Articles

Evidence for strong genetic structure in European populations of the little owl *Athene noctua*

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The little owl *Athene noctua* is a widespread species in Europe. This mainly sedentary owl experienced reduction in population sizes in some areas due to habitat loss and modification of the landscape. To assess the genetic structure of the populations of western and central Europe, we analysed 333 specimens from 15 geographical areas at 13 microsatellite loci. Statistical analyses and Bayesian clustering procedures detected two major genetically distinct clusters, the first distributed from Portugal to the Czech Republic and the second from the Balkans to Italy. The second cluster was further split into three groups, located in Italy, Sardinia and the Balkans. These groups match four previously-described mtDNA haplogroups, and probably originated from the isolation of little owl populations in Sardinia and in three glacial refugia (Iberia, south Italy and Balkans) during the ice ages. High genetic admixture was recorded in central and northern Europe, probably as a consequence of the expansion from the refugia during interglacial. The main colonization route originated from the Iberian Peninsula towards central and northern Europe. Contact zones with colonization events from Italy and the Balkans were detected respectively in northern Italy and central Europe. Genetic indices show the existence of moderate levels of genetic variability throughout Europe, although evidence of recent evolutionary bottlenecks was found in some populations. Estimation of migration rates and approximate Bayesian computations highlighted the most likely phylogeographical scenario for the current distribution of little owl populations.

Animal populations can vary in abundance and range distribution over time. Population changes can be driven by natural or competition events, although the impact of human activities has become decisive for the survivor of species in the last millennia, representing the main cause of habitat loss, isolation and fragmentation for many species and populations. In the last decades also global climate changes contributed to altered environmental conditions. Several ornithological studies have documented the effects of global changes on local population dynamics such as phenology (many bird species reproduce or arrive earlier to breeding habitats on high latitude breeding grounds (Jiguet et al. 2010), changes of distribution (Crick 2004), and altitudinal limits (Jiguet et al. 2010).

Population genetics is an useful tool to investigate population dynamics at present and in the past, inferring the occurrence of events such as bottlenecks, expansions, movements and related gene flow (Allendorf et al. 2012). Microsatellites represent the most popular and versatile marker type (Sunnucks 2000, Selkoe and Toonen 2006) for ecological applications. Besides individual identification,

parentage assignment and kinship analyses (Jones and Ardren 2003, Ringler 2012), or sex identification (Nesje and Roed 2000), microsatellites have been employed to investigate population structure (Procházka et al. 2011), genetic flow between different areas (Zink and Barrowclough 2008, Chaves et al. 2012), effective population size during bottleneck events (Barton and Wisely 2012), colonization, selection and drift processes (Estoup and Clegg 2003, Clegg and Phillimore 2010, Spurgin et al. 2014). Also mtDNA is often used to investigate the demographic changes or colonization events in wild populations (Selkoe and Toonen 2006, Fuchs et al. 2007). Although mtDNA and nDNA analyses sometimes lead to contrasting results (Brito 2005, 2007), the literature supports using a multigene approach in order to obtain reliable results from phylogeographic studies and produce complementary data that enable reconstructing a species phylogeographic history.

Several genetic and paleontological studies on plants and animals revealed the existence of three major glacial refugia in Europe during the Last Glacial Maximum: south Iberia, south Italy and south Greece (Taberlet et al. 1998, Cheddadi

et al. 2006, Sommer and Nadachowski 2006, Liepelt et al. 2009). Furthermore several extra-Mediterranean (Schmitt and Varga 2012) and north African refugia have been identified (Godoy et al. 2004, Habel et al. 2011). Postglacial colonization from southern refugia followed different patterns depending on dispersal behaviour which is influenced by the biology and ecology of the species, barriers in the landscape and geomorphology (Taberlet et al. 1998, Hewitt 2004, Gómez and Lunt 2007). Taxa expanding northward from a glacial refugia came into contact with taxa expanding from other glacial refugia in areas known as suture zones, characterized by genetic admixture (Hewitt 2011). Phylogenetic analyses have confirmed the existence of three major glacial refugia and suture zones for many European avian species, e.g. green woodpecker *Picus viridis* (Perktas et al. 2011, Pons et al. 2011), Savi's warbler *Locustella luscinioides* (Neto et al. 2012), the blue tit *Cyanistes* complex (Illera et al. 2011) and several other passerine bird species (Aliabadian et al. 2005). North Europe was recolonized from glacial refugia by bird species expanding from different refugia: from the Balkans (e.g. tawny owl *Strix aluco*, Brito 2005, 2007), from south Italy (e.g. dipper *Cinclus cinclus*, Hourlay et al. 2008), or from the Iberian Peninsula (e.g. little owl *Athene noctua*, Pellegrino et al. 2014). Despite historical isolation should have favoured population structuring, in some bird species molecular analysis showed no phylogeographic structure (Hung et al. 2013, Kraus et al. 2013, Perktas and Quintero 2013). This suggests that geographical barriers did not play the same role for all species and that dispersal behaviour could vary due to social as well as ecological factors.

The little owl is a small nocturnal species, with short distance dispersal (Cramp 1985, Van Nieuwenhuyse et al. 2008). It is distributed throughout the Palaearctic regions, from Iberia to China, in north Africa and Arabia (Cramp 1985). It is strongly associated with open agricultural landscapes. In northern and central Europe, populations size are rapidly declining likely due to environmental modification, habitat loss, degradation and pollution (Brink et al. 2003, Šálek and Schröpfer 2008, Šálek and Lövy 2012, Thorup et al. 2013). The species is listed as a 'SPEC 3' species (i.e. a species whose global populations are not concentrated in Europe, but which have an unfavourable conservation status in Europe; Tucker and Heath 1994). A previous study on the species using mtDNA markers (control region and COI) found four distinct European clades distributed respectively in the three main glacial refugia (Iberian, Italian and Balkan Peninsulas) and in Sardinia (Pellegrino et al. 2014), with contact zones in north Italy and in Hungary. The molecular analyses revealed the existence of a strong phylogeographic pattern in another European strigid, twany owl (Brito 2005, 2007), while no structure was observed in Tengmalm's owl *Aegolius funereus* (Broggi et al. 2013), snowy owl *Bubo scandiacus* (Marthinsen et al. 2009) and Ural owl *Strix uralensis* (Hausknecht et al. 2014). Tawny owl and little owl survived in the same three major refugia during the last ice age (south Italy, Balkans, and Iberian Peninsula), but they experienced different recolonization routes. Tawny owl expanded mainly from the Balkans and little owl mainly from the Iberian Peninsula. Contact zones between clades were found in Iberia and in France for tawny owl and in central Europe and north Italy for little owl.

In this study thirteen microsatellites loci were used with the aim: 1) to describe the genetic variability in European little owl; 2) to examine the structure of European little owl populations; 3) to obtain insights on past bottleneck events and gene flow; 4) to analyse admixture between clusters and 5) to compare the distribution of the present genotypes with the pattern of postglacial recolonization previously inferred from mtDNA (Pellegrino et al. 2014). Our results will yield an understanding of the genetic structure and level of genetic variation in this species that experience a decrease in population size in many European countries and can be used by wildlife managers for conservation and restoration plan purposes.

Methods

Sample collection and DNA extraction

A total of 333 samples were collected from 15 countries across western and central Europe (Fig. 1, Supplementary material Appendix 1, Table 1). Tissues (blood, plucked feathers, or muscle) were collected from natural history
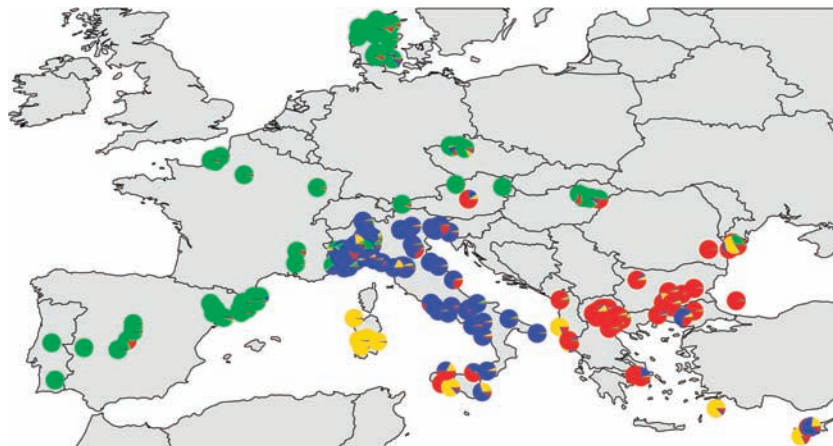

Figure 1. Sampling sites and genotype structure of individuals. Pie charts show the proportion of the four clusters calculated by the structure analysis ($K = 4$, same colours as Fig. 3) in each sampled population.

Table 1. Groups and population used in the analysis, including origin and number of individuals in each pool.

Group	Number of individuals	Population	Country	Number of individuals
Iberia	47			
		PT	Spain	18
		ES	Portugal	29
North central Europe	102			
		FR	France	16
		DK	Denmark	20
		CZ	Czech Republic	11
		AT	Austria	16
		HU	Hungary	19
		RO	Romania	20
Balkan	60			
		BG	Bulgaria	14
		MK	Macedonia	14
		MK	Albania	7
		GR	Greece	20
		GR	Cyprus	5
Italy	124			
		IT_S	South Italy	23
		IT_C	Central Italy	33
		IT_Sar	Sardinia island	12
		IT_N	North Italy	51
		IT_N	Switzerland	5

museums, recovery centres, road killed individuals, or during bird banding. Tissue and feathers samples were individually stored in ethanol at 20°C, blood samples were kept in Longmire Buffer (Longmire et al. 1997) and preserved at 2–4°C.

Tissue and blood genomic DNA was isolated using a NucleoSpin®Tissue kit (Macherey-Nagel, Düren, Germany). For feathers, the calamus was placed in a lysis buffer (FLB Macherey-Nagel, Düren, Germany, designed for small DNA quantities), exposed to thermal shock in liquid nitrogen and left for prelysis at 56°C overnight.

DNA amplification and genotyping

Each individual was genotyped at 13 polymorphic microsatellite loci (Supplementary material Appendix 2). We used the six most polymorphic primers, previously isolated in little owl (Aurelle 2010), while other seven primers were selected after testing for cross-amplification from those previously isolated in other Strigiform species (Thode et al. 2002, Hsu et al. 2003, Proudfoot et al. 2005, Burri et al. 2008).

We utilized the following protocol: (94°C × 5 min), 29–32 cycles at (94°C × 45 s) (TD°C × 45 s) (72°C × 45 s), and a final extension at 72°C for 7 min. We applied a 'touch-down' thermal protocol where the annealing temperature was lowered 0.5°C per cycle, starting from 60°C until a temperature of 55°C for Ath3-4-5-7-9-10 primers, and starting from 55°C until a temperature of 50°C for 15A6, Oe045, Oe085, FEP043, Oe053, Ta212 and Ta216 primers. Amplifications were performed in a Bio-Rad C1000 thermal cycler. Negative controls were included for both the extraction and amplification procedures. After PCR, loci tagged with two different labels were combined and run on an ABI-3130xl automated sequencer using rox350 size. Results were analysed in GeneMapper ver. 4.0 (Applied Biosystems).

Data analysis

In order to perform the analyses, individuals were gathered in four groups representing four European areas separated from geographical barriers (Table 1, column 1) and in 15 populations, (Table 1, column 3), corresponding to areas sampled. Adopted grouping is detailed for each analysis.

Allele frequencies, standard diversity indices, observed heterozygosity (H_O) and expected heterozygosity (H_E) for each locus and population were calculated using GenAlex ver. 6 (Peakall and Smouse 2006) and patterns of differentiation were visualized by a factorial correspondence analysis (FCA) of individual multilocus scores using Genetix 4.05 (Belkhir et al. 2004).

We investigated the occurrence of population structure calculating a discriminant analysis of principal components (DAPC) with Adegenet 1.2-8 (Jombart et al. 2010) package in R (R Core Team). This approach reduced genetic data to principal components to ensure that variables used in the discriminant analysis are uncorrelated. The inference of the most likely number of clusters was based on the Bayesian information criterion (BIC; Schwarz 1978). DAPC plot and Bayesian information criterion (BIC) graphs were obtained by retaining a number of principal components (PCs) representing 100% of the total variation of the sample set.

We investigated genetic structure also using multilocus genotypes and Bayesian clustering procedures. We used Structure 2.3 (Pritchard et al. 2000, Falush et al. 2003) to infer the number of K unknown populations (genetic clusters) in which the sampled multilocus genotypes could be subdivided. We run admixture model with correlated allele frequencies as suggested by Falush et al. (2003). Analyses were performed with $K = 1$–10, 50×10^5 iterations following a burn-in period of 50×10^4 iterations, and all simulations were independently replicated ten times for each K. In order to assess the best K value supported by the data, we used the LnP(D) and Evanno et al. (2005) methods in the software Structure Harvester 0.6.93 (Earl and von Holdt 2012). To align the cluster membership coefficients of the five structure runs and to display the results, we used CLUMPP ver. 1.1.2 (Jakobsson and Rosenberg 2007) and Distruct ver. 1.1 (Rosenberg 2003).

Population differentiation was tested between all population pairs and among all populations, at each locus and over all loci, using FSTAT 2.9.3.2. (Goudet 1996). Departures from Hardy–Weinberg equilibrium (HWE) at each locus and within each population, linkage disequilibrium for all pairwise combinations of loci and unbiased estimates of F_{ST} (Weir and Cockerham 1984) were computed, using exact tests, in Genepop 3.4 (Raymond and Rousset 1995, Rousset 2008) with Markov chain parameters left at the default settings (Raymond and Rousset 1995).

ARLEQUIN 3.5 (Excoffier and Lischer 2010) estimated the genetic variance within and between the 15 sampled populations through a hierarchical analysis of molecular variance (AMOVA, Excoffier et al. 1992), and the presence of

isolation-by-distance through a Mantel test, running 1000 bootstrap iterations. The geographic distance connecting samples was represented by Euclidean (linear geographic) distances computed in QGis (QGIS Development Team 2014).

We detected the occurrence of genetic bottlenecks using two different approaches. First, we applied the Wilcoxon's test to compute three mutational models in the program BOTTLENECK 1.2.02 (Cornuet and Luikart 1996): the infinite allele model (IAM, Maruyama and Fuerst 1985), the two phase model (TPM, Di Rienzo et al. 1994) and strict stepwise mutation model (SMM, Ohta and Kimura 1973). These methods detect departure from mutation–drift equilibrium as assessed by heterozygosity excess or deficiency, enabling detection of recent genetic bottlenecks. Then, we computed the M ratio test, using the software M_P_Val (Garza and Williamson 2001). The significance of an observed M value is determined by comparing it to a distribution of M values calculated from theoretical populations in mutation–drift equilibrium; the M_c was calculated using Critical_M (Garza and Williamson 2001). The analysis was run assuming a TPM (Di Rienzo et al. 1994) with proportion of one-step mutations, $p_s = 0.88$, average size of non one-step mutations, $\Delta g = 2.8$ and Theta $\theta = 10$ as suggested by Garza and Williamson (2001).

Identification of admixture areas and gene flow

Identification of contact zones, admixture areas and existence of gene flow was detected using STRUCTURE. Genotypes clustering with an individual membership value lower than 0.90 were considered admixed (Barilani et al. 2007). To identify samples showing discordance between nuclear and mitochondrial DNA, we compared the mtDNA control region haplotypes previously analyzed by Pellegrino et al. (2014), with genotypes resulting from the present study (data available for 269 out of 333 individuals). Migration rate was estimated using the Bayesian inference approach implemented in BayesAss 3.0.3 and 1.3 (Wilson and Rannala 2003). In keeping with the authors' recommendation, we

performed 5 independent runs of 3×10^6 iterations including a burn-in of 10%, and a sampling frequency of 2000 to ensure convergence of the MCMC. Delta values were varied for all parameters to obtain the acceptance rates between 40 and 60% of the total iterations (Wilson and Rannala 2003). Mixing and convergence of MCMCs were visually assessed using Tracer 1.6 (Rambaut et al. 2014).

Finally we used STRUCTURE, with prior population information model (USEPOPINFO = 1; MIGR-PRIOR = 0.05) under the admixture model with allele frequencies uncorrelated, to investigate the movement of individuals between predefined geographical populations. Approximate Bayesian computation was used to interpret the routes of recolonization from glacial refugia in DIYABC 2.0.3 (Cornuet et al. 2010). The four groups formerly identified (Iberia, central Europe, Balkan and Italy) were analyzed and a total of 3×6^{10} simulated dataset was generated using the default set mutation model of STRs. Population sizes were set as uniform and times of split and merge were $t3 > t2 > t1$. Three scenarios of recolonization of central Europe were tested: 1) recolonization from Iberia Peninsula; 2) recolonization from Balkans; 3) recolonization from both Iberia and Balkans.

Results

Genetic variability

All loci were polymorphic in every population. The number of alleles per locus ranged from a maximum of 23 in Oe045 locus to 4 in Ta212. Average number of alleles per locus varied from 4 in Sardinia to 7.8 in north Italy (Table 2). We found 26 private alleles in 12 populations, the number of private alleles per population ranging from zero in three different populations to four in the populations from Sardinia and north Italy.

Observed heterozygosity ranged from 0.454 in Austria to 0.699 in Czech Republic (mean 0.593), while expected heterozygosity varied from 0.507 in Sardinia to 0.688 in

Table 2. Genetic diversity in sampling populations. N = sample size, Ao = average number of alleles per locus, Ae = average number of effective alleles per locus, Ap = number of private alleles, Ar = allelic richness, Ho = observed heterozygosity, He = expected heterozygosity, F = fixation index. Standard errors in brackets.

Pop	N	Ao	Ae	Ap	Ar	Ho	He	F
PT	18	4.923 (0.525)	3.152 (0.326)	1	4.502	0.585 (0.074)	0.605 (0.067)	0.024 (0.058)
ES	29	6.000 (0.588)	3.382 (0.401)	0	4.746	0.537 (0.076)	0.606 (0.074)	0.120 (0.060)
FR	16	5.077 (0.593)	3.373 (0.450)	3	4.693	0.587 (0.081)	0.625 (0.054)	0.078 (0.082)
DK	20	4.923 (0.537)	3.151 (0.344)	3	4.410	0.592 (0.066)	0.618 (0.053)	0.042 (0.059)
CZ	11	5.077 (0.560)	3.478 (0.373)	1	5.077	0.699 (0.069)	0.653 (0.050)	–0.050 (0.078)
AT	16	4.462 (0.386)	2.378 (0.240)	3	4.077	0.454 (0.067)	0.516 (0.055)	0.178 (0.089)
HU	19	5.846 (0.750)	3.789 (0.515)	1	5.195	0.683 (0.071)	0.647 (0.063)	–0.062 (0.044)
RO	20	5.615 (0.626)	3.509 (0.422)	0	4.984	0.563 (0.070)	0.621 (0.071)	0.079 (0.036)
BG	14	5.154 (0.541)	3.077 (0.434)	1	4.845	0.588 (0.088)	0.571 (0.070)	–0.013 (0.062)
MK	21	6.000 (0.784)	3.721 (0.504)	2	5.115	0.616 (0.084)	0.613 (0.083)	–0.018 (0.035)
GR	25	6.154 (0.831)	4.019 (0.597)	2	5.307	0.587 (0.088)	0.620 (0.084)	0.109 (0.059)
IT_S	23	6.231 (0.907)	4.023 (0.516)	0	5.371	0.579 (0.076)	0.656 (0.072)	0.142 (0.049)
IT_C	33	7.000 (0.906)	4.254 (0.582)	1	5.431	0.619 (0.066)	0.684 (0.060)	0.102 (0.044)
IT_Sar	12	4.000 (0.543)	2.720 (0.377)	4	3.959	0.550 (0.092)	0.507 (0.080)	–0.081 (0.046)
IT_N	56	7.769 (1.014)	4.584 (0.685)	4	5.825	0.650 (0.069)	0.688 (0.066)	0.086 (0.037)
Total mean	333	5.615 (0.186)	3.507 (0.122)	–	6.202	0.593 (0.019)	0.615 (0.017)	0.049 (0.015)

northern Italy (mean 0.615). Estimates of Wright's fixation index (F_{IS}) revealed that populations from Austria, Denmark, France, Italy, Romania, Greece and Spain had significantly greater than zero values, indicating widespread departure from HWE (Table 2), while those from Bulgaria, Hungary, Sardinia, Macedonia, Portugal and Czech Republic were in Hardy–Weinberg equilibrium. No evidence for linkage disequilibrium was found between loci and populations (after Bonferroni correction).

Genetic structure

Results of a discriminant analysis of principal components (DAPC) showed that the first three DA eigenvalues and the first 100 retained PCs explained the observed variance (Supplementary material Appendix 4). Bayesian information criterion (BIC) found three-five clusters best representing the genetic subdivision of the dataset. Clusters at $K = 3$ (data not shown) grouped respectively: 1) Spain, Portugal, France, Denmark, and Austria, 2) Italy and Sardinia, 3) Balkan regions. At $K = 4$ (Fig. 2a) Austrian samples grouped separately in a cluster and at $K = 5$ (Fig. 2b) Sardinian and some Italian individuals split from Italy. A DAPC scatterplot of the first two principal components (Fig. 2c) showed three distinct groups, the first including Sardinia and Italy; the second Spain, Portugal, France, Denmark, and Austria; the third all the Balkan populations (Romania, Bulgaria, Greece, and Macedonia) and Czech Republic. Hungary was plotted between the Balkans and north-western European populations. The scatterplot (Fig. 2d) of the second and third principal component axis evidenced the separation of Sardinia. The FCA plotting of individual genotypes supported the same clear separation between the four little owl groups (figure not shown).

Results from STRUCTURE supported the presence of 3 genetic clusters (greater ΔK, Supplementary material Appendix 4). We explored the individuals assignment from $K = 2$ to $K = 5$ in order to examine the sequential splits. At $K = 2$ (Fig. 3a) the first cluster included individuals from Spain, Portugal, France, Denmark, Austria, and Czech Republic, the second included the samples from Italy, Sardinia, Switzerland, Greece, Romania, Macedonia, and Bulgaria (Fig. 3a). At $K = 3$ (Fig. 3b), this second cluster was split in two groups, and the individuals from Greece, Bulgaria, and Romania were separated from Italian, Sardinian and Swiss individuals. At $K = 4$ (Fig. 3c and Fig. 1), Sardinian
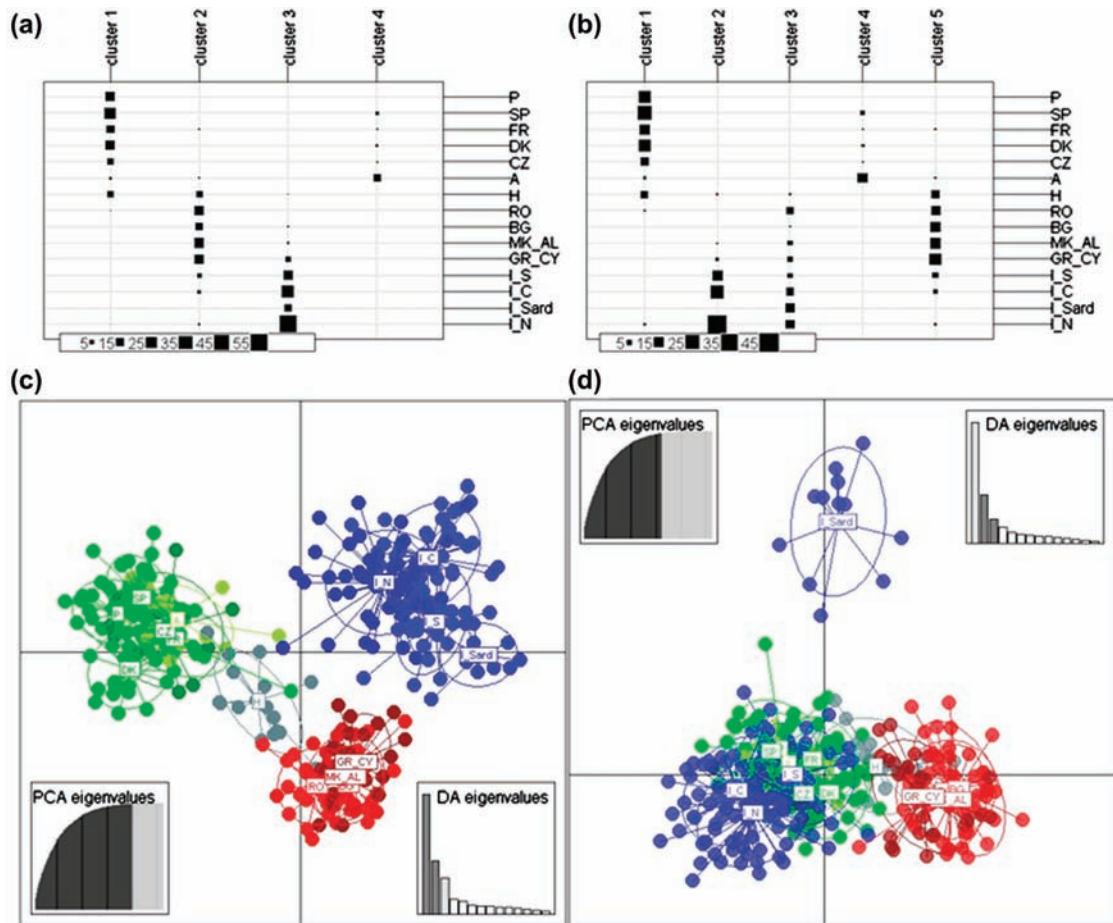

Figure 2. Results from multivariate analyses DAPC for populations of little owl. (a) and (b) individuals assignment to each identified cluster $K = 4$ and $K = 5$; (c) and (d) scatterplot of the multivariate analysis DAPC for populations of little owl. Populations (groups) are displayed by different colours and inertia ellipses. Dots represent individuals. (c) First two principal components of the DAPC. (d) DAPC axes 1 and 3.
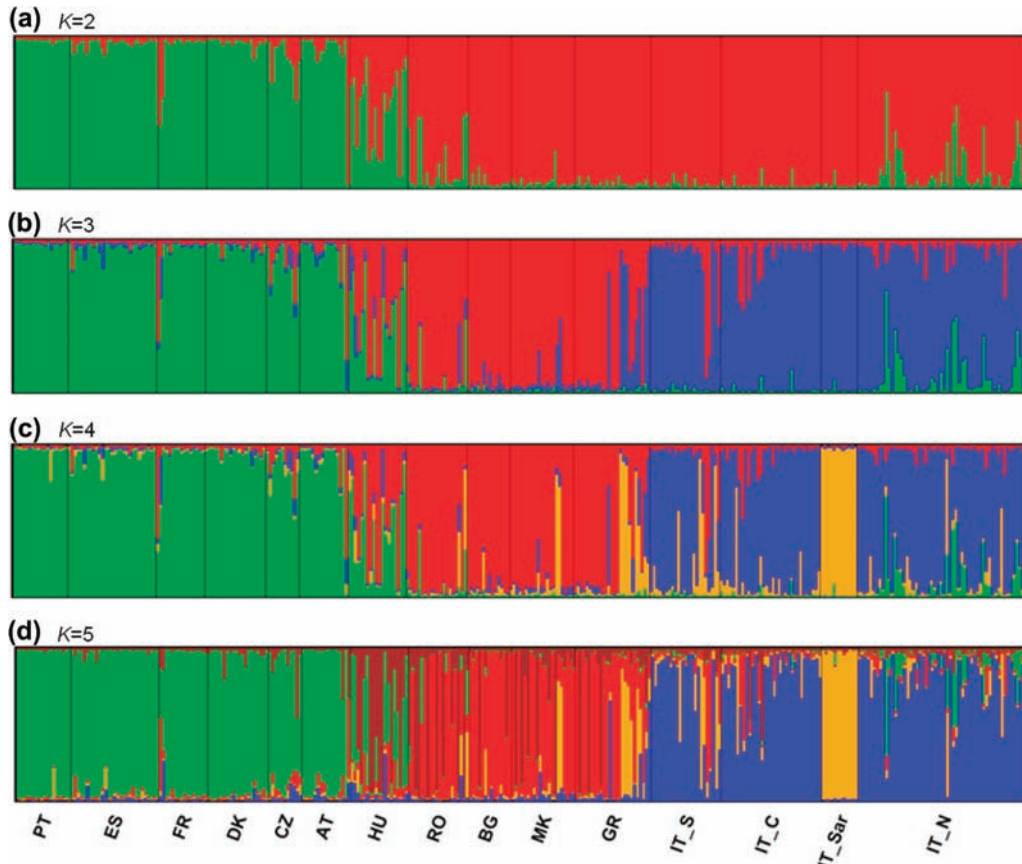
Figure 3. Individual assignment probabilities for $K = 2$ (a), $K = 3$ (b), $K = 4$ (c), and $K = 5$ (d) found by structure analyses and computed in CLUMPP from 5 independent runs. Each vertical line represents one individual and shows its inferred cluster membership. Each colour represents a single genetic cluster. Colour legend: green: western cluster; red: Balkan cluster; blue: south Italian cluster; yellow: Sardinian cluster.

individuals were clustered in a separate group. Considering $K = 5$ (Fig. 3d) we noted that some individuals were assigned to the fifth cluster, distinct from the Sardinian, and without a significant geographic structure.

Population differentiation and analysis of population variance

AMOVA analysis revealed that 81.6% of the total genetic variance was distributed within individuals, while 10.2 and 8.2% was distributed respectively among populations and individuals within populations. These values agree with the results obtained analysing the samples in four groups (Table 3).

Among populations F_{ST}-values were mostly significant (average value = 0.156; Supplementary material Appendix 3). Lowest and highest differentiations were found respectively within and between the main genetic groups (Balkans, north-western Europe, Sardinia, and Italy). The largest differentiation was found between Denmark and Sardinia, while minimum differentiation was found between north east Spain and Portugal.

In the Sardinia population, allelic richness, the mean number of alleles per polymorphic locus, and heterozygosity observed were similar to those found in other populations (Table 2); thus there is no strong evidence of founder effect.

Identification of admixture areas and gene flow

Individuals were plotted on a geographic map and coloured differently according to the cluster assignment (Fig. 1). Birds from Hungary always showed admixed genotypes: 49.4% were assigned to the Balkans cluster and 42.6% to

Table 3. Results from analysis of molecular variance (AMOVA) for different grouping methods.

	15 populations			4 groups		
Source of variation	Sum of squares	Variance components	Percentage variation	Sum of squares	Variance components	Percentage variation
Among populations	358.09	0.480	10.17	196.79	0.382	7.98
Among individuals within populations	1470.53	0.387	8.21	1631.84	0.555	11.60
Within individuals	1282.00	3.850	81.62	1282.00	3.850	80.43
Total	3110.63	4.717	100	3110.63	4.787	100

the NW European cluster. At $K=2$, Swiss and north Italian samples showed clear admixture between Balkan and north-west European clusters. At $K=3$ and $K=4$, the same genotypes were shared between the Italian and north-west European clusters. Moreover, at $K=3$ and $K=4$ the samples from central and south Italy were associated to both Balkan and NW European clusters. Outside of Sardinia, the highest percentage of membership to the Sardinian cluster (q_i ranging from 0.74 and 0.89%) was detected in two individuals from south Italy (Sicily), two from Albania, and two from Greece.

Comparison between nuclear and mtDNA (Pellegrino et al. 2014) showed discordance in 54 Italian samples previously ascribed by mtDNA to the Balkan haplogroup: 47 samples were assigned by microsatellites to the Italian cluster and seven showed Italian, Balkan, Sardinian, NW European or admixed genotypes (Fig. 1). Discordance was observed also in eight samples with the south Italian haplotype (seven from Italy and one from Switzerland) that showed Sardinian, Balkan or mixed genotypes. Five individuals from north Italy belonging to the NW European haplogroup were assigned by nuclear DNA to the south Italian cluster. We detected no discordance between nuclear and mtDNA for samples collected in Sardinia. All samples from France, Spain, Portugal, Denmark, and Czech Republic (except one) showed concordance between haplotypes and genotypes. Some samples from Hungary, one from Austria, Romania, and Czech

Republic showed discordance between nuclear and mtDNA data, with admixed or NW Europe genotypes.

BayesAss allowed the estimation of recent migration events. Inferred mean non-migration rate was 0.75 (95% confidence intervals: 0.68–0.99). Migration rate values indicated the presence of gene flow from: 1) south and central Italy to north Italy; 2) Spain and France to Portugal; 3) Czech Republic to Denmark; 4) Bulgaria and Romania to Macedonia.

Results from STRUCTURE migrants analysis partly complied with those obtained by BayesAss; structure detected low proportion of migrants (4.2–6.9%) between Portugal and Spain, and also revealed gene flow between Czech Republic, Austria, Hungary, and Macedonia. These data confirmed the absence of gene flow between Balkan and Iberian areas.

Post-probabilities detected with the logistic approach in DIYABC revealed that central Europe was recolonized from both western and eastern Europe, although the post probability is smoothly higher in the first scenario (recolonization from Iberian peninsula) than in the second (Fig. 4). Post probability values were 0.05 [0.04–0.05] for the first scenario, 0.02 [0.02–0.03] for the second and 0.93 [0.93–0.94] for the third.

Isolation by distance and recent bottleneck events

The Mantel test on geographic and genetic distances yielded a significant correlation coefficient between geographic and genetic distances ($r_{xy}=0.271$, p $<$ 0.01).
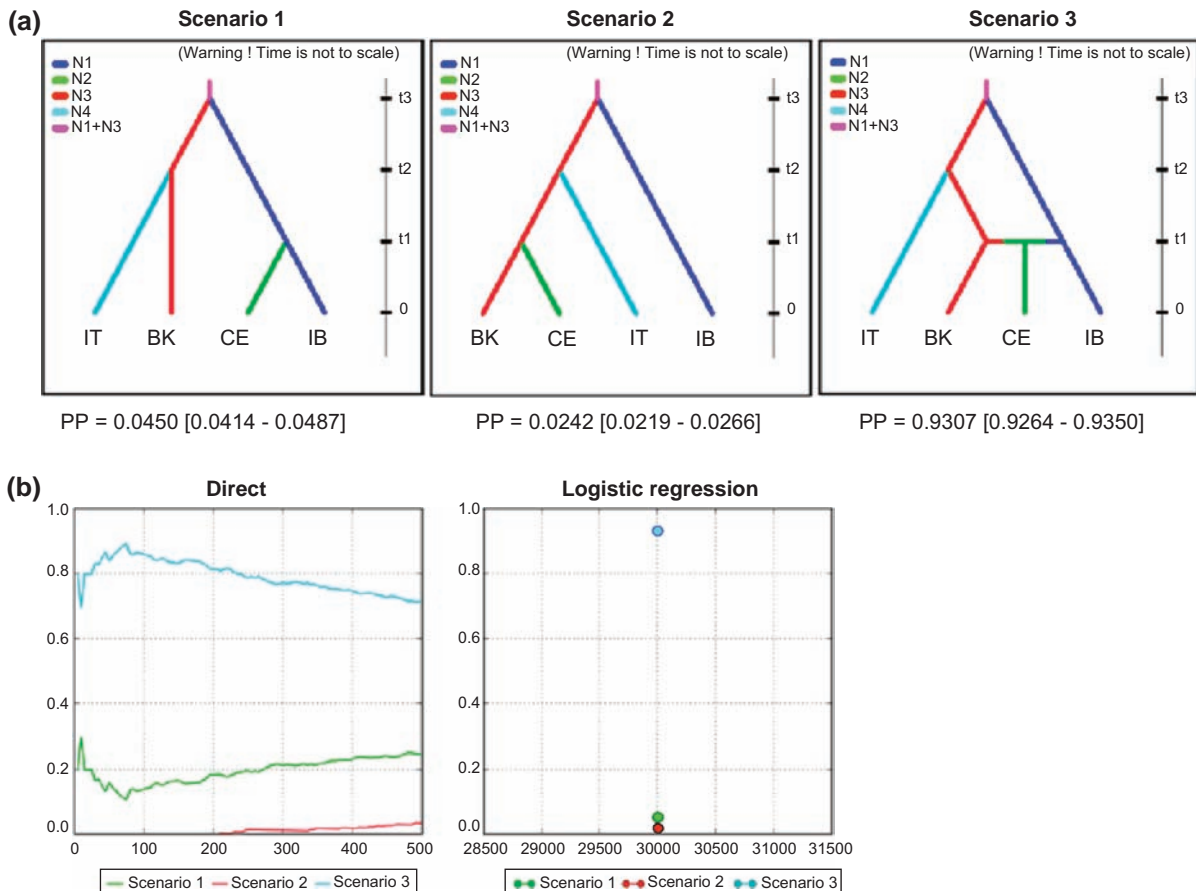

Figure 4. (a) Scenarios explored in DIYABC. In the first, colonization of central Europe is from Iberian Peninsula, in the second, from Balkans region, in the third, from both the glacial refugia. PP = posterior probabilities for each scenario. (b) Direct and logistic regression plots. Posterior probabilities are reported on the y axis.

Table 4. Bottleneck and M-Ratio results in little owl. Values are shown for each population and for four groups. Listed by column are p-values (TPM, IAM, SMM), M-ratio and MC for population bottleneck testing. Significant p-value are reported in bold.

	Sampling regions	IAM p-values	TPM p-values	SMM p-values	M-ratio	M_C
Populations	Portugal	0.0522	0.2661	0.9097	0.91	0.489
	Spain	0.08771	0.8501	**0.0167**	0.78	0.53
	France	**0.0012**	0.0681	0.4973	0.85	0.47
	Denmark	**0.0006**	**0.0327**	0.8394	0.88	0.50
	Czech Republic	0.0681	0.1677	0.6848	0.87	0.43
	Austria	0.6848	0.0803	**0.0040**	0.71	0.47
	Hungary	**0.0012**	0.0574	0.4973	0.71	0.49
	Romania	0.0681	0.5417	0.2439	0.76	0.50
	Bulgaria	0.5693	0.3013	**0.0342**	0.81	0.46
	Macedonia	**0.0134**	0.1763	0.3394	0.77	0.51
	Greece	0.0342	0.0923	0.7334	0.89	0.52
	South Italy	**0.0007**	**0.0134**	0.9097	0.69	0.51
	Central Italy	**0.0012**	0.0681	0.1698	**0.54**	0.55
	Sardinia	0.2334	0.5185	0.6772	0.84	0.44
	North Italy	**0.0067**	0.0574	0.7513	0.83	0.59
Groups	Iberian Peninsula	**0.0081**	0.6221	0.1099	**0.17**	0.62
	N-C Europe	**0.0002**	0.0803	**0.0052**	0.68	0.64
	Balkans	0.0640	0.9697	0.0342	0.74	0.60
	Italy	**0.0017**	0.1099	0.0681	**0.49**	0.65

Bottleneck events were tested both under the assumptions of IAM, TPM and SMM by two-tailed Wilcoxon test for heterozygote excess or deficiency. Evidence for genetic bottlenecks was found by IAM in seven populations (p < 0.01): north, central and south Italy, Hungary, Macedonia, France, Denmark. Deficiency was detected under TPM in populations from south Italy and Denmark. SMM provided evidence of bottleneck events in Spain, Austria, Bulgaria, and in the north central Europe group (Table 4).

The observed M-ratio values was lower than the commonly used bottleneck threshold (0.68; Garza and Williamson 2001) ranging from 0.43 (Czech Republic) to 0.59 (north Italy). All the populations, except for central Italy, had an upper 95% CI above the threshold, indicating a lack of severe bottleneck events. Central Italy showed M = 0.54 and Mc = 0.55 (Table 4). When we analyzed the samples subdivided in the four groups, we found that the Iberian group showed a low M-ratio (0.17).

Discussion

Genetic variability

Results obtained in this study show moderate levels of genetic variation in the European population of little owl despite the fact that the species has experienced a significant decline in population sizes across Europe. The average heterozygosity was similar to those reported in two other European strigiformes: from 0.57 to 0.70 in tawny owl (Brito 2007) and from 0.59 to 0.71 in barn owl *Tyto alba* (Antoniazza et al. 2010). Comparable values were found also for some Palearctic birds of prey, such as the white-tailed sea eagle *Halieetus albicilla* (Honnen et al. 2010) and griffon vulture *Gyps fulvus* (Le Gouar et al. 2008).

In our study, the highest heterozygosities were found in populations from Czech Republic, Hungary and north Italy, corresponding to the contact areas of haplogroups described by Pellegrino et al. (2014). A similar result was also found in tawny owl, where populations sampled close to postglacial contact zones showed the highest values (Brito 2007). The lowest heterozygosity values were observed in little owls from Austria, but here we cannot rule out that sampling may have affected the result as samples from Austria were collected from a single locality. The highest number of alleles was found in central and north Italy, corresponding to areas in which Italian, Iberian and Balkan haplogroups are present (Pellegrino et al. 2014).

Refugial populations should have a higher genetic diversity than populations established after the most recent glacial cycle (Hewitt 2000). However, as in tawny owl (Brito 2007), we did not find a pattern of decreasing genetic diversity with latitude. This could be related to a mixture of genomes in northern areas, possibly due to a secondary contact between populations originating from different southern refugia (Hewitt 2001).

Genetic structure

Our data revealed the existence of a genetic structure, although a different number of genetic groups was retrieved when different statistical approaches were applied. DAPC identified five clusters while STRUCTURE only identified three. These retrieving are not necessary in contradiction because the two genetic approaches estimate the genetic structure at different levels: STRUCTURE identifies the highest-level of the genetic structure, while DAPC scrutinizes the finest level of genetic structure (Jombart et al. 2010).

The identification of five groups in DAPC may be influenced by the presence of an Austrian group. However, this may not be due to a real genetic difference of this population, since samples were collected in the same locality and their allele frequencies could have affected the estimation method. Conversely, the identification of four clusters supported the phylogeographic structure previously found by Pellegrino et al. (2014). At $K = 4$ populations were divided

in four main geographical groups: 1) the NW Europe cluster, ranging from Portugal to Austria; 2) the Italian cluster, comprising continental Italy and Switzerland; 3) the Sardinian cluster; and 4) the Balkan cluster, ranging from Cyprus to Czech Republic. These four clusters included Sardinia and the Iberian, Italian, and Balkan refugia. Several studies on the phylogeography of European species have described the same three major glacial refugia in plants (Médail and Diadema 2009, Tzedakis et al. 2013) and animal species (Joger et al. 2007, Lehtonen et al. 2009).

In this study, the postglacial colonization of central Europe originated both from Iberian Peninsula and Balkans, although a main contribution of western Europe was detected. This pattern was exemplified by Taberlet et al. (1998) on the brown bear *Ursus arctos*, and similar patterns was found in other vertebrate (Hewitt 1999, Ruedi and Castella 2003, Michaux et al. 2004) and invertebrate species (Habel et al. 2005, 2009). In Strigiformes, a study on tawny owl highlighted the same three major refugia (Balkans, south Italy and Iberian Peninsula) but, unlike little owl, the postglacial colonization of north Europe originated from Balkan areas (Brito 2005, 2007). Postglacial expansion patterns may have been influenced by elevation and relief, palaeoecological conditions after the last glacial maximum, and different habitat preferences (Schmitt 2007). Indeed, the little owl breeds in open areas while tawny owl prefers wooden areas (Cramp 1985, Šálek and Lövy 2012). However, proof that the postglacial expansion of the two owls mirrored the expansion of forest and open areas is still lacking.

The Pyrenees, Alps and Scandinavian mountains often acted as barriers or suture zones in Europe (Hewitt 2000). Our data suggest that the Alps represented the main obstacle to the expansion of little owl. This barrier probably prevented the diffusion of Italian genomes out of the Peninsula and strongly limited the penetration of NW European genotypes from the west. The genetic flow between Italy and Dalmatia seems currently hampered by the Julian and Dinaric Alps, but gene flow could have occurred more easily along the Adriatic coast during the recent glacial maximum, when the Adriatic sea receded several hundred kilometres southwards (Zonneveld 1996, Taberlet et al. 1998). The current geographical analysis of microsatellite data agrees with previous findings based on mtDNA (Pellegrino et al. 2014), the two markers showing a concordant geographical pattern.

Admixed and contact zones

Postglacial expansion from refugia can lead populations that diverged in distant geographical areas to meet and merge. The encounter can generate a genetic admixture that can be detected in contact zones (Hewitt 2001, 2011). In little owl, we found several hybrid individuals in Hungary, north-central Italy, and some coastal Mediterranean localities. In particular, Hungarian samples showed mixed NW Europe and Balkan genotypes and occupied an intermediate position between these two main groups in the multivariate analysis plot. Mitochondrial DNA confirmed the pattern of admixture that was found by microsatellite analysis. Future sampling of neighbouring areas would be useful to shed light on the extent of the contact zone between NW European and Balkan genomes, and to test whether the expansion of

NW European genomes towards the Balkan Peninsula and vice versa could be hampered by ecological selection pressures such as habitat differences (Hewitt 2001, Dorken and Pannell 2007, Antoniazza et al. 2010).

In Italy, nuclear and mitochondrial DNA described different genetic compositions. Particularly, in northern and central Italy 56% of the individuals assigned by nuclear DNA to the Italian cluster showed Balkan mtDNA. This discrepancy could be due to differential movements in cold and warm ages. Colder periods probably contributed to the expansion of Balkan individuals into northern and central Italy, while warmer ages contributed to a northward expansion from southern Italy. As suggested by various authors (Barton 1993, Chan and Levin 2005), asymmetrical mating could have produced an asymmetrical mtDNA introgression. A similar discordance between mtDNA and nuclear data was found in tawny owl in northern Italy by Brito (2007), and in hares *Lepus* sp. in Spain by Melo-Ferreira et al. (2009). The contact zone between Italian and Balkan genomes may be wider than we report here, and perhaps extends through Slovenia and Croatia. More detailed and focused samples collected across Adriatic areas are needed for characterizing the genetic structure in this contact area.

Considering other European species a similar contact zone located in north Italy was found in the pine marten *Martes martes* (Ruiz-González et al. 2013), while in the dipper *Cinclus cinclus* a contact zone ranging from Luxemburg to Hungary was detected (Hourlay et al. 2008). A focused sampling on German populations of little owl may reveal a similar pattern also for our study species. In other European Strigiformes, clear-cut hybrid zones were absent because genetic structure was lacking (Marthinsen et al. 2009, Broggi et al. 2013, Hausknecht et al. 2014). In tawny owls different pathways resulted in different contact zones, and maximum admixture was found on the Iberian Peninsula (Brito 2007).

Sardinian genotypes were clearly associated with a single cluster, suggesting an isolation process that prevented gene flow with the continent. However, traces of Sardinian and Italian genotypes were found in some distant Mediterranean areas (Fig. 3). There are several non-exclusive explanations for the clustering of these individuals in the Sardinian genetic group. Sardinian genotypes could have originated from a Mediterranean population located in an area that was not sampled in this study. An alternative explanation involves ancient human translocations. It is well known that several species inhabiting Sardinia were introduced to the island during historical times (Vigne 1992, Scandura et al. 2009). Little owl could have been introduced by hunters: the species was traditionally employed as live decoy to catch small birds in Italy since Roman times (Bianchi Bandinelli 1970). Alternatively, ancient human-mediated translocation could have occurred because little owl was considered a symbol of protection and victory in Athens and Roman myths (Van Nieuwenhuyse et al. 2008). Diodorus reported that in 301 BC. Agathocles released many little owls to galvanize his troops before defeating the Carthaginians (Tillyard 1908). Finally, the presence of Sardinian genotypes in other Mediterranean areas could be related to occasional long-distance movements that could have contributed to shared genetic pools.

Bottleneck events and gene flow

The population bottleneck analysis revealed a deficiency of heterozygosity in 7 populations out of 15 under the IAM (France, Denmark, Hungary, Macedonia, north, central and south Italy, no Sardinia). The TPM detected deficiency in 2 populations only (S-Italy and Denmark) and SMM revealed evidence of bottlenecks in Spain, Austria, and Bulgaria. Generally we did not find evidence of bottlenecks in populations that are currently decreasing in size or that encountered a severe reduction in last few decades (i.e. Czech Republic, Šálek and Schröpfer 2008, Šálek 2014; Netherlands, Gouar et al. 2011; Switzerland, Juillard 1989; Poland, Żmihorski et al. 2009). In Denmark, where the little owl is also facing a strong decline (Thorup et al. 2013), a recent study did not reveal evidence of bottlenecks under TPM while IAM showed evidence of bottlenecks (Pertoldi et al. 2012). The discrepancy between models could be related to the different ability of the mutation models to detect bottleneck events. Empirical data suggest that the SMM is the most appropriate model for microsatellite loci with 3- to 5-bp repeats, while IAM is more suitable for shorter repeats (Cornuet and Luikart 1996).

Despite the evidence for recent bottlenecks found by BOTTLENECK analyses, no population size reduction events were detected, using M-ratio tests, with the exception of the owl population in central Italy. These tests provided evidence of population decline over different time scales. M-ratio may not detect recent population size reductions because populations recently in decline will not have had time to recover from the genetic signatures associated with bottleneck detection methods (Garza and Williamson 2001).

The Mantel test on geographic and genetic distances yielded a significant correlation, indicating a relationship between geographic and genetic distances. This finding is in line with the typical pattern of isolation by distance, where individuals that are geographically close tend to be genetically more similar than individuals that are far apart (Meirmans 2012).

Bayesian analysis on migration rates showed high non-migration rates. This is expected in a sedentary species like the little owl (Cramp 1985, Van Nieuwenhuyse et al. 2008, Abadi et al. 2010, Newton 2010). Gene flow may be related to accidental movements, juvenile dispersal or unknown migratory movements as suggested by Holroyd and Trefry (2011). However some authors demonstrated that this species tends to make only short distance dispersal (Schaub et al. 2006, Sunde et al. 2009). Italian areas showed low dispersal rates and the flows were limited to populations within the Italian Peninsula. There was no recent emigration from Italy and Sardinia toward other European regions.

F_{ST} values showed that most populations differ from each other, while we detected no significant F_{ST} values between neighbouring populations (i.e. Bulgaria-Macedonia and Hungary-Austria). This high differentiation supports a strong phylogeographic structure and a gene flow limited to neighbouring areas only. Our results also confirm that the maximum dispersal distances of the little owl do not exceed a few hundred kilometres, as suggested from bird ringing data (Spina and Volponi 2008, Van Nieuwenhuyse et al. 2008, Holroyd and Trefry 2011). Our result is likely linked to the short distance dispersal for this species. A similar pattern was found in another European sedentary species like the rock ptarmigan *Lagopus lagopus* (Bech et al. 2009).

Systematic evaluations

Our data on microsatellites and a previous study on mtDNA (Pellegrino et al. 2014) clearly identified four separate European genetic groups of little owl. These findings can be compared to the subspecific distribution proposed by different ornithologists (Vaurie 1960, Cramp 1985, del Hoyo et al. 1999) for European subspecies: *A. n. noctua* in Italy and central Europe; *A. n. vidalii* in western and NW Europe, and *A. n. indigena* in the Balkan regions, south Russia, Caucasus, south-west Siberia, Turkey and Middle East. Genetic data were thus somewhat at odds with morphology-based subspecies taxonomy and phylogeographic distribution. In north west and central Europe we detected no trace of south Italian genomes, and we found a contact zone between three main genetic groups in northern Italy that was not described in the literature. Indeed, little owl subspecies are difficult to distinguish using classic plumage and biometric criteria because geographical variation is small and there is a clear effect of habitat on plumage colour (Cramp 1985). Available data on vocal features are of little help for subspecies identification: this species is difficult to study due to a high variety of vocalizations (22 different calls, Exo and Scherzinger 1989). Although differences in calls and songs between certain populations have been documented (Exo 1990, Hardouin et al. 2006), a complete picture of the vocal characteristics of the various potential subspecies is still lacking. More studies on plumage, biometric, and vocal characteristics are needed to establish clear and reliable criteria for subspecific identification. The subspecies *A. n. sarda* was described for Sardinia, but the validity is debated (del Hoyo et al. 1999, König et al. 2008). Our results do not disprove the validity of this subspecies. To confirm the taxonomic status it would be worth broadening the genetic analyses to include a larger number of Sardinian samples and individuals from Corsica, where the species has begun to breed recently (Yeatman-Berthelot and Jarry 1995). Future studies should investigate genetic differentiation in a wider area embracing the entire distributional range of the species with particular attention to Mediterranean north Africa in order to complete the knowledge of genetic diversity, especially in not yet sampled declining populations. This is an important step for effective management efforts and species conservation.

Conclusions

Our findings showed a strong genetic structure and no evidence of genetic depletion in European little owl populations, although in several countries the species is currently declining. The genetic data presented here will be useful for restocking programs of endangered populations: stable populations, as those in Italy, Portugal, Greece or Romania could in the future be a source of individuals. Genetic characterization provides useful information in order to identify populations that are genetically suitable to prevent negative genetic consequences in endangered populations and in species management (Crandall et al. 2000). The strong genetic structure and diversity of European little owls suggest that

their management units should encompass areas of limited extension, and prospective restocking programs should be set up from nearby stable populations. Many reintroduction programs are based on birds bred in captivity and it is important that these birds are of local origin.

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Supplementary material (Appendix JAV-00679 at <www.avianbiology.org/readers/appendix>). Appendix 1–4.