Adaptive selection and reshaping of the genetic structure of an immune gene in invasive Lithobates catesbeianus

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Abstract

When alien species enter new environments, they face new challenges. They encounter new predators, parasites, or pathogenic bacteria, which leads to the rapid evolution of invasive species to adapt to new habitats, where immune-related genes play a significant role. Lithobates catesbeianus is one of the worst invasive species in the world; it rapidly spread worldwide in the 19th century and invaded China in the 1950s. We predicted the possible transmission routes of bullfrogs in China through microsatellite loci and clustered the populations at different points. We studied the adaptive selection and drift reshaping of the genetic structure of an immune genetic major histocompatibility complex (MHC) in invasive bullfrogs in 23 Chinese invasive populations (13 island and 10 mainland sites) and two American populations (Kansas and California). The Chinese bullfrog populations were divided into 6 clusters by microsatellite structure, and the MHC diversity in each cluster was different. The genetic diversity of both microsatellite and MHC genes decreased due to the bottleneck effect and the rate of diversity loss at functional and neutral loci, and there was no significant difference. We found that two MHC alleles were endemic in Chinese populations, which corresponded to distinct functional supertypes. We also analyzed the impact of environmental factors and island effects on the diversity of MHC genes. These results indicate that rapid evolution plays an important role in maintaining functionally important MHC variation during the bullfrog invasion process and provide evidence that low genetic diversity can result in successful invasion.

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Abstract

When alien species enter new environments, they face new challenges. They encounter new predators, parasites, or pathogenic bacteria, which leads to the rapid evolution of invasive species to adapt to new

habitats, where immune-related genes play a significant role.*Lithobates catesbeianus* is one of the worst invasive species in the world; it rapidly spread worldwide in the 19th century and invaded China in the 1950s. We predicted the possible transmission routes of bullfrogs in China through microsatellite loci and clustered the populations at different points. We studied the adaptive selection and drift reshaping of the genetic structure of an immune genetic major histocompatibility complex (MHC) in invasive bullfrogs in 23 Chinese invasive populations (13 island and 10 mainland sites) and two American populations (Kansas and California). The Chinese bullfrog populations were divided into 6 clusters by microsatellite structure, and the MHC diversity in each cluster was different. The genetic diversity of both microsatellite and MHC genes decreased due to the bottleneck effect and the rate of diversity loss at functional and neutral loci, and there was no significant difference. We found that two MHC alleles were endemic in Chinese populations, which corresponded to distinct functional supertypes. We also analyzed the impact of environmental factors and island effects on the diversity of MHC genes. These results indicate that rapid evolution plays an important role in maintaining functionally important MHC variation during the bullfrog invasion process and provide evidence that low genetic diversity can result in successful invasion.

Keywords: major histocompatibility complex, microsatellite, alien species, rapid evolution, genetic drift, population bottleneck

INTRODUCTION

Invasive species are threatening biodiversity at an accelerating rate worldwide and exerting increasingly negative economic impacts (Pimentel, Lach, Zuniga, & Morrison, 2000; Pimentel, Zuniga, & Morrison, 2005). Moreover, invasions can lead to the extinction of native species (Doherty, Glen, Nimmo, Ritchie, & Dickman, 2016). Successful invasion requires an invasive species to overcome environmental factors such as climate, seasonal changes, available resources and mortality risk caused by natural enemies (Seiter & Kingsolver, 2013). Increased genetic variation and evolutionary potential aid the success of invasive species (Seiter & Kingsolver, 2013). Since pathogens are one of the major sources of mortality in natural populations, rapid adaptive evolution of immune genes plays a crucial role in defending against pathogenic bacteria and parasites during the invasion process (Eizaguirre, Lenz, Kalbe, & Milinski, 2012; Pruter et al., 2020). Although there have been some studies on immune genes in alien species (Martin et al., 2017), there is little comparison of the genetic diversity of populations in invasive areas versus the origin and how island effects and environmental factors affect the major histocompatibility complex (MHC) diversity.

The MHC, which is one of the most polymorphic regions of the vertebrate genome (Piertney & Oliver, 2005), plays a critical role in the expansion of invasive species by protecting against pathogens (Trowsdale, 2004; Wang et al., 2017) and mediating mate choices (Ejsmond, Radwan, & Wilson, 2014; Milinski, 2006). Extremely high polymorphism coupled with evidence of strong positive selection has made MHC genes a textbook model in studying the role of natural selection in maintaining genetic variation (Radwan, Babik, Kaufman, Lenz, & Winternitz, 2020). The evolution of the number of MHC genes in the genome is driven by a complex interaction among three factors: pathogen richness, the intrinsic cost of expressing additional MHC variants, and the pathogen mutation rate (Bentkowski & Radwan, 2019). The gene structure of MHC may be more diverse due to the spatially heterogeneous distribution of pathogenic bacteria (Christophe et al., 2017). Over 20 years of research, empirical studies offer different conclusions of the relative roles of different evolutionary forces, selection and genetic drift acting on MHC genes during population bottlenecks. Empirical analyses of MHC and neutral genetic variation have proven equivocal under different selection pressures, with MHC variation eroded at a rate that exceeds (Lillie et al., 2015), equals (Miller, Allendorf, & Daugherty, 2010) or is less than that of neutral loci (Oliver & Piertney, 2012; Sutton, Nakagawa, Robertson, & Jamieson, 2011). Differences in genetic diversity of functional and neutral loci may reflect differences in mutation rates, coalescence times (Slatkin, 1995) and historical selection pressures (Radwan, Biedrzycka, & Babik, 2010). Information on historical patterns of genetic diversity provides an important baseline for understanding the changes in genetic variation following population bottlenecks. Identifying the factors that affect the genetic structure of invasive species is important to understand the process of successful establishment and evolution and to efficiently protect local populations by preventing the spread and eradication of

alien species (Lee, 2002; Prentis, Wilson, Dormontt, Richardson, & Lowe, 2008). Environmental factors and human activities can affect the genetic variation, such as the hunting pressure, residence time, and number of farms (Wang et al., 2019).

Structural regions of MHC genes commonly include antigen-binding sites (ABSs) and other sites (non-ABS) (Bjorkman et al., 1987). ABSs are characterized by an excess of nonsynonymous substitutions over synonymous substitutions, which is a signature of positive selection. In contrast, non-ABSs are largely neutral or potentially even under negative selection (Hughes & Yeager, 1998). A comparison of genetic diversity between ABS and non-ABS MHC genes from the same populations will provide important insights into how positive selection and nearly neutral processes shape the geographical patterns of genetic variation (E et al., 2020; Spurgin & Richardson, 2010; Sutton et al., 2011).

Rapid evolution plays an important role in predicting and managing biological invasion by conducting successful translocations, introductions, and reintroductions of species (Benson, 2010; Colautti & Lau, 2015). Many forms of rapid evolution have been demonstrated in the spread of population invasions, including adaptation to new habitats (Colautti & Lau, 2015), the ability to spread (Lombaert et al., 2014; Phillips, Brown, Webb, & Shine, 2006), and the evolution of geographic clines along climatic gradients (Colautti & Barrett, 2013; Huey, 2000). Recent studies revealed that variation in specific immune vectors, which may be important for invasion success, might lead to higher variance and enable invasive species to reduce the overall physiological cost of immunity while maintaining the ability to efficiently defend against novel parasites encountered (Pruter et al., 2020).

The American bullfrog (*Lithobates catesbeianus*) is one of worst invaders in the world (Luque, 2014). The invasion of bullfrogs has led to the decimation of some native amphibian populations due to their high reproductive ability and high densities or predation on native amphibians (Liu et al., 2018). Bullfrogs can also spread the chytrid fungus *Batrachochytrium dendrobatids* , which is the proximate cause of rapid amphibian declines across diverse biomes (Garner et al., 2006). Previous results from a study in Zhoushan, China, showed that the density and species richness of native frogs were negatively correlated with the post-metamorphosis bullfrog density (Li, Ke , Wang, & Tim, 2011). Bullfrogs are intentionally introduced for aquaculture or as pets (Kraus, 2009), and wild populations of bullfrogs are established by farm escapes or artificial releases (Ficetola, Thuiller, & Miaud, 2007; Liu, McGarrity, Bai, Ke, & Li, 2013; Wang et al., 2009).

Bullfrog invasions in China provide an ideal model system to examine genetic variation in populations of alien species between invaded areas and origins (Bai, Ke, Consuegra, Liu, & Li, 2012). Bullfrogs were introduced for aquaculture from Cuba to Hanshou, Hunan Province, China, in the late 1950s through a single introduction, and the population from Cuba is native to North America (Li et al., 2011; Liu & Li, 2009). Feral bullfrog populations are established by bullfrogs escaping from farms or by frogs released by people (Liu et al., 2013). Permanent water bodies are critical to the physiological and ecological needs of bullfrogs at all stages of their life history, associated with their establishment and dispersal (Liu & Li, 2009); thus, there are geographical barriers to bullfrog transmission between Chinese provinces that prevent genetic exchange between bullfrogs. We compared the environmental factors in different provinces to perform correlation analysis on genetic diversity in this study.

We hypothesized that the rapid evolution and bottleneck effect of invasive bullfrog species had a profound effect on genetic variation. First, the diversity of the bullfrogs in microsatellites and MHC was lost in the invasion area relative to the origin area due to the bottleneck effect. Second, due to selection pressures and rapid evolution, new MHC genotypes may appear in the invasion zone, and the frequency of genotypes may change. Third, we suspect that environmental factors influenced the genetic diversity of MHC. Finally, we predict the impact of the island effect on MHC diversity.

MATERIALS AND METHODS

2.1 Study area and sampling for bullfrogs

In total, 25 bullfrog populations were collected during the nonbreeding seasons of 2010-2015: two American populations in Kansas and California, where the frog originated, which were provided by the American Museum (Sternberg Museum and the University of California, Berkeley Museum of Vertebrate Zoology); 23 Chinese populations, including 10 mainland regions and 13 Zhoushan Archipelago regions (China) (see Table S1 and Fig S4 for details). We randomly drew 20 individuals from 500 frogs detected per site for MHC genotyping. The captured frogs were released alive after clipping off the tip of the third toe of the right foot. The sampled toe tips were preserved in 95% ethanol and stored at -20 °C in the laboratory.

2.2 DNA extractions, MHC genes and multilocus microsatellite genotyping

We followed a standard procedure of DNA extraction (Wang et al., 2019). The tips of bullfrogs' toes (approximately 3 mg) were clipped and placed in a 2-ml centrifuge tube with 100 μl of lysis buffer containing 0.01 M NaCl, 0.1 M EDTA, 1 mg/ml proteinase K, 0.01 M Tris–HCl (pH 8.0) and 0.5% Nonidet P-40. The tube was vortexed for 1 min at ambient temperature, centrifuged to recover all material from the bottom of the tube, and incubated at 50 °C for 120 min and at 95 °C for 20 min. Then, the tube was centrifuged at 12,000 rpm for 3 min at a cold temperature, and the extract was diluted to one tenth of its original concentration to amplify the DNA by polymerase chain reaction (PCR).

The MHC class-II β gene in the DNA extract was amplified using the primers ForN ('5-GTTCTCTCCCCGCAGATGATTTC-3') and RevA (5 -GCATAGCAGACGGAGGAGT-3') (barcode-ForN and barcode-RevA) (Mulder et al., 2017). In total, 20 pairs of barcodes were used to distinguish different individuals in each population. PCRs were run with Pfu DNA polymerase (TransGen, Beijing, China), including a denaturing step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 20 s, 51 °C for 20 s, and 72 °C for 30 s, and a final extension of 5 min at 72 °C. The amplification products were sequenced on an Illumina HiSeq2500 platform (Novogene Bioinformatics Technology Company, Beijing, China) using a 250-bp paired-end strategy. All samples were run on a single lane.

The bullfrog populations were genotyped using nine nuclear microsatellite loci (BF1, BFD11, and GenBank accessions AY323928-AY323933) (Wang et al., 2019). Primer sequences and the procedures used during DNA amplification were based on previously published data (Austin, Davila, Lougheed, & Boag, 2003; Oosterhout, Hutchinson, Wills, & Shipley, 2004) (Table S1, Supporting Information). All primers were tagged with 5'-fluorescein bases (TAMRA, FAM or HEX). PCRs were performed as previously described. The amplification conditions consisted of an initial denaturation at 94 degC for 3 min followed by 35 cycles of 10 s at 94 degC, 30 s at the annealing temperature (Table S2), and 30 s at 72 degC and a final 10-min extension at 72 degC. Then, the PCR products were separated by 2% agarose gel electrophoresis. Following amplification, the PCR products were resolved using an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Next, GENESCAN version 3.7 (Applied Biosystems) and GeneMarker version 1.71 (SofGenetics) were used to score the microsatellite fragments.

2.3 Statistical analysis of multilocus microsatellites

We applied MICRO-CHECKER 2.2.3 (Oosterhout et al., 2004) to quantify the scoring errors resulting from factors such as the large allele dropout, stuttering or null alleles. GENEPOP version 4.0 was employed to test the linkage disequilibrium and Hardy-Weinberg equilibrium (Raymond & Rousset, 1995). We used Bonferroni corrections to test multiple comparisons (Rice, 1989).

We applied GenAlEx 6.5 to quantify the expected heterozygosity (He), observed heterozygosity (Ho), and mean number of alleles (Na) for each population (Peakall & Smouse, 2006). Asymmetric migration rates were estimated using the MIGRATE-N 3.2.7 program (Beerli, Peter, Felsenstein, & Joseph, 1999).

We examined the genetic structure of the sampled bullfrogs using STRUCTURE (Pritchard &Stephens, 2000). The optimal number of clusters (the best K) was determined using the method of reference (Evanno, Regnaut, & Goudet, 2005) and was implemented in STRUCTURE HARVESTER (Earl & vonHoldt, 2011). STRUCTURE 2.3.4 was used to calculate the K value of the cluster. In our research, the admixture model and correlated allele frequency model were used. STRUCTURE was run with 10 repetitions of 1,000,000

iterations of MCMC simulation, following a burn-in of 200,000 iterations at K=1-10.

2.4 Statistical analysis of MHC genes

The sequences for all experimental samples were processed using AmpliSAT tools to automatically genotype MHC genes from high-throughput sequencing data (Sebastian, Herdegen, Migalska, & Radwan, 2016). AmpliCHECK was used with default parameters to establish the length of the desired PCR products (markers), percentage of errors in variants and a threshold frequency to decide whether a variant was real or only an artifact. AmpliSAS was used to filter the obtained sequences using default parameters to discard sequences of the wrong length that did not blast to MHC, have stop codons and only retain alleles that are recovered from at least 2 different individuals (Simone Sommer, Alexandre Courtiol, & Mazzoni, 2013).

Arlequin 3.11 software was used to calculate the pairwise FSTs of MHC genotype diversity between populations (Laurent Excoffier, Guillaume Laval, & Schneider, 2005). Relationships among the genotypes were visualized by reconstructing a median-joining network by Network 5.0.1.1 (Bandelt, Macaulay, & Richards, 2000). Nucleotide diversity is frequently used to investigate the spatial patterns of genetic diversity. We measured the genetic diversity at exon 2 of MHC II for bullfrogs using the average nucleotide diversity by the DnaSP6 software (Sommer, Courtiol, & Mazzoni, 2013).

Antigen-binding sites (ABS) were inferred from the structure of ABS in human leucocyte antigen (Bjorkman et al., 1987). Tests for positive selection at all sites, ABS and non-ABS of exon 2 of MHC II, were globally and separately performed for each population by comparing the number of nonsynonymous substitutions per nonsynonymous codon site (dN) to the number of synonymous substitutions per synonymous codon site (dS). A Z test with Jukes–Cantor correction was performed to determine if $dN > dS$ using MEGA 5 (Kumar, Stecher, & Tamura, 2016).

Nonrecombinant MHC class II β alleles were divided into functional supertypes using a measurement of functional diversity developed for the MHC, which has been increasingly used in human disease studies and evolutionary biology (Schwensow, Fietz, Dausmann, & Sommer, 2007; Sepil, Lachish, & Sheldon, 2013). Codon-based positive selection was determined using three models: single-likelihood ancestor counting (SLAC), random-effects likelihood (REL) (Pond & Frost, 2005) and mixed-effect model of evolution (MEME) (Murrell et al., 2012) using an online website (Datamonkey Adaptive Evolution Server). Codons identified as being under positive selection by two or more models were retained as amino acid residues for translation (Lillie et al., 2015). Amino acids under positive selection were first characterized using Z descriptors (Sandberg, Eriksson, Jonsson, Sjostrom, & Wold, 1998) and subsequently clustered into supertypes using various clustering methods (hierarchical clustering; K-means clustering; multidimensional scaling) and the "ADEGENET" package in R (Doytchinova & Flower, 2005; Sutton, Robertson, & Jamieson, 2015) (Table S4).

The lengths of a sequence and the ABS in the sequence have important effects on the estimation of nucleotide diversity and inference of natural selection at MHC genes (Li et al., 2020; Yang, Wong, & Nielsen, 2005). We used the human MHC II DRB gene (Tong et al., 2006) and alignment of the β1 domain of MHC class II of anurans (Kiemnec-Tyburczy, Richmond, Savage, & Zamudio, 2010) as the reference sequence, and we obtained 45 bp of ABS and 228 bp of non-ABS sequence fragments (Li et al., 2020). All sequence alignments were performed using Clustal W with the default settings in MEGA7 (Kumar et al., 2016).

2.5 Predictor variables

In this work, we focus on four predictor variables of the MHC genetic diversity.

1. Diversity of microsatellites. We predict whether functional sites are associated with neutral sites during rapid evolution. We calculated the expected (He) and observed heterozygosity (Ho) and the mean number of alleles (Na) of microsatellites for each population.
2. Number of amphibian species. Because amphibians carry pathogens, we predicted whether the MHC diversity was related to the amphibian density. For each location, we counted the number of amphibian species by using GIS maps of amphibian species ranges available at the IUCN Global Amphibian

Assessment. Sampling sites were divided into 10 × 10 km rasters, and the average number of amphibian species in the raster nearest to the sampling site was taken for the analysis.

3. Human population density. Because of the impact of human activities on amphibians, we predicted whether the human density would affect the MHC diversity. For each population, we determined the density of the human population using GIS maps at the Resource and Environment Science and Data Center (http://www.resdc.cn/DOI).
4. Biome. Different biomes have differences in the ecological environment and available resources, so we predict whether biome will affect the MHC diversity. We used the World Wildlife Fund and GIS to extract information about the biome for each population.

2.6 Island effect on MHC diversity

Data on the approximate date of invasion of bullfrogs and environmental features were obtained from Wang et al. (2017, 2019) (Table S5). We used the log10X-transformed island area, number of island bullfrogs raised, distance of the island from the mainland and period of bullfrog presence on the island as variables to measure the island effects (Wang et al., 2017). We evaluated the relative importance of island features on the genetic diversity of MHC across insular populations using single-variable regression. We performed hierarchical partitioning (HP) analysis (Nally, 2002; Wang et al., 2017) to quantify the unique contribution of each predictor to MHC diversity. The HP analysis compares all potential models to estimate the independent and shared contributions of each predictor to a dependent variable (Nally, 2002). HP was implemented using the "hier.part" function, and significance testing was performed using the "rand.hp" function in R (Wang et al., 2017).

RESULTS

3.1 Microsatellite genetic structure in Chinese bullfrog populations

Based on the ΔK value, the most likely structure clustering was K= 6 (Fig 3). Figure shows the admixture frequency of populations. The bullfrog population in China mainly consists of six clusters: the populations of Tibet and the United States; the mainland populations of Guangdong, Hunan, Shandong, Sichuan and Shiping; the mainland populations of Luguhu and Anhui; three clusters of the remaining island populations, which comprised admixture populations.

The expected heterozygosity (He) and observed heterozygosity (Ho) of microsatellites for native populations were 0.69 ± 0.04 and 0.60 ± 0.06, respectively, and those for invasive populations were 0.69 ± 0.08 and 0.65 ± 0.07. The mean number of alleles (Na) was 7.39 ± 0.08 for the native populations and 5.38 ± 0.89 for the invasive populations. Overall, the US populations had a higher microsatellite allelic richness (Na) than the Chinese populations (one-way ANOVA, F= 5.846, P= 0.024).

3.2 Genetic diversity of MHC in Chinese bullfrog populations

We compared the results of AmpliCHECK and AmpliSAS with the exclusion of artifacts or chimeras. Ten genotypes were retained with a length of 273 bp. Three alleles were identical to those previously recovered in bullfrogs (Mulder et al., 2017) (GenBank Accession nos: KY587180, KY587181, and KY587170). Comparing the distribution of all genotypes in bullfrog populations, two genotypes are endemic to Chinese populations, two genotypes are endemic to American populations, and the remaining genotypes are common to Chinese populations and American populations. (fig 1)

We identified 10 alleles in total in the 273 bp of the MHC II β gene region in 500 individuals, none of which contained indels or stop codons. Populations carried 3.17±0.53 MHC IIβ alleles, ranging from a minimum of 2 alleles at the Cezi site to a maximum of 4 alleles at the California site. We found that only one genotype was common between mainland China (Anhui, Xizang) and two American populations in bullfrog, two genotypes were restricted to the origin of California and Kansas in the United States, and two genotypes were restricted to China (Anhui, Zhejiang, Yunnan, Suzhou, LH, XS, ZS, ZJJ). Five alleles were found in two or more populations. The three highest frequency alleles (H1, H2, H3) were shared among all 25 populations. Seven alleles were shared between island and mainland sites. The bulk of the alleles shared between China

and the United States populations (6/10, 60%) were located at central nodes in the phylogenetic network of MHC sequences, which supported their shared ancestry. (Fig S1)

We identified 6 MHC II supertypes. Populations carried 2.29±0.70 supertypes (Table S2, Figure S2), ranging from a minimum of 2 supertypes in most populations to 5 supertypes in California (Table 1). Two supertypes were common to all populations. Both Chinese and American populations have unique supertypes, and four supertypes were shared.

The population genetic diversity of MHC and microsatellites was significantly higher in America than in China: the average was 7.39 ± 0.08 microsatellite alleles and 3.95 ± 0.07 MHC II alleles in American populations; the average was 5.38 ± 0.89 microsatellite alleles and 3.11 ± 0.49 MHC II alleles in Chinese populations (Mann–Whitney U test: df= 23, microsatellite: Z= 2.31, p= 0.007; MHC II alleles: Z= 2.30, p= 0.007). The number of supertypes (ST) was also higher for America than for China: the average 4.5 ± 0.71 supertypes in American populations and 2.1 ± 0.19 supertypes in Chinese populations (Mann–Whitney U test: df= 23, Z= 2.49, p= 0.007) (Table 1).

The observed alleles contained 91 polymorphic nucleotide sites, with average nucleotide differences (k)= 29.178, gene diversity (Hd) (± SD) = 1 ± 0.000 and nucleotide diversity (Pi) (± SD) = 0.028 ± 0.023 (Table 1). We detected significant positive selection at MHC II in each population (dN/dS range: 2.28-4.74; p< 0.05 for each population) (Table S2). Significant positive selection was detected for ABS (dN/dS range: 5.069-17.085; p <0.05 for each population) and non-ABS (dN/dS range: 1.534-3.021; p <0.05 for each population). The dN/dS ratios were higher in invasive populations (mainland and islands) than in native populations for both ABS (13.203 ± 2.626 in mainland, 13.043 + 2.144 in islands vs. 5.309 ± 0.34) and non-ABS (2.293 ± 0.578 in mainland, 2.243 ± 0.389 islands vs. 1.735 ± 0.285) (Mann–Whitney U test: all sites: df= 23, Z= 2.23, p= 0.026. ABS: Z = 2.33, p= 0.020; non-ABS: Z= 1.72, p= 0.085).

3.3 Factors affecting the invasive population genetic diversity of MHC

The model averaging analysis shows that the diversity of MHC was positively correlated with the amphibian species density (average value of P <0.05) but not significantly correlated with the microsatellite diversity, human population density or biome (Table 2; Fig 4). The model-averaged 95% confidence intervals for these variables also excluded zero.

3.4 Island effect on the MHC genetic diversity

The number of MHC genotypes and supertypes of bullfrogs was positively correlated with the residence time, island area, and number of bullfrogs raised. (Pearson correlation, number of genotypes: R^2= 0.417, p=0.017 for the island area; R^2= 0.446, p= 0.01 for the residence time; R^2= 0.624, p= 0.001 for the number of bullfrogs raised, supertypes: R^2= 0.280, p= 0.063 for the island area; R^2= 0.566, p= 0.003 for the residence time; R^2= 0.497, p= 0.007 for the number of bullfrogs raised) (Fig 5). There was no significant relationship between MHC gene diversity and island distance to the mainland.

The HP analysis shows that the bullfrog residence time, island area, and number of bullfrogs raised accounted for statistically significant and unique portions of MHC variation (Na and ST). The distance to the mainland was not significant for the MHC diversity (Table 3).

DISCUSSION

The results showed lower genetic diversity at both MHC class-IIβ and neutral microsatellites in Chinese populations of bullfrogs, which suggests that population bottlenecks following species invasion have led to the loss of genetic diversity at both adaptive and neutral loci through drift (Prediction 1). We detected new haplotypes and supertypes of MHC for bullfrogs in both mainland and island populations of China (Prediction 2). Among the predicted environmental factors, the MHC diversity was positively correlated with the amphibian density (Prediction 3). Due to the island effect, the genetic diversity of bullfrogs in the Zhoushan Archipelago was affected by many factors such as the island area, number of rearing bullfrogs and residence time (Prediction 4). Our study provides rare information on the impact of rapid evolutionary and

genetic drift on the genetic diversity of immune gene MHC and microsatellite loci in invasive bullfrog species in invasion areas.

Several studies have proven the important function of immune genes in the process of alien species invasion. For example, Martin (Martin et al., 2017) detected that TLR-4 expression may influence house sparrow expansion in Kenya. Pruter (Pruter et al., 2020) found that variations in specific immune effectors, which may be important for invasion success, may lead to higher variance and enable invasive species to reduce the overall physiological cost of immunity while maintaining the ability to efficiently defend against novel parasites encountered. In our study, we found that invasive species could successfully invade and spread even when the MHC diversity was reduced due to bottleneck effects.

Although two haplotypes were first identified in China, there are three potential sources. First, the new haplotypes may be a rare haplotype in the United States, and our samples (2 populations) in the United States are insufficient. Second, the new haplotypes could have originated in Cuba as a result of mutation and were introduced to China in the late 1950s. According to the records, bullfrogs were widely farmed in Cuba in the early 1940s for trade and food supply, and genetic variation could have occurred in Cuba and spread to China (Zeng, 1998). Finally, after bullfrogs entered China, new genotypes arose over dozens of generations of genetic drift due to the bottleneck effects and rapid evolution. Although specific reasons for the emergence of new haplotypes are unknown, rapid evolution and genetic drift play a crucial role in bullfrog populations in China.

Supertypes provide a functional classification for different MHC alleles and can help clarify links between MHC sequences and disease resistance (Acevedo-Whitehouse & Cunningham, 2006). Several recent studies have identified a relationship between specific supertypes and disease infection and resistance (Savage & Zamudio, 2016; Wang et al., 2019). For example, Wang found that the representation of key functional supertypes was positively correlated with the abundance of specific viruses in the environment. Similarly, Savage and Zamudio (Savage & Zamudio, 2016) identified an MHC II supertype in *Lithobates yavapaiiensis* associated with increased susceptibility to amphibian chytridiomycosis, a disease believed to be responsible for recent declines in many amphibian species. In our study, we found a new supertype in the Chinese population, which provides theoretical support for further research on amphibian resistance to pathogens.

Chinese bullfrog populations have significantly reduced genetic diversity in both MHC and microsatellites compared to American populations. Although the California bullfrog is also an invasive area, the genetic diversity of bullfrogs and the Kansas population in the place of origin are not very different, which should be related to the time of invasion and the route of introduction. Serious reductions in genetic diversity in invading areas have been commonly hypothesized to be a result of low propagule pressures and the founder effect, especially the lack of multiple introductions from different native regions (Bai et al., 2012; Dlugosch & Parker, 2008). Our results are consistent with the documented fact that bullfrogs were introduced to China only once from Cuba in the late 1950s (Liu & Li, 2009). However, the rates of diversity loss of microsatellites and MHC were similar when American populations were used as a reference (Fig 6), which is not consistent with our initial prediction of more diversity loss from MHC. Our results suggest that invasive populations may mutate in novel environments at both functional and neutral loci.

In model averaging, the results show that the diversity of microsatellites, human population density and biome hardly affect the genetic diversity of MHC, but the MGC was positively correlated with amphibian density (Table 2). Pathogen-mediated selection is the absolute major driver of immune gene MHC evolution, and amphibians are the main carriers of the pathogen (Wang et al., 2019). Future work is necessary to quantify the dynamics of the rapid evolution of MHC on pathogenic bacteria during successful invasion.

We quantified the unique contribution of island characteristics to the MHC diversity of bullfrog populations in Zhoushan Islands and provided additional evidence for a relationship between the rapid evolution of MHC and the island characteristics. Our results suggest that the number of haplotypes and supertypes was positively correlated with the area of the islands, residence time of bullfrogs and number of bullfrogs raised. The positive relationship between MHC diversity and island features is consistent with predictions

from neutral theory (Raymond & Rousset, 1995), which indicates that genetic drift also affected the MHC variation.

Enhanced genetic variation may increase the potential for adaptive evolution, which can lead to rapid evolution by natural selection in novel environments (Jason J et al., 2008; Lee, 2002). Our results support that a successful invasion will reduce the genetic diversity (Tsutsui, Suarez, & Holway, 2000), but it will not damage the colonization and spread of invasive species. The results of this study provided sufficient data for the evolution of immune genes in the MHC of invasive species, and the results show that invasive species can successfully establish and spread even when the MHC diversity is low. We also clustered Chinese bullfrog populations and provided theoretical support to control the invasion and spread of bullfrogs, which can help protect local species.

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DATA ACCESSIBILITY

MHC sequences and microsatellite data are available at DRYAD entry*https://orcid.org/0000-0002-5251-2601*

AUTHOR CONTRIBUTIONS

Y.L. and J.Z. designed the study, S.W., X.L., C.X., collected samples, J.Z., C.X., S.W., X.H., X.Y., performed experiments, J.Z. analysed data, and J.Z. wrote manuscript.

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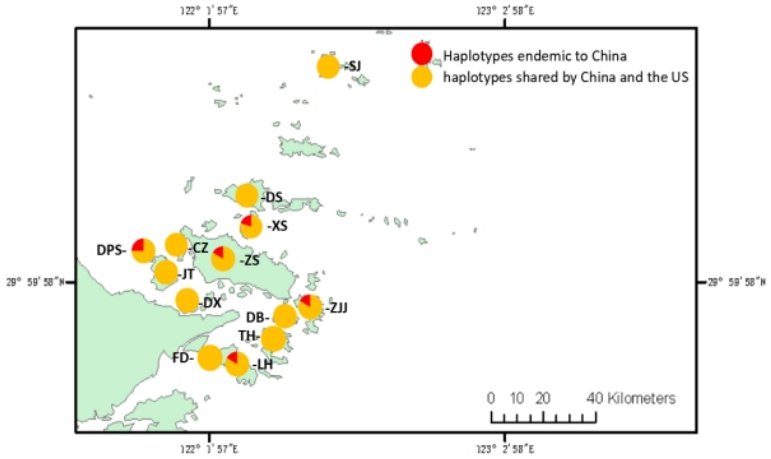
Fig 1

Map of *Lithobates catesbeianus* populations sampled on the Zhoushan Archipelago (a) and the mainland(b), China. Proportion of private and shared MHC alleles (a) for each population is shown by the pie charts. Circle size is proportional to the number of haplotypes. Population abbreviations are as follows: AH, Anhui; GD, Guangdong; HN, Hunan; SD, Shandong; SC, Sichuan; XZ, Xizang; JS, Jiangsu; SP, Shiping; ZJ, Zhejiang; LGH, Luguhu; CZ, Cezi; DB, Dengbu; DPS, Dapengshan; DS, Daishan; DX, Daxie; FD, Fodu; JT, Jintang; LH,Liuheng; SJ, Sijiao; TH, Taohua; XS, Xiushan; ZJJ, Zhujiajian; ZS, Zhoushan.



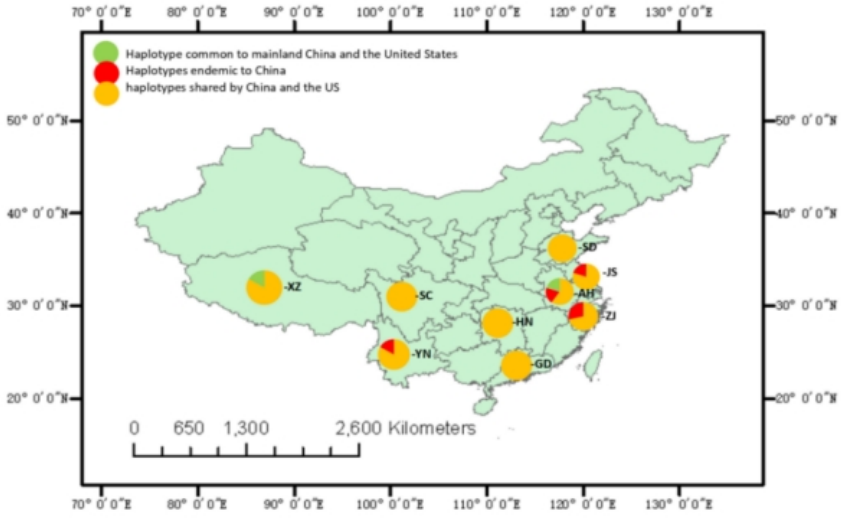


Fig 2

Median-Joining network of MHCIIβ haplotypes of *Lithobates catesbeianus* . The size of the circles is proportional to haplotype frequency. Each color represents locality country.

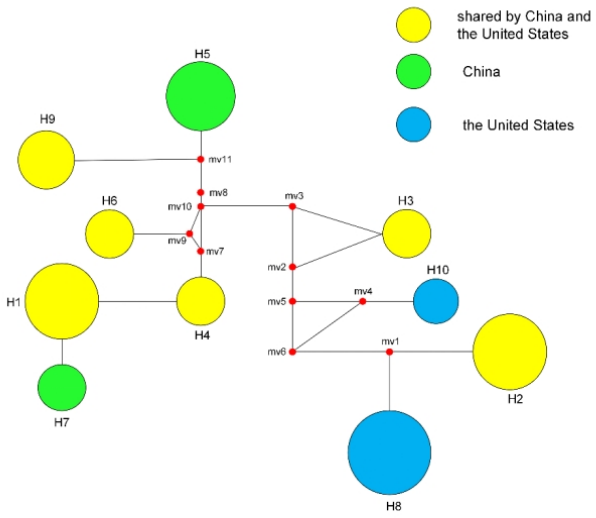


Fig 3

The fraction of the cluster was estimated by STRUCTURE (K=6). The length of color bars expresses a fraction of the cluster (Q value). Population abbreviations are as follows: MC, California; MK, Kansas; AH, Anhui; GD, Guangdong; HN, Hunan; SD, Shandong; SC, Sichuan; XZ, Xizang; JS, Jiangsu; SP, Shiping; ZJ, Zhejiang; LGH, Luguhu; CZ, Cezi; DB, Dengbu; DPS, Dapengshan; DS, Daishan; DX, Daxie; FD, Fodu; JT, Jintang; LH,Liuheng; SJ, Sijiao; TH, Taohua; XS, Xiushan; ZJJ, Zhujiajian; ZS, Zhoushan.

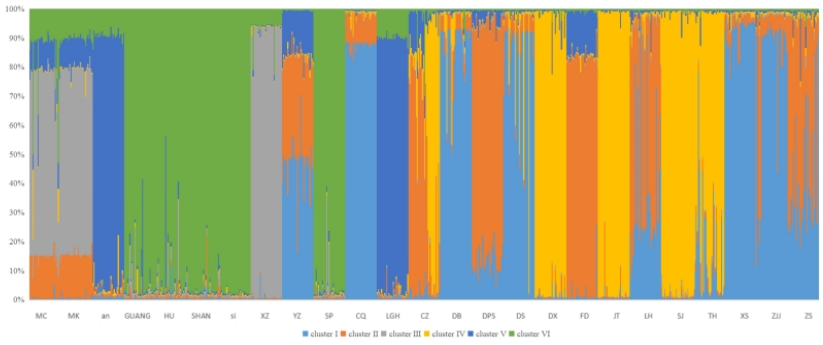


Fig 4

the diversity of MHC was positively correlated with the amphibian species density

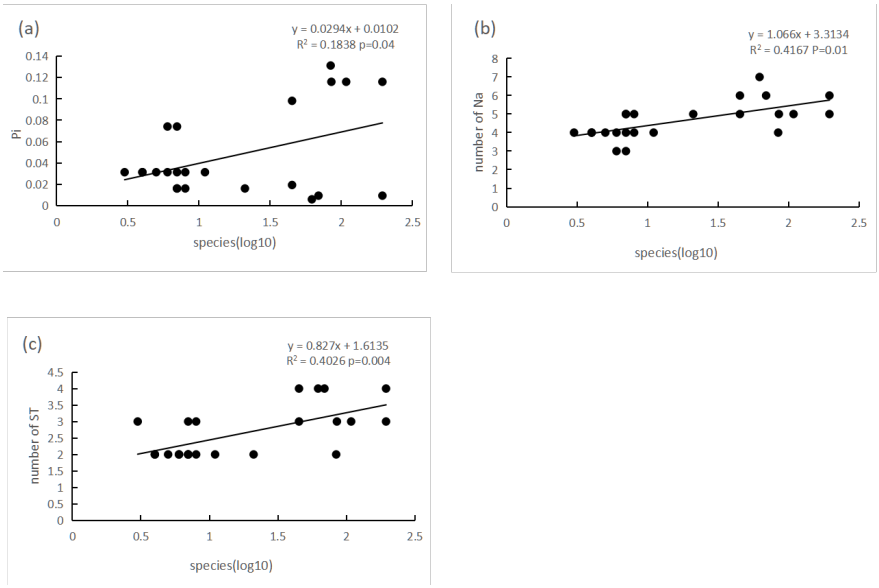
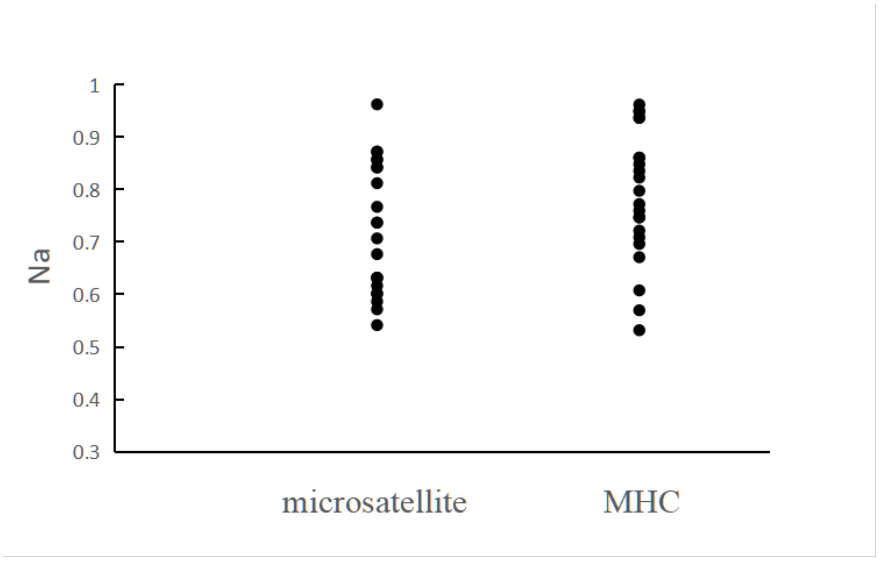


Fig 5

Island effects on MHC diversity. The correlation between the total number of MHC found on each island and the characteristics of the island, Figure A shows the relationship between the residence time of bullfrog invasion and the number of haplotypes. Figure B shows the relationship between the number of bullfrogs raised and the number of haplotypes on the islands. Figure C shows the relationship between the island area and the number of haplotypes on the islands.

Fig 6



Comparisons of standardized genetic diversity (Na) at MHC and microsatellite loci in invasive populations. Standardized diversity is calculated using American populations as a reference. Na = number of alleles.

Table 1

Data on genetic diversity of MHC and microsatellites in bullfrog populations. N, sample size; Na-mic, number of alleles for microsatellites; Na, number of alleles for MHC; ST, number of supertypes; Pi, Nucleotide diversity of

MHC; Ho, observed heterozygosity; He, expected heterozygosity;

		N	Na-mic	Na-MHC	ST	Pi	Ho	He
American population	MC	20	7.44	4	4	0.006	0.64	0.72
	MK	20	7.33	3.9	5	0.016	0.56	0.67
Chinese mainland populations	AH	20	4.44	3.25	2.2	0.098	0.7	0.68
	GD	20	4.67	3.3	2.05	0.016	0.58	0.64
	HN	20	5.78	3.25	2	0.031	0.61	0.64
	SD	20	6.22	3.15	2	0.031	0.72	0.69
	SC	20	5.00	3.7	2.1	0.016	0.73	0.68
	XZ	20	4.22	2.95	2.1	0.009	0.56	0.49
	YZ	20	4.67	2.4	2	0.016	0.7	0.67
	SP	20	6.22	3.4	2	0.016	0.67	0.71
	CQ	20	4.00	3.7	2.8	0.006	0.84	0.64
	LGH	20	6.44	3.4	2.15	0.009	0.7	0.65
Chinese island populations	CZ	20	5.67	2.1	2	0.031	0.72	0.68
	DB	20	5.22	2.85	2	0.031	0.7	0.68
	DPS	20	4.56	2.2	2	0.031	0.71	0.65
	DS	20	6.33	3.05	2	0.031	0.72	0.69
	DX	20	4.67	2.65	2	0.031	0.62	0.55
	FD	20	5.44	2.65	2.1	0.031	0.74	0.68
	JT	20	4.44	2.95	2	0.074	0.57	0.54
	LH	20	6.44	3.75	2	0.016	0.7	0.71
	SJ	20	4.33	2.8	2	0.074	0.51	0.51
	TH	20	5.44	3.8	2	0.031	0.68	0.65
	XS	20	7.11	3.8	2.15	0.016	0.71	0.73
	ZJJ	20	6.00	3.3	2.2	0.016	0.71	0.67
	ZS	20	6.33	2.95	2.45	0.009	0.78	0.74
Overall average			5.54 ± 1.02	3.17 ± 0.53	2.29 ± 0.70	0.0278 ± 0.023	0.68 ± 0.08	0.65

Table 2

A summary of the standard estimates and 95% confidence intervals of regression coefficients based on model averaging. The full model is a GLMM with a beta error structure and a logit link function, and with the within-species genetic diversity as the response variable and 4 variables as predictor variables (fixed effects). Na, number of alleles for MHC; ST, number of supertypes; Pi, Nucleotide diversity of MHC;

			95% Confidence interval	95% Confidence interval	95% Confid
Variables		Standard estimates (β*)	2.50%		97.50%
Pi					
	species(log10)	0.0191366	-0.009412327		0.01110446
	mic	-0.0051474	-0.024376933		0.00148331
	population(log10)	0.0075454	-0.000320955		0.01597899
	biome	0.001167	-0.00071345		0.00322386
Na					
	species(log10)	0.78946	-0.02672832		0.11379498
	mic	0.144	-0.00265757		0.04382238

			95% Confidence interval	95% Confidence interval	95% Confi
	population(log10)	-0.06043	-0.07126609		0.02107682
	biome	-0.05982	-0.01807614		0.00199364
ST					
	species(log10)	0.16877	0.15439739		0.2138681
	mic	-0.036664	-0.22268961		0.13901397
	population(log10)	-0.04224	-0.1257746		0.06708722
	biome	-0.012436	-0.05291872		0.02919755

Table 3

The unique contribution of island features to genetic diversity in MHC gene of bullfrog by HP analysis on China.

	Independent deviance explained (%)	Independent deviance explained (%
Variables	Na	ST
island area km2, log-transformed)	33.63*	16.49*
number of bullfrogs raised log- ransformed)	13.64*	36.31*
Residence time (year)	48.16*	45.10*
Distance to mainland (km, log-transformed)	4.57	2.11

Na, number of alleles; ST, number of supertypes.

*Significant at 0.05.