**Mitochondrial DNA and microsatellite markers evidence a different pattern of hybridization in red-legged partridge (Alectoris rufa) populations from NW Italy.**



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# Mitochondrial DNA and microsatellite markers evidence a different pattern of hybridization in red-legged partridge *(Alectoris rufa)* populations from NW Italy.

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**Abstract.**
Genetic introgression with exotic genomes represents a major conservation concern for the red-legged partridge *(Alectoris rufa, Phasianidae).* In particular, massive releases of chukar partridges *(Alectoris chukar)* and/or red-legged x chukar partridge hybrids for hunting purposes have cast serious doubts on the Italian present-day occurrence of wild *A. rufa* populations not affected by introgressive hybridization. This study investigates the genetic structure of red-legged partridges populations in two ecologically different areas in Northern Italy. Analysis of maternal mitochondrial DNA and biparental microsatellite markers excluded the presence of hybridization in a typical agricultural habitat where hunting and release of reared birds are strictly banned. By contrast, signs of chukar introgression were detected in a perifluvial habitat unusual for the red-legged partridge in Italy. The present study documents the first red-legged partridge population with no genetic evidences of recent chukar introgression presently living in Italy. We recommend that urgent conservation actions are taken to preserve the genetic integrity of this population from the risk of hybridization with farm-reared birds and to support its long-term conservation.

**Keywords** *Alectoris*, Introgressive hybridization, Microsatellites, mtDNA control region, Self-sustaining wild populations

## Introduction

Genetic homogenization is recognized as one of the most prominent threats to biodiversity, together with habitat loss and/or fragmentation, human disturbance, alien/invasive species (McKinney and Lockwood 1999), overexploitation of natural resources (Mack et al. 2000; Olden et al. 2004), and climate change (Thomas et al. 2004; Jiguet et al. 2010). The evolutionary role of natural hybridization as a source of new adaptive variations is still controversial (Barton 2001; Seehausen 2004). Nevertheless, most authors agree on the negative effects on regional biodiversity of anthropogenic introgressions of non-indigenous into native gene pools (Mooney and Cleland 2001; Mallet 2005; Allendorf and Luikart 2007).
In some cases, hybridization occurs naturally due to overlapping habitat and insufficient genetic barriers. This is most frequent in birds (Grant and Grant 1992), e.g., in Scandinavian populations of rock ptarmigan *Lagopus mutus* and willow ptarmigan *Lagopus lagopus* (Quintela et al. 2010), in Central European populations of greater spotted eagle *Aquila clanga* and lesser spotted eagle *Aquila pomarina* (Vali et al. 2010), and between house sparrow *Passer domesticus* and Eurasian tree sparrow *Passer montanus* in Norway (Solberg et al. 2006). Natural hybridization is frequent in Galliformes (McCarthy 2006); indeed, all the native grouse species in Britain can hybridize with at least one other species (Millais 1894). The hybrids between black grouse *Tetrao tetrix* and capercaillie *Tetrao urogallus* are the most common patterns of grouse hybridization (Porkert et al. 1997).
A growing body of evidence suggests that the introduction of exotic species and the translocation of captive-reared animals or artificial hybrids have a negative impact on the integrity of local populations, leading to disruption of coadapted gene complexes and loss of local adaptations (Allendorf et al. 2001; Randi 2005, 2008; Williams et al. 2005; Barilani et al. 2007b; Barbanera et al. 2010; De Haan et al. 2010). These detrimental effects may result in reduced fitness and demographic decline (outbreeding depression; Templeton 1986), concurring to the disappearance of wild populations. The risk of genetic pollution by introgressive hybridization evoked growing concern, especially for game species with high socioeconomic value. For example, wild Galliformes populations are frequently restocked with captive-

reared birds in order to sustain the hunting pressure (Randi et al. 2003; Barbanera et al. 2009, Sokos et al. 2009). It is well-known that captive breeding conditions can induce genetic deteriorations and alter antipredator or mating behavior (Ford 2002). A drastic reduction of survival in the wild has been previously shown (Woodworth et al. 2002; Frankham 2008), particularly when management disregards genetic similarity or geographical origin of farmed stocks (Barbanera et al. 2010). Galliformes is now one of the most threatened groups of birds, affected by overexploitation and genetic pollution (Keane et al. 2005; Romagosa et al. 2009).

The genus *Alectoris* (Galliformes, *Phasianidae)* is present in the Mediterranean basin with four of the seven usually recognized species, showing a basically allopatric distribution. Natural hybridization is known to occur only in a few areas of parapatric contacts between red-legged *(Alectoris rufa)* x rock *(Alectoris graeca)* partridge (Bernard-Laurent 1984; Randi and Bernard-Laurent 1999; Randi et al. 2003), and *Alectoris chukar* x *A. graeca* (Dragoev 1974). By contrast, the separate distributions of the species should prevent natural hybrids of *A. rufa* x *A. chukar.*

The red-legged partridge is the most important small game bird in southwestern Europe (Blanco-Aguiar et al. 2003; Martinez-Fresno et al. 2008). Its natural distribution range encompasses the Iberian Peninsula, France, North Western and Central Italy, including the Mediterranean islands of Corsica, the Balearics, and the Tuscan Archipelago. *A. rufa* has successfully been introduced to the UK in the eighteenth century (Potts 1989). The rock partridge *(A. graeca)* is distributed in the Apennines and the Alps at altitudes ranging from 400 to 3,000 m, and between 50 and 2,500 m in Albania, Greece, and Sicily. The wider distribution of *A. chukar* extends from Eastern Balkans, including Greek islands and Turkey, to central Asia up to Northern China.

*A. rufa* have declined since the second half of the twentieth century throughout its distribution range, due mainly to deterioration of the natural habitats and overexploitation of the native populations for hunting purposes. At present, the red-legged partridge is considered threatened under European Union Legislation (79/409 CEE Ap.2/1, 3/1 BERN Ap.3) and it is included in the list of Species of European Conservation Concern. Several European countries have counterbalanced the decline of wild populations by means of the yearly release of millions of farm-bred partridges (Negro et al. 2001; Casas et al. 2012). These inadequate management practices have seriously compromised the integrity of native gene pools, contributing to the decline of many local populations in Europe (Barilani et al. 2007a). A further problem in restocking programs is the use of hybrids obtained from cross-breeding of *A. rufa* with closely related species, in particular *A. chukar.* Western European countries forbid the release of chukar partridges and their cross *A. rufa* hybrids. Nevertheless, the production of *A. rufa* x *A. chukar* hybrids has become a common practice aimed to improve the tolerance to captivity of *A. rufa*, to increase laying period and produce heavier and tame birds (Baratti et al. 2004; Tejedor et al. 2008). Moreover, the difficult detection of hybrids on the basis of morphological and plumage criteria (Johnsgard 1988; Martinez-Fresno et al. 2008) beyond the earliest backcross generations, leads to their the uncontrolled production and trade by many unaware breeders. Therefore, the assessment of introgressive hybridization rates between wild populations and captive-bred relatives is a fundamental prerequisite to optimize the management of *A. rufa.* At present, only molecular techniques based on the combined use of maternal and biparentally inherited nuclear markers allow the detection of introgressed individuals and hybridizing populations. In the last decade, the development of hypervari-able genomic markers (i.e., microsatellites) and new statistical methods (Bayesian models) has significantly improved population structure assessments, admixture analyses, and testing for individual assignment (Beaumont and Rannala 2004; Barilani et al. 2007b; Randi 2008; Scandura et al. 2010).

Recent reports indicated the widespread introgression of chukar genes in most European red-legged partridge populations studied so far across the native distribution range in Iberia, France, and Italy (Tejedor et al. 2007; Barilani et al. 2007a; Martinez-Fresno et al. 2008; Blanco-Aguiar et al. 2008; Sevane et al. 2009; Barbanera et al. 2009, 2011; Ferrero et al. 2011; Casas et al. 2012). Indeed, mitochondrial DNA (mtDNA) haplotypes distribution revealed a decreasing gradient of *A. rufa* x *A. chukar* hybridization from Italy to Portugal (Barbanera et al. 2010). In Italy, the introgres-sion process has dramatically contributed to decline wild populations, now estimated at only 1,500-2,000 pairs (Brichetti and Fracasso 2004). Accordingly, these red-legged partridge populations can be considered near extinction (Meriggi et al. 2007).

The historical range of Italian red-legged partridges included hill and low mountain areas (between 200 and 900 m above sea level) from the French border to Central Italy, including the introduced populations in some islands of the Tuscan Archipelago National Park. Preferred habitat was well exposed and drained agricultural mosaic, with hedgerows and margins covered by herbaceous vegetation, alternated to fallow fields and rolling hill country with scrubby gullies (Spagnesi and Serra 2004).

Nowadays, the species mainly survives in small and highly fragmented populations in protected areas (Meriggi et al. 2007). Most frequently, the occurrence of *A. rufa* results from continuous restocking rather than the existence of self-sustaining groups (Spano 2010). Releases of reared birds in unsuitable areas also lead to alterations of the species biogeography, and small introduced populations are now expanding in lowland areas where *A. rufa* had never been reported in the past (Spano 2010). Restocking activities may have seriously compromised the integrity of the gene pool and conservation status of native Italian populations.

In Piedmont (NW Italy), *Alectoris rufa* was historically limited to the hilly and low-mountain areas of the southern half of the region (between 300 and 800 m asl) in the districts of Cuneo, Asti, and Alessandria (Fig. 1). Recent management strategies have been undertaken with the aim to sustain residual wild stocks. In particular, the suspension of hunting seemed to improve the stabilization of wild populations (Boano and Pulcher 2003). At present, hunting partridge is performed only in Cuneo districts. Indeed, most of the releases approved by Piedmont Region from 2000 to 2006 occurred in this province (94.4 %; 35.969 individuals). By contrast, only 5.3 % (1,902 individuals) of releases occurred in the area of Alessandria. In this latter case, red partridge restocking operations have been stopped since 2003 (Fig. 1, data from: http://www.regione.piemonte.it/ agri/areatecnicoscientifica/osservfaun/index.htm). In this study, we aimed to investigate two self-sustaining wild populations of *A. rufa* in NW Italy, characterized by different life history and habitat utilization. Genetic composition and presence of introgressive hybridization with *A. chukar* have been evaluated by mtDNA control region sequences and Bayesian admixture analyses of multilocus genotypes determined at eight microsatellite loci.

## Materials and methods

Study areas and sample collection

One hundred seventy-six animals were captured during winter 2010 (Fig. 1) in two protected areas (about 14 km apart) of the province of Alessandria (NW Italy), where release of partridges is not allowed. In the same study area, Tizzani et al. (2012) observed the presence of self-sustaining wild populations. The first site (Brignano-Casasco, BC; about 1,000 ha) is a typical habitat for the red-legged partridge in Italy, consisting of hills (220-180 m asl) characterized by the presence of crops (85 %) alternating with wooded areas (14 %) and shrubs (1 %; CORINE Land Cover Project; http://www.eea.europa.eu/data-and-maps/data/). This area has been protected for about 70 years, formerly (since the late 1930s) as private reserve then as "protection reserve" and currently as "restocking and capture zone" (ZRC), and no restocking of red-legged partridges has been documented in that time (Pellegrino 2011). Presently, the estimated population amounts to 23 nesting couples, with a density of about 3.8 pairs/km$^2$ (Tizzani et al. 2012). The second area has been protected since the early 1990s. It is a perifluvial habitat of Scrivia stream a right tributary of the Po River including Sites of Community Interest (SCI) under the Habitat and Birds European Directive (92/43/CEE; 79/409/CEE) located between built-up areas of Cassano Spinola and Tortona (Fig. 1). Land use is characterized by the presence of shrubs (6 %), crops (75 %), wooded (4 %), and urban areas (4 %). River banks cover 8 % of the area (CORINE Land Cover). Notwithstanding the foregoing literature (Spano 2010), we herein report a rapid colonization of the river basin southwards occurred over the last 7 years. Multiple capture-recapture survey of marked individuals performed from 2009 to 2011 within the SCI showed dispersion movements within 4-5 km (unpublished data). The first occasional captures of partridges have been performed before 2004 in cage traps for pheasants near of built-up areas of Arquata Scrivia and Serravalle Scrivia (Silvano, personal communication). Actually, the latest releases farm-reared partridges authorized by the province of Alessandria were performed mainly in the hilly areas of the district (Fig. 1). These partridges probably originated from natural populations inhabiting the surrounding hills, then subjected to repeated restocking operations since early 1980s up until 2003 (Spano et al. 1987). The total population consist of 23 nesting couples, with a density of about 2.1 bird pairs/km$^2$ (Tizzani et al. 2012). Partridges from this second area originated from three capture sites along the river (Serravalle Scrivia (SS), Cassano Spinola (CA), Rivalta Scrivia (RV)) and a single site in the neighboring hill (Serravalle Scrivia-Montei (SM)).

Same sampling strategies were performed in both study areas. Partridges were captured by grain-baited cage traps. Animals were then individually ringed, measured, and classified on the basis of morphological criteria (i.e., garget and flank feathers color) according to Cramp and Simmons (1980). We collected contour feathers and blood samples by venipunction of the brachial vein (~40 µl). Samples were stored separately in test tubes containing 95 % ethanol (feathers) or Longmire buffer (Longmire et al. 1997; blood sample), and preserved at -20 °C until analyzed. Partridges were released within 30 min in the same capture site.

Laboratory methods

Total genomic DNA was isolated from blood (n=122) or feathers (n=54) with a commercially available NucleoSpin kit (Macherey-Nagel) according to the manufacturer's instructions. Sequences of hypervariable domain I of mitochondrial DNA control region (mtDNA CR-IA) were amplified by PCR using primers PHDL and PH-H521 (Randi and Lucchini 1998). Reactions were performed in a volume of 15 Hi including 10-15 ng of total DNA, 1 U of Taq DNA polymerase (Sigma-Aldrich, Germany), 6 pmol of each primer (0.24 HM), lx reaction buffer, 1.7 mM MgCi2, and 0.125 mM of each dNTP. Selective amplification was carried out in a Bio-Rad C1000 thermal cycler by the following protocol: 2 min at 94 °C, followed by 30 cycles of three steps of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, before a final extension of 10 min at 72 °C. Amplicons were sequenced on an ABI 3130XL automated sequencer using primer PHDL and then analyzed with Sequencing Analysis version 5.3 and SeqScape version 2.5 (Applied Biosystems).

Analysis of mtDNA data

Sequence alignment was performed with BioEdit version 7.0.9 (Hall 1999) and haplotypes were identified using Collapse version 1.2 (Khromov-Borisov et al. 1999). Unrooted networks were drawn to infer haplotype relationships and species identification using the median-joining network procedure (Bandelt et al. 1999), as implemented in Network version 4.5.1.0 (© 2004-2009 Fluxus Technology). Sequences of *A. rufa* (AJ222739; AJ222740 Lucchini and Randi 1998) and .4. *chukar* (AJ222728; AJ222729; Lucchini and Randi 1998) have been included as references, DNASP version 5.00.07 (Librado and Rozas 2009) was used to estimate mtDNA haplotype diversity («), nucleotide diversity (π), and the mean number of pairwise differences *(k)* in the sampled populations. Phylogenetic relationships among haplotypes were indirectly obtained by blasting (Altschul et al. 1990) the mtDNA CR-IA sequences produced in this study against 57 homologous A *rufa* sequences (319 bp) published by Ferrero et al. (2011).

Analysis of microsatellite data

A set of samples (107 individuals) was also genotyped by PCR amplification at eight microsatellite loci *(MCW118*, *MCW135*, *MCW152*, *MCW225*, *MCW276*, *MCW280*, *MCW295*, and *MCW323)*, as previously described in Barilani et al. (2007a). Samples were compared with specimens of A *rufa* (51 individuals) and *A. chukar* (111 individuals) collected from native populations in Spain and Portugal *(A. rufa)*, China, and Israel *(A. chukar)*, and used as reference individuals by Barilani et al. (2007a, b).

All PCR products were analyzed automatically with an ABI 3130XL sequencer and the softwares GeneScan version 3.7 and Genotyper version 2.1 (Applied Biosystems). We calculated micro satellite allelic richness *(Ac)* with HP-RARE 1.0 (Kalinowski 2005) and tested for linkage disequilibrium with FSTAT 2.9.3 (Goudet 2001). Genetix version 4.05.2 (Belkhir et al. 1996-2004) was used to compute allele frequencies, expected $(H_e)$ and observed $(H_o)$ heterozygosities, *F* statistic by Wright (1965), deviations from Hardy-Weinberg equilibrium (HWE). Bonferroni sequential correction (Rice 1989) was applied to evaluate statistical significance where multiple tests were performed. Patterns of differentiation among multilocus genotypes were visualized by factorial correspondence analysis (FCA). The hierarchical distribution of genetic diversity within and among Italian populations was investigated by analysis of molecular variance (AMOVA; Excoffier et al. 1992) as implemented in Arlequin version 3.0 (Excoffier et al. 2005).

Bayesian clustering and hybrid identification

Maternal introgression was firstly detected by discordance between mtDNA haplotypes and morphological classification (i.e., *A. chukar* CR-IA haplotype and *A. rufa* morphology). Bayesian clustering procedure was performed in Structure version 2.1 (Pritchard et al. 2000; Falush et al. 2003) to infer the number of *K* unknown populations (genetic clusters) in which the dataset of multilocus genotypes could be subdivided. Analyses were performed with all the Italian samples and the reference red-legged and chukar partridges (^= 1—10). The procedure simultaneously estimates the allele frequencies at each locus in each population and probabilistically assigns the individuals to the population of origin or proportionally to more than one population if they are admixed. The "admixed model" and "/ model" (independent allele frequencies) were performed without any prior population information. Structure was run with four repetitions of $3.5xlO^5$ iterations following a burn-in period of $3.5xlO^4$ iterations. The most likely number of subpopulations *(K)* was assessed basing on *AK* statistic, as described in Evanno et al. (2005). We assessed the average proportion of membership *(Qj)* of the populations to the inferred clusters. Then, each individual was assigned to a cluster with a proportion of membership (<7,)>0.95 and 90 % credible intervals (90 %CI) falling within the range 0.9-1.0. If an individual did not fit this requirements, it was classified as putative hybrid. A more rigorous threshold with respect to Barilani et al. (2007b) was chosen to improve the accuracy of hybrid detections (Vaha and Primmer 2006). In so doing, we used Structure to estimate the posterior probability that each individual belonged to each species or that it had fractions of its genome from two parental species.

Structure was subsequently run to infer the genetic structure of populations in the province of

Alessandria. Besides
local partridges, genotypes of red-legged partridges from Spain and Portugal were also included in the analysis in order to get more clues on the presence of Iberian genomes. Independent allele frequencies and admixture model were run using 3.5x $10^5$ iterations after a burn-in period of 3.5x $10^4$. Analyses were performed with *K=l-l0* and four replicated runs were carried out for each value of *K.*

**Results**

Genetic variation in partridges and species identification

All samples were morphologically assigned to *A. rufa* based on border of the black gorget with black spotting, and flank feathers with only one black bar. The alignment of 176 sequences of mtDNA CR (467 nucleotides) showed 12 different haplotypes (Table 1) defined by 34 polymorphic sites and 26 informative sites, with a total number of 39 mutations and 3 indels. An unrooted median joining network (Fig. 2) was drawn using our dataset and the reference sequences. Two clusters were connected by a minimum of 24 mutations: the *A. rufa* group *(Ar;* clade A) with nine haplotypes, and the chukar group *(Ac;* clade B) with three haplotypes (GenBank ID: JN655844-JN655855).

The mean net genetic distance between the two clusters was 0.050 (±0.010 SD), whereas the mean genetic distance within both groups was 0.002 (±0.001 SD). Three haplotypes identified in this study did not show nucleotide identity with any sequence in Genbank. They are *Ar4*, *Ar6* haplotypes for *A. rufa*, and *Ac3* haplotype for chukar.

Mitochondrial identification fitted phenotype in all individuals from the BC and SM subpopulations, whereas mtDNA chukar haplotypes were detected in 34 partridges sampled along the river (CA, SS, and RV populations; Table 1, Fig. 1). *Ac2* was the most common chukar haplotype, found in all the introgressed subpopulations. Haplotype *Ad* was found in CA *(n=2)* and SS («=11), while *Ac3* was detected in only one individual from the SS population. The *Arl rufa* haplotype was widespread in all sampled groups, with a high frequency (0.6). Haplotypes *Ar2* and *Ar4* were found in the SS population, while *Ar6* and *Ar7* were only observed in the BC and SM populations respectively. Five haplotypes *(Ar2*, *Ar3*, *Ar7*, *Ar8*, and *Ar9)* showed nucleotide identity with *A. rufa* sequences from Iberian Peninsula and Balearic islands, whereas two *(Arl* and *Ar5)* corresponded to French-Italian haplotypes identified by Ferrero et al. (2011; Table 1).

The estimates of genetic diversity resulting from mtDNA control region sequencing and Bayesian admixture analyses of multilocus genotypes determined at eight micro satellite loci are shown in Table 2. The mitochondrial haplotype diversity *(h)* ranged from *h=0.49* to *h=0.70* (mean *h*=0.61), whereas nucleotide diversity values ranged from тт= 0.002 to 7T=0.023 (mean тт=0.017). Average number of pairwise differences between haplotypes was *k*= 1.003 in the BC subpopulation, *k*= 10.886 in partridges in the CA subpopulation, and £=7.882 in the entire sample (Table 2). All microsatellites analyzed in the five populations were polymorphic. The only exception was the locus MCW 118, which was monomorphic in the BC, CA, and RV populations. These loci showed a total of 39 alleles and an average of 4.9 alleles per locus. Allelic diversity ranged from$^{\wedge}_0$=2.7 (CA) to $^{\wedge}_O$=3.9 (SS). Allelic richness showed values between $A_c$= 1.7 *and* $A_c$*=2.2.* Partridges from the BC group exhibited the highest number of private alleles (m=3) in MCW225, MCW295, MCW323 loci, with frequencies of 0.054, 0.013, and 0.27, respectively. Only the RV group failed to show any unique alleles. Observed and expected heterozygosity were moderate, with similar values in the whole population (mean $i/_o$=0.34, $i/_e$=0.36). Heterozygosity was comparable among the five populations and range between $H_O$*=Q29* (CA) to 0.38 (SM). Significant departures from HWE were observed when the entire Italian sample was analyzed, suggesting a possible population sub structuring (Wahlund's effect; Wahlund 1928). All subpopulations were in HWE when the entire sample was split into the five geographical groups, with the exception of SS. Linkage disequilibrium was not observed between any pair of loci after Bonferroni correction.

AMOVA was computed in Arlequin and showed that 91.8 % of the total genetic variance in Italian partridges was significantly distributed within populations (p<0.001). Overall $F_{ST}$ from AMOVA was 0.076.

FCA plot (Fig. 3) of individual microsatellite genotypes showed a sharp separation of Alessandria partridges from *A. chukar* reference samples, with high _$F_{ST}$ values (0.56) between the two clusters (Italian *A. rufa* and *A. chukar* partridges). Italian specimens showed partial overlapping with the Iberian reference *A. rufa* cluster, although _$F_{ST}$ computation between the Iberian and Italian samples showed limited differentiation (mean _$F_{ST}$=0.09). Comparison between Italian populations revealed slightly lower values (i*sT=0.07), with BC and SS showing highly significant differentiation (p<0.001). SM, CA, and RV displayed lower significance (p=0.02). Individuals 137, 138, 143, 151, 152, and 206 plotted slightly towards the chukar group.

Hybrids identification

Structure analysis of all samples, including reference chukar and red-legged partridges, showed a most likely genetic subdivision with *K=2* (Fig. 4). *A. rufa* individuals were assigned to cluster I and chukar were

included in cluster II, both with *Qi=99 %.* We assumed that hypothesis of introgression cannot be discarded for individuals having values of ^<0.95 and/or 90 %CI outside the range 0.9-1.0. All specimens from Alessandria, except seven, were unequivocally attributed to cluster I, with individual $q_t$> 0.95 (indicating that over 95 % of their genome was assigned to *A. rufa)* and with 90 %CI values ranging between 0.9 and 1.00 as in all reference samples. Only two partridges from the SS group (nos. 151, 152) showed an individual proportion of membership below the threshold of 0.95 (0.91<^<0.95). These individuals also had large CI intervals, with 90 %CI values <0.90 (Table 3). Other five partridges (nos. 137, 138, 187, 153, andl60) having ^<0.95 but 90 %CI below the admitted range (0.9-1.0) have been identified in SS and SM.

Four out of the six putative admixed individuals (nos. 137, 138, 151, and 152), distinctly shown as outliers using the FCA, were identified as hybrids in structure. Fifteen specimens with chukar mtDNA haplotypes were not detected as hybrids by Bayesian analyses, whereas all partridges identified as admixed in Structure showed red-legged mtDNA. No individuals from the BC population showed discordant mtDNA or admixed genotypes, whereas maternal and/or nuclear introgressions were identified in the remaining groups (Table 4).

Bayesian clustering of Italian populations

Sub structuring of the Alessandria population was assessed by running Structure without reference chukar samples and no prior information on sampling locations (Fig. 5). The LnP(D) values increased sharply with *K* from 2 to 5 where they reached a plateau. *AK* (Evanno et al. 2005) computed for all *K* indicated a strong signal for *K=3* (Fig. 5). With *K*— 3, populations were differently associated with clusters, but low values were shown by individual $q_h$ indicating high admixture and elevated gene flow among populations

Iberian samples from Spain and Portugal were associated with cluster 2 with g/=0.698. BC was associated with cluster 1 with *Qj=0.76l.* SM, CA and SS were assigned to cluster 3 with g/=0.667, 0.670, and 0.794, respectively. RV was associated with cluster 1 with g/=0.572. No population was unequivocally associated with a single cluster. No individual of the CA, SS, and SM subpopulations was significantly assigned to cluster 1, with the exception of two individuals from Serravalle Montei which had $q_t$ values >80 %. Twenty-four individuals of BC were assigned to cluster 1 with $q_t$ values >80 %. Only six individuals of RV were significantly associated (^>0.80) with cluster 1 whereas 5 individuals were assigned to cluster 3 with $q_t$ value >70 %.

**Discussion**

Genetic admixture and introgression of "alien" genomes are main conservation concerns for many threatened species. Introduction of invasive species and translocation of captive-breed stocks or artificial hybrids may lead to loss of local adaptations contributing to drop of fitness and decline in hybridizing native populations (Randi 2008). These phenomena appear pronounced in overhunted game species. As concerns the red-legged partridge, wild populations are constantly supplemented by commercial stocks of captive-bred individuals (often using *A. chukar* or *A. rufa* x *A. chukar* hybrids) aimed to contrast their numerical decline (Bernard-Laurent et al. 2001; Barilani et al. 2007a).

Recent molecular studies on nuclear and/or maternal markers show a significant incidence of chukar genes in most of the *A. rufa* populations investigated so far. Chukar introgression *(A. rufa* x *A. chukar* hybridization) was detected in 45 % of wild populations in the Iberian Peninsula (Blanco-Aguiar et al. 2008), and in 17.2 % in Majorca Island (Tejedor et al. 2007). Introgression *of A. chukar* nuclear or mitochondrial DNA have been also described in 30 % *of A. rufa* populations investigated in southern France (Vallance et al. 2006), in 28 % of natural red-legged x rock partridge hybrids in the French Alpes-Maritimes (Barilani et al. 2007a), and in Corsican *A. rufa* populations with frequencies up to 67 % (Barbanera et al. 2010). Despite their endangered status, the genetic make-up of residual red-legged partridge populations in Italy remains partially unknown. Actually, genetic investigation of *A. rufa* has focused on reintroduced/restocked populations in the islands of the Tuscan Archipelago, and on captive partridges from three Tuscan farms (Baratti et al. 2004; Barbanera et al. 2005, 2009; Guerrini and Barbanera 2009). However, genetic data on wild mainland populations are still needed. To that extent, lack of viable self-sustaining populations for reintroduction/restocking programs is a critical issue for the conservation of this species in Italy. It is well-known that captive-born birds are prone to difficulties in acclimatization due to adverse genetic and behavioral factors and that they are susceptible to health issues. These are reflected in a shorter survival compared to wild individuals, mainly through easy predation (Meriggi and Mazzoni della Stella 2004; Perez et al. 2004, 2010; Alonso et al. 2005; Casas et al. 2012).

The present study provides insights into the genetic structure of self-sustaining and expanding red-legged partridges living in two areas of NW Italy (Fig. 1), where restocking or introduction operations

have never been documented. The mtDNA and micro satellite markers agreed in excluding recent signs of introgressive hybridization with exotic partridges in the BC subpopulation. Conversely, we detected mitochondrial and/or nuclear introgression in the four subpopulations living along the river and the surrounding hill, although all individuals were morphologically assigned to *A. rufa.* In these cases, we detected introgression frequencies, ranging from 17.6 to 32.2 % for mtDNA and from 16.7 to 21.4 % for micro satellite markers (Table 4).

Our results highlight the importance of an integrated approach based on the use of mitochondrial and biparental

nuclear data in the identification of interspecific hybridization events and gene flow between domesticated and wild animals. However, our data also pointed out the limits of both markers. For instance, if maternal DNA analysis was missing, no evidence of the presence of the chukar genome would have appeared in the CA and RV groups. On the other hand, only microsatellite markers revealed the occurrence of hybrids in the SM population. The benefits and limits of DNA-based markers with uniparental modes of inheritance (e.g., maternally inherited mtDNA) in the detection of introgressive hybridization are well-known (Latch et al. 2006). As regards the assignment of hybrid samples or admixed populations, the potential of Bayesian statistical analyses of multilocus genotypes is difficultly definable, especially when a limited number of markers is used (Vaha and Primmer 2006). A literature survey revealed that the number of microsatellite loci employed in population genetics studies is often lower than ten (Koskinen et al. 2004). Previous Bayesian analysis of empirical and simulated data showed that eight unlinked microsatellite markers can

allow the 100 % correct identification of simulated Fl and F2 hybrid partridges if the parental populations markedly diverge (^x>0.50) (Barilani etal. 2007a). However, the same authors highlighted that these loci lead to approximately 10 % underestimation of the proportion of first backcrosses. As suggested by Randi (2008), the use of a too high number of micro-satellites (at least 50-100) or the application of additional hypervariable markers such as SNPs are required to detect admixed ancestries whereas parental allele frequencies are not fixed or unknown (Morin et al. 2004; Sevane et al. 2009). Uncertainty in the assignment of admixed individuals can be reduced by defining strict threshold values of the individual proportion of membership $(q_t)$ and their 90 % credible intervals (90 %CI), which are expectedly larger in admixed individuals (Randi 2008). In order to improve the accuracy of hybrid detection, we adopted a more stringent $q_t$ threshold value (>95 %) with respect to other studies (Randi et al. 2003; Baratti et al. 2004; Barilani et al. 2007a, b) and a 90 % CI within the range 0.9-1.0. The Bayesian clustering procedure identified seven putative introgressed individuals (6.5 % of the total sample) in comparison to 34 cases of maternal introgression (19.3 % of the total sample) directly found by discordant mtDNA and morphological traits. As previously reported by Barilani et al. (2007b), the low levels of nuclear introgression (all specimens showed ^>90 %) and the presence of chukar haplotypes suggest that the admixed individuals could have had a hybrid ancestry with exotic partridges over the second past generation. In the BC subpopulation, both markers suggest the exclusion of recent admixture events with the chukar genome. However, historical admixtures cannot be excluded. In a natural red-legged x rock partridge hybrid zone in the southern French Alps (Alpes Maritimes), the diffusion of hybrids was found to be constrained by natural selection as a consequence of differential selective and/or competitive pressures among hybrids and their parental populations (Randi and Bernard-Laurent 1999; Barilani et al. 2007a). In the same study area, Barilani et al. (2007a) observed a similar introgression cline also into hybrid rock partridge populations showing chukar genome.

The BC area could represent an "extreme" and unsuitable habitat for hybrids, considering that individuals used to restock neighboring areas are for "put-and-take" partridge hunting (Byers and Burger 1979), with low chances of survival into the wild in the long term. However, in a wild population located in central Spain, Casas et al. (2012) found that *A. rufa* x *A. chukar* hybrids may breed as well as or even better than "pure" red-legged partridges although their feeding behaviors result in a lower survival rate. Thus, the lower survival rate of hybrids could be compensated by their higher ability to hatch. Nevertheless, in the same report the authors do not exclude that the persistence in the wild of hybrid genomes could be due to repeated releases of farmed hybrids conducted over many years and that the observed phenomena might be due, in part, to domestication effects rather than effects associated with the introgression of chukar genes. Conversely, in our case BC area is not subjected to repeated restocking for hunting purpose although the occurrence of occasional contacts with captive-bred individuals released in surrounding zones cannot be excluded. The presence of Iberian haplotypes in BC population seems to corroborate the latter hypothesis. In addition, both Alessandria wild populations are expanding, even if the last authorized release of farmed birds in nearby areas dates back to 2003 and management strategies based on provision of drinking and feeding stations are not performed (Tizzani et al. 2012). Further investigations using additional biparentally

inherited nuclear markers, such as SNPs, will help to identify possible remnants of isolated episodes of genetic contamination occurring over longer time scale.
In the river basin and surrounding hill, we found wild partridge subpopulations, in which both nuclear and maternal introgressive hybridization were detected. Historical surveys and recent field data seem to confirm that these partridges are from natural populations living in neighboring hills of built-up areas of Arquata Scrivia and Serravalle Scrivia, which were restocked from the 1980s (Spano et al. 1987) until 2003.
The recaptures of previously marked partridges performed in 2009-2011 showed high dispersion movements. The observed colonization occurred mostly along the perifluvial areas (unpublished data). These hybrid partridges are able to survive in wild, taking advantage of perifluvial area that can be considered an ecological corridor. It should be noted that riverside areas represent atypical habitats for native red-legged partridge populations (Spano 2010). In fact, the presence of A.*rufa* in Italian river habitats was never reported before 2004 and no introductions have been carried out in these areas as they are considered unsuitable for the species. Investigations on hybrid zones suggested that admixed individuals can show higher fitness in novel or previously underexploited habitats (Burke and Arnold 2001; Seehausen 2004). Baratti et al. (2004) evoked hybrid superiority to partially explain the successful adaptation of a red-legged partridge population affected by chukar introgression in a xeric environment of Pianosa Island (Tuscany), which is not particularly suitable for .4. *rufa.* Other factors, such as reduced predator pressure, poaching control, and a strict ban on hunting have probably favored the rapid demographic expansion of admixed partridges along the river basin investigated in our study. However, it is important to note that all Italian reintroduction plans performed with farm-reared birds have failed in absence of repeated restocking, also in strictly managed protected areas (Barbanera et al. 2010).
We found comparable levels of genetic variation among the five populations as previously reported in other natural populations of the genus *Alectoris* (Randi et al. 2003; Baratti et al. 2004; Barilani et al. 2007a, b). Significant departure from HWE was observed only in the SS subpopulation and when the whole sample was considered, suggesting the occurrence of true subpopulations (Wahlund's effect, Wahlund 1928). Conversely, both the AMOVA showing that up to 91.8 % of the total genetic variability was distributed within populations and the Bayesian analysis differentiated the Iberian from the Italian populations and identified two main clusters in the latter. The geographical separation and the apparent lack of a contact zone between the two areas could limit the admixture events and the present-day gene flow. Thus, the low levels of genetic structuring might be due to the persistence, in introgressed subpopulations, of wild genomes inherited from local ancestors in common with individuals living in the BC area. In fact, although we detected the presence of Iberian haplotypes in all sampled populations, the French-Italian haplotype *Ar*\ was the most common in both study areas.
Conversely, we cannot completely exclude the existence of gene flow between subpopulations, and that the substructure which we managed to find is a present-day view only. In the absence of geographical or ecological barriers hampering the natural gene flow between populations, the hypothesis that the two gene pools will homogenize in the future cannot be entirely rejected. Further suggestions include the existence of a biased gene flow from BC sub-population to outwards that have molded the actual genetic structure and determined the absence of both exotic introgressed genes or hybrids. Additional studies on these populations will permit to clarify. a metapopulation approach (VV.AA. 2007). According to Allendorf et al. (2001), a non-introgressed natural population requires further attention and management to favor its maintenance and possible diffusion into other ecologically suitable areas.
Thus, releases of farmed partridges should be strictly avoided in the study area and in the neighboring zones. We rather suggest a management policy based on habitat improvement, e.g., provision of drinking and feeding stations coupled to amelioration of threatening factors (mainly predator and poaching control). With regard to the individuals inhabiting the river area, we suggest that their translocation in other areas should be avoided. In a context of the widespread genetic pollution and in absence of stable wild stocks, it can be reasonable to make efforts to manage population with low levels of introgression but able to self-sustaining in the wild. Moreover, eradication plans of hybrids could be useless in scenarios of widespread introgressive hybridization, as in A *rufa.* Therefore we suggest a program to monitor genetic and ecological changes and to manage these populations as part of hunting and action plans.

**Conclusions**

In Italy, many native *Alectoris* populations are affected by genetic pollution due to introgressive hybridization with exotic taxa (Barbanera et al. 2009) and are mostly unable to survive in the wild over long time, in absence of repeated releases of farmed birds. Nowadays, highly suitable habitat areas are

still available (Meriggi and Mazzoni della Stella 2004). However, wild useful stocks for restocking or re-introduction plans as valuable alternative to farmed birds are lacking. We believe that partridges from Brignano-Casasco can be considered of primary conservation interest. To our knowledge, this is the first genetic investigation suggesting the existence of a self-sustaining red-legged population without signs of recent introgression of exotic chukar genes, presently living in Italy. Because of this peculiarity, further studies on the BC population are warranted to fully comprehend its origin (native or not) and demography, in order to verify its suitability as a possible source for restocking and reintroduction plans. Urgent conservation actions should be promoted to preserve the genetic integrity of this population from the risk of hybridization with captive-reared birds and to support its demographic expansion using.

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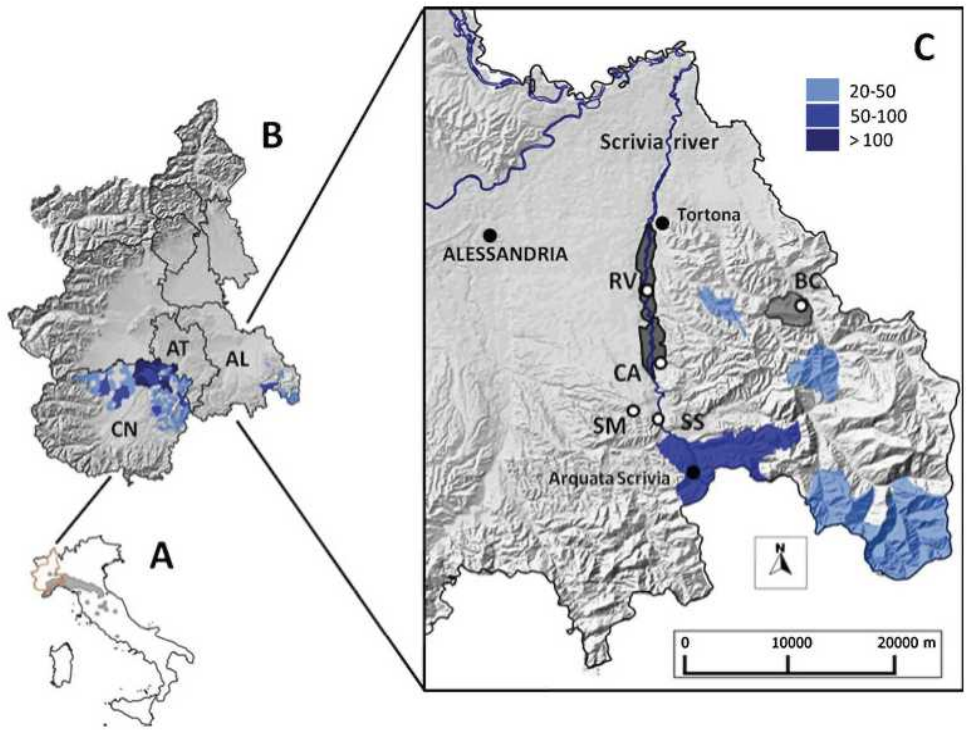


Fig. 1 Maps showing *a* the distribution range *(shaded)* of *A. rufa* in Italy, *b* provinces and districts of Piedmont (NW Italy) i captive farm-bred birds were released between 2000 and 2005 (in *blue;* average number of individuals/years), and *c* the locations *(white dots)* of red-legged partridge specimens in Alessandria province and boundary of Sites of Community Int *(dark gray). CN* Cuneo province, *AT* Asti province, *AL* Alessandria province, *RK* Rivalta Scrivia, *CA* Cassano Spinola, *SS* Serra *SM* Serravalle Montei, *BC* Brignano-Casasco.

**Table 1** Distribution of the mtDNA CR haplotypes (467 base pair) in the sampled populations: *BC* Brignano-Casasco, *SM* Serravalle Montei, *SS* Serravalle Scrivia, *CA* Cassano Spinola, _RKRivalta Scrivia

| Haplotype | Bc | SM | SS | CA | RV | Frequency (%) | *A. rufa* references (Ferrero et al. 2011) |
|---|---|---|---|---|---|---|---|
| Ar1 | 28 | 10 | 41 | 15 | 12 | 60.2 | AJ586225 (French–Italian hapylotype) |
| Ar2 | | | 1 | | | 0.6 | HQ621761 (Northwestern Spain hapylotype) |
| Ar3 | 10 | | | 4 | 1 | 8.5 | HQ621775 (Baleriac hapylotype) |
| Ar4 | | | 1 | | | 0.6 | AJ586225 (French–Italian hapylotype) |
| Ar5 | | | 4 | 2 | 1 | 3.9 | AJ586226 (French–Italian hapylotype) |
| Ar6 | 1 | | | | | 0.6 | |
| Ar7 | | 1 | | | | 0.6 | AMB50842 (Southwestern Spain hapylotype) |
| Ar8 | 7 | 1 | | | | 4.5 | HQ621773 (Central–eastern Spain hapylotype) |
| Ar9 | | 2 | | | | 1.1 | HQ621797 (Central–eastern Spain hapylotype) |
| Ac1 | | | 11 | 2 | | 7.4 | |
| Ac2 | | | 9 | 8 | 3 | 11.4 | |
| Ac3 | | | 1 | | | 0.6 | |
| Total | 48 | | 68 | 31 | 17 | 176 | |

The relative observed frequency is given (in percentage). The correspondence with haplotypes (319 base pair) from Ferrero et al. (2011) is reported in the last column. GenBank accession number of sequences is reported

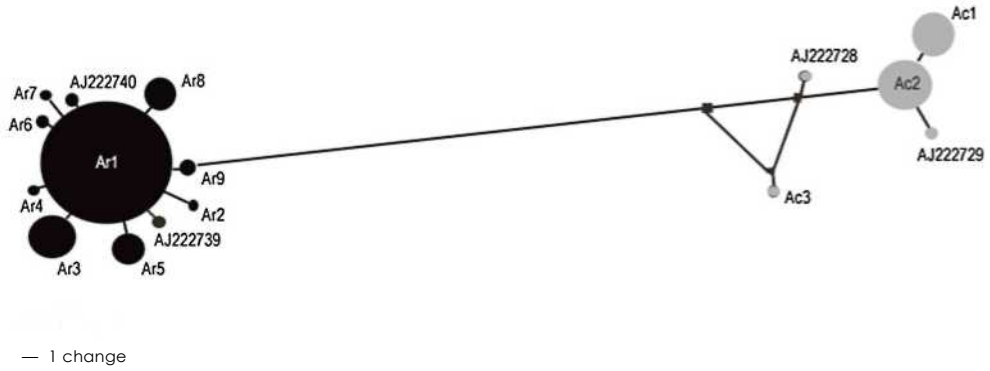


**Fig.** 2 Median joining network connecting the mtDNA haplotypes obtained in this study *(Ar, A. rufa; Ac, A. chukar)* implemented with NETWORK 4.5.1.0. F'our *Alectoris* reference sequences were included: two *A. rufa* and two *A. chukar* (AJ222739-40, AJ222728-29 Lucchini and Randi 1998). *Circle size* is proportional to haplotype frequency in the province of Alessandria, *while line length* is proportional to the number of mutations between the two connected haplotypes. For reference, haplotypes *Arl* and *Ac2* are separated by 23 mutations.

**Table 2** Summary of gene diversity at mtDNA control region sequences and at eight micro satellite loci (STR) in the red legged partridge populations from Alessandria (NW Italy).

| | mtDNA | | | | Microsatellite (STR) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Population | $(n_i/n_h)$ | $h$ | $\pi$ | $k$ | $n_i$ | $A_o$ | $A_c$ | $n_a$ | $H_o$ | $H_e$ | $F_{is}$ |
| BC | 46/4 | 0.571 (0.063) | 0.002 (0.0003) | 1.003 | 38 | 3.7 | 2.2 | 3 | 0.36 (0.09) | 0.38 (0.11) | 0.058 |
| SM | 14/4 | 0.495 (0.151) | 0.003 (0.001) | 1.319 | 14 | 3.4 | 1.9 | 2 | 0.38 (0.13) | 0.35 (0.10) | −0.019 |
| SS | 68/7 | 0.597 (0.058) | 0.023 (0.0023) | 10.543 | 24 | 3.9 | 2 | 2 | 0.35 (0.09) | 0.38 (0.09) | 0.099* |
| CA | 31/5 | 0.697 (0.062) | 0.023 (0.003) | 10.886 | 17 | 2.7 | 1.7 | 1 | 0.29 (0.09) | 0.30 (0.10) | 0.063 |
| RV | 17/4 | 0.493 (0.131) | 0.016 (0.006) | 7.39 | 14 | 2.9 | 2 | / | 0.33 (0.11) | 0.36 (0.10) | 0.118 |
| Total | 176/12 | 0.611 (0.039) | 0.017 (0.0018) | 7.882 | 107 | 4.9 | 2.2 | / | 0.34 (0.04) | 0.36 (0.04) | 0.128* |

$n_i$ number of genotyped individuals, $n_h$ number of observed mtDNA haplotypes, $h$ haplotype diversity, $\pi$ nucleotide diversity, $k$ average number of nucleotide differences, $A_o$ mean number of alleles per locus, $A_c$ allelic richness (rarefaction to 24 genes), $n_a$ number of unique alleles, $H_o$ observed heterozygosity, $H_e$ expected heterozygosity, $F_{is}$ fixation index

*$p<0.05$, significant departures from Hardy–Weinberg equilibrium; standard deviation was given in brackets

**Fig.** 3 Factorial correspondence analysis (computed using Genetix 4.05.2) showing relationships among the multilocus genotypes of individual Italian partridges and *A. rufa* and *A. chukar* reference samples. $F_{ST}$ the proportion of total genetic variation between samples from *Alessandria* and chukar individuals. *A. rufa* outliers are marked.

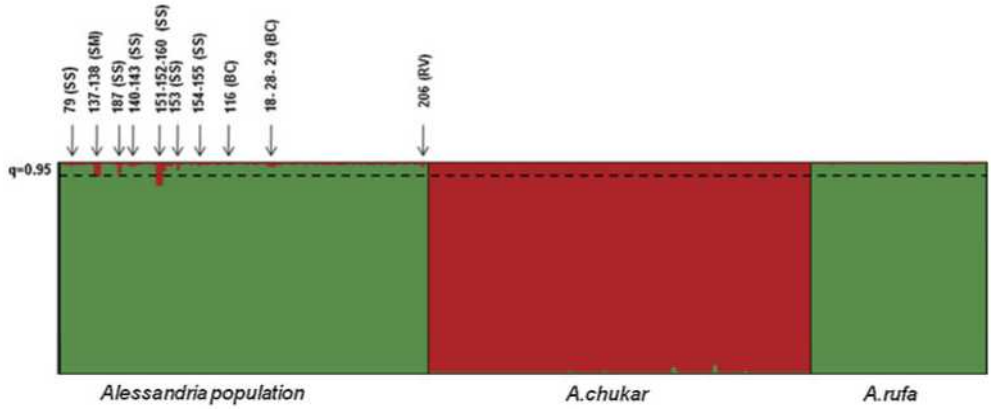


**Fig. 4** Bayesian admixture analyses: Individual proportion of membership *(q,)* of Italian partridges and *Alectoris (A. rufa* and reference samples, estimated using Structure 2.1 with *K=2.* Each individual is represented as a *vertical bar* partitioned into two clusters. Putative hybrids are marked.

**Table 3** List of red legged partridges which showed individual proportion of membership to the *A. rufa* cluster </,<0.95 or 90 % credible interval out of the range 0.90-1.00.

| Sample ID | Population | Cluster I *A. rufa* $Q_I$=0.99 | Cluster II *A. chukar* $Q_I$=0.99 | 90 %CI |
|---|---|---|---|---|
| 151 | Serravalle Scrivia (SS) | 0.916 | 0.084 | 0.695–1.000 |
| 152 | Serravalle Scrivia (SS) | 0.915 | 0.085 | 0.693–1.000 |
| 137 | Serravalle Montei (SM) | 0.956 | 0.044 | 0.747–1.000 |
| 138 | Serravalle Montei (SM) | 0.956 | 0.044 | 0.747–1.000 |
| 187 | Serravalle Montei (SM) | 0.962 | 0.038 | 0.805–1.000 |
| 153 | Serravalle Scrivia (SS) | 0.971 | 0.029 | 0.833–1.000 |
| 160 | Serravalle Scrivia (SS) | 0.977 | 0.023 | 0.863–1.000 |

**Table 4** Summary of detected introgression in *Alessandria* populations using nuclear and mitochondrial markers. The last column shows contrasting individual assignments detected with mtDNA sequences and structure analysis.

| Population | mtDNA | | Structure | | Contrasting assignment *N* individuals |
|---|---|---|---|---|---|
| | *n* | *A. chukar* | *n* | *A. rufa* × *A. chukar* | |
| Brignano Casasco (BC) | 46 | 0 | 38 | 0 | 0 |
| Cassano Spinola (CA) | 31 | 10 (32.2 %) | 17 | 0 | 5 |
| Rivalta Scrivia (RV) | 16 | 3 (17.6 %) | 14 | 0 | 3 |
| Serravalle Scrivia (SS) | 14 | 21 (30.9 %) | 24 | 4 (16.7 %) | 12 |
| Serravalle Montei (SM) | 87 | 0 | 14 | 3 (21.4 %) | 3 |

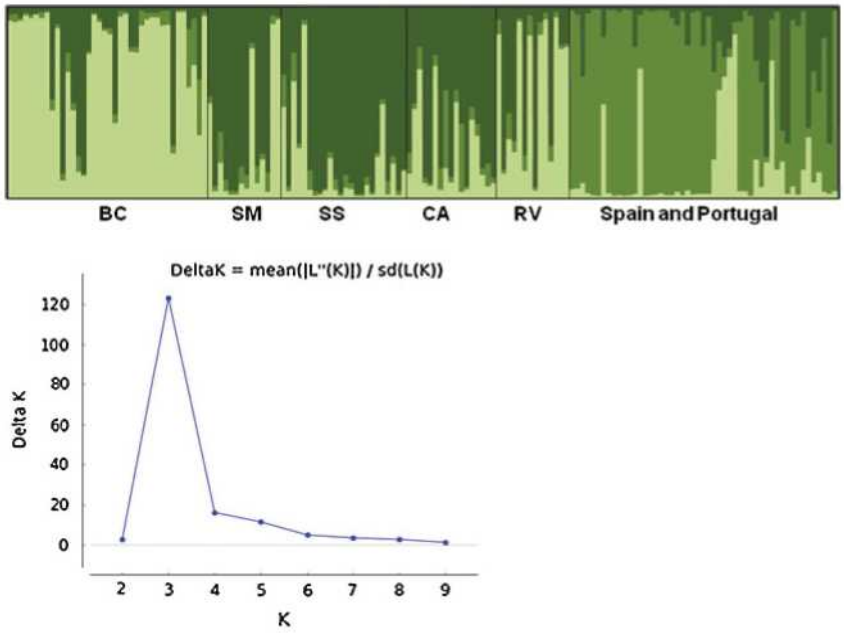


Fig 5 Individual membership of Italian and Iberian partridges to the *K=3* clusters inferred by Structure 2.1. The Delta-K plot (Evanno et al. 2005) is reported below.