Cytological evidence for population-specific sex chromosome heteromorphism in Palaearctic green toads (Amphibia, Anura)

G Odierna[1,*], G Aprea[1], T Capriglione[2], S Castellano[3] and E Balletto[3]

[1]*Dipartimento di Biologia Strutturale e Funzionale, Università di Napoli Federico II, Via Cinthia 6, 80126 Naples, Italy*
[2]*Dipartimento di Scienze Biologiche, Università di Napoli Federico II, Via Mezzocannone 8, 80134 Naples, Italy*
[3]*Dipartimento di Biologia Animale e dell'Uomo, Università di Torino, Via Accademia Albertina 13, 10123 Turin, Italy*

**Corresponding author (Fax, 39 081 679233; Email, gaetano.odierna@unina.it)*

A chromosome study was carried out on a number of European and Central Asiatic diploid green toad populations by means of standard and various other chromosome banding and staining methods (Ag-NOR-, Q-, CMA_3-, late replicating [LR] banding pattern, C- and sequential C-banding + CMA_3 + DAPI). This study revealed the remarkable karyological uniformity of specimens from all populations, with the only exception being specimens from a Moldavian population, where one chromosome pair was heteromorphic. Though similar in shape, size and with an identical heterochromatin distribution, the difference in the heteromorphic pair was due to a large inverted segment on its long arms. This heteromorphism was restricted to females, suggesting a female heterogametic sex chromosome system of ZZ/ZW type at a very early step of differentiation.

[Odierna G, Aprea G, Capriglione T, Castellano S and Balletto E 2007 Cytological evidence for population-specific sex chromosome heteromorphism in Palaearctic green toads (Amphibia, Anura); *J. Biosci*. **32** 763–768]

1. Introduction

Toads of the *Bufo viridis* (Laurenti 1768) complex (*Pseudepidalea viridis*, following Frost *et al* 2006), have a very broad distribution, ranging from Morocco and South Sweden to Mongolia and northern Xinjiang, across all the ex-Soviet Central and 'middle' Asiatic Republics (Kazakhstan, Kyrgyzstan, Uzbekistan, Tajikistan, Turkmenistan) and the Arabian Peninsula. Green toads are unique within their family, as well as among vertebrates in general, in possessing bisexual diploid, triploid (fertile) and tetraploid populations (*see* Odierna *et al* 2004 for refer,ences). A previous study (Odierna *et al* 2004) conducted on a number of European, Near Eastern, North African and Central Asiatic green toad populations demonstrated that specimens, independently of the sex, provenance and ploidy levels, do not show any difference in their chromosome and chromatinic characteristics, including the number and shape of chromosomes, as well as the Ag-NOR- and CMA_3- phenotype and patterns of Q- and C-bands. More recently, Stöck *et al* (2005) have shown that some Middle Asiatic tetraploids exhibit clear differences in the 6th chromosome quartets, which are divisible into pairs of chromosomes, either having or lacking an intense Q-positive band on their short arms. Thus, Middle Asiatic tetraploids are allopolyploid. Since the Iranian diploid populations lack paracentromeric Q-positive bands on their sixth chromosome pair, Stöck *et al* (2005) consider them possible candidates for one of the two parent populations.

In this paper, we provide evidence for the existence of population-specific heteromorphism, which we found in a Moldavian green toad population, during an extensive inter-populational chromosome analysis, aimed at studying the rise and evolution of tetraploidy in this group (Odierna *et al* 2004).

2. Materials and methods

A number of European and Asiatic green toad populations were sampled during our 1993–2003 field surveys (details in Odierna *et al* 2004), totalling 465 karyotyped

Keywords. Sex chromosome differentiation; inversion heteromorphism; green toads; *Bufo viridis* complex; Anura

specimens. In this paper, we have focused on the results of a chromosome study carried out on those diploid populations where specimens of either both sexes or only females were sampled, totalling 102 karyotyped specimens (table 1). In addition, we recently had the opportunity to enlarge our sampling of the Calarasi (Moldavia) population from the initial two to seven pairs.

Metaphase plates were obtained by means of blood cultures, performed according to the method described by Nishioka *et al* (1993, 1994). In addition to the standard staining method (5% Giemsa at pH 7), the following banding techniques were used: Ag-NOR (Howell and Black 1980); chromomycin A_3/methyl green (CMA_3) (Sahar and Latt 1980); Q- (Schmid 1978), C- (Sumner 1972), the sequential C- + CMA_3 + DAPI (Odierna *et al* 1999) and late replication (LR) banding. For LR, 30 μg/ml BrdU was added to blood cultures during the last 6 h and differential staining was revealed by staining 4-day-old slides with 4% Giemsa solution in 2% 4Na-EDTA (Nishioka *et al* 1993; Nishioka *et al* 1994; Miura 1995) for 20 min.

For each specimen, five mitotic figures under each staining method were examined, apart from the LR-banding of the Moldavian sample, in which ten metaphase plates were analysed.

3. Results

Irrespective of their origin and sex, all investigated specimens displayed a karyotype of $2n = 22$ biarmed chromosomes, with the first 6 pairs distinctively bigger than the last 5 pairs. Chromosomes of both sexes also showed very similar patterns of CMA_3, C-banding and sequential C-banding + CMA_3 + DAPI banding, as evidenced by a comparison of male and female haploid sets. CMA_3 (figure 1A) and Ag-NOR (Odierna *et al* 2004) showed two positive loci only, on the peritelomeric regions of the long arms of the 6th chromosome pair. Bright Q-bands were observed on the short arms of chromosome pairs 6–11 (figure 1B). Furthermore, the 7th and 11th pairs also showed a paracentromeric Q-band on their long arms. Dark C-bands were observed on the centromeric regions of all the chromosomes (figure 1C). The sequential staining of C-banding + CMA_3 + DAPI showed only CMA_3-positive fluorescence in the peritelomeric regions on the long arm of the 6th chromosome pair (figure 1D), whereas centromeric C-bands were DAPI-positive (figure 1E).

LR staining procedures produced cross-longitudinal dark and light bands along the chromosomes. Their number ranged from 40 in the chromosomes of the first pair down

Table 1. Number, sex and origin of the green toad specimens investigated

Locality	Country	Latitude	Longitude	Number and sex	Ploidy	Year
Europe						
Calarasi	Moldavia	47° 16′ N	28° 19′ E	2 ♂♂+ 2 ♀♀	2n	1995
Calarasi	Moldavia	47° 16′ N	28° 19′ E	5 ♂♂+ 5 ♀♀	2n	2005
Tula	Russia	54° 12′ N	37° 36′ E	1 ♀	2n	1997
Cirindinu	France – Corse	41° 44′ N	9° 27′ E	2 ♂♂+ 4 ♀♀	2n	1993
Marina d′Alberese	Italy	42° 17′ N	11° 23′ E	3 ♂♂ + 2 ♀♀	2n	1993
Berchidda	Italy – Sardinia	40° 46′ N	9° 07′ E	6 ♂♂+ 1 ♀	2n	2001
Isonzo	Italy	45° 19′ N	13° 39 E	9 ♂♂ +1 ♀	2n	2000
Central Asia						
Ciokesu	Kazakhstan	47° 14′ N	61° 04′ E	12 ♂♂+ 1♀	2n	2002
Kizilkul	Kazakhstan	43° 44′ N	69° 30′ E	5 ♂♂+ 1♀	2n	2002
Khantau	Kazakhstan	44° 16′ N	74° 07′ E	5 ♂♂+ 1♀	2n	2002
Balkash	Kazakhstan	45° 36′ N	73° 20′ E	1 ♀	2n	1997
Ala Medin	Kyrgyzstan	42° 25′ N	74° 02′ E	6 ♀♀+8 ♂♂	2n	1999
Bishek	Kyrgyzstan	42° 52′ N	74° 37′ E	2 ♂♂+ 2 ♀♀	2n	1999
Lake Sudochye	Uzbekistan	43° 10′ N	58° 24′ E	6 ♂♂+ 1♀	2n	2003
Karatereng	Uzbekistan	43° 30′ N	60° 25′ E	1 ♂+ 4 ♀♀	2n	2003
Kanal	Uzbekistan	43° 04′ N	61° 24′ E	2 ♀♀	2n	2003
Kuyu Mazar Canal	Uzbekistan	39° 54′ N	64° 42′ E	1 ♂+ 3 ♀♀	2n	2003
Nuratau Mt Ridge	Uzbekistan	40° 56′ N	66° 27′ E	2 ♂♂+ 1♀	2n	2003
North Africa						
Marrakesh	Morocco	31° 39′ N	8° 03′ W	8 ♂♂ +1 ♀	2n	2000

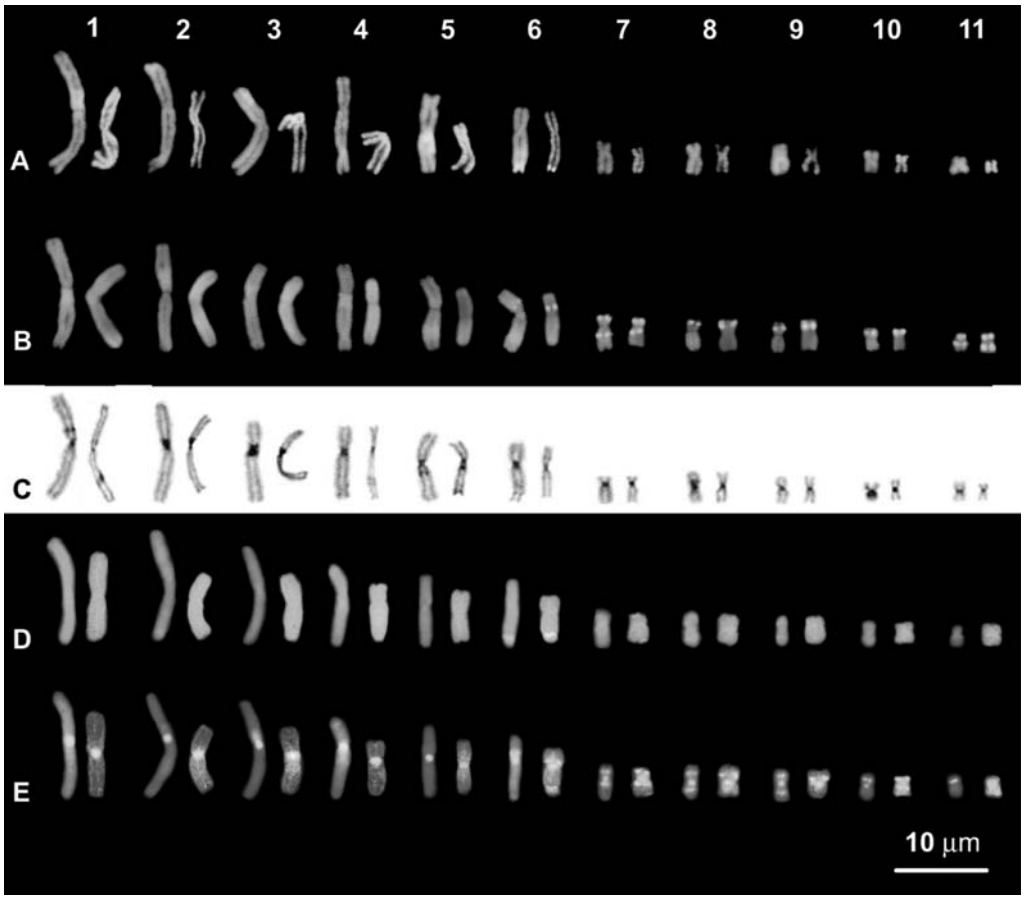

Figure 1. Comparison between male (chromosomes on the left) and female (chromosomes on the right) haploid chromosome sets, stained with CMA_3- (**A**; male from Berchidda, female from Kizlkul), Q- (**B**; male from Marrakesh, female from Bishek), and C-banding (male and female from Calarasi) + sequential staining with Giemsa (**C**), CMA_3(**D**) and DAPI (**E**).

to 4 in the 11th pair. Dark and light band profiles were chromosome-specific, making homologue identification and pairing easy. An identical LR banding pattern was conserved in all specimens, apart from females from Calarasi (Moldavia). Their 4th chromosome pair was heteromorphic because of a large inverted segment on the long arms of one of the two chromosomes (figure 2). The probable mode of inversion is schematized in figure 3.

4. Discussion

Specimens from all the studied populations did not show any difference in their chromosome and chromatinic characteristics, with the remarkable exception of females from Calarasi (Moldavia), whose chromosomes of the 4th pair appear heteromorphic. Since heteromorphism is fixed in females of this population, it strongly suggests a Z and W sex chromosome system. Both sex reversal experiments and breeding tests performed in various anuran families invariably prove the existence of genetic sex determination (Schmid *et al* 1991; Hayes 1998; Sumida and Nishioka 2000; Schmid and Steinlein 2001; Eggert 2004). Though evolving sex chromosomes represent an important, perhaps inevitable, step in the evolution of sex determination (Bull 1983), heteromorphic sex chromosomes (HSCs) occur in relatively few anuran species. Karyological studies, performed in about 900 out of the almost 5200 species recognized so far (Frost *et al* 2006), have documented HSCs in18 species only (King 1990 and references therein; Schmid and Steinlein 2001; Schmid *et al* 2002). They display either simple or complex male (XX/XY; $XXAA/XAA^y$) or female (ZZ/ZW; 00/0W) sex chromosome systems, where Y (W) is completely or partially heterochromatic, homomorphic or heteromorphic to X(W). Different sex chromosome systems can be found at various taxonomic levels, such as between families, genera and even between populations of the same species (Schmid *et al* 1990; Nishioka *et al* 1993; Nishioka *et al* 1994; Sumida and Nishioka 1994; Miura 1996; Schmid and Steinlein 2001; Schmid *et al* 2002). In other words, sex

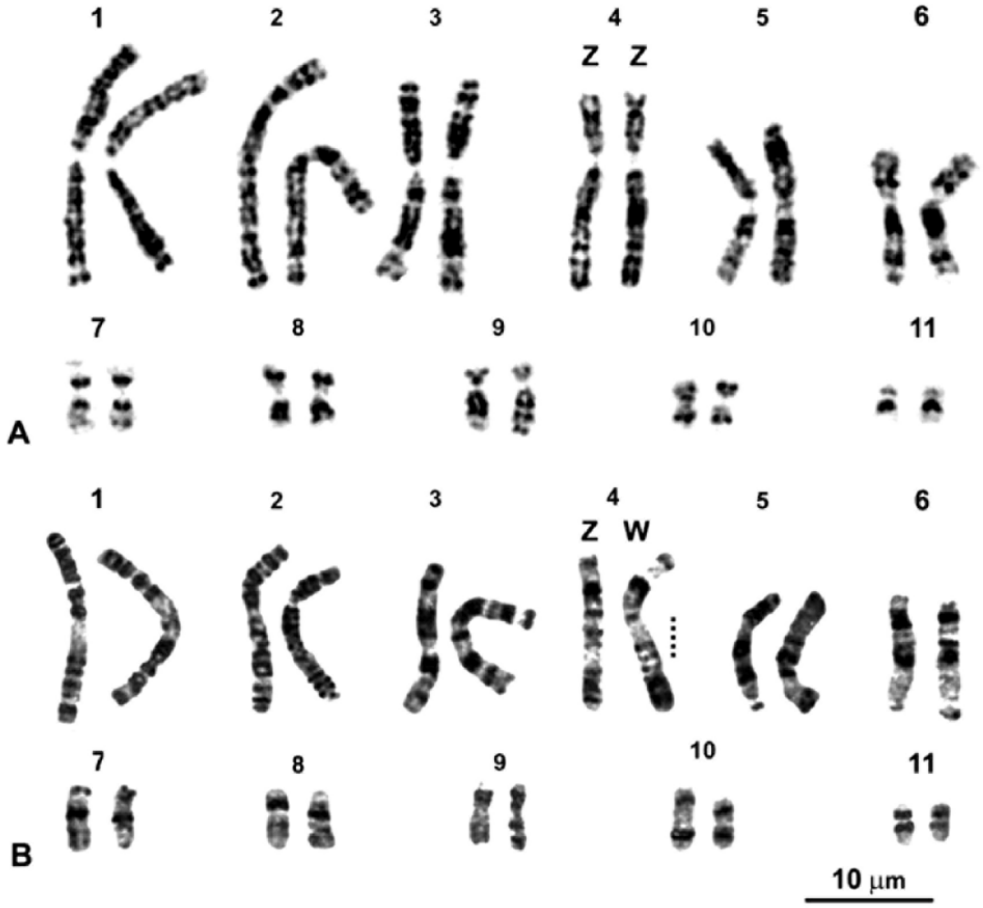

Figure 2. Male (**A**) and female (**B**) Late-replicating banding patterns of green toads from Calarasi (Moldavia). **N**ote: In **B**, the 4th chromosome heteromorphic pair corresponds to the ZW sex chromosomes. W differs from Z because of an inverted segment (hatched) along its long arm.

chromosomes in anurans may not be directly correlated with evolutionary history (Schmid and Steinlein 2001).

Our results, however, suggest that the number of anuran species possessing sex chromosomes may be larger than known so far. The W chromosome of Moldavian females is morphologically similar to Z, being as long as this chromosome, and having equal heterochromatin distribution and composition. It differs from the Z chromosome only in a large segment of the long arm of the W chromosome, which has probably undergone a paracentric inversion. This kind of rearrangement, which keeps the chromosome morphology unmodified, is detectable in amphibians by early (ER)- or late-replicating (LR) banding pattern techniques only. G-bands, in fact, are notorious for being non-inducible, or very difficult to induce, in this class of vertebrates (Schmid 1978). The detection of an XX/XY sex chromosome system in *R*. klep. *esculenta* has been made possible only by the incorporation of BrdU into the chromosomes during the synthetic (S) phase of the cells (ER- or LR-banding techniques). Y and X chromosomes are homomorphic, apart from having a large asynchronous LR band on the long arm of the Y chromosome (Schempp and Schmid 1981).

The Moldavian Z and W sex chromosomes appear to be at an early stage of morphological differentiation. Future analysis of additional Moldavian green toad populations will show whether the Z and W sex chromosomes are present.

Two contrasting models have been proposed to explain how the suppression of recombination between X (Z) and Y (W) segments, containing sex-opposing genes, may be ensured.

(i) According to Ohno's model (1967), suppression occurs by structural rearrangements such as inversions. Other changes, involving the accumulation of repetitive sequences, are thought to arise later in the process.

(ii) Jones (1984, 1991) proposed that events of heterochromatinization or displacements during replication precede structural rearrangements.

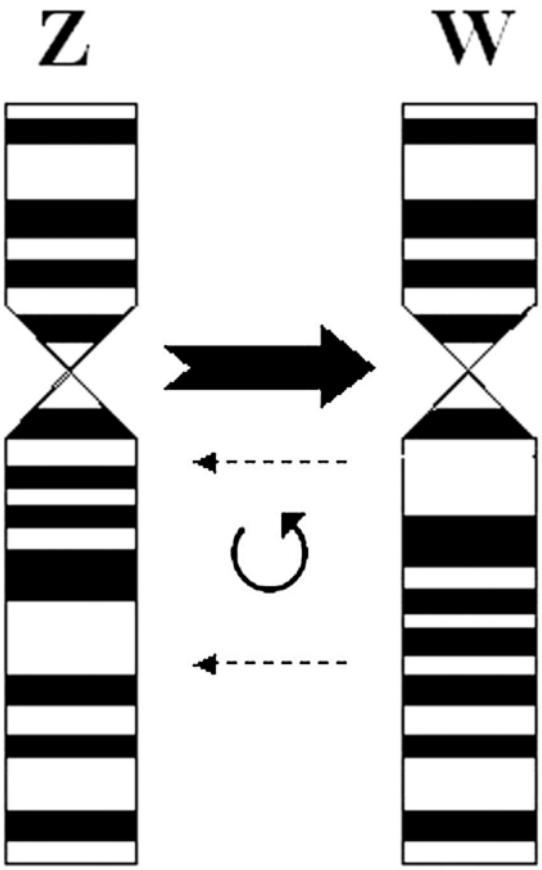

Figure 3. Late replicating pattern idiograms of Z and W sex chromosomes showing the schematic representation of the inversion of the long arm segment (contained between the two hatched arrows) of the Z chromosome, which arose from the W sex chromosome in the green toad females from Calarasi.

Both patterns appear to be operative in anurans. The XX/XY sex system of *R*. klep. *esculenta* supports the model proposed by Jones (1984), since X and Y chromosomes are homomorphic and differ in a large, LR segment on the long arm of the Y chromosome (Schempp and Schmid 1981). The model proposed by Ohno (1967) is supported by the ZZ/ZW sex chromosomes of our Moldavian population, whose W is homomorphic to Z and differs only in having a large inverted segment on its long arm.

Acknowledgments

This research was supported by grants from MIUR and ARAL. We are grateful to L Borkin, N Bressi, G Dujsebayev, T Dujsebayeva, V Eremchenko, C Giacoma, V Kolbintzev, T Kozar, D Malakov in providing us with many specimens.

References

Bull J J 1983 *Evolution of sex determining mechanisms* (London and Amsterdam: Benjamin Cummings)

Eggert C 2004 Sex determination: the amphibian models; *Reprod. Nutr. Dev.* **44** 539–549

Frost D R, Grant T, Faivovich J and Bain R H *et al* 2006 The amphibian tree of life; *Bull. Am. Mus. Nat. Hist.* **297** 1–370

Hayes T B 1998 Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms; *J. Exp. Zool.* **281** 373–399

Howell W M and Black D A 1980 Controlled silver staining of nucleolus organiser regions with a protective colloidal developer: 1-step method; *Experientia* **36** 1014–1015

Jones K W 1984 The evolution of sex chromosomes; in *Chromosomes today Vol 8* (eds) M D Bennett, A Gropp and U Wolf (Hemel Hempstead, England: George Allen and Unwin) pp 241–255

Jones K W 1991 Sex genes and chromosome inactivation; in *Symposium on the evolution of terrestrial vertebrates*; *Selected symposia and monographs U Z I Vol 4* (eds) G Ghiara, F Angelini, E Olmo and L Varano (Modena, Italy: Mucchi) pp 269–277

King M 1990 Amphibia; in *Animal cytogenetics Vol 4* (eds) B John and C Gwent (Berlin: Gebrüder Borntraeger) pp vi+241

Miura I 1995 The late replication banding patterns of chromosomes are highly conserved in the genera *Rana*, *Hyla*, and *Bufo* (Amphibia: Anura); *Chromosoma* **103** 567–574

Miura I, Ohtani H, Kashiwagi A, Hanada H and Nakamura M 1996 Structural differences between XX and ZW sex lampbrush chromosomes in *Rana rugosa* females (Anura: Ranidae); *Chromosoma* **105** 237–241

Nishioka M, Hanada H, Miura I and Ryuzaki M 1994 Four kinds of sex chromosomes in *Rana rugosa*; *Sci. Rep. Lab. Amphibian Biol. Hiroshima Univ.* **13** 1–34

Nishioka M, Miura I and Saitoh K 1993 Sex chromosomes of *Rana rugosa* with special reference to local differences in sex-determining mechanism; *Sci. Rep. Lab. Amphibian Biol. Hiroshima Univ.* **12** 55–81

Odierna G, Aprea G and Capriglione T 1999 Chromosomal and molecular analysis of some repeated families in *Discoglossus* Otth, 1837 (Anura, Discoglossidae): taxonomic and phylogenetic implications; *It. J. Zool.* **66** 275–283

Odierna G, Aprea G, Capriglione T, Castellano G and Balletto E 2004 Evidence for chromosome and *Pst* I satellite DNA family evolutionary stasis in the *Bufo viridis* group (Amphibia, Anura); *Chromosome Res.* **12** 671–681

Ohno S 1967 *Sex chromosomes and sex-linked genes* (Berlin: Springer)

Sahar E and Latt S A 1980 Energy transfer and binding competition between dyes used to enhance staining differentation in metaphase chromosomes; *Chromosoma* **79** 1–28

Schempp M and Schmid M 1981 Chromosome banding in amphibia VI. BrdU-replication patterns in anura and demonstration of XX/XY chromosomes in *Rana esculenta*; *Chromosoma* **83** 697–710

Schmid M 1978 Chromosome banding in amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*; *Chromosoma* **66** 361–388

Schmid M, Steinlein C, Nanda I and Epplen J T 1990 Chromosome banding in amphibia; in *Cytogentics of amphibians and reptiles* (ed.) E Olmo (Basel: Birkhäuser) pp 21–45

Schmid M, Nanda I, Epplen J T, Steinlein C, Kausch K and Haaf T 1991 Sex-determining mechanisms and sex chromosomes in amphibia; in *Amphibian cytogenetics and evolution* (eds) D M Green and S S Sessions (San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press) pp 393–430

Schmid M and Steinlein C 2001 Sex chromosomes, sex-linked genes, and sex determination in vertebrate class amphibia; in *Genes and mechanisms in veterbrates sex determination* (eds) G Scheer and M Schmid (Basel: Birkhäuser) pp 143–176

Schmid M, Feichtinger W, Steinlein, Rupprecht A, Haaf T and Kaiser H 2002 Chromosome banding in amphibia. XXIII. Giant W sex chromosomes and extremely small genomes in *Eleutherodactylus euphronides* and *Eleutherodactylus shrevei* (Anura, Leptodactylidae); *Cytogenet*. *Genome Res*. **97** 81–94

Stöck M, Steinlein C, Lamatsch D K, Schart M and Schmid M 2005 Multiple origins of tetraploid taxa in the Eurasian *Bufo viridis* subgroup; *Genetica* **124** 255–272.

Sumida M and Nishioka M 1994 Geographic variability of sex-linked loci in the Japanese brown frog *Rana japonica*; *Sci*. *Rep*. *Lab*. *Amphibian Biol*. *Hiroshima Univ*. **13** 173–195

Sumida M and Nishioka M 2000 Sex-linked genes and linkage maps in amphibians; *Comp*. *Biochem*. *Physiol*. *B* **126** 257–270

Sumner A T 1972 A simple technique for demonstrating centromeric heterochromatin; *Exp*. *Cell Res*. **75** 304–306

MS received 24 October 2006; accepted 14 March 2007

*e*Publication: 2 April 2007

Corresponding editor: Subhash C Lakhotia