

Genetic population structure of the vulnerable bog fritillary butterfly

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Populations of the bog fritillary butterfly *Proclossiana eunomia* (Lepidoptera, Nymphalidae) occur in patchy habitat in central and western Europe. *P. eunomia* is a vulnerable species in the Belgian Ardennes and the number of occupied sites has significantly decreased in this region since the 1960s. RAPD (random amplified polymorphic DNA) markers were used to study the consequences of habitat loss and fragmentation on the genetic population structure of this species. Gene diversity was lower in populations with smaller population sizes. Genetic subdivision was high ($F_{st} = 0.0887$) considering the small spatial scale of this study (150 km²). The most geographically isolated population was also the most genetically differentiated one. The genetic population structure and genetic differentiation detected in this study were explained by (1) differences in altitude of the sampled locations and, (2) lower dispersal propensity and dispersal rate in fragmented landscapes versus continuous landscapes. Results from the RAPD analyses were compared with a previous allozyme based study on the same populations. The results of this study suggest that increased fragmentation has led to a greater genetic differentiation between remaining *P. eunomia* populations.

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Habitat destruction, the leading cause of species extinction (PIMM and RAVEN 2000), is becoming increasingly common as human population growth continues. Habitat loss poses the greatest threat to the long term survival of species on earth and has three major components: straightforward destruction of habitat, increasing fragmentation and deterioration of habitat quality. Habitat fragmentation, i.e. the reduction of continuous habitat into several smaller spatially isolated remnants, decreases species richness, increases edge effects, decreases density and abundance of species, alters interspecific interactions and ecological processes, and decreases connectivity (DEBINSKI and HOLT 2000). Following the theory of island biogeography, species richness in habitat fragments is expected to be a function of island size and degree of isolation. Smaller, more isolated fragments are expected to retain fewer species than larger, less isolated patches. Comparable to the effects of area on species richness, one might expect to observe area effects on genetic diversity within species; smaller fragments having a lower genetic diversity compared to larger fragments. Genetic diversity is essential in conservation biology for at least two reasons. First of all, the fundamental theorem of natural selection (FISHER 1930) tells us that “the rate of increase in fitness of any organism at any time is equal to its genetic variance in fitness at that time”, i.e. the rate of evolutionary change in a population is proportional to the amount of genetic diversity available. Consequently, loss of genetic diversity reduces future evolutionary options (FRANKEL

et al. 1995; LANDE 1995). Secondly, decreasing genetic diversity (partly measured by heterozygosity, or the state of having one or more pairs of non-identical alleles) increases the extinction risk of populations due to a decline in fitness of individuals (MEFFE and CARROLL 1994; OOSTERMEIJER et al. 1995; SACCHERI et al. 1998; SHIKANO and TANIGUCHI 2002). That genetic diversity is inversely proportional to isolation and that it is directly correlated with population size has been proven empirically numerous times. A review by FRANKHAM (1996), including 23 allozyme studies, showed that in 22 of these studies genetic variation within a species was correlated with population size. A study by SCHMITT and SEITZ (2002) on the butterfly *Polyommatus coridon*, detected a tendency of genetic erosion in smaller populations and found that gene flow was much higher in regions with high habitat densities than in areas with very fragmented habitats, i.e. populations where isolation was greatest had significantly reduced amounts of gene flow which is thought to be a trigger for genetic erosion in isolated populations. Another butterfly study, using microsatellite markers, found that larger populations support significantly higher levels of genetic diversity than do small populations, but did not detect an influence of isolation on genetic diversity (HARPER et al. 2003). The authors believe that this was largely due to the closed population structure, i.e. very little gene flow occurring even between closely situated habitats.

In this study, we use RAPDs (random amplified polymorphic DNA) to analyse the genetic diversity and population structure of a butterfly species in which dispersal and demography are exceptionally well known (PETIT et al. 2001; SHTICKZELLE et al. 2002; SHTICKZELLE and BAGUETTE 2003, 2004). In the Belgian Ardennes, the bog fritillary butterfly, *Proclossiana eunomia* Esper, is present in several clusters of suitable habitat patches. The current distribution of favourable habitats for this vulnerable species is influenced by two factors. First, since soil conditions required for the species' presence are particular (seasonal alternation of flooding and dry periods), its habitat (wet hay meadows) is naturally patchy along river valleys or in peat bogs on uplands. Secondly, important changes in agricultural practices, i.e. the drainage of meadows and afforestation of peat bogs by Norway spruce *Picea abies* Karst, which have taken place since the 1950s, have augmented the degree of fragmentation, thereby increasing the isolation of remnant populations. The population structure resulting from this particular occupancy pattern has been investigated previously in the bog fritillary butterfly using both capture—mark—recapture and allozyme data. From these analyses, it was concluded that topographical barriers shaped dispersal and that stepping stone type movements allow effective gene flow between local populations (NÈVE et al. 1996). However, these allozyme based analyses were limited by the very low level of genetic variability detected in allozymes: only 3 out of the 10 polymorphic loci known to exist in Europe were polymorphic in the study area (NÈVE et al. 1996, 2000). Since RAPDs generate numerous polymorphic loci they may provide a more reliable picture of the population structure compared to allozymes, especially when loci of the latter marker are rare and display little polymorphism (VANDEWOESTIJNE and BAGUETTE 2002). Additionally, several of the populations that were thought to serve as stepping stones between populations sampled in the previous study have since gone extinct (Fig. 1). Therefore, a decade after the study by NÈVE et al. (1996) we re-analyse the genetic structure of these populations using a more powerful genetic marker. We have attempted to explain variation in the amount of genetic diversity and the degree of genetic population differentiation with differences in habitat size and isolation. The principle aim of the work reported here was to estimate the current connectivity between persisting bog fritillary populations. This will help identify factors that are essential for the long – term survival of this species in the Belgian Ardennes.

MATERIAL AND METHODS

Species and study area

Proclossiana eunomia is a specialist species occurring in unfertilised wet hay meadows along rivers or in peat bogs, where the unique larval and adult host plant of this butterfly, *Polygonum bistorta*, is found. In Europe, the distribution of the bog fritillary is typical of a boreo-montane species with a central, continuous part in Fennoscandia, and marginal parts widely scattered on uplands in western Europe and mountainous parts of central and southern Europe. In the southern part of Belgium, this butterfly is widely but patchily distributed. The distribution in Belgium has not changed since the 1960s in terms of occupied 10×10 km grid squares, but the number of occupied sites has significantly decreased (BAGUETTE et al. 1992).

The study area is found in the southern part of Belgium. Four populations of *P. eunomia* were sampled in 2001 and 2002 (Fig. 1). The Prés de la Lienne is situated at 350 m altitude, whereas the remaining three populations are found on the Taille upland, where elevations reach 450–550 m. The Prés de la Lienne is a patchy population composed of nine patches found in a highly fragmented landscape, i.e. the patches are separated from each other by artificial matrix (spruce plantations and fertilised pastures, BAGUETTE and NÈVE 1994). The Pisserotte, Sommerain and Bovigny populations are found in more continuous landscapes where patches are separated from each other by natural matrix, i.e. peat bogs. These four particular populations were chosen because they were previously analysed by NÈVE et al. (1996) in the beginning 1990s. The authors of this study identified three genetic units that are identified by dotted contours in Fig. 1. The population of Bihain was not sampled in the present study since it has gone extinct since the work by NÈVE et al. (1996). Other populations, situated between the Prés de la Lienne, Pisserotte, Sommerain and Bovigny, have also become extinct over the past decade (Fig. 1).

Genetic analyses

Random amplified polymorphic DNA (RAPD) markers (WILLIAMS et al. 1990) were used to describe the population structure of *P. eunomia* in this study. DNA was extracted using the same phenol-chloroform protocol as the one described in VANDEWOESTIJNE and BAGUETTE (2002). DNA was quantified using a VersaFluor fluorometer (Bio-Rad) and samples were diluted to obtain identical concentrations before amplification reactions. Reactions were standardised and care was taken to create identical experimental

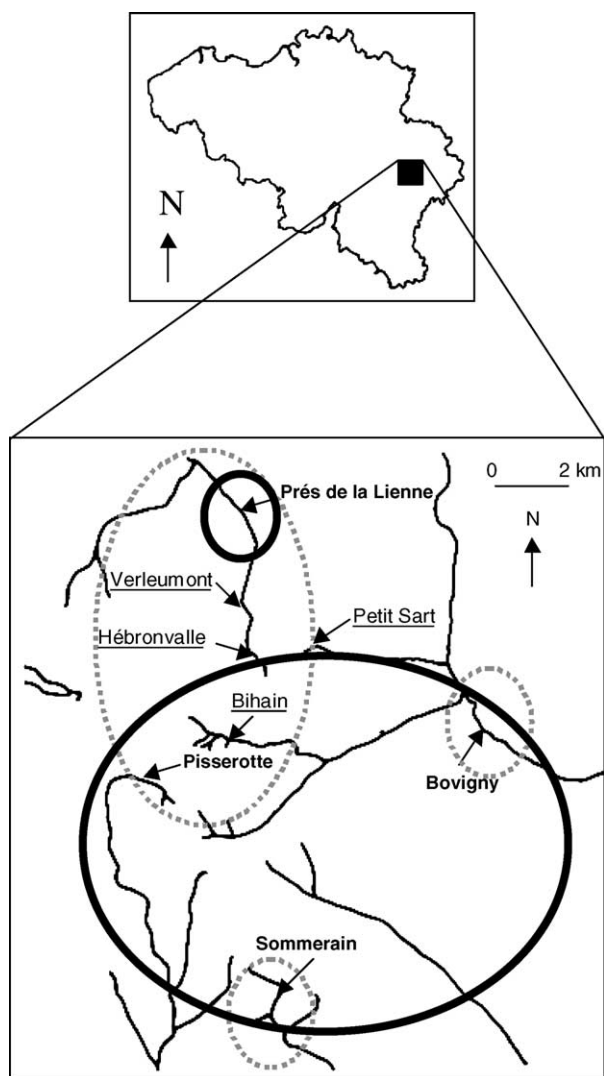


Fig. 1. Localisation of the study site within Belgium. Map of the study sites with river system. Study sites sampled for the current genetic study are indicated in bold, while sites that have gone extinct during the 1990s are underlined. The genetic units identified based on an allozyme study (NÈVE et al. 1996) are indicated by a discontinuous line, while those identified by the current study, using RAPDs, are identified by a continuous line. The map has been reproduced from NÈVE et al. 1996.

conditions for all samples (PCR reactions were run on the same thermal cycler, identical products and concentrations were used for each run, etc.). Negative controls were used continuously to check for contamination and amplifying the same samples on different days tested reproducibility. For every 25 μ l reaction, 20 ng of DNA, 0.4 μ M of primer, 3 mM of $MgCl_2$, 200 μ M of dNTP and 0.5 U of AmpliTaq polymerase (Perkin-Elmer) were included. The following parameters were used for the amplifications: 4 min denaturation at 94°C, followed by 35 cycles of 1 min

at 94°C, annealing at 36°C for 1 min, and elongation at 72°C for 2 min. An additional 5 min period for elongation at 72°C followed this cycle. PCR products were separated on 1.6% agarose gels (TBE buffer) that were run for 225 min at 100V. Several DNA size standards were run on every gel to aid in identifying the target bands. Ethidium bromide staining was used to visualize band patterns that were recorded on disc using GelDoc (Bio-Rad). Five RAPD primers (OPB12, 5'CCTTGA CGCA3'; OPB17, 5'AGGGAACGAG3'; UBC23, 5'CCCGCCTTCC3'; A18, 5'AGGTGACCGT3'; and BAM, 5'TAGGATCCGC3') out of 50 were selected based on the polymorphism and reproducibility of the bands that they generated. Only primers with 100% reproducible bands were considered.

Statistical analyses

Allele frequencies were calculated from null homozygote frequencies assuming panmixia and corrected for dominance according to LYNCH and MILLIGAN (1994) using TFPGA 1.3 (MILLER 1997). Using these allele frequencies, gene diversity, expected heterozygosity (calculated from the null allele frequency and based on Hardy-Weinberg equilibrium), the percentage of polymorphic loci (95% criterion) and genetic structuring (F_{st}) were calculated using the same program. We also used genotype data directly to obtain the average gene diversity over all loci (the mean number of differences between all pairs of genotypes divided by the number of loci) and derive F_{st} from the variance components (Amova) using Arlequin (SCHNEIDER et al. 2000). Hickory v0.7 (HOLSINGER et al. 2002) was used to estimate the partitioning of genetic variation (F_{st} and F_{is}), using a Bayesian hierarchical model, without assuming that genotypes are found in Hardy-Weinberg proportions and without treating RAPD phenotypes as haplotypes. These analyses were run five times to ensure the relative merits of the hypotheses. An exact test of population differentiation was carried out with a Markov Chain Monte Carlo approach for an approximation of the exact probability of the observed differences in allele frequencies using TFPGA 1.3 (MILLER 1997). Additionally, Fisher's combined probability test (FISHER 1954; SOKAL and ROHLF 1995) was employed as a global test over loci to determine the overall significance. A Mantel test was used to assess the association between NEI's unbiased (1978) genetic distance matrix and the euclidian distance matrix using TFPGA 1.3. A Mantel test (Mantel v2.0, LIEDLOFF 1999) was also used to test the association between F_{st} and total pairwise distances (altitudinal and Euclidian). To obtain this combined value we used

values for Euclidian distances in kilometres and values for altitudinal differences in decimetres. This was done so that both types of distances were of the same order of magnitude and to give more weight to altitudinal differences which also represent greater ecological and climatic variations. The combined distance was calculated as follows:

$$\text{Combined altitudinal/Euclidian distance} = \sqrt{[(\text{pairwise altitudinal difference})^2 + (\text{pairwise Euclidian distance})^2]}.$$

We produced an UPGMA (i.e. unweighted pair-group method using an arithmetic average) tree, based on Nei's unbiased (1978) genetic distance corrected for small sample size, to further examine the relationship between geographic and genetic distances (TFPGA 1.3). To generate increased confidence in the tree constructed by the original data, bootstrapping was performed with 1000 permutations.

RESULTS

Primers OPB12, OPB17, UBC23, A18 and BAM produced 5, 5, 5, 4 and 5 reproducible and polymorphic bands respectively (a RAPD pattern on agarose gel is shown in Fig. 2). For pooled data, all of these loci exhibited overall frequencies of band presence of less than 1–3/N (where N is the number of individuals sampled), reducing potential bias in the allele frequency estimates stemming from low counts

of band absence phenotype (LYNCH and MILLIGAN 1994). For individual population data, results were very similar, if not identical, when loci with a frequency of less than 1–3/N were excluded from the analyses. Therefore, we only report results for those analyses including all loci. The average unbiased heterozygosity, often used as an indicator of the genetic variability, for all populations was 0.3848 and varied between 0.3482 (Prés de la Lienne) and 0.371 (Pisserotte) (Table 1). The average gene diversity over all loci was 0.3720 ± 0.1952 . The percentage of polymorphic loci (using the 95% criterion) varied from 79.83 to 91.67% with the Prés de la Lienne having the lowest amount of polymorphism and Pisserotte the highest (Table 1).

F_{st} values of 0.0887 ± 0.0225 [0.0465, 0.1340], of 0.08602 and of 0.0845 ± 0.0216 [0.0477, 0.1318], based on unbiased allele frequency, on genotype data and on the Bayesian method respectively, were high (considering the spatial scale of this study, i.e. 150 km²) but very similar to one another. Pairwise comparisons revealed significant genetic differentiation for those pairs of populations that included the Prés de la Lienne (Table 2). F_{st} values ranged from 0.0512 ± 0.0182 to 0.1253 ± 0.0533 (Table 2), with the higher values, i.e. greater genetic differentiation, including the Prés de la Lienne. F_{is} values, estimated using the Bayesian method were 0.6999 ± 0.1685 [0.3405, 0.9733], indicating a high degree of inbreeding. The Bayes factor analysis gave similar results for the five runs.

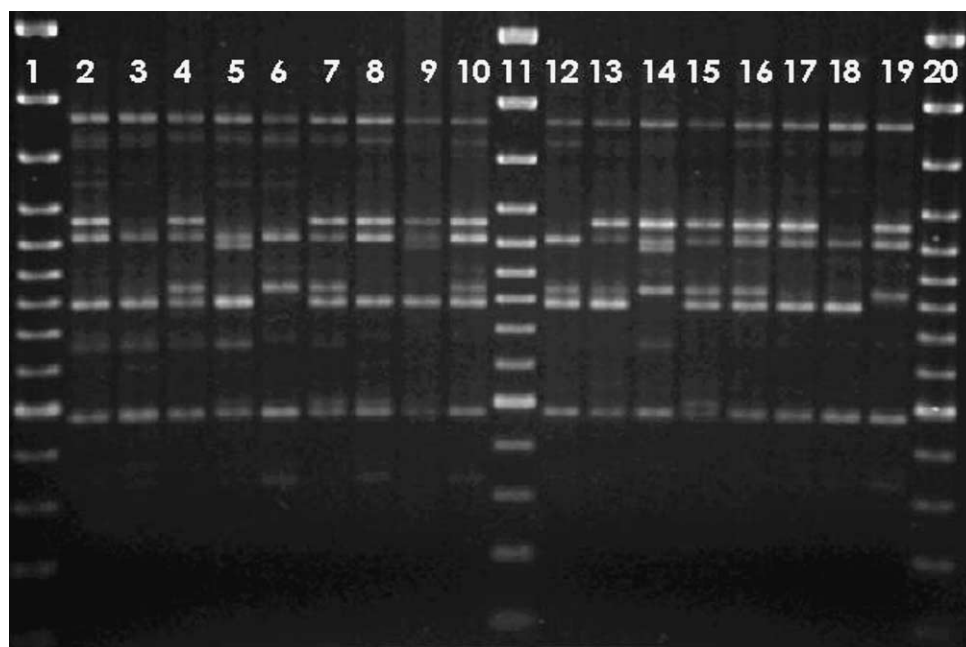


Fig. 2. An agarose gel with RAPD PCR products revealed by ethidium bromide staining. DNA size standards are found in lanes 1, 11 and 20.

Table 1. Sample size and genetic diversity indices for all sampled populations separately and combined. Average unbiased heterozygosity calculated from the null homozygote frequencies and percentage of polymorphic loci (95% criterion).

	Sample size (no. of individuals)	Average unbiased heterozygosity	Percentage of polymorphic loci
Bovigny	13	0.3584 (0.000–0.5212)	83.33
Sommerain	14	0.3485 (0.000–0.5176)	83.33
Pisserotte	19	0.3713 (0.000–0.5125)	91.67
Prés de la Lienne	21	0.3481 (0.000–0.5125)	79.83
All populations	67	0.3848	100.00

These analyses may suggest that inbreeding is occurring in the sampled populations.

No significant correlation was found between pairwise genetic differentiation and geographic (Euclidian) distances ($P=0.1680$, $r=0.5215$). However, combined altitudinal and Euclidian pairwise distances resulted in a significant correlation with pairwise F_{st} values ($P<0.05$, $r=0.7519$). The UPGMA clustering isolated the population of Prés de la Lienne from the populations of Sommerain, Pisserotte and Bovigny with a 70.7% bootstrap value (Fig. 3).

DISCUSSION

Demographic studies on *P. eunomia* have shown that the population size of Pisserotte is about ten times larger than that of Prés de la Lienne (SCHTICKZELLE 2003). Theory predicts that smaller populations will be characterised by a lower amount of genetic diversity than larger populations. This was confirmed in this study. Indeed, the Pisserotte population was characterised by a higher genetic diversity compared to the Prés de la Lienne population (for both the average unbiased heterozygosity and the percentage of polymorphic loci, Table 1).

The pairwise comparisons, the F_{st} estimates and the UPGMA dendrogram reveal that the Prés de la Lienne population forms a genetic unit different from the one including Sommerain, Bovigny and Pisserotte. These populations, which are genetically not significantly differentiated from each other, form the other unit in this study. Two explanations can be found for this observation. First of all, the Prés de la Lienne population is situated at least 150 meters lower

in altitude than the other sampled populations, increasing the distance a butterfly needs to fly to reach neighbouring populations. All of the other studied populations are found at similar altitudes. Altitude definitely plays an important role in the genetic differentiation between populations, as indicated by the significant Mantel test between pairwise F_{st} values and combined Euclidian/altitudinal distances. Another explanation, which could interact with the first one, are the behavioural differences observed between Prés de la Lienne butterflies and Pisserotte butterflies (SCHTICKZELLE and BAGUETTE 2003). This study reported on a difference in the behaviour of butterflies at habitat patch boundaries. Indeed, butterflies tended to come back into a patch at a significantly higher rate in the fragmented system (Prés de la Lienne) versus the more continuous system (Pisserotte). Mortality during dispersal was also higher in the fragmented system versus the more continuous one as estimated using the virtual migration (VM) model (HANSKI et al. 2000). Altogether, these observations suggest that there exists a 'behavioural' barrier to migration in Prés de la Lienne. This, and the difference of altitude, may have led to the genetic isolation and subsequent differentiation of this population from the others. The absence of significant genetic differentiation between Sommerain, Bovigny and Pisserotte can be explained by the fact that they are all situated at similar altitudes and characterised by a more or less continuous habitat, and therefore a higher and more successful dispersal rate than the Prés de la Lienne population.

Although only inferences can be made concerning the magnitude of within-population inbreeding when

Table 2. F_{st} values between sampled populations above and significance of pairwise population differentiation below.

	Bovigny	Sommerain	Pisserotte	Prés de la Lienne
Bovigny	–	0.0580±0.0269	0.0608±0.0270	0.1253±0.0533
Sommerain	NS	–	0.0512±0.0182	0.1178±0.0369
Pisserotte	NS	NS	–	0.0972±0.0337
Prés de la Lienne	0.0004	0.0001	0.0001	–

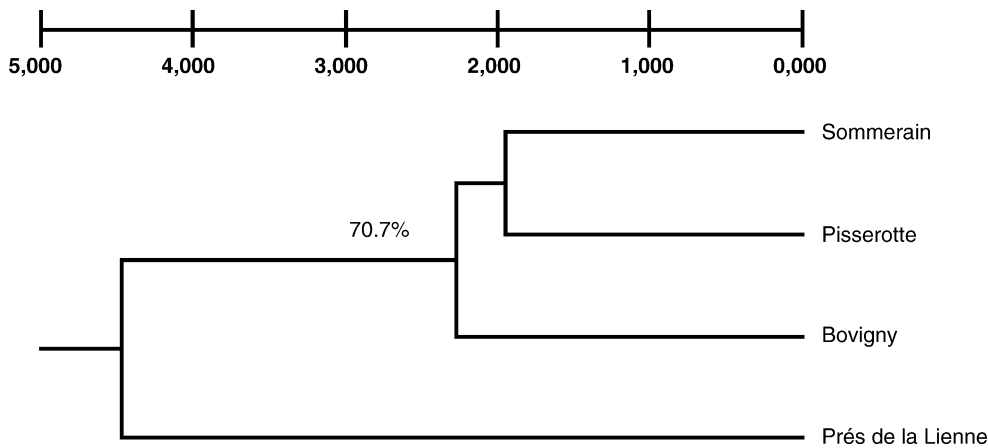


Fig. 3. UPGMA dendrogram based on Nei's unbiased minimum distance (NEI 1978). Only bootstrap values greater than 50% are indicated.

using the Bayesian hierarchical model, all results seemed to indicate a high amount of inbreeding. With the allozyme data NÈVE et al. (2000) also observed heterozygote deficiencies in Prés de la Lienne, Pisserotte and Bovigny, most likely having resulted from nonpanmictic pairing. Besides the lack of long-distance migration events resulting in reproduction between closely related butterflies, inbreeding may also be occurring due to fluctuations in population size. During the long term monitoring of the Prés de la Lienne patchy population (11 generations), values ranging between 119 (SE = 8) and 1046 (SE = 45) adult butterflies were recorded (SCHTICKZELLE et al. 2002, SCHTICKZELLE 2003). These variations may be due to (1) the high levels of endoparasitism (WAEYENBERGH and BAGUETTE 1996)-since this species lays its eggs by clutches, it is possible that during a particular season, the offspring of only a very limited number of females survive; and (2) the high variation in fecundity between females – indeed some females produce up to eight times as many eggs as others (SCHTICKZELLE 2003).

The population genetic structure identified in this study differs from the one in NÈVE et al. (1996) (Fig. 1). Several facts could explain this incongruence. Most importantly, the Bihain population and others (Fig. 1) have gone extinct since the study by NÈVE et al. (1996). It is likely that these populations served as a stepping-stone between the Prés de la Lienne and the populations on the upland. Since they've gone extinct, gene flow between Prés de la Lienne and the other populations has become increasingly difficult. Another difference between this and the earlier study is the molecular marker employed. Due to the inherently high polymorphism of RAPDs, phenomenon such as reduced gene flow and population differentiation are easier to detect with RAPDs than

with only a few polymorphic allozyme loci. That conclusions drawn from enzyme data may be misleading when very few polymorphic allozyme loci are available was demonstrated in a study on the closely related *Boloria aquilonaris* butterfly in the same landscape (VANDEWOESTIJNE and BAGUETTE 2002). In that study, the RAPD-based UPGMA tree seemed to reflect more truly the present spatial configuration of favourable habitat for this species while the allozyme-based UPGMA tree rather reflected the configuration of favourable habitat at the end of the 18th century (VANDEWOESTIJNE and BAGUETTE 2002). Additionally, a high congruence in dispersal rates between the demographic and genetic-based estimates in this species also indicates that RAPD is a reliable molecular technique (VANDEWOESTIJNE and BAGUETTE 2004).

Gene diversity (0.3720 ± 0.1952) and genetic subdivision ($F_{st} = 0.0887 \pm 0.0225$) values were much like those obtained for *B. aquilonaris*, a closely related butterfly inhabiting wet acid peat bogs, at a comparable spatial scale ($F_{st} = 0.0887 \pm 0.0218$, VANDEWOESTIJNE and BAGUETTE 2002). The resemblance of these values could be explained by the fact that although the number of favourable habitat patches is much sparser for *B. aquilonaris* than for *P. eunomia*, movements observed for the former species are longer than those observed for the latter. It is also interesting to note that, for *P. eunomia*, the F_{st} value obtained in this study (landscape scale) using RAPDs are similar to the F_{st} value obtained at a regional scale using allozymes ($F_{st} = 0.080$, NÈVE et al. 2000). The degree of population subdivision was also much higher for RAPDs than for allozymes in the study of *B. aquilonaris* (VANDEWOESTIJNE and BAGUETTE 2002).

Again, we believe that the higher variability inherent to RAPDs makes this marker more sensitive to

processes such as genetic drift and reduced gene flow, resulting in a more accurate and recent 'picture' of the genetic population structure analysed. Although microsatellites are often considered as the marker of choice for population genetic studies, they are inherently difficult to use in Lepidoptera (NÈVE and MEGLÉCZ 2000). Indeed, even though microsatellites are occasionally isolated in Lepidoptera (ANTHONY et al. 2001; KEYGHOBADI et al. 2002), primer design is time-consuming (up to more than one year) and primer pairs are generally useless on other species (Megléc and Roland, pers. comm.). Therefore, even though RAPDs are dominant markers, considering the ease with which they produce polymorphic loci they may be the more logical choice as the molecular marker to study the genetic aspects of metapopulation dynamics in butterflies.

CONCLUSIONS

Like many endangered species living in highly fragmented western European landscapes, the long term persistence of *P. eunomia* depends on the species ability to disperse, allowing gene flow and (re) colonisation of favourable habitat (MAES and VAN DYCK 2001). However, a recent study has demonstrated that the fragmentation of the species habitat decreases both its dispersal propensity and dispersal rate (SCHTICKZELLE and BAGUETTE 2003). Here we suggest that this "dispersal depression", i.e. this decrease in mobility, has deep genetic effects including an increase in genetic differentiation. Ensuring the long-term survival of the endangered bog fritillary butterfly will therefore necessitate the conservation of as large a number of habitat patches as possible, however small they may be.

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