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Isozymes and Karyotypes of *Bufo* Species

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두꺼비 종류의 아이소자임과 염색체

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적 요

두꺼비와 물두꺼비 종류의 아이소자임과 염색체를 조사 비교 하였다. 전기영동법에 의하여 23가지 종류의 효소성 및 비효소성 단백질을 분석하였으며, 두꺼비 종류는 23가지중 6종류의 단백질이 다형이었고 물두꺼비 종류에서는 23가지중 4종류의 단백질만이 다형이었다.

23가지 종류의 단백질중 11가지는 두꺼비와 물두꺼비 사이의 종간차이가 없었으며 12가지는 뚜렷한 종간차이를 보였다.

두꺼비의 이형율은 4.8%이었고 물두꺼비의 이형율은 3.7%이었다. 두꺼비와 물두꺼비의 유전적 유사성은 0.5 이하였다. 두꺼비와 물두꺼비는 모두 22개의 염색체를 가지고 있었으며 서로 유사한 핵형이었으나 두꺼비에 있어서는 한쌍의 염색체에 부수 염색체가 있었다.

본 실험결과 두꺼비와 물두꺼비는 그들의 핵형은 서로 유사하나 유전자 구성에는 큰 차이가 있으며 진화학상 비교적 오래전에 2종이 서로 분화 하였음을 알 수 있었다.

INTRODUCTION

Since Nuttall (1901) used a serological precipitation method for separating more than 500 different species and trying to determine their inter-relationships with development of the starch gel electrophoresis, inter-and intra-specific variations have been studied by numerous workers (Imam and Allard, 1965, Lewontin, 1967; Carter *et al.*, 1968; Harris, 1969; Selander, 1970 : Johnson and Selander 1971; Gorman and Kim, 1975; Kim *et al.*, 1976).

Recent improvements in electrophoretic and biochemical staining techniques (Hunter and Markert, 1957) have allowed a quantification of levels of genetic similarity between various species and stimulated renewed interests in role of speciation in converting intraspecific to interspecific variation (Lewontin, 1967).

It has been reported only one *Bufo* species, *Bufo bufo gargarizans* Cantor until Kang and Yoon (1965) found another new species named *Bufo kangii* Yoon. Because frog species are readily available and also easily maintained in the laboratory for experimental purpose, more extensive embryological and physiological studies have been done on frogs than other animals. However, the *Bufo* species (Family *Bufonidae*) are not well known from genetical studies as well as other studies. The *B. kangii* species are strongly modified for land and watery life, and these members occupy only the river and river side at high mountain valley of North-Eastern area in Korea. However, the *B. bufo* species are widely distributed in Korea.

The techniques of gel electrophoresis (Selander *et al.*, 1971) and chromosomal preparation (Ford and Hamerton, 1956; Rothfedls and Siminovitch, 1958) provided powerful tools for investigating genetic and chromosomal variation in natural population of animals.

Using starch gel electrophoresis and chromosomal preparation techniques, the authors have gathered data on allele frequencies at 23 loci controlling enzymatic and other proteins as well as karyotypes in wild population of *Bufo* of the family *Bufonide*. The purposes of this study were (1) to obtain estimates of the degree of heterozygosity in species of *Bufo*, (2) to compare the karyotypes between *B. bufo* and *B. kangii* species, (3) to compare the genetic variations between *B. bufo* and *B. kangii*, and (4) to compare the gentic similarities between these two species.

MATERIALS AND METHODS

A total of 40 specimens (20 *B. bufo* and 20 *B. kangii*) were used for biochemical studies employing electrophoretic techniques, and 10 of each species were used for chromosome analysis.

Field work for *B. bufo* proceeded irregularly in August, 1975 and July, 1976. *B. kangii* was also sampled irregularly in March, 1975 and April, 1976. The sample localities are shown in Fig. 1.

Preparation of Samples for Electrophoresis

Blood samples were obtained from live specimens by the throat bleeding in a heparinized small beaker. Once the blood was obtained, it was centrifuged

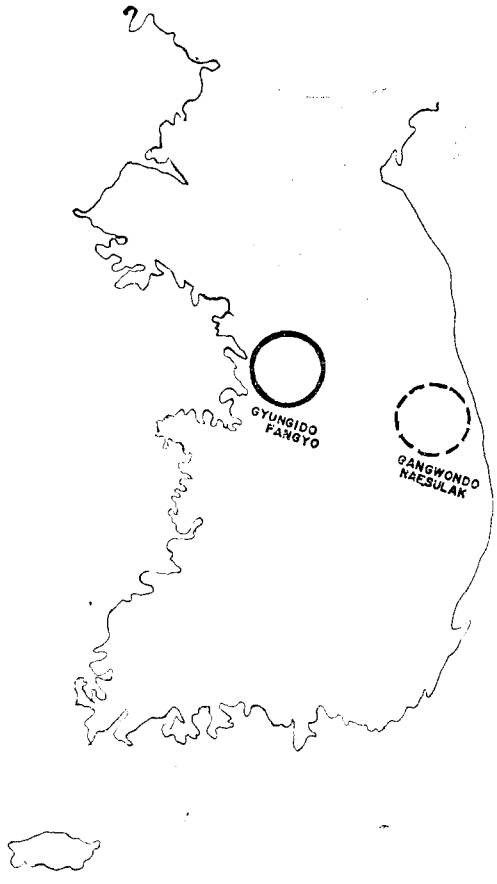

Fig. 1. sample collecting areas. *B. bufo* (circle), *B. kangii* (broken line circle)

at 2,500 G at 4°C, after which the plasma was pipetted out and stored in ultracold freezer (−40°C) until electrophoresis was performed. The blood cells were then suspended and washed twice with 0.85% saline. After the second wash, the cells were lysed by addition of an equal volume of deionized water to whole blood and then vibrated by means of an electric shaker for about 30 seconds, after this the samples were placed in ultracold freezer for about 15 minutes to lyse the blood cells thoroughly. The samples were then centrifuged at 14,000 G for 30 minutes. The supernatant was pipetted out and used for electrophoresis or stored at −40°C in ultracold freezer. All of the animals killed by throat bleeding or alive were frozen in ultracold freezer. For electrophoretic samples, kidney, liver or muscle tissues were individualy homogenized in two-fold greater volume of grinding solution using glass grinder tubes. The homogenates were then centrifuged at 14,000 G for 30 minutes. The supernatant was then pipetted out and placed in a glass culture tube. All of the samples were stored in a ultracold freezer until electrophoresis was performed. Enzymes in tissues that were processed as described above showed the same electophoretic patterns as did enzymes from fresh, unfrozen extracts processed in the same manner.

Electrophoretic and Staining Techniques

Horizontal starch-gel electrophoresis was used in this study as described by Selander *et al.* (1971). All buffers and stains are listed in the Appendix. Electro starch Lot 371 (Otto Hiller, Madison Wisconsin, U.S.A.) was used in these determinations at 12.5% concentration.

Chromosome Preparations

The animals sampled alive were injected intraperitoneally according to their body size with 0.2 to 0.5 m*l* of 1% colchicine solution. Twenty-four hours after the injection, the animals were sacrificed by brain damage and the femur of hind legs were removed. Marrow cells were then flushed out into a hypotonic solution by use of 1 m*l* syringe equipped with a No. 22 G needle. The marrow cells were then treated with the hypotonic solution for 10 minutes using a modification of the hypotonic citrate technique of Ford and Hamerton (1956). The marrow cells were then centrifuged at 400 rpm for 5 minutes at room temperature. All of the supernatant was removed, and Carnoy's solution was added gently without disrupting the cell pellet. Samples were kept in a refrigerator at 5°C for 30 minutes. This cell pellet was suspended by pipetting it in and out using 1 m*l* syringe equipped with a No. 22 G needle. The samples were then centrifuged again at 400 rpm for 5 minutes and the supernatant was removed. The cells were resuspended in fresh Carnoy's solution by shaking the tubes gently and centrifuged again as described above. This procedure was repeated twice to obtain fine samples, and after the final centrifugation, an appropriate amount of fresh Carnoy's solution was added according to the volume of cell pellet. Then, the tubes were agitated gently to suspend the cells. After the fine cell suspensions were obtained, chromosome preparations were made using the air-drying technique of Rothfelds and Siminovitch (1958). The slides were stained with Giemsa stain for about 10 minutes, and the chromosomes were obsesrved with a Nikon phase contrast microscope. Once suitable metaphase cells were obtained, a diploid number of chromosomes for each specimen was determined by counting at least 20, and often more than 30 selected metaphase cells. For each specimen a selected, well-spread metaphase was photographed.

RESULTS

A total of 23 enzyme systems was investigated. With regard to the degree of phenotypic variability, six proteins were variable within *B. bufo* species and four within *B. kangii* species. The most extreme cases were represented by hemoglobin, Mdh-2, Got-2, Ipo-1, Pept, 6-Pgd and Pgi, which were neither polymorphic nor interspecially different. Ldh-A, Idh-1 and Pgm-1 were polymorphic only in *B. bufo* and Mdh-1, Mpi, and Got-1 were polymorphic in both species. Among 23 enzymes encoded by structural gene 12 enzyme systems were genetically different between two species.

Lactate Dehydrogenase (Ldh)

Two lactate dehydrogenase systems, Ldh-A and Ldh-B both migrating anodally, were found in liver extracts on Tris citrate gels. As in other vertebrates (Markert and Massaro 1966), polypeptides of Ldh-A and Ldh-B in *Bufo* combine to produce a five-banded pattern (Fig. 2-A).

Three alleles, Ldh-A^F, Ldh-A^M and Ldh-A^S have been detected in *B. bufo* at frequencies of 0.075, 0.05 and 0.875, respectively. In *B. kangii*, all of the samples examined were fixed for Ldh-A^{SI} allele. At the Ldh-B locus, no polymorphic populations were found. However, all of the *B. bufo* and *B. kangii* species were fixed for different allele, Ldh-B^M and Ldh-B^S, respectively.

Malate Dehydrogense (Mdh)

Two forms of NAD-dependent malate dehydrogenase have been detected in liver extracts on Tris citrate gels. Both systems, Mdh-1 and Mdh-2 were migrated anodally (Fig. 2-C). Mdh-1 was polymorphic in both species. In *B. bufo*, three alleles, Mdh-1^F, Mdh-1^M, and Mdh-1^S were found at Mdh-1 locus at frequencies of 0.05, 0.09 and 0.05, respectively. Two alleles, Mdh-1^F and Mdh-1^M were detected in *B. kanii* at frequencies of 0.05 and 0.90, respectively. At Mdh-2 locus, only one allele, Mdh-2^M, which is common one in both species was found.

Isocitrate Dehydrogenase (Idh)

Two forms of isocitrate dehydrogenase, Idh-1 and Idh-2, were found in kidney and liver extracts on Tris citrate gels (Fig. 4-A). Both of these enzyme systems were NADP-dependent. Only one heterozygote was found at Idh-1 locus in *B. bufo* samples and all of the other samples were monomorphic and common allele between two species. However, Idh-2^S was the fixed allele for *B. bufo*, and *B. kangii* sampes were fixed for Idh-2^S.

6-Phosphogluconate Dehydrogenase (6-Pgd)

6-Phosphogluconate dehydrogenase was examined in liver extracts as a single band migrating anodally on Tris Maleic EDTA gels. No polymorphism was detected in all samples and one allele, 6-Pgd^M, was a common allele between two species. In these species, the 6-Pgd bands were not clear and fuzzy (Fig. 4-B). However, the reason is not known.

Glutamate Oxaloacetate Transaminase (Got)

In the extracts of liver and kidney analyzed electrophoretically on lithium hydroxide gels, two Got systems, Got-1 and Got-2, were demonstrated. One is an anodally migrating soluble enzyme (Got-1), and the other is a cathodally migrating mitochondrial enzyme system (Got-2).

Fig. 3-D shows the relative mobilities of hetero-and homozgote of alleles.

Two alleles, Got-1^M, and Got-1^S were detected at the Got-1 locus, however, only one allele, Got-2^M was found at the Got-2 locus in both species. Got-1^M and Got-1^S were found in *B. bufo* at frequencies of 0.05 and 0.95, respectively. In *B. kangii* samples Got 1^M and Got-1^S were detected at frequencies of 0.725 and 0.275, respectively. On the other hand, Got-2^M was the common and fixed allele in all *Bufo* samples examined.

Heterozygotes at the Got-1 locus were represented by three-banded patterns. Similar three-banded patterns were also found in the mitochondrial form of the enzyme (Got-2) in rodents (Delorenzo and Ruddle, 1970; Kim *et al.*, 1976) and in the soluble form (Got-1) in the rodents (Selander *et al.*, 1971).

Phosphoglucomutase (Pgm)

Three Pgm systems appearing in *Bufo* samples were analyzed by electrophoresis on Tris citrate gels. The loci are designated in order of increasing anodal mobility, Pgm-1, Pgm-2, and Pgm-3. However, Pgm-1 was too irregular and weakly staining to be scored and, therefore, was excluded from further consideration.

Three alleles, Pgm-2^F, Pgm-2^M, and Pgm-2^S, were demonstrable at the Pgm-2 locus in *B. bufo*. The allele observed and their relative mobilities are shown in Fig. 2-D. The allele frequencies were 0.05, 0.05 and 0.90, respectively. On the other hand, all of the *B. kangii* samples studied were fixed for Pgm-2^M.

At the Pgm-3 locus, *B. bufo* samples and *B. kangii* were fixed for Pgm-3^M and Pgm-3^F, respectively.

Indophenol Oxidase (Ipo)

Indophenol oxidase, which was analyzed on Tris versene borate gels, showed two anodally migrating bands (Fig. 4-C). The Ipo systems were represented by white bands on dark-blue background. The Ipo-1 was common system between two species. However, at Ipo-2 locus, *B. bufo* and *B. kangii* were fixed for Ipo-2^M and Ipo-2^S, respectively.

Esterase (Es)

Two forms of esterase, Es-1 and Es-2 have been detected in liver extracts on lithium hydroxide gels and Fast Blue RR salt stain, and was seen as a double-banded system migrating anodally (Fig. 3-B). Similar double-banded systems have been reported by Selander *et al.* (1971) for *Peromyscus*. No heterozygote was found in both species examined. However, each species were fixed for different allele. Es-1^M and Es-2^M were fixed allele for *B. bufo* and Es-1^S and Es-2^S for *B. kangii* species.

General Protein (Gp)

Non enzymatic proteins, general protein, were analyzed on lithium hydroxide gels and Amido black stain. Three anodally migrating systems, Gp-1, Gp-2 and Gp-3, were demonstrated at the Gp locus. The systems detected and their relative mobilities are shown in Fig. 3-A. As was shown at the esterase locus, no polymorphic loci were observed in all samples examined. However, the two species were fixed for different allele. *B. bufo* and *B. kangii* samples were fixed for $Gp\text{-}1^{M}$, $Gp\text{-}2^{M}$, $Gp\text{-}3^{S}$, and $Gp\text{-}1^{S}$, $Gp\text{-}2^{S}$, $Gp\text{-}3^{M}$, respectively.

Phosphoglucose Isomerase (Pgi)

Phosphoglucose isomerase was demonstrable in liver extracts analyzed on Poulik gels. One anodally migrating system was found at the Pgi locus. No polymorphic and no specific differences were found at Pgi locus.

Mannose Phosphate Isomerase (Mpi)

In extracts of liver analyzed on Tris citrate gels, an anodally migrating Mpi system was demonstrable.

In *B. bufo*, three alleles, Mpi^{M}, Mpi^{S}, and Mpi^{S1} were found at Mpi locus at frequencies of 0.13, 0.80 and 0.07, respectively. In *B. kangii*, two alleles, Mpi^{M}, and Mpi^{F} were found at Mpi locus at frequencies of 0.78 and 0.22, respectively. Mpi locus was most highly polymorphic locus in both species. Fig. 2-B shows the relative mobilities of hetero-and homozygote alleles.

Triose Phosphate Isomerase (Tpi)

Anodally migrating triose phosphate isomerase system was demonstrable in liver extracts analyzed on Poulik gels (Fig. 3-C). Tpi^{M} allele was the fixed allele for *B. bufo*. However, two alleles, Tpi^{M} and Tpi^{F}, were found in *B. kangii* at Tpi locus at frequencies of 0.95 and 0.05, respectively.

Peptidase (Pept)

Two peptidase systems, Pept-1 and Pept-2, were analyzed on Poulik gels. Both systems were migrated anodally, However, Pept-2 system was too weakly stained to be scored and, therefore, was excluded from further consideration (Fig. 4-E). Neither polymorphic nor specific differences were observed at Pept-1 locus.

Hemoglobin (Hb)

Hemoblobin, which was analyzed on Tris HCl gels, showed one anodally migrating bands (Fig. 4-D).

Karyotypes

In all specimens examined, the chromosome count accepted as the diploid

number was obtained in more than 95% of the cells observed in each of the animals. The rest of the cells, less than 5% are believed to have suffered defects during preparation. Certain chromosomes at different stages of contraction were frequently observed to present different arm-ratio or lengths. It is believed that once cell division has been arrested by colchicine, the chromosomes continue to contract one arm of a chromosome, or one chromosome of a pair may contract more rapidly the other, thus changing the arm-ratio.

Both species of the *Bufo* revealed a diploid chromosome number of 22, with 6 pairs of large meta- or submetacentric and 5 pairs of small meta- or submetacentric chromosomes. Both species had the same karyotype except one pair of large chromosomes which showed sat-chromosome at it's long arm in *B. bufo* species. Fig. 5 is showing the idiograms of the two species. The idiogram obtained for *B. kangii* is in good agreement with the result reported by Kang and Sunwoo (1973).

DISCUSSION

The 23 proteins considered in estimating the degree of genetic variation in the population of *Bufo bufo* and *B. kangii* are listed in Table 1, in which indicated also are the proportion of loci known to be polymorphic for each controlling locus and the total number of alleles detected. In *B. bufo*, 6 of the 23 loci were considered polymorphic loci and 17 were monomorphic. On the other hand, in *B. kangii*, 4 of the 23 loci were polymorphic and 19 were monomorphic. Obviously, *B. bufo* species had more polymorphic loci than did *B. kangii* species. However, the authors believe that, if these values are considered in terms of the number of samples and population sampled, the difference in the extent of polymorphic loci is likly to be less than that observed.

In *B. bufo* species, 4 loci had three alleles, while no loci had more than 2 alleles in *B. kangii* samples. All of these results indicated much smaller number of alleles per locus than occured in other animals. In *Peromyscus polionotus*, 12 of 32 loci examined were reported to have three or more alleles (Selander *et al.*, 1971). However, in *Dipodomys* no population of any of 11 species was found to have more than three alleles segregating at any locus (Johnson and Selander, 1971).

Twenty-six percent of 23 loci was polymorphic in *B. bufo* samples and 17% in *B. kangii*. The total number of alleles found at 23 locus in *B. bufo* and *B. kangii* were 33 and 27, respectively. On the other hand, the heterozygosity of *B. bufo* and *B. kangii* species was 4.8% and 3.7%, respectively. The number of alleles,

polymorphic loci and hetorozygosity were higher in *B. bufo* samples than were in *B. kangii*. It has been noted by many authors that populations occupying a relatively large variety of subhabitat types or exploiting a relatively large variety of types of food will maintain higher levels of genetic variation than does the population relatively constrained ecologically. Evidence for such a relationship has been reported for species of *Geomyidae* (Durrant, 1946; Miller, 1964), for *kangaroo* rats (Johnson and Selander, 1971), for *insular* species of birds (Van Valen, 1965), for *Drosophila* (Beardmore, 1970), and for *Sepea* snails (Cain and Sheppard, 1954). As mentioned in introduction, *B. bufo* occupies more extensive range, with *B. kangii* inhabiting only in a restricted area in North-Eastern high mountain valley in Korea. The genic variation also shows the same trend.

Among 23 loci examined 12 loci were common loci between two species and

Table 1. Biochemical variation in proteins in *Bufo bufo* and *B. kangii*.

Proteins	Proportion of population polymorhic		Total number of alleles	
	B.bufo	*B.kangii*	*B.bufo*	*B.kangii*
Hb	0	0	1	1
Ldh-A	0.22	0	3	1
Ldh-B	0	0	1	1
Pgm-2	0.17	0	3	1
Pgm-3	0	0	1	1
Mdh-1	0.17	0.13	3	2
Mdh-2	0	0	1	1
Idh-1	0.04	0	2	1
Idh-2	0	0	1	1
Got-1	0.09	0.22	2	2
Got-2	0	0	1	1
Ipo-1	0	0	1	1
Ipo-2	0	0	1	1
Mpi	0.26	0.30	3	2
Pept	0	0	1	1
6-Pgd	0	0	1	1
Es-1	0	0	1	1
Es-2	0	0	1	1
Pai	0	0	1	1
Tpi	0	0	1	2
Gp-1	0	0	1	1
Gp-2	0	0	1	1
Gp-3	0	0	1	1

11 loci were different. These results and others indicate that the genetic similarity between the two species is less than 0.5. This is a fairly low value. For example, for the allopatric species, *Sigmodon hispidus* and *S. arizonae*, the mean value of similarity was 0.76 (Johnson and Selander 1971). For 11 species of *Dipodomys*, the mean value of similarity was 0.61 (Johnson and Selander, 1971). Similar values close to those reported here have been found for subspecies of *Mus* from Denmark, the mean value was 0.50 (Selander *et al.*, 1969). The low value of genetic similarity of *Bufo* species was not consistent with the karyotypic variation.

The levels of interspecific similarity, together with the other evidence, suggest that these two *Bufo* species are established long times ago without karyotypic changes.

SUMMARY

Isozymes and chromosomes of *Bufo bufo* and *B. kangii* were studied by starch gel electrophoresis and bone marrow air-drying method. Twenty-three enzymes and nonenzymatic proteins in *Bufo* species collected in Korea provided a basis for the estimating the proportion of polymorphic loci in the genum.

Of 23 loci controlling the proteins examined, 26% and 17% were polymorphic in *B. bufo* and *B. kangii*, respectively.

In *B. bufo*, 4 loci had 3 alleles and no loci had more than 2 alleles in *B. kangii*.

Both species had same karyotypes with 22 chromosomes except one pair of chromosome which had sat-chromosomes in *B. bufo* karyotypes.

The genetic similarity was approximately 0.5.

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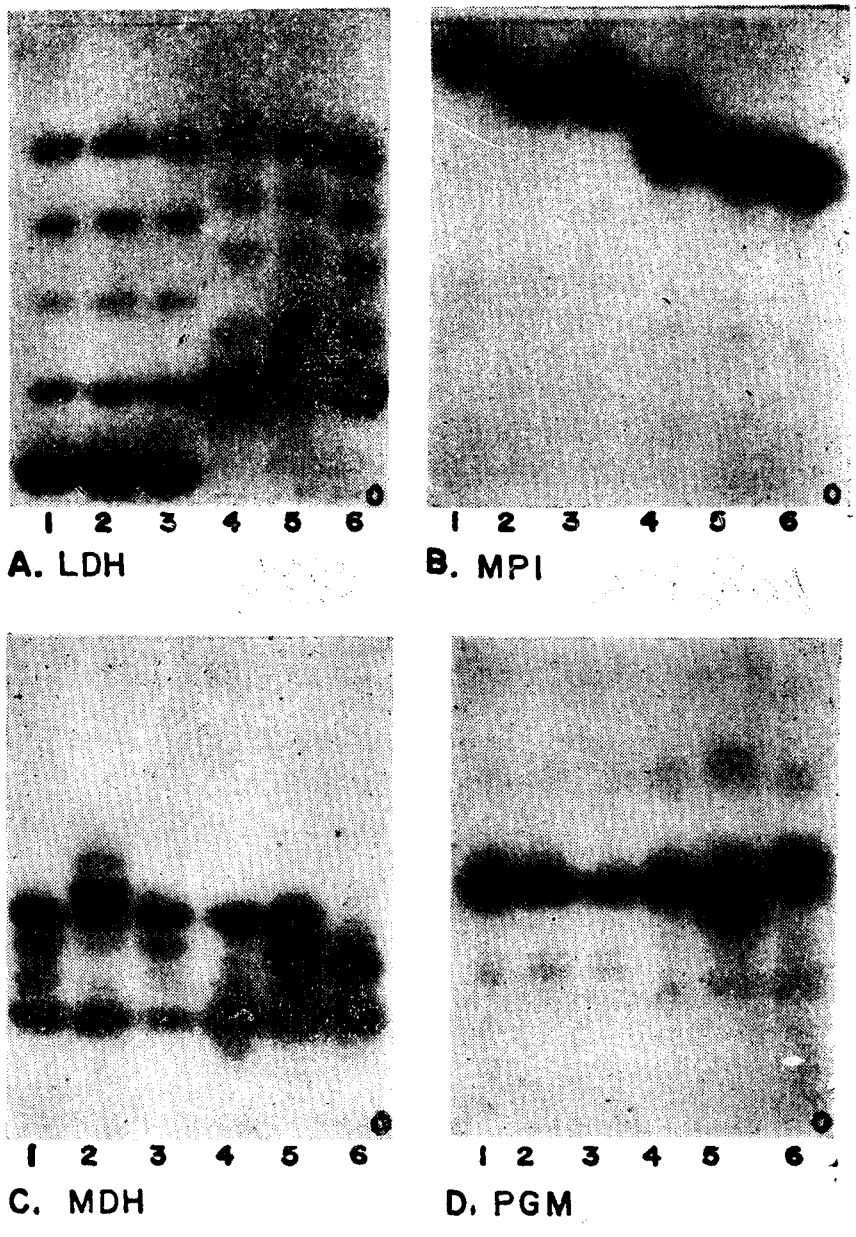

Fig. 2. Starch gel electrophoretic phenotypes of *Bufo bufo* and *B. kangii* species. The starting point is indicated by O, and the anodal directionis upward. 1,2,3, indicate *B. bufo* and 4,5,6, indicate *B. kangii*. A; LDH-A (bottom) and LDH-B (top), 5 is showing LDH-A heterozygote. B; 3 and 4 is showing two banded MPI heterozygote. C; MDH—1 (top) and MDH—2 (bottom), 2 and 5 is showing three banded MDH—1 heterozygote. D; PGM—1 (top), PGM—2 (middle) and PGM—3 (bottom), 5 and 6 is showing two banded heterozygote.

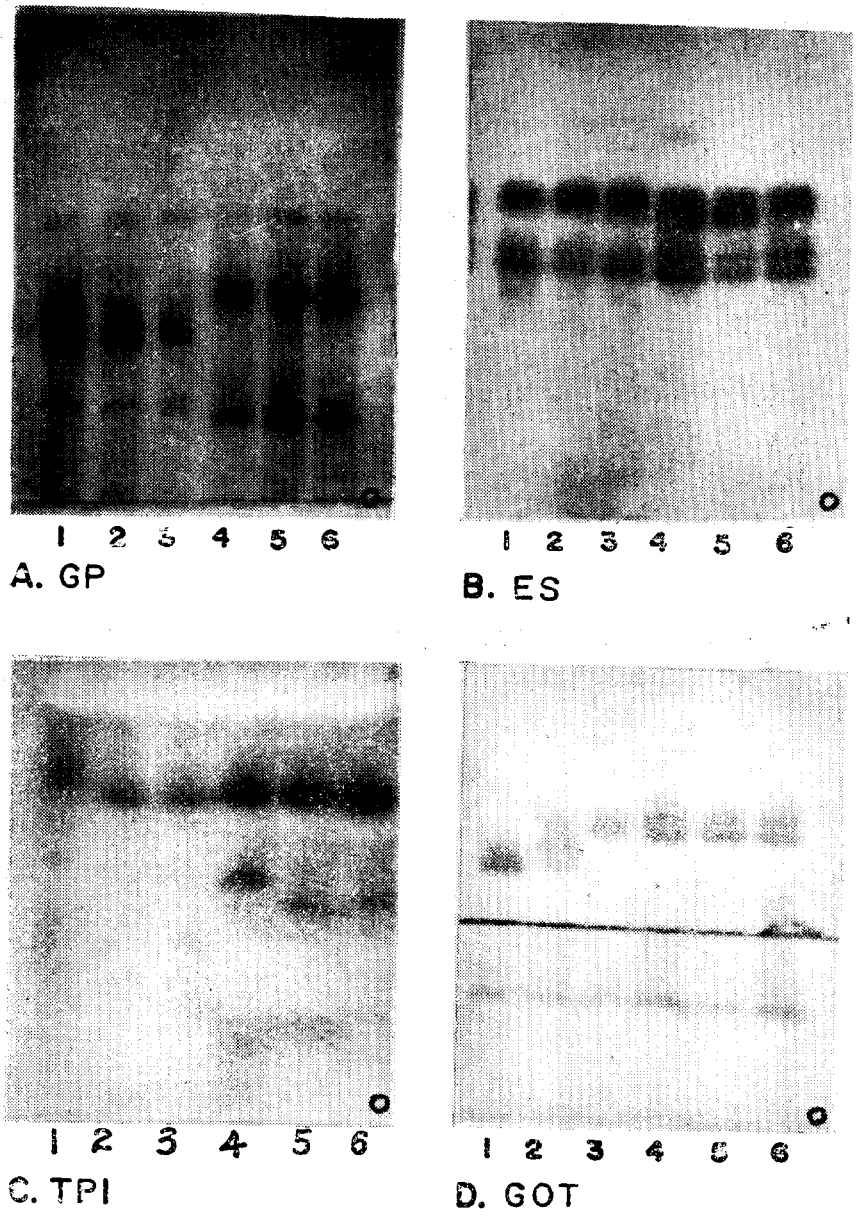

Fig. 3. Starch gel electrophoretic phenotypes of *Bufo bufo* and *B. kangii*. The starting point is indicated by O, and the anodal direction is upward. 1,2,3, indicate *B. bufo* and 4,5,6, indicate *B. kangii*. A; GP—1 (top), GP—2 (middle) and GP—3 (bottom). Note the protein mobilities. B; ES—1 (two banded top) and ES—2 ((two banded bottom). Note the protein mobilities. C; TPI. 1 is showing three banded heterozygote. D; GOT—1 (anodal) and GOT—2 (cathodal), 2 is showing GOT—1 heterozygote.

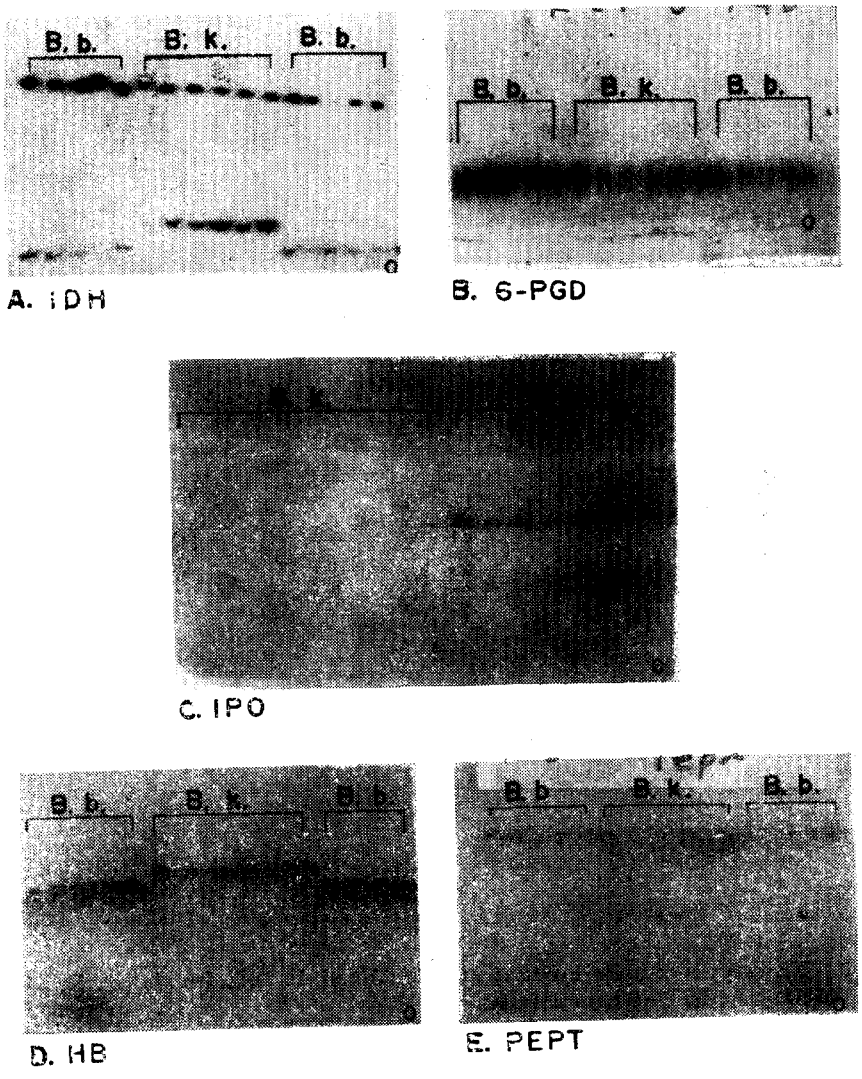

Fig. 4. Starch gel electrophoretic phenotypes of *Bufo bufo* and *B. kangii* species. The starting point indicated by O, and anodal is upward. *B. b.* indicates *Bufo bufo; B.k.* indicates *Bufo kangii.* A; IDH—1 (top) and IDH—2 (bottom), 4th from left is showing IDH—1 heterozygote. Note the IDH—2 mobilities. B; 6—PGD. All bands are fuzzy. C; IPO—1 (top) and IPO—2 (bottom) IPO—2 is stained very weakly.D; HB. Note the protein mibilities. E; PEPT—1 (top) and PEPT—2 (bottom), PEPT—2 is stained weakly.

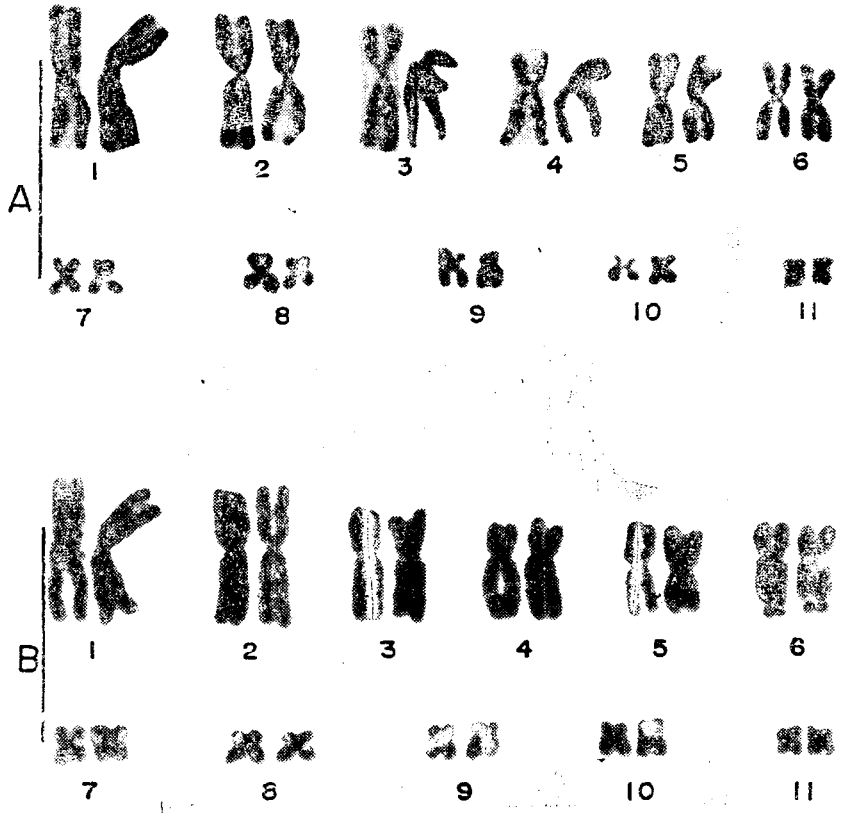

Fig. 5. Karyotypes of a *Bufo kangii* female (A) and *B. bufo* female (B). Sat-chromosomes are seen in B—6 chromosomes.