Ardeola 53(1), 2006, 107-113

DIFFERENTIATION AMONG SPANISH SOUTHERN GREAT SHRIKE *LANIUS M. MERIDIONALIS* POPULATIONS USING TANDEM REPEATS IN mtDNA CONTROL REGION

Francisco GUTIÉRREZ-CORCHERO*, María Angeles HERNÁNDEZ** [1], Francisco CAMPOS*** and Ana AMEZCUA**

SUMMARY.—*Differentiation among Spanish southern great shrike* Lanius m. meridionalis *populations using tandem repeats in mtDNA control region.*

Aims: The aim of this work is to discover a) the existence of specimens of southern grey shrike with two, three or 2+3 tandem repeats in areas further away within the Iberian Peninsula, b) what proportion of specimens with this genetic character exist in any one population, and c) if there is genetic flow between neighboring populations.

Location: Four populations in Iberian Peninsula (three populations from Navarre and one population from Cáceres).

Methods: A small blood sample was obtained from 242 captured shrikes. An analysis has been carried out of the control region for the mitrocondrial DNA. The DNA was extracted and a fragment was amplified using primers DLL2 and FTPH2. The results were analysed by means of the Arlequin program for haplotype frequencies.

Results: Birds with two, three and 2+3 tandem repeats were recorded. The percentage of shrikes with two or three repeats did not differ either between populations or sexes. The presence of heteroplastic shrikes in two of the four populations was few frequent (1.6 % of the 242 birds studied).

Conclusions: Data suggest the existence of a genetic flow between populations, so they maintain the equilibrium conditions for this characteristic.

Key words: Control region, *Lanius meridionalis meridionalis*, mitochondrial DNA, southern grey shrike, Spain, tandem repeats.

RESUMEN.—*Diferencias entre las poblaciones españolas de alcaudón real* Lanius m. meridionalis *utilizando repeticiones en tandem de la región control del ADN mitocondrial.*

Objetivos: El objetivo de este trabajo es descubrir a) la existencia de ejemplares de alcaudón real de la península Ibérica con dos, tres o 2+3 repeticiones en tandem en la región control del ADN mitocondrial, b) la proporción de aves con cada uno de estos caracteres en cada población, y c) si hay flujo genético entre poblaciones vecinas.

Localidad: Tres poblaciones de Navarra y una de Cáceres.

* Joan Baptista La Salle 30, E-17002 Girona, Spain.

** Departamento de Zoología y Ecología, Facultad de Ciencias, Universidad de Navarra, E-31080 Pamplona, Spain.

*** Universidad Europea Miguel de Cervantes, C/ Padre Julio Chevalier, 2, E-47012 Valladolid, Spain.

[1] Corresponding author: mahermin@unav.es

Métodos: Una pequeña cantidad de sangre fue extraída de 242 alcaudones anillados. Se analizó la región control del ADN mitocondrial. El ADN fue extraído de la sangre y se amplificó un fragmento usando los primers DLL2 y FTPH2. Los resultados se analizaron mediante el programa Arlequin para frecuencias de haplotipos.
Resultados: Se encontraron ejemplares con dos, tres y 2+3 repeticiones en tandem. Los porcentajes de alcaudones con dos y tres repeticiones no difirieron ni entre poblaciones ni entre sexos. La presencia de alcaudones heteroplásticos en dos de las cuatro poblaciones fue esporádica (1,6 % de 242 ejemplares estudiados).
Conclusiones: Los datos sugieren la existencia de flujo genético entre poblaciones, por lo que se mantienen en equilibrio respecto a este carácter.

Palabras clave: ADN mitocondrial, alcaudón real, España, *Lanius meridionalis meridionalis*, región control, repeticiones en tandem.

Introduction

The repetition of elements in a sequence of DNA makes complex structures, as when a short fragment (a few hundred base pairs) is multiplied several times to form a tandem. Sometimes these repeats are due to mutations, which can affect protein coding and expression (Weiss and Buchaman, 2004).

Inter-specific differences in the number of tandem repeat sequences can cause length polymorphisms (according to the number of repeats) and heteroplasmy (different numbers of of mtDNA control region in mitochondria from the same individual). This information can be used to help differentiate species (Fridez *et al.*, 1999) or compare populations of the same species (Ketmaier and Bernardini, 2005). The variation in tandem repeat lengths is especially high in the control region of mitochondrial DNA (mtDNA).

Mundy *et al.* (1996) found a 128 bp tandem repeat sequence in the mtDNA control region of the loggerhead shrike *Lanius ludovicianus*, with two, three and heteroplasmic 2+3 repeats. Since there are no repeats in the fiscal shrike *Lanius collaris*, this technique can be used to differentiate between species and subspecies of shrikes. Hernández *et al.* (2004) found differences in the tandem repeat of mtDNA control region between the southern grey shrike *Lanius meridionalis*, in Spain and some Eurasian subspecies and the great grey shrike *Lanius excubitor*.

Mundy and Helbig (2004) also studied the tandem repeat region of the mtDNA for eight species and 14 subspecies of shrikes, of which only three showed tandem repeats: the loggerhead shrike, great grey shrike and southern grey shrike. The three southern grey shrikes from Spain analysed by Mundy and Helbig (2004) had three repeats in the tandem repeat mtDNA control region, as had the great grey shrike specimens, which apparently differentiates them from loggerhead shrikes that can have two, three and 2+3 repeats. This data disagrees with Hernández *et al.* (2004), who analysed 71 southern grey shrikes from northern Spain and found two and three repeats, as well as different tandem repeats among Eurasian populations of southern grey shrikes and great grey shrikes.

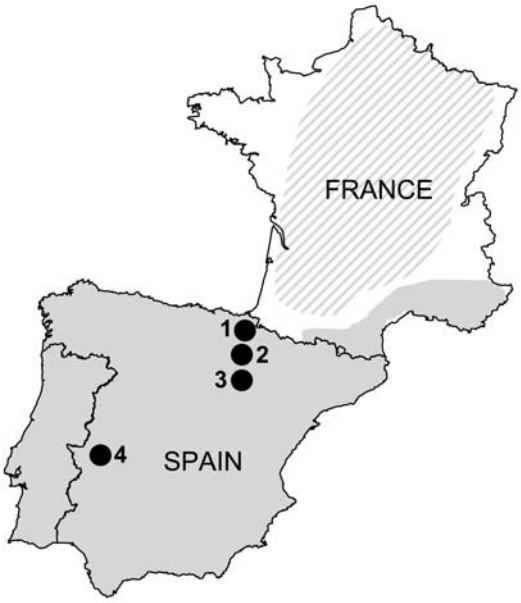
Nonetheless, it is unknown whether populations of the same subspecies of southern grey shrike *L. m. meridionalis* are genetically similar for this character in a large geographic area (such as the Iberian Peninsula).

The southern grey shrike is a different species from the great grey shrike (BOU, 1997; British Birds, 2000). The southern grey shrike is a territorial and socially monogamous species, without conspicuous sexual dimorphism. Ten

subspecies are recognized and are distributed mainly through Afro-Asia. The nominal subspecies can be found in the Iberian Peninsula and southwest France (Lefranc and Worfolk, 1997; Harris and Franklin, 2000). The breeding areas of the southern grey shrike and the great grey shrike are very close in the southeast of France, although they do not overlap (Isenmann and Bouchet, 1993) (Fig. 1). Therefore it would seem necessary to investigate further into the genetic characteristics of its population and into the genetic difference with other species.

The aim of this work is to discover a) the existence of specimens of southern grey shrike with two, three or 2+3 tandem repeats in areas further away within the Iberian Peninsula, b) what proportion of specimens with this genetic character exist in any one population, and c) if there is genetic flow between neighboring populations.

FIG. 1.—Populations of southern grey shrike sampled in Iberian Peninsula (solid circles) 1: Pamplona. 2: Olite. 3: Bardenas. 4: Cáceres. Shaded area: area occupied by the southern grey shrike. Lined area: area occupied by the great grey shrike, according to Lefranc and Worfolk (1997).
[Poblaciones de alcaudón real muestreadas en España (círculos rellenos). Zona sombreada: área ocupada por el alcaudón real. Zona rayada: área ocupada por el alcaudón norteño, según Lefranc and Worfolk (1997).]



Material and methods

Analysis was carried out on 242 southern grey shrikes from four populations found in two areas of the Iberian Peninsula (one in the north and another in the west, Fig. 1). Three of these populations are from the Province of Navarra, and have different climatic conditions, vegetation and bird density: 1) Pamplona (42º 48' N, 01º 38' W), located in the Eurosiberian region, in upper mountainous terrain with an annual precipitation of 870 mm and an average annual temperature of 12.3 ºC; 2) Olite (42º 29' N, 01º 39' W), 35 km south of Pamplona, in meso-Mediterranean terrain, with an average annual precipitation of 525 mm and an average annual temperature of 13.3 ºC; the dominant vegetation is cereal crops, vineyards and fruit trees; 3) Bardenas (42º 15' N, 01º 37' W), 40 km southeast of Olite, on meso-Mediterranean terrain, with an average precipitation of 381 mm and an average annual temperature of 14.0 ºC; mostly cereal crops. In the second area, only one population was sampled close to Cáceres (39º 29' N, 06º 22' W), approximately 500 km southwest of Pamplona, on meso-Mediterranean terrain, with an average annual precipitation of 491 mm and an average annual temperature of 16.3 ºC; the dominant vegetation was meadow-like pasture. It was chosen as a region for comparison with the northern populations because it had a density of shrikes higher than was found in the northern region (*pers. obs.*). The external groups used were red-backed shrike *Lanius collurio* and the woodchat shrike *Lanius senator*.

A small blood sample was obtained from all captured shrikes by puncturing the brachial

vein and storing on FTA Classic Cards®. The DNA was extracted according to Gutiérrez-Corchero *et al.* (2002). Birds were sexed by molecular genetic techniques (Griffiths *et al.*, 1998).

The DNA fragment was amplified using primers DLL2 (control region: 5'-ATG-CACTTTTACCCCATTCATGGTGG-3') and FTPH2 (Phe tRNA: 5'-CCATCTTGA-CATCTTCAGTGCCATGC-3'), designed by Mundy *et al.* (1996). The PCR amplification was performed on a GeneAmp PCR system 2400 (Applied-Biosystems). The volume of the sample was 40 µl, including 0.5 units of Taq polymerase (Bioline), 10 x PCR buffer, dNTPs 10 mM, 1.5 mM MgCl2 and 20 µM of each primer. The parameters of the thermocycler were 1 x 94 ºC, 3 min; 40 x 94 ºC, 30 s, 60 ºC, 60 s, 72 ºC, 90 s; 1 x 72 ºC, 10 min. The PCR product was run on a 1.5 % agarose gel and stained with ethidium bromide. The DNA marker was ϕ-phage DNA digested with Hae III, which has 11 fragments from 72 to 1353 pb (I.X174 marker HaeIII, Sigma). Each gel was photographed with a camera linked to a computer and an image analyzer (Molecular Analysis software, BIO-RAD).

The results were analysed by means of the Arlequin (Schneider *et al.*, 2000) program for haplotype frequencies. A comparison of pairs of population samples was carried out, using the population pairwise FST. Also, we used the χ^2 test to check for significant differences in the frequencies of tandem repeats. Due to the small sample size, individuals with three repeats were grouped with heteroplasmic individuals with 2+3 repeats. The Yates correction was applied when necessary (Sokal and Rholf, 1981).

Results and discussion

The four populations studied presented shrikes with two and three repeats in the control region of the mtDNA. In the populations in Pamplona and Olite individuals were found with 2+3 repeats (heteroplasmic), although the percentage was low (8.9 % of the population in Pamplona and 1.2 % in that of Olite; n = 242), but this did not come up in the populations further south (Bardenas) or in that of Cáceres.

The percentage of birds with two repeats decreased from Olite to Cáceres. The same percentage in Pamplona was between that of Cáceres and Bardenas (Table 1). The percentage of birds with three repeats increased from Olite to Cáceres, while those from Pamplona were similar to Olite. Nonetheless, these

Table 1

Percentage of southern grey shrikes with 2, 3 and 2+3 tandem repeats according to the sex (M = males, F = females) in four Spanish populations (Pamplona, Olite, Bardenas, Cáceres). n = sample size.
[Porcentaje de individuos con 2, 3 y 2+3 repeticiones en tandem, separados según el sexo (M = machos, F = hembras), en las poblaciones de alcaudón real de Pamplona, Olite, Bardenas y Cáceres. n *= tamaño de muestra.]*

	Pamplona			Olite			Bardenas			Cáceres		
	M	F	Total	M	F	Total	M	F	Total	M	F	Total
2	61.5	43.7	48.9	62.5	52.5	57.5	48.4	50.0	49.2	42.2	45.5	42.9
3	30.8	46.9	42.2	35.0	47.5	41.3	51.6	50.0	50.5	57.8	54.5	57.1
2+3	7.7	9.4	8.9	2.5	0	1.2	0	0	0	0	0	0
n	13	32	45	37	43	80	31	30	61	45	11	56

TABLE 2

Values of FST of pairwise population comparing four populations of southern grey shrike (Pamplona, Olite, Bardenas, Cáceres) and one population of great grey shrike (Poland). *P*: probability of FST per 35 permutations. Significance level $P = 0.05$.
[Valores de la FST de pairwise comparando cuatro poblaciones de alcaudón real (Pamplona, Olite, Bardenas, Cáceres) y una de alcaudón norteño (Polonia). P*: probabilidad de FST para 35 permutaciones. Nivel de significación* P *= 0,05.]*

	Pamplona	Olite	Bardenas	Cáceres
Olite	-0.0049 $P = 0.3333$			
Bardenas	0.0017 $P = 0.3611$	0.0144 $P = 0.1388$		
Cáceres	0.0112 $P = 0.0833$	0.0304 $P = 0.1388$	-0.0146 $P = 0.8333$	
Poland	0.2258 $P < 0.0001$	0.28838 $P < 0.0001$	0.1758 $P < 0.0001$	0.1468 $P < 0.0001$

variations were not statistically significant (Table 2).

The distribution of tandem repeats within each sex was described per location (Table 1). In males and females there was the same tendency: a decrease in the percentage of individuals with two repeats and an increase in the percentage of three repeats from Olite to Cáceres. Statistically these differences were not significant in either sex ($\chi^2_3 = 3.954$ for males, $\chi^2_3 = 0.688$ for females, both $P > 0.05$). However, in the Pamplona population there were more heteroplasmic females than males; these females were captured in winter and disappeared from the population in the breeding season (*pers. obs.*). In Olite, only one heteroplasmic male was found (Table 1).

In the loggerhead shrike, Mundy *et al.* (1996) found very different percentages of heteroplasmic individuals in several parts of the USA (range 0 - 28.5 %), but the sample sizes were lower than in our case. Hernández *et al.* (2004) mentioned the presence of heteroplasmic individuals in the great grey shrike and in two subspecies of the southern grey shrike (*L. m. koenigi* y *L. m. aucheri*), but they found no heteroplasmic specimens in 71 birds of the nominal subspecies southern grey shrike present in Iberian Peninsula.

According to the results, it can be stated that the three populations of southern grey shrike located in the northern area of Iberian Peninsula were homogeneous for the percentages of birds with two and three repeats, which may indicate a high genetic flow among them. The population in Cáceres also had a similar percentage of birds with two and three repeats, but it is difficult to claim that this similarity is the result of genetic flow due to the movements of individuals, as there is a considerable distance between the two sampled areas and the southern grey shrike does not fly great distances (Hernández, 1999; *pers. obs.*) However, it is very interesting to note the presence of heteroplamic shrikes in the northern area, although the percentage is much lower than that found by Hernández *et al.* (2004) for the great grey shrike (1.6 % *vs.* 8.7 %). In Pamplona, as already stated, females were only seen and captured outside the breeding season. Thus, it may be concluded that a) the Pamplona population may receive specimens

from other geographical areas outside the breeding season, which would explain why there are heteroplasmic females in this population only; b) during the breeding season these females move to their places of origin or to closer locations, which were not sampled. Since mtDNA is only transferred by females, heteroplasmic males found in the Olite population could only have come from a heteroplasmic female from Pamplona (where there are 2+3 females) or from another place further north since there were no heteroplasmic individuals to the south, as no heteroplasmic specimens were found in the Bardenas population.

On the other hand, these data do not agree with Mundy and Helbig (2004), who only found three repeats in southern grey shrikes from Spain. Probably the small sample size used by those authors decreased the chances of finding birds with two repeats and heteroplasmic individuals. Likewise, this study confirms the results obtained by Hernández *et al.* (2004) on the presence of two and three repeats in Spanish shrikes, as well as heteroplasmic individuals, which have not been reported previously. It appears that large sample sizes should be used when considering the tandem repeat region, since neither Mundy and Helbig (2004) with three samples, nor Hernández *et al.* (2004) with 71, were able to find heteroplasmic southern grey shrikes in Spain.

This study demonstrates the importance of comparing samples from different areas within Spain. Verifying the proportions of tandem repeats can help to clarify the evolution of the southern grey shrike in the Iberian Peninsula and confirm the existence of high genetic flow. The genetic flow leads to equilibrium conditions between the two genotypes (two or three tandem repeats), which fact may be due to continuous migration between sub-populations which are close to the areas occupied by this species. Gene flow and migration rates (Beebee and Rowe, 2004) and to greater genetic stability.

ACKNOWLEDGEMENTS.—The Ministry of Education and Culture of the Government of Navarre helped finance this study via Funds for Research Projects of Special Interest in Navarre (2001-2002). The Government of Navarre and the Government of Extremadura kindly provided sampling permits.

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Francisco Gutiérrez-Corchero did a PhD on the breeding ecology of southern grey shrikes, and is currently working on steppe birds in Girona, northern Spain. **Dr. María Angeles Hernández** and **Dr. Francisco Campos** are Professors at the University of Navarre and the European University Miguel de Cervantes, respectively. Currently they are working on taxonomy and ecology of shrikes, mainly the grey shrikes group. **Ana Amezcua** is a member of the Department of Zoology and Ecology at the University of Navarre. She is working on molecular techniques applied to birds.

[Recibido: 25-05-05]
[Aceptado: 01-03-06]