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Genetic structure of Bufo bankorensis distinguished by amplified restriction fragment length polymorphism of cytochrome b

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Abstract

Background: *Bufo bankorensis* is an endemic species in Taiwan, and its populations are geographically and reproductively isolated. However, the distinction of Taiwanese *B. bankorensis* as a separate species from the Chinese *Bufo gargarizans* remains in dispute.

Results: A primer set was designed to explore the mitochondrial (mt)DNA cytochrome (Cyt) *b* sequence (700 bp) of *B. bankorensis* in 148 individuals collected from 12 locations in Taiwan. After a polymerase chain reaction and sequencing, we found that the nucleotide sequence of Cyt *b* contained two restricted enzyme sites of *Bam*HI and *Tsp*RI. Following *Bam*HI enzyme digestion, samples of *B. bankorensis* were divided into two clades: western (which were undigested) and eastern (which were digested) clades. Additionally, Cyt *b* of the western clade of *B. bankorensis* was not cut by *Bam*HI, while it was cut by *Tsp*RI into two sublineages. The result infers that at least two broadly divergent phylogroups of *B. bankorensis* exist in Taiwan and are not morphologically distinguishable. Based on the divergent sequence of Cyt *b* and cutting restriction enzymes, these populations were classified into three distinct phylogroups.

Conclusion: Genetically, one (western group 1, uncut by *Bam*HI and cut by *Tsp*RI) is most likely *B. gargarizans*, a second one (western group 2, uncut by both *Bam*HI and *Tsp*RI) is *B. bankorensis*, and a third one (eastern clade, cut by *Bam*HI but not cut by *Tsp*RI) could be a new subspecies. All three phylogroups were found in some areas, suggesting that they are sympatric, not allopatric.

Keywords: Cytochrome *b*; Restriction enzyme; Sympatry; *Bufo bankorensis*; *B. gargarizans*

Background

There are many species of Anura in Taiwan. They are identified and classified into five families: the Bufonidae, Hylidae, Microhylidae, Ranidae, and Rhacophoridae. The Bufonidae is one of the most species-rich families of anurans with more than 550 species in approximately 50 recognized genera (Frost 2011). As is known from the literature, *Bufo bufo* is in Europe, *Bufo gargarizans* is in mainland Asia, *Bufo japonicus* is restricted to Japan, and *Bufo miyakonis* is found in Miyako Island, Japan (Igawa et al. 2006). Interestingly, in the Bufonidae, only two species (*Bufo bankorensis* and *Bufo melanostictus*) are found in Taiwan (Li et al. 2006). *B. melanostictus* is a common toad in Asia. *B. bankorensis* is widely distributed in the island of Taiwan at 0 ~ 3,000 m in elevation. According to the classification of previous studies (Kawamura et al. 1980, 1982; Nishioka et al. 1990), two subspecies of *B. gargarizans*, i.e., *B. gargarizans* and *B. bankorensis*, are found in Taiwan. Typically, *B. bankorensis* is placed in the *B. gargarizans* species complex, but no morphological distinction exists (Inger 1972; Matsui 1984; Liu et al. 2000; Fu et al. 2005). The *B. gargarizans* complex is one of the most common and widely distributed amphibian groups in eastern Asia. Based on reproductive isolation mechanisms elucidated by crossing experiments, toads from Japan, China, and Taiwan are classified as the

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subspecies group *Bufo gargarizans japonicus* (Kawamura et al. 1980, 1982). *B. bankorensis* was reclassified as a distinct endemic species in Taiwan although similar to allopatric populations of *Bufo andrewsi* (Matsui 1986). Moreover, *B. bankorensis* is one of three clades of *B. japonicus* (the other two are *Bufo japonicus miyakonis* in Miyako Island, Japan and the eastern and western groups of the Japanese *Bufo japonicus japonicus* subspecies group, and *B. gargarizans* in China) (Igawa et al. 2006). Thus, the *Bufo* taxa of Taiwan, *B. bankorensis* and *B. gargarizans*, remain unclear.

Mitochondrial (mt)DNA can be a powerful molecular marker for reconstructing evolutionary lineages of animals (Avise 1994; Kocher and Stepien 1997; Zhao et al. 2011). Many recent phylogenetic studies also applied mtDNA markers to infer the histories of animals with respect to geography, geology, and paleoclimatology (Macey et al. 1998; Mulcahy and Mendelson 2000). Cytochrome (Cyt) *b*, a region of mtDNA, is used to determine phylogenetic relationships between organisms due to its sequence variability (Castresana 2001). In a phylogenetic study of *B. bufo* based on mtDNA (Cyt *b*, transfer (t) RNAs, 12S ribosomal (r)RNA, and 16S rRNA), gene sequences suggested that one group is *B. bufo* in Europe and the other is *B. japonicus* in the Far East. *B. japonicus* was later divided into four major clades corresponding to a group consisting of *B. gargarizans* in China, *B. bankorensis* in Taiwan, *B. miyakonis* in Miyako Island, and eastern and western groups of the Japanese *B. j. japonicus* subspecies group (Igawa et al. 2006). The taxonomic status of *B. bankorensis* has been widely debated, and various names, e.g., *B. bufo*, *B. gargarizans*, and *Bufo vulgaris* var. *asiatica*, have been either recognized as distinct species (Frost 1985; Matsui 1986; Zhao and Adler 1993) or synonymized with *B. gargarizans* which is widely distributed in China (Lue and Chen 1982). The taxonomic status and phylogenetic relationships among populations in eastern Asia are still unclear, and to understand the effects that past geological events had on the evolutionary history, further investigation is necessary (Fu et al. 2005). The debate as to whether the Taiwanese *B. bankorensis* is a species or subspecies, however, is still ongoing. This study was conducted to analyze the mtDNA Cyt *b* of *B. bankorensis* collected from various locations in Taiwan to verify the genetic structure and taxonomic status.

Methods

Animal collection

B. bankorensis and *Buergeria robusta* treatment and capture procedures were performed with permission from the Taroko National Park Administration (permit nos. 0990010921, 0990011963, 1000011365, and 1010011630), approved by the Committee for Animal Experimentation of National Dong Hwa University, and conformed to guidelines of the International Association for the Study of Pain. In total, 148 adult *B. bankorensis* were collected from 12 locations including Jhuzihu ($n = 20$), Chilan ($n = 7$), Guanwu ($n = 8$), Shueili ($n = 6$), Tungpu ($n = 15$), Tatachia ($n = 7$), Walami ($n = 9$), Motian ($n = 21$), Chukou ($n = 8$), Nanzihsien River ($n = 3$), Meishankou ($n = 32$), and Kenting ($n = 12$) (Table 1, Figure 1). In addition, *Buergeria robusta* and *Rana swinhoana* were collected from Shakadang Creek, a low-elevation area in Taroko National Park (Hualien, Taiwan). *B. melanostictus* was collected on our school campus in National Dong-Hwan University (Hualien, Taiwan). *B. gargarizans* was obtained from Shangai (China). *Buergeria robusta* and *R. swinhoana* were used as outgroups. In our previous study, we determined that the population of

Table 1 Summary of the sample size in each location

	Location	Latitude and longitude	Sample size	Western clade	Eastern clade
1	Jhuzihu	25.18°N, 121.54°E	20	20	
2	Chilan	24.58°N, 121.38°E	7	7	
3	Guanwu	24.50°N, 121.11°E	8	8	
4	Shueili	23.81°N, 120.85°E	6	6	
5	Tungpu	23.55°N, 120.91°E	15	15	
6	Tatachia	23.49°N, 120.89°E	7	7	
7	Walami	23.35°N, 121.19°E	9	1	8
8	Kenting	21.94°N, 120.80°E	12	12	
9	Motian	23.19°N, 121.02°E	21		21
10	Chukou	23.44°N, 120.59°E	8	6	2
11	Nanzihsien River	23.45°N, 120.90°E	3	3	
12	Meishankou	23.27°N, 120.83°E	32	30	2
	Total		148	115	33

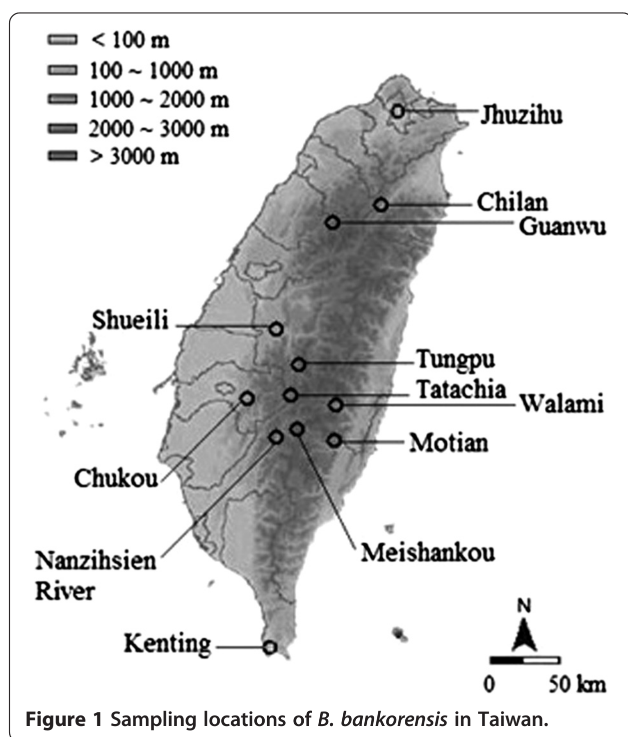

Figure 1 Sampling locations of *B. bankorensis* in Taiwan.

B. bankorensis could be divided into two major clades (western and eastern clades) following a phylogenetic analysis of haplotypes from a control region (D-loop) sequence (unpublished data).

Preparation of genomic DNA by B. bankorensis mtDNA extraction

Animals were first placed on ice in a bucket causing them to pass out in accordance with the Animal Protection Law for animal welfare. Next, 20 ~ 30 mg of muscle tissue was excised from each animal. The Quick-Extract^{TM} DNA Extraction Solution (Epicentre, Madison, WI, USA) was used to extract the muscle tissue following the manufacturer's instructions. The muscle was cut into pieces in GT buffer, 20 μL of proteinase K was added, and then it heated to 60°C for 10 min. Next, 500 μL of GBT buffer was added to the vial and heated to 60°C for 10 min, followed by the addition of 500 μL of 100% absolute ethanol to precipitate the DNA. Finally, 750 μL of the mixture was loaded onto a GD column (Epicentre) for filtering, and the filtrate was centrifuged at 13,000 rpm for 1 min. The previous steps were repeated on the remaining mixture. Then, 400 μL of W1 buffer was added to the GD column, and the filtrate was centrifuged at 13,000 rpm for 30 s. Next, 600 μL of wash buffer (ethanol added) was added to the GD column, the filtrate was centrifuged at 13,000 rpm for 30 s again, and the mixture was subjected to further centrifugation at 13,000 rpm for 30 min. The filtrate of the GD column was put into a new 1.5-mL Eppendorf vial, and 100 μL of elution buffer or double-distilled (dd)H_2O (previously preheated at 60°C) was added to dissolve the DNA. After standing for 5 min, the vial was centrifuged at 13,000 rpm for 30 s. The remaining solution contained the DNA. The DNA solution was stored at −20°C, or polymerase chain reaction (PCR) amplification was carried out immediately.

PCR amplification

In total, 25 μL in the PCR vial contained 1 μL of genomic DNA, 1 μL of 10 pmol primers (Table 2), 2.5 μL of 10× PCR buffer, 2 μL of dNTP, and 1 U Taq (R001A, Takara Bio, Otsu, Japan) with ddH_2O added to reach 25 μL with adequate vortexing and centrifuging. The vial was heated to 94°C for 5 min, followed by 35 cycles of heat denaturation at 94°C for 30 s, primer annealing at 45°C for 30 s, and DNA extension at 72°C for 1 min in a PCR machine (ASTEC PC802, GMB, Banciao, New Taipei City, Taiwan), with a final amplification step at 72°C for 10 min. Moreover, 1.5% agarose gel electrophoresis was employed to analyze the products, which were visualized by SYBR Safe DNA gel stain (S33102, Invitrogen^{TM}, Life Technologies, Taipei, Taiwan).

Sequencing Cyt b

After gel electrophoresis, PCR products were extracted with a DNA gel extraction mini kit (Geneaid, Agoura Hills, CA, USA) and selected for DNA sequencing analysis (Genomics BioSci & Tech, Taipei, Taiwan). The isolated and sequenced nucleotide fragments ($n = 148$, 700 bp) of *B. bankorensis* were blasted by a Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD, USA) to confirm validation of the cloned sequences. Based on these sequences, restriction enzyme digestion of DNA (vers. 711, www.biophp.org) was used to find restriction sites such as *Bam*HI and *Tsp*RI.

BamHI and TspRI restriction sites confirmed with an agarose gel electrophoresis analysis

In total, 10 μL of enzymatic digestion reaction contained 4 μL of PCR products, 1 μL of 10× buffer E, l μL of 10× bovine serum albumin, and 1 μL of *Bam*HI (BioLab, Taigen, Taipei, Taiwan) or *Tsp*RI (BioLab), and ddH_2O was added to reach 10 μL with vortexing. The digestion reaction was conducted at 37°C for 2 h (*Bam*HI) or 65°C for 4 h (*Tsp*RI). After digestion, 5 μL of products was electrophoresed on a 1.5% agarose gel for 30 min at 100 V, photographed, and archived.

Table 2 Sequences of cytochrome (Cyt) *b* primers

Primer	Sequence	Amplicon size
Bu_Cyb-F	5′-ACCGCCGACACATCCATAGC-3′	700 bp
Bu_Cyb-R	5′-CGAGAATAGCATAGGCGAACAAGA-3′	

Accession number: AY247260.

(A) Alignments

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Majority          CATATCTGCCGAGATGTAAACAACGGCTGGCTGCTTCGCAACCTTCATGCAAATGGCGCCTCATTCTTCTTCATCTGCATCTACCTCCACATCGGACGGG
                           10        20        30        40        50        60        70        80        90       100
B.bankorensis 1   CATATCTGCCGAGATGTAAACAACGGCTGGCTGCTTCGCAACCTTCATGCAAATGGCGCCTCATTCTTCTTCATCTGCATTTACCTCCACATCGGACGGG 100
B.bankorensis 2   CATATCTGCCGAGATGTAAACAACGGCTGGCTGCTTCGCAACCTTCATGCAAATGGCGCCTCATTCTTCTTCATCTGCATCTACCTCCACATCGGGCGGG 100
B.gargarizans     CATATCTGCCGAGATGTAAACAACGGCTGGCTGCTTCGCAACCTTCATGCAAATGGCGCCTCATTCTTCTTCATCTGCATCTACCTCCACATCGGACGGG 100

Majority          GTATATACTATGGCTCCTTCTTATTCAAAGAAACTTGAAATATTGGTGTCATTCTCCTATTTCTGGTCATAGCTACAGCATTCGTGGGCTACGTCCTTCC
                          110       120       130       140       150       160       170       180       190       200
B.bankorensis 1   GTATGTACTATGGCTCCTTCTTATTCAAAGAAACTTGAAATATTGGTGTCATTCTCCTATTTCTGGTCATAGCTACGGCATTCGTAGGCTATGTCCTTCC 200
B.bankorensis 2   GTATATACTATGGCTCCTTCTTATTCAAAGAAACTTGAAATATTGGTGTCATTCTCCTATTTCTGGTCATAGCTACAGCATTCGTGGGCTACGTCCTTCC 200
B.gargarizans     GTATATACTATGGCTCCTTCTTATTCAAAGAAACTTGAAATATTGGTGTCATTCTCCTATTTCTGGTCATAGCTACAGCATTCGTGGGCTACGTCCTTCC 200

Majority          ATGGGGACAAATATCTTTCTGGGGGGCAACTGTTATTACAAACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGC
                          210       220       230       240       250       260       270       280       290       300
B.bankorensis 1   ATGGGGACAAATATCTTTCTGAGGGGCAACCGTTATTACAAACCTTCTCTCCGCTGCCCCCTATATTGGAACTGAACTTGTTCAATGAATCTGAGGGGGC 300
B.bankorensis 2   ATGGGGACAAATATCTTTCTGGGGGGCAACTGTTATTACAAACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGG 300
B.gargarizans     ATGGGGACAAATATCCTTCTGGGGGGCAACTGTTATTACAAACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGC 300

Majority          TTTTCAGTAGACAACGCAACTCTAACACGATTTTTTACATTTCACTTTATCCTGCCGTTTATTATTGCAGGCGCTTCCATGCTTCACCTTCTATTTTTAC
                          310       320       330       340       350       360       370       380       390       400
B.bankorensis 1   TTTTCAGTAGACAACGCAACTCTAACACGATTTTTTACATTTCACTTTATCCTACCGTTTATCATTGCAGGCGCCTCCATACTTCACCTTCTATTTTTAC 400
B.bankorensis 2   TTTTCAGTAGACAACGCAACTCTAACACGATTTTTTACATTTCACTTTATCCTGCCGTTTATTATTGCAGGCGCTTCCATGCTTCACCTTCTATTTTTAC 400
B.gargarizans     TTTTCAGTAGACAACGCAACTCTAACACGATTTTTTACATTTCACTTTATCCTGCCGTTTATTATTGCAGGCGCTTCCATGCTTCACCTTCTATTTTTAC 400

Majority          ATCAAACAGGGTCCTCTAACCCAACAGGCCTTAACCCCAACTTTGACAAGATCCCCTTCCACGCCTATTACTCCTACAAAGATCTCTTCGGCTTCGCAAT
                          410       420       430       440       450       460       470       480       490       500
B.bankorensis 1   ATCAAACAGGATCCTCTAACCCAACAGGCCTTAACCCCAACTTTGACAAGATCCCTTTCCACGCCTATTACTCCTACAAAGATCTCTTCGGCTTCGCAAT 500
B.bankorensis 2   ATCAAACAGGGTCCTCTAACCCAACAGGCCTTAACCCCAACTTCGACAAGATCCCCTTCCACGCCTATTACTCCTACAAAGATCTCTTCGGCTTCGCAAT 500
B.gargarizans     ATCAAACAGGGTCCTCTAACCCAACAGGCCTTAACCCCAACTTTGACAAGATCCCCTTCCACGCCTATTACTCCTACAAAGATCTCTTCGGCTTCGCAAT 500

Majority          TATGCTTGCCCTACTTGCCTTACTATCCACTTTCGCCCCCAACCTCTTAGGTGACCCAGACAACTTCACACCAGCTAACCCCTTGGTCACCCCACCACAC
                          510       520       530       540       550       560       570       580       590       600
B.bankorensis 1   TATACTTGCCCTACTTGCCTTACTATCCACCTTCGCCCCCAACCTCTTAGGTGACCCAGACAACTTCACACCGGCTAACCCCTTGGTCACCCCACCACAC 600
B.bankorensis 2   TATGCTTGCCCTACTTGCCTTACTATCCACTTTCGCCCCCAACCTCTTAGGTGACCCAGACAACTTCACACCAGCTAACCCCTTGGTCACCCCACCACAC 600
B.gargarizans     TATGCTTGCCCTACTTGCCTTACTATCCACTTTCGCCCCCAACCTCTTAGGTGACCCAGACAACTTCACACCAGCTAACCCCTTGGTCACCCCACCACAC 600

Majority          ATCAAGCC

B.bankorensis 1   ATCAAGCC                                                                                             608
B.bankorensis 2   ATCAAGCC                                                                                             608
B.gargarizans     ATCAAGCC                                                                                             608
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(B) BamHI site

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Majority          ATCAAACAGGGTCCTCTAACCCAACAGGCCTTAACCCCAACTTTGACAAGATCCCCTTCCACGCCTATTACTCCTACAAAGATCTCTTCGGCTTCGCAAT
                          410       420       430       440       450       460       470       480       490       500
B.bankorensis 1   ATCAAACAGGATCCTCTAACCCAACAGGCCTTAACCCCAACTTTGACAAGATCCCTTTCCACGCCTATTACTCCTACAAAGATCTCTTCGGCTTCGCAAT 500
B.bankorensis 2   ATCAAACAGGGTCCTCTAACCCAACAGGCCTTAACCCCAACTTCGACAAGATCCCCTTCCACGCCTATTACTCCTACAAAGATCTCTTCGGCTTCGCAAT 500
B.gargarizans     ATCAAACAGGGTCCTCTAACCCAACAGGCCTTAACCCCAACTTTGACAAGATCCCCTTCCACGCCTATTACTCCTACAAAGATCTCTTCGGCTTCGCAAT 500
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Figure 2 Alignments of nucleotide sequence and the *Bam*HI site. Alignments of nucleotide sequence of Cyt *b* of *B. bankorensis* and *B. gargarizans* **(A)** and the *Bam*HI site (GGATCC) of the Cyt *b* sequence **(B)**. The alignments were performed with DNA Star. *B. bankorensis* 1 and 2 are from the Walami area (labeled location number 7 in Table 1).

Phylogenetic analysis

A haplotype genealogy was constructed using an unrooted neighbor-joining (NJ) algorithm and a bootstrap method with 1,000 replicates using the software MEGA vers. 4.0 (Biodesign Institute, Center for Evolutionary Functional Genomics, Tempe, AZ, USA). *B. japonicus* was used as an outgroup.

Results

The Cyt *b* primer set was applied to run the PCR with DNA of *B. bankorensis*, *B. melanostictus*, *B. gargarizans*, *Buergeria robusta*, and *R. swinhoana*. Figure 2 illustrates alignments of nucleotide sequences of Cyt *b* of *B. bankorensis*, *B. gargarizans*, and *Buergeria robusta*. Only PCR

products of *B. bankorensis*, *B. gargarizans*, and *Buergeria robusta* were amplified, and the size of the band was about 700 bp, but *R. swinhoana* was not amplified. Figure 3A presents the results of Cyt *b* PCR products. Based on the Cyt *b* sequence, the *Bam*HI restriction site was found at 410 ~ 420 of 700 bp (Figure 3B). After digestion with the *Bam*HI restriction enzyme, the mtDNA of *B. bankorensis* was divided into two types, and electrophoretic results are shown in Figure 3B. *B. bankorensis* populations were divided into two types based on whether the sequence was cut or not. *B. bankorensis* 1 (Bu1) and Bu3 were the eastern group, and Bu2 was the western group. The distinction between the eastern and western groups depended upon geography because samples were collected from both western to eastern parts of the island of Taiwan. The western group was not cut and had a size of about 700 bp, while the eastern group was digested into two bands of about 400 and 300 bp. In a comparison of Cyt *b* sequences among eastern and western groups of *B. bankorensis* with *B. gargarizans*, only the eastern group of *B. bankorensis* contained the *Bam*HI restriction site. Additionally, comparison of variations in Cyt *b* sequences revealed that 99% similarity was observed for western group 1 of *B. bankorensis* with *B. gargarizans* (Figure 4A), and 97% similarity was found for western group 2 of *B. bankorensis* with *B. gargarizans* (Figure 4B). The *Tsp*RI site of Cyt *b* among *B. gargarizans* and *B. bankorensis* is depicted in Figure 5A. *B. gargarizans* DNA was digested by the *Tsp*RI enzyme. Moreover, the western clade of *B. bankorensis* was further digested by *Tsp*RI and defined as western clade 1, while the other one (western clade 2) was not (Figure 5B). Therefore, the Cyt *b* sequence of *B. bankorensis* could be cut by *Tsp*RI which was 99% similar to *B. gargarizans*, whereas the uncut type was only 97% similar. Figure 6 illustrates phylogenetic relationships. Surprisingly, western group 1 differed from *B. gargarizans* and formed a cluster with *B. gargarizans* at 99% similarity. This result suggests that two *B. bankorensis* populations (western and eastern clades) exist in Taiwan. Moreover, the western clade had two phylogroups, one of which was possibly genetically related to *B. gargarizans* (western clade 1) but morphologically differed, and the other which was still named *B. bankorensis* (western clade 2). The eastern clade may be a new subspecies in the genetic structure.

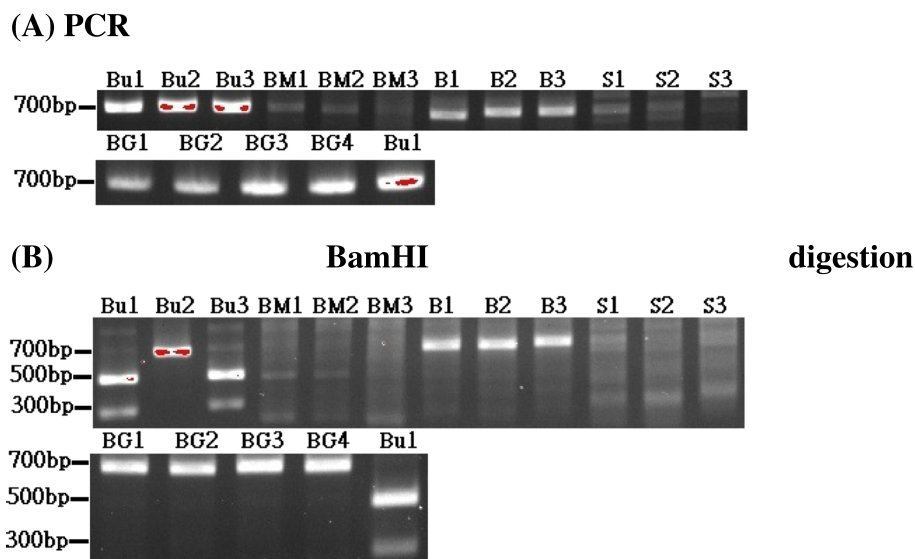

Figure 3 PCR products of various toads and frogs (A), and digestion of *Bam*HI (B). *B. bankorensis* (Bu), *B. melanostictus* (BM), *B. gargarizans* (BG), *Buergeria robusta* (B), and *R. swinhoana* (S). *Buergeria robusta* (B) and *R. swinhoana* (S) were used to test the specificity of the primer set. No specific bands were found for *B. melanostictus* or *R. swinhoana*, and no further sequencing was performed. Bu1 and Bu3 are the eastern clade. Bu2 is the western clade. Bu1, Bu2, Bu3, BM1, BM2, BM3, B1, B2, B3, S1, S2, S3, BG1, BG2, BG3, and BG4 represent different individual samples in each group.

Discussion

After the *Bam*HI assay of the Cyt *b* sequence of *B. bankorensis*, the presence of two genotypes was observed: one was the Cyt *b* genotype of the western clade (which was not cut), and the other was the Cyt *b* genotype of the eastern clade (which was cut). This result was similar to that for the Japanese common toad *B. japonicus* which is classified into two subspecies, *B. j. japonicus* and *Bufo japonicus formosus* of western and eastern regions of Japan, respectively (Hase et al. 2012). Interestingly, the similarity of the Cyt *b* nucleotide sequence

(A) Western group 1

Score	Expect	Identities	Gaps	Strand
1101 bits(596)	0.0	604/608(99%)	0/608(0%)	Plus/Plus

```
Query  1    CATATCTGCCGAGATGTAAACAACGGCTGGCTGCTTCGCAACCTTCATGCAAATGGCGCC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    CATATCTGCCGAGATGTAAACAACGGCTGGCTGCTTCGCAACCTTCATGCAAATGGCGCC  60

Query  61   TCATTCTTCTTCATCTGCATCTACCTCCACATCGGGCGGGGTATATACTATGGCTCCTTC  120
            ||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  61   TCATTCTTCTTCATCTGCATCTACCTCCACATCGGACGGGGTATATACTATGGCTCCTTC  120

Query  121  TTATTCAAAGAAACTTGAAATATTGGTGTCATTCTCCTATTTCTGGTCATAGCTACAGCA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TTATTCAAAGAAACTTGAAATATTGGTGTCATTCTCCTATTTCTGGTCATAGCTACAGCA  180

Query  181  TTCGTGGGCTACGTCCTTCCATGGGGACAAATATCTTTCTGGGGGGCAACTGTTATTACA  240
            ||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  181  TTCGTGGGCTACGTCCTTCCATGGGGACAAATATCCTTCTGGGGGGCAACTGTTATTACA  240

Query  241  AACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| 
Sbjct  241  AACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGC  300

Query  301  TTTTCAGTAGACAACGCAACTCTAACACGATTTTTTACATTTCACTTTATCCTGCCGTTT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TTTTCAGTAGACAACGCAACTCTAACACGATTTTTTACATTTCACTTTATCCTGCCGTTT  360

Query  361  ATTATTGCAGGCGCTTCCATGCTTCACCTTCTATTTTTACATCAAACAGGGTCCTCTAAC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  ATTATTGCAGGCGCTTCCATGCTTCACCTTCTATTTTTACATCAAACAGGGTCCTCTAAC  420

Query  421  CCAACAGGCCTTAACCCCAACTTCGACAAGATCCCCTTCCACGCCTATTACTCCTACAAA  480
            ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  421  CCAACAGGCCTTAACCCCAACTTTGACAAGATCCCCTTCCACGCCTATTACTCCTACAAA  480

Query  481  GATCTCTTCGGCTTCGCAATTATGCTTGCCCTACTTGCCTTACTATCCACTTTCGCCCCC  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GATCTCTTCGGCTTCGCAATTATGCTTGCCCTACTTGCCTTACTATCCACTTTCGCCCCC  540

Query  541  AACCTCTTAGGTGACCCAGACAACTTCACACCAGCTAACCCCTTGGTCACCCCACCACAC  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  AACCTCTTAGGTGACCCAGACAACTTCACACCAGCTAACCCCTTGGTCACCCCACCACAC  600

Query  601  ATCAAGCC  608
            ||||||||
Sbjct  601  ATCAAGCC  608
```

(B) Western group 2

Score	Expect	Identities	Gaps	Strand
1007 bits(545)	0.0	587/608(97%)	0/608(0%)	Plus/Plus

```
Query  1    CATATCTGCCGAGATGTAAACAACGGCTGGCTGCTTCGCAACCTTCACGCAAATGGCGCC  60
            ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  1    CATATCTGCCGAGATGTAAACAACGGCTGGCTGCTTCGCAACCTTCATGCAAATGGCGCC  60

Query  61   TCATTCTTCTTCATCTGCATATACCTCCACATCGGACGGGGTATGTATTATGGCTCCTTC  120
            |||||||||||||||||||| ||||||||||||||||||||||| || ||||||||||||
Sbjct  61   TCATTCTTCTTCATCTGCATCTACCTCCACATCGGACGGGGTATATACTATGGCTCCTTC  120

Query  121  TTATTCAAAGAAACTTGAAATATTGGTGTCATTCTCCTATTTCTGGTCATAGCTACGGCA  180
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  121  TTATTCAAAGAAACTTGAAATATTGGTGTCATTCTCCTATTTCTGGTCATAGCTACAGCA  180

Query  181  TTCGTAGGCTACGTCCTTCCATGGGGACAAATATCTTTCTGAGGGGCAACCGTTATTACA  240
            ||||| ||||||||||||||||||||||||||||| ||||| |||||||| |||||||||
Sbjct  181  TTCGTGGGCTACGTCCTTCCATGGGGACAAATATCCTTCTGGGGGGCAACTGTTATTACA  240

Query  241  AACCTTCTCTCCGCTGCCCCCTATATTGGAACTGAGCTTGTTCAATGAATCTGAGGGGGG  300
            |||||||| ||||||||||||||||| |||||||| |||||||| |||||||||||||| 
Sbjct  241  AACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGC  300

Query  301  TTTTCAGTAGACAACGCAACTCTAACACGATTTTTTACATTTCACTTTATCCTGCCGTTT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TTTTCAGTAGACAACGCAACTCTAACACGATTTTTTACATTTCACTTTATCCTGCCGTTT  360

Query  361  ATCATTGCAGGCGCCTCCATGCTTCACCTTCTATTTTTACATCAAACAGGGTCCTCTAAC  420
            || ||||||||||| |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  ATTATTGCAGGCGCTTCCATGCTTCACCTTCTATTTTTACATCAAACAGGGTCCTCTAAC  420

Query  421  CCAACAGGCCTTAATCCCAACTTTGACAAGATCCCTTTCCACGCCTATTACTCCTACAAA  480
            |||||||||||||| |||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  421  CCAACAGGCCTTAACCCCAACTTTGACAAGATCCCCTTCCACGCCTATTACTCCTACAAA  480

Query  481  GATCTCTTCGGCTTCGCAATTATACTTGCCCTACTTGCCTTACTATCCACCTTCGCCCCC  540
            ||||||||||||||||||||||| |||||||||||||||||||||||||| |||||||||
Sbjct  481  GATCTCTTCGGCTTCGCAATTATGCTTGCCCTACTTGCCTTACTATCCACTTTCGCCCCC  540

Query  541  AACCTCTTAGGCGACCCAGACAACTTCACACCAGCTAACCCCTTGGTCACCCCACCACAC  600
            ||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  AACCTCTTAGGTGACCCAGACAACTTCACACCAGCTAACCCCTTGGTCACCCCACCACAC  600

Query  601  ATCAAGCC  608
            ||||||||
Sbjct  601  ATCAAGCC  608
```

Figure 4 Alignments of Cyt *b* sequences. Alignments of Cyt *b* sequences between western clade 1 of *B. bankorensis* and *B. gargarizans* **(A)**, and western clade 2 of *B. bankorensis* and *B. gargarizans* **(B)**. The alignment of nucleotide sequences was prepared using Mega 4 and NCBI blast.

(A) TspRI site

```
Majority          ATGGGGACAAATATCTTTCTGGGGGGCAACTGTTATTACAAACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGC
                                     210       220       230       240       250       260       270       280       290       300
B. bankorensis 7  ATGGGGACAAATATCTTTCTGAGGGGCAACCGTTATTACAAACCTTCTCTCCGCTGCCCCCTATATTGGAACTGAACTTGTTCAATGAATCTGAGGGGGC 300
B. bankorensis 8  ATGGGGACAAATATCTTTCTGAGGGGCAACCGTTATTACAAACCTTCTCTCCGCTGCCCCCTATATTGGAACTGAACTTGTTCAATGAATCTGAGGGGGC 300
B. bankorensis 9  ATGGGGACAAATATCTTTCTGGGGGGCAACTGTTATTACAAACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGG 300
B. bankorensis 10 ATGGGGACAAATATCTTTCTGAGGGGCAACCGTTATTACAAACCTTCTCTCCGCTGCCCCCTATATTGGAACTGAACTTGTTCAATGAATCTGAGGGGGC 300
B. bankorensis 11 ATGGGGACAAATATCTTTCTGGGGGGCAACTGTTATTACAAACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGG 300
B. bankorensis 12 ATGGGGACAAATATCTTTCTGGGGGGCAACTGTTATTACAAACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGG 300
B. gargarizans 1  ATGGGGACAAATATCCTTCTGGGGGGCAACTGTTATTACAAACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGC 300
B. gargarizans 2  ATGGGGACAAATATCCTTCTGGGGGGCAACTGTTATTACAAACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGC 300
B. gargarizans 3  ATGGGGACAAATATCCTTCTGGGGGGCAACTGTTATTACAAACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGC 300
B. gargarizans 4  ATGGGGACAAATATCCTTCTGGGGGGCAACTGTTATTACAAACCTTCTTTCCGCTGCCCCCTATATCGGAACTGAACTTGTTCAGTGAATCTGAGGGGGC 300
```

(B) TspRI Digestion

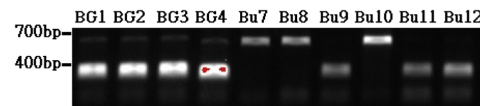

Figure 5 The *Tsp*RI site and digestion of *Tsp*RI. (A) The *Tsp*RI site (NNCASTGNN in the green box; N = A, T, C, G; S = C, G) of cytochrome *b* in the western clade and *B. gargarizans*. **(B)** Digestion of *Tsp*RI in *B. bankorensis* (Bu) and *B. gargarizans* (BG). BG1, BG2, BG3, BG4, Bu7, Bu8, Bu9, Bu10, Bu11, and Bu12 represent different individual samples in each group. BG1, BG2, BG3, BG4 are from China. Bu7, Bu8, Bu9, Bu10, Bu11, and Bu12 are from the Jhuzihu area, Taiwan (labeled location number 1 in Table 1).

was 99% between the western clade 1 and *B. gargarizans*, and they were further digested by *Tsp*RI, whereas the other western clade 2 was 97% similar with *B. gargarizans* and was not cut by *Tsp*RI. Recent molecular phylogenetic studies (Liu et al. 2000; Fu et al. 2005) supported the two-species classification by synonymizing *B. bankorensis* with *B. gargarizans*. Our data (Figure 6) revealed that *B. bankorensis* was proposed to be *B. japonicus* which was divided into three major clades corresponding to a group consisting of *Bufo japonicus gargarizans* in China, *Bufo japonicus bankorensis* in Taiwan, and *B. j. miyakonis* in Miyako Island, Japan (Igawa et al. 2006); *B. bankorensis* was also postulated to be an endemic species in Taiwan (Fu et al. 2005). From ecological view, the range of certain species is associated with habitat conditions. In the current study, the size and age at metamorphosis of tadpoles vary among individuals from the same species in different habitats in a relatively small area, suggesting that distributions of different species depend upon adaptation to extreme conditions (Goldberg et al. 2012). Moreover, we found that *bankorensis* toads formed a complex from the restriction enzyme and phylogenetic relationship, but more detailed assessments including morphology and physiology need to be carried out in the future work to identify subspecies or new species existing.

A previous report found that *B. japonicus* in the Far East was divided into two groups, one of which became two subspecies: *B. j. gargarizans* in China and Taiwan

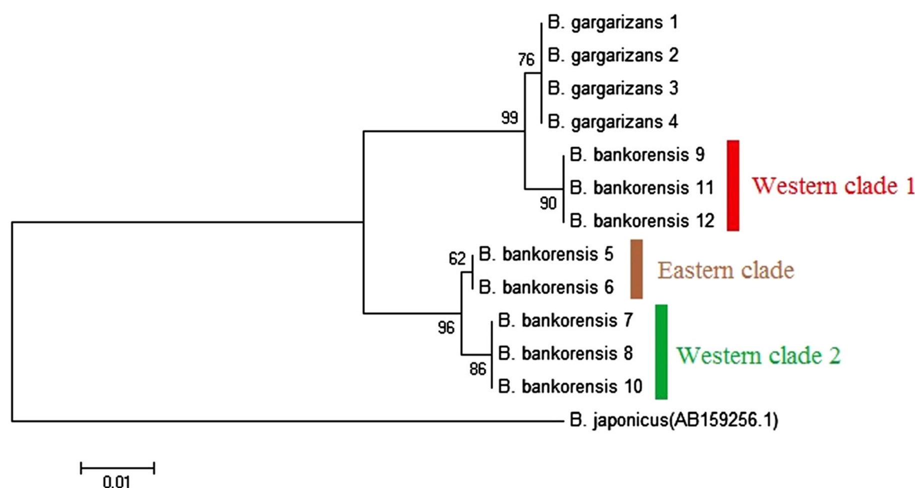

Figure 6 Phylogenetic tree. The neighbor-joining phylogenetic tree of cytochrome *b* of the eastern and western clades of *B. bankorensis*, *B. gargarizans*, and *B. japonicus* (sequence AB159256.1 from NCBI GenBank). The phylogenetic tree was analyzed by Mega 4. *B. japonicus* was used as an outgroup. *B. gargarizans* (BG1, BG2, BG3, and BG4) was from China. The eastern (Bu5 and Bu6) and western clades (Bu7, Bu8, Bu9, Bu10, Bu11, and Bu12) were from the Jhuzihu area, Taiwan (labeled location number 1 in Table 1).

and *B. j. miyakonis* in Miyako Island, Japan, while the other group became four subspecies on the eastern and western groups of Japanese *B. j. japonicus*, Hakodate, and Yaku Island, Japan (Nishioka et al. 1990). However, in past phylogenetic studies, sampling was limited to a small sample size (mostly one or two locations and did not include samples from eastern Taiwanese B. Bankorensis populations). Given that populations resembling *B. gargarizans* occur on an isolated island (Taiwan Island), there are two alternative hypotheses that might explain this pattern: (1) *B. bankorensis* populations are from a single lineage of *B. gargarizans* that subsequently dispersed to the island and became isolated, or (2) *B. bankorensis* independently evolved multiple times as *B. gargarizans* colonized the island. The present work proved the existence of two species (*B. gargarizans* and *B. bankorensis*) in Taiwan using a simple restriction enzyme (*Tsp*RI). From our data and according to previous reports, there are two subspecies, *Bufo gargarizans gargarizans* and *Bufo gargarizans bankorensis*, in Taiwan (Kawamura et al. 1980, 1982; Nishioka et al. 1990). Furthermore, *B. bankorensis* was classified in the *B. gargarizans* species complex (Inger 1972; Matsui 1984; Liu et al. 2000; Fu et al. 2005), one of the most common and widely distributed amphibian groups in eastern Asia. The eastern and western groups of *B. japonicus* were divided into several subclades that tended to reflect the region-specific geographic distribution of all localities except *B. j. japonicus* from Hakodate, Japan (Igawa et al. 2006). The western clade of *B. bankorensis* can be distinguished by restriction fragment length polymorphism and the phylogenetic tree, but not by nucleotide homology. This viewpoint suggests that the tree represents homoplasy. This region-specific subclade of the toad, such as western groups 1 and 2, was also found in Taiwanese *B. bankorensis*. This finding is in agreement with a report by Hase et al. (2012) that an admixed population was observed in the urban Tokyo area that consisted of both native and non-native *B. japonicus* subspecies. Conversely, *Bufo tibetanus* is a morphologically identified species but not genetically diagnosable, which was inferred to be a junior synonym of *B. gargarizans* (Zhan and Fu 2011). Our results propose that two populations (western and eastern clades) of *B. bankorensis* appear in Taiwan and are classified into three distinct phylogroups: the first is genetically related to *B. gargarizans*, the second should be *B. bankorensis*, and the third may be considered a new subspecies. In the same area (Tungpu, Taiwan), we also identified two different clades: western clades 1 and 2. The molecular phylogenetic studies supported *B. gargarizans* and *B. bankorensis* being synonymized, and *B. bankorensis* only being a lineage of *B. gargarizans* (Liu et al. 2000; Fu et al. 2005). This reflects a previous study (Fu et al. 2005) showing that *B. gargarizans* and *B. bankorensis* may be subspecies if samples were collected at certain locations. The present study postulates that *B. bankorensis* (western clade 1) is only a lineage of *B. gargarizans* because they are only genetically identifiable in contrast to having different morphologies. Hence, *B. bankorensis* is one species and is restricted to Taiwan. Based on our data, all three distinct phylogroups were observed in the wild and were found in some locations, further suggesting that they are sympatric, not allopatric.

Conclusions

Genetically, one (western group 1, uncut by *Bam*HI and cut by *Tsp*RI) is most likely *B. gargarizans*, a second one (western group 2, uncut by both *Bam*HI and *Tsp*RI) is *B. bankorensis*, and a third one (eastern clade, cut by *Bam*HI but not cut by *Tsp*RI) could be a new subspecies. *B. bankorensis* is recognized as one species having different morphologies compared with *B. gargarizans* and is restricted to Taiwan. All three phylogroups were found in some areas, suggesting that they are sympatric, not allopatric.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

C-CC, K-WL, P-YS, and Yi-WT carried out the molecular genetic studies and participated in the sequence alignment. T-LY and L-HC participated in the design of the study and performed the statistical analysis. K-JH and C-FW conceived the study and participated in its design and coordination and drafted the manuscript. All authors read and approved the final manuscript.

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