Seasonal Genetic Structure Analysis of a Resident Population of European Robin

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Abstract: This study is based on the microsatellite DNA analysis of 160 birds from the resident population of the European robin (*Erithacus rubecula*) in South Italy. It used assignment procedures to study population genetic structure on a seasonal and spatial scale. This population undergoes, twice each year, an altitudinal and intraregional short-distance migration. The present analysis corroborates inference on short-distance altitudinal migratory phenomenon in a resident robin population and suggests that the resident population investigated by us is "faithful" to its intraregional breeding and wintering areas. Our data further indicate that the resident population may represent a distinct genetic entity and suggest that regional ecological features along this geographical area as well as some behavioural characteristics of birds may concur in the maintenance of population separation.

Keywords: *Erithacus rubecula*, population genetic structure, phenology, microsatellite.

INTRODUCTION

The European robin (*Erithacus rubecula*) is a small passerine bird that breeds in higher and mid-latitudes of the Western Palearctic [1, 2]. Like other passerine species, the European robin shows a variegated pattern of movements in the non-breeding period. There are long-distance migrating populations of robins in Europe and there are other populations, generally in sourthern Europe, that only migrate short distances between their breeding and wintering grounds [3, 4, 5]. The latter populations are referred to as resident populations in this manuscript. Continental Europe, including the entire Mediterranean area, is traversed by at least four allohiemic robin populations that differ from each other in the geographical location of their winter quarters and migration routes as well as in migration timing [6]. These long-distance migrants make brief stop-overs, to rest and refuel, in the Mediterranean basin.

An adjacent area of the Palinuro Peninsula in South Italy (ca. 350 km south of Rome) is an important stop-over site, to rest and refuel in spring, for one of the long-distance migratory robin populations. Notably, in this area there are resident populations of robin that breed in the mountain and winter in the adjacent river valley and coastal area (our unpublished data under the guidance of late Prof. M. Milone) [7]. Ringing, as standard monitoring technique, has demonstrated that such resident robins exhibit strong fidelity to there wintering and breeding territories as well in North and South Italy [8] as in several other localities of the Mediterranean basin [9, 10, 11]. What may have caused the presence of several extant resident populations of the European robin along the Mediterranean basin has been discussed by several researchers [12, 13, 14, 15, 16, 17, 18, 19]. These studies suggest that early occupation of the most suitable feeding areas during wintering by some long-distance migratory populations may have induced them to remain and become resident populations. Although still a matter of active debate as to how and why, such geographically-defined resident robin populations do persist despite the annual arrival in such areas of relatively large numbers of long-distance migrant conspecifics. The migrant conspecifics may cause a substantial population-admixture with resident populations during the migratory periods [20].

Studies of this sort require the recognition of individual birds of a given population at each capture. Bird migration and bird population dynamics have been studied by a variety of techniques of which ringing is undoubtedly the oldest and probably the most common. Colour marking, use of radar, radio- and satellite tracking and use of stable hydrogen isotopes are some of the newer techniques employed in such studies [21]. Some of these methods are based on the recognition of single individuals whose morphological characters (such as body size, body mass, wing length) become part of the whole scheme in making population structure analysis. The availability of some recent molecular techniques allows genetic fingerprinting of single individuals. Such techniques are non-invasive and represent a highly efficient tool to reconstruct complex population structures, parental relationships and geographical dispersion or aggregation.

The objective of the present study was to assess the population genetic structure of the European robin across a resident population in a small, geographically well-defined, area in South Italy (Cilento Mountain range, Mingardo river valley

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and estuary within the Cilento & Vallo di Diano National Park). It has been verified repeatedly, by ringing methods, that the European robin populations in Campania Region primarily display altitudinal and short-distance migration, mountain-valley-mountain. During their total absence in the mountain breeding area, they are seen in an adjacent valley/coastal area. The Mingardo river valley area was selected because it is here that the majority of individuals from the mountain breeding population descend for wintering and the long-distance migrant conspecifics make a stop-over during their North-bound migration in spring (Fig. **1**). Within the study area breeding population gathers at an altitude of approximately 900 meters or more and the wintering population settles at near sea level in the river valley and the contiguous coastal area . The investigated area therefore provides an interesting situation to study population structure on a seasonal scale and to compare genetic diversity, if any, between mountain and Mingardo River Valley residents. In the present study we used polymorphic microsatellite markers (extracted and purified from a single feather from each robin) and assignment procedures to investigate gene flow and population structure of *E. rubecula* at two different spatial scales within a fragmented mountain-valley landscape. This study will complement previous morphological and population survey work and may provide evidence for any genetic admixture between individuals of the resident population and the long-distance migrant conspecifics of the European robin.

MATERIAL AND METHODOLOGY

1. Study Area and Sampling Strategy

During the years 2005 and 2006 we sampled a resident population from the breeding (mountain – ca. 900 – 1200 m altitude) and wintering grounds. Sampling locations are shown in Fig. (**1**). *E. rubecula* is endemic to the region and is not classified as vulnerable; nonetheless, our study was conducted under the necessary authorizations. We mist-netted, measured and ringed a total of 160 robins in the study area belonging to the Cilento & Vallo di Diano National Park in South Italy, using standard protocol of identification [22]. Only adult birds (males and females) were used in this study. A single feather was plucked from each bird for the extraction of genomic DNA. These robins undertake short distance migration, twice-a-year, in response to changing photoperiod and seasonal surplus or scarcity of food (unpublished data) [23]. During the breeding period (May – July) practically all resident birds move to the mountains of Cilento, at an altitude of approx. 900 – 1200 m. Sixty of these adults (M; n = 60) were sampled in an area of approx. 105 km^2, between late June and early July 2005. We define the breeding robins sampled by us as a population because it is a large group of individuals separated from the neighbouring breeding groups by deep and large valleys. In autumn the mountain population descends in the nearby valley and its contiguous coastal area of the National Park. Valley- and coastal area-dwelling robins were sampled in a ca. 5-km X 6-km area, at the end of Octo-

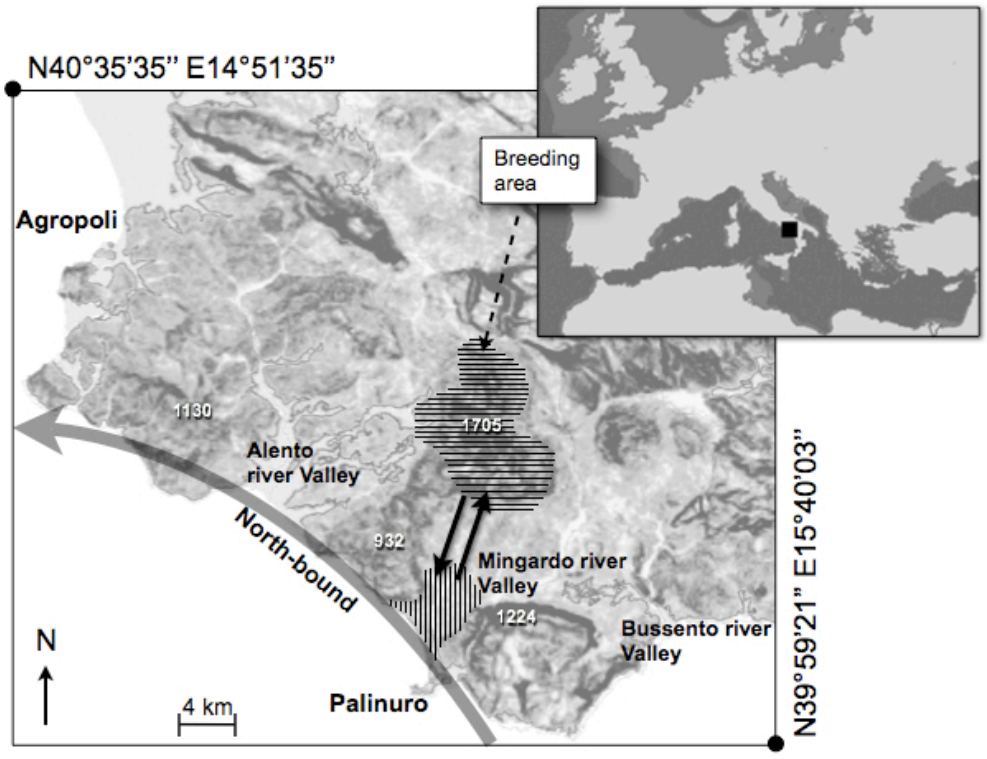

Fig. (1). Geographical location of the Cilento & Vallo di Diano National Park in South Italy. Sampling areas are shown by vertical hatching (wintering area) and horizontal hatching. The main altitudinal short-distance migratory movements of the resident population studied by us is shown by black arrows. Grey arrow indicates the North -bound stream of the long-distance migrant conspecifics.

ber 2005 (V_O; n = 20) as well as in late March - early April 2006 (V_A; n = 20) when the long-distance migrant conspecifics flock in this area. Sampling in December 2005 (V_D; n = 20), January 2006 (V_J; n = 20) and February 2006 (V_F; n = 20) covered the typical wintering months of this species in Campania Region of Italy.

For the field preservation of feathers we used a preservation solution (20% DMSO, 25% EDTA 0.25 M pH 8.0) saturated with NaCl (5 M) and thymol in traces. Birds were released after ringing, body measurements and feather sample collection.

2. Genetic Characterization

Genomic DNA was extracted from each feather by using Chelex (Bio-Rad) synthetic resins. DNA extraction was processed according to Taberlet and Bouvet with some minor modifications [24]. Each feather was cut transversely from the base; this was cut longitudinally and put in a tube for digestion with proteinase K. Digestion was carried out overnight at 50°C with gentle shaking in 5% Chelex 100. Each digested sample was vortexed and again incubated at 80°C for 10 min in Chelex. Subsequently, the sample was centrifuged at 13.000 rpm to separate supernatant from Chelex spherules. The upper phase was filtered through a Bio-101 tube to eliminate Chelex residuals and then utilized for PCR amplification after quantification.

Microsatellite analysis was performed on six polymorphic loci. The flanking region of microsatellites may be retained in closely related taxa [see 25, 26, 27, 28] and as such, microsatellite primers developed for one species may be useful for amplification and analysis of other species. In this heterologous amplification, the primers annealing probability in the retained loci decreases as evolutionary distance between the examined species increases [27, 29]. Ten microsatellite couples of primers were screened and only six of these, related to six polymorphic loci, were selected: Ck.1B5D and Ck.4A3G described in *Corvus kubary* [30]; LOX1 and LOX2 described in *Loxia scotica* [31]; HrU2 and HrU7 described in *Hirundo rustica* [27].

PCR amplification was performed in a final reaction volume of 25 µl containing 85 ng of genomic DNA and 0.5 µl of each primer (1 µM). Each reaction mixture included 1 U of *Taq* polymerase, PCR buffer (160 mM $(NH_4)_2SO_4$; 670 mM Tris-HCl pH 8.8); 15 mM $MgCl_2$; 0.1% Tween 20, 0.2 mM dNTP and 2 mM $MgCl_2$. All PCRs were performed in an Eppendorf thermocycler. Amplification thermal profile for each microsatellite primer was tested and standardized. PCR conditions for Ck.1B5D and Ck.4A3G involved annealing at 50°C ; for primers LOX1 and LOX2 involved annealing at 54°C; for primers HrU2 and HrU7 involved annealing at 55°C. Each reaction ended with a final 5-min extension at 72°C. In order to verify the presence of tandem repeat, the amplification products were purified with Amersham PCR purification kit and sequenced using the BigDye™ Terminator Cycle Sequencing chemistry (Applied Biosystem™ product) protocols. The sequences were recorded with an ABI3100 automated sequencing instrument (Perkin-Elmer™). For the detection and sizing of allele fragments, one primer from each pair was end-labeled with a fluorescent dye (FAM and HEX, MWG Biotech™) and each microsatellite locus was screened with capillary electrophoresis in ABI3100.

3. Elaboration

Pair-wise linkage disequilibrium was computed using Linkage Disequilibrium test for all pairs of loci [32]. Population genetic structure was analyzed in several ways: the level of variation using mean number of allele (MNA), expected heterozygosity (He), observed heterozygosity (Ho) per population and pair-wise genetic differences [33, 34]. These analyses were done with ARLEQUIN version 2.0 [35]. Patterns of differentiation were visualized by a Factorial Correspondence Analysis (FCA) of individuals' multilocus score using GENETIX version 4.04 [36]. This multivariate analysis is based on a $\chi 2$ value computed by comparing the observed distribution of alleles in genotypes with that expected if alleles were randomly assigned to individuals. The computed $\chi 2$ were elaborated and assigned to genotypes, and usually only the first two variables were plotted [37, 38].

An identification of population affinity of individual samples was done using STRUCTURE version 2.1 [39, 40], assuming no prior information, the admixture model with correlated alleles, and a burn-in phase of 10 000 iterations followed by a run phase of 100 000 iterations. The number of populations (K) ranging from 2 to 13 was tested in three independent runs to establish consistency. The posterior probability was then calculated for each value of K to chose the most likely K. We also used a model with essentially the same parameters as earlier, but providing prior information of population membership, to identify immigrants if any, or in-

Table 1. Genetic Diversity in the Valley Temporal Populations (V) and Mountain Breeding Populations (M)

Population	N	MNA(SE)	Lower	Upper	He(SD)	Ho(SD)
V_O	20	3.33(0.42)	2.24	4.41	0.440(0.2)	0.480(0.2)
V_D	20	3.66(0.33)	2.80	4.52	0.347(0.3)	0.507(0.2)
V_J	20	4.50(1.38)	0.94	8.05	0.479(0.2)	0.634(0.1)
V_F	20	3.33(0.49)	2.06	4.60	0.333(0.2)	0.453(0.2)
V_A	20	2.16(0.16)	1.73	2.59	0.142(0.1)	0.250(0.2)
M	60	4.53(1.89)	0.19	9.52	0.487(0.1)	0.653(0.1)

MNA: mean number of allele, SE: standard error, He: expected heterozygosity, Ho: observed heterozygosity., SD: standard deviation. Lower and Upper 95% confidence interval is reported.

Table 2. Pair-Wise Conventional F-Statistics from Temporal Groups in the Valley (V) and Breeding Population in the Mountain (M)

	V_O	V_D	V_J	V_F	V_A	M
V_O	-					
V_D	0.047*	-				
V_J	0.055*	0.000	-			
V_F	0.076*	0.008	0.031*	-		
V_A	0.170*	0.118*	0.136*	0.086*	-	
M	0.054*	0.004	0.007	0.000	0.111*	-

*indicates significant distance level, $P < 0.05$.

dividuals who have recent immigrant ancestry [34]. This approach is a powerful tool to analyze population admixture when it is suspected that some samples might have contributed to the genetic composition of the "admixed" population.

RESULTS AND DISCUSSION

All six microsatellite loci were polymorphic, with the mean number of alleles per locus being to the tune of 3.58 (Table **1**). Comparatively, however, in the sample of 20 individuals collected in the month of April in Mingardo river valley the number of alleles was the least variable and that of the breeding (M) and January wintering (V_J) samples the most variable. Similarly, the expected heterozygosity across all samples was variable and ranged from 0.142 to 0.487 (mean = 0.370 ± 0.18). Population-level and global tests of linkage disequilibrium failed to detect the instances of significant linkage among the six microsatellite markers in the majority of individuals. Genetic diversity analysis shows high genetic variability as inferred from the rather high mean values of allele numbers and heterozygosity, particularly high among individuals of the breeding population (M) and among those collected in high winter (V_J) (Table **1**). Among all samples, the lowest genetic variability was observed in the V_A (valley, April) sample.

The pair-wise genetic distance values (using Fst; ARLEQUIN version 2.0) are relatively high between the mountain breeding population (M) and the April valley sample (V_A and V_O) when the long-distance migratory individuals might have become a part of the sample (Table **2**). In contrast, these values are comparatively lower among the breeding M sample and the wintering December, January and February valley (V_D, V_J, V_F) samples. Highest such values were observed for the V_A sample as compared to all other samples, i.e., M, V_F, V_J, V_D, V_O (Table **2**).

By plotting the individual scores, the genotype factorial analysis (Fig. **2**) shows that practically all individuals sampled from the mountain breeding population are clustered in a central portion of the X- and Y-axis crossing. Individual scores from all bird samples of the valley population during

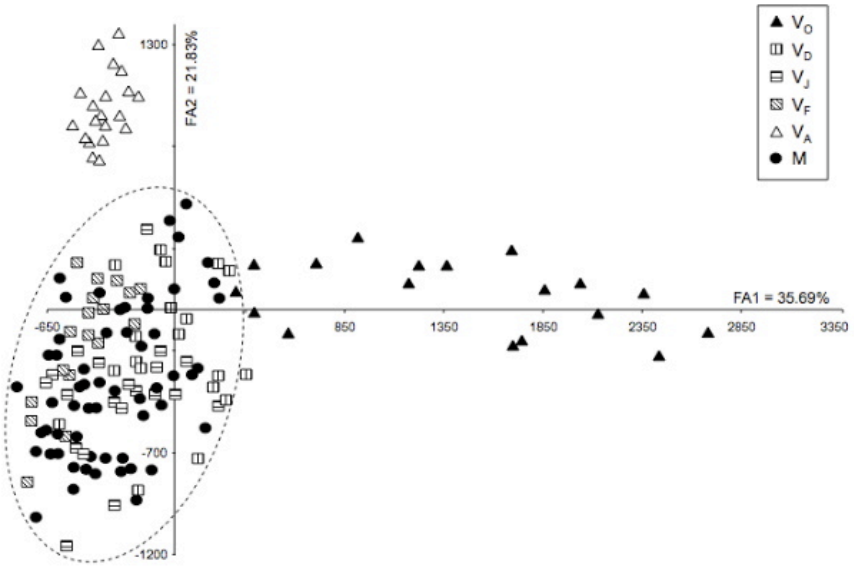

Fig. (2). Factorial Correspondence Analysis of the 160 individual genotypes. Symbols indicate the different spatial and temporal samples.

Table 3. Assignment analysis with Bayesian clustering approaches. The number of clusters is indicated by K. Probability of the data is in the Ln, probability of K clusters column and the variance of the probability is presented in the variance Ln column. Alpha values (α) indicate the admixture value

K	Ln probability of K clusters	Variance Ln	α
2	-1256.3	64.7	0.1010
3	-1228.1	171.1	0.0656
4	-1175.5	189.3	0.0541
5	-1147.0	230.4	0.0488
6	-1148.9	299.5	0.0477
7	-1158.9	371.5	0.0436
8	-1186.0	448.1	0.0315
9	-1170.5	570.1	0.0399
10	-1226.6	653.3	0.0390
11	-1271.5	709.5	0.0390
12	-1282.9	726.1	0.0382
13	-1301.0	725.1	0.0388

the winter months (V_D, V_J, V_F) also cluster within this area as shown in Fig. (**2**). However, individual genotype scores from V_A and V_O are distinct and they cluster separately, respectively along the Y-axis and the X-axis, well apart from each other and from the rest of the groups (Fig. **2**).

In the assignment analysis with Bayesian clustering approach, structuring of the data was performed by inferring different numbers of genetic clusters (K) or populations ranging from K = 2 to K = 13 (Table **3**). The probability of the number of populations (K) was estimated in each case (Ln: probability of data) without using any prior population information so that each individual was assigned to a cluster based upon its multilocus genotype profile. The admixture parameter (α) detected with K value was also estimated. Data was obtained from six population samples, from the mountain and the river valley area. Of these, birds in the mountain made one sample, whereas in the valley they were sampled in five different months. The highest probability of the data (Ln = -1147.0; α = 0.0488; Table **3**) was found with clusters set at 5. Instead, the lowest α value was found with cluster set at 8 (Table **3**). This suggests that the number of clusters defined by allele frequencies is lower than 8 but higher than 5. By K = 6 analysis, some individuals become strongly assigned to one of the six inferred groups (i.e., genetic population clusters). The genetic contribution (y-axis) of each inferred group or cluster (shown by colours) into the individual genotype is shown in the histogram (Fig. **3**). Each bar corresponds to one individual genotype.

In general, in all samples there were individuals that were assigned genetically to cluster #1 (eighteen out of sixty in the mountain sample, and six, four, two, four and eight respectively in the October, December, January, February and April sample). Comparatively fewer individuals were assigned to the genetic cluster #5 but only from the breeding sample and from that collected in January. Genetic cluster #3 was represented only in the breeding and October samples, whereas only four individuals in December sample were assigned to the genetic cluster #4. No individual from the whole lot of animals investigated showed assignment probabilities of greater than 60% for the genetic cluster #6 (Fig. **3**). In the April sample, twelve robins, out of twenty, were assigned genetically to the cluster #2 with assignment probabilities and posterior probabilities of greater than 50%. No other individual in any other period or area of sampling was assigned to this cluster (Fig. **3**).

In the mountain breeding population an average of 47% of the individuals could not be assigned to any of the genetic cluster. Similarly, in October, December, January and February samples there occurred a highly variable percentage of individuals not assigned to any cluster being, respectively 40%, 75%, 70% and 80%. Only in the April sample 100% of the individuals could be assigned to a genetic cluster (Fig. **3**). In this sample, a 60% proportion was assigned to a source, most likely the long-distance migrant conspecifics. In fact, in April 2006, there occurred a conspicuous flooding of the sampling area on part of the long-distance migrant conspecifics; interestingly, in this sample it was observed that twelve out of twenty individuals showed a genetic assignment probability of 60 % to a cluster (#2) to which no other individual from any other sample was assigned, a fact that might corroborate their status as migrants.

It seems that this population does not experience significant gene flow during the invasion of its feeding grounds on part of the long-distance migrant conspecifics. In the April sample, the high value of pair-wise genetic distance suggests an apparent separation between the short- and long-range populations.

Our data are also suggestive of the existence of loop migration for the long-distance migrants which is consistent with the probability assignment results clearly showing the

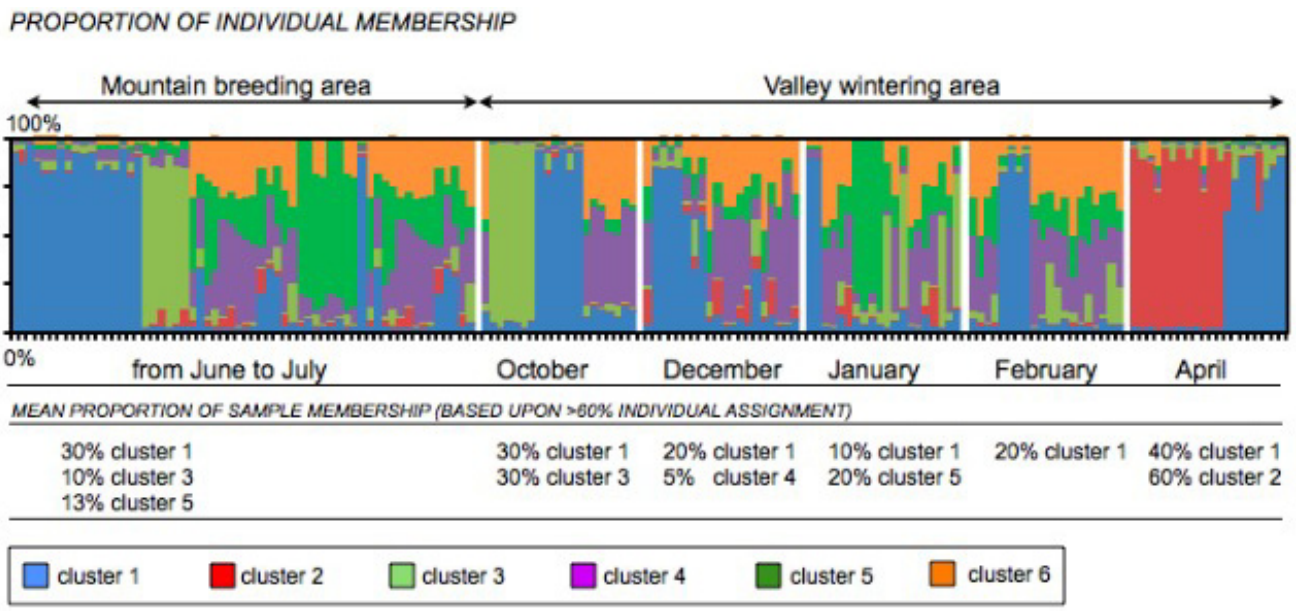

Fig. (3). Genetic Clustering assignment by STRUCTURE version 2.1. In the bar plot, individuals are grouped according to sampling month and each of the 160 individuals is represented by a vertical bar indicating its estimated proportion of assignment to each genetic cluster. Genetic population clusters are coded with different colours and the fraction of colour for each individual represents the probability of assignment to the cluster with that colour. Under the bar plot is indicated the mean proportion of assignment of each sample to each cluster where individuals with greater than 60% assignment to a cluster are counted.

appearance of a genetic cluster entirely different only in the April sample. The long-distance migrants are required to make this stop-over to refuel and store energy while migrating to their Northern breeding grounds; short-distance migrants return to their breeding grounds directly without calling at any intermediate stop-over site. This population can be considered located in a putatively "isolated" Mediterranean area and it apparently shows evidence of only restricted gene flow with other nearby populations.

Relatively high levels of genetic diversity in the mountain sample (M) and a valley sample (V_J) makes it plausible that they are part of the same breeding population where gene flow appears high. Wthether and to what extent there could be out-breeding type of gene flow is not possible to analyze.

From a population perspective, each of the samples examined in this study may together represent a population that may be predominantly self-recruiting having a quite similar genetic composition. It can be argued that there is a relatively high level of within population gene flow which is sufficient to prevent within population divergence. From our data we can not determine explicitly whether there is any genetic admixture between long-distance migrants and local individuals. Bigger and repeated sampling is required to clarify this point. It may be expected that such "isolated" populations may have high in-breeding levels and increased genetic differentiation with the neighbouring populations. It is also plausible that though our population and the neighbouring populations are practically isolated from each other during wintering because of the presence of high peaks between valleys, there is in all probability some gene flow when these birds return to the breeding areas high up in the mountains.

Could the presence of a mountain range between the river valleys be considered a significant barrier to movement of birds from one valley to the other during wintering? It would signify that resident birds from different nearby river valleys, all separated by high peaks, will remain isolated from each other and as such the genetic structure of any one of these populations could maintain its identity without any significant gene flow from outside (except perhaps on part of the long-distance migrants; but these migrants usually make a brief stop over and then go away). Further investigations are required to fill these lacunae in our understanding of the population dynamics in a geographical area where there are more than one resident populations that are separated by physical barriers during wintering. As yet, all statistical methods utilized here for inferring phenology dectected the occurrence of a local population conservation.

In the end, the data on population genetic structure becomes a useful source of information for future management plans. Further studies on some neighbouring populations are warranted.

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