DNA barcoding of Iberian Peninsula and North Africa Tawny Owls *Strix aluco* suggests the Strait of Gibraltar as an important barrier for phylogeography

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Abstract

Eight subspecies have been proposed within the Tawny Owl *Strix aluco*. 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* – *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 some 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 last years bird barcoding has boosted the reassessment of within-species taxonomy, and three quarters of proposed subspecies have been unsupported by barcoding (e.g. 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; Hebert et al. 2004, Brown et al. 2012, Puillandre et al. 2012, Ratnasingham et al. 2013) or coalescence theory (Pons et al. 2006, Masters et al. 2011). This has improved the integration between molecular and classical taxonomic approaches (i.e. integrative taxonomy, Dayrat 2005, Will et al. 2005, Padial et al. 2010).

The Tawny Owl species (*Strix aluco*) comprises eight recognized subspecies distributed from North Africa to Asia (Holt et al. 1999, Fig. 1). Subspecies are differentiated by plumage colour and size, but often overlap in these characters (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 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* (Whiterby 1905), 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 grey-brown (Holt et al., 1999), in contrast to *sylvatica* that presents rufous and grey 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 (Cramp 1985, Coles and Petty, 1997). 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 subspecies complexes (Besansky et al. 2003, Smith et al. 2006, Hajibabaei et al. 2007, Masters et al. 2011, Prévot et al. 2013, Wilson et al. 2013). This allowed, for the first time, to: (1) re-evaluate 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, Fig 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 following the method described in Malago et al. (2002) 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 uL with 1 μL DNA template, 1x PCR buffer, 2 mM MgCl2, 200 μM dNTPs, 0.4 μmol/μL. PCR cycles followed an initial denaturation step of 5 min at 95ºC, 30 cycles of 30s at 94ºC, 30s at 55ºC, 30s at 72ºC, a final extension step of 7 min. We used the primers ND6Z (5'-ACAACCCCATAATACCGCGAAGG-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 by Sanger method by MACROGEN Europe Inc. 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 and Stanley, 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 and Drummond 2007). We also calculated the haplotype and nucleotide diversities with DnaSP v5.10.01 (Librado et al., 2009) and tested the genetic structure among subspecies with an AMOVA in Arlequin v3.5.1.2 (Excoffier et al. 2010).

Results

20 individuals from both subspecies were morphologically identified (Supplementary material Table S1). We obtained a final alignment of 678 bp. We found 17 haplotypes, with an haplotype 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 Fst = 0.81 ($p < 0.00001$).

The frequency distribution of pair-wise sequence distances revealed a clear barcoding gap (Fig. 2) with a mean interspecific distance (±SD) of 9 % ± 0.88 and a mean intraspecific

distance of 1 % ± 1 (Fig. 2). The threshold value separating intra from interspecific distances was ca. 5% (Fig. 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 this 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 and 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 and Double 2003, Burg and 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. 2014). 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]; dx.doi.org/10.5883/DS-JD2FJ1

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

Acknowledgments

Special thanks to all GOES (The Ornithological Group of the Strait of Gibraltar) members for the economic support for the genetic analysis. Especially to Ricardo Campos for field work logistics. Many thanks to GOES people who collected Iberian samples (in alphabetical order): Ricardo Campos, Francisco Jiménez-Cazalla, Alejandro Colorado, David Cuenca, Darío Delgado, Francisco Delgado, José Luis Garzón, Javier Espinosa, Jaime González, Alberto González, Javier González, Juan Manuel Jiménez, José Manuel Pérez, Antonio Sepúlveda. We also want to thank all CHAGRA ringing group members who collaborated collecting North African samples. Many thanks for Andalusian and Ceutan species recovery centres. 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). Finally, thanks to Isabel Afán (LAST, EBD) for her help with Figure 1. Jorge Doña and Francisco J. Ruiz-Ruano contributed equally to this study.

References

Abbott, C.L., Double, M.C. 2003. Phylogeography of shy and white-capped albatrosses inferred from mitochondrial DNA sequences: implications for population history and taxonomy. *Mol Ecol* **12**: 2747 – 2758.

Barrowclough, G. F., Gutiérrez, R. J., and Groth, J. G. 1999. Phylogeography of spotted Owl (Strix occidentalis) populations based on mitochondrial DNA sequences: gene flow, genetic structure, and a novel biogeographic pattern. *Evolution* **53**: 919 - 931.

Besansky, N.J, Severson, D.W., Ferdig, M.T. 2003. DNA barcoding of parasites and invertebrate disease vectors: what you don't know can hurt you. *Trends in Parasitology* **19**: 545 – 546.

Boykin, L. M., Armstrong, K. F., Kubatko, L., and De Barro, P. 2012. Species delimitation and global biosecurity. *Evol Bioinform Online* **8**: 1 - 37.

Brito, P. H. 2005. The influence of Pleistocene glacial refugia on Tawny Owl genetic diversity and phylogeography in western Europe. *Mol ecol* **14**: 3077 - 3094.

Brown, S. D., Collins, R. A., Boyer, S., Lefort, M. C., Malumbres-Olarte, J. A. G. O. B. A., Vink, C. J., and Cruickshank, R. H. 2012. Spider: An R package for the analysis of species identity and evolution, with particular reference to DNA barcoding. *Mol Ecol Resour* **12** : 562 - 565.

Burg, T.M., Croxall, J.P. 2001. Global relationships amongst black-browed and grey-headed albatrosses: analysis of population structure using mitochondrial DNA and microsatellites. *Mol Ecol* **10**: 2647 – 2660.

Burg, T.M., Croxall, J.P. 2004. Global population structure and taxonomy of the wandering albatross species complex. *Mol Ecol* **13**: 2345 – 2355.

Coles, C, Petty, S. 1997. Dispersal behavior and survival of juvenil Tawny Owls (*Strix aluco*) during the low point in a vole cycle. In: *Biology and Conservation of Owls of the Northern Hemisphere. Second International Symposium*. USDA Forest Service NC-**190**: 111–118.

Coyne, J.A., Orr, H. A. 2004. *Speciation*. Sinauer Associates, Sunderland, MA.

Cramp, S. 1985. *The Birds of the Western Palearctic*. Vol. 4. Oxford Univ. Press, Oxford.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D .2012. jModelTest 2: more models, new heuristics and parallel computing. *Nat methods* **9**: 772.

Dayrat, B. 2005. Towards integrative taxonomy. *Biol J Linnean Soc* **85**: 407-415.

Drummond, A. J., Ashton, B., Cheung, M., Heled, J., and Kearse, M. 2009. Geneious 4.8. Biomatters, Auckland, New Zealand.

Eda, M., Koike, H., Kuro-o, M., Mihara, S., Hasegawa, H., and Higuchi, H. 2012. Inferring the ancient population structure of the vulnerable albatross *Phoebastria albatrus*, combining ancient DNA, stable isotope, and morphometric analyses of archaeological samples. *Conserv Genet* **13**: 143 - 151.

Eda, M,. and Higuchi, H. 2012. Does the Short-tailed Albatross *Phoebastria albatrus* consist of two species? *Japanese Journal of Ornithology* **61**: 263 - 272.

Excoffier, L., and Lischer, H. E. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol Resour* **10**: 564-567.

Galeotti, P. & Cesaris, C. 1996. Rufous and grey colour morphsin the Italian Tawny Owl: geographical and environmental influences. J *Avian Biol* **27**: 15–20.

Garcia Mudarra, J.L., Ibáñez, C., and Juste, J. 2009. The Straits of Gibraltar: barrier or bridge to Ibero Moroccan bat diversity? *Biol J Linn Soc* **96**: 434 - 450.

Hajibabaei, M., Singer, G.A.C., Hebert, P.D.N., Hickey, D.A. 2007. DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends Genet* **23**: 167 – 172.

Hebert, P. D., Stoeckle, M. Y., Zemlak, T. S., & Francis, C. M. (2004). Identification of birds through DNA barcodes. *PLoS Biol* **2**: 1657-1663.Holt, W., Berkley, R., Deppe, C., Enríquez

Rocha, P., Petersen, J.L., Rangel Salazar, J.L., Segars, K.P. and Wood, K.L. 1999. *Tawny Owl* (*Strix aluco*). In: del Hoyo, J., Elliott, A., Sargatal, J., Christie, D.A. and de Juana, E. (eds.) (2014). Handbook of the Birds of the World Alive. Lynx Edicions, Barcelona.

Husemann, M., Schmitt, T., Zachos, F. E., Ulrich, W., & Habel, J. C. 2014. Palaearctic biogeography revisited: evidence for the existence of a North African refugium for Western Palaearctic biota. *J Biogeogr* **41**: 81 - 94.

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol biol evol* **30**: 772 - 780.

Kerr, K. C., Stoeckle, M. Y., Dove, C. J., Weigt, L. A., Francis, C. M., & Hebert, P. D. 2007. Comprehensive DNA barcode coverage of North American birds. *Mol Ecol Notes* **7**: 535-543.

Kress, W. J., García-Robledo, C., Uriarte, M., and Erickson, D. L. 2015. DNA barcodes for ecology, evolution, and conservation. *Trends Ecol Evol* **30**: 25-35.

Librado, P., and Rozas, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 145 - 1452.

Malagó, W., Franco, H. M., Matheucci, E., Medaglia, A., and Henrique-Silva, F. 2002. Large scale sex typing of ostriches using DNA extracted from feathers. *BMC Biotechnol* **2**: 19.

Masters, B. C., Fan, V. and Ross, H. A. 2011. Species Delimitation–a Geneious plugin for the exploration of species boundaries. *Mol Ecol Resour* **11**: 154 – 157.

Meier, R., Shiyang, K., Vaidya, G., & Ng, P. K. (2006). DNA barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. *Syst. Biol*, **55**: 715-728.

Padial, J. M., Miralles, A., De la Riva, I., & Vences, M. 2010. The integrative future of taxonomy. *Front Zool* **7**: 1-14.

Phillimore, A. B., and Owens, I. P. 2006. Are subspecies useful in evolutionary and conservation biology? *P Roy Soc Lond B Bio* **273**: 1049 - 1053.

Pons, J., Barraclough, T. G., Gomez-Zurita, J., Cardoso, A., Duran, D. P., Hazell, S., & Vogler, A. P. (2006). Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Syst. Biol*. **55**: 595-609.

Puillandre, N., Lambert, A., Brouillet, S., & Achaz, G. (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Mol Ecol* **21**: 1864-1877.

Prévot, V., Jordaens, K., Sonet, G., and Backeljau, T. 2013. Exploring species level taxonomy and species delimitation methods in the facultatively self-fertilizing land snail genus Rumina (Gastropoda: Pulmonata). *PloS one* **8**: e60736.

Rains, D., Weimerskirch, H., Burg, T.M. 2011. Piecing together the global population puzzle of wandering albatrosses: genetic analysis of the Amsterdam albatross *Diomedea amsterdamensis. J Avian Biol* **42**: 69 – 79.

Rambaut, A., Drummond, A.J. 2007. Tracer v1.4. Available from http://beast.bio.ed.ac.uk/Tracer.

Ratnasingham, S., & Hebert, P. D. (2013). A DNA-based registry for all animal species: The Barcode Index Number (BIN) System. *PloS one* **8**: e66213.

Rosenberg, N. A. 2007. Statistical tests for taxonomic distinctiveness from observations of monophyly. *Evolution* **61**: 317 – 323.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D.L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P. 2012. MrBayes 3.2: Efficient Bayesian

phylogenetic inference and model choice across a large model space. *Syst Biol* **61**: 539 – 542.

Salisbury, C. L., Seddon, N., Cooney, C. R., and Tobias, J. A. 2012. The latitudinal gradient in dispersal constraints: ecological specialisation drives diversification in tropical birds. *Ecol Lett* **15**: 847 - 855.

Smith, M,A., Woodley, N.E, Janzen, D.H., Hallwachs, W., Hebert, P.D.N. 2006. DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). *P Natl Acad Sci Usa* **103**: 3657 – 3662.

Svensson, L., Grant, P. J., and Mullarney, K. 2009. *Collins Bird Guide.* HarperCollins.

Will, K. W., Mishler, B. D., & Wheeler, Q. D. 2005. The perils of DNA barcoding and the need for integrative taxonomy. *Syst. Biol.* **54**: 844-851.

Wilson, J. J., Sing, K. W., and Sofian-Azirun, M. 2013. Building a DNA Barcode Reference Library for the True Butterflies (Lepidoptera) of Peninsula Malaysia: What about the Subspecies? *PloS one* **8**: e79969.

Declaration of Interest

The authors declare that they have no conflict of interest.

Figure Legends

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 centres. This, jointly with the approximate map by Holt et al. (1999) explains why two localities are apparently outside the distribution of the species.

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%.

Figure 1

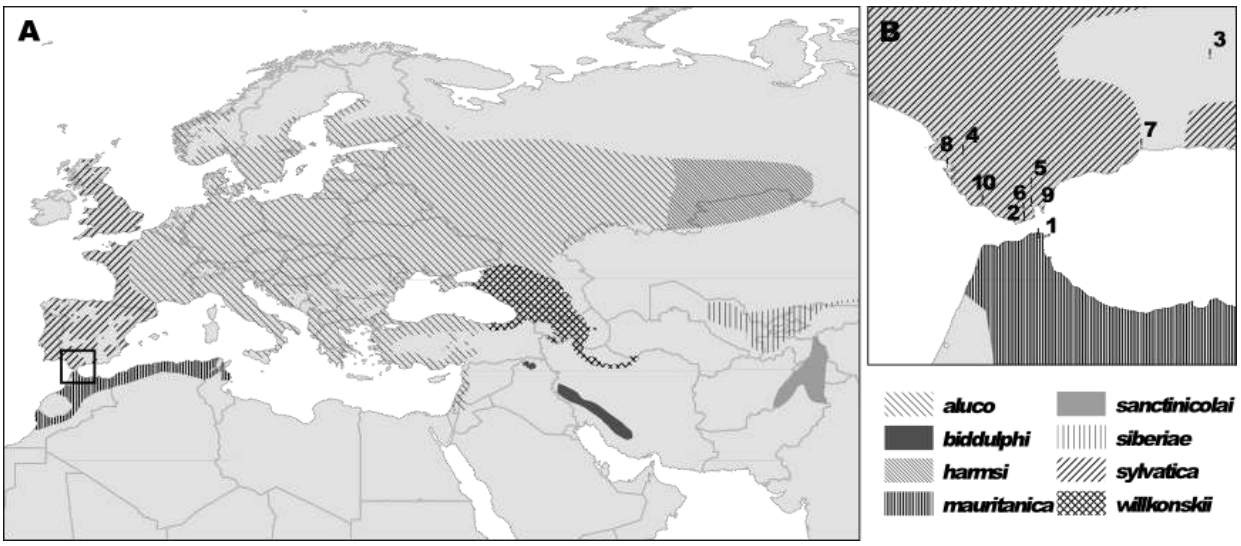
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Figure 2