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Phylogeographic analysis of two cold-tolerant plants with disjunct Lusitanian distributions does not support *in situ* survival during the last glaciation

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- 1 ABSTRACT
- 2

Aim We used a combination of modelling and genetic approaches to investigate whether *Pinguicula grandiflora* and *Saxifraga spathularis*, two species which exhibit disjunct
Lusitanian distributions, may have persisted through the Last Glacial Maximum (LGM, *ca*.
21 ka) in separate northern and southern refugia.

8 Location Northern and eastern Spain and southwestern Ireland

9

Methods Palaeodistribution modelling using MAXENT was used to identify putative refugial areas for both species at the LGM, as well as to estimate their distributions during the Last Interglacial (LIG, *ca.* 120 ka). Phylogeographic analysis of samples from across both species' ranges was carried out using one chloroplast and three nuclear loci for each species.

15 **Results** The palaeodistribution models identified very limited suitable habitat for either 16 species during the LIG, followed by expansion during the LGM. A single, large refugium 17 across northern Spain and southern France was postulated for *P. grandiflora*. Two suitable 18 regions, one in Northern Spain which corresponds to the eastern part of the species' present-19 day distribution in Iberia, as well another on the continental shelf off the west coast of 20 Brittany, south of the limit of the British-Irish ice sheet, were identified for S. spathularis. 21 Phylogeographic analyses indicated extremely reduced levels of genetic diversity in Irish 22 populations of *P. grandiflora* relative to those in mainland Europe, but comparable levels of 23 diversity between Irish and mainland European populations of S. spathularis, including the 24 occurrence of private hapotypes in both regions.

25

26	Main conclusions Modelling and phylogeographic analyses indicate that <i>P. grandiflora</i>
27	persisted through the LGM in a southern refugium, and achieved its current Irish distribution
28	via northward dispersal after the retreat of the ice sheets. Although the results for S.
29	spathularis are more equivocal, a similar recolonization scenario also seems the most likely
30	explanation for the species' current distribution.
31	
32	Keywords
33	Large-flowered butterwort, Last Glacial Maximum, Lusitanian flora,
34	palaeodistribution modelling, Pinguicula grandiflora, phylogeography, refugia,

35 Saxifraga spathularis, St. Patrick's cabbage.

36 INTRODUCTION

37

38 The present day distributions of many Northern Hemisphere temperate species are largely the 39 result of the climatic fluctuations that occurred throughout the Pleistocene (1.8 Ma – 10 ka; Webb & Bartlein, 1992; Hewitt, 2003). During the extended glacial periods, many of these 40 41 species persisted in climatically suitable refugia, usually south of the ice sheets, and 42 recolonized formerly glaciated areas following the retreat of the ice during the interglacials, 43 achieving their current distributions by the Holocene (10 ka – present; Taberlet et al. 1998; 44 Hewitt 1999). In recent years, however, this simple "expansion-contraction" paradigm of 45 species' persistence throughout the glacial periods has been challenged by palynological and 46 phylogeographic evidence, which suggests that some species might have persisted in 47 "cryptic" refugia further north than had previously been considered (Bennett and Provan 48 2008; Provan and Bennett 2008).

49 The distribution of the so-called "Lusitanian" flora, a group of about a dozen plant species 50 that are found only in southern and western Ireland and northern Iberia, has long puzzled 51 biogeographers. Some botanists proposed that this disjunct distribution was the result of 52 persistence in separate northern and southern refugia during the ice ages, whilst others 53 insisted that not even the hardiest of cold-tolerant plants could survive through the Last 54 Glacial Maximum (LGM; ca. 21 ka) in situ (Forbes 1846; Reid 1913; Praeger 1939; Webb 55 1983). Recently, the first phylogeographic study on a Lusitanian plant species, the heath 56 Daboecia cantabrica, suggested that the species had been confined to southern refugia during 57 the LGM, and had achieved its Irish distribution following the deglaciation (Beatty and 58 Provan 2013). D. cantabrica has minute seeds conducive to dispersal, and it may be that its 59 glacial history is not typical of the Lusitanian flora as a whole, particularly for cold-tolerant 60 species which may have been more likely to survive in northern refugia.

61 In the present study, we have used a combination of palaeodistribution modelling and 62 phylogeographic analysis to determine whether two of the more cold-tolerant Lusitanian 63 plant species, Pinguicula grandiflora Lam. (Large-flowered butterwort) and Saxifraga 64 spathularis Brot. (St. Patrick's cabbage), might have survived in northern refugia during the 65 LGM. S. spathularis has a typical Lusitanian distribution, being found only in northwestern 66 Spain and southwest Ireland, whilst P. grandiflora has a wider distribution in Spain, being 67 found across the north of the country and into the Pyrenees, as well as sporadically in the French and Italian alps, and in southwest Ireland (Figures 1a and 1b). If either species had 68 69 persisted in northern refugia, Irish populations would exhibit comparable genetic diversity to 70 those from Spain whilst harbouring unique genotypes, the two key phylogeographic 71 signatures of long-term persistence (Provan and Bennett 2008).

72 MATERIALS AND METHODS

73

74 Sampling and DNA extraction

Samples were obtained for both species from across their distribution ranges either through
collection in the field or from herbarium collections (See Appendices S1a and S1b for
details). In total, between 149 and 160 samples of *Pinguicula grandiflora* from 38 locations,
and between 161 and 176 samples of *Saxifraga spathularis* from 41 locations were analysed.
Differences in sample numbers were due to lack of amplification in several individuals at one
or more loci. DNA was extracted from field-collected material using a modified CTAB
protocol (Doyle & Doyle 1987) and from herbarium samples using the Qiagen DNeasy kit.

82

83 Palaeodistribution modelling

Palaeodistribution modelling was carried out to determine suitable climate envelopes for both 84 85 species during the last interglacial (LIG, ca. 120 ka) and at the last glacial maximum (LGM, 86 ca. 21 ka) using the maximum entropy approach implemented in the MAXENT software 87 package (V3.3.3; Phillips et al. 2006). Species occurrence data between 1950 and 2000 (269 88 and 385 occurrences for *P. grandiflora* and *S. spathularis* respectively) were downloaded 89 from the Global Biodiversity Information Facility data portal (www.gbif.org). Current-day 90 climatic data (1950-2000; Hijmans et al. 2005) at 2.5 minute resolution were clipped to the 91 approximate distribution area of the species (i.e. Western Europe 13 °W to 10 °E, and 35°N 92 to 60°N) to reduce potential problems associated with extrapolation. Models were generated 93 using cross-validation of ten replicate runs under the default MAXENT parameters. Model 94 performance was assessed based on the area under the receiver operating characteristic curve 95 (AUC). Models were projected onto reconstructed climate data for the LGM (two models: CCSM and MIROC) and the LIG (WorldClim www.worldclim.org). Outputs from the two 96

97 LGM models were averaged to give a single consensus model. To identify areas where the
98 model has extrapolated beyond current climatic conditions, which could lead to unreliable
99 predictions, we carried out a multivariate environmental similarity surfaces (MESS) analysis
100 (Elith *et al.* 2010) in MAXENT.

101

102 **DNA sequencing**

103 Sequence data were obtained from one chloroplast locus (the *trnL-trn*F intergenic spacer for 104 *P. grandiflora* and the *trn*S-*trn*G intergenic spacer for *S. spathularis*) and three anonymous 105 single-copy nuclear loci for each species (details and primer sequences are given in Appendix 106 S2). Primers to amplify anonymous single-copy nuclear DNA loci were developed using the 107 ISSR-cloning method described in Beatty et al. (2010). For herbarium samples from which 108 the complete chloroplast product could not be amplified in a single PCR, the region was 109 amplified as two or three overlapping fragments using internal primers (Appendix S3). PCR 110 was carried out on a MWG Primus thermal cycler (Ebersberg, Germany) using the following 111 parameters: initial denaturation at 94 °C for 3 min followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C (52 °C for S. spathularis trnS-G intergenic spacer) for 30 s, 112 extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. PCR was carried out in 113 114 a total volume of 20 µl containing 200 ng genomic DNA, 10 pmol of each primer, 1x PCR 115 reaction buffer, 200 µM each dNTP, 2.5 mM MgCl₂ and 0.5 U GoTaq Flexi DNA 116 polymerase (Promega, Sunnyvale, CA). Five µl PCR product were resolved on 1.5% agarose 117 gels and visualised by ethidium bromide staining, and the remaining 15 µl were EXO-SAP 118 purified and sequenced in both directions using the BigDye sequencing kit (V3.1; Applied 119 Biosystems) and run on an AB 3730XL DNA analyser (Life Technologies; Carlsbad, 120 California, USA).

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Page | 7

122 **Phylogeographic analysis**

123 DNA sequences were aligned in BIOEDIT (V7.0.9.0; Hall, 1999). For the single-copy 124 nuclear loci, haplotypes were resolved for individuals exhibiting two or more heterozygous 125 positions using the PHASE program (V2.1; Stephens & Donnelly 2003) implemented in the 126 DnaSP software package (V5.10; Librado & Rozas 2009). Pinguicula grandiflora is 127 tetraploid (Heslop-Harrison 2004), but to avoid difficulties with estimation of allele dosage, 128 all heterozygotes were scored as 50:50 i.e. similar to treatment of diploid loci. Given the 129 extremely low levels of heterozygosity observed in Irish populations relative to continental 130 European populations, this should not unduly affect our conclusions regarding the glacial 131 history of the species. Potential recombination was assessed using the Hudson & Kaplan 132 (1985) test in DnaSP. Median-joining networks for all loci were constructed using the 133 NETWORK software package (V4.5.1.6; www.fluxus-engineering.com). Any reticulations in 134 the networks were broken following the rules described in Pfenninger & Posada (2002). 135 Levels of haplotype diversity (H) and nucleotide diversity (π) were calculated using DnaSP. 136 To account for differences in sample sizes, particularly in the case of S. spathularis, levels of 137 haplotype richness (R_h) were calculated using HAPLOTYPE ANALYSIS (V1.05; Eliades & 138 Eliades 2009).

139 To assess potential geographical structuring of genetic variation in continental European 140 populations associated with persistence in multiple glacial refugia, we performed a spatial 141 analysis of molecular variance (SAMOVA) using the SAMOVA software package (V1.0; 142 Doupanloup et al. 2002) for each of the eight data sets. The program uses a simulated annealing approach based on genetic and geographical data to identify groups of related 143 144 populations. The program was run for 10,000 iterations for K = 2 to 10 groups from 200 145 initial conditions, and the most likely structure was identified using the maximum value of $\Phi_{\rm CT}$ that did not include any groups of a single population. 146

147 **RESULTS**

148

149 Palaeodistribution modelling

150 For all models, AUC values were high (*P. grandiflora* mean AUC = 0.944 SE = 0.008; *S. spathularis* mean AUC = 0.973 SE = 0.005). Palaeodistribution modelling of species 151 152 distributions suggested far more restricted ranges during the LIG compared to current 153 distributions, particularly for S. spathularis (Figures 1c and 1d). The models suggested that 154 both species had larger potential distribution ranges during the LGM. For *P. grandiflora*, 155 extensive regions of suitable habitat coincided largely with the species' current distribution in 156 continental Europe, with additional areas in southern France where the species is currently 157 absent (Figure 1e). Two main areas of suitable habitat during the LGM were identified for S. 158 spathularis, one in Northern Spain which corresponds to the eastern part of the species' 159 present-day distribution in Iberia, as well another on the continental shelf off the west coast 160 of Brittany, south of the limit of the British-Irish ice sheet (Figure 1f).

161

162 **Phylogeographic analysis**

163 Between three (trnL-F) and twelve (Pg-C01 and Pg-F10) haplotypes were found in the four 164 loci analysed in *P. grandiflora* (Table 1 and Figure 2). Populations from continental Europe 165 harboured far higher levels of diversity than those from Ireland, with total of 37 haplotypes 166 across the four loci, including 31 private haplotypes, compared to six haplotypes (one 167 private) in Irish populations. The frequency of private alleles ranged from 0.09 (Pg-C02) to 168 0.46 (trnL-F) in continental Europe, whilst the sole private allele in Irish populations, at locus 169 Pg-F10, was only found at a frequency of 0.01. Levels of haplotype richness (R_h) , haplotype 170 diversity (H), and nucleotide diversity (π) ranged from 2.000 (trnL-F) to 9.087 (pg-F10), from 0.179 (Pg-C01) to 0.606 (trnL-F), and from 0.0007 (Pg-F10) to 0.0030 (Pg-F02) 171

172 respectively in continental European populations, and from zero (trnL-F and Pg-C02) to

173 0.480 (Pg-F02), from zero (*trn*L-F and Pg-C01) to 1.000 (Pg-F02 and Pg-F10), and from zero

174 (*trn*L-F and Pg-C01) to 0.0036 (Pg-F02) respectively in Irish populations.

175 Evidence for recombination was detected at all three nuclear loci analysed in *S*.

- 176 spathularis. Consequently, subsequent analyses were carried out using only the largest non-
- 177 recombining portion of each locus (see Appendix S3 for details). The chloroplast *trn*S-G
- 178 intergenic spacer region exhibited eleven haplotypes in Spain ($R_h = 10.000, H = 0.872, \pi =$

179 0.0020), but only a single haplotype in Ireland (Table 1 and Figure 3). For three nuclear loci,

180 values for R_h , H and π ranged from 3.000 (Ss-G04) to 5.000 (Ss-C02), from 0.238 (Ss-G04)

181 to 0.557 (Ss-G07), and from 0.0026 (Ss-G04) to 0.0076 (Ss-C02) respectively in Spain, and

- 182 from 1.000 (Ss-G04) to 4.491 (Ss-C02), from 0.162 (Ss-G04) to 0.581 (Ss-C02), and 0.0017
- 183 (Ss-G04) to 0.0084 (Ss-C02) respectively in Ireland. All four loci exhibited private
- 184 haplotypes in Spain, whilst two of the four (Ss-C02 and Ss-G07) had private haplotypes in
- 185 three of the Irish populations studied.
- 186 For both species, none of the SAMOVA analyses (four loci for each species) indicated
- 187 any obvious geographical structuring of genetic variation (data not shown).

188 **DISCUSSION**

189

190 Phylogeographic studies are increasingly being used in conjunction with palaeodistribution 191 modeling to provide insights into the response of species to the glacial periods of the late 192 Pleistocene (for reviews see Chan et al. 2011; Alvaredo-Serrano & Knowles 2014). It is 193 important, however, to appreciate the potential pitfalls and problems of such modeling 194 approaches, particularly for species with restricted and/or limited distributions, as is the case 195 for the Lusitanian flora. The main drawback with ecological niche models based on 196 correlative approaches is that these approaches assume species / environment equilibrium, a 197 condition which is frequently violated when examining range-shifts such as those associated 198 with the glaciations (Menke et al. 2009; Elith et al. 2010). The incorporation of multivariate 199 environmental similarity surface (MESS) methods (Elith et al. 2010) into the most recent 200 versions of the MAXENT modeling software package (V3.3.2 onwards) allows identification 201 of areas in the model where extrapolation is greatest, and consequently where prediction may 202 be less reliable e.g. areas that lie under the ice sheets at the LGM (Figure S3, Additional 203 Supporting Material). When applied to the LIG and LGM models for both of our study 204 species, the only modeled area that was associated with strongly negative (i.e. less than -10) 205 MESS values, and consequently may reflect unreliable prediction, was the northeastern part 206 of the modeled LGM range for P. grandiflora in eastern Aquitaine and the Midi-Pyrénées 207 regions of France. The apparently larger distributions for both species at the LGM relative to 208 the LIG may reflect the fact that they are cold-tolerant to some degree (Webb 1983), and 209 many cold-tolerant species have been suggested to have larger distributions during glacial 210 periods (reviewed in Bennett & Provan 2008; Stewart et al. 2010). Nevertheless, although 211 the observed patterns of genetic variation in *P. grandiflora* are consistent with the occurrence 212 of a single large refugium (see below), caution should be exercised when trying to make

213 inferences on past ranges for species with restricted distributions (Elith *et al.* 2010).

214 Although all models had high AUC values, which generally indicates good predictive power,

these values tend to be inflated for species which occupy a limited part of the area analyzed,

- as typified by species that exhibit disjunct distributions where large areas are unoccupied.
- 217

218 Pinguicula grandiflora – the classic southern refugium paradigm

219 Both the palaeodistribution modelling and the phylogeographic evidence indicate that P. 220 grandiflora persisted throughout the LGM in a southern refugium and recolonized Ireland 221 following the retreat of the ice sheets. Unlike *Daboecia cantabrica*, the only other plant with 222 a Lusitanian distribution on which phylogeographic analysis has been carried out (Beatty & 223 Provan, 2013), and which survived the LGM in two southern refugia, the lack of 224 geographical structuring of genetic variation in continental European populations of P. 225 grandiflora suggests the occurrence of a single refugium. This is consistent with the large, 226 mostly continuous area of suitable habitat at the LGM indicated by the palaeodistribution 227 modelling. The extremely low levels of genetic diversity observed in Irish populations, 228 coupled with the occurrence of only a single, low frequency private haplotype, are indicative 229 of the founder effects associated with postglacial recolonization (Hewitt 1999; Provan & 230 Bennett 2008). This is in contrast to Webb (1983), who could conceive no mechanism 231 whereby P. grandiflora could have reached Ireland from the Pyrenees, and concluded that the 232 species must have persisted *in situ* since the interglacial. Such persistence seems unlikely, 233 since although hibernacula can withstand low temperatures, rosettes are extremely 234 susceptible to frost (Heslop-Harrison 1962; Grace 1987). Seeds of P. grandiflora are minute 235 and dust-like (as are those of *D. cantabrica*), a feature which could facilitate long-distance 236 dispersal following the retreat of the ice after the LGM.

237

238 Saxifraga spathularis – evidence for persistence in a northern refugium?

239 Whilst P. grandiflora exhibits the classic "southern richness vs. northern purity" pattern of 240 genetic diversity consistent with survival in southern refugia (Hewitt 1999), the distribution 241 of genetic diversity in S. spathularis initially appears incompatible with such a scenario. 242 Levels of haplotype diversity in Irish populations are comparable to those found in Spain for 243 the three nuclear loci studied, and private haplotypes are present in Ireland at two of these 244 loci, although after rarefaction to account for differences in sample size, levels are lower for 245 all three loci in Ireland. This is in contrast, however, to the data from the chloroplast *trn*S-G 246 intergenic spacer, which suggest an extreme bottleneck in Irish populations, effectively ruling 247 out the possibility that Iberian populations might have originated in a northern refugium. The 248 discrepancy between the chloroplast and nuclear markers is most likely due to the smaller 249 effective population size of the haploid, uniparentally inherited chloroplast genome, which 250 will be more susceptible to effects of genetic drift during the population fluctuations 251 associated with climatic changes during the Pleistocene.

252 Despite the comparable levels of nuclear diversity and occurrence of private haplotypes, it 253 seems unlikely that S. spathularis persisted through the LGM in separate northern and 254 southern refugia, as suggested by the palaeodistribution model, since this would lead to 255 phylogenetic structuring which would be reflected geographically. The opposite is apparent 256 for locus Ss-G07, where the two private haplotypes found in Ireland, depicted in vellow and 257 white, are two of the most phylogenetically divergent haplotypes, being separated by four 258 mutations. It is possible that these represent localized recent mutations, since they are present 259 at very low frequencies. Alternatively, it might be that the lower number of plants analyzed 260 from Spain means that these haplotypes are present there, but were simply not sampled. 261 The patterns of genetic variation observed in S. spathularis are similar to those reported for *Meconopsis cambrica*, an herbaceous species with a disjunct distribution similar to that 262

263 exhibited by the Lusitanian flora, being found in Northern Spain and the Massif Central, as 264 well as in north Devon, Somerset, Wales and Ireland (Valtueña et al. 2012). Populations of 265 *M. cambrica* from Wales shared a single chloroplast haplotype with samples from 266 northeastern Spain, but also displayed comparable levels of nuclear diversity to some 267 continental European populations, including private AFLP alleles. It was suggested that 268 these populations might have originated from a separate northern refugium, but not as far 269 north as mainland Britain. Unlike in *M. cambrica*, however, *S. spathularis* populations are 270 not separated into "northern" and "southern" groups, and Irish populations most likely shared 271 a refugial area with present-day Spanish populations. The lack of correspondence with the 272 palaeodistribution model, which did suggest the potential existence of separate northern and 273 southern refugial areas, is probably a consequence of the aforementioned problems associated 274 with extrapolating ranges of species with restricted distributions, as is the case with S. 275 spathularis (Elith et al. 2010). This could also explain the extremely low levels of predicted 276 suitable habitat during the LIG.

277 An alternative explanation for the occurrence of private haplotypes in Ireland could be introgression from the congeneric S. hirsuta. Both species occur sympatrically in Cos. Cork 278 279 and Kerry, where they hybridize to form S. x polita (Webb, 1951). Although S. hirsuta is 280 much less common than S. spathularis, introgression of species-specific SNP alleles from the 281 former into the latter has been observed, even in populations of *S. spathularis* from Co. 282 Galway where S. hirsuta is now absent (G.E. Beatty & J. Provan, unpublished results). Such 283 introgression is consistent with the high levels of recombination detected at all three nuclear 284 loci, but would not affect the chloroplast genome. Unfortunately, though, it was not possible 285 to amplify the two orthologous loci of Ss-C02 and Ss-G07 in S. hirsuta to determine whether 286 the alleles had been introgressed into S. spathularis from its congener.

287 CONCLUSIONS

288

- 289 The findings of the present study, together with the only previously published
- 290 phylogeographic study on a member of the Lusitanian flora, Daboecia cantabrica (Beatty &
- 291 Provan, 2013), suggest that the original theory of persistence in separate northern and
- southern refugia cannot explain these species' puzzling distribution. Both *Pinguicula*
- 293 grandiflora and Saxifraga spathularis, like D. cantabrica, are likely to have persisted through
- the LGM in southern refugia and colonized Ireland after the retreat of the ice sheets.
- 295 Nevertheless, the differing refugial histories of the three species suggest that no one scenario
- 296 can account for the present-day distribution of the Lusitanian flora, and that several southern
- 297 refugia facilitated these species' survival during the LGM.

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Page | 17

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391 BIOSKETCHES

392

393 Gemma Beatty is a Postdoctoral Research Fellow at Queen's University Belfast. Her PhD 394 research compared how postglacial recolonization and range-edge effects have shaped the 395 genetic diversity of several northern hemisphere Monotropoideae species. She is interested 396 in using genetic approaches to study the effects of past and present climate change on the 397 distribution ranges of natural populations, and the various factors that determine these ranges. 398 Jim Provan is a Reader in Evolutionary Genetics at Queen's University Belfast. His 399 research interests focus on how genetic variation is distributed across species ranges, and on 400 the effects of past, present and future climate change on levels and patterns of intraspecific 401 diversity. 402 403 Author contributions: JP conceived the study; both authors collected and analysed the data, 404 and wrote the manuscript. 405

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Species	Locus	Europe			Ireland					GenBank				
species	Locus	N	h	Freq _p	R_h	Н	π	N	h	Freq _p	R_h	Н	π	Accessions
P. grandiflora	<i>trn</i> L-F	92	3 (2)	0.46	2.000	0.606	0.0010	68	1 (0)	-	-	-	-	
	Pg-C01	180	12 (11)	0.09	8.951	0.179	0.0011	140	1 (0)	-	-	-	-	
	Pg-F02	180	11 (9)	0.14	8.042	0.455	0.0030	118	2 (0)	-	1.000	0.480	0.0036	
	Pg-F10	166	11 (9)	0.10	9.087	0.190	0.0007	136	2 (1)	0.01	1.000	0.015	0.0001	
S. spathularis	trnS-G	39	11 (10)	0.85	10.000	0.872	0.0020	134	1 (0)	-	-	-	-	
	Ss-C02	78	6 (2)	0.21	5.000	0.532	0.0076	244	8 (4)	0.02	4.491	0.581	0.0084	
	Ss-G04	76	4 (2)	0.03	3.000	0.238	0.0026	274	2 (0)	-	1.000	0.162	0.0017	
	Ss-G07	80	5 (3)	0.06	4.000	0.557	0.0051	272	4 (2)	0.02	2.257	0.500	0.0048	

Table 1 Diversity statistics by region. N – number of individuals; h – number of haplotypes observed (number of private haplotypes in parentheses); Freq_p – frequency of private haplotypes; R_h – Haplotype richness; H – haplotype diversity; π - nucleotide diversity.

Figure Legends

Figure 1 Present-day distributions of (a) *Pinguicula grandiflora*, and (b) *Saxifraga spathularis* (shaded; based on Webb 1982 and the Global Biodiversity Information Facility [data.gbif.org]) in Europe. Palaeodistribution models for (c,e) *P. grandiflora*, and (d,f) *S. spathularis* during the Last Interglacial (*ca.* 120 ka) and the Last Glacial Maximum (*ca.* 21 ka) repectively. Darker areas in (c-f) show most suitable modelled habitat. The limits of the British-Irish ice sheet (after Sejrup *et al.* 2005) and Alpine and Pyrenean glaciers (after Ehlers & Gibbard 2004) at the LGM are also indicated.

Figure 2 Haplotype distributions for (a) chloroplast *trn*L-F intergenic spacer, (b) nuclear Pg-C01 region, (c) nuclear Pg-F02 region, and (d) nuclear Pg-F10 region for *Pinguicula grandiflora*. Pie chart sizes are approximately proportional to sample size, with the smallest circles representing N = 1 (chloroplast locus) or N = 2 (nuclear loci) and the largest representing N = 8 (chloroplast locus) or N = 16 (nuclear loci). In the haplotype networks, black circles represent unique haplotypes found in a single individual, and open diamonds indicate missing (unsampled) haplotypes.

Figure 3 Haplotype distributions for (a) chloroplast *trn*S-G intergenic spacer, (b) nuclear Ss-C02 region, (c) nuclear Ss-G04 region, and (d) nuclear Ss-G07 region for *Saxifraga spathularis*. Pie chart sizes are approximately proportional to sample size, with the smallest circles representing N = 1 (chloroplast locus) or N = 2 (nuclear loci) and the largest representing N = 8 (chloroplast locus) or N = 16 (nuclear loci). In the haplotype networks, black circles represent unique haplotypes found in a single individual, and open diamonds indicate missing (unsampled) haplotypes.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1a Samples of *Pinguicula grandiflora* analysed

Appendix S1b Samples of Saxifraga spathularis analysed

Appendix S2 PCR / sequencing primers used in this study.

Figure S3 Results of the multivariate environmental similarity surfaces (MESS) analysis for (a) LIG, and (b) LGM models. Darker blue areas highlight regions with more highly negative MESS values, indicating areas where the model has extrapolated beyond current climatic conditions, which could lead to unreliable predictions.











Country	Location	Code*	Lat (N)	Long (W)	п			
					trnL-F	Pg-C01	Pg-F02	Pg-F10
France	Pyrénées-Atlantiques	G-00308102	43.2503	0.8794	1	1	1	1
	Bagnère-de-Luchon	G-00308101	43.1183	-1.2436	1	1	1	1
	Près Lajoux	G.00308100	46.7392	-5.7289	1	1	1	1
Ireland	Lough Cloon 1		51.9573	9.8530	6	6	4	5
	Lough Cloon 2		51.9547	9.8525	8	8	8	8
	Gap of Dunloe		51.9892	9.6562	8	8	2	8
	Galway Bridge		51.9628	9.5793	7	8	8	8
	Gougane Barra		51.8333	9.3450	7	8	7	8
	Killarney National Park		52.0155	9.2461	8	8	6	7
	Priest's Leap		51.7781	9.4564	6	6	6	6
	Healy Pass		51.7152	9.7270	6	6	6	6
	Slieve Mish		52.1881	9.8773	6	6	6	6
	Burren		53.1258	9.2699	6	6	6	6
Spain	Valdés	FCO-15016	43.46	6.56	6	6	6	6
	Villariño del Sil	LEB-15742	42.8737	6.4289	4	4	4	4
	Puerto de Leitariegos	LEB-13532	42.9637	6.4252	4	4	4	4
	Yernes y Tameza	FCO-23468	43.24	6.12	3	2	1	2
	Puerto de la Ventana	SEV-68044	43.0568	6.0106	-	2	2	2
	Mirantes de Luna	LEB-3760	42.9099	5.88	5	5	5	4
	Branillín	LEB-63747	43.0044	5.7606	6	6	6	6
	Puerto de San Isidro	LEB-62112	43.0541	5.3502	5	5	5	5
	Cangas de Onís	FCO-26683	43.24	5.34	2	2	2	2
	Covadonga	SEV-25704	43.3088	5.0544	4	4	4	4
	Cabrales	FCO-13569	43.19	4.80	8	7	8	6
	Valdelateja	SEV-18909	42.7743	3.7687	7	6	8	7
	Merindad de Montija	SALA-103945/104063	43.13	3.43	3	3	3	2

Appendix S1a Samples of *Pinguicula grandiflora* analysed

Appendix S1a (continued)

Country	Location	Code*	Lat (N)	Long (W)	n			
					trnL-F	Pg-C01	Pg-F02	Pg-F10
Spain	Valgañón	SALA-100469	42.32	3.10	3	3	3	2
	Ezcaray	SALA-100470	42.25	2.98	2	2	2	2
	Riopar	SEV-53522	38.499	2.417	2	1	-	1
	Aia	SALA-88654	43.23	2.17	2	2	2	2
	Valle del Roncal	SEV-91312	42.9265	1.6515	4	4	3	3
	Roncesvalles	SEV-224332	43.0135	1.3157	2	2	2	-
	Sallent de Gállego	FCO-7678/SEV-69895	42.79	0.33	7	6	4	5
	Beceite	BC-905331	40.8311	-0.1796	2	1	2	2
	Roquetes	BC-905330	40.8207	-0.5018	2	1	2	1
	Valle de Arán	SALA-10903	42.61	-0.68	3	3	3	2
	Arcalis	BC-912895	42.3542	-1.0838	3	2	3	3
	Queralbs	SEV-92951	42.3505	-2.1710	-	4	3	3
					160	160	149	151

* Herbarium codes: G - Université de Genève; FCO - Universidad de Oviedo Herbario; LEB - Universidad de León Herbario; SEV - Universidad de Sevilla Herbario; SALA - Universidad de Salamanca Herbario; BC - Herbari BC, Institut Botànic de Barcelona..

Country	Location	Code*	Lat (N)	Long (W)	n			
-				-	trnS-G	Ss-C02	Ss-G04	Ss-G07
Ireland	Croaghaun Mountain	DBN-6031	53.9789	10.2047	1	1	1	1
	Lough Bunafreva	DBN-6033	54.0000	10.1803	2	1	2	2
	Streamstown	DBN-6010/6017/6018/6021	53.5267	10.0438	8	7	8	8
	Connemara National Park		53.5509	9.9454	4	4	4	4
	Dingle Peninsula		52.2168	9.9000	3	3	3	3
	Lough Cloon		51.9547	9.8653	8	6	8	8
	Caherconree	DBN-6020	52.2030	9.8537	1	-	1	1
	Ballaghbeama Gap		51.9432	9.8128	7	7	7	7
	Glancuttaun		52.045	9.775	8	2	8	8
	Leenaun		53.5972	9.7134	8	8	8	8
	Mamean		53.505	9.679	3	6	6	6
	Gap of Dunloe		52.0466	9.6500	8	7	8	8
	Galway Bridge		51.9665	9.5794	8	8	8	8
	Glengariff		51.7500	9.5667	8	6	8	8
	Lady Bantries Lookout		51.7415	9.5565	3	3	3	2
	Druidsview		51.80	9.55	8	8	8	8
	Nephin Mountain	DBN-6026	54.0138	9.3684	2	2	2	2
	Gougane Barra 1		51.8333	9.3450	8	8	8	8
	Gougane Barra 2		51.8340	9.2954	8	7	8	8
	Coomdorragh		51.8691	9.2658	8	8	8	8
	Burren		53.0352	9.0769	4	4	4	4
	Curragraig		52.1049	7.8657	8	8	8	8
	Mahon Falls		52.2324	7.5470	8	8	8	8

Appendix S1b Samples of Saxifraga spathularis analysed

Appendix S1b (continued)

Country	Location	Code*	Lat (N)	Long (W)	п			
					trnS-G	Ss-C02	Ss-G04	Ss-G07
Portugal	Sierra de la Estrella	MGC-13014	40.3281	7.6327	1	1	-	1
Spain	Mazaricos	SANT-110427	42.9389	8.9922	4	4	4	4
	Los Peares	SALA-99560	42.36	7.79	1	1	1	1
	Cebreiro	SALA-115510	42.70	7.08	2	2	3	2
	La Coba	SALA-59413	43.21	6.97	3	3	3	3
	Peña Trevinca	FCO-15020	42,16	6.82	2	2	2	2
	Villar de Acero	LEB-48167	42.7286	6.8135	1	1	1	1
	Toreno	LEB-78833	42.7322	6.5044	1	1	-	1
	Cueto de Arbás	LEB-92470	42.9923	6.4299	4	4	4	4
	Villariño del Sil	LEB-17789	42.8737	6.4289	2	2	2	2
	Salas	FCO-29610	43.40	6.32	2	2	2	2
	Murias de Paredes	LEB-44527	42.7810	6.3105	1	1	2	3
	Pico la Berza	FCO-24445	43.18	6.21	2	2	1	2
	Caso	FCO-22519	43.10	5.33	3	3	3	3
	Pinar de Lillo	MGC-12919	43.0594	5.2832	3	3	3	3
	Puerto del Pontón	LEB-86557	43.0637	5.0878	2	2	2	2
	Portilla de la Reina	SALA-114146	43.04	4.85	4	4	4	3
	Polaciones	MGC-61618	43.04	4.29	1	1	1	1
					173	161	175	176

* Herbarium codes: DBN - National Botanic Gardens of Ireland, Glasnevin; MGC - Universidad de Málaga Herbario, SANT - Universidad de Santiago de Compostela Herbario; SALA - Universidad de Salamanca Herbario; FCO - Universidad de Oviedo Herbario; LEB - Universidad de León Herbario

Species	Primer	Sequence (5' – 3')	Size (bp) ^b	Source
P. grandiflora	trnLF-F	GCTGTTCTAACAAATGGGGTTG	712	GenBank AF482623
	trnLF-R	CTGAGCTATCCCGACCATTC		
	trnLF-IN-R ^a	GAGAAACATTTTGGGAGTCAAATAG		
	trnLF-IN-F ^a	CTATTTGACTCCCAAAATGTTTCTC		
	Pg-C01-F	AGCAAGAGAAGGAAAATAAGAGTTT	206	This study
	Pg-C01-R	GCTCATAGACTACTGATACTTGAGCA		
	Pg-F02-F	TCTTGCATGGGTAGTTGGTG	400	This study
	Pg-F02-R	GTTGGCGTATGAAATTGTTGC		
	Pg-F10-F	ATAGGCCCGTGGCTGAAGT	292	This study
	Pg-F10-R	ACAATGGAATCCCCGACAG		
S. spathularis	trnSG-F	GCCGCTTTAGTCCACTCAGC	882-914	Hamilton (1999)
	trnSG-R	GAACGAATCACACTTTTACCAC		<i>Mol. Ecol.</i> 8 , 521-523
	trnSG-IN-R1 ^a	GATAAACGTGATATATTTGTATC		
	trnSG-IN-F1 ^a	GATACAAATATATCACGTTTATC		
	trnSG-IN-R2 ^a	ATACTTGAATTCCCTATCATAG		
	trnSG-IN-F2 ^a	CTATGATAGGGAATTCAAGTAT		
	Ss-C02-F	CACCCCATTACCTCATTCTTAGG	400 (84)	This study
	Ss-C02-R	TCGGCCACTATAAAGTTTTTCC		
	Ss-G04-F	TCCCTCTCTGAATAACACACGA	372 (95)	This study
	Ss-G04-R	TGGGAACGTAACCACAAACA		
	Ss-G07-F	CACGCCCCTAAAATAGAAGAAA	400 (211)	This study
	Ss-G07-R	ACGACTAAATCAACAATGGAGTC		

Appendix S2 PCR / sequencing primers used in this study.

^a Internal primer ^b Figure in parenthesis indicates largest non-recombining portion used for analysis

