1 **Morphological evidence for introgressive hybridization between *Feirana quadranus* and**
2 ***Feirana taihangnica* in Qinling Mountains, China**

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13

14 **Abstract**

15   **Background.** *Feirana quadranus* and *Feirana taihangnica*, two species of frogs
16 inhabiting in waterbodies in the Qinling (Tsinling) Mountains, China, are believed to be sister
17 species that diverged 4.6 million years ago. In their sympatric area, morphological variations
18 found between the two species imply that the two species had inter-bred. Additionally, *F.*
19 *taihangnica*'s polyandrous breeding behavior, without amplexus, would not hinder the
20 potential hybridization.
21   **Methods.** To verify the hybridization, 117 specimens of *F. quadranus* and *F. taihangnica*
22 were collected from eight sampling sites in their sympatric area, and 110 of the specimens
23 were classified morphologically into VV, vw&wv, and ww, representing the putative parental
24 and suspected hybrid types. Their maternal bloodlines were identified using a phylogenetic
25 tree based on a region of the mitochondrial *16S rRNA* gene.
26   In total, 34 morphometric indices were selected to analyze the morphological variation
27 between 16S-types or among morphotypes. A principal component analysis (PCA) and linear
28 discriminant analysis (LDA) were conducted on total or partial indices for females, males,
29 and total specimens, as well as simulated populations with falsified morphotypes. The most
30 important indices for differentiation among morphotypes were revealed with the assistance of
31 heat-maps.
32   **Results.** In the mitochondrial DNA tree, most of the VV were in the same clade as the
33 reference *F. quadranus*, labeled as Q, while most of the ww and vw&wv were grouped with
34 the reference *F. taihangnica*, labeled as T. According to the PCA, there was a clear
35 differentiation between VV and ww, while vw&wv specimens were in the middle area close
36 to ww. According to the LDA, VV, vw&wv, and ww were clustered into three separate
37 groups. An ambiguous differentiation between Q and T was shown both in mtDNA tree and
38 in multivariate analyses. Seven of the specimens with conflicting classifications blurred the
39 morphological boundary between Q and T. In both the PCA and LDA, indices that were
40 based on the extent of bumps and skin coloration discriminated VV, vw&wv, and ww better
41 than ratio indices that were derived from measurements.
42   **Discussion.** The distribution of VV, vw&wv, and ww in multivariate spaces, especially
43 vw&wv being scattered between VV and ww, demonstrated an introgressive hybridization
44 pattern. The extents of bumps in the shape of an inverted "V" between the shoulder blades,
45 spot pattern on the back, and large bumps above the anal region were the most important
46 characteristics for differentiating between three morphotypes or between *F. quadranus* and *F.*
47 *taihangnica*.

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1 Introduction

1.1 Discovery and classification history of the genus Feirana

Genus *Feirana* (Dubois, 1992) belonging to Tribe Paini Dubois, 1992, of subfamily Dicroglossinae, Anderson, 1871, of family Ranidae, Rafinesque-Schmaltz, 1814 (Amphibia, Anura), contains *Feirana* (*Rana*) *quadranus* (Liu, Hu & Yang, 1960), *Feirana taihangnica* (Chen & Jiang, 2002) and *Feirana kangxianensis* (Yang *et al*., 2011) to date. They are widely distributed in the areas of the southern Taihang Mountains, the Zhongtiao, Funiu, and Qinling Mountains, the eastern Minshan Mountains, the Longmen, Micang, Daba, and Wushan Mountains and the northern Wuling Mountains (Fei, 1999; Fei *et al*., 2005; Fei, Ye & Jiang, 2010; Wang, 2007; Wang, *et al*., 2007) (Fig. 1).

F. (*Rana*) *quadranus*, in Chinese named "Longgang", meaning "swollen vent", was firstly described by Liu, Hu & Yang (1960), who found a group of frogs with bubble-like vesicles around the anus, living in the streams of the Wushan Mountains, and named the species as *Rana quadranus*. Later on, it was determined that only adult males have swollen vents. The nomenclature *Feirana* was proposed by Dubois (1992) originally as a subgenus name, and this taxon was upgraded to generic rank later as the number of group members increased (Fei *et al*., 2005).

After Liu's report (Liu, Hu & Yang, 1960), similar frogs were discovered in the Qinling (Fang, 1983; Li, 1992) and Taihang (Wu & Qu, 1984) Mountains, and they were considered "swollen vent" frogs despite having small morphological differences. Fang (1983) and Li (1992) reported that 5–10% of the "swollen vent" frogs in the Tsingling Mountains had cream mid-dorsal lines. Li (1992) pointed out that individuals in the Tsingling Mountains were apparently different from those reported by Liu, Hu & Yang (1960) in the Wushan Mountains. For example, they were speckled in black and brown-yellow, forming a water-wave-like coloration pattern, instead of consistent brown, and adult male vents were not swollen. The diversity of "swollen vent" frogs was also revealed by chromosomal studies among populations in the Minshan (Yang, Zhao & Gao, 1986), Wushan (Li, Fei & Ye, 1994), Taihang (Li & Hu, 1996; Chen *et al*., 2006) and Funiu (Chen *et al*., 2006) Mountains.

Chen & Jiang (2002; 2004) compared the morphometric parameters of specimens from the Taihang Mountains with those from the Wushan Mountains and were convinced that the differences between the two groups had reached the species level. Accordingly, they established a new species, *F. taihangnica* Chen & Jiang, 2002, representing the group from the Taihang Mountains. Further molecular taxonomy using mitochondrial *12S* and *16S rRNA* genes confirmed the morphological classification (Jiang *et al*, 2005).

Wang *et al*. (2007) gathered the morphometric traits of samples on a large scale, which revealed the complexity of geographic populations of the genus *Feirana*. Frogs from the Zhongtiao and Taihang Mountains were allocated to *F. taihangnica*. To determine the evolutionary relationship between *F. quadranus* and *F. taihangnica*, Wang *et al*. (2009; 2012) studied mitochondrial *12S*, *16S* and *ND2* genes. He believed these were sister species that diverged 4.6 million years ago and that the Qinling Mountains was a large contact zone for the two species.

Yang *et al*. (2011) focused on a group of frogs in Kang County, Gansu Province, which had originally been identified as *F. taihangnica* but Wang *et al*. (2009) found that they significantly diverged from other populations of *F. taihangnica*. An analysis of morphometric traits and the mitochondrial *ND2* gene from more specimens confirmed that this group should be assigned to a single species, named *Feirana kangxianensis*.

1.2 Living and breeding habits

According to our field observations and to relevant references (Liu, Hu & Yang, 1960; Fei, 1999; Huang, Gong & Zhang, 2011; Yang, 2011; Zhang *et al*., 2012), the living habits of

99   frogs among the genus *Feirana* are indistinguishable. They inhibat, and are basically limited
100   to, waterbodies, such as creeks, brooks, streams, and rivers in mountainous areas at altitudes
101   of 500 m to 2,500 m. They prey, hibernate, and breed mostly in water, and are hardly seen on
102   the land unless there is enough rain or moisture.

103   Underwater hibernation varies with the local climate, but takes place in October and
104   November, and resumes in March and April, with breeding occuring from April to early June.
105   Spawns are often found under large stones in sun-exposed, slow-flowing and shallow stream
106   sections (Zhang *et al.*, 2012). Consistent with ecological observations, physiological studies
107   on the ovaries (Lei, 2003) and testes (Li, 2003) of specimens (*F. quadranus* or *F.*
108   *taihangnica*)[1], collected monthly from Zhouzhi County in the Qinling Mountains, indicated
109   that ovulation and ejaculation must occur between April and June.

110   Their reproductive activities were very secretive, progressing under large stones in the
111   water, without conspicuous courtship calls. Even local villagers had never observed their
112   breeding. After several years of seeking and following oviposition sites, Chen *et al*. (2011)
113   reported on the breeding biology of *F. taihangnica*, including the time of the breeding season,
114   spawning site preperences, the size of egg clutches and other data on reproductive ecology.
115   Zhang *et al*. (2012) observed unique breeding behaviors in this species. Without amplexus, a
116   female frog deposits sticky eggs beneath a rock under water, and multiple males release
117   semen on to the spawn. Additionally, Wang *et al*. (2014) identified three spawns of *F.*
118   *kangxianensis* using microsatellite markers, one of which was oviposited by two females and
119   fertilized by three males.

120   Owing to the lack of courtship calls and amplexus, the unique reproductive behaviors
121   avoid sexual selection. The asynchrony between oviposition and fertilization makes eggs
122   available for any possible sperms. These factors could facilitate the potential hybridization
123   between two cohabiting species.

1.3 Cohabitation in overlapping ranges and morphological variation between *F. quadranus* and *F. taihangnica*

126   *F. quadranus* ranges from the southern Taihang Mountains, throughout the Zhongtiao
127   Mountains and Funiu Mountains, and into the Qinling Mountains; *F. taihangnica* ranges from
128   the northern Wuling and Wushan Mountains, throughout the Daba Mountains and Micang
129   Mountains, into the Qinling Mountains, with the western range reaching the eastern Minshan
130   and Longmen Mountains (Fei *et al*., 2005; Fei, Ye & Jiang, 2010; Wang, 2007; Wang, *et al*.,
131   2007) (Fig.1).

132   According to Yang (2011), their ranges overlapped in three areas of the Tsingling
133   Mountains, one area is in Zhouzhi County and another is in Ningshan County.

134   We noticed the cohabitation of the two species in Hua'erping, Zhouzhi County, and in
135   Xunyangba, Huoditang, and Huodigou, Ningshan County. They could be found underwater in
136   the daytime in the same brooks or pools and could be observed sitting about stones and
137   waiting for their prey at night. Morphological variations were observed among the cohabitants
138   (Song, 2010), with some resembling *F. quadranus* or *F. taihangnica*, and some having traits
139   of both species (Fig. 2).

140   The purpose of our study was to find evidence through a morphological analysis to
141   verify the suspected hybridization between *F. quadranus* and *F. taihangnica* in their shared
142   habitat.

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[1] It should be noted that the two articles of Lei (2003) and Li (2003) used the dated nomen, *Rana*, instead of *Feirana*, which was confusing. It is possible that they did not know the new taxonomy when sampling took place, which was between April and November, 2002, the same year that Chen & Jiang (2002) published *F. taihangnica* as a new species. Both species (*F. quadranus* and *F. taihangnica*) exist in Zhouzhi County, so their samples may contain *F. quadranus*, *F. taihangnica*, or both.

2 Materials & Methods

2.1 Sampling

The *Feirana* specimens used in this study were collected during fieldwork in the early summer (May to July) between 2009 and 2011. They were mostly captured by electrofishing in the daytime, and by bare hands at night. Artificial hybridization in the lab failed because the frogs failed to survive long enough. Hence, we had 117 specimens.

All of the eight sampling sites were located in the contact zone between *F. quadranus* and *F. taihangnica* (Fig. 1, Table1). Five sampling sites (XYB, PHL, HDT, HDG, and LJZ) in Ningshan County were chosen along the National Highway 210, as well as along the Xunhe River flowing throughout the Qinling Mountains; two sites (HRP and LXC) in Zhouzhi County, with secluded environments, were at the south foot of Mount Taibai, the highest peak of the Qinling Mountains; and the site FP in Foping County was a convenient site.

After death, specimens were given voucher numbers, then dehydrated through an ethanol series (50%, 70%, 90%, and 95%), and finally, preserved in 95% ethanol. A piece of muscle was torn from the thigh and preserved separately. Preserved specimens were photographed dorsally (Photographs of the 117 specimens are available at URL: https://figshare.com/s/a76953fe8b682d7d1220). Only a small number of frogs with distinct morphological traits can be traced back to their live photos. Morphometric characteristics and indices were measured or evaluated (see 2.2, and the sexes were identified by anatomy.

Samples were accidently mingled with two corpses of *Rana rugosa* (LN1, 2) which were a peer's study subjects, being raised in the same room with the *Feirana*. Corpses and thigh muscles of these two subjects were preserved through the same procedure for genetic analyses (see section 2.3).

Ethics Statement

All the species included in our study (*F. quadranus*, *F. taihangnica* and *R. rugosa*) are not endangered or protected species according to the "Law of the People's Republic of China on the Protection of Wildlife" and "Regulations for the Implementation of the People's Republic of China on the Protection of terrestrial Wildlife" (State Council Decree [1992] No. 13); and our eight simpling sites were not in core conservation areas. With the permission for sampling frog specimens issued by the College of Life Science, Shenyang Normal University (Approval No. SNY-LS-2009001), the Forestry Department of Shaanxi Province, China, approved of the field work orally.

2.2 Morphotypical classifications

Specimens were assigned to morphotypes (VV, VV+, vw, wv and ww) (Fig. 2) based on the criteria below, which were compiled from references (Fei *et al*., 2009; Wang, 2007) and our observations.

Typical traits of *F. quadranus*:

The trunk appears as narrow as the head; the back is olive brown in colored; there are wart-like granular bumps above the anal region, which are relatively large and sparse; there is a group of wart-like granular bumps between the shoulder blades that forms an inverted "V" shape; and the vents of the male adults are swollen (Fig.2A).

Typical traits of *F. taihangnica*:

The trunk appears wider than the head; the back has brown, yellow and black spotted, like a mosaic of light and shadow created by waves and ripples (Fig. 2E); above anal region, there are inconspicuous wart-like granular bumps, which are small and thick (dense); there are no inverted V-shaped granules between the shoulder blades; and the vents of the male adults are not swollen.

Specimens with typical *F. quadranus* traits were labeled "VV"; the variation of *F.*

192 *quadranus* with a cream-colored mid-dorsal line was labeled "VV+" (Fig. 2B); frogs with
193 typical traits of *F. taihangnica* were labeled as "ww"; and intermediates with mixed traits
194 were labeled as "vw" or "wv", depending on their similarities to typical *F. quadranus* or *F.*
195 *taihangnica*. For example, some intermediates have half the extent of the dorsal spot pattern;
196 some intermediates have no inverted V-shaped granules between the shoulder blades but do
197 have an inverted V-shaped black spot at that position; some frogs labeled as "vw" look like
198 "VV", only without granular bumps above the anus (Fig. 2C); and some labeled as "wv" (Fig.
199 2D) look like "ww", only with too many granular bumps on the back. For the convenience of
200 analysis, three-morphotypes classifications (VV, vw&wv and ww) were employed, where
201 "VV" and "VV+" were both noted as "VV", and "vw" and "wv" as "vw&wv", representing
202 putative parents and suspected hybrids, respectively.

203   Out of 117 specimens, 110 produced morphological results. XYB117–123, which were
204 newly metamorphosed frogs, were too young to be morphotypically identified (Table 1).

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206 **2.3 16S classification**

207   **2.3.1 Laboratory work**

208   The mitochondrial *16S rRNA* gene was used to genetically classify 117 frog samples by
209 maternal bloodline. Two specimens of *R. rugosa* went through the same procedures as an
210 outgroup of *Feirana*.

211   Genomic DNA was isolated from ethanol-preserved muscle tissues using the genomic
212 DNA purification kit (Axygen, Hangzhou). A region of the *16S rRNA* gene (~547bp) was
213 amplified by the primer pair P7 (forward, 5′-CGC CTG TTT ACC AAA AAC AT-3′) and P8
214 (reverse, 5′-CCG GTC TGA ACT CAG ATC ACG T-3′) (Simon *et al.*, 1994), which were
215 also used in Jiang *et al.* (2005) and Wang *et al.* (2009). Amplifications were performed under
216 the following conditions: 94°C for 4 min, 35 cycles of 94°C for 40 s, 53°C for 40 s, 72°C for
217 70 s, and 72°C for 8 min. Purified PCR products were sent to biotechnology companies
218 (Sangon, Shanghai; Majorbio, Shanghai) to be sequenced in one direction, 113 *Feirana* and
219 two *R rugosa* samples by P7, and four *Feirana* samples by P8.

220   **2.3.2 Data analysis**

221   The trimmed sequences of 113 *Feirana*, 2 *R. rugosa*, and 4 *Feirana* were submitted to
222 GenBank's NCBI database, under the following accession numbers: KU865180–KU865181
223 (*R. rugosa*), KU865182–KU865185 (*F. taihangnica*, sequenced by the reverse primer), and
224 KU865186–KU865298 (*F. quadranus* and *F. taihangnica*, sequenced by the forward primer),
225 respectively.

226   The sequences of the 117 specimens were compared with sequences of 2 *R. rugosa*, and
227 the reference sequences of 32 *F. quadranus*, 15 *F. taihangnica*, and 2 *F. kangxianensis*
228 downloaded from GenBank (Table 2), which were also amplified by the primers P7 and P8
229 (Che *et al.*, 2009; Wang *et al.*, 2009).

230   In Unipro UGENE 1.21.0 (Okonechnikov *et al.*, 2012), 168 sequences were aligned with
231 MUSCLE mode (Edgar, 2004), leaving the other parameters as default, and then trimmed to
232 the same length, 495 bp.

233   A phylogenetic analysis was conducted in MEGA 6.06 (Tamura *et al.*, 2013). Several
234 statistical methods, including maximum likelihood (ML), neighbor-joining,
235 minimum-evolution, (unweighted pair group with arithmetic mean and maximum-parsimony,
236 were tested.

237   The tree shown (Fig. 3) was inferred by ML using the best model (K2+G) estimated to
238 have the lowest Bayesian information criterion value (Schwarz, 1978). It was a combination
239 of the Kimura 2-parameter nucleotide substitution model (Kimura, 1980) and a discrete
240 gamma distribution of five categories to model evolutionary rate differences among sites. The

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241   initial tree for the heuristic search was obtained by applying the neighbor-joining method to a
242   matrix of pairwise distances estimated using the Maximum Composite Likelihood approach.
243   A bootstrap test was performed with 500 replications. All of the gaps and missing data were
244   included.

245   **2.3.3 Divergence (p-distance)**
246   Evolutionary divergence over sequence pairs (means ± standard errors of p-distances)
247   between and within groups were estimated. The number of base differences per site from the
248   averaging over all of the sequence pairs between and within groups are shown (Table S1).
249   Standard error estimates (s.e.) were obtained by a bootstrap procedure with 500 replicates. All
250   of the positions with less than 95% site coverage were eliminated. That is, fewer than 5%
251   alignment gaps, missing data, and ambiguous bases were allowed at any position. There were
252   a total of 491 positions in the final dataset.

253   **2.3.4 Phylogenetic classification**
254   For the 117 specimens, those in the same branch as the reference *F. quadranus* or *F.*
255   *taihangnica* were classified as "Q" or "T", respectively. All of the morphotype "VV" are
256   genetically "Q", and all of the morphotypes "ww" and "vw&wv" are genetically "T", except
257   four specimens (see pink and blue rectangles in Fig.3). One specimen (HDT102) was labelled
258   "Q-vw", and three specimens (HDT101, HDT113, and HRP125) were labelled with "T-VV"
259   (Fig. 6F).

260   **2.4 Morphometric indices and statistical analyses**
261   **2.4.1 Chosing and designing morphometric indices**
262   To evaluate the morphological variation and differentiation among the putative parents,
263   "VV" and "ww", and the suspected hybrids, "vw&wv" (see 4 for explaination), 34 indices
264   were employed. Originally, 32 morphometric characteristics based on Wang (2009) and Fei *et*
265   *al*. (2009) were measured on preserved specimens using Vernier calipers. 13 with significant
266   measurement errors (i.e. nostril-snout distance, width of outer web of first toe) or that were
267   disproportional to body size (i.e. tympanum horizontal diameter, distance between internal
268   nares, size of vomerine teeth, length and width of inner metacarpal tubercle, length and width
269   of inner metatarsal tubercle) were removed because Hayek, Heyer & Gascon (2001) warned
270   against measurement errors and data transformation. The remaining measured characteristics
271   were divided by snout-vent length (SVL) to eliminate body size effects.
272   Nine ratios were derived from certain measured characteristics, which together with 18
273   ratios of measured characters to SVL were called ratio indices. The name of a ratio index is
274   composed as the pattern "dividend_divisor", e.g. HL_SVL represents HL/SVL. Seven extent
275   indices, based on extent of bumps and coloration patterns on the skin were given values
276   between 0 and 1, or 0 and 2. In the end, 34 indices, including 27 ratio indices and 7 extent
277   indices, remained for analysis (Table S3-Table S5).
278   **Abbriviations of characters or indices with descriptions**
279   **Measured characters**
280   SVL: snout-vent length, used to eliminate body size effects;
281   HL: head length, from posterior end of mandible to tip of snout;
282   HW: head width, measured at corners of the mouth;
283   SL: snout length, distance between anterior edge of orbit and tip of snout;
284   NED: nostril-to-eye distance, distance between centre of nostril and anterior edge of orbit;
285   IND: internarial distance, distance between inner ends of nostrils;
286   IOD: interorbital distance, shortest distance between inner edges of upper eyelids;
287   IAE: distance between anterior corners of eyes;
288   IPE: distance between posterior corners of eyes;
289   LHL: length of lower arm and hand, from elbow to tip of third finger;

290 HAL: hand length, from base of outer palmar tubercle to tip of third finger;
291 TEL: femur length, from vent to knee;
292 TL: tibia length;
293 TFL: tibiofibula length (length of tarsus and foot), from base of tarsus to tip of fourth toe;
294 FL: foot length, from proximal end of inner metatarsal tubercle to tip of fourth toe;
295 T5FFL: length of free flap of the fifth toe, length of cutaneous fringe along the outer margin
296 of the fifth toe;
297 F1L: first finger length, from proximal end of thenar tubercle to tip of first finger;
298 F3L: partial 3rd finger length, distance between basal border of third finger to tip of third
299 finger;
300 F4L: fourth finger length, from proximal end of thenar tubercle to tip of fourth finger.
301   **Extent indices** (scored between 0, indicating the trait was not seen, and 1, indicating the
302 maximum extent):
303 BBE: extent of big bumps above the anal region;
304 SBE: extent of small bumps above the anal region;
305 VBE: extent of bumps in the shape of inverted "V" between shoulder blades;
306 VSE: extent of patch in the shape of inverted "V" between shoulder blades;
307 LBE: extent of line-shaped bumps on the back; BSE: the extent of spot pattern on the back;
308 LSE: extent of strip or or spot pattern on legs (scored between 0, indicating no obvious
309 pattern, 1, indicating pure strip pattern, and 2, indicating pure spot pattern).
310

311   **2.4.2 Description of morphometric data**
312   Morphometric raw data was in Table S2. Means and standard deviations (mean ± s.d.) of
313 34 indices were calculated in five groupings, with each set containing two or three groups:
314 16S (Q and T), morp3 (VV, vw&wv, and ww), sex (female and male), F_morp3 (females' VV,
315 vw&wv, and ww) and M_morp3 (males' VV, vw&wv, and ww) (Table S3). To reveal the
316 differences in means between or among groups in each set, statistical tests were performed on
317 the R 3.2 platform (R Core Team, 2015). The function "t.test" was employed to execute t-tests
318 of sets containing two groups (16S and sex sets). For the sets containing three groups, firstly,
319 "bartlett.test" (Bartlett, 1937) was used to check the homogeneity of the variances; and if the
320 p-value generated by Bartlett's test was above 0.05, meaning variances were homogeneous,
321 then an ANOVA was applicable, and the function "avo" (Chambers, Freeny & Heiberger,
322 1992) was then used to compare the means of the groups, otherwise "kruskal.test" for the
323 Kruskal-Wallis test (Myles & Douglas, 1973), which applies to extreme non-normal
324 distributions of sample values, was used instead.
325   In Excel 2011 for mac, profiles of the p-values for the five sets in Table S4 were plotted.
326 P-values were ordered from highest at the bottom, to lowest at the top), and a logarithmic
327 scale with base 10 was applied to the *y* axis to magnify the high-degree differences
328 represented by p-values near 0, and minimize the low-degree differences represented by
329 p-values near 1 (Fig. 4).
330   **2.4.3 Multivariate analyses**
331   Two types of multivariate analyses were performed on the R 3.2 platform (R Core Team,
332 2015) to estimate the morphometric variation among morp5 set (VV, VV+, vw, wv, and ww),
333 morp3 set (VV, vw&wv, and ww), 16S set (Q and T), or 16S_versus_morp set (Q, Q-vw,
334 T-VV, and ww) (Fig. 5F). The function "prcomp" with "scale = TRUE" was used for the
335 principal component analyses (PCA; see 4 for interpretation of PCA), clustering individuals
336 in the multivariate space of the first two principal components (PC1 and PC2); and the
337 function "lda" in package "MASS" (Venables & Ripley, 2002) was used for the linear
338 discriminant analysis (LDA; see 4 for interpretation of LDA).
339   Considering the possible sexual dimorphism, the females (Fig. 5A; Fig. 6A) and the

340   males (Fig. 5E; Fig. 6E) were analyzed separately, as well as together (Fig. 5B, F; Fig. 6B, F).
341   The independent impacts of ratio indices (Fig. 5D; Fig. 6D) or extent indices (Fig. 5H; Fig.
342   6H) on the PCA and LDA were explored.
343   To test the reliability of the morphotype-based classifications, morphotypical information
344   was simulated in two ways using falsified data sets from two populations (see Table S2).
345   Based on the real data, for the first simulated population, we changed a small proportion of
346   VV into vw&wv and a small proportion of ww into vw&wv or VV, and remixed the original
347   vw&wv with all three types (Fig. 5C; Fig. 6C); for the second simulated population, the three
348   morphotypes (VV, vw&wv, and ww) were randomly assigned to specimens (Fig. 5G; Fig.
349   6G).

350   **2.4.4 Analyses of indices**
351   To explore which indices are important in each PC or for each LD function, heat-maps
352   implemented by the function "aheatmap" of the package "NMF" (Gaujoux *et al*. 2010) were
353   employed to visualize weighted or not-weighted rotation matrices in the PCA and coefficients
354   matrices in the LDA. The function "aheatmap" defaults to a complete linkage clustering
355   method, using a Euclidean distance measure to hierarchically cluster rows and columns. To
356   emphasize the highly contributing PCs or LD functions, the absolute values of the respective
357   rotations were multiplied by the corresponding proportion of explained variance for each PC
358   and the absolute values of the respective coefficients were multiplied by the corresponding
359   proportion of explained discriminability for each LD. For matrices with small proportions of
360   explained discriminability for LD2, which would weaken its coefficients too much to be
361   measurable, the absolute values of the coefficients were not multiplied by the corresponding
362   proportion of the explained discriminability.

363   **3 Results**

364   **3.1 16S**

365   The applications of several statistical methods produced similar phylogenetic trees. The
366   ML tree with the highest log likelihood (-1398.4472) is shown (Fig.3). The outgroup, *R.*
367   *rugosa*, and isolates from *Feirana* (*F. kangxianensis*, *F. quadranus*, and *F. taihangnica*) had a
368   high bootstrap support value of 100%. *Feiranus* divides into two major clades, one containing
369   all of the reference *F. quadranus* (bootstrap value of 94%), and the other (bootstrap value of
370   69%) containing all the reference *F. taihangnica* (bootstrap value 83%), and reference *F.*
371   *kangxianensis* (bootstrap value 97%) branched off shallowly. Most of the VV are in the same
372   clade as the reference *F. quadranus*, while most of the ww and vw&wv are in the same clade
373   as reference *F. taihangnica*. The only four exceptions are HDT101, HDT113 and HRP125,
374   which are morphotypically "VV", and HDT102, which is morphotypically "vw".

375   **3.2 Morphometric analysis**

376   **3.2.1 PCA**

377   According to the PCA, in both females (Fig. 5A) and males (Fig. 5E), there is a clear
378   differentiation between VV and ww. The distribution of wv&vw is closer to ww. In males
379   (Fig. 5E) and total specimens (Fig. 5B), most of the wv&vw are mixed with ww, or in the
380   area between ww and VV, and a small proportion are mixed with VV. In females (Fig. 5A),
381   limited samples of vw&wv are near the borderline of the ww zone.
382   The incomplete differentiation between Q and T is shown by the genetic classification
383   (Fig. 5F). The three border-crossers, HRP108, HDT106, and HDT110, being genetically T,
384   appear in VV's territory. The four specimens with controversial classifications (see 2.3.4),
385   T-VV (HDT111, HDT113, and HRP125), which are genetically T but morphotypically VV,
386   and Q-vw (HDT102), which is genetically Q but morphotypically vw, appear in the
387   ambiguous zone between Q and T (Fig. 5F). The positions of the four specimens in the

388 multivariate spaces of females (Fig. 5A) and males (Fig. 5E) are also close to the borderline
389 between VV and ww.
390   Ratio indices failed to differentiate between VV and ww (Fig. 5E); however, extent
391 indices differentiate solely using PC1, and vw&wv are perfectly scattered along the boundary
392 zone between VV and ww (Fig. 5H).

393   **3.2.2 LDA**
394   Based on the LDA, VV, vw&wv, and ww are clustered into three separate groups in both
395 females (Fig. 6A) and males (Fig. 6E). The differentiation between vw&wv and VV or ww is
396 less complete in total specimens (Fig. 6B).
397   Similar to the PCA results, extent indices differentiate the three morphotypes more
398 completely than the ratio indices (Fig. 6D, H).

399   **3.2.3 Simulated data**
400   For the first simulated population, as the number of falsified morphotypes increased, the
401 differentiation between VV and ww became indistinct in the PCA (compare Fig. 5C with Fig.
402 5B) and spaces among the three morphotypes narrowed in the LDA (compare Fig. 6C with
403 Fig. 6B). The PCA (Fig. 5G) and LDA (Fig. 6G) of the second simulated population, with
404 random morphotypical data, exhibited an increased degree of disorder.

405   **3.2.4 Analyses for importance of indices**
406   A full version of heat-maps are shown in Fig. S2.
407   In the LDA, it seems that, to discriminate three morphotypes or five morphotypes from
408 the total specimens, finger lengths and other length indices were the most contributive
409 characters (Fig. S2E-H). Finger lengths, however, are not crucial for discriminating between
410 morphotypes. When F1L_SVL, F3L_SVL and F4L_SVL were eliminated from the
411 morphometric data, plots of the LDA for each set stayed the same, only the indices originally
412 ranked after F1L_SVL, F3L_SVL, and F4L_SVL upgraded their contributions to each LD
413 (data not shown). Contrarily, the seven extent indices seemed to be minimally involved in
414 discriminating between morphotypes. However, when these seven extent indices were
415 excluded, leaving only 27 ratio indices, the three morphotypes could not be easily
416 distinguished (Fig. 6D); or when the 27 ratio indices were excluded, leaving only the seven
417 extent indices, the distribution pattern of three morphotypes stay the same (Fig. 6H).
418 Therefore, we decided not to use coefficients matrix of LDA to analyze importance of indices.
419   In the PCA, generally speaking, indices on limb and finger lengths contribute most to
420 PC1, while indices involving bumps and coloration patterns contribute most to PC2. The
421 weighted rotation matrix of 34 indices for the first 10 PCs (eigenvalues > 1), accounting for
422 78.11% (total specimens) of the variation is shown here (Fig. 7A). The most important indices
423 in PC1 were TL_SVL, (tibia length)/SVL; LHL_SVL, (length of lower arm and hand)/SVL;
424 and TFL_SVL, (tibiofibula length)/SVL. The most important indices in PC2 were BSE, the
425 extent of spots on the back and BBE, the extent of big bumps above the anal region.
426   The weighted rotation matrix of seven extent indices is shown for the first two PCs
427 (eigenvalues > 1), accounting for 63.80% of the variation (Fig. 7B). VBE, the extent of
428 bumps in the shape of an inverted "V" between the shoulder blades; BSE, the extent of spots
429 on the back; and BBE, the extent of big bumps above the anal region, account for most of the
430 PC1 variance, which clearly differentiated the three morphotypes (Fig. 5H).

431 **4 Discussion**

432   Introgressive hybridization is often identified by the presence of morphological
433 intermediates in the contact zone between two parental species (Anderson, 1949; Hubbs, 1955;
434 Arnold, 1992). We devised the three morphotypical classification to represent two "parents"
435 and their suspected "hybrid", a simplified hybridization pattern, which, however, did not

436 mean that each vw&wv was a hybrid, or that each VV or ww was a pure parent, especially for
437 samples at sites inhabited by two or three morphotypes (e.g. HDT, HRP, and XYB). Based on
438 the theory of introgressive hybridization (Anderson, 1949), frogs at these sites may have been
439 intercrossed and backcrossed for many generations, leading to limited pure "parents", and
440 these morphotypical "parents", VV and ww, may only have been more back-crossed than the
441 morphotypical "hybrids" (Lehtinen *et al*., 2016). Another possible hypothesis is that
442 hybridization does not necessarily equally (50%) affect the hybrids' appearances because
443 there are genetic and developmental buffers between a frog's genotype and its phenotype,
444 such as hybridogenesis (Holsbeek & Jooris, 2010; Mikulíček *et al*., 2014), genomic
445 imprinting (Tunner, 2000), pleiotropy, dominance, epistasis (Gallez & Gottleib, 1982), and
446 epigenetic phenomenon. Therefore, this simplified hybridization pattern was only adopted for
447 the convenience of verifying possible introgression.
448   PCA and LDA are often used to detect or estimate hybridization, especially in
449 morphology (e.g. Albert, D'Antonio & Schierenbeck, 1997; Wu *et al*., 2011). In PCA theory,
450 PCs are uncorrelated linear combinations of rotated indices, and analyzing the entire data is
451 reduced to only considering the first several PCs that explain the majority of the variation
452 (Crawley, 2009). The most common visual way is to place individuals on a scatterplot of the
453 first two PC axes, and use group-based symbols to represent individuals. The closer two
454 points are, the more similar their corresponding indices. This allows one to see whether
455 individuals of one group are clustered in the space and whether they are isolated from
456 individuals of the other group.
457   Similar to the PCA, the LDA seeks the best linear functions to discriminate between
458 predefined groups instead of between individuals (Selvin, 1994). The grouping information
459 for each individual is preset, and coefficients of indices are estimated for LD functions which
460 is one less than the number of groups. Consequently, between-group differences are
461 maximized in a scatter plot of the first two LD functions, exhibiting how well pre-defined
462 groups of individuals can be separated by multivariate measurements (Lihová *et al*., 2007).
463   The expected pattern is presented in the multivariate space of the first two PCs of the
464 PCA (Fig. 5A, B, E, and H) and of the first two LD functions of the LDA (Fig. 6A, B, E, and
465 H). A further analysis on the simulated two populations, which were created based on the
466 actual population by falsifying morphotypical data (see 2.4.3) confirmed the reliability of the
467 hybridization pattern established by the three morphotypes.
468   In the mtDNA's phylogenetic tree (Fig. 3), wv and most vw's are intermixed with ww in
469 the clade *F. taihangnica*. Could this suggest that *F. taihangnica* might be the maternal species
470 of suspected "hybrids", vw and wv, with VV being the paternal species? Considering the
471 unique breeding behavior of *F. taihangnica*, which is simultaneous polyandry with multiple
472 males not engaged in amplexus (Zhang *et al*., 2012) (see 1.2), it seems more likely that
473 female *F. taihangnica* ley eggs on rocks and then male *F. quadranus* and/or *F. taihangnica*
474 fertilize them.
475   In the morphometric analysis, vw&wv are often intermixed with ww (Fig. 5A, B, E). In
476 particular, when excluding the seven extent indices, leaving only 27 ratio indices, which were
477 derived from measured characteristics (see 2.4.1), vw&wv were completely intermixed with
478 ww (Fig. 6D). However, when leaving only the seven extent indices, which describe typical
479 traits of *F. quadranus* and *F. taihangnica* (see 2.2), the vw&wv's proneness to ww
480 disappeared (Fig. 5H). Could this suggest that mtDNA have an influence on the measurable
481 characteristics instead of extent characteristics? Since backcrossing causes the offsprings to
482 resemble the recurrent parental species (Anderson, 1949), could this imply the hybrids' have a
483 preference for backcrossing with ww? Or could it be the genome-dosage effects that were
484 often seen between diploid and triploid frogs (e.g. Borkin *et al*., 2004; Plötner, 1994)?
485   Although morphological evidence has historically been used in studies of hybridization

and introgression (Rieseberg & Wendel, 1993), as Hubbs (1955) stated it is "an almost universally valid rule that natural interspecific hybrids are intermediate between their parental species in all characters in which those species differ", there are still alternative explanations for morphological intermediacy. For instance, shared ancestral traits (Muir & Schlötterer, 2005), phenotypic plasticity in the parental species (Gibbs, 1968; Birch & Vogt, 1970), relictual genes in the gene pool before the divergence, or less likely, parallel mutations, could result in the common characteristics of the two species (Albert *et al*., 1997). Findings that morphometric analyses of genetically identified hybrids can misclassify groups of hybrids as pure parents emphasizes the limitations inherent in describing hybrid classes solely by morphological criteria (Lamb & Avis, 1987; Pagano & Joly, 1999).

Since there have not been any other reports supporting hybridization between *F. quadranus* and *F. taihangnica*, we did not have enough confidence in our conclusion until evidence from nuclear gene markers (Song *et al*., unpublished results) was found.

5 Conclusion

The mtDNA, *16S rRNA* gene, identified specimens as genetically *F. quadranus* (labeled as Q) or genetically *F. taihangnica* (labeled as T), while a morphological classification grouped specimens into three morphotypes (VV, vw&wv, and ww), representing putative parents and suspected hybrids. Four exceptional specimens with conflicting classifications on the mtDNA tree and three genetically Q having T phenotypes by morphometric analysis, blurred the morphological boundary between Q and T.

The multivariate analyses of measured characteristics, and characteristics related to the extent of bumps and coloration patterns, demonstrated a hybridization pattern where the suspected hybrids, vw&wv, were intermediate between putative parents, VV and ww, with a proneness to ww. vw&wv were also intermixed with ww in the mtDNA tree. vw&wv's proneness to ww in the morphometric analysis disappeared when measured indices were excluded.

Indices on the extents of bumps and coloration patterns, such as BSE, BBE, and LBE, were better at discriminating among suspected hybrids and putative parental types than the measured indices. Because this is the first report on hybridization between *F. quadranus* and *F. taihangnica*, cautions regarding the use of solely morphological evidence in identifying hybridization require evidence from nuclear gene markers.

Acknowledgement

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701   **Tables and Figures**

702

703   **Table 1** The information for eight sampling sites in this study

Sampling sites	Abbr.	Morphotypes		16S types		Location Coordinates	
		N[a]	VV/vw&wv/ww	n	Q/T	Longitude (E, °)	Latitude (N, °)
Laoxiancheng, Zhouzhi County, Shaanxi Prov.	LXC	1	0/1/0	1	0/1	107.7568	33.8030
Hua'erping, Zhouzhi County, Shaanxi Prov.	HRP	24	4/11/9	24	3/21	107.8290	33.8349
Pengjiagou, Foping County, Shaanxi Prov.	FP	20	20/0/0	20	20/0	107.9557	33.4461
Liangjiazhuang, Ningshan County, Shaanxi Prov.	LJZ	14	14/0/0	14	14/0	108.3743	33.3976
Huoditang, Ningshan County, Shaanxi Prov.	HDT	26	11/6/9	26	10/16	108.4534	33.4322
Huodigou, Ningshan County, Shaanxi Prov.	HDG	3	1/1/1	3	1/2	108.4845	33.4567
Pingheliang, Ningshan County, Shaanxi Prov.	PHL	3	0/0/3	3	0/3	108.5045	33.4733
Xunyangba, Ningshan County, Shaanxi Prov.	XYB	19	10/2/7	26	11/15	108.5459	33.5522
Total	8	110	60/21/29	117	59/58	/	/

704   [a] n represents the sample size from each site.

705   **Table 2** Information on *16S rRNA* reference sequences

No.	GenBank No.	Voucher No.	Species name in reference article[a]	Species name in new nomenclature	Locality (village/county/city, province)	Latitude (N, °)	Longitude (E, °)	Reference article
1	GQ225974	CIBKangxian01	*"Feirana". taihangnica*	*F. kangxianensis*	Kangxian, Gansu	105.4367	33.2804	Wang *et al*., 2009
2	GQ225975	CIBKangxian02	*"Feirana". taihangnica*	*F. kangxianensis*	Kangxian, Gansu	105.4367	33.2804	Wang *et al*., 2009
3	GQ225907	CIB20060644	*Feirana quadranus*	*F. quadranus*	Anxian, Sichuan	104.1856	31.6316	Wang *et al*., 2009
4	GQ225908	CIB20070336	*Feirana quadranus*	*F. quadranus*	Beichuan, Sichuan	104.1262	31.795	Wang *et al*., 2009
5	GQ225909	CIB20060509	*Feirana quadranus*	*F. quadranus*	Qingchuan, Sichuan	104.7541	32.5778	Wang *et al*., 2009
6	GQ225910	CIB20060533	*Feirana quadranus*	*F. quadranus*	Wenxian, Gansu	105.1842	32.7354	Wang *et al*., 2009
7	GQ225911	CIBHuixian01	*Feirana quadranus*	*F. quadranus*	Huixian, Gansu	105.8702	33.8964	Wang *et al*., 2009
8	GQ225912	CIB20060463	*Feirana quadranus*	*F. quadranus*	Nanjiang, Sichuan	106.6751	32.5883	Wang *et al*., 2009
9	GQ225913	CIBNanzheng02	*Feirana quadranus*	*F. quadranus*	Nanzheng, Shaanxi	106.8261	32.8446	Wang *et al*., 2009
10	GQ225914	CIB20060469	*Feirana quadranus*	*F. quadranus*	Fengxian, Gansu	106.5649	34.0983	Wang *et al*., 2009
11	GQ225915	CIBFengxian03	*Feirana quadranus*	*F. quadranus*	Fengxian, Gansu	106.5649	34.0983	Wang *et al*., 2009
12	GQ225916	CIBLiuba03	*Feirana quadranus*	*F. quadranus*	Liuba, Shaanxi	107.0848	33.7031	Wang *et al*., 2009
13	GQ225917	CIB20060340	*Feirana quadranus*	*F. quadranus*	Changan, Shaanxi	108.7731	33.7628	Wang *et al*., 2009
14	GQ225918	CIB20060353	*Feirana quadranus*	*F. quadranus*	Zhouzhi, Shaanxi	107.9742	33.7747	Wang *et al*., 2009
15	GQ225919	CIB200503551	*Feirana quadranus*	*F. quadranus*	Fuping, Shaanxi	107.9491	33.6986	Wang *et al*., 2009
16	GQ225920	CIBNingshan01	*Feirana quadranus*	*F. quadranus*	Ningshan, Shaanxii	108.4452	33.4344	Wang *et al*., 2009
17	GQ225921	CIBShanyang02	*Feirana quadranus*	*F. quadranus*	Shanyang, Shaanxi	109.9675	33.6501	Wang *et al*., 2009
18	GQ225922	CIBShanyang03	*Feirana quadranus*	*F. quadranus*	Shanyang, Shaanxi	109.9675	33.6501	Wang *et al*., 2009
19	GQ225923	CIBLangao01	*Feirana quadranus*	*F. quadranus*	Langao, Shaanxi	106.3042	34.0021	Wang *et al*., 2009
20	GQ225924	CIBZhengba02	*Feirana quadranus*	*F. quadranus*	Zhengba, Shaanxi	107.9339	32.5774	Wang *et al*., 2009
21	GQ225925	CIB20070187	*Feirana quadranus*	*F. quadranus*	Wanyuan, Sichuan	108.2387	32.0877	Wang *et al*., 2009
22	GQ225926	CIB20060716	*Feirana quadranus*	*F. quadranus*	Shennongjia, Hubei	110.5101	31.8211	Wang *et al*., 2009
23	GQ225927	CIBFangxian0203	*Feirana quadranus*	*F. quadranus*	Fangxian, Hubei	110.3231	31.925	Wang *et al*., 2009
24	GQ225928	CIB20060387	*Feirana quadranus*	*F. quadranus*	Wushan, Chongqing	109.9074	31.3721	Wang *et al*., 2009
25	GQ225929	CIBWanzhou34	*Feirana quadranus*	*F. quadranus*	Wuxi, Chongqing	109.9026	31.4804	Wang *et al*., 2009
26	GQ225930	CIBWanzhou41	*Feirana quadranus*	*F. quadranus*	Fengjie, Chongqing	109.4298	30.6169	Wang *et al*., 2009
27	GQ225931	CIB20060715	*Feirana quadranus*	*F. quadranus*	Lichuan ,Hubei	109.0946	30.5244	Wang *et al*., 2009
28	GQ225932	CIBB20010018	*Feirana quadranus*	*F. quadranus*	Sangzhi, Hunan	109.9232	29.6346	Wang *et al*., 2009
29	GQ225976	CIBLaoxiancheng01	*"Feirana". taihangnica*	*F. taihangnica*	Old city of Zhouzhi, Shaanxi	107.4032	33.4832	Wang *et al*., 2009
30	GQ225977	CIBTaibai03	*"Feirana". taihangnica*	*F. taihangnica*	Taibai, Shaanxi	107.5421	34.0573	Wang *et al*., 2009

31	GQ225978	CIB2871K	"Feirana". taihangnica	F. taihangnica	Changan, Shaanxi	108.8389	33.8846	Wang et al., 2009
32	GQ225979	CIB20060316	"Feirana". taihangnica	F. taihangnica	Ningshan, Shaanxi	108.5425	33.5482	Wang et al., 2009
33	GQ225980	CIB2874K	"Feirana". taihangnica	F. taihangnica	Ningshan, Shaanxi	108.5425	33.5482	Wang et al., 2009
34	GQ225981	CIB2876K	"Feirana". taihangnica	F. taihangnica	Zhashui, Shaanxi	108.8367	33.7837	Wang et al., 2009
35	GQ225982	CIBHuashan03	"Feirana". taihangnica	F. taihangnica	Huashan, Shaanxi	109.8083	34.4672	Wang et al., 2009
36	GQ225983	CIB20060325	"Feirana". taihangnica	F. taihangnica	Qinshui, Shanxi	112.0184	35.4302	Wang et al., 2009
37	GQ225984	CIB20060320	"Feirana". taihangnica	F. taihangnica	Qinshui, Shanxi	112.015	35.4302	Wang et al., 2009
38	GQ225985	CIB20060346	"Feirana". taihangnica	F. taihangnica	Jiyuan, Henan	112.0902	35.2649	Wang et al., 2009
39	GQ225986	CIB20070485	"Feirana". taihangnica	F. taihangnica	Songshan, Henan	112.2176	33.9305	Wang et al., 2009
40	GQ225987	CIB0408II012	"Feirana". taihangnica	F. taihangnica	Neixiang, Henan	111.8575	33.4984	Wang et al., 2009
41	GQ225988	CIB20060349	"Feirana". taihangnica	F. taihangnica	Luanchuan, Henan	112.2963	33.7311	Wang et al., 2009
42	DQ118514	KizYP215	Chaparana quadranus	F. quadranus	Maowen Co., Sichuan	/	/	Che et al., 2009
43	DQ118515	KizYP016	Chaparana quadranus	F. quadranus	Guanyang,Wushan Co., Chongqing	/	/	Che et al., 2009
44	EU979831	SCUM20030031GP	Chaparana quadranus	F. quadranus	An Co., Sichuan	/	/	Che et al., 2009
45	EU979832	YNU-HUJJ7	Chaparana quadranus	F. quadranus	Sangzhi, Hunan	/	/	Che et al., 2009
46	EU979842	KIZ-HN0709001	Paa taihangnica	F. taihangnica	Taihangshan, Jiyuan, Henan	/	/	Che et al., 2009
47	EU979843	KIZ-HN0709002	Paa taihangnica	F. taihangnica	Taihangshan, Jiyuan, Henan	/	/	Che et al., 2009
48	DQ118516	KizYP216	Chaparana quadranus	F. quadranus	/	/	/	Hu et al., not published in articles
49	EU979833	YNU-HU20025113	Chaparana quadranus	F. quadranus	/	/	/	Hu et al., not published in articles

a Due to taxonomic chaos in tribe Paini (Che et al., 2009), *Feirana quadranus* was also named *Chaparana quadranus* (Jiang et al., 2005; Ohler & Dubois, 2006; Che et al., 2009; Wang et al., 2009), and *Feirana taihangnica* was named *Paa taihangnica* (Jiang et al., 2005; Ohler & Dubois, 2006; Ye et al., 2013).
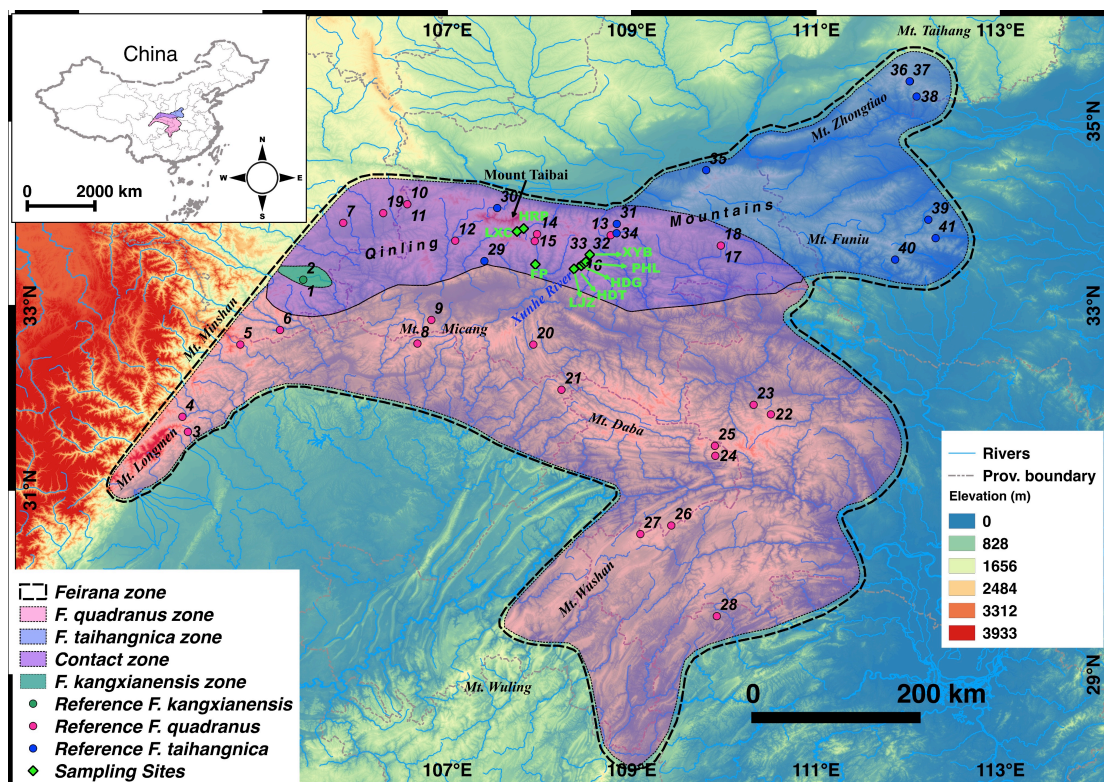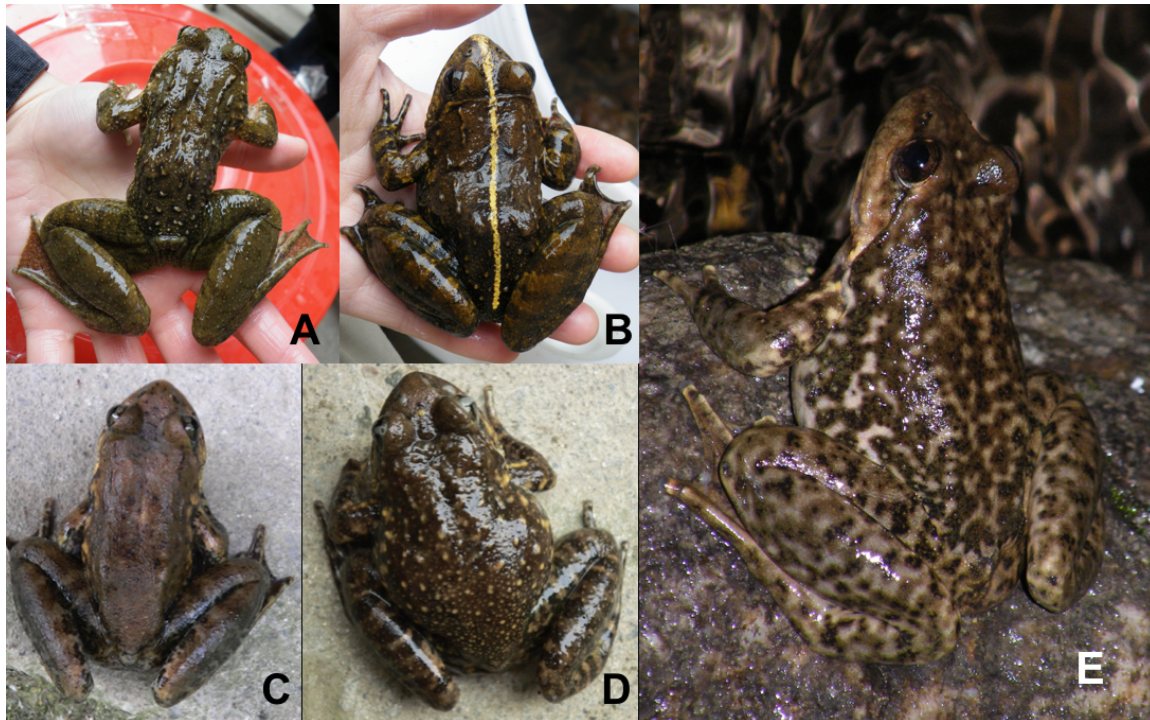
Fig. 1 Distribution of the 8 sampling sites from which 117 specimens were collected. Abbreviations for sampling sites (light green diamonds) correspond to those in Table 1. Reference sampling sites (green, pink, and blue spots), 1–41, correspond to the numbers in Table 2. Distribution zones were drawn according to Fig. 1 in Wang *et al*. 2009.

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720
721 Fig. 2 Examples of the five morphotypes of *F. quadranus* and *F. taihangnica*. (A) VV; (B)
722 VV+; (C) vw, looks like VV, only without granular bumps above the anus; (D) wv, looks like
723 ww, only with too many granular bumps on the back; (E) ww. Photo credit: Yang Song & Xin
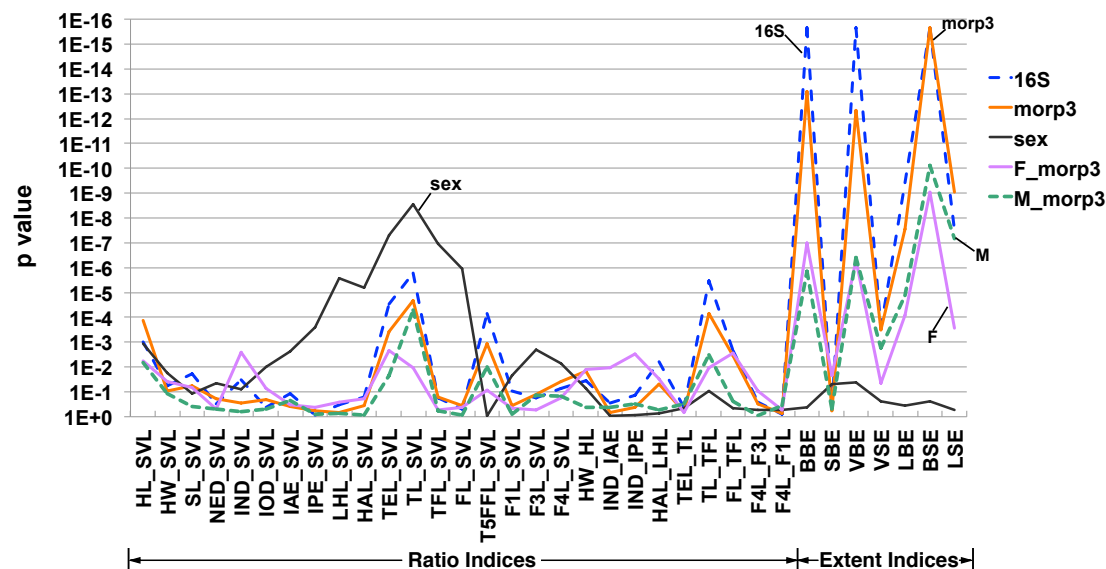724 Sui.

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Fig. 3 Compressed maximum likelihood (ML) phylogenetic tree based on *16S rRNA* gene
partial sequences. The bootstrap support values are shown below branches. Scale bar indicates
an evolutionary distance of 0.05 nucleotides per position in the sequence. The four grey
rectangles on the compressed tree correspond to four close-up shots along the right side,
which are abstracted from Fig. S1, a full version of the ML tree. In the close-up shots,
Feirana specimens are named by a combination of voucher number and corresponding
morphotype, the *F. quadranus*, *F. taihangnica* and *F. kangxianensis* references are named by
a combination of GenBank number and species name; pink and blue rectangles indicate four
specimens with conflicting morphotypical classifications.

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739 Fig. 4 Profile plots of p-values for the five groupings in Table S3, with the vertical scale being
740 logarithmic in base 10. The blue dashed line labelled "16S", indicates the Q and T set; the
741 orange solid line labelled "morp3", indicates the VV, vw&wv, and ww set; the black solid
742 line labelled "sex", indicates the female and male set; the pink solid line labelled "F_morp3"
743 or "F" indicates the female VV, vw&wv, and ww set; and the green dashed line labelled
744 "M_morp3" or "M", indicates the male VV, vw&wv, and ww set.
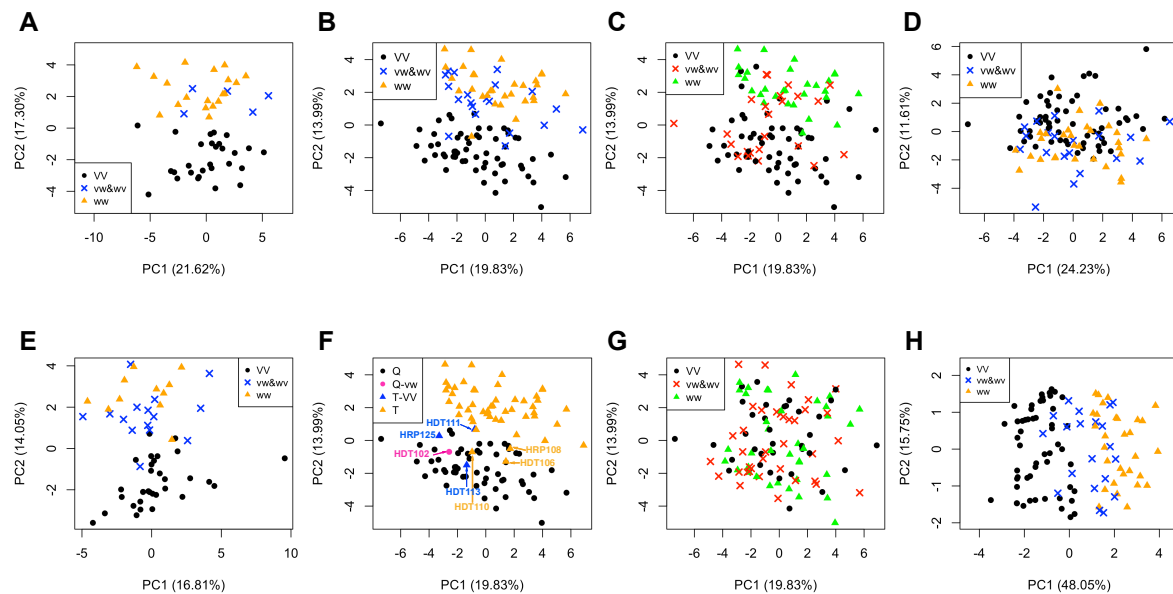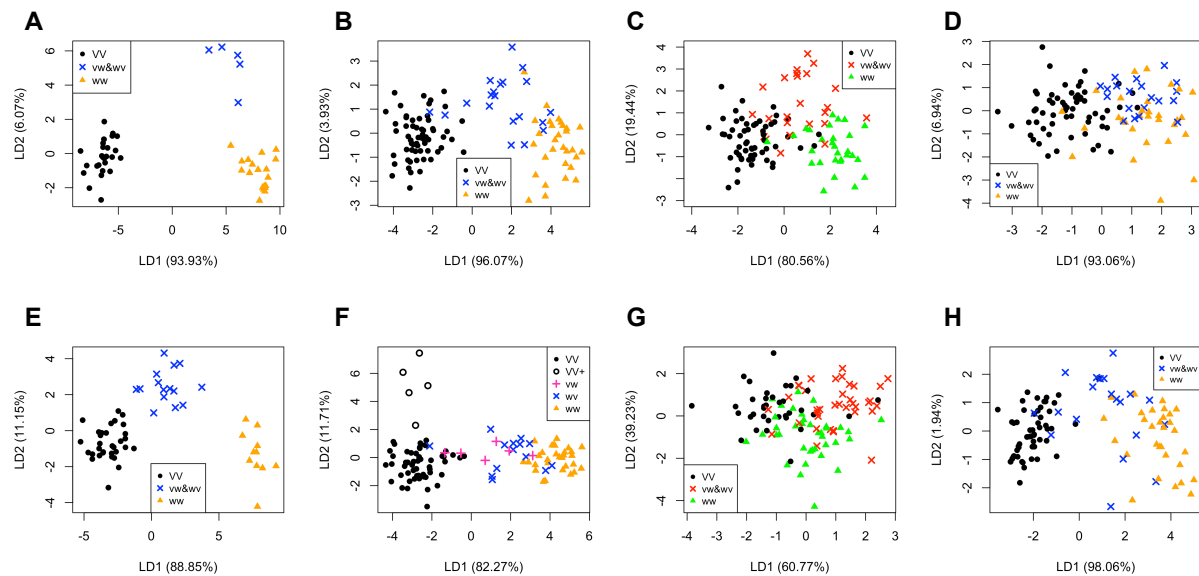
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747 Fig. 5 Results of the PCA. Scatterplots for the first two principal components, PC1 and PC2.
748 (A, E) PCA for 52 females and 58 males, respectively, grouped by the three morphotypes; (B,
749 F) PCA for the total 110 specimens, grouped into the three morphotypes and four
750 16S_versus_morphotypes, respectively; (C, G) PCA for the two simulated populations, the
751 different palettes signify the data's distance from reality; (D, H) PCA for the 110 individuals
752 based on the 27 ratio indices and on the 9 extent indices, independently.
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757 Fig. 6 Results of the LDA. Scatterplots for the first two linear discriminant functions, LD1
758 and LD2. (A, E) LDA for 52 females and 58 males, respectively, grouped by the three
759 morphotypes; (B, F) LDA for the total 110 specimens, grouped into the three morphotypes
760 and five morphotypes, respectively; (C, G) LDA for the two simulated populations' three
761 morphotypes, the different palettes signify the data's distance from reality; (D, H) LDA for
762 the total 110 specimens based on the 27 ratio indices and 7 extent indices, independently.
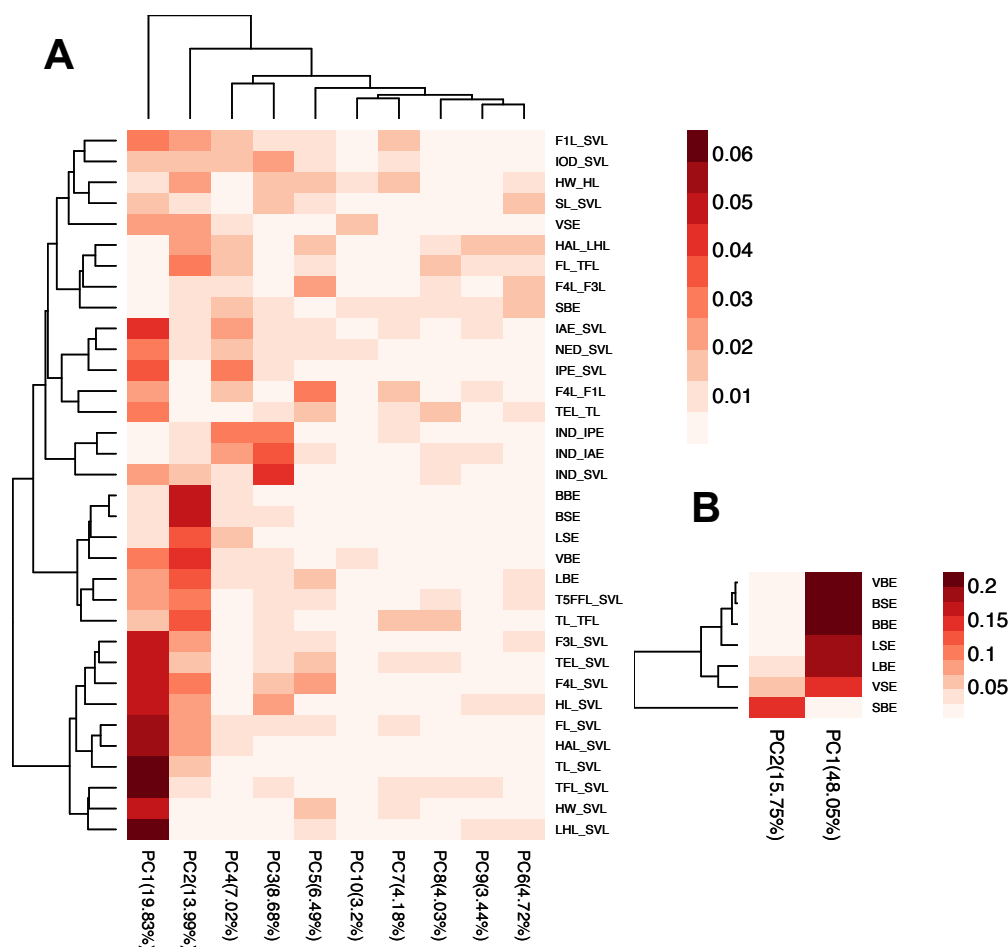
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765 Fig. 7 Heat-maps of weighted rotation matrices of the PCA. In the weighted (multiplier)
766 matrix, the corresponding proportion of explained variance for each PC is in parenthesis. (A)
767 The first 10 PCs for the total specimens, corresponding to Fig. 5B; (D) The first two PCs of
768 the extent indices for the total specimens, corresponding to Fig. 5H.
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