1   **Unconstrained gene flow between populations of a widespread epiphytic lichen *Usnea***

2   ***subfloridana* (Parmeliaceae, Ascomycota) in Estonia**

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15

16   **Abstract**

17

18   Few studies have investigated the genetic diversity of populations of common and widespread

19   lichenized fungi using microsatellite markers, especially the relationships between different

20   measures of genetic diversity and environmental heterogeneity. The main aim of our study was

21   to investigate the population genetics of a widespread and mainly clonally reproducing *Usnea*

22   *subfloridana* at the landscape scale, focusing on the comparison of lichen populations within

23   hemiboreal forest stands. Particular attention has been paid to the genetic differentiation of lichen

24   populations in two geographically distinct regions in Estonia and the relationships between forest

25   characteristics and measures of genetic diversity. We genotyped 578 *Usnea* thalli from eleven

26   lichen populations using seven specific fungal microsatellite markers. Measures of genetic

27   diversity (allelic richness, Shannon's information index, Nei's unbiased genetic diversity, clonal

28   diversity, the number of multilocus genotypes, the number of private alleles, and the minimum

29   number of colonization events) were calculated and compared between *Usnea* populations.

30   Shared haplotypes, gene flow and AMOVA analyses suggest that unconstrained gene flow and

31   exchange of multilocus genotypes exist between the two geographically remote regions in

32   Estonia. Stand age, mean circumference of the host tree, size of forest site and tree species

33   composition did not show any significant influence on allelic richness, Shannon's information

34   index, Nei's unbiased genetic diversity, clonal diversity, the number of private alleles, and the

35   minimum number of colonization events of *U. subfloridana* populations. Therefore it was

36   concluded that other factors of habitat heterogeneity could probably have a more significant

37   effect on population genetics of *U. subfloridana* populations.

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39   *Keywords*: forest age, genetic diversity, lichenized fungi, microsatellites, population genetics

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42   **1. Introduction**

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44   The genetic diversity, an important part of overall biodiversity, enables evolutionary processes,

45   which provide the raw material for adaptation to changing environments, and ensure healthy

46   populations (Helm et al. 2009; Frankham et al. 2010). The genetic diversity of natural

47   populations results from cumulative effects of historical and present-day processes (Hewitt 2000;

48   Frankman et al. 2010); the latter include, for example, changes in the current habitat conditions

49   of the environment, which may influence dispersal, growth and vitality of species. Estimating the

50   genetic variability within and among populations, and revealing genetic patterns of populations,

51   improves out understanding of the population history, genetic differentiation and gene flow

52   among populations (Werth et al. 2015). Population genetics also contributes to our knowledge of

53   evolutionary processes, ecology, and conservation biology; for example, knowledge of genetic

54   structure and variation of natural populations could be helpful in predicting the population fate in

55   fluctuating environment (e.g., climate change or forest management) or estimating the effective

56   population size of populations (Scheidegger and Werth 2009; Ouborg 2010).

57

58 Previously published studies regarding genetic structure and diversity of lichen-forming fungi
59 led to different conclusions for each species studied and scale of geographical distribution (e.g.,
60 Werth 2010; Scheidegger et al. 2012; Alors et al. 2017). The genetic diversity of a lichen
61 population could be shaped by different factors of environmental heterogeneity. Habitat quality,
62 measured as age or diameter of the host tree, is one of the most important factors affecting the
63 genetic patterns of lichen-forming fungi populations (Otalora et al. 2011; Scheidegger et al.
64 2012). For example, Jüriado et al. (2011) found a higher genetic diversity in populations of the
65 *Lobaria pulmonaria* (L.) Hoffm. and more juvenile thalli in old-growth forests compared with
66 managed forests and wooded meadows. Furthermore, different types of disturbance (Werth et al.
67 2006a), environmental and microclimatic factors (Nadyeina et al. 2014a; Otalora et al. 2015)
68 could also be significant in explaining the genetic structure and the distribution of gene pools of
69 lichen populations.
70
71 Microsatellites or simple sequence repeats (SSR) are considered the most promising markers for
72 investigating the genetic variation and population structure of highly clonal organisms such as
73 lichens (Werth 2010). The microsatellites are highly polymorphic, species-specific, and
74 selectively neutral markers with the a high mutation rate, which provides a more powerful
75 resolution for estimating genetic diversity and variability among populations than former
76 sequence-based method (Selkoe and Toonen 2006; Werth 2010). To date, microsatellite primers
77 have been designed for several lichenized fungi (e.g., Prieto et al. 2015; Lutsak et al. 2016;
78 Lagostina et al. 2017) and SSR markers have been successfully applied for studying the genetic
79 diversity, phylogeographic structure, gene flow and genetic differentiation of lichen populations
80 (e.g., Walser et al. 2003; Otalora et al. 2011; Nadyeina et al. 2014a). The majority of previous
81 studies which have considered the population genetic variability of lichen-forming fungi using
82 microsatellite markers have used the threatened, regionally rare or narrowly distributed lichens
83 (e.g., Nadyeina et al. 2014a; Jones et al. 2015; Prieto et al. 2015), but only a few studies have
84 reviewed the microsatellite diversity of common and widely distributed lichenized fungi and
85 genetic structure of their populations (Mansournia et al. 2012; Degtjarenko et al. 2016; Alors et
86 al. 2017).

87

88 In the current research we studied the population genetics of a common and widespread

89 lichenized fungus at the landscape scale, focusing on a comparison of lichen populations within

90 hemiboreal forest stands. To achieve this, the genetic variation at seven microsatellite loci in the

91 mycobiont of the epiphytic lichen *Usnea subfloridana* Stirt. in Estonia, Northern Europe was

92 investigated. The main objectives of this study were: (i) to study the genetic differentiation of *U.*

93 *subfloridana* populations in two separate regions of Estonia; and (ii) to investigate the

94 relationships between habitat characteristics and measures of genetic diversity of *U. subfloridana*

95 populations.

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98 **2. Material and methods**

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100 **2.1 Studied species**

101

102 *Usnea subfloridana* is an epiphytic fruticose macrolichen with a wide distribution across Eurasia,

103 Macaronesia, and North America (Nash et al. 2007; Randlane et al. 2009; Smith et al. 2009). It is

104 is very frequent and one of the most commonest *Usnea* species in Estonia, occurring mostly on

105 Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*) and Silver birch (*Betula pendula*),

106 and more rarely on other deciduous trees and lignum (Tõrra and Randlane 2007; Randlane et al.

107 2011). *Usnea subfloridana* is not protected locally, and is red-listed in Estonia as Least

108 Concerned (LC) (Randlane et al. 2008). This species reproduces asexually by symbiotic

109 propagules, soralia and isidia, but could also propagate sexually, but specimens with apothecia

110 are very rarely observed (Tõrra and Randlane 2007; Randlane et al. 2011). Recent phylogenetic

111 studies indicate that *U. subfloridana* is not a monophyletic entity but forms an intermixed clade

112 with *U. florida* (L.) Weber ex F.H. Wigg., which is considered the primary, fertile counterpart of

113 the sterile *U. subfloridana* (e.g., Articus et al. 2002; Saag et al. 2011; Mark et al. 2016). The

114 apotheciate *U. florida* reproduces exclusively sexually and always lacks vegetative propagules;

115 furthermore, it has distinct ecological requirements, preferring old deciduous trees in areas with a

116 high atmospheric humidity (Randlane et al. 2009; Smith et al. 2009), while *U. subfloridana* is

117 less ecologically demanding. To date, *U. florida* has not been recorded from Estonia (Tõrra and

118 Randlane 2007).

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120

121 **2.2 Study area**

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123 The study area is situated in two separate regions of Estonia, in Põlva County, in the southeastern

124 region (hereafter SE), and in Lääne-Viru County, in the northern region (hereafter N) of Estonia,

125 Northern Europe (Fig. 1); the maximum distance between the two studied areas is 184 km. The

126 study area has a characteristic temperate climate with a mean annual temperature of 6°C; the

127 mean annual precipitation is 672 mm, and the mean wind is 3,7 m/s (Estonian Weather Service

128 2018). The vegetation of Estonia belongs to the hemiboreal forest zone, lying in the transitional

129 area, where the southern taiga forest subzone changes into the spruce-hardwood subzone (Ahti et

130 al. 1986; Laasimer and Masing 1995). The two study sites (SE and N) are both located within the

131 hemiboreal forest zone but in the different vegetation subdivisions, N Estonia being situated in

132 the slightly oceanic to indifferent section, and SE Estonia in the indifferent to slightly continental

133 section according (Ahti et al. 1986) and also in different regions according to the classifications

134 based on sedimentary bedrock (Viiding 1995) and soils (Reintam 1962). The study was carried

135 out in *Pinus sylvestris*-dominated boreal forests, belonging to the *Oxalis–Vaccinium myrtillus*,

136 the *Vaccinium myrtillus*, and the *Vaccinium vitis-idaea* forest site types. These forest types are

137 also widely distributed in other Baltic states (Kairiūkštis 1966; Bušs 1997), in Fennoscandia

138 (Dierβen 1996), and in northwest Russia (Fedorchuk et al. 2005).

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140

141 **2.3 Sampling**

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143 Fieldwork was carried out during the summer of 2011 (in SE Estonia) and the autumn of 2014

144 (in N Estonia). The potential localities for sampling were chosen from Forest Public Registry

5

145 maps using comparable forest characteristics from their forest survey (Forest Public Registry

146 2017). In total, *Usnea subfloridana* populations were sampled from eleven localities; eight

147 populations from SE and three populations from N (Fig 1; Table 1). In each locality or lichen

148 population, 30–62 samples were randomly collected from Norway spruce up to 6 m from the

149 ground using a tree pruner (Table 1). On average, three *Usnea* thalli were taken from a host tree;

150 if there were less than three thalli, only one or two specimens were sampled, while in other cases

151 more than tree specimens were collected for balancing the sampling. *Usnea* populations were

152 defined according to the boundaries of forest sites sharing the same values of forest survey data

153 (forest site type, age of trees and proportion of trees in forest stand) according to Forest Public

154 Registry (2017). The tree circumference (BHC) was recorded for each sampled tree at breast

155 height (1.3 m). Other habitat characteristics (stand age, the proportion of pines and birches in

156 forest stands, and size of forest site sharing the same values of data from forest survey) were

157 provided by from Forest Public Registry (2017). Geographical coordinates were recorded per

158 sampled tree with a GPS receiver Garmin GPSMAP 60C.

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161 **2.4 Chemical and molecular analyses**

162

163 All collected *Usnea* thalli were air dried, cleaned to remove other lichen specimens, and

164 examined under a stereomicroscope. Thin layer chromatography (TLC) with solvent A (Orange

165 et al. 2001) was used to confirm the identification of collected *Usnea* species. According to

166 morphology and chemical characteristics (Halonen et al. 1999; Randlane et al. 2009; Clerc

167 2011), 578 specimens were identified as *U. subfloridana* and used in further molecular analyses;

168 another 79 specimens were removed from the sampling as they belonged to other, similar *Usnea*

169 species (*U. glabrescens* (Vain.) Vain. or *U. wasmuthii* Räsanen). Then, the 50 mg of each *U.*

170 *subfloridana* specimen was maintained in 1.5 mL microtubes at –20°C until the molecular

171 analyses.

172

173   The total genomic DNA was extracted using PowerPlant® Pro DNA Isolation Kit (MO BIO

174   Laboratories, Inc., Qiagen, USA) according to the manufacturer's protocol. Seven fungal

175   microsatellite loci (*Us02*, *Us03*, *Us04*, *Us05*, *Us06*, *Us08*, and *Us09*) were amplified in two

176   different multiplex PCR using QIAGEN Multiplex PCR Kit following the instructions described

177   in Tõrra et al. (2014) and Degtjarenko et al. (2016). Fragment lengths of PCR products were

178   determined on a 3730xl DNA Analyzer (Applied Biosystems) with LIZ-500 as the internal size

179   standard. The alleles were sized and genotyped using GeneMapper® Software v5 (Applied

180   Biosystems).

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182

183   **2.5 Statistical analyses**

184

185   The basic measurements of population statistics (the total number of alleles, mean number of

186   alleles per locus, Nei's unbiased genetic diversity (H), Shannon's information index (I), allelic

187   richness (A)) for *Usnea subfloridana* populations were calculated in the GenAlex ver 6.5

188   (Peakall and Smouse 2012) and the Microsatellite Analyzer ver 2.65 (MSA) (Dieringer and

189   Schlötterer 2003). The number of private alleles (P) per population were calculated using

190   software HP-Rare (Kalinowski 2005). Measurements of A and P were standardized using the

191   rarefaction method implemented in the software HP-Rare (Kalinowski 2005) and MSA

192   (Dieringer and Schlötterer 2003) respectively. The number of multilocus genotypes (G), the

193   percentage of multilocus genotypes, i.e clonal diversity or genotypic diversity (M; the proportion

194   of different genotypes in the population, G/N), the minimum number of colonization events (C)

195   per population, and total number of multilocus genotypes from all populations were calculated in

196   the software R (R Core Team 2013) using the R script by Werth et al. (2006a). The number of

197   shared multilocus genotypes between populations was calculated in the software ARLEQUIN

198   ver 3.5 (Excoffier and Lischer 2010). Hierarchical analyses of molecular variance (AMOVA)

199   with 999 permutations to estimate genetic differentiation were performed using GenAlex ver 6.5

200   (Peakall and Smouse 2012). The rate of gene flow (Nm) across seven loci between 11

201   populations was also estimated using GenAlex ver 6.5 (Peakall and Smouse 2012). General

202 regression model (GRM) analysis in the STATISTICA ver 7.1 (StatSoft, Inc. 2005) was used to

203 study the relationship between different measurements of genetic diversity (A, I, H, G, M, P, and

204 C) and the characteristics of forest stands. Each population was characterized by the following

205 explanatory variables: (1) stand age (the square root of oldest tree age per forest stand); (2) mean

206 BHC of the host tree per population, values log-transformed; (3) the number of sample size (the

207 square root of collected specimens per population); (4) the size of forest site sharing the same

208 values of forest survey data (forest site type, age of trees and proportion of trees in forest stand);

209 (5) the proportion of pines and birches in forest stands.

210

211

212 **3. Results**

213

214 **3.1 Genetic variation of *Usnea subfloridana* in Estonia**

215

216 In total, 66 alleles at seven microsatellite loci in 578 specimens from eleven *Usnea subfloridana*

217 populations were detected (Table 1). All microsatellite loci were polymorphic. The minimum

218 number of alleles was six in locus *Us04* and the maximum number of alleles was 14 in locus

219 *Us03*, and, on average, 3.2–9.5 alleles were found per locus across eleven populations. The mean

220 number of alleles per population was comparatively similar in both regions, varying from 4.9 to

221 5.4 in populations from N region, and from 5.4 to 7.0 in populations from SE region. We found

222 283 different multilocus genotypes across 578 specimens in eleven lichen populations. Allelic

223 richness (A) ranged from 4.86 to 6.45 across all lichen populations. Nei's unbiased genetic

224 diversity (H) varied from 0.58 to 0.65 (Table 1). All lichen populations, except no 3, had private

225 alleles (Table 1). Other detailed measurements of genetic variation per population are given in

226 Table 1. The AMOVA results indicated that most of the total variation (99%) was found within

227 populations, i.e. among individuals, followed by significant variation among populations within

228 one region (0.5%; Table 2). The molecular variation between populations from distinct regions

229 (0.5%) was also statistically significant (Table 2). The mean gene flow (Nm) for all populations

230 across seven loci was 8.52. The analyses for checking shared haplotypes among populations in

231 the software ARLEQUIN ver 3.5 (Excoffier and Lischer 2010) revealed that all *Usnea*

232 populations shared the identical multilocus genotypes with other populations as well as within

233 both regions (Fig. 2; Appendix A.1).

234

235

236 **3.2 Genetic diversity of *Usnea subfloridana* in relation to forest stand heterogeneity**

237

238 The relationships between different measurements (A, I, H, G, M, P and C) of genetic variation

239 and characteristics of forest stands were studied. The results of GRM showed that stand age,

240 mean BHC of the host tree per population, the number of sample size, the size of forest site

241 sharing the same values of data from forest survey, and proportion of pines and birches in forest

242 stands did not reveal any significant influence on A, I, H, M, P and C (Appendix A.2). There was

243 a statistically significant association between the number of multilocus genotypes (G) and

244 sample size (i.e the number of collected/studied specimens per population; SS=321.3; F=160.6;

245 p=0.0002; Appendix A.2).

246

247

248 **4. Discussion**

249

250 Seven microsatellite loci were used to study the intra-species genetic diversity of the common

251 and widespread lichen-forming fungus *Usnea subfloridana*. The genetic differentiation of *Usnea*

252 populations from two geographically remote regions in Estonia and the relationships between

253 characteristics of forest stands and measures of genetic variation per *U. subfloridana* populations

254 were investigated; the mainly clonally distributed specimens exhibited relatively high levels of

255 genetic diversity (H=0.62; SD=0.02; Table 1). Populations of the pendulous and clonally

256 reproducing *Bryoria capillaris* (Ach.) Brodo & D. Hawksw. and *B. fuscescens* (Gyeln.) Brodo &

257 D. Hawksw also revealed similar high levels of genetic diversity, H=0.71 and H=0.79,

258 respectively (Nadyeina et al. 2014b). Previous studies have also observed high levels of genetic

259 variation in other clonally reproducing cryptogams such as the bryophyte *Pleurochaete*

9

260   *squarrosa* (Brid.) Lindb. (Spagnuolo et al. 2007). The populations of predominantly asexually

261   reproducing lichen-forming fungus *Lobaria pulmonaria* exhibited slightly lower levels of genetic

262   diversity (H=0.46; SD=0.15) in Spain (Otalora et al. 2011) and in Central Europe (Walser et al.

263   2005; Werth et al. 2006a). Population of strictly outcrossing lichen-forming fungus *Parmelina*

264   *carporrhizans* (Taylor) Poelt & Vězda revealed very high levels of genetic diversity (H=0.74–

265   0.90) and complete absence of clonality in the Mediterranean region (Alors et al. 2017). Our

266   results support the view that mainly clonally reproducing species can have comparable high

267   levels of genetic variation, as have normally sexually reproducing species (Vrijenhoek 1990).

268   *Usnea subfloridana* usually reproduces asexually by symbiotic propagules, soralia and isidia, but

269   rarely bears a few (single or a couple) apothecia as well (Clerc 2011) indicating the possibility of

270   only limited sexuality. These rare apothecia have also been noticed in Estonian material (Tõrra

271   and Randlane 2007), but not in the study samples.

272

273   The AMOVA results demonstrated that most of the total genetic variation (99%) was due to

274   differences among individuals within studied *Usnea* populations; it also revealed a low

275   proportion (0.5%) of genetic variation attributed to regional differences (Table 2). High levels of

276   gene flow (Nm=8.52) or genetic similarity between all studied *U. subfloridana* populations was

277   demonstrated. The low dissimilarity could reflect the dominance of clonal spread between

278   populations (Walser 2004). Moreover, it was found that lichen populations shared common

279   identical multilocus genotypes between all populations and also between the two regions (Fig. 2;

280   Appendix A.1). The maximum geographical distance among the populations sharing the

281   identical multilocus genotypes was 183 km, which was observed between population no 2 from

282   N region and population no 4 from SE region of Estonia.

283

284   Lichen-forming fungi reproducing via ascospores generally have long distance dispersal, while

285   clonally distributed species via isidia/soredia or thallus fragments may have a restricted dispersal

286   and distinct genetic structure (Werth 2010; Werth et al. 2006b). Previous SSR-based studies on

287   population genetics of lichenized fungi showed that populations of predominantly asexually

288   reproducing *Lobaria pulmonaria* were highly clonal and structured by limited dispersal capacity

289   of vegetative propagules (Walser 2004; Werth et al. 2006b; Dal Grande et al. 2012). The

290   effective dispersal distance of *L. pulmonaria* has been observed to remain very low, ranging

291   from 15–30 m (Jüriado et al. 2011) to 140–200 m (Walser 2004; Werth et al. 2006b). Our results

292   indicated that in case of *U. subfloridana*, exchange of lichen individuals or their vegetative

293   propagules should exist between the populations even from different regions of Estonia, which

294   are located at almost 200 km from each other. The long distance between northern and

295   southeastern populations did not seem to be a barrier for the dispersal of this species. The

296   morphological peculiarity (growth form of the thallus) probably relevant to the efficient long-

297   range dispersal of pendulous taxa by thallus fragments. Moreover, it has been suggested that

298   drastic events such as storms or long-distance vectors (birds) may also play an important role in

299   the distribution of vegetative propagules that are heavier than sexual propagules (Walser 2004);

300   for example, Högberg et al. (2002) suggested that migration of the epiphytic lichen *Letharia*

301   *vulpina* (L.) Hue from western North America to Europe occurred via lightweight soredia,

302   overcoming the long distances between continents. Therefore, the long-distance transport of

303   lichen propagules by birds or strong winds and storms seems not impossible in distribution of a

304   common and widespread pendant lichen. Further research should focus on determining the long-

305   range propagule dispersal by pendulous *U. subfloridana*.

306

307   Unconstrained gene exchange among populations may also indicate the connectedness of forest

308   patches in different regions, or at least historically. The significance of landscape-scale

309   parameters (including, for example, historic woodland structure or distances from the study area

310   to the nearest contemporary or historic forest) on the colonization and richness of lichens has

311   been demonstrated earlier (Ellis and Coppins 2007; Randlane et al. 2017). Currently, about half

312   of the territory in Estonia is covered by forests (Raudsaar et al. 2016); prior to agricultural

313   activity (ca. 3000 years ago) this figure was most probably more than 80%, while in the 1920–

314   1930s it was the most restricted (Pärt 2011). Our study areas in both SE and N Estonia belong to

315   forested territories that have existed since 1935–1939 according to historical topographic maps

316   (1:50 000) of the Estonian Land Board (2017). It is possible that well-connected populations of

317   lichens shared the individuals or propagules without ecological barriers and accumulated clones

318   over many generations (Frankham et al. 2010).

319   No significant effect of forest characteristics and other explanatory variables of lichen

320   populations on the minimum numbers of colonization events (C) and the number of private

321   alleles (P) were found in the studied *U. subfloridana* populations (Appendix A.2). These findings

322   suggest that all observed *Usnea* populations had developed by multiple independent immigration

323   events from a large, genetically diverse source of populations by rapid clonal spread. Neither the

324   age of forest stands (based on the oldest trees in the stands) nor circumference of host trees had a

325   significant effect on C and P. We also recorded high values (0.77−0.90; SD=0.04; Table 1) of

326   clonal diversity (M) in *U. subfloridana* populations and found no significant relationships

327   between the clonal diversity and forest stand characteristics (Appendix A.2). Thus the studied

328   lichen populations probably belonged to the same demographic phase.

329

330   Given the fact that epiphytic lichen richness and abundance generally increase with forest stand

331   age and environmental heterogeneity (McCune 1993; Marmor et al. 2011), we hypothesized that

332   the genetic variability of *U. subfloridana* populations could be influenced by the habitat maturity

333   (estimated as age of forest stands or circumference of host tree), and tree species composition

334   could be important in maintaining the genetic diversity of epiphyte populations. However, the

335   GRM analyses showed that the age of forest stands and circumference of host tree did not

336   demonstrate a significant effect on any measurement of genetic variability for *U. subfloridana*

337   populations (Appendix A.2), although the positive trend was observed between Nei's unbiased

338   genetic diversity (H) and age of forest stands. These results are consistent with those of

339   Degtjarenko et al. (2016) who did not reveal a significant effect of the average age of lichen

340   phorophyte on the genetic variation of *U. subfloridana* populations. A possible explanation for

341   our result might be that variation in age of a forest stand (92−174; SD=34.1; Table 1) was not

342   sufficient to reveal a difference between young and old-growth forests; for example, Jüriado et

343   al. (2011) showed that genetic diversity of *L. pulmonaria* populations was significantly higher in

344   habitats of old-growth forests than in managed forests and wooded meadows; furthermore, the

345   relationship between stand age and genetic diversity of lichen populations within the habitats

346   was significantly positive only for wooded meadows. Gjerde et al. (2012) also demonstrated that

347   no significant differences were detected in haplotype richness (based on data from two

348   microsatellite loci) of *L. pulmonaria* collected in either young (40–120 years) or old (140–200

349   years) forests.

350

351   Our observations support the hypothesis that stand age or tree species composition are not of

352   great importance in explaining the genetic patterns of *U. subfloridana*; for example, Boudreault

353   et al. (2009) showed that the relationships between tree age and epiphytic biomass of *Usnea*

354   species was not linear in boreal forest: the biomass of *Usnea* species tended to decrease in 150

355   year old forest stands. The abundance of *Usnea* thalli could be explained by the availability of

356   branches on spruces, regardless of tree age (Rolstad and Rolstad 1999). It is also possible that

357   microclimatic heterogeneity (e.g. humidity, wind speed, canopy openness) could have a more

358   consequential influence on *U. subfloridana* populations than habitat age and tree species

359   composition; for instance, annual precipitation has an effect on genetic diversity of *Lobaria*

360   *pulmonaria* populations in the Iberian Peninsula (Otalora et al. 2015). Further research involving

361   microclimatic measurements may determine the most important factors in shaping the genetic

362   patterns of *U. subfloridana* populations.

363

364

365   **5. Conclusions**

366

367   Our study was aimed at studying the population genetics of the widespread epiphytic *Usnea*

368   *subfloridana* in hemiboreal forest stands in Estonia. The results indicated that populations of

369   mainly clonally reproducing *U. subfloridana* exhibited relatively high levels of genetic diversity,

370   revealing a spatially unrestricted dispersal of individuals. Only a negligible genetic

371   differentiation of *U. subfloridana* populations between two geographically remote regions of

372   Estonia was found. Therefore it was concluded that unconstrained gene flow and multilocus

373   genotypes exchange occurs among *U. subfloridana* populations between the two geographical

374   regions or had occurred at least in the past. In our study, the stand age, mean circumference of

375   the host tree, size of forest site sharing the same values of forest survey data and the proportion

376 of birches and pines in the forest stand did not reveal any significant influence on allelic richness

377 (A), Shannon's information index (I), Nei's unbiased genetic diversity (H), clonal diversity (M),

378 the number of private alleles (P), and the minimum number of colonization events (C) of *U.*

379 *subfloridana* populations. We suggest that stand age or tree species composition is not of great

380 importance in explaining the genetic patterns of the pendulous epiphytic lichen *U. subfloridana.*

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382

383 **Acknowledgements**

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560 **Figure legend**

561

562 Fig 1 – Distribution map of *Usnea subfloridana* in Estonia (marked with light grey squares), and

563 study populations (dark grey circles) in the southeastern and the northern regions of Estonia.

564

565 Fig 2 – Counts of shared haplotypes between populations of *Usnea subfloridana* in the

566 southeastern (SE) and the northern regions (N) of Estonia; the thickness of lines reflects the

567 number of shared haplotypes between populations.

Table 1 – Overview of the studied *Usnea subfloridana* populations from the northern (1−3) and the southeastern (4−11) regions of Estonia: sample size, geographical coordinates, forest stand variables, and measurements of genetic variation. Populations, the number of population; Specimens, the number of collected thalli per population; Trees, the number of host trees from which thalli were collected in each population; Latitude, latitudinal coordinates of the centre of forest site; Longitude, longitudinal coordinates of the centre of forest site; Age, the stand age (based on the oldest trees in the stands); BHC, mean circumference (cm) of the host tree per population (measured from each sampled tree at breast height 1.3 m); Betula, proportion of *Betula pendula* in each forest site; Pinus, proportion of *Pinus sylvestris* in each forest site; Size, the size of forest site (ha) sharing the same values of forest survey (age, proportion of trees in forest stands, forest site type); H, Nei's unbiased genetic diversity per population; A, standardized allelic richness per population; I, Shannon's information index per population; G, the number of multilocus genotypes per population; M, clonal diversity per population; C, the minimum number of colonization events per population; P, the number of private alleles per population.

Variables	Region	Northern			Southeastern								Total
	Population	1	2	3	4	5	6	7	8	9	10	11	
Sample size	Specimens	46	30	36	56	61	60	59	62	58	58	52	578
	Trees	11	10	11	21	21	21	21	21	21	21	20	199
Coordinates	Latitude N	59°33′9.1″	59°35′54.6″	59°34′31.1″	58°6′13.5″	58°6′28.2″	58°7′13.2″	58°7′23.8″	58°7′23.0″	58°8′51.8″	58°8′29.3″	58°7′54.7″	
	Longitude E	25°48′4.1″	25°45′23.8″	25°55′47.7″	27°4′28.9″	27°2′50.4″	27°3′2.8″	26°59′6.0″	26°59′20.3″	27°3′16.2″	27°3′2.3″	27°2′28.3″	
Forest stand variables	Age	97	146	131	164	164	99	92	162	94	94	174	
	BHC	92.9	118.6	90.4	125.2	117.2	135.9	76.8	119.3	84.2	119.2	107.3	
	Betula	0.01	0	0	0.05	0.03	0.04	0.05	0.05	0.2	0.15	0.05	
	Pinus	0.78	0.8	0.81	0.85	0.65	0.5	0.65	0.6	0.4	0.6	0.55	
	Size	0.7	1.9	4.3	13.6	11.6	8.6	4.4	1.9	8.8	2.9	4	
													Mean
Genetic variation	H	0.58	0.60	0.63	0.65	0.62	0.65	0.62	0.62	0.64	0.61	0.64	0.62
	A	5.33	4.86	5.26	6.22	5.31	5.71	5.60	5.37	6.45	5.39	5.78	5.57
	I	1.16	1.16	1.23	1.34	1.23	1.33	1.26	1.26	1.35	1.22	1.30	1.26
	G	38	27	31	46	51	46	46	51	46	50	44	
	M	0.83	0.90	0.86	0.82	0.84	0.77	0.78	0.82	0.79	0.86	0.85	0.83
	C	9	9	10	9	10	10	9	10	11	9	9	
	P	0.25	0.18	0	0.45	0.11	0.12	0.22	0.11	0.52	0.22	0.12	

Table 2 – Hierarchical analysis of molecular variance (AMOVA) for eleven populations of *Usnea subfloridana* according to seven microsatellite loci. Bold-faced values of P represent significant effect.

Source of variation	d.f.	Sum of squares	Variance	Percentage	P
Among regions	1	4.778	0.011	0.5%	**0.014**
Among populations within region	9	25.576	0.012	0.5%	**0.015**
Within populations	567	1253.054	2.210	99%	**0.002**
Total	577	1283.408	2.233		

N

SE
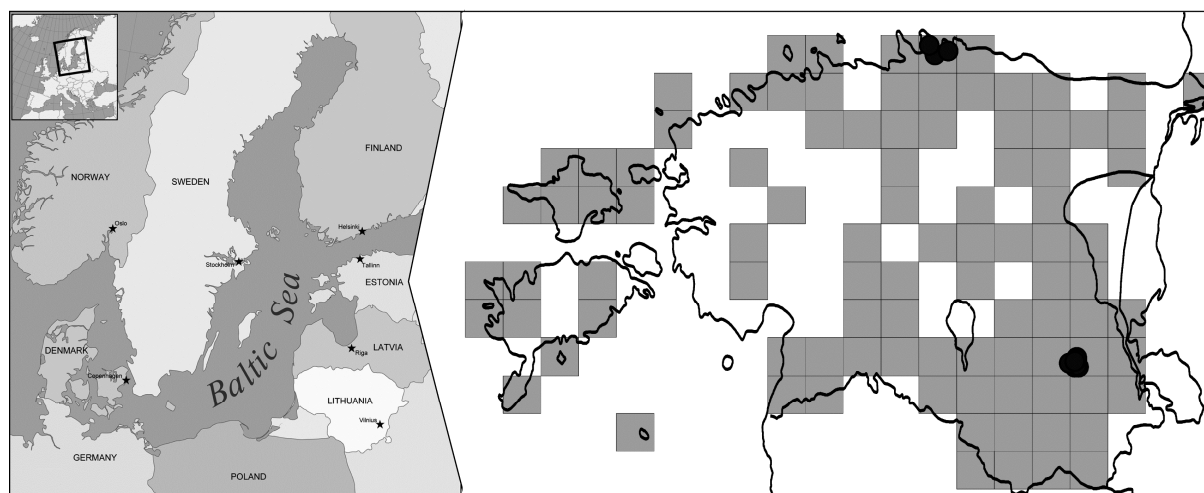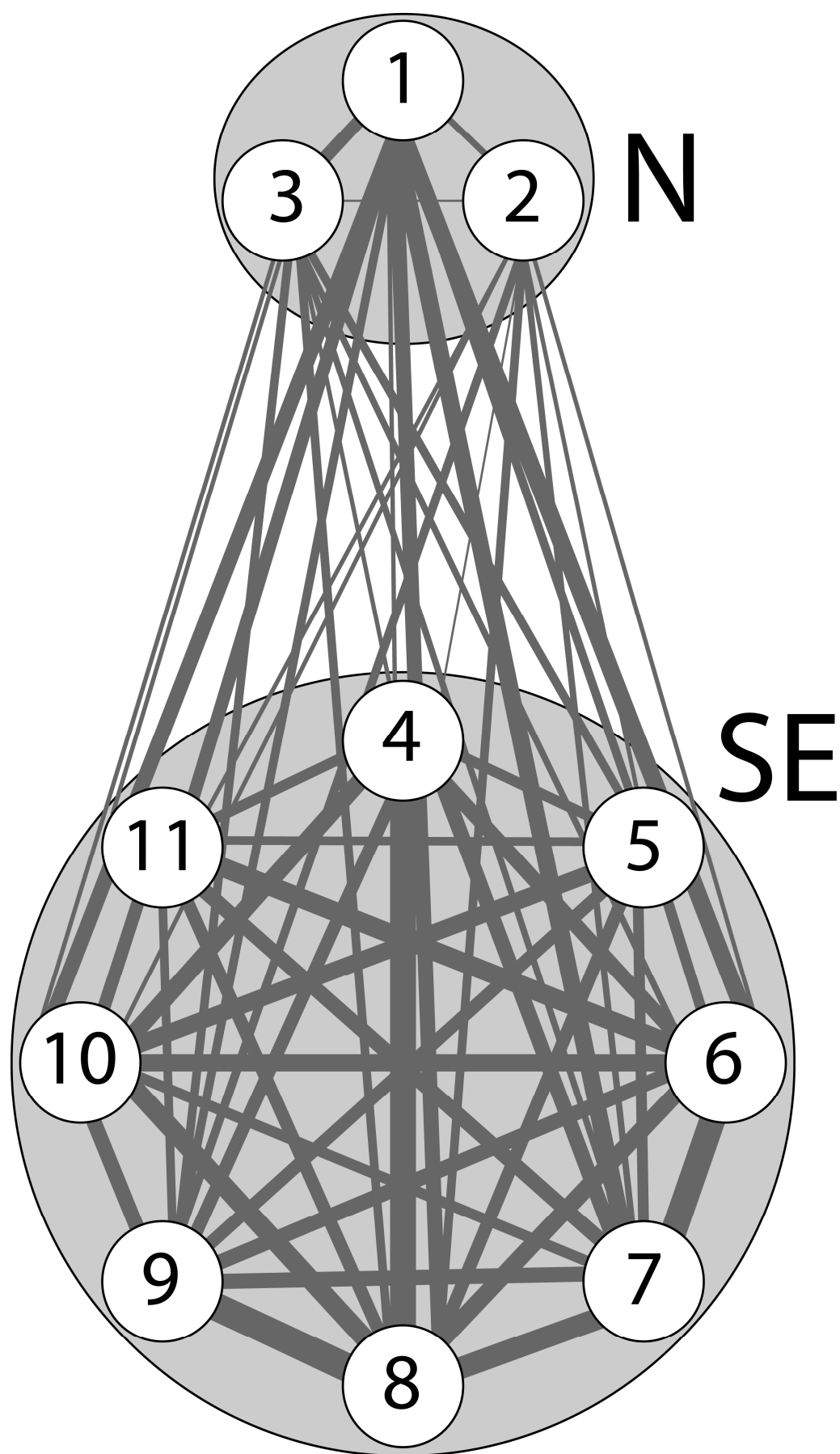
1 **Highlights**
2
3 • Microsatellite diversity of *Usnea subfloridana* was studied in hemiboreal forests.
4 • Populations of *U. subfloridana* exhibited high levels of genetic diversity.
5 • Populations from remote regions of Estonia shared common multilocus genotypes.
6 • A high level of gene flow must have occurred between *U. subfloridana* populations.