EVALUATION OF GENETIC VARIABILITY IN BROWN HARE POPULATIONS FROM TWO PROTECTED AREAS IN NORTHERN ITALY

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ABSTRACT - The brown hare (*Lepus europaeus*) is widely distributed throughout Europe where it constitutes an important game species. However, there is concern about its conservation because the number of European hares has drastically declined in Europe since the 1960s and the species is now considered at low risk of extinction. During the last decades, several countries have carried out restocking programs with the introduction of allochtonous individuals. We analyzed 109 blood samples from two brown hare populations captured in two protected areas in northern Italy, where no animals have been released in the last 20 years, to assess genetic variability and inbreeding status for management and conservation purposes. For this study, eight microsatellite markers were selected from those described in the literature and two multiplex PCR reactions were optimised. The number of alleles per locus, allelic frequencies, observed and expected heterozygosity, and inbreeding coefficient were then calculated.
Our results revealed good genetic variability in both populations. Analysis of brown hare populations in a larger number of protected areas would be useful for improving their management through wider genetic characterization of populations for restocking programs, more accurate measurement of their genetic distances, evaluation of the reduction in their genetic variability and gene flows among adjacent areas, and perhaps detection of the introduction of allochtonous animals.

Key words: Lepus europaeus, genetic variability, microsatellites, Italy

INTRODUCTION

The brown hare (*Lepus europaeus*) is widely distributed throughout Europe where it represents an important game species. Its current Eurasian distribution extends from the northern prov- inces of Spain to the United Kingdom and from southern Europe and the northern portions of the Middle East to southern Scandinavia. Natural expansion eastwards to Siberia has been observed. The brown hare can be found in habitats ranging from sea level up to

2600 m. The species is present throughout Italy with the exception of Sardinia and Sicily (Trocchi and Riga 2005).

Since the 1960s, the number of brown hares in Europe has drastically declined (Smith et al. 2005). The observed decrease in hare populations has been attributed to intensification of agriculture, with reduction in habitat heterogeneity, field enlargement, and decreased crop diversity (Smith et al. 2005; Delibes-Mateos 2009). Additional factors contributing to its decline include increased predation (Reynolds et al. 2010), overhunting, changes in climate conditions (Smith et al. 2005), and landscape fragmentation by roads (Roedenbeck and Voser 2008).

Owing to its decline in Europe, the brown hare is now considered a low risk of extinction species and is listed on the International Union for Conservation of Nature (IUNC) Red List (http://www.iucnredlist.org/apps/redlist/details/41280/0) as a "Least Concern" species, and under Appendix III of the Convention on the Conservation of European Wildlife and Natural Habitats (Vaughan et al. 2003). Moreover, some countries (Norway, Germany, Austria and Switzerland) have placed *L. europaeus* on their Red Lists as "near threatened" or "threatened" species (Smith and Johnston 2008). During the past decades, several countries have carried out restocking programs with the introduction of allochtonous individuals mainly for hunting purposes (Meriggi and Verri 1990; Trocchi and Riga 2005).

As elsewhere in Europe, in Italy the brown hare populations have also decreased primarily because of overhunting. In areas where hunting is not permitted, hare populations can reach very high densities in relation to habitat suitability (Meriggi and Verri 1990; Vidus Rosin et al. 2009). In Italy, hare hunting is not subject to harvest planning; instead, hare populations are ordinarily managed by annual restocking in winter after the hunting season has ended. Hares for restocking are in part imported from Eastern Europe and in part translocated from areas, located in the same territory, where hunting is prohibited in order to enhance hare numbers (named restocking areas). These protected areas are set aside for the natural reproduction of wildlife, with the aim of capturing and translocating wild individuals which are then released in hunting districts. Management by restocking and translocation from protected areas has influenced the present conservation status of the species throughout the country. Abundant populations can be found in protected areas with suitable habitats, whereas very low densities are recorded in hunting grounds (0-5 hares per km^2) (Trocchi and Riga 2005). In this context, the northern Italian network of protected areas can play a role in the natural dispersion of vital brown hare populations into the surrounding territory. On the other hand, the high hunting pressure causes the extinction of hares in the territories located between protected areas, resulting in the isolation of some hare populations in protected areas. This may trigger serious consequences: high inbreeding rates and loss of genetic diversity because of the reduced gene flow between populations (Trizio et al. 2005; Frankham et al. 2006; Fulgione 2009). Inbreeding depression is

characterized by a decline in reproductive success, decreased disease resistance and, in general, by reduced fitness which can dramatically affect population survival (Allendorf et al. 2001; Goldberg et al. 2004). To counteract the potential inbreeding depression in protected areas, allochtonous individuals or hares from other protected areas are released occasionally or regularly. Restocking programs are burdened by three main problems: introduction of allochtonous individuals that compete with the indigenous population; outbreeding (Edmands 2007; Randi 2008; Houde et al. 2011); and health threats associated with the release of animals carrying infectious diseases, especially tularemia, pasteurellosis, and European Brown Hare Syndrome (EBHS) (Tizzani et al. 2002; Frölich and Lavazza 2008). For example, hare restocking has been cited as a threat to regional gene pools in Greece (Mamuris et al. 2001) and to the Cantabric population in Spain (Estonba et al. 2006).

In the present study, we analysed populations from two different protected areas in northern Italy where no animals have been released in the last 20 years. The aim was to assess the genetic variability and inbreeding status of both populations in order to draw conclusions about the effects that management practices have had on the areas and also in light of consistent demands by hunters to release hares imported from other countries. Moreover, given the important role that protected areas play in the conservation of brown hares in Italy, and the differences in management practices and policies, the study results could be useful for informing guideline development to harmonize the governance of such areas throughout the country.

MATERIALS AND METHODS

1. Study areas

The study was carried out in two protected areas in northern Italy in the southwestern part of the Po River plain: Martina di Castelnuovo Scrivia ([MCS], 44°58'49.17''N, 8°52'40.58''E, 15.46 km^2) and Casal Cermelli-Frugarolo ([CCF], 44°50'02.45''N, 8°37'43.17''E, 14.90 km^2) (*Fig. 1*). Land-use is mainly agricultural; winter wheat and maize crops predominate (MCS, 82.8%; CCF, 81.8%) . Besides croplands, the landscape of the study areas comprises woodlands (MCS, 5.1%; CCF, 3.9%), meadows (MCS, 1.2%; CCF, 5.2%), rivers (MCS, 4.7%; CCF, 3.3%), and urbanized areas (MCS, 5.5%; CCF, 0.3%). The two study areas form part of a network of protected areas for the production of hares and pheasants for restocking hunting grounds. The average distance from the nearest surrounding protected areas is 2541 m (SE=919.7) for MCS and 3688 m (SE=600.3) for CCF; the MCS study area is contiguous with other protected areas, while the minimum distance between the CCF study area and the nearest protected area is 1079 m. The density of hares estimated by spot-light counts in November 2009 was 30.0 hares per km^2 at MCS and 34.5 per km^2 at CCF. Taking into account the intense hare hunting in the surrounding areas and considering that the maximum dispersal distance of hares in these habitats is 1138.6 m (Pella 2001), the CCF study area can be regarded as isolated with low or absent hare flow, whereas the hare population in the MCS study area cannot be considered as being separated from surrounding populations.

The hare populations in the two study areas originated from the release of autochthonous hares captured in other protected areas

in the province of Alessandria and of reared hares from several stock farms. All releases ended in 1990.

2. Sampling and genetic analysis

A total of 109 brown hares (35 males and 74 females) were captured by nets in January 2010 (MCS, n=53; CCF, n=56). DNA was isolated from 109 EDTA-treated 1-ml blood samples using silica columns (Pure-Link™ Genomic DNA Mini Kit, Invitrogen™) following the manufacturer's instructions.

Eight microsatellite loci were selected from those described in the literature (*Tab. 1*). Five (Sat5, Sat12, Sat13, Sol08 and Sol33) were isolated in rabbit (*Oryctolagus cuniculus*) (Rico et al. 1994; Mougel et al. 1997; Surridge et al. 1997) and three (Lsa1, Lsa2 and Lsa6) were identified in *L. capensis* and *L. saxatilis* (Kryger et al. 2002); all were used and assessed to be polymorphic in several hare species (Surridge et al. 1997; Andersson et al. 1999; Thulin et al.2006). In order to detect and size the allelic fragments, one primer from each pair was labeled at the 5' end with fluorescent dyes (FAM, VIC, NED); simplex PCRs were performed to assess correct amplification of each locus, then two multiplex PCR reactions were optimised for simultaneous analysis of the samples: one containing primers for the Sat5, Sat12, Sat13, Lsa2 and Sol8 loci and the other with primers for Lsa1, Lsa6 and Sol33. PCR was performed in a 10-μl reaction containing 2 μl DNA template (about 50 ng/μl), 1X PCR buffer, 1.5 mM MgCl$_2$, 0.4 mM dNTPs, 0.4 mM of each primer, 0.4 unit of polymerase (FastStart Taq DNA polymerase, Roche). A no template control was added to each PCR to check for cross contamination. Two multiplex PCR cycles with different annealing temperatures were set up consisting of an initial denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation (95°C for 30 s), annealing at 58°C (multiplex 1) or 53°C (multiplex 2) for 1 min, elongation (72°C, for 1 min), and a final elongation step at 72°C for 30 min on temperature gradient cyclers (GeneAmp 9700, Applied Biosystems).
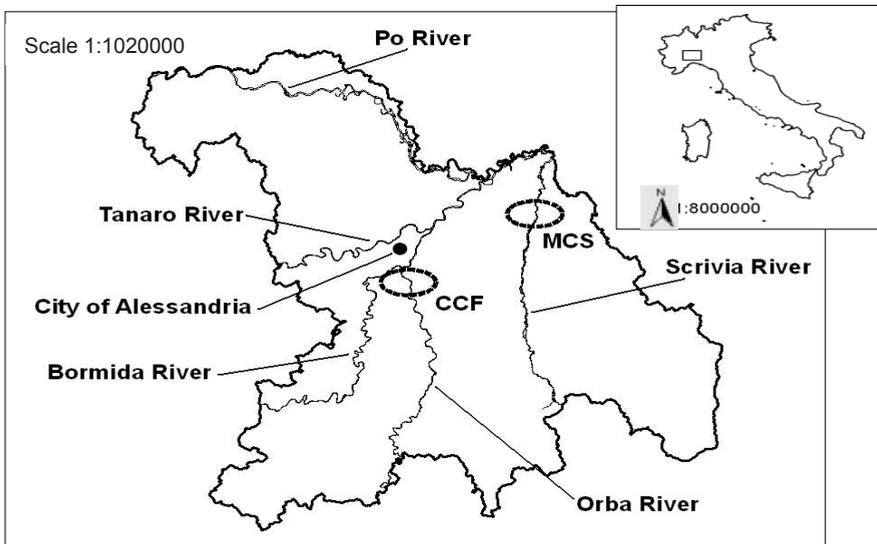

Figure 1 - Location of the two study areas (CCF and MCS) in the Province of Alessandria (northern Italy).

Table 1 - Characteristics of each locus analyzed, sequence and labeling of the primer pair used for amplification, and reference study; references: 1 = Mougel et al. 1997; 2 = Kryger et al. 2002; 3 = Rico et al. 1994; 4 = Surridge et al. 1997.

Locus	Primer sequence	No. of alleles	Allele size (bp)	References	Dye
Lsa1	F-CCTTGCAGGTTTTCAGCCTC R-GCTGTAGAAAATGAGAGGGAC	5	150-174	1	FAM
Lsa2	F-GGTACTCTATTAGGGAACCCG R-GCTAGTTGCCATTAGCTCCC	12	230-260	1	FAM
Lsa6	F-CCTAAGATGAAATGGATAAGTT R-CTCTTCTGTTTTCTGGAGCA	2	162-178	1	VIC
Sat12	F-CTTGAGTTTTAAATTCGGGC R-GTTTGGATGCTATCTCAGTCC	7	106-138	2	FAM
Sat13	F-CAGTTTTGAAGGACACCTGC R-GCCTCTACCTTTGTGGGG	6	110-130	2	VIC
Sat5	F-GCTTCTGGCTTCAACCTGAC R-CTTAGGGTGCAGAATTATAAGAG	13	174-234	2	VIC
Sol33	F-GAAGGCTCTGAGATCTAGAT R-GGGCCAATAGGTACTGATCCATGT	4	185-225	3	NED
Sol8	F-GGATTGGGCCCTTTGCTCACACTTG R-ATCGCAGCCATATCTGAGAGAACTC	8	100-130	4	NED

Amplification products were screened on a 3130 Genetic Analyzer and sizing was performed by fragment analysis with Genemapper™ software (Applied Biosystems) using ROX™ 500 size standard (Applied Biosystems).

Homozygote samples were amplified three times to reduce the large allelic drop-out, also known as short allele dominance (Wattier et al. 1998), which can result in deviations from Hardy-Weinberg equilibrium and an apparent deficiency in heterozygotes.

3. Statistical analysis

The MStools software application (Park 2001) was used to check data for identical or near identical genotypes to ensure that the same animal was not sampled two or more times. This occurrence was excluded and all samples were differenced by at least 4 alleles. Data analysis was performed using Genepop version 4.0 (Rousset 2008), FSTAT version 2.9.3 (Goudet 2001), and Genetix version 4.05 (Belkhir 2004). Descriptive statistics for each locus (mean number of alleles per locus, polymorphic information content [PIC], private alleles, allelic frequencies, observed [Ho] and expected heterozygosity [He] and estimated inbreeding based on heterozygosity ratio [Fis]) were computed.

The PIC value is commonly used as a measure of the informativeness of polymorphism for a marker locus in population genetics; it was calculated according to Bolstein et al. (1980) using MStools (Park 2001). The number of alleles per locus and allelic frequencies were calculated using Genetix version 4.05 (Belkhir et al. 2004). The deviations from Hardy-Weinberg equi-

librium (HWE) for each locus and the significance of the lack of heterozygosity at each locus were evaluated through the Weir and Cockerham (1984) estimates of Fis using a Markov Chain simulation (20 batches, 5000 iterations per batch, and a 10.000 dememorization number) (Guo and Thompson 1992) and the complete enumeration (Louis and Dempster 1987) methods as implemented in Genepop version 4.0 (Rousset 2008); FSTAT version 2.9.3 (Goudet 2001) was employed to calculate observed (Ho) and expected heterozygosity (He) (Nei 1988), the Fst coefficient following Weir and Cockerham (1984) and to estimate the p-value of Wright's fixation index (Wright 1969; Wright 1978) for each population (Petit et al. 2001).

RESULTS AND DISCUSSION

All microsatellite markers were observed to be polymorphic in both populations, and a total of 57 alleles were found (CCF= 52, MCS= 46). The number of alleles detected for each marker was comparable to that reported for *L. europaeus* (Surridge et al. 1997; Andersson et al. 1999; Estonba et al. 2006; Thulin et al. 2006); the highest number of alleles (13) was observed for Sat5, while the Lsa6 locus showed the lowest variability (2 alleles) (*Tab. 1*). The number of alleles per locus ranged from 2 (Lsa6) to 11 (Lsa2) (mean, 6.5 ±standard deviation [SD] 2.88) for CCF and from 2 (Lsa6) to 10 (Sat5) (mean, 5.75±2.87) for MCS (*Tab. 2* and *3*). These results were similar to those described for wild populations of *L. europaeus* (6.6 in Andersson et al. 1999; from 6.5 to 8 in Estonba et al. 2006), although the panels, number of markers, loci, and sample size in those studies differed from ours. The mean

number of alleles was higher than that reported for *L. granatensis* (3.2) and *L. castroviejoi* (2.3) (Estonba et al. 2006) and lower than that recorded for *L. americanus* (13.4) (Burton et al. 2002) and *L. timidus* (12.7) (Hamill et al. 2006). The observed heterozygosity (Ho) value ranged from 0.34 to 0.80 (mean, 0.60±0.19) for CCF and from 0.25 to 0.81 (mean, 0.60±0.18) for MCS. The expected heterozygosity (He) ranged from 0.28 to 0.80 (mean, 0.67±0.18) for CCF and from 0.22 to 0.77 (mean, 0.63±0.19) for MCS. In the latter population, five out of eight loci had higher Ho than He values, showing an excess of heterozygotes that was not significant. In both populations, the lowest Ho and He values were observed for the Lsa6 locus, which was also the marker with the smallest number of alleles; the Sat5 marker also showed much lower Ho than He values. This occurrence has been reported in *L. europaeus*, *L. timidus* and *L. americanus* (Andersson et al. 1999; Burton et al. 2002; Estonba et al. 2006; Thulin et al 2006) and can be due to the Wahlund effect or it can be caused by mutations in the priming site of the microsatellite that results in non amplification of the alleles (null alleles). This occurrence can increase when primers are developed for one species, as in our study, and then used in a related one. In our opinion, a likely explanation for the deficit of the heterozygotes is the presence of null alleles, taking into account the presence of null genotype for the same locus equally distributed in the two populations and considering that a significant deficit was found for only one marker. The medium to high values of Ho and He detected for the other

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Table 2 - Characteristics of the hare population from the Casal Cermelli Frugarolo area (NA= Number of alleles, H_E= Expected heterozygosity, H_O= Observed heterozygosity, PIC= Polymorphism Information Content, AU= Unique alleles, Fis =Fixation index, SD= standard deviation, * $p<0.05$, † values calculated after SAT5 exclusion).

	NA	AU	H_O	H_E	PIC	F_{IS}	*p*-value
Lsa1	5	1	0.71	0.75	0.70	0.05	0.332
Lsa2	11	4	0.75	0.75	0.73	0.01	0.191
Lsa6	2	0	0.34	0.28	0.24	-0.19	1.000
Sat12	7	0	0.77	0.79	0.75	0.03	0.433
Sat13	6	0	0.68	0.76	0.72	0.11	0.198
Sat5	9	3	0.36	0.70	0.66	0.48	0.000*
Sol33	4	2	0.43	0.52	0.40	0.18	0.101
Sol8	8	1	0.80	0.8	0.76	-0.01	0.430
Mean (SD)	6.5 (2.88)		0.64† (0.02)	0.67† (0.07)	0.62† (0.21)		
Total		11				0.0402†	0.0971†

Table 3 - Characteristics of the hare population from the Martina di Castelnuovo Scrivia area (NA= Number of alleles, H_E= Expected heterozygosity, H_O= Observed heterozygosity, PIC= Polymorphism Information Content, AU= Unique alleles, Fis =Fixation index, SD= standard deviation, * $p<0.05$, † values calculated after SAT5 exclusion)

	NA	AU	H_O	H_E	PIC	F_{IS}	*p*-value
Lsa1	4	0	0.66	0.64	0.59	-0.03	0.740
Lsa2	8	1	0.64	0.67	0.64	0.05	0.215
Lsa6	2	0	0.25	0.22	0.19	-0.13	1.000
Sat12	7	0	0.68	0.75	0.7	0.09	0.252
Sat13	6	0	0.81	0.75	0.70	-0.08	0.875
Sat5	10	4	0.49	0.77	0.74	0.36	0.000*
Sol33	2	0	0.53	0.50	0.37	-0.06	0.755
Sol8	7	0	0.77	0.76	0.71	-0.02	0.578
Mean (SD)	5.75 (2.87)		0.62† (0.02)	0.61† (0.07)	0.56† (0.2)		
Total		5				-0.0111†	0.6494†

(11, 21.1%) than the MCS population (5, 10.9%), which may reflect the different origins of the two populations.

Genetic markers showing PIC values higher than 0.5 are normally considered informative in population genetic analyses (Botstein et al. 1980). In both populations, the mean PIC values were above this level (*Tab. 2* and *3*). Since previous studies investigating the same markers as ours did not give PIC data, no comparison was possible. Deviation from Hardy-Weinberg equilibrium (HWE) was calculated for each locus. Fis ranged from -0.19 to 0.48 in CCF hares and from -0.13 to 0.36 in MCS hares; only Sat5 showed a statistically significant Fis value (0.48 and 0.36, respectively) (p <0.0001) in both populations. These findings are in line with the low He values possibly linked to the presence of blank genotypes in the dataset (possible null-null homozygote genotypes due to non amplification), suggesting the existence of null alleles at this locus that could lead to false observation of homozygotes and account for deviations from HWE of this marker; thereafter the locus was excluded from further analysis. Two markers in the CCF population (Lsa6 and Sol8) and five markers in the MCS population (Lsa1, Lsa6, Sat13, Sol33 and Sol8) showed negative though not statistically significant Fis values, indicating an excess of heterozygotes mainly in the MCS population.

The global Fis value was 0.0402 (p= 0.0971) for the CCF hares and -0.0111 (p= 0.6494) for the MCS hares, revealing the absence of inbreeding in both populations.

The Fst coefficient was 0.036 (Jackknife Standard error 0.012, p<0.001), showing a slight differentiation between the two populations. However, these results can be explained by the different origins of the founders of the two populations or by the geographic distance between the two populations (Burton et al. 2002; Fulgione et al. 2009).

In conclusion, the genetic analysis revealed good genetic variability in both hare populations. The geographical and historical characteristics of the protected areas under study corroborate findings about slight genetic differentiation and presence of private alleles: they are located in distant areas and were founded by subjects from different stock farms.

Moreover the CCF area is geographically isolated, far from other protected areas and located inside a fragmented landscape since agricultural land-use and highways surround the protected area. In contrast, the MCS area is located near other protected areas where there are no barriers to hare migration. Despite these differences our results show that currently both populations are self-sustaining and viable. However, the potential of inbreeding due to geographical isolation should not be neglected since fragmentation can create barriers to movement because an unfavorable habitat does not provide cover against predators or because distances between suitable patches are greater than those that species are able to cross in one step. Consequently, the movement ability of animals, and particularly their faculty to disperse, may be altered by landscape fragmentation. This alteration can have dramatic consequences on populations, partly because of the reduction in gene flow be-

tween populations, which leads to higher inbreeding rates and loss of genetic diversity (Frankham et al. 2006; Fulgione 2008). From this point of view the MCS area has an optimal situation since hare dispersal from surrounding protected area can be supposed due to the absence of geographical barriers.

Studies involving a larger number of protected areas could allow broader investigation of the genetic characteristics of hare populations used for restocking programs, measurement of their genetic distances, and evaluation of reduced genetic variability and gene flows among adjacent areas. Since reintroductions of brown hare in northern Italy probably modified the genetic composition of the Italian populations, the presence of hybrid individuals is likely. A probabilistic approach applied to wider genetic characterization could be useful to detect the introduction of allochtonous individuals. Indeed, precisely because of the substitution of autochthonous with hybrid individuals, mitochondrial DNA analysis cannot be applied to differentiate between Italian and Central European samples (restricted female gene flow) (Trocchi and Riga 2005; Barilani et al. 2007).

Given the importance of protected areas for brown hare conservation in Italy, the management of the populations inhabiting these areas needs to be improved. Genetic diversity should be periodically assessed and related to the population size, and strict controls on the release of allochtonous individuals adopted. Our study reveals that even though the last release of allochtonous hares was done a long time ago, the gene diversity is conserved. Hence, releasing hares that can spread diseases or threaten the conservation of specific genetic feature of local populations seems useless. Conservation efforts need to be focused on preventing the isolation of protected areas and improving the connectivity between subpopulations characterized by low gene flow. This can be achieved by ensuring the retention of a viable populating of hares in hunting districts, by preventing overhunting, and also by reducing the distance between protected areas.

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