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Gene flow and genetic divergence among mainland and insular populations across the south-western range of the Eurasian treecreeper (*Certhia familiaris*, Aves)

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The Eurasian treecreeper (*Certhia familiaris*) comprises two mitochondrial lineages that diverged during the mid-Pleistocene. One palaeoendemic 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 mitochondrial DNA signal, the results for microsatellites clearly demonstrate that the Corsican population (*Certhia familiaris corsa*) is strongly divergent from nearby mainland populations (*Certhia familiaris 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 might 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.

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

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*, *Cytb* and *ND2*), suggested that Eurasian treecreepers found in Corsica belong to a palaeoendemic lineage that has an allopatric range, restricted to this island and the Caucasus region. Corsican and Caucasian treecreepers are currently assigned to two distinct morphological subspecies, *Certhia familiaris corsa* E. J. O. Hartert, 1905 and *Certhia familiaris caucasica* Buturlin, 1907 respectively. All other sampled subspecies (N = 6), including *Certhia familiaris 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).

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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 reflect its evolutionary history correctly. The phylogeographical 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 phylogeographical pattern, with 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) alone with independent nuclear markers has been pointed out (see Ballard & Whitlock, 2004; Toews & Brelsford, 2012). Contrasting results across molecular markers having distinct modes of inheritance, such as mtDNA and microsatellites, might be very useful to infer evolutionary relationships among populations that diverged recently and for which gene flow might not be interrupted entirely (Brito, 2007; Pons *et al.*, 2014; Tritsch *et al.*, 2018).

Phylogenetic relationships among *C. familiaris* subspecies obtained by Pons *et al.* (2015) using three nuclear introns are generally poorly resolved, with the exception of a well-supported sister relationship between Corsican and western European populations. This result, in agreement with geographical distances, is in strong conflict with the mtDNA inferences that support a deeply divergent clade grouping Corsican and Caucasian treecreeper populations. Such mito-nuclear discordance may be explained by several non-exclusive hypotheses, including retention of ancestral polymorphism from the ancestor of the Eurasian and Corsican lineage, positive selection on mtDNA variants, male-mediated gene flow with no consequence for maternally inherited mtDNA, and hybrid sterility in the heterogametic female sex according to Haldane's rule (see Ballard & Whitlock, 2004; Toews & Brelsford, 2012). Pons *et al.* (2015) suggested that retention of ancestral polymorphism might be the most probable hypothesis explaining mito-nuclear discordance in phylogenetic relationships of *C. f. corsa* with its closest relatives. To obtain further insights regarding the evolutionary history of the Eurasian treecreeper and the causes of this mito-nuclear discrepancy, in the present study we investigated the relationships among mainland and insular populations of the Eurasian treecreeper using much larger population samples than those of Pons *et al.* (2015) and fast-evolving nuclear markers.

Our present study compares mtDNA and microsatellite results on population structure, genetic diversity and gene flow between insular and mainland populations of the Eurasian 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 the 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 suggested initially.

We aim specifically to answer the following questions:

1. 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?
2. Are there differences in nuclear and mtDNA gene flow between Corsican and mainland populations?
3. Is genetic variation of Corsican and mainland French populations geographically structured?
4. 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 by Pons *et al.* (2015) is discussed, taking account of the present results. We 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 are 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 2011 and 2016, except birds from the Apennines sampled in 1992 by Guido Tellini during an independent field session. The male 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 playback experiments. The presence of the cloacal protuberance was checked for each bird. We mist-netted 258 males and three females, which were not included in the analyses. Six

discrete geographical populations were sampled in France (Alps, Jura, Pyrenees, Massif Central, Eastern France and Western France, $N = 122$) and one isolated population in Central Italy (Apennines, $N = 26$; Fig. 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 (Supporting Information, Fig. S1). More individuals were sampled in Northern Corsica, where most forest areas are located. Information on exact localities and collectors' names is reported in the Supporting Information (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 and SpuL6-16, Haas *et al.*, 2009; SS1-6, SS1-11, SS1-12, SS2-32, SS2-52, SS2-80, SS2-106, SS2-130 and 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 Biosystems) using GeneScan500 LIZ (Applied Biosystems) as an internal lane size standard. Results were visualized using Peak Scanner (Applied Biosystem) and analysed with Geneious v.9.1.8 (http://www.geneious.com; Kearse *et al.*, 2012).

COI *amplification and sequencing*

The mitochondrial cytochrome *c* oxidase subunit I (*COI*) was amplified and sequenced using primers COIext/FISH1R (Ward *et al.*, 2009; Johnsen *et al.*, 2010).

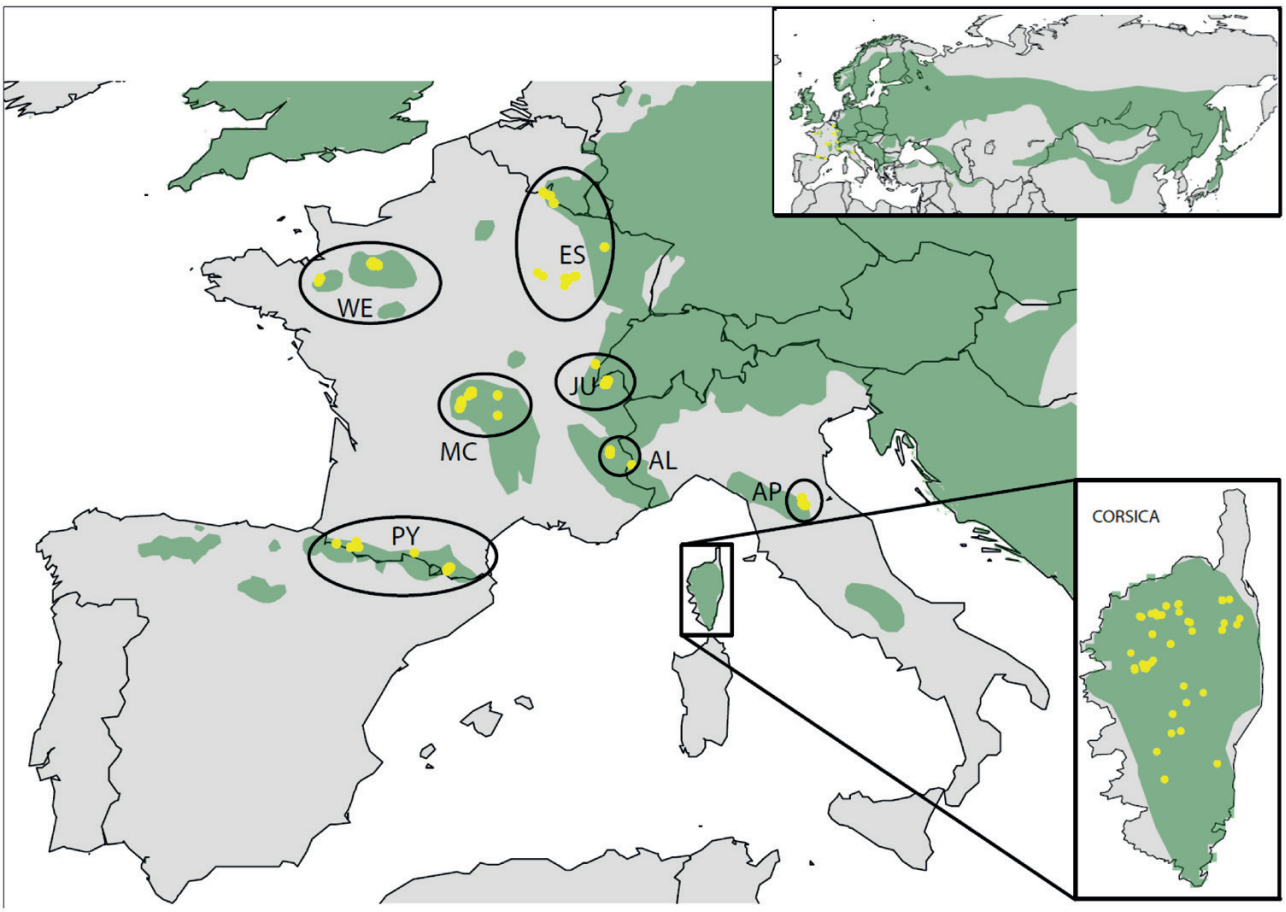

Figure 1. Map showing sampling localities (yellow dots) and the distribution of the Eurasian treecreeper. Mainland France: AL, Alps; ES, eastern France; JU, Jura; MC, Massif Central; PY, Pyrenees; WE, western France; and Central Italy: AP, Apennines. The map was made using the IUCN distributions (NatureServe and IUCN, 2018). Maps were made using the following R packages *maps* and *mapdata* (Becker and Wilks 2013), *maptools* (Bivand and Lewin-Koh 2014) and *scales* (Wickham 2014).

Standard amplification and sequencing protocols were followed. The *COI* sequences were aligned using Bioedit v.7.0.9 (Hall, 1999). New sequences were deposited in GenBank with the accession numbers MK125306–MK125323.

Data analyses

Microsatellites

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

The fixation index (F_{ST}) values between pairs of populations were calculated in Arlequin, with sequential Bonferroni corrections. We performed Bayesian analyses using STRUCTURE v.2.3.4 (Pritchard *et al.*, 2000) to determine the optimal group structure from the microsatellite loci. For the complete data set, the number of K clusters was set from two to ten, with ten iterations per number of clusters and 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 log probability of data (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 and Central Italy) using two approaches that differ in their time frames: BayesAss 3.0.3 (Wilson & Rannala, 2003), which estimates recent (two or three last generations) migration rates between populations using Markov chain Monte Carlo (MCMC), and MIGRATE v.3.6 (Beerli, 2009), which uses an MCMC-based maximum-likelihood approach based on an expansion of the coalescence model (from the present to the most recent common ancestor). In BayesAss, after a few preliminary runs a chain length of ten million iterations with a burn-in of one million iterations and a thinning interval of 1000 was chosen to run the program. Delta values (parameters a, f and m, defining the size of the proposed change to the parameter values at each iteration) set to 0.3 in the final analyses were used for migration rates. Two independent runs were performed, with unique random seeds, to assess convergence. In MIGRATE, the runs consisted of ten short chains (sampling 10 000 trees) and three long chains (sampling 100 000 trees), with a burn-in period of 10 000 trees. All runs were repeated five times to verify consistency of results. To avoid applying a unique mutation rate to the microsatellite loci, we inferred the number of immigrants per generation (N_i) by multiplying the mutation-scaled effective population size (Θ) by the mutation-scaled effective immigration rate (M).

Nei genetic distances among mainland and insular populations were calculated using GENETIX (Belkhir *et al.*, 1996) and visualized with a neighbour-joining network constructed using PHYLIP (Felsenstein, 2005).

Mitochondrial COI

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

Mean Kimura 2-parameter (K2P) pairwise genetic distances among populations were estimated by MEGA6 (Tamura *et al.*, 2013). Standard diversity indices (haplotype diversity, nucleotide diversity and the number of polymorphic sites) were calculated using ARLEQUIN v.3.5 (Excoffier & Lisher, 2010). We used Fu's F_S and Tajima's 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 F_S and Tajima's D represent reliable indicators of whether a population has experienced a population size change. We computed pairwise F_{ST} for all pairs of populations to assess the level of geographical structuring of the genetic variability.

We generated a median-joining network with NETWORK v.4.6.1.2 (Bandelt *et al.*, 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 Eck *et al.*, 2011: fig. 13, p. 76), and bill length at the nostrils (BLN), tarsus length (TL) and hind claw length (HCL) were measured to the nearest 0.1 mm using callipers.

Morphometric analyses

All statistics were performed in R (R Development Core Team, 2018). The 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 ANOVA or the 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 components analysis (PCA) using a correlation matrix was performed with the package FactoMineR (Husson *et al.*, 2018) to visualize how individuals from different geographical origins and admixed Corsican treecreepers were distributed in multivariate space. Given that biometric measurements were obtained from several ringers, we controlled potential measurement biases statistically using a linear mixed-effects model with the package *lme4* (LME4 Authors, 2018) and we also performed a PCA using a covariance matrix (following the recommendation of Perktas & Gosler, 2010). Principal component 1 (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 and SS2-130) were removed from the analysis. The genetic diversity for the six remaining microsatellite loci in each population is summarized in the Supporting Information (Table S2). The allelic richness varied from 1.4 to 7.9. Deviation from the Hardy–Weinberg equilibrium ($\alpha = 0.05$) was found in two cases (Corsica for locus 2, and eastern France for locus 6), but null allele frequencies estimated by FreeNa for these loci and populations were < 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 were close to one (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 (Peery *et al.*, 2012). Based on the expected heterozygosity (H_e), the genetic variation appeared moderate and fairly constant between the samples (0.5–0.7; Supporting Information, Table S2), except for Corsica and Central Italy, which had the lowest H_e (0.3–0.4).

Population differentiation and genetic structure

Values for pairwise F_{ST} 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; mean F_{ST} Corsica vs. Central Italy = 0.30; mean F_{ST} France vs. Central Italy = 0.17; Table 1). In contrast, no significant differentiation was observed among the populations located in France (15 pairwise comparisons). The neighbour-joining network (Fig. 2) clearly shows that Corsican, Central Italian and mainland French populations form three separated groups, with the Corsican population being the farthest one.

Results from STRUCTURE indicated that the Corsican and Italian populations were significantly

Table 1. Pairwise F_{ST} of microsatellites, averaging the variance components over loci

			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

Abbreviations: AL, Alps; ES, eastern France; F_{ST}, fixation index; JU, Jura; MC, Massif Central; PY, Pyrenees; WE, western France; F_{ST}, fixation index.
*Significantly different from zero after Bonferroni correction.

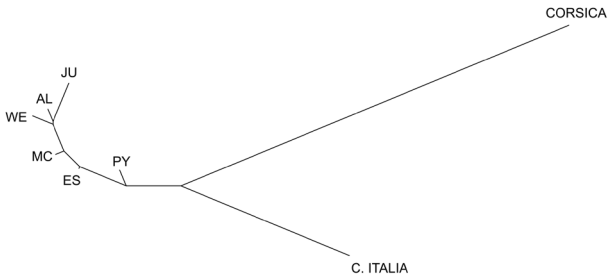

Figure 2. Network based on microsatellites, showing genetic distances among insular and mainland populations of *Certhia familiaris*. Abbreviations: AL, Alps; C. ITALIA, Central Italy (Apennines); ES, eastern France; JU, Jura; MC, Massif Central; PY, Pyrenees; WE, western France.

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 data set (Fig. 3; Supporting Information, Fig. S2). No further genetic substructure was observed within Corsica or within continental France (Supporting Information, Fig. S2). Five individuals sampled in Corsica (numbers 266, 284, 1010, 1040 and 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). Likewise, 34 individuals sampled in continental France also showed significant admixture, whereas no samples from Central Italy presented another inferred ancestry.

Contemporary gene flow among populations

The effective sample sizes for all parameters were all > 200, and the two independent BayesAss3 runs gave very similar results regarding migration rates among the three primary populations (Supporting Information, Table S3), suggesting that our analyses reached convergence.

Estimates for the fraction of the Corsican individuals being migrants from other populations were very low (0.7% from mainland France and 0.4% from Italy), suggesting that migration from the mainland is very rare (Supporting Information, Table S3). Estimates for the fraction of the Italian individuals being migrants from other populations were slightly higher but still very low (1.44% from Corsica and 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 were < 0.5%. Overall, these results suggest that contemporary gene flow between insular and mainland lineages is very low. Given that the confidence intervals of gene flow estimates were very large and overlapping, our results did not allow any detailed comparisons of the contemporary gene flow among populations to be made (Supporting Information, Table S3).

Historical gene flow

MIGRATE results suggested asymetrical past gene flow between the three groups (Fig. 4), with 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 was low but not null (close to one immigrant per generation). This asymmetry was also reflected in the estimation of population size, which was six times larger in mainland France ($\Theta = 9.24$) than in Corsica ($\Theta = 1.49$) and Central Italy ($\Theta = 1.7$). Additionally, we enforced a more realistic migration model, in which Corsica was 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 (Fisher's exact test, $P = 1$). Our results did 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 were significant (Table 2). Genetic diversity parameters (H and π) varied from moderate to low values depending on geographical populations (Table 2). Our results did not suggest any differences in genetic diversity between the insular and mainland populations. The most striking result was a complete lack of genetic variability for the Italian population. In France, haplotype diversity was highest in mountainous populations (Alps, Jura and Pyrenees).

Genetic divergence

The K2P genetic distances among mainland populations from France and Central Italy were very low, varying from 0.03 to 0.06% (Supporting Information, Table S4). In contrast, the K2P genetic distances between the insular population from Corsica and mainland populations from France and Central Italy were much higher, varying from 2.3 to 2.4%. Accordingly, pairwise population F_{ST} values between the Corsican population and mainland populations were highly significant and close to one (Table 3),

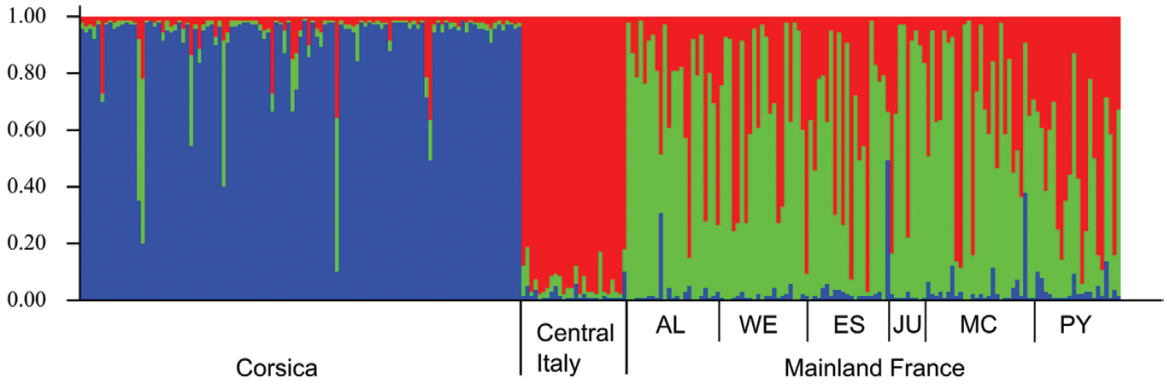

Figure 3. Bayesian clustering analysis of microsatellite data with individual assignment probabilities for $K = 3$. Five individuals sampled in Corsica (numbers 266, 284, 1010, 1040 and 1064) showed significant admixture, i.e. a non-Corsican inferred ancestry with a probability > 50%. Abbreviations: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

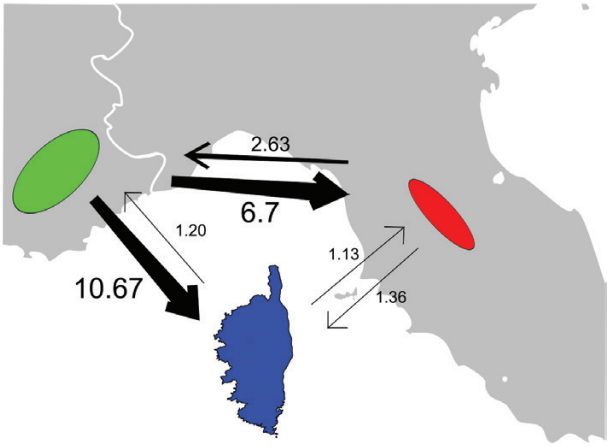

Figure 4. MIGRATE results of past gene flow estimations among insular and mainland French and central Italian populations (Apennines). Numbers refer to the inferred number of immigrants per generation between the three groups.

indicating a near-complete geographical partitioning of the genetic variation. In contrast, among mainland populations our results showed a lack of genetic differentiation except for the Pyrenean population, which was slightly but significantly differentiated.

Median-joining network

In accordance with the genetic results described above, the median-joining network (Fig. 5) showed that Corsican treecreepers did not share any haplotypes with mainland treecreepers sampled in either France or Italy. Likewise, no Corsican haplotypes were found among mainland populations from France and Central Italy. The lack of geographical mixing of insular and mainland haplotypes clearly suggests that female-mediated gene flow across the Mediterranean Sea is non-existent or so low that much larger sample sizes would be necessary to detect it.

MORPHOMETRY

Univariate analyses

One-way analyses indicated that there were highly significant differences between the insular and mainland populations for most morphological variables. In particular, the insular population had a longer BLN and a shorter HCL (Supporting Information, Table S5) than both Italian and French mainland populations.

Multivariate analyses

More than 68% of the total variance was explained by the first two principal components (Fig. 6). Component loadings of the variables are reported in the Supporting Information (Table S6). Bill length at the nostrils, tarsus length and hind claw length were highly correlated to the first axis (PC1), which can be interpreted as a size axis. ANOVA using a linear mixed-effects model, with 'ringer' as a random factor, performed on the first axis of the covariance matrix PCA showed highly significant differences among the three populations ($F_{2,240} = 17.63$, $P < 0.0001$). Nevertheless, there was no clear separation among the three populations according to principal component 2 (PC2; Fig. 6). Insular birds from Corsica formed a morphological cluster fairly well separated from French and Italian clusters despite some overlap. Confidence ellipses of each geographical population were clearly separated from each other (Fig. 6), with the Italian population occupying an intermediate position between French and Corsican. Clearly, more samples from Central Italy would be necessary to assess morphological variation

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* (667 bp)

		France (mainland)						
Parameter	Corsica	MC	PY	WE	ES	JU	AL	Italy
N_i	108	27	23	21	20	9	23	26
N_h	3	3	3	2	4	4	4	1
N_p	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	
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 F_S	−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.

Abbreviations: AL, Alps; ES, eastern France; JU, Jura; MC, Massif Central; PY, Pyrenees; WE, western France. N_i, number of individuals N_h, number of haplotypes; N_p, number of polymorphic sites; n.a., not applicable.
*Significant values supporting population expansion. Fu's F_S statistic was considered to be significant if the P-value was < 0.02 (Excoffier & Lischer, 2010).

Table 3. Mitochondrial DNA pairwise populations F_{ST} among insular and mainland populations from Corsica, France and Central Italy (N = 257)

		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

Abbreviations: AL, Alps; ES, eastern France; F_{ST}, fixation index; JU, Jura; MC, Massif Central; PY, Pyrenees; WE, western France.
*F_{ST} values significant at the 5% level.

of the population of the Apennines more rigorously. Nevertheless, our results clearly suggest that the Corsican population is morphologically divergent from the nearby mainland populations. Of the five admixed Corsican treecreepers possessing > 50% of continental alleles, three were located with other 'pure' Corsican birds. Two admixed individuals (individual numbers 266 and 1064; dark blue and red squares, respectively, in Fig. 6) were close to mainland Italian and French birds. However, the lack of a clear gap between insular and continental morphological clusters allows us to conclude only that admixed birds are probably more similar morphologically to Corsican than to mainland treecreepers.

DISCUSSION

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 fivefold 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 (K2P genetic distance > 2%, pairwise F_{ST} > 0.90). In accordance with mtDNA, our microsatellite 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 (Fig. 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 in addition to microsatellite alleles never found in nearby mainland populations. Interestingly, five Corsican treecreepers (i.e nearly 5% of our sample) possessed > 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.

Although no mitochondrial gene flow across the Mediterranean Sea was detected in the present study (no mixing of insular and mainland populations in any

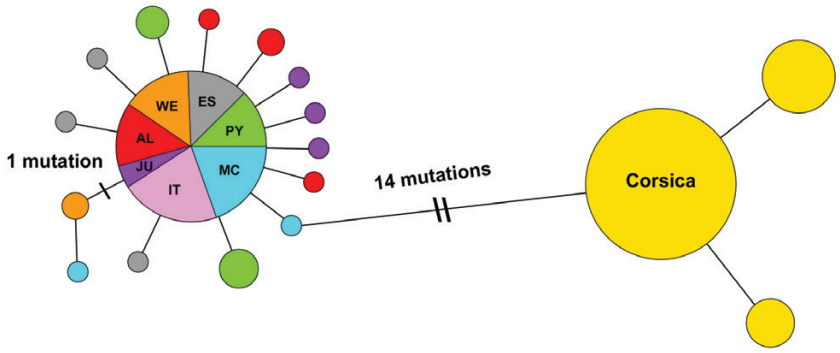

Figure 5. Median-joining network showing relationships among *COI* haplotypes for mainland and insular populations of *Certhia familiaris* (*N* = 257, 667 bp). The size of each circle is proportional to haplotype frequency. Abbreviations: AL, Alps; ES, eastern France; IT, Central Italy (Apennines); JU,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

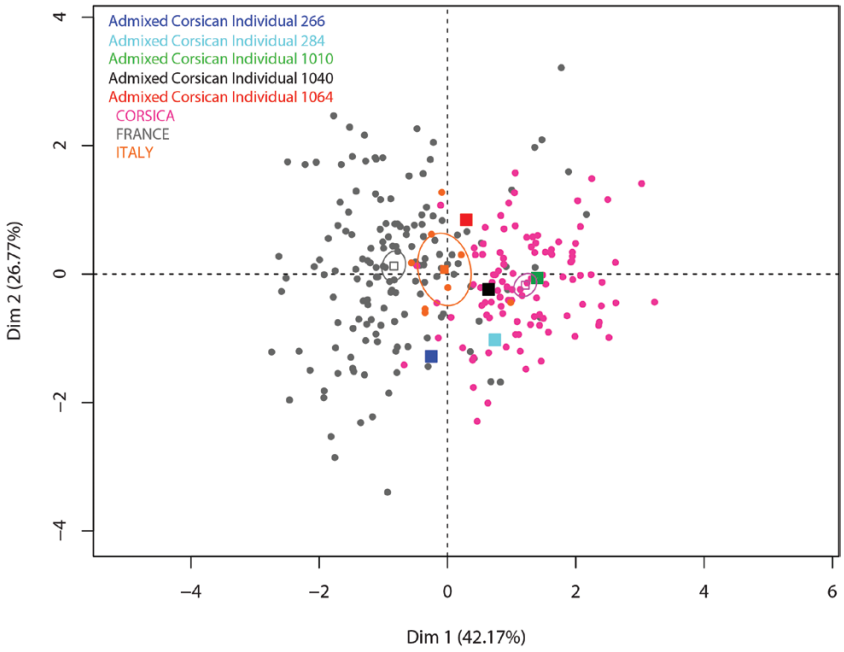

Figure 6. Scatter plot of the first two principal components resulting from a principal components analysis performed on four biometric variables (BLN, bill length at nostrils; HCL, hind claw length; TL, tarsus length; WL, wing length). Five admixed Corsican individuals that possessed > 50% of continental alleles are represented by coloured squares in dark blue, light blue, green, black and red. Confidence ellipses show significant differences between the three main populations (grey ellipse, France; orange ellipse, Central Italy; pink ellipse, Corsica).

sampled populations), our results for the microsatellites 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 also supported by the detection of five Corsican treecreepers possessing both insular mtDNA haplotypes and microsatellite alleles mostly of continental origin. Such mito-nuclear discordance can arise from several non-exclusive mechanisms (reviewed by 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 (Li & Merilä, 2010; Both *et al.*, 2012; Dobson, 2013). If males are the main long-distance dispersers in the Eurasian treecreepers, this might 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 Eurasian treecreepers. Another possible explanation is selection against hybrids of the heterospecific sex (female in birds), as expected if 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 result solely in the introgression of continental nuclear alleles into the Corsican population. Furthermore, incomplete lineage sorting of microsatellite alleles, attributable to longer coalescent times of nuclear markers compared with 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 slightly 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 provided 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 might occasionally reach Corsica.

We did not detect any significant genetic structure within Corsica for the Eurasian treecreeper; this situation is in contrast to what has been found for *Sitta whiteheadi* (Thibault *et al.*, 2016), another forest passerine endemic to Corsica. This difference between the two species might be explained by their distinctive habitat requirements, the Corsican nuthatch occurs only in mature scattered forest 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 J.-C., 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 with 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).

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, possessing only the most widespread, and thus probably the most ancestral, haloptype found all over Europe. Consequently, mtDNA pairwise F_{ST} values with the northern French populations were low and most often not significant. In contrast, our microsatellite results showed significant genetic divergence (F_{ST} = 0.16), with 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 also have favoured nuclear genetic admixture, yet most Italian treecreepers are not admixed (Fig. 3). The high mutation rate of microsatellites compared with mtDNA, associated with the low demographic size of the Apennine population, might 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 geographical range, isolated from the northern Alpine and southern Abruzzi populations (Meschini & Frugis, 1993), with numbers estimated at only a few hundred birds (Tellini *et al.*, 1997). Owing to the small geographical range and the founder effect, the Eurasian treecreeper population from the Apennines most probably had a small effective population size, allowing microsatellite differentiation despite the longer coalescence time of nuclear markers compared with mtDNA (Zink & Barrowclough, 2008).

Both the lack of mitochondrial variability and the low diversity of microsatellites of the Apennine population might be explained if we consider that it was founded from a subset of the ancestral genetic lineage (Hewitt, 1996). In this scenario, Central Italy was colonized from northern European populations that would have been expanding rapidly 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 biogeographical scenario is also 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. Nevertheless, more samples, covering a larger geographical area in Italy, would be required to test this phylogeographical pattern robustly.

Another phylogeographical scenario might be possible, in which the Apennine population would have diverged in the Italian peninsula during the Pleistocene. In this scenario, the 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 have highlighted three Mediterranean primary refugia not covered by ice masses (the Iberian Peninsula, the Italian Peninsula and the Balkans), where populations persisted during the cooling periods and were able to colonize northern areas during the warming periods (Hewitt, 2000; Weiss & Ferrand, 2007). Several studies report genetic divergence of Italian vertebrate lineages dating back to the Pleistocene (Brito, 2005; Ruedi *et al.*, 2008; Lo Brutto *et al.*, 2011). However, our genetic data do not give strong credence to such a phylogeographical scenario for the Eurasian treecreeper, because in the case of ancient bottleneck events, we would expect at least some mtDNA differentiation.

Mainland French populations

The six geographically isolated populations found in France are not genetically differentiated, except for the Pyrenean population, which is significantly divergent based on our mtDNA results ($0.06 < F_{ST} < 0.13$). This lack of genetic divergence was expected for the 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). Given that these populations are more-or-less connected, their genetic pools are most probably homogenized continuously by ongoing gene flow. The western population was discovered only in the late 1970s (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. Lack of genetic divergence of this small population might be explained both by its probable very recent foundation and by 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). Likewise, the Rhone valley is probably not an effective dispersal barrier, and therefore, gene flow between the Massif Central and Alpine populations is likely to be 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 *C. f. 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 (~1 Mya). According to Pons *et al.* (2015), *C. f. corsa* does not share a common recent evolutionary history with the nearby mainland treecreeper populations, which all belong to a widespread lineage that probably arrived recently in Western Europe. Owing to its spatiotemporal 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 (mtDNA K2P ~2%, nuclear F_{ST} ~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). *Certhia familiaris 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 might 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. Nevertheless, we 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 *C. f. corsa* before proposing any systematic arrangement.

Conservation issues

Based on its significant genetic distinctiveness and low genetic diversity highlighted in the present

study, in addition to its small population size and isolated geographical distribution, we suggest that the Eurasian treecreeper population from the Apennines warrants treatment as a distinct management unit (*sensu* Moritz, 1994). Information on the 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, such as birds (i.e. *Sylvia cantillans*, Brambilla *et al.*, 2008; *Carduelis corsicana*, Pasquet & Thibault 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 on the 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* and *Fagus sylvatica*) found only in mountainous areas of the island (Thibault & Bonnacorsi, 1999; Thibault J.-C., unpublished results.). The relatively small size of the breeding population, estimated at 5000–10 000 pairs (Thibault J.-C., unpublished results), should be managed independently of the continental populations, which have an extremely large range that encompasses 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. Owing 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 in Annex 1 of the European Birds Directive that lists endangered species and subspecies in Europe.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. List of specimens and localities included in the study.
Table S2. Estimators of genetic diversity in each population for the five microsatellite loci. Abbreviations: G-W, Garza–Williamson index; H_e and H_o, expected and observed heterozygosity; N_a, number of alleles; N, number of individuals. Allelic richness is also given for each locus.
Table S3. Fraction of individuals in population 1 that are migrants derived from population 2 (per generation) as estimated by BayesAss+. Results from the two runs are presented. Numbers between brackets represent the 95% highest posterior density.
Table S4. Mitochondrial DNA genetic distances (K2P, as a percentage) among populations of Corsica, France and Central Italy (N = 257).
Table S5. Morphological variations in hind claw length (HCL) and bill length at nostrils (BLN) among insular (Corsica, CO) and mainland populations (French, FR; Central Italy, IT) of the Eurasian treecreeper. Insular birds have a longer bill length and shorter hind claws than mainland birds.
Table S6. Results of a standardized principal components analysis using a correlation matrix performed on Eurasian treecreepers with four biometric variables.
Figure S1. Sampling localities and sample size per locality (in brackets) in Corsica.
Figure S2. Results of ΔK statistics for the complete data set (A; best K = 3), the analysis with only the Corsican samples (B) and the analysis with only the samples from continental France (C). The curves in B and C show several modal values for ΔK, indicating that no groups were detected within these data sets.