

# The role of founder effects on the genetic structure of the invasive bullfrog (*Lithobates catesbeianus*) in China

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**Abstract** Founder effects and genetic drift can reduce the genetic diversity and alter the genetic composition of introduced species during the processes of population establishment and spread. Thus, founder effects are of particular concern for introduced commercial populations (usually founded from few individuals) and for the natural populations they interact with. Bullfrogs were initially introduced in China for aquaculture purposes and escapes from farms have established many feral populations. Most of the bullfrog farms currently operative have been founded from a limited number of descendants from the original introductions, providing an excellent framework to elucidate the importance of founder effects and genetic diversity in the establishment and persistence of

invasive species introduced for commercial purposes. We sequenced a region of the mitochondrial cytochrome *b* gene in 510 samples collected from feral and farm individuals across China and compared them to populations in their native range. Only two haplotypes (H43 and H7) were identified, and H43 identified in this study for the first time was present at high frequency in both feral and commercial populations. We show a significant difference in the relative frequency of the two identified haplotypes in commercial and feral populations, and suggest that sequential founding events are responsible for the emerging widely distributed new haplotype and the observed differences in genetic structure between bullfrog populations. Our findings indicate that lack of genetic diversity does not necessarily impair the colonizing ability of invasive species and highlight the potential threat posed by introduced commercial populations, given their unique genetic makeup.

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Propagule pressure · Farm populations

## Introduction

Biological invasions pose one of the most serious threats to biodiversity in both natural and agricultural ecosystems (Pimentel et al. 2005; Sakai et al. 2001; Simberloff and Stiling 1996; Strayer et al. 2006).

The occurrence of non-native invasion has accelerated to an extraordinary degree due to the globalization of economy and caused serious economic loss (Floerl et al. 2009; Vitousek et al. 1996; Westphal et al. 2008). In the United States approximately \$120 billion are lost each year as a consequence of the 50,000 nonnative species (Pimentel et al. 2005). In many cases, the invasive process involves population bottlenecks and founding events, especially in species introduced for biological control or agriculture purposes with a limited number of initial founders (Hufbauer et al. 2004; Simberloff 2009). Furthermore, subsequent dispersals (for example as seedings or stock individuals for mass-production) can result in additional founder events. This stochastic sampling effect can lead to distinct genetic compositions between introduced and source populations and among the introduced populations themselves (Delaney et al. 2009; Hinamoto et al. 2006). In contrast, in cases where the founders come from multiple introductions, the genetic diversity can be higher than in the original native populations and hybridization among individuals from different sources can help the invasive process by facilitating the adaptation to new conditions (Kolbe et al. 2008).

The American bullfrog, *Lithobates catesbeianus* (also known as *Rana catesbeiana*), is listed among the “100 of the World’s Worst Invasive Alien Species” in the Global Invasive Species Database (ISSG 2008). Native to eastern North America, bullfrogs have been widely introduced throughout the world, and established populations have been reported in many regions (Lever 2003). Bullfrogs can affect native amphibian populations through predation and competition for food and habitat (Kats and Ferrer 2003; Kiesecker and Blaustein 1998; Kiesecker et al. 2001; Lawler et al. 1999; Pearl et al. 2004; Li et al. 2011a). Furthermore, recent studies have also shown that bullfrogs are asymptomatic carriers of the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) that is deadly to other frog species (Daszak et al. 2004; Garner et al. 2006), the continuous trading of bullfrogs contributing to the spread of *Bd*. (Fisher and Garner 2007; Garner et al. 2006). In fact, the chytrid has been detected both in bullfrogs sampled in the field and in captivity in China (Bai et al. 2010).

Bullfrogs were first introduced into mainland China in the late 1950s from Cuba and Japan as a source of food (Li and Xie 2002), although the founders

introduced from Japan failed to reproduce (Liu et al. 2009). As these were the only recorded introductions, bullfrogs in China are assumed to have source single original source (Cuba) (Liu et al. 2009). Within a period of 50 years after the first documented introduction, bullfrog aquaculture has gone through 3 waves of development (Bai 1989; Zeng 1998). The first wave was in the early 1960s following the first introduction of bullfrogs. The duration of this boom was short and interrupted for social reasons in 1966 when all bullfrog farms were shut down for about twenty years (Bai 1989; Zeng 1998). In spite of that, some bullfrogs escaped from farms established feral populations in Hanshou county of Hunan Province (Zeng 1998). The second wave of growth was in 1980s, when bullfrog aquaculture became a big business benefiting from improved husbandry techniques and economic reforms in China. In this case, the founders were not newly introduced but originated from descendants of the feral bullfrog populations established in 1960s in the Hunan Province (Bai 1989; Zeng 1998). During this period simple enclosures were employed by many households (Liu et al. 2009), favoring frequent escapes and the establishment of new feral bullfrog populations in different regions of China (Liu and Li 2009). With the development of aquaculture, these small scale breeding farms were eliminated gradually, and the bullfrog aquaculture ran into the third and mature period. Currently, the remaining bullfrog farms consist of elaborate enclosures, usually surrounded by brick and concrete wall, more effective in preventing escapes (Liu and Li 2009).

Previous studies in Europe and USA have shown that invasive bullfrogs are able to establish successful populations, even from a limited number of founders with low genetic variation (Ficetola et al. 2008; Funk et al. 2011). Unlike invasive bullfrogs in Europe and USA, the bullfrogs were introduced in China for aquaculture purposes only (Li and Xie 2002; Zeng 1998). However, limited information is available about the origin, frequency, numbers of individuals introduced and the subsequent spread of bullfrogs and across China. Furthermore, historical data cannot reveal the genetic changes occurring during the range expansion. Here, we sequenced a part of the mitochondrial cytochrome *b* gene of Chinese wild and farm bullfrogs, and compared them with populations from the bullfrog native range (Austin et al. 2004). We then

used a combination of genetic data and historical records to (a) test the hypothesis that the genetic diversity of both feral and farm populations is limited and smaller than that in the natural range of the species as a consequence of the limited numbers of original founders (founder effect) and (b) to identify the potential source population of the bullfrogs in China.

## Materials and methods

### Sample collection

Surveys were conducted at 112 sites in six provinces of China during 2007–2010 (Fig. 1). Sampling sites were distributed at an altitudinal range of 12–2,700 m and a latitude range of 22.0°–30.3°N from the eastern to southwestern China (Fig. 1). All sites were in regions recorded as having bullfrog introductions for breeding purposes. To determine whether a site had been invaded by bullfrogs, we surveyed all accessible water bodies in each site for three consecutive nights with line transects (Li et al. 2011b). The third toe of the right hind-foot from each post-metamorphosed bullfrog was clipped if they were available (Bai et al. 2010). Tissue samples were then preserved separately in 70% EtOH and stored at –20°C in the laboratory. Since bullfrogs are known to be one of the main vectors of the deadly amphibian pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Daszak et al. 2004; Fisher and Garner 2007; Garner et al. 2006), all the equipment, including boots and capture tools were disinfected before entering each site to prevent the spread of live *Bd* spores across sites.

Samples from captive bullfrogs were collected from six bullfrog farms and 23 food markets in 12 provinces and two municipalities during 2007–2011 (Fig. 1). At least five samples were collected per food market and twenty per bullfrog farm.

### Laboratory analysis

DNA was extracted following the procedure described by Goka et al. (2009) with some modifications. In brief, approximately 3 mg (wet weight) of each toe-clip or mouthparts were placed in a 2 mL disposable centrifuge tube, 100  $\mu$ L of lysis buffer containing 1 mg/mL proteinase K, 0.01 M NaCl, 0.1 M EDTA, 0.01 M Tris–HCl (pH 8.0) and 0.5% Nonidet P-40 was

added to the tube. The microtube was shaken for 1 min using vortex mixer at room temperature (about 20°C), after a brief centrifugation to recover all material from the bottom of the tube, the tube was incubated at 50°C for 120 min and at 95°C for 20 min. Finally, the microtube was centrifuged at 12,000 rpm for 3 min in cold temperatures, and the extract was diluted one-tenth of its original concentration for polymerase chain reaction (PCR) amplification (Goka et al. 2009). A 591-bp segment of the cytochrome *b* mitochondrial gene was amplified as described in Ficetola et al. (2008). The PCR products were then separated on agarose gel electrophoresis (2% agarose gels). Finally, PCR products were directly sequenced using the same primers used for the amplification (Beijing Genomics Institute, Beijing, China). The sequenced fragment includes the 408-bp amplified by Austin et al. (2004) in the native range bullfrogs.

### Data analysis

To make our results comparable with previous phylogeography analyses of bullfrogs in their native range (Austin et al. 2004), we used only 408-bp of the sequenced 591-bp for the following analysis. Sequences were aligned using Clustal X (Thompson et al. 1997), and unique haplotypes were identified using DnaSP 5.10 (Rozas et al. 2003). Number of haplotypes, haplotype diversity (Hd) and nucleotide diversity ( $\pi$ ) within each introduced populations were estimated by ARLEQUIN ver3.5 (Excoffier and Lischer 2010).  $\phi$ ST values were calculated to measure the genetic differentiation among feral bullfrog populations. Field sampling sites from each island in Zhoushan Archipelago were merged into a single biological meaningful population for their geographical proximity (Yang 2003).

Two basic methods were used to infer the most likely population(s) from which the invasive bullfrogs in China originated (Muirhead et al. 2008; Wares et al. 2005). Firstly, the putative native source was identified from the relationships among haplotypes between native and invasive populations based on the minimum spanning network. Networks were constructed based on statistical parsimony implemented in TCS 1.21 (Clement et al. 2000). In order to make a full comparison between our data and previously published work we also included haplotype information from the invaded Europe and Oregon range in the

analysis Secondly, the similarity of haplotype frequencies among four native groups and invasive populations in China was assessed through Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992), and the native group with the highest level of similarity with invasive bullfrogs was considered the most likely population of origin. For AMOVA, the native range of bullfrogs was divided into four biologically meaningful groups based on the geographical distribution of *cyt b* clade groups (Austin et al. 2004; Ficetola et al. 2008). These four areas were the western group (clade 3–1 and subclades 2–1 and 2–2; native populations 19, 20, 21, 31, 32, 34 in Austin et al. 2004), the eastern group (clade 3–2 and subclades 2–3 and 2–5; populations 5, 7, 9, 25, 26, 28, 35, 36, 37, 38, 39, 40, 41, 42), the northeastern group (presence of the subclade 2–5 only within the clade 3–2; populations 1, 2, 3, 4, 6, 15, 16, 17, 24), and the area of overlap between western and eastern haplotypes (contemporary presence of haplotypes from both clades 3–1 and 3–2, and several different subclades; populations 8, 10, 11, 12, 13, 14, 18, 22, 23, 27, 29, 30, 33). The geographical distribution of the *cyt b* clades and geographic distribution of the populations is showed in Fig 3 and Fig 8 respectively in Austin et al. (2004).

## Results

Bullfrogs were detected in 64 out of 112 surveyed sites. In total 288 bullfrog samples were collected from 42 invaded sites covering three provinces in China: 152 samples originated from 32 sites on 7 islands in the Zhoushan Archipelago of Zhejiang Province in eastern China and 136 samples came from 10 sites of Sichuan Province and Yunnan Province in southwestern China (Fig. 1, Table 1, Appendix 1—Electronic Supplementary Material). In addition, 222 samples were collected from captive bullfrogs: 133 individuals from 23 food markets and 89 from six bullfrog farms (Fig. 1, Table 2).

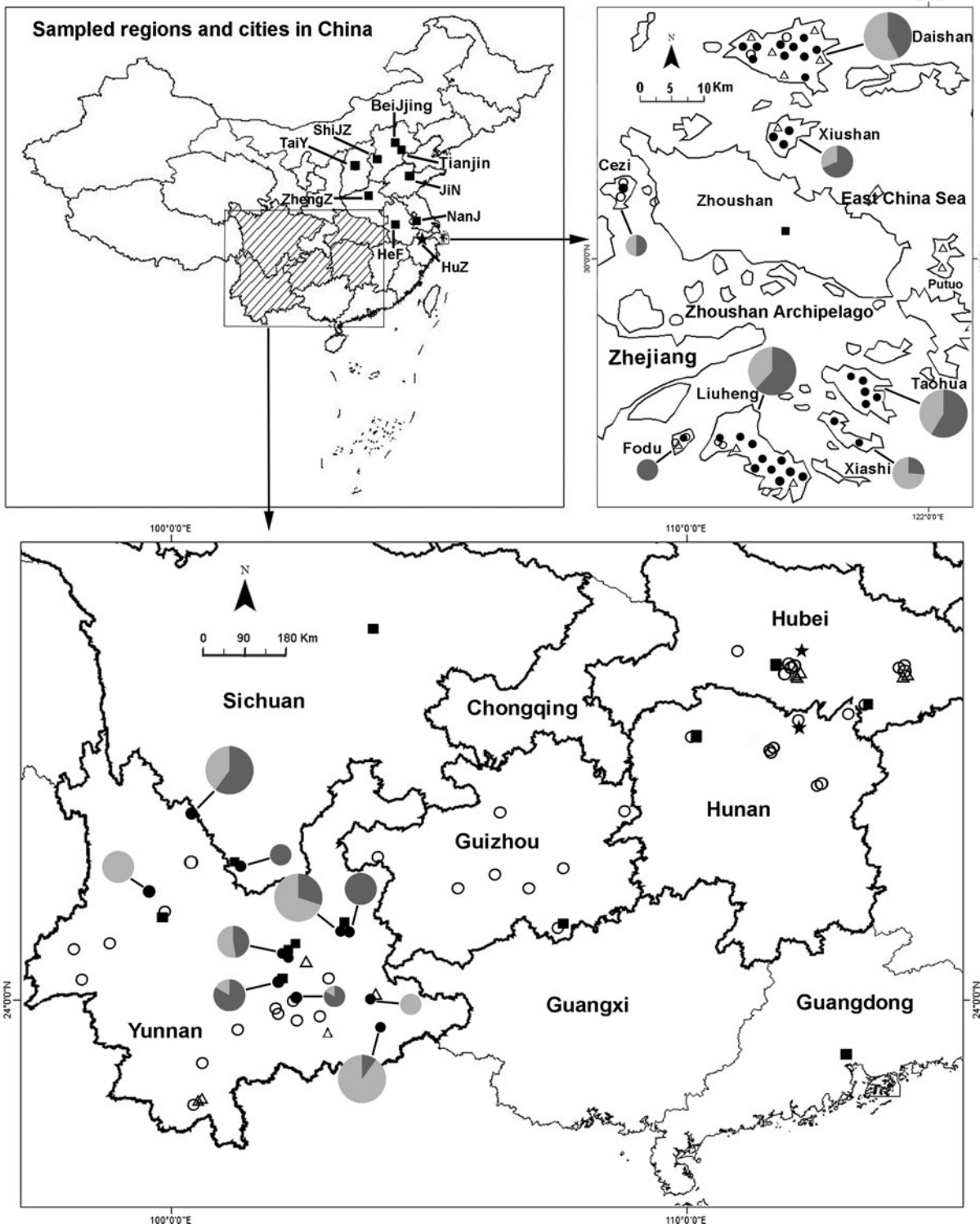
Only two haplotypes were found in the 510 samples (288 from feral populations and 222 from captive individuals) collected across China, while 41 haplotypes were identified from 215 samples collected from the native ranges and at least two haplotypes were detected in almost all native populations where more than one individual was sampled (Austin et al. 2004).

**Fig. 1** Map showing feral and captive populations sampled in this study. *Open circles* denote sites where *Lithobates catesbeianus* invaded but no individuals were captured, *closed circles* denote sites where *L. catesbeianus* invaded and some individuals were captured; *open triangles* denote uninvaded sites; *closed squares* denote sampled food markets; *closed stars* denote sampled *L. catesbeianus* farms. Some points are superimposed. Population assignment of feral individuals to the two haplotypes is represented by the *shaded portion* of the circle (H43: dark grey, H7: light grey; *small circle*: 1 ≤ sample size <10, *maddile-sized circle*: 10 ≤ sample size <20, *big circle*: 20 ≤ sample size)

Nucleotide diversity in feral bullfrogs from China was significantly lower than in those from the native ranges (Mann–Whitney  $U_{15, 40} = 154$ ,  $P = 0.006$ ; populations where only a single individual was sampled were excluded from analysis).

Two closely related haplotypes (H43 and H7) were identified in both feral and captive bullfrogs, with only one silent nucleotide substitution different between them. Haplotype H7 was one of the most frequent in both the native and the other two invasive (Europe and Oregon) populations, while H43 (GenBank Accession no. JQ241268) had not been previously identified (Austin et al. 2004; Ficetola et al. 2008; Funk et al. 2011). Furthermore, H43 was present in high frequencies in samples from bullfrog farms, food markets and feral populations, amounting 98.9, 76.7 and 53.1% respectively. Among the samples collected in the farms, in five out of six farms a single haplotype (H43) was found and the only farm with variability had a low frequency of the haplotype H7 (1/18) (Table 2). The proportion of H43 in bullfrogs from the three different sources (bullfrog farms, food markets and feral populations) differed significantly (Chi-squared test,  $\chi^2 = 71.90$ ,  $df = 2$ ,  $P < 0.001$ ). The proportion of H43 also differed significantly between bullfrogs from farms and food markets with frequencies of 98.9 and 76.7% respectively, (Chi-squared test,  $\chi^2 = 20.03$ ,  $df = 1$ ,  $P < 0.001$ ). The proportion of H43 in food markets was also higher than that in wild populations (Chi-squared test,  $\chi^2 = 20.16$ ,  $df = 1$ ,  $P < 0.001$ ).

Pairwise estimates of  $\phi_{ST}$  were not significant among six feral populations in Zoushan Archipelago after Bonferroni correction (italicised area in Table 3), suggesting they might derive from the same source of rearing stock, and thus the commercial populations that once existed in early 1990s on these island. In contrast, significant population differentiation was found among some sites of southwestern China,



**Table 1** Feral populations sampled, and summary results of genetic survey of *Lithobates catesbeianus* in China

Provinces/ sites	Number of sampled individuals	Number of haplotype 43	Number of haplotype 7	Hd (haplotype diversity) $\pm$ SD	$\pi$ (nucleotide diversity) $\pm$ SE
Zhejiang					
Daishan	40	17	23	0.501 $\pm$ 0.029	0.0012 $\pm$ 0.0012
Cezi	2	1	1	1.000 $\pm$ 0.500	0.0025 $\pm$ 0.0035
Fodu	1	1	0	1.000 $\pm$ 0.000	0.0000 $\pm$ 0.0000
Liuheng	42	26	16	0.483 $\pm$ 0.039	0.0012 $\pm$ 0.0012
Taohua	29	17	12	0.503 $\pm$ 0.040	0.0012 $\pm$ 0.0012
Xiashi	19	5	14	0.409 $\pm$ 0.100	0.0010 $\pm$ 0.0011
Xiushan	19	13	6	0.456 $\pm$ 0.085	0.0011 $\pm$ 0.0012
Sichuan					
PanZH	7	7	0	0.000 $\pm$ 0.000	0.0000 $\pm$ 0.0000
Yunnan					
QiuB	1	0	1	1.000 $\pm$ 0.000	0.0000 $\pm$ 0.0000
HuaN	6	5	1	0.333 $\pm$ 0.215	0.0008 $\pm$ 0.0011
YanS	20	2	18	0.190 $\pm$ 0.108	0.0005 $\pm$ 0.0007
ErY	15	0	15	0.000 $\pm$ 0.000	0.0000 $\pm$ 0.0000
QingL	19	9	10	0.526 $\pm$ 0.040	0.0013 $\pm$ 0.0013
YuX	18	15	3	0.294 $\pm$ 0.119	0.0007 $\pm$ 0.0009
LuGH	20	12	8	0.505 $\pm$ 0.056	0.0012 $\pm$ 0.0012
QuJ1	20	20	0	0.000 $\pm$ 0.000	0.0000 $\pm$ 0.0000
QuJ2	10	3	7	0.467 $\pm$ 0.132	0.0011 $\pm$ 0.0012

implying that multiple rearing stocks with different genetic compositions had been introduced into this region. However, we couldn't rule out the possibility that the genetic differentiation among these populations were simply due to genetic drift.

Statistical parsimony network showed that haplotypes identified in the east and west native region were divided into two main groups (Fig. 2). The H43 and H7 haplotypes found in China clustered in the group of western haplotypes, suggesting that the invasive bullfrogs most likely originated from the western native region. Consistent with these results, AMOVA analysis also showed that the percentage of among group variation was smallest for western native populations (39.43%) when compared the four native groups with wild China (Table 4). The percentages of among group variations between Northeast, East and Overlap of native rage and wild China were larger, amounting 92.38, 86.62 and 64.29% respectively (Table 4). Overall, these results indicated that western native population has the highest probability to be the potential origin of invasive bullfrogs in China and that the genetic make-up of introduced bullfrogs has

changed relative to their source populations during the process of introduction and spread.

## Discussion

Only two haplotypes were identified both from wild ( $n = 288$ ) and captive ( $n = 222$ ) samples in China, while 41 haplotypes were present in 215 samples from native populations (Austin et al. 2004). This indicated that the molecular genetic diversity of invasive bullfrogs in China has decreased drastically compared to that of conspecifics in the native populations. Serious reductions of genetic diversity in invading areas have been commonly hypothesized as a result of low propagule pressures, especially the lack of multiple introductions from different native regions (Dlugosch and Parker 2008; Simberloff 2009). Our results match the historical records of bullfrog introductions in China, according to which bullfrogs were introduced only once during the late 1950s (Liu et al. 2009). Correspondingly, five haplotypes were recently identified in 174 samples from invasive feral



**Table 2** Captive individuals sampled, and summary results of genetic survey of *Lithobates catesbeianus* in China

Provinces	Sites	Number of sampled individuals	Number of haplotypes 42	Number of haplotypes 7	Hd (haplotype diversity) ± SD	π (nucleotide diversity) ± SE
Hubei	CiB	5	5	0	0.000 ± 0.000	0.0000 ± 0.0000
	HongG <sup>a</sup>	18	17	1	0.111 ± 0.096	0.0003 ± 0.0005
	ShaS	5	5	0	0.000 ± 0.000	0.0000 ± 0.0000
Hunan	HuaR <sup>a</sup>	20	20	0	0.000 ± 0.000	0.0000 ± 0.0000
	ZhangJJ	5	5	0	0.000 ± 0.000	0.0000 ± 0.0000
Guizhou	LiB	5	5	0	0.000 ± 0.000	0.0000 ± 0.0000
Yunnan	KunM	5	5	0	0.000 ± 0.000	0.0000 ± 0.0000
	QingL	5	4	1	0.400 ± 0.237	0.0010 ± 0.0012
	YuX	5	1	4	0.400 ± 0.237	0.0010 ± 0.0012
	QuJ	4	3	1	0.500 ± 0.265	0.0012 ± 0.0015
	DaL	9	0	9	0.000 ± 0.000	0.0000 ± 0.0000
Sichuan	PanZH	5	5	0	0.000 ± 0.000	0.0000 ± 0.0000
	ChengD	5	1	4	0.400 ± 0.237	0.0010 ± 0.0012
Zhejiang	ZhouS	5	5	0	0.000 ± 0.000	0.0000 ± 0.0000
	HuZ1 <sup>a</sup>	14	14	0	0.000 ± 0.000	0.0000 ± 0.0000
	HuZ2 <sup>a</sup>	14	14	0	0.000 ± 0.000	0.0000 ± 0.0000
	HuZ3 <sup>a</sup>	13	13	0	0.000 ± 0.000	0.0000 ± 0.0000
	HuZ4 <sup>a</sup>	10	10	0	0.000 ± 0.000	0.0000 ± 0.0000
Beijing	QingH	4	4	0	0.000 ± 0.000	0.0000 ± 0.0000
	JingG	5	4	1	0.400 ± 0.237	0.0010 ± 0.0012
	DaZS	5	3	2	0.600 ± 0.175	0.0015 ± 0.0016
Tianjin	TianJ	5	2	3	0.600 ± 0.175	0.0015 ± 0.0016
Hebei	ShiJZ	4	4	0	0.000 ± 0.000	0.0000 ± 0.0000
Henan	ZhengZ	5	5	0	0.000 ± 0.000	0.0000 ± 0.0000
Shandong	JiN	5	3	2	0.600 ± 0.175	0.0015 ± 0.0016
Jiangshu	NanJ	5	3	2	0.600 ± 0.175	0.0015 ± 0.0016
Anhui	HeF	5	4	1	0.400 ± 0.237	0.0010 ± 0.0012
Shanxi	TaiY	5	5	0	0.000 ± 0.000	0.0000 ± 0.0000
Guangdong	GuangZ	22	21	1	0.091 ± 0.081	0.0002 ± 0.0005

<sup>a</sup> Six bullfrog farms sampled in this study

populations in Europe (Ficetola et al. 2008), where at least 25 different introductions occurred between 1930s and late 1990s (Ficetola et al. 2007). The differences in genetic diversity of invasive bullfrogs between Europe and China is consistent with the hypothesis that multiple introductions can mitigate the loss of genetic diversity loss in invaders (Dlugosch and Parker 2008; Kolbe et al. 2004). Additionally, our results confirmed the ability of invasive bullfrogs to create new populations even with very low levels genetic diversity (Ficetola et al. 2008; Funk et al. 2011).

Three factors could have contributed greatly to the successful establishment of bullfrogs in China despite extreme low genetic diversity. Firstly, the high fertility of bullfrogs may allow them to survive demographic bottlenecks, as demonstrated by invasive bullfrogs in Europe where the most invasive populations derived from less than six females (Ficetola et al. 2008). Secondly, the invasive species characterized by initial small population size can experience an increase in the frequency of recessive homozygotes, which could increase the efficiency of selection against deleterious mutants, and thus the potential to purge inbreeding

**Table 3** Pairwise  $\varphi$ ST (lower triangle) and associated  $P$  values (upper triangle) between introduced wild populations of *Lithobates catesbeianus*

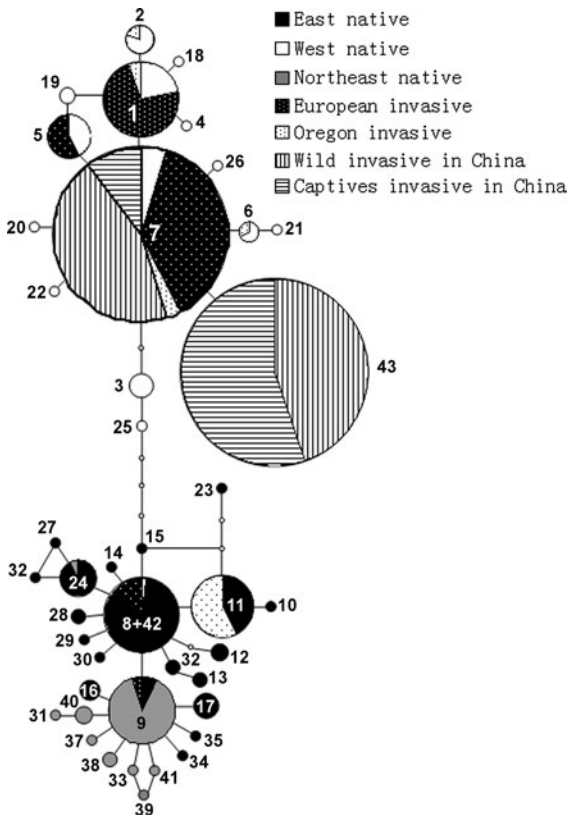
	DaiS	CeZ	LiuH	TaoH	XiaS	XiuS	PanZH	HuaN	YanS	EY	QingL	YuX	LuGH	QuJ1	QuJ2
DaiS		0.9990	0.1172	0.2178	0.2627	0.0850	0.0098	0.0889	0.0186	0.0029	0.7734	0.0029	0.2803	<b>0.0000</b>	0.7578
CeZ	0.0000		0.9990	0.9990	0.9990	0.9990	0.2148	0.9990	0.2578	0.1133	0.9990	0.3516	0.9990	0.0801	0.9990
LiuH	0.0496	0.0000		0.7998	0.0147	0.7676	0.0918	0.4316	<b>0.0000</b>	<b>0.0000</b>	0.4170	0.1348	0.9990	0.0020	0.0850
TaoH	0.0216	0.0000	0.0000		0.0479	0.5488	0.0781	0.3906	0.0010	<b>0.0000</b>	0.5557	0.1152	0.9990	<b>0.0000</b>	0.1523
XiaS	0.0164	0.0000	0.1914	0.1528		0.0147	0.0010	0.0273	0.2334	0.0615	0.3154	<b>0.0000</b>	0.0459	<b>0.0000</b>	0.9990
XiuS	0.0902	0.0000	0.0000	0.0000	0.2631		0.1582	0.6426	<b>0.0000</b>	<b>0.0000</b>	0.3311	0.4141	0.7197	0.0088	0.0703
PanZH	0.4037	0.5882	0.2070	0.2454	0.6256	0.1623		0.4512	<b>0.0000</b>	<b>0.0000</b>	0.0225	0.5273	0.0606	0.9990	0.0098
HuaN	0.2000	0.0000	0.0031	0.0268	0.4179	0.0000	0.0278		0.0010	<b>0.0000</b>	0.1670	0.9990	0.3770	0.2197	0.1182
YanS	0.1851	0.2961	0.3953	0.3697	0.0371	0.5044	0.8468	0.7008		0.5273	0.0186	<b>0.0000</b>	0.0020	<b>0.0000</b>	0.3018
EY	0.3074	0.7761	0.5046	0.4937	0.1943	0.6383	1.0000	0.8864	0.0323		0.0039	<b>0.0000</b>	<b>0.0000</b>	<b>0.0000</b>	0.0547
QingL	0.0000	0.0000	0.0043	0.0000	0.0404	0.0362	0.3766	0.1360	0.2559	0.4112		0.0400	0.5381	<b>0.0000</b>	0.4463
YuX	0.2538	0.0526	0.0634	0.0902	0.4641	0.0048	0.0278	0.0000	0.6873	0.8093	0.2055		0.1553	0.1045	0.0098
LuGH	0.0229	0.0000	0.0000	0.0000	0.1639	0.0000	0.2426	0.0076	0.4011	0.5411	0.0000	0.0753		0.0029	0.2656
QuJ1	0.4854	0.8253	0.2871	0.3463	0.7279	0.2847	0.0000	0.2258	0.8947	1.0000	0.5075	0.1283	0.3684		<b>0.0000</b>
QuJ2	0.0000	0.0000	0.1305	0.0898	0.0000	0.1967	0.6196	0.3529	0.0642	0.2905	0.0000	0.4207	0.0972	0.7614	

The italicised area contains pairs of feral populations in Zhoushan Archipelago, Zhejiang Province

The pairwise  $\varphi$ ST values significant after Bonferroni correction (105 comparisons,  $P < 0.00048$ ) appear in bold

Only sites with  $N > 1$  were included





**Fig. 2** TCS 95% confidence cytochrome *b* haplotype parsimony network of *Lithobates catesbeianus*. Circle size is proportional to the number of individuals, each line represents a single mutational change, and empty circles represent unsampled or extinct haplotypes

depression (Ficetola et al. 2011). Lastly, high propagule pressure due to repeated escapes from commercial populations. Liu and Li. (2009) found the establishment of bullfrog populations is positively correlated with the presence of simple aquaculture

enclosures and the numbers of bullfrogs raised. The rare occurrences of feral bullfrogs in central China (Hubei and Hunan Provinces), where bullfrog aquaculture industry have flourished, is probably related to high hunting pressure (Liu and Li 2009), which may reduce bullfrog survival and the breeding chance of females because of higher hunting pressure on males that is easy to find by call identification in the field (Li et al. 2006). A recent study on invasive rainbow trout in Chile by Consuegra et al. (2011) found that propagule pressure—measured as the cumulative weighted distance to every salmonid farm within a 100-km radius—was a good predictor of the incidence of farm escapees, and that escapees could promote both the establishment and spread of feral population. These results not only underscore the ecological threats posed by aquaculture industry to wild populations, but also provide an alternative explanation of the well-known genetic paradox of why invaders become establishing successfully despite low genetic variation. The other identified potential solutions to the dilemma including high reproductive rates, purging deleterious alleles that cause inbreeding depression as mentioned above and multiple introduction events (Frankham 2005).

The different proportion of H43 in samples from captivity (85.6%) and wild (53.1%) indicated that extreme founding events had occurred during the mass-production process in captivity after 1990s, given that the majority of feral bullfrogs were descents of those escaped from farms with simple enclosures in 1980s–1990s (Liu and Li 2009; Zeng 1998). Subsequent founding events and population bottlenecks have probably occurred in commercial populations since 1990s as a consequence of the fluctuations of the bullfrog aquaculture industry. Thus, while only one or

**Table 4** Summary of AMOVA results for variation between feral invasive *Lithobates catesbeianus* (from China) and different native groups

Source of variation/ significance tests	Four native groups compared to feral populations in China			
	Northeastern native and China	Eastern native and China	Overlap native and China	Western native and China
Among groups	92.38%	86.62%	64.29%	39.43%
Among populations	1.78%	4.33%	10.00%	22.22%
Within populations	5.84%	9.06%	25.71%	38.35%
$F_{CT}$	0.92	0.87	0.64	0.39
$P$ value	<0.0001	<0.0001	<0.0001	0.0001

a few bullfrog farms were under operation when the bullfrog aquaculture was in depression, their descents were dispersed to many farms around the country at the flourishing period of the frog farming industry (Bai 1989). Although our results suggest that most farm populations are dominated by the H43 haplotype, the presence of H7 in many market samples such as food markets in Yuxi and Dali in Yunnan Province and Chengdu in Sichuan Province suggest that there must be some remaining genetic diversity in some of the unsampled farms, particularly in southwestern China. Thus, the results implied that the genetic composition of commercial bullfrog populations varied among farms, as for other introduced commercial populations such as the honey bee (*Hymenoptera: Apidae*) in the United States (Delaney et al. 2009). We cannot discard that the extremely high frequency of H43 found in farm populations could be an artifact of insufficient sampling, since only six farms were sampled and four of them were managed by the same aquaculture company in Huzhou, Zhejiang Province. However, 23 food markets distributed across 21 cities of 14 provinces were sampled (Table 2), and bullfrog farms are the only source of bullfrogs in food markets. Thus, samples from food markets could provide a better representation of commercial populations of bullfrog.

Both AMOVA and phylogeographic analyses indicate that western native populations are the most likely origin of invasive bullfrogs in China. However, a significant genetic difference still existed between bullfrog populations in western native and those in caught in the field in China ( $P = 0.0001$ ) (Table 4). We suggest that the widely distributed new haplotype (H43) should be responsible for this differences, because AMOVA is more sensitive to the sequence and frequency changes of haplotypes (Wares et al. 2005). A recent study on the invasive *Anolis sagrei* in St Vincent also found the absence or lower frequency in the native range of particular haplotypes that were nearly fixed in the invasive range due to founder effects during the introduction and secondary establishment process, making it hard to identify the original population (Thorpe and Eales 2010). Both the intensity of sampling and the resolution of the molecular markers can strongly influence the accuracy of source population assignment (Dlugosch and Parker 2008; Muirhead et al. 2008). For this study, samples have been collected across large areas of both the native range and the invasive range. However, only a

single locus in the mitochondrial cytochrome *b* gene was analysed, and the differentiation among some populations might not be fully resolved. The application of multiple variable markers would help in the finer discrimination among populations (Muirhead et al. 2008). Furthermore, since Cuba seems to be the initial origin of bullfrogs in China, the comparison of the genetic structure of samples from Cuba could provide a better understanding of the source and introduction history of bullfrogs in China.

Although H43 has been firstly identified in China, there are three potential sources of H43. Firstly and most importantly, H43 could have been present as a rare haplotype in the United States and introduced to Cuba in 1916–1917 (Zeng 1998). The reasons why it was not detected by Austin et al. in 2004 could be either that it disappeared due to genetic drift or that it was only present at low frequencies. Secondly, H43 could have originated in Cuba as a result of mutation and have been introduced to China in late 1950s. Given that bullfrogs had been widely cultured across Cuba since early 1940s for food supply and international trade (Zeng 1998), founding events are also likely in Cuban commercial populations. Lastly, H43 could have derived from a mutation in H7 after bullfrogs were introduced to China and then increased in frequency due to several founder effects. Although the origin of H43 is still under debate, there is no doubt that sequential founding events should be responsible for the high frequency of H43 both in wild and captive populations in China.

Sequential founding events related to introduced commercial populations have important implications for the understanding and study of biological invasions. Firstly, rare alleles/haplotypes will be lost or overrepresented in these populations compared to the initial founders, making it difficult the inferences of the origin of the source populations through the analysis of genetic variation (Muirhead et al. 2008; Wares et al. 2005). Thus the interpretation of our results should be taken cautiously. Secondly, commercial populations can be critical as a source of continuing propagule pressure (Consuegra et al. 2011; Naylor et al. 2005). Delaney et al. (2009) found that the genetic makeup of commercial honey bees changed over a 10-year span due to artificial selection, and the commercial populations of *Orius strigicollis* in Japan were always genetically different from nearby wild populations (Hinomoto et al. 2006). These

examples would lead us to expect that the commercial populations may act as genetic pools to the invasive colorizations, just as multiple introductions can do to promote the establishment and spread of invasive species (Allendorf and Lundquist 2003; Forsyth and Duncan 2001; Kolar and Lodge 2001; Lavergne and Molofsky 2007; Lockwood et al. 2005; Roman and Darling 2007; Simberloff 2009; Von Holle and Simberloff 2005). Our results reaffirm the difficulty of inferring the original source of invasive populations based on genetic analysis alone as a result of sequential founding events, and the potential threats posed by introduced commercial bullfrog populations, given their unique genetic makeup compared to feral populations.

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