

The influence of Pleistocene glacial refugia on tawny owl genetic diversity and phylogeography in western Europe

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Abstract

The glacial refugia hypothesis indicates that during the height of the Pleistocene glaciations the temperate species that are today widespread in western Europe must have survived in small and climatically favourable areas located in the southern peninsulas of Iberia, Italy and Balkans. One such species is the tawny owl, a relatively sedentary, nonmigratory bird presently distributed throughout Europe. It is a tree-nesting species closely associated with deciduous and mixed coniferous woodlands. In this study I used control region mtDNA sequences from 187 individuals distributed among 14 populations to determine whether current genetic patterns in tawny owl populations were consistent with postglacial expansion from peninsular refugia. European, North African and Asian tawny owls were found to represent three distinct lineages, where North Africa is the sister clade to all European owls. Within Europe, I found three well-supported clades that correspond to each of the three allopatric refugia. Expansion patterns indicate that owls from the Balkan refugium repopulated most of northern Europe, while expansion out of Iberia and Italy had only regional effects leading to admixture in France. Estimates of population divergence times between refugia populations are roughly similar, but one order of magnitude smaller between Greece and northern Europe. Based on a wide range of mutation rates and generation times, divergence between refugia appears to date to the Pleistocene.

Keywords: divergence time, gene flow, glacial refugia, phylogeography, Pleistocene, *Strix*

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Introduction

Quaternary climatic fluctuations have been widely recognized as the main historical process influencing the genetic diversity of natural populations of the temperate Northern Hemisphere (e.g. Frenzel 1973; Hewitt 1996, 2004). During the late Pleistocene the climate fluctuated from full-glacial conditions to full-interglacial. The last glacial maximum (LGM) occurred in Europe around 18 000 before present (BP) and was characterized by an extensive decrease of the average temperatures that led to the formation of the Scandinavian ice sheet (coverings parts of Britain and northern Europe), and ice caps on the top of such major mountain ranges as the Pyrenees, the Alps and the Caucasus (Frenzel 1973; Nilsson 1983). Between the main ice sheet in the north and the southern mountains, Europe was covered by tundra and cold steppe (Prentice

et al. 2000; Tzedakis *et al.* 2002). The term 'glacial refugia' was used to describe the only suitable localities where temperate fauna and flora could have existed during full-glacial conditions (Hewitt 1996, 1999). The reconstruction of palaeo-vegetation maps has indicated three main glacial refugia of deciduous temperate forest located in the southern peninsulas of Iberia, Italy, and Balkans (Frenzel 1973; Hewitt 1996; Tzedakis *et al.* 2002).

Due to the severe climatic oscillations, temperate flora and fauna are expected to have gone through many contractions and range expansions, which are expected to have left signatures in the geographical distribution and genetic diversity of extant populations (Bennett *et al.* 1991; Slatkin 1993; Avise 2000; Furlong & Brookfield 2001). Refugial populations that evolved in allopatry are expected to have accumulated independent genetic differences that may be used as genetic markers to trace expansion routes. It is also expected that genetic variability would be higher in refugial populations than in recent populations, since the later are characteristically formed by a subset of the original gene pool.

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Phylogeographic studies in western Europe have used the glacial refugia hypothesis to interpret results of geographical structure and gene flow (e.g. Nesbø *et al.* 1999; Weiss *et al.* 2000; Nilsson *et al.* 2001), as well as subspecies distribution and speciation (e.g. Hewitt 1996, 2001; Newton *et al.* 1999). In general, studies of temperate species where all three southern refugia as well as northern populations were sampled show phylogeographic patterns that are congruent with the three putative Mediterranean refugia (Hewitt 2004), although alternative hypotheses about the demographic history of the populations are usually not explicitly addressed.

The majority of avian phylogeographic studies where the Pleistocene refugia hypothesis for Europe was analysed have been restricted to subsets of the three putative refugia, either because of restricted sampling (e.g. Merilä *et al.* 1997; Bensch & Hasselquist 1999; Kvist *et al.* 1999), or due to constraints in species range (e.g. Kvist *et al.* 2001; Randi *et al.* 2003; Gay *et al.* 2004). One exception is the study of common chaffinches by Griswold & Baker (2002), where all three potential refugia as well as northern European populations were sampled but highly structured clades reflecting geographical structure compatible with the refugia hypothesis were not recovered.

In the present study, mitochondrial sequences were used to study the phylogeography of the tawny owl (*Strix aluco*) in Europe. Specifically, these data were used to examine whether the Pleistocene glaciations helped shape the genetic diversity currently found in tawny owl populations. Several features make the tawny owl a good model organism for this purpose. These owls are closely associated with temperate woodland forest, so it is a reasonable assumption that these birds could not have survived outside refugia during the height of the glaciations. Also, their current widespread distribution throughout Europe suggests that populations from northern Europe had an origin in at least one of those glacial refugia. Tawny owls are non-migratory and breeding adults are sedentary and remain on territory all year, while juvenile dispersal only occurs within a few kilometres of the natal site (Cramp 1985; Coles & Petty 1997). This limited dispersal propensity should facilitate the detection of phylogeographic patterns of population expansion by reducing the homogenizing effects of long-distance dispersal. Current taxonomy classifies tawny owl as a widespread species with two main allopatric groups. One in Europe (n nominate *aluco*) including six subspecies and another in Asia (*nivicola*) with five subspecies (König *et al.* 1999). Taxonomic status of some forms is uncertain and species limits are re-evaluated in a molecular phylogeny of the genus *Strix* (Brito in prep.). The present study analyses tawny owls from two contiguous subspecies (*Strix aluco sylvatica* and *Strix aluco aluco*) that cover all western Europe from the Iberian Peninsula to western Russia.

Three putative refugia as well as northern European localities that could not have harboured tawny owl populations during the LGM were sampled. Current genetic diversity within and among populations is characterized. The likely refugial populations and expansion routes are identified, and used to locate the geographical origin of northern European populations. Finally, it is investigated whether the inferred processes are compatible with a late Pleistocene time frame.

Materials and methods

Sample collection

Population sample locations were chosen to cover the entire distribution of the tawny owl in western Europe (Appendix, Fig. 1). When possible, the regions where the putative refugia were located were sampled more intensively, e.g. Iberia was represented by three populations: Portugal, Madrid (Spain), and Bilbao (Spain); Italy was sampled in Sicily, and in northern Italy; the Balkans was only represented by one population, Greece, since, at present, the regions immediately north of Greece are not easily sampled. Population samples were obtained through research teams carrying out projects with tawny owls, local rehabilitation centres for raptors, and natural history museums. Tissue samples were taken from growing contour feathers or by puncturing the brachial vein from live birds. Muscle samples and pieces of the toe pads were removed from carcasses and museum study skins, respectively. In addition to the tawny owls from western Europe, three individuals from North Africa (*Strix aluco mauritanica*), one from Nepal (*Strix aluco nivicola*), one from Taiwan (*Strix aluco yamadae*), and one Ural owl (*Strix uralensis*) were also sampled. The Ural owl was used as outgroup in the phylogenetic analyses.

DNA sequencing

As has been described for other avian taxa, e.g. *Strix varia* (Barrowclough & Groth, unpublished data), *Amazona* spp. (Eberhard *et al.* 2001), and *Buteo buteo* (Haring *et al.* 2001), the tawny owl has two copies of the control region in their mtDNA. These copies are located between the cytochrome *b* and 12 S genes in the following order: cytochrome *b*/tRNA-Thr/control region 1/tRNA-Pro/ND6/tRNA-Glu/control region 2/tRNA-Phe/12 S. The control region fragments sequenced in this study correspond to the highly variable domain I and part of domain II from each of the two control regions (Baker & Marshall 1997).

Extractions of total genomic DNA were carried out with commercial kits (DNeasy Tissue Kit, QIAGEN, Inc.) following manufacturer's instructions for animal tissues. Polymerase chain reaction (PCR) amplifications and gene

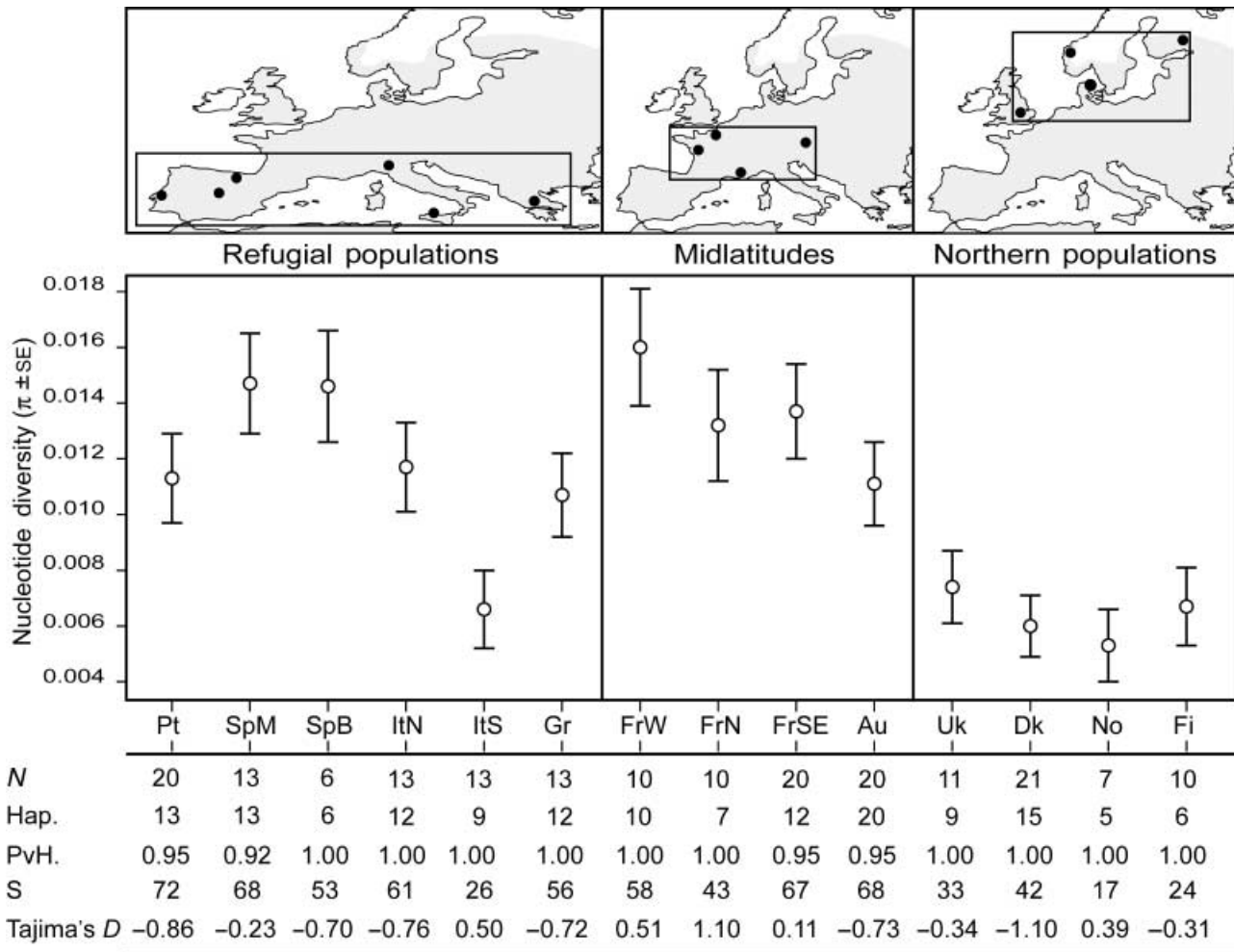


Fig. 1 Genetic diversity within populations. The maps on the top indicate current geographical distribution of tawny owls in western Europe (shaded area) and approximate locations of sampled populations. The graph shows estimates (and standard errors) of nucleotide diversity within populations. Below the graph are sample size (*N*), number of unique haplotypes (Hap.), proportion of private haplotypes (PvH.), number of segregating sites (*S*), and Tajima's *D* statistic. Populations are represented by their abbreviations (see Appendix for full names), and within each group populations and data are ordered with longitude.

sequencing followed protocols from Barrowclough *et al.* (1999), and detailed information is available in Brito (2005). Briefly, the control region 1 was amplified in a two-step process that included a first PCR amplification with primers N1/D16 (Table 1), followed by a subsequent PCR where the forward primer was coupled with an internal reverse primer (N1/D20). Control region 2 was PCR amplified in one step using primer combinations (ND6Z/D20). Although the same reverse primer was used to amplify fragments of both control regions, accurate homology was assured by using forward primers with high specificity to the tRNA-Thr and ND6 genes, respectively. Both light and heavy strands were sequenced, and contigs were assembled and edited using SEQUENCHER 3.1.1 (Gene Codes). Amplification from museum study skins required additional primers (Table 1) that were used in different combinations

depending on the quality of the DNA extracts. In general, both control regions were amplified in four fragments of 200–250 bp, having a minimum of 50–80 bp of overlap. Extractions, genomic PCR, and GENECLEAN purification (performed with Glassmilk kits, Bio 101) for the three North African skins (collected in 1897, 1907, and 1920) were done in a different laboratory using new QIAGEN kits, primers, and other laboratory reagents. Hotstart *Taq* was used in all genomic amplifications and, in general, changing the annealing temperature, annealing time, and the number of cycles was sufficient to troubleshoot all PCRs.

Data analysis

DNA sequences were aligned using CLUSTAL_x (Thompson *et al.* 1997) and confirmed by eye in BIOEDIT (Hall 1999)

Table 1 Primers used to amplify both fragments of the mitochondrial control region

| | Primer | Sequence (5'–3') | Source |
|------------------|------------------|----------------------------|---------------------------------|
| Control region 1 | | | |
| Forward | N1 | AACATTGGTCTTGTAAGCCAA | Barrowclough <i>et al.</i> 1999 |
| | DL165 | GCCGCTTGGGATGTATAATTG | present study |
| Reverse | DL383 | CACCCTAATTCATGATCAACCG | present study |
| | DL532 | CCAAATCACAATCCATCCATGCC | present study |
| | D16 | AGTGCATCAGTGTCTAGGTGATTC | Barrowclough <i>et al.</i> 1999 |
| | D12 | TAGGCGGGACTATTACTTGAAT | Barrowclough <i>et al.</i> 1999 |
| | D20* | GTGATGGATCTTACTAACACC | Barrowclough <i>et al.</i> 1999 |
| | DH365 | GGGTGTTTTTGGTACATGCAGAG | present study |
| | DH469 | GGGCATGGATTATATATCCG | present study |
| | DH622 | GGCTAACTTAAGGTGGGACCACTACT | present study |
| | DH724 | CAGCTGCGCCAGATGTC | present study |
| | Control region 2 | | |
| Forward | ND6Z | ACAACCCCATTAATACCGGAAGG | present study |
| | DL2 | GAAACCCCTACCAGGGCA | present study |
| | DL4 | ACATACCATTTCATGCCCA | present study |
| | DL4a | TACCTTCCACCGATCACAAGG | present study |
| Reverse | DH1a | TAATGCACACCAGTACATCCTC | present study |
| | DH3a | CATGGATTATATATCCGGTTGAC | present study |
| | DH3 | GCTAACTTAAGGTGGGACCA | present study |

*Also used to amplify CR2.

where they were concatenated to form the final data set. Genetic diversity within populations was characterized by the number of unique haplotypes, proportion of private haplotypes (haplotypes found only in one population) and number of segregating sites using *DNASP* version 4.0 (Rozas *et al.* 2003). Nucleotide diversity (Nei 1987) was estimated with *MEGA* version 2.1 (Kumar *et al.* 2001) and standard errors were estimated using a bootstrap with 1000 replications. Tajima's *D* statistic (Tajima 1989) was computed in *ARLEQUIN* version 2.000 (Schneider *et al.* 2000) to test for selective neutrality; its significance was estimated by generating random samples under the hypothesis of selective neutrality and population equilibrium, using a coalescent simulation adapted from Hudson (1990). Analyses of molecular variance (*AMOVA*; Excoffier *et al.* 1992) were performed in *ARLEQUIN* using a Jukes–Cantor correction (Jukes & Cantor 1969). This analysis partitions the total genetic variance into among- and within-population components that were used to compute a fixation index, hereafter F_{ST} . Two analyses were performed, one with 14 populations within one group, and another with six populations arranged in a hierarchical structure that reflect the three hypothetical refugia. This later analysis was done to estimate the partitioning of the total genetic variance among a hierarchical structure of three refugia and six populations distributed within those refugia. Because the numbers of populations sampled within refugia were unequal, the design was unbalanced. *ARLEQUIN* addresses

the significance of the fixation indices by permuting haplotypes among populations.

All phylogenetic analyses were performed using *PAUP** version 4.0b10 for Windows (Swofford 2001). Parsimony analyses were run with a heuristic search and tree-bisection–reconnection (TBR) branch swapping; starting trees were obtained by random addition and gaps were treated as a fifth state. This search was repeated 100 times, and each replicate was run for 2 h on a 2.00 GHz computer. The strict consensus was computed for all most-parsimonious trees and taken as the best phylogeographic hypothesis for the recent history of the tawny owl in western Europe. Nodal support was estimated using 100 replicates of a nonparametric bootstrap using the same search parameters as above. Because the inclusion of distant outgroups may decrease nodal support, bootstrap analysis was also run with the unrooted network. The significance of geographical structure was estimated by testing the independence between 'geographical location' and 'genealogical clade location' with a chi-squared (χ^2) contingency test for samples for putative refugial areas. The significance of the test was evaluated by estimating exact *P* values by Monte Carlo simulation ($\alpha = 0.01$; $N = 10\,000$) as implemented in *SAS* version 8.02; and following the suggestions of Roff & Bentzen (1989). This test statistic has the advantage of taking into account the genetic correlation due to common genealogical history when testing for geographical structure.

The principle that gene flow can be detected using co-ancestry of alleles (Hudson 1990; Slatkin 1993) was used to identify and trace expansion routes out of the refugia. For example, if a population is derived by range expansion from one unique refugium then their haplotypes will coalesce with haplotypes from the same refugial population before they coalesce with haplotypes from other refugial populations. Levels of gene flow were estimated with both N_{ST} (Lynch & Crease 1990), using the program DNASP, and a maximum-likelihood method based on the coalescent, as implemented in MIGRATE (Beerli & Felsenstein 1999, 2001). For the latter, a stepping-stone model of population structure was designed, which reduced the number of parameters being estimated, but did not compromise the determination of the geographical origin of northern European populations. A parallel version of MIGRATE-N (1.7.6.1) was used with the following search parameters: 20 short chains of 50 000 steps followed by 1 long chain of 50 000 000 steps, each chain was sampled every 100 steps and an initial burn-in of 10 000 steps was used. Adaptive heating with the following initial relative temperatures {1; 1.1; 1.3; 2} was included, where acceptance–rejection swaps were tried with every step. In addition, each run applied the Gelman's convergence criterion that extends the last run until the convergence criterion is satisfied. Nucleotide frequencies were estimated from the data, and initial estimates of theta and gene flow were obtained using F_{ST} (Beerli 1997–2002). The program was run twice with different random numbers and results were averaged. Populations from Bilbao and Norway were excluded from this analysis due to their small sample sizes (less than 10).

MDIV (Nielsen & Wakeley 2001) was used to distinguish between recent gene flow and the retention of ancestral polymorphism. This program uses a Bayesian approach to simultaneously estimate population divergence times and migration rates between pairs of populations that are assumed to have diverged from a common ancestral population. MDIV was run multiple times with different random seeds and prior distributions to assess the stability of the results. Final results were obtained using the following parameters: Hasegawa–Kishino–Yano (HKY) model (Hasegawa *et al.* 1985) with the transitions/transversion ratio estimated directly from the data; Markov chain simulation for 5 000 000 steps, where the first 500 000 were discarded as burn-in; and uniform prior distributions between 0 and 10 for both M and t_{pop} . MDIV measures divergence time in units of effective population time ($N_e t$), that can be calibrated into generations, and hence years when a specific mutation rate and generation time are assumed. The modes of the posterior distribution for both population divergence time and θ (where $\theta = 2N_e\mu$, and μ is the mutation rate per sequence per generation) were used to estimate divergence times between refugial populations, and to explore the probability that the signatures of

population segregation and range expansion were congruent with late Pleistocene glaciations. The sensitivity of the final results to specific mutation rates and generation times was explored.

Results

Sequence variation

The first 724 base pairs of control region 1 (CR1), and a fragment of 701 bp that included the last 32 bp of ND6, the 74 bp of the complete tRNA-Glu, and 595 bp of control region 2 (hereafter CR2) were sequenced. With the exception of the three owls from North Africa, the sequences of both fragments of the two control regions were obtained for all individuals (GenBank Accession nos: DQ086865–7169).

The combined data comprises a total of 1425 bp for 187 individual tawny owls distributed among 14 populations. This resulted in 148 unique haplotypes and 215 polymorphic sites, of which 136 were parsimony informative, when gaps were treated as a fifth state. The sequences varied in length from 1411 to 1418 bp and the final alignment included 17 indels that varied from 1 to 6 bp. For one individual (It25, Appendix) three nucleotide positions were identified with pyrimidine heteroplasmy. These three polymorphic sites comprised the only differences between this individual and another (It23) from the same population (Sicily) so I took the conservative measure of considering them identical haplotypes in the phylogenetic analyses. The substitution rate was slightly higher in fragment CR2 than CR1; it contained approximately 60% of the total polymorphic sites, and although both fragments had an equal number of informative sites, CR2 had three times as many autapomorphies. With the inclusion of outgroups, the alignment required additional indels, this resulted in a final sequence length of 1430 bp, comprising 25 indels and 283 informative sites. The high percentage of haplotypes to individuals sequenced (ca 80%) and the rapid substitution rate observed for both fragments of the control region argue against the likelihood of having sequenced slower evolving nuclear copies (Sorenson & Fleischer 1996; Pereira & Baker 2004).

The two copies of the control region both appear to have functional components; however, they average 17% divergent within individuals, whereas copies of the same control region average only 1.55% divergence across all Europe. If there were extensive concerted evolution of control region copies within individuals, then the two copies would be expected to be nearly identical. Therefore, polymorphisms in the two CRs were treated as independent characters.

Genetic diversity in these tawny owl populations was substantial (Fig. 1). No haplotype was geographically

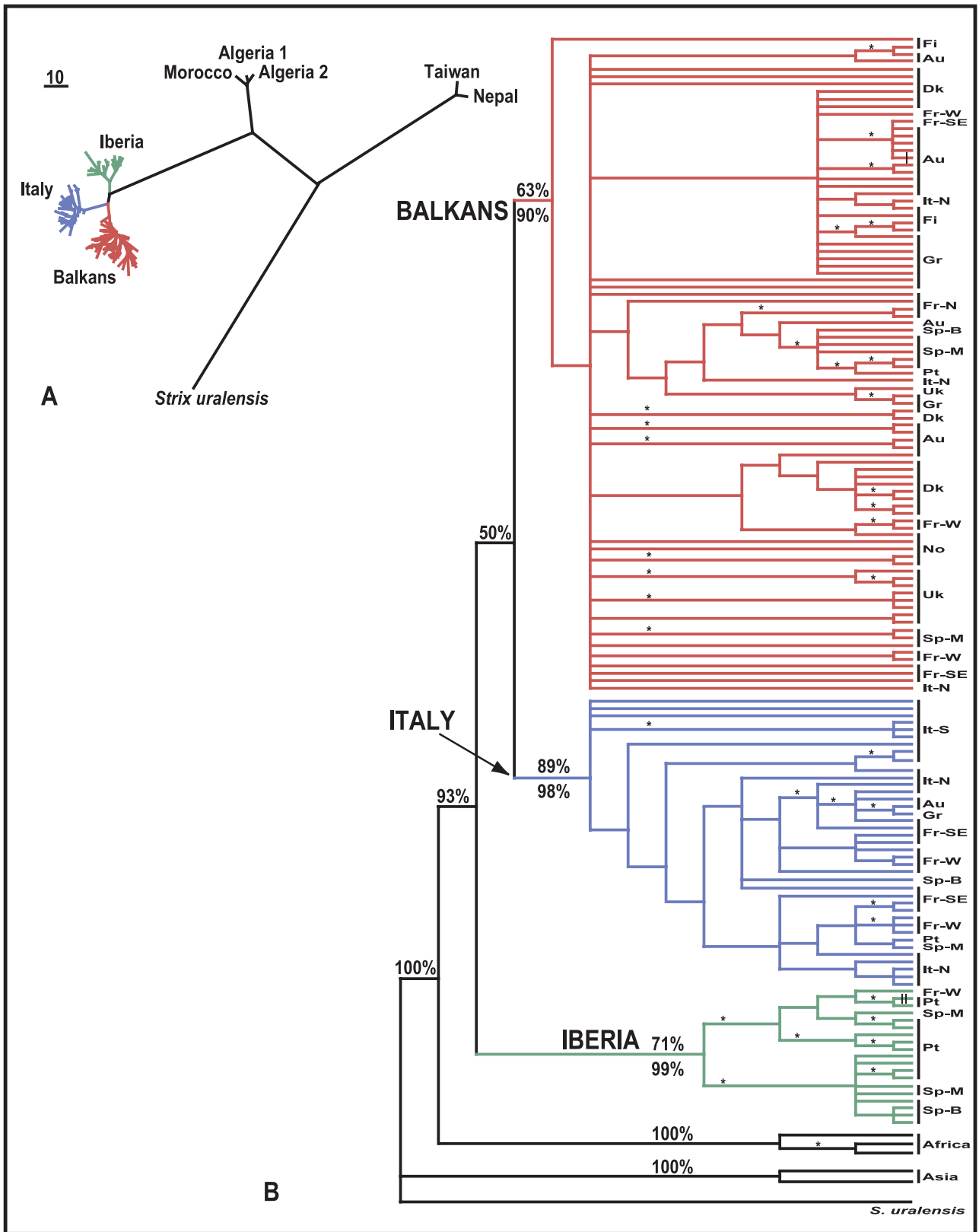


Fig. 2 Phylogenetic relationships among tawny owl haplotypes. (A) Phylogram of one of the most parsimonious trees indicating a monophyletic European tawny owl; branch lengths are drawn to scale. (B) Strict consensus of all most parsimonious trees. Numbers on the branches are bootstrap support for the basal nodes, above the lines when the outgroups are included in the analysis, and below for the unrooted network. *Indicate other nodes with bootstrap support higher than 50%. Terminals are identified by population of origin; one bar over an Austrian terminal indicates a shared haplotype between Austria and SE France. Two bars over a Portuguese terminal indicate a shared haplotype between Portugal and Madrid.

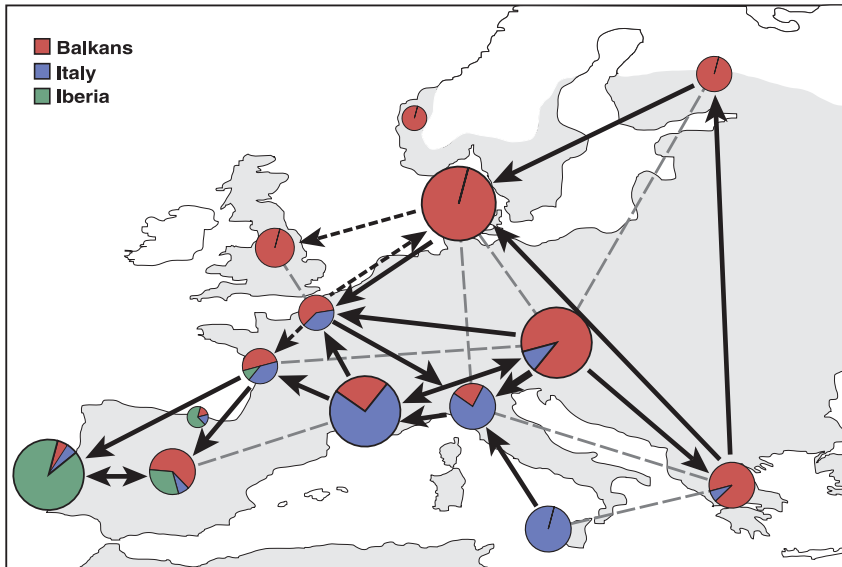


Fig. 3 Haplotype distribution and gene flow among tawny owl populations. Pie charts represent the proportion of individuals in each of the three major clades (Fig. 2); size of pie charts is proportional to sample size. Arrows and dotted lines represent results of MIGRATE. Arrows indicate directional migration whenever $N_e m > 1$; dotted arrows represent directional migration when $0.8 < N_e m < 1$; dotted lines connect populations whose $N_e m$ were, in both directions, less than 0.8. The shaded area indicates current geographical distribution of tawny owls in western Europe.

widespread and only two were shared between populations, one between Portugal and Madrid, and the other between Austria and France-SE (Appendix). Nucleotide diversity ranged between 0.016 in France-W to 0.005 in Norway (Fig. 1). Although northern populations have lower genetic diversity than midlatitudes or refugial populations, nucleotide diversity did not show a clear decreasing pattern with latitude (Fig. 1). The southern populations of Iberia, northern Italy, and Greece had nucleotide diversities that were similar to the ones found in the three French populations and Austria. In addition, Sicily had as low a nucleotide diversity as the northern populations of England, Denmark, Norway, and Finland. In no population did the Tajima's D statistic differ significantly from the expectation under neutrality.

Phylogenetic analyses and geographical distribution of haplotypes

Data sets of DNA sequences for phylogeographic analyses often result in numerous alternate most-parsimonious trees (MPT) due to the large sample sizes and the low levels of genetic divergence among individuals. This study was no exception and the parsimony analysis of all unique haplotypes and outgroups resulted in 351 576 MPT (length 913; CI = 0.54; RI = 0.84). This analysis consisted of 100 replicates with TBR branch swapping that were run for 2 h each. It is possible that if the time limit had been increased or more replicates had been run, the number of MPT would have been even higher. However, in addition to the search reported, I also applied the ratchet, tree-drifting, and tree-fusing algorithms available on TNT (Goloboff *et al.* 2000) and in none of these additional searches were parsimonious trees of shorter length found. Since the

purpose of the phylogenetic analysis was to determine the number of basal clades of haplotypes, and not the particular relationship of every haplotype to each other, it was not necessary to complete TBR searches. Rather, the robustness of the clades was determined by the bootstrap analysis.

The strict consensus recovered a monophyletic European tawny owl with the three North African birds (*Strix aluco mauritanica*) as a sister taxon (Fig. 2). Mean uncorrected sequence divergence between Europe and the outgroups ranged from 7.64%, to the African clade, to 14.20% to *Strix uralensis*, whereas sequence divergence within Europe was 1.55% (SE = 0.18%). Tawny owl specimens from Asia and North Africa represent distinct lineages.

The strict consensus recovered a basal phylogeographic structure of three major clades with high bootstrap support, reflecting haplotype distributions that correspond to recognizable refugial populations (Figs 2b and 3). For this reason I assigned each clade the name of the putative refugia that it reflects; an Iberian clade contains 18 out of the 20 individuals sampled in Portugal (Iberia), a mainly Italian and French clade contains 23 out of 26 individuals sampled in Italy (Italy), and a clade that contains 12 out of 13 individuals sampled in Greece (Balkans). In the strict consensus, Iberia is sister to the other two clades but this relationship has low bootstrap support (50%).

The three major clades of the tawny owl showed significant geographical structure ($\chi^2 = 95.90$; d.f. = 10; $P \ll 0.001$; exact P value estimated by Monte Carlo simulations; alpha = 0.01; $N = 10\ 000$). This analysis rejected the hypothesis that the observed proportion of individuals found in each clade could be obtained by chance alone. The relative frequencies of individuals representing each clade per population were plotted in pie charts and superimposed on geography (Fig. 3).

Table 2 Hierarchical AMOVA computed with the six refugial populations

| Source of variation | d.f. | Sum of squares | Variance components | Percentage of variation |
|----------------------------------|------|----------------|---------------------|-------------------------|
| Among refugia | 2 | 266.951 | 4.081 | 28.37 |
| Among populations within refugia | 3 | 99.635 | 2.052 | 14.27 |
| Within populations | 72 | 594.206 | 8.253 | 57.37 |
| Total | 77 | 960.792 | 14.386 | |

F_{ST} : 0.426, $P << 0.001$.

Table 3 Pairwise N_{ST} estimates (above diagonal) and corresponding migration rates $N_e m$ (below diagonal)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|--------------|------|------|------|--------|------|-------|-------|------|------|-------|------|------|------|------|
| 1. Portugal | | 0.21 | 0.08 | 0.42 | 0.59 | 0.45 | 0.30 | 0.38 | 0.40 | 0.46 | 0.52 | 0.57 | 0.60 | 0.54 |
| 2. Sp-Madrid | 1.88 | | 0.14 | 0.26 | 0.47 | 0.14 | 0.08 | 0.10 | 0.22 | 0.15 | 0.26 | 0.26 | 0.31 | 0.24 |
| 3. Sp-Bilbao | 6.11 | 3.18 | | 0.33 | 0.49 | 0.35 | 0.21 | 0.29 | 0.29 | 0.35 | 0.38 | 0.45 | 0.50 | 0.45 |
| 4. Italy N | 0.69 | 1.45 | 1.04 | | 0.24 | 0.34 | 0.08 | 0.13 | 0.00 | 0.35 | 0.48 | 0.48 | 0.51 | 0.46 |
| 5. Italy S | 0.35 | 0.57 | 0.52 | 1.61 | | 0.59 | 0.32 | 0.40 | 0.25 | 0.59 | 0.68 | 0.69 | 0.71 | 0.69 |
| 6. Greece | 0.60 | 3.00 | 0.94 | 0.98 | 0.35 | | 0.12 | 0.13 | 0.28 | 0.01 | 0.23 | 0.13 | 0.26 | 0.05 |
| 7. France-W | 1.16 | 5.77 | 1.90 | 5.43 | 1.05 | 3.65 | | 0.04 | 0.06 | 0.14 | 0.24 | 0.22 | 0.26 | 0.24 |
| 8. France-N | 0.80 | 4.42 | 1.23 | 3.42 | 0.74 | 3.29 | 12.92 | | 0.13 | 0.15 | 0.30 | 0.27 | 0.32 | 0.25 |
| 9. France-SE | 0.75 | 1.76 | 1.20 | 128.03 | 1.50 | 1.29 | 7.57 | 3.40 | | 0.29 | 0.41 | 0.41 | 0.45 | 0.40 |
| 10. Austria | 0.59 | 2.74 | 0.93 | 0.93 | 0.35 | 81.87 | 3.20 | 2.92 | 1.23 | | 0.25 | 0.14 | 0.26 | 0.04 |
| 11. England | 0.46 | 1.39 | 0.83 | 0.55 | 0.24 | 1.68 | 1.55 | 1.17 | 0.71 | 1.52 | | 0.30 | 0.43 | 0.31 |
| 12. Denmark | 0.38 | 1.42 | 0.62 | 0.54 | 0.23 | 3.30 | 1.78 | 1.37 | 0.72 | 2.97 | 1.16 | | 0.24 | 0.24 |
| 13. Norway | 0.34 | 1.12 | 0.50 | 0.48 | 0.20 | 1.44 | 1.39 | 1.05 | 0.62 | 1.39 | 0.67 | 1.62 | | 0.39 |
| 14. Finland | 0.42 | 1.55 | 0.62 | 0.59 | 0.22 | 9.00 | 1.61 | 1.52 | 0.74 | 10.68 | 1.10 | 1.55 | 0.78 | |

The phylogeography of tawny owl haplotypes also revealed that all individuals sampled in the northern European populations of England, Denmark, Norway, and Finland had haplotypes associated with the Balkans. France was composed of 40% Balkan haplotypes, 55% Italian haplotypes, and 5% Iberian haplotypes. The Iberian haplotypes were not detected further east than the western region of France, while the Balkan haplotypes were found with high frequency as far west as Madrid.

Partitioning of genetic variation

Overall F_{ST} among 14 populations was 0.35 ($P << 0.001$); this indicates substantial genetic structure (Wright 1978, pp. 82–86). A hierarchical analysis of molecular variance (AMOVA) performed with just the six refugial populations recovered similar results (Table 2): $F_{ST} = 0.43$ ($P << 0.001$), but most of the total genetic variance is explained by variance within populations (57.37%).

Gene flow

Results from pairwise N_{ST} analyses of genetic differentiation and levels of gene flow are presented in

Table 3. N_{ST} between Greece and the four most northern European populations of England, Denmark, Norway, and Finland were generally low, varying between 0.05 and 0.26, implying little genetic differentiation between those populations. In contrast, those four northern European populations were significantly structured relative to the Italian and Portuguese populations, having N_{ST} that varied between 0.52 and 0.71 and corresponding low levels of gene flow. N_{ST} analyses suggested a complex phylogeographic history for the Iberian and French regions. On one hand, Iberia is represented by a very homogeneous population in the west (Portugal), but also by a population (Madrid) that is connected by significant gene flow ($Nm > 1$) with northern Europe, northern Italy, and Greece. France, on the other hand, is represented by three populations with differing genetic patterns: northern France shows a similar pattern of gene flow as Austria; the southeast of France is genetically very similar to northern Italy; and western France is the only population that shows $Nm > 1$ with every other population.

Maximum-likelihood estimates of gene flow (MIGRATE) were used to infer directional migration. In most cases MIGRATE resulted in asymmetrical estimates of gene flow between population pairs (Fig. 3). For example, there was

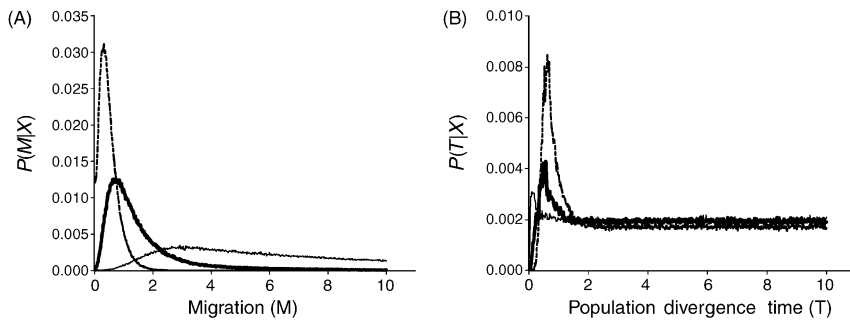


Fig. 4 Relative effects of migration and population divergence time among tawny owl populations. MDIV results for the posterior distribution of M (migration) and t_{pop} (population divergence time) for the following population pairs: Portugal–Greece (dashed line), Madrid–Greece (bold line), and Greece–Finland (fine line).

| Pop. 1 | Pop. 2 | T_{MRCA} | t_{pop} | θ | Population divergence time (T_{pop}) | | | | |
|--------|--------|-------------------|------------------|----------|---|---------|---------|---------|---------|
| | | | | | 2% Myr | 5% Myr | 10% Myr | 15% Myr | 20% Myr |
| Pt | Gr | 1.30 | 0.62 | 27.640 | 300 643 | 120 257 | 60 129 | 40 086 | 30 064 |
| Pt | ItS | 1.74 | 1.32 | 19.216 | 445 002 | 178 001 | 89 000 | 59 334 | 44 500 |
| Gr | ItS | 1.72 | 0.94 | 19.316 | 318 549 | 127 419 | 63 710 | 42 473 | 31 855 |
| Gr | Fi | 1.75 | 0.10 | 17.469 | 30 648 | 12 259 | 6 130 | 4 086 | 3 065 |
| Pt | Africa | 3.47 | 2.50 | 11.386 | 499 391 | 199 756 | 99 878 | 66 585 | 49 939 |

Table 4 MDIV estimates of divergence time (in generations) among refugial populations calibrated for different mutation rates

T_{MRCA} and t_{pop} are measured in units of $2N_e \mu$; generations; $\theta = 2N_e \mu$, and μ is the mutation rate per sequence per generation.

directional gene flow from southern Italy to western and northern France via northern Italy and southeastern France; from Greece to the northern European populations of Finland and Denmark; and, from western France into Iberia. Both MIGRATE and N_{ST} did not detect significant levels of gene flow between southern Italy and Greece, nor between northern Italy and Denmark. In only two situations did MIGRATE fail to detect significant gene flow when the corresponding N_{ST} -based estimate of Nm was greater than 1; these were between Madrid and southeastern France, and western France and Austria. This is because MIGRATE, as opposed to N_{ST} , is not a pairwise estimation but it takes into account the entire network of possible migrations.

MIGRATE produces better estimates of gene flow than F_{ST} -based approaches because it not only uses information from the tree (co-ancestry of alleles) but also accounts for uncertainty in the tree estimation (Beerli & Felsenstein 2001). However, MIGRATE is still unable to distinguish between short divergence times with low levels of gene flow (incomplete lineage sorting) from longer divergence times with moderate gene flow. MIGRATE recovered gene flow from France into Iberia because a high proportion of Madrid haplotypes clustered within the Balkan clade. MDIV was used to determine if those haplotypes are better explained as the result of recent gene flow from France into Iberia, or as the retention of ancestral polymorphism in the Madrid population. Figure 4 shows the posterior distributions for migration (Fig. 4A) and population divergence

time (Fig. 4B) between Madrid and Greece. Results obtained for two other population pairs are also plotted and used as controls: Portugal–Greece, represents two refugial populations assumed to have been separated during the peak of the glaciations; and Finland–Greece, represents populations that have a very recent common ancestry. MDIV estimated migration between Madrid and Greece (mode ≈ 1) that is very different from migration between Portugal and Greece (mode $\ll 1$) or between Greece and Finland (mode ≈ 3). Conversely, population divergence time between Madrid and Greece is similar to divergence time between Portugal and Greece, indicating that those events must have occurred at nearly the same time. Thus, the posterior distributions for both migration and divergence times favour the hypothesis that the Balkan haplotypes found in Madrid are due to recent gene flow and not to incomplete lineage sorting.

Divergence times

MDIV estimates of population divergence time among refugial population samples were used to determine if the temporal divergence of those populations was consistent with a Pleistocene timescale. To calibrate population divergence time in generations before present (T_{pop}), I computed the mode of the posterior distributions for t_{pop} and θ (Table 4), and computed T_{pop} using the equality:

$$T_{\text{pop}} = [(t_{\text{pop}} \cdot \theta) / 2L] / \mu;$$

where L is the sequence length (1425 bp) and μ the mutation rate per site per generation. For a wide range of plausible mutation rates for mtDNA control regions (5%/Myr to 20%/Myr) and considering generation time 1 year, estimated divergence times between refugia date to the late Pleistocene. On the other hand, divergence time between Greece and Finland is one order of magnitude smaller and postdates the LGM.

Discussion

Phylogeographic patterns — glacial refugia

This phylogeographic analysis of tawny owl haplotypes in western Europe corroborates the hypothesis that this species survived the Pleistocene glaciations in three allopatric refugia. In identifying refugial populations I assumed that if a population sampled in one putative refugium forms a highly supported clade with significant geographical structure, then that population must have evolved in allopatry, and by consequence, in one glacial refugium; this is Avise's phylogeographic category I (Avise 2000, p. 169), but in this case there is the added signature of admixture (e.g. Petit *et al.* 2003).

The phylogenetic analysis recovered three highly supported clades in which populations from Iberia, Italy, and the Balkans showed significant geographical structure (Fig. 2). Nucleotide diversities in Portugal, the two populations from Spain (Madrid and Bilbao), northern Italy, and Greece (Fig. 1) were high as expected in refugial populations that have evolved in allopatry (Hewitt 1996). F_{ST} also indicated substantial genetic structure; in the hierarchical AMOVA performed with just the six refugial populations, 57% of the total genetic variance was within populations, while 28% was among refugia. The existence of three glacial refugia for the tawny owl falls in the general pattern that has been obtained for pan-European species (Taberlet *et al.* 1998; Hewitt 2000, 2004).

Phylogeographic patterns — postglacial expansion

The four most northern populations were collected in locations that during the LGM were either covered by ice or tundra vegetation (Tzedakis *et al.* 2002) and could not have sustained a forest-dependent species like the tawny owl. My analyses demonstrated that tawny owls currently found in those locations originated from range expansion from a refugium located in the Balkan region. The proportion of individuals per clade in each population (pie charts in Fig. 3) shows that all individuals sampled in northern Europe are part of the Balkan clade. Nucleotide diversities for England, Denmark, Norway, and Finland are low as expected in recent populations (Fig. 1). Population expansion out of the Balkan region is corroborated by the

low genetic structure found among northern European populations and Greece that can only be explained by ongoing gene flow or recent ancestry.

Phylogeographic results from a wide variety of European taxa were summarized into three patterns of postglacial expansion — *grasshopper*, *bear*, and *hedgehog* — that reflect different contributions from each refugium to the northern European populations (Hewitt 1999, 2000). These differential contributions may be due to the relative effectiveness of the southern mountain ranges (Pyrenees and Alps) as barriers to gene flow or/and to different timings and pacing of postglacial expansion. Tawny owl postglacial expansion is most similar to the common pattern of range expansion in Europe — *the grasshopper paradigm* — that describes species where the Balkan refugium is solely responsible for colonization of all northern Europe (Hewitt 1999, 2000).

More recently, a fourth glacial refugia has been proposed in the Caspian/Caucasus region (Hewitt 2004; Deffontaine *et al.* 2005). Tawny owls that currently occupy that area belong to another subspecies (*Strix aluco wilkenskii*) and preliminary cytochrome *b* data suggest that this subspecies is highly divergent from tawny owls of western Europe (Brito in prep.). In addition, if northern Europe had been repopulated from an unsampled refugium, a fourth clade would have been recovered in the phylogeographic analysis.

Other phylogeographic patterns

Data from the French populations recovered a very complex phylogeographic history for France: here expansions from all three putative refugia converge. This explains the high genetic diversity detected in all French populations as well as in Austria. Populations from France and Austria, although not included in current hypotheses of refugial location, have nucleotide diversities that are as high as the putative refugia populations. Instead of postulating additional refugia locations, this high genetic diversity is better explained as a consequence of the confluence of lineages of multiple origin (Petit *et al.* 2003).

In contrast, Sicily has unexpectedly low genetic diversity for a refugial population. Its nucleotide diversity is as low as that of England and Finland (Fig. 1). However, the hypothesis that Sicily was part of the Italian refugia is substantiated by the presence of temperate woodland during the LGM (Frenzel 1973), land connection with continental Italy (Nilsson 1983), and fossils of *Strix aluco* dated from the early late Pleistocene (Pavia 2001). This low nucleotide diversity could instead be due to a recent bottleneck. Alternatively, Hewitt (1996) hypothesized that refugial populations that colonized northern Europe may now have reduced genetic diversity due to reduction in population size at the southern ends of their distribution while their

range shifts northwards. The Sicilian population was also found to be very structured relative to Greece, suggesting that the Adriatic and Ionian seas have been effective barriers to gene flow and no significant interchange occurred between these two refugia.

The Madrid sample also has a complex phylogeographic history. Unlike the Portuguese population with 90% of its individuals in the Iberian clade, Madrid only had 31%. More than 60% of the individuals sampled in Madrid clustered within the Balkan clade. However, as the MIGRATE and MDIV results indicate, it is high gene flow from France into Iberia and not expansion out of Iberia into northern Europe that is responsible for the high proportion of Balkan haplotypes in the Madrid population. Other studies have detected complex phylogeographic histories for Iberian populations (Alexandrino *et al.* 2000; Branco *et al.* 2002; Paulo *et al.* 2002). However, unlike this interpretation of recent gene flow into Iberia, those studies have suggested multiple allopatric refugia within the Iberian Peninsula.

Species-specific patterns of postglacial expansion have to be explained by a contribution of orography, palaeoecological conditions after the LGM, and individual natural history traits. The reason why Balkan tawny owls had time to reach Iberia while Iberian owls only reached western France must be related to the efficiency of the Pyrenees and the Alps as barriers to northward expansion from the two other refuges. However, given enough time and unchanging climatic conditions, it is possible that tawny owls from Iberia and Italy will eventually reach northern Europe through gene flow and introgression.

Divergence times

The glacial refugia hypothesis for western Europe makes explicit predictions for divergence times among refugial populations and between refugia and northern European populations. It is expected that refugium populations diverged during the late Pleistocene or earlier, before the LGM, while divergence times between recent northern populations and source refugia populations should postdate the LGM, and occur sometime after the beginning of the interglacial period (approx. 16 000 Myr).

Since at present there is no well-supported, independent calibration of DNA substitution rates for Strigidae, I evaluated a range of possible rates. These varied from the widely used 2% per Myr (cytochrome *b*) to 20.8% for the domain I of the CR (Quinn 1992). With the exception of the 2% rate, divergence times obtained for different population pairs of tawny owl are congruent with the Pleistocene glaciations (Table 4). MDIV dates the divergence time between the refugial populations to the late Pleistocene before the LGM, and the divergence time between Greece and Finland reflects their very recent common

ancestry and suggests a northward expansion after the LGM. Comparisons between cytochrome *b* and control region sequences for the same individuals (P. H. Brito, unpublished) imply that the fragments of control region sequenced in this study evolve on average 2.26 times faster than cytochrome *b* sequences. Therefore, if the widely used substitution rate of 2% per Myr is correct for cytochrome *b* of tawny owl, then this section of the control region should have a substitution rate close to 4.5% per Myr. This agrees with estimates for other species. Drovetski (2003) calibrated a molecular clock for four grouse genera using the complete control region at between 4.54% and 12.45% per Myr, while Quinn (1992) estimated a mutation rate of 20.8% per Myr for domain I of the lesser snow goose control region.

The results presented (Table 4) assume that generation time for tawny owls is 1 year. It is not clear how generation time of a long-lived organism with overlapping generations should be used to calibrate units of coalescent time, measured in N_e generations, to real time units measured in years. Tawny owls are known to occasionally breed at 1 year of age, but more frequently at 2–3 years old (Delmée *et al.* 1978). On the other hand, generation time for tawny owls was estimated as 4.95 years (Barrowclough & Coats 1985) using a life table based on original data of Southern (1970). If divergence times are calibrated for generation time using a factor of approximately 5, then the Pleistocene hypothesis can only be supported if the substitution rate is 20% per Myr or higher. Otherwise, divergence between Greece and Finland will predate the LGM and hence reject the late Pleistocene hypothesis. In either case, divergence between refugial populations date to the Pleistocene, and consequently my data support the Avise & Walker (1998) conclusion that the Pleistocene was important for creating phylogeographic subdivisions within species.

The T_{MRCA} between population pairs of tawny owl in Europe are all roughly similar (Table 4) as opposed to t_{pop} that is one order of magnitude smaller for Greece–Finland than it is between refugial populations. This difference is expected between populations that have very recent co-ancestry (Edwards & Beerli 2000; Rosenberg & Feldman 2002), and it indicates that population divergence time, and not gene lineage divergence, is the parameter of interest in most phylogeographic studies. The relative divergence times among the three refugial populations as well as the shape of the phylogram (Fig. 1A) and the low bootstrap support for the relationship [Iberia (Italy, Balkans)] (Fig. 1B) suggest that the splitting into the three refugial populations occurred due to two nearly simultaneous events. The calibrated divergence time between Portugal and North Africa is only slightly greater than between Portugal and Sicily although t_{pop} is twice as high (Table 4). This result may be due to an underestimate of θ due to the small sample size of the North Africa population ($N = 3$).

The two copies of the mitochondrial control region provided sufficient data to fully recover the late Pleistocene and recent history of tawny owls in western Europe. By sampling all three putative refugia as well as several populations across northern Europe, the study design assured that signatures of refugia population and postglacial expansion routes would be revealed if still present. However, due to the haploid, maternal inheritance, mitochondrial genomes provide only a single realization of the evolutionary history of a species (e.g. McVean 2001). However, the high genealogical concordance of codistributed species with similar habitat requirements (Hewitt 2000, 2004) suggests shared historical biogeographical factors in shaping these phylogeographies (Avice 2000, pp. 215–223).

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This work is part of Patrícia H. Brito's doctoral thesis research on the phylogeography and population genetics of the tawny owl in Europe. Her interests concern the application of phylogenetic and population genetic methods to the study of speciation, geographical variation, and gene flow in natural populations.

Appendix

List of localities and accession numbers from samples used in this study; samples are sorted by population: Austria (Au), Denmark (Dk), England (Uk), Finland (Fi), France-north (Fr_N), France-west (Fr_W), France-southeast (Fr_{SE}), Greece (Gr), Italy-north (It_N), Italy-Sicily (It_S), Norway (No), Portugal (Pt), Spain-Bilbao (Sp_B), Spain-Madrid (Sp_M)

| Sample code | Locality/Region | Clade* | Identical haplotypes | Hap. codet | |
|-------------|---|--------|----------------------|------------|------|
| | | | | CR1 | CR2 |
| Au02 | Stopfenreuth, Niederosterreich, Austria | Bk | — | 6865 | 7019 |
| Au05 | Schrems, Niederosterreich, Austria | Bk | — | 6866 | 7020 |
| Au06 | Sinabelkirchen, Steiermark, Austria | It | — | 6867 | 7021 |
| Au07 | Leobersdorf, Niederosterreich, Austria | Bk | — | 6868 | 7022 |
| Au08 | Haringsee, Niederosterreich, Austria | Bk | — | 6869 | 7023 |
| Au09 | Parndorf, Burgenland, Austria | Bk | — | 6870 | 7024 |
| Au10 | Haringsee, Niederosterreich, Austria | Bk | — | 6871 | 7025 |
| Au11 | Haringsee, Niederosterreich, Austria | Bk | — | 6872 | 7026 |
| Au12 | Haringsee, Niederosterreich, Austria | It | — | 6873 | 7027 |
| Au13 | Parndorf, Burgenland, Austria | Bk | — | 6874 | 7028 |
| Au14 | Haringsee, Niederosterreich, Austria | Bk | — | 6875 | 7029 |
| Au15 | Parndorf, Burgenland, Austria | Bk | — | 6876 | 7030 |
| Au16 | Parndorf, Burgenland, Austria | Bk | — | 6877 | 7031 |
| Au17 | Parndorf, Burgenland, Austria | Bk | Fr _{SE} 88 | 6878 | 7032 |
| Au18 | Wels, Oberosterreich, Austria | Bk | — | 6879 | 7033 |
| Au19 | Goldwörth, Oberosterreich, Austria | Bk | — | 6880 | 7034 |
| Au20 | Schwertberg, Oberosterreich, Austria | Bk | — | 6881 | 7035 |
| Au22 | Sigharting, Oberosterreich, Austria | Bk | — | 6882 | 7036 |
| Au24 | Niederspaching, Oberosterreich, Austria | Bk | — | 6883 | 7037 |
| Au27 | Maria Laah, Oberosterreich, Austria | Bk | — | 6884 | 7038 |
| Dk01 | Hillerød, Zeeland, Denmark | Bk | Dk26 | 6885 | 7039 |
| Dk02 | Hillerød, Zeeland, Denmark | Bk | — | 6886 | 7040 |
| Dk03 | Hillerød, Zeeland, Denmark | Bk | — | 6887 | 7041 |
| Dk04 | Hillerød, Zeeland, Denmark | Bk | Dk21 | 6888 | 7042 |
| Dk05 | Hillerød, Zeeland, Denmark | Bk | — | 6889 | 7043 |
| Dk06 | Hillerød, Zeeland, Denmark | Bk | Dk07 | 6890 | 7044 |
| Dk07 | Hillerød, Zeeland, Denmark | Bk | Dk06 | 6890 | 7044 |
| Dk08 | Viborg Amt, Jutland, Denmark | Bk | Dk15, 17 | 6891 | 7045 |
| Dk10 | Viborg Amt, Jutland, Denmark | Bk | — | 6892 | 7046 |
| Dk12 | Frederiksvaerk, Jutland, Denmark | Bk | — | 6893 | 7047 |
| Dk13 | Rodskov, Jutland, Denmark | Bk | — | 6894 | 7048 |
| Dk14 | Hobro, Jutland, Denmark | Bk | — | 6895 | 7049 |
| Dk15 | Løgster, Jutland, Denmark | Bk | Dk08, 17 | 6891 | 7045 |
| Dk16 | Bajlum, Jutland, Denmark | Bk | — | 6896 | 7050 |
| Dk17 | Arden, Jutland, Denmark | Bk | Dk08, 15 | 6891 | 7045 |
| Dk18 | Djursland, Jutland, Denmark | Bk | — | 6897 | 7051 |
| Dk20 | Hillerød, Zeeland, Denmark | Bk | Dk23 | 6898 | 7052 |
| Dk21 | Hillerød, Zeeland, Denmark | Bk | Dk04 | 6888 | 7042 |
| Dk23 | Hillerød, Zeeland, Denmark | Bk | Dk20 | 6898 | 7052 |
| Dk26 | Hillerød, Zeeland, Denmark | Bk | Dk01 | 6885 | 7039 |
| Dk40 | Jutland, Denmark | Bk | — | 6899 | 7053 |
| Uk01 | Stoke-on-Trent, Trentham, UK | Bk | — | 6900 | 7054 |
| Uk02 | Huntingdon, Cambridgeshire, UK | Bk | — | 6901 | 7055 |
| Uk03 | Huntingdon, Cambridgeshire, UK | Bk | — | 6902 | 7056 |
| Uk04 | Huntingdon, Cambridgeshire, UK | Bk | — | 6903 | 7057 |
| Uk05 | Huntingdon, Cambridgeshire, UK | Bk | — | 6904 | 7058 |
| Uk06 | Huntingdon, Cambridgeshire, UK | Bk | Uk08 | 6905 | 7059 |
| Uk07 | Huntingdon, Cambridgeshire, UK | Bk | — | 6906 | 7060 |
| Uk08 | Huntingdon, Cambridgeshire, UK | Bk | Uk06 | 6905 | 7059 |
| Uk09 | Huntingdon, Cambridgeshire, UK | Bk | Uk10 | 6907 | 7061 |
| Uk10 | Huntingdon, Cambridgeshire, UK | Bk | Uk09 | 6907 | 7061 |

Appendix *Continued*

| Sample code | Locality/Region | Clade* | Identical haplotypes | Hap. codet | |
|---------------------|--|--------|---------------------------------|------------|------|
| | | | | CR1 | CR2 |
| Uk11 | Huntingdon, Cambridgeshire, UK | Bk | — | 6908 | 7062 |
| Fi01 | Junakkala, Finland | Bk | — | 6909 | 7063 |
| Fi02 | Nokia, Finland | Bk | — | 6910 | 7064 |
| Fi03 | Hauho, Finland | Bk | Fi05, 09 | 6911 | 7065 |
| Fi04 | Mikkeli, Finland | Bk | Fi06, 08 | 6912 | 7066 |
| Fi05 | Helsinki, Finland | Bk | Fi03, 09 | 6911 | 7065 |
| Fi06 | Espoo, Finland | Bk | Fi04, 08 | 6912 | 7066 |
| Fi07 | Porvoo, Finland | Bk | — | 6913 | 7067 |
| Fi08 | Imatra, Finland | Bk | Fi04, 06 | 6912 | 7066 |
| Fi09 | Espoo, Finland | Bk | Fi03, 05 | 6911 | 7065 |
| Fi10 | Helsinki, Finland | Bk | — | 6914 | 7068 |
| Fr _N 06 | Roncq, Lille, Nord Pas de Calais, France | It | Fr _N 11 | 6915 | 7069 |
| Fr _N 07 | St. Omer, Lille, Nord Pas de Calais, France | Bk | Fr _N 12, 13 | 6916 | 7070 |
| Fr _N 08 | Masnieres, Lille, Nord Pas de Calais, France | Bk | — | 6917 | 7071 |
| Fr _N 09 | Lille, Nord Pas de Calais, France | It | — | 6918 | 7072 |
| Fr _N 10 | Houdaim, Lille, Nord Pas de Calais, France | Bk | — | 6919 | 7073 |
| Fr _N 11 | Lille, Nord Pas de Calais, France | It | Fr _N 06 | 6915 | 7069 |
| Fr _N 12 | Lille, Nord Pas de Calais, France | Bk | Fr _N 07, 13 | 6916 | 7070 |
| Fr _N 13 | Lille, Nord Pas de Calais, France | Bk | Fr _N 07, 12 | 6916 | 7070 |
| Fr _N 14 | Lille, Nord Pas de Calais, France | Bk | — | 6920 | 7074 |
| Fr _N 15 | Lille, Nord Pas de Calais, France | It | — | 6921 | 7075 |
| Fr _W 22 | Poitou Charente, France | Bk | — | 6922 | 7076 |
| Fr _W 23 | Poitou Charente, France | It | — | 6923 | 7077 |
| Fr _W 24 | Poitou Charente, France | It | — | 6924 | 7078 |
| Fr _W 25 | Poitou Charente, France | Bk | — | 6925 | 7079 |
| Fr _W 26 | Poitou Charente, France | Bk | — | 6926 | 7080 |
| Fr _W 27 | Poitou Charente, France | Bk | — | 6927 | 7081 |
| Fr _W 28 | Poitou Charente, France | It | — | 6928 | 7082 |
| Fr _W 29 | Poitou Charente, France | Ib | — | 6929 | 7083 |
| Fr _W 30 | Poitou Charente, France | Bk | — | 6930 | 7084 |
| Fr _W 31 | Poitou Charente, France | It | — | 6931 | 7085 |
| Fr _{SE} 71 | Orange, Vaucluse, PACA, France | Bk | — | 6932 | 7086 |
| Fr _{SE} 73 | Bollene, Vaucluse, PACA, France | It | — | 6933 | 7087 |
| Fr _{SE} 74 | Cavaillon, Vaucluse, PACA, France | It | — | 6934 | 7088 |
| Fr _{SE} 75 | Pernes les Fontaines, Vaucluse, PACA, France | Bk | Fr _{SE} 81 | 6935 | 7089 |
| Fr _{SE} 76 | Cavaillon, Vaucluse, PACA, France | It | Fr _{SE} 82, 93 | 6936 | 7090 |
| Fr _{SE} 78 | Pernes les Fontaines, Vaucluse, PACA, France | It | Fr _{SE} 79, 80, 83, 87 | 6937 | 7091 |
| Fr _{SE} 79 | Pernes les Fontaines, Vaucluse, PACA, France | It | Fr _{SE} 78, 80, 83, 87 | 6937 | 7091 |
| Fr _{SE} 80 | La Tour D'Aigues, Vaucluse, PACA, France | It | Fr _{SE} 78, 79, 83, 87 | 6937 | 7091 |
| Fr _{SE} 81 | La Tour D'Aigues, Vaucluse, PACA, France | Bk | Fr _{SE} 75 | 6935 | 7089 |
| Fr _{SE} 82 | Merindol, Vaucluse, PACA, France | It | Fr _{SE} 76, 93 | 6936 | 7090 |
| Fr _{SE} 83 | Carpentras, Vaucluse, PACA, France | It | Fr _{SE} 78, 79, 80, 87 | 6937 | 7091 |
| Fr _{SE} 84 | Violes, Vaucluse, PACA, France | It | — | 6938 | 7092 |
| Fr _{SE} 85 | Bollene, Vaucluse, PACA, France | It | — | 6939 | 7093 |
| Fr _{SE} 86 | Monteux, Vaucluse, PACA, France | It | — | 6940 | 7094 |
| Fr _{SE} 87 | Carpentras, Vaucluse, PACA, France | It | Fr _{SE} 78, 79, 80, 83 | 6937 | 7091 |
| Fr _{SE} 88 | Cadenet, Vaucluse, PACA, France | Bk | Au17 | 6878 | 7032 |
| Fr _{SE} 89 | Morieres les Avignon, Vaucluse, PACA, France | Bk | — | 6941 | 7095 |
| Fr _{SE} 91 | Saignon, Vaucluse, PACA, France | It | — | 6942 | 7096 |
| Fr _{SE} 92 | Mane, Vaucluse, PACA, France | Bk | — | 6943 | 7097 |
| Fr _{SE} 93 | St. Martin de la Brasque, Vaucluse, PACA, France | It | Fr _{SE} 76, 82 | 6936 | 7090 |
| Gr05 | Greece | Bk | — | 6944 | 7098 |
| Gr06 | Greece | Bk | — | 6945 | 7099 |
| Gr07 | Greece | Bk | — | 6946 | 7100 |
| Gr08 | Arachova, Greece | It | — | 6947 | 7101 |

Appendix Continued

| Sample code | Locality/Region | Clade* | Identical haplotypes | Hap. code† | |
|--------------------|--|--------|------------------------|------------|------|
| | | | | CR1 | CR2 |
| Gr09 | Greece | Bk | — | 6948 | 7102 |
| Gr10 | NW Athens, Greece | Bk | Gr16 | 6949 | 7103 |
| Gr11 | Tripoli, Arkadias, Greece | Bk | — | 6950 | 7104 |
| Gr12 | Greece | Bk | — | 6951 | 7105 |
| Gr13 | Thessaloniki, Greece | Bk | — | 6952 | 7106 |
| Gr14 | Kalamata, Peloponese, Greece | Bk | — | 6953 | 7107 |
| Gr15 | Ilieia, Peloponese, Greece | Bk | — | 6954 | 7108 |
| Gr16 | Greece | Bk | Gr10 | 6949 | 7103 |
| Gr17 | Katerini, Greece | Bk | — | 6955 | 7109 |
| It _N 01 | Parma, Italy | It | — | 6956 | 7110 |
| It _N 02 | Cremona, Italy | It | — | 6957 | 7111 |
| It _N 03 | Sarzana, Italy | It | — | 6958 | 7112 |
| It _N 04 | Cremona, Italy | It | It _N 07 | 6959 | 7113 |
| It _N 05 | Parma, Italy | Bk | — | 6960 | 7114 |
| It _N 06 | Piacenza, Italy | It | — | 6961 | 7115 |
| It _N 07 | Tizzano, Italy | It | It _N 04 | 6959 | 7113 |
| It _N 08 | Cremona, Italy | It | — | 6962 | 7116 |
| It _N 09 | Cremona, Italy | It | — | 6963 | 7117 |
| It _N 10 | Piemonte, Italy | Bk | — | 6964 | 7118 |
| It _N 11 | Venaria Reale, Turin, Italy | Bk | — | 6965 | 7119 |
| It _N 12 | Cuneo, Italy | It | — | 6966 | 7120 |
| It _N 13 | Carignano, Turin, Italy | It | — | 6967 | 7121 |
| It _S 15 | Messina, Sicily, Italy | It | — | 6968 | 7122 |
| It _S 16 | Gratteri, Palermo, Sicily, Italy | It | It _S 26 | 6969 | 7123 |
| It _S 17 | Ficuzza Wood, Palermo, Sicily, Italy | It | — | 6970 | 7124 |
| It _S 18 | Monreale, Palermo, Sicily, Italy | It | It _S 19, 22 | 6971 | 7125 |
| It _S 19 | Giacalone, Palermo, Sicily, Italy | It | It _S 18, 22 | 6971 | 7125 |
| It _S 20 | Ficuzza, Palermo, Sicily, Italy | It | — | 6972 | 7126 |
| It _S 21 | Pollina, Palermo, Sicily, Italy | It | — | 6973 | 7127 |
| It _S 22 | Gibilmanna, Palermo, Sicily, Italy | It | It _S 18, 19 | 6971 | 7125 |
| It _S 23 | Palermo, Sicily, Italy | It | It _S 25 | 6974 | 7128 |
| It _S 24 | Dintorni di Palermo, Sicily, Italy | It | — | 6975 | 7129 |
| It _S 25 | Giacalone, Palermo, Sicily, Italy | It | It _S 23 | 6974 | 7128 |
| It _S 26 | Parco Della Favourita, Palermo, Sicily, Italy | It | It _S 16 | 6969 | 7123 |
| It _S 27 | Traponi, Sicily, Italy | It | — | 6976 | 7130 |
| No01 | Landås, Bergen, Hordaland, Norway | Bk | — | 6977 | 7131 |
| No02 | Risnes, Bergen, Hordaland, Norway | Bk | No05 | 6978 | 7132 |
| No03 | Sløgstadmarka, Standa, Møre og Romsdal, Norway | Bk | No06 | 6979 | 7133 |
| No04 | Bergen Domkerke, Bergen, Hordaland, Norway | Bk | — | 6980 | 7134 |
| No05 | Fanahammeren, Bergen, Hordaland, Norway | Bk | No02 | 6978 | 7132 |
| No06 | Bjordal, Høyanger, Sogn og Fjordane, Norway | Bk | No03 | 6979 | 7133 |
| No07 | Norway | Bk | — | 6981 | 7135 |
| Pt01 | Lisbon, Portugal | Ib | — | 6982 | 7136 |
| Pt02 | Sintra, Lisbon, Portugal | Ib | — | 6983 | 7137 |
| Pt03 | Mora, Évora, Portugal | Bk | — | 6984 | 7138 |
| Pt04 | Lisbon, Portugal | Ib | — | 6985 | 7139 |
| Pt05 | Olhão, Faro, Portugal | Ib | Pt06, 07, 08 | 6986 | 7140 |
| Pt06 | Monsanto, Lisbon, Portugal | Ib | Pt05, 07, 08 | 6986 | 7140 |
| Pt07 | Portugal | Ib | Pt05, 06, 08 | 6986 | 7140 |
| Pt08 | Portugal | Ib | Pt05, 06, 07 | 6986 | 7140 |
| Pt09 | Portugal | Ib | Pt11, 15 | 6987 | 7141 |
| Pt10 | Portugal | It | — | 6988 | 7142 |
| Pt11 | Portugal | Ib | P09, 15 | 6987 | 7141 |
| Pt12 | Chaves, Vila Real, Portugal | Ib | — | 6989 | 7143 |
| Pt13 | Portugal | Ib | Pt18 | 6990 | 7144 |

Appendix Continued

| Sample code | Locality/Region | Clade* | Identical haplotypes | Hap. codet | |
|--------------------|---|--------------------------------|----------------------|------------|------|
| | | | | CR1 | CR2 |
| Pt15 | Portugal | Ib | Pt09, 11 | 6989 | 7141 |
| Pt16 | Penamacor, Castelo Branco, Portugal | Ib | Sp _M 12 | 6991 | 7145 |
| Pt17 | Portugal | Ib | — | 6992 | 7146 |
| Pt18 | Évora, Portugal | Ib | Pt13 | 6990 | 7144 |
| Pt19 | Portalegre, Portugal | Ib | Pt21 | 6993 | 7147 |
| Pt20 | Évora, Portugal | Ib | — | 6994 | 7148 |
| Pt21 | Coruche, Santarém, Portugal | Ib | Pt19 | 6993 | 7147 |
| Sp _B 01 | Vizcaya, Spain | Bk | — | 6995 | 7149 |
| Sp _B 02 | Vizcaya, Spain | Ib | — | 6996 | 7150 |
| Sp _B 03 | Vizcaya, Spain | It | — | 6997 | 7151 |
| Sp _B 04 | Asturias, Spain | Ib | — | 6998 | 7152 |
| Sp _B 05 | Vizcaya, Spain | Ib | — | 6999 | 7153 |
| Sp _B 06 | Vizcaya, Spain | Ib | — | 7000 | 7154 |
| Sp _M 09 | Villamanta, Madrid, Spain | Bk | — | 7001 | 7155 |
| Sp _M 10 | Soria, Spain | It | — | 7002 | 7156 |
| Sp _M 11 | Rozas de Puerto Real, Madrid, Spain | Bk | — | 7003 | 7157 |
| Sp _M 12 | Moce Jón, Toledo, Spain | Ib | Pt16 | 6991 | 7145 |
| Sp _M 13 | Polan, Toledo, Spain | Bk | — | 7004 | 7158 |
| Sp _M 14 | Mostoles, Madrid, Spain | Bk | — | 7005 | 7159 |
| Sp _M 15 | Sevilla la Nueva, Madrid, Spain | Bk | — | 7006 | 7160 |
| Sp _M 16 | Madrid, Spain | Ib | — | 7007 | 7161 |
| Sp _M 17 | Madrid, Spain | Bk | — | 7008 | 7162 |
| Sp _M 18 | Madrid, Spain | Ib | — | 7009 | 7163 |
| Sp _M 19 | San Sebastián de los Reyes, Madrid, Spain | Ib | — | 7010 | 7164 |
| Sp _M 20 | Tres Cantos, Madrid, Spain | Bk | — | 7011 | 7165 |
| Sp _M 21 | Navalcarnero, Madrid, Spain | Bk | — | 7012 | 7166 |
| NAfr1 | Tangier, Morocco | <i>Strix aluco mauritanica</i> | | 7013 | — |
| NAfr2 | Batra, Algeria | <i>Strix aluco mauritanica</i> | | 7014 | — |
| NAfr3 | Tizi Ouzou, Algeria | <i>Strix aluco mauritanica</i> | | 7015 | — |
| Asia1 | Taiwan | <i>Strix aluco yamadae</i> | | 7016 | 7167 |
| Asia2 | Gonga, Nepal | <i>Strix aluco nivicola</i> | | 7017 | 7168 |
| Ural | Keuruu, Finland | <i>Strix uralensis</i> | | 7018 | 7169 |

*Clade reflects phylogenetic results from Fig. 2; †hap. code corresponds to the last four digits of GenBank Accession no.; prefix for all haplotypes is DQ08. CR1 and CR2 correspond to the two fragments of the control region.