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Phylogeographic analysis of the *Bufo gargarizans* species complex: A revisit

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Abstract

Using mtDNA sequencing and allozyme electrophoresis data, we tested the "vicariance followed by dispersal" hypothesis of the *Bufo gargarizans* species group and re-evaluated the species status in the general lineages species concept. A phylogenetic analysis suggested that dispersal, instead of vicariance, dominated the history of the species group. There was a general trend of west to east dispersal, while some lineages from the east subsequently returned to the west. The secondary admixture of those previously allopatric lineages produced substantial levels of sympatric genetic diversity, often as high as 7.0% pairwise difference within populations. The phylogenetic hypothesis does not support the current two species designation. Neither *B. andrewsi* nor *B. gargarizans* represents an independent evolutionary lineage, and monophyletic groups did not correspond to geographically discrete groups. Allozyme data also failed to reveal any fixed allelic difference among the populations. Therefore, we recommend regarding the complex as a single species, *Bufo gargarizans*, without subspecies division.

Keywords: Vicariance; Dispersal; Phylogeography; mtDNA sequence; Allozyme; *Bufo gargarizans*; Amphibia

1. Introduction

Phylogeographic analysis provides a powerful tool for dissecting a species' evolutionary history, offering significant insight into the formation of present day biogeographic and systematic patterns (Avise, 2000). Using a phylogeographic approach and mitochondrial DNA (mtDNA) sequence data, Macey et al. (1998a) presented a "vicariance followed by dispersal" hypothesis for the *Bufo gargarizans* species complex, one of the most common and widely distributed amphibian groups of eastern Asia. Classically, four species have been placed in this group: *B. gargarizans* is widely distributed in the lowlands from the Amur River basin and Sakhalin Island, through Korea, to most of China; *B. andrewsi* and *B. minshanicus* are in the mountains of western China with the latter being constrained to only a few locations; *B. bankorensis* is exclusive to the island of Taiwan (Frost, 2004). Macey et al. (1998a) concluded that the common ancestor of the *B. gargarizans* species complex was divided by a vicariance event approximately 5–10 million years ago, producing *B. andrewsi* at high elevations and *B. gargarizans* at low elevations. One lineage of *B. gargarizans* (= *B. minshanicus sensu lato*) subsequently invaded the elevated areas, and *B. minshanicus* was a synonym of *B. gargarizans*. The fourth member of the *B. gargarizans* species group, *B. bankorensis*, was not considered in their study. More recently, a molecular phylogenetic study by Liu et al. (2000) lent further

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support to the two-species classification by synonymizing *B. bankorensis* with *B. gargarizans*. These two molecular studies provide an interesting framework for investigating the influence of past geological events on the evolutionary history of this ecologically important species group. However, for several reasons, this group requires further investigation.

For instance, other biogeographic studies do not corroborate Macey et al.'s (1998a) "vicariance followed by dispersal" hypothesis. One example of a contrary biogeographic scenario is the case of the *Batrachuperus* salamanders, which are also distributed throughout western China yet do not display a similar phylogeographic pattern (Fu et al., 2001). Macey et al. estimated that high elevation populations of the ancestral *Bufo gargarizans* group separated from low elevation ones approximately 5–10 million years ago, which coincides with the third phase of uplift of the Tibetan Plateau. However, most of the high elevation populations of *B. gargarizans* occur at the eastern periphery of the Tibetan Plateau, which were formed at 45–50 million years ago during the first phase uplift of the Tibetan Plateau (Ren et al., 1980). This phase of uplift left many signatures in the local freshwater fauna at high taxonomic level, for example, the vicariant separation of the fish subfamilies Barbinae and Schizothoracinae (Li, 1981; Wu and Wu, 1992).

The two-species classification is not widely accepted either. Frost (2004) listed all four as valid species, while Fei and Ye (2000) treated *Bufo andrewsi* and *B. minshanicus* as subspecies of *B. gargarizans*. The lack of consensus generated by the two molecular studies (Macey et al., 1998a; Liu et al., 2000) may be due, at least partially, to the small number of specimens examined from the *B. gargarizans* species group (9 and 5, respectively).

It is of little surprise that species from the eastern periphery of the Tibetan Plateau would attract the attention of biologists. This region has the most complex river system on earth and a profoundly complex and dynamic geological history, making it an excellent model system for biogeographic studies. The area is also a biodiversity hotspot (Conservation International, 2004), and elucidating the evolutionary history and clarifying the taxonomic status of its flora and fauna are critically important for directing biodiversity conservation efforts. As such, we undertook this study with two objectives: (1) to thoroughly re-evaluate the biogeographic history of the *Bufo gargarizans* species group and Macey et al.'s "vicariance followed by dispersal" hypothesis, and (2) to clarify the group's taxonomy from a phylogenetic perspective. To these ends, we extensively sampled individuals from populations throughout the group's range, at both high and low elevations, and used mitochondrial DNA sequence data to create a gene tree for phylogeographic inference. Additionally, we used allozyme electrophoresis data to reinforce the assessment of the species boundaries suggested by the gene tree.

2. Materials and methods

A total of 90 specimens from 33 locations across the distribution range of the *Bufo gargarizans* species complex were collected (Fig. 1, Table 1). Sampling effort was particularly concentrated in the western region where *B. gargarizans* and *B. andrewsi* are in contact, and the proposed vicariance event occurred (Macey et al., 1998a). Specimens were euthanized in the field and tissues were immediately removed and frozen in liquid nitrogen (liver, heart, and skeletal muscle) or preserved in 95% ethanol (skeletal muscle). Among all tissues collected, 56 were frozen, and the rest were preserved in ethanol. For phylogenetic reconstruction, four closely related species, *B. exsul*, *B. verrucosissimus*, *B. tibetanus*, and *B. tuberculatus* (Graybeal, 1997; Macey et al., 1998a; Liu et al., 2000), were chosen as outgroups. All outgroup sequences, except those of *B. tibetanus*, were obtained from GenBank. All voucher specimens are deposited in the herpetological collections of the Chengdu Institute of Biology, the Kunming Institute of Zoology, and the University of Guelph.

DNA sequence data were used for phylogenetic reconstruction and for the evaluation of genetic divergence. Two fragments from the mitochondrial genome were selected for sequencing. The first fragment was the first half of the control region, which was used in a previous study of Asian *Bufo* and shown to be highly variable and informative at the population level (Liu et al., 2000). The second fragment, including part of the ND1 and ND2 genes and the three tRNA genes between them, had a mix of relatively variable (ND1 and ND2) and conservative parts (tRNA genes), and provided excellent resolution of divergence at greater depths. This fragment has been used extensively in amphibians and reptiles (e.g., Macey et al., 1998a,b).

DNA extraction was accomplished using the Promega Wizard genomic DNA extraction protocols. A standard polymerase chain reaction (PCR) was used to amplify the genomic DNA. PCR products were purified using Qiaquick protocols (Qiagen), and were directly sequenced with BigDye-labeled terminator sequencing kits (Perkin–Elmer) on an ABI 377 automatic sequencer. The primers, cytb-A and control B (Goebel et al., 1999) were used to amplify and sequence the control region fragment. The primers L-int (5′ CGA GCA TCC TAC CCA CGA TTT CG 3′, this study), L4437 and H4980 (Macey et al., 1998a) were used to amplify and sequence the ND1–ND2 fragment. The annealing temperature of primer L-int was optimized at 50 °C for both PCR and cycle sequencing. Sequence editing and alignment were conducted with the computer program Sequencher (version 3.1.1).

For the control region, four specimens were sequenced for most populations with sample size of four or more. For populations with samples fewer than four, all specimens were sequenced. Only a subset of samples was

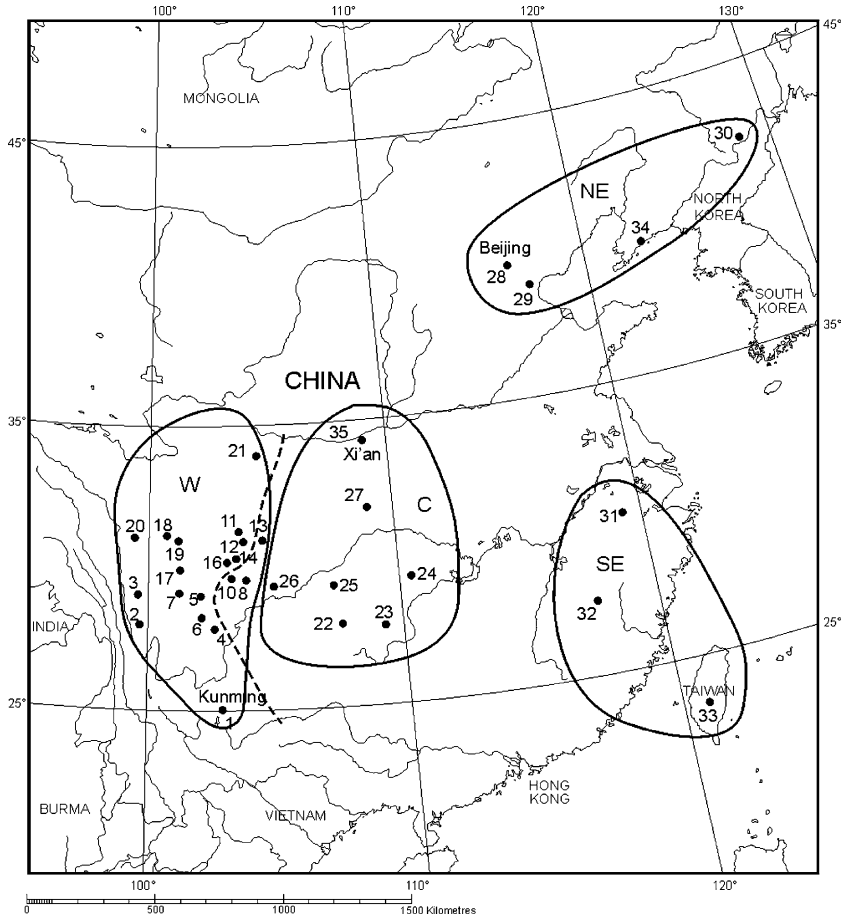

Fig. 1. Map of the sample sites. Site names and coordinates are listed in Table 1. Site 9 = site 8, site 15 = site 14. For convenience, the sampling sites were grouped into four geographical units according to their geographical distances and topologies. W (west) includes sites 1 to 21; C (central) includes sites 22–27 and 35; NE (northeast) includes sites 28–30 and 34; SE (southeast) includes sites 31–33. The inclusion of site 26 in the central unit is because its landscape (farmland) is the same as that of other sites in the central unit, but contrary to that of the sites in the west unit (mountain forest). Sites 34 and 35 represent sequences obtained from GenBank. Sites on the left side of the dashed line are from high elevations; sites on the right side of the dashed line are from low elevations.

sequenced for the ND1–ND2 region. Our efforts were to sequence samples that represented the major lineages identified from an initial phylogenetic reconstruction of the control region data, and the data from the ND1–ND2 region were primarily used to confirm the deeper phylogenetic relationships. We stopped sequencing when we were convinced that more ND1–ND2 sequence would not improve the phylogenetic resolution significantly.

For phylogenetic reconstruction, both maximum parsimony and Bayesian approaches were used. The control region sequences and the ND1–ND2 region sequences were analyzed alone and combined. For maximum parsimony analysis, each unique haplotype was treated as an operational taxonomic unit and each nucleotide site was treated as a character. All characters were unordered and equally weighted. Tree search processes were conducted using PAUP (version 4.0b10; Swofford, 2002). All phylogenetically uninformative characters were excluded from the analysis. A heuristic search was conducted using TBR branch-swapping with 1000 random step-wise addition replicates. Bootstrap proportions (BSP; Felsenstein, 1985) were used to estimate the support of the recovered nodes. For the Bayesian analysis, all nucleotide sites were included and a likelihood ratio test (Goldman, 1993) was first conducted to select an evolutionary model that best fits the observed data. Based on the results from the computer program Modeltest (version 3.06; Posada and Crandall, 1998), the HKY85 + I + G model (Hasegawa et al., 1985) was chosen for the control region data and the TrN + G model (Tamura and Nei, 1993) was chosen for the ND1–ND2 fragment data. For the combined analysis, the data from the two fragments were separated into two partitions, each following its own model. The Bayesian analysis was conducted using MrBayes (version 3.01; Huelsenbeck and Ronquist, 2002) in conjunction with PAUP. Four Markov chains were used and the data set was run for four million generations to allow for adequate time of convergence. Trees were sampled every 100 generations and we used the last 10,000 sample trees to estimate the

Table 1
Sample information and haplotype designation

Locality	Coordinates	Elevation (m)	Control region			ND1–ND2 region			Allozyme
			n	Haplotype	GenBank Accession No.	*n*	Haplotype	Genbank Accession No.	*n*
Bufo andrewsi sensu lato (*n*=60)									
(1) Kunming	N25.0°, E102.7°	2000	2	1A/3A, 1B	AF190230, AY924312	2	1A, 1B	AY936841, 66	
(2) Zhongdian	N27.7°, E99.7°	2600	1	2A	AY924310	1	2A	AY936840	
(3) Derong	N28.7°, E99.2°	4000	2	1A/3A, 3B	AY924311, 13	1	3A	AY936865	
(4) Xichang	N27°52.177′, E102°30.947′	3050	2	4AB/6AB	AY924317–18				
(5) Yele	N28°55.614′, E102°11.404′	2600	1	5A	AY924314				
(6) Mianning	N28.5°, E102.1°	3000	2	4AB/6AB	AY924315–16	2	6AB	936842-43	
(7) Jiulong	N29.0°, E101.5°	3000	1	7A	AY924339				
(8) Omei Mt.-I	N29°35′, E103°17′	1410	1	(8A+)	AY924356	1	8A	AY936858	
(9) Omei Mt.-II	N29°35′, E103°17′	1300	6	9A, (8A+), 9E 16B/9C, 9F	AY924320, 21, 24, 25, 55, 57	1	9A	AY936869	6
(10) Hongya	N29°39.111′, E102°57.065′	1300	2	(8A+), 10B	AY924328, 54	1	10A	AY936857	2
(11) Maoxian	N31°41.893′, E103°52.729′	1765	4	11AB, 11C, 11D	AY924333–36	2	11AB	AY936847–48	12
(12) Pengxian	N31°14′, E103°45′	2000	1	12A	AY924361	1	12A	AY936868	1
(13) Zhongjiang	N31.0°, E104.6°	500	4	(8A+), 13D 22A/13C	AY924337, 52, 53, 60	2	13AB	AY936855–56	6
(14) Qionglai	N30°14.817, E103°05.182	1800	4	14A, 14B, 14CD	AY924329–32	2	14A, 14B	AY936845–6	6
(15) Dayi	N30°37.915′, E103°10.243′	2060	2	15A, 15B	AY924326–27				2
(16) Baoxing	N30.3°, E102.8°	2000	3	16AC, 16B/9C	AY924319, 22–23	1	16B	AY936844	3
(17) Xinduqiao	N30°01.950′, E101°28.496′	3500	1	17A/18A	AY924368	1	17A	AY936861	
(18) Luhou	N31°24.082′, E100°37.465′	3450	1	17A/18A	AY924369	1	18A	AY936862	
(19) Daofu	N30°29.505′, E101°28.372′	3880	2	19A, 19B	AY924370–71				
(20) Baiyu	N30°48.690′, E99°35.494′,	4080	2	20A, 20B	AY924372–73				
(21) Wengxian	N33°03.408′, E104°41.225′	2200	3	21A, 21B, 21C	AY924340–42	3	21A, 21B, 21C	AY936849–51	
Bufo gargarizans sensu lato (*n*=30)									
(22) Suiyang	N28°13.581′, E107°09.593′	1350	1	22A/13C	AY924359	1	22A	AY936875	
(23) Jiangkou	N27°53.772′, E108°42.918′	810	1	23A	AY924343	1	23A	AY936852	
(24) Zhangjiajie	N29.3°, E110.4°	1430	2	24A, 24B	AY924346–47	2	24A, 24B	AY936871–72	
(25) Nanchuan	N29°04.077′, E107°11.679′	1554	1	(8A+)	AY924351	1	25A	AY936854	1
(26) Fushun	N29.2°, E105.0°	200	2	26B, (8A+)	AY924358, 63				
(27) Wangyuan	N32°03.836′, E108°10.257′	1300	2	27A, 27B	AY924338, 62	1	27B	AY936876	
(28) Baihuashan	N39°47.067′, E115°24.038′	360	2	28A, 28B	AY924364–65	2	28A, 28B	AY936859, 63	
(29) Jixian	N40.1°, E117.2°	600	2	29A, 29B	AY924366–67	2	29A, 29B	AY936860, 64	
(30) Antu	N42°32.731′, E128°18.043′	900	2	30AB	AY924344–45	2	30A, 30B	AY936867, 70	3
(31) Lin'an	N30.2°, E119.7°	1000	2	31A, 31B	AY924348–49	2	31A, 31B	AY936853, 73	7
(32) Guadun	N27°43.89′, E117°39.36′	1170	2	32A, 32B	AF190234, AY924350	1	32A	AY936874	7
(33) Taiwan	No detailed location data		1	33A	AF190231				
(34) Zhuanghe	No detailed location data		1	34A	AF190233				
(35) Xi'an	No detailed location data		1	35A	AF190235				
Bufo tibetanus (*n*=2)									
Zhongdian	N27.7°, E99.7°		2	A, B	AF190249, AY924309	2	A, B	AY936838–39	

Numbers in front of location names are location numbers in Fig. 1. Coordinates with minutes are GPS readings, and others were obtained from maps. Haplotypes are named with their location numbers and letters A–F, and a letter designates a specific specimen. When specimens from multiple locations share the same haplotype, the locations numbers are separated by a "/". For example, 4AB/6AB indicates that two specimens from location 4 and two specimens from location 6 share the same haplotype. Haplotype (8A+)=8A/9BD/10A/13AB/25A/26A. Sequences AF190233–5 were obtained from Genbank.

consensus tree and the Bayesian posterior probabilities. Templeton's test and the Shimodaira–Hasegawa test were used to evaluate alternative tree topologies (Shimodaira and Hasegawa, 1999; Templton, 1983). To further resolve relationships within major haplotype clusters, a statistical parsimony approach was employed (Templeton et al., 1992), which was carried out using computer program TCS (version 1.18; Posada, 2004). Ambiguous connections in the haplotype network were resolved with the following criteria: (1) haplotypes are more likely to be connected to common than to rare haplotypes; and (2) haplotypes are more likely to be connected to interior than to exterior haplotypes (Templeton and Sing, 1993). Pairwise comparisons of the sequences were also conducted to estimate the magnitude of variation among populations and major lineages.

To test Macey et al.'s (1998a) "vicariance followed by dispersal" hypothesis, elevations of the populations were mapped on the phylogenies. The lowest sampling site in Macey et al.'s "high elevation" group was 1600 m, and therefore, we defined sampling sites at 1600 m or above as high elevation. Macey et al. (1998a) considered their sampling site from Omei Mt. as low elevation (no elevation data available). The two sampling sites from Omei Mt. in this study (8 and 9 with elevations of 1300 and 1410 m, respectively) are geographically very close to Macey et al.'s site, and we therefore categorized them, as well as any other sites below 1410 m, as low elevation. Furthermore, site 24 (1430 m) and site 25 (1554 m) were also considered as low elevation because they are located in central China among the low land sites. In addition, sampling sites were grouped into four geographical units for convenience (west, central, southeastern, and northeastern), according to their geographical distances and topologies, which were also mapped onto the phylogenies.

Allozyme electrophoresis data were used as supplementary data to evaluate population structure and potential reproductive isolation among lineages. Only samples with frozen tissues could be subjected to allozyme evaluation. Homogenates of liver, muscle, and heart were used. Procedures, protocols, and enzyme and allelic nomenclature followed Murphy et al. (1996) with minor modification. Allozymes were separated by 11% horizontal starch gel electrophoresis. To maximize resolution, two buffer systems were used for each allozyme system whenever possible. The computer program GDA (Lewis and Zaykin, 2001) was used for population genetic analysis. Due to the small sample size, the data were only evaluated for population substructuring using *F*-statistics, and for reproductive isolation by fixed allelic differences.

3. Results

We sequenced 68 individuals for the control region and 39 for the ND1–ND2 region, including two outgroup specimens (*Bufo tibetanus*). We also obtained six other sequences from previous studies (AF004524, 26, Macey et al., 1998a; AF190233–5, 50, Liu et al., 2000). All sequences have been deposited at GenBank (Table 1).

There were 37 specimens of the *B. gargarizans* complex for which we had sequences from both the control region and the ND1–ND2 fragment (Table 1). These sequences produced a combined data set of 35 haplotypes. The data set had a total of 1451 nucleotide sites of which 278 were variable and 207 were phylogenetically informative among the ingroup members. Surprisingly, in both the parsimony and Bayesian analyses, the four outgroup species each provided a different root for the trees while the relationships among the ingroup members remained nearly identical (Fig. 2). When used in combination, some outgroup species became part of the ingroup. Among the outgroup members, several independent studies suggested that *B. tibetanus* is most

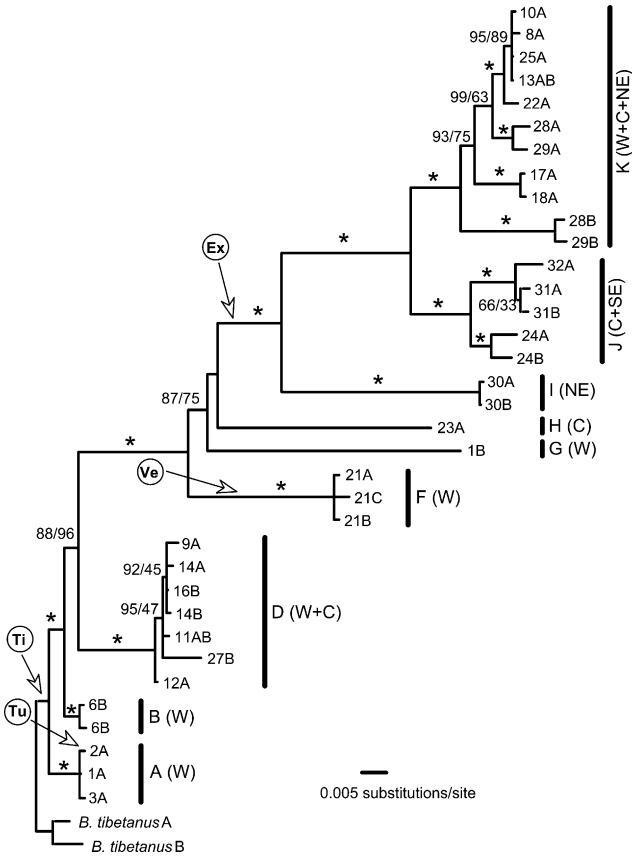

Fig. 2. Phylogenetic tree derived from the Bayesian analysis of the combined data of the control region sequences and the ND1–ND2 fragment sequences. Taxa are haplotypes represented by their location numbers and letters A–F (Table 1). A ∗Indicates a node with a 100 Bayesian posterior probability (BPP) and a great than 95 bootstrap proportion (BSP). Numbers above nodes are BPPs/BSPs. Vertical bars indicate the clade assignment (A–K) and geographical unit assignment (W, western; C, central; NE, northeastern; and SE, southeastern). The arrows Ti, Tu, Ve, and Ex indicate the different roots designated by outgroup species *B. tibetanus*, *B. tuberculatus*, *B. verrucosissimus*, and *B. exsul*, respectively.

closely related to the *B. gargarizans* complex (e.g., Hu et al., 1984; Liu et al., 2000). After considering all evidence, we adopted the root provided by *B. tibetnus*, and excluded all other outgroup species in the subsequent analyses. The Bayesian 50% majority consensus tree for the combined data set is presented in Fig. 2. The parsimony analysis resulted in 60 equally most parsimonious trees with 414 steps, a consistency index of 0.6329 and a retention index of 0.9051. The strict consensus tree had a nearly identical topology to the majority consensus of the Bayesian inference. In the parsimony consensus tree, the positions of 12A and 27B and the position of 1B and 23A were reversed. Most recovered nodes, particularly the ones defining the major lineages (A–K), were strongly supported by the data (Fig. 2).

The control region data included 69 sequences and 51 unique ingroup haplotypes (Table 1). The data set had 654 characters, of which 158 were variable and 118 were phylogenetically informative. Using *B. tibetanus* as outgroup, a 50% majority consensus tree was generated from the Bayesian analysis (Fig. 3). The parsimony analysis resulted in 32247 equally most parsimonious trees with 288 steps, a consistency index of 0.5278 and a retention index of 0.8957. A strict consensus produced a phylogeny similar to that of the Bayesian analysis. However, the parsimony solution placed clades B, C, D, E, and (F + G + H + I + J + K) in a polytomy, while the Bayesian solution was partially resolved. Additionally, the parsimony consensus tree resolved the relationships among clades F, G, H, and (I + J + K); clades (I + J + K), G, H, and F were sequentially nested in each other. All other relationships were identical between the two consensus trees. The Bayesian posterior probabilities are mapped on Fig. 3. We did not conduct a bootstrap analysis because of the calculation time constraints. Overall, the topologies derived from the control region data were nearly identical to those derived from the combined data. However, those derived from the control region data provided more detailed geographic information because they included more specimens. A separate analysis of the ND1–ND2 region data yielded an essentially identical tree to that of the combined data (tree not shown).

The statistical parsimony analysis of the control region data produced 14 independent networks. Most of them contained only 1–3 haplotypes, which were hardly informative. Two networks, however, which included all haplotypes of clade D and most haplotypes of clade K, respectively, provided complementary information to the bifurcating trees (Fig. 3). While the bifurcating trees were unable to resolve most nodes within these two clades, the networks provided hypothetical evolutionary relationships of the haplotypes.

The phylogenetic hypotheses derived from both the control region data and the combined data revealed several interesting aspects of the evolutionary history of the *Bufo gargarizans* species complex (Figs. 2 and 3). First, all haplotypes were grouped into several major clades (A–K) that were well supported with high values from both the Bayesian posterior probability and bootstrap analyses. Most of the relationships among the major clades were also well resolved and well supported. Second, while most samples from the same location were grouped within the same clade, there were several exceptions; the two samples from Kunming (site 1) were separated into clade A and G, and samples from Omei (site 9), Wanyuan (site 27), and Hongya (site 10) were separated between clades D and K. These samples diverged substantially; for instance, for the ND1–ND2 region, the two samples from Kunming (site 1) had a 7% pairwise difference, while two samples from Omei (site 9; 9A vs. 9B) had a 5.4% pairwise difference. Furthermore, samples from close geographical proximities were not always phylogenetically closely related. Populations from the western distribution (sites 1–21) revealed vastly different evolutionary histories, and were found throughout most clades (Fig. 3). Third, phylogenetically closely related haplotypes were often distributed over a wide geographic range. This is particularly true for clade K, which include haplotypes from every major geographical unit (W, C, SE, and NE), from the extreme southeast [Taiwan (33)] to the far west [Baiyu (20), Luhou (18), and Daofu (19)]. Within the clade, closely related haplotypes covered huge geographical distances, e.g., haplotypes from Beihuashan (site 28) and Jixian (site 29) grouped with a haplotype from Taiwan (site 33), while other haplotypes from Beihuashan (site 28) and Jixian (site 29) grouped with a haplotype from Xi'an (site 35) (Fig. 3). One common haplotype of the control region, 8A/9BD/10A/13AB/25A/26A [=(8A+)], was shared by eight individuals from six populations (8, 9, 10, 13, 25, and 26) separated by distances up to 400 km (Fig. 1).

The "vicariance followed by dispersal" hypothesis predicts that all populations would group into two major clades. One clade would consist of populations from high elevations and the other would primarily consist of populations from low elevations. Some populations from the latter clade might occur at high elevations, as a result of low to high elevation dispersal events. To test this hypothesis, alternative tree topologies were examined. For instance, clade D and clade (J + K) include populations from both high and low elevations. Inserting or removing any one taxon from these two clades results in a significantly less optimal tree topology (monophyly test using the combined data set with Templeton's test and the Shimodaira–Hasegawa test, $P < 0.05$; Schulte et al., 1998). Clade (F + G + H + I + J + K) also received significant support from the monophyly tests. This suggests that the associations of populations from both high and low elevations in these clades are not random. All other attempts to make the high elevation populations monophyletic or to make populations of

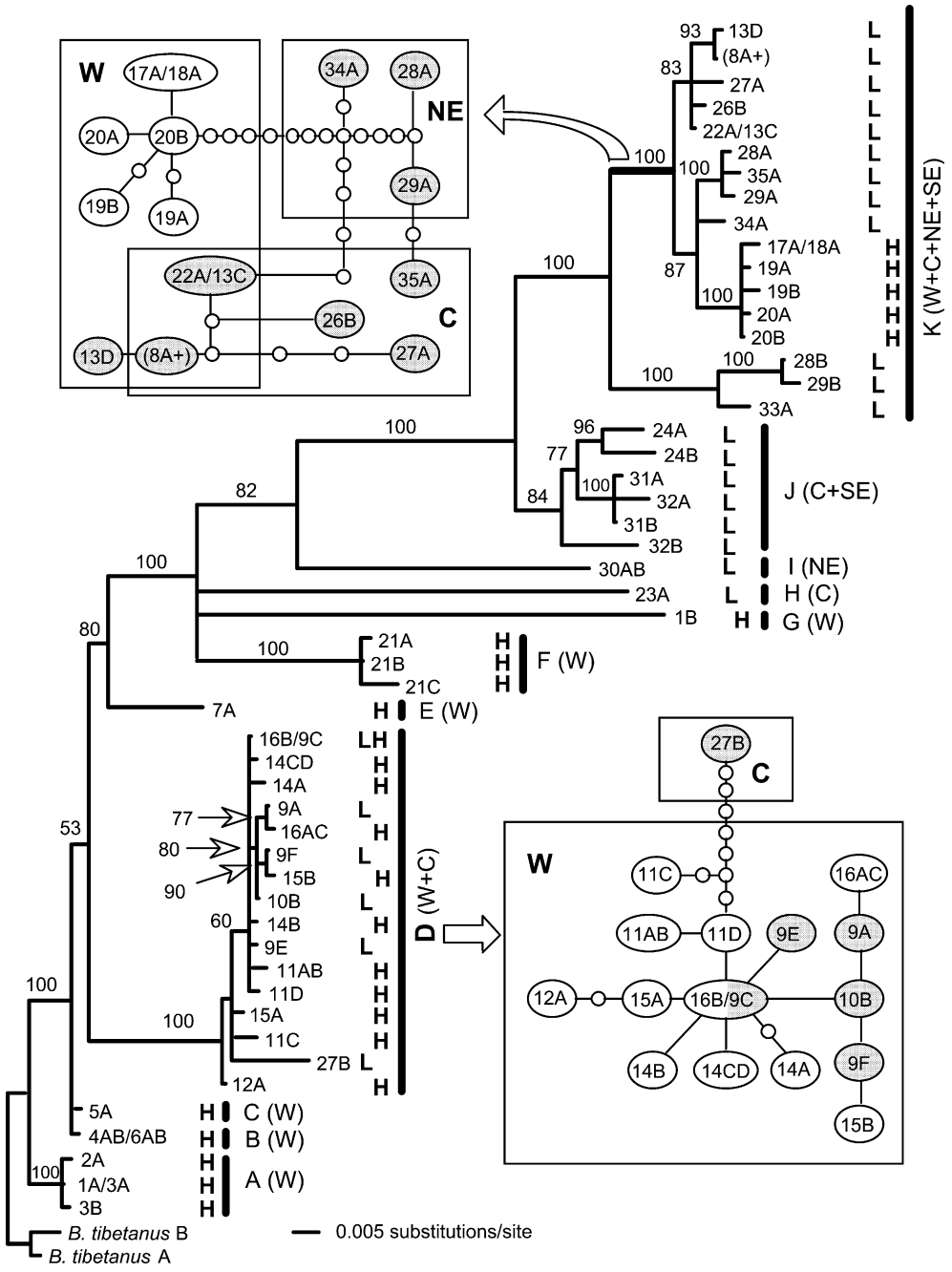

Fig. 3. Phylogenetic tree derived from the Bayesian analysis of the control region sequences. Taxon and vertical bar denotation are the same as in Table 1 and Fig. 2. Haplotype (8A+) is 8A/9BD/10A/13AB/25A/26A. Numbers above nodes are Bayesian posterior probabilities. "H" indicates elevations equal or higher than 1600 m, and "L" indicates elevations lower than 1600 m. The inserts are networks of clade D and part of clade K, generated by statistical parsimony with TCS. Shaded haplotypes are from low elevation and unshaded ones are from high elevation. Large boxes indicate geographical units (W, C, and NE) and empty circles represent haplotypes that are necessary intermediates but were not present in the samples.

"*B. andrewsi*" monophyletic were also rejected ($P < 0.001$, Templeton's test; $P < 0.001$, Shimodaira–Hasegawa test). Similar patterns were found for the control region data set.

The pairwise differences (uncorrected *p*-distance) revealed a large amount of genetic divergence within this species complex. For the ND1–ND2 fragment, the largest pairwise difference between haplotypes was 8.5% (3A vs. 32A), and the highest within population difference ranged from 0 (site 14) to 7.0% (site 1). The differences among the major clades (A–K) ranged from 1.0 (A vs. B) to 8.5% (A vs J). Within the major clades, maximum differences ranged from 0.13 (F) to 2.4% (K). For the control region fragment, the largest pairwise difference was 10.3% between 29B and 21C, and the highest within population difference ranged from 0 (sites 4 and 6) to

8.2% (sites 1 and 27). The differences among the major clades (A–K) ranged from 1.9 (A vs. B) to 10.3% (F vs K). The highest within major clade differences ranged from 0.15 (A) to 9.4% (K). Consistent with the trees, the "super" clade K revealed the largest intra-cladal variation under both data sets; 2.4% for the ND1–ND2 fragment, and 9.4% for the control region. The pairwise variation between the ingroup and outgroup (*B. tibetanus*) ranged from 1.1 to 7.5% for the ND1–ND2 region, and 1.1 to 9.7% for the control region.

A total of 56 specimens from 12 populations were examined with allozyme electrophoresis. Populations from all geographic units were included, although most were from the western region. Alleles from 22 presumptive loci were confidently resolved and recorded. Among them, three loci, ADA-A, βGLUR, and sSOD-A, were monomorphic while the other 19 loci were polymorphic. The genotypic data of the polymorphic loci are listed in Appendix A.

The *F*-statistics indicated a significant deviation from random mating and that considerable genetic substructuring exists within the species group (Table 2). To reduce the influence of population substructuring caused by geographically distant populations, an evaluation with only the western populations (9, 10, 11, 12, 13, 14, 15, and 16) that were in close geographical proximity was conducted. Again, the high *F*-statistics suggested considerable genetic substructuring and significant non-random mating among and within each of the western populations (Table 2). In particular, a co-ancestry index (θ, equivalent to the traditional F_{ST}) of 0.426 among the western populations indicated a "very great" genetic differentiation (Wright, 1978). All statistical measurements were significant at the 95% confidence level.

Among the 12 populations with allozyme data, the eight western populations were grouped into two clades on the phylogenetic trees, clade D and clade K (Figs. 2 and 3). The genotype data between members of the two clades was compared (Appendix A). At four loci, sACOH-A, sIDH-A, mIDH-A, and PK-A, the two clades possessed different common alleles. In all but one case, all alleles observed in clade K were also found in clade D. This is not surprising given that clade D had a much larger sample size. The one exception was at locus sIDH-A; clade K possessed a common allele *b* while clade D possessed a common allele *c* without allele *b*. In any case, no fixed allelic differences were observed between the two clades or among any of the populations.

Table 2
F-statistics for the *Bufo gargarizans* populations

	$f(F_{IS})$	$F(F_{IT})$	$\theta(F_{ST})$
All populations			
Overall	0.574	0.800	0.531
Upper bound	0.698	0.867	0.671
Lower bound	0.395	0.687	0.338
Western populations only			
Overall	0.533	0.732	0.426
Upper bound	0.704	0.834	0.573
Lower bound	0.312	0.573	0.240

The upper and lower bound were calculated from 10,000 replicates of bootstrapping over loci at 95% confidence interval.

4. Discussion

4.1. The tree root and the monophyly of the Bufo gargarizans complex

One interesting observation in this study is that the four outgroup members each designated a different root, and when use in combination, some outgroup species became part of the ingroup. This observation raised the question whether the *Bufo gargarizans* complex is monophyletic, however, other evidence overwhelmingly supports the monophyly of the complex. Both Macey et al. (1998a) and Liu et al. (2000) provided unambiguous molecular evidence supporting the complex's monophyly, and morphologically, members of the complex are very similar and in many cases, indistinguishable. Geographically, the *B. gargarizans* complex is restricted to eastern Asia, while *B. verrucosissimus* only occurs in the Caucasus Mountains and *B. exsul* only occurs in California. Furthermore, our allozyme data failed to find any fixed allelic difference among populations of *B. gargarizans* complex, lending further support to the monophyly of the complex (as a single species).

If the complex is monophyletic, which root represents the best hypothesis? All four outgroup species have been suggested as being closely related to *Bufo gargarizans* in different studies. Graybeal's (1997) phylogenetic review of bufonid toads, which included eight Eurasian *Bufo* species, found *B. gargarizans* to be closely related to *B. exsul* and several other American toads. No other close relatives of *B. gargarizans* were included in the study. Macey et al. (1998a) used *B. viridis* and *B. exsul* as outgroups and found that *B. verrucosissimus* is most closely related to the *B. gargarizans* complex. No other Asian species were included in their analysis. Liu et al. (2000) presented the most comprehensive phylogenetic study of eastern Asian bufonids to date, and found that *B. tibetanus* and *B. tuberculatus* are mostly closely related to the *B. gargarizans* complex. Other studies also corroborated Liu et al.'s conclusion. For example, Hu et al.'s (1984) morphological reviews suggested that *B. tibetanus* is the most closely related species to *B. gargarizans* complex. Considering all the available evidence, a sistergroup relationship between *B. tibetanus* and *B. gargarizans* is best supported and therefore*B. tibetanus* is the preferred outgroup in our analysis.

We currently do not have an explanation as to why multiple outgroups resulted in multiple roots, but the

most likely answer is related to the evolutionary history of the genus *Bufo*. *Bufo* is one of largest amphibian genera, with 255 species (Frost, 2004), and multiple rapid radiation events may dominate its evolutionary history. Phylogenetic reconstruction within the genus is notoriously difficult, and homoplasy is commonly found among behavioral and morphological traits (Graybeal, 1997). Except among very closely related species, the rampant homoplasy may distort the phylogenies due to phenomena such as long branch attraction. For this reason, the species *B. verrucosissimus* and *B. excel*, which may not be close enough to the ingroup, are likely inappropriate and misleading choices as outgroup.

4.2. Vicariance or dispersal

Our preferred phylogenetic hypothesis clearly rejects the "vicariance followed by dispersal" hypothesis (Macey et al., 1998a). Our trees disagree with the predictions of the hypothesis, such as a tree with two major clades consisting of either high or low elevation populations. Furthermore, populations of "*B. andrewsi*" failed to form a monophyletic group. As such, an alternative explanation is required. Since the patterns on both the bifurcating trees and networks suggest that dispersal events between high and low elevations or between geographic units were commonplace (Fig. 3), dispersal, rather than vicariance, might dictate the history of the species group. This is consistent with the general biology of the species group. *Bufo gargarizans* is an ecological generalist, present in many different habitats, such as farmland, mountainous forest, and even semi-desert areas. Rivers, low elevation mountains, and hostile habitats, such as farmland, also seem to impose no barrier to their dispersal (Huang, 1990). The symmetric gene tree that Macey et al. used for phylogeographic analysis was most likely a result of limited sample size. For example, when our ND1–ND2 data were added to Macey et al.'s data set, several samples from high elevation (e.g., 17A, 18A, and 1B from populations 17, 18, and 1, respectively) became nested in their low elevation clade, while several samples from low elevations (e.g., 9A from population 9) became nested in their high elevation clade.

Phylogenetic branching patterns can also suggest dispersal routes. This has been demonstrated successfully by many empirical cases, particularly among island populations (e.g., Carranza et al., 2000). Within the *Bufo gargarizans* species group, our trees suggested a general west to east dispersal trend, with the common ancestor of the group originating in the west (in the Hengduan Mountains area). This is evidenced by the fact that most of the basal clades are western populations, and the eastern populations (clades I, J, and K) are nested higher in the phylogeny (Fig. 3; Futuyma, 1998). Furthermore, the sister groups of *B. gargarizans* species group, *B. tibetanus*, and *B. tuberculatus* (Liu et al., 2000) occur only in the Hengduan Mt. area of western China. This hypothesis is consistent with the area's geological history. During the Pleistocene glaciation period, the river valleys were never completely glaciated, and the deep river valleys provided opportunities for short distance vertical migration (Yang, 1993). Consequently, the area preserved a wide range of ancient lineages, such as the giant panda and many megophryid frogs (Yang, 1993). The west to east dispersal likely occurred multiple times. Clade (I + J + K) may represent a major dispersal event, with the central occurrence of 27B of clade D and 23A of clade H representing additional, independent dispersal events (Fig. 3). Furthermore, several western populations nested in clade K, which is dominated by eastern populations, suggests that at least three lineages within this clade returned to the central and western regions (network of clade K; Fig. 3).

According to our phylogenetic hypothesis for the *Bufo gargarizans* species group, members of several lineages must have dispersed great distances within relatively short time frames. Macey et al. (1998a) estimated an evolutionary rate of 0.69% pairwise differences per lineage per million years for the ND1–ND2 fragment of the *Bufo bufo* complex (including *B. gargarizans* species group), using the geological event that separated the European and Asian stock of the complex. Using this calibration, clade K is approximately 1.5 million years old, yet members of the clade are present in every geographic unit (W, C, SE, and NE). Furthermore, some specimens from Beihuashan (site 28) and Jixian (site 29) are more closely related to each other than they are to others from their own population (Fig. 3), despite being separated by a distance of approximately 130 km. Additionally, haplotypes from Beihuashan (site 28) and Jixian (site 29) closely resemble haplotypes from populations 33 (approximately 1700 km away) and 35 (approximately 900 km away). Throughout the eastern and central portions of the group's distribution, plains and agricultural land dominate the landscape, providing little barrier to migration. Additionally, natural and human assisted dispersal may facilitate gene flow among populations.

Our study also revealed pronounced mtDNA genetic divergence. The intraspecific divergence is higher than has been reported in the same DNA fragment of other species. For example, Macey et al. (1998b) found only 3.5% pairwise differences of ND1–ND2 region among populations of *Laudakia caucasia* (Reptilia: Agamidae) and Lu et al. (2004) reported no intraspecific variation of ND2 among populations of six *Paramesotriton* species (Amphibia: Caudata). The pairwise differences revealed in this study were as high as 8.5% for the same fragment of DNA. In particular, sympatric individuals of *Bufo gargarizans* displayed unusually high levels of mtDNA divergence; for example, the two specimens from Kunming (1) have a 7.0% pairwise difference of the

ND1–ND2 region and a 8.2% difference of the control region. Although many of these lineages are currently sympatric, much of the divergence may have evolved in isolation. The observation in the *Bufo gargarizans* group is consistent with a category II intraspecific phylogeographic pattern (Avise, 2000), where major lineages display pronounced divergence and members of the lineages are broadly sympatric. Most empirical data suggest that such patterns involve secondary admixture of allopatrically evolved lineages, such as in the case of snow geese (Baker and Marshall, 1997).

In summary, the current observed distribution of the *Bufo gargarizans* species group is best explained by multiple dispersal events. Although vicariance also likely played a role, these vicariance patterns were swamped by rampant dispersal events. We acknowledge that a dispersal explanation is by nature descriptive and often labeled as an ad hoc explanation (Futuyma, 1998). However, such hypotheses are falsifiable. If a new phylogenetic analysis reveals a symmetric tree with two distinct clades, which are dominantly composed of western or eastern populations, respectively, a vicariant explanation would be preferred. Or, an asymmetric tree with eastern populations locating at the base and the western populations nesting among the western populations, would suggest east to west dispersal.

4.3. Taxonomic implications

Taking the evolutionary history of the *Bufo gargarizons* species complex into account, and given that our allozyme analysis showed no reason for us to conclude that gene flow was blocked, we suggest the *Bufo gargarizans* complex be considered a single species with the name, *Bufo gargarizans*, including *B. andrewsi*, *B. minshanicus*, and *B. bankorensis* as junior synonyms. Although what constitutes a species is a subject of intense debate, there is a general agreement that species are segments of evolutionary lineages (deQueiroz, 1998). Neither the currently designated *B. andrewsi* nor *B. gargarizans* represent independent evolutionary lineages; haplotypes from respective "species" failed to form monophyletic groups, and allozyme data failed to reveal any fixed allelic differences among examined populations (Appendix A). Subspecies division is also meaningless since these geographic population assemblages have little in common with evolutionary history.

The characters previously used to diagnose the four "species" were primarily based on morphology. Liu and Hu (1961) first detailed the differences between three "subspecies"*Bufo g. gargarizans*, *B. g. andrewsi*, and *B. g. minshanicus*. They claimed that *B. g. minshanicus* differed from the others by its smaller adult body size and the presence of large warts on the back of the heads, while *B. g. gargarizans* and *B. g. andrewsi* were discriminated by differences in tadpole morphology and some minor adult color differences (Liu and Hu, 1961). Despite these differences, it is often impossible to distinguish them. Local herpetologists often use location data and habitat information to identify them, i.e., specimens from streams and forest areas of mountainous western China are *B. g. andrewsi*, and specimens from ponds and low-lying plain areas (mostly agricultural land) are *B. g. gargarizans* (G. Wu, personal communication). *Bufo g. minshanicus* is restricted to a few locations in northwestern Sichuan and in the adjacent Gansu province. One possible explanation for the observed differences is that the morphological variation represents intraspecific polymorphisms. For example, polymorphisms generated as a result of adaptation to different breeding sites may explain the two types of tadpoles described by Liu and Hu (1961). Investigating these putative discriminating characters in a phylogenetic context should shed light on these issues and aid herpetologists in properly identifying specimens without relying on geography or habitat preference.

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Appendix A

Genotype data of the populations of the *Bufo gargarizans* species complex

Population:	31	32	11	12	14	30	13	25	10	9	15	16	Clade D	Clade K
n:	7	7	12	1	6	3	6	1	2	6	2	3	29	10
sAAT-A	b	b	bb(11) ab(1)	b	bb(3) ab(3)	bb(2) cc(1)	b	b	bb(1) dd(1)	b	ab(2)	aa(2) ab(1)	aa(2)bb(19) dd(1)ab(7)	b
mACOH-A	b	b	b	b	b	b	bb(2) bc(3) cc(1)	b	aa(1) bb(1)	bb(2) bc(4)	b	b	aa(1)bb(25) bc(3)	bb(5)cc(1) bc(4)

(*continued on next page*)

Appendix A (*continued*)

Population:	31	32	11	12	14	30	13	25	10	9	15	16	Clade D	Clade K
n:	7	7	12	1	6	3	6	1	2	6	2	3	29	10
sACOH-A	b	b	c	c	c	bb(2) ??(1)	b	b	aa(1) bb(1)	cc(2) bc(4)	c	c	aa(1)cc(25) bc(3)	bb(8)cc(1) bc(1)
CK-A	aa(1) cc(4) dd(1) cd(1)	aa(1) cc(5) cd(1)	d	d	d	d	d	d	d	d	d	d	d	d
EST	aa(4) bb(2) ??(1)	a	a	a	a	a	a	a	aa(1) bb(1)	a	a	a	aa(28)bb(1)	a
βGLUS	bb(4) cc(2) ?(1)	bb(6) bd(1)	aa(5) bb(5) cc(1) ab(1)	bc	aa(1) bb(5)	bb(3)	bb(5) cc(1)	b	bb(1) ??(1)	bb(4) cc(1) bc(1)	a	bb(2) bc(1)	aa(8)bb(15) cc(2)ab(1) bc(2)?(1)	bb(8)cc(1) bc(1)
GPI-A	b	b	b	b	b	ab(2) bb(1)	b	b	bb(1) ??(1)	b	b	b	b	b
GTDH-A	a	a	a	a	a	a	a	a	aa(7) bb(1)	a	a	a	aa(28)bb(1)	a
mIDH-A	bb(6) ab(1)	bb(6) dd(1)	a	a	a	c	b	a	b	aa(2) bb(2) ab(2)	a	a	aa(26)bb(2) ab(1)	aa(1)bb(8) ab(1)
sIDH-A	b	bb(4) bd(3)	c	c	c	d	b	b	cc(1) bb(1)	c	c	cc(2) ac(1)	cc(28)ac(1)	bb(8)cc(2)
LDH-B	a	a	a	a	a	a	a	a	aa(1) bb(1)	a	a	a	aa(28)bb(1)	a
sMDH-A	a	a	a	a	a	a	a	a	aa(1) bb(1)	a	a	a	aa(28)bb(1)	a
mMDH-A	a	a	aa(5) bb(1) ab(6)	ab	a	a	a	a	aa(6) ??(1)	aa(5) ab(1)	aa(1) ab(1)	a	aa(18)bb(1) ab(9)?(1)	a
mMDHP-A	bb(3) cc(4)	bc(2) cc(4) dd(1)	bb(9) ab(3)	b	ab(1) bb(5)	b	bb(1) cc(5)	b	bb(1) cc(1)	bb(5) cc(1)	aa(1) bb(1)	b	aa(1)bb(23) cc(1)ab(4)	bb(4)cc(6)
sMDHP-A	bb(6) bc(1)	b	b	b	bb(4) ab(2)	b	b	b	bb(1) cc(1)	b	b	b	bb(26)cc(1) ab(2)	b
PEP-A	b	bb(6) ab(1)	b	b	b	b	b	b	b	b	b	b	bb(28)cc(1)	b
PEP-B	a	a	a	a	a	b	a	a	a	a	a	a	a	a
PGM-A	a	a	a	a	a	a	a	a	aa(1) bb(1)	a	a	a	aa(28)bb(1)	a
PK-A	bb(2) cc(4) bc(1)	cc(5) dd(1) ??(1)	aa(1) bb(8) bc(3)	b	b	e	e	e	ee(1) ff(1)	bb(3) dd(2) be(1)	b	b	aa(1)bb(21) dd(2)ff(1) bc(3)be(1)	bb(2)ee(8)

Three loci (ADA-A, βGLUR, and sSOD-A) were monomorphic. Population numbers are site numbers in Fig. 1 and Table 1. For analysis, populations from Maoxian (11) and Pengxian (12), Hongya (10) and Omei (9), and Baoxing (16) and Dayi (15) were pooled together. 31, Lin'an; 32, Guandun; 14, Qionglai; 30, Antu; 13, Zhongjiang; and 25, Nanchuan.

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