BIOLOGICAL LETT. 2004, 41(2): 95–101
Available online at http://www.biollett.amu.edu.pl

Using microsatellites to obtain genetic structure data for Red-backed Shrike (*Lanius collurio*): a pilot study

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(*Received on 28th August 2003; Accepted on 9th September 2004*)

Abstract: The declining Western European population of the Red-backed Shrike (*Lanius collurio*) is hypothesised to be a part of the same metapopulation as the Dutch population, at the edge of the contracting western range. Due to habitat fragmentation the Dutch population is mainly situated in the Bargerveen reserve (40–60 pairs) with a few local populations of 1–5 pairs present in other parts of The Netherlands. Previous studies showed that all locations are complemented by a high number of immigrants. The origin of the immigrants and the level of genetic variation in this population, which acts as a sink, can be investigated by molecular markers. In this pilot study we investigated the possibility of using 4 microsatellite markers to study the genetic structure of the Red-backed Shrike populations in Bargerveen and in the north of Denmark, on the basis of 10 individuals per population. All loci were variable, and both populations were comparable in mean number of alleles and distinct private alleles. In both populations, the observed heterozygosity was relatively low, and the inbreeding coefficient high. In the future, this project should be continued at a larger scale to obtain data for the genetic structure of the Western European Red-backed Shrike across a larger part of its range. The analysis needs to be enlarged with more loci and more independent individuals per population.

Key words: Red-backed Shrike, *Lanius collurio*, DNA, microsatellites, population genetics

INTRODUCTION

Due to anthropogenic habitat fragmentation, a formerly continuous population can become a metapopulation, with subpopulations connected with each other by dispersal (OPDAM 1987, HANSKI & GILPIN 1991, SEGELBACHER et al. 2003). The rate of dispersal, and thus the genetic exchange between those populations, depends partly on the population size and partly on the physical aspects of the surrounding landscape (OPDAM 1987, HANSKI 1999).

In the core of a metapopulation, dispersal originates from all surrounding populations. More isolated populations, however, or populations at the edge of their range, have fewer neighbouring populations, and thus fewer dispersal possibilities (SEGELBACHER & STORCH 2002). Restricted dispersal to these populations can result in population decline (SCHIEGG et al. 2002), increased levels of inbreeding (SACCHERI et al. 1998), loss of genetic diversity (GILPIN 1991) and genetic differentiation from other (core) populations (BUSCH et al. 2000).

Frequently, a decline of a species coincides with the contraction of the species' range (CEBALLOS & EHRLICH 2002) and the populations at the periphery are more susceptible to extinction than populations at the core of the range (SEGELBACHER & STORCH 2002). These populations may function as sinks, which can only persist thanks to continuous immigration (OPDAM 1987, LARSSON et al. 2003, SEGELBACHER et al. 2003). In this way much of the genetic diversity may be preserved (GAGGIOTTI 1996, HANSKI 1999).

The Red-backed Shrike (*Lanius collurio*) has been subject to a dramatic decline in most Western European countries (HUSTINGS & BEKHUIS 1993, HAGEMEIJER & BLAIR 1997). Due to human activities, the bird's breeding habitat became highly fragmented during the 20th century. In The Netherlands the Red-backed Shrike almost disappeared, as from the estimated 5 000–15 000 pairs around 1900 it decreased to about 150 pairs at the end of the 20th century (HUSTINGS & BEKHUIS 1993). Currently the largest population is in the Bargerveen reserve (40–60 pairs). In addition, a few local populations (1–5 pairs) are found in other parts of The Netherlands.

Restoration management leading to high food availability (ESSELINK et al. 1995), helped to increase the Bargerveen population two decades ago (GEERTSMA et al. 2000), and it was hoped that this population could act as a source to (re)colonise the local populations. However, a colour-ringing program showed no exchange between Bargerveen and the other populations, especially between populations that are separated by more than 30 km. It seems that the dispersing Dutch birds are not able to find the fragments of habitats in the surrounding 'desert'.

On the other hand, from results on reproductive success and return rates of juveniles and adults, a high immigration rate is estimated at >30 individuals each year (GEERTSMA et al. 2000 and 2004 unpublished results). Many unringed birds, found in all (local) populations, support our conclusion that birds from larger populations near The Netherlands complement the Dutch populations. Our populations, including Bargerveen, seem to act as a sink at the edge of the Western European metapopulation.

We were interested in the origin of the immigrant birds that help to maintain the Dutch population. Furthermore, we wanted to know the level of genetic diversity in the Bargerveen population compared to a Red-backed Shrike population from the dunes of Denmark. Hence, in this pilot study we used microsatellite markers, which are highly variable, selectively neutral markers, thus making them ideal for dispersal and metapopulation structure studies (GOLDSTEIN & POLLOCK 1997, HARPER et al. 2003). Microsatellites are tandem repeats of nucleotides in the DNA sequence, such as $(CA)_n$, that are distributed all over the genome. Four microsatellite loci published for the Loggerhead Shrike (*Lanius ludovicianus*) (MUNDY & WOODRUFF 1996) gave polymorphic results in the Red-backed Shrike and were therefore used in this research.

The goal of this pilot study was to design an optimal protocol for microsatellite-PCR and to investigate the possibilities of using these loci in a genetic study of the Red-backed Shrike populations in Bargerveen and in the dunes of Denmark. We hoped to be able to assess whether it is feasible to set up a large-scale project to study the genetic structure of the Red-backed Shrike metapopulation of Western Europe.

METHODS

Red-backed Shrike populations

Two Red-backed Shrike populations were studied: one in The Netherlands and another in the dunes of northern Denmark, Skagen (Fig. 1A and 1B). The Dutch population is mainly located in the Bargerveen reserve, but a few other places have also been identified as breeding sites in the Netherlands, for example Cottessen and Elperstroom. During ecological research, 30 feather samples originating from ten different nests were collected in Skagen, Denmark. Although about 350 samples are available from the Netherlands, only a comparable set of 30 samples originating from 11 Bargerveen nests was used in this study. Since statistical analysis requires input of independently collected samples, the final population size was only 10 individuals per population for the F-statistic calculations (see microsatellite analysis).

Fig. 1A. Locations of Red-backed Shrike populations in The Netherlands
(Only samples from Bargerveen were used in this study.)

Fig. 1B. Location of the sampled population in the north of Denmark

DNA isolation

Growing feathers were collected during the ringing of about 9-day-old nestlings. The fluid-filled base of a feather contains enough DNA for our genetic analy-

sis. After sampling, each feather was stored in an Eppendorf tube containing EDTA storage buffer (0.01 M Na-EDTA, 0.01 M Tris, 1% lauroyl sarcosine, 0.01 M NaCl, pH=8.0). When the vane of a feather was larger than 3 mm, it was removed and only the remaining tip was used for DNA extraction. DNA was isolated with the DNeasy 96 Tissue Kit (Qiagen, Hilden, Germany). Lysis time was at least 24 hrs and the DNA was resuspended in 200 µl AE buffer (as delivered with DNeasy Tissue Kit). Before use, DNA was diluted 10 times.

Microsatellite analysis

The samples were examined for 4 microsatellite loci of the Loggerhead Shrike, LS1, LS2, LS3 and LS4 (MUNDY & WOODRUFF 1996). To 2 µl of diluted DNA template, we added: 0.067 µM labelled (IRD700 or IRD800, LI-COR, Lincoln, Nebraska, USA) forward primer, 0.6 µM unlabelled forward primer, 0.67 µM reverse primer, 0.15 units Platinum Taq Polymerase (Invitrogen), 1× PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 50 µM dNTPs each and 1.5 mM $MgCl_2$ in a total volume of 15 µl. The PCR reactions were run in a Biometra T-gradient thermocycler (Whatman, Göttingen, Germany) with the following cycle parameters: 3 min denaturation at 94ºC, 24 cycles of 30 sec at 94ºC, 60 sec at 55ºC, 60 sec at 72ºC, and a final extension of 2 min at 72ºC. Electrophoresis on polyacrylamide gels (6.5% KB Plus gel matrix from LI-COR) was carried out using a LI-COR Global IR^2 DNA analyser. In the LI-COR e-Seq software the following electrophoresis conditions were chosen, genotyping, volts: 1500, current: 40, power: 40, temperature: 45ºC, scan speed: 3-moderate, time prerun: 15 min, time run: 1:45 h.

Microsatellite bands were scored with $SAGA^{GT}$ software (LI-COR, Lincoln, Nebraska, USA). Statistic analyses were performed with Fstat version 2.9.3.2 (http://www2.unil.ch/izea/softwares/fstat.html) (GOUDET 1995). With this program we calculated allele frequencies per locus and population, allelic richness, heterozygosity (expected heterozygosity H_{exp}, and observed heterozygosity H_{obs}), basing on the Hardy-Weinberg equilibrium, and the inbreeding coefficient $F_{IS} = 1\text{-}H_{obs}/H_{exp}$.

RESULTS

The PCR reaction was optimised by varying parameters, such as dilution of the DNA template, amount of (labelled) primer, number of cycles in the PCR program, and type of Taq polymerase. This resulted in gels of sufficiently good quality to score with $SAGA^{GT}$ software, an example of which is shown in Figure 2.

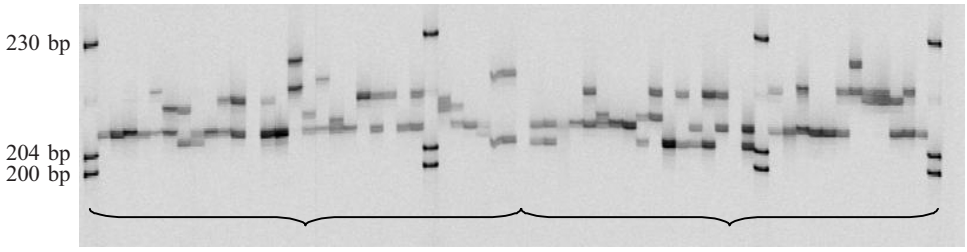

Fig. 2. Samples from Bargerveen (left) and Denmark (right). Primer combination: LS1

In the samples of 10 individuals per population, all loci turned out to be polymorphic, with 9, 12, 11, and 3 alleles for LS1, LS2, LS3, and LS4 respectively. Bargerveen and Denmark both had the same mean number of alleles per locus, 6.75; and the same allelic richness, 6.75. Some alleles were present in just one population – the private alleles – of which Bargerveen and Denmark had the same number per locus as well, i.e. eight (Table 1). We calculated the heterozygosity and the inbreeding coefficient by using Fstat. The overall observed heterozygosity (H_{obs}) was lower than the expected heterozygosity (H_{exp}), in both Bargerveen and Denmark. The inbreeding coefficient F_{IS} was mostly high (Table 1).

Table 1. Mean number of alleles per locus (A), allelic richness (R), number of private alleles (PA), observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}), and inbreeding coefficient (F_{IS}) for the Bargerveen and the Danish populations of Red-backed Shrikes. Sample size: 10 individuals per population

Location	A	R	PA	H_{obs}	H_{exp}	F_{IS}
Bargerveen	6.75	6.75	8	0.625	0.727	0.141
Denmark	6.75	6.75	8	0.675	0.752	0.129

We also counted the alleles for 30 individuals per population. Since these individuals were not sampled independently, they could not be used in the F-statistics. In this case, Bargerveen seemed to have a higher mean number of alleles per locus than Denmark: 8.75 and 7.5, respectively. The number of private alleles per locus in Bargerveen was higher as well: 8 and 3, respectively.

DISCUSSION

In both populations the microsatellites were highly variable. H_{obs} was lower than H_{exp} in both populations, with a relatively high F_{IS}, which might indicate inbreeding. However, a high F_{IS} could also be a result of sex-biased dispersal, a feature of population decline, or a result of the breeding system. Bargerveen showed a slightly higher F_{IS} than Denmark. However, due to the low sample size, these data should be interpreted carefully.

Most likely, immigration can still maintain the population for the time being. However, during the last few years the Bargerveen population and most other Dutch populations were declining. This will probably lead to extinction if there is no conservation management for this species in the Netherlands.

In this study, the mean number of alleles per locus was exactly the same as the allelic richness, due to the comparable sample size of 10 individuals in each population. The Danish population had about the same values for heterozygosity, allelic richness and number of private alleles per locus as the Bargerveen population. Unfortunately there is no long-term ringing program in the Danish population. Consequently, it is unknown whether the number of immigrants in the Danish population is comparable to that in the Bargerveen population.

The slightly higher mean number of alleles and private alleles found in 30 individuals of the Bargerveen population compared to the Danish population might point to certain trends. However, these samples are not independent and further research is needed for verification.

As a pilot study, this research was successful in showing the possibilities concerning the genetic variation analyses. For further research the random test should be enlarged in two ways: (1) more loci; and (2) more independent individuals per population. Furthermore, the metapopulation should be defined in order to be able to take significant samples to study the metapopulation structure with genetic methods.

In this hypothesised metapopulation structure, it is important to investigate a number of populations in the core of the metapopulation, and many populations at the edge. In this way genetic diversity can be compared, population differentiation can be investigated, and the origin of the immigrants may become clear.

To expand the analyses, other statistic programs to test microsatellites, besides Fstat, have to be investigated, for example POPGENE and GENEPOP. Moreover, because LS1, LS2, LS3, and LS4 are the only available loci now, new loci have to be developed by sequencing significant parts of the genome and designing primers for the sequence outside the target bases of microsatellites. Another valuable technique in population genetics is AFLP. With this technique more markers are visible per gel, therefore it is very valuable for example in showing the differentiation between populations (BREYNE et al. 1999).

Results of a larger project may give strong data to support better the conservation of the Red-backed Shrike habitats. Larger, continuous habitat patches and dispersal stepping stones between habitat fragments can give a higher chance for successful dispersion. In this way the metapopulation will be able to persist and local populations will be less susceptible to extinction.

Acknowledgements: We thank Hugh JANSMAN for help with the first set-up of this pilot study, Joop OUBORG for advice on the statistical approach, and Judith HANSSEN for assistance in the difficulties of the English language. We are also grateful to the Bargerveen team for collecting samples; and to the shrikeologists who supported us by e-mail or telephone: Miriam HERNANDEZ, Wolfgang STAUBER, and Heinz KOWALSKI. Special thanks are due to Gernot SEGELBACHER who helped us understand population genetics.

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