INTRODUCTION

Amphibians are considered to be good indicators of environmental changes because they depend on both terrestrial and aquatic environments to complete their life cycles (Blaustein et al., 1994). Due to their vulnerability to numerous environmental stressors, population declines and species extinctions among amphibians are common across the globe. Of the 7,219 currently recognized amphibian species, about 33.87% are classified as vulnerable, endangered or critically endangered according to the red list maintained by the International Union for Conservation of Nature (IUCN) (IUCN, 2020). In fact, amphibians have been estimated to be more...
threatened than mammals and birds (Stuart et al., 2004) and, thus, have been receiving increased attention in the midst of the current biodiversity crisis (Adams et al., 2013; Houlanah et al., 2000; Stuart et al., 2004; Wake, 2012; Wake & Vredenburg, 2008). Habitat loss resulting from environmental alterations has been implicated as one of the primary factors affecting amphibian diversity and numbers (Blaustein et al., 1994, 2010; Cushman, 2006). For example, natural habitats have been fragmented and degraded through the continuous modification of landscapes by humans (Lindenmayer & Fischer, 2006). This has had negative effects on demographics and genetic characteristics of amphibian populations (Beebee, 2005), including their adaptation to local environments (Johansson et al., 2007).

In recent two decades, genetic data have become increasingly useful in informing amphibian conservation (reviewed in: McCartney-Melstad & Shaffer, 2015). The importance of preserving population genetic diversity has long been recognized to influence long-term viability of population as it influences populations' ability to adapt to changes in environmental conditions (Allendorf & Leary, 1986; Chapman et al., 2009; Reed & Frankham, 2003). Moreover, inbreeding can influence fitness of amphibians by reducing genetic diversity within populations (Allentoft & O'Brien, 2010; Rowe & Beebee, 2003; Rowe et al., 1999). In particular, small effective population sizes (N_e) can lead to loss of diversity and inbreeding, which can also decrease the populations' adaptive potential. Levels of genetic diversity and patterns of population differentiation in many amphibians have now been successfully assessed using genetic markers, typically microsatellites (Andersen et al., 2004; Beebee, 2005; Burns et al., 2004; Jehle et al., 2005; Nair et al., 2012; Nair et al., 2012;Yang et al., 2016; Zheng et al., 2021). As such, these studies have typically been limited to a few genetic loci, although studies based on a larger number of markers have started to emerge (e.g., Funk et al., 2018; Guo, Lu, et al., 2016; Hardy et al., 2021; Thörn et al., 2021). Yet, the genetic underpinnings of adaptations in natural populations of amphibians are largely unknown, although quantitative genetic methods have revealed adaptive differentiation in important life history traits (e.g., Berven, 1982; Laugen et al., 2003; Laurila et al., 2002; Palo et al., 2003).

The relative paucity of examples for adaptive differentiation among amphibian populations in any traits other than embryonic or larval traits (but see: Alho et al., 2010; Bonin et al., 2006; Guo, Lu, et al., 2016; Richter-Boix et al., 2011; Weber et al., 2002) can be understood considering two facts. Firstly, it is difficult to conduct common garden experiments with amphibians due to their long and complex life histories. Secondly, amphibians have large and complex genomes (Gregory, 2002), and genomic resources for amphibians are scarce (but see: Cano et al., 2011; Hellsten et al., 2010; Nürnberger et al., 2003; Palomar et al., 2017; Smith et al., 2005; Sun et al., 2015; Yang et al., 2016). Furthermore, transfer of genomic resources from one amphibian species to another is challenging as amphibians exhibit high levels of genetic divergence even within genera (Johns & Avise, 1998; Primmer & Merilä, 2002; Vences et al., 2005). However, studies on adaptive differentiation among amphibian populations based on genome-wide characterizations of genetic variability using single-nucleotide polymorphisms (SNPs; Allendorf et al., 2010; Davey & Blaxter, 2010; Davey et al., 2011; Nielsen et al., 2011; Stampley et al., 2010) have started to emerge (e.g., Bell et al., 2015; Bewick et al., 2013; Brelsford, Dufresnes, et al., 2016; Brelsford, Rodrigues et al., 2016; Funk et al., 2018; Gamble & Zarkower, 2014; Guo, Lu, et al., 2016; Streicher et al., 2014; Wei et al., 2020). In particular, sequencing of pooled DNA samples (Pool-seq; Futschik & Schlötterer, 2010; Kofler, Orozco-terWengel, et al., 2011; Rubin et al., 2010; Schlötterer et al., 2014; Turner et al., 2010) provides affordable means to conduct population genomic studies.

Many amphibian species have become endangered in China due to rapid socio-economic development, population growth, urbanization and climate change. Although Chinese amphibians face the serious threats due to habitat loss, pollution, alien species invasions and overharvesting (Liao et al., 2015; Liu et al., 2018; Xie et al., 2007), little is known about their genetic structuring (but see: Guo, Lu, et al., 2016; Wei et al., 2020; Yang et al., 2016). The ornate chorus frog (Microhyla fissipes) is a case in point. It is a small-sized anuran, and its wide distribution area in China covers two biodiversity hotspots: Indo-Burman and mountains of south central China. The species occurs at altitudes ranging from 0 to 1,400 m and is an ideal model species to study local adaptation across environmental gradients. The species is commonly found on land around rice fields, bogs, ponds and ditches (Fei et al., 2009). It emerges from hibernation at early April and breeding commences in mid-May in mainland China.

In recent years, rapid urbanization following socio-economic development and climate change have reduced their habitats and population sizes (Fei et al., 2009). Hence, from the conservation point of view, it would be useful to know whether this widely distributed species comprises genetically differentiated, locally adapted populations, and whether there is any evidence of reduced genetic diversity in its population isolates.

The aim of this study was to investigate genome-wide genetic variation and differentiation and their possible association with variation in temperature and precipitation among M. fissipes populations to see whether there is genetic evidence for local adaptation. To this end, the restriction site-associated DNA sequencing (RAD-seq) approach (Bell et al., 2015; Brelsford, Dufresnes, et al., 2016; Guo, Lu, et al., 2016; Streicher et al., 2014) with the pool-sequencing strategy (Kofler, Orozco-terWengel, et al., 2011; Schlötterer et al., 2014), was used to identify SNPs from 10 localities spanning 1,398 km long latitudinal, temperature and precipitation gradient (Figure 1). We first used a suite of different outlier tests to identify the putative signatures of directional and/or balancing selection on the SNPs. We then looked for associations with key environmental factors (viz., temperature and precipitation) using landscape genomic methods. In addition, we explored the patterns of genome-wide genetic differentiation and variability associated with geography to get insights into the levels of genetic differentiation on different geographic scales. Finally, we evaluated the relative importance of environmental factors and geography in explaining the distribution of genetic variation across M. fissipes populations.
2 | METHODS

2.1 | Specimen sampling

The specimens used in this study were collected with permission from the China West Normal University Ethical Committee for Animal experiments (CWNU-17002), and the experimental protocols adhered to the current laws of China concerning animal experimentation. The specimens were collected at 10 sites from the south-western and southern China subject to different temperature and precipitation regimes (Figure 1). Molecular voucher specimens were preserved in the Laboratory of Animal Molecular Ecology and Evolution, College of Life Sciences, China West Normal University.

The sampling covered four population clusters situated ≥210 km from each other; populations within these clusters were separated from each other by a distance of ≤180 km (Figure 1). The four population clusters are referred to as (a) SC, which included three populations from Sichuan Province: Xindu (XD, 496 m.a.s.l), Nanchong (NC, 370 m.a.s.l) and Guanghan (GH, 478 m.a.s.l); (b) GX, which included two populations from Guangxi Province: Beihai (BH, 36.1 m.a.s.l) and Yulin (YL, 73 m.a.s.l); (c) EH, which included two populations from the east of Hainan Province: Wanning (WN, 15 m.a.s.l) and Haikou (HK, 15 m.a.s.l); and (d) WH, which included three populations from the west of Hainan Province: Nanbin (NB, 17 m.a.s.l), Ledong (LD, 10 m.a.s.l) and Changjiang (CJ, 126 m.a.s.l; Figure 1: Table S1 in Appendix S1). All sampling was conducted in breeding seasons of 2014 and 2015 by collecting an arbitrary sample of adults in breeding condition by hand. All animals were sacrificed using the single-pithing method, and a piece of muscle tissue was collected and preserved in 95% ethanol (Lüpold et al., 2017; Yang et al., 2018; Yu et al., 2018). Based on the findings of a previous study, accurate allele frequency estimation should include at least 10 or more individuals (Mita et al., 2013). Hence, 200 specimens, 20 individuals (10 males and 10 females) from each breeding site were used for sequencing in this study.

2.2 | DNA extraction, RAD library construction and sequencing

A standard phenol–chloroform method (Sambrook et al., 1989) was used to extract whole genomic DNA from muscle tissues. DNA quality was assessed by visualizing it on 1% agarose gels. To avoid possible unequal representation of individuals within sequencing pools caused by unequal DNA quality, DNA samples exhibiting degradation on agarose gels were re-extracted and re-examined until high quality DNA was obtained. All non-degraded samples were further quantified using both a Qubit® fluorometer and NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific). Thereafter, individual samples were diluted to 10 ng/μl and pooled within each of the ten sampling locations. Pooled samples were re-quantified twice using both the fluorometer and spectrophotometer and equalized to 10 ng/μl for constructing of the RAD-seq libraries.

RAD library construction and sequencing were conducted by Gene Denovo Biotechnology Co., Ltd, following the protocol of Guo, Lu, et al. (2016). In brief, restriction enzyme EcoRI was used to fragment DNA followed by adding a P1 adapter with sequencing primer, forward amplification primer and barcode to each of digested DNA pools. Barcoded samples were pooled and randomly sheared. After this, a P2 adapter was added to the sheared DNA fragments, followed by enrichment of DNA with a P1 adapter by PCR amplification. Finally, DNA fragments at a size range of 300–500 bp were gel-purified, and the barcoded RAD samples were sequenced on the Illumina HiSeq X platform with 150-bp paired-end strategy. All DNA pools were sequenced on a single sequencing lane.
2.3 | Data processing, assembly and alignment

We first used an in-house Perl script which allowed for no mismatches between the sequenced barcode and its sequence to de-multiplexed sequences. To reduce the sequencing error rate, we end-trimmed raw reads to a length of 90 bp using a FASTX toolkit (see: http://hannonlab.cshl.edu/fastx_toolkit/). We then discarded reads containing one or more bases with a Phred quality score below 10 or if more than 5% of the positions had score below 20.

We de novo-assembled the samples from all 10 sampling sites using Velvet 1.2.02 (Zerbino & Birney, 2008). After testing odd k-mer lengths from 49 to 73, 100 being the minimum length parameter and 400 being the insert length parameter, we used K-mer of 55 as the optimum. After this, the de novo-assembled sequences from each sampling site were further assembled using CAP3 with default parameters (Huang & Madan, 1999). In the following analyses, we used the singleton and contig sequences based on the assembly with CAP3 as the reference sequences.

We also used BWA 0.6.1 (Li & Durbin, 2009) to align quality-filtered reads from all sampling sites to the reference sequences (Xenopus tropicalis UCB_Xtro_10.0; NCBI). The maximum edited distance for each read was two, and the maximum number of alignments for reads paired properly was one. The maximum insert size for a read pair was 500. The maximum insert size was 500 for a read pair. We used SAMtools 0.1.18 (Li et al., 2009) to convert the mapping results in SAM format into BAM format and filtered them for a read pair. We used SAMtools 0.1.18 (Li et al., 2009) to convert the mapping results in SAM format into BAM format and filtered them for a minimum mapping quality of 20. We then removed duplicate reads using Picard Tools v1.133 (http://broadinstitute.github.io/picard/). We then discarded reads containing one or more bases with a Phred quality score below 10 or if more than 5% of the positions had score below 20.

The maximum insert size was 500 for a read pair. We used SAMtools 0.1.18 (Li et al., 2009) to convert the mapping results in SAM format into BAM format and filtered them for a minimum mapping quality of 20. We then removed duplicate reads using Picard Tools v1.133 (http://broadinstitute.github.io/picard/). Finally, BAM files were converted into “mpileup” format with maximum 1,000 reads at a position per BAM file using SAMtools 0.1.18 (Li et al., 2009).

2.4 | Estimation of genome-wide genetic variation and differentiation

Nucleotide diversity (Tajima’s π), Tajima’s D (Tajima, 1989) and population mutation rate (Watterson’s Theta, θW; Watterson, 1975) were estimated to characterize patterns of genome-wide genetic variation with PoPoolation 1.2.2 (Kofler, Orozco-terWengel, et al., 2011). We firstly defined SNPs across the entire genome based on several stringent criteria, namely, as sequence coverage has a strong effect on the accuracy of allele frequency estimates in sequencing of pooled samples, we sought to improve the accuracy of these parameter estimates using large sliding windows and high sequence coverage (Kofler et al., 2011). To estimate x and θW, we required the identified SNPs to possess a minimum minor allele count of six and coverage of 24 to 1,000 reads for each sampling site. Because Tajima’s D is sensitive to variation in coverage, it was estimated using only SNPs with a coverage of 48 reads in all sampling sites. A non-overlapping 200-bp sliding window was used to estimate Tajima’s π, Tajima’s D and θW across the reference sequences using minimum base Phred quality of 20 for the analysed sites. We calculated FST values for each pairwise population comparison to estimate of the degree of population differentiation. For these calculations, a minimum count of the minor allele six across all the 10 populations and coverage between 24 and 1,000 reads for each sampling site were analysed using PoPoolation2 (Kofler et al., 2011).

2.5 | Detection of selection footprints

We identified SNPs being likely differentiated as result selection using PoPoolation2 following Guo, Lu, et al. (2014). We employed two independent methods to identify selection. Firstly, we used PoPoolation2 to calculate FST values for each SNPs within each pairwise comparison of populations. Following the empirical outlier detection approach (Akey et al., 2010), we considered SNPs falling into 99% of the empirical distribution within each pairwise FST as potentially differentiated SNPs. Secondly, we exported all identified SNPs using PoPoolation2 in GenePop format to BayeScan 2.1 (Foll & Gaggiotti, 2008) to estimate the posterior probability that balancing selection/purifying selection (negative alpha coefficient) or directional selection (positive alpha coefficient) had an effect on a given locus. Briefly, we identified the top candidates of selected loci using prior odds of 100 and ran the reversible-jump MCMC chains of 55,000 with a thinning interval of 10, following 20 pilot runs of 5,000 iterations each and burn-in length of 50,000. SNPs identified by both of the two approaches outlined before were considered as truly differentiated loci. Finally, we also performed the BayeScan analyses within each of the population clusters separately.

2.6 | Detecting genetic differentiation associated with environmental parameters

Using all SNPs identified by PoPoolation2, we examined the relationships between genetic differentiation and environmental parameters using a Bayesian approach as implemented in Bayenv (Coop et al., 2010). Bayenv accounts for demographic effects when evaluating the relationship between genetic differentiation and environmental parameters. We first estimated neutral covariance matrix (estimated from markers declared as neutral by BayeScan) and then evaluated the relationship between genetic differentiation and environmental parameters (viz. latitude, average annual temperature and precipitation; Table S1). Annual temperature and precipitation data were retrieved from https://www.meteoblue.com. Each environmental parameter was standardized by subtracting the mean from daily values and dividing them by the standard deviation of the parameter among populations (Coop et al., 2010). To confirm that the results were not sensitive to stochastic errors, we ran three independent runs with different random seeds.
2.7 | SNP annotation and gene ontology analysis

To examine genetic differentiation across genome features, the reference sequences were annotated by searching matching protein-coding sequences of *Xenopus tropicalis* from the Ensembl database, with an E-value cut-off of \(1 \times 10^{-5}\) using BLAST (Altschul et al., 1997). Gene ontology (GO) terms of the reference sequences were retrieved based on their orthologs using BioMart (Kasprzyk, 2011). We also performed the GO term enrichment analysis using g: profiler (Uku et al., 2019).

2.8 | Detection of population structure

We characterized the population structure on the basis of matrices of pairwise \(F_{ST}\) values among populations using PoPoolation2. To visualize the multilocus patterns of population differentiation, we first generated a principal coordinate analysis (PCoA) plot based on average pairwise \(F_{ST}\) values in the R package of “labdsv” (http://ecology.msu.montana.edu/labdsv/R/). To further explore patterns of population differentiation, neighbour-joining (NJ) trees were constructed on the basis of pairwise \(F_{ST}\) values (Latter, 1972) of each SNPs using the data on GenePop format including all identified SNPs with PoPoolation2, as well as restricting the used SNPs to the SNPs identified as outliers by Populations 1.2.32 (http://bioinformatics.org/~trypphon/populations/). In both cases, we estimated tree topology and branch-support values using 1,000 bootstrap replicates.

2.9 | Relationships among population differentiation, geographic distance and environmental parameters

Isolation by distance (IBD; Wright, 1943) is expected to be observed in most amphibians because of their poor dispersal capabilities. A relationship between linearized \(F_{ST}\) values \([F_{ST}/(1-F_{ST})]\) and log (distance) is considered a powerful test of IBD (Rousset, 1997). Therefore, we used the Mantel test to test for IBD for all populations in the “VEGAN” R package (Oksanen et al., 2015). Geographic distances between populations were estimated using Google Earth (Table S2 in Appendix S1). Moreover, we carried out separate analyses to test whether IBD could be observed at a finer geographic scale (≤70 km) than that covering the total sampling area (>1,400 km) for each of the population clusters (Figure 1).

Besides the poor dispersal capabilities resulting in low rates of gene flow among populations, environmental variables (i.e., average annual temperature and precipitation) can potentially act as drivers of population differentiation in *M. fissipes*. Therefore, to compare pairwise genetic distance matrices and pairwise environmental variables, we performed a partial Mantel test to analyse isolation by environment (IBE; Wang & Bradburd, 2014) using the R package in “VEGAN” (Oksanen et al., 2015). We used partial Mantel tests to rule out false-positive relationships stemming from effects of IBD by repeating IBE tests holding geographic distances constant. As the partial Mantel tests are known to have high Type I error rates (Guillot & Rousset, 2013), we also conducted a conditional independence test to identify cause–effect associations between genetic distances, geographic distances and environmental variables (i.e., average annual temperature and precipitation) in the R package “pcalg” (Kalisch et al., 2012). We performed IBE tests for all 10 populations together, as well as for each of the three population clusters separately.

To detect signals of IBD and IBE, we used six different SNP data sets both across all 10 populations, as well as within each of the three population clusters: (a) all SNPs identified using PoPoolation2; (b) the SNPs identified using BayeScan to be under directional selection; (c) the SNPs with variation in allele frequency linked to latitudinal position, either across all 10 populations analysed together or in any of the three clusters; (d) the SNPs with variation in allele frequency linked to annual temperature variation, either across all 10 populations or in any of the three clusters; (e) the SNPs with variation in allele frequency linked to annual precipitation variation, either across all 10 populations or in any of the three clusters; and (f) the neutral SNPs on the basis of the fact that BayeScan did not identify these as outliers, and environmental parameters in Bayenv analyses were not associated with allele frequency variation in these SNPs. Evidence for IBE in the first five data sets could arguably be regarded dubious and even circular; a signal of IBE in the sixth data set should give the most honest and also a conservative test of IBE.

3 | RESULTS

3.1 | RAD-seq data set and the assembled reference sequences

We obtained 275.3 million reads, with their numbers ranging from 19.1 to 41.9 million for each population (Table 1). A total of 1,931,696 unique sequences >200 bp were produced by the de novo assembly, and these sequences were used as references for alignment. The mean size of reference sequences was 269 bp (median =273 bp), ranging from 200 to 1,509 bp (Figure S1 in Appendix S1). A total of 258.1 million reads were aligned to the reference sequences, with the number of aligned reads being ranging from 17.8 to 39.6 million for each population (Table 1). The number of mapped reads was strongly and positively correlated with the number of raw reads within each population \(r_s \geq 0.9636, p < .0001\).

3.2 | Genome-wide genetic variation

The number of SNPs in a given population identified by PoPoolation ranged from 213,934 to 567,341 (Table 1). The expected heterozygosity for all SNPs ranged from 0.3839 to 0.4006 within each population, with a mean of 0.3916 across the populations. Genome-wide average Tajima’s \(\pi\) ranged from 0.4102 to 0.4312 for each population, with a mean of 0.4202 across the populations.
Table 1: Summary statistics of RAD data, including the number of SNPs identified and their average heterozygosity in each population.

| Population code | No. of clean reads | No. of mapped reads | No. of SNPs | Average heterozygosity | Tajima’s π | Watterson’s θ |
|-----------------|--------------------|---------------------|-------------|------------------------|------------|--------------|
| BH              | 21,602,992         | 20,173,624          | 411,707     | 0.3948                 | 0.4245     | 0.2455       |
| GH              | 19,139,414         | 17,765,222          | 213,934     | 0.3929                 | 0.4312     | 0.2423       |
| YL              | 31,091,374         | 29,643,338          | 388,123     | 0.3858                 | 0.4167     | 0.2368       |
| HK              | 23,911,896         | 21,167,264          | 488,030     | 0.3959                 | 0.4220     | 0.2447       |
| LD              | 22,775,474         | 21,706,050          | 485,651     | 0.3935                 | 0.4193     | 0.2442       |
| NB              | 25,919,790         | 24,247,070          | 481,162     | 0.3938                 | 0.4192     | 0.2426       |
| NC              | 35,019,458         | 33,485,054          | 280,350     | 0.3878                 | 0.4204     | 0.2323       |
| WN              | 23,726,758         | 21,906,298          | 504,673     | 0.4006                 | 0.4257     | 0.2424       |
| XD              | 41,898,570         | 39,621,566          | 259,580     | 0.3839                 | 0.4126     | 0.2266       |
| CJ              | 30,243,238         | 28,412,370          | 567,341     | 0.3868                 | 0.4102     | 0.2406       |

Note: The ten populations include Xindu (XD), Nanchong (NC), Guanghan (GH), Beihai (BH), Yulin (YL), Wanning (WN), Haikou (HK), Nanbin (NB), Ledong (LD) and Changjiang (CJ).

(3.3) Genome-wide Tajima’s π ranged from 0 to 0.50, and the genome-wide θ_W was between 0 and 0.26 (Figure 2a, b, Figure 2S in Appendix S1). The island populations tended to exhibit less genetic variability than mainland populations (Table 2), but this difference was not significant (Kruskal–Wallis test, df = 4, p = .7540).

3.3 Genome-wide genetic differentiation

A total of 20,572 SNPs across the ten populations were identified for estimation the genome-wide genetic differentiation. The polymorphism index (= the proportion of SNPs which had read support for both alleles for each population) ranged from 0.1362 to 0.2761. The overall average pairwise F_ST across all populations was 0.090 (SE = 0.0747), ranging from 0 to 0.7233 with median at 0.0766 (Figure 2c). Many SNPs displayed an elevated level of divergence. For example, 7,323 SNPs exhibited an overall average pairwise F_ST > 0.1 across all populations, and 205 SNPs occurred at least once in the top 1% of pairwise F_ST values.

A total of 360 SNPs were identified as the outliers at a false discovery rate threshold of 0.01 in the global outlier test across ten populations. Of these, 212 SNPs were indicated to be under balancing selection and 148 under diversifying selection (Figure 2d). However, as 15 and 28 of these SNPs were correlated with variation in latitude and altitude, respectively, we excluded them from the further analyses reducing the data set to 317 SNPs.

3.4 Genetic differentiation associated with environmental parameters

The global Bayenv analyses based on the subset of 317 SNPs across 10 populations revealed that allele frequencies in the number of SNPs were strongly correlated with variations in annual average temperature and/or precipitation. Of these, variation in an allele frequency of 69 SNPs was associated with variation in annual average temperature (Figure 2e), and variation in an allele frequency of 248 SNPs was associated with variation in annual average precipitation (Figure 2f). The variation in allele frequency in seven SNPs was associated with variations in both annual average temperature and precipitation (Figure 3a).

3.5 Candidate genes for local adaptation

A BLAST search against the genome of X. tropicalis found 23 candidate genes for local adaptation using the 360 SNPs identified as outliers (Figure 3b; Table S4 in Appendix S1). These genes were classified under 60 GO terms and were markedly enriched in functional categories “biological processes” and “molecular functions” (Benjamini-Hochberg FDR, p < .05) as compared with all genes in X. tropicalis (Figure 3b).
3.6 | Population structure

Average pairwise $F_{ST}$ among the 10 populations based on the 20,572 SNPs was 0.219, ranging from 0.136 (between BH and NB) to 0.443 (between GH and XD; Table S5 in Appendix S1). The principal component analysis (PCoA) plot revealed clear geographically ordered population structuring (Figure 2g). The first PCA-axis differentiated the western Hainan populations (NB, LD and CJ) from the Sichuan populations (GH, XD and NC), as well as from the eastern Hainan populations (HK and WN) and the Guangxi populations (YL and BH; Figure 2g). The second PCA axis additionally differentiated the Guangxi populations (YL and BH) from the western Hainan populations (NB, LD and CJ), as well as from the Sichuan populations (NC, XD and GH) and the eastern Hainan populations (HK and WN; Figure 2g). The pattern in an NJ tree was very similar to the geographically ordered patterns with high bootstrap support values (Figure 2h). NJ trees based only on outlier SNPs under diversifying selection also detected the same population structure as the PCoA analysis. This was true also if all SNPs were used (Figure 2i).

3.7 | Isolation by distance (IBD) and environment (IBE)

No IBD was detected in analysis of all populations using all 20,572 SNPs (Mantel test, $r = -0.101$, $p = .514$). The IBD was detected
FIGURE 3  Number of SNPs whose allele frequency variations are associated with variation in temperature, precipitation and/or altitude in Bayenv analysis (a), and GO-term enrichment analyses of outliers (b)
among populations within the western Hainan cluster (Mantel test, \( r_s = -1.000, p < .001 \)) but not in the Sichuan cluster (Mantel test, \( r_s = -0.500, p = .349 \)). No IBD was detected within the entire Hainan cluster (Mantel test, \( r_s = -0.236, p = .484 \); Table 3). However, we found that the IBD was detectable across all populations in the three SNP data sets (viz., the 360 SNPs under diversifying selection, SNPs associated with latitude and temperature; Table 3). IBD was detectable in two SNP data sets across local populations from the Sichuan cluster (SNPs associated with latitude and precipitation) and the western Hainan cluster (SNPs associated with precipitation and neutral SNPs; Table 3). For the Hainan population cluster, we only detected IBD in one of the SNP data sets (SNPs associated with precipitation; Table 3).

Population differentiation was associated with environmental temperature across all populations if all 20,572 SNPs were used, but this was not the case with environmental precipitation (Table 4). Population differentiation was significantly associated with temperature within the Hainan population cluster but not in others (Table 4). The relationship between population differentiation and environmental temperature tended to approach significance in the other five different SNP data sets too (Mantel tests, \( r_s ≥0.118, p < .062 \); Table 4). The relationship between population differentiation and environmental precipitation was significant in the SNPs identified to be associated with latitude (Bayenv analysis; partial Mantel test, \( r_s = -0.292, p = .001 \); Table 4). Geographic distance displayed a significant relationship with population differentiation across all populations (conditional independence: \( p < .0001 \)) but not with local population clusters (\( p = 1.00 \)). No cause/effects of temperature and precipitation on population differentiation were found neither in the analysis of across all populations nor within those conducted with local population clusters (in both \( p = 1.00 \)).

### 4 | DISCUSSION

We found that allele frequency variations in numerous SNPs were significantly associated with variation in average annual temperature and precipitation among *M. fissipes* populations. This suggests that these two environmental factors, or factors in strong association with them, are likely to have been important shaping forces behind the observed population genetic differentiation providing evidence for local adaptation. The findings further indicate moderate levels of genome-wide genetic differentiation across *M. fissipes* populations both at short (<100 km) and long (>500 km) geographic distances. To this end, our results align with the earlier findings from low coverage population genetic studies, suggesting that anuran amphibians typically exhibit substantial population structuring that often exceeds that observed in other classes of vertebrates (Ward et al., 1992).

#### 4.1 | Local adaptation in *M. fissipes*

Spatially heterogeneous selection is the driver of local adaptation (Blanquart et al., 2013; Qi et al., 2019; Saijuntha et al., 2020; Savolainen et al., 2013; Tantrawatpan et al., 2021). Earlier studies of local adaptation in amphibians have relied mostly on classical common garden experiments (e.g., Alho et al., 2010; Beveren, 1982; Berven & Gill, 1983; Beveren et al., 1979; Laugen et al., 2003; Luquet et al., 2015; Mlaud & Merilä, 2001), or combination of them with population genetic approaches (e.g., Palo et al., 2003). More recently, population genomic approaches have started replacing the aforementioned approaches in studies of local adaptation (e.g., Campbell et al., 2012; Guo et al., 2015; Guo, Li, et al., 2016; Laurent et al., 2016; Luikart et al., 2003; Tiffin & Ross-Ibarra, 2014), including amphibians (Bonin et al., 2006; Dufresnes et al., 2013; Guo, Lu, et al., 2016; Richter-Boix et al., 2011). In line with the results of a limited number of amphibian studies which have looked for evidence of local adaptations with population genomic approaches, we found the evidence for local adaptation in *M. fissipes*. Specifically, we found that allele frequency differentiation in a relatively large number of SNPs exceeded what one would expect if the differentiation was driven by neutral processes. Further evidence for adaptive nature of this differentiation was provided by the fact that the patterns of differentiation in a number of outlier loci aligned with variation in environmental temperature and precipitation both in Bayenv and IBE analyses. Such associations are not surprising given that amphibians are ectothermic animals whose biology is strongly affected by moisture and temperature.

### TABLE 3 Isolation-by-distance (IBD) tests with different SNP data sets

| Population cluster | Full data set of 20,572 SNPs | 360 SNPs under diversifying selection identified by BayeScan | 5 SNPs associated with latitude | 69 SNPs associated with temperature | 248 SNPs associated with precipitation | 19,880 neutral SNPs |
|-------------------|-----------------------------|----------------------------------------------------------|-------------------------------|-----------------------------------|----------------------------------------|-------------------|
|                   | \( r_s \) | \( p \)            | \( r_s \) | \( p \)            | \( r_s \) | \( p \)            | \( r_s \) | \( p \)            | \( r_s \) | \( p \)            |
| All               | -0.101 | .514        | -0.301 | .046        | -0.388 | .010        | -0.357 | .018        | -0.028 | .853        | -0.090 | .549        |
| SC                | -0.500 | .349        | -0.500 | .322        | -1.000 | <.001       | -0.500 | .316        | -1.000 | <.001       | -0.500 | .336        |
| WH                | -1.000 | <.001       | -0.500 | .341        | -0.500 | .327        | -0.500 | .323        | -1.000 | <.001       | -1.000 | <.001       |
| HN                | -0.236 | .484        | -0.565 | .083        | -0.103 | .767        | -0.018 | .940        | -0.721 | .019        | -0.207 | .563        |

Note: \( r_s \) = Spearman rank correlation coefficient, \( p \) refers to empirical significance level from 1,000 permutations.
Genetic differentiation among *M. fissipes* populations

The patterns of genetic differentiation were heterogeneous across the ten study populations, suggesting a varying but generally fairly high degree of genomic differentiation among *M. fissipes* populations. The mean $F_{ST}$ between local *M. fissipes* populations separated by geographic distances less than 110 km was around 0.285. Such clear population structuring is not unusual for temperate zone amphibians (e.g., Chan & Zamudio, 2009; Guo, Lu, et al., 2016) and likely to result from their limited dispersal ability and high degree of philopatry (Smith & Green, 2005; Ward et al., 1992). One manifestation of limited dispersal ability of amphibians is the generally high IBD among amphibian populations (Brelsford, Rodrigues, et al., 2016; Dodd, 2009; Guo, Lu, et al., 2016). The current study failed to find evidence for IBD at the global scale of sampling. However, we found significant IBD within the local population clusters. The observed discontinuity in IBD at different geographical scales could owe, for instance, to historical discontinuities among different population clusters or to dispersal barriers of natural and/or anthropogenic origin. Whatever the reason, dispersal among local (and global) populations of *M. fissipes* seems to be limited.

### TABLE 4 Isolation-by-environment (IBE) tests with different SNP data sets

|                | Temperature | Precipitation |
|----------------|-------------|---------------|
|                | Mantel test | Partial Mantel test | Mantel test | Partial Mantel test |
|                | $r_s$ | $p$ | $r_s$ | $p$ | $r_s$ | $p$ | $r_s$ | $p$ |
| Full data set of the 20,572 SNPs | | | | | | | | |
| All            | 0.120 | .048 | 0.089 | .108 | 0.048 | .250 | 0.023 | .347 |
| SC             | 0.500 | .500 | NaN   | NA   | 1.000 | .167 | 1.000 | NA   |
| WH             | 1.000 | .167 | 1.000 | NA   | -1.000 | 1.000 | -1.000 | NA   |
| HN             | 0.476 | .009 | 0.478 | .013 | -0.184 | .903 | -0.185 | .907 |
| The 360 SNPs under diversifying selection identified by BayeScan | | | | | | | | |
| All            | 0.105 | .062 | 0.056 | .187 | 0.035 | .298 | 0.002 | .441 |
| SC             | 0.500 | .500 | NaN   | NA   | 1.000 | .167 | 1.000 | NA   |
| WH             | -1.000 | 1.000 | -1.000 | NA   | 1.000 | .167 | 1.000 | NA   |
| HN             | -0.080 | .658 | -0.083 | .673 | 0.127 | .193 | 0.129 | .205 |
| The 69 SNPs associated with temperature | | | | | | | | |
| All            | 0.258 | .002 | 0.251 | .001 | 0.088 | .135 | 0.065 | .210 |
| SC             | 0.500 | .500 | NaN   | NA   | 1.000 | .167 | 1.000 | NA   |
| WH             | 0.500 | .500 | 1.000 | NA   | -0.500 | .833 | -1.000 | NA   |
| HN             | 0.789 | .001 | 0.795 | .001 | -0.190 | .913 | -0.194 | .926 |
| The 248 SNPs associated with precipitation | | | | | | | | |
| All            | 0.155 | .037 | 0.105 | .072 | 0.116 | .089 | 0.083 | .165 |
| SC             | 1.000 | .167 | NaN   | NA   | 0.500 | .500 | NaN   | NA   |
| WH             | 1.000 | .167 | 1.000 | NA   | -1.000 | 1.000 | -1.000 | NA   |
| HN             | -0.082 | .694 | -0.069 | .680 | -0.192 | .900 | -0.209 | .912 |
| The 28 SNPs associated with altitude | | | | | | | | |
| All            | 0.099 | .061 | 0.004 | .455 | 0.329 | .001 | 0.292 | .001 |
| SC             | 0.500 | .500 | NaN   | NA   | 1.000 | .167 | 1.000 | NA   |
| WH             | -0.500 | .833 | NaN   | NA   | 0.500 | .500 | NaN   | NA   |
| HN             | 0.306 | .032 | 0.316 | .027 | 0.250 | .089 | 0.246 | .088 |
| The 19,880 neutral SNPs | | | | | | | | |
| All            | 0.118 | .062 | 0.090 | .093 | 0.037 | .295 | 0.014 | .390 |
| SC             | 0.500 | .500 | NaN   | NA   | 1.000 | .167 | 1.000 | NA   |
| WH             | 1.000 | .167 | 1.000 | NA   | -1.000 | 1.000 | -1.000 | NA   |
| HN             | 0.521 | .004 | 0.524 | .003 | -0.177 | .887 | -0.178 | .882 |

*Note:* The outcomes NaN (not a number) and NA (not applicable) in the partial Mantel tests occurred because variance in some tests was effectively zero, and test statistics could not be estimated. Given are simple (Mantel test) and tests accounting geographic distance among localities (partial Mantel). $r_s$ = Spearman rank correlation coefficient. $p$ refers to empirical significance level obtained from 1,000 permutations.
The IBE analyses revealed some positive associations between genetic and environmental distances (temperature and precipitation) independent of geographic distance, providing further evidence for the role of environmental factors driving patterns of genetic differentiation in certain genomic regions. In our tests, IBE was detected mainly in cases where the analyses were restricted to outlier loci indicated to be associated with variation in temperature and precipitation, corroborating the conjecture that these environmental factors have been drivers of genetic differentiation among M. fissipes populations.

4.3 | Genetic variability within M. fissipes populations

The levels of genetic variability within local population of M. fissipes, as assessed by different measures of diversity, were generally moderate to high. However, the island populations tended to exhibit less genetic diversity than the mainland populations. This aligns with the results of numerous previous studies which have found reduced genetic diversity in insular as compared with mainland populations (Bromham & Woolfit, 2004; Frankham, 1997; García-Verdugo et al., 2009; Mason et al., 2011; Wang et al., 2014; Yamada & Maki, 2012). Reduced genetic diversity in island populations can be understood based on the fact that they are closed populations subject to stronger effects of inbreeding and genetic drift than that experienced by outbred mainland populations. However, in our case, the differences in genetic diversity between island and mainland populations were not significant.

4.4 | Implications for conservation

The steep global population decline of amphibians has received increasing attention in the scientific community (Alroy, 2015; Blaustein et al., 1994, 2010; Collins & Storfer, 2003; Cushman, 2006; Ficetola et al., 2015; Grant et al., 2020; Houlaahan et al., 2000; Lips et al., 2005; Stuart et al., 2004; Wake, 1991, 2012; Wake & Vredenburg, 2008), and genetic studies can inform conservation of amphibian populations (Neal et al., 2020; Shaffer et al., 2015; Storfer et al., 2009). Although M. fissipes is a species which is not considered threatened, it occurs in areas experiencing heavy anthropogenic activity and rapid socio-economic development, as many other Chinese amphibians (Luo et al., 2015). Furthermore, it also occurs on island isolates, which are known to be generally particularly vulnerable to loss of genetic diversity (Frankham, 1997). The moderate levels of genome-wide genetic differentiation among M. fissipes populations, and in particular, the evidence for adaptive differentiation suggests that different populations of this species cannot be considered a homogenous entity. The significant adaptive differentiation among local populations should be taken into consideration by the conservation and management actions pertinent to them. The adaptive differentiation among local populations suggests that possible conservation or management efforts should target local populations, rather than the entire species, and the island populations should be of particular concern as they are likely to be first ones to suffer negative effects of loss of genetic variability and suffer reduced ability to adapt to future environmental changes (Chapman et al., 2009).

4.5 | Methodological considerations

It is now widely recognized that many commonly used genome scan approaches are prone to false-positive findings of local adaptation (reviewed in: Hoban et al., 2016). Approaches to reduce the rate of false-positive findings include the use of null models incorporating information on the demographic history of the focal populations, as well as the use of relatedness among populations for correcting for a neutral population structure (Hoban et al., 2016). Similarly, access to a reference genome may markedly improve the inference of genetic local adaptation by reducing errors due to incomplete information (Li & Durbin, 2009; McKenna et al., 2010). One can also summarize correlated environmental parameters with a dimensionality reduction analysis such as principal component analysis (Lasky et al., 2012), and correlate allele frequencies with the obtained principal components. Moreover, composite measures of selection can improve one’s ability to filter selection signals, but they are not a panacea and univariate statistics they summarize can limit their power (Lotterhos et al., 2017). Unfortunately, most of these approaches are not implementable with data consisting of pooled DNA samples.

Theoretical studies have suggested that the pool-seq strategy can be more effective in SNP discovery and can provide more accurate estimates in allele frequency than individual sequencing (Guo, Lu, et al., 2016; Schlötterer et al., 2014). Nevertheless, other studies have pointed out shortcomings in the pool-seq strategy (Anderson et al., 2014; Lynch et al., 2014; Schlötterer et al., 2014). These shortcomings are related to the facts that (a) pipetting or errors of DNA quantification may lead to differential representation of individuals and bias allele frequency estimation; (b) it is more difficult to identify improper alignments of short reads based on the reference sequences in pool-seq than in individual sequencing. Despite these possible shortcomings, pool-seq has been recognized as a valid approach to study population adaptive differentiation among animal populations (Corander et al., 2013; Guo et al., 2015; Guo, Li, et al., 2016; Guo, Lu, et al., 2016; Rellstab et al., 2013). Furthermore, by following the fairly stringent protocol of Schlötterer et al. (2014), we believe that our results and inferences should be reliable and not subject to biases stemming from the issues listed before.

5 | CONCLUSION

By utilizing 20,572 SNP loci, we explored genome-wide genetic variability and differentiation within and among M. fissipes populations...
covering a large geographic area. The overall degree of genetic differentiation was moderate to high. Numerous SNPs were found to be in strong association with variation in average annual temperature and/or precipitation across the sampled locations, suggesting that ecological factors play an important role in driving genetic differentiation among local M. fissipes populations. In general, our results provide strong genomic support for the view that adaptive differentiation among amphibian populations can be considerable, and we suggest that this heterogeneity should be factored into planning conservation and management of amphibian species and populations. We further note that although the Indo-Burma and the mountains of south central China have been already recognized as important biodiversity hotspots, our results show that they may contain yet unrecognized genetic biodiversity as evidenced by intraspecific genetic heterogeneity uncovered in this study.

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CONFLICT OF INTEREST
The authors claim no conflict of interests.

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