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Restocked and non-restocked populations genetic composition: a case study in red-legged partridge (*Alectoris rufa*)

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Release of captive-bred red-legged (*Alectoris rufa*) partridges is used to reinforce hunting areas where wild populations have decreased in southern European countries. However, breeders have often used other species to improve acclimation to captivity (e.g. *Alectoris chukar*), producing different degrees of hybridized individuals. In this study, three hunting reserve partitions, characterized by the different likelihood of contact with captive-reared partridges, were sampled and genotyped with 22 microsatellite markers to check for the existence of *A. rufa* × *A. chukar* hybridization and to compare the genetic composition of restocked and non-restocked red-legged partridge populations. Our results reveal the efficiency of the marker set used to differentiate among closely related *A. rufa* partridge populations, and the different genetic composition between captive-reared individuals and wild ones, but also the hybridization with *A. chukar* partridges on cynegetic farms. These facts must be taken into account and genetic controls of farm breeding stocks should be performed before restocking, both to avoid introgression in wild populations and to guarantee the reintroduction of partridges of known genetic origin in each area.

Key words: introgression, genetic structure, hybridization, microsatellite.

INTRODUCTION

Although the role of natural hybridization in generating biodiversity is controversial, scientists have agreed that human-mediated hybridization, with or without introgression, threatens native populations (Rhymer & Simberloff, 1996; Allendorf *et al*., 2001). Introduced species can generate a genetic extinction by hybridization (interbreeding of individuals from genetically distinct populations) and introgression (gene flow between populations whose individuals hybridize) with native fauna (Rhymer & Simberloff, 1996; Avise, 2004).

The red-legged partridge (*Alectoris rufa)* is an example of a threatened species, being classified as a Species of European Conservation Concern (SPEC) (Aebischer & Lucio, 1996; Meriggi & Mazzoni della Stella, 2004). This species is in danger for many reasons although over-hunting and intensification of agriculture are the main ones (Aebischer & Lucio, 1996; Negro *et al.*, 2001; Meriggi & Mazzoni della Stella, 2004). In order to sustain the heavy hunting pressure, millions of captive-reared partridges are released every year, especially in southern European countries such as Spain or Italy (Negro *et al.*, 2001; Baratti *et al.*, 2005). Given that other partridge species, particularly *Alectoris chukar*, adapt better to captivity and have higher performances than *A. rufa* (Nadal, 1992), farmers have crossed these two species. Although early studies suggested *Alectoris graeca* introgression in *A. rufa* during captive rearing (Nadal, 1992), no *A. rufa* × *A. graeca* hybrids were identified in such conditions and recent data clearly point to extended hybridization of *A. rufa* with *A. chukar* (Blanco-Aguiar *et al.*, 2008; Martínez-Fresno *et al.*, 2008; Barbanera *et al.*, 2009, 2010). The use of *A. rufa* × *A. chukar* hybrids for restocking purposes may lead to the erosion of the gene pool of wild populations (Meriggi & Mazzoni della Stella, 2004; Baratti *et al*., 2005; Tejedor *et al.*, 2008). When controlling restocking operations, identification of hybrids is often difficult on morphological grounds alone, particularly beyond the first backcross generation (Negro *et al*.,

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2001; Barilani *et al.*, 2007). This fact, along with the scarce control of genetic integrity on cynegetic farms, has made allochthonous lineages common in restocked areas (Rhymer & Simberloff, 1996; Negro *et al.*, 2001; Barilani *et al*., 2007; Blanco-Aguiar *et al.*, 2008; Martínez-Fresno *et al.*, 2008; Randi, 2008; Barbanera *et al.*, 2009, 2010).

Although captive-reared red-legged partridges seem to be of inferior quality to wild ones regarding anti-predatory and mating behavior (Nadal, 1992), there are data about successful reintroduction, establishment and reproduction of these partridges in the wild, reaching breeding densities and reproductive performances similar to those of wild partridges (Duarte & Vargas, 2004; Meriggi & Mazzoni della Stella, 2004; Meriggi *et al.*, 2007). Therefore farm hybrids can backcross to wild red-legged populations in geographical areas periodically reinforced with captive reared partridges.

In this case study we sampled three different estate partitions of a Ciudad Real (Castile La Mancha, Spain) hunting reserve characterized by the different probability of contact with captive-reared partridges, and used the information provided by 22 microsatellite markers. We used these highly variable molecular markers given their successful application in red-legged partridge to detect introgression with chukar (Baratti *et al.*, 2005; Barilani *et al.*, 2007; Tejedor *et al.*, 2007) and to analyze the genetic variability within *A. rufa* populations (Tejedor *et al.*, 2008). The reserve managers delimited these three estate partitions on the basis of the estimated percentage of partridges restocked (Fig. 1): partition (A) with 0% restocking, considered as a wild red-legged autochthonous area; partition (B) where ~70% of partridges were restocked, being considered an intermediate area between autochthonous and restocked partridges; and finally, partition (C) where all partridges had their origin in cynegetic farms. Partridge hunting and restocking only took place in partition C, where the goal was to provide birds for shooting and only 50% of captive-reared partridges gets hunted (Rada, personal communication). Restocked partridges of partition B came from the spread of the individuals released in C given that, even if 50% of birds were hunted, the periodic release of around three to five thousands of captive-reared animals per year made possible that some of them disseminated to neighboring areas. Additionally, we genotyped chukar partridges from farms and commercial meat markets based on the consideration that hybridization occurs with farmed animals and not with wild ones. We assumed partition A as reference of wild *A. rufa* in this case study due to its lower probability of contamination with *A. chukar* genes compared with partitions B and C.

Considering partition A and the farmed chukar samples as references of *A. rufa* and *A. chukar* species respectively, the aims of this case study were to compare the genetic composition of restocked and non-restocked red-legged partridge populations and to check for the existence of *A. rufa* × *A. chukar* hybridization in the A, B and C partition populations using 22 microsatellite markers.

MATERIALS AND METHODS

Sample collection and DNA extractions

The entire sample included 102 individuals. Red-legged partridge populations were sampled throughout a hunting reserve from Ciudad Real (Castile La Mancha, Spain) that covered an area of 12,603 hectares. Three partitions have been created by the managers of the area on the basis of the estimated percentage of partridges restocked (Fig. 1): partition A (n = 24) with 0% restocking, so we could consider it a wild red-legged autochthonous area; in partition B (n = 22) ~70% of total individuals were restocked, and it was considered as an intermediate area between autochthonous and restocked partridges; finally, all partition C (n = 26) partridges came from cynegetic farms.

Around three to five thousands of partridges from several farms were annually released in partition C during the last 11 years and around 50% of the birds were shot just after their release. Ringed individuals were released for a period of two years (2007-08) to estimate the rate of survival and spread of the captive-reared animals. After that period, managers did not detect any ringed partridge in partition A whereas 70% of partridges in B were ringed.

Samples were gathered between October 2008 and March 2009. Feathers were collected from partitions A and B using trap cages at different locations to avoid taking more than one sample from the same flock. In partition C partridges were hunted and liver samples were obtained. In the case of partition B, we only collected feathers from non-ringed individuals in order to determine the genetic structure of the animals that managed to establish and reproduce successfully in this area.

Additionally we genotyped blood samples of chukar partridges (n = 30) from farms (Spanish and Greek) and commercial meat markets. All samples were con-

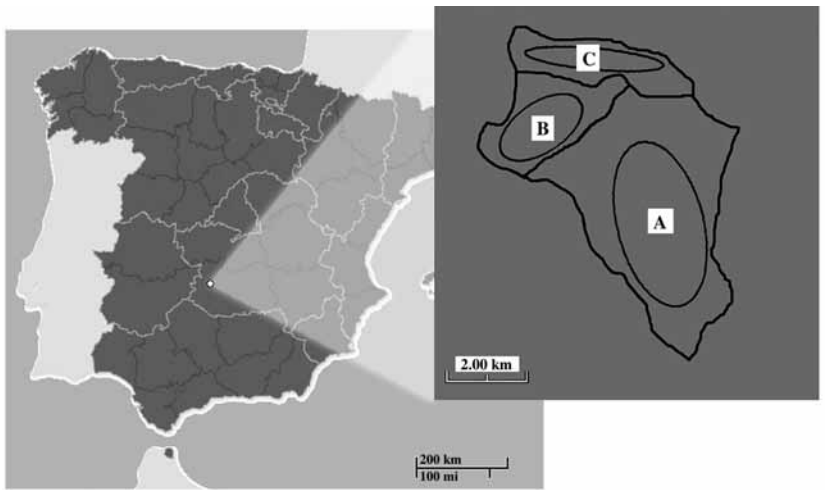

FIG. 1. Red-legged partridge reserve partitions in Ciudad Real (Castile La Mancha, Spain) on the basis of the estimated percentage of partridges restocked.

served in MagicBuffer® (BIOGEN Diagnóstica, Spain) at 5°C and DNA was extracted using a standard phenol-chloroform method (Sambrook *et al.*, 1989).

Laboratory methods

Samples were genotyped by PCR amplifications of 22 microsatellites. Eight of them were originally isolated at Wageningen University from chicken (*Gallus gallus*) genome: MCW118 (PCR annealing temperature T = 55°C), MCW135 (T = 55°C), MCW152 (T = 50°C), MCW225 (T = 45°C), MCW276 (T = 60°C), MCW280 (T = 55°C), MCW295 (T = 55°C), MCW323 (T = 55°C). We also used nine chicken microsatellites previously described by Baratti *et al.* (2005): MCW0043 (T = 50°C), MCW0044 (T = 50°C), MCW0104 (T = 55°C), MCW0121 (T = 55°C), MCW0127 (T = 50°C), MCW0146 (T = 50°C), MCW0199 (T = 55°C), MCW0212 (T = 60°C), MCW0215 (T = 55°C). Finally, we added five partridge microsatellites described by González *et al.* (2005): Aru1.9 (T = 60°C), Aru1.19 (T = 60°C), Aru1.23 (T = 60°C), Aru1.27 (T = 60°C), Aru1.29 (T = 60°C). Reactions started at 94°C for 4 min followed by 34 cycles of 50 s at 94°C, annealing at either primer annealing temperature for 50 s, 50 s at 72°C, and final extension for 10 min at 72°C. PCR amplifications were composed of 1.5 mM $MgCl_2$, 0.25 U of Taq Polymerase (Biotools, Spain), 0.3 mM dNTPs, 0.5 mM of each primer and 10 ng of DNA in a total volume of 5 ml. PCR products were separated by electrophoresis in 8% polyacrylamide gels under denaturing conditions, followed by silver staining (Bassam *et al.*, 1991).

Data analysis

Allele frequencies and observed and expected heterozygosities were obtained using the software GenePop v4.0.7 (Raymond & Rousset, 1995). Fisher's exact test for Hardy-Weinberg (HW) equilibrium across loci and populations was performed using the Markov chain method, as implemented in GenePop v4.0.7. Wright's indices (F_{IT}, F_{IS} and F_{ST}) and mean number of alleles (MNA) were calculated using the GENETIX v4.05 program (Belkhir *et al.*, 2004). The FSTAT software (Goudet, 2001) was used to calculate the allelic richness (AR) per population. The presence of null alleles was calculated for each locus using MICRO-CHECKER v2.2.3 (Van Oosterhout *et al.*, 2004). Unbiased F_{ST} was estimated using the ENA method described in Chapuis & Estoup (2007), as implemented in the software FreeNA.

STRUCTURE 2.2 software (Pritchard *et al.*, 2000) was used to infer population substructure in each partridge population with the admixture model and uncorrelated allele frequencies. This program allows the identification of subpopulations (K) with distinctive allele frequencies from the full dataset without prior information of sampling groups. We made five independent runs for each value of the putative number of subpopulations (K) between 2 and 6, with a burn-in period of 10,000 followed by 100,000 MCMC repetitions.

Factorial correspondence analysis (FCA) was performed to visualize patterns of differentiation between populations using the GENETIX v4.05 program (Belkhir *et al.*, 2004).

RESULTS

Genetic diversity

A total of 159 alleles were detected at the 22 microsatellite loci assessed in the 102 individuals genotyped, with a mean value of 4.9 alleles (ranging from 4.68 for partition A to 5.27 for partition C, Table 1). Among them, 47 private alleles were identified; most of them were species-specific alleles since 25 were found on *A. chukar* partridges. Table 1 shows the values for the observed heterozygosity, the expected heterozygosity under HW equilibrium and the measure of F_{IS} per population. The main microsatellite characteristics and summary statistics per microsatellite are shown in Table S1 (see online supplementary material). The markers for the entire population showed a high number of alleles per marker (mean = 7.4), varying from 2 to 25. Allele frequencies are listed in Table S2 (see online supplementary material).

The results of the Fisher's exact test for HW equilibrium showed significant deviations for all populations. Significant deviations from HWE were detected in markers MCW118, MCW0199, Aru1.23, Aru1.27, MCW0212, MCW280, MCW0043, MCW0044, MCW225, MCW323, MCW135, and MCW146. The null allele test revealed that several loci (MCW0199, MCW0121, MCW0215, Aru1.9, Aru1.19, Aru1.29, MCW295, MCW0127, MCW0043, MCW276, MCW323, and MCW146) showed evidence of null alleles.

Genetic distances

Since the presence of null alleles can underestimate the genetic diversity within populations and, conversely, increase F_{ST} and genetic distance values (Dakin & Avise, 2004), we estimated unbiased F_{ST} using the ENA method described in Chapuis & Estoup (2007) among the four partridge populations (Table 2). The *A. chukar* samples were the most distant with an average distance of 0.37 to all the other populations.

Population structure

The overall genetic differentiation estimated through the F_{ST} value was 0.265 (95% confidence interval, 0.19-0.36), which means that an important amount of the genetic variability is explained by the populations included into the analysis, due mainly to the two species considered. When the Wright's F statistics were calculated without the *A. chukar* samples, the F_{IS} value across loci and populations was 0.084 (95% confidence interval, 0.01-0.16) and the F_{ST} value dropped to 0.054 (95% confidence interval, 0.03-0.09).

The Bayesian analysis used to infer the population structure showed the maximum likelihood of the data when K = 4, matching up with the initial number of

TABLE 1. Number of samples, observed (H_o) and expected (H_e) heterozygosities, *p* values for HW exact tests (SE, standard error), F_{IS} values, mean number of alleles (MNA), allele richness (AR), and unique allele number (UAN) for each of the four partridge populations across 22 microsatellite loci

Sample	Sample size	H_o	H_e	*p*-value	SE	F_{IS}	MNA	AR	UAN
Partition A	24	0.454	0.460	0.0012	0.0003	0.041*	4.68	3.28	5
Partition B	22	0.474	0.500	0.0000	0.0000	0.075*	4.86	3.35	11
Partition C	26	0.432	0.478	0.0000	0.0000	0.128*	5.27	3.28	6
A. chukar	30	0.461	0.537	0.0000	0.0000	0.154*	4.95	3.23	25

Markov chain parameters: Demorization: 1000; Batches: 100; Iterations per batch: 1000
*Values different from 0 at $p < 0.05$

TABLE 2. Unbiased F_{ST} estimates using the ENA method described in Chapuis & Estoup (2007) for each pair of populations (CI 95% in parentheses)

	Partition B	Partition C	*A. chukar*
Partition A	0.032 (0.017-0.049)	0.085 (0.040-0.135)	0.391 (0.291-0.512)
Partition B		0.040 (0.011-0.080)	0.363 (0.261-0.482)
Partition C			0.361 (0.273-0.469)

TABLE 3. Proportion of membership of each partridge population assigned to each cluster when K = 2, 3 and 4

Sample	2 inferred clusters		3 inferred clusters			4 inferred clusters			
	1	2	1	2	3	1	2	3	4
Partition A	0.998	0.002	0.938	0.003	0.059	0.131	0.803	0.064	0.003
Partition B	0.997	0.003	0.699	0.003	0.298	0.547	0.188	0.263	0.003
Partition C	0.985	0.015	0.105	0.010	0.885	0.088	0.067	0.832	0.012
A. chukar	0.005	0.995	0.005	0.988	0.007	0.005	0.006	0.007	0.982

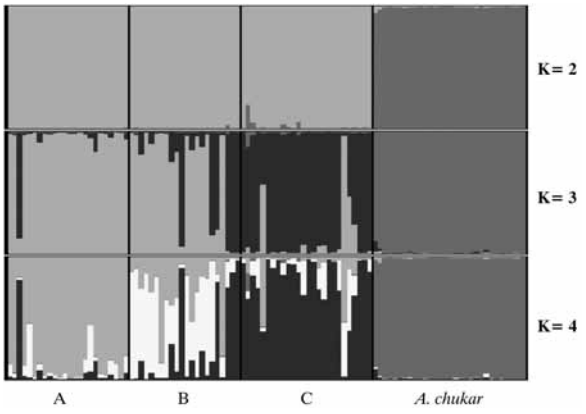

FIG. 2. Membership of each population to K inferred clusters (K = 2-4) using the STRUCTURE software.

populations assumed. The estimated membership fractions of populations for K = 2, 3 and 4 were represented in Figure 2. With K = 4, chukar partridges were assigned to an exclusive cluster with an average proportion of membership of 0.98, similar to those obtained when K = 2 and 3 (Table 3). Regarding red-legged populations, samples from partition C formed clusters on their own when K = 3 and K = 4. Partitions A and B shared one genetic origin when the number of inferred clusters was 3. However, when 4 clusters were considered, the previous common genetic origin split into two clusters, whereas the percentage of membership to the partition C cluster remained constant for both A and B (0.059 *vs* 0.064 and 0.298 *vs* 0.263, respectively) (Table 3).

Applying a similar criterion to that of Vähä & Primmer (2006), in our case with CI 85% and genome percentages > 85%, the information derived from the molecular data analyzed, using the clustering model-based method (Pritchard *et al.*, 2000) and assuming 2 clusters (K = 2), allows us to evaluate the existence of hybridization between *A. rufa* and *A. chukar* partridges. With this criterion only one partridge belonging to partition C shows a hybrid genome (*A. rufa* genome percentage of 80%).

Factorial Correspondence Analysis

The first two axes contributed 77.5% and 14.1% of the total inertia, respectively (Fig. 3). Axis 1 separated the red-legged populations from the chukar partridges, while red-legged populations span across Axis 2. Taking into account the position of the chukar partridges, which had an inertia of 70.3%, we repeated the analysis excluding them. This caused a radical change in the results (Fig. 4), which created a zooming-in effect on the red-legged populations and thus facilitated our ability to interpret the findings. In this case, Axes 1 and 2 contributed 100% of the total inertia, with 63% and 37% respectively, and the A, B and C partitions followed one another through Axis 1, with partition B appearing closer to partition A than to C.

DISCUSSION

In the present case study, genetic diversity and population structure of four populations belonging to two partridge species have been inferred using the molecular information derived from 22 microsatellite loci.

The four populations studied have observed heterozygosity values ranging from 0.43 to 0.47, similar to those reported by Chen *et al.* (2006) in *A. magna* natural populations, and slightly higher than those reported by Randi *et al.* (2003) in *A. graeca* natural populations or by Baratti *et al.* (2005) in an *A. rufa* reintroduced population. There are no outstanding differences between the values of allele richness among populations and these are consistent with gene diversity.

Partition C and *A. chukar* samples showed positive F_{IS} values; however, those shown by partitions A and B, even if statistically significant, are not far from zero, indicating that mating is close to panmixia. There are several causes, not mutually exclusive, to explain the significant departures from Hardy-Weinberg equilibrium such as the presence of null alleles, strong

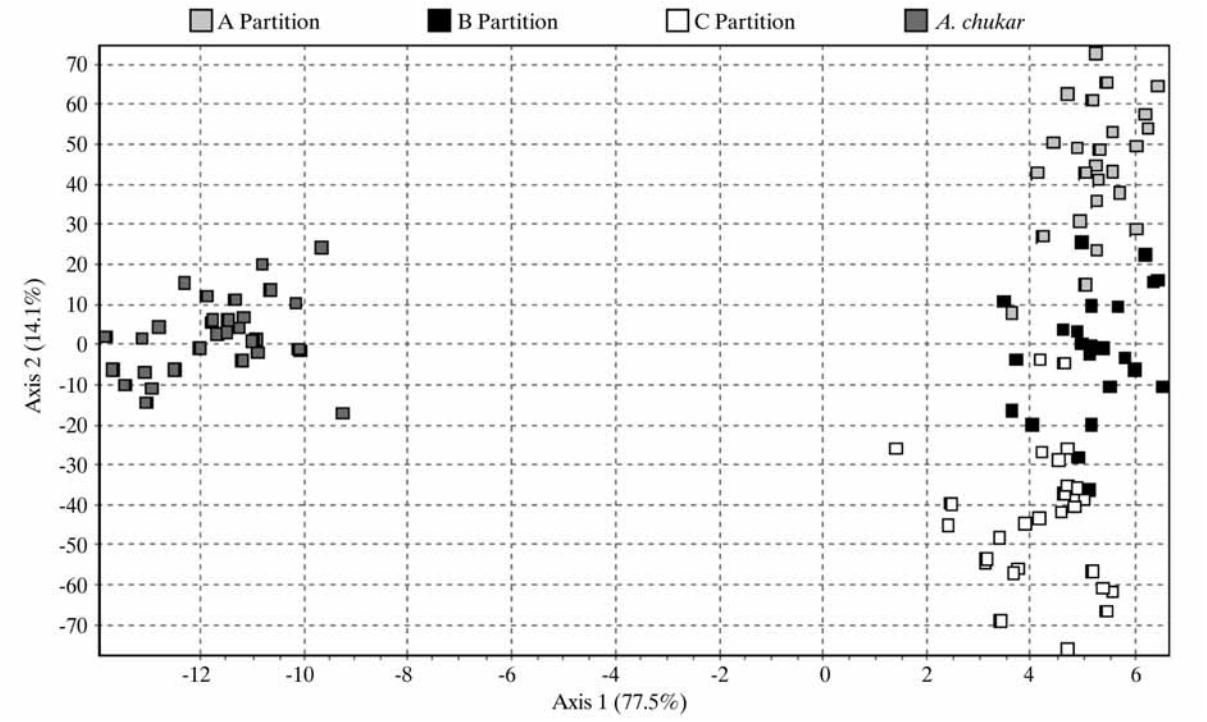

FIG. 3. Spatial representation of the factorial correspondence analysis (FCA) of individual genotypes from four partridge populations obtained with FCA 3D by population (GENETIX v4.05).

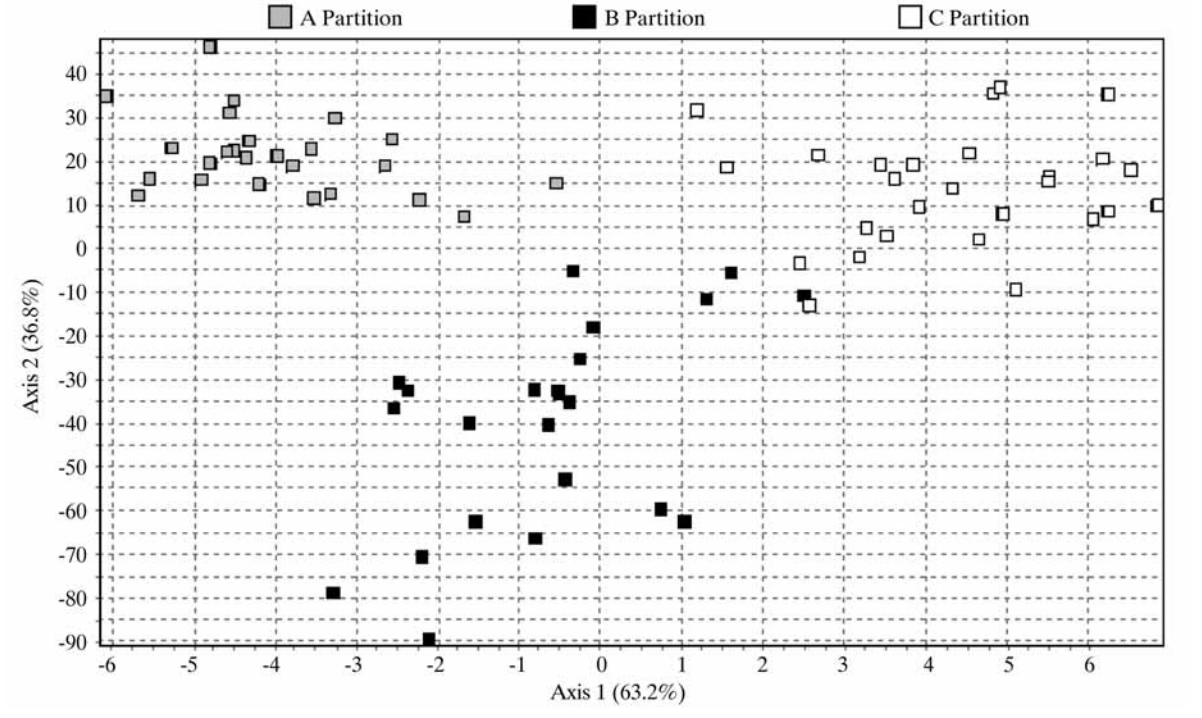

FIG. 4. Spatial representation of the factorial correspondence analysis of individual genotypes from three *A. rufa* populations obtained with FCA 3D by population (GENETIX v4.05).

selection effects affecting restocked partridges, periodic demographic fluctuations and/or re-introductions.

The information obtained with the Bayesian clustering procedure gives an idea of the gene flow between the studied populations. The number of groups that maximize the likelihood of the data with the STRUCTURE program was 4, one for each population sampled *a priori*. The allelic frequencies of the partition C population always keep this group apart from the others, as expected for a captive-reared stock. When the number of clusters was reduced from 4 to 3, partitions A and B clustered together, showing higher genetic proximity between them than between any of them and partition C. These results were confirmed by the FCA which is a multivariate exploratory method, since no genetic model is assumed, such as the Hardy-Weinberg equilibrium or the models that assume the absence of linkage disequilibrium (Moazami-Goudarzi & Laloe, 2002) that allows the representation of genetic relationships among populations taking into account the effects of admixtures between branches (Lebart *et al.*, 1984). This analysis places the *A. rufa* populations far away from the *A. chukar* samples, although some partition C partridges plot towards the chukar distribution (Fig. 3). Partitions A and C are clearly separated whereas samples from B partition appear widespread across Axis 1 (Fig. 4) but mainly plotting towards partition A distribution, which is coincident to the results obtained with the Bayesian analysis and allows more robust conclusions.

Despite managers estimation for restocked partridges in partition B (~ 70%, which would correspond to the ringed individuals released throughout 2007 and 2008 in partition C and disseminated to B), we recognize proximity of partition B to A which indicates the reduced level of hybridization between the wild and the restocked individuals. The molecular data inferred from partition C shows some degree of genetic isolation from wild partridges, demonstrating high genetic distance to partition A and clustering on their own. The occurrence of the *A. chukar* genome in this population supports the hypothesis of a certain level of hybridization with that of foreign species on cynegetic farms.

CONCLUSIONS

These results reveal the efficiency of the marker set used to detect hybridization between two *Alectoris* species, and to differentiate among closely related *A. rufa* partridge populations. Although the results obtained in this case study correspond to a particular situation and can probably not be applied to all other hunting reserves, it seems that restocking produced for shooting purposes has little genetic impact on the partitions as demonstrated by the reduced hybridization in partition B which allows it to be closer to A than to C. This suggests that most of the restocked partridges introduced in C do not survive and do not hybridize to the wild autochthonous individuals. Nevertheless, care should be taken when applying genetic control of reproductive-bred individuals in hunting estates and on farms before restocking to avoid a possible, although small, introgression (specially due to the evidence of hybridization with *A. chukar* partridges on cynegetic farms); the control and hybrids removal in farms is recommended to guarantee the reintroduction of partridges of known genetic origin in each area and decrease any possibility of introgression in the wild populations.

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