

# Relationships between mycobiont identity, photobiont specificity and ecological preferences in the lichen genus *Peltigera* (Ascomycota) in Estonia (northeastern Europe)

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## ABSTRACT

We studied the genotype diversity of cyanobacterial symbionts in the predominately terricolous cyanolichen genus *Peltigera* (Peltigerales, Lecanoromycetes) in Estonia. Our sampling comprised 252 lichen specimens collected in grasslands and forests from different parts of the country, which represented all common *Peltigera* taxa in the region. The cyanobacteria were grouped according to their tRNA<sup>Leu</sup> (UAA) intron sequences, and mycobiont identities were confirmed using fungal ITS sequences. The studied *Peltigera* species associated with 34 different “*Peltigera*-type” *Nostoc* trnL genotypes. Some *Peltigera* species associated with one or a few trnL genotypes while others associated with a much wider range of genotypes. Mycobiont identity was the primary factor that determined the presence of the specific *Nostoc* genotype within the studied *Peltigera* thalli. However, the species-specific patterns of cyanobiont selectivity did not always reflect phylogenetic relationships among the studied fungal species but correlated instead with habitat preferences. Several taxa from different sections of the genus *Peltigera* were associated with the same *Nostoc* genotype or with genotypes in the same habitat, indicating the presence of functional guild structure in the photobiont community. Some *Nostoc* trnL genotypes were only found in the *Peltigera* species of moist and mesic forest environments, while another set of *Nostoc* genotypes was typically found in the *Peltigera* species of xeric habitats. Some *Nostoc* trnL genotypes were only found in the *Peltigera* taxa that are common on alvars and may have specialized to living in this unusual and threatened habitat type.

## 1. Introduction

Lichen symbioses always include at least one primary fungal symbiont, the mycobiont, and one or more photosynthetic partners, the photobionts. The photobionts of bipartite lichens are either green algae or cyanobacteria, while tripartite lichen thalli include both major types of photobionts (Rikkinen, 2002; Henskens et al., 2012). Besides the primary mycobiont and the photobionts, also many associated fungi and bacteria take part in lichen-symbiotic consortia (Grube et al., 2009; Sigurbjornsdottir et al., 2015; Aschenbrenner et al., 2016; Grube and Wedin, 2016; Spribille et al., 2016).

Approximately 10% of all known lichen-symbiotic fungi associate with cyanobacterial photobionts (Rikkinen, 2015, 2017). Most fungi in the order Peltigerales (Ascomycota) invariably associate with cyanobacteria, most commonly with symbiotic representatives of *Nostoc* (Nostocales). These fungi include all species of *Peltigera* (Vitikainen, 1994, 2007). Lichen-symbiotic *Nostoc* cannot presently be named to species, but symbiotic genotypes can be identified by using DNA markers such as the cyanobacterial trnL intron, rbcLX and 16S rDNA genes (Rikkinen, 2013; Kaasalainen et al., 2015; Joneson and O'Brien, 2017; Magain et al., 2017a).

Some of the earliest molecular studies on cyanolichens focused on the *Nostoc* cyanobionts of *Peltigera* in northern Europe and western North America (Paulsrud et al., 1998, 2000, 2001). In these studies, only one *Nostoc* genotype was typically detected in each bipartite *Peltigera* thallus and in different cephalodia of the tripartite *Peltigera* species. These findings have not been challenged by the results of more recent studies, with the exception that some tripartite *Peltigera* species have sometimes been found to house different *Nostoc* genotypes within different cephalodia of single thalli (Paulsrud et al., 2000; Kaasalainen et al., 2009; Rikkinen, 2013). Several studies on green algal lichens, have challenged the dogma of single photobiont genotypes or taxa within a single lichen thallus, and suggest the presence of mixed photobiont populations in many cases (e.g. Guzew-Krzeminska, 2006; Casano et al., 2010; Onuț;-Brannstrom et al., 2018). However, Paul et al. (2018) compared Sanger sequencing and high-throughput sequencing for determining photobiont diversity in lichens and proposed that Sanger technology consistently yields the most abundant photobiont sequence in the lichen sample.

The level of photobiont specificity in lichen-forming fungi can be determined by elucidating the number of photosynthetic partners that are utilized by one mycobiont species (Yahr et al., 2006; Otalora et al., 2010; Magain et al., 2017a). The symbiont specificity expressed by lichen-forming fungal species and their main photobionts varies widely and they range from strict

specialists, i.e. those associating with only one species, through moderate specialists, i.e., those associating consistently with a few species, to broad generalists, i.e. those associating with many different species with little or no apparent selectivity (Magain et al., 2017a; Lu et al., 2018). The fact that lichen mycobionts generally tend to associate with only one or a few photobiont genera (green algae or cyanobacteria), suggests inherent deep phylogenetic constraints in partner compatibility (Rolshausen et al., 2018). In rare cases, single mycobionts can form different lichen morphotypes in symbiosis with compatible green algal and cyanobacterial photobionts, respectively, and such disparate morphs can either combine into one compound thallus or live separately (Rikkinen, 2015).

The patterns of photobiont specificity are scale dependent regarding the various phylogenetic scales (e.g. genotype, species or higher taxonomical levels) and as well as the spatial scale. An analysis of photobiont association patterns within the green algal lichen family Parmeliaceae at the scale of ecoregions indicated that the generic identity of fungal hosts was a better predictor of photobiont association than ecological predictors (Leavitt et al., 2015). Likewise, a study of photobiont specificity in *Peltigera* section *Polydactylon* revealed very high specificity even at the smallest spatial scales analysed (Chagnon et al., 2018).

However, according to several studies on green algal lichens (Fernandez-Mendoza et al., 2011; Sadowska-Deś et al., 2014; Leavitt et al., 2015, 2016; Williams et al., 2017), climatic factors can also play a role in shaping photobiont distributions and association patterns, even at a global scale (Singh et al., 2017; Magain et al., 2017a). While reciprocal (one-to-one) specificity by both symbiotic partners has been reported for some mycobionts (Otalora et al., 2010; Magain et al., 2017a, b), many fungal species associate with several different *Nostoc* (Fedrowitz et al., 2012; Magain et al., 2017a), or *Trebouxia* haplotypes (Leavitt et al., 2015). It is suggested that association patterns among lichen symbionts at a very fine level would be environmentally structured rather than phylogenetically constrained and switching between photobiont ecotypes with distinct environmental preferences has been hypothesized as an adaptive strategy for lichen-forming fungi (Rikkinen, 2003; Rolshausen et al., 2018). Photobiont switches within a single fungal species have indeed been identified along both latitudinal and altitudinal gradients (Muggia et al., 2008; Fedrowitz et al., 2012; Vargas Castillo and Beck, 2012; Magain et al., 2017a; Dal Grande et al., 2018).

Although community scale patterns of photobiont diversity are most likely influenced by the environment, few molecular studies have so far addressed ecological segregation between closely

related lichen photobionts. Distinguishing the influence of different ecological factors on photobiont populations or on mycobiont selectivity is complicated by the fact that many ecological factors tend to be strongly intercorrelated. In green algal lichens the role of habitat or substratum as a determinant of the photobiont type seems to vary among different lichens and environments (Beck et al., 2002; Blaha et al., 2006; Muggia et al., 2008, 2013; Leavitt et al., 2013). For example, Peksa and Škaloud (2011) found that the green algal photobionts of the crustose lichen *Lepraria* (Lecanorales) were clearly differentiated based on their substrate and climatic preferences; the photobionts of the epiphytic pendulous lichen *Ramalina menziesii* showed significant structure according to the ecoregion and phorophyte species (Werth and Sork, 2014); and photobionts associating with the epigeic fruticose lichen *Cladonia subtenuis* exhibited population subdivision according to the ecoregion and habitat (Yahr et al., 2006). There is evidence that the substratum of lichenized fungi can play some role in determining photobiont association patterns (e.g. Elvebakk et al., 2008), although others have suggested that differences in substrate preferences do not have major influence (O'Brien et al., 2005, 2013; Stenroos et al., 2006; Ojala et al., 2010). Ortiz-Alvarez et al. (2015) found that the selection of cyanobacterial photobionts in two closely related maritime species of *Lichina* (Lichinales) was linked to contrasting environmental conditions in their closely situated coastal niches.

Several studies have indicated that species of lichen-forming fungi that only reproduce sexually are often less selective in their choice of photobionts compared to related fungi that reproduce via symbiotic diaspores (Blaha et al., 2006; Ojala et al., 2010, 2013; Fedrowitz et al., 2012; Muggia et al., 2013, 2014; Leavitt et al., 2015). At the community scale, functional lichen guilds exist in which appropriate photobiont genotypes are shared among coexisting mycobiont species and in some cases even between fungi and bryophyte hosts (Costa et al., 2001; Rikkinen, 2002, 2017; Rikkinen et al., 2002; Rikkinen and Virtanen, 2008; Lucking et al., 2009; Dal Grande et al., 2014; Cornejo and Scheidegger, 2016). Lichen guilds can involve mycobiont species with different dispersal modes: core species can effectively disperse photobionts in symbiotic diaspores, while sexually reproducing fringe species can benefit from this activity (Rikkinen, 2003).

Our recent analysis of 252 *Peltigera* specimens from different habitats in Estonia revealed 31 putative fungal taxa (OTUs), confirming that the genus includes many insufficiently known species (Juriado et al., 2017). Multivariate analysis revealed habitat-specific segregation between the different species along a gradient from humid eutrophic forests to dry oligotrophic forests and grasslands and along a soil pH gradient from alkaline soils of alvar grasslands to acidic soils of

conifer forests. The species diversity of *Peltigera* was the highest on roadsides and dunes and the lowest in alvar habitats, which supported a unique assemblage of undescribed taxa. Deciduous broad-leaved forests, too, included several undescribed or rare and red-listed species, demonstrating that many *Peltigera* species have narrow habitat requirements and are threatened by habitat loss and degradation (Juriado et al., 2017).

Here we extend our treatment of Estonian *Peltigera* species through analysing a large new dataset of their *Nostoc* photobionts. The cyanobacterial photobiont (cyanobiont) of each lichen specimen was determined by using cyanobacterial tRNA<sup>Leu</sup> (UAA) intron (trnL) sequences as a genetic marker. As trnL is easy to amplify and shows sufficient variability, especially in the P6b region, it has been widely employed for DNA based identification of symbiotic *Nostoc* genotypes (e.g. Paulsrud et al., 1998, 2000, 2001; Fedrowitz et al., 2011, 2012; O'Brien et al., 2005, 2013). In addition, the more conserved parts of trnL intron have been used to assess phylogenetic relationships, often alongside the 16S rRNA gene and other markers (e.g. Summerfield et al., 2002; Kaasalainen et al., 2015).

To determine possible correlations between habitat specificity and photobiont selectivity, we compared photobiont diversity in *Peltigera* specimens collected from different habitat types and substrata, including grassland and forest types. We hypothesize that cyanobacterial photobionts are not randomly distributed along the complex environmental gradient, but their distributions correlate both with identity of the mycobiont as well as with growth conditions.

## **2. Material and methods**

### *2.1. Study region and sampling*

Lichen specimens were collected in 2012–2016 from 107 localities in Estonia; some additional specimens from the collections of the University of Tartu (TU) were also included in the study. The study sites were distributed over the whole country and represented three wooded habitat types (oligotrophic forests, eutrophic forests, and park stands) and three grassland types (alvars, dunes, and roadsides). In addition to the habitat type, each lichen specimen was assigned one of three substratum types (tree, rock, and ground). For additional information on the field sites and sampling, see Juriado et al. (2017).

In ecological analyses, the substratum and the habitat variables were combined ('ground\_alvar', 'ground\_dune', 'ground\_eutrophic forest', 'tree\_eutrophic forest', 'rock\_roadside', 'rock\_eutrophic forest' etc.). The habitats collectively represent a natural gradient of decreasing atmospheric humidity from grasslands to mesotrophic forest, and increasing soil pH, from acidic soils of oligotrophic forest to basic alvar grassland soils. At each study site, up to three specimens of each morphologically distinguishable *Peltigera* taxon were collected for DNA analysis. Phylogenetic analyses of Internal Transcribed Spacer (ITS) sequences allowed to delimit 31 putative *Peltigera* taxa (OTUs) of 252 *Peltigera* specimens, some of them undescribed (e.g. *P. "neorufescens"*, *P. "fuscoponojensis"*, *P. "neocanina"*, Juriado et al., 2017).

## 2.2. Molecular data

Well-developed lobes of *Peltigera* thalli without visible symptoms of fungal infection were selected for molecular analyses. For DNA extraction, tiny thallus fragments containing both the cyano- and mycobiont from terminal parts of the lobes were placed under a dissecting microscope. DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific) following the manufacturer's protocol for Gram-Negative Bacteria. Amplification of cyanobacterial trnL was performed with the primer pair tRNA Leu outF and tRNA Leu outR (Paulsrud and Lindblad, 1998). The amplification reaction was prepared for a 50- $\mu$ l final volume containing 2  $\mu$ l genomic DNA, 37.5  $\mu$ l of sterile distilled water, 5  $\mu$ l of 10 $\times$ reaction buffer, 1 $\mu$ l dNTP (10mM), 1  $\mu$ l tRNA<sub>Leu</sub> outF (50mM), 1  $\mu$ l tRNA<sub>Leu</sub> outR (50mM), 1.25  $\mu$ l BSA (20mg/ml) (Thermo Scientific) and 1.25  $\mu$ l Dynazyme II (2 U/ $\mu$ l) (Thermo Scientific). The heating cycle was the following: initial denaturation of 3min at 94 °C followed by 4 cycles of 30 s at 94 °C, 30 s at 55 °C, and 2min at 72 °C. This was followed by 26 cycles of 30 s at 94 °C, 30 s at 60 °C, and 2min at 72 °C, with a final extension of 10min at 72 °C. The amplification products were purified with the GeneJET PCR Purification Kit (Thermo Scientific). Sequencing was performed by Macrogen Inc. in Europe with the same primers. The chromatograms of all sequences were checked and aligned using the program CodonCode Aligner 6.0.2 (CodonCode Corporation, Dedham, MA, USA). The alignment of the entire tRNA<sub>Leu</sub> intron sequences (354–374 bp) was used in the analyses. All newly obtained sequences are deposited in the European Nucleotide Archive (<http://www.ebi.ac.uk/ena/data/view/LS998801-LS999057>) (Table S1). The mycobiont ITS sequences of the same lichen sample are stored in the NCBI GenBank database (LT852805-LT853056) (Juriado et al., 2017). The

voucher specimens are deposited in the lichenological herbarium of the Natural History Museum at the University of Tartu (TU).

### 2.3. Data analyses

The program Network 5.0.0.1 (Bandelt et al., 1999) was used to reconstruct the median-joining networks of the *Nostoc* trnL genotypes based on nucleotide differences in the trnL sequences. The *Nostoc* genotypes are denoted by letters and numbers (Fig. 1). Different letters were assigned to genotypes that differ by a minimum of 6 nucleotides. To illustrate the associations between the cyanobiont trnL genotypes and the fungal OTUs, a bipartite interaction network was constructed using R 3.3.3 (R Core Team, 2017) and the 'bipartite' package (Dormann et al., 2008). By using the program DnaSP 5.10.01 (Librado and Rozas, 2009), the diversity of the *Nostoc* trnL genotypes among the substratum-habitat groups was calculated.

Variation partitioning analysis (VPA) in the program package CANOCO 5 (ter Braak and Šmilauer, 2012; Šmilauer and Lepš, 2014) was employed to partition variation in the symbiotic *Nostoc* genotypes associated with the studied *Peltigera* samples. Rare *Nostoc* genotypes appearing only once or twice in the dataset were removed prior to analysis. Two subsets of explanatory variables ('Mycobiont species' and 'Habitat') were used to test the unique effects of both variable sets and the shared proportion of variation explaining the distribution of the *Nostoc* genotypes. 'Mycobiont species' represented 18 *Peltigera* taxa, including widely used traditional species and some undescribed taxa (i.e. *P. "neorufescens"*, *P. "fuscoponojensis"*, *P. aff. "neocanina"* according to Juriado et al., 2017, Table S1). The variable set 'Habitat' included the combined substratum and habitat variables. Significance was assessed using permutation tests.

The symmetric co-correspondence analysis (symmetric CoCA, ter Braak and Schaffers, 2004) in the program package CANOCO 5 was used to relate two different kinds of biotic community (i.e. *Peltigera* taxa and *Nostoc* trnL genotypes) recorded over identical sets of locations. CoCA finds the ordination axes (gradients) along which the weighted co-variance among case scores for the two compared communities is maximized. Statistically significant compositional co-variation between the two communities is tested by the permutation test. Effectiveness of the ordination is expressed by eigenvalues (variance in the community matrix attributed to a particular axis) and by total inertia (sum of the eigenvalues or total "variance" in the species data). The environmental variables were used as supplementary variables to help describe the ecological gradients that were common for both communities. The delimitation of the mycobiont taxa follows the

description in Juriado et al. (2017). Rare taxa, appearing only once or twice in the dataset, and redundant specimens from the same site with identical mycobiont and photobiont sequences were removed prior to analysis. As a result, 22 *Peltigera* taxa were obtained, which were used in the first step of ordination analyses (Table S1). In the second step of ordination analysis, three taxa were excluded as the outliers.

### 3. Results

#### 3.1. Distribution of *Nostoc* genotypes in different habitats

In total, 244 *Nostoc* trnL sequences were obtained from the 252 sampled *Peltigera* specimens (Tables S1 and S2). Most of these sequences (229) had a Class 2 repeat motif in the P6b region (Costa et al., 2002; Kaasalainen et al., 2015) and represented 30 different *Nostoc* genotypes (genotypes A1–A23, B, C1–C3, D, J, K in Fig. 1).

Fifteen sequences of *Nostoc* from four *Peltigera* species had a Class 1 repeat motif in the P6b region (Costa et al., 2002; Kaasalainen G, H in Fig. 1). Three *Nostoc* genotypes (B, A1 and A2) were very common and widely distributed, accounting for 24, 21 and 18 percent of all sequences, respectively. Nearly half (47%) of all *Nostoc* sequences, from less than 10 *Peltigera* thalli, belonged to the other genotypes, with 15 *Nostoc* genotypes found only once.

Genotypes A1 and A2 were very similar, differing in only one nucleotide site (Fig. 1). These two genotypes, like many other highly similar *Nostoc* genotypes marked with letter A, were largely confined to terricolous *Peltigera* species. Genotype A1 was most commonly found in *Peltigera* specimens from alvars (37%), dunes (29%), and roadside grasslands (14%). Genotype A2 was most commonly found in *Peltigera* specimens from dunes (30%), oligotrophic forest (22%), and parks (18%). Genotypes A3 and A4 were found mostly, and genotypes A10, A14, A17 and A20, exclusively, in the lichens growing on alvar grasslands (Fig. 1). Genotypes A7, A12 and A16 were only found in lichens from dunes, and genotypes A5, A13 and A12 were only found from lichens from roadsides (Fig. 1). *Nostoc* genotype D was largely restricted to terricolous *Peltigera* species that grew in oligotrophic forests and on dunes (Fig. 1).

A substantial majority (66%) of the sequences representing *Nostoc* genotype B were obtained from *Peltigera* specimens growing on mossy tree bases or on logs in eutrophic forests. Another 12% of these sequences were obtained from lichens of mossy rocks in park stands and



still another 12%, from lichens growing on ground in eutrophic forests. Also *Nostoc* genotypes C1, C2 and C3 were obtained from eutrophic forests and park stands, where they were mainly found in *Peltigera* taxa that grew on mossy rocks or ground (Fig. 1). *Nostoc* genotypes E, F, G, H with a Class 1 repeat motif in the P6b region, were found mostly in *Peltigera* species growing on the ground in dunes, parks, roadsides and oligotrophic forests as well as on mossy rocks in the same habitats (Fig. 1).

Among all studied substrata in the different habitat types, the total number of symbiotic *Nostoc* genotypes was the largest in *Peltigera* species that grew on the ground in alvar grasslands and on dunes. However, the diversity values recorded for the roadsides as well as for the ground and rock in eutrophic forests were even slightly higher (Table 1). The smallest number of *Nostoc* genotypes and the lowest genotype diversity values were recorded for tree bases in eutrophic forests (Table 1).

### 3.2. Distribution of *Nostoc* genotypes in different *Peltigera* taxa

The bipartite interaction network (Fig. 2) shows associations between the different *Peltigera* species and different *Nostoc* genotypes. *Nostoc* genotype A1 was found in 13 different *Peltigera* taxa (OTUs), most frequently in *P. "neorufescens"* and *P. rufescens* (Table S1). *Nostoc* genotype A2 was found from 15 different *Peltigera* taxa, most frequently in *P. rufescens* and *P. canina* s. lat. (Table S1). Most species of the *Peltigera* section *Peltigera* associated with these two genotypes, but also *P. leucophlebia* (section *Chloropeltigera*) and *P. neckeri* (section *Horizontales*), associated with the same two *Nostoc* genotypes (Table S1, Fig. 2). As a whole, all *Nostoc* genotypes assigned with letter A (A1–A23) had similar distributions among the same set of *Peltigera* species.

*Nostoc* genotype B was found in eight different *Peltigera* taxa, most commonly *P. polydactylon* (section *Polydactylon*), *P. praetextata*, *P. canina* II and III, and *P. aff. neocanina* (section *Peltigera*). *Nostoc* genotype C2 was only found in *P. degenii* (section *Peltigera*) and *P. neopolydactyla* (section *Polydactylon*). *Peltigera membranacea* (section *Peltigera*) associated with *Nostoc* genotype C1 and *P. hymenina* (section *Polydactylon*) associated with genotypes C1 and C3. *Nostoc* genotype D was mostly found in *P. extenuata* and in one case in *P. didactyla*. *Peltigera aphthosa* and *P. malacea* (section *Peltidea*) always associated with Class 1 type *Nostoc* genotypes (E and F, respectively), and the sole specimen of *P. collina* had its own, unique *Nostoc* genotype. (Table S1, Fig. 2).

### 3.3. Relationships between *Peltigera* taxa, *Nostoc* genotypes and ecological factors

The results of variation partitioning analysis (VPA) showed that the two variable sets ('Mycobiont identity' and 'Habitat') together explained 65.3% of the variation in the symbiotic *Nostoc* genotypes associated with different *Peltigera* taxa (Table 2). The identity of mycobiont species was the most important factor, explaining 45.6% in the total variation. Of the total variation, 7.2% was explained by the habitat, while the co-effect of mycobiont identity and habitat type explained 12.6% of the total variation.

Permutation tests revealed that the relationship between the *Peltigera* taxa and symbiotic *Nostoc* genotypes in symmetric co-correspondence analysis CoCa was significant ( $p=0.004$ ) for all ordination axes. The cross-correlation values of the first two ordination axes were 0.91 and 0.94 and the corresponding eigenvalues were 0.71 and 0.59, respectively. The total inertia values (the sum of all eigenvalues) for the *Peltigera* taxa and the *Nostoc* genotypes were 11.2 and 12.5, respectively. The response scores are presented in the dual ordination diagrams of the CoCa axes of Fig. 3. Also the environmental variables are presented as overlays on the subplots.

*Peltigera degenii* (Fig. 3, subplot A) occurred in eutrophic forests and park stands and associated with *Nostoc* genotype C2 (Fig. 3, subplot B). *Peltigera membranacea* and *P. hymenina* occurred mainly on ground or mossy stones in eutrophic forests (Fig. 3, subplot A) and associated most frequently with *Nostoc* genotypes C1 and C3, respectively (Fig. 3, subplot B). As these three *Peltigera* species differed clearly from all the rest with respect to both habitat preferences and cyanobiont composition (Figs. 1 and 3), they were excluded from the next step of the ordination analysis (Fig. 4).

Also in the second CoCA analysis, permutation tests revealed that the relationship between the *Peltigera* taxa and symbiotic *Nostoc* genotypes was significant ( $p=0.003$ ) for all ordination axes. The cross-correlation values of the first two ordination axes were 0.89 and 0.78 and the corresponding eigenvalues were 0.65 and 0.36, respectively. The total inertia values for the *Peltigera* taxa and for the *Nostoc* genotypes were 10.7 and 12.1, respectively. The response scores are presented in the dual ordination diagrams of the CoCa axes of Fig. 4. Also the environmental variables are presented as overlays on the sub-plots.

*Peltigera polydactylon* grew on mossy tree trunks in eutrophic forests (Fig. 4, subplot A) and associated only with *Nostoc* genotype B (Fig. 4, subplot B). Also *P. aff. "neocanina"*, *P. praetextata*, *P. canina II*, *P. canina III* and *P. didactyla II* grew mainly on tree bases and mossy rocks in eutrophic forests (Fig. 4, subplot A) and associated with the same *Nostoc* genotype B (Fig. 3, subplot B).

*Peltigera extenuata* grew on soil in oligotrophic forests and on dunes (Fig. 4, subplot A) and always associated with its own *Nostoc* genotype D.

Most terricolous *Peltigera* taxa that grew in park stands, oligotrophic forests, dunes, and roadsides, incl. *P. canina* I, *P. didactyla* I and III, *P. neckeri* (Fig. 4, subplot A), associated mostly with a group of closely related *Nostoc* genotypes, most commonly the frequent genotype A2, but also A1, A3, A9 and A12 (Fig. 4, subplot B). *Peltigera* “*fuscoponojensis*”, *P. ponojensis* I and II, and *P. rufescens* (Fig. 4, subplot A) associated with the same group of closely related *Nostoc* genotypes, most commonly the frequent genotype A1 (Fig. 4, subplot B). *Peltigera* “*neorufescens*” and *P. “neorufescens”* agg. III grew only on alvar grasslands (Fig. 4, subplot A) and always associated with their own distinct selection of *Nostoc* genotypes (Fig. 4, subplot B).

#### 4. Discussion

The degree of photobiont specificity of cyanolichen-forming fungi belonging to different taxonomic groups has been extensively studied. For cyanolichens with *Nostoc* cyanobionts, the results have been quite variable depending on the set of taxa studied (e.g. Paulsrud et al., 2000; Myllys et al., 2007; Elvebakk et al., 2008; Otalora et al., 2010; Fedrowitz et al., 2012; Ortiz-Alvarez et al., 2015; Magain et al., 2017a,b). Some *Peltigera* species seem to be highly specialized and only associate with one or a few selected photobiont genotypes, while others are more promiscuous and associate with a range of different *Nostoc* genotypes (e.g. Paulsrud et al., 1998, 2001; O'Brien et al., 2005, 2013; Miadlikowska et al., 2014; Zuniga et al., 2015; Magain et al., 2017a; Chagnon et al., 2018). Some widely distributed *Peltigera* species can associate with different *Nostoc* cyanobionts in different parts of their range (Manoharan-Basil et al., 2016; Magain et al., 2017a). Thus, photobiont specificity may often be scale-dependent, and a strict specificity detected at a local scale does not necessarily hold on larger geographical scales (Magain et al., 2017a; Lu et al., 2018). These general patterns were also evident in our data set, with some Estonian *Peltigera* species (e.g. *P. ponojensis*, *P. rufescens*, *P. neckeri*) associated with several *Nostoc* genotypes while other species (e.g. *P. apthosa*, *P. malacea*, *P. extenuata*, *P. polydactylon*) were always confined to one specific *Nostoc* genotype.

Many of the *Nostoc* trnL genotypes detected in Estonian *Peltigera* specimens are new; others have previously been found from other parts of Europe. For example, the three *Nostoc*

genotypes (A1, A2, B) that were most frequently found in Estonia were previously known from other regions. According to the sequences in GenBank, *Nostoc* genotypes A1 and A2 have previously been found in cephalodia of *Peltigera leucophlebia* in southern Norway, and in *Peltigera canina* in Spain and in Poland; *Nostoc* genotype A2 has been found also in a *Peltigera rufescens* specimen in southern Finland (Table S3). *Nostoc* genotype B has been detected repeatedly in different *Peltigera* species in Europe, typically in taxa that grow on forest soil, rotten logs, or bryophyte covered tree trunks (Table S3). Even more interestingly, sequences of identical *Nostoc* cyanobionts have also been found in western North America and East Asia, indicating that this *Nostoc* genotype is widely distributed in suitable habitats across the boreal and temperate zones of the northern hemisphere. Also the rare *Nostoc* genotypes, for example *Nostoc* genotype F, which was found in *Peltigera malacea* specimens in Estonia, has previously been detected in the same *Peltigera* species in central Finland (Kaasalainen et al., 2015). *Nostoc* genotype K from *Peltigera collina* has previously been found in the same species in Scotland and has also been cultured from *Lobaria pulmonaria* and epiphytic mosses in central Finland (Rikkinen et al., 2002; Fedrowitz et al., 2012). *Nostoc* genotype E from *Peltigera apthosa* specimens has previously been found in *Nephroma arcticum* in northern Finland (Fedrowitz et al., 2012).

One may expect that many of the novel *Nostoc* genotypes now reported from Estonia are more widely distributed than presently known. Sampling in most previous studies has mainly focused on the muscicolous *Peltigera* species of boreal forests and bordering temperate and subalpine habitats, while the terricolous *Peltigera* communities of calcareous soils have not received equal attention. Hence, the *Nostoc* cyanobionts of the lichens of such habitats are probably underrepresented in GenBank and other databases. This finding is in accordance with the overall bioclimatic conclusions of Magain et al. (2017a) and suggests co-specialization between certain mycobionts and cyanobionts that are both specifically adapted to living on calcareous soils in temperate regions.

Our current findings demonstrate that mycobiont identity is the most important factor determining the presence of a specific *Nostoc* genotype within the *Peltigera* thallus. However, also an independent effect of the habitat was detected. The habitat specific spectra of different *Peltigera* taxa in different habitat types explained a major proportion of variation in the distribution of symbiotic *Nostoc* genotypes. For example, *P. canina* II and III, growing on tree bases and logs, were more likely to associate with *Nostoc* genotype B than with any of the other *Nostoc* genotypes that were typically found in soil-dwelling members of the *Peltigera canina* group. A

similar pattern of substrate specific selectivity was also detected in *P. didactyla* s. lato. Thus, specimens of *P. didactyla* II growing on mossy logs or rock in forests associated with *Nostoc* genotype B, while terricolous specimens of *P. didactyla* I and III housed different *Nostoc* genotypes (Fig. 4). Our results indicate that some groups of *Nostoc* genotypes are largely confined to specific habitat types (e.g. grasslands versus forests) and/or certain substrata (e.g. ground versus tree bases). Such symbionts are shared between closely related fungal taxa, but also between distantly related species representing different sections of the genus *Peltigera*. For example, in xerophytic habitats there was rampant cyanobiont sharing, not only among terricolous taxa of the section *Peltigera* (e.g. *P. ponojensis* and *P. rufescens*), but also between ecologically similar species from other sections (*P. neckeri* from Horizontales and *P. leucophlebia* from Chloropeltigera). Another such mixed group of *Peltigera* species preferred mesic forests and centered on a group of closely related *Nostoc* genotypes (genotypes C1-C3, Figs. 2 and 3). Their cyanobionts were never found from among the other *Peltigera* species in the region. As a further example, *Nostoc* genotype B was common in *Peltigera* species that grew on mossy tree bases or logs in shaded forest habitats (Figs. 2 and 4). *Peltigera polydactylon* (section *Polydactylon*) relied on this *Nostoc* genotype, and *P. praetextata* and several other taxa of the section *Peltigera* also frequently housed the same cyanobiont.

The habitat specific selection of the trnL *Nostoc* genotypes found in this study indicates the presence of a guild structure similar to that found in many other cyanolichens (Rikkinen et al., 2002; Myllys et al., 2007; Elvebakk et al., 2008; Kaasalainen et al., 2013; O'Brien et al., 2013; Joneson and O'Brien, 2017) and green algal lichens (Beck et al., 1998; Peksa and Škaloud, 2011; Dal Grande et al., 2014). Photobiont-mediated guilds are communities of lichenized fungi often occurring in the same habitat, and are horizontally linked through photobiont sharing (Rikkinen, 2003; Dal Grande et al., 2014). In Estonia, certain *Nostoc* genotypes were shared by *Peltigera* species that only occurred in mesic forests, while others were shared by species growing on xerophytic open grasslands. Such division of the photobiont association according to the habitat and substratum type indicates that the guild structure is linked not only to the environmental requirements of mycobionts but also to those of the *Nostoc* cyanobionts. On a functional level, some lichen guilds have been suggested to involve two types of mycobionts: core species that effectively disperse photobionts in their symbiotic diaspores, and fringe species that exploit these photobionts but mainly disperse via fungal spores. Thus, core species can effectively maintain a viable population of photobionts at the local scale, and these photobionts are also exploited and

incorporated into fringe species via horizontal transmission (Rikkinen et al., 2002; Rikkinen, 2003; Dal Grande et al., 2014). Considering the common occurrence of *Nostoc* genotype B in Estonian forests, *P. praetextata*, which often produces symbiotic diaspores (phylidia), may possibly function as a core species. Other *Peltigera* species, which also depend on *Nostoc* genotype B but only reproduce via ascospores, may behave as fringe species that directly benefit from cyanobionts dispersed by *P. praetextata*.

Ramirez-Fernandez et al. (2013) reported that in the Chilean Patagonian region (South America), the diversity of *Peltigera* cyanobionts was higher in native forests with low or medium disturbance intensity than in grasslands with high disturbance intensity. When comparing the diversity of the symbiotic *Nostoc* genotype between different habitats and substrata in Estonia, high diversity of *Nostoc* genotypes associated with *Peltigera* taxa was found in many different habitats: on ground in roadside grasslands, on dunes or eutrophic forests, and on mossy rocks in eutrophic forests (Table 1).

A comparatively high diversity of *Nostoc* genotypes was found also associating with *Peltigera* specimens collected from alvar grasslands. The Estonian grasslands are usually characterized by high soil pH and they support high diversity of different *Peltigera* taxa (Juriado et al., 2017). The alvars are the most peculiar and extreme grassland habitats in this region, both with respect to soil type and their properties (Koster and Kolli, 2016) and land-use history (Eriksson et al., 2002; Partel et al., 2007). Thin-soil alvars typically support biological soil crusts with highly specific lichen communities (Leppik et al., 2013, 2015; Budel et al., 2014). Two *Peltigera* taxa (*P. "neorufescens"* agg.) that were typically found in alvar grasslands (Juriado et al., 2017) are phylogenetically well defined (Miadlikowska et al., 2003; Juriado et al., 2017) and will potentially represent new species. In addition to their preference for nutrient-rich calcareous soil, they seem only to associate with a specific group of *Nostoc* genotypes (Fig. 4).

A comparatively low diversity of *Nostoc* genotypes was found in *Peltigera* specimens collected from mossy tree bases and logs (Table 1). Accordingly, Fedrowitz et al. (2011) found only five closely related *Nostoc* genotypes in 232 epiphytic *Nephroma* thalli representing three different species. Such findings may suggest that the pool of compatible *Nostoc* genotypes on tree trunks or logs may be more limited compared to those occurring on moss-covered rocks, mossy ground or bare soil (Zuniga et al., 2017). On the other hand, epiphytic bryophytes on tree trunks and logs may often act as a reservoir of compatible *Nostoc* genotypes (e.g. Rikkinen et al., 2002). For example, epiphytic mats of the liverwort *Frullania asagrayana* harbors lichen symbiotic

*Rhizonema* strains and seems to provide nursery beds for the establishment and growth of *Erioderma pedicellatum* (Cornejo and Scheidegger, 2016).

In conclusion, we established that mycobiont identity was the most important factor determining the presence of specific *Nostoc* genotypes within the *Peltigera* thalli. However, the pattern of cyanobiont selectivity also correlated with environmental variables. In eutrophic and mesic forests the widespread and prolific sharing of some *Nostoc* genotypes between several different *Peltigera* taxa indicates that guild interactions are important in the habitat ecology of these lichens. The same applies to certain groups of terricolous *Peltigera* species on calcareous soils, including the two *Peltigera* species that are adapted to the unique environmental conditions of alvar grasslands. As several phylogenetically defined taxa (Juriado et al., 2017) showed a distinct pattern of *Nostoc* genotype specificity, which was also in correlation with habitat conditions, our findings of symbiont specificity may help delimit the undescribed species.

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## **Appendix A. Supplementary data**

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.funeco.2018.11.005>.

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## TABLES

**Table 1.** Number of *Peltigera* taxa, number of *Nostoc* sequences and genotypes, and genotype diversity (Hd) in combined substratum and habitat groups. Abbreviations of combined variable of substratum and habitat: 'Ground\_alvar'=ground in alvars, 'Ground\_dune'=ground on dunes, 'Ground\_road'=ground in roadside grasslands, 'Ground\_eutr'=ground in eutrophic forests, 'Ground\_oligotr'=ground in oligotrophic forests, 'Ground\_park'=ground in park stands, 'Rock\_eutr'=rocks in eutrophic forests, 'Rock\_park'=rocks in park stands, 'Tree\_eutr'=trees in eutrophic forests.

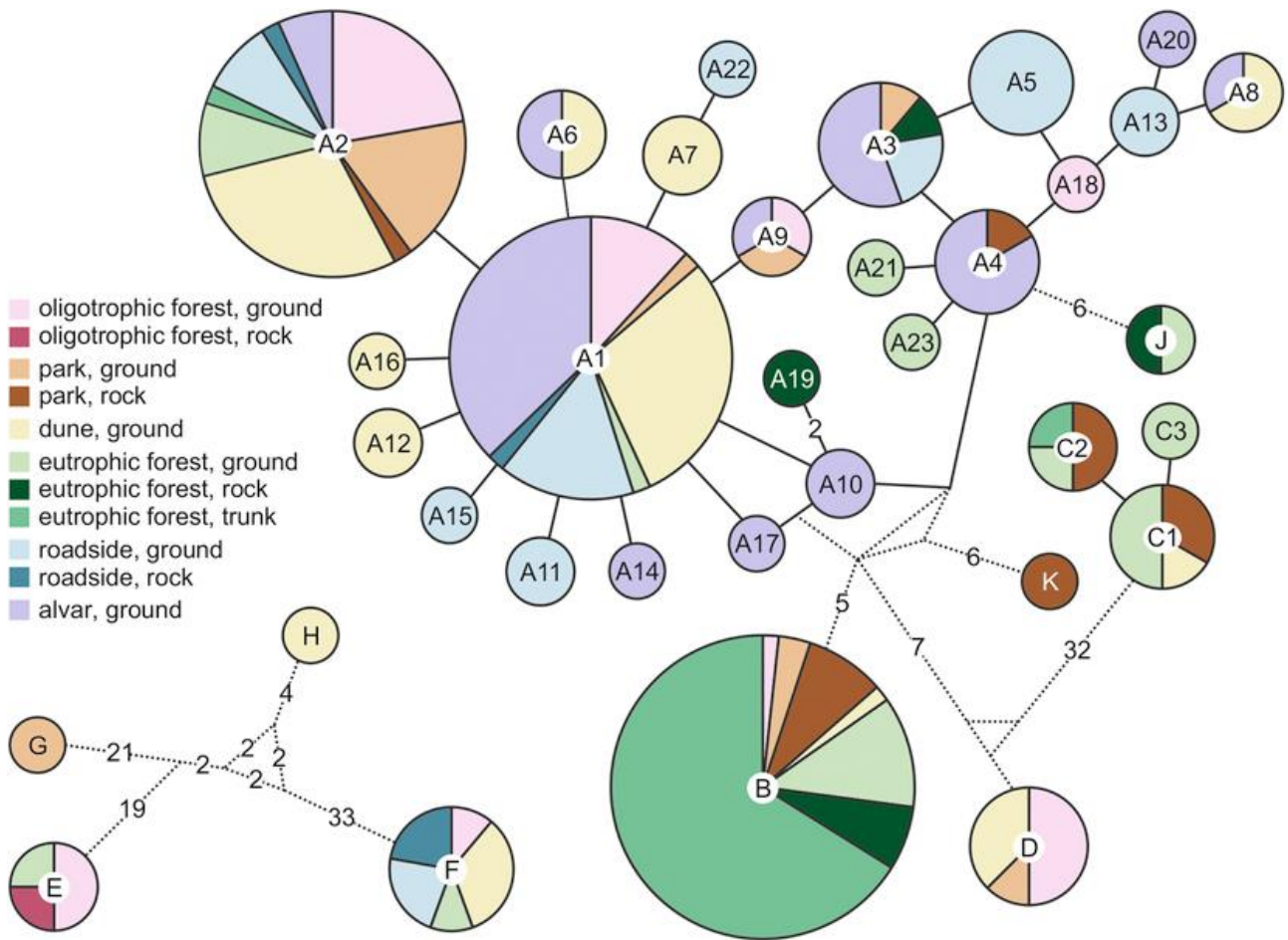
Substratum and habitat	No. of <i>Peltigera</i> taxa	No. of <i>Nostoc</i> sequences	No. of <i>Nostoc</i> genotypes	Hd
Ground_alvar	10	40	10	0.75
Ground_dune	15	43	10	0.79
Ground_road	12	26	7	0.83
Ground_eutr	12	19	8	0.82
Ground_oligotr	9	24	7	0.76
Ground_park	8	14	5	0.67
Rock_eutr	8	9	5	0.81
Rock_park	8	10	5	0.75
Tree_eutr	7	42	4	0.14

**Table 2.** Results from variation partitioning analysis (VPA), partitioning variance in *Nostoc* genotypes (presence/absence) found in *Peltigera* onto two variable sets of ‘Mycobiont species’ and ‘Habitat’. ‘Mycobiont species’ – 18 taxa of *Peltigera* (see Supplementary Table 1), ‘Habitat’ – the combined variables of substratum and habitat (see Table 1).

<b>Component</b>	<b>Variance explained</b>	<b>% variance explained</b>	<b>% of total variation</b>	<b>F</b>	<b>P</b>
Unique effect of ‘Mycobiont species’	4.30	69.8	45.6	5	0.001
Unique effect of ‘Habitat’	0.67	11.0	7.2	1.5	0.001
Shared effect of ‘Mycobiont species’ and ‘Habitat’	1.18	19.2	12.6	4.7	0.001
Total explained	6.15	100	65.3		
All variation	9.42		100		

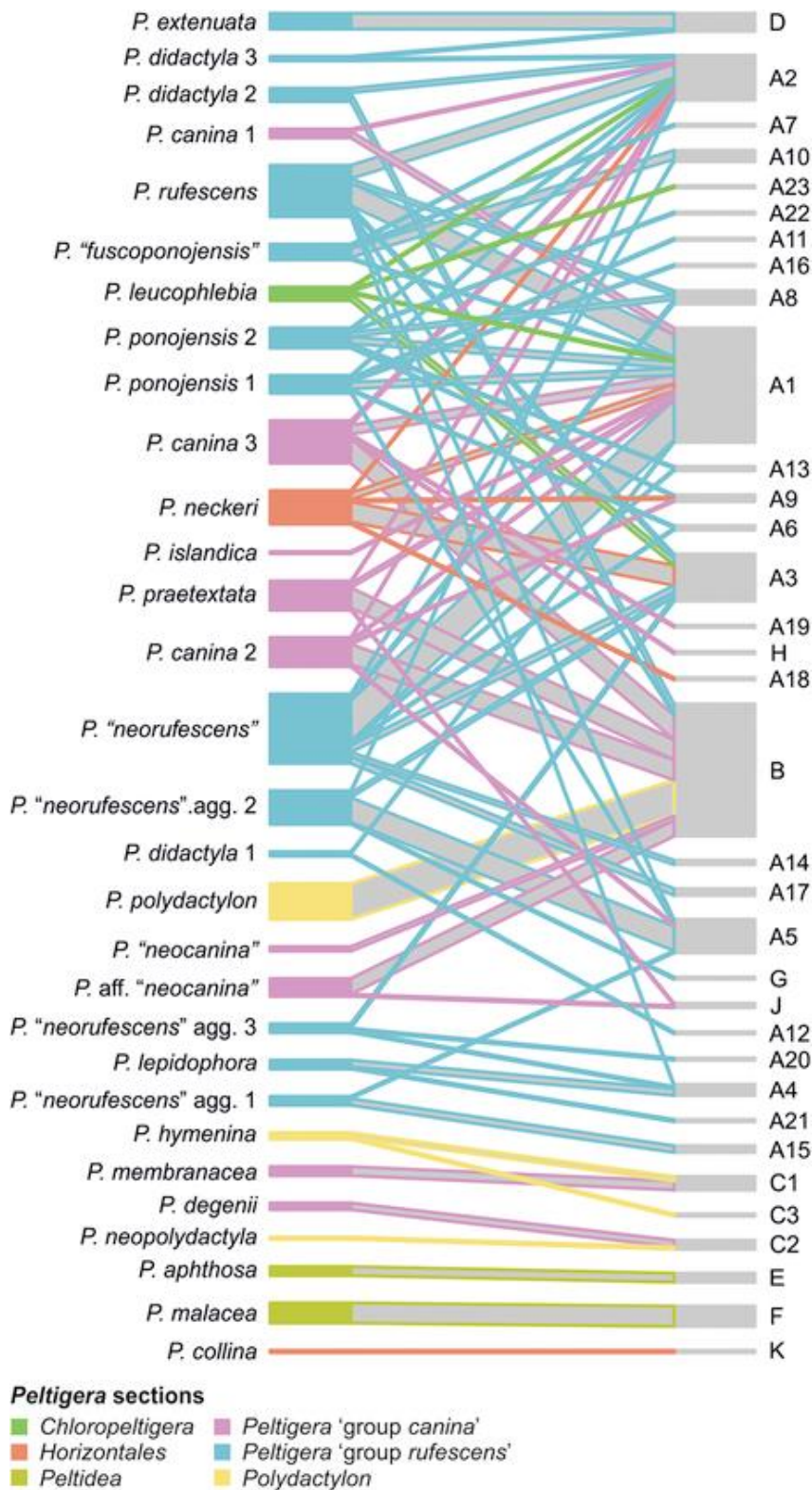
F – F-criterion value, P – significance level.

FIGURES

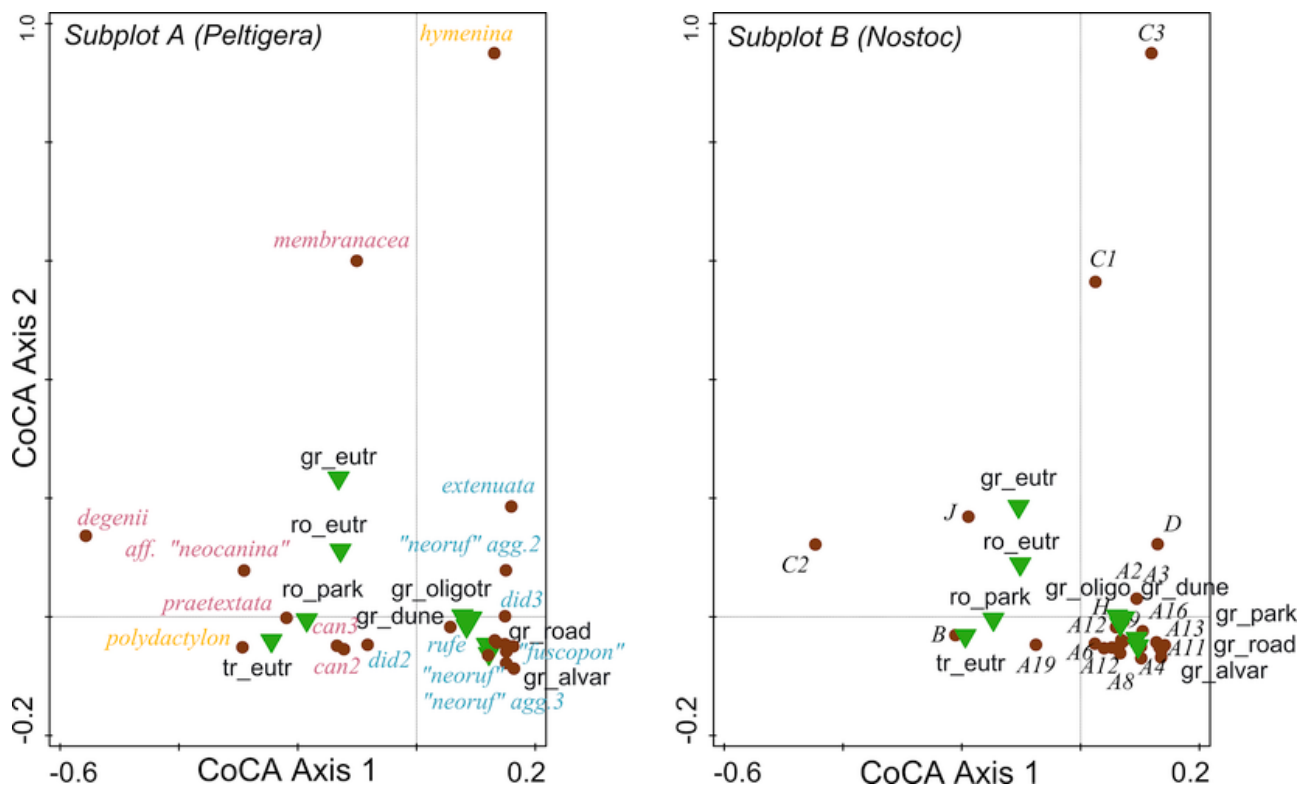


**Fig. 1.** *Nostoc* trnL genotype networks. trnL sequences with Class 1 (small network on lower left) and Class 2 (large, on right) P6b regions were analysed separately. The number of single nucleotide differences is shown on connecting lines; genotypes separated by six or more differences are connected via dashed lines and denoted by different letters. The size of each pie chart is proportional to the number of specimens (1–59); the colours of the slices represent different habitats and substrata (light shades indicate ground and dark shades indicate rock).

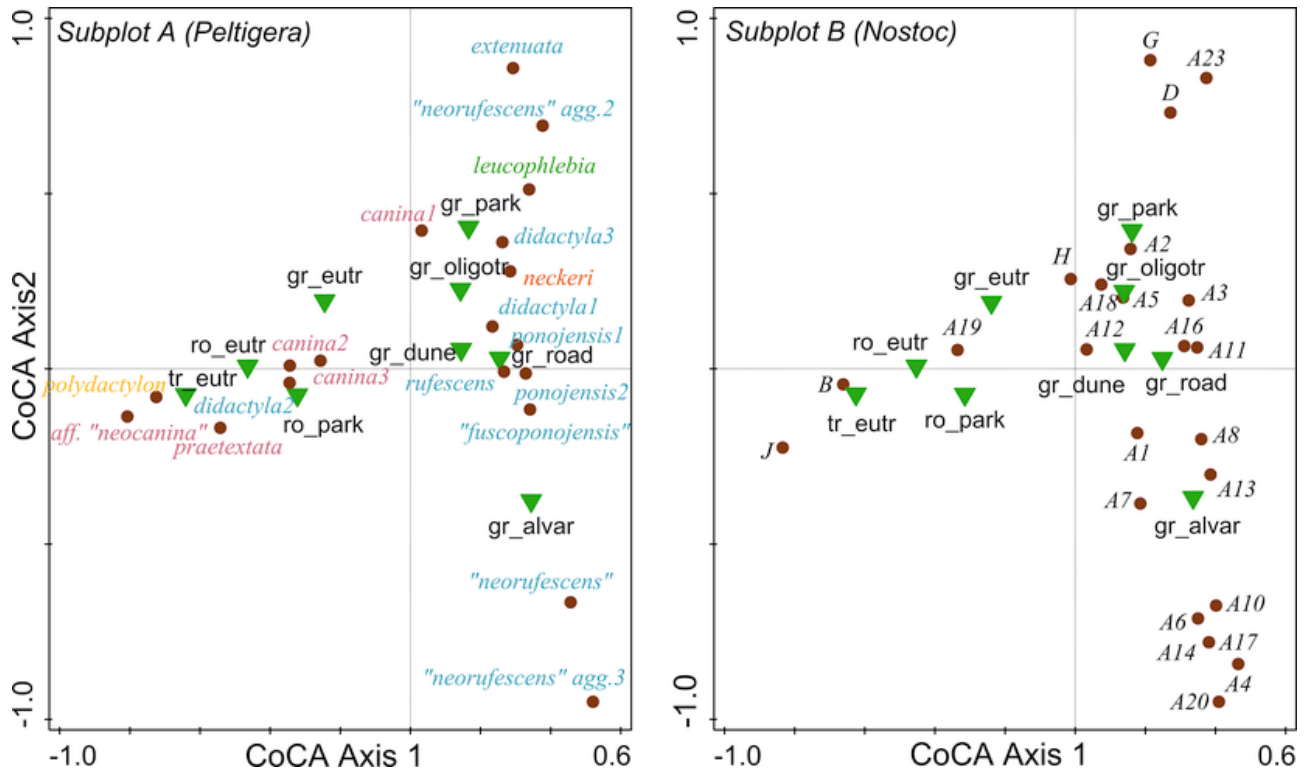




**Fig. 2.** A bipartite interaction network of *Peltigera* taxa (on left) and cyanobiont trnL genotypes (on right). Different sections of *Peltigera* (Miadlikowska and Lutzoni, 2000) and the groups 'canina' and 'rufescens' within the section *Peltigera* (Juriado et al., 2017) are separated by using different colours.



**Fig. 3.** Symmetric co-correspondence (CoCA) analysis of 22 *Peltigera* taxa (subplot A) and 26 symbiotic *Nostoc* genotypes (subplot B). The subplots of the dual diagram show the first two axes (axes 1 and 2), and the environmental descriptors are passively projected into the subplots as filled triangles. Abbreviations of the substrata 'gr'=ground, 'ro'=rock, 'tr'=tree. Abbreviations of the habitat types 'alv'=alvar, 'dune'=dunes, 'eutr'=eutrophic forests, 'oligo'=oligotrophic forests, 'park'=park stands, 'road'=roadside grasslands. Abbreviations of the *Peltigera* taxa (see Table S1) 'can'=*P. canina*, 'did'=*P. didactyla*, 'fuscopon'=*P. "fuscoponjensis"*, 'neoruf'=*P. "neorufescens"*, 'ruje'=*P. rufescens*. Different sections of *Peltigera* (Miadlikowska and Lutzoni, 2000) and the groups 'canina' and 'rufescens' within the section *Peltigera* (Juriado et al., 2017) are separated by using different colours as in Fig. 2.



**Fig. 4.** Symmetric co-correspondence (CoCA) analysis of 19 *Peltigera* taxa (subplot A) and 24 symbiotic *Nostoc* genotypes (subplot B). The subplots of the dual diagram show the first two axes (axes 1 and 2), and the environmental descriptors are passively projected into the subplots as filled triangles. Abbreviations of the substrata and habitat types as in Fig. 3. Different sections of *Peltigera* (Miadlikowska and Lutzoni, 2000) and the groups ‘*canina*’ and ‘*rufescens*’ within the section *Peltigera* (Juriado et al., 2017) are separated by using different colours as in Fig. 2.