Species identification method by a new non-invasive technique in Korean endangered terrestrial snail, Koreanohadra koreana (Castropoda: Mollusca)

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Abstract

*Koreanohadra koreana* (K. koreana) is an endemic species in South Korea that is listed as endangered. While the ecology and phylogenetics of *K. koreana* have been studied, its morphological similarity to the related species *Koreanohadra kurodana* (K. kurodana), can make species identification difficult. Furthermore, this has led to confusion when determining essential habitat information for the conservation of *K. koreana*. To bypass this issue, we have developed a noninvasive species identification method that can genetically differentiate between them. While there are already various noninvasive genomic DNA (gDNA) extraction methods that utilize the mucus from mollusks, they are limited as they require the target species to be physically located. To address this, in this investigation a method of extracting gDNA from the feces of snails was developed. The method utilized a primer set to amplify a cytochrome b fragment from *K. koreana* but not *K. kurodana* or other terrestrial snails. The feces of terrestrial snails could thus be used to obtain gDNA to a genetically usable level if collected within 5 days of excretion. This noninvasive species identification method using feces will help to facilitate genetic research without harming the endangered species and if the target species is not physically in the habitat. Moreover, *K. koreana* and *K. kurodana* could perhaps be further distinguished, using their habitat information to help facilitate essential conservation measures.

Introduction

*Koreanohadra koreana* belongs to Bradybaenidae family and is an endemic terrestrial snail species in Hong-do and Hatae-do islands of southwestern South Korea. Species with limited habitats are especially vulnerable to sudden environmental change, such as those that occur with climate, habitat destruction, and human disturbance (Malcolm et al. 2006; Mullu 2016). To this end, *K. koreana* has been designated and protected as an endangered species by the Ministry of Environment of South Korea since 2012.

Another Korean terrestrial snail belonging to the genus *Koreanohadra, K. Kurodana*, is morphologically similar to *K. koreana* (Lee and Kwon 1993) and their phylogenetic analyses are thus also very similar (Kimura et al. 2022). Consequently, comparisons of the morphological characteristics (such as umbilical width, aperture height and width, body whorl height) cannot be used to effectively distinguish between these species. This limits the identification of these species within the habitat if taxon experts are not involved. Due to the nature of endangered species, it is important to identify and protect the habitats of *K. koreana*, but as noted, defining their habitats is confusing due to their external similarity with *K. kurodana*, which creates a major hurdle in facilitating appropriate protection. Furthermore, accurate species identification methods also help to monitor populations and track changes in distribution and abundance over time.

Recently, various genetic analyzes using environmental DNA (eDNA) that comes from organisms has been studied to identify their presence or absence in the habitat (Weltz et al. 2017; Mizumoto et al. 2020; Ruiz-Ramos et al. 2023) and to verify host plants (Milla et al. 2022). These studies confirmed that large amounts of residual gDNA from organisms exists in the environment, and on this basis it was established that genetic research could be sufficiently conducted using eDNA. The use of eDNA has led to the development of noninvasive methods for the collection of DNA samples from mollusks using their mucus without damaging the target organisms (Armbuster et al. 2005; Palmer et al. 2008; Régnier et al. 2011). The noninvasive method can be used for genetic research (genetic diversity, phylogeography analyses) without fatal effects on survival when collecting DNA samples from *K. koreana* tissue. The noninvasive technique for species identification is expected to bring a remarkable advancement in securing genetic analysis for the conservation of *K. koreana*, as it is not restricted by duration, area, or number of capture and re-capture according to "The Regulation on Captive Propagation of Endangered Wildlife (Ministry of Environment's Regulation No. 621)".

Considering the above circumstances and conditions, we aimed to develop an identification method for *K. koreana* to distinguish it from the morphologically indistinguishable from *K. kurodana* using noninvasive methods with non-tissue materials (feces and mucus). Then, like the eDNA method, we have determined whether the noninvasive method using feces can verify the habitat of *K. koreana* without visually identifying the target species. Finally, we aimed to determine whether it was possible to conduct genetic studies such as population genetics for *K. koreana* using the noninvasive method.

Materials and Methods

Species collecting and rearing

Two types of terrestrial snails were collected for an indoor experiment from the following locations: *Koreanohadra koreana* was collected from the boundary between a village and a forest at Hong-do island (34.691438N, 125.198160E) and Hatae-do island (34.389398N, 125.297561E) which are located far southwest of South Korea. *Koreanohadra kurodana*, the comparative species for *K. koreana*, was collected from Mt. Cheonggye (37.415741N, 127.041662E), a mountain located near Seongnam-si in South Korea. All terrestrial snails (*Achatina fulica, Euhadra herklotsi, Nesiohelix samarangae, Acusta despecta, and Euhadra dixonii*) in this study, including the above two species, were reared in acryl cases with perforated plastic lid, respectively. All rearing cases were filled by substrate (coconut bark), up to 10 cm in height. Cabbage and carrot were provided as well a calcium source (i.e. egg shell) every 3 days. All rearing cases were placed at 24°C with 90% relative humidity with a 16:8 hours light:dark period until further use in the experiment. *Koreanohadra koreana* is designated as a second-degree endangered species in South Korea. All capture activities and rearing were thus conducted after obtaining appropriate permits from the Yeongsangang River Basin Environmental Office (Permission No. 2020-7) and the Daegu Regional Environment Agency (Permission No. 2022-2).

**DNA extraction from feces, mucus, and foot tissue**

To isolate the residual gDNA originating from the epithelial cells included in the mucus from *K. koreana* and *K. kurodana* (Kawai et al. 2004), the mucus was collected by smoothly rubbing a sterile cotton swab 20 times between the foot and inner surface of the shell that meets snail’s flesh. In succession, the heads of swabs were cut off and put into 1.5 mL tubes with 180 µL of ATL buffer and 20 µL of proteinase K solution (DNeasy Blood & Tissue kit, QIAGEN, Germany) and then incubated for 1 hour at 56°C. Other steps were followed by manufacturer's instructions except for the final elution volume of 25 µL of AE buffer. 5 µL
of the eluted solution was used as PCR template. The feces were collected from rearing cases in the laboratory. 40 mg (wet weight) of feces from each rearing case was put into a 1.5 mL tube and disrupted with plastic pestle. The gDNA was extracted in the extraction method used for mucus as above the described. Moreover, to compare the DNA extraction method with another extraction method, gDNA from the mucus and the feces was also extracted by DAP buffer method (Cha et al. 2020). The mucus and the feces was then collected as previously described. Then, 200 µL of DAP buffer (20 mM Sodium Hydroxide, 5% Polyethylene glycol 200, and 5% Dimethyl Sulfoxide) was put into a 1.5 mL tube which includes a swab head or 40 mg of feces. The tube with the swab was vortex briefly and the tube with feces was disrupted using a plastic pestle. The tube was then placed at room temperature (20–25°C) for 10 min to dissolve the epithelial cells. After lysis, 5 µL of the lysate was used as a PCR template. Finally, to compare the efficiency of the gDNA extraction between non-tissue materials and tissue materials, gDNA was extracted from 20 mg of the snail foot tissue using a proteinase K solution method. All gDNA isolated from the foot tissue were used in a total 20 ng concentration as the PCR template.

**Procedure of the PCR amplification**

Primers were designed to only amplify the 624 base pairs partial fragment of cytchrome b from *K. koreana* to distinguish between *K. koreana* and other terrestrial snails (Fig. 1). The primer sequences KK_FS_CYTB_F2 (forward direction) 5'-TACATATTAGCGGAGGTG-3' and KK_FS_CYTB_R3 (reverse direction) 5'-ATATACCTACGGTTGAAATGC-3' were used in PCR procedures. PCR was performed using AccuPower® PCR premix (Bioneer, South Korea) according to the manufacturer's instructions. Specifically, a total of 50 µL of PCR reagent consisting of 5 µL of gDNA from the mucus or the feces of snails extracted by DNeasy Blood & Tissue kit or 5 µL of the lysate from the mucus or the feces of snails was extracted by DAP buffer, 10 pmol of each primer pair, and 42.6 µL of nuclease-free water. The PCR reagent was then put into an enzyme pellet tube and mixed briefly. The PCR amplification procedure was conducted for 3 min at 95°C and 50 thermal cycles with 30 sec at 95°C, 30 sec at 57°C, and 1 min at 72°C, and a final extension period of 3 min at 72°C. In total 10 µL of PCR products was used for electrophoresis with 1.8% of agarose gel to determine the PCR success or failure. Furthermore, to confirm the reproducibility of the gDNA extraction with PCR methods, PCR amplifications were performed on feces and mucus samples from different *K. koreana* individuals. Finally, PCR products were sequenced to confirm whether the existing nucleotide sequence variation in the cytchrome b fragment of each individual snail or not. All the Cytocrome b sequence used in this study are deposited in Genbank. Genbank accession numbers for 16 sequences of *K. koreana* (from OQ852448 to OQ852463) and for one sequence of *K. kurodana* (OQ852464), respectively. Two internal transcribed spacer 1 (ITS1) from *K. koreana* (OQ789637) and *K. kurodana* (OQ789638) are also deposited in Genbank.

**Verification of primer specificity**

To verify primer specificity, PCR with gDNA from the foot tissue of *K. koreana* and other terrestrial snails was conducted to determine whether a nonspecific amplification reaction occurred in the other terrestrial snails. The PCR amplification was carried out as previously described, except that a total of 20 ng of gDNA was used from each snail as the PCR template, and the terrestrial snail species were used as negative controls, and they were as follows: *Koreanohadra kurodana*, *Achatina fulica*, *Euhadra herklotsi*, *Nesiohelix samarangae*, *Acusta despecta*, and *Euhadra dixoni*.

**Limit of detection time from feces after excretion**

To determine how long after excretion from the snails the gDNA in feces could still be amplified using a common PCR method, each 20 mg of the feces from *K. koreana* was collected immediately after excretion and placed on a cell culture dish (4 cm diameter x 1 cm height) which was filled with substrates (sand or coconut bark). All culture dishes were placed at 25°C, 45% relative humidity (RH), and with a 16:8 hours light: dark period, for 30 days. The limit of detection time was then confirmed by PCR amplification of the gDNA from the feces at intervals of 5 days. PCR amplification every 5 days was performed three times for each substrate.

**Phylogenetic analysis**

A total of 16 partial sequences (seven from feces, eight from mucus, and one from foot tissue) of cytochrome b from *K. koreana* with one reference out-group using cytchrome b from *K. kurodana* sequenced in this study were used for phylogenetic analyzes to determine the presence of *K. koreana* haplotypes. All sequences were aligned and trimmed using BioEdit (Hall 1999), and jModelTest 2.1.7 was used to select the best fit model TPM3uf + I under Akaike information criterion (AIC) (Darriba et al. 2012). For the Maximum likelihood (ML) and Bayesian inference (BI) analyzes, the FASTA file was converted to NEXUS and PHYLIP files using MEGA 7.0 (Tamura et al. 2011), and the jModelTest results (Invariable sites: 0.6960, freqG: 0.2404, freqC: 0.1868, freqG: 0.1867, freqT: 0.3861, AC = CG: 0.1850, AG = CT: 2.1313, AT = GT 1.0000) were used. ML analysis was conducted using PhyML version 3.1 and 1,000 bootstrap replicates (Guindon et al. 2010). BI assessment was conducted using MrBayes 3.2.2 (Ronquist et al. 2012) with a chain length of 1,000,000 generations. Trees were sampled every 100 generations with a prior burn-in of 2000 trees. A neighbor-joining test was carried out using MEGA v11.11 (Tamura et al. 2021) with the Kimura 2-parameter distance option (Kimura 1980) with 1,000 bootstrapping. Molecular trees were visualized using the FigTree v1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/, written by A. Rambaut).

**Results and discussion**

**Efficiency of gDNA extraction using non-tissue materials**

To compare the efficiency of gDNA extraction using DAP buffer and Proteinase K from non-tissue material, feces and mucus samples were obtained from *K. koreana* and *K. kurodana*, and foot tissue was used as a positive control. The foot tissues of mollusks are considered one of their best sources of DNA, as they are the largest and most accessible organ in living animals, but also because other tissues (such as digestive glands) are often contaminated with microorganisms (Sokolov 2000). The results for the efficiency of gDNA extraction using non-tissue materials (feces and mucus, KFQ in Fig. 2A and KMQ in Fig. 2B) showed that PCR amplification when using tissue material (foot tissue, KTQ in Fig. 2) was greater than with the non-tissue materials. The reason for this may be because of PCR inhibitors, such as mucopolysaccharides (Adema 2021) in the mucus and humic acid in the feces (Schrader et al. 2012), as well
as low concentration of residual gDNA in the non-tissue materials. In addition, the direct gDNA extraction method (using DNeasy Blood & Tissue kit) had shown significantly different efficiencies for PCR amplification. Specifically, PCR amplicon from gDNA of the non-tissue materials extracted using the direct extraction method were only weakly amplified or were not amplified more when compare with the gDNA extracted using column filter method. These results indicate that more impurities (mucopolysaccharides, humic acid, etc.) remain in the lysate of the direct extraction method, and that the degree of PCR inhibition for PCR using gDNA extracted by the direct extraction method is higher than the column filter method. The used of the snail mucus with CTAB (hexadecyltrimethylammonium bromide) could effectively overcome the PCR inhibition caused by mucopolysaccharides (Goodacre and Wade 2001), and increasing the foot tissue rubbing from the snail can help to increase the amount of DNA obtained from mucus (Armbruster et al. 2005). Nevertheless, the PCR using gDNA from non-tissue materials showed less amplification than PCR using gDNA extracted from the foot tissue, but the amplification was sufficient for the identification of K. koreana (KFQ in Fig. 2A and KMQ in Fig. 2B) and K. kurodana (BFQ in Fig. 2A and BMQ in Fig. 2B).

**Specificity and reproducibility of PCR amplification using non-tissue materials**

*Koreanohadra koreana* and *K. kurodana* are highly similar species morphologically and genetically, as was previously discussed. Even their internal transcribed spacer 1 (ITS1) two nucleotide sequences, which are 980 bp, differ by just two base pairs among the two species (Genbank accession number: OQ789637 for *K. koreana*, and OQ789638 for *K. kurodana*). In addition to the genetic similarities between two species, identification using their morphological characteristics via the naked eye is also very difficult and even experts can confuse the two species. If specific molecular markers for target species, *K. koreana* were identified, however, then this should provide a solution to this issue.

To date, various noninvasive techniques for obtaining gDNA from mollusks for genetic studies have been developed (Huelskens et al. 2011, Jaksch et al. 2016). Despite the advantage of being non-harmful physically to target species, the noninvasive species identification method for the classification of species that are morphologically indistinguishable has not yet been widely developed.

In this study, the verification of primer specificity using closely related terrestrial snails showed that a pair of primers for the 624 base pair fragment of cytochrome b from *K. koreana* had high levels target specificity and only amplified the gDNA from *K. koreana* (Fig. 3). This result indicates that a set of primers could be used to distinguish *K. koreana* from terrestrial snails including *K. kurodana* which show high levels of genetic similarity, using the gDNA extracted from non-tissue materials (feces and mucus). In addition, the primer's that showed high levels of species specificity, also showed high levels of reproducibility when using fresh non-tissue materials. (Fig. 4).

**Detection limits of residual DNA in the feces from terrestrial snails**

While mucus can only be obtained when the snail is identified within their habitat, their feces can be collected via habitat survey, and thus without locating the actual snail. However, the duration of time after feces excretion cannot be confirmed when collected from the field. To address this, artificial environmental condition (using substrates such as sand or soil, 25°C, and 45% RH) mimicking the actual snail. However, the gDNA from the shrunken feces could still be extracted and amplified. The results showed that gDNA in the feces could be amplified 30 days after excretion from the snail. In detail, PCR with gDNA from feces which is placed in the artificial environmental condition was not amplified constantly but was amplified according to an individual sample (Fig. 5). The reason for this seems to be due to the original amount of gDNA in the feces as well as various factors such as the damage to the gDNA due to factors such as the light source and free radicals (Dizdaroglu et al. 2002; Sinha and Häder 2002). Moreover, the gDNA from the feces placed on the soil tended to result in less amplification than when it was placed on the sand, and the phenomenon seems to be related to soil-based PCR inhibitors (Braid et al. 2003).

To help limit the hurdles that occur with the use of feces for genetic analyzes, it should be collected in as fresh a condition as possible. Furthermore, collecting feces from sand, stone, or tree branches rather than the soil seems to help prevent PCR inhibition due to the inhibitors found in the soil.

**Presence or absence of genetic variation in cytochrome b from K. koreana**

Mitochondrial DNA (mtDNA) markers offer an effective approach for deducing the phylogeny and evolutionary history of closely related organisms because of their rapid rates of evolution, limited or negligible meiotic recombination, and predominantly uniparental inheritance (Avise 2000). The cytochrome b region, specifically, is recognized for having noteworthy intra-specific variability, which allows for extensive molecular identification at detailed taxonomic levels, such as within a species complex (Yokoyama et al. 2001; Biswas et al. 2003), and for population genetic assessments (Chen et al. 2007). The results of the phylogenetic analyzes indicate that partial sequences of cytochrome b between interspecies (*K. koreana* and *K. kurodana*) differed by 4.9 – 5.4% (differences in 21 to 26 of the 483 base pair of sequences). Furthermore, the intraspecies sequence differences for the cytochrome b of *K. koreana* were found to ranges from 0%-1.5% in 16 individuals (Table 1). The results of the phylogenetic analysis generated using the Maximum-likelihood tree, showed that there were two haplogroups which consisted of four haplotypes that were observed in the sample population for *K. koreana* with high supporting values (Fig. 6) and this was thought to be due to the differences in the genetic characteristics between the individuals that were isolated in each habitat (Hong-do and Hatae-do islands are 35 km apart in the straight line) which is surrounded by a sea barrier to dispersal (Jensen et al. 2013). However, further studies that utilized specimen samples or non-tissue materials, and the comparison of other genes will be required to elucidate whether the genetic differences between intraspecies are due to the genetic isolation caused by their habitats. Although many genetic issues still need to be resolved, the results of phylogenetic analyses have indicated that gDNA using non-tissue materials could be used in genetic studies.
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The authors declare no conflict of interest.
Author contributions Conceptualization, D.C. and JY. K.; Data curation, D.C. and KS. K; Formal analysis, D.C.; Investigation, D.C. and JY. K.; Methodology, D.C. and KS. K; Resources, JY. K.; Supervision, JY. K. and YJ. K; Visualization, D.C. and KS. K; Writing—original draft, D.C. and JY. K.; Writing—review and editing, D.C., JY. K., KS. K. and YJ. K. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement All the Cytochrome b sequence used in this study are deposited in Genbank. Genbank accession numbers for 16 sequences of K. korean (from OQ852448 to OQ852463) and for one sequence of K. kurodana (OQ852464), respectively. Two internal transcribed spacer 1 (ITS1) from K. korean (OQ789637) and K. kurodana (OQ789638) are also deposited in Genbank. All sequence data will be released on May 31 in 2024.

References


Figures

![Figure 1](image_url)
Nucleotide sequence alignment for cytochrome b from *K. koreana* and *K. kurodana*. Locations of the KK_FS_CytB_F2 and KK_FS_CytB_R3 primers are marked with a red and green colored boxes, respectively. Sequence identity is shown by blue colored gradations.

Figure 2

Efficiency of gDNA extraction using non-tissue materials for *K. koreana*. To determine the efficiency of gDNA extraction, PCR with a set of specific primers for *K. koreana* was conducted using non-tissue materials (feces vs. mucus), with two extraction methods (DAP buffer vs. Proteinase K), and gDNA from two species (*K. koreana* vs. *K. kurodana*). Comparison of the gDNA extraction efficiency with each extraction method and species for the feces (a) and mucus (b) materials from snails. The notation for the PCR amplification products according to non-tissue materials, species, and the extraction methods is as follows: BFD (feces of *K. kurodana*, DAP buffer), KFD (feces of *K. koreana*, DAP buffer), BFQ (feces of *K. kurodana*, Proteinase K), KFQ (feces of *K. koreana*, Proteinase K), BMD (mucus of *K. kurodana*, DAP buffer), KMD (mucus of *K. koreana*, DAP buffer), BMQ (mucus of *K. kurodana*, Proteinase K), KMQ (mucus of *K. koreana*, Proteinase K), BTQ (foot tissue of *K. kurodana*, Proteinase K), and KTQ (foot tissue of *K. koreana*, Proteinase K). gDNA from the foot tissue of *K. kurodana* and *K. koreanawere used as positive controls, respectively. Mk: 100 base pairs DNA ladder size marker. Black arrowheads indicate the target PCR amplicons for cytochrome b.

Figure 3

Specificity results for the *K. koreana* cytochrome b primers. The specificity test was performed using 20 ng gDNA extracted from *K. koreana* (K), *K. kurodana* (B) which is the closest relative species with *K. koreana*, and six terrestrial snails: *Euhadra herklotsi* (C), *Euhadra dixoni* (N), *Acusta despecta* (M), *Neso helix*...
samarangae (D), and Achatina fulica(A), respectively. Mk: 100 base pairs DNA ladder size marker. Black arrowheads indicates the target PCR amplicon of cytochrome b.

Figure 4
Reproducibility results for the non-invasive method with non-tissue material type. The reproducibility test was conducted using gDNA from the feces (a) and mucus (b) of K. koreana extracted using the proteinase K method. Each non-tissue material was collected from each individual and a total of 16 K. koreana individuals were used (1-8 in each panel). Mk: 100 base pairs DNA ladder size marker. Black arrowheads indicate the target PCR amplicons of cytochrome b.

Figure 5
Detection of residual DNA in K. koreana feces on two types of substrates. Electrophoresis data of the PCR amplicon using feces which were incubated at 25°C and 45% relative humidity. The PCR with feces was conducted once 5 days for a month and repeated three times for each substrate type for each PCR performance. Red colored circles indicate feces of K. koreana which were incubated for each substrate type. Repeat 1, 2, and 3 indicate PCR amplicon using each gDNA extracted from feces for each substrate type. Sand and soil, the substrate on which the feces is placed, represent river sand and coconut bark, respectively. Mk: 100 base pairs DNA ladder size marker. Black arrowheads indicate the target PCR amplicons of cytochrome b.
Figure 6

Phylogenetic relationship of *K. koreana* populations from Hong-do and Hatae-do islands. Maximum likelihood (ML), Bayesian inference (BI), and Neighbor-joining (NJ) analyzes based on cytochrome b sequences. Numbers at nodes indicate the bootstrap values of ML and NJ out of 1000 replicates, the posterior probability of BI. The scale bar corresponds to two substitutions per 100 nucleotide positions. Cytochrome b sequence data from foot tissue of *K. kurodana* (*BKKFT*) was used as an out-group. H1-H4 = haplotype 1-4; HG1, 2 = haplogroup 1, 2; M1-M8 = mucus-derived DNA sequences; F1-7, FT = foot tissue-derived DNA sequences.