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A genetic characterization of European Woodcock (*Scolopax rusticola*, Charadriidae, Charadriiformes) overwintering in Italy

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Abstract
Overwintering and stop-over areas of long-distance migrants need to be identified and studied to ensure effective year-round protection measures. Here, genetic and biogeochemical markers were used to describe the structure and to infer the origin of Italian overwintering stocks of Eurasian Woodcock (*Scolopax rusticola,* Charadridae, Charadriiformes). A 771 base pairs fragment of mitochondrial cytochrome *b* gene was analyzed in 260 specimens sampled in Italy and in Scotland, Croatia, Bulgaria, Hungary and Greece. In addition, nuclear β-Fibrinogen Intron 7 gene (898 base pairs) was analyzed in a subset of 34 samples. Hydrogen/deuterium isotopic ratio was measured in 29 individuals and compared with a map of isotopic values of European rainfalls. The mitochondrial DNA analysis revealed the presence of two different haplogroups in the analyzed sample, occurring sympatrically in many sampling sites. Conversely, nuclear DNA did not show a similar pattern of differentiation. Analysis of synonymous/non-synonymous substitutions ratio in cytochrome *b* data set revealed a striking difference between the two disclosed haplogroups, suggesting the existence of two diverging evolutionary lineages. Genetic traces of demographic expansions were revealed for both mitochondrial lineages. Isotopic data did not suggest a clear separation of the two mtDNA haplogroups in the source breeding range but hinted at the presence of a migration pattern from the north-easternmost portion of the breeding range to the southernmost one of the wintering range. Overall, we proved the existence of two different mitochondrial lineages in the Italian overwintering stock, as well as in the other European winter samples, both characterized by a demographic increase in the recent past. Our results can be considered a valid tool to study migratory connectivity in this species, in comparison with the increasing availability of genetic data from the breeding range.

Keywords: Scolopax rusticola, *mtDNA and nuclear markers*, *stable isotope analysis*, *migratory connectivity*, *population genetics*

Introduction

Scientifically sound flyway conservation strategies require solid information on connectivity between breeding, staging and wintering areas; therefore, the non-breeding areas of long-distance migrants need to be identified when trying to assess conservation actions aiming to ensure effective year-round protection for the species in countries hosting wintering populations (Webster & Marra 2005). The Eurasian Woodcock (*Scolopax rusticola*, Charadriidae, Charadriiformes) breeds widely in central, northern and eastern Europe and winters in much of the western, central and southern portions of the continent (Cramp 1985). This species is a popular game bird which has been experiencing strong hunting pressure across all its range. It has been listed as a species with a vulnerable status in winter (Tucker & Heath 1994). However, population trends and conservation status are still debated (Hagemeijer & Blair 1997; Wetlands International 2002). This debate originates from the extremely large breeding range of the species and the difficulties in properly assessing the status at the European level. European Woodcock decline is known in some countries (e.g. Russia), within a situation of overall moderate decline (>10%). However, population size is evaluated as

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'extremely large' and hence does not approach the thresholds for Vulnerable under the population size criterion (<10,000 mature individuals with a continuing decline estimated to be >10% in 10 years or three generations, or with a specified population structure evaluate; Birdlife International 2009).

The winter season is a critical phase for migrants due to higher densities of birds and a potentially harsh climate, which might also reduce food resources. Winter is also the focal period for additive vs. compensatory mortality, as considered by different and sometimes conflicting hypotheses regarding the effects of harvest on survival (Burnham & Anderson 1984; Conroy et al. 2002). The importance of protected areas in maintaining population stability of wintering Woodcock stocks has been highlighted by Aradis et al. (2008). Ringing data showed that Woodcocks recovered in Italy have been ringed in a large area across Europe and Russia, the latter being the most represented country. Recovery sites in Italy are widely distributed across the country, with areas of concentration along the Prealps and the western coasts of the Peninsula and data confirming sea-crossing strategies during the autumn movements. In this period adult birds ringed in Italy show increasing average wing length, suggesting the presence of birds of different geographical origins (Spina & Volponi 2008).

In the last years, molecular methods have been widely applied to study bird phylogeography and migration (Webster et al. 2002). In particular, the use of mitochondrial DNA markers may provide additional information concerning demographic connectivity between breeding and overwintering sites. However, the resolution at a broad geographic scale often limits information gained from phylogeographic approaches based on mtDNA (Kimura et al. 2002; Lovette et al. 2004). The analysis of rapidly evolving nuclear markers (Fleischer et al. 2008) or the combination of direct (ringing recovery analysis), genetic and biogeochemical methods may provide resolution at a finer geographic scale (Webster et al. 2002). Studies based on combined methodologies have successfully recognized population connectivity in the Wilson Warbler (*Wilsonia pusilla*, Clegg et al. 2003) or a temporal segregation pattern of Dunlin (*Calidris alpina*) populations in Western Europe non-breeding areas (Lopes et al. 2006). The application of biogeochemical markers (stable isotope analysis) to study bird migration can be used as an effective complement to genetic data. In fact, stable isotopes ratios of natural elements (e.g. carbon, hydrogen, nitrogen, oxygen, etc.) in bird feathers mainly reflect the environmental isotopic concentrations of the molting areas (Hobson 1999). These concentrations fluctuate in predictable patterns across Europe. In particular, hydrogen/deuterium (H/D) ratio shows a particular distribution with a depleting gradient from south-west to north-east (Hobson et al. 2004). In long-distance migrants, molting occurs before migration and feathers sampled in overwintering or stop-over areas should reflect isotopic ratios of the breeding sites (Kelly & Finch 1998). Significant correlation of H/D ratios in rainfalls and locally grown feathers was described in North America (Hobson & Wassenaar 1997) as well as in Europe (Hobson et al. 2004). This correlation has been used when studying population connectivity in several migratory birds (e.g. Meehan et al. 2001; Rubenstein et al. 2002).

We attempt to infer the genetic structure of the Italian overwintering stock of Eurasian Woodcock and to discuss its relationship with the breeding areas. The analysis of both mitochondrial and nuclear DNA, implemented by the analysis of H/D stable isotope ratio, may provide useful information to deal with this species and to develop a pan-European conservation strategy. A characterization of woodcock genetic variability at a European scale is in progress (Yves Ferrand, pers. comm., 2009) and our results can be considered as a solid counterpart to study migratory connectivity in this species.

Materials and methods

Sample collection and analysis

We analyzed 260 specimens of *Scolopax rusticola*, sampled in Italy ($n = 189$), Scotland ($n = 19$), Croatia ($n = 2$), Bulgaria ($n = 17$), Hungary ($n = 30$) and Greece ($n = 3$; Figure 1; see supplementary material for sample data, available online via the Supplementary Content tab of the article's online page at http://dx.doi.org/10.1080/11250003.2010.547877). Some samples were provided by authorized ringers following the standardized national field protocols. The outer rectrice (T6) was plucked and preserved in 70% alcohol solution. Tissue samples were also collected by hunters with regular licenses. Genomic DNA was extracted from wing feathers, specifically from the blood clot located in the superior umbilicus of the feather shaft and the terminal portion of the calamus (Segelbacher 2002). Then a fragment of the mitochondrial cytochrome *b* gene (Cyt-*b*: 771 base pairs) was amplified and sequenced for each of the 260 specimens; in addition, Intron 7 of the nuclear β-Fibrinogen gene sequence (β-Fib: 898 base pairs) was analyzed in a sub-sample of 34 specimens. Moreover, a small fragment of mtDNA Control Region (CR: 247 base pairs) was amplified and sequenced in a sub-sample of 29 specimens

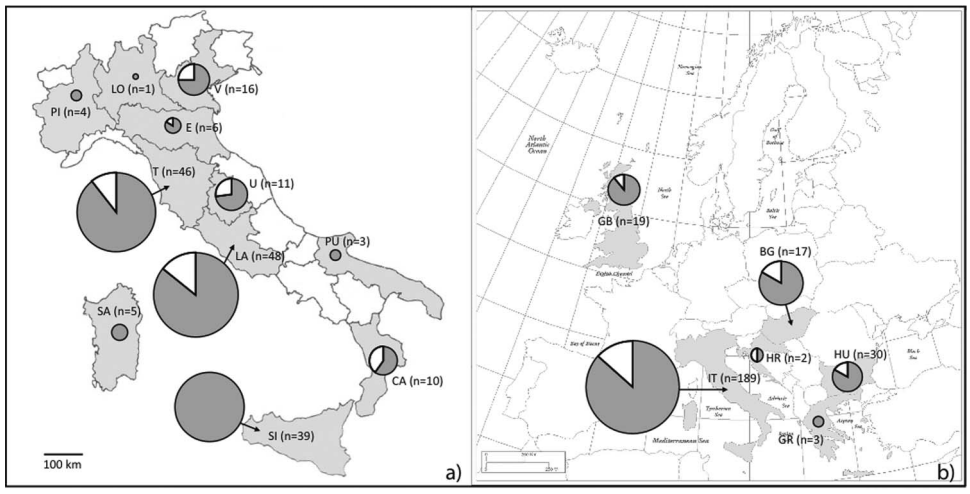

Figure 1. Geographic location and sample size of the analyzed specimens and haplogroup assignment according to Cyt-*b* gene. Circle area is proportional to sample size. County (**a**) and Country (**b**) boundaries are used to summarize samples distribution. County ID is indicated in the name of the sample (prefix). Details about origin of the samples are reported in the supplementary material (available online).

in order to confirm the pattern retrieved in Cyt-*b* diversity.

DNA extraction was carried out with the Sigma-Aldrich GenElute^{TM} Mammalian Genomic DNA Miniprep Kit following the manufacturer's instructions. By analyzing Charadriiformes Cyt-*b* sequences available in GenBank, a new primers pairs (Sr.F1: 5-CTG CAG ACA CAA CCC TAG CC and Sr.F3: 5-CTG TGA TGG GGA AAA GGA CT) was designed in order to amplify a fragment of this mtDNA gene. PCR amplifications were performed under the following conditions: the mix contained 12.5 μm of MasterMix (Promega), 2 μm of each primer (2.5 mM), and 1 μm DNA template in a final volume of 25 μl. Amplifications were performed on Applied Biosystems GeneAMP® PCR System 9700 with the following settings: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 40 s, and a final extension at 72° for 7 min. The sequence of β-Fib gene was amplified using published primers (FIB-BI7U 5-GGA GAA AAC AGG ACA ATG ACA ATT CAC and FIB-BI7L 5-TCC CCA GTA GTA TCT GCC ATT AGG GTT; Prychitko & Moore 1997), with the following settings: 94°C for 4 min, followed by 35 cycles of 94°C for 45 s, 52°C for 45 s, 72°C for 1 min and 15 s, and a final extension at 72° for 10 min. CR fragment was amplified according to Memoli and Paffetti (2007) using the following primers pair: CR-F 5-TGT CCT GCG TTA CTA GCT TCA G and CR-R 5-AGG ACG CCA CGC ACG AGA TGC TC. Each individual was double-stranded sequenced and each singleton was re-sequenced thus excluding Taq polymerase errors. Moreover, as regards Cyt-*b*, different primer pairs were used in order to exclude pseudo-genes and nuclear mtDNA inserted fragment (numt) amplification and each haplotype was checked for open reading frame producing working protein. Purification of PCR products and sequencing on Applied Biosystem Genetic Analyzer 96-capillary were performed by external service (DNA Analysis Facility on Science Hill at Yale University; http://dna-analysis.research.yale.edu//#).

Molecular statistics and phylogenetic analysis

Sequences were aligned using the CodonCode Aligner^{TM} Algorithm (2002–2006, CodonCode Corporation, version 1.6.3). Haplotype diversity, segregating sites, nucleotide diversity averaged per site (Nei 1987) and synonymous/non-synonymous ratio (Cyt-*b* gene) were calculated using Dnasp 4.0 (Rozas et al. 2003). All newly recognized haplotypes were submitted to GenBank database (Accession Numbers: HQ540434–HQ540506; see supplementary material available online). Genealogical relationships among Cyt-*b* and β-Fib haplotypes were calculated with Network 4.502 (Bandelt et al. 1999) using the Median-Joining algorithm. This algorithm is designed for non-recombining bio-molecules, thus an extra step was required in order to analyze the β-Fib data: we used Phase 2.1 software package (Stephens et al. 2001; Stephens & Scheet 2005) that implements a Bayesian statistical method for reconstructing haplotypes from population genotype data. Applying the recombination model (MR option), 5 independent runs were performed (1000 iterations; 100 burn-in) and the convergence of results was checked.

Population demography

The mtDNA Cyt-*b* sequences were analyzed in order to recognize the past demographic trend in *Scolopax rusticola* populations. Two different neutrality tests were used: Fu's Fs (Fu 1997) statistics were calculated, as implemented in Arlequin 3.0 (Excoffier et al. 2005) and R^2 (Ramons-Onsins & Rozas 2002) as implemented in Dnasp 4.0. The significance of Fs was assessed by 10,000 randomizations, while the significance of R^2 was determined by means of 10,000 coalescent simulations based on observed number of segregating sites in the sample. The mismatch distribution of pairwise differences between sequences was examined to find genetic traces of the demographic trend (demographic and spatial expansion). The sum-of-squared-differences (SSD) statistic was used to test the goodness-of-fit between the observed mismatch distribution and that expected under a sudden expansion model. The significance of SSD was assessed by 10,000 parametric bootstrap resamplings (Schneider & Excoffier 1999) using Arlequin 3.0. The McDonald-Kreitman test (McDonald & Kreitman 1991), as implemented in Dnasp, was used to test the null hypothesis of selective neutrality of the Cyt-*b* (*Scolopax minor* sequence, GenBank Acc. AF172384, was included as outgroup) and, then, to interpret the Fu's Fs and R^2 significant negative values as actual population size increase. Significance of the results was determined by means of 10,000 coalescent simulations on the basis of observed number of segregating sites in the sample.

The past population demography was also investigated with a Bayesian Skyline Plot (BSP; Drummond et al. 2005), as implemented in Beast 4.8 (Drummond & Rambaut 2007). This coalescent-based approach calculates the effective breeding population size (N_{ef}) through time directly from sampled sequence data and not from a previously recognized phylogeny. The analysis was done using an 8-groups time segmentation (past N_{ef} points) and HKY + I substitution model (Hasegawa et al. 1985). Two independent runs of 100×10^6 iterations were performed. In addition, the null hypothesis of a constant population size over time was tested. Three independent runs of 30×10^6 iterations were performed, using a coalescent tree prior of constant population size. Convergence of chains, effective sample size, estimates and credible intervals for each parameter and demographic reconstructions were analyzed with the software Tracer 1.4 (Drummond & Rambaut 2007). The Bayesian Skyline Plot model and the null hypothesis of constant population size were compared each other to assess their relative fit to the data. The comparisons were made under Tracer 1.4 workspace by approximating the marginal likelihood ratio between the models (Bayes Factor calculation). The best approximation to the marginal likelihood comparison was found by calculating the Bayes Factor on the treeLikelihood trace (Suchard et al. 2001). Beast runs were performed on the Cornell University Bioinformatic Facility (BioHPC; http://cbsuapps.tc.cornell.edu/beast.aspx).

Stable isotope analysis

We measured H/D ratio only in a subset of 29 samples, because most of our samples had the terminal portion of rachis cut off, making isotopic analysis unfeasible. Feathers H/D ratios were compared with a European rainfalls H/D ratio map (Hobson et al. 2004) to infer the breeding origin of Italian samples. Analysis of H/D ratio was performed following Clegg et al. (2003) protocol at the Geoscience and Georesources Institute of National Research Council, Pisa, Italy. Feathers were washed in SDS 4% and rinsed thoroughly to remove dirt and residual detergent and were then oven-dried at 100°C to remove water. A small piece (0.1–0.2 mg) of the distal end of a feather was taken and wrapped in a silver capsule. This capsule was dropped into a high-temperature reduction furnace (Finnigan TC/EA) interfaced with a mass spectrometer (Finnigan MAT Delta XP). Feather samples were burnt in the reduction furnace at 1450°C. We express the ratio of stable hydrogen isotopes (H/D) in a sample as a deviation from standard mean ocean water expressed in parts per thousand and reported in delta notation: $\delta D = (\text{sample H/D/standard mean ocean water} - 1)$.

Results

The Cyt*b* fragment was amplified in all samples. We found 58 polymorphic sites and 47 different haplotypes (Table II). Twelve substitutions were

Table I. Demographic expansion and selective neutrality tests. Significant values are marked with an asterisk. McD–K: McDonald–Kreitman test, investigated group vs. outgroup (*Scolopax minor*); MD: mismatch distribution.

	Hap-A	Hap-B
Fu's Fs	$-73.9^{\star}$ ($P < 0.0001$)	$-11.22^{\star}$ ($P < 0.0001$)
*R*2 Rozas	$0.0097^{\star}$	$0.0455^{\star}$
Sum-of-Squared-Deviation (MD)	$0.0007^{\star}$	$0.0003^{\star}$
Demographic expansion	($P = 0.69$)*	($P = 0.936$)*
Spatial expansion	$0.0002^{\star}$ ($P = 0.74$)	$0.003^{\star}$ ($P = 0.88$)
τ (MD)	$0.246^{\star}$	$0.827^{\star}$
Fisher Exact Test (McD–K)	$0.035^{\star}$	0.353

Table II. Polymorphic sites, number of individuals (N), GenBank Accession Number and geographic origin of Cyt-*b* haplotypes (Country and County ID correspond to those used in Figure 1).

Hap_ID	N	GenBank ID	003	009	030	033	039	061	097	135	144	156	165	168	176	177	216	226	231	241	249	250	265	288	297	318	325	328	330	336	366	384	396	408	420	448	487	496	502	525	528	549	582	609	612	630	639	654	655	657	663	666	678	679	699	732	750	754	756	762	Geographic ID
Hap_A	182	HQ540434	C	A	T	T	C	C	T	C	A	C	G	T	C	C	T	T	A	A	G	A	G	T	A	C	C	T	A	T	C	T	C	C	T	A	C	A	G	A	A	A	G	C	C	T	G	C	T	A	T	C	C	T	C	T	C	A	T	C	IT (CA, E, LA, LO, PI, PU, SA, SI, T, U, V), BG, GB, GR, HU
Hap_1	1	HQ540435	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (CA)
Hap_6	1	HQ540439	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (E)
Hap_7	3	HQ540440	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (E, LA), HU
Hap_9	1	HQ540464	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (LA)
Hap_10	1	HQ540441	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (LA)
Hap_11	1	HQ540463	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (LA)
Hap_12	2	HQ540442	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (LA), HU
Hap_13	1	HQ540462	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (LA)
Hap_14	1	HQ540461	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (LA)
Hap_15	1	HQ540443	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (LA)
Hap_17	1	HQ540444	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (PI)
Hap_18	1	HQ540445	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (PI)
Hap_19	2	HQ540448	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	IT (SI)
Hap_20	1	HQ540447	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	IT (SI)
Hap_21	2	HQ540472	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	IT (SI)
Hap_22	2	HQ540476	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	IT (SI)
Hap_23	1	HQ540473	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (SI)
Hap_24	1	HQ540477	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	IT (T)
Hap_25	2	HQ540453	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (T, U)
Hap_26	1	HQ540449	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (T)
Hap_27	1	HQ540478	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (T)
Hap_28	1	HQ540479	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (T)
Hap_29	1	HQ540450	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (T)
Hap_30	1	HQ540451	-	G	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (T)
Hap_31	1	HQ540480	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (T)
Hap_33	2	HQ540452	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	IT (U), HU
Hap_35	1	HQ540457	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IT (V)
Hap_36	1	HQ540458	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	IT (V)
Hap_39	1	HQ540475	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	BG
Hap_41	1	HQ540437	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	HR
Hap_42	1	HQ540470	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GB
Hap_43	1	HQ540446	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GB
Hap_45	1	HQ540454	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	HU
Hap_46	1	HQ540455	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	HU
Hap_B	24	HQ540436	-	-	-	-	-	T	C	T	-	T	-	-	-	T	-	-	-	-	-	-	C	-	-	-	C	-	-	T	-	-	C	-	-	-	-	-	-	-	-	-	T	-	-	-	T	-	-	-	-	-	G	-	T	-	-	G	-	T	IT (CA, LA, T, U, V), BG, GB, HR, HU
Hap_4	1	HQ540465	-	-	-	C	-	T	C	T	-	T	-	-	-	T	-	-	-	-	-	-	C	-	-	-	C	-	-	T	-	-	C	-	-	-	-	-	-	-	-	-	T	-	-	A	T	-	-	-	-	-	G	-	T	-	-	G	-	T	IT (CA)
Hap_5	1	HQ540466	-	-	-	-	-	T	C	T	-	T	A	-	-	T	-	-	-	-	-	-	C	-	-	-	C	-	-	T	-	-	C	-	-	-	-	-	-	-	-	-	T	-	-	-	T	-	-	-	-	-	G	-	T	-	-	G	-	T	IT (CA)
Hap_8	1	HQ540438	-	-	-	-	-	T	C	T	-	T	-	-	-	T	-	-	-	-	-	-	C	-	-	-	C	-	-	T	A	-	C	-	-	-	-	-	-	-	-	-	T	-	-	-	T	-	-	-	-	-	G	-	-	-	-	G	-	T	IT (E)
Hap_16	1	HQ540471	-	-	-	-	-	T	C	T	-	T	-	C	-	T	-	-	-	-	-	-	C	-	-	-	C	-	-	T	-	-	C	-	-	-	-	-	-	-	-	-	T	-	-	-	T	-	-	-	-	-	G	-	T	-	-	G	-	T	IT (LA)
Hap_32	2	HQ540468	-	-	-	-	-	T	C	T	-	T	-	-	-	T	-	-	-	-	-	-	C	-	-	-	C	-	-	T	-	-	C	-	-	-	-	-	-	G	-	-	T	-	-	-	T	-	-	-	-	-	G	-	T	-	-	G	-	T	IT (T, U)
Hap_34	1	HQ540467	-	-	-	-	-	T	C	T	-	T	-	-	-	T	-	-	-	-	-	-	C	-	-	-	C	-	-	T	-	-	C	-	-	-	-	-	-	-	-	-	T	-	-	-	T	-	-	C	-	-	G	-	T	-	-	G	-	T	IT (U)
Hap_37	1	HQ540460	-	-	-	-	-	T	C	T	-	T	-	-	-	T	-	-	-	-	-	-	C	-	-	-	C	-	-	T	-	-	C	-	-	-	-	-	-	-	-	-	T	G	-	-	T	-	-	-	-	-	G	-	T	-	-	G	-	T	IT (V)
Hap_38	1	HQ540459	-	-	-	-	-	T	C	T	T	T	-	-	-	T	-	-	-	-	-	-	C	-	-	-	C	-	-	T	-	-	C	-	-	-	-	-	-	-	-	-	T	G	-	-	T	-	-	-	-	-	G	-	T	-	-	G	-	T	IT (V)
Hap_40	1	HQ540474	-	-	-	-	-	T	C	T	-	T	-	-	-	T	-	-	-	-	-	-	C	-	-	-	C	-	-	T	-	-	C	-	T	C	-	-	-	-	-	-	T	-	-	-	T	-	-	-	-	-	G	-	T	-	-	G	-	T	BG
Hap_44	1	HQ540469	-	-	-	-	-	T	C	T	-	T	-	-	-	T	-	-	-	-	-	-	C	-	-	-	C	-	-	T	-	-	C	G	-	-	-	-	-	-	-	-	T	-	-	-	T	-	-	-	-	-	G	-	T	-	-	G	-	T	GB
Hap_47	1	HQ540456	-	-	-	-	-	T	C	T	-	T	-	-	-	T	-	-	G	-	-	-	C	-	-	-	C	-	-	T	-	-	C	-	-	-	-	-	-	-	-	-	T	-	-	-	T	-	-	-	-	-	G	-	T	-	-	G	-	T	HU

non-synonymous and, among polymorphic sites, 29 were parsimony informative. Haplotype diversity and nucleotide diversity were 0.508 and 0.0053, respectively. Two main haplogroups (Hap-A, Hap-B), separated by 12 substitutions, were identified by means of Median-Joining Network analysis (Figure 2). Both haplogroups have a star-like topology and are characterized by 0.347 and 0.560 of haplotype diversity, and 0.0006 and 0.0011 of nucleotide diversity, respectively. As shown in Figure 1, a geographic structure of genetic diversity cannot be recognized in the Italian wintering population as well as in those sampled in Scotland or Eastern Europe. Variability of CR fragment was very low with only one polymorphic site forming two haplotypes (GenBank Accession numbers: HQ540432–HQ540433) in agreement with Cyt-*b* results (Figure 2).

Sequences of 890–898 bp from nuclear β-Fib were aligned in a subset of 34 *Scolopax rusticola* samples. First 26 genotypes were identified and, after gametic phase resolution, 23 polymorphic sites (21 parsimony informative) and 22 haplotypes were recognized in the data set (Table III). An 8-base pairs duplication between position 735 and 742 was detected in 19 samples. Haplotype diversity and nucleotide diversity were 0.928 and 0.0081, respectively. The recombination analysis among different

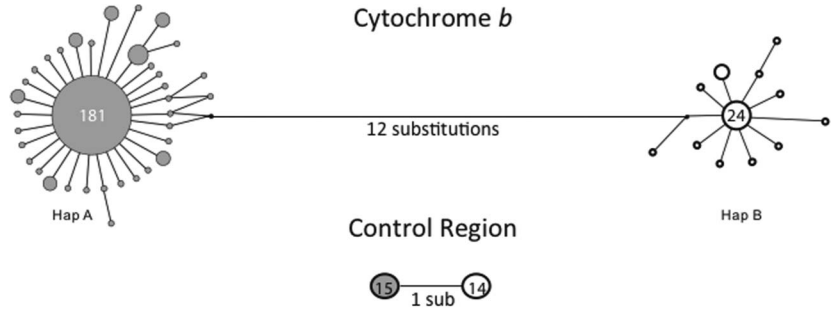

Figure 2. Median-Joining network of Cyt-*b* and CR gene sequences. Circle area is proportional to sample size. Branch length is proportional to nucleotide substitutions. Colors of CR network correspond to Cyt-*b* haplogroups.

Table III. Polymorphic sites, number of individuals (N), GenBank Accession Number of β-Fib genotypes.

Gen_ID	N	GenBank ID	034	040	056	160	174	205	243	277	307	308	318	349	412	474	514	559	636	699	736	744-751	787	788	808	818	843
Genotype4	1	HQ540484	A	G	C	A	G	A	G	A	C	A	G	G	G	G	T	C	G	A	G	del	C	G	C	G	T
Genotype1	1	HQ540481	-	-	-	-	-	-	-	-	S	R	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-
Genotype2	1	HQ540482	-	-	-	-	-	-	-	-	-	R	R	-	-	-	-	-	-	R	-	ins	-	S	-	R	Y
Genotype3	3	HQ540483	R	-	M	G	-	-	-	-	-	-	A	T	T	A	-	A	R	-	S	-	Y	-	-	-	Y
Genotype5	1	HQ540485	-	-	A	R	-	R	-	G	-	-	A	T	-	A	C	A	-	G	-	ins	-	C	Y	-	C
Genotype6	4	HQ540486	-	-	M	R	-	-	-	-	-	-	R	K	K	R	-	M	R	-	-	-	-	-	-	-	-
Genotype7	1	HQ540487	-	-	A	R	-	-	-	G	-	-	A	T	-	A	C	A	-	G	-	ins	-	C	Y	-	C
Genotype8	1	HQ540488	-	-	M	-	-	-	-	-	-	-	A	K	-	R	Y	M	-	G	-	ins	-	C	-	-	C
Genotype9	1	HQ540489	-	-	M	R	-	-	-	-	-	R	R	K	K	R	-	M	-	-	-	-	-	-	-	R	-
Genotype10	1	HQ540490	R	-	-	R	-	-	-	-	-	R	R	K	K	R	-	M	-	-	S	-	Y	-	-	R	Y
Genotype11	1	HQ540491	R	-	-	R	-	-	-	-	-	-	A	T	K	R	-	M	-	-	S	-	Y	-	-	-	Y
Genotype12	1	HQ540492	R	-	M	G	-	-	-	-	-	-	A	T	T	A	-	A	A	-	S	-	Y	-	-	-	Y
Genotype13	1	HQ540493	-	-	A	-	-	-	-	R	-	-	A	T	-	A	C	A	-	G	-	ins	-	C	-	-	Y
Genotype14	1	HQ540494	-	-	-	-	-	-	-	-	-	-	R	K	-	-	-	-	-	-	-	-	-	-	-	-	-
Genotype15	1	HQ540495	-	-	M	-	-	-	K	-	-	-	R	K	-	-	-	-	-	-	-	-	-	-	-	S	-
Genotype16	2	HQ540496	-	-	M	-	-	-	K	R	-	R	R	K	-	R	Y	M	-	R	-	ins	-	S	-	R	Y
Genotype17	1	HQ540497	R	-	Y	R	-	-	-	-	-	R	R	K	K	R	-	M	-	-	S	-	Y	-	-	R	Y
Genotype18	1	HQ540498	-	-	A	G	-	-	-	-	-	-	A	T	T	A	-	A	A	-	-	-	-	-	-	-	-
Genotype19	2	HQ540499	-	-	A	R	-	R	-	R	-	-	R	K	-	R	Y	M	-	R	-	ins	-	S	Y	S	Y
Genotype20	1	HQ540500	-	R	M	R	-	R	-	R	-	-	R	K	-	R	Y	M	-	R	-	ins	-	S	Y	-	Y
Genotype21	1	HQ540501	-	-	A	R	-	-	-	-	-	-	A	T	K	A	Y	A	R	R	-	ins	-	S	-	-	Y
Genotype22	1	HQ540502	-	-	M	-	A	-	-	-	S	-	R	K	-	R	-	M	R	-	-	ins	-	S	Y	-	Y
Genotype23	1	HQ540503	G	-	-	R	-	-	-	-	-	-	A	T	K	R	-	M	-	-	C	-	Y	-	-	-	Y
Genotype24	1	HQ540504	-	-	M	-	-	-	-	R	-	R	R	K	-	R	Y	M	-	R	-	ins	-	S	-	R	Y
Genotype25	1	HQ540505	-	R	-	-	-	-	-	-	-	-	R	K	-	-	-	-	-	-	-	-	-	-	-	-	-
Genotype26	2	HQ540506	-	-	-	-	-	-	-	-	S	-	R	-	-	-	-	-	-	R	-	ins	-	S	-	-	Y

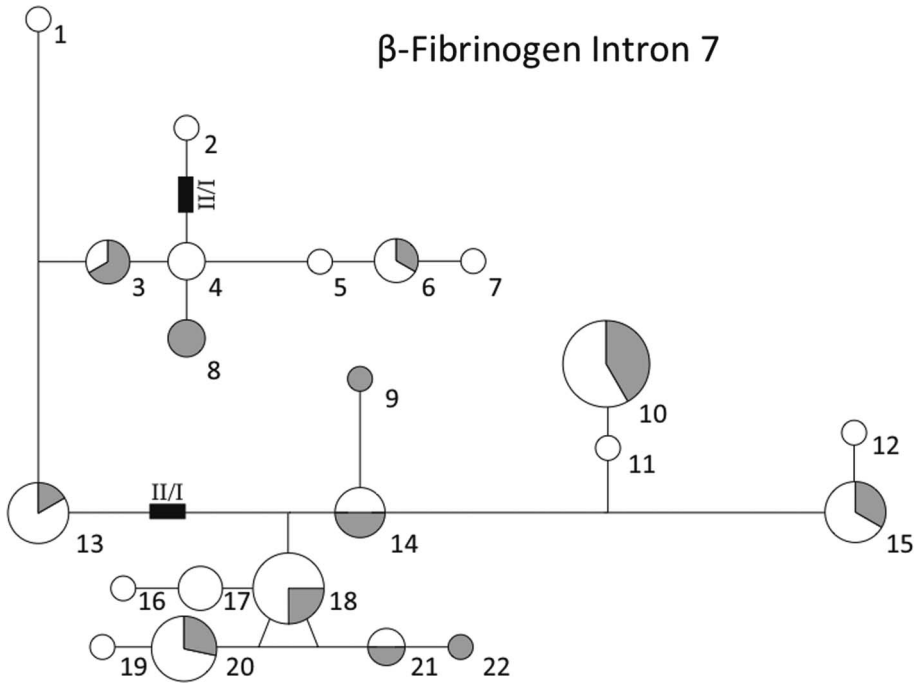

Figure 3. Median-Joining network of β-FIB gene sequences. Circle area is proportional to sample size. Branch length is proportional to nucleotide substitutions. Colors refer to Cyt-*b* gene structure in haplogroup-A (gray) and -B (white). Black rectangle indicates the state of indel mutation: I or II repeats. Samples repartition per haplotype as follows. 1: IT (U); 2: IT (LA); 3: IT (LA, U), GR; 4: BG, HR; 5: HR; 6: IT (T, U); 7: BG; 8: IT (PU); 9: IT (U); 10: IT (E, LA, SI, T, U, V), BG, GB; 11: GB; 12: IT (LA); 13: IT (CL, U, V), GR; 14: IT (LA, V), HU; 15: IT (U, T), HU, GB; 16: IT (U); 17: IT (CL, V); 18: IT (E, LA, SI, U, V), BG; 19: IT (T); 20: IT (CL, PU, U), GB, HU; 21: IT (T), BG; 22: IT (LA).

allelic copies suggested the existence of a minimum number of recombination events Rm = 5 in the data sample (95% CR between 1 and 6; with *P* [Rm <= 5.0000]: 0.93200). The Median-Joining Network of β-Fibrinogen (Figure 3) neither reflects a geographic structure in the wintering individuals, nor correlates with the genetic structure recognized in the mitochondrial cytochrome *b* gene.

Stable H/D isotope analysis was performed on 29 samples (Figure 4). Although with few data corresponding to Hap-B, feathers belonging to both haplogroups showed H/D ratio corresponding to a wide range (from –86.8 to –33.8) of European rainfalls H/D values as mapped in Hobson et al. (2004). However, the isotopic analysis highlighted a geographic differentiation of wintering individuals on the basis of their breeding origin: a clear separation between Northern (including Croatian samples) and Southern Italy (including Bulgarian samples) appeared. Central Italy samples (Latium region) showed H/D ratio values, which correspond to a wide geographic range.

The analysis of synonymous/non-synonymous substitutions ratio revealed a striking difference between the two Cyt-*b* haplogroups: 25/11 and 13/0 for haplogroup A and haplogroup B, respectively. Indeed, the McDonald–Kreitman's test rejected neutral evolution of Cyt-*b* sequences belonging to haplogroup A ($P = 0.035$ with Fisher's exact test); as for the haplogroup B sequences, the test failed to reject neutral evolution ($P = 0.353$ with Fisher's exact test). According to these results, the two haplogroups were considered separately in further demographic analyses.

Genetic signature of past demographic expansion were highlighted by Fu's Fs and Rozas's R^2 in Hap-A as well as in Hap-B. Significant values of these parameters indicate the excess of recent mutation typical of a population size expansion (Table I). The observed mismatch distribution was unimodal and fitted the expected curve of a demographic expansion model (Figure 5); values of τ and the results of the goodness-of-fit test are reported in Table I. Bayesian Skyline Plot showed a substantial population growth and was largely preferred in comparison with the null hypothesis of a constant population size (Figure 5). Concerning haplogroup A sequences, ln Bayes Factor was 3.143, while for haplogroup B, ln Bayes Factor was 3.882. As regards the time elapsed since expansion (in substitutions/site), the Bayesian analysis partially agreed with mismatch distribution: demographic expansion of haplogroup A

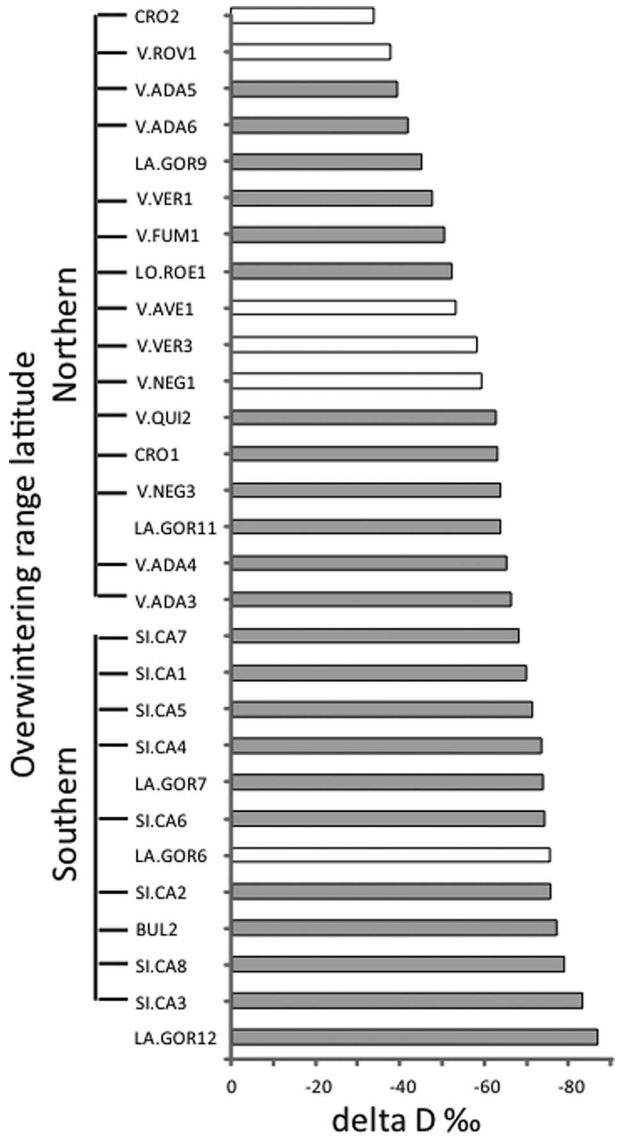

Figure 4. Feathers hydrogen/deuterium ratio expressed as a deviation from standard mean ocean water in parts per thousand and reported in delta notation (delta D‰). Colors refer to Cyt-*b* gene structure in haplogroup-A (gray) and -B (white). Details about origin of the samples are reported in the supplementary material (available online).

was two- to threefold closer in the past than expansion of haplogroup B. The median estimate of the extant populations size provided by Bayesian analysis ($\theta = 2N_{ef}\mu$, where N_{ef} is the effective female population size and μ is the substitution rate) was 0.07 and 0.04 for haplogroup A and B, respectively (95% credible intervals are shown in Figure 5).

Discussion

Mitochondrial DNA analysis revealed two clearly diverging haplogroups. However, the genetic diversity of the Italian populations does not seem to be related to the geographic origin of the samples. Indeed the two haplogroups occur together in different sampling sites. This could result from the presence, within the wintering stock, of individuals originating from different breeding populations. Otherwise, it could be the consequence of a secondary contact between formerly isolated populations, which may have occurred in the breeding range. Having the same mitogenetic pattern, Cyt-*b* sequences of samples collected in England, Croatia, Bulgaria, Hungary and Greece did not show any phylogeographical structure. Lack of phylogeographical structure was also disclosed in the American Woodcock (*Scolopax minor*) by mtDNA analysis performed across the species' range (Rhymer et al. 2005).

Conversely, the analysis of the nuclear β-Fib marker highlighted a different genetic structure between nuclear and mitochondrial genomes. The association of the same β-Fib alleles with both mitochondrial haplogroups and the presence of several recombination events can be explained with an admixture of two formerly differentiated populations. However, a sex-specific dispersal pattern, characterized by a male-mediated gene flow, could influence the genetic structure of biparentally inherited markers (Gay et al. 2004). Despite this, genetic structure of a nuclear marker could also be the result of genetic diversity generated before geographical segregation and preserved through time. It could be the consequence of slow fixation rate of nuclear alleles due to greater N_{ef} and to the possibility of recombination among different alleles.

Even though our analysis relied on a relatively small sample (in particular concerning haplogroup B), H/D stable isotope values of feathers collected in Italy may suggest, to some extent, an overlap between the breeding areas of origin of each mitochondrial haplogroup. H/D feathers ratios proved to be good indicators of H/D ratio detected in sites where feathers were grown (Wunder et al. 2005; Langin et al. 2007). Feathers belonging to haplogroup A showed extremely different H/D ratio values. This means that Italian Hap-A specimens come from geographically different breeding sites, according to the map of geographical distribution of H/D rainfall values across Europe (Hobson et al. 2004). This result suggests the lack of geographic structure in the genetic diversity of source breeding populations of stocks wintering in Italy. The geographical structure of H/D values (high in Southern and low in Northern Italy) deserves further investigations but it could suggest that individuals coming from the north-easternmost breeding range (Baltic and Scandinavian regions) winter in southernmost

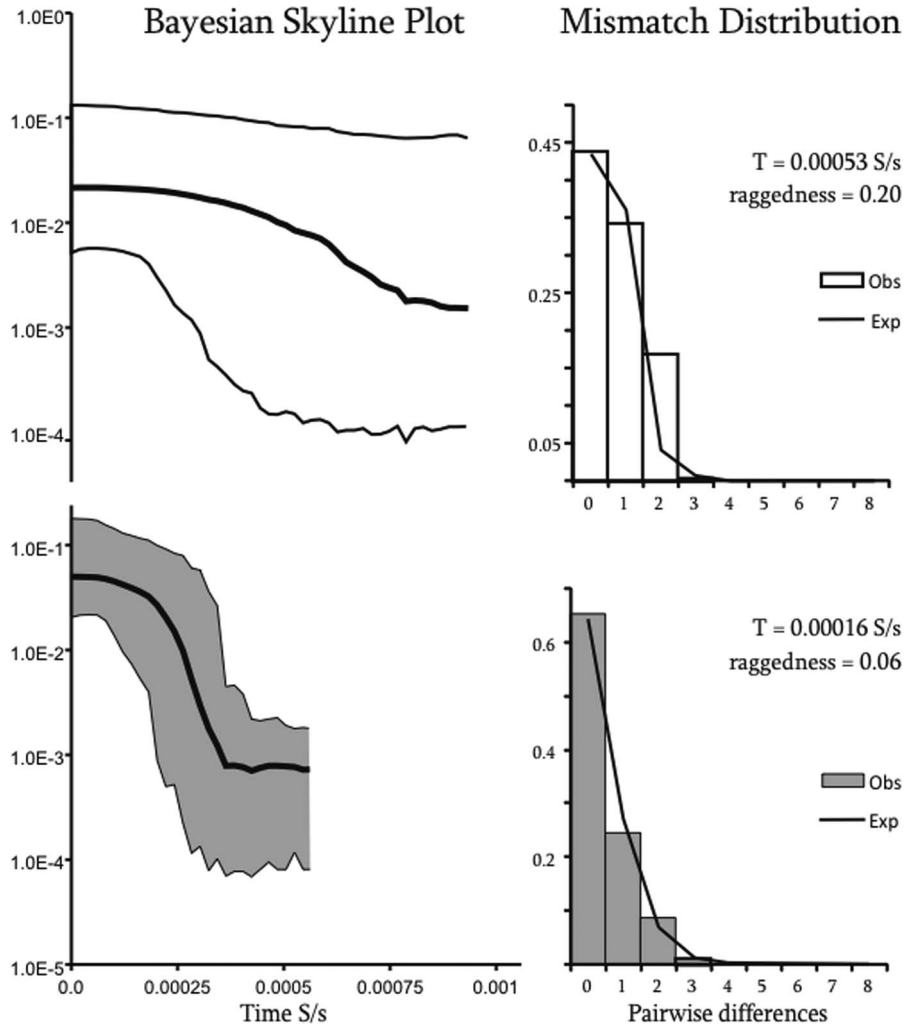

Figure 5. Demographic trend analyses: Bayesian skyline plot and mismatch distribution of the pairwise differences. T: time since expansion; S/s: substitutions per site. Colors (gray and white) refer to Cyt-*b* gene structure in haplogroup-A and -B.

Europe. This is consistent with evidences provided by RAPD (Random Amplification of Polymorphic DNA) analysis in European Woodcock samples coming from Scandinavia, Russia, Italy and Turkey (Burlando et al. 1996) and with the migration pattern. Interestingly, all Sicilian samples, belonging to haplogroup-A, showed low H/D values typical of Baltic and Scandinavian latitudes.

The strong difference observed in synonymous/non-synonymous substitutions ratio further supports a different evolutionary history for the two mitochondrial haplogroups. In particular, haplogroup A showed an excess of non-synonymous substitutions if compared with both haplogroup B and an outgroup (*Scolopax minor*). Many non-synonymous substitutions are expected to be slightly deleterious mutations that will be eliminated by purifying selection after some generations (Gerber et al. 2001) so that synonymous/non-synonymous ratio is expected to be slightly lower in intraspecific polymorphism than in interspecific divergence. However, sudden population size changes (in particular, size increases) can greatly affect this expectation (Charlesworth & Eyre-Walker 2007). By this perspective, the different values recognized for synonymous/non-synonymous parameter in polymorphism of haplogroup A and B strongly suggest that they have experienced separate evolutionary and demographic histories. These findings suggest that mitochondrial structure recognized in the Woodcock samples may be due to a geographical separation of at least two breeding populations. Nonetheless, our preliminary isotopic analysis suggests the co-existence of the two haplogroups at same sites. Given the result of the synonymous/non-synonymous analysis, their contact must have been quite recent for not having affected their differentiation in the molecular substitutions pattern.

Genetic traces of demographic expansions were confirmed by all analyses for both sequence sets belonging to each mitochondrial lineage. However, time elapsed since demographic expansion of haplogroup A resulted two- to threefold closer in the past than that of haplogroup B. Very recent expansion of haplogroup A may be related to the colonization of Scandinavian and Baltic regions after the melting of the last glaciation ice sheet (12,000–14,000 years ago; Rinterknecht et al. 2006) and the appearance of a suitable forest vegetation (10,000–12,000 years ago; Huntley & Birks 1983). In fact, this region hosts the largest European breeding Woodcock populations. The winter presence of individuals belonging to haplogroup-A and coming from different European localities (H/D isotope ratio pattern) in Italy could be the result of a dispersal behaviour after the demographic and, probably, spatial expansion of the northernmost populations as discussed in the American Woodcock (*Scolopax minor*; Rhymer et al. 2005). The possibility of a contact at the wintering grounds (i.e. Latium region) and of a mixed back-migration needs to be investigated further.

Conclusions

Although some clues of a unique European interbreeding population exist, genetic and isotopic data strongly suggest a past separation between two populations, followed by a very recent admixture. We then hypothesize a different evolutionary scenario with two formerly isolated populations and a secondary contact caused by a recent demographic and spatial expansion of one of them. In particular, the haplogroup A seems to be responsible for such recent expansion all over Europe. In this article we clarified some aspects of the genetic structure of *S. rusticola* overwintering stock in Italy. The ongoing genetic characterization of the breeding range at the European level will provide a solid framework to study migratory connectivity in this species and to test our evolutionary and demographic hypotheses.

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