

Phylogeography of the rock partridge (*Alectoris graeca*)

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Abstract

We used mitochondrial DNA control-region and microsatellite data to infer the evolutionary history and past demographic changes in 332 rock partridges (*Alectoris graeca*) sampled from throughout the species' distribution range, with the exception of the central Balkans region. Maternal and biparental DNA markers indicated concordantly that rock partridge populations are structured geographically (mtDNA $\phi_{ST} = 0.86$, microsatellite $F_{ST} = 0.35$; $R_{ST} = 0.31$; $P < 0.001$). Phylogenetic analyses of 22 mtDNA haplotypes identified two major phylogroups (supported by bootstrap values = 93%), splitting partridges from Sicily vs. all the other sampled populations at an average Tamura–Nei genetic distance of 0.035, which corresponds to 65% of the average distance between closely related species of *Alectoris*. Coalescent estimates of divergence times suggested that rock partridges in Sicily were isolated for more than 200 000 years. This deep subdivision was confirmed by multivariate, Bayesian clustering and population assignment analyses of microsatellite genotypes, which supported also a subdivision of partridges from the Alps vs. populations in the Apennines, Albania and Greece. Partridges in the Apennines and Albania–Greece were probably connected by gene flow since recently through a late Pleistocene Adriatic landbridge. Deglaciated Alps were probably colonized by distinct and, perhaps, not yet sampled source populations. Bottleneck and mismatch analyses indicate that rock partridges have lost variability through past population declines, and did not expand recently. Deglaciated areas could have been recolonized without any strong demographic expansion. Genetic data partially supported subspecies subdivisions, and allowed delimiting distinct conservation units. Rock partridges in Sicily, formally recognized as *A. g. whitakeri*, met the criteria for a distinct evolutionary significant unit.

Keywords: *Alectoris graeca*, demographic changes, microsatellites, mtDNA control-region, phylogeography, population structure, rock partridge

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Introduction

Pleistocene climate changes have shaped the patterns of geographical distribution, demographic history and genetic diversification in a number of plant and animal species in the Palaearctic (Avice 2000; Hewitt 2000). During cold-dry glacial periods arctic tundra ecosystems covered most of northern and central Europe. For thousand of years the Alps, Pyrenees and parts of the Apennines

were glaciated. In these periods populations adapted to temperate climates were forced to retreat to southern refuges. During glacials the Mediterranean Sea level was 100–120 m lower than present, many islands were interconnected or united to the mainland and a wide landbridge filled the gap between the eastern and western Adriatic coasts (Dawson 1996: 35–36), thus providing occasions for dispersal of animal populations (Fig. 1).

Glaciations predictably shaped the patterns of genetic diversity in Mediterranean species that inhabit mid-latitude temperate zones or formerly glaciated mountains. Among them, the *Alectoris* partridges (Aves, Galliformes) constitute an interesting case study. The fossil record documents the presence of *Alectoris* in Europe in the

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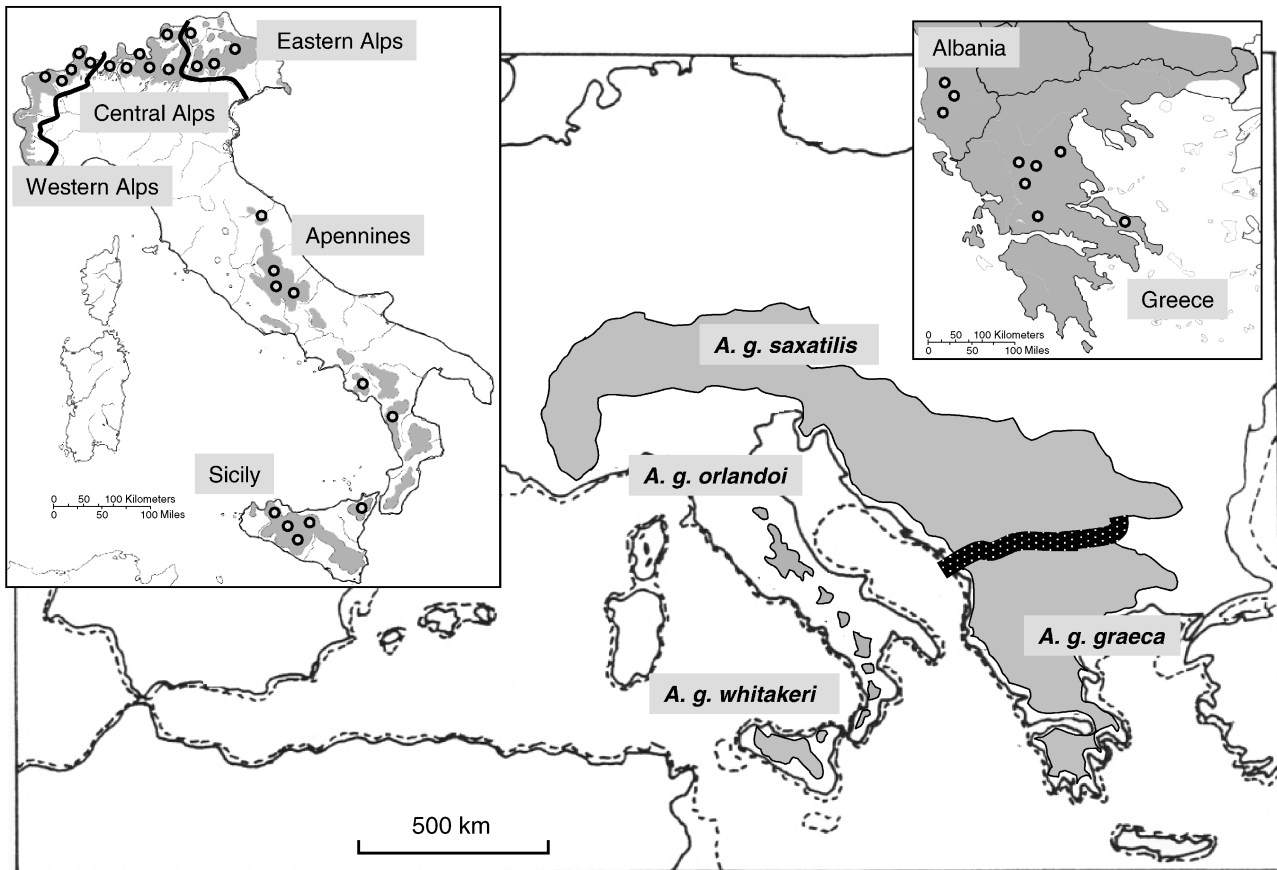


Fig. 1 Distribution range (shaded) of the four subspecies of rock partridge (*Alectoris graeca*) and sampling localities (circles) in Greece, Albania, the Italian Alps, the Apennines and Sicily. The interrupted line describes the level of the Mediterranean Sea at the Last Glacial Maximum, showing interisland connections and the expansion of the north Adriatic landbridge (Dawson 1996). The thick black strip indicates the presumptive distribution border between *A. g. graeca* and *A. g. saxatilis* in central Balkans.

Pleistocene (Mourer-Chauviré 1975). Their current distributions include both glacial refuges (southern Iberia, Italy and the Balkans), and mountains that were glaciated until the Holocene (c. 10 000 years ago). Indeed, genetic data suggested that patterns of speciation and population diversification of *Alectoris* were affected by past climate and habitat changes (Lucchini & Randi 1998; Randi & Lucchini 1998; Randi *et al.* 1998; Randi & Bernard-Laurent 1999).

The rock partridge *A. graeca* has four described subspecies: *A. g. graeca* (distributed in Greece, Albania and southwestern Balkans), *A. g. saxatilis* (northwestern Balkans, Dinaric Alps and Alps), *A. g. orlandoi* (limited to the central-southern Italian Apennines; Priolo 1984) and *A. g. whitakeri* (endemic to the island of Sicily) (Cramp & Simmons 1980; Fig. 1). Rock partridges are distributed patchily in open and hilly habitats, at altitudes of between 50 and 2500 m in Albania, Greece and Sicily, or between 400 and 3000 m in the Apennines and the Alps (Bernard-Laurent & De Franceschi 1994).

The historical biogeography of the west Mediterranean region provides us with a priori expectations of the phylogeographical structure of *A. graeca*. Rock partridges could have not populated glaciated Alps and parts of northern Apennines throughout the last glaciation since the Holocene, and should have survived Pleistocene glaciations in refuge areas. Partridges in Sicily could have been separated repeatedly from mainland populations due to fluctuating contacts between the island and the western tip of peninsular Italy. Partridge populations from the eastern and western sides of the Adriatic could potentially have been interconnected through the late Pleistocene landbridge.

The rock partridge is declining in most of its range (Lups 1981; Bernard-Laurent & De Franceschi 1994). Partridges use open woodlands, pastures and vineyards that were created in the last few thousand years by agriculture and livestock grazing (Cramp & Simmons 1980). The abandonment of traditional agriculture, reforestation and over-hunting led to the eradication of many populations in the

Apennines, Sicily and Greece (Sarà 1989; Bernard-Laurent & De Franceschi 1994; Handrinos & Akriotis 1997). In hunting areas the population decline was contrasted unsuccessfully by the massive release of captive-reared rock partridges, often hybrids with closely related *Alectoris* species (Dias 1992; Randi & Bernard-Laurent 1999; Negro *et al.* 2001). The concomitant decline of natural populations, overhunting and the risk of genetic pollution raised concerns about the conservation of the rock partridge (Tucker & Heath 1994).

A prerequisite for adequate conservation and management plans is the correct definition of the taxonomic rank and geographical distribution of the populations (Moritz 1994; Avise 2000). Current taxonomy and subspecies distinction in rock partridge is uncertain, because it is based exclusively on subtle plumage colour and size variation (Cramp & Simmons 1980; Priolo 1984). The taxonomic identity of Apennine rock partridge populations and the distribution borders between *A. g. graeca* and *A. g. saxatilis* are unclear. Plumage colour variation might evolve rapidly if shaped by natural selection and/or by nongenetic factors, and may describe the manifold effects of contemporary ecological factors. In contrast, the evolutionary history of populations, the genetic consequences of vicariance, dispersal and secondary contacts, are more reliably described by the geographical variation of selectively neutral gene lineages (phylogeography vs. ecogeography; Avise 2000).

Phylogeographical patterns have been reconstructed mainly using maternally inherited mitochondrial DNA sequences (Avise 2000). More recently, the development of hypervariable genomic markers, microsatellites (Goldstein & Schlotterer 1999) and new statistical methods based on coalescent theory allowed the inferring of additional details on evolutionary processes, demographic history and population structuring (Balding *et al.* 2001). Lucchini & Randi (1998) used mtDNA sequences to study the maternal genetic structure in a limited sample of rock partridge populations. In this study we sequenced the left half of the mtDNA control region, and genotyped eight microsatellites in rock partridges sampled throughout the species distribution range. We aimed to: (1) assess whether maternal mtDNA and biparental microsatellite markers described concordant population subdivisions; (2) infer the evolutionary and past demographic processes that probably affected the current population structure; and (3) identify evolutionary units and develop conservation guidelines based on the phylogeography of the rock partridge.

Materials and methods

Sample collection and DNA extractions

We collected 343 tissue and feather samples from five geographical regions across the rock partridge distribu-

tion range in the Alps, Apennines and Sicily in Italy, in Albania and Greece (Fig. 1). Samples were stored individually at -20°C in test tubes containing 95% ethanol. Total DNA was extracted using a guanidinium thiocyanate and diatomaceous earth protocol (Gerloff *et al.* 1995). After DNA genotyping and population assignment analyses, 11 samples were identified as interspecific hybrids between *A. graeca* and red-legged (*A. rufa*) or chukar (*A. chukar*) partridges, and were not considered further. Excluding the hybrids, in this study we used 332 rock partridges. Rock partridges were sampled far from the contact zone with red-legged partridges in the Western French Alps (Randi & Bernard-Laurent 1999). Although samples were collected as far as possible from known areas of intensive restocking, we cannot exclude that some captive-reared birds have been included in the sample collection.

Laboratory methods

The entire mitochondrial DNA control region (mtDNA CR) was polymerase chain reaction (PCR)-amplified using the external primers PHDL (tRNA^{Glu}; 5'-AGGACTACGGCTTGAAAAGC-3') and PHDH (tRNA^{Phe}; 5'-CATCTTGGCATCTTCAGTGCC-3'). We automated sequenced 450 nucleotides from the left side of the mtDNA CR, including the hypervariable domain CR-IA (Randi & Lucchini 1998), using primer PHDL. Sequences were aligned and unique haplotypes were identified using COLLAPSE 1.0 (D. Posada; http://bioag.byu.edu/zoology/crandall_lab/programs.htm). All samples were genotyped by PCR amplifications of eight microsatellites that were isolated originally at Wageningen University from the chicken (*Gallus gallus*) genome: MCW118 (annealing temperature = 50°C), MCW225 (45°C), MCW280 (55°C), MCW252 (60°C), MCW276 (60°C), MCW295 (50°C), MCW135 (45°C) and MCW323 (60°C). Primer sequences and information on MCW markers can be retrieved at <http://www.zod.wau.nl/abg/index.html>. All PCR products were analysed automatically using an ABI 3100 sequencer and programs GENESCAN 3.7 and GENOTYPER 2.1. Details of laboratory protocols are available upon request.

Phylogenetic trees and analysis of the mtDNA sequences

Phylogenetic trees were reconstructed using: (1) the neighbour-joining procedure (NJ; Saitou & Nei 1987) with pairwise Tamura & Nei's (TN93) genetic distance model, which is appropriate to describe the evolution of CR sequences (Tamura & Nei 1993); (2) the maximum-parsimony procedure (MP; Swofford *et al.* 1996), with random haplotype additions and 10 replications per run, multiple minimal trees swapped by tree bisection and reconnection, and collapsed zero length branches, as implemented in PAUP* 4.0b2a (Swofford 2002). Multiple

MP trees were collapsed to obtain a 50% majority-rule consensus tree. Support for the internodes in NJ tree was assessed by bootstrap percentages (BP; Felsenstein 1985) after 10 000 resampling steps. Phylogenetic trees were rooted using homologous CR sequences of red-legged and chukar partridges. Unrooted networks were obtained using a minimum-spanning network (MSN) procedure implemented in ARLEQUIN 2.0b8 (Schneider *et al.* 2002; <http://anthropologie.unige.ch/arlequin>).

ARLEQUIN was used to estimate average and population haplotype diversity (h), mean number of pairwise differences (k), nucleotide diversity (π) and the parameter $\theta_{(k)} = Nu$ (Tajima 1983). The partition of genetic diversity within and among populations was analysed by analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992), using ϕ analogues of Wright's (1965) F -statistics. Pairwise F_{ST} and interpopulation divergence times allowing for unequal population sizes (Gaggiotti & Excoffier 2000) were also computed. The assumption of neutrality of mutations was tested by Tajima's procedure (Tajima 1989).

Microsatellites: analyses of genetic diversity within and among populations

Commonly used summary population genetic statistics were computed for each locus and population using GENETIX 4.03 (Belkhir *et al.* 2001; <http://www.univ-montp2.fr/~genetix/genetix.htm>). Significance of differences in heterozygosity between two populations was tested using Wilcoxon's signed-ranks test. The observed allele numbers were corrected for sample size variations, computing the allelic richness (A_C) based on a minimum size of 12 individuals per sample, using FSTAT 2.9.1 (Goudet 1995; <http://www.unil.ch/izea/softwares/fstat.html>). Deviations from Hardy-Weinberg equilibrium (HWE) for each locus and each population were assessed using the complete enumeration method (Louis & Dempster 1987) for loci with less than five alleles, and Markov's chain method (Guo & Thompson 1992) for the other loci, as implemented in GENEPOP 3.2a (Raymond & Rousset 1995a; <http://www.cefe.cnrs-mop.fr>). Deviations from allelic linkage equilibrium (LE) for all pairwise locus combinations were tested using the exact probability test in GENEPOP.

ARLEQUIN was used to test the significance of differences in allele frequency distributions between samples (exact test of population differentiation; Raymond & Rousset 1995b), to estimate populations pairwise weighted mean F_{ST} (Weir & Cockerham 1984) and R_{ST} (Slatkin 1995), and perform AMOVAS with analogues of F_{ST} or R_{ST} (Michalakis & Excoffier 1996). Critical levels in simultaneous tests of significance were adjusted using Hochberg's procedure (Legendre & Legendre 1998: 18), as implemented in ADJUSTED P-VALUES (P. Legendre 2002; <http://www.fas.umontreal.ca/biol/legendre>).

Structure and patterns of populations diversification

ARLEQUIN was used to test for patterns of population diversification through isolation-by-distance (Mantel test; Rousset 1997). We subdivided the Alpine samples into three subgroups, corresponding to western ($n = 25$), central ($n = 23$) and eastern Alps ($n = 11$), and measured the geographical distances between eight partridge populations assuming the following linear connections: Greece, Albania, eastern Alps, central Alps, western Alps, Apennines and Sicily (see Fig. 1). This model corresponds to a hypothetical dispersal from a single source population (Greece).

Patterns of differentiation were visualized by factorial correspondence analysis (FCA; Benzécri 1973) of individual multilocus scores computed using GENETIX. Population structure was also inferred using a Bayesian clustering procedure implemented in STRUCTURE (Pritchard *et al.* 2000; <http://pritch.bsd.uchicago.edu>), designed to identify the K (unknown) populations of origin of the sampled individuals and assign the individuals simultaneously to the populations. This procedure assumes that neutral unlinked molecular markers are in Hardy-Weinberg and linkage equilibrium in the populations. Population structure within a data set is detected by estimating departures from HWE and LE, which could result by recent admixtures, migration or hybridization. The sample is subdivided into a number of different subpopulations (clusters) that minimize departures from HWE and LE and, simultaneously, individuals are assigned probabilistically to one (the population of origin) or more than one cluster (the parental populations) if their genotypes indicate that they are admixed. In this study, we used STRUCTURE to infer the existence of genetically distinct clusters and assign the individuals to the clusters, using or not using the available previous population information (option USEPOPINFO = 1, or = 0), with 100 000 iterations, following a burn-in period of 10 000 iterations. Populations or individuals were assigned to one cluster if their proportion of membership (q_i) to that cluster was equal to or larger than an arbitrary threshold of 0.800.

Analyses of bottleneck, mismatch distributions and coalescence time

Evidence for a population bottleneck was assessed using the M -ratio test (Garza & Williamson 2001; <http://www.pfeg.noaa.gov/tib/carlos.htm>). The probability of the observed M -values were estimated by simulation, using the two-phase mutation model (TPM; Di Rienzo *et al.* 1994), with proportions of one-step mutations $p_s = 90\%$ or 95% , average size of non-one-step mutations $\Delta g = 3.5$, and $\theta = 4N_e\mu$ equal to 5 or 10 (N_e = effective population size at equilibrium before the bottleneck; μ = mutation rate). We used also the two-tailed Wilcoxon's signed-ranks and the mode-shift

Haplotypes	Variable sites	Number(frequency)	Sampling locations(sample size)
	111122222222222222223333333		
	599900122334444556990000389		
	215603706784569387690357234		
H1	TATCCACATGCTTCCCCGAAAAGTCTC	33 (0.102)	Italian Alps (33)
H2	T.....	1 (0.003)	Italian Alps (1)
H4	T..T.T.....	4 (0.012)	Italian Alps (4)
H5	G.....	1 (0.003)	Italian Alps (1)
H6	T.T...G.....	4 (0.012)	Italian Alps (4)
H14	..C.....CT.T.....C.	3 (0.009)	Italian Alps (3)
H15	T.T..G.....	1 (0.003)	Italian Alps (1)
H3	T.T.....	23 (0.071)	Italian Alps (8), Apennines (15)
H8	T.T.....C.	37 (0.114)	Apennines (19), Albania (3), Greece (15)
H10	CT.T.....C.	146 (0.452)	Albania (6), Greece (140)
H11	C...CT.T.....C.	1 (0.003)	Albania (1)
H13	...T.....CT.T.....C.	15 (0.046)	Albania (2), Greece (13)
H12	T.....	3 (0.009)	Apennines (1), Albania (1), Greece (1)
H23	..-.....CT.T.....C.	9 (0.028)	Apennines (1), Greece (8)
H24	CT.T.....A..C.	6 (0.018)	Apennines (1), Greece (5)
H27	...T.....CT.T.....G...C.	7 (0.022)	Greece (7)
H28	C.....CT.T.....C.	1 (0.003)	Greece (1)
H29	T.CT.T.....C.	2 (0.006)	Greece (2)
H17	...T.G.G.A.CCT..AA.C..C.TCT	17 (0.053)	Sicily (17)
H18	..T.GTG.A.CCT..AA.C..C.TCT	3 (0.009)	Sicily (3)
H19	...T.G.C.A.CCT..AA.C..C.T.T	5 (0.015)	Sicily (5)
H20	...T.G.G.A.CCT..AA.C..CCTCT	1 (0.003)	Sicily (1)

Fig. 2 Sampling locations, numbers and frequency in the total population of the 22 mtDNA CR haplotypes found in rock partridges sampled from five regions (see Fig. 1). Haplotype positions are aligned with nucleotide n. 1 of the complete mtDNA CR sequence of *Alectoris* (Randi & Lucchini 1998). Haplotype sequences are available from GenBank (Accession nos AJ555843–AJ555864).

tests (Cornuet & Luikart 1996), implemented in BOTTLENECK 1.2.02 (Piry *et al.* 1999; <http://www.ensam.inra.fr/URLB>), using the TPM with 90% or 95% single-step mutations.

The distributions of pairwise genetic differences between individual haplotypes were analysed using the sudden expansion model (Rogers & Harpending 1992). Goodness-of-fit tests (sum of squared deviations and Harpending's raggedness index) of the observed to the estimated mismatch distributions were computed using ARLEQUIN (Schneider & Excoffier 1999).

The coalescent time of the mtDNA CR haplotypes in the rock partridges were computed following Rooney *et al.* (2001). We counted the number of transversions between rock partridges and the chukar outgroups, and estimated the number of substitutions per site (s/s) using the formula $d = (tv + tvR)/m$ (R = transition/transversion ratio in the rock partridges; m = length of the sequence). The rate of s/s per lineage per year is $\lambda = d/2T$, where T is the divergence time between the ingroup and the outgroup species ($T = 2.0$ million years, Myr; Lucchini & Randi 1998). The s/s per generation is $\mu = \lambda g$, where g is the generation time ($g = 2.05$ years in rock partridges; Bernard-Laurent & Leonard 2000). The s/s per haplotypes is $u = m\mu$, and the coalescence time in generations is $t = \tau/2\mu$ (Rogers & Harpending 1992).

Results

Mitochondrial DNA sequence description and variability

The mtDNA CR sequence alignment (431 nucleotides) showed 22 different haplotypes, defined by 27 polymorphic sites including 28 substitutions (24 transitions and four transversions), and one insertion/deletion (Fig. 2). Some haplotypes were restricted to unique geographical populations: seven haplotypes were sampled only in rock partridges from the Alps, three exclusively in partridges from Greece, one is known only from Albania and four haplotypes were sampled only in Sicily. The other seven haplotypes were shared among populations sampled from the Alps, Apennines, Albania and Greece (Fig. 2). All transversions were observed between the haplotypes from Sicily and the other haplotypes.

Haplotype diversity was similar across the five local populations (average $h = 0.760$ in the total sample; min = 0.541 and max = 0.756 in the local populations; Table 1). Nucleotide diversity was low in all sampled populations (average $\pi = 0.008$; min = 0.001, max = 0.003). The pairwise divergence between haplotypes (average $k = 3.504$) was lowest ($k = 0.498$) in partridges from Greece,

Table 1 Rock partridge (*Alectoris graeca*) collection sites (mapped in Fig. 1), number of genotyped samples (*n*) and mtDNA CR sequence diversity in five populations and in the total sample

Population	<i>n</i>	Haplotypes	<i>h</i>	<i>k</i>	π	$\theta_{(k)}$
Italy Alps	55	8	0.615 (0.068)	1.348 (0.848)	0.003 (0.002)	2.34 (1.06–4.85)
Italy Apennines	37	5	0.612 (0.048)	0.802 (0.595)	0.002 (0.001)	1.78 (0.72–4.10)
Italy Sicily	26	4	0.541 (0.097)	0.940 (0.666)	0.002 (0.002)	1.06 (0.36–2.90)
Albania	13	5	0.756 (0.097)	0.980 (0.708)	0.002 (0.002)	2.49 (0.85–6.96)
Greece	192	9	0.546 (0.456)	0.498 (0.427)	0.001 (0.001)	1.81 (0.89–3.47)
Total	323	22	0.760 (0.022)	3.504 (1.790)	0.008 (0.004)	5.18 (3.24–8.03)

Haplotype diversity (*h*), mean pairwise differences (*k*), nucleotide diversity (π) and θ (from *k*) were computed using ARLEQUIN. Standard deviations and 95% confidence limits (for θ) are in brackets.

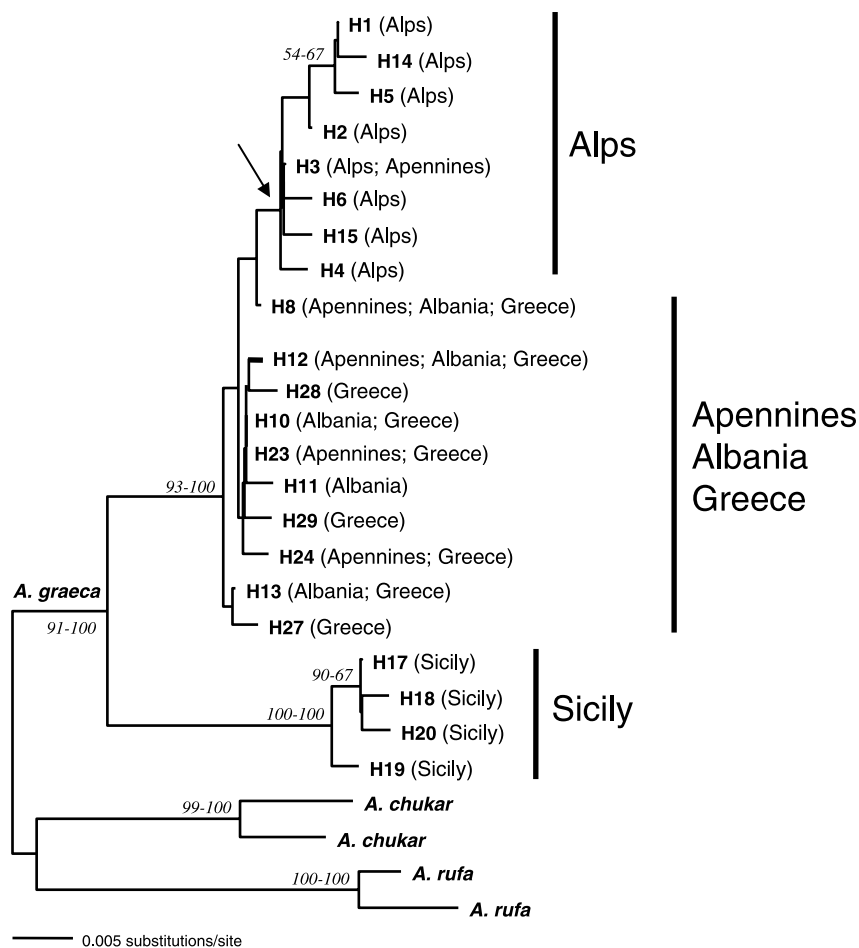


Fig. 3 Neighbour-joining tree computed by PAUP* using Tamura & Nei's (1993) genetic distances among the aligned 432 nucleotides of mtDNA CR sequences of rock partridges (*Alectoris graeca*; 22 haplotypes). The phylogenetic tree was rooted using homologous sequences of chukar (*A. chukar*) and red-legged partridges (*A. rufa*). Numbers above the internodes indicate all the bootstrap percentage values > 50% computed in neighbour-joining (first numbers), and the consensus percentage values computed in maximum-parsimony (second numbers) trees. The arrow indicates a clade including the mtDNA CR haplotypes sampled in rock partridges from the Alps.

and highest ($k = 1.348$) in partridges from the Alps. Values of $\theta_{(k)}$ (5.18 in the total sample) ranged from $\theta_{(k)} = 1.06$ (Sicily) to 2.49 (Albania). Tajima's *D* was -0.418 in the total sample ($P = 0.36$), and ranged from $D = -1.254$ ($P = 0.10$; Greece) to $D = -0.288$ ($P = 0.41$; Sicily). The null hypothesis of neutral evolution of these mtDNA CR sequences was not rejected in the total as well as in the local samples.

Phylogenetic structure of the mtDNA haplotypes

The NJ tree, computed with TN93 distances and rooted using homologous CR sequences from red-legged and chukar partridges, grouped all rock partridge haplotypes into a monophyletic cluster (supported by BP = 91%), which included two deeply divergent haplogroups

(Fig. 3). The first haplogroup included only the haplotypes sampled from the partridges that were collected in Sicily (BP = 100%). The second haplogroup (BP = 93%) included the haplotypes sampled from all the other populations, the root being intermediate between the two clusters. All the haplotypes sampled from the Alps joined into a single cluster (shown by an arrow in Fig. 3), which was not supported by bootstrap, and that included also the haplotype H3 that was sampled in the Alps and the Apennines.

The MP procedure produced three equally parsimonious trees obtained from 38 parsimony informative characters (length $L = 63$; consistency index $CI = 0.762$, retention index $RI = 0.878$). The 50% consensus MP tree showed the same topology as the NJ tree (Fig. 3). The MSN showed that haplotypes sampled from the Apennines, Albania and Greece group together, stemming from H10 that was at the centre of a star-like topology (Fig. 4). The haplotypes sampled from the Alps were linked to H3, the haplotype shared with the Apennines, and were connected directly to the haplotypes from Sicily. All the haplotypes sampled from Sicily grouped together at a minimum of 13 mutations from all the other haplotypes.

The average TN93 genetic distance between haplotypes from Sicily and the other haplotypes was $d = 3.5\%$ (min 3.1%; max 4.1%), corresponding to about 65% of the average interspecific distance between *A. graeca*, *A. rufa* and *A. chukar* (average interspecific distance = 5.4%). The average TN93 distance between partridges from the Alps and the Apennines–Albania–Greece was $d = 0.9\%$; that is, six times lower than the average interspecific distance.

Microsatellites genetic diversity, Hardy–Weinberg and linkage equilibrium

The eight microsatellites were polymorphic in the partridge samples, with the exception of locus MCW252, which was monomorphic in the Italian Alps (Table 2). These loci showed 63 alleles in total, with an average of 7.9 alleles per locus. Allelic diversity ranged from 3.2 to 5.5 in the partridges from Sicily and Greece, respectively. Allelic richness ranged from a minimum $A_C = 2.42$ in Sicily to a maximum $A_C = 3.59$ in Albania ($P = 0.012$; Wilcoxon's signed-rank test), and the number of private alleles ranged from zero (in the Apennines) to seven (in Sicily). Partridges from Sicily showed more private alleles than partridges from Greece, notwithstanding a much larger sample size in Greece (i.e. $n = 35$ vs. 186; Table 2). Values of heterozygosity ranged from 0.20 (H_O in Sicily) to 0.48 (H_E in Albania), and were not correlated significantly to sample size (H_O : $r = -0.10$, $P = 0.88$; H_E : $r = -0.02$, $P = 0.97$; Z-test). Partridges from Sicily showed the lowest values of H_E and H_O , significantly different from heterozygosity values of all the other samples ($P = 0.05$; Wilcoxon's signed-rank test).

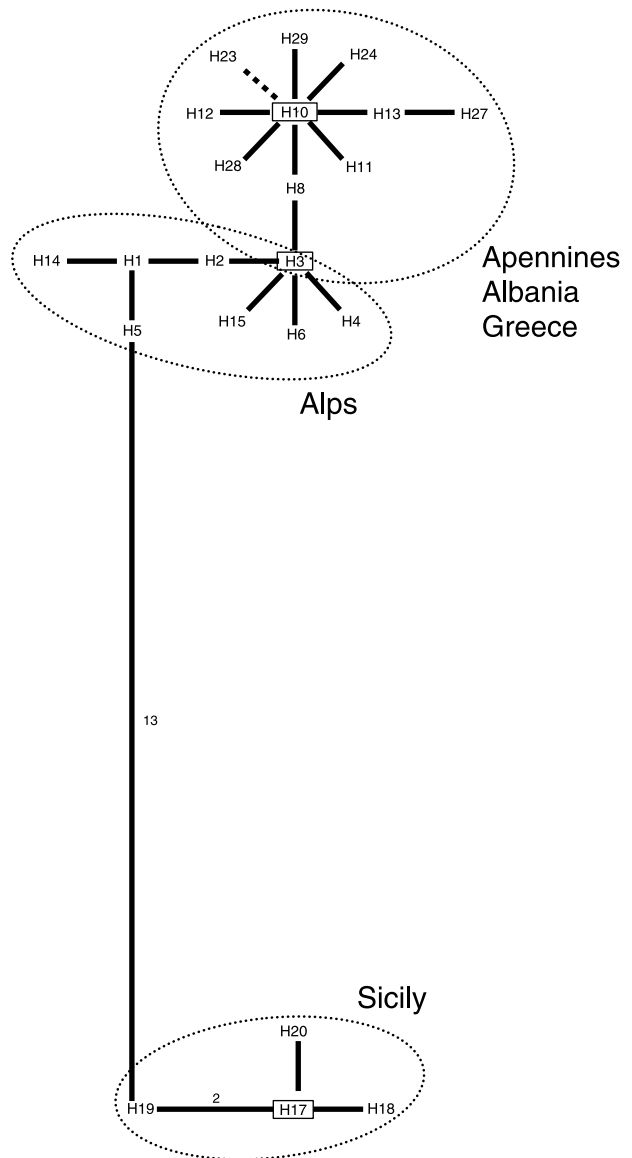


Fig. 4 Minimum-spanning network computed by ARLEQUIN using the number of different mutations among 22 mtDNA CR haplotypes of rock partridges. Ellipses were drawn to indicate groups including the haplotypes sampled in partridges from Sicily, the Alps and from the Apennines, Albania and Greece. Haplotype H3 was sampled in the Alps and in the Apennines. Distances between linked haplotypes correspond to one mutation, except when indicated by numbers. Haplotype H23 differs from H10 only for a deletion at position n. 191 (see Fig. 2).

The average value of $H_O = 0.32$ was smaller than $H_E = 0.48$, and the average $F_{IS} = 0.339$ was significantly positive ($P < 0.0001$), showing that there were less heterozygotes than expected in the total sample (Table 2). The multilocus test (Rousset & Raymond 1995) performed across all populations showed that the heterozygote deficit was highly significant ($P = 0.0000$). Probability tests for

Population	<i>n</i>	<i>A</i>	<i>A_C</i>	<i>na</i>	<i>p</i>	<i>H_O</i> (SD)	<i>H_E</i> (SD)	<i>F_{IS}</i> (95% CI)
Italy Alps	59	4.1	2.8	2	7	0.28 (0.25)	0.31 (0.28)	0.100 (0.001; 0.187)
Italy Apennines	39	3.5	2.9	0	8	0.37 (0.19)	0.41 (0.15)	0.099 (−0.029; 0.191)
Italy Sicily	35	3.2	2.4	7	8	0.20 (0.24)	0.22 (0.24)	0.098 (−0.055; 0.220)*
Albania	13	3.6	3.6	1	8	0.44 (0.35)	0.48 (0.26)	0.084 (−0.188; 0.192)*
Greece	186	5.5	3.3	6	8	0.33 (0.29)	0.38 (0.33)	0.126 (0.071; 0.174)*
Total	332	7.9	—	—	8	0.32 (0.26)	0.48 (0.27)	0.339 (0.293; 0.381)*

Number of genotyped partridges (*n*), mean number of alleles per locus (*A*), allelic richness (*A_C*), number of unique alleles (*na*) found in a population when it is compared to all the other populations, number of polymorphic loci (*p*) in the populations and in the total sample.

*Significant ($P < 0.001$) departures from HWE tested by multilocus probability test using GENEPOP. SD = one standard deviation, 95% CI = 95% confidence interval, H_E = unbiased average heterozygosity.

Table 2 Summary of genetic diversity in rock partridges genotyped at eight microsatellite loci

Table 3 Pairwise values of F_{ST} (microsatellites/mitochondrial DNA; above the diagonal) and R_{ST} (microsatellites; below the diagonal) among populations of rock partridges. All pairwise values are significantly different from zero ($P = 0.05$; 16 000 permutations), except for microsatellite R_{ST} and mtDNA F_{ST} (distance method: TN93) between Albania and Greece

Population	Alps	Apennines	Sicily	Albania	Greece
1 Italy Alps		0.184/0.535	0.637/0.922	0.136/0.666	0.171/0.806
2 Italy Apennines	0.076		0.574/0.942	0.038/0.412	0.186/0.605
3 Italy Sicily	0.432	0.470		0.581/0.933	0.572/0.960
4 Albania	0.385	0.197	0.509		0.068/0.032
5 Greece	0.367	0.234	0.312	0.019	

departure from HWE, performed in each population, showed that one locus in the Alps (MCW323), one in the Apennines (MCW295) and three loci (MCW118, MCW252, MCW135) in Greece were not in equilibrium ($P < 0.001$). F_{IS} values were positive in each population, and the multilocus test showed that heterozygote deficit was significant ($P < 0.001$) in partridges from the Alps, Sicily and Greece (Table 2). Heterozygote deficit might, in part, be a consequence of null alleles. All locus combinations were in LE in the pooled sample set and in each population, except for one combination in partridges from Albania and one in partridges from the Apennines.

Genetic divergence and geographical population structure

An AMOVA performed with the total sample subdivided into five populations (Table 1) showed that 13.5% of the total mtDNA genetic variability was distributed within, and 86.5% among populations ($\phi_{ST} = 0.86$, computed using TN93 interhaplotype molecular distances; $P < 0.001$). Pairwise F_{ST} values and a Fisher's exact test showed that all populations were significantly differentiated one from each other ($P < 0.001$), except for the Albanian and Greek populations ($F_{ST} = 0.032$, $P = 0.109$; Fisher's exact P -value of nondifferentiation = 0.018; Table 3).

Microsatellite genetic diversity was also significantly partitioned among the five partridge populations (average

multilocus $F_{ST} = 0.35$, $R_{ST} = 0.31$, $P < 0.001$). All pairwise F_{ST} and R_{ST} values among populations were significant ($P < 0.001$), except for the R_{ST} comparison between partridges from Albania and Greece (Table 3). Excluding partridges sampled in Sicily, the proportions of gene diversity among populations were: mtDNA $\phi_{ST} = 0.71$, microsatellite $F_{ST} = 0.16$; microsatellite $R_{ST} = 0.28$ (all values significant: $P < 0.001$).

Mantel tests, performed to check for isolation-by-distance using $F_{ST}/(1 - F_{ST})$, as recommended by Rousset (1997), derived from mtDNA and microsatellites, showed the following matrix correlations: $r_{mtDNA} = 0.58$ ($R^2 = 0.33$; $P = 0.027$; 1000 permutations), $r_{microsatellites} = 0.46$ ($R^2 = 0.21$; $P = 0.079$). A null hypothesis of isolation-by-distance could be rejected at the 0.01 level, but not at the 0.05 level for mtDNA distances. Geographical distances explained about 20–30% of the genetic distances among populations. Isolation-by-distance plottings (Fig. 5) showed that partridges from Sicily were more differentiated from all the other populations than expected based only on distances. In contrast, partridge from the Apennines and Albania–Greece were less differentiated than expected from geographical distances.

The FCA plotting of individual microsatellite genotypes (Fig. 6) once again supported a clear distinction of partridges from Sicily, which grouped separately from all the other partridges on the first factorial component FC-I

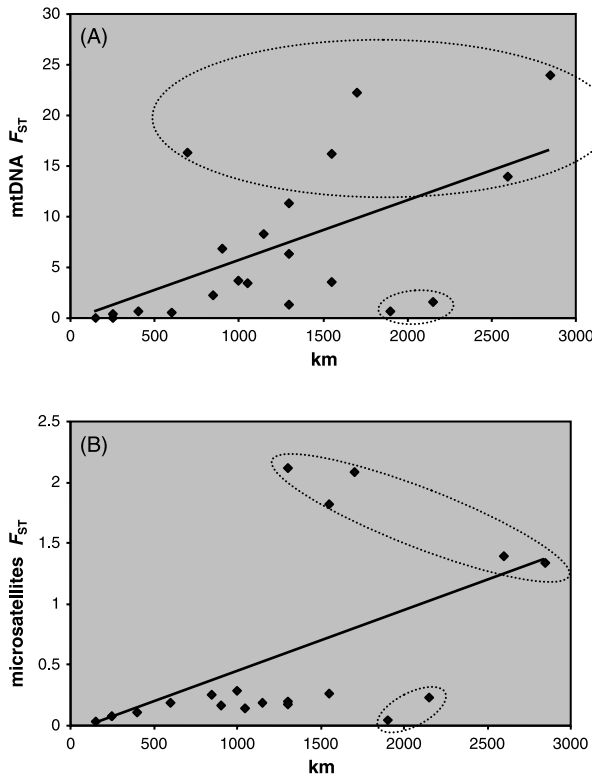


Fig. 5 Isolation-by-distance plotting (Rousset 1997) showing the relationships between pairwise geographical distances (in km) and genetic distances, estimated from mtDNA (A) and microsatellites (B), in five populations of rock partridges. Ellipses (dotted) were drawn to indicate pairwise comparisons involving partridges sampled from Sicily (upper ellipses) and partridges from the Apennines and Albania–Greece (lower ellipses).

(which explained 12.53% of the total genetic variation). Partridges from the Alps, Apennines and Greece were almost completely distinct on FC-II and FC-III (which explained 4.80% and 3.58% of the total variation, respectively). Samples from Albania were admixed mainly with partridges from Greece and the Apennines.

Bayesian assessment of population structure

We performed STRUCTURE using the total sample set (332 samples, eight microsatellite loci, five sampled populations) and $K = 1 - 6$. The probability of the data was maximum with $K = 5$, suggesting that partridges can be split into five distinct genetic clusters. The proportions of membership of each sampled population in five inferred clusters are reported in Table 4. Partridges from the Alps were assigned to cluster V ($q_V = 0.905$); partridges from Sicily were assigned to cluster III ($q_{III} = 0.983$); partridges sampled from the Apennines were assigned to cluster I ($q_I = 0.815$). Partridges from Albania and Greece were not assigned to any single cluster with $q = 0.800$, but were split into two (Greece) or more (Albania) clusters.

The assignment of the individuals to the five clusters, performed without using prior population information, showed that all the individuals from Sicily and 95.1% of the individuals from the Alps were assigned correctly to their own clusters (Table 4). The number of assigned individuals was in the range of 54% to 72% in the other populations. Using the previous population information (option USEPOPINFO = 1), i.e. indicating the population from whence each individual was sampled, 100% of the

Table 4 Bayesian clustering analysis in rock partridges performed using STRUCTURE (Pritchard *et al.* 2000) with the total sample set (332 partridges; eight microsatellite loci; five sampled populations). The posterior probability of the number of populations in the sample set was maximum with $K = 5$. The table shows the proportion of membership (q) of each predefined sampled population in each of five inferred clusters. Each sampled population was assigned to a single cluster if its q_i ($i = I-V$) was equal or larger than 0.800, or to two or more clusters when the cumulative q_i values were equal or larger than 0.800. The number and percentage of individuals assigned to their original population with a probability threshold of 0.800 are indicated in parentheses. The unassigned individuals were assigned to more than one cluster with probability lower than 0.800

Population (n)	Cluster					Unassigned individuals	% of assigned individuals
	I	II	III	IV	V		
Italy Alps (59)	0.032 (0)	0.030 (0)	0.005 (0)	0.028 (0)	0.905 (54)	5	91.5
Italy Apennines (39)	0.815 (28)	0.078 (0)	0.023 (0)	0.025 (0)	0.059 (0)	11	71.8
Italy Sicily (35)	0.004 (0)	0.004 (0)	0.983 (35)	0.004 (0)	0.004 (0)	0	100
Albania (13)	0.248 (3)	0.454 (3)	0.018 (0)	0.133 (0)	0.147 (1)	6	53.8
Greece (186)	0.040 (0)	0.444 (51)	0.007 (0)	0.465 (61)	0.044 (1)	73	60.7

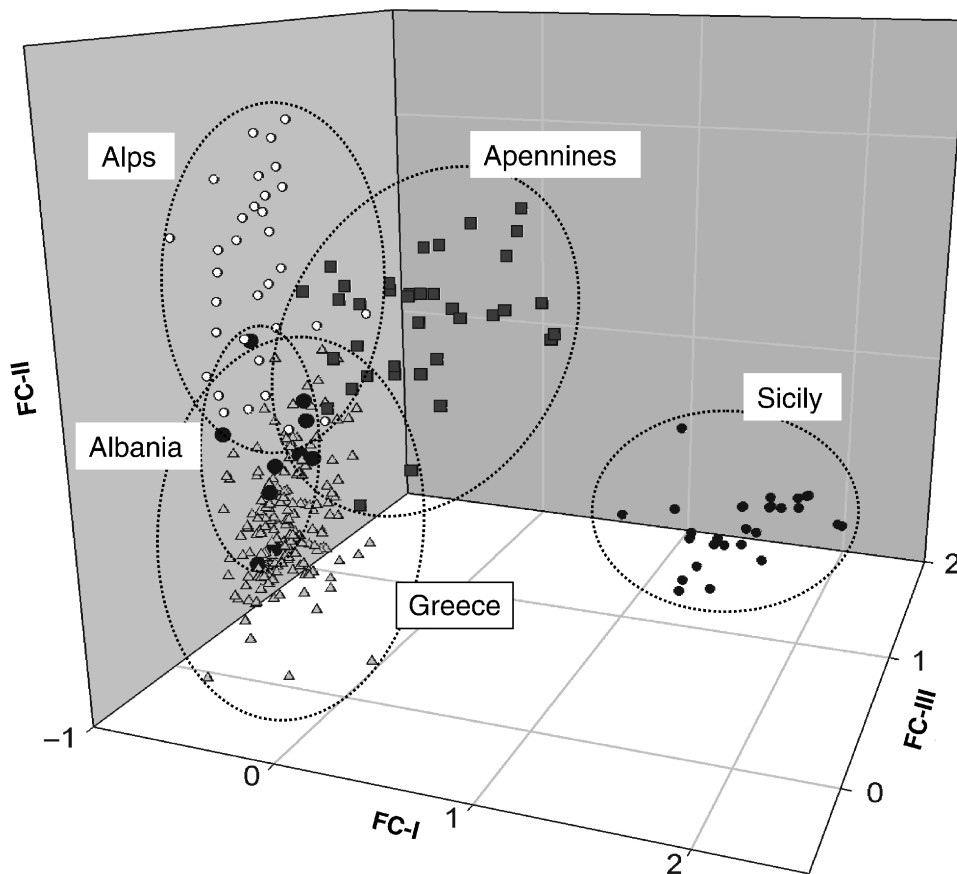


Fig. 6 Factorial correspondence analysis (Benzécri 1973), computed using GENETIX, showing relationships among the multilocus genotypes of individual rock partridges. Ellipses (dotted) were drawn by hand to include the distributions of partridges sampled in Sicily (small dots), the Apennines (filled squares), the Alps (circles), Albania (large dots) and Greece (triangles). FC-I, FC-II and FC-III are, respectively, the first, second and third principal factors of variability.

partridges sampled in the Alps, Apennines, Sicily and Greece were assigned correctly to their own cluster with individual $q_i = 0.800$. The global performance of STRUCTURE in this case allowed assigning $329/332 = 99.01\%$ of the individuals.

Bottlenecks, mismatch distributions and coalescence time

Values of average M were significantly lower than expected ($P < 0.001$), suggesting evidence of recent declines in all partridge populations, except in Sicily ($P = 0.176$). In contrast, values of the Wilcoxon's signed-ranks test were not significant ($P > 0.05$) for all populations except for Sicily ($P = 0.016$). All mode-shift tests were not significant except for Albania which had, however, a low sample size ($n = 13$). Overall, results of the bottleneck tests suggest that rock partridge populations were recently affected by demographic declines. Different values of p_s (90% or 95%) and θ (5 or 10) did not change the results of bottleneck tests.

The mismatch distribution for the total sample was not bell-shaped as expected under the sudden expansion

model, but showed a skewed exponential shape due to excess frequencies of small pairwise differences. However, the observed distribution was not ragged, and the goodness-of-fit statistics were not significant (sum of squared deviations = 0.0108, $P = 0.52$; Harpending's raggedness index = 0.0220, $P = 0.94$). Thus, a fitting of the observed to the expected distribution under the sudden expansion model cannot be rejected. The estimated value of θ after the expansion ($\theta_1 = 50.9$; 95% CI = 3.5–4980.9) was 24 times higher than θ before the expansion ($\theta_0 = 2.1$; 95% CI = 0.0–6.1). The value of $\tau = 0.25$ (time to the expansion) was very small. Mismatches in the local samples also showed an excess of small pairwise differences. The values of θ_0 and θ_1 were very similar to each other in rock partridges from the Alps ($\theta_0 = 0.00$; $\theta_1 = 2.00$) and Sicily ($\theta_0 = 0.00$; $\theta_1 = 1.41$), and only the sample from Greece showed instances of sudden ($\theta_0 = 0.00$; $\theta_1 = 56.13$) but recent ($\tau = 0.625$) population growth.

There were 11 observed transversions between rock partridge and chukar haplotypes. In the rock partridge the ts/tv ratio was $R = 8.7$ (as estimated by PAUP* using

maximum-likelihood with the TN93 model, shape parameter of a gamma distribution $\alpha = 0.55$ and proportion of invariant sites $I = 0.75$). The average number of s/s of the mtDNA CR alignment was $d = (11 + 11 \times 8.7)/431 = 0.25$. The rate of s/s per lineage per year was $\lambda = 0.25/(2 \times 2 \times 10^6) = 6.25 \times 10^{-8}$ (assuming an interspecific divergence time $T = 2.0$ Myr), and the rate of s/s per generation was $\mu = 2 \times 6.25 \times 10^{-8} = 1.25 \times 10^{-7}$ (assuming a generation time of 2.0 years). The mutation rate for the entire haplotype was $u = 1.25 \times 10^{-7} \times 431 = 5.39 \times 10^{-5}$. The coalescent time $t = \tau/2\mu$, corresponding to the time to the most recent common ancestor (TMRCA), was estimated from $\tau = 2ut = 12.84$ (coalescence of the rock partridge haplotypes allowing for unequal population size). The divergence time for the split between partridges in Sicily and elsewhere was $t_g = 12.84/2 \times 5.39 \times 10^{-5} = 118\,889$ generations, corresponding to $t = 237\,778$ years. The divergence time of Alpine and Albania–Greece populations was $\tau = 1.85$, corresponding to $t = 17\,130$ years. The divergence time between the Apennines and Albania–Greece populations was $\tau = 0.41$, corresponding to $t = 3796$ years.

Discussion

Population structure and phylogeography of rock partridges

Mitochondrial DNA and microsatellite data revealed significant genetic structuring in rock partridges, showing concordantly a deep genetic distinction between partridges in Sicily and the other populations sampled in the Alps, Apennines, Albania and Greece. The mtDNA trees grouped all the Sicilian haplotypes into a fully supported monophyletic clade and showed a distinction of the Alpine rock partridges, which shared a common ancestral haplotype (H3), suggesting *in situ* diversification after postglacial recolonization (see also Lucchini & Randi 1998). AMOVAS, pairwise ϕ_{ST} , F_{ST} , R_{ST} values and Fisher's exact tests confirmed population subdivisions. Bayesian clustering also showed that almost all partridges sampled in Sicily and the Alps were assigned correctly to unique clusters. Some local populations showed departures from HWE, which can in part be due to null alleles, but that should not affect the output of STRUCTURE. This procedure does not need perfect HWE to cluster individuals, but attempts to minimize departures from HWE within groups. Thus, two genetically distinct populations will be split anyway, and also if each one is not in HWE, simply as consequence of the Wahlund effect.

According to the estimated coalescent time of CR haplotypes rock partridges diverged in isolation in Sicily for more than 200 000 years, i.e. since before the onset of the Riss glaciation (200 000–135 000 years ago). We may speculate that Sicily and Greece were refuges for rock par-

tridges during the last glaciation. The coalescent theory (Crandall & Templeton 1993) predicts that the most frequent and widespread haplotypes are ancestral and indicate the location of refugial populations. The two most frequent mtDNA haplotypes, which have a cumulative frequency of 57%, are both present in Greece and Albania (H10 and H8) and in the Apennines (H8). Partridges in Greece, which also showed the largest values of heterozygosity, allelic richness and signals of population expansion, could have contributed to the postglacial recolonization of northern Balkans and, perhaps, of the Apennines. In contrast, the deep genetic distinction of Sicilian partridges suggests that this population did not contribute to the recolonization of the Apennines and Alps.

Alpine rock partridges are distinct from a genetically homogeneous assemblage including partridges in the Apennines, Albania and Greece. These populations might have diverged from partridges in the Alps for about 17 000 years, i.e. since the end of the Würm glaciation. However, partridges probably colonized the Alps more recently, about 6000–8000 years ago (Randi & Bernard-Laurent 1999). Hence, divergence date is compatible with the idea that other partridge populations recolonized the Alps, either from the southeast (i.e. from the northern Dinaric Alps), the southwest (northern Apennines) or from both directions. Military events in the Balkans prevented us collecting samples from the Dinaric Alps, while natural partridge populations in the northern Apennines were eradicated totally during the last century. Therefore, the question of which source population/s recolonized the deglaciated Alps remains unanswered. Weak genetic distinctions suggest that partridges in the Apennines and southeastern Adriatic regions were interconnected since recently, perhaps throughout a corridor provided by the Adriatic landbridge. Partridges in the Apennines and Albania–Greece might have been interconnected until the beginning of the Holocene (10 000 years ago), when ice caps definitely melted and the Mediterranean was raised, again flooding the north Adriatic landbridge.

The haplotype mutation rate ($u = 5.39 \times 10^{-5}$), corresponding to 0.125 s/s/Myr, is in the range of other 'phylogenetically' derived rates in birds (i.e. Milot *et al.* 2000), but it is much lower than 'evolutionary' rates of 0.4–1.4 s/s/Myr, which were estimated in penguins (Lambert *et al.* 2002). Higher mutation rates mean proportionally shorter divergence times. In contrast, our estimates could have been deflated by a longer generation time in Mediterranean partridge populations living in less extreme and more stable habitats than the Alps.

Divergence times based on mtDNA data are corroborated roughly by estimates computed using microsatellites. A divergence time of 106 250 generations (221 500 years) between Sicily and the other populations is obtained from $t = d_A/2\mu$, where $d_A = 3.4$ is Nei's average net number

of differences (Nei & Li 1979), and $\mu = 1.6 \times 10^{-5}$ is the microsatellite mutation rate (Estoup & Cornuet 1999). The microsatellite divergence time between the Alpine and Balkan populations is $t = 0.48/2 \times 1.6 \times 10^{-5} = 15\,000$ generations (30 000 years), about twice the time computed from mtDNA. However, divergence between the Apennines and Balkan populations is $t = 0.38/2 \times 1.6 \times 10^{-5} = 12\,031$ generations (24 062 years), about six times the estimate obtained using mtDNA. We do not know, of course, the average long-term mutation rate of these microsatellites, nor can we estimate the effects of past demographic fluctuations on mtDNA or microsatellite population divergence. Microsatellite mutation rates could be higher than 10^{-5} , homoplasy might have deflated microsatellite distances due to the longlasting evolution of Sicilian partridges or, in contrast, bottlenecks might have inflated microsatellite distances among recently isolated populations. Thus, estimated divergence times remain hypothetical.

Inferring historical demography

The geographical subdivision of genetic diversity exhibited by mtDNA haplotypes is larger than diversification observed at microsatellite loci. The ratio of mtDNA ϕ_{ST} to microsatellite F_{ST} is $0.86/0.35 = 2.46$ in rock partridges. Excluding Sicily the ratio is $0.71/0.16 = 4.43$. These results are expected, because the mtDNA genome has 1/4 the effective population size of nuclear genes, and a decline in mtDNA diversity should be faster in bottlenecked or declining populations (Birky *et al.* 1989). Sex-biased dispersal and female philopatry can enhance further mtDNA subdivisions (Tiedemann *et al.* 2000). However, field data on Alpine rock partridge populations suggest that dispersal is female-biased (Bernard-Laurent pers. comm.). The observed differences among mtDNA and microsatellite diversification thus indicate that rock partridges might have experienced periods of population fragmentation and low effective population size.

Bottleneck tests showed that extant partridge populations are not in genetic equilibrium, presumably due to past population declines. The M -ratio test evidenced bottlenecks in all populations except Sicily, while the Wilcoxon's signed-ranks test was not significant in all populations except Sicily. It is not clear, at present, if procedures by Garza & Williamson (2001) and Cornuet & Luikart (1996) have different power or may detect bottleneck signals in different time windows, although Garza & Williamson suggested that M should reach a new equilibrium slower than H_E . It is possible, but difficult to demonstrate, that population decline in Sicilian rock partridges was more recent than in other populations.

The mtDNA mismatch distribution showed a skewed exponential shape due to excess of identical or similar haplotypes but it was, nevertheless, compatible with an expansion model.

However, the estimated expansion sizes were small for all populations, except for partridges in Greece. Eventual expansions in Greece would have been recent, as suggested by the high frequency of a few haplotypes, low haplotype diversity and small pairwise differences. We computed $\tau = 2ut = 0.625$ in partridges from Greece, a value that can be used to estimate a time since population expansion of $t = \tau/2\mu = 0.625/2 \times 5.39 \times 10^{-5} = 5798$ generations (11 596 years). Rock partridges might have recolonized deglaciated areas without any strong population expansion. The species is sedentary, although altitudinal and migratory movements were recorded in the Alps (Bernard-Laurent 1991). Notwithstanding a high fecundity, populations might experience large interannual fluctuations and severe declines due to unpredictable weather conditions in the mountains, which affect their breeding success or winter survival (Bernard-Laurent & Leonard 2000). Past population fluctuations might account for both observed low genetic diversity and inferred demographic declines.

Evolutionary units and conservation genetics of rock partridges

Phylogeography and population genetic data are only partially concordant with formal intraspecific taxonomy of *A. graeca*. The taxonomic identity of the Apennine rock partridge (*A. g. orlandoi*) is not supported by genetic data, and this subspecies should probably be merged within *A. g. graeca*. However, the small sample size of partridges from Albania we used in this study, and the lack of critical samples from the Balkans and Dinaric Alps, left still unsolved the assessment of genetic distinction and the identification of distribution borders of *A. g. graeca* and *A. g. saxatilis*. The unsatisfactory assessment of genetic diversification of partridge populations from these areas makes it difficult to evaluate the extent of genetic distinction of partridges in the Apennines. Nowadays, partridges in the Apennines are isolated and demographically independent from all the other populations. Therefore, we suggest considering the Apennine partridges as a distinct management unit (MU; Moritz 1994), pending future additional genetic analyses.

Phylogeographical and genetic data indicate concordantly that Sicilian partridges (*A. g. whitakeri*) met the criteria for an evolutionary significant unit (ESU Moritz 1994); that is, monophyly of mtDNA haplotypes and significant divergence of allele frequencies at nuclear loci. We recognize that splits in single-gene trees do not coincide usually with separations in populations or species trees. Phylogenetic partitions in mtDNA trees might be from 10% to 50% deeper than historical subdivisions in population trees (Edwards & Beerli 2000). However, in this study we used an approach that takes into account both intrapopulation variability and unequal population size (Gaggiotti & Excoffier 2000). Also assuming that the estimated divergence time of

rock partridges in Sicily is upwardly biased by 50%, the mtDNA data still support an isolation more ancient than 100 000 years. Moreover, independent nuclear markers, the microsatellites, confirmed the deep mtDNA subdivision, lending support to organismal subdivision under the principle of genealogical concordance (Avice 2000).

A strict biological species concept (BSC; Mayr 1963) is not appropriate for *Alectoris*, because even formally recognized species can interbreed and produce fertile offspring in nature and in captivity. Natural populations of *Alectoris* are mainly alloparapatric and, when in contact, reproductive isolation is not intrinsic but due to ecological or behavioural constraints to gene flow (e.g. differential selective and/or competitive pressures on rock and red-legged partridges in the Alpine contact zone; Randi & Bernard-Laurent 1999). The phylogenetic species concept (PSC; Cracraft 1983) seems more appropriate for partridges. Under a strict PSC, the Sicilian rock partridge population represents the smallest diagnosable cluster and is a distinct monophyletic lineage.

Although the data available suggest that hybridization and introgression are not widespread in natural partridge populations (Randi & Bernard-Laurent 1999), the massive release of captive-reared birds can produce detrimental consequences to prey–predator equilibria, habitat resources and diffusion of parasitic and infectious diseases (Lups 1981). Conservation plans should control and limit the use of captive-reared birds for population restocking, should not allow the release and mixing of partridges from different biogeographical areas, and avoid completely any kind of uncontrolled captive-breeding and release of partridges in Sicily.

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