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ORIGINAL PAPER

Human-mediated introgression of exotic chukar (*Alectoris chukar*, Galliformes) genes from East Asia into native Mediterranean partridges

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Abstract Mediterranean red-legged (*Alectoris rufa)* and rock (*Alectoris graeca*) partridge populations are affected by genetic pollution. The chukar partridge (*Alectoris chukar*), a species only partly native to Europe, is the most frequently introgressive taxon detected in the genome of hybrid partridges. Both theoretical (evolutionary) and practical (resources management) matters spur to get insight into the geographic origin of the *A. chukar* hybridizing swarm. The phenotypic *A. rufa* populations colonizing the easternmost part of the distribution range of this species, the islands of Elba (Italy) and Corsica (France), were investigated. The analysis of both mitochondrial (mtDNA: Cytochrome-*b* gene plus Control Region: 2,250 characters) and nuclear (Short Tandem Repeats, STR; Random Amplified Polymorphic DNA, RAPD) genomes of 25 wild (Elba) and 20 captive (Corsica) partridges, disclosed spread introgression of chukar origin also in these populations. All mtDNA haplotypes of Elba and Corsica partridges along with those we obtained from other *A. rufa* (total, $n = 111$: Italy, Spain, France) and *A. graeca* ($n = 6$, Italy), were compared with the mtDNA haplotypes of chukars ($n = 205$) sampled in 20 countries. It was found that the *A. chukar* genes detected in red-legged ($n = 43$) and rock partridges ($n = 4$) of Spain, France and Italy as well as in either introduced (Italy) or native (Greece, Turkey) chukars ($n = 35$) were all from East Asia. Hence, a well-defined geographic origin of the exotic chukar genes polluting the genome of native Mediterranean *A. rufa* and *A. graeca* (inter-specific level) as well as *A. chukar* (intra-specific level), was demonstrated.

Keywords *Alectoris chukar* · *Alectoris graeca* · *Alectoris rufa* · Anthropogenic hybridization · Mitochondrial DNA · Partridges · RAPD · STR · Translocated species

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Abbreviations

CR	Control Region
Cyt-*b*	Cytochrome-*b* gene
MtDNA	Mitochondrial DNA
RAPD	Random Amplified Polymorphic DNA
STR	Short Tandem Repeats

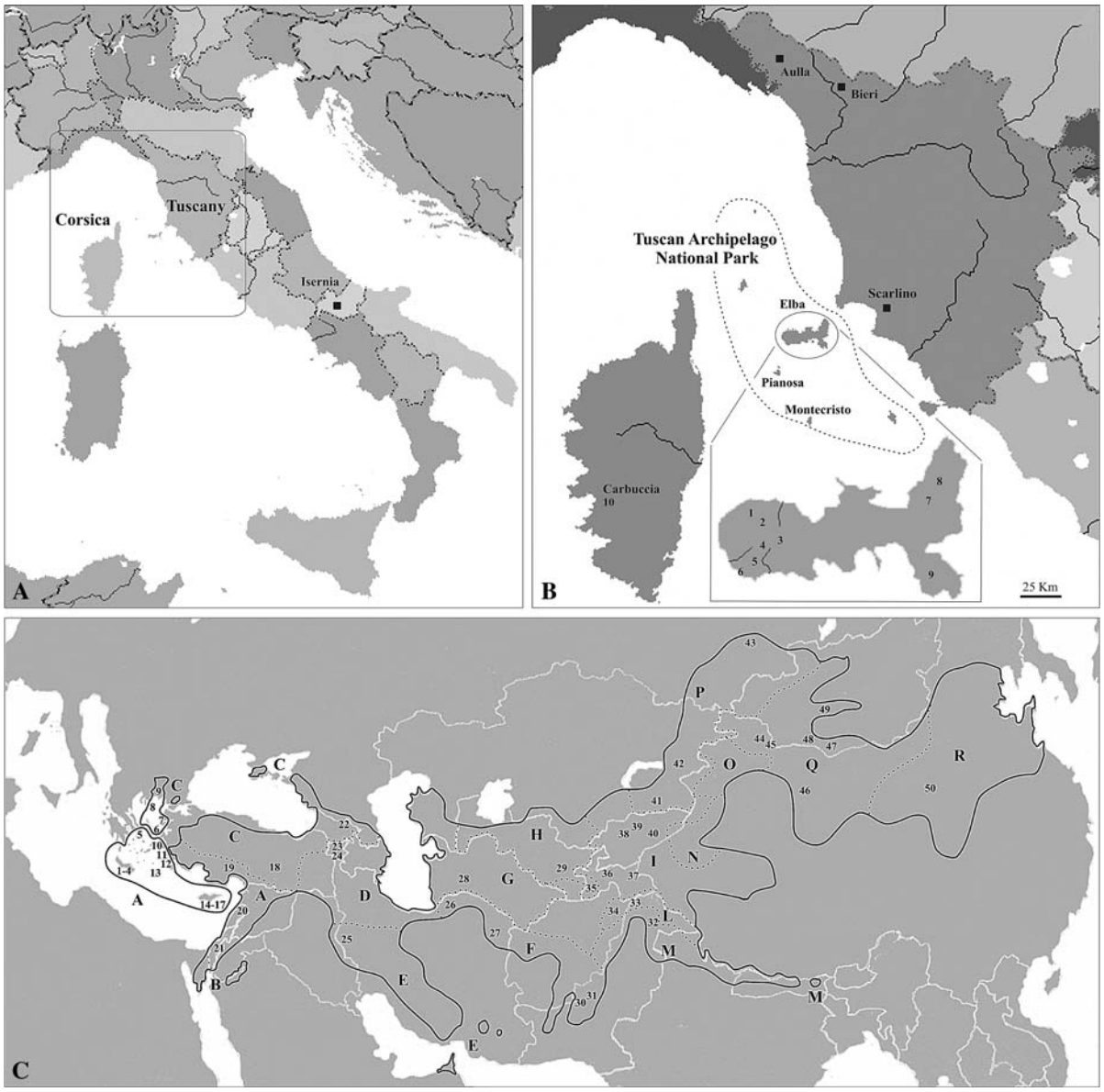
Introduction

In the last centuries there has been a considerable displacement of species around the globe resulting from trade and landscape alterations by human activity. Hybridization between invasive or translocated organisms and representatives of local populations is the second most important threat to biodiversity after habitat degradation (Mack et al. 2000; Allendorf and Luikart 2007). Even though the risk is particularly relevant in taxa comprising wild and domesticated or captive-reared relatives, it often remains underestimated because the process cannot be detected without a DNA-based monitoring (Darling and Blum 2007; Randi 2008).

The red-legged partridge, *Alectoris rufa*, is a member of the order Galliformes whose native range of distribution extends from Iberian Peninsula throughout France up to North Western (NW) Italy, including Corsica and Tuscan Archipelago (Madge and McGowan 2002; Fig. 1a, b). Since the past century both hunting pressure and fast transformation of the rural landscapes have induced the species' decline, which was not balanced by intensive yet genetically uncontrolled restocking. This practice led to the spreading of allochthonous genes within the species. Many *A. rufa* populations suffered introgressive hybridization in Italy, France and Spain because of release of hybrids between red-legged and chukar partridge, *Alectoris chukar* (Barbanera et al. 2005; Barilani et al. 2007; Tejedor et al. 2007). The distribution range of this species extends from Aegean Sea to Central (C) and Eastern (E) Asia (Madge and McGowan 2002). Although Negro et al. (2001) provided a set of molecular markers to detect hybrids between red-legged and rock partridge (*Alectoris graeca*), a species native to the Mediterranean (Italian and Balkan Peninsulas), *A. rufa* × *A. graeca* specimens were identified only where the ranges of distribution of the two species get into contact and natural interbreeding occurs (SE France: Bernard-Laurent 1984). Hence, their native distribution restricted to the E basin, notwithstanding the *A. chukar* genes are the most frequent in the hybrid genome of Mediterranean partridges (Barbanera et al. 2007).

In this work, definitive evidence that a spread *A. rufa* × *A. chukar* hybridisation characterizes even the populations colonizing the easternmost part of the *A. rufa* distribution range, the islands of Elba (Italy) and Corsica (France) (Fig. 1a, b), was produced. Hence, both theoretical (evolutionary) and practical (resources management) matters spurred to get insight into the geographic origin of the *A. chukar* introgressive swarm affecting Mediterranean partridges. To draw an overall picture of this genetic admixture, past (Barbanera et al. 2005, 2007) and present results produced on this matter by the authors were pooled together. A comparison with data obtained from *A. chukar* representatives sampled

Fig. 1 (**a**) The studied area. (**b**) *A. rufa* sampling areas at Elba (1–9) and in Corsica (10). (**c**) The *A. chukar* distribution range (thick line) including subspecies (dotted line) (A: *A. c. cypriotes*; B: *A. c. sinaica*; C: *A. c. kleini*; D: *A. c. kurdestanica*; E: *A. c. werae*; F: *A. c. koroviakovi*; G: *A. c. shestoperovi*; H: *A. c. subpallida;* I: *A. c. falki*; L: *A. c. pallescens*; M: *A. c. chukar*; N: *A. c. fallax*; O: *A. c. pallida*; P: *A. c. dzungarica*; Q: *A. c. potanini*; R: *A. c. pubescens*). The sample type (dried blood spots, DBS; feathers, F; liver, L), the captive (c) or wild (w) nature, the sampling area and the number of specimens are as follows: Greece (L, w: 1–4, Crete, $n = 16$; 5, Andros, $n = 6$; 6, Chios, $n = 6$; 7, Lesvos, $n = 10$; 8, Limnos, $n = 9$; 9, Mepaika, $n = 1$; 10, Lipsi, $n = 1$; 11, Leros, $n = 1$; 12, Kalimnos, $n = 1$; 13, Karpathos, $n = 1$), Cyprus (L, w: 14, Paphos, $n = 6$; 15, Larnakas, $n = 6$; 16, Karpasia, $n = 6$; F, c: 17, Stavrouvoni, $n = 7$), Turkey (F, w: 18, Adiyaman, $n = 1$; 19, Mersin, $n = 2$), Lebanon (F, w: 20, Aammiq, $n = 3$), Israel (L, w: 21, $n = 7$), Georgia (L, w: 22, Kahetia, $n = 2$), Armenia (DBS, c: 23, Garni, $n = 2$; L, w: 24, Yeghegnadzor, $n = 4$), Iran (F, w: 25, Ilam, $n = 4$; 26, N Khorasan, $n = 4$; 27, Razavi Khorasan, $n = 4$), Turkmenistan (DBS, w: 28, Garrygala, $n = 5$), Uzbekistan (DBS, c: 29, Baysun, $n = 1$), Pakistan (F, w: 30, Quetta, $n = 2$; 31, Loralai and Berg, $n = 3$; 32, Kashmir, $n = 7$; 33, Chitral, $n = 4$), Afghanistan (F, w: 34, Kabul, $n = 1$), Tajikistan (DBS, c: 35, Kurgan-Tyube, $n = 1$; 36, E Turkistan, $n = 2$; 37, E Pamirs, $n = 2$), Kyrgyzstan (F, w: 38, Ak-Suu river, $n = 2$; 39, Shamsi river, $n = 6$; 40, Kyzyl-Ompol, $n = 3$), Kazakhstan (L, w: 41, Tien Shan, $n = 2$; DBS, c: 42, Tarbagatay, $n = 2$), Russia (DBS, c: 43, Krasnoyarsk Krai, $n = 2$), China (F, w: 44, Aibi, $n = 2$; 45, Baytag, $n = 5$; DBS, c: 46, Qi-Lian, $n = 2$; F, w: 50, Zheng, $n = 9$), Mongolia (L, w: 47–48, Omnogovi, $n = 5$; F, w: 49, Ovorkhangai, $n = 1$). Others were collected in USA (Nevada, L, w, four different counties, $n = 9$; Washington, L, w, $n = 1$) and Italy (Montecristo, F, w, $n = 16$). *Alectoris graeca* samples are from Italy (Isernia, L, w, $n = 6$); Spanish and French mainland *A. rufa* sampling sites are not reported (see Table 1).



throughout the entire distribution range of the species was done (Fig. 1c). The introgression of exotic chukar genes from East Asia into native Mediterranean partridges was demonstrated.

Methods

Biological sampling

We performed non-invasive sampling of wild *A. rufa* on Elba Island given its current low population density (1–2 pairs/km^2; R. Giombini pers. com.). Dry faeces (n = 25) were individually collected during winter at well-distant sampling sites (Fig. 1b). No chemicals were added and no extra drying was carried out before samples were stored at –40°C. One scat per sampling site was analyzed to avoid duplicate samples. Other *A. rufa* samples were collected in Italy (n = 32), Spain (n = 29) and France (n = 25) (Table 1). Among these latter, the captive population of Carbuccia (n = 20, Corsica) was selected as it is the only farm of the island committed to the partridges' management. Indeed, it provides birds

Table 1 The *A. rufa* (n = 111), *A. graeca* (n = 6) and *A. chukar* samples are indicated

Species	Country	Locality	Population type	Samples (n)	Tissue type	Literature record
A. rufa	Italy	Elba	Wild	25	Faeces	This study
A. rufa	Italy	Pianosa	Wild	10	Feather	Barbanera et al. (2005)
A. rufa	Italy	Bieri farm	Captive	10	Feather	Barbanera et al. (2005)
A. rufa	Italy	Scarlino farm	Captive	10	Feather	Barbanera et al. (2005)
A. rufa	Italy	Aulla farm	Captive	2	Feather	This study
A. rufa	France (Corsica)	Carbuccia farm	Captive	20	Feather	This study
A. rufa	France	Atlantic Loire	Wild	5	Liver	This study
A. rufa	Spain	Andalusia	Wild	7	Muscle	This study
A. rufa	Spain	Andujar farm	Captive	10	Feather	This study
A. rufa	Spain	Mallorca	Wild	6	Muscle	Barbanera et al. (2005)
A. rufa	Spain	Ciudad Real	Wild	2	Muscle	Barbanera et al. (2005)
A. rufa	Spain	Catalonia	Wild	2	Liver	This study
A. rufa	Spain	Extremadura	Wild	2	Liver	This study
A. graeca	Italy	Isernia	Wild	6	Liver	This study
A. chukar	From 11 countries	See Fig. 1c	See Fig. 1c	81	See Fig. 1c	Barbanera et al. (2007)
A. chukar	From 20 countries	See Fig. 1c	See Fig. 1c	124	See Fig. 1c	This study

Detailed *A. chukar* sampling data are reported in Fig. 1

for the 70% of the restocking activity, the residual 30% being from French mainland farms (C. Pietri pers. com.). Also n = 6 Italian *A. graeca* specimens were studied (Fig. 1a). Further, the *A. chukar* distribution range was reconstructed according to the literature records reporting a total of 16 subspecies (Dementiev et al. 1952; Abdusalyamov 1971; Ming 2001; Zheng 1987; Madge and McGowan 2002; Clements 2007; A. Ostaschenko pers. com.). Whereas in a former paper only 81 *A. chukar* specimens had been studied (Barbanera et al. 2007), in this study the samples size was largely increased (n = 205) either in the natural or introduced distribution range of the species (Fig. 1c; Table 1). Samples (n = 19) of chukars from zoos were collected only when managers provided precise information about their origin in the wild.

DNA extraction

DNA was extracted from liver and feathers as reported by Barbanera et al. (2005), and from dried blood spots on filter paper (Whatman, UK) using the Puregene® Genomic DNA Isolation Kit (Gentra Systems, USA).

DNA was isolated from faecal samples using the QIAmp DNA Stool Mini-kit (Qiagen, Germany). A whole scat (~250 mg, on average) was used in each extraction following the manufacturer's instructions. All extractions from faeces were done in a laboratory free of partridge DNA and two blanks (no scat) were included in each session. For the first PCR (see below), 3 µl of the final elution (200 µl, total volume) were used.

Mitochondrial DNA

The partial Cytochrome-*b* (Cyt-*b*, 1,092 bp) and the entire Control region (CR, about 1,155 bp) of the mitochondrial DNA (mtDNA) were amplified for all specimens following Barbanera et al. (2005) (Table 2). Only with faecal DNA templates no bands were visualised after gel electrophoresis. Nevertheless, the PCR products were purified using Genelute PCR Clean-up Kit (final volume, 40 µl; Sigma Aldrich, Italy) and 1 µl was re-amplified by means of a semi-nested PCR (snPCR), which is similar to a nested PCR except that one of the primers is used in the first PCR (Table 2). The general profile of the snPCR was the same as the first PCR but annealing was set at 60°C instead of 55°C (cf., Barbanera et al. 2005). For each mtDNA marker, two semi-nested reactions were prepared to get two overlapping fragments. Each intermediate primer (Table 2) was added to the reaction since its onset whereas the other

Table 2 The primers used in this study: (1) to amplify the partial (1,092 bp) Cyt-*b* and the entire CR in the first PCR (Cyt-*b*: CytL/CytH; CR: PHDL/H1321); (2) to get 575 bp (Cyt-*b* I) and 734 bp (Cyt-*b* II) long Cyt-*b* fragments (CytL/SEMC575 and SEMC409/CytH, respectively) in the snPCR; (3) to get 621 bp (CR I) and 689 bp (CR II) long CR fragments (PHDL/SEMD621 and SEMD467/H1321, respectively) in the snPCR; (4) to sequence the Cyt-*b* (SEMC409, SEMC575, SEQC519, SEQC1047); (5) to sequence the CR (SEQD83, SEQD484, SEQD604, SEQD1120)

Primer	5′–3′ sequence	First PCR	snPCR	Sequencing
Cyt-*b*				
F: CytL	ATGGCACCTAATATCCGAAAATCT	Yes	Yes	No
R: CytH	TTAGTAGTTGAGAATTTTATTTTCAAG	Yes	Yes	No
F: SEMC409	GGCCAAATATCCTTCTGRGGG	No	Yes	Yes
R: SEMC575	CCTGCGATTACGAAGGGGA	No	Yes	Yes
F: SEQC519	CCCAACCCTTACCCGATTCTT	No	No	Yes
R: SEQC1047	GATGAAGGGGTGTTCTACTGGTT	No	No	Yes
CR				
F: PHDL	AGGACTACGGCTTGAAAAGC	Yes	Yes	No
R: H1321	TAGYAAGGTTAGGACTRAGTCTT	Yes	Yes	No
F: SEMD467	CCTCGGTCAGGCACATCC	No	Yes	No
R: SEMD621	AACCTGTGAAGAAGCCCCAGA	No	Yes	No
F: SEQD83	TATATTTATATGCCCCATATATATG	No	No	Yes
R: SEQD484	GGATGTGCCTGACCGAGG	No	No	Yes
F: SEQD604	GGGGCTTCTTCACAGGTT	No	No	Yes
R: SEQD1120	AATAGTATTTGTTTGTGGGG	No	No	Yes

With the exception of CytL, CytH, H1321 (Barbanera et al. 2005) and PHDL (Fumihito et al. 1995), the nomenclature of the primers refers to the position of their 5′ terminal nucleotides in the Cyt-*b* and CR sequences published by Randi (1996) and Randi and Lucchini (1998), respectively. Legend: F, forward; R, reverse

only after five cycles. The above-mentioned changes guaranteed a higher specificity in the snPCR (Andreoli 2006). After the snPCR, the products were always visualized after gel electrophoresis, purified as above and sequenced on both DNA strands. All sequencing reactions were done in total reaction volumes of 10 µl containing 4 µl of template, 2 µl of both BigDye® Terminator v. 3.1 Cycle Sequencing mix, 2 µl of Cycle Sequencing Buffer 5X (Applied Biosystems, USA) and 2 µl (~3 pmol) of each primer (Table 2). The reactions were analyzed on an ABI Prism® 310 sequencer (Applied Biosystems). A careful check of either purity (negative control: no DNA) or workability of the reagents (positive control: DNA from feather) was performed during standard PCR and snPCR. The sequences of all of the haplotypes were deposited at the GenBank (accession codes: AM850716–AM850851).

The alignment of *A. rufa* (n = 111), *A. graeca* (n = 6) and *A. chukar* (n = 205) Cyt-*b* and CR joint sequences was performed with CLUSTAL 1.81 (Thompson et al. 1994). The sequences were analysed as a unique mtDNA data set (Partition-homogeneity test, P = 0.98, PAUP* 4.0b10: Swofford 2002). Phylogenetic relationships were inferred using PAUP* with both Neighbour Joining (NJ: Saitou and Nei 1987) and Maximum Parsimony (MP: Swofford et al. 1996) methods. The GTR + I + G algorithm was selected using MODELTEST 3.6 (Posada and Crandall 1998) and following the Akaike Information Criterion (Posada and Buckley 2004). The MP procedure was set-up as in Barbanera et al. (2007). Two out of six *A. graeca* specimens ($Apps_{1,2}$) were used as outgroup and the statistical support was evaluated by bootstrapping (BP, 1,000 resampling steps: Felsenstein 1985). The partition of the genetic diversity was investigated by AMOVA with ARLEQUIN 3.01 (Excoffier et al. 2005) using F_{ST} pairwise distances (1,000 permutations). ARLEQUIN was also used (i) to calculate the mean number of pairwise differences and the haplotype

diversity (h), (ii) to construct the Minimum-spanning tree and (iii) to check for neutral evolution of the mtDNA sequences (Tajima test, D). MEGA 3.1 (Kumar et al. 2004) was used to compute the nucleotide diversity (π), the transitions/transversions ratio (Ti/Tv) and the average sequence divergence.

Short Tandem Repeats (STR)

Both Elba (n = 25) and Carbuccia (n = 20) partridges were STR genotyped together with Spanish *A. rufa* and Cypriot *A. chukar* representatives (n = 25 each: Fig. 1; Table 4). Spanish and Cypriot partridges were used as genetically homogeneous controls (n = 4 Spanish specimens with *A. chukar* mtDNA lineage were excluded, see Results; Barbanera et al. 2005, 2007; Guerrini et al. 2007). Five STR loci originally isolated from either chicken (Wageningen University, The Netherlands: MCW 104, MCW 118, MCW 280) or red-legged partridge genome (Gonzalez et al. 2005: Aru 1.23, Aru 1.27) were investigated. The following PCR thermal profile was performed: (94°C × 2′) + [(94°C × 45″) + (Ta_1 × 45″) + (72°C × 1′)] × 5 cycles + [(94°C × 45″) + (Ta_2 × 45″) + (72°C × 1′)] × 25 cycles + 72°C × 10′ (Table 3). When faecal DNA was used, each locus was amplified five times per specimen to reduce allelic dropout or false alleles (Regnaut et al. 2006): allele size was assigned after at least three identical results. The expected (H_e, under the Hardy–Weinberg Equilibrium, HWE) and observed heterozigosity (H_o) were computed with ARLEQUIN and GENEPOP 3.4 (Raymond and Rousset 1995). The Bayesian clustering analysis (admixture model with independent allele frequencies among populations) was applied using STRUCTURE 2.0 (Pritchard et al. 2000). All simulations were run with 10^6 iterations, following a burn-in period of 10^5 iterations, and replicated four times per each K-value. The optimal K-value was found using the information provided by the program (Evanno et al. 2005): (i) the formula $\Delta_{\ln P(D)} = [\ln P(D)_k - \ln P(D)_{k-1}]$ (ΔK; Garnier et al. 2004), (ii) ln $P(D)_k$ itself and (iii) the α value. Hence, STRUCTURE was employed to estimate the posterior probability for each specimen to belong to one parental species or to have fractions (q_i) of its genome originating from two parental species. According to Vaha and Primmer (2006), an identification threshold was selected ($q_i = 0.90$), assigning each individual to one cluster if $q_i \geq 0.90$ (i.e., *A. rufa* or *A. chukar*) or jointly to two clusters if $q_i < 0.90$ (i.e., *A. rufa* × *A. chukar*).

Random amplified polymorphic DNA (RAPD)

RAPD markers (Welsh and McClelland 1990; Williams et al. 1990), which are well known for their reliability in disclosing hybrid specimens (Rieseberg and Gerber 1995; Anttila et al. 2000; Negro et al. 2001; Barbanera et al. 2005, 2007), were used to study only the Carbuccia representatives (n = 20). The faecal samples of Elba *A. rufa* did not allow their use because of the risk of contamination with exogenous DNA, as they require low annealing temperature in PCR. The amplifications were performed using four *A. rufa* versus *A. chukar* species-specific primers (Operon, Germany) selected

Table 3 The characteristics of the STR loci are reported. F, forward; R, reverse; T_a (°C), annealing temperature; TD, touch-down PCR

Locus	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	Size range (bp)
MCW 104	$(TG)_4$	F: TAGCACAACTCAAGCTGTGAG	TD 62/55	80–110
		R: AGACTTGCACACATGTGACC		
MCW 118	$(TA)_2(TATG)_3$ [TA $(TATG)_2]_2$ $(TA)_3$ (TATG)	F: ATGATGAAGCATTTAGTCTAAG	TD 60/55	143–155
		R: CACTGCATCTCTGAGTAAATTG		
MCW 280	$(AT)_8$	F: TGAATGGTTTTATGCATTGT	TD 60/55	174–178
		R: AGCAACATATCCATAAGTGT		
Aru 1.23	$(TG)_{13}$	F: GTAAACTTGCCCCCTGCTGTTC	TD 62/55	173–187
		R: CTTCTCTGGGCAGCTGTGTC		
Aru 1.27	$(TG)_{13}$	F: GTTCTGGCTTTAAAGAGCTTGG	TD 60/55	170–200
		R: TGAGAATGCAGGACAGGAGATA		

according to a large number of controls for both taxa and already employed to detect either to *A. rufa* or *A. chukar* backcrosses (Barbanera et al. 2005, 2007). The protocol was as in Barbanera et al. (2005). The concurrent presence of distinctive *A. rufa* and *A. chukar* bands in the RAPD profile of a given specimen was considered occurrence of *A. rufa* × *A. chukar* hybridization. Each specimen was tested three times for each primer. The species-specific bands were scored only when present in all replicates.

Results

Mitochondrial DNA

The alignment of Cyt-*b* and CR joint sequences of 322 specimens (outgroup included) defined a set of 2,250 characters, indels included. There were 232 variables sites: among these, 175 were parsimony informative. The Ti/Tv ratio was 3.8 (outgroup excluded). The sequences evolved neutrally (Tajima's $D = 0.535$, $P = 0.78$) and their mitochondrial nature was assessed by comparison with those obtained from isolated mtDNA (Barbanera et al. 2005, 2007). A total of 134 haplotypes were found, 23 (R) belonging to the *A. rufa* and 111 (C) to the *A. chukar*. Unique haplotypes (122) were mostly sampled, for the *A. rufa*, in Spain ($n = 12$), and for the *A. chukar*, in Greece ($n = 25$), Cyprus ($n = 17$) and Pakistan ($n = 9$). Both the NJ (GTR + I + G algorithm, $\alpha = 0.810$) and MP (length, $L = 550$; consistency index, CI = 0.462; retention index, RI = 0.898) reconstructions clustered the haplotypes in two groups corresponding to the *A. rufa* and *A. chukar* species (both BP = 100%). Among the 23 *A. rufa* haplotypes, 13 (R1–R13) were detected only in Spanish representatives, with the exception of R7, which was shared with specimens from Elba. Haplotypes R14–R16 (Elba) and R17 (Carbuccia) were unique. Representatives from Italy (Scarlino) and Corsica shared the haplotype showed by partridges from Atlantic Loire (R19). The average sequence divergence was 0.27% (± 0.07) between Spanish ($n = 25$) and either Corsican ($n = 11$) or Tuscan ($n = 28$) *A. rufa*, in turn, differentiated by 0.15% (± 0.04). Within the *A. chukar* clade, both NJ and MP identified two groups, whose average sequence divergence was 0.58% (± 0.18). The first group (clade-A: BP = 60%, NJ; BP = 61%, MP) included 80 haplotypes sampled from E Mediterranean to C Asia, whereas the second (clade-B: BP = 91%, NJ; BP = 86%, MP) included 31 haplotypes. Among these, 20 were detected in birds from E Asia, whereas 11 (C1, C38, C69–C75, C110, C111: Fig. 2) were found in (i) 43 red-legged partridge sampled in Spain (Catalonia, Extremadura: C38, C69, C70; $n = 4$), France (Carbuccia, Atlantic Loire: C1, C69, C73; $n = 10$) and Italy (C1, C38, C69–C72, C74, C75; $n = 29$), (ii) four Italian rock partridge (Apennines: C1, C38), and (iii) 35 either native (Greece: C1, C38, C74: Crete, $n = 1$; Andros, $n = 4$; Chios, $n = 1$; Lesvos, $n = 1$; Turkey, C1: Mersin, $n = 2$) or introduced (Italy, C70: Montecristo, $n = 16$; USA, C74, C110, C111: Nevada and Washington, $n = 10$) chukars. The *A. chukar* clade-A ($n = 80$) showed values of haplotype diversity (0.990 ± 0.003), mean number of pairwise differences (6.57 ± 3.12) and nucleotide diversity (0.32 ± 0.05) higher than those of the clade-B (0.910 ± 0.015, 2.96 ± 1.56, 0.13 ± 0.04, respectively, $n = 31$; Student's *t*-test: $P < 10^{-3}$, each comparison). Finally, C38 was the most frequent *A. chukar* haplotype (11.5%: Spain, Italy, Greece, China, USA); the Montecristo population showed only one haplotype (C70). The Minimum-spanning tree marked out the divergence between *A. rufa* and *A. chukar* haplotypes (86 mutations; Fig. 3), and within the *A. chukar* group eight changes accounted for the separation between clade-A and clade-B. According to the reconstructions of Fig. 2, AMOVA indicated that 70.8% of the total mitochondrial variance was partitioned among-groups (clade-A versus clade-B, $F_{CT} = 0.71$), 9.8% among populations within groups ($F_{SC} = 0.33$) and 19.4% within populations ($F_{ST} = 0.80$) (all $P < 10^{-5}$).

Short Tandem Repeats

STR results were reported in Table 4. The 76% of faecal samples from Elba gave rise to complete genetic profiles, whereas the 24% was genotyped at four loci (alleles not-determined: 4.8%). Populations from Spain and Elba showed significant deviations from HWE due to heterozygotes deficiency (Fisher test: Spain, $P = 0.0031$; Elba, $P < 10^{-5}$). The 35.3% of the total genetic variability was distributed among populations (F_{ST} between *A. rufa* and *A. chukar* controls = 0.59), 14.9% among individuals within populations, and 49.8% among all individuals (AMOVA, all $P < 10^{-5}$).

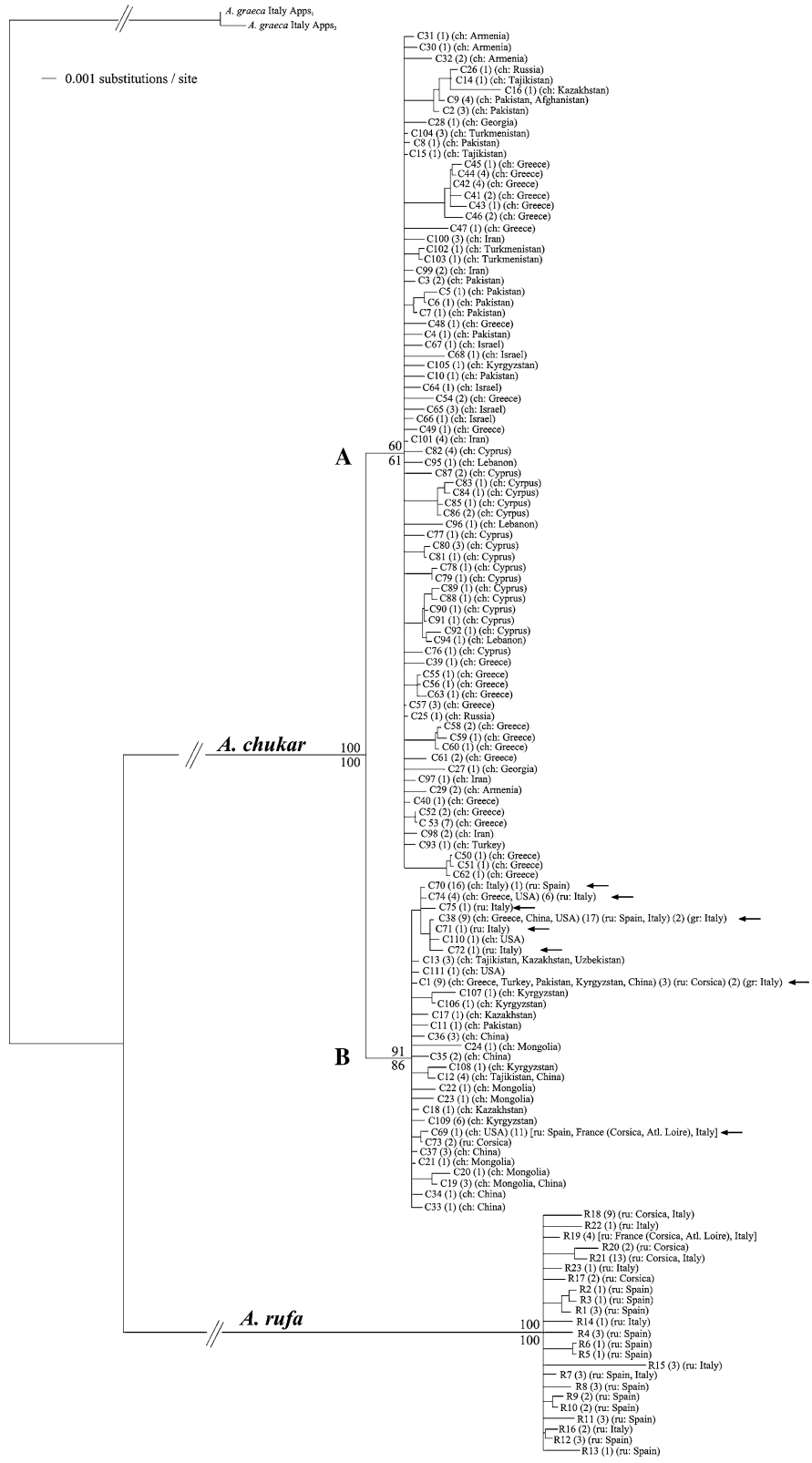


Fig. 2 NJ tree computed by PAUP* for the aligned 134 haplotypes using the GTR + I + G algorithm ($\alpha = 0.810$). Numbers at the internodes indicate main bootstrap percentage values computed in both the NJ (above internodes) and 50% majority-rule consensus MP (below internodes) tree. The phylogenetic trees are rooted using *A. graeca*. Haplotype (R, *A. rufa*; C, *A. chukar*) number and total occurrence, partridge phenotype (ru: *A. rufa*; gr: *A. graeca*; ch: *A. chukar*) and sampling area are reported. Arrows indicate all *A. chukar* haplotypes found in partridges morphologically assigned to *A. rufa* or *A. graeca* species

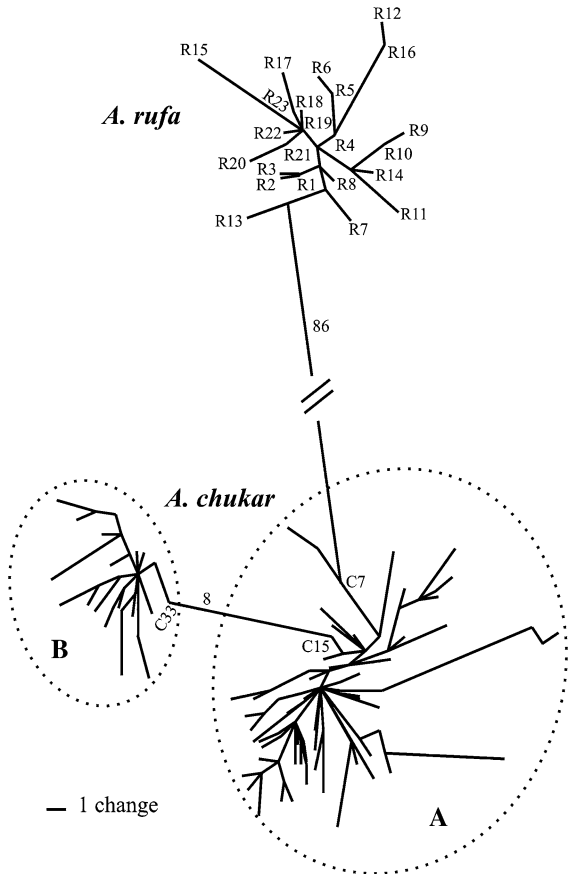


Fig. 3 Minimum-spanning tree computed by ARLEQUIN using the number of mutational changes among all the *A. rufa* (from R1 to R23) and *A. chukar* (from C1 to C111) haplotypes. Circles indicate groups including the haplotypes of clade-A and clade-B (Fig. 2). For the sake of clarity, the *A. chukar* haplotypes are not indicated with the exception of those connecting clade-A, clade-B and *A. rufa* clade

STRUCTURE (K = 2; clusters I and II) compared Elba and Carbuccia partridges with *A. rufa* and *A. chukar* controls (Fig. 4). Individual membership (q_i) revealed that there was no alleles admixture between parental species: no birds with $q_i < 0.90$ were found. The average population assignment values were, for the *A. rufa* control (Spain), Q_i (I) = 0.97 and Q_i (II) = 0.03, for the *A. chukar* control (Cyprus), Q_i (II) = 0.97 and Q_i (I) = 0.03: hence, they were assigned to cluster I and II, respectively. The values computed for experimental populations were Q_i (I) = 0.62 and Q_i (II) = 0.38 (Elba), and Q_i (I) = 0.85 and Q_i (II) = 0.15 (Carbuccia).

Random Amplified Polymorphic DNA

The RAPD banding profile of four Carbuccia specimens, six *A. rufa* and six *A. chukar* controls is given in Fig. 5. Two birds (8, 19) were identified as *A. rufa* × *A. chukar* hybrids; the remaining two (11, 14) hold only the *A. rufa* band. No birds were found to be hybrid for all primers; three (1, 2, 9) showed only the *A. rufa* band, the remaining ones were *A. rufa* × *A. chukar* hybrids with intermediate patterns. The overall genetic profile (mtDNA, STR, RAPD) for all Elba and Carbuccia specimens was reported in Table 5.

Discussion

In the context of the widespread *A. chukar* genetic pollution affecting Mediterranean *A. rufa* and *A. graeca* populations (Barbanera et al. 2005; Barilani et al. 2007), it is essential to make efforts to manage not only the allegedly pure resources but also those actually assessed to be hybrid to avoid a generalized genetic homogenization. Hence, the geographic origin of the introgressive chukar genes detected in the genome of Mediterranean partridges represents an important issue, as the admixture of unknown genomes would hinder any reliable management program. Our study aimed to get insight into potentially different, exotic *A. chukar* gene pools, providing useful information to game bird managers to bring the commerce of Mediterranean partridges into line with knowledge of their genetic kinship.

Genetic admixture in Mediterranean *Alectoris* partridges

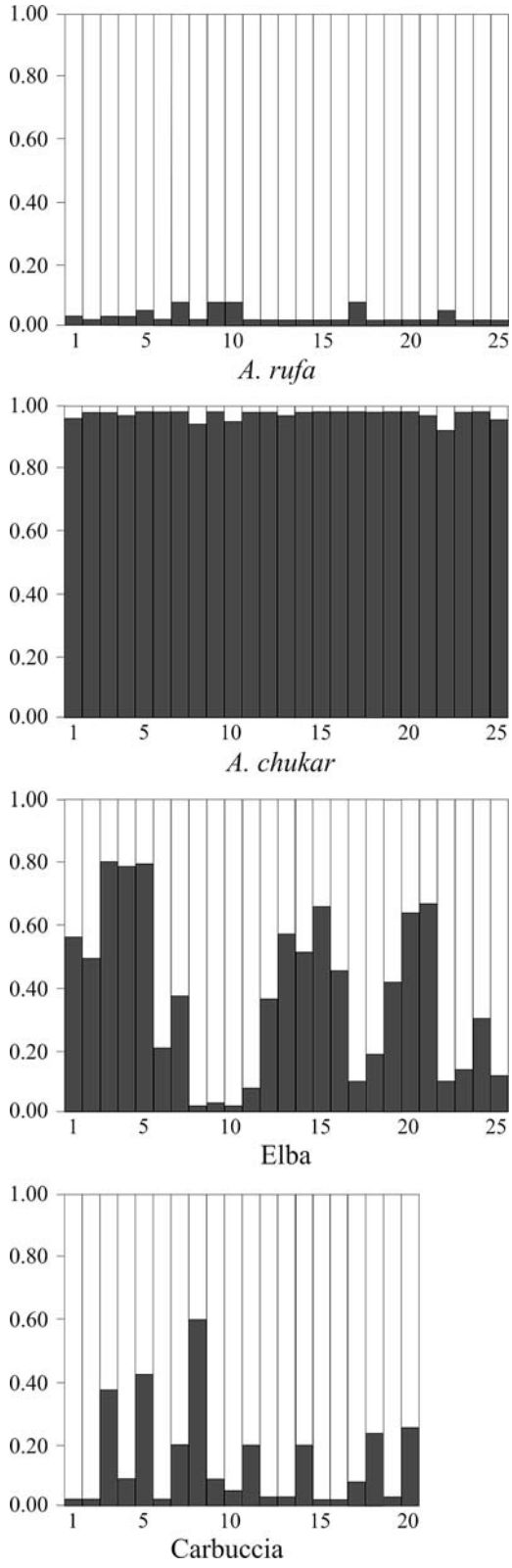
The genetic structure of the last-allegedly-native, wild, Italian *A. rufa* population of Elba Island as well as that of the farmed, Corsican *A. rufa* population from Carbuccia, were investigated. Mixed ancestry was found in both populations, a large part of the specimens (Elba, 68%; Carbuccia, 45%) showing mtDNA haplotypes discordant with their red-legged partridge phenotype and corresponding to that of an exotic species, the *A. chukar* (Fig. 2). A high percentage of *A. rufa* × *A. chukar* hybrids was disclosed in both Elba (80%, by STR) and Carbuccia (85%, by STR and RAPD) populations (Figs. 4 and 5; Table 5). Moreover, *A. chukar* mtDNA haplotypes

Table 4 The genetic variability of the STR loci for each population: *n*, sample size; n_A, number of alleles per locus; *n*a, number of unique alleles; Ar, allelic richness; H_o, observed heterozigosity; H_e, expected heterozigosity; *P* HWE, probability value for the Hardy–Weinberg Equilibrium test; SD, standard deviation for the *P* value; Chi^2 test with relative degrees of freedom (df) (Fischer global test, all loci)

Population	Locus	*n*	n_A	*n*a	Ar	H_o	H_e	*P* HWE	SD	Chi^2 (df)
A. rufa (Spain)	MCW 104	25	10	1	9.7	0.680	0.868	$<10^{-5}$	$<10^{-5}$	–
	MCW 118	25	1	0	1.0	Monomorphic		–	–	–
	MCW 280	25	3	0	2.8	0.440	0.411	1	$<10^{-5}$	–
	Aru 1.23	25	3	0	3.0	0.360	0.577	0.012	0.001	–
	Aru 1.27	25	10	2	9.1	0.720	0.625	0.986	0.006	–
	Average	25	5.4	0.6	5.1	0.550	0.620	$<10^{-5}$	–	∞ (8)
A. chukar (Cyprus)	MCW 104	25	7	1	6.2	0.680	0.652	0.758	0.014	–
	MCW 118	25	2	0	1.9	0.120	0.115	1	$<10^{-5}$	–
	MCW 280	25	1	0	1.0	Monomorphic		–	–	
	Aru 1.23	25	1	0	1.0	Monomorphic		–	–	
	Aru 1.27	25	5	0	4.8	0.600	0.709	0.126	0.007	–
	Average	25	3.2	0.2	2.9	0.467	0.492	0.584	–	4.7 (6)
Elba (Italy)	MCW 104	25	8	1	7.7	0.560	0.800	0.006	0.002	–
	MCW 118	23	2	0	2.0	0.043	0.241	0.007	$<10^{-3}$	–
	MCW 280	22	3	0	3.0	0.227	0.661	$<10^{-5}$	$<10^{-5}$	–
	Aru 1.23	25	4	1	4.0	0.320	0.673	0.001	$<10^{-3}$	–
	Aru 1.27	24	9	0	8.7	0.458	0.879	$<10^{-5}$	$<10^{-5}$	–
	Average	23.8	5.2	0.4	5.1	0.322	0.651	$<10^{-5}$	–	∞ (10)
Carbuccia (France)	MCW 104	20	9	1	9	0.800	0.825	0.722	0.019	
	MCW 118	20	2	0	4	0.100	0.229	0.235	0.006	
	MCW 280	20	4	1	6	0.400	0.617	0.138	0.006	
	Aru 1.23	20	4	1	2	0.300	0.318	1	$<10^{-5}$	
	Aru 1.27	20	6	0	4	0.650	0.822	0.197	0.009	
	Average	20	5	0.6	5	0.450	0.562	0.377	–	10.7 (10)

were also detected in 12 Italian, four Spanish, one French (Atlantic Loire) red-legged and four Italian rock partridge (Fig. 2). Following these results, Italian *A. rufa* populations can be now considered as entirely polluted with *A. rufa* × *A. chukar* hybrids, while Corsican, wild *A. rufa* populations are endangered by restocking programs employing Carbuccia partridges.

Only scant studies dealt with DNA analysis from avian scats, and all referred to large-sized birds producing large-sized faeces where endogenous DNA may be guessed to be more abundant although still in trace amounts (Idaghdour et al. 2003; Regnaut et al. 2006; Maki-Petays et al. 2007). The faecal DNA of the red-legged partridge, a species producing small-sized scats, was successfully investigated. Although extracting amplifiable DNA from faeces is challenging because of potential cocktail of nucleases and PCR inhibitors (Kohn et al. 1995), their use was compulsory for Elba partridges. The snPCR worked reliably and the STR genotyping was weakly hindered by DNA degradation due to the reduced size range of the studied loci (<200 bp; cf., Buchan et al. 2005; Tables 3 and 4). Less than 5% of the alleles were not amplified, a frequency similar to that of analogous studies (Frantzen et al. 1998; Frantz et al. 2003). According to Vaha and Primmer (2006), five loci provided adequate power to detect hybrid birds as STR controls were well differentiated (F_{ST} = 0.59), much more, for instance, than those (F_{ST} = 0.23) used by Williams et al. (2005) to detect hybridization within Anseriformes using an identical number of loci. Nevertheless, the employ of either maternal or biparental markers proved to be essential (Table 5). Within Elba population, specimens with



◀**Fig. 4** Bayesian admixture analysis of *A. rufa* (Spain), *A. chukar* (Cyprus), Elba (Italy) and Carbuccia (Corsica) population genotypes computed by STRUCTURE with K = 2 (white, *A. rufa*; grey, *A. chukar*). Each individual is represented as a vertical bar partitioned in K segments, whose length is proportional to the estimated membership in the K clusters

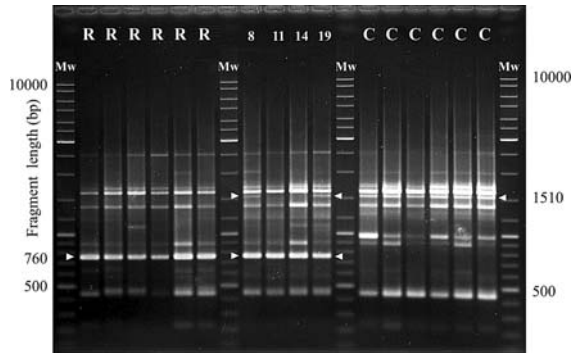


Fig. 5 The banding profile of a RAPD marker (OP-C-08; cf., Barbanera et al. 2005) is shown. From left to right: six Spanish *A. rufa* controls (R: 1st and 2nd, Mallorca; 3rd and 4th, Seville; 5th and 6th, Andujar); four Carbuccia partridges (8, 11, 14, 19); six *A. chukar* controls (C: 1st, Kazakhstan; 2nd, Mongolia; 3rd, Armenia; 4th, Cyprus; 5th, Pakistan; 6th, China). Species-specific fragments are 760 bp and 1,510 bp long for *A. rufa* and *A. chukar*, respectively (white arrows; cf., Barbanera et al. 2005). Mw, molecular weight marker

A. rufa haplotype (R14, R15, R16) were identified as *A. rufa* × *A. chukar* hybrids by STR genotyping (Fig. 4: 1–6, 13, 14), whereas others (Fig. 4: 8–11, 22) with *A. rufa* q_i value ≥ 0.90 showed the *A. chukar* mtDNA lineage (Fig. 2): hence, all of these birds were genetically introgressed with genes of chukar origin. Similarly, eight Carbuccia specimens with *A. rufa* haplotype were identified as *A. rufa* × *A. chukar* hybrids by STR and RAPD markers, as well as all those (n = 9) with *A. chukar* haplotype. Only three Carbuccia specimens (1, 2, 9: Table 5) showed the *A. rufa* genetic profile whatever the marker employed for their analysis could be (mtDNA, STR, RAPD). Nonetheless, we felt confident in considering these farmed birds too as hybrids. Indeed, crossing among offspring from different broods is a common practice to work against inbreeding depression. Likely, they represent very high-category backcrosses to *A. rufa* (Barbanera et al. 2005). In conclusion, it must be noted that within Carbuccia population four RAPD markers detected

Table 5 The overall genetic profile (mtDNA, STR, RAPD) for all Elba and Carbuccia specimens is reported

Specimen	Elba (Italy)			Carbuccia (France)		
	mtDNA	STR	RAPD	mtDNA	STR	RAPD
1	R	H	–	R	R	R
2	R	H	–	R	R	R
3	R	H	–	R	H	H
4	R	H	–	R	R	H
5	R	H	–	C	H	H
6	R	H	–	C	R	H
7	C	H	–	C	H	H
8	C	R	–	C	H	H
9	C	R	–	R	R	R
10	C	R	–	R	R	H
11	C	R	–	R	H	H
12	C	H	–	C	R	H
13	R	H	–	C	R	H
14	R	H	–	R	H	H
15	C	H	–	R	R	H
16	C	H	–	C	R	H
17	C	H	–	R	R	H
18	C	H	–	C	H	H
19	C	H	–	C	R	H
20	C	H	–	R	H	H
21	C	H	–			
22	C	H	–	STR	40%	
23	C	H	–	RAPD		85%
24	C	H	–	STR + RAPD		85%
25	C	H	–			
	STR	80%	–			

The assignation to the *A. rufa* (R), *A. chukar* (C) or to the *A. rufa* × *A. chukar* (H) profile is indicated. The percentage of *A. rufa* × *A. chukar* specimens is computed only on the basis of the STRs for Elba partridges, and of STRs and/or RAPDs for Carbuccia partridges

much more hybrids than five STR loci (85% vs. 40%, respectively: Table 5). Notwithstanding these latter may certainly provide comprehensive genetic information on the structure of a given population, any marker system has its drawbacks and advantages. In this work, it was proved that our *A. rufa* versus *A. chukar* species-specific RAPD markers, the only currently available, can disclose hybrid partridges not only categorically, namely without the probabilistic assessment provided by the Bayesian statistics, but also more efficiently than STRs.

Diversity and origin of the *A. chukar* genes introgression

Recently, Barbanera et al. (2007) argued that invasive *A. chukar* genes detected in the genome of Mediterranean partridges originated from E Asia. This hypothesis followed the study of the introduced chukar population of Montecristo Island (Italy), which weighted for a fair relationship with Chinese and Mongolian representatives of the same species. However, introgression of *A. chukar* genes in both red-legged and rock partridge was not analysed, and the overall *A. chukar* samples size ($n = 81$) appeared not-entirely reliable when weighted against the huge range of distribution of this species. In order to sweep over the possible shortcomings of the previous study, in this work two mtDNA markers (Cyt-*b*, CR) were employed, providing a larger data set (2,250 characters) than in former studies where only the CR was considered (Barbanera et al. 2007: ~1,155; Barilani et al. 2007: 431). Also the red-legged ($n = 111$) and rock ($n = 6$) partridge were studied, and the *A. chukar*

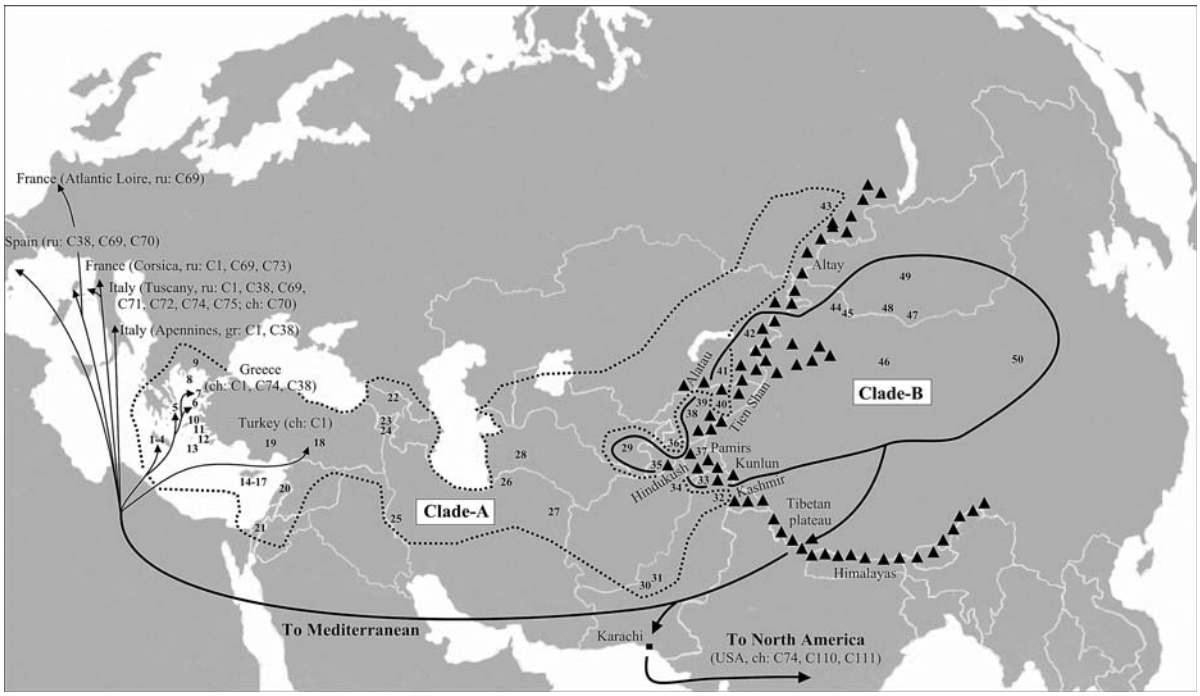


Fig. 6 The *A. chukar* sampling localities are reported (from 1 to 50, see Fig. 1). The clade-A (dotted line) and clade-B (thick line) here include only phenotypic chukars (i.e., *A. chukar*—introgressed *A. rufa* and *A. graeca* are excluded). The arrows show the origin of the *A. chukar* haplotypes found in Mediterranean and North American partridges. Black solid triangles mark out the main mountain chains in Asia. Their average altitude ranges from 2,500 m (Altay), to 3,500 m (Alatau), to 4,500 m (Hindukush, Pamirs, Tien Shan), 5,500 m (Kunlun, Kashmir) and up to over 8,000 m (Himalayas). Chukar (C) haplotypes sampled in the Mediterranean and in North America are indicated. Phenotype legend: ru, *A. rufa*; gr, *A. graeca*; ch, *A. chukar*

samples size was significantly increased (n = 205). Framing the *A. chukar* genetic diversity across its whole distribution range, only two groups occurred (Figs. 2 and 3). The clade-A included the chukar haplotypes sampled from E Mediterranean to C Asia, a large, not-homogeneous territory including several islands. A large variability due to the genetic drift likely affecting the small island populations may be assumed. The values computed for the haplotype diversity, the mean number of pairwise differences and the nucleotide diversity occurring in the clade-A, when compared with those of the clade-B, were in agreement with the above-mentioned genetic variability. The clade-B comprised all of the chukar representatives from E Asia, a territory more homogeneous than that colonized by clade-A populations. It can be hypothesized that the mountain ranges extending from Nepal to C Asia and to Siberia might have represented the natural barrier accounting for the genetic differentiation between the two *A. chukar* groups (Fig. 6). Indeed, the split between European and Asian *Alectoris* lineages should have occurred at the Pliocene/Pleistocene limit (Randi 1996), when the uprising of the Himalayas became an important regulator of the diversification of the Asian environment (Yu et al. 2004). Particularly, N Pakistan as well as E Tajikistan, Kyrgyzstan and extreme E Kazakhstan run well through this partition of haplotypes as their *A. chukar* representatives belong to either clade-A or clade-B (Fig. 6: e.g., Pakistan: 33, Chitral, clade-A versus 32, Kashmir, clade-B).

Literature reports that first *A. chukar* introduction into N America was in 1893. A mix of specimens of different Asian subspecies was exported from Karachi (True 1937; Cottam et al. 1940). In this study, the US chukars sampled in Nevada and Washington showed only haplotypes belonging to the clade-B (C74, C110, C111: Fig. 2). When the *A. chukar* haplotypes disclosed in phenotypic red-legged (Italy, Spain, France) and rock (Italy) partridge were compared with the total of the chukar ones, a strong evolutionary affinity with those

sampled in E Asia was found (Figs. 2 and 3). The haplotype C74, hold by Italian representatives (Bieri, Aulla: Fig. 1), was an exception. However, due to its occurrence also in one specimen from Nevada, we felt confident in considering it too as originating from E Asia. This conclusion is similar to that of Martinez-Fresno et al. (2008) about the origin of the *A. chukar* mtDNA introgression detected in Spanish *A. rufa* specimens.

Since first centuries A.D. people were used to carry and breed chukars along the Silk Road to get food on the way to Europe (M. Ming pers. com.). Nevertheless, the introduction of *A. chukar* is fundamentally a recent phenomenon (since World War II until 1980s: Goodwin 1986; Dias 1992; Scalera 2001). Although people translocated chukars along different routes to Mediterranean and N America (Fig. 6), it was found that their geographic origin was quite homogeneous. Further, it is particularly remarkable that three *A. chukar* haplotypes (C1, C38, C74) sampled in morphologic chukars from E Mediterranean (Greece: Andros, $n = 4$; Lesvos, Chios, Crete, $n = 1$ each; Turkey, Mersin, $n = 2$), clustered in the clade-B, whereas the large majority did not (clade-A: Greece, $n = 45$; Turkey, $n = 1$; all from Cyprus, Israel and Lebanon: Fig. 2). We had to conclude that these haplotypes belong to not endemic, Asian specimens dispersed within native, Mediterranean *A. chukar* populations. It follows that the genetic pollution is now threatening also native *A. chukar* (intra-specific level) other than *A. rufa* and *A. graeca* (inter-specific level). In this context, it is extremely urgent to pose constraints to a rapid attainment of an even more generalized homogenization. Hence, the strict employment of only genetically controlled *A. chukar* stocks, such as that from the island of Cyprus (Fig. 4; cf., Guerrini et al. 2007), represents the first step to get a reliable management of this important game resource.

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