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The origin of invasion of an alien frog species in Tibet, China

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Abstract

Identifying the origins of alien species has important implications for effectively controlling the
spread of alien species. The black-spotted frog *Pelophylax nigromaculatus*, originally from East
Asia, has become an alien species on the Tibetan Plateau (TP). In this study, we collected 300 indi-
viduals of *P. nigromaculatus* from 13 native regions and 2 invasive regions (including Nyingchi and
Lhasa) on the TP. To identify the source region of the TP introductions, we sequenced portions of
the mitochondrial *cyt b* gene. We sequenced a ∼600-bp portion of the mitochondrial *cyt b* gene to
identify 69 haplotypes (124 polymorphic sites) in all sampled populations. According to the net-
work results, we suggest that the *P. nigromaculatus* found on the TP was most likely originated
from Chongqing by human introduction. Furthermore, we found that the genetic diversity was sig-
nificantly lower for invasive than for native sites due to founder effects. Our study provides genetic
evidence that this alien species invaded the cold environment of high elevations and expanded the
distribution of *P. nigromaculatus* in China.

Key words: alien species, amphibians, chytridiomycosis, cold environment, invasion genetics, invasion route, Tibetan Plateau.

Invasive species are responsible for changes to native biological di-
versity, the extinction of many native species around the globe, and
the disruption of ecosystem functions (Lockwood et al. 2013). Their
presence can inflict huge economic costs (Mack et al. 2000). Several
methods for measuring how species invade, establish, and spread
have been proposed to provide information to prevent or manage in-
vasive species (Hulme et al. 2008). Molecular approaches are among
the most important methods and have become widely used to better
understand the invasion process and the relationship between inva-
sive and source populations (Bai et al. 2012; Liebl et al. 2015;
Moule et al. 2015; Rius et al. 2015). Exploring invasion pathways
and sources can contribute to identifying the ecological characteris-
tics and physiological tolerances of source populations (Ficetola

et al. 2008). Invasion pathways and source populations can be used
to simulate potential distributions or predict future expansion (Liebl
et al. 2015).

Previous studies have suggested that regions of high elevation are
viewed as resistant to biological invasions because of an extreme cli-
mate and limited accessibility (Bennett et al. 2015; Pauchard et al.
2016). However, the risk of biological invasions is increasing due to
land-use change, climate warming, and increasing globalization in
these regions (Li et al. 2013). Non-native species move into regions
of high elevation and may change the composition of the community
(Bennett et al. 2015; Pauchard et al. 2016). These changes can be
viewed as with both positive (increasing biodiversity) and negative
(decreasing biodiversity) consequences. In contrast to the large

number of studies conducted in low-elevation regions (Rollins et al. 2015; Sherwin et al. 2015; Wang et al. 2016), non-native species invading regions of high elevation represent a problem in invasive biology that remains to be addressed.

The Tibetan Plateau (TP) is the most extensive (covering an area of 2.5 million km^2) and highest (approximately 4500 m above sea level on average) plateau in the world (Zhou et al. 2006). The TP is a conservation priority because the plateau and its adjacent areas cross 3 biodiversity hotspots: Indo-Burma, the Himalayas, and the mountains of southwestern China (Mittermeier et al. 2011). The extensive variation in the topography and climate of the TP generates a number of different habitats and supports abundant species diversity (Mittermeier et al. 2011; Li et al. 2016). There are 56 amphibian species on the TP, including 3 caudata species (*Batrachuperus tibetanus*, *Andrias davidianus*, and *Batrachuperus karlschmidti*) and 53 anuran species (Amphibia China 2016). Furthermore, against the background of global climate change, the TP is experiencing faster warming than low-elevation regions at the same latitude (Liu and Chen 2000; Qin et al. 2009; Wei and Fang 2013). This faster warming pattern may increase the risk of biological invasions and facilitate the rapid dispersal of disease vectors on the TP (Di Rosa et al. 2007; Liu et al. 2013).

The black-spotted frog *Pelophylax nigromaculatus*—native to East Asia, including low-elevation regions of northern, eastern, central, and southwestern mainland China; the Korean Peninsula; Japan; and far-eastern Russia—has become an invasive species in areas of western China, such as the Xinjiang Uyghur Autonomous Region (Wang et al. 2016). Wang et al. (2016) showed that the invasion of the alien *P. nigromaculatus* population in Yining originated from the Beijing and Chongqing area based on an mtDNA analysis. Prior to the present study, the black-spotted frog was never recorded in the TP (Li et al. 2010). The TP is at a higher elevation than Yining (average elevation 620 m); for example, the elevations of the Chabalang Wetland and Nyingchi City are, respectively, 3,600 m and 3,000 m. Such different environments may be the cause of different invasion processes.

In this study, we used an mtDNA marker to investigate the genetic patterns underlying the expansion of *P. nigromaculatus* population on the TP. We 1) compared the genetic diversity and examined the genetic structure of *P. nigromaculatus* in its 13 native ranges in China and in the 2 invaded territories of the TP, 2) identified the source region(s) of the TP introductions, 3) discuss possible potential damage caused by the invasion of *P. nigromaculatus* on the TP, and 4) discuss the application of our results to the planning of suitable control measures.

Materials and Methods

Surveying and sampling of *P. Nigromaculatus*

We estimated the introduction range of *P. nigromaculatus* by line transect methods in the TP (Heyer et al. 1994; Li et al. 2011). We suggested that *P. nigromaculatus* breeding populations had been established in this site when both adult and sub-adult *P. nigromaculatus* (and tadpoles) were found at survey sites (Li et al. 2011). We obtained information on the introduction history of *P. nigromaculatus* in the TP using a questionnaire survey (Li et al. 2006, 2011). We usually interviewed 2 or 3 residents living near the sampled water bodies. The residence time was based on the time the first sighting by the resident of tadpoles, eggs, or juvenile or adults of *P. nigromaculatus* or heard calls. If the interviewees gave different answers on the residence time of *P. nigromaculatus* invasion for a water body,

we used the average value (year) of these answers. The longest value for all surveyed sites in a region was defined as the residence time.

We collected 260 adult individuals from different locations (20 samples per locality) in 13 native ranges (Figure 1) in 2012. These locations encompassed most of the distribution of this species in northeastern, northern, central, northwestern, southeastern, and southwestern China. We collected 40 frogs from the 2 different regions of introduction (Nyingchi and Lhasa) on the TP (Figure 1) between 2014 and 2015. To determine whether a site has been invaded successfully by *P. nigromaculatus*, we searched for tadpoles of *P. nigromaculatus* using line transects that surveyed all accessible water bodies at each site for 3 consecutive nights. The third toes of individuals of *P. nigromaculatus* were collected, and then the tissue samples were preserved separately in 95% ethanol and stored at −20°C in the laboratory.

DNA extraction and amplification

Total genomic DNA was extracted from the toe tissue following the standard method published previously (Wang et al. 2014; Shine et al. 2016). A 695-bp segment of the mitochondrial cytochrome *b* (cyt *b*) gene from all specimens was amplified using the primers RanaLeuF5d (5′-AA T MCC GWA AA T CTC ACCCCC T-3′) and RanacytbB1 (5′-GCT GGT GTAAA T TGT CTG GGT C-3′) (Yang et al. 2003). The PCR protocol was initiated with an initial step of denaturing of 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing of 56°C for 30 s, extension of 72°C for 30 s, and a final extension step of 72°C for 10 min. The PCR products were subjected to electrophoresis on 2% agarose gels and directly sequenced using the same forward and reverse primers used for amplification (Beijing Genomics Institute, Beijing, China).

Data analysis

We used Clustal X in MEGA 6 (Tamura et al. 2013) to align and edit the mitochondrial *cyt b* gene sequences. To identify unique haplotypes in all sampling populations, we used DnaSP 5.10 to define these sequences (Rozas et al. 2003). Genetic diversity was assessed by calculating the number of haplotypes (*Hn*), haplotype diversity (*Hd*), and nucleotide diversity (*π*) within each sampling population using ARLEQUIN ver3.5 (Excoffier and Lischer 2010). A neighbor-joining tree of mtDNA was constructed from the Kimura 2-parameter nucleotide distances using Mega 6 (Tamura et al. 2013). Branch support was calculated by the bootstrap method according to 1,000 replicates. To identify the origin of the TP population, we utilized the software package TCS 1.21 (Clement et al. 2000) to construct cladogram networks of *P. nigromaculatus cyt b* haplotypes by statistical parsimony. We compared differences in the number of haplotypes (*Hn*) between native and invasive populations using the independent samples *t*-test (R Development Core Team 2012).

Results

We identified only 2 invasion sites (Nyingchi and Lhasa) for *P. nigromaculatus* that has established breeding populations in the TP. The residence time for *P. nigromaculatus* invasion is approximately 15 years (since the start of this century) in Nyingchi and 10 years (since 2005) in Lhasa. We determined that alien *P. nigromaculatus* in the TP originated from accidental introduction by fish farming.

In total, 300 individuals of *P. nigromaculatus* were collected from the 2 invasive regions and 13 native regions (Figure 1) and
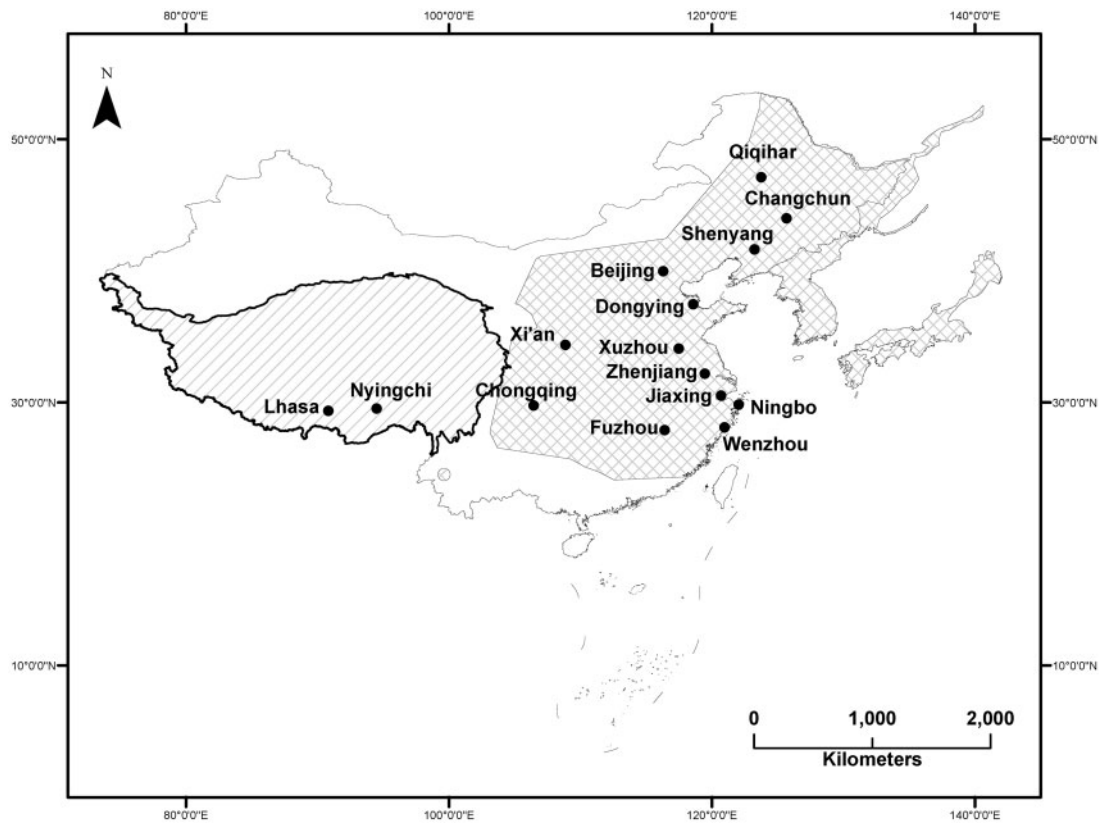
Figure 1. Sampled areas for *P. nigromaculatus* in China. Backward diagonal areas indicate the Tibet Plateau. Diagonal cross areas indicate the distribution area in Asia. Closed circles denote the sampling sites.

Table 1. Sampling information and genetic diversity indices of *P. Nigromaculatus*

Population	Abbreviation	N	Hn	Hd	π
Nyingchi	LZ	20	2	0.526	0.01692
Lhasa	LS	20	1	0	0
Chongqing	CQ	20	4	0.742	0.01762
Xi'an	XA	20	3	0.195	0.00032
Jiaxing	JX	20	9	0.795	0.00748
Beijing	BJ	20	10	0.863	0.00674
Dongying	DY	20	14	0.963	0.00763
Ningbo	NB	20	5	0.442	0.00127
Zhenjiang	ZJ	20	12	0.926	0.00651
Qiqihar	QQ	20	7	0.732	0.00162
Changchun	CC	20	9	0.832	0.01631
Shenyang	SY	20	6	0.621	0.00987
Xuzhou	XZ	20	13	0.932	0.00721
Wenzhou	WZ	20	6	0.579	0.00542
Fuzhou	FZ	20	2	0.1	0.00016
Total		300	69	0.952	0.02461

Note: N, number of samples sequenced; *hn*, number of haplotypes; *hd*, haplotype diversity; π, nucleotide diversity.
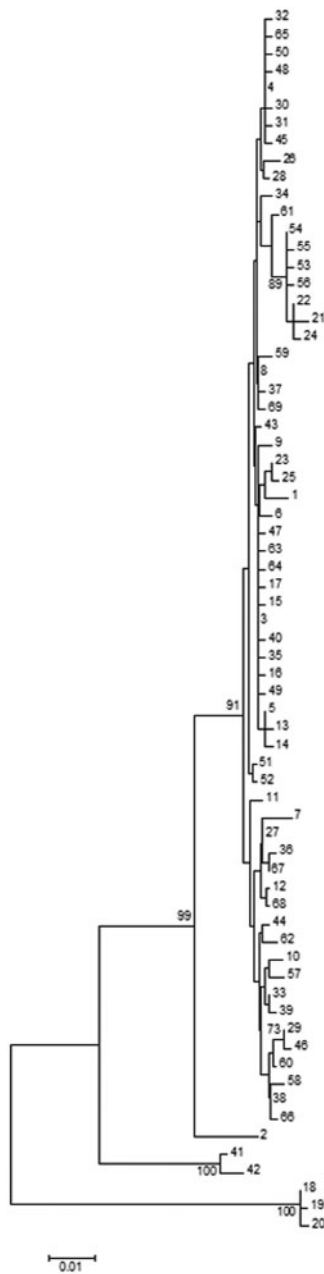
yielded a 622-bp DNA sequence for *cyt b* gene. All 69 haplotypes (H1–69) were identified by the 124 polymorphic sites in all sampled populations (Table 1). A list of their distributions is provided in the Appendix, and the phylogenetic relationships are shown in Figure 2. Collectively, 49 unique haplotypes and 20 haplotypes are shared among sampled populations (Appendix). Haplotypes H1 and H2 are not found in locations other than Chongqing and the introduced populations. *Hn*, *Hd*, and π ranged, respectively, from 1 to 14, 0 to 0.963, and 0 to 0.01762 among the sampled populations (Table 1). We found that the H1 and H2 haplotypes occurred in the Tibet (including Nyingchi and Lhasa) and Chongqing populations, suggesting that the *P. nigromaculatus* found in Tibet most likely originated from Chongqing (Figure 3). The number of haplotypes (*Hn*) was significantly higher for native than for invasive sites (native vs introduced populations: $df = 13$, $t = 2.21$, $P = 0.046$).
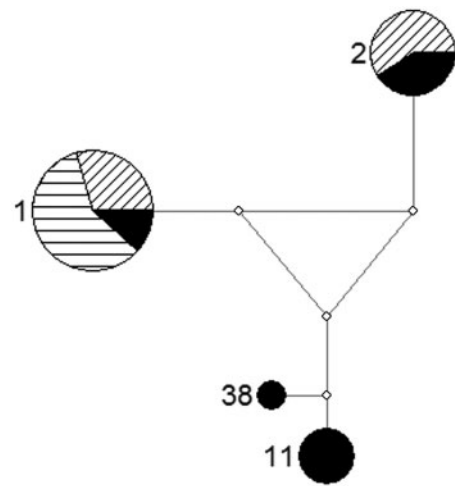
Discussion

Our results suggest that the alien *P. nigromaculatus* population on the TP originated from a single native-range source region (Chongqing population). Our data provide new evidence that low genetic diversity does not impede successful amphibian invasion on the TP. Our study also provides a new case of a non-native species invading high-elevation environments due to human activities and raises awareness of the growing importance of the expansion of non-native species in high-elevation cold environments. Furthermore, the new record from the TP extends the known distribution range of *P. nigromaculatus* in Asia by approximately 1,000 km from its ancestral area (Fei et al. 1999).

Our study shows that the recently established populations of *P. nigromaculatus* on the TP have reduced genetic variability in

Figure 2. Phylogenetic relationships among mtDNA haplotypes from *P. nigromaculatus* collected in both native and introduced regions. Only bootstraps of 70 or greater are shown. The numbers correspond to the haplotype numbers in the Appendix.

Figure 3. Statistical parsimony cladogram network representing the relationships among mtDNA haplotypes from *P. nigromaculatus* collected in both origin and introduced regions. Haplotype circle size is proportional to the number of individuals, and the numbers correspond to the haplotype numbers in the Appendix. The backward diagonal represents the Nyingchi population. The horizontal represents the Lhasa population. Black represents the Chongqing population.

We found that *P. nigromaculatus* has successfully invaded the high-elevation (>3,000 m) regions (Nyingchi and Lhasa) on the TP. Furthermore, other studies have discovered that a number of non-native species have successfully invaded these regions (Fan et al. 2016). For example, Fan et al. (2016) found that 13 non-native fish species have successfully invaded the Lhasa River of Tibet. These studies are not in accordance with previous hypotheses that cold environments of high elevations are often regarded as resistant to biological invasions due to an extreme climate and limited accessibility. Therefore, it is important that we conduct more research on invasion biology in regions of high elevation, such as Tibet.

Our study suggests that the alien *P. nigromaculatus* on the TP stemmed from Chongqing. A previous study found that the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Zhu et al. 2014, 2016), which is a lethal pathogen responsible for declines in amphibians worldwide, was detected in *P. nigromaculatus* in Chongqing. Furthermore, *Bd* has been found in other regions of high elevations, such as the Andes (Seimon et al. 2007), the Rocky Mountains (Pilliod et al. 2010), and the Sierra Nevada (Vredenburg et al. 2010). Although some studies suggest that the cold temperatures of high elevations can limit *Bd* (Muths et al. 2008; Pilliod et al. 2010), Knapp et al. (2011) suggest that the cold environments of high elevations do not necessarily limit this pathogen (Knapp et al. 2011). Therefore, to prevent the introduction of *Bd* to native amphibians, we suggest that the government control the spread of *P. nigromaculatus* from Chongqing to Tibet (such as developing a real-time monitoring system).

Humans may facilitate the spread of alien species across biogeographical borders such as high elevations, which could generate positive and negative conservation outcomes depending on these species and the invaded community (Bennett et al. 2015). As globalization increases, there will not only be an intensification of biological invasions, but the risk of pathogenic species being introduced as contaminants of their hosts may rise (Pauchard et al. 2016). Based on our study, schemes to prevent the invasion of *P. nigromaculatus*

comparison to native populations. The haplotype diversity in the 2 recently established populations is significantly lower than that in the area of origin, presumably due to founder effects during the colonization of Nyingchi and Lhasa. Frankham (2005) suggested that mechanisms (such as multiple introduction events, purging deleterious alleles, and high reproductive rates) can overcome the genetic dilemma that causes invasive populations to often show low genetic diversity and inbreeding in the invasive region (Frankham 2005). Previous studies have shown that *P. nigromaculatus* has high reproductive rates (Wang et al. 2008), which may be an important factor in the successful invasion of *P. nigromaculatus* on the TP.

on the TP should be prioritized based on those likely to have the greatest impact. Management should be more directed toward preventing the arrival of this species or catch it in the early stages of invasion. Other types of management could include developing early detection and rapid response programs and increasing educational outreach and public awareness.

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References

AmphibiaChina, (2016). The database of Chinese amphibians.Kunming, Yunnan. [cited 2016 June 10] Available from: http://www.amphibiachina.org/.

Bai CM, Ke ZW, Consuegra S, Liu X, Li YM, 2012. The role of founder effects on the genetic structure of the invasive bullfrog *Lithobates catesbeianaus* in China. *Biol Invasions* 14:1785–1796.

Bennett JR, Shaw JD, Terauds A, Smol JP, Aerts R et al., 2015. Polar lessons learned: long-term management based on shared threats in Arctic and Antarctic environments. *Front Ecol Environ* 13:316–324.

Clement M, Posada D, Crandall KA, 2000. TCS: a computer program to estimate gene genealogies. *Mol Ecol* 9:1657–1659.

Di Rosa I, Simoncelli F, Fagotti A, Pascolini R, 2007. Ecology: the proximate cause of frog declines?. *Nature* 447:E4–E5.

Excoffier L, Lischer HEL, 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol Resour* 10:564–567.

Fan L, Liu H, Lin J, Pu Q, 2016. Non-native fishes: distribution and assemblage structure in the Lhasa river basin, Tibet, China. *Acta Hydrobiol Sin* 40:295–303.

Fei L, Ye CY, Huang YZ, Liu MY, 1999. *Atlas of Amphibians of China*. Zhengzhou: Henan Science and Technology Press.

Ficetola GF, Bonin A, Miaud C, 2008. Population genetics reveals origin and number of founders in a biological invasion. *Mol Ecol* 17:773–782.

Frankham R, 2005. Resolving the genetic paradox in invasive species. *Heredity* 94:385–385.

Heyer WR, Donnelly MA, McDiarmid RW, Hayek L-AC, Foster MS, 1994. *Measuring and Monitoring Biological Diversity: Standard Methods for Amphibians*. Washington (DC): Smithsonian Institution Press.

Hulme PE, Bacher S, Kenis M, Klotz S, Kühn I et al., 2008. Grasping at the routes of biological invasions: a framework for integrating pathways into policy. *J Appl Ecol* 45:403–414.

Knapp RA, Briggs CJ, Smith TC, Maurer JR, 2011. Nowhere to hide: impact of a temperature-sensitive amphibian pathogen along an elevation gradient in the temperate zone. *Ecosphere* 2:1–26.

Li PP, Zhao EM, Dong BJ, 2010. *Amphibians and Reptiles of Tibet*. Beijing: Science Press.

Li X, Liu X, Kraus F, Tingley R, Li Y, 2016. Risk of biological invasions is concentrated in biodiversity hotspots. *Front Ecol Environ* 14:411–417.

Li YM, Wu ZJ, Duncan RP, 2006. Why islands are easier to invade: human influences on bullfrog invasion in the Zhoushan archipelago and neighboring mainland China. *Oecologia* 148:129–136.

Li YM, Cohen JM, Rohr JR, 2013. Review and synthesis of the effects of climate change on amphibians. *Integr Zool* 8:145–161.

Li YM, Ke ZW, Wang YH, Blackburn TM, 2011. Frog community responses to recent American bullfrog invasions. *Curr Zool* 57:83–92.

Liebl AL, Schrey AW, Andrew SC, Sheldon EL, Griffith SC, 2015. Invasion genetics: lessons from a ubiquitous bird, the house sparrow *Passer domesticus*. *Curr Zool* 61:465–476.

Liu X, Rohr JR, Li YM, 2013. Climate, vegetation, introduced hosts and trade shape a global wildlife pandemic. *Proc Biol Sci* 280:20122506.

Liu XD, Chen BD, 2000. Climatic warming in the Tibetan Plateau during recent decades. *Int J Climatol* 20:1729–1742.

Lockwood JL, Hoopes MF, Marchetti MP, 2013 *Invasion Ecology*. Oxford (UK): Blackwell Publishing.

Mack RN, Simberloff D, Lonsdale WM, Evans H, Clout M et al., 2000. Biotic invasions: causes, epidemiology, global consequences, and control. *Ecol Appl* 10:689–710.

Mittermeier RA, Turner WR, Larsen FW, Brooks TM, Gascon C, 2011. Global biodiversity conservation: the critical role of hotspots. In: Zachos FE, Habel JC, editors. *Biodiversity Hotspots*. Berlin: Springer, 3–22.

Moule H, Chaplin K, Bray RD, Miller KA, Thompson MB et al., 2015. A matter of time: temporal variation in the introduction history and population genetic structuring of an invasive lizard. *Curr Zool* 61:456–464.

Muths E, Pilliod DS, Livo LJ, 2008. Distribution and environmental limitations of an amphibian pathogen in the Rocky mountains, USA. *Biol Conserv* 141:1484–1492.

Pauchard A, Milbau A, Albihn A, Alexander J, Burgess T et al., 2016. Non-native and native organisms moving into high elevation and high latitude ecosystems in an era of climate change: new challenges for ecology and conservation. *Biol Invasions* 18:345–353.

Pilliod DS, Muths E, Scherer RD, Bartelt PE, Corn PS et al., 2010. Effects of amphibian chytrid fungus on individual survival probability in wild boreal toads. *Conserv Biol* 24:1259–1267.

Qin J, Yang K, Liang SL, Guo XF, 2009. The altitudinal dependence of recent rapid warming over the Tibetan Plateau. *Clim Change* 97:321–327.

R Development Core Team, 2012. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation Statistical Computing. ISBN 3-900051-07-0. Available from: http://www.R-project.org/.

Rius M, Bourne S, Hornsby HG, Chapman MA, 2015. Applications of next-generation sequencing to the study of biological invasions. *Curr Zool* 61:488–504.

Rollins LA, Whitehead MR, Woolnough AP, Sinclair R, Sherwin WB, 2015. Is there evidence of selection in the dopamine receptor D4 gene in Australian invasive starling populations? *Curr Zool* 61:505–519.

Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R, 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19:2496–2497.

Seimon TA, Seimon A, Daszak P, Halloy SRP, Schloegel LM et al., 2007. Upward range extension of Andean anurans and chytridiomycosis to extreme elevations in response to tropical deglaciation. *Glob Change Biol* 13:288–299.

Sherwin WB, Frommer M, Sved JA, Raphael KA, Oakeshott JG et al., 2015. Tracking invasion and invasiveness in Queensland fruit flies: from classical genetics to 'omics'. *Curr Zool* 61:477–487.

Shine R, Wang S, Madani G, Armstrong K, Zhang L et al., 2016. Using genetic data to predict the vulnerability of a native predator to a toxic invader. *Endang Species Res* 31:13–17.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S, 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30:2725–2729.

Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ, 2010. Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proc Natl Acad Sci USA* 107:9689–9694.

Wang S, Liu C, Zhu W, Gao X, Li Y, 2016. Tracing the origin of the black-spotted frog, *Pelophylax nigromaculatus*, in the Xinjiang Uyghur Autonomous Region. *Asian Herpetol Res* 7:69–74.

Wang SP, Zhu W, Gao X, Li XP, Yan SF et al., 2014. Population size and time since island isolation determine genetic diversity loss in insular frog populations. *Mol Ecol* 23:637–648.

Wang Y, Wu Z, Lu P, Zhang F, Li Y, 2008. Breeding ecology and oviposition site selection of black-spotted pond frogs *Rana nigromaculata* in Ningbo, China. *Front Biol China* **3**:530–535.

Wei YQ, Fang YP, 2013. Spatio-temporal characteristics of global warming in the Tibetan Plateau during the last 50 years based on a generalised temperature zone-elevation model. *PLoS ONE* **8**:e60044.

Yang Y, Zhang D, Li Y, Ji Y, 2003. Mitochondrial DNA diversity and preliminary biogeographic inference of the evolutionary history of the black-spotted pond frog *Rana nigromaculata* populations in China. *Curr Zool* **50**:193–201.

Zhou SZ, Wang XL, Wang J, Xu LB, 2006. A preliminary study on timing of the oldest Pleistocene glaciation in Qinghai–Tibetan Plateau. *Quat Int* **154**:44–51.

Zhu W, Bai C, Wang S, Soto-Azat C, Li X et al., 2014. Retrospective survey of museum specimens reveals historically widespread presence of *Batrachochytrium dendrobatidis* in China. *EcoHealth* **11**:241–250.

Zhu W, Fan L, Soto-Azat C, Yan S, Gao X et al., 2016. Filling a gap in the distribution of *Batrachochytrium dendrobatidis*: evidence in amphibians from Northern China. *Dis Aquat Org* **118**:259–265.

Appendix

Population distribution of mtDNA haplotypes of *P.nigromaculatus*

Haplotypes	LZ	LS	CQ	XA	JX	BJ	DY	GJ	ZJ	QQ	CC	SY	XZ	WZ	FZ
1	10	20	4												
2	10		7												
3						7	1		1	9	7	12			
4						3	3						5		
5						2				6	2	1			
6						1									
7						2									
8					1	1	1	1	1						
9						1									
10						1									
11			7		2	1			1						
12						1			2				1		
13										1					
14										1					
15										1					
16										1	1				
17										1					
18				18											
19				1											
20				1											
21														1	
22														13	
23														1	
24														2	
25														2	
26														1	
27									2				1		
28													2		
29							2						1		
30													1		
31													1		
32													1		
33							1						2		
34							1						2		
35							1						1		
36							1						1		
37													1		
38			2		2										
39											5	4			
40											1				
41											1				
42											1	1			
43											1				
44									2		1				
45							1								
46							1								

Appendix (Continued)

Haplotypes	LZ	LS	CQ	XA	JX	BJ	DY	GJ	ZJ	QQ	CC	SY	XZ	WZ	FZ
47							2								
48							2								
49							1								
50							2		1						
51															19
52															1
53								2							
54								15							
55								1							
56								1							
57					9										
58					1										
59					2										
60					1										
61					1										
62					1										
63												1			
64												1			
65									1						
66									2						
67									5						
68									1						
69									1						
Total	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20