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SHORT COMMUNICATION

DNA barcoding of Iberian Peninsula and North Africa Tawny Owls *Strix aluco* suggests the Strait of Gibraltar as an important barrier for phylogeographyJorge Doña^{1*}, Francisco J. Ruiz-Ruano^{2*}, and Roger Jovani¹¹Department of Evolutionary Ecology, Estación Biológica de Doñana (CSIC), Avda. Americo Vespucio S/N, Sevilla, Spain and ²Departamento de Genética, Universidad de Granada, Granada, Spain**Abstract**

Eight subspecies have been proposed within the Tawny Owl (*Strix aluco*) species. However, recent molecular data have challenged this view, encouraging further work in this species complex. Here we reevaluated the taxonomic status between the North-Western African Tawny Owl, *S. a. mauritanica*, and its closest Iberian Tawny Owl population (from the *S. a. sylvatica* to *S. a. aluco* clade) separated by the Strait of Gibraltar. The Tawny Owl is a non-migratory and territorial species, and juvenile dispersal is restricted to a few kilometers around the natal site. This limited dispersal and the barrier imposed by the Strait of Gibraltar predicted a strong differentiation between the two populations. We tested this using DNA barcoding, Bayesian phylogenetic and species delimitation analysis. We found that an 81.1% of variation is due to the intergroups variation. In addition, the inter-intraspecific distances distribution revealed a barcoding gap among the two subspecies. Also, posterior probabilities and the P_{AB} value allowed to reject the hypothesis that observed degree of distinctiveness is due to random coalescence processes. These findings clearly support the Strait of Gibraltar as an isolating barrier for this species. The subspecific status is confirmed and species status is even suggested for *S. a. mauritanica*.

Introduction

During the past years, bird barcoding has boosted the reassessment of within-species taxonomy, and three quarters of proposed subspecies have been unsupported by barcoding (Kerr et al., 2007). In parallel, more robust tools for molecular species delimitation have been developed based, for instance, on the existence of a greater genetic distance between interspecific than intraspecific sequences (the barcoding gap concept; Brown et al., 2012; Hebert et al., 2004; Puillandre et al., 2012; Ratnasingham & Hebert, 2013) or coalescence theory (Masters et al., 2011; Pons et al., 2006). This has improved the integration between molecular and classical taxonomic approaches (i.e. integrative taxonomy, Dayrat, 2005; Padial et al., 2010; Will et al., 2005).

The Tawny Owl species (*Strix aluco*) comprises eight recognized subspecies distributed from North Africa to Asia (Holt et al., 1999, Figure 1). The subspecies are differentiated by plumage color and body size, but often overlap in these characters due to large individual variation (Holt et al., 1999). In fact, Brito (2005) found that *S. a. sylvatica* and *S. a. aluco* differentiation was not supported by molecular data. Instead, she found three genetic

Keywords

Bird taxonomy, DNA barcoding, mitochondrial control region, phylogeography, subspecies

History

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clades among European Tawny Owls, which could be explained by three glacial refugia in Iberia, Italy and Balkans (Brito, 2005). This shows that taxonomic status of Tawny Owl subspecies should be rethought.

The North-west African Tawny Owl, *S. a. mauritanica*, is the only representative of the Tawny Owl in Africa (Holt et al., 1999). Interestingly, the closest Tawny Owl population (from the Iberian clade, according to Brito (2005), and classically termed as belonging to *S. a. sylvatica*) is located in the European side of the Strait of Gibraltar. These two populations show morphological differences, being African birds larger (wingspan up to 20% larger) and always gray-brown (Holt et al., 1999), in contrast to *sylvatica* that presents rufous and gray morphs (with intermediate variants) (Galeotti & Cesaris, 1996, Holt et al., 1999).

The biogeographic relevance of the Strait of Gibraltar for most species inhabiting its margins remains unknown (Husemann et al., 2014). For the Tawny Owl, the distance between the two margins of the strait (14.4 km) is within the dispersal range of juveniles (Coles & Petty, 1997; Cramp, 1985). This would suggest that there could be regular gene flow between the two continents and thus likely little genetic differentiation. However, previous morphological evidence (see above) and preliminary results by Brito (2005) would suggest the opposite scenario.

Here, we studied the differentiation between *S. a. mauritanica* and *S. a. sylvatica-aluco*. We explored the genetic divergence presented by Brito (2005) but using a new molecular marker, individuals from both sides of the Strait of Gibraltar, DNA barcoding and species delimitation analysis as has been used in recent studies involving species discovery and delimitation in

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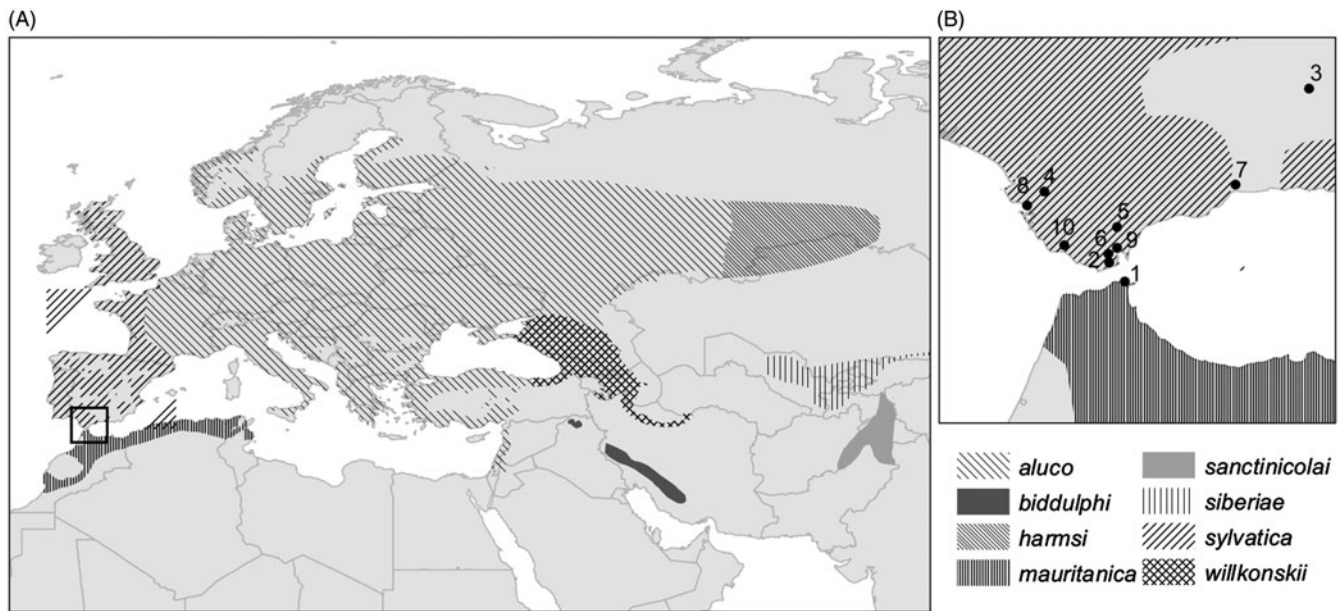


Figure 1. (A) Tawny Owl world distribution. Distribution of subspecies follows Holt et al. (1999) and it is approximate. (B) Sampling localities in Iberian Peninsula (*Strix aluco sylvatica-aluco* clade, following Brito 2005) and North African individuals (*S. a. mauritanica*) used in the current study. Numbers correspond to locality numbers from Supplementary material, Table S1. Note that localities 3 and 7 are wildlife recovery centers. This, jointly with the approximate map by Holt et al. (1999) explains why two localities are apparently outside the distribution of the species.

subspecies complexes (Besansky et al., 2003; Hajibabaei et al., 2007; Masters et al., 2011; Prévot et al., 2013; Smith et al., 2006; Wilson et al., 2013). This allowed, for the first time, to: (1) reevaluate the taxonomic status of *S. a. mauritanica* using a DNA barcoding approach and a species delimitation analysis, and (2) investigate the degree of genetic structure between *S. a. mauritanica* and the Iberian clade of *S. a. sylvatica-aluco* subspecies in their closest populations.

Materials and methods

Sampling

We collected feathers from 16 Tawny Owls from nine locations in South Iberia (Iberian clade of *S. a. sylvatica-aluco*) and four *S. a. mauritanica* individuals from two locations in Ceuta, North Africa (Supplementary material Table S1, Figure 1). Eight *S. a. sylvatica-aluco* and two *S. a. mauritanica* individuals were collected from wildlife recovery centers. The rest of individuals were captured in the field with mist nets. Subspecies were identified based on morphological characters (Svensson et al., 2009). We collected one primary body feather from each individual and stored it into individually labeled plastic bags with silica gel until their utilization for DNA analysis.

PCR amplification and DNA sequencing

We extracted DNA from each sample separately following the method described by Malagó et al. (2002) and used the resulting product as template to amplify the CR2 of the mitochondrial DNA. This marker presents resolution to resolve little differences among species in the *Strix* genera and is variable enough for phylogeographic studies (Brito, 2005). PCR were performed in 25 μ L reaction volumes with 1 μ L DNA template, 1 \times PCR buffer, 2 mM $MgCl_2$, 200 μ M dNTPs, 0.4 μ mol/ μ L of each primer. PCR cycles followed an initial denaturation step of 5 min at 95 $^{\circ}C$, 30 cycles of 30 s at 94 $^{\circ}C$, 30 s at 55 $^{\circ}C$, 30 s at 72 $^{\circ}C$, a final extension step of 7 min. We used the primers ND6Z (5'-ACAACCCATAATACCGCGAAGG-3') and D20 (5'-GTGATGGAT CTTACTAACACC-3') getting fragments of

ca. 700 bp (Barrowclough et al., 1999; Brito, 2005). PCR products were sequenced in both directions using Sanger method by Macrogen, Europe (Holland). Sequences were submitted to GenBank with accession numbers KP977552-KP977571.

Data analysis

Sequences were visually edited with Geneious v4.8 (Drummond et al., 2009) and aligned with MAFFT v7.029b (Katoh & Standley, 2013) applying LINSI options. The final alignment included *Strix uralensis* (GenBank acc. no. DQ087169.1) as an outgroup taxon.

We computed a matrix of pairwise distances using the Kimura 2-parameter (K2P) models with the *sppDistMatrix* function from the R package SPIDER v1.3–0 (Brown et al., 2012). We then performed a barcoding gap analysis and threshold calculations with the *local minima* function (Brown et al., 2012). The specimen identification accuracy was calculated using the “best close match” (BCM) method presented by Meier et al. (2006) with the *bestCloseMatch* function.

We performed a species delimitation analysis with the Genious v4.8 plug-in (Masters et al., 2011) to calculate the Rosenberg’s P_{AB} . This parameter indicates the probability of reciprocal monophyly of the lineage of interest and its nearest defined lineage, under a random branching model (Rosenberg, 2007). We determined the appropriate model of sequence evolution with the JMODELTEST 2 program (Darriba et al., 2012). Then, we performed a Bayesian phylogenetic analysis with MrBayes v3.2 (Ronquist et al., 2012). Convergence of each analysis was evaluated using Tracer v1.4.1 (Rambaut & Drummond, 2007). We also calculated the haplotype and nucleotide diversities with DnaSP v5.10.01 (Librado & Rozas, 2009) and tested the genetic structure among subspecies with an AMOVA in Arlequin v3.5.1.2 (Excoffier & Lischer, 2010).

Results

Twenty individuals from both subspecies were morphologically identified (Supplementary material Table S1). We obtained a final alignment of 678 bp. We found 17 haplotypes, with a haplotype

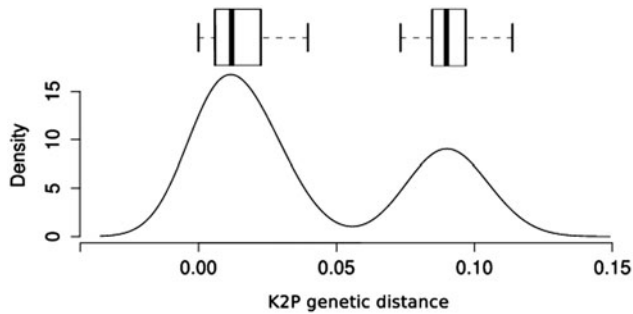


Figure 2. Boxplot of interspecific and intraspecific genetic distances. The median is indicated by the line inside the box and whiskers show data range. The density plot shows the determination of the threshold genetic distance for species identification. The transition between intra- and interspecific distances is the dip in the density graph, here approximately at 5%.

diversity of 0.97. We found 124 polymorphic sites, with an average number of differences of 25.5 and a nucleotide diversity of 0.038. The AMOVA analysis showed that an 82% of variation is due to the intergroup variation (18% intragroup variation), showing an $F_{st} = 0.81$ ($p < 0.00001$).

The frequency distribution of pair-wise sequence distances revealed a clear barcoding gap (Figure 2) with a mean interspecific distance (\pm SD) of $9\% \pm 0.88$ and a mean intraspecific distance of $1\% \pm 1$ (Figure 2). The threshold value separating intra from interspecific distances was *ca.* 5% (Figure 2). In addition, the specimen identification accuracy for both subspecies was 100%.

The Bayesian tree showed the African and the Iberian individuals as belonging to two distinctive clusters with high support (Posterior probabilities = 1) (Supplementary material; Figure S3). In addition, the species delimitation analysis rejected the null hypothesis of a random coalescent process ($P_{AB} < 0.01$).

Discussion

Here we provide the first verification of the DNA barcoding accuracy and its potential utility for Tawny Owl taxonomy. Moreover, our results even suggest that in this case, *mauritanica* could be a sister species of the *aluco-sylvatica* main European clade. Hence, our results support the hypothesis that the Strait of Gibraltar has acted as an important geographic barrier during the recent history of the Tawny Owl phylogeography. In a previous study, Brito (2005) showed that European populations of Tawny Owl comprise three connected clades, and concluded that the Iberian Peninsula acted as refugia during Pleistocene glaciations. Our results clarify the taxonomic issues among these two subspecies, suggesting that *mauritanica* clearly differentiated during this time because of the geographic barrier of the Strait of Gibraltar. However, our results have to be considered as preliminary until a more exhaustive sampling, especially of *S. a. mauritanica*, will be performed.

Here we obtained a genetic distance of 9% between the two studied subspecies analyzing interspecific CR2 distances. This is close to the 11.5% value obtained with the same molecular marker in short-tailed tentative sister albatross (*Phoebastria albatrus*) species, which led to consider them as different species (Eda et al., 2012; Eda & Higuchi, 2012). Indeed, the 9% distance reported here is also higher than the inter-specific distances obtained for CR1 among sister species of albatrosses, which ranged from 4.5 to 7.2% (Abbott & Double, 2003; Burg & Croxall, 2001, 2004; Rains et al., 2011). Moreover, K2P genetic distances followed by posterior probabilities of Bayesian trees and P_{AB} of Rosenberg's have been considered as the most restrictive

parameters for single locus based species delimitation analysis (Boykin et al., 2012). Thus, our results support the subspecies status of this morphological subspecies and even suggest that the North African *mauritanica* subspecies could be a sister species of *sylvatica-aluco* European clade. Further molecular studies addressing taxonomic issues on this species are encouraged because this will likely change the taxonomy of the group, with obvious implications in conservation biology.

Data accessibility

DNA sequences: GenBank accessions: KP977552-KP977571

BOLD public project-ID: Tawny Owl subspecies [TOSP]; <http://dx.doi.org/10.5883/DS-JD2FJ1>

Supplementary material: Figure S3, Table S1 and the alignment are deposited in Figshare; <http://dx.doi.org/10.6084/m9.figshare.1466715>

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Declaration of interest

The authors declare that they have no conflicts of interest. Special thanks to all GOES (The Ornithological Group of the Strait of Gibraltar) members for the economic support for the genetic analysis. JD and RJ were supported by the Spanish Ministerio de Economía y Competitividad (Severo Ochoa scholarship SVP-2013-067939 and Ramón y Cajal research contract RYC-2009-03967, respectively).

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Supplementary material available online