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Genome survey and characterization of new microsatellite markers in *Hynobius amjiensis* (Caudata: Hynobiidae)

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Abstract. The Anji Salamander, *Hynobius amjiensis* Gu, 1992, is a critically endangered amphibian only known from a few mountain tops in the Zhejiang and Anhui Provinces of China. Along with tremendous efforts to breed this species in captivity, there have been attempts to understand the causes for its endangered status from a genetic perspective, which was limited to a few markers from earlier studies. Here we used next-generation sequencing technology on the DNASEQ platform to investigate the characteristics of the salamander's genome. Based on k-mer analysis, the 19-mer frequency distribution yielded the optimal estimation that suggested a genome size of ~17.54 Gb, 70.77% of which consist of repetitive sequences. Filtered sequences were assembled in 8,852,165 contigs with N50 at 1052 bp; GC rate was 45.3%. We identified 1,441,045 microsatellite loci across the assembled partial genome. Mono-nucleotide microsatellites accounted for 61.4% of all loci, and 2 to 6-base repeat motifs are present at a frequency of 19.59.67.91.5and 0.06%, respectively. PCR primers were developed for 98 microsatellite loci with 2 to 6-base motifs. Our work provides an overview of the genome characteristics of *H. amjiensis* that can serve subsequent studies on the evolutionary history of this endangered salamander.

Key words. Anji Salamander, endangered species, genome size, microsatellite.

Introduction

The Anji Salamander, *Hynobius amjiensis* (Fig. 1), belongs to the Caudata family Hynobiidae and is found in the *Sphagnum* moss and herbage of the montane swamps on Longwang Mountain (Gu 1992). Being endemic to China, it has since been classified in the top ten critically endangered amphibians by the International Union for Conservation of Nature (IUCN), and as critically endangered in the Red List of China's Biodiversity Amphibians (Jiang & Xie 2015). It has also been given Class I protection according to the most recent list of Wild Animals under Special State Protection in China in 2021. This salamander is currently only known from four neighbouring sites in China, which are Longwang Mountain in Anji, Qingliangfeng, and Baizhangling Mountain in Linan, Zhejiang Province, and on Anhui Qingliangfeng Mountain, Anhui Province.Here, increasing human activities and adverse changes in climate have been making survival ever more difficult for it, as both lead to the shrinking of *Sphagnum* moss-covered swanps and a reduction in the number of breeding sites. As a result, the reproductive population of *H. amjiensis* has constantly been declining. Since the estimated effective population comprises merely 600 adults (Chen et al. 2016),it is necessary to study the mechanisms causing its endangerment and manage and if possible expand the suitable habitat for this population. To the latter end, Chinese authorities have set up the Anji Salamander National Nature Reserve in which the Anji Salamander may find a less disturbed habitat. Meanwhile, throughout this reserve, we devised and started off multiple strategies to help this population recover, including protection and restoration of habitat alongside with captive breeding. However, various major questions have as yet remained unsolved. For instance, how large is the genome of *H. amjiensis*, what kind of genetic structure exists in the population, and what is the number of actual subpopulations, and to what extent is gene exchange still taking place, to name only some. Answers to these key questions should be helpful to the protection of *H. amjiensis*.

Due to its rarity, only a few groups of researchers have thus far been able to conduct basic studies on *Hynobius amjiensis*, such as breeding characteristics (Gu et al. 1999) and the influence of size class, population density, and food availability during its larval development (Fu et al. 2003a, Fu et al. 2003b). Also, Ye (2012) studied the ecological factors of its microhabitat that had the largest impact on

winter habitat selection. Yang et al. (2016) were the first to study the population structure and genetic diversity of *H. amjiensis* and found no evidence of geographic partitioning between populations; Bayesian skyline plots also revealed no dramatic change in population size (Yang et al. 2016). However, this work was limited to mitochondrial DNA data, which had minimal intraspecific divergence and thus were not suitable for a more in-depth study of population demographics. It was only recently that Kan et al. (2021) used double-digest restriction-site associated DNA (ddRAD) sequencing to develop 33 single nucleotide polymorphism (SNP) markers, which are useful for genetic assessments at population level and hence the conservation of species (Kan, 2022, Kan et al. 2021), including the one dealt with here. However, these works were limited to sectional DNA data, which had minimal intraspecific divergence and thus were not suitable for a more in-depth study of population demographics either.

With the advent of next-generation sequencing, genome scanning of non-model organisms has become more feasible and less expensive. This approach can provide an overview of the whole genome of the study organism and build a foundation for subsequent genomic studies. Because the genome of *Hynobius amjiensis* has not been assessed to date, we performed a genome scan using k-mer analysis to generate a genomic reference for this critically endangered salamander. We compared its estimated genome size to those of other species of the orders Caudata and Anura. We also recovered more than one million microsatellite markers, which constitute powerful indicators that can be used for both historical and contemporary estimations of population demographics (Choi et al. 2021). Microsatellite analysis can reveal the evolutionary and ecological causes that have led to the current endangered status of *H. amjiensis* and these important insights may then help with its protection.

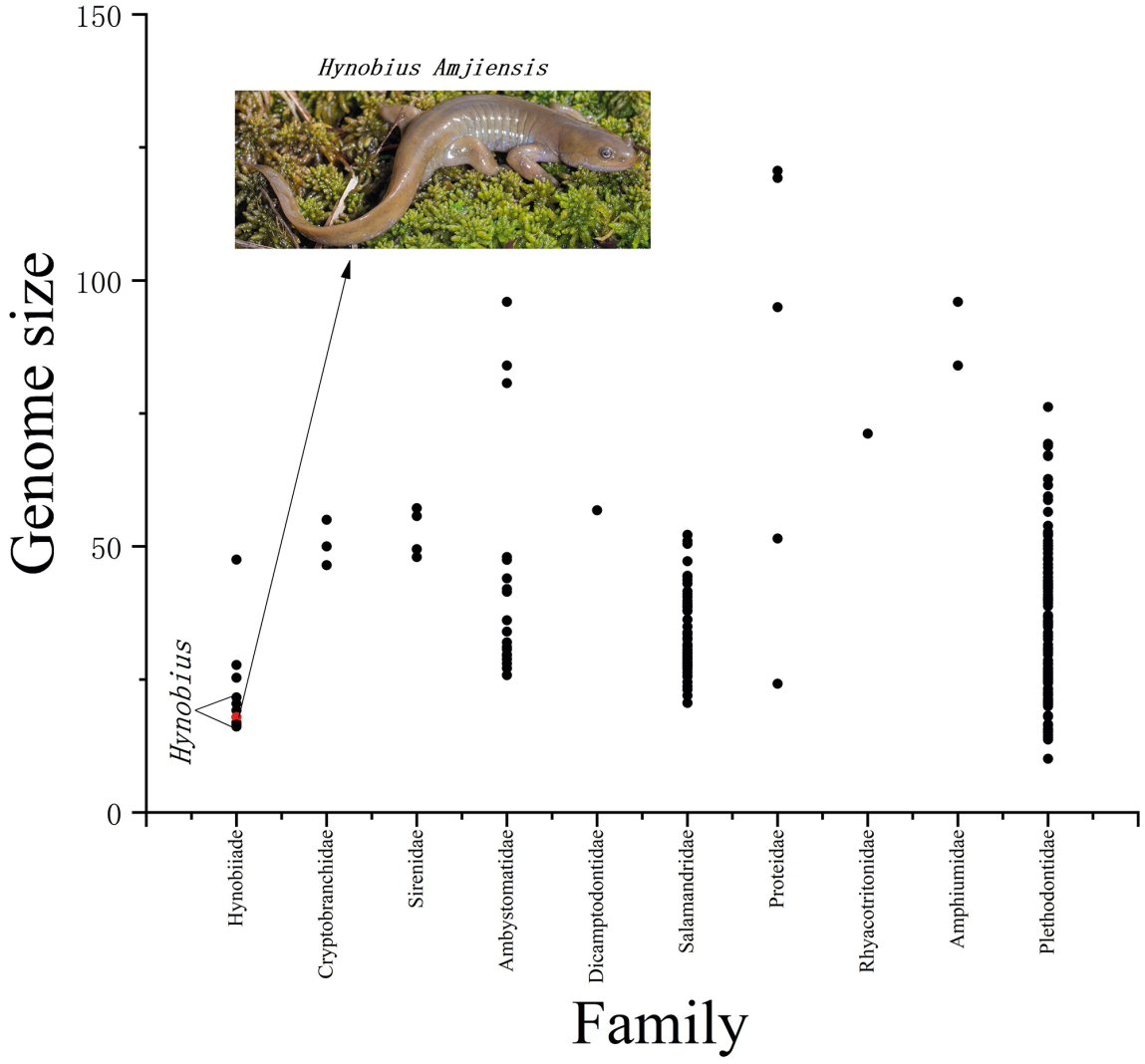

Figure 1. Genome size estimation (gigabases) using a k-mer approach in *Hynobius amjiensis*. Genome size estimates were retrieved from www.genomesize.com (last accessed on 8 April 2022). The inset at the top depicts a specimen of *H. amjiensis,* Photo: Cangsong Chen).

Materials and methods

Sample collection and DNA extraction

To avoid injuring breeding adults of *H. amjiensis*, several larvae were collected for obtaining tissue samples. They were caught in a swamp at Qianmutian on Longwang Mountain during the breeding season and stored in the freezer. We subsequently used 200 mg of tissue from one larva (AA0000810M1, vouchered at the Zhejiang Museum of Natural History) that was cut up into pieces and incubated with 1 ml lysis buffer containing 2 mg proteinase K at 56°C for 1–2 hours. DNA was extracted as per the phenol/chloroform/isopentanol (25:24:1) protocol by BGI Genomics, China, and eluted in 30 μl TE buffer. DNA quality was analyzed through electrophoresis on 1.5% of agarose (PowerPac, Bio-rad, US). DNA quantity was measured by an ultraviolet-visible spectrophotometer (NanoDrop 2000, Thermo Scientific, USA) to ensure samples contained at least 2 μg of genomic DNA.

Library preparation and sequencing

A DNA library was compiled according to the standard protocol of the PCR-free DNBSEQTM library (BGI Genomics, China). Briefly, 1 μg of genomic DNA was randomly sheared with the Covaris System (Woburn, Massachusetts, USA) and size-selected with the Agencourt AMPure XP-Medium (Beckman Coulter Inc., California, USA) kit to an average size of 300–400 bp. Selected fragments were end-repaired and 3'adenylated, then the adaptors were ligated to the ends of these 3'adenylated fragments. DNA fragments were then purified by Agencourt AMPure XP-Medium kit and heat-denatured into single strands and circularized by the splint oligo sequence. The single-stranded circular DNA (ssCir DNA) was formatted as the final library, forming DNA nano-balls (DNB) and undergoing rolling-circle amplification (RCA). The final qualified libraries were sequenced with BGISEQ-500. Finally, pair-end 100-bp readings were obtained by combined Probe-Anchor Synthesis (cPAS) at BGI Genomics, China.

K-mer analysis, genome assembly, and microsatellite analysis

Prior to the k-mer analysis, raw readings were filtered to remove sequencing adapters, contamination, and low-quality readings (e.g., ambiguous characters "N" and readings with more than 10% Q < 20). Filtering was conducted with SOAPnuke 1.5.6 (https://github.com/BGIflexlab/SOAPnuke), applying the following parameters: -n 0.01 -l 20 -q 0.1 -i -Q 2 -G -M 2 -A 0.5 -d. Filtered readings were k-mer analyzed using Jellyfish (Marçais & Kingsford 2011) and GenomeScope (Vurture et al. 2017). We then assembled the genome with MaSuRCA (Zimin et al. 2013) and used MISA v2.1 to identify microsatellite loci, which were called only when the motif was repeated more than ten times for a single base, more than six times for two bases, and more than five times for 3–6 bases.

Primer design and validation

To develop PCR primers for identified microsatellite loci, we first filtered out mono-nucleotide repeat motifs and any motifs that appeared fewer than 10 times in the assembly. We then discarded microsatellites near the end of the contigs and must have at least 150 bp in up- and downstream sequences, which ensured enough sequences for identifying primer locations. We furthermore filtered out microsatellites that were situated within 100 bp of each other, because these loci are essentially linked and can violate the linkage disequilibrium in subsequent analyses. Primers were developed as per the Primer3 tool (p3_in.pl, p3_out.pl, primer3-2.5.0) incorporated in MISA v2.1 (http://pgrc.ipk-gatersleben.de/misa/). Lastly, we used PANDAseq (Masella et al. 2012) to confirm that designed primer sequences were present in the assembly. From the final primer pairs, 24 pairs were experimentally validated with PCR prior to using them further. Each test was performed in triplicate. The volume of each PCR reaction system was 20 μl, comprising 1 μl genomic DNA, 10 μl 2 × Super PCR Mix Pro, 0.5 μl each of forward and reverse primers, and 8 μl ddH_2O. The PCR reaction program was set up under the following PCR conditions: 95°C for 4 min; 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s for 35 cycles; and 72°C for 7 min. The PCR products were separated using 4% agarose gel electrophoresis, and the BGI D2000 Plus DNA ladder (BGI, China) was used to estimate sizes.

Results and discussions

Sequencing data summary

DNA Nano Ball-sequencing (DNB-seq) generated 595.45 Gb of raw data for *Hynobius amjiensis*. After filtering, we obtained 2,372,006,372 clean readings for reading 1 and 1,386,500,244 clean readings for readings 2 that totalled 563.78 Gb of filtered data. The GC content of clean readings was estimated to be 46.84%, which is within the range of 30–50% that is widely accepted (Cheung et al. 2011, Zhou et al. 2013, Shangguan et al. 2013).

Genome assessment and k-mer analysis

We performed k-mer analysis by evaluating seven k-mer values from 19 to 31. Results show that k-mer = 19 produced the optimal estimate (Table 1, Fig. 2) (Marçais & Kingsford 2011), because it had the lowest error rate at a heterozygosity rate close to 1. The estimated genome size of *Hynobius amjiensis* was about 17.54 Gb (read depth = 32X), which is smaller than in other salamander species for which genome sequencing data is available. For example, *Ambystoma mexicanum* (Ambystomatidae) has a genome

Table 1. K-mer statistics.

K-mer	n k-mer	Used base	Genome size	Heter rate	Repeat rate	Err rate	Depth
19	486,539,139,441	562,097,417,035	17,543,380,785	1.01%	70.77%	0.3%	32.04
21	480,620,686,817	561,735,433,384	17,746,536,093	1.04%	63.87%	0.36%	31.65
23	474,361,759,556	561,656,713,342	17,804,958,084	1.02%	60.73%	0.38%	31.54
25	467,859,957,717	561,635,983,299	17,845,504,353	0.99%	58.35%	0.38%	31.47
27	461,183,308,916	561,627,746,531	17,873,834,662	0.96%	56.23%	0.38%	31.42
29	454,362,831,498	561,626,872,679	17,895,949,323	0.94%	54.3%	0.38%	31.38
31	447,426,522,039	561,629,855,054	17,911,969,466	0.91%	52.54%	0.38%	31.36

of about 32 Gb (Keinath et al. 2015), and *Pleurodeles waltl* (Salamandridae) one of about 20 Gb (Elewa et al. 2017). However, our estimate is in line with those for other species of the genus *Hynobius*, which likewise have relatively small genomes in terms of C-values, i.e., between 16.16 and 20.45 pg (Fig. 1) (Olmo 1973), equivalent to 15.8 to 20 Gb (assuming 1 pg = 978 Mb).

Amongst the ten recognized families in the order Caudata, C-values estimated by feulgen densitometry or flow cytometry (www.genomesize.com, last accessed on 8 April 2022) showed that the average genome size is 35.3 pg. However, there is considerable variation, ranging from 10.1 pg in *Gyrinophilus porphyriticus* (Plethodontidae) (Goin et al. 1968) to as much as 120.6 pg in *Necturus lewisi* (Proteidae) (Olmo 1973). Although the Hynobiidae is an old phylogenetic group, genome size seems not to correlate with its age. Indeed, this family generally has a smaller genome compared to the other nine families. The other basal

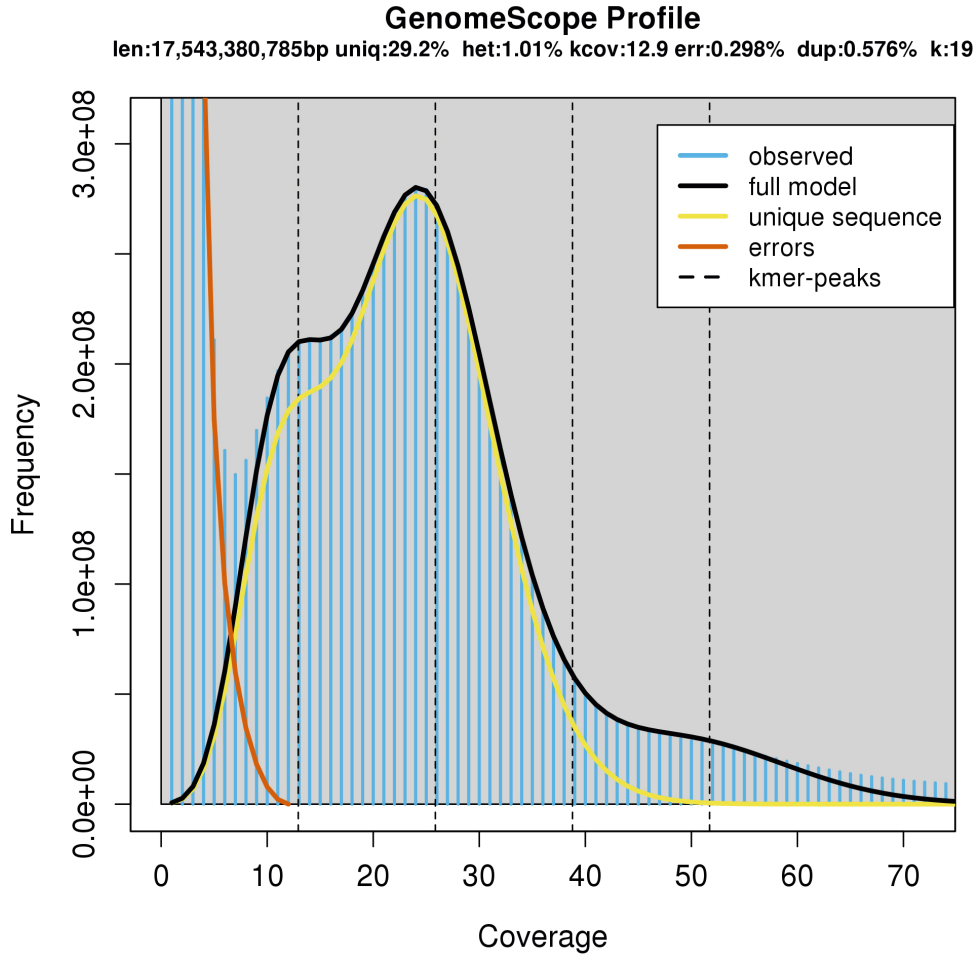

Figure 2. K-mer analysis (K = 19) of *Hynobius amjiensis*. The x-axis represents coverage, and the y-axis the frequency at each depth.

Table 2. Six repeat types of microsatellite statistics.

Repeat type	Number	Proportion (%)	Average distance (bp)
1	885011	61.41	488.52
2	281559	19.54	128.09
3	138721	9.63	256.27
4	113496	7.88	469.16
5	21431	1.49	358.73
6	827	0.06	200.97

lineage in the order Caudata, Cryptobranchidae, has much large genome sizes of around 50 pg (Gregory 2022). On the other hand, frogs and toads in the order Anura mostly possess small genomes of less than 10 pg, and many species have a genome size of as little as about 5 pg (Gregory 2022). It has been known that polyploidy can dramatically increase genome size in plants, but it remains unclear how salamanders acquired such large genomes. Having a large genome could benefit the organism by having extra genetic material for mutation, which increases the likelihood of adaptation through genetic selection.

Another notable feature of the *Hynobius amjiensis* genome is the high level (70.77%) of repetitive sequences. In contrast, *Ambystoma mexicanum* is estimated to have only about 40% of its genome composed of repetitive sequences (Keinath et al. 2015). After genome assembly, we obtained 8.85 million contigs, and the longest contig approached 62.15 kb. The N50 was 1052 bp, and L50 was 2190151 bp. The total length of assembled contigs was 7.7 Gb, representing 43.9% of the genome.

Microsatellite discovery and primer design

Among microsatellites with motif lengths between 1–6 bases, there were 1,441,045 loci identified in the assembled partial genome of *Hynobius amjiensis*, the total length of

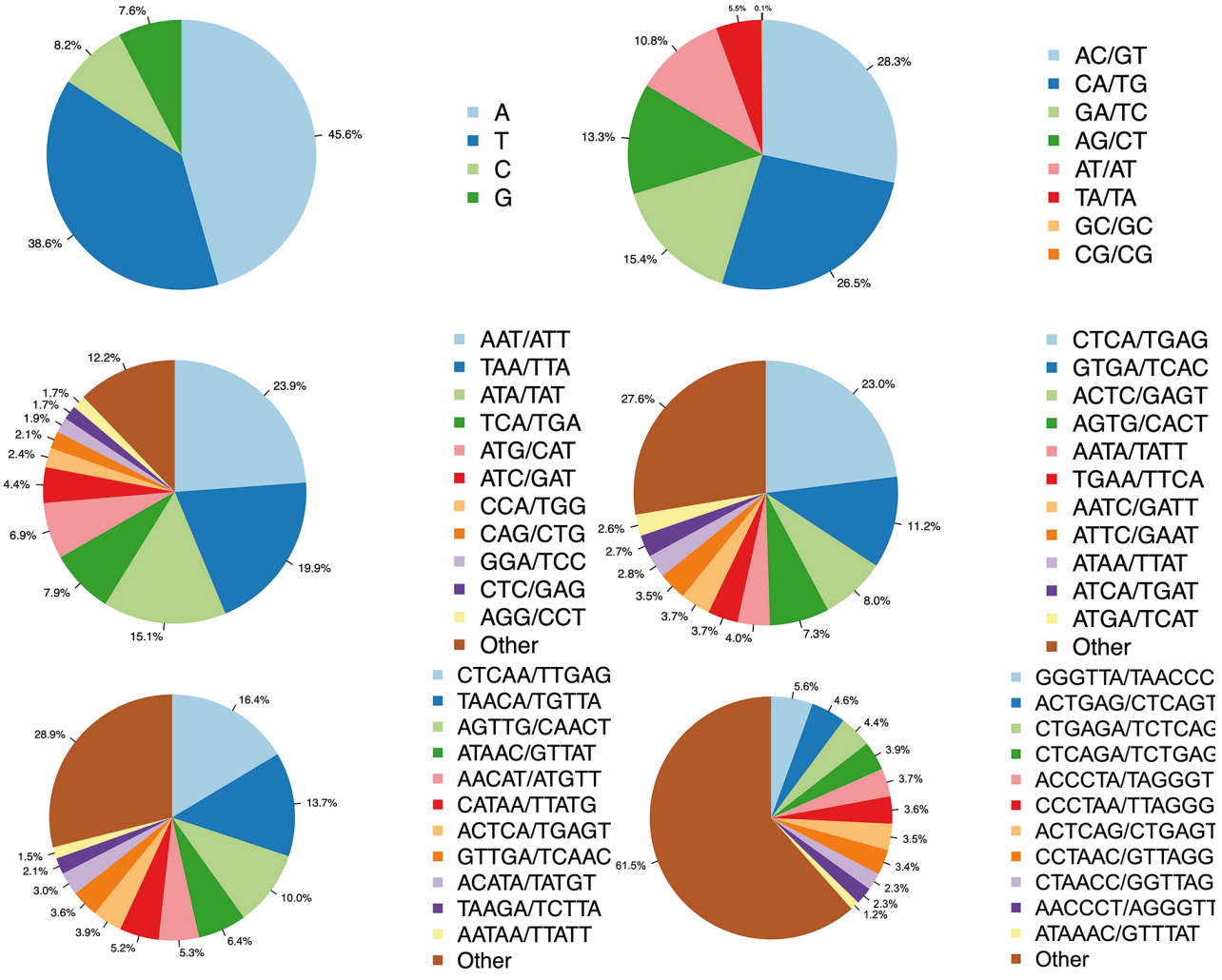

Figure 3. Frequencies of microsatellites in *Hynobius amjiensis*.

Table 3. Overview of bands: A–H represent plate rows, and 1–9 represent plate columns for each primer pair tested in this study. AJx means one primer pair, and -1, -2, 3 specify the repeat groups.

	1	2	3	4	5	6	7	8	9
A	AJ3-1	AJ6-3	AJ11-2	AJ23-1	AJ44-3	AJ47-2	AJ86-1	AJ88-3	AJ95-2
B	AJ3-2	AJ8-1	AJ11-3	AJ23-2	AJ45-1	AJ47-3	AJ86-2	AJ89-1	AJ95-3
C	AJ3-3	AJ8-2	AJ21-1	AJ23-3	AJ45-2	AJ48-1	AJ86-3	AJ89-2	AJ96-1
D	AJ5-1	AJ8-3	AJ21-2	AJ24-1	AJ45-3	AJ48-2	AJ87-1	AJ89-3	AJ96-2
E	AJ5-2	AJ10-1	AJ21-3	AJ24-2	AJ46-1	AJ48-3	AJ87-2	AJ94-1	AJ96-3
F	AJ5-3	AJ10-2	AJ22-1	AJ24-3	AJ46-2	AJ85-1	AJ87-3	AJ94-2	AJ97-1
G	AJ6-1	AJ10-3	AJ22-2	AJ44-1	AJ46-3	AJ85-2	AJ88-1	AJ94-3	AJ97-2
H	AJ6-2	AJ11-1	AJ22-3	AJ44-2	AJ47-1	AJ85-3	AJ88-2	AJ95-1	AJ97-3

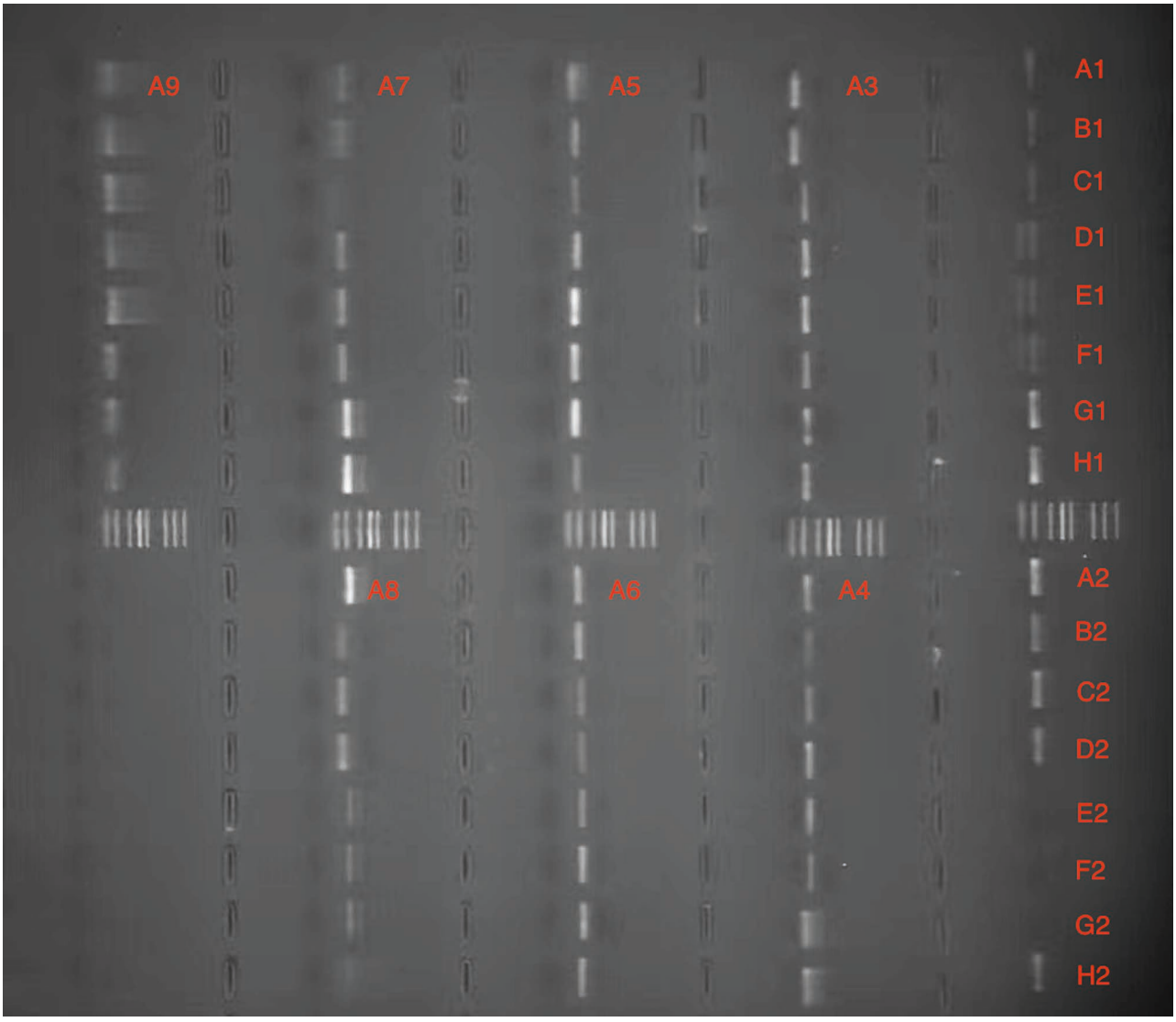

Figure 4. Bands showing PCR test results for the 24 primer pairs used in PCR amplifications for *Hynobius amjiensis* using 4% agarose gel electrophoresis. There are 72 bands, including three missing ones. The mean primer AJ10 (E2, F2, G2) proved useless.

which represented 0.12% of the whole genome. Genome assembly data that support the findings of this study have been deposited at the CNGB Sequence Archive (CNSA) (Guo et al. 2020) of the China National GeneBank DataBase (CNGBdb) (Chen et al. 2020) under accession number CNP0002813. In comparison, microsatellites make up a much greater percentage in some other animals, such as 3% in human (Subbaya et al. 2003), 2.85% in *Mus musculus* (Tong et al. 2006), 1.41% in *Rattus norvegicus* (Tu et al. 2015), and 0.77% in the Japanese puffer fish (*Takifugu rubripes*) (Cui et al. 2006). It is likely that the low prevalence of microsatellites in *H. amjiensis* is associated with low genetic diversity in this species. Amongst those microsatellites, mono-nucleotide motifs accounted for 61.4% (885,011) of identified loci, followed by 19.5% (281,559) dinucleotide repeat motifs, 9.6% (138,721) tri-nucleotide repeat motifs, and 7.9% (113,496) tetra-nucleotide repeat motifs (Table 2). Penta- and hexa-nucleotide motif repeats occurred at much lower frequencies. We identified 4, 12, 60, 212, 514, and 368 types of microsatellites for mono-, di-, tri-, tetra-, penta-, and hexa-nucleotide motif repeats, respectively. The reason for fewer types of hexa- than penta-nucleotide microsatellites is likely due to the lower frequency of, and an increased mutation rate associated with, longer motifs (Katti et al. 2001). Microsatellite loci with the highest frequencies are illustrated in Figure 3. Interestingly, the majority of microsatellites are heavily biased towards nucleotides A and T. After excluding mono-nucleotide repeats, the most frequent repeat motif was the AC/GT one. The average number of repeats per microsatellite loci, relative frequency (RF), and relative abundance (RA) were summarized for loci that had at least 700 copies in the assembly (Supplementary Table S1). Lastly, after applying the filtering criteria, we obtained 98 microsatellite markers with repeat motifs between 2–6 bases, and primer pairs were designed for those markers (Supplementary Table S2). Those included 8, 35, 32, 15, and 8 primer pairs for di-, tri-, tetra-, penta-, and hexa-nucleotide motif repeats, respectively. Our newly designed primers can be used to assess the population structure and evolutionary history of *H. amjiensis*, but it is likely that they may also work for other species of *Hynobius*. From these primer sets, we randomly selected 24 primer pairs for PCR amplification with *H. amjiensis*, 23 of which produced one clear band (Fig. 4, Table 3); this will help us to clone microsatellite loci genes for future studies, for instance, for parentage tests.

Conclusions

For the first time, we assessed the genomic characteristics of *Hynobius amjiensis*, a critically endangered salamander endemic to a small area in eastern China. Its genome size was estimated to be 17.54 Gb with 70.77% as repetitive sequences. We further assembled 7.7 Gb of its genome sequences, which can serve as a foundation for future genomic studies to understand the small population size as well as how to conserve this evolutionary lineage that is facing extinction. A total of 98 pairs of microsatellite primers were designed. Our work thus provides very important guidelines and methods to assess the population structure and demographic history of *H. amjiensis* and possibly other species of this genus.

Acknowledgements

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References

Choi, E., S. H. Kim, S. J. Lee, E. Jo, J. Kim, J. H. Kim, S. J. Parker, Y. M. Chi & H. Park (2021): A first genome survey and genomic SSR marker analysis of *Trematomus loennbergii* Regan, 1913. – Animals. **11**(11): 3186.

Cheung, M. S., T. A. Down, I. Latorre & J. Ahringer (2011): Systematic bias in high-throughput sequencing data and its correction by BEADS. – Nucleic Acids Research, **39**: e103.

Cui, J. Z., X. Y. Shen, G. P. Yang, Q. L. Gong & Q. Q. Gu (2006): The analysis of simple sequence repeats in *Takifugu rubripes* Genome. – Periodical of Ocean University of China, **36**(2): 249–254.

Chen, C. S., Y. Jia, Y. K. Wu, Z. Y. Fan, W. W. Lu, S. H. Chen & L. P. Yu (2016): The breeding ecology of a Critically Endangered Salamander, *Hynobius amjiensis* (Caudata: Hynobiidae), endemic to Eastern China. – Asian Herpetological Research, **7**: 53–58.

Chen, F. Z., L. J. You, F. Yang et al. (2020): CNGBdb: China National GeneBank DataBase. – Hereditas, **42**(08): 799–809.

Elewa, A., H. Wang, C. Talavera-López, A. Joven, G. Brito et al. (2017): Reading and editing the *Pleurodeles waltl* genome reveals novel features of tetrapod regeneration. – Nature Communications, **8**: 2286.

Fu, C. Z., R. Rao, J. H. Wu, J. K. Chen & G. C. Lei (2003): Effects of density and food availability on growth and cannibalism in basin-raising larval salamanders (*Hynobius amjiensis*). – Zoological Research, **24**(3): 186–190.

Fu, C. Z., X. F. Sun, X. Y. Wang, J. K. Chen & J. H. Wu (2003): Effects of size class and group size structure on cannibalism in basin-raising larval salamanders, *Hynobius amjiensis*. – Zoological Research, **24**(6): 407–412.

Gu, H. Q. (1992): A new species of *Hynobius* – *Hynobius amjiensis*. – Animal Science Research. Vol. Celebrate Prof. M. Chang: 39–43.

Gu, H. Q., X. M. Ma, Y. Wang, Z. H. Du & X. Q. Lou (1999): Research on population number and dynamics of *Hynobius amjiensis*. – Sichuan Journal of Zoology, **18**(3): 104–106.

Gregory, T. R. (2022): Animal Genome Size Database. – www.genomesize.com, accessed on 8 April 2022.

Guo, X. Q., F. Z. Chen, F. Gao et al. (2020): CNSA: a data repository for archiving omics data. – Database (Oxford), 2020: baaa055.

Goin, O. B., C. J. Goin & K. Bachmann (1968): DNA and amphibian life history. – Copeia, 1968: 532–540.

Jiang, J. P. & F. Xie (2015): China's red list of biodiversity: Vertebrates, 1st ed. – Science Press, China, Beijing.

Kan, X. (2022): The conservation genomics analysis of the critically endangered *Hynobius amjiensis*. – M. D., Zhejiang Normal University, Jinhua, China.

Kan, X., G. L. Wang, L. L. Zhang & X. Zhao (2021): Development and characterization of 33 SNP markers in a critically endangered *Hynobius amjiensis* using ddRAD sequencing. – Conservation Genetics Resources, **13**: 425–428

Keinath, M. C., V. A. Timoshevskiy, N. Y. Timoshevskaya, P. A. Tsonis, S. R. Voss et al. (2015): Initial characterization of the large genome of the salamander *Ambystoma mexicanum* using shotgun and laser capture chromosome sequencing. – Scientific Reports, **5**: 16413.

Katti, M. V., P. K. Ranjekar & V. S. Gupta (2001): Differential distribution of simple sequence repeats in eukaryotic genome sequences. – Molecular Biology and Evolution, **18**: 1161–1167

Marçais, G. & C. A. Kingsford (2011): Fast, lock-free approach for efficient parallel counting of occurrences of k-mers. – Bioinformatics, **27**: 764–770.

Masella, A. P., A. K. Bartram, J. M. Truszkowski, D. G. Brown & J. D. Neufeld (2012): PANDAseq: Paired-end assembler for Illumina sequences. – BMC Bioinformatics, **13**: 31.

Olmo, E. (1973): Quantitative variations in the nuclear DNA and phylogenesis of the amphibia. – Caryologia, **26**: 43–68.

Shangguan L., J. Han, E. Kayesh, X. Sun, C. Zhang, T. Pervaiz, X. Wen & J. Fang (2013): Evaluation of genome sequencing quality in selected plant species using expressed sequence tags. – PLoS ONE, **8**: e69890.

Subramanian, S., V. M. Madgula & R. George et al. (2003): Triplet repeats in human genome: distribution and their association with genes and other genomic regions. – Bioinformatics, **19**(5): 549–552.

Tong, X. L., F. Y. Dai, B. Li, Q. Y. Yu, Q. Y. Xia & C. Lu (2006): Microsatellite repeats in mouse: abundance, distribution, and density. – Dongwu Xuebao (Acta Zoologica Sinica), **52**(1): 138–152.

Tu, F. Y., X. H. Liu, L. M. Du, C. C. Yan & X. F. Huang (2015): Distribution characteristics of microsatellites in the rat genome. – Jiangxi Nongye Daxue Xuebao (Acta Agriculturae Universitatis Jiangxiensis), **37**(4): 708–711.

Vurture, G. W., F. J. Sedlazeck, M. Nattestad, C. J. Underwood, H. Fang, J. Gurtowski & M. C. Schatz (2017): GenomeScope: Fast reference-free genome profiling from short reads. – Bioinformatics, **33**: 2202–2204.

Ye, L. J. (2012): Research of the survival status and habitat characteristics of Anji salamander during the breeding season. – M. D. Zhejiang Normal University, Jinhua, China.

Yang J., S. H. Chen, S. H. Chen, P. Ding, Z. Y. Fan, Y. W. Lu, L. P. Yu & H. D. Lin (2016): Population genetic structure of critically endangered salamander (*Hynobius amjiensis*) in China: Recommendations for conservation. – Genetics and Molecular Research, **15**(2): gmr.15027733.

Zimin, A. V., G. Marçais, D. Puiu, M. Roberts, S. L. Salzberg & J. A. Yorke (2013): The MaSuRCA genome assembler. – Bioinformatics, **29**: 2669–2677.

Zhou, W., Y. Hu, Z. Sui, F. Fu, J. Wang, L. Chang, W. Guo & B. Li (2013): Genome survey sequencing and genetic background characterization of *Gracilariopsis lemaneiformis* (Rhodophyta) based on next-generation sequencing. – PLoS ONE, **8**: e69909.

Supplementary data

The following data are available online:

Supplementary Table S1. Characteristic statistics of microsatellite that had at least 700 copies.

Supplementary Table S2. PCR primers for a selection of microsatellites in *Hynobius amjiensis*.