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Japanese Hard Ticks (*Ixodes nipponensis*) Parasitizing on the Endangered Leopard Cat (*Prionailurus bengalensis euptilura*) in the Republic of Korea

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ABSTRACT

Because of the potential negative influence on their hosts, ecto-parasites are of prime importance to numerous species. Ticks are among these, distributed worldwide, and potentially transmitting diseases while sucking blood of diverse hosts. The leopard cat (*Prionailurus bengalensis euptilura* Elliot, 1871) is the only felid left in the Republic of Korea following widespread anthropogenic disturbances that have resulted in the extinction of both *Panthera* species: the Siberian tiger (*Panthera tigris altaica* Temminck, 1844) and Amur leopard (*P. pardus orientalis* (Schlegel, 1857)). This study identifies ticks collected from a roadkill leopard cat retrieved in Seosan area in the Republic of Korea. Two ticks attached to the facial area of the carcass were identified as Japanese hard ticks, *Ixodes nipponensis*, based on mitochondrial cytochrome oxidase I. The matching sample was from Japan with 99.7% similarities, and the only available sequence on GenBank. This study reconfirms that *I. nipponensis* parasitizes the endangered leopard cat *P. bengalensis euptilura*.

Keywords: *Ixodes nipponensis*, *Prionailurus bengalensis euptilura*, cytochrome oxidase subunit I, Republic of Korea

INTRODUCTION

Parasites have co-evolved with their hosts through the evolutionary times, and very few organisms are free of parasites (Brook, 1979). Parasites rarely kill their hosts, but they often have a negative impact on their fitness through mechanical damage, disease transmission, toxic production and immunodepression (Holdenried et al., 1951; Sokhna et al., 2004; Kamal and El Sayed Khalifa, 2006). Mammals are typically parasitized by worms like tapeworms or hookworms, ticks and flukes. Among ecto-parasites, ticks are common and obtain their blood meal by piercing the skin and sucking blood from their host (Elstron, 2010).

Ticks also often act as the main vector for disease transmission. For instance, ixodid (hard-bodied) ticks transmit the lyme borreliosis disease, a common tick-borne infection in the United States and Europe (Lane et al., 1991), and *Amblyomma* spp. transmits the rickettsial disease (Spolidorio et al., 2012). Ticks have been reported, although not common, from the Republic of Korea (Cho et al., 1994). Compared to areas with similar climates and topography, the lower prevalence is expected to be a low detection rate due to infrequent research. The first report of thrombocytopenia syndrome, the tick-to-human virus transmission on the Korean Peninsula was in 2013, and transmissions resulted in a higher fatality rate than typical for viruses (Shin et al., 2015).

Ticks are usually found on wild animals, especially mammals, and at least six tick species (*Haemaphysalis longicornis* Neumann, 1901, *H. flava* Neumann, 1897, *H. japonica* Warburton, 1908, *Ixodes nipponensis* Kitaoka & Saito, 1967, *I. pomerantzevi* Serdyukova, 1941, and *I. persulcatus* Schulze, 1930) have been confirmed to parasitize nine mam-

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mals in Korea (*Tamias sibiricus* (Laxmann, 1769), *Mogera robusta* Nehring, 1891, *Nyctereutes procyonoides* (Gray, 1834), *Hydropotes inermis* (Swinhoe, 1870), *Capreolus pygargus* (Pallas, 1771), *Felis catus* Linnaeus, 1758, *Mustela sibirica* Pallas, 1773, *Meles leucurus* Hodgson, 1847, and *Prionailurus bengalensis euptilura* Elliot, 1871) (Kim et al., 2011). Among the tick species, *I. nipponensis* have been reported as one of the main vectors to transmit tick-borne pathogens such as *Borrelia afzelii* Canica et al., 1994 and *B. valaisiana* Wang et al., 1997, and tick bites for humans in South Korea recently (Ryu et al., 1988; Part et al., 1993; Masuzawa et al., 1999; Ko et al., 2002; Chang et al., 2006). Among the host animals, the leopard cat (*P. bengalensis euptuilura*) is classified as endangered species in the Republic of Korea, and they are considered as one of the top predator. Despite of their crucial role to maintain the ecosystem balanced, there are only two research papers which mentioned about the leopard cat and ticks in the Republic of Korea. Here, we report the presence of *I. nipponensis* parasitizing the endangered leopard cat (*P. bengalensis euptilura*).

MATERIALS AND METHODS

The collection was conducted in Aejeong-ri, Seosan-si, South Chungcheong Province, 36.72372°N, 126.409916°E, on 20 Nov 2016. We collected the body of a roadkill leopard cat on the verge of a road (permit number Geum river area 2017-10). The muscles of the corpse were stiff, in rigor mortis, presumably a few hours (estimated *circa* 6 h) after death. The left side of the skull had been crushed, presumably because of automobile accident. Except for the skull,

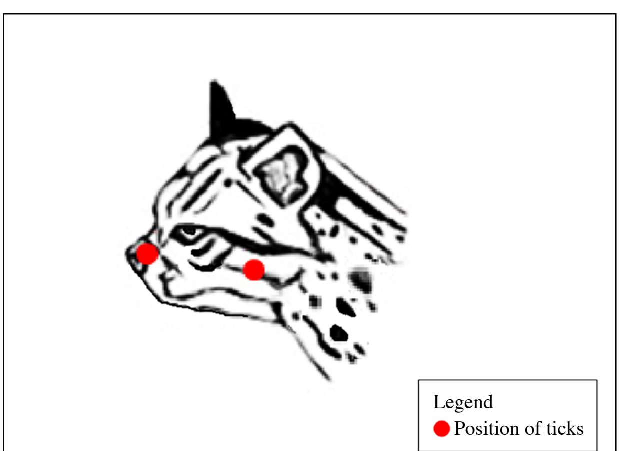

Fig. 1. Schematic representation of the position of the two ticks on the facial part of the dead body of the leopard cat.

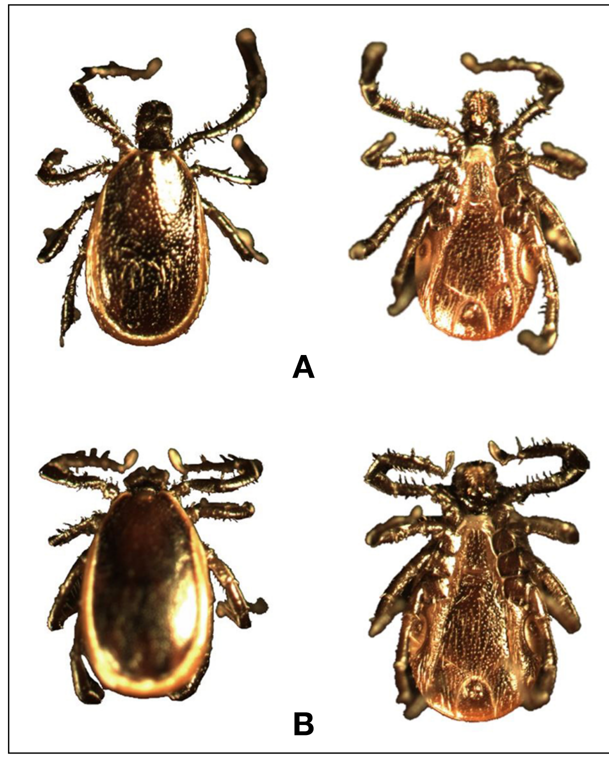

Fig. 2. The picture of two ticks (*Ixodes nipponensis*) were taken by Infinity1-1C (serial number 0172878) microscope camera (Lumenara Corporation) with software Infinity Capture. Both ticks collected are identified as female. A, B, The two different ticks collected.

no external lesion was visible. We collected the body in a closed plastic bag and brought it to the laboratory for further investigation. The search for ecto-parasites revealed the presence of two ticks attached to the right cheek and the muzzle area (Figs. 1, 2). We detached the ticks using tweezers, and immersed them individually in 2 mL Eppendorf tube filled with 100% ethyl alcohol. After crushing the body of the ticks, we extracted genomic DNA from the whole organism following the DNeasy Blood & Tissue kit protocol (catalog number 69504, Qiagen, USA). We selected the mitochondrial DNA primers (COI-F: 5′-ATCATAAAKAY HTTGG-3′ and COI-R: 5′-GGGTGACCRAARAAHCA-3′) as described in Lv et al. (2014), because the cytochrome oxidase subunit 1 (COI) region is one of the most commonly used for tick species identification. All DNA samples were adjusted to 40 ng DNA/μL before PCRs and the primers were used with each PCR reaction containing 14.5 μL of distilled water, 2.0 μL of (10×) buffer, 1.6 μL of dNTPs (final concentration of 0.07 mM), 0.4 μL of each primer, 0.1 μL of Takara Taq polymerase and 1 μL of DNA. We used a touchdown PCR protocol with a range of 52 to 46°C, with minus two degrees increment for the annealing temperature.

The protocol was set as follows: initial denaturation (94°C, 5 min); followed by five cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 1 min; five cycles of 94°C for 30 s, 50°C for 30 s, and 68°C for 1 min; five cycles of 94°C for 30 s, 48°C for 30 s, and 68°C for 1 min; 25 cycles of 94°C for 30 s, 46°C for 30 s, and 68°C for 1 min; finally followed by a final extension step of 68°C for 5 min. PCRs were carried on a SimpliAmp Thermal Cycler (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). Samples were sent to Cosmogentech (Seoul, Korea) for direct sequencing with both forwards and reverse primers on an ABI PRISM 3100 automatic sequencer (Applied Biosystem Inc., USA) to prevent ambiguities. Sequences were aligned and analyzed using Geneious 9.1.6 (v9.04, Biomatters Limited, Auckland, New Zealand). Finally, we analyzed the genetic variations between the sequences obtained and the matching sequences from GenBank through BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with *n* related sequences.

RESULTS

The ticks collected from the leopard cat were identified as *Ixodes nipponensis* from both molecular and morphological examinations. First, the BLAST search for the sequences obtained from the two ticks was 99.7% matching with the only record of *I. nipponensis*. *Ixodes asanumai* Kitaoka, 1973 also showed high matching result of 98.3%; however, the rest of the species were $<91\%$ match. Species identification as *I. nipponensis* was also assigned as *I. asanumai* has so far been confirmed in Japan but never in Korea. Both sequences were uploaded on GenBank under accession number: KY606283 and KY606284. Comparison between the two 768 bp COI sequences obtained and the matching Japanese *I. nipponensis* from Genbank revealed high conservativeness. There were only three nucleotides differences at the positions 357, 570 and 654. Second, we also identified the collected ticks by comparing morphological characteristics of with previous studies. *Ixodes nipponensis* has similar appearance with *I. persulcatus*, but *I. nippoensis* has a smaller body and can be distinguished with other characters (Yamaguti et al., 1971). Thus, based on molecular and morphological characteristics, we identified the ticks as *I. nipponensis*.

DISCUSSION

Our results reconfirm parasitism by Japanese Hard Ticks, *Ixodes nipponensis*, on the endangered *Prionailurus bengalensis euptilura* in the Republic of Korea as reported by Kim et al. (2011). Despite the important role of leopard cats to maintain balanced ecosystems in the Republic of Korea, the species is exposed to various threats, including roadkill and parasitism.

Our findings highlight that, there is a need for a broader assessment of the impacts of ecto-parasites on Korean wildlife, and for determining if the low prevalence is due to low parasitic infection, or due to low detection because of scant natural history knowledge. It is important as ticks can carry diseases potentially lethal to their hosts, and thus may significantly impact species with small population sizes. Furthermore, the absence of sequences in GenBank to which the sequences extracted here could align indicates the lack of research on basic ecology and evolution of ticks. Despite medical interest to the disease-causing microorganisms hosted by *I. nipponensis* (Park et al., 2014), a general information of the species is poorly understood in South Korea. For instance, previous studies draw opposite conclusion. Park et al. (2014) reported a rare occurrence of 0.3% for *I. nipponensis* in the Republic of Korea, from a study based on 13,053 collected ticks, whereas Cho et al. (1994) concluded that *I. nipponensis* is the species, leading to most tick bites in Korea.

The identification of the ticks collected through COI sequencing leads to only three base pair transitions compared to Japanese samples. The extremely low variation within the DNA sequences may result from the COI locus being conservative, or because of geographic isolation of populations between the two countries. As leopard cats do not occur in Japan, at the exception of Tsushima Island, the closest island to the Korean shores (Masuda and Yoshida, 1995), another host species would be the dispersion link. Besides, the occurrence of glacial refugium during the last glacial maximum and the potential for dispersion between refugia by ticks' hosts prevents hypothesizing the origin of the founding populations (Kim et al., 2013). Arbitrarily, considering Korean individuals as originating from Japanese populations, the nucleotide on position 357 clearly indicated a base transition (A to G) between Japanese and Korean individuals.

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