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The Sicilian rock partridge: latest data on genetic integrity from four different relict areas

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Abstract: Sicily (Italy) hosts a "relict", endemic population of the birds *Alectoris graeca whitakeri* commonly known as Sicilian Rock Partridge. In the last decades, due to the risk of restocking with other European and Asiatic species for hunting purpose, a study was carried out to investigate the potential risk of hybridisation. The mtDNA control-region and nuclear microsatellites were genotyped. Due to the importance of the species, samples were mainly characterized by feather and stool samples, and rarely by carcasses found in the environment, from year 2011 to 2012. A panel of 7 microsatellite loci was validated. Three multiplexes that allowed the simultaneous amplification of 3 microsatellites, and 2 for other two microsatellites, for a total of 7 markers, were utilized. Results showed the occurrence of hybridization both towards the Middle Eastern species, *A. chukar* and the Northern European species, *A. rufa*. A total of 18.5% of the samples were collected from the wild environment showed a high degree of hybridization. This fact, even if linked to a small number of samples, highlights a potential risk of hybridization in 4 Sicilian provinces and underlines the importance of further investigations to understand the entity of the problem.

Key words: *Alectoris*, genetic diversity, microsatellites, Sicily

1. Introduction

The genus *Alectoris* includes seven recognized species distributed in southern Europe, Northern Africa, Asia, and Arabian Penisula (Madge & McGowan, 2002; del Hoyo et al., 2014). Three species are native to Italy: *A. barbara* in Sardinia, *A. rufa* (red-legged partridge) occurring the northern Apennines and central Italy and *A. graeca* (the rock partridge) whose distribution includes the Alps, central Italy, and southern Italy. While those from the Alps prefer high altitudes, its distribution areas in southern regions are rocky areas and barren plains. Sicily (Italy) hosts a "relict", endemic subspecies as *A. graeca whitakeri* (Schiebel, 1934), commonly known as Sicilian Rock Partridge (Randi and Lucchini, 1998; Randi et al., 2003, Randi, 2006). *A. graeca* is considered 'near threatened' by the International Union for Conservation of Nature (IUCN), but the Sicilian subspecies is even more endangered based on the IUCN Red List rating of endangered species (Rondinini et al., 2013). *A graeca whitakeri* has been included in Annex I of the 'Birds Directive' (79/409/EEC) as well as Annex III of the Berne Convention, and, in recent years, all subspecies of *A. graeca* have been included in Annex I of the 'Birds Directive' (2009/147/EEC).

The Sicilian Rock partridge has been declining over the last 50 years due to land use changes (intensification of agricultural practices, urban expansion), overharvesting (illegal hunting) and increasing frequency of wildfires (Palumbo and Lo Valvo, 2002). Following the huge increase of boar *Sus scrofa* populations recorded in recent years, this species has been indicated as possible source of impact on rock partridge (Monaco et al., 2010). During rooting activities, this mammal might cause the loss of brood and eggs of birds nesting on the ground such as rock partridge.

Rock partridges are sedentary birds, they do not move/fly for long distances and suffer from habitat fragmentation due to urbanisation. This results in decreasing gene flow and, hence, increasing genetic drift (Sorace et al., 2013). Also, Sicilian Rock partridges suffer from genetic pollution due to hybridization with other partridges of the *Alectoris* genus raised in captivity (Randi et al., 2003); these include Chukars and Rock partridges illegally introduced for hunting purposes.

In the past, in Sicily, with the aim to increase the bird population for hunting purpose, repeated restocking actions, not utilizing the endemic species but with other *A.*

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graeca farmed in the mainland, occurred. These nonnative *Alectoris* could potentially mate with the local subspecies, generating hybrids, and this can cause a big problem in term of conservation of autochthonous/local genotype.

Asian rock partridge (*A. chukar*) was referred to be the species mainly utilized for constant hunting restocking in some areas of Sicily and, as a matter of fact, that it is the species most frequently identified in the genome of hybrid rock partridges (Barbanera et al., 2009). Several studies reported introgression of *A. chukar* gene in most European red-legged partridge populations (Tejedor et al., 2007; Barilani et al., 2007; Martínez-Fresno et al., 2008; Barbanera et al., 2009; Casas et al., 2012).

Today, the few remaining couples are a unique example of biodiversity heritage of the Sicilian fauna, which is protected by special conservation actions with the hope to increase a relict population very close to extinction. To prevent the species decline, precise actions concerning the conservation of the local population has started during the 2010 within projects as the SPA "ITA010029 Monte Cofano, Capo San Vito e Monte Sparagio", with the financial support of LIFEplus (LIFE09 NAT/IT/000099-SICALECONS)-"Urgent actions for the conservation of *Alectoris graeca whitakeri*".

As part of LIFE project, a laboratory action to investigate the genetic integrity of this population was implemented. The aim of this paper is to investigate the Sicilian Rock Partridge population using microsatellites data for deepening their genetic status, in terms of variability, genetic structure, and essential condition for future survival of this relict *Alectoris* population.

2. Materials and methods

2.1. Sampling

The study was carried out between 2011 and 2012. It concerned two years collection of samples of feathers and feces of Sicilian rock partridge in Monte Cofano, Capo San Vito and Monte Sparagio located in the northwestern coast of Sicily (Trapani province) or from dead individuals coming from the same protected areas. From this area, 10 samples were collected during the winter and considered for the study. Moreover, 17 previous sampled Sicilian rock partridge populations belonging to five different districts (Agrigento, Caltanissetta, Enna, Messina and Palermo) and two samples of *A. chuckar* from Palermo were considered for comparison purpose. Samples taken in the Sicilian territory will be referred to as population one (POP1), while the two samples of *A. chukar* as population two (POP2).

2.2. Laboratory procedures

DNA was extracted from feathers and tissues by PureLink Genomic DNA Mini Kit (Invitrogen) and from feces samples by QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The entire mitochondrial DNA control-region (mtDNA CR) was PCR-amplified using the external primers PHDL and PHDH (Randi and Lucchini, 1998, Randi et al., 2003).

In order to obtain data on the current genetic diversity of species, molecular step was carried out through a set of microsatellites as specific molecular markers. The protocol concerned a preliminary phase to optimize a panel of 7 microsatellite loci (MCW118, MCW135, MCW152, MCW225, MCW276, MCW295, MCW323) originally isolated from the genome *Gallus gallus* (http://www.zod.wau.nl/abg/index.html) (Table 1). This approach was obliged because of the lack of specific references on microsatellites test available for the species *Alectoris graeca whitakeri*. For this reason, whole study was based on what previously published by Barilani et al. (2007).

Specifically, after determination of the amplification conditions for each single marker, we tested the composition of several microsatellites into multiplex either for amplification and for loading, depending on temperature of annealing, amplification range and specific fluorescence dye linked to the primer. Marking different microsatellites with different fluorochromes allowed their discrimination according to the number of loci grouped in the electrophoretic run (multiplex), also in case of the range overlaps. At the end, 3 multiplexes that allowed the simultaneous amplification of 3 microsatellites, and 2 for other 2 microsatellites, for a total of 7 markers, were implemented. Amplification tests using the AmpliTaq Gold DNA Polymerase (Applied Biosystems) were allowed to define the following thermal profile: 10 min at 95 °C followed by 40 cycles of 45 s at 95 °C, 50 s at 50 °C, 1 min at 72 °C, and finally 20 min at 60 °C. The size standard used was the GeneScan 500 ROX dye size standard (Applied Biosystems). The PCR products were subsequently analyzed by direct sequencing in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), and results were analyzed by GeneMapper v.3.7 software (Applied Biosystems). All 7 microsatellites showed positive amplification and subsequently were enrolled for the analysis. All loci were compared to each other in all populations and demonstrated to be in linkage equilibrium.

2.3. Microsatellite data analyses

Data obtained from the analysis of electrophoretic samples were used to estimate the genetic diversity within the Sicilian Rock Partridge population. Summary population genetic statistics, as total number of observed alleles and the mean number of alleles for locus, observed and expected heterozygosity, polymorphic information content (PIC) were estimated in the two populations using Cervus version 3.0.3 (Kalinowski et al., 2007). The deviation from Hardy–Weinberg equilibrium (heterozygote deficiency)

Table 1. Microsatellite markers with corresponding fluorescent dye, fragment size, and annealing temperature (Ta).

Locus	Dye	Size	Ta (°C)
MCW118	FAM	140-170	55 °C
MVW152	FAM	180-200	50 °C
MCW295	FAM	70-100	50 °C
MCW276	HEX	210-240	60 °C
MCW323	HEX	110-130	50 °C
MCW135	NED	110-130	55 °C
MCW225	NED	170-190	45 °C

was calculated with the GENEPOP package version 4.0.11 (Raymond and Rousset, 1995) using a Markov Chain method (dememorization 10,000, batches 100, and iterations per batch 5000). STRUCTURE version 2.3.1 (Pritchard et al., 2000) was used to analyze the genetic structure and identify the true number of populations (clusters) and assign the individuals to each cluster. The program estimates the natural logarithm of the probability that a given genotype (G) is part of a given population (K). The model used assumed admixture and correlated allele frequencies as suggested by several authors (Pritchard et al., 2000; Falush et al., 2003). The Ln Pr(G|K) was calculated for K ranging from 1 to 4, without prior information on the breed of origin, to estimate the most likely number of clusters in the dataset. All simulations were replicated 10 times, each one with 105 iterations, following a burn-in period of 104 iterations. We determined the best K using the Evanno method (Evanno et al., 2005), based on ΔK calculated using pophelper package (Francis, 2017). Individuals were assigned to one cluster if their proportion of membership estimate (q) to that cluster was equal to or greater than 0.9. Otherwise, they are presumed to be admixed with other clusters. Finally, Genetix version 4.03 (Belkhir et al., 1996) was used to perform factorial correspondence analysis (FCA) based on the individual multilocus and genetic distances (Nei minimum distance).

3. Results

Number of alleles, observed and expected heterozygosity and PIC for each marker and overall loci are shown in Table 2. All markers were polymorphic with number of alleles ranged from 2 to 7 for MCW152 and MCW276, respectively. MCV135 and MCW152 showed an observed heterozygosity equal to 0, while it was between 0.04 and 0.87 for MCW295 and MCW276 with an overall mean of 0.27.

Assignment test was performed using the STRUCTURE software with the number of expected population (K) ranging from 1 to 4. The most likely number of K was estimated by comparing the log-likelihood of each K-value. For K=2, the Ln Pr(G|K) was maximized and also mean variance of the Ln Pr(G|K) estimates was the lowest one (Figures 1 and 2). The proportion of membership of each sampled bird is showed in Table 3. A total of 17 out of 27 (63%) partridges sampled from Sicily were assigned to cluster 2 with a proportion ranging from 0.96 to 0.99. In the same cluster of *A. chukar*, five samples (18.5%) were assigned with values ranging from 0.95 to 0.99. The five remaining individuals (18.5%) from the Sicilian partridge's samples were not assigned to a specific cluster, thus they were considered to be hybrids. These hybrid birds were sampled in the provinces of Palermo (n = 3), Agrigento (n = 1), and Trapani (n = 1). For a hybrid sample taken in the province of Palermo with a proportion of membership of 0.35 and 0.65 to cluster 1 and 2, respectively, the sequence of domain 1 of the mtDNA control region was available (Randi et al., 2003), and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for homologous sequences in nucleotide databases. A total of 134 sequences

Table 2. Genetic variability measures in rock partridge across 7 microsatellite markers.

Locus	Allele Range	# of alleles	H_{obs}	H_{exp}	PIC	F null
MCW118	145–157	4	0.33	0.55	0.45	0.2309
MVW152	181–183	2	0.00	0.08	0.08	0.6831
MCW295	83–91	3	0.04	0.41	0.36	0.8399
MCW276	198–215	7	0.87	0.74	0.68	–0.1112
MCW323	109–125	4	0.33	0.43	0.38	0.0778
MCW135	115–121	3	0.00	0.45	0.40	0.9983
MCW225	154–180	6	0.32	0.72	0.66	0.3750
Overall mean		4.14	0.27	0.48	0.43	0.4419
st. dev.		1.77	0.31	0.22	0.20	0.4110

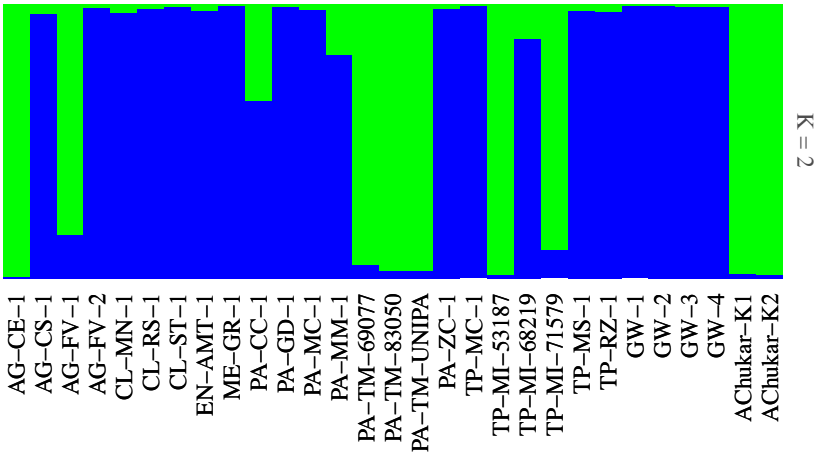

Figure 1. Estimated proportion of membership for each individual represented by a single vertical line into *K = 2* predefined cluster

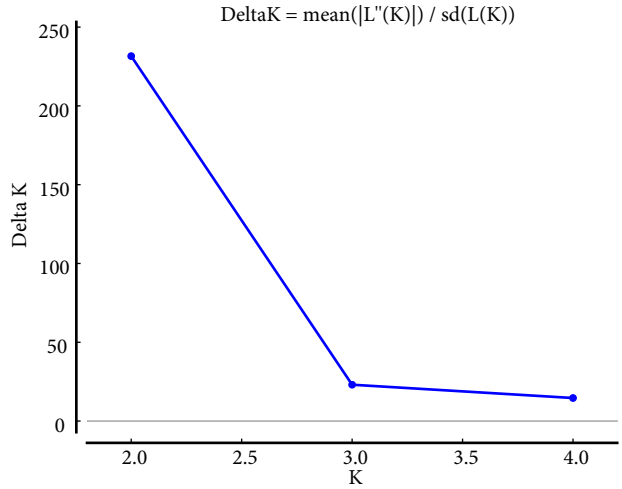

Figure 2. Plot of delta *K*-values for each *K* from 2 to 4.

belonging to the species *Alectoris* were found, of these 83 were classified as *A. chukar*, 25 as *A. rufa*, while only 8 as *A. graeca*.

Finally, genetic relationship was investigated by FCA that was performed including all samples and using the corresponding individual multilocus genotypes. The three principal factors explained the 49.43% of the overall variation. The results showed that the two populations formed nonoverlapping clusters. Axis 1 that explains the 23.20% of total variation clearly separates one sample of partridge (AG-CE) collected in the Agrigento province. Also, for this sample, the sequence of domain 1 of the mtDNA control region was available, and BLAST was used to search for homologous sequences in nucleotide databases. A total of 144 sequences belonging to the species *Alectoris* were found, and 127 of these were classified as *A. chukar*, 5 as *A. rufa* and 10 as A. rufa x *A. chukar*.

4. Discussion

Observed heterozygosity of MCW295 and MCW276 was in contrast with that reported by Tejedor et al. (2008) who measured values of 0.60 and 0.30 in 4 captive-reared red-legged partridges. Same authors (Tejedor et al., 2007) for MCW295 and MCW276 microsatellites reported an observed heterozygosity other than zero in a population of *Alectoris* rufa in the Island of Majorca. In this study, each microsatellite showed a different value of PIC ranged from 0.08 and 0.68 for MCW152 and MCW276, respectively, with an overall value of 0.48. The eight microsatellites analyzed by Randi et al. (2006) were also polymorphic, comprising 63 alleles in total, with an average of 7.9 alleles per locus. Heterozygosity was in the range of 0.20 (HO in Sicily) to 0.48 (HE in Albania). Partridges from Sicily showed the lowest values of HE and HO, suggesting that they evolved in isolation at small effective population size for many generations.

Table 3. Proportion of membership (q) for each of sampled partridge for K = 2 (AG: Agrigento, CL: Caltanissetta; EN: Enna; ME: Messina; PA: Palermo; TP: Trapani).

Sample	Localities	Cluster 1	Cluster 2
1	AG	**0.993**	0.007
2	AG	0.040	**0.960**
3	AG	0.838	0.162
4	AG	0.017	**0.983**
5	CL	0.033	**0.967**
6	CL	0.019	**0.981**
7	CL	0.010	**0.990**
8	EN	0.026	**0.974**
9	ME	0.008	**0.992**
10	PA	0.352	0.648
11	PA	0.010	**0.990**
12	PA	0.023	**0.977**
13	PA	0.183	0.817
14	PA	**0.946**	0.054
15	PA	**0.970**	0.030
16	PA	**0.970**	0.030
17	PA	0.017	**0.983**
18	TP	0.008	**0.992**
19	TP	0.008	**0.992**
20	TP	0.010	**0.990**
21	TP	0.011	**0.989**
22	TP	0.009	**0.991**
23	TP	**0.983**	0.017
24	TP	0.131	0.869
25	TP	0.890	0.110
26	TP	0.024	**0.976**
27	TP	0.027	**0.973**
A. chukar		**0.985**	0.015
A. chukar		**0.988**	0.012

The birds used for restocking the Sicilian population are either *A. chukar* or the continental subspecies of *A. graeca*. Besides *A. rufa* is a potential candidate for hybridization with the native Sicilian partridge to the presence of this species in Italy (Tejedor et al., 2007; Barilani et al., 2007; Martínez-Fresno et al., 2008; Barbanera et al., 2009; Casas et al., 2012). In the current study, there are only two *A. chukar* samples apart from the Sicilian supposedly *A. graeca* because of the difficulties on getting additional samples. In order to detect hybridization and the origin of these events, it would have been easier to have reference samples from all potential candidate species and subspecies involved. (Randi, 2006, Tejedor et al., 2008)

Although a reduced number of samples was considered in this study, considering the high scientific significance of the species classified as endangered by IUCN, the results showed the presence of hybridization both towards the Middle Eastern species *A. chukar* and the endemic one of the Northern Europe *A. rufa*. In particular, 5 out of 27 samples (18.5%) were found to be hybrids. And these individuals came both as carcasses and biological samples from the wild environment (nature reserves, protected areas, and in some cases phenotypically selected as potential reproducers for the repopulation of the species). This fact, even if linked to a small number of samples, highlights a potential risk of hybridization in 4 Sicilian provinces and hopes for further investigations to better understand the extent of the problem.

Molecular methods proved invaluable in the identification of the hybrid *Alectoris* because identification of hybrids based on phenotypic external appearances can be misleading. Areas where different populations of partridge are sympatric need to be assessed for the level and extent of hybridization occurring and, thus, need to be managed in order to protect the genetic integrity of 'pure' Sicilian Rock Partridge populations.

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Conflicts of interest
The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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