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SPATIAL PATTERNS OF GENETIC DIVERSITY IN MEDITERRANEAN EAGLE OWL *BUBO BUBO* POPULATIONS

PATRONES ESPACIALES DE DIVERSIDAD GENÉTICA EN POBLACIONES MEDITERRÁNEAS DE BÚHO REAL *BUBO BUBO*

Mario León-Ortega[1] *, Mercedes González-Wangüemert[2], José E. Martínez[1] and José F. Calvo[1]

Summary.—Little information is available on the patterns of genetic connectivity in owls. We studied the genetic structure of the eagle owl *Bubo bubo* (Linnaeus, 1758) in southeastern Spain at two different spatial scales. Seven microsatellites previously described for this species were used, although only six loci amplified correctly. The observed low genetic variation could be explained by the short dispersal distance, high mortality rate and high degree of monogamy shown by this large nocturnal predator. As expected, the highest genetic isolation was detected in the geographically most isolated population. Significant genetic differentiation was found among study units separated by less than 50 km. The territorial analysis showed interesting connectivity patterns related with the gene flow and turnover rate of the breeding individuals. The lowest genetic diversity was found in the region with the largest population, which could imply incipient inbreeding.

Key words: connectivity pattern, genetic diversity, Iberian Peninsula, inbreeding, microsatellites.

Resumen.—Hay poca información disponible sobre los patrones de conectividad genética en rapaces nocturnas. Nosotros estudiamos la estructura genética del búho real *Bubo bubo* (Linnaeus, 1758), a dos escalas espaciales diferentes en el sureste de España. Siete microsatélites previamente descritos para esta especie fueron utilizados, aunque solo seis *loci* amplificaron correctamente. La baja variabilidad genética observada podría ser explicada por la baja distancia de dispersión, la alta tasa de mortalidad y el alto grado de monogamia mostrada por este gran depredador nocturno. Como se esperaba, el mayor aislamiento genético fue detectado en la población geográficamente más aislada. Se encontró diferenciación genética significativa entre unidades de estudio separadas por menos de 50 km. El análisis territorial mostró interesantes patrones de conectividad relacionados con el flujo génico y la tasa de renovación de los reproductores. La menor diversidad genética fue encontrada en la región que muestra el mayor tamaño poblacional, lo que podría implicar una incipiente endogamia.

Palabras clave: diversidad genética, endogamia, microsatélites, patrón de conectividad, península Ibérica.

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Introduction

A thorough knowledge of the genetic diversity, structure and connectivity of a species is crucial for understanding its population dynamics and evolutionary potential, and for determining units of management for future wildlife conservation programmes (Agudo *et al*., 2011). It is assumed that genetic variation is required for individuals to adapt to environmental changes and for populations to remain viable (Reed and Frankham, 2003; Bourke *et al*., 2010). In fact, severe reductions in population size, whether from natural or anthropogenic-related mortality, may lead to a loss of genetic diversity due to drift and to an increase in the frequency of rare deleterious recessive alleles, resulting in reduced fitness through inbreeding depression (Brook *et al*., 2002).

Small populations showing little or no gene flow could eventually show adaptive divergence, in which different alleles arising from genetic drift become established, or may suffer local extinction (Athrey *et al*., 2012 and references therein). Low genetic variation resulting from historically small population sizes, in contrast to that resulting from a recent bottleneck, may have different outcomes for the species (Athrey *et al*., 2012, and references therein). If populations have been isolated or persist at small sizes, they could accumulate inbreeding or lose adaptive genetic potential, interfering with future evolution (Athrey *et al*., 2012). On the other hand, habitat loss and the resulting fragmentation can have many impacts on wildlife populations such as reduction of gene flow, loss of genetic diversity, increased inbreeding, significant changes in genetic structure among isolated populations and reduction in population size (Athrey *et al*., 2012). The effects of fragmentation vary according to many factors: the size, configuration and age of habitat patches; the dispersive capacity of the species, and the characteristics of the matrix between patches. For species that remain widely distributed across fragmented landscapes, connectivity and gene flow between populations may be reduced. In addition, the loss of genetic diversity within isolated patches can lead to a decrease in the ability to adapt to environmental change (Delaney *et al*., 2010).

These effects may be more marked and profound within species whose effective population size is low, such as large birds of prey. Among these, the Eurasian eagle owl *Bubo bubo* (Linnaeus, 1758) is an interesting species that has received little attention from a genetic point of view. The distinctiveness of this species lies in the following: (1) it is the largest owl in the world, with a wide geographical distribution throughout the Palaearctic region, occupying a wide variety of habitats, ranging from Mediterranean scrublands and steppes to boreal forests, including cliffs and deserts (Mikkola, 1994); (2) it is a monogamous long-lived species (Penteriani *et al*., 2010); (3) it has a sedentary and territorial nature, being a dominant predator in the terrestrial ecosystem (Solonen, 2011); (4) it has suffered a demographic decline during the last century in many European countries (Penteriani *et al*., 2002; Zuberogoitia *et al*., 2003), even being completely eradicated in some areas (Dalbeck and Heg, 2006; Schaub *et al*., 2010); and (5) it was considered "vulnerable" (according to Annex II of the CITES, Convention on International Trade of Endangered Species of Wild Fauna and Flora and Annex I of the Council Directives 2009/147/EC on the conservation of wild birds, Birds Directive), but is now classified of "Least Concern" according IUCN criteria (IUCN, 2013), following recent population recovery and range expansion in many regions.

Despite its singularity no population genetic study has considered wild populations of the eagle owl, although many as yet

unresolved questions about its population dynamics, reproduction ecology and dispersal patterns could be answered using molecular markers. Isaksson and Tegelstrom (2002) published the development of seven microsatellites for this species although they were only tested on a limited number of captive individuals from Sweden.

Considering this background, we focused our genetic study on eagle owls in the province of Murcia (SE Spain), where this species shows the highest density and reproductive success observed throughout its European distribution (Penteriani *et al*., 2010). We aimed: (1) to assess spatial patterns of genetic variability by analysing possible differences between the three study units defined and between territories; (2) to understand the genetic connectivity patterns (gene flow); and (3) to analyse the effects of the apparent monogamy of this species on its genetic structure. To reach these objectives, 285 individuals, including adults of both sexes and chicks, were analysed, using seven microsatellites as molecular markers.

We expected to find high genetic variability as result of high gene flow between our study units and also from outside our study area. The long-term persistence of our study populations could also favour their genetic diversity, considering the large number of territorial pairs, their high reproductive success, the abundance of food resources and the availability of optimal habitat.

Material and methods

Study area

This study was carried out in the province of Murcia (southeastern Spain). This area is a quaternary sedimentary basin surrounded by two mountain systems (fig. 1). In the northern limit, a mountain chain extends from northeast to southwest, with altitudes ranging from 40 to 646 m a.s.l. It includes two protected areas: "El Valle y Carrascoy" Regional Park and "Monte El Valle y Sierras de Altaona y Escalona" Special Protection Area (SPA: Site Code: ES0000269). The southern limit of the basin is formed by a coastal massif running west to east (0-629 m a.s.l) that includes the "Calblanque, Monte de las Cenizas y Peña del Águila" Regional Park and the "Sierra de la Fausilla" (ES0000199) and "La Muela-Cabo Tiñoso" SPAs (ES0000264).

Sample collection

We defined three study units of eagle owls within our study area (fig. 1), on the basis of the ecological features of the habitat and the availability of the main prey species, the rabbit *Oryctolagus cuniculus,* close to the nests (Hiraldo *et al*., 1975; Serrano, 1998). The first study unit is situated in the north (N) of the sampling area, including 43 territories (40 nesting sites/100 km^2) and characterised by an agroforestry habitat of Aleppo pines *Pinus halepensis* and scrubland, low hills with gentle slopes and both dry and irrigated croplands (Penteriani *et al*., 2010). The second study unit, SW, harbouring 15 territories, was in hilly land dominated by scrubland and scattered trees. The third study unit, SE, includes 10 territories in a landscape heavily modified by human practices, with extensive abandoned mines replacing natural vegetation and showing local contamination by heavy metals (Gómez-Ramírez *et al*., 2011; Espín *et al*., 2014). Rabbits were relatively scarce in the SE and SW study units but abundant in the N study unit (Sánchez *et al*., 2004).

As stated, each study unit harbours several territories. A territory is defined as the home range used by a couple, including the hunting and nesting areas. Between 2004 and 2009, 66 eagle owl territories were moni-

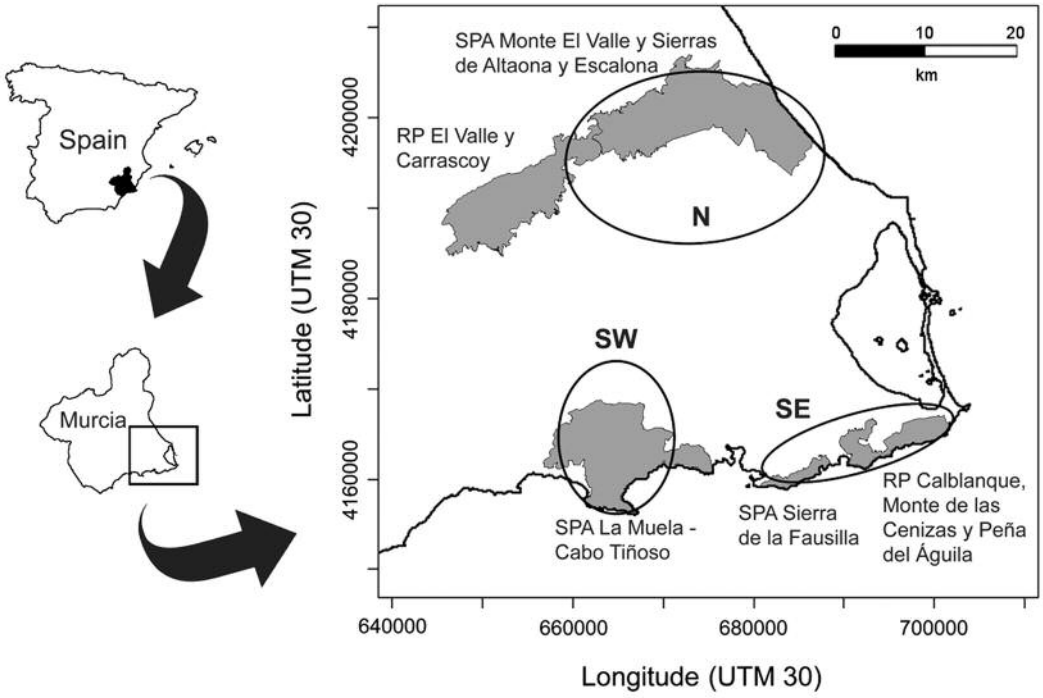

FIG. 1.—Location map of the sampling area, showing the three eagle owl study units (N, SE and SW). The shaded areas represent Regional Parks (RP) and Special Protection Areas (SPAs).
[Mapa de localización del área de muestreo, mostrando las tres unidades de estudio de búho real (N, SE y SO). Las áreas sombreadas representan Parques Regionales (PR) y Zonas de Especial Protección para las Aves (ZEPA).]

tored on a yearly basis, recording occupancy and reproductive parameters. Each year, a number of adult individuals (30 in total during our study period) were trapped in their territories by simulating a territorial intrusion using a mounted owl and a mist net placed strategically close to the nest (Penteriani *et al*., 2007). In addition, 490 chicks were sampled in their nests (1-4 per nest) when they were at least 35-40 days old, so that blood could be extracted safely. Chicks and adults were sampled simultaneously in only 14 of the 151 nests (9.2%). Only one chick was sampled in 39.1% of the nests, 27.5% nests provided two samples and 18% three samples. In total, we sampled 151 nests in 66 territories over seven years. All trapped adult birds and chicks were marked with a metal ring preventing any pseudoreplication, with a mean of 1.54 ± 0.75 sampled individuals per nest and year.

DNA extraction, PCR amplification and microsatellite screening

Blood samples were extracted from the radial vein of eagle owl chicks and adults and stored in absolute ethanol, Li-Heparin, Na-Heparin or frozen. Total genomic DNA was extracted from 80 µl volume of blood following the protocol based on Sambrook

TABLE 1

Characterisation of the six microsatellite loci used to genotype *Bubo bubo* individuals from SE Spain. (T_a = annealing temperature).
[Caracterización de los seis microsatélites loci *usados para genotipar individuos de* Bubo bubo *del SE España. (T_a = temperatura de anillamiento).]*

PCR Multiplex	Locus	Repeat motif	Accession number	Primer sequence (5'-3')	No. of alleles	Allele-size (bp)	T_a (ºC)
BUBO1	Bb131	$(AC)_2C(AC)_6GC(AC)_{11}$	AF32098	F: FAM-AAATGCACTGATTCTTCACTG R-AAACATGCCAGATGCTGTAGC	3	151-155	53
BUBO2	Bb145	$(CA)_4TGCACTT(AC)_{10}$	AF32099	F: HEX-CAAGCTGAAAATACACATACGCAC R-CACAATAGCAGCAGCAGAGTACAG	3	196-200	44
	Bb42	$(CA)_{20}$	AF32093	F: FAM-TTGCATGACATAAAAGAGTG R-AATAAGCCAAGGAAAAGTAG	6	298-310	44
BUBO3	Bb101	$(AC)_{10}$	AF32095	F: FAM-AATAACCCCAATAGAAGC R-ACCAGAAGGAGATGAGACC	5	170-178	50
	Bb111	$(AG)_{10}$	AF32096	F: HEX-CTTTGTCAGTTTTCCCTGTAG R-ATCAGTCAAGTCATCACCAATA	2	199-201	50
	Bb126	$(GA)_{15}$	AF32097	F: NED-TCTCCAGAAGGGTTGTCATC R-TGCTAAAACCTTACAGAATAACAG	7	195-213	50

et al. (1989). Samples were screened for variations in each of the seven microsatellite loci, previously isolated and characterised by Isaksson and Tegelstrom (2002); however, only six of them amplified correctly. Polymorphisms of these six microsatellite loci were tested by two multiplex PCRs (Bubo2 and Bubo3) and one single PCR (Bubo1) designed by our team (table 1) and performed in 10 µl total volume, which included 50 ng of DNA, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 130 µM dNTP's, buffer and 0.1 U *Taq* polymerase. The reaction conditions were: an initial denaturation step of 5 min at 94º C, 30 cycles consisting of 30 s at 94º C, 30 s at 44º C (Bubo2), 50º C (Bubo3) and 50 s at 53º C (Bubo1) annealing temperature, 45 s at 72º C then a final extension step of 5 min at 72º C.

Individuals were genotyped by assessing allele size on an ABI 3700 automated sequencer, using forward primers labelled with FAM (SIGMA), HEX (SIGMA) and NED (Applied Biosystems). Allele scoring was carried out using STRand v. 2.3.94 (Toonen and Hughes, 2001) and the MsatAllele package (Alberto, 2009) of the R statistical software (R Core Team, 2013). Some problems were found in discriminating alleles from Bb131 and Bb145 loci using the chromatograms on STRand, which were resolved through their sequencing.

Statistical analysis

Genetic information was analysed considering two spatial scales, the previously defined study units and territories. Of the 520 individuals sampled, only 285 with complete information from the six microsatellite loci were considered for these analyses: 213 from unit N (23 adults and 190 chicks), 33 from unit SE (2 adults and 31 chicks) and 39 from unit SW (2 adults and 37 chicks).

Allele frequencies, mean and total allelic richness, number of private alleles, and expected (H_e) and observed (H_o) heterozygosity were calculated using GENETIX v. 4.1 (Belkhir *et al*., 2004) and ARLEQUIN v. 3.11 (Excoffier *et al*., 2005). Linkage disequilibrium, i.e. the non-random association of alleles at two or more loci, was also tested for each locus-population combination using the same software, employing a Markov chain method with 10,000 iterations, following the algorithm of Guo and Thompson (1992). Deviations from the Hardy-Weinberg equilibrium (HWE) were characterised by inbreeding index (F_{IS}) and tested using the exact test. In instances where the observed genotype frequencies deviated significantly from HWE, the MICRO-CHECKER v. 2.2.3 software (Van Oosterhout *et al*., 2004) was used to infer the most probable causes of such deviations.

The spatial genetic structure was studied by a set of statistical approaches. Genetic differences between the three study units and territories were compared using Correspondence Analysis on the allelic frequencies (González-Wangüemert *et al*., 2009, 2010, 2012), performed with the BiodiversityR package of the R software. Differences between study units and territories were quantified by F_{ST} (using the estimator θ of Weir and Cockerham, 1984) and tested for allele-frequency heterogeneity using exact tests (20,000 steps). The null hypothesis of no genetic differentiation was tested by permutation methods using GENETIX. Tests of genic and genotypic differentiation (*G*-based) for all pairs of study units and territories were performed using GENEPOP v. 4.0 (Rousset, 2008). Also, Cavalli-Sforza distances were computed and the Bonferroni correction was applied to F_{ST} values and genetic distances. The correlation between genetic and geographic distances was assessed using the Mantel test (1,000 permutations) implemented in Genetix software. The geographical distances (km) were computed as the straight geographical distance between the centroids of each study units. A structured analysis of molecular variance (AMOVA) was also carried out using ARLEQUIN v. 3.11, to assess the component of genetic diversity attributable to: (i) variance between study units; (ii) variance among territories within units; (iii) variance within territories.

To understand the directionality of gene flow, a maximum likelihood approach was used to calculate the effective population sizes (Q) and asymmetrical migration rates between study units. For this analysis, we used the MIGRATE v. 3.0.3 software (Beerli and Felsenstein, 2001). MIGRATE uses a Markov Chain Monte Carlo based (MCMC) approach to explore all possible gene genealogies to provide maximum likelihood estimates of the population size and migration rates compatible with the data. These estimates are computed as $\theta = Ne\mu$ for population size and $M = m/\mu$ as the mutation-scaled migration rate for migration, where *Ne* is the effective population size, *m* is the fraction of the new immigrants in the population per generation and μ is the mutation rate of the gene. The first MCMC run consisted of 10 short chains (sampling 20,000 trees) and one long chain (sampling 10,000 trees) with a burn-in period of 10,000 trees. The Bayesian approach was implemented, enforcing a full migration model, with three replicates run for each dataset (Beerli, 2009). Each analysis was performed with four con-

nected chains, using static heating (1,000,000, 3, 1.5, 1), a burn-in period of 10,000 steps, followed by 90,000 steps, and parameters were recorded every 100 steps. In order to obtain estimates of migration rates per generation (and not scaled by mutation) one general mutation rate was used: 0.1% per generation.

Individual probabilities (Cornuet *et al*., 1999) were also considered in order to determine the most likely geographic origin of each individual sampled and to assign the origin of eagle owls based on genotypic data alone. We assigned each individual to the sample to which it had the highest "probability of belonging" (GENECLASS v. 1.0; Cornuet *et al*., 1999).

Taking into consideration the life patterns of eagle owls with respect to their supposed monogamy, we also tested the presence of bottlenecks in samples. Bottlenecks can be detected from the depletion of both allele numbers and heterozygosity excess. We used the Sign and Wilcoxon tests implemented in the BOTTLENECK v. 1.2.2 software (Piry *et al*., 1999). Computations were based on the infinite allele model (IAM) and the two-phased model of mutation (TPM).

Results

Study units

The highest number of alleles was found in northern study unit (N) (table 2), although that unit includes the highest number of sampled individuals, which would increase

Table 2

Estimates of genetic diversity of the study units of *Bubo bubo* based on 6 microsatellite markers. (N, number of individuals; A, number of alleles; AP, number of private alleles; Ho, observed heterozygosity; He, expected heterozygosity; F_{IS}, inbreeding index, measured deviation from Hardy-Weinberg equilibrium. *p < 0.05, **p < 0.01, ***p < 0.001).
[Estimas de diversidad genética de las unidades de estudio de Bubo bubo *basadas en 6 marcadores de microsatélites. N, número de individuos; A, número de alelos; AP, número de alelos privativos; Ho, heterocigosis observada; He, heterocigosis esperada; F_{IS}, índice de endogamia, desviación medida del equilibrio Hardy-Weinberg. *p < 0.05, **p < 0.01, ***p < 0.001.]*

	North (N = 213)					South-east (N = 33)					South-west (N = 39)				
Locus	A	AP	Ho	He	F_{IS}	A	AP	Ho	He	F_{IS}	A	AP	Ho	He	F_{IS}
Bb101	4	1	0.4554	0.4396	–0.0359	2	0	0.6666	0.4960	–0.3512	4	1	0.2820	0.3899	0.2793*
Bb111	2	0	0.0516	0.0593	0.1295	1	0	0.0000	0.0000	–	2	0	0.1538	0.1438	–0.0704
Bb126	7	0	0.6948	0.6653	–0.0444	6	0	0.6969	0.7090	0.0174	7	0	0.7948	0.6859	–0.1612
Bb145	3	0	0.0798	0.0775	–0.0288	3	0	0.1515	0.1449	–0.0458	3	0	0.1282	0.1235	–0.0383
Bb42	6	3	0.6291	0.6196	–0.0153***	3	0	0.5151	0.6568	0.2184	3	0	0.5128	0.4612	–0.1136
Bb131	3	1	0.3896	0.3886	–0.0026	2	0	0.5454	0.4512	–0.2126	2	0	0.3076	0.2637	–0.1692
Total	25	5	0.3834	0.3750	–0.0224	17	0	0.5151	0.4916	–0.0486	21	1	0.3632	0.3447	–0.0545

*p < 0.05, **p < 0.01, ***p < 0.001

the probability of finding private alleles. Indeed, study unit N had the highest number of private alleles (Bb101-178, Bb42-298, Bb42-306, Bb42-310 and Bb131-155), unit SW only had one (Bb101-172), while unit SE did not have any (table 2). Only three alleles (Bb101-170, Bb111-199 and Bb126-213) were shared between study units N and SW. The observed and expected heterozygosity between these sampling sites at the different loci were not always equal, unit SE showing the highest values (table 2). Linkage disequilibrium was detected in two loci pairs, Bb101/Bb111 and Bb101/Bb131, but not in all study units. Therefore all the loci were used for the subsequent analysis. Departures from the Hardy-Weinberg equilibrium were observed in the three study units, with SW showing the lowest value indicating the greatest deviation from HWE (F_{IS} = –0.0545), although these departures were not significant. When all loci were analysed separately, the departure was mainly due to three loci (Bb131, Bb126 and Bb111). The MICRO-CHECKER software did not detect the presence of null alleles in any locus.

To detect a possible genetic structure among the three study units considered, a Correspondence Analysis was performed using the allele frequencies. Components I and II explained 66.79% of the variance, with a gradient being detected along component

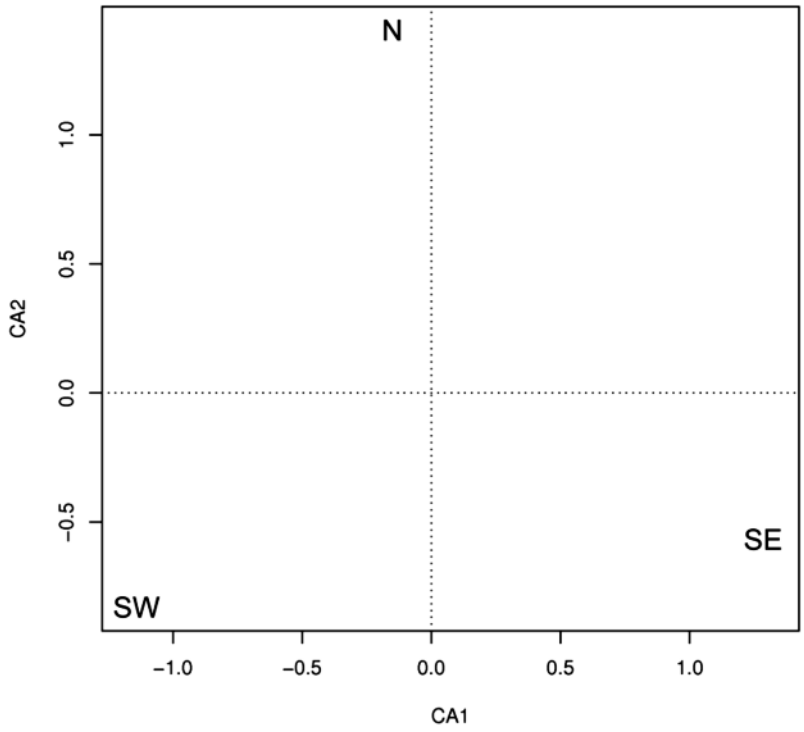

FIG. 2.—Correspondence analysis of genotype frequencies of three populations of eagle owl in SE Spain. (N: North; SW: South-west; SE: South-east).
[Análisis de correspondencias de las frecuencias genotípicas de tres poblaciones de búho real en el SE de España. (N: norte; SW: suroeste; SE: sureste).]

I that sets SE on one side and N and SW on the opposite. Component II differentiated between these two last units, situated at the extremes of the gradient (fig. 2). Similar results were obtained when we used the exact test ($P = 0.0175$; 20 000 Markov chains), genic and genotypic differentiation (*G*-based) and Cavalli-Sforza distances (results not shown), pointing to significant differences among study units. F_{ST} values also showed significant genetic differentiation ($P < 0.001$ in all cases) among the three study units with values ranging from 0.0721 (SW-SE) to 0.0179 (N-SW), the F_{ST} value between N-SE being an intermediate 0.0214. According to these significant results, the three study units can be considered different populations. In fact, the gene flow estimate using Migrate software detected limited gene flow between the three populations (fig. 3). N showed higher connectivity with SW (8.72 migrants) than with SE (2.62 migrants); however,

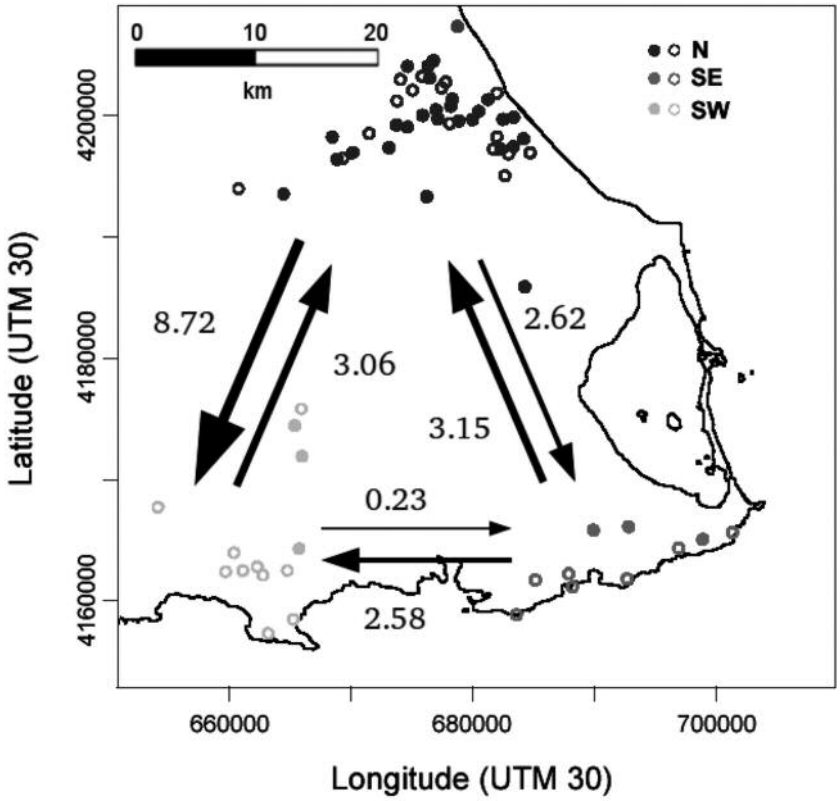

FIG. 3.—Study units and territory distribution of eagle owls in the study area. Each circle represents a territory: the solid circles indicate the territories used for the genetic analysis. Numbers and arrows show the gene flow (number of migrants per generation) among study units obtained using Migrate software. *[Unidades de estudio y distribución territorial de los búhos reales en el área de estudio. Cada círculo representa un territorio: los círculos rellenos indican los territorios usados en el análisis genético. Los números y flechas muestran el flujo génico (número de migrantes por generación) entre unidades de estudio obtenido usando el software Migrate.]*

gene flow between SW and SE was low and mainly in the direction SE to SW (2.58 migrants).

The assignment tests demonstrated that over 50% of individuals were correctly classified into the population from which they originated (fig. 4). It is important to stress that population SE showed the highest self-assignation values (72.72%) which could imply a lower gene flow with the other localities. Also, populations SE and SW had a higher assignation score with population N, demonstrating increased gene flow with this locality. These results corroborated the pattern of gene flow obtained using Migrate software.

The sign and Wilcoxon tests using IAM and TPM models did not detect any bottleneck in our owl populations.

Territory scale

We analysed 34 territories which contributed at least four individuals to the study over the whole period, all them without missing data for the six microsatellite loci (table 3). The highest observed heterozygosity corresponded to TR15-N (H_o = 0.5560) and the lowest to TR10-N (H_o = 0.2000). However, the territory with the highest average allele number was TR31-SW (A = 2. 7) while TR50-SW showed the lowest value (A = 1.5). We also detected a genetic pattern in the southern territories: TR51, TR24 and TR48, from population SE, showed higher heterozygosity than TR31, TR50 and TR30, from population SW, despite the greater number of alleles found in these last territories (except TR50).

The AMOVA identified significant differences between the three populations (4.04%, P = 0.0019). Hierarchical analysis of molecular variance revealed that most of the genetic variance was attributable to variations within the sampled territories (84.5%, P < 0.001), although part of the variance was also explained by the significant differences between territories within populations (11.06%, P < 0.001).

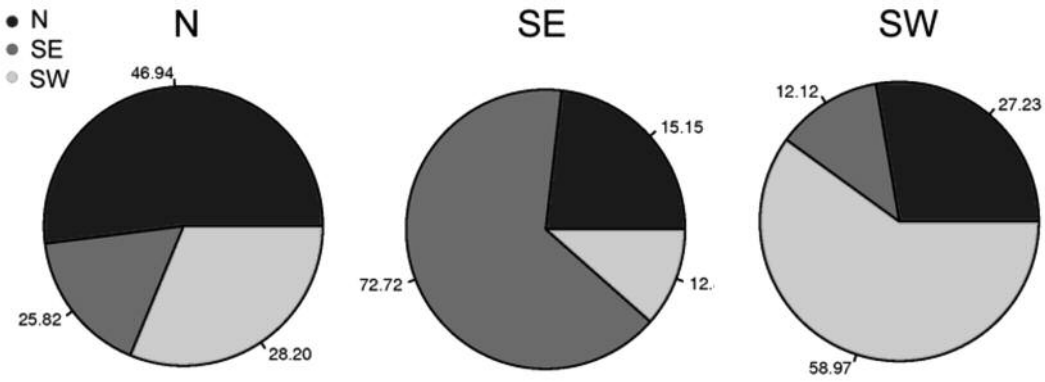

FIG. 4.—Assignment test of eagle owl individuals based on 6 microsatellite loci. The circular charts indicate the adjusted mean probability of the assigned individual actually belonging to a given population, based on the exclusion-simulation test.
[Test de asignación de los individuos de búho real basado en 6 loci *microsatélites. Los gráficos circulares indican la probabilidad de asignación media ajustada de los individuos pertenecientes a una población dada, basada en la prueba de exclusión-simulación.]*

When F_{ST} values were considered to test the genetic differentiation between territories, TR50-SW had high and significant F_{ST} values compared with all the rest. Similar features were observed in territories TR19, TR15, TR10 and TR20 from population N and TR48 from population SE, which presented significant genetic differences with all territories except three. However, other territories, such as TR22, TR25, TR42 and TR47 from population N, showed a high degree of similarity with the other territories (negative or low and non-significant F_{ST} values). The remaining territories did not show any clear connectivity pattern.

Finally, when the existence of bottlenecks was tested, only one territory (TR48-SE) showed significant values for the two tests (sign and Wilcoxon tests) using the IAM and TPM models, although several other territories had significant values for some of tests but not for all simultaneously.

Discussion

Genetic diversity

In general, low genetic variation was found in our eagle owl populations. The number of alleles and the expected heterozygosity showed low values compared with results from other owl species (Thode *et al*., 2002; Henke, 2005; Koopman *et al*., 2007a; Funk *et al*., 2010). The observed heterozygosity was similar to previous findings on other raptor species (Gautschi *et al*., 2003; Martínez-Cruz *et al*., 2004, 2007; Rudnick *et al*., 2005; Literák *et al*., 2007; Banhos *et al*., 2008; Le Gouar *et al*., 2008; Takaki *et al*., 2009; Bourke *et al*., 2010). However, it is important to highlight that there are limitations on interspecific comparisons of genetic diversity parameters, due to different ecological and biological features of the species (including effective population size, number of broods and dispersal patterns) which could affect these parameters. For example, populations with little dispersal and/or decreasing numbers of broods and breeders, could eventually fix some alleles (drift) and lose others, especially those present at low frequency, decreasing their total genetic diversity. Nevertheless, it is important to stress that the earlier work on our target species (Isaksson and Tegelstrom, 2002), could not have established the correct levels of genetic diversity because it used a limited number of reared or captive individuals.

The eagle owl populations studied show low genetic diversity, even in the northern population which harbours a large number of individuals at densities of around 40 pairs per 100 km^2 (Penteriani *et al*., 2010). Certain biological features of eagle owls could explain these results: i) distances and directions of natal dispersal seem to be determined by the location and characteristics each nesting place (Penteriani and Delgado, 2011); ii) juvenile/adult mortality rates are high because of natural or anthropogenic causes (Marchesi *et al*., 2002; Sergio *et al*., 2004; Martínez *et al*., 2006; Schaub *et al*., 2010); and iii) owls exhibit a high degree of monogamy (Marks *et al*., 1999; Muller *et al*., 2001; Arsenault *et al*., 2002; Koopman *et al*., 2007b; Saladin *et al*., 2007).

Bottlenecks were not detected in our populations but these results do not necessarily imply that they did not exist. Although the methods used to detect bottlenecks have tremendous potential for detecting changes in effective population sizes, their power and limitations in natural populations are still being explored (Funk *et al*., 2010). One potential limitation is that bottleneck tests may not have the power to detect relatively slow, steady rates of population decline, as has been found in some other raptors (e.g. northern spotted owls, *Strix occidentalis caurina*; Funk *et al*., 2010) and as may be happening in our eagle owl populations.

TABLE 3

Estimates of genetic diversity of *Bubo bubo* territories based on six microsatellite loci. (N: number of individuals; Ho: observed heterozygosity; He: expected heterozygosity; A: average number of alleles per territory).

		LOCUS							
		Bb101		Bb111		Bb126		Bb145	
TERRITORY	N	He	Ho	He	Ho	He	Ho	He	Ho
TR1N	6	0	0	0	0	0.611	1.000	0	0
TR2N	7	0.133	0.143	0.133	0.143	0.694	1.000	0	0
TR3N	7	0.459	0.714	0	0	0.541	0.429	0	0
TR4N	9	0.401	0.333	0.105	0.111	0.370	0.444	0	0
TR5N	6	0	0	0	0	0.486	0.500	0.278	0.333
TR6N	5	0.480	0.800	0	0	0.420	0.600	0.000	0.000
TR7N	8	0.492	0.875	0	0	0.555	0.750	0.117	0.125
TR9N	8	0.305	0.375	0	0	0.469	0.750	0.219	0.250
TR10N	5	0.460	0.600	0	0	0.460	0.600	0	0
TR11N	9	0.346	0.444	0.198	0.222	0.549	0.667	0.401	0.556
TR13N	5	0.620	0.800	0	0	0.620	0.800	0	0
TR15N	6	0.444	0.667	0	0	0.764	0.833	0.375	0.500
TR19N	4	0.500	1.000	0	0	0.656	1.000	0.219	0.250
TR20N	7	0.500	0.714	0	0	0.520	0.714	0	0
TR21N	11	0.496	0.727	0.484	0.636	0.533	0.727	0	0
TR22N	5	0.320	0.400	0	0	0.580	0.600	0	0
TR25N	4	0.375	0	0	0	0.719	0.750	0	0
TR40N	4	0	0	0	0	0.625	0.750	0	0
TR41N	10	0.180	0.200	0.095	0.100	0.535	0.500	0	0
TR42N	5	0.320	0.400	0	0	0.660	1.000	0	0
TR47N	6	0.486	0.167	0	0	0.611	0.833	0	0
TR49N	10	0.095	0.100	0	0	0.545	0.600	0	0
TR55N	4	0.375	0.500	0	0	0.719	1.000	0	0
TR59N	6	0.486	0.500	0	0	0.653	0.833	0	0
TR61N	7	0.337	0.429	0	0	0.643	0.714	0	0
TR62N	7	0.561	0.714	0	0	0.500	0.429	0	0
TR68N	6	0.444	0.667	0	0	0.653	0.500	0	0
TR69N	4	0.656	1.000	0	0	0.375	0.500	0	0
TR24SE	4	0.500	1.000	0	0	0.219	0.250	0	0
TR30SW	4	0.406	0.500	0	0	0.531	0.500	0.219	0.250
TR31SW	7	0.531	0.429	0	0	0.704	0.857	0.133	0.143
TR48SE	7	0.459	0.714	0	0	0.622	1.000	0.337	0.429
TR50SW	8	0	0	0.375	0.500	0.500	1.000	0	0
TR51SE	7	0.408	0.571	0	0	0.602	0.571	0.133	0.143

TABLE 3 (cont.)

[Estimas de diversidad genética de territorios de Bubo bubo *basadas en seis* loci *microsatélites (N: número de individuos; Ho: heterocigosis observada; He: heterocigosis esperada; A: número medio de alelos por territorio).]*

LOCUS								
Bb42		Bb131		Total				
He	Ho	He	Ho	He	Ho	A	N	TERRITORY
0.611	0.833	0.500	0.333	0.287	0.361	1.833	6	TR1N
0.337	0.429	0.490	0.857	0.298	0.429	2.167	7	TR2N
0.357	0.429	0	0	0.226	0.262	1.833	7	TR3N
0.593	0.444	0.475	0.556	0.324	0.315	2.167	9	TR4N
0.569	0.500	0.486	0.833	0.303	0.361	2.000	6	TR5N
0.460	0.600	0.480	0.400	0.307	0.400	1.833	5	TR6N
0.625	0.875	0.633	0.625	0.404	0.542	2.333	8	TR7N
0.648	0.875	0.539	0.500	0.363	0.458	2.167	8	TR9N
0	0	0	0	0.153	0.200	1.667	5	TR10N
0.438	0.333	0	0	0.322	0.370	2.167	9	TR11N
0.180	0.200	0.420	0.600	0.307	0.400	2.000	5	TR13N
0.500	1.000	0.278	0.333	0.394	0.556	2.333	6	TR15N
0.406	0.500	0	0	0.297	0.458	2.167	4	TR19N
0.571	0.857	0.133	0.143	0.287	0.405	2.000	7	TR20N
0.430	0.455	0	0	0.324	0.424	2.000	11	TR21N
0.620	0.800	0.180	0.200	0.283	0.333	2.167	5	TR22N
0.625	0.750	0.500	0	0.370	0.250	2.167	4	TR25N
0.500	1.000	0	0	0.188	0.292	1.500	4	TR40N
0.635	1.000	0.495	0.500	0.323	0.383	2.333	10	TR41N
0.580	0.600	0.320	0.400	0.313	0.400	2.167	5	TR42N
0.500	0.667	0	0	0.266	0.278	1.667	6	TR47N
0.595	0.800	0.320	0.400	0.259	0.317	2.000	10	TR49N
0	0	0.469	0.750	0.260	0.375	1.833	4	TR55N
0.653	1.000	0.375	0.500	0.361	0.472	2.167	6	TR59N
0.571	0.571	0.459	0.714	0.335	0.405	2.167	7	TR61N
0.459	0.429	0.500	1.000	0.337	0.429	2.000	7	TR62N
0.611	1.000	0.444	0.667	0.359	0.472	2.000	6	TR68N
0.375	0.500	0.500	0.500	0.318	0.417	1.833	4	TR69N
0.500	1.000	0.469	0.750	0.281	0.500	1.667	4	TR24SE
0	0	0.219	0.250	0.229	0.250	2.000	4	TR30SW
0.449	0.571	0.245	0.286	0.344	0.381	2.667	7	TR31SW
0.408	0	0.500	0.714	0.388	0.476	2.000	7	TR48SE
0.375	0.500	0	0	0.208	0.333	1.500	8	TR50SW
0.561	0.857	0.459	0.714	0.361	0.476	2.333	7	TR51SE

Differentiation among populations

Our analysis shows that the three populations differed significantly, with F_{ST} values similar to those detected in other raptor populations separated by hundreds of kilometres (Martínez-Cruz *et al*., 2004; Funk *et al*., 2008; Le Gouar *et al*., 2008; Takaki *et al*., 2009; Hull *et al*., 2010; Agudo *et al*., 2011). Population SE showed the highest F_{ST} values with regard to the other populations, the highest heterozygosity indexes, the lowest number of alleles, no private alleles, the highest assignation index and the lowest connectivity with the other populations. All these features demonstrate that this is the population with the greatest genetic isolation, probably due to its geographical isolation. Population SE is limited by the Mediterranean Sea to the east and south (fig. 1), while to the northward side, several anthropogenic elements (e.g., power lines, roads and fences), may increase the mortality of dispersing individuals. Pesticide use and hunting are also common in this area. All these features involve a high-risk of mortality and a decreasing gene flow in our target species.

The dispersal features of the eagle owl would also explain the observed isolation of population SE. The dispersal pattern of this species, with its anisotropic flow of individuals, is asymmetrical and influenced by the spatial structure and connectivity of patches in the landscape (the number of available settlement areas) and the direction of local winds at the start of dispersal (Penteriani and Delgado, 2009; Delgado *et al*., 2010). Population SE is the least connected by patches with the other two populations and the winds blow predominantly from the west at the beginning of dispersal time.

On the other hand, the comparatively low level of anthropogenic influence could explain the highest gene flow between populations N and SW, increasing the survival chance during dispersal of the young owls in these areas.

Territorial analysis

AMOVA showed that the territorial spatial scale constitutes an important component to explain the genetic variation within populations. In fact according to the F_{ST} values, we found three different genetic patterns among territories. The first is represented by TR50-SW which was significantly different from the rest, showing low gene flow and one low frequency allele (Bb111-199), which persisted in this territory. Such features could be explained considering the persistence of the same breeding pair in this territory throughout the years studied. The second pattern includes five territories (TR19, TR15, TR10, TR20 from population N and TR48 from population SE), which differed from most of the others in their high genetic isolation and low gene flow. All these territories showed the presence of one or two low frequency alleles (Bb145-198 in TR19-N and TR48-SE; Bb126-207 and Bb145-200 in TR15-N; Bb101-170 and Bb126-211 in TR10-N) or common alleles combined in homozygosity with low genotypic frequency (Bb101-176 in TR15.N; Bb42-300 in TR19-N; Bb42-302 in TR20-N; Bb131-153 in TR48-SE). These characteristics could be explained by limited gene flow and a lower turnover rate of breeding individuals, or by the existence of gene flow with individuals from territories outside the study area. Finally the third group consisted of four territories (TR22, TR25, TR42, TR47 from population N) which showed high similarity with the other ones and the most common alleles (Bb101-174, Bb111-201, Bb126-203, Bb126-195, Bb145-196, Bb42-308 and Bb131-151). This scenario suggests high territorial connectivity by means of a high migration rate or substantial changes

of individuals between these territories over the years, leading to their homogeneity. The remaining territories show no clear connectivity pattern.

Concluding remarks

Our results suggest that the effect of environmental features, such as landscape structure and geographical isolation, on the dispersal characteristics of eagle owls could explain the significant genetic differentiation found in several populations at small spatial scales (10–10^2 km). Therefore, care should be taken when designing sampling and treating collection data in owls to carry out genetic studies, because the wrong design could generate biased results and conclusions. It is also important to stress the need to carry out genetic research at the territory level when owls are the target species. This scale provides interesting information about real connectivity patterns, relationships between individuals and turnover rates. This study provides baseline levels of genetic diversity in high-density eagle owl populations that could be used as a reference in other population genetic studies of this species.

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