Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ. 8: 95–108 (1986)

# Phylogenetic Relationships of Several Japanese Frog Species as Determined by a Serological Method

By

Akihiko KASHIWAGI

*Laboratory for Amphibian Biology, Faculty of Science, Hiroshima University, Hiroshima 730, Japan*

(With 4 Text-figures)

## INTRODUCTION

Since more than 40 years ago, phylogenetic divergence of Japanese anurans has been studied by KAWAMURA (1943, 1950), KAWAMURA and KOBAYASHI (1959, 1960), KOBAYASHI (1962a, b), KAWAMURA and NISHIOKA (1962, 1963, 1981), *etc.* in brown frogs, and by MORIYA (1951, 1954), KAWAMURA and NISHIOKA (1975, 1978, 1979), KAWAMURA, NISHIOKA and KURAMOTO (1972), *etc.* in pond frogs. All of these authors made crossing experiments in each frog group and confirmed that the members are isolated from each other by postgametic isolating mechanisms such as hybrid inviability and hybrid sterility. From these hybridization experiments, it became evident that two species, such as *Rana nigromaculata* and *Rana brevipoda*, or *Rana japonica* and *Rana ornativentris*, are closely related in reproduction, as they produce viable hybrids, although they are completely or incompletely sterile.

Hybridization experiments in various anurans other than those in the brown or pond frog species have been reported by KAWAMURA (1956) and KURAMOTO (1974). According to these authors, no cleavage usually occurred in intergeneric crossings except those between closely allied genera such as *Rana* and *Rhacophorus*. In many interspecific crossings, no cleavage occurred or fertilized eggs died at the blastula or gastrula stage. Thus, it was almost impossible to surmise the phylogenetic relationships between these different anuran species or genera on the basis of crossing experiments.

The karyotypes of allied species were compared with each other by NISHIOKA, OKUMOTO and RYUZAKI (1986) in pond frogs and by NISHIOKA, OKUMOTO, UEDA and RYUZAKI (1986) in brown frogs from Japan, Korea, Taiwan, Europe and America. OKUMOTO (1977) compared the karyotypes of four *Rhacophorus* species distributed in Japan with one another. The karyotypes of most of the other Japanese anurans remarkably differ from those of the above species.

The present author attempted to clarify the phylogenetic relationships of eight anuran species by utilizing the immunological method. These species are closely or remotely related to one another in taxonomy.

## MATERIALS AND METHODS

The frogs used in the present study are eight species belonging to three families. Their specific names, numbers of frogs used and the localities where the frogs were collected are shown in Table 1. Of these frogs, *Rana nigromaculata* and *Rana brevipoda* are very closely allied pond frog species, while *Rana japonica* and *Rana ornativentris* are brown frog species. *Rana rugosa* and *Rana limnocharis* are small, dark frog species. They are somewhat similar to each other in appearance, while they distinctly differ from the pond frog and brown frog species in appearance. All the frogs other than *Bombina orientalis* are distributed in Honshu, Shikoku and Kyushu of Japan. *Bombina orientalis* is distributed in Korea and eastern China.

TABLE 1
Species names, localities and numbers of specimens

| Family | Species | Locality | Number | |
|---|---|---|---|---|
| | | | Female | Male |
| Ranidae | *Rana nigromaculata* | Bingo-ochiai | 10 | 1 |
| | *Rana brevipoda* | Kurashiki | 7 | 6 |
| | *Rana japonica* | Hiroshima | 8 | 7 |
| | *Rana ornativentris* | Ooita | 8 | 8 |
| | *Rana rugosa* | Hiroshima | 10 | 10 |
| | *Rana limnocharis* | Hiroshima | 10 | 10 |
| Hylidae | *Hyla arborea japonica* | Bingo-ochiai | 10 | 10 |
| Discoglossidae | *Bombina orientalis* | Kodongsan, Korea | 10 | 10 |

Blood samples are obtained from the hearts of frogs under ether anesthesia by drawing the blood into a pipette which contains a small quantity of heparin solution. The blood is centrifuged at 3,000 r.p.m. for 10 minutes at room temperature to separate serum. The serum is preserved at −20°C until use.

### 1. Preparation of four kinds of immunizing antigens from *Rana nigromaculata*

Albumin is prepared from 0.8 ml of serum removed from a single male according to a modification of the method described by PETERS (1962). (1) The precipitate obtained from a serum fraction without globulins by lowering the pH with acetic acid is dissolved in RINGER's solution for the adult amphibian and dialyzed against the same solution until a negative test for sulfate ion is obtained with $BaCl_2$. (2) The dialyzed fraction is concentrated by suction filtration through a collodion bag (Membranefilter Gesellschaft, Göttingen). (3) Further purification of albumin is accomplished by acrylamide gel disc electrophoresis (ORNSTEIN, 1964; DAVIS, 1964). (4) After electrophoresis, the albumin bands are cut out with a razor and the albumin is eluted with a small amount of 0.01 M phosphate buffer of pH 7.0 containing 0.85% NaCl.

$\alpha_1$-Acid glycoprotein is separated from 0.8 ml of serum of a single male by fractional saltingout with ammonium sulfate according to WEIMER, MEHL and

WINZLER (1950). The precipitate is dissolved in a small amount of phosphate buffer, dialyzed and concentrated.

Transferrin is prepared from 0.7 ml of serum of a single male by rivanol precipitation (SUTTON and KARP, 1965) and thin layer acrylamide gel electrophoresis (MORIWAKI, SADAIE and HIRASAWA, 1974). After electrophoresis, the transferrin band is cut out and the transferrin is eluted with phosphate buffer.

Gamma globulin is prepared from 0.8 ml of serum of a single male by fractional saltingout with ammonium sulfate according to the method of SAKAGUCHI (1970). The precipitate is dissolved in phosphate buffer, dialyzed and concentrated.

### 2. Preparation of antisera

Eight healthy female rabbits are used to produce antisera. They are divided into four groups each consisting of two animals. The rabbits of each group are injected with one of the four serum protein extracts. Thus, they are immunized to a protein prepared from a single male *Rana nigromaculata*. Just before use, the extracts are cleared by 0.45 $\mu$ millipore filters. After obtaining the control blood, each rabbit receives an initial intramuscular injection of 0.6 ml of the protein mixed with 0.6 ml of the complete FREUND's adjuvant (Difco Co.). Three weeks later, it receives a 0.5 ml subcutaneous booster injection of the protein. Three weeks after the booster injection, a blood sample is obtained from the marginal vein of the ear. The sample is then allowed to clot for three hours at room temperature. Antiserum is separated by centrifugation and stored at $-20°C$ after addition of sodium azide to a final concentration of 0.1%.

### 3. Diffusion in agar-gel

Double diffusion in agar-gel is performed following the technique of GOODMAN and MOORE (1971). To each OUCHTERLONY reaction plate 0.1 ml of rabbit antisera and 0.1 ml of frog sera both of which have been diluted to $1/6 \sim 1/32$ in protein concentration are added. Plates are kept at room temperature and reactions are observed for two days under fluorescent light. After reactions are completed, unreacted proteins are removed by washing the gels in several changes of phosphate buffer for 24 hours. Gel diffusion patterns are interpreted according to GOODMAN (1962a, 1963).

## OBSERVATION

### *I. Albumin*

A total of 144 gel diffusion tests were made for serum albumin. A single distinct precipitin line was produced in the homologous reaction between antiserum and antigen (Fig. 1). The lines of *Rana nigromaculata* and *Rana brevipoda* antigens were completely fused with each other. Each of these antigens formed a medium spur in reaction with *Rana rugosa* antigen, while they each formed long spurs against *Rana japonica* and *Rana ornativentris* antigens. *Rana rugosa*

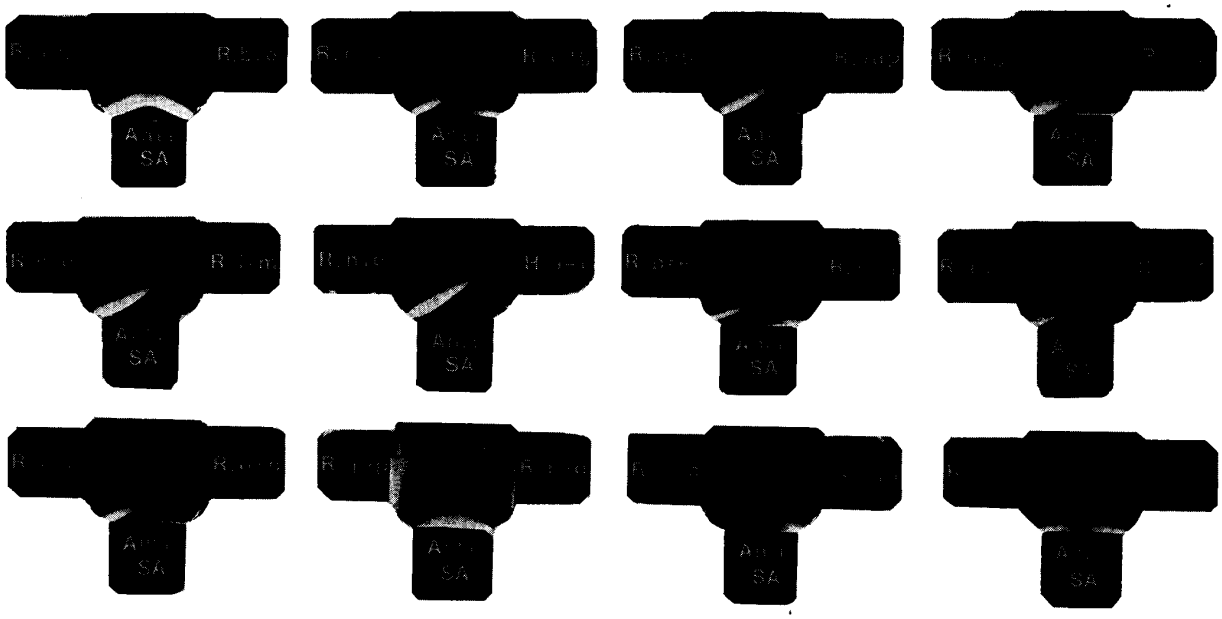

Fig. 1. Comparison between the albumins of two frog species in an OUCHTERLONY reaction plate, developed by rabbit antiserum to *Rana nigromaculata* serum albumin. ×1

Anti-SA, Rabbit antiserum to *Rana nigromaculata* serum albumin. R. nig, *Rana nigromaculata* serum. R. bre, *Rana brevipoda* serum. R. rug, *Rana rugosa* serum. R. jap, *Rana japonica* serum. R. orn, *Rana ornativentris* serum. R. lim, *Rana limnocharis* serum. H. a–j, *Hyla arborea japonica* serum.

antigen formed medium spurs against *Rana japonica* and *Rana ornativentris* antigens, while the latter two showed complete fusion. The albumins of *Rana limnocharis*, *Hyla arborea japonica* and *Bombina orientalis* produced no discernible reactions with each other as well as with any antigen of the preceding species.

These results seem to show that *Rana nigromaculata* is serologically identical with *Rana brevipoda*, and that *Rana rugosa* is intermediate in relationship between these two species and *Rana japonica* and *Rana ornativentris*. The latter two species are very similar to each other. *Rana limnocharis* is most distantly related to the other five *Rana* species. No relationship exists between the six species of Ranidae and the two other species, *Hyla arborea japonica* and *Bombina orientalis*.

## *II. $\alpha_1$-Acid glycoprotein*

A total of 108 gel diffusion tests were made. One upper distinct precipitin line and three lower, fainter precipitin lines were observed in each of the homologous reactions (Fig. 2). The presence of such double precipitin lines may be a result of impurity of the $\alpha_1$-acid glycoprotein used as the immunizing antigen. The upper of these lines was selected as the basis of comparison. The lines of *Rana nigromaculata* and *Rana brevipoda* antigens were completely fused with each other. Each of them formed a medium spur in reaction with *Rana rugosa* antigen, while each formed long spurs against *Rana japonica* and *Rana ornativentris* antigens. A very long spur was formed against *Rana limnocharis* antigen. On the other hand, the upper line of *Rana rugosa* antigen formed medium spurs against *Rana*

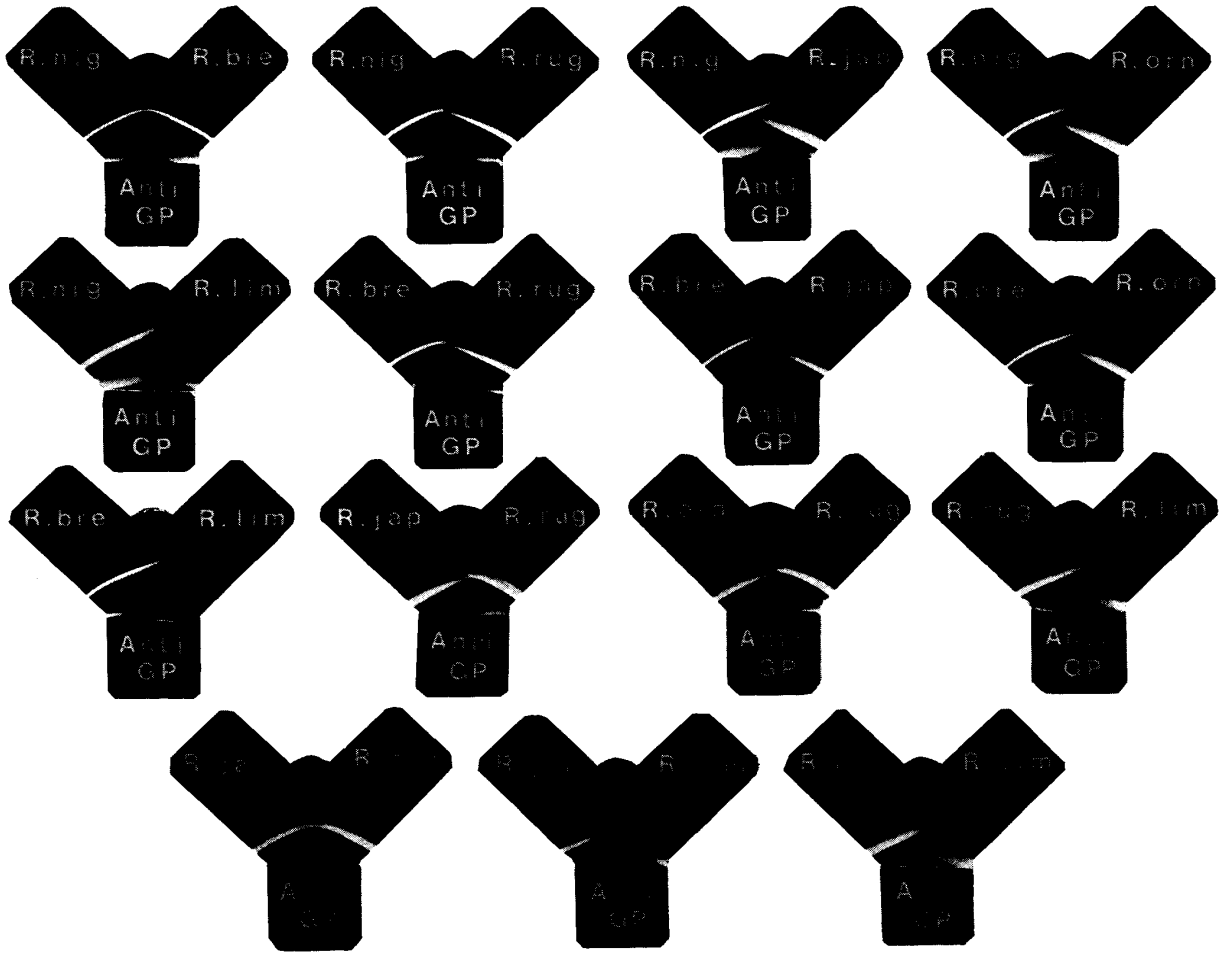

Fig. 2. Comparison between the $\alpha_1$-acid glycoproteins of two frog species in an OUCHTERLONY reaction plate, developed by rabbit antiserum to *Rana nigromaculata* serum $\alpha_1$-acid glycoprotein. ×1

Anti-GP, Rabbit antiserum to *Rana nigromaculata* serum $\alpha_1$-acid glycoprotein. R. nig, *Rana nigromaculata* serum. R. bre, *Rana brevipoda* serum. R. rug, *Rana rugosa* serum. R. jap, *Rana japonica* serum. R. orn, *Rana ornativentris* serum. R. lim, *Rana limnocharis* serum.

*japonica* and *Rana ornativentris* antigens and a very long spur against *Rana limnocharis* antigen. The lines of *Rana japonica* and *Rana ornativentris* antigens were completely fused with each other, while they formed a very long spur against *Rana limnocharis* antigen. The $\alpha_1$-acid glycoproteins of *Hyla arborea japonica* and *Bombina orientalis* produced no discernible reactions between each other or with those of the six *Rana* species.

These results seem to show that *Rana nigromaculata* is serologically identical with *Rana brevipoda* and somewhat differs from *Rana rugosa*. *Rana japonica* and *Rana ornativentris* are closely related to each other, while they are somewhat remotely related to *Rana rugosa* and more remotely related to *Rana nigromaculata* and *Rana brevipoda*. *Rana limnocharis* is most remotely related to the other *Rana* species. *Hyla arborea japonica* and *Bombina orientalis* completely differ from all the six *Rana* species examined.

## *III. Transferrin*

A total of 134 diffusion tests were made. A single precipitin line was observed in each of the homologous reactions (Fig. 3). The lines of *Rana nigromaculata* and *Rana brevipoda* antigens were completely fused with each other, while each of them formed long spurs against *Rana limnocharis*, *Rana japonica*, *Rana ornativentris* and *Rana rugosa* antigens. The line of *Rana limnocharis* antigen formed medium spurs against *Rana japonica*, *Rana ornativentris* and *Rana rugosa* antigens. The lines of *Rana japonica* and *Rana ornativentris* antigens were completely fused with each other, while they formed a short spur against *Rana rugosa* antigen. The transferrins of *Hyla arborea japonica* and *Bombina orientalis* showed no discernible reactions with those of the six *Rana* species.

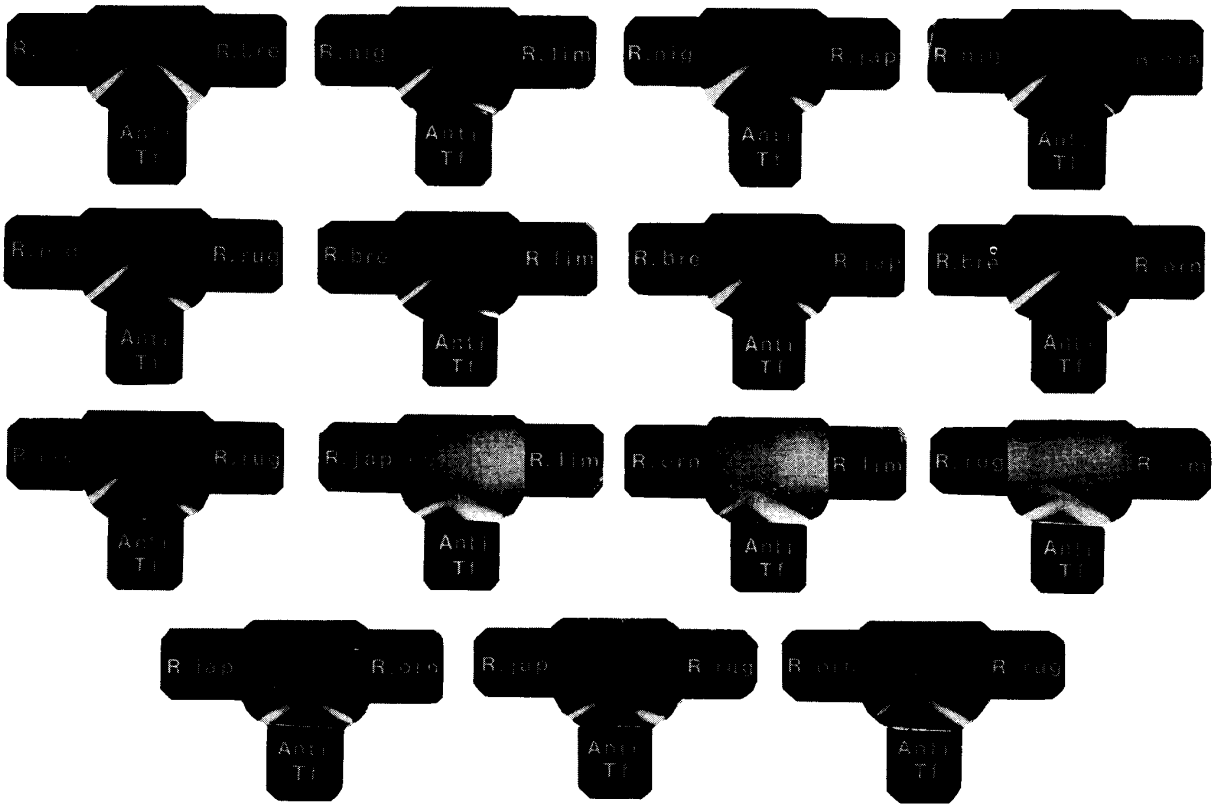

Fig. 3. Comparison between the transferrins of two frog species in an OUCHTERLONY reaction plate, developed by rabbit antiserum to *Rana nigromaculata* serum transferrin. ×1

Anti-Tf, Rabbit antiserum to *Rana nigromaculata* serum transferrin. R. nig, *Rana nigromaculata* serum. R. bre, *Rana brevipoda* serum. R. lim, *Rana limnocharis* serum. R. jap, *Rana japonica* serum. R. orn, *Rana ornativentris* serum. R. rug, *Rana rugosa* serum.

These results seem to show that *Rana nigromaculata* is serologically identical with *Rana brevipoda*. *Rana limnocharis* is intermediate in relationship between these two species and *Rana japonica* and *Rana ornativentris*. The latter two species are serologically very similar to each other and somewhat differ from *Rana rugosa*. With respect to transferrin, *Rana rugosa* is most remotely related to the other *Rana*

species. *Hyla arborea japonica* and *Bombina orientalis* completely differ from all the six *Rana* species examined.

### *IV. Gamma globulin*

A total of 160 gel diffusion tests were made. Seven precipitin lines were observed in each of the homologous reactions (Fig. 4). Five of these lines were distinct, while the other two were very faint. The presence of multiple precipitin lines is probably ascribable to incomplete purification of the gamma globulin used as the immunizing antigen. The outer three of the five distinct lines were

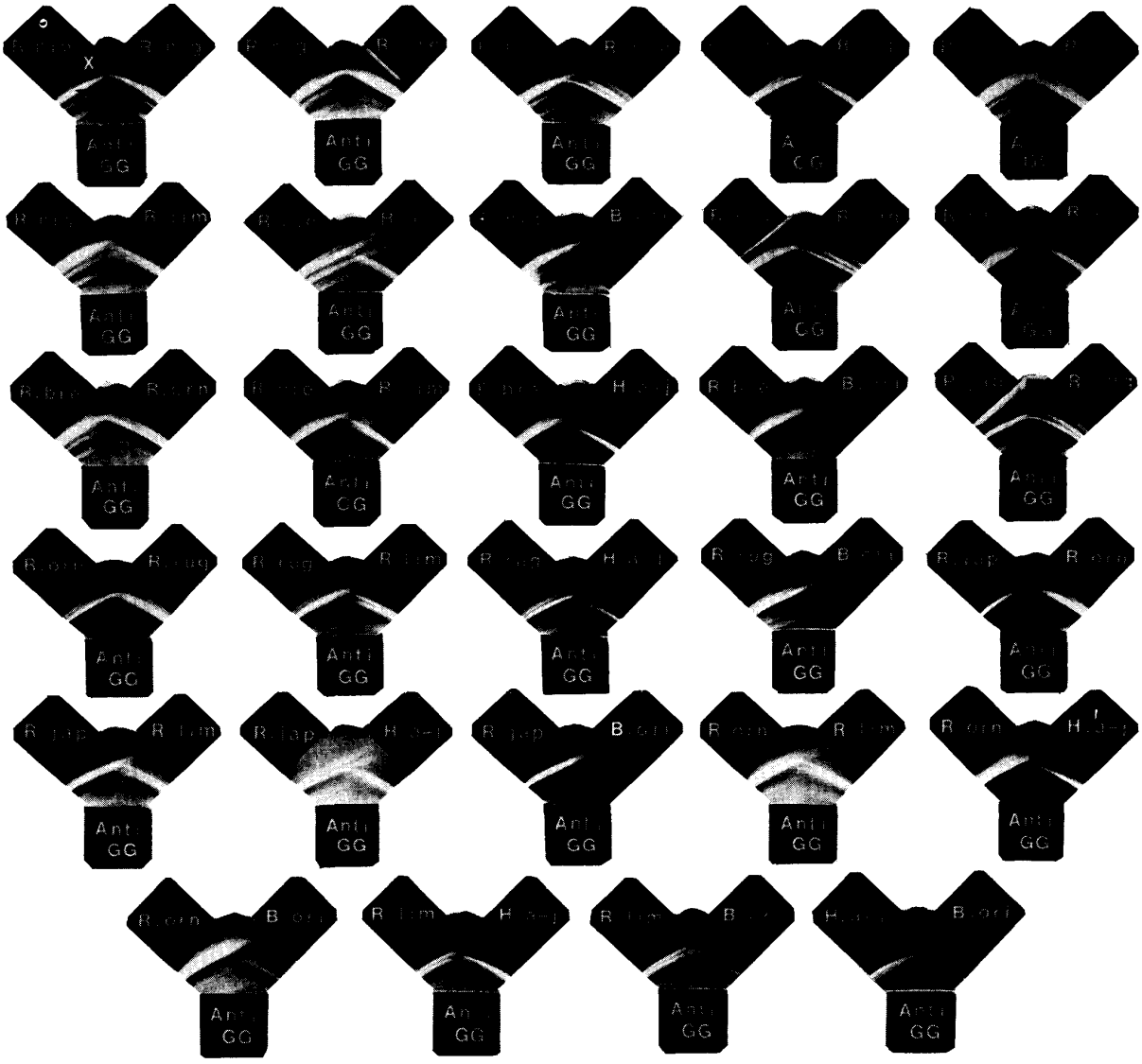

Fig. 4. Comparison between the gamma globulins of two frog species in an OUCHTERLONY reaction plate, developed by rabbit antiserum to *Rana nigromaculata* serum gamma globulin. ×0.8

Anti-GG, Rabbit antiserum to *Rana nigromaculata* serum gamma globulin. R. nig, *Rana nigromaculata* serum. R. bre, *Rana brevipoda* serum. R. rug, *Rana rugosa* serum. R. jap, *Rana japonica* serum. R. orn, *Rana ornativentris* serum. R. lim, *Rana limnocharis* serum. H. a-j, *Hyla arborea japonica* serum. B. ori, *Bombina orientalis* serum.

selected for comparison and designated from the outer to the inner as X, Y and Z for convenience' sake.

The outermost precipitin line X of *Rana nigromaculata* antigen formed very short spurs against *Rana brevipoda*, *Rana rugosa* and *Rana ornativentris* antigens. The lines of *Rana brevipoda* and *Rana ornativentris* antigens were completely fused with that of *Rana rugosa*. The line of *Rana brevipoda* antigen formed a very short spur against *Rana ornativentris* antigen. The antigens of *Rana japonica*, *Rana limnocharis*, *Hyla arborea japonica* and *Bombina orientalis* all failed to react with each other.

The middle precipitin line Y of *Rana nigromaculata* was completely fused with that of *Rana brevipoda*, while it formed spurs against all the other antigens. The line of *Rana brevipoda* was completely fused with the lines of the other five *Rana* species, and formed spurs against *Hyla arborea japonica* and *Bombina orientalis* antigens. The line of *Rana rugosa* was completely fused with those of *Rana brevipoda*, *Rana japonica* and *Rana ornativentris* antigens, while it formed spurs against *Rana limnocharis*, *Hyla arborea japonica* and *Bombina orientalis* antigens. The line of *Rana japonica* was completely fused with those of *Rana brevipoda*, *Rana rugosa* and *Rana ornativentris* antigens, while it formed spurs against *Rana limnocharis*, *Hyla arborea japonica* and *Bombina orientalis* antigens. The line of *Rana ornativentris* antigen was completely fused with those of *Rana brevipoda*, *Rana rugosa* and *Rana japonica* antigens, while it formed spurs against *Rana limnocharis*, *Hyla arborea japonica* and *Bombina orientalis* antigens. The line of *Rana limnocharis* antigen was completely fused with that of *Rana brevipoda* antigen, while it formed spurs against *Hyla arborea japonica* and *Bombina orientalis* antigens. The line of *Hyla arborea japonica* antigen formed a spur against *Bombina orientalis* antigen. The antigen of *Bombina orientalis* showed very faint precipitin lines in all combinations with the other frog species.

The innermost precipitin line Z of *Rana nigromaculata* antigen was completely fused with those of *Rana brevipoda* and *Rana rugosa* antigens, while it formed spurs against all the other antigens. The line of *Rana brevipoda* antigen was completely fused with that of *Rana nigromaculata* antigen, while it formed spurs against *Rana japonica*, *Rana ornativentris*, *Rana limnocharis*, *Hyla arborea japonica* and *Bombina orientalis* antigens. *Rana brevipoda* and *Rana rugosa* antigens formed small bilateral spurs. The spur of *Rana brevipoda* antigen was slightly longer than that of *Rana rugosa* antigen. The line of *Rana rugosa* antigen was completely fused with those of *Rana nigromaculata*, *Rana japonica* and *Rana ornativentris* antigens, while it formed spurs against *Rana limnocharis*, *Hyla arborea japonica* and *Bombina orientalis* antigens. The line of *Rana japonica* antigen was completely fused with those of *Rana rugosa* and *Rana ornativentris* antigens, while it formed spurs against *Rana limnocharis*, *Hyla arborea japonica* and *Bombina orientalis* antigens. The line of *Rana ornativentris* antigen was completely fused with those of *Rana rugosa* and *Rana japonica* antigens, while it formed spurs against *Rana limnocharis*, *Hyla arborea japonica* and *Bombina orientalis* antigens. The lines of *Rana limnocharis* and *Hyla arborea japonica* antigens were completely fused with each other, while each of these antigens formed a spur against *Bombina orientalis* antigen. On the whole, the precipitin lines of

*Bombina orientalis* antigen were very faint.

The foregoing results seem to show that on the basis of antigens represented by precipitin line X, *Rana nigromaculata* somewhat differs from *Rana brevipoda*, while *Rana brevipoda* and *Rana rugosa* are very similar to each other. *Rana nigromaculata* is more closely related to *Rana ornativentris* than to *Rana japonica* and *Rana limnocharis*. All the six *Rana* species completely differ from *Hyla arborea japonica* and *Bombina orientalis*. On the other hand, the serum antigens represented by precipitin lines Y and Z seem to show that *Rana nigromaculata* is serologically identical with *Rana brevipoda*, that *Rana rugosa*, *Rana japonica* and *Rana ornativentris* are very similar to one another and are intermediate between *Rana nigromaculata* and *Rana limnocharis*, and that *Hyla arborea japonica* is more closely related to the *Rana* species than to *Bombina orientalis*.

## DISCUSSION

Biochemical and immunological methods have been applied to solve various problems in amphibian taxonomy and evolution (DESSAUER and FOX, 1956, 1964; DESSAUER, FOX and HARTWIG, 1962; HEBARD, 1964). Electrophoretic analysis of blood proteins furnishes reliable information on hybridization and introgression in the genus *Bufo* (GUTTMAN, 1967, 1969; BROWN and GUTTMAN, 1970). NISHIOKA, UEDA and SUMIDA (1981) have examined the electrophoretic patterns and allele frequencies at each locus of five enzymes in *Rana nigromaculata*, *Rana brevipoda brevipoda* and *Rana brevipoda porosa* distributed in Japan and assumed that introgression is occurring. UZZELL and GOLDBLATT (1967) investigated the serum proteins of four species of the *Ambystoma jeffersonianum* complex, including two diploid and two triploid species, and confirmed that the latter are allotriploids evolved between the former two species. The assumption that *Rana esculenta* is a hybrid "species" between *Rana ridibunda* and *Rana lessonae* was made by electrophoretic analyses of plasma and tissue proteins (TUNNER, 1972, 1973; ENGELMANN, 1972, 1974; UZZELL and BERGER, 1975; TUNNER and DOBROWSKY, 1976; VOGEL and CHEN, 1976, 1977; EBENDAL, 1977).

Phylogenetic relationship in amphibians has been examined by the microcomplement fixation technique in which antisera were all directed to purified serum proteins and tissue enzymes (SALTHE and KAPLAN, 1966; WALLACE, KING and WILSON, 1973; WAKE, MAXON and WURST, 1978). This technique developed by WASSERMAN and LEVINE (1961) has been utilized in taxonomic investigations as it has several advantages. It requires 100 to 1,000 times less antigen and antiserum than conventional methods, and moreover it can detect minor structural differences between antigens.

The agar-gel precipitin technique used in the present study is also valuable in providing information about phylogenetic relationships (GOODMAN, 1960, 1962a, b, 1963, 1964; MOORE and GOODMAN, 1968; GOODMAN and MOORE, 1971).

From the present study using the agar-gel precipitin technique, the following results were obtained. The rabbit antisera prepared against albumin, $\alpha_1$-acid

glycoprotein, transferrin and gamma globulin of *Rana nigromaculata* show that these antigens are identical with those of *Rana brevipoda*, except for one component of gamma globulin. Two brown frog species, *Rana japonica* and *Rana ornativentris*, are also very similar to each other in these four antigens. The antigens of *Rana rugosa* are intermediate in affinity with *Rana nigromaculata* between these two groups of species. The antigens of *Rana limnocharis* most remarkably differ from those of all the other *Rana* species. The antigens of *Hyla arborea japonica* seem to be closer to those of Ranidae than to those of *Bombina orientalis*. These results are in well accord with those reported by WALLACE, KING and WILSON (1973), who clarified albumin differences among many ranid frogs by micro-complement fixation with antisera against albumin. They also indicated that the antigens of *Rana limnocharis* remarkably differ from those of *Rana nigromaculata*, *Rana brevipoda*, *Rana rugosa* and *Rana japonica*. However, the transferrin of *Rana limnocharis* is comparatively similar to that of *Rana nigromaculata* in contrast to the other antigens, while the transferrin of *Rana rugosa* remotely differs from that of *Rana nigromaculata* in contrast to the other antigens. This seems to indicate that *Rana limnocharis* shares some antigenic configuration with *Rana nigromaculata* which *Rana rugosa* lacks.

*Rana nigromaculata* and *Rana brevipoda* are very closely allied. OKADA (1931) first classified these two species as subspecies of a single species. On the basis of MORIYA's extensive work (1954, 1960a, b), KAWAMURA (1962) proposed that they are different species. Karyotypes of these two sibling species have been examined by NISHIOKA (1972). The results showed that there are minute differences between them in numerical values of centromere positions as well as relative lengths of some chromosomes. According to OHTANI (1975), some lampbrush chromosomes of *Rana nigromaculata* are somewhat different from those of *Rana brevipoda* in relative length and the kind and position of landmarks. In the present study, it was found that *Rana nigromaculata* are serologically very similar to *Rana brevipoda* in albumin, $\alpha_1$-acid glycoprotein and transferrin, while *Rana nigromaculata* somewhat differs from *Rana brevipoda* in gamma globulin.

GOODMAN (1960, 1962a, b, 1964) and GOODMAN and MOORE (1971) used immunodiffusion plate comparisons of serum proteins to examine phylogenetic relationships among Primates and confirmed that albumin does not show large divergencies in antigenic structure or a high degree of species specificity. On the other hand, gamma globulin exhibits marked divergences and high degree of species specificity. Thus albumin is defined as a slow-evolving protein (S), and gamma globulin as a rapid-evolving protein (R).

The same seems to be found in the study of phylogenetic relationships in anurans. The albumin of *Rana nigromaculata* shows no divergency, a medium divergency and a large divergency from that of *Rana brevipoda*, that of *Rana rugosa* and those of *Rana japonica* and *Rana ornativentris*, respectively, and reveals a very large divergency from or no relationship to the albumins of *Rana limnocharis*, *Hyla arborea japonica* and *Bombina orientalis*. Concerning two gamma globulin components represented by precipitin lines Y and Z, the gamma globulin of *Rana*

*nigromaculata* shows no divergency from *Rana brevipoda*, and slight divergencies from *Rana rugosa*, *Rana japonica* and *Rana ornativentris*. Moreover, the gamma globulin of *Rana nigromaculata* shows a medium divergency from *Rana limnocharis* and also a medium to a large divergency from *Hyla arborea japonica* and *Bombina orientalis*. According to GOODMAN's classification, the two gamma globulin components Y and Z seem to be S proteins which have evolved at a much slower rate than albumin. The remaining component X of the gamma globulin remarkably differs from the other components in behavior. The component X of *Rana nigromaculata* shows a clear divergency from that of *Rana brevipoda* as well as from those of *Rana rugosa* and *Rana ornativentris*. This component X shows very large divergencies from those of *Rana japonica* and *Rana limnocharis* and has no relationship with those of *Hyla arborea japonica* and *Bombina orientalis*. Thus, it can be said that component X is R protein which has evolved at a more rapid rate than the albumin.

## SUMMARY

1. The agar-gel precipitin technique was used to examine the serological relationships among six species of Ranidae, one species of Hylidae and one species of Discoglossidae. Four different kinds of antisera were produced in rabbits from the albumin, $\alpha_1$-acid glycoprotein, transferrin and gamma globulin of *Rana nigromaculata* serum. The production of the antisera was first stimulated by intramuscular injection of the serum proteins and complete FREUND's adjuvant, and then completed by a subcutaneous booster injection of serum proteins only.

2. Comparisons of albumin, $\alpha_1$-acid glycoprotein and gamma globulin show that within the family Ranidae, *Rana brevipoda* is most closely related to *Rana nigromaculata*, while *Rana rugosa* is the second, *Rana ornativentris* and *Rana japonica* are the third in affinity with *Rana nigromaculata*. *Rana limnocharis* is the most remotely related species to *Rana nigromaculata*. *Hyla arborea japonica* is somewhat more closely related to Ranidae than *Bombina orientalis*. Comparison of transferrin, on the other hand, shows that *Rana rugosa* is more remotely related to *Rana nigromaculata* than *Rana limnocharis* is, in contrast to the results of experiments performed with antisera against the remaining three kinds of proteins.

3. It was found that albumin differs from gamma globulin in the rate of evolution, and also that there are two types of gamma globulin components differing in the rate of evolution.

## ACKNOWLEDGMENTS

The author is especially indebted to Emeritus Professor Toshijiro KAWAMURA and Professor Midori NISHIOKA for encouragement and guidance during the course of this work and critical review of the manuscript.

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