FULL PAPER

Wildlife Science

Individual identification of Eurasian otters (*Lutra lutra*) in South Korea (Sincheon River, Daegu) by microsatellite markers

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ABSTRACT. The objective of this study was to determine the number of Eurasian otters (*Lutra lutra*) that occupied the Sincheon River in Daegu, South Korea. Twenty-seven spraints collected from February to May 2016 at four sites (Jangam Bridge approximately 6.1 km from the Gachang Dam, Docheong Bridge approximately 13.5 km, Chimsan Bridge approximately 15.1 km and Nogoek Bridge approximately 18 km) along the Sincheon River (approximately 27.06 km) were analyzed using 12 microsatellite markers. The analyses resulted in the identification of 16 (59.3%) individual Eurasian otters in the Sincheon River based on the 27 spraints. Of the 16 individual Eurasian otters, seven were male, and nine were female. Groups were centered at the Jangam Bridge (3 males and 2 females), Chimsan Bridge (2 males and 3 females) and Docheong Bridge (2 males and 4 females). Thus, the 16 Eurasian otters formed three genetically related groups in each sampling area. The number of alleles per locus varied from three to seven, with a mean value of 5.08 alleles.

KEY WORDS: Eurasian otter, individual identification, microsatellite marker, spraints

The Eurasian otter is an internationally protected species by the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List and Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), because of population declines caused by pollution, habitat loss and roadkills resulting from industrialization [15]. This species is also protected as an endangered species I and national monument No. 330 designated by the Korean government in 1982 [9].

The otter belongs to the order Carnivora, family Mustelidae [1], which also includes weasels and badgers. Otters are further classified into subfamily Lutrinae, which is specific to otters. The otter is widespread in Europe, Africa and Asia. The original classification system for otters had 19 species with 63 subspecies; however, Corbet and Hill classified four genera (*Lutra, Pteronura, Aonyx* and *Enhydra*) with 13 species [2]. More recently, a classification system having five genera with 13 species, also including the genus *Lontra*, was established. Today, 13 otter species worldwide, including the Eurasian otter, *Lutra lutra* [15, 17], are recognized in the subfamily Lutrinae and the IUCN Red List [10].

The number of individuals of Eurasian otters in South Korea is unknown. The distribution of the otter in Korea is wide and encompasses the river where it originates inland in the Baekdudaegan Mountain range and the southern coast with its many small islands having shores with gentle waves [7, 20].

The length of the Sincheon River is approximately 27.06 km. The Gachang Dam is located upstream, and the Sincheon River joins with the Geumho River downstream. The first sighting of an otter in the Sincheon River was reported in 2005, and thereafter, eight otters were confirmed to inhabit the river by DNA typing in 2010 [15]. Research on otters in South Korea has primarily focused on their ecological environment and molecular biology [15, 20].

The detection of mammals, including the Eurasian otter, can be challenging, because of low densities and/or elusive behavior. Furthermore, traditional capture mark recapture approaches can cause practical and ethical difficulties [14]. DNA-based assays of noninvasive genetic samples (e.g., from spraints) have become reliable tools for species identification, molecular sex typing and individual identification by microsatellite genotyping using DNA [4, 6, 8, 12, 13, 18]. Microsatellite DNA typing has been applied to various fields in molecular genetic research on humans and animals using polymerase chain reaction (PCR) techniques that require only a small amount of DNA for analysis, which can come from blood, oral epithelioid cells, hair roots or feces [1].
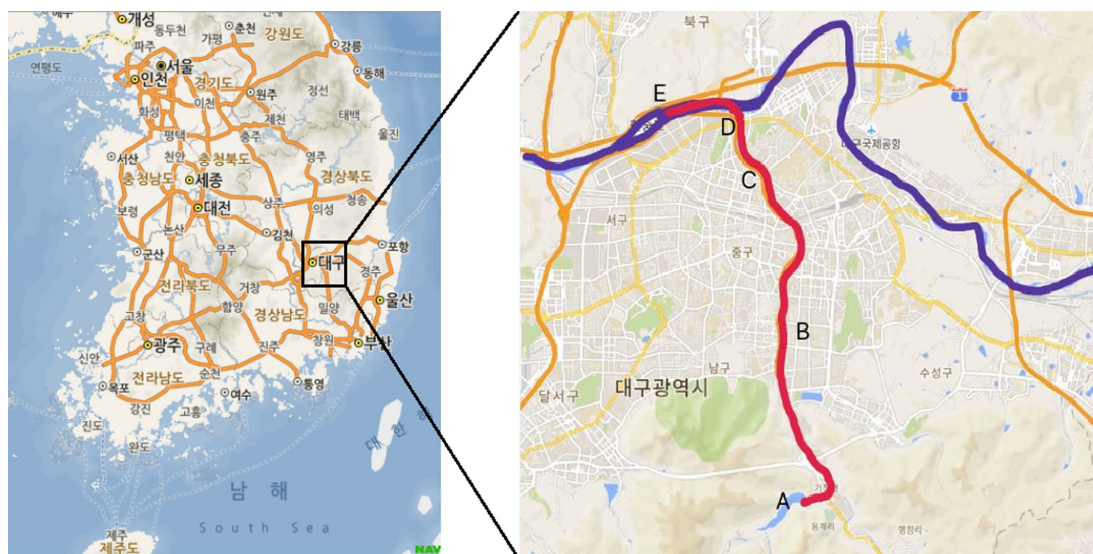
Our research team hypothesized that the number of otters in the Daegu Sincheon River increased gradually since their first

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Fig. 1. Location of study site, Daegu Sincheon River, South Korea. The length of the Sincheon River (Red color) is approximately 27.06 km. The Gachang Dam is located upstream, and it joins with the Geumho River (Blue color) downstream. A, Gachang Dam; B, Jangam Bridge; C, Docheong Bridge; D, Chimsan Bridge; E, Nogoek Bridge (Citation in caption Maps from Google Maps, Google).

report of their occurrence in 2005. The goal of this study was to identify individuals and their sex based on genetic analysis of DNA isolated from feces (spraints) collected along the Daegu Sincheon River, South Korea.

MATERIALS AND METHODS

Sample collection and DNA extraction

This study area was located between 35°47′54″N, 128°38′55″E and 35°54′16″N, 128°35′30″E (Fig. 1). Spraints were collected from February to May 2016 at four sites (Jangam Bridge approximately 6.1 km from the Gachang Dam, Docheong Bridge approximately 13.5 km, Chimsan Bridge approximately 15.1 km and Nogoek Bridge approximately 18 km) along the Sincheon River (approximately 27.06 km), Daegu, Korea. Twenty-seven spraints (10 at Jangam Bridge, seven at Docheong Bridge, eight at Chimsan Bridge and two at Nogoek Bridge) were collected. As fresh as possible feces of otters were collected from under bridges. Genomic DNA was extracted from samples using a QIAGEN QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

### Microsatellite marker and analysis

Before genotyping, to determine whether the genomic DNA was actually from an otter, we used otter specific primers reported by Park *et al*. [15], LutcytF (5′-CCACAATCCTCAACAACTCGC-3′) and LutcytR (5′-CTCCGTTTGGGTGTATGTATCG-3′), which were designed to amplify the partial cytochrome *b* sequence of otters. For the PCR, template DNA 2 μl, 10 pmol forward and reverse primer 1 μl each, and sterile distilled water 3 μl were mixed with PCR Premix buffer 3 μl (Qiagen), adjusted to 10 μl total, and then amplified by the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, U.S.A.). PCR amplification was as follows: denaturation for 5 min at 94°C, followed by 35 cycles at 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec. An extension step at 72°C for 7 min was conducted after the final cycle [15].

Amplified products were analyzed using 2.5% agarose gel electrophoresis, stained with ethidium bromide and visualized on a UV transilluminator [15]. The band intensities of the fecal PCR products were compared to those of DNA from otter tissues. Twelve microsatellite markers (Lut 435, Lut 453, Lut 457, Lut 604, Lut 615, Lut 701, Lut 715, Lut 717, Lut 733, Lut 782, Lut 818 and Lut 833) reported by Dallas and Piertney [3] and Park *et al*. [15] were used for PCR genotyping. For the PCR, template DNA 2 μl, 10 pmol forward and reverse primer 1 μl each, and sterile distilled water 8 μl were mixed with PCR Premix buffer 3 μl (Qiagen), adjusted to 15 μl total, and then amplified by the GeneAmp PCR system 9700 (Applied Biosystems). PCR was performed using an ABI 9700 with a 95°C denaturing step for 15 min, followed by 20 cycles at 95°C for 45 sec, 59°C for 45 sec, 72°C for 45 sec, 20 cycles at 95°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec, with a final extension step at 72°C for 30 min. Single PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems). PCR products were denatured with formamide, and electrophoresis was conducted on an ABI 3130xL Genetic Analyzer (Applied Biosystems) using the recommended protocols. We repeated the PCR for genotyping a minimum of four independent times to minimize mistyping errors caused by allelic dropout and false alleles. Sizes of alleles were scored using GeneMapper Software Ver. 4.0 (Applied Biosystems). Lastly, to determine sex of each individual otter, we used the method recommended by Dallas *et al*. [4]. If ambiguities arose, further replicates were conducted until a consensus was reached.

Table 1. Number of alleles, heterozygosity and PIC values of the 12 microsatellite loci in 16 the Eurasian otters

Marker	No. of alleles	Ho	He	PIC
Lut 435	4	0.7500	0.5907	0.4888
Lut 453	5	0.3750	0.6129	0.5223
Lut 457	4	0.5625	0.5444	0.4422
Lut 604	6	0.6250	0.7157	0.6518
Lut 615	6	0.3125	0.7319	0.6743
Lut 701	4	0.3750	0.5060	0.4545
Lut 715	6	0.1875	0.5202	0.4814
Lut 717	7	0.3750	0.7540	0.6916
Lut 733	3	0.6250	0.5948	0.4864
Lut 782	7	0.8125	0.7581	0.6974
Lut 818	4	0.3125	0.6331	0.5502
Lut 833	5	0.7500	0.8065	0.7470
Mean	5.08	0.5052	0.6473	0.5740

Ho, Observed heterozygosity; He, Expected heterozygosity; PIC, Polymorphism Information Content.
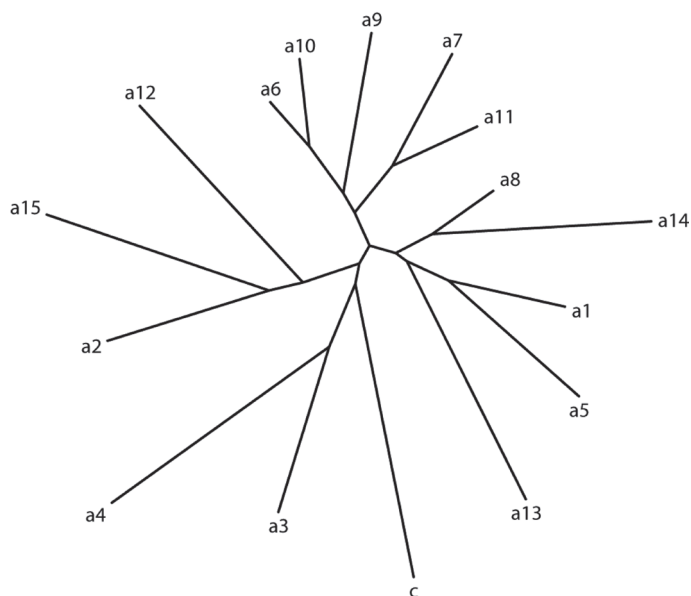
Fig. 2. Phylogenetic tree showing allele-sharing distances among 16 individuals in Eurasian otters. a6, a7, a9, a10, a11, Jangam Bridge; a1, a5, a8, a13, a14, Chimsan Bridge; c, a2, a3, a4, a12, a15, Docheong Bridge.

Statistical analysis

Allelic frequencies, the number of alleles per locus, observed heterozygosity, expected heterozygosity and polymorphic information content (PIC) were calculated using the Microsatellite Toolkit software [16]. After examining the relationship between the expected allele and genotype frequency, the Chi-square value and p-value were evaluated to determine the compatibility of the observed genetic distribution with the Hardy-Weinberg equilibrium. The clustering method [19] was used to construct a phylogenetic tree based on the genetic distance matrix in the Phylip package version 3.67 [5].

RESULTS

Analysis of the genetic diversity of the Eurasian otter

A total of 27 Eurasian otter spraint samples from four different locations (Jangam Bridge, Docheong Bridge, Chimsan ridge and Nogoek Bridge) along the Sincheon River were genetically analyzed using 12 microsatellite markers. Of the 27 samples, 16 were successfully amplified. Five of the 10 samples (50.0%) collected from the Jangam Bridge, six of seven (85.7%) from Docheong Bridge, five of eight (62.5%) from Chimsan Bridge and zero of two (0.0%) from Nogoek Bridge were genotyped. Unfortunately, of the 27 samples, 11 were genotyped with only nine to 11 microsatellite markers, because of the low quality of genomic DNA or cross contamination. These samples were excluded from individuals identification. Based on the results, at least 16 (59.3%) individuals existed in the Sincheon River. Of the 16 individual otters, seven were males, and nine were females. As shown in Table 1, there were 5.08 alleles out of the 12 microsatellite markers in the 16 Eurasian otter individuals. The Lut 717 and Lut 782 locus had a comparably high number of genotypes, whereas the Lut 733 locus had the fewest genotypes of the three alleles. The mean value of observed heterozygosity was 0.5052, with the highest value at the Lut 782 locus (0.8125) and the lowest value at the Lut 715 locus (0.1875). The average value of expected heterozygosity was 0.6473, with the highest value at the Lut 833 locus (0.8065) and the lowest value at the Lut 715 locus (0.5202). The mean microsatellite marker PIC value was 0.5740. Seven microsatellite markers had a PIC value higher than 0.5000, and among these values, the Lut 833 locus (0.7470) had a PIC value higher than 0.7000. In addition, no statistically significant deviations from the Hardy-Weinberg equilibrium were found when calculated separately for each locus.

Analysis of the phylogenetic relationships of the Eurasian otter

A phylogenetic dendrogram was created for the population using the Phylip Ver.3.67 statistics program. As shown in Fig. 2, clustering analysis, combined with biological and geographical data, divided the otters into three subgroups: one centered on the Jangam Bridge (3 male and 2 female otters), one centered on the Chimsan Bridge (2 male and 3 female otters) and another on the Docheong Bridge (2 male and 4 female otters).

DISCUSSION

By using microsatellite markers, we confirmed that 16 individual otters inhabited the area around the Sincheon River in Daegu.

The study included the area along the Sincheon River downstream of the Gachang Dam, which provided good habitat for otters, including weak currents and abundant prey, such as carp and catfish, although there were many artificial islands comprised of grass and gravel adjacent to the Geumho River. We confirmed that the number of otters in the Daegu Sincheon River has increased gradually since the first reports of their occurrence in 2005 [15], because of improvements in their habitable environment owing to the efforts of the government and citizens.

The activity radius (home range) of the otter is large, usually more than 10 km [11]. According to the findings of Han [7], male otters have a 15 km territory, and females have a territory of approximately 7 km. In addition, the territories of male otters encompass those of 2–3 females. Our results of analysis of the phylogenetic relationships of otters showed that they belonged to three main groups. One centered at the Jangam Bridge (3 males and 2 females), another centered at the Chimsan Bridge (2 males and 3 females) and the third at the Docheong Bridge (2 males and 4 females). Otters are highly social animals and tend to form groups. A lead male has 2–3 otters in the group, including one territorial female, and they mark their territory with excrement. Our findings are in agreement with those of previous studies [7].

The average number of alleles was 5.08, with the number of alleles distributed from three to seven. The PIC was higher than 0.5000 for seven microsatellite markers. Thus, these markers could be useful for individual identification and genetic parentage verification of otters. Allele number and PIC may be influenced by sample size. Unlike DNA from blood or tissue collected directly from captured animals, fecal DNA has limitations in population genetic studies of otters because of a low credibility level because of low-quantity or poor-quality DNA. Such samples may result in genotyping errors, such as allelic dropout and false alleles. Hence, when using feces as a DNA source, it is important to avoid genotyping errors.

The allelic peak appeared to vary in the microsatellite marker analysis when samples suffered from cross contamination or low-quality DNA. This might cause serious problems in precision and interpretation. To overcome this problem, fresh feces samples and only one sample per tube/case should be collected.

A non-invasive analytic approach using otter spraints successfully provided information about genetic diversity and relationships among otters living in this area. However, additional research is needed, including mitochondrial DNA analysis for full siblings or half siblings inhabiting this area, including areas along the Geumho River adjacent to the Sincheon River in Daegu, South Korea. Thereafter, we need to determine how each otter family in this study moves among habitats, including the Geumho River and the nearby Sincheon River. Other population demographics, including mortality and determination of family relationships for otters through offspring identification, are needed for otter preservation and conservation. Therefore, it is necessary to determine gene flow and genetic relationships between otter populations in this area and those living outside. Continuous monitoring will also be important.

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