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ABSTRACT

The Eurasian treecreeper (*Certhia familiaris*) comprises two mitochondrial lineages that diverged during the mid-Pleistocene. One paleoendemic lineage has an allopatric range currently restricted to the Island of Corsica and the Caucasus region, whereas the second one has a very large Eurasian range. Here we used microsatellites (N = 6) and mitochondrial DNA (COI) to assess the genetic structure of insular and mainland populations from Corsica, mainland France and Central Italy (N = 258) and the level of mitochondrial and nuclear gene flow among these populations. Concordant with the mtDNA signal, microsatellites results clearly demonstrate that the Corsican population (*C. f. corsa*) is strongly divergent from nearby mainland populations (*C. f. macrodactyla*). Microsatellite data also support significant divergence and low gene flow between the Central Italian and mainland French populations. Our results suggest low nuclear gene flow from the mainland into Corsica and no mitochondrial gene flow. Sporadic gene flow from the nearby mainland may explain the presence of continental nuclear alleles in the genome of 5% of sampled insular birds. Our study confirms the existence of an endemic Corsican treecreeper lineage with important conservation value. Our results also imply that Eurasian treecreepers from Central Italy constitute a distinct management unit.

KEYWORDS: Corsica, Mediterranean islands, microsatellites, mitochondrial DNA, population structure, gene flow.

INTRODUCTION

The Eurasian treecreeper (*Certhia familiaris*) is a forest passerine found over a very large Palaearctic range from the British Isles to Japan and northern China. Ten morphological subspecies are currently recognized based on slight clinal variation in plumage colour (Harrap, 2018). In a previous study, Pons *et al.* (2015) using **mitochondrial markers (COI, Cyt b, ND2)** suggested that Eurasian treecreepers found in Corsica belong to a paleoendemic lineage which has an allopatric range, restricted to this island and the Caucasus region. Corsican and Caucasian treecreepers are currently assigned to two distinct morphological subspecies, *C. f. corsa* E. J. O. Hartert, 1905 and *C. f. caucasica* Buturlin, 1907 respectively. All other **sampled** subspecies (N = 6), including *C. f. macrodactyla* C. L. Brehm, 1831, which is found in **mainland** France and Italy, belong to a more recent and widespread lineage distributed over Eurasia (Pons *et al.*, 2015). Phylogeography of the Eurasian treecreeper was thus more strongly influenced by Pleistocene climatic oscillations leading to population contractions and expansions rather than by geography, and its current subspecific treatment does not correctly reflect its evolutionary history. The phylogeographic pattern depicted for the Corsican treecreeper (Pons *et al.*, 2015) differs from the one known for most Corsican subspecies of forested passerines which are closely related to their nearest mainland counterparts (i.e. Blue tit *Cyanistes caeruleus ogliastrae*, Kvist *et al.*, 2004; Great tit *Parus major corsus*, Kvist *et al.*, 2003; Coal tit *Periparus ater sardus*, Pentzold *et al.*, 2013; Tritsch *et al.*, 2018). In contrast, it is worth noting that the endemic Corsican nuthatch (*Sitta whiteheadi*) shares with the Corsican treecreeper a similar phylogeographic pattern, its sister species being the eastern Palaearctic Chinese *Sitta villosa*, while the genus *Sitta* is also represented by several intervening species in the western Palaearctic (Pasquet *et al.*, 2014). The importance of testing previously established hypotheses about population history and genetic structure based on mitochondrial DNA (mtDNA hereafter) alone with independent

93 nuclear markers has been pointed out (see Ballard & Whitlock, 2004; Toews & Brelsford,
94 2012). Contrasting results across molecular markers having distinct mode of inheritance, such
95 as mtDNA and microsatellites, may be very useful to infer evolutionary relationships among
96 populations that recently diverged and for which gene flow may not be entirely interrupted
97 (Brito, 2007; Pons *et al.*, 2014; Tritsch *et al.*, 2018).
98 Phylogenetic relationships among *C. familiaris* subspecies obtained in Pons *et al.* (2015)
99 using three nuclear introns are generally poorly resolved with the exception of a well-
100 supported sister relationship between Corsican and western European populations. This result,
101 in agreement with geographic distances, is in strong conflict with the mtDNA inferences that
102 support a deeply divergent clade grouping Corsican and Caucasian treecreeper populations.
103 Such mito-nuclear discordance may be explained by several non-exclusive hypotheses
104 including retention of ancestral polymorphism from the ancestor of the Eurasian and Corsican
105 lineage, positive selection on mtDNA variants, male mediated gene flow with no consequence
106 for maternally herited mtDNA, and hybrid sterility in the heterogametic female sex according
107 to Haldane's rule (see Ballard & Whitlock, 2004; Toews & Brelsford, 2012). Pons *et al.*
108 (2015) suggested that retention of ancestral polymorphism might be the most probable
109 hypothesis explaining mito-nuclear discordance in phylogenetic relationships of *C. f. corsa*
110 with its closest relatives. To obtain further insights on the evolutionary history of the Eurasian
111 treecreeper and the causes of this mito-nuclear discrepancy, we investigated in the present
112 study the relationships among mainland and insular populations of the Eurasian treecreeper
113 using much larger population samples than in Pons *et al.* (2015) and fast evolving nuclear
114 markers.
115 Our present study compares mtDNA and microsatellite results on population structure,
116 genetic diversity and gene flow between insular and mainland populations of the Eurasian
117 treecreeper (*Certhia familiaris* Linnaeus, 1758) across its south-western range. More than 250

individuals from Corsica and nearby France and Italy were sampled to investigate population genetics of south-western populations of the Eurasian treecreeper. In addition, we also analysed morphological variation to assess whether the Corsican population differs from its mainland counterparts, as was initially suggested.

We specifically aim to answer the following questions:

- Is endemism of the Corsican population highlighted by previous studies on the basis of plumage variation (Harrap, 2018) and mtDNA (Pons *et al.*, 2015) confirmed by microsatellite data?
- Are there differences in nuclear and mtDNA gene flow between Corsican and mainland populations?
- Is genetic variation of Corsican and mainland French populations geographically structured?
- Do mainland populations from France and Italy belong to the same nuclear genetic cluster as suggested by their mtDNA relationships?

The mito-nuclear discordance highlighted in Pons *et al.* (2015) will be discussed taking account of the present results. We will discuss the systematic status of the Corsican treecreeper in the light of new information on gene flow and population divergence brought by the present study and conservation issues will be evoked.

MATERIAL AND METHODS

Sampling

We obtained feather samples **(two secondary feathers per bird)** from 258 individuals. **All birds were mist-netted from February to June between 2011and 2016, except birds from the Apennines sampled in 1992 by Guido Tellini during an independent field session.**The male of the Eurasian treecreeper is an aggressively territorial bird, singing and approaching in

response to playback of conspecific songs. In this study, all birds were caught using male song play-back experiments. **The presence of the cloacal protuberance was checked for each bird. We mist-netted 258 males and 3 females which were not included in the analyses.** Six discrete geographical populations were sampled in France (Alps, Jura, Pyrenees, Massif Central, Eastern France, Western France; N = 122) and one isolated population in Central Italy (Apennines, N = 26) (Figure 1). One individual sampled in the Italian Alps near the French border was included in the "Alps" population. In Corsica (N = 109), the whole range of the subspecies has been sampled (Figure S1). More individuals were sampled in Northern Corsica where most forest areas are located. Information on exact localities and collectors' names is reported in Table S1.

Laboratory work

DNA extraction

DNA was isolated from feathers using the QIAamp DNA Micro Kit (Qiagen, Valencia, CA, USA**) following the standard QIAamp protocol**.

Microsatellite genotyping

We genotyped 12 microsatellite loci, eight of which were originally developed for passerines (SpuL5-22, SpuA6, SpuL6-16, Haas *et al.*, 2009; SS1-6, SS1-11, SS1-12, SS2-32, SS2-52, SS2-80, SS2-106, SS2-130, SS3-42C, Rubenstein, 2005). Microsatellite fragments were amplified using 12 fluorescent primers and multiplex PCRs, following standard protocols. Genotyping was conducted on an ABI 3700 Genetic Analyzer (Applied Biosystem) using GeneScan500 LIZ (Applied Biosystems) as internal lane size standard. Results were visualized using Peak Scanner (Applied Biosystem) and analyzed with Geneious version 9.1.8 (http://www.geneious.com, Kearse *et al.*, 2012).

COI amplification and sequencing

The mitochondrial cytochrome c oxidase subunit 1 (COI) was amplified and sequenced using primers COIext/FISH1R (Ward, Hanner& Hebert, 2009; Johnsen *et al*., 2010). Standard amplification and sequencing protocols were followed. COI sequences were aligned using Bioedit version 7.0.9 (Hall, 1999). New sequences were deposited in Genbank with the accession numbers MK125306-125323.

Data analyses

Microsatellites

We used MSA 4.05 (Dieringer & Schötterer, 2003), Arlequin 3.11 (Excoffier *et al.*, 2005), and FreeNa 1.0 (Chapuis & Estoup, 2007) to quantify the genetic diversity of the microsatellite loci for allelic richness, Garza-Williamson index (M-ratio test), observed and expected heterozygosity, and to test for deviation from Hardy-Weinberg genotype frequency equilibrium. PGDSpider 2.0.3.0 (Lischer & Excoffier, 2012) was a very useful tool for converting our data set into different formats.

The Fst values between pairs of population were calculated in Arlequin, with sequential Bonferroni corrections. We performed Bayesian analyses using STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) to determine the optimum group structure from the microsatellite loci. For the complete dataset, the number of K clusters was set from 2 to 10, with 10 iterations per number of clusters, a burn-in of 100,000 followed by 900,000 iterations while allowing for admixture. We also conducted analyses for the Corsican samples and for the populations from continental France separately, to check for population structure within these areas. We used Structure Harvester (Earl & von Holdt, 2012) to choose the best estimate of K based on the LnP(D) and ΔK ad hoc statistics (Evanno *et al.*, 2005).

We estimated the nuclear migration rates among the three groups highlighted in STRUCTURE (Corsica, mainland France, Central Italy) using two approaches that differ in their time frames: BayesAss3.0.3 (Wilson & Rannala, 2003) that estimates recent (2-3 last

192 generations) migration rates between populations using Markov chain Monte Carlo (MCMC),
193 and MIGRATE 3.6 (Beerli, 2009) that uses a MCMC-based maximum-likelihood approach
194 based on an expansion of the coalescence model (from present to the most recent common
195 ancestor). In BayesAss, after a few preliminary runs a chain length of ten million iterations
196 with a burn-in of one million iterations and a thinning interval of 1000 was chosen to run the
197 program. Delta values (parameters *a*, *f*, *m*, defining the size of the proposed change to the
198 parameter values at each iteration) set to 0.3 in the final analyses were used for migration
199 rates. Two independent runs were performed, with unique random seeds, to assess
200 convergence. In MIGRATE, the runs consisted of 10 short chains (sampling 10 000 trees) and
201 three long chains (sampling 100 000 trees) with a burnin period of 10 000 trees. All runs were
202 repeated five times to verify consistency of results. To avoid applying a unique mutation rate
203 to the microsatellite loci, we inferred the number of immigrants per generation N_i by
204 multiplying the mutation-scaled effective population size Θ to the mutation-scaled effective
205 immigration rate M.
206 Nei Genetic distances among mainland and insular populations were calculated using
207 GENETIX (Belkhir *et al.*, 1996) and visualized with a neighbour joining network constructed
208 using PHYLIP (Felsenstein, 2005).
209 *Mitochondrial COI*
210 We used the McDonald-Kreitman test (MK) (McDonald & Kreitman, 1991), as implemented
211 in DnaSP ver. 5.0 (Librado & Rozas, 2009), to test if selection was acting on the COI protein-
212 coding gene used to infer population genetic patterns. MK tests were performed between the
213 insular and the mainland lineages.
214 Mean K2P pairwise genetic distances among populations were estimated by MEGA6 (Tamura
215 *et al.*, 2013). Standard diversity indices (haplotype diversity, nucleotide diversity, number of
216 polymorphic sites) were calculated using ARLEQUIN 3.5 (Excoffier & Lisher, 2010). We

used Fu's Fs and Tajima' D tests (1000 replicates) to detect signatures of population expansion. These two tests were initially developed as selection tests; it has been shown that they are in fact very sensitive to demographic fluctuations, with significant negative values being a signature of population expansion (Fu, 1997). Hence, in the absence of selection, as evidenced by the MK test, Fu's Fs and Tajima' D represent reliable indicators as to whether a population experienced a population size change. We computed pairwise Fst for all pairs of populations to assess the level of geographical structuring of the genetic variability.

We generated a median-joining network with NETWORK 4.6.1.2 (Bandelt, Forster & Rohl, 1999) to visualize relationships among COI haplotypes and to check for the possible presence of insular haplotypes in mainland populations and vice versa.

Morphometric differentiation

Biometric data

Four biometric variables were included in the analyses to assess morphological variations among the French, Italian and Corsican populations. **All Measurements were made according to Eck *et al.* (2011) and Demongin (2016)**. Wing-length (WL) was measured to the nearest 0.5 mm using a ruler **(see Figure 13, page 76; Eck et al., 2011)**, bill length at the nostrils (BLN), tarsus length (TL) and hind claw length (HCL) to the nearest 0.1 mm using a calliper.

Morphometric analyses

All statistics were performed in R (R development Core Team, 2018). Normality of quantitative variables was first checked using the one sample Kolmogorov-Smirnov test and homoscedasticity using the Bartlett test. Biometric data were compared using one-way parametric analysis of variance (ANOVA) or Kruskal-Wallis non-parametric test if data were not normally distributed. We used the Tukey test to perform multiple comparisons among populations or multiple non-parametric tests using the package

pgirmess (Giraudoux et al., 2018). A standardized Principal component analysis (PCA) using a correlation matrix was performed with the package FactoMineR (Husson *et al.*, 2018) to visualize how individuals from different geographic origins and admixed Corsican treecreepers were distributed in multivariate space. As biometric measurements were obtained from several ringers, we statistically controlled potential measurement biases using a linear mixed-effects model with the package lme4 (LME4 Authors, 2018) and we also performed a PCA using covariance matrix (following the recommendation of Perktas & Gosler, 2010). PC1 scores were compared using an ANOVA with "ringer" considered as a random factor and "region" as fixed factor.

RESULTS

Microsatellites

Six non-polymorphic loci (SpuA6, SS1-6, SS2-32, SS2-52, SS2-106, SS2-130) were removed from the analysis. The genetic diversity for the six remaining microsatellite loci in each population is summarized in the supplementary material (Table S2). The allelic richness varied from 1.4 to 7.9. Deviation from HWE ($\alpha = 0.05$) was found in two cases (Corsica for locus 2, and France-East for locus 6), but null allele frequency estimated by Free Na for these loci and populations were inferior to 0.15, suggesting that the presence of null alleles for these loci should have little effect on the outcome of Bayesian assignment analyses (Carlsson, 2008). The values of the Garza-Williamson index are close to 1 (average of 0.84), indicating that recent bottlenecks cannot be detected in the populations, although the power of this test might be impeded by the low number of loci used (Perry *et al.*, 2012). Based on the expected heterozygosity (HE), the genetic variation appeared moderate and fairly constant between the samples (0.5-0.7; Table S2) excepting Corsica and Central Italy which has the lowest HE (0.3-0.4).

Population differentiation and genetic structure

Pairwise Fst were highly significant after Bonferroni correction for 13 pairs of populations, all involving the populations located in Corsica and Central Italy (mean F_{st} Corsica vs France = 0.27; F_{st} Corsica vs Central Italy = 0.30, mean F_{st} France vs Central Italy = 0.17; Table 1). By contrast, no significant differentiation was observed among the populations located in France (15 pairwise comparisons). The neighbour joining network (Figure 2) clearly shows that Corsican, Central Italian and mainland French populations form three separated groups, the Corsican population being the farthest one.

Results from STRUCTURE indicate that the Corsican and Italian populations were significantly differentiated from all populations from France, with K=3 as the most likely number of genetic clusters when all populations were analysed as a single dataset (Figure 3, Figure S2). No further genetic substructure was observed within Corsica or within continental France (Figure S2). Five individuals sampled in Corsica (numbers 266, 284, 1010, 1040, 1064) showed significant admixture, i.e. a non-Corsican inferred ancestry with a probability > 50%, suggesting potential genotypic introgression from the continental populations. These five individuals were sampled in different localities in Corsica (Rospa Sorba, Tova, Fratte, Albertacce and Bastelica). Similarly, 34 individuals sampled in continental France also showed significant admixture, whereas no samples from Central Italy present another inferred ancestry.

Contemporary gene flow among populations

The effective sample size for all parameters were all above 200 and the two independent BayesAss+ runs gave very similar results regarding migration rates among the three primary populations (Table S3), suggesting that our analyses reached convergence.

Estimates for the fraction of the Corsican individuals being migrants from others populations were very low (0.7%) from mainland France and 0.4% from Italy suggesting that migration

from the mainland is very rare (Table S3). Estimates for the fraction of the Italian individuals being migrants from others populations were slightly higher but still very low (1.44% from Corsica; 2.2% from mainland France) suggesting that migration into Italy from Corsica or mainland France is rare. Estimates for the fraction of the mainland France individuals being migrants from Corsica was less than 0.5%. Overall these results suggest that contemporary gene flow between insular and mainland lineages is very low. As confidence intervals of gene flow estimates are very large and overlapping, our results do not allow any detailed comparisons of the contemporary gene flow among populations (Table S3).

Historical gene flow

Migrate results suggested asymetrical past gene flow between the three groups (Figure 4), mainland France providing the largest number of immigrants to Corsica (N_i = 10.67) and to Central Italy (N_i = 6.17). Gene flow between Corsica and Central Italy is low but not null (close to 1 immigrant per generation). This asymmetry was also reflected in the estimation of population size, which is six times larger in mainland France (Θ = 9.24) than in Corsica (Θ = 1.49) and Central Italy (Θ = 1.7). Additionally, we enforced a more realistic migration model in which Corsica is treated as a sink, with no migrants to mainland France or Central Italy. Using this model, the migration to Corsica remained three times more important from mainland France (N_i = 8.07) than from Central Italy (N_i = 2.85).

COI

Genetic variation

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 test, P = 1). Our results do not support any clear patterns of population expansion except for populations from eastern France and the Alps for which both Tajima's D and Fu's tests are significant (Table 2). Genetic diversity parameters (H, π) varied from moderate to low values depending on

317 geographical populations (Table 2). Our results did not suggest any differences in genetic
318 diversity between the insular and mainland populations. The most striking result is a complete
319 lack of genetic variability for the Italian population. In France, haplotype diversity was
320 highest in mountainous populations (Alps, Jura and Pyrenees).
321 *Genetic divergence*
322 K2P genetic distances among mainland populations from France and Central Italy were very
323 low, varying from 0.03% to 0.06% (Table S4). By contrast, the K2P genetic distances
324 between the insular population from Corsica and mainland populations from France and
325 Central Italy were much higher, varying from 2.3% to 2.4 %. Accordingly, pairwise
326 population Fst values between the Corsican population and mainland populations were highly
327 significant and close to 1 (Table 3), indicating a near complete geographical partitioning of
328 the genetic variation. On the other hand, among mainland populations our results show a lack
329 of genetic differentiation except for the Pyrenean population which is slightly **but**
330 **significantly** differentiated.
331 *Median joining network*
332 In accordance with above genetic results, the median joining network (Figure 5) shows that
333 Corsican treecreepers do not share any haplotypes either with mainland treecreepers sampled
334 in France or Italy. Likewise, no Corsican haplotypes were found among mainland populations
335 from France and Central Italy. The lack of geographic mixing of insular and mainland
336 haplotypes clearly suggests that female-mediated gene flow across the Mediterranean Sea is
337 non-existent or so low that much larger sample sizes would be necessary to detect it.
338 **Morphometry**
339 *Univariate analyses*
340 One-way analyses indicated that there were highly significant differences between the insular
341 and mainland populations for most morphological variables. In particular, the insular

342 population had a longer bill length at nostrils (BLN) and a shorter hind claw length (HCL)
343 (Table S5) than both Italian and French mainland populations.
344 *Multivariate analyses*
345 More than 68% of the total variance was explained by the first two principal components
346 (Figure 6). **Component loadings of the variables are reported in Table S6**. Bill length at
347 nostrils (BLN), tarsus length (TL) and HCL were highly correlated to the first axis **(PC1)**
348 which can be interpreted as a size axis. **ANOVA using a linear mixed effects model with**
349 **"ringer" as a random factor performed on the first axis of the covariance matrix PCA**
350 **showed highly significant differences among the 3 populations ($F_{(2, 240)} = 17.63$, $P < 0.0001$). On the other hand, there was no clear separation among the three populations**
352 **according to PC2 (Figure 6).** Insular birds from Corsica formed a morphological cluster
353 fairly well separated from French and Italian clusters despite some overlap. Confidence
354 ellipses of each geographical population were clearly separated from each other (Figure 6),
355 the Italian population occupying an intermediate position between French and Corsican.
356 Clearly more samples from Central Italy would be necessary to soundly assess morphological
357 variation of the population of the Apennines. Nevertheless our results clearly suggest that the
358 Corsican population is morphologically divergent from its nearby mainland populations.
359 Among the five admixed Corsican treecreepers possessing more than 50% of continental
360 alleles, three were located with other "pure" Corsican birds. Two admixed individuals
361 (individual number 266, 1064, dark blue and red squares respectively, Figure 6), were close to
362 mainland Italian and French birds. However, the lack of a clear gap between insular and
363 continental morphological clusters only allows us to conclude that admixed birds are probably
364 morphologically more similar to Corsican than to mainland treecreepers.
365
366

DISCUSSION

The Corsican population

In this study, we used genetic data from both microsatellites and mtDNA to examine the levels of divergence and diversity within and among insular and mainland populations of the Eurasian treecreeper. Our mtDNA results based on a five-fold increase in sample size of Corsican treecreepers when compared with Pons *et al.* (2015), confirm the high genetic distinctiveness of the Corsica population with respect to mainland populations from France and Central Italy (DK2P > 2%, Pairwise $F_{st} > 0.90$). In accordance with mtDNA, our microsatellites results also support high genetic divergence ($0.31 < F_{st} > 0.16$) consistent with an endemic Corsican lineage. Bayesian inferences of population structure based on microsatellites support the presence of three genetic clusters (Figure 3). The first cluster includes all individuals from France, the second includes all treecreepers from Central Italy and the third one all insular individuals from Corsica.

Overall, our results strongly support the endemism of the Corsican population which possesses private and highly differentiated mtDNA haplotypes as well as microsatellite alleles never found in nearby mainland populations. Interestingly, five Corsican treecreepers (i.e nearly 5% of our sample) possessed more than 50% of continental nuclear alleles suggesting slight genetic admixture. This result highlights the importance of sample size in detecting low levels of gene flow between populations.

While no mitochondrial gene flow across the Mediterranean Sea was detected in the present study (no mixing of insular and mainland populations in any sampled populations), our microsatellites results obtained with MIGRATE suggest the existence of moderate gene flow from continental France into Corsica. Gene flow from mainland populations into the Corsican population is further supported by the detection of five Corsican treecreepers possessing both insular mtDNA haplotypes and microsatellites alleles mostly of continental origin. Such mito-

nuclear discordance can arise from several non-exclusive mechanisms (reviewed in Toews & Brelsford, 2012). Sex-biased dispersal is often considered as a possible mechanism that may explain mito-nuclear discordance in gene flow (Petit & Excoffier, 2009; Pons *et al.*, 2014). In birds, several recent studies suggest that female biased dispersal is not a general rule contrary to what was classically suggested (Dobson, 2013; Li & Merilä, 2010; Both, Robinson & van der Jeugd, 2012). If males are the main long-distance dispersers in the Eurasian treecreepers, this may explain the introgression of mainland microsatellite alleles in the Corsican population while at the same time no introgression of mtDNA haplotypes is expected. Unfortunately, information on sex-specific dispersal distances is currently lacking for the Eurasian treecreepers. Another possible explanation is selection against hybrids of the heterospecific sex (female in birds) as expected if the Haldane's rule applies to the Eurasian treecreeper. If hybrid females are counter-selected while hybrid males are able to backcross with treecreepers belonging to the insular lineage, this would solely result in the introgression of continental nuclear alleles into the Corsican population. Furthermore, incomplete lineage sorting of microsatellite alleles due to longer coalescent times of nuclear markers compared to mtDNA (Zink, 2010) cannot be excluded and might explain the presence of some continental alleles in the genetic pool of the Corsican populations. Finally, the slight mixed genetic composition of the Corsican treecreeper population might be the result of both incomplete lineage sorting and recent gene flow from occasional arrivals in Corsica of a small number of continental dispersers Pons *et al.* (2015) found that the main conflict between mitochondrial and nuclear phylogenetic trees was the sister relationship of the Corsican population with nearby mainland populations of Western Europe recovered in the nuclear species tree obtained with three nuclear introns whereas the Corsican and the geographically distant Caucasian lineages formed a strongly supported monophyletic group in the mtDNA tree. Pons *et al.* (2015) favoured the sharing of ancestral poplymorphism as the most probable process

explaining mito-nuclear phylogenetic discordance and dismissed the dispersal hypothesis. New information brought by the present study also supports occasional gene flow from the continent as a possible explanation for the presence of continental nuclear alleles in the genome of insular birds. The Eurasian treecreeper is known to occur as a rare vagrant to the Channel Islands, Mallorca and the Faroe Islands (Cramp & Perrins, 1993; Harrap & Quinn, 1996); it is thus plausible that long-distance dispersers may occasionally reach Corsica.

We did not detect any significant genetic structure within Corsica for the Eurasian treecreeper; this situation is in contrast with what has been found for *Sitta whiteheadi* (Thibault *et al.*, 2016), another forested passerine endemic to Corsica. Such a difference between both species might be explained by their distinctive habitat requirements, the Corsican nuthatch occurring only in mature scattered forested patches of *Pinus nigra laricio* (Thibault *et al.*, 2016) whereas the Eurasian treecreeper occupies a larger variety of tree species and is more continuously distributed over Corsica, although still in mature forests (Thibault *et al.*, unpublished results); moreover, birds regularly disperse outside their breeding range into secondary habitats.

The lower **microsatellite** variability of the Corsican population **found here might result from** its geographical isolation and its smaller effective population size compared to mainland European populations allowing genetic drift and lineage sorting to be more effective. Low genetic variation was found in a wide range of insular taxa (Frankham, 1997) and in several small insular bird populations (Pons *et al.*, 2016).

The Apennine population

Mitochondrial and nuclear markers provide different patterns of genetic structure for the Italian treecreeper population from the Apennines. The sampled population was characterized by a lack of mitochondrial variation, only possessing the most widespread, and thus probably the most ancestral haloptype found all over Europe. Consequently, mtDNA pairwise Fst

values with the northern French populations were low and most often not significant. By contrast, our microsatellites results showed significant genetic divergence (Fst = 0.16), the Italian population being assigned to a specific genetic cluster with STRUCTURE. Sex-biased gene flow as a possible explanation of mito-nuclear discordance in the Apennine population is not supported by our data because maternal gene flow would have also favoured nuclear genetic admixture, yet most Italian treecreepers are not admixed (Figure 3).The high mutation rate of microsatellites compared to mtDNA, associated with low demographic size of the Apennine population, may have permitted the detection of a genetic cluster of too recent origin to be detected by mtDNA alone. In the Apennine Mountains, the Eurasian treecreeper occupies a small geographic range, isolated from northern Alpine and southern Abruzzi populations (Meschini & Frugis, 1993), with numbers estimated at only a few hundred birds (Tellini *et al.*, 1997). Due to small geographic range and founder effect, the Eurasian treecreeper population from the Apennines had most probably a small effective population size allowing microsatellite differentiation despite longer coalescence time of nuclear markers compare to mtDNA (Zink & Barrowclough, 2008).

Both lack of mitochondrial variability and low microsatellites diversity of the **Apennine** population may be explained if we consider that it was founded from a subset of the ancestral genetic lineage (Hewitt, 1996). Under such a scenario, Central Italy was colonized from northern European populations that would have been rapidly expanding from a unique refuge located in the Eastern part of the *C. familiaris* range (Pons *et al.*, 2015), and **thus** did not play any role as a glacial refuge for the Eurasian treecreeper. This biogeographic scenario is further supported by our STRUCTURE microsatellite results suggesting that most treecreepers from Central Italy are not admixed and harbour less genetic diversity than the more northern French populations. More samples covering a larger geographical area in Italy would nevertheless be required to firmly test this phylogeographic pattern. **Another**

phylogeographic scenario in which the Apennine population would have diverged in the Italian peninsula during the Pleistocene may be possible. Under this scenario, low genetic diversity of the extant Apennines population might result from one or several bottlenecks that would have occurred in Italy during cold periods. Indeed, in Europe, several comparative genetic surveys highlighted three Mediterranean primary refugia not covered by ice masses: the Iberian Peninsula, the Italian Peninsula and the Balkans, where populations persisted during cooling periods and were able to colonize northern areas during warming periods (Hewitt, 2000; Weiss & Ferrand, 2007). Several studies report genetic divergence of Italian vertebrates lineages dating back to the Pleistocene (Brito, 2005; Lo Brutto *et al*., 2011; Ruedi *et al*., 2008). However, our genetic data do not give strong credit to such a phylogeographic scenario for the Eurasian treecreeper, because in the case of ancient bottleneck events, we would expect at least some mtDNA differentiation.

The mainland French populations

The six geographically isolated populations found in France are not genetically differentiated, except the Pyrenean population which is significantly divergent based on our mtDNA results ($0.06 < Fst < 0.13$). This lack of genetic divergence was expected for treecreeper populations found in the Alps, Jura and Eastern France which are geographically close to each other and to very large populations found across northern and eastern Europe (Cramp & Perrins, 1993; Nissa & Muller, 2015). As these populations are more or less connected, their genetic pools are most probably continuously homogenized by ongoing gene flow. The western population has been discovered only in the late 1970's (Nissa & Muller, 2015). **Our genetic results do not favour the hypothesis of a small and old isolated population that would have been overlooked by local ornithologists. L**ack of genetic divergence of this small population may be explained both by its probable very recent foundation and ongoing gene flow with eastern

populations. The latter seems particularly likely given that westwards range expansion of north-eastern populations is currently observed in France (Nissa & Muller, 2015). Similarly, the Rhone valley is probably not an effective dispersal barrier, and therefore gene flow between the Massif Central and Alpine populations is likely sufficient to prevent genetic differentiation. The most distinctive population found in the Pyrenees is also the most isolated one. The Pyrenean population is situated at the south-western limit of the Eurasian treecreeper range, thus probably limiting the occurrence of gene flow with other northern populations sampled in France.

Systematic issues

Hartert (1905) described the subspecies *corsa* on the basis of its slightly larger size than the mid-European form, its long bill and more distinct markings on the upperparts. Based on mtDNA, *C. f. corsa* belongs to a lineage that is also found in the Caucasus region, and that most probably disappeared from the rest of Europe during the mid-Pleistocene (c. 1 Mya). According to Pons *et al.* (2015), *C. f. corsa* does not share a common recent evolutionary history with the nearby mainland treecreepers populations that all belong to a widespread lineage that probably arrived recently in Western Europe. Due to its spatio-temporal isolation and insularity, the Corsican population evolved distinct phenotypic and genetic characters that warrant its subspecific rank. The present study based on an expanded sampling of individuals and additional nuclear markers confirms the genetic distinctiveness of Corsican treecreepers (mtDNAK2P ~ 2 %, nuclear Fst ~ 0.28) and morphological differentiation (longer bill, shorter hind claw) with respect to nearby mainland populations currently assigned to *C. f. macrodactyla* **(see Tietze & Martens, 2009)**. *C. f. corsa* also differs from all continental subspecies by vocal characters (Tietze *et al.*, 2008). From an evolutionary perspective, sporadic gene flow from the mainland supported by the present study did not prevent the Corsican population from acquiring specific characters that may result from insular selection

pressures and/or phenotypic plasticity. It thus makes sense to question whether assigning a specific rank to this insular population would not be a better taxonomic option. We nevertheless suggest keeping the current systematic arrangement assigning a subspecific rank to the Corsican treecreeper population because of its fairly recent splitting and lack of information on the efficiency of pre and post mating isolating barriers with mainland treecreepers. Most importantly, it would be crucial to assess the level of nuclear genetic divergence achieved by the Caucasus population with respect to both northern European populations and *corsa* before proposing any systematic arrangement.

Conservation issues

Based on significant genetic distinctiveness and low genetic diversity highlighted in this study, as well as small population size and isolated geographic distribution, we suggest that the Eurasian treecreeper population from the Apennines warrants treating as a distinct management unit (sensu Moritz, 1994). Information on genetics of the population located in the Abruzzi is also much needed, in order to assess the conservation status of the Eurasian treecreeper in Central and southern Italy.

Several recent studies have highlighted the existence of endemic genetic lineages in Corsica and other western Mediterranean islands even for highly mobile organisms like birds (i.e. *Sylvia cantillans*, Brambilla *et al.*, 2008; *Carduelis corsicana*, Pasquet *et al.*, 1997; *Burhinus oedicnemus*, Mori *et al.*, 2017; *Sitta whiteheadi*, Pasquet *et al.*, 2014; *Muscicapa striata*, Pons *et al.*, 2016; *Periparus ater*, Tritsch *et al.*, 2018). For these species an important part of the genetic variability is located in Mediterranean islands. Therefore these islands harbour an important part of Mediterranean biodiversity to be conserved. The present study adds further support to the originality of the Corsican avifauna that requires specific management decisions. Among the insular endemic lineages mentioned above, only the Corsican nuthatch and the Corsican treecreeper lineages are strictly limited to Corsica (Pons *et al.*, 2015). The

breeding habitat of the Corsican treecreeper is mainly restricted to the mature and dense forests of Corsican pines (*Pinus nigra laricio*), Holm oaks (*Quercus ilex*) and several deciduous trees (*Castanea sativa, Fagus sylvatica*) only found in mountainous areas of the island (Thibault & Bonnacorsi, 1999; Thibault *et al.,* unpublished results.). Its relatively small breeding population size estimated at 5000-10 000 pairs (Thibault, unpublished results) should be managed independently of the continental populations which have an extremely large range encompassing a large part of Eurasia and comprise 40 000 000-80 000 000 individuals (Birdlife international, 2018).

The Corsican population is currently subject to several threats such as fire and logging that are major causes of reduction and fragmentation of its habitat. Due to this adverse ecological context, its unique evolutionary history, small insular range and breeding population size, we argue that the Corsican treecreeper should be registered on the Annex 1 of the European bird directive that lists endangered species and subspecies in Europe**.**

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TABLES

Table 1: Microsatellites pairwise F_{st} averaging the variance components over loci. Values in bold differ significantly from zero after Bonferroni correction. MC = Massif Central, PY= Pyrenees, WE = Western France, ES= Eastern France, JU = Jura, AL = Alps

			FRANCE (Mainland)					
	CORSICA	ITALY	AL	WE	ES	JU	MC	PY
CO	0							
IT	**0.30**	0						
AL	**0.29**	**0.17**	0					
WE	**0.31**	**0.22**	0.00	0				
ES	**0.22**	**0.13**	0.01	0.02	0			
JU	**0.28**	**0.16**	-0.04	-0.02	-0.03	0		
MC	**0.24**	**0.18**	0.00	0.00	-0.00	-0.01	0	
PY	**0.22**	**0.16**	0.03	0.03	0.02	0.01	0.02	0

Table 2: Number of haplotypes, haplotype diversity (H), nucleotide diversity (π), Tajima's D and Fu's statistics of selective neutrality obtained for insular and mainland populations from Corsica, France and Central Italy (N = 257) using the mitochondrial gene COI (667bp). Ni = number of individuals, Nh = number of haplotypes, Np = number of polymorphic sites. MC= Massif Central, PY= Pyrenees, WE = Western France, ES = Eastern France, JU= Jura, AL= Alps. In bold significant values supporting population expansion. Fu's Fs statistic was considered as significant if P-value is below 0.02 (Excoffier and Lischer 2010).

		FRANCE (Mainland)						
	CORSICA	MC	PY	WE	ES	JU	AL	ITALY
Ni	108	27	23	21	20	9	23	26
Nh	3	3	3	2	4	4	4	1
Np	2	3	2	1	3	3	3	0
H	0.29	0.15	0.49	0.18	0.28	0.58	0.32	0
π	0.0005	0.0003	0.0008	0.0003	0.0005	0.001	0.0005	0
Tajima's D	- 0.29	- 1.73	-0.017	-0.617	- 1.72	- 1.51	- 1.48	0
p-value	0.41	**0.02**	0.42	0.23	**0.03**	0.06	**0.05**	1
Fu's Fs	-0.24	- 1.49	0.024	- 0.14	- 2.75	- 1.89	- 2.32	0
p-value	0.36	0.03	0.43	0.20	**0.0001**	**0.008**	**0.01**	N.A.

Table 3: mtDNA pairwise populations F_{st} among insular and mainland populations from Corsica, France and Central Italy (N = 257). F_{st} values significant at 5% level are in bold. MC = Massif Central, PY= Pyrenees, WE = Western France, ES = Eastern France, JU = Jura, AL = Alps.

		FRANCE (Mainland)					
	CORSICA	MC	PY	WE	ES	JU	AL
CO	0						
MC	**0.98**	0					
PY	**0.98**	**0.09**	0				
WF	**0.98**	0.01	**0.10**	0			
EF	**0.98**	0.002	**0.08**	0.02	0		
JU	**0.98**	0.05	**0.07**	0.07	0.03	0	
AL	**0.98**	0.02	**0.08**	0.03	0.01	0.03	
ITALY	**0.98**	0.001	**0.13**	0.06	0.01	**0.13**	0.03

LEGENDS OF FIGURES

Figure 1: Map showing sampling localities and the distribution of the Eurasian treecreeper. Mainland France; WE = western France, ES = eastern France, MC = Massif Central, JU = Jura, AL = Alps, PY = Pyrenees; Central Italy, AP = Apennines. Distribution was made using the IUCN distributions (NatureServe and IUCN 2018).

Figure 2: Network based on microsatellites showing genetic distances among insular and mainland populations of *Certhia familiaris*. AL = Alps, WE = Western France, ES = Eastern France, JU = Jura, MC = Massif Central, PY = Pyrenees, C. Italia = Central Italy (Apennines).

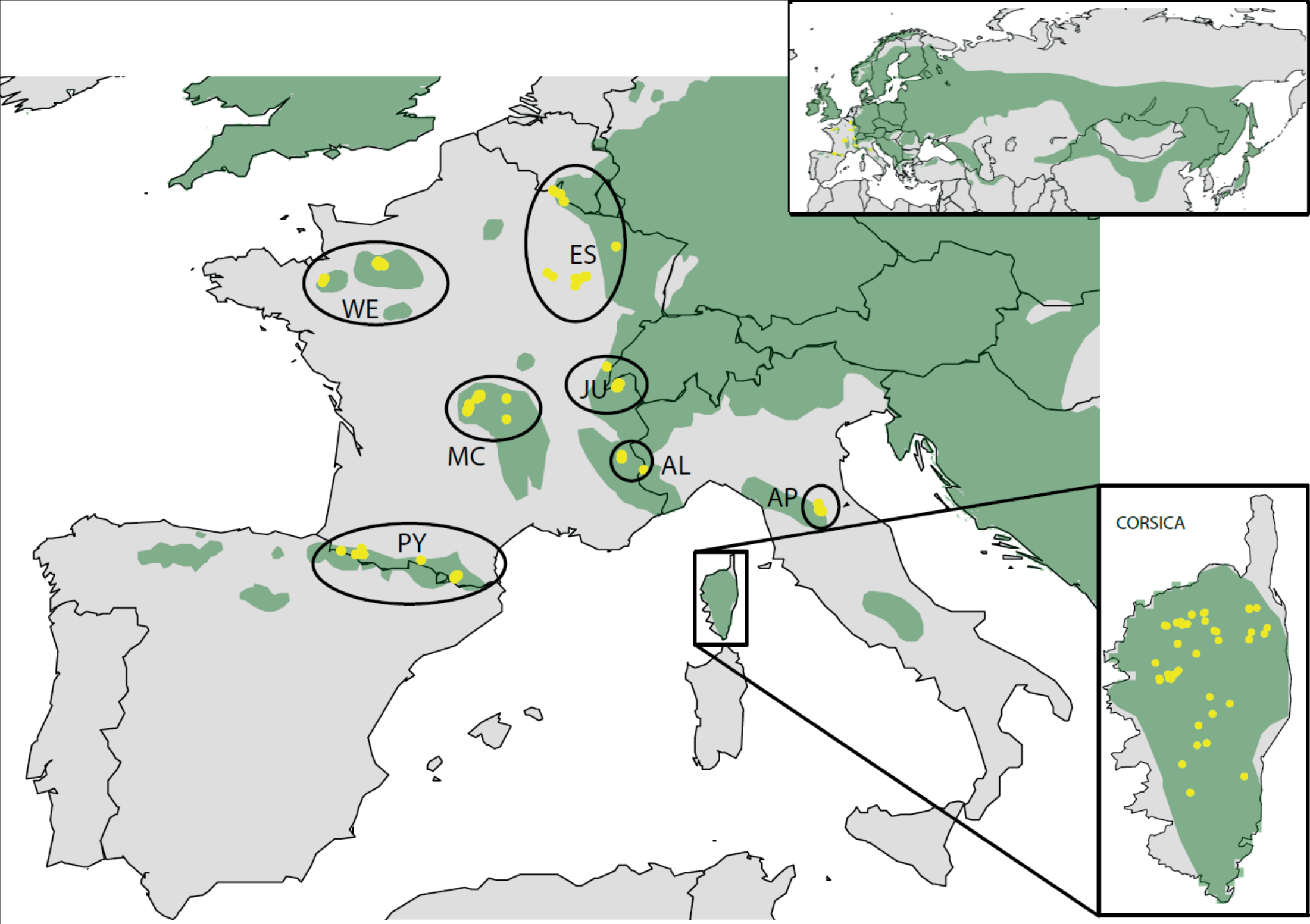
Figure 3: Bayesian clustering analysis of microsatellite data with individual assignment probabilities for K = 3. Five individuals sampled in Corsica (numbers 266, 284, 1010, 1040, 1064) showed significant admixture, i.e. a non-Corsican inferred ancestry with a probability > 50%. AL = Alps, WE = Western France, ES = Eastern France, JU = Jura MC = Massif Central, PY = Pyrenees.

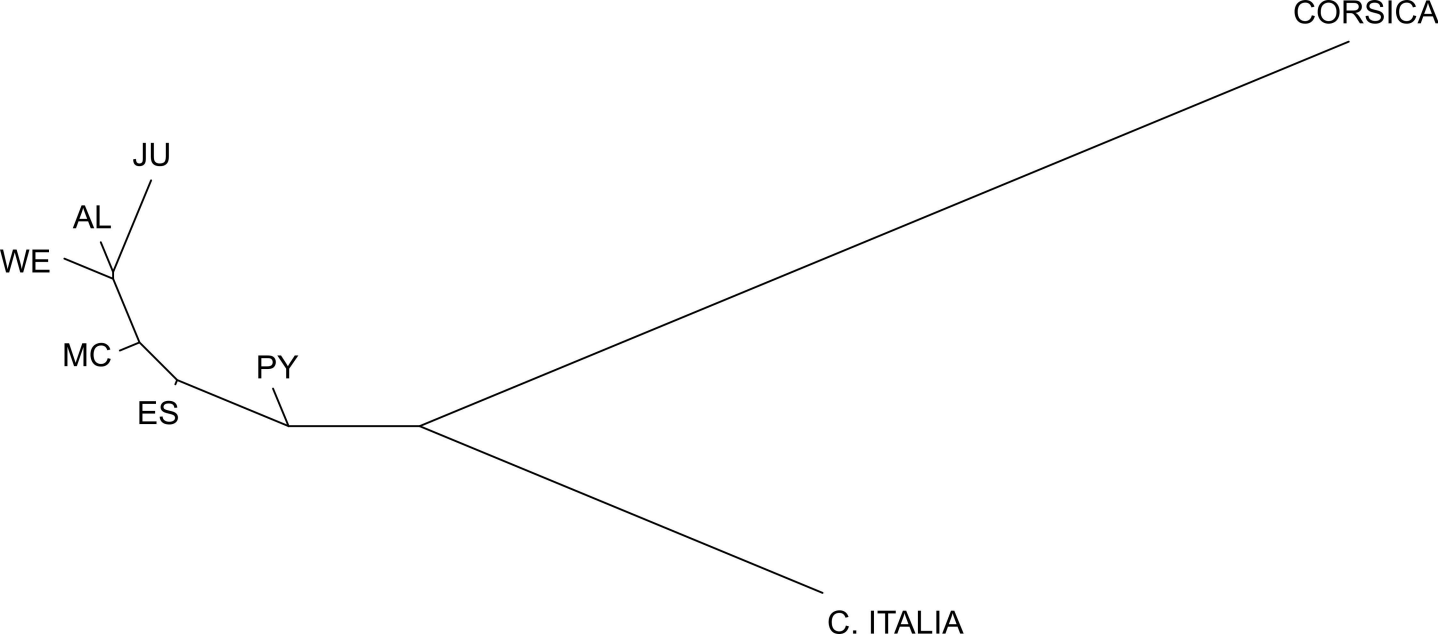
Figure 4: Migrate results of past gene flow estimations among insular and mainland French and central Italian populations (Apennines). Numbers refer to the inferred number of immigrants per generation between the three groups.

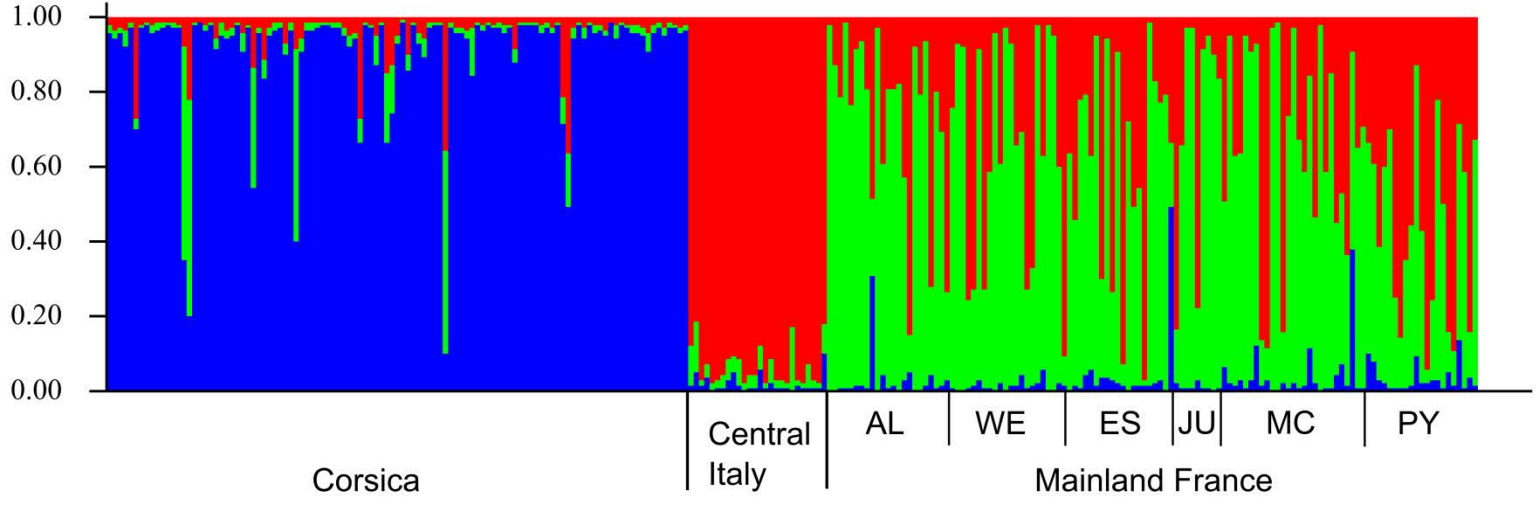
Figure 5: Median joining network showing relationships among COI haplotypes for mainland and insular populations of *Certhia familiaris* (n = 257, 667 bp). The size of each circle is proportional to haplotype frequency. AL = Alps, WE = Western France, ES = Eastern France, PY = Pyrenees, MC = Massif Central, Central Italy (Apennines) = IT, JU = Jura. There is no mixing of insular and mainland haplotypes.

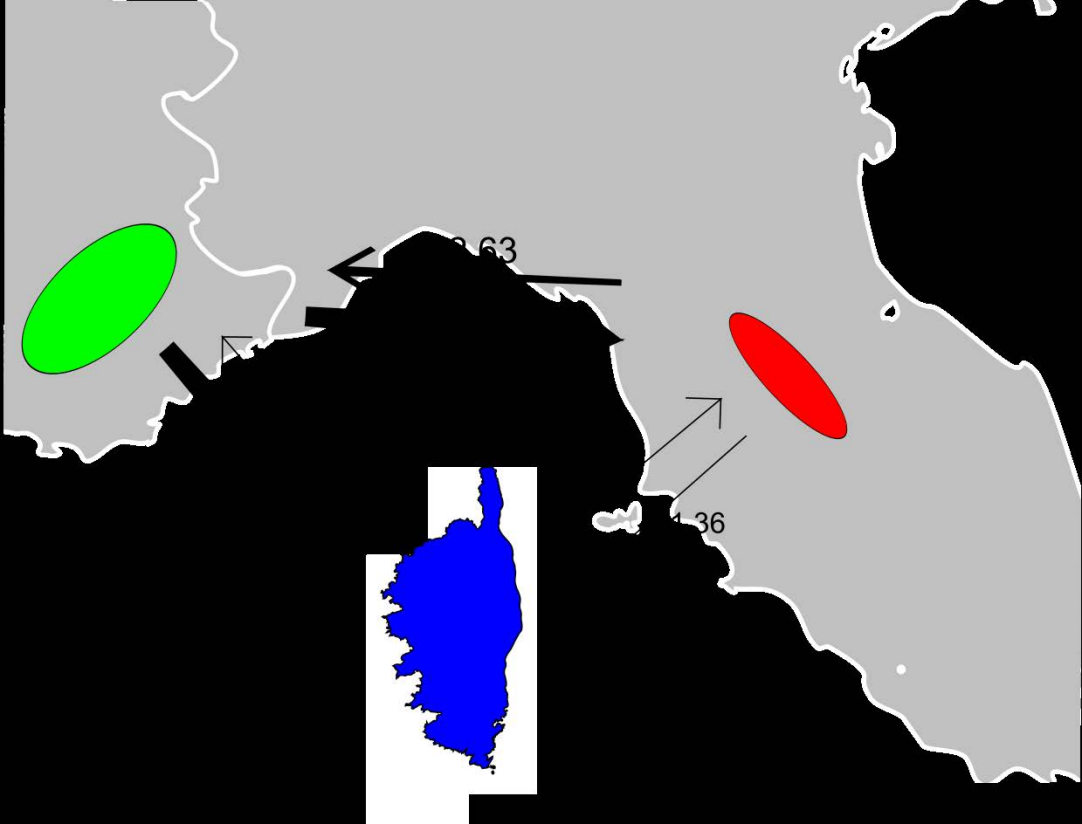
Figure 6: Scatter plot of the first two principal components resulting from a PCA performed on four biometric variables (Wing length, WL; Tarsus length, TL; Bill length at nostrils, BLN; Hind claw length, HCL). Five admixed Corsican individuals that possessed more than

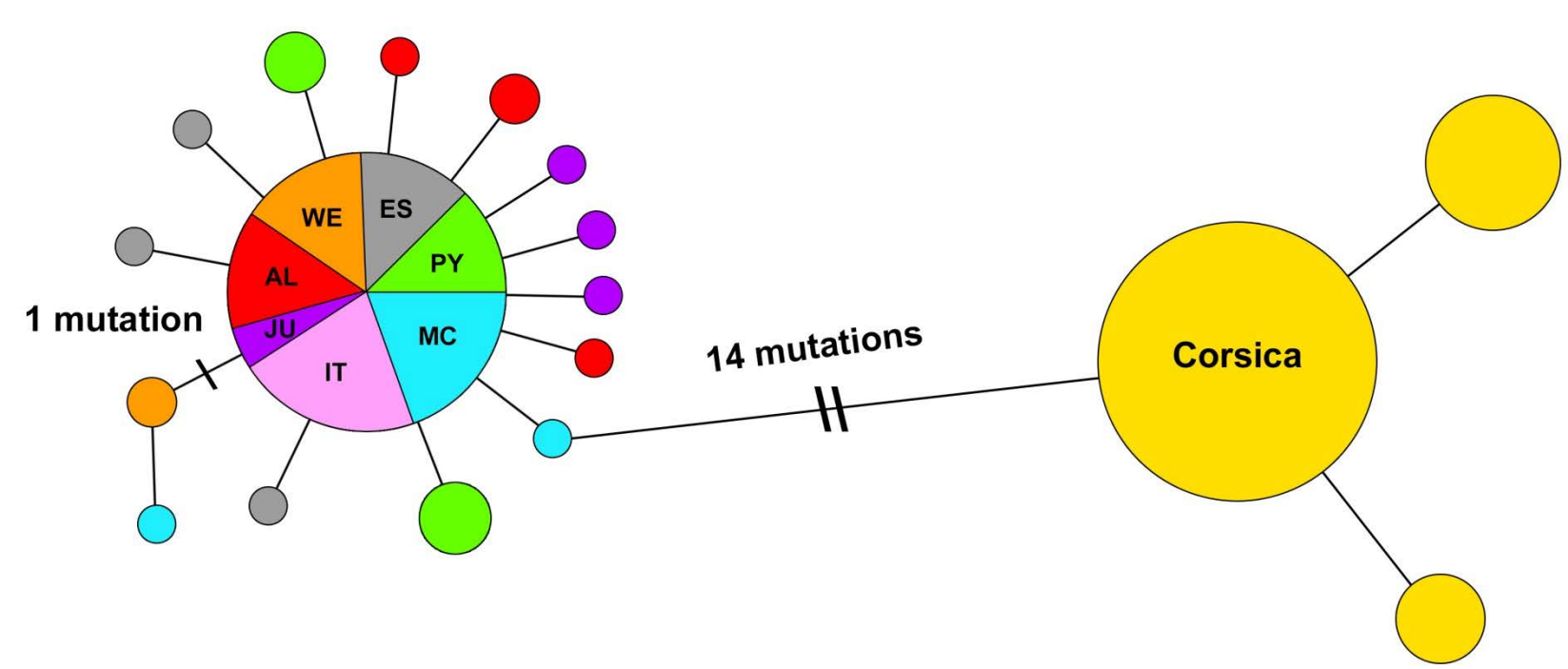
50% of continental alleles are represented by colored squares in Dark blue, Light blue, Green, Black and Red. Confidence ellipses show significant differences between the three main populations; Grey circle = France; Orange circle = Central Italy; Pink circle = Corsica.

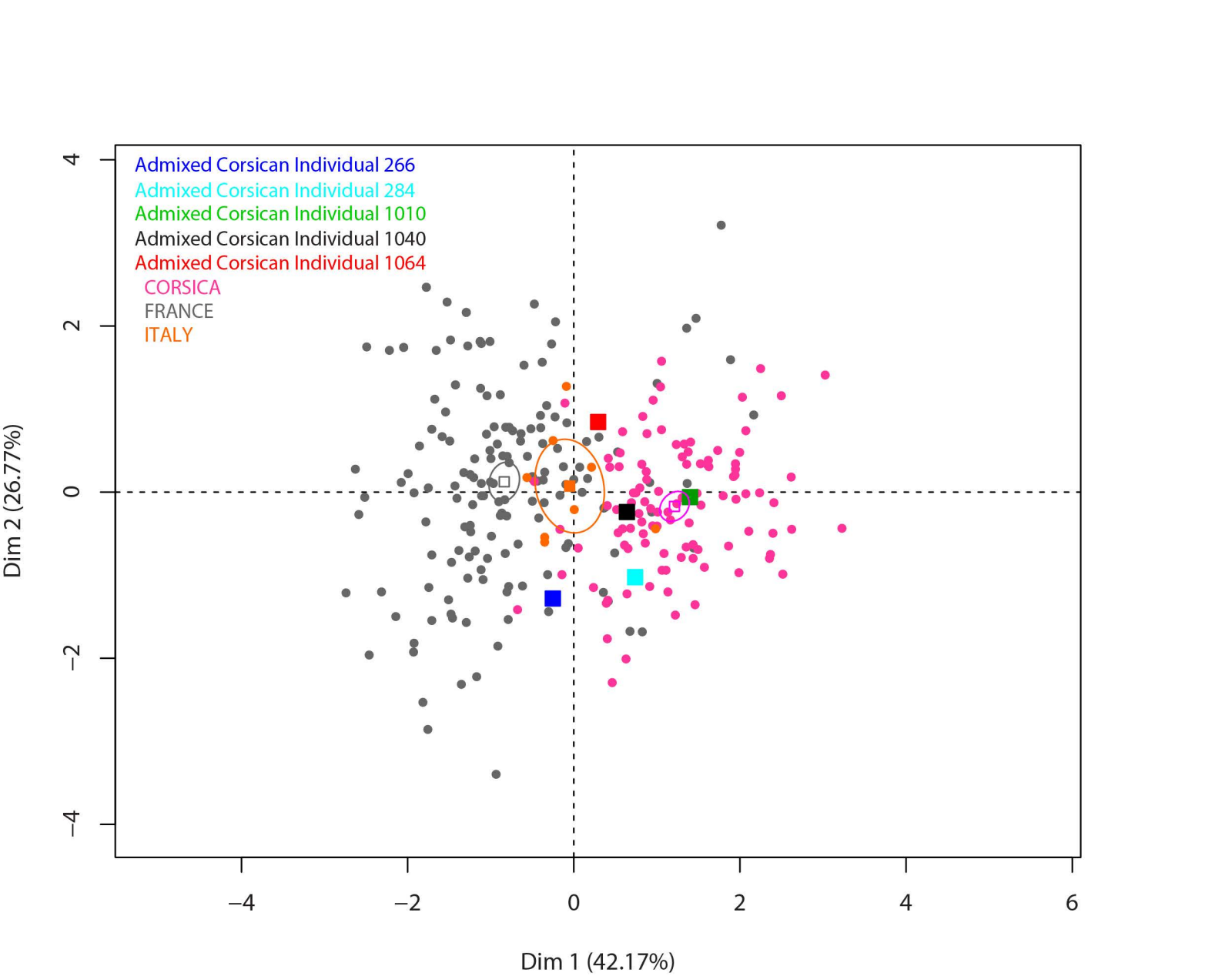

WE
ES
JU
MC
AL
AP
PY
CORSICA


CORSICA
JU
AL
WE
MC
ES
PY
C. ITALIA


1.00
0.80
0.60
0.40
0.20
0.00
Corsica
Central Italy
AL
WE
ES
JU
MC
PY
Mainland France


63
36


WE
ES
PY
AL
JU
MC
IT
1 mutation
14 mutations
Corsica


Admixed Corsican Individual 266
Admixed Corsican Individual 284
Admixed Corsican Individual 1010
Admixed Corsican Individual 1040
Admixed Corsican Individual 1064
CORSICA
FRANCE
ITALY
Dim 2 (26.77%)
Dim 1 (42.17%)
4
2
0
−2
−4
−4
−2
0
2
4
6