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 individuals 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 network 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 significantly 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.
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 karlschmidtii) 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. 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).

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). 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.

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.

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 (Roza 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 2003) 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
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

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**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.*

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**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.
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.

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...
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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### 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          | 3  | 3  | 1  | 9  | 7  | 12 |    |    |    |    |    |    |    |    |    |
| 4          | 2  | 2  | 6  | 2  | 1  |    |    |    |    |    |    |    |    |    |    |
| 5          | 1  | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 6          | 1  | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 7          | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 8          | 7  | 2  | 1  | 1  |    |    |    |    |    |    |    |    |    |    |    |
| 9          | 1  | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 10         | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 11         | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 12         | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 13         | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 14         | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 15         | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 16         | 1  | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 17         | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 18         | 18 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 19         | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 20         | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 21         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 22         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 23         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 24         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 25         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 26         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 27         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 28         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 29         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 30         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 31         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 32         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 33         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 34         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 35         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 36         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 37         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 38         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 39         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 40         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 41         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 42         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 43         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 44         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 45         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 46         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

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|-----------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 47        |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 2  |
| 48        |    |    |    |    | 1  |    |    |    |    |    |    |    |    |    |    |
| 49        | 2  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 50        |    | 1  |    |    | 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 |