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ORIGINAL ARTICLE

Population Size, Genetic Diversity and Molecular Evidence of a Recent Population Bottleneck in *Hynobius chinensis*, an Endangered Salamander Species

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Abstract Severe population declines can reduce species to small populations, offering permissive conditions for deleterious processes. For example, following such events, species can become prone to inbreeding and genetic drift which can lead to a loss of genetic diversity and evolutionary potentials. *Hynobius chinensis* is a poorly studied very rare and declining endangered amphibian species endemic to China in Changyang County. We investigated adult census population size by monitoring breeding populations from 2015 to 2018, developed microsatellite markers from the transcriptome and used them to investigate genetic diversity, and a population bottleneck in this species. We found *H. chinensis* in 4 different localities in a total area of 2.18 km^2 and estimated the overall adult census population size at 386–404 individuals. The adult census size (mean ± SE) per breeding pond ranged from 44 ± 6 to 141 ± 8 individuals and appeared smaller than that reported in closely related species in undisturbed habitats. We developed and characterized 13 microsatellite markers in total. Analysis of data at 7 loci (N = 118) in Hardy-Weinberg equilibrium gathered from the largest population showed that genetic diversity level was low. The average number of alleles per locus was 2.14. The observed and expected heterozygosities averaged 0.38 and 0.40, respectively. The inbreeding coefficient was –0.06. All tests performed to investigate a population bottleneck, i.e. The Garza-Williamson test, Heterozygosity excess test, Mode shift test of allele frequency, and effective population size estimates detected a population bottleneck. The contemporary and the historical effective population sizes were estimated at 36 and 234 individuals, respectively. We argue that as bottleneck effects, the studied population may have become prone to genetic drift and inbreeding, losing microsatellite alleles and heterozygosity. Our results suggest that populations of *H. chinensis* may have been extirpated in the study area.

Keywords transcriptome, microsatellites, adult census population size, effective population size, genetic drift, inbreeding

1. Introduction

Elucidating factors and processes affecting genetic diversity in species of conservation concern can help understand the ecology and evolution of such species. It can also help formulate appropriate conservation strategies (Amos and Balmford, 2001; Frankham, 2005).

Theoretical and empirical works show that severe population declines or population bottlenecks can affect genetic diversity in several ways (Amos and Balmford, 2001; Frankham, 2005; Hoffmann *et al*., 2017; Willi and Hoffmann, 2009). For examples, by reducing species to small populations, population bottlenecks can reduce the standing genetic diversity and impair the ability

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to fix novel adaptive genetic mutations (Frankham, 2005; Hoffmann *et al*., 2017). Still, these demographic events can offer permissive conditions for inbreeding and random drift, which can cause a continuous loss of genetic diversity and of individual fitness or inbreeding depression (Amos and Balmford; Frankham, 2005; Heredia-Bobadilla *et al*., 2017; Jordan *et al*., 2009; Spear *et al*., 2006; Storfer *et al*., 2014; Willi and Hoffmann, 2009). Due to these effects, affected species can lose their ability to respond to novel selection pressures and adapt novel environmental conditions (Frankham, 2005; Hoffmann *et al*., 2017; Willi and Hoffmann, 2009). Population bottlenecks can also render species vulnerable to extinction due to stochastic and catastrophic events (Amos and Balmford, 2001; Frankham, 2005; Willi and Hoffmann, 2009).

Severe population bottlenecks cause the values of different population genetics parameters, including heterozygosity and allele frequency at selectively neutral loci such as microsatellites to differ from those expected at Hardy-Weinberg equilibrium across several generations (Garza and Williamson, 2001; Luikart *et al*., 1998; Peery *et al*., 2012). Different tests based on the analysis of multilocus microsatellite data within populations allow detecting severe population bottlenecks that occurred in the recent history of species by detecting violation of theoretical expectations under this equilibrium (Garza and Williamson, 2001; Luikart *et al*., 1998; Peery *et al*., 2012). These include the Mode shift test, the Garza-Williamson test (the updated version of the Garza *M*-ratio test), and tests of Heterozygosity excess (Garza and Williamson, 2001; Luikart *et al*., 1998; Peery *et al*., 2012).

Potential factors causing population bottlenecks and loss of genetic diversity may vary between organisms (Rivera-Ortíz *et al*., 2015). For amphibians, anthropogenic destruction and degradation of natural habitats is probably among the most important (Allentoft and O'Brien, 2010; Rivera-Ortíz *et al*., 2015). Studies that analyze microsatellite data in amphibian species whose natural habitats have been considerably reduced and degraded by human activities show that such species have undergone fragmentation in gene pools. They have reduced census and effective sizes and exhibit molecular signs of recent population bottlenecks, inbreeding and loss of microsatellite alleles and heterozygosity (Allentoft and O'Brien, 2010; Heredia-Bobadilla *et al*., 2017; McCartney-Melstad and Shaffer, 2015; Rivera-Ortíz *et al*., 2015; Storfer *et al*., 2014; Sugawara *et al*., 2015).

The Chinese salamander, *Hynobius chinensis* (Caudata, Hynobiidae) is an amphibian species endemic to China (Fei and Ye, 2016; Frost 2018). Classified as Endangered by the IUCN Red List (IUCN, 2018), literature shows that *H. chinensis* is threatened by anthropogenic habitat degradation and destruction, causing habitat loss for populations (Fei and Ye, 2016). As effects of these threats, this species has undergone population declines and is very rare (Fei and Ye, 2016; IUCN, 2018). It is restricted to mountain forests in Changyang County in Yichang (Hubei province) -Note that Changyang County covers 3 412 km^2 (Source: Wikipedia)- at altitudes ranging from 1 400 to 1 500 m (Fei and Ye, 2016; Frost, 2018).

H. chinensis is a poorly known and studied species. Data on many aspects of Biology, including landscape use, genetic diversity, current population size, and demographic history are scarce. *H. chinensis* has been mostly involved in studies addressing systematic and taxonomic issues (Fei and Ye, 2016; Frost, 2018; Wang *et al*., 2007).

The nature of threats facing *H. chinensis*, the level of rarity, and the declining population trend reported in the literature let suspecting that this species might have experienced severe population bottlenecks and lost genetic diversity in the recent past (Apodaca *et al*., 2012; Heredia-Bobadilla *et al*., 2017; Sugawara *et al*., 2016; Sugawara *et al*., 2015; Storfer *et al*., 2014; Wang *et al*., 2017). If the history of *H. chinensis* conforms to this hypothesized trajectory, we should expect to see representative populations of this species exhibiting molecular signs of population bottlenecks detectable by microsatellite markers, low levels of genetic diversity and reduced sizes (Apodaca *et al*., 2012; Heredia-Bobadilla *et al*., 2017; Storfer *et al*., 2014; Sugawara *et al*., 2016; Sugawara *et al*., 2015; Wang *et al*., 2017).

We investigated (i) adult census population size, developed microsatellite markers and used them to investigate (ii) genetic diversity, and (iii) molecular signatures of a population bottleneck in *H. chinensis*. Our aim was to contribute to our understanding of the demography and genetic viability of this endangered species.

2. Materials and Methods

2.1. Study area and field procedure Published literature shows that *H. chinensis* has one breeding period per year which begins in November (Fei and Ye, 2016). During the breeding period, adult individuals aggregate, mate and reproduce in ponds (Fei and Ye, 2016). An individual female lays eggs in a pair of sacs, with an egg sac's length

measuring tens of cm (Fei and Ye, 2016).

From November 2015 to April 2016, we searched for *H. chinensis* individuals and egg sacs in ponds at altitudes ranging from 1300 to 1600 m in Changyang (Fei and Ye, 2016; Frost, 2018). Egg sacs were considered as a precursory sign of the presence of *H. chinensis* as this species is the only Hynobiid salamander distributed in Changyang (Fei and Ye, 2016). We found breeding populations of *H. chinensis* in at different sites of different localities.

Data from *H. nebulosus* showed that the number of adult male individuals could be accurately determined by examining breeding aggregations because a male individual would spend several weeks in a breeding pond (Kusano, 1980). We examined a breeding population of *H. chinensis* at a breeding pond found in one locality: Dongtoutang. The pond was small and shallow (Table 1). And, the pond's water was enough clear to observe objects present in the pond. Based on our previous field experience, and on information provided by local people, we were expecting to observe the largest breeding population at this pond. At the landscape scale, the area was at 50% covered by a degraded natural deciduous forest of *Quercus* sp. (Fagaceae). The pond's surrounding area was covered by various herbs (mainly Poaceae, Oxalidaceae, and Polygonaceae). The minimum distance to a forest patch was 25 m.

H. chinensis individuals were carefully collected from the pond, using a net (Heyer *et al*., 1994). The salamanders were sexed based on the morphology of the cloacae (Fei and Ye, 2016), marked by toe-clipping and released (Heyer *et al*., 1994). The number of captured individuals was recorded for each sex. After a time period ranging from of 1 to 4 weeks, the population was re-examined. For each sex, the number of "captured", i.e. non-toe-clipped, and the number of "recaptured" i.e. toe-clipped individuals were recorded (Heyer *et al*., 1994). Pairs of egg sacs were progressively individually tagged as they were spawned and their total number was determined at the end of the breeding season (Chen *et al*., 2016; Kusano, 1980). The investigation was finished on March 25, 2016. There was no additional novel pair of egg sacs since March 2; and no unmarked male individual since March 8, 2016. Then, the number of adult females, *F* and the number of adult males, *M* were estimated as the number of pairs of egg sacs (Chen *et al*., 2016; Kusano and Miyashita, 1984; Kusano, 1980) and toe-clipped male individuals, respectively (Heyer *et al*., 1994). The sex ratio, *SR* was estimated as $SR=M/F$ and the adult census population size, N_c as $N_c = M+F$.

To minimize the impact on the populations (McCartney-Melstad and Shaffer, 2015), as the value of *SR* was close to that reported in closely related species (Kusano, 2012), for the breeding populations found in other localities and for the remaining study period, the values of N_c were indirectly estimated. In summary, for each population, the number of adult females, F_n was estimated based on egg clutch number as previously explained. And, N_{cn} was estimated as: $N_{cn} = F_n + (F_n*SR)$, where *n* represents a given population. For all the localities, the values of N_c was determined for 2015–2016; 2016–2017 and 2017–2018 breeding seasons, at the same ponds and following this method.

From 22 different egg clutches, 22 *H. chinensis* hatched larvae samples were randomly collected. All these larvae and the toe clips were kept in anhydrous (98%) ethanol at –20 °C in the laboratory for DNA-based experiments.

During the fieldwork, to identify threats facing *H. chinensis* in the study area, local people were interviewed for a better understanding of the evolution of landscapes and of the usage of the species by humans. The species' mating behavior was occasionally observed at the breeding pond found in Dongtoutang.

2.2. Development of microsatellite markers An adult *H. chinensis* individual was brought to the laboratory, euthanized by injection of MS-22 (3g/l), and dissected. A tissue sample was taken from the liver. Total RNA was extracted (Omega Bio-Tek, USA) with TRIzol Regent® (Invitrogen, CA, USA). The purity, concentration and integrity of RNA were assessed by Nanodrop, Qubit 2.0 and Agilent 2100 bioanalyzer, respectively. Using 1μg of total RNA as input, a library for Illumina sequencing was prepared following the NEBNext Ultra™ RNA protocol. The library preparation was sequenced on an Illumina HiSeq 2500 platform (pair-end reads, 125 bp).

Sequencing results were analyzed and reads containing adaptors, reads containing poly-N, and low-quality reads (i.e. with more than 50% $Q \leqslant 10$ bases) were filtered out. And, the clean reads (i.e. the remaining reads) were *De novo* assembled, using Trinity v2.0.6 software (Grabherr *et al*., 2011).

The Simple Sequence Repeat Identification Tool available at http://archive.gramene.org/db/markers/ssrtool/ was used to identify microsatellite loci in the assembled *H. chinensis*' unigene DNA sequences. The following searching criteria were applied: (i) the minimum number of repeats: 5 times; (ii) the repeat type: tetranucleotide; and (iii) flanking sequence length: $\geqslant 18$ bp. Using the software Primer v3.0, 74 microsatellite

loci were screened and 119 primer pairs were designed, applying the following criteria: (1) primer length 18–23 bp, with 20 bp as optimum; (2) product size (bp): 100–250 bp; (3) primer melting temperature: 55–69 ℃ , with 60 ℃ as optimum and (4) GC% content: 40–70%, with 50% as optimum, and (5) GC clump: 2.

Genomic DNA was extracted from all the toe tissue and larvae samples, using TIANamp Genomic Kit (TIANGEN). The 119 primer pairs were synthesized (Applied Biosystems, Beijing, China) and tested by gradient PCR on the standard agarose gel. Individual PCR amplification was performed as following: an initial denaturation at 94 °C for 5 min was followed by 35 cycles of denaturation at 94 °C and at a primer's specific annealing temperature for 30 sec, and an extension at 72 °C for 45 sec and finally at 72 °C for 8 min. PCR was performed in a reaction volume of 10 µl containing 0.6 µl of template DNA, 5 µl of Taq DNA polymerase (Premix), of 0.3 µl of forward primer, 0.3 µl of reverse primer, and 3.8 µl of ddH_2O. In total, 51 primer pairs consistently amplified at optimal annealing temperature and produced clear bands in the expected size range. These primer pairs were retained for polymorphism test.

The 5' end of each forward primer was labeled with one of the fluorescent dyes *FAM*, *TAMRA* or *HEX*, and polymorphism was tested, using data from the 22 larvae samples. In summary, PCR amplification of the 51 primer pairs was performed, following the protocol above described. For each individual larva, genotyping at the 51 microsatellite loci was performed with an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Beijing, China). Microsatellite alleles were scored, using GeneScan ROX 400 size standard and GeneMapper v4.0 software (Applied Biosystems, Beijing, China). The existence of genotyping errors (i.e. stuttering, large allele dropout) and of null alleles within the microsatellite data ($N = 22$) was checked, using the software Micro-Checker v2.2.3 (Van Oosterhout *et al*., 2004).

An exact test for Hardy-Weinberg equilibrium (HWE) for each locus and a test for potential linkage disequilibrium (LD) between all pairs of loci were implemented in the online Genepop software (Raymond and Rousset, 1995), using Bonferroni-corrected *P*-values (Rice, 1989). Exact *P*-values were determined by Markov Chain method, using the following settings: dememorization: 10 000; batches: 100; iteration per batch: 1 000 (Raymond and Rousset, 1995). The values of basic genetic diversity indices, exclusion probabilities, and polymorphism information content (*PIC*) were determined for each locus and for overall loci, using Cervus v3.0 software (Kalinowski *et al.*, 2007). Genotypes data were analyzed and the sampled *H. chinensis* adult individuals were genotyped at polymorphic loci in HWE, without LD, null alleles and/or genotyping errors (Table 2), following the procedure above described.

2.3. Analysis of genetic diversity and bottleneck effects

For the adult samples' genotype data, the observed heterozygosity (H_o), expected heterozygosity (H_e), and the number of alleles (A) were calculated for each locus and for the overall loci, using Arlequin v3.5 software (Excoffier and Lischer, 2010). The inbreeding coefficient, F_{is} was calculated for each locus and for the overall loci, using FSTAT v2.9.3 software (Goudet, 2001).

Different approaches were used to investigate genetic signatures of a population bottleneck. First, the signature of a population bottleneck was tested by Garza-Williamson test. In summary, Garza-Williamson index, *GW* was estimated for each locus as $GW=k/(r+1)$, where k is the number of alleles, and r the allele size range (Excoffier and Lischer, 2010). And, the overall *GW* value was compared to the theoretical critical value for rejecting the null hypothesis of demographic stability that is 0.68 (Garza and Williamson, 2001; Peery *et al*., 2012). The value of *GW* is expected to be below this critical value in bottlenecked populations. The core idea behind Garza-Williamson test is that during population bottleneck, reduction in k is proportionally important than the reduction in r. Hence, a mall value of *GW* can be considered a genetic signature of a population bottleneck (Excoffier and Lischer, 2010; Garza and Williamson, 2002). The Garza-Williamson statistics were computed in the software Arlequin v3.5 (Excoffier and Lischer, 2010).

Second, the signature of a population bottleneck was investigated based on heterozygosity excess (Cornuet and Luikartt, 1996), using Bottleneck v1.2.02 software (Luikart and Cornuet, 1999). In Bottleneck v1.2.02, three different mutation models, namely the infinite allele model (IAM), the stepwise mutational model (SMM), and the two-phase mutational model (TPM); and three different statistical tests namely the Sign test, the Standardized difference test and the Wilcoxon signed-rank test are available for heterozygosity excess testing (Luikart and Cornuet, 1999). Considering the number of used loci, heterozygosity excess was tested by the Wilcoxon signed-rank test, under all mutation models. The following settings were applied: TPM with SMM accounting for 95% of mutations, 5% SMM, a variance among multiple steps of 12 000 and 10 000 iterations (Luikart and Cornuet, 1999).

Third, genetic signature of population bottleneck

was investigated by Mode-shift test (Luikart *et al*., 1998), using the software Bottleneck v1.2.02 (Luikart and Cornuet, 1999). A putatively stable population is expected to have a peak of allele number at the lowest frequency class, resulting in an L-shaped distribution of allele frequency. Population bottleneck displaces the peak at other frequency classes, causing distortion of this graphical pattern or a shifted mode of allele frequency distribution (Luikart *et al*., 1998).

Finally, the trajectory of the effective population size on long-term was investigated by comparing the historical and the contemporary values. Assuming mutation-drift equilibrium, the historical effective population size N_e was estimated based on heterozygosity data as $N_e = (1/[1-H_e]^2-1)/8\mu$, where H_e is the average expected heterozygosity and μ the mutation rate per generation (Nei, 1987). For μ, 10^{-3}, the value reported in other amphibians was used (Peery *et al*., 2012). The contemporary N_e was estimated based on LD, using the single-sample method as implemented in NeEstimator v2.0 software (Do *et al*., 2014). A potential bias from rare alleles was controlled and a *pcritic* value of 0.01 was used (Do *et al*., 2014). LD estimates are based on the expectation that as N_e decreases, genetic drift systematically associates alleles at different loci. Hence, in bottlenecked populations, the level of LD reflects N_e (Do *et al.*, 2014).

2.4. Ethic statement Access to the focal species and the fieldwork did not require any formal research permit. The animal manipulation and sampling protocols were previously approved by the Animal Welfare Committee of the Central China Normal University.

3. Results

3.1. Adult census population size We found *H. chinensis* at 4 sites of different localities (Figure 1; Table 1), in a total area of 2.18 km^2. These sites were located north-east of Yichang city (Figure 1). The overall adult census population size in the area slightly varied between breeding seasons. It ranged from 386 to 404 individuals (Table 1). Reproduction was completed in permanent ponds of varying sizes (Table 1). These ponds occurred at altitudes ranging from 1 408 to 1 582 m (Table 1) and were distant from 1.01 to 3.2 km.

In Dongtoutang during the 2015–2016 breeding season, we identified 62 *H. chinensis* adult females and 78 adult males, resulting in an N_c of 140 individuals and a sex ratio of 1.26:1. We sampled 118 adult individuals in total, including 40 females and the 78 males. *H. chinensis* individuals appeared in the breeding pond found in this locality on November 15. The number of male individuals found in the pond increased from the first (November 26, 2015) to the third (January 4, 2016) census, reaching a peak of 60 individuals (Figure 2). The peak lasted till the sixth (February 20, 2016) census with minor fluctuation (Figure 2). The number of male individuals found in the pond then decreased till the last (March 8, 2016) census (Figure 2).

As expected, we obtained a high recapture rate of male individuals (Figure 2). Out of the 78 male individuals identified in total, 67 (85.90%) were already identified and toe clipped on January 4, 2016, i.e. with the third census (Figure 2). Since the fourth census on January 13, 2016, 97.30–100% of all male individuals found in the breeding pond were individuals that had already been identified and toe clipped (Figure 2). As expected, together, this high recapture rate and the evolution pattern of the number of male individuals found in the pond along the breeding season suggest that male individuals spent several days in the breeding pond. They confirmed the accuracy of the method used to estimate the number of adult males and N_c.

The average value (± SE) of N_c over the study period (i.e. 2015–2018) varied between localities or breeding ponds. It ranged from 44 ± 6 to 141 ± 8 individuals (Table 1). And, as expected, the maximum value of N_c was observed in Dongtoutang (Table 1). The sex ratio was 1.26: 1.

3.2. Microsatellite markers We obtained 27 721 722 (91.33%) clean reads in total. The Trinity assembly resulted in 77 111 unigenes. The size of the unigenes ranged from 201 to more than 3 000 bp (Figure 3). The total length of the unigenes and N50 were 46 797 297 bp and 951 bp, respectively. Among the unigenes, 46 378 (61.14%) were not more than 400 bp in length. A total of 20 599 (26.71%) unigenes were in the range of 401–1000 bp. Unigenes longer than 3 000 bp were 1 507 (2.04%) in total (Figure 3).

The transcriptomic sequencing raw data have been deposited into the NCBI Sequence Read Archive (SRA) database under the accession number SRP150396. They are accessible from https://www.ncbi.nlm.nih.gov/sra/SRP150396.

Microsatellite loci were identified in 3 516 unigenes. Among these unigenes, 2 999 (85.10%) contained 1 or 2 microsatellite loci. Only 524 (14.90%) contained over 2 microsatellite loci. We identified 4 297 microsatellite loci in total. The loci had repeat motifs varying from di- to tetra-. Among them, 3 254 (75.73%) loci had dinucleotide repeat motifs. Trinucleotide repeat motifs were observed

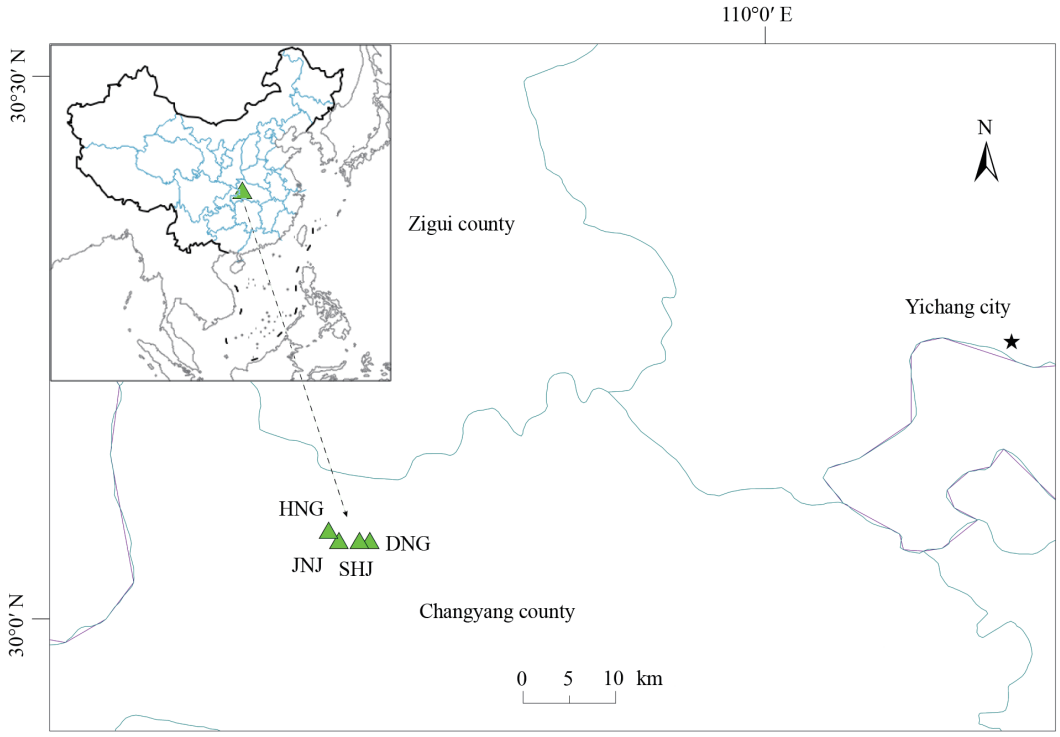

Figure 1 Locations of study sites of *Hynobius chinensis* in Changyang county. Sites are distinguished by the names of the corresponding localities. DNG: Dongtoutang; SHJ: Shuijingwan; JNJ: Jiangjunao; HNG: Hengtun.

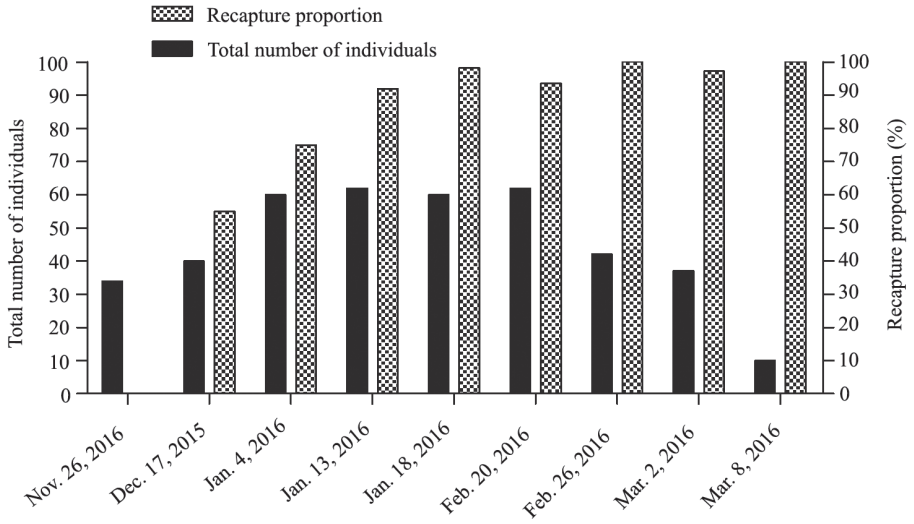

Figure 2 Number of *Hynobius chinensis* male individuals present in a breeding pond at different dates in 2015–2016 in Dongtoutang (Changyang county, Hubei).

Table 1 Adult census population size of *Hynobius chinensis* estimated at different breeding ponds in different localities in Changyang County.

Locality	Longitude	Latitude	Elevation (m)	PS (m^2)	N_c
Dongtoutang	110°41'49" E	30°34'42" N	1548	7	141 ± 8
Shuijingwan	110°41'24" E	30°35'04" N	1582	25	91 ± 12
Jiangjunao	110°40'25" E	30°34'59" N	1482	20	44 ± 6
Hengtun	110°39'34" E	30°35'16" N	1408	15	111 ± 10

N_c: adult census population size; PS: pond size. For each sex, and for N_c, values are given in terms of average (± standard error) over the study period, i.e. 2015–2018.

in 223 (5.2%) loci. Tetranucleotide repeat motifs were observed in 120 (2.79%) loci.

We developed and characterized 13 polymorphic microsatellite loci. These loci were in HWE and there were no evidence of significant LD between pairs of loci, of null alleles or genotyping errors in the 22 sample dataset. Among the 13 microsatellite loci, 7 (54%) loci have tetranucleotide repeat motifs and 6 (46.15%) have dinucleotide repeat types. The remaining locus has a trinucleotide repeat motif. The number of repeats range from 5 to 9 and the length of the loci range from 105 to 253 bp (Table 2).

The microsatellite DNA sequences have been deposited in GenBank. The characteristics and the accession number of each locus are shown in Table 2.

3.3. Genetic diversity and signature of a population bottleneck Genotype data at the 13 microsatellite loci have been obtained for all the 118 sampled *H. chinensis* adult individuals (see Table S1 in Appendix). As observed during the development of the microsatellite markers, the analyses in Micro-checker v2.2.3 provided no evidence of either null alleles or genotyping errors. And, there were no cases of linkage disequilibrium among the loci. Among the 13 loci, the locus *HC64* revealed a relatively low *PIC* (Table 2) and 5 loci, i.e. *HC23*, *HC47*, *HC54*, *HC85* and *HC116* significantly deviated from HWE, after Bonferroni correction ($P < 0.0042$). All these 5 loci and the locus *HC64* were therefore removed from further analyses. As a result, 7 microsatellite loci (Table 3) were used in the analyses.

For the 118 samples and 7 loci, 15 different alleles were identified, resulting in 2.14 alleles per loci or average *A* of 2.14 (Table 3). In other terms, the value of *A* was 2 for 6 (85.71%) out of the 7 loci (Table 3). Allele frequency ranged from 0.013 to 0.87. The maximum number of alleles (46.15%) was observed for the frequency range of 0.4–0.6 (Figure 4). 23.07% of alleles were found in the lowest frequency range, i.e. 0–0.2 (Figure 4). Average H_o and H_e were 0.38 and 0.40, respectively. The average value of the inbreeding coefficient, F_{is} was –0.06 (Table 3).

The value of *GW* varied between loci, ranging from 0.17 to 1, and with an average value of 0.61 (Table 3). Comparison of each locus'*GW* to the critical value for rejecting the null hypothesis of demographic stability (i.e. 0.68) showed that *GW* was below this value for 4 (57%) loci (Table 3). The Wilconxon signed-rank test of heterozygosity excess yielded significant (One tail for heterozygosity excess) results ($P < 0.05$) as well as under IAM ($P = 0.004$), SMM ($P = 0.004$) and STM

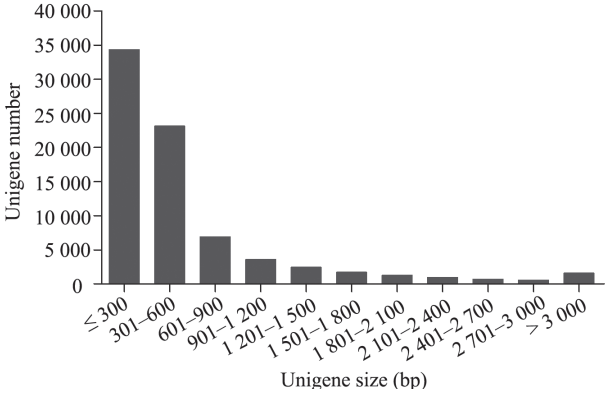

Figure 3 Distribution of unigenes by size range in the transcriptome of *Hynobius chinensis*.

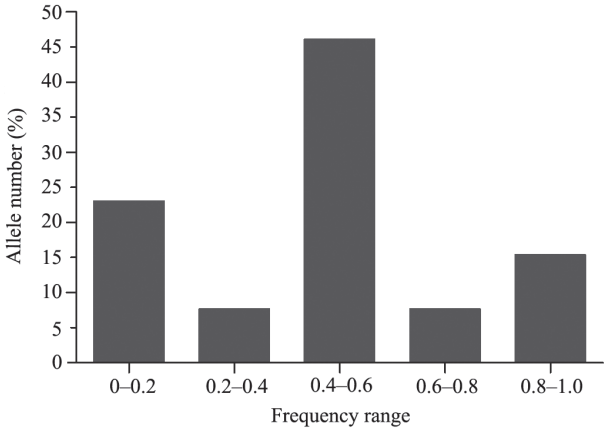

Figure 4 Distribution of alleles at seven microsatellite loci by frequency range in *Hynobius chinensis* ($N = 118$).

($P = 0.027$). In the Sing test, heterozygosity excess was detected for all the 7 loci under IAM. Under TPM and also under SMM, it was detected for 5 loci namely *HC19*, *HC28*, *HC33*, *HC103* and *HC114*. A significant distortion of the typical L-shaped distribution of allele frequency of putatively stable populations was detected. The historical N_e was estimated at 234 individuals. The contemporary N_e was found to be 36 (95% CI: 13.1–124.6) individuals.

4. Discussion

With an average value of 0.61, the results of Garza-Williamson test indicate that the studied population of *H. chinensis* has experienced a severe reduction in size or population bottleneck. The results of Wilconxon signed-rank test and Mode shift test also evidence signatures of a population bottleneck.

Detection of a recent population bottleneck in *H. chinensis* seems to not a surprising finding (Fei and Ye, 2016; Wang *et al*., 2007). Literature shows that *H. chinensis* may have experienced a severe demographic

Table 2 Characteristics of thirteen novel microsatellite markers for the Chinese salamander, *Hynobius chinensis*.

Locus	Primer sequence (5'–3')	Repeat motif	Labeling dye	*Ta* (°C)	*PIC*	*NE-1P*	*NE-2P*	*NE-PP*	*NE-I*	*NE-SI*	GenBank ac. No.
HC19	F: GGGACGTCTAGGGGTAAAGG R: GGACTCGGCAGCTGATAGTC	$(CATC)_6$	TAMRA	64	0.305	0.93	0.848	0.761	0.461	0.678	MC107119
HC23	F: GCCACCTCTTAACAGTGCCT R: CCATTCACCCACAGTCCCTC	$(GAAA)_5$	HEX	64	0.356	0.893	0.822	0.731	0.396	0.618	MC107120
HC28	F: CAGTTGAGGGCCTGGAAACT R: ATACAGCTGGGCCAATCACC	$(CATC)_5$	TAMRA	64	0.374	0.876	0.813	0.719	0.376	0.594	MC107121
HC33	F: ACATCTACGGGGCAGGTCAT R: AGCTCTTATGTCCCCAGCTTT	$(AATC)_5$	TAMRA	64	0.362	0.906	0.83	0.74	0.415	0.637	MC107122
HC47	F: AGAGAGGCCTGTTCATAGCA R: TCTCTGAAGCTGAAGACCCA	$(TCAA)_5$	FAM	60	0.362	0.888	0.819	0.727	0.389	0.61	MC107123
HC49	F: CCCAAGCGTTTCTGTAATGT R: GCAGTGGGGAGCATGATTGA	$(AATC)_5$	HEX	58	0.181	0.98	0.909	0.843	0.658	0.814	MC107124
HC54	F: CGGCTGAGAGTTCCAGAGAA R: TTACCATGCCTGCCTGCC	$(CAGG)_5$	HEX	64	0.318	0.93	0.848	0.761	0.461	0.678	MC107125
HC63	F: CATCGGCATCTTCCTCCTCC R: GCCCATGGACACTGGAGAAA	$(TCC)_7$	FAM	64	0.27	0.954	0.857	0.761	0.518	0.727	MC107126
HC64	F: TCTACCCTCCTCCTCCTCCT R: GCTGTGGTCATAATGGGCCT	$(CCT)_7$	HEX	64	0.043	0.999	0.978	0.958	0.914	0.956	MC107127
HC85	F: CGTCAACCACTGCTTTACTGT R: TGCACTGAGAATCCATGCTGT	$(AC)_9$	FAM	64	0.625	0.737	0.57	0.389	0.156	0.45	MC107128
HC103	F: AAACAAGCTGCACAGTTCCG R: GTTGGGAGGAGGGAAGTTGG	$(TA)_9$	HEX	64	0.253	0.944	0.846	0.747	0.487	0.705	MC107129
HC114	F: ATGCTTTGCCACCACCTCAT R: AAGCAGCTAGAAGGGAGTGC	$(CA)_9$	TAMRA	64	0.374	0.876	0.813	0.719	0.376	0.594	MC107130
HC116	F: ACACGCACAGCAAGAAAAGG R: GGTCAAACATGCACATTCCAAAA	$(AC)_9$	FAM	52	0.232	0.888	0.819	0.727	0.389	0.61	MC107131
Combined non-exclusion probabilities						0.274	0.08	0.015	$0.18*10^{-4}$	$0.43*10^{-2}$	

PIC: polymorphic information content; *NE*-: non-exclusion probability; *1P*: first parent; *2P*: second parent; *PP*: parent pair; *I*: identity; *SI*: sib identity ($N = 22$); *Ta*: annealing temperature; ac. No: accession number.

Table 3 Values of genetic diversity indices and Garza-Williamson statistics (N = 118)

Locus	A	H_o	H_e	F_{is}	Size range (bp)	GW
HC19	2	0.318	0.384	–0.143	182–198	0.4
HC28	2	0.591	0.511	0.042	133–177	0.167
HC33	2	0.591	0.485	–0.001	173–177	1
HC49	2	0.136	0.206	0.102	249–253	1
HC63	3	0.273	0.312	–0.152	105–111	1
HC103	2	0.364	0.304	–0.147	212–222	0.333
HC114	2	0.409	0.511	–0.009	161–169	0.4
Mean	2.14	0.383	0.395	–0.06		0.614

A: number of alleles; H_o: observed heterozygosity; H_e: expected heterozygosity; F_{is}: inbreeding coefficient; GW: Garza-Williamson index.

crisis in the last two centuries (Wang *et al*., 2007). For example, Wang *et al*. (2007) reported that after its description at the end of the 19th century, *H. chinensis* became irretrievable till 2005 in the current location of the Yichang city (Wang *et al*., 2007).

With no more than 2.14 alleles per locus, and with 0.38 and 0.40 as respective average values of H_o and H_e, the genetic diversity revealed by *H. chinensis* seems obviously low in comparison to that reported in Least Concern (IUCN, 2018) pond-breeding Hynobiid salamanders (Matsunami *et al*., 2015; Yoshikawa *et al*., 2013). For examples, the study of small samples from 2 populations (N = 16 and N = 8) revealed 6.63 and 3.53 alleles per locus in *H. nebulosus* (Yoshikawa *et al*., 2013). Another study of small samples (N = 20) from two populations of *H. retardatus* found that the average numbers of alleles per locus were 4.25 and 3.83. The average H_o for these populations was 0.64 and 0.57; and the average H_e was found to be 0.59 and 0.49 (Matsunami *et al*., 2015).

The level of genetic diversity revealed by *H. chinensis* in the present study seems close to that reported in other threatened and narrowly distributed (IUCN, 2018) closely related species such as *H. dunni* (Sugawara *et al*., 2015), *H. maoershanensis* (Lin *et al*., 2015), *and H. tokyoensis* (Sugawara *et al*., 2015). A recent study of 12 populations of *H. dunni* reported an average A of 2.53 and 0.34 as the value of H_o and H_e (Sugawara *et al*., 2015). In a another recent large-scale study of *H. tokyoensis*, a forest-dwelling Hynobiid salamander threatened by habitat loss and fragmentation due to land use changes as it might be in the focal species (Fei and Ye, 2016) which involved 46 populations from 12 different geographic regions, Sugawara *et al*. (2016) found comparable results. These authors showed that the maximum average value of A was 3.4. They also showed that average A was < 2.0 in 39.13% of populations and between 2.0 and 2.40 in 32.61% of populations (Sugawara *et al*., 2016). H_e ranged from 0.02 to .51 and averaged 0.27 for all the populations.

Our demographic and genetic diversity results seem to corroborate findings of earlier studies, using microsatellite data, which report population bottlenecks (Apodaca *et al*., 2012; Heredia-Bobadilla *et al*., 2017; Wang *et al*., 2017), and levels of genetic diversity similar to that revealed by *H. chinensis* in this study (Jordan *et al*., 2009; Spear *et al*., 2006) in a variety of threatened salamanders, including Ambystomatids, and Phlethodontids.

Inbreeding is one of the mechanisms expected to lead to loss of genetic diversity after population bottlenecks in threatened and narrowly distributed amphibians (Ficetola *et al*., 2011; Storfer *et al*., 2014). However, with an average value of –0.06, the inbreeding coefficient, F_{is} provides evidence of moderate level of inbreeding in the present study.

Analyses of microsatellite data in Genepop v1.2 software detected departures from HWE for 5 microsatellite loci (i.e. *HC23*, *HC47*, *HC54*, *HC85* and *HC116*). These departures cannot be attributed to null alleles because a significant occurrence of such alleles in the dataset has not been confirmed by analyses in Micro-Checker v2.2.3. In comparison to loci in HWE, these loci exhibited heterozygosity deficiency (Results not shown). Hence, though inbreeding coefficient F_{is} estimated without these loci revealed a moderate level of inbreeding, the departures from HWE can be considered as a consequence of inbreeding following the inferred population bottleneck (Apodaca *et al*., 2012; Storfer *et al*., 2014). Inbreeding may have caused loss of heterozygosity, which resulted in the observed low level of heterozygosity (Storfer *et al*., 2014). The level of inbreeding may have been brought at a moderate level by selection against inbreeding (Ficetola *et al*., 2011) and by polyandry (Slatyer *et al*., 2012). Few inbreed individuals may reach maturity (Ficetola *et al*., 2011). During this study, at all observation opportunities (8 in total), we found that an individual female's eggs were seemingly fertilized by several males (2–7). Such a mating pattern may result in a reduced probability of inbreeding (Slatyer *et al*., 2012).

The low number of alleles revealed by *H. chinensis* in this study may have been a consequence of loss of rare alleles by genetic drift following the inferred population bottleneck (Garza and Williamson, 2001). Loss of such alleles may have been facilitated by fragmentation of populations caused by forced exclusion of *H. chinensis* from proportions of natural habitats (Allentoft and O'Brien, 2010; Apodaca *et al*., 2012; Sugawara *et al*., 2016). Also, a low density of breeders (i.e. 386–404 breeders per 2.18 km^2) may have contributed to fragmentation of populations (Allentoft and O'Brien, 2010).

According to interviewed people, environmental conditions have considerably changed in the study area since 1960s. Since that time period, the local forest that is also the habitat of *H. chinensis* was intensively harvested. From valleys toward mountain tops, cultivated lands expanded rapidly, reaching almost the current area in 1970s. The forest rapidly regressed towards mountain tops and became fragmented. As larger trees were selectively cut, the density of the canopy rapidly decreased and the forest become degraded. Cultivated lands rapidly became infertile, and the use of fertilizers became intensive in 1970s.

Interviewed people indicated that fire was largely used to clear forest areas for land cultivation, suggesting a possible massive and repeated decimation of terrestrial life stages of *H. chinensis*. Human-induced forest reduction, fragmentation and degradation were evident in all the study localities. Exposure of *H. chinensis* (both aquatic and terrestrial stages) to agrochemicals was also suspected.

These human-induced changes in the potential habitat may have affected gene flow and population growth rate and caused population fragmentation and declines, triggering loss of alleles and heterozygosity in *H. chinensis* (Allentoft and O'Brien, 2010; Apodaca *et al*., 2012; Heredia-Bobadilla *et al*., 2017; Rivera-Ortíz *et al*., 2015; Sugawara *et al*., 2016).

In addition, interviewed people revealed that during breeding seasons, adult *H. chinensis* individuals are collected from breeding ponds and used as a source of proteins. This finding suggests that overharvesting may be another contributing factor to population declines and loss of genetic diversity (Hauser *et al*., 2002) in the study system.

The values of adult census population size, N_c revealed by *H. chinensis* in the present study appears to be smaller than values reported by earlier studies of close related species under suitable habitat conditions (Fu *et al*., 2003; Gu *et al*., 1999; Ma and Gu, 1999), suggesting a possible reduced abundance of *H. chinensis* in the study area due to threats mentioned above (Chen *et al*., 2016; Fei and Ye, 2016). For example, survey data from *H. amjiensis* gathered from 1992 to 1998 (Gu *et al*., 1999), from *H. yiwuensis* -identified by the authors as *H. chinensis*- gathered in 1985, 1988 and 1998 (Ma and Gu, 1999) and personal observation of *H. yiwuensis* by Fu *et al*. (2003) show that in natural and less anthropogenically disturbed habitats, the number of female breeders per breeding pond should exceed 50, even 100 individuals (Chen *et al*., 2016; Fu *et al*., 2003; Gu *et al*., 1999; Ma and Gu, 1999).

Comparison of the contemporary N_e to the historical N_e shows that the contemporary N_e is more than 6 orders of magnitude smaller than the historical N_e, evidencing a long-term reduction in population size. This suggests that *H. chinensis* may have undergone population declines and loss of genetic diversity prior to the anthropogenic threats listed above (Jordan *et al*., 2009). And, long-term declines may have also significantly contributed to fragmentation in gene pools and loss of genetic diversity (Jordan *et al*., 2009).

We report data on N_c in *H. chinensis* from three different localities collected over three successive breeding period. Despite a relative stability during the study period, as N_c may naturally highly fluctuate, long-term data may be required to know the exact N_c in the study localities (Beebee and Griffiths, 2005; McCartney-Melstad and Shaffer, 2015). The observed variability in N_c between localities suggests that local factors may govern the distribution and abundance of *H. chinensis* (Beebee and Griffiths, 2005; Gu *et al*., 1999). And, this should also be true for genetic diversity (Beebee and Griffiths, 2005; Sugawara *et al*., 2016). Evidence from closely related species such as *H. amjiensis* shows that variation in N_c may be caused by spatial variation in human-induced changes in ponds' water quality, and physical characteristics (Gu *et al*., 1999).

We sampled the largest population found, i.e. Taungtotang's population expecting to see a better genetic variability and demographic history (Hoffmann *et al*., 2017). But our results show that despite its bigger size, this population is genetically poor and bottlenecked. And, as expected for amphibians (Allentoft and O'Brien, 2010; Beebee and Griffiths, 2005), N_e was several orders of magnitudes smaller than N_c. Note that, in 2015, we recorded 140 *H. chinensis* adult individuals in Taungtotang, resulting in a N_e/N_c ratio of 0.26 (or 36:140). Though literature shows that higher values can be reached in some species (e.g. *Georcrina vitellina*,

Rana sylvatica), the value of this ration is < 0.2 in many amphibians, including anurans and Caudata (Beebee and Griffiths, 2005). As N_c was smaller in the other localities than in Taungtotang, this relationship suggests that N_e and genetic diversity must be more reduced in those localities (Hoffmann *et al*., 2017), suggesting a possible extirpation of *H. chinensis*' populations.

As mentioned above, information on landscape use lacks for *H. chinensis*. The results of this study demonstrate the paucity of the existing information. For example, while expected to occur at altitudes ranging from 1400 to 1500 m (Fei and Ye, 2016), we found two (50%) breeding populations, including the largest breeding populations beyond this range, at altitudes of 1548 m and 1582 m precisely. The lack of such information prevents us from appreciating the sampling effort of this study and hence the significance of the results at the species level (Allentoft and O'Brien, 2010; Beebee and Griffiths, 2005).

In conclusion, this study makes available thirteen novel microsatellite markers for *H. chinensis*. It provides information on the exact location of populations and breeding sites. This study provides molecular evidence of a population bottleneck in *H. chinensis*, which is provided by the Garza-Williamson test, Heterozygosity excess tests, Mode shift test and estimates of effective population sizes. It shows that *H. chinensis* may exist in small-sized populations, which retain low microsatellite genetic diversity. Together, our results suggest that populations of *H. chinensis* may have been extirpated in the study area.

For further understanding of the demography and genetic viability of *H. chinensis*, future studies should also investigate the distribution and sizes of the representative populations of this endangered species. They should investigate fitness effects of inbreeding and specific causes of population declines while considering gene flow and populations genetic structuring. Conservation initiatives should aim to conserve and restore the habitats and regulating the exploitation of the species. They should aim to maintain the extant genetic variation.

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Appendix

Table S1 The microsatellite genotype data (13 loci) from adults individuals of *Hynobius chinensis* used ($N = 118$)

ID	Sex	Locus 1		Locus 2		locus 3		Locus 4		Locus 5		Locus 6		Locus 7		Locus 8		Locus 9		Locus 10		Locus 11		Locus 12		Locus 13	
		HC19a	HC19b	HC23a	HC23b	HC28a	HC28b	HC33a	HC33b	HC47a	HC47b	HC49a	HC49b	HC54a	HC54b	HC63a	HC63b	HC64a	HC64b	HC85a	HC85b	HC103a	HC103b	HC114a	HC114b	HC116a	HC116b
1	Male	182	198	241	249	133	177	173	177	113	153	253	253	211	219	105	105	175	175	143	143	212	222	161	161	109	109
2	Male	198	198	241	249	133	133	177	177	113	153	253	253	219	227	105	111	175	175	143	145	212	212	161	169	109	127
3	Male	198	198	241	249	133	133	173	173	113	153	253	253	211	219	105	105	175	175	141	141	212	212	161	169	109	127
4	Male	198	198	241	249	133	177	173	177	113	153	253	253	219	219	105	105	175	175	145	145	212	222	161	169	109	109
5	Male	198	198	241	249	133	133	173	173	113	153	253	253	219	219	105	105	175	175	139	145	212	222	169	169	109	109
6	Male	182	198	241	249	133	177	173	177	113	153	253	253	219	227	105	105	175	175	139	145	212	222	161	169	109	109
7	Male	198	198	241	249	133	177	173	173	113	153	249	253	211	219	105	105	175	175	139	143	212	212	169	169	109	127
8	Male	182	198	249	249	177	177	173	177	113	153	253	253	219	227	105	105	175	175	139	143	212	212	161	169	109	109
9	Male	182	198	241	249	133	133	173	177	113	153	253	253	211	219	105	111	175	175	143	145	212	212	169	169	109	109
10	Male	198	198	241	249	133	133	173	177	113	153	253	253	211	219	105	105	175	175	143	145	212	212	169	169	109	127
11	Male	182	198	241	249	177	177	173	173	113	153	253	253	211	219	105	111	175	175	143	145	212	222	169	169	109	109
12	Male	182	198	241	249	133	177	173	177	113	153	249	253	211	219	105	108	175	175	143	145	212	222	161	169	109	127
13	Male	182	198	249	249	133	133	177	177	113	153	253	253	211	219	105	105	175	175	143	143	212	212	169	169	109	127
14	Male	182	198	241	249	177	177	173	177	113	153	253	253	211	219	105	105	175	175	139	143	212	212	161	169	109	109
15	Male	182	182	241	249	133	177	173	177	113	153	253	253	211	219	105	105	175	175	143	145	212	212	161	169	109	127
16	Male	182	198	241	249	133	177	173	177	113	153	253	253	219	219	105	105	175	175	139	145	212	212	169	169	109	127
17	Male	198	198	241	249	177	177	173	177	113	153	253	253	211	219	105	105	175	175	143	145	212	222	161	169	109	109
18	Male	182	198	241	249	133	177	173	173	113	153	253	253	211	219	105	105	175	175	143	143	212	212	161	169	109	127
19	Male	198	198	241	249	133	177	173	177	113	153	253	253	211	219	105	111	175	175	143	145	212	222	169	169	109	109
20	Male	182	198	241	249	133	133	173	177	153	153	253	253	211	219	105	105	175	175	143	145	212	212	161	169	109	109
21	Male	182	198	241	249	133	133	173	173	113	153	253	253	219	219	105	105	175	175	143	145	212	222	161	169	109	109
22	Male	182	198	241	249	133	133	173	177	113	153	253	253	211	219	105	111	175	175	143	145	212	212	161	161	109	109
23	Male	198	198	249	249	133	133	173	173	113	153	253	253	211	219	105	108	175	175	139	145	212	212	161	169	109	109
24	Male	182	182	241	249	133	177	173	173	113	153	253	253	219	219	105	105	175	175	143	145	212	212	161	169	109	109
25	Male	182	198	249	249	133	177	173	173	113	153	253	253	211	219	105	105	175	175	139	143	212	212	161	169	109	127
26	Male	198	198	241	249	133	177	177	177	113	153	253	253	211	219	105	111	175	175	143	145	212	212	161	161	109	109
27	Male	198	198	241	249	133	133	173	177	113	153	249	253	211	219	105	111	175	175	139	143	212	222	161	169	109	109
28	Male	182	198	241	249	133	177	177	177	113	153	253	253	219	227	105	105	175	175	143	145	212	212	161	169	109	109
29	Male	182	198	241	249	133	177	173	177	113	153	253	253	211	219	105	105	175	175	143	145	212	212	161	169	109	127

Continued Table S1

ID	Sex	Locus 1		Locus 2		locus 3		Locus 4		Locus 5		Locus 6		Locus 7		Locus 8		Locus 9		Locus 10		Locus 11		Locus 12		Locus 13	
		HC19a	HC19b	HC23a	HC23b	HC28a	HC28b	HC33a	HC33b	HC47a	HC47b	HC49a	HC49b	HC54a	HC54b	HC63a	HC63b	HC64a	HC64b	HC85a	HC85b	HC103a	HC103b	HC114a	HC114b	HC116a	HC116b
30	Male	198	198	241	249	133	133	177	177	153	153	253	253	211	219	105	111	175	175	143	145	212	222	169	169	109	127
31	Male	182	198	249	249	133	133	173	177	153	153	253	253	211	227	105	111	175	175	139	143	212	222	161	161	109	127
32	Male	182	182	241	249	133	177	173	173	113	153	253	253	211	219	105	111	175	175	143	145	212	212	161	169	109	109
33	Male	198	198	249	249	133	133	173	173	153	153	253	253	211	219	105	111	175	175	137	141	212	212	161	169	109	127
34	Male	198	198	241	249	133	133	173	177	113	153	253	253	219	227	105	111	175	175	139	145	212	212	169	169	113	113
35	Male	182	198	241	249	133	133	173	173	113	153	253	253	219	219	105	111	175	175	143	145	212	212	169	169	109	109
36	Male	182	198	241	249	133	133	177	177	113	153	253	253	211	219	105	111	175	175	143	145	212	222	169	169	109	109
37	Male	182	198	241	249	133	133	173	173	153	153	253	253	219	219	105	105	175	175	139	143	212	222	161	169	109	127
38	Male	198	198	241	249	133	133	173	177	153	153	253	253	211	219	105	105	175	175	143	145	212	212	161	161	109	109
39	Male	198	198	241	249	133	133	177	177	153	153	249	253	211	219	105	111	175	175	143	145	212	212	161	169	109	127
40	Male	198	198	241	249	133	133	173	177	113	153	253	253	211	219	105	105	175	175	143	143	212	212	161	169	109	127
41	Male	182	198	241	249	133	177	173	173	153	153	253	253	211	219	105	105	175	175	143	145	212	212	161	169	109	127
42	Male	182	198	241	249	177	177	173	177	153	153	253	253	219	219	105	111	175	175	145	145	212	222	161	161	109	109
43	Male	182	198	241	249	133	177	173	177	153	153	253	253	219	227	105	105	175	175	143	145	212	212	169	169	109	109
44	Male	182	182	241	249	177	177	177	177	153	153	249	253	219	219	105	111	175	175	139	143	212	212	161	161	109	127
45	Male	182	198	241	249	133	177	173	173	153	153	253	253	219	219	105	105	175	175	139	145	212	222	169	169	109	109
46	Male	182	182	241	249	177	177	173	177	113	153	253	253	211	219	105	105	175	175	143	145	212	212	161	161	109	127
47	Male	182	198	241	249	133	133	173	177	113	153	253	253	219	219	105	105	175	175	143	145	212	212	161	169	109	127
48	Male	182	198	241	249	133	133	177	177	153	153	253	253	211	219	105	105	175	175	143	143	212	212	161	169	109	109
49	Male	198	198	241	249	133	133	177	177	113	153	253	253	211	219	105	105	175	175	143	143	212	212	161	161	109	109
50	Male	198	198	241	249	133	177	177	177	113	153	253	253	211	219	105	111	175	175	143	143	212	212	161	169	109	109
51	Male	182	182	241	249	133	133	173	177	153	153	249	253	211	219	105	105	175	175	143	145	212	222	161	161	109	109
52	Male	198	198	241	249	133	133	173	177	113	153	253	253	219	219	105	105	175	175	143	143	212	212	161	169	109	127
53	Male	182	198	241	249	133	177	173	177	113	153	253	253	219	227	105	105	175	175	145	145	212	212	161	169	113	113
54	Male	182	198	241	249	133	177	177	177	153	153	253	253	219	219	105	111	175	175	143	143	212	212	161	161	109	109
55	Male	182	198	241	249	133	133	173	177	113	153	253	253	211	219	105	105	175	175	143	145	212	212	161	161	109	109
56	Male	182	198	241	249	133	177	173	177	113	153	253	253	219	219	105	105	175	175	145	145	212	212	161	169	109	109
57	Male	198	198	241	249	133	177	177	177	153	153	253	253	219	227	105	105	175	175	139	143	212	212	161	169	109	109
58	Male	182	198	249	249	133	177	173	177	113	153	253	253	211	219	105	105	175	175	139	143	212	212	161	161	109	127
59	Male	182	198	249	249	133	177	173	173	113	153	249	253	211	219	105	105	175	175	139	143	212	212	169	169	109	127
60	Male	182	198	241	249	177	177	173	177	113	153	253	253	211	219	105	105	175	175	143	145	212	212	161	169	109	109

Continued Table S1

ID	Sex	Locus 1		Locus 2		locus 3		Locus 4		Locus 5		Locus 6		Locus 7		Locus 8		Locus 9		Locus 10		Locus 11		Locus 12		Locus 13	
		HC19a	HC19b	HC23a	HC23b	HC28a	HC28b	HC33a	HC33b	HC47a	HC47b	HC49a	HC49b	HC54a	HC54b	HC63a	HC63b	HC64a	HC64b	HC85a	HC85b	HC103a	HC103b	HC114a	HC114b	HC116a	HC116b
61	Male	182	198	249	249	133	133	173	173	153	153	249	249	219	219	105	105	175	175	143	145	212	212	169	169	109	109
62	Male	182	198	241	249	133	177	173	177	153	153	253	253	219	219	105	105	175	175	143	143	212	212	169	169	109	127
63	Male	182	198	241	249	133	177	177	177	113	153	249	253	219	219	105	105	175	175	139	143	212	222	161	161	109	109
64	Male	182	182	241	249	133	177	173	173	153	153	253	253	211	219	105	105	175	175	143	145	212	212	161	169	109	109
65	Male	182	198	249	249	133	133	173	173	113	153	249	253	219	227	105	105	175	175	139	139	212	212	161	169	109	109
66	Male	182	198	249	249	133	177	173	177	153	153	253	253	211	219	105	111	175	175	139	145	212	212	161	169	109	109
67	Male	182	198	241	249	177	177	173	177	113	153	253	253	219	227	105	105	175	175	143	145	212	212	161	169	109	109
68	Male	182	198	241	249	133	133	173	177	113	153	253	253	219	219	105	105	175	175	143	143	212	212	169	169	109	127
69	Male	198	198	241	249	177	177	173	177	113	153	253	253	219	227	105	105	175	175	143	145	212	212	161	169	109	127
70	Male	198	198	241	249	133	177	173	177	153	153	253	253	211	219	105	111	175	175	143	143	212	222	169	169	109	109
71	Male	182	182	241	249	133	133	173	177	153	153	253	253	211	219	105	105	175	175	143	145	212	212	161	169	109	109
72	Male	198	198	241	249	133	133	173	173	153	153	249	253	211	219	105	108	175	175	143	145	212	222	161	169	109	127
73	Male	182	198	241	249	133	177	173	177	113	153	253	253	211	219	105	105	175	175	139	145	212	212	161	169	109	127
74	Male	198	198	241	249	133	133	177	177	113	153	253	253	211	219	105	111	175	175	143	143	212	212	161	161	109	109
75	Male	198	198	241	249	133	177	177	177	113	153	253	253	211	219	105	105	175	175	143	145	212	212	161	169	109	127
76	Male	182	182	241	249	177	177	173	173	153	153	253	253	211	219	105	111	175	175	139	145	212	212	161	169	109	109
77	Male	182	198	241	249	133	177	173	177	113	153	253	253	211	219	105	105	175	175	139	143	212	212	161	169	109	109
78	Male	198	198	241	249	133	177	173	177	153	153	253	253	211	219	105	105	175	175	143	145	212	212	161	161	109	127
79	Female	182	182	241	249	133	133	173	177	113	153	249	253	211	219	105	105	175	175	143	145	212	222	161	161	109	109
80	Female	182	198	241	249	133	133	173	177	113	153	249	253	219	219	105	105	175	175	143	145	212	212	161	161	109	109
81	Female	198	198	241	249	133	177	177	177	113	153	249	253	211	219	105	105	175	175	143	143	212	212	161	161	109	127
82	Female	198	198	241	249	133	133	173	177	113	153	253	253	211	219	105	105	175	175	143	145	212	212	169	169	109	109
83	Female	182	198	241	249	133	177	173	177	113	153	253	253	211	219	105	108	175	175	143	143	212	212	161	169	109	109
84	Female	198	198	241	249	177	177	177	177	153	153	253	253	211	219	105	111	175	175	143	145	212	212	161	169	109	109
85	Female	182	198	249	249	177	177	173	173	113	153	253	253	211	219	105	105	175	175	139	143	212	212	169	169	109	127
86	Female	182	198	241	249	133	133	173	177	113	153	249	249	211	219	105	105	175	178	143	145	212	212	161	169	109	109
87	Female	182	198	241	249	133	177	177	177	153	153	253	253	211	219	105	111	175	175	143	143	212	222	161	169	109	127
88	Female	198	198	241	249	133	177	177	177	113	153	253	253	211	219	105	111	175	175	143	145	212	222	161	169	109	109
89	Female	198	198	241	249	133	177	173	177	113	153	253	253	211	219	105	111	175	175	139	145	212	222	161	169	109	127
90	Female	182	198	241	249	177	177	177	177	113	153	253	253	219	219	105	111	175	175	139	143	212	222	169	169	109	127
91	Female	198	198	241	249	133	177	173	177	153	153	253	253	211	219	105	111	175	175	145	145	212	212	161	169	109	109

Continued Table S1

ID	Sex	Locus 1		Locus 2		locus 3		Locus 4		Locus 5		Locus 6		Locus 7		Locus 8		Locus 9		Locus 10		Locus 11		Locus 12		Locus 13	
		HC19a	HC19b	HC23a	HC23b	HC28a	HC28b	HC33a	HC33b	HC47a	HC47b	HC49a	HC49b	HC54a	HC54b	HC63a	HC63b	HC64a	HC64b	HC85a	HC85b	HC103a	HC103b	HC114a	HC114b	HC116a	HC116b
92	Female	182	198	241	249	177	177	173	177	113	153	253	253	211	219	105	111	175	175	139	143	212	212	161	169	109	109
93	Female	182	198	241	249	177	177	177	177	113	153	253	253	211	219	105	105	175	175	143	145	212	212	161	169	109	109
94	Female	182	198	241	249	133	177	177	177	153	153	249	253	211	219	105	111	175	175	143	143	212	222	161	161	109	109
95	Female	182	198	241	249	133	177	173	177	113	153	253	253	211	219	105	111	175	175	137	143	212	222	169	169	109	127
96	Female	198	198	241	249	133	177	177	177	153	153	253	253	219	219	105	105	175	175	143	145	212	212	161	161	109	127
97	Female	198	198	241	249	133	177	177	177	113	153	253	253	211	219	105	111	175	175	143	145	212	212	161	169	109	109
98	Female	198	198	241	249	133	177	177	177	153	153	253	253	211	219	105	111	175	175	141	141	212	222	169	169	109	109
99	Female	198	198	241	249	177	177	177	177	113	153	253	253	211	219	105	105	175	175	143	145	212	212	161	169	109	109
100	Female	198	198	241	249	133	177	177	177	113	153	249	253	211	219	105	105	175	175	143	143	212	212	161	169	109	127
101	Female	182	198	249	249	133	133	173	177	113	153	253	253	211	219	105	105	175	175	143	143	212	222	161	169	109	127
102	Female	198	198	241	249	133	133	173	173	113	153	253	253	211	219	105	105	175	175	143	143	212	212	161	169	109	109
103	Female	182	198	249	249	133	177	173	173	113	153	253	253	219	219	105	105	175	175	139	139	212	212	169	169	109	109
104	Female	182	182	249	249	177	177	173	177	113	153	253	253	211	219	105	105	175	175	139	139	212	212	169	169	109	127
105	Female	182	198	241	249	133	177	177	177	153	153	253	253	211	219	105	105	175	175	143	143	212	212	161	169	109	127
106	Female	182	198	241	249	133	177	173	177	113	153	253	253	211	219	105	105	175	175	143	145	212	212	161	161	109	109
107	Female	182	198	241	249	133	177	177	177	113	153	253	253	211	219	105	105	175	175	143	143	212	212	161	169	109	127
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110	Female	182	198	241	249	177	177	173	177	113	153	253	253	211	219	105	111	175	175	145	145	212	222	161	161	109	109
111	Female	182	182	241	249	133	177	177	177	153	153	249	253	211	219	105	108	175	175	143	143	212	212	161	169	109	127
112	Female	198	198	241	249	133	133	173	177	113	153	253	253	219	219	105	111	175	175	143	145	212	212	161	161	109	127
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115	Female	198	198	241	249	133	133	177	177	153	153	249	253	211	219	105	108	175	175	143	145	212	212	169	169	109	127
116	Female	182	198	241	249	177	177	173	173	113	153	253	253	219	219	105	105	175	175	143	145	212	212	161	169	109	127
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