

POPULATION GENETIC EFFECTS OF FOREST FRAGMENTATION IN FLANDERS (BELGIUM) ON *COELOTES TERRESTRIS* (WIDER) (ARANEAE: AGELENIDAE) AS REVEALED BY ALLOZYMES AND RAPD

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Abstract

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Due to an ever-increasing urbanisation, industrialisation, development of road infrastructure and an intensive agriculture, forests in Flanders have become heavily fragmented. In general, organisms bound to small forest fragments have a reduced population size and are highly isolated from other populations. To assess the population genetic effects of forest fragmentation, we chose *Coelotes terrestris* (WIDER, 1834) as a model organism, because it is strongly bound to forest habitats. A first attempt to reveal the population genetic structure of this species was made by using allozyme electrophoresis. Only one enzyme (PGI) however showed good interpretable variation. This low degree of polymorphism together with the sometimes-questioned neutrality of allozyme markers made us choose genetic marker (RAPD). Ten forests, with a variable degree of isolation and a variable size were investigated. The majority (allozymes) and all (RAPD) pairwise comparisons of population allele/marker frequencies were significantly different, implying a very high degree of genetic isolation between the spider populations inhabiting the forests. No significant correlation could be found between the genetic diversity of the populations and the size of the forest in which they predominate.

Introduction

Natural ecosystems in Flanders (the northern part of Belgium) have become severely fragmented over the years. In general, the development of roads is known to be one of the causes of habitat fragmentation (SPELLERBERG, 1998), but also more intensive agricultural and industrial activities and increasing urbanisation are causative agents in this process. In

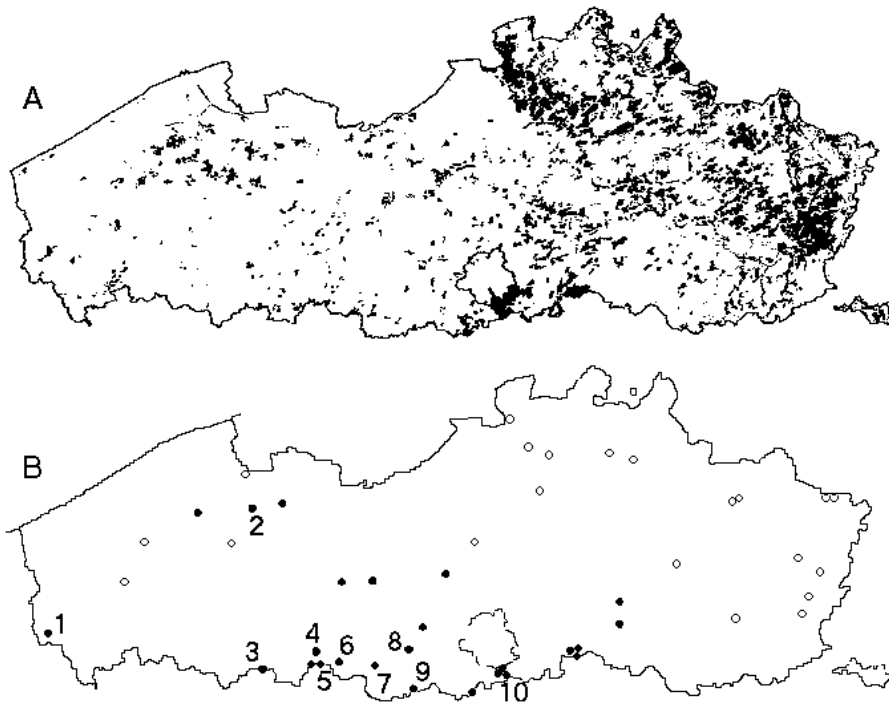


Fig. 1. A. Distribution of forests (shaded parts) in Flanders (Belgium), B. Geographic location of the sampled forests (open/filled circles represent absence/presence of *C. terrestris*, names of forests in Table 1).

the framework of the Flemish Impulse Program for Nature Conservation (VLINA) “Genetic-ecological research for nature conservation”, a collaboration between several universities and institutes was started, to study the effects of habitat fragmentation in Flanders. This collaboration between research groups, each with their own specific area of expertise, will make it possible to assess fragmentation effects for different habitat types (e.g. dunes, marshes and forests), as well as for organisms belonging to different groups, ranging from invertebrates and vertebrates to plants. This can be useful since the effects of habitat fragmentation seem to differ between species, in relation to differences in their body size and vagility (GASTON, BLACKBURN, 1996; DESENDER et al., 1998).

In this study, genetic and ecological effects of forest fragmentation are investigated. Forests cover only about 10% of the total surface of Flanders and are highly fragmented (Fig. 1A). Only two forests possess a surface size that is larger than 800 ha, whereas 50% of the remaining forests are not larger than 60 ha (VAN DEN MEERSCHAUT, LUST, 1994). The high degree of forest fragmentation implies that populations of organisms bound to this habitat type are forced to live in several smaller and spatially isolated remnants (YOUNG et

al., 1996). Especially in these fragments, population size is often reduced and a loss of genetic diversity may occur because of a lack of gene flow and/or the prevalence of inbreeding and genetic drift (GIBBS et al., 1994; SIMBERLOFF, 1998). This can result in a lowered adaptability to changing environmental conditions and in a higher risk of extinction (SIMBERLOFF, 1998). Belonging to a metapopulation structure (HOOPEs, HARRISON, 1998) can be the answer to maintain sufficient genetic diversity.

The model organism we chose to conduct this study is *Coelotes terrestris* (WIDER, 1834) (Agelenidae, Araneae). Because of its method of prey capture and web building, it is strongly bound to forest habitats (SEGERs, MAELFAIT, 1990), rendering it suitable for this kind of research. This particular species was chosen because it is a rather large spider, which is highly abundant and easy to catch during the whole year. Furthermore, a large amount of literature is available concerning its ecology (e.g. TRETZEL, 1954; DE BLAUWE, BAERT, 1981; MAELFAIT et al., 1991; DE KNIJF, 1993; HÄNGGI et al., 1995) and its life cycle (SEGERs, 1986; SEGERs, MAELFAIT, 1990; DE BAKKER, 1995).

The population genetic structure of *C. terrestris* was studied by using two techniques: cellulose acetate electrophoresis and RAPD (WILLIAMS et al., 1990). RAPD has been used in arachnological studies only recently. A population genetic study was started on *Masoncus pogonophilus* CUSHING (Linyphiidae) by CUSHING (1998), whereas HETTLE et al. (1996) used it mainly to identify siblings and to discriminate between individuals of different broods of *Brachypelma albopilosa* VALERIO. A'HARA et al. (1998) focused on protocols and conditions for specimen and DNA storage, DNA extraction and RAPD profiling of spiders.

The goal of this project is 1) to contribute to the knowledge of the population genetic effects of forest fragmentation; 2) to try to develop a bio-indicating system which can be used to monitor the gains (or losses) of nature development measures that aim to connect otherwise isolated forest fragments. Connecting habitat fragments without any knowledge of the genetic structure of the inhabiting organisms may, indeed, lead to the occurrence of outbreeding depression if local adaptation has occurred in these specific forest fragments (TEMPLETON, 1986).

Material and methods

Sample collection

Individuals were caught by hand and, prior to analysis, stored at either -80°C or in liquid nitrogen (allozymes) or at -20°C (RAPD). For both techniques, a minimum of 30 individuals per population were investigated.

The sampled forests (Fig. 1B, Table 1) were chosen following a large inventory study conducted by the IBW (Institute of Forestry and Game Management). Those woodlands possessing a high abundance of *C. terrestris* were selected for allozyme analysis. Ten forests were chosen for RAPD analysis based on the results of a first study by MAELFAIT, HENDRICKX (1998). All forests occur on sandy loam and loamy soils and are mainly beech (*Fagus sylvatica*) forests.

For RAPD analysis, only adult females were used. This was necessitated partly by the timing of the sampling campaign: the life cycle of this species is such that mainly females occur in spring. Another reason was that, hitherto, there was no knowledge about any possible sex-specific bands.

Table 1. Location and size of the sampled forests.

| No. | Code | Name | Location | Size (ha) |
|-----|------|---------------|----------------|-----------|
| 1 | HEL | Helleketelbos | Poperinge | 42 |
| 2 | DRO | Drongengoed | Knesselare | 560 |
| 3 | KLU | Kluisbos | Kluisbergen | 295 |
| 4 | BUR | Burreken | Zegelsem | 16 |
| 5 | BRA | Brakelbos | Brakel | 205 |
| 6 | PAR | Parikebos | Parike | 9 |
| 7 | RAS | Raspaillebos | Geraardsbergen | 145 |
| 8 | NEI | Neigembos | Ninove | 77 |
| 9 | EDI | Bos Ter Rijst | Edingen | 280 |
| 10 | ZON | Zoniën | Hoeilaart | 4380 |

Allozymes

The cephalothorax was homogenised in distilled water. Cellulose acetate electrophoresis was performed following the procedures of HEBERT, BEATON (1989). Twenty enzymes were tested for polymorphism: adenilate kinase (AK), aldehyde oxidase (AO), alkaline phosphatase (ALP), arginine phosphokinase (APK), fumarate hydratase (FUM), glucose-6-dehydrogenase (G6PDH), glyceraldehyde-3-phosphate dehydrogenase (G3PDH), glycerol-3-phosphate dehydrogenase (GPDH),

hexokinase (HEX), isocitrate dehydrogenase (IDH), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), malic enzyme (ME), mannose phosphate isomerase (MPI), peptidase A (PEP-A), peptidase N (PEP-N), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PGDH), phosphoglucose isomerase (PGI), trehalase (TREH).

Enzyme allelic frequencies and deviances from Hardy-Weinberg equilibrium were tested with the software program TFPGA (MILLER, 1997). A Fisher exact test, using a contingency table approach (Fisher's RxC test) (see SOKAL, ROHLF, 1995), was performed to test for differences in allele frequencies between populations and an UPGMA dendrogram was constructed based on the genetic similarity index of NEI (1972), as implemented by the TFPGA program.

DNA extraction

Sterile needles were used to isolate muscle tissue from the spider's cephalothorax. The use of tissue was preferred over the whole cephalothorax to avoid any possible contamination by external parasites. This body part was also chosen over the spider's abdomen to avoid contamination by foreign DNA that could be present in the digestive organs. A similar approach was used by A'HARA et al. (1998).

Genomic DNA was isolated with the commercially available "Puregene" kit (Gentra Systems, Inc.). The instructions of the manufacturer were followed and yielded DNA of high molecular weight and good purity. Quantification of the DNA was performed with a Biospec 1601E Shimadzu spectrophotometer and the samples were diluted to a final concentration of 5 ng/μl.

RAPD procedures

Twenty RAPD primers (primer kit A – Operon Technologies Inc., Alameda, CA) were tested and a selection was made on the basis of the degree of polymorphism and the reproducibility of the banding profiles. This procedure yielded two primers to perform the population genetic analysis: OPA-01 and OPA-20 with respective sequences 5'-CAGGCCCTTC-3' and 5'-GTTGCGATCC-3'.

RAPD was performed on a TECHNE GENIUS thermal cycler, by using volumes of 25 μl containing 2.5 μl 10x PCR buffer (+ 1.5 mM MgCl₂) (Qiagen), 0.5 μl 25mM MgCl₂ (Qiagen), 100μM dNTP mix (Eurogentec), 25 picomoles of primer (Operon), 0.5 units *Taq*-polymerase (Qiagen) and 25 ng genomic DNA. Negative controls, in which the genomic DNA was replaced by water, were added during each PCR-reaction. The PCR-reaction consisted of an initial denaturation of 2 min at a temperature of 94°C, followed by 45 cycles of 1 min at 94°C, 2 min at 36°C (primer annealing step) and 2 min at 72°C (primer extension step). A final extension of the fragments was possible for 10 min at 72°C.

PCR products (5 μl) were size-separated on a 2 % TBE agarose gel for 53 min at 110 V and stained with ethidium-bromide. The banding profiles were visualised under ultraviolet light and the gel image was saved on computer with the ColorVision I software.

The interpretation of the banding pattern was conducted with the computer program GelCompar 4.2 (Applied Maths, Kortrijk, Belgium; VAUTERIN, VAUTERIN, 1992). The bands were sized against a Low Ladder (purchased from BIOzymTC BV, Landgraaf, the Netherlands) and scored as binary data: present (1) or absent (0).

RAPD data were analysed with the TFPGA program developed by MILLER (1997). Allele frequencies were estimated based on the square root of the frequency of the null (recessive) allele (WEIR, 1990) assuming that genotype frequencies were in Hardy-Weinberg equilibrium. UPGMA (Unweighted Pair Group Method using Arithmetic means) cluster analysis was performed using Nei's (1972) original distance. Like with the allozymes, a Fisher exact test showed the degree of differentiation between the studied populations. Geographical patterns in the data were tested with a Mantel test (MANTEL, 1967) of genetic distance versus log geographic distance. Pearson correlations were made with Statistica for Windows, release 5.1 (Statsoft, Inc., Tulsa, USA).

Results

Allozymes

A first attempt to unravel the genetic structure of *C. terrestris* populations in fragmented woodlands was made by using allozyme electrophoresis. Twenty enzymes were screened for possible polymorphism; only one (PGI) showed good interpretable allelic variation. UPGMA-clustering (Fig. 2) shows a deviant genetic structure of the two sampled sites (1 and 2) in the Zoniën forest. A second group is formed by the forests belonging to the Flemish Ardennes. Neigembos, although also being a forest of this geographic area is clustered together with the isolated forests Helleketelbos and Drongengoed in a third group. The pattern of this dendrogram can be explained by the allelic frequencies of PGI that occur in these forests. For this enzyme, three different alleles (S: slow, M: medium and F: fast) could be distinguished. By determining the genotype of a sufficiently large number of individuals per population, the allelic frequencies of these populations could be estimated (Fig. 3). The largest forest (Zoniën) possessed the three alleles in comparable amounts, whereas populations inhabiting the smaller forest fragments of the Flemish Ardennes had only two alleles. Populations in Neigembos, Helleketelbos and Drongengoed had also two alleles, of which one was present only in a very small amount.

A Fisher exact test showed that not all populations were significantly different from each other (Table 2). All populations were in Hardy-Weinberg equilibrium, so this assumption, made to analyse the RAPD data, was correct.

RAPD

The two primers yielded 30 good interpretable bands that were used in this analysis. Their sizes ranged between 400 and 1650 bp. 87% of the studied loci were polymorphic.

The constructed UPGMA-tree (Fig. 4) divided the forests into two clusters for which no geographic (or other) pattern could be found ($r=0.06$, $P=0.38$, Mantel test). All populations showed a highly significant genetic differentiation ($P<0.001$ in all pairwise combinations, Fisher exact test). Although these results are still preliminary, it appears that the patterns found with the allozymes are not supported by this molecular technique.

Table 2. Genetic differentiation between the 10 populations based on allozyme data (with **: <0.01, *: <0.05).

| | ZON1 | ZON2 | PAR | KLU | RAS | NEI | EDI | BRA | BUR | DRO | HEL |
|------|------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ZON1 | X | | ** | ** | ** | ** | ** | | ** | ** | ** |
| ZON2 | | X | ** | ** | ** | ** | ** | * | ** | ** | ** |
| PAR | | | X | ** | ** | ** | ** | | ** | ** | ** |
| KLU | | | | X | | * | * | * | | ** | ** |
| RAS | | | | | X | ** | * | * | | ** | ** |
| NEI | | | | | | X | ** | ** | ** | | |
| EDI | | | | | | | X | | | ** | ** |
| BRA | | | | | | | | X | | ** | ** |
| BUR | | | | | | | | | X | ** | ** |
| DRO | | | | | | | | | | X | |
| HEL | | | | | | | | | | | X |

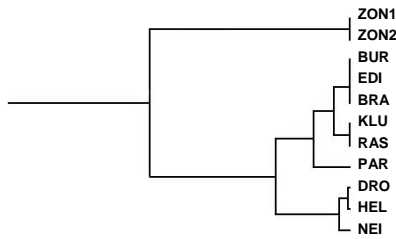


Fig. 2. UPGMA-dendrogram of *C. terrestris* populations based on allozyme data.

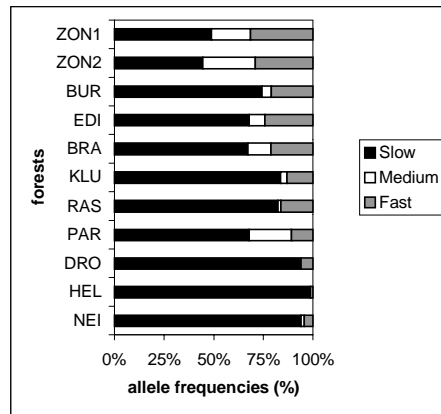


Fig. 3. Allele frequencies of the enzyme PGI in the 10 sampled populations.

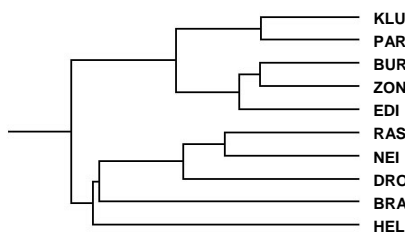


Fig. 4. UPGMA-dendrogram of *C. terrestris* populations based on RAPD data.

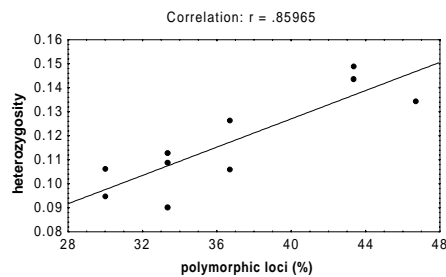


Fig. 5. Correlation between the percentage of polymorphic loci and heterozygosity.

Two different measures were used to study the genetic diversity of the sampled populations, namely the percentage of polymorphic loci and the average heterozygosity (Table 3). The lowest percentage of polymorphic loci (30%) was found in the populations of Burreken and Helleketelbos; the lowest average heterozygosity (0.09) was reached in Burreken and Parike. Based on these two measures, the spider population in Burreken had the lowest genetic diversity. The highest number of polymorphic loci were found in the population of Brakelbos (46.67%). The highest heterozygosity (0.15) appeared in the population of Raspaillebos. The percentage of polymorphic loci was correlated with the heterozygosity in all populations ($r=0.86$, $P<0.01$; Fig. 5). However, there appeared to be no correlation between the size of the forest and the degree of polymorphism in its populations ($r=0.43$, $P=0.21$).

Table 3. Percentage of polymorphic loci and heterozygosity based on RAPD data.

| Forest | Average heterozygosity | % polymorphic loci |
|--------|------------------------|--------------------|
| KLU | 0.11 | 36.67 |
| PAR | 0.09 | 33.33 |
| BUR | 0.09 | 30.00 |
| ZON | 0.14 | 43.33 |
| EDI | 0.11 | 33.33 |
| RAS | 0.15 | 43.33 |
| NEI | 0.11 | 33.33 |
| DRO | 0.13 | 36.67 |
| BRA | 0.13 | 46.67 |
| HEL | 0.11 | 30.00 |

Discussion

Although allozymes offer a quick and easy method to investigate the genetic structure in populations, the number of loci and alleles per locus is sometimes too low, rendering them only suitable to detect large-scale genetic patterns (GROSBURG et al., 1996). The low degree of polymorphism in *Coelotes terrestris*, together with the sometimes questioned neutrality of allozymes (e.g. RIDDOCH, 1993; CONGDON, 1994), made it necessary to search for another technique. BLACK (1993) noted that PCR techniques (SAIKI et al., 1988) have revealed polymorphisms in insect taxa that lacked allozyme polymorphisms. Apart from this higher degree of polymorphism, another advantage of PCR techniques is that they reveal variation on the DNA-level, whereas allozymes only offer variation on the gene-product level (STEWART, EXCOFFIER, 1995).

However, a lack of repeatability of the banding patterns is considered to be one of the drawbacks of the RAPD technique (HEDRICK, 1992; HARRY et al., 1998). Being a PCR-based technique, RAPD is very sensitive to the amplification conditions. Models of thermocyclers, changes in annealing temperatures, primer and template concentrations, dNTP concentration, Mg^{2+} concentration, are known to cause unreliable and inconsistent amplifications (see GROSBURG et al., 1996). Nevertheless, this technique is widely used and many authors stated that a standardisation of the reaction conditions is enough to ensure reproducibility of the amplification products (HADRY et al., 1992; BLACK, 1993; HARRY et al.,

1998). We can confirm this statement since banding profiles were reproducible within, as well as between, different PCR reactions.

The higher degree of polymorphic markers generated by RAPD makes it possible to study the population genetic structure of our study species more thoroughly. However, in contrast with BUSO et al. (1998) and DE WOLF et al. (1998), RAPD and allozyme markers did not reveal the same or similar patterns. The populations of the ten sampled forests appear to have undergone a significant differentiation; furthermore, no geographical pattern could be found. This implies that they are not part of a metapopulation and that they each possess different gene pools.

In future, more populations will be investigated by RAPD analysis, by using more individuals per population, as well as more primers. A selected number of populations will be checked for temporal variation and also effects of forest fragmentation on a smaller scale will be assessed (e.g. between adjacent forest patches and within larger forest complexes that are subdivided by road infrastructure). Possible co-variation between genetic diversity and forest age or degree of isolation will be studied.

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