PHYLOGEOGRAPHY AND POPULATION GENETICS OF THE TAWNY OWL (*STRIX ALUCO*) IN WESTERN EUROPE: CONTRAST BETWEEN MITOCHONDRIAL AND NUCLEAR MARKERS

by

PATRÍCIA H. BRITO

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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____

Date Chair of the Examining Committee
Dr. George F. Barrowclough, American Museum of Natural History

____

Date Executive Officer
Dr. Richard L. Chappell

____

Dr. Robert Rockwell, City College of New York

____

Dr. Rob DeSalle, American Museum of Natural History

____

Dr. John H. Wahlert, Baruch College

____

Robert M. Zink, University of Minnesota

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

ABSTRACT

PHYLOGEOGRAPHY AND POPULATION GENETICS OF THE TAWNY OWL (*STRIX ALUCO*) IN WESTERN EUROPE: CONTRAST BETWEEN MITOCHONDRIAL AND NUCLEAR MARKERS

by

Patrícia H. Brito

Adviser: Dr. George F. Barrowclough

The glacial refugia hypothesis indicates that during the height of the Pleistocene glaciations the temperate species that are today widespread across western Europe must have survived in small and climatically favorable areas located in the southern peninsulas of Iberia, Italy and the Balkans. This hypothesis was investigated with the tawny owl (*Strix aluco*), a relatively sedentary, nonmigratory bird that is currently widespread throughout Europe. Populations from all putative refugia as well as from northern Europe were sampled and individuals were analyzed using both mitochondrial and nuclear markers. A total of 1425 bp of mitochondrial control region sequences and seven microsatellite loci were sequenced and genotyped from 187 individual owls distributed among 14 populations. High congruence was obtained in the detection of three groups of tawny owls in western Europe that corroborate the three Pleistocene refugia, although additional assumptions were required with the microsatellite data. Both mitochondrial and microsatellites recovered the Balkans' origin of northern populations of tawny owls due to post-glacial expansion, as well as extensive gene flow between northern Italy and

adjacent mid-latitude populations. Estimates of population divergence times between refugial populations, as computed with mtDNA data, were 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. Two hybrid zones, corresponding to the meeting of the different refugial populations (Iberia-Balkans) and (Italy-Balkans) were detected with both marker classes but the precise location of the contact zones differed between markers. When corrected for differences in effective population size, mtDNA showed less structure than did the microsatellites, which is consistent with female-biased dispersal, typical of avian taxa. The mitochondrial and microsatellite results were largely congruent, and when they differed it was often (but not always) due to differences in natural history, behavior, and molecular biology.

DEDICATION

To my parents Margarida and Fernando Brito

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GENERAL INTRODUCTION

The discipline of phylogeography is heuristically placed between the fields of historical biogeography and population genetics (Avise 2000). First defined in 1987 (Avise *et al.* 1987; Avise 1998), phylogeography applies the tools of molecular systematics to study patterns of intraspecific genetic variation. Gene genealogies that relate individual haplotypes within and among populations are overlapped on geography, and analyzed through the principles of coalescence theory. Ultimately, patterns of historical demography are interpreted and related with the geographic distribution of the genealogical lineages (Avise *et al.* 1987). Phylogeography shares with population genetics the goal of understanding how mutation, natural selection, random drift, and gene flow interact to produce the genetic diversity that is observed in natural populations. The emphasis on genealogies, as opposed to summary statistics, is what separates these two fields of evolutionary biology (Hey & Machado 2003). However, phylogeography is currently undergoing major methodological advances and it is moving from single gene tree analyses to multilocus data, hypothesis testing, and highly sophisticated statistical analyses meant to deal with stochastic variance due to sampling and drift (review in Hey & Machado 2003; Knowles 2004). However, a major feature remains: the importance of assessing congruence across sequence characters, gene trees, species within the same geographic area, and with independently generated information such as paleoecological and bioclimatic data.

In the northern hemisphere it has been recognized that the main historical processes with potentially major influence on the current genetic structure of populations

are the Quaternary climatic fluctuations (Frenzel 1973; Hewitt 1996, 2004). Paleoclimatic research has shown that the Earth's climate over the past million years has been dominated by major ice ages with roughly 100 kyr cycles interrupted by relatively short and warm interglacials (Hays *et al.* 1976; Imbrie *et al.* 1993). This alternation of glacial and interglacial climates was recognized to be a consequence of the regular variation in the Earth's orbital geometry that is explained by the Milankovitch theory (Hays *et al.* 1976; Paillard 1998). During the Pleistocene, climate changed from full ice age to full interglacial conditions, and the height of the last glaciation (LGM) is dated to have occurred around 18 000 BP (Prentice *et al.* 2000). In the northern hemisphere, the Scandinavian ice sheet reached 52°N covering parts of Britain and northern Europe (Hewitt 1996). The main European mountain ranges, the Pyrenees, the Alps and the Caucasus, were also completely glaciated and covered by ice. Between the main ice sheet in the north and the southern mountains, Europe was a plain of permafrost, tundra, and cold steppe (Elenga *et al.* 2000; Prentice *et al.* 2000).

The term 'glacial refugia' was coined to describe the only localities where temperate fauna and flora could have existed during the full-glacial conditions (Hewitt 1996; Taberlet *et al.* 1998; Hewitt 1999). The reconstruction of paleovegetation maps based on pollen-abundance profiles from pond or lake sediment cores has located three main refugia of deciduous temperate forest located in western Europe: (1) southwest Iberia, (2) southern Italy, and (3) Greece, southern Balkans, and northern Turkey (Hewitt 1996; Tzedakis *et al.* 2002) but see Stewart and Lister (2001). The glacial refugia hypothesis predicts that animal and plant species that are today widespread across temperate Europe were restricted to these refugia during the ice age. It is expected that

when the climate warmed those species would have expanded from the refugia and colonized northern Europe. The severe climatic oscillations must have produced great changes in species distributions. Species are expected to have gone through many contractions and expansions of range, characterized by extinctions of the northern populations when the temperature decreased followed by a northward expansion when the climatic conditions became more favorable. These range contractions and expansions are expected to leave signatures in the geographic distribution and genetic diversity of the extant populations (Bennett *et al.* 1991; Slatkin 1993; Avise 2000).

In the current research project, the tawny owl (*Strix aluco* Linnaeus, 1758) was used as a species-model to investigate how the Pleistocene glaciations have shaped the genetic diversity of temperate species in Europe. The tawny owl is a typical medium-sized wood owl of the family Strigidae. This species comprises the most polytypic species-group in the genus *Strix* and includes 11 described subspecies (Vaurie 1965). Current taxonomy of the Tawny owl is composed of a European group (nominate *aluco*) including up to six subspecies and an Asian (or *nivicola*) group with five subspecies (Figure 1). These birds are markedly polymorphic across their geographic range, especially in coloration, and display gray, brown, and rufous morphs that seem to vary in a clinal way, although they may coexist sympatrically (Vaurie 1965).

This study analyses tawny owls from two contiguous subspecies (*Strix aluco sylvatica* and *S. a. aluco*) that cover all western Europe from the Iberia Peninsula to western Russia. Due to specificities of its life history, the tawny owl offered a unique opportunity to analyze the glacial refugia hypothesis. These birds have a widespread distribution in western Europe that covers all three proposed refugia as well as the

northern adjacent areas. They occur from the edge of the boreal zone through temperate regions and steppe to Mediterranean and related mountain zones. These birds are typically found in semi-open deciduous and mixed forest with clearings. They may also occupy open landscapes with wooded patches, parks, and rocky areas with sufficient tree and bush cover (Cramp 1985). Another feature that makes tawny owl a good model system for phylogeographic analyses is its limited dispersal ability. Tawny owls are non-migratory and breeding adults are sedentary and remain in territory all year, while juveniles disperse between August-November but usually only within radius of a few kilometers of the natal site (Cramp 1985; Coles & Petty 1997). These characteristics facilitate the detection of phylogeographic patterns of population expansion by reducing the homogenizing effects of long-distance dispersal.

General aims of this research were (1) to investigate whether the Pleistocene glaciations helped shape the genetic diversity currently found among tawny owl populations; (2) to assess levels of congruence between mitochondrial and nuclear markers in defining patterns of geographic structure, quantifying gene flow, and inferring historical processes, such as evolution in allopatry and re-colonization after expansion from refugia; and finally (3) to propose a new analytical framework to study the influence of glacial refugia on temperate species from western Europe. To address these aims, tawny owls were sampled from the three putative refugia as well as from northern European localities that could not have harbored temperate species during the LGM. A total of 187 individual owls, distributed in 14 populations, were analyzed for 1425 bp of mitochondrial control region sequences and seven microsatellite loci.

Chapter 1 describes mitochondrial phylogeography, and proposes the first hypotheses for refugia populations and post-glacial expansion routes of tawny owls in western Europe. Also investigated is whether the inferred processes are compatible with a late Pleistocene timeframe.

Chapter 2 analyses data from seven microsatellite loci and contrasts mitochondrial and microsatellite results.

Finally, the concluding remarks address the advantages and limitations of mitochondrial sequences and microsatellite loci as genetic markers for phylogeographic studies and population genetic analyses.

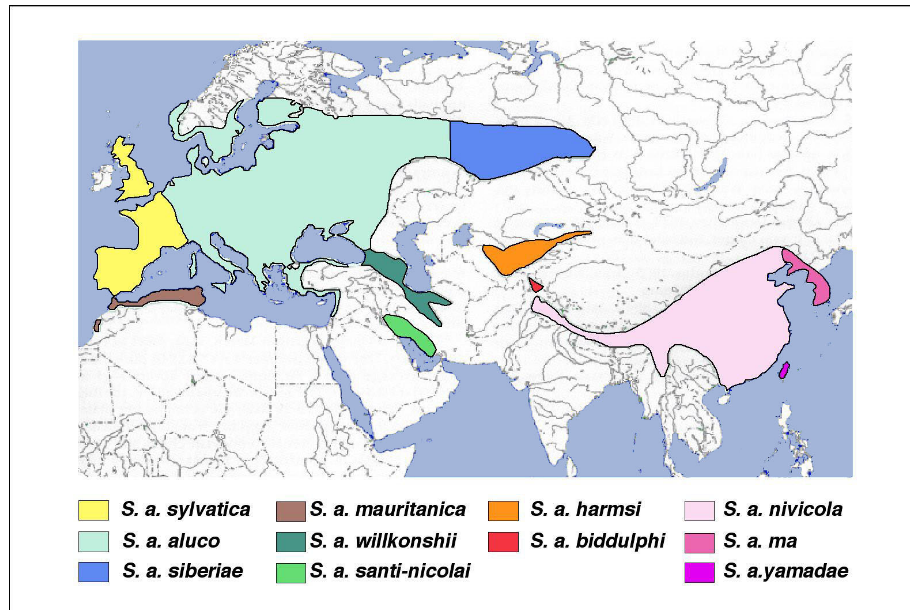


Figure 1 Geographic limits of the tawny owl (*Strix aluco*) subspecies. Map is adapted from König et al (1999).

CHAPTER 1. THE INFLUENCE OF GLACIAL REFUGIA ON TAWNY OWL GENETIC DIVERSITY: PHYLOGEOGRAPHY IN WESTERN EUROPE

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 favorable areas located in the southern peninsulas of Iberia, Italy and Balkans. One such species is the tawny owl, a relativel**Y** sedentary, non-migratory 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 186 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 Balkans 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.

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 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 paleo-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 geographic 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.

Phylogeographic studies in Western Europe have used the glacial refugia hypothesis to interpret results of geographic 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; Newton *et al.* 1999; Hewitt 2001). 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 analyzed 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 and Baker (2002), where all three potential refugia as well as northern European populations were sampled but highly structured clades reflecting geographic 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 kilometers 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 (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 *S. a. aluco*) that cover all Western Europe from the Iberia Peninsula to western Russia.

Three putative refugia as well as northern European localities that could not have harbored 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 geographic origin of northern European populations. Finally, it is investigated whether the inferred processes are compatible with a late Pleistocene time frame.

MATERIAL AND METHODS

SAMPLE COLLECTION

Population sample locations were chosen to cover the entire distribution of the tawny owl in Western Europe (Appendix 1, 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 centers for raptors, and natural history museums. Tissue samples were taken from growing contour feathers or by puncturing the brachial vein from live birds. Muscles 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 and Groth, unpubl. 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 mitochondrial DNA. 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 / 12S. 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., Valencia CA) following manufacturer's instructions for animal tissues. 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. Genomic amplification profiles were similar for both control region fragments: initial denaturation, 95°C for 15 min, 38 cycles of denaturation at 95°C for 30 sec, annealing at 50-54°C for 5 sec, and extension at 72°C for 40 sec; followed by a final extension of 72 for 1 min. Reamplications profiles were: initial denaturation, 94°C for 1 min, 40 cycles of denaturation at 94°C for 20 sec, annealing at 53-56°C for 5 sec, and extension at 72°C for 30 sec; followed by a final extension of 72 for 1 min. Each reaction was checked by electrophoresing 5 µl in an agarose gel with ethidium bromide staining. Successful reactions were purified with Geneclean purification kits (Bio 101, Vista CA), cycle sequenced using d-Rhodamine chemistry,

ethanol precipitated, and electrophoresed on either an ABI 377 or an ABI 3100 automated sequencer (ABI, Foster City CA). Both light and heavy strand 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 of 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, Vista CA) for the three North African skins (collected in 1897, 1907, and 1920) were done in a different lab using new Qiagen kits, primers, and other lab reagents. Hot start *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 PCR reactions.

DATA ANALYSIS

DNA sequences were aligned using Clustal X (Thompson *et al.* 1997) and confirmed by eye in BioEdit (Hall 1999) where they were concatenated to form the final dataset. 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 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 two hours 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 non-parametric 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 geographic structure

was estimated by testing the independence between "geographic location" and "genealogical clade location" with a chi-square contingency test for samples for putative refugial areas. The significance of the χ^2 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 and Bentzen (1989). This test statistic has the advantage of taking into account the genetic correlation due to common genealogical history when testing for geographic 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 geographic 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 5 000 000 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: HKY model (Hasegawa *et al.* 1985) with the transition/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 ($N_{e♀}$), 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 33 bp of ND6, the 73 bp of the complete tRNA-Glu, and 599 bp of control region II (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 numbers: 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 bp to 1418 bp and the final alignment included 17 indels that varied from 1 to 6 bp. For one individual (It25, Appendix 1) 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 1431 bp,

comprising 25 indels and 283 informative sites. The high percentage of haplotypes to individuals sequenced (80%), and the rapid substitution rate observed for both fragments of the control region argues 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 widespread and only two were shared between populations, one between Portugal and Madrid, and the other between Austria and France-SE (Appendix 1). Nucleotide diversity ranged between 0.016 in France-W to 0.005 in Norway (Fig. 1). Although northern populations have lower genetic diversity than mid-latitudes 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 Geographic Distribution of Haplotypes

Datasets 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 two hours 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 (Fig. 2b, Fig. 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 geographic structure ($\chi^2 = 95.90$; df=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).

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% Balkans haplotypes, 55% Italian haplotypes, and 5% Iberian haplotypes. The Iberian haplotypes were not detected further east than the western region of France, while the Balkans 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 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 directional gene flow from southern Italy to western and northern France via northern Italy and SE France; from Greece to the northern European populations of Finland and Denmark; and, from W 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 Nst-based estimate of Nm was greater than 1; these were between Madrid and SE France, and W 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 Fst-based approaches because it not only uses information from the tree (coancestry 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 Balkans 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. Fig. 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, represent two refugial populations assumed to have been separated during the peak of the glaciations; and Finland-Greece, represent 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<<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 favors the hypothesis that the Balkans 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: $T_{pop} = [(t_{pop} * \theta) / 2L]1/\mu$; 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%/MY - 20%/MY) and considering generation time one 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 post-dates the last glacial maximum (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 geographic 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, pp169), 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 geographic 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 - POST-GLACIAL 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 Balkans 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 Balkans clade. Nucleotide diversities for England, Denmark, Norway, and Finland are low as expected in recent populations (Fig. 1). Population expansion out of the Balkans 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 post-glacial 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 mountains ranges (Pyrenees and Alps) as barriers to gene flow or/and to different timings and pacing of post-glacial expansion. Tawny owl post-glacial expansion is most similar to the common pattern of range expansion in Europe –

the grasshopper paradigm - that describes species where the Balkans 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 (*S. a. wilkonskii*) and preliminary cytochrome *b* data suggest that 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: 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 Balkans 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 Balkans 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 post-glacial expansion have to be explained by a contribution of orography, paleo-ecological 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 last glacial maximum (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 MY).

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 use 2% per MY (cytochrome *b*) to 20% for the domain I of the CR (Marshall & Baker 1997). With the exception of the 2% rate, divergence times obtained for different populations 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 (Brito unpubl. data) 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 MY 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 MY. 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 MY, while Baker and Marshall (1997) estimated a mutation rate of 20.8% per MY for domain I of the lesser snow goose control region.

The results presented (Table 4) assume that generation time for tawny owls is one 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 one 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 MY 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 and 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 coancestry (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 post-glacial 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 co-distributed species with similar habitat requirements (Hewitt 2000; 2004) suggests shared historical biogeographic factors in shaping these phylogeographies (Avise 2000, pp 215-223).

TABLES

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

Region	Direction	Primer	Sequence (5' - 3')	Source
Control Region 1	Forward	N1	AACATTGGTCTTGTAAGCCAA	Barrowclough et al 1999
		DL165	GCCGCTTGGGATGTATAATTG	*present study*
		DL383	CACCCTAATTCATGATCAACCG	*present study*
		DL532	CCAAATCACAATCCATCCATGCC	*present study*
	Reverse	D16	AGTGCATCAGTGTCTAGGTGATTC	Barrowclough et al 1999
		D12	TAGGCGGGACTATTACTTGAAT	Barrowclough et al 1999
		D20[1]	GTGATGGATCTTACTAACACC	Barrowclough et al 1999
		DH365	GGGTGTTTTTGGTACATGCAGAG	*present study*
		DH469	GGGGCATGGATTATATATCCG	*present study*
		DH622	GGCTAACTTAAGGTGGGACCATTACT	*present study*
		DH724	CAGCTGCGCCAGATGTC	*present study*
Control Region 2	Forward	ND6Z	ACAACCCCATAATACCGCGAAGG	*present study*
		DL2	GAAACCCCTACCAGGGCA	*present study*
		DL4	ACATACCATTCATGCCCA	*present study*
		DL4a	TACCTTCCACCGATCACAAGG	*present study*
	Reverse	DH1a	TAATGCACACCAGTACATCCTC	*present study*
		DH3a	CATGGATTATATATCCGGTTGAC	*present study*
		DH3	GCTAACTTAAGGTGGGACCA	*present study*

1 - Also used to amplify CR2

TABLE 2. Hierarchical AMOVA computed with the six refugial populations.

Source of variation	df	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, $p \ll 0.001$				

TABLE 3. Pairwise N_{ST} estimates (above diagonal) and corresponding migration rates N_em (below diagonal).

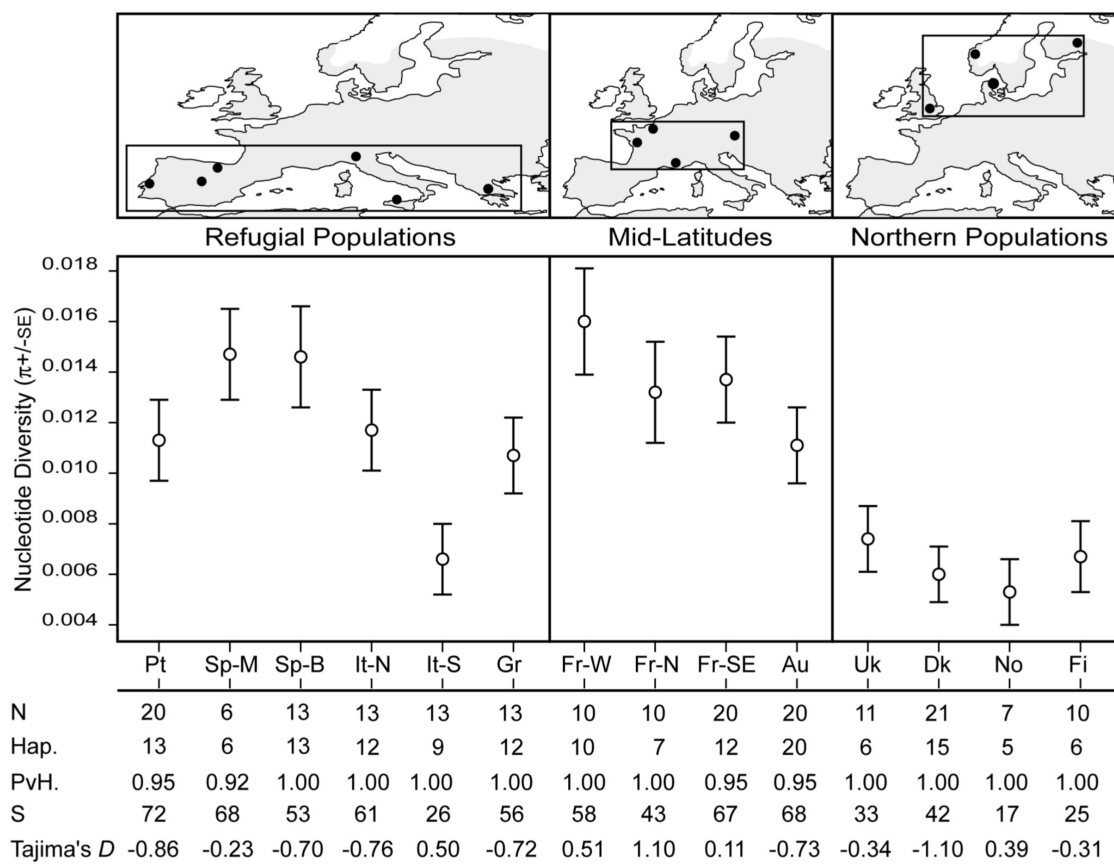
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Portugal		0.21	0.08	0.42	0.59	0.45	0.30	0.38	0.40	0.46	0.52	0.57	0.60	0.54
2. Sp-Madrid	1.88		0.14	0.26	0.47	0.14	0.08	0.10	0.22	0.15	0.26	0.26	0.31	0.24
3. Sp-Bilbao	6.11	3.18		0.33	0.49	0.35	0.21	0.29	0.29	0.35	0.38	0.45	0.50	0.45
4. Italy-N	0.69	1.45	1.04		0.24	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	

TABLE 4. MDIV Estimates of divergence time (in generations) among refugial populations calibrated for different mutation rates.

Pop.1	Pop.2	T_{MRCA}	t_{pop}	θ	Population Divergence time (Tpop)				
					2% MY	5% MY	10% MY	15% MY	20% MY
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

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

FIGURES



	Pt	Sp-M	Sp-B	It-N	It-S	Gr	Fr-W	Fr-N	Fr-SE	Au	Uk	Dk	No	Fi
N	20	6	13	13	13	13	10	10	20	20	11	21	7	10
Hap.	13	6	13	12	9	12	10	7	12	20	6	15	5	6
PvH.	0.95	0.92	1.00	1.00	1.00	1.00	1.00	1.00	0.95	0.95	1.00	1.00	1.00	1.00
S	72	68	53	61	26	56	58	43	67	68	33	42	17	25
Tajima's *D*	-0.86	-0.23	-0.70	-0.76	0.50	-0.72	0.51	1.10	0.11	-0.73	-0.34	-1.10	0.39	-0.31

Figure 1. Genetic diversity within populations. The maps on the top indicate current geographic 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.

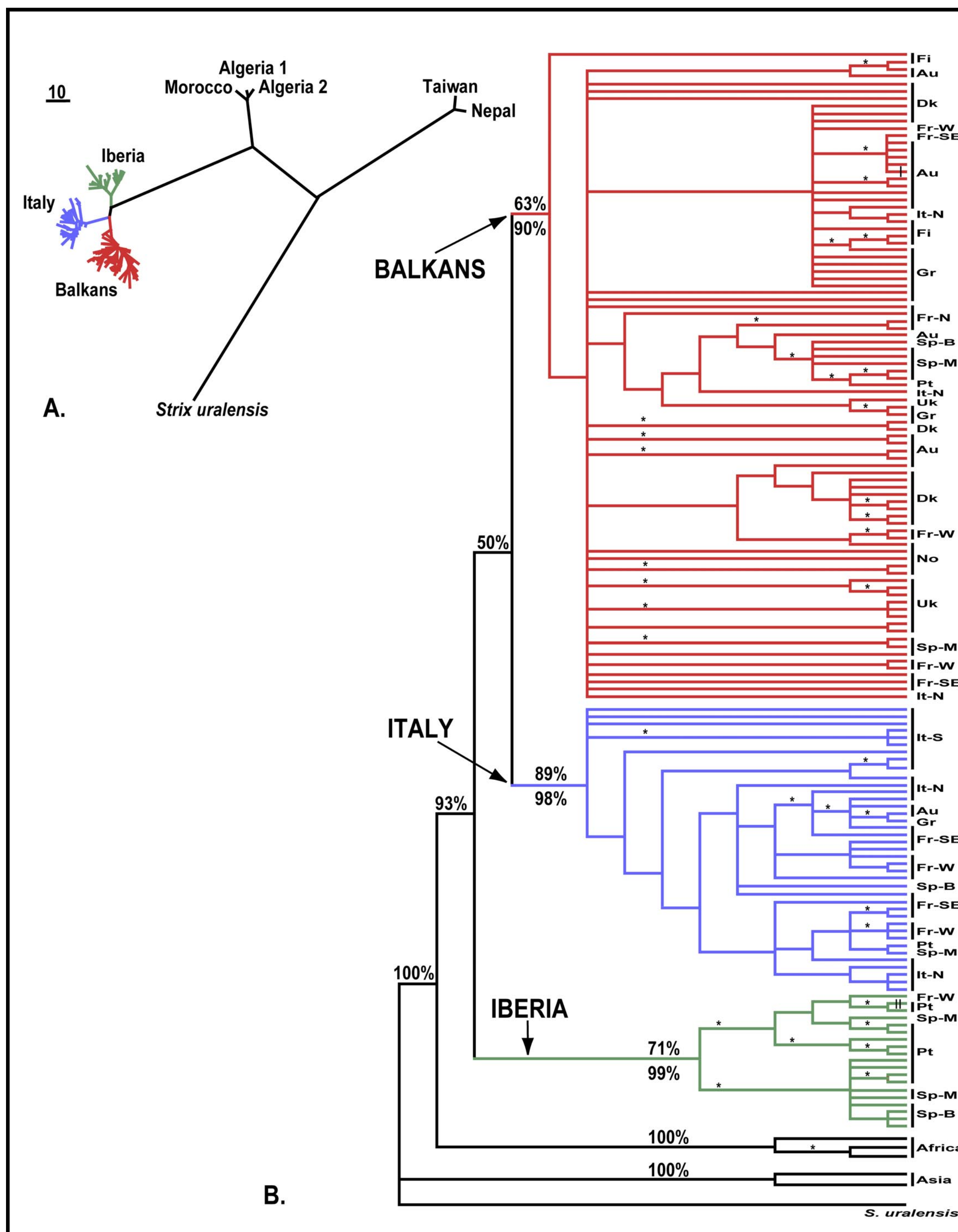


Figure 2. Phylogenetic relationships among tawny owl haplotypes (a) Phylogram of one of the most parsimonious trees indicating a monophyletic European tawny owl; branch lengths are drawn to scale. (b) Strict consensus of all most parsimonious trees. Numbers on the branches are bootstrap support for the 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

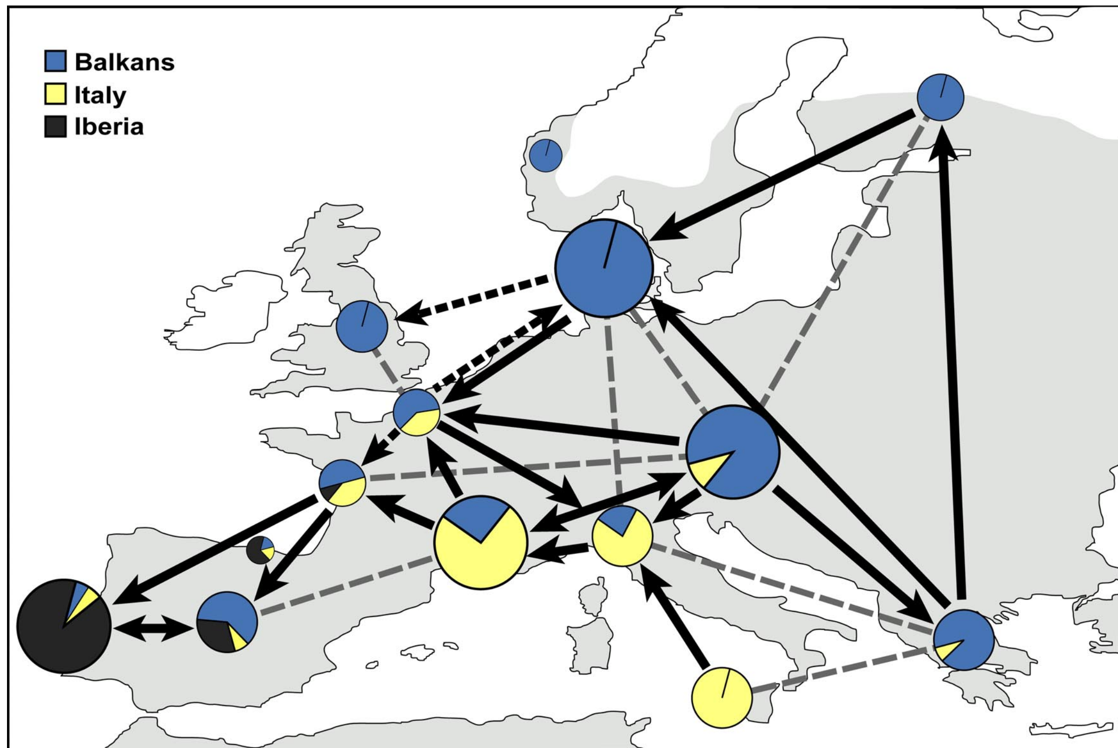


Figure 3. Haplotype distribution and gene flow among tawny owl populations. Pie charts represent the proportion of individuals in each of the three major clades (Fig. 2); size of pie charts is proportional to sample size. Arrows and dotted lines represent results of Migrate. Arrows indicate directional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

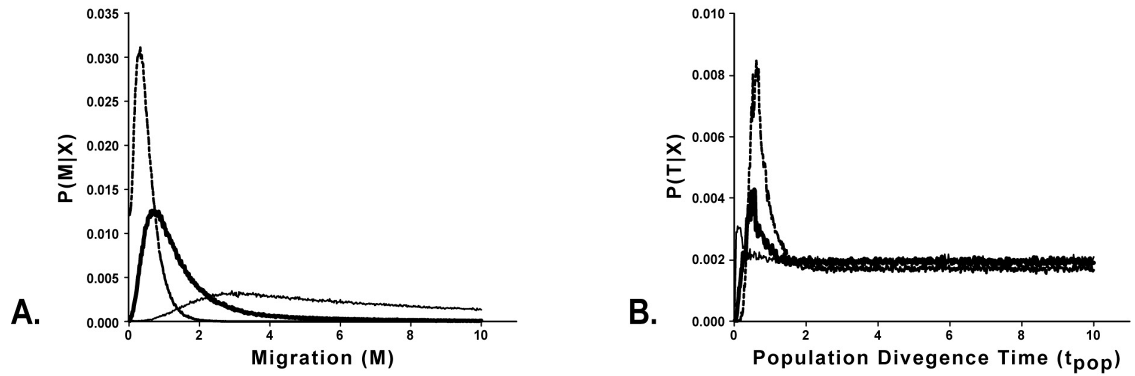


Figure 4. Relative effects of migration and population divergence time among tawny owl populations. MDIV results for the posterior distribution of M (migration) and t_{pop} (population divergence time) for the following population pairs: Portugal_Greece (dashed line), Madrid-Greece (bold line), and Greece-Finland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

APPENDIX 1. TAWNY OWL SAMPLES USED IN THIS STUDY

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 (FrN), France-west (FrW), France-southeast (FrSE), Greece (Gr), Italy-north (ItN), Italy-Sicily (ItS), Norway (No), Portugal (Pt), Spain-Bilbao (SpB), Spain-Madrid (SpM).

Sample code	Locality/Region	Clade[1]	Identical haplotypes	Hap. Code[2]	
				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	Løgstør, 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
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_N06	Roncq, Lille, Nord Pas de Calais, France	It	Fr_N11	6915	7069
Fr_N07	St. Omer, Lille, Nord Pas de Calais, France	Bk	Fr_N12, 13	6916	7070
Fr_N08	Masnières, Lille, Nord Pas de Calais, France	Bk	-	6917	7071
Fr_N09	Lille, Nord Pas de Calais, France	It	-	6918	7072
Fr_N10	Houdaim, Lille, Nord Pas de Calais, France	Bk	-	6919	7073
Fr_N11	Lille, Nord Pas de Calais, France	It	Fr_N06	6915	7069
Fr_N12	Lille, Nord Pas de Calais, France	Bk	Fr_N07, 13	6916	7070
Fr_N13	Lille, Nord Pas de Calais, France	Bk	Fr_N07, 12	6916	7070
Fr_N14	Lille, Nord Pas de Calais, France	Bk	-	6920	7074
Fr_N15	Lille, Nord Pas de Calais, France	It	-	6921	7075

Fr_W22	Poitou Charente, France	Bk	-	6922	7076
Fr_W23	Poitou Charente, France	It	-	6923	7077
Fr_W24	Poitou Charente, France	It	-	6924	7078
Fr_W25	Poitou Charente, France	Bk	-	6925	7079
Fr_W26	Poitou Charente, France	Bk	-	6926	7080
Fr_W27	Poitou Charente, France	Bk	-	6927	7081
Fr_W28	Poitou Charente, France	It	-	6928	7082
Fr_W29	Poitou Charente, France	Ib	-	6929	7083
Fr_W30	Poitou Charente, France	Bk	-	6930	7084
Fr_W31	Poitou Charente, France	It	-	6931	7085
$Fr_{SE}71$	Orange,Vaucluse, PACA, France, 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
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_N01	Parma, Italy	It	-	6956	7110
It_N02	Cremona, Italy	It	-	6957	7111
It_N03	Sarzana, Italy	It	-	6958	7112
It_N04	Cremona, Italy	It	It_N07	6959	7113
It_N05	Parma, Italy	Bk	-	6960	7114
It_N06	Piacenza, Italy	It	-	6961	7115
It_N07	Tizzano, Italy	It	It_N04	6959	7113
It_N08	Cremona, Italy	It	-	6962	7116
It_N09	Cremona, Italy	It	-	6963	7117
It_N10	Piemonte, Italy	Bk	-	6964	7118
It_N11	Venaria Reale, Turin, Italy	Bk	-	6965	7119

It_N12	Cuneo, Italy	It	-	6966	7120
It_N13	Carignano, Turin, Italy	It	-	6967	7121
It_S15	Messina, Sicily, Italy	It	-	6968	7122
It_S16	Gratteri, Palermo, Sicily, Italy	It	It_S26	6969	7123
It_S17	Ficuzza Wood, Palermo, Sicily, Italy	It	-	6970	7124
It_S18	Monreale, Palermo, Sicily, Italy	It	It_S19, 22	6971	7125
It_S19	Giacalone, Palermo, Sicily, Italy	It	It_S18, 22	6971	7125
It_S20	Ficuzza, Palermo, Sicily, Italy	It	-	6972	7126
It_S21	Pollina, Palermo, Sicily, Italy	It	-	6973	7127
It_S22	Gibilmanna, Palermo, Sicily, Italy	It	It_S18, 19	6071	7125
It_S23	Palermo, Sicily, Italy	It	It_S25	6974	7128
It_S24	Dintorni di Palermo, Sicily, Italy	It	-	6975	7129
It_S25	Giacalone, Palermo, Sicily, Italy	It	It_S23	6974	7128
It_S26	Parco Della Favorita, Palermo, Sicily, Italy	It	It_S16	6969	7123
It_S27	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	Pt09, 15	6987	7141
Pt12	Chaves, Vila Real, Portugal	Ib	-	6989	7143
Pt13	Portugal	Ib	Pt18	6990	7144
Pt15	Portugal	Ib	Pt09, 11	6987	7141
Pt16	Penamacor, Castelo Branco, Portugal	Ib	Sp_M12	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_B01	Vizcaya, Spain	Bk	-	6995	7149
Sp_B02	Vizcaya, Spain	Ib	-	6996	7150
Sp_B03	Vizcaya, Spain	It	-	6997	7151
Sp_B04	Asturias, Spain	Ib	-	6998	7152
Sp_B05	Vizcaya, Spain	Ib	-	6999	7153
Sp_B06	Vizcaya, Spain	Ib	-	7000	7154
Sp_M09	Villamanta, Madrid, Spain	Bk	-	7001	7155
Sp_M10	Soria, Spain	It	-	7002	7156
Sp_M11	Rozas de Puerto Real, Madrid, Spain	Bk	-	7003	7157
Sp_M12	Moce Jón, Toledo, Spain	Ib	Pt16	6991	7145
Sp_M13	Polan, Toledo, Spain	Bk	-	7004	7158
Sp_M14	Mostoles, Madrid, Spain	Bk	-	7005	7159

Sp_M15	Sevilla la Nueva, Madrid, Spain	Bk	-	7006	7160
Sp_M16	Madrid, Spain	Ib	-	7007	7161
Sp_M17	Madrid, Spain	Bk	-	7008	7162
Sp_M18	Madrid, Spain	Ib	-	7009	7163
Sp_M19	San Sebastián de los Reyes, Madrid, Spain	Ib	-	7010	7164
Sp_M20	Tres Cantos, Madrid, Spain	Bk	-	7011	7165
Sp_M21	Navalcarnero, Madrid, Spain	Bk	-	7012	7166
NAfr1	Tangier, Morocco		*Strix aluco mauritanica*	7013	-
NAfr2	Batra, Algeria		*Strix aluco mauritanica*	7014	-
NAfr3	Tizi Ouzou, Algeria		*Strix aluco mauritanica*	7015	-
Asia1	Taiwan		*Strix aluco yamadae*	7016	7167
Asia2	Gonga, Nepal		*Strix aluco nivicola*	7017	7168
Ural	Keuruu, Finland		*Strix uralensis*	7018	7169

[1] Clade reflects phylogenetic results from Fig. 2; [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

CHAPTER 2. CONTRASTING PATTERNS OF GEOGRAPHIC STRUCTURE AND GENE FLOW AMONG WESTERN EUROPEAN POPULATIONS OF TAWNY OWLS USING MITOCHONDRIAL AND MICROSATELLITES MARKERS

ABSTRACT

A previous study of mitochondrial phylogeography of tawny owls in western Europe indicated that these birds survived the peak of the Pleistocene glaciations in three allopatric refugia located in southern Europe in Iberia, Italy, and the Balkans. It also suggested that post-glacial expansion from the Balkans refugium was the predominant source of colonization of northern Europe. In the present study, analyses of seven microsatellite loci were performed to assess levels of congruence between these markers and the earlier mtDNA study. The major results of the mitochondrial study were verified with microsatellites, although additional assumptions were required; microsatellites recovered the existence of three major refugia and the Balkans origin of northern populations. When corrected for differences in effective population size, microsatellites and mtDNA yield generally congruent overall estimates of population structure (N_{ST}*=0.11 vs R_{ST}=0.16); however, in detail the agreement was not good (r^2=0.19), and there was substantial heterogeneity among the various microsatellite estimates. When corrected for effective population size differences, the mitochondrial DNA marker showed less structure than did the microsatellites; this is consistent with greater female gene flow (hence dispersal) than male gene flow, which is typical of many birds. Populations representing the Balkans postglacial expansion interact with populations from the other two refugia in hybrid zones near the Alps and Pyrenees; the apparent position of those zones differ by several hundred km between mtDNA and microsatellite markers. As expected, the mitochondrial study found lower nucleotide diversity in northern, recently populated regions than in refugia and mid-latitude populations; the

microsatellite results indicated heterozygosity in most northern populations was of the same magnitude as that of refugial populations. This might be anticipated for markers with relatively high mutation rates. The mtDNA and microsatellite results were largely congruent; when they differed, many – but not all – of the instances were consistent with differences in natural history, behavior, and molecular biology.

INTRODUCTION

The study of the genetic structure of natural populations describes and quantifies how total genetic variation is distributed geographically across populations. This genetic structure is dependent on several demographic and stochastic processes such as mutation, genetic drift, and gene flow (Slatkin 1985). In birds, gene flow is usually the result of juveniles dispersing from their natal areas and, less frequently, adults changing their breeding locations (Johnson & Gaines 1990). Estimates of gene flow based on allozyme frequencies have detected little geographic variation in temperate birds, particularly when compared with other vertebrates (Barrowclough 1983). The use of fast-evolving markers, such as the mitochondrial control region, has indicated much higher levels of genetic differentiation (Zink 1997), and mitochondrial phylogeographic studies typically identify several clades with significant geographic information (e.g. Wenink *et al.* 1996; Barrowclough *et al.* 2004, 2005). Crochet (2000) has suggested that this difference between allozymes and mitochondrial results is due to a difference in effective population size between those markers, and proposed that the same levels of gene flow could produce the distributions of the *F*-statistics that are usually observed for those markers. Exceptions to the high geographic structure estimated with mtDNA are not infrequent, but these examples are usually interpreted as due to recent range expansions and non-equilibrium populations (e.g. Zink *et al.* 2002; Zink *et al.* 2003).

Microsatellites are popular markers for the study of geographic structure and gene flow because of their high levels of polymorphism and biparental inheritance (Jarne & Lagoda 1996). Microsatellites are codominant and inherited in a Mendelian fashion

and, like allozymes, provide an easy way to screen multiple nuclear loci for geographic variation in natural populations. Avian studies where microsatellites and mitochondrial results are compared frequently recover a pattern of reduced nuclear genetic structure (e.g. Helbig *et al.* 2001; Crochet *et al.* 2003; Eggert *et al.* 2004), although exceptions have also been reported (e.g. Burg & Croxall 2001; Johnson *et al.* 2003).

The importance of assessing the congruence among independent genetic markers has been widely discussed in the literature (e.g. Avise 2000). Combined analysis of multiple and independent loci are usually advocated because they reduce the variance in the estimated parameters that are due to random effects of sampling and lineage sorting (e.g. Takahata 1989; Hudson 1990). Also, contrasting results from marker classes with distinct modes of inheritance such as microsatellites and mitochondria may also provide a better understanding of the underlying evolutionary processes by revealing events of sex-biased dispersal (Prugnolle & Meeus 2002).

In this study microsatellites are contrasted with previous results from a phylogeographic study based on mitochondrial control region sequences (Brito 2005). The tawny owl (*Strix aluco*) is a tree-nesting bird associated with temperate forest that has a widespread distribution throughout western Europe. Due to its inability to survive outside temperate refugia, populations of this owl must have experienced extensive contractions and range expansions associated with the dramatic climatic fluctuations of the late Pleistocene. Brito (2005) showed that this species is composed of three major evolutionary lineages in western Europe that evolved in allopatry in the three glacial refugia located in Iberia, southern Italy, and the Balkans. The latter was assumed to be the sole source of post-glacial expansion since all individuals sampled in the four most

northern populations had haplotypes that coalesced within the Balkans clade. Considerable secondary intergradation was also detected in mid-latitude populations located in Austria, France, and Spain.

This study follows a novel comparative format in contrasting mitochondrial and microsatellite loci in defining patterns of geographic structure, quantifying gene flow, and inferring historical processes for tawny owls in western Europe. General conclusions about the applicability and limits of these markers are discussed.

MATERIAL AND METHODS

SAMPLE COLLECTION

A total of 184 Tawny owl samples was collected from 14 populations in western Europe. These populations cover the three southern peninsulas of Iberia (Portugal, Spain-Bilbao, and Spain-Madrid), Italy (northern Italy and Sicily), and the Balkans (Greece) as well as four populations in northern Europe (England, Denmark, Norway, and Finland), three in France (France-north in Lille, France-West in Poitou Charente, and France – Southeast in Vaucluse), and one in Austria (Fig. 1). Details on the specific geographic location of each sample are in Brito (2005), with the exception of three samples (Fr_W30, It_S21, and It_S26) all other individuals were analyzed in this study. Fresh tissues (blood, growing contour feathers, or muscle) were collected whenever possible, but for the population in southern Italy (Sicily) six samples were also obtained from toe pads of museum study skins that dated from 1982 to 1997.

MICROSATELLITE GENOTYPING

Seven microsatellite loci originally developed for *Strix occidentalis* (15A6, 1C6, 8G11, 4E10, and 4E10.2; Thode *et al* (2002)) and *Bubo bubo* (Bb111, Bb131, Isaksson and Telgelström (2002)) were genotyped. Microsatellite fragments were amplified using a touch-down PCR protocol designed to reduce nonspecific priming and subsequent arbitrary fragment amplification. Annealing temperatures in the first touch-down cycle were eight degrees above the final annealing temperature, which were 50°C (15A6, 1C6, 4E10, and Bb111), 48°C (Bb131), and 39°C (4E10). Hot start *Taq* was used in all genomic amplifications. PCR reactions were confirmed by electrophoresis of 5 µl of PCR product in an agarose gel with ethidium bromide. Samples were diluted depending on the brightness of the PCR bands. Diluted PCR products were combined with an internal labeled size standard (500 ROX, ABI, Foster City, CA), and loaded for GeneScan in an ABI 3700 automated sequencer (ABI, Foster City, CA). Multiplex GeneScans, when used, were done with microsatellites labeled with different fluorescent dyes. Microsatellites fragments were scored using GENOTYPER version 2.0 (PE Biosystems). All samples were PCR amplified and scored at least twice, and the six samples extracted from toe pads were scored three times to reduce the chances of having null alleles. Due to the recent age of those study skins it was found that doing the multiple tubes approach of Navidi et al (1992) and Taberlet *et al* (1996) was not necessary.

DNA SEQUENCING

Because these microsatellite loci were developed for other species and had not been previously tested in tawny owls, each locus was sequenced for selected homozygous individuals to confirm that the amplified repeats were similar to the ones described in the original literature. Repeats for each locus were confirmed after sequencing between two and eight individuals representing a minimum of two distinct populations. PCR's were carried out as previously indicated and sequencing was performed using standard procedures described in Brito (2005).

DATA ANALYSIS

MICRO-CHECKER software (Oosterhout *et al.* 2004) was used to assess the presence of genotyping errors, such as non-amplified alleles, short allele dominance, and scoring of stutter peaks. Microsatellite genetic diversity was quantified as absolute and mean number of alleles per locus (A and a respectively), observed heterozygosity (Ho), unbiased gene diversity (He), frequency of most common allele (Xc), and number of private alleles; these statistics were estimated using MS-Toolkit (Park 2001). Linkage disequilibrium between loci, and deviations from Hardy-Weinberg genotype frequency equilibrium (HWE) were tested with GENEPOP (Raymond & Rousset 1995). For all these analyses, significance was estimated by Fisher exact tests where p-values were estimated by applying a Markov chain method with the following parameters (10 000 dememorization; 200 batches; 5 000 interactions per batch) as described in Raymond and

Rousset (1995). Population genetic structure was estimated with both F_{ST} (Weir & Cockerham 1984) and R_{ST} (Slatkin 1995) using the programs FSTAT (Goudet 1995) and RST-CALC (Goodman 1997) respectively. The later is particularly useful for microsatellite data because it assumes a stepwise mutation model; also RST-CALC standardizes the variance of allele size prior to calculation, making loci with different variances comparable. Sequential Bonferroni corrections were applied to correct for type I error when appropriate (Rice 1989).

STRUCTURE version 2 (Pritchard *et al.* 2000) applies a Bayesian method to infer the number, K, of clusters without using prior information of the individual sampling locations. This program distributes individuals among K clusters based on their allelic frequencies, and estimates the posterior probability of the data given each particular K. To estimate the K with highest probability, STRUCTURE was run for K=1 to K=14 clusters. Each run was pursued for 1 000 000 MCMC interactions, with an initial burn-in of 100 000, and an ancestry model that allows for admixture, with same Alpha (Dirichelet parameter for degree of admixture) for all populations. Five independent simulations were run for each K to check for stability. The final posterior probability of K was computed as suggested by Pritchard (2000), with the runs with highest probability for each K used. An additional ad hoc statistic (ΔK) was also estimated because it was shown to provide a better predictor of the number of groups (i.e., K) at the uppermost hierarchical level (Evanno *et al.* 2005). The sensitivity of the final result to specific prior assumptions of Alpha and independence of allelic frequencies was also computed.

The ability of microsatellite allele frequencies to detect structure among the population samples was further analyzed by performing a principal component analysis

on the allelic frequencies. The data for each population were arcsine square-root-transformed (angular transformation) before analysis and used as input data for the principal component analysis on the covariance matrix. These analyses were performed with SAS version 8.02 using procedure PRINCOMP, and the angular transformation was chosen due to its applicability to percentage and proportion data (Sokal & Rohlf 1995, pp: 419).

Gene flow ($N_e m$) was estimated from pairwise R_{ST} (Slatkin 1995) using RST-CALC (Goodman 1997). Pairwise estimates of gene flow and geographic distances were then used to infer patterns of isolation-by-distance as well as to identify possible events of recent population expansion (Slatkin & Maddison 1990; Slatkin 1993). In this study, pairwise $N_e m$ was plotted against geographic distance in kilometers, and the correlation coefficient was estimated. Because sample points are not independent, significance of the correlation coefficient was estimated with Mantel tests (Mantel 1967), 1 000 replicates, as performed in GENEPOP (Raymond & Rousset 1995). Equality of regression slopes was estimated in SAS, version 8.02, using procedure SYSLIN. $N_e m$ was estimated from both pairwise R_{ST} (microsatellites) and N_{ST} (mtDNA), and the program INVERSE, available from the National Geodetic Survey, US Department of Commerce (www.ngs.noaa.gov) was used to estimate geographic distances between pairs of locality coordinates. Isolation-by-distance plots were made for specific population groups that reflect the Balkans expansion (Greece, Austria, France-N, England, Denmark, and Finland), the Iberia-Balkans hybrid zone (Portugal, Spain-Madrid, France-W, France SE, Austria, and Greece), and the Italy-Balkans hybrid zone (Italy-Sicily, Italy-N, Austria, and Greece). Populations sampled in Norway and Bilbao (Spain) were excluded due to

their small sample sizes (6 and 7 respectively). To reflect geography in western Europe, geographic distances with England and Sicily were estimated using a stepping-stone model that passes through France-N and Italy-N respectively (see Fig. 1).

RESULTS

MICROSATELLITE GENE DIVERSITY

Fourteen microsatellite loci originally developed for *Strix occidentalis* (Thode *et al.* 2002) and *Bubo bubo* (Isaksson & Telgelström 2002) were initially survey for this study. Seven of those 14 loci were not included in the analyses due to difficulties in the PCR amplifications (6H8, Bb100, and Bb145), lack of polymorphism (Bb42, Bb101, and Bb126), or because homology between alleles and repeat numbers could not be established (13D8). Sequencing of loci 15A6, 8G11, 4E10.2, 1C6, 4E10, and Bb111 confirmed that their repeats were similar to that described in the literature. The microsatellite Bb131, originally described as a complex of three dinucleotide repeats, was assumed to behave as a single dinucleotide repeat in tawny owls, after sequencing 18 individuals representing five distinct populations; these 18 individuals differed among each other by mutations of the same dinucleotide repeat (data not shown).

All seven microsatellite loci analyzed were polymorphic although levels of genetic diversity were variable (Table 1). Loci 4E10.2 and 4E10 showed high levels of polymorphism with 5 to 20 alleles per population and expected heterozygosities that ranged from 0.67 to 0.97. Loci 15A6, 8G11, and 1C6 showed medium levels of

polymorphism with 3 to 8 alleles per population and expected heterozygosities that ranged from 0.54 and 0.85. Finally, loci Bb111 and Bb131 had low levels of polymorphism with only 1 to 5 alleles per population and expected heterozygosities ranging from 0.00 to 0.67. Overall, the mean number of alleles per locus averaged 5.92, ranging from 4.29 in Italy-Sicily to 8.43 in Austria, and expected heterozygosity averaged across loci ranged from 0.57 (Italy-Sicily) to 0.70 (Spain-Madrid). Two pairs of individuals from Denmark had identical alleles for all seven loci, but only one of those pairs shared identical mtDNA haplotypes. No population was fixed for any allele, and private alleles did not account for more than 15% of the allelic frequency in a population; in general, rare alleles were rare everywhere. Allele frequencies for all seven loci are shown in the Appendix.

Microsatellites did not show a pattern of decreasing genetic diversity (He) with latitude (Fig. 2); the four most northern populations (England, Denmark, Norway, and Finland) had genetic diversities that were not distinguishable from the remaining populations. Microsatellites and mtDNA mostly agreed in their assessment of the genetic diversity of refugial and mid-latitude populations (r^2=0.66, p=0.004). However, the correlation between those two parameters decreased substantially when populations from northern Europe were included in the analysis (r^2=0.23, p=0.085). The unbiased gene diversity (He) was used here because of its relative insensitivity to sample sizes. Exact tests of genotypic disequilibrium between loci (either global or for each population sample) were not significant suggesting that loci were independent. Departures from Hardy-Weinberg equilibrium were only found in two cases: France_SE for locus 8G11,

and Denmark for locus 4E10. Global tests across populations for each locus revealed significant departure from equilibrium frequencies at two loci 8G11 and 4E10.

POPULATION GENETIC STRUCTURE

F-Statistics

Levels of population genetic structure varied considerable depending on the *F*-statistic and on the molecular marker (Fig. 3). R_{ST} averaging over all seven loci was 0.16, indicating substantial genetic structure (Wright 1978; Hartl & Clark 1997), whereas R_{ST} for each locus ranged from 0.04 (4E10.2) to 0.29 (4E10) (Table 2). Averaged F_{ST} was considerable smaller (0.06) and showed less variability across loci, although no simple correspondence with R_{ST} (Table 2). N_{ST}, estimated after sequencing 1425 bp of the mitochondrial control region was 0.35 (Brito 2005). However, due to their different effective population sizes, *F*-statistics derived from mitochondrial and nuclear data have different expectations under equilibrium, and hence need to be calibrated before comparison. Assuming an infinite island model, at mutation-drift equilibrium, with no sex-biased dispersal and sex-ratio of one, the F_{ST} equalities derived for nuclear (Wright 1951) and mitochondrial data (Birky *et al.* 1983) can be compared using the expression (see also Crochet 2000): $F_{ST}(nuc) = F_{ST}(mt) / [4\text{-}3F_{ST}(mt)]$. The expected N_{ST}, (hereafter N_{ST}*) if nuclear markers had been used is then 0.11 (Fig. 3).

Bayesian Analysis

A Bayesian clustering method available in STRUCTURE was used to infer substructure in the data set. The program was run several independent times and the natural logarithm of the posterior distribution of the data given K (number of hypothetical subgroups or clusters) was plotted in Fig 4A. The number of subgroups that gave the highest probability to the data was three. As K gets higher the variance of the estimates increased substantially, which indicates that for K>5 there is probably not a single preferred clustering solution. The posterior probability of K was essentially one for K=3, and zero for all remaining K. Evanno et al (2005) suggested that this log probability of the data does not provide the best estimation of the number of clusters, and therefore they proposed a new heuristic statistic (ΔK) to infer the number of different clusters. ΔK was computed for all K and also indicated a strong signal for K=3 (Fig. 4B). Changing the assumptions of ‘equal alpha for each population’ and ‘correlated allele frequencies’ did not change this final result. Although there was a strong signal for K=3, the proportion of membership of each pre-defined population in each of the three clusters did not have a simple geographic interpretation. Whereas Iberian populations had on average 80% of their individuals in a single cluster, all other populations were of mixed origin between the two remaining clusters that could not be associated with specific populations nor geographic regions.

Multivariate Analysis

A principal component analysis (PCA) was performed on the population allelic frequencies to determine whether the information from all loci could be used

simultaneously to segregate the populations in units of similar allelic frequencies. This analysis revealed that the 14 tawny owl populations sampled in western Europe are structured in three groups that correspond to the three Pleistocene refugia (Fig. 5). Iberia, southern Italy (Sicily), and Greece are well differentiated in the first and second principal components, which together explained 39% of the total genetic variance. All mid-latitude and northern European populations formed a single cluster with Greece and none of those had PC scores similar to either Iberian or Sicilian populations. Interestingly, the northern Italy population was similar to Greece rather than to southern Italy. In addition, the first two eigenvectors' coefficients indicate that the alleles with most influence in the discrimination of the three clusters are not lineage specific alleles but rather widespread alleles that have distinct frequencies in the three clusters (e.g. alleles 151 and 155/locus Bb131; allele 129/locus 8G11; and allele 205/locus 4E10; Fig. 1). These alleles were not concentrated in a single locus but were distributed across loci; with the exception of locus 4E10.2, all loci had at least one allele with major influence in the first principal component.

HYBRID ZONES

Both mtDNA and microsatellite loci identified signatures of three refugia for tawny owls in southern Europe. However, they differed in their identification of the genetic composition of the populations from Madrid and northern Italy. In particular, the two data sets differed in their placement of the hybrid zones between Balkans genotypes and those of Iberia and Italy (Fig. 6). In a transect from Portugal to Greece, mtDNA

showed a transition between the populations of Portugal and Madrid, whereas microsatellites placed the contact zone farther east, close to the Pyrenees. In the transect between southern Italy and Greece the hybrid zone moved from the southern Italian peninsula (microsatellites) to the Alps (mtDNA). These differences correspond to offsets of approximately 645 and 390 kilometers respectively. The offsets were estimated by finding the difference, in kilometers, between the two geographic points that correspond to the midpoints in the Y-axes. Those are the PC1 scores for microsatellites and the percentage of Balkans haplotypes for mtDNA.

Population Differentiation: Effects of Drift and Gene Flow

Pairwise R_{ST}

Pairwise estimates of geographic structure (R_{ST}) and gene flow (N_em) corroborated the PCA results in that they also segregated Iberia and Sicily from all remaining European populations (Table 3). R_{ST} indicated significant levels of gene flow within clusters and almost negligible gene flow between them, with the exception of southern Italy that had $N_em>1$ with the three Iberian populations, but see below. Greece had, in general, low R_{ST} with all northern European (England, Denmark, Norway, and Finland) and mid-latitude populations (three populations from France and Austria), as well as with northern Italy. R_{ST} between these populations varied from negative values (Greece - Austria) suggesting essentially panmictic populations, and 0.02 (Greece - Italy-N), 0.04 (Greece - Norway) to a maximum of 0.13 (Greece – Denmark). Northern Italy and Greece had very similar gene flow patterns; they were connected with substantial

levels of gene flow (N_em>1) with the same group of populations. The population from Bilbao (Spain) was not significantly different from the majority of the other populations, but this result was likely due to its small sample size. The Iberian populations sampled in Portugal and Madrid were relatively divergent from populations east of the Pyrenees, with the exception of the population sampled in Sicily. However, this result is likely due to convergence because Sicily was divergent from both northern Italy (0.25) and SE France (0.26) but had lower R_{ST} with Norway (0.21), and France-W (0.14), as well as with all three Iberian populations (0.12, 0.09, 0.17).

R_{ST} was significantly but weakly correlated with pairwise N_{ST}* (r^2=0.19, $p<0.01$; Fig. 7) where slightly higher levels of population structure were observed with microsatellites, i.e. more points were observed above the dashed line in Fig. 7 than below it. Levels of gene flow measured with pairwise R_{ST} were also significantly correlated between markers (r^2=0.17, $p<0.01$; not shown) and for the majority of the population-pairs corresponded to moderate levels of gene flow (Table 3); N_em was largely between one and four although there was a tendency for higher levels of mitochondrial gene flow. In general, there was no contradiction between marker-classes in identifying substantial levels of gene flow (N_em>1), but when disagreement occurred it was because microsatellites found no gene flow where mitochondrial DNA estimated N_em>1. For example, microsatellite results differed from mtDNA results in estimating lower levels of gene flow (N_em<1) between Madrid (Spain) and other European populations east of the Pyrenees.

Isolation by Distance

Pairwise comparison of gene flow estimates (N_em) and geographic distance when plotted in a log-log scale can be used to depict patterns of isolation-by-distance in equilibrium and non-equilibrium populations (Slatkin 1993). In this study, this approach was applied to specific groups of tawny owl populations that reflect the Balkans expansion (Figure 8a), and the two hybrid zones of Iberia-Balkans (Figure 8b), and Italy-Balkans (Figure 8c). Scatter plots for the Balkans group were congruent between marker classes in showing no detectable pattern of isolation-by-distance (r^2=0.009, p>0.05 and r^2=0.034, p>0.05 for mitochondrial and nuclear markers respectively) among populations connected by substantial levels of gene flow; in this group of populations all pairwise gene flow estimates were higher than one, although significant differences were found between the two regression slopes (F=8.39, df=1, p<0.01). In contrast, scatter plots for populations across the two hybrid zones depict an interesting contrast between markers. Although mitochondrial data showed decreasing gene flow with increasing geographic distance, with microsatellites population pairs are discriminated in two groups depending on whether they exchange significant amounts of gene flow (black circles) or had $N_em<1$ (white squares). The former group corresponds to population pairs located on the same side of the hybrid zone, whereas the latter group includes populations from different sides of the hybrid zones.

DISCUSSION

CONGRUENT PATTERNS

The combined analyses of the seven nuclear microsatellite loci have corroborated the major findings of the mitochondrial phylogeography of tawny owls in western Europe (Table 2). Both mitochondrial and nuclear markers have shown the existence of three groups of tawny owl populations that correspond to the three proposed Pleistocene refugia of Iberia, Italy, and Balkans. The Balkans has also been confirmed as the main source of postglacial colonization of northern Europe, and two contact zones with some introgression were detected with both markers, between Iberia and Balkans, and Italy and Balkans. However, the historical signal across the microsatellite loci was not uniform, and levels of polymorphism as well as signatures of population structure varied considerably (Table 2). The congruence between mitochondrial and nuclear markers was obtained in the combined analysis and with two of the seven microsatellite loci analyzed.

The ability to detect a consistent signal across different markers depends on marker specific factors, such as the degree of homoplasy, the mutation mechanism and rates, as well as on the analytical methods applied. Mitochondrial conclusions are the result of genealogical analyses of control region sequences. Glacial refugia and postglacial expansion routes were inferred after the detection of highly supported clades that showed significant geographic structure. Those clades were identified with outgroup analysis, and geographic structure determined by the proportion of individuals from each

population in each clade. Microsatellite conclusions, on the other hand, were inferred from multivariate (PCA) and Bayesian analyses designed to find clusters of populations with similar allele frequencies. The PCA identified three groups of populations (Iberia, southern Italy, and Balkans, the later with mid-latitude and northern European populations) but the relationship among those groups was unknown and assumed to be hierarchical. That is, the corroboration of the three refugia hypothesis assumed that those microsatellite clusters are equivalent to three monophyletic clades defined by outgroup analysis. Therefore without mtDNA results, one could not necessarily interpret the microsatellite data historically. The Bayesian approach uses individuals as the unit of analysis but although it has identified three clusters, only Iberia was clearly distinct from the remaining European populations. STRUCTURE allocated all remaining individuals to a mixed origin, as if there were no geographic structure between those two clusters. Due to the lack of an a priori definition of the population limits, it is possible that this method would not perform as well along gradients of allele frequencies such as the ones created by wide contact zones, because this method's only assumption is the maintenance of Hardy-Weinberg equilibrium frequencies in local populations. The hybrid zone between Iberian and Balkan genomes is much steeper (see below) and for that reason presented no difficulty to the Bayesian approach in the identification of an Iberian cluster.

In conformity with the mitochondrial data, microsatellite loci provided independent support of a Balkans origin of northern European populations, although only three of the seven loci showed an unequivocal signal (Table 2). Genetic signatures of range expansion were identified for both mitochondrial and nuclear data from analyses of isolation-by-distance (IBD). In the Balkans expansion (Figure 8a) relatively large values

of gene flow were estimated among populations but gene flow was independent of geographic distance, suggesting that populations had recently colonized the area (Slatkin 1993). With time, as populations reach equilibrium between drift and gene flow, patterns of isolation-by-distance are expected to occur (Wright 1943, Slatkin 1993), and the regression slope of gene flow with geographic distance should indicate this process by becoming progressively more negative. In the Balkans expansion, the significant difference detected between the mitochondrial and nuclear regression slopes is an indication of slightly higher levels of mitochondrial gene flow because effects of drift are dependent on the effective population size, and therefore expected to be greater among mitochondrial genomes.

CONTRASTING PATTERNS

Microsatellites and mitochondrial loci showed a striking difference in the genetic diversity of northern European populations. Expected heterozygosity and nucleotide diversity computed for all 14 populations were weakly correlated suggesting that microsatellite data could not be used to predict mitochondrial genetic diversity, and vice versa. However, if northern European populations were excluded from the analysis those statistics became strong and significantly correlated, indicating that either mitochondrial data have underestimated the genetic diversity of those populations, or microsatellites had no power to distinguish different levels of genetic diversity. Lower genetic diversity in northern European populations is expected due to their recent origin (Hewitt 1996; Brito 2005). Conversely, the higher diversity estimated with microsatellites

can be explained by their higher mutation rates because recovery rate of neutral variation from bottlenecks is a function of the mutation rate (Nei *et al.* 1975; Lande & Barrowclough 1987). The higher mutation rate of microsatellites would then lead to faster recovery rates.

Overall population structure estimated with microsatellites showed extensive variability between statistics and across loci. The later is expected under conditions of substructure in the dataset or due to local abundance of newly arisen neutral alleles (Singh & Rhomberg 1987). Yet differences in the *F*-statistics may be due to the underlying mutation model. F_{ST} assumes an infinite allele model (IAM) and it will tend to underestimate the true level of genetic differentiation if used with markers under mutation models that generate homoplasy, such as the stepwise mutation model (SMM) (Slatkin 1995; Rousset 1996). R_{ST}, on the other hand, assumes a SMM and it was shown to perform better than F_{ST} whenever the mutation model carries some memory of the mutation process although deviations from a strict SMM are known to decrease estimates of R_{ST} and increase its variance (e.g. Balloux & Lugon-Moulin 2002). For this reason, reporting both R_{ST} and F_{ST} has been advocated by several authors (Balloux *et al.* 2000; Balloux & Goudet 2002; Balloux & Lugon-Moulin 2002). In this study, R_{ST} estimates were in general higher than F_{ST}, which was taken as an indication that the underlying mutation model carried some memory of the mutation process, and therefore R_{ST} estimates were preferred.

Overall R_{ST} was substantially smaller than mitochondrial N_{ST} as expected from nuclear markers due to their differences in effective population size (Birky *et al.* 1983; Chesser & Baker 1996; Crochet 2000). However, after calibrating N_{ST} to its expected

result if nuclear genes had been sampled (N_{ST}*) these two measures of overall geographic structure became quite similar. This result does not support the general expectation that microsatellites may underestimate overall genetic structure due to their typical high mutation rates that may violate assumptions made in the analyses of the *F*-statistics (Balloux *et al.* 2000; Epperson 2005).

Levels of polymorphism were not a good indicator of the effectiveness of each microsatellite locus to detect genetic structure among populations as might be expected if differentiation were a function of population variability. The two loci with highest genetic diversity (4E10.2 and 4E10) were at the extremes of the R_{ST} scale suggesting that even if some ascertainment bias was committed in the decision to analyze the more polymorphic loci, that bias was independent of the final geographic structuring level. Also, the two loci with higher R_{ST} were the same two with significant departures from overall Hardy-Weinberg equilibrium frequencies, indicating that those departures are due to substructure in the data and not to other population process such as natural selection.

Congruence between mitochondrial and microsatellite loci was found in the identification of two hybrid zones of tawny owls in western Europe but those markers contrasted in the location of the contact zones. Major differences between mitochondrial and nuclear markers occurred in Italy-N and Spain-Madrid populations; this made the contact zones shift south-north and west-east respectively. Mitochondrial results have identified an Italian clade, although recent migration was detected between northern Italy and populations associated with the Balkans clade. Principal component analysis of microsatellite data, on the other hand, grouped northern Italy with the Balkans group instead. Because PCA treats populations as the unit of analysis, the results for each

population reflect the average of their individuals. High gene flow between northern Italy and mid-latitude populations originated by Balkans expansion (i.e. Austria) could have produced this result, especially if gene flow between Sicily and northern Italy was reduced.

In the Iberia-Balkans hybrid zone the genetic composition of the Madrid population varied considerably depending on the genetic marker analyzed. Although mitochondrial data have identified extensive gene flow from France into Iberia, nuclear data identified a hybrid zone between Iberian and Balkans genotypes across the Pyrenees. Pairwise estimation of geographic structure and gene flow also corroborated this result that indicates a much steeper hybrid zone than the one identified between Italian and Balkans genomes. A much larger offset between mitochondrial and nuclear contact zones was estimated for the Italian-Balkans hybrid zone but only a denser sampling strategy across the Italian and Iberian peninsulas could test the precision of those estimates.

The isolation-by-distance analyses recovered a different pattern for the Balkans expansion than for the analyses of the transects across the two hybrid zones. In the first, similar results were obtained from both markers but populations across the two transects showed contrasting patterns between markers. With microsatellites, the variation of pairwise estimates of gene flow with geographic distance segregate population-pairs according to their location in the hybrid zones. Across both transects, all values of gene flow between populations of opposite sides of the hybrid zones were small and independent of the geographic distance whereas gene flow among populations from the same side of the hybrid zone was high in general. The same striking pattern was not

observed with mitochondrial data, which might suggest higher mitochondrial introgression.

A major difference distinguishes the Balkans expansion from populations across the two transects. In the Balkans expansion, populations expanded their range into areas that were not previously occupied; this process could be envisaged as the movement of an advancing wave that faced no major obstructions. Across the transects, population expansion is better understood as the introgression of one genome group into an area already occupied by another. Hewitt (1993) has described this introgression as postglacial hybridization due to a series of advances and retreats with the consequent formation of small pockets of pioneers and refugees. The two marker classes differed in the location of the two contact zones by showing different positions of nuclear and mitochondrial introgression. This difference may be due to chance and/or error, or it may have a historical or biological explanation. Population genetics theory predicts that hybrid zones tend to move to natural barriers characterized by low density and dispersal (Barton & Hewitt 1985). In Europe such areas are likely to be the Pyrenees and the Alps for temperate species such as the tawny owls. Hence, mitochondrial results are more consistent with theoretical expectations in the location of the Italy-Balkans contact zone, whereas microsatellite recovered more congruent results in the placement of the Iberia-Balkans contact zone. The comparison between pairwise estimates of microsatellite and mitochondrial gene flow, also suggested a general bias for higher mitochondrial gene flow. This difference in the level of genetic structure between the two markers with contrasting modes of inheritance is expected when female biased dispersal occurs (Prugnolle & Meeus 2002).

TABLES

Table 1 Summary of genetic variation at the seven microsatellite loci scored from the tawny owl populations in western Europe: Sample size (N), average no. of alleles (a),observed and expected heterozygosity (Ho and He, SD is standard deviation); number of alleles per locus (A); most common allele and its frequency (C (xC)), and number of private alleles (P).

	Averaged over all loci				15A6					8G11					4E10.2				
	N	a	Ho	He (SD)	A	C (x_C)	P	Ho	He	A	C (x_C)	P	Ho	He	A	C (x_C)	P	Ho	He
Refugial Populations																			
Portugal (Pt)	20	6.43	0.596	0.667 ± 0.113	6	145 (0.40)	2	0.7000	0.7718	5	129 (0.33)	0	0.4500	0.7500	13	220 (0.20)	0	0.7500	0.9077
Spain-Madrid (Sp_M)	13	5.86	0.565	0.703 ± 0.070	5	141 (0.35)	0	0.6923	0.7662	5	129 (0.42)	0	0.6154	0.7138	12	235 (0.27)	1	0.7692	0.8985
Spain-Bilbao (Sp_B)	6	5.29	0.738	0.697 ± 0.110	4	137 (0.50)	0	0.8333	0.7273	5	141 (0.42)	0	0.8333	0.8030	10	170/235 (0.17)	0	0.8333	0.9697
Italy-North (It_N)	13	6.14	0.604	0.628 ± 0.122	5	145 (0.35)	0	0.9231	0.7631	5	145 (0.42)	0	0.4615	0.7015	9	225 (0.27)	0	0.8462	0.8585
Italy-Sicily (It_S)	11	4.29	0.633	0.574 ± 0.116	5	117 (0.32)	1	0.8182	0.7879	4	149 (0.36)	0	0.7273	0.7403	6	215 (0.27)	0	0.8182	0.8312
Greece (Gr)	13	6.29	0.615	0.640 ± 0.125	6	145 (0.35)	0	0.9231	0.7815	4	145 (0.35)	0	0.6154	0.7538	10	215 (0.23)	0	0.8462	0.8954
Mid-Latitudes																			
France_W (Fr_W)	9	5.86	0.619	0.669 ± 0.093	6	141 (0.44)	0	0.8889	0.7712	3	149 (0.50)	0	0.3333	0.5817	10	220 (0.33)	1	0.7778	0.8824
France_N (Fr_N)	10	5.43	0.543	0.632 ± 0.100	5	141 (0.40)	0	0.6000	0.7263	5	141 (0.45)	0	0.5000	0.6947	8	215 (0.35)	2	0.8000	0.8474
France_SE (Fr_{SE})	20	7.43	0.586	0.684 ± 0.102	6	141 (0.38)	0	0.6500	0.7449	5	145/149 (0.30)	0	**0.3000**	0.7654	11	195 (0.18)	2	0.8500	0.8987
Austria (Au)	20	8.43	0.628	0.665 ± 0.121	6	145 (0.40)	0	0.6000	0.7462	8	145 (0.35)	1	0.8000	0.8013	11	210 (0.30)	1	0.8500	0.8667
Northern Populations																			
England (UK)	11	4.86	0.571	0.591 ± 0.132	5	137 (0.41)	0	0.8182	0.7489	3	149 (0.41)	0	0.4545	0.6797	8	210/220 (0.27)	0	0.8182	0.8398
Denmark (Dk)	21	7.00	0.618	0.667 ± 0.104	6	141 (0.50)	0	0.7143	0.6957	6	141 (0.57)	2	0.4762	0.6353	10	210 (0.33)	0	0.6667	0.8316
Norway (No)	7	4.57	0.674	0.628 ± 0.114	5	145 (0.29)	0	1.0000	0.8352	4	137 (0.36)	0	0.8571	0.7802	5	220 (0.57)	0	0.7143	0.6703
Finland (Fi)	10	5.00	0.729	0.701 ± 0.081	5	145 (0.45)	0	0.8000	0.7579	4	145 (0.35)	0	0.9000	0.7316	7	225 (0.25)	0	0.8000	0.8842

In bold are Ho that significantly deviate from HWE after Bonferroni correction has been applied

Table 1 (*cont.*)

1C6					4E10					Bb111					Bb131				
A	C (x_C)	P	Ho	He	A	C (x_C)	P	Ho	He	A	C (x_C)	P	Ho	He	A	C (x_C)	P	Ho	He
5	105 (0.38)	0	0.8500	0.7718	12	205 (0.23)	0	0.8500	0.8974	2	200 (0.98)	0	0.0500	0.0500	3	155 (0.55)	0	0.6000	0.5295
6	105 (0.27)	0	0.6154	0.8369	7	205 (0.45)	1	0.7273	0.7532	3	200 (0.81)	0	0.3077	0.3354	3	151 (0.46)	0	0.4615	0.6185
5	99/105/108 (0.25)	0	1.0000	0.8485	8	215 (0.25)	0	1.0000	0.9242	2	200 (0.92)	0	0.1667	0.1667	3	151 (0.75)	0	0.5000	0.4394
6	105/108 (0.31)	0	0.7692	0.7908	14	220 (0.19)	0	0.8462	0.9323	2	200 (0.85)	0	0.3077	0.2708	2	151 (0.96)	0	0.0769	0.0769
3	105 (0.59)	0	0.8182	0.5411	6	205 (0.38)	0	1.0000	0.7833	1	200 (1.00)	0	0.0000	0.0000	5	151 (0.82)	3	0.2727	0.3377
6	108 (0.38)	0	0.7692	0.7631	14	275 (0.19)	1	0.7692	0.9354	2	200 (0.85)	0	0.3077	0.2708	2	151 (0.96)	0	0.0769	0.0769
5	105/108/114 (0.22)	0	0.7778	0.8431	11	205 (5/18)	0	0.8889	0.9085	3	200 (0.78)	0	0.3333	0.3856	3	151 (0.83)	0	0.3333	0.3072
4	105 (0.45)	0	0.7000	0.7000	11	215 (0.25)	0	0.8000	0.9158	3	200 (0.80)	0	0.2000	0.3526	2	151 (0.90)	0	0.2000	0.1895
6	111 (0.43)	0	0.8000	0.7462	19	225 (0.18)	0	0.9000	0.9385	3	200 (0.63)	0	0.5500	0.5526	2	151 (0.93)	0	0.1500	0.1423
8	105 (0.30)	1	0.8000	0.8103	20	285 (0.13)	0	0.9474	0.9616	4	200 (0.75)	0	0.3500	0.4218	2	151 (0.98)	0	0.0500	0.0500
6	111 (0.32)	1	0.9091	0.7965	8	215 (0.23)	1	0.8182	0.8918	2	200 (0.95)	0	0.0909	0.0909	2	151 (0.95)	0	0.0909	0.0909
7	111 (0.26)	0	0.9524	0.8130	14	275 (0.15)	0	**0.8500**	0.9321	4	200 (0.43)	0	0.5714	0.6678	2	151 (0.95)	0	0.0952	0.0929
5	105 (0.36)	0	0.7143	0.7912	9	265 (0.29)	0	1.0000	0.9121	2	200 (0.93)	0	0.1429	0.1429	2	151 (0.86)	0	0.2857	0.2637
6	105 (0.35)	0	0.9000	0.8053	9	270 (0.20)	0	0.8000	0.9105	2	200 (0.65)	0	0.5000	0.4789	2	151 (0.80)	0	0.4000	0.3368

Table 2 Summary of the major results reached with mitochondrial and microsatellite loci.

RESULTS	MTDNA	MICROSATELLITES								
		Mean	p^1	15A6	8G11	4E10.2	1C6	4E10	Bb111	Bb131
Iberian Refugium	yes	yes	4 / 7	yes	yes	yes	no	no	no	yes
Italian Refugium	yes	yes	4 / 7	yes	no	yes	yes	no	yes	no
Balkans Refugium	yes	yes	3 / 7	yes	no	yes	no	yes	no	no
Source of Northern Europe	Balkans	Balkans	3 / 7	Balkans	not Iberia	Balkans	not Sicily	Balkans	not Sicily	not Iberia
F-statistics	0.34*	0.16 / 0.06		0.14 / 0.05	0.22 / 0.05	0.04 / 0.03	0.17 / 0.04	0.29 / 0.04	0.15 / 0.12	0.10 / 0.23

p1 indicates the proportion of times that each conclusion was reach with single microsatellite loci; * mitochondrial FST is 0.11 if converted to expected value for a nuclear locus.

Table 3 Pairwise R_{ST} estimates (above diagonal), and corresponding migration rates N_em (below diagonal)*. R_{ST} significantly different from zero (after Bonferroni correction) are in bold

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Portugal		0.05	-0.01	**0.28**	0.12	**0.35**	**0.23**	**0.30**	**0.31**	**0.25**	**0.37**	**0.28**	**0.23**	**0.30**
2. Sp-Madrid	4.57		0.03	**0.24**	0.17	**0.31**	**0.23**	0.23	**0.23**	**0.23**	**0.28**	**0.20**	0.25	**0.26**
3. Sp-Bilbao	V.High	8.09		0.15	0.09	**0.24**	0.08	0.16	0.17	0.15	0.19	0.16	0.17	0.19
4. Italy-N	0.64	0.79	1.43		**0.25**	0.02	0.06	0.00	0.06	0.00	0.09	0.10	0.03	0.06
5. Italy-S	1.80	1.19	2.41	0.73		**0.30**	0.14	0.23	**0.26**	**0.22**	**0.28**	**0.23**	0.21	**0.25**
6. Greece	0.46	0.55	0.81	12.83	0.59		0.11	0.10	0.07	-0.01	0.12	**0.13**	0.04	0.06
7. France-W	0.85	0.83	2.91	3.66	1.48	2.00		0.08	0.05	0.05	0.07	0.10	0.13	0.05
8. France-N	0.58	0.82	1.29	V.High	0.84	2.15	3.08		0.09	0.06	0.07	0.11	0.11	0.09
9. France-SE	0.56	0.82	1.21	3.98	0.73	3.51	4.31	2.51		0.02	0.08	0.00	0.15	0.03
10. Austria	0.73	0.82	1.47	V.High	0.88	V.High	4.83	3.78	10.21		0.11	0.06	0.02	0.03
11. England	0.42	0.65	1.04	2.57	0.65	1.76	3.14	3.10	3.02	2.06		**0.13**	0.24	0.15
12. Denmark	0.63	1.03	1.29	2.19	0.84	1.72	2.34	1.93	V.High	4.01	1.65		0.17	0.07
13. Norway	0.83	0.74	1.23	7.08	0.96	6.07	1.62	2.09	1.40	14.26	0.79	1.19		0.10
14. Finland	0.58	0.73	1.07	4.11	0.75	3.84	5.30	2.48	9.56	9.75	1.45	3.51	2.16	

* Negative R_{ST} were translated into Nm that were too high as to be quantifiable using this method.

FIGURES

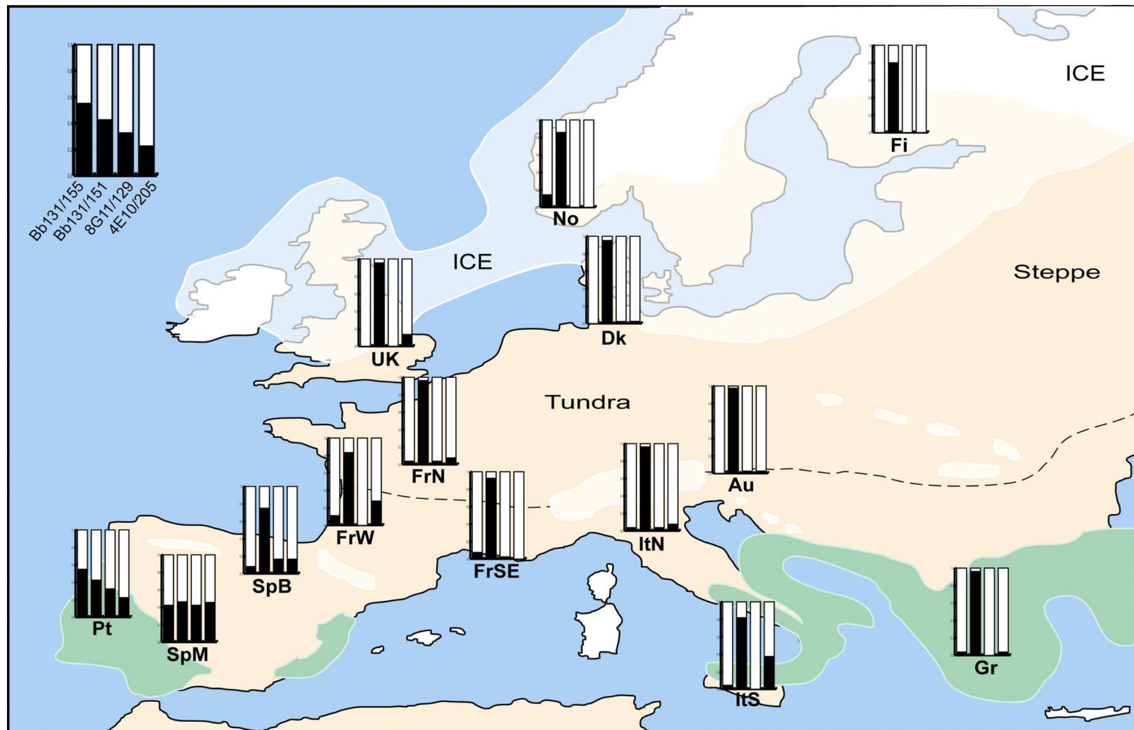


Fig. 1 Geographic variation of tawny owl microsatellites across western Europe. Histograms show the frequency of the four most influential alleles in the principal component analysis. Histograms' positions point to approximate locations of sampled populations. Map was adapted from Hewitt (1996, Fig. 5), and Hewitt (1999, Fig. 1B), to indicate suitable habitat during the last glacial maximum. Ice coverage is indicated in white, and location of potential refugia in green. Dashed line shows the southern limit of the permafrost

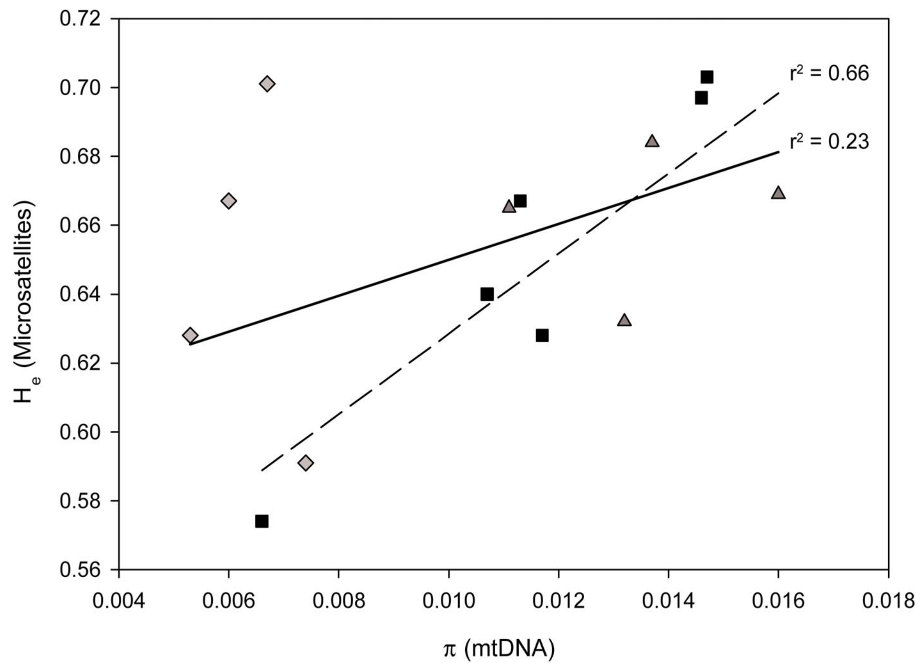


Fig. 2 Tawny owl genetic diversity in western Europe; contrast between microsatellites and mitochondria loci. Each population is characterized by its expected heterozygosity (microsatellites), and nucleotide diversity (mtDNA). The regression line and coefficients of determination are shown for all populations (full line), and for the refugial and mid-latitude populations only (dashed line). Squares, triangles, and diamonds represent refugial, mid-latitudes, and northern populations respectively.

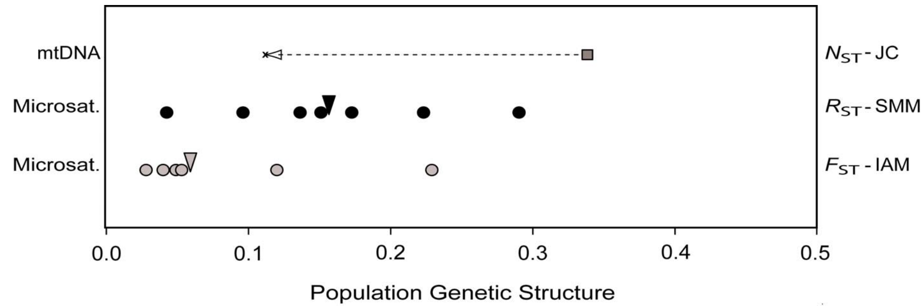


Fig. 3 Overall population genetic structure of tawny owls. *F*-statistics estimated from microsatellite loci, R_{ST} and F_{ST}, assume a stepwise mutation model (SMM) and an infinite allele model (IAM) respectively; *F*-statistic estimated with mtDNA, N_{ST}, uses a Jukes and Cantor (JC) model of evolution. Black and gray circles indicate R_{ST} and F_{ST} results for each locus, respectively, and inverted triangles correspond to mean values across loci. The mtDNA arrow indicates corresponding N_{ST} value (N_{ST}*) calibrated for nuclear effective population size.

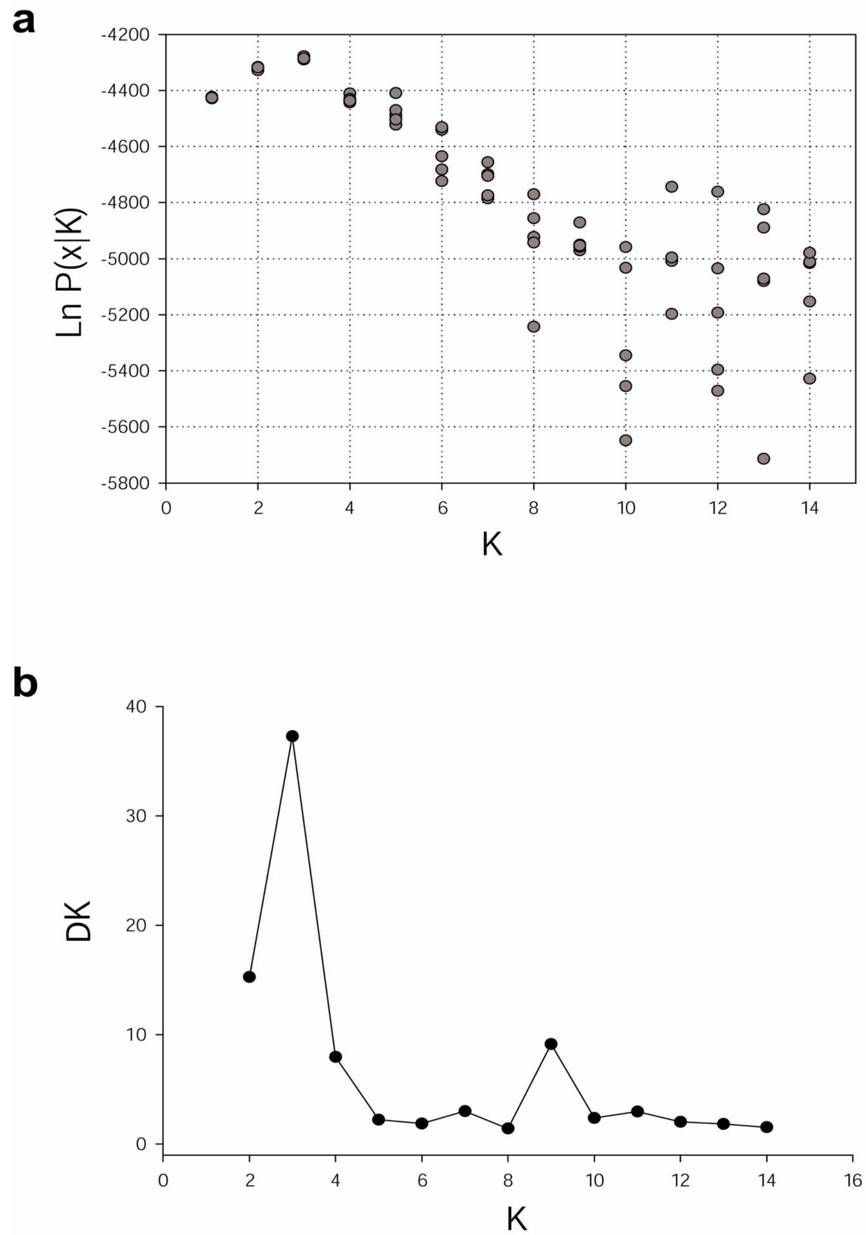


Fig. 4 Bayesian inference of the number of clusters (K) of tawny owls. K was estimated using the (a) the posterior probability of the data given each K (five replicates), and (b) the distribution of ΔK.

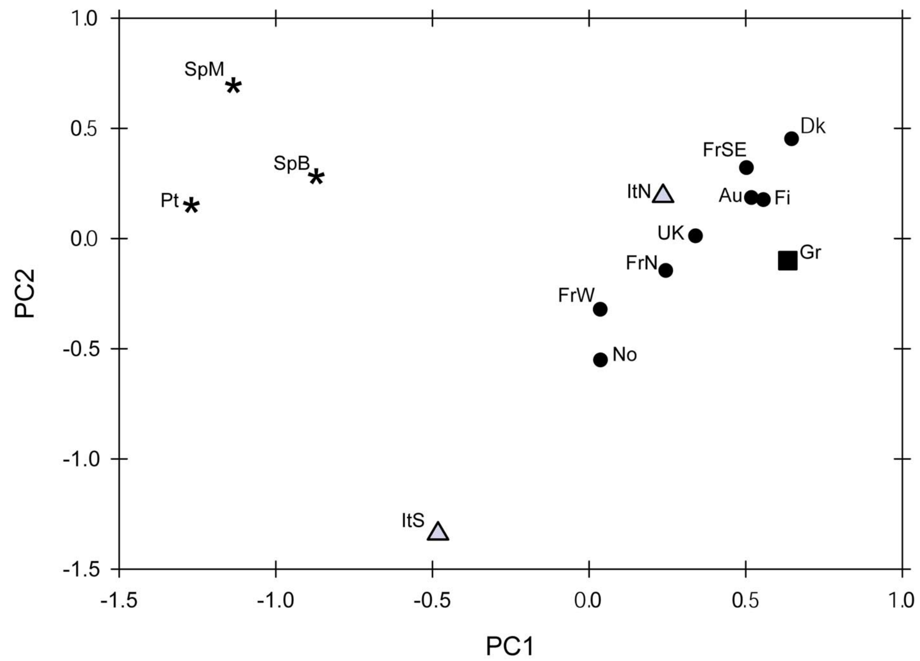


Fig. 5 Scores of 14 populations of tawny owl on the first two principal components extracted from a matrix of 91 scored alleles from seven microsatellite loci. Populations are represented by different symbols to indicate geography: Iberia (asterisks), Italy (triangles), Greece (square), and non-refugial populations (circles). Abbreviations as in Table 1.

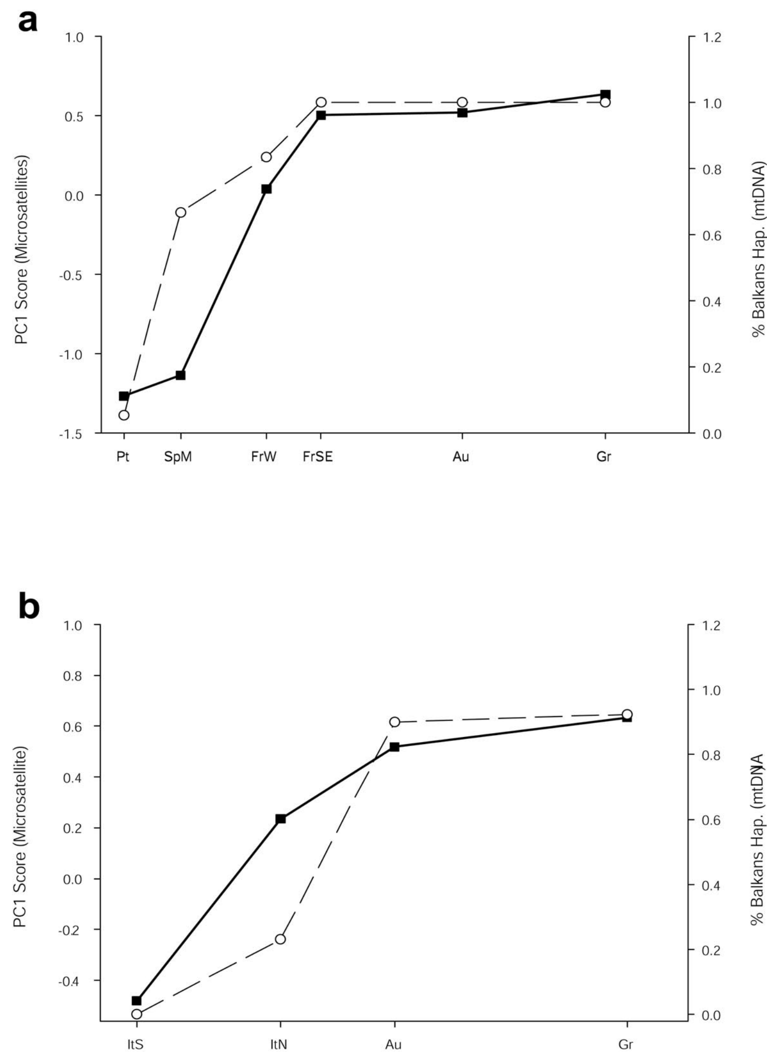


Fig. 6 Microsatellite vs. mitochondrial profiles across two hybrid zones in the tawny owl. Replacement of (a) Iberian and (b) Italian genomes by Balkans genomes across western Europe as measured by seven nuclear microsatellite loci (black squares) and control region mtDNA sequences (white circles). Microsatellite loci are measured by PC1 scores, and mtDNA by the proportion of Balkans haplotypes over (a) Iberian and (b) Italian haplotypes in each population. The distance between population-pairs is drawn to geographic scale.

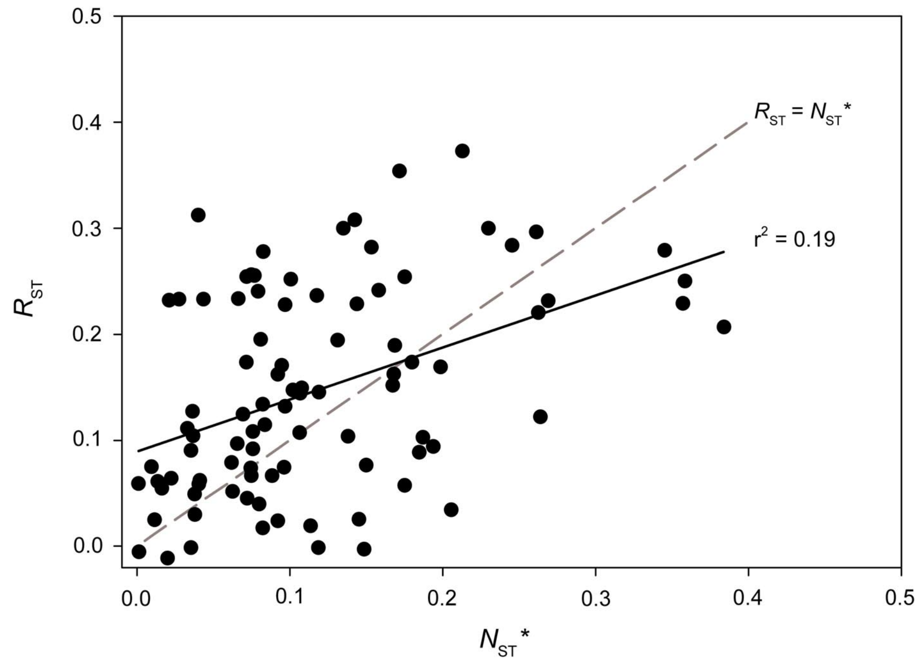


Fig. 7 Scattergram of mitochondrial N_{ST} (calibrated to its expected nuclear values, N_{ST}*) and microsatellite $R_{ST.}$ The regression line (solid) and the coefficient of determination are shown. The dashed line corresponds to perfect correlation ($R_{ST} = N_{ST}$*).

Appendix

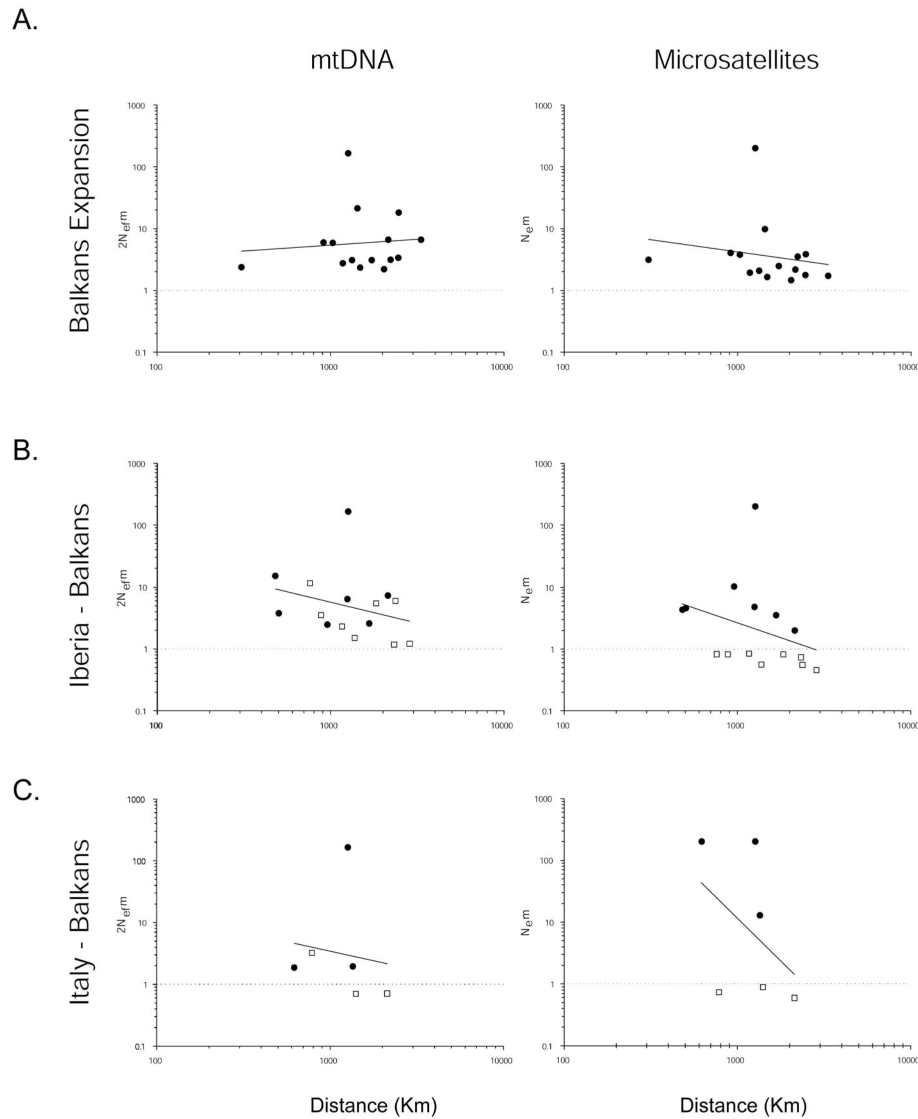


Fig. 8 Analyses of isolation by distance for mitochondrial (graphs on the left) and microsatellite loci (graphs on the right). $N_e m$ is plotted against geographic distance (Km) on a log-log scale. Comparison were made for populations in the 'Balkans expansion' (a), and across the transects across Iberia-Balkans hybrid zone (b), and Italy-Balkans hybrid zone (c). In graphs b and c black circles indicate results from populations sampled on the same side of the hybrid zone, while white squares are population-pairs located in opposite sides of the hybrid zone

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APPENDIX 1. MICROSATELLITE ALLELE FREQUENCIES

Microsatellite allele frequencies across loci and populations of tawny owl.

Locus	Allele	Portugal	Spain-M	Spain-B	Italy-N	Italy-S	Greece	France_W	France_N	France_SE	Austria	England	Denmark	Norway	Finland	Total
15A6	117	0.000	0.000	0.000	0.038	0.318	0.115	0.056	0.000	0.025	0.100	0.000	0.119	0.071	0.000	0.063
	121	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003
	125	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003
	129	0.150	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016
	133	0.175	0.192	0.167	0.000	0.273	0.000	0.111	0.050	0.075	0.025	0.000	0.071	0.214	0.150	0.098
	137	0.375	0.308	0.500	0.308	0.136	0.115	0.167	0.150	0.225	0.225	0.409	0.071	0.214	0.150	0.231
	141	0.225	0.346	0.167	0.115	0.227	0.308	0.444	0.400	0.375	0.225	0.182	0.500	0.214	0.150	0.291
	145	0.050	0.077	0.167	0.346	0.000	0.346	0.167	0.350	0.275	0.400	0.273	0.214	0.286	0.450	0.242
	149	0.000	0.077	0.000	0.192	0.000	0.077	0.056	0.050	0.025	0.025	0.091	0.024	0.000	0.100	0.049
	153	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.000	0.005
8G11	125	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.005
	129	0.325	0.423	0.167	0.038	0.000	0.000	0.000	0.000	0.025	0.025	0.000	0.024	0.000	0.000	0.082
	133	0.000	0.038	0.083	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.008
	137	0.100	0.000	0.000	0.038	0.091	0.154	0.000	0.050	0.150	0.075	0.000	0.095	0.357	0.000	0.082
	141	0.300	0.308	0.417	0.346	0.227	0.308	0.000	0.450	0.225	0.225	0.364	0.571	0.286	0.300	0.315
	145	0.250	0.192	0.167	0.423	0.318	0.346	0.444	0.100	0.300	0.350	0.227	0.190	0.143	0.350	0.277
	149	0.025	0.038	0.167	0.154	0.364	0.192	0.500	0.350	0.300	0.175	0.409	0.071	0.214	0.300	0.209
	153	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.050	0.000	0.075	0.000	0.000	0.000	0.050	0.016
	157	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.005
4E10.2	150	0.000	0.038	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003
	155	0.000	0.000	0.000	0.000	0.000	0.000	0.111	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005
	160	0.050	0.077	0.083	0.000	0.000	0.000	0.056	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.022
	165	0.025	0.000	0.083	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005
	170	0.125	0.000	0.167	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.022

	175	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.000	0.005
	180	0.000	0.038	0.083	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005
	185	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.003
	190	0.000	0.000	0.000	0.038	0.000	0.038	0.000	0.050	0.000	0.025	0.000	0.000	0.000	0.000	0.011
	195	0.025	0.077	0.000	0.077	0.000	0.038	0.111	0.000	0.175	0.025	0.045	0.048	0.000	0.100	0.057
	200	0.100	0.000	0.000	0.000	0.136	0.038	0.000	0.000	0.000	0.075	0.000	0.024	0.000	0.100	0.038
	205	0.125	0.115	0.083	0.038	0.227	0.077	0.056	0.000	0.075	0.075	0.045	0.071	0.000	0.000	0.076
	210	0.050	0.038	0.083	0.115	0.000	0.077	0.056	0.150	0.150	0.300	0.273	0.333	0.143	0.150	0.152
	215	0.025	0.038	0.083	0.231	0.273	0.231	0.056	0.350	0.075	0.125	0.182	0.214	0.143	0.150	0.149
	220	0.200	0.154	0.083	0.154	0.227	0.192	0.333	0.100	0.150	0.125	0.273	0.048	0.571	0.150	0.177
	225	0.125	0.038	0.083	0.269	0.091	0.115	0.056	0.100	0.150	0.100	0.091	0.048	0.071	0.250	0.114
	230	0.025	0.038	0.000	0.038	0.045	0.115	0.111	0.150	0.100	0.025	0.045	0.095	0.000	0.100	0.065
	235	0.100	0.269	0.167	0.038	0.000	0.000	0.000	0.000	0.025	0.100	0.000	0.024	0.071	0.000	0.057
	240	0.000	0.077	0.000	0.000	0.000	0.077	0.000	0.000	0.000	0.000	0.000	0.095	0.000	0.000	0.022
	245	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.003
	255	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.003
	260	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.003
	270	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.003
1C6	96	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.024	0.000	0.000	0.005
	99	0.225	0.077	0.250	0.038	0.045	0.000	0.167	0.000	0.000	0.025	0.000	0.000	0.286	0.050	0.068
	102	0.175	0.077	0.000	0.077	0.000	0.038	0.000	0.000	0.000	0.125	0.000	0.024	0.000	0.050	0.052
	105	0.375	0.269	0.250	0.308	0.591	0.269	0.222	0.450	0.225	0.300	0.227	0.238	0.357	0.350	0.310
	108	0.100	0.192	0.250	0.308	0.364	0.385	0.222	0.300	0.050	0.275	0.045	0.095	0.214	0.150	0.196
	111	0.125	0.192	0.167	0.192	0.000	0.077	0.167	0.200	0.425	0.150	0.318	0.262	0.071	0.250	0.198
	114	0.000	0.192	0.083	0.077	0.000	0.192	0.222	0.050	0.150	0.050	0.273	0.238	0.071	0.150	0.125
	117	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.125	0.050	0.091	0.119	0.000	0.000	0.041
	120	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.000	0.003
	123	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.003
>4E10	160	0.000	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003
	185	0.075	0.045	0.083	0.000	0.000	0.000	0.000	0.050	0.025	0.000	0.000	0.000	0.000	0.000	0.020
	190	0.100	0.045	0.167	0.038	0.000	0.000	0.056	0.000	0.075	0.000	0.136	0.000	0.000	0.000	0.042
	195	0.025	0.000	0.000	0.000	0.125	0.000	0.056	0.000	0.000	0.000	0.000	0.100	0.000	0.000	0.023

	200	0.175	0.227	0.083	0.077	0.000	0.038	0.000	0.050	0.050	0.053	0.000	0.075	0.000	0.000	0.068
	200	0.175	0.227	0.083	0.077	0.000	0.038	0.000	0.050	0.050	0.053	0.000	0.075	0.000	0.000	0.068
	205	0.225	0.455	0.167	0.077	0.375	0.038	0.278	0.150	0.000	0.026	0.136	0.025	0.000	0.000	0.121
	210	0.100	0.091	0.083	0.038	0.063	0.038	0.000	0.050	0.025	0.053	0.000	0.125	0.071	0.150	0.065
	215	0.075	0.000	0.250	0.000	0.313	0.038	0.111	0.250	0.125	0.026	0.227	0.100	0.000	0.050	0.099
	220	0.075	0.091	0.083	0.192	0.000	0.000	0.000	0.050	0.000	0.079	0.000	0.025	0.000	0.000	0.045
	225	0.050	0.000	0.000	0.000	0.063	0.000	0.056	0.100	0.175	0.079	0.000	0.075	0.071	0.000	0.056
	230	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.000	0.025	0.053	0.091	0.075	0.143	0.150	0.040
	235	0.000	0.000	0.000	0.038	0.000	0.000	0.056	0.000	0.025	0.026	0.000	0.000	0.071	0.000	0.014
	240	0.000	0.000	0.000	0.077	0.063	0.000	0.000	0.000	0.000	0.026	0.000	0.075	0.000	0.000	0.020
	245	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.006
	250	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.008
	255	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.091	0.000	0.000	0.000	0.006
	260	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000	0.025	0.053	0.000	0.050	0.000	0.000	0.017
	265	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.050	0.025	0.000	0.000	0.000	0.286	0.000	0.020
	270	0.050	0.000	0.000	0.000	0.000	0.115	0.000	0.000	0.075	0.026	0.091	0.000	0.000	0.200	0.042
	275	0.000	0.000	0.000	0.038	0.000	0.192	0.000	0.100	0.025	0.053	0.000	0.150	0.000	0.100	0.054
	280	0.000	0.000	0.083	0.000	0.000	0.038	0.056	0.000	0.025	0.026	0.182	0.000	0.000	0.100	0.031
	285	0.025	0.000	0.000	0.038	0.000	0.077	0.167	0.100	0.075	0.132	0.000	0.075	0.071	0.150	0.068
	290	0.025	0.000	0.000	0.115	0.000	0.115	0.000	0.000	0.025	0.053	0.045	0.025	0.071	0.050	0.040
	295	0.000	0.000	0.000	0.154	0.000	0.000	0.000	0.000	0.025	0.053	0.000	0.000	0.071	0.000	0.023
	300	0.000	0.000	0.000	0.038	0.000	0.115	0.056	0.000	0.100	0.079	0.000	0.000	0.000	0.000	0.034
	305	0.000	0.000	0.000	0.000	0.000	0.077	0.000	0.050	0.000	0.053	0.000	0.000	0.000	0.000	0.014
	310	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.000	0.000	0.026	0.000	0.000	0.143	0.050	0.014
	315	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.026	0.000	0.000	0.000	0.000	0.006
	320	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003
Bb111	200	0.975	0.808	0.917	0.846	1.000	0.846	0.778	0.800	0.625	0.750	0.955	0.429	0.929	0.650	0.780
	202	0.000	0.154	0.000	0.000	0.000	0.000	0.000	0.150	0.000	0.025	0.045	0.119	0.000	0.000	0.038
	204	0.000	0.000	0.083	0.154	0.000	0.154	0.167	0.050	0.175	0.125	0.000	0.381	0.000	0.000	0.111
	206	0.025	0.038	0.000	0.000	0.000	0.000	0.056	0.000	0.200	0.100	0.000	0.071	0.071	0.350	0.071
Bb131	151	0.425	0.462	0.750	0.962	0.818	0.962	0.833	0.900	0.925	0.975	0.955	0.952	0.857	0.800	0.826
	155	0.550	0.423	0.083	0.038	0.045	0.038	0.111	0.100	0.075	0.000	0.000	0.000	0.143	0.000	0.125

157	0.025	0.115	0.167	0.000	0.000	0.000	0.056	0.000	0.000	0.025	0.045	0.048	0.000	0.200	0.041
163	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003
165	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003
167	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003

CONCLUDING REMARKS

The contrast between mitochondrial and microsatellite markers in the study of tawny owl populations in Europe has uncovered general features about these markers' usefulness and applicability to the study geographic structure, gene flow, and historical processes in natural populations. The most important difference between these markers lies in the information-bearing quality of the data. Mitochondrial sequence data are character-based data and hence appropriate for hierarchical genealogical analyses, which are most useful for historical inference; here individuals can be assigned to clades that are defined by shared common ancestry. Microsatellite data, on the other hand, are characteristically analyzed as allele frequency data and clusters of individuals are defined by distances that reflect similarity, which may or may not result from common history. Additionally, microsatellites are highly homoplasious with a mutation process that is not well understood (Palsbøll *et al.* 1999; Rubinsztein *et al.* 1999; Weetman *et al.* 2002). As a result, the analyses of microsatellite data have to include more assumptions about the marker's molecular evolution than the analyses of mitochondrial sequence data.

In spite of this, microsatellites have several advantages over mitochondrial data that derive from being part of the nuclear genome. These are: (1) microsatellites track historical processes for both males and females, while mtDNA only enable the inference of the females' history; (2) microsatellites are diploid and each individual yields twice as much information as would a haploid marker; (3) microsatellites are commonly screened for multiple linkage units, where each unit enables an independent inference of the coalescent history of the populations; therefore multiple-locus analyses can decrease the

effects of stochastic variance due to drift and sampling; and finally (4) microsatellites are inherited in a Mendelian fashion and hence deviations from Hardy-Weinberg equilibrium can be used to uncover signals of population substructure.

As a consequence of the differences between the two markers, advantages might accrue by examining both mitochondrial and microsatellite data. The ability to infer population historical information from genetic markers is a result of the interaction between their effective population size and mutation rates. On one hand, the presence of signatures of population processes is dependent on the random effects of drift, which are greater in mitochondrial genomes due to their small N_e (Hudson 1990). Mutation rates are also important because they are what make those historical signatures readable. Also, since historical information is preserved, on average, four times as long in nuclear markers, then high mutation rates on these markers may lead to very high levels of homoplasy that could potentially confound history. This is more important in microsatellites as identical alleles are considered identical-by-descent in all analyses. Finally, the use of markers with distinct modes of inheritance enables the identification of sex-biased gene flow (e.g. Prugnolle & Meeus 2002).

In the present study of tawny owls in western Europe, high congruence was obtained between mitochondrial and nuclear markers in the identification of three refugial populations, and in the Balkans postglacial colonization of northern regions. This congruence added confidence concerning the general conclusions since it is highly unlikely that the same pattern would be obtained from both nuclear and mitochondrial markers if there were no common historical process (Avise 2000). However, the contrast between the two markers also brought some incertitude, especially in the location of the

two contact zones that differed between markers. Although the reason for this conflict is unknown, the single locus nature of mitochondrial data, and the difficulty of extracting information with microsatellites have complicated the analyses. The contrast of mitochondrial and nuclear markers added two major advantages to the study of tawny owl populations; (1) it was possible to infer female-biased dispersal due to the markers contrasting mode of inheritance, and (2) their different mutation rates allowed detection of the recent recovery of northern populations from bottlenecks, which occur faster in markers that have higher evolutionary rates (Nei *et al.* 1975; Lande & Barrowclough 1987).

This research constitutes the first attempt to study the glacial refugia hypothesis proposed for Europe (e.g. Hewitt 1996; Hewitt 2000) with an avian taxon where populations from all putative refugia as well as northern European were sampled for both mitochondrial and nuclear markers. This is also the first time that these two markers are so explicitly contrasted in their abilities and limitations to extract information useful to answer phylogeographic and population genetics questions.

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CHAPTER 1

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