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Assessing the impact of alkaline dust pollution on the genetic variation of populations of a widespread epiphytic lichen, *Usnea subfloridana* (Parmeliaceae, Ascomycota)

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Abstract

Very little is known whether and how air pollution impacts genetic diversity of lichenized fungi that are well-known indicators of environmental quality. We studied the genetic variation of eight *Usnea subfloridana* populations in *Pinus sylvestris*-dominated boreal forest stands in southern Estonia, Northern Europe; four of these populations were exposed to long-term dust pollution released from unpaved road. The mean bark pH of lichen-phorophyte differed considerably between polluted and unpolluted forest stands. We genotyped 274 *Usnea* thalli using nine specific fungal microsatellite markers. Genetic variation measures were calculated and compared between populations from different habitats. Allelic richness, Shannon's information index, and genetic diversity of lichen populations were significantly higher in unpolluted forest sites than in polluted forest sites. We conclude that environmental disturbances caused by alkaline dust pollution had negative impact on the genetic variation of *U. subfloridana*, a common species of lichenized fungi.

Keywords: lichenized fungi, population genetics, particulate matter, dust pollution, Estonia.
1. Introduction

Environmental pollution (including air pollution) is a consequential threat to global biodiversity. The effects of environmental pollution on species diversity have been intensively studied (Ellenberg 2009; Smith 1990), while pollution impact at the genetic diversity of species has received much less attention (van Straalen & Timmermans 2002). The variation at the genetic level is important as it enables evolutionary processes, provides the raw material for adaptation to changing environments and ensures healthy populations (Frankham et al. 2010; Helm et al. 2009; Lacy 1997). Therefore, the loss of genetic diversity, caused by anthropogenic disturbances, could be a serious hazard to natural populations. The knowledge of changes or declines in genetic variation of populations may be used as a warning to vulnerability of populations to environmental changes (Bickham et al. 2000).

The total emission of many air pollutants (e.g. SO$_2$, CO, C$_6$H$_6$) has decreased in Europe, but particulate matter (PM) is still a problematic pollutant as it poses a significant harm to human health and environment (Guerreiro et al. 2014). Primary PM$_{10}$ (larger dust particles, hereafter dust pollution) is released to environment from rock quarrying, combustion processes, kiln grinding or from surfaces of gravel roads by intensive traffic. As larger particles are dispersed by the wind not far from their source, the dust pollutants deposit generally in the vicinity of power plants, cement industries, limestone quarries or unpaved roads, and have a considerable impact on local environment (Gilbert 1976; Mandre 1995; Paal & Degtjarenko 2015; Paoli et al. 2014). Emissions of alkaline dust pollution increase the pH value and change chemical composition of soil and other substrates, hence altering the composition of plant communities and species richness (Farmer 1993). For example, soil long-term alkalization evokes the ‘nemoralization’ of pine forest ecosystems, increasing species richness and increasing the frequency of non-common plants, which are generally lacking in the natural ecosystems (Paal et al. 2013).

Lichens, being composite associations, are widely used as indicators of air pollution (Nimis et al. 2002) due to their physiological and metabolic peculiarities and
differential sensitivity to specific air pollutants (Nash III 2008). Alkaline dust pollution may impact lichens directly, changing element concentration in lichen thalli (Kortesharju & Kortesharju 1989), inducing chlorophyll degradation of photobionts (Zaharopoulou et al. 1993) or causing necrotic damages of thalli (Jóźwiak & Jóźwiak 2009). Long-term alkaline dust pollution, which increases the pH value of tree bark, influences species richness and composition of epiphytic lichen communities. For example, acidophytic lichens are replaced by neutro- and nitrophytic species, and reduction of fruticose species is compensated by an increase of foliose and crustose species along a dust pollution gradient (Kortesharju & Kortesharju 1989; Loppi & Pirintsos 2000; Marmor et al. 2010; Paoli et al. 2014).

Given the fact that environmental pollution generally affects mortality and size of lichen populations, we hypothesize that dust pollution influences the genetic structure of lichen populations as well. Only one previous study addresses genetic variation of lichen symbionts in conditions of air pollution, viz., Ohmura et al. (2006) demonstrated that the genetic diversity of Parmotrema tinctorum (Despr. ex Nyl.) Hale photobionts was lower in the urban area than in suburbs and mountainsides. In addition, pollution stress may drastically change habitat size and habitat quality of species (Lovett et al. 2009), and therefore potentially affects genetic structure of lichen populations. Previous population studies of a widespread, although regionally rare and threatened lichen-forming fungus, Lobaria pulmonaria (L.) Hoffm. have shown that genetic diversity of its populations was higher in old-growth than in younger forests (Jüriado et al. 2011), and in primeval than in managed forests (Scheidegger et al. 2012). Habitat quality (host tree age) appeared more important in affecting the genetic pattern of Lobaria populations than spatial distance (Otalora et al. 2011), and different types of habitat disturbances (fires, logging) also impacted the genetic diversity of its populations (Werth et al. 2006).

There are different techniques for DNA-based population studies of which microsatellite markers as highly polymorphic and not affected by selection are considered most promising for estimating genetic variation and population structure of often clonally reproducing organisms such as lichenized fungi (Werth 2010). The high variability of microsatellite loci provides more power to estimate the genetic diversity than sequence-based method. However, using microsatellite markers has
A number of pitfalls – in order to be able to work with new taxa, usually new primers have to be developed, and primer development and testing are relatively expensive and time-consuming (Selkoe & Toonen 2006; Werth 2010). Till now, microsatellite primers have been developed for several lichenized fungi and allied species: *Bryoria capillaris* (Ach.) Brodo & D. Hawksw. (Nadyeina et al. 2014), *Buellia frigida* Darb. (Jones et al. 2012), *Lobaria pindarensis* Räsänen (Devkota et al. 2014), *L. pulmonaria* (Dal Grande et al. 2010; Walser et al. 2003; Werth et al. 2013), *Lobarina scrobiculata* (Prieto et al. 2015), *Nephroma laevigatum* Ach. and *N. parile* (Ach.) Ach. (Belinchon et al. 2014), *Parmelina carporrhizans* (Taylor) Poelt & Vězda (Alors et al. 2014), *Parmotrema tinctorum* (Mansournia et al. 2012), *Peltigera dolichorhiza* complex (Nyl.) Nyl. (Magain et al. 2010), *Protoparmeliopsis muralis* (Schreb.) Choisy (Guzow-Krzenieńska & Stocker-Wörgötter 2013), and *Usnea subfloridana* Stirt. (Tőrre et al. 2014). Just a few of those species (*B. capillaris, L. pulmonaria, P. muralis,* and *U. subfloridana*) are widely distributed in northern hemisphere, and of these few only two taxa, *B. capillaris* and *U. subfloridana*, are common in the boreal zone, inhabiting coniferous trees. We chose the latter as the target species for evaluating the genetic variation of lichen populations in conditions of air pollution in boreal forests because the species boundaries in *Bryoria sect. Implexae* are still disputable (Myllys et al. 2011; Myllys et al. 2014).

We aimed to study the effects of limestone dust pollution from unpaved roads on the genetic variation of *Usnea subfloridana* populations using fungus-specific microsatellite markers. We examined and compared the various variables of genetic variation of *U. subfloridana* populations from polluted and unpolluted forest sites, hypothesizing that genetic variation was lower in polluted than in unpolluted populations of this lichenized fungi. To our knowledge, no previous study has investigated the microsatellite variation of lichen populations under long-term air pollution, especially under alkaline dust pollution. This is also the first research where recently developed fungus-specific microsatellite markers of *U. subfloridana* (Tőrre et al. 2014) have been used to evaluate the genetic diversity of this common epiphytic lichen-forming fungus.

### 2. Material and methods
2.1 Study species

*Usnea subfloridana*, a shrubby corticolous lichen, is widely distributed throughout Europe (Randlane et al. 2009; Smith et al. 2009). It is very frequent in Estonia and has been recorded in most regions growing mainly on coniferous trees – Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*), but also on Silver birch (*Betula pendula*) (Tõrra & Randlane 2007). *Usnea subfloridana* is not protected in Estonia, and belongs to the category Least Concerned in the Red List of Estonia (Randlane et al. 2008). It propagates asexually by symbiotic propagules such as soredia and isidia; sexual reproduction is possible as well, however, apothecia occur extremely rarely. Phylogenetic studies suggest that *U. subfloridana* is not a monophyletic entity, but may constitute a single species together with *Usnea florida* (L.) Weber ex F.H. Wigg., which reproduces sexually (Articus et al. 2002; Kelly et al. 2011; Saag et al. 2011; Mark et al. 2016). *Usnea florida* is typically richly fertile and is lacking vegetative propagules; furthermore, its ecological preferences differ from those of *U. subfloridana* as the former prefers to grow on old deciduous trees in areas with high atmospheric humidity (Randlane et al. 2009; Smith et al. 2009). *Usnea florida* is not known in Estonia (Tõrra & Randlane 2007).

2.2 Study area

The study was carried out in Estonia, which has a temperate climate; the mean annual temperature is 5°C, and the total annual precipitation is 770 mm (Estonian Weather Service 2014). The vegetation of Estonia belongs to the hemiboreal subzone of the boreal forest zone, lying in the transitional area, where the southern taiga forest subzone changes into the spruce-hardwood subzone (Ahti et al. 1968; Laasimer & Masing 1995). The study area is located in *Pinus sylvestris*-dominated boreal forests in southern Estonia, Põlva county (58°8′N 27°2′E), where the target species is abundant (Fig 1), and unpaved limestone gravel roads still exist. Forests belonging to the *Oxalis-Vaccinium myrtillus* site type, the *Vaccinium myrtillus* site type, and the *Vaccinium vitis-idaea* site type were selected for this study. Those forest site types are also widely distributed in other Baltic states (Bušs 1997; Kairiūkštis 1966), in
According to the historical map (Historical Map Collection 2014), the studied forest stands have been recorded as forests at least from the end of 19th / beginning of 20th century (earlier maps not available). The unpaved road, which is the source of limestone dust pollution in our study, has been indicated as a road on the map from the same period, however, serious pollution started about 60 years ago, in 1960s when motor vehicles, including agricultural machines, became widely used (Fig 2).

**Figure legend**

**Fig 1** – The distribution map of *Usnea subfloridana* in Estonia, and the locality of study area (marked with red circle) in southern Estonia, Põlva county (58°8’N 27°2’E).

**Fig 2** – Dust pollution caused by a motor vehicle on a typical unpaved road in Estonia (photo Pauli Saag).

### 2.3 Sampling

Fieldwork was carried out during summer 2011 and autumn 2012. Eight *U. subfloridana* populations were studied; four of them within polluted forest stands, which were adjacent to unpaved roads, and four populations within undisturbed forest stands, which were situated more than 180 meters from the source of dust pollution, as reference data (Table 1). *Usnea* populations were delimited according to the boundaries of forest sites having different average age of the lichen phorophyte (Forest Public Registry 2012). A total of 310 shrubby *Usnea* thalli were randomly sampled from Norway spruce in forest stands with the average age of spruces (between 70–114 years) (Table 1). The samples were collected up to six meters from the ground using tree pruner. On average, three thalli were randomly taken from a tree. If there were less than three thalli, only one or two specimens were sampled while in other cases more than three (but not more than five) specimens were collected per tree to balance sampling. In small populations (with not required three
specimens available per tree and with only a few trees inhabited by the study species), the scarce sampling relatively reflected the population size.

2.4 Chemical and molecular analyses

Specimens were air dried, cleaned from other lichen thalli and examined under a stereomicroscope; all collected thalli were sterile, i.e. without apothecia. Secondary compounds of 310 specimens were identified using thin layer chromatography (TLC) with solvent A (Orange et al. 2001). According to morphological and chemical characters, 274 specimens were identified as *U. subfloridana*, and 36 specimens were eliminated from further analyses as they belonged to other *Usnea* species (*U. glabrescens* (Vain.) Vain. or *U. wasmuthii* Räsänen). Then, 50 mg of each *U. subfloridana* specimen was kept frozen in 1.5 mL microtubes at -20°C until further treatment.

Total DNA was isolated using PowerPlant® Pro DNA Isolation Kit (MO BIO Laboratories, Inc., USA) according to the manufacturer’s protocol. Nine unlinked fungus-specific microsatellite loci (*Us01, Us02, Us03, Us04, Us05, Us06, Us07, Us08, Us09*) were analyzed and amplified in three different multiplex PCR following the protocol described in Tõrra et al. (2014) except the reverse primer sequences, which had erroneously been presented as direct sequences from the genome. Therefore, we converted the reverse primer sequences by Tõrra et al. (2014) using the Reverse Complement software (Reverse Complement; Table 2). Additionally, the final primer concentration of *Us01* (multiplex I) was increased to 0.05 µM to get PCR amplification (see Tõrra et al. (2014) for other primer concentration). Fragment lengths of PCR products were determined on a 3730xl DNA Analyzer (Applied Biosystems, Zurich, Switzerland). The electropherograms were analyzed using GENEMAPPER v5 (Applied Biosystems) using LIZ-500 (all multiplexes) as size standard.

2.5 Bark pH measurement
Two bark pieces from five random spruces were collected at the height of 120 cm from the ground in each forest site (altogether from 40 trees); in polluted forest sites one piece of bark was taken from the northern side (N), which was adjacent to unpaved road, and second piece from the opposite, southern, side (S) of spruce tree. In unpolluted forest sites, bark pieces were analogously collected, keeping the same cardinal direction. The bark pH was measured in laboratory using a flathead pH meter Consort C532. To allow rapid solution of hydrogen ions, 0.5 mL of 0.1 M KCl was dripped on the bark 1 min before the measurement following Schmidt et al. (2001). The mean bark pH of every tree was expressed as an arithmetic mean of two measurements of bark pH values (all calculations of mean pH were based on mean hydrogen ion concentrations and then transformed back into pH value).

2.6 Statistical analyses

The total number of alleles, the mean number of alleles, the maximum and the minimum number of alleles, the number of private alleles (P), heterozygosity, i.e. genetic diversity (H), and Shannon's information index (I) for eight Usnea subfloridana populations were calculated in the GenAlEx ver 6.5 software (Peakall & Smouse 2012) and the Microsatellite Analyzer (MSA) software (Dieringer & Schlötterer 2003). As P is strongly related to the sample size (Kalinowski 2004), this measure was standardised using the rarefaction procedure implemented in the software HP-Rare (Kalinowski 2005). The number of multilocus genotypes (G), the percentage of multilocus genotypes, i.e. clonal diversity (M; G divided by the number of collected thalli in each population), and the minimum number of colonisation events (C; the number of alleles at the most variable locus, in this case ignoring potential mutations) per population were estimated in the R software (R Core Team 2012) using the script written by Werth et al. (2006). The clonal diversity is the probability that the next sampled specimens will be a different genotype. The allelic richness (A) was measured using the software MSA (Dieringer & Schlötterer 2003). The measures of A were also corrected for variation in sample size using the rarefaction method as applied in the software MSA. Spearman's rank correlation was used to test correlation between the number of collected thalli in each population, i.e. sample size (N) with G, M, C, A, I, P, and H. Generalized linear model (GLZ)
analysis with normal distribution and “identity” link function in the software

STATISTICA 7 (StatSoft 2004) was applied to find an effect of dust pollution on the
G, M, C, A, I, H, and P of *U. subfloridana* populations; the square root of average age
of spruces in forest sites (sqrt AGE) and the square root of sample size (sqrt N) were
taken as continuous predictor variables. To estimate genetic differentiation among
populations from polluted and unpolluted habitats, hierarchical analyses of molecular
variance (AMOVA) with 999 permutations was performed using the GenAIEx ver 6.5
software (Peakall & Smouse 2012). T-test was used for detecting the differences
between polluted and unpolluted forest stands (according to bark pH value of spruces)
using the software STATISTICA 7 (StatSoft 2004). To measure bottlenecks of
population (to undergone the significant reductions in size or bottleneck effect), the
Wilcoxon signed-rank test and the allele frequency distribution test (mode-shift test)
were performed using the software BOTTLENECK (Piry et al. 1999). For two-phase
mutation model (TPM), we set *ps* = 0.9 (the frequency of single step mutations), and
the variance of those mutations as 12, which is considered relevant for microsatellite
data (Piry et al. 1999).

3. Results

3.1 Bark pH

The bark pH was measured on 40 spruces, from two different sides of each tree. The
absolute pH value per one side of a tree varied between 3.27 and 7.00, and the mean
pH value per forest site ranged between 3.49 and 6.63 (Table 1). According to the
results of t-test, the mean bark pH of spruces significantly differed between polluted
and unpolluted forest stands (t=14.6; df=6; p<0.00001).

3.2 Genetic variation of *Usnea subfloridana* in the study area

In total, we analysed and genotyped 274 specimens from eight *Usnea subfloridana*
populations (Table 1). We found 72 alleles at nine microsatellite loci, which provided
168 different genotypes across eight lichen populations. All microsatellite loci were
highly polymorphic. Allele size distribution per loci varied from 106 bp in locus *Us06* to 368 bp in locus *Us09*. The allele sizes of *Us02* and *Us05* were not in agreement with those recently described in Törra et al. (2014; Table 2). The allele sizes in loci *Us03*, *Us06*, and *Us08* were longer than analogous allele sizes according to Törra et al. (2014; Table 2). The minimum total number of alleles per loci was three (*Us04*) and the maximum total number of alleles per loci was 14 (*Us01*). The mean number of alleles per loci ranged from 2.63 to 7.50 (mean 5.1; Table 3). The clonal diversity varied between 0.76 and 0.91 (Table 1). The minimum number of colonisation events per population (C) ranged from five to ten (Table 1). Other detailed genetic variables and number of alleles per each marker and population are given in Tables 1 and 3.

### 3.3 Genetic variation of *Usnea subfloridana* in relation to the studied variables

The results of Spearman's correlation analyses showed that several measures of genetic variation were positively correlated with the number of collected samples (N): minimum number of colonisation events per population (C), number of multilocus genotypes (G), allelic richness (A), Shannon's information index (I), and number of private alleles (P). However, N had no significant effect on genetic diversity (H) and clonal diversity (M) (results of analyses not shown). The generalized linear model (GLZ) showed that the presence of dust pollution and sqrt N revealed significant influence on allelic richness (A); this measure was higher in unpolluted forest sites than in polluted forest sites (Table 4). Additionally, the presence of dust pollution revealed the same significant impact on the Shannon's information index per population (I) and genetic diversity (H), but sqrt N revealed significant influence only on I (Table 4). GLZ also showed that the number of multilocus genotypes (G) and the number of private alleles (P) were significantly depending on the sample size (sqrt N), but did not vary among polluted and unpolluted forest sites (Table 4). There was no statistically significant association between clonal diversity (M) and dust pollution, and M did not correlate with sqrt N (Table 4). There was also no statistically significant interaction between C and the presence of dust pollution (data not shown). The average age of spruces in forest sites (sqrt AGE) did not reveal significant influence on any measures of genetic variation in all CLZ analyses (Table 4, results of analyses shown partially). Hierarchical analyses of molecular variance (AMOVA)
results revealed no genetic differentiation between studied *U. subfloridana* populations from polluted and unpolluted forest sites (1% differentiation; PhiRT=0.01; P=0.06). The proportion of alleles showed a ‘shifted mode’ distribution in population 1 and 5 (Table 5). The results of Wilcoxon signed-rank test under all tree mutation models per population are given in Table 5.

4. Discussion

DiBattista (2008) has suggested that the effects of environmental pollution could influence the genetic variation of vascular plants, invertebrates and vertebrates; pollution could both decrease and increase the genetic variation in populations of different organisms. Very little is known about the effects of environmental pollution on the genetic pattern of cryptogams. Extensive urban pollution lowered the genetic diversity of the epiphytic bryophyte *Leptodon smithii* (Spagnuolo et al. 2007a); moreover, gene diversity was correlated with airborne trace element content in moss tissue (Spagnuolo et al. 2009). No studies have been performed about the genetic variation of lichenized fungi in polluted conditions; however, lower genetic diversity of the photobionts of the lichenized fungus *Parmotrema tinctorum* was demonstrated in the urban area compared to that in suburbs and mountainsides (Ohmura et al. 2006).

We studied the genetic variation of eight *Usnea subfloridana* populations using nine fungus-specific microsatellite markers. Four of those populations were exposed to the source of long-term moderate alkaline dust pollution released from unpaved road, and other four were from undisturbed forest stands. Our results indicated that the number of multilocus genotypes (168 different genotypes across all populations) was relatively high for the lichenized fungus *U. subfloridana*, which commonly reproduces asexually by symbiotic propagules (apothecia indicating sexual reproduction were not observed in any of our specimens). This could refer to the large regional population with high levels of genetic diversity and long-range dispersal. *Usnea subfloridana* is, indeed, one of the most common *Usnea* species in Estonia, with very frequent occurrence in southern Estonia (Tõrra & Randlane 2007), where the study area is situated (Fig 1). Previous studies have also demonstrated cases of
high genetic variability in other clonal cryptogams such as the bryophyte *Pleurochaete squarrosa* (Spagnuolo et al. 2007b). We demonstrated that the number of multilocus genotypes did not differ in *U. subfloridana* populations among polluted and unpolluted forest sites, however, the sample size had a significant effect on this measure, i.e. G values were higher in large lichen populations than in small ones (Tables 1 and 4). We also found no significant differences between the minimum numbers of colonisation events (C) in populations among different habitats. Therefore, we hypothesize that the studied *U. subfloridana* populations developed from multiple colonization events from large, genetically diverse source populations.

We also detected that clonal diversity revealed high values (M=0.76–0.91, Table 1) in all eight *U. subfloridana* populations and no significant differences were recorded in *U. subfloridana* populations among polluted and unpolluted forest sites. The observed similarity of clonal diversity indicates that there were no differences in population dynamics; *U. subfloridana* populations from unpolluted and polluted forest sites belonged to the same demographic stage, obviously reflecting the first generation of thalli after their establishment on spruce trees. We found that the number of private alleles (P) did not significantly differ among *U. subfloridana* populations from unpolluted and polluted forest stands (Table 4). We suggest that detected similarity of P between populations from different habitats indicates that specified alleles have not been lost from populations exposed to road dust pollution.

We recorded several measures of genetic variation that were significantly different in *U. subfloridana* populations in polluted and unpolluted habitats. Populations that developed under long-term alkaline dust pollution exhibited significantly lower values of the allelic richness (A), Shannon's information index (I) and genetic diversity (H) than populations in unpolluted forest sites (Table 4). There are some possible explanations for this result. Recent studies have demonstrated that habitat quality was a crucial factor influencing the genetic variation of lichen populations (Jüriado et al. 2011; Otalora et al. 2011; Werth et al. 2006). Long-term alkaline dust pollution changes drastically local environmental conditions in the vicinity of the source of dust pollution and influences species richness and composition of epiphytic lichen communities (Marmor et al. 2010; Paoli et al. 2014). Indeed, our results also indicated the increased mean bark pH value (5.9–6.6) of spruces growing near the
source of alkaline dust pollution, while natural pH value of spruce bark is about 3.3–3.8 in unpolluted sites of Estonia (Marmor et al. 2010; 3.49–3.56 in our sites without dust pollution). At the same time, Usnea species generally prefer a lower bark pH (Marmor & Randlane 2007), and are usually sensitive to alkaline dust pollution (Martin & Nilson 1992). Moreover, alkaline dust pollution can promote drier microclimatic conditions (Loppi & Pirintsos 2000), and thus decreases habitat quality for Usnea species even more. Therefore, we suggest that reduced habitat quality increased Usnea mortality, which could cause a decline in population size (not directly measured in this study). Hence, Usnea populations in polluted habitats may have experienced a continuing bottleneck, which may reduce the allelic richness (A) of Usnea populations in polluted habitats but not yet other examined measures of genetic variation in this study (for instance M or P). The measure of A is usually more sensitive to the impact of harsh bottleneck and is reduced by bottlenecks faster than other commonly reported measure of genetic variation (Kalinowski 2004; Leberg 2002). The observed measures of I and H support our results (Table 4), although, those measures were not corrected for sample size as A. The bottleneck analysis indicated a strong evidence of the bottleneck only in one lichen population from polluted habitat in this study (Table 5). Recently, it has been suggested that microsatellite-based bottleneck tests often failed to detect bottleneck in populations known to have experienced reductions or detected frequently bottlenecks in putatively stable populations (Peery et al. 2012). For example, the significant heterozygosity excess was also detected under infinite alleles mutation model (IAM) in stable populations (Luikart & Cornuet 1998). Considering the imperfection of microsatellite-based bottleneck tests, we hypothesize that probable population bottleneck (although detected only in one population from polluted habitat), which is caused by considerable changes of local environment due to the presence of alkaline dust pollution, may reduce the genetic variation of U. subfloridana in this study. The changes in genetic variation observed in U. subfloridana populations that were exposed to unpaved roads may theoretically have also experienced the negative edge effect of habitat, which could disrupt spore dispersal and exchange of individuals or genes among populations, resulting in genetic drift, enhancing the genetic differentiation between populations, and reducing the genetic variation (Frankham et al. 2010; Holderegger & Di Giulio 2010). In this study, however, we found an evidence that exchange of individuals still exists between the populations as AMOVA
analysis showed no genetic differentiation (1% differentiation; PhiRT=0.01; P=0.06) among the lichen populations from polluted and unpolluted habitats.

We tested also the possible influence of the average age of spruces in each forest site on the genetic variation of *U. subfloridana* populations as previous studies have shown that habitat quality estimated as the age of forest patches was important in explaining the genetic diversity in populations of another lichen-forming fungus, *Lobaria pulmonaria* (Jüriado et al. 2011; Otalora et al. 2011). In our study, the average age of the lichen phorophyte had no significant effect on the genetic variability of lichen populations (Table 4). This result may be related to the fact that studied spruce forests, both in polluted and unpolluted sites, were relatively young (70–114 and 85–93 years accordingly; Table 1).

5. Conclusions

Our study sheds light on the influence of dust pollution on genetic diversity of lichen population. We recorded for the first time that long-term alkaline dust pollution had a negative impact on allelic richness, Shannon's information index, and genetic diversity of lichen populations, while some other estimates of genetic variation (e.g. clonal diversity or the number of private alleles per population) were not significantly affected by the alkaline dust pollution. Thus, more research is needed to better understand population demographics influenced by dust pollution. We suggest that probable population bottleneck caused by changes of local environment due to the presence of alkaline dust pollution could result in the reduction of genetic variation of *Usnea subfloridana* in this study. Changes in genetic structure and loss of genetic diversity in populations of common lichen-forming fungi may be used as a warning to vulnerability of populations to environmental disturbances. Moreover, this knowledge of negative effect on genetic variation of populations might be functional for conservation prospects in future, for example, when estimating viable effective population size.

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References


Table 1 – Overview of the studied *Usnea subfloridana* populations in polluted forest sites (1–4) and unpolluted forest sites (5–8): sample size, geographic coordinates, phorophyte variables, and measures of genetic variation. Populations, the number of population; Specimens, the number of collected thalli in each population; Trees, the number of trees from which lichens were collected in each forest site; Latitude, latitudinal coordinates of the centre of forest site; Longitude, longitudinal coordinates of the centre of forest site; Age, the average age of Norway spruce in each forest site; Bark pH, the mean bark pH per each forest site; A, allelic richness per population; I, Shannon's information index per population; H, gene diversity per population; G, the number of multilocus genotypes per population; M, clonal diversity per population; C, the minimum number of colonisation events per population; P, the number of private alleles per population.

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<tr>
<th>Variables</th>
<th>Site disturbance</th>
<th>With dust pollution</th>
<th>Without dust pollution</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Populations</td>
<td>1  2  3  4  5  6  7  8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample size</td>
<td>Specimens</td>
<td>11  63  23  10  19  38  50  60</td>
<td>274</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trees</td>
<td>4   19  8   3   7   14  17  21</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>Coordinates</td>
<td>Latitude N</td>
<td>58°08'40.2''  58°08'39.9''  58°08'45.4''  58°08'50.3''  58°08'51.3''  58°08'52.8''  58°08'29.3''  58°07'13.1''</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Longitude E</td>
<td>27°01'49.4''  27°01'53.2''  27°02'13.7''  27°02'21.8''  27°03'23.5''  27°03'14.9''  27°02'57.9''  27°03'03.9''</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phorophyte variables</td>
<td>Age</td>
<td>95  114  70  80  85  90  85  93</td>
<td>3.49  3.55  3.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bark pH</td>
<td>6.63  6.56  5.92  5.97  3.56  3.49  3.55  3.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic variation</td>
<td>A</td>
<td>3.88  4.66  4.08  3.56  4.42  4.66  4.46  4.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>1.05  1.31  1.13  1.07  1.26  1.29  1.25  1.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>0.56  0.64  0.58  0.6  0.64  0.62  0.62  0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>10   48   21   8   17   31   38   51</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>0.91  0.76  0.91  0.8  0.89  0.82  0.76  0.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>6    10   8    5    7    9    8    10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.11  0.23  0.14  0    0.13  0.22  0.19  0.37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2 – Sequences of reverse primers and allele size ranges for microsatellite loci (Us01–Us09) developed for lichenized fungus *Usnea subfloridana* (see Tõrra et al. (2014)) for forward primers.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequence (5’-3’)</th>
<th>Allele size range (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Us01</td>
<td>R: TATCATGGAGACGATGGATC</td>
<td>181–303</td>
</tr>
<tr>
<td>Us02</td>
<td>R: GTGCGTCCTTGTCTTTCTAC</td>
<td>167–179</td>
</tr>
<tr>
<td>Us03</td>
<td>R: ATTTGCCAAAGGATAAGGG</td>
<td>192–233</td>
</tr>
<tr>
<td>Us04</td>
<td>R: CTAAGCTCGAGAAGGATACG</td>
<td>181–196</td>
</tr>
<tr>
<td>Us05</td>
<td>R: TATCTGTGGCTTTCCGCAG</td>
<td>322–346</td>
</tr>
<tr>
<td>Us06</td>
<td>R: GTAATGACGCTGAGTGTGTC</td>
<td>106–130</td>
</tr>
<tr>
<td>Us07</td>
<td>R: AGAGAGATGCTGAGTCTTCT</td>
<td>123–135</td>
</tr>
<tr>
<td>Us08</td>
<td>R: GACGTGATTGGACTGGGATTG</td>
<td>192–223</td>
</tr>
<tr>
<td>Us09</td>
<td>R: TACCCGTTGCGCCATTTCT</td>
<td>341–372</td>
</tr>
</tbody>
</table>

Table 3 – The number of alleles per each microsatellite marker and population in the studied *Usnea subfloridana* populations. Populations, the number of population; Min/Max, the minimum and maximum number of alleles per loci across populations; Mean, the mean number of alleles per loci across populations.

<table>
<thead>
<tr>
<th>Loci</th>
<th>Populations</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>Min/Max</th>
<th>Mean</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Us01</td>
<td></td>
<td>4</td>
<td>10</td>
<td>7</td>
<td>5</td>
<td>6</td>
<td>8</td>
<td>9</td>
<td>11</td>
<td>4/11</td>
<td>7.5</td>
<td>14</td>
</tr>
<tr>
<td>Us02</td>
<td></td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>4/6</td>
<td>5.25</td>
<td>33</td>
</tr>
<tr>
<td>Us03</td>
<td></td>
<td>6</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>9</td>
<td>4/9</td>
<td>7.38</td>
<td>12</td>
</tr>
<tr>
<td>Us04</td>
<td></td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2/3</td>
<td>2.63</td>
<td>3</td>
</tr>
<tr>
<td>Us05</td>
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<td>2</td>
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<td>4</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>2/7</td>
<td>5.25</td>
<td>33</td>
</tr>
<tr>
<td>Us06</td>
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<td>5</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>4/6</td>
<td>5.25</td>
<td>8</td>
</tr>
<tr>
<td>Us07</td>
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<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3/4</td>
<td>3.63</td>
<td>4</td>
</tr>
<tr>
<td>Us08</td>
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<td>4</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>4/7</td>
<td>5.38</td>
<td>8</td>
</tr>
<tr>
<td>Us09</td>
<td></td>
<td>3</td>
<td>7</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>2/7</td>
<td>4.13</td>
<td>8</td>
</tr>
</tbody>
</table>

**Total** | 35 | 56 | 41 | 32 | 42 | 53 | 52 | 56 |  | 5.1 | 72  |
Table 4 – Results of generalized linear model (GLZ) for the allelic richness (A), Shannon’s information index (I), the genetic diversity (heterozygosity) (H), the number of multilocus genotypes (G), the clonal diversity (M), and the number of private alleles (P) in examined *Usnea subfloridana* populations. df, degrees of freedom; F, Wald-type F-statistic; p, significance level; sqrt Age, the square root of average age of spruces in each forest site; sqrt N, the square root of number of collected thalli in each population; Pollution, the presence of dust pollution. Bold-faced values represent significant effect.

<table>
<thead>
<tr>
<th>Effect</th>
<th>A</th>
<th></th>
<th></th>
<th>I</th>
<th></th>
<th></th>
<th>H</th>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
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<td>F</td>
<td>p</td>
<td>df</td>
<td>F</td>
<td>p</td>
<td>df</td>
<td>F</td>
<td>p</td>
<td>df</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
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<td>0.21</td>
<td>1</td>
<td>1.26</td>
<td>0.26</td>
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<td>0.32</td>
<td>0.57</td>
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<tr>
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<td>18.41</td>
<td>&lt;0.0001</td>
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<td>1.89</td>
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<tr>
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<td>0.002</td>
<td>1</td>
<td>12.30</td>
<td>&lt;0.001</td>
<td>1</td>
<td>4.17</td>
<td>0.04</td>
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<td></td>
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<tr>
<td>sqrt Age</td>
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<td>0.18</td>
<td>0.67</td>
<td>1</td>
<td>0.21</td>
<td>0.64</td>
<td>1</td>
<td>0.09</td>
<td>0.76</td>
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<tr>
<td>sqrt N</td>
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<td>247.6</td>
<td>&lt;0.00001</td>
<td>1</td>
<td>1.75</td>
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<td>10.68</td>
<td>0.001</td>
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<tr>
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<td>1</td>
<td>1.30</td>
<td>0.25</td>
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</tr>
</tbody>
</table>
Table 5 – Results of the bottleneck analyses: allele frequency distribution and heterozygote excess under all tree mutation models, infinite alleles (IAM), two-phase (TPM), and the step-wise mutation model (SMM). Populations, the number of population; L-shaped, normal L-shaped allele frequency distribution; Shifted, shifted mode allele frequency distribution; NS, nonsignificance ($\alpha=0.05$).

<table>
<thead>
<tr>
<th>Populations</th>
<th>Allele frequency distribution</th>
<th>Mutation model</th>
<th>Heterozygote excess</th>
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<tr>
<td></td>
<td></td>
<td>TPM</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SMM</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>L-shaped</td>
<td>IAM</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TPM</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SMM</td>
<td>NS</td>
</tr>
<tr>
<td>3</td>
<td>L-shaped</td>
<td>IAM</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TPM</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SMM</td>
<td>NS</td>
</tr>
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<td>Shifted</td>
<td>IAM</td>
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<td></td>
<td>TPM</td>
<td>0.01</td>
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<tr>
<td></td>
<td></td>
<td>SMM</td>
<td>0.01</td>
</tr>
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<td>IAM</td>
<td>0.003</td>
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<td></td>
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<td>NS</td>
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<td></td>
<td>SMM</td>
<td>NS</td>
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<td>NS</td>
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<td>NS</td>
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<td>SMM</td>
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</tr>
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<td>NS</td>
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<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SMM</td>
<td>NS</td>
</tr>
</tbody>
</table>
Highlights

• Genetic diversity of *Usnea subfloridana* was analyzed using microsatellite markers.
• Reverse primers for 9 microsatellite loci of *Usnea subfloridana* were rectified.
• Dust pollution had negative impact on genetic variation of *Usnea subfloridana*.
• Allelic richness of populations was lower in polluted than in unpolluted sites.