FISH of 5S rDNA and telomeric (TTAGGG)_n repeats in normal and translocated populations of the frog *Quasipaa boulengeri* (Anura, Ranidae)

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Quasipaa boulengeri, a spiny frog, is widely distributed in the low mountain regions, around Sichuan Basin. Our previous study revealed five karyotypes, caused by a translocation, that are randomly distributed throughout different populations. 5S rDNA and telomere sequence (TTAGGG)_n are potential good markers for chromosome identification and karyological evolution. In this study, we examined the sequences of 14 populations using fluorescence *in situ* hybridization (FISH) to detect if there is any variation between karyologically normal and translocated populations. 5S rDNA loci were located at the same position on chromosomes 1 in 7 translocated populations. In two of the seven normal populations, 5S rDNA also occurred on chromosome 5 in addition to chromosome 1. Our findings further indicate that the 5S rDNA on No. 1 most likely represents the ancestral condition, while the minor loci represent the derived state. Signal density variations of the 5S rDNA were observed between homologous chromosomes or sister chromatids of pair 1 in both normal and translocated populations. Telomere sequences were identically located on all ends of the 26 chromosomes in seven rearranged populations, however, no ITSs were observed on the translocated chromosomes 1 and 6. Two of the six normal populations were found to contain ITSs which indicates that populations with translocation events diverged prior to those with ITSs rearrangements. In the KKS and BF populations, the ITSs of chromosome 3 are not always found on both homologues. Inter-chromosomal signal strength of telomeric sequences commonly differs within all populations.

***Quasipaa boulengeri*, fluorescence *in situ* hybridization (FISH), 5S rDNA, telomere (TTAGGG)_n sequence, intraspecific**

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The 5S rDNA array is an excellent marker for chromosome identification and the detection of karyological evolution [1–3]. The 5S rDNA multigene family comprises a highly conserved coding sequence of 120 bp forming arrays of hundreds to thousands of tandem repeats which are separated by variable nontranscribed spacers (NTS) [4]. The 5S cluster has been chromosomally mapped in only a few species of amphibians [5,6]. Generally, the numbers and locations for 5S rDNA vary between species. For instance, in genus *Rana*, distinctive 5S rDNA sites are located on No. 12 and No. 7 in *Rana catesbeiana* and *R. temporaria*, re-

spectively. In the genus *Triturus*, the 5S clusters are present on No. 10 in *Triturus carnifex* and on No.11 in *T. helveticus* [1,7,8]. Nevertheless, in some genera, different species show identical 5S rDNA patterns. For example, the 5S clusters are consistently localized in the telomeric regions of chromosomes 8 and 12 in six species of the genus *Hydromantes* [9,10]. Additionally, intraspecific investigations of *Alytes obstetricans*, *Bufo bufo*, *Eleutherodactylus maussi*, *Rana esculenta*, *Triturus vulgaris* (L.) *meridionalis* showed that 5S rDNA sites are identical [1,11,12]. This evidence suggests that the chromosomal locations of 5S rDNA cis-trons are conserved in different populations of the same species. However, investigations at the population level are

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scarce, so further studies are needed to detect whether the 5S rDNA sites are intraspecifically conserved.

According to a study by Meyne et al. [13], intrachromosomal telomeric sequences (ITSs) are useful in estimating the original time of chromosome rearrangement. A telomere is a region of repetitive nucleotide sequences at the end of a chromosome. In vertebrates, it consists of tandem repeats of the hexanucleotide sequence (TTAGGG/CCCTAA)_n [14]. The locations of telomeres are routinely at the chromosomal termini, although they also occur at internal sites of the chromosome, the so called intrachromosomal telomeric sequences [5]. The ITSs may originate from rearrangement and amplification, and the presence of ITSs is related to the evolutionary status of the species. That is, ancestral species tend to have only telomeric (TTAGGG)_n sites, whereas highly evolved species may not only display telomeric sites but also non-telomeric sites [13]. In amphibians, interspecific variations have been reported. In some species, exclusively telomeric signals were observed [5,13,15,16], while in others, both telomeric sites and ITSs were detected [17–19]. Intraspecific variations have also been observed. In population studies of *Eleutherodactylus maussi*, *E. euphronides*, *Bufo terrestris*, *B. oblongus oblongus*, *Leptodactylus ocellatus*, no ITSs were observed [11,16,20–22], while in the species *Hylidae versicolor* and *H. chrysoscelis* ITSs were recorded [17,18]. However, these studies en-

compass only seven species with very limited populations. Thus, further intraspecific investigations are needed to clarify the variation of ITSs.

Recently, a reciprocal translocation between pairs 1 and 6 was found in the spiny frog *Quasipaa boulengeri* [23]. Our subsequent studies illustrate a fascinating case of translocation polymorphism involving five different karyomorphs, i.e. types I, II, III, IV, and V randomly occurring in different populations of this species. The type I represents the normal/non-translocated karyotype, while the others display rearrangements/translocations [24]. In the present study, we performed 5S rDNA and telomere (TTAGGG)_n FISH in 14 populations of *Q. boulengeri* and explored the intraspecific variation of these two sequences. Specifically, our objective was to test for variation between the populations with normal and those with rearranged karyotypes.

1 Materials and methods

1.1 Mitotic chromosome preparation

Adults of *Quasipaa boulengeri* used in the present study were collected from 14 localities in Sichuan Province, Guizhou Province, Chongqing Municipality, Hunan Province and Hubei Province, China. The sampling localities and numbers of individuals are shown in Table 1. Mitotic metaphases

Table 1 Collection localities, chromosomal locations and signal variation of 5S rDNA and ITSs^{a)}

Localities	Telomere FISH		5S FISH	
	Number of animals (ITSs)	Terminal signal intensity varied	Number of animals (major 5S/minor 5S)	No. 1 signal variations: homologs/sister chromatids
HKX (I)	1(0)	Yes	1 (No.1q/–)	No/No
HKX (III)	1(0)	Yes	1 (No.1q/–)	No/No
QCHS (I)	–	–	1 (No.1q/–)	No/No
QCHS (IV)	1(0)	Yes	1 (No.1q/–)	No/No
XLZ (I)	1(0)	No	2 (No.1q/–)	No/No
XLZ (IV)	–	–	1 (No.1q/–)	No/No
GTS (IV)	2(0)	Yes	3 (No.1q/–)	No/No
PLZ (II)	1(0)	Yes	1 (No.1q/–)	No/No
HSH (I)	3(0)	Yes**	5(No.1q/–)	No/Yes**
HSH(II)	1(0)	Yes	1 (No.1q/–)	No/Yes**
HSH (V)	1(0)	Yes	2 (No.1q/–)	No/No
DJD (I)	2(0)	No	3 (No.1q/–)	No/Yes
BF (I)	7 (No.3q**#)	No	2 (No.1q/No. 5 q #)	No/No
EM (I)	–	–	2 (No.1q/–)	No/Yes**
YY (I)	2 (0)	Yes	2 (No.1q/–)	Yes/No
KKS (I)	3 (No.3q**#)	Yes	3 (No.1q/–)	No/No
LGS (I)	4(0)	Yes**	2 (No.1q/–)	No/Yes
XFS (I)	1(0)	Yes	2 (No.1q/–)	No/Yes
BZ (I)	2(0)	Yes	4 (No.1q/No.5 q #)	Yes/No

a) Hybridizations have been conducted in five different karyotypes with chromosome No.1/No.6: I, MM/mm; II, MM/mSt; III, MT/mm; IV, MT/mSt; V, MT/StSt (M=large metacentric chromosome; m=small metacentric chromosome; St=large submetacentric chromosome; T=large telocentric chromosome). HKX=Hongkouxiang, Dujiangyan City, Sichuan, China; QCHS=Qingchenghoushan (Mt. Qingcheng), Sichuan, China; XLZ=Xilingzhen, Dayi Co., Sichuan, China; GTS=Gaotangsi, Dayi Co., Sichuan, China; PLZ=Pinglezhen, Qionglai Co., Sichuan, China; HSH=Huashihai, Mt. Tiantai-1, Qionglai Co., Sichuan, China; DJD=Dajiudian, Mt. Tiantai-2, Qionglai Co., Sichuan, China; BF=Bifeng Valley, Yaan City, Sichuan, China; EM=Mt. Omei, Sichuan, China; YY=Xinglongzhen, Youyang Co., Chongqing, China; KKS=Kuankuoshui, Suiyang, Guizhou, China; LGS=Leigongshan, Guizhou, China; XFS=Xuefengshan, Hunan, China; BZ=Baozhen, Hejiapingzhen, Changyang Co., Hubei, China. “–” No specimen was used for FISH or no hybridization signal was detected. “***” Parts of the investigated individuals or metaphases are present the corresponding characters showing in this table. “#” hybridization signals are very faint.

were prepared from bone marrow by the air-dry method as described by Schmid et al. [5]. The slides were stored at room temperature for 2–4 d, dehydrated in an ethanol series (70%, 90%, 100%, 3 min each) and stored at -20°C until used for fluorescence *in situ* hybridization.

1.2 5S rDNA and telomere probe construction and fluorescence *in situ* hybridization (FISH)

Genomic DNA was extracted from liver or muscle tissues by the standard Proteinase K method [25]. 5S rDNA probes were prepared using forward 5'-GCCTACGGCCACACCAC-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. [26] with small modification. The PCR program included 3 min initial denaturation, 30 cycles of $94^{\circ}\text{C}/50^{\circ}\text{C}/72^{\circ}\text{C}$ for 30 s/30 s/1 min followed by a 10 min extension at 72°C . The telomeric probes (TTA-GGG)₇ were commercially synthesized and end-labeled with Biotin at both 3' and 5' positions (Invitrogen) [27].

The hybridization was conducted following the protocol of Zhang et al. [28] and Bi et al. [26] with minor changes. For telomere FISH, the chromosomes were treated with 0.005% pepsin in 0.01 mol L^{-1} HCl for approximately 45 min at 37°C before hybridizing. The hybridization mix contained $10\text{ ng }\mu\text{L}^{-1}$ 5S rDNA probe or $100\text{ ng}/\mu\text{L}$ telomere probe, 10% dextran sulphate, 0.1% SDS, $1\times$ Denhardt's, 50% deionized formamide in $2\times$ SSC, 83°C for 7 min and cooled down by immediately placing on ice for at least 10 min. 5S rDNA and telomere probes were detected with anti-digoxigenin-fluorescein fab fragments (Roche) or fluorescein-labeled avidin DCS (Vector) at 37°C for approximately 1 h and 15 min. Then, the chromosomes were counter-stained with propidium iodide (PI) in antifade solution (Vector) at 37°C for about 45 min. Hybridization signals were observed under a Leica DMRA2 fluorescent microscope equipped with appropriate filter sets. Images were digitized by using a Leica DFC490 CCD camera with Qwin V3 and Qgo software.

2 Results

2.1 5S rDNA FISH

FISH with 5S rDNA probe showed that the hybridization signals were exclusively located near the centromeric region on the long arm of pair 1 in seven karyologically changed populations, including three populations (HKX, XLZ and HSH) reported in our previous study (Table 1, Figure 1(c)–(f)). In seven karyologically normal populations, 5S rDNA is exclusively mapped on the long arm of No. 1 near the centromeric region (Figure 1(b)), except for in BZ and BF populations. In these two populations, in addition to the bright signals on pair 1, very faint signals were also found

Figure 1 *In situ* hybridization of 5S rDNA to mitotic metaphase chromosomes. The 5S rDNA signals appear yellow. The five karyotypes with chromosome No.1/No.6 and population names are designated as in Table 1. (a) Karyomorph I: bright signals on No. 1 (arrow heads) and very faint signals in the terminal region of No. 5 (long arrows). Signal intensity varied in homologous chromosomes of No. 1. The spread is from the BZ population. (b) Karyomorph I: Light signals observed on No. 1 (arrow heads). Signal intensity variations on sister chromatids of No. 1. The spread was from the XFS population. (c)–(f) Karyomorphs II, III, IV, V: 5S rDNA were exclusively located on No. 1 (arrow heads). Spreads were from HSH, HKX, GTS and HSH populations respectively.

in the telomeric position on the long arm of chromosome 5 (Minor 5S rDNA were found in $2\text{♀}2\text{♂}$, 21 metaphases and $1\text{♀}1\text{♂}$, 7 metaphases of BZ and BF populations, respectively) (Figure 1(a)).

Signal intensity differences of 5S rDNA on chromosome 1 were observed either in homologous chromosomes or sister chromatids in the specimens collected from HSH, DJD, EM, YY, LGS, XFS and BZ. In YY and BZ populations, individuals possessed unequal intensity of 5S rDNA signals in pair 1 homologues while the sister chromatids had no variation in 5S rDNA size (Table 1, Figure 1(a)); in populations from HSH, DJD, EM, LGS and XFS, however, variations of the 5S rDNA signal intensity existed on the sister chromatids, but not on homologues of no. 1 (Table 1, Figure 1(b)). In populations HKX, QCHS, XLZ, GTS, PLZ, BF and KKS, 5S rDNA were observed without any variation on homologous chromosomes and sister chromatids (Table 1, Figure 1(d) and (e)). On chromosome 5, 5S rDNA signal intensity variation was not observed.

2.2 Telomere FISH

Telomere sequences (TTAGGG)_n detected by FISH were located on all ends of the chromosomes in seven populations which randomly possessed translocated karyotypes (HKX, QCHS, XLZ, GTS, PLZ, HSH, DJD populations) (Table 1, Figure 2(d)–(g)). In six karyologically normal populations, telomere sequences were also exclusively situated on all ends of the chromosomes except for in the BF and KKS populations (Table 1, Figure 2(c)). In these two populations,

Figure 2 *In situ* hybridization of telomere sequences (TTAGGG)_n to mitotic metaphase chromosomes. The telomere signals appear yellow. All of the chromosome ends display fluorescent signals. The five different karyotypes with chromosome No.1/No.6 and population names are designated as in Table 1. (a) Very faint ITSs (long arrows) are shown on the long arm of chromosome 3 from the BF population with karyotype I. (b) Very faint ITSs (long arrow) are on one of the homologs, but absent on the other one of No.3 from the KKS population with karyotype I. (c) The signals on some small chromosomes were more intense than those on large chromosomes from the YY population with karyotype I. (d)–(g) No ITSs are observed in karyomorphs II, III, IV, and V from the PLZ, HKX, GTS and HSH populations. Signal intensity varies between large and small chromosomes.

both the telomeric pattern, and very minor signals for interstitial telomeric sites (ITSs) were detected on the long arm of chromosome 3 in some individuals (Table 1). In the BF population, some of the individuals possessed faint ITSs in both homologous chromosomes of pair 3 (Figure 2(a)), while others did not have these ITSs; in the KKS population, very weak signals for ITSs were investigated on only one of the homologues of chromosome 3 in two individuals (Figure 2(b)). In a third specimen, no ITSs were detected.

For the terminal (TTAGGG)_n sequences, signal size varied in karyologically normal and translocated populations, including HKX, QCHS, GTS, PLZ, HSH, YY, KKS, LGS, XFS, and BZ populations, i.e. signal intensity on some small chromosomes was much stronger than those on large chromosomes (Table 1, Figure 2(c)–(g)).

3 Discussion

3.1 5S rDNA FISH

(i) The 5S rDNA loci were identical in translocated populations. The positions of 5S rDNA are identical in all seven populations from the Western Sichuan Basin, regardless of the types of rearranged karyomorphs the population contains. 5S rDNA loci are located at the same sites of pair 1 (Table 1). This result suggests that translocated karyotypes arise from common descent. The 5S loci have been mapped on chromosomes in only a few species of amphibians. As in *Alytes obstetricans*, *Rana esculenta*, *Triturus vulgaris* (L.) *meridionalis*, *Notophthalmus viridescens*, *Eleutherodactylus maussi*, intraspecific variation for 5S rDNA is not observed [1,11,12,29]. The 5S rDNA sites appear to be conservative

at the intraspecific level in amphibians.

(ii) The 5S rDNA loci differ in karyologically normal populations. Variations in the positions and numbers of 5S rDNA sites were found in populations with normal karyotypes including seven populations from BF, EM, YY, KKS, LGS, XFS, and BZ (Table 1). In five of these populations, only one major 5S rDNA site was found which is positioned very close to the centromere on the long arm of pair 1, while in the BZ and BF populations, signals occur at the major sites as well as faint signals at the terminal regions of the long arm of pair 5 (Table 1, Figure 1(a)). This indicates that the locations and numbers of 5S rDNA clusters are not as conservative in amphibians as previously assumed. The presence of additional 5S rDNA loci can be interpreted as resulting from the reduplication of 5S genes [30], translocation of a part of the 5S rDNA cluster to another chromosome [7] or nonhomologous crossing-over [3]. These mechanisms may account for the minor 5S rDNA loci present in *Q. boulengeri*. Furthermore populations of *Q. boulengeri* with normal and rearranged karyomorphs share the major 5S loci on chromosome 1. As in some fish species [4,31], this most likely represents the ancestral condition, while the existence of one minor ribosomal gene loci represents to represent the derived condition.

(iii) There is variation in the signal densities of 5S rDNA on No.1. As expected, there is variation in the signal density of 5S rDNA on homologous chromosomes or sister chromatids of pair 1 in both karyologically normal and translocated populations of *Quasipaa boulengeri*. A similar phenomenon has frequently been reported in other species of amphibians and fish [4,5,32]. Such heteromorphisms might be related to variations in the copies of 5S rDNA

array repeats. Some authors propose that the heteromorphisms of the homologous NORs, which contain the main ribosomal RNA genes, 18S+5.8S+28S rDNA in eukaryotes, may result from unequal or non-reciprocal meiotic crossing over between homologous chromosomes [31,33], or unequal exchanges between sister chromatids [34]. Heteromorphisms of 5S rDNA may be explained by the same mechanisms.

3.2 Telomere FISH

(i) The locations of telomere sites were identical in translocated populations. In *Quasipaa boulengeri*, the locations of telomere (TTAGGG)_n sequences were identically located at both ends of all 26 chromosomes in seven karyologically rearranged populations (Table 1, Figure 2(d)–(g)). No ITSs were observed on translocated chromosomes 1 and 6 in these populations. Two predictions may explain the absence of ITSs. First, if the translocation was not accompanied by a reversion of the exchanged chromosome segments between No. 1 and No. 6, the telomere sequence would not move to the fusion sites, thus the intrachromosomal sites will not be detected. Second, if a reversion is involved, tandem translocations have the capacity to internalize telomeric repeats which will give rise to ITSs [5,13]. If a reversion is involved, it is expected that ITSs will be found in *Q. boulengeri*. However, no ITSs were detected in the translocated chromosomes. The deficiency of ITSs on the rearranged chromosomes was also observed in some other species of mammals, birds and amphibians. The absence of ITSs in these species may be explained by the excise of (TTAGGG)_n repeats at the time of fusion, the gradual shortening and degradation of nonfunctional arrays at the fusion point, or the number of the repeats at these sites is below the threshold of detectability [11,35,36].

(ii) The locations of telomere sites vary in karyologically normal populations. Compared to the karyologically translocated populations, interstitial signals were found on pair 3 in two out of six populations which contain normal karyotypes (Table 1, Figure 2(a) and (b)). Meyne et al. [13] have suggested that the presence of telomeric sequences at non-telomeric sites is related to the evolutionary status of the species. That is, the more ancestral species only have telomeric (TTAGGG)_n sequences, while the species that are intermediate or considered to be more derived have ITSs [37]. Assuming the ITSs on chromosome 3 were the result of chromosomal rearrangement, then it follows that at least two chromosomal rearrangements occurred in the populations of *Q. boulengeri*: 1) the translocation between chromosomes 1 and 6, and 2) the rearrangement showing on chromosomes 3. As proposed by Meyne et al. [13], *Q. boulengeri* populations with translocation events most likely diverged earlier than the populations with ITSs rearrangements.

(iii) ITSs vary in homologous chromosomes. Variations exist in the presence or absence of ITSs on one or two

homologs of No. 3 in KKS and BF populations (Table 1, Figure 2(a) and (b)). Similar phenomenon was also found in *Hyla chrysoscelis* [17], and the authors predicted that unequal crossing-over, submicroscopic deletion, or differential amplification may have played a role in the evolution of interstitial locations of (TTAGGG)_n sequences in general. The variations of interstitial telomere sites found on No. 3 in *Q. boulengeri* can be interpreted by these mechanisms. In addition, the weak interstitial signal on pair 3 indicates that the number of (TTAGGG)_n repeats is quite low and probably not easy to detect.

(iv) Signal size varies in terminal (TTAGGG)_n sequences. The inter-chromosomal signal size of telomeric sequences differed both in karyologically normal and translocated populations, and is a common phenomenon in *Quasipaa boulengeri* (Table 1, Figure 2(c)–(g)). Inter-chromosomal intensity differences of the telomere hybridization signals have also been observed in other species such as in birds and amphibians [5,17,36,38]. These can be attributed to the difference in the number of (TTAGGG)_n repeats present on the chromosomes. Schmid et al. [5] predicted that the size variability of the telomeric repeats is possibly the result of transposable elements moving freely between the telomeric regions. Mobile elements can remove parts of the repetitive telomeric DNA sequences of one chromosome and insert them in others to decrease or increase the number of repetitive telomeric DNA sequences and thus the FISH labeling intensity. This may explain the telomere signal variations in *Q. boulengeri*.

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