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Genetic variation in the horsetail *Equisetum variegatum* Schleich., an endangered species in the Parisian region

NATHALIE MACHON[1,*], JEAN-MICHEL GUILLON[1,2], GAUTHIER DOBIGNY[3], SOLENN LE CADRE[1] and JACQUES MORET[1]

*[1]Conservatoire Botanique National du Bassin Parisien, Muséum National d'Histoire Naturelle, 61, rue Buffon, F-75005 Paris; [2]Laboratoire Ecologie, Systématique et Evolution, UPRESA 8079 CNRS, Bâtiment 362, Université Paris-Sud, F-91 405 Orsay cedex; [3]Present adress: Laboratoire de Zoologie Mammifère et Oiseau, Muséum National d'Histoire Naturelle, 55, rue Buffon, F-75005 Paris, France; *Author for correspondence (e-mail: machon@mnhn.fr; fax: +33-1-40793553)*

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Abstract. *Equisetum variegatum* Schleicher is a circumboreale species of horsetail. In France, it typically grows at high elevations but is very rare in lowlands. The genetic variation of these populations is described using isozyme electrophoresis and PCR-RFLP of chloroplast DNA. Sampled sites were chosen to represent central vs. marginal and/or endangered parts of the distribution area. Extensive clonal multiplication of plants together with the absence of local recruitment by sexual reproduction seem to be responsible for the low genetic diversity observed within populations. Since adaptive response to environmental changes ultimately relies on the presence of genetic variability, clonal populations of *E. variegatum* may be particularly vulnerable to disturbance. Moreover, in lowland populations, isolation gives no chance to recover new genotypes through migration events. The preservation of the two endangered populations is proposed by propagation by cuttings of all extant genetic individuals. In the case of a disappearance of one genotype in the field, a replacement will be possible. This plan may be sufficient to preserve *E. variegatum* in the French lowland for several years.

Key words: chloroplast DNA, clonal reproduction, conservation, *Equisetum variegatum*, isozymes

Introduction

Genetic studies are more and more employed for plant conservation purposes: first to identify and evaluate the threats that endanger a species and secondly to help design optimal conservation programs. The lack of genetic variability, even when estimated at selectively neutral genetic markers, such as allozymes, would be able to decrease population viability (Vrijenhoek 1994). Indeed, a parameter such as allelic richness is highly dependent on effective population size (Nei et al. 1975) and should be a good indicator of past demographic changes (Petit et al. 1998). Loveless and Hamrick (1984) showed how the mode of reproduction of the species had an effect on genetic structure within and among populations. For species reproducing mainly asexually, the distribution of genetic markers can show how clonal a population is (Ellstrand and

Roose 1987). Most of the time, a population contains many different clones but at the extreme some populations may consist of a single genetic individual (Alpert et al. 1993). In asexually reproducing plants, the degree of clonality or number of different clones within populations is important in conservation biology because populations with few genetic individuals, regardless of ramet number, are subject to genetic processes that make them particularly vulnerable to new evolutionary challenges imposed by diseases, competition or changing environmental conditions (Ellstrand and Elam 1993). Because of the risk due to demographic stochasticity, the threat is more serious if, in addition, the ramet number is low.

In conservation programs, the knowledge of the distribution of genetic diversity provides a guide to the wise management of the genetic resources of a species (Barrett and Kohn 1991). *In situ* conservation consists of a set of measures aimed at restoring the maximum evolutionary potential of a population including reinforcement, creation of new populations and corridors, together with ecological management. *Ex situ* creates collections containing the maximum genetic diversity possible. These measures are possible only if the genetic structure of species and populations are known. Moreover, the use of genetic markers can also show the possible genetic uniqueness of a particular population and thus provide objective reasons to preserve it.

Equisetum variegatum Schleicher is a circumboreale species of horsetail (Figure 1). In France, it commonly grows in high elevations. In the Alps and Pyrenees, *E. variegatum* is mainly found along rivers. Populations are numerous and are representing a part of its central distribution area. In contrast, only two populations are known to be growing in lowland habitats (Prelli and Boudrie 1992). A large population of *E. variegatum* was discovered in Ile-de-France, 10 km from Paris in 1896, in the forest of Marly (Figure 2), (Jeanpert 1896). Numerous authors reported on the continued existence of this same population (Dupuis and Rapilly 1953, 1956, 1965, 1973; Guffroy 1935; Jovet 1936; Rapilly and Dupuis 1955; 1958). This population still exists but contains only 9 distinct ramets. It is the only extant population in the Parisian region. In 1991, another lowland population was found in northern France, (200 km from Paris), near Dunkerque (Figure 2), (F. Truant, pers. comm.). The population grows in a dune pan and contains more than 100 ramets, all growing on a 50 $\times$ 20 m area. This population is the only French representative population of *E. variegatum* from the Flemish coast, but another location is known in Belgium.

In the present paper, we describe the distribution of genetic variation in French populations of *E. variegatum*, a species for which no such studies had been reported. Two complementary methods were used: isozyme electrophoresis as an indicator of nuclear genetic diversity and PCR-RFLP as a marker of chloroplast polymorphism. Specifically, we address the following questions: What are the patterns of genetic diversity within and among population? What are the threats to the lowland populations? Which conservation steps should be taken given the observed genetic diversity?

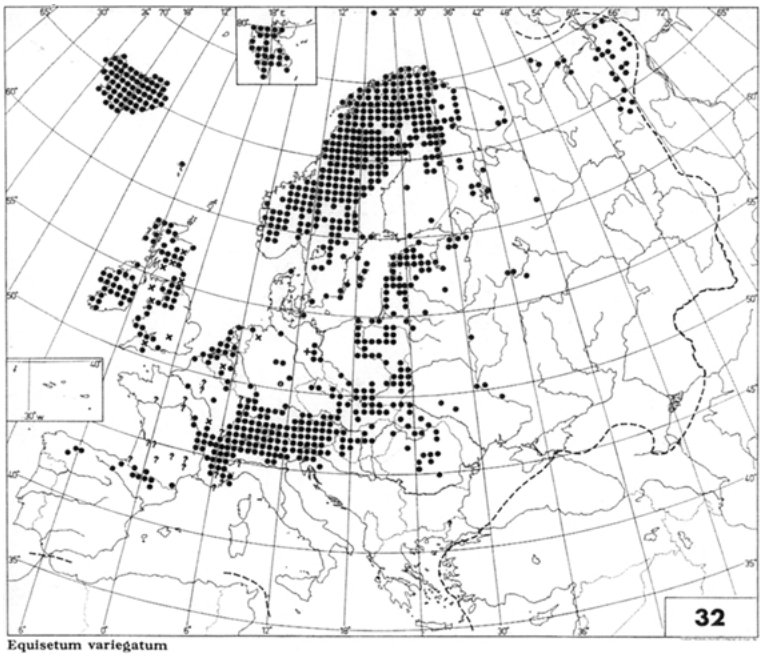

Figure 1. Equisetum variegatum natural range in Europe (from Tuttin et al. 1993).

Figure 2. Equisetum variegatum sampled populations.

Materials and methods

A total of 122 *E. variegatum* samples was collected from 6 populations (Figure 2). Marly (10 km west of Paris) where nine ramets are growing in an area of 50 m^2; Dunkerque (300 km north of Paris), plants are growing in an area of 1000 m^2 but there are grouped in 8 areas of 2 to 20 m^2 where the density is of about 10 ramets per m^2 for a population of more than 1000 ramets; four populations in the Alps (600 km south-east of Paris): the two populations 'Clarée2', 5 m^2 and 'Clarée3', 50 m^2 have a very high density in plants that constitute a uniform carpet of uncountable plants (may be 100 plants per m^2). These two populations are situated on opposite sides at the same location of the river Clarée, 'Clarée1', 5 m^2, 20 plants per m^2, is located 3 km upstream Clarée2 and Clarée3. 'Guisane', located 10 km from the Clarée populations is composed of 17 plants in 5 m along the river Guisane. Clarée and Guisane are two tributaries of the river Durance. The Marly and Guisane populations were sampled exhaustively. In the other populations, 8 to 34 ramets were uniformely selected every meter along transects that cross the populations (Table 1). Sporophytic ramets were collected in the field, transported on ice to the laboratory where they were planted in pots. Genetic analysis were performed on shoot samples of the potted plants.

Isozyme electrophoresis

The following enzymes were analyzed: DIA, E.C.1.6.9.9. Diaphorase; MDH, E.C.1.1.1.40. Malate dehydrogenase; IDH, E.C.1.1.1.42., Isocitrate dehydrogenase; ADH, E.C.1.1.1.1., Alcohol dehydrogenase; PGM, E.C.2.7.5.1., Phosphoglucomutase; GOT, E.C.2.6.1.1., Glutamate oxaloacetate transaminase; SKDH, E.C.1.1.11.25., Shikimate dehydrogenase; 6PGD, E.C.1.1.1.49., Glucose 6 phosphate dehydrogenase; PGI, E.C.5.3.1.9., Phosphoglucoisomerase; LAP, E.C.3.4.11.1., Leucine amino peptidase.

Samples were prepared and electrophoresis conducted following general methods of Soltis et al. (1983). The tris-HCl grinding buffer-PVP solution of these authors was used. Gel buffer and staining techniques were as described by Pasteur et al. (1987) and Soltis and Soltis (1989).

The Tools For Population Genetic Analyses program was used to calculate the following parameters: allele frequencies, allele numbers, effective allele numbers (Hartl and Clark 1989), polymorphic loci, observed and expected heterozygosity under random mating (Nei 1973), *F*-statistics (Weir 1990) (Table 2). We produced a matrix based on pairwise Fst estimates calculated by the TFPGA program and performed an exact test to evaluate the genetic differences within each pair of populations (Sokal and Rohlf 1995) (Tables 3 and 4).

For the population Clarée3, a chi-square test was performed to test if two and three adjacent plants in a transect were more often identical than would be expected by chance.

Table 1. Allelic frequencies for polymorphic loci in the *Equisetum variegatum* populations. Results are given for each population, for the grouped populations of Alps (Clardée1, Guisane and Val) and for the total sample. A, B and C represent different alleles for each locus.

Population	Sample size	Allele	PGM	6PGD1	ADH
Marly	9	A	0	0	0.5
		B	0.78	1	0.5
		C	0.22		
Dunkerque	34	A	0	–	0
		B	1	–	1
		C	0		
Lowlands	43	A	0	0	0.10
		B	0.95	1	0.90
		C	0.05		
Guisane	17	A	0	0	0.5
		B	0.71	1	0.5
		C	0.29		
Clarée1	20	A	0.15	0	0.45
		B	0.85	1	0.55
		C	0		
Clarée2	8	A	0	0	0.5
		B	1	1	0.5
		C	0		
Clarée3	34	A	0.33	0.12	0.34
		B	0.67	0.88	0.66
		C	0		
The Alps	79	A	0.18	0.06	0.40
		B	0.75	0.94	0.60
		C	0.07		
Total population	122	A	0.12	0.05	0.28
		B	0.82	0.95	0.72
		C	0.06		

Table 2. Indices of genetic diversity within and among populations of *Equisetum variegatum* sampled in France. *n* is the sample size for each population. NG is the number of genets in each population. P is the polymorphic rate. A is the mean number of alleles per locus. Ae is the mean effective number of alleles per locus. He is the expected heterozygosity. Ho is the observed heterozygosity.

	Marly	Dunkerque	Lowlands	Guisane	Clarée1	Clarée2	Clarée3	Alps	Total
n	9	34	43	17	20	8	34	79	122
NG	2	1	3	2	3	1	6	6	6
P (%)	15.38	0	15.38	15.38	15.38	7.69	23.08	23.08	23.08
A	1.15	1	1.15	1.17	1.15	1.08	1.25	1.31	1.31
Ae	1.12	1	1.02	1.14	1.10	1.08	1.16	1.13	1.09
He	0.07	0	0.02	0.08	0.06	0.04	0.09	0.08	0.06
Ho	0.11	0	0.02	0.13	0.09	0.08	0.13	0.11	0.08

Table 3. *F*-statistics (Weir 1990) per locus and per populations.

		PGM	ADH	6PGD	Over all loci
Marly	Fis	−0.29	−1	–	
Dunkerque	Fis	–	–	–	
Lowlands	Fis	−0.29	−1	–	−0.71
	Fit	−0.12	−0.33	1	−0.26
	Fst	0.12	0.33	1	0.26
Guisane	Fis	−0.42	−1	–	
Clarée 1	Fis	−0.18	−0.82	–	
Clarée 2	Fis	–	−1	–	
Clarée 3	Fis	−0.5	−0.5	−0.14	
Alps	Fis	−0.39	−0.84	−0.14	−0.60
	Fit	−0.17	−0.81	−0.03	−0.47
	Fst	0.16	0.02	0.09	0.08
Total	Fis	−0.37	−0.87	−0.14	−0.65
	Fit	−0.14	−0.62	0.88	−0.26
	Fst	0.17	0.14	0.89	0.23

Table 4. Matrix of Fst estimates within each pair of populations.

	Marly	Dunkerque	Guisane	Clarée1	Clarée2	Clarée3
Marly						
Dunkerque	0.2625*					
Guisane	0.0030	0.2628*				
Clarée1	0.0242	0.2142*	0.0385*			
Clarée2	0.0367	0.3333*	0.0673	0.0243		
Clarée3	0.0626*	0.3863*	0.0655*	0.0335	0.0986	

*Significant differences ($P < 1\%$).

Chloroplast DNA PCR-RFLP

Total DNA extraction was performed according to Edwards et al. (1991). PCR primers and procedures were as described in Demesure et al. (1995) and in Dumolin-Lapegue et al. (1997). Five pairs of chloroplast versatile primers were chosen for their efficiency in PCR amplification of fern DNA: CD, FV, HK, SfM and ST (Dumolin-Lapegue et al. 1997). PCR products were ethanol precipitated, resuspended in pure water and an aliquot was analysed by agarose gel electrophoresis. Afterward, 0.5 μg of ST, 0.5 μg of HK and 1 μg of FV amplified fragments were restricted by 5 units of *Hha*I plus *Hinf*I, or *Hha*I plus *Msp*I, or *Hinf*I, respectively, in 20 μl for 3 h at 37 °C. We loaded 0.2–0.4 μg of the restriction products on a 4% denaturing acrylamide gel. Electrophoresis was performed for 2–4 h at 1800V in 1X TBE, and the DNA fragments were silver stained following Cho et al. (1996). DNA fragment sizes were estimated from parallel migration of a molecular weight marker ($\Phi \times 174$/*Hae*III).

Results

The 10 enzyme systems used in the present study revealed 13 loci (two for 6PGD, for GOT and for DIA). In the total sample, *E. variegatum* was monomorphic at 10 loci surveyed and thus polymorphic at 23% of the 13 loci analyzed. The polymorphic loci were ADH, PGM and one locus of 6PGD (Table 1). For locus $6PGD_1$, allele A is rare (f(A) = 5%). For the whole sample, the number of alleles per locus (averaged over 13 loci) was 1.31, the effective allele number was 1.09 and the expected heterozygosity equaled 0.06. A very high heterozygosity is observed for each polymorphic locus in every populations (Fis < 0) and also globally for the whole sample (Fis = −0.65).

The mean Fst over all *E. variegatum* populations was 0.23, and differentiation between Alps populations and lowland ones is Fst = 0.094, both significantly different from zero. The exact test showed no differences among Clarée populations, but did detect a significant differences between two Clarée populations and the other alpine populations (Table 4). However, the Marly population, which is geographically very distant from the Alps also was not significantly different from three of the four alpine populations. Dunkerque significantly differentiated from every other populations.

Only one genotype was found for Clarée2 and Dunkerque. Two genotypes were observed among the 9 ramets of Marly and also within the population of Guisane. Clarée3 presented six distinct genotypes. It is the most diverse and also the largest mountain population sampled. For this last population, a chi-square test showed that identical genotypes are significantly spatially grouped ($\chi^2 = 13.99$, ddl = 1, $P < 0.001$). Observations for several years in the two lowlands populations showed that, contrarily to alpine populations, no cones have been produced by the plants in these two locations, indicating that these populations of *E. variegatum* do not reproduce sexually.

SfM and CD chloroplast primers failed to amplify *E. variegatum* DNA. The three remaining pairs of primers allowed the amplification of 2 kbp (FV), 1.5 kbp (HK) and 1.3 kbp (ST). Enzymes were chosen in order to yield small sized restriction fragments (<550 bp) that could be easily separated by denaturing polyacrylamide gel electrophoresis. Only one polymorphism was revealed, a 2 or 3 bp insertion/deletion in the HK fragment, defining two haplotypes (Light, and Heavy). The Heavy haplotype was present in all samples from Marly, Dunkerque, Clarée1, Clarée2 and Clarée3 populations. The two haplotypes cohabited in the population from Guisane, in which their distribution exactly matched the two clones identified with isozyme markers.

Discussion

Genetic diversity of French E. variegatum *populations*

Despite the high number of chromosomes found in this genus: 2n = 216 (Löve et al. 1977), *E. variegatum* exhibited isozyme patterns typical of diploids like other members of this genus (Soltis 1986; Haufler 1987; Haufler and Soltis 1986).

The genetic diversity found in *E. variegatum* species is low compared with other *Equisetum* species and even more with plant species in general. We detected polymorphism at 3 of 13 loci and the average P among populations ($P = 0.128$) was lower than those found in *E. arvense* ($P = 0.191$ in Soltis et al. 1988; $P = 0.214$ in Korpelainen and Kolkkala 1996) and in *E. hyemale* ($P = 0.292$ in Korpelainen and Kolkkala 1996). The average number of alleles per locus among populations (A = 1.13) was similar to that reported in *E. arvense* (A = 1.16; Soltis et al. 1988) as was the average expected heterozygosity He = 0.09 in *E. variegatum*, (He = 0.092 in *E. arvense*; He = 0.134 in *E. hyemale*; see Korpelainen and Kolkkala 1996). According to the general review made by Hamrick and Godt (1990), species that reproduce both sexually and asexually, exhibit a mean polymorphism rate of 0.44, while mean values of P and A for early successional and weedy plant species are 0.297 and 1.60, respectively (Hamrick et al. 1979). Extensive clonal multiplication of individual genets together with the absence of local recruitment by sexual reproduction after foundation (Duckett 1979; Duckett and Duckett 1980; Milton and Duckett 1985) could be responsible for the comparatively lower diversity found within *Equisetum* populations.

The data show that the genetic differentiation found among the populations of *E. variegatum* is high (Fst = 0.23). These data seem correspond to those found in other plant species since the mean value found for perennial plants, are Gst = 0.213 to 0.233 and plants reproducing both sexually and asexually Gst = 0.213 (Hamrick and Godt 1990). Actually, the high differentiation observed on *E. variegatum* is only due to the particular genetic structure of the Dunkerque population. Differentiation is very low among the alpine populations (Fst = 0.080). This result is different from those shown by Korpelainen and Kolkkala (1996) on *E. arvense* and *E. hyemale* which are very common species of horsetail. Their study was conducted on nine enzyme systems resolving 14 loci and showed high Fst values among Finnish populations of *E. arvense* (Fst = 0.232) and *E. hyemale* (Fst = 0.269), which came from territories as extended as the Alps. The difference in Fst values between our study and theirs could be explained if our alpine populations are connected by gene flow, are of recent origin, all issued from one same ancestor population. *E. variegatum* are perennial and long-lived plants, so recent or recently isolated populations may not have had the time necessary to differentiate via mutation, selection and drift (Lokki 1976). Lowland populations are to small (1 and 2 genotypes) to perform unbiased F-statistics but the exact test showed that contrarily to Marly, Dunkerque population is significantly different from the alpine ones. This population could be issued from northern eastern European populations whereas Marly could be originated from alpine ones. However, the alleles in the Dunkerque population are all found in every population that has been looked at. Indeed, its multilocus genotype could easily come from single sexual event from virtually every population in the study. Clonal reproduction represents difficulties of interpretation of F-statistics. Allele frequency estimates and measures based on them can be misleading when clonal growth is prevalent. This idea is reinforced by the results found on cpDNA diversity.

As recently shown, chloroplasts are maternally inherited through sexual reproduction in *E. variegatum* (Guillon and Raquin 2000). Only two chloroplast haplotypes have been found in our survey of *E. variegatum* populations. In particular, no differences were found between Dunkerque and the others. This seemingly low level of cpDNA diversity still is in the range reported for other plants on which PCR-RFLPs studies have been conducted at a similar sampling scale (Rumsey et al. 1996; Demesure et al. 1996; El Mousadik and Petit 1996; Hollingsworth et al. 1999). Indeed, the Light haplotype is not restricted to the population from Guisane. Preliminary studies revealed its presence in samples from two other distant localities from Northern Alps and Pyrénées (Guillon unpublished results). Because France is at the southern limit of *E. variegatum* distribution in Europe, it would be worthwhile to study populations from Northern Europe. Such a study could tell us if French populations are descended from local ice-age populations and have contributed to post-glacial recolonization.

Within-population genetic structure and reproductive system

Data give several evidences that *E. variegatum* mainly reproduce asexually. Even if the number of genotypes we found with 10 isozyme loci is a underestimation of the actual number of clones in populations, this number is not related to the number of ramets investigated and is too small for a sexually reproducing species in both mountain populations and isolated lowland populations. The data show a large excess of heterozygotes for all loci, particularly for ADH and all populations, and particularly in three out of five populations (Marly, Guisane, and Clarée 2) (Table 3). High heterozygosity was also found in *E. arvense* and *E. hyemale* populations (Soltis et al. 1988; Korpelainen and Kolkkala 1996). This overrepresentation of heterozygotes could be explained by the chance success of genets that happen to be heterozygous at the loci that have been studied or it could equally be due to a more efficient asexual reproduction of heterozygotes as has been proposed by Oostermeijer et al. (1995) and Korpelainen and Kolkkala (1996). Other studies are needed to test this hypothesis in *E. variegatum*. In the same population, however, the spatial proximity of ramets of the same genotype suggests their clonality. For instance in Clarée3, statistical tests showed that the same genotypes were grouped together. These results suggest that vegetative reproduction is common within *E. variegatum* populations and that recruitment of new ramets to the population occurs almost exclusively vegetatively. Clonal spread enables some genets to cover considerable areas and to maintain when environmental conditions are unfavorable for sexual reproduction (in lowlands). In a population such as Marly, extant genets could be the same as those that were present a long time ago. Comparisons of DNA fingerprints obtained from herbarium samples and modern samples could be used to demonstrate their genetic identity.

The demographic and genetic structure of *E. variegatum* populations are threats for this species in the regions where isolation gives no chance to recover new genotypes through migration events. This is the case for Marly which is the only populations in the Parisian region and for Dunkerque. Once more abundant in the northern France, this horsetail was considered to have disappeared from this region so the Dunkerque population may be a last French Flemish relic. From a geographical point of view, the lowland populations can be considered as both marginal and isolated. Such isolation is a common phenomenon among perennial plant that can multiply vegetatively (Johannsson 1993) and could be explained by changing climatic conditions and destruction of habitats in combination with limited dispersal properties. Dispersal ability differs between marginal and central populations of *E. variegatum* (Johannson 1993). When marginal populations do not reproduce sexually, the only means of dispersal is by rhizome or stalks fragments and would need a running river. In the central populations, however, spores can be dispersed between water-systems by wind or animals. This might explain a higher regional abundance in the Alps, as compared to Marly and Dunkerque, where water systems are absent or stagnant, preventing colonization of other locations. Since adaptive response to environmental changes ultimately relies on the presence of genetic variability, isolated and clonal populations of *E. variegatum* may be particularly vulnerable to disturbance.

The preservation of all genetic individuals is an optimal goal for the conservation of any rare species (Sipes and Wolf 1997). For perennial plants like *E. variegatum*, which can be propagated by cuttings, this objective is particularly easy to achieve. The lowland populations, Dunkerque and Marly, appear to be the most vulnerable, and contain one and two distinct genotypes respectively. A conservatory garden comprising several replicates of the three genotypes and other ramets from the Alps is located in Paris at the Conservatoire Botanique National du Bassin Parisien in the Muséum National d'Histoire Naturelle. In the case of a disappearance of one genotype in the field, a replacement will be possible. Also, *in situ* management of the threatened populations has begun. Trees shading the populations have been cut to provide more light and water to give *E. variegatum* a chance to favorably evolve. A reinforcement of the Marly population with new ramets from Guisane could also be proposed.

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