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Analysis of the genetic structure of red-legged partridge (*Alectoris rufa*, Galliformes) populations by means of mitochondrial DNA and RAPD markers: a study from central Italy

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Abstract

The strong hunting pressure on the red-legged partridge, *Alectoris rufa*, warranted its inclusion into the list of species of European conservation concern. During the last decades, restocking plans with farmed specimens have counterbalanced the hunting drawings from wild populations. Our concern was the study of *A. rufa* in the easternmost part of its range, the central Italy, to gain insights into the effects of this compensation practice on the genetic structure of its populations. Partridges from both a geographically isolated, long-time protected, wild population (Pianosa island, Tuscan Archipelago National Park) and two Tuscan farms (Bieri and Scarlino) were investigated. All the specimens were very similar in outward appearance, looking much like to *A. rufa*. Ninety-six sequences of both Cytochrome *b* and D-loop Control Region of mitochondrial DNA (mtDNA) were analysed to get evidence of ancestry at the population level, whereas, the Random Amplified Polymorphic DNA (RAPD) technique was employed to get fingerprinting at the individual level. Pianosa and Bieri populations showed both the *A. rufa* and *Alectoris chukar* mtDNA lineages, whereas the Scarlino one only the *A. rufa*-mtDNA line. However, a spread overall pattern of *A. rufa* × *A. chukar* hybridisation among specimens, whatever their mtDNA lineage could result to be, was disclosed by means of RAPD species-specific markers. This is the first genetically documented record of the *A. rufa* × *A. chukar* hybrids. The occurrence of the pure, native *A. rufa* genome in the easternmost part of the species' geographical range may be guessed to be virtual.

Keywords: Chukar; Genetic introgression; Hybridisation; Molecular markers; Numt; Restocking plans

1. Introduction

The red-legged partridge (RLP) (*Alectoris rufa*, Galliformes) is a native species of south western Europe, occurring naturally in Portugal, Spain (Balearics included), France, north western and central Italy. It has been successfully introduced in other European countries, including the United Kingdom (Aebischer and Lucio, 1997).

The RLP is included in the list of the species of European conservation concern (SPEC), being considered vulnerable as far as its threat status is concerned (Tucker and Heath, 1994). Since 1960s, RLP started to decline throughout its distribution range as it represented one of the most important game species. Indeed, the demand of high amounts of birds by the rising hunting pressure induced the release of an ever-increasing number of farmed specimens. This encouraged a level of exploitation that wild populations were not able to sustain. The mechanisation of the agriculture, the intensification of the pesticide use, and the abandonment of farming

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were other factors contributing to the species decline (Rands, 1986).

It is known that RLP hybridises with rock partridge (*Alectoris graeca*) throughout their natural overlapping ranges, as it occurs in the southern French Alps (Bernard-Laurent, 1984; Randi and Bernard-Laurent, 1999). Hybridisation occurs also between rock partridge and chukar (CHK) (*Alectoris chukar*), as it happens in Thrace south of the Rhodope Mountains (Dragoev, 1974). Chukar has a very wide distribution, ranging from east Balkans and the adjacent Mediterranean islands to central Asia up to north eastern China (Aebischer, 1997). Provided their natural, sharply bordered, distribution ranges, *A. rufa* × *A. chukar* hybrids should not occur. However, in the last decades, CHK have been released for shooting alongside RLP, especially in the United Kingdom, France and Italy, and hybrids occur now in the wild (Goodwin, 1986). Moreover, captive-bred birds are produced by illegal crossbreeding between *A. rufa* × *A. chukar*. Farmers are spurred to do this by the reward following the flourishing state of the much looking RLP hybrids, which are sold for restocking or as meat for human consumption. A few morphological pointers can be used to identify *A. rufa* × *A. chukar* first-generation hybrids, but uncertainty remains with backcross individuals (Wilkinson, 1987, 1991). Hence, when farmed birds are released to reinforce the impoverished autochthon populations, the risk of genetic pollution is strong, as restocking usually occurs without any genetic control (Negro et al., 2001).

The Italian RLP population (about 1500 bird pairs: Spanò, 1992) occurs across the Apennines and central regions, Pianosa island included. This island (10.2 km^2) belongs to the Tuscan Archipelago National Park (PNAT), which is sited in the Tyrrhenian sea (Fig. 1). The National Park is the largest European marine park and an important area for both non-migratory and migratory avifauna. Since 1856, Pianosa had been a even strictly security penal colony, forbidden to free people exchange until the end of 1998, when the prison was used no longer and the island passed under the authority of the National Park. This long isolation allowed the preservation of both the flora and fauna of the island. Pianosa RLP population rapidly flourished, even following a relaxed predatory pressure. At present, total population amounts to 150–250 nesting couples (Arcamone and Sposimo, 2002), corresponding to a density of about 15–25 bird pairs/km^2 (cf. Rands, 1986: 5–9 bird pairs/km^2).

This work deals with RLP conservation status in central Italy by means of genetic analysis of both wild and captive populations. Pianosa wild population was chosen for both its history of preservation and healthy looking status, representing a stock susceptible to be employed in future restocking plans. Captive populations from the Tuscan stock-farms of Bieri and Scarlino (Fig. 1) were selected, as they are major sources for RLP restocking in central Italy.

As in many genetic studies on animal populations, mitochondrial DNA (mtDNA) was investigated to getting evidence of ancestry at the population level (Avise, 2000; for a review about mtDNA recombination, see Rokas et al., 2003). Specifically, Cytochrome *b* gene (Cyt-*b*) and D-loop Control Region (D-loop) were employed as markers (Randi, 1996; Randi and Lucchini, 1998). In addition, total genomic DNA (tgDNA) was analysed as well by means of Random Amplified Polymorphic DNA (RAPD: Welsh and McClelland, 1990;

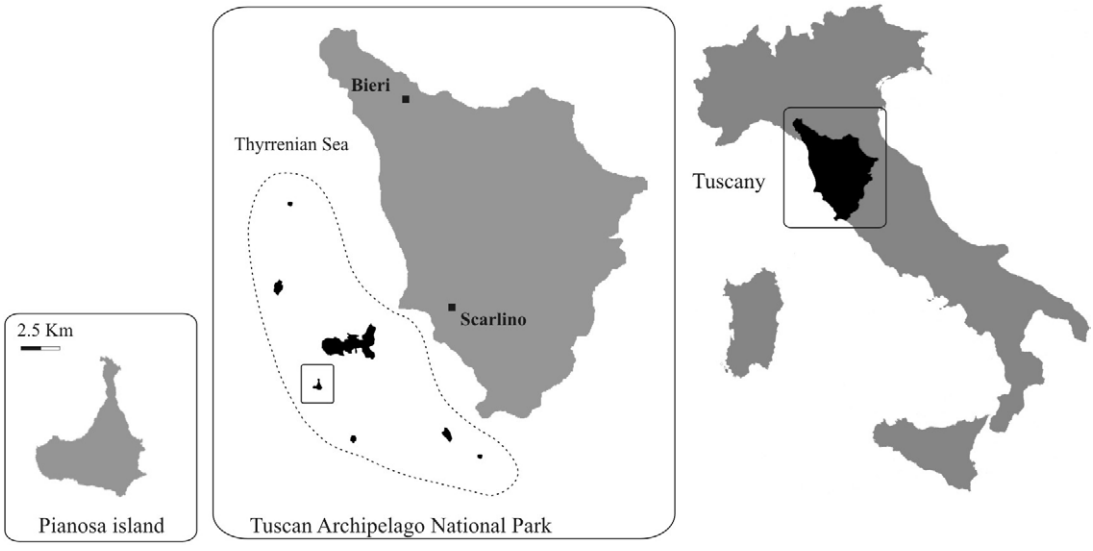

Fig. 1. Right part: Tuscany, central Italy: the area of this study. Central panel: the geographical position of both Bieri and Scarlino stock-farms is showed. Tuscan Archipelago National Park (PNAT) stretches out alongside the coast: its marine border is indicated by the dotted line. Left panel: Pianosa island is the fifth island of PNAT in order of width (10.2 km^2).

Williams et al., 1990). This fingerprinting technique proved to be effective as species-specific tool in hybridisation studies (Fristch and Rieseberg, 1996; Rieseberg and Swensen, 1996; Anttila et al., 2000). Indeed, the RAPD technique was successfully used as a rapid molecular method for detecting *A. rufa* × *A. graeca* hybrids in Spanish partridge populations, allowing hybrids eradication up to the F2 and 3rd backcross generations, which was essential as these are the individuals generally released for restocking purposes (Negro et al., 2001).

2. Methods

2.1. Samples

Partridges were captured on Pianosa island and transferred to the Bieri Experimental Stock-farm of the Italian Forest Service (Fig. 1). As regards the two captive populations, the first one, called Bieri population (Bie), was from the homonymous stock-farm. The second one, called Scarlino population (Sca), was obtained from the "Public Centre for the Game Production" of Scarlino (Fig. 1). Partridges from Pia ($n = 14$), Bie ($n = 14$) and Sca ($n = 11$) populations were ringed and sampled: for each specimen some feathers were pulled up and put into a test tube containing 95% ethanol. All the biological material was stored at −80 °C.

To assess the genetic status of our Tuscan RLP populations, a total of ten Spanish (Spa) specimens of *A. rufa*, six from Mallorca, Balearics (from P_1 to P_6), two from Ciudad Real (RP_{546}, RP_{548}), and two from Seville (A_1, A_2), were used as pure controls (Negro et al., 2001). Muscular tissue samples from Spanish partridges were 95% ethanol preserved. As a counterpart of hypothetical introgressive events, the involvement of *A. chukar* representatives was considered the most likely. Hence, a total of 10 *A. chukar* specimens, three from Mongolia (Ömnogövi Aymag, Gobi Desert: Mgl), two from Kazakhstan (Almaty Oblysy, Tien Shan: Kzh), two from Georgia (Kahetia: Geo), and three from Israel (Isr), were chosen as the other parental control. Muscular tissue samples from Mongolia ($UWBM_{57853}$, $UWBM_{57857}$, $UWBM_{57859}$) and Kazakhstan ($UWBM_{46402}$, $UWBM_{46516}$) were provided by the Burke Museum of Natural History, University of Washington, Seattle, USA (museum acronym: UWBM). Liver samples of specimens from Georgia were recovered by C. Valentini (Rome, Italy). All samples were 95% ethanol preserved. Finally, tgDNA samples from Israel were provided by E. Randi (INFS, Ozzano nell'Emilia, Italy).

2.2. Total genomic DNA extraction

Total genomic DNA from Pia, Bie and Sca partridges was extracted from feathers. A fragment (3 mm long) from the base of the quill was employed (Sorenson and Fleischer, 1996). Each fragment was plunged down in 500 μl of sterile distilled water, adding 150 μl of NDS (0.5 M EDTA, 0.1 M Tris–HCl pH 9.5, 1% SDS), 65 μl of proteinase K (1 mg/ml), 30 μl of dithiothreitol (100 mg/ml), and incubated overnight at 55 °C. Then, a RNAse treatment was followed by phenol:chloroform extraction. Total genomic DNA was extracted from muscular tissue (50 mg) of both Spanish RLPs and Asian CHKs, using Puregene® Genomic DNA Isolation Kit (Gentra Systems). DNA concentration and purity were determined spectrophotometrically.

2.3. PCR amplification and sequencing

Almost the entire length of Cyt-*b* (1092 bp) was amplified using the primers CytL (5′-ATG GCA CCT AAT ATC CGA AAA GC-3′) and CytH (5′-TTA GTA GTT GAG AAT TTT ATT TTC AAG-3′), whose their 5′ terminal nucleotides correspond to the positions No. 1 and No. 1143 of the *A. rufa* sequence published by Randi (1996), respectively. The entire D-loop was amplified using the PHDL primer (5′-AGG ACT ACG GCT TGA AAA GC-3′) of Fumihito et al. (1995), together with the primer H1321 (5′-TAG YAA GGT TAG GAC TRA GTC TT-3′), whose 5′ terminal nucleotide binds to position No. 1321 of the chicken mitochondrial genome (Desjardins and Morais, 1990). Cyt-*b* and D-loop were amplified from 10 birds of each Tuscan population, from Spanish *A. rufa* ($n = 10$) and Asian *A. chukar* ($n = 8$). Amplifications (50 μl) were performed with 1 μl Polymed Taq (1 U/μl), 2 μl of 50 mM $MgCl_2$, 5 μl of 10X Polymed PCR reaction buffer, 5 μl of each 2.5 mM dNTP, and 20 ng of DNA. The amplification profile in a 2400-Gene Amp PCR System (Applied Biosystems) was the same for both Cyt-*b* and D-loop: 7 min at 94 °C, 30 cycles of 1 min at 94 °C, 2 min at 55 °C and 1 min at 72 °C, and 7 min at 72 °C as final extension. For each cycle, a 10% ramp was added between annealing and extension. PCR products were purified using PCR Kleen Spin Columns (BioRad). Both DNA strands were directly sequenced on an ABI 310 (Applied Biosystems) automated sequencer (Genelab, ENEA, Roma, Italy).

2.4. Checking for mitochondrial pseudogenes

Mitochondrial pseudogenes or "Numts" – nuclear sequences of mitochondrial origin (Lopez et al., 1994) – can easily contaminate PCR-based mitochondrial studies (Sorenson and Quinn, 1998; Bensasson et al., 2001; Williams and Knowlton, 2001). Due to the shortage of mitochondrial content, blood samples were discriminated vs. feathers as DNA source. To assess the mitochondrial authenticity of all our sequences, a comparison with those obtained from purified mtDNA

was performed for each population (see below). Moreover, specifically for Cyt-*b*, possible contamination was checked looking for Numts properties such as stop codons and equal rates in all codon site positions (Sheldon et al., 2000).

2.5. Mitochondrial DNA extraction

Mitochondrial DNA was extracted from liver (or muscle) of the following specimens: *A. rufa* P_1, *A. chukar* $UWBM_{57857}$, *A. chukar* $UWBM_{46402}$, *A. chukar* Geo_1, Pia_5, Pia_8, Bie_{40}, Bie_{50}, Sca_{11}. A piece of tissue (250 mg) was homogenized in a cold medium (0.25 M saccharose, 5 mM HEPES, 1 mM EDTA, pH 7.2), and then centrifuged at 4 °C for 10 min (500*g*) to separate the nuclear fraction. The supernatant was recovered and centrifuged at 4 °C for 10 min (9400*g*). Mitochondria were recovered and suspended in a 4 °C buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris–HCl pH 8.0), and then mtDNA was isolated with an alkaline extraction (Palva and Palva, 1985). Finally, mtDNA concentration and purity were determined spectrophotometrically.

A PCR for the amplification of a definite nuclear marker was run to assess possible contamination into the mtDNA extractions. The spindlin gene fragment (250 bp) was chosen, as it is present on both W and Z chromosomes (Itoh et al., 2001). For each extraction, the mtDNA (20 ng) was tested using both the primers (CPE 15F, CPE 15R) and amplification profile reported by Itoh et al. (2001). The mtDNA extractions reliably produced a quite pure mitochondrial withdrawal, the expected amplification of the 250-bp nuclear marker never occurred. As a positive control spindlin gene amplification was performed also with tgDNA, and the 250-bp band was always observed.

2.6. Phylogenetic data analysis

Both Cyt-*b* and D-loop from 48 specimens were amplified for a total of 96 sequences. Alignments were performed with ClustalW (Thompson et al., 1994). Nucleotides composition and pattern of substitutions were computed using the program MEGA 2.1 (Kumar et al., 2001). Phylogenetic relationships were inferred using both distance and parsimony methods. The neighbour-joining procedure (NJ: Saitou and Nei, 1987) with Tamura–Nei distance (TN93) as implemented in PAUP 4.0b10 (Swofford, 2002), was used for both the Cyt-*b* and D-loop, the latter with pairwise deletion of gaps. The TN93 algorithm, specifically developed for the Control Region (Tamura and Nei, 1993), was here used also for Cyt-*b* because it takes into account inequality of nucleotide frequencies and variable substitution rates among the four nucleotides (Irwin et al., 1991), distinguishing between transitions (Ts) and transversions (Tv). The gamma distribution was applied and, according to Rooney et al. (2001), a maximum-likelihood based estimation of the α shape parameter was performed with the method of Sullivan et al. (1995). Maximum parsimony procedure (MP: Swofford et al., 1996) as implemented in PAUP was performed with the following settings for both Cyt-*b* and D-loop (Kimball et al., 1999; Randi et al., 2003): unweighted, with random addition sequence with a number of rearrangements limited to 10 for each bootstrap replicate, tree bisection and reconnection swapping algorithm, collapsed zero length branch. Multiple MP trees were collapsed to obtain a 50% majority-rule consensus tree. For both kinds of phylogenetic reconstructions, statistical support for internodes was evaluated by bootstrapping percentage (BP) after 1000 resampling steps (Felsenstein, 1985).

2.7. GenBank nucleotide sequences

Cyt-*b* and D-loop phylogenetic trees were constructed using *Alectoris barbara*, *Alectoris melanocephala*, *A. graeca*, *Alectoris philbyi*, *A. rufa* and *Alectoris magna* GenBank sequences. Their accession codes are reported in Table 1, together with those of the sequences of this study. Nucleotide positions reported in this paper refer to sequences published by Randi and Lucchini (Randi, 1996; Randi and Lucchini, 1998).

2.8. RAPD

Amplifications for RAPD markers were performed according to Negro et al. (2001). Decanucleotide RAPD primers from Operon Technologies Inc. were employed (Kit C and Kit H). The reaction mixture contained 10 ng of template DNA, 18.75 pmol of a single primer, 0.2 mM of each dNTP, 3 mM $MgCl_2$, and 0.6 U of Taq polymerase in the reaction buffer provided by the manufacturer (Operon Technologies Inc.). Sterile distilled water was added to a final volume of 25 µl. Amplification was programmed as it follows: 3 min at 94 °C, 45 cycles of 30 s at 94 °C, 30 s at 36 °C and 1 min at 72 °C; then, a final extension of 5 min at 72 °C. A 50% ramp was added between annealing and extension. Amplification products were run on 1.4% agarose gel at 100 V for 75 min (constant voltage), along with a GeneRuler™ DNA ladder-mix as molecular weight marker (Fermentas), stained with ethidium bromide (15 min) and photographed under UV light. Finally, band pattern analysis was performed using BioRad Quantity One software. Primers giving the same band pattern for both *A. rufa* and *A. chukar* controls were discarded, as well as those producing a species-specific band but not simultaneously in all the individuals from different geographical areas. To verify the reliability of single RAPD profiles, all the *A. rufa* and *A. chukar* controls

Table 1
The GenBank accession codes for both Cyt-*b* and D-loop sequences are reported together with both sample locality and literature record

Specimen	Locality	Cyt-*b*	D-loop	Literature record
A. barbara	Sardinia, Italy	Z48771	–	Randi (1996)
A. melanocephala	Taif, Saudi Arabia	Z48772	–	Randi (1996)
A. graeca	Albania	Z48773	–	Randi (1996)
A. philbyi	Taif, Saudi Arabia	Z48774	–	Randi (1996)
A. rufa	Bombarral, Portugal	Z48775	–	Randi (1996)
A. magna	Lanzhou, China	Z48776	–	Randi (1996)
A. barbara	Sardinia, Italy	–	AJ222727	Randi and Lucchini (1998)
A. melanocephala	Taif, Saudi Arabia	–	AJ222736	Randi and Lucchini (1998)
A. graeca	Alps, Italy	–	AJ222730	Randi and Lucchini (1998)
A. graeca	Sicily, Italy	–	AJ222731	Randi and Lucchini (1998)
A. philbyi	Taif, Saudi Arabia	–	AJ222737	Randi and Lucchini (1998)
A. rufa	Portugal	–	AJ222739	Randi and Lucchini (1998)
A. rufa	Spain	–	AJ222740	Randi and Lucchini (1998)
A. magna	Lanzhou, China	–	AJ222732	Randi and Lucchini (1998)
A. chukar	Xichuan, China	–	AF435559	Wei et al., unpublished data
A. chukar	Xichuan, China	–	AF435560	Wei et al., unpublished data
A. rufa P_1	Mallorca, Spain	AJ586141	AJ586190	This study
A. rufa P_2	Mallorca, Spain	AJ586142	AJ586191	This study
A. rufa P_3	Mallorca, Spain	AJ586143	AJ586192	This study
A. rufa P_4	Mallorca, Spain	AJ586144	AJ586193	This study
A. rufa P_5	Mallorca, Spain	AJ586145	AJ586194	This study
A. rufa P_6	Mallorca, Spain	AJ586146	AJ586195	This study
A. rufa A_1	Seville, Spain	AJ586147	AJ586196	This study
A. rufa A_2	Seville, Spain	AJ586148	AJ586197	This study
A. rufa RP_{546}	Ciudad Real, Spain	AJ586149	AJ586198	This study
A. rufa RP_{548}	Ciudad Real, Spain	AJ586150	AJ586199	This study
A. chukar $UWBM_{57853}$	Gobi Desert, Mongolia	AJ586151	AJ586200	This study
A. chukar $UWBM_{57857}$	Gobi Desert, Mongolia	AJ586152	AJ586201	This study
A. chukar $UWBM_{46402}$	Tien Shan, Kazakhstan	AJ586153	AJ586202	This study
A. chukar $UWBM_{46516}$	Tien Shan, Kazakhstan	AJ586154	AJ586203	This study
A. chukar Geo_1	Kahetia, Georgia	AJ586155	AJ586204	This study
A. chukar Geo_2	Kahetia, Georgia	AJ586156	AJ586205	This study
A. chukar Isr_{63}	Israel	AJ586157	AJ586206	This study
A. chukar Isr_{64}	Israel	AJ586158	AJ586207	This study
Pia_5	Pianosa, Italy	AJ586164	AJ586227	This study
Pia_5^*	Pianosa, Italy	AJ586169	–	This study
Pia_6	Pianosa, Italy	AJ586165	AJ586229	This study
Pia_7	Pianosa, Italy	AJ586166	AJ586230	This study
Pia_8	Pianosa, Italy	AJ586159	AJ586208	This study
Pia_9	Pianosa, Italy	AJ586160	AJ586209	This study
Pia_{10}	Pianosa, Italy	AJ586163	AJ586210	This study
Pia_{11}	Pianosa, Italy	AJ586161	AJ586211	This study
Pia_{12}	Pianosa, Italy	AJ586167	AJ586231	This study
Pia_{13}	Pianosa, Italy	AJ586162	AJ586212	This study
Pia_{14}	Pianosa, Italy	AJ586168	AJ586232	This study
Bie_{37}	Bieri, Italy	AJ586175	AJ586233	This study
Bie_{38}	Bieri, Italy	AJ586170	AJ586213	This study
Bie_{39}	Bieri, Italy	AJ586171	AJ586214	This study
Bie_{40}	Bieri, Italy	AJ586176	AJ586234	This study
Bie_{41}	Bieri, Italy	AJ586172	AJ586217	This study
Bie_{42}	Bieri, Italy	AJ586177	AJ586235	This study
Bie_{45}	Bieri, Italy	AJ586173	AJ586215	This study
Bie_{46}	Bieri, Italy	AJ586178	AJ586236	This study
Bie_{48}	Bieri, Italy	AJ586179	AJ586239	This study
Bie_{50}	Bieri, Italy	AJ586174	AJ586216	This study
Sca_1	Scarlino, Italy	AJ586180	AJ586218	This study
Sca_2	Scarlino, Italy	AJ586181	AJ586219	This study
Sca_3	Scarlino, Italy	AJ586182	AJ586220	This study
Sca_4	Scarlino, Italy	AJ586183	AJ586221	This study
Sca_5	Scarlino, Italy	AJ586184	AJ586222	This study
Sca_6	Scarlino, Italy	AJ586185	AJ586223	This study
Sca_7	Scarlino, Italy	AJ586186	AJ586224	This study

(*continued on next page*)

Table 1 (*continued*)

Specimen	Locality	Cyt-*b*	D-loop	Literature record
Sca_8	Scarlino, Italy	AJ586187	AJ586225	This study
Sca_9	Scarlino, Italy	AJ586188	AJ586226	This study
Sca_{11}	Scarlino, Italy	AJ586189	AJ586228	This study

Cyt-*b* and D-loop GenBank sequences of the same specimen are not available into the literature records. Pia_5^* AJ586169 sequence is a Numt (see Section 3).

as well as each Tuscan RLP were tested at least three times for each selected primer. For each specimen bands were positively scored only when they were present in all of the replicates.

3. Results

3.1. Morphological traits

The large majority of Tuscan RLPs showed black lores and straw ear coverts. The collar was cleanly demarcated with a heavily streaked necklace. Flank feathers had pale cream sub-terminal bar, bordered terminally by single black bar and chestnut tip. Ambiguous specimens, showing middle brown colour of ear coverts and slightly reduced streaking from the collar, were rarely observed. According to Cramp and Simmons (1980) our partridges were assigned to the taxon *A. rufa*.

3.2. Cytochrome b: sequences and phylogenetic data

The length of Cyt-*b* sequences in this study was 1092 bp, and so almost the entire Cyt-*b* gene (1143 bp) was analysed. Unequal nucleotide composition was found: 27.2% of adenine, 24.8% of thymine, 35.8% of cytosine, and 12.2% of guanine. The average number of Ts was 7.8 higher than Tv.

Genetic distance analysis (TN93 γ-distance, $\alpha = 0.318$) produced the phylogeny represented by the NJ tree of Fig. 2. Spanish *A. rufa* and Asian *A. chukar* specimens clustered in two distinct groups, each supported by BP = 100%. All of Pia and Bie partridges were equally distributed into these two clades (five each). Partridges from Sca population were all within the *A. rufa* group.

Into the *A. rufa* clade, the *A. rufa* Por Z48775 sequence diverged from all the other RLPs (BP = 89%). It differed for eight nucleotides (7 Ts and 1 Tv) from the consensus sequence of the RLP group with BP = 86%. Two of these substitutions were into 1st codon position (pos. 127: from G to A; pos. 699: from C to T), and determined two aminoacidic changes, from V to I and from L to F, respectively. Within the *A. rufa* group, Pia_5^* sequence, which was obtained from Pia_5 tgDNA, revealed to be a Numt, with a T-deletion in a conserved site (pos. 920), causing a frame shift mutation and early stop codon. This was the only nucleotide change with respect to the other mtDNA Cyt-*b* sequences of the same group (BP = 62%).

As far as the *A. chukar* group is concerned, Pia and Bie sequences clustered together with those from Mongolia and $UWBM_{46402}$ sequence from Kazakhstan (BP = 81%). A single nucleotide change occurred between Pia and Bie sequences.

Maximum parsimony produced a tree from 91 parsimony informative characters. The 50% consensus MP tree (length, $L = 251$; consistency index, CI = 0.745; retention index, RI = 0.941) showed substantially the same topology as the NJ tree (Fig. 2), with *A. rufa* (BP = 100%) and *A. chukar* (BP = 100%) groups. Within the *A. rufa* clade, RLP sequences clustered together with the exception of *A. rufa* Por Z48775 (BP = 94%).

3.3. D-loop control region: sequence and phylogenetic data

D-loop sequences were 1156 bp long in *A. rufa* and 1154 bp in *A. chukar* (only $UWBM_{46402}$ sequence was 1155 bp long). Nucleotide composition was, on average: 32.1% of thymine, 27.0% of cytosine, 26.6% of adenine, and 14.3% of guanine. The number of variables sites was 64, indels included. The average number of Ts was 1.7 higher than Tv.

Genetic distance analysis with TN93 γ-pairwise distance algorithm ($\alpha = 0.121$) produced the phylogeny represented by the NJ tree of Fig. 3. Spanish *A. rufa* and Asian *A. chukar* specimens clustered in two distinct groups with BP = 100%. Partridges from Pia and Bie populations were equally distributed into these two clades (five each). All the Sca sequences grouped within the *A. rufa* cluster. Within the *A. rufa* clade Pia, Bie and Sca partridges grouped together (BP = 69%). Sequences of Pia and Bie partridges were identical. Spanish *A. rufa* specimens from Mallorca clustered as one (BP = 88%) with the exception specimen P_5. At least six Ts separated *A. rufa* Spa AJ222740 sequence from Spanish *A. rufa* specimens of this study.

As far as the *A. chukar* clade is concerned, sequences of Pia and Bie populations clustered with both specimens from Mongolia and $UWBM_{46402}$from Kazakhstan (BP = 96%). This group diverged (BP = 86%) from that one with specimens from Georgia, Israel and Kazakhstan ($UWBM_{46516}$).

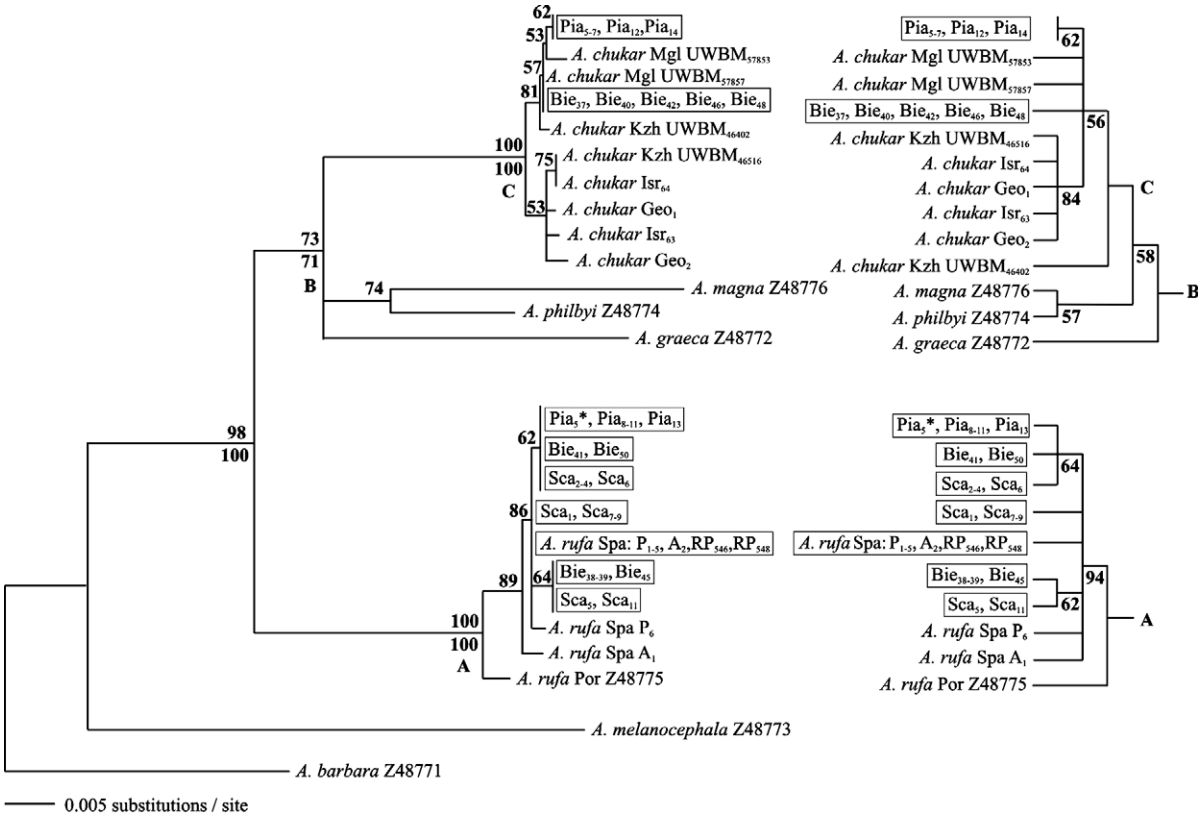

Fig. 2. Neighbour-joining tree computed by PAUP using Tamura and Nei's (1993) γ-genetic distances (α = 0.318, four rate categories) among the aligned 1092 nucleotide of mtDNA Cyt-*b* sequences of partridges from Pianosa (Pia, *n* = 10), Bieri (Bie, *n* = 10), Scarlino (Sca, *n* = 10), Spain (*A. rufa*, Spa, *n* = 10), Asia (*A. chukar*: Mongolia, Mgl, *n* = 2; Kazakhstan, Kzh, *n* = 2; Georgia, Geo, *n* = 2; Israel, Isr, *n* = 2). Other abbreviations: Chi, China; Por, Portugal. GenBank sequences were used as reported in Section 2. Numbers at the internodes indicate all the bootstrap percentage values computed in both the neighbour-joining (NJ: above internodes) and 50% majority-rule consensus maximum parsimony (MP: below internodes) tree. Capital letters "A", "B" and "C" indicate corresponding internodes between NJ and MP trees when their topologies diverge: in this case, MP tree is showed onto the right side. Rectangular boxes show identical sequences. The asterisk (*) indicates the Pia_5 Numt sequence. The phylogenetic trees were rooted using *A. barbara* Z48771 GenBank sequence.

Maximum parsimony produced a tree from 85 parsimony informative characters. The 50% consensus MP tree (length, $L = 253$; consistency index, CI = 0.640; retention index, RI = 0.911) showed closely the same topology as the NJ tree (Fig. 3), with *A. rufa* (BP = 100%) and *A. chukar* (BP = 100%) groups.

Within the *A. rufa* clade, *A. rufa* Spa RP_{546} diverged by BP = 81%. Partridges from Pia, Bie and Sca populations grouped with all the other sequences, with the exception of *A. rufa* Spa A_1 (BP = 58%). *A. rufa* sequences from Mallorca ($P_{1–4}$, P_6) grouped together (BP = 83%).

As far as the *A. chukar* clade is concerned, Pia and Bie sequences grouped with those from Mongolia and with $UWBM_{46402}$ from Kazakhstan (BP = 89%), diverging from the *A. chukar* group with specimens from Israel and Georgia (BP = 92%).

3.4. RAPD banding patterns

RAPD primers were first tested on 10 pure *A. rufa* (Negro et al., 2001) from three Spanish areas (Mallorca, Ciudad Real, Seville), and 10 Asian *A. chukar* (from Mongolia, Kazakhstan, Georgia and Israel). Among all of the primers we tested (*n* = 15), only those reliably amplifying partridge DNA were selected (*n* = 12). A further selection led to singling out four species-specific primers (Table 2). Specimens used as parental control revealed homogeneous RAPD profiles (Fig. 4), and all the scored species-specific bands always showed up in all of the replicates. Hence, experimental partridges from Pia (*n* = 14), Bie (*n* = 14) and Sca (*n* = 11) populations were tested. Electrophoresis of PCR products produced patterns comprising 2–11 well-defined bands in the 200–3500 bp range, plus a variable number of not scorable bands. RAPD banding profile of marker OP-C-09 for four Pia partridges and six controls for both *A. rufa* and *A. chukar* is reported in Fig. 4. As it is shown, three out of four Pia birds (Pia_7, Pia_{10}, Pia_{12}) were hybrids, showing simultaneously both *A. rufa* and *A. chukar* specific bands, the fourth (Pia_{11}) having only the *A. rufa* marker. A synopsis of the results obtained from RAPD banding profiles for the three populations is shown in Table 3. It was found that 93%, 86%

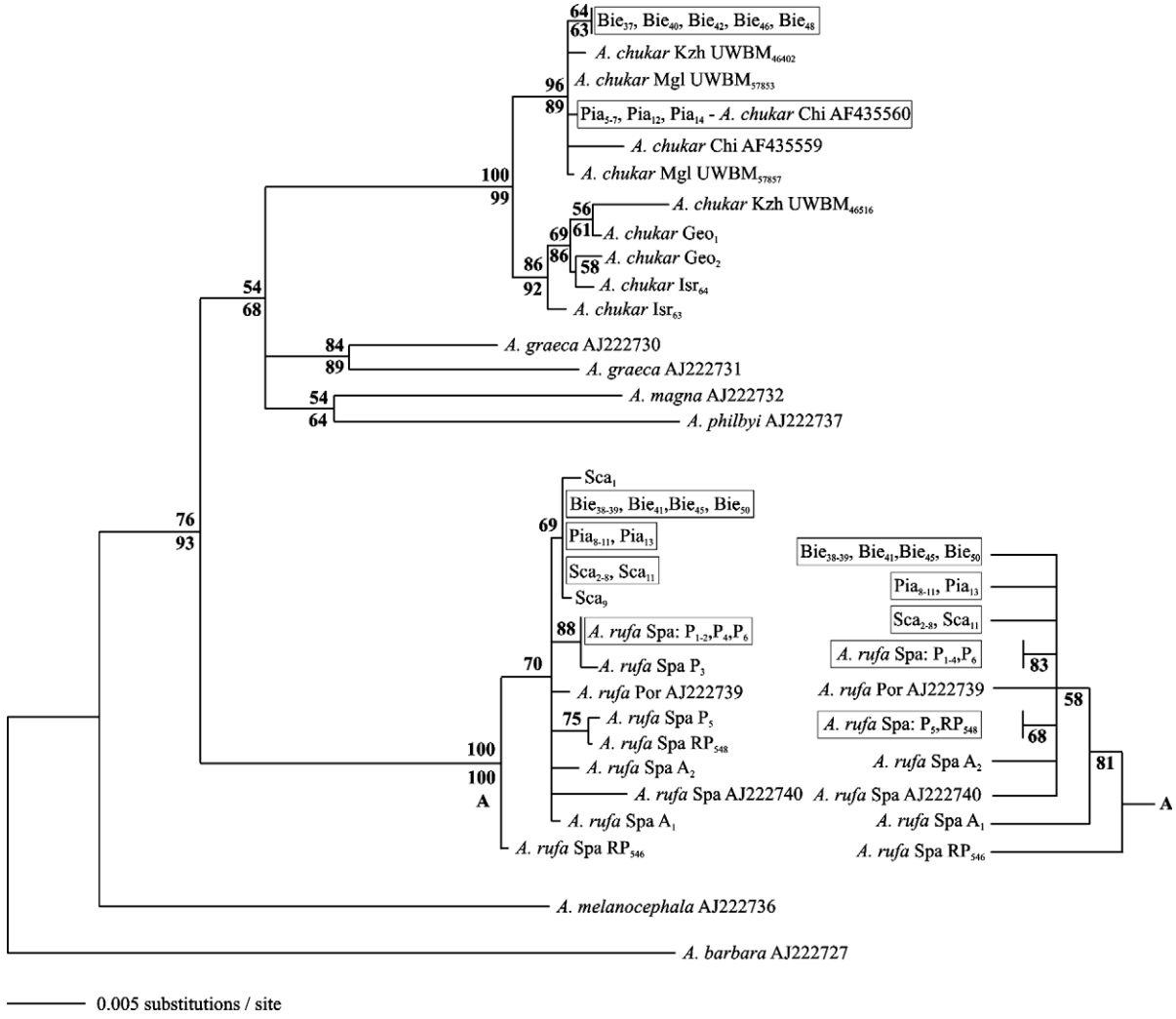

Fig. 3. Neighbour-joining tree computed by PAUP using Tamura and Nei's (1993) γ-genetic distances (α = 0.121, four rate categories) among the aligned 1167 nucleotide of mtDNA D-loop sequences of partridges from Pianosa (Pia, *n* = 10), Bieri (Bie, *n* = 10), Scarlino (Sca, *n* = 10), Spain (*A. rufa*, Spa, *n* = 10), Asia (*A. chukar*: Mongolia, Mgl, *n* = 2; Kazakhstan, Kzh, *n* = 2; Georgia, Geo, *n* = 2; Israel, Isr, *n* = 2). Other abbreviations: Chi, China; Por, Portugal. GenBank sequences were used as reported in Section 2. Numbers at the internodes indicate all the bootstrap percentage values computed in both the neighbour-joining (NJ: above internodes) and 50% majority-rule consensus maximum parsimony (MP: below internodes) tree. Capital letter "A" indicates corresponding internode between NJ and MP trees when their topologies diverge: in this case, MP tree is showed onto the right side. Rectangular boxes show identical sequences. The phylogenetic trees were rooted using *A. barbara* AJ222727 GenBank sequence.

Table 2
The RAPD primers with sequence and specific marker fragment length as selected from *A. rufa* and *A. chukar* controls

Primer code	Sequence (5′-3′)	Marker fragment length (bp)	
		A. rufa	*A. chukar*
OP-C-08	TGGACCGGTG	760	1510
OP-C-09	CTCACCGTCC	3360	920
OP-C-20	ACTTCGCCAC	2050	210
OP-H-12	ACGCGCATGT	920	200

and 64% of the partridges from Pia, Bie and Sca populations, respectively, were hybrid birds, showing simultaneously the *A. rufa* and *A. chukar* bands for at least one out of four primers. It was found that one bird (Pia_7) was hybrid for all of the four primers, whereas one bird from Pianosa (Pia_6), two from Bieri (Bie_{38}, Bie_{46}) and four from Scarlino (Sca_5, Sca_8, Sca_9, Sca_{10}) showed only the *A. rufa* species-specific band independ-

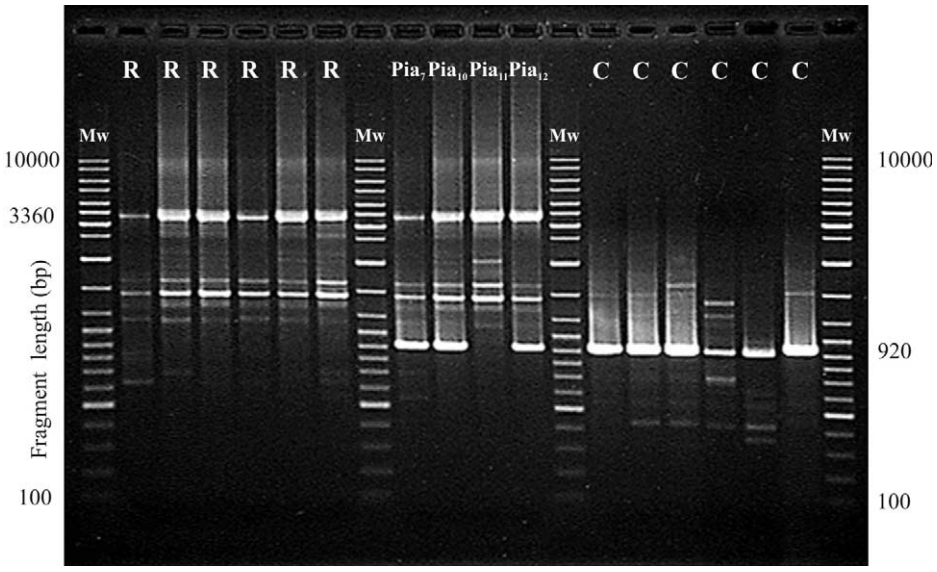

Fig. 4. RAPD banding profile of marker OP-C-09 is shown as it was obtained from the gel electrophoresis. For the sake of precision, molecular weight marker (Mw, base pairs) was run four times. As for single specimens, from left to right: six Spanish RLPs as *A. rufa* controls (R: 1st, 2nd and 3rd from Mallorca; 4th and 5th from Ciudad Real; 6th from Seville); four experimental RLPs from Pia population (Pia_7, Pia_{10}, Pia_{11}, Pia_{12}); six Asian CHKs as *A. chukar* controls (C: 1st and 2nd from Mongolia; 3rd from Kazakhstan; 4th and 5th from Israel; 6th from Georgia). Species-specific fragments are 3360 bp and 920 bp long for *A. rufa* and *A. chukar*, respectively. Bands analysis was performed using BioRad Quantity One software.

Table 3
The results of RAPD banding profiles as obtained for Pianosa, Bieri and Scarlino populations

Pianosa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	(H, %)
OP-C-08	R	R	R	H	R	R	H	R	R	R	H	H	R	R	93
OP-C-09	R	R	R	R	R	R	H	R	R	H	R	H	R	H	
OP-C-20	R	H	H	R	H	R	H	R	R	H	H	H	H	H	
OP-H-12	H	H	H	H	R	R	H	H	H	H	H	R	H	H	
Bieri	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**45**	**46**	**47**	**48**	**50**	**(H, %)**
OP-C-08	R	R	H	R	R	H	R	R	R	H	R	R	H	R	86
OP-C-09	H	R	R	R	R	R	R	R	R	R	R	R	R	R	
OP-C-20	R	H	H	R	H	H	R	H	H	H	R	R	R	H	
OP-H-12	H	H	R	R	R	H	H	R	R	R	R	H	H	R	
Scarlino	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**				**(H, %)**
OP-C-08	R	R	R	R	R	R	R	R	R	R	R	–	–	–	64
OP-C-09	R	R	R	H	R	R	R	R	R	R	R	–	–	–	
OP-C-20	H	R	R	R	R	R	R	R	R	R	H	–	–	–	
OP-H-12	H	H	H	R	R	H	H	R	R	R	H	–	–	–	

The occurrence either of the only *A. rufa* marker (R) or of both the *A. rufa* and *A. chukar* markers (H) is reported. The percentage of hybrid partridges (H, %) for each population is indicated at the right margin.

ently upon the selected primers. The remaining RLPs of this study were hybrids with intermediate band patterns (i.e., from one to four primers).

4. Discussion

As far as birds are concerned, literature records many instances of naturally occurring breakdown of isolating mechanisms established to prevent interbreeding of a species with others (Mayr, 1977; Grant and Grant, 2002). Hybridisation (*sensu* Short, 1969) occurs also in the genus *Alectoris* (Gray, 1958). Nevertheless, it has only been demonstrated so far between *A. rufa* and *A. graeca* (Randi and Bernard-Laurent, 1999; Negro et al., 2001); *A. rufa*, *A. graeca* and *A. chukar* have been suggested to hybridise among each other (Bernard-Laurent and De Franceschi, 1994), and hybridisation

has allegedly been assumed to occur between *A. magna* and *A. chukar* (Cheng et al., 1999).

The genetic conservation status of the species *A. rufa* in the easternmost area of its geographical range, the central Italy, was analysed comparing both wild and captive populations. Wild population from Pianosa island (Tuscan Archipelago National Park) was chosen, as it was both the most historically preserved and healthy looking in central Italy, along with captive populations from two Tuscan stock-farms (Bieri and Scarlino), the most important public Institutions in providing partridges for restocking plans.

Phenotype inspection of the studied populations was performed using a few morphological pointers (Wilkinson, 1987, 1991) and partridges were assigned to the taxon *A. rufa*. Notwithstanding an extensive morphological uniformity, maternally inherited mtDNA Cyt-*b* and D-loop markers clearly demonstrated a mixed ancestry in both Pianosa and Bieri populations: kinship of representatives comprising each of these populations resulted to be 50% *A. rufa* and 50% *A. chukar*, whereas the Scarlino partridges uniquely showed the *A. rufa*-mtDNA line (cf. Figs. 2 and 3). Biparentally inherited nuclear markers such as those revealed by RAPD, unequivocally identified hybrid individuals, suggesting that *A. rufa* × *A. chukar* hybridisation was largely spread in the studied populations (cf. Table 3). According to Tranah et al. (2003), species identification exclusively based on morphological characters revealed to be an uncorrected measurement. Correspondence between ambiguous morphological traits and genetically identified hybrids was not found, stressing that a molecular approach was essential to detect not pure specimens.

Cyt-*b* and D-loop mtDNA phylogenetic reconstructions concurrently assigned partridges from each Tuscan population to either *A. rufa* or *A. chukar* clade (cf. Figs. 2 and 3). Accurate checking to evaluate any possible Numt contamination of our sequences, led us to demonstrate that, within the *A. rufa* clade, Pia_5^* Cyt-*b* sequence actually met such a distinctive connotation. It was concluded that Pia_5^* *A. rufa*-like sequence, which was obtained from Pia_5 tgDNA, was a Numt transferred to the nucleus before *A. rufa* × *A. chukar* hybridisation occurred. Indeed, both Pia_5 Cyt-*b* and D-loop sequences from purified mtDNA showed to be *A. chukar*-like. Excluding any contamination, as other sequences identical to Pia_5^* were never found, heteroplasmy too was ruled out. The only bird species recorded as a heteroplasmic instance is the razorbill (Moum and Bakke, 2001). However, the nucleotide changes ($n = 52$) occurring between Pia_5 *A. chukar*-like and Pia_5^* *A. rufa*-like sequences, were too many to be explained by somatic mutation within an individual. Moreover, PCR products from Pia_5 purified mtDNA never revealed to be a mixture of *A. rufa* and *A. chukar*-like sequences. This, in turn, rejected any hypothesis about mtDNA paternal leakage too (Gyllestein et al., 1991; Schwartz and Vissing, 2002; Kvist et al., 2003). Finally, it is remarkable that, as Pia_5^* Numt sequence nested into the *A. rufa* mtDNA clade, its transfer to the nucleus should not have been occurred so far in the past, as Numts usually evolve slower than their functional mitochondrial counterparts (Bensasson et al., 2001).

As far as Cyt-*b* trees are concerned, NJ and MP reconstructions showed largely overlapping branching patterns. Within the *A. rufa* clade, sequences from Pia, Bie and Sca populations clustered with those from Spain. The *A. rufa* Por Z48775 (from Portugal: Randi, 1996) clearly diverged in both NJ (BP = 100%) and MP (BP = 94%) trees. Although sampling size was reduced, genetic distance due to geographical variation may be hypothesised. *A. rufa* is a polytypic species with differences involving only colour patterns. Three subspecies are recognised: *A. rufa hispanica* (Seoane, 1894) in northern and central Portugal and northwestern Spain; *A. rufa intercedens* (A E Brehm, 1858) in southern and eastern Spain; *A. rufa rufa* (Linnaeus, 1758) in Italy and France. In addition, RLPs from Balearics are intermediate between *A. r. rufa* and *A. r. intercedens* (Cramp and Simmons, 1980). According to this morphological outline, clustering between sequences from central Italy and Balearics was found to be tighter than that between Balearics and Portugal (cf. Fig. 2).

As far as D-loop is concerned, NJ and MP trees were substantially identical. Within the *A. chukar* clade, the group (NJ tree, BP = 81%; MP tree, BP = 89%) comprising sequences produced by Mongolia, Kazakhstan ($UWBM_{46402}$) and China (AF435559, AF435560) representatives diverged from that (NJ tree, BP = 86%; MP tree, BP = 92%) including sequences from Israel, Georgia and Kazakhstan ($UWBM_{46516}$). However, a larger sampling collection is needed to individuate distinctive geographical haplotypes.

The RAPD analysis has proved beyond any doubt the hybridisation between *A. rufa* and *A. chukar*. The analysis of the Tuscan RLP populations was performed with markers selected using *A. rufa* and *A. chukar* controls from different geographical areas, in order to have more reliable results (cf. Fig. 4). This was particularly important for *A. chukar*, because of its very wide range of geographical distribution (Aebischer, 1997).

As discussed by Boecklen and Howard (1997), the probability of having false negatives (i.e., true hybrids considered as RLPs) using the four RAPD primers employed, was calculated. When a single marker is considered, the probability of not detecting a true hybrid is 0.5, 0.75 and 0.875 in the 1st (BC-1), 2nd (BC-2) and 3rd (BC-3) backcross, respectively. When markers are combined, the probability is reduced in a multiplicative way.

With four markers the estimated probability are: $(0.5)^4 = 0.062$ (6.2%) for BC-1, $(0.75)^4 = 0.316$ (31.6%) for BC-2, $(0.875)^4 = 0.586$ (58.6%) for BC-3. RAPD markers show dominant Mendelian inheritance and they are supposed to always show up in F1 hybrids. However, this is not the RAPD banding profile typical of our RLP populations. Indeed, all degrees of genetic introgression with *A. chukar* species were found, namely a given partridge showed up as hybrid in the case of some markers but not in others (cf. Table 3). Such an overall result agreed with the fact that all of partridges of this study closely resembled just one of the two parental species (i.e., *A. rufa*). More in particular, as far as both Pia_6 and Bie_{46} are concerned (cf. Table 3), taking into account their *A. chukar*-like mtDNA lineages (cf. Figs. 2 and 3), we feel confident in considering them as *A. rufa* × *A. chukar* hybrids. However, as for Bie_{38} and a few Sca partridges (Sca_5, $Sca_{8–10}$), we can only presume their hybrid status. As these birds originate from the same farms as well as those disclosed to be hybrids, it is likely that only the relatively low number of selected primers hindered the revealing of their possible hybridisation (cf. Table 3). Although we cannot classify the hybrid birds of this study into specific backcross categories, we feel confident in concluding that they are *A. rufa* × *A. chukar* backcrosses to the *A. rufa* species.

As far as single populations are concerned, the different percentage of hybrid partridges that was found deserves a comment. Regarding to the Pia population, we hypothesised that the releasing of 10 captive-bred RLP pairs made by the University of Pisa (1987), the only one occurred onto the island during its long-lasting period of surveillance (P. Mani, pers. comm.), could have been responsible for the genetic introgression with *A. chukar* alleles. The stock-farm that was used as partridge's source was in Umbria (central Italy), and it is not longer operating. Since 1985 the Bieri Experimental Stock-farm was used to exchange animals with other farms in central Italy, included that one mentioned above (F. Cappelli, pers. comm.). It is believed that a commercial stock of *A. chukar*-introgressed *A. rufa* partridges genetically polluted both the Pia and Bie populations, although no definitive proof is available. As far as the Sca birds, they were from the "Public Centre for the Game Production" of Scarlino, which recently started (1999) a selective program aimed to purify the *A. rufa* genome. Following their backcrossing activity, both the selected *A. rufa* mitochondrial line and fairly lowest percentage of hybrid partridges were found. Sca hybrids disclosure, in turn, confirmed both the power and usefulness of the RAPD technique, as backcrosses are partridges that are usually released for restocking (Negro et al., 2001). Considering that hybridisation largely affects even a geographically isolated and long-time protected RLP population, other than farmreared specimens aimed to restocking activities, the occurrence of the pure, native *A. rufa* genome in the easternmost part of the species' geographical range, may be guessed to be virtual.

According to Frankham et al. (2002), management of a species subjected to hybridisation involve identification and eradication of the hybrids, and expanding of the number of the pure individuals. However, supportive breeding can pose risks to wild populations because of possible, deleterious genetic consequences due to the use of captive specimens (Poteaux et al., 1998; Ford, 2002). Indeed, genetic integrity of a range of many taxa is threatened by introgression (for a review, cf. Rhymer and Simberloff, 1996). Markers can be used to detect hybrids and RAPD technique showed to reliably work in many populations (Anttila et al., 2000). Once species-specific primers are established, RAPD represented both a time-saving and comparatively low cost method to test as many captive founders as possible, together with a fraction of their chicks (1–5%, depending upon to the number of the birds to be restocked; cf. Schulz, 2000; Negro et al., 2001). As genetic introgression with allochthon species actually represents a broad-scale phenomenon in Galliformes (Gray, 1958; for hybridisation in quails, cf. Deregnacourt et al., 2002), a policy dealing with a RAPD-based genetic screening is here recommended to rule out the main core of the genome pollution.

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