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Development and characterization of nine microsatellite loci from the Korean hare (*Lepus coreanus*) and genetic diversity in South Korea

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The Korean hare, *Lepus coreanus,* is an important mammal in ecosystem food chains, and is distributed across the entire Korean peninsula and northeastern China. Polymorphic microsatellite loci were developed using the biotin-enrichment technique for use in population genetics studies. Five trinucleotide and four dinucleotide microsatellite loci were selected and tested on 22 Korean hare specimens collected from Gangwon Province and Gyeongsangbuk Province in South Korea. The number of alleles across the two sampling regions ranged from three to nine with a mean of 6.1. Mean observed and expected heterozygosities and polymorphic information content were 0.540, 0.627 and 0.579, respectively. Only one locus, *Lc*06, showed departure from Hardy-Weinberg equilibrium after applying the Bonferroni correction. Four microsatellites, *Lc*01, *Lc*03, *Lc*12, and *Lc*19, satisfied the criteria to serve as a core set of markers recommended for population genetics studies. These new microsatellite markers will be widely applicable to future genetic studies for management and conservation of the Korean hare and related species, including assessment of the genetic diversity and population structure of *L. coreanus.*

Keywords: Leporidae; Korean hare; *Lepus coreanus*; microsatellite; magnetic bead enrichment

Introduction

In Leporidae, the genus *Lepus* includes approximately 12 to over 30 species depending on the classification scheme (Flux and Angermann 1990). Clear taxonomic status of species in *Lepus* remains unresolved. In particular, the taxonomic classification of five hare species (*L. sinensis*, *L. mandshuricus*, *L. capensis*, *L. timidus*, and *L. brachyurus*) from eastern Asia near the Korean peninsula, along with the Korean hare, *L. coreanus*, is under considerable debate (Waltari et al. 2004; Wu et al. 2005; Koh and Jang 2010).

According to Flux and Angermann (1990), *L. coreanus* might be most closely related to the Manchurian hare, *L. mandshuricus*, which has a similar body size. Also *L. mandshuricus* was often misidentified as *L. coreanus* because of overlapping distribution in northern Korea (Jones and Johnson 1965). Subsequently, molecular data from mitochondrial DNA (mtDNA) has been used to infer *Lepus* species phylogenies. Analysis of partial cytochrome *b* gene (655 bp) suggested that *L. coreanus* is distinct from the other five East Asian hare species (Koh et al. 2001). In contrast, Waltari et al. (2004) and Wu et al. (2005) concluded that *L. coreanus* is more closely related to *L. timidus* than to other *Lepus* species, based on molecular comparisons of the mitochondrial DNA fragments. More recently, analyses of the nuclear DNA thyroglobulin gene and mtDNA control region among East Asian *Lepus* species suggested that *L. coreanus* has no close relationship with *L. timidus* (Koh and Jang 2010). However, all of these phylogenetic studies among *Lepus* species were conducted in a limited area and based on analyses of only a few specimens.

The occurrence of interspecific hybridization and introgression between the different *Lepus* species has been observed in Europe (Thulin and Tegelstrom 2002; Melo-Ferreira et al. 2005). Although, Waltari et al. (2004) suggested that genetic discontinuities were found from the three closely related arctic hares (*L. arcticus*, *L. othus*, and *L. timidus*) in the Beringia, no obvious genetic relationship of *Lepus* species has been clearly elucidated so far. It is unclear whether there is a possible biological barrier affecting gene flow in *L. coreanus* and other closely distributed *Lepus* species populations.

In Korea, *L. coreanus* is considered as an indigenous species (Jones and Johnson 1965). It is not only distributed throughout the entire Korean peninsula, but also inhabits Jillin, Liaoning and Heilongjiang provinces in northeastern China (Hoffmann and Smith 2005). Though distribution and demography of *L. coreanus* have not been comprehensively investigated, Korean hares are frequently encountered in mountainous areas and less commonly in hilly districts (Won et al. 2001). *L. coreanus* has historically been recognized as one of the common species of South Korea, yet its population has been steadily declining in recent years as a result of indiscriminate hunting and habitat destruction (Won and Smith 1999). Despite its

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continuing decline, *L. coreanus* is currently listed as an animal of low concern in the International Union for Conservation of Nature and Natural Resources (IUCN) red list, and there is a lack of zoological information, including demographic research. To effectively manage *L. coreanus*, clarification of population relationships and demographic research is of primary importance for providing a foundation for practical conservation of this indigenous species.

Herbivores stimulate energy flow through different trophic levels in food-chains (Feldhamer et al. 2007). They perform the role of primary consumer by feeding on autotrophs, which manufacture their energy through photosynthesis (Mukhopadhyay and Bhattacharyya 2007). As a key herbivorous animal in Korean ecosystems, *L. coreanus* usually feeds on herbs, bark and buds. In turn, they are important food items for predators such as leopard cats and Eurasian eagle owls (Won et al. 2004). Multi-trophic levels of interaction in the food chain can affect the relationship between population diversity and ecosystem function (Finke and Snyder 2010). Since *L. coreanus* is an important component of trophic interactions in the ecosystem as a primary consumer and as prey for carnivores, there is a need for identifying its contribution to biodiversity and for the development of a management strategy for this animal.

In this study, we report isolation and characterization of novel microsatellite markers that will be useful in future population, demographic, and phylogenetic studies of *L. coreanus* and other *Lepus* species. We apply these markers to provide initial insights into the population genetic structure of *L. coreanus* in South Korea.

Materials and methods

DNA samples

In total, 22 specimens of Korean hare, provided by Conservation Genome Resource Bank for Korean Wildlife, were analyzed. Eleven samples were collected from each of two localities, one from Gangwon Province in the northeastern South Korea, and the other from Gyeongsangbuk Province in southeastern South Korea. Genomic DNA was isolated from muscle tissue samples preserved at 70°C using the DNeasy Tissue Kit (QIAGEN, USA).

Microsatellite marker development

Polymorphic microsatellites were isolated using the biotin-enrichment technique (Ronald et al. 2000; Kim and Sappington 2004). The extracted DNA was electrophoresed on a 1.2% agarose gel in 0.5 × TBE buffer and digested with the restriction enzyme *Nde*II. We excised and purified 250 ~ 700 bp DNA fragments using the DNA Clean & Concentrator-5^{TM} (Zymo research, USA). Following the methods of Rozen and Skaletsky (2000), we ligated 1 μg of the *Nde*II linkers, EP-1 and EP-2 to the size-selected genomic DNA using 20 μl of T4 DNA ligase (Promega, USA). Through EP-1, EP-2 linker ligation, priming sites were provided for the first polymerase chain reaction (PCR) amplification using primer EP-3. PCR was performed in a TaKaRa PCR Thermal Cycler with 1.5 U i-star Taq DNA polymerase (iNtRON Inc, Korea), 1 × PCR buffer, 200 μM of each dNTP, 2 mM of $MgCl_2$ and 1.6 μM of EP-3 in a total of 30 μl. To remove the detached residual linkers below 100 bp in length, we used the Microcon 100 centrifugal filter (Millipore Corporation, USA).

The biotinylated capture probe was annealed to the linkered DNA. 89 μl of 5 × SSC, 10 μl of amplified DNA, and 1 μl each of 5′ biotinylated capture probes $(CA)_{15}$, $(CT)_{15}$ and $(AGC)_7$ were combined at different temperatures. The mixture was heated at 95°C for 10 min, cooled on ice for 30 sec, then incubated for 5 min at room temperature. Capture hybridization on magnetic beads was accomplished through compounding the annealed DNA with 100 μl of washed magnetic beads (1 mg/ml), followed by incubation for 15 min at room temperature. After incubation, the beads were washed three times with 200 μl of 2 × SSC at room temperature, and three times with 200 μl of 1 × SSC at the appropriate temperature (CA, 65°C; AGC, 67°C; CT, 61°C) for 3 min. Captured DNA was extracted from the magnetic beads into 50 μl of distilled water and incubated for 5 min at 95°C. A second amplification of the captured hybrid DNA was performed using the EP-3 primer as described for the first PCR.

We ligated the prepared PCR product to the pGEM-T vector and transformed *E. coli* JM109 (Promega) with the vector. After incubation of the *E. coli* colony, 527 positive white colonies were selected by eye. The plasmid DNAs were isolated by amplification with M13 forward and reverse primers and repeat priming with internal primer according to the method described by Schuelke (2000). Following electrophoresis of the PCR product, we screened 294 positive colonies that clearly showed a smear band, and 112 of the clones were sequenced. Based on flanking repeat elements, a total of 47 primer pairs were designed using Primer 3 (Rozen and Skaletsky 2000), and of these, 22 sets of primer pairs were subsequently screened.

Genotyping

Amplification for genotyping was performed with 1 ~ 5 ng of template, 1 × PCR buffer, 4 pmol of fluorescence

dye labeled forward primer and non-labeled reverse primer, 0.5 unit of i-star Taq DNA polymerase (iNtRON Inc), 1.5 mM $MgCl_2$, and 200 μM each dNTP in a 10 μl of total volume. We used a touchdown profile for PCR performed at 94°C for 3 min, followed by 20 cycles at 94°C for 1 min, 60°C for 1 min with a decrease of 0.5°C per cycle, 72°C for 1 min, followed by 20 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min in a TaKaRa PCR Thermal Cycler. Sizes of alleles were determined using an ABI Prism 3730 XL DNA analyzer (Applied Biosystems, USA) with size standard GENESCAN-500 [Rox] and GeneMapper v3.7 (Applied Biosystems).

Data analysis

To isolate suitable microsatellite markers for successful population genetics studies, data analyses were conducted to examine characteristics under five criteria; polymorphism, readability and repeatability, null alleles, selective neutrality, and linkage relationships (Kim et al. 2008; An et al. 2010a). Observed (H_O) and expected (H_E) heterozygosities, and polymorphism information content (PIC) were estimated using Cervus v3.0 (Kalinowski et al. 2007) and allelic diversity was calculated using GenAlEx v6.1 (Peakall and Smouse 2006) to characterize the polymorphism of each marker. We incorporated positive controls while making allele size calls and genotyping. The plausible occurrence of null alleles was tested using the program MICROCHECKER (Oosterhout et al. 2004). The presence of null alleles is suggested when significant excess homozygosity is dispersed equally across all alleles at a locus. Arlequin 3.11 (Excoffier et al. 2005) was employed to test selective neutrality of each locus using the Ewens-Watterson-Slatkin exact test (Slatkin 1996) of allele frequency distribution.

Deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were tested using Genepop v4.0 (Rousset 2008). To examine the population structure pattern and differentiation, we applied Structure 2.3.2 using structure simulation by the Bayesian clustering method (Pritchard and Rosenberg 1999) and FSTAT 2.9.3.2 to calculate pairwise *F*st (Weir and Cockerham 1984).

Results

Of the 22 microsatellite loci tested, 13 were monomorphic or not amplified clearly. The remaining nine loci were polymorphic among the 22 individuals sampled from two regions in South Korea (Table 1). These markers, therefore, were further employed to obtain population genetics parameters for each location, as well as the total for combined samples (Table 2).

Table 1. Sampling localities of Korean hare in this study.

No.	CGRB ID	Year of collection	Locality
1	2530	2005	Chuncheon, Gangwon Province
2	2538	2005	Chuncheon, Gangwon Province
3	2542	2005	Chuncheon, Gangwon Province
4	2906	2006	Chuncheon, Gangwon Province
5	2907	2006	Chuncheon, Gangwon Province
6	3115	2006	Gangwon Province
7	3652	2006	Gangneung, Gangwon Province
8	3680	2006	Gangneung, Gangwon Province
9	3727	2006	Gangwon Province
10	3737	2006	Hwacheon, Gangwon Province
11	3755	2006	Chuncheon, Gangwon Province
12	278	2003	Uljin, Gyeongsangbuk Province
13	543	2004	Yeongcheon, Gyeongsangbuk Province
14	1790	2004	Bonghwa, Gyeongsangbuk Province
15	1841	2005	Bonghwa, Gyeongsangbuk Province
16	1842	2005	Bonghwa, Gyeongsangbuk Province
17	1965	2005	Andong, Gyeongsangbuk Province
18	3668	2006	Andong, Gyeongsangbuk Province
19	3921	2006	Yeongju, Gyeongsangbuk Province
20	4059	2007	Yeongju, Gyeongsangbuk Province
21	6162	2008	Pohang, Gyeongsangbuk Province
22	6735	2008	Gunwi, Gyeongsangbuk Province

CGRB, Conservation Genome Resources Bank for Korean Wildlife.

A total of 55 alleles were observed in the 22 Korean hares for the nine polymorphic microsatellite markers, with 44 alleles observed in the individuals from Gangwon Province and 46 alleles in the individuals from Gyeongsangbuk Province. In the individuals from Gangwon Province, the number of alleles per locus ranged from three to eight. *Lc*03 had the highest number of alleles, which included 10 pairs of

Table 2. Characteristics of nine microsatellite loci developed for *Lepus coreanus*.

Locus	Clone ID	Primer sequence	5' Fluorescent label	Repeat motif	Size (bp)	Accession no.	Polymorphism[a]	Null allelesb		Selective neutralityc		Linkage between locid
								Presence	Brookfield frequency	Presence	Slatkin's exact P value	
*Lc01**	25-a12	F-AAGGCAGGAAGCTAGTTGGA R-CAGACATGAAGTGGCAGCAG	6FAM	(AGC)6	165–180	GU471251	Moderate	No	0.0424	Yes	0.057	*Lc06, Lc11*
Lc02	25-b7	F-TGCATCATGGAAACCCAGTA R-GTGCACTGAGAGGTTGATGC	6FAM	(AGC)7	149–158	GU471252	Low	No	0	Yes	0.398	–
*Lc03**	25-b10	F-GTTTGGCCACTTTTTCTGGA R-GTCAACATTGGAGGCAGGAG	6FAM	(TGC)7	155–185	GU471253	Moderate	No	0	Yes	0.849	–
Lc06	25-e10	F-AGCAGCGTTTGTCAACAAGG R-AAGGCAGGAAGCTAGTTGGA	6FAM	(GCT)12	150–168	GU471254	Moderate	Yes	0.197	Yes	0.528	*Lc01, Lc11*
Lc11	22-h6	F-AAGGCAGGAAGCTAGTTGGA R-GCGTAAGAGGAAAAGCTTCG	HEX	(AGC)12	240–258	GU471255	Moderate	Yes	0.12	Yes	0.624	*Lc01, Lc06*
*Lc12**	36-b7	F-CCATGAATGCACACTCCAAA R-GTAGTGAACCCTCGGATGGA	6FAM	(AG)8	142–152	GU471256	Moderate	No	0.0713	Yes	0.706	–
Lc13	50-e6	F-AGTCCCTGGGAGGATAAGGA R-GGTGAACCACAGGAAGGAAG	6FAM	(GA)17	144–164	GU471257	High	No	0.0107	Yes	0.067	–
*Lc19**	7-c11	F-TGACACATGAGGGGTCTTCA R-TGAGGTTTGCTGTGGATTGA	HEX	(GT)14 (GA)10	201–215	GU471259	Moderate	No	0	Yes	0.966	–
Lc22	8-h3	F-TGCATCATTGGCAGTTGATAG R-GGAGAAGCTGTGAAGCCATC	6FAM	(CA)11	144–150	GU471260	Moderate	Yes	0.0906	No	0.02	–

* Indicates the core set of microsatellites of *L. coreanus* tested on 22 Korean hare specimens.
[a] Based on the expected heterozygosity (H_E); 0 < low (L) < 0.4, 0.4 ≤ moderate (M) < 0.8, 0.8 ≤ high (H) < 0.9.
[b] Presence of null allele discovered based on family analysis.
[c] 'Yes' denotes the null hypothesis of selective neutrality against the presence of selection was not rejected for that locus at $P = 0.05$, and 'No' denotes the null hypothesis of neutrality was rejected for that locus.
[d] Linkage disequilibrium detected at $P = 0.05$ after Bonferroni correction for multiple testing.

heterozygotes and one homozygote. Mean allele frequency of *Lc*03 was 0.11, which was the lowest value among polymorphic loci. The highest mean allele frequency, 0.33, was exhibited by *Lc*02. In this cluster, mean H_O, H_E, and PIC were 0.535, 0.616, and 0.550, respectively. H_O was higher than H_E at four loci (*Lc*02, 03, 12, and 19), and the H_O values of *Lc*02 and *Lc*12 were less than 0.5. *Lc*01 and *Lc*06 showed the highest value of H_E, which implies higher gene diversity.

The number of alleles per locus in Korean hares from Gyeongsangbuk Province ranged from three to seven, and mean H_O, H_E, and PIC were 0.545, 0.630, and 0.567, respectively. Similar to the individuals from Gangwon Province, four loci (*Lc*01, 02, 13, and 19) had higher values of H_O than H_E, and that of *Lc*02 was less than 0.5. Locus *Lc*13 showed the highest level of H_O and allele diversity. Seven alleles were detected from *Lc*13 and mean allele frequency was 0.11. *Lc*01 also showed the highest mean allele frequency (0.33). Low H_O values compared to H_E in loci could be generated by non-random mating or sampling error. None of the loci showed significant deviation from HWE for the populations tested after the Bonferroni correction (adjusted significance [5%] threshold = 0.00138). Gene diversity of Korean hares in the two regions was similar (0.616 vs. 0.630 in mean H_E).

When Korean hares from the two regions were combined, the number of alleles ranged from three to nine (mean = 6.1), and mean H_O, H_E, and PIC were 0.540, 0.627, and 0.579, respectively. Only one locus, *Lc*06, showed departure from Hardy-Weinberg equilibrium after applying the Bonferroni correction for multiple tests. This might reflect an underestimation of observed heterozygosity caused by a Wahlund effect. Three pairs of loci (*Lc*01 vs. *Lc*06, *Lc*06 vs. *Lc*11, *Lc*11 vs. *Lc*01) were in significant linkage disequilibrium, indicating non-independence among the three loci ($P < 0.05$).

F_{ST} values for each locus ranged from 0.026 for *Lc*01 to 0.051 in *Lc*22 (Table 3). Overall F_{ST} values across loci showed no significant genetic differentiation between the two sampling regions of Korean hares ($F_{ST} = 0.008$, *P*-value = 0.65). Furthermore, the Structure analysis using seven independent loci (*Lc*01, *Lc*02, *Lc*03, *Lc*12, *Lc*13, *Lc*19, and *Lc*22) showed no evidence of population structure, as indicated in the bar plot (Figure 1). The highest value of mean log likelihood distribution was obtained when K was set to one (Table 4).

Four of the microsatellite loci, *Lc*01, *Lc*03, *Lc*12, and *Lc*19, satisfied the criteria for a core set of population genetic markers suggested by Kim et al. (2008) (Table 1). These four markers showed no evidence for the presence of null alleles, selection, or linkage disequilibrium between loci. In addition, these markers revealed non-ambiguous PCR products with a moderate level of polymorphism ($0.4 < H_E < 0.8$).

Discussion

Population genetics parameters using nine newly developed markers in this study were measured for Korean hares from two locations in the eastern part of South Korea. An F_{ST} test and Structure analysis revealed that no clear geographical barrier exists between the Korean hares from Gangwon Province and Gyeongsangbuk Province. Compared with the results when all nine

Table 3. Descriptive statistics for 22 samples of Korean hares from two populations.

Locus	Gangwon province ($n = 11$)[a]					Gyeongsangbuk province ($n = 11$)[a]					Total ($n = 22$)[a]					
	k[b]	H_O[c]	H_E[d]	PIC[e]	*P*[f]	k[b]	H_O[c]	H_E[d]	PIC[e]	*P*[f]	k[b]	H_O[c]	H_E[d]	PIC[e]	*P*[f]	F_{ST}
*Lc*01	6	0.545	0.810	0.742	0.038	5	0.818	0.753	0.674	0.154	6	0.682	0.774	0.722	0.045	0.026
*Lc*02	3	0.364	0.329	0.292	1.000	3	0.455	0.385	0.326	1.000	3	0.409	0.352	0.314	1.000	0.021
*Lc*03	8	0.909	0.788	0.722	0.936	6	0.545	0.667	0.595	0.124	9	0.727	0.720	0.670	0.419	0.020
*Lc*06	5	0.364	0.714	0.630	0.005	5	0.273	0.584	0.527	0.014	6	0.318	0.656	0.603	0.0001	0.004
*Lc*11	5	0.455	0.719	0.635	0.042	5	0.364	0.472	0.428	0.109	6	0.409	0.615	0.565	0.002	0.047
*Lc*12	3	0.273	0.255	0.228	1.000	5	0.364	0.584	0.527	0.014	5	0.318	0.429	0.399	0.056	0.030
*Lc*13	7	0.727	0.831	0.765	0.348	7	0.909	0.870	0.808	0.993	9	0.818	0.857	0.818	0.602	0.013
*Lc*19	3	0.636	0.481	0.387	0.629	6	0.636	0.589	0.535	0.411	7	0.636	0.532	0.479	0.237	0.001
*Lc*22	4	0.545	0.619	0.547	0.499	4	0.545	0.766	0.681	0.156	4	0.545	0.716	0.649	0.095	0.051
Mean	4.89	0.535	0.616	0.550	0.500	5.11	0.545	0.630	0.567	0.331	6.11	0.540	0.628	0.580	0.307	0.008

*Indicates the locus with significant departure from Hardy-Weinberg equilibrium.
[a]Number of individuals.
[b]Number of alleles.
[c]Observed heterozygosity.
[d]Expected heterzoygosity.
[e]Polymorphism information content.
[f]*P*-value for heterozygote deficit.

Figure 1. Bar-plot (K = 2) derived from the *Q*-matrix using the population structure analysis for the Korean hares (sampling region 1: Gangwon Province; sampling region 2: Gyeongsangbuk Province).

markers were used, the core set of markers alone showed an even lower F_{ST} (0.004) between Korean hares from the two regions, confirming no genetic differentiation between them. In this study, we determined that Korean hares from two different geographic locations were clustered together as a single population, but future researchers must consider unknown barriers that may affect genetic structure (Evanno et al. 2005).

Although our sample size was small in this study, we can infer that the generally flat geographic topography of South Korea does not pose a significant biological barrier to gene flow among Korean hares. To develop the conservation management strategies and to understand the evolutionary history of *L. coreanus*, studies of phylogenetic relationships, genetic diversity and population genetics using more individuals from more geographic locations are required. The microsatellite markers developed in this study will facilitate future population genetics studies of not only Korean hares but also other closely related species, depending on the results of future cross-species amplification studies (An et al. 2010b). The genetic data from this study will contribute to strategies for management and conservation of *L. coreanus* in Korea and China.

The markers we developed in this study will be useful in predicting the evolutionary history of *Lepus* species particularly in East Asia and pattern of gene flow among hare populations at regional scales, eventually contributing to establishing the management strategy of this species.

Table 4. Rate of log likelihood distribution (Ln) depending on the number of iterations (1 to 10) using seven independent loci (*Lc*01, *Lc*02, *Lc*03, *Lc*12, *Lc*13, *Lc*19, and *Lc*22).

	Ln		
No. of iterations	K = 1	K = 2	K = 3
1	−420.7	−421.8	−423.9
2	−421.5	−421.9	−421.4
3	−421	−420.2*	−422.5
4	−420.6*	−423.7	−422
5	−420.7	−420.9	−422.2
6	−420.8	−425.6	−422.8
7	−420.7	−425.2	−421.9
8	−422.2	−427.2	−422.6
9	−420.7	−421.6	−423.8
10	−420.7	−422.7	−423.2
mean	−420.96**	−423.08	−422.63

* Indicates highest rate of log likelihood distribution.
** Indicates highest mean log likelihood distribution rate.

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