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Enzymes of *Rana nigromaculata*, *Rana brevipoda*, Reciprocal Hybrids and Auto- and Allotriploids

By

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(With 6 Text-figures)

INTRODUCTION

Recently, electrophorctic analysis of proteins such as blood component and various enzymes has been extensively conducted for the purpose of clarifying the relationship between allied animal species and their speciation process. In amphibians, there are many reports on electrophoretic analysis of some proteins in *Acris*, *Bufo*, *Rana*, etc. (DESSAUER and NEVO, 1969; ROGERS, 1973; ENGELMANN, 1974; GUTTMAN, 1975; UZZELL and BERGER, 1975; UZZELL, BERGER and GÜNTHER, 1975; TUNNER and DOBROWSKY, 1976; VOGEL and CHEN, 1976, 1977). The present authors produced reciprocal hybrids and auto- and allotriploids from two sibling species, *Rana nigromaculata* and *Rana brevipoda*, and electrophoretically examined the enzymes extracted from these individuals as well as their parental species in order to clarify the biochemical affinity of these two species and the expression of genes under abnormal combinations of nuclei and cytoplasm.

In the preliminary experiment, the present authors analyzed the following 16 kinds of enzymes extracted from skeletal muscles or liver tissues of five male and five female *Rana nigromaculata* and five male and five female *Rana brevipoda*.

1. Lactate dehydrogenase (LDH)
2. Malate dehydrogenase (MDH)
3. α-Glycerophosphate dehydrogenase (α-GDH)
4. Isocitrate dehydrogenase (IDH)
5. Aspartate aminotransferase (AAT)
6. Creatine kinase (CK)
7. Phosphoglucomutase (PGM)
8. Glucose-phosphate isomerase (GPI)
9. Esterase
10. Superoxide dismutase (SOD)
11. Adenylate kinase (AK)
12. Alcohol dehydrogenase (ADH)
13. Glucose-6-phosphate dehydrogenase (G-6-PD)
14. Hexokinase (HK)

15. Glutamate dehydrogenase (GaDH)
16. Pyruvate kinase (PK)

The results showed that some differences were found between the two species in the electrophoretic patterns of eight enzymes, that is, LDH, MDH, α-GDH, IDH, G-6-PD, SOD and esterase from skeletal muscles and ADH from liver tissues. In the electrophoretic patterns of seven other enzymes, that is, AAT, CK, PK, AK, PGM and GPI from skeletal muscles and GaDH from liver tissues, no difference could be demonstrated between the two species. In the remaining HK, the existence of differences between the two species could not be determined due to obscureness of their electrophoretic patterns. Electrophoretic analyses of the two species, reciprocal hybrids and auto- and allotriploids were carried out in terms of six enzymes, LDH, MDH, α-GDH, IDH, SOD and esterase, as there were especially distinct differences in these enzymes between the two species.

In this paper, the results of electrophoretic analysis of the six kinds of enzymes from the eight kinds of individuals are reported.

MATERIALS AND METHODS

The two sibling species, *Rana nigromaculata* HALLOWELL and *Rana brevipoda* ITO were collected from the suburbs of Hiroshima and Konko-cho, Okayama Prefecture, respectively. Ovulation was accelerated by injection of frog pituitary suspension. All the matings were performed by artificial fertilization. Autotriploids (N)NNN of *Rana nigromaculata* were produced as follows: eggs fertilized with sperm of the same species were put into PETRI dish filled with water 10 minutes after fertilization, and 10~12 minutes later they were refrigerated at 1~2°C for 3~3.5 hours to suppress the extrusion of the second polar body. Allotriploids (N)NNB consisting of two genomes of *Rana nigromaculata* and one genome of *Rana brevipoda* were produced as follows: eggs of *Rana nigromaculata* fertilized with sperm of *Rana brevipoda* were refrigerated at 1~2°C for 3~3.5 hours, 20~22 minutes after fertilization. Autotriploids (B)BBB of *Rana brevipoda* and allotriploids (B)BBN consisting of two genomes of *Rana brevipoda* and one genome of *Rana nigromaculata* were produced by the same methods as those of (N)NNN and (N)NNB, except that the eggs of *Rana brevipoda* were refrigerated at 1~2°C for 2~2.5 hours.

The ploidy of interspecific hybrids and auto- and allotriploids was confirmed by counting the chromosomes by the squash method in the tail-tips removed at the tadpole stage. Each individual was reared until sexual maturity in the laboratory.

The methods of analyzing five of the six enzymes excluding SOD were the same as those described by BREWER (1970) and NISHIOKA, OHTANI and SUMIDA (1980). For the analysis of SOD, a tris-borate-EDTA buffer system of pH 8.0 (2.1 M tris, 2.0 M boric acid and 0.068 M EDTA) was used by diluting to one part in 100 for making starch-gel, and to one part in ten for the bridge.

Electrophoretic patterns of each enzyme were examined in five males and

five females of each of ten kinds of frogs, that is, field-caught *Rana nigromaculata* and *Rana brevipoda,* offspring of these frogs, reciprocal hybrids, autotriploids and allotriploids.

OBSERVATION

I. Lactate dehydrogenase (LDH)

1. *Rana nigromaculata* and *Rana brevipoda*

Electrophoretic patterns of LDH were examined in 20 *Rana nigromaculata* and 20 *Rana brevipoda.* The group of each species contained five male and five female field-caught frogs and five male and five female offspring produced from them in the laboratory. The electrophoretic pattern of LDH in *Rana nigromaculata* as well as *Rana brevipoda* consisted of five bands that migrated toward the anode (Fig. 1). Although there were no differences between males and females, remarkable differences were found between the two species. When the five bands of LDH in each of *Rana nigromaculata* and *Rana brevipoda* were named LDH-1, LDH-2, LDH-3, LDH-4 and LDH-5 in order of mobility from fast to slow, the four faster bands, LDH-1 ~ LDH-4, in *Rana nigromaculata* were distinctly faster than those in *Rana brevipoda,* while there was no difference in the mobility of band LDH-5 between the two species.

2. Reciprocal hybrids

Electrophoretic patterns of LDH were examined in ten (N)NB hybrids between female *Rana nigromaculata* and male *Rana brevipoda* and ten (B)BN hybrids of the reciprocal combination. It was found that five bands in total appeared at and between the LDH-1 sites of *Rana nigromaculata* and *Rana brevipoda* (Fig. 1). These bands were arranged at equal intervals. Two distinct bands appeared at the LDH-4 sites of the two species. Several faint bands also appeared at and between the LDH-2 sites and at and between the LDH-3 sites of the two species. Thus, 12 or 13 bands in total were found in reciprocal hybrids. Some differences were found in bands around the LDH-4 and LDH-5 sites between reciprocal hybrids.

3. Auto- and allotriploids

The LDH extracted from ten autotriploids (N)NNN of *Rana nigromaculata,* ten autotriploids (B)BBB of *Rana brevipoda,* ten allotriploids (N)NNB consisting two genomes of *Rana nigromaculata* and one genome of *Rana brevipoda* and ten of the reciprocal allotriploids (B)BBN were analyzed. The electrophoretic patterns of the two kinds of autotriploids, (N)NNN and (B)BBB, were nearly the same as those of diploid *Rana nigromaculata* and *Rana brevipoda,* respectively, although their bands were generally more intense than those of the latter. The electrophoretic pattern of allotriploids (N)NNB differed from that of the reciprocal

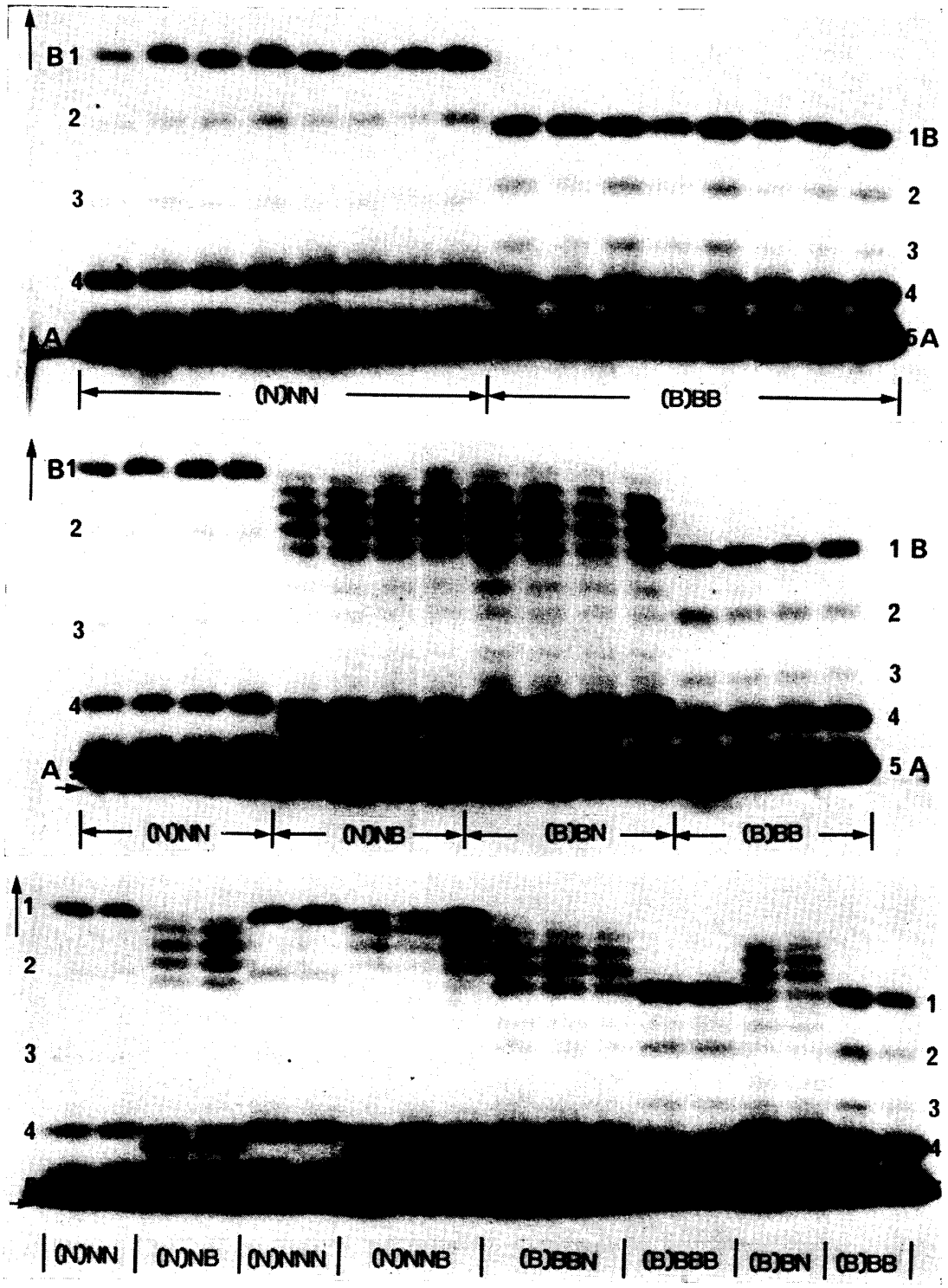


Fig. 1. Electrophoretic patterns of LDH from normal diploids, reciprocal hybrids, autotriploids and reciprocal allotriploids in *Rana nigromaculata* and *Rana brevipoda*.

(N) NN, diploid *Rana nigromaculata*
(B) BB, diploid *Rana brevipoda*
(N) NB, diploid hybrid, *Rana nigromaculata* ♀ × *Rana brevipoda* ♂
(B) BN, diploid hybrid, *Rana brevipoda* ♀ × *Rana nigromaculata* ♂
(N) NNN, autotriploid *Rana nigromaculata*
(B) BBB, autotriploid *Rana brevipoda*
(N) NNB, allotriploid consisting of two *Rana nigromaculata* genomes and one *Rana brevipoda* genome
(B) BBN, allotriploid consisting of two *Rana brevipoda* genomes and one *Rana nigromaculata* genome

allotriploid, (B)BBN, as the bands controlled by two genomes of one species were remarkably more intense than those controlled by one genome of the same species (Fig. 1).

II. Malate dehydrogenase (MDH)

1. *Rana nigromaculata* and *Rana brevipoda*

MDH was analyzed in 20 *Rana nigromaculata* and 20 *Rana brevipoda*. The group of each species contained five male and five female field-caught frogs and five male and five female offspring produced from them in the laboratory. Electrophoretic patterns of MDH in the two species consisted of four bands, two major and two subordinate, which migrated toward the anode and showed remarkable differences between the two species (Fig. 2). However, there were no differences between males and females. When the four bands of MDH in each species were named MDH-1, MDH-2, MDH-3 and MDH-4 in order of mobility from fast to slow, the MDH-4 bands of the two species were the same in terms of mobility. The other three bands of *Rana nigromaculata* were somewhat faster than those of *Rana brevipoda* and differed from the latter in intensity of each band. While bands MDH-2 and MDH-4 were major in *Rana nigromaculata,* bands MDH-3 and MDH-4 were major in *Rana brevipoda.*

2. Reciprocal hybrids

Electrophoretic patterns of reciprocal hybrids were examined in five male and five female (N)NB hybrids and five male and five female (B)BN hybrids. It was found that they always consisted of four bands (Fig. 2). The slowest of these four bands was the same as band MDH-4 of each species. Of the other three bands, one was situated at the MDH-2 site of *Rana nigromaculata* and another was at the MDH-3 site of *Rana brevipoda,* while the remaining one was a hybrid band formed between the MDH-2 and MDH-3 sites. The fastest MDH-1 bands of reciprocal hybrids were half of band MDH-2 of *Rana nigromaculata* in intensity and appeared at the site of the latter, while the MDH-2 bands of reciprocal hybrids were the sum of half of band MDH-2 of *Rana nigromaculata* and half of band MDH-3 of *Rana brevipoda* in intensity and situated at the site between these two bands. The MDH-3 bands of reciprocal hybrids were half of band MDH-3 of *Rana brevipoda* in intensity and situated at the site of the latter.

The four bands of the (N)NB hybrids were the same as those of the reciprocal hybrids in mobility. However, band MDH-1 of the (N)NB hybrids was slightly more intensive than that of the reciprocal hybrids, while band MDH-3 of the (B)BN hybrids was slightly more intensive than that of the reciprocal hybrids.

The four subordinate bands, MDH-1 and MDH-3 of *Rana nigromaculata* and MDH-1 and MDH-2 of *Rana brevipoda,* were so faint that they could hardly be recognized in reciprocal hybrids.

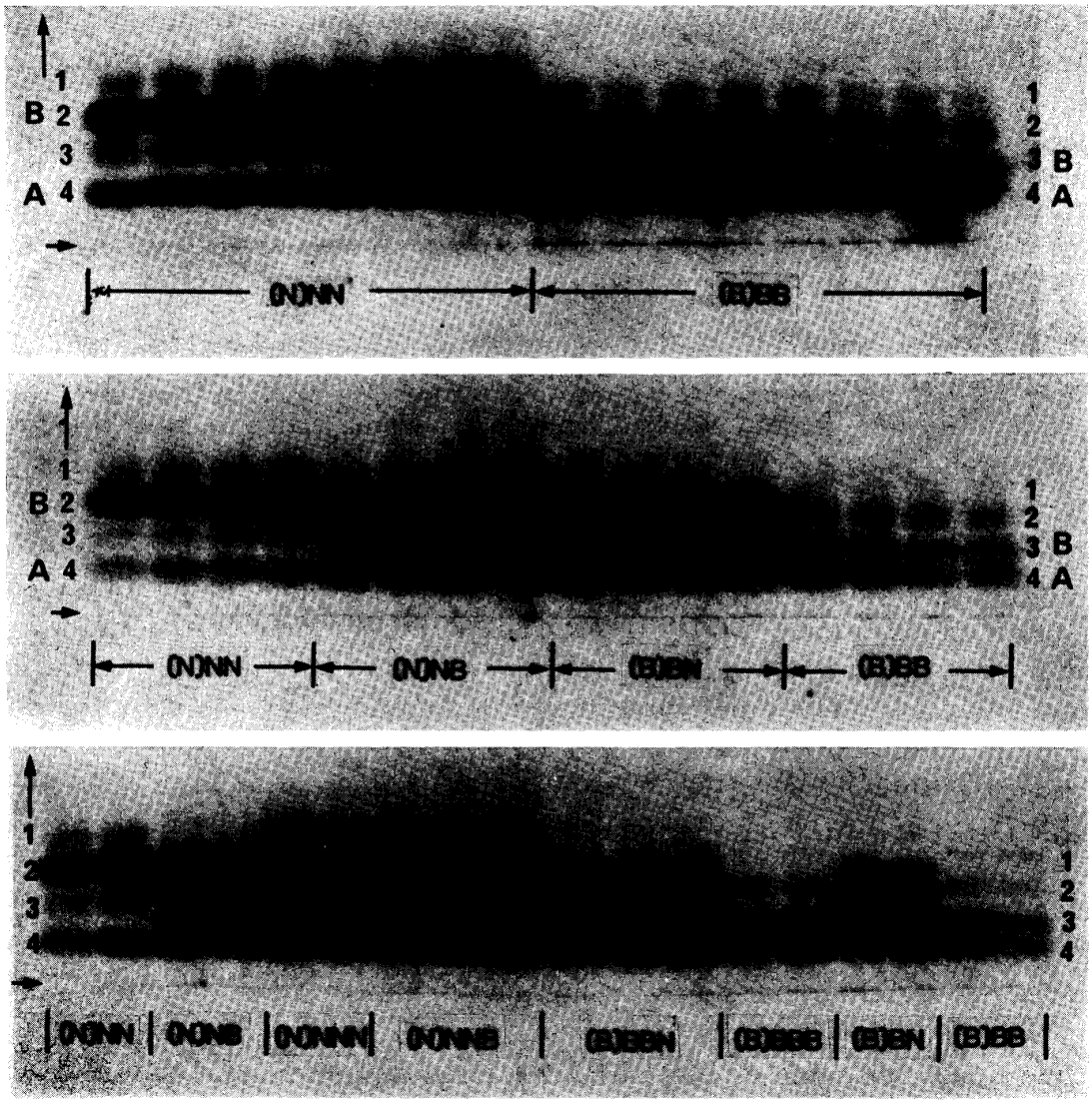


Fig. 2. Electrophoretic patterns of MDH from normal diploids, reciprocal hybrids, autotriploids and reciprocal allotriploids in *Rana nigromaculata* and *Rana brevipoda*.

(N) NN, diploid *Rana nigromaculata*
(B) BB, diploid *Rana brevipoda*
(N) NB, diploid hybrid, *Rana nigromaculata* ♀ × *Rana brevipoda* ♂
(B) BN, diploid hybrid, *Rana brevipoda* ♀ × *Rana nigromaculata* ♂
(N) NNN, autotriploid *Rana nigromaculata*
(B) BBB, autotriploid *Rana brevipoda*
(N) NNB, allotriploid consisting of two *Rana nigromaculata* genomes and one *Rana brevipoda* genome
(B) BBN, allotriploid consisting of two *Rana brevipoda* genomes and one *Rana nigromaculata* genome

3. Auto- and allotriploids

Electrophoretic patterns of MDH were examined in ten (N)NNN autotriploids of *Rana nigromaculata,* ten (B)BBB autotriploids of *Rana brevipoda,* ten (N)NNB allotriploids consisting of two genomes of *Rana nigromaculata* and one genome of *Rana brevipoda* and ten (B)BBN allotriploids of the reciprocal combination. The electrophoretic patterns of the two kinds of autotriploids, (N)NNN and (B)BBB,

were nearly the same as those of *Rana nigromaculata* and *Rana brevipoda,* respectively. Those of the two kinds of allotriploids, (N)NNB and (B)BBN, were also nearly the same as those of the two kinds of hybrids, (N)NB and (B)BN, in number and sites of bands. However, the bands controlled by two genomes of one species were more intense than those controlled by one genome of this species (Fig. 2).

III. α-Glycerophosphate dehydrogenase (α-GDH)

1. *Rana nigromaculata* and *Rana brevipoda*

Electrophoretic patterns of α-GDH were examined in 20 *Rana nigromaculata* including five male and five female field-caught frogs and five male and five female offspring of the latter, and 20 *Rana brevipoda* including five male and five female field-caught frogs and five male and five female offspring of the latter. It was found that the electrophoretic pattern of each species consisted of four bands, which were arranged at equal intervals and named α-GDH-1, α-GDH-2, α-GDH-3 and α-GDH-4 in order of mobility from fast to slow (Fig. 3). Of the four bands of *Rana nigromaculata,* bands α-GDH-2, α-GDH-3 and α-GDH-4 were comparatively intense, while the other band α-GDH-1 was faint. Of the four bands of *Rana brevipoda,* band α-GDH-4 was faint, while the other three bands were comparatively intense. The three bands, α-GDH-1, α-GDH-2 and α-GDH-3, of *Rana nigromaculata* were situated at the sites corresponding to those of the three bands, α-GDH-2, α-GDH-3 and α-GDH-4, of *Rana brevipoda,* respectively.

2. Reciprocal hybrids

Electrophoretic patterns of α-GDH were examined in 20 reciprocal hybrids, (N)NB and (B)BN. It was found that there were five distinct bands in the electrophoretic pattern of each kind of hybrids (Fig. 3). When these bands were named α-GDH-1, α-GDH-2, α-GDH-3, α-GDH-4 and α-GDH-5 in order of mobility from fast to slow, the three middle bands, α-GDH-2, α-GDH-3 and α-GDH-4, corresponded to bands α-GDH-1, α-GDH-2 and α-GDH-3 of *Rana nigromaculata,* on one side, and to bands α-GDH-2, α-GDH-3 and α-GDH-4 of *Rana brevipoda* on the other side, respectively. Thus, each of these three bands of reciprocal hybrids was the sum of two corresponding bands of the two species in intensity. Band α-GDH-1 of reciprocal hybrids corresponded to band α-GDH-1 of *Rana brevipoda,* while band α-GDH-5 of reciprocal hybrids corresponded to band α-GDH-4 of *Rana nigromaculata.* The two kinds of hybrids somewhat differed from each other in intensity of bands α-GDH-1, α-GDH-3 and α-GDH-4.

3. Auto- and allotriploids

Electrophoretic patterns of α-GDH were examined in ten frogs each of (N)NNN autotriploids, (B)BBB autotriploids, (N)NNB allotriploids and (B)BBN allotriploids. The two kinds of autotriploids, (N)NNN and (B)BBB, were nearly

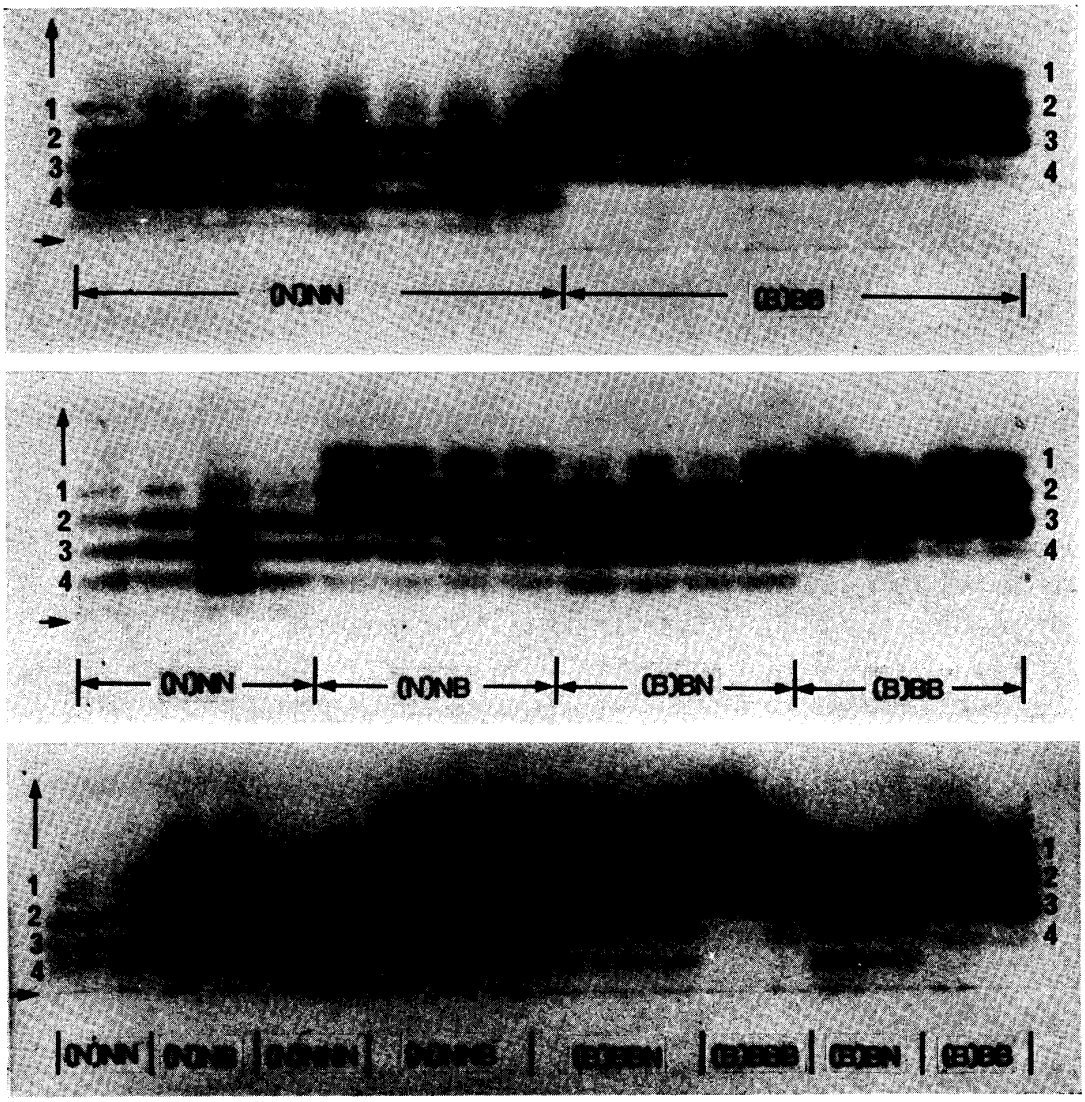


Fig. 3. Electrophoretic patterns of α-GDH from normal diploids, reciprocal hybrids, autotriploids and reciprocal allotriploids in *Rana nigromaculata* and *Rana brevipoda*.

(N) NN, diploid *Rana nigromaculata*
(B) BB, diploid *Rana brevipoda*
(N) NB, diploid hybrid, *Rana nigromaculata* ♀ × *Rana brevipoda* ♂
(B) BN, diploid hybrid, *Rana brevipoda* ♀ × *Rana nigromaculata* ♂
(N) NNN, autotriploid *Rana nigromaculata*
(B) BBB, autotriploid *Rana brevipoda*
(N) NNB, allotriploid consisting of two *Rana nigromaculata* genomes and one *Rana brevipoda* genome
(B) BBN, allotriploid consisting of two *Rana brevipoda* genomes and one *Rana nigromaculata* genome

the same as the two diploid species, (N)NN and (B)BB, in electrophoretic pattern, respectively, except that the bands of the autotriploids were generally more intense than those of the diploids (Fig. 3). However, one of the ten (B)BBB frogs was a biochemical mutant, in which the three bands, α-GDH-2, α-GDH-3 and α-GDH-4, were situated at the sites corresponding to those of bands α-GDH-1, α-GDH-2 and α-GDH-3 of the other nine (B)BBB frogs, respectively. Band α-GDH-1 of this mutant migrated faster than that of the other frogs. The four

bands of this mutant were nearly the same as those of the other (B)BBB frogs in intensity.

The electrophoretic patterns of the reciprocal allotriploids, (N)NNB and (B)BBN, consisted of five bands. The two kinds of allotriploids differed from each other in that bands α-GDH-3, α-GDH-4 and α-GDH-5 of the (B)BBN frogs were somewhat fainter than those of the (N)NNB.

IV. Isocitrate dehydrogenase (IDH)

1. *Rana nigromaculata* and *Rana brevipoda*

Electrophoretic patterns of IDH were examined in 20 *Rana nigromaculata* and 20 *Rana brevipoda*. In the frogs of each species, there were 10 males and 10 females. It was found that the electrophoretic patterns of *Rana nigromaculata* consisted of two band groups, IDH-A and IDH-B, which moved toward the anode (Fig. 4). Band group IDH-A of *Rana nigromaculata* consisted of three bands and was the same as that of *Rana brevipoda* in number, site and intensity of constitutive bands. Band group IDH-B of *Rana nigromaculata* showed three kinds of phenotypes which were a homozygous B^bB^b, a homozygous B^eB^e and a heterozygous B^bB^e in genotype. While homozygous alleles B^bB^b or B^eB^e always showed one band, heterozygous alleles B^bB^e produced three bands, of which the middle band was more intense than the other two. In all the 20 *Rana brevipoda,* the site corresponding to band group IDH-B was occupied by only one band, which was a homozygous B^fB^f in genotype. This band was slower in mobility than those of *Rana nigromaculata.*

2. Reciprocal hybrids

The electrophoretic patterns of IDH were examined in ten (N)NB and ten (B)BN hybrids. Band group IDH-A in the two kinds of hybrids was the same as the parental species in number, site and intensity of bands. Band group IDH-B of reciprocal hybrids consisted of three bands, which were controlled by heterozygous alleles B^bB^f or B^eB^f (Fig. 4). In the (N)NB hybrids, the hybrid band controlled by two heterozygous genes was always more intense than the band controlled by one parental gene. In the reciprocal hybrids, the band controlled by one maternal gene was intense like the hybrid band controlled by two heterozygous genes, while the band controlled by one paternal gene could be scarcely recognized (Fig. 4).

3. Auto- and allotriploids

The electrophoretic patterns of IDH were examined in ten (N)NNN, (B)BBB, (N)NNB or (B)BBN frogs. The two kinds of autotriploids, (N)NNN and (B)BBB, were nearly the same as the diploid *Rana nigromaculata* and *Rana brevipoda* in electrophoretic pattern, respectively. The two kinds of allotriploids, (N)NNB and (B)BBN, were also the same as the two kinds of hybrids, (N)NB and (B)BN,

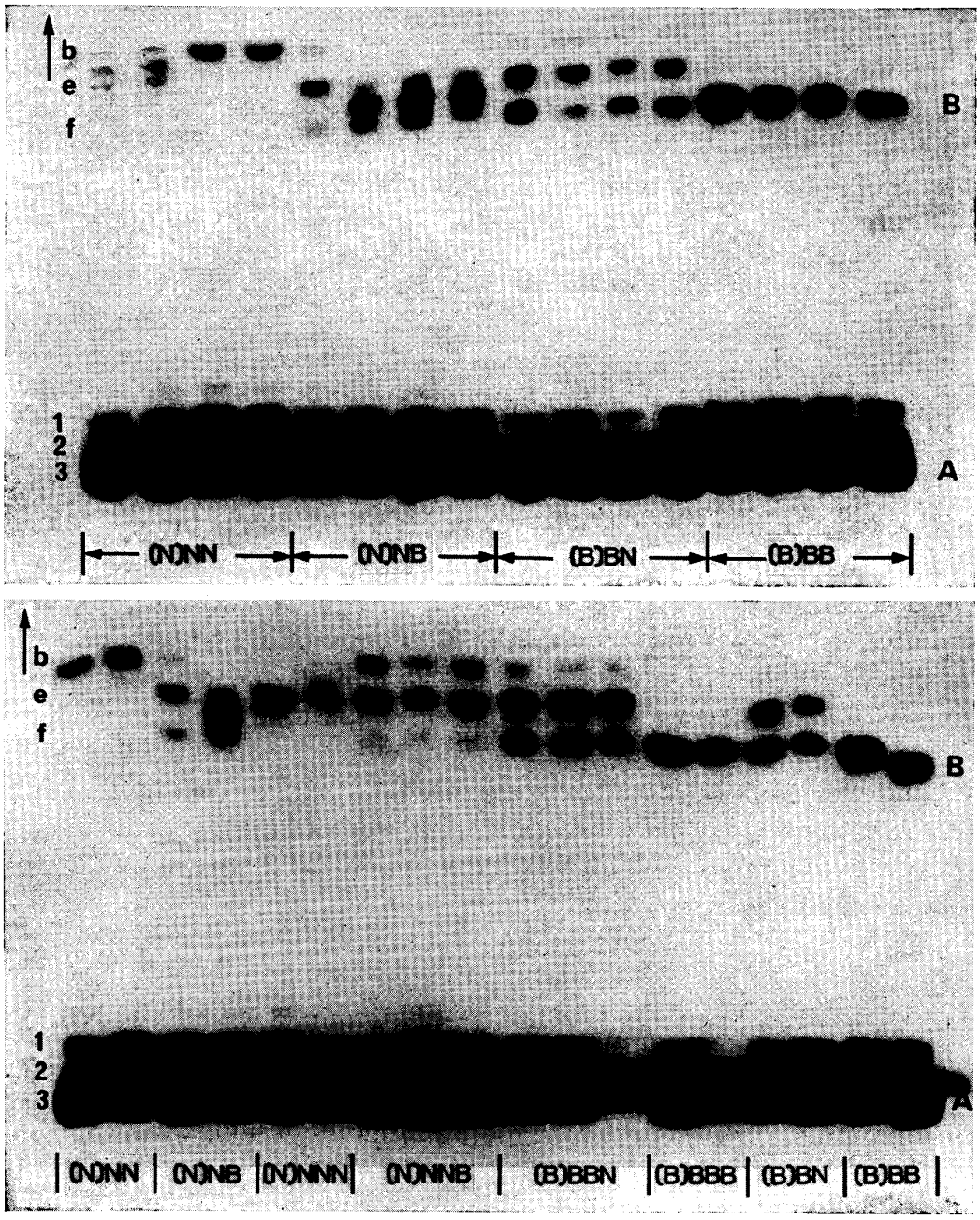


Fig. 4. Electrophoretic patterns of IDH from normal diploids, reciprocal hybrids, autotriploids and reciprocal allotriploids in *Rana nigromaculata* and *Rana brevipoda*.

(N) NN, diploid *Rana nigromaculata*
(B) BB, diploid *Rana brevipoda*
(N) NB, diploid hybrid, *Rana nigromaculata* ♀ × *Rana brevipoda* ♂
(B) BN, diploid hybrid, *Rana brevipoda* ♀ × *Rana nigromaculata* ♂
(N) NNN, autotriploid *Rana nigromaculata*
(B) BBB, autotriploid *Rana brevipoda*
(N) NNB, allotriploid consisting of two *Rana nigromaculata* genomes and one *Rana brevipoda* genome
(B) BBN, allotriploid consisting of two *Rana brevipoda* genomes and one *Rana nigromaculata* genome

in number and mobility of bands. However, the band controlled by two genomes of one species was remarkably more intense than that controlled by one genome of this species (Fig. 4).

V. Superoxide dismutase (SOD)

1. *Rana nigromaculata* and *Rana brevipoda*

Electrophoretic patterns of SOD were examined in 20 *Rana nigromaculata* and 20 *Rana brevipoda*. While *Rana nigromaculata* always showed two bands, 3 and 4, which were controlled by homozygous alleles S^bS^b, *Rana brevipoda* revealed three kinds of electrophoretic patterns (Fig. 5). One of them consisted of the same two bands as those of *Rana nigromaculata*, 3 and 4. Another consisted of two bands, 1 and 2, which were controlled by homozygous alleles S^aS^a. These bands migrated faster than bands 3 and 4. The remaining electrophoretic pattern consisted of four bands 1, 2, 3 and 4, which were controlled by heterozygous alleles S^aS^b.

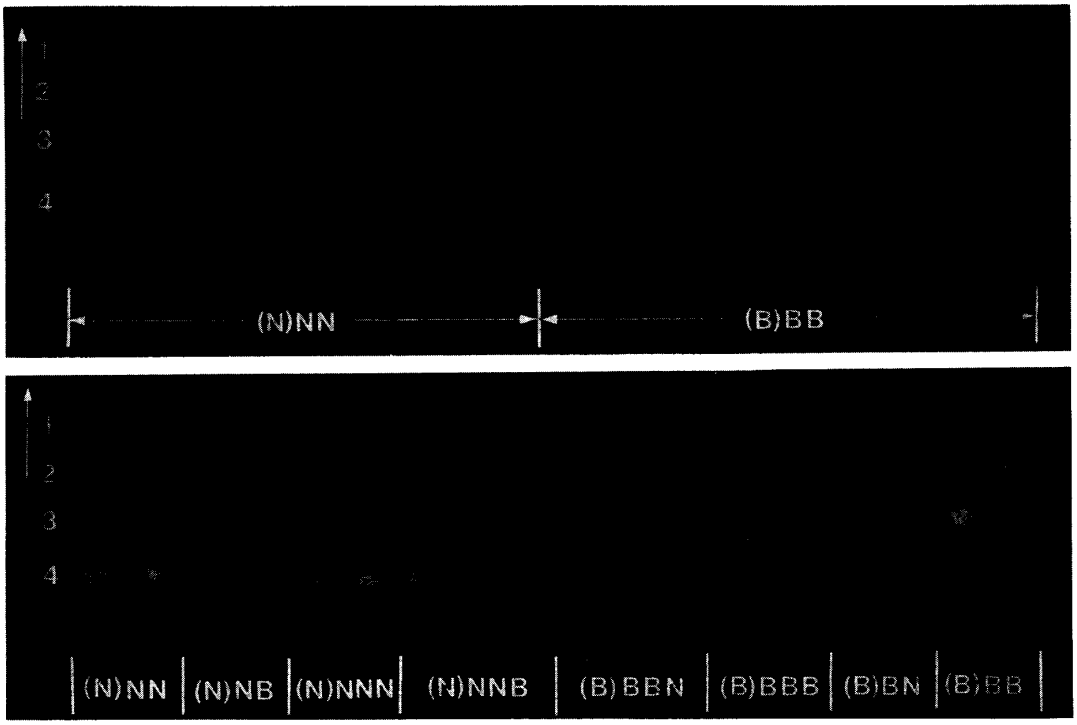


Fig. 5. Electrophoretic patterns of SOD from normal diploids, reciprocal hybrids, autotriploids and reciprocal allotriploids in *Rana nigromaculata* and *Rana brevipoda*.

(N) NN, diploid *Rana nigromaculata*
(B) BB, diploid *Rana brevipoda*
(N) NB, diploid hybrid, *Rana nigromaculata* ♀ × *Rana brevipoda* ♂
(B) BN, diploid hybrid, *Rana brevipoda* ♀ × *Rana nigromaculata* ♂
(N) NNN, autotriploid *Rana nigromaculata*
(B) BBB, autotriploid *Rana brevipoda*
(N) NNB, allotriploid consisting of two *Rana nigromaculata* genomes and one *Rana brevipoda* genome
(B) BBN, allotriploid consisting of two *Rana brevipoda* genomes and one *Rana nigromaculata* genome

2. Reciprocal hybrids

Electrophoretic patterns of SOD were examined in ten (N)NB and ten (B)BN hybrids. When both parents had bands 3 and 4, reciprocal hybrids had also bands 3 and 4. When the parental *Rana brevipoda* had bands 1 and 2, reciprocal hybrids had bands 1, 2, 3 and 4. When the former had four bands, 1, 2, 3 and 4, half of reciprocal hybrids had also four bands, 1, 2, 3 and 4, while the other half had two bands, 3 and 4. In any case when there were four bands, bands 2 and 3 were more intense than bands 1 and 4 (Fig. 5).

3. Auto- and allotriploids

Electrophoretic patterns of SOD were examined in ten frogs of (N)NNN, (B)BBB, (N)NNB or (B)BBN. The (N)NNN and (B)BBB frogs revealed the same bands as those of the diploid *Rana nigromaculata* and *Rana brevipoda,* respectively (Fig. 5). However, a faint band appeared at the site of band 2 of *Rana brevipoda* in the electrophoretic pattern of the (N)NNN frogs. The appearance of such a subordinate band seemed to be attributable to an increase in quantity of a subliminal substance by autotriploidy. A similar increase in quantity was also found in bands 3 and 4 of the (N)NNN frogs.

The two kinds of allotriploids, (N)NNB and (B)BBN, were nearly the same as the two kinds of hybrids, (N)NB and (B)BN, in electrophoretic pattern, although the bands controlled by two genomes of one species were more intense than those controlled by one genome of this species.

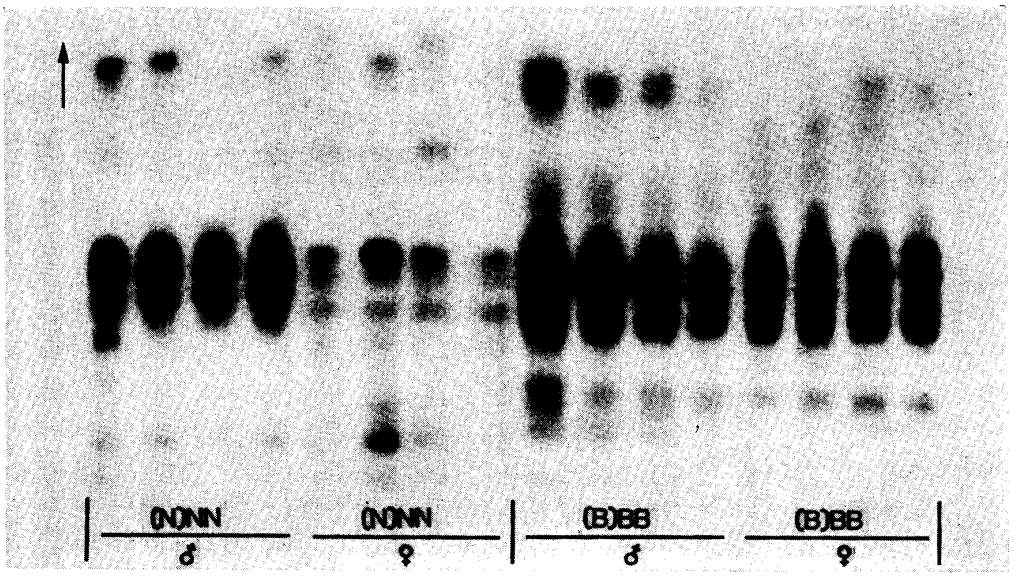
VI. Esterase

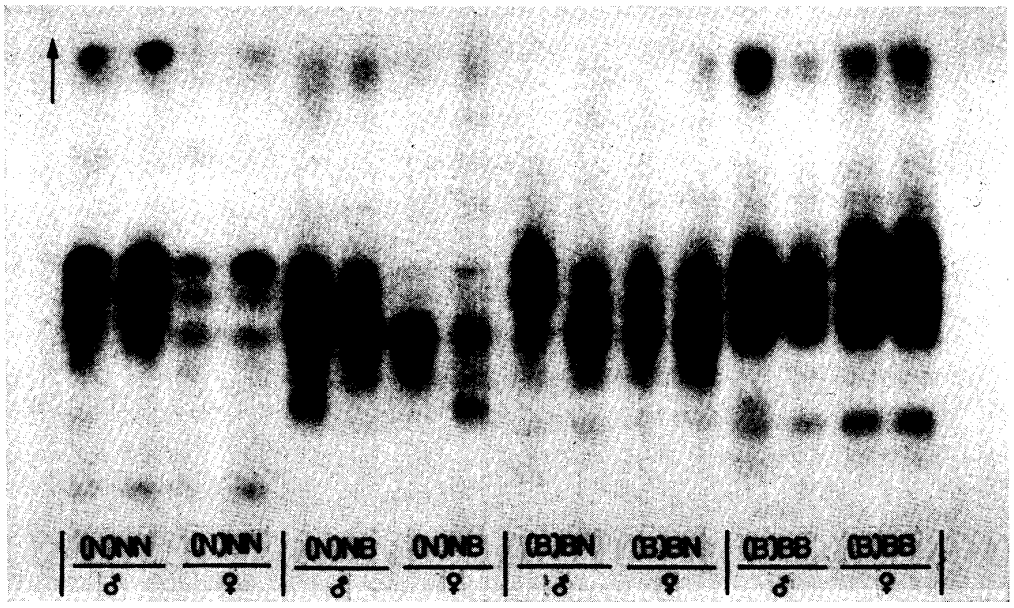
1. *Rana nigromaculata* and *Rana brevipoda*

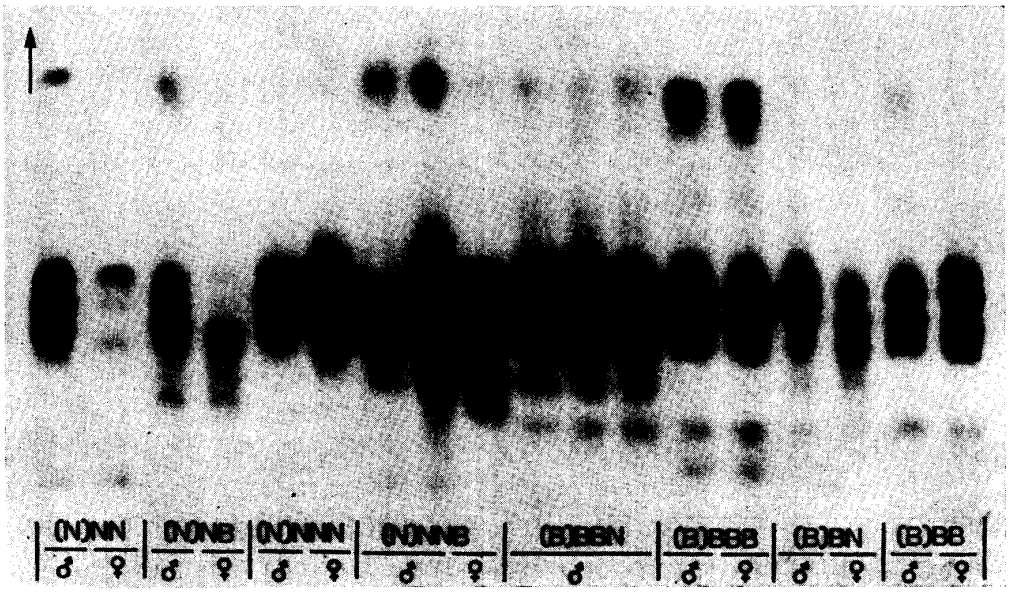
Electrophoretic patterns of esterase were examined in 20 *Rana nigromaculata* and 20 *Rana brevipoda.* Each species contained five male and five female field-caught frogs and five male and five female offspring produced from them in the laboratory. It was found that each electrophoretic pattern consisted of several bands which moved toward the anode. Three or four major bands in the middle

Fig. 6. Electrophoretic patterns of esterase from normal diploids, reciprocal hybrids, autotriploids and reciprocal allotriploids in *Rana nigromaculata* and *Rana brevipoda.*

(N) NN, diploid *Rana nigromaculata*
(B) BB, diploid *Rana brevipoda*
(N) NB, diploid hybrid, *Rana nigromaculata* ♀ × *Rana brevipoda* ♂
(B) BN, diploid hybrid, *Rana brevipoda* ♀ × *Rana nigromaculata* ♂
(N) NNN, autotriploid *Rana nigromaculata*
(B) BBB, autotriploid *Rana brevipoda*
(N) NNB, allotriploid consisting of two *Rana nigromaculata* genomes and one *Rana brevipoda* genome
(B) BBN, autotriploid consisting of two *Rana brevipoda* genomes and one *Rana nigromaculata* genome


(N)NN
♂
(N)NN
♀
(B)BB
♂
(B)BB
♀


(N)NN
♂
(N)NN
♀
(N)NB
♂
(N)NB
♀
(B)BN
♂
(B)BN
♀
(B)BB
♂
(B)BB
♀


(N)NN
♂ ♀
(N)NB
♂ ♀
(N)NNN
♂ ♀
(N)NNB
♂ ♀
(B)BBN
♂
(B)BBB
♂ ♀
(B)BN
♂ ♀
(B)BB
♂ ♀

part of the electrophoretic pattern of *Rana nigromaculata* somewhat differed from those of *Rana brevipoda*. Some subordinate bands were found around the major bands in both species. There was a remarkable sexual difference in the middle major bands of *Rana nigromaculata,* while no sexual difference was found in those of *Rana brevipoda* (Fig. 6).

2. Reciprocal hybrids

Electrophoretic patterns were examined in five male and five female (N)NB hybrids and five male and five female (B)BN. It was found that the bands of hybrids were not the simple sum of those of the two parental species (Fig. 6). The two kinds of hybrids differed from each other in electrophoretic pattern and, moreover, there were many individual variations. The three major bands of male (N)NB hybrids were similar to those of male diploid *Rana nigromaculata* but differed from those of female (N)NB hybrids. While three major bands appeared in the reciprocal hybrids, their derivation was not always evident, since there were many individual variations in electrophoretic pattern.

3. Auto- and allotriploids

Electrophoretic patterns were examined in ten frogs of (N)NNN, (B)BBB, (N)NNB or (B)BBN. The electrophoretic patterns of the two kinds of autotriploids, (N)NNN and (B)BBB, were nearly the same as those of diploid *Rana nigromaculata* and *Rana brevipoda,* respectively. The ten allotriploids, (B)BBN, were all mutually the same in electrophoretic pattern and closely resembled the (B)BN hybrids, although the bands controlled by two genomes were remarkably intense. In the electrophoretic patterns of the (N)NNB frogs, the derivation of each major band was not always evident, since there were individual variations as well as sexual differences (Fig. 6).

DISCUSSION

Electrophoretic studies on proteins in various species of anurans have been reported by many investigators. Of these studies, analysis of enzymes has been actively carried out since about ten years ago, while analysis of blood components such as hemoglobin, transferrin and serum albumin commenced far earlier. DESSAUER and NEVO (1969) analyzed blood esterase, liver esterase and six other enzymes extracted from liver tissues, besides hemoglobin, transferrin and albumin in *Acris crepitans* and *Acris gryllus* distributed in the United States. They found local variations of *Acris crepitans* in liver esterase, hemoglobin and transferrin, and also polymorphism of two kinds of enzymes in a local population of this species. ENGELMANN (1974) analyzed LDH and α-naphthylacetate esterase of European *Rana ridibunda, Rana esculenta* and *Rana lessonae* and confirmed BERGER's theory (1968) that *Rana esculenta* is a hybrid of the other two species. UZZELL and BERGER (1975) and UZZELL, BERGER and GÜNTHER (1975) verified the hybrid nature of *Rana esculenta* on the basis of the results obtained from analysis of

five kinds of enzymes, AAT, α-GDH, GPI, PGM and LDH, extracted from skeletal muscles of *Rana ridibunda, Rana esculenta* and *Rana lessonae.* The hybrid nature of *Rana esculenta* was also verified by TUNNER and DOBROWSKY (1976) from analysis of blood serum and PGM, and by VOGEL and CHEN (1976, 1977) from analysis of LDH.

ROGERS (1973) and GUTTMAN (1975) examined enzymes of North American toads. GUTTMAN analyzed ten kinds of enzymes extracted from liver and kidney tissues in 25 populations of *Bufo americanus* distributed in the northeastern part of North America, and found biochemical polymorphism in these populations. He discovered that the populations differed from one another in gene frequency of each kind of enzyme.

The present authors analyzed 16 kinds of enzymes mainly extracted from skeletal muscles of the two sibling species, *Rana nigromaculata* and *Rana brevipoda.* The results showed that the two species distinctly differed from each other in the electrophoretic patterns of six kinds of enzymes, LDH, MDH, α-GDH, IDH, SOD and esterase. Then, these six kinds of enzymes were also analyzed in reciprocal hybrids, two kinds of autotriploids and reciprocal allotriploids produced from the two species. The electrophoretic patterns of reciprocal hybrids indicated that LDH was probably a tetramer enzyme controlled by alleles at two different loci, LDH-A and LDH-B, that MDH was probably a dimer enzyme controlled by alleles at two different loci, MDH-A and MDH-B, that α-GDH was probably a monomer enzyme controlled by alleles at a single locus, α-GDH-A, that IDH was probably a dimer enzyme controlled by alleles at two different loci, IDH-A and IDH-B, that SOD was probably a dimer enzyme controlled by alleles at a single locus, SOD-A, and that esterase was probably controlled by alleles at more than two loci. *Rana nigromaculata* and *Rana brevipoda* differed from each other in alleles of loci LDH-B, MDH-B, α-GDH-A, IDH-B and SOD-A, and also in alleles of a locus for esterase. It was noteworthy that there were slight differences between reciprocal hybrids of the two species in the electrophoretic patterns of LDH, MDH and α-GDH in spite of equality of genomes. Such differences seemed to be attributable to the difference of cytoplasm.

The electrophoretic pattern of autotriploids was very similar to that of diploids of the same species in number and site of bands. The bands of autotriploids were often more intense than those of diploids. This was probably attributed to the existence of three genomes. The difference in electrophoretic pattern between reciprocal allotriploids was usually far more remarkable than that between reciprocal diploid hybrids, as expected. The bands controlled by two genomes of one species were usually more intense than those controlled by one genome of the same species.

From the findings stated above, it is evident that *Rana nigromaculata* and *Rana brevipoda* are in very close affinity with each other, as the alleles controlling various kinds of enzymes in one species can similarly function in other species. The slight difference between reciprocal hybrids in the electrophoretic patterns of the three enzymes seems to indicate that the two species are none other than sibling species.

SUMMARY

1. Biochemical differences between *Rana nigromaculata* and *Rana brevipoda* were examined by starch-gel electrophoresis using 16 kinds of enzymes extracted from skeletal muscles and liver tissues. The results showed that there were some differences in eight kinds of enzymes. Of the latter, the electrophoretic patterns of six kinds of enzymes, LDH, MDH, α-GDH, IDH, SOD and esterase, revealed especially distinct differences between the two species.

2. The relationship between *Rana nigromaculata* and *Rana brevipoda* were examined by analyzing the above six kinds of enzymes extracted from skeletal muscles of reciprocal hybrids, two kinds of autotriploids and reciprocal allotriploids as well as the two species.

3. LDH seemed to be a tetramer enzyme controlled by alleles at two different loci, LDH-A and LDH-B. The two species differed from each other in alleles of locus LDH-B.

4. MDH seemed to be a dimer enzyme controlled by alleles at two different loci, MDH-A and MDH-B. The two species differed from each other in alleles of locus MDH-B.

5. α-GDH seemed to be a monomer enzyme controlled by alleles at a single locus, α-GDH-A. The two species differed from each other in alleles of this locus.

6. IDH seemed to be a dimer enzyme controlled by alleles at two different loci, IDH-A and IDH-B. The two species differed from each other in alleles of locus IDH-B.

7. SOD seemed to be a dimer enzyme controlled by alleles at a single locus, SOD-A. The two species differed from each other in alleles of this locus.

8. Esterase seemed to be controlled by alleles at more than two loci. The two species distinctly differed from each other in alleles of one locus. There was a remarkable sexual difference in the middle major bands of *Rana nigromaculata*.

9. There were slight differences between reciprocal hybrids of the two species in the electrophoretic patterns of LDH, MDH and α-GDH in spite of equality of genomes. These differences seemed to be attributable to the difference of cytoplasm.

10. The electrophoretic pattern of autotriploids was very similar to that of diploids of the same species, although the bands of the former often appeared to be more intense than those of the latter.

11. The electrophoretic pattern of reciprocal allotriploids differed from each other in that the bands controlled by two genomes of one species were more intense than those controlled by one genome of the same species.

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LITERATURE

BERGER, L. 1968. Morphology of the F_1 generation of various crosses within *Rana esculenta*-complex. Acta Zool. Cracov. (Kraków) **13**: 301–324.

BREWER, G. J. 1970. An Introduction to Isozyme Techniques. Academic Press (New York and London).

DESSAUER, H. C. and E. NEVO 1969. Geographic variation of blood and liver proteins in cricket frogs. Biochem. Genet. **3**: 171–188.

ENGELMANN, W. E. 1974. Vergleichende Enzymuntersuchungen der Serum mitteleuropäischer Raniden (Amphibia, Anura). Experientia **30**: 870–872.

Guttman, S. I. 1975. Genetic variation in the genus *Bufo*. II. Isozymes in northern allopatric populations of the American toads, *Bufo americanus*. Isozymes. Vol. IV. Genetics and Evolution, edited by C. L. MARKERT. pp. 679–697. Academic Press (New York).

NISHIOKA, M., H. OHTANI and M. SUMIDA 1980. Detection of the chromosomes bearing the loci for seven kinds of proteins in Japanese pond frogs. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ. **4**: 127–184.

ROGERS, J. S. 1973. Protein polymorphism, genic heterozygosity, and divergence in the toads *Bufo cognatus* and *B. speciosus*. Copeia 1973: 322–330.

TUNNER, H. G. und M.-Th. DOBROWSKY 1976. Zur morphologischen, serologischen und enzymologischen Differenzierung von *Rana lessonae* und der hybridogenetischen *Rana esculenta* aus dem Seewinkel und dem Neusiedlersee (Österreich, Burgenland). Zool. Anz. **197**: 6–22.

UZZELL, Th. and L. BERGER 1975. Electrophoretic phenotypes of *Rana ridibunda, Rana lessonae*, and their hybridogenetic associate, *Rana esculenta*. Proc. Acad. Nat. Sci. Phila. **127**: 13–24.

UZZELL, Th., L. BERGER and R. GÜNTHER 1975. Diploid and triploid progeny from a diploid female of *Rana esculenta* (Amphibia, Salientia). Ibid. **127**: 81–91.

VOGEL, P. und P. S. CHEN 1976. Untersuchungen über die Isozyme der Lactatdehydrogenase (LDH) beim *Rana esculenta*-Komplex. Revue suisse Zool. **83**: 944–947.

——— 1977. A further study of LDH isozymes in the *Rana esculenta*-complex. Experientia **33**: 1285–1287.