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RH: Genetic landscape of red deer in Andalusia

The genetic landscape of the Iberian red deer (*Cervus elaphus hispanicus)* after 30 years of big-game hunting in southern Spain.

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ABSTRACT

The Iberian red deer (*Cervus elaphus hispanicus*) suffered a striking collapse of its populations during the first half of the 20th century due to excessive hunting. In Andalusia, southern Spain, re-colonization took place from a few relict populations through natural dispersal, and through artificial reintroductions for big-game hunting. It is unclear how the population decline impacted genetic diversity, and what is its current distribution after the re-colonization and intensive hunting practices. Here, we address these questions by analysing nuclear microsatellite variability from 58 red deer populations distributed throughout Andalusia. Our results showed a relatively high genetic variability spatially structured into five clusters, corresponding to the locations of relict populations. This suggests that the red deer's current genetic background has presumably retained much of the genetic variation present in those relict populations. We also found that an important portion (32%) of the populations displays some degree of inbreeding. We suggest that new herds should be established using individuals from the different genetic clusters, and a careful monitoring of the breeder's genetic background to prevent further inbreeding and inadvertent hybridisation. Failure to do so could lead to loss of genetic diversity and the dilution of the genetic identity of the Iberian red deer.

Key Words: Andalusia, *Cervus elaphus*, Microsatellite, Genetic diversity, big-game, Hunting, Red Deer.

The red deer (*Cervus elaphus L.*) is one of the most important and widely distributed big-game species in Europe today, with an intensive anthropogenic management of its populations throughout its history and distribution (Milner et al. 2006). In the Iberian peninsula, one of its subspecies; the Iberian red deer (*Cervus elaphus hispanicus*) suffered a severe decline of its populations during the first half of the 20th century due to excessive hunting (De Leyva 2002). Only a few marginal populations remained unaltered in Montes de Toledo, central Spain, and Sierra Morena, Andujar, Despeñaperros, and the Doñana National park in Andalusia, southern Spain (Soriguer et al. 1994, Crespo 2013). After a significant economic growth during the 1960s, and the introduction of a hunting law in 1970, re-colonization began throughout Andalusia through natural dispersal, but also through anthropogenic reintroductions motivated by an emerging big-game hunting economy (Soriguer et al. 1994). Presently, hunting enclosures comprise 75% of the areas dedicated for big-game hunting in Andalusia (Andalucia 2009). Hence, the current distribution of red deer in Andalusia is the product of both, natural and artificial expansion processes experienced during the last three decades.

It is unclear how the population collapse impacted genetic diversity, and if intensive management has contributed to reduce genetic variation. It has been shown that enclosures, and other anthropogenic activities such as forest clearings, and motorways can be major threats to red deer's genetic diversity (Harris et al. 2002, Hartl et al. 2003, Milner et al. 2006). Reductions in genetic diversity can have important consequences such as inter-population divergence, and a reduced potential to cope with environmental changes (Frankham 1995). Therefore, determining the levels of genetic diversity of reintroduced or recovering populations is of great importance in informing conservation-management strategies (Hajji et al. 2008, Cronin et al. 2009). Moreover, identifying the spatial

distribution of such genetic diversity allows managers to delineate discrete conservation and management units (Manel et al. 2003). Here, we aim to evaluate the levels of genetic diversity of the Iberian red deer throughout Andalusia, and to identify the current spatial distribution of its genetic diversity.

STUDY AREA

Samples were obtained from 1309 adult Iberian red deer shot over three hunting seasons (2003-2006) along different points of Andalusia (Fig. 1). In total, 58 pre-defined populations were analysed from different locations throughout Andalusia with a mean of 22.6 samples/population. Sampling effort was focused along the Sierra Morena system (Huelva, Sevilla, Córdoba and Jaen provinces), Doñana National Park and Cazorla Natural Reserve, as well as in the mountains of Cadiz where the density of red deer populations and hunting activity are the highest (Table 1). We also obtained samples from two populations of the province of Granada where the red deer is currently expanding (Granados et al. 2001)

METHODS

Two types of tissue were collected: tongue (1270 samples) and antler bone (39 samples). Genomic DNA was extracted from tongue tissue through a Hot Sodium and Tris (HotSHOT) protocol (Truett et al. 2000) and from antler bone following a Silica protocol (Milligan 1998). Genotyping was performed at 11 microsatellite loci previously isolated in other ungulates: TGLA94 (Georges et al. 1992), OarFCB193, OarFCB304 (Buchanan and Crawford 1993) CSSM43 (Barendse et al. 1994) , BM302, BM203 (Bishop et al. 1994), RT1, RT13 (Wilson et al. 1997) , NVHRT48, NVHRT73 (Røed and Midthjell 1998), MB25 (Vial et al. 2003). These markers were co-amplified using four multiplex polymerase chain reactions (PCR) as described in (Sánchez-Fernández et al. 2008). Fragments were resolved

on an ABI Prism 3100 Genetic Analyser (Applied Biosystems) and scored using GENEMAPPER v 3.7 software (Applied Biosystems).

Deviations from Hardy-Weinberg expectations (HWE) and linkage disequilibrium were evaluated according to the level of significance determined by means of 10,000 MCMC iterations using GENEPOP software v.3.4 (Raymond and Rousset 1995). Bonferroni corrections were applied for multiple comparisons (Rice 1989). The software MICROCHECKER (van Oosterhout et al. 2004) was used to infer the most probable cause of departures from HWE (null alleles, large allele dropouts or stutter bands). The level of genetic diversity within each population was characterized by calculating expected heterozygosity (HE) using Arlequin v.2.0 (Schneider et al. 2000) , as well as by inbreeding coefficients (*FIS*) calculated in GENEPOP v.3.4, and allelic richness (RS), which quantifies the number of alleles independently of sample size using FSTAT (Goudet 1995).

To characterise the spatial distribution of genetic diversity throughout Andalusia we used GENELAND (Guillot et al. 2005). This program makes use of a geographically constrained Bayesian model to estimate the number of populations (*K*) taking into account the spatial position of sampled multilocus genotypes without any prior information on the number of populations and degree of differentiation between them. Geographic coordinates for each population were determined by GPS and digital maps. Individual coordinates were then assigned to each sample by allowing a 5 km. coordinate uncertainty when running the clustering algorithm. The Dirichlet distribution was set as prior for allele frequencies with 40,000 MCMC iterations using spatial information only. Then, the algorithm was rerun with an additional 40,000 MCMC iterations, setting the Poisson processes equal to the number of samples. The results were graphically displayed by fitting the map of posterior membership

probabilities to a geographic map of Andalusia using the mapping toolbox in MATLAB (Mathworks).

RESULTS

Measures of genetic diversity calculated from observed allele frequency distributions are presented in Table 1. The locus CSSM43 was removed from further analysis due to stuttering issues. A small percentage (8.5%) of the tongue tissue samples had to be re-amplified due to technical errors during batch pipetting. The DNA recovered from this tissue, however, was of good quality and quantity, as verified by agarose gel electrophoresis. On the other hand, recovered DNA from the antler bone tissue was of inferior quality and quantity. Therefore, all samples (39) were genotyped twice at all loci to check for consistency in amplification. Discrepancies between scorings of both amplification rounds were observed in two samples, for which, all loci were amplified individually (i.e. not in multiplex) and scored. In the final database, 85 samples (6.4%) were missing data from one locus, and only two samples (0.15%) were missing data from two loci.

We found no linkage disequilibrium between any locus pair. However, significant deviations from HWE within populations and loci were observed. Out of 580 tests performed, 40 remained significant after Bonferroni correction, 13 of which occurred at locus RT13. Departures from HWE may be caused by several factors such as inbreeding, population sub-structuring (i.e. Wahlund effect) and the presence of null alleles caused by technical issues. Inbreeding or population sub-structuring should be reflected in consistent deviations across most or all loci, whereas null alleles caused by technical causes such as misscoring or poor amplification should result in variable deviations across loci and

158 populations (Purcell et al. 2006). Results from Microchecker software indicated the presence
159 of null alleles occurring at one locus (RT13) across all populations. Therefore, this locus was
160 removed from all subsequent analysis. The rest of loci showed random patterns of deviation
161 across populations and thus were kept in the final marker set. Overall, genetic diversity was
162 relatively high. The mean number of alleles/locus/population ranged from 5.5-9.6, whereas
163 allelic richness ranged form 5.5 to 8.5 effective alleles/locus/population and the expected
164 heterozygosity/population ranged from 0.696 to 0.829 (Table 1). Estimates of F_{IS} ranged
165 from -0.010 to 0.127 (Table 1) with 32% of the populations showing significant values
166 (Table 1).
167
168 The Bayesian clustering algorithm showed a clear mode at K=5 along the MCMC
169 chain with the highest mixing occurring around this value (Figs. 1S,2S supporting
170 information). This indicates five different genetic clusters present in the dataset. The
171 populations of Ag, Rb, Cc, Ng, Pd, Tj, Al, Am, Jt, and Ps formed one cluster around the
172 province of Cadiz (Fig. 1). Populations along the Sierra Morena were longitudinally divided
173 into three different clusters. The oriental part of Cordoba province (Co, Gm, Oz), and part of
174 Jaen province (Tm, Sm, Aa, Sd, Fn) comprise a single cluster, including populations from
175 the natural reserves of Cardeña-Montoro and Andujar. Interestingly, the population from
176 Huelva (Ae) was also assigned to this cluster. The main cluster in the Sierra Morena
177 included populations from the province of Seville together with the occidental and central
178 parts of Cordoba (Ac, Cq, Cu No, En, Cr, Gt, Cd, Pi, Nb, Pa, Nh, Cs, Pl, Ad, Ct, Lc, Aj, Ab,
179 Ms, Mn Au, Hl, Pt, Ht). Two populations from Granada (Ca, Fr) and one from oriental Jaen
180 (Cz) also clustered within this main cluster. A separate cluster was formed by the
181 populations from Despeñaperros (Sn, Ti, Jn, Ch, Vz) , in the province of Jaen, whereas the
182 population from the Doñana national park (Dn) formed its own cluster (Fig. 1).

DISCUSSION

The results from our study indicate that allele diversities and expected heterozygosities are relatively high in Andalusia and within the range of values reported for red deer (Kuehn et al. 2003, Feulner et al. 2004, Hmwe et al. 2006, Zachos et al. 2007, Queiros et al. 2014). However, the high heterozygosity observed in the majority of the populations analysed differed from a previous microsatellite-based study performed in the Extremadura region (Southwest Spain), where most of the Iberian red deer populations analysed revealed a heterozygosity deficit (Martinez et al. 2002). A possible explanation for such a discrepancy may be found in the low number of markers analysed (6) as well as in the reduced number of populations (17) sampled by the previous study. However, our results are concordant with a more recent study carried out in the Extremadura and Andalusia regions (Pérez-González et al. 2012), where the Andalusia populations showed similar heterozygosity levels to those found here.

On the other hand, both Pérez-González et al. (2012) and Martinez et al. (2002), found moderate (23%) and high (88%) inbreeding levels in their respective populations analysed. In the present study, we found that 32% of the populations showed signs of inbreeding. This shows that an important proportion of red deer populations in southern Spain have experienced some degree of inbreeding during the last decade. This is most likely due to the small number of relict populations that remained after the collapse (see below), and the short time since expansion processes begun.

Overall, genetic diversity was spatially structured. Genetic structuring appears to be a common feature of red deer, as other studies have shown (Polziehn et al. 2000, Kuehn et al.

2003, Frantz et al. 2006, Hmwe et al. 2006, Pérez- Espona et al. 2008, Haanes et al. 2010). However, the processes influencing structuring patterns may differ between populations and areas. In our case, after the red deer's severe decline, only one marginal population remained in Montes de Toledo, central Spain, and another four populations in Andalusia; Sierra Morena mountain range, Andujar between Córdoba and Jaén provinces, Despeñaperros in northern Jaén, and the Doñana National in Huelva (Soriguer et al. 1994, Crespo 2013). Accordingly, our results showed that the red deer's genetic diversity is distributed in this geographical manner along Andalusia forming five discrete clusters (Fig. 1). This could indicate that the genetic variability remnant in those regions during the decline is still represented in Andalusia. Further investigations of current and historical samples (i.e. before the collapse) are needed to corroborate this.

The biggest genetic cluster found in the Sierra Morena system may be the result of both, natural range expansions after the decline, and anthropogenic introductions. For instance, the two populations from Granada (Ca,Fr), and the population of Cazorla (Cz) in Jaen, were established by breeders introduced from Sierra Morena (Granados et al. 2001). Similarly, the majority of the populations from Cadiz (southernmost genetic cluster), were re-established by introducing individuals from Montes de Toledo (Soriguer et al. 1994).

In the case of the Despeñaperros, the special topography of this area with high vertical cliffs likely prevents incoming gene flow, maintaining the genetic homogeneity of this cluster. In the neighbouring Andujar, the private nature of its hunting areas could have contributed to conserve populations during the decline, and this is now reflected as a separate genetic cluster. Interestingly, the population Ae from Huelva clustered with populations of Andujar. This is most likely due to undocumented reintroductions and

warrants further investigation. Finally, decades of governmental protection in the Doñana National Park, with strict surveillance and conservation management, could be the reason of its genetic differentiation from the rest.

MANAGEMENT IMPLICATIONS

Despite intensive management and the severe decline of its populations, the red deer's genetic diversity in Andalusia appears to be in good condition overall. Nevertheless, managers are advised to carefully evaluate the genetic background of breeders in order to avoid further inbreeding of Andalusian populations. New herds should preferentially be established using individuals from the different genetic clusters identified here. This approach would help prevent loss of genetic diversity while preserving the genetic identity of the Iberian red deer.

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Figure Captions

Figure 1. Study area in Andalusia showing 58 Iberian red deer sampling sites. Different colours represent the different genetic clusters observed based on multi-locus Bayesian inference.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s website.

376 Table 1. Iberian red deer genetic variables. Population code, sample size, type of system,
377 mean number of alleles (A), allelic richness (R_S), Expected heterozygosity (H_E), Inbreeding
378 coefficient (F_{IS}). Asterisk represents $P < 0.005$ after Bonferroni correction
379

Population	Ind Genotyped	A	R_S	H_E	F_{IS}
Aa	20	7.44	6.850	0.783	0.048
Ab	25	6.88	6.140	0.767	0.000
Ac	20	8.22	7.480	0.820	0.009
Ad	20	6.11	5.768	0.773	0.000
Ae	22	7.22	6.612	0.765	0.067
Ag	24	7.66	5.967	0.735	0.052
Aj	16	6.33	6.113	0.716	0.051
Al	32	8.22	6.785	0.758	0.036
Am	25	7.00	6.202	0.732	-0.043*
Au	15	7.00	6.864	0.788	0.065
Ay	26	6.77	6.054	0.769	0.002
Br	23	8.77	8.160	0.799	-0.041
Ca	15	6.33	6.226	0.743	0.024
Cc	25	8.33	7.040	0.776	-0.011
Cd	25	8.44	7.369	0.801	0.114*
Ch	20	7.77	7.021	0.734	0.071
Co	23	7.55	6.656	0.779	0.014
Cq	23	8.88	6.986	0.798	0.035
Cr	24	8.33	7.348	0.814	0.110*
Cs	20	9.66	8.576	0.826	0.086*
Cu	16	8.55	8.198	0.829	0.099*
Cz	18	7.44	6.917	0.771	0.089*
Dn	52	6.55	5.873	0.745	0.038
En	20	8.00	7.314	0.808	0.028
Fn	27	8.55	7.132	0.788	0.004
Fr	15	5.55	5.532	0.766	0.033
Ft	20	7.66	6.510	0.751	0.109
Gm	29	8.22	6.901	0.770	0.069
Gt	25	7.33	6.453	0.771	0.054
Hl	18	8.22	7.524	0.798	0.051*
Ht	18	6.66	6.169	0.696	-0.100
Jn	32	9.11	7.424	0.787	0.046
Jt	25	8.11	6.783	0.736	0.127*
Lc	30	7.33	6.383	0.764	0.040
Mn	15	7.77	7.664	0.801	-0.016
Ms	25	7.44	6.711	0.781	0.030
Nb	25	8.66	6.949	0.793	0.078
Ng	23	7.88	6.948	0.769	0.093*
Nh	25	7.22	6.490	0.784	0.105*
No	25	8.77	7.509	0.811	0.090*

Ns	21	7.88	7.293	0.807	0.028*
Oz	20	7.77	7.086	0.788	0.042
Pa	23	7.22	6.459	0.766	0.066
Pd	25	7.77	6.682	0.760	0.011
Pi	17	7.11	6.815	0.800	0.001
Pl	19	7.00	6.518	0.798	0.088*
Ps	16	6.33	6.128	0.737	0.076
Pt	25	8.00	6.967	0.785	-0.010*
Rb	25	7.11	6.174	0.735	-0.039
Re	21	7.00	6.480	0.765	0.120*
Sd	20	7.66	7.047	0.798	0.022*
Sm	21	7.33	6.469	0.745	0.049
Sn	21	9.55	8.513	0.804	0.087*
St	25	7.22	6.414	0.767	0.007
Ti	25	8.22	7.021	0.745	0.044
Tj	24	7.66	6.717	0.758	0.121*
Tm	16	6.77	6.555	0.776	0.023
Vz	19	7.00	6.473	0.752	0.049*

380