Experimental Hybridization between Toads from Kyoto and Toads from Miyako Is. and France

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ABSTRACT

Intersubspecific hybridizations among common toads were made, and the morphological characters of the hybrids thus obtained were examined. Females of *Bufo bufo japonicus* from Momoyama, Kyoto were crossed experimentally with males of *B. b. miyakonis* from Miyako Is., Ryukyu Archipelago and a male of *B. b. spinosus* from Banyuls, Southern France. Early development of hybrid embryos proceeded in the same normal way as the control embryos, although the percentages of normally cleft eggs were slightly lower in the former than in the latter. Most of the normally cleft hybrid eggs became normal swimming tadpoles and attained the completion of metamorphosis. The *japonicus* ♀ × *miyakonis* ♂ hybrid tadpoles had longer tails than the control, while the hybrid *japonicus* ♀ × *spinosus* ♂ tadpoles had the smallest tails. Immediately after metamorphosis, the hybrid young toads had shorter hindlegs than the controls and the older young hybrids of two to four months after metamorphosis differed markedly from the controls in this respect. Some of the male hybrids of *japonicus* ♀ × *miyakonis* ♂ attained sexual maturity in the fall. Histological examinations of the young toads revealed that both male and female gonads of *japonicus* ♀ × *miyakonis* ♂ were developing normally, whereas no normal spermatogonia were developing in the testes and the ovarian structures were completely destroyed in the *japonicus* ♀ × *spinosus* ♂ hybrids. These results seem to indicate that the genetical isolation is somewhat advanced between Japanese *japonicus* and French *spinosus*, and that they could be treated as two distinct species.

INTRODUCTION

The common Toad, *Bufo bufo*, and its allies are distributed widely from northwest Africa through most of the Eurasian Continent with Japan their easternmost limit. Though extensive descriptive studies have been made on this toad group in the past years, there still remain many unsolved questions as to the status and relation of not a few known forms. In particular, as to the relation between Asian and European common

toads, our knowledge is still almost at the same stage as in Stejneger's era (Stejneger, 1907; Matsui, 1974a, 1975a, 1976). There have been increasing studies on the systematics of Anurans in which the results of the cross experiments, together with other morphological and non-morphological characters, play a great role in determining the taxonomic relation of one form to another. This kind of experimental study on the genus *Bufo* was initiated by A. P. Blair, (1941) on the North American toad species, and recently W. F. Blair and his co-workers have compiled a vast amount of data on this genus (Blair, 1972). Even in this context, the knowledge of Eurasian toads, compared with those of America, is far from complete. With regards to Japanese anurans, studies of hybridization have been made by several authors (Daito, 1968; Kawamura, 1942-1960; Kuramoto, 1967, 1974; Moriya, 1951, 1960). Most of their studies, however, are confined to true frogs (chiefly of the genus *Rana*), and actually no study on toads has been made in Japan so far. Taking all these circumstances into consideration, the author is undertaking hybridization experiments among the known forms of Eurasian common toads. The results of the hybridization experiments between toads of Kyoto and those of Miyako Is., and Southern France are reported in this article.

MATERIALS AND METHODS

For crossing experiments among three subspecies of common toads, 7 females and 2 males of *Bufo bufo japonicus* SCHLEGEL from Momoyama, Kyoto, 3 males of *B. b. miyakonis* OKADA from Miyako Is., Ryukyu Archipelago and one male of *B. b. spinosus* DAUDIN from Banyuls, Southern France were used.

The laboratory hybridizations were performed during the breeding season of *japonicus* at Momoyama, March in 1974 and 1975. Male toads from Miyako Is. and Banyuls had been reared for 2 months before crossing, and were induced to clasp by Gonadotrophin injection ($150 \text{ IU} \times 2$ times for each animal). Immediately after being collected at the breeding pond, the *japonicus* females were carried to the laboratory for crossing. The eggs of *japonicus* females were fertilized not artificially (Rugh, 1934) but by the natural process. The eggs were inseminated by the male (during amplexus) as they emerged from the cloaca of the female. No difficulty was encountered in inducing males of *miyakonis* and *spinosus* to clasp females of *japonicus*.

The procedures employed in rearing the embryos were essentially similar to those used in other work of this type (Blair, 1955; Volpe, 1956). The egg strings from each fertilization were cut into small groups. Approximately 50 eggs were placed in a tank containing 2000cc of water and were raised at 13°C — 21°C (Mean 14.6°C). Tadpoles were fed boiled spinach. The water was changed every day and the dead embryos were removed. Thus, all kinds of tadpoles were reared under like conditions. Juvenile toads were fed fruit flies (*Drosophila* spp.), termites (*Reticulitermes speratus*) and meal-worm larvae (*Tenebrio molitor*). Some individuals were marked by the ordinary toe clipping

methods and released on the campus of the Zoological Institute of Kyoto University. Most of these toads were recaptured in two to four months. The developmental stages of tadpoles were determined following the table of Limbaugh and Volpe (1957). All the sample specimens were fixed in 10% formalin and stored in 70% ethanol. Measurements to the nearest 0.05mm were made with dial calipers and under a binocular dissecting microscope equipped with an ocular micrometer. The gonads of young and some matured toads were fixed in Bouin's fluid or 10% formalin, sectioned by the ordinary paraffin method ranging 5 to 8 μ in thickness and stained with haematoxylin and eosine.

OBSERVATIONS

I. Production of hybrid embryos

The results of the crossing experiments are shown in Table 1. Though the lack of

Table 1. Results of hybridization experiments

Crosses* female male	No. eggs	No. cleft eggs(%)**	No. Hatched (%)**	No. normal tadpoles(%)**	No. metamor- phosed(%)
<i>jap</i> #751 $\times$ <i>jap</i> #758	123	123 (100)	123 (100)	109 (88.6)	} 74/75 (98.7)
<i>jap</i> #752 $\times$ <i>jap</i> #759	127	126 (99.2)	126 (100)	115 (91.3)	
<i>jap</i> #741 $\times$ <i>miy</i> #741	252	236 (93.7)	—	—	
<i>jap</i> #753 $\times$ <i>miy</i> #751	500	493 (98.6)	489 (99.2)	448 (91.6)	} 74/75 (98.7)
<i>jap</i> #754 $\times$ <i>miy</i> #752	508	345 (67.9)	328 (95.1)	268 (81.7)	
<i>jap</i> #756 $\times$ <i>spi</i> #751	481	49 (10.2)	35 (7.4)	1 (2.9)	0 (0)
<i>jap</i> #757 $\times$ <i>spi</i> #751	718	702 (97.8)	632 (90.0)	618 (97.8)	75/75 (100)

* *jap*, *miy* and *spi* are abbreviations for *Bufo bufo japonicus*, *B. b. miyakonis* and *B. b. spinosus* respectively.
** With the number given in the preceding column being 100.

gravid females of *miyakonis* and *spinosus* prevented operating intrasubspecific control experiments of these two forms, the males of both subspecies were considered to be potent, since 68 to 99 percent of eggs inseminated by these males cleaved normally in the crossing experiments between *japonicus* females. In all the three series of *japonicus* females mated with *miyakonis* males, percentages of normally cleft eggs were slightly lower than in the control series; 68 to 99 percent of eggs in the experiment series cleaved normally in contrast with 99 to 100 percent in the control. Most of the nomally cleft eggs became normal swimming larvae in the experimental series as in the control. There was no marked difference in the growth rate between these hybrids and controls.

In one of the two experimental crosses between *japonicus* females and the *spinosus* male, only 10 percent of eggs cleaved normally. This might be due not to the defect of *spinosus* sperm but to the bad physiological condition of *japonicus* female #756, since in the other series, 98 percent of the eggs, also inseminated by the same *spinosus* male, cleaved normally, which value is almost the same as that in the control series. Moreover,

the shape of the eggs oviposited in the former case was abnormally triangular, which fact strongly suggested poor fertilizability of the eggs. The small number of cleaved eggs became abnormal swimming larvae, none of which reached metamorphic stages. On the contrary, in the latter experimental series of female *japonicus* #757 with the same *spinosus*, most of the eggs cleaved normally and became quite normal swimming larvae. They grew almost at the same pace as the controls, and the metamorphosis took place about 50 days after insemination.

II. External characters of hybrid tadpoles

The body color of hybrid tadpoles, both *jap.* ♀ × *miy.* ♂ and *jap.* ♀ × *spi.* ♂, was almost identical with that of the control, but there were some differences in the body shape of each tadpole. As the body shape of tadpoles changes with the growth, the comparisons were made with individuals of the same stages of development. The dimensions of each body part are shown in Tables 2 and 3, 25 days and 35 days after insemination, respectively.

Table 2. Measurements (mean and one standard deviation, in mm) of the hybrids and controls of the toad tadpoles, 25 days after insemination.

Parents	<i>jap</i> ♀ × <i>jap</i> ♂	<i>jap</i> ♀ × <i>miy</i> ♂	<i>jap</i> ♀ × <i>spi</i> ♂
Sample size	11	17	10
Devel. Stage	30—31	30—31	30—31
Snout-Vent length	9.06±0.69	9.43±0.60	9.54±0.56
Body depth	4.66±0.51	4.83±0.40	5.09±0.58
Body breadth	5.47±0.59	5.66±0.40	5.94±0.62
Tail length	15.11±1.42	15.96±0.77	14.37±0.70
Tail depth	4.71±0.43	4.74±0.33	4.54±0.47
Tail breadth	1.39±0.20	1.44±0.19	1.57±0.12
Depth of muscular portion	1.97±0.21	1.96±0.15	1.80±0.10
Hind-limb length	1.03±0.17	0.99±0.13	0.99±0.11
Snout to spiracle	6.06±0.43	6.24±0.44	6.35±0.44
Oral disc breadth	2.46±0.20	2.34±0.16	2.56±0.22
Interorbital space	2.48±0.18	2.68±0.16	2.68±0.16
Tail length Snout-Vent length ×100	166.61±6.55	169.62±8.47	151.02±10.05

In the 25-day tadpoles (stages 30-31), there were no statistically significant differences in the snout-vent length (S-VL) of the three groups, although there was a tendency for the hybrids *jap.* ♀ × *miy.* ♂ to be larger than those *jap.* ♀ × *spi.* ♂, which were in turn larger than the control tadpoles of *jap.* ♀ × *jap.* ♂. The most remarkable difference was the relative length of the tail. In the *jap.* ♀ × *miy.* ♂ hybrids, the tail was long and the tail fins were low, compared with the control tadpoles. On the contrary, the *jap.* ♀ × *spi.* ♂ hybrids had shorter tails than the controls. Thus, the hybrid tadpoles of *jap.* ♀ ×

miy. ♂ could easily be discriminated by their long tails (169.6% of the S-VL) from the short-tailed (151.0% of S-VL) *jap.* ♀ × *spi.* ♂ hybrids. Furthermore, the ratio of the tail depth to the body depth was the smallest in the *jap.* ♀ × *spi.* ♂ hybrids, indicating poor tail development in that group. The body shape of the hybrids *jap.* ♀ × *spi.* ♂ somewhat differed from that of the other two groups: the body of the former was broader and higher than that of the latter. In the hybrids *jap.* ♀ × *miy.* ♂, the mouth parts were

Table 3. Measurements (mean and one standard deviation, in mm) of the hybrids and controls of the toad tadpoles, 35 days after insemination.

Parents	<i>jap.</i> ♀ × <i>jap.</i> ♂	<i>jap.</i> ♀ × <i>miy.</i> ♂	<i>jap.</i> ♀ × <i>spi.</i> ♂
Sample size	10	16	10
Devel. Stage	33—36	34—36	34—36
Snout-Vent length	11.42±1.13	11.44±0.58	11.39±0.60
Body depth	5.57±0.54	5.12±0.39	5.42±0.56
Body breadth	6.80±0.80	6.70±0.42	7.01±0.57
Tail length	18.56±1.98	19.53±1.60	17.91±0.89
Tail depth	5.61±0.58	5.77±0.43	5.46±0.41
Tail breadth	2.12±0.27	2.12±0.15	2.06±0.17
Depth of muscular portion	2.43±0.29	2.59±0.17	2.35±0.11
Hind-limb length	2.32±0.59	2.64±0.54	2.25±0.44
Snout to spiracle	7.57±0.82	7.48±0.42	7.58±0.51
Oral disc breadth	3.18±0.30	2.88±0.18	2.95±0.27
Interorbital space	3.27±0.25	3.21±0.18	3.25±0.22
$\frac{\text{Tail length}}{\text{Snout-Vent length}} \times 100$	162.69±8.52	170.59±10.51	157.38±6.71

relatively smaller than those of the other two groups. These tendencies, characteristics of 25-day tadpoles, held true in the older tadpoles of 35 days after insemination (stages 33–36). There was no marked difference in the body length of the 35-day tadpoles of the three groups. Hybrids *jap.* ♀ × *miy.* ♂ had longer tails, lower bodies and smaller mouth parts than the *jap.* ♀ × *spi.* ♂ hybrids and controls. The relative tail length was smaller (157.4% of S-VL) in the *jap.* ♀ × *spi.* ♂ hybrids (162.7% in the controls and 170.6% in the *jap.* ♀ × *miy.* ♂ hybrids).

III. External characters of hybrid toads

Hybrid young toads, *jap.* ♀ × *miy.* ♂, immediately after metamorphosis, tended to be larger (S-VL 11.2mm) than the controls (10.4mm), but the individual variations within each group was great and the difference was not statistically significant. *Jap.* ♀ × *miy.* ♂ hybrids had slightly smaller heads than the other two groups as shown by the relative values (in terms of 100th of S-VL) of head width and eye margin distance.

The most remarkable difference in the body proportion of the three groups was seen in the length of the hind-leg (Table 4). Since the exact measurements of the total

Table 4. Measurements (Mean and one Standard Deviation, in mm) of the hybrid and normal toads immediately after metamorphosis.

Parents	<i>jap</i> ♀ × <i>jap</i> ♂	<i>jap</i> ♀ × <i>miy</i> ♂	<i>jap</i> ♀ × <i>spi</i> ♂
Sample size	11	10	10
Snout-Vent length	10.4±0.7	11.2±0.6	10.6±0.6
Head width	4.4±0.3	4.4±0.2	4.5±0.3
Distance between outer margins of upper eyelids	4.1±0.2	4.2±0.3	4.1±0.2
Tibia length	4.0±0.3	3.9±0.4	3.7±0.2

length of the hind-leg were difficult to make on the fixed young toads, the greatest length of the tibia was taken. The relative lengths of the tibia were similar in the *jap*. ♀ × *miy*. ♂ and *jap*. ♀ × *spi*. ♂ hybrids (34.5% and 34.9%, respectively), but they were slightly smaller than that of the controls (38.9%). As is evident from these values, the hybrids had shorter legs than the controls. Control young toads, *jap*. ♀ × *jap*. ♂, were wholly black at the time of metamorphosis. The back became coppery in a few days and the black color of the belly became paler. The young toads about a month after metamorphosis were coppery on the back with two rows of chevron-shaped bands. The back of the thigh and the tibia was mottled with dark bars. The number and degree of these bands varied individually. Dark stripes on the flank became clearer and the belly was dirty gray with many dark speckles. Hybrids, *jap*. ♀ × *miy*. ♂ and *jap*. ♀ × *spi*. ♂, were almost the same in their coloration as the controls. In the *jap*. ♀ × *spi*. ♂ young, the back was more brilliantly coppery and the bars on the hind-legs were more sharply marked than in the controls. Moreover, the belly of these toads was light gray with less dark speckles than in the control.

The growth rate varied remarkably among individuals, and the young toads about four months after metamorphosis were about 35 to 60mm in S-VL (Fig.1). Hybrids, especially *jap*. ♀ × *miy*. ♂, tended to grow more rapidly than the controls. In contrast to the metamorphosed stages, half-grown toads of three to four months were morphologically well differentiated. The toads of the three groups were distinguished from each other both in the body proportions and in the coloration. As the body proportions change with growth in anurans, it is undesirable simply to compare ratios of each part of the body against S-VL. Each individual here treated, however, belongs to immature stages of less than one year (except some *jap*. ♀ × *miy*. ♂ hybrids). Thus, the comparison of body proportions were made on the basis of percentage ratios of each measurement against S-VL (Table 5). The smallness of the sample size limited generalized conclusions, but as is evident from the Table 5, the two hybrid groups had similar proportions and were slightly different from the controls in several dimensions. Hybrids had shorter extremities than the controls. Parotoid glands of *jap*. ♀ × *spi*. ♂ hybrids were shorter and broader than those of *jap*. ♀ × *miy*. ♂ hybrids and the controls. The number and the develop-

Fig. 1. Dorsal (left rows) and ventral (right) views of young toads three to four months after metamorphosis. In each figure, the female is on the left side and the male on the right. The Black lines are scales of 10mm. a, b. Controls, *japonicus* ♀ × *japonicus* ♂ ; c, d. Hybrids, *japonicus* ♀ × *miyakonis* ♂ ; e, f. Hybrids, *japonicus* ♀ × *spinosus* ♂ .

mental conditions of dorsal warts and coarse ventral granules were more remarkable in the hybrids than in the controls. Further, the top of the warts was more sharply spiny, and the webs of the hind feet were more developed and their edges more sharply den-

Table 5. Measurements of young toads, three to four months after metamorphosis. Snout-vent lengths (S-VL) are actual values (in mm) with means followed by extremes in parenthesis, and all other dimensions are represented by relative values with means and one standard errors. Abbreviations: PL (Parotoid Length), PW (Parotoid Width), DT (Diameter of Tympanum), ET (Eye to Tympanum), FLL (Fore-Leg Length), HLL (Hind-Leg Length), FL (Foot Length), TL (Tibia Length).

Combination Sex Sample size	<i>jap</i> ♀ × <i>jap</i> ♂ male 3	<i>jap</i> ♀ × <i>miy</i> ♂ male 5	<i>jap</i> ♀ × <i>spi</i> × male 1	<i>jap</i> ♀ × <i>jap</i> ♂ female 1	<i>jap</i> ♀ × <i>miy</i> ♂ female 5	<i>jap</i> ♀ × <i>spi</i> ♂ female 2
S-VL	41.0	37.9	39.2	33.2	47.1	46.0
(Range)	(37.6—45.8)	(27.0—53.5)	—	—	(42.2—62.5)	(40.2—51.7)
PL/PW	3.2±0.2	3.0±0.2	2.6	3.0	3.0±0.2	2.6
DT/ET	1.3±0.1	1.5±0.1	1.3	2.0	1.5±0.1	1.7
FLL/S-VL×100	67.8±0.2	60.7±1.8	60.7	69.0	59.9±0.9	62.4
HLL/S-VL×100	126.2±0.8	113.5±2.2	113.3	128.0	113.9±1.5	115.5
FL/S-VL×100	40.9±0.3	36.6±0.7	35.2	39.8	37.5±0.7	38.2
TL/S-VL×100	37.3±0.3	32.2±0.6	32.1	37.4	33.2±0.2	33.9

ticulate in the hybrids. In contrast to the metamorphosed stages, young toads of four months varied individually in their coloration. The back of the controls was pinkish brown and the black horny layer on the wart poorly developed. The *jap.* ♀ × *miy.* ♂ hybrids were orangish brown on the back with chevron-shaped orange markings behind the parotoids and on the sacral regions. The top of the warts was surmounted with a black spine. There were dark bands on the surface of the extremities. The belly was dirty yellowish white, with several dark spots, which varied among individuals in number and distinctness. Broad dark stripes extended posteriorly on the flank from behind the parotoid glands. The *jap.* ♀ × *spi.* ♂ hybrids were similar to *jap.* ♀ × *miy.* ♂ hybrids and were somewhat different from the controls. For the sake of comparison, characters of the parental populations, *japonicus* from Momoyama, *miyakonis* from Miyako Is. and *spinosus* from Banyuls are also shown in Table 6. As there were few individuals of *jap.*

Table 6. Measurements of adult toads. Snout-vent lengths are actual mean values (in mm), and all other dimensions are represented by relative mean values. Abbreviations are the same as those used in the Table 5.

Subspecies Sex Sample size	<i>japonicus</i> male 23	<i>miyakonis</i> male 13	<i>spinosus</i> male 1	<i>japonicus</i> female 20	<i>miyakonis</i> female 37	<i>spinosus</i> female 1
S-VL	99.4	72.1	31.5	108.9	86.7	99.2
PL/PW	3.0	2.7	2.2	3.0	2.6	2.5
DT/ET	1.2	1.5	1.9	1.2	1.4	1.7
FLL/S-VL×100	66.2	60.3	67.5	64.4	56.1	63.0
HLL/S-VL×100	134.4	121.5	136.4	124.0	114.5	128.2
FL/S-VL×100	45.9	39.6	46.4	40.4	36.7	42.5
TL/S-VL×100	38.0	34.6	39.0	36.1	33.3	36.1

♀ × *jap.* ♂ controls and *jap.* ♀ × *spi.* ♂ hybrids, and further, the parental *spinosus* were but one male and one female, it was impossible to generalize the results of the observations. It was clear, however, that some characters of the hybrids were maternal, and some others paternal, while the remaining ones were intermediate between both parents. The relative length of the extremities, showing marked differences among young toads, is the character which discriminates adult *miyakonis* from *japonicus*, and the *jap.* ♀ × *miy.* hybrids resembled their paternal population in this character. There was no difference in the relative length of extremities between the adult male population of *japonicus* and *spinosus*, but this might be due to the fact that only one *spinosus* was used for the present crossing. Therefore, which parental source affected the hybrids in this character is unknown. By contrast, the wide black bands on the flanks and the dirty markings on the belly of the hybrids, though their degree varied among individuals, were doubtless from their maternal source.

IV. Structure of gonads

Some of *jap.* ♀ × *miy.* ♂ hybrids reached sexual maturity, having well-developed nuptial asperities, and uttered cries when handled. The structures of the gonads of the hybrids and controls were as follows.

A control male, *jap.* ♀ × *jap.* ♂ (Fig. 2a): The Bidder's organs were surrounded by follicles, suggesting the possible development of eggs. The testes were not yet fully ripened. Seminiferous tubules were developing and melanin granules were densely distributed among them. Seminiferous tubules were mostly filled with spermatogonia, but fairly large numbers of first spermatocytes were also seen. In another individual, a few spermatids were seen. A control female, *jap.* ♀ × *jap.* ♂ (Fig. 2b): Both the Bidder's organs and the ovaries were normally developing. A matured hybrid male, *jap.* ♀ × *miy.* ♂ (Fig. 2c): The Bidder's organs were normally developed. Fairly large numbers of sperm were seen, indicating rather active sperm formation. A hybrid female, *jap.* ♀ × *miy.* ♂ (Fig. 2d): The connective tissue was developing in the Bidder's organ and the eggs were slightly degenerating. The ovaries were normal and were developing in the same degree as those in the control females. A hybrid male, *jap.* ♀ × *spi.* ♂ (Fig. 2e): The poor state of preservation prevented close examinations. The Bidder's organs were normally developing, while the condition of the testes were slightly different from that of the control males. The testes were not yet ripened. Seminiferous tubules were rather thin. A few abnormal spermatogonia were observed. A hybrid female, *jap.* ♀ × *spi.* ♂ (Fig. 2f): The Bidder's organs were mostly filled with connective tissue, and the eggs were degenerating. There were abundant follicle cells where debris of degenerated eggs was absorbed. Few normally developing eggs were observed in the ovaries, which suggested the degeneration and absorption of the eggs after they had partly developed. The tissues near the outer margins of the ovaries were similar to the cortex tissue seen in the immature ovaries. The conditions found in the Bidder's organs and the ovaries of this hybrid were

Fig. 2. Cross sections of testes (a, c, e) and ovaries (b, d, f). The black lines are scales denoting 50μ . a, b. Controls, *japonicus* ♀ × *japonicus* ♂ ; c, d. Hybrids, *japonicus* ♀ × *miyakonis* ♂ ; e, f. Hybrids, *japonicus* ♀ × *spinosus* ♂ .

identical with those generally observed in the early and middle stages of amphibian sex reversion from female to male. Since no testicular structures were seen in these ovaries, it was impossible to say whether they would change into testes or not. Even so, that the ovarian structures were completely destroyed was quite evident.

DISCUSSION

Blair (1972) made extensive hybridization studies on toad species collected from many localities of the world. According to him, F_1 is sometimes obtained between two geographically remote species. Further, F_1 is easily obtained within the same species group. In the latter case, however, the results vary in the two reciprocals. For example, females of the *americanus* group easily produce metamorphosed young with other species, but males of the same group rarely produce viable hybrids with females of other species. Blair went so far as Eurasian *Bufo*, and he reported that the male *B. bufo* he used produced a few metamorphosed hybrids with *B. viridis* females. Moreover, intercontinental crosses between the former and species of North American *B. americanus* group sometimes produced metamorphosed F_1 .

Although Blair's description is extensive, his results were rather superficial, especially when he treated Eurasian species. Further, no studies on genera other than *Bufo*, comparable to his report, are available at present. Consequently, we are at present unable to generalize the genetic features of *Bufo*, but it is probable that genetic differentiation is preceded by morphological differentiation in this genus. There have been lots of arguments on the utility of genetical results when we carry on systematic studies of some group of animals. Generally speaking, we regard two forms as the same species when they actually or potentially interbreed with each other with fertile offspring. But we may regard two forms as distinct species when pre-mating isolating mechanisms inhibit natural hybridizations, notwithstanding the availability of experimental fertile hybrids. In fact, hybrids are sometimes found under natural conditions (Blair, 1941), but such cases are not unlimited. There are many closely related and taxonomically problematical species of animals distributed in the western and eastern parts of the Eurasian Continent, i. e. Europe and Asia. Besides, several morphologically related forms of animals are found allopatrically on the continent and its adjacent islands, such as Japan and Formosa. One author treats the taxonomic position of these forms quite differently from another. Genetical evidence may play a great role in clarifying taxonomic problems on these superficially related forms.

In the case of anurans, Kawamura's extensive studies (Kawamura and Kobayashi, 1960; Kawamura, 1962) revealed that the European common frog, *Rana temporaria*, and the Japanese brown frogs, *R. chensinensis* and *R. ornativentris* are genetically completely isolated in spite of their close morphological similarities. Similarly, Daito (1968) showed that the European and the Japanese tree frogs (*Hyla sarda* and *H. japonica*) are not in a subspecific relation as formerly considered, but are distinct species on the basis of F_1 sterility. When two faunal groups, distributed between Japan and continental Asia or between Asia and Europe, are considered, the above examples of the genera of anurans other than *Bufo* seem to suggest that genetical isolation may be proceeding between continental and Japanese common toads. Up to the present, about 20 taxa are known in

the *B. bufo* species group, including both currently accepted subspecies and morphologically related species, and their taxonomic status and phylogenetic relations are still poorly understood. Actually, the present situation is almost same as at the beginning of this century when Boulenger (1883) dealt badly with the Japanese common toads and Stejneger (1907) put the taxonomic problems on this group in order. *B. b. japonicus* is distributed in the western half of Japan, and *B. b. miyakonis* in the Miyako Is. of the Ryukyu Archipelago. The latter is now introduced on Minami- and Kita-Daito Is. of the same Archipelago (Matsui, 1975b), but many problems are involved in its origin and taxonomic position. Many workers consider it identical with the Chinese subspecies, *B. b. gargarizans* (Inger, 1947; Nakamura and Uéno, 1963), but I am of the opinion that there are clear morphological differences between these two forms (Matsui, 1974b). *B. b. spinosus* is distributed in the mediterranean part of Europe, Asia Minor and Northern-west Africa, and is considered to be a geologically older subspecies than *B. b. bufo* (Nikol'ski, 1918; Ternt'ev and Chernov, 1949). Some authors consider these two forms not clearly separated morphologically (De Lange, 1972), but I consider them as two separate subspecies because they are different in their breeding habits (Hotz, 1970). The three subspecies treated in this article are discriminated from each other notwithstanding their great intrasubspecific morphological variations. As the results of crossing experiments, *japonicus* ♀ and *miyakonis* ♂ showed close genetic compatibility, while *japonicus* ♀ × *spinosus* ♂ produced sterile F₁. As mentioned previously, the results might differ in the reciprocal, but it is conceivable that at least in *spinosus* and *japonicus*, genetical isolation is somewhat advanced. Further, in the case of *miyakonis*, the result of a reciprocal cross might be different from that obtained in the present work, since the morphological characters of this subspecies resemble *spinosus* rather than *japonicus*. And even if fertile F₁ is obtained in the both reciprocals, the existence of premating isolating mechanisms must be examined. *B. b. japonicus* and *B. b. spinosus* are the two extreme forms of the common toad, distributed in the peripheral regions of the ranges covered by this species group. Consequently, geographically intermediate forms, such as *B. b. bufo* of northern Europe, should be investigated in future studies, but the results shown in this paper strongly suggested that *B. b. japonicus* may be separated as a full species from *B. b. spinosus*.

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