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Phylogeography and Pleistocene refugia of the Little Owl *Athene noctua* inferred from mtDNA sequence data

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Pleistocene glaciations greatly affected the distribution of genetic diversity in animal populations. The Little Owl is widely distributed in temperate regions and could have survived the last glaciations in southern refugia. To describe the phylogeographical structure of European populations, we sequenced the mitochondrial cytochrome *c* oxidase I (COI) and control region (CR1) in 326 individuals sampled from 22 locations. Phylogenetic analyses of COI identified two deeply divergent clades: a western haplogroup distributed in western and northwestern Europe, and an eastern haplogroup distributed in southeastern Europe. Faster evolving CR1 sequences supported the divergence between these two main clades, and identified three subgroups within the eastern clade: Balkan, southern Italian and Sardinian. Divergence times estimated from COI with fossil calibrations indicate that the western and eastern haplogroups split 2.01–1.71 Mya. Slightly different times for splits were found using the standard 2% rate and 7.3% mtDNA neutral substitution rate. CR1 sequences dated the origin of endemic Sardinian haplotypes at 1.04–0.26 Mya and the split between southern Italian and Balkan haplogroups at 0.72–0.21 Mya, coincident with the onset of two Pleistocene glaciations. Admixture of mtDNA haplotypes was detected in northern Italy and in central Europe. These findings support a model of southern Mediterranean and Balkan refugia, with postglacial expansion and secondary contacts for Little Owl populations. Central and northern Europe was predominantly recolonized by Little Owls from Iberia, whereas expansion out of the Balkans was more limited. Northward expansion of the Italian haplogroup was probably prevented by the Alps, and the Sardinian haplotypes remained confined to the island. Results showed a clear genetic pattern differentiating putative subspecies. Genetic distances between haplogroups were comparable with those recorded between different avian species.

Keywords: central western Europe, control region, cytochrome *c* oxidase, postglacial expansion.

Phylogeographical studies indicate that Quaternary climate change played a significant role in shaping contemporary patterns of genetic diversity and the geographical distribution of many Palaearctic plant and animal species (Taberlet *et al.* 1998, Hewitt 2000, Avise 2009). This effect was most pronounced during the late Pleistocene when at least 10 glaciation events occurred in the course of 1 million years (Tzedakis *et al.* 2002). Climatic change induced by glacial and interglacial cycles led to contraction and expansion of species'

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distributions, with the extinction of northern populations when the temperatures decreased, followed by waves of northward expansion from southern refugia when temperatures rose (Hewitt 2011).

During glacial periods, Arctic tundra reached most of central Europe and ice caps covered the Alps, the Pyrenees, part of the Apennines and the Balkans. During such periods, fragmented populations of temperate-adapted species survived in geographically isolated southern refugia, where they evolved distinct genetic traits that may have facilitated local adaptation (Avise & Walker 1998, Stewart *et al.* 2010). In contrast, land-bridge connections due to lower sea levels promoted gene flow among populations from different areas, for instance by connecting Mediterranean islands to the mainland (e.g. Sicily and the southwestern tip of Italy: Bonfiglio *et al.* 2002) or coastlines (e.g. the northeastern and northwestern Adriatic coasts; Taberlet *et al.* 1998). Comparative phylogeographical studies of plant and animal species have identified at least three main glacial refugia in Europe, located in southern Iberia, southern Italy and the Balkans (e.g. Hewitt 1999), although a number of cryptic northern refugia have also been postulated (Stewart & Lister 2001). Postglacial recolonization waves followed a number of different routes, which in some cases caused populations to meet and admix at hybrid zones as a consequence of secondary contact between two or more lineages expanding from separate refugia (Hewitt 2011). The location and genetic structure of some hybrid zones is well known, with lineages from several taxa meeting in central Scandinavia, across central Europe and towards the lower borders of the Pyrenees and the Alps (Taberlet *et al.* 1998, Hewitt 2000). Birds constitute a particularly well-studied group in Europe. The locations of refugia in southern Europe have been documented in several species (Brambilla *et al.* 2008, Hourlay *et al.* 2008, Lehtonen *et al.* 2009) and a pattern of 'refugia within refugia' has been found in some cases (e.g. in Savi's Warbler *Locustella luscinioides*; Neto *et al.* 2012).

Bird populations presently situated in central and northern Europe primarily originate from postglacial expansion out of Iberia (e.g. Red Kite *Milvus milvus*, Roques & Negro 2005) or from southern Italy or the Balkans (e.g. European Green Woodpecker *Picus viridis*, Perktas *et al.* 2011, Pons *et al.* 2011). Postglacial colonization may lead to relatively wide secondary contact zones, as reported for the Coal Tit *Periparus ater* (Pentzold *et al.* 2013). In other species, little population genetic structure at the continental scale has been detected. This is probably a consequence of rapid population expansion from refugia or irruptive and loop migration, as suggested for Great Spotted Woodpecker *Dendrocopos major* (Perktas & Quintero 2013) and Eurasian Reed Warbler *Acrocephalus scirpaceus* (Procházka *et al.* 2011).

The population genetic structure and phylogeography of Palaearctic owls (Strigiformes) is still largely unknown. A mitochondrial DNA (mtDNA) phylogeny for the Tawny Owl *Strix aluco* (Brito 2005, 2007) suggested that this species survived the Pleistocene glaciations in three allopatric refugia located in Iberia, Italy and the Balkans, with the Balkans probably being the predominant source of postglacial recolonization of northern Europe. Northward recolonization from glacial refugia has also been reported in other owl species in North America (Newton 2003). In contrast, no phylogeographical structure was detected in the circumpolar Snowy Owl *Bubo scandiacus*, suggesting the historical persistence of a single panmictic population (Marthinsen *et al.* 2009). The Little Owl *Athene noctua* is a sedentary species, widely distributed throughout temperate and warm Palaearctic regions, from Iberia to China, North Africa and Arabia (Cramp 1985). The species is closely associated with warm arid areas, including arid lands, pastures, steppes, stony deserts, farmland and open woodland (van Nieuwenhuyse *et al.* 2008). The Pleistocene fossil record documents the wide distribution of the Little Owl around the Mediterranean (Tyrberg 1998), including the main islands (Menorca, Mallorca, Tylos, Karpathos, Sardinia and Sicily; Pavia & Mourer-Chauviré 2002). The breeding range of the Little Owl might have only recently expanded to central Europe (Schönn *et al.* 1991), following deforestation and agricultural practices, which created suitable habitat for the species (large pasture fields with perches, breeding cavities) and increased abundance of its insect and small mammal prey (Goldewijk & van Drecht 2006). The Little Owl is described as a single polytypic species (Cramp 1985, del Hoyo *et al.* 1999), including a controversial 7–13 subspecies that differ in body size (especially tarsus-length: Cramp 1985) and plumage colour (Vaurie 1960). Three subspecies of *Athene noctua* are usually recognized in Europe: *A. n. noctua* (Scopoli 1769), widespread in central Europe (including southern Italy, Sardinia and Sicily); *A. n. vidalii* (Brehm 1857), distributed

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mainly in western and northwestern Europe (from the Balearic Islands to northwest Russia); and *A. n. indigena* (Brehm 1855) in the Balkans, southern Ukraine, southern Russia, Caucasus and southwest Siberia, Crete, Turkey and the Middle East. A fourth subspecies, *A. n. sarda*, was proposed for the population of Sardinia (Kleinschmidt 1907), but it is not widely accepted (del Hoyo *et al.* 1999).

Adaptation to temperate and warm climates, and limited dispersal (Cramp 1985, van Nieuwenhuyse *et al.* 2008), may have forced Little Owl populations to survive in isolation in southern refugia during the glacial periods in Europe, as found in the Tawny Owl (Brito 2005). A southern refugia phylogeographical model could be tested through the use of molecular analyses and phylogenetic methods. In this study, mtDNA sequences were used to reconstruct the phylogeography of Little Owl populations sampled throughout the species range in central and southwestern Europe. This study aims to identify glacial refugia, reconstruct the colonization pattern and establish the putative routes of Little Owl expansion during the Pleistocene in Europe, which is needed to fully understand the patterns of connectivity among populations in agricultural and urbanized habitats, data that could contribute to the conservation of this species (Tucker & Heath 1994). Moreover, a molecular analysis is needed to improve the phylogeny of owls (Randi *et al.* 1991, Desmond *et al.* 2001, Wink *et al.* 2004) and to delineate a framework for improved subspecies identification.

METHODS

Sample collection and DNA extraction

Sampling locations were chosen to cover as much as possible the entire distribution of the Little Owl in Europe (Appendix S1). We obtained 326 Little Owl samples from natural history museums, wildlife rehabilitation centres, road-killed individuals or during bird-ringing activities. Two Tawny Owls from northwestern Italy were used as outgroups in phylogenetic analyses of the hypervariable domain I of the mtDNA Control Region (CR1), and a Tawny Owl from northwestern Italy was used as an outgroup for the cytochrome *c* oxidase subunit I (COI) analyses. Mainly feathers, but also muscle tissues (83 samples) and blood (19 samples), were used as a source of DNA. Muscle samples were removed from carcasses and museum skins, and blood was collected by puncturing the brachial vein of living birds. Tissue and feather samples were individually stored in the lab at −20 °C in 95% ethanol; blood samples were stored in Longmire Buffer (Randi *et al.* 2002) and preserved at 2–4 °C. Total genomic DNA was isolated by a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). The extraction protocol was modified for feather samples: feather tips were placed in a lysis buffer designed for small DNA yield (FLB buffer; Macherey-Nagel), and exposed to thermal shock in liquid nitrogen.

PCR amplification and DNA sequencing

A 681-bp region near the 5′ COI terminus was PCR-amplified in a sample set of 276 specimens collected through Europe (Appendix S1) using the universal barcoding primers BirdF1 and BirdR2 (Hebert *et al.* 2004). Furthermore, we amplified domains I and II of the first part of the mtDNA CR in 10 individuals chosen to cover the entire distribution of the Little Owl in Western Europe using primers N1 and D16 (Barrowclough *et al.* 2005). The first 494 bp of the sequences (CR1) contained 95.3% of the variation present in the amplified region, and hence we designed a new forward primer D11AL (GCTCGGGATGTAT AAATGTG – modified from Barrowclough *et al.* 2005) and reverse primer Ath547 (TGTTCTTCA-GAAACCGGAAC) for the PCR amplification of 326 samples (Appendix S1). Both CR1 and COI PCR amplifications were performed in a Bio-Rad C1000 thermal cycler using the following protocol: 94 °C for 5 min, followed by 29–32 cycles at: 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 7 min. PCR products were cleaned with the NucleoSpin Extra II kit (Macherey-Nagel) and were sequenced on an ABI 3730XL (Applied Biosystems, Foster City, CA, USA) automated DNA sequencer using primer D11AL at the 'BMR genomics' lab (Padova, Italy). Haplotype sequences were deposited in GenBank (COI: accession numbers KF452050–KF452083; CR1: accession numbers KF452085–KF452230, *Strix aluco* accession numbers COIKF452084, CR1KF452231, KF452232).

Data analysis

Sequences were aligned using BIOEDIT 7.0.9 (Hall 1999). Unique haplotypes and their nucleotide composition, polymorphic and parsimony-informative

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sites were analysed with TCS 1.13 (Clement *et al.* 2000) and DNASP v. 5.1 (Librado & Rozas 2009). Phylogenetic analyses were performed on COI (681 bp; $n = 276$ sequences + one outgroup), on CR1 (494 bp; $n = 326$ sequences + two outgroups) and concatenated sequences COI + CR1 (1175 bp; $n = 276$ + one outgroup) using four methods: neighbour-joining, maximum likelihood, maximum parsimony and a Bayesian procedure.

The neighbour-joining method (NJ; Saitou & Nei 1987), clustering pairwise Tamura–Nei's (TN93, Tamura & Nei 1993) genetic distances between haplotypes, was performed with MEGA 5.0 (Tamura *et al.* 2011); support was assessed by 1000 bootstrap pseudo-replicates (BP; Felsenstein 1985). Maximum likelihood (ML) and maximum parsimony (MP) trees were obtained through the DNAML, CONSENSE and DNAPARS programs in PHYLIP 3.67 (Felsenstein 2005). Bootstrap values were based on 1000 pseudo-replicates, and the topologies of the trees were visualized with FIGTREE 1.3.1 (Rambaut 2009). Bayesian trees were obtained by the Markov Chain Monte Carlo (MCMC) method implemented in MRBAYES 3.1.2 (Huelsenbeck & Ronquist 2001), which was applied with the following parameters: (1) COI: substitution model = HKY + G, base frequencies: A = 0.2454, C = 0.3405, G = 0.1700, T = 0.2441, $k = 5.9326$, gamma shape = 0.1740 (MCMC chains were run for 2×10^6 generations sampled every 100; burnin = 5000); (2) CR1: substitution model = TIM2 + I + G, base frequencies: A = 0.2921, C = 0.3158, G = 0.1328, T = 0.2593, gamma shape = 0.3570 (MCMC chains were run for 4×10^6 generations sampled every 100; burnin = 10 000). The substitution models and parameters were selected using JMODELTEST (Posada 2008), based on the Akaike information criterion (AIC; Posada & Buckley 2004). A haplotype network was obtained using the median-joining method (MJ; Bandelt *et al.* 1999) in NETWORK 4.5.1.0 (http://www.fluxus-engineering.com/sharenet_rn.htm).

The partitioning of mtDNA diversity within and among the sampled geographical populations and haplogroups as defined by phylogenetic analyses was assessed by AMOVA (analysis of molecular variance) in ARLEQUIN 3.5 (Excoffier & Lischer 2010) using *phi*ST, an estimator of F_{ST}. The significance of *phi*ST was assessed through 1000 permutations. Signals of population expansion were tested in the haplogroups using Harpending's raggedness index (*r*; Schneider & Excoffier 1999). The expansion time was estimated under a model of pure demographic expansion (Rogers & Harpending 1992) with parameters set to default values in ARLEQUIN. The parameter of demographic expansion Tau was estimated according to Schneider and Excoffier (1999). The validity of the expansion model was tested using the sum of square deviations (SSDs) between the observed and expected mismatches as implemented in ARLEQUIN. DNASP was used to test Fu's F_S statistic for neutrality (Fu 1996) in each single geographical population and haplogroup previously defined by MEGA and PHYLIP analyses. Isolation-by-distance was tested on the CR1 dataset using a non-parametric Mantel test and spatial autocorrelation analysis in GENALEX (Peakall & Smouse 2006) and in AIS (alleles in space, Miller 2005). We used BEAST 1.6 (Drummond & Rambaut 2007) to calculate Bayesian Skyline Plots (BSPs). Bayesian skyline analyses were run under the coalescent tree prior (Bayesian Skyline) with the piecewise constant model applied and the number of groups set to 10. The length of the Markov chain was set to 1×10^7 generations, log parameters were sampled every 1000 generations and the 'auto optimize' option was activated. BSPs were generated with TACER 1.4 (Drummond & Rambaut 2007).

We also utilized BEAST to estimate haplotype/haplogroup divergence dates, with both strict and relaxed molecular clock models (Ho 2007). We used as calibration points the following fossil data: the first record of *A. noctua* (1.8 Mya: Mlíkovský 2002), the first record of Strigidae (22 Mya: *Mioglaux poirrieri* Mlíkovský 1998), and the first record of Tytonidae (*Necrobyas* spp. 37 Mya: Mourer-Chauviré 1987). These values are proxies for times to the most recent common ancestor and so were specified in node age constraints. To obtain the internal nodes for the calibrations we utilized seven owl COI DNA sequences from GenBank (GU572113, GU571703, GU481385, GU571703, GU571285, GU572155, JQ173911), and two CR1 sequences (EU410491, EU344979). We also calculated a rough estimate of divergence time using the standard 0.02 substitutions per site per million years for COI (Ho & Larson 2006) and a molecular clock rate estimated from fourfold degenerated sites to be 0.073 substitutions per site per million years with a 95%, highest posterior density interval of 0.025–0.123 substitutions per site per million years (Subramanian *et al.* 2009).

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RESULTS

Variability at mtDNA sequences

We obtained COI sequences of 681 bp from 276 Little Owls (Table 1), which included 34 distinct haplotypes that were defined by 57 polymorphic sites (44 transitions and 13 transversions); 45 of them were parsimony informative. Haplotype diversity (H_d = 0.819 ± 0.018) and nucleotide diversity (π = 0.0243 ± 0.0011) were high; the average number of mutations per haplotype was also high (k = 16.52; Table 1, Appendix S2).

CR1 sequences of 494 bp were obtained from 326 samples. In total, 146 haplotypes were defined by 167 polymorphic sites (123 transitions and 41 transversions); 141 of them were parsimony informative (Appendix S3). Haplotype diversity was very high (H_d = 0.976 ± 0.004) in the total sample (Table 2) and in all the populations (from 0.80 in Albania to 1.00 in southern France, central Spain and Portugal), with the exception of Little Owls sampled in Austria, which had low variability (H_d = 0.167 ± 0.134). Nucleotide diversity was also high, with a maximum π = 0.0879 in southern France (Table 2). Little Owls from Albania and Sardinia had the lowest values of nucleotide diversity (π = 0.0058 and 0.0095, respectively).

Phylogenetic analyses

A Bayesian tree, computed with the concatenated sequence alignment (COI + CR1, 1175 bp long; 115 haplotypes; 276 individuals) suggested that *A. noctua* clustered in two major clades (Fig. 1). A western clade included all sequences sampled from Iberia, Denmark and the Czech Republic, 15 of 16 individuals from France, and 24 of 28 individuals from Austria and Hungary. The eastern clade included all the Little Owls from the Balkans, southern Italy, Sardinia and 64 of 66 individuals from northern Italy. Exceptions were two individuals from northern Italy and another individual from southern France, which were assigned to clades not congruent with their sampling locations (those individuals are marked with an asterisk on Fig. 1). The trees generated with other tree-building methods (MP, ML, NJ; not shown) recovered very similar topologies. The trees obtained using each dataset independently (COI and CR1) recovered the same general topology, although the COI trees identified only the two main haplogroups (Fig. S1).

The haplotype median-joining networks (Fig. 2) were concordant with the phylogenetic tree topology. The western clade included sequences sampled from Iberia to Denmark and was highly divergent from the eastern clade, which comprised the southern Italian, Balkan and Sardinian haplogroups. The geographical frequency distributions of haplogroups are reported in Figure 3.

Overall the Tamura–Nei sequence divergence within groups was lower than the divergence among groups (Table 3). The divergence computed among COI haplogroups was high (0.053). Genetic divergence for CR1 was high between the western and eastern clades (> 0.209 for all possible combinations; Table 3), whereas divergences among Balkan, southern Italian and Sardinian groups were less pronounced (range 0.031–0.041).

Partition of genetic diversity and mismatch analyses

The partition of diversity within and among all populations for the full dataset (COI and CR1) indicated significant genetic subdivisions ($P < 0.001$). In the hierarchical AMOVA, 20.70% of the total genetic variance was within haplogroups, whereas 79.30% was among haplogroups.

The unimodal mismatch distributions for COI (Fig. 4) recovered for both the western and the eastern haplogroups indicated demographic expansions at τ = 2.410 (95% confidence interval (CI) = 0.000–6.053) for the western clade, and τ = 0.328 (95% CI = 0.088–0.994) for the eastern clade. The mismatch analysis indicated stationarity in the eastern clade (SSD = 0.199, $P < 0.001$; τ = 0.328), and demographic expansion in the western clade (SSD = 0.0354, P = 0.15; τ = 2.410). The low and non-significant raggedness indices, r = 0.121 (western clade, P = 0.199 n.s.) and r = 0.046 (eastern clade, P = 1.0), indicate a good fit to a model of population expansion. Fu's F_S values (Table 1) were significantly negative for the two haplogroups, consistent with demographic expansion. Reconstructions of population size (N_e) through time for COI and CR1 are also consistent with population expansion (Fig. 3).

The unimodal mismatch distributions for CR1 (Fig. 4) indicated demographic expansions for the western (τ = 12.88; 95% CI = 8.71–15.24), Balkan (τ = 9.26; 95% CI = 5.35–11.68), southern

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Table 1. Genetic diversity of mtDNA COI in Little Owl populations across Europe.

Population	*n*	Polymorphic sites	Singleton (all)	Parsimony-informative sites	Haplotypes	Private haplotypes	Haplotype diversity (H_d)	Nucleotide diversity (π)	Average number of differences (k)	Fu's F_S
Albania	6	1	1	0	2	0	0.333 (0.215)	0.00049 (0.00032)	0.33333	−0.003
Austria	10	38	35	3	3	0	0.511 (0.164)	0.01207 (0.00802)	8.22222	7.068
Bulgaria	9	5	2	6	5	0	0.806 (0.120)	0.00302 (0.00049)	2.05556	−0.787
Cyprus	3	1	1	0	2	1	0.667 (0.314)	0.00098 (0.00046)	0.66667	0.201
Denmark	7	1	1	0	2	0	0.286 (0.196)	0.00042 (0.00029)	0.28571	−0.095
France N	11	2	0	2	2	1	0.327 (0.153)	0.00096 (0.00045)	0.65455	1.454
France S	5	37	37	0	4	2	0.900 (0.161)	0.02173 (0.01207)	14.80000	2.255
Greece	21	5	5	2	5	0	0.486 (0.124)	0.00119 (0.00042)	0.80952	−1.600
Hungary	18	37	1	36	5	0	0.680 (0.074)	0.01628 (0.00599)	11.08497	8.144
Italy N	66	41	3	38	9	3	0.560 (0.057)	0.00582 (0.00249)	3.96270	2.140
Italy C	30	5	2	3	6	1	0.632 (0.059)	0.00148 (0.00033)	1.00690	−1.627
Italy S	16	2	1	1	3	1	0.342 (0.140)	0.00053 (0.00023)	0.35833	−0.979
Italy Sardinia	10	4	0	4	3	1	0.689 (0.104)	0.00297 (0.00042)	2.02222	2.010
Macedonia	9	8	2	6	6	2	0.917 (0.073)	0.00457 (0.00058)	3.11111	−1.076
Portugal	10	2	1	1	6	1	0.600 (0.131)	0.00098 (0.00027)	0.66667	−0.272
Czech Rep.	5	5	5	0	3	1	0.700 (0.218)	0.00294 (0.00114)	2.0000	0.644
Romania	11	5	1	4	6	0	0.873 (0.071)	0.00342 (0.00032)	2.32727	−1.166
Spain C	6	4	2	2	3	1	0.600 (0.215)	0.00255 (0.00087)	1.73333	0.758
Spain NE	17	7	5	2	5	3	0.533 (0.142)	0.00142 (0.00047)	0.96667	−2.467*
Switzerland	5	3	0	3	4	0	0.900 (0.161)	0.00264 (0.00059)	1.80000	−1.195
Western clade	87	16	9	7	15	–	0.698 (0.036)	0.00228 (0.00017)	1.55306	−7.066*
Eastern clade	189	15	6	10	19	–	0.678 (0.034)	0.00204 (0.00018)	1.39086	−10.574**
All samples	276	57	12	45	34	–	0.819 (0.018)	0.02426 (0.00108)	16.52295	5.564

n, number of individuals. Fu's F_S test and significance (*$P < 0.05$; **$P < 0.001$) are reported.

Table 2. Genetic diversity of the mtDNA control region in Little Owl populations from Europe.

Population	*n*	Polymorphic sites	Singleton (all)	Parsimony-informative sites	Haplotypes	Private haplotypes	Haplotype diversity (H_d)	Nucleotide diversity (π)	Average number of differences (k)	Fu's F_S
Albania	6	8	7	1	4	3	0.800 (0.172)	0.00583 (0.0026)	2.867	0.153
Austria	12	92	92	0	2	0	0.167 (0.134)	0.03142 (0.0253)	15.333	16.468
Bulgaria	12	25	8	17	10	5	0.955 (0.057)	0.01503 (0.0020)	7.394	−2.308
Cyprus	3	5	5	0	3	3	–	–	–	−0.077
Czech Rep.	11	30	8	22	7	4	0.873 (0.089)	0.02385 (0.0031)	11.636	1.791
Denmark	7	27	10	17	4	3	0.810 (0.130)	0.02571 (0.0043)	12.571	4.070
France NE	11	38	12	26	8	6	0.927 (0.066)	0.02647 (0.0032)	12.945	0.873
France S	5	101	91	10	5	3	1.000 (0.026)	0.08791 (0.0403)	42.900	1.432
Germany	1	–	–	–	1	0	–	–	–	–
Greece	22	34	10	24	11	7	0.913 (0.035)	0.01797 (0.0016)	8.840	0.676
Netherlands	2	–	–	–	1	0	–	–	–	–
Hungary	21	109	7	102	10	6	0.900 (0.039)	0.06389 (0.0191)	31.176	8.413
Italy N	71	115	8	106	21	11	0.838 (0.030)	0.03951 (0.010)	19.282	5.811
Italy C	31	32	15	17	14	7	0.852 (0.052)	0.01045 (0.0019)	6.520	−1.131
Italy S	25	37	10	27	19	15	0.977 (0.018)	0.01374 (0.0014)	8.573	−5.723*
Italy Sardinia	11	17	12	5	7	7	0.909 (0.909)	0.0095 (0.0028)	4.691	−0.504
Macedonia	14	25	9	16	10	5	0.945 (0.045)	0.01543 (0.0028)	7.593	−1.177
Portugal	11	41	21	20	11	11	1.000 (0.039)	0.02573 (0.0027)	12.582	−3.467*
Romania	16	105	87	18	9	3	0.925 (0.039)	0.03689 (0.0185)	18.000	3.467
Spain C	8	32	20	12	8	7	1.000 (0.063)	0.02242 (0.0038)	10.964	−1.985
Spain NE	21	41	13	28	18	17	0.971 (0.030)	0.02178 (0.0014)	10.629	−5.653*
Switzerland	5	16	3	13	4	0	0.900 (0.161)	0.01829 (0.0046)	9.000	1.432
Balkan clade	157	61	13	48	50	50	–	0.01243 (0.01243)	6.113	−14.752*
Iberian clade	112	73	18	55	65	65	–	0.02334 (0.02334)	11.367	−23.294**
S-Italian clade	46	33	12	21	24	24	–	0.01048 (0.00087)	5.156	−9.995*
Sardian clade	11	17	12	5	7	7	–	0.00953 (0.00276)	4.691	−0.504
All samples	326	165	22	143	146	–	0.976 (0.004)	0.09651 (0.003)	46.906	−18.706

n, number of individuals. Fu's F_S test statistic and significance (*$P < 0.05$; **$P < 0.001$) are reported.

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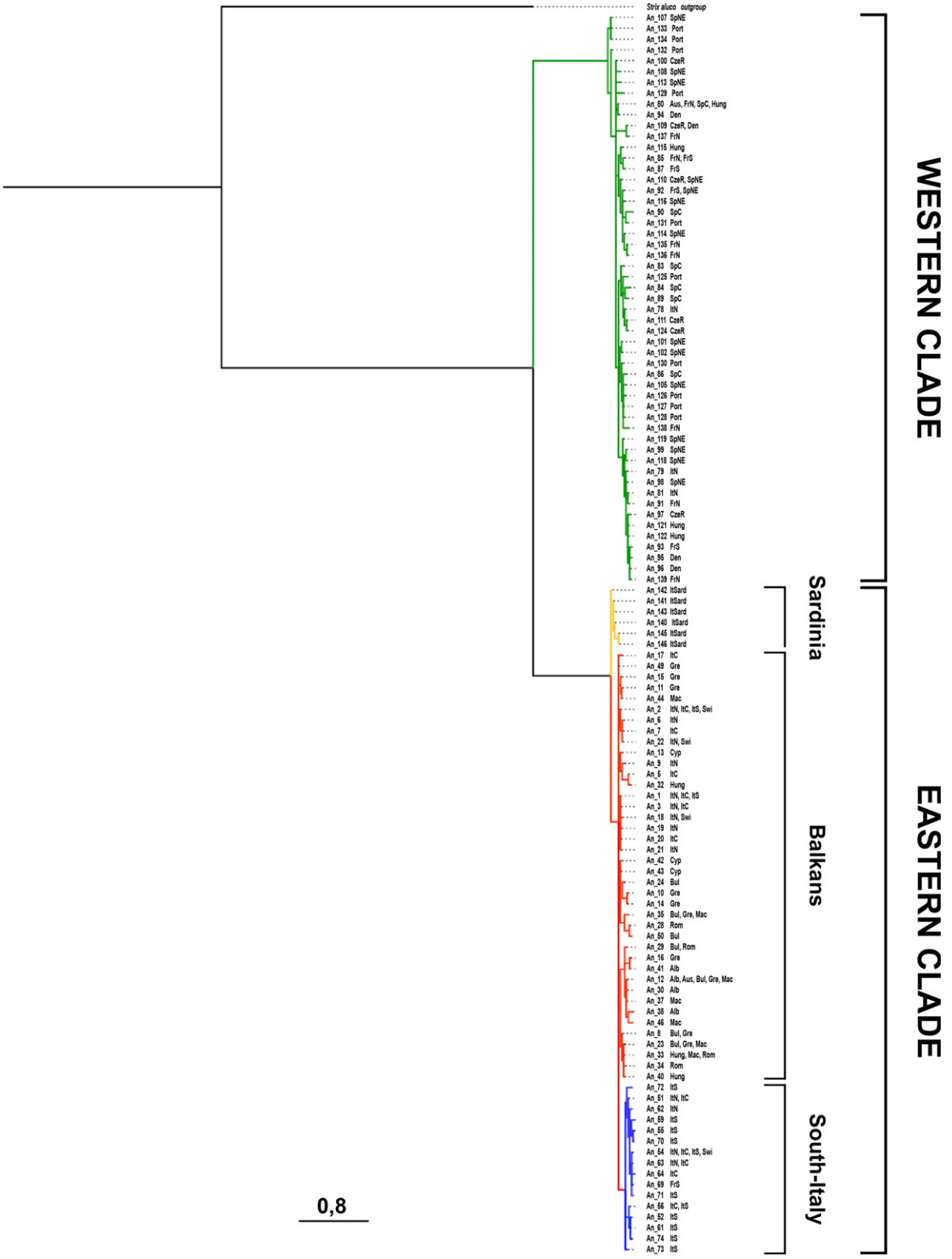

Figure 1. The phylogenetic tree obtained with Bayesian inference (partitioned by gene) from the analysis of the CR1 + COI dataset.

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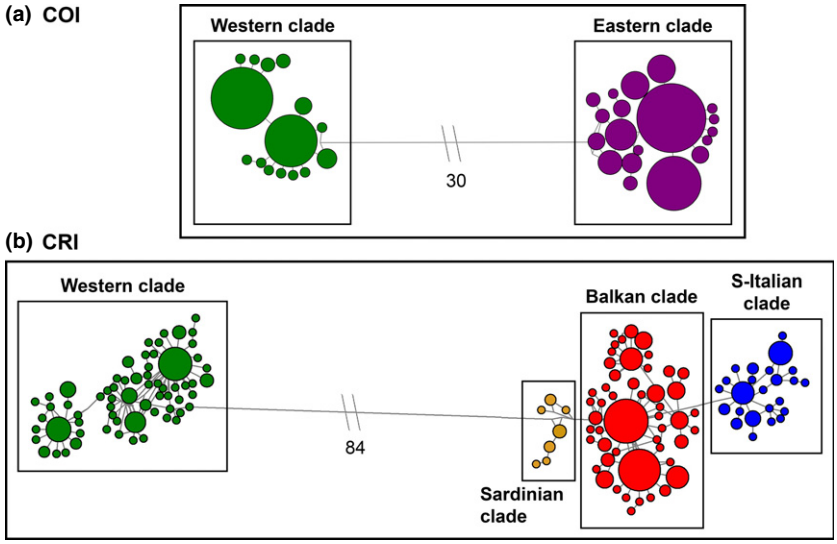

Figure 2. Median-joining network of the Little Owl (a) COI and (b) CR1 haplotypes. The size of the node indicates the relative frequency of the corresponding haplotype in the whole dataset. The COI network recovered two major clades broadly consistent with a western and eastern geographical division, separated by 30 mutations. The CR1 network identified two major clades: a western clade and an eastern clade, further subdivided into Sardinian, Balkan and Southern Italian subnetworks. For CR1, the western and eastern clades were separated by 84 mutations.

Italian (τ = 7.31; 95% CI = 3.31–11.41) and Sardinian groups (τ = 1.66; 95% CI = 0.16–14.33). All SSDs between the observed and expected mismatches had non-significant *P*-values. The raggedness indices, $r = 0.006$ (Balkan haplogroup), $r = 0.019$ (southern Italian haplogroup), $r = 0.004$ (western haplogroup) and $r = 0.156$ (Sardinian haplogroup), were small and non-significant for all haplogroups ($P > 0.10$). Fu's F_S values (Table 2) were significantly negative for all three main clades, consistent with demographic expansion, but non-significant ($P = 0.362$) for the Sardinian clade. The CR1 BSP was consistent with stationarity through time in three eastern subclades, with a weak historical population expansion in the southern Italian clade (Fig. 3). Mantel test and autocorrelation analyses were not significant when computed considering all sampled individuals or when considering only the Italian samples. Mantel test plots are consistent with a pattern of isolation in allopatry for the south Italian haplogroup followed by post-glacial recolonization of Italy (Fig. S2).

COI divergence times estimated using both fossil calibrations and the 2% or fourfold 7.3% substitution rates suggest that the haplogroups diverged during the Pleistocene, between 1 and 2 Mya (Table 4). CR1 divergence times estimated in BEAST indicated that the western haplogroup diverged from the other haplogroups in the Pleistocene (strict molecular clock: 2.08 to 1.69 Mya; relaxed molecular clock: from 2.05 to 1.66 Mya). The split between the Sardinian group and southern Italian–Balkan haplogroups occurred about 1.04–0.26 Mya and the split between the southern Italian and Balkan haplogroups at 0.72–0.21 Mya (Table 4).

DISCUSSION

Phylogeographical structure and glacial refugia of the Little Owl

Pleistocene climate change in Europe moulded the distribution of species and the genetic composition of populations (Hewitt 2000). Major Mediterranean glacial refugia have been identified in the southern Iberian, Italian and Balkan peninsulas (Taberlet *et al.* 1998, Provan & Bennett 2008). Additional northern refugia have been hypothesized for several vertebrate and invertebrate species (e.g. around the Carpathian Mountains, and near the Caucasus at the border with the Black Sea; Hewitt 2004, Deffontaine *et al.* 2005). As has been documented for many animal species adapted to temperate climates (Avise 2000), the results of

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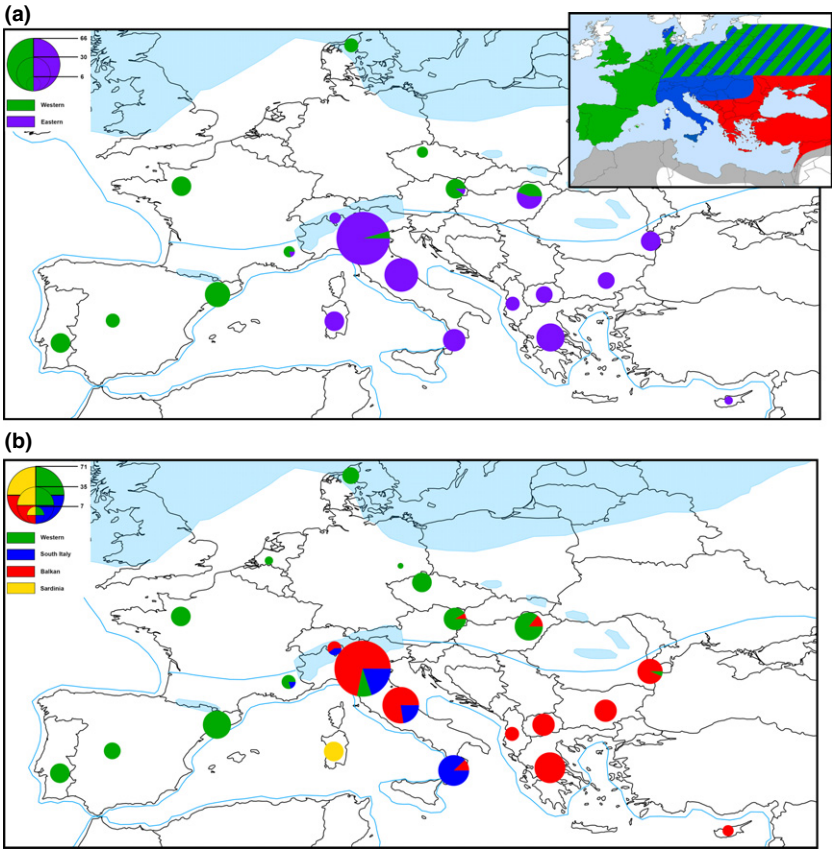

Figure 3. Putative subspecies distributions according to Cramp (1985) and Vaurie (1960) are shown on the top right: green: *Athene noctua vidalii*; blue: *Athene noctua noctua*; red: *Athene noctua indigena*. In the hatched area, Vaurie (1960) reports *A. n. noctua* and Cramp (1985) *A. n. vidalii*. In Cyprus, the literature suggests the presence of *Athene noctua lilith*. (a) COI haplogroups distribution at sampled sites. Pie charts represent the proportion of individuals in the western and eastern clades, respectively. (b) CR1 haplogroup distribution. Pie charts represent the proportion of individuals in each of the four clades recovered. The shaded area indicates the extension of ice sheets during the last glacial maximum (redrawn from Taberlet *et al.* 1998): lowered seashore is depicted by a thinner line at the 100-m submarine contour. A full colour version of this figure is available at *Ibis* online.

our phylogeographical study support the hypothesis that *A. noctua* survived the Pleistocene glaciations in southern European refuges. Future studies could clarify whether North Africa, and in particular Morocco, served as a glacial refugium and contributed to the recolonization of Europe through the Iberian peninsula (Griswold & Baker 2002, Perktas & Quintero 2013).

Phylogenetic analyses of the mtDNA sequences identified two main monophyletic clades, the first primarily including individuals from western and northwestern Europe (from Iberia to Denmark and the Czech Republic), and the second individuals sampled from localities in central and southeastern Europe. Kerr *et al.* (2007, 2009) and Johnsen *et al.* (2010) barcoded hundreds of North American, South American and Scandinavian bird species, showing that the average interspecific COI distance was 7.9% (range 0–17.05%), whereas the average intraspecific distance was 0.24% (range 0–5.08%). In these studies, taxa showing high intraspecific sequence variation (> 2%) were considered good candidates for further taxonomic splitting. In our study, the COI genetic distance estimated between the two primary Little Owl haplogroups falls towards the upper end of the range for avian interspecific distances using the barcoding COI locus. Although genetic distances alone rarely can be used to derive taxonomic conclusions, the wide COI divergence assessed between the western and eastern clades suggests that genetic structure among Little Owl populations traces well back into the Pleistocene. The absence of haplotype admixture in the eastern and western portions of

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Table 3. Genetic distances (TN93, Tamura & Nei 1993) within and among different groups of the COI and CR1 mtDNA datasets.

	Genetic distances within haplogroups	Genetic distance among haplogroups			
COI		Eastern	Western		
Eastern	0.005 ± 0.001	–			
Western	0.005 ± 0.002	0.053 ± 0.009	–		
CR1		Balkan	Italian	Iberian	Sardinian
Balkan	0.012 ± 0.002	–			
Italian	0.011 ± 0.003	0.031 ± 0.006	–		
Western	0.023 ± 0.004	0.231 ± 0.024	0.221 ± 0.024	–	
Sardinian	0.009 ± 0.002	0.031 ± 0.007	0.041 ± 0.008	0.209 ± 0.022	–

the geographical distribution also suggests that Little Owl populations may not have recently admixed, although sampling the nuclear genome is needed to further test this hypothesis.

The eastern haplogroup was further split into three subclades distributed in the Balkans, in southern Italy and in Sardinia. Consistent moderate estimates of divergence were recovered among these three minor haplogroups, indicating genetic structure within the eastern lineage (Hutchinson & Templeton 1999). AMOVAs attributed most of the total genetic variance to that among haplogroups, with only a small percentage of variation within clades. Such levels of genetic divergence could perhaps best be explained by long-term allopatric divergence of Little Owl populations among different glacial refugia (Nosil *et al.* 2009).

The phylogeographical structure described in *A. noctua* fits one of the major phylogeographical patterns observed in temperate plant and animal species in Europe (Bhagwat & Willis 2008). According to the Mediterranean refuge model (Hewitt 2004), the extant phylogeographical patterns of species are compatible with a northward postglacial colonization of central Europe, with routes starting from three distinct glacial refugia in the Iberian, Italian and Balkan peninsulas (Comes & Kadereit 1998, Schmitt 2007). With a few exceptions (Brito 2005, Marthinsen *et al.* 2009), the phylogeography of Palaearctic Strigiformes is not known. The phylogeography of the Little Owls is broadly similar to that reported for the Tawny Owls (Brito 2005), with some exceptions (see below). In these two non-migratory species, the southern European peninsulas (Iberia, Italy, Balkan) acted as refugia during the climatic extremes of the Pleistocene ice ages. In contrast, the Snowy Owl is a nomadic species, with circumpolar distribution, and phylogeographical analyses did not reveal substantial genetic structure across its breeding range (Marthinsen *et al.* 2009).

High haplotype and nucleotide diversities were found in all Little Owl populations, and genetic variability did not vary significantly with latitude. The high nucleotide diversity in northern populations does not agree with a previous study in which a northward decreasing gradient in genetic diversity was described in the Tawny Owl (Brito 2005) and with studies of several European taxa where high genetic variation was only recovered from populations located within refugia (Provan & Bennett 2008). In *A. noctua*, the lack of a northward decreasing pattern in genetic diversity suggests the absence of a founder effect, bottleneck or undue influence of genetic drift (Hewitt 1996), and could be due to recolonization of northern Europe with many individuals from southern Europe. The case of Sardinia, where a high number of haplotypes was recovered, deserves further investigation. The island was mostly isolated until the Holocene and inhabited by endemic taxa (Masini *et al.* 2008). The presence of *A. noctua* on the island has been documented from around 10 000 years BP (Louchart 2002); before this point in time the island was inhabited by the now extinct species *Athene angelis*, which was endemic to Corsica and Sardinia (Louchart 2002, Abbazzi *et al.* 2004).

Postglacial expansion

The availability of suitable habitats during and after the ice age should be considered in order to understand the pattern of postglacial expansion

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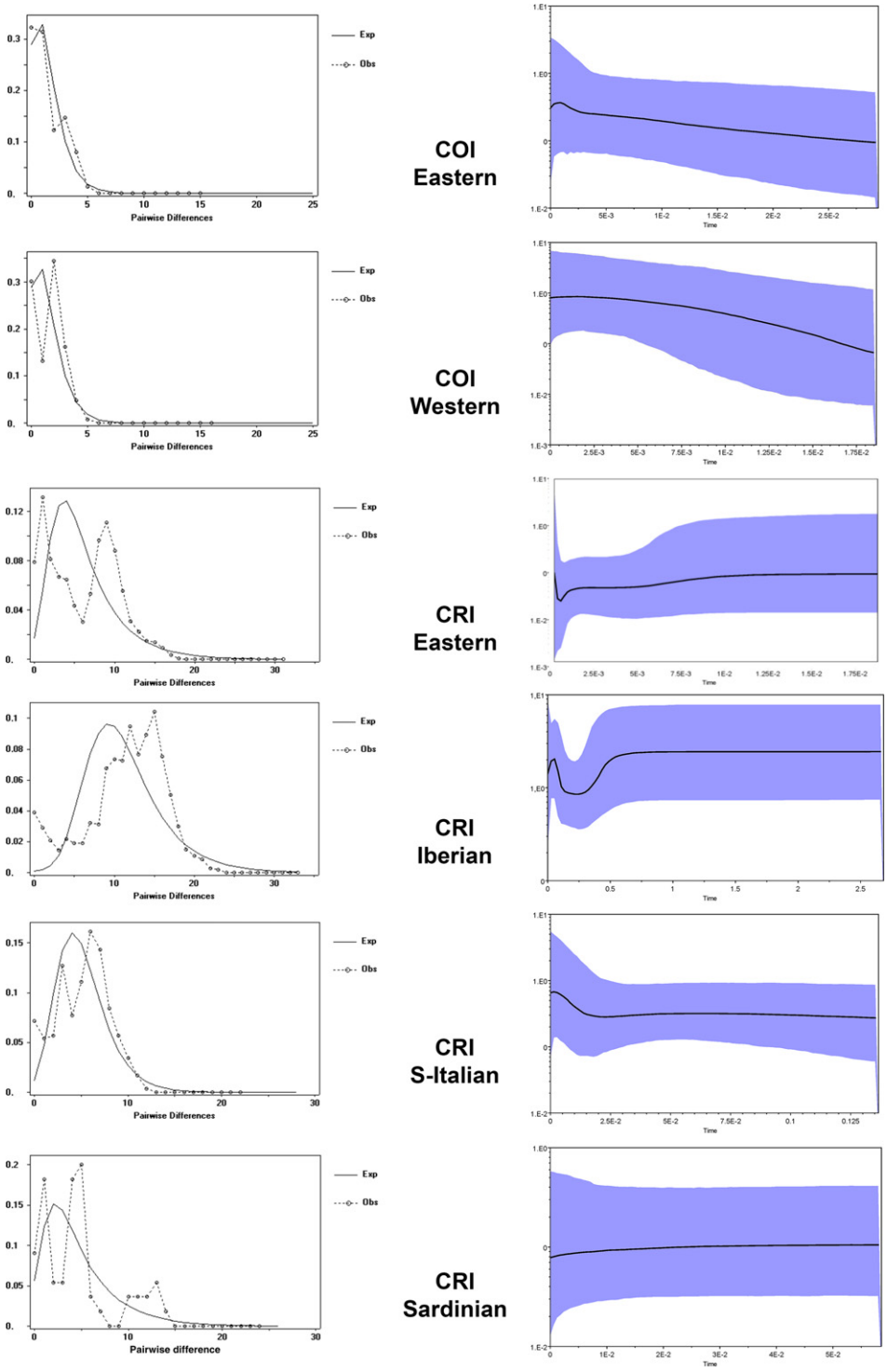

Figure 4. Mismatch distribution analyses (left) and Bayesian skyline plots (right) for COI and CR1 haplogroups. BSPs depict the median population size through time for each clade as well as the upper and lower 95% higher posterior density.

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Table 4. Divergence times (Mya) (95% highest posterior density) obtained using different priors for the molecular clock model (strict clock or uncorrelated lognormal), fossil calibration or substitution rate.

	CR1		COI				
Clades	Fossil calibration Strict clock	Fossil calibration Lognormal relaxed clock	Fossil calibration Strict clock	Fossil calibration Lognormal relaxed clock	2% Rate Strict clock	2% Rate Lognormal relaxed clock	7.3% Rate Lognormal relaxed clock (COI fourfold)
Western–Balkan	3.5079 (2.4977–4.7261)	3.5979 (2.4312–4.7956)	1.9015 (1.717–2.0944)	1.9556 (1.6342–2.0114)	1.6144 (1.0569–2.2689)	1.5950 (0.9238–2.4286)	1.0405 (0.3674–1.9948)
Balkan–Sardinian	0.4966 (0.3304–0.6921)	0.4682 (0.2785–0.6077)	–	–	–	–	–
Italian–Balkan	0.4105 (0.2782–0.3573)	0.5468 (0.3251–0.7079)	–	–	–	–	–

(Goldewijk *et al.* 2011). Little Owl habitats include arid areas, pastures, steppes, stony deserts, farmland and open woodland (van Nieuwenhuyse *et al.* 2008). Climatic shifts of alternating cold and warm periods in Europe, associated with glacial and interglacial periods, contributed to the disjointed distribution of savanna, grass and other xeric habitats (Tzedakis *et al.* 2002). This probably led to a cyclical reduction and expansion of European populations of *A. noctua*. According to Schönn *et al.* (1991), the past breeding areas did not include central Europe, which was colonized after deforestation by humans (Foley *et al.* 2005). Few Pleistocene records of *A. noctua* have been reported from the UK, Germany, Central France and the Czech Republic, while in the same period the species was widespread in southern Europe (Tyrberg 1998).

Our data indicate that Little Owls currently distributed in northern France, the Netherlands, Denmark and the Czech Republic originated through range expansion from a refugium located in the Iberian region. All individuals sampled in these areas are part of the western clade. The western haplotypes reached the Balkans and were found together with Balkan haplotypes in Hungary and Romania. All individuals sampled in Greece, Macedonia, Bulgaria, Cyprus and Albania pertained to the Balkan haplogroup, which extended as far as Austria in the north. In Italy, this clade spread along the whole peninsula but was not found in Sicily. This suggests that the Adriatic was not an effective barrier to gene flow, probably because of geographical proximity and connections when sea level was lower during cold periods (Taberlet *et al.* 1998). The southern Italian haplogroup has a smaller geographical range. In Sicily, all samples pertained to this clade that reached northern Italy but did not cross the southern slopes of the Alps. This haplogroup was also found in Switzerland, again in the southern part of the Alps, and in southern France close to the Italian border (Maritime Alps). The Sardinian clade was found exclusively in Sardinia. Future work should determine whether this haplogroup is also present in Corsica, where the species has only bred in recent years (Yeatman-Berthelot & Jarry 1995). In Cyprus, literature reports the presence of the *lilith* subspecies (Cramp 1985, del Hoyo *et al.* 1999). All three samples from Cyprus pertain to the Balkan haplogroup. Further analysis of more individuals can confirm this result or

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determine whether there exists another haplogroup on the island. Northern Italy is an interesting area because there is a mixture of three haplogroups, probably reflecting secondary contact as a consequence of convergent expansion from all three putative refugia.

Phylogeographical results from a wide variety of European taxa were summarized into three patterns of postglacial expansion that reflect different contributions from each refugium to the northern European populations (Hewitt 2000, 2004). Little Owl postglacial expansion is most similar to the common pattern of range expansion in Europe that describes species for which the Iberian refugium is responsible for colonization of all northern Europe until the Balkans (Bear paradigm; Hewitt 2004). Species-specific patterns of postglacial expansion have to be explained by a combination of orography, palaeoecological conditions after the last glacial maximum and individual natural history traits. The reason why Iberian Little Owls had time to reach Central Europe, whereas Balkan and southern Italian owls only reached the Alps, could be related to the efficiency of the Alps as a barrier to northward expansion from the two other refuges. However, it seems the Pyrenees did not act as a barrier for individuals expanding from the Iberian refugium, as found in other species (Michaux *et al.* 2003). For instance, the phylogeography of the Tawny Owl (Brito 2005) showed a different genetic pattern, with the Balkan refugium contributing mainly to recolonization of Europe. Unlike the Little Owl, Tawny Owls are associated with woodland and closed habitats, and this characteristic could explain the difference in how the two owl species responded to Pleistocene range expansion cycles.

Unimodal mismatch distributions as well as SSDs and raggedness indices supported the hypothesis of expansion for all haplogroups. This pattern of demographic expansion was suggested also by Fu's F_S test, with the exception of the Sardinian clade, for which the non-significant value indicated the absence of either a population expansion or a population bottleneck. BSP indicated more recent expansion in the western and eastern COI clades, and a trend of more constant population size was recovered for the CR1 clades, with the exception of a weak signal of historical population expansion in the southern Italian clade.

Divergence times

In the absence of a fossil record, a rate of 2% sequence divergence per million years is commonly applied to calibrate divergence times between and within species (Brown *et al.* 1979, Hansson *et al.* 2008, Weir & Schluter 2008). Although many calibrations have been shown to cluster around 2%, it was also demonstrated that the rates of molecular evolution within the last million years tend to be accelerated relative to older events (Garcìa-Moreno 2004, Ho & Larson 2006, Subramanian *et al.* 2009). Moreover, it is well known that mtDNA genes evolve at different rates (Seo *et al.* 2005). Indeed, the mutation rate in the mitochondrial Control Region is higher than in protein-coding genes, with estimates ranging from 5% (Milá *et al.* 2007) to 20% per million years (Godoy *et al.* 2004).

In this study, taking advantage of the availability of dated fossils of members of the Strigidae (Mourer-Chauviré 1987, Mlíkovský 1998, 2002), we used a fossil record calibration. The molecular clock analysis for COI revealed that the split between the two major haplogroups (western and eastern) occurred about 2.09–1.63 Mya during the Early Pleistocene, much earlier than the last glacial maximum. A similar split occurring before the main Pleistocene glaciations was recovered when the CR1 dataset was analysed (2.05–1.69 Mya). A rough estimate of divergence time using the COI genetic distance between haplogroups and a molecular rate of 2% would date the split between Iberian and Balkan haplogroups at 2.4286–0.9238 Mya. As postulated by Ho (2007), the application of a 2% rate would overestimate the divergence time. An estimate using the neutral substitution rate provided by Subramanian *et al.* (2009) for fourfold degenerate sites would date the split between the two haplogroups at 1.0405 Mya (95% higher posterior density (HPD): 0.3674–1.9948), possibly underestimating divergence times. In summary, the estimates obtained using the traditional rate and the fossil calibration were twofold older than the dates obtained using the neutral substitution rate and corresponding 95% HPDs were tighter than in the last model. We suggest the divergence time estimates using the fourfold neutral rate to be more reliable than the traditional 2% rate because the first *A. noctua* fossil was dated to around 1.8 Mya (Mlíkovský

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2002). Indeed, the use of the 2% traditional rate has been criticized and compelling evidence suggests that this rate should not be broadly applied to birds due to large variation across lineages and loci (e.g. Warren *et al.* 2003, Arbogast *et al.* 2006, Pereira & Baker 2006).

The fossil calibration on CR1 recovered older divergence times than on COI. This would probably be due to the influence of mutational saturation in the analysis. Multiple changes that can occur in CR1 sites may lead to an underestimation of the substitution rate.

The estimated divergence times suggest that a long period of isolation occurred to separate all haplogroups and that gene flow during the Pleistocene glacial cycles was probably limited to areas of secondary contact. Isolation of Sardinian and southern Italian haplogroups appears to be predate the last glacial maximum.

The subspecies problem

The mtDNA genomes of the Little Owl belong to four distinct clades in Europe. The phylogeographical pattern described in this study is consistent with the existence of four subspecies described in the southern part of Europe, i.e. the widely accepted *A. noctua noctua*, *A. n. vidalii*, *A. n. indigena*, and the questioned *A. n. sarda*. However, the geographical boundaries among subspecies distributions are unclear and difficult to assess using morphological traits. Indeed, it has been shown that the correlations between genetic patterns and morphological variation of subspecies are often weak (Zink 2004). These comparisons are further complicated by the cryptic plumage variation of nocturnal species (Roulin *et al.* 2001). Geographical variation in the Little Owl is complex, and involves the coloration of the upperparts and the extent of streaking on the underparts and the size of white spots on the crown, mantle and scapulars. Variation is clinal and boundaries between subspecies are indistinct, with integration across wide areas. Individual variation in colour is marked in some areas (birds often tend to be dimorphic, some more rufous, others more olive-grey), but virtually absent in others. Division based on size is also uncertain. Of the range considered in our study, a short tarsus is characteristic of the subspecies *lilith*, whereas all European populations have similar tarsus length (Cramp 1985). Future studies considering the whole distributional range of the species and both mtDNA and other molecular markers (e.g. microsatellite DNA, Müller *et al.* 2001, Aurelle *et al.* 2010) could clarify whether distribution boundaries at the subspecies level differ from those reported in the literature (Vaurie 1960, Glutz von Blotzheim & Bauer 1980, Cramp 1985). Information from both uniparental and biparental markers will help to confirm the existence of genetic differentiation among subspecies, to identify evolutionary taxonomic units and secondary contact zones, and to detect the presence of admixture at the boundary of the distribution.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a) COI Neighbour-Joining tree based on Tamura–Nei distance. Bootstrap values > 50% are indicated. (b) CR1 neighbor-joining tree based on Tamura–Nei distance. Bootstrap values > 50% are indicated, 1000 pseudo-replicates.

Figure S2. Results of the isolation-by-distance CR1 analysis for (a) complete dataset ($r = 0.02486$) and (b) Italian populations ($r = -0.0255$).

Appendix S1. List of sampled individuals used in this study.

Appendix S2. Distribution of the 34 COI haplotypes found in 276 Little Owls from 20 European sites.

Appendix S3. Distribution of 146 CR haplotypes found in 326 Little Owls from 22 European sites.

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