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Studies on the Speciation of Pond Frogs in East Asia

By

Mitsuru KURAMOTO*

Department of Biology, Fukuoka University of Education, Munakata, Fukuoka 811–41, Japan

(With 3 Text-figures)

INTRODUCTION

Palearctic pond frogs include six species; *Rana nigromaculata, R. plancyi* and *R. brevipoda* of East Asia and *R. ridibunda, R. lessonae* and their hybrid, *R. esculenta*, of Europe. TING (1939) first reported the artificial hybrids in this group, those between *R. nigromaculata* and *R. plancyi plancyi*, and MANDEVILLE and SPURWAY (1949) reported those between *R. ridibunda* and *R. esculenta*, both of which were viable. Many hybridization experiments were performed thereafter among Asiatic species (MORIYA, 1951, 1960; TING, TSAI and LIU, 1965), among European species (BERGER, 1970, 1973; GÜNTHER, 1973), and among Asiatic and European species (KAWAMURA and NISHIOKA, 1975; KAWAMURA, NISHIOKA and KURAMOTO, 1972).

Evaluation on the post-mating isolation mechanisms revealed by hybridization experiments is essential in clarifying the species problems in general and provides one of the most useful clues in determining phylogenetic relationships between related species. In this study, the developmental compatibility and reproductive capacity of the hybrids between four members of the Asiatic pond frogs were examined with the aim of elucidating the speciation process in this group of frogs.

MATERIALS AND METHODS

The frogs used in this study are *Rana nigromaculata* HALLOWELL from Hiroshima, Japan and Seoul, Korea, *R. brevipoda brevipoda* ITO from Okayama, Japan, *R. plancyi fukienensis* POPE from Changhua (Zhanghua), Taiwan and *R. plancyi chosenica* OKADA from Seoul, Korea. The following abbreviations are used to indicate the combinations of female and male parent, with abbreviation of the female preceding that of the male: N, *R. nigromaculata* from Japan; K, *R. nigromaculata* from Korea; B, *R. brevipoda brevipoda*; F, *R. plancyi fukienensis*; C, *R. plancyi chosenica*. For instance, NC means the hybrid combination between female *R. nigromaculata* from Japan and male *R. plancyi chosenica*.

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* Visiting Professor, Laboratory for Amphibian Biology, Faculty of Science, Hiroshima University, Hiroshima 730, Japan

Ovulation of female *R. nigromaculata* and *R. brevipoda brevipoda* was induced by pituitary transplantation. A part of the egg batch from a single female was inseminated with the conspecific sperm and the other parts were inseminated with the sperm of different species or subspecies. Embryos and tadpoles were reared at room temperature (18~22°C) in PETRI dishes (18 cmϕ) and enameled pans (32×22×6 cm). The stages of embryonic development were recorded after RUGH's table (1948). Tadpoles were fed with boiled spinach. Metamorphosed froglets were kept in outdoor tanks (95×65×20 cm) and fed with mosquitoes and flies.

Gonads of some metamorphosed froglets and mature males were fixed in NAVASHIN's solution, sectioned at 10 μm thickness and stained with HEIDENHAIN's iron haematoxylin to check the histological structure. Fertility of mature individuals was tested by backcross experiments.

OBSERVATION

I. Development of hybrids

1. Developmental compatibility

Table 1 shows the development of control and hybrid embryos involving three females of Japanese *R. nigromaculata*. Fertilization rates were high in three combinations, NN, NK and NB. Most of these embryos developed into normal

TABLE 1
Development of control and hybrid embryos produced from females of Japanese *Rana nigromaculata*

Parents*		No. of eggs	No. of cleaved eggs (%)	No. of dead embryos**				No. of swimming tadpoles	
				C-B	Gast	Neur	T-H	Abnormal	Normal (%)
N♀I	× N♂	66	62 (94)	2	6	0	6	9	39 (63)
	× K♂	92	77 (84)	0	5	1	7	8	56 (73)
	× B♂	116	91 (78)	1	10	6	4	9	61 (67)
	× F♂	110	8 (7)	0	0	0	6	0	2 (25)
	× C♂	101	5 (5)	0	0	0	0	0	5 (100)
N♀II	× N♂	94	92 (98)	0	0	0	0	6	86 (93)
	× K♂	96	86 (90)	1	0	1	0	5	79 (92)
	× B♂	88	85 (97)	0	0	0	0	6	79 (93)
	× F♂	110	11 (10)	0	0	0	0	0	11 (100)
	× C♂	106	49 (46)	0	0	0	0	0	49 (100)
N♀III	× N♂	127	125 (98)	1	0	1	1	0	122 (98)
	× K♂	100	100 (100)	1	0	1	0	0	98 (98)
	× B♂	97	71 (73)	1	0	0	1	0	69 (97)
	× F♂	98	2 (2)	0	0	0	0	0	2 (100)
	× C♂	113	0 (0)	—	—	—	—	—	— (—)

* N, Japanese *R. nigromaculata* K, Korean *R. nigromaculata* B, *R. brevipoda brevipoda* F, *R. plancyi fukienensis* C, *R. plancyi chosenica*

** C-B, Cleavage to blastula stage Gast, Gastrula stage Neur, Neurula stage T-H, Tailbud to hatching swimming tadpoles and nearly all of them completed their metamorphoses. The fertilization rates in the hybrid combinations, NF and NC, were very low. These low rates seemed to result from partial fertilization block because the rates in BF and BC combinations were normal as described below. In spite of the low fertilization rates, the majority of embryos developed normally to the swimming tadpoles and completed their metamorphoses. No hybrid inviability was observed in the hybrids between females of Japanese *R. nigromaculata* and males of the other four kinds of frogs.

Artificial hybridization involving a female *R. nigromaculata* from Korea gave similar results (Table 2). KK, KN and KB embryos were normal in the rates of fertilization and normal tadpoles. Fertilization rates of KF and KC were lower than those of control embryos, but the rates of normal tadpoles were not so remarkably inferior compared to the controls. Since more than 60% of tadpoles completed their metamorphoses in each of the five crosses, there were no hybrid inviability in the hybrids between a female of Korean *R. nigromaculata* and males of the other four kinds of frogs.

TABLE 2

Development of control and hybrid embryos produced from a female of Korean *Rana nigromaculata*

Parents*	No. of eggs	No. of cleaved eggs (%)	No. of dead embryos**				No. of swimming tadpoles	
			C-B	Gast	Neur	T-H	Abnormal	Normal (%)
K♀I × K♂	280	273 (98)	13	0	0	0	1	259 (95)
× N♂	384	316 (82)	23	1	0	0	0	292 (92)
× B♂	354	312 (88)	21	0	0	1	0	290 (93)
× F♂	391	91 (23)	14	0	0	0	11	66 (73)
× C♂	381	89 (23)	14	0	0	0	0	75 (84)

*, ** Same as in Table 1

The experimental results using three female *R. brevipoda brevipoda* were the same as those using female *R. nigromaculata* except for rather high fertilization rates in BF and BC hybrid combinations (Table 3). The fertilization rates and percentages of normal tadpoles in all combinations derived from B♀II were exceedingly low, apparently due to the inadequate maturity of the eggs. The hybrid embryos from the other two females developed to swimming tadpoles as normal as their control embryos.

In spite of the normal development in embryonic stages, all of BF and BC hybrids died. The tadpoles became abnormal in appearance soon after free swimming stage and all of them died within two weeks after hatching. Morphological abnormalities observed were edematous syndrome and malformation of the tail and eyes. Some tadpoles were normal in morphology but they became immobile, did not take food, and eventually died. From this observation, it is concluded that the genomes of *R. brevipoda brevipoda* and two subspecies of *R. plancyi* are, to a considerable extent, incompatible in the hybrid combinations of BF and BC. On the other hand, BN and BK tadpoles metamorphosed normally as their control tadpoles.

TABLE 3
Development of control and hybrid embryos produced from females of *Rana brevipoda brevipoda*

Parents*		No. of eggs	No. of cleaved eggs (%)	No. of dead embryos**				No. of swimming tadpoles	
				C-B	Gast	Neur	T-H	Abnormal	Normal (%)
B♀I	× B♂	91	81 (89)	0	5	3	0	12	61 (75)
	× N♂	80	74 (93)	0	3	7	1	6	57 (77)
	× K♂	99	75 (76)	1	7	4	3	10	50 (67)
	× F♂	101	61 (60)	0	2	4	2	23	30 (49)
	× C♂	94	67 (71)	0	5	7	3	6	46 (69)
B♀II	× B♂	124	29 (23)	27	0	0	0	1	1 (3)
	× N♂	143	60 (42)	42	5	4	0	0	9 (15)
	× K♂	127	54 (43)	33	9	0	0	0	12 (22)
	× F♂	111	34 (31)	25	2	2	5	0	0 (0)
	× C♂	123	45 (37)	25	10	1	7	2	0 (0)
B♀III	× B♂	24	23 (96)	1	0	0	0	0	22 (96)
	× N♂	21	12 (57)	4	0	0	0	1	7 (58)
	× K♂	17	16 (94)	0	0	0	0	0	16 (100)
	× C♂	29	27 (93)	1	1	0	0	2	23 (85)

*, ** Same as in Table 1

2. Development rate and growth rate

Differences in the development rate among various combinations of embryos from the females of Japanese *R. nigromaculata* could not be detected until the late gastrula stage. The development of hybrid embryos retarded somewhat at the neurula stage. The five kinds of embryos entered stage 15 (closure of neural folds) in the following order.

$$NN \geqq NK > NB \geqq NC = NF$$

NF embryos entered stage 15 about 1.5 hr later than NN embryos. Similarly, the following order was observed in development rate of embryos involving a female of Korean *R. nigromaculata*.

$$KK = KN > KB > KC = KF$$

In the hybrid embryos, KB, KC and KF, retardation became evident after stage 12 (mid-gastrula), and KF embryos entered stage 15 about 2.5 hr later than KK embryos. At later embryonic stages, no developmental retardation was noticed in the hybrids involving females of Japanese and Korean *R. nigromaculata*.

In the embryos from female *R. brevipoda brevipoda*, BC and BF embryos showed developmental retardation after stage 12. The order of development rate observed at stage 15 was:

$$BB = BN = BK > BC = BF$$

The time difference between BB and BF embryos at stage 15 was 1 to 1.5 hr. In contrast to NF, KF, NC and KC embryos, BF and BC embryos showed developmental retardation in the late embryonic stages.

The growth of embryos did not differ significantly from one another except inviable BF and BC hybrids. However, NF, KF, NC and KC hybrids tended to grow and metamorphose faster than the other combinations. BK tadpoles metamorphosed earlier than BN, and the latter metamorphosed earlier than BB. There were no significant differences in snout-vent lengths of various kinds of froglets measured at the end of tail absorption. No hybrid vigor nor hybrid weakness was recognized in the growth rate of metamorphosed frogs.

II. Reproductive capacity of hybrid frogs

1. Gonads of metamorphosed froglets

The gonads of the control and intraspecific hybrid forglets of *R. nigromaculata*, NN, KK, NK and KN, had differentiated normally into ovaries or testes at metamorphosis (Table 4). The ovaries had many growing auxocytes (Fig. 1a) and the testes contained many spermatogonia and rete cells (Fig. 1b). Sex ratio did not differ from the expected 1: 1 ratio in NN, KK and NK frogs ($P>0.05$, χ^2-test), but differed significantly in KN frogs ($P<0.005$). The female preponderance in the KN frogs probably indicates some genetic differentiation of Japanese and Korean populations of *R. nigromaculata*.

The males of NB, KB, BN and BK hybrids had normal testes, while most of the females had underdeveloped ovaries (Table 4). The ovaries had narrow ovarian cavities but contained a few auxocytes in the inner part and small oocytes in the peripheral parts (Fig. 1c). Sex ratio in these hybrids did not differ significantly from 1: 1 ratio ($P>0.05$).

TABLE 4
Gonads of froglets immediately after metamorphosis

Parents*	No. of individuals examined	Females with normal ovaries	Females with underdeveloped ovaries	Males with normal testes
N♀ × N♂	21	9	0	12
× K♂	13	7	0	6
× B♂	11	0	7	4
× F♂	3	0	1	2
× C♂	15	0	6	9
K♀ × K♂	23	13	0	10
× N♂	22	20	0	2
× B♂	26	0	17	9
× F♂	24	1	12	11
× C♂	28	2	15	11
B♀ × N♂	6	0	3	3
× K♂	7	1	1	5

* Same as in Table 1

Structures of the gonads of NF, KF, NC and KC hybrids were similar to those of the hybrids described just above (Table 4). Their testes were normal (Fig. 1d), while the ovaries were underdeveloped (Fig. 1e), although a very few indi-

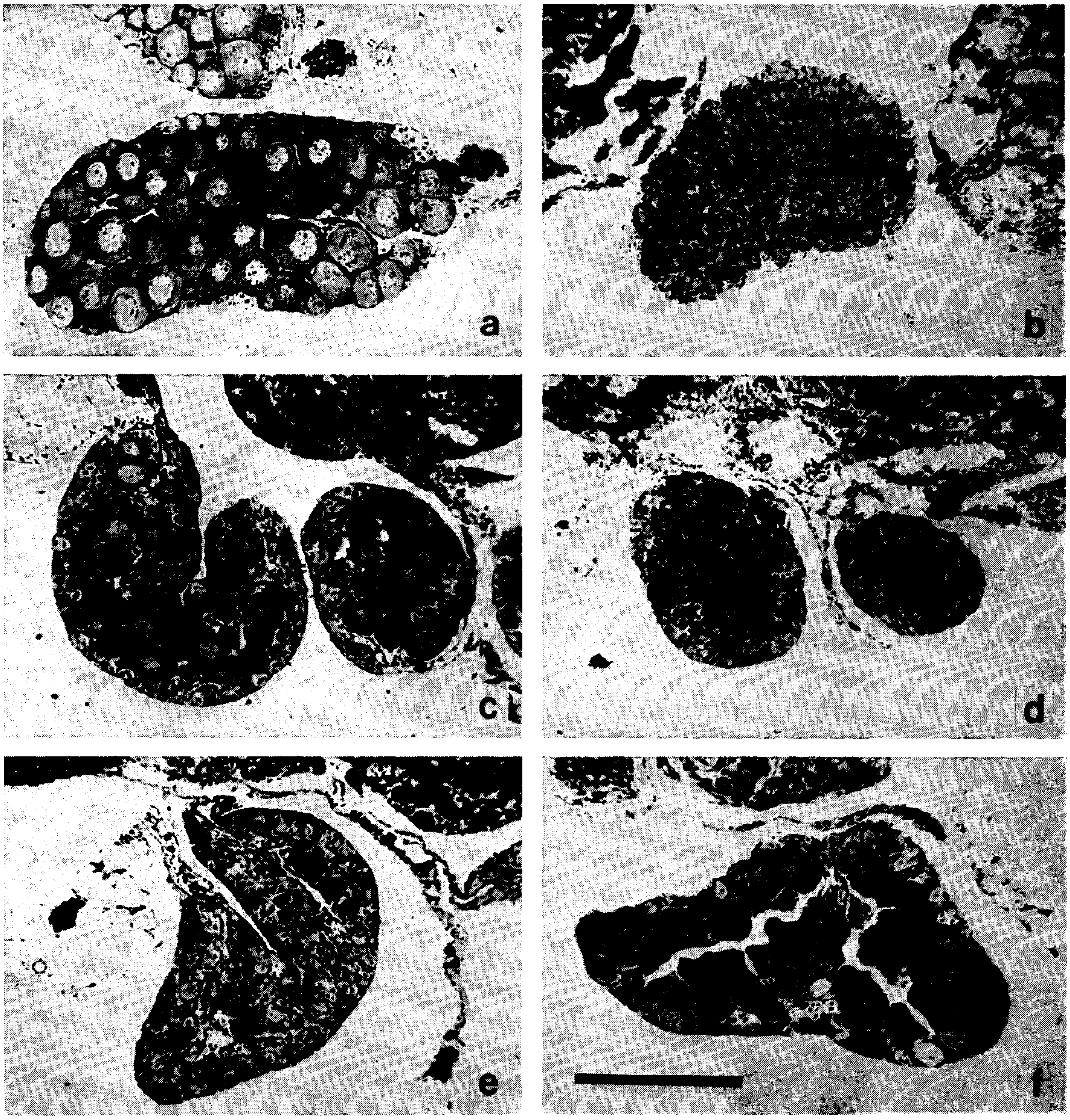

Fig. 1. Cross sections of the gonads of metamorphosed frogs.

a. *R. nigromaculata* (Korea) ♀ × *R. nigromaculata* (Japan) ♂, normal ovary b. *R. nigromaculata* (Korea) ♀ × *R. nigromaculata* (Japan) ♂, normal testis c. *R. nigromaculata* (Korea) ♀ × *R. brevipoda brevipoda* ♂, abnormal ovary d. *R. nigromaculata* (Japan) ♀ × *R. plancyi chosenica* ♂, normal testis e. *R. nigromaculata* (Japan) ♀ × *R. plancyi fukienensis* ♂, abnormal ovary f. *R. nigromaculata* (Korea) ♀ × *R. plancyi fukienensis* ♂, normal ovary Scale, 300 μm

viduals had nearly normal ovaries (Fig. 1f). Sex ratio did not differ from 1: 1 ratio ($P>0.05$).

The gonads of 15 NC hybrids (8 males and 7 females) which died accidentally about 2 months after metamorphosis were examined. In the testes, there were many well-formed seminiferous tubules. However, the spermatogenetic process showed apparently abnormal features resembling those found in the mature hybrids which will be described below. In contrast, all the ovaries had numerous growing auxocytes in the inner parts. This observation showed that the hybrid testes did not differentiate normally regardless of their normal structure at metamorphosis, while the hybrid ovaries differentiated normally from the apparently abnormal state at metamorphosis.

2. Backcross experiments

The results of backcross experiments are summarized in Table 5. KN males were fertile; the embryos from NN females and KN males developed into normal tadpoles. BN, BK and KB males were nearly completely sterile, and only a few eggs of NN, BB or BK females cleaved when inseminated with sperm of these hybrid males. However, the cleaved eggs developed normally into swimming tadpoles. The sterility of BK and KB males was more complete than that of BN males, corresponding to the abnormal structure of BK and KB testes compared to BN testes.

NF, NC and KC males were completely sterile; not an egg of *R. nigromaculata* cleaved when inseminated with sperm suspensions prepared from these hybrid males.

Two BK hybrid females were proved to be fertile. Their eggs inseminated with sperm of BB and KK males developed normally (Table 5).

TABLE 5
Development of backcross embryos

Parents*		No. of eggs	No. of cleaved eggs (%)	No. of dead embryos**			No. of swimming tadpoles	
				Gast	Neur	T-H	Abnormal	Normal (%)
NN♀ (2)	×NN♂ (3)	269	232 (86)	0	1	3	4	224 (97)
	×KN♂ (2)	226	169 (75)	1	1	3	7	157 (93)
	×BN♂ (4)	336	1 (0)	0	0	0	0	1 (100)
	×BK♂ (3)	235	0 (0)	—	—	—	—	— (—)
	×KB♂ (5)	421	0 (0)	—	—	—	—	— (—)
	×NF♂ (4)	1206	0 (0)	—	—	—	—	— (—)
	×NC♂ (2)	514	0 (0)	—	—	—	—	— (—)
	×KC♂ (3)	401	0 (0)	—	—	—	—	— (—)
BB♀ (6)	×BB♂ (2)	435	283 (65)	3	2	5	13	260 (92)
	×BN♂ (3)	1010	33 (3)	3	2	0	1	27 (82)
	×BK♂ (2)	819	4 (0)	4	0	0	0	0 (0)
	×KB♂ (2)	1168	1 (0)	0	0	0	0	1 (100)
BK♀ (2)	×BB♂ (4)	490	444 (91)	3	16	25	44	356 (80)
	×KK♂ (1)	288	284 (99)	8	4	26	11	235 (83)
	×BN♂ (3)	308	3 (1)	0	0	0	1	2 (67)
	×BK♂ (2)	249	0 (0)	—	—	—	—	— (—)
	×KB♂ (2)	435	0 (0)	—	—	—	—	— (—)

*, ** Same as in Table 1. Figures in parentheses are the number of individuals used in the matings

3. Structure of the testes of mature frogs

The size of testes of the hybrid males used in the backcross experiment did not

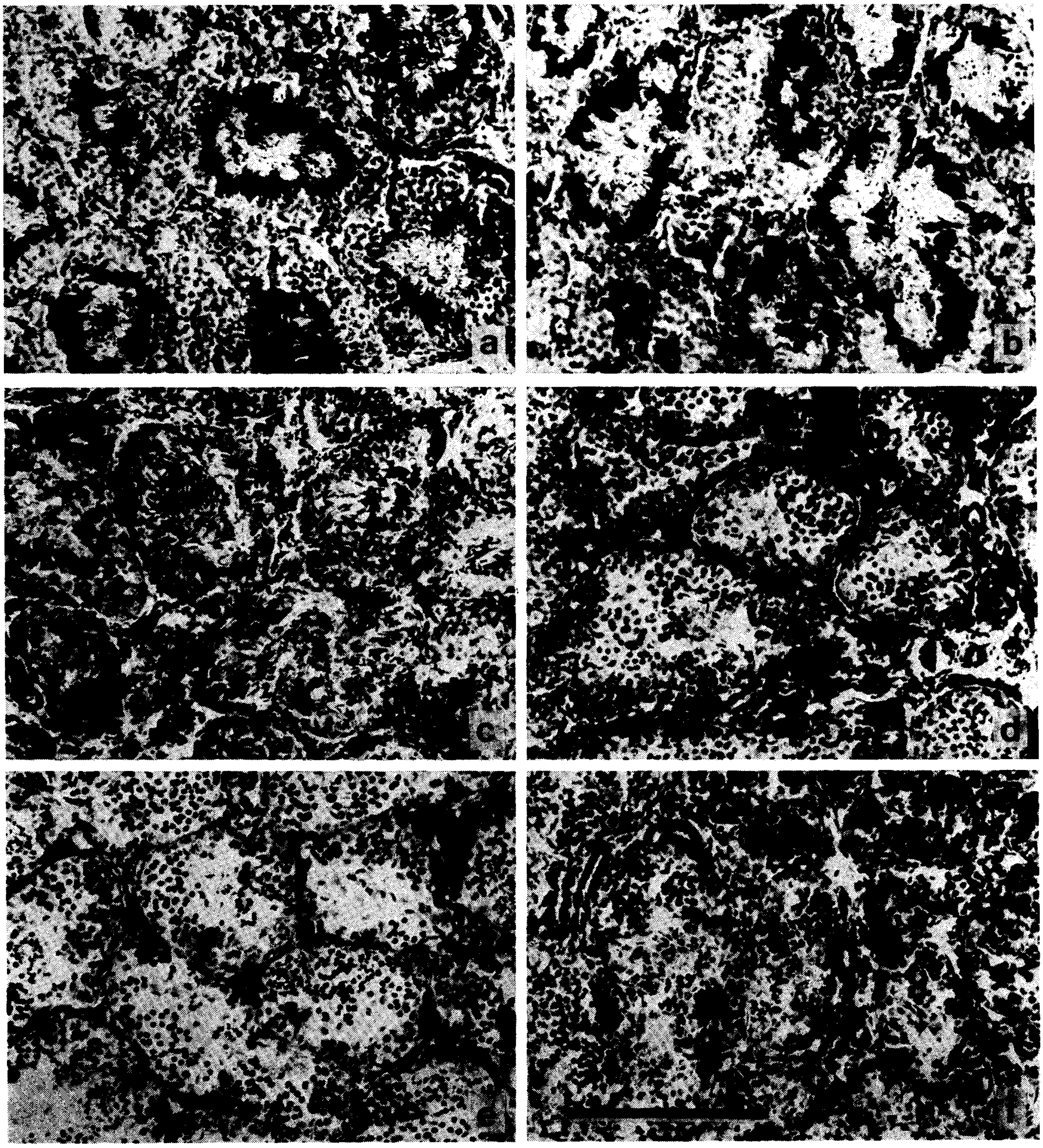

Fig. 2. Cross sections of the testes of mature frogs.

a. *R. brevipoda brevipoda* ♀ × *R. brevipoda brevipoda* ♂ b. *R. nigromaculata* (Korea) ♀ × *R. nigromaculata* (Japan) ♂ c. *R. brevipoda brevipoda* ♀ × *R. nigromaculata* (Japan) ♂ d. *R. brevipoda brevipoda* ♀ × *R. nigromaculata* (Korea) ♂ e. *R. nigromaculata* (Japan) ♀ × *R. plancyi fukienensis* ♂ f. *R. nigromaculata* (Japan) ♀ × *R. plancyi chosenica* ♂ Scale, 300 μm

differ from that of control males. Of two testes from each individual, one was used to prepare sperm suspension for the backcross experiment and another one was histologically examined. Relative abundance of different kinds of male germ cells is listed in Table 6.

The testes of NN, KK, BB and KN males were identical in histological structure

TABLE 6
Relative amount of male germ cells in mature testes*

Individual number**	First spermatocytes					Second spermatocytes	Spermatids	Spermatozoa
	Growing stages	Leptotene	Synaptene Pachytene	Diplotene Diakinesis	Metaphase Anaphase			
NN ♂ 1	+	+	−	−	−	−	+	++
♂ 2	+	+	−	−	−	−	+	++
BB ♂ 1	+	+	+	+	−	−	+	++
♂ 2	+	+	+	+	−	−	+	++
KK ♂ 1	+	+	+	+	−	−	++	++
KN ♂ 1	+	+	+	−	−	−	+	++
♂ 2	+	+	+	+	−	+	+	++
BN ♂ 1	+	+	−	−	−	− −	$+^{\alpha}$	$+^{\alpha}$
♂ 2	+	+	−	−	−	− −	$+^{\alpha}$	$+^{\alpha}$
♂ 3	++	+	+	−	−	− −	$+^{\alpha}$	$+^{\alpha}$
♂ 4	+	+	−	++	++	− −	$+^{\alpha}$	−
BK ♂ 1	+	+	++	−	−	− −	−	−
♂ 2	+	+	+	+	−	− −	−	−
♂ 3	+	+	−	+	++	− −	−	−
KB ♂ 1	+	+	++	−	−	− −	$+^{\alpha}$	$+^{\alpha}$
♂ 2	+	+	++	−	−	− −	$+^{\alpha}$	$+^{\alpha}$
♂ 3	++	+	−	+	++	− −	−	−
♂ 4	+	+	−	+	++	− −	−	− −
♂ 5	+	+	−	++	++	− −	−	− −
♂ 6	+	+	++	−	−	− −	−	−
♂ 7	−	+	++	−	−	− −	− −	− −
NF ♂ 1	+	+	++	+	+	− −	− −	− −
♂ 2	+	+	++	−	+	− −	−	−
♂ 3	−	+	++	+	+	− −	− −	− −
♂ 4	−	+	++	+	+	− −	−	−
NC ♂ 1	−	+	−	+	++	− −	− −	− −
♂ 2	+	+	+	+	+	− −	−	−
KC ♂ 1	−	+	−	+	+	− −	$+^{\alpha}$	$+^{\alpha}$
♂ 2	+	+	++	+	+	− −	$+^{\alpha}$	$+^{\alpha}$
♂ 3	+	+	+	−	+	− −	−	$+^{\alpha}$

* ++, abundant +, many −, few − −, very few α, abnormal
** Symbols are the same as in Table 1.

(Figs. 2a, 2b and 3a). Numerous spermatozoa were arranged in bundles along the inner surface of the seminiferous tubules. In the wall of the tubule there were a number of spermatogonia and first spermatocytes and a few second spermatocytes. KN testes did not show any abnormal features in the spermatogenetic process.

Relatively numerous spermatozoa were found in the testes of BN hybrids but they did not form compact sperm bundles (Fig. 2c). Almost all of the spermatozoa were abnormal in that the heads of spermatozoa were slightly longer and much thicker than those of the BB and NN males; mean head size was 12.8×1.75 μm in BN (N=80), while 10.3×1.36 μm in BB (N=40) and 10.4×1.01 μm in NN (N=40). In BK and KB hybrids the structure of testes was more abnormal than in BN hybrids (Fig. 2d). Spermatozoa were very few and scattered in the tubules without forming bundles. The mean size of sperm head was as large as

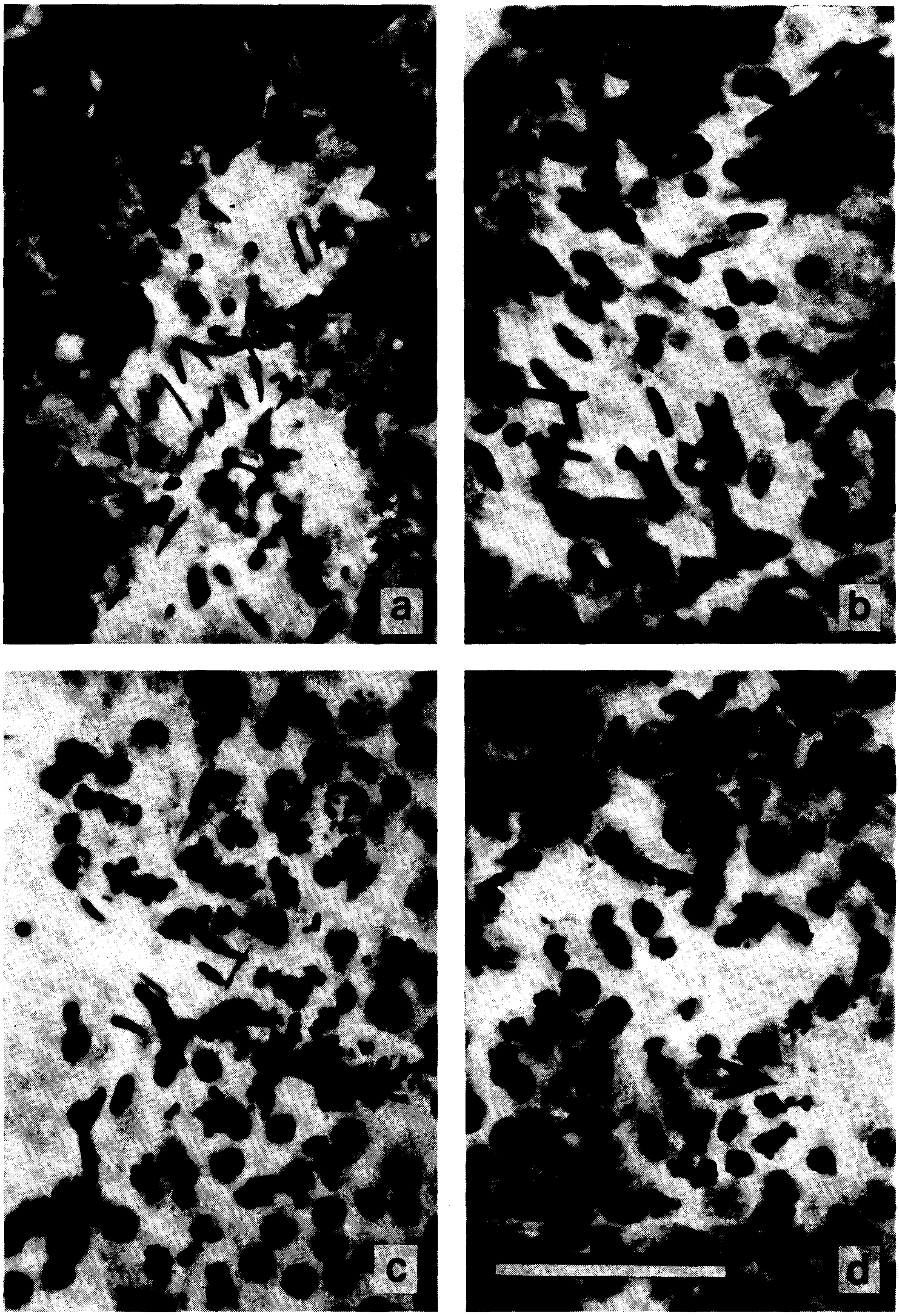

Fig. 3. Cross sections of the testes of mature frogs.

a. *R. nigromaculata* (Korea) ♀ × *R. nigromaculata* (Japan) ♂ b. *R. nigromaculata* (Korea) ♀ × *R. brevipoda brevipoda* ♂ c. *R. nigromaculata* (Japan) ♀ × *R. plancyi fukienensis* ♂ . d. *R. nigromaculata* (Japan) ♀ × *R. plancyi chosenica* ♂ Scale, 50μm in the BN hybrids (Fig. 3b). In these hybrids, including BN, first meiotic division seemd to be repressed at synaptene stage in some males and at first metaphase or anaphase in the other males as shown in the differences in relative abundance of germ cells (Table 6).

Spermatozoa were very few in NF males (Fig. 2e) and many sections of seminiferous tubules contained no spermatozoa. The shape and size of sperm head were abnormal (12.0×1.97 μm, N=80). Tubules were filled with numerous first spermatocytes, but the first meiotic division proceeded abnormally; chromosome bridges and irregularly shaped chromosome masses were frequently observed (Fig. 3c). Meiosis seemed to stop at first metaphase. No second spermatocytes were found.

In NC and KC hybrid males, spermatogenetic process showed abnormalities as in the other interspecific hybrids mentioned above. Spermatozoa were few in KC and very few in NC males (Fig. 2f), meiotic figures were irregular (Fig. 3d), and sperm heads were large (12.9×1.75 μm in NC, N=40, and 13.5×1.87 μm in KC, N=60). As in the other kinds of hybrid males, these abnormal spermatozoa may be formed without second meiotic division.

At diakinesis, 13 bivalent chromosomes were observed in the spermatocytes of NN, KK and BB males. Since the same number of chromosomes was observed in KN males, it is apparent that the homologous chromosomes of Japanese and Korean *R. nigromaculata* pair regularly. On the other hand, chromosome numbers were 17~20 in BN, BK and KB males, 14~21 in NF, 16~20 in NC, and 13~17 in KC. This observation showed the irregularities in chromosome pairing during hybrid spermatogenesis probably due to the partial lack of chromosomal homology.

DISCUSSION

Moriya (1960) studied the development and reproductive capacity of interspecific hybrids between Japanese *R. nigromaculata* and two subspecies of *R. brevipoda* and found that the hybrids were viable, females were fertile and males were almost sterile. The present results confirmed his conclusion. The poor reproductive capacity of the hybrid males results from the failure in first meiotic division probably due to differences in chromosome homology as in Moriya's results.

Hybridization experiments using male and female *R. plancyi chosenica* were reported by Kawamura and Nishioka (1975), while details of hybridization using males of *R. plancyi fukienensis* were reported here for the first time. So far as the present results are concerned, the fertilization rate was higher in BF than in NF or KF combinations, suggesting the presence of weak gametic isolation between *R. plancyi fukienensis* and *R. nigromaculata*. On the other hand, Ting, Tsai and Liu (1965) reported normal fertilization rate in the reciprocal hybridization between *R. plancyi fukienensis* and *R. nigromaculata*, both from Fukien Province, China. Whether this discrepancy is based on the local genetic differences of *R. nigromaculata* or of *R. plancyi fukienensis* remains to be clarified.

No hybrid inviability was observed in the hybrids between female *R. nigroma-*

culata and male *R. plancyi fukienensis* or *R. plancyi chosenica*, but the males of these hybrids were sterile. In contrast, the hybrids between female *R. brevipoda brevipoda* and male *R. plancyi fukienensis* or *R. plancyi chosenica* died at early tadpole stages. Since KAWAMURA and NISHIOKA (1975) observed relatively high viability in BC hybrids through metamorphosis, the hybrid inviability in this kind of hybrid is not complete. They reported also that CN, CK and CB hybrids were viable and all of these showed nearly complete male sterility and incomplete female sterility except BC and CB females which were nearly completely sterile.

The mechanism of male sterility in these hybrids is essentially identical with that of the hybrids between *R. nigromaculata* and *R. brevipoda brevipoda*. All Asiatic members of the pond frogs have 13 pairs of chromosomes (TING, 1939; SETO, 1965; NISHIOKA, 1972; LIN and HUANG, 1979; KURAMOTO, 1980). According to OKUMOTO (1980) who studied meiosis of NB and BN hybrids, various numbers of univalents were observed at diakinesis or first metaphase of spermatocytes. The numbers of chromosomes counted in the present study were also variable at diakinesis. Undoubtedly this means irregularities of pairing between different kinds of chromosome sets. From the range of chromosome counts in the hybrids, it seems reasonable to conclude that *R. nigromaculata* is more similar to two subspecies of *R. plancyi* than to *R. brevipoda brevipoda*.

These experimental results, together with our present knowledge on morphology, ecology and distribution, suggest a schema on the speciation process of the Asiatic pond frogs. A polytypic species should be older than monotypic species having similar ecological requirements and distribution range. Presently three subspecies are recognized in *R. plancyi*; *R. plancyi plancyi* in eastern parts of China, *R. plancyi chosenica* in southwestern Korea and *R. plancyi fukienensis* around Fukien Province of China and Taiwan. Based on the data of KAWAMURA and NISHIOKA (1977, 1979), *R. plancyi chosenica* and Taiwanese *R. plancyi fukienensis* differentiated to the extent that the two should be regarded as distinct species. On the other hand, there are few morphological differences in *R. nigromaculata* throughout its vast range in China, Korea and Japan. Although SCHMIDT (1927) and POPE (1931) distinguished three subspecies in Chinese *R. nigromaculata*, the differences are trivial and this subdivision has not been accepted (FANG and CHANG, 1931; BORING, 1938; POPE and BORING, 1940). These facts support the hypothesis that *R. plancyi* originated earlier than *R. nigromaculata* and, assuming the monophyletic origin of Asiatic pond frogs, that the latter should be derived from the former stock.

The distribution range of *R. brevipoda* is limited to central Japan. Based on the comparison of habitats and on the possible origin of the subspecies of *R. brevipoda*, MORIYA (1960) argued that *R. brevipoda* was derived from *R. plancyi* stock before *R. nigromaculata* invaded Japan. The present author agrees with his explanation. Since there are remarkable morphological differences and nearly complete reproductive isolation between *R. plancyi* and *R. brevipoda*, the divergence of *R. brevipoda* from *R. plancyi* stock should be the first event in the speciation process of Asiatic pond frogs. Probably the peripheral nature of the original Japanese population promoted the rapid differentiation of *R. brevipoda*.

In contrast to our results, TING (1948) reported the normal fertility in a hybrid male between Chinese *R. nigromaculata* and *R. plancyi plancyi*. This may suggest a close relationship of the two. Possibly, *R. nigromaculata* has its origin somewhere in China. After differentiated in an isolated population, it extended the range rapidly because of its superior adaptability to cold habitats as well as to warm ones. As proved in this study, the genomes of Korean and Japanese *R. nigromaculata* are compatible with each other and there are few indications of the genetic divergence between them. NISHIOKA, UEDA and SUMIDA (1981) found minor differentiation of Korean and Japanese *R. nigromaculata* in the allelic frequencies of several enzymes but it does not seem to exceed the range of variation between different populations of Japan.

R. plancyi chosenica is very similar to *R. plancyi plancyi* in external morphology and the differentiation seems to have taken place rather recently, probably by the advance of Yellow Sea which separated their distribution range. *R. plancyi fukienensis* has intermediate morphological characteristics of *R. plancyi plancyi* and *R. nigromaculata*. Although the evidence is not available as yet, it is probable that *R. plancyi fukienensis* has a hybrid origin as suggested by MORIYA (1954, 1960) to explain the origin of *R. brevipoda porosa*. Along with more extensive hybridization experiments, morphometric and acoustic comparisons, such as those made by MORIYA (1954) and KURAMOTO (1977) on Japanese pond frogs, will shed light on this problem.

SUMMARY

1. Artificial hybridization experiments were performed using males and females of *R. nigromaculata* and *R. brevipoda brevipoda*, and males of *R. plancyi fukienensis* and *R. plancyi chosenica*, from Japan, Korea and Taiwan.

2. Reciprocal hybrids between Japanese and Korean *R. nigromaculata* were quite normal in development and fertility except female preponderance in the hybrid between a Korean female and Japanese males.

3. Reciprocal hybrids between *R. nigromaculata* and *R. brevipoda brevipoda* developed normally. The females were fertile but the males were nearly completely sterile.

4. Incomplete fertilization block was observed in the cross *R. nigromaculata* ♀ × *R. plancyi fukienensis* or *R. plancyi chosenica* ♂, but not in *R. brevipoda brevipoda* ♀ × *R. plancyi fukienensis* or *R. plancyi chosenica* ♂. Male hybrids from these crosses were completely sterile.

5. Male sterility in the interspecific hybrids results from irregular chromosome pairing in the first meiotic division, apparently due to partial lack of chromosome homology. Spermatozoa were few or very few and abnormally large.

6. A possible speciation process of the Asiatic pond frogs is suggested based on the evidence available to date.

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LITERATURE

BERGER, L. 1970. Some characteristics of the crosses within *Rana esculenta* complex in postlarval development. Ann. Zool. **27**: 373–416.

——— 1973. Systematics and hybridization in European green frogs of *Rana esculenta* complex. J. Herpetol. **7**: 1–10.

BORING, A. M. 1938. Studies in variation among Chinese Amphibia. II. Variation in five wide-ranging common salientia. Peking Nat. Hist. Bull. **13**: 89–110.

FANG, P. W. and M. L. Y. CHANG 1931. Amphibia of Nanking. Contr. Biol. Lab. Sci. Soc. China **7**: 65–114.

GÜNTHER, R. 1973. Über die verwandtschaftlichen Beziehungen zwischen den europäischen Grünfröschen und dem Bastardcharakter von *Rana esculenta* L. (Anura). Zool. Anz. **190**: 250–285.

KAWAMURA, T. and M. NISHIOKA 1975. On the pond frogs in the Palearctic region, with special reference to the isolating mechanisms between different species. Proc. Jap. Soc. Syst. Zool. **11**: 61–78.

——— 1977. Aspects of the reproductive biology of Japanese anurans. The Reproductive Biology of Amphibians, edited by D. H. TAYLOR and S. I. GUTTMAN. Plenum Press (New York and London). pp. 103–139.

——— 1979. Isolating mechanisms among the water frog species distributed in the Palearctic region. Mitt. Zool. Mus. Berlin **55**: 171–185.

KAWAMURA, T., M. NISHIOKA and M. KURAMOTO 1972. Interspecific hybrids between Japanese and European pond frogs. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ. **1**: 277–301.

KURAMOTO, M. 1977. Mating call structures of the Japanese pond frogs, *Rana nigromaculata* and *Rana brevipoda* (Amphibia, Anura, Ranidae). J. Herpetol. **11**: 249–254.

——— 1980. Karyotypes of several frogs from Korea, Taiwan and the Philippines. Experientia **36**: 826–827.

LIN, F. Y. and C. C. HUANG 1979. Karyological studies of five *Rana* species in Taiwan (Amphibia: Salientia). Proc. Natl. Sci. Council, Taiwan **3**: 429–436.

MANDEVILLE, L. C. and H. SPURWAY 1949. The development of hybrids between *Rana esculenta* LINN. and *Rana ridibunda* PALLAS. Brit. J. Herpetol. **1**: 39–50.

MORIYA, K. 1951. On isolating mechanisms between the two subspecies of the pond frog, *Rana nigromaculata*. J. Sci. Hiroshima Univ., Ser. B, Div. 1, **12**: 47–56.

——— 1954. Studies on the five races of the Japanese pond frog, *Rana nigromaculata* HALLOWELL. I. Differences in the morphological characters. J. Sci. Hiroshima Univ., Ser. B, Div. 1, **15**: 1–21.

——— 1960. Studies on the five races of the Japanese pond frog, *Rana nigromaculata* HALLOWELL. III. Sterility in interracial hybrids. J. Sci. Hiroshima Univ., Ser. B. Div. 1, **18**: 125–156.

NISHIOKA, M. 1972. The karyotypes of the two sibling species of Japanese pond frogs, with special reference to those of the diploid and triploid hybrids. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ. **1**: 319–337.

NISHIOKA, M., H. UEDA and M. SUMIDA 1981. Genetic variation of five enzymes in Japanese pond frogs. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ. **5**: 107–153.

OKUMOTO, H. 1980. Studies on meiosis in male hybrids and triploids in the *Rana nigromaculata* group. I. Interspecific hybrids between *Rana nigromaculata* and *Rana brevipoda*. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ. **4**: 201–216.

POPE, C. H. 1931. Notes on amphibians from Fukien, Hainan, and other parts of China. Bull. Am. Mus. Nat. Hist. **61**: 397–611.

POPE, C. H. and A. M. BORING 1940. A survey of Chinese Amphibia. Peking Nat. Hist. Bull. **15**: 13–86.

RUGH, R. 1948. Experimental Embryology. Burgess Publ. Co., Minneapolis.

SCHMIDT, K. P. 1927. Notes on Chinese amphibians. Bull. Am. Mus. Nat. Hist. **54**: 553–575.

SETO, T. 1965. Cytogenetic studies in lower vertebrates. II. Karyological studies of several species of frogs (Ranidae). Cytologia **30**: 437–446.

TING, H. P. 1939. A study of the reciprocal hybrids of two species of frogs, *Rana nigromaculata* and *Rana plancyi*. Peking Nat. Hist. Bull. **13**: 181–200.

——— 1948. A study of the two-year-old hybrids of *Rana nigromaculata* and *Rana plancyi* with notes on a backcross experiment. Lingnan Sci. J. **22**: 115–119.

TING, H. P., M. C. TSAI and C. J. LIU 1965. Hybridization experiments of 16 species of anurans from Fukien Province. J. Fujian Normal Univ. **1**: 65–114.