Animal Conservation

Animal Conservation. Print ISSN 1367-9430

Assessment of game restocking contributions to anthropogenic hybridization: the case of the Iberian red-legged partridge

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Keywords
Alectoris chukar; *Alectoris rufa*; cytochrome *b*; game management; introgression; supplemental stocking.

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Received 7 September 2007; accepted 18 September 2008

doi:10.1111/j.1469-1795.2008.00212.x

Abstract

Anthropogenic hybridization in wildlife has been identified as one of the main causes of genetic homogenization, highlighting the need for identification and evaluation of populations at risk. Relocation of wildlife for game management purposes is a widespread practice that may promote the admixing of genetically different populations, subspecies or species. We undertook a large-scale study on the Iberian Peninsula to assess the extent of hybridization in red-legged partridge *Alectoris rufa* populations, which have been subject to extensive restocking of farm-reared individuals. Using a polymerase chain reaction-restriction fragment length polymorphism technique to assess the prevalence of individuals with mtDNA from other species, we examined samples of *A. rufa* from museum specimens (229), extant wild populations (955) and game farms (530). We found widespread occurrence of chukar partridge *Alectoris chukar* mtDNA lineages in samples obtained from game farm partridges (63% of game farms) and from wild partridges (45% of populations), but no allochthonous mtDNA lineages were found in museum partridges. We also found that the probability of occurrence and the number of partridges with allochthonous lineages was higher in localities where recent restocking had occurred. In addition, investigation of trends in bag records and the numbers of game farms over the past 30 years suggests that the general decline of wild populations has been accompanied by an increase in game farm facilities. These results suggest that supplemental stocking practices are threatening the integrity of the wild population gene pool. We recommend that rural development policies and associated wildlife management programs focused on maintaining high stock densities for hunting also need to consider the impact of game management on the genetic integrity of game populations.

Introduction

Hunting has become not only a means of obtaining animal protein but also an important sport and recreational activity. Sport hunting is a traditional economic activity that may be compatible with conservation of game species. However, management of populations for hunting can create new conservation problems and threaten biodiversity if sustainable practices are not established (Leopold, 1986). Relocation of wildlife species for the purpose of introduction, re-introduction or supplementation is one of the most commonly used techniques in game management (Griffith *et al.*, 1989; Wolf *et al.*, 1996; Fischer & Lindenmayer, 2000). However, the lack of specific scientific criteria for relocations and the use of captive-reared stocks involving artificial hybrids may promote admixing of populations, threatening the integrity of wild populations.

Some of the factors that have promoted hybridization of game species include management for increased game species densities or quality of trophies, the reduction of production costs for game farms and the absence of effective markers to identify hybrids (e.g. Carranza *et al.*, 2003; Vernesi *et al.*, 2003; Barilani *et al.*, 2005). There is evidence that the genetic integrity of a large number of game species is likely have been eroded by anthropogenic hybridization as a consequence of relocation. For example, in Scotland, the native red deer *Cervus elaphus* has hybridized with the introduced sika deer *Cervus nippon* (Goodman *et al.*, 1999). Re-introduction of wild boar from central Europe to Italy has induced hybridization between the subspecies *Sus scrofa*

majori and *Sus scrofa scrofa* (Vernesi *et al*., 2003). Admixing of the wild rabbit subspecies, *Oryctolagus cuniculus cuniculus* and *Oryctolagus cuniculus algirus*, has occurred in Spain as a consequence of game translocations (Delibes-Mateos *et al*., 2008). Mass release of domesticated quail *Coturnix coturnix japonica* in Europe may threaten the common wild quail *Coturnix coturnix coturnix* (Barilani *et al*., 2005). Extensive translocation of wild turkeys *Meleagris gallopavo* through North America has led to concerns for the taxonomic integrity of a number of wild turkey subspecies (Latch, Applegate & Rhodes Jr, 2006*a*; Latch *et al*., 2006*b*). Given these examples, it is essential to detect and monitor introgression to assess the impacts of relocations and hybridizations on the genetic integrity of native populations.

Hybridization may also result in the loss of unique genetic, morphological, behavioral or ecological characteristics that have evolved in local populations over time, leaving hybrid populations less well adapted to local environments (Rhymer & Simberloff, 1996; Allendorf *et al*., 2001). As a result, outbreeding depression and disruption of the co-adapted gene complexes may manifest in a number of different ways, including reduced fertilization success, reduced embryonic survival, lower disease or pathogen resistance, ineffective foraging ability, reduced predation survival and reduced capacity to tolerate physiological stress (e.g. Vamosi, Hatfield & Schluter, 2000; Edmands, 2007; Hutchings & Fraser, 2008).

From the point of view of wildlife managers and conservation biologists, hybridization should be of special concern because hybrids may erode the gene pool of local populations, and may have reduced fitness, negatively impacting autochthonous population dynamics. Therefore, the management of wild species should be directed at selecting suitable source stock so that relocated individuals are adapted to local environment conditions, and are not a threat to the genetic integrity of native populations (e.g. Latch *et al*., 2006*a*,*b*). Assessment of introgressive hybridization and identification of admixed individuals is essential in evaluating the risk of biodiversity losses due to genetic homogenization.

In the present study, we focused on the genetic status of *Alectoris rufa*, a game species that breeds naturally in Spain, Portugal, south-eastern France and north-western Italy, and was introduced to the UK in the 18th century (Snow & Perrins, 1998). Three species, *A. rufa*, the rock partridge *Alectoris graeca* and the chukar partridge *Alectoris chukar*, range naturally in Europe. They are listed as threatened under European Union legislation (79/409 CEE Ap.2/1, 3/I; BERN Ap.3) and are classified as Species of European Conservation Concern (BirdLife International, 2004). During recent decades, increases in hunting activity and declines of wild populations have resulted in supplemental stocking from game farms as a way of reinforcing wild populations and providing financial return in rural areas (REGHAB, 2002; Keane, Brooke & McGowan, 2005). Introductions have extended the distribution of some *Alectoris* species, sometimes resulting in unnatural inter-species contacts (Barbanera *et al*., 2005; Barilani *et al*., 2007). In addition, captive-reared stocks also have been managed to produce artificial hybrids (Negro, Torres & Godoy, 2001).

The first records of anthropogenic hybridization of *Alectoris* species are from the early 1970s, when releases of *A. chukar* × *A. rufa* hybrids were discovered in the UK (Potts, 1989). *Alectoris* hybrids have also been detected recently in Italy and Greece using molecular tools (Baratti *et al*., 2005; Barbanera *et al*., 2005; Barilani *et al*., 2007). The occurrence of hybrids (*A. chukar* or *A. graeca* × *A. rufa*) on the Iberian Peninsula also has been suggested (Negro *et al*., 2001; González, Castilla & Zardoya, 2005). Natural hybridization on the peninsula is impossible due to the disjunct distributions of the species (Madge & McGowan, 2002). Because neither *A. chukar* nor *A. graeca* have been released on the Iberian Peninsula (Martí & del Moral, 2003), the presence of hybrids or advanced backcrosses in this area is most likely the result of the release of hybrid birds or advanced backcrosses produced in captivity, a practice suggested, for example, by Negro *et al*. (2001).

The red-legged partridge is the most important gamebird on the Iberian Peninsula, where over 80% of the natural range of the species occurs (Snow & Perrins, 1998). Populations of *A. rufa* began to decline at the end of the 1960s as a consequence of intensification of agricultural activities and unregulated harvest, but in Spain, the status of *A. rufa* is classified as 'data deficient' (Blanco-Aguiar, Virgós & Villafuerte, 2004), and the genetic quality of farm-reared and wild partridges has not been previously assessed. Given that the conservation and management of Iberian populations of *A. rufa* are essential for the viability of the species worldwide, there is an urgent need to investigate the genetic status of the species in this area.

The aims of this study were to (1) investigate the occurrence of allochthonous mtDNA lineages (mtDNA from the rock or chukar partridges) in farm-reared stocks and wild populations of the *A. rufa* and (2) assess the influence of supplemental stocking with game farm birds on the occurrence of allochthonous mtDNA lineages in extant wild populations. Results from this study are evaluated in light of the relative growth of partridge breeding facilities and hunting bag numbers in Spain during the period 1969–2003.

Materials and methods

Study area and sample collection

We collected samples from 1714 *A. rufa* from the Iberian Peninsula and Mallorca Island, identified on the basis of morphological and plumage criteria. Four categories of samples were collected (see Fig. 1 for the geographical distribution of samples): (1) extant wild partridges (Table 1; $n = 955$), this group contains 949 partridges taken during the 2001–2004 hunting seasons from extant wild populations on the Iberian Peninsula, and six *A. rufa* obtained from the UK; (2) breeding farm partridges (see Table S1; $n = 440$), those from farm-reared populations raised for hunting (offered voluntarily by game farm owners); (3) inspection partridges (Table S1; $n = 90$), those sampled

Figure 1 Geographical sample distribution of *Alectoris rufa* on the Iberian Peninsula. Stars (☆) represent partridge breeding facilities where samples were offered voluntarily, gray dots represent collection sites for museum samples (•) and squares represent extant wild populations. Dotted squares (▣) represent populations with non-mixed lineages, and filled squares (■) represent populations with allochthonous mtDNA (mixed) lineages.

during government inspections of farm-reared partridges before release (mandatory request; specific geographic information not available but primarily from central Spain, with $n = 9$ known to originate from a game farm in France); (4) museum partridges (see Table S2; $n = 229$) (housed in the Estación Biológica de Doñana museum collection, collected from wild *A. rufa* on the Iberian Peninsula 1960–1980, when supplemental stocking was not commonplace).

We also obtained samples of 90 *A. chukar* from the wild in China (five from Gansu province) and Greece (18 from Ikaria Island, five from Kos Island, eight from Karpathos island), and 54 from private bird collections. An additional 118 *A. graeca* were obtained from the wild in Greece (18 from Peloponnese, 37 from Thessaly, 26 from Epirus, three from Central Greece, 18 from Macedonia and 16 from private facilities in Italy).

DNA isolation and polymerase chain reaction (PCR) conditions

Muscle tissue or blood samples were taken from hunted and live partridges, and skin or small pieces of tissue were sampled from toe pads of museum specimens. We isolated total DNA using a proteinase K digestion followed by phenol:chloroform extraction (Sambrook, Fritsch & Maniatis, 1989). DNA was isolated from museum specimens using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) in a dedicated ancient DNA laboratory, and extraction blanks were included to assure the absence of contamination.

We designed two sets of primer pairs to amplify fragments from the mitochondrial cytochrome *b* gene (mtDNA Cyt-*b*), based on published *Alectoris* sequences held in GenBank (Randi, 1996; Barbanera *et al.*, 2005). The first primer set (upstream primer Aru-Cyt-*b* F; 5′-CATCAAA CATCTCYGCCT-3′; downstream primer Aru-Cyt-*b* R; 5′-TGTTCTACTGGTTGGCTTCC-3′; 62 °C annealing temperature) amplified a 964 base pair (bp) fragment including a number of species-specific endonuclease restriction sites useful for diagnostic purposes. We designed a second set of internal primers for amplification of a smaller DNA fragment. The second primer set (upstream primer Aroja-Cyt-*b* F; 5′-CTCCGCCTGATGAAACTT-3′; downstream primer Aroja-Cyt-*b* R; 5′-AATGAGGCGCCGTTTGCATG-3′; 58 °C annealing temperature) amplified a 186 bp fragment. These fragments included a number of species-specific endonuclease restriction sites useful for diagnostic purposes.

The PCR reaction mixture (20 μL final volume) contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.01% Tween-20, 25 mM $MgCl_2$, 0.5 U of Taq DNA polymerase (Bioron GmbH, Ludwigshafen, Germany), 0.5 μM of each primer, 0.2 mM of each dNTP and 50 ng of genomic DNA. We performed PCR amplification under the following conditions: one cycle of 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature, 1 min at 72 °C and a final extension of 5 min at 72 °C. We included extraction blanks as negative controls in all reactions to test for contamination.

Restriction enzyme analysis

We aligned *Alectoris* sequences published in GenBank (Randi, 1996; Barbanera *et al.*, 2005) and performed a search for endonuclease cutting sites using the software BioEdit 7.0 (Hall, 1999) and visual inspection, choosing a set of enzymes on the basis of a specific predictable pattern that would enable species identification (*A. rufa*, *A. chukar* and *A. graeca*). The 964 bp fragment (Aru-Cyt-*b* F–R) was sequentially digested with *BsrG*I (5′-T ↓ GTACA-3′), *Bst*NI (5′-CC ↓ WGG-3′) and *Tsp45*I (5′- ↓ GTSAC-3′), according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). The 186 bp amplicon (Aroja-Cyt-*b* F–R) was digested with *BsrG*I (5′-T ↓ GTACA-3′) and *Tsp45*I (5′- ↓ GTSAC-3′). DNA fragments were electrophoresed in 2% (w/v) agarose gels and detected by staining with ethidium bromide.

Sequencing and phylogenetic data analysis

To differentiate mtDNA clades within *Alectoris* and illustrate the utility of the restriction fragment length polymorphism (PCR-RFLP) technique, we performed a phylogenetic analysis of a set of previously published and newly characterized sequences of *Alectoris* mtDNA Cyt-*b*. We aligned *Alectoris* sequences published in GenBank: *A. chukar*: AJ586151, AJ586153–58, AM08456–57, AM084568, AM084576, AM 084582–83, AM492950; *A graeca*: Z48772, AM492951–52; *A. rufa*: Z48775, AJ586141, AJ586147, AJ586149; *Alectoris barbara*: Z48771; *Alectoris philbyi*: Z48774; *Alectoris melanocephala*: Z48773 and *Alectoris magna*: Z48776 (Randi, 1996; Barbanera *et al.*, 2005; Guerrini *et al.*, 2007). In addition, we selected and sequenced a set of samples characterized by PCR-RFLP as allochthonous mtDNA lineages ($n = 19$; EF638920–38, coded as *A. rufa* × *A. chukar*), *A. rufa* ($n = 10$; EF638907–16, coded as Aru), *A. graeca* ($n = 3$; EF638917–19,

coded as Agr) or *A. chukar* ($n = 10$; EU526097–106, coded as Ach). These sequences were included in the phylogenetic analysis.

For the newly reported sequences, we purified mtDNA Cyt-*b* PCR-amplified products using MicroSpin™ S-400 columns (Amersham Biosciences, Piscataway, NJ, USA), and sequenced samples using BigDye™ 3.1 kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA). Sequence alignment was performed with BioEdit 7.0 (Hall, 1999) and nucleotide composition and the pattern of substitution were computed using the program MEGA 3.1 (Kumar, Tamura & Nei, 2004). A phylogenetic tree was constructed using MEGA 3.1 with the neighbor-joining procedure (NJ) and Tamura–Nei's evolutionary model (Tamura & Nei, 1993). Support for internodes was assessed using 10 000 bootstrap re-sampling steps.

Supplemental stocking information

Supplemental stocking with game farms birds is one of the primary game management strategies in Spain (Angulo, 2003). To assess the possible influence of supplemental stocking on the occurrence of allochthonous lineages, we used a χ^2 test to assess differences in the percentage of individuals with allochthonous lineages among museum specimens, game farm raised individuals, inspection individuals and extant wild *A. rufa*. We also assessed differences in the percentage of populations or farms with allochthonous lineages between game farm partridges and extant wild *A. rufa*. Data from inspection and museum partridges were not considered in the latter analyses because of the low sample size per geographic area for these groups. In addition, we interviewed gamekeepers overseeing management in each of the 38 wild sample locations (see Table 1) to determine whether they, or the managers of neighboring estates (game management units scaled between 500 and 5000 ha), had restocked partridges from game farms during the previous year or in the 5 years before sample collection. We used Kruskal–Wallis ANOVAs to assess the probability that localities with and without supplemental stocking were different with respect to the number of *A. rufa* with allochthonous lineages.

We also analyzed temporal trends in the number of *A. rufa* breeding facilities and changes in hunting bag numbers during the past three decades. Official data on the temporal trends of game restocking and the number of game farms were not available for the evaluation of temporal changes in the number of game farms. Instead, we reviewed hunting journals (Caza y Pesca and Federcaza) and recorded the number of game farms that advertised the availability of partridges during the categorical periods: 1969–1970, 1976–1977, 1982–1983, 1988–1989, 1996–1997 and 2002–2003. We randomly selected 13 monthly issues from each period for examination. Analysis was performed using a general linear model (GLM) with a Poisson's distribution and a log link function (Crawley, 1993). The number of game farms that advertised the availability of partridges during each monthly issue was introduced as the dependent variable, whereas the 'categorical periods' variable was entered as a fixed factor.

We used historical hunter take data as an indicator of population trends. Indirect measures of population abundance, such as hunting harvest data, must be corrected for

Table 1 Red-legged partridge *Alectoris rufa* collection sites and sample sizes (*n*) of extant wild populations

Regions	Localities	*n*	Ach mit	R5	R1
Ciudad Real	Picón	30	4	+	+
Ciudad Real	Ciudad Real	28	1	+	+
Ciudad Real	Santa Cruz de Mudela	23	1	+	+
Toledo	Corral de Almaguer	30	7	+	+
Álava	El ciego	37	5	+	–
Badajoz	Badajoz	23	4	+	–
Burgos	Santa María del Campo	2	1	+	–
Ciudad Real	Poblete	30	0	+	–
Ciudad Real	Berrinches	29	2	+	–
Ciudad Real	Moral de Calatrava	39	1	+	–
Gerona	Palau Saverdera	17	1	+	–
Madrid	Algete	21	0	+	–
Murcia	Lorca	20	0	+	–
Álava	Salinas de Añana	40	0	–	–
Albacete	La Gineta	18	0	–	–
Alicante	Castell de Castells	25	0	–	–
Almería	Purchena	30	0	–	–
Asturias	Cangas de Narcea	11	0	–	–
Cádiz	Medina Sidonia	47	0	–	–
Ciudad Real	Alcolea de Calatrava	35	2	–	–
Ciudad Real	Alhambra	38	0	–	–
Gerona	Cadaqués	10	0	–	–
Granada	Baza	28	1	–	–
Huelva	Villalba del Alcor	25	0	–	–
Jaén	Linares	10	1	–	–
León	Palacios del Sil	20	0	–	–
Lugo	Cervantes	17	0	–	–
Madrid	Fuente el Saz	22	5	–	–
Mallorca	Mallorca	6	1	–	–
Navarra	Estena	21	1	–	–
Tarragona	Vimbodi	16	0	–	–
Tarragona	Conca del Barbera	21	0	–	–
Toledo	Quintanar de la Orden	21	1	–	–
Toledo	Méntrida	29	0	–	–
Toledo	Huecas	25	0	–	–
Toledo	Villatobas	17	0	–	–
Valladolid	Medina de Rioseco	31	0	–	–
Zaragoza	Moneva	23	0	–	–
Portugal	Mourela	3	1	*	*
Portugal	Mogadouro	19	0	*	*
Portugal	Sabugal	8	1	*	*
Portugal	Tavira-Castro Marim	10	0	*	*
UK	Stroud (Gloucestershire)	6	1	*	*
	Total	955	41		

The number of individuals with allochthonous mitochondrial lineages (Ach mit, *Alectoris chukar* mitochondrial) for each extant wild population is specified, as are populations where supplemental stocking occurred (+) or did not occur (–) in the last year (R1) or during the last 5 years (R5), or where no interview data were available (*).

Table 2 Restriction patterns found for identification of different *Alectoris* species (*A. chukar*, *A. graeca* and *A. rufa*) based on the cytochrome *b* (Cyt-*b*) PCR-RFLP method

Base reference[a]	129	757	220	498	885	
Endonucleases	*Tsp45*I	*Tsp45*I	*BsrG*I	*BstN*I	*BstN*I	mtDNA lineage
Fragment sizes	91,153	–	146,818	–	–	*A. chukar*
Aru-Cyt-*b* F–R primers	–	681,283	–	–	–	*A. graeca*
	–	681,283	–	–	154,810	*A. graeca*
	91,153	681,283	–	422,542	–	*A. rufa*
	91,153	681,283	–	–	–	*A. rufa*
Fragment sizes	43,143	–	13,650	–	–	*A. chukar*
	–	–	–	–	–	*A. graeca*
Aroja-Cyt-*b* F–R primers	43,143	–	–	–	–	*A. rufa*

These patterns illustrate that the fragment sizes [base pairs (bp)] associated with independent restriction digests can be used to identify the *Alectoris* species using two different primers sets: Aru-Cyt-*b* F–R (964 bp) and Aroja-Cyt-*b* F–R (186 bp).
[a]Nucleotide positions of restriction sites are numbered following Randi (1996).
PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; –, no digest.

the hunting effort applied (Cattadori *et al*., 2003). Therefore, the 'bag-record index' in each Spanish province was expressed as total take per hunting license issued. Bag-records and license information were obtained from the Spanish Ministry of Agriculture for the period 1973–2002, from which provincial data were analyzed for the categorical periods: 1973, 1976–1977, 1982–1983, 1988–1989, 1995–1996 and 2001–2002. Analysis was performed using a general linear mixed model (GLMM) (Littell *et al*., 1996). The bag-record index in each Spanish province was introduced as the dependent variable. For each province, we used the mean bag-record index value from 2 years in each time category. The variable 'categorical periods' was entered as the fixed factor, and provincial identity was introduced as a random factor. The bag-record index met normal identity and homoscedasticity requirements.

Results

Molecular results

Sequence comparisons of *Alectoris* published in GenBank showed a number of polymorphisms useful for species identification (see sequencing and phylogenetic data analysis). We confirmed the effectiveness of these polymorphic sites for species characterization by sequencing *A. rufa* ($n = 10$), *A. graeca* ($n = 3$) and *A. chukar* ($n = 10$). One *BsrG*I recognition site was unique to *A. chukar* sequences (a third codon positions). The second endonuclease, *Tsp45*I, had single recognition sites (a third codon positions) in both *A. graeca* and *A. chukar*. For *A. rufa* there were two *Tsp45*I recognition sites. Finally, *BstN*I had single recognition sites (a third codon positions) in both *A. rufa* and *A. graeca*. However, we also found individuals from *A. rufa* and *A. graeca* without recognition sites for *BstN*I, which were differentiated through *Tsp45*I enzyme (Table 2).

In order to describe the large-scale pattern of hybridization in Iberian Peninsula, we amplified samples using the Aru-Cyt-*b* primers and digested the PCR products with *BstN*I and *BsrG*I enzymes, or amplified using the Aroja-Cyt-*b* primers and digested the products with *Tsp45*I and *BsrG*I (see Table 2). The banding pattern resulting from digestion of the Cyt-*b* fragments showed distinct profiles (Fig. 2) for each of the three species studied (*A. rufa*, *A. graeca* and *A. chukar*).

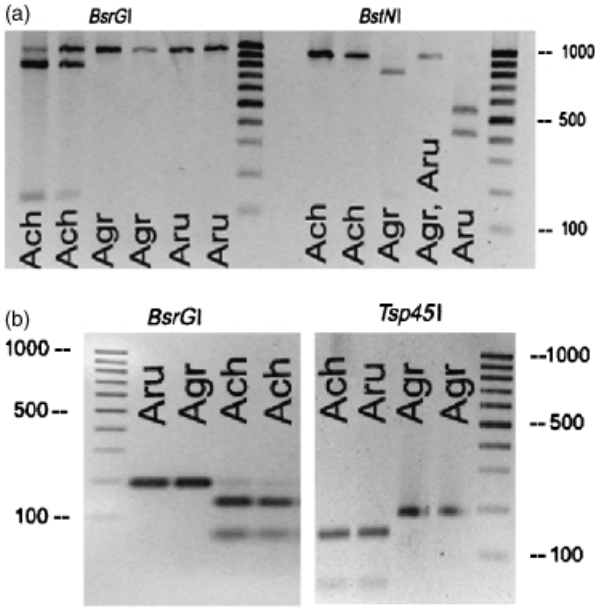

Figure 2 Diagnostic polymerase chain reaction-restriction fragment length polymorphism agarose gel banding profiles of *Alectoris chukar*, *Alectoris graeca* and *Alectoris rufa* obtained through independent digestions of mtDNA cytochrome *b* (Cyt-*b*) sequences with *BstN*I, *BsrG*I or *Tsp45*I enzymes. *BstN*I and *BsrG*I were used with the Aru-Cyt-*b* primers (a) and *BsrG*I and *Tsp45*I were used with the Aroja-Cyt-*b* primers (b). Agr, *A. graeca*; Aru, *A. rufa*; Ach, *A. chukar*. The molecular ladder is shown in base pairs. *Note*: Agr–Aru haplotype with *BstN*I was present in both species.

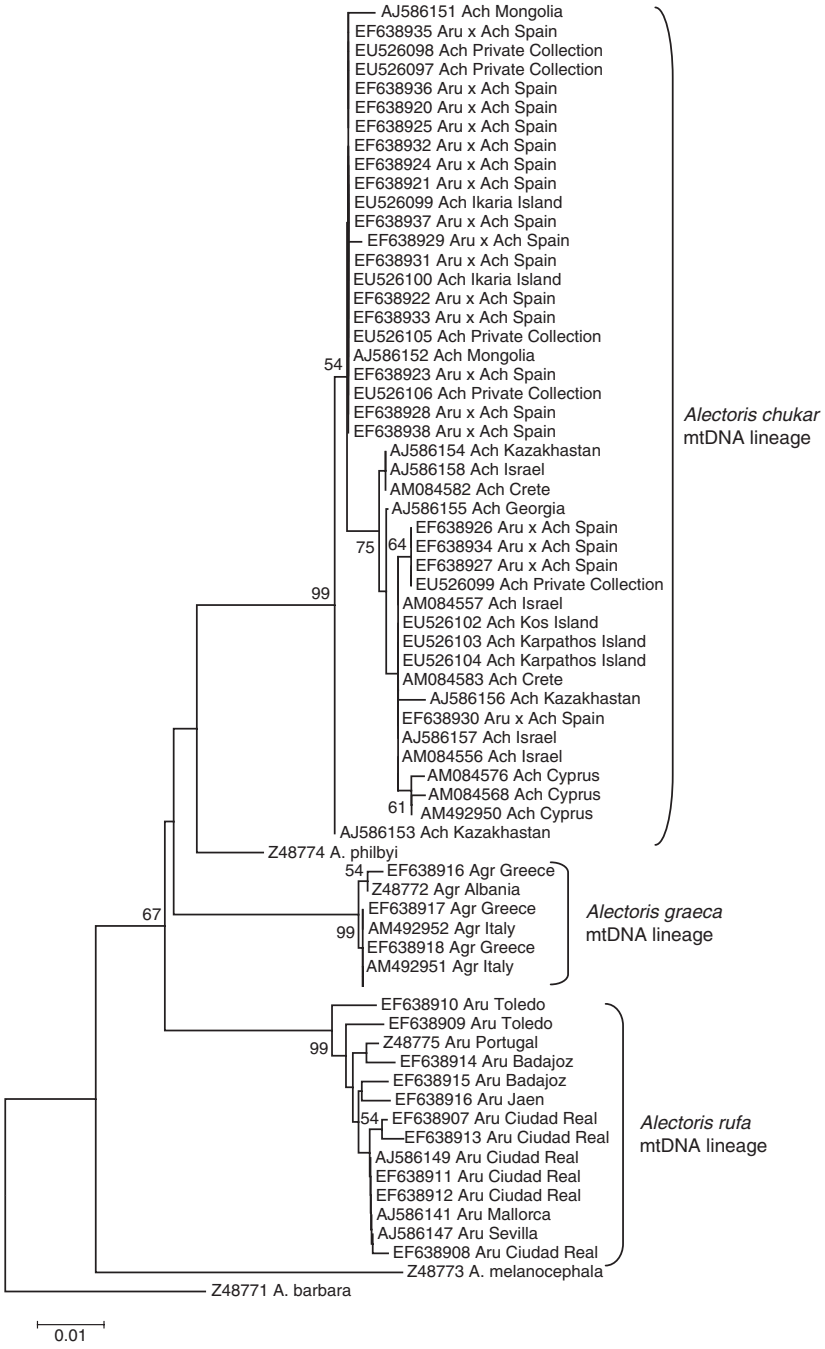

Figure 3 Neighbor-joining tree computed by MEGA using Tamura and Nei's genetic distances (Tamura & Nei, 1993) among the aligned partridge mtDNA Cyt-*b* sequences. The tree was rooted using the *Alectoris barbara* Z48771 GenBank sequence. Number at the internodes indicates bootstrap percentages (>50). Agr, *Alectoris graeca*; Aru, *Alectoris rufa*; Ach, *Alectoris chukar*; and *Aru* × *Ach*, samples classified morphologically as *A. rufa* but which contain allochthonous mitochondrial lineages via the polymerase chain reaction-restriction fragment length polymorphism analysis.

The Cyt-*b* alignment (515 bp) yielded a NJ tree (Fig. 3) in which *A. rufa*, *A. graeca* and *A. chukar* clustered into three well-supported groups (bootstrapping percentage = 99%). All the partridges showing an allochthonous chukar PCR-RFLP phenotype clustered in the *A. chukar* clade, highlighting the practical utility of the PCR-RFLP approach for diagnosing samples with allochthonous lineages. As mtDNA is inherited maternally, hybrids with a *A. rufa* female parent would not be identified as hybrids in our mitochondrial analysis if they had *A. rufa* phenotypes, and therefore our survey probably underestimated the number of hybrid individuals.

Study samples (wild, game farm, inspection and museum *A. rufa*) were examined using the PCR-RFLP method. We detected a total of 93 *A. chukar* mtDNA haplotypes among Iberian *A. rufa* (see Table 3), but no *A. graeca* mtDNA; we also found one *A. rufa* with *A. chukar* mtDNA from six UK samples (Table 1). In order to evaluate historical levels of introgression/hybridization, we analyzed 229 museum *A. rufa* specimens using the 186 amplicon primer set

Table 3 Number of individuals and populations analyzed that showed allochthonous (*Alectoris chukar*) mtDNA lineages in each sample group

	Individuals		Populations	
	Sample size	Allochthonous mtDNA lineage	Population number	Allochthonous mtDNA lineage
Extant wild partridges	955	41 (4.3%)	42	19 (45.2%)
Breeding farm partridges	440	26 (5.9%)	11	7 (63.6%)
Inspection partridges	90	26 (28.9%)	–	–
Museum partridges	229	0 (0%)	–	–

Numbers in parentheses are the percentages of individuals and populations with *A. chukar* mtDNA lineages.

(Aroja-Cyt-*b* F–R). Taking into account the lack of *A. graeca* haplotypes within the extant wild *A. rufa* samples studied, the 186 amplicon was designed to contain the restriction site specific for *A. chukar* (using *BsrG*I endonuclease). If the estimated prevalence in museum samples was the same as in extant wild populations (4.3%, see Table 3), then we would expect to sample about 10 allochthonous haplotypes in our sample of 229 individuals. Remarkably, we did not find allochthonous lineages in any museum samples, even though these were from widely distributed locations on the Iberian Peninsula (Fig. 1).

The spatial distribution of PCR-RFLP phenotypes (Fig. 1) showed widespread occurrence of wild populations with allochthonous lineages on the Iberian Peninsula. There were significant differences in the percentage of samples with allochthonous lineages between museum individuals and extant wild *A. rufa* (Yates corrected $\chi^2 = 8.9$, d.f. = 1, $P = 0.003$; see Table 3), and when museum samples were compared with either game farm or inspection partridges (Yates corrected $\chi^2 > 11$, d.f. = 1, $P < 0.0004$; see Table 3). There was no significant difference in the percentage of samples with allochthonous lineages from game farm and wild *A. rufa* ($\chi^2 = 1.72$, d.f. = 1, $P = 0.19$; see Table 3). Similarly, the percentage of populations showing allochthonous mitochondrial lineages was not significantly different between farm facilities and the wild (Table 3; Yates corrected $\chi^2 = 0.56$, d.f. = 1, $P = 0.45$). However, there was a significant difference in the percentage of samples with allochthonous lineages from inspection and game farm partridges (Table 3; $\chi^2 = 32.35$, d.f. = 1, $P < 0.0001$).

Supplemental stocking information

Interviews with game managers indicated that supplemental stocking with game farm birds occurred in 34.2% of localities during the last 5 years, and in 11.7% of localities in the last year (Table 1). Mitochondrial introgression was more frequent in localities in which *A. rufa* were released in the last 5 years (77%) than in localities where there was no documentation of *A. rufa* release from game farms (28%). We confirmed that the number of partridges analyzed in each studied estate did not vary significantly between localities with and without supplemental stocking with game farm birds during the last year (Kruskal–Wallis ANOVA, $H = 1.36$, $P = 0.24$) or in the last 5 years (Kruskal–Wallis ANOVA, $H = 0.79$, $P = 0.37$). We found that the number of hybrids detected was significantly higher in localities where there were supplemental stocking in the previous year (Kruskal–Wallis ANOVA, $H = 6.28$, $P < 0.012$), or in the previous 5 years (Kruskal–Wallis ANOVA, $H = 9.02$, $P = 0.002$).

In our analysis of partridge farm industry trends and bag records in Spain, we observed an increase in the number of game farm facilities advertising and a decrease in *A. rufa* populations. The evaluation of trends in the game farm industry using a GLM showed that there were significant differences between periods [$F_{(5,72)} = 123.01$; $P < 0.0001$], with an increase in the number of game farms with advertisements per journal issue in each period (Fig. 4). Analysis of bag records using a GLMM test and fitting province as a random term revealed significant differences in the bag-record index between categorical periods [$F_{(5,218)} = 5.59$; $P < 0.0001$]. There was a decline in the number of *A. rufa* taken in the first five categorical periods but a slight upward turn in the last period, as evidenced by the number of *A. rufa* shot per hunting license. Although this pattern does not infer causation, it follows the trend of increasing number of game farm facilities with advertisements in the same period (see Fig. 4).

Discussion

Our results suggest widespread incidence of hybridization in Iberian populations of *A. rufa*, likely facilitated by supplemental stocking programs. Mitochondrial introgression was detected by the presence of discordant mtDNA haplotypes in individuals that were morphologically identified as *A. rufa*. Natural interbreeding between *A. graeca* and *A. rufa* may be possible because there is a parapatric region in the southern French Alps where the species may hybridize (Randi & Bernard-Laurent, 1999). However, in none of our samples, we did find mtDNA haplotypes of *A. graeca*.

The distributions of *A. chukar* and *A. rufa* are highly disjunct and natural contact in the past seems improbable. *Alectoris rufa* is distributed in western Europe and speciated about 2.4–3.8 million years ago, whereas *A. chukar* is a Eurasian species, which originated more recently about 1.8–2 million years ago (Randi, 1996). During glaciations, the distributions of *A. rufa* and *A. chukar* contracted to southern independent refugia (Randi *et al.*, 2003). Deforestation and agriculture favored a rapid expansion of habitat suitable for *Alectoris* species, which resulted in expanded

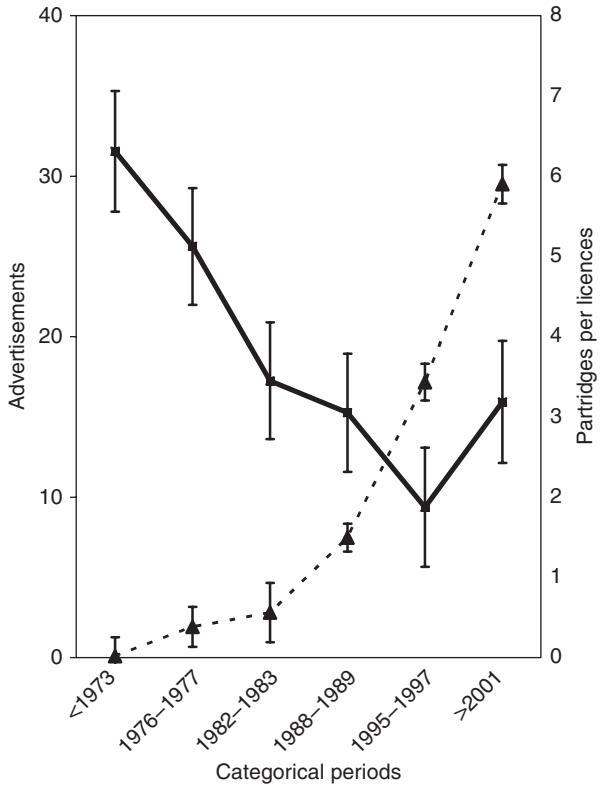

Figure 4 Number of *Alectoris rufa* game farms with advertisements in Spain in hunting journals during 1969–2003 (▲, left scale). Change in the mean number of partridges taken per hunting license in each Spanish province during 1973–2002 (■, right scale) is presented (whisker: ± SE).

ranges in contrast to refugia, but *A. chukar* and *A. rufa* have no extant contact zones. The combination of disjunct ranges for *A. rufa*, *A. graeca* and *A. chukar*, and the absence of allochthonous mtDNA lineages in the museum specimens of *A. rufa* make it improbable that sharing of ancestral mtDNA haplotypes between *A. rufa* and the other species has resulted from natural hybridization or unsorted mitochondrial lineages. Rather, these data suggest that hybridization has occurred since the mid-1980s when supplemental stocking with game farm birds rose exponentially.

The decline of Iberian *A. rufa* populations and the desire to maintain hunting quotas likely created the demand for game stocking and the rise of game farm facilities. Although the use of hybrid partridges has been banned under Spanish law since 1975, the competitiveness of markets may have promoted the illegal use of hybrids as a way of reducing production costs. For example, *A. rufa* breeders have indicated that their activities are more profitable when they interbreed *A. rufa* with allochthonous partridge species, because these hybrid partridges have larger clutch sizes and animal handling and rearing are easier (Padrós, 1991). In fact, *A. chukar* has been used for a long time in meat production, and it is likely that this species has been selected for hybridization with *A. rufa* by game breeders because its behavior and productivity in captivity is better than that of *A. graeca*.

The lack of genetic monitoring programs has favored the wide spread of allochthonous lineages in both wild and captive populations. We detected a high percentage of partridges in inspected facilities and a high percentage of game farms (from voluntary samples) with allochthonous lineages despite having relatively low sample sizes for many facilities. Samples obtained from government authority inspections of game farms exhibited much higher levels of introgression than did samples provided voluntarily by game farms. This suggests a pronounced difference in the rate of introgression among game farms or a potential bias in the manner in which samples are provided to inspectors. It is possible that some farmers could be involved in intentional hybridization whereas other farmers are not certain of the genetic quality of their breeding sources. Although we do not know the criteria used by authorities when selecting game farms for inspection, it is possible that they select game farms (e.g. based on facility size or infringement licenses) that are more likely to be involved in hybridization. Alternatively, inspection samples may have come mainly from central Spain where we detected a high density of game farms with hybrid populations (see Fig. 1). Such a sampling bias may have skewed the result when we compared our results with game farms distributed across Spain.

We also observed that the occurrence of populations and the number of *A. rufa* with allochthonous lineages was higher in localities where release activities were known to have occurred; for example, in south-central Spain (Fig. 1), indicating that game management activities may have important consequences for the genetic integrity of wild populations. Various studies have shown poor survival of released *A. rufa* (Gortázar, Villafuerte & Martín, 2000; Pérez *et al.*, 2004), but evidence also exists for their reproduction and survival after restocking in the wild (Duarte & Vargas, 2004; Barbanera *et al.*, 2005). Although interviews with game managers must be interpreted with caution, our data showed that *A. rufa* from localities with no record of supplemental stocking in the last 5 years did exhibit *A. chukar* mtDNA, which suggests current admixing of farm-reared and wild *A. rufa* in the wild or persistent mitochondrial introgression from past introductions.

The introduction of allochthonous lineages of *Alectoris* spp. is not geographically restricted to the Iberian Peninsula, it also has been described in Italy with *A. rufa* (Barbanera *et al.*, 2005) and Greece with *A. graeca* (Barilani *et al.*, 2007). The detection of *A. chukar* mtDNA lineages in game farm partridges during government inspections of French facilities, the occurrence of allochthonous mitochondrial lineages in the UK, and the possibility that admixed stocks from Spanish game farms have been introduced in Portugal and vice versa makes the restocking of partridges an international conservation problem. It must also be considered that most of the birds raised in farm facilities come originally from other farm facilities and that there is an international trade of both eggs and chicks as a source of birds for

game farms. Consequently, cooperation between countries in promoting the effective control of illegal relocation of wildlife between and within countries is urgently required.

Most countries where the *A. rufa* is distributed (France, Italy, Portugal, Spain, UK) have developed regulations to avoid the releases or breeding of allochthonous gamebirds (Arroyo & Beja, 2002). However, there are no accurate records of the number of gamebirds released, and the effort undertaken to monitor genetic quality of restocked partridges is largely anecdotal, suggesting inadequate oversight of regulations (Arroyo & Beja, 2002). As consequence of *A. rufa* decline, more than four million farm-reared partridges are released annually in Spain for hunting purposes. If we consider the high hybrid prevalence in game farm facilities observed in this study, the enforcement of regulations (e.g. ban of hybrid releases) would likely bring significant economic losses to farmers and the hunting sector, both of which are very important in the regional economies of southern Europe. Thus, local governments may not be motivated to increase genetic monitoring.

This study has implications beyond this particular game species and, because of the absence of adequate regulation and monitoring, a lack of legal control on the genetic quality of species is a general characteristic of game management in Europe. Relocations of game species have been widespread in European countries, but the criteria used to conduct such programs primarily have been oriented toward economic rather than conservation goals. Some game species, including European rabbit, red deer and wild boar, are crossed with other related or domesticated species because handling of domesticated lineages in captivity is often easier, litter sizes are larger, or some characteristics preferred by hunters, such as antler size, have been artificially selected in captive stock (Carranza *et al*., 2003; Piorno, 2006).

One of the European Union's priorities is rural development (Council Decision 2006/144/EC of 20 February 2006), and policies have promoted hunting as an economic alternative to agriculture in rural areas (CARD, 1996). European policies promoting hunting as a tool for diversification of the rural economy should consider the quality and impact of supplemental stocking practices if alteration of the gene pool of species is to be avoided. We recommend that governmental regulations and programs more actively protect the genetic integrity of game species through close monitoring of game farm facilities.

In brief, we detected a remarkably high number of wild and game farm populations with allochthonous lineages but found no such lineages among museum samples. Further, it will be important to investigate the success of hybrids in the wild, the consequences of hybridization on fitness and its potential implications for *A. rufa* conservation. In addition, we have shown that PCR-RFLP analysis of *Alectoris* mtDNA Cyt-*b* is an effective tool for large-scale surveys and assessment of populations with mixed ancestries, providing a potentially cheaper and less time-consuming alternative to sequencing. As mtDNA is inherited maternally, our survey probably underestimated the number of hybrid individuals and thus, the overall impact of hybridization. Testing with nuclear markers would allow the assessment of paternal hybridization that may exist as a result of male releases.

Acknowledgments

We deeply appreciate the collaboration of institutions that helped in sampling collection, in particular: Fundación para el Estudio y la Defensa de la Naturaleza y la Caza (Real Federación Española de Caza), provincial delegations of environmental and game agencies, and staff from Instituto de Investigación en Recursos Cinegéticos, Estación Biológica de Doñana and Centro de Investigação em Biodiversidade e Recursos Genéticos. We also acknowledge sample contributors from Giannis Kouvelos, Naifa Liu, and many hunter associations and anonymous hunters who kindly contributed samples to this study. We also appreciate the helpful comments of Dr Karen Mock, Dr Beatriz Arroyo and two anonymous referees. This research is a contribution to the projects PREG-05-17 and PREG-05-22 funded by Junta de Comunidades de Castilla la Mancha (JCCM) and project CGL2005-02340 funded by Ministerio de Educación y Ciencia. J.A.B.-A. and M.E.F. were supported by JCCM fellowships and European Social Fund.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Red-legged partridge (*A. rufa*) farm facilities, sample sizes (N), number of individuals with *A. chukar* mitochondrial lineages (Ach = *A. chukar*), and percentage of Ach mitochondrion detections for each farm facility.

Table S2. Red-legged partridge (*A. rufa*) collection sites and sample sizes (N) from museum specimens (Estación Biológica de Doñana). Allochthonous mitochondrial lineages were absent from all samples.

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