1 **Little hope for the polyploid endemic Pyrenean Larkspur (*Delphinium***

2 ***montanum*): evidences from population genomics and Ecological Niche**

3 **Modelling**

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19

20 **Abstract**

21 Species endemic to restricted geographical ranges represent a particular conservation issue,

22 be it for their heritage interest. In a context of global change, this is particularly the case for

23 plants which belong to high-mountain ecosystems and, because of their ecological

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24   requirements, are doomed to survive or disappear on their 'sky islands'. The Pyrenean

25   Larkspur (*Delphinium montanum*, Ranunculaceae) is endemic to the Eastern part of the

26   Pyrenees (France and Spain). It is now only observable at a dozen of localities and some

27   populations show signs of decline, such as a recurrent lack of flowering. Implementing

28   population genomic approach (e.g. RAD-seq like) is particularly useful to understand

29   genomic patterns of diversity and differentiation in order to provide recommendations in

30   term of conservation. However, it remains challenging for species such as *D. montanum* that

31   are autotetraploid with a large genome size (1C-value > 10 pg) as most methods currently

32   available were developed for diploid species. A Bayesian framework able to call genotypes

33   with uncertainty allowed us to assess genetic diversity and population structure in this

34   system. Our results show evidence for inbreeding (mean G_{IS} = 0.361) within all the

35   populations and substantial population structure (mean G_{ST} = 0.403) at the metapopulation

36   level. In addition to a lack of connectivity between populations, spatial projections of

37   Ecological Niche Modelling analyses under different climatic scenarios predict a dramatic

38   decrease of suitable habitat for *D. montanum* in the future. Based on these results, we

39   discuss the relevance and feasibility of different conservation measures.

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41   **Keywords** Sky islands, Inbreeding, Tetraploid, Restriction Site Associated DNA Sequencing

42   (RAD-seq),   Species   Distribution   Modelling,   Genotyping   By   Sequencing   (GBS)

43   **Introduction**

44   Extinction risk associated to climate change is expected not only to increase but to

45   accelerate for every degree rise in global temperatures (Urban, 2015). In this context, the

46   number of threatened species by reduced population sizes, habitat degradation and habitat

47   fragmentation is increasing. Small and fragmented populations are particularly prone to be

48   affected by drift and inbreeding depression and generally display decreased levels of genetic

49   diversity (e.g. Bergl et al. 2008; Dixo et al. 2009). The increase of anthropogenic and

50   environmental pressures, both being intimately bound, represents an unprecedented threat

51   for vulnerable species. In particular, populations from species of high-mountain ecosystems

52   might not be able to move upward rapidly enough to keep within their thermal tolerances

53   and might be doomed on their 'sky islands' (see McCormack et al. 2009, e.g. Kidane et al.

54   2019, De Gabriel Hernando et al. 2021). Thus, species endemic to specific mountain ranges

55   represent a major conservation issue, if only for their heritage interest. It is critical to

56   characterize the distribution of genetic diversity within- and between- their populations to

57   evaluate the inbreeding risk and study population structure to better understand how

58   isolated they really are, in order to undertake effective conservation actions (Calevo et al.

59   2021).

60   The threatened Pyrenean larkspur *Delphinium montanum* DC., 1815 perfectly

61   illustrates this situation. This plant (Family Ranunculaceae), endemic to the Eastern part of

62   the Pyrenees (France and Spain), is now only observable at a dozen of localities (two already

63   got extinct over the 20th century) and its global population size was estimated to range

64   between 7 800 to 10 300 individuals in total (Aymerich et al. 2020). It occurs between alpine

65   and subalpine levels, between 1 600 and 2 400 m (Lopéz-Pujol et al. 2007).*D. montanum* is

66   vulnerable as a result of increasing levels of multiple threats. Some of its populations are

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67   located nearby touristic sites (*i.e.* mountain resorts or hiking trails) and are negatively

68   impacted by trampling (Simon et al., 2001). Flowers and fruits predation by herbivores,

69   particularly Pyrenean chamois/isards (*Rupicapra pyrenaica pyrenaica*) was also shown to be

70   responsible for the seed bank's decrease in some populations (Simon et al. 2001, Aymerich

71   2003). Importantly, differences in population dynamics are observable from one population

72   to another. Some populations are stable, whereas others show decrease in population size

73   (4-Pedraforca) and/or absence of flowering individuals since 2011 (2-Nohèdes). Pioneering

74   work of Simon et al. (2001) and Lopéz-Pujol et al. (2007) also suggested relatively low

75   effective size as well as, evidence of inbreeding within-populations and significant

76   population structure. However, these two studies were based on a limited number of

77   genetic markers (*i.e.* 7 and 14 allozyme *loci*, respectively) and populations studied (*i.e.* 2 and

78   7, respectively).

79   Implementing a population genomic approach on *D. montanum* could be particularly

80   fruitful to inform conservation strategies by providing a substantial increase in the resolution

81   of the genetic marker dataset and genome complexity reduction protocols such as Reduction

82   site-associated DNA sequencing (or RAD-seq, Baird 2008) allow to get such information on

83   tens to hundreds of individuals. However, the features of the genome of the species make

84   *Delphinium montanum* particularly challenging from a technical and methodological point of

85   view. *D. montanum* is an autotetraploid species (Simon et al. 2001; Lopéz-Pujol et al. 2007).

86   Our recent flow cytometry estimates of 10.32 pg/1C for this species (*i.e.* about twice the 1C-

87   value reported for several other diploid *Delphinium* species) corroborates previous

88   knowledge and confirms the large genome size of *D. montanum* (Bertrand et al. unpublished

89   data). A large genome size can be considered has a limitation as itself, be it because in

90   requires higher sequencing efforts and costs to carry out conservation studies. The difficulty

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91   to infer genotype and allele frequencies on polyploid species with RAD-seq techniques as

92   well as the restricted set of methods available to analyze such data (see Meirmans and van

93   Tienderen 2013; Dufresne et al. 2014; Meirmans et al. 2018) is also likely to explain the

94   relatively low number of population genomics studies dealing with RAD-seq like techniques

95   on non-model polyploid organisms (Clevenger et al. 2015; van de Peer et al. 2017, Ahmad

96   2021).

97   Out the handful of studies that have addressed population genomic questions on

98   polyploid organisms without reference genome, some have considered the genotypes as

99   diploid-like data, an assumption that allows to use classical variant calling tools with default

100   settings (e.g. Brandrud et al. 2017, see also Brandrud et al. 2019; 2020 and Záveská et al.

101   2019). Consensus allelic information may also be coded in the form of ambiguous sites at

102   heterozygous positions (e.g. Wagner et al. 2020). At last, a couple of methods are able to

103   incorporate ploidy-aware calling algorithms and use genotype likelihoods to infer tetraploid

104   genotypes (e.g. Záveská et al. 2019; Brandrud et al. 2020; Karbstein et al. 2020; Ahmad et al.

105   2021). In studies for which both kind of methods were used, the latter one generally

106   outputted a higher number of variable sites but signals were found to be overall congruent

107   (Záveská et al. 2019; Brandrud et al. 2020).

108   The recent studies of Záveská et al. (2019), Brandrud et al. (2020) and Ahmad et al.

109   (2021) used the RAD-seq loci from the catalog generated in the popular pipeline Stacks

110   (Catchen et al. 2011; Catchen et al. 2013) to generate a synthetic reference onto which the

111   raw reads are mapped back before to infer tetraploid genotypes with the approach

112   implemented in EBG (Empirical Bayes Genotyping in Polyploids, Blischak et al. 2018, see also

113   Blischak et al. 2016). As a putative alternative, Clark et al. (2019) proposed a package called

114   PolyRAD to call genotypes with uncertainty from polyploid data in an R environment.

115   PolyRAD can deal with multiple levels of ploidy and is able to use for example population

116   structure, linkage disequilibrium and/or self-fertilization as priors to estimate genotype

117   probabilities. To our best knowledge, this promising and convenient method has been rarely

118   used to implement a population genomic approach on natural populations of polyploid non-

119   model organisms.

120   In this study, we first aimed at reevaluating the conservation status of *Delphinium*

121   *montanum* following such population genomics approach. We used a protocol called

122   normalized Genotyping By Sequencing (nGBS) that provided us with thousands of genetic

123   markers (SNPs) from which we inferred patterns of diversity and differentiation with an

124   unprecedented resolution in order to quantify inbreeding and level of connectivity between

125   populations. We then used an Ecological Niche Modelling (ENM) approach to better

126   understand the bioclimatic features that constraint the current geographic distribution of *D.*

127   *montanum*. Based on several Global Climate Models (CGM), we also predicted the putative

128   evolution of the geographic distribution of the species in the future, at different time periods

129   (2011-2040, 2041-2070 and 2071-2100) and under different, more or less pessimistic but still

130   realistic scenarios of evolution of global warming (or RCPs for Representative Concentration

131   Pathways).

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133   **Materials and Methods**

134   **Geographic distribution and population sampling**

135   The Pyrenean Larskpur (*Delphinium montanum*) geographic distribution ranges from Serra

136   del Cadí-Pedraforca (Fig. 1, localities 4, 5, 8, and 9) up to the Puigmal massif (localities 1, 3

137   and 6) via Tosa d'Alp massif (locality 7). There, the distance between the most distant sites is

138   about 60 km, and only one locality is located outside this area: Nohèdes (locality 2), in the

139   Madres massif. This latter locality is separated from the Puigmal massif populations by the

140   pit of Cerdagne-Conflent, which could have formed a historical barrier to gene flow. In terms

141   of population size, Cadí-Pedraforca is the most important with up to 70% of the global

142   population size (5 450-6 700 individuals). The sites of the Puigmal massif represent 25 to

143   30% of the global population (2 200-2 700 individuals). Localities of Madres massif (2) and

144   Tosa d'Alp (7) are numerically low (less than 300, from which none are actually flowering

145   and 35 individuals, respectively).

146   We sampled a total of 106 individuals from 9 localities representative of the

147   geographic distribution of *Delphinium montanum* (in the Eastern Pyrenees: 3 sites in France

148   and 6 in Spain) between July and October 2020 (Table 1, Fig. 1, see also Supplementary

149   Appendix S1). At each locality, we collected on individual distant from at least 1 meter from

150   each other, about 1-2 cm^2 of leaf tissues that were stored in 90% ethanol at 4°C until DNA

151   extraction. *Delphinium montanum* is not legally protected in France but is considered as

152   vulnerable (VU) on the Red List of threatened species of IUCN France (UICN France, FCBN,

153   AFB, MNHN, 2018). It is legally protected in Catalonia (Decret 172/2008. Catàleg de flora

154   amenaçada de Catalunya) and Spain (Orden AAA/1771/2015. Modificación del catálogo de

155   especies amenazadas). In France, for all sampling done in natural reserves, special

156   authorizations were granted by the 'Direction Départementale des Territoires et de la Mer

157   66' (DDTM 66). In Spain, we asked for and obtained special permits from the Servei de Fauna

158   i Flora de la Generalitat de Catalunya and Natural Parks (Cadí-Moixeró and Ter-Freser).

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160   **Molecular procedures**

161   Genomic DNA extraction and genotyping were subcontracted to LGC Genomics GmbH

162   (Berlin, Germany). Genotyping-By-Sequencing (GBS) was performed following a specific

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163　protocol called nGBS for 'normalised GBS'. This double digest Restriction site-Associated

164　DNA seq (or RAD-seq) like protocol relies on two restriction enzymes (PstI and ApeKI, in our

165　case) to reduce *D. montanum* genome complexity and includes a normalization step that

166　aims at avoiding repetitive regions. The resulting 106 individually barcoded libraries were

167　sequenced in paired-end mode (2 x 250 bp) on an Illumina NovaSeq 6000 with an

168　expectation of a minimum number of 1.5 million read pairs per sample.

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170　**Genomic data processing**

171　We used Stacks v.2.41 (Catchen et al. 2011; 2013) to build loci from Illumina reads, *de novo*

172　(*i.e.* without aligning reads to a reference genome). Stacks consists of a wrapper of several

173　scripts that are usually run sequentially including: *process_radtags* to demultiplex and clean

174　reads, *denovo_map.pl* to build loci within individuals, create a catalog and match all samples

175　against it and *populations* to further filter the SNPs obtained at the population level and

176　compute basic population genetic statistics. We first optimised several key parameters of

177　the pipeline: -*m* (the minimum number of identical raw reads required to form a putative

178　allele), -*M* (the number of mismatches allowed between alleles to form a locus) and -*n* (the

179　number of mismatches allowed between loci during construction of the catalog) on a subset

180　of 12 individuals representative of the whole data set (*i.e.* geographic origin and coverage).

181　As recommended by several authors, we varied *M* and *n* (fixing *M* = *n*) while keeping *m* = 3

182　(see Paris et al. 2017; Rochette & Catchen 2017). The combination -*m* 3, -*M* 3 and -*n* 3 was

183　found to be the most suitable to maximize the number of SNPs, assembled and polymorphic

184　loci in our case and was used to run Stacks on the whole data set (see Supplementary

185　Appendix S2). As *D. montanum* is a tetraploid species, we also specified the option '-*X*

186　*ustacks:--max_locus_stacks* 5' (in the *ustacks* script's) to allow the number of alleles per site

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187   to reach the number of 5 instead or 3 by default. However, as the *populations* script is

188   unable to deal with polyploid genotypes, we followed a different approach to call variants

189   and complete genomic data processing.

190   We then used polyRAD (Clark et al. 2019), a method implemented in an R-package to

191   call genotypes with uncertainty from sequencing data in polyploids. The Bayesian genotype

192   caller implemented in polyRAD is able to import read depth from the catalog and matches

193   files from Stacks through a specific function called polyRAD::read_stacks() to further

194   estimate the probability of each possible genotype for each individual and each locus, taking

195   into account features such as possible levels of ploidy and population structure as priors. In

196   order to get similar filtering parameters than with Stacks and following the polyRAD author's

197   guidelines, we used the following arguments *-min.ind.with.minor. alleles= 5, -*

198   *min.ind.with.reads= 84* and a ploidy of 4. We then plotted the distribution of the ratio of the

199   individual over expected heterozygosity H_{ind}/H_E. The peak of the distribution of H_{ind}/H_E by

200   locus (0.2) was considered to represent well-behaved markers from which inbreeding (F)

201   was estimated at 0.73. From this estimate of F, we then simulated the expected distribution

202   of H_{ind}/H_E, if all markers were behaving in a Mendelian fashion. Based on the distribution, we

203   defined minimum and maximum thresholds of 0 and 0.406 comprising 95% of the

204   observations and filtered out markers for which H_{ind}/H_E were outside this interval. We then

205   tested overdispersion (*i.e.* in our case, how much does read depth distribution deviate from

206   what would be expected under binomial distribution) and adjusted its parameter to 6 (from

207   a range of 2 to 14). This information was used to call genotypes while taking into account

208   population genetic structure. The most likely genotypes of the remaining 5 095 loci were

209   then exported in several formats such as genind objects or Structure (.str).

210

211   **Population genomic analyses**

212   To get an overview of the overall genetic diversity and differentiation among individuals and

213   populations, we first performed a Principal Component Analysis (PCA) based on a matrix of

214   106 individuals (as rows) and 5 095 SNPs (as columns) coded as a genind object with the R-

215   package 'adegenet' (Jombart 2008). We then used Genodive v.3.04 (Meirmans 2020) to

216   compute expected and observed heterozygosity (H_E and H_O, respectively) as well as the

217   number of alleles (A) and the number of private alleles was computed with the R-package

218   'poppr' (Kamvar et al. 2014). The deviation from panmixia was evaluated by computing G_{IS}

219   and the statistical significance of the obtained values was estimated based on 10 000

220   permutations, also in Genodive. Overall and pairwise genetic differentiation was also

221   assessed based on G-statistics (G_{ST}, Nei 1987) as implemented in Genodive in a similar

222   manner. Pattern of Isolation By Distance (IBD) was assessed by examining the relationship

223   between linearized G_{ST}s values (*i.e.* ln(G_{ST}/(1- G_{ST})) and ln-geographical distances (after

224   Rousset 1997) and its statistical significance was tested with a Mantel test between matrices

225   of pairwise G_{ST} and geographical distance.

226   To further investigate population structure and characterize putative

227   migration/admixture event, we used sNMF (Frichot et al. 2014) as implemented in the R-

228   package LEA (Frichot & François 2015) to estimate individual ancestry coefficients based on

229   sparse non-negative matrix factorization algorithms. sNMF is particularly suitable for our

230   genotype dataset as it can deal with polyploid data and is fast enough to be applied on

231   hundreds of individuals and thousands of markers with reasonable computation time. The

232   number of genetic clusters was varied from K = 1 to 10, and analyses were run with 10

233   replicates at each value of K.

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235   **Ecological Niche Modelling**

236   In order to assess the future trends of spatial dynamics of *D. montanum* distribution in a

237   context of global change, we conducted Ecological Niche Modelling (ENM) analyses based on

238   known occurrences of the species. Based on 19 bioclimatic layers we downloaded from

239   CHELSA v.2.1 (Karger et al. 2017), we aimed to i) model current environmental niche and ii)

240   predict the spatial evolution of the niche in the future. The bioclimatic layers available in

241   CHELSA are similar to those available in WORLDCLIM (Fick et al. 2017) in a way that they

242   consist of downscaled Global Climate Models (GCM) output temperature and precipitation

243   estimates at a maximal resolution of 30 arc seconds (approximately 1 km). In the current

244   study, we used the most recent version of CHELSA as the methodology and bias correction it

245   implements have been shown to outperforms WORLDCLIM in mountainous environments

246   (Karger et al. 2017; Bobrowski et al. 2021). Current climate data correspond to time

247   averaged variables over the period 1981-2010.

248   Future climates correspond to estimates from phase 3b of the Inter-Sectoral Impact

249   Model Intercomparison Project (ISIMIP3b) based on output of phase 6 of the Coupled Model

250   Intercomparison Project (CMIP6). They consist of the output of five GCMs (GFDL-ESM4,

251   UKESM1-0-LL, MPI-ESM1-2-HR, IPSL-CM6A-LR and MRI-ESM2-0) and three climate scenario

252   specifiers (ssp126, ssp370, ssp585). Climate scenarios specifiers reflect different

253   Representative Concentration Pathways (RCPs) associated with different radiative forcing

254   values: 2.6, 7.0 and 8.5 W/m^2 that can be considered as a plausible range of greenhouse gas

255   concentrations that appear to be optimistic, very likely and pessimistic in term of climate

256   change, respectively. Annually averaged data were downloaded for three future time

257   periods: 2011-2040 ("2035"), 2041-2070 ("2055") and 2071-2100 ("2085").

258 The ENM analyses were conducted thanks to the R-package ENMwizard v.0.3.7

259 (Heming et al. 2018, see also Gutiérrez et al. 2019; Bagley et al. 2020). We first spatially

260 filtered occurrences to keep only those that were at least 1 km away from each other using

261 the R-package 'spThin' (Aiello-Lammens et al. 2015). Due to the relatively small spatial scale

262 of the area under study, this resulted in considering a total of 9 out the 106 occurrences

263 initially available. We then examined raw values for the 19 bioclimatic variables extracted at

264 each occurrence point and noticed that the variable bio19 ("precipitation of coldest

265 quarter") displayed obviously aberrant (*i.e.* negative) values. This variable bio19 was thus

266 discarded from subsequent analyses. From the 18 remaining variables (bio1 to bio18), we

267 selected the less correlated ones (Person correlation coefficient < 0.75) thanks to the R-

268 package 'caret' (Kuhn 2019) and kept five variables: bio5 ("mean daily maximum air

269 temperature of the warmest month"), bio7 ("annual range of air temperature"), bio14

270 ("precipitation amount of the driest month") and bio15 ("precipitation seasonality") for

271 further analyses.

272 The calibration area for the models was created as a buffer of 1.5° around the

273 minimum convex polygon encompassing all occurrences. We used the maximum entropy

274 method (implemented in MaxEnt ver. 3.4.1, Phillips et al. 2006, 2017) to calibrate model and

275 evaluated model performance thanks to the package ENMeval (Muscarella et al. 2014) as

276 implemented in ENMwizard. We evaluated models using a geographic partition scheme of

277 type "block" and optimized two parameters of MaxEnt: the Regularization Multipliers (RM)

278 and the Feature Classes (FC). RM was varied from 0.5 to 4.5, incremented by 0.5 whereas a

279 suite of 15 FCs (L, for Linear, P, for Product, Q, for Quadratic and H for Hinge) or combination

280 of them were evaluated: L, P, Q, H, LP, LQ, LH, PQ, PH, QH, LPQ, LPH, LQH, PQH, LPQH,

281 resulting in a total of 135 models. Model selection was done by computing the corrected

282 Akaike Information Criterion ("LowAIC") and the Area Under the receiver operating

283 characteristic Curve ("AUC") in the function ENMWizard::calib_mdl_b(). The relative

284 importance of the different variables was evaluated with the function

285 ENMWizard::get_cont_permimport() . The best selected model was then spatially projected

286 as current niche. For future predictions, a spatial projection of the consensus of the five

287 CGMs was performed for each time period and each RCPs. Area calculations were carried

288 out after applying a 10-percentile (x10ptp) and a Maximum training sensitivity plus

289 specificity (mtss) threshold rules.

290

291 **Results**

292 **Genetic diversity and differentiation**

293 We obtained a total of 159 million read pairs across the 106 individuals (2 283 178 to 6 266

294 452 of raw reads per ind., mean 3 761 722, s.d. = 873 055). We assembled a total of 210 756

295 loci including 22 911 SNPs with Stacks. Average read depth ranged from 27X to 53X based on

296 the combination of parameters we used. After filtering the data with polyRAD, we finally

297 obtained 32 728 loci including 5 095 SNPs.

298   The two first PCA axes (PC1 and PC2) represented 14.85% and 9.21% of the total

299 genetic variance, respectively (Fig. 2A). This analysis is consistent with limited amount of

300 within-population differentiation and a clear pattern of between-population differentiation.

301 Overall, populations are arranged according to geography, especially along PC1. In term of

302 genetic diversity, all populations display a significant deviation from panmixia: mean G_{IS} =

303 0.361, $p < 0.05$ and population G_{IS} values ranging from 0.315 to 0.426 (all $p < 0.05$). However,

304 G_{IS} values appear as similar from one population to another (this holds true for the number

305   of total alleles A, too, see Table 1). The number of private alleles Ap is consistent with the

306   relative level of geographic isolation of the populations.

307   The degree of genetic differentiation was found to be relatively high overall (mean

308   G_{ST} value = 0.403, p < 0.05) and pairwise (with values ranging from 0.156 to 0.527,

309   Supplementary Appendix S3). There is a clear and significant pattern of Isolation By Distance

310   across the dataset (r = 0.601, p = 0.001, Fig. 3) indicating that gene flow is limited in space at

311   the scale of the $D.$ $montanum$ geographic range. The sNMF analysis is also consistent with a

312   strong population structure arranged with geography. Cross-entropy was the lowest for K= 6

313   (Fig. 2B and 2C). Each cluster corresponds to a sampling site with two exceptions. The

314   individuals from the locality of 1-Orri de Baix were grouped together with the close locality

315   of 6-Cambre d'Aze, and the Western localities of the Serra del Cadí (8-Bastanist), 5-Serra

316   Pedregosa and to a lesser extent 4-Pedraforca) formed another group. Only the individuals

317   of 4-Pedraforca showed mixed ancestry coefficients.

318   **Suitable habitats for $D.$ $montanum$ and prediction of its spatial dynamics**

319   ENMeval analyses identified RM = 2 and a combination of Quadratic and Hinge feature (QH)

320   classes as the best-performing parameters for calibrating the final ENM following the

321   "LowAIC" and M=2 and a combination of Product and Hinge feature (PH) classes following

322   the "AUC" optimality criteria. The first model had the best corrected Akaike Information

323   Criterion (AICc) score (152.25) and an AICc weight of 0.33 whereas the second one had an

324   AICc score of 174.63 and an AICc weight of $4.53.10^{-6}$. These two models had mean omission

325   rates of 0.375 and 0.25 for the 10th percentile and the lowest presence training thresholds,

326   respectively, as well as a mean test AUC of 0.988. Values of these diagnostic metrics are

327   provided for all candidate models in Supplementary Appendix S4. Both models (RM= 2/PH

328   and RM=2/QH) considered bio15 ("precipitation seasonality") as being the most important

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329   explanatory variable with a contribution of 88.6% and 90.0%, respectively, followed by bio7

330   ("Temperature annual range (bio5-bio6))" with 6.2% and 7.1% and bio5 "Maximum

331   temperature of the warmest month" with 5.2 and 3.0%; the contribution of bio14

332   "Precipitation of the driest month", being 0% with both models. Current suitable area for *D.*

333   *montanum* was found to be 2 670 and 2 365 km^2 following "LowAIC" and "AUC" selection

334   model criteria, respectively (the values were identical whatever the threshold method used).

335   All future consensus spatial projections predict a dramatic decrease of the suitable area

336   (Table 2 and Fig. 4). For RCPs 7.0 and 8.5, the suitable area would have completely

337   disappeared for the time period 2071-2100 (but not for RCP 2.6). We also notice some

338   slightly different outcomes depending on the selection model criteria with RCP 8.5 scenarios

339   giving sometimes higher values than RCP 7.0, and even RCP 2.6 (e.g. time period 2011-2040,

340   "Low AIC" criterion).

341

342   **Discussion**

343   **Conservation genomics on a polyploid species with large genome size**

344   The use of RAD-seq like (nGBS) data with the method implemented in PolyRAD allowed us to

345   successfully address the challenge of conducting a population genomic approach on a plant

346   species that is both polyploid (autotetraploid) and has a large genome size (> 10 Gbp). It

347   confirms the convenience of the workflow we used, specifically calling genotypes in an R-

348   environment, as an alternative to the ones followed in the handful of studies recently

349   published on similar systems (e.g. Záveská et al. 2019; Brandrud et al. 2020; Karbstein et al.

350   2020; Ahmad et al. 2021). From the > 5 000 SNPs we could rely on, we were able to get an

351   unprecedented genomic picture of the diversity and the differentiation of the species in

352   order to propose appropriate conservation recommendations. The results we obtained at

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353   the genome scale are overall consistent with previous knowledge on population genetics of

354   *D. montanum* (see Simon et al. 2001; López-Pujol 2007).

355

356   **A set of populations isolated on sky islands**

357   Our results confirm that populations of *D. montanum* can be seen as a set of island-like

358   dwelling systems separated from each other by a matrix of unsuitable habitats and that gene

359   flow has little to no chance to be efficiently maintained within this metapopulation. All

360   populations considered in this study were found to display deficit in heterozygotes (G_{IS} >

361   0.315, p < 0.05). These results confirm previous findings of Simon et al. (2001) and López-

362   Pujol et al. (2007) and show that the autotetraploid status of the species does not impede

363   relatively low levels of heterozygosity (H_O < 0.17). Polyploidy is expected to be associated

364   with increased genetic variation, a characteristic that provides more diversity to adapt

365   (especially to extreme environments such as mountainous ones) and allows buffering effect

366   against deleterious mutations (see van de Peer et al. 2021 and references therein). From a

367   conservation point of view, polyploidy does not seem to be associated with increased risk of

368   extinction even though plant with big genome size may be more prone to be under threat

369   (Vinogradov 2003; Pandit et al. 2011). As two other closely related congeneric species of

370   *Delphinium montanum*: *D. dubium* and *D. oxysepalum*, that are endemic to the Alps and the

371   Carpathians, respectively, are also tetraploid (López-Pujol et al. 2007), we speculate that

372   polyploidy may have allowed to these species to adapt and survive into these mountainous

373   environments. The open question is whether current levels of genetic diversity may be

374   sufficient or not to allow *D. montanum* populations to overcome inbreeding depression and

375   provide sufficient variation for adaptation in a context of global change. It should be noted

376   that levels of genetic diversity are overall similar among the populations investigated and we

377   could not associate obvious signs of decline of some of them (e.g. Nohèdes, Pedraforca) to

378   decreased levels in genetic diversity or evidence for deviation from panmixia. This suggests

379   that the evidences of possible decrease in effective size may be too recent to be detectable

380   at the genetic level (see Peery et al. 2012). Unfortunately, the limited number of available

381   methods for polyploids makes effective size estimation and test for bottleneck events

382   cumbersome to perform in such systems.

383   The PCA, the clustering analyses, the F_{ST} values as well as the pattern of Isolation By

384   Distance, further confirm limited gene flow between populations and the existence of a

385   population structure that is consistent with geography. The most likely number of clusters

386   found in the sNMF analysis (K=6) globally distinguishes populations from the Serra del Cadí

387   (8-Bastanist, 5-Serra Pedregosa and 4-Pedraforca) from those of the Puigmal range (1-Orri

388   de Baix and 6-Cambre d'Aze) plus the satellite populations of 3-Vallter, 2-Nohèdes, 7-Tosa

389   d'Alp and 9-Torreta de Cadí (which is the westernmost locality, a bit distant from the others

390   in the Serra del Cadí). Only the locality of 4-Pedraforca shows some ambiguity in population

391   assignment that could indicate a mixed origin. At K=2, the intermediate status of 7-Tosa

392   d'Alp (and to a lesser extent of 4-Pedraforca) are also in agreement with its intermediate

393   geographic position and may reflect an ancestral split between Northern and Southern

394   populations. Altogether, these results support that the insect pollinators (mainly

395   bumblebees) and the natural dispersal of seeds produced (barochoric) are not able to

396   maintain gene flow over the geographic distances involved.

397

398   **Climate change as the main threat for *Delphinium montanum***

399   The two previous studies of Simon et al. (2001) and López-Pujol et al. (2007) have listed

400   several threats to explain the decline of *D. montanum*, at least in some particular

401 populations. According to Simon et al. (2001), natural disturbance of habitats due to rock

402 falls and avalanches, trampling because of an overall increase in human visits and animal

403 predation (e.g. caterpillar, Pyrenean chamois/isards and perhaps red and roe deers) as well

404 as competition for insect pollinators with simultaneously blooming species (e.g. *Aconitum*

405 spp.) have thus been considered as more or less serious problems for *D. montanum*. The

406 most recent study of López-Pujol et al. (2007) also emphasized on the consequences of the

407 erosion of genetic diversity, especially the loss of rare alleles associated with weak possibility

408 of gene flow between localities that could be however compensated by relatively high

409 effective size in several populations. Without ruling out all these threats on *D. montanum*

410 populations, our results rather support climate change as the main threat for this species.

411 The future predictions of the area of habitat suitability all agree that the spatial extent of the

412 ecological niche of *D. montanum* is very likely to dramatically decrease, and even disappear

413 by the end of the 21th century, with some differences based on the various scenarios we

414 considered. The current suitable habitat for *D. montanum* has an area that was estimated at

415 about 2500 km^2, an area that would decrease to about 500 km^2 (*i.e.* -75 %), by 2040

416 whatever the scenario envisaged. By 2070, this area would again have decreased to about

417 150 km^2 based on RCP 2.6 and up to less than 50 km^2 based on the two other scenarios (RCP

418 7 and RCP 8.5). A Representative Concentration Pathway of 2.6 W/m^2 has to be considered

419 as a rather optimistic pathway (that would keep global temperature rise below 2°C by 2100).

420 It would require that CO_2 emissions start declining by 2020 and go to zero by 2100 (as well as

421 declines in emission of other greenhouses gases, such as CH_4, SO_2). RCP 7 is usually

422 considered as a baseline rather than a mitigation target. According to RCP 8.5, emissions

423 would continue to rise throughout the 21st century and can be considered as a pessimistic

424 scenario (with an increase in average temperature in the order of 4°C by 2100). The various

18

425 output of these scenarios likely explains why the bioclimatic niche of *D. montanum* would

426 have completely disappeared by 2100 for RCP 7 and 8.5 but could have persisted and even

427 started to increase again to about 10% of its current suitable area following the RCP 2.6

428 scenario.

429 Environmental changes as the main threat for *D. montanum* populations is also

430 supported from field observations. The population of Nohèdes (2) have not flowered since

431 2011. The individuals there are very small (*i.e.* a few centimetres high) and remain at a

432 vegetative stage. On this site, we could not notice any evidence of avalanche or landslide

433 that could have altered the habitat. In addition, other plants species present in the vicinity

434 (e.g. *Orchis spitzelii, Maianthemum bifolium* and *Convallaria majalis*) seem to suffer from

435 flowering default, further supporting that this phenomenon could be non-specific to *D.*

436 *montanum* and perhaps caused by bioclimatic or at least environmental modifications.

437 There, output from Regional Climate Models (RCMs) predict an increase in mean annual

438 temperature that could reach 3.1 to 4.5°C (+1.5°C already observed since the mean of the

439 period 1961-1990) associated with a decrease in precipitation of up to 25% and perhaps

440 more compared to present-day conditions (Lespinas et al. 2014). According to the same

441 study, precipitations would mostly decrease in summer (-40%) and in spring (-20%) but

442 would remain similar the rest of the year. This in accordance with our ENM predictions

443 suggesting that bio15 "Precipitation seasonality", the annual coefficient of variation of

444 rainfall may be by far, the most important variable in explaining the bioclimatic niche of *D.*

445 *montanum*.

446

447 **Recommendations for conservation**

448   *Ex-situ* conservation would be a way to preserve the genetic diversity of *D. montanum*, but

449   also a bridge for future *in-situ* conservation efforts. A seed collection of different populations

450   of *D. montanum* could be stored in a bank. However, this measure would be of limited

451   interest if the decline's cause of *D. montanum* is actually climate change as keeping dormant

452   seeds will probably not allow this species to adapt to future global changes. Alternatively,

453   conserving genetic diversity in a botanical garden may be a good option to test whether and

454   if so, leave time to plants to adapt to rising temperatures and rainfall modifications.

455   Nevertheless, this approach has potential drawbacks like artificial selection and habitat

456   conversion if *D. montanum* adapts to *ex-situ* environment, which may differ from their initial

457   natural environment (Ren et al. 2014). Moreover, if, growing individuals from the all 9

458   localities studied in an appropriate botanical garden could be a way to set-up *in-situ*

459   conservation, preliminary experiments have showed that *cultivating D. montanum* under

460   experimental conditions in a greenhouse is very difficult because of its environmental

461   requirements (Aymerich, pers. comm.). This confirms that if any, botanical garden would

462   have to be set up at a relatively high elevation (*i.e.* > 1500 m), where environmental

463   conditions are similar to the ecological optimum of the species.

464   We can identify three main methods of *in-situ* conservation that could be envisaged

465   for *D. montanum* (see Godefroid et al. 2011; Ren et al. 2014; Mashinski & Albrecht 2017):

466   reinforcement, translocation and managed relocation (or assisted migration). The first one is

467   reinforcement, which consists in adding *ex-situ* grown individuals in their natural population

468   of origin to increase the total number of individuals, increase the spatial occupancy of the

469   species and thereby reduce extinction risk. On the one hand, the increase in population size

470   provided by reinforcement could contribute to counteract the effect of genetic drift. On the

471   other hand, it would have a limited impact to impede inbreeding as all the individuals would

472 originate from the same genetic pool. The second method consists in translocating

473 individuals (seeds, seedlings or adult plants) from a population to another one with the same

474 ecogeography, to increase genetic diversity (the so-called "genetic rescue") and thus

475 increase the likelihood of population persistence in a changing climate (Mashinski and

476 Albrecht 2017). Our results show that *D. montanum* populations harbor substantial levels of

477 pairwise genetic differentiation which suggest that they have exchanged few or no genetic

478 migrants for many generations and have possibly evolved distinct adaptative differences. As

479 a consequence, gene pool mixing may not be appropriate, since the risk of outbreeding

480 depression could be non-negligible between individuals from the different part of the

481 geographic distribution of *D. montanum*. It could be thus careful to first experiment *ex-situ*

482 crossings to check if hybrids are viable, and do not suffer from outbreeding depression.

483 However, the genetic benefits of mixing source populations during genetic rescue have been

484 shown to generally outweigh the risks of outbreeding depression and loss of local adaptation

485 (see Whiteley et al. 2015; Frankham 2015; Ralls et al. 2018, see also Liddell 2021). For

486 example, translocations with mixed source populations of *Arnica montana* showed that

487 genetic variation remained similar or increased across successive environments and some

488 authors documented a 10-fold increase in population size following gene flow between wild

489 Trinidadian guppy populations (Albretch and Edwards 2020; Fitzpatrick et al. 2020).

490   Finally, the third method consists in introducing a new population in a suitable new

491 ecosystem, where *D. montanum* has not been observed yet. This method is called managed

492 relocation or assisted migration (Mashinski and Albrecht 2017). However, it may represent a

493 risk of modifying the ecosystem, by creating competition for space and pollinators with

494 other plants naturally present, or by introducing new pathogens. Thus, these three methods

495 of reinforcement, translocation, and managed relocation can present risks of outbreeding

496   depression and ecosystem destabilization, and their benefits may not outweigh those risks.

497   Moreover, since the main threat for *D. montanum* is climate change, on a longer-scale, all

498   those efforts could be unsuccessful. Keeping individuals in a botanical garden seems to be

499   the least harmful and risky method for natural populations today and it could be a good start

500   for crossing experiments between populations.

501   For the very peculiar situation of 2-Nohèdes's locality, which seems to be the

502   population the most at risk of extinction at the moment, there is still the question of the lack

503   of flowering. To test whether this problem may be associated with the absence of a specific

504   environmental stimulus, we may propose to either i) experimentally modify

505   temperature/water supply, *in situ*, ii) translocate some individuals to localities where *D.*

506   *montanum* seem healthy (e.g. Cadí range) and see if flowering can be restored and/or iii)

507   experimentally modify temperature/water supply in the lab to better understand the

508   conditions required for flowering and figure out the limiting factor in the wild. Alternatively,

509   it may be due to the fact that that this locality is formed by immature individuals and that

510   the local population itself may be recovering from an important mortality event. At the

511   moment, we have no further arguments to support this scenario.

512

513   **Conclusion**

514   Overall, our results confirm that the remaining populations of *D. montanum* show evidence

515   of decreased genetic diversity and evidence of inbreeding at a genomic scale. The degree of

516   genetic structure observable is consistent with a set of strongly isolated populations no

517   longer able to maintain gene flow between their sky islands. In this context, *in-situ*

518   conservation methods are likely to have an effect on the status of the species.

519   Reinforcement would help counteracting decrease in local population sizes, and

22

520   translocation would help increasing genetic diversity, buffering inbreeding depression and

521   perhaps, bring the variation required for *D. montanum* to adapt. This being said, *ex-situ*

522   crossings are first recommended to verify the absence of outbreeding depression as local

523   genetic pools were found to differ substantially. As our results support that environmental

524   change may represent the main risk of extinction for *D. montanum* all these measures may

525   be however considered as of limited interest on the long term, if the ecological niche of the

526   species disappears.

527

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537

538   **Authors' Contributions and Conflict of Interest**

539   PS, VH, JAMB analysed the data and wrote the manuscript. PAB, JP, AVB, MM, CC, JML and

540   JAMB contributed to fieldwork. MM, CC and VH coordinated the FLORALAB network and/or

541   project aspects related to this work and JAMB contributed to the conception of the study. All

542   authors have read and approved the manuscript. The authors declare no conflict of interest.

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Data Accessibility Statement

Sequencing data have been submitted to the European Nucleotide Archive (ENA; https://www.ebi.ac.uk.ena) under Study with accession n°PRJEB46773 and samples accession n°ERS7180281 (SAMEA9457265) and n°ERS7643565 (SAMEA9965271) to n°ERS7643669 (SAMEA9965375).

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721

722   **Tables**

723

724   **Table 1** Geographic coordinates (in °) and elevation (in m above sea level) of the sampling

725   localities, sample size (N), observed (H_O) and expected (H_E) levels of heterozygosity,

726   deviation from panmixia (G_{IS}), total number of alleles (A) and number of private alleles (A_p)

727   per locality.

	Locality	Latitude	Longitude	Elevation	N	H_O	H_E^*	G_{IS}	A	A_p
1	Orri de Baix	42.444	2.119	2115	12	0.117	0.171	**0.343**	17 549	275
2	Nohèdes	42.615	2.263	1758	12	0.101	0.146	**0.336**	19 570	3,704
3	Vallter	42.426	2.264	2141	12	0.095	0.147	**0.378**	16 862	3,483

31

4	Pedraforca	42.237	1.704	2366	10	0.144	0.244	**0.426**	22 090	1,069
5	Serra Pedregosa	42.277	1.688	2586	12	0.159	0.237	**0.346**	22 456	524
6	Cambre d'Aze	42.451	2.129	2637	12	0.134	0.209	**0.380**	27 075	668
7	Tosa	42.315	1.886	2153	12	0.125	0.177	**0.315**	21 782	3,857
8	Bastanist	42.289	1.688	1979	12	0.165	0.242	**0.332**	26 822	310
9	Torreta de Cadí	42.283	1,574	2358	12	0.122	0.191	**0.376**	17 876	4,537
					106	**0.129**	**0.196**	**0.361**		

728 Geographic coordinates are in WGS84.

729 Population genetic statistics were computed based on 5 095 SNPs. Statistically significant values are indicated

730 in bold ($p < 0.05$).

731 *H_E: unbiased expected heterozygosity measured following Nei (1987) in Genodive.

732

733 **Table 2** Current and future *Delphinium montanum* suitable area (in km^2) as modelled and

734 predicted from Ecological Niche Modelling under different model selection criteria (LowAIC

735 and AUC) and thresholding methods (10th percentile of training presence, x10ptp and

736 Maximum training sensitivity plus specificity, mtss. Future predictions are reported for

737 several time periods (2011-2040, 2041-2070 and 2071-2100) and various Representative

738 Concentration Pathways (RCP 2.6, 7.0 and 8.5).

Model selection criterion	Thresholds methods	Current	2011-2040			2041-2070			2071-2100		
			RCP 2.6	RCP 7.0	RCP 8.5	RCP 2.6	RCP 7.0	RCP 8.5	RCP 2.6	RCP 7.0	RCP 8.5
LowAIC	x10ptp mtss	2670	523	492	526	137	17	24	272	0	0
AUC	x10ptp mtss	2365	459	455	446	161	25	43	287	0	0

739

740 **Figures**

32

741

742 **Figure 1** Map representing the sampling localities (pink dots) within the extant geographic
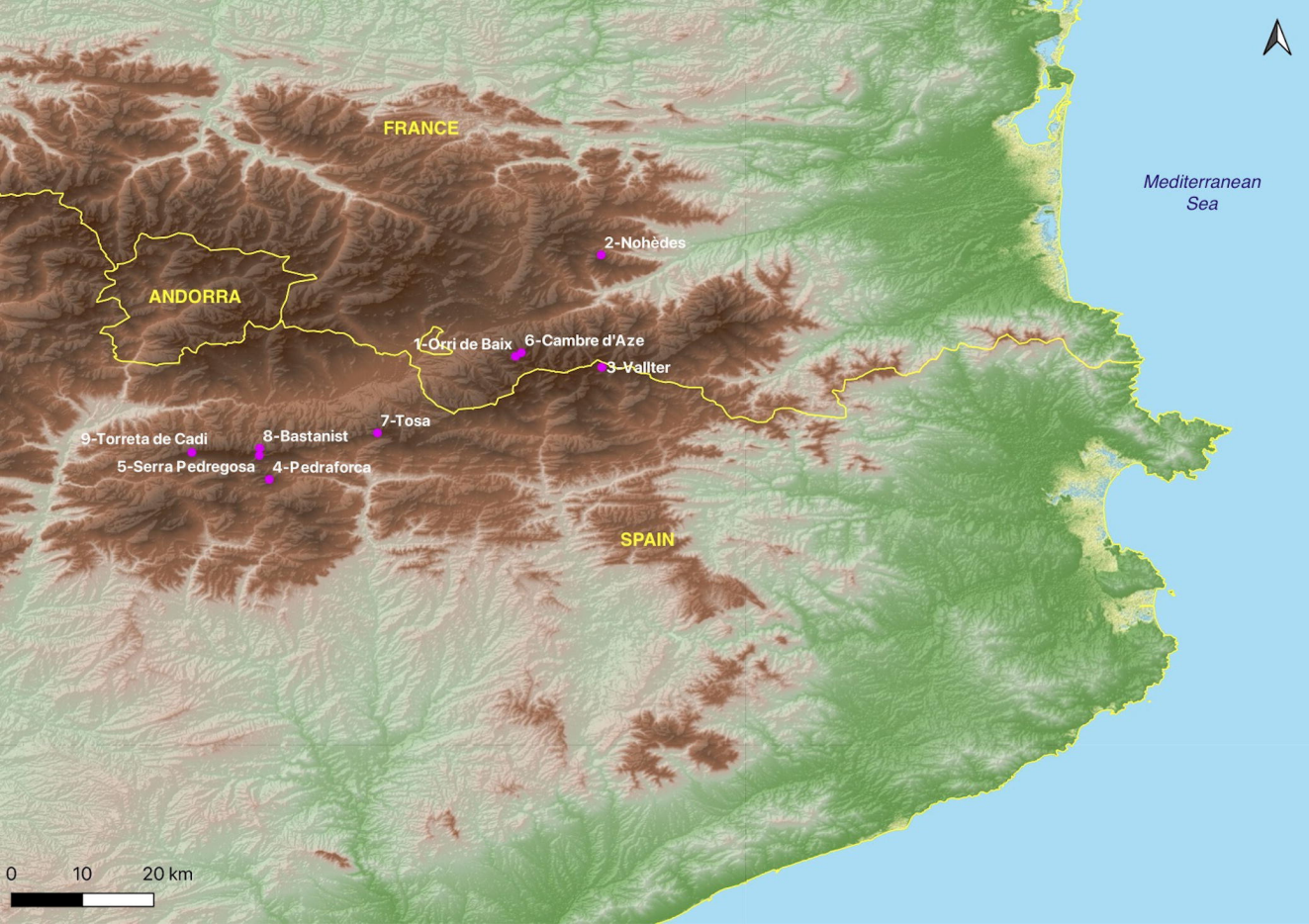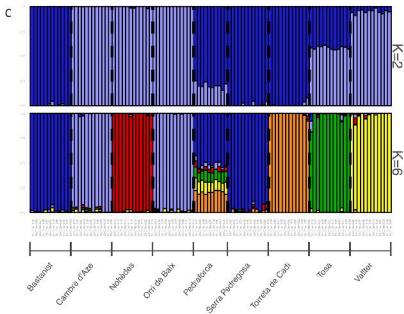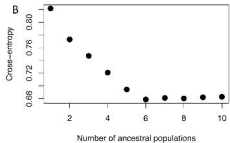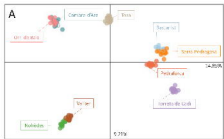
743 distribution of *Delphinium montanum*.

744

745 **Figure 2 A)** Principal Component Analysis (PCA) displaying the two first axes (PC1 and PC2)

746 representing 14.85% and 9.21% of the total genetic variance. PCA was computed based on

747 the 106 individuals of *D. montanum* genotyped at 5 095 SNPs whose colours represent

748 sampling localities. **B)** Values of the cross-entropy criterion for a number of clusters ranging

749 from K = 1 to 10 (10 sNMF runs each). The optimal number of K was found to be 6. **C)** Barplot

750 of ancestry coefficients obtained from sNMF for 106 individuals for K = 2 and K = 6, based on

751 5 095 SNPs.

752

753 **Figure 3** Pattern of Isolation By Distance (IBD) across the data set. Linearized G_{ST}s values (*i.e.*

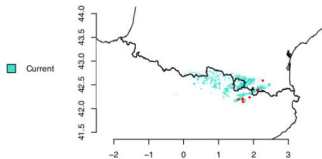754 ln(G_{ST}/(1- G_{ST})) are plotted against ln-geographical distances.

755 **Figure 4** Geographic areas holding suitable climatic conditions for *Delphinium montanum*

756 with projection under present climatic conditions (average for 1981-2010) and consensus

757 projection (from 5 Global Climate Models (CGMs): GFDL-ESM4, UKESM1-0-LL, MPI-ESM1-2-

758 HR, IPSL-CM6A-LR and MRI-ESM2-0), for various plausible climatic scenarios for years 2035

759 (average for 2011-2040), 2055 (average for 2041-2070) and three Representative

760 Concentration Pathways (RCPs): RCP 26, RCP 70 and RCP 8.5. The time period 2071-2100 is

761 not represented since the suitable area would have completely disappeared for RCPs 7.0 and

762 8.5. The Barplot displays current and future area values under various plausible climatic

763 scenarios. These values correspond to "LowAIC" model ("AUC" model being very similar).
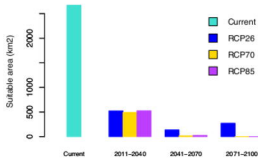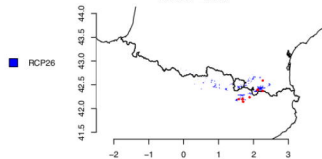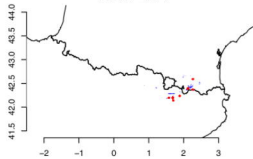
A

Cambon d'Aixe	Tora
LMT-480,480	Teu de la
	Serra Pedregosa
	Peidafonca
Teu lgir	Torroria de Cordi
Neofoldos	

B

C

K=2

K=6

Bastanist · Cambon d'Aixe · Neofoldos · Orri de Baix · Peidafonca · Serra Pedregosa · Torroria de Cordi · Tosta · Vallter
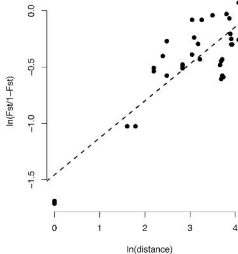
Current

Species total suitable area

2011-2040

2041-2070

2011-2040

2041-2070