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Lopes, Ricardo J.; Alves, Jose A.; Gill, Jennifer A.; Gunnarsson, Tomas G.; Hooijmeijer, Joslyn; Lourenco, Pedro M.; Masero, Jose A.; Piersma, Theun; Potts, Peter M.; Rabacal, Bruno

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ORIGINAL ARTICLE

Do different subspecies of Black-tailed Godwit *Limosa limosa* overlap in Iberian wintering and staging areas? Validation with genetic markers

Ricardo J. Lopes · José A. Alves · Jennifer A. Gill · Tómas G. Gunnarsson · Jos C. E. W. Hooijmeijer · Pedro M. Lourenço · Jose A. Masero · Theunis Piersma · Peter M. Potts · Bruno Rabaçal · Sandra Reis · Juan M. Sánchez-Guzman · Francisco Santiago-Quesada · Auxiliadora Villegas

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Abstract Resolving the migratory connectivity (identifying non-breeding grounds) of migrating bird populations that are morphologically similar is crucial for an understanding of their population dynamics and ultimately their conservation. Such is the case in Black-tailed Godwits *Limosa limosa*, where the Iceland-breeding subspecies *islandica* shows overlap during the non-breeding season with the continental-Europe-breeding *limosa*. On the basis of variation in the control region of mitochondrial DNA, it was already shown that there is a clear geographic structure in their phylogeography and a clear discrimination between the haplotypes of the two subspecies. We can thus assign subspecies of non-breeding individuals on the basis of a molecular assay. Here we validated this approach using samples of 113 birds with known breeding origin, and on the basis of haplotype variation, all birds were properly assigned to each subspecies. We then tested for overlap during non-breeding season using a sample of 278 birds from an Iberian wintering and staging area, the inland rice fields in southwest Iberia (Extremadura, Spain). We showed that even in this inland area, 6.5 % of the birds belonged to *islandica* subspecies, thus demonstrating the usefulness of genetic markers as an alternative or supplementary method to the most common approach, individual colour-ringing.

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R. J. Lopes (✉) · B. Rabaçal · S. Reis
CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal
e-mail: riclopes@me.com

J. A. Alves · J. A. Gill
School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, UK

T. G. Gunnarsson
South Iceland Research Centre, University of Iceland, Tryggvagata 36, IS-800 and Gunnarsholt IS-851, Hella, Iceland

J. C. E. W. Hooijmeijer · P. M. Lourenço · T. Piersma
Animal Ecology Group, Centre for Ecological and Evolutionary Studies (CEES), University of Groningen, P.O. Box 11103, 9700 CC Groningen, The Netherlands

P. M. Lourenço
CEAI, Centro de Estudos de Avifauna Ibérica, Rua do Raimundo 119, Apartado 535, 7002-506 Évora, Portugal

J. A. Masero · J. M. Sánchez-Guzman · F. Santiago-Quesada · A. Villegas
Conservation Biology Research Group, Zoology, University of Extremadura, Avenida de Elvas s/n, 06071 Badajoz, Spain

T. Piersma
Department of Marine Ecology, Royal Netherlands Institute for Sea Research (NIOZ), P.O. Box 59, 1790 AB Den Burg, Texel, The Netherlands

P. M. Potts
Solent Court Cottage, Chilling Lane, Warsash, Southampton SO31 9HF, UK

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Zusammenfassung

Überschneiden sich verschiedene Unterarten der Uferschnepfe *Limosa limosa* in den iberischen Winter- und Rastgebieten? Eine Bestätigung anhand genetischer Marker

Die Aufklärung der Zugkonnektivität (Ermittlung der Überwinterungsgebiete) von morphologisch ähnlichen Zugvogelpopulationen ist entscheidend für das Verständnis ihrer Populationsdynamik und somit letzten Endes auch für ihren Schutz. Ein solcher Fall ist die Uferschnepfe *Limosa limosa*, bei der die in Island brütende Unterart *islandica* außerhalb der Brutzeit mit der in Kontinentaleuropa brütenden Nominatform *limosa* zusammentrifft. Anhand der Variation in den Kontrollregionen mitochondrialer DNA konnte bereits gezeigt werden, dass die beiden Unterarten eine klare geografische Struktur in ihrer Phylogeografie und eine deutliche Trennung zwischen den Haplotypen aufweisen. Somit kann man mit molekularen Methoden auch außerhalb der Brutzeit Individuen einer Unterart zuordnen. Hier prüften wir diesen Ansatz anhand einer Stichprobe von 113 Vögeln bekannter Brutherkunft, von denen alle auf der Grundlage der Haplotyp-Variation korrekt der Unterart zugeordnet werden konnten. Anschließend testeten wir mittels einer Stichprobe von 278 Vögeln aus einem iberischen Rast- und Überwinterungsgebiet - den im Inland gelegenen Reisfeldern im Südwesten der Iberischen Halbinsel (Extremadura, Spanien) - auf eine Überlappung außerhalb der Brutzeit. Es zeigte sich, dass selbst in dieser Binnenlandregion 6,5 % der Vögel zur Unterart *islandica* gehörten, wodurch die Eignung genetischer Marker als alternative oder zusätzliche Methode zur individuellen Farbberingung als dem verbreitetsten Ansatz belegt wird.

Introduction

In many migratory species, populations from different breeding areas mix during winter and migration (Boulet and Norris 2006). Such *synhiemy* is opposed to *allohiemy*, a situation in which breeding populations segregate on wintering quarters, either by spatial, demographic or ecological isolation (Salomonsen 1955). These definitions are closely linked with the recent definitions of weak or strong *migratory connectivity* (Webster et al. 2002; Boulet and Norris 2006). We need to know such patterns of population connectedness to understand population dynamics and gene flow, carry-over effects, life-history strategies, and evolution of migratory patterns. This knowledge is also critical for the design of effective conservation plans.

This is certainly relevant for the case of the Black-tailed Godwit *Limosa limosa*, a widespread shorebird that has a population (*islandica* subspecies) that is currently growing and a population (*limosa* subspecies) that is in steep decline (Gill et al. 2007). The latter now qualifies as Near-Threatened (BirdLife International 2010).

The East Atlantic is the only migratory flyway used by both subspecies (Kirby and Scott 2009). The *islandica* breeds predominantly in Iceland, with much smaller numbers in the Faeroe Islands, Shetland (UK) and the Lofoten Islands (Norway). The *limosa* breeds across a wide area extending from Western and Central Europe to central Asia and Asiatic Russia as far east as the river Yenisey, although only the western populations use the East Atlantic flyway. The *islandica* winter mainly in Britain, Ireland, western France, Iberia and Morocco, while *limosa* mainly winter south of Sahara (e.g. Senegal, Guinea-Bissau, Gambia, Mali) although some areas are coincident, namely Iberia, west France and Morocco (Fig. 1a).

Black-tailed godwits are gregarious and make long-distance flights between relatively few staging and wintering areas (Fig. 1a). During northward migration *limosa* birds use staging areas that are used in winter by *islandica* (Lourenço et al. 2010). It was generally assumed that at these overlapping sites the two subspecies use different types of habitat (*islandica* using brackish habitats such as salt marshes and mudflats, and *limosa* using freshwater habitats such as rice fields and natural wetlands). However, analyses of resightings of individually colour-marked birds suggest that this is not the case even in areas with only one type of habitat (Masero et al. 2009; Alves et al. 2010).

Genetic markers (Wink 2006; Lopes et al. 2008) can be used to distinguish different populations. The analysis of a segment of 334 bp from the control region of mitochondrial DNA (mtDNA) already showed that there is clear discrimination of haplotypes between *limosa* and *islandica*, which are private for each subspecies (Höglund et al. 2009) (Fig. 1b).

Here we first test the use of these genetic markers to distinguish *limosa* and *islandica* individuals in non-breeding season, to then quantify the percentage of mixing that occurs during winter and migration in an important inland freshwater stopover in southwest Iberia, assumed to only hold *limosa*. This approach also provides a robust method to validate previous and future results from colour-marking methodology.

Methods

The results from Höglund et al. (2009) indicate that we can assign the subspecies of individuals caught on non-breeding

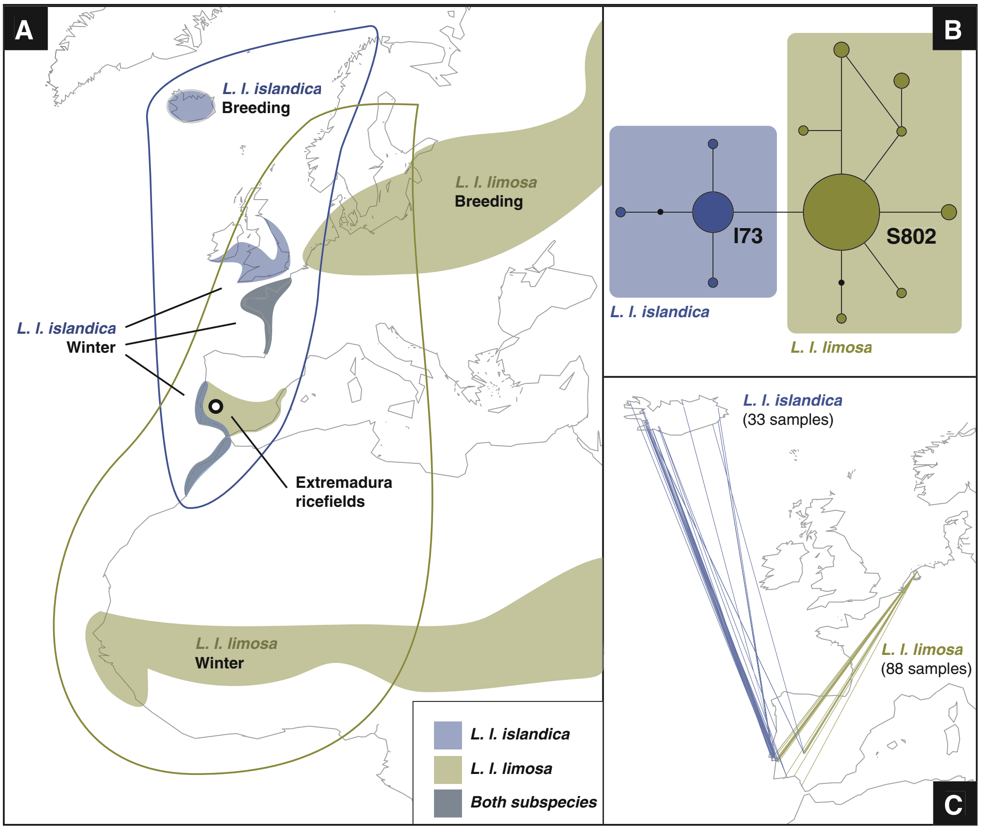

Fig. 1 **a** The most important breeding and wintering areas of the two Black-tailed Godwit subspecies that use the East-Atlantic flyway. Our wintering and staging study site is also shown; **b** Statistical parsimony network of mtDNA control region haplotypes from breeding grounds (Höglund et al. 2009), calculated using TCS 1.2.1 (Clement et al. 2000). *Black dots* are missing haplotypes; **c** Records of color-ringed birds that were used for testing the reliability of genetic assignment

grounds and clearly estimate the proportion of each subspecies in wintering and migrating flocks. As a precautionary test, we verified the accuracy of assignment for Iberia before applying this methodology routinely. Birds from the two subspecies were blood-sampled in breeding areas in Iceland and the Netherlands. Since these birds were all colour-ringed, we were able to select samples of 113 birds that were also registered in Iberia during the non-breeding season (Fig. 1c). This allowed us to analyse their haplotypes in order to test the accuracy of genetic markers.

To test our major hypothesis, the occurrence of mixing of both subspecies during winter and migration, we used samples of birds caught in rice fields of Extremadura, in Southwest Spain (Masero et al. 2011) during winter and migration (January–March), from 2005 to 2008 (Fig. 1a). Birds that roost during the night in a single flock were captured with mist nets at night, and a blood sample was taken from the brachial vein before release. We tested for sex and year differences on the proportion of *islandica* birds using chi-square goodness of fit tests and estimated 95 % confidence intervals for the proportion of *islandica* birds (Zar 2009). We tested for time effect on the proportion of *islandica* birds by dividing the data in 15 days periods (starting at the first day of the year) and using a generalized linear model (GLM) with binomial distribution (Zar 2009). All analysis were performed in R 2.12.1 (R Development Core Team 2011).

The mtDNA was extracted mostly from whole blood and some from feathers using the DNeasy Blood & Tissue Kit and the QIAamp DNA Micro Kit (Qiagen, The Netherlands), respectively. It was then amplified by polymerase chain reaction (PCR), with the primers L438 and H772 (Höglund et al. 2009). The PCR reaction contained 1 μL DNA, 1 μL of each primer (10 μm), 5 μL Phusion® Flash High-Fidelity PCR Master Mix (Finnzymes, Finland) and 3 μL of dH_2O. It included one cycle of 30 s at 98 °C, 35 cycles (3 s at 98 °C, 3 s at 57 °C, and 5 s at 72 °C), and a final extension at 72 °C for 5 min. Sequencing reactions were run in a 3730xl DNA Analyzer (Applied Biosystems, USA) in Macrogen (Seoul, Korea) and results were edited and assembled in Geneious 5.1 (Biomatters, New Zealand). They were then compared to the haplotypes (GenBank accession numbers EU233757–EU233769) found by Höglund et al. (2009). The last 50 samples were assessed after the PCR using a different but alternative method that uses a restriction enzyme that only cuts *islandica* haplotypes. PCR products were digested using the restriction enzyme MboII, incubated at 37 °C for 6 min and at 65 °C for 5 min. The digestion reaction contained 4 μL of PCR product and 0.5 μL enzyme (Fermentas, Life Sciences), 1 μL buffer and 8.5 μL of dH_2O.

The sex of black-tailed godwits is difficult to determine in the field with biometrics and plumage characteristics (Schroeder et al. 2008; Gunnarsson et al. 2006b), but can be assessed using PCR based methods. DNA from the sex chromosomes was amplified using the primers CHD1F and CHD1R (Lee et al. 2010). The reaction contained 0.5 µL DNA, 0.25 µL of each primer (10 µm), 2.5 µL HotStarTaq Master Mix Kit (Qiagen, The Netherlands) and 1.5 µl dH_2O. It included 5 min at 95 °C, 37 cycles (30 s at 94 °C, 20 s at 56 °C, and 40 s at 72 °C), and a final extension at 72 °C for 5 min. The PCR products were separated by electrophoresis in a 2 % agarose gel.

Results

To test the accuracy of our methodology we analysed 33 *islandica* and 80 *limosa* (Fig. 1c). All *islandica* were assigned to haplotype I73 (GenBank accession numbers EU233766), diagnostic of *islandica* subspecies, and all *limosa* birds were assigned to haplotype S802 (GenBank accession numbers EU233757), diagnostic of *limosa* subspecies.

To test our main hypothesis we analysed 278 birds (95 % were adults) and we found 6.5 % (95 % confidence limits: upper = 10.0; lower = 4.1) birds with *islandica* haplotypes. No sex (chi square = 1.29, $p = 0.256$) and no year (chi-square = 0.93, $p = 0.817$) effects were observed in the proportion of *islandica* birds. The proportion of *islandica* birds did not show any significant trend in relation with fortnight (fortnight estimate = 0.06, $p = 0.717$). Fortnight proportions ranged between 3 and 8 % (in the last fortnight no *islandica* bird was found). The highest proportion of *islandica* birds occurred in the fifth fortnight (Fig. 2b) while the peak of abundance of birds occurred in the third fortnight (beginning of February) (Fig. 2a).

Discussion

The initial test clearly confirmed the accuracy of our methodology. Interestingly, only the two most abundant haplotypes (I73 and E802) were found, while the rare haplotypes (Fig. 1b) were not found, confirming that in each subspecies one haplotype is predominant (Höglund et al. 2009). The small *islandica* populations of Faeroe Islands, Shetland and the Lofoten Islands were not sampled in the initial phylogeographic analysis and therefore it is not possible to disregard the possibility that these populations share the two haplogroups. Nevertheless, even considering that possibility, the number of birds that use these breeding sites is very small in relation to the major breeding site, Iceland, and for this reason it is very low the probability that they are among the sampled Iberian birds.

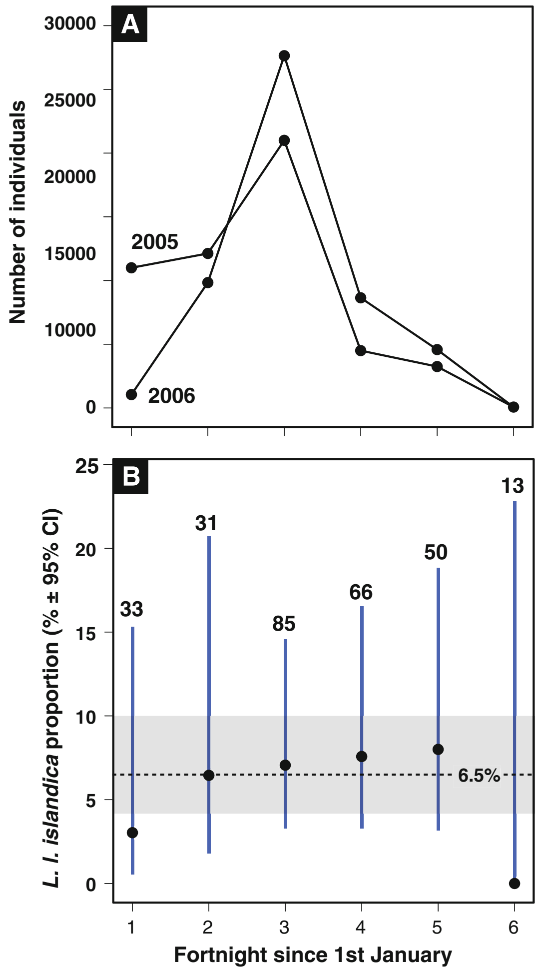

Fig. 2 **a** Number of Black-tailed Godwits in Badajoz rice fields during 2005–2006 (Maximum number of birds recorded per fortnight; see details in Masero et al. (2009); **b** Proportion of *islandica* godwits in Extremadura rice fields per fortnight, showing also 95 % confidence limits and sample sizes. The overall proportion is shown as a *dashed line* with 95 % confidence limits as *a shaded area*

A small but constant proportion (6.5 %) of *islandica* birds in Extremadura rice fields was estimated using genetic markers, which is very near the proportion (7.9 %) estimated using colour-ring data from the same period. However, this estimate is derived from two estimates, which ranged from 14.5 % (117 birds ringed in breeding grounds and registered in Extremadura) to 3.1 % (161 birds ringed in Extremadura and registered in breeding grounds) (Masero et al. 2009). Therefore, it is possible that there is over-estimation of the subspecies proportion when only resightings of breeding birds (the most common methodology) are used. This is probably a result of the different probabilities of resighting (mainly dependent on the real-time proportion of marked birds in the total population), the resighting effort (e.g., number of observers and total time looking for rings), the type of habitat (e.g., density of birds in breeding grounds) and on other smaller biases (e.g., type and quality of the ringing protocols). In 2006 the total estimate of birds ringed in the previous half-decade was estimated to be 1,639 *islandica* and 384 *limosa* (Alves et al. 2010) and the percentage of the total Icelandic population marked with colour-rings (1–2 %) was very high in comparison to the Western European population (Gunnarsson et al. 2006a). Therefore the probability of resighting in wintering grounds is biased towards *islandica* while the probability of resighting in breeding grounds is biased towards *limosa,* since the amount of observers in breeding grounds is higher in continental Europe. One way to reduce the impact of these biases is to calculate the number of colour-ringed godwits in each year from the total of ringed birds and survival rates (Alves et al. 2010), but we do not have data from genetic markers to validate that approach.

The use of genetic markers can therefore validate the estimates from the colour-ring method, and we propose that they can be used in conjunction, since each can balance the other's weaknesses, such as: (1) colour-ring methodology require the marking of birds on breeding sites of both subspecies but requires very little fieldwork in a non-breeding site, while genetic assignment requires the capture of birds in a given site and requires the analysis of samples on a molecular biology laboratory; (2) colour-ringing depends on the continuous ringing of new birds to maintain a reasonable proportion of ringed birds in the populations of both subspecies, while genetic assignment can be done throughout many years with no reliance on additional fieldwork on breeding grounds—in our study our sample size (278 birds) provides a more robust estimate than the estimates based on resightings of breeding birds (117 birds); (3) colour-ringing can be used to estimate proportions on many sites with very little manpower, while genetic assignment requires many ringing teams in order to obtain a reliable sample size.

Concerning our main hypothesis, the presence of this small but constant proportion of *islandica* birds in Extremadura rice fields confirms that overlap of both subspecies in Iberia may be more common than it was supposed. Our study area was chosen because it is an example of an extreme situation, inland freshwater habitats (rice fields) with no adjacent suitable habitats, yet despite that, *islandica* birds were found throughout the whole study period. The small temporal differences on the proportion of *islandica* (Fig. 2b) did not show any significant trend but, if future data unequivocally confirms this trend, this may indicate that *limosa* depart earlier than *islandica*. Colour-ring data for the Tagus estuary (Portugal) also suggests that the estimated proportion of *islandica* birds using rice fields is higher in February (Alves et al. 2010; Lourenço et al. 2010).

It is not clear if the continuous presence of *islandica* birds in inland freshwater habitats is a long term wintering and migrating strategy, or a recent strategy driven by competition of resources. Due to the drainage of wetlands all over Iberia and Maghreb, rice fields are now the main wintering and staging freshwater habitat for black-tailed godwits. Extremadura rice fields span 30,000 ha that were gradually created in the 1960's and 1970's and now support more than 14 % of the western Europe *limosa* population (Masero et al. 2011). Supposedly the presence of *islandica* birds would be more unlikely here than in the other important Iberian locations near the coast, such as Tagus & Sado estuaries, Lower Guadalquivir valley, Ria Formosa and Cadiz Bay, where most of the *islandica* birds occur. Although this may indicate that in these habitats the proportion of mixing may be even higher, the availability of other roosting (salt marshes and saltpans) and feeding habitats (intertidal mudflats) could prevent this mixing, such mixing cannot be avoided in inland rice fields. Despite all this, in the Tagus estuary (Portugal), colour-ring data shows that *islandica* (10 %) were present in rice fields, and that *limosa* (24 %) were also present in estuarine mudflats during winter (Alves et al. 2010). Many wintering populations, despite sharing the same wintering range with resident birds or other wintering populations, do not share ecological niches, given that when niches overlap, competition for resources may occur (Perez-Tris and Telleria 2002). Population overlap in the main freshwater habitat available for foraging Black-tailed Godwits in Iberia can have important conservation implications, especially in the context of the recent population changes that may benefit the increase of *islandica* birds in habitats where both subspecies overlap and where *limosa* birds are declining.

In conclusion, we clearly show that genetics can be used to assign the subspecies of non-breeding black-tailed godwits and that it can be used as an alternative or supplementary method to colour-ringing approaches. We also show that our data and other studies (Alves et al. 2010; Masero et al. 2009) clearly indicate that subspecies overlap occurs in important wintering and migrating sites all over Iberia, and also point out the importance of more detailed studies on the future trends of non-breeding habitat use of these subspecies.

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