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Electrophoretic Study on the Hemoglobin of Japanese Pond Frogs

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(With 2 Text-figures)

INTRODUCTION

It has been reported by KAWAMURA (1962) that Japanese pond frogs consist of *Rana nigromaculata nigromaculata*, *Rana brevipoda brevipoda* and *Rana brevipoda porosa*. While *Rana nigromaculata* is widely distributed on three of the four main islands of Japan, that is, Honshu, Shikoku and Kyushu, *Rana brevipoda brevipoda* is found together with *Rana nigromaculata* in the Tokai and Kinki districts of Honshu and along the shores of the eastern side of the Inland Sea. *Rana brevipoda porosa* occurs in the Kanto and Sendai plains and in most of the areas along the Shinano River. The fact that the two species are incompletely isolated reproductively from each other was elucidated by MORIYA (1954, 1960a, b), who divided Japanese pond frogs into five races mainly on the basis of their morphological characteristics. KAWAMURA and NISHIOKA (1977) have assumed that *Rana brevipoda porosa* is a population derived from *Rana brevipoda brevipoda* by introgression of genes from *Rana nigromaculata*. Although *Rana brevipoda porosa* is similar in appearance to the hybrids between *Rana nigromaculata* and *Rana brevipoda brevipoda*, it is completely fertile in contrast with the hybrids. KAWAMURA and NISHIOKA (1978) examined in detail the reproductive capacity of reciprocal hybrids and their backcross offspring in both restoration and substitution lines. During the experiments they found that some triploids were produced from female hybrids by mating with males of the parental species.

In order to elucidate biochemically the phylogenic relationship among the three forms, *Rana nigromaculata*, *Rana brevipoda brevipoda* and *Rana brevipoda porosa*, as well as the function of gene dosages in hybrids and triploids, the present author analyzed the hemoglobin of the three forms and reciprocal hybrids of the two species at various developmental stages and of auto- and allotriploids by the electrophoretic method.

MATERIALS AND METHODS

Rana nigromaculata HALLOWELL were collected from the suburbs of Hiroshima, *Rana brevipoda brevipoda* ITO from Konko-cho, Okayama Prefecture, and *Rana*

brevipoda porosa from Odawara, Kanagawa Prefecture. Ovulation was accelerated by injecting frog pituitaries. Oviducal eggs were artificially inseminated late in May. Auto- and allotriploids were produced by refrigeration of fertilized eggs at 1°C for 2.5 to 3 hours from 20 minutes after insemination (NISHIOKA, 1972). The triploidy of the tadpoles raised from these eggs was confirmed by counting the chromosome number by the squash method (MAKINO and NISHIMURA, 1952) in the tail-tip of each tadpole. For the purpose of electrophoretic analysis, tadpoles at nine developmental stages were prepared. Eight of these nine stages corresponded to stages II, V, VIII, XII, XVIII, XX, XXII and XXV of TAYLOR and KOLLROS' table, while the remaining was the stage three months after metamorphosis.

Hemoglobin samples of tadpoles and frogs were principally prepared according to Moss and INGRAM's method (1968a) with the following modifications. Five to three hundred tadpoles at each developmental stage were anesthetized in 1: 3000 solution of MS222, and then their blood was collected into a vessel containing a small amount of heparinized RINGER's solution from their heart by cutting the truncus arteriosus. The blood of juvenile and adult frogs was collected after injecting heparinized RINGER's solution into their abdominal cavity, anesthetizing them and cutting the truncus arteriosus. Blood cells were washed 3 times with RINGER's solution and frozen at −70°C. One volume of packed blood cells was lysed by adding 2 to 3 volumes of distilled water and this was centrifuged at 3000 r.p.m. for 3 minutes. The supernatant was recentrifuged at 10000 r.p.m. for 20 minutes at 1°C and then subjected to electrophoretic analysis.

Starch gel electrophoresis was carried out by SMITHIES' method (1959) with a modification suggested by IUCHI and YAMAGAMI (1969). For the starch gel electrophoresis in the present study, hydrolysed starch (Connaught, Lot No. 328–2) was utilized. The TEB buffer containing 0.9 M tris, 0.5 M boric acid and 0.02 M EDTA, pH 8.6, was used as the stock solution. This stock solution was diluted 1: 20 with distilled water for gel buffer, while it was diluted 1: 8 with distilled water for bridge buffer. A trace of KCN (0.01%) was added to each buffer. Each hemoglobin sample was previously converted to a cyanomethemoglobin by adding 0.1 to 0.2 volume of 2% $K_3Fe(CN)_6$ and 5% KCN. The electrophoretic run was made at a constant voltage of 300 V for 6 hours at 2°C. Upon completion of electrophoresis, the gel was sliced into two parts, which were stained with amido black 10B for protein and with o-dianisidine for hemoglobin, respectively. The electrophoretic patterns stained with amido black 10B were essentially the same as those stained with o-dianisidine.

The following abbreviations are used in the present report.

N A set of *Rana nigromaculata* chromosomes
(N) *Rana nigromaculata* cytoplasm
B A set of *Rana brevipoda brevipoda* chromosomes
(B) *Rana brevipoda brevipoda* cytoplasm
B^p A set of *Rana brevipoda porosa* chromosomes
(B^p) *Rana brevipoda porosa* cytoplasm

OBSERVATION

I. Frog hemoglobin

The electrophoretic patterns of adult hemoglobin of *Rana nigromaculata* (N)NN, *Rana brevipoda brevipoda* (B)BB, *Rana brevipoda porosa* (B^P)B^PB^P are shown in Figs. 1a and 2a. All these hemoglobin banbs migrated exclusively towards the anode. The hemoglobin pattern of *Rana nigromaculata* consisted of two component bands, that is, a fast migrating band that was weakly stained and a slowly migrating band that was intensely stained. The hemoglobin pattern of *Rana brevipoda brevipoda* also consisted of two bands, which were situated between the two bands of *Rana nigromaculata*. *Rana brevipoda porosa* showed the same hemoglobin pattern as that of *Rana brevipoda brevipoda*. These species and subspecies showed no sexual dimorphism in their hemoglobin patterns. The hemoglobin patterns of reciprocal hybrids between *Rana nigromaculata* and *Rana brevipoda brevipoda* consisted of four bands that were the total of the bands of both parental species. There was no difference in hemoglobin pattern between the reciprocal hybrids. No hybrid bands were found in these patterns.

The electrophoretic patterns of hemoglobin of auto- and allotriploids which were produced from *Rana nigromaculata* and *Rana brevipoda brevipoda* are shown in Figs. 1b and 2a. The hemoglobin patterns of autotriploids (N)NNN and (B)BBB closely resembled those of the diploid controls (N)NN and (B)BB, respectively. The hemoglobin patterns of reciprocal allotriploids (N)NNB and (B)BBN consisted of four bands derived from their parental species, although the two bands derived from doubled genomes were more intensely stained than the other two bands derived from a single genome.

II. Tadpole hemoglobin

The electrophoretic patterns of hemoglobin of *Rana nigromaculata, Rana brevipoda brevipoda*, reciprocal hybrids of these two species and *Rana brevipoda porosa* at four developmental stages are shown in Figs. 1 and 2. At stage VIII, middle tadpole stage, the hemoglobin pattern of *Rana nigromaculata* consisted of two bands, that is, a fast migrating band stained intensely and a slowly migrating band stained weakly, while the hemoglobin patterns of both *Rana brevipoda brevipoda* and *Rana brevipoda porosa* consisted of four bands, that is, two fast migrating and two slowly migrating bands. While the band with the highest mobility was intensely stained, the other three bands were very weakly stained. The two bands with intermediate mobility appeared to be very similar in mobility to the two bands of *Rana nigromaculata*. The hemoglobin patterns at stage VIII of reciprocal hybrids between *Rana nigromaculata* and *Rana brevipoda brevipoda* showed the total of those of both parental species; they consisted of two fast migrating bands which stained intensely and two slowly migrating bands which stained weakly (Figs. 1c and 2b). The reciprocal hybrids resembled each other in hemoglobin pattern.

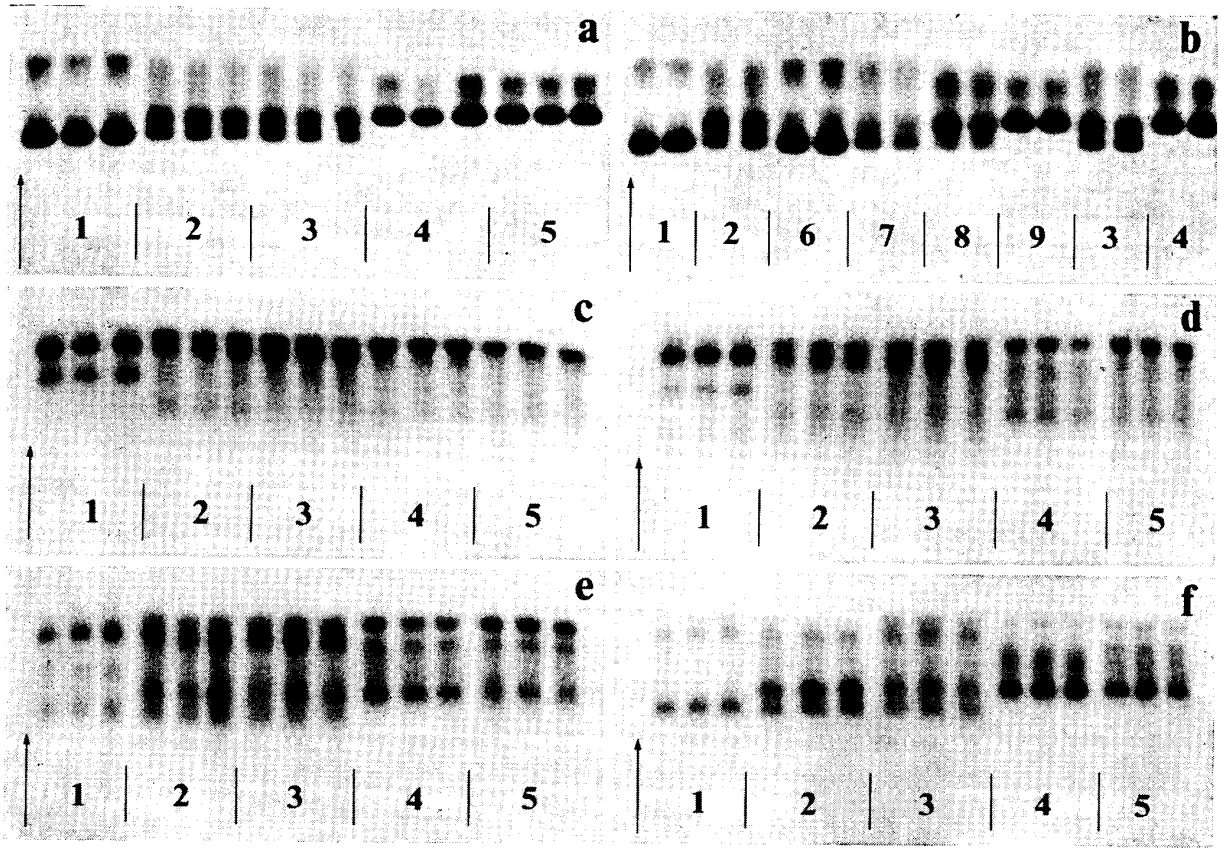

Fig. 1. Electrophoretic patterns of hemoglobin from adult frogs and tadpoles with normal and abnormal combinations of nuclear and cytoplasmic systems at four developmental stages in *Rana nigromaculata*, *R. brevipoda brevipoda* and *R. brevipoda porosa*.

a, b. Adult frogs c. Tadpoles at stage VIII d. Tadpoles at stage XX
e. Tadpoles at stage XXII f. Tadpoles at stage XXV
1, (N)NN 2, (N)NB 3, (B)BN 4, (B)BB 5, $(B^p)B^pB^p$ 6, (N)NNN 7, (N)NNB
8, (B)BBN 9, (B)BBB

The same hemoglobin patterns as found in the two species and their reciprocal hybrids at stage VIII were also observed at stages II, V, XII and XVIII.

At stage XX, immediately after the protrusion of forelegs, the slowly migrating band of *Rana nigromaculata* became somewhat weakly stained, while a weakly stained band that migrated more slowly than the preexisting two bands newly appeared (Figs. 1d and 2c). This new band was characteristic of *Rana nigromaculata* and found in the hemoglobin patterns of reciprocal hybrids. In the hemoglobin pattern of *Rana brevipoda brevipoda*, the slowest band became somewhat intensely stained at this stage.

At stage XXII, when the tail became shorter than the hind legs, the hemoglobin pattern of *Rana brevipoda brevipoda* or *Rana brevipoda porosa* consisted of three bands similar to that of *Rana nigromaculata*. Of the two slowly migrating bands of *Rana brevipoda* at stage XX, the weak but somewhat fast band disappeared at stage XXII. The other band which was the slowest in mobility among the three bands became intensely stained at this stage. The slowest band of *Rana nigro-*

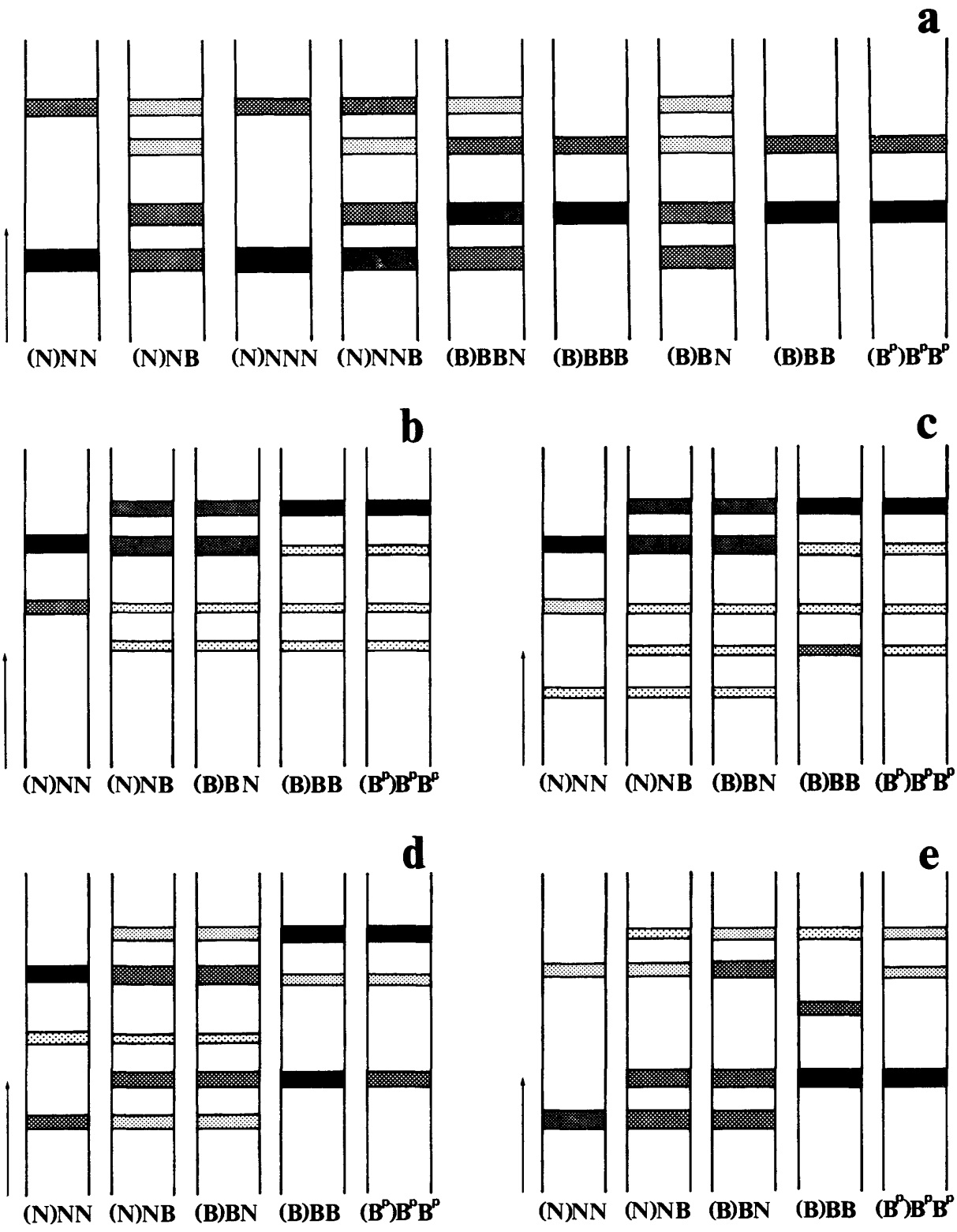

Fig. 2. Schematic representation of electrophoretic patterns of hemoglobin from adult frogs and tadpoles with normal and abnormal combinations of nuclear and cytoplasmic systems at four developmental stages in *Rana nigromaculata*, *R. brevipoda brevipoda* and *R. brevipoda porosa*.

a. Adult frogs b. Tadpoles at stage VIII c. Tadpoles at stage XX
d. Tadpoles at stage XXII e. Tadpoles at stage XXV

maculata became somewhat intensely stained, too. In reciprocal hybrids, the hemoglobin patterns showed the total of those of the two species (Figs. 1e and 2d). They consisted of five bands, as one of the three bands of one species was the same in mobility as that of the other species. There was no difference between the hemoglobin patterns of the reciprocal hybrids. No hybrid bands were found in these hemoglobin patterns.

At stage XXV, just at the completion of metamorphosis, the hemoglobin pattern of each species somewhat resembled that at the adult stage (Figs. 1f and 2e). The middle of the three bands observed in the hemoglobin pattern of *Rana nigromaculata* at stage XXII almost completely disappeared. While the fast migrating band became somewhat weakly stained, the slowly migrating band became most intensely stained. A large change also occurred in the hemoglobin patterns of *Rana brevipoda brevipoda* and *Rana brevipoda porosa* by stage XXV. The fastest band that was intensely stained became weakly stained, while the slowest band became most intensely stained. Moreover, in *Rana brevipoda brevipoda* at stage XXV, the weakly stained middle band found at stage XXII disappeared and the fast migrating band of the adult frog began to appear newly in place of this band at stage XXV. In contrast, this new band could not yet be found in *Rana brevipoda porosa* at stage XXV.

In reciprocal hybrids, there were two fast migrating bands and two slowly migrating bands. No middle band of *Rana brevipoda brevipoda* was found in the hemoglobin patterns of reciprocal hybrids. In contrast with the hybrids described earlier, there was a slight difference in hemoglobin pattern between the two kinds of hybrids at stage XXV. The two bands with the fast mobility were more weakly stained in hybrid (N)NB than those in the reciprocal hybrid (B)BN.

Three months after metamorphosis, the hemoglobin patterns of *Rana nigromaculata, Rana brevipoda brevipoda, Rana brevipoda porosa* and reciprocal hybrids between the former two forms were the same as those found at their adult stage.

DISCUSSION

GILLESPIE and CRENSHAW (1966) reported that *Rana pipiens* differed from *Rana ultricularia* (=*Rana pipiens sphenocephala*) in electrophoretic pattern of the hemoglobin. The hemoglobin of *Rana pipiens* was split into two subunits. PLATZ and PLATZ (1973) and PLATZ (1976) examined hemoglobin samples from numerous populations of *Rana pipiens* in Arizona and found that there were three distinct phenotypes correlated with morphological differences. On the basis of morphological and electrophoretic findings, they have reported that the *Rana pipiens* complex consists of several species. Electrophoretic analysis of hemoglobin has also been made in the genera *Bufo* and *Acris* by several authors. FOX, DESSAUER and MAUMUS (1961) examined the hemoglobin of two North American species, *Bufo fowleri* and *Bufo valliceps*, and their natural F_1 hybrids, and found that each species possessed a single hemoglobin. All electrophoretic fractions present in the parental species occurred in the hybrid. MARSCHLEWSKA-KOJ (1963) re-

ported that the hemoglobin of two European species, *Bufo viridis* and *Bufo bufo*, was the same, and noted that another European species, *Bufo calamita*, had also an identical component. GUTTMAN (1967) clarified the natural hybridization and introgression between *Bufo regularis* and *Bufo rangeri* in South Africa from electrophoretic analyses of hemoglobin and transferrin. He (1969, 1972, 1973) examined the hemoglobin of the *Bufo americanus* group and found ten hemoglobin components in individuals belonging to six species. While hybrids typically had two hemoglobin components, one from each parental species, the majority of hybrid toads possessed either one hemoglobin from one parent or a "hybrid" hemoglobin unique to either species. BROWN and GUTTMAN (1970) reported on the hemoglobin of Argentine toads, *Bufo arenarum* and *Bufo spinulosus*, and of the natural hybrid between these species. While each of the two species had one electrophoretic band, the hybrid had also only one band which was the same in mobility as that found in some individuals of the parental species. Similar cases of hybrids with only one hemoglobin band resembling one of the parental species were found in artificial hybrids between *Bufo perplexus* and *Bufo bocourti*, between *Bufo speciosus* and *Bufo arenarum* and between *Bufo arenarum* and *Bufo valliceps*. In cricket frogs, DESSAUER and NEVO (1969) described that the hemoglobin of *Acris gryllus* was electrophoretically distinguishable from that of *Acris crepitans*.

How the hemoglobin of tadpoles differs from that of frogs in electrophoretic mobility has been reported by many investigators in various anurans (FRIEDEN, 1961 and HERNER and FRIEDEN, 1961 in *Rana grylio, Rana catesbeiana, Rana heckscheri* and *Xenopus laevis*; CHIEFFI, SINISCALCO and ADINOLFI, 1960, in *Rana esculenta*; BAGLIONI and SPARKS, 1963, MOSS and INGRAM, 1965, 1968a, b, and BENBASSAT, 1970, 1974, in *Rana catesbeiana* and BENBASSAT, 1970 in *Rana pipiens*). A remarkable transition from tadpole to frog hemoglobin occurs during metamorphosis. As observed in these anurans, the tadpole hemoglobin of *Rana nigromaculata* and *Rana brevipoda brevipoda* distinctly differed from the frog hemoglobin of these species, respectively. The transition from tadpole to frog hemoglobin also occurred during metamorphosis.

The hemoglobin of *Rana nigromaculata* showed an electrophoretic pattern differing from that of *Rana brevipoda brevipoda* at each developmental stage from early tadpole to adult frog. The two subspecies, *Rana brevipoda brevipoda* and *Rana brevipoda porosa*, were the same in electrophoretic pattern of hemoglobin at the tadpole and frog stages except for the stage just at the completion of metamorphosis. The relationship among the hemoglobin patterns of the three forms of Japanese pond frogs seems to support the classification of these forms proposed by KAWAMURA (1962). At the middle tadpole stage, the hemoglobin patterns of the two subspecies of *Rana brevipoda* showed four bands, while that of *Rana nigromaculata* had two bands. Two of the four bands of *Rana brevipoda* were very similar in mobility to the two bands of *Rana nigromaculata*, although they were weakly stained in contrast with the latter. The fact that the two Japanese pond frog species have similar bands at the tadpole stage seems to indicate a close affinity between them.

The hemoglobin patterns of reciprocal hybrids between *Rana nigromaculata* and *Rana brevipoda brevipoda* at each developmental stage consisted of four bands that were the total of the bands of both parental species. No difference was observed in hemoglobin pattern between the reciprocal hybrids. Neither appearance of a hybrid band nor absence of the band belonging to one of the parental species occurred in the electrophoretic patterns of reciprocal hybrids. These findings of the hybrids between the two Japanese pond frog species were the same as those found in the hybrids between Northern and Southern types or between Southern and Lowland types of the *Rana pipiens* complex in Arizona (PLATZ and PLATZ, 1973), and differed from those found usually in the hybrids between different *Bufo species* (BROWN and GUTTMAN, 1970; GUTTMAN, 1972). The electrophoretic patterns of reciprocal allotriploids between *Rana nigromaculata* and *Rana brevipoda brevipoda* were similar to those expected from the combinations of the two kinds of genomes as those of reciprocal hybrids of these species. The finding that the genes for hemoglobin could typically function in accordance with the kind and number of its components in the individuals with abnormal combinations of nuclear and cytoplasmic systems seemed to show an intimate affinity of the two species.

SUMMARY

1. The hemoglobin of the following specimens was analyzed by starch gel electrophoresis in order to elucidate biochemically the phylogenic relationship among the three forms of Japanese pond frogs.

a. *Rana nigromaculata, Rana brevipoda brevipoda, Rana brevipoda porosa* and reciprocal hybrids between the former two forms at various developmental stages from early tadpole to adult frog.

b. Autotriploids and reciprocal allotriploids of *Rana nigromaculata* and *Rana brevipoda brevipoda.*

2. The hemoglobin pattern of *Rana nigromaculata* remarkably differed from those of *Rana brevipoda brevipoda* and *Rana brevipoda porosa,* while there was almost no difference between those of the two subspecies.

3. Reciprocal hybrids between *Rana nigromaculata* and *Rana brevipoda brevipoda* showed the same hemoglobin pattern, in which the electrophoretic bands consisted of the total of those of the two species.

4. The hemoglobin of auto- and allotriploid frogs showed electrophoretic patterns which were in accord with the constitution of their genomes.

5. The hemoglobin patterns of *Rana nigromaculata, Rana brevipoda brevipoda* and *Rana brevipoda porosa* at the tadpole stage differed from those at the frog stage. The transition from tadpole hemoglobin to frog hemoglobin occurred during metamorphosis. The hemoglobin patterns of reciprocal hybrids at various developmental stages consisted of the total of the electrophoretic bands of the parental species, except those just at the completion of metamorphosis.

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