,6], and species of the angiosperm genus Gunnera [7–9]. Symbiotic Nostoc genotypes, or cyanobionts, can be assigned to two main groups. One clearly defined, monophyletic lineage includes Nostoc cyanobionts that are exclusively shared by a wide variety of lichen-forming Ascomycota that are collectively called the Nephroma guild. The other Nostoc cyanobionts represent a much more heterogeneous assemblage and do not constitute a monophyletic clade. This group includes cyanobionts of various cyanolichens, particularly Peltigera species, cyanobionts of plants, and many non-symbiotic Nostoc genotypes [10–15].
Group 1 introns are RNA enzymes that catalyze their own splicing [16–18]. The cyanobacterial group 1 tRNAleu (UAA) intron (trnL) interrupts the tRNA gene and is generally believed to be of ancient origin [19]. It is present in most cyanobacterial lineages as well as in the plastids of many eukaryotic algae and all green plants [20–22]. The intron is, however, missing from some modern cyanobacteria and its mosaic-like distribution has been explained by one acquisition and several subsequent losses [19].

The self-splicing ability of trnL is dependent on the RNA secondary structure consisting of highly conserved sequence regions [17,18]. In addition, the intron contains more variable regions, like the P6b stem-loop and P9 region in the trnL intron of genus Nostoc [21]. The considerable sequence length variation observed in the Nostoc trnL P6b stem-loop is mainly caused by processes other than random mutations [21,23]. The stem-structure of the stem-loop is always built upon one of two distinct repeat motifs: either the Class I repeat motif TDNGATT or the Class II motif NNTGAGT [21]. The distribution of these two motifs does not correspond precisely with the phylogeny of Nostoc, and a similar dichotomy of the same two repeat motifs has also been identified from Calothrix, another genus of Nostocalean cyanobacteria [24].

Nostoc trnL sequences with a Class I P6b region can be further divided into two groups on the basis of signature characters both in the P6b stem-loop and the more conserved parts of the intron [12,24]. The clade of Nephroma guild lichen cyanobionts all have a Class I repeat motif, AATCTTC in the P6b central loop and a characteristic 71T in the P5b stem-loop. The sequence evolution of this group of Nephroma-type Nostoc cyanobionts is fairly well understood [23]. The lichen cyanobiont clade with Class I P6b regions that fall outside this clade do not share the above-mentioned signature characters [12,24], and are collectively referred to as Collema-type Nostoc cyanobionts.

The phylogenetic classification of prokaryotes is largely based on the phylogeny of the small subunit ribosomal RNA, even though the 16S rRNA gene has been shown to be prone to horizontal gene transfer (discussed for example in [25,26]). The 16S rRNA gene is routinely used in constructing phylogenies also in cyanobacteria [27–30]. As the trnL intron is easy to amplify and shows sufficient variability especially in the P6b region, it is widely utilized for DNA based identification of symbiotic Nostoc genotypes. In addition, the more conserved parts of the trnL intron have been used to construct phylogenies, often alongside the 16S rRNA gene, but also with other markers including the genes encoding the ribulose bisphosphate carboxylase (rbcL), homocitrate synthase (nifV), and RNA polymerase (rpoC), and/or the internal transcribed spacer (ITS) between the 16S and 23S rRNA genes (e.g. [31,32]).

In this study we clarify the evolution of the trnL P6b region in lichen-symbiotic Nostoc. We compare the congruence of the trnL intron, 16S rRNA gene, rbcLX, nifV1, and rpoC2, and evaluate the aptitude of Saguaro, a recently published application for the genome-wide detection of distinct local relationship patterns [33], to detect the evolutionary patterns between and within genetic markers.

**Material and Methods**

**Taxon sampling and molecular markers**

The data was compiled to give an extensive representation of the variation of the 16S rRNA gene and trnL markers in lichen-symbiotic Nostoc. Our new sequence data represent lichen cyanobionts of 52 lichen taxa representing 11 different genera. This data was complemented with data acquired from the NCBI GenBank and the full data set contained 78 lichen symbiotic Nostoc genotypes. Three lichen-symbiotic genotypes of Rhizonema [34,35] were used as out-group in the phylogenetic analyses (S1 Table).
Either fresh or dried lichen specimens were used for DNA extractions, and two genomic regions were sequenced for all specimens: the approximately 1,700 nt 16S rRNA gene and the 270–372 nt trnL intron. DNA isolation, amplification and sequencing of the 16S rRNA gene and trnL intron were performed following Fedrowitz et al. [15,36]. Sequences were edited with Bioedit v7.0.9.0 [37] or PhyDE v1.0 [38] and primer sequences were eliminated. All sequences were submitted to GenBank, and accession numbers are listed in S1 Table together with corresponding voucher information. The collection permits for the fresh material were issued by Special Forest Products Coordinator John Poet, USDA Forest Service, and Medford District Manager Mary Smelcer, U.S. Department of the Interior Bureau of Land Management, for Oregon, and Forest Officer Julie K. Nelson, USDA Forest Service, and Natural Heritage Manager Carol Pehl, California Department of Parks and Recreation, for California localities.

In addition to the 16S rRNA gene–trnL sequence set, we included another data set comprised of four gene regions sequenced from lichen-symbiotic Nostoc [31]: rbcLX consisting of the rbcX and the intergenic spacer between rbcL and rbcX, nifV1, rpoC2, and trnL. All genotypes with data from all four genes in GenBank were included. After removing the P6b region from trnL sequences and the 74–158 nt long intron present in three rbcLX genotypes, all four genes were compiled into a single data set and duplicates removed resulting a data set of 21 unique Nostoc genotypes (S2 Table).

Alignment and phylogenetic analyses

Alignment of the sequence data was performed manually in PhyDE v1.0, based on the criteria laid out by Kelchner [39]. As the P6b region does not always reflect true phylogenetic relationships in broad taxonomic studies [23,24,40,41], this region was omitted from the phylogenetic analyses.

Bayesian analyses were performed with MrBayes v3.2.1 [42] first for the 16S rRNA gene and trnL separately, and then for the combined data set. To allow possible deviating substitution models for the different regions, the data set was divided in a partition of subsets according to the markers. The best fitting nucleotide substitution models were selected by jModelTest using Akaike and Bayesian information criteria [43]. For the 16S rRNA gene the General Time Reversible nucleotide substitution model [44] with Gamma distributed rate variation among sites and proportion of Invariable sites (GTR+Γ+I) was applied. For the trnL the GTR+Γ model was used. The a priori probabilities supplied were those specified in the default settings of the program. Posterior probability distributions of trees were calculated using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) method and the search strategies suggested by Huelsenbeck et al. [45,46]. Four runs with four chains (1 x 10^7 generations each) were run simultaneously, with the temperature parameter set to 0.1 but otherwise with default settings. Chains were sampled every 1,000 generations and calculations of the consensus tree and of the posterior probability of clades were performed based upon the trees sampled after the generation 2,500,000. The convergence of the chains was confirmed with Tracer v1.5 [47].

Maximum likelihood (ML) analyses were performed with Garli v2.0 [48]. For the 16S rRNA gene–trnL data set ML analyses with 500 replicates were run with default settings. Bootstrap (BS) analysis was performed with 10,000 BS replicates. All analyses of the 16S rRNA gene–trnL data set with MrBayes and Garli were performed on Bioportal [49].

For the combined data set of rbcLX, nifV1, rpoC2, and trnL, the ML analyses were performed on CIPRES Science Gateway [50]. The data set was divided in a partition of subsets according to the boundaries of the combined genes. Two times eight search replicates were run using GTR model for all regions. The BS analyses were performed with 100 BS replicates. The
results of the BS analyses were summarized using SumTrees v3.3.1 [51] and all the trees were visualized using TreeGraph2 [52] and edited manually.

**Ancestral character state reconstruction**

The evolutionary history of the Class II and Collema-type Class I trnL P6b regions was reconstructed by determining the posterior probability for their occurrence in the ancestral species. The Markov chain model implemented in BayesTraits v1.0 [53] was used to estimate the posterior probability distributions of ancestral states at selected nodes of the Bayesian 16S rRNA gene–trnL tree. For the analysis the presence of Class II trnL P6b region was set as one state of character and Class I (Collema- and Nephroma-types) as another. A set of 600 best trees found by MrBayes was used and the three outgroup species were pruned from the trees using R v3.0.0 package APE [54,55]. The rate at which parameters get changed (’ratedev’) was tested and set to 2.75 so that the acceptance rate of the proposed changes ranged between 20 and 40%. A uniform distribution with a range of 0–100 was used as prior. Rate coefficients and ancestral character states were sampled every 1,000 generations to ensure independence from successive samplings. The chain was run for 5,050,000 generations. In order to circumvent issues associated with the fact that not all of the trees necessarily contain the internal nodes of interest, reconstructions were performed using a ‘most recent common ancestor’ approach. The approach identifies the most recent common ancestor to a group of species and reconstructs the state at the node for each tree and then combines this information across trees [53].

**Haplotype networks and secondary reconstruction of the trnL P6b regions**

The haplotype network was calculated with Network v4.6.0.0 [56] using median-joining and the resulting network was edited manually. The network was calculated for the Collema-type trnL P6b regions to illustrate the sequence similarity of the regions. The constructed network and the results of the phylogenetic analysis were used to formulate a hypothesis about the sequence evolution in the Collema-type P6b regions.

Secondary structure reconstructions of the Collema-type and Class II trnL P6b regions were calculated from the RNA equivalent of a DNA sequence with NUPACK [57–59] using the NUPACK web server [60]. The folding temperature was set in 20°C.

**Saguaro analyses**

To further elucidate the congruence between and within different markers we used the software Saguaro [33]. By combining Hidden Markov Model (HMM) with a Self-Organising Map (SOM), Saguaro compares sections within the alignment, detects sections with separate phylogenies, and gives an estimation of relations of the separate sections. The analysis results provide ‘cacti’, which are distance matrices representing a topology supported by one or several alignment segments. If the alignment includes incongruent regions, in other words alignment segments with sufficient support for dissimilar topologies, the analysis results several cacti. In addition to cacti, the analysis produces a topology reflecting the similarity of the different cacti. Saguaro analyses were run for forty iterations with default settings except for skipping positions in which all calls are identical. Separate analyses were run for the 16SrRNA gene–trnL and the rbcLX–nifV1–rpoC2–trnL data sets.

Based on the Saguaro analyses, additional phylogenetic analyses were performed. The 16S rRNA gene and trnL segments supporting the two most supported Saguaro topologies (cacti 39 and 2) were analyzed by Bayesian methods first separately and then together. For the combined rbcLX–nifV1–rpoC2–trnL data set, segments supporting all three supported Saguaro topologies
Recombination tests

To detect potentially conflicting phylogenetic signals in the gene regions, parsimony split analyses using SplitsTree4 [61] were performed. As implemented in the program, a Pairwise Homoplasy Index (PHI) [62] was calculated to test for the role of past recombination in generating allelic variation. In addition, the Genetic Algorithm Recombination Detection (GARD) approach with $\beta$-$\Gamma$ rate distribution was used to further evaluate recombination and identify recombination breakpoints for each gene [63]. The GARD analyses were run on Datamonkey webserver (http://www.datamonkey.org/GARD) [64]. All data sets (S3 Table) were first analyzed with the model selection tool to define the correct nucleotide substitution bias model for the GARD analysis. GARD tests for the 16S rRNA gene were performed with and without outgroup sequences. Because of the webserver time limit for each analysis, the 16S rRNA gene analyses stopped before reaching convergence. For this reason the analyses were also performed with reduced taxon sampling. In the end, we were able to successfully run the complete analyses with 15 sequences including an outgroup and with 16 sequences without an outgroup (S3 Table). With the reduced 16S rRNA gene data set and the complete trnL data set the analysis was also run with rate classes set to five. GARD test for the trnL gene was performed for all sequences together without the P6b region and in addition including the P6b region for four different sequence sets: sequences with Class II repeat motifs, sequences with Collema-type Class I repeat motifs, sequences with Nephroma-type Class I repeat motifs, and all sequences with Class I, Nephroma- or Collema-type repeat motifs. For the sequences from the rbclX–nifV1–rpoC2–trnL data set the recombination tests were performed separately for four different regions: for rbclX with and without the long insert in the intergenic spacer region, for nifV1, and for rpoC2.

Results

The alignment and phylogenetic analyses of the genetic regions

The length of the 16S rRNA gene–trnL alignment was 1680 nt of which 1477 nt were 16S rRNA gene and 203 nt trnL (P6b region excluded). The alignment included 148 variable positions in the 16S rRNA gene and 41 in the trnL region. The full sequence set of 78 Nostoc genotypes contained 61 variable 16S rRNA gene and 71 (P6b region included) trnL sequences. The differences include both single nucleotide polymorphism and length variation. Of the 78 Nostoc trnL sequences included in this study, 34 had a Peltigera Class II type P6b region (33 different genotypes), 22 had a Collema Class I type P6b region (20 different genotypes), and 22 had a Nephroma Class I type P6b region (18 different genotypes).

The separate Bayesian analyses of the 16S rRNA gene and trnL sequences resulted in trees with relatively poor resolution (S1 Fig). Visual comparison of the resolved parts of the trees revealed very little conflict between the two loci on the inspection threshold of Posterior Probability (PP) 0.8. In addition, when the regions were combined for Bayesian analysis, it resulted in a tree with higher resolution and more supported clades than either of the two individual analyses. This suggested that the phylogenetic signals from the 16S rRNA gene and trnL are not in conflict, and the combined data set could be subjected for further analyses. The Bayesian analysis of the combined 16S rRNA gene–trnL data set identified altogether 30 well supported (PP ≥ 0.95) clades among the lichen-symbiotic Nostoc (Fig 1). The ML analysis found the best tree with identical topologies 22 times, and the topology was mostly congruent with the final
tree obtained by Bayesian analysis. The crown group of the Bayesian tree consisted of the Nostoc cyanobionts of Nephroma guild lichens that have Nephroma-type Class I trnL P6b regions (PP 1 / BS 87). Conversely, the Nostoc cyanobionts with Collema-type P6b regions are dispersed over ten different clades, and frequently occur mixed with Nostoc genotypes with Class II P6b regions.

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In the ML analyses of the $rbcLX$–$nifV1$–$rpoC2$–$trnL$ data set, the same best tree topology was found in eight of the altogether 16 runs (S2A Fig). The analysis found seven well supported (BS $\geq 90$) groups, of which one was formed by the two included Nephroma-type Nostoc cyanobionts (BS 100). The 14 Nostoc genotypes with Collema-type $trnL$ P6b regions were divided into two strongly supported groups separated by a clade including the five Nostoc genotypes with Class II $trnL$ P6b regions.

**Ancestral character state analysis and haplotype networks**

The ancestral character reconstruction analysis estimates that the probability of the common ancestor of the Nostoc clade having a Class II p6b region is more than 99% (Fig 2). The probability of the other analyzed ancestral nodes having a Class II P6b region varied from 93 to over 99%, except for the final 17th node. The 17th node is the common ancestor of the Nephroma guild Nostoc cyanobionts with Nephroma-type Class I P6b regions and their sister group with Collema-type Nostoc P6b regions, and it was the most probable analyzed ancestor with a Class I P6b region with a probability of 79%.

The single nucleotide similarity based relations between the Collema-type Nostoc $trnL$ P6b regions do not follow the phylogenetic reconstruction of the group (Fig 3A). However, if each indel event is only counted as one change and the haplotype network modified according to the obtained phylogeny, a simpler network is obtained (Fig 3B): the latter network is 15 changes shorter (altogether 40 changes) than the network constructed by the program (altogether 55 changes). The only major change seen in the latter network is represented by the long branch between Nostoc sequence 16 and the rest of the Peltigera aphthosa group Nostoc sequences (13–15).

**$trnL$ P6b region secondary structure reconstruction analyses**

The Class I P6b regions of Collema-type Nostoc $trnL$ genotypes are more variable in length (varying from 50 to 126 nt) and both by calculated free energies of the whole P6b structure (from -53.25 to -18.70 kcal/mol) and by calculated free energy per nucleotide (from -0.426 to -0.274 kcal/mol) than the Nostoc $trnL$ genotypes with Class II P6b regions (with corresponding values of 52–83 nt, -39.44... -22.04 kcal/mol, and -0.460... -0.323 kcal/mol/nt, respectively; S4 Table). In addition to being more variable, the Collema-type P6b structure is also less stable when the free energy is calculated per nucleotide (average -0.35 in Collema-type and -0.40 in Class II).

If the phylogenetic groups that have Collema-type P6b regions are inspected individually, adding an insertion normally lowers the overall free energy, also when calculated per nucleotide, hence stabilizing the structure (Fig 4, S4 Table). Also, most of the indel events happen in the same position of the folded structure causing an emergence of a side stem-loop structure(s) (Fig 4). Only in the Peltigera aphthosa group (sequences 13–16) the indel events have happened in different parts of the structure and included two separate 14 nt insertions of the basic Class I repeat motif, this resulting in a long stem-structure without side stem-loops (Fig 5).

In Class II P6b regions only one insertion causes the formation of a side stem-loop (Fig 6A). In addition, three indel combinations involving different numbers of the basic Class II repeat motifs cause length variation in the basic stem-structure without side stem-loops (Fig 6B–6E). Two of the three indel types include events in both sides of the stem implying two separate indel events (Fig 6B and 6D), and one has only lost or gained one seven nucleotide repeat on the one side of the stem, resulting in a large head loop (Fig 6D).

When the single nucleotide differences are compared, the alignment of Class II P6b regions include altogether 33 variable single nucleotide positions (Fig 6C), while the Collema-type P6b regions include only 19 (Fig 5B). Of these variable positions eleven (58%) in the Collema-type
Fig 2. *trnL* P6b region ancestral character state reconstruction. A cladogram from the bayesian analysis of the combined 16S rRNA gene–*trnL* data set with reconstructed ancestral character states for the *trnL* P6b region. Black specimens have a Class II and red specimens *Collema*-type Class I *trnL* P6b region. The pie charts show the probability of the presence of Class II (white) or Class I (*Collema* and *Nephroma* types; red) *trnL* P6b region for the ancestral nodes. The clade formed by the cyanobionts of *Nephroma* guild lichens has been collapsed to a single branch for the figure.

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sequences and 26 (79%) in the Class II sequences are situated in the unpairing loop regions of the structures.

Saguaro analyses and phylogenetic analyses based on Saguaro segments

Saguaro analysis of the 16S rRNA gene and trnL identified seven different cacti supported by the data set. The alignment segments supporting the cacti included 164 of the 189 variable nucleotide positions present in the alignment (Fig 7). The most supported topology, cactus 39, was supported by 11 separate alignment segments. These segments included 71 variable positions in the 16S rDNA and 30 variable positions in the trnL region, covering 48 and 73% of all variable nucleotide positions of the regions, respectively. Also the second most supported topology, cactus 2, was supported by alignment segments present in both 16S rRNA gene and trnL regions, including altogether 38 variable positions distributed among nine separate segments. The remaining five cacti were all supported by less than ten variable nucleotide positions, and all these within the 16S rRNA gene.

The topology reflecting the topological similarities of the detected cacti divided the seven cacti into three different clades (Fig 7C). One clade was formed by the most supported topology cactus 39 alone, the second by the second most supported topology cactus 2 together with three other cacti, and the third by two cacti supported by altogether 12 variable positions. The most supported topologies cacti 39 and 2 have several differences (Fig 7B): cactus 39 mainly distinguishes the Rhizonema sequences (outgroup) from the Nostoc sequences (ingroup) while
Fig 4. Insertions leading to side-pin structures in the secondary structure reconstruction of the Collema-type P6b regions. The folding of the P6b region sequences of specimens (A) 31 and 32, (B) 30, (C) 11, (D) 12, (E) 52 and 54, (F) 56, (G) 53, and (H) 55 were folded with NUPACK at 20°C. The color of each position reflects the stability of the structure, dark red being the most stable, and beneath is the calculated Gibbs free energy (ΔG) for each structure. Black arrow in (A), (C), and (E) show the position of the insertion leading to the structures shown in (B), (D), (G), and (H), respectively.

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cactus 2 divides all the sequences into four groups, then also mixing in- and outgroup sequences.

The Bayesian trees constructed separately of the alignment segments supporting topologies cacti 39 and 2 included relatively few supported groups. They did not show drastic incongruence between the different regions, but only relatively minor differences in the placements of single taxa (S3A and S3B Fig). Also the Bayesian trees show that the first topology mainly supports the division between out- and ingroup taxa and the second shows more variation within the ingroup. When the segments supporting these two topologies were analyzed together, Bayesian inference produced a tree with 27 strongly supported (PP ≥ 0.95) clades (S3C Fig).

The tree includes some topological differences in comparison to the tree constructed of the whole gene regions (Fig 1), the most essential being the joining of the Peltigera aphthosa cyanobiont group (13–16) together with the gelatinous lichen cyanobionts (52–56) to form a strongly supported (PP = 0.99) sister clade to the Nephroma guild cyanobionts.

In the Saguaro analysis of the rbcLX–nifV1–rpoC2–trnL data set, three separate supported topologies were detected, of which two, cacti 1 and 3, were by far the most strongly supported (Fig 8). The third topology, cactus 34, was only supported by six variable positions in the intergenic spacer region of the rbcLX. The topologies produced by Saguaro did not show any conflict between the most supported cacti 1 and 3, but also very little resolution. When separate ML trees were constructed from the segments supporting the different topologies, all three
Recombination analyses

For the trnL sequences, the PHI test did not find statistically significant evidence for recombination (S3 Table). Recombination was neither detected from the conserved parts of the trnL when all the sequences were tested together with the outgroup taxa nor when the P6b region was included in different sets of sequences.

With the 16S rRNA gene, the PHI test indicated a significant degree of recombination (p-value 0.0 with and without outgroup; S3 Table). Furthermore, the GARD approach confirmed the existence of significant recombination in 16S rRNA gene as several recombination breakpoints with significant topological incongruence between the segments were found (S3 Table). Analysis of a reduced data set of 17 sequences detected four recombination breakpoints even though the analysis did not reach convergence because of the server time limit (Fig 7). It is possible that if the analysis had continued, more breakpoints would have been detected and their exact positions might have changed.

The PHI test detected recombination also from the rbcLX (p-value 3.7E-5 with the insert and 2.0E-4 without), nifV1 (p-value 5.5E-4), and rpoC2 (p-value 0.04; S3 Table). However, GARD analysis did not detect recombination breakpoints from any other region than rbcLX when analyzed with the insertion (S3 Table; Fig 8).
Discussion

The evolution of the trnL P6b region in lichen-symbiotic Nostoc

Visualization of the distribution of the different trnL P6b region types in lichen-symbiotic Nostoc shows that the Class I P6b regions mainly occur in scattered groups of a few genotypes, with the exception of the large crown group of the phylogenetic tree (taxa 52–78, Fig 1). The crown group is formed by two well supported sister clades of Nostoc cyanobionts, which all harbour Class I trnL P6b regions. Based on the phylogeny, the lichen cyanobionts with Nephroma-type Class I trnL P6b regions clearly represent their own monophyletic lineage, as has been suggested by the results of several previous studies (e.g. [8,24,31,65,66]). The results also confirm that the Nephroma and Collema-type Class I trnL sequences can reliably be
distinguished based on the characteristic sequence patterns mentioned in the introduction. However, the defining difference in the P6b central loop sequence, introduced as AATCTTC for the Nephroma-type, seems to be only the second character. In the light of the new results, the Nephroma-type loop varies as AA(T/-)TCTTY and Collema-type loop as WG(-)TBTWH.

The results of the ancestral character reconstruction of the Class I and II trnL P6b regions show that the scattered distribution of the Collema type Class I P6b regions is a consequence of several independent replacements of the Class II P6b region with a Class I region (Fig 2). Also, even though the ML tree constructed of the rbcLX, nifV1, rpoC2, and trnL gene regions is
considerably smaller and lacks resolution in some parts, it shows a well supported division of the Nostoc genotypes with Collema-type trnL P6b regions, pointing towards the independent origin of the regions (S2A Fig). The ancestral character state analysis also suggests that the replacement has always been unidirectional. Since the Collema type Class I P6b region has repeatedly replaced the Class II type P6b region in different Nostoc lineages, and the Nephroma type Class I P6b regions are not found outside the monophyletic group of Nephroma guild Nostoc cyanobionts, the evolutionary changes from a Collema type P6b region to a Nephroma type P6b region appears to only have occurred once.

The repeated unidirectional replacements of Class II type P6b regions with Class I type P6b regions suggest that some structural features of Class II P6b regions increase the likelihood of such substitutions. This structural tendency may be reflected in the above average free energies of the Class II P6b secondary structures in several phylogenetic groups with Collema-type P6b regions (genotypes 3, 4, 6, 7, 9, and 17; S4 Table). In addition to the replacements of Class II P6b region with Class I, also replacements of one Collema-type P6b region with another are probable: The most parsimonious explanation for the two different Collema-type P6b regions present in the monophyletic Peltigera aphthosa group (13–16) is the replacement of the entire P6b region, and not by several indel and single nucleotide mutation events within the region. If the phylogenetic reconstruction based only on the more generally supported segments of the 16S rRNA gene–trnL alignment is believed to be more trustworthy (S3 Fig), at least three independent replacements have occurred within the group including cyanobionts 13–16 and 52–56, most probably between different Collema-type P6b regions. Thus, if mobile elements were to exist in the P6b regions of lichen-symbiotic Nostoc genotypes, they should most likely be found from Collema-type sequences.

While the nucleotide sequence of the P6b stem-loop in Class II and Class I types is very different the secondary structure is quite similar. The most important feature defining the similar structure are the two seven nucleotide repeat motifs that ensure the correct pairing and sufficient stability. While the evolutionary origins of the complementary repeat classes are not known, the presence of only one of the two repeat motifs in Nostoc genotypes suggests that both motifs can fulfil the same functional role. From this perspective, it is interesting that the insertions causing the side stem-loop structure tend to occur in the same position in both Collema-type Class I and in Class II P6b regions. The secondary structure reconstructions of the different P6b regions show, that Class II P6b regions are generally more stable and include less length variation than the Collema-type P6b regions. The phylogenetic reconstruction also suggests, that within lineages with Collema-type trnL P6b regions, the number of repeats is a conserved character inherited from the common ancestral P6b region of each phylogenetic group. As a whole, the Class II P6b regions include much more single nucleotide variation than the Collema-type P6b regions and of this variation the major part is found in the unpaired regions of the stem-loop structure, minimizing its effect on structural stability. According to the neutral theory of molecular evolution [67], the mutations in the unpaired loop regions could perhaps be used as relative molecular clocks, providing independent evidence for the ancestry of Class II P6b regions. It is also feasible that the ancestral Class II P6b structures have evolved to relatively stable states through time, while the derived Collema-type P6b regions are still in the process of balancing between the stabilizing benefits of long side stem-loop insertions and the costs of maintaining such structures.

Saguaro and congruence of the markers

We tested the suitability of the program Saguaro [33] to detect evolutionary patterns between and within genetic markers. In both separate data sets, Saguaro detected that all tested markers
supported more than one incongruent topologies. The results were confirmed by other analyses: Recombination breakpoints were detected from the 16S rRNA gene and from the rbcLX. The Bayesian and ML trees constructed from the alignment segments supporting each topology also indeed proved to be incongruent, even though they partly differed from the cacti produced by Saguaro using a fast neighbour-joining method.

The recombination breakpoints detected in the 16S rRNA gene do not separate the segments supporting the two most supported topologies cactus 39 and cactus 2, but are situated between the segments supporting other topologies. The segments situated closely together in the beginning of 16S rRNA gene supporting cacti 31 and 36, the only segment supporting cactus 27, the second segment supporting cactus 31, the only segment supporting cactus 40, and the only segment supporting cactus 26 are all separated by recombination breakpoints. On the other hand, almost all such parts include segments supporting both cactus 39 and cactus 2. This implies that there may not be much incongruence between the two topologies. However, it must be kept in mind that as the GARD analysis for the whole 16S rRNA gene sequence set could not finish, the gene region may potentially include even more recombination breakpoints. The position of the detected \textit{rbcLX} breakpoint almost coincides with the end of the only segment supporting topology cactus 34 in the Saguaro analysis and it is in the close proximity of the end of the intergenic spacer. There are also other reports of recombination from this position in \textit{Nostoc} [28].

Generally, incongruence was mostly detected within genetic markers rather than between the different genetic regions. The conserved parts of the \textit{trnL} sequences did not include phylogenetic signals that were not present in the other tested markers, 16S rRNA gene, \textit{rbcLX}, \textit{nifV1}, and \textit{rpoC2}. Also, no recombination was detected from the \textit{trnL}, even when different combinations of sequences with the P6b region were analyzed. However, the \textit{trnL} gene region is very short and this may hamper the detection of conflicting signals. 16S rRNA gene, on the other hand, included segments supporting several incongruent topologies separated also by recombination breakpoints. Most of the incongruence seems to concentrate in short alignment segments including only a few variable nucleotides, and the vast majority of the variation present is in congruence with the conserved parts of the \textit{trnL}. Previously, when the congruence of 16S rRNA gene and other genetic markers have been evaluated, \textit{rpoC1}, \textit{hetR}, \textit{rbcLX} have been found to be incongruent with 16S rRNA gene [28]. However, the comparison was made based on gene tree topologies, and no further analysis of the reason of the incongruence was made. Here, \textit{rbcLX}, \textit{nifV1}, and \textit{rpoC2} were mostly mutually congruent and also congruent with \textit{trnL}. Also these markers included segments supporting two different topologies, but such segments were present in all three markers. Only the intergenic spacer in the beginning of \textit{rbcLX} supported a topology not supported by other parts of the gene regions. It was also separated by a recombination breakpoint and perhaps should therefore be omitted from phylogenetic analyses.

The 16S rRNA gene has been widely used in constructing cyanobacterial phylogenies. However, in our study of lichen-symbiotic \textit{Nostoc} the 16S rRNA gene data alone could not produce a well-supported and resolved phylogenetic tree. The phylogenetic trees inferred from the 16S rRNA gene have often lacked resolution and been weakly supported also in previous studies. This ambiguity is most probably caused by the conflicting signals resulting from several recombination events, even though the conflicting alignment segments seem to be very short, comprising only a few variable nucleotides each. Many studies have found evidence of recombination events in different cyanobacterial genes [28,68–70] and the bacterial 16S rRNA gene in general has been shown to be prone to horizontal gene transfer [25,26]. The various 16S rRNA gene alignment segments supporting variable topologies and the amount of detected
recombination breakpoints inside the phylogenetically relatively closely related taxa suggests that horizontal gene transfer may be relatively common in this group of cyanobacteria.

Conclusions

Our results confirm the monophyly of the *Nephroma* guild cyanobionts with *Nephroma* type Class I P6b regions, the polyphyly of *Nostoc* cyanobionts with *Collema* type Class I P6b regions, and the clear phylogenetic distinction between these two groups. The sporadic distribution of the *trnL* P6b region types Class I and II in the lichen-symbiotic *Nostoc* is explained by several independent replacements of Class II P6b region with a Class I region. The ancestry of Class II P6b region is also supported by the total and relative amounts of the single nucleotide mutations in the more neutral, unpaired loop regions of the stem-loop secondary structure.

The 16S rRNA gene was shown to contain several recombination breakpoints and segments supporting variable phylogenetic topologies. Also other gene regions commonly used in constructing cyanobacterial phylogenies contained signs of possible horizontal gene transfer. When examining the congruence of genetic regions, trees based on the separate markers only show the possible presence of incongruence. This can lead to misjudgments, since, like in both data sets analyzed here, a lot of incongruence seems to occur within individual marker regions. In our study Saguaro proved to be a valuable tool for clarifying incongruence. In addition to recognizing conflicting signals, it helped to pinpoint the incongruent regions that need to be identified prior to phylogenetic analyses. In general, the amount of detected incongruence especially in the 16S rRNA gene and the very complex evolution of the *trnL* P6b region, emphasize the importance of such protocols, and suggests that horizontal transfer of genetic material have had a great influence in evolution also in the lichen-symbiotic *Nostoc*.

Supporting Information

S1 Fig. Separate Bayesian trees of 16S rRNA gene and *trnL*. Bayesian trees constructed of the 16S rRNA gene (left) and *trnL* (without the P6b region; right) for the visual inspection of the congruence of the markers. The nodes have been collapsed to a threshold of PP > 0.8. (EPS)

S2 Fig. Maximum likelihood (ML) trees constructed of *rbcL* and *trnL* sequences. (A) ML tree constructed of all four gene regions excluding only the long introns in the beginning of *rbcL* and the P6b region in *trnL*. Numbers in parenthesis after the accession number refer to specimens in our material having an identical *trnL* sequence (including P6b region). ML trees constructed of the alignment segments supporting topology (B) cactus 1 (the best tree was found once in hundred search replicates), (C) cactus 3 (the best tree was found four times in hundred search replicates), and (D) cactus 34 (the best tree was found only once in hundred search replicates) in the Saguaro analysis. In all trees nodes with bootstrap BS support less than 50 have been collapsed. BS values equal or greater than 75 are shown on branches, and thick branches have BS equal or greater than 90. Specimens having a Class II-type *trnL* P6b region are marked black, a *Collema*-type Class I P6b region red, and a *Nephroma*-type Class I P6b region white on red background. The shown GenBank accession numbers belong to the *rbcL* gene. (EPS)

S3 Fig. Bayesian trees constructed from 16S rRNA gene and *trnL* segments supporting different topologies in the Saguaro analysis. Bayesian trees constructed of the 16S rRNA gene and *trnL* segments supporting topologies (A) cactus 39, (B) cactus 2, and (C) cacti 39 and 2 in the 16S-*trnL* Saguaro analysis. Posterior probability (PP) values equal or over 0.75 are shown.
on the branches. Thick branches have PP equal or greater than 0.95. Specimens having a Class II-type trnL P6b region are marked black, a Collema-type Class I P6b region red, and a Nephroma-type Class I P6b region white on red background.

S1 Table. Specimen information. List of specimens used in the study including collection information and NCBI GenBank accession numbers for the sequences. The sequences generated for this study are bolded. The collector and voucher information is presented only for newly generated sequences.

S2 Table. Data set of rbcLX, nifV1, rpoC2, and trnL from O’Brien et al. [31]. The numbering follows the numbering of the original Supporting Information S1 Table in O’Brien et al. [31]. The ‘Identical trnL’ column refers to the identical trnL sequences in the 16S rRNA gene–trnL data set.

S3 Table. Results of the SplitsTree and GARD recombination tests. P is the p-value of the PHI-test and RC the number of rate classes used in the GARD analysis. KH refers to Kishino Hasegawa test and * in the end of the row indicates that the GARD analysis stopped before convergence. 16S refers to the 16S rRNA gene.

S4 Table. trnL P6b region secondary structure reconstruction. ID tells the taxa in which the P6b region is present. The secondary structure reconstruction and calculations were made with a sequence where two nucleotides outside the region were included in both ends, and in the ones marked with the * six nucleotides from the beginning and seven from the end outside the P6b region were included to ensure the correct folding [17].

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Author Contributions

Conceived and designed the experiments: UK SO JR. Performed the experiments: UK. Analyzed the data: UK SO. Contributed reagents/materials/analysis tools: JR. Wrote the paper: UK SO JR.

References

1. Rikkinen J. Cyanolichens: an evolutionary overview. In: Rai AN, Bergman B, Rasmussen U, editors. Cyanobacteria in Symbiosis. Dordrecht: Kluwer Academic Publisher; 2002. pp. 31–72.
2. Rikkinen J. Cyanolichens. Biodivers Conserv. 2015; 24: 973–993.
3. Adams DG, Duggan PS. Cyanobacteria-bryophyte symbioses. J Exp Bot. 2008; 59: 1047–1058. doi:10.1093/jxb/ern005 PMID: 18267939
4. Rikkinen J, Virtanen V. Genetic diversity in cyanobacterial symbionts of thalloid bryophytes. J Exp Bot. 2008; 59: 1007–1011. doi:10.1093/jxb/ern004 PMID: 18319239
5. Costa JL, Romero ME, Lindblad P. Sequence based data supports a single Nostoc strain in individual coralloid roots of cycads. FEMS Microbiol Ecol. 2004; 49: 461–487. doi:10.1016/j.femsec.2004.05.001 PMID: 19712296
6. Thajuddin N, Muralitharan G, Sundaramoorthy M, Ramamoorthy R, Ramachandran S, Akbarsha MA, et al. Morphological and genetic diversity of symbiotic cyanobacteria from cycads. J Basic Microbiol. 2010; 50: 254–265. doi: 10.1002/jobm.200900343 PMID: 20473963

7. Nilsson M, Bergman B, Rasmussen U. Cyanobacterial diversity in geographically related and distant host plants of the genus Gunnera. Arch Microbiol. 2000; 173: 97–102. PMID: 10795680

8. Bergman B. Nostoc-Gunnera symbiosis. In: Rai AN, Bergman B, Rasmussen U, editors. Cyanobacteria in Symbiosis. Dordrecht: Kluwer Academic Publisher; 2002. pp. 207–232.

9. Svenning MM, Eriksson T, Rasmussen U. Phylogeny of symbiotic cyanobacteria within the genus Nostoc based on 16S rDNA analyses. Arch Microbiol. 2005; 183: 19–26. PMID: 15549268

10. Rikkinen J, Oksanen I, Lohtander K. Lichen guilds share related cyanobacterial symbionts. Science 2002; 297: 357. PMID: 12130774

11. Rikkinen J. Ecological and evolutionary role of photobiont-mediated guilds in lichens. Symbiosis 2003; 34: 443–450.

12. Rikkinen J. Ordination analysis of tRNALeu (UAA) intron sequences from lichen-forming Nostoc strains and other cyanobacteria. Symb Bot Ups. 2004; 34: 377–391.

13. Rikkinen J. Molecular studies on cyanobacterial diversity in lichen symbioses. Mycokeys 2013; 6: 3–32.

14. Elvebakk A, Papaefthimiou D, Robertsen EH, Laimer A. Phylogenetic patterns among Nostoc cyanobionts within bi- and tripartite lichens of the genus Pannaria. J Phycol. 2008; 44: 1049–1059.

15. Fedrowitz K, Kaasalainen U, Rikkinen J. Geographic mosaic of symbiont selectivity in a genus of epiphytic cyanolichens. Ecol Evol. 2012; 2: 2291–2303. doi: 10.1002/ecs3.343 PMID: 23139887

16. Kruger K, Grabowski PJ, Zaug AJ, Sands J, Gottschling DE, Cech TR. Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of Tetrahymena. Cell 1982; 31: 147–157. PMID: 6297745

17. Cech TR. Conserved sequences and structures of group-I introns: building an active site for RNA catalysis—a review. Gene 1988; 73: 259–271. PMID: 3072259

18. Cech TR, Damberger SH, Gutek RR. Representation of the secondary and tertiary structures of group I introns. Nat Struct Biol. 1994; 1: 273–280. PMID: 7545072

19. Paquin B, Kathe SD, Nierzwicki-Bauer SA, Shub DA. Origin and evolution of group-I introns in cyanobacterial tRNA genes. J Bacteriol. 1997; 179: 6798–6806. PMID: 9352932

20. Besendahl A, Qiu Y- L, Lee J, Palmer JD, Bhattacharya D. The cyanobacterial origin and vertical transmission of the plastid tRNALeu group-I-intron. Curr Genet. 2000; 37: 12–23. PMID: 10672439

21. Costa JL, Paulsrud P, Lindblad P. The cyanobacterial tRNALeu (UAA) intron: evolutionary patterns in a genetic marker. Mol Biol Evol. 2002; 19: 850–857. PMID: 12032241

22. Simon D, Fewer D, Friedl T, Bhattacharya D. Phylogeny and self-splicing ability of the plastid tRNA-Leu group I intron. J Mol Evol. 2003; 57: 710–720. PMID: 14745540

23. Olsson S, Kaasalainen U, Rikkinen J. Reconstruction of structural evolution in the trnL intron P6b loop of symbiotic Nostoc (Cyanobacteria). Curr Genet. 2012; 58: 49–58. doi: 10.1007/s00294-011-0364-0 PMID: 22210193

24. Oksanen I, Lohtander K, Sivonen K, Rikkinen J. Repeat-type distribution in tRNA introns does not correspond with species phylogeny: comparison of the genetic markers 16S rRNA and tRNA intron in heterocystous cyanobacteria. Int J Syst Evol Microbiol. 2004; 54: 785–772. PMID: 15140022

25. Mclnemey JO, Cotton JA, Pisani D. The prokaryotic tree of life: past, present... and future? Trends Ecol Evol. 2008; 23: 276–281. doi: 10.1016/j.tree.2008.01.008 PMID: 18367290

26. Kitahara K, Miyazaki K. Revisiting bacterial phylogeny: Natural and experimental evidence for horizontal gene transfer of 16S rRNA. Mol Genet Elements 2013; 3: e24210. PMID: 23734299

27. Papaefthimiou D, Drouzek P, Mugnai MA, Lukesova A, Turicchia S, Rasmussen U, et al. Differential patterns of evolution and distribution of the symbiotic behaviour in nostocacean cyanobacteria. Int J Syst Evol Microbiol. 2008; 58: 553–564. doi: 10.1099/ijs.0.05312-0 PMID: 18319454

28. Han D, Fan Y, Hu Z. An evaluation of four phylogenetic markers in Nostoc: implications for cyanobacterial phylogenetic studies at the intrageneric level. Curr Microbiol. 2009; 58: 170–176. doi: 10.1007/s00284-008-9302-x PMID: 18972163

29. Kaasalainen U, Fewer DP, Jokela J, Wahlsten M, Sivonen K, Rikkinen J. Cyanobacteria produce a high variety of hepatotoxic peptides in lichen symbiosis. Proc Natl Acad Sci U S A. 2012; 109: 5886–5891. doi: 10.1073/pnas.1202791109 PMID: 22451908

30. Schirrmeister BE, de Vos JM, Antonelli A, Bagheri HC. Evolution of multicellularity coincided with increased diversification of cyanobacteria and the Great Oxidation Event. Proc Natl Acad Sci U S A. 2013; 110: 1791–1796. doi: 10.1073/pnas.1209927110 PMID: 23319632
31. O’Brien HE, Miadlikowska J, Lutzoni F. Assessing population structure and host specialization in lichenized cyanobacteria. New Phytol. 2013; 198: 557–566. doi: 10.1111/nph.12165 PMID: 23406441

32. Micheli C, Cianchi R, Paperi R, Belmonte A, Pushparaj B. Antarctic Cyanobacteria Biodiversity Based on ITS and TrnL Sequencing and Its Ecological Implication. Open J Ecol. 2014; 4: 456–467.

33. Zamani N, Russell P, Lantz H, Hoeppner MP, Meadows JR, Vijay N, et al. Unsupervised genome-wide recognition of local relationship patterns. BMC Genomics 2013; 14: 347. doi: 10.1186/1471-2164-14-347 PMID: 23706020

34. Lücking R, Lawrey JD, Sikaroodi M, Gillevet PM, Chaves JL, Sipman HJM, et al. Do lichens domesticate photobionts like farmers domesticate crops? Evidence from a previously unrecognized lineage of filamentous cyanobacteria. Am J Bot. 2009; 96: 1409–1418. doi: 10.3732/ajb.0800258 PMID: 21628288

35. Lücking R, Barrie FR, Genny D. Dictyonema coppinsii, a new name for the European species known as Dictyonema interruptum (Basidiomycota: Agaricales: Hygrophoraceae), with a validation of its photobiont Rhizoma (Cyanoprokaryota: Nostocales: Rhizonomataceae). Lichenologist 2014; 46: 261–267.

36. Fedrowitz K, Kaasalainen U, Rikkinen J. Genotype variability of Nostoc symbionts in association with three epiphytic Nephroma species in a boreal forest landscape. Bryologist 2011; 114: 220–230.

37. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999; 41: 95–98.

38. Müller K, Quandt D, Müller J, Neinhuis C. PhyDE 0.995: Phylogenetic Data Editor. 2005. Available: www.phyde.de.

39. Kelchner SA. The evolution of non-coding chloroplast DNA and its application in plant systematics. Am Mo Bot Gard. 2000; 87: 482–498.

40. Costa JL, Paulsrud P, Rikkinen J, Lindblad P. Genetic diversity of Nostoc symbionts endophytically associated with two bryophyte species. Appl Environ Microbiol. 2001; 67: 4393–4396. PMID: 11526056

41. Linke K, Hemmerich J, Lumbsch HT. Identification of Nostoc cyanobionts in some Pettigera species using a group I intron in the rRNALeu gene. Bibl Lichenol. 2003; 86: 113–118.

42. Huelsenbeck JP, Ronquist F. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 2001; 17: 754–755. PMID: 11524383

43. Posada D. jModelTest: phylogenetic model averaging. Mol Biol Evol. 2008; 25: 1253–1256. doi: 10.1093/molbev/msn083 PMID: 18397919

44. Tavaré S. Some probabilistic and statistical problems in the analysis of DNA sequences. In: Miura RM, editor. Some Mathematical Questions in Biology—DNA Sequence Analysis. Providence: American Mathematical Society; 1986. pp. 57–86.

45. Huelsenbeck JP, Ronquist F, Nielsen R, Bollback JP. Bayesian inference of phylogeny and its impact on evolutionary biology. Science 2001; 294: 2310–2314. PMID: 11743192

46. Huelsenbeck JP, Larget B, Miller RE, Ronquist F. Potential applications and pitfalls of bayesian inference of phylogeny. Syst Biol. 2002; 51: 673–688. PMID: 12396583

47. Rambaut A, Drummond AJ. Tracer v1.5. 2009. Available: http://beast.bio.ed.ac.uk/Tracer.

48. Zwickl DJ. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. Thesis, The University of Texas at Austin. 2006. Available: http://repositories.lib.utexas.edu/handle/2152/2666.

49. Kumar S, Skjæveland Å, Orr RJ, Enger P, Ruden T, Mevik BH, et al. (2009) AIR: A batch-oriented web program package for construction of supermatrices ready for phylogenomic analyses. BMC Bioinformatics 2009; 10: 357. doi: 10.1186/1471-2105-10-357 PMID: 19863793

50. Miller MA, Pfeiffer W, Schwartz T. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE). 14 Nov 2006. pp. 1571–1572.

51. Sukumaran J, Holder MT. DendroPy: A Python library for phylogenetic computing. Bioinformatics 2010; 26: 1569–1571. doi: 10.1093/bioinformatics/btp228 PMID: 20421198

52. Stöver BC, Müller KF. TreeGraph 2: Combining and visualizing evidence from different phylogenetic analyses. BMC Bioinformatics 2010; 11: 7. doi: 10.1186/1471-2105-11-7 PMID: 20051126

53. Pagel M, Meade A, Barker D. Bayesian estimation of ancestral character states on phylogenies. Syst Biol. 2004; 53: 673–684. PMID: 15545248

54. Paradis E, Claude J, Strimmer K. APE: analyses of phylogenetics and evolution in R language. Bioinformatics 2004; 20: 289–290. PMID: 14734327

55. R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing. 2011. Available: http://www.R-project.org/. doi: 10.1016/j.neuroimage.2011.01.013 PMID: 21238596
56. Bandelt HJ, Forster P, Röhl A. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol. 1999; 16: 37–48. PMID: 10331250
57. Dirks RM, Pierce NA. A partition function algorithm for nucleic acid secondary structure including pseudoknots. J Comput Chem. 2003; 24: 1664–1677. PMID: 12926009
58. Dirks RM, Pierce NA. An algorithm for computing nucleic acid base-pairing probabilities including pseudoknots. J Comput Chem. 2004; 25: 1295–1304. PMID: 15139042
59. Dirks RM, Bois JS, Schaefer JM, Winfree E, Pierce NA. Thermodynamic analysis of interacting nucleic acid strands. SIAM Rev. 2007; 49: 65–88.
60. Zadeh N, Steenberg CD, Bois JS, Wolfe BR, Khan AR, et al. NUPACK: analysis and design of nucleic acid systems. J Comput Chem. 2011; 32: 170–173. doi: 10.1002/jcc.21596 PMID: 20645303
61. Huson DH, Bryant D. Application of phylogenetic network in evolutionary studies. Mol Biol Evol. 2006; 23: 254–267. PMID: 16221896
62. Bruen TC, Philippe H, Bryant D. A simple and robust statistical test for detecting the presence of recombination. Genetics 2006; 172: 2665–2681. PMID: 16489234
63. Kosakowsky Pond SL, Posada D, Gravenor MB, Woelk CH, Frost SDW. GAR D: a genetic algorithm for recombination detection. Bioinformatics 2006; 22: 3096–3098. PMID: 17110367
64. Delport W, Poon AF, Frost SDW, Kosakovsky Pond SL. Datamonkey 2010: a suite of phylogenetic analysis tools for evolutionary biology. Bioinformatics 2010; 26: 2455–2457. doi: 10.1093/bioinformatics/btq429 PMID: 20671151
65. Myllys M, Stenroos S, Thell A, Kuusinen M. High cyanobiont selectivity of epiphytic lichens in old growth boreal forest of Finland. New Phytol. 2007; 173: 621–629. PMID: 17244057
66. Magain N, Serusiaux E. Do photobiont switch and cephalodia emancipation act as evolutionary drivers in the lichen symbiosis? A case study in the Pannariaceae (Peltigerales). PLOS ONE 2014; 9: e89876. doi: 10.1371/journal.pone.0089876 PMID: 24587091
67. Kimura M. The neutral theory of molecular evolution: A review of recent evidence. Jpn J Genet. 1991; 66: 367–386. PMID: 1954033
68. Rudi K, Skulberg OM, Jakobsen KS. Evolution of cyanobacteria by exchange of genetic material among phylogenetically related strains. J Bacteriol. 1998; 180: 3453–3461. PMID: 9642201
69. Tanabe Y, Kaya K, Watanabe MM. Evidence for recombination in the microcystin synthetase (mcy) genes of toxic cyanobacteria Microcystis spp. J Mol Evol. 2004; 58: 633–641. PMID: 15461420
70. Shishido TK, Kaasalainen U, Fewer DP, Rouhiainen L, Jokela J, Wahlsten M, et al. Convergent evolution of [D-Leucine] microcystin-LR in taxonomically disparate cyanobacteria. BMC Evol Biol. 2013; 13: 86. doi: 10.1186/1471-2148-13-86 PMID: 23601305