Conserv Genet (2007) 8:343–354
DOI 10.1007/s10592-006-9174-1

ORIGINAL PAPER

Detecting introgressive hybridisation in rock partridge populations (*Alectoris graeca*) in Greece through Bayesian admixture analyses of multilocus genotypes

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Received: 10 January 2006 / Accepted: 22 May 2006 / Published online: 16 September 2006

Abstract The nominal subspecies of rock partridge (*Alectoris graeca graeca*) is widely distributed in Greece, where populations are declining due to overhunting and habitat changes. Captive-reared chukars (*A. chukar*) have been massively released throughout the country, raising fear that introgressive hybridisation might have disrupted local adaptations leading to further population declines. In this study we used mtDNA control-region sequences and Bayesian admixture analyses of multilocus genotypes determined at eight microsatellite loci, to assess the extent of introgressive hybridisation in 319 wild rock partridges collected in Greece. A neighbour-joining tree split the mtDNA haplotypes into three strongly supported clades, corresponding to rock, red-legged (*A. rufa*) and chukar partridges. We did not detect any case of maternal introgression. In contrast, admixture analyses of microsatellite genotypes identified from four to 28 putative hybrids (according to different assignment criteria), corresponding to 1.2–8.8% of the samples, which were widespread throughout all the country. Power and limits of admixture analyses were assessed using simulated hybrid genotypes, which revealed that a small number of markers can detect all first and second generation hybrids (F_1 and F_2), and up to 90% of the first generation backrossess. Thus, the true proportion of recently introgressed rock partridges in Greece might be ca. 20%. These findings indicate that introgressive hybridisation is widespread, suggesting that released captive-bred partridges have reproduced and hybridised in nature polluting the gene pool of wild rock partridge populations in Greece.

Keywords *Alectoris graeca* and *chukar* · Rock and chukar partridges · Introgressive hybridisation · mtDNA Control-region · Microsatellites · Bayesian admixture analysis

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Introduction

The evolutionary role of introgressive hybridisation in nature is controversial (Seehausen 2004). Research in stable tension zones often revealed various kinds of hybrid unfitness, which could drive hybrids to evolutionary dead ends, while, in other biological frameworks, hybridisation may have the potential to generate genetic diversity and boost novel adaptive radiations (Barton 2001). Less controversial, and mostly negative, are the consequences of anthropogenic hybridisation (Allendorf et al. 2001; Olden et al. 2004). Captive-bred animals, derived from native, alien or hybrid stocks, are often massively released in nature aiming to sustain exploited wild populations. The goals of restocking programmes are not frequently fulfilled. Restocking may fail due to ecological, ethological or genetic factors, which limit the survival of translocated animals in nature (Storfer 1999; Fischer and

Lindenmayer 2000). When translocations succeed, the breakdown of coadapted gene systems may led to loss of local adaptations and fitness decline in hybridising local populations (outbreeding depression; Templeton 1986; Edmands and Timmerman 2003). Risks of deep genetic pollution by introgressive hybridisation are threatening both endangered and common species (Rymer and Simberloff 1996; Allendorf et al. 2001). The negative impact of introgressive hybridisation on regional biodiversity, and the effects of outbreeding depression in restocked populations have been well documented in fish (Perry et al. 2002; Hallerman 2003), but threatens to local gene pools are alarmingly high also in game birds, as galliforms and waterfowl, which are restocked to contrast the decline of over-hunted populations (Randi et al. 2003; Mank et al. 2004).

Partridges of the genus *Alectoris* (Galliformes, Phasianidae) offer interesting cases to study the consequences of natural and anthropogenic hybridisation. The seven extant species of *Alectoris* are distributed allopatrically in Eurasia, China and Arabia, with the exception of two partially sympatric species in southern Arabia (Johnsgard 1988). Molecular studies suggest that the *Alectoris* speciated recently in allopatry and did not achieve reproductive isolation and ecological compatibility (Randi 1996; Randi and Lucchini 1998; Randi et al. 1998). The red-legged (*A. rufa*), rock (*A. graeca*) and chukar (*A. chukar*) partridges hybridise naturally in a few areas of parapatric contacts, e.g. between red-legged and rock partridges in the southern French Alps, and between chukar and rock partridges in the Rhodope Mountains, at the border between Bulgaria and Greece (Randi and Bernard-Laurent 1998). Introgression of red-legged allozyme and mtDNA markers was detected in Alpine rock partridge populations up to about 150 km far from the contact zone (Randi and Bernard-Laurent 1999). However, gene introgression across the Alps seems to be contrasted by natural selection against the hybrids, suggesting outbreeding depression (Randi and Bernard-Laurent 1998). Red-legged, rock and chukar partridges have been reproduced in captivity since the end of the 1950', and chukars or hybrids (mainly with chukars) have been massively released for hunting purposes in Europe (Bernard-Laurent et al. 2001). Field observations (Potts 1989; Duarte and Vargas 2004) and genetic data (Negro et al. 2001) reveal that released chukars or hybrids can mate with local wild populations, raising concerns about the conservation of *Alectoris* in Europe (Tucker and Heath 1994). However, the genetic structure of partridge populations is largely unknown, and the consequences of restocking operations are still undocumented.

Greece is the core distribution area of the nominate subspecies *A. graeca graeca* (Randi et al. 2003). Rock partridges are hunted throughout the country and chukars have been massively released for years to contrast the decline of wild populations (Handrinos and Akriotis 1997). Here, we report results of a study planned to detect the extent of introgressive hybridisation in rock partridges in Greece, and assess power and limits of a procedure (Bayesian admixture analyses of multilocus genotypes) that is being increasingly used to detect hybridisation in natural populations. Detecting introgression through hybridisation is problematic if the parental populations cannot be sampled (unlike in classical stable hybrid zones), or if released animals have low reproductive success and hybridisation is rare (Goodman et al. 1999). However, the use of hypervariable microsatellite markers and new statistical methods (Beaumont and Rannala 2004), have dramatically improved the assessment of cryptic population structure, admixture analyses and individual assignment testing, although the power of markers and models in empirical case-studies remains to be determined (Vähä and Primmer 2006). Here we used standard phylogenetic analyses of mtDNA control-region sequences, and Bayesian admixture analyses (STRUCTURE 2.1; Falush et al. 2003) of multilocus microsatellite genotypes, to assess the presence of chukar genes in rock partridges in Greece. We also used simulated genotypes to assess the power of microsatellite markers and admixture analyses to detect F_1, F_2 and first generation backcrosses. Estimating introgression rates is a first descriptive step, which might allow evaluating the survival rates and reproductive success of the released species, the risk of outbreeding depression in the local populations, and estimate costs/benefits of restocking programs.

Materials and methods

Sampling, genotyping and DNA analyses

In 1999–2004 we collected 319 samples from 23 locations across the distribution range of the rock partridge in Greece (Table 1; Fig. 1). Chukar partridges were collected from natural populations in the islands of Karpathos ($n = 8$) and Kos ($n = 7$). Released chukars were collected in Spilia ($n = 15$). We used also the following additional reference samples: red-legged partridges (*A. rufa*) collected from various locations in Portugal ($n = 18$) and south Spain ($n = 37$), chukar partridges collected from Israel ($n = 47$) and central China ($n = 41$). Tissue samples were individually stored

Table 1 Gene diversity at mitochondrial DNA control-region sequences (mtDNA) and eight microsatellite loci (STR) in rock partridge (*Alectoris graeca*) populations from Greece (see Fig. 1). Samples from Dirfi ($n = 4$; n. 22 in Fig. 1) and Nafpaktia ($n = 1$; n. 23 in Fig. 1) were not used in these analyses. h = mtDNA haplotype diversity; H_0 = STR observed heterozygosity; H_e = STR unbiased expected heterozygosity; [a]numbers in parenthesis indicate the sampling locations mapped in Fig. 1

Population group[a]	Sample size	Region	mtDNA h	STR H_0	STR H_e
1—Drama (1)	12	Eastern Macedonia	0.44	0.37	0.37
2—Kavala (2)	8	Eastern Macedonia	0.67	0.30	0.33
3—Serres (3)	9	Eastern Macedonia	0.39	0.34	0.31
4—Taigetos (4), Ziria (5), Napflio (6)	11	Peloponnese	0.65	0.26	0.23
5—Mainalo (7)	17	Peloponnese	0.84	0.27	0.20
6—Spilia (8)	59	Spilia	0.23	0.34	0.34
7—Olympos (9), Elassona (10)	14	Olympos	0.74	0.35	0.37
8—Koziakas (11), Tzoumerka (12), Olitsika (13)	28	Pindos	0.63	0.34	0.35
9—Kalabaka (14), Peristeri (15), Mitsikeli (16)	11	Pindos (part)	0.49	0.39	0.37
10—Desi (17)	108	Pindos	0.52	0.34	0.32
11—Agrafa (18)	16	Pindos	0.64	0.32	0.30
12—Grammos (19), Nemertsika (20), Filiates (21)	16	Grammos	0.61	0.36	0.36

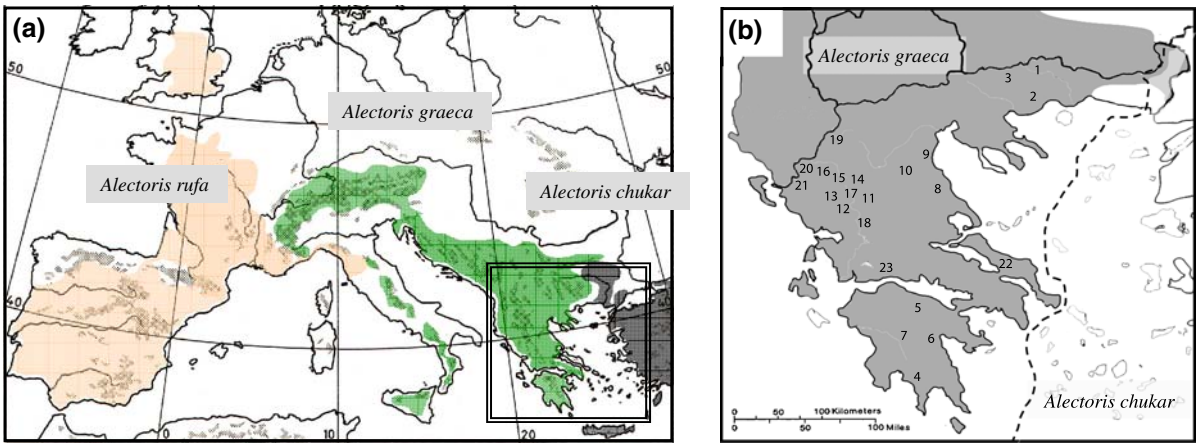

Fig. 1 Approximate distribution ranges of red-legged (*Alectoris rufa*), rock (*A. graeca*) and chukar (*A. chukar*) partridges in Europe (**a**), and sampling locations of the studied rock partridge populations in Greece (**b**)

at – 20°C in 95% ethanol. Total DNA was extracted using guanidine thiocyanate (Gerloff et al. 1995).

The 5′ half of the mitochondrial DNA control-region (mtDNA CR) was PCR-amplified using the external primer PHDL ($tRNA^{Glu}$; 5′-AGGACTACG GCTTGAAAAGC-3′) and the internal primer PH-H521 (5′-TTATGTGCTTGACCGAGGAACCAG-3′), and sequenced using primer PHDL (Randi and Lucchini 1998). All samples were also genotyped at eight microsatellites originally isolated at the Wageningen University (http://www.zod.wau.nl/abg/index.html) from the chicken (*Gallus gallus*) genome: MCW118 (PCR annealing temperature $T_a = 55°C$), MCW135 ($T_a = 55°C$), MCW152 ($T_a = 50°C$), MCW 225 ($T_a = 45°C$), MCW276 ($T_a = 60°C$), MCW280 ($T_a = 55°C$), MCW295 ($T_a = 50°C$), MCW323 ($T_a = 50°C$). PCRs were done using the following thermal cycle: $(94° \times 2′) + [(94° \times 30″) + (T_a \times 30″) + (72° \times 30″)] \times$ 40 cycles + $(72° \times 2′)$. The amplicons were analysed using an ABI 3100 automated sequencer and programs GENESCAN 3.7 and GENOTYPER 2.1. Details of laboratory protocols are available upon request.

A neighbour-joining tree (NJ; Saitou and Nei 1987), clustering pair wise Tamura-Nei's (TN93; 1993) genetic distances between the aligned individual mtDNA CR haplotypes, was constructed using MEGA 3.0 (Kumar et al. 2004). Support for the internodes in NJ tree was assessed by bootstrap percentages (BP; Felsenstein 1985) after 1,000 resampling steps. We used also other distance methods (i.e., genetic distances computed using β-distributions of variable sites), and a Bayesian procedure (MRBAYES 3.04; Huelsenbeck and Ronquist 2001), which produced results very similar to the NJ tree. Smaller sample sizes in population genetic analyses were avoided by pooling samples collected

from geographically close locations in 12 distinct groups (Table 1). ARLEQUIN 2.0b8 (Schneider et al. 2002) was used to estimate average and population mtDNA haplotype diversity (h), mean number of pair wise differences (k), nucleotide diversity (π). The partition of mtDNA and microsatellite genetic diversity within and among populations was analysed by AMOVA (Excoffier et al. 1992), using ϕ analogues of Wright's (1965) F-statistics. Summary population genetic statistics (microsatellite allelic frequencies, heterozygosity and deviations from Hardy–Weinberg equilibrium), and a Factorial Component Analysis (FCA), were computed using GENETIX 4.03 (Belkir et al. 2001).

Species identification and admixture analyses

All partridges were classified using diagnostic morphologic traits, before genetic analyses. Genetic identifications were based first on the structure of the mtDNA tree. Maternal hybridisation was assessed searching for mtDNA haplotypes discordant with morphologic classifications. Then, species distinction and individual ancestry of each microsatellite genotype was inferred using a Bayesian genetic model implemented in STRUCTURE 2.1 (Falush et al. 2003). This method was designed to identify the K (unknown) populations of origin of the samples, and simultaneously assign the individuals to the populations (clusters). Population structure is detected by departures from Hardy–Weinberg (HWE) and linkage equilibrium (LE), which could result by recent admixture, migration or hybridisation. The total sample is subdivided into K populations, which maximize HWE and LE. Simultaneously, individuals are assigned probabilistically to one (the population of origin), or more than one population (the parental populations) if their genotypes indicate that they are admixed. In this study we performed STRUCTURE using reference red-legged and chukar partridges, and all rock partridges sampled in Greece, the ''admixture model'' and the ''I-model'' (independent allele frequencies), without using any prior population information. All simulations were replicated four times; each one was run with 10^5 iterations, following a burn-in period of 10^4 iterations. We selected the optimal K values using the formula [Ln $P(D)_k$ – Ln $P(D)_{k\text{-}1}$] (Garnier et al. 2004), where Ln $P(D)$ is the estimated posterior probability of the data conditional to K. For each selected K value, we assessed the average coefficient of membership (Q) of each sampled population (chukar, red-legged and rock partridges) to the inferred clusters. Then, we assigned each genotype to the inferred clusters, based on threshold values of the individual proportion of membership (q_i) and its confidency interval (90%CI). Predictably, the threshold values will strongly affect both efficiency and accuracy of hybrid groups identifications (Vähä and Primmer 2006). Following empirical and simulation results (see: Results) we used an inclusive identification threshold assigning each individual to one cluster if q_i and 90%CI > 0.90 (parental individuals), or jointly to two clusters if the proportion of membership to each one was $q_i < 0.90$ (admixed individuals). In this way we used STRUCTURE to estimate the posterior probability for each individual to belong to one parental species, or to have fractions q_i of its genome originating from two parental species. STRUCTURE was also run with $K = 3$ and USEPOPINFO and POPFLAG options active flagging reference chukars and red-legged partridges, so asking the software to assign the rock partridges to the sampled, first or second past generation in one or more than one of the parental species. In this way we used a more stringent hybrid identification threshold, i.e., STRUCTURE run with USEPOPINFO and POPFLAG options active, $q_i < 0.90$ and no use of CI values.

Assessing the power of admixture analyses

The power of admixture analyses to detect F_1, F_2 hybrids and backcrosses, given the number of markers (eight microsatellites) and the level of genetic differentiation between parental populations (estimated by F_{ST}), was assessed by simulations. We randomly selected 50 parental chukars (from Greece and Israel), 50 red-legged partridges (from Portugal and Spain) and 50 rock partridges from Greece to generate 100 of each F_1, F_2 and backcross genotypes with the software HYBRIDLAB (Nielsen et al. 2001). Hybrid genotypes are created by random sampling alleles from their frequency distributions in the parental populations, assuming neutrality, linkage equilibrium and random mating. The parental genotypes were selected from populations located as far as possible from known area of hybridisation, and among individuals with $q_i > 0.90$ aiming to exclude recent hybrids. This procedure was replicated 10 times. The simulated genotypes were used to carry out admixture analyses with STRUCTURE 2.1 using the admixture and I models, and no prior population information. Results were evaluated aiming to assess the efficiency of admixture analyses, and compute the proportion of hybrid individuals that were correctly identified as hybrids in the simulated data set (Vähä and Primmer 2006).

Results

Genetic variation and species distinction in partridges

The mtDNA alignment (492 sequences of 430 nucleotides) showed 82 distinct haplotypes, which were split into three strongly supported clades by the NJ procedure, corresponding to *A. chukar* (40 haplotypes; bootstrap support BP = 97), *A. graeca* (20 haplotypes; BP = 99) and *A. rufa* (22 haplotypes; BP = 100; Fig. 2). The same three strongly supported clades were generated by NJ using other genetic distances, or by the MRBAYES procedure (not shown). The average TN93 genetic distance between red-legged and chukar or rock partridge haplotypes was $d = 6.2\%$, between rock and chukar partridges was $d = 5.3\%$. In contrast, the 20 rock partridge haplotypes sampled in Greece differed from 1 to 5 nucleotide substitutions only, the average sequence divergence being 0.6% (ranging from 0.2 to 0.9%), suggesting that these mtDNA lineages originated recently. There were 10 distinct haplotypes in the chukar partridges sampled in Greece, which joined the *A. chukar* clade (Fig. 2). There was no red-legged mtDNA haplotype among the partridges sampled in Greece. All samples from Greece that were phenotypically identified as rock or chukar partridges had rock or chukar mtDNA haplotypes, and we did not detect any individual with the mtDNA of the other species, thus ruling out maternal introgression.

Mitochondrial DNA diversity was moderately high in the 12 rock partridge groups, ranging from $h = 0.40$ to 0.80, with the exception of populations n. 6 (Spilia), which showed the lowest value of $h = 0.23$ (Table 1). The eight microsatellite loci were also moderately variable in rock partridges in Greece, showing an average of 5.5 alleles per locus, $H_O = 0.33$ and unbiased $H_E = 0.37$. Values of observed and expected heterozygosity were very similar in the total population and in the 12 groups, which were in HWE, except for population n. 5 (Mainalo) that showed a slightly significant excess of observed heterozygotes ($P = 0.05$). Heterozygosity was very similar among the 12 population groups (Table 1). The proportion of total genetic variation that is distributed among the 12 groups was $F_{ST} = 0.19$ (as estimated from mtDNA haplotype frequencies using AMOVA), or $F_{ST} = 0.31$ (as estimated from pair wise sequence difference), that is 2–3 times greater than interpopulation differences estimated using microsatellites ($F_{ST} = 0.09$). An FCA plotting of individual microsatellite genotypes, including *A. chukar* and *A. rufa* reference samples, showed that the three species plot in sharply distinct clusters separated by highly significant F_{ST} values ($F_{ST} = 0.53$, 0.55 and 0.60; Fig. 3). All chukars from Greece plotted within the *A. chukar* cluster. The rock partridges sampled in Greece plot in a tight cluster, although there were some slightly outlier individuals (evidenced in Fig. 3), which plot towards the chukar cluster.

Bayesian assessment of admixture and hybridisation

The ancestry of rock partridges sampled in Greece was assessed using STRUCTURE with reference chukar and red-legged partridges. The probability of the data increased sharply from a minimum with $K = 1$ to $K = 3$, thereafter reaching a plateau, and the maximum increase in Ln $P(D)$ was achieved with K from 2 to 3 (Fig. 4). Splitting the samples in three clusters allowed assigning the individuals to their species, being the chukars assigned to cluster I with average proportion of membership $Q_I = 99\%$, the rock partridges assigned to cluster II with $Q_{II} = 98\%$, and the red-legged partridges assigned to cluster III with $Q_{III} = 99\%$ (Table 2). However, while all chukar and red-legged partridges were assigned to their own cluster with individual $q_i > 0.90$ (indicating that >0.90% of their genomes was assigned to a single species), there were four red-legged partridges with $q_i < 0.90$, that is n. 926 sampled from population n. 2, Kavala; n. 940 and n. 943 from population n. 1, Drama; and n. 884 from population n. 13, Olitsika (Fig. 5). Individuals n. 926, 940 and 884 were identified as outliers also in the FCA plotting (Fig. 3). STRUCTURE run with USEPOPINFO and POPFLAG options active (the stringent procedure) identified two strongly admixed rock partridges: n. 926, showing $q_i = 0.78$ in the second past generation of the *A. chukar* population; and n. 940, showing $q_i = 0.72$ in the second past generation of *A. rufa*. In these analyses, none of the red-legged and chukar partridges showed significant admixture signals. Assuming that values of $q_i < 0.90$ indicate genetic admixture, there will be four over 319 rock partridges sampled in Greece (i.e., 1.2%) that showed signals of hybridisation.

Uncertain assignment of admixed samples

While most of the 90%CI values ranged between 0.90 and 1.00 in rock partridges, the four putative admixed samples (nos. 926, 940, 943 and 884) showed 90%CI larger than usual, ranging in the interval 0.56–0.98 (Table 2). There were 24 additional rock partridges showing wider CI ranges, with their lower 90%CI values <0.90 (Fig. 6a). The average coefficient of

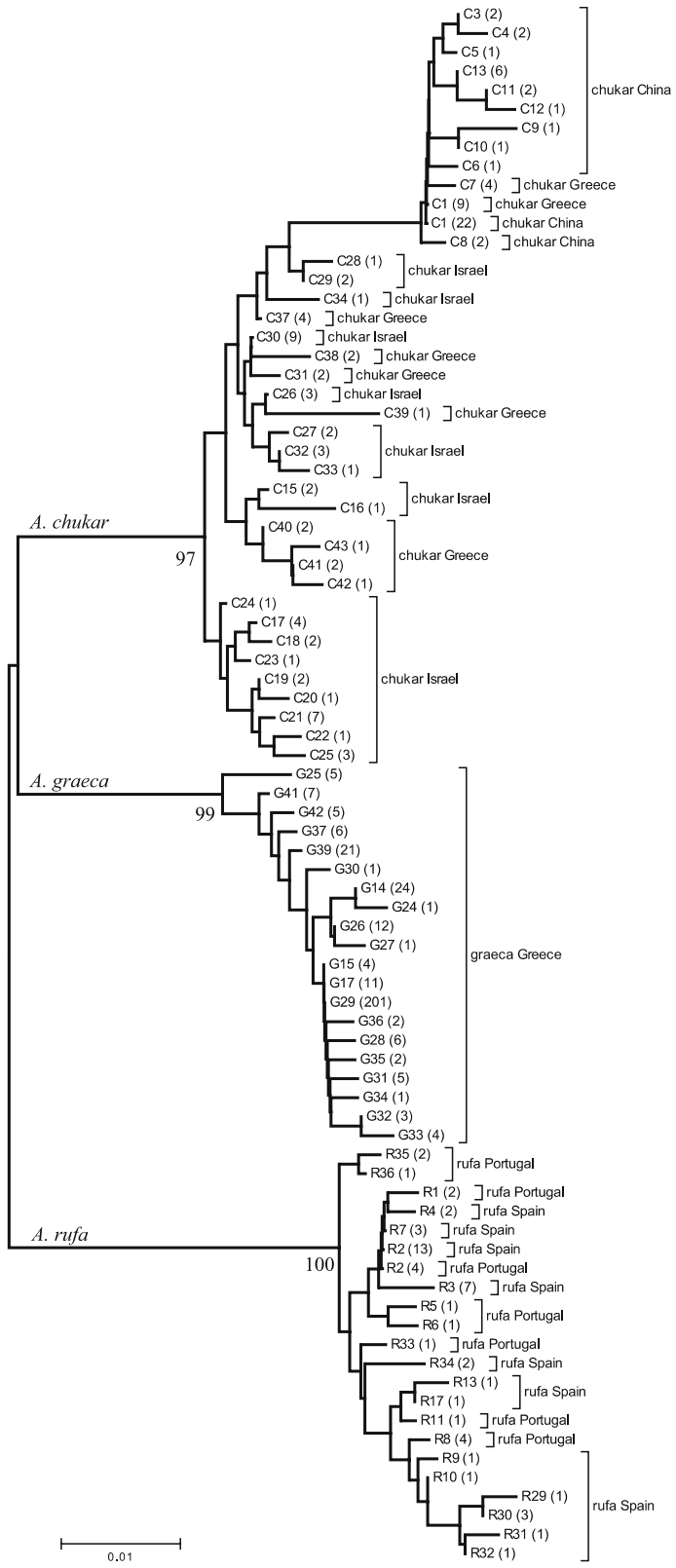

Fig. 2 Neighbour-joining tree computed by MEGA (Kumar et al. 2004) using Tamura-Nei (1993) genetic distances among the aligned mtDNA control region haplotypes of red-legged (*A. rufa*), rock (*A. graeca*) and chukar (*A. chukar*) partridges. The phylogenetic tree was mid-point rooted. Haplotype identifications and sampling regions are indicated. Haplotypes were aligned with nucleotide n. 1 of the complete mtDNA control region sequence of *Alectoris* (Randi and Lucchini 1998). These sequences are available at GenBank (accession numbers DQ 6794 74–75; DQ 6794 76–77; AJ 222 730–731)

membership of these samples to the *A. graeca* cluster was $q_i = 0.946$ (SD = 0.059), which is significantly lower (*t*-test, $P = 0.0002$) than the average coefficient of membership of all the other rock partridges ($q_i = 0.994$; SD = 0.002). All these samples had complete genotypes, identified at all the eight

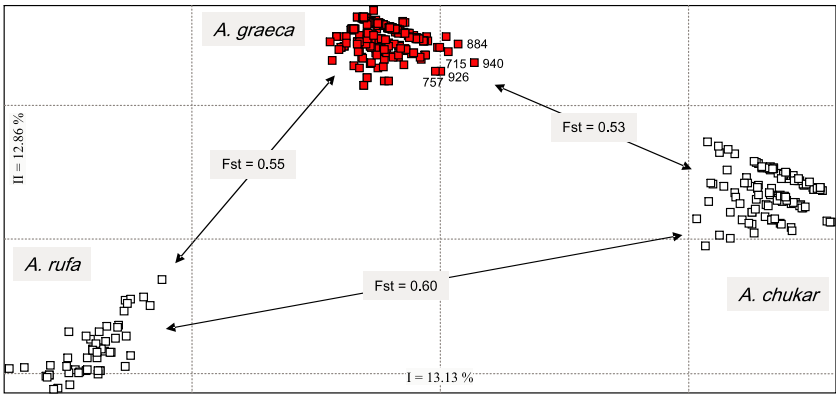

Fig. 3 Factorial Correspondence Analysis (computed using GENETIX; Belkhir et al. 2001) of individual microsatellite genotypes of rock partridges sampled in Greece (*A. graeca*), plotted with reference *A. rufa* and *A. chukar* samples. Outlier rock partridges are numbered: n. 940, sampled from population n. 1, Drama; n. 926 from population n. 2, Kavala; n. 715 from population n. 15, Desi; n. 757 from population n. 8, Spilia, and n. 884 from population n. 13, Olitsika (see Fig. 1, Table 1)

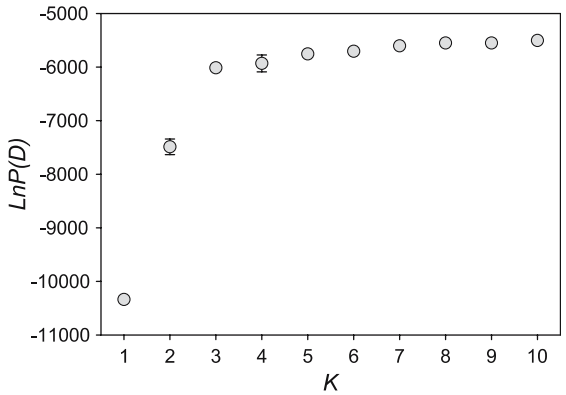

Fig. 4 Posterior probability of the data, Ln $P(D)$, against the number of K clusters computed using STRUCTURE for K = 1–10, in microsatellite genotypes of *A. graeca*, *A. rufa* and *A. chukar* samples

microsatellite loci, thus ruling out that missing data could have generated 90%CI wider than usual. It is well known that CI values may be wider in admixed genotypes, particularly if the true parental populations were not sampled (Pritchard et al. 2000), as in this case study. Thus, assuming that CI values <0.90 indicate genetic admixture (the inclusive procedure), a total of 28 over 319 rock partridges sampled in Greece (i.e., 8.8%) might show signals of hybridisation. However, although all samples from the parental species (that should have no admixed ancestry) had $q_i > 0.90$, there were four red-legged and 12 chukar partridges showing 90%CI < 0.90 (Fig. 6b, c). These wider CI values suggest that the assignment of some samples is uncertain, perhaps due to their less informative genotypes (Vähä and Primmer 2006). Assuming that reference red-legged and chukar samples were not admixed, uncertain assignments might be due to genetic heterogeneity in these samples, which are composed by red-legged partridges collected in Portugal and Spain, and by chukars collected in Israel and China.

Assessing the power of admixture analyses

A randomly selected subset of 50 chukars (from Greece and Israel), 50 red-legged partridges (from Portugal and Spain) and 50 rock partridges from Greece (excluding the 28 putative admixed individuals showing q_i and 90%CI < 0.90) was used to generate 100 of each of the following genotypes: F_1 *chukar* × *rufa*, F_1 *chukar* × *graeca*, two F_2 sets from each of the two F_1, two F_1 × parental *chukar* backcrosses (B_2 and B_4), and two F_1 × parental *graeca* (B_3) and *rufa* (B_1) backcrosses, with HYBRIDLAB (Nielsen et al. 2001). The simulated hybrid genotypes were used for admixture analyses with STRUCTURE ($K = 3$), without prior population information. At a threshold $q_i = 0.90$ STRUCTURE recognised very efficiently all the F_1 and F_2 genotypes (Fig. 7a): 100% of the simulated F_1 and F_2 were correctly identified as admixed (Fig. 7b, c). However, a proportion of 10% of the backcrosses showed $q_i > 0.90$ and could not be distinguished from their parentals (Fig. 7d). Thus, analyses of the simulated data set suggested that 100% of the F_1 and F_2 could have been identified in the empirical data set using eight microsatellites and STRUCTURE with $q_i > 0.90$, but that the true proportion of first generation backcrossed rock partridges could have been underestimated by ca. 10%. The 90%CI in all the simulated hybrids were very wide (0.20–0.80), suggesting that CI values may be wider in admixed genotypes also if the true parental populations have been sampled.

Table 2 List of the 28 rock partridges sampled in Greece which showed individual proportions of membership to cluster II (*A. greca*) smaller than 0.90, or that showed values of the 90% credibility intervals to cluster II (90%CI-II) larger than 0.90–1.00. Samples ID, sampling region (see Fig. 1), average (Q_I) and individual proportion of membership to cluster I (*A. chukar*), II (*A. greca*) and III (*A. rufa*), and the number of diagnostic alleles, are indicated

	Sample ID	Population	Cluster I *A. chukar* $Q_I = 0.99$	Cluster II *A. greca* $Q_{II} = 0.98$	Cluster III *A. rufa* $Q_{III} = 0.99$	90%CI-II	Diagnostic alleles
1	926	2—Kavala	0.207	0.787	0.007	0.565,0.952	2
2	940	1—Drama	0.026	0.835	0.002	0.566,0.966	0
3	884	13—Olitsika	0.163	0.835	0.002	0.639,0.970	2
4	943	1—Drama	0.139	0.858	0.003	0.676,0.982	2
5	740	8—Spilia	0.097	0.901	0.002	0.631,1.000	1
6	891	11—Koziakas	0.079	0.919	0.002	0.754,1.000	1
7	927	2—Kavala	0.072	0.925	0.003	0.761,1.000	2
8	719	8—Spilia	0.071	0.925	0.004	0.768,1.000	1
9	757	8—Spilia	0.049	0.948	0.003	0.737,1.000	2
10	706	8—Spilia	0.039	0.958	0.003	0.782,1.000	2
11	715	15—Desi	0.038	0.959	0.003	0.764,1.000	3
12	822	22—Dirfi	0.027	0.971	0.002	0.821,1.000	1
13	650	11—Koziakas	0.014	0.977	0.009	0.854,1.000	0
14	761	15—Desi	0.020	0.978	0.002	0.853,1.000	1
15	841	9—Olympos	0.007	0.980	0.013	0.866,1.000	1
16	854	10—Elassona	0.007	0.981	0.012	0.871,1.000	1
17	842	9—Olympos	0.007	0.981	0.013	0.867,1.000	1
18	849	9—Olympos	0.007	0.981	0.012	0.867,1.000	1
19	753	8—Spilia	0.014	0.982	0.004	0.883,1.000	0
20	919	3—Serres	0.002	0.983	0.015	0.891,1.000	0
21	923	8—Spilia	0.002	0.983	0.015	0.889,1.000	0
22	618	15—Desi	0.002	0.983	0.015	0.891,1.000	0
23	681	15—Desi	0.002	0.983	0.015	0.888,1.000	0
24	705	15—Desi	0.002	0.983	0.015	0.890,1.000	0
25	770	15—Desi	0.002	0.983	0.015	0.889,1.000	0
26	776	15—Desi	0.002	0.983	0.015	0.887,1.000	0
27	671	15—Desi	0.002	0.983	0.014	0.892,1.000	0
28	629	8—Spilia	0.012	0.983	0.005	0.886,1.000	0

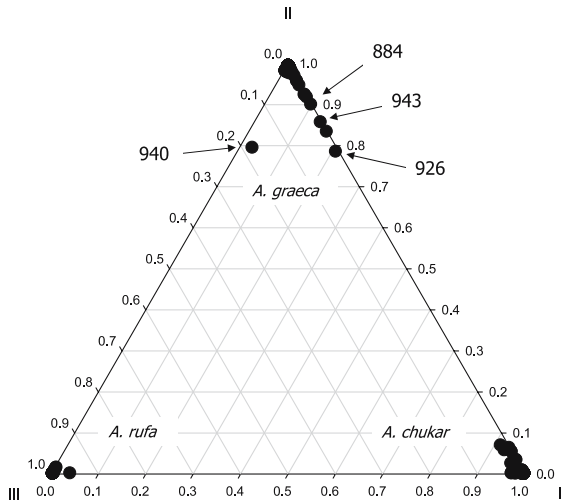

Fig. 5 Ternary plot of individual proportions of membership computed by STRUCTURE with $K = 3$, the admixture and *I* models, and eight microsatellite loci in *A. graeca*, *A. rufa* and *A. chukar* samples. Samples plotting at the apices have proportion of membership close to $q_i = 1.0$ to cluster I (*A. chukar*), II (*A. graeca*) or III (*A. rufa*). Admixed Greek rock partridges (at threshold $q_i = 0.90$) are labelled

Discussion

Power and limits of admixture analyses carried out using a small number of marker loci

In this study we performed admixture analyses of empirical and simulated data sets (multilocus microsatellite genotypes) to assess the extent of introgressive hybridisation in rock partridges sampled in Greece from areas where captive-bred chukar or red-legged partridges have been massively released. The mtDNA and microsatellite markers concordantly allowed us to identify chukar, rock and red-legged partridge individuals without any ambiguity, using only genetic information. The mtDNA results did not reveal any case of maternal introgression in rock partridges. In contrast Bayesian admixture analyses of the microsatellite genotypes suggested that at least 8.8% of the rock partridges sampled in Greece could have hybrid ancestry with chukar or red-legged partridges. Using a stringent hybrid identification procedure (STRUCTURE run

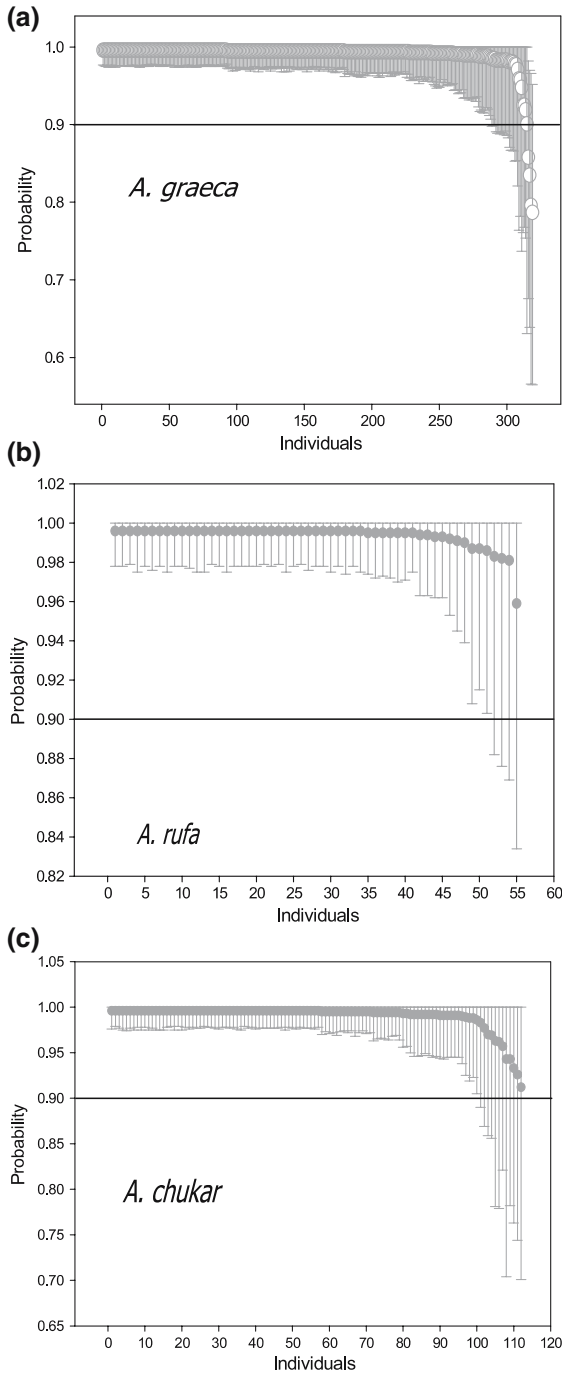

Fig. 6 Distributions of the q_i (dots) and 90%CI (bars) values in *A. graeca*, *A. rufa* and *A. chukar* samples computed by STRUCTURE with $K = 3$, the admixture and *I* models, and eight microsatellite loci

with USEPOPINFO and POPFLAG options active, $q_i < 0.90$ and no use of CI values), we identified only two strongly admixed rock partridges (i.e., 0.6% of the total sample). Using a more inclusive procedure (STRUCTURE run without prior population information, q_i and 90% *CI* < 0.90), we identified 28 putative hybrids over 319 rock partridges sampled in Greece. Simulation analyses showed that there could be 10% undetected backcrosses, corresponding to an additional 29 admixed samples, for a total of ca. 20% recently introgressed rock partridges in Greece. Appropriate threshold values could be defined through analyses of empirical results of experimental crosses (but we are not aware of any study of this kind published so far), or by simulating hybrid genotypes (i.e., Nielsen et al. 2001; Vilà et al. 2003; Vähä and Primmer 2006).

In this research we used only eight microsatellites, originally isolated from the chicken genome, and that, perhaps for this reason, are moderately variable in partridges. The number of microsatellite loci normally used in published population genetic studies is less than 10 (Koskinen et al. 2004). Small numbers of markers have been efficiently used to split species, or cluster genetically distinct populations, also if F_{ST} values are moderate (Falush et al. 2003), but might have limited resolution power in the assessment of admixed ancestries (Vähä and Primmer 2006). A small number of fixed genetic markers between parental species are sufficient to identify F_1, F_2 and first generation backcrosses (Boecklen and Howard 1997). However, detecting past generation hybrids when parental allele frequencies are not fixed, or unknown, require the use of many more markers. For instance, Rosemberg et al. (2003) suggested that at least 50–100 microsatellites might be necessary to estimate admixed ancestries.

Bayesian analyses of empirical and simulated data in this study lend support to a simulation study recently published by Vähä and Primmer (2006), which showed that efficient detection of first generation hybrids could be achieved with 12–24 loci and pair wise F_{ST} = 0.21–0.12. The detection of backcrosses would, however, need at least 48 loci. In this study we generated simulated hybrids starting from real parental genotypes, which were randomly sampled from a set of *A. rufa* and *A. chukar* collected as far as possible from known areas of hybridisation, and that did not show any obvious signal of admixture. The data sets analysed here included parentals and hybrids in fixed proportions, an approach supported by Vähä and Primmer's results, which showed that the efficiency of hybrid identification was not correlated to the proportion of hybrids in the samples. The number of alleles per locus (average = 5.5), and heterozygosity (H_O = 0.33, H_E = 0.37) in our study were lower than in Vähä and Primmer's simulated populations. However, lower genetic diversity was compensated by larger F_{ST} values among the parental *Alectoris* groups ($F_{ST} > 0.50$). Results of both studies concordantly showed that 100% of the simulated F_1 and F_2 were identified at a

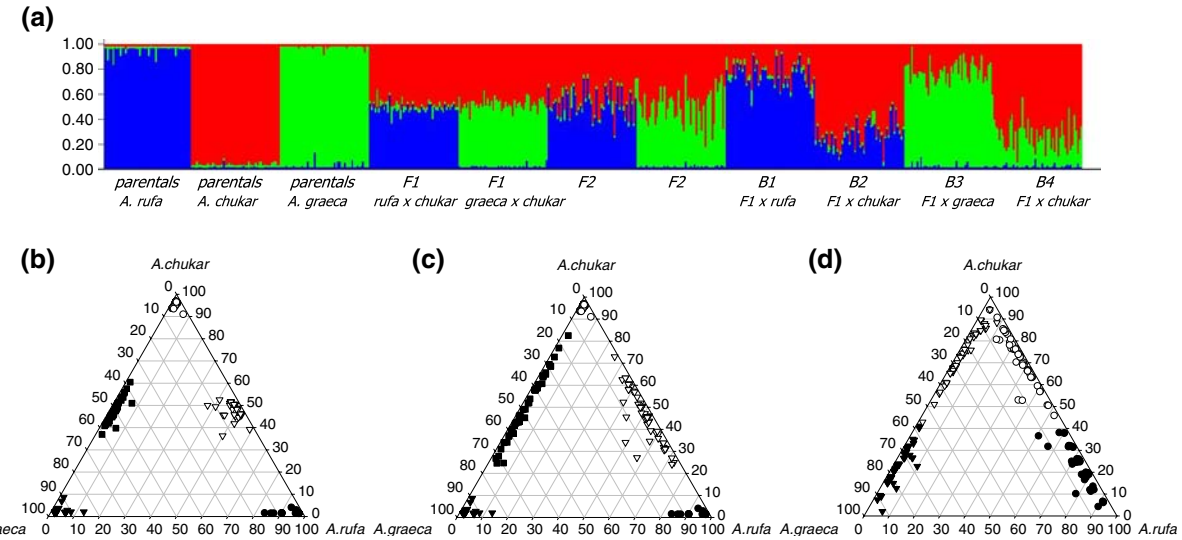

Fig. 7 Admixture analyses of *A. graeca*, *A. rufa* and *A. chukar* observed parental, and simulated F_1, F_2 and backcross genotypes computed by STRUCTURE with $K = 3$, the admixture and *I* models, and eight microsatellite loci (**a**). Each individual is represented as a vertical bar partitioned into *K* segments, whose length is proportional to the estimated membership in the *K* clusters. Ternary plot of individual proportions of membership of parental and simulated F_1 genotypes (**b**), F_2 genotypes (**c**), and backcrosses (**d**). Simulated genotypes were generated using the software HybridLab (Nielsen et al. 2001). Samples plotting at the apices have proportion of membership close to $q_i = 1.0$ to cluster I (*A. rufa*), II (*A. chukar*) or III (*A. graeca*)

threshold q_i-value = 0.10, but that the identification of backcrosses was more problematic. From 10 to 20% backcrosses were misclassified using $q_i = 0.10$, 24 or 48 loci and $F_{ST} = 0.21$ in Vähä and Primmer's simulations. Larger F_{ST} among parental *Alectoris* species could explain why here we missed a similar 10% proportion of backcrosses using only eight markers.

As clearly stated by Vähä and Primmer, there is a trade-off between efficiency and accuracy: using narrow threshold q_i-values (for instance, $q_i = 0.05$ or = 0.01) efficiency increased, but accuracy decreased. A trade-off is also clearly shown in our simulations: using $q_i = 0.05$ would have increased the efficiency of hybrid identification vs. $q_i = 0.10$ or 0.20, at the cost of decreased accuracy, because backcross groups would have included a larger number of parentals (Fig. 7). This trade-off in admixture analyses may be reduced increasing the number of markers, which now is possible in *Alectoris* (Gonzalez et al. 2005). However, in deeply introgressed populations that hybridised for many generations, it will be difficult to balance efficiency and accuracy also with realistically large numbers of loci. It is, therefore, important to define explicitly the assignment thresholds and rules, and assess the uncertainty of admixture analyses. Additional studies, using either empirical or simulated data sets, are needed particularly to define how to use CI values, that is to understand if CI wider than usual should be interpreted as signals of admixture or uncertainty. The use of linked loci in species were linkage maps are available is promising, because modelling the ''admixture linkage disequilibrium'' (Stephens et al. 1994) should allow detecting older admixture events (Falush et al. 2003), as suggested, for instance, by recent research on hybridising wild × domestic cats (Lecis et al. 2006), and wolves × dogs (Verardi et al. 2006).

Introgressive hybridisation and conservation genetics of rock partridges in Greece

Results of this study reveal that from ca. 1 to 20% (according to different hybrid identification thresholds) rock partridges showed signals of introgressive hybridisation. Introgressed partridges were widespread across all the populations sampled in Greece, ranging between 7% (in Desi) and 29% (in Olympos). The mtDNA tree allowed excluding the presence of chukar mtDNA haplotypes, suggesting that released female partridges reproduced poorly in nature and that introgression was mainly unidirectional, originating by backcrossing with resident female rock partridges. Genetic data indicates that not only chukar, but also red-legged partridges have been released in Greece: samples n. 926 and 940, which showed the stronger hybridisation signals, were identified as second generation hybrids with *A. chukar* and with *A. rufa*, respectively. These results highlight the limit of mtDNA screenings, and confirm the limited power of visual identifications as performed by FCA vs. model-based assignment procedures in analysing introgressed

populations (see also Pritchard et al. 2000; Randi et al. 2001; Randi and Lucchini 2002). Limited resolution of FCA may be due to the absence of F_1 genotypes, which should be easily detected because they are expected to map intermediately between the parental groups. In contrast, introgressed individuals with low occurrence of diagnostic alleles in their genotypes, may not have genetic distances large enough to allow the FCA splitting them apart.

Massive restocking has been carried out for years in Greece, with the annual release of tens of thousands of captive-reared partridges. Also if captive-reared birds are poorly fitted to survive in nature, released stocks might outnumber resident populations. Limited survival and reproduction success may nevertheless produce significant introgressive hybridisation, thus threatening the integrity of local gene pools, destroy local adaptations and eventually led to outbreeding depression. The occurrence of outbreeding depression, arising from hybridisation with translocated or migrating animals from different environments, or from genetic adaptations to domestication (Ford 2002), has been clearly documented in fish (Hallerman 2003; Miller et al. 2004; Rubidge and Taylor 2004), and in avian populations (Marr et al. 2002). Although inbreeding depression was never demonstrated in partridges, well-known ecological and sanitary risks, and the pollution of local gene pools by anthropogenic hybridisation (Simberloff 1996) strongly suggest recommending the interruption of massive and uncontrolled restocking programs. In this particular case study, the detectable extent of introgression in rock partridges in Greece is around 10–20%, meaning that populations in the entire core distribution of the nominate rock partridge subspecies *A. graeca graeca* have already been genetically polluted.

Acknowledgements We deeply appreciated the collaboration of everybody who helped in sampling collection, and in particular: P. Alkon, S. Kark and N. Liu for sampling chukars in Israel and China; D. Dias and J.A. Perez Garrido for red-legged partridges from Portugal and Spain; P. Birtsas, the hunters and Forest Service personnel for sampling in Greece. Fundings for laboratory analyses were provided to ER by the INFS, Ministero per le Politiche Agricole e Forestali, and Ministero per l'Ambiente e la Tutela del Territorio, Direzione Conservazione della Natura. Special thanks to Lars Bach which sent us copy of the software HybridLab, to C. Primmer and two anonymous referees that greatly contributed to improve a first version of this paper.

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