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Original Article

Development of an eDNA metabarcoding tool for surveying the world's largest amphibian

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Abstract

Due to the overexploitation of farming, as well as habitat destruction, the wild population of Chinese giant salamander (CGS) *Andrias davidianus*, a species with seven genetically distinct lineages, has decreased by over 80% in the past 70 years. Traditional survey methods have proven to be unsuitable for finding this rare and elusive species. We evaluated the efficacy of environmental DNA (eDNA) sampling to detect CGS indirectly from its aquatic environment. We developed several species-specific primer sets; validated their specificity and sensitivity; and assessed their utility in silico, in the laboratory, and at two field sites harboring released farm-bred CGS. We detected the presence of CGS DNA by using polymerase chain reaction and Sanger sequencing. We also sequenced an amplicon mixture of seven haplotype-represented samples using high-throughput sequencing. Our eDNA methods could detect the presence of CGS at moderate densities reported across its range, proving them as a cost-effective way to establish broad-scale patterns of occupancy for CGS. In addition, our primers enabled the detection of mitochondrial lineage mixture or introduced individuals from geographically isolated populations of CGS.

Keywords: *Andrias davidianus*, Chinese giant salamander, eDNA, metabarcoding, population survey

Chinese giant salamander (CGS) *Andrias davidianus* is the largest amphibian in the world. As a classic living fossil, its anatomical structure has remained almost unchanged for 160 million years (Gao and Shubin 2003). Genetic data suggests that CGS consists of seven genetically distinct, but morphologically similar, lineages (Yan et al. 2018; Liang et al. 2019), some of which warrant species-level recognition (Turvey et al. 2019). This species was once widely distributed in the watersheds of Pearl, Yangtze, and Yellow Rivers in central and southern China (Fei et al. 2006). However, due to the overexploitation of farming, as well as habitat loss and degradation, the wild population has decreased by over 80% in the past 70 years (Zhang et al. 2002; Turvey et al. 2018). In contrast, 2,080 CGS farms were licensed, and up to 12,490,000 individuals were farmed across the country (China Aquatic Wildlife Conservation 2015). Government-promoted conservation translocations have been conducted in 16 provinces or municipalities since 2002 (Shu et al. 2021). Distinct genetic lineages that historically occupied different geographic ranges have become admixed in the wild, raising concerns about the loss of genetic diversity within wild populations (Wang 2015; Yan et al. 2018; Shu et al. 2021). CGS is listed as critically endangered on the IUCN Red List and China's Vertebrates Red List (Jiang et al. 2016), and it has also been listed as national Grade II protected wild animal since 1989. Globally, CGS has drawn the world's conservation concern as one of the top Evolutionarily Distinct and Critically Endangered animals (Isaac et al. 2012). There is an urgent need for population monitoring and genetic risk assessment of CGS.

The traditional labor-intensive methods for surveying CGS populations, including snorkeling, bow-hooking, trapping, and mist-netting (Browne 2011), rely on visual counts of individuals, and they have proven to be unsuitable for finding rare giant salamanders. No wild ones were observed in an 82-day survey conducted in core habitats in the period 2000–2001 (Wang et al. 2004). Fifteen years later, only one juvenile was found in a 68-day survey by spot-light snorkeling and trapping in Sichuan province and Chongqing city (Wang 2015), and only 24 individuals were found in a 4-year range-wide survey (97 sites) (Turvey et al. 2018). Therefore, the development of an efficient survey method is highly desirable.

The use of environmental DNA (eDNA) and metabarcoding has been shown to offer increased sampling resolution for species detection and biodiversity assessment (Sepulveda et al. 2020). Sources of eDNA include sloughed hair and skin, urine, feces, gametes, saliva, mucus, decomposing carcasses, and animal-feeding invertebrate samplers (Harrison et al. 2019; Drinkwater et al. 2021), and could consist of both free molecules (extracellular DNA) and free cells (Turner et al. 2014; Sassoubre et al. 2016; Moushomi et al. 2019; Jo et al. 2019a). Furthermore, eDNA collected from water samples has highly sensitive detection capabilities, is non-invasive

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to sampled biota, and is not limited to physical environmental conditions (e.g., hydrogeomorphic features or substrate types). It is important to note that eDNA capture can differ between environment types (Collins et al. 2018; Seymour et al. 2018) and within sites (Bista et al. 2017), due to seasonal variation, ecology, or random sample variation. Coupled with high-throughput sequencing (HTS) applications, such as metabarcoding, eDNA-based sampling is rapidly being integrated into the standard ecological monitoring of reclusive, inaccessible, or dangerous taxa when other survey methods are impractical (Jerde et al. 2011; Deiner et al. 2016; Boussarie et al. 2018).

eDNA primers have been developed for surveying closely related species, including the Japanese giant salamander (JGS) *Anadrias japonicus* and the hellbender *Cryptobranchus alleganiensis* (Olson et al. 2012; Fukumoto et al. 2015; Takahashi et al. 2018; Wineland et al. 2019), as well as non-native populations of CGS that exist in Japan (Fukumoto et al. 2015). However, an eDNA primer set for detecting all lineages of CGS is still deficient. Here, we developed one and evaluated its efficiency in silico, in the laboratory, and at field sites. Our primer set is the first to allow the identification of all lineages of CGS across its range. This metabarcoding tool will be invaluable for the population monitoring and conservation of the world's largest, elusive, and endangered amphibian.

Materials and Methods

Primer development and evaluation

To develop primers that would universally amplify all lineages of CGS, we built a large database of mitochondrial sequences that represented the overall genetic background of wild populations. We downloaded 720 nucleotide sequences whose definition contained the words "*Andrias davidianus*" and "mitochondrial" or "mitochondrion" from the GenBank database of the National Center for Biotechnology Information (NCBI). Most of these sequences, for wild-born or historical specimens, were originally collected to clarify the spatial genetic structure and taxonomy of CGS (Murphy et al. 2000; Wang et al. 2017; Yan et al. 2018; Liang et al. 2019; Turvey et al. 2019). We manually checked the voucher numbers of specimens and filtered out 16 duplicate sequences. The final dataset consisted of 704 sequences, including 29 complete and 17 partial mitogenomes.

To assess mitogenome variation, a complete mitogenomic sequence with the GenBank accession code of NC_004926 (Zhang et al. 2003) was selected as a reference. The other 703 sequences in the dataset were mapped to this reference using bowtie2 in "-very-sensitive-local" mode (Langmead and Salzberg 2012), and the depth option of Samtools v1.9 (Li et al. 2009) was used to assess the sequence coverage at each site. Next, sequences were mapped to the reference using BWA-MEM v0.7.17 (Li 2013) and then called single-nucleotide polymorphism (SNP) density using bcftools v1.9 (Danecek and McCarthy 2017) and VCFtools v0.1.16 (Danecek et al. 2011). Mitogenomic architecture, sequence coverage, and SNP density were plotted using Circos v0.69.9 (Krzywinski et al. 2009).

The mitochondrial Cytochrome *b* (Cytb) gene, with a medium rate of evolution (Zhang et al. 2003; Mueller 2006), is widely targeted for the development of eDNA-based assays in amphibians (Thomsen et al. 2012; Spear et al. 2015; Evans et al. 2016; Wineland et al. 2019). A suite of primer sets with amplicon lengths of 90–250 base pairs (bp) was developed using the software Primer5 (Lalitha 2000). Primer sets that targeted annealing sites containing SNPs were abandoned.

Non-specific targets of primers were checked using the primer-BLAST option in NCBI and by cross-species amplification of sympatric amphibians (Supplementary Table S1). Additionally, the reliability and universality of primers were tested on 22 DNA samples from the Gutian Mountain National Nature Reserve (GMNNR), Kaihua County, Zhejiang province (Supplementary Table S2).

For each primer set, the efficiency and lowest threshold of amplification were tested by triple polymerase chain reaction (PCR) trials with a concentration of template DNA ranging from 0.1 to 1 × 10^{-11} ng·μL^{-1}. The primer set of Ad_Cytb_e1 was advanced for use in the remainder of the study as it provided the most efficient and reliable amplification. The best PCR profile was 25 μL in total, including 10 μL mix (TransGen Biotech Co., Ltd., Beijing); 12 μL ddH_2O; 0.5 μL forward and reverse primer, respectively; and 2 μL template DNA. The reaction was pre-PCR for 4 min at 94°C; 35 cycles of denaturation at 95°C for 40 s, annealing at 51.2°C for 60 s, and extension at 72°C for 30 s; and post-PCR at 72°C for 8 min.

Laboratory trial

Prior to the field trial, the eDNA methods were tested on water samples from the Pidu Captive-Breeding Farm of CGS. In total, one hundred 3-year-old individuals with snout–vent lengths of 20–30 cm were raised in each pool (physical dimensions = 1 m × 1 m × 0.9 m; water volume based on fill depth ~ 400 L). Each pool had a constant input–output flow, resulting in one total water change per 24 h. Two 4.5 L water samples were collected from each pool using polyethylene terephthalate drinking bottles. Such containers, which are cheap and readily available in China, are sterilized following standards for food processing.

Samples from each pool were combined for filtering using a sterile 0.45-μm pore size cellulose acetate and cellulose nitrate mixed membrane and a vacuum pump system (Tianjin Jinteng Experiment Instrument Co., Ltd.) during 6 h of sampling (Maruyama et al. 2014). Filtration occurred in areas where no CGS existed, and we changed gloves between each filtering. The filter membrane was then kept in 95% ethanol in a 2 mL EP tube and stored at –20°C in the laboratory until DNA extraction.

DNA was extracted from each membrane using the TIANamp Genomic DNA Kit (TIANGEN Biotech Co., Ltd., Beijing) following the manufacturer's instructions. For each water sample, we assayed for the presence of eDNA by scoring 5 PCRs: 3 replicate reactions incorporating the sample being tested, 1 negative control reaction in which we added water in place of the template, and 1 positive control reaction in which we added 0.01 ng of muscle-derived CGS DNA. PCR products were visualized on 1.2% (w/v) BBI agarose gels (Sangon Biotech Co., Ltd., Shanghai). DNA extraction and amplification were conducted in different laboratories where no CGS experiments had been conducted previously (Taberlet et al. 1999).

Field test

The Dujiangyan Release Base (DRB) and the GMNNR, which harbored released captive-bred CGS, were selected for our field test. Seventy salamanders were released in the period

2017–2018 along a 1 km length of stream (stream width 5–8 m) at DRB, and 4,532 salamanders were released in the period 2015–2016 along a 2 km length of stream (stream width 2–10 m) in GMNNR. Salamanders at both sites were monitored monthly by a line-transect survey, radio-telemetry tracking, and trapping. A total of 22 and 9 individuals were captured in September of 2018 and 2019 in GMNNR, respectively (Liu et al. 2021). The density of salamanders was calculated as the number of individuals found per m^2 (i.e., stream length × width) (Olson et al. 2012).

Water samples (each is two 4.5 L combined) were collected in September 2019, from DRB (n = 7) and GMNNR (n = 15), every 150 m and 100 m, respectively. The containers were submerged 1–2 inches below the surface of the stream for sampling. The samples were then assayed in the same way as in laboratory trials.

Quality assurance

As contamination is a concern in presence–absence genetic studies, in particular eDNA studies, ultrapure water was filtered in the field and tested along with other filters to allow us to identify any equipment or background contamination. All equipment was sterilized using a 5-min exposure to a 10% bleach solution before sampling. Scissors, tweezers, EP tubes, pipettes, tips, and glass cores of vacuum pumps were sterilized at 121°C for 30 min before use. The work area was cleaned using RNase, DNase, RNA, and DNA Away Reagent (Beyotime Biotechnology, Shanghai).

Each eDNA sample was PCR-amplified in triplicate to account for stochasticity in amplifications of low-quality/quantity DNA. A clear gel band in any replicate indicated a positive amplification, which was purified and then subjected to Sanger sequencing. The likely origin of each amplified sequence was determined by a BLAST search in the NCBI non-redundant database, with the top-hit species (based on e-value) representing its origin.

Next-generation sequencing of a lineage-mixed sample

A mock sample was used to test whether all CGS lineages could be detected simultaneously with HTS. First, the cytb gene of 341 tissue or swab samples, which were either collected in this study or previously (Wang et al. 2013, 2017; Yan et al. 2018), were sequenced following the procedure of Tao et al. (2006). These sequences, together with those retrieved from GenBank, were aligned by ClustalW implemented in Mega v7.0 (Kumar et al. 2016), truncated by Ad_Cytb_e1 primers, and then collapsed to identify haplotypes. Second, seven genomic DNA samples that could represent the haplotypes and mitochondrial lineages of CGS (Yan et al. 2018; Liang et al. 2019) were selected and amplified using primers with sample-specific 6 bp barcodes. Third, 2 μg of PCR products for each sample was mixed as a mock sample and sequenced using paired-end 150 bp reads in a NovaSeq 6000 System. Fourth, the paired-end reads were merged using PEAR software (Zhang et al. 2014) with the parameters of -v 30 -n 236 -m 236 -t 86, filtered using the fastq_quality_filter function (-q 30 -p 90), and split into original samples using the fastx_barcode_splitter.pl function of fastx_toolkit (http://hannonlab.cshl.edu/fastx_toolkit/download.html). Fifth, target or non-target sequences of each haplotype were counted with seqkit software (Shen et al. 2016), and their likely origin was determined using a BLAST search in GenBank.

All specimen sampling and field surveys were carried out following Animal Use Protocols approved by the Animal Ethics and Welfare Committee of Chengdu Institute of Biology (permit code 2016-AR-JJP-02).

Results

Of the 704 mitochondrial sequences that were retrieved from GenBank, 570 were identified as containing a whole or partial Cytb gene. Compared to the other genes, Cytb had the highest sequence coverage and modest SNP density (Supplementary Figure S3), and was chosen as the target for eDNA amplification as its primer-binding sites were conserved across, and largely unique to, CGS (Supplementary Figure S4).

Only 5 primer sets, including 5 forward and 2 reverse primers, passed the initial tests and were retained for downstream analysis (Table 1). The primer set Ad_Cytb_e1 showed the most efficient amplification, with the lowest eDNA concentration for detection of 1.3×10^{-7} ng·μL^{-1} (Figure 1). It had a degenerate site (A/C) in the forward primer (Supplementary Figure S4).

Primer-BLAST analysis showed that all GenBank sequences that have 100% identity with Ad_Cytb_e1 were from CGS. Two sequences of JGS (GenBank accession codes: AB445781

Table 1. eDNA primer sets for the Chinese giant salamander

Primer	Sequence (5′ –3′)	Annealing temperature (°C)	Product length (bp)
Ad_Cytb_e1_f	TCTTCAGCATTTTCATCMGTGG	51.2	224
Ad_Cytb_e1_r	GGAAGGACATAACCAACAAAAGC		
Ad_Cytb_e2_f	GATGTAAACTATGGCTGG	47.5	158
Ad_Cytb_e2_r	ATTACTAAGAATAGGAGAAC		
Ad_Cytb_e3_f	GAGATGTAAACTATGGCT	47.5	160
Ad_Cytb_e2_r	ATTACTAAGAATAGGAGAAC		
Ad_Cytb_e4_f	TGCCGAGATGTAAACTAT	47.5	164
Ad_Cytb_e2_r	ATTACTAAGAATAGGAGAAC		
Ad_Cytb_e5_f	CCGAGATGTAAACTATGG	47.5	162
Ad_Cytb_e2_r	ATTACTAAGAATAGGAGAAC		

Ad_Cytb_e1 showed superior performance and was used for all downstream analyses.
M: indicates A or T.

and AB445779) were identified with one or two nucleotide mismatches in the forward binding region and could be amplified by Ad_Cytb_e1 (Supplementary Figure S5). The seven sympatric amphibian species were not amplified by Ad_Cytb_e1. The detection probability was 100% for all four ponds in the Pidu Farm (Table 2), but decreased to 85.7% (n = 7) at DRB, and further decreased to 20% (n = 15) in GMNNR.

Eleven haplotypes were identified for the amplified fragments of Ad_Cytb_e1 (Supplementary Figure S3). HTS of the amplicon mixture indicated that all the seven haplotypes were detected (Figure 2 and Table 3). Each haplotype was sequenced with 89,744–252,789 paired-end reads, covering a proportion of 8.6–24.2% in total. Target sequences covered 91.49 ± 2.87% (mean ± standard deviation) of reads in each sample (Figure 2), showing that Ad_Cytb_e1 has a high degree of generality in amplifying different lineages of CGS. Non-target sequences had a range of 4.9–13.2% of reads for each sample and 1–27 bp mismatches to the true haplotype (Supplementary Table S6), indicating PCR or sequencing errors.

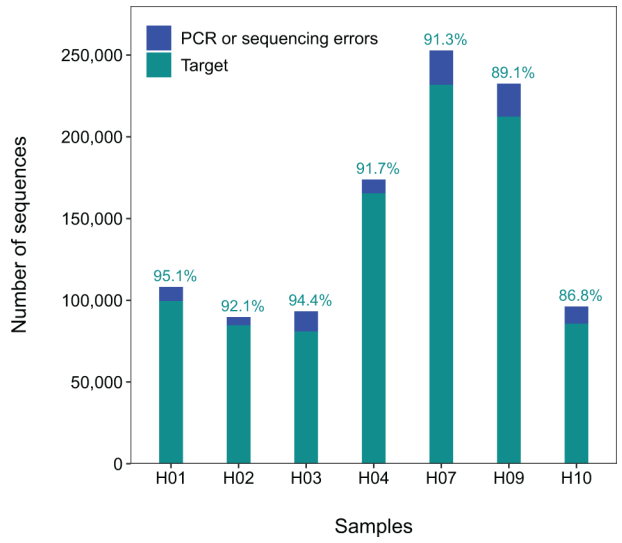

Figure 1. Detection probability of eDNA primer set Ad_Cytb_e1 based on DNA concentration (5 PCR replicates).

Discussion

The identification of DNA sequences derived from environmental samples heavily depends on reliable reference databases (Thomsen and Willerslev 2015). CGS has a wide geographic range and high genetic variability (Wang et al. 2017; Yan et al. 2018; Liang et al. 2019), and one of its mitochondrial lineages was regarded as a separate species, *Andrias sligoi* (Turvey et al. 2019). Fortunately, a large number of Cytb sequences were deposited in GenBank and collected from a much wider range (Murphy et al. 2000; Yan et al. 2018; Turvey et al. 2019). Therefore, in addition with our own 341 Cytb sequences as a reference, we could develop an eDNA tool to strike the balance between the specificity (targeting CGS solely) and generality (targeting all divergent CGS lineages) of detection.

Our eDNA primer set is an improvement over that of Fukumoto et al. (2015), which targeted the mitochondrial NADH1 gene and was designed to survey the introduced CGS and its hybrids with the indigenous JGS in Japan. The NADH1 primer binding sites matched many, but not all known sequences of CGS. Our eDNA primer set may be able to detect some populations of JGS, as sequences that differ in 1 or 2 bp across the primer-binding sites could be amplified (Supplementary Figure S5). Since JGS has not been documented to exist in China, this will not diminish the utility of our eDNA primer set for the detection of CGS across its native range.

Both abiotic and biotic conditions could influence eDNA persistence and concentration (Barnes and Turner 2016). For example, no correlation was found between eDNA estimates and the abundance or biomass of hellbender, but eDNA concentrations and thus detection probability was highest during the September breeding season (Spear et al. 2015; Takahashi et al. 2018). Water temperature facilitated eDNA degradation and accumulation simultaneously: the higher eDNA decay rates could reflect the activity and abundance of microbes and extra-organism nucleases in the water, and the higher eDNA shedding rates might due to the higher metabolism and physiological activity of organisms (Jo et al. 2019b). In this study, we revealed the presence of higher negative reactions in GMNNR than at DRB. The possibility of lower population densities (Table 2) as well as lower eDNA concentration of CGS in GMNNR should be subjects of future studies.

eDNA-based surveys can lead to underestimating the distribution of a species due to erroneous detection during field work (e.g., water sampling) or during laboratory analysis

Table 2. Aquatic eDNA detection for CGS in a farm and two field sites

Sampling sites	Water temperature (°C)	pH value	Elevation(m)	Riverbed or pool bottom	Density of CGS (indiv./m^2)	Sampling date	Sampling strategy	Detection probability
Pidu Farm	22.0–22.9	8.0–8.0	540	Ceramic tile	100	2018.10.17	1 sample per pool	100% (n = 4)
Dujiangyan Release Base (DRB)	17.5–18.3	8.5–8.6	789–829	Cobble	0.014[a]	2019.9.18	Every 150 m	85.7% (n = 7)
Gutian Mountain National Nature Reserve (GMNNR)	19.0–22.5	7.0–8.8	362–465	Cobble, humus	0.002[b]	2019.9.5–10	Every 100 m	20% (n = 15)

[a]Estimated according to releasing records (70 individuals released in 1-km stream length; stream width: 5–8 m).
[b]Estimated according to our survey records (9 individuals found in 2-km stream length; stream width: 2–10 m).

(e.g., PCR). Filtration with a 0.2-μm pore size was the best strategy for maximizing eDNA and minimizing non-target eDNA (Turner et al. 2014), but this may increase the filtration time and risk of filter clogging. Humic acids or humic substances, co-extracted with DNA in environmental samples, strongly inhibit enzymes, such as Taq Polymerase used in PCR amplification (Matheson et al. 2010; Sidstedt et al. 2015). Quantitative PCR and droplet digital PCR appear to be more sensitive than conventional PCR for single species detection (Wilcox et al. 2013; Doi et al. 2015). In the future, we will continue to optimize this eDNA technique and assess whether improved sensitivity could be achieved using other protocols, for example, by changing water volume and/or pore size, the use of ATL as an eDNA storage buffer, and the use of PCR inhibitor removal kits. Considering the low detection rate of 20% in some sites such as Gutian Mountain, a minimum of five samples in a field site and the adoption of appropriate statistical methods, such as site occupancy models (Schmidt et al. 2013), should increase the likelihood of occupancy detection for CGS across its range.

Translocations were proposed as a way of conserving biodiversity, particularly in the management of threatened and keystone species, with the aims of maintaining biodiversity and ecosystem function under the combined pressures of habitat fragmentation and climate change (Weeks et al., 2011). To protect the world's largest amphibian and the apex predator in freshwater ecosystem, government-promoted translocations have been conducted across its range since 2002 (Shu et al. 2021). However, the geographically distinct evolutionary lineages were brought into contact, and lineage mixtures have been found both in farms (Yan et al. 2018) and in the wild (Shu et al. 2021). Our eDNA primer successfully detected four of the seven genetically distinct lineages simultaneously (Figure 2), demonstrating that it could be used to identify the presence of mitochondrial lineage admixtures in captive and wild populations of CGS. This will facilitate genetic screening and genetically informed conservation efforts when combined with the metabarcoding tool of multi-copy nuclear eDNA (e.g., ribosome RNA genes) in the future.

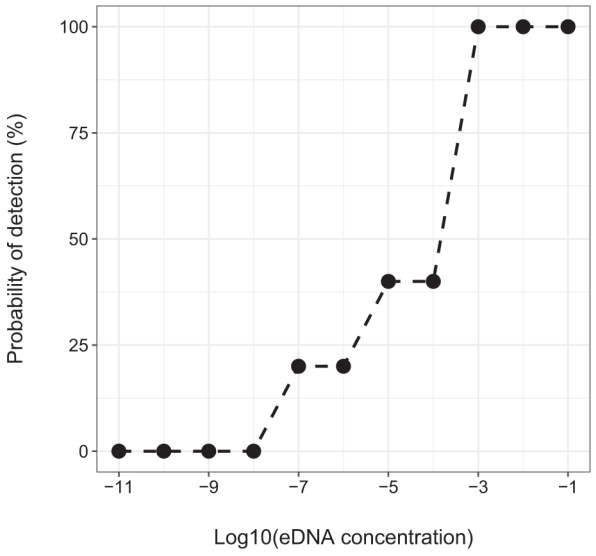

Figure 2. Composition of amplicons detected by HTS. Each of the seven samples has one target haplotype (in cyan color), whereas blue color indicates non-target sequences (i.e., errors introduced during PCR or sequencing). H10 corresponds to the lineage D in Yan et al. (2018) and *Andrias sligoi* in Turvey et al. (2019).

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Table 3. Haplotypes and corresponding lineages detected in the mock sample using HTS

Lineage[a]	Haplotype[b]	DNA sample	Collection site	Barcode sequence for forward primer	Barcode sequence for reverse primer	GenBank accession code	Paired-end reads
C	H04	SCDJY4	Pidu, Sichuan province	ATCACG	ACTGAT	MZ291454	173,892
B	H01	SCDJY8	Pidu, Sichuan province	CGATGT	ATGAGC	MZ291455	108,112
B	H02	ZJGTS2	Gutian, Zhejiang province	TTAGGC	ATTCCT	MZ291457	89,744
B	H07	ZJGTS5	Gutian, Zhejiang province	ACAGTG	CAACTA	MZ291458	252,789
U1	H09	ZJGTS10	Gutian, Zhejiang province	GCCAAT	CACCGG	MZ291459	232,457
D	H10	GD19	Guiding, Guizhou province	CAGATC	CACGAT	MZ291460	96,134
B	H03	SXLY6538	Lueyang, Shaan'xi province	GGCTAC	CATTTT	MZ291456	93,215

[a]Lineage is identified as Yan et al. (2018). Lineage D corresponds to *Andrias sligoi* in Turvey et al. (2019).
[b]Haplotype is identified as Supplementary Figure S4.

Authors' contributions

This study is P.L.'s MSc thesis work under the supervision of J.W. and F.X. P.L. conducted field survey, laboratory work, and data analysis. J.C. and C.L. contributed to data analysis. J.W., F.X. and J.J. contributed to conceptualization, formal analysis, and writing. All authors have read and approved the manuscript for submission.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Data Availability Statement

The raw high-throughput sequence data has been deposited in the Genome Sequence Archive at the National Genomics Data Center (CRA004266) and is publicly accessible at https://bigd.big.ac.cn/gsa.

Supplementary Material

Supplementary material can be found at https://academic.oup.com/cz.

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