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GENETIC DIVERSITY AND ECOLOGICAL DIFFERENTIATION IN THE ENDANGERED FEN ORCHID (*LIPARIS LOESELII*)

Running Title: *Liparis loeselii* conservation genetics

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ABSTRACT

Liparis loeselii is a rare and endangered orchid occurring in Europe and north-east America. Genetic diversity and structure of this species in north-west France and the United Kingdom were investigated using amplified fragment length polymorphisms (AFLPs). Although clonality and autogamy are common in *L. loeselii*, we found moderate to important variability within populations. We observed a significant genetic differentiation between populations occurring in dune slacks and fens. This may be correlated with leaf shape as dune slack individuals are sometimes treated as the distinct variety *L. loeselii* var. *ovata*. Genetic differentiation between populations was generally low suggesting that gene flow can occur over long distances and possibly across the English Channel. These results show that populations from dune slacks and fens should be managed separately and that geographically distant populations may be equivalent.

INTRODUCTION

Appropriate conservation of biodiversity requires evaluation of the conservation value of populations, species, ecosystems and areas, and a determination of priorities at each scale. The species is the most commonly used measure for biodiversity (Purvis & Hector 2000) and the main unit in conservation. However the conservation of wild species requires a good knowledge of their delimitation and distinctiveness from their closest relatives (Vane-Wright et al. 1991). Systematics is an important issue to take into account in conservation (May 1990) so that cryptic species are not neglected (e.g. for the tuatara, Daugherty et al. 1990) or to clarify the status of taxa of dubious distinctiveness (e.g. the red wolf, Wayne & Gittleman 1995).

In spite of extensive work, European orchids are still the subject of major taxonomic issues, as shown by the differences between treatments by Delforge (1994) (2001) and, for example, Pedersen (1998), with substantial differences in the number of species recognised. Because of the ease of hybridisation, morphological variability within species and their popularity, taxonomic splitting may have been particularly common in orchids. Taxonomic clarification in this charismatic group is therefore an essential condition for efficient conservation plans. Taxonomic issues have already been raised in the British Isles concerning the conservation of rare species in taxonomically difficult genera such as *Dactylorhiza* (Bateman 2001, Hedrén 2001, Pillon et al. 2006) and *Epipactis* (Squirrell et al. 2002).

The fen orchid, *Liparis loeselii*, is a declining species throughout its distribution, a large part of Europe and North America. This species occupies two types of habitats: dunes slack on the coast and neutral to alkaline fens in plains and mountains. Suitable

habitats have become scarce due to coastal urbanisation and the draining of wetlands. The species is protected in most European countries where it occurs and is listed on Annex II of the European directive 92/43/EEC on the conservation of natural habitats and of wild fauna and floras.

In Britain, the species is now only known to occur at three sites in eastern England (Wheeler et al. 1998) and four in southern Wales (Jones 1998). In France, *L. loeselii* was the first plant species to be the subject of a national conservation plan (Hendoux et al. 2001). Compared with other European countries, the species is still relatively well represented in France, especially on the coast of Northern France and in the Alps. However, most populations have drastically reduced in size, and the majority of continental lowland populations have completely disappeared.

Within the species, Welsh populations in dunes are characterised by rounded leaves and have been described as *Liparis loeselii* var. *ovata* (Riddlesdell 1907). This variety was later identified in Brittany, and coastal populations of northern France may also belong to this taxon (Hendoux et al. 2001). However, the delimitation of the var. *ovata* and var. *loeselii* remains unclear. There is continuous variation between ovate and more lanceolate shapes (Hendoux et al. 2001), and leaf shape has not been compared critically across populations under controlled conditions. Therefore leaf shape variation could be the result of environmental variance due to biotic (e.g. interspecific competition for light) or abiotic (e.g. soil trophic richness) differences among habitats. However, seedlings germinated in vitro appear to maintain differences in leaf shape (M. Ramsay, pers. comm.).

Because vegetative reproduction and autogamy are common in *Liparis loeselii*, genetic drift probably affects diversity within populations. The severe fragmentation of its habitat is expected to contribute to genetic impoverishment as well as differentiation between populations. Also, because in the area investigated here, the English Channel region, *L. loeselii* occurs in two readily distinguishable habitats (dune slacks and alkaline fens), genetic differentiation may be expected between the two habitats.

A good knowledge of the genetic diversity and structure is a necessary prerequisite for the conservation of a species as it reflects the status and survival potential of populations (Lande 1988). This requires the use of molecular markers, which can also reveal the dispersal capacity of a species (Ouborg et al. 1999) and infraspecific structure (Soltis & Gitzendanner 1999). Amplified fragment length polymorphisms (AFLPs) are recently developed multilocus markers (Vos et al. 1995) that have already shown their usefulness in population genetics of rare or endangered species (Juan et al. 2004; Maunder et al. 2001; Travis et al. 1996). Although *L. loeselii* probably has a relatively large genome (the congeneric *L. rostrata* has a C-value of 9.7 pg, Bennett & Leitch 2003) that could affect the quality of AFLP results(Fay et al. 2005), they have been previously used successfully in *L. loeselii* (Qamaruz-Zaman 2000).

In the present study, we investigated genetic variation within *L. loeselii* with a sampling covering Britain and northwestern France and including both varieties and habitat types. We tested for genetic differentiation between varieties and habitats and a possible match between them. We searched for putative geographical structure of genetic diversity and evaluated the genetic differentiation between populations revealing the eventual capacity of the species to disperse in a fragmented habitat.

MATERIAL AND METHODS

The plant species

Liparis loeselii is a perennial plant that can live for up to eight years but generally less (Jones 1998; Wheeler et al. 1998). Individual plants consist of a pseudobulb, one or generally two leaves in adult plant and a central inflorescence with up to twenty green scentless flowers. Vegetative reproduction is achieved though the development of one or two small pseudobulbs from an adult one. The species is generally considered to be self-pollinated and rain drops may facilitate self fertilization (Catling 1980). As for most orchids, *Liparis* seeds are small (less than one millimetre long) and produced in great number, probably several thousand per fruit (Arditti & Ghani 2000). *Liparis loeselii* is generally associated with early succession stages of vegetation in dune slacks and fens. Population demography can be irregular, but dormancy is believed to be negligible in this species (Jones 1998, Wheeler et al. 1998).

Sampling

Our sampling covers 12 populations (see figure 1): the three remaining English populations (Catfield, Sutton and Upton), two southern Welsh populations (Whiteford and Kenfig), four populations from northern France (Merlimont, Stella, Villiers and Wimereux), two populations from Brittany (Guidel and Guisseny) and one population from Michigan (North America), used as an outgroup. In the northern French population of Stella, two subpopulations were distinguished corresponding to two non-adjacent dune slacks.

Our sampling includes dune slack and fen populations and regions where plants are classically referred to as var. *loeselii* (England) and var. *ovata* (Wales). A total of 155 individuals were included, with an average of 12 individuals per population, but this varies from one to 31, as small populations could not be extensively sampled. No more than half a leaf was collected from two-leaved adult plants. Observations on greenhouse grown plants showed that this had no apparent deleterious effect on the plants; sampling was less drastic than snail or rabbit predation frequently observed in the field. Leaves were dried in silica gel (Chase & Hills 1991).

Molecular Procedure

DNA was extracted using a 2×CTAB protocol or with the Nucleospin® 96 Plant kit (Macherey-Nagel) and a Microlab Star robot (Hamilton). Genomic DNA was quantified on agarose gel using λ DNA dilution, and 300 ng of DNA was used for the AFLP reaction using the standard protocol recommended by LI-COR. Two pairs of selective primers were used as they showed appropriate levels of polymorphism (i.e. allowing detection of within and between population variations) in an earlier study of *L. loeselii* (Qamaruz-Zaman 2000): E-AGC and M-CTG, E-ACT and M-CAA. Fragments were separated on a 41 cm denaturing acrylamide gel in a LI-COR sequencer. Fragment lengths were measured using the software AFLP-SCAN; only bands between 50 and 500 bp were scored as present (1) or absent (0). Electrophoregrams were read twice to guarantee the accuracy of the results.

Data analysis

A principal coordinate analysis (PCOA) was used to look for groupings of the different genotypes observed using the R package for Multivariate Analysis version 4.0

(Casgrain & Legendre 1999) using Jaccard's coefficient (Jaccard 1908). To reconstruct relationships between populations, genetic distances between each pair of populations were calculated using AFLP-SURV (Vekemans 2002). Allele frequencies were calculated with the assumption that the species was fully autogamous as this has generally been observed (Catling 1980). A neighbor-joining tree was then built using PHYLIP (Felsenstein 1993) and NJPLOT (Perrière & Gouy 1996). Genetic diversity within populations were measured using the Nei and Shannon diversity indices (based on allele frequencies), as calculated with POPGENE 1.31 (Yeh et al. 1999). To take into account the sampling effects we used a rarefaction procedure to compare the populations that were unevenly sampled (Kalinowski 2004). For populations for which more than 10 individuals were analysed, ten samples were randomly chosen and the numbers of genotypes and of polymorphic loci were measured and averaged for one hundred replicates.

Genetic structure was tested by AMOVA (Excoffier et al. 1992) with the software ARLEQUIN 2.0 (Schneider et al. 2000). We tested for genetic differentiation between the four areas sampled (England, Wales, northern France and Brittany), between Britain and France and between dune slack and fen populations.

Overall genetic differentiation (F_{ST}) was estimated using AFLP-SURV with 1000 permutations. Genetic isolation through distance was tested by the mean of a Mantel test using the software PASSAGE 1.0 (Rosenberg 2002).

The Upton population, for which only a single individual was analysed, was excluded from all analyses except AMOVA.

RESULTS

Amplifications for both pairs of primers tested were successful in 155 individuals. With the primer pair E-AGC M-CTG and E-ACT M-CAA an average of 37 and 39 bands per individual was revealed. In total 108 bands were scored. Bands that were either present or absent in single accessions were excluded as they were likely to be artefactual. With both primer pairs, a total of 44 unambiguous polymorphic bands were selected, of which four were polymorphic above the 5% level. When combining these 44 bands, we could distinguish 51 genotypes.

The numbers of genotypes observed, number of polymorphic loci, Nei's diversity index and Shannon's diversity index are given for each population in Table 1. Often one genotype was dominant within the populations, but all the populations for which at least four individuals were sampled showed variability.

Genetic diversity was particularly high for the American population from Michigan and the northern French population of Merlimont. In all the other populations the most frequent genotype was found in half or more of the individuals. The number of genotypes observed per population and genotypic Shannon index were significantly correlated with the number of accessions analysed (Spearman's rank test, $n=12$, $p<0.01$ and $p<0.05$). The correlation between the number of polymorphic loci and the number of accessions was also close to significance ($0.05<p<0,1$). However the number of genotypes and the number of polymorphic loci were no more correlated to sample size after rarefaction ($n=8$; $p>0;1$). Neither Nei's nor Shannon's diversity index showed significant correlation with sampling effort ($p>0.1$) or with estimated population size ($n=9$, $p>0.1$).

The relationships between the 51 genotypes observed according to the PCOA are shown in Figure 2. The American accessions were clearly distinguishable from the European individuals. Three genotypes were present on both sides of the English Channel. One genotype was found in both fen and dune slack populations, but otherwise genotypes from these two habitats tended to cluster in two groups, although there are exceptions.

The neighbor-joining tree showing the relationships between the populations (Fig. 3) indicates that the dune slack populations form a cluster, to which the fen populations form successive branches, but no geographical structure was apparent. For instance, although the fen population of Villiers is only ca. 10 km away from the dune slack population of Merlimont, it is genetically closer to the fen populations in England. AMOVA indicates a significant genetic differentiation between dune slack and fen populations ($p=0.023$). With AMOVA we did not find any significant differentiation between Britain and France ($p=0.23$) or between the four regions sampled (northern France, Brittany, Wales and England; $p=0.44$). When both fen and dune slack populations were considered separately, the differentiation between the regions was again not significant ($p=0.52$ and $p=0.91$).

There was a significant genetic differentiation between populations overall ($F_{ST}=0.382$, $p<0.01$). When considering the two habitat types separately genetic differentiation was high between fen populations ($F_{ST}=0.370$, $p<0.01$) but non-significant between dune slack populations ($F_{ST}=0.146$; $p>0.05$).

The Mantel test did not reveal any evidence of genetic isolation through distance ($p=0.073$). When the structure of the sampling into two habitat types was taken into account (partial Mantel test), the correlation was even weaker ($p=0.25$).

DISCUSSION

Genetic diversity within *L. loeselii*

The genetic variability revealed by our markers is relatively limited as only 40 percent of the bands scored were polymorphic and only four bands were polymorphic above the 5% level. Trials of other primer pairs showed fewer bands and less variability than the two presented here (data not shown). However the primer pairs used were sufficient to distinguish clearly all the individuals from the population of Michigan. Therefore the lack of variability observed may be explained by a limited genetic diversity of the European populations, which is not unexpected considering the biology of the species. Although the limited variability of the markers may affect the power of the analyses, it was sufficient to reveal some significant genetic structure (i.e. between fen and dune slack populations). However the variability was not sufficient to reveal any significant biogegraphic structure.

Although *L. loeselii* is known to be autogamous, capable of vegetative reproduction and in decline, we found a higher level of genetic variation than expected. More than one genotype was found in all populations that had been appropriately sampled. This observation is consistent with the non-negligible genetic diversity observed in other clonal plants (Ellstrand & Roose 1987). Generally one genotype was dominant in most populations, with a frequency often exceeding 50%. This could be interpreted as evidence of extensive clonality in *Liparis* populations, although the genetic variability observed could be explained by founding by multiple individuals or gene flow via seeds.

At equal population size, fen populations may be less variable than dune slack populations, but our sampling did not allow to test this. Before rarefaction, the numbers of genotypes and polymorphic loci were clearly correlated with sampling effort and therefore these raw figures should not be used to compare the diversity between populations. After rarefaction the difference between unevenly sampled populations was greatly diminished and only the Merlimont population remained clearly more polymorphic than the others.

Although poorly sampled, the only North American population sampled here displays a comparatively high level of variability. The distribution of *L. loeselii* does not overlap with any other species of the genus containing over 600 species (World Checklist of Monocots 2004) mostly found in the Tropics. The nearest species in distribution is probably *L. lilifolia*, found in North America in more southern locations than *L. loeselii* (Luer 1975). A close relationship between the two species is further supported by molecular data (Cameron 2005). Therefore a North American origin of *L. loeselii* seems reasonable and could explain the higher genetic diversity found there.

Genetic differentiation between habitats

Our markers reveal that the populations cluster according to habitat type rather than geographical location, with dune slack populations from Northern France clustering with dune slack populations from Britain rather than with the northern France fen populations. The separation is not perfect at the individual level (Fig. 2) as one genotype (A) was found in both habitats (and in Britain and France: Merlimont, Villiers, Catfield and Kenfig). Therefore the separation between these two groups is probably recent or

some gene flow may still be occurring between the two forms. The genetic differentiation between these two putative ecotypes could potentially be linked with adaptation to dune slack and fen environments and the concomitant differences in nutrients, light and humidity or association with different mycorrhizal fungi (Bidartondo et al. 2004, Taylor & Bruns 1999). Distinct races only found in dune slacks have also been described in other European orchid genera, e.g. *Dactylorhiza* (Pedersen 2001) or *Epipactis* (Squirrell et al. 2002), but so far genetic studies do not support their distinctiveness from commoner varieties or species (Hedrén et al. 2001, Squirrell et al. 2002, respectively).

The differentiation between dune slack and fen populations matches in some way the distinction between var. *ovata* and var. *loeselii*. *Liparis loeselii* var. *ovata* was originally described from Wales and then recorded from Brittany in the 1990s. Morphometric studies based on ratios of leaf width and length (Hendoux et al. 2001) indicate that all dune slack populations from Northern France and Northern Brittany have broad leaves and could be unambiguously attributed to var. *ovata*. The fen population of Villiers in Northern France, and some continental French fen populations have longer leaves and should be placed in var. *loeselii*, along with the English fen populations. Thus leaf shape, similarly to genetics, tends to separate fen and dune forms. All the *Liparis* populations in Brittany are coastal and occur in dunes. The two populations analysed here, Guisseny and Guidel (from southern and northern Britanny, respectively) both cluster genetically with other dune populations. However, morphometric data indicate that Guisseny plants have narrower leaves typical of var. *loeselii* and represent an exception among the sampled populations. However, Hendoux et al.(2001) expressed

some concerns regarding the accuracy and the potential bias in their data due to the observer as leaf length cannot be clearly defined in *L. loeselii*.

More precise data are needed to clarify the status of the var. *ovata* and var. *loeselii*, for example with the application of Fourier's ellipses (Jensen et al. 2002). Furthermore, studies under controlled conditions would be necessary to rule out direct effects of biotic and abiotic factors in the different habitats on the morphology of the plants.

Genetic structure within habitats

Beyond the split between habitats, the genetic differentiation between populations is limited and not significant for dune populations, and the limited variability of the markers used may explain this lack of the structure. The F_{ST} value observed for this habitat (0.146) is below any other value obtained with similar markers (AFLPs or RAPDs) in orchids (Forrest et al. 2004). Similarly no obvious geographical structure was observed (Fig. 3) and we did not find any evidence for isolation through distance. Although this lack of differentiation may be linked with the limited variability that we observed, this may have a real biological explanation. As for most orchids, the seed of *L. loeselii* are minute (Arditti & Ghani 2000). Because this species is often found in coastal environments, dispersal by wind is even more likely. The populations sampled in this study could also have a recent and common origin, rendering any structure invisible. Genetic differentiation was greater and more significant in the fen populations; this may be due to limited exposure to winds and/or higher habitat fragmentation, although our sampling for this habitat is more limited, thus making any conclusions more speculative.

Implications for conservation and perspectives.

Our study reveals a genetic distinction between the dune slack and the fen forms of *L. loeselii*. These two forms should thus be considered separately in any conservation plan in Britain and nortwestern France at least. Further morphological studies are needed to clarify the delimitation of var. *ovata* and var. *loeselii*, and whether or not they match the two ecological forms. Further sampling is also desirable, for instance on the coast of the North Sea and the Baltic Sea, the Alps region and North America. Our results emphasize the need for preserving the fenland form, which has undergone the most dramatic decline and for which population sizes are generally small. As for the dune slack form, the lack of genetic differentiation between populations studied here suggests a possible exchangeability of individuals within this area, which could facilitate reintroduction efforts, if required.

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Figure 1: Map of southern Britain and north-western France showing the location of the populations sampled. Rounds (●) indicate dune slack populations and squares (□) fen populations.

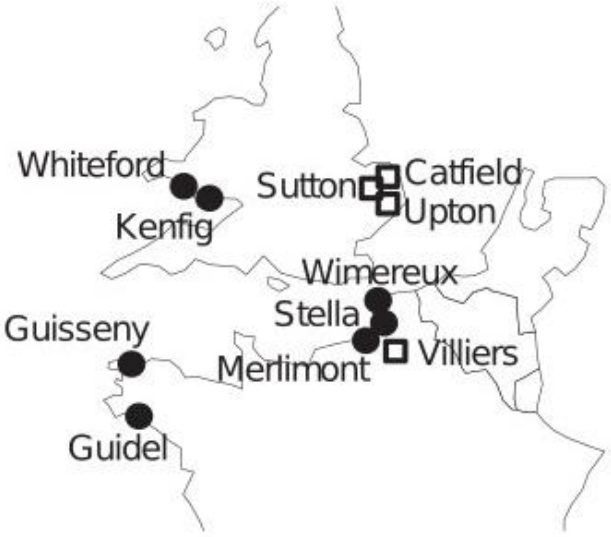


Figure 2. Principal Component Analysis based on AFLP data separating the 51 genotypes obtained. AG is the most common genotype in dune slack populations (45 % of the individuals). The genotypes A and H are the most common in fen populations (36% and 27 % of the individuals). The genotype A is the only one found in both fen and dune slack populations. Axis 1 and Axis 2 represent each 23% and 12% of the variation.

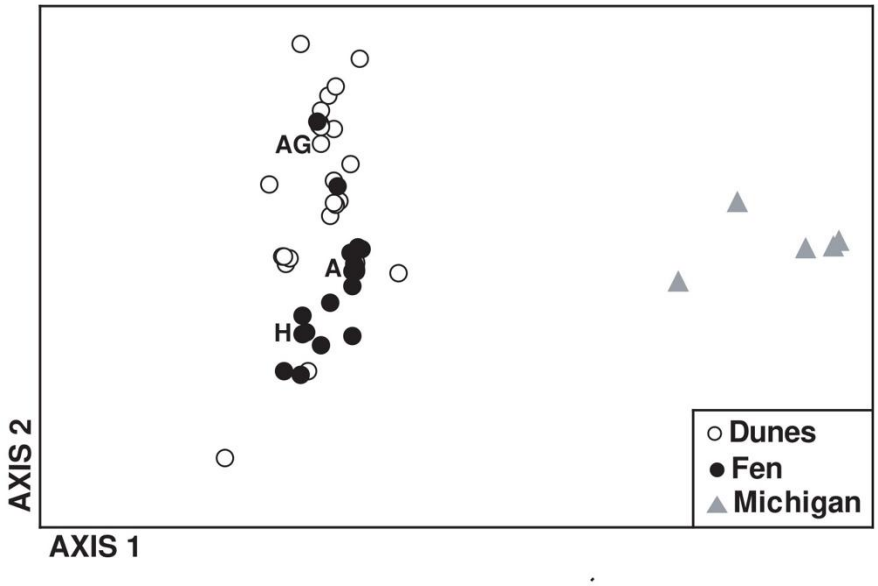


Figure 3. Neighbor joining tree depicting relationships between the populations studied, based on Nei's genetic distance.

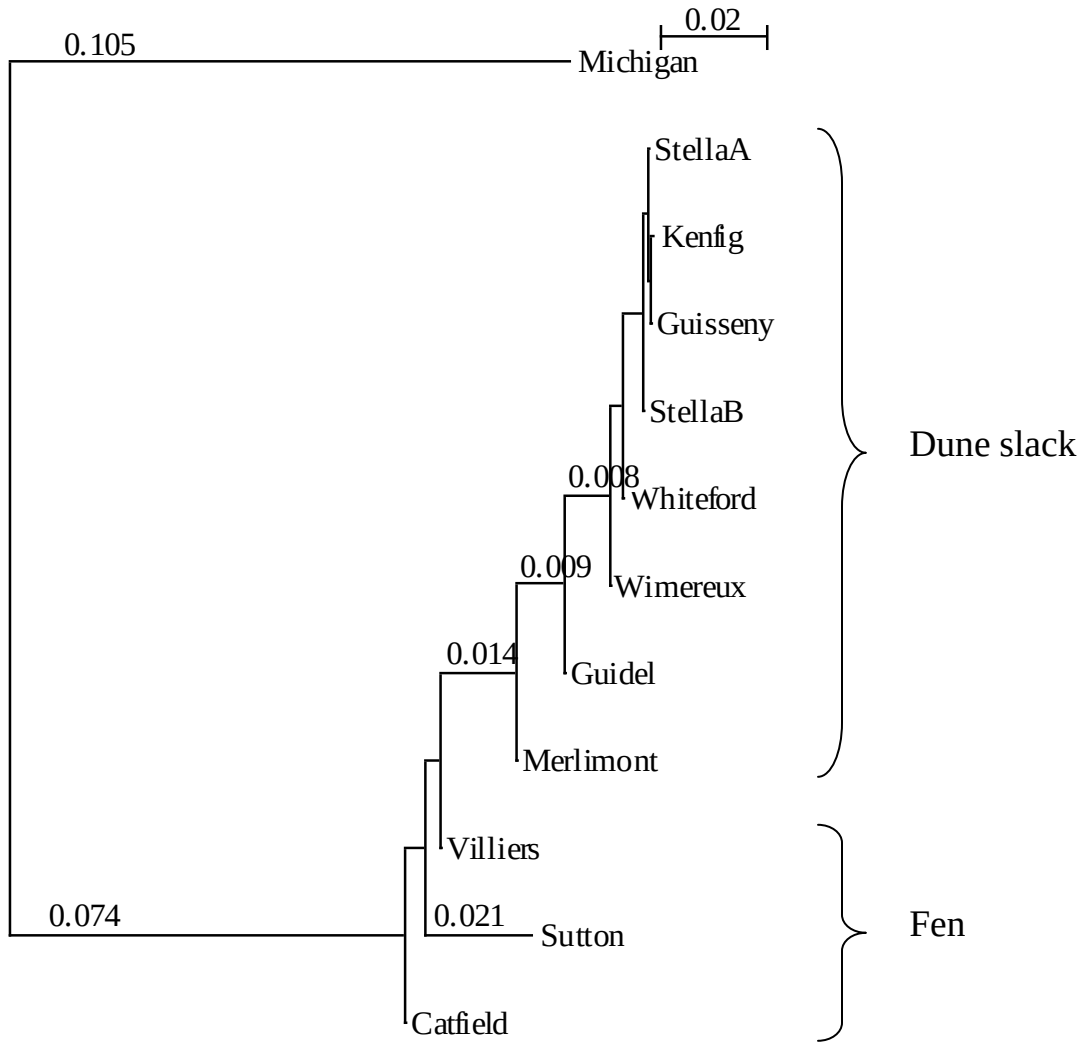


Table 1: Results of the genetic analysis for each population. For populations for which more than ten accessions had been analysed the number of genotypes and the number of polymorphic loci were recalculated after rarefaction (random selection of ten individuals with one hundred replicates).

Population	Estimated size	Habitat	Number of accessions	Number of genotypes	Frequency of main genotype	Number of polymorphic loci	Number of genotypes after rarefaction	Number of polymorphic loci after rarefaction	Nei's index (allelic)	Shannon's index (allelic)	Shannon's index (genotypic)
Michigan	unknown	opening in forest	5	5	1	16	-	-	0,146	0,213	2,32
Guidel	unknown	dune slack	2	2	1	3	-	-	0,034	0,047	1
Guisseny	unknown	dune slack	3	1	3	0	-	-	0	0	0
Merlimont	>10000 (2004)	dune slack	31	15	6	18	7.3	9.4	0,063	0,109	3,51
Stella A	272 (2002)	dune slack	12	4	9	10	3.4	8.0	0,038	0,069	1,21
Stella B	~ 60 (2004)	dune slack	15	4	11	4	3.2	2.8	0,046	0,084	1,24
Wimereux	~ 144 (2004)	dune slack	14	6	7	6	4.9	5.4	0,042	0,064	2,06
Kenfig	22000 (1992)	dune slack	10	5	6	8	5	8	0,039	0,067	1,77
Whiteford	90 (1992)	dune slack	4	2	3	2	-	-	0,017	0,026	0,81
Villiers	94 (2003)	fen	18	6	11	6	4.2	4.0	0,02	0,038	1,79
Catfield	147 (1998)	fen	17	6	10	7	4.3	4.6	0,024	0,045	2,23
Sutton	24 (1998)	fen	23	7	14	9	4.2	4.6	0,023	0,044	1,86
Upton	2 (1999)	fen	1	1	1	0	-	-	0	0	0