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ORIGINAL RESEARCH

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A single multiplex of twelve microsatellite markers for the simultaneous study of the brown hare (*Lepus europaeus*) and the mountain hare (*Lepus timidus*)

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Abstract

The management of hunted species is challenging, as it must conciliate the conservation of species and their sustainable exploitation. Nongenetic tools are widely used in this context but they may present limitations notably when species can hybridize or when large-scale spatial monitoring is required to establish optimal management actions. This is why genetic tools have been more and more integrated in wildlife management practices. However, the markers proposed are often amplified in small multiplexes when larger ones could allow to better cope with the small quantities of DNA obtained with noninvasive sampling methods. Here, we propose a unique multiplex of 12 autosomal microsatellite markers for the study of two hare species that exist in sympatry in some areas in Europe and are hunted notably in France: the brown hare *Lepus europaeus* and the mountain hare *L. timidus*. We tested 17 markers previously used in these two species or other lagomorph species, from which 12 were included in this single multiplex. Diversity was between 4 and 30 alleles per locus totaling 126 alleles, and we showed that these markers possess appropriate genetic resolution for individual and species identification for the populations under study. This multiplex panel represents the largest number of microsatellites amplified in one reaction proposed for these two hare species and provides a cost-effective and valuable tool for further hybridization studies and the management of hares.

KEYWORDS

hares, hybridization, individual identification, microsatellites, species identification

1 | INTRODUCTION

The two most widespread species of hares in Europe are the brown hare (*Lepus europaeus*), present in most parts of Europe, and the mountain hare (*L. timidus*) that can be found in northern Europe and in the Alps. Both species have been classified by the International Union for the Conservation of Nature as "Least Concern" (Smith & Johnston, 2008). In France, the brown hare is one of the most hunted species on the territory (the national bag is of about 600,000 hares; estimations between 587,080 and 667,207 individuals considering a 95% confidence interval for the hunting season 2013–2014; Aubry et al., 2016), whereas mountain hare hunting is limited to the Alps (the national bag is of several hundred; ONCFS 2011). This hunting dimension adds sustainable exploitation issues to the classic conservation questions and makes management plans even more essential to ensure the viability of hare populations in France. Sustainable management tools, such as

wildlife reserve and limitation of hunting bag or of hunting season, have already been developed for the brown hare and have led to an adequate hunting management of this species (Péroux, Bray, Mauvy, Lartiges, & Marboutin, 2005; Smith, Jennings, & Harris, 2005). In contrast, the impact of hunting on mountain hare populations is uncertain and the direct assessment of the mountain hare demography through in situ observations and using the same tools encounters practical problems due to the harshness of its habitat. Furthermore, morphological differentiation between the two species is error prone due to extensive resemblance and to the possibility of hybridization within contact zones (Thulin, Stone, Tegelström, & Walker, 2006). New tools, based on genetic data, are thus required to improve the specific management of these hare species and to address large-scale issues like the spatial range and structure of populations, their genetic diversity, or the monitoring of individuals over time. These are urgent questions for the mountain hare as climate change may threaten its habitat range, particularly in the Alps (Acevedo, Jiménez-Valverde, Melo-Ferreira, Real, & Alves, 2012; Bisi, Wauters, Preatoni, & Martinoli, 2015; Gobiet et al., 2014).

The genetics of hares species has first been studied using mitochondrial DNA notably in order to unravel the phylogenetic relationships between these species (Alves, Ferrand, Suchentrunk, & Harris, 2003; Stamatis et al., 2009; Wu et al., 2005). Results showed that the history of hares, both past and present, has been influenced by different processes (genetic drift, anthropogenic introductions, and translocations, post-glacial recolonizations from refuges—Kasapidis, Suchentrunk, Magoulas, & Kotoulas, 2005; Suchentrunk et al., 2006; Stamatis et al., *2009*) which have promoted encounters between species and the occurrence of hybridizing events (Melo-Ferreira et al., 2007, 2014; Thulin, Stone, et al., 2006; Zachos, Ben Slimen, Hackländer, Giacometti, & Suchentrunk, 2010). As a result, the mitochondrial genome of hares is characterized by multiple introgressions, which led many authors to turn to nuclear markers to identify species or assess levels of ongoing hybridization (Alves et al., 2003, 2006; Estonba et al., 2006). Several types of nuclear markers can be applied and microsatellites, notably thanks to their high polymorphism, allow to reach a high resolution to answer wildlife genetics issues (Morin et al., 2012).

No microsatellite has been specifically designed either for the brown hare or for the mountain hare. However, such markers have already been applied to both species, thanks to the possibility for cross-amplification of microsatellites (Barbará et al., 2007). The microsatellites used in both species were developed for the European rabbit (*Oryctolagus cuniculus*) and the scrub hare (*L. saxatilis*) (Andersson, Thulin, & Tegelström, 1999; Antoniou, Magoulas, Platis, & Kotoulas, 2013; Campos, De Bellocq, Schaschl, & Suchentrunk, 2011; Canu et al., 2013; Djan, Popović, & Veličković, 2014; Estonba et al., 2006; Fickel et al., 2005; de Freitas, 2006; Fulgione, Maselli, Pavarese, Rippa, & Rastogi, 2009; Hamill, Doyle, & Duke, 2006; Melo-Ferreira et al., 2014; Mengoni, Mucci, & Randi, 2015; Surridge, Bell, Rico, & Hewitt, 1997; Thulin, Fang, & Averianov, 2006; Thulin, Malmsten, & Laurila, 2012; Thulin, Stone, et al., 2006; Zachos et al., 2010). None of these studies used more than eight markers in the same multiplex. However, given the increasing use of noninvasive genetics and the subsequent search for an optimized use of available DNA, larger multiplexes are expected as they use of a lower quantity of DNA (Beja-Pereira, Oliveira, Alves, Schwartz, & Luikart, 2009).

In this study, we developed a 12-microsatellites multiplex and assessed its utility in French populations of brown and mountain hares (1) to validate the specific identification performed based on morphologic data, (2) to assess the power of discrimination between individuals of the same species, and (3) to detect putative clues of an ongoing hybridization between the two hare species within the contact zone.

2 | MATERIALS AND METHODS

2.1 | Samples collecting and DNA extraction

Tissue samples (N = 532 for brown hares, N = 141 for mountain hares) from hunted hares were collected between 2003 and 2009 in southeastern France (Figure 1). The sampling area included regions where only the brown hare was present, consisting mostly of plains below 1,500 m altitude (see supplementary, Figure S1). The sampling area also encompassed regions above 2,000 m altitude where mountain hares were predominantly observed. Both species were observed between 1,500 and 2,000 m. Hare species identification was made in the field by hunters. DNA was extracted from ear tissues using purification column kits (Nucleospin 96 Tissue kit, Macherey-Nagel) following the manufacturer procedure.

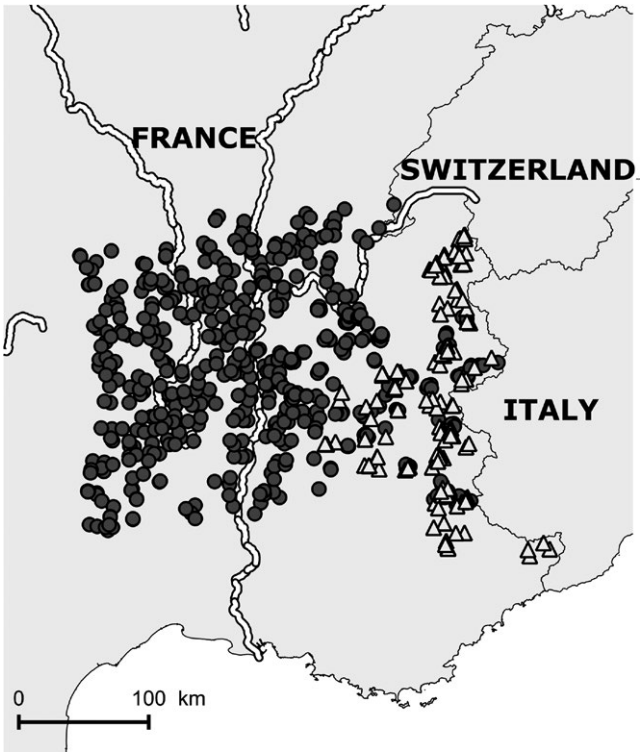

FIGURE 1 Geographical locations of sampling areas. Gray circles and white triangles represent brown and mountain hares, respectively

2.2 | Microsatellite markers selection

We selected 17 microsatellite markers described in the literature: SAT2, SAT3, SAT5, SAT08, SAT12, SAT13 (Mougel, Mounolou, & Monnerot, 1997), SOL30, SOL33, SOL8 (Rico et al., 1994; Surridge et al., 1997), OCELAMB, OCELS1B (van Haeringen, den Bieman, van Zutphen, & van Lith, 1996), D0UTR4 (Korstanje et al., 2001), D7UTR4 (Korstanje et al., 2003) developed for the European rabbit and LSA2, LSA3, LSA6, LSA8 (Kryger, Robinson, & Bloomer, 2002) developed for the scrub hare. Sixteen of them have already been used in both the brown and/or the mountain hare. As for D7UTR4, it has been developed for the European rabbit and have not been applied to any species of hare as far as we know.

We found 23 studies using microsatellites for the study of the brown and/or the mountain hare (see supplementary, Table S1). The 17 markers we tested over the 31 available were ranked among the most used and all had been reported as polymorphic. Overall, no more than eight microsatellites were amplified in a single multiplex (mean = 5.5, *SD* = 2.23) over the 23 studies.

2.3 | Primers design and multiplex design

A first simplex step was performed in order to check whether all loci were successfully amplified. Only loci correctly amplified were tested for the multiplex construction. A pigtail (Brownstein, Carpten, & Smith, 1996) was added to all reverse primers in order to avoid the addition of adenine nucleotides by the polymerase at the end of the amplification. This first step also allowed us to estimate the allele size range of each locus as well as to assess their polymorphism. The primers of loci with overlapping alleles were dyed with different color fluorescent dyes. At least 10 base pairs (bp; 15–20 bp for loci with high polymorphism) separated loci labeled with the same fluorescent dye. Several adjustment steps followed in order to reduce the number of artifacts on electrophoregrams and to maximize the results readability.

2.4 | Multiplex genotyping

PCR reactions were performed step-by-step following a unidirectional workflow starting in a clear room with positive air pressure to prepare sensitive reagents (enzymes, primers) and then in a pre-PCR room to assembly DNA and reagents. The PCR reaction occurred a primer solution containing 5 µl of mastermix Taq polymerase (Type-it, QIAGEN), 1.35 µl of 12 primers pairs at a final concentration of 0.08–0.6 µmol/L and 30 ng of DNA to be amplified. Each pair of primers was coupled with a fluorescent dye label (details in Table 1). PCR amplifications happened in 96-well microplates in a post-PCR area with negative air pressure. The samples were first denatured at 95°C during 5 min. Then, thirty cycles followed (denaturation step: 95°C, 30 s; annealing step: 55.9°C, 90 s; elongation step: 72°C, 30 s) and a final elongation step at 60°C during 30 min. PCR products were resolved on a capillary sequencer ABI PRISM 3130XL (Applied Biosystem) with formamide (denaturing conditions) and an internal size marker (600 liz; Applied Biosystem) in one migration. The electrophoregrams were analyzed using GENEMAPPER 4.1 (Applied Biosystem/Life Technologies) independently twice by different operators. Results were then compared, and ambiguous loci were set to missing data.

2.5 | Statistical analysis

2.5.1 | Data selection

The power of resolution for the individual identification was assessed by calculating the probability of identity P(ID) and the probability of identity between siblings P(ID)sib using GenAlEx 6.501 (Peakall & Smouse, 2012). These probabilities also allowed us to determine the minimum number of amplified loci required to have no ambiguity for individual identification. According to Waits, Luikart, and Taberlet (2001), we used 0.01 as threshold for the P(ID). We removed from our dataset any individual typed under the minimum number of loci determined.

Species identification for each individual was determined using the Bayesian software STRUCTURE 2.3.4 (Falush, Stephens, & Pritchard, 2003; Pritchard, Stephens, & Donnelly, 2000). We used an admixture model with correlated allele frequencies between populations and the option POPINFO = 0. The program was run with a Monte Carlo Markov chain length of 1.000,000 after a burn-in of 100,000 iterations, and from one to four genetic clusters (*K* = 1–4). Ten independent runs were carried out for each value of *K*, and the information from the outputs of the 10 runs for each *K* was compiled using CLUMPP (Jakobsson & Rosenberg, 2007). We determined the number of clusters (*K*) that best describe the data following the method of Evanno, Regnaut, and Goudet (2005) implemented in STRUCTURE HARVESTER online web 0.6.94 (Earl & VonHoldt, 2011). We then chose to discard any individual for which the genetic assignment did not match the morphologic identification (individuals had to show a probability of assignment higher than 0.9 to the opposite cluster to which they had been morphologically assigned).

Once the species of each individual was determined, null alleles for each locus in each species were looked for with the software Micro-Checker 2.2.3 (Van Oosterhout, Hutchinson, Wills, & Shipley, 2003). The significance of null allele frequencies was then assessed using a binomial exact test following de Meeüs, Béati, Delaye, Aeschlimann, and Renaud (2002). Dropout probability per locus was assessed according to De Meeûs, Humair, Grunau, Delaye, and Renaud (2004). All subsequent analyses were performed with and without the loci for which we detected significant signs of null allele frequencies or dropout. Finally, we tested for deviation from Hardy–Weinberg equilibrium and linkage disequilibrium for each pair of loci separately for brown hares and mountain hares with FSTAT 2.9.3.2 (Goudet, 1995). Significance was assessed applying Bonferroni's correction.

2.5.2 | Detection and characterization of hybrids

In each cluster, we selected the eighty individuals best assigned. All selected individuals reached a posterior probability larger than 0.99. They were then used to simulate parental and hybrid (F1, F2,

TABLE 1 Description of the microsatellite markers of the 12-plex. The accession number in NCBI is given for each locus if the information is available. The pigtails are reported in bold in the reverse sequence of the primers. The given concentration corresponds to the final concentration of each primer in the primer solution

LOCUS	Accession number	Amorces 5′–3′	Fragment length	Dye	Concentration (µmol/L)	Reference
LSA2	AF491763	F:GGTACTCTATTAGGGAACCCG R: **GTGTCTT**GCTAGTTGCCATTAGCTCCC	236–254	FAM	0.4	Kryger et al. (2002)
LSA3	AF491764	F: GGATATCAAAGAACATGCCC R: **GTGTCTT**TGCCTTAAAGGCTGATTCTG	199–211	FAM	0.3	Kryger et al. (2002)
LSA6	AF491766	F: CCTAAGATGAAATGGATAAGTT R: **GTGTCTT**CTCTTCTGTTTTCTGGAGCA	163–171	FAM	0.2	Kryger et al. (2002)
LSA8	AF491768	F: AAGGTATTAAATTGGGCACTC R: **GTGTCTT**GACTGAAATTGATGTGCTACC	171–189	VIC	0.6	Kryger et al. (2002)
OCELAMB		F:AGTCACATTTGGCATTTCGTGA R: **GTGTCTT**TCCTTTGAATTTAGGATCCACAGC	107–119	NED	0.12	van Haeringen et al. (1996)
OCELS1B		F: TCAGGTATTTGGAAAGTGAATCCC R: **GTGTCTT**ACTGCTATATCAAAGGCATGACCC	143–167	PET	0.3	van Haeringen et al. (1996)
SAT8	X99889	F: CAGACCCGGCAGTTGCAGAG R: **GTGTCTT**GGGAGAGAGGGATGGAGGTATG	88–98	VIC	0.08	Mougel et al. (1997)
SAT12	X99891	F: CTTGAGTTTTAAATTCGGGC R: **GTGTCTT**GTTTGGATGCTATCTCAGTCC	108–136	FAM	0.08	Mougel et al. (1997)
SAT13	X99892	F: GCCTCTACCTTTGTGGGG R: **GTGTCTT**CAGTTTTGAAGGACACCTGC	109–115	VIC	0.2	Mougel et al. (1997)
SOL30	X79215	F: TGCAGCACTTCATAGTCTCAGGTC R: **GTGTCTT**CCCGAGCCCCAGATATTGTTACCA	155–201	NED	0.3	Rico et al., 1994; Surridge et al. (1997)
SOL33	X94683	F: GGGCCAATAGGTACTGATCCATGT R: **GTGTCTT**GAAGGCTCTGAGATCTAGAT	197–215	PET	0.4	Rico et al., 1994; Surridge et al. (1997)
SOL8	X79217	F: ATCGCAGCCATATCTGAGAGAACTC R: **GTGTCTT**GGATTGGGCCCTTTGCTCACACTTG	106–122	PET	0.2	Rico et al., 1994; Surridge et al. (1997)

Backcrosses) genotypes with the function *hybridize* implemented in the R package *adegenet* 2.0.1 (Jombart, 2008). A total of 1,800 simulated genotypes were generated and analyzed using NewHybrids 1.1 (Anderson & Thompson, 2002) in order to estimate the resolution power of the 12-plex for the detection of hybridization. We considered that an individual was assigned to a genotypic class when the probability for this class exceeded 0.90 (Godinho et al., 2011).

2.5.3 | Species diversity and genetic structure

Observed and expected heterozygosity, mean number of alleles per locus, number of private alleles were assessed using GenAlEx. Estimations of F_{st} and F_{is} per locus, between and among populations were calculated using Weir and Cockerham's estimators (1984) implemented in GENETIX 4.05.2 (Belkhir, Borsa, Chikhi, Raufaste, & Bonhomme, 1996).

3 | RESULTS

3.1 | Characteristics of the multiplex

The final marker set consisted of 12 multiplexed microsatellite markers (LSA2, LSA3, LSA6, LSA8, OCELAMB, OCELS1B, SAT8, SAT12, SAT13, SOL30, SOL33, SOL8). Of the 17 markers originally considered, two (SAT2 and SAT5) were excluded because their amplification was not satisfactory. SAT3 was also eliminated because of its low polymorphism. Finally, D7UTR1 and D0UTR4 proved impossible to multiplex with the other markers and were thus discarded. Two to four markers were allocated to the same fluorescent dye (see Table 1) with an average spacing of 28 bp between two markers of the same dye.

3.2 | Data selection

According to the values of P(ID) ($2.9.10^{-10}$ for the brown hare, $1.5.10^{-07}$ for the mountain hare, see supplementary material, Table S2) and P(ID)sib (0.00015 and 0.0013, respectively), individuals could be reliably identified with 12 microsatellite loci. In fact, nine markers were enough to reach the threshold of 0.01 (P(ID)sib for eight markers equal to 0.0004 and 0.01 for the brown and the mountain hare, respectively).

The most likely number of clusters was $K = 2$. More than 97% of the individuals were assigned to one of the clusters with a posterior probability higher than 0.9; 81% were assigned with probabilities higher than 0.99 (Figure 2). F_{st} estimate indicated significant differentiation between the two clusters ($F_{st} = 0.19$; CI 95%: 0.13–0.25). According to the microsatellite markers, errors of morphologic identification of the species by hunters were detected for 11 individuals. These individuals as well as all individuals genotyped with less than nine markers were removed from the analysis. The final dataset, after PI calculations and STRUCTURE analysis, resulted in 521 brown hares and 122 mountain hares.

Deviation from Hardy–Weinberg equilibrium varied among loci within both species. Some loci that were out of HW equilibrium: SAT12 and LSA8 in the mountain hare, SOL33 in the brown hare, LSA3, SAT13, and SOL30 in both species. There was no linkage disequilibrium between any pair of loci after the Bonferroni correction. Only the locus LSA3 showed significant signs of null alleles. We also found signs of allelic dropout for the loci LSA6 and SAT12. In following analyses, we compared the results using 12 markers and nine markers (LSA3, LSA6, and SAT12 removed). The same 11 errors of species assignation also appeared with the set of nine markers. The posterior probabilities of assignation were similar to the ones obtained with 12 markers (see Figure 2). The F_{st} value calculated over nine markers was slightly higher than what was obtained with the whole panel ($F_{st} = 0.21$; CI 95%: 0.14–0.28). The confidence interval overlapped widely the one obtained with the 12 markers.

3.3 | Detection and characterization of hybrids

The profiles obtained with 12 or nine microsatellites were very similar (see Figure 3). Overall, each hybrid category was mostly assigned to its real class by the computer program. Using 12 microsatellites, the mean assignment of F1 and F2 to parental classes was null. On average, 85.6% of the F1 and 64.6% of the F2 were assigned as such, respectively. Thus, these hybrid classes were reliably detected using the microsatellite panel. On the contrary, the distinction between

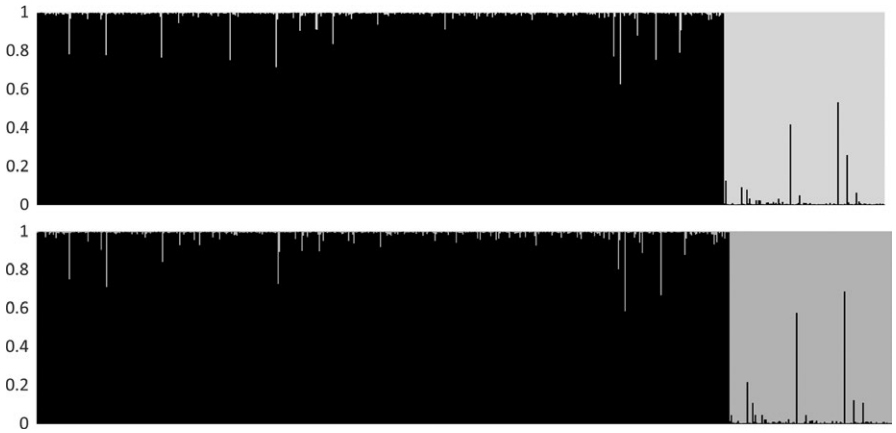

FIGURE 2 Posterior assignation probabilities obtained within the STRUCTURE analysis for the sampled hares with 12 (top plot) and nine (bottom plot) markers and $K = 2$. Brown hare membership is represented in black while mountain hare assignation is in gray. The 11 dubious individuals are not represented

backcross and parental classes was not always clear. The mean assignment of the backcross hybrids to the parental classes was equal to 7.4% on average and, inversely, the mean assignment of parental genotypes to backcross classes reached 5.3% on average. When only nine markers were used, F2 hybrids and backcrosses tended to be considered as parental individuals more often (<1% of F2 hybrids and 12%–16% of the backcrosses). This loss of resolution was expected as we reduced the number of loci.

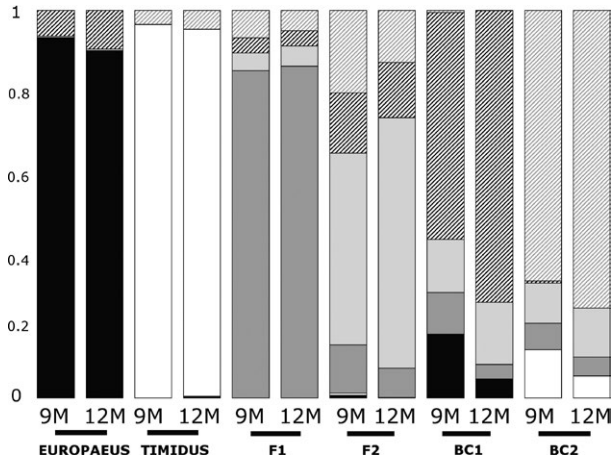

FIGURE 3 Mean probability of assignment of each simulated categories to the six parental and hybrid classes tested with NEWHYBRIDS. Both results obtained with the whole microsatellite panel (12M) and the reduced panel of 9 markers (9M) are represented. Black bars represent brown hares, white bars mountain hares, dark gray stands for F1, light gray for F2, black stripes for F1*brown hare hybrids (BC1), and gray stripes for F1*mountain hare hybrids (BC2)

3.4 | Species diversity and genetic structure

All loci were polymorphic with a mean number of alleles per locus of 10.5 (standard error = 2.19) in brown hare and 6.08 (*SE* = 1.06) in mountain hare (see Table 2). When considering only alleles present at frequency higher than 5%, the mean numbers were much closer (brown hare: mean = 3.83, *SE* = 0.53; mountain hare: mean = 2.91, *SE* = 0.39) as were the numbers of private alleles in the two species when considering frequencies higher than 5% (four for the brown hare, five for the mountain hare). The brown hare showed a lower F_{is} (F_{is} = 0.11; CI95%:0.092–0.312) than the mountain hare (F_{is} = 0.20; CI95%: 0.15–0.24). Using the panel of nine loci, F_{is} estimates were lower (F_{is} = 0.078; CI95%: 0.057–0.097 for the brown hare and F_{is} = 0.13; CI95%:0.083–0.18 for the mountain hare). However, the order of magnitude between the two values remained comparable.

4 | DISCUSSION

Our goal was to generate a set of microsatellite markers in brown and mountain hares that could be used cost-effectively to distinguish species and hybrids, and to survey populations and individuals. In this study, we found that 12 markers could be amplified reliably in a single PCR reaction in the two target species.

4.1 | Characterization of the French hare populations and individuals

All 12 loci were polymorphic although two of them (LSA6 and SAT08) showed only one allele present at a frequency higher than 5%. The

TABLE 2 Estimates of the genetic diversity estimators for the brown hare and the mountain hare. *N* stands for the number of alleles; H_o and H_e are the observed and expected heterozygosity, respectively. The expected frequencies of null alleles are given in the column "Null". Loci in Hardy–Weinberg disequilibrium are indicated with a "*" for the brown hare and a "#" for the mountain hare. Statistically significant null alleles frequencies are indicated with "**". The last column indicates dropout *p*-value for both species

	Lepus europaeus					Lepus timidus					
Locus	*N*	F_{is}	H_o	H_e	Null	*N*	F_{is}	H_o	H_e	Null	Dropout *p*-value
LSA2	18	0.025	0.69	0.71	0.012	13	0.051	0.7	0.73	0.016	.99
LSA3*#	6	0.47	0.35	0.66	0.22**	7	0.54	0.33	0.71	0.26**	.52
LSA6	4	0.074	0.21	0.22	0.031	2	0.66	0.008	0.024	0.082	.028
LSA8#	6	0.073	0.56	0.60	0.029	4	0.55	0.17	0.39	0.22	.67
OCELAMB*	8	0.10	0.43	0.48	0.044	4	0.048	0.28	0.29	0.038	.36
OCELS1B	14	0.0095	0.85	0.86	0.004	12	0.064	0.67	0.72	0.038	.11
SAT08	4	0.040	0.37	0.38	0.015	2	0	0.008	0.008	−0.0041	.073
SAT12#	13	0.022	0.81	0.83	0.0089	7	0.27	0.52	0.7	0.14	.038
SAT13*#	9	0.059	0.74	0.79	0.028	4	0.19	0.5	0.61	0.087	.076
SOL30*#	30	0.095	0.62	0.68	0.047	9	0.18	0.44	0.54	0.10	.32
SOL33*	4	0.37	0.29	0.46	0.17	3	0.13	0.35	0.4	0.057	.092
SOL08	10	0.036	0.77	0.80	0.016	6	−0.015	0.61	0.59	−0.012	.29
Mean	10.5	0.11	0.56	0.62	–	6.08	0.2	0.38	0.47	–	
Variance	2.19	0.0018	0.064	0.057	–	1.08	0.0043	0.068	0.07	–	

substantial null allele frequency that we have found in both species for LSA3 was observed by Mengoni et al. (2015). Thus, in the future studies, it may be relevant to conduct analyses including or excluding this marker even if its impact on the results appears limited especially in our hybridization study. For both species, the estimated mean number of alleles, including rare alleles, fell within the range of values found in other European populations of brown hares (11 in Fickel et al., 2005 and Mengoni et al., 2015 vs. 10.6 in our study) and mountain hares (between 5 and 9 in Hamill et al., 2006; Zachos et al., 2010 and Rehnus & Bollmann, 2016 vs. 6.1 in our study). Such polymorphism made the discrimination of individuals possible based on nine markers over the 12 available in the multiplex. This set of optimized microsatellite markers represents a powerful tool for future noninvasive genetic approaches (e.g., based on feces), often associated with a higher rate of amplification failure. The analysis of a complementary dataset (unpublished data) made of noninvasive samples (feces) of mountain hares (n = 38) showed a microsatellite amplification success reaching 97.71%, quite similar to the amplification success obtained in Rehnus and Bollmann (2016) on noninvasive samples (feces). The estimates of allelic dropout (0.17 across loci) and false allele (0.014) were consistent with the ranges obtained in previous noninvasive genetic studies (Frantz et al., 2003; Steyer, Simon, Kraus, Haase, & Nowak, 2012). In particular, this panel may be used to estimate other population parameters such as population size and survival rates when more common tools (such as direct counts or telemetry for example) reveal impractical (Kohn et al., 1999; Lukacs & Burnham, 2005). More widely, this panel of microsatellites should allow investigating the population genetic structures of the two hare species.

4.2 | Specific identification of hares and hybrids detection

The punctual inconsistencies between the morphological and the genetic identification (1.4% and 6.8% of individuals wrongly assigned to the brown hare and the mountain hare, respectively, when considering exclusively individuals sampled in regions were both species are observed) show that morphologically similar individuals can belong to distinct species. The panel of microsatellites might then come to validate morphological identifications, in particular in mountain areas, where the accurate estimation of the species distribution over time is crucial in the context of climate change.

Moreover, this panel allowed to detect reliably first and second generation hybrids, as well as a majority of backcrossed individuals. Hybridization has been reported in several occasions (Thulin, Fang, et al., 2006; Thulin, Stone, et al., 2006) and is expected to happen more and more notably due to the fragmentation of the landscape and the change in populations ranges caused by climate change (Parmesan & Yohe, 2003). Thus, it might represent a growing issue for the conservation of the mountain hare. The present data show signs of hybridization as one individual appears clearly as a hybrid (assigned to several of the hybrid classes of Newhybrids but never to the parental classes, using nine or 12 markers). This individual was located in the contact zone (see supplementary, Figure S2). Four other individuals showed signs of hybridization and were mostly assigned to the backcrossed categories. However, given the resolution of the microsatellite panel for this class of hybrids, we cannot know for sure whether they are hybrids. The presence of such suspicious individuals reinforces the need for a genetic tool to further investigate to what extent hybridization might represent a threat for the conservation of this species in the Alps. Thus, the 12-plex hereby proposed represents an interesting tool for the quantification of interspecific reproduction events although it proved efficient mainly for the detection of recent events of hybridization. Its resolution power remains limited for older events, and more markers are required to reliably detect backcrosses.

4.3 | Using multiplexes in wildlife management

The multiplexing of several markers is always a challenge because of the interactions between primers that may prevent correct amplification of some loci and potentially lead to genotyping errors such as allelic dropout. However, a careful primer selection and multiple adjustments allow for the coamplification of a substantial number of microsatellites (Guichoux et al., 2011; Hill, Butler, & Vallone, 2009). The 12-plex described in this study is the largest, to our knowledge, to have been proposed for the simultaneous study of the brown hare and the mountain hare. The multiplexing approach is particularly appropriate considering the development of noninvasive genetic studies for which DNA extracted from samples is invariably in extremely small quantities (Beja-Pereira et al., 2009). However, multiplex genotyping panels rarely consist of more than eight markers (Guichoux et al., 2011). Larger multiplexes have been proposed, for example, in humans (26 microsatellites, Hill et al., 2009) or in apricots (20 microsatellites, Campoy, Martínez-Gómez, Ruiz, Rees, & Celton, 2010), but they remain few. The use of next-generation sequencing methodologies should promote their development as the technology allows for the simultaneous detection of many markers (Gardner, Fitch, Bertozzi, & Lowe, 2011), increasing the probability to find compatible microsatellite markers (i.e., markers that do not overlap when attributed to the same dye).

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CONFLICT OF INTEREST

None declared.

DATA ACCESSIBILITY

The data is available from the dryad repository: http://dx.doi.org/10.5061/dryad.d4368.

AUTHOR CONTRIBUTION

J-SG and JL designed the study and coordinated field data sampling. LM, CK, and GQ developed the panel of microsatellite markers. MPB conducted the analysis and prepared the initial draft of the manuscript. GQ and DP supervised the analysis and writing of the manuscript. JL and JSG contributed to the writing of and critically revised the manuscript. All authors approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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