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Novel non-invasive molecular identification method for two tree frogs, *Dryophytes suweonensis* and *Dryophytes japonicus*, based on high resolution melting (HRM) analysis

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Abstract: Two tree frogs, *Dryophytes suweonensis* and *Dryophytes japonicus*, inhabiting Korea, are morphologically similar and share the same habitats. Therefore, they are identified mainly through their calls, especially for males. *Dryophytes suweonensis* is registered as an endangered (IUCN: EN grade) and protected species in South Korea. Thus, it is necessary to develop a method to rapidly identify and discriminate the two species and establish efficient protection and restoration plans. We identified significant genetic variation between them by sequencing a maternally-inherited mitochondrial 12S ribosomal DNA region. Based on the sequence data, we designed a pair of primers containing 7 bp differences for high resolution melting (HRM) analysis to rapidly and accurately characterize their genotypes. The HRM analysis using genomic DNA showed that the melting peak for *D. suweonensis* was 76.4±0.06°C, whereas that of *D. japonicus* was 75.0±0.05°C. The differential melt curve plot further showed a distinct difference between them. We also carried out a pilot test for the application of HRM analysis based on immersing *D. suweonensis* in distilled water for 30 min to generate artificial environmental DNA (eDNA). The results showed 1.10–1.31°C differences in the melting peaks between the two tree frog samples. Therefore, this HRM analysis is rapid and accurate in identifying two tree frogs not only using their genomic DNA but also using highly non-invasive eDNA.

Keywords: endangered species, HRM analysis, eDNA, non-invasive, tree frogs

INTRODUCTION

Dryophytes japonicus is widely distributed across North-East Asia, including South Korea, whereas *Dryophytes suweonensis* is known to inhabit only the western parts of the Korean Peninsula based on the Taebaek mountain range (Do *et al.* 2017). Between the two tree frog species, *D. suweonensis* is an indigenous species of South Korea, and its population size has decreased owing to a continuous loss of habitats. The species has been designated as a Class I Endangered Wildlife Species in South Korea and a IUCN Red List Endangered (EN) Species (IUCN 2017) due to various causes such as hybridization. Meanwhile, *D. suweonensis* and its close relative *D. japonicus* are sympatric species sharing the same habitats (Roh *et al.* 2014). *D. suweonensis* has been reported to differ from *D. japonicus* in terms of its call and facial shape, but it is difficult to visually discriminate the two species based solely on morphological differences. Notably, in the case of female frogs that do not call, accurate species identification is extremely diffi-

cult (Kuramoto 1980; Borzée *et al.* 2013). Therefore, the first step in a series of studies toward the conservation and restoration of *D. suweonensis* is accurate species identification.

Previous studies on the genetic species identification of land animals including amphibians have, to date, applied a variety of methods including DNA sequence analysis and comparisons, restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP), random amplified polymorphic DNA (RAPD), single-strand conformation polymorphisms (SSCP), and species-specific PCR analysis (Bertolini *et al.* 2015; Kanthaswamy 2015). Among these methods, DNA sequence analysis and comparisons were often involved with regions of mitochondrial 12S and 16S ribosomal DNA (rDNA), D-loops, cytochrome *b* (CYB), and cytochrome *c* oxidase I (COI), as well as nuclear 28S rDNA, in studies investigating inter- or intra-species variation (Kocher *et al.* 1989; Bataille *et al.* 1999; Bellis *et al.* 2003; Girish *et al.* 2005; Berry and Sarre 2007; Imaizumi *et al.* 2007; Tobe and Linacre 2008; Robertson *et al.* 2009; Fitzcharles 2012). Compared to nuclear DNA, mitochondrial DNA has a higher copy number to allow for easier analysis; this is associated with the advantage of the accumulation of a large number of genetic mutations between species or individuals owing to a relatively high rate of mutation. The species identification of *D. suweonensis* versus *D. japonicus* is mostly conducted via the sequencing of mitochondrial DNA (Lee and Park 1992; Lee *et al.* 1999; Borzée *et al.* 2017; Lee *et al.* 2017), which requires considerable time and cost. High resolution melting (HRM), however, allows for the immediate detection and analysis of the differences in dissociation temperatures of the double helix DNA chains, as steadily increasing temperatures are applied to the amplified PCR products. This leads to the rapid and accurate characterization of genotypes of different species with varying sequence data, without the PCR product purification and DNA sequencing steps (Reed *et al.* 2007).

There are also non-invasive DNA sampling methods using environmental DNA (eDNA), such as feces, urine, or mucus, without direct capture of the target species (Goldberg *et al.* 2015; Sigsgaard *et al.* 2015). Recent studies have shown that the eDNA, found as DNA fragments of organisms in various environments, such as soil and water, is highly useful in species identification (Thomsen and Willerslev 2015). This method generally involves the collection of samples from water or soil to detect a rare or endangered species. It has been actively applied to numerous studies worldwide (Jerde *et al.* 2011; Thomsen *et al.* 2012; Pilliod *et al.* 2013; Cardás *et al.* 2020). HRM analysis using eDNA leads to more rapid, accurate, and cost-effective results than conventional methods (Martinou *et al.* 2010). In this study, a rapid and accurate method of HRM analysis was developed for species identification of the two tree frog species (*D. japonicus* and *D. suweonensis*) that inhabit South Korea, with verification of the potential use of this novel non-invasive method using eDNA for species identification.

MATERIALS AND METHODS

1. Sample collection and genomic DNA extraction

D. suweonensis and *D. japonicus* were captured from April to June, 2021, in the following regions (the number in parentheses indicates the number of captured animals): *D. suweonensis* (2) and *D. japonicus* (5) in Asan, Chungcheongnam-do; *D. suweonensis* (3) and *D. japonicus* (3) in Chungju, Chungcheongbuk-do; *D. suweonensis* (1) in

Fig. 1. Photographs of two frog species with similar morphology (left: *Dryophytes suweonensis* and right: *D. japonicus*).

Pyeongtaek, Gyeonggi-do; *D. suweonensis* (2) in Wanju, Jeollabuk-do. The direct capture of animals was performed at night when *D. suweonensis* was active. The animals that were either calling or mating were captured while the investigator walked along the rice paddies, the main habitat of the species. To extract the genomic DNA (gDNA), each frog was made to hold a sterile swab in its mouth for approximately 1 min to collect oral epithelial cells (Goldberg *et al*. 2003). From the sterile swabs with oral epithelial cells, the gDNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manual. The quantity of the extracted gDNA was analyzed using a spectrophotometer (DeNovix DS-11 FX, DeNovix, USA).

2. Sample preparation for eDNA analysis

For non-invasive species identification using eDNA, three frogs each of *D. japonicus* captured in Jeollabuk-do and *D. suweonensis* captured in Chungcheongnam-do were used. First, plastic containers (6.5 × 6.5 × 20 cm) were rinsed in bleach to remove any residual DNA and washed clean with sterile tertiary distilled water. Each frog was washed with sterile tertiary distilled water and placed in a plastic container with 200 mL of tertiary distilled water. The control was tertiary distilled water without a frog. To prevent cross-contamination, the investigator wore a mask and lab gloves for replacing each frog. After 30 min of immersion in tertiary distilled water, 200 mL of the immersion water was collected from each container. The eDNA from the immersion water was filter-concentrated using a cellulose nitrate filter (Φ0.45 μm, Whatman, Germany). Next, 20 μL of proteinase K and 280 μL of ATL buffer of the DNeasy Blood & Tissue Kit (QIAGEN, Germany) were added, and the filter was completely destroyed by adding three stainless steel beads of 2.4 mm in diameter (OMNI International, Kennesaw GA, USA) and using the Bead Ruptor Elite (OMNI International, USA). The same volume of AL buffer was added, and the mixture was incubated at 56°C for 12 h. The subsequent steps were carried out as described in the manual, and eDNA was ultimately extracted.

3. PCR primer design and DNA sequencing

To design the PCR primers for mitochondrial 12S rDNA of *D. japonicus* and *D. suweonensis*, the data of congeneric species were downloaded from the GenBank database of the National Center for Biotechnology Information (NCBI, USA). The DNA sequence matrix was drawn using BioEdit 7.2.5 (https://www.mbio.ncsu.edu/bioedit/bioedit.html; Biological Sequence Alignment Editor) (Hall 1999), and the PCR primer combinations with high conservation were designed using Sequence Manipulation Suite ver. 2 (http://www.bioinformatics.org/). Here, their length was set to the 19–21 mer range, GC content was set to 36.84–47.62%, and T_m was set to 56.8–65°C. To perform the PCR amplification of the mitochondrial 12S rDNA, the *AccuPower*® PCR PreMix (Bioneer, Korea) was used. The PCR reaction solution consisted of 10 ng of gDNA, 5 μM of forward primer (ANU-MT-00018f: 5′-AAAGCRTAG CACTGAAAATG-3′), 5 μM of reverse primer (ANU-MT-01017r: 5′-TCGGTGTAAGCGAGATGCTTT-3′), and sterile distilled water to make up a final volume of 20 μL. Using the ProFlex PCR System (Life Technologies Corporation, Carlsbad, CA, USA), the PCR amplification comprised an initial 1 min of initial denaturation at 95°C and 35 cycles that consisted of 20 s denaturation at 95°C, 20 s binding at 55°C, and 1 min extension at 72°C, with a final extension for 5 min at 72°C. Here, hot-start PCR was performed, wherein the PCR tube that contained all PCR reactants was placed in the PCR device to start the amplification reaction when the temperature (95°C) of the initial denaturation was reached. The resulting PCR products were purified using the *AccuPrep*® PCR Purification Kit (Bioneer, Korea) according to the manual. The BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and DNA Analyzer 3730*xl* (Thermo Fisher Scientific) were used for DNA sequence decoding. Here, the primers used in the cycle sequencing were the same as the forward and reverse primers in the PCR reaction. After error correction using BioEdit 7.2.5, the unnecessary parts were adequately cut to complete the contig, and finally, the DNA sequence was decoded.

4. HRM analysis

The mitochondrial 12S rDNA of *D. japonicus* and *D. suweonensis* was analyzed to explore the regions that exhibit an adequate level of interspecies genetic variation. A new set of PCR primers was prepared for the flanking regions of such sites (Fig. 2). For the HRM analysis, the MeltDoctor™ HRM Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) was used according to the manual; 10 μL of MeltDoctor™ HRM Master Mix, 10 ng of gDNA or 2 μL of eDNA, 5 μM of forward primer (HYL-12S-0250f: 5′-GTTACACCACGAGGCTCA-3′), 5 μM of reverse primer (HYL-12S-0343r: 5′-TGAGTTTCTTAAGAA

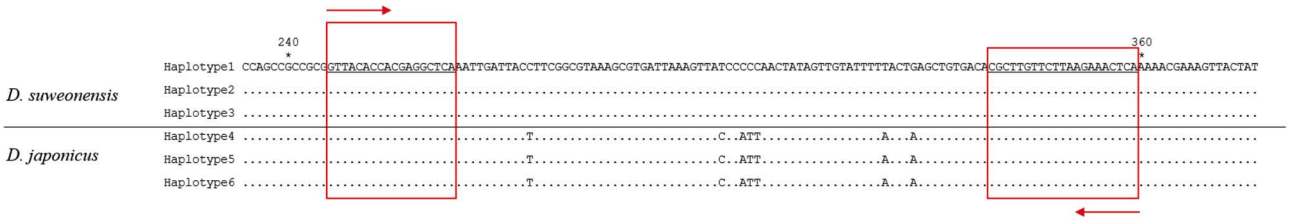

Fig. 2. DNA sequence matrix that shows the differences between *Dryophytes suweonensis* and *D. japonica* and PCR primer sites for high resolution melting (HRM) analysis.

CAAGCG-3′), and 6 μL of sterile distilled water comprised the PCR reactant solution with a 20 μL final volume. Using the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA), the PCR reaction began with an initial 10 min denaturation at 95°C, followed by 40 cycles that consisted of 15 s denaturation at 95°C and 1 min binding/extension at 60°C. The HRM analysis was performed through 10 s denaturation at 95°C and 1 min binding at 60°C for the melt curve and dissociation steps, and through 15 s denaturation at 95°C and 15 s binding at 60°C for the HRM.

RESULTS

Through the novel design of a PCR reaction in this study, to amplify the mitochondrial 12S rDNA region of *D. japonicus* and *D. suweonensis* using PCR primers, a product with the predicted size was detected for all 16 frogs. Subsequent DNA sequencing identified 915 bp in *D. suweonensis* and 916–917 bp in *D. japonicus*. A comparison of the two sequences allowed for the discrimination of the two species based on an indel (insertion or deletion) mutation of 2 bp, and a species-specific insertion or deletion was found in one of eight *D. japonicus* frogs. Between the two species, 46 bp of mutations were identified in total; of these, 45 bp comprised a region of interspecific variation between *D. suweonensis* and *D. japonicus* and 1 bp was a region of intraspecific variation for *D. suweonensis*. Based on this, the intraspecific haplotype was analyzed, and *D. suweonensis* and *D. japonicus* each showed three haplotypes, of which sequences were registered in the GenBank database (GenBank No. OK156170-OK156175).

The HRM analysis in this study using the novel primer design showed that PCR amplification of the gDNA of all frogs was successful. The melting peak of the *D. suweonensis* sample was 76.4 ± 0.06°C, whereas that of the *D. japonicus* sample was 75.0 ± 0.05°C, suggestive of a mean interspecies variation of 1.31°C. In addition, the aligned melt curve and difference plot melt curve led to the easy discrimination of the two species (Fig. 3), in agreement with the DNA sequence result. Thus, the PCR primer design

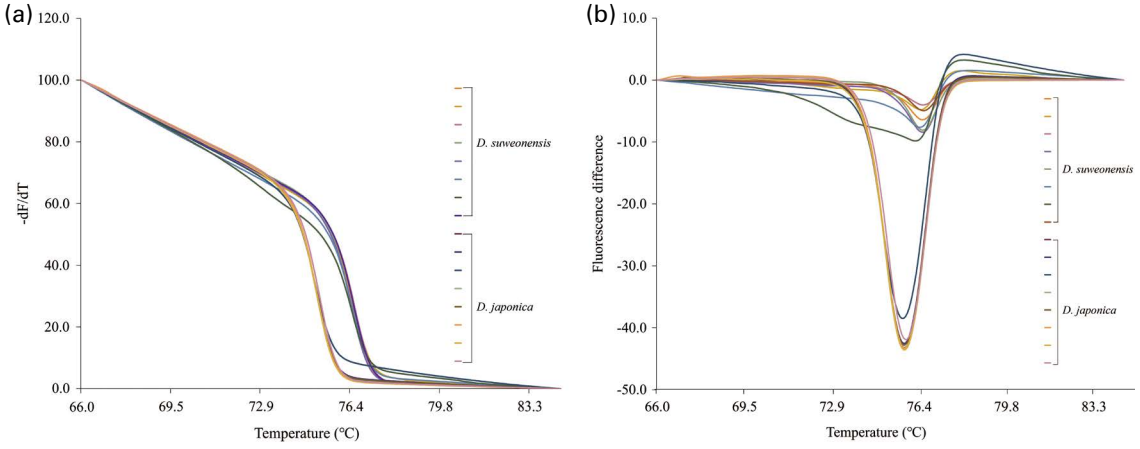

Fig. 3. Melting peak profiles after high resolution melting (HRM) analysis of the genomic DNAs of *Dryophytes suweonensis* and *D. japonicus*; (a) Normalized and shifted melting curves and (b) Difference curves.

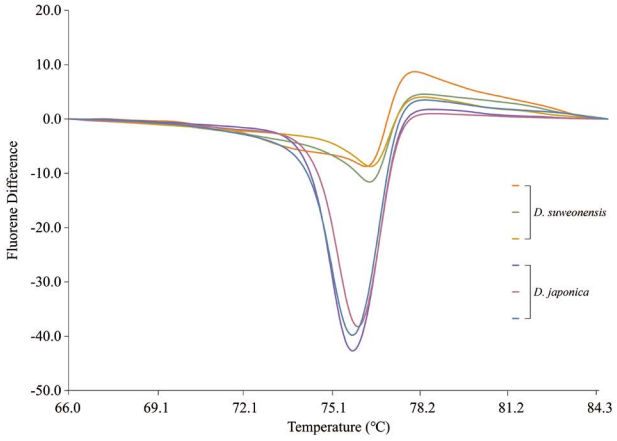

Fig. 4. Difference curves after high resolution melting (HRM) analysis of *Dryophytes suweonensis* and *D. japonicas*-immersed distilled water.

for HRM analysis produced in this study based on the mitochondrial 12S rDNA was shown to be highly useful for the differentiation of *D. japonicus* and *D. suweonensis*.

Moreover, HRM analysis using eDNA obtained from the immersion experiment showed a difference of 1.10–1.31°C in the melting curve analysis for *D. japonicus* and *D. suweonensis*. For distilled water, used as a negative control, melting peaks were not observed. In other words, the melting peaks of *D. suweonensis* were all identical at 76.3°C, whereas those of *D. japonicus* were 75.0 ± 0.14°C (Fig. 4). This result thus coincided perfectly with the HRM analysis using gDNA.

DISCUSSION

In previous studies, *D. suweonensis* and *D. japonicus* were found to have 12.90–15.66% variation in the mitochondrial *CYB* region (Yang *et al.* 1997; Lee *et al.* 1999), whereas the mitochondrial 12S rDNA region showed 7.17% genetic distance based on 68 bp point mutations of 938 bp (Lee *et al.* 1999). Moreover, the region comprises a highly conserved region (Arif and Khan 2009). In this study, the two species were found to have 4.91% variation, which deviated from the report of Lee *et al.* (1999), but the resolution was determined to be adequate for differentiating the maternal properties of the two species. In performing HRM analysis, the use of a PCR primer combination that includes a greater area of differential regions could increase the efficiency of species identification (Perini *et al.* 2020). In this study, the PCR primer design contained 7 bp interspecies point mutation regions from 246 to 359 bp, indicating a 6.19% genetic point mutation rate. The selected region thus exhibited a higher mean interspecies point mutation rate than the 12S rDNA region.

DNA sequencing requires considerable time and cost as it involves a series of steps, including the PCR reaction, PCR product purification, DNA sequence decoding, and sequence alignment (Garritano *et al.* 2009). In contrast, HRM analysis can be completed within approximately 2 h, allowing for rapid analysis, as the gDNA extraction and PCR amplification are immediately followed by the melting curve analysis (Erali *et al.* 2008; Garritano *et al.* 2009). In addition, HRM analysis is associated with a very low risk of cross-contamination to allow for accurate analysis, as the PCR amplification and melting curve analysis can be performed simultaneously within a single container (Erali *et al.* 2008; Garritano *et al.* 2009). The cost is also far lower than that of conventional DNA sequencing. Thus, the method of molecular identification developed in this study using the novel PCR primer designed for HRM analysis to discriminate between *D. japonicus* and *D. suweonensis* led to the same result as DNA sequencing but was faster, more accurate, and cost-effective.

Recently, various studies have been conducted regarding the use of eDNA to detect amphibian species (Bedwell *et al.* 2021). In general, the skin of amphibians is moist with granular glands that show the characteristic secretion of intestinal fluid, toxic granules, or mucus mixtures (Mills and Prum 1984; Toledo and Jared 1995; Clarke 1997). As amphibian species have this mucus-secreting skin, it is likely that the immersion of two different species in distilled water for only 30 min will allow for the secretion of eDNA at an adequate quantity for the HRM analysis.

Non-invasive species identification is widely applied in conservation genetics as there is no need to directly capture wildlife species. As it is especially useful to study small and rare species (Rees *et al.* 2014), related methods have been developed for different species (e.g. Eggert *et al.* 2001; Palomares *et al.* 2002). The non-invasive method of sample collection for anuran amphibians has mostly relied on the collection of oral epithelial cells (Pidancier *et al.* 2003; Broquet *et al.* 2007). However, this method is also not suitable in terms of minimizing the stress imparted on the animals, as their mouths must be forced open while the body of the animal is restrained in the hand of the investigator. The method in this study, however, involves a simple, 30 min immersion of the animal (*D. japonicus* and *D. suweonensis*) in distilled water to obtain the eDNA for subsequent

molecular identification, which is thought to sufficiently reduce the stress that might arise during the handling of the animal. This non-invasive method is likely to minimize stress upon species identification, the most basic process in the conservation and management of endangered species.

The results in this study suggested that the HRM analysis using eDNA is a non-invasive method for the rapid and accurate identification of two morphologically similar tree frog species, *D. japonicus* and *D. suweonensis*. With the accumulation of melting curve profiles of sympatric species and their availability in databases, this method will be applicable for molecular identification in the future. Further studies should investigate the level of eDNA generation and its detection with respect to various factors such as immersion time and animal size.

ETHICAL APPROVAL

All applicable international, national, and/or institutional (NIEIACUC-2020-012) guidelines for the care and use of animals were followed.

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