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Phylogenetic Relationships of the *Rana esculenta* Group Clarified by Electrophoretic Analyses of Serum Transferrins

By

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ABSTRACT

In order to clarify the phylogenetic relationships of *Rana nigromaculata*, *R. brevipoda*, *R. plancyi chosenica*, *R. p. fukienensis*, *R. lessonae*, *R. ridibunda* and *R. esculenta* distributed in the Palearctic region, serum transferrins obtained from field-caught animals as well as interspecific hybrids and allotriploids were analyzed by acrylamide-gel electrophoresis. Transferrins of *R. nigromaculata* were found to have nine phenotypes determined by four alleles, while those of *R. p. chosenica* had five phenotypes determined by three alleles. *R. esculenta* had two phenotypes, which were identical to those of the other two European species. Transferrins of *R. brevipoda*, *R. p. fukienensis*, *R. lessonae* and *R. ridibunda* each showed one phenotype, controlled by one gene. These genes controlled transferrin bands differing in mobility. Bands moved faster in the two *R. plancyi* subspecies than in the five frog species from Japan and Europe. The electrophoretic patterns of interspecific hybrids and allotriploids completely corresponded to the kind and number of their genes. These findings demonstrated that these frogs belonging to the *R. esculenta* group were all closely related to each other, and that the two Japanese species, the two Taiwan and Korean subspecies and the three European species composing three subgroups differentiated systematically.

INTRODUCTION

Since SMITHIES (1959) discovered transferrin polymorphism in human blood by starch-gel electrophoresis, it has been studied in various kinds of animals. In amphibians, however, reports on transferrin polymorphism and its heredity are comparatively few. In anurans, DESSAUER, FOX and HARTWIG (1962), GUTTMAN (1967, 1969, 1972), BROWN and GUTTMAN (1970) and GUTTMAN and WILSON (1973) in *Bufo*, DESSAUER and NEVO (1969) in *Acris*, KASHIWAGI (1981) in *Rana* and SCHONNE, PETIT, LIZEN and PICARD (1988) in *Xenopus*, respectively, discovered the transferrin polymorphism and clarified its inheritance. In urodeles, CHALUMEAU-LE FOULGOC, FINE and L. GALLIEN (1966, 1972) and FINE, CHALUMEAU-LE FOULGOC and AMOUCH (1967) in *Pleurodeles waltl* reported that transferrin phenotypes were controlled by codominant alleles *a* and *b*. CHALUMEAU-LE FOULGOC and C. L. GALLIEN (1967) and CHALUMEAU-LE FOULGOC (1968, 1969) confirmed

that the bands produced by *a* and *b* differed in mobility between *P. waltl* and *P. poireti*. The present study was performed as a part of the work to elucidate the phylogenetic relationships of frogs belonging to the *Rana esculenta* group distributed in the Palearctic region by analyzing electrophoretically the transferrins of natural strains as well as those of reciprocal hybrids and allotriploids produced in the laboratory.

MATERIALS AND METHODS

Specimens used in this study, collection sites and numbers were as follows:

1. *Rana nigromaculata* HALLOWELL, from Hiroshima and Okayama Prefectures, Japan, 160 specimens.
2. *R. brevipoda* ITO, from Okayama Prefecture, Japan, 32 specimens.
3. *R. plancyi fukienensis* POPE, from Chiayi, Taiwan, 8 specimens.
4. *R. p. chosenica* OKADA, from the vicinity of Suwon, Korea, 22 specimens.
5. *R. lessonae* CAMERANO, from a suburb of Luxembourg, 4 specimens.
6. *R. ridibunda* PALLAS, from Adana, Turkey, 2 specimens.
7. *R. esculenta* L., from Florence, Italy, 3 specimens and from Roscoff, France, 2 specimens.

Ovulation was induced by injecting suspension of acetone-dried bullfrog pituitaries into the body cavity of mature females. Fertilization was carried out artificially. Triploids were produced by the following procedure: following insemination, eggs were kept at room temperature for about 20 minutes and then immersed in water at 1~2°C for two to three hours in order to suppress extrusion of the second polar body (NISHIOKA, 1972). The ploidy was determined by counting chromosomes in the tail-tips of tadpoles (NISHIOKA, 1972), or by measuring the size of resting nuclei.

Separation and identification of serum transferrin were made following the method described by MORIWAKI, SADAIE and HIRASAWA (1974). Blood samples were collected from the heart. After centrifugation, the supernatant was treated with acrinol and ethanol and then subjected to acrylamide-gel electrophoresis.

OBSERVATION

1. Field-caught frogs

a. *Rana nigromaculata*, (N)NN

A total of 160 *Rana nigromaculata* was used for transferrin analysis. Nine transferrin phenotypes controlled by four alleles, *a* , *b* , *c* and *d*, were detected (Fig. 1). Electrophoretic pattern of each individual displayed one band indicating a homozygous phenotype or two bands indicating heterozygous ones.

Ten kinds of phenotypes controlled by four alleles were presumably found. Of the individuals analyzed, two had AA, 30 had BB, 50 had CC, three had DD,

seven had AB, three had AC, 34 had BC, eight had BD, and 23 had CD. No specimens of AD were discovered.

b. *Rana brevipoda*, (B)BB

All the 32 *Rana brevipoda* analyzed displayed one phenotype controlled by one gene (Fig. 1). This band was very close in mobility to the BB band controlled by allele *b* of *R. nigromaculata*, although it migrated somewhat slower.

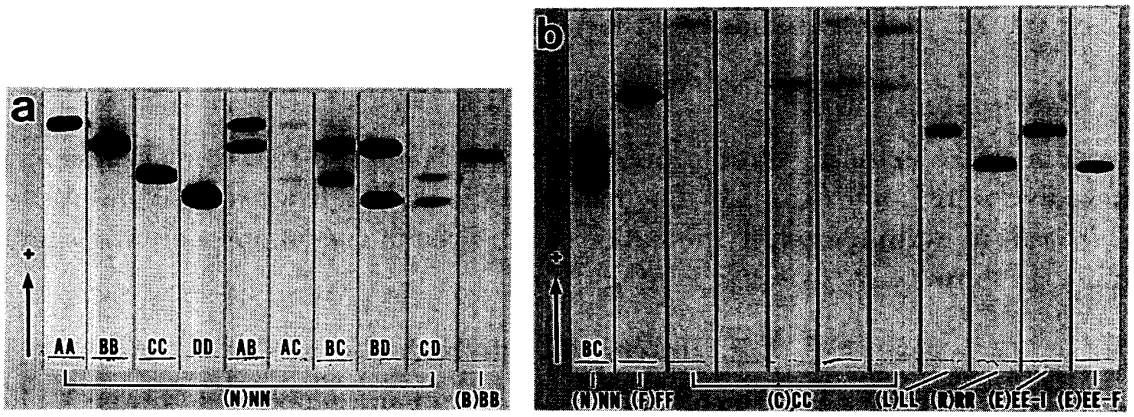

Fig. 1. Transferrin patterns of seven Palearctic pond-frog species and subspecies.
(N)NN, *Rana nigromaculata* (B)BB, *Rana brevipoda*
(F)FF, *Rana plancyi fukienensis* (C)CC, *Rana plancyi chosenica*
(L)LL, *Rana lessonae* (R)RR, *Rana ridibunda*
(E)EE-I, *Rana esculenta* from Italy
(E)EE-F, *Rana esculenta* from France

c. *Rana plancyi fukienensis*, (F)FF

Analyses were made in eight frogs which had been used for crossing experiments. There was one phenotype determined by one gene (Fig. 1). The mobility of this band was greater than that of the AA band controlled by allele *a* in *R. nigromaculata*.

d. *Rana plancyi chosenica*, (C)CC

A total of 22 frogs examined revealed five transferrin phenotypes determined by three codominant alleles (Fig. 1). The bands showed greater mobility than those of *R. nigromaculata*, *R. brevipoda* and *P. p. fukienensis*. While six phenotypes were expected to be controlled by three alleles, one frog showed a homozygous band having the fastest mobility, three frogs displayed homozygous bands with intermediate mobility, seven individuals had homozygous bands with the slowest mobility, four had heterozygous bands including the fastest and slowest bands, and seven frogs displayed heterozygous bands including the intermediate and slowest bands. There were no individuals which had heterozygous bands consisting of a combination of the fastest and intermediate bands.

e. *Rana lessonae*, (L)LL

Transferrins were examined in four individuals. One band controlled by one gene was found (Fig. 1). This band showed a mobility intermediate between the AA and BB bands of *R. nigromaculata*.

f. *Rana ridibunda*, (R)RR

Transferrins were analyzed in only two individuals. These showed one band controlled by one allele (Fig. 1). The mobility of this band was intermediate between the BB and CC bands in *R. nigromaculata*.

g. *Rana esculenta*, (E)EE

Analyses were made in three individuals from Italy and two from France. All of these five were homozygous. The bands of the Italian frogs were equal to those of *R. lessonae* in mobility, while the bands of the French specimens were the same in mobility as those of *R. ridibunda* (Fig. 1).

2. Interspecific hybrids

Transferrin analyses were made in 10 inter- and intraspecific hybrids. These included reciprocal hybrids, (N)NB and (B)BN, between *R. nigromaculata*, (N)NN, and *R. brevipoda*, (B)BB, the hybrid, (N)NF, between female *R. nigromaculata*, (N)NN, and male *R. p. fukienensis*, (F)FF, the hybrid, (N)NC, between female *R. nigromaculata*, (N)NN, and male *R. p. chosenica*, (C)CC, the hybrid, (B)BF, between female *R. brevipoda*, (B)BB, and male *R. p. fukienensis*, (F)FF, the hybrid, (B)BC, between female *R. brevipoda*, (B)BB, and male *R. p. chosenica*, (C)CC, reciprocal hybrids, (F)FC and (C)CF, between *R. p. fukienensis*, (F)FF, and *R. p. chosenica*, (C)CC, the hybrid, (L)LR, between female *R. lessonae*, (L)LL, and male *R. ridibunda*, (R)RR, and the hybrid, (L)LE, between female *R. lessonae*, (L)LL, and male *R. esculenta*, (E)EE, from France. It was found that all the hybrids had two bands derived from the parents (Fig. 2). Some of the (B)BN, (F)FC and (C)CF specimens had bands that appeared single (Fig. 2a, g). This happened when the bands of the parents were similar in mobility. Such bands were considerably widened.

3. Allotriploids

Transferrin analyses were conducted in reciprocal allotriploids, (N)NNB and (B)BBN, between *R. nigromaculata*, (N)NN, and *R. brevipoda*, (B)BB, the allotriploids, (N)NNF and (N)NNC, between female *R. nigromaculata* and male *R. p. fukienensis* and *R. p. chosenica*, and the allotriploids, (B)BBF and (B)BBC, between female *R. brevipoda* and male *R. p. fukienensis* and *R. p. chosenica*. The results showed that the phenotype of each allotriploid corresponded to the kind and number of genes coming from their parents, and that the bands derived from two genes were darker than those derived from one gene (Fig. 2). The band displayed by (B)BBN appeared to be single, because the bands of the *R. nigromaculata* and *R.*

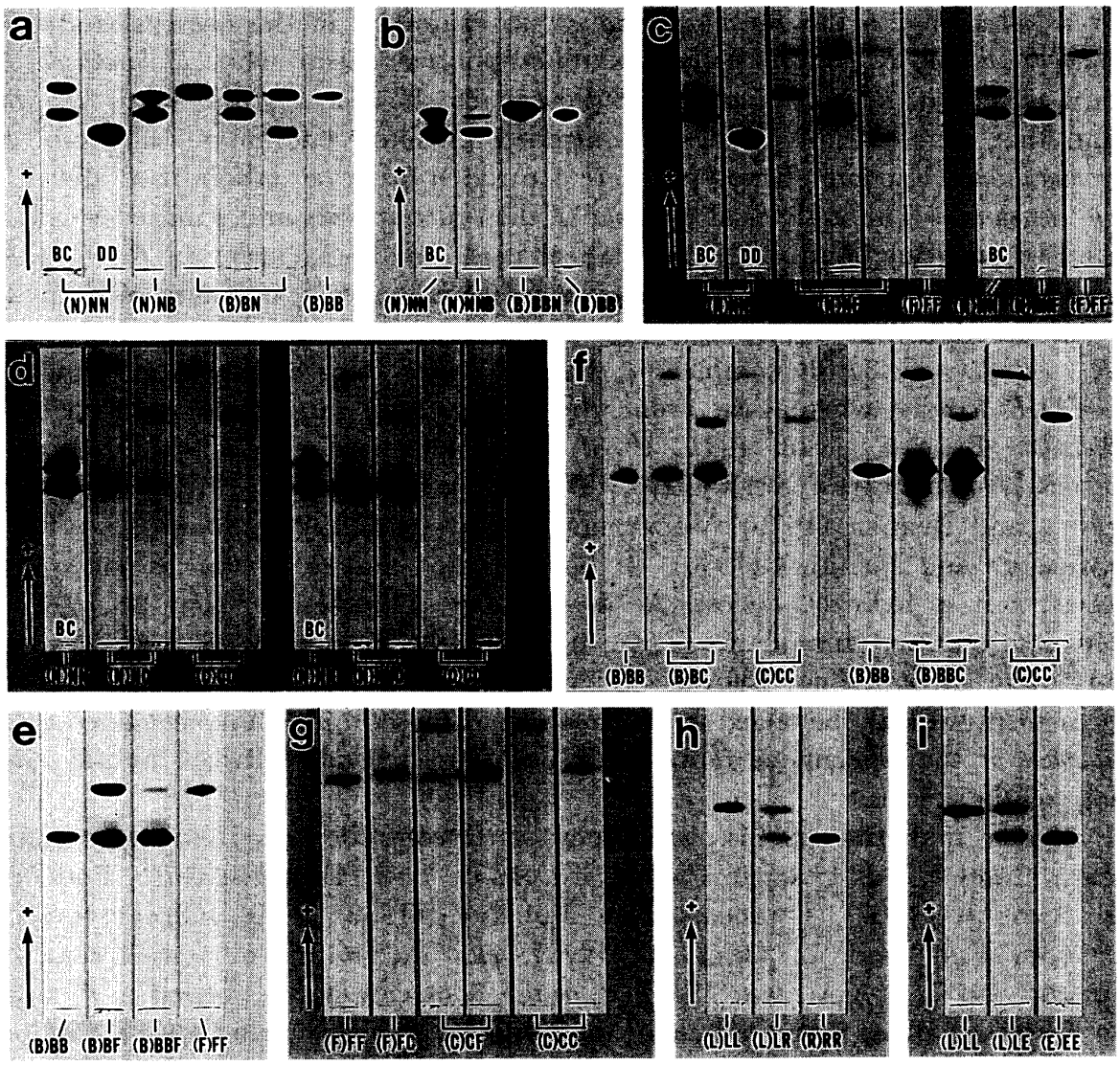

Fig. 2. Transferrin patterns of diploid and triploid hybrids in seven Palearctic pond-frog species and subspecies.

a. *Rana nigromaculata*, *R. brevipoda* and their hybrids
b. *Rana nigromaculata*, *R. brevipoda* and their allotriploid hybrids
c. *Rana nigromaculata*, *R. plancyi fukienensis* and their diploid and triploid hybrids
d. *Rana nigromaculata*, *R. plancyi chosenica* and their diploid and triploid hybrids
e. *Rana brevipoda*, *R. plancyi fukienensis* and their diploid and triploid hybrids
f. *Rana brevipoda*, *R. plancyi chosenica* and their diploid and triploid hybrids
g. *Rana plancyi fukienensis*, *R. plancyi chosenica* and their hybrids
h. *Rana lessonae*, *R. ridibunda* from Turkey and their hybrid
i. *Rana lessonae*, *R. esculenta* from France and their hybrid

brevipoda parents were similar in mobility. This band was wide and stained dark (Fig. 2b).

DISCUSSION

Of the five species from Japan and Europe and two subspecies from Taiwan and

Korea belonging to the *Rana esculenta* group examined in the present study, *R. nigromaculata* and *R. plancyi chosenica* exhibited polymorphism, while *R. brevipoda*, *R. plancyi fukienensis*, *R. lessonae* and *R. ridibunda* each showed only one band. It was found that nine phenotypes were controlled by four codominant alleles in *R. nigromaculata*, while five phenotypes were controlled by three co-dominant alleles in *R. p. chosenica*. The individual number of the other species and subspecies which possessed only one band was very small, that is, only 2~8, except for *R. brevipoda*. Accordingly, if a larger number of specimens were analyzed, polymorphism might be discovered in these species and subspecies.

R. esculenta was slightly complex, in that the Italian frogs had the same bands as those of *R. lessonae*, whereas the French frogs showed the same bands as those of *R. ridibunda*. BERGER (1968, 1971, 1976, 1977), GÜNTHER (1970, 1973), BLANKENHORN (1973, 1977), TUNNER (1970, 1972, 1973), ENGELMANN (1972, 1973, 1974), UZZELL and BERGER (1975), VOGEL and CHEN (1976, 1977), EBENDALL (1977), KAWAMURA and NISHIOKA (1986), *etc.* have insisted that *R. esculenta* is not a pure species, but a hybrid between *R. lessonae* and *R. ridibunda*. In the light of electrophoretic patterns of transferrin, however, the *R. esculenta* examined in this study were certainly not hybrids. A possible conclusion was that *R. esculenta* specimens from Italy belonged to *R. lessonae*, and those from France belonged to *R. ridibunda*, or that specimens represented backcross hybrids between hybrids and the parental species.

Evidence that transferrin phenotypes were controlled by two codominant alleles, *a* and *b*, was given in *Pleurodeles waltl* by FINE, CHALUMEAU-LE FOULGOC and AMOUCH (1967), CHALUMEAU-LE FOULGOC (1968, 1969), CHALUMEAU-LE FOULGOC, FINE and L. GALLIEN (1972), *etc.* According to CHALUMEAU-LE FOULGOC and C. L. GALLIEN (1967), *P. poireti*, a species closely related to *P. waltl*, also possessed alleles *a* and *b*, but the mobilities of the transferrin bands attributed by these genes were slightly lower than the bands of *P. waltl*. A similar phenomenon was noted in *Acris crepitans* and *A. gryllus* by DESSAUER and NEVO (1969). These species displayed one or two transferrin bands. Five codominant alleles were present in *A. crepitans*, while *A. gryllus* had only two. When these species were compared with each other in the mobility of the bands, two bands of *A. gryllus* migrated faster than any of the bands of *A. crepitans*. The transferrin polymorphism was also investigated in two allied species of *Xenopus*, *X. laevis* and *X. borealis*, and their hybrids (SCHONNE, PETIT, LIZEN and PICARD, 1988). *X. laevis* showed one or two phenotypes controlled by two codominant alleles at one locus, while *X. borealis* exhibited two phenotypes coded by one or two homozygous genes. Hybrids of them displayed three transferrin phenotypes.

The most detailed studies of transferrin polymorphism have been made on *Bufo* (GUTTMAN, 1972). BROWN and GUTTMAN (1970) and GUTTMAN (1973), who examined transferrin components of *Bufo arenarum* and *B. spinulosus*, as well as those of natural and artificial hybrids, found that *B. arenarum* showed two transferrin bands resulting from a combination of four codominant alleles, while *B. spinulosus* showed one band produced by one allele. The hybrids of these two species

showed two bands derived from the two parents. According to GUTTMAN and WILSON (1973), the populations of the *B. americanus* group distributed in southwestern Ohio showed 36 phenotypes controlled by 13 codominant alleles. The electrophoretic pattern of natural hybrids between two species invariably possessed the bands of the parents, and that of the hybrids between the species groups showed the same phenomenon. However, when the parents were remote in affinity, the hybrids often displayed bands of one parent or sometimes showed bands which were not found in either of the parents (GUTTMAN, 1972).

All the transferrin bands of various hybrids produced from the five species of Japan and Europe and two subspecies of Korea and Taiwan belonging to the *R. esculenta* group exhibited the bands derived from the parents as expected. All allotriploids produced between frogs from Japan, Korea and Taiwan showed transferrin bands corresponding to the genomes making up the individuals. Therefore, the fact that the genes of the foregoing five species and two subspecies of the *R. esculenta* group functioned in hybrids, as in the pure species, was the same as the observations between two species of each of *Pleurodeles* and *Acris*, and also between two comparatively closely related species of *Bufo* or *Xenopus*. A close relationship between the foregoing five species and two subspecies of the *R. esculenta* group was confirmed from these findings not only by comparison of morphological characters and crossing experiments, but also by biochemical techniques. It is interesting to note, however, that the bands of the subgroups including two subspecies from Korea and Taiwan displayed greater mobility as compared with the bands of the two subgroups of the two Japanese species and the three European species. A future investigation of *R. nigromaculata* and *R. p. plancyi* distributed widely in the eastern China will undoubtedly uncover several new codominant alleles, which will be useful in resolving phylogenetic relationships among the three subgroups of the *R. esculenta* group.

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