Genetic consequences of long-term isolation for the last French population of Eryngium viviparum 1

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ABSTRACT

Eryngium viviparum (Apiaceae) is an endangered endemic plant of the Atlantic region of Europe, growing in seasonally flooded sites. The species is characterized by a highly disjunct distribution. Indeed, it occurs only in a few sites in the North-West part of the Iberian Peninsula and in a single locality in France. In order to improve the conservation status of *Eryngium viviparum* in France, a conservation program has been implemented, which plans reintroduction actions. Before considering such an operation, genetic studies are conducted in order to determine the genetic status of the last French population and to identify the genetic source that should be considered for the best reintroduction strategy. Using microsatellite markers, we documented the genetic structure of the last French population, and compared its genetic diversity with ten Iberian populations, which cover the

three geographic regions where the species occurs. As expected, the French population of *Eryngium viviparum* present a very low genetic diversity due to bottleneck and to geographical isolation. The evolutionary potential appears very low, with no private allele in this population. Furthermore, this population is highly differentiated from the Iberian populations, both for genetic variation and ecological niche. These results imply new questions about the conservation of *Eryngium viviparum* in France, especially for management and reintroduction, which are aimed to favor genetic diversity and to avoid extinction.

KEYWORDS

Conservation genetics - Endangered species - *Eryngium viviparum* - Isolated population - Microsatellites

INTRODUCTION

Ongoing habitat destruction, mainly induced by human activities, is described as "the most serious threat to biological extinction crisis" by causing a reduction and fragmentation of species geographic range (Wilcox & Murphy, 1985; Saunders, Hobbs & Margules, 1991; Sala *et al.*, 2000). Consequently, natural populations became smaller and more isolated in anthropogenic landscapes (McGarigal & Cushman, 2002; Fahrig, 2003), and more sensitive to demographic and genetic stochasticity (Young, Boyle & Brown, 1996; Lowe *et al.*, 2005; Ouborg, Vergeer & Mix, 2006; Honnay & Jacquemyn, 2007).

As gene flow appears to be restricted in fragmented species, an erosion of genetic diversity within isolated populations and an increase of the genetic divergence with other populations are expected (Young & Clarke, 2000; Newman & Tallmon, 2001). In the initial stage after the isolation, both the number of polymorphic loci and the number of alleles per locus decrease, due to bottleneck and genetic drift (Ellstrand & Elam, 1993; Young *et al.*, 1996). If isolation persists, the population can experience a significant decrease of vigor and fecundity due to the increased inbreeding and the accumulation of deleterious alleles (Lynch, Conery & Burger, 1995; Higgins & Lynch, 2001; Keller & Waller, 2002; Reed & Frankham, 2003). In the short-term, the fragmented population becomes more vulnerable face to environmental stochasticity, and population extinction risk can drastically increase (Huenneke, 1991; Young *et al.*, 1996). On the long-term, genetic depletion reduces the population ability to adapt to any environmental change (Barrett & Kohn, 1991). Genetic drift, consequent to isolation, also contributes to increase the genetic differentiation between the isolated population and populations in the core distribution of the species (Pironon *et al.*, 2016). Small populations are often more particularly affected (Leimu *et al.,* 2006*;* Richards, 2000), which can sometimes lead to the extinction of the population (Lande & Barrowclough, 1987; Spielman, Brook & Frankham*,* 2004). The species vulnerability face to these negative consequences also varies according to any life-history trait that reduces the effective population size such as a short-life cycle (Young *et al.,* 1996), and selfing

(Hamrick & Godt, 1989). In contrast, the ability to reproduce clonally may buffer the genetic events (Gitzendanner & Soltis, 2000; Honnay & Bossuyt, 2005).

Integrating genetic variation has become a key element of practical conservation and adapted management (Holsinger & Gottlieb, 1991; Hamrick & Godt, 1996; Rieseberg & Swensen, 1996; Escudero, Iriondo & Torres, 2003) and is particularly relevant when reintroduction or reinforcement are planned (Mistretta, 1994; Havens, 1998; Falk *et al.*, 2006; Neale, 2012). For example, genetic studies are used to identify the most appropriate source of plant material for reintroduction operations (Haig, 1998; Petit, Mousadik & Pons, 1998; Lawrence & Kaye, 2011), giving the advantage to suitable level of genetic diversity (Breed *et al.*, 2013). Using the closest geographic population as source material for reinforcement is often recommended (McKay *et al.*, 2005), while using multiple source populations appears relevant when populations exhibit a low genetic variability (Vergeer *et al.*, 2005; Maschinski *et al.*, 2013). However, it should be noted that, despite guidelines and recommendations, a few number of reintroduction success, or reinforcement, have been recorded yet in plant species (Godefroid & Vanderborght, 2011).

Eryngium viviparum J. Gay, is a *priority species* of the European Habitats Directive and the Bern Convention. It occurs in vernal pools, highly specific of these open habitats maintained by the temporary flooded system and traditional farming practices (Jovet, 1939). In the 1980's, *Eryngium viviparum* experienced a rapid population decline throughout its natural range, especially in France, leading to a highly fragmented Ibero-Armorican distribution (Fig. 1). The reduction of suitable habitats, due to change in farming practices and urbanization, is a significant threat for the survival of the species (Magnanon, Hardegen & Guillevic, 2013). In France, it is considered as *critically endangered* (Olivier, 1995) as only one population remains. For these reasons, several protection measures has been applied since 1987, including annual monitoring, demographic studies, and conservation management of the population. More recently, a National Action Plan (NAP) was implemented, which supports the long-term conservation of *Eryngium viviparum* in France (Magnanon *et al.*, 2013). This program includes notably the restoration of extinct populations. This latter aspect implies to define the best

genetic sources to be reintroduced. Despite the strong isolation of the French population and its reduced spatial distribution (0.1 ha), annual census showed an increasing population between 1994 and 2016 (from 1 500 to 10 000 individuals), including an average of 30% of flowering plants over the summer (Guillevic, unpublished data). However, considering the clonal ability of this species, the effective population size should be significantly lower than the demographic one.

The aim of this study is to investigate the genetic status of the remaining *Eryngium viviparum* French population. Using microsatellites markers, specifically designed for the study, we compared the within population genetic diversity between the French population and the Iberian populations (Spain and Portugal), to infer consequences of isolation. According to the theoretical models in population genetics, it is expected that this population has a low genetic diversity. We also identified which Iberian population is the most genetically related to the French one, in order to provide practical recommendations for the reintroduction plan. Under these objectives, ecological differentiation between *Eryngium viviparum* populations is also evaluated, based on climatic and vegetation data.

MATERIAL AND METHODS

STUDY SPECIES

Eryngium viviparum J. Gay (Apiaceae) is a diploid species, occurring in temporary ponds. These monocarpic species combines sexual and clonal reproduction. The flowers are hermaphroditic and pollinated by insects. Numerous clonal rosettes emerge at the root plate level of the mother plants and on the flower stems. Seed germination and clonal plants development occur mostly in Autumn. The dispersion appears limited as clonal individuals and seedlings are mainly observed at the foot of the mother plants, causing a patchy distribution.

The species is distributed in the north-west of France (Brittany) and north-west part of the Iberian peninsula in three distinct geographical groups (North, Central and South, Fig. 1). Only one population is known in France, occurring in the protected area of "les Quatre-chemins" (Belz, department of

115 Morbihan). This population is nowadays strongly isolated, whereas in the 80's, about 40 populations
116 were known (Magnanon *et al*., 2013). The North Iberian populations also experienced a decline in the
117 last decades, due to habitat modification (Romero, Ramil & Rubinos, 2004).
118
119 *SAMPLING DESIGN AND MICROSATELLITE ANALYSES*
120
121 Leaves were sampled in 2014 on 247 individuals from 11 populations (Fig. 1). Sampled individuals were
122 sufficiently spaced from each other, to reduce the probability of sampling identical genotypes. Samples
123 were dried and stored in silica gel. The number of samples per population ranged from 15 to 27
124 individuals for the Iberian populations, depending on population size, and 37 individuals were sampled
125 from the French population (Table 1).
126
127 Genomic DNA was extracted from dried leaves using the CTAB protocol from Doyle & Doyle (1990).
128 The genotypes of each individual were characteized using 7 operational microsatellite markers (Table
129 2) specifically developed for *Eryngium viviparum* by the biotechnology company "Genoscreen" (Lille,
130 France). Microsatellite loci were isolated by Titanium pyrosequencing (Malausa *et al.*, 2011) and
131 designed using QDD pipeline (Meglécz *et al.*, 2009). The PCR were processed performing two reactions,
132 multiplexing markers based on size compatibility, and using fluorescent labeling of the forward primers
133 (Applied Biosystems). The PCR were carried out in a final volume of 10 µL, including 1 µL of the
134 extracted DNA, 0.2 µL of the forward and reverse primers, and 2X QIAGEN Multiplex Master Mix (5 µL,
135 QIAGEN, France). All the microsatellites amplifications were performed using a thermocycler
136 (Eppendorf Pro) under the following temperature conditions: initial denaturation at 95°C for 15 min,
137 followed by 30 cycles of 94°C for 30 seconds (denaturation), 90 seconds at 60°C (annealing) and 60
138 seconds at 72°C (elongation), finished by the final extension step at 60°C during 30 min. The sizes of

PCR products were analysed by electrophoresis using a 24 capillary Genetic analyser (ABI3500XL, Applied Biosystems). The raw data were visualized with GeneMapper 5.0 (Applied Biosystems).

POPULATION SIZE AND ECOLOGY

Individual censuses were performed each year in the French population, but no comparable estimation exists for Iberian populations. Therefore population size was estimated using the population surface and the abundance of *Eryngium viviparum* individuals evaluated by Glemarec *et al.* (2017), and was summarized in 5 classes: 1: < 500 individuals; 2: 500 to 2 000 individuals; 3: 2 000 to 10 000 individuals; 4: 10 000 to 50 000 individuals; 5: > 50 000 individuals. Geographic distance to the nearest existent population was estimated from GPS coordinates and used as connectivity indice (Table 1).

Ecological distances among populations were considered using both climatic and vegetation data. Three climatic data were extracted from WorldClim 2 database (Hijmans *et al.*, 2005; Trabucco & Zomer, 2009) with 30 arc second spatial resolution (about 1km): maximum and minimum annual temperature (T_{MAX} and T_{MIN}, respectively) and annual precipitation (PP, Table 1). Mean values between 1970 and 2000 were used for each population.

For eight of the sampled populations (S_1, S_2, C_1, C_3, C_4, N_1, N_2 and FRENCH), vegetation communities have been characterized by Glemarec *et al*. (2017) according to the phytosociologial approach. From this dataset, presence/absence of co-occuring species have been extracted (Supplementary Material, Table S1), as they appeared relevant to characterized global ecological factors (Gillet, 2000). These qualitative data have been preferred to abundance/dominance values since species abundances vary according to the management regime which is very heterogeneous among *Eryngium viviparum* populations.

DATA ANALYSES

MICROSATELLITE POLYMORPHISM AND DIVERSITY PARAMETERS

The presence of null alleles was checked using Micro-Checker 2.2.3 (Van Oosterhout *et al.*, 2004). Linkage disequilibrium for each pair of loci within each population and the conformity to Hardy-Weinberg equilibrium were tested with a significance level of 5% using GENEPOP 4.0 (Rousset, 2008). When multiple tests were involved the sequential Bonferroni correction was applied to adjust significance values (Rice, 1989).

Several genetic diversity parameters, including average number of alleles per locus, the observed heterozygosity, the unbiased expected heterozygosity, and the fixation index were computed using GENETIX 4.05 (Belkhir *et al.*, 1996). Correlations between population size and diversity parameters, and between connectivity and diversity, were tested using Spearman ranks correlations (significance level of 0.05).

PAST DEMOGRAPHY DYNAMICS

Demographic changes in effective population sizes were inferred using the Migraine software (Version 0.5, Leblois *et al*., 2014). A detailed procedure can be found in Zenboudji *et al*. (2016). Briefly, we estimated two parameters: the ancestral $\theta_{anc}=4N_{anc}\mu$, and the actual $\theta_{act}=4N_{act}\mu$, where N_{act} is the current effective population size, N_{anc} is the ancestral population size, and μ is the mutation rate per locus per generation. The parameter $Nratio=N_{act}/N_{anc}$ allows to detect, either a reduction (ratio <1) or an expansion (ratio >1) in population size. For each Nratio estimated, its 95 % confidence intervals was used to test for significant difference with 1. All Migraine runs were done using the pGSM model for

188 mutation model (Leblois *et al*., 2014), with 2000 trees per iteration and 512 points per tree and 9
189 iterations.
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191 *DIFFERENTIATION AMONG POPULATIONS*
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193 Pairwise F_{ST} values were calculated with GENEPOP 4.0 (Rousset, 2008). Isolation by distance was tested
194 using Mantel test between $F_{ST}/(1-F_{ST})$ and log (linear distance), with a significance level of 5%.
195 Furthermore, we used Nei's D_A distances (Nei, Tajima & Tateno, 1983), to study the relationship among
196 populations and groups of populations. Overall genetic differentiation was evaluated using a principal
197 components analysis (PCA) using the "adegenet" R package (Jombart, 2008). Bayesian analysis of
198 genetic structure was also applied using STRUCTURE v.2.3.4 (Pritchard, Stephens & Donnelly, 2000).
199 The admixture models were performed for eleven independant runs (K=1 to 11), with 10 replicates at
200 each value of K. Each run consisted of 5 000 Markov Chain Monte Carlo (MCMC) repetitions following
201 a burn-in period of 5 000 iterations. The optimum value of K was determined according to the Δ(K)
202 method developped by Evanno, Regnaut & Goudet (2005) with the STRUCTURE HARVESTER tool (Earl
203 & vanHoldt, 2012).
204
205 Ecological data were used to compute a dissimilarity matrix among the 8 concerned populations using
206 the R "Vegan" package (Oksanen *et al*., 2016; R Development Core Team 2016). We used the Gower
207 coefficient (Gower, 1971), since it allows to combine continuous variables (climatic data) and binary
208 data (vegetation data).
209
210 Correlations among genetic distances ($F_{ST}/(1- F_{ST})$ and Nei's D_A), geographical distances (log-
211 transformed, and obtained from GPS coordinates) and ecological distances were tested, at an alpha
212 level of 0.05, using Mantel tests implemented in the R "Vegan" package (R Development Core Team,

2016; Oksanen *et al.*, 2016) with 1000 permutations. Following Guillot & Rousset (2013), we did not perform partial Mantel tests.

RESULTS

MICROSATELLITE POLYMORPHISM

The number of alleles per locus varied from 5 (pmEV01) to 19 (pmEV04). Only the locus pmEV05 showed sign of null alleles with frequencies ranging from 0.08 to 0.41. This locus is the only one showing a significant heterozygote deficiency ($P < 0.01$, Table 2).

Among the 231 tests used to detect for linkage disequilibrium between loci, only three were significant after the Bonferroni correction: pmEV04/pmEV17, pmEV04/pmEV09, and pmEV05/pmEV09 ($P < 0.001$). Hardy-Weinberg equilibrium was tested at each locus for each population and only three tests (out of 77) showed a significant deviation from Hardy-Weinberg expectations after Bonferroni correction for loci pmEV04 and pmEV05 in the N_3 population and locus pmEV05 in N_2 population ($P < 0.001$). This is congruent with presence of null alleles for pmEV05.

GENETIC DIVERSITY WITHIN POPULATIONS

As expected, the French population showed the lowest values for the three parameters estimating the within-population diversity (Table 3). The French population was the only one with a significant heterozygote deficit ($F_{IS}=0.17$, $P < 0.05$, Table 3). Genetic diversity was quite similar among the Iberian populations, with the exception of the smallest population (S_2), which had the lowest genetic diversity (Table 3), but which was nevertheless twice that in the French population. These results are congruent

with the ratio between the number of multilocus genotypes and the sample size (Table 3). The French population showed the lowest value and all the Iberian populations showed high values. However, no similar multilocus genotype was detected across populations.

None of the genetic diversity parameters were correlated with population size ($P > 0.05$), with or without the French population. In contrast, distance to the closest population, used as a connectivity index, was correlated to N_A, H_{OBS} and F_{IS} ($R=-0.61$, -0.66, and 0.69 respectively, $P < 0.05$) when considering the French population, but not anymore when excluding it ($P > 0.05$).

PAST DEMOGRAPHIC DYNAMICS

According to the N ratio estimates produced by Migraine, no population showed a significant demographic expansion ($P > 0.05$). A bottleneck (N ratio> 1) was detected in most of the sampled populations, and all of them have recovered allelic diversity (Table 3). Consistent with the low diversity, a bottleneck was also detected for the French population (Table 3).

DIFFERENTIATION AMONG POPULATIONS

Genetic differentiation

Global F_{ST} value (0.29) indicated high differentiation among populations. For the Iberian populations, the genetic differentiation was also high ($F_{ST}=0.24$). Pairwise F_{ST} values ranged from 0.04 to 0.6, and 26 values out 55 were > 0.25 (Table 4). Isolation by distance tested using the correlation between $F_{ST}/(1-F_{ST})$ and log(geographical distance) was significant considering the 11 populations ($R=0.65$, $P < 0.01$, Fig. 2), and was also significant excluding the French population ($R=0.41$, $P=0.01$). Correlation between

261 Nei D_A and log (geographical distance) was also significant whether the French population was
262 included, or not, in the analysis ($R > 0.58$, $P < 0.001$).

263

264 The PCA also suggested differentiation between the FRENCH and Iberian populations (Fig. 3A). This
265 observation is also consistent with the structure analysis. According to the Δ(K) method, the most
266 appropriate value of K given for our data was K=4 (Fig. 3B). These four clusters of individuals match the
267 four geographical regions were *Eryngium viviparum* occurs (FRENCH, North, Central and South of
268 Iberian peninsula, Fig. 3C).

269

270 *Ecological differentiation*

271

272 Gower distances ranged from 0.19 (N_2 & N_1) to 0.67 (French & S_1). Genetic and Gower distances were
273 positively correlated using Nei D_A values ($R=0.59$, $P < 0.001$), but not when using linearized F_{ST} ($R=0.43$,
274 $P > 0.05$). These correlations, using both Nei D_A and linearized F_{ST}, were not significant when
275 considering only the Iberian populations ($P > 0.05$).
276 Among populations, the plant communities share common species, characterized by pioneer species
277 growing in seasonally flooded open habitats, such as *Exaculum pusillum* (Lam.) Caruel (Gentianaceae),
278 *Chamaemelum nobile* (L.) All. (Asteraceae), *Baldellia ranunculoides* (L.) Parl. (Alismataceae) and
279 *Eleocharis multicaulis* (Sm.) Desv (Cyperaceae). However some ecological vicariances were observed
280 among *Eryngium viviparum* co-occurring species. For example, *Agrostis canina* L. (Poaceae) was
281 observed only in the French population, while *Agrostis hesperica* Romero Garcia, Blanca López &
282 Morales Torres was identified in North and Central Iberian population, and *Agrostis pourrettii* Willd.
283 only in South Iberian populations.

284

Considering only the presence/absence of species, the French population seems closer to North Iberian populations, sharing several Atlantic species, such as *Aristavena setacea* (Huds.) F.Albers & Butzin (Poaceae) and *Galium debile* Desv. (Rubiaceae), which are not reported in the other two clusters. S_1 is the furthest population from the French one, characterized by the occurence of Mediterranean species such as *Agrostis pourrettii* (Poaceae), *Pulicaria paludosa* Link. (Asteraceae) and *Mentha cervina* L. (Lamiaceae).

DISCUSSION

ASSESSMENT OF GENETIC DIVERSITY FOR THE ONLY FRENCH POPULATION

Among the 11 studied populations, a reduction of effective size was detected in eight populations. These bottlenecks are consistent with the historical data of the species, known from the botanical literature and herbarium specimens (Magnanon *et al.*, 2013). For instance, in France, since the first description of the species in 1839, 36 populations had been recorded in a limited area in Brittany, and all of them except one, have disappeared. However, the genetic consequences of these bottlenecks appear very different according to the geographic area. Indeed, all the Iberian populations, even the smallest one (<500 individuals) appear to have recovered genetic diversity. In contrast, the French population shows an extremely low level of genetic diversity and an evidence of inbreeding.

Moreover, only one rare allele was found for the French population for the pmEV09 locus (frequency <5%), and no private allele was observed, suggesting an absence of specificity for this population considering these seven microsatellites. Conversely, some Iberian populations display private alleles and a global high multi-locus allelic diversity, except for S_2, the smallest one, which appeared twice

310 diversified than the French population. The level of genetic diversity detected in the French population
311 appeared especially lower than those noticed in other endangered Apiaceae, such as its congeneric
312 *Eryngium alpinum* L. (Gaudeul & Till-Bottraud, 2008), or other species under similar isolated context
313 (Wiberg *et al.*, 2016; Tamaki, Setsuko & Tomaru, 2016; Aavik *et al.*, 2017).

314 In this study, population size is not correlated with genetic diversity, while genetic diversity
315 and heterozygosity are commonly expected to be positively correlated with population size (Ellstrand
316 & Elam, 1993; Frankham, 1996; Leimu *et al.*, 2006). Among the eleven populations studied, the French
317 population showed the lowest genetic variability, despite a population size estimated to 10 000
318 individuals in 2016, which was considered as intermediate when compared to Iberian populations.
319 Therefore, according to our results, isolation appeared to have more negative influence than
320 population size on the genetic diversity for *Eryngium viviparum*, and connectivity seems to reduce the
321 effect of bottleneck. This isolation effect has been reported in some genetic studies on other plant
322 species (*Eucalyptus albens* Benth., Prober & Brown, 1994; *Anthyllis vulneraria* L., Honnay *et al.*, 2006),
323 which suggest to avoid population fragmentation when possible or to reestablish connectivity among
324 populations. The demographic size of the French population reveals the efficiency of the management
325 strategy to sustain the population. However, the absence of genetic diversity due to the combined
326 effect of bottleneck and spatial isolation could cast doubt on the long-term persistence of the
327 population.

328 The species is also characterized by clonal propagation, which often contributes to decrease genetic
329 diversity (Hamrick & Godt, 1989; Watkinson & Powell, 1993; Young *et al.*, 2002; Vallejo-Marín, Dorken
330 & Barrett, 2010). It could ultimately lead to a monoclonal genotypic pattern (Balloux, Lehmann & De
331 Meeûs, 2003; Honnay & Bossuyt, 2005). However, in some cases, clonal propagation may benefit
332 species under isolated context, by maintaining allelic diversity, polymorphism and heterozygosity, but
333 only on the short-term (Ellstrand & Roose, 1987; Auge *et al.*, 2001; Meloni *et al.*, 2013). The monocarpy
334 (short-lived species) and the poor dispersal ability observed for *Eryngium viviparum* are also factors
335 that are well-known to induce a loss of genetic diversity (Young *et al.*, 1996).

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338

339 *STRONG STRUCTURE BETWEEN GEOGRAPHIC GROUPS*

340

341 Consistent with the low dispersal ability of the species, but despite the putative contribution of genetic
342 drift, isolation by distance revealed that geographical distance appears to be a major component of
343 the genetic differentiation among populations. The French population appears less distant from the
344 North Iberian populations, as suggested by Rodriguez-Gacio *et al*. (2009) with RAPD analyses. This
345 relative genetic proximity is probably the result of a common biogeographical history. Nevertheless,
346 the F_{ST} showed a high differentiation between the French population and the Iberian ones, which is
347 consistent with the spatial isolation. Such high F_{ST} levels are regularly found for rare plants (Maguire *et*
348 *al.*, 2000).
349 Consistently with the geographical distances, bioclimatic data also revealed that the French population
350 is distant from all other populations. This population occurs on temperate hyperoceanic domain, while
351 northern and central Iberian populations are located respectively under temperate oceanic and
352 temperate submediterranean bioclimate (Rivas-Martínez, Rivas-Sáenz & Penas-Merino, 2011).
353 However, Glemarec *et al.* (2017) considered that the French population and the northern Iberian
354 populations occur under the same bioclimatic region, as temperate oceanic, and highlight similar plant
355 associations. In these two regions, *E. viviparum* communities are characterized by Atlantic wetland
356 species which belong to the floristic associations of *Eleocharitetum multicaulis* Allorge 1922 ex Tüxen
357 1937 and *Deschampsio setaceae-Agrostietum caninae* Lemée 1937. The populations S_1 and S_2 located
358 in the southern limit of the *E. viviparum* distribution range, are characterized by a Mediterranean
359 pluvioseasonal oceanic bioclimate (Rivas-Martínez *et al.*, 2011) and the occurrence of Atlantic and
360 Mediterranean species. According to Glemarec *et al*. (2017), these floristic composition may be linked

to the associations of *Pulicario uliginosae-Agrostietum salmanticae* Rivas Goday 1956 and *Periballio laevis-Illecebretum verticillati* Rivas Goday 1954. These two populations also appeared more genetically differentiated from the other Iberian populations. Therefore, both ecological and genetical data confirm the differentiation between the French population and the Iberian populations, and also between the three Iberian clusters.

IMPLICATIONS FOR CONSERVATION

With its strong isolation (<500 km), excluding any genetic exchange with other populations, the French population of *Eryngium viviparum* is a suitable model to discuss implications of genetic studies for conservation purposes. The very low genetic diversity, on the edge of monoclonality, can reduce the environmental adaptability of the species (Frankham, 2005). This low allelic diversity can also lead to inbreeding depression and affect the viability of individuals by fixation of deleterious alleles (Lynch *et al.*, 1995; Higgins & Lynch, 2001; Keller & Waller, 2002; Reed & Frankham, 2003). Correlation between population size and reduction in fitness is a common pattern for plants (Reed, 2005). Indeed, a decrease of the viability of both seeds and seedlings seems already to occur in the French population (Guillevic, pers. com.). Moreover, *ex situ* germination experiments show lower germination rates for seeds sampled on French individuals (30%) than seeds collected on Iberian individuals (80%, Gautier, pers. com.). These observations combined to our results imply that restoring the genetic diversity in the French population should be considered.

However, introduction of new genotypes in an endangered population always remains problematic for stakeholders concerned with the species conservation, arguing that it will alter the genetic identity of the local population (Maurice *et al.*, 2013). An alternative solution is to reestablish connectivity by restoring populations, as planned within the framework of the NAP (Magnanon *et al.*, 2013). The long-term and ambitious objective is to re-create a sustainable metapopulation of *E. viviparum* in Brittany.

Nevertheless, guidelines for reintroduction suggest avoiding low genetic diversity (Montalvo *et al*., 1997; Weeks *et al*., 2011), and studies have shown that reintroducing material from multiple populations can increase translocation success (Vergeer *et al.*, 2005; Maschinski *et al.*, 2013) and restore significantly the genetic diversity (Zavodna *et al.*, 2015). This would suggest that in absence of other French sources, reintroduction could be implemented using Iberian genetic material. It is also generally recommended choosing source material from the nearest populations (Brown & Marshall, 1995; McKay *et al.*, 2005; Bottin *et al.*, 2007), in order to avoid environmental maladaptation. As the French population clearly appears strongly ecologically distant from the Iberian populations, it should be necessary to test for a possible outbreeding depression between French and Iberian populations before any reintroductions (Yardeni *et al.*, 2016). Populations from the Northern part of the Iberian distribution range should be favored, as they present the lesser distance for both ecological and genetic data. Experimental crossing should also be considered beforehand, to test the viability of first generations, and their adaptability to survive in the natural habitat of *E.viviparum*.

CONCLUSION

This study brings new elements of the genetic status of *Eryngium viviparum,* an important requirement for the French National Action Plan. Seven microsatellites markers were developed and validated specifically for the genetic characterization of *E. viviparum*. Our results clearly contribute to guide management and restoration operations for the species, and suggest that the restoration of extinct populations is a priority. Experimental reintroductions, using only the French genotypes, had already occurred in order to optimize technical modalities and to identify factors that influence the success of *E. viviparum* reintroductions (Rascle *et al.*, 2018). However, the origin of the material must be clarified by some complementary studies in order to test the viability and the environmental adaptation of offspring from controlled crosses.

411

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431

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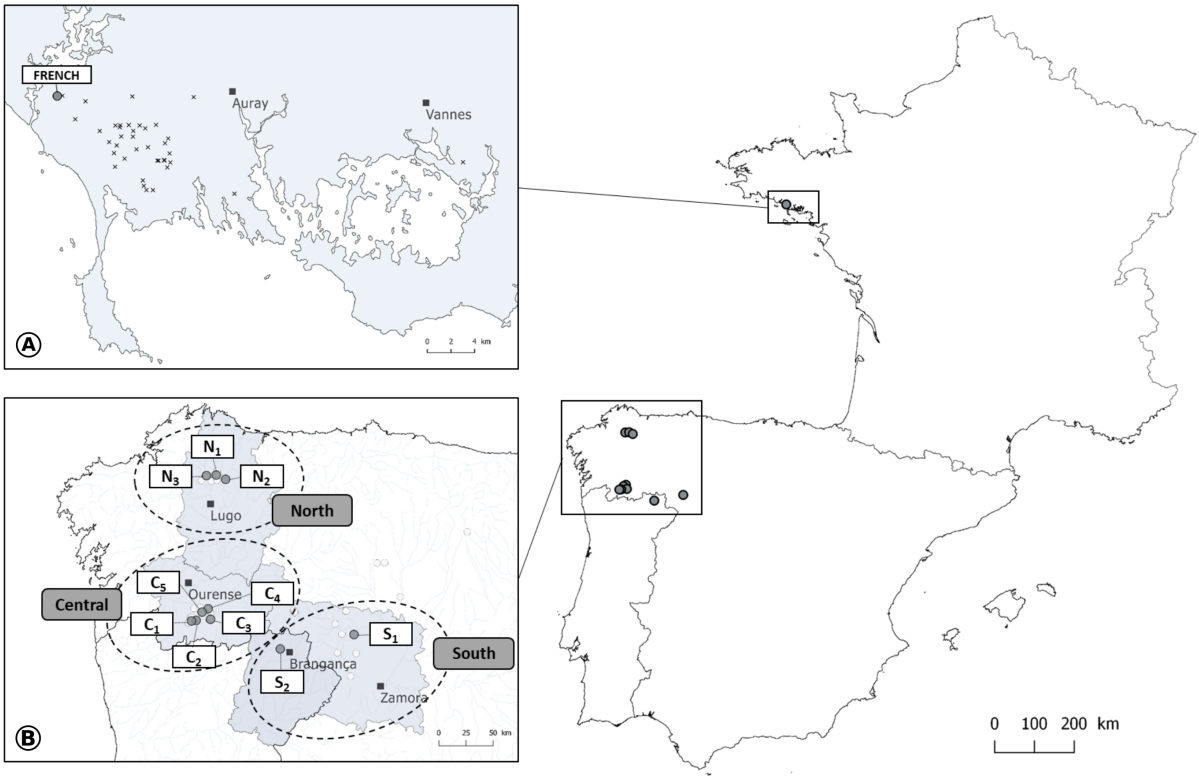
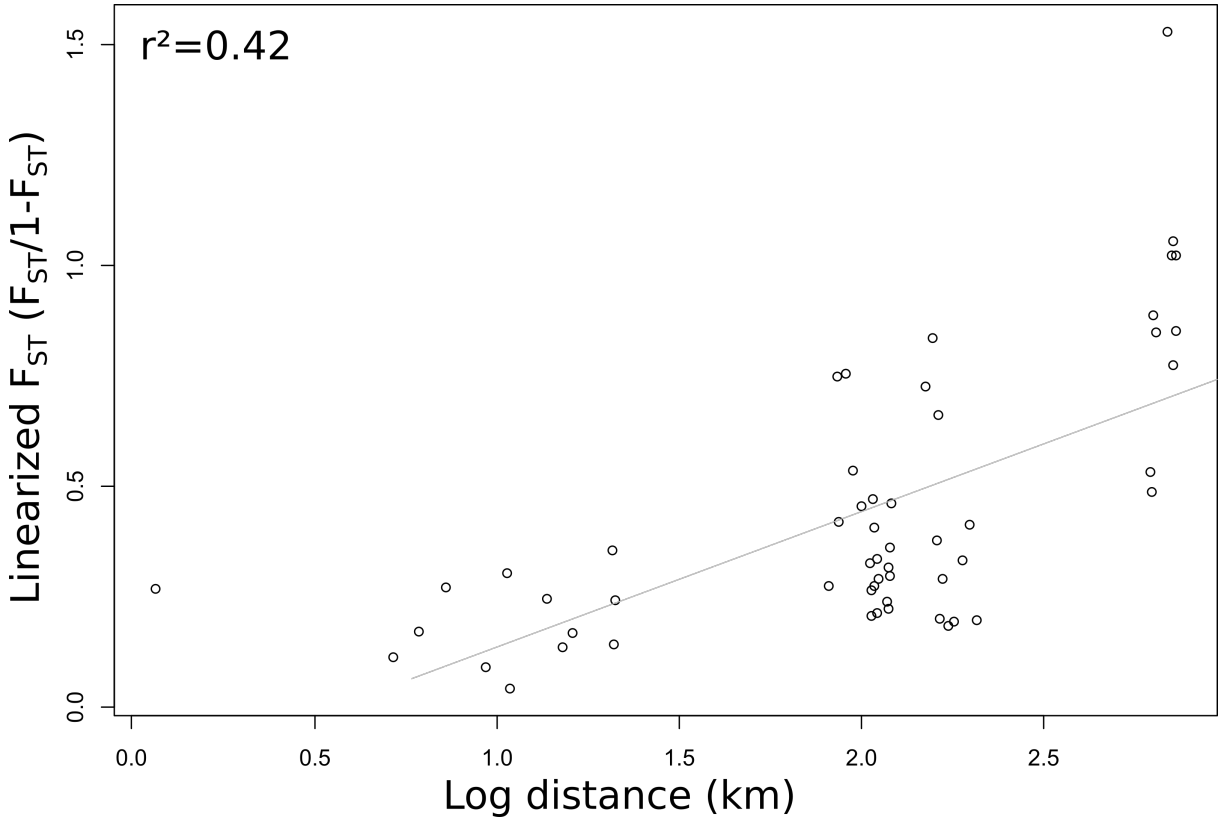


Fig. 1. Global distribution and location of the 11 sampled populations of *E. viviparum*. A. Actual and historic distribution in France. B. Actual distribution in the Iberian Peninsula. Dotted line ellipses delimit the three population geographical clusters.



664

665 **Fig. 2**. Correlation between F_{ST} (F_{ST}/1- F_{ST}) and log(geographical distance, in km) for pairwise
666 comparisons of the ten *E. viviparum* Iberian populations. r^2 indicates the coefficient of correlation from
667 the Mantel test.

668

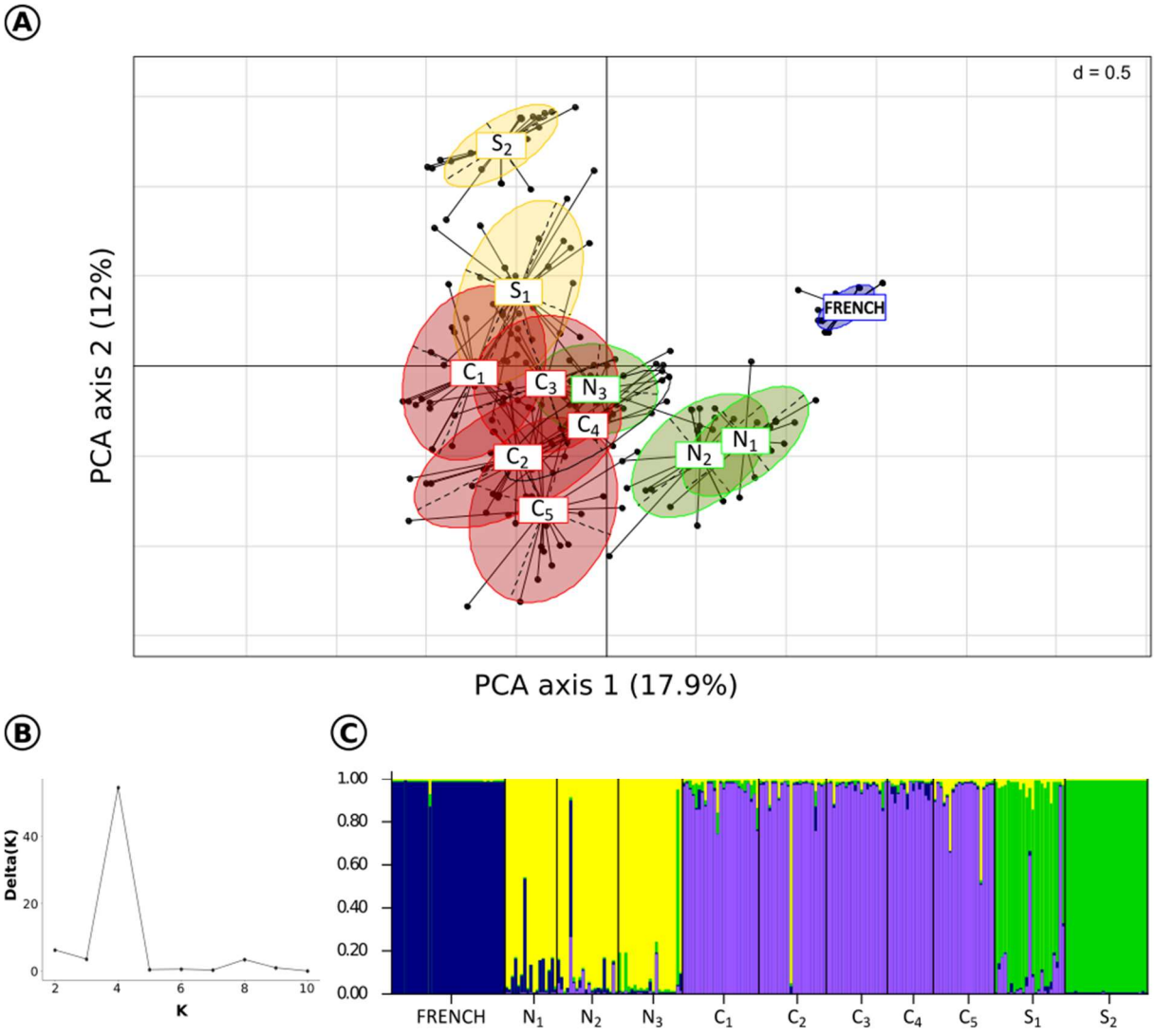


Fig. 3. Population structure analyses. a. First plan of the principal components analysis (PCA) of *Eryngium viviparum's* genetic structure among eleven populations and based on seven microsatellites multilocus genotypes. Each point represent the individual genotypes and are connected by lines to the centroid of the 95% confidence interval ellipse of each population. b. Delta(K) values from K=1 to 11. c. Average assignment probability of *Eryngium viviparum* individuals for K=4.

Table 1. Population location, sample size and estimated size (see text for details), distance to the nearest population and ecological characteristics. The four climatic data (mean annual temperature=T_M, maximum annual temperature=T_{MAX}, minimum annual temperature=T_{MIN} and annual precipitation=PP) were extracted from Worldclim 2 (Hijmans *et al.*, 2005) based on 30-years average values (from 1970 to 2000). The number of co-occuring species was extracted from Glemarec *et al*. (2017). The populations N_3, C_2 and C_5 were not included in this study. Exact GPS coordinates are not indicated considering the rarity of *Eryngium viviparum.* NA notifies data are not available.

Location	Herbarium voucher	ID population	Sampled size	Population size category	Distance to the nearest existant population (km)	Elevation (m)	Climatic values from Worldclim v.2				N° of co-occuring species
							T_M (°C)	T_{MAX} (°C)	T_{MIN} (°C)	PP (mm)	
France											
Morbihan	*No voucher*										
Belz		**FRENCH**	37	3	516	11	12.1	21.1	3.5	893	20
NORTH (Spain, Galicia, Lugo) :											
Lagoa de Cospeito	*J.Amigo, P.Ramil, M.Rodriguez & J.Izco 39497* [SANT 38490]	N_1	17	2	2.9	457	12.2	22.5	1.6	1357	15
Bexan	[LUGO 773]	N_2	20	3	8	401	12.4	22.4	1.5	1329	13
Fontefria	[LUGO 772]	N_3	21	2	6	413	12.4	22.4	1.9	1382	*NA*
CENTRAL (Spain, Galicia, Ourense) :											
O Toxal	*I.Pulgar* [SANT 45414]	C_1	25	4	2.4	613	12.2	23.1	0.9	1602	12
O Foxos		C_2	22	5	2.7	616	12.5	24.3	0.4	1638	*NA*
Vilaseca	*I.Pulgar* [SANT 45969]	C_3	20	5	0.6	617	12.5	24.5	0.4	1718	19
Veiga de Gomareite		C_4	15	5	0.7	618	12.4	24.4	0.4	1719	17
Cardeita	*I.Pulgar* [SANT 45413]	C_5	20	4	4.6	618	12.5	24.4	0.2	1698	*NA*
SOUTH :											
Ferreira de Abajos (Spain, Castilla y Léon, Zamora)	*P. Bariego 2480* [SANT 60815]	S_1	23	3	1.3	804	11.6	24.8	-2.8	750	19
Tras-o-Montes (Portugal, Bragança)	*No voucher*	S_2	27	1	33.3	963	11	23.2	-1.9	943	7

Table 2. Characteristics of the seven microsatellites primers used for genotyping *Eryngium viviparum* populations. T_a: annealing temperature. N_A: average number of alleles. H_{OBS}: observed heterozygosity. H_{EXP}: expected heterozygosity. F_{IS}: intrapopulation fixation index. F_{IS} values statistically different from zero, at the 0.05 level, appeared in bold.

Locus	GenBank accession number	Repeat arrays	Primer sequences (5'-3')	T_a (F/R)	Size range (bp)	Global diversity indices among all populations			
						N_A	H_{OBS}	H_{EXP}	F_{IS}
pmEv01	MK319936	ACA_{22}	F : AGTATTACTTCTGCCTTTAATATTTCG R : CATGATTAATTAGATGCTTGAAGATG	60.7/60	219 -295	5	0.19	0.24	-0.0517
pmEv02	MK319937	GT_{21}	F : TTAGTGTCCGAATGAGCAGC R : GCACCGTTTCCTGTTGGTAT	58.4	75-125	9	0.4	0.52	0.0707
pmEv04	MK319938	GA_{21}	F : TTGGTGAGGGTTTCGATTTG R : TCACCTCGATTCTTGTGCAT	56.4	124-170	19	0.72	0.91	0.0206
pmEv05	MK319939	GT_{20}	F : CGCAAGAAATTGCTCCCATA R : TGTTGCCAATATGACAGTAACG	56.4/58.4	108-160	11	0.5	0.85	**0.1195***
pmEv09	MK319940	$ATGT_{17}$	F : CCCACGATTGATCTGCATAG R : TCAGAGGATGTCTCCCACAA	58.4	250-340	15	0.43	0.77	0.0455
pmEv10	MK319941	CA_{17}	F : GTTATGTCACACTTCATGCTGC R : TGCTTCTGTCCTGTTATCCTCA	60.3	149-178	11	0.52	0.79	-0.0132
pmEv17	MK319942	AC_{16}	F : ATAAGAGGGGGAAAAGGTGG R : TTAATTGTGTATTCAATGAACTTTCC	58.4	213-227	7	0.58	0.72	-0.0526

Table 3. Genetic diversity (number of alleles per locus=N_A , observed heterozygosity=H_{OBS}, unbiased expected heterozygosity=H_{EXP}, fixation index=F_{IS}) within each population of *Eryngium viviparum* and Migraine outputs of past demographic analyses expressed by N ratio (current effective population size =N_{act}/ancestral population size=N_{anc}, the 95% confidence intervals are given into brackets). Populations with an N ratio significantly different from 1 are in bold. F_{IS} values statistically different from zero, at the 0.05 level, are in bold.

Population	N_A	H_{OBS}	H_{EXP}	F_{IS}	No. of multilocus genotypes/ Sample size	No. of private alleles	N ratio	Demographic event
FRENCH	1.57	0.15	0.18	**0.17***	16/37	0	**2.2e-11 [1.64e-11 -- 5.46e-06]**	bottleneck
N_1	4.14	0.56	0.53	-0.03	15/17	0	3.69e-05 [2.66e-05 -- 72.18]	stable
N_2	4.86	0.56	0.59	0.07	18/20	0	**8.43 e-04 [9.88e-05 - 0.182]**	bottleneck
N_3	5.28	0.53	0.56	0.08	15/21	0	0.0771 [1.02e-05 -- 3.454]	stable
C_1	5.71	0.57	0.56	-0.006	22/25	2	**2.6e-11 [10e-12 – 2.9e-9]**	bottleneck
C_2	6.00	0.69	0.63	-0.07	21/22	2	1.092 [6.22e-05 – 1.955]	stable
C_3	6.14	0.69	0.67	-0.005	19/20	4	**0.00477 [0.000132 -- 0.816**]	bottleneck
C_4	4.00	0.48	0.50	0.005	15/15	1	**0.01 [0.003 -- 0.053]**	bottleneck
C_5	4.00	0.55	0.55	0.02	20/20	0	**5.88e-03 [8.07e-05 -- 0.142]**	bottleneck
S_1	5.00	0.59	0.57	-0.02	21/23	2	**1.54e-19 [1.65e-20 -- 9.06e-11]**	bottleneck
S_2	2.86	0.30	0.32	0.08	25/27	3	**7.14e-05 [3.16e-05 – 0.132]**	bottleneck

Table 4. Pairwise F_{ST} values for seven microsatellites markers between the 11 *Eryngium viviparum* populations studied. All values are significantly different from zero at $P < 0.05$.

	FRENCH	N1	N2	N3	C1	C2	C3	C4	C5	S1
N1	0.3273									
N2	0.3476	0.0393								
N3	0.4695	0.2317	0.1946							
C1	0.5055	0.3154	0.2647	0.2393						
C2	0.4597	0.2279	0.1825	0.1925	0.1018					
C3	0.4365	0.2148	0.1696	0.2097	0.1238	0.1184				
C4	0.5053	0.2883	0.2451	0.3204	0.2622	0.1438	0.2105			
C5	0.5127	0.2254	0.1745	0.2504	0.1966	0.0822	0.1466	0.213		
S1	0.4582	0.2917	0.249	0.165	0.1627	0.1553	0.1666	0.2744	0.2241	
S2	0.6045	0.4546	0.4204	0.3973	0.3123	0.3481	0.2944	0.4274	0.4298	0.2154

Table S1. Presence/absence data of *Eryngium viviparum* co-occurring species among the French population and seven Iberian populations extracted from Glemarec *et al*. (2017).

		French	Iberian peninsula						
			NORTH		CENTRAL			SOUTH	
Species	Family	Belz	N_1	N_2	C_1	C_4	C_3	S_2	S_1
Chamaemelum nobile (L.) All.	Asteraceae	1	1		1	1	1	1	1
Leontodon saxatilis Lam.	Asteraceae	1	1	1	1	1	1		1
Trocdaris verticillatum (L.) Raf.	Apiaceae	1	1	1		1	1	1	1
Exaculum pusillum (Lam.) Caruel	Gentianaceae	1		1		1		1	
Mentha pulegium L.	Lamiaceae	1		1			1	1	
Juncus bulbosus L.	Juncaceae	1	1	1	1	1	1		
Baldellia ranunculoides (L) Parl.	Alismataceae	1		1	1	1	1		
Ranunculus flammula L.	Ranunculaceae	1	1	1	1		1		
Eleocharis multicaulis (Sm.) Desv.	Cyperaceae	1	1	1			1		
Carex demissa Vahl. ex Hartm.	Cyperaceae	1	1	1					
Aristavena setacea (Huds.) F. Albers & Butzin	Poaceae	1	1	1					
Galium debile Desv.	Rubiaceae	1	1						
Molinia caerulea (L.) Moench	Poaceae	1				1	1		
Littorella uniflora (L.) Asch.	Plantaginaceae	1					1		1
***Agrostis canina* L.**	**Poaceae**	**1**							
Cicendia filiformis (L.) Delarbre	Gentianaceae	1							
Hydrocotyle vulgaris L.	Araliaceae	1							
Lotus corniculatus L.	Fabaceae	1							
Potentilla erecta (L.) Räusch.	Rosaceae	1							
Scorzonera humilis L.	Asteraceae	1							
Juncus acutiflorus Ehrh. Ex Hoffm.	Juncaceae		1	1		1			1
***Agrostis hesperica* Romero García, Blanca & C. Morales**	**Poaceae**		**1**	**1**		**1**	**1**		
Carex flacca Schreb.	Cyperaceae		1						
Hypericum elodes L. Huds.	Hypericaceae		1						
Lythrum salicaria L.	Lythraceae		1						
Scutellaria galericulata L.	Lamiaceae		1						
Salix repens L.	Salicaceae			1					
Antinoria agrostidea Parl.	Poaceae				1	1	1	1	1
Lythrum borystenicum (Schrank) Litv.	Lythraceae				1	1	1	1	1
Illecebrum verticillatum L.	Caryophyllaceae				1	1	1		1
Eleocharis palustris (L.) Roem. & Schult.	Cyperaceae				1		1	1	1
Juncus pygmaeus Rich. Ex thuill.	Juncaceae					1	1	1	1
Spergula rubra (L.) D. Dietr.	Caryophyllaceae				1	1	1		
Agrostis truncatula Parl.	Poaceae				1				
Myosotis sicula Guss.	Boraginaceae				1				
Corrigiola littoralis L.	Molluginaceae					1			
Molineriella leavis (Brot.) Rouy	Poaceae					1			
Radiola linoides Roth	Linaceae					1			
Juncus capitatus Weigel	Juncaceae						1		
Lotus subbiflorus Lag.	Fabaceae						1		
***Agrostis pourretti* Willd.**	**Poaceae**								**1**
Cynodon dactylon (L.) Pers.	Poaceae								1
Deschampsia cespitosa (L.) P.Beauv.	Poaceae								1
Herniaria glabra L.	Caryophyllaceae								1
Isoetes velata A. Braun	Isoetaceae								1
Juncus bufonius L.	Juncaceae								1
Mentha cervina L.	Lamiaceae								1

Polypogon viridis (Gouan) Breistr.	Poaceae	1
Pulicaria paludosa Link	Asteraceae	1