Morphological evidence for introgressive hybridization between *Feirana quadranus* and *Feirana taihangnica* in Tsinling Mountains, China

Yang Song, Xin Sui, Yuhong Bian, Junfang Zhang, Junqiang Zheng, Pipeng Li

Background. *Feirana quadranus* and *Feirana taihangnica*, two species of frogs inhabiting in waterbodies in the Tsinling Mountains, China, are believed to be sister species that diverged 46,000 years ago. In their sympatric area, morphological variations found between the two species imply that the two species had inter-bred. Additionally, *F. taihangnica*'s polyandrous breeding behavior, without amplexus, would not hinder the potential hybridization.

Methods. To verify the hybridization, 117 specimens of *F. quadranus* and *F. taihangnica* were collected from eight sampling sites in their sympatric area, and 110 of the specimens were classified morphologically into VV, vw&wv, and ww, representing the putative parental and suspected hybrid types. Their maternal bloodlines were identified using a phylogenetic tree based on a region of the mitochondrial *16S rRNA* gene. In total, 34 morphometric indices were selected to analyze the morphological variation between 16S-types or among morphotypes. A principal component analysis (PCA) and linear discriminant analysis (LDA) were conducted on total or partial indices for females, males, and total specimens, as well as simulated populations with falsified morphotypes. The most important indices for differentiation among morphotypes were revealed with the assistance of heat-maps.

Results. In the mitochondrial DNA tree, most of the VV were in the same clade as the reference *F. quadranus*, labeled as Q, while most of the ww and vw&wv were grouped with the reference *F. taihangnica*, labeled as T. According to the PCA, there was a clear differentiation between VV and ww, while vw&wv specimens were in the middle area close to ww. According to the LDA, VV, vw&wv, and ww were clustered into three separate groups. An ambiguous differentiation between Q and T was shown both in mtDNA tree and in multivariate analyses. Seven of the specimens with conflicting classifications blurred the morphological boundary between Q and T. In both the PCA and LDA, indices that were based on the extent of bumps and skin coloration discriminated VV, vw&wv, and ww better than ratio indices that were derived from measurements.

Discussion. The distribution of VV, vw&wv, and ww in multivariate spaces, especially vw&wv being scattered between VV and ww, demonstrated an introgressive hybridization pattern. The extents of bumps in the shape of an inverted "V" between the shoulder blades, spot pattern on the back, and large bumps above the anal region were the most important characteristics for differentiating between three morphotypes or between *F. quadranus* and *F. taihangnica*.

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Abstract

Background. *Feirana quadranus* and *Feirana taihangnica*, two species of frogs inhabiting in waterbodies in the Tsinling Mountains, China, are believed to be sister species that diverged 46,000 years ago. In their sympatric area, morphological variations found between the two species imply that the two species had inter-bred. Additionally, *F. taihangnica*'s polyandrous breeding behavior, without amplexus, would not hinder the potential hybridization.

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In total, 34 morphometric indices were selected to analyze the morphological variation between 16S-types or among morphotypes. A principal component analysis (PCA) and linear discriminant analysis (LDA) were conducted on total or partial indices for females, males, and total specimens, as well as simulated populations with falsified morphotypes. The most important indices for differentiation among morphotypes were revealed with the assistance of heat-maps.

Results. In the mitochondrial DNA tree, most of the VV were in the same clade as the reference *F. quadranus*, labeled as Q, while most of the ww and vw&wv were grouped with the reference *F. taihangnica*, labeled as T. According to the PCA, there was a clear differentiation between VV and ww, while vw&wv specimens were in the middle area close to ww. According to the LDA, VV, vw&wv, and ww were clustered into three separate groups. An ambiguous differentiation between Q and T was shown both in mtDNA tree and in multivariate analyses. Seven of the specimens with conflicting classifications blurred the morphological boundary between Q and T. In both the PCA and LDA, indices that were based on the extent of bumps and skin coloration discriminated VV, vw&wv, and ww better than ratio indices that were derived from measurements.

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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 Tsinling 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 Tsinling (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 46,000 years ago and that the Tsinling 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 frogs among the genus *Feirana* are indistinguishable. They inhibat, and are basically limited to, waterbodies, such as creeks, brooks, streams, and rivers in mountainous areas at altitudes of 500 m to 2,500 m. They prey, hibernate, and breed mostly in water, and are hardly seen on the land unless there is enough rain or moisture.

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

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

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

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

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

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

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

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

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 Tsinling 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 Tsinling 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. quadranus* with a cream-colored mid-dorsal line was labeled “VV+” (Fig. 2B); frogs with typical traits of *F.*

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

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

2.3 16S classification

2.3.1 Laboratory work

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

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

2.3.2 Data analysis

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

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

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

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

The tree shown (Fig. 3) was inferred by ML using the best model (K2+G) estimated to have the lowest Bayesian information criterion value (Schwarz, 1978). It was a combination of the Kimura 2-parameter nucleotide substitution model (Kimura, 1980) and a discrete gamma distribution of five categories to model evolutionary rate differences among sites. The initial tree for the heuristic search was obtained by applying the neighbor-joining method to a matrix of

pairwise distances estimated using the Maximum Composite Likelihood approach. A bootstrap test was performed with 500 replications. All of the gaps and missing data were included.

2.3.3 Divergence (p-distance)

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

2.3.4 Phylogenetic classification

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

2.4 Morphometric indices and statistical analyses

2.4.1 Chosing and designing morphometric indices

To evaluate the morphological variation and differentiation among the putative parents, "VV" and "ww", and the suspected hybrids, "vw&wv" (see 4 for explaination), 34 indices were employed. Originally, 32 morphometric characteristics based on Wang (2009) and Fei *et al*. (2009) were measured on preserved specimens using Vernier calipers. 13 with significant measurement errors (i.e. nostril-snout distance, width of outer web of first toe) or that were disproportional to body size (i.e. tympanum horizontal diameter, distance between internal nares, size of vomerine teeth, length and width of inner metacarpal tubercle, length and width of inner metatarsal tubercle) were removed because Hayek, Heyer & Gascon (2001) warned against measurement errors and data transformation. The remaining measured characteristics were divided by snout-vent length (SVL) to eliminate body size effects.

Nine ratios were derived from certain measured characteristics, which together with 18 ratios of measured characters to SVL were called ratio indices. The name of a ratio index is composed as the pattern "dividend_divisor", e.g. HL_SVL represents HL/SVL. Seven extent indices, based on extent of bumps and coloration patterns on the skin were given values between 0 and 1, or 0 and 2. In the end, 34 indices, including 27 ratio indices and 7 extent indices, remained for analysis (Table S3-Table S5).

Abbriviations of characters or indices with descriptions

Measured characters

SVL: snout-vent length, used to eliminate body size effects;
HL: head length, from posterior end of mandible to tip of snout;
HW: head width, measured at corners of the mouth;
SL: snout length, distance between anterior edge of orbit and tip of snout;
NED: nostril-to-eye distance, distance between centre of nostril and anterior edge of orbit;
IND: internarial distance, distance between inner ends of nostrils;
IOD: interorbital distance, shortest distance between inner edges of upper eyelids;
IAE: distance between anterior corners of eyes;
IPE: distance between posterior corners of eyes;
LHL: length of lower arm and hand, from elbow to tip of third finger;
HAL: hand length, from base of outer palmar tubercle to tip of third finger;

TEL: femur length, from vent to knee;
TL: tibia length;
TFL: tibiofibula length (length of tarsus and foot), from base of tarsus to tip of fourth toe;
FL: foot length, from proximal end of inner metatarsal tubercle to tip of fourth toe;
T5FFL: length of free flap of the fifth toe, length of cutaneous fringe along the outer margin of the fifth toe;
F1L: first finger length, from proximal end of thenar tubercle to tip of first finger;
F3L: partial 3rd finger length, distance between basal border of third finger to tip of third finger;
F4L: fourth finger length, from proximal end of thenar tubercle to tip of fourth finger.

Extent indices (scored between 0, indicating the trait was not seen, and 1, indicating the maximum extent):
BBE: extent of big bumps above the anal region;
SBE: extent of small bumps above the anal region;
VBE: extent of bumps in the shape of inverted "V" between shoulder blades;
VSE: extent of patch in the shape of inverted "V" between shoulder blades;
LBE: extent of line-shaped bumps on the back; BSE: the extent of spot pattern on the back; LSE: extent of strip or or spot pattern on legs (scored between 0, indicating no obvious pattern, 1, indicating pure strip pattern, and 2, indicating pure spot pattern).

2.4.2 Description of morphometric data

Morphometric raw data was in Table S2. Means and standard deviations (mean ± s.d.) of 34 indices were calculated in five groupings, with each set containing two or three groups: 16S (Q and T), morp3 (VV, vw&wv, and ww), sex (female and male), F_morp3 (females' VV, vw&wv, and ww) and M_morp3 (males' VV, vw&wv, and ww) (Table S3). To reveal the differences in means between or among groups in each set, statistical tests were performed on the R 3.2 platform (R Core Team, 2015). The function "t.test" was employed to execute t-tests of sets containing two groups (16S and sex sets). For the sets containing three groups, firstly, "bartlett.test" (Bartlett, 1937) was used to check the homogeneity of the variances; and if the p-value generated by Bartlett's test was above 0.05, meaning variances were homogeneous, then an ANOVA was applicable, and the function "avo" (Chambers, Freeny & Heiberger, 1992) was then used to compare the means of the groups, otherwise "kruskal.test" for the Kruskal-Wallis test (Myles & Douglas, 1973), which applies to extreme non-normal distributions of sample values, was used instead.

In Excel 2011 for mac, profiles of the p-values for the five sets in Table S4 were plotted. P-values were ordered from highest at the bottom, to lowest at the top), and a logarithmic scale with base 10 was applied to the *y* axis to magnify the high-degree differences represented by p-values near 0, and minimize the low-degree differences represented by p-values near 1 (Fig. 4).

2.4.3 Multivariate analyses

Two types of multivariate analyses were performed on the R 3.2 platform (R Core Team, 2015) to estimate the morphometric variation among morp5 set (VV, VV+, vw, wv, and ww), morp3 set (VV, vw&wv, and ww), 16S set (Q and T), or 16S_versus_morp set (Q, Q-vw, T-VV, and ww) (Fig. 5F). The function "prcomp" with "scale = TRUE" was used for the principal component analyses (PCA; see 4 for interpretation of PCA), clustering individuals in the multivariate space of the first two principal components (PC1 and PC2); and the function "lda" in package "MASS" (Venables & Ripley, 2002) was used for the linear discriminant analysis (LDA; see 4 for interpretation of LDA).

Considering the possible sexual dimorphism, the females (Fig. 5A; Fig. 6A) and the males (Fig. 5E; Fig. 6E) were analyzed separately, as well as together (Fig. 5B, F; Fig. 6B, F). The

independent impacts of ratio indices (Fig. 5D; Fig. 6D) or extent indices (Fig. 5H; Fig. 6H) on the PCA and LDA were explored.

To test the reliability of the morphotype-based classifications, morphotypical information was simulated in two ways using falsified data sets from two populations (see Table S2). Based on the real data, for the first simulated population, we changed a small proportion of VV into vw&wv and a small proportion of ww into vw&wv or VV, and remixed the original vw&wv with all three types (Fig. 5C; Fig. 6C); for the second simulated population, the three morphotypes (VV, vw&wv, and ww) were randomly assigned to specimens (Fig. 5G; Fig. 6G).

2.4.4 Analyses of indices

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

3 Results

3.1 16S

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

3.2 Morphometric analysis

3.2.1 PCA

According to the PCA, in both females (Fig. 5A) and males (Fig. 5E), there is a clear differentiation between VV and ww. The distribution of wv&vw is closer to ww. In males (Fig. 5E) and total specimens (Fig. 5B), most of the wv&vw are mixed with ww, or in the area between ww and VV, and a small proportion are mixed with VV. In females (Fig. 5A), limited samples of vw&wv are near the borderline of the ww zone.

The incomplete differentiation between Q and T is shown by the genetic classification (Fig. 5F). The three border-crossers, HRP108, HDT106, and HDT110, being genetically T, appear in VV's territory. The four specimens with controversial classifications (see 2.3.4), T-VV (HDT111, HDT113, and HRP125), which are genetically T but morphotypically VV, and Q-vw (HDT102), which is genetically Q but morphotypically vw, appear in the ambiguous zone between Q and T (Fig. 5F). The positions of the four specimens in the multivariate spaces of females (Fig. 5A) and

males (Fig. 5E) are also close to the borderline between VV and ww.

Ratio indices failed to differentiate between VV and ww (Fig. 5E); however, extent indices differentiate solely using PC1, and vw&wv are perfectly scattered along the boundary zone between VV and ww (Fig. 5H).

3.2.2 LDA

Based on the LDA, VV, vw&wv, and ww are clustered into three separate groups in both females (Fig. 6A) and males (Fig. 6E). The differentiation between vw&wv and VV or ww is less complete in total specimens (Fig. 6B).

Similar to the PCA results, extent indices differentiate the three morphotypes more completely than the ratio indices (Fig. 6D, H).

3.2.3 Simulated data

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

3.2.4 Analyses for importance of indices

A full version of heat-maps are shown in Fig. S2.

In the LDA, it seems that, to discriminate three morphotypes or five morphotypes from the total specimens, finger lengths and other length indices were the most contributive characters (Fig. S2E-H). Finger lengths, however, are not crucial for discriminating between morphotypes. When F1L_SVL, F3L_SVL and F4L_SVL were eliminated from the morphometric data, plots of the LDA for each set stayed the same, only the indices originally ranked after F1L_SVL, F3L_SVL, and F4L_SVL upgraded their contributions to each LD (data not shown). Contrarily, the seven extent indices seemed to be minimally involved in discriminating between morphotypes. However, when these seven extent indices were excluded, leaving only 27 ratio indices, the three morphotypes could not be easily distinguished (Fig. 6D); or when the 27 ratio indices were excluded, leaving only the seven extent indices, the distribution pattern of three morphotypes stay the same (Fig. 6H). Therefore, we decided not to use coefficients matrix of LDA to analyze importance of indices.

In the PCA, generally speaking, indices on limb and finger lengths contribute most to PC1, while indices involving bumps and coloration patterns contribute most to PC2. The weighted rotation matrix of 34 indices for the first 10 PCs (eigenvalues > 1), accounting for 78.11% (total specimens) of the variation is shown here (Fig. 7A). The most important indices in PC1 were TL_SVL, (tibia length)/SVL; LHL_SVL, (length of lower arm and hand)/SVL; and TFL_SVL, (tibiofibula length)/SVL. The most important indices in PC2 were BSE, the extent of spots on the back and BBE, the extent of big bumps above the anal region.

The weighted rotation matrix of seven extent indices is shown for the first two PCs (eigenvalues > 1), accounting for 63.80% of the variation (Fig. 7B). VBE, the extent of bumps in the shape of an inverted "V" between the shoulder blades; BSE, the extent of spots on the back; and BBE, the extent of big bumps above the anal region, account for most of the PC1 variance, which clearly differentiated the three morphotypes (Fig. 5H).

4 Discussion

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

a hybrid, or that each VV or ww was a pure parent, especially for samples at sites inhabited by two or three morphotypes (e.g. HDT, HRP, and XYB). Based on the theory of introgressive hybridization (Anderson, 1949), frogs at these sites may have been intercrossed and backcrossed for many generations, leading to limited pure "parents", and these morphotypical "parents", VV and ww, may only have been more back-crossed than the morphotypical "hybrids" (Lehtinen *et al*., 2016). Another possible hypothesis is that hybridization does not necessarily equally (50%) affect the hybrids' appearances because there are genetic and developmental buffers between a frog's genotype and its phenotype, such as hybridogenesis (Holsbeek & Jooris, 2010; Mikulíček *et al*., 2014), genomic imprinting (Tunner, 2000), pleiotropy, dominance, epistasis (Gallez & Gottleib, 1982), and epigenetic phenomenon. Therefore, this simplified hybridization pattern was only adopted for the convenience of verifying possible introgression.

PCA and LDA are often used to detect or estimate hybridization, especially in morphology (e.g. Albert, D'Antonio & Schierenbeck, 1997; Wu *et al*., 2011). In PCA theory, PCs are uncorrelated linear combinations of rotated indices, and analyzing the entire data is reduced to only considering the first several PCs that explain the majority of the variation (Crawley, 2009). The most common visual way is to place individuals on a scatterplot of the first two PC axes, and use group-based symbols to represent individuals. The closer two points are, the more similar their corresponding indices. This allows one to see whether individuals of one group are clustered in the space and whether they are isolated from individuals of the other group.

Similar to the PCA, the LDA seeks the best linear functions to discriminate between predefined groups instead of between individuals (Selvin, 1994). The grouping information for each individual is preset, and coefficients of indices are estimated for LD functions which is one less than the number of groups. Consequently, between-group differences are maximized in a scatter plot of the first two LD functions, exhibiting how well pre-defined groups of individuals can be separated by multivariate measurements (Lihová *et al*., 2007).

The expected pattern is presented in the multivariate space of the first two PCs of the PCA (Fig. 5A, B, E, and H) and of the first two LD functions of the LDA (Fig. 6A, B, E, and H). A further analysis on the simulated two populations, which were created based on the actual population by falsifying morphotypical data (see 2.4.3) confirmed the reliability of the hybridization pattern established by the three morphotypes.

In the mtDNA's phylogenetic tree (Fig. 3), wv and most vw's are intermixed with ww in the clade *F. taihangnica*. Could this suggest that *F. taihangnica* might be the maternal species of suspected "hybrids", vw and wv, with VV being the paternal species? Considering the unique breeding behavior of *F. taihangnica*, which is simultaneous polyandry with multiple males not engaged in amplexus (Zhang *et al*., 2012) (see 1.2), it seems more likely that female *F. taihangnica* ley eggs on rocks and then male *F. quadranus* and/or *F. taihangnica* fertilize them.

In the morphometric analysis, vw&wv are often intermixed with ww (Fig. 5A, B, E). In particular, when excluding the seven extent indices, leaving only 27 ratio indices, which were derived from measured characteristics (see 2.4.1), vw&wv were completely intermixed with ww (Fig. 6D). However, when leaving only the seven extent indices, which describe typical traits of *F. quadranus* and *F. taihangnica* (see 2.2), the vw&wv's proneness to ww disappeared (Fig. 5H). Could this suggest that mtDNA have an influence on the measurable characteristics instead of extent characteristics? Since backcrossing causes the offsprings to resemble the recurrent parental species (Anderson, 1949), could this imply the hybrids' have a preference for backcrossing with ww? Or could it be the genome-dosage effects that were often seen between diploid and triploid frogs (e.g. Borkin *et al*., 2004; Plötner, 1994)?

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.

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675 **Tables and Figures**

676 **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	/	/

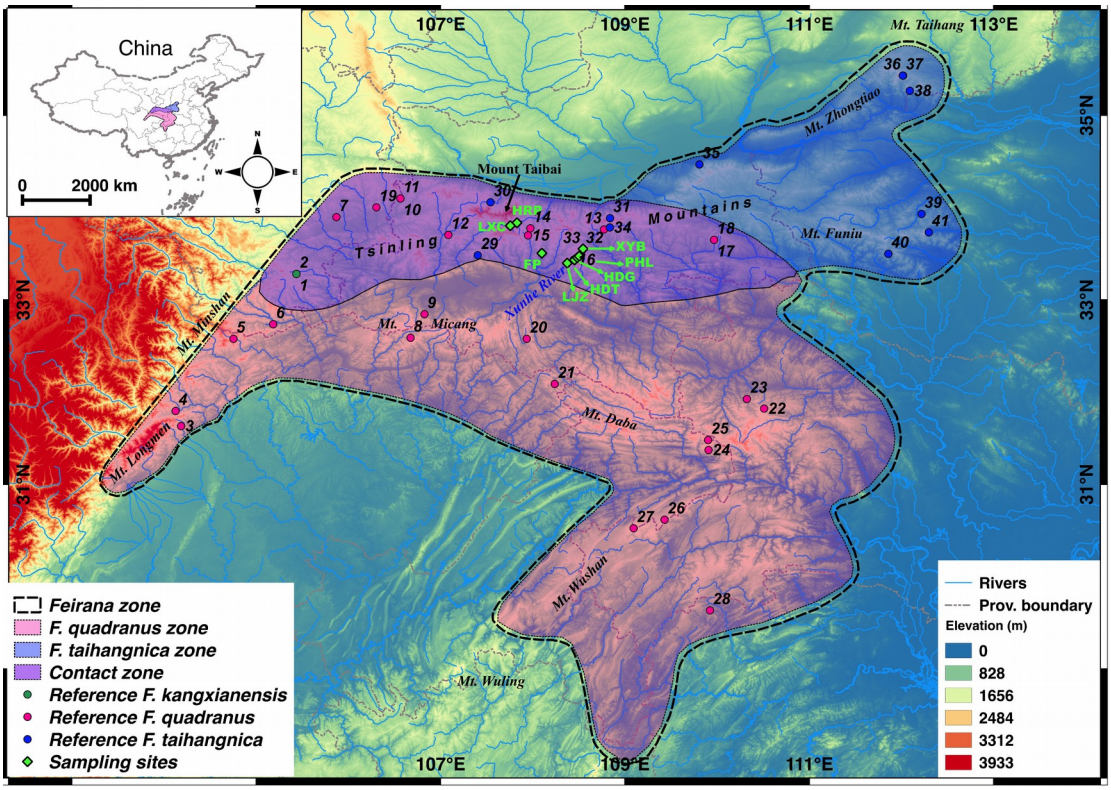
677 [a] n represents the sample size from each site.

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

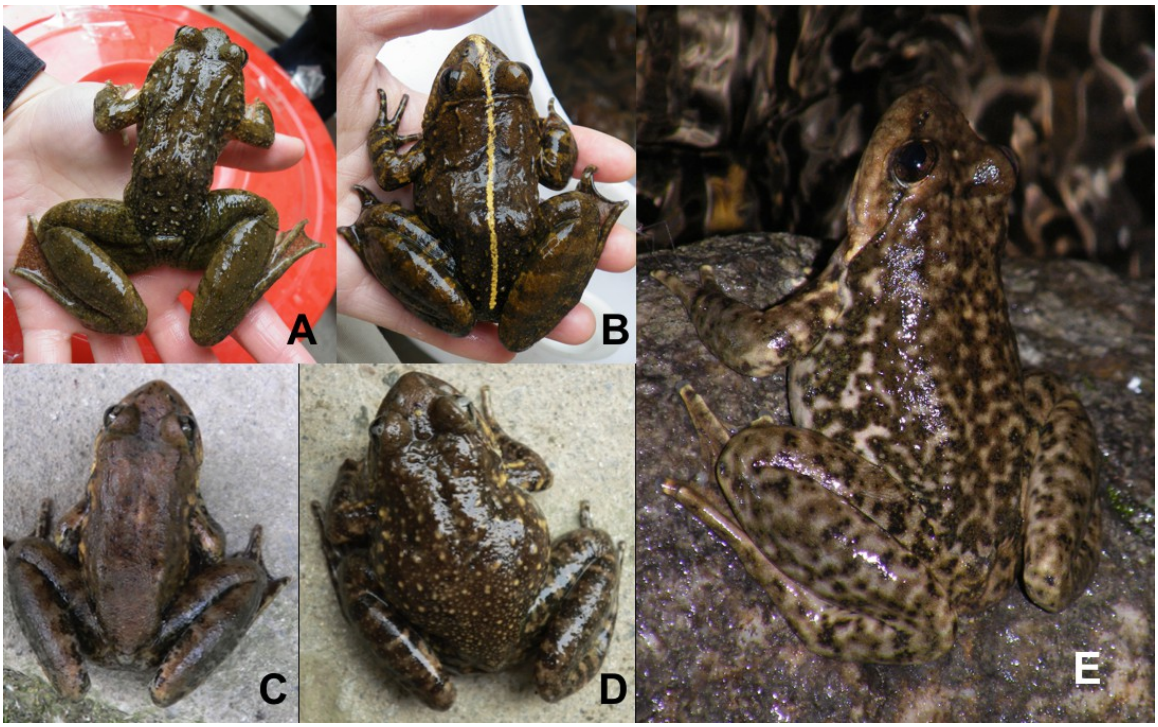
No.	GenBank No.	Voucher No.	Species name in reference article[a]	Species name in new nomenclature	Locality (village/county/city, province)	Latitude (N, °)	Longitude (E, °)	Reference article
1	GQ225974	CIBKangxian01	*"Feirana". taihangnica*	*F. kangxianensis*	Kangxian, Gansu	105.4367	33.2804	Wang *et 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

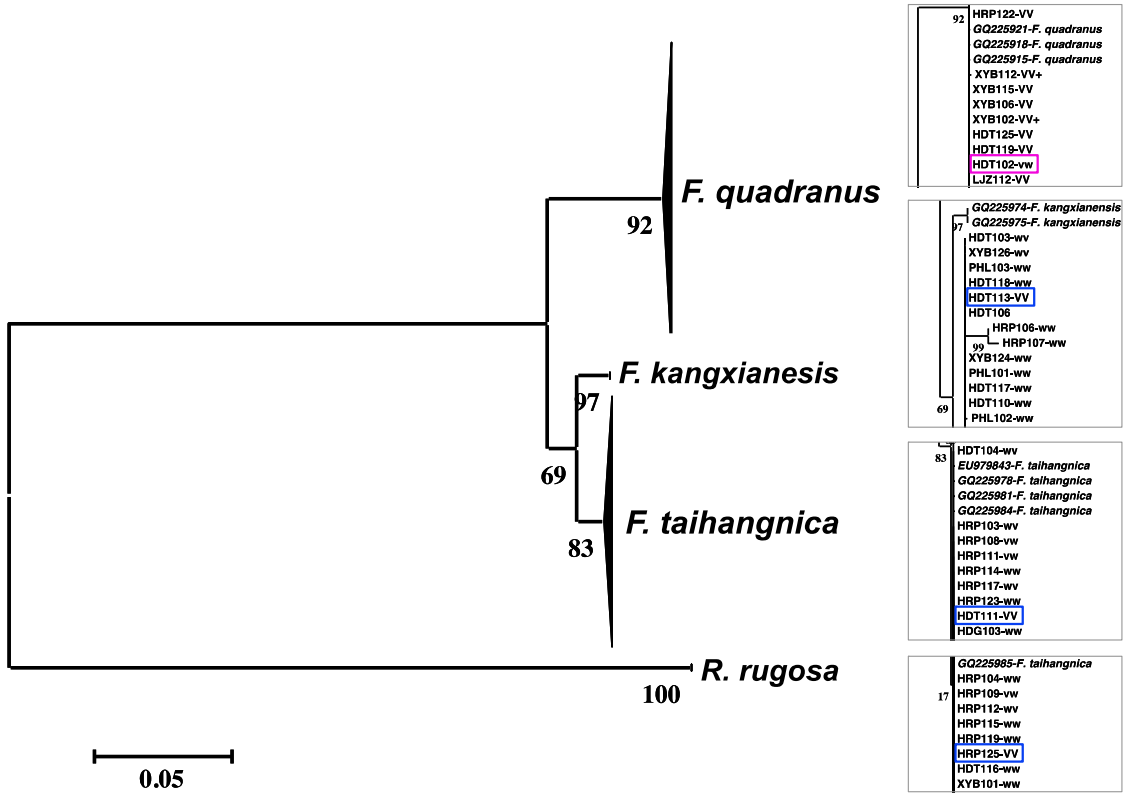
[a] Due to taxonomic chaos in tribe Paini (Che *et al*., 2009), *Feirana quadranus* was also named *Chaparana quadranus* (Jiang *et al*., 2005; Ohler & Dubois, 2006; Che *et al*., 2009; Wang *et al*., 2009), and *Feirana taihangnica* was named *Paa taihangnica* (Jiang *et al*., 2005; Ohler & Dubois, 2006; Ye *et al*., 2013).



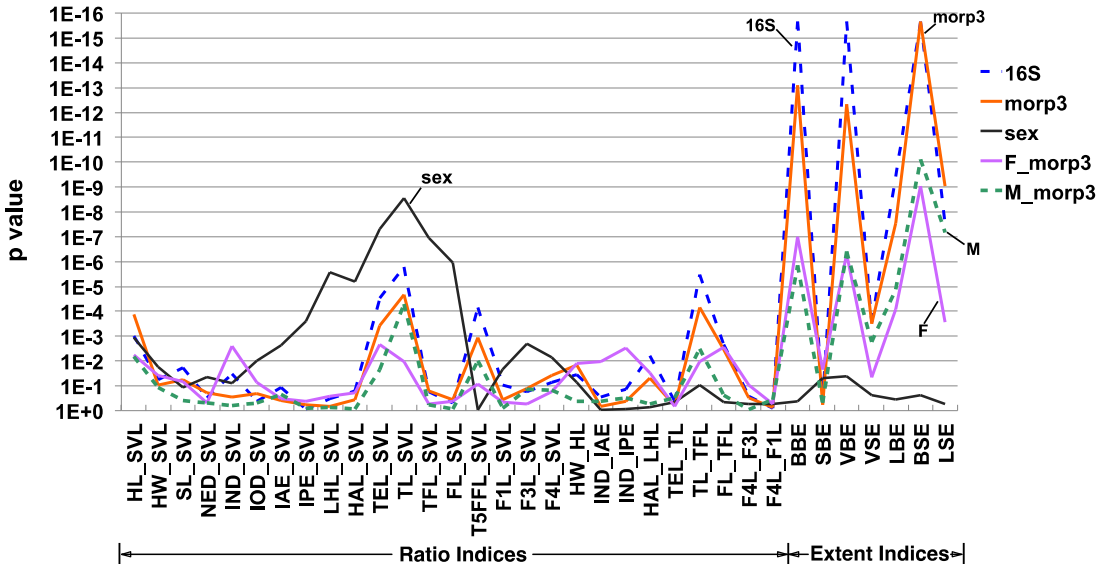
683 Fig. 1 Distribution of the 8 sampling sites from which 117 specimens were collected.
684 Abbreviations for sampling sites (light green diamonds) correspond to those in Table 1.
685 Reference sampling sites (green, pink, and blue spots), 1–41, correspond to the numbers in Table
686 2. Distribution zones were drawn according to Fig. 1 in Wang *et al*. 2009.



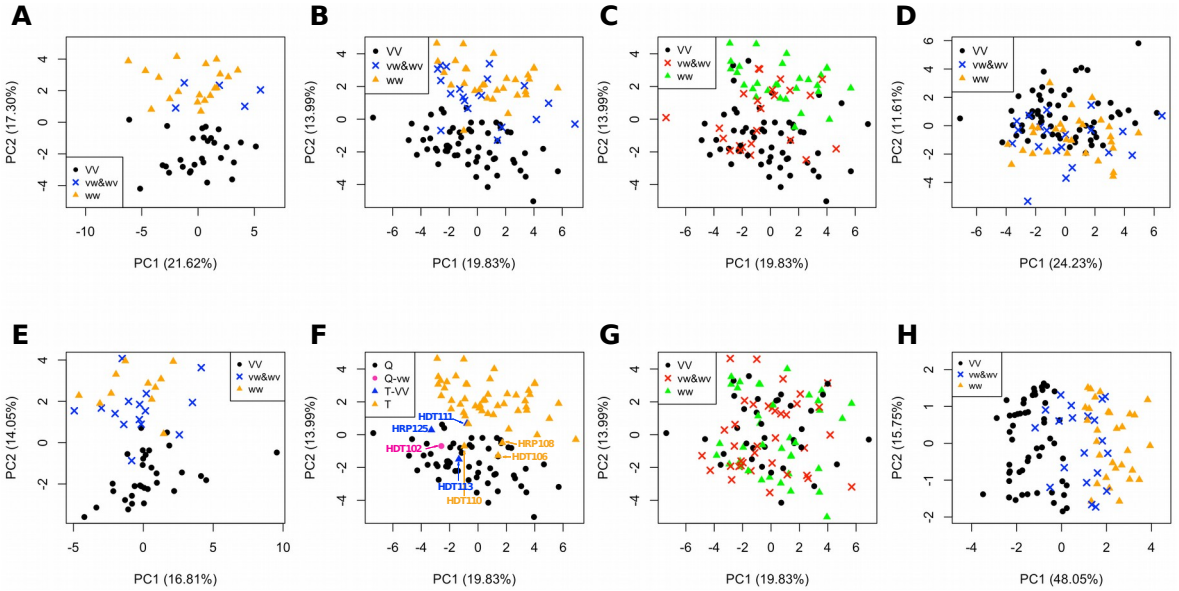
687 Fig. 2 Examples of the five morphotypes of *F. quadranus* and *F. taihangnica*. (A) VV; (B) VV+;
688 (C) vw, looks like VV, only without granular bumps above the anus; (D) wv, looks like ww, only
689 with too many granular bumps on the back; (E) ww. Photo credit: Yang Song & Xin Sui.



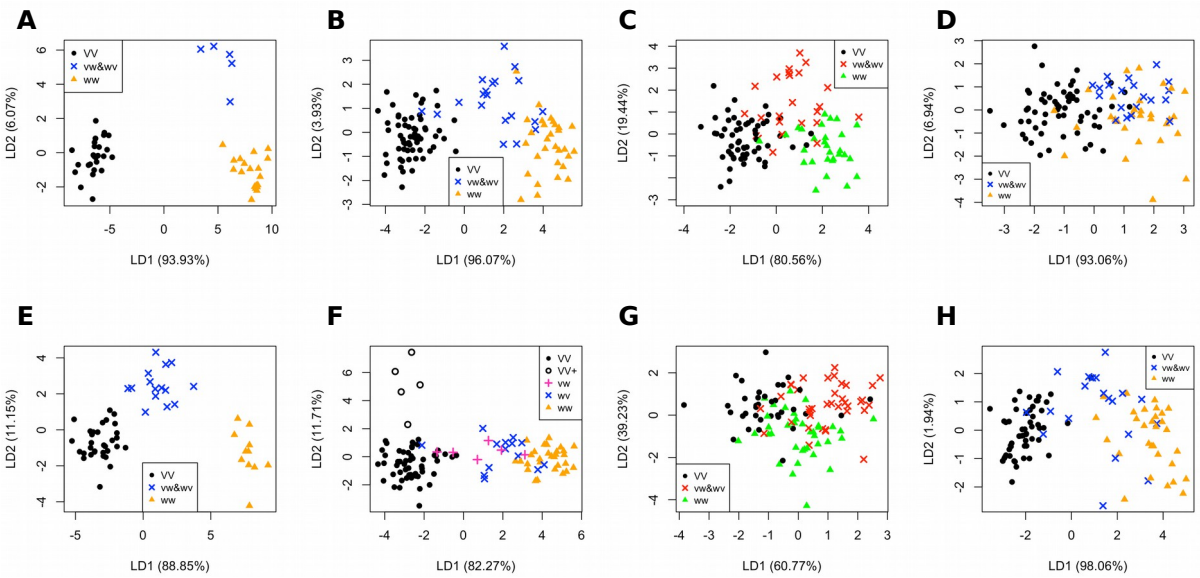
690 Fig. 3 Compressed maximum likelihood (ML) phylogenetic tree based on *16S rRNA* gene partial
691 sequences. The bootstrap support values are shown below branches. Scale bar indicates an
692 evolutionary distance of 0.05 nucleotides per position in the sequence. The four grey rectangles
693 on the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



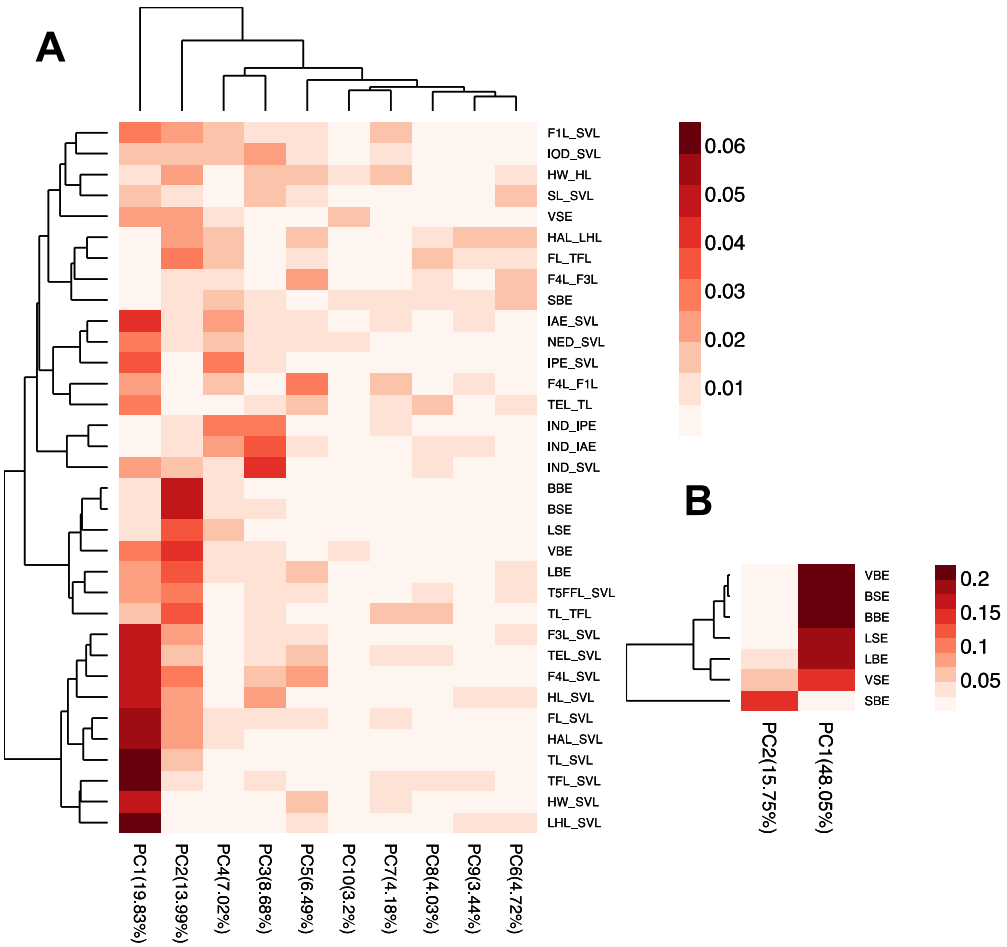
699 Fig. 4 Profile plots of p-values for the five groupings in Table S3, with the vertical scale being
700 logarithmic in base 10. The blue dashed line labelled "16S", indicates the Q and T set; the orange
701 solid line labelled "morp3", indicates the VV, vw&wv, and ww set; the black solid line labelled
702 "sex", indicates the female and male set; the pink solid line labelled "F_morp3" or "F" indicates
703 the female VV, vw&wv, and ww set; and the green dashed line labelled "M_morp3" or "M",
704 indicates the male VV, vw&wv, and ww set.



705 Fig. 5 Results of the PCA. Scatterplots for the first two principal components, PC1 and PC2. (A,
706 E) PCA for 52 females and 58 males, respectively, grouped by the three morphotypes; (B, F) PCA
707 for the total 110 specimens, grouped into the three morphotypes and four
708 16S_versus_morphotypes, respectively; (C, G) PCA for the two simulated populations, the
709 different palettes signify the data's distance from reality; (D, H) PCA for the 110 individuals
710 based on the 27 ratio indices and on the 9 extent indices, independently.



711 Fig. 6 Results of the LDA. Scatterplots for the first two linear discriminant functions, LD1 and
712 LD2. (A, E) LDA for 52 females and 58 males, respectively, grouped by the three morphotypes;
713 (B, F) LDA for the total 110 specimens, grouped into the three morphotypes and five
714 morphotypes, respectively; (C, G) LDA for the two simulated populations' three morphotypes,
715 the different palettes signify the data's distance from reality; (D, H) LDA for the total 110
716 specimens based on the 27 ratio indices and 7 extent indices, independently.



717 Fig. 7 Heat-maps of weighted rotation matrices of the PCA. In the weighted (multiplier) matrix,
718 the corresponding proportion of explained variance for each PC is in parenthesis. (A) The first 10
719 PCs for the total specimens, corresponding to Fig. 5B; (D) The first two PCs of the extent indices
720 for the total specimens, corresponding to Fig. 5H.