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1 **Sensitivity to habitat fragmentation across European landscapes in three temperate forest**

2 **herbs**

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26

27 **Abstract**

28 Context. Evidence for effects of habitat loss and fragmentation on the viability of temperate forest  
29 herb populations in agricultural landscapes is so far based on population genetic studies of single  
30 species in single landscapes. However, forest herbs differ in their life histories, and landscapes have  
31 different environments, structures and histories, making generalizations difficult.

32 Objectives. We compare the response of three slow-colonizing forest herbs to habitat loss and  
33 fragmentation and set this in relation to differences in life-history traits, in particular their mating  
34 system and associated pollinators.

35 Methods. We analysed the herbs' landscape-scale population genetic structure based on  
36 microsatellite markers from replicate forest fragments across seven European agricultural  
37 landscapes.

38 Results. All species responded to reductions in population size with a decrease in allelic richness and  
39 an increase in genetic differentiation among populations. Genetic differentiation also increased with  
40 enhanced spatial isolation. In addition, each species showed unique responses. Heterozygosity in the  
41 self-compatible *Oxalis acetosella* was reduced in smaller populations. The genetic diversity of  
42 *Anemone nemorosa*, whose main pollinators are less mobile, decreased with increasing spatial  
43 isolation, but not that of the bumblebee-pollinated *Polygonatum multiflorum*.

44 Conclusions. Our study indicates that habitat loss and fragmentation compromise the long-term  
45 viability of slow-colonizing forest herbs despite their ability to persist for many decades by clonal  
46 propagation. The distinct responses of the three species studied within the same landscapes confirm  
47 the need of multi-species approaches. The mobility of associated pollinators should be considered an  
48 important determinant of forest herbs' sensitivity to habitat loss and fragmentation.

49

50 **Keywords**

51 connectivity; genetic differentiation; genetic diversity; mating system; pollinator mobility; population  
52 size

53 **Declarations**

54

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64

65 **Conflicts of interest**

66 We have no conflict of interest to declare.

67

68 **Ethics approval**

69 We have no ethical concerns to declare.

70

71 **Consent to participate**

72 All authors contributed to this study and this manuscript of their own accord.

73

74 **Consent for publication**

75 All authors agree with the content of this manuscript and its publication in Landscape Ecology.

76

77 **Availability of data and material**

78 The microsatellite allele tables for all species and populations as well as population locations will be  
79 made available on DRYAD (doi:10.5061/dryad.tb2rbp00k; still kept private).

80

81

82 **Authors' contributions**

83 TN, SIJH and KK conceived and designed the study. All authors were involved in site selection, field  
84 work and sampling. JTF, SH, TN and KK performed the molecular lab work and did the allele calling.  
85 TN analyzed the data and wrote the first draft of the manuscript with contributions of JTF, SIJH, SH  
86 and KK. All authors contributed to revisions.

87

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92 picardie.fr/smallforest/uk/](https://www.u-picardie.fr/smallforest/uk/)).

93

94 **1. Introduction**

95

96 Large parts of the Earth are covered by human-modified, agricultural landscapes, in which only small  
97 remnants of (semi-)natural habitats are left (Kennedy et al. 2019). Habitat loss and fragmentation  
98 are, therefore, considered among the most important threats to biodiversity (Wilson et al. 2016). In  
99 the temperate zones of the Northern Hemisphere, agricultural landscapes are dominant (Kennedy et  
100 al. 2019). Many organisms are not adapted to live in small populations restricted to isolated habitat  
101 fragments. Typical temperate forest herbs, for instance, evolved during times when forests were  
102 much more common and connected than today (Honnay et al. 2005) and therefore exhibit traits  
103 associated with long-term stable conditions, such as a high age of first flowering, the production of  
104 few and heavy seeds and the absence of long-distance seed dispersal mechanisms (Whigham 2004).  
105 These traits limit the species' ability to colonize new habitat patches (Verheyen et al. 2003) and their  
106 populations nowadays are often spatially isolated (Jamoneau et al. 2012; Naaf and Kolk 2015).

107 Small, isolated populations are increasingly prone to local extinction due to environmental,  
108 demographic and genetic stochasticity (Shaffer 1981). Theoretically, genetic diversity in small and  
109 isolated populations is eroded through random genetic drift and enhanced inbreeding (Young et al.  
110 1996), reducing both the populations' fitness and their potential to adapt to environmental changes  
111 (Jump et al. 2009). Empirically, however, forest herbs often appear to be able to survive in small,  
112 spatially isolated populations for many decades (Vellend et al. 2006; Valdés et al. 2015). Many forest  
113 specialist herbs are long-lived and able to propagate vegetatively, thus exhibiting long generation  
114 times. This delays fragmentation effects through genetic drift and results in extinction debts (Honnay  
115 et al. 2005). Thus, it remains unclear how susceptible temperate forest herbs are to detrimental  
116 effects of habitat loss and fragmentation in the long term.

117 Population genetics offers tools to answer this question by assessing both genetic diversity  
118 within and genetic connectivity among spatially isolated populations. There are also some population  
119 genetic studies for temperate forest herbs (see below). Interestingly, the majority of these focus on

120 species relying solely on sexual reproduction, despite the fact that most temperate forest herbs are  
121 clonal (Whigham 2004). A reduced genetic diversity in smaller, more isolated forest herb populations  
122 compared to larger, better connected populations has been found in a number of case studies  
123 (Vellend 2004; Jacquemyn et al. 2006; Kolb and Durka 2013; Schlaepfer et al. 2018). However, this  
124 pattern is not ubiquitous: the genetic diversity of some self-compatible forest herbs appears to be  
125 unaffected by habitat loss and fragmentation (Culley et al. 2007; Toma et al. 2015). High levels of  
126 among-population genetic differentiation independent of among-population spatial distance  
127 (absence of isolation-by-distance) were found in some case studies (Jacquemyn et al. 2006; Schmidt  
128 et al. 2009; Zomlefer et al. 2018; Toczydlowski & Waller 2019), indicating that drift dominates over  
129 gene flow and populations are genetically isolated. But also low levels of genetic differentiation  
130 among fragmented populations have been observed, when drift was either compensated by gene  
131 flow (Tomimatsu and Ohara 2003; Jacquemyn et al. 2009) or impeded by very limited sexual  
132 reproduction (Honnay et al. 2006).

133         Almost all of the above-mentioned studies focus on a single species in a single landscape or  
134 region (but see Schmidt et al. 2009 for a multi-landscape study). Any generalization is therefore  
135 difficult (Segelbacher et al. 2010). One way to draw general conclusions are meta-analyses across  
136 various plant species. These have shown that the susceptibility of plant populations to negative  
137 effects of habitat loss and fragmentation varies with their life-history traits (Leimu et al. 2006; Aguilar  
138 et al. 2008; Duminil et al. 2007; Honnay and Jacquemyn 2007; Gonzalez et al. 2020). The decrease in  
139 genetic diversity in response to habitat fragmentation is more pronounced in predominantly  
140 outcrossing species than in selfing species (Leimu et al. 2006; Honnay and Jacquemyn 2007; Aguilar  
141 et al. 2008). Populations of species with gravity-dispersed seeds show generally a higher genetic  
142 differentiation than populations of species with wind- or animal-dispersed seeds (Duminil et al.  
143 2007). The loss of genetic diversity through drift also depends on the number of generations elapsed  
144 since fragmentation took place (Young et al. 1996; Aguilar et al. 2008). We should therefore assume



145 that long-lived clonal plants with limited seedling recruitment, such as many forest herbs, lose  
146 genetic diversity more slowly than annuals and short-lived plants (Honnay et al. 2005).

147 An important element of the mating strategy that has been neglected so far in meta-analyses  
148 on genetic effects of habitat fragmentation is the pollinator group associated with the species. The  
149 contribution of pollen flow to gene flow can be essential in plants (Auffret et al. 2017). Therefore,  
150 pollinator mobility may affect susceptibility to fragmentation effects (Breed et al. 2015). On the one  
151 hand, the contribution of pollen flow to total gene flow among forest herb populations is generally  
152 considered low (Honnay et al. 2005) because most pollinator species do not travel far enough to  
153 cross the agricultural matrix between spatially isolated herb populations (Wilcock and Neiland 2002).  
154 Pollinator visits are perceived to be relatively rare in forest herbs, and habitat loss and fragmentation  
155 are assumed to limit pollination service even more because small and sparse populations appear less  
156 attractive for pollinators (Kwak et al. 1998; Kolb 2008). On the other hand, some pollinator groups,  
157 including bumblebees and some solitary bees, have foraging distances of as much as several hundred  
158 meters (Gathmann and Tschardt 2002; Redhead et al. 2016). These groups are also important  
159 pollinators for many temperate forest herbs (e.g., *Phyteuma* spp.: Kolb 2008, *Polygonatum* spp.:  
160 Hasegawa and Kudo 2005, or *Stachys sylvatica*: Fussell and Corbet 1991). Sensitivity to habitat loss  
161 and fragmentation in these species might therefore be lower than expected.

162 Moreover, the above-mentioned meta-analyses cannot account for various interactions  
163 between life-history traits and landscape context, which finally determine the regional population  
164 genetic structure (Aparicio et al. 2012). Also, accounting for time since fragmentation is difficult in  
165 meta-analyses (Schlaepfer et al. 2018). A powerful approach would therefore be to compare the  
166 population genetic structure of multiple species with contrasting traits within the same landscape  
167 (Bolliger et al. 2014). Such studies are, however, extremely rare and have so far not dealt with  
168 temperate forest herbs.

169 Here, we compared the population genetic structure of three slow-colonizing, clonal forest  
170 herb species: *Anemone nemorosa* L., *Oxalis acetosella* L. and *Polygonatum multiflorum* (L.) All. (Fig.

171 1). These species differ in their mating strategy, which comprises in our understanding not only the  
172 mating system *s. str.* (outcrossing vs. selfing), but also the associated pollinator group. To enhance  
173 the generality of our results, we compared the three species over six to seven agricultural landscapes  
174 across temperate Europe from North France to Central Sweden and Estonia. Our objective was to  
175 assess the species' sensitivity to habitat loss and fragmentation (i.e., the degree by which genetic  
176 diversity decreases and among-population genetic differentiation increases in response to a reduced  
177 population size and an increased spatial isolation). We tested the following hypotheses: (i) All three  
178 species show general responses to habitat loss and fragmentation that are consistent across  
179 landscapes. (ii) As a regular selfing species, we expect *O. acetosella* to be less sensitive to habitat loss  
180 and fragmentation than the mostly outcrossing *A. nemorosa* and *P. multiflorum*. (iii) Given its  
181 pollination by far-flying bumblebees, we expect *P. multiflorum* to be less sensitive to habitat loss and  
182 fragmentation than *A. nemorosa*, which is associated with less mobile pollinators.

183

184

## 185 **2. Methods**

186

### 187 *2.1 Studied species*

188 The selected forest herbs (Fig. 1) are common species in European temperate deciduous forests.

189 They have a similar life history in that they are all known as slow-colonizing forest specialists

190 (Verheyen et al. 2003; Schmidt et al. 2014), concurrently flower in spring (Klotz et al. 2002), are

191 pollinated by insects (Klotz et al. 2002) and propagate vegetatively, besides regular seedling

192 recruitment (Holderegger et al. 1998; Berg 2002; Kosiński 2012). They all have a relatively broad

193 ecological amplitude in terms of soil conditions and frequently co-occur at mesic, slightly to

194 moderately acidic and moderately nutrient-rich sites (Leuschner and Ellenberg 2017).

195 However, the three species differ in their mating strategy (Table 1). While *O. acetosella* has a

196 mixed mating system and produces most of its seeds from cleistogamous flowers (Berg and Redbo-

197 Torstensson 1998), *A. nemorosa* and *P. multiflorum* are considered mainly and strictly self-  
198 incompatible, respectively (Müller et al. 2000; Kosiński 2012). The two outcrossing species differ in  
199 their associated pollinators. *Anemone nemorosa* is pollinated by several groups, including flies, bees,  
200 small beetles and thrips (Shirreffs 1985), however, according to standardized pollinator observations  
201 (unpublished data, see Appendix S1 in the Online Resource), the most important groups are  
202 hoverflies and solitary bees. The most important hoverfly pollinators of *A. nemorosa*, such as  
203 *Melanostoma scalare* or *Syrphus torvus*, show a clear preference for deciduous forest (Speight et al.  
204 2016) and are therefore expected to cross the agricultural matrix between forest patches rarely. The  
205 maximum foraging distances of solitary bees do not exceed 600 m (Gathmann and Tschardt 2002).  
206 In contrast, *P. multiflorum* is pollinated by long-tongued bumblebees (Hasegawa and Kudo 2005;  
207 Kosiński 2012), which are generally more mobile than solitary bees (Redhead et al. 2016). Our  
208 observations confirmed that *Bombus pascuorum* and *B. pratorum* with maximum forage distances of  
209 >1800 m (Redhead et al. 2016) and >670 m (Knight et al. 2005), respectively, are the most important  
210 pollinators of *P. multiflorum* (Appendix S1).

211         Apart from their mating strategy, the three species also differ in some other traits such as  
212 ploidy level and age of first flowering, which might affect their response to habitat loss and  
213 fragmentation (Table 1). In contrast to *O. acetosella* and *P. multiflorum* that are diploid, *A. nemorosa*  
214 is an aneuploid tetraploid with  $2n = 30 = 4x-2$  throughout Central Europe (Baumberger 1971).  
215 Hexaploid individuals have been reported from southern Sweden (Bernström 1946). Since our raw  
216 data did not show any evidence for differences in ploidy among regions and more than four allele  
217 peaks were only exceptionally observed across all regions, we treated *A. nemorosa* as tetraploid.

218

## 219 2.2 Study design and population fragmentation

220 We studied the landscape-scale population genetic structure of the three plant species in seven  
221 (*A. nemorosa*) and six (*O. acetosella* and *P. multiflorum*)  $5 \times 5$  km<sup>2</sup> landscape windows, which cover a  
222 geographical gradient across temperate Europe from North France, over Belgium, West Germany,

223 East Germany and South Sweden up to Central Sweden and Estonia (Fig. 2).  
224 *Polygonatum multiflorum* is fairly rare in Central Sweden and did not occur within the landscape  
225 window. *Oxalis acetosella* had too few occurrences in the Belgian landscape window to be included.  
226 The landscape windows have previously been extensively studied to quantify the plant species  
227 diversity in all forest fragments (Valdés et al. 2015; Vanneste et al. 2019). Therefore, the locations of  
228 all forest herb populations in these landscapes are well documented. All landscape windows  
229 represent agricultural landscapes, in which forest fragments are embedded in an agricultural matrix.  
230 Forest cover ranges between 6 and 28% (Fig. 2; Table S3).

231 In each landscape window, we aimed at selecting six populations for each species. For  
232 *O. acetosella*, only five populations were available in France and Central Sweden (Table S6). Thus, the  
233 total number of surveyed populations amounted to 42, 34 and 36 for *A. nemorosa*, *O. acetosella* and  
234 *P. multiflorum*, respectively. We defined a population as a spatially distinct group of shoots >100 m  
235 apart from other shoots. Typically, populations covered the whole forest patch, but were in some  
236 cases restricted to certain parts of a forest patch if habitat conditions were heterogeneous. We  
237 selected the populations within landscape windows according to the following criteria: (a) the  
238 corresponding forest patches should be older than 200 years, as assessed from old maps (Valdés et  
239 al. 2015) to avoid potential founder effects, which can occur in young populations; (b) the  
240 populations had to represent the full range of available population sizes; (c) they should be  
241 maximally distributed across the entire landscape window; (d) the populations of all three species  
242 should preferably be in the same forest patches; and (e) more than one population within the same  
243 forest patch was only accepted if these populations were separated by >200 m and if no other  
244 populations were available in other forest patches.

245 For each selected population, we estimated census population size and its degree of spatial  
246 isolation. Population size was either determined by counting all flowering shoots during the time of  
247 full flowering or, if this was not feasible, estimated as the product of population area and population  
248 density (i.e., the mean number of flowering shoots per square meter). If the population area did not

249 correspond to the forest patch area, it was demarcated in the field by marking the outermost shoots  
250 of the population with a GPS device and afterwards measured in GIS. To estimate population density,  
251 we counted 40 flowering shoots along each of five randomly placed transects of 2 m width and  
252 measured transect length (Appendix S4). For *O. acetosella*, we took only the easily visible  
253 chasmogamous flowers into account, which are produced in spring.

254 The degree of spatial isolation was measured based on the connectivity of a population to all  
255 other populations in each landscape window (including those we did not survey) using the incidence  
256 function model derived from Hanski (1994):  $C_i = \sum_{j \neq i} A_j^b \cdot e^{-\alpha d_{ij}}$ , where  $C_i$  is the connectivity of  
257 population  $i$ ,  $A_j$  is the population size of population  $j$ ,  $d_{ij}$  is the edge-to-edge distance between  
258 populations  $i$  and  $j$ , and  $\alpha$  and  $b$  are calibration parameters. The parameter  $\alpha$  scales the effect of  
259 distance to dispersal (i.e., with a larger  $\alpha$ , migration between habitats becomes more difficult). We  
260 estimated  $1/\alpha$  for each species as the mean nearest-neighbour distance among all populations in a  
261 landscape window, averaged across landscape windows. This resulted in  $\alpha$  values for *A. nemorosa*,  
262 *O. acetosella* and *P. multiflorum* of  $3.65 \text{ km}^{-1}$ ,  $3.16 \text{ km}^{-1}$  and  $3.59 \text{ km}^{-1}$ , respectively. The parameter  $b$   
263 was set to 0.5 as recommended by Moilanen and Nieminen (2002). Population size of non-surveyed  
264 populations was estimated based on data from an earlier study (Appendix S5; Valdés et al. 2015).  
265 Population areas and edge-to-edge distances among populations were calculated using ARCGIS 10.6.1  
266 (ESRI 2017).

267

### 268 2.3 Sampling, DNA extraction and genotyping

269 From each plant population, we randomly collected leaf material from up to 20 flowering, healthy  
270 individuals spread across the population for DNA extraction. A minimum distance of 10 m between  
271 selected plants was set to avoid sampling of clones. Fewer than 20 samples per population were  
272 available in 29.5% of the populations (Tables S6 and S7.1) either due to genotyping failures or due to  
273 a very small population size. Leaf samples were dried and stored using silica gel. In total, 2,159 leaf  
274 samples were used in subsequent genetic analyses. Total genomic DNA was extracted with the

275 innuPREP Plant DNA Kit (Analytik Jena AG, Germany). We genotyped our samples based on sets of  
276 microsatellite markers (Appendix S8) that either had been developed for congeneric species  
277 (*A. nemorosa* and *P. multiflorum*) or were newly developed for *O. acetosella* by AllGenetics & Biology  
278 SL (Spain) on demand. The applied marker sets comprised six, nine and six markers with a total  
279 number of 102, 61 and 149 alleles for *A. nemorosa*, *O. acetosella* and *P. multiflorum*, respectively  
280 (Tables S8.1-3). The corresponding primers were multiplexed and amplified with PCR following  
281 different protocols (Appendix S8). Fragment analysis was performed on a 3730XL DNA analyzer  
282 (Applied Biosystems, USA) by Macrogen Europe (Amsterdam, Netherlands) with GeneScan 500 LIZ  
283 (Applied Biosystems) for *A. nemorosa* and *P. multiflorum* and GeneScan 350 ROX (Applied  
284 Biosystems) for *O. acetosella* as an internal size standard. Alleles were manually scored using  
285 GENEMAPPER 5 (Applied Biosystems). For a stratified random subsample of 10% from all landscapes,  
286 we repeated genotyping to estimate the multilocus genotyping error rate, which turned out to be  
287 4.8%, 2.8% and 5.7% for *A. nemorosa*, *O. acetosella* and *P. multiflorum*, respectively. The number of  
288 repeated multilocus genotypes (MLG) was low in *A. nemorosa* (10 out of 814 samples) and  
289 *P. multiflorum* (44 out of 669 samples), reflecting our efforts to avoid sampling of clones. In  
290 *O. acetosella*, the number of repeated MLG was higher (144 out of 663), likely as a result of the lower  
291 degree of polymorphism in the applied marker set (Appendix S9). Nevertheless, the repeated MLG  
292 were randomly distributed across the regions (Appendix S9) and 519 unique MLG remained for our  
293 population genetic analyses. For further analyses, we excluded all repeated MLG.

294 For all three species, we tested for linkage disequilibrium using the function `pair.lia` in the  
295 R package `poppr` 2.8.3 (Kamvar et al. 2014). Although significant in some pairs of loci, the  
296 standardized index of association was always  $\leq 0.12$  in all three species, indicating that linkage among  
297 loci was negligible.

298 While the calculation of allele frequencies is straightforward in diploid species, the  
299 distribution of alleles in partially heterozygous polyploids is ambiguous (Meirmans et al. 2018).  
300 Therefore, we used the function `deSilvaFreq` from the R package `polysat` 1.7-4 (Clark and

301 Jasieniuk 2011) to estimate allele frequencies according to De Silva et al. (2005) assuming a selfing  
302 rate of 0.06 (Müller et al. 2000) and polysomic inheritance (Stehlik and Holderegger 2000) for  
303 *A. nemorosa*.

304

#### 305 *2.4 Genetic diversity and differentiation*

306 We calculated several measures of genetic diversity and differentiation. These are mostly not directly  
307 comparable among species, given their different ploidy and the varying level of polymorphism in the  
308 applied marker sets. However, the aim of this study was to compare species in terms of their relative  
309 responses to population size and connectivity (section 2.5) rather than in terms of absolute values.

310 To quantify genetic diversity within populations, we calculated allelic richness ( $A_r$ ), unbiased  
311 expected heterozygosity ( $H_e$ ), observed heterozygosity ( $H_o$ ) and the inbreeding coefficient  $F = 1 - H_o/H_e$   
312 for each population. Allelic richness is only comparable among populations when based on the same  
313 sample size. However, nine populations had genotype numbers <10 (Table S7.1). Therefore, rarefying  
314 on the minimum sample size would mean losing a lot of information and to down-weight rare alleles.  
315 Furthermore, low genotype numbers were not due to limited sampling effort, but very small  
316 population sizes (i.e., more genotypes could not be found in the field). As a trade-off, we therefore  
317 rarefied allelic richness based on the mean sample size across populations corresponding to 19, 15  
318 and 17 samples in *A. nemorosa*, *O. acetosella* and *P. multiflorum*, respectively. Heterozygosity  
319 measures were calculated using standard formulas in the diploid species (Nei and Chesser 1983). For  
320 *A. nemorosa*, we calculated unbiased  $H_e$  using equation 8 in Hardy (2016) and  $H_o$  using the concept of  
321 gametic homozygosity (Moody et al. 1993). The latter is the observed homozygosity when drawing  
322 random diploid gametes from individuals, and thus comparable among ploidy levels. The probability  
323  $\Phi$  of choosing two alleles that are identical in state from a given tetraploid sample depends on the  
324 allele dosage information, which was missing. Therefore, we assembled all possible unambiguous  
325 genotypes for each phenotype and averaged  $\Phi$  across these genotypes using the genotype

326 probabilities as weights. Unambiguous genotype probabilities were calculated using code from the  
327 function `meandistance.matrix2` of the `polysat` package (Appendix S10).

328 To quantify genetic differentiation among populations, we used two measures,  $G''_{ST}$   
329 (Meirmans and Hedrick 2011) and  $D_{PS}$  (Bowcock et al. 1994). While  $G''_{ST}$  is based on heterozygosity  
330 (like the traditional  $F_{ST}$  or  $G_{ST}$ ) and is the recommended genetic differentiation measure with  
331 microsatellite markers (Meirmans and Hedrick 2011),  $D_{PS}$  is equal to 1 minus the proportion of shared  
332 alleles and therefore facilitates an intuitive interpretation. We also tested two other commonly used  
333 differentiation measures,  $D_{est}$  (Jost 2008) and  $\rho$  (Ronfort et al. 1998), which were, however, highly  
334 correlated ( $r \geq 0.92$ ) with  $G''_{ST}$  and gave very similar results (Appendix S15). To calculate  $G''_{ST}$ , we  
335 used the original formula for diploid species based on the average ( $H_S$ ) and total expected  
336 heterozygosity ( $H_T$ ). For *A. nemorosa*, we used formulas for  $H_S$  and  $H_T$  analogous to equations 15 and  
337 16, respectively, in Nei and Chesser (1983), but replaced the harmonic mean of the number of  
338 samples in the populations by the harmonic mean of the number of genomes in the populations to  
339 allow for polyploidy (Clark and Jasieniuk 2011). We calculated both global statistics ( $G''_{ST}$ ) for each  
340 landscape window (tested with permutation tests) and pairwise statistics ( $G''_{ST}$  and  $D_{PS}$ ) for each  
341 population pair within each landscape window. In addition, we calculated for each population a site-  
342 level differentiation value as the average pairwise differentiation of a focal population to all other  
343 populations in the landscape.

344

## 345 *2.5 Data analysis*

346 In a first step, we tested for differences in population size, connectivity, genetic diversity, geographic  
347 distance and genetic differentiation among species using stratified two-samples permutation tests as  
348 implemented in the R package `permuter` 0.1 (Ottoboni 2020). Permutation was done  
349 independently within landscape windows. Next, we used linear mixed models (LMMs) to model  
350 genetic diversity and site-level genetic differentiation as a function of population size, connectivity  
351 and their interaction with species identity, using forest patch ID nested within landscape window ID



352 as random intercept terms. Collinearity of population size and connectivity was low as indicated by a  
353 generalized variance-inflation factor < 3.0 for all predictors in all models. In order to compare specific  
354 responses to the degree of population fragmentation among species regardless of absolute  
355 differences in species-level genetic diversity and differentiation, we standardized all quantitative  
356 variables to mean = 0 and s.d. = 1 separately for each species. Prior to standardization, all variables  
357 were Box-Cox transformed (Box and Cox 1964) to make them approach normality. We fitted separate  
358 models for each genetic diversity and differentiation variable. All LMMs were fitted with the R  
359 function `lme` (Pinheiro et al. 2019).

360 Finally, we used two approaches to test for isolation-by-distance. First, we regressed pairwise  
361 genetic differentiation against geographic distance and its interaction with species using maximum-  
362 likelihood population-effects models (MLPE, Clarke et al. 2002). These models take the dependence  
363 of two pairwise distances involving a common population into account and were fitted using the  
364 function `corMLPE` (Pope 2020) to define a correlation structure within the `lme` function. Landscape  
365 window was included as a random intercept term. A positive relationship between genetic  
366 differentiation and geographic distance is often found only over a limited extent of the spatial range,  
367 usually reflecting gene dispersal distances (Slatkin 1993). Therefore, we used an autocorrelation  
368 analysis as a second approach to test for isolation-by-distance. We produced Mantel correlograms  
369 (Borcard and Legendre 2012) separately for each species, but with data from all landscape windows  
370 combined, to test, up to which distance nearby populations are genetically more similar to each  
371 other than more distant populations. For this purpose, we used the R function `eco.cormante1`  
372 (Roser et al. 2017) and restricted permutations to occur within landscape windows only. Whether  
373 significant spatial autocorrelation is found at a certain distance depends largely on the defined  
374 distance interval and thus the number of population pairs included in the test. Therefore, we tested a  
375 sequence of distance intervals from 600 m to 2,000 m to find the interval that yielded the highest  
376 Mantel statistic  $r_M$  at the first distance lag that was significant according to a permutation test with  
377 9,999 permutations. All calculations and analyses were done in R 3.6.2 (R Core Team 2019).

378

379

### 380 **3. Results**

381

#### 382 *3.1 Differences in population attributes among species*

383 Census population size and connectivity were much higher in *A. nemorosa* than in *P. multiflorum*,  
384 with *O. acetosella* taking an intermediate position. The range of values covered several orders of  
385 magnitude in all three species (Fig. 3a, b; Table S7.2). Genetic diversity measures were generally  
386 higher in *A. nemorosa* and *P. multiflorum* than in *O. acetosella* (Fig. 3c-e), reflecting the different  
387 degree of polymorphism in the applied marker sets. Differences between expected and observed  
388 heterozygosity were highest in *A. nemorosa*, for which the inbreeding coefficient was highest (Fig. 3f)  
389 and significantly positive (i.e., indicating inbreeding) in 41 out of 42 populations (Fig. S11a). For  
390 *O. acetosella*, the *F*-value was significantly positive in 21 out of 34 populations (Fig. S11b), whereas  
391 for *P. multiflorum*, *F*-values were mostly negative, indicating outbreeding (significant in 10 out of 36  
392 populations; Fig. S11c). The geographic distance among surveyed populations was similar between  
393 species (Fig. 3g) as intended by our sampling design. The degree of pairwise genetic differentiation,  
394 however, differed among species (Fig. 3h, i). In general, genetic differentiation was highest in  
395 *P. multiflorum*, while the ranking of *A. nemorosa* and *O. acetosella* depended on the applied  
396 differentiation measure. Global  $G''_{ST}$  statistics were generally significant, indicating population  
397 genetic structure, for all species and all landscapes except for *A. nemorosa* in France (Tables S12).

398

#### 399 *3.2 Effects of population size and connectivity on genetic diversity and site-level differentiation*

400 Allelic richness increased significantly with population size in all three species (Table 2; Fig. 4a). This  
401 effect corresponded to a loss of allelic richness between the largest and smallest populations studied  
402 of 20%, 41% and 44% for *A. nemorosa*, *O. acetosella* and *P. multiflorum*, respectively. In  
403 *O. acetosella*, also expected and observed heterozygosity increased significantly with population size

404 (Fig. 4b, c). The loss of heterozygosity between the largest and smallest population amounted to  
405 60%. In *A. nemorosa* and *P. multiflorum*, heterozygosity was unaffected or weakly affected (only  $H_e$ )  
406 by population size, respectively. The inbreeding coefficient was unrelated to population size in all  
407 three species. Site-level genetic differentiation decreased with increasing population size for all  
408 species and differentiation measures (Table 2).

409 Connectivity only affected the genetic diversity of *A. nemorosa*. Specifically, allelic richness,  
410 expected heterozygosity and the inbreeding coefficient showed a positive response to connectivity  
411 (Table 2; Fig. 4d, e). The loss of allelic richness between the least and most spatially isolated  
412 population amounted to 30%. In all three species, a high connectivity reduced site-level genetic  
413 differentiation (Table 2). This effect was significantly stronger in *A. nemorosa* than in *P. multiflorum*  
414 (Fig. 4f).

415 In general, the amount of variation explained by population size and connectivity, as  
416 expressed by the marginal  $R^2$ , was moderate to low (8-31%; Table 2). The conditional  $R^2$  was  
417 generally much higher (20-77%), indicating differences in genetic diversity and differentiation among  
418 forest patches and landscapes.

419

### 420 3.3 Effects of geographic distance on genetic differentiation

421 Geographic distance had no significant effect on pairwise genetic differentiation in MLPE models  
422 (Tables S14). However, spatial autocorrelation analysis revealed for all species that genetic  
423 differentiation at the first distance lag was significantly lower than expected by chance, indicating  
424 isolation-by-distance (Fig. 5). The lag distance yielding the highest Mantel statistic ( $r_M$ ) was lowest in  
425 *O. acetosella* (700 m), intermediate in *A. nemorosa* (1,300-1,400 m) and highest in *P. multiflorum*  
426 (1,600 m). The  $r_M$ -value at the first distance lag was highest for *O. acetosella* (0.20-0.21),  
427 intermediate for *A. nemorosa* (0.12-0.17) and lowest for *P. multiflorum* (0.06-0.07).

428

429

## 430 4. Discussion

431

432 Our study demonstrates that clonal, slow-colonizing forest herbs are in fact sensitive to habitat loss  
433 and fragmentation. All three species responded to reductions in population size with a decrease in  
434 allelic richness and an increase in genetic differentiation among populations. Genetic differentiation  
435 also increased with enhanced spatial isolation. For the first time, we show this by using simultaneous  
436 population genetic analysis of three forest herbs across multiple landscapes. At the same time,  
437 however, our results reveal unique responses for each species, reflecting differences in life-history  
438 traits.

439

### 440 4.1 Differences in the inbreeding coefficient between species

441 While most genetic variables were not comparable among species in absolute terms, the relative  
442 difference between observed and expected heterozygosity, as expressed by the inbreeding  
443 coefficient, should be independent of allele numbers and thus comparable among species.  
444 Surprisingly, the mainly outcrossing *A. nemorosa* (median 0.29) rather than the regularly selfing  
445 *O. acetosella* (0.20) showed the highest *F*-values (Fig. 3f). In a study on 20 Central European  
446 populations of *A. nemorosa*, *F*-values were lower (mean 0.21) and significantly >0 in only half of the  
447 populations (Stehlik and Holderegger 2000). Although *A. nemorosa* is not strictly self-incompatible,  
448 seed set is strongly reduced when open pollination is excluded (Müller et al. 2000). We think,  
449 therefore, that mostly other factors than occasional selfing caused the homozygosity excess, in  
450 particular biparental inbreeding (Stehlik and Holderegger 2000), null alleles and double reduction  
451 (i.e., the erroneous inheritance of twice the same gene copy during meiosis; Hardy 2016). In fact, the  
452 null allele frequencies in our *A. nemorosa* samples (inherently estimated by the function  
453 `deSilvaFreq`, section 2.5) were relatively high, amounting to  $0.237 \pm 0.183$  (grand mean across  
454 loci and populations  $\pm$  s.d.). An observation that supports the idea that null alleles and double  
455 reduction contributed to the homozygosity excess in *A. nemorosa* is the unexpected positive

456 relationship between the inbreeding index and connectivity (Table 2). It likely results from the fact  
457 that expected heterozygosity increased with connectivity, while observed heterozygosity remained  
458 low independent of connectivity. This pattern is unlikely to be caused by uni- or biparental  
459 inbreeding, which are usually enhanced in more isolated populations (Young et al. 1996).

460         The range of  $F$ -values in *O. acetosella* was large compared to *A. nemorosa* and values at the  
461 upper extreme exceeded those in *A. nemorosa*, indicating that levels of inbreeding can be higher in  
462 *O. acetosella* than in *A. nemorosa*. However, average  $F$ -values were unexpectedly low compared to  
463 other selfing forest herbs (*Geum urbanum*: 0.92 [Vandepitte et al. 2007], *Viola grypoceras*: 0.87  
464 [Toma et al. 2015]). In their thorough study on the recruitment strategy in *O. acetosella*, Berg and  
465 Redbo-Torstensson (1998, 2000) found that the contribution of seedlings originating from cross-  
466 fertilized flowers to the adult plant population can be substantial, amounting to 30% on average  
467 though varying greatly between years and sites. This number might be even underestimated  
468 considering that their study was conducted near Uppsala in Sweden, which is located further north  
469 than any of our landscape windows. Thus, a lower pollinator availability might have enhanced the  
470 relative importance of selfing in their study sites (Moeller et al. 2017), while our results suggest that  
471 *O. acetosella* is mostly outcrossing.

472         In *P. multiflorum*,  $F$ -values close to zero were expected, given its self-incompatibility (Kosiński  
473 2012). The tendency towards negative values (Fig. 3f; Fig. S11c) indicates that sexual reproduction  
474 occurs more often between individuals from different populations than between nearby individuals  
475 (outbreeding). A mechanism that could explain this pattern is the fact that bumblebees, on which the  
476 pollination of *P. multiflorum* flowers depends, prefer to fly across the open landscape rather than  
477 through dense forest (Kamm et al. 2010).

478

#### 479 4.2 Does the mating system matter?

480 Allelic richness was positively related to population size in all three species (Table 2; Fig. 4a). In  
481 contrast to our second hypothesis, this relationship was not more pronounced in the mainly

482 outcrossing species. Meta-analyses have shown a stronger positive relationship between population  
483 size and the number of alleles in self-incompatible than in self-compatible species (Leimu et al. 2006;  
484 Honnay and Jacquemyn 2007; Aguilar et al. 2008). As discussed above, the high outcrossing rate  
485 observed in the self-compatible *O. acetosella* explains the lack of this pattern in our results. In terms  
486 of heterozygosity, *O. acetosella* showed the strongest relationship with population size (Fig. 4b, c). It  
487 was the only species, in which observed heterozygosity was significantly reduced in smaller  
488 populations. Although not unexpected (Frankham 1996), a positive relationship between observed  
489 heterozygosity and population size has to our knowledge not been reported in any other study on  
490 fragmented forest herb populations. The most obvious mechanism that could lead to decreased  
491 heterozygosity in small populations is selfing or biparental inbreeding (Young et al. 1996). However,  
492 the inbreeding index was not related to population size in *O. acetosella* (Table 2). Genetic drift can  
493 also increase homozygosity and might occur faster in *O. acetosella* than in *A. nemorosa* and  
494 *P. multiflorum* given its shorter generation time. Although all three species exhibit strong clonal  
495 growth, *O. acetosella* relies more on sexual reproduction than the other two species. Seedling  
496 production in *O. acetosella* may exceed ramet production (Berg 2002). Both seedlings that have  
497 survived their first winter as well as new ramets may already flower in their first year as adults (Berg  
498 2002). Therefore, the generation time is probably much shorter in *O. acetosella* than in *A. nemorosa*  
499 and *P. multiflorum*, which reach their generative phase mostly after ten or more years (Shirrefs 1985;  
500 Kosiński 2015).

501 In accordance with our second hypothesis, genetic diversity ( $A_r$  and  $H_e$ ) decreased with  
502 increasing spatial isolation in the mainly outcrossing *A. nemorosa*, but not in the self-compatible  
503 *O. acetosella* (Table 2; Fig. 4d). However, given the low selfing signal in *O. acetosella* (and the  
504 apparent high inbreeding signal in *A. nemorosa*), we cannot be sure that this distinct response is due  
505 to the originally assumed difference in mating system. Furthermore, there was no effect for the  
506 other outcrossing species, *P. multiflorum* (Table 2). The different response between *A. nemorosa* and  
507 *O. acetosella* indicates that the inflow of new alleles is reduced in more isolated *A. nemorosa*

508 populations, but not much impeded in more isolated *O. acetosella* populations. In plants, gene flow  
509 takes place through seed or pollen dispersal. However, both dispersal pathways do not clearly differ  
510 between the two species. Both species are strongly dispersal-limited (Table 1) and have similarly low  
511 colonization capacities (Verheyen et al. 2003). According to anecdotal knowledge (Knuth 1898;  
512 Redbo-Torstensson and Berg 1995), *O. acetosella* is pollinated by a similar range of pollinators as  
513 *A. nemorosa*, including flies, beetles, thrips, bees and bumblebees. Moreover, further results of our  
514 analysis argue against a higher seed or pollen dispersal distance in *O. acetosella* than in *A. nemorosa*.  
515 First, in both species, site-level genetic differentiation increased with decreasing population size and  
516 decreasing connectivity at similar rates (Table 2; Fig. 4f). Second, the Mantel correlograms indicated  
517 that isolation-by-distance is effective over a shorter distance in *O. acetosella* than in *A. nemorosa*  
518 (Fig. 5). We therefore conclude that the answer to the question, whether and why genetic diversity is  
519 more sensitive to spatial isolation in *A. nemorosa* than in *O. acetosella*, requires further investigation.  
520 So far, the difference in mating system seems to be the best explanation despite the unexpectedly  
521 high and low inbreeding coefficients in *A. nemorosa* and *O. acetosella*, respectively.

522

#### 523 *4.3 Does the mobility of the associated pollinators matter?*

524 The higher mobility of the pollinators associated with *P. multiflorum* compared to *A. nemorosa* is  
525 reflected in some of our results. For *A. nemorosa*, the spatial proximity to other populations was  
526 important to maintain high levels of genetic diversity, whereas for *P. multiflorum* this was not the  
527 case (Table 2; Fig. 4d). Also, the degree of genetic differentiation was stronger determined by  
528 connectivity in *A. nemorosa* than in *P. multiflorum* (Table 2; Fig. 4f). The isolation-by-distance signal  
529 was stronger in *A. nemorosa* (higher  $r_M$ ) and restricted to a lower distance compared to  
530 *P. multiflorum* (Fig. 5). These results are in line with our assumption that the main pollinators of  
531 *A. nemorosa* (i.e., hoverflies and solitary bees), either prefer deciduous forest as habitat (Appendix  
532 S1; Speight et al. 2016), or cover shorter forage distances than the bumblebee pollinators of  
533 *P. multiflorum* (Gathmann and Tschardt 2002; Redhead et al. 2016). Alternatively, a higher seed

534 dispersal potential in *P. multiflorum* than in *A. nemorosa* would explain the weaker response of  
535 *P. multiflorum* to increased spatial isolation. The fleshy berries of *P. multiflorum* appear to be  
536 destined for endozoochorous dispersal. They are, however, toxic and in fact hardly dispersed by birds  
537 or large mammals (Ehrlén and Eriksson 1993; Schaumann and Heinken 2002). Also, both plant  
538 species show a strong affinity to ancient forests, indicating their low colonization capacity (Graae  
539 2000; Schmidt et al. 2014; Naaf and Kolk 2015). We think, therefore, that the higher mobility of the  
540 bumblebees associated with *P. multiflorum* is a more likely explanation for the observed patterns  
541 than a higher seed dispersal potential.

542         Other results were not in line with our expectations. In terms of genetic diversity, both  
543 species were similarly sensitive to habitat loss (i.e., a reduced population size; Table 2; Fig. 4a, b).  
544 This suggests that pollen-mediated gene flow between populations via bumblebees is not able to  
545 compensate for genetic erosion in small populations of *P. multiflorum*. This is also reflected in a  
546 relatively high level of genetic differentiation among *P. multiflorum* populations (Fig. 3h, i). Small  
547 populations of *P. multiflorum* may have difficulties to attract bumblebees and thus to sustain pollen  
548 flow. The flowering shoot density in *P. multiflorum* is relatively low compared to, for instance, that in  
549 *A. nemorosa*. Although a single *P. multiflorum* shoot may have more than 20 flowers, only three of  
550 these flowers are open at the same time (Kosiński 2012). In another self-incompatible forest herb  
551 that is pollinated by bumblebees, *Phyteuma spicatum*, seed production was significantly reduced in  
552 smaller compared to larger populations, but not in more isolated populations (Kolb 2005).  
553 Furthermore, pollen limitation in small populations of plants pollinated by bumblebees has been  
554 frequently demonstrated through differences in seed or fruit set between hand-pollinated and open-  
555 pollinated patches (Sih and Baltus 1987; Kwak et al. 1998; Kolb 2005).

556         The relatively weak response of genetic diversity in *A. nemorosa* to a reduced population size  
557 (i.e., not significantly stronger than in *P. multiflorum* as hypothesized) may have two reasons. First,  
558 *A. nemorosa* is tetraploid, which means that genetic erosion through drift happens more slowly  
559 (Meirmans et al. 2018). Second, although the main pollinator groups associated with *A. nemorosa*



560 may be less mobile than those associated with *P. multiflorum*, they might be attracted at higher  
561 rates. Even small populations of *A. nemorosa* build carpets of conspicuous flowers, of which each  
562 offers pollen for approximately one week (Sherriffs 1985). Moreover, *A. nemorosa* offers pollen early  
563 in the spring, when few other floral resources are available.

564

#### 565 *4.4 General patterns across temperate Europe*

566 Population size and connectivity as well as geographic distance explained only small to moderate  
567 proportions of the variation in genetic response variables. The conditional  $R^2$  was always much  
568 higher than the marginal  $R^2$  (Table 2), indicating that considerable amounts of variation in genetic  
569 response variables could be explained by the different landscapes. Although all landscapes represent  
570 agricultural landscapes of temperate Europe and in this sense were regarded as replicates in this  
571 study, we know from previous research that our landscapes differ in many respects including the  
572 macroclimate (Valdés et al. 2015), the degree of forest cover and fragmentation (Vanneste et al.  
573 2019), the composition and configuration of the agricultural matrix (Vanneste et al. 2019), the  
574 position within the species' distribution range (Reinecke et al. 2016) and the species' ecological  
575 behaviour (De Frenne et al. 2009; Reinecke et al. 2016). While all these factors might directly or  
576 indirectly affect the species' population genetic structure, analysing their influence and relative  
577 importance would require larger  $N$  and is out of scope in this study. We want to stress, however, that  
578 despite these differences among landscapes, we were able to find general patterns of species'  
579 responses to habitat loss and fragmentation (including differences among species) that were  
580 consistent across landscapes. In this regard, our study extends and goes beyond population genetic  
581 case studies that focus on a single species in a single landscape (Segelbacher et al. 2010).

582

583

## 584 **5. Conclusions**

585

586 From our results, we draw three main conclusions. First, despite their long life spans and ability to  
587 delay fragmentation effects through clonal propagation (Honnay et al. 2005), all three forest herb  
588 species are sensitive to habitat loss and fragmentation. This will compromise their long-term viability,  
589 particularly in small and isolated populations. Second, each species has its own life-history, which led  
590 to distinct responses to habitat loss and fragmentation. Results for a single species may be difficult to  
591 assess without reference to other species with known differences in life-history traits studied within  
592 similar landscapes. Multi-species comparisons across replicate populations in multiple landscapes are  
593 thus needed to assess the generality of patterns found in independent case studies. Third, our results  
594 indicate that the mobility of associated pollinators may strongly affect forest herbs' sensitivity to  
595 habitat loss and fragmentation beyond the commonly considered effects of mating system and  
596 clonality.

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841

842 **Tables**

843

844 **Table 1** Differences in life-history traits for three forest herb species that might affect their response

845 to habitat loss and fragmentation. Superscripts indicate sources of information, which are provided in

846 Appendix S2

	<i>Anemone nemorosa</i>	<i>Oxalis acetosella</i>	<i>Polygonatum multiflorum</i>
Mating system	mostly outcrossing <sup>9</sup>	mixed <sup>2</sup>	outcrossing <sup>7</sup>
Associated pollinators	bees, hoverflies, bumblebees and others <sup>14, 15, 17</sup>	flies, beetles, thrips, bees <sup>6, 12</sup>	bumblebees <sup>7, 17</sup>
Seed dispersal mode	blastochory, myrmecochory <sup>10</sup>	ballochory <sup>10</sup>	barochory <sup>5, 10, 13</sup>
Seed dispersal distance	<15 m <sup>10, 16</sup>	<5 m <sup>3</sup>	<1 m <sup>10, 16</sup>
Ploidy	tetraploid <sup>1</sup>	diploid <sup>11</sup>	diploid <sup>7</sup>
Age of first flowering	10 years <sup>14</sup>	≥1 year <sup>4</sup>	10 years <sup>8</sup>

847

848

849 **Table 2** Effects of census population size and connectivity on measures of genetic diversity ( $A_r$ ,  $H_e$ ,  $H_o$   
850 and  $F$ ) and site-level differentiation ( $G''_{ST}$  and  $D_{PS}$ ) according to linear mixed modelling results. Given  
851 are standardized regression coefficients for the species *Anemone nemorosa* (An), *Oxalis acetosella*  
852 (Ox) and *Polygonatum multiflorum* (Po) and marginal (only fixed effects) and conditional (fixed and  
853 random effects)  $R^2$ . Significance of regression coefficients is indicated by asterisks: <sup>n.s.</sup> $P > 0.1$ , (\*) $P \leq 0.1$ ,  
854 \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ . Significant differences ( $\alpha = 0.1$ ) in slopes among species are  
855 indicated with lower-case letters. See Table S13 for complete T tables

		$A_r$	$H_e$	$H_o$	$F$	$G''_{ST}$	$D_{PS}$
Pop. size	An	<b>0.35**</b> ,a	0.04 <sup>n.s.</sup> ,a	0.00 <sup>n.s.</sup> ,a	0.04 <sup>n.s.</sup> ,a	<b>-0.24*</b> ,a	<b>-0.16</b> (*) <sup>n.s.</sup> ,a
	Ox	<b>0.54***</b> ,a	<b>0.54**</b> ,b	<b>0.53***</b> ,b	-0.07 <sup>n.s.</sup> ,a	<b>-0.36**</b> ,a	<b>-0.18</b> <sup>n.s.</sup> ,a
	Po	<b>0.61***</b> ,a	<b>0.27</b> (*) <sup>n.s.</sup> ,ab	0.02 <sup>n.s.</sup> ,a	0.22 <sup>n.s.</sup> ,a	<b>-0.32**</b> ,a	<b>-0.35**</b> ,a
Connectivity	An	<b>0.46**</b> ,a	<b>0.34</b> (*) <sup>n.s.</sup> ,a	-0.10 <sup>n.s.</sup> ,a	<b>0.38*</b> ,a	<b>-0.58***</b> ,a	<b>-0.48***</b> ,a
	Ox	0.22 <sup>n.s.</sup> ,ab	0.04 <sup>n.s.</sup> ,a	-0.13 <sup>n.s.</sup> ,a	0.01 <sup>n.s.</sup> ,ab	<b>-0.47**</b> ,ab	<b>-0.31*</b> ,a
	Po	0.00 <sup>n.s.</sup> ,b	0.05 <sup>n.s.</sup> ,a	0.19 <sup>n.s.</sup> ,a	-0.18 <sup>n.s.</sup> ,b	<b>-0.25*</b> ,b	<b>-0.42***</b> ,a
Marginal $R^2$		0.31	0.14	0.09	0.08	0.24	0.21
Conditional $R^2$		0.62	0.42	0.40	0.20	0.75	0.77

856

857

858 **Figures**

859

860

861 **Fig. 1** The three temperate forest herbs surveyed in the present study. From left to right:  
862 *Anemone nemorosa* L. visited by a hoverfly (*Sphaerophoria* spec.; photo: J.T. Feigs), *Oxalis acetosella* L.  
863 (photo: T. Naaf), *Polygonatum multiflorum* (L.) All. visited by *Bombus pascuorum* Scopoli (photo: J.T.  
864 Feigs)

865

866

867 **Fig. 2** Seven 5 × 5 km<sup>2</sup> landscape windows used to study the regional population genetic structure of  
868 three forest herb species: (a) North France, (b) Belgium, (c) West Germany, (d) East Germany, (e) South  
869 Sweden, (f) Central Sweden and (g) Estonia. Landscape window locations across temperate Europe are  
870 shown in the top-left panel. Locations of the surveyed populations within each landscape window are  
871 indicated by the initial of the corresponding species name: *Anemone nemorosa*, *Oxalis acetosella*,  
872 *Polygonatum multiflorum*

873

874

875 **Fig. 3** Differences in population attributes among the studied species *Anemone nemorosa* (An),  
876 *Oxalis acetosella* (Ox) and *Polygonatum multiflorum* (Po): (a) population size, b) connectivity, (c) allelic  
877 richness ( $A_r$ ), (d) expected heterozygosity ( $H_e$ ), (e) observed heterozygosity ( $H_o$ ), (f) inbreeding  
878 coefficient ( $F$ ), (g) geographic distance, (h) and (i) measures of pairwise genetic differentiation (link  
879 level). Boxplots are standard boxplots. Significant differences ( $P \leq 0.05$  after controlling the false-  
880 discovery rate) according to pairwise stratified two-samples permutation tests (cf. Table S7.2) are  
881 indicated with lower-case letters

882

883

884 **Fig. 4** Partial effects of population size (a-c) and connectivity (d-f) on measures of genetic diversity and  
885 site-level differentiation as resulting from linear mixed models (cf. Table 2). All variables are scaled in  
886 standard deviation units. Shown are regression lines and 95% confidence bands (only for significant  
887 slopes). Lower-case letters denote significant differences ( $\alpha = 0.1$ ) in slopes between species. See Fig.  
888 S13 for plots with partial residuals

889

890

891 **Fig. 5** Mantel correlograms showing the spatial autocorrelation of genetic differentiation measured by  
892 the Mantel statistic  $r_M$  in relation to geographic distance. Marks represent the mean distance for the  
893 corresponding distance lag. The distance intervals differ for the species and/or the genetic  
894 differentiation metrics and amount to 1,400 m ( $G''_{ST}$  in *Anemone nemorosa*), 1,300 m ( $D_{PS}$  in *A.*  
895 *nemorosa*), 700 m (*Oxalis acetosella*) and 1,600 m (*Polygonatum multiflorum*). A maximum of four lags  
896 was used to test for isolation-by-distance

897