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Development of identification methods for purebred parental species of *Dryophytes suweonensis* and *D. japonicus*, and their hybrids

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Abstract

Background

The Suwon treefrog, *Dryophytes suweonensis*, is an endangered wildlife species in Korea. This species shares its habitat and often hybridizes with the common treefrog, *D. japonicus*. Because hybridization can reduce biodiversity or cause extinction it is important to identify purebred parental species and their hybrids prior to conservation plans such as for *D. suweonensis*. In particular, *D. suweonensis* and *D. japonicus*, and their hybrids often have abnormal ovaries and gonads, which are known to be a source of extinction threat.

Methods

We collected 57 individuals from six localities in which *D. suweonensis* has been known to be distributed. We first performed a high-resolution melting curve (HRM) analysis of the mitochondrial 12S ribosomal RNA gene to determine their maternal species. Thereafter, we analyzed DNA sequences of five nuclear genes (*SIAH*, *TYR*, *POMC*, *RAG1*, and *C-MYC*) to determine their parental species and hybrids.

Results

The HRM analysis showed that the melting temperature of *D. suweonensis* was in the range of 79.0-79.3°C, and that of *D. japonicus* was 77.7-78.0°C, which clearly distinguished the two treefrog species. DNA sequencing the five nuclear genes revealed a total of 37 single nucleotide polymorphism (SNP) sites between them, and STRUCTURE analysis inferred from the variant sites showed a delta K of two. We showed no double peaks in the purebred parental sequences with Q values ≥ 0.995, which clearly distinguished the two treefrog species from their hybrids; eleven individuals were *D. suweonensis*, eight were *D. japonicus*, and the other 38 were hybrids.

Conclusion

Therefore, it was possible to unambiguously identify the parental species and their hybrids using the HMR analysis and DNA sequencing methods we applied in this study, which will provide fundamental information for *D. suweonensis* conservation and restoration research.

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64 **Keywords**

65 Mitochondrial gene, nuclear genes, hybridization, endangered species, *Dryophytes*
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1. Introduction

The Suwon treefrog, *Dryophytes suweonensis*, is an endangered wildlife species due to a number of factors, including population fragmentation and continued habitat loss (Borzée 2018; Zhang et al. 2019). As a result, this species is designated as a Class I endangered wildlife in Korea and listed as Endangered (EN) on the IUCN red list (IUCN, 2017). While the common treefrog, *D. japonicus* uses a variety of habitats including forests and wetlands as well as rice fields and is widely distributed in Asia, *D. suweonensis* is mainly found in lowland rice field wetlands and known to be endemic to the Korean Peninsula (Do et al. 2017). *Dryophytes suweonensis* diverged from *D. japonicus* between 6.4 mya and 5.1 mya and is characterized by very low genetic diversity compared to *D. japonicus* (Chun et al. 2012; Li et al. 2015).

Hybridization is the reproduction of two genetically different species (Barton & Hewitt 1985). It is primarily caused by human activities such as the introduction of plant or animal species, or habitat fragmentation and modification. The more rapidly these activities interact, the more rapidly hybridization occurs (Rhymer & Simberloff 1996). It can cause outbreeding depression, which in severe cases can lead to species extinction and reduced biodiversity (Hoffmann et al. 2015; Huff et al. 2011). In addition, hybrids may be less healthy than purebreds due to interspecific incompatibilities or various negative effects (Coyne & Orr 2004; Moulia 1999). Hybrid individuals that have inherited half genes from each parental species are often morphologically indistinguishable from their parents (Leary et al. 1996). It is currently estimated that hybridization occurs in about 10% of animals, although the actual percentage is likely higher because most hybrids are difficult to identify in the wild (Maloy & Hughes 2013). Interspecific hybridization is common in frogs (Berger 1968; Kierzkowski et al. 2013; Peek et al. 2019). Identifying hybrids is important because populations can be restored by removing hybrid individuals or by captive breeding if a population contains a sufficient number of parental individuals without hybrids (Allendorf et al. 2001).

Identification of purebred parental species and their hybrids has been identified using a variety of analytical methods, including mitochondrial DNA (mtDNA) sequencing, microsatellite analysis, single nucleotide polymorphism (SNP) analysis, Restriction-site associated DNA capture (Rapture) sequencing, and so forth (Iwaoka et al. 2021; Simoes et al. 2012; Melville et al. 2017; Peek et al. 2019). A previous study reported that hybridization has also occurred between *D. suweonensis* and *D. japonicus* in their wild populations by

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analyzing both mitochondrial cytochrome o oxidase I (COI) and microsatellite markers (Borzée et al. 2020). mtDNA is widely used in population genetics to measure genetic variation of various wildlife animals to assess the population differentiation and habitat conservation strategies (Avise et al. 1987; Moritz 1994). It is also useful in phylogenetic studies because its mutation rates are ten times faster than those of nuclear DNA (nuDNA) and it shows low recombination rates (Brown et al. 1979; Masuda & Yoshida 1994). However, there are limitations in determining hybridization using mtDNA alone because it only provides information on maternal inheritance (Sato & Sato 2013). In addition, use of microsatellite markers from different species can cause errors due to the high probability of null allele occurrences as the taxonomic distance between species increases (Wan et al. 2004).

In this study, we employed the high-resolution melting curve (HRM) technique to identify the two treefrog species, *D. suweonensis* and *D. japonicus*, and their hybrids based on the mitochondrial 12S ribosomal RNA (rRNA) gene, which allowed us to identify their maternal parentage (Yoo et al. 2022). Additionally, we newly designed primer sets for five nuclear genes, E3 ubiquitin protein ligase 1 (*SIAH*), tyrosinase (*TYR*), proopiomelanocortin (*POMC*), V(D)J recombination-activating protein 1 (*RAG1*), and transcriptional regulator Myc-like (*C-MYC*), and detected single nucleotide polymorphism (SNP) sites by sequencing their amplicons to determine their parentage and hybridization. This integrated approach facilitated the unambiguous identification of the purebred parental species and their hybrids, proving to be a valuable information prior to conservation and restoration research of *D. suweonensis*.

2. Materials & Methods

Sampling and DNA extraction

This study was performed in accordance with the recommendations of the Animal Ethics Review Committee of National Institute of Ecology (NIEIACUC-2020-012). We had approval for captive and management wildlife from Han River Basin Environmental Office (No. 2020-24), Geum River Basin Environmental Office (No. 2020-24), Jeonbuk Regional Environmental Office (No. 2020-22), and Won-ju Regional Environmental Office (No. 2020-24) by Wildlife Protection and Management Act (https://elaw.klri.re.kr/kor_service/lawTwoView.do?hseq=49116).

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From April to June 2021, we sampled a total of 57 individuals of treefrogs from six localities in South Korea, including Suwon and Pyeongtaek cities in Gyeonggi-do, Chungju city in Chungcheongbuk-do, Asan city in Chungcheongnam-do, and Iksan and Wanju counties in Jeollabuk-do, where *D. suweonensis* has been known to occur (Fig. 1). Surveys were conducted during the day time when treefrogs were active, and they were captured randomly by walking around the rice field banks in the vicinity of rice field wetlands, the main habitats of this species (Kim et al. 2012). For sample collection for molecular experiments, oral epithelial cells were non-invasively obtained according to Goldberg et al. (2003), i.e., by gently swabbing a sterile cotton swab (Han ChangMedic, City, Korea) inside the frog's mouth for about 30 seconds to 1 minute. Genomic DNA (gDNA) was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's manual. The amount of extracted gDNA was determined using a spectrophotometer (DeNovix DS-11 FX, DeNovix Inc., Wilmington, DE USA).

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PCR primer design and DNA sequencing

To design primer sets for PCR amplification of five nuclear genes, *SIAH*, *TYR*, *POMC*, *RAG1*, and *C-MYC*, we downloaded the nucleotide sequence information of treefrog species available in GenBank database in National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/).

The nucleotide sequence information of the five nuclear genes was subjected to multiple sequence alignment using ClustalW (Thompson et al. 2003) in BioEdit 7.2 (www.mbio.ncsu.edu/BioEdit/bioedit.html), and five primer sets were newly designed based on their information around the highly conserved regions (Table 1). To validate the primer sets, the PCR reactions were carried out with 10 µl of Platinum Hot Start PCR Master Mix 2X (Invitrogen, Waltham, MA USA), 100 ng of gDNA, 1 µl of each primer at 5 µM, and the final volume was adjusted to 20 µl using sterilized tertiary distilled water. The PCR reaction consisted of an initial denaturation at 94°C for 2 minutes, followed by 38 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds. Finally, after an elongation step at 72°C for 1 minute, the success of the PCR reaction was confirmed by electrophoresis on a 2% agarose gel stained with GelRed (Invitrogen, USA).

The amplified PCR products were purified using the *AccuPrep*® PCR Purification Kit (Bioneer, Daejeon, Korea) following the user manual. For DNA sequencing, the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the DNA Analyzer 3730*xl* (Thermo Fisher Scientific) were utilized. The forward and reverse primers used in the PCR reaction for each nuclear gene were used for cycle sequencing. Subsequently, the raw data of each nuclear gene were aligned using SEQUENCHER version 5.4.6 (Nishimura 2000), and unnecessary parts were appropriately trimmed to complete the contigs.

HRM analysis

HRM analysis of the mitochondrial 12S rRNA gene was performed using the method described by Yoo et al. (2022). Briefly, a total volume of 20 µl PCR reaction was prepared, containing 10 µl of MeltDoctor™ HRM Master Mix (Thermo Fisher Scientific), gDNA (10 ng/µl), and 2 µl of a primer set at 5 µM (HYL-12S-0250f: 5′-GTTACACCACGAGGCTCA-3′ HYL-12S-0343r: 5′-TGAGTTTCTTAAGAACAAGCG-3′), with 6 µl of sterile distilled water. The PCR reaction was performed using the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) with an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute for ligation/extension. The Meltcurve and dissociation steps for HRM analysis were conducted at 95°C for 10 seconds for denaturation and 60°C for 1 minute for binding. Subsequently, high-resolution melting was performed at 95°C for 15 seconds, followed by 60°C for 15 seconds for binding.

For an individual that did not show reliable melting temperuature, its gDNA was PCR amplified using the forward primer 5′-AAAGCRTAGCACTGAAAATG-3′ (ANU-MT-00018f) and the reverse primer 5′-TCGGTGTAAGCGAGATGCTTT-3′ (ANU-MT-01017r) according to Yoo et al. (2022). The amplified PCR products were then sequenced using the same method as mentioned above, and identification was performed using BLASTn in NCBI.

STRUCTURE analysis

To identify patterns in the degree of hybridization between *D. suweonensis* and *D. japonicus*, we conducted a STRUCTURE analysis using the Bayesian clustering algorithm. For this

analysis, we created a nucleotide sequence matrix that included both SNP sites representing interspecific differences between the two treefrog species and SNPs identifying individual variations. The SNP sites were then analyzed using STRUCTURE v. 2.3.4. (Pritchard et al. 2000) with 100,000 burn-in and 500,000 simulations. Additionally, posterior probabilities (LnP(D)) values were calculated using the delta K (ΔK) method through STRUCTURE HARVESTER (Evanno et al. 2005) to determine the optimal K value (Earl & VonHoldt 2012).

3. Results

HRM analysis

The HRM analysis of the mitochondrial 12S rRNA gene revealed distinct melting temperatures of 79.0-79.3℃ for *D. suweonensis* and 77.7-78.0℃ for *D. japonicus*, enabling reliable species identification (Fig. 3). However, one individual (SJ02_5) showed a melting temperature of 78.6°C, which made it challenging to identify the species accurately. As a result, the species identification success rate using HRM analysis was approximately 97.88%. Its DNA sequencing identified SJ02_5 as *D. japonicus* with 99.78% identity with the nucleotide information in GenBank database (GenBank accession number: OK156173).

DNA sequencing

The DNA sequencing of the five nuclear genes sampled from 57 individuals of *D. suweonensis* and *D. japonicus* revealed specific sequence lengths for each gene; 267 bp in *SIAH*, 361 bp in *TYR*, 372 bp in *POMC*, 561 bp in *RAG1*, and 301 bp in *C-MYC*. When comparing the variable sites and parsimony informative sites (PIs) of each gene in the two treefrog species and their hybrids, no variable sites and PIs were identified in *TYR* and *SIAH* for *D. suweonensis*, and no PIs were identified in *C-MYC* and *SIAH* for *D. japonicus*. The nuclear genes with the most variable sites overall were *RAG1*, while those with the most PIs were *TYR* and *RAG1* (Table 2).

The DNA sequencing of the five nuclear genes revealed that their sequence chromatograms displayed double peaks in the SNP sites between the two treefrog species in numerous individuals (Fig. 2). For instance, the hybrid individuals showed a double peak of

(G/A) at 175 bp in *SIAH*, a double peak of (T/C) at 202 bp in *TYR*, double peaks of (G/C) and (G/A) at 91 bp and 93 bp positions in *POMC*, respectively, and a double peak of (A/T) in 97 bp in *RAG1*.

STRUCTURE analysis

STRUCTURE analysis was performed to calculate the optimal number of groups based on the Q values calculated repeatedly using the STRUCTURE HARVESTER, and the highest delta K value was found at K = 2 (Fig. 3a). Using the optimal number of groups, K = 2, the graph with the lowest maximum likelihood value (K = 2, Est. Ln prob. of data = 1232.8) was selected (Fig. 3b).

In the STRUCTURE analysis, if the Q value, which means the estimated probability that each individual belongs to a specific species or population, is 0.800 or higher based on one species, 16 hybrids were determined with a rate of 28.07%. If the Q value is 0.900 or higher based on one species, 23 hybrids were determined with a rate of 40.35%. On the other hand, when the Q value, which is the criterion that no double peaks appeared at the heterozygous mutation positions, which is a sequence that shows SNPs between species among the five nuclear gene sequences, was determined to be 0.995 or higher, eleven individuals were determined to be purebred *D. suweonensis*, eight individuals purebred *D. japonicus*, and 38 individuals hybrids (Fig. 3b). Individuals with a Q value of 0.750 or more for one species and a Q value of less than 0.250 for the other species in the STRUCTURE analysis can be assumed to be backcrosses (Weetman et al. 2014). Therefore, applying the above criteria, a total of 32 hybrid individuals were backcrosses. 13 individuals were assumed to be backcrosses with the maternal parentage of *D. japonicus*, and 19 of *D. suweonensis*, representing 56.14% of the total individuals.

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For most individuals, HRM analysis of mitochondrial 12S rRNA gene and the ratio of Q values from STRUCTURE analysis of the five nuclear genes were consistent for their maternal parentage determination. For example, most of the individuals determined as maternal inheritance with *D. japonicus* had a Q value of 0.721 or higher for the corresponding species, and most of the individuals with *D. suweonensis* had a Q value of 0.716 or higher for the corresponding species. On the other hand, two individuals, SJ02_5 and JN02 were determined to be *D. japonicus* as maternal inheritance, but the STRUCTURE

analysis showed that their Q values of 0.406 and 0.346, respectively, were attributed to *D. japonicus*, indicating a cyto-nuclear discordance.

4. Discussion

In general, nuDNA is stably transmitted to offspring and characterized by biparental inheritance. Methods that analyze both mtDNA and nuDNA for species and hybrid identification have been shown to significantly increase accuracy in determining their parental lineage (McKay & Zink 2010; Sun & Pang 2013; Toews & Brelsford 2012; Whittaker et al. 1994; Funk & Omland 2003). Studies identifying hybrids through mtDNA-nuDNA comparative analysis have been used to identify introgressive hybrids to elucidate the process of introgressive hybridization, and to understand the level of genetic diversity (Zhang et al. 2018). These methods have been used in amphibians research, including the identification of potential polyploid hybrids and backcrosses (Correa et al. 2012; Stöck et al. 2010; Velo-Antón et al. 2021). In this study, we used the mitochondrial 12S rRNA gene of *D. suweonensis* and *D. japonicus*, and their hybrids to identify the maternal parentage by HRN analysis, and also applied the DNA sequencing of the five nuclear genes that contains SNP sites, thereby greatly improving the identification accuracy of the purebred parental species and hybrids.

Sequence chromatograms from the five nuclear genes have the advantage of being able to reconstruct parental sequences for DNA segments from heterozygotes and interspecies hybrids for multiple linked points through the identification of SNP sites and double peak patterns (Sousa-Santos et al. 2005). While interspecific F_1 hybrid individuals are commonly characterized by double peaks at all SNPs where the heterozygous mutation between the two species occurs (Depaquit et al. 2019; Sousa-Santos et al. 2005), the individuals analyzed in this study showed an irregular pattern; the double peak was not consistently observed only in the interspecific SNP sequences of the F_1 hybrid, making it challenging to identify the purebred parental species and their hybrids based on the presence of a simple double peak.

Vähä& Primmer (2006) employed two Bayesian-based programs, STRUCTURE and NEWHYBRIDS, to effectively detect hybrids. They determined the optimal genetic differentiation threshold based on three key aspects: efficiency, accuracy, and overall performance, with a Q value of 0.900 or higher. In a previous study, hybrids of *D.*

suweonensis were classified as such when the assignment probability was below 90.0% (Borzée et al. 2020). In contrast, our study proposed a Q value threshold of 0.995 for the absence of double peaks at heterozygous mutation sites between *D. suweonensis* and *D. japonicus* across the five nuclear genes. While reducing the threshold may result in a larger number of individuals being classified as the purebred parental species, we argue that a stricter threshold aligns more closely with the criteria essential for determining the parental species, particularly in the context of conserving endangered wildlife species (De Hert et al. 2012; Yan et al. 2017).

Admixture analysis can be used to identify F_1 and F_2 hybrids, and first-generation backcrosses, which are characterized by a decrease in the admixture rate (Q) of the species by approximately one-half with each new backcross generation (Vähä& Primmer 2006; Weetman et al. 2014). In this study, we were able to accurately identify hybrids between *D. suweonensis* and *D. japonicus*, and the proportion of individuals that could be presumed to be reverse hybrids was very high at 56.14% of the total individuals. In particular, the fact that there were no F_1 hybrids identified between *D. suweonensis* and *D. japonicu*s suggests that hybridization between the two treefrog species has occurred over a long period of time and hybrids can interbreed with one of their parental species that shares the same habitats. Therefore, as mentioned above, it emphasizes the need to apply strict threshold values when separating the two treefrog species and their hybrids by Q values in STRUCTURE analysis using the method developed in this study.

Hybridization, recognized as a significant driver of extinction, can imperil endangered wildlife species through processes like hybridization suppression or genetic assimilation. The impact of hybridization on species can be detrimental as it facilitates the gene flows between different species, potentially leading to reduced biodiversity through direct and indirect pathways or even culminating in species extinction (Levin 2002; Rieseberg & Carney 1998). Our findings indicate that substantial occurrences of hybridization or backcrosses between *D. suweonensis* and *D. japonicus* could markedly diminish their population. Of greater concern, however, is the fact that 81.58% of these hybrids are backcrosses. Backcrosses tend to exhibit a higher imbalance in mitochondrial/nuclear ratios, which detrimentally influences their survival (Vilaça et al. 2023). In this study, we identified two *D. japonicus* individuals displaying cyto-nuclear discordance. Similar instances of genetic mismatches between mtDNA and nuDNA have

been also observed in other amphibian species (Ambu et al. 2023; Cairns et al. 2021; Eto et al. 2013). The occurrence of cyto-nuclear discordance may suggest factors such as hybridization through introgression (Lee-Yaw et al. 2019), sex-biased dispersal (Seixas et al. 2018), or shifts in hybrid zones (Wielstra 2019). Notably, amphibians have demonstrated differential growth in individuals based on their mitochondrial type (mitotype), with lower growth rates occurring in instances of cyto-nuclear discordance (Lee-Yaw et al. 2014). Hence, it is plausible that individuals displaying cyto-nuclear discordance among hybrids between the two treefrog species also exhibit growth disparities compared to the purebred individuals from their respective mitotypes.

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Conclusions

Previous studies have underscored that hybridization between *D. suweonensis* and *D. japonicus* constitute a primary driver behind the extinction threat faced by the former species (Borzée et al. 2018; Borzée et al. 2020). Given the substantial identification of hybrid individuals in this study, it becomes imperative to explore strategies for curbing hybridization to safeguard this endangered wildlife species. Hence, the crucial course of action involves elucidating the mechanisms underlying its hybridization and promoting population stabilization (Bohling 2016). The strategy of the DNA sequencing of five nuclear genes applied through this study is expected to offer several benefits. It can counteract potential data bias attributed to null alleles when solely employing microsatellite markers for hybridization analysis. Additionally, the nuclear gene markers can enable a stringent determination of the purebred parental species with a higher resolution, thus significantly aiding in unraveling the mechanisms of hybridization. This research lays the groundwork for systematic investigations, enhancing the precise identification of the purebred parental species and their hybrids of *D. suweonensis* and *D. japonicus*. Such advancements serve as a fundamental framework for guiding efforts toward the restoration of reproductive processes, a critical endeavor during times necessitating the conservation and restoration of the endangered *D. suweonensis*.

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Author Contributions

Nakyung Yoo performed the experiments, analyzed the data, prepared figures and/or tables,

authored or reviewed drafts of the article, and approved the final draft.

Ju-Duk Yoon conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.

Keun-Yong Kim performed the experiments, authored or reviewed drafts of the article, and approved the final draft.

Jeongwoo Yoo analyzed the data, prepared figures and/or tables, and approved the final draft.

Jung Soo Heo analyzed the data, authored or reviewed drafts of the article, and approved the final draft.

Keun-Sik Kim conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.

Data Availability

The following information was supplied regarding data availability: Q value from STRUCTURE analysis, results of mitochondrial DNA analysis using melting temperature from HRM analysis, species identification through nuDNA-mtDNA comparison of *D. suweonensis* and *D. japonicus,* and their hybrids are available in the Supplemental Files. All nuDNA data are available in the NCBI: OR474555 – OR474832

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Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers): Han River Basin Environmental Office (No. 2020-24), Geum River Basin Environmental Office (No. 2020-24), Jeonbuk Regional Environmental Office (No. 2020-22), Won-ju Regional Environmental Office (No. 2020-24) and the IACUC at National Institute of Ecology approved this research (NIEIACUC-2020-012).

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Conflict of interest

The authors declare no conflicts of interest.

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Tables and Figures legends

Table 1. Primer sets newly designed in this study to identify purebred *Dryophytes suweonensis*, *D. japonicus*, and their hybrids.

Table 2. Sequence nucleotide polymorphism (SNP) sites and parsimony informative sites (PI) in five nuclear genes of purebred *Dryophytes suweonensis*, *D. japonicus*, and their hybrids.

Figure 1. Sampling localities of individuals of *Dryophytes suweonensis*, *D. japonicus*, and their hybrids in South Korea.

Figure 2. Examples of double peaks at sequence nucleotide polymorphism (SNP) sites in sequencing chromatograms of five nuclear genes of *Dryophytes suweonensis*, *D. japonicus*, and their hybrids. (a-c) *SIAH*, (d-f) *TYR*, (g-i) *POMC*, (j-l), *RAG1* and (m-o), and *C-myc* of *D. suweonensis* and *D. japonica*, and their hybrids. (a, d, g, j, m) homozygous peaks in *D. japonica*, (b, e, h, k, n) heterozygous peaks in hybrids between *D. japonicus* and *D. suweonensis*, and (c, f, I, l, o) homozygous peaks in *D. suweonensis*.

Figure 3. Identification results of purebred *Dryophytes suweonensis*, *D. japonicus*, and their hybrids. (a) The best suitable K of *D. suweonensis* and *D. japonicus* obtained by the delta K (ΔK) method in STRUCTURE Harvester. The value of ΔK was highest at 2 (ΔK: 1681.844). (b) Results of mitochondrial 12S rRNA using HRM analysis (mtDNA) and probabilistic assignment to genetic clusters (K = 2) using the STRUCTURE software. A vertical column represents each individual, and the length of each column indicates the proportional membership (Q value) in each cluster (*D. suweonensis* is green, *D. japonicus* is blue, and red box represents cyto-nuclear discordant individuals).

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