A De Novo Case of Floating Chromosomal Polymorphisms by Translocation in *Quasipaa boulengeri* (Anura, Dicroglossidae)

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**Abstract**

Very few natural polymorphisms involving interchromosomal reciprocal translocations are known in amphibians even in vertebrates. In this study, thirty three populations, including 471 individuals of the spiny frog *Quasipaa boulengeri*, were karyotypically examined using Giemsa stain or FISH. Five different karyomorphs were observed. The observed heteromorphism was autosomal but not sex-related, as the same heteromorphic chromosomes were found both in males and females. Our results indicated that the variant karyotypes resulted from a mutual interchange occurring between chromosomes 1 and 6. The occurrence of a nearly whole-arm translocation between chromosome no. 1 and no. 6 gave rise to a high frequency of alternate segregation and probably resulted in the maintenance of the translocation polymorphisms in a few populations. The translocation polymorphism is explained by different frequencies of segregation modes of the translocation heterozygote during meiosis. Theoretically, nine karyomorphs should be investigated, however, four expected karyotypes were not found. The absent karyomorphs may result from recessive lethal mutations, position effects, duplications and deficiencies. The phylogenetic inference proved that all populations of *Q. boulengeri* grouped into a monophyletic clade. The mutual translocation likely evolved just once in this species and the dispersal of the one karyomorph (type IV) can explain the chromosomal variations among populations.

**Introduction**

Very few natural polymorphisms involving interchromosomal reciprocal translocations are known in animal populations [1,2]. The reasons are obvious because this kind of rearrangement can lead to reduction of fertility and reduced fitness for the carrier. Translocation heterozygotes will result in the formation of rings or chains of four chromosomes during meiosis and if they orientate with the adjacent centromeres passing to same poles (adjacent segregation) instead of to the opposite poles (alternate segregation), aneuploid gametes carrying either a duplication or a deficiency will be produced and would result in lower fecundity [3].

In amphibians, there are no reports of fixed translocation polymorphisms in natural populations but rare spontaneous translocations have been documented. Morescalchi (as cited in Chiarelli and Capanna, 1973, p326) [3] described a possible case of a simple translocation based on an analysis of oocyte lampbrush chromosomes from a female toad (*Pelodytes punctatus*). In 2004, Schmid et al. [4] observed a low frequency (2.7%) of a non-reciprocal translocation between autosomes 3 and 11 that were identified by the BrdU/dT replication banding patterns in aging cultured fibroblast cells of *Gastrotheca riobambae*. Schmid et al. (2010), [5] found only three of 2,548 individuals (0.001%) of terraranan frogs that demonstrated a constitutional reciprocal translocation. Non-fixed mutual translocations have been found by Siqueira Jr et al. (2004) [6] in *Hedduas bisinatus*, also a species of terraranan frogs, however, few individuals were examined so translocation polymorphisms at a population level are not known.

Recently, a fascinating case of interchange translocation polymorphisms was discovered in the Asian spiny frog, *Quasipaa boulengeri*. This species is widely distributed in low mountainous regions along the edges of Sichuan Basin and nearby areas in southern China [7] (Fig. 1). No heteromorphic chromosomes had been found in previous studies that examined several populations [8–13]. In a Xuanzhouhen population, Wang (2006) [14] discovered two pairs of heteromorphic chromosomes in a single female and these chromosome pairs were homomorphic in males (3♂, 1♀, Table 1). He speculated that female sex-related heteromorphism exists, and the heteromorphic chromosomes were multiple sex chromosomes of a *Z₁Z₁W₁Z₁Z₁W₁Z₁Z₁W₁Z₁* configuration. Further, he predicted that there exists a heterozygous reciprocal translocation between these two pairs of heteromorphic chromosomes.

To understand this rare karyotype, we have cytogenetically re-examined the species *Quasipaa boulengeri* at a population level since 2006. We examined 33 populations and 471 individuals. Our results revealed a de novo case of reciprocal translocation polymorphisms in amphibians that involves the most complicated
multiple karyotypes so far known to exist in amphibians. Herein, we have reconstructed the phylogeny of the species *Q. boulengeri* based on DNA sequences of three mitochondrial genes (12S, 16S and COI) to test if this species is monophyletic, and then we try to elucidate these polymorphic karyotypes in different populations, and to decipher the origin of the chromosomal polymorphisms between populations in this frog.

**Results**

**Mitotic Chromosomes**

All specimens from all 33 populations had a diploid number of 2n = 26 chromosomes, and inter- and intrapopulation karyotype variations were found in several populations. At least five different karyomorphs (Type I–V) were observed in the pooled populations (Figs. 2, 3; Tables 1, 2). All karyotypic differences found in these populations were chromosome types that involved pairs no.1 and no.6.

**Type I (MM/mm).** The normal karyotype. Chromosome numbers 1 and no. 6 consisted of two large homomorphic metacentric (MM) chromosomes and two small homomorphic metacentric (mm) chromosomes, respectively (Figs. 2-I, 3a).

**Type II (MM/mSt).** This type was characterized by having a large pair of homomorphic metacentric no. 1 (MM) and a pair of heteromorphic no. 6 chromosomes, which consisted of a small metacentric chromosome (m) and a large subtelocentric chromosome (St) (Figs. 2-II, 3b).

**Type III (MT/mm).** Heteromorphic chromosome no. 1 and homomorphic chromosome no. 6 comprised type III karyomorph. The first chromosome pair was composed of a large metacentric (M) and a large telocentric chromosome (T). And the latter chromosome pair consisted of two homomorphic small metacentric chromosomes (mm) (Figs. 2-III, 3c).

**Type IV (MT/mSt).** This type was also designated as translocation heterozygotes. It has two pairs of heteromorphic chromosomes (no. 1 and no. 6). Chromosome no. 1 is comprised of a large metacentric chromosome (M) and a large telocentric chromosome (T). Chromosome no. 6 consisted of a small metacentric chromosome (m) and a large subtelocentric chromosome (St) (Figs. 2-IV, 3d).

**Type V (MT/StSt).** Chromosome no. 1 was heteromorphic with a large metacentric chromosome (M) and a large telocentric chromosome (T). The no. 6 was homomorphic with two large subtelocentric chromosomes (StSt) (Figs. 2-V, 3e).

Figure 1. Map showing the sites of this and previous study around Sichuan Basin, China. The site names designated as in Table 1. doi:10.1371/journal.pone.0046163.g001
### Table 1. Synopsis of the karyomorph types.

| Localities                          | Number of animals | Number of | Number of | References      |
|-------------------------------------|-------------------|-----------|-----------|-----------------|
|                                     |                   | $\varphi$ | $\sigma$  |                 |
|                                     |                   | I         | II        | III            | IV      | V      | I       | II      | III     | IV      | V       |          |
| Hongkouxiang, Dujiangyan City, Sichuan (1) | 6                  | 5         | 1         | 5               | –       | –      | –       | –       | –       | –       | 1       | –       | –       | This study  |
| * Xuankouzhen, Wenchuan Co., Sichuan (2) | 8                  | 1         | 7         | –               | –       | –      | 1       | –       | 1       | –       | –       | 6       | –       | –       | This study  |
| *Mt. Qingcheng, Sichuan (3)         | 13                 | 3         | 10        | 2               | –       | –      | 1       | –       | 5       | –       | –       | 5       | –       | –       | This study  |
| Wushanxiang, Dayi Co., Sichuan (4)  | 21                 | 11        | 10        | 11              | –       | –      | –       | –       | 9       | –       | –       | 1       | –       | –       | This study  |
| Xilingzhen, Dayi Co., Sichuan (5)   | 10                 | 6         | 4         | 5               | –       | –      | 1       | –       | 4       | –       | –       | –       | –       | –       | This study  |
| * Xieyuanzhen-1, Dayi Co., Sichuan (6) | 7                  | 4         | 3         | 4               | –       | –      | –       | –       | 3       | –       | –       | –       | –       | –       | This study  |
| Hemingxiang-1, Dayi Co., Sichuan (7) | 3                  | 2         | 1         | 2               | –       | –      | –       | –       | 1       | –       | –       | –       | –       | –       | This study  |
| Hemingxiang-2, Dayi Co., Sichuan (8) | 10                 | 3         | 7         | 3               | –       | –      | –       | –       | –       | –       | –       | –       | 7       | –       | –       | This study  |
| * Jinxingxiang-1, Dayi Co., Sichuan (9) | 16                | 11        | 5         | 10              | –       | –      | 1       | –       | 2       | –       | –       | 3       | –       | –       | This study  |
| * Jinxingxiang-2, Dayi Co., Sichuan (10) | 55                | 14        | 41        | 9               | –       | –      | 5       | –       | 21      | –       | 1       | 19      | –       | –       | –       | This study  |
| Jinxingxiang-3, Dayi Co., Sichuan (11) | 21                | 7         | 14        | 7               | –       | –      | –       | –       | 14      | –       | –       | –       | –       | –       | This study  |
| *Gaotangsi, Dayi Co., Sichuan (12)  | 27                 | 8         | 19        | –               | –       | –      | –       | –       | 8       | –       | 1       | –       | –       | 18      | –       | –       | This study  |
| Xinchangzhen, Dayi Co., Sichuan (13) | 8                  | 4         | 4         | 4               | –       | –      | –       | –       | 4       | –       | –       | –       | –       | –       | This study  |
| Datongxiang, Qionglai Co., Sichuan (14) | 16               | 9         | 7         | 9               | –       | –      | –       | –       | 7       | –       | –       | –       | –       | –       | This study  |
| Shuikuizhen-2, Qionglai Co., Sichuan (15) | 4                 | 3         | 1         | 3               | –       | –      | –       | –       | –       | –       | 1       | –       | –       | –       | –       | This study  |
| Nanbaoxiang-1, Qionglai Co., Sichuan (16) | 11                | 9         | 2         | 9               | –       | –      | –       | –       | 2       | –       | –       | –       | –       | –       | This study  |
| Huojingzhen, Qionglai Co., Sichuan (17) | 15                | 6         | 9         | 6               | –       | –      | –       | –       | 9       | –       | –       | –       | –       | –       | This study  |
| Gaohanzhen-2, Qionglai Co., Sichuan (18) | 7                 | 2         | 5         | 2               | –       | –      | –       | –       | 5       | –       | –       | –       | –       | –       | This study  |
| Daoruoxiang-1, Qionglai Co., Sichuan (19) | 29                | 4         | 25        | 4               | –       | –      | –       | –       | 25      | –       | –       | –       | –       | –       | This study  |
| Daoruoxiang-2, Qionglai Co., Sichuan (20) | 18                | 4         | 14        | 4               | –       | –      | –       | –       | 14      | –       | –       | –       | –       | –       | This study  |
| Pinglezhen, Qionglai Co., Sichuan (21) | 2                  | –         | 2         | –               | –       | –      | –       | –       | 2       | –       | –       | –       | –       | –       | This study  |
| Mt. Tianlai-1, Qionglai Co., Sichuan (22) | 19                | 6         | 13        | 6               | –       | –      | –       | –       | 8       | 1       | –       | 4       | –       | –       | This study  |
| Mt. Tianlai-2, Qionglai Co., Sichuan (23) | 9                  | 1         | 8         | 1               | –       | –      | –       | –       | 8       | –       | –       | –       | –       | –       | This study  |
| Bifeng Valley, Yaan City, Sichuan (24) | 11                 | 6         | 5         | 6               | –       | –      | –       | –       | 5       | –       | –       | –       | –       | –       | This study  |
| Huotouzhen, Jiajiang Co., Sichuan (25) | 21                 | 3         | 18        | 3               | –       | –      | –       | –       | 18      | –       | –       | –       | –       | –       | This study  |
| Mt. Emei, Sichuan (26)              | 14                 | 7         | 7         | 7               | –       | –      | –       | –       | 7       | –       | –       | –       | –       | –       | This study  |
| Shuangfuzhen, Emeishan City, Sichuan (27) | 26                | 8         | 18        | 8               | –       | –      | –       | –       | 18      | –       | –       | –       | –       | –       | This study  |
| Luomuzhen, Emeishan City, Sichuan (28) | 17                 | 3         | 18        | 3               | –       | –      | –       | –       | 18      | –       | –       | –       | –       | –       | This study  |
| Xinglongzhen, Youyang Co., Chongqing (29) | 4                  | 2         | 2         | 2               | –       | –      | –       | –       | 2       | –       | –       | –       | –       | –       | This study  |
| Kuankouzhen, Suyang, Guizhou (30)   | 14                 | 7         | 7         | 7               | –       | –      | –       | –       | 7       | –       | –       | –       | –       | –       | This study  |
| Leigongshan, Guizhou (31)           | 4                  | 2         | 2         | 2               | –       | –      | –       | –       | 2       | –       | –       | –       | –       | –       | This study  |
| Xuefengshan, Hunan (32)             | 14                 | 7         | 7         | 7               | –       | –      | –       | –       | 7       | –       | –       | –       | –       | –       | This study  |
| Hejiapingzhen, Changyang Co., Hubei (33) | 11                | 5         | 6         | 5               | –       | –      | –       | –       | 6       | –       | –       | –       | –       | –       | This study  |
| **Total**                           | 471               | 173       | 298       | 156             | –       | –      | 17      | –       | 230     | 3       | 2      | 59      | 4       | –       | –       | This study  |
| Localities | Number of animals | Number of \( \sigma/\varphi \) | \( \sigma \) | \( \varphi \) | \( \sigma \) | \( \varphi \) | \( \sigma \) | \( \varphi \) | \( \sigma \) | \( \varphi \) | \( \sigma \) | \( \varphi \) | \( \sigma \) | \( \varphi \) | References |
|------------|------------------|------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| Xuankouzhen, Wenchuan Co., Sichuan | 4 | 3 | 1 | 3 | – | – | – | – | – | – | 1 | – | Wang 2006 |
| Mt. Emei & Leshang City, Sichuan | 8 | 4 | 4 | 4 | – | – | – | – | 4 | – | – | – | Chen et al. 1983 |
| Mt. Emei, Sichuan | 10 | 5 | 5 | 5 | – | – | – | – | 5 | – | – | – | Wang et al. 1983 |
| Mt. Emei, Sichuan | 3 | 1 | 1 | 1 | – | – | – | – | 2 | – | – | – | Wang 2006 |
| Shuicheng Co., Guizhou | 8 | 5 | 3 | 5 | – | – | – | – | 3 | – | – | – | Zhang et al. 1997 |
| Zunyi Co., Guizhou | ? | ? | ? | ? | – | – | – | – | ? | – | – | – | Li and Hu 1999 |
| Pingbian Co., Yunnan | 3 | 1 | 2 | 1 | – | – | – | – | 2 | – | – | – | Li and Hu 1996 |
| Pingbian Co., Yunnan | ? | ? | ? | ? | – | – | – | – | ? | – | – | – | Hu 2004 |

Roman numerals represent different karyomorphs with chromosome no.1/no.6: I, MA/mm; II, MM/mm; III, MT/mm; IV, MT/mSt; V, MT/StSt (M = large metacentric chromosome; m = small metacentric chromosome; St = large subtelocentric chromosome; T = large telocentric chromosome).

* Populations with heteromorphic chromosomes in both males and females.

** Specimens from Mt. Qingchengshan reported by Wang (2006) are actually from Xuankouzhen (personal communication).

*** The numbers of experimental animal are not shown in the references. Numbers in parentheses stand for the collecting localities.

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Karyomorphs Type I (MM/mm) and Type IV (MT/mSt) are the most (82.0%) and second most (16.1%) common karyomorphs in *Quasipaa boulengeri*. The other three karyomorphs only made up about 1.8% of the karyomorphs (Table 3).

5S rDNA FISH Karyotypes

In all five karyomorphs, 5S rDNA sites were located on no.1 homologues regardless the karyotypic morphology (Figs. 2, 4).

**Type I.** 5S rDNA were detected near the centromeric region of the largest homomorphic chromosome pair (no. 1, MM) (Figs. 2-I, 4a). No signal was found on other homomorphic chromosomes.

**Type II.** Even with one pair of heteromorphic chromosome no. 6, 5S rDNA was only found in the centromeric region of chromosome no. 1 (MM) (Figs. 2-II, 4b).

**Type III.** 5S rDNA sites were distributed in the centromeric region of a large metacentric chromosome (M) and a large telocentric chromosome (T) (Figs. 2-III, 4c). Besides the chromosome size, the location of 5S rDNA provided further proof that these two heteromorphic chromosomes were homologous pairs composed of chromosome no. 1 (MT).

**Type IV.** In this karyomorph, 5S rDNA signals were also observed close to the centromere of a pair of heteromorphic chromosomes no. 1 (MT) like in karyotype III (Figs. 2-IV, 4d).

**Type V.** 5S rDNA positions in this karyotype were at exactly the same location of no. 1 like type III and type IV (Figs. 2-V, 4e).

In conclusion, combined with the chromosome size, 5S rDNA can be used as a good marker to identify the homologous chromosomes. We re-collected specimens from Xuankouzhen (1♂, 7♀), and the same heteromorphic chromosomal pairs, as reported by Wang (2006) [14], were found in one male and six females (Table 1). Subsequent analysis of four other populations has shown a similar situation to Xuankouzhen. In Gaotangsi (8♂ of 27, 18♀ of 27) and Jinxingxiang-2 (5♂ of 55, 19♀ of 55) populations, the male and female rates involving heteromorphic chromosomes were both higher (Table 1). Obviously, the heteromorphic chromosomes are not related to sex heteromorphism and do demonstrate autosomal heteromorphic variation.

**Phylogenetic analysis**

The results of the separate analyses based on different data sets were mostly mutually compatible. The alignment for the COI fragment was straightforward. But the alignment of several loop regions of the rRNA genes was ambiguous, and therefore, 68 and 7 sites with questionable homology of the rRNA + COI and COI + rRNA alignments were excluded from the rest of the analysis, respectively.

The rRNA+COI data set had 29 haplotypes and 1456 nucleotide sites (Table S1; Fig. 5A). In both ML and Bayesian analysis, all *Quasipaa boulengeri* samples formed a well supported clade with *Q. robertingeri* which was resolved as a synonym of the former [15]. Furthermore, the specimens possessing heteromorphic and homomorphic karyomorphs from Western Sichuan Basin belonged to a strongly support clade with extremely low sequence diversity. The COI+rRNA data set had 22 haplotypes and 1517 sites, and the Bayesian analyses with different model parameters produced an identical topology and similar posterior probabilities.
Figure 3. Mitotic metaphase and five different karyomorphs. Left row: mitotic metaphase; middle row: karyotypes; right row: idiograms of no.1 and no. 6. (a) Karyotype I obtained homomorphic biarmed chromosomes no. 1 (M/M) and no. 6 (m/m), the metaphase spread was taken from one male of Mt. Omei population; (b) Karyotype II had homomorphic biarmed chromosomes no. 1 (M/M), heteromorphic biarmed chromosomes no. 6 (m/Sm), the metaphase spread was taken from one female of Mt. Omei population; (c) Karyotype III was found to possess heteromorphic biarmed chromosomes no. 1 (M/T), homomorphic biarmed chromosomes no. 6 (m/m), the metaphase spread was taken from one female of Jinxingxiang-2 population; (d) Karyotype IV had heteromorphic biarmed chromosomes no. 1 (M/T), no. 6 (m/St), the metaphase spread was taken from one male of Gaotangsi population; (e) Karyotype V was characterised with heteromorphic biarmed chromosomes no. 1 (M/T), homomorphic biarmed chromosomes no. 6 (St/St), the metaphase spread was from one female of Mt. TianTai-1 population. Long arrows indicated chromosomes no. 1; short arrows indicated chromosomes no. 6. Scale bar = 10 μm.

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Similarly, on both ML and Bayesian trees, samples from Western Sichuan Basin belonged to a highly supported clade with extremely low diversity (Table S1; Fig. 5B).

Discussion

Evidence for a reciprocal translocation

Reciprocal translocations can be identified using relative length and centromeric indices of the chromosomes [14,16]. We measured and compared the chromosome arm lengths of heteroand homomorphic chromosomes no.1 and no.6 which were chosen from the karyotypes of different individuals in several populations (Table 4), our results coincided with Wang’s which were based on only one individual from a single population [14].

It is important to know how the four homologues of the two pairs of translocated chromosomes match in a pair. Wang (2006) analyzed R-bands of the heteromorphic karyotypes and showed that the largest metacentric (M) and the largest telocentric chromosome (T) are a homologous pair composing the no. 1 chromosomes, while the other two heteromorphic chromosomes (m and St) are homologous chromosomes no. 6 [14]. Usually, such multiple bandings as R-bands are not definitive and can result in ambiguous assignments [17,18]. In the present study, 5S rDNA FISH clearly demonstrates that a reciprocal translocation exists between heteromorphic chromosomes no. 1 and no. 6. By comparing the five hetero- and homomorphic karyotypes in different populations of Quasipaa boulengeri, 5S rDNA markers were separately located on the long arm of the largest metacentric as well as the largest telocentric chromosome, which provides strong evidence that these two heterochromosomes exactly match homologous chromosome pair of no. 1 (Fig. 4).

Translocation polymorphisms

The polymorphic karyotypes within and between different populations of Quasipaa boulengeri, are likely caused by a mutual translocation involving alternate and adjacent segregation in meiosis. A translocation heterozygote (type IV, MT/mSt) is expected to form a quadrivalent during meiosis. Three segregation modes (alternate, adjacent-1, and adjacent-2) could produce six types of gametes [19], i.e. M/m and T/St for alternate, M/St and m/T for adjacent-1, M/T and m/St for adjacent-2 modes (Fig. 6). If all these gametes are functional, 19 karyologically different offspring are expected to be observed in populations of this species. In the present study, however, based on the analysis of 471 individuals from 33 populations (Table 1), we have only observed five karyotypes (i.e. type I, II, III, IV, and V). They represent, respectively, MM/mm (I), MM/mSt (II), MT/mm (III), MT/mSt (IV), and MT/StSt (V) chromosomal pairs. These combinations can be attributed to the formation of only four possible gametes, i.e. M/St, m/T, M/m, and T/St (Fig. 6), which are associated with alternate and adjacent-1 meiotic segregation modes, and suggest that there is no adjacent-2 segregation from these translocations in this species.

The possible explanation for higher frequencies of type I (81.8%) and IV (16.3%) karyomorphs is that alternate segregation predominates over adjacent-1 segregation. Alternate segregation can produce genetically normal [M/m] and complementary [T/St] gametes, which are genetically balanced without any duplications or deficiencies. When these gametes fuse with each other, three karyomorphic progenies would be expected: normal individuals (type I, MM/mm); individuals that are translocation heterozygotes (type IV, MT/mSt); and individuals that are translocation homozygotes (type IX, TT/StSt) (Figs. 6, 7). This is in accordance with our observations in most populations of Quasipaa boulengeri although translocation homozygotes (type IX) were not found in any population (Tables 1, 3). Similar cases with high alternate frequencies have been previously found in plants and animals, such as Clarkia speciosa [20], Isotoma petraea [21], Rye grass [22,23], Kalotermes approximates [24] and Periplaneta americana [25], and especially in the genus Oenothera where segregation is always in the alternate mode [19,26].

Table 2. Constitution of the five karyomorphs.

| no. | Type | 1–1 | 1–2 | 2 | 3 | 4 | 5 | 6–1 | 6–2 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|-----|------|-----|-----|---|---|---|---|-----|-----|---|---|---|---|---|---|---|
| I   | M    | M   | Sm  | Sm | M | M | m | m   | m   | sm|m | m | m | m | m |
| II  | M    | M   | Sm  | Sm | M | M | m | St  | m   | m | m | m | m | m |
| III | M    | T   | Sm  | Sm | M | M | m | m   | sm | m | m | m | m | m |
| IV  | M    | T   | Sm  | Sm | M | M | m | St  | m   | sm|m | m | m | m | m |
| V   | M    | T   | Sm  | Sm | M | M | St | St  | sm | m | m | m | m | m | m |

Roman numerals (I–V) represent five karyomorph types. The bold numbers and letters indicate the homologues of no. 1 and no. 6 and their centromere position, respectively. M = large metacentric chromosome; m = small metacentric chromosome; Sm = large submetacentric chromosome; St = large subtelocentric chromosome; T = large telocentric chromosome. Variations exist in the types of chromosome no. 1 and no. 6.

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Table 3. Karyotype frequencies in each and pooled populations.

| Localities | I | II | III | IV | V |
|------------|---|----|-----|----|---|
| Hongkouxiang, Duijiangyan City, Sichuan (1) | 83.3% | – | 16.7% | – | – |
| *Xuankouzhen, Wenchuan Co., Sichuan (2) | 12.5% | – | – | 87.5% | – |
| Mt. Qingcheng, Sichuan (3) | 53.8% | – | – | 46.2% | – |
| Wushanxiang, Dayi Co., Sichuan (4) | 95.2% | – | 4.8% | – | – |
| Xilingzhen, Dayi Co., Sichuan (5) | 90.0% | – | 10.0% | – | – |
| Xieyuanzhen-1, Dayi Co., Sichuan (6) | 100.0% | – | – | – | – |
| Hemeixiang-1, Dayi Co., Sichuan (7) | 100.0% | – | – | – | – |
| *Hemingsiang-2, Dayi Co., Sichuan (8) | 30.0% | – | – | 70.0% | – |
| Jinxingiang-1, Dayi Co., Sichuan (9) | 75.0% | – | – | 25.0% | – |
| Jinxingiang-2, Dayi Co., Sichuan (10) | 54.5% | – | 1.8% | 43.6% | – |
| Jinxingiang-3, Dayi Co., Sichuan (11) | 100.0% | – | – | – | – |
| *Gaotangsi, Dayi Co., Sichuan (12) | 3.7% | – | – | 96.3% | – |
| Xinchangzhen, Dayi Co., Sichuan (13) | 100.0% | – | – | – | – |
| Datongxiang, Qionglai Co., Sichuan (14) | 100.0% | – | – | – | – |
| Shuikouxiang-2, Qionglai Co., Sichuan (15) | 100.0% | – | – | – | – |
| Nanbaoxiang-1, Qionglai Co., Sichuan (16) | 100.0% | – | – | – | – |
| Huojingzhen, Qionglai Co., Sichuan (17) | 100.0% | – | – | – | – |
| Gaohezhen-2, Qionglai Co., Sichuan (18) | 100.0% | – | – | – | – |
| Dazouxiang-1, Qionglai Co., Sichuan (19) | 100.0% | – | – | – | – |
| Dazouxiang-2, Qionglai Co., Sichuan (20) | 100.0% | – | – | – | – |
| Pinglezhen, Qionglai Co., Sichuan (21) | – | 100.00% | – | – | – |
| Mt. Tianzai-1, Qionglai Co., Sichuan (22) | 73.7% | 5.3% | – | – | 21.1% |
| Mt. Tianzai-2, Qionglai Co., Sichuan (23) | 100.0% | – | – | – | – |
| Bifeng Valley, Yaan City, Sichuan (24) | 100.0% | – | – | – | – |
| Huatouzhen, Jiajiang Co., Sichuan (25) | 100.0% | – | – | – | – |
| Mt. Omei, Sichuan (26) | 100.0% | – | – | – | – |
| Shuangfuzhen, Omeishan City, Sichuan (27) | 100.0% | – | – | – | – |
| Luomuzhen, Omeishan City, Sichuan (28) | 100.0% | – | – | – | – |
| Xinglongzhen, Youyang Co., Chongqing (29) | 100.0% | – | – | – | – |

Table 3. Cont.

| Localities | I | II | III | IV | V |
|------------|---|----|-----|----|---|
| Kuankuojiu, Sulyang, GuiZhou (30) | 100.0% | – | – | – | – |
| Leigongshan, GuiZhou (31) | 100.0% | – | – | – | – |
| Xuefengshan, Hunan (32) | 100.0% | – | – | – | – |
| Hejiapengzhen, Changyang Co., Hubei (33) | 100.0% | – | – | – | – |
| Total | 82.0% | 0.6% | 0.4% | 16.1% | 0.8% |

Locality names and numbers are consisted with those in Table 1; Roman numerals (I~IX) represent different karyotypes; * Populations with more than 50% type IV.

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The frequency of the adjacent-1 segregation mode was found to be extremely low in this species. Karyotype II (MM/mSt), III (MT/mm), and V (MT/StSt), involving gametes M/St and m/T yielded by adjacent-1 mode, were found in few individuals in few populations (Tables 1, 3). Theoretically, the gametes derived from the adjacent-1 mode are genetically unbalanced for duplications or deficiencies, which could lead to lethality and infertility. This is, however, not always true. Occurrences of adjacent-1 mode have been found in plants and in invertebrates. It appears at a low frequency in Hordeum vulgare, and Cochliomyia hominivorax [27,28]. This mode may even predominate as observed in Allium atropurpureum, A. consanguinum, Chorthippus brunneus, Gomphocerus sibiricus [29–32]. If gametes from both the adjacent-1 and alternate segregation fuse with each other in Quasipaa boulengeri (Fig. 6, 7). Only three (Type II, III, and V) were found, which suggests that the adjacent-1 mode is uncommon or genetically detrimental in Q. boulengeri. Adjacent-2 segregation must not be viable in Q. boulengeri. Gametes resulting from the adjacent-2 mode would possess more duplications and/or deficiencies of chromosome segments. Some authors advocate that the adjacent disjunction that results in the movement of homologous centromeres to the same pole is extremely infrequent [31,33,34]. This may indicate that adjacent-2 orientations rarely occur during the meiosis. It should be also noticed that the meiotic pairing between some segments (Fig. 6) which can frequently convert the quadrivalent to a chain (rather than a ring) and make adjacent-2 segregations very unlikely [35].

Absence of anticipated karyotypes

Theoretically, the union of the four occurring gametes (M/St, m/T, M/m, and T/St) produced by alternate and adjacent-1 segregation can form nine kinds of karyologically different progeny, i.e. type I (MM/mm), type II (MM/mSt), type III (MT/mm), type IV (MT/mSt), V (MT/StSt), VI (MM/mSt), VII (TT/mm), VIII (TT/mSt) and IX (TT/StSt) (Figs. 6, 7). Four anticipated karyotypes (VI, VII, VIII, and IX) were not found in any population. The absence of type IX is particularly confusing. Type IX (i.e. translocation homozygotes), would result from the fusion of translocated gametes (T/St), are genetically balanced without any duplication or deficiency, and can theoretically survive. Verified
cases of homozygously fixed reciprocal translocations have been reported to occur in both plants and animals, such as Lewis rats [36], mice [37], different species of Secale [38,39] and species in the genus Datura [40]. But, translocation homozygotes with lethal effects have also been observed in Caenorhabditis elegans [41,42], and some species of Drosophila [43–45]. Some authors predicted that lethality of the translocation homozygosity (induced following "X-rays") found in Drosophila may have one of two causes: recessive lethal mutations at the points where chromosomes broke and the position effect of the rearrangement [43,45]. These two assumptions may explain the absence of type IX, together with type VII and VIII in Quasspia boulengeri (Fig. 8A, B). In addition, it should be also noticed that either the occurring of adjacent-segregation can reduce the chances of segregating a balanced translocation into a gamete, or the translocation homozygotes were not found, just by chance. The absence of type VI, together with the rarity of types II, III, and V are possibly caused by the genetic duplication or deficiency (Fig. 7). These two mechanisms can cause the reduction of the viability of the individuals and this is probably why only a very few or totally no frogs with these karyomorphs were found.

**Origin of chromosomal polymorphisms**

Why do translocation polymorphisms tend to be common in the species Quasspia boulengeri? Interchanges are rare in nature in the heterozygous condition, and most translocations that occur in vertebrates are generally observed in single individuals [1,3]. However in present study, there is a high frequency of the translocation heterozygotes (type IV) within and between different populations of *Q. boulengeri*. The reciprocal translocations can probably become evolutionarily fixed only when the interchanged regions are so minute that genetic unbalance involved in aneuploidy is not deleterious or the chromosomes regularly undergo alternate segregation. The latter is only likely to be the case when the chromosomes have interchanged virtually entire chromosome arms and then have distally localized chiasmata where it can be terminalized without difficulties [1]. Such as in the black flies, i.e. the genus Prosimulium and Tsiminnia, and midges, i.e. the genus Chironomus, whole-arm translocation have been evolutionarily fixed in various species [46]. Sometimes, similar translocation polymorphisms do become fixed in a species, such as the bird, Megalaima zeylanica caniceps. In that species, a translocation occurred involving the exchange of a large segment of chromosome no. 1 and a small terminal region of a microchromosome and a chromosome chain formed during meiosis. In a limited sample of 11 individuals, five normal individuals (2♂,3♀), five translocation heterozygotes (3♂, 2♀) and one translocation homozygote (1♀) were found [16]. Obviously, these three karyomorphs resulted from the union of gametes produced by alternate segregation and this segregation type may dominate in this bird. Similarly, in *Q. boulengeri*, the exchange involved almost the whole short arm of chromosome 1 and a very small chromosome segment from the long arm of chromosome 6 which is very similar to that of *Megalaima zeylanica caniceps* (Fig. 9). It is possible that a chain quadrivalent form and alternate segregation dominate in *Q. boulengeri* which would result in the high frequencies of type IV such as the populations from Xuankouzhen, Hemingxiang-2 and Gaotangsi (Table 3). The whole-arm translocation probably resulted in the maintenance of the translocation polymorphisms in a few populations of *Q. boulengeri*.
Western Sichuan Basin (heteromorphic and homomorphic karyomorphs) and Eastern Sichuan Basin (homomorphic karyomorphs)

Southern and Eastern Sichuan Basin (homomorphic karyomorphs)

Eastern Sichuan Basin (homomorphic karyomorphs)

Western Sichuan Basin (heteromorphic and homomorphic karyomorphs) and Eastern Sichuan Basin (homomorphic karyomorphs)

Southern and Eastern Sichuan Basin (homomorphic karyomorphs)
How does the translocation disperse among natural populations in *Quasipaa boulengeri*? The phylogenetic inference proved that all populations of *Q. boulengeri* grouped into a monophyletic clade with high support value, sister to *Q. spinosa* (COI+rRNA) or a clade containing *Q. spinosa* and *Q. cf. boulengeri* (rRNA+COI). In fact, the populations possessing translocated karyomorphs and those without any heteromorphic karyotypes did not diverge significantly from each other in DNA sequences sampled (Figs. 5A, B). We believe that the mutual translocation independently evolved just once in this species. The first step is possibly that a translocation rearrangement between chromosome no.1 and no.6 randomly occurred in a single individual, then this translocation heterozygote (type IV, MT/mSt) individual mated with a normal one (type I, MM/mM) and produced more translocation heterozygote animals in the population. When the translocation heterozygotes mate with each other, all five karyomorphs can be produced in the same population (Fig. 10). Theoretically, an individual possessing each of karyotypes can disperse to a different population. Supposing an individual of type II (MM/mSt) moves into another population with type I individuals (normal), obtaining possible gametes of M/m and M/St during meiosis. Only hybrid offspring of type I and II will be produced after several generations. Similarly, if a type III migrates, two types of I and III will be produced in the future population. When each of these individuals disperses, the populations with two fixed types are anticipated to appear in the species. But this is not true in our data, which might suggest that the frequencies of both two types are so low that the opportunity for the dispersal of these types would be severely reduced. Significantly, if an individual with type IV (MT/mSt) or type V (MT/StSt) spreads, and mates with normal individual with gametes of M/m (type I), type IV must yield in F1 progenies (Fig. 10). Further, whenever type IV shows up in the population, the occurrences of all the five types of I, II, III, IV, and V are excepted except VI, VII, VIII, and IX (death) in next progenies. Thus, it seems that the dispersal of each of both type IV and V is probably the mechanism for the translocation variations among populations. Virtually, for far lower frequency of the type V, the more reasonable explanation is that just the type IV is dispersing in various populations in the species.

### Materials and Methods

#### Animals

Four hundred and seventy two adult frogs from 33 populations were used for karyological investigation in present study. Frogs were collected from 2006 to 2011 and consisted of 173 males and 298 females. The sampling localities, numbers of frogs and sexual ratios are provided in Table 1 and Fig. 1. All the specimens were obtained in the breeding season so that male specimens could be used to confirm identity of the species. Males and females were distinguished from each other both by morphology and by dissection to observe gonads. All animal work in this paper has been conducted according to relevant national and international guidelines. All animal care and experimental procedures were approved by the Chengdu Institute of Biology Animal Care and Use Committee.

#### Mitotic Chromosome preparation

Mitotic metaphases were prepared from bone marrow technique described by Schmid et al. (2010) [5]. Chromosome preparations were examined using a Leica DMRA2 microscope.

Individuals with karyotype I (6q, 4q from five populations) and karyotype IV (5q, 5q from four populations) were chosen to measure the chromosome relative length and arm ratio, respectively (Tables 2, 5). The chromosomes were described following the nomenclature for centromeric position on chromosomes defined by Leven et al. (1964) [47]. Idiograms were also made using these measurements (Fig. 2). T tests were performed between the sum of the length of the four heteromorphic chromosomes from chromosome no. 1 and no. 6 designed as $Z_1+Z_2$ by Wang (2006) [14] and the sum of the length of the two normal homologues designed as $(Z_1+Z_2)/2$ (Table 4). Other slides were stored at room temperature for 2–4 days, dehydrated in ethanol series(70%, 90%, 100%, 3 min each)and stored at −20°C until used for fluorescence in situ hybridization.

### Table 4. The lengths of four heterorphic chromosomes in ten cells(μm).

| Chrom. No| Cell no. | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | Mean |
|-----------|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| 1–1       | 5.38    | 9.07| 7.60| 9.58| 7.19| 6.02| 5.02| 5.71| 4.70| 4.99|     |
| 1–2       | 3.13    | 5.82| 4.45| 6.46| 4.25| 3.60| 3.40| 3.91| 3.17| 3.46|     |
| 6–1       | 2.24    | 3.00| 3.03| 3.38| 2.81| 2.39| 2.06| 2.05| 2.00| 2.15|     |
| 6–2       | 3.36    | 6.48| 5.08| 6.59| 4.98| 4.30| 3.63| 3.49| 3.25| 4.02|     |
| (1–1)+(1–2)+(1–1)+(1–2) | 14.11 | 24.37| 20.17| 26.01| 19.23| 16.31| 14.12| 15.16| 13.11| 14.62| 17.72 |
| [(1–1)+(6–1)]×2 | 15.25 | 24.14| 21.26| 25.92| 20.00| 16.80| 14.16| 15.52| 13.39| 14.28| 18.07 |

The ten cells are respectively from ten individuals (5q, 5q') with karyotype IV from different populations. The T test is made between the sum of the length of the four heteromorphic chromosomes from chromosome no. 1 and no. 6 designed as $(Z_1+Z_2)/2$, p > 0.05. "1–1" and "1–2" refer to the length of two homologues of chromosome no. 1, respectively. "6–1" and "6–2" represent the length of two homologues of chromosome no.6, respectively.

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Figure 6. Hypothetical segregation of reciprocal translocation involving chromosome 1 and 6 during meiosis. Alternate segregation results in production of normal or balanced chromosomes. The separation of homologous centromeres (adjacent-1) or nonhomologous centromeres (adjacent-2) results in production of gametes with unbalanced chromosome. Horizontal lines represent the breakpoints.
doi:10.1371/journal.pone.0046163.g006

Figure 7. Diagrammatic representation of the nine expected karyomorphs resulting from the combination of four gametes produced by alternate and adjacent-1 segregations. Progenies with karyotypes in the grey box are absent (type VI, VII, VIII, IX) while those in white box are viable (type I, II, III, IV, V). For both gametes and progenies, only no. 1 and no. 6 chromosomes are given.
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5S rDNA probe construction and fluorescence in situ hybridization (FISH)

Genomic DNA was extracted from liver or muscle tissues by standard Proteinase K method [48]. 5S rDNA was amplified using forward 5'-GCCTACGGGCACACCAC-3' and reverse 5'-AAGCCTACGACACCTGGTATTC-3' primers. Probes were labeled by PCR with biotin-16-Dutp (Roche) or Digoxigenin-11-dUTP (Roche) following the procedure described by Bi et al. (2009) [49] with small modification. The PCR program included 3 min initial denaturation, 30 cycles of 94\(^\circ\)C/50\(^\circ\)C/72\(^\circ\)C for 30 s/30 s/1 min followed with a 10 min extension at 72\(^\circ\)C.

Mitotic chromosomes were used for 5S rDNA FISH analysis following the procedure reported by Zhang et al (2007) [50] and Bi et al., (2009) [49] with a few modifications. The PCR program included 3 min initial denaturation, 30 cycles of 94\(^\circ\)C/50\(^\circ\)C/72\(^\circ\)C for 30 s/30 s/1 min followed with a 10 min extension at 72\(^\circ\)C.

Mitotic chromosomes were used for 5S rDNA FISH analysis following the procedure reported by Zhang et al (2007) [50] and Bi et al., (2009) [49] with a few modifications. The PCR program included 3 min initial denaturation, 30 cycles of 94\(^\circ\)C/50\(^\circ\)C/72\(^\circ\)C for 30 s/30 s/1 min followed with a 10 min extension at 72\(^\circ\)C.

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Phylogenetic analysis

A total of 133 specimens were sampled for molecular phylogenetic analysis, including 116 Quasipaa boulengeri individuals from 20 collecting sites and 17 individuals belonging to 13 species closely related to Q. boulengeri. Three fragments from the mitochondrial genome were selected for sequencing (Table S1). The first fragment is part of the COI gene, and was 628 bp in length. The other two fragments are part of the 12S and part of the 16S genes, and were approximately 362 bp and 534 bp before alignment, respectively. The COI fragment of 96 specimens and both the 12S and 16S fragments of 52 specimens were sequenced in this study. Other sequences were obtained from previous studies [15,51,52]. Details of the sampling and polymerase chain reaction primers are presented in Tables S1 and S2, respectively. Sequence alignment was conducted with ClustalX version 1.83 [53] and checked by eye. The amino acid sequences for coding regions and the rRNA secondary structures of Xenopus laevis [54] were used for checking.

For phylogenetic reconstruction, the three fragments were combined, and two data sets were analyzed separately. The rRNA+COI data set covered a relatively wider phylogenetic range, and all haplotypes in this data set contained the 12S and 16S sequences. The COI+rRNA data set was a more comprehensive sampling of Quasipaa boulengeri, especially the populations found in Western Sichuan Basin which possessed both heteromorphic and homomorphic karyotypes. All haplotypes in this data

Figure 8. Diagram illustrates the possible mechanisms for parts of the absent karyomorphs. A. “recessive lethal mutation” mechanism, if one gene “a” locates at the translocation breakpoint (arrow indicated) and when the translocation occurs, “a” mutates to a recessive lethal gene “a”’, and the progenies with genotype “aa” will die while with “aa” will be viable. B. “position effect” mechanism. The breakpoint locates between two genes “a” and “b” on chromosome 1. When the translocation occurs, “b” will translocate to chromosome 6 and the relative position of “a” and “b” will be changed, then neither “a” nor “b” can function effectively and give rise to the death of progenies with either separated gene “a” or “b”. Once a progeny obtain a chromosome with normal gene order of “a” and “b”, it will be viable. doi:10.1371/journal.pone.0046163.g008

Figure 9. Diagrammatic representation of a reciprocal translocation occurring between one member of the chromosome pair 1 (AB) and pair 6 (CD) giving rise to two new chromosomes (BD and AC). doi:10.1371/journal.pone.0046163.g009
set contained the COI fragment sequence. Three and one species were selected as outgroups for the rRNA+COI and COI+rRNA data sets based on the current understanding of their phylogenetic relationships, respectively [15]. A three-partition strategy was applied to partition both data sets. It defined each of the 12S, 16S, and COI genes as a separate partition. Both maximum likelihood (ML) and Bayesian approaches were conducted on these data sets. The Bayesian information criterion (BIC) and corrected Akaike information criterion (AICc) implemented in jModelTest version 0.1.1 [55] were used to select and evolutionary model that best fit each data partition [56,57]. Models selected are provided in Table S3.

The ML analysis was conducted using RAxML version 7.2.6 [58]. This program applies one substitution model (GTR+G or GTR+I+G) to all DNA data partitions. As the selected models are relatively simple (Table S3), the GTR+G model was used. The rapid hill-climbing algorithm [59] was used and 200 inferences were executed. To estimate nodal support, nonparametric bootstrap proportions [60] with 1000 replicates were used. The Bayesian analysis was conducted using MrBayes version 3.1.2 [61]. For the rRNA+COI data set, a same set of model parameters were chosen by both BIC and AICc. While for the COI+rRNA data, different sets of parameters were chosen by the two criterions. Consequently, the Bayesian analysis was conducted on the COI+rRNA data with parameters chosen by BIC or AICc, separately, and the results were compared [57]. For the rRNA+COI data set, a same set of model parameters were chosen by both BIC and AICc. While for the COI+rRNA data, different sets of parameters were chosen by the two criterions. Consequently, the Bayesian analysis was conducted on the COI+rRNA data with parameters chosen by BIC or AICc, separately, and the results were compared [57]. Four Markov chains were used and the data was run for 5 million generations to allow adequate time for convergence. Trees were sampled every 500 generations and the last 5000 sample trees were used to estimate the consensus tree and the Bayesian posterior probabilities.

Supporting Information

Table S1 Sampling information for molecular phylogenetic analysis. For some individuals, not all the three fragments were sequenced, causing one sequence be categorized into more than one haplotype. * All rRNA+COI haplotypes contained the 12S and 16S sequences, and all COI+rRNA haplotypes contained the COI sequence.

(DOC)

Table S2 Primers used in PCR and sequencing of Quasipaa boulengeri in this study.

(DOC)

Table S3 Models selected for data partitions by the Bayesian information criterion (BIC) and corrected Akaike information criterion (AICc) * All rRNA+COI haplotypes contained the 12S and 16S sequences, and all COI+rRNA haplotypes contained the COI sequence.

(DOC)
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Author Contributions

Conceived and designed the experiments: LQ YX YZ XZ. Performed the experiments: LQ. Analyzed the data: LQ ZY YX XZ. Contributed reagents/materials/analysis tools: LQ. Wrote the paper: LQ YZ XZ.

References

1. White MJ (1979) Animal cytology and evolution London: Cambridge University Press. 933 p.
2. Futuyma D (1997) Evolutionary Biology. Sinauer Associates: Sunderland, Massachusetts. 763 p.
3. Chiarelli A, Capanna E (1973) Cytotoxicity and vertebrate evolution. London: New York: Academic Press. 780 p.
4. Schmid M, Steinlein C, Hahl T (2004) Chromosome banding in Amphibia. Karyotype aberrations in cultured fibroblast cells. Cytogenetic and Genome Research 104: 277–282.
5. Schmid M, Steinlein C, Bogart JP, Feichtinger W, Loim P, et al. (2010) The chromosomes of terraraman frogs. Insights into vertebrate cytogenetics. Cytogenetic and Genome Research 130-131: 1–568.
6. Siqueira Jr S, Annaia F, Recco-Pimentel S (2004) Cytogenetics of three Brazilian species of Eleutherodactylus (Amura, Leptodactylidae) with 22 chromosomes and re-analysis of multiple translocations in E. bananicense. Genetics and Molecular Biology 27: 363–372.
7. Fei L, Hu SQ, Ye CY, Huang YZ (2009) Fauna Sinica. Amphibia Vol. 3 Anura Ranidae. Beijing: Science Press. 957 p.
8. Chen W, Wang ZS, Wang YZ, Yang YH, Sun QL (1983) A Comparative Study of the Karyotypes From six Species of Frogs in Sichuan. Zoological Research 4: 83–88.
9. Wang ZS, Wang XZ, Chen WY (1983) A comparative study on constitutive heterochromatin and nucleus-organization regions (NORs) of three species of the genus Rana. Acta Herpetologica Sinica 2: 1–6.
10. Li SS, Hu JS (1999) The karyotype evolution and infraspecies variation of geographical population of anuran genus of Ps from China. China Zoological Society Zoological Studies in China. Beijing: Chinese Forestry Publishing House. 976-982.
11. Li SS, Hu JS (1996) The study on the karyotypes, C-bandning and AgNORs of four Ps species in China. Zoological Research 17: 86–88.
12. Zhang JY, Gu XM (1997) A study on karyotype and Ag-NORs of Ps bufofeeding from Shanshiang of Guizhou. Journal Guizhou Normal University (Natural Science) 15: 48–51.
13. Hu JS (2004) Molecular phylogenetic studies on Spinosa group (genus Ps) in China (Amphiban, Anura, Ranidae). Kungming: Yunnan University. 97 p.
14. Wang D (2006) Chromosome Research on Ps bufoventing and Ps yunnanensis (Ranidae: Ps). Chenghong: Sichuan University. 48 p.
15. Che J, Hu J, Zhou W, Murphy RW, Papadus JT, et al. (2009) Phylogeny of the Asian spiny frog tribe Paini (Family Dicroglossidae) sensu Dubois. Molecular Phylogenetics and Evolution 50: 59–73.
16. Kaul D, Asriel A (1987) Chromosomal polymorphism in a natural population of the northern green harbet, Megalatina zeylanica caniceps (Franklin)(Piciformes: Aves). Genetic 54: 241–245.
17. Nai WH, Liu RQ, Chen YZ, Wang JH (1999) A Study of Chromosome Translocation of Francois’ Monkey by Fluorescence in situ Hybridization (FISH). Hereditas (Beijing) 32: 1–3.
18. Kingston HM (2002) ABC of Clinical Genetics. London: BMJ Books. 120 p.
19. Hartl DL, Jones EW (1998) Genetics: principles and analysis. Sudbury, Massachusetts: Jones and Bartlett Publishers. 1367 p.
20. Lawrence C (1963) The orientation of multiple associations resulting from the effect of selection on the disjunction frequency of interchange associations. Heredity 25: 53–78.
21. Hartl DL, Jones EW (1998) Genetics: principles and analysis. Sudbury, Massachusetts: Jones and Bartlett Publishers. 1367 p.
22. Lawrence C (1963) The orientation of multiple associations resulting from the effect of selection on the disjunction frequency of interchange associations. Heredity 25: 53–78.
23. Lawrence C (1963) The orientation of multiple associations resulting from the effect of selection on the disjunction frequency of interchange associations. Heredity 25: 53–78.
24. Syren RM, Luykx P (1981) Geographic variation of sex-linked translocation rates of chromosomal rearrangement in Pectinidae (Bivalvia: Pteriomorphia) implied based on chromosomal localization of histone H3 gene in four scallops. Genetics 130: 193–198.
25. Che J, Chen HM, Yang JX, Jin JQ, Jiang K, et al. (2012) Universal COH primers for DNA barcoding amphibians. Molecular Ecology Resources 12: 247–250.
26. Zhou Y, Zhang JY, Zheng RQ, Yu BG, Yang G (2009) Complete nucleotide sequence and gene organization of the mitochondrial genome of Ps spinosa (Anura: Ranolidae). Gene 474: 86-96.
27. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876-4882.
28. Posada D (2008) ModelTest: phylogenetic model averaging. Molecular biology and evolution 25: 1253–1256.
29. Posada D, Buckley TR (2004) Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Systematic Biology 53: 793–808.
57. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution 28: 2731–2739.

58. Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688–2690.

59. Stamatakis A, Blagojevic F, Nikolopoulos DS, Antonopoulos CD (2007) Exploring new search algorithms and hardware for phylogenetics: RAxML meets the IBM cell. The Journal of VLSI Signal Processing 48: 271–286.

60. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution: 783–791.

61. Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.