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The Karyotype of *Hyla arborea japonica* with Some Remarks on Heteromorphism of the Sex Chromosome^{1), 2)}

By

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(With 11 Text-figures)

Chromosomes of the Japanese tree frog, *Hyla arborea japonica*, have been previously studied by Iriki (1930) in the sectioned testicular material, Yosida (1957) in spermatogonial cells with a squash technique, and Matsuda (1963) by means of a tissue culture method. These authors have insisted on the existence of recognizable sex chromosomes of either XX- or XY-type in males. However male or female heterogamety has not been convincingly determined cytologically in amphibians except a recent report on a primitive anuran, *Xenopus laevis*, in which Weiler and Ohno (1962) have described a female heterogamety. Cases of male heterogamety in anurans reported hitherto seemed to be rather rare (Witschi 1959). The studies as cited above have remained without precise karyotype analyses in both male and female individuals for determining a heteromorphism of chromosomes, except the study of *Xenopus*.

The purpose of this study was (1) to investigate the karyotypes of male and female frogs as accurate as possible using a tissue culture technique, and (2) to demonstrate heteromorphic chromosomes, if any, in either sex. Because of the unclarified system of amphibian sex-determination, the clear-cut karyotypes of both the sexes of *Hyla* would be significant for reconsideration of inconvincible conclusion on male heterogamety.

Before going further, the author wishes to express his sincere gratitude to Professor Sajiro Makino for his keen interest in the subject and improvement this manuscript for publication.

Material and Methods: *Hyla arborea japonica* Günther were collected during the period June to July, in the campus of the Hokkaido University and in suburbs of Sapporo city. Preparations for somatic chromosomes were made in part by the tissue culture method

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2) This paper is dedicated to Professor Atsuhiko Ichikawa, in honor of his sixtieth birthday, May 20, 1964.

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and by direct squash technique according to Weiler and Ohno (1962). Adult male and female frogs were killed immediately after collection. Kidney and lung tissues from the animals were removed and transferred into sterile amphibian Ringer's solution, and cut into small pieces of approximately 1-2 mm square. Several such explants were placed on the bottom of a culture flask, which was coated by plasma clot. Nutrient medium used was Shah's amphibian culture medium (Shah 1962). The cultures were incubated at 26°C for 8 to 10 days. After colchicine treatment (30 µg/ml) for 48 hours, cells of the outgrowth together with the original tissue fragments were dislodged with the trypsin solution (0.5 per cent trypsin in amphibian Ringer's solution). After removal of the tissue fragments the cellular suspension was centrifuged at 1500 rpm for 5-10 minutes and the supernatant was decanted. Cells were resuspended in distilled water for 20 minutes. A drop of the cell suspension was placed on the slide, and after staining with acetic dahlia the cells were squashed.

Another technique was also employed for observing somatic chromosomes *in vivo*. The animal was injected intraperitoneally with 0.5 ml of 0.5 per cent colchicine solution four hours before being sacrificed. Various tissues were removed and minced in the amphibian culture medium. The material was then centrifuged and resuspended in distilled water and large fragments of the tissue were separated from the cell suspension.

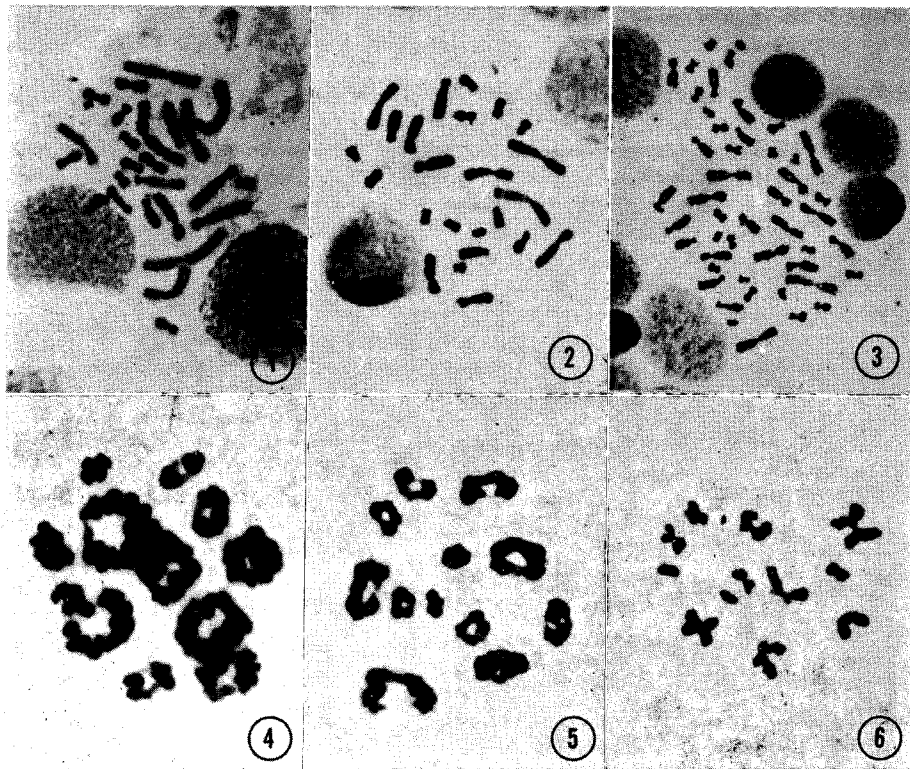
Cytological preparations were made with routine acetic dahlia squash method as described above.

Meiotic chromosomes of adult male were studied by means of the water-pretreatment squash technique (Makino and Nishimura 1952).

Observations

Somatic chromosomes: The diploid chromosome number of this species was determined as 24, confirming the earlier reports (cf. Makino 1956). Representative metaphase plates are shown in Figure 1 (male) and Figure 2 (female). The former was derived from the testis *in vivo* and the latter from the female kidney *in vitro*. Twenty-four chromosomes were arranged lengthwise in the descending order. Every series of the chromosomes of the male (Figs. 7 and 8) as well as the female frogs (Figs. 9 and 10) was easily classified into two major groups. Large metacentric, submetacentric and subtelocentric chromosomes were aligned on the top, while short meta- or submetacentric elements were arranged beneath them. For the karyotype analysis the chromosomes were assorted into 12 pairs and numbered in order of the length.

Chromosomes nos. 1 and 2 are large metacentric being easily distinguishable from others by the size and the centromere position. Nos. 3-5 are similar-sized chromosomes with submedian centromeres. Only no. 6 pair is subtelocentric having satellites at the tip of the short arms. It is rather difficult to demonstrate clearly the satellites, particularly in female chromosomes, possibly due to technical difficulty. Nos. 7-12 consist of two small metacentrics and 4 submetacentrics approximately identical in length. No. 7 is the largest submetacentric of this group and nos. 8 and 9 are metacentric. Numbers 10 and 11 are smaller chromosomes with submedian centromeres. Their similarity in size and the centromere position makes it difficult to distinguish one from the other. Number 12 is a pair of the smallest size in the complement having submedian centromere. Numbers



Figures 1-6. Photomicrographs of chromosomes of the Japanese tree frog, *Hyla arborea japonica*, from squash preparations with acetic dahlia.

Fig. 1. Mitotic metaphase from an adult male testicular tissue.

Fig. 2. Mitotic metaphase from an adult female kidney.

Fig. 3. Metaphase from a tetraploid cell of a female.

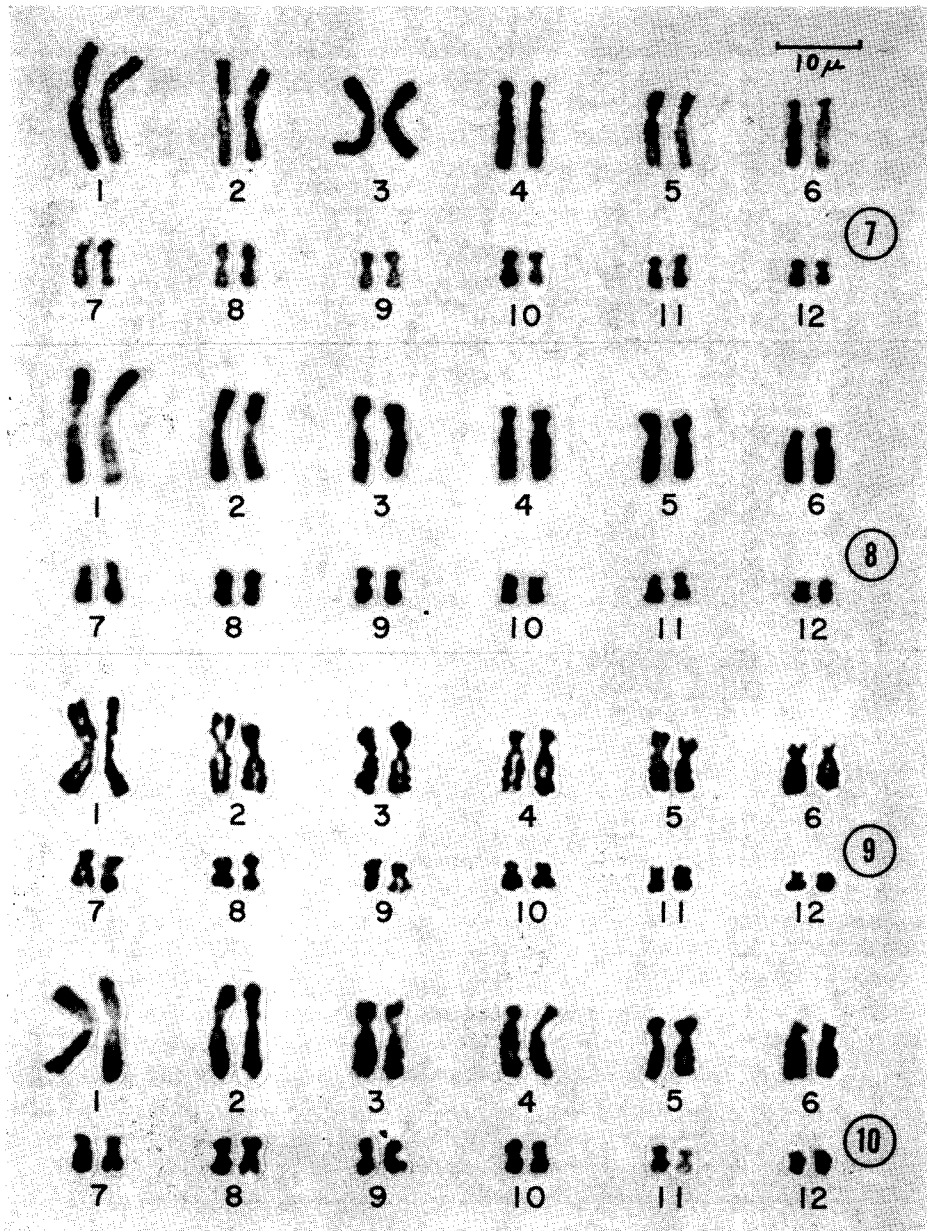
Fig. 4. Late prophase cell of the first spermatocyte.

Fig. 5. Metaphase of the first spermatocyte.

Fig. 6. Second metaphase from an adult male.

1, 2, 6 and 12 chromosomes could be unequivocally identified. Two largest pairs, nos. 1 and 2, are outstanding in feature, clearly different from others in the length and arm ratio, no. 2 being more submetacentric than no. 1. Number 6 is easy to recognize, not only by its having a satellite but also by its being most telomeric chromosome among all the members. Specially noticeable is the fact that in both sexes the members of no. 6 pair are slightly unequal in length. This size difference was found consistently in cells so far observed in male and female animals.

Details on the heteromorphism found in no. 6 chromosomes were published another paper (Seto and Makino 1964).



Figures 7-10. Karyotypes of male and female *Hyla arborea japonica*. Figs. 7 and 8, from male diploid cells. Figs. 9 and 10, from female diploid cells.

It is evident from the comparison of karyotypes in both sexes that the somatic chromosomes are apparently the same numerically as well as morphologically (Figs. 7-10). This finding was confirmed from the karyotype analysis in tetraploid cells found in the female kidney tissue (Fig. 11).

The evidence presented above excludes altogether, the possibility of heteromorphism of sex chromosomes in males.

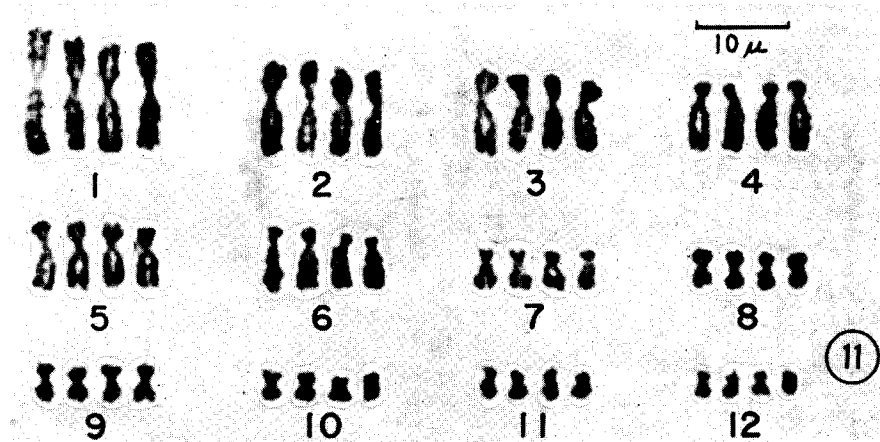


Figure 11. Karyotype of a female tetraploid cell from the cultured kidney tissue.

Meiotic chromosomes: In order to confirm further the occurrence of sex chromosomes of XY-type, the first meiotic chromosomes were analysed in squashed male germ-cells. As shown in Figures 4 and 5, the spermatocytes exhibited no particular figure characteristic of a sex-bivalent at prophase-I and metaphase-I: there were consistently 12 bivalents in each cell. This result is in concordance with the negative finding for the heteromorphic sex-element as described earlier. At diakinesis ring-shaped chromosomes were commonly found. Each of these chromosomes consisted of two homologous mates widely separated in the middle but united at both ends. No single interstitial chiasma could be observed in any bivalent. An interesting feature was that every bivalent showed a spiral structure (Fig. 4). There was absence of heteropycnotic element in meiotic prophase nuclei.

Chromosomes in the second meiotic metaphase were also 12 in number; there were four metacentrics and eight submetacentrics in a haploid set in every metaphase cells. There was no heteropycnotic element in the haploid set.

In conclusion therefore the above findings failed to detect any heteropycnotic or heteromorphic element corresponding to the Y chromosome in both cultured and squashed materials, contrary to the results of Yosida (1957) and Matsuda (1963).

Discussion

The nature of the sex chromosomes in amphibians has long been a matter of controversy. And claims for and against the existence of heteromorphic (sex) chromosomes have been presented by many investigators. For instance, Witschi (1924, 1933) and Bushnell *et al.* (1939) claimed that the male in family *Ranidae* contains a pair of sex chromosomes; an X and Y could be seen in spermatogenesis. But detailed work of Makino (1932), Saez *et al.* (1936) and Wickbom (1945) failed to detect such elements in many species of frogs. Recently Weiler and Ohno (1962) reported on chromosomes of a primitive anuran, *Xenopus laevis*, that a distinct heteromorphic element exists in female. Di Berardino (1962) and Freed (1962) reached the conclusion, on the basis of extensive karyotype analyses, that no unequal pair of elements was found in *Rana pipiens*.

There are three papers dealing with the chromosomes at studies in the Japanese tree frog, *Hyla arborea japonica*. Iriki (1930) who studied meiotic chromosomes from sectioned material emphasized male homogamety on account of the fact that the two largest V-shaped elements represented the sex chromosomes of an XX-type. On the other hand, Yosida (1957) concluded, in the study with squashed germ-cells, that the male had heteromorphic XY chromosomes, and that the Y element was often positive heteropycnotic at the spermatogonial prophase. Further, recently, Matsuda (1963) also reported a heteromorphic pair in male cells derived from kidney cultures. The difference in size of the X and Y, according to Yosida (1957) and Matsuda (1963), is extremely small. Their conclusion, however, is not decisive, because they did not investigate the spermatocyte chromosomes in detail, and further they did not carry out accurate karyotype analyses in both sexes.

A comparison between the karyotypes presented by Yosida (1957) and by the present author showed to some extent a remarkable similarity, especially in the relative length of chromosomes and the arm ratio. But there are some discrepancies between these two studies. The most obvious dissimilarity deals with the pairing of chromosomes in nos. 6-9 for the male: in Yosida's presentation two unequal elements rank between 6 and 7, and 7 and 8, while the present study has only detected a slight difference between the members of no. 6 pair in both sexes. Thus the present study has revealed a striking similarity in karyotype between males and females. Further there was a marked correspondence between the diploid and tetraploid chromosomes in female cells.

It is a well-known fact in mammals that the sex bivalent shows a strikingly different behavior from autosomal bivalents at prophase and metaphase of the first meiotic division. The present observations, however, failed to detect the existence of a bivalent which takes a particular behavior at the first metaphase and anaphase. Makino (1947) made a comparative study of mitotic chromosomes in two sexes of the urodelan, *Hynobius retardatus* with sectioned material, and reported that the constitution of the chromosomes is identical

between the two sexes and there is no indication for the existence of any particular chromosome distinguishable in form and behavior from the other elements.

In the spermatogonial tissue of the rat, Tjio and Levan (1956) demonstrated that the Y chromosome was heteropycnotic through the whole cycle of mitosis, while X element showed no such behavior. Following their conclusion Yosida (1957) described the existence of the Y chromosome which was often positive heteropycnotic at the spermatogonial prophase. Based on squashed male germ-cells, the present author made a precise investigation to demonstrate any heteropycnotic chromosome through spermatogonial prophase to metaphase, but failed to find such pycnotic element.

At the present status of investigation, a number of investigators, except a report by Weiler and Ohno (1962), has failed to demonstrate a heteromorphic pair in male cells. Makino (1947) and White (1954) expressed a view that the sex chromosomes of amphibians are very little differentiated from autosomes and hence they are not distinguishable morphologically.

Summary

The karyotype of the tree frog, *Hyla arborea japonica*, was analyzed with squash materials of adult male and female tissues *in vivo* and *in vitro*. The diploid chromosome number was 24, containing 4 pairs of metacentrics, 7 pairs of submetacentrics, and a pair of subtelocentrics. Most of the chromosomes can be identified individually according to their size and morphology. Number 6 chromosomes were found to be satellited ones.

A comparison of the karyotypes of male and female frogs showed no significant variation in morphology. A slight size difference was found to occur in no. 6 pair of both sexes.

First meiotic metaphase showed no characteristic bivalent which can be referred to as the sex chromosome. No heteropycnotic element was observed in the spermatogonial prophase.

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