BIOLOGICAL CONSERVATION 137 (2007) 57–69

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Hybridisation with introduced chukars (*Alectoris chukar*) threatens the gene pool integrity of native rock (*A. graeca*) and red-legged (*A. rufa*) partridge populations

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ARTICLE INFO

Article history:
Received 30 August 2006
Received in revised form
2 January 2007
Accepted 5 January 2007
Available online 6 March 2007

Keywords:
Alectoris
Introgression
Outbreeding depression
Admixture analysis
mtDNA control-region
Microsatellites
Partridge conservation genetics

ABSTRACT

The decline of over-hunted red-legged (*Alectoris rufa*) and rock (*A. graeca*) partridge populations has been contrasted with massive releases of captive-reared birds, often hybrids with non-indigenous *A. chukar*. Released interspecific hybrids raise the risks of introgressive hybridisation, and can contribute to further depress the fitness of native populations. Aiming to assess the extent of hybridisation, we genotyped the mtDNA control-region and eight nuclear microsatellites in 671 red-legged, rock and chukar partridges and hybrids, identified by phenotypic traits. Results reveal a diffuse occurrence of hybridisation: (1) 39 samples (6.2%) show mtDNA haplotypes discordant with their phenotypes, indicating red-legged and chukar mtDNA introgression in native rock partridges; (2) admixture analyses of the microsatellite genotypes identified 32 additional rock partridges (5.1%) hybridised mainly with chukars. We analysed also 39 samples collected from a presumed natural red-legged x rock partridge hybrid zone in the French Alps. Surprisingly, 28% birds showed typical chukar mtDNAs, indicating hybridisation with introduced chukars or hybrids. This hybrid zone led to an introgression cline of chukar alleles into neighbouring Alpine rock partridges detectable up to 100 km, which was shorter than expected by neutral genetic theory, and that suggested natural selection against hybrids. These findings indicate that introgressive hybridisation may disrupt local adaptations in natural red-legged partridge and rock partridge populations, and call for strict control of farming and restocking operations.

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doi:10.1016/j.biocon.2007.01.014

1. Introduction

The role of hybridisation in generating biodiversity patterns, as well as the conservation value and the legal status of hybrids in populations that are protected by law are controversial (Allendorf et al., 2001; Mallet, 2005). Various models have been developed to capture the complexities of hybrid zones (Barton and Hewitt, 1985; Moore, 1987; Harrison, 1986), and to evaluate the evolutionary consequences of hybridisation (Barton, 2001). Often, but not always, hybrids found in stable hybrid zones have low fitness, and their temporal persistence and evolutionary fate are unclear (Barton, 2001). Consequently, hybrid zones are sometimes regarded to as evolutionary dead ends, or, in contrast, as genetic melting pots that can potentially foster the emergence of evolutionary novelties (Seehausen, 2004).

Anthropogenic habitat changes, invasion of alien species and translocation of captive-reared stocks or artificial hybrids raise risks of genetic pollution and extinction of natural populations via hybridisation (Rhymer and Simberloff, 1996). Genetic admixture and introgression of "alien" alleles can disrupt local adaptations, and can eventually lead to fitness and population declines (i.e., outbreeding depression; Templeton, 1986). Risks of genetic pollution by introgressive hybridisation are threatening both endangered and common species (Rhymer and Simberloff, 1996; Allendorf et al., 2001). The occurrence of natural hybridisation is relatively frequent in birds (Grant and Grant, 1992), and anthropogenic hybridisation has been documented in game bird species, particularly in galliforms and waterfowl (Rhymer and Simberloff, 1996; Mank et al., 2004; Barilani et al., 2007).

The *Alectoris* partridges include seven closely related interfertile species that are distributed in Eurasia, China and southern Arabia (Johnsgard, 1988). Their distributions are largely allopatric, with the exception of two partially sympatric species in Arabia. Natural hybridisation in parapatric *Alectoris* contact zones was described in the southern French Alps (Bernard-Laurent, 1984), in Thrace (Greece; Dragoev, 1974); and in central China (Chen et al., 1999). A red-legged (*Alectoris rufa*) x rock partridge (*A. graeca*) hybrid population, distributed over an area ca. 15 km wide along the southern edge of the French Alps, showed shorter than expected introgression of red-legged allozyme and mtDNA markers in Alpine rock partridge populations distributed up to ca. 150 km from the contact zone. This suggests that the diffusion of hybrid partridges in nature could be contrasted by outbreeding depression (Randi and Bernard-Laurent, 1998, 1999).

Rock partridges have declined in the second part of the last century in most of their native range, due to habitat changes and over-hunting, which have led to the extinction of local populations in parts of the Alps, Italian Apennine and Greece (Bernard-Laurent and De Franceschi, 1994; Handrinos and Akriotis, 1997). Population decline has been contrasted by massive releasing of captive-reproduced partridges, often using chukars (*A. chukar*) or hybrids with chukars (Randi et al., 2003; Barilani et al., 2007). Red-legged partridges are massively hunted and restocked throughout the entire species range in Iberia, France and Italy (Aebischer and Potts, 1994). Earlier genetic analyses indicate that chukar mtDNA haplotypes are widespread in most of the red-legged partridge populations studied (Negro et al., 2001; Barbanera et al., 2005; Baratti et al., 2004). Thus, the genetic integrity of red-legged and rock partridges might be at risk of widespread introgressive hybridisation (Potts, 1989; Bernard-Laurent et al., 2001; Barilani et al., 2007). The concomitant decline of natural populations and the risk of genetic pollution with captive-reared hybrids are raising concerns about the conservation of the declining partridges in Europe (Tucker and Heath, 1994).

Detecting the presence of hybrids or admixed populations can be problematic, particularly if the parental taxa are morphologically similar (as some species of the *Alectoris* partridges; Johnsgard, 1988), or if a limited number of diagnostic markers is used in genetic screening (Boecklen and Howard, 1997; Vähä and Primmer, 2006). However, nowadays, mitochondrial and abundant nuclear hypervariable DNA markers (e.g., microsatellites) and new Bayesian statistical methods have dramatically improved the assessment of cryptic population structure, population admixture analyses and individual assignment testing (Beaumont and Rannala, 2004). In this study we aimed to investigate the extent of hybridisation in red-legged and rock partridge populations sampled in Iberia, France and Italy using Bayesian admixture analyses of multilocus individual genotypes. We sequenced the hypervariable part of the mtDNA control-region (Randi and Lucchini, 1998) and genotyped eight microsatellites (Barilani et al., 2007). Specifically, we aimed to: (1) probabilistically assign individual partridges to one of the sampled parental species or to hybrid groups of natural or artificial origin, using genetic data; (2) assess the extent of natural or anthropogenic hybridisation in Alpine, Apennine and Sicilian rock partridge populations; and (3) propose conservation guidelines based on the assessment of hybridisation risk in European partridges.

2. Materials and methods

2.1. Sample collection and morphological identification

We collected 671 tissue samples from nine geographic regions across the native distribution range of the red-legged, rock and chukar partridges (Table 1; Fig. 1). Red-legged partridges ($n = 123$) were collected in Portugal, Spain and France between 1990 and 2002. Rock partridges ($n = 416$) were collected from the French and Italian side of the Alps, the Italian Apennines and Sicily between 1989 and 1999. We collected also 39 partridges from the natural hybrid zone in the southern French Alps (Alpes Maritimes; Figs. 1 and 2). Artificial hybrids have been produced in captivity mainly by crossing red-legged and rock partridges with chukars. Therefore, we also analysed 93 chukars sampled in 1990–2002 from native populations in Greece, Israel and China.

Samples were assigned to one of the three *Alectoris* species, or were identified as hybrids, based on diagnostic morphological traits and geographic distributions, independently of the genetic data. Red-legged partridges are easily identified by the discontinuous border of the black gorget with black spotting, and flank feathers with only one black band. Rock partridges and chukars can be distinguished mainly by lore color (black vs. white), ear-covert colors (black

Table 1 – List of the species, population of origin and geographic location of the studied Alectoris samples

Species	Population	Region	Samples provided by
A. rufa	Portugal	Alentejo	D. Dias (Lisboa, Portugal)
	Spain	Andalucia	J. A. Perez Garrido (León, Spain) J. J. Negro (Sevilla, Spain) E. Randi (Bologna, Italy)
Hybrid zone	France	Alpes-Maritimes (Cipières, Coursegoules)	A. Bernard-Laurent (Nice, France)
	France	Alpes-Maritimes (mid Valley of Tinée and Valley of Cians)	A. Bernard-Laurent
A. graeca	France	Alpes-Maritimes (high Valley of Tinée)	A. Bernard-Laurent
	France	Hautes-Alpes (Champsaur, Queyras)	A. Bernard-Laurent
	France	Isère	A. Bernard-Laurent
	France	Savoie	A. Bernard-Laurent
	Italy, Alps	Cuneo Province	E. Randi
	Italy, Alps	Valle d'Aosta Region	E. Randi
	Italy, Alps	Novara, Vercelli provinces	E. Randi
	Italy, Alps	Como, Lecco, Sondrio, Brescia provinces	E. Randi
	Italy, Alps	Trento Province	E. Randi, M. Paganin
	Italy, Apennines	Abruzzo Region	E. Randi
	Italy, Sicily	Sicilia Region	E. Randi
A. chukar	North Israel	Galilee	P. Alkon (Las Cruces, USA) S. Kark (Jerusalem, Israel) E. Randi
	Central China	Gansu	Zhou Tianlin, L. Naifa (Lanzhou, China)
	Greece	Kos and Karpathos Islands	A. Sfougaris (Volos, Greece) A. Giannakopoulos (Volos, Greece)

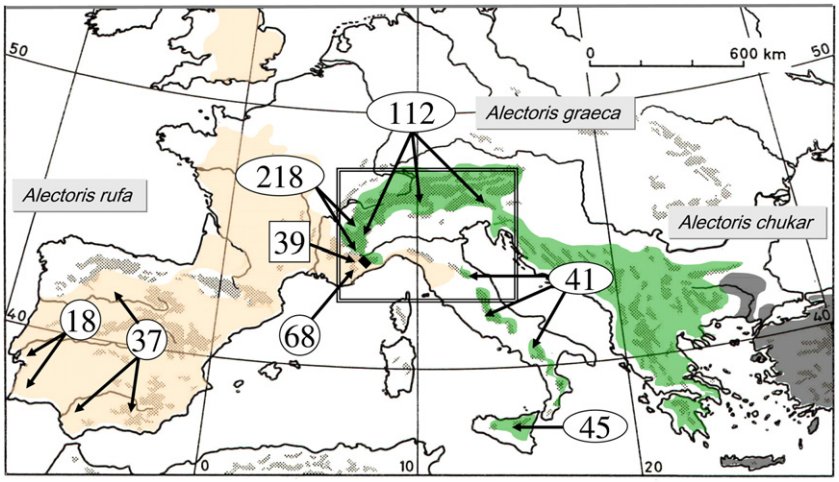

Fig. 1 – Distribution ranges, sampling areas and sample sizes of red-legged (*Alectoris rufa*; circles), rock (*A. graeca*; ellipses) and chukar (*A. chukar*) partridges in Europe, and the *A. rufa* × *A. graeca* hybrid zone (square). Details on populations sampled in the framed region are shown in Fig. 2.

with yellow extremities vs. chestnut) and by the narrower width of the two black bands on the flank feathers in rock partridge (Johnsgard, 1988). Partridges in the natural hybrid zone showed variable combinations of red-legged and rock partridge feather patterns (described by Bernard-Laurent, 1984; sampling location 2 in Fig. 2). Partridges collected outside the hybrid zone did not show morphologic traces of hybridisation and are assumed to be genetically "pure". In the French Alps (but not in Italy), commercial farming and restocking of rock partridges has always been forbidden. However, red-legged partridges close to the rock partridge range were restocked until the beginning of the 1990s, when tissues for this study were collected in the French Alps. Since the mid 1990s, restocking of red-legged partridges close to the distribution range of the French rock partridge is also forbidden (Bernard-Laurent et al., 2001). Red-legged partridges collected in Portugal and Spain and chukars did not show any morphological or genetic sign of hybridisation (see Section 3).

2.2. *Laboratory methods*

Tissue samples were individually stored at −20 °C in 95% ethanol. Total DNA was extracted using guanidine thiocyanate (Gerloff et al., 1995). The 5′ half of the mitochondrial DNA

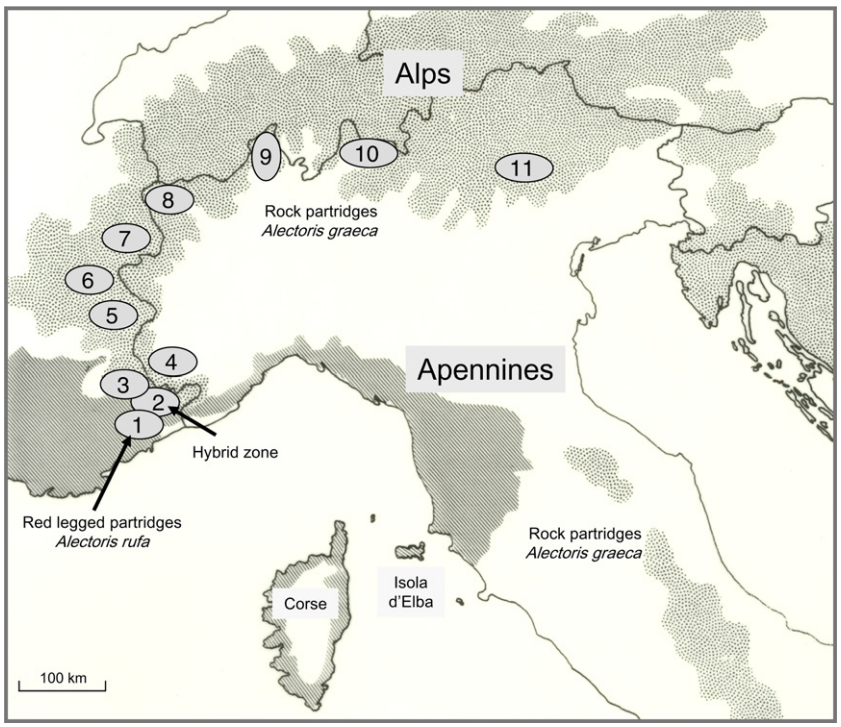

Fig. 2 – Distribution of red-legged (dark grey areas) and rock partridges (light grey areas) in south-eastern France and northern Italy. Sampled populations: (1) red-legged partridges, Cipières, Coursegoules (Alpes-Maritimes, France), 20–24 km from the hybrid zone; (2) partridges collected within the hybrid zone, mid Valley of Tinée (Alpes-Maritimes). Rock partridges sampled in: (3) high Valley of Tinée (Alpes-Maritimes, 14–28 km); (4) Cuneo, (Italy, 30–40 km); (5) Champsaur and Queyras (Hautes Alpes, France 75–85 km); (6) Isère (France, 80–90 km); (7) Savoie (170–190 km); (8) Valle d'Aosta (Italy, 130–150 km); (9) Novara and Vercelli (Italy, 250 km); (10) Como, Lecco, Sondrio, Brescia (Italy, 350 km); (11) Trento (Italy, 550 km).

control-region (mtDNA CR) was PCR-amplified using the external primer PHDL ($tRNA^{Glu}$; 5′-aggactacggcttgaaaagc-3′) and the internal primer PH-H521 (5′-ttatgtgcttga-ccgaggaac-cag-3′), and sequenced using primer PHDL (Randi and Lucchini, 1998). All samples were also genotyped at eight microsatellites (Barilani et al., 2007) originally isolated in Wageningen University (http://www.zod.wau.nl/abg/index.html) from the chicken (*Gallus gallus*) genome: MCW118 (PCR annealing temperature $T_a = 55$ °C), MCW135 ($T_a = 55$ °C), MCW152 ($T_a = 50$ °C), MCW225 ($T_a = 45$ °C), MCW276 ($T_a = 60$°C), MCW280 ($T_a = 55$ °C), MCW295 ($T_a = 50$ °C), MCW323 ($T_a = 50$ °C). PCRs were done using the following thermal cycle: $(94° \times 2') + [(94° \times 30'') + (T_a \times 30'') + (72° \times 30'')] \times 40$ cycles + $(72° \times 2')$. The amplicons were analysed using an ABI 3100 automated sequencer and programs GENESCAN 3.7 and GENOTYPER 2.1. Details of laboratory protocols are available upon request.

2.3. Analyses of sequence and microsatellite variability

Phylogenetic trees were obtained using MEGA 2.1 (Kumar et al., 2001), with the neighbor-joining procedure (NJ; Saitou and Nei, 1987) and Tamura-Nei's (TN93; 1993) genetic distance model, which is appropriate to describe the evolution of CR sequences. Support for the internodes in NJ tree was assessed by bootstrap percentages (BP; Felsenstein, 1985) after 10,000 resampling steps. We used also other distance methods (i.e., genetic distances computed using β-distributions of variable sites), and a Bayesian procedure (MRBAYES 3.04; Huelsenbeck and Ronquist, 2001), which produced results very similar to the NJ tree. Commonly used summary population genetic statistics (allelic frequencies, heterozygosity and deviations from Hardy–Weinberg equilibrium) were computed for each locus and population, and patterns of differentiation were visualized by a factorial correspondence analysis (FCA) of individual multilocus scores using GENETIX 4.03 (Belkhir et al., 2001).

2.4. Admixture and hybridisation analyses

Maternal hybridisation was directly detected by discordant mtDNA and morphologic traits, while biparental multilocus genotypes were analyzed using a Bayesian clustering procedure implemented in STRUCTURE2 (Pritchard et al., 2000; Falush et al., 2003), which was designed to identify the *K* (unknown) populations of origin of the sampled individuals, and assign the individuals to the populations. Population clusters are constructed by minimizing the departures from Hardy–Weinberg equilibrium (HWE) and linkage equilibrium (LE), which could result from recent admixtures, migration or hybridisation. The samples are subdivided into a number of different sub-populations (clusters) and, simultaneously, individuals are assigned probabilistically to one (the population of origin) or more than one cluster (the parental populations) if their genotypes are admixed. STRUCTURE does not need perfect genet-

ic equilibrium to cluster individuals, but attempts to minimise departures from HWE and LE within the inferred clusters.

In this study we applied STRUCTURE with the admixture model and allele frequencies uncorrelated. All simulations were run with 10^5 iterations, following a burn-in period of 10^4 iterations, and were replicated four times. The optimal K values were selected using the formula: $\Delta_{\ln P(D)} = [\ln P(D)_k - \ln P(D)_{k-1}]$ (Garnier et al., 2004), where $\ln P(D)$ is the estimated posterior probability of the data conditional to K. We performed explorative analyses with $K = 1$–15, using all the samples that were reliably genotyped at eight microsatellites ($n = 668$), or excluding the known hybrids (i.e., 39 partridges with discordant mtDNA and 39 samples from the hybrid zone; $n = 590$). Results showed that the probability of the data, and the value of $\Delta_{\ln P(D)}$, increased sharply from $K = 1$ to $K = 4$, then very weakly up to asymptotic values that were obtained with $K = 9$–10 (not shown). Therefore, we used STRUCTURE with the following search strategy:

1. Assessment of the global genetic subdivision and identification of the cryptic hybrids. We analysed the 590 presumptive non-hybrid samples, both using and not using their mtDNA haplotypes as additional characters (the haplotypes were coded as 1, *rufa*; 2, *graeca*; 3, *chukar*, based on result of mtDNA phylogenetic analyses), without prior population information (option USEPOPINFO = 0), and $K = 1$–9. For the selected K value, we assessed the average coefficient of membership (Q) of each sampled population (chukar, red-legged and rock partridges) to the inferred clusters. Then, we assigned each genotype to the inferred clusters, based on threshold values of the individual proportion of membership (q_i). Predictably, the threshold values will affect the accuracy of hybrid identifications (Vähä and Primmer, 2006). Following empirical and simulation results (Barilani et al., 2007) we selected an identification threshold $q_i = 0.90$, assigning each individual to one cluster (species) if $q_i \geqslant 0.90$ (parental individuals), or jointly to two clusters if the proportion of membership to each one was $q_i < 0.90$ (admixed individuals). In this way we used STRUCTURE to estimate the posterior probability for each individual to belong to one parental species, or to have fractions q_i of its genome originating from two parental species.
2. Identification of the admixed partridges. STRUCTURE was run with $K = 4$, and option USEPOPINFO active, that is indicating the reference population from where each individual was sampled (POPFLAG = 1), except for all the individuals that showed a mtDNA haplotype discordant with prior species identification, or that showed admixed genotypes (POPFLAG = 0). In this way we evaluated the probability to assign, with individual proportion of membership $q_i \geqslant 0.90$, each putative hybrid to its sampled populations, or to the sampled (0), first (1) or second (2) past generation in another one or in more than one population.
3. Genetic composition of the Alpine hybrid zone. STRUCTURE was run with all the samples ($n = 668$), with $K = 4$ and option USEPOPINFO active. The genetic composition of individual genotypes was assessed using the identification threshold $q_i = 0.90$, as described above. Moreover, the software NEWHYBRIDS (Anderson and Thompson, 2002) was used for computing the posterior probability for each individual to belong to each of six genotypic classes that originate after two generations of hybridisation, that is: two parentals (P_0,P_1), first generation hybrids (F_1), second generation hybrids (F_2), backcrosses of F_1 with the first parental (B_0); backcrosses of F_1 with the second parental (B_1). Posterior distributions were evaluated after 10^5 Monte Carlo Markov Chains, without using any individual or allele frequency prior information. STRUCTURE can identify admixtures among any number K of parental populations, while NEWHYBRIDS assumes that hybrid classes originated after admixture of two parental species. Therefore, we assessed hybridisation in the rock partridges using either the red-legged or the chukar partridges as parentals, and excluding rock partridges from Sicily, as they were divergent from all the other populations and were identified as a distinct group by the software.

3. Results

3.1. Mitochondrial DNA sequence diversity, species distinction and identification of maternal introgression

The mtDNA CR sequence alignment (431 nucleotide long) showed 92 distinct haplotypes, including 36 haplotypes in red-legged partridges, 21 in rock partridges and 35 in chukar partridges, defined by 63 polymorphic sites (62 nucleotide substitutions and one insertion/deletion). The NJ clustering grouped these haplotypes into three monophyletic clades supported by BP $\geqslant$ 89%, and largely corresponding with morphologic red-legged, rock and chukar partridges (Fig. 3). The average interspecific TN93 sequence divergence was $d = 0.042$ (SD = 0.007). The species clades included also haplotypes (indicated by arrows in Fig. 3) of birds with phenotypes not corresponding with the mitochondrial identification. Six red-legged haplotypes (R15, R17, R22, R24, R26 and R27) were found in 27 rock partridges sampled in the French ($n = 21$) and Italian Alps ($n = 2$), Apennines ($n = 2$) and Sicily ($n = 2$). Six chukar haplotypes (C1, C2, C7, C14, C26 and C35) were found in French red-legged ($n = 6$) and rock partridges ($n = 3$), as well as in rock partridges from the Italian Alps ($n = 2$) and Sicily ($n = 1$).

Partridges sampled within the Alpine hybrid zone (indicated with HYZ in Fig. 3) showed mtDNA haplotypes both from red-legged (11) and rock partridge (16) parentals, as expected. However, there were also 11 hybrid birds that unexpectedly showed chukar mtDNA haplotypes, which could not originate from natural introgression, but from hybridisation with released captive-bred female chukars or hybrids. The proportions of chukar, red-legged and rock partridge mtDNAs that were found in the Alpine populations are shown in Fig. 4a. This plotting indicates that the frequency of red-legged mtDNA haplotypes introgressed in the Alpine rock partridges sharply falls below 10% within 100 km distance from the Alpine hybrid zone.

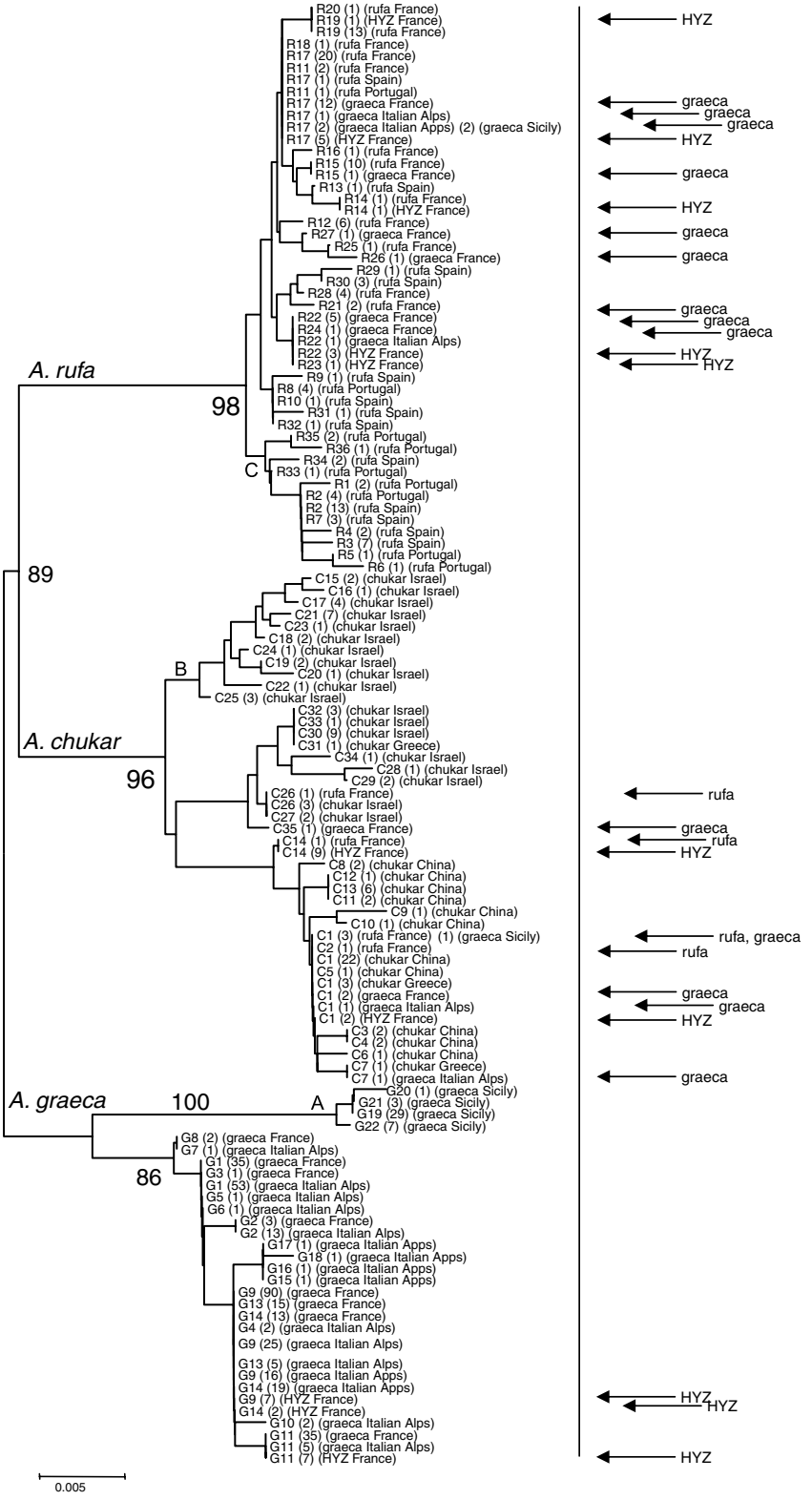

Fig. 3 – Mid-point rooted neighbor-joining tree computed using MEGA and Tamura-Nei genetic distances between mtDNA control-region haplotypes of rock, chukar and red-legged partridges. Bootstrap values of the main clades are indicated. The arrows indicate all discordant haplotypes that were found in partridges phenotypically identified as *A. rufa*, *A. chukar* and *A. graeca*, or that were sampled in the hybrid zone (HYZ; location no. 2 in Fig. 2). Haplotype numbers and sampling locations are indicated.

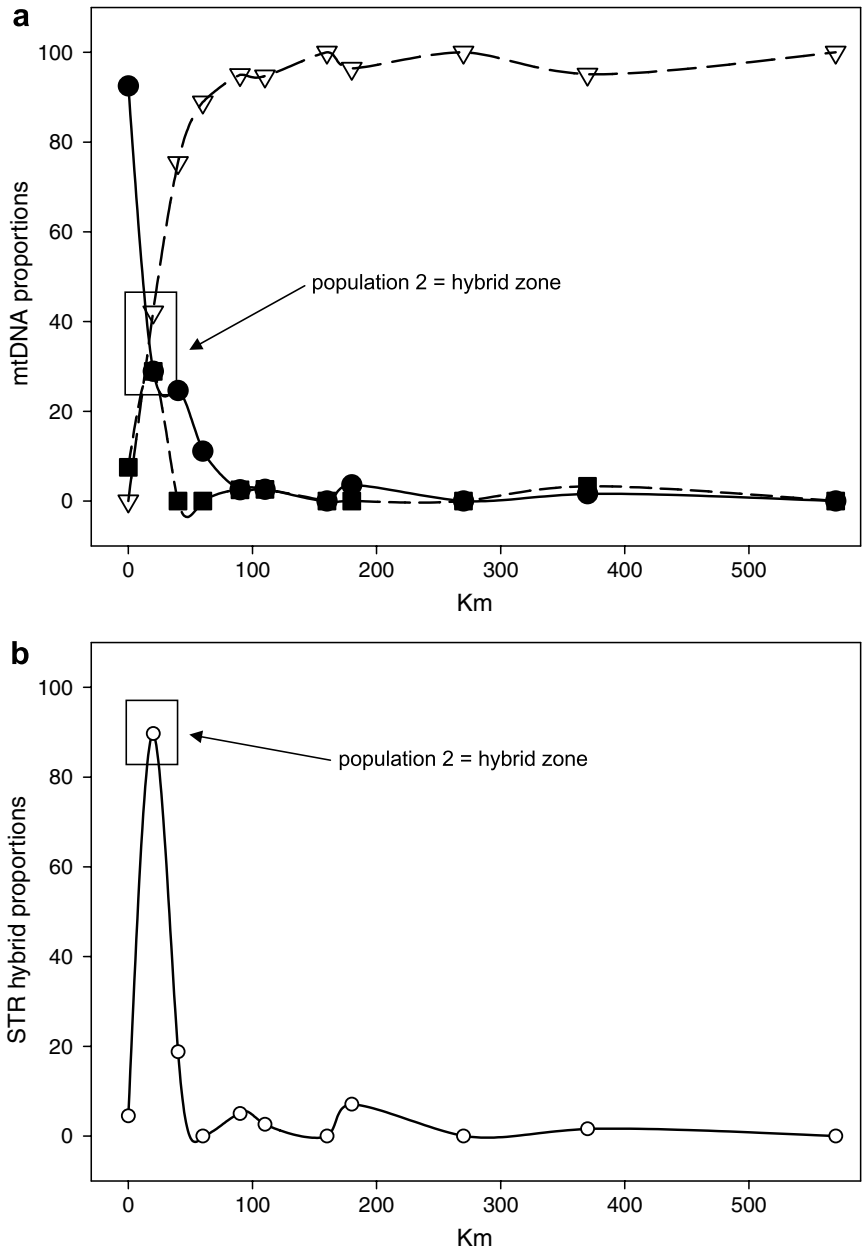

Fig. 4 – (a) Proportions of *A. graeca* (empty triangles), *A. chukar* (filled squares) and *A. rufa* (filled circles) mtDNA haplotypes found in Alpine partridges, plotted against distances (measured in km across the Alpine ridge) starting from the red-legged population in Cipières (population 1 in Fig. 2). Note that the proportions of *A. chukar* and *A. rufa* mtDNA haplotypes in population 2, the hybrid zone, are identical. (b) Proportion of admixed microsatellite (STR) genotypes in the same populations. Admixture proportions were computed as the percent of individuals that had admixed ancestry after the assignment procedure (STRUCTURE, $K = 4$; see Section 3).

3.2. *Genetic diversity and species distinction at microsatellite loci*

The eight microsatellite loci showed 62% (in Chinese chukars) to 100% (in the hybrid zone) polymorphic loci (at the 95% level), and from 2.7 (in Chinese chukars) to 4.9 (in the hybrid zone) alleles per locus, on average. Expected heterozygosity was lowest in Sicilian rock partridges ($H_E = 0.26$), and highest in partridges of the Alpine hybrid zone ($H_E = 0.55$), which showed the highest levels of variability, an expected outcome of genetic admixture. Observed and expected heterozygosities were similar in the samples, except in Sicily, where H_O was lower than expected, and deviations from HWE were significant ($P < 0.05$; estimated by permutations using GENETIX). Microsatellite diversity was significantly partitioned among the 11 sampled populations (Weir and Cockerham's multilocus $F_{ST} = 0.49$, $P < 0.001$). The FCA plotting of individual genotypes showed that chukars grouped separately from the other partridges on the first factorial component CA-I, while the red-legged and rock partridges were separated on CA-II (Fig. 5). Partridges sampled in the hybrid zone plotted intermediately, partially overlapping with both the red-legged

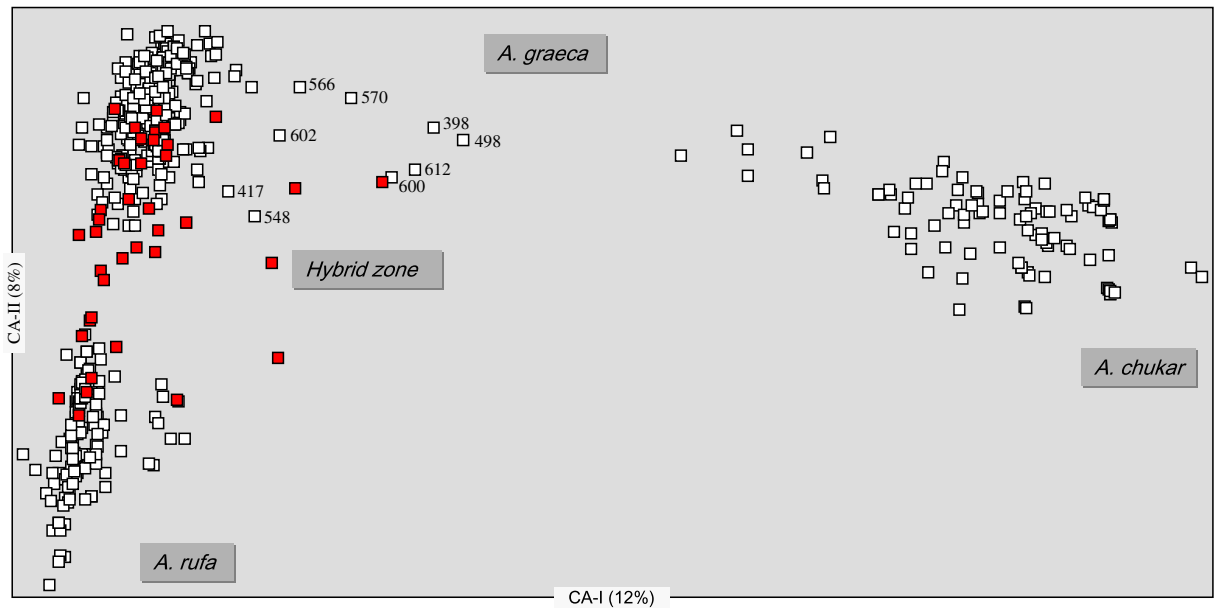

Fig. 5 – Factorial correspondence analysis of individual microsatellite genotypes. Dark squares indicate partridges sampled in the hybrid zone. Some outlier *A. graeca* are marked.

and rock partridge distributions. Some rock partridges (labelled in Fig. 5) and hybrids plotted towards the chukar distribution.

3.3. *Bayesian assessment of genetic subdivisions and identification of cryptic hybrids*

STRUCTURE was run with 590 presumptive non-hybrid samples, both with and without their mtDNA haplotypes, USEPOPINFO = 0, and $K = 1$–9. Optimal genetic subdivisions were obtained with $K = 3$ or 4 (Fig. 6a). Almost all the samples were univocally assigned to single clusters (with individual $q_i \geqslant 0.90$), supporting the morphological species identifications, and some admixed genotypes were identified (Fig. 6b and c). With $K = 3$, chukars were assigned to cluster 1 with an average proportion of membership $Q_1 > 0.99$; red-legged partridges were assigned to cluster 2 with $Q_2 = 0.97$ (samples from France), or 99% (Portugal and Spain).The rock partridges were assigned to cluster 3 with $Q_3 > 0.98$ (Fig. 6b). Very similar results were obtained with $K = 4$, with the rock partridges from Sicily being assigned to their own cluster 4($Q_4 = 97\%$), whereas rock partridges from the Apennines were assigned to both clusters 3 and 4 (cumulative $Q_3 + Q_4 = 95\%$; Fig. 6c). In these analyses we identified 28 putative admixed samples (mainly in French rock and red-legged partridges; Fig. 6b and c), which were jointly assigned to two clusters with individual proportion of membership to each one $q_i < 0.90$. All the other samples were assigned to their specific cluster with individual $q_i \geqslant 0.90$.

STRUCTURE was then run with all the samples, including those with discordant mtDNA and the 28 putative admixed samples, except the partridges collected in the hybrid zone ($n = 629$; using or not their mtDNA haplotypes, USEPOPINFO = 1, and $K = 4$). Results confirmed the occurrence of 32 admixed individuals, which are listed in Table 2. There were no discordant mtDNAs or admixed genotypes among the red-legged partridges sampled in Iberia. In contrast, nine presumed red-legged partridges from France showed chukar or red-legged mtDNAs. Five red-legged partridges with chukar mtDNAs were not identified as hybrids by STRUCTURE. One rock partridge showing a red-legged mtDNA haplotype was sampled within the red-legged partridge population (number 1 in Fig. 2), and could be a hybrid migrating out of the hybrid zone. Sixteen rock partridges sampled close to the hybrid zone (population 3 in Fig. 2) showed red-legged mtDNAs, and seven of them were identified as red-legged x rock partridge hybrids also by STRUCTURE. Moreover, in population 3 there were seven birds with rock partridge mtDNAs that were identified as admixed by STRUCTURE. Admixed partridges were identified also in populations 5–7, located far from the hybrid zone. Three of them showed chukar mtDNAs. Ten additional birds with rock partridge mtDNAs were identified as red-legged x rock partridge hybrids. Two birds were identified as hybrids with chukar by STRUCTURE. Eight of the 12 hybrids found among the Italian Alpine, Apennine and Sicilian rock partridges were also hybrids with chukars. Genetic analyses discovered three possibly mislabelled individuals that were labelled as rock partridges, but that showed red-legged mtDNA and microsatellite genotypes. Most of the admixed rock partridges had ancestry in the red-legged partridge or in the chukar second past generation. Two samples from the central Italian Alps, two from southern Apennines, and three admixed samples from Sicily can result from released captive-bred hybrids with chukars. Using the mtDNA as an additional locus did not change these results.

3.4. *Genetic composition of the Alpine hybrid zone*

STRUCTURE with $K = 4$ split the 39 partridges sampled in the hybrid zone between the rock partridge cluster 1 (with $Q_1 = 0.58$), and the red-legged partridge cluster 4 (with $Q_4 = 0.37$). Individual assignments further revealed that 77% of the samples

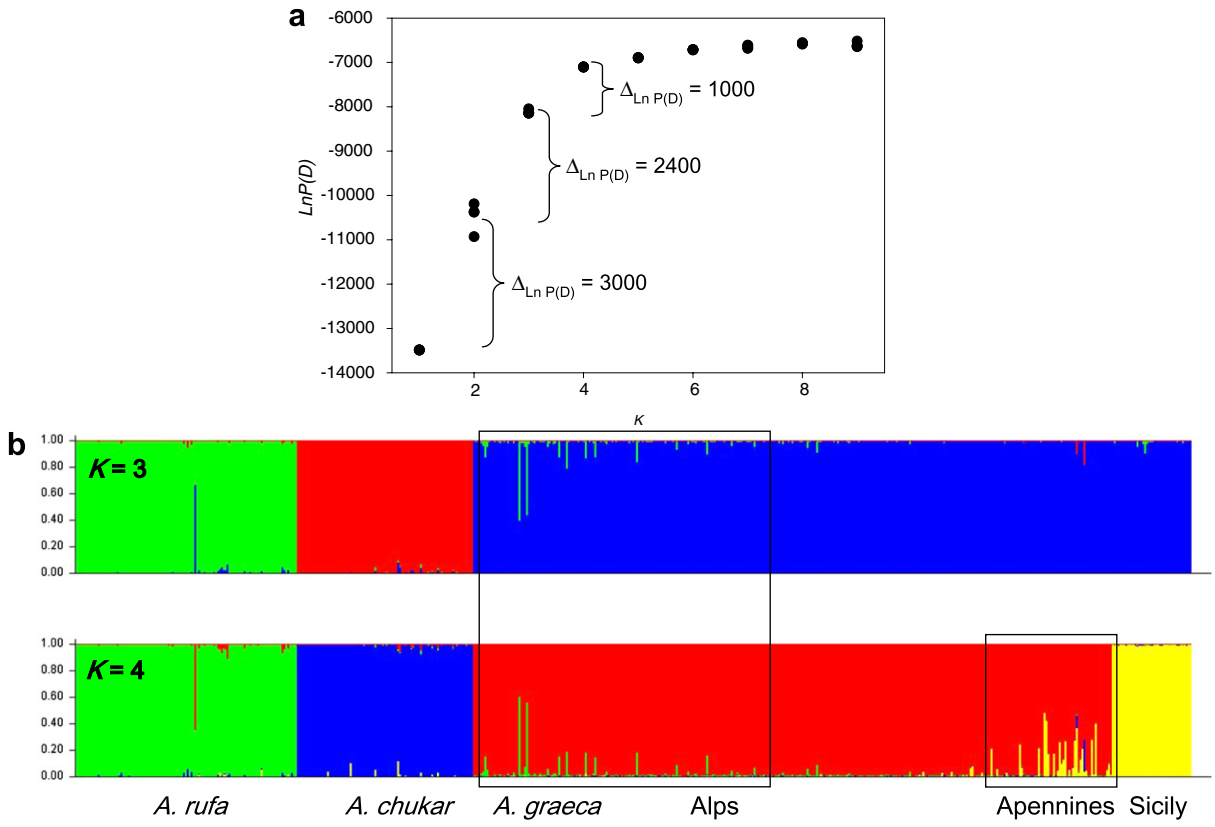

Fig. 6 – Results of STRUCTURE analyses. (a) Plot of ln P(D) and $\Delta_{\ln P(D)} = [\ln P(D)_k - \ln P(D)_{k-1}]$ (computed following Garnier et al., 2004) values for K = 1–9. (b) Assignment of individuals to population clusters for K = 3 and 4. Rock partridges (*A. graeca*) sampled from the French Alps and the Italian Apennines are boxed.

Table 2 – Number of admixed red-legged (*A. rufa*) and rock (*A. graeca*) partridges, which showed discordant mtDNA haplotypes, or that did not join their specific clusters with $q_i \geqslant 0.90$

Admixed samples	*n*	mtDNA	STRUCTURE				Mislabelled
		chukar	*graeca*	*rufa*	*rufa* × *graeca*	*chukar* × *graeca*	
A. rufa France	9	6	0	3	3	0	0
A. graeca France	41	3	17	21	20	2	2 *rufa*
A. graeca Alps	5	2	1	2	0	1	1 *rufa*
A. graeca Apennines	4	0	2	2	1	2	0
A. graeca Sicily	3	1	0	2	0	3	0

Assignments were obtained using STRUCTURE with POPFLAG option not active, with eight microsatellites and the mtDNA as an additional locus and K = 4. Mislabelled individuals are three samples which were labelled as *A. graeca*, but that showed *A. rufa* mtDNA and microsatellite genotypes.

show detectable signals of admixture (Fig. 7): only three birds (34, 38 and 39; 7.7%) could be assigned to the parental red-legged partridges; six birds could be parental rock partridges (1, 2, 4, 5, 6 and 7; 15.4%). All the other samples showed discordant nuclear/mtDNA assignments (i.e., sample 3, which could be identified as a rock partridge with red-legged partridge mtDNA haplotype R17), or they were strongly admixed, showing individual $q_i < 0.90$. Among the admixed birds, only six partridges (22–27; 15.4%) showed the intermediate q_i values ($0.40 < q_i < 0.60$), which are expected in first generation hybrid (F_1). All the other partridges (24/39 = 61.5%) showed prevalent rock or red-legged partridge genotypes, suggesting that they are backcrosses (Fig. 7). Results obtained with NEWHYBRIDS also suggests that partridges in the hybrid zone included mainly F_2 and backcrosses: No hybrid partridge was assigned to the F_1 genotypic class, but there were four F_2 (sample 1, 2, 14 and 31), and three backcrosses with rock partridges (7, 8 and 22). Fourteen samples showing hybrid ancestry with STRUCTURE were classified as parental rock partridges by NEWHYBRIDS. However, five of them had chukar (17, 37) or red-legged partridge (20, 32, 39) mtDNAs. Three samples (12, 16, 18) were concordantly identified as parental red-legged partridges. The other samples were partially identified as F_2 or backcrosses.

This hybrid zone likely led to introgression of red-legged partridge mtDNA haplotypes and microsatellite alleles into the adjacent Alpine rock partridge populations (Fig. 2). The proportion of admixed genotypes, computed as the percent of individuals that had admixed ancestry after the assignment

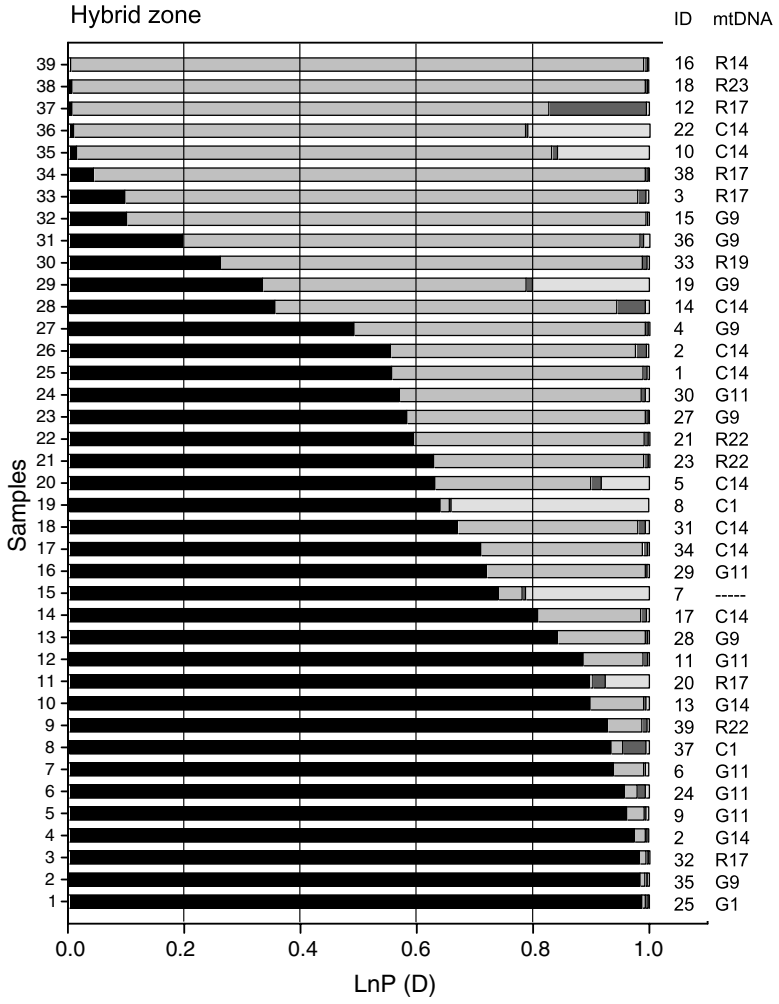

Fig. 7 – Genetic composition of partridges sampled in the Frenc hybrid zone (Alpes-Maritimes), estimated using STRUCTURE with *K* = 4 genetic clusters. ln *P*(*D*) is the probability of each individual genotype (samples) to be assigned to one of four genetic clusters. Light grey bars indicate the proportion of red-legged genes; dark grey bars indicate the proportion of rock partridge genes, intermediate grey bars indicate the proportion of chukar genes. Individual mtDNA haplotypes (labelled as R = *A. rufa*; C = *A. chukar*; G = *A. graeca*) are indicated.

procedure (STRUCTURE, *K* = 4, PF = 1, mtDNA) in the sampled Alpine populations is plotted in Fig. 4b, which shows that the peak of hybrids in population 2 (partridges sampled within the hybrid zone) quickly drops down close to zero in rock partridge populations less than 150 km away from the contact zone.

4. Discussion

Invasive species and translocated populations are threatening native populations by hybridisation, raising risks of genetic extinction, loss of local adaptations or outbreeding depression (Templeton, 1986; Rhymer and Simberloff, 1996; Allendorf et al., 2001). Worldwide translocations of non-indigenous populations are threatening a number of species of anseriforms (such as the New Zealand grey duck, *Anas superciliosa* superciliosa; Rhymer et al., 1994; the white-headed duck, *Oxyura leucocephala*; Munoz-Fuentes et al., 2007) and galliforms (such as the Italian subspecies of grey partridge, *Perdix perdix italica*; Liukkonen-Attila et al., 2002; and wild populations of the common quail, *Coturnix c. coturnix*, threatened by restocking with domesticated Japanese quail, *Coturnix c. japonica*; Barilani et al., 2005).

Genetic data in this and other studies indicate that hybridisation with chukars partridges is widespread across the entire distribution range of the red-legged partridge (Tejedor et al., 1994; Negro et al., 2001), as well as in introduced red-legged partridges in Britain (Potts, 1989) and central Italy (Baratti et al., 2004; Barbanera et al., 2005). Between the 1960s and the 1980s chukars, or hybrids with chukars, were massively released to restock hunted red-legged populations in Iberia (Dias, 1992; Arruga et al., 1996), France (Goodwin, 1986), and Italy (Priolo, 1970), leading to widespread genetic pollution of native populations. Hybrids, mainly with chukars, were found also across the entire native distribution of the rock partridge.

4.1. *Widespread hybridisation in rock partridges*

Alectoris partridges speciated in allopatry during the Pleistocene period, and did not evolve strong intrinsic mechanisms

of reproductive isolation since then (Randi and Bernard-Laurent, 1998). This is examplified by the naturally hybridising populations of red-legged and rock partridges in the French contact zone (Bernard-Laurent, 1984; Randi and Bernard-Laurent, 1999), and by the occurrence of hybrids in other natural (Barilani et al., 2007) or introduced populations (Baratti et al., 2004; Barbanera et al., 2005). While natural hybridisation is strictly limited to three small contact zones (Randi and Bernard-Laurent, 1999; Chen et al., 1999), the anthropogenic diffusion of artificial hybrids seems to be much more widespread, and can occur in most of the restocked populations throughout the distribution range of red-legged and rock partridges (Dias, 1992; Bernard-Laurent et al., 2001; Negro et al., 2001; Barilani et al., 2007). Field observations showed that released red-legged partridges can mate in nature (Potts, 1989; Duarte and Vargas, 2004), and that interspecific hybrids are fertile (Bernard-Laurent, 1990). However, genetic data suggest that survival and diffusion of natural hybrids in Alpine habitats might be constrained by natural selection (e.g., as consequences of differential selective and/or competitive pressures between hybrids and their parental populations; Randi and Bernard-Laurent, 1999).

In this study, we identified the presence of chukar mtDNAs and admixed microsatellite genotypes in red-legged and rock partridges that were sampled close to the hybrid zone in the southern French Alps. This hybrid zone contains a high proportion (28%) of chukar mtDNA haplotypes, indicating gene introgression from chukars, or from hybrids with chukars, released to restock the red-legged partridge populations close to the hybrid zone. Rock partridges in this study were sampled between 1989 and 1994, that is before wildlife conservation laws prohibited the introduction of non-indigenous animal species since 1995 in France, and 1997 in Italy. It would be interesting to analyse again partridges originating from the same study areas of the French Alps to assess if chukar genes are still present in these populations today.

Chukar mtDNA and admixed genotypes were found also in 5.1% of rock partridges sampled in the Italian Alps, Apennines and in Sicily. In principle, the presence of chukar alleles in the Italian Alps might be due to occasional long distance dispersal or gene flow from the French hybrid zone. However, the limited introgression of mtDNA haplotypes and admixed microsatellite genotypes across the Alpine rock partridge populations (Fig. 4a and b), suggests strong constraints to gene flow. Thus, the admixed rock partridge genotypes most probably originated as a result of releases of captive-reared chukars or hybrids. The diffuse presence of chukar genes in other parts of the rock partridge range (such as in Greece; Barilani et al., 2007), and in translocated red-legged partridge populations in central Italy (Baratti et al., 2004; Barbanera et al., 2005), calls for stricter control of captive-breeding of *Alectoris* partridges in Europe.

The Bayesian models (STRUCTURE and NEWHYBRIDS) used in this study assume that individuals are genotyped at neutral unlinked molecular markers, which should be in Hardy–Weinberg and linkage equilibrium in the reference populations. The small number of genetic markers used in this study would limit the power of the assignment analyses to the first two-three generations of hybridisation and backcrossing (Pritchard et al., 2000; Anderson and Thompson, 2002). These simulations suggest that the proportion of admixed rock partridges detected in this study in the Alps and Apennines (about 5.0–6.0%) has been underestimated. These limitations can, in principle, be overcome by increasing the number of microsatellite (Gonzalez et al., 2005) or other markers (i.e., SNP; Morin et al., 2004). Fast and cheap RAPD markers can be used in genetic screenings (Barbanera et al., 2005), although dominant markers are less useful for detecting hybrids, and the unknown nature of RAPD variation makes it difficult any genetic interpretation of the results.

4.2. *Genetic admixture and introgression in hybridising partridges*

The partridges sampled in the French hybrid zone included 77% admixed genotypes, most of which (61.5%) were not F_1, but F_2 or some kind of backcross. The hybrid zone seems to be predominantly composed by hybrids originated within the hybrid zone, and not by migrants flowing from parental red-legged or rock partridges. A sporadic occurrence of hybridisation events, and a low frequency of F_1 are typical of most natural hybrid zones where extensive hybridisation and gene introgression are prevented by various behavioural or genetic mechanisms, as predicted by the "tension zone" model (Barton and Hewitt, 1985).

The genetic structure of the hybrid zone is in agreement with plumage trait variation, showing that 86% of partridges have admixed color patterns, while only 10% have typical rock partridge plumage, and 4% have pure red-legged partridge phenotypes (Bernard-Laurent unpublished). In this study, we morphologically identified eight putative rock partridges among the 39 samples collected within the hybrid zone. However, two of them showed chukar mtDNA haplotypes, and one of these two originated in the red-legged population. Therefore, these two birds are probably hybrids that retained introgressed chukar mtDNA. The other six rock partridges, which had *graeca* mtDNA and were identified as *graeca* by STRUCTURE or NEWHYBRIDS, could be tentatively identified as parental *graeca*. Three apparently pure red-legged partridges were assigned to *rufa* by the assignment procedures, and could be considered as parentals migrating into the hybrid zone. The high frequency of chukar mtDNA haplotypes in the hybrid zone suggests that hybridising red-legged partridges were admixed with released captive-bred chukars or hybrids. Thus, the consequences of restocking with captive-reared partridges can be detected also in the hybrid zone, which, from this point of view, should not be considered as a natural one.

The composition of the hybrid zone as inferred from microsatellite data is in agreement with previously published allozyme data (Randi and Bernard-Laurent, 1999). Introgression of red-legged mtDNA CR haplotypes or microsatellite alleles into the rock partridge Alpine populations is detectable up to ca. 100 km away from the hybrid zone (Fig. 4), in agreement with allozyme (see Randi and Bernard-Laurent, 1999; their Figs. 1 and 4), and mtDNA findings (Randi and Bernard-Laurent, 1998). The geographic diffusion of hybrid genomes is lower than expected by neutral genetic cline models. Randi and Bernard-Laurent (1999) estimated that neutral clines generated after post-glacial secondary contact between red-legged and rock partridges in the Alps should be about 1120–2750 km wide,

much wider than the observed clines of 100–150 km. Introgression could be constrained by natural selection against hybrid partridges. If hybrids are unfit and their survival and dispersal are constrained by natural selection, we expect negative impacts from the restocking of natural populations.

4.3. *Management and conservation implications and suggestions*

Red-legged and rock partridge populations are declining in parts of their range. Uncontrolled restockings with captive-reared red-legged and rock partridge, or their hybrids with chukars, is leading to massive introgressive hybridisation, in Iberia and France, and in parts of Italy and Greece. Urgent conservation actions should include the implementation of an officially accepted analytical-based protocol, aimed to identify genetically pure or hybrid populations (both in nature and in captivity), using DNA markers and admixture analyses. Despite their morphologic similarity, the *Alectoris* partridges are genetically well differentiated and can be identified by molecular methods (Randi et al., 1998, 2003; Barilani et al., 2007), by diagnostic plumage and vocalization traits (Ceugniet et al., 1999). The mitochondrial and microsatellite markers used in this paper and in Barilani et al. (2007), analysed with the appropriate statistical procedures, can be applied to assess the extent of hybridisation and gene introgression in partridge populations. Population genetic analyses should be used: (1) to map the distribution of pure natural populations of partridges, and support their conservation in the wild; (2) to enforce strict controls of the genetic status of partridge stocks reproduced in breeding farms and used for restocking or any other hunting activity. These actions, coupled with strict limitations to restocking operations, which should be allowed only under control, and in presence of technically sound releasing and monitoring programs, should help in preserving the gene pools of *Alectoris* species in Europe.

Acknowledgement

We deeply appreciate the collaboration of many people who helped in sampling collection: D. Dias (for sampling in Portugal), P. Alkon and N. Liu (for help during sampling in Israel and China), I. Artuso, P. De Franceschi, A. Gentile, M. Paganin, M. Pandolfi, M. Pellegrini (for sampling in Italy). A. Bernard-Laurent was supported by the Office National de la Chasse et de la Faune Sauvage. Funding for laboratory analyses were provided to E. Randi by the Ministero per le Politiche Agricole e Forestali, and the Ministero per l'Ambiente e la Tutela del Territorio, Direzione Conservazione della Natura.

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