Cytogenetic Studies in Lower Vertebrates, II Karyological studies of several species of frogs (Ranidae)¹

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Anurans of the family Ranidae are widely distributed rather commonly all over the world. The frogs, because of the favorite animals for biological experiments, have been subjected to cytological investigations during the past several decades. Notwithstanding that a large amount of work has been published (cf. Makino's list 1956), precise chromosome analyses in those animals have still remained rather insufficient. Before the new technical innovations opened, it was a difficult task to analyze in detail the morphological characteristics of the chromosomes through classical sectioning procedures or the squash method without pretreatment. Recent methodological advances in cytogenetics involving tissue cultures have facilitated accurate and reliable studies of chromosomes in mammals including man. This led to a tremendous increase of knowledge on cytogenetics of man and some other mammals. Under this situation, reinvestigation has seriously been requested on the chromosomes of lower vertebrates, particularly of amphibians, in order to provide more meaningful data for various studies.

Incidentally, the author has developed a simple method for studying chromosomes from bone marrow specimens by modifying the technique of Tjio and Whang (1962). The present paper deals with the karyotype analysis of several species of the Ranidae, very common in Japan, with special regard to the relationship between the karyological features and taxonomical information.

Materials and methods

The following species provided materials for the study of the diploid chromosome complement in adult males and females. 1) *Rana temporaria temporaria* L., collected in suburbs of city of Sapporo, Hokkaido. 2) *Rana temporaria ornativentris* Werner, morphologically not identical with *R. t. temporaria*, being reproductively isolated from each other; collected in Hirosaki, Aomori-ken. 3) *Rana japonica* Günther, obtained in Urawa, Saitama-ken. 4) *Rana nigromaculata* Hallowell, collected in Yonago, Tottori-ken. 5) *Rana rugosa* Schlegel, secured in Kuroishi, Aomori-ken. 6) *Rana catesbiana* Shaw, obtained in suburbs of Sakura, Chiba-ken.

Cytological preparations were made through a modified technique of Tjio and Whang (1962) for bone marrow chromosomes as follows: Bone marrow cells in femur and tibia of adult frogs were aspirated by a small syringe and were suspended in 10 ml of the amphibian Ringer's solution or of the Shah's medium (1960), containing colchicine at the dosage of 5 μ g/ml in final concentration. After collecting metaphasic cells for 2 to 3 hours at 26°C, the solution was replaced with distilled water. The cells were treated in distilled water for 30 minutes, and lightly fixed with acetic alcohol (1:3) prior to centrifugation.

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This paper is dedicated to Professor Sajiro Makino, Zoological Institute, Hokkaido University, Sapporo, in honor of his sixtieth birthday, June 21, 1966.

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The cells were then refixed with fresh fixative for 30 minutes. Fixed cells were spread onto clean slides according to a air-drying technique (Rothfels and Siminovitchi 1958). Giemsa's solution (1 part of stock solution to 20 parts of tap water) was used as stain. Karyotypes were prepared from chromosomes cut from enlarged photomicrographs of metaphase plates. Both male and female karyotypes were analyzed on the basis of 10 to 15 well-spread metaphasic cells in each species.

Results

1. Comparison of karyotypes in two subspecies of *Rana temporaria*

Two subspecies of *Rana temporaria temporaria* and *R. t. ornativentris* are somewhat different from each other in general bodily characters. The latter is distributed in Honshu, Shikoku and Kyushu, while *R. t. temporaria* is found in Hokkaido. Kawamura and Kobayashi (1959) have reported that the two subspecies are completely isolated from each other on account of hybrid sterility. No precise comparison has been made on their karyotypes between the two subspecies, except a report by Kobayashi (1962).

Both *R. t. temporaria* and *R. t. ornativentris* was found to have the diploid number of 24. The individual chromosomes were numbered in order of descending length, and divided tentatively into two main groups which consist of 5 pairs of large-sized elements (nos. 1-5) and 7 pairs of small ones (nos. 6-12). The karyotypes of *temporaria* and *ornativentris* are presented for comparison in Figs. 1 to 4. Without any possible exception, individual chromosomes are morphologically identical in the two subspecies regardless of the sex. Group 1 of the complement contains 5 pairs of large-sized chromosomes which are easily identified. Chromosomes nos. 1 and 5 are metacentric. Chromosomes nos. 2, 3, and 4 are submetacentric and each carries a slight secondary constriction in its short arms, though rather difficult in demonstration. Group 2 includes smaller submetacentric and subtelocentric elements. No. 6 chromosomes are submetacentric in appearance being most remarkable in the group. No. 9 pair is represented by subtelocentric chromosomes. As seen in Fig. 4 chromosomes no. 7 seem to be satellited, though clear demonstration of the satellite was difficult. A remarkable feature was seen in no. 11 chromosomes; a gross secondary constriction in the longer arm was found in every specimen from both *temporaria* and *ornativentris*.

Kobayashi (1962) reported, based on the squash preparations of spermatogonia, that the karyotype of *temporaria* showed a slight difference from that of *ornativentris*. Careful inspection of the karyotype of the two subspecies here considered revealed no significant difference between them.

2. Chromosome morphology of four different species of Ranidae

Male and female karyotypes of *R. japonica*, *R. nigromaculata*, *R. rugosa* and *R. catesbiana* are presented in Figs. 5 to 12.

Makino's chromosome-number list (1956) referred to a considerable amount of work on the chromosomal study of the Ranidae carried out by

Figs. 1-4. Male and female karyotypes of *Rana temporaria*, prepared from bone marrow cells. Giemsa stain. 1 and 2, *Rana temporaria temporaria*. 1, male. 2, female. 3 and 4, *Rana temporaria ornativentris*. 3, male. 4, female.

classical techniques. The diploid chromosome number of four species here concerned was described as 26 by previous authors: Kawamura (1939a, 1943)

and Kobayashi (1962) for *japonica*, Iriki (1932) and Kawamura (1939b) for *nigromaculata*, Iriki (1932) for *rugosa*, and Makino (1947a) for *catesbiana*. Some of these authors described that the chromosome morphology of the four species was similar in general appearance, but the karyotype analysis has remained in most cases rather insufficient, being not available for comparison with the results derived from the modern technique.

The present investigation made it clear that the diploid complements of the four species were characterized by 26 chromosomes which consisted of 13 pairs of homologues (Figs. 5-12). According to the examination of the size and shape, the chromosomes were tentatively classified into two groups: five large and 8 small pairs. The members of the diploid complements of the four species were arranged and numbered in order of the length in Figs. 5 to 12.

Group 1: All pairs of chromosomes in this group were apparently identical in morphology in the four species. No. 1 chromosomes are metacentric in appearance being easily identified in the complement on account of its remarkable large size. Nos. 2 and 3 chromosomes are submetacentric, similar in size, no. 3 elements being more telomeric than no. 2. Nos. 4 and 5 chromosomes are metacentric; no. 5 pair is easily identifiable because of comparatively smaller size than other elements of this group.

Group 2: With two possible exceptions, the morphological characters of chromosomes in this group are generally similar in the four species. The minor variation was observed in centromere positions. No. 6 chromosomes which are the largest element in this group are metacentric except in *nigromaculata* in which no. 6 chromosomes are possibly submetacentric (Figs. 7-8). Nos. 7, 8, 10 and 12 chromosomes are undoubtedly identified as submetacentrics, although there are some variations in arm ratio among them. Nos. 9 and 11 chromosomes are of submetacentric or subtelocentric nature. Since it is quite difficult to determine with certainty the centromere positions of nos. 9 and 11 chromosomes, their morphological features do not clearly fall into either category. So far as the present observations are concerned, no. 9 chromosomes of *japonica* is most probably of submetacentric nature, but those of the other three species are subtelocentric in appearance. No. 11 chromosomes of *japonica* and *nigromaculata* are mostly telomeric elements in each complement, while those of *rugosa* and *catesbiana* are rather submetacentric. No. 13 chromosomes are the smallest in the whole complement; in three species, *japonica*, *nigromaculata* and *rugosa*, they are obviously submetacentric, while those of *catesbiana* appear as small metacentrics.

Noticeable is the fact that the longer arms of no. 7 chromosomes of *japonica* and *nigromaculata* are characterized by the secondary constriction in each, while those of the other two species, *rugosa* and *catesbiana*, show no such structure. Recently Di Berardino (1962) and Hennen (1964) have demonstrated in *R. pipiens* and *R. sylvatica* a clear secondary constriction

on the longer arm of the corresponding submetacentric chromosomes.

Figs. 5-8. Male and female karyotypes of *Rana japonica* and *Rana nigromaculata*, prepared from bone marrow cells. Giemsa stain. 5 and 6, *Rana japonica*. 5, male; 6, female. 7 and 8, *Rana nigromaculata*. 7, male; 8, female.

Discussion

General similarity in the chromosome complements occurring among

Figs. 9-12. Male and female karyotypes of *Rana rugosa* and *Rana catesbiana*, prepared from bone marrow cells. Giemsa stain. 9 and 10, *Rana rugosa*. 9, male; 10, female. 11 and 12, *Rana catesbiana*. 11, male; 12, female.

different species of *Rana* has been pointed out by Wickbom (1945) based on the sectioned materials. The present study revealed that all species

examined here contained five pairs of prominently large-sized chromosomes without exception, although there are minor variations in centromere positions. Recent studies on the karyotypes of *Rana pipiens* by Di Berardino (1962), of *R. temporaria* and *R. japonica* by Kobayashi (1962) and *R. sylvatica* by Hennen (1964) demonstrated similar features in squashed preparations of embryonic tissues or spermatogonial cells. It is apparent in the light of the present study that *R. japonica*, *R. nigromaculata*, *R. rugosa* and *R. catesbiana* are categorized into a cytologically closely related group. Similar feature was reported to occur in *R. pipiens* and *R. sylvatica* by other investigators.

A matter of special attraction was the chromosome number of two subspecies of *R. temporaria* which were characterized by the diploid number of 24. There is a confusion on the chromosome number of *R. temporaria*, since many authors reported 26 as the diploid number of this species during the past several decades (cf. Makino's list 1956). Early papers by Witschi (1924, 1933) for the European *R. temporaria* and by Makino (1932) for the Japanese *R. temporaria* described 26 chromosomes in the diploid complement. In contrast, Kawamura (1943) reported 24 as the diploid number for the Japanese *R. temporaria*, and recently a supplementary result was presented by Kobayashi (1962). Spermatogonial cells drawn by Makino (1932, 1946) from sectioned materials showed 2 rod-shaped and 2 dot-like chromosomes. He stated that, in 83 per cent of cells analyzed, these small chromosomes were located very closely. The present study by means of current technique revealed that the rod-shaped chromosome and dot-like one were not separate entities, but one single chromosome which had a gross secondary constriction. The chromosomes discussed by Makino (1932, 1946) seem to correspond to no. 11 chromosomes described in this paper. In all probability this situation causes miscounting of the chromosome number in *R. temporaria*. Such a remarkable secondary constriction was detected in each member of no. 11 pair in two subspecies of *R. temporaria*. Further, the present study demonstrated that there was a striking similarity of karyotype between *R. t. temporaria* and *R. t. ornativentris*, in contrast to the view of Kobayashi (1962) who concluded that the karyotypes were slightly different from each other.

Recently, a question was raised whether the Japanese *R. temporaria* in Hokkaido is exactly the same as the European *temporaria*, or not, and whether *R. t. temporaria* in Hokkaido and *R. t. ornativentris* from Honshu belong, or not, to the same species (Witschi *et al.* 1958, Kawamura and Kobayashi 1959, and others). Witschi *et al.* (1958) insisted that a gross morphological study with measurements revealed a considerable difference between the Japanese *R. temporaria* and the European species, and that the Japanese species was very closely allied to *R. arvalis* in Europe. Of interest is that the chromosome number of the European *R. temporaria* is 26 (Witschi

1924, Wickbom 1945, Witschi *et al.* 1958), while that of the Japanese *R. temporaria* is 24, and morphological characters are of the *arvalis* type. Further, the chromosome number, 24, so far reported in the Ranidae is only found in *arvalis* and in the Japanese *R. temporaria*. According to extensive studies by Kawamura and his colleagues on reproductive isolation, *R. t. temporaria* from Hokkaido is completely isolated from the European *R. temporaria* by means of gametic isolation and hybrid inviability, and *R. t. ornativentris* also reproductively isolated from the European *R. temporaria*, as well as from the Japanese *R. t. temporaria* (Kawamura and Kobayashi 1960). On the above basis Kawamura (1962) proposed a change of the name of some Japanese frogs; *R. t. temporaria* should be provisionally changed into *R. chensinensis* David, and *R. t. ornativentris* be placed as a valid species *R. ornativentris*. Witschi *et al.* (1958) suggested a close relationship between the Japanese *R. temporaria* and *R. arvalis*, whereas Kawamura and Kobayashi (1959, 1960) reported that no fertilization occurred between *ornativentris* (♀) and *arvalis* (♂). Evidence presented in this study is in favor of the view of Kawamura for renaming of frogs here considered.

The chromosome number of *R. japonica*, *R. nigromaculata*, *R. rugosa* and *R. catesbiana* was established as 26 in diploid number based on classical techniques (Iriki 1932, Kawamura 1939a 1939b, 1943, Makino 1947a, and others). The present study with new technique has made clear that the karyotypes of the four species in general are indistinguishable from one another without actual analysis, but a careful inspection detected two noticeable features as follows: (1) no. 6 chromosomes of *R. nigromaculata* are of submetacentric nature, while the corresponding ones of the other three species are obviously metacentric in appearance; (2) the smallest chromosomes of *R. catesbiana* (no. 13) appear as metacentrics, while those of the other three species are small submetacentrics. It should be mentioned that the karyotypes of *R. japonica* and *R. rugosa* are basically the same. The karyotype of *R. japonica* has recently been described by Kobayashi (1962), but the results of the present study have failed to supplement those of Kobayashi, though precise comparison is impossible.

Finally, some comments should be made on the sex chromosomes in amphibians. The sex-determining mechanism of amphibians has long been a matter of controversy, involving the presence or absence of a heteromorphic pair of (sex) chromosomes. For instance, the presence of sex chromosomes was insisted by Witschi (1929, 1933), while Makino (1932) failed to demonstrate them. Makino (1947b) in an urodelan, *Hynobius retardatus*, reported the absence of a heteromorphic pair of chromosomes in cells from both sexes. Recent investigations of Freed and Cole (1961) on *Rana pipiens*, of Seto *et al.* (1964) on *Necturus maculosus*, and of Seto and Makino (1964) on *Hyla arborea japonica* have shown that there was no identifiable sex chromosomes in either male or female karyotypes, while female heterogamety

has been reported to occur in *Xenopus laevis* by Weiler and Ohno (1962). Precise karyotype analyses of six members of the Ranidae in the present study revealed the absence of unequal pair of chromosomes in both sexes.

Summary

The karyotype analysis was undertaken in six members of the Ranidae based on current technique with adult bone marrow cells, with special regard to the relationship between the karyotype and taxonomical feature.

Karyotypes of two subspecies of *R. temporaria temporaria* and *R. t. ornativentris* were basically identical with each other.

The karyotypes of *R. japonica*, *nigromaculata*, *rugosa* and *catesbiana* were generally similar from one another in both number and morphology, though two identifiable features were found to occur in *nigromaculata* and *catesbiana*.

Comparison of the karyotypes in both sexes demonstrated no significant morphological difference, no heteromorphic pair having been detected either in males or in females.

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