Articles

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The role of western Mediterranean islands in the evolutionary diversification of the spotted flycatcher *Muscicapa striata*, a long-distance migratory passerine species

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We investigated the evolutionary history of the spotted flycatcher *Muscicapa striata*, a long distance migratory passerine having a widespread range, using mitochondrial markers and nuclear introns. Our mitochondrial results reveal the existence of one insular lineage restricted to the western Mediterranean islands (Balearics, Corsica, Sardinia) and possibly to the Tyrrhenian coast of Italy that diverged from the mainland lineages around 1 Mya. Mitochondrial genetic distance between insular and mainland lineages is around 3.5%. Limited levels of shared nuclear alleles among insular and mainland populations further support the genetic distinctiveness of insular spotted flycatchers with respect to their mainland counterparts. Moreover, lack of mitochondrial haplotypes sharing between Balearic birds (*M. s. balearica*) and Corso-Sardinian birds (*M. s. tyrrhenica*) suggest the absence of recent matrilineal gene flow between these two insular subspecies. Accordingly, we suggest that insular spotted flycatchers could be treated as one polytypic species (*Muscicapa tyrrhenica*) that differs from *M. striata* in morphology, migration, mitochondrial and nuclear DNA and comprises two subspecies (the nominate and *M. t. balearica*) that diverged recently phenotypically and in mitochondrial DNA and but still share the same nuclear alleles.

This study provides an interesting case-study illustrating the crucial role of western Mediterranean islands in the evolution of a passerine showing high dispersal capabilities. Our genetic results highlight the role of glacial refugia of these islands that allowed initial allopatric divergence of insular populations. We hypothesize that differences in migratory and breeding phenology may prevent any current gene flow between insular and mainland populations of the spotted flycatcher that temporarily share the same insular habitats during the spring migration.

The western Mediterranean islands (Balearics, Corsica, Sardinia) lie a few tens or hundreds kilometers from the nearby mainland (Spain, France, Italy, northern Africa) from which they were isolated since the end of the Messinian salinity crisis (5.3 Mya) (Maldonado 1985, Bover et al. 2008) or intermittently connected for very short periods of time (Corsica, Sardinia; Ketmaier and Caccone 2013).

Variations in the level of endemism achieved by organisms on western Mediterranean islands may be explained by several ecological factors among which the probability of colonization is of major importance (Blondel and Aronson 1999, Voelker and Light 2011). Pre-Pleistocene endemics have been documented on these islands for many unrelated organisms (e.g. plants, invertebrates, non-flying vertebrates) with low potential for long distance over-sea dispersal (Pereira and Salotti 2002, Ketmaier and Caccone 2013, Jeanmonod et al. 2015, Salvi et al. 2015).

In the case of animals showing higher over-sea dispersal abilities (e.g. bats, birds), repeated colonization events of an island close to the mainland are much more probable than

for terrestrial organisms (e.g. reptiles, amphibians). Consequently, the level of endemism achieved by these flying organisms is generally lower in these continental islands (that was once connected to the mainland; Blondel and Aronson 1999, Newton 2003). For instance, specific endemism for passerine birds in western Mediterranean islands, has already been documented for a limited number of taxa including the Corsican nuthatch *Sitta whiteheadii* (Pasquet et al. 2014), the Corsican finch *Carduelis corsicana* (Förschler et al. 2009 but see Pasquet and Thibault 1997) and three *Sylvia* warblers: the Marmora's warbler *Sylvia sarda* (Corsica and Sardinia; Aymí and Gargallo 2006); the Balearic warbler *Sylvia balearica* (Aymí and Gargallo 2006, Voelker and Light 2011) and the Moltoni's warbler *Sylvia subalpina* (Corsica, Sardinia, Brambilla et al. 2008). All these species are sedentary except for the Moltoni's warbler which winters in sub-Saharan Africa. In contrast to this low endemism at the species level, several endemic subspecies have been recognized in the western Mediterranean islands, especially in Corsica and to a lesser extent in the Balearics (Pons and Palmer 1996, Prodon et al. 2002, de Juana and Garcia 2015). Among passerine birds, most of the Corsican and Balearic endemic subspecies described to date are not long-distance migrants but forest birds with sedentary or short-distance migratory habits (Prodon et al. 2002, de Juana and Garcia 2015, Pons et al. 2015).

The present study aims to evaluate the importance of Corsica, Sardinia and the Balearic islands in the evolutionary history and the present day genetic structure of the spotted flycatcher *Muscicapa striata*, a widespread Palaearctic species with conserved morphology. The spotted flycatcher is a common long-distance migratory species wintering throughout sub-Saharan Africa. The breeding distribution of the species encompasses a very large longitudinal range, from the British Isles to Mongolia (Cramp and Perrins 1993, Aymí and Gargallo 2006). It is commoner in northern than in southern Europe, except on main western Mediterranean islands where the species is abundant, especially in towns and villages (Hagemeijer and Blair 1997). The European breeding population, estimated at 14–22 million pairs (BirdLife International 2015), has declined by roughly 40% over the last 30 yr (Vickery et al. 2014).

Two insular subspecies have been described in the Mediterranean basin. *Muscicapa s. tyrrhenica* breeds in Corsica, Sardinia and the Tuscan archipelago (Viganò and Corso 2015) whereas its possible presence along a narrowband of the Tyrrhenian coast (Italy) needs to be confirmed (Tellini et al. 1997, Brichetti and Fracasso 2008). *Muscicapa s. balearica* has a geographic distribution restricted to the Balearic Islands (Mallorca, Minorca, Ibiza, and Formentera). One mainland subspecies nominate *striata* is distributed across Europe as far as Ural and in the Maghreb (north Africa). All these subspecies have been described on the basis of differences in plumage features (Vaurie 1959) and significant variations in body size (Gargallo 1993, Taylor 2006, Martínez 2011, Viganò and Corso 2015). However their genetic distinctiveness and relationships have not been addressed so far. Vocalizations have not been used by avian systematists to address subspecific taxonomy of the spotted flycatcher probably because it is a fairly silent bird species.

Each spring, huge numbers of spotted flycatchers cross the Mediterranean Sea on a wide front to reach their breeding grounds (Cramp and Perrins 1993, Taylor 2006, Martínez 2011). The western Mediterranean islands offer suitable habitat which is used as stopover sites by many migrant spotted flycatchers as well as breeding habitat which is used by local breeders (Thibault and Bonaccorsi 1999, Martínez 2011). Hence, mainland migrants and local insular breeders potentially temporarily share the same habitats during the pre-breeding (April–May) migration. This situation raises an interesting question about isolating barriers that may prevent or limit gene flow between mainland and insular populations.

In this study, we assess the phylogenetic relationships of *M. striata* using mitochondrial and nuclear DNA sequence data and investigate the phylogeographic pattern within the species using one mitochondrial marker and a relevant geographical sampling of birds assigned to insular and mainland subspecies. More specifically, we assess the level of genetic distinctiveness achieved by *M. s. balearica* from the Balearic Islands and *M. s. tyrrhenica* from Corsica/Sardinia with respect to mainland birds from Europe and north Africa. We also propose a time frame for the diversification of the primary lineages.

The present study highlights a unique pattern showing that western Mediterranean islands played a pivotal role in the evolutionary history of a common and widespread long-distance migratory passerine species. In the discussion, we present an evolutionary scenario that may explain our results and discuss the possible role played by migration phenology in impeding gene flow between insular and mainland populations. To conclude, implications of our study for systematics and the conservation status of insular spotted flycatchers are exposed.

Material and methods

Sampling

We sampled 29 individuals from the western Mediterranean Islands and 25 individuals from mainland Eurasia and north Africa distributed over the western part of the spotted flycatcher's range (Fig. 1 and Supplementary material Appendix 1, Table A1 for details on exact localities). In addition, we further retrieved 12 mitochondrial sequences from Genbank (Supplementary material Appendix 1, Appendix A1, <www.ncbi.nlm.nih.gov>) to our data set. Four out of the five subspecies currently recognized for *M. striata* and found in the western Palaearctic were included in the present study. We used 22 individuals to assess the phylogenetic relationships among subspecies using three mitochondrial and four nuclear markers. One Asian species (*Muscicapa muttui*) and four African species (*Muscicapa adusta*, *Muscicapa sethsmithi*, *Muscicapa cassini*, *Muscicapa aquatica*) known to be more closely related to *Muscicapa striata* than Eurasian *Muscicapa* species (Sangster et al. 2010, Voelker et al. 2015) were used as outgroups.

DNA extraction, amplification and sequencing

DNA was isolated from blood samples using a DneasyBlood and Tissue kit (Qiagen, Valencia, CA, USA) and from

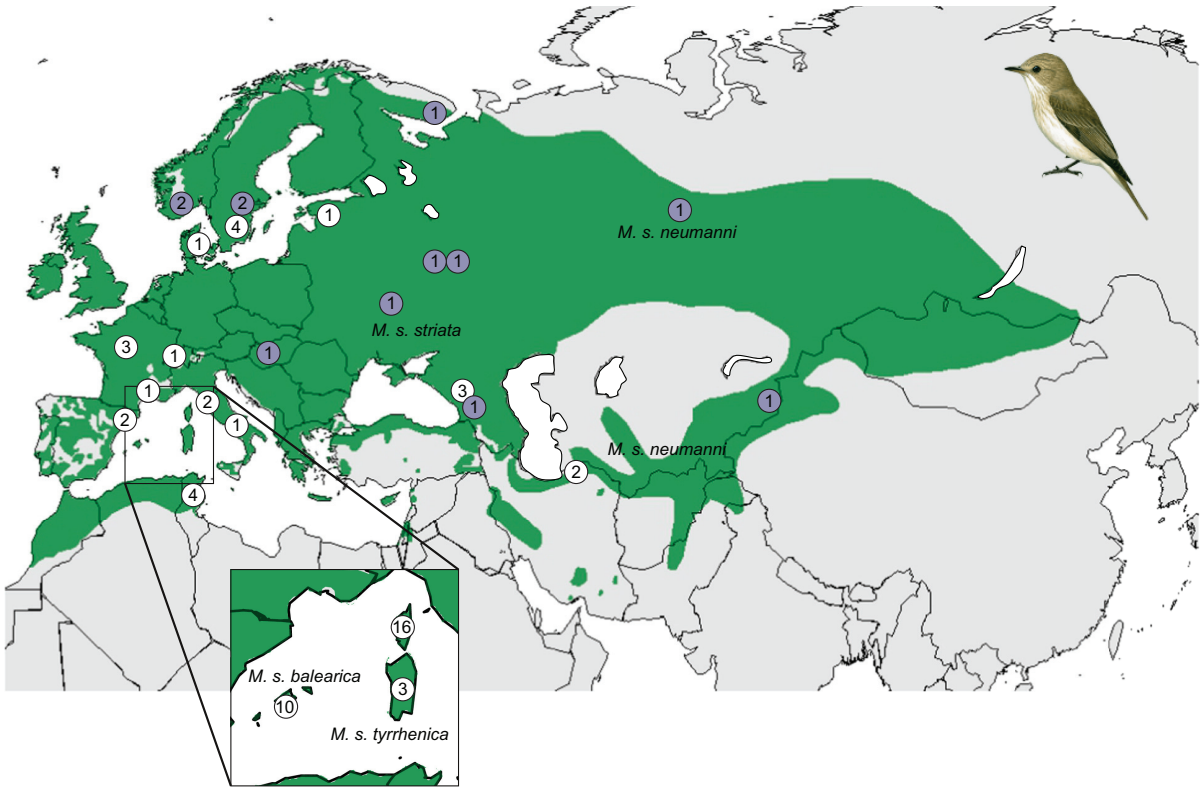

Figure 1. Breeding distribution map of the spotted flycatcher. Dots give size samples and approximate localities of spotted flycatchers included in the study. Precise sampling localities are reported in the Supplementary material Appendix 1, Table A1. White dots indicate localities for which new mitochondrial and nuclear DNA sequences have been obtained. Blue dots indicate COI sequences retrieved from Genbank and included in the phylogeographic analyses. Distribution data are from BirdLife International and NatureServe (2013). Maps were made using the R (R CoreTeam) libraries 'maps' and 'mapdata' (Becker et al. 2013), 'maptools' (Bivand and Lewin-Koh 2014) and 'scales' (Wickham 2014). Wintering range (not shown) covers a large part of sub-Saharan Africa up to the southern tip of the continent.

feathers using the QIAamp DNA Micro Kit (Qiagen). We obtained sequences data from three mitochondrial coding genes [NADH dehydrogenase subunit 2 (ND2), cytochrome *b* (Cyt b) and cytochrome oxidase subunit 1 (COI)] and four autosomal nuclear markers [lactate dehydrogenase intron 3 (LDH), transforming growth factor β-2 intron 5 (TGFb2), myoglobin intron 2 (MB2), fibrinogene intron 5 (FGB5)]. ND2, Cyt b and COI were amplified and sequenced using primers L5219/H6313 (Sorenson et al. 1999), L14967/H15503 (Pons et al. 2004) and COIext/FISH1R (Ward et al. 2009, Johnsen et al. 2010), respectively. For the nuclear introns we used the following primers: LDH: B1/B4 (Helbig et al. 2005), TGFb2: tgf5/tgf6 (Bures et al. 2002), MB2: [MYO2/MYO3F, Slade et al. 1993, Heslewood et al. 1998] and FGB [FGB5/FGB6, Marini and Hackett 2002]. Standard amplification and sequencing protocols were followed. Sequences were aligned and heterozygotes sites were checked by eye using Bioedit ver. 7.0.9 (Hall 1999). Sequences were deposited in Genbank with details on the accession numbers (KT861636-KT861721) reported in Supplementary material Appendix 1, Appendix A2.

Analyses

Selection on the mitochondrial COI

We used the McDonald–Kreitman test (MK) (McDonald and Kreitman 1991), as implemented in DnaSP ver. 5.0 (Librado and Rozas 2009) to determine whether selection was acting on the COI protein-coding gene used to infer population genetics patterns. MK tests were performed between *M. striata* insular and mainland lineages.

Determining the phase of alleles and phylogenetic reconstruction

We used Phase ver. 2.1.1 (Stephens et al. 2001), as implemented in DNAsp 5.0 (Librado and Rozas 2009), to infer the alleles for each nuclear locus. Three runs were performed and results were compared across runs. We used the recombination model and ran the iterations of the final run 10 times longer than for the initial runs.

Gene tree reconstructions of the unique haplotypes were performed using Bayesian inferences (BI), as implemented in MrBayes 3.2 (Ronquist et al. 2012). The best-fitting partitioning strategy for the concatenated mitochondrial data set (nine putative partitions; by gene and codon position) was selected using PartitionFinder ver. 1.1.1 (Lanfear et al. 2012). We used the lset nst = mixed rates = invgamma option to take into account model uncertainty in the phylogenetic analyses and performed the analyses using four different branch length priors (unconstrained, exponential mean of 10, 50, 100, and 500).

Four Metropolis-coupled Markov chain Monte Carlo (MCMC) iterations (one cold and three heated) were run for

10×10^6 iterations with trees sampled every 1000 iterations. The first 10^6 iterations (1000 trees) were discarded ('burn-in' period) and the posterior probabilities were estimated for the remaining sampled generations. Two independent Bayesian runs initiated from random starting trees were performed. We ensured that the potential scale reduction factor approached 1.0 for all parameters and that the average standard deviation of split frequencies converged towards zero. We also used the program Tracer ver. 1.5 (Rambaut and Drummond 2009) to check that we reached convergence for the posterior distributions of the parameter estimates and that our effective sample size of the underlying posterior distribution was large enough (>200) for a meaningful estimation of parameters.

Divergence times

We used Beast 1.8 (Drummond et al. 2006, Drummond and Rambaut 2007) with both strict molecular clock and an uncorrelated molecular clock model and a speciation yule tree prior to estimate the divergence times among the Muscicapa clades. We compared the divergence time estimates obtained using 1) the neutral four-fold rate from Subramanian et al. (2009); 0.073 substitutions per site per lineage per million year (s/s/l/Myr) (95% HPD: 0.025–0.123) and 2) the substitution rates proposed by Lerner et al. (2011) for the three mitochondrial loci (ND2: 0.029 s/s/l/Myr, 95% HPD: 0.024–0.033; COI: 0.016 s/s/l/Myr, 95% HPD: 0.014–0.019; cytochrome b: 0.014 s/s/l/Myr, 95% HPD: 0.012–0.016). For the four-fold degenerated sites, our settings differ slightly from the original settings of Subramanian et al. (2009) in that we used a yule speciation tree prior (instead of coalescent constant) and a TrNef substitution model instead of a GTR. We ran the Markov chain Monte Carlo for 10^8 generations and sampled trees and parameters every 1000 generations. We used the program Tracer ver. 1.6 (Rambaut and Drummond 2009) to check that our effective sample size of the underlying posterior distribution was large enough (>200) for a meaningful estimation of parameters.

Multilocus network

We used Pofad ver. 1.03 (Joly and Bruneau 2006) and SplitsTree ver. 4.0 (Huson and Bryant 2006) to build a multi-locus network. We only included individuals for which all five loci (the three mitochondrial genes were considered a single locus) were available ($n = 20$). We used uncorrected *p*-distances as input for Pofad and made use of the standardized matrix for network reconstruction.

Mitochondrial genetic variation and median-joining networks

The substitution model that best fit our COI data ($n = 66$ sequences) was selected with TOPALi ver. 2.5 (Milne et al. 2004). Mean pairwise genetic distances within and among subspecies were estimated by Mega6 (Tamura et al. 2013). Standard diversity indices (haplotype diversity, nucleotide diversity, number of polymorphic sites) were calculated using Arlequin 3.5 (Excoffier and Lischer 2010). We used Fu's Fs and Tajima' D tests (1000 replicates) to detect signatures of population expansion. We also generated an extended Bayesian skyline plot (EBSP; Heled and Drummond 2008) for the Eurasian sub-lineage using all the available COI sequences ($n = 29$). We used a HKY model and enforced a strict molecular clock (0.016 s/s/l/Myr, 95% HPD: 0.014–0.019). We ran three independent analyses, each of 50×10^6 generations, as implemented in Beast 1.8 (Drummond et al. 2006, Drummond and Rambaut 2007).

We computed pairwise Fst for all pairs of insular and mainland populations to assess the level of geographical structuring of the genetic variability. The significance of variance components was tested with 110 permutations.

We generated a median-joining network to visualize relationships among COI haplotypes ($n = 66$) and Cyt b and ND2 haplotypes for a subsample of 21 and 19 individuals respectively using NETWORK 4.6.1.2 (Bandelt et al. 1999).

Results

Phylogenetic relationships

Mitochondrial trees

The mitochondrial phylogenetic tree based on three coding genes strongly supported the monophyly of *M. striata* with respect to its closest African relatives (Fig. 2). Two reciprocally monophyletic divergent lineages were highlighted with high support within *M. striata.* One lineage included individuals that belong to insular *balearica* and *tyrrhenica* subspecies. Both insular subspecies do not share any haplotypes and are thus reciprocally monophyletic. Two mainland birds breeding along the Tyrrhenian coastline were grouped with Corsican and Sardinian birds. The other lineage has a wide continental distribution ranging from France and Italy to eastern Russia and extending southwards to North Africa. This lineage which consisted of birds traditionally assigned to *M. s. striata* or *M. s. neumanni* subspecies was further divided into two sub-lineages. The first sub-lineage was only found in north Africa and Spain whereas the second one had a large range that encompassed a large part of Eurasia.

Nuclear gene trees

The FGB5 nuclear gene tree supported the monophyly of *M. striata* with respect to African species whereas phylogenetic relationships were not resolved for the 3 other nuclear markers. In a similar way, within *M. striata*, phylogenetic relationships among alleles were generally poorly resolved due to low variation. Expectedly, the proportion of shared alleles between insular and mainland individuals varied according to each intron but it was generally low. For instance there was no allele sharing for the FGB intron (Fig. 3A), only one shared allele among 12 alleles for MYO2 (Fig. 3B) and only one among 13 for TGFb2 (Supplementary material Appendix 1, Fig. A1). The proportion of alleles sharing was highest for LDH (Supplementary material Appendix 1, Fig. A2). Such a low proportion of shared alleles for three out of four introns most probably may be explained by lack of recent gene flow between insular and mainland lineages and indicates that sorting of ancestral polymorphism is well advanced but still not completed.

Individual locus and multilocus network

In accordance with gene trees, allele networks showed that the proportion of alleles sharing between insular and

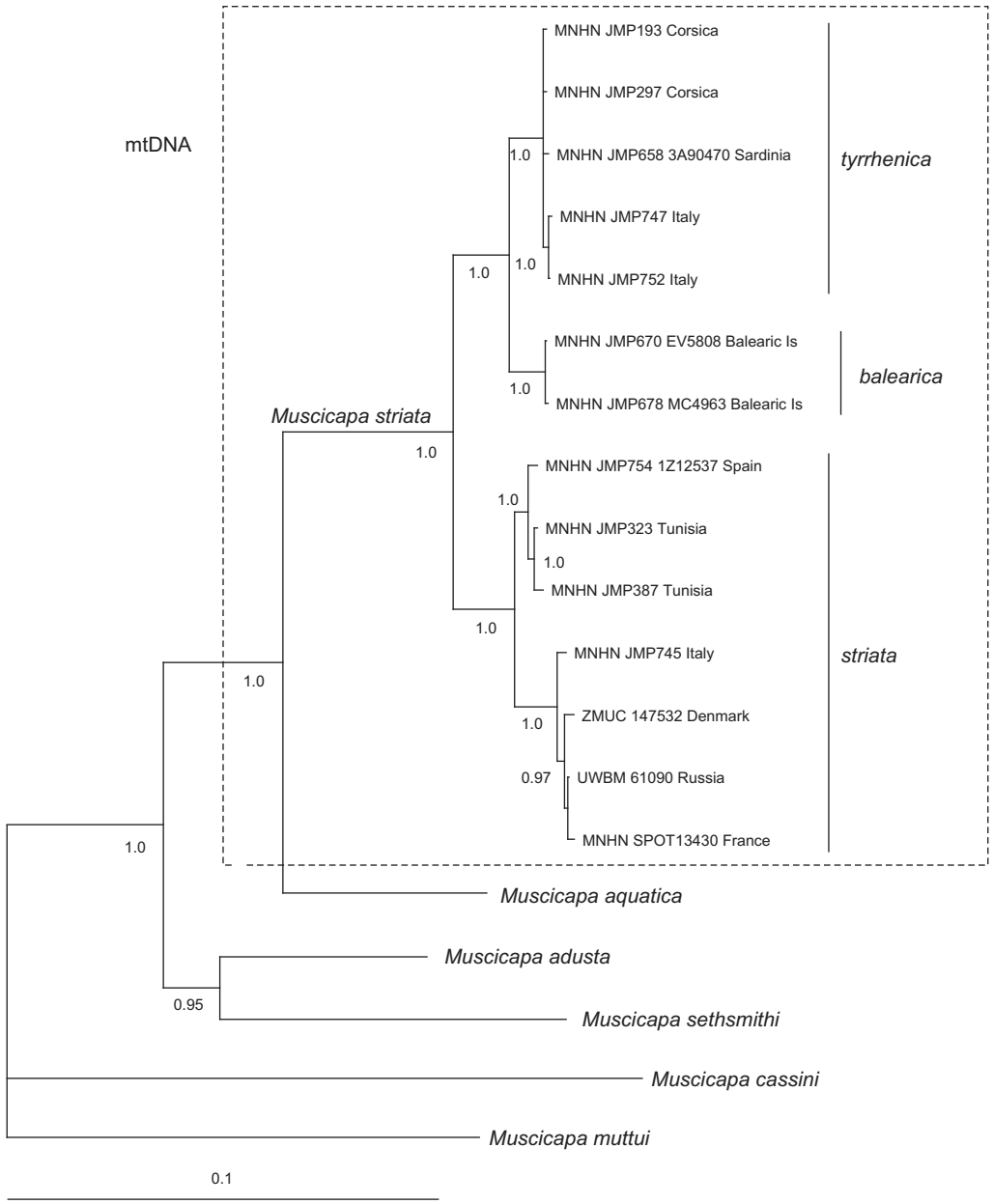

Figure 2. 50% majority-rule consensus tree obtained from the Bayesian analyses of the three concatenated mitochondrial markers (COI, ND2, Cyt b). Only unique haplotypes from twenty-two individuals belonging to the main lineages were included in the matrix. Values close to nodes represent Bayesian posterior probabilities.

mainland populations varied among loci but was generally low and that insular and mainland formed different subparts of the networks (Fig. 4).

The multilocus network obtained using mitochondrial and nuclear sequences revealed strong genetic divergence between insular (*balearica*, *tyrrhenica*) and mainland subspecies (*striata*) (Fig. 4).

Mitochondrial divergence times

Our divergence time estimates were very similar across analyses; the choice of the molecular clock prior (strict clock or uncorrelated) had no effect on the absolute values of the estimates (Table 1). The only important difference concerned the most ancient split between *M. striata* and *M. aquatica* estimated at 1.8 (0.8–3.2) Mya using the neutral substitution rate, and 2.6 (2.1–3.2) Mya using mitochondrial markers substitution rates estimated by Lerner et al. 2011. Our divergence time analyses suggested that the mainland lineage *striata* diverged from the insular lineage *tyrrhenica-balearica* about 1.1 Mya. The split between insular lineages occurred about 0.5 Mya, which was also the timing of the split between north Africa-Spain and Eurasian lineages (Table 1).

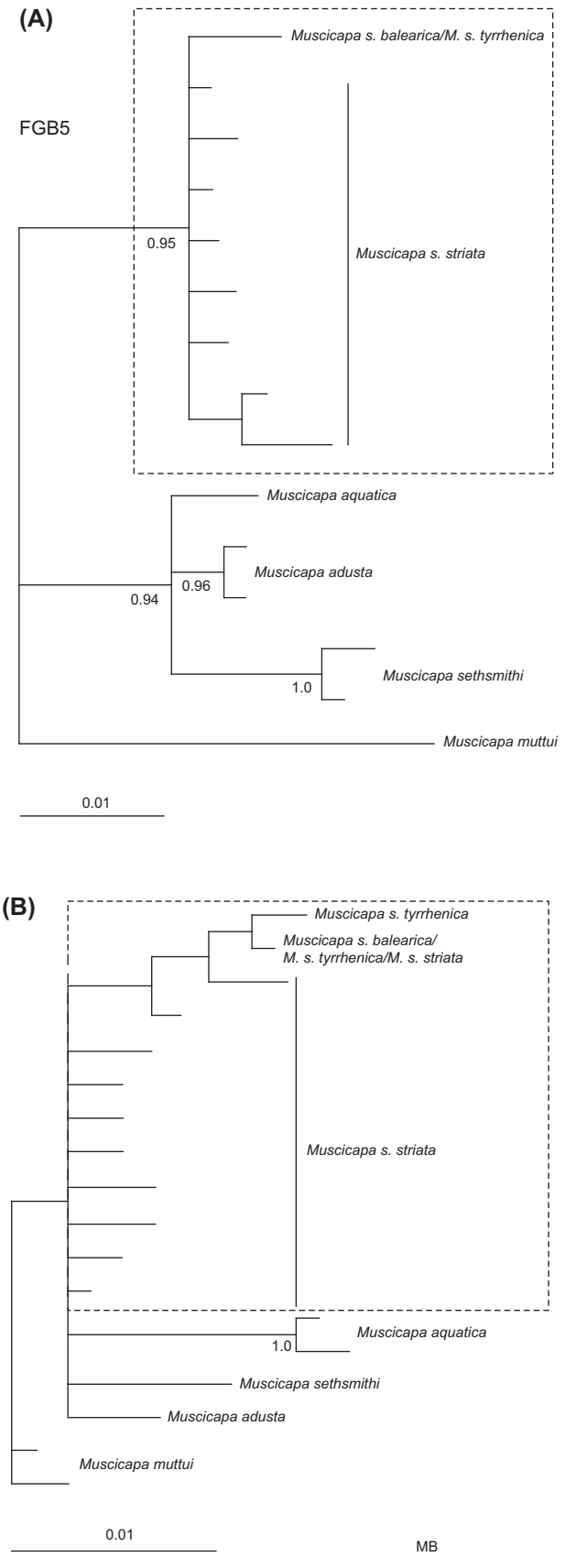

Figure 3. Gene trees obtained from the Bayesian analyses of autosomal introns FIB5 (A) and MYO2 (B). Alleles from five insular and fourteen mainland spotted flycatchers were included in the analyses.

Phylogeographic patterns

Median joining networks

Median joining networks based on mitochondrial markers (COI, n = 66 spotted flycatchers; Cyt b, n = 21; ND2, n = 19) are reported on Fig. 5. As expected the three mitochondrial networks shared the same structure that consists of two main lineages that are further structured into two sub-networks. The insular lineage was separated from the mainland lineage by 21 mutation steps in the COI network which is based on the complete data set. Within the insular lineage, the unique *balearica* haplotype was separated from its *tyrrhenica* counterparts (n = 5 haplotypes) by 10 mutation steps. The mainland network displayed a star-like shape with a common central haplotype having a wide geographical distribution at the centre of the network and derived haplotypes radiating from the ancestral haplotype. Such a pattern is commonly observed in the case of recent population expansion. North African and mainland Iberian spotted flycatchers diverged from Eurasian spotted flycatchers by 6 substitutions. Western Eurasian COI haplotypes belonging to *M. s. striata* and eastern haplotypes from *M. s. neumanni* were weakly differentiated from each other and mixed within the same sub-network.

Genetic variation and selection (COI)

The MK tests did not detect any significant evidence of selection in the COI gene when comparing the insular lineage with the mainland lineage (Fischer's exact tests, $p = 0.31$).

The genetic diversity was higher in mainland populations than in insular populations. Haplotype diversity was lower in insular *tyrrherica* than in mainland subspecies (*striata*/*neumanni*) whereas no difference in nucleotide diversity was detected among them (Table 2). Noticeably, *M. s. balearica* was characterized by an absence of genetic variability in COI, all individuals (n = 10) possessing the same haplotype. Tajima's D and Fu's tests detected strong evidence of population expansion only in the case of the Eurasian *striata* lineage (Table 2). This corroborates the star-like shape obtained with the Eurasian haplotypes in the median-joining network (Fig. 5) as well as the extended Bayesian skyline plot which supports effective population growth (Supplementary material Appendix 1, Fig. A3).

Genetic divergence (COI)

We examined the level of genetic distinctiveness achieved among and within *M. striata* subspecies using pairwise population comparisons (Fst). All Fst pairwise comparisons were higher than 0.80 and highly significant ($p < 0.0001$, Table 3). The north African-Iberian lineage was significantly differentiated from its Eurasian counterpart as well as from the two insular subspecies. *Muscicapa s. tyrrhenica* and *M. s. balearica* did not share any haplotypes and genetic divergence between these two insular subspecies was highly significant.

Among populations mean Tamura–Nei genetic distances ranged from 1.3 to 4.2% whereas within population genetic distance did not exceed 0.7%. The largest genetic distance recorded was between the insular *balearica* population and the mainland north African/Iberian population (D = 4.2%,

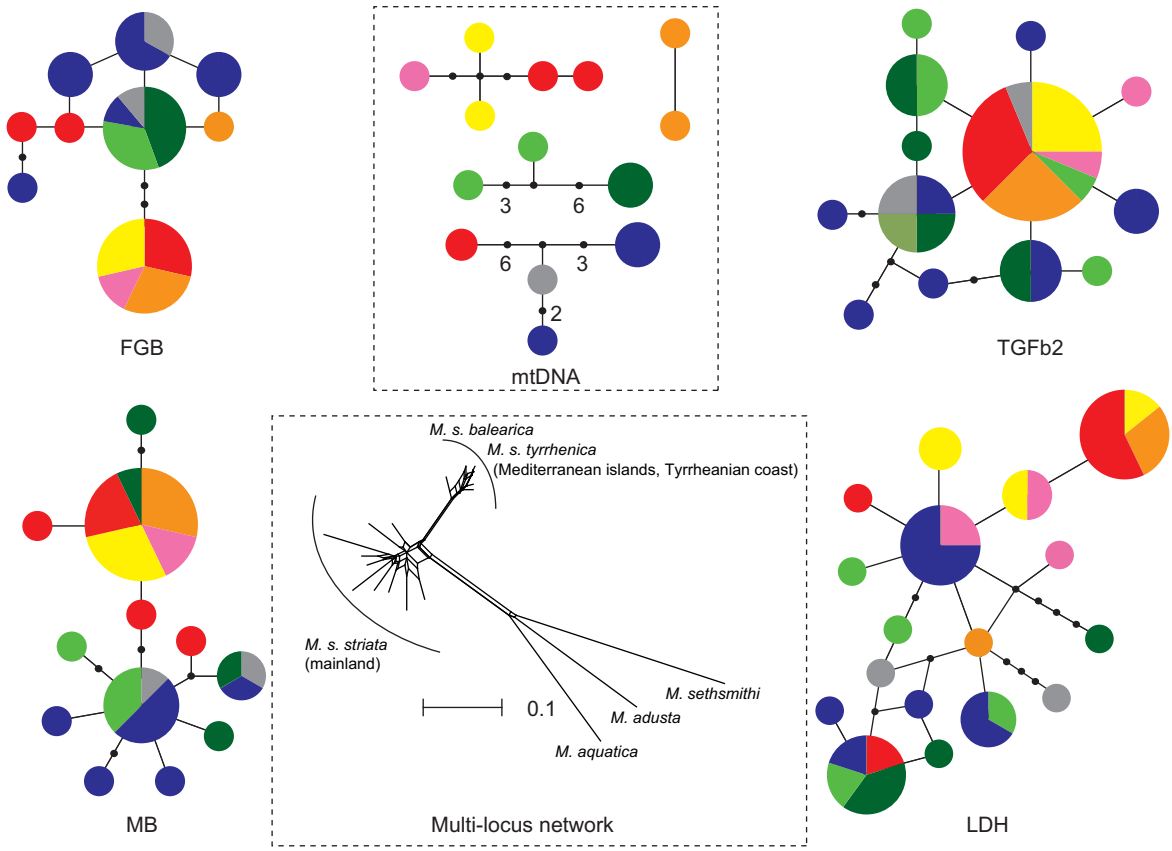

Figure 4. 95% minimum spanning network obtained using TCS (Clement et al. 2000). Small black circles represent unsampled or extinct alleles/haplotypes (numbers next to the circles indicate the number of substitutions, if different from one). The multilocus network was obtained from the phased four autosomal nuclear introns (MB, FGB, LDH, TGFb2) and three concatenated mitochondrial markers (COI, ND2, Cyt b) showing genetic relationships among *M. s. striata*, *M. s. balearica*, *M. s. tyrrhenica* (based on traditional definition) and three African flycatcher species. Dark blue = western Europe, Scandinavia, western Russia; grey = southern Russia; red = Italy; dark green = north Africa; light green = Spain; yellow = Corsica; pink = Sardinia; orange = Balearic islands.

Table 3). The lowest genetic distance was found between the two mainland *striata* lineages (D = 1.3%).

Discussion

Phylogenetic relationships

Phylogeny results obtained in the present study strongly support the monophyly of *M. striata* with respect to its most closely related counterparts found in sub-Saharan Africa (Sangster et al. 2010, Voelker et al. 2015). One of the putative closest species of *M. striata*, *M. gambagae* (Gambaga flycatcher) could only be included in the Cytochrome b analyses. The Gambaga flycatcher was sister to the swamp flycatcher *M. aquatica* (Supplementary material Appendix 1, Fig. A4) and not directly related, or nested within *M. striata* and thus we did not find any support for a conspecific relationship with *M. striata.* Consequently, the superspecies hypothesis suggested by Taylor (2006) to depict systematic

Table 1. Divergence time estimates in million years among main lineages (95% highest posterior density) using substitution rates estimated by Lerner et al. (2011) for each mitochondrial marker and the neutral four-fold degenerated sites rate from Subramanian et al. (2009).

	ND2	COI	Cyt b	Combined data	Four-fold rate
Clock					
M. striata/M. aquatica	2.2 (1.6–2.8)	2.7 (2.0–3.6)	2.8 (2.0–3.7)	2.6 (2.1–3.2)	1.8 (0.8–3.2)
M. s. striata/tyrrhenica-balearica	0.9 (0.7–1.2)	1.1 (0.8–1.5)	1.2 (0.8–1.6)	1.1 (0.9–1.4)	0.95 (0.4–1.7)
M. striata striata:					
North Africa-Spain/western Palaearctic	0.4 (0.25–0.5)	0.5 (0.3–0.6)	0.5 (0.3–0.7)	0.4 (0.3–0.6)	0.4 (0.2–0.7)
M. s. tyrrhenica/M. s. balearica	0.4 (0.25–0.5)	0.5 (0.3–0.7)	0.5 (0.3–0.7)	0.45 (0.3–0.6)	0.3 (0.1–0.6)
Uncorrelated					
M. striata/M. aquatica	2.2 (1.6–3.0)	2.7 (1.9–3.6)	2.9 (1.9–4.0)	2.7 (2.1–3.4)	1.9 (0.8–3.6)
M. s. striata/tyrrhenica-balearica	0.95 (0.7–1.3)	1.2 (0.8–1.6)	1.2 (0.8–1.7)	1.2 (0.9–1.5)	1.0 (0.4–2.0)
M. striata striata:					
North Africa-Spain/western Palaearctic	0.4 (0.2–0.5)	0.5 (0.3–0.7)	0.5 (0.3–0.7)	0.45 (0.3–0.6)	0.4 (0.1–0.9)
M. s. tyrrhenica/M. s. balearica	0.4 (0.2–0.6)	0.5 (0.3–0.7)	0.5 (0.3–0.8)	0.48 (0.3–0.6)	0.4 (0.1–0.8)

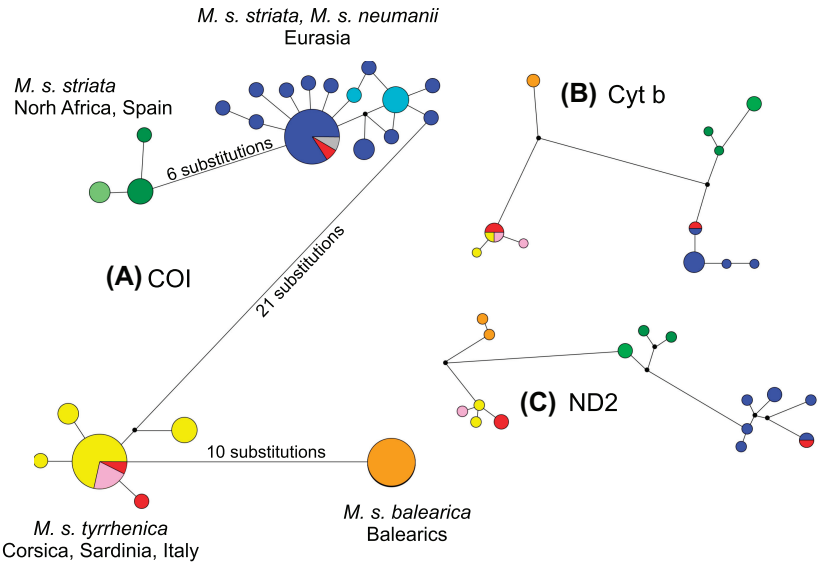

Figure 5. Median joining networks showing relationships among mitochondrial markers for *Muscicapa striata* subspecies. As expected, (A) – COI (n = 66), (B) – Cyt b (n = 21) and (C) – ND2 (n = 19) networks display the same overall structure and highlight two mainland and two insular lineages. Dark blue = western Europe, Scandinavia, western Russia; grey = southern Russia; light blue = Kazakhastan, Iran; red = Italy; dark green = north Africa; light green = Spain; yellow = Corsica; pink = Sardinia; orange = Balearic islands. The size of each circle is proportional to haplotype frequency. The small black circles correspond to extinct or unsampled haplotypes.

relationships between *striata* and *gambagae* is not supported by our analyses.

Phylogenetic relationships among mitochondrial haplotypes indicate the existence of two clades within *M. striata*. The first one includes two insular subspecies (*balearica* and *tyrrhenica*) while the second one correspond to the mainland subspecies (*striata*). The insular group is further divided into two subgroups that match current subspecies delimitation. In contrast, the phylogeographic structure within *M. s. striata* revealed cryptic diversity by highlighting a strongly supported north African/Iberian subgroup well separated from the other mainland Palaearctic populations. Within *M. striata*, limited levels of shared nuclear alleles for 3 out of 4 introns and one distinctive FGB5 insular allele gave additional support to the genetic distinctiveness of insular spotted flycatchers with respect to their mainland counterparts highlighted with mitochondrial markers.

Phylogeographic patterns

Our genetic survey reveals a unique phylogeographic pattern, highlighting the major importance of western Mediterranean islands in the present day genetic structure of the spotted flycatcher. With exception of Mediterranean *Sylvia* warblers, for which Mediterranean islands played a major role in speciation patterns (Brambilla et al. 2008, Voelker and Light 2011), most endemism cases documented to date for passerine birds on these islands concerns sedentary or short-distance migratory species (Pasquet and Thibault 1997, Förschler et al. 2009, *Carduelis*; Pasquet et al. 2014,

Table 2. Number of haplotypes, haplotype diversity (H), nucleotide diversity (π), Tajima's D and Fu's statistics of selective neutrality obtained for mtDNA (COI) lineages of *Muscicapa striata*. NS ($p > 0.05$), *($p < 0.05$), **($p < 0.001$).

	striata Eurasia	*striata* Tunisia, Spain	*tyrrhenica* Corsica, Italy	*balearica* Mallorca
Number of individuals	27	6	21	10
Number of haplotypes	14	3	5	1
Number of polymorphic sites	13	3	5	0
H	0.77 ± 0.09	0.73 ± 0.15	0.54 ± 0.12	0
π	0.002 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	0
Tajima D's	–1.83*	–0.44 (NS)	–1.09 (NS)	–
Fu's Fs	–11.44**	–0.11 (NS)	–1.36 (NS)	–

Table 3. Genetic distances (Tamura–Nei average distance and standard error expressed in percentages, above the diagonal) and pairwise population comparisons (FSTs, below the diagonal) between *Muscicapa striata* populations (COI). *$p < 0.0001$, all pairwise populations' comparisons are highly significant.

	striata Eurasia	*striata* Tunisia, Spain	*tyrrhenica* Corsica, Italy	*balearica* Mallorca
striata (Eurasia)	0	1.3 (0.4)	3.4 (0.7)	4.1 (0.8)
striata (Tunisia, Spain)	0.81*	0	3.5 (0.7)	4.2 (0.8)
tyrrhenica (Corsica, Italy)	0.95*	0.96*	0	1.7 (0.5)
balearica (Mallorca)	0.96*	0.98*	0.94*	0

Sitta; Pons et al. 2015, *Certhia*). There is no clear geographical structure for the very widespread Eurasian (that is Iberian Peninsula and north Africa excluded) clade for which a western subspecies (*striata*) and an eastern subspecies (*neumanni*) were traditionally recognized. The recent spatial expansion of the *striata/neumanni* lineage is suggested by negative values of Tajima's D and Fu's, and the extended Bayesian skyline plot. Interestingly, the three Italian individuals included in the network are not closely related. One individual, which was sampled in inland Tuscany, is part of the mainland *striata* lineage, whereas the other two individuals, sampled along the Tyrrhenian coast, possess insular *tyrrhenica* haplotypes. The presence of *tyrrhenica* along the Tyrrhenian coast was previously suspected (Tellini et al. 1997, Brichetti and Fracasso 2008). A similar situation has been described for the subalpine warbler with the *subalpina* lineage occurring both on the Italian mainland and in Corsica (Brambilla et al. 2008). More samples would be needed to precisely delimit the geographical distribution of mainland and insular lineages in Italy as well as the location of their potential contact zones.

The Mid-Pleistocene played a pivotal role in the differentiation of many western Palaearctic bird species (Hung et al. 2012, *Sitta europea*; Pons et al. 2011, *Picus viridis*; Pellegrino et al. 2014, *Athene noctua*). According to our divergence time analyses, the insular lineage probably splitted from the mainland lineage around 1 Mya, most probably during a cold climatic period that could have led to a contraction of the spotted flycatcher's breeding range. At the same time a peak of aridification occurred in Africa (Demenocal 1995) that possibly led to a fragmentation of its wintering quarters in a way that mainland and insular populations might have been isolated from each other during all their annual life cycle. Brambilla et al. (2008) did not estimate divergence date for the split between the *S. subalpina* lineage from Corsica and Sardinia and mainland lineages of the *S. cantillans* complex. This precludes any direct comparisons with the present study. It is nevertheless worth noting that they found a genetic distance between insular and mainland lineages (Cyt b, around 4%), which is very similar to the inter-lineage Cyt b genetic distance (D = 4.2%) we found for the spotted flycatcher.

Divergence event between *M. s. balearica* (Balearic Islands) and *M. s. tyrrhenica* (Corsica, Sardinia and Tyrrhenian coast) from western Mediterranean islands occurred during the late Pleistocene (around 0.4 Mya) as well as the split between the widespread Eurasian lineage and the Iberian-north African lineage. The late-Pleistocene was characterized by accentuation of amplitude and duration of climatic cycles that most probably favoured allopatric divergence of many Palaearctic bird species (Guillaumet et al. 2008, Pellegrino et al. 2014, Pons et al. 2015). Yet, given the broad 95% HPD and the uncertainties associated with the molecular rates, it is not possible to attribute any of these diversification events to a particular climatic oscillation.

In addition to western Mediterranean islands, our results suggest that the spotted flycatcher survived repeated glacial periods in at least two mainland glacial refugia. The most ancestral and widespread Eurasian haplotype has been found in the Italian Peninsula and Caucasus, two regions known to have constituted glacial refugia for bird taxa (Hung et al. 2012, Pellegrino et al. 2014, Pons et al. 2015). The phylogeographic pattern and divergence date estimates recovered in this study favour the existence of several refugia located in western Mediterranean islands, southern Europe, and north Africa and/or the Iberian Peninsula. However, further sampling would be necessary to precisely identify geographical regions selected by spotted flycatcher populations as Pleistocene glacial refugia.

Genetic variation and divergence

Lack of variation in mitochondrial COI and small number of nuclear introns alleles compared to mainland *striata* (Table 2, Fig. 4) suggests a very low level of genetic variation in *M. s. balearica*. Although more individuals and genetic markers would be necessary to assess the level of genetic variability of this taxa at the genome level, low variability of mitochondrial and nuclear markers included in the present study matches genetic impoverishment often detected in small insular bird populations (Barrientos et al. 2009, *Bucanetes githagineus*; Chan et al. 2011, *Pomarea dimidiate*; Jensen et al. 2013, *Passer domesticus*; Pentzold et al. 2013, *Periparus ater*). Compared to Corsica and Sardinia, Mallorca is a small island (3625 km^2 vs 8690 km^2 and 24 090 km^2 respectively). Hence, *M. s. balearica* should have fluctuated around smaller population size levels throughout the late Pleistocene climatic oscillations in comparison to *M. s. tyrrhenica*. Low genetic variability of *M. s. balearica* may result from repeated bottleneck events and/or genetic drift, which is known to play a preponderant role in shaping the genetic composition of small islands populations (Jensen et al. 2013). Haplotype diversity was lower in the *M. s. tyrrhenica* population than in mainland populations whereas no difference in nucleotide diversity was detected.

All population pairwise comparisons ($p < 0.0001$) revealed strong geographical structure in the genetic variation of the spotted flycatcher, not only between insular and mainland lineages but also between Eurasian and north African/Iberian populations. We found a large COI genetic distance between insular and mainland lineages (3.4–4.2%) which is of the same order of magnitude as interspecific genetic distances found among four black and white *Ficedula* flycatchers species distributed around the Mediterranean basin (Sætre et al. 2001). Females are often the furthest dispersing sex in passerine birds (Clarke et al. 1997). Thus the relatively high genetic distance (1.7%) between *M. s. balearica* and *M. s. tyrrhenica* together with the absence of mitochondrial haplotypes sharing indicate that these two insular taxa probably act as incipient species. Sharing of most nuclear alleles is best explained by retention of ancestral polymorphism due to recent divergence event.

Winter range, morphology, migration phenology

The spotted flycatcher winters in sub-Saharan Africa. Yet, the wintering range of each subspecies is poorly known. The scarcity of available data suggests that nominate *striata* individuals are widely distributed in west Africa as well as in southern Africa. Insular *balearica* individuals were recorded from Ivory Coast, Namibia and possibly Cameroon whereas no reliable winter records are currently available for

the Corsican and Sardinian spotted flycatchers (Cramp and Perrins 1993, Taylor 2006).

Spotted flycatchers found in the Balearics possess a very pale and lightly streaked plumage and thus are clearly different in plumage from their mainland and Corso-Sardinian counterparts. *Muscicapa s. tyrrhenica* exhibits warmer coloured upper parts than both *M. s. striata* and *M. s. balearica* and is less streaked on the breast than the nominate subspecies with streaks replaced by coalescing spots (Cramp and Perrins 1993, Taylor 2006). Spotted flycatchers from the Balearics have significantly shorter wings than the mainland spotted flycatchers (*balearica*, L = 81.2 mm ± 1.71, n = 33; *striata*, L = 85.0 mm ± 2.05, n = 96, Gargallo 1993), as well as a different wing shape. In Balearic birds, the second primary (counted ascendantly) typically falls between the fifth and the sixth and not between the fourth and the fifth as is the case for mainland flycatchers (Gargallo 1993). As a consequence, insular individuals have a more rounded wing than their mainland counterparts suggesting that their migration route is shorter (Baldwin et al. 2010). In a similar way, the Sardinian spotted flycatchers have 5% shorter wings when compared to mainland birds, as well as more rounded wings (Viganò and Corso 2015). Hence, even if a morphological comparison remains to be done between *tyrrhenica* and *balearica*, available published studies do suggest that *tyrrhenica* and *balearica* share a similar wing shape that significantly distinguishes them from the mainland nominate subspecies.

In the Balearics, approximately half of the spring migrants mist-netted on stopover sites belong to the mainland subspecies and the other half to *balearica* (Martínez 2011). Based on a large sample size (n = 1507 mist-netted flycatchers), Martínez (2011) found an approximatively 10 days difference between median dates of both subspecies, with mainland birds migrating later than local insular breeders. Ringing data available for Corsica also show that a large number of mainland flycatchers stopover on the island during spring migration (Faggio and Jolin 2008). Yet, study on migration phenology of local Corsican breeders and mainland migrants using Corsican stopover sites remains to be done.

Insular evolution

The most original point revealed by the present study is the great importance of western Mediterranean islands in the evolution of the spotted flycatcher which is an abundant, long-distance migratory bird species. Insular populations were isolated from nearby mainland populations for a relatively long period (approximately 1 Mya) and accordingly evolved independently in response to insular selective pressures and drift that shaped their phenotypic and behavioural traits.

Available data suggest that both *M. s. balearica* and *M. s. tyrrhenica* evolved rounder and shorter wings and that *M. s. balearica* individuals tend to start spring migration earlier than mainland birds do (Gargallo 1993, Martínez 2011, Viganò and Corso 2015). Several studies demonstrated that wing pointness correlates with migratory behaviour (Copete et al. 1999, *Emberiza schoeniclus*; Baldwin et al. 2010, *Saxicola torquata*; Förschler and Bairlein 2011, *Carduelis citrinella*). Moreover, Voelker and Light 2011 suggested for *S. sarda* and *S. balearica,* two insular Mediterranean *Sylvia* warblers, that island endemic distributions were derived from continental distributions as a consequence of the loss of migration. Regarding the spotted flycatcher, we suggest that short-winged insular birds modified their migratory habits and thus may be geographically isolated from mainland birds not only at their breeding sites but also at their wintering quarters.

Remarkably, thousands of mainland migrants use spring stopover sites in the Balearics (Martínez 2011) and in Corsica (Faggio and Jolin 2008) and are thus temporarily sympatric to local insular breeders. Our results, albeit based on a small sample size, did not reveal any mixing of lineages among Corsican breeders. These results suggests that pre-zygotic reproductive barriers may operate to prevent gene flow between insular and mainland flycatchers. We thus hypothesize that temporal segregation of migration documented for *M. s. balearica* may constitute a pre-zygotic barrier through competition for accessing nest sites and/or female partners because most mainland migrants stopover in the Balearics when local breeders are already occupying their breeding territories. In addition, recent studies demonstrated that migratory phenology and breeding phenology are under genetic control (Caprioli et al. 2012, Saino et al. 2015). It is thus possible that migrant birds may not be able to start breeding when they stopover in Mediterranean islands for genetic and behavioural reasons. Furthermore, if secondary contact zones between *tyrrhenica* and *striata* exist in western Italy as suggested by our preliminary results, this would provide a good opportunity to investigate how pre-zygotic barriers may act in a mainland context to reduce or impede inter-lineages gene flow.

Further studies are needed to assess in more details potential gene flow between insular and mainland spotted flycatchers and efficiency of isolating barriers that could reduce or prevent genetic exchanges among lineages. Our results, as well as significant differences in morphological and behavioural traits previously highlighted among mainland and insular subspecies (Gargallo 1993, Martínez 2011, Viganò and Corso 2015), suggest that such mechanisms are currently operating.

Systematic issues

Our results show a clear genetic differentiation of insular subspecies with regard to *M. s. striata* in mitochondrial DNA, and to a lesser extent, in nuclear alleles. Furthermore, available data suggest that insular subspecies differ in terms of morphology (plumage and wing shape), breeding phenology and migration patterns. Given that insular breeders are temporarily sympatric with mainland migrants (see above) without any apparent admixture of lineages, we may infer the existence of pre-mating isolating barriers that prevent gene flow between local breeders and migrants. Accordingly, we suggest that current taxonomic treatment of insular spotted flycatchers should be changed to more adequately take into account new genetic information provided here. The insular spotted flycatchers could be treated as one polytypic species (*Muscicapa tyrrhenica*) that differs from *M. striata* in morphology, migration, mitochondrial and nuclear DNA and would consist of two subspecies (the nominate

and *M. t. balearica*) that recently diverged in mitochondrial DNA and phenotypically but still extensively share the same nuclear alleles.

Conservation issues

The spotted flycatcher is currently classified as 'least concern' by the IUCN because it has an extremely large geographical range even if the population trends appear to be decreasing (Birdlife International 2015). The present study clearly indicates that spotted flycatchers from the Mediterranean islands are on their own evolutionary trajectories and are likely demographically independent from related mainland populations. Hence, their conservation status should be reassessed accordingly.

Special attention should be devoted to insular flycatchers from the Balearics which occupies the smallest range and probably possess a low level of genetic variation. Low genetic variability, a feature found in a wide range of insular taxa (Frankham 1997), could hamper the ability of many insular endemics to cope with current environmental changes (Frankham et al. 2002).

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Supplementary material (Appendix JAV-00859 at <www.avianbiology.org/appendix/jav-00859>). Appendix 1.