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Interspecific Hybrids between Japanese and European Pond Frogs

By

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(With 2 Text-figures and 2 Plates)

INTRODUCTION

In the palearctic region there seem to be five species of the *Rana esculenta* group. While two of them, *R. esculenta* and *R. ridibunda* are mainly distributed in Europe, the other three, *R. brevipoda, R. nigromaculata* and *R. plancyi*, are principally in Far East. MANDEVILLE and SPURWAY (1949) produced reciprocal interspecific hybrids between *R. esculenta* and *R. ridibunda*. Concerning the Chinese species, TING (1939) obtained healthy hybrids from reciprocal crosses of *R. nigromaculata* and *R. plancyi*. Later, he (1948) described about those which could live for two years. The hybridization experiments between the two Japanese sibling species, *R. brevipoda* and *R. nigromaculata* were made by MORIYA (1951, 1960). In those days *Rana brevipoda* had been situated at the position of a subspecies of *R. nigromaculata* (ITO, 1941), but later given the rank of a species by KAWAMURA (1962). The results of hybridization experiments performed by the above authors showed that there were no or feeble gametic isolation and hybrid inviability among the members of the *esculenta* group.

In the present research reciprocal crosses were made between the two species of Japanese pond frogs and the European *Rana esculenta* to clarify their biological differences. Hybridization experiments were repeated during the years from 1959 to 1965. A preliminary report was made by KAWAMURA and KURAMOTO in 1960.

MATERIAL AND METHODS

The two species of Japanese pond frogs, *Rana nigromaculata* HALLOWELL and *R. brevipoda* ITO, were collected in the suburbs of Hiroshima and Okayama, respectively. The European pond frogs, *R. esculenta* L. which were used for the experiments in 1959 and 1960 were sent to our laboratory by Dr. Friedrich PÖLZ, Harburg, West Germany by his courtesy. The present authors were informed by him that the frogs had been collected from the vicinity of Dresden, Saxony, East Germany. As the material in the two years, a female and 2 males of *R. esculenta,* 8 females and 5 males of *R. nigromaculata* and 11 females and

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5 males of *R. brevipoda* were utilized. For the crosses performed in the years 1961～1965, 5 females and 4 males of *Rana esculenta*, 14 females and 5 males of *Rana brevipoda* and 7 females and 3 males of *Rana nigromaculata* were used as the material. The European pond frogs were collected from the suburbs of Luxemburg by Mr. Robert THORN, who sent them to our laboratory by his courtesy.

The crosses were performed by the method of artificial fertilization. Eggs were obtained from females whose ovulation had been accelerated by pituitary transplantation. Fertilized eggs were reared in the laboratory under room temperatures. After hatching, tadpoles were kept in outdoor aquaria; they were fed on boiled spinach. Metamorphosed frogs were fed on mosquitos at their youngest stage and afterwards on flies until their sexual maturity.

The gonads were fixed in NAVASHIN's fluid. They were sectioned at 12μ and stained with HEIDENHAIN's iron hematoxylin.

RESULTS

I. *Production of reciprocal hybrids*

1. Experiments in 1959

a. Crosses of female *nigromaculata* and male *esculenta*

The crossing experiments were made on June 10 and 25, 1959. On June 10, eggs of three female *nigromaculata* Nos. I～III were inseminated with sperm of a male *esculenta* No. I and two male *nigromaculata* Nos. I and II. On June

TABLE 1

Results of crosses between female *Rana nigromaculata* and male *Rana esculenta* (in 1959)

Parents: Female	Parents: Male	No. of eggs	No. of cleaved eggs	No. of dead embryos: Cleaving eggs & Blastulae	No. of dead embryos: Gastrulae	No. of dead embryos: Neurulae & Tail-bud embryos	No. of normally hatched tadpoles
R. nigr.,	*R. nigr.*, Nos. I, II	209	201(96.2%)	0	0	0	201(96.2%)
No. I	*R. escul.*, No. I	169	98(58.0%)	0	6(3.6%)	92(54.4%)	0
R. nigr.,	*R. nigr.*, Nos. I, II	237	229(96.6%)	7(3.0%)	0	2(0.8%)	220(92.8%)
No. II	*R. escul.*, No. I	245	144(58.8%)	6(2.4%)	2(0.8%)	100(40.8%)	0
R. nigr.,	*R. nigr.*, Nos. I, II	150	150(100 %)	5(3.3%)	3(2.0%)	16(10.7%)	63(42.0%)
No. III	*R. escul.*, No. I	207	178(86.0%)	29(14.0%)	19(9.2%)	56(27.1%)	2(1.0%)
R. nigr.,	*R. nigr.*, Nos. III, IV	189	188(99.5%)	3(1.6%)	1(0.5%)	0	181(95.8%)
No. IV	*R. escul.*, No. II	181	90(49.7%)	6(3.3%)	0	84(46.4%)	0
R. nigr.,	*R. nigr.*, Nos. III, IV	235	231(98.3%)	0	0	0	229(97.4%)
No. V	*R. escul.*, No. II	232	190(81.9%)	6(2.6%)	0	184(79.3%)	0
R. nigr.,	*R. nigr.*, Nos. III, IV	119	100(84.0%)	6(5.0%)	0	12(10.1%)	71(59.7%)
No. VI	*R. escul.*, No. II	72	6(8.3%)	3(4.2%)	1(1.4%)	2(2.8%)	0
Total							
R. nigr.	*R. nigr.* (4)	1139	1099(96.5%)	21(1.8%)	4(0.4%)	30(2.6%)	965(84.7%)
(6)	*R. escul.* (2)	1106	706(63.8%)	50(4.5%)	28(2.5%)	518(46.8%)	2(0.2%)

25, eggs of three other female *nigromaculata* Nos. IV～VI were inseminated with sperm of another male *esculenta* No. II and two other male *nigromaculata* Nos. III and IV. The main results are presented in Table 1.

In the experimental series, 706(63.8%) of 1106 eggs of all the six female *nigromaculata* cleaved normally, while 1099(96.5%) of 1139 eggs of the same females did in the control series. In the latter, 55(5.0%) of the cleaved eggs died by the tail-bud stage and 79(7.2%) were abnormal, although attained the hatching stage. The remaining 965(87.8%) embryos hatched normally and became normal swimming tadpoles. In contrast with this, a majority of the cleaved eggs in the experimental series became abnormal and died by the tail-bud stage; that is, 50(7.1%), 28(4.0%) and 518(73.4%) of the 706 eggs died at the cleavage or blastula, the gastrula and the neurula or tail-bud stages, respectively. Of the remaining 110 embryos, only two (0.3%) hatched normally, while 108(15.3%) became edematous or showed an abnormality such as curvature of the body at the hatching stage. One of the hatched hybrids completed its metamorphosis at the age of 45 days, while the other died at the tadpole stage.

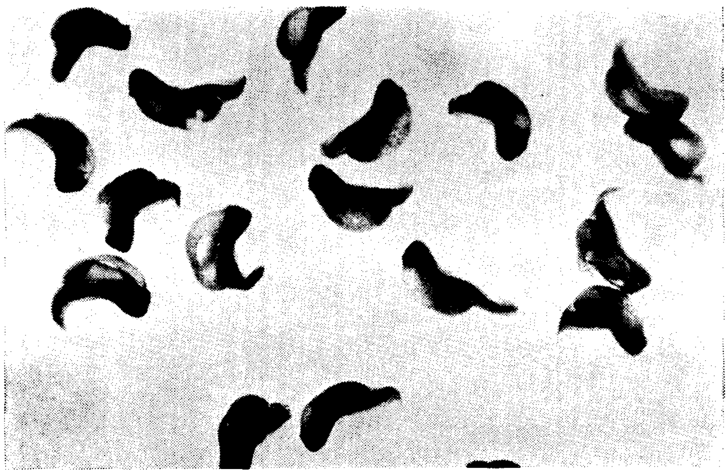

Fig. 1. Inviable hybrids between female *Rana nigromaculata* and male *Rana esculenta*, (N)NE, at the embryonal stage.

b. Crosses of female *brevipoda* and male *esculenta*

On June 10, 1959, eggs of five female *brevipoda* were inseminated with sperm of a male *esculenta* No. I and two male *brevipoda* Nos. I and II. On the 25th of the same month, eggs of three female were inseminated with sperm of a male *esculenta* No. II and two male *brevipoda* Nos. III and IV. The two male *esculenta* utilized in these crosses were the same as those in the crosses of female *nigromaculata* and male *esculenta* (Table 2).

In the experimental series 977(63.3%) of 1543 eggs in total cleaved normally, while in the control series 1423(80.9%) of 1759 eggs did. The cleaved eggs in the control series mostly developed normally and hatched; 110(7.7%), 19(1.3%) and 55(3.9%) of them died at the cleavage or blastula, the gastrula, and the neurula or tail-bud stages, respectively, and the remaining 1239(87.1%) embryos

TABLE 2

Results of crosses between female *Rana brevipoda* and male *Rana esculenta* (in 1959)

Parents		No. of eggs	No. of cleaved eggs	No. of dead embryos			No. of normally hatched tadpoles
Female	Male			Cleaving eggs & Blastulae	Gastrulae	Neurulae & Tailbud embryos	
R. brev.,	*R. brev.,* Nos. I, II	82	58(70.7%)	1(1.2%)	1(1.2%)	0	55(67.1%)
No. I	*R. escul.,* No. I	96	72(75.0%)	13(13.5%)	12(12.5%)	14(14.6%)	29(30.2%)
R. brev.,	*R. brev.,* Nos. I, II	327	280(85.6%)	12(3.7%)	13(4.0%)	3(0.9%)	221(67.6%)
No. II	*R. escul.,* No. I	345	181(52.5%)	34(9.9%)	55(15.9%)	13(3.8%)	69(20.0%)
R. brev.,	*R. brev.,* Nos. I, II	242	195(80.6%)	19(7.9%)	4(1.7%)	5(2.1%)	162(66.9%)
No. III	*R. escul.,* No. I	237	154(65.0%)	11(4.6%)	26(11.0%)	24(10.1%)	88(37.1%)
R. brev.,	*R. brev.,* Nos. I, II	340	303(89.1%)	19(5.6%)	0	10(2.9%)	265(77.9%)
No. IV	*R. escul.,* No. I	199	102(51.3%)	9(4.5%)	9(4.5%)	6(3.0%)	44(22.1%)
R. brev.,	*R. brev.,* Nos. I, II	186	121(65.1%)	11(5.9%)	0	33(17.7%)	73(39.2%)
No. V	*R. escul.,* No. I	91	72(79.1%)	5(5.5%)	17(18.7%)	18(19.8%)	17(18.7%)
R. brev., No. VI	*R. brev.,* Nos. III, IV	180	142(78.9%)	35(19.4%)	0	2(1.1%)	100(55.6%)
	R. escul., No. II	200	113(56.5%)	15(7.5%)	0	4(2.0%)	75(37.5%)
R. brev., No. VII	*R. brev.,* Nos. III, IV	162	110(67.9%)	9(5.6%)	0	1(0.6%)	96(59.3%)
	R. escul., No. II	205	160(78.0%)	8(3.9%)	17(8.3%)	10(4,9%)	98(47.8%)
R. brev., No. VIII	*R. brev.,* Nos. III, IV	240	214(89.2%)	4(1.7%)	1(0.4%)	1(0.4%)	205(85.4%)
	R. escul., No. II	170	123(72.4%)	2(1.2%)	7(4.1%)	7(4.1%)	99(58.2%)
R. brev. (8)	*R. brev.*(4)	1759	1423(80.9%)	110(6.3%)	19(1.1%)	55(3.1%)	1177(66.9%)
	R. escul.(2)	1543	977(63.3%)	97(6.3%)	143(9.3%)	96(6.2%)	519(33.6%)

attained their hatching. However, 1177(82.7%) embryos hatched normally and became normal swimming tadpoles. The other 62(4.4%) were abnormal at this stage. In the experimental series, 97(9.9%), 143(14.6%) and 96(9.8%) of the cleaved eggs died at the cleavage or blastula, the gastrula, and the neurula or tail-bud stages, respectively. Although the remaining 641(65.6%) embryos attained their hatching, 122(12.5%) were edematous or showed curvature of the body, and 519(53.1%) embryos became normal swimming tadpoles.

2. Experiments in 1960

Crosses of female *esculenta* and male *nigromaculata* or *brevipoda* were performed on June 9, 1960 by the use of one female *esculenta*. Eggs of this female were inseminated with sperm of a male *nigromaculata* and a male *brevipoda*. As the control series eggs of two female *nigromaculata* and three *brevipoda* were inseminated with sperm of the same *nigromaculata* and *brevipoda* as used in the experimental series. Regret to say, there was no male *esculenta* available.

In the experimental series, 153(72.9%) of 210 eggs inseminated with sperm of the *nigromaculata* and 152(75.2%) of 202 eggs inseminated with sperm of the *brevipoda* cleaved normally. Since more than 82% of eggs cleaved normally in the control series, it was sure that the male *nigromaculata* and *brevipoda* were nearly normal in fertilizing capacity. In the combination of *esculenta* and *brevipoda*, 123

TABLE 3

Results of crosses between a female *R. esculenta* and male *R. nigromaculata* or *R. brevipoda* (in 1960)

Parents		No. of eggs	No. of cleaved eggs	No. of dead embryos			No. of normally hatched tadpoles
Female	Male			Cleaving eggs & Blastulae	Gastrulae	Neurulae & Tail-bud embryos	
R. escul., No. I	*R. nigr.*, No. V	210	153(72.9%)	20(9.5%)	104(49.5%)	26 (12.4%)	0
	R. brev., No. V	202	152(75.2%)	25(12.4%)	88(43.6%)	10 (5.0%)	20(9.9%)
R. nigr., Nos. VII, VIII	*R. nigr.*, No. V	431	355(82.4%)	50(11.6%)	0	62 (14.4%)[1]	99
R. brev., Nos. IX~XI	*R. brev.*, No. V	978	843(86.2%)	21(2.1%)	0	141 (14.4%)[2]	165

1) 117 out of 243 embryos were abandoned.
2) 515 out of 681 embryos were abandoned.

(80.9%) of the cleaved eggs died of various abnormalities by the tail-bud stage. Although 29 embryos reached the hatching stage, nine of them became abnormal at this stage and 20(13.2%) hatched normally. In contrast with this, the cleaved eggs produced from the cross of *esculenta* and *nigromaculata* all died of various abnormalities by the hatching stage; although only three reached the hatching stage, these were abnormal in appearance and died soon afterwards. As compared with the cleaved eggs of the control series, it was characteristic of those of the experimental series that a majority of them died at the gastrula stage. As a matter of fact, 68.0% cleaved eggs in the cross of *esculenta* and *nigromaculata* and 57.9% in the cross of *esculenta* and *brevipoda* died, while in the control series there were no eggs which died at this stage (Table 3).

Of the 20 tadpoles produced from the cross of *esculenta* and *brevipoda*, 11 grew normally and completed their metamorphosis, while the others became abnormal and died in the tadpole stage.

3. Experiments in 1961 and 1962

In the seasons of the years 1961 and 1962 some reciprocal crosses between *Rana esculenta* and *R. nigromaculata* or *brevipoda* were made. These crossing experiments were those utilized as the control at the production of nucleo-cytoplasmic hybrids between these species in the same years.

a. Reciprocal crosses between *brevipoda* and *esculenta* in 1961

As material, two male and two female *brevipoda*, and a male and two female *esculenta* were used. The results of crosses are presented in Table 4. In the control series, 160(91.4%) of 175 normally cleaved *brevipoda* eggs and 185(87.3%) of 212 normally cleaved *esculenta* ones developed into normal swimming tadpoles. Of these tadpoles 158 *brevipoda* and 153 *esculenta* completed their metamorphosis. In the experimental series, a large number of the cleaved eggs died at the embryonal stages, although a fairly good percentage of eggs cleaved normally. Ultimately, 86(52.4%) of 164 *brevipoda* ♀ × *esculenta* ♂ eggs and 25(20.7%) of 121 *esculenta*

TABLE 4

Results of crosses between *Rana esculenta*

Parents		No. of eggs	No. of cleaved eggs
Female	Male		
R. brev., Nos. I, II	*R. brev.*, Nos. I, II	224	175(78.1%)
	R. escul., No. I	250	164(65.6%)
R. escul., Nos. I, II	*R. brev.*, Nos. I, II	167	121(72.5%)
	R. escul., No. I	213	212(99.5%)
R. brev., Nos. III, IV	*R. brev.*, No. III	213	176(82.6%)
	R. escul., No. II	205	143(69.8%)
R. escul., No. III	*R. brev.*, No. III	202	165(81.7%)
	R. escul., No. II	224	196(87.5%)
	R. nigr., No. I	65	44(67.7%)
R. nigr., No. I	*R. escul.*, No. II	121	86(71.1%)
	R. nigr., No. I	103	94(91.3%)

TABLE 5

Results of crosses between *Rana esculenta*

Parents		No. of eggs	No. of cleaved eggs
Female	Male		
R. escul., Nos. IV, V	*R. escul.*, No. III	80	72(90.0%)
	R. brev., No. IV	252	194(77.0%)
	R. nigr., No. II	75	71(94.7%)
R. brev., Nos. V~VIII	*R. brev.*, No. IV	213	166(77.9%)
	R. escul., No. III	163	109(66.9%)
R. nigr., Nos. II, III	*R. nigr.*, No. II	170	165(97.1%)
	R. escul., No. III	242	183(75.6%)

TABLE 6

Results of crosses between female *Rana brevipoda*

Parents		No. of eggs	No. of cleaved eggs
Female	Male		
R. brev., Nos. IX~XIV	*R. brev.*, No. V	658	630(95.7%)
	R. escul., No. IV	737	689(93.5%)
R. nigr., Nos. IV~VII	*R. nigr.*, No. III	348	331(95.1%)
	R. escul., No. IV	294	174(59.2%)

♀ × *brevipoda* ♂ ones became normal swimming tadpoles. Most of these tadpoles died afterwards and only 17 and 9 completed their metamorphosis, respectively.

b. Reciprocal crosses among *brevipoda, esculenta* and *nigromaculata* in 1962

A male and two female *brevipoda*, a male and a female *esculenta* and a male and a female *nigromaculata* were used as material for hybridization experiments. While in the control series, 82.6% of *brevipoda* eggs, 87.5% of *esculenta* and 91.3% of *nigromaculata* cleaved normally, 69.8% of *brevipoda* eggs with *esculenta* sperm, 81.7% of *esculenta* eggs with *brevipoda* sperm, 67.7% of *esculenta* eggs with *nigromaculata* sperm, and 71.1% of *nigromaculata* eggs with *esculenta* sperm did normally. These figures show that the hybridized eggs were inferior to the control ones in the rate of normal fertilization.

and *Rana brevipoda* or *Rana nigromaculata* (in 1961, 1962)

No. of dead embryos			No. of normally hatched tadpoles	No. of metamorphosed frogs
Blastulae & Gastrulae	Neurulae	Tail-bud embryos		
2(0.9%)	6(2.7%)	2(0.9%)	160(71.4%)	158(70.5%)
3(1.2%)	9(3.6%)	39(15.6%)	86(34.4%)	17(6.8%)
21(12.6%)	46(27.5%)	22(13.2%)	25(15.0%)	9(5.4%)
5(2.3%)	5(2.3%)	9(4.2%)	185(86.9%)	153(71.8%)
12(5.6%)	4(1.9%)	0	152(71.4%)	137(64.3%)
14(6.8%)	22(10.7%)	4(2.0%)	58(28.3%)	19(9.3%)
39(19.3%)	30(14.9%)	43(21.3%)	17(8.4%)	4(2.0%)
4(1.8%)	5(2.2%)	0	181(80.8%)	177(79.0%)
9(13.8%)	19(29.2%)	16(24.6%)	0	0
22(18.2%)	43(35.5%)	16(13.2%)	5(4.1%)	0
2(1.9%)	0	1(1.0%)	90(87.4%)	87(84.5%)

and *Rana brevipoda* or *Rana nigromaculata* (in 1963)

No. of dead embryos			No. of normally hatched tadpoles	No. of metamorphosed frogs
Blastulae & Gastrulae	Neurulae	Tail-bud embryos		
0	0	0	72(90.0%)	72(90.0%)
91(36.1%)	29(11.5%)	22(8.7%)	35(13.9%)	24(9.5%)
22(29.3%)	32(42.7%)	17(22.7%)	0	0
35(16.4%)	1(0.5%)	3(1.4%)	125(58.7%)	77(36.2%)
19(11.7%)	6(3.7%)	11(6.7%)	43(26.4%)	31(19.0%)
3(1.8%)	0	0	161(94.7%)	160(94.1%)
47(19.4%)	21(8.7%)	112(46.3%)	0	0

or *Rana nigromaculata* and a male *Rana esculenta* (in 1965)

No. of dead embryos			No. of normally hatched tadpoles	No. of metamorphosed frogs
Blastulae & Gastrulae	Neurulae	Tail-bud embryos		
0	5(0.8%)	4(0.6%)	610(92.7%)	564(85.7%)
113(15.3%)	226(30.7%)	81(11.0%)	129(17.5%)	30(4.1%)
0	0	0	330(94.8%)	327(94.0%)
10(3.4%)	0	164(55.8%)	0	0

In the control series, 16 *brevipoda*, nine *esculenta* and three *nigromaculata* died by the hatching stage, and 152 *brevipoda*, 181 *esculenta* and 90 *nigromaculata* became normal swimming tadpoles. A great majority of these tadpoles completed their metamorphosis, that is, 137 *brevipoda*, 177 *esculenta* and 87 *nigromaculata* became young frogs. In the experimental series, all or most embryos became abnormal and died by the hatching stage. Reciprocal hybrids between *nigromaculata* and *esculenta* were remarkably inferior in viability to those between *brevipoda* and *esculenta*. Out of 143 normally cleaved eggs of *brevipoda*♀ × *esculenta* ♂ 58 and 19 became normal tadpoles and frogs, respectively, and out of 165 normally cleaved eggs of the reciprocal cross only 17 and four became normal tadpoles and frogs, respectively. On the other hand, nearly all the 44 and 86 normally cleaved eggs obtained from reciprocal crosses between *esculenta* and *nigromaculata* became abnormal and died by the hatching stage and no feeding tadpoles were obtained.

4. Experiments in 1963

Reciprocal crosses between *esculenta* and *brevipoda* or *nigromaculata* were carried out by using a male and two female *esculenta*, a male and four female *brevipoda* and a male and two female *nigromaculata*. The results were nearly the same as those in the previous year, as presented in Table 5.

While more than 77.9 percent of *esculenta, brevipoda* and *nigromaculata* eggs in the control series cleaved normally, more than 66.9% of eggs of these species did also normally after being inseminated with foreign sperm. However, most embryos produced from reciprocal crosses between *esculenta* and *brevipoda* or *nigromaculata* became abnormal and died, in contrast to the controls. In the control series, all the normally cleaved *esculenta* eggs grew into normal tadpoles and completed their metamorphosis. One hundred and twenty-five and 77 of 166 normally cleaved *brevipoda* eggs as well as 161 and 160 of 165 normally cleaved *nigromaculata* grew into normal tadpoles and metamorphosed frogs, respectively. In the hybrids *esculenta* ♀ × *brevipoda* ♂, 35 and 24 of 194 normally cleaved eggs became normal tadpoles and frogs, respectively. Forty-three and 31 out of 109 normally cleaved eggs of the reciprocal cross became also normal tadpoles and frogs, respectively. Differing from the hybrids between *esculenta* and *brevipoda,* nearly all the reciprocal hybrids between *esculenta* and *nigromaculata* became abnormal and died in their embryonal stage. No normal tadpoles were produced from them.

5. Experiments in 1965

Hybridization experiments were performed between female *brevipoda* or *nigromaculata* and a male *esculenta*. As material, a male and six female *brevipoda* and a male and four female *nigromaculata* were used, besides a male *esculenta*. The results of experiments are presented in Table 6.

In the control series, more than 95 percent of eggs cleaved normally. In the experimental series, 174(59.2%) of 294 *nigromaculata* eggs cleaved normally with *esculenta* sperm, while 689(93.5%) of 737 *brevipoda* eggs did normally with the same sperm. In the control series, 610 of 630 normally cleaved *brevipoda* eggs hatched normally and afterwards 564 completed their metamorphosis, while 330 out of 331 cleaved *nigromaculata* eggs became normal tadpoles and 327 were metamorphosed. In the experimental series, 129 out of 689 normally cleaved eggs of *brevipoda* ♀ × *esculenta* ♂ hatched normally and became swimming tadpoles. Thirty of the latter completed their metamorphosis afterwards. Differing from this kind of hybrids, all the 174 normally cleaved eggs of *nigromaculata* ♀ × *esculenta* ♂ became abnormal and died by the hatching stage.

TABLE 7

Developmental stages of various kinds of embryos at their definite ages in hour (in 1962, ca. 25°C)

Kind \ Age \ Date	July 3				July 4		July 5	July 7	July 8
	15h.	20h.	25h.	30h.	40h.	45h.	66h.	112h.	136h.
BB	11M	12M	12L	14M	16L	18	19	20	21
EE	11M	12M	12L	14M	16L	18	19	20	21
NN	11E	12M	12L	14E	16L	18	19	20	21
BE	11E	12E	12M	12L	16M	17	18	19	20
EB	10L	12E	12M	12L	16M	17	18	19	20
EN	10L	12E	12M	12L	14L	15M	17	—	—
NE	10L	12E	12M	12L	13L	14L	17	—	—

E, Early M, Middle L, Late

BB — *Rana brevipoda* ♀ × *Rana brevipoda* ♂
EE — *Rana esculenta* ♀ × *Rana esculenta* ♂
NN — *Rana nigromaculata* ♀ × *Rana nigromaculata* ♂
BE — *Rana brevipoda* ♀ × *Rana esculenta* ♂
EB — *Rana esculenta* ♀ × *Rana brevipoda* ♂
EN — *Rana esculenta* ♀ × *Rana nigromaculata* ♂
NE — *Rana nigromaculata* ♀ × *Rana esculenta* ♂

TABLE 8

Developmental stages of various kinds of embryos at their definite ages in hour (in 1963, 21~25°C)

Kind and series \ Age \ Date		June 11		June 12		June 13	June 15	June 16
		20h.	28h.	42h.	50h.	72h.	108h.	153h.
BB	I	12E	12L	16M	17	19	20	21
	II	12E	12L	16M	17	19	20	21
	III	12E	12L	16M	17	19	20	21
EE	I	12E	12L	16M	17	19	20	21
	II	12E	12L	16M	17	19	20	21
NN	I	11L	12M	15L	17	19	20	21
	II	11L	12M	15L	17	19	20	21
BE	I	11M	12M	14M	16	18	19	20
	II	11M	12M	14M	16	18	19	20
	III	11M	12M	14M	16	18	19	20
	IV	11M	12M	14M	16	18	19	20
EB	I	11E	12M	13L	16	17	19	20
	II	11E	12M	13L	16	17	19	20
EN	I	11E	12M	13M	14	15	17	—
	II	11E	12M	13M	14	15	17	—
NE	I	11M	12E	13E	14	15	17	—
	II	11M	12E	13E	14	15	17	—

E, Early M, Middle L, Late

BB — *Rana brevipoda* ♀ × *Rana brevipoda* ♂
EE — *Rana esculenta* ♀ × *Rana esculenta* ♂
NN — *Rana nigromaculata* ♀ × *Rana nigromaculata* ♂
BE — *Rana brevipoda* ♀ × *Rana esculenta* ♂
EB — *Rana esculenta* ♀ × *Rana brevipoda* ♂
EN — *Rana esculenta* ♀ × *Rana nigromaculata* ♂
NE — *Rana nigromaculata* ♀ × *Rana esculenta* ♂

II. Development and characters of hybrids

1. Embryos

The reciprocal hybrids between *esculenta* and *brevipoda* or *nigromaculata* were compared with the control *brevipoda* and *nigromaculata* in terms of developmental velocity during the embryonal stage. Such comparison was made several times in the breeding seasons of the years 1959~1965. The results of observations performed in 1962 and 1963 are presented in Tables 7 and 8. The stage numbers in the tables correspond to those of SHUMWAY's table. The letters, E, M and L represent early, middle and late, respectively.

In the year 1962, seven kinds of fertilized eggs produced from *brevipoda* ♀ × *brevipoda* ♂, *esculenta* ♀ × *esculenta* ♂, *nigromaculata* ♀ × *nigromaculata* ♂, *brevipoda* ♀ × *esculenta* ♂, *esculenta* ♀ × *brevipoda* ♂, *esculenta* ♀ × *nigromaculata* ♂ and *nigromaculata* ♀ × *esculenta* ♂, were synchronously developed at the room temperature which was about 25°C, and their developmental velocities were observed for about six days. During the period of these six days the control *brevipoda* and *esculenta* were quite the same in the rate of development, while the control *nigromaculata* was slightly retarded until the stage 14 or 15. After the stage 16, however, the three kinds of controls were in the same stage with one another. In the experimental series, all the four kinds of hybrids were a little delayed in development, as compared with the controls. The *esculenta* ♀ × *brevipoda* ♂ hybrids were slightly delayed at the mid-gastrula as compared with the reciprocal ones, but soon afterwards both kinds of hybrids became the same in developmental velocity. The reciprocal hybrids between *esculenta* and *nigromaculata* were quite the same as those between *esculenta* and *brevipoda* until the stage 12. However, they began to retard after the stage 13 or 14, and moreover, their gastrulation was incomplete and the yolk plug remained long. Owing to such an abnormality, all the reciprocal hybrids between *esculenta* and *nigromaculata* died sooner or later, and could not advance further than the stage 17.

In the breeding season of the year 1963, the seven kinds of fertilized eggs were compared with one another in terms of developmental velocity. In this year eggs of two to four females of each species were fertilized with sperm of a male of each species. Each kind of fertilized eggs was simultaneously developed with the others at the room temperature which was 21~25°C.

The results of observations are presented in Table 8. They were nearly the same with those obtained in the year 1962. In the control series, the *nigromaculata* embryos were slightly delayed during the period until the stage 16, as compared with the others. On the other hand, the four kinds of hybrids were delayed in development since the stage of gastrulation, as compared with the controls. The reciprocal hybrids between *esculenta* and *nigromaculata* became more distinct in retardation at the stage of neural plate formation than those between *esculenta* and *brevipoda*. Moreover, the former became more and more abnormal in shape after the stage of neural folds. All of them became abnormal at the stage 17 and could not proceed further than this stage. Differing from them the reciprocal hybrids between *esculenta* and *brevipoda* attained the hatching stage, although they were somewhat delayed in development.

2. Metamorphosis

Although the *brevipoda* ♀ × *esculenta* ♂ hybrids were fairly retarded in the development during the embryonal stage, as compared with the control *brevipoda*, they generally completed their metamorphosis earlier than the latter, as shown in Fig. 2. In the experiments performed by the use of the female *brevipoda* Nos. I～VIII in 1959, there was a distinct peak of metamorphosis, except a few cases.

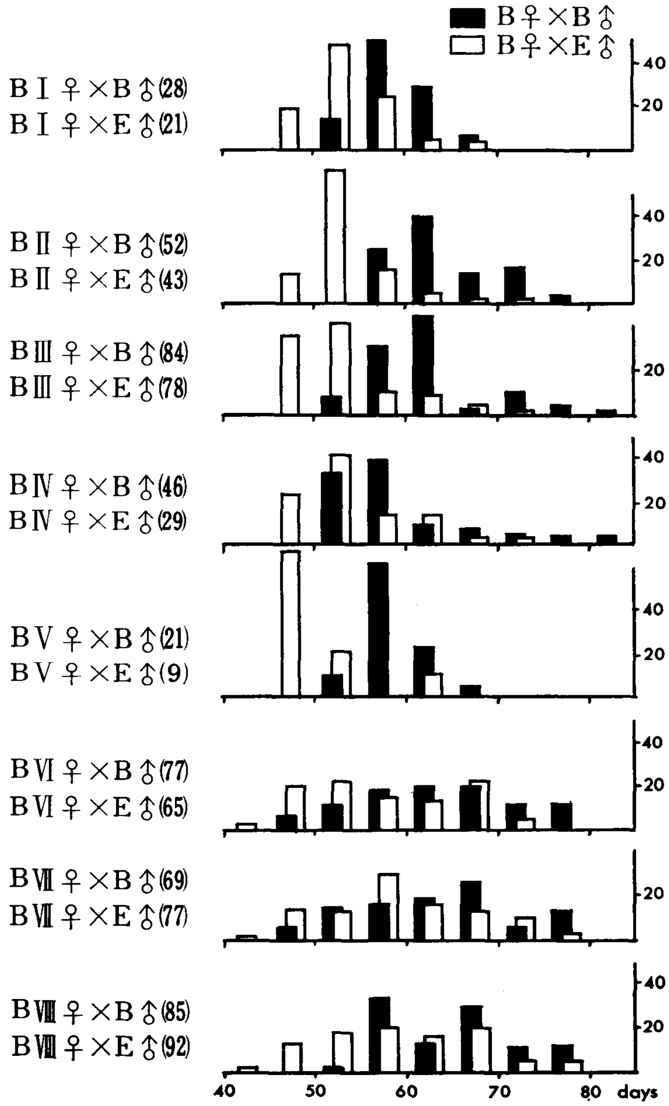

Fig. 2. Ages in day of hybrids between female *Rana brevipoda* and male *Rana esculenta* and the controls at the time of completion of metamorphosis.
B, *Rana brevipoda* E, *Rana esculenta* I～VIII, Individual number of females. Parentheses, no. of frogs.

Such a peak of metamorphosis in the hybrids was always situated at an earlier age than that of the control *brevipoda*. In this year, it was impossible to compare the age of metamorphosis of the hybrids with that of *esculenta*, because there were no control *esculenta*.

In the experiments performed in 1962 and 1963, reciprocal hybrids between *brevipoda* and *esculenta* were compared with individuals of both species in the age of metamorphosis. Among these four kinds of individuals the *esculenta* were the earliest and the *brevipoda* the latest in metamorphosis. The reciprocal hybrids were intermediate between the two controls and rather resembled the control *esculenta* (Table 9).

TABLE 9

Ages of hybrids, *R. brevipoda* ♀ × *R. esculenta* ♂, and the controls at the time of metamorphosis

Parents		No. of metamorphosed frogs	Age at the protrusion of forelegs	
Female	Male		Range (days)	Mean (days)
R. brev.	*R. brev.*	77	44–54	49.7
R. brev.	*R. escul.*	31	40–49	44.2
R. escul.	*R. brev.*	24	42–45	43.5
R. escul.	*R. escul.*	72	37–49	42.3

The *brevipoda* ♀ × *esculenta* ♂ hybrids were very similar in body length to the control *brevipoda* immediately after the completion of metamorphosis, as presented in Table 10. The mean body length of 382 hybrid frogs was 18.09 mm., while that of the 455 controls was 18.84 mm. However, there were remarkable differences among the eight experimental series as well as the eight control ones. Although these differences seemed mostly to have their origin in the rearing conditions, such as the density of tadpoles, the comparison between the experimental and the control series was possible.

TABLE 10

Body lengths of hybrids, *R. brevipoda* ♀ × *R. esculenta* ♂, and the controls immediately after metamorphosis

Male / Female	*R. brevipoda*, Nos. I ~ IV		*R. esculenta*, Nos. I, II	
	No. of frogs	Mean body length (mm.)	No. of frogs	Mean body length (mm.)
R. brevipoda, No. I	28	21.22 ± 0.30	21	18.99 ± 0.36
〃 No. II	52	18.78 ± 0.21	43	18.05 ± 0.22
〃 No. III	84	17.78 ± 0.10	78	16.82 ± 0.13
〃 No. IV	45	18.90 ± 0.22	29	18.66 ± 0.46
〃 No. V	21	22.55 ± 0.36	8	22.80 ± 0.43
〃 No. VI	75	18.48 ± 0.11	57	18.52 ± 0.15
〃 No. VII	65	18.57 ± 0.16	57	18.81 ± 0.18
〃 No. VIII	85	18.18 ± 0.12	89	17.88 ± 0.12
	455	18.84	382	18.09

The *esculenta* ♀ × *brevipoda* ♂ hybrids were also similar in body length to the control *brevipoda*, immediately after the completion of metamorphosis (Table 11).

TABLE 11
Body lengths of hybrids, *R. esculenta* ♀ × *R. brevipoda* ♂, and the controls immediately after metamorphosis

Parents		No. of frogs	Mean body length (mm.)
Female	Male		
R. esculenta, No. I	*R. brevipoda,* No. V	10	22.35±0.57
R. brevipoda, No. IX	〃 No. V	13	20.18±0.32
〃 No. X	〃 No. V	20	22.46±0.47
R. nigromaculata, No. VII	*R. nigromaculata,* No. V	15	21.12±0.27

TABLE 12
Body lengths of hybrids, *R. brevipoda* ♀ × *R. esculenta,* ♂, and the controls one year after metamorphosis

Series	Parents		No. of frogs	Mean body length (mm.)
	Female	Male		
Control	*R. brev.,* No. VI	*R. brev.,* Nos. III, IV	11	48.7
	〃 No. VII	〃 Nos. III, IV	13	47.2
	〃 No. VIII	〃 Nos. III, IV	11	47.0
			35	47.6
Experimental	*R. brev.,* No. VI	*R. escul.,* No. II	6	49.7
	〃 No. VII	〃 No. II	10	46.1
	〃 No. VIII	〃 No. II	17	42.4
			33	44.8

One year after metamorphosis the *brevipoda* ♀ × *esculenta* ♂ hybrids were compared with the control *brevipoda* in terms of body length. They were 44.8 mm. on the average, in contrast to 47.6 mm. of the control *brevipoda* (Table 12). This smaller size of the hybrids was mainly due to the fact that the frogs produced from the cross between the female *brevipoda* No. VIII and the male *esculenta* were very poor in growth, that is, the 17 frogs were 42.4 mm. on the average in body length. The ten hybrids from the female *brevipoda* No. VII were also rather smaller than the control frogs. In contrast with these series, the hybrids from the female *brevipoda* No. VI were not only larger than those of the other two experimental series, but rather larger than those of the control frogs. The six hybrids produced from the female *brevipoda* No. VI were 49.7 mm. on the average in body length.

3. Color patterns

The three species, *Rana esculenta, R. nigromaculata* and *R. brevipoda,* and the three kinds of hybrids, *nigromaculata* ♀ × *esculenta* ♂, *brevipoda* ♀ × *esculenta* ♂, and *esculenta* ♀ × *brevipoda* ♂, were compared with one another in terms of the color pattern of frogs. The latter are shown in Table 13.

The *esculenta* ♀ × *brevipoda* ♂ hybrids were quite similar in color pattern to some of the reciprocal ones. Concerning the dorsal ground color there were two types, bluish green and brown, among the *brevipoda* ♀ × *esculenta* ♂ hybrids. The frequency of each type among these hybrids is given in Table 14. In each experimental series as well as in total, bluish green frogs and brown ones were nearly equal in number. Accordingly, it was clear that the bluish green of the dorsal ground color was inherited by a single dominant gene, and moreover, that the male *esculenta* had this gene in a heterozygous condition.

TABLE 13
Color patterns of hybrid frogs and the controls

Item \ Kind	*Rana esculenta*	*Rana nigromaculata*	*Rana brevipoda*	*R. nigr.* ♀ × *R. escul.* ♂	*R. brev.* ♀ × *R. escul.* ♂	*R. escul.* ♀ × *R. brev.* ♂
Dorsal ground color	Yellow-green	Light brown	Dark brown	Light brown	Bluish green or brown	Bluish green
Dorsal markings	A small number of small black spots	Black spots connected irregularly	A small number of large round black spots	A small number of longish black spots	A small number of small round black spots	A small number of small round black spots
Dorso-median stripe	Light yellow-green (faint)	Yellow-brown or yellow-green	None	Yellow-brown	Bluish green (faint) or yellow-brown	Bluish green (faint)
Dorso-lateral stripes	Light brown	Light brown	Light brown	Light brown	Light brown	Light brown
Ventral markings	None or black marble-like	None	Grey marble-like	None	None or speckles on the throat	None

TABLE 14
Inheritance of dorsal ground colors

Parents		Green		Brown	
Female	Male	No. of frogs	%	No. of frogs	%
R. brev., No. I	*R. escul.*, No. I	5	35.7	9	64.3
〃 No. II	〃 No. I	10	45.5	12	54.5
〃 No. III	〃 No. I	11	45.8	13	54.2
〃 No. IV	〃 No. I	11	45.8	13	54.2
〃 No. V	〃 No. I	4	50.0	4	50.0
〃 No. VI	〃 No. II	26	45.6	31	54.4
〃 No. VII	〃 No. II	27	47.4	30	52.6
〃 No. VIII	〃 No. II	37	41.6	52	58.4
		131	44.4	164	55.6

The dorsal markings of the reciprocal hybrids were intermediate between those of *esculenta* and *brevipoda*. The dorso-median stripe appeared in all the hybrids, in contrast to the absence of this stripe in the control *brevipoda*. Accordingly, it was sure that the presence of this stripe was inherited by a single dominant gene, and that the male *esculenta* was homozygous for this gene. The color of the dorso-median stripe were related to the dorsal ground color. Concerning the ventral markings, there were some frogs with dark speckles on their throat among the *brevipoda* ♀ × *esculenta* ♂ hybrids, while in the others the undersides were quite white.

III. Sex and gonads of hybrids

1. *R. brevipoda* ♀ × *R. esculenta* ♂ hybrids

a. Frogs immediately after metamorphosis

The gonads of 220 hybrids of female *brevipoda* and male *esculenta* and 295 control *brevipoda* were examined immediately after metamorphosis. Although there were nearly equal numbers of males and females in the hybrids of the experimental series, as well as in the control *brevipoda,* there were no typical females similar to those of the control series (Tables 15, 16). The gonads of the hybrids were divided into the following four types, according to their inner structures.

TABLE 15
The gonads of *R. brevipoda* ♀ × *R. esculenta* ♂ hybrids, immediately after metamorphosis

Parents		No. of females		No. of males		Total
Female	Male	Type 1a	Type 1b	Type 2a	Type 2b	
R. brev., No. I	*R. escul.,* No. I	1	1	5	0	7
〃 No. II	〃 No. I	5	8	3	2	18
〃 No. III	〃 No. I	8	19	24	2	53
〃 No. IV	〃 No. I	3	3	1	0	7
〃 No. VI	*R. escul.,* No. II	5	9	6	11	31
〃 No. VII	〃 No. II	3	14	11	8	36
〃 No. VIII	〃 No. II	3	25	24	16	68
Total		28 (12.7%)	79 (35.9%)	74 (33.6%)	39 (17.7%)	220
		107 (48.6%)		113 (51.4%)		

TABLE 16
The gonads of *Rana brevipoda,* immediately after metamorphosis

Parents		No. of females	No. of males	Total
Female	Male			
R. brev., No. I	*R. brev.,* Nos. I, II	2	6	8
〃 No. II	〃	17	15	32
〃 No. III	〃	30	34	64
〃 No. IV	〃	15	10	25
〃 No. V	〃	0	1	1
〃 No. VI	*R. brev.,* Nos. III, IV	29	26	55
〃 No. VII	〃	26	19	45
〃 No. VIII	〃	32	33	65
Total		151(51.2%)	144(48.8%)	295

Type 1a, ill-developed ovary, having wide ovarian cavities. In the cortices there were many oogonia and a small number of oocytes at the early stage. There were no growing auxocytes.

Type 1b, degenerative ovary, having narrow ovarian cavities, and there were a small number of germ cells, mostly oogonia, in the cortices.

Type 2a, nearly typical testis, in which spermatogonia and rete cells were more or less radially arranged.

Type 2b, ill-developed testis, having a small number of spermatogonia surrounded with rete cells.

Among the 220 *brevipoda* ♀ × *esculenta* ♂ hybrids there were four types 1a, 1b, 2a and 2b, of gonads in the rate of 12.7, 35.9, 33.6 and 17.7 (Table 15). In the control series the ovaries of females were quite typical, having many auxocytes, while the testes of males were quite or nearly typical.

b. Frogs, ca. 12 or 21 months after metamorphosis

On September 29, 1960, 13 hybrid frogs of the experimental series in 1959 were killed together with 13 control *brevipoda*. About seven months later, on May 1, 1961, other 13 hybrid frogs were killed. The gonads of all these frogs were sectioned and stained.

Among the 13 control *brevipoda* there were five females and eight males. The ovaries of the females contained a lot of perfectly matured ova, while in the testes of the males bundles of normal spermatozoa were abundantly formed in seminal tubules.

The gonads of all the 26 hybrid frogs were very abnormal; there were no ovaries and testes similar to those of the control frogs. However, they could be divided into ovaries and testes, owing to the presence or the absence of rete appa-

TABLE 17

The gonads of *R. brevipoda* ♀ × *R. esculenta* ♂ hybrids, ca. 12 or 21 months after metamorphosis

Individual no.	Months after metamorph.	Body length (mm.)	Hind-limb length (mm.)	Color of back	Size of gonads (mm.)		Type of gonads
					Left	Right	
BVI E 161	21	45	64.5	Green	Very small	0.7×0.5	2b
BVI E 162	21	52	74.5	Brown	1.7×0.9	1.1×1.4	2a
BVI E 163	(21)	55	71				
BVI E 164	21	52	74		Very small	Very small	1b*
BVI E 165	(21)	54.5	68.5	Green			
BVII E 71	12	49	72.5	Brown	1.0×0.7	1.2×0.5	2b
BVII E 72	12	50.5	69		3.1×1.8	3.1×2.1	1a
BVII E 73	12	44	62		2.1×1.5	2.0×1.1	1a
BVII E 74	12	42.5	60.5		Very small	Very small	2b*
BVII E 171	21	42	56	Green	Very small	Very small	2b
BVII E 172	(21)	55	76	Brown			
BVII E 173	21	58.5	64.5		Very small	1.2×1.5	1b, 1a
BVII E 174	21	51	70.5		1.0×0.9	1.0×1.2	2b*
BVII E 175	(21)	49	63.5	Green			
BVIIIE 81	12	46	66.5	Brown	1.4×1.1	1.4×1.1	2a
BVIIIE 82	12	43	64.9		0.6×0.3	Very small	1b
BVIIIE 83	12	47.5	69		4.6×2.4	2.3×1.6	1a
BVIIIE 84	12	40.5	59.5		Very small	Very small	2b*
BVIIIE 85	12	40.5	59.5		1.6×0.9	1.3×0.9	2a
BVIIIE 86	12	46	68	Green	3.0×1.9	1.8×0.7	1a
BVIIIE 87	12	41	64.5		Very small	Very small	2b*
BVIIIE 88	12	45	68		1.7×1.5	Very small	2a, 2b
BVIIIE 89	12	37	54.5		Very small	Very small	2b*
BVIIIE 181	21	38	53	Brown	0.6×0.5	0.7×0.4	2b
BVIIIE 182	21	34	45		Very small	Very small	2b
BVIIIE 183	21	42	63	Green	0.4×0.3	Very small	2b*
BVIIIE 184	21	48.5	60		2.8×1.9	2.7×1.9	1a
BVIIIE 185	21	43	59		4.2×3.3	0.7×0.4	1a, 1b
BVIIIE 186	21	44.5	61		4.2×1.9	1.4×1.9	1a
BVIIIE 187	21	53	77	Brown	0.6×0.4	2.3×1.9	2b, 2a

* No germ cells ( ) ···Size of gonads was not measured.

ratus. For convenience' sake they were divided into four types, corresponding to those of the gonads of the frogs immediately after metamorphosis. Among the 26 hybrids there were 10 females and 16 males. The size and type of the gonads of each hybrid frog are shown in Table 17, together with the body length, hind-limb length and the color of the back.

i) Type 1a, ill-developed ovary. The gonads of eight hybrids were of this type, although in two of these frogs the gonad of one side was of type 1b. The presence of wide or narrow cavities surrounded with squamous epithelium and the absence of well-developed rete apparatus seemed to indicate that these gonads derived from such ovaries as those of type 1a found in hybrid frogs immediately after metamorphosis. There were no growing auxocytes in the gonads of six hybrids. In the gonads of the other two there were a few auxocytes, which were less than 150 μ in diameter. Some of them were deformed and degenerating. There were masses of secondary oogonia or first oocytes at the early stage; they were surrounded by the walls of slit-like ovarian cavities. Besides, there were oogonia with polymorphic nuclei and oocytes at the early stage. In some gonads germ cells were solitarily or in small groups surrounded with many follicular cells which had increased in number as compensation for degenerated germ cells. A large number of germ cell nuclei were pycnotic and degenerating. Moreover, there were often some intercellular spaces filled with fluid containing vestiges of degenerated cells (Plate I, 1).

ii) Type 1b, very degenerative ovary. Both gonads of each of two hybrids, the right gonad of one hybrid and the left of another were of this type, while the gonads of the other sides in the latter two were of type 1a. One of the former two hybrids, No. BVIE164, had no definite gonads; along the proximal part of each fat body there was a small mass of loose connective tissue which seemed to be a trace of degenerated ovary. The gonads of type 1b were very small and degenerative. It was the most distinct characteristic of these gonads that there were no remains of rete apparatus. In both gonads of one hybrid, No. BVIIIE82, the left gonad of No. BVIIE173 (Plate I, 2) and the right of No. BVIIIE185, there were a few germ cells which seemed to be oogonia.

iii) Type 2a, ill-developed testis. Five hybrid frogs had gonads of this type. However, two of these hybrids had a gonad of type 2b on one side. The gonads of type 2a were generally smaller than those of type 1a and 1b; they were characterized by the presence of ramified rete apparatus and many seminal tubules. The rete apparatus were extraordinarily conspicuous, owing to under-development of seminal tubules. The latter were mostly filled with masses of secondary spermatogonia and first spermatocytes. Along the walls of seminal tubules there were solitary primary spermatogonia. The meiosis usually did not proceed beyond the first maturation division. There were no second spermatocytes, spermatids and spermatozoa. However, in the testes of two hybrids there were a few large abnormal heads of spermatozoa and many pycnotic nuclei which seemed to have been derived from first spermatocytes after abnormal first maturation divisions. The amount of secondary spermatogonia and first spermatocytes was remarkably variable with the frog or the gonad. In the gonads of No. BVIE162 there were only a few spermatogonia in each seminal tubule. Moreover, some spermatogonia were degenerating at their proper sites along the walls or in the midst of the lumens. In such a gonad there was plenty of connective tissue around the rete apparatus near the hilum (Plate I, 3).

iv) Type 2b, very degenerative testis. Both gonads of each of eleven hybrids, the right of one hybrid and the left of another were of this type. The other gonads of the latter two hybrids were of type 2a. Both gonads of each of six hybrids and the right gonad of another had no germ cells, while those of the remaining hybrids had a few germ cells, i.e. spermatogonia. It was a characteristic of the gonads of type 2b that the ductules of rete apparatus were surrounded with plenty of connective tissue. Generally speaking, the very small gonads shown in Table 17 had a few degenerative ductules and half of them had no seminal tubules and spermatogonia. In the latter gonads the connective tissue surrounding degenerative ductules was often altered into adipose tissue (Plate I, 4).

c. Three-year-old frogs

The gonads of five 3-year-old *brevipoda* ♀ × *esculenta* ♂ hybrids produced in the breeding season of the year 1961 were histologically observed. There were one female and four males. The ovaries of the female were very small and degenerative, being somewhat similar to degenerating BIDDER's organs of the toad in inner structure. They contained many oogonia with polymorphic nuclei and a small number of oocyte-like cells with spherical nuclei, besides degenerating germ cells. The oocyte-like cells seemed to be a kind of degenerating oogonia which were a little larger than usual ones. The ovarian cavities were completely filled with adipose tissue.

The four males had very degenerative testes which had no germ cells. The testes of two males were comparatively large, as compared with those of the other two and were filled with seminal tubules, slender ducts of rete apparatus and a small amount of connective tissue. The testes of another male were also filled with seminal tubules and slender ducts of rete apparatus. A part of the outer portions of each testis was changed into adipose tissue. The testes of the remaining male were most degenerative. There were only a few remains of slender ducts of rete apparatus and several vasa efferentia. The testes were mostly changed into adipose tissue.

2. *R. esculenta* ♀ × *R. brevipoda* ♂ hybrids

The sex and the gonads of 37 hybrid frogs produced during the years 1961～1963 were examined. There were 17 females and 20 males. Their gonads were very similar to those of the reciprocal hybrids in size, shape and structure.

Out of nine hybrids developed in the year 1961, three were killed immediately after metamorphosis to examine their gonads. Among them there were one female and two males. While the testes of the males were nearly typical in structure, the ovaries of the female contained no growing auxocytes, differing from those of the *brevipoda* and *esculenta* controls, although they were nearly normal in size.

One frog was preserved at the age of one year. This was a male which had revealed the secondary sexual characters, such as thumb pads, on the fore-legs. However, the testes consisted of seminal tubules and rete apparatus and there were no germ cells.

The remaining five frogs were preserved at the age of three years. Two were females and the other three were males. The females had ill-developed ovaries which were of type 1a found in those of one- or two-year-old *brevipoda* ♀ × *esculenta* ♂ hybrids. The ovaries had slit-like ovarian cavities which were surrounded with squamous epithelium. The right ovary of a female had many secondary oogonia and a small number of primary oogonia, while in the left there were a comparatively small number of germ cells, which were mostly primary oogonia surrounded with multiplied follicular cells. The ovaries of the other female were divided into many lobules and contained a small number of primary oogonia surrounded with multiplied follicular cells. Besides, there were some degenerating germ cells here and there in the ovaries (Plate II, 5, 6).

One of the three males had ill-developed testes which were of type 2a found in those of one- or two-year-old *brevipoda* ♀ × *esculenta* ♂ hybrids. The seminal tubules were thick and filled with germ cells, which were primary and secondary spermatogonia, first spermatocytes, abnormal figures of the first reduction division, pycnotic nuclei and very abnormal spermatozoa. There were some parts of seminal tubules which contained comparatively numerous pycnotic nuclei or abnormal spermatozoa. The remaining two males had very degenerative testes which were of type 1b. Although the testes were comparatively large, they were filled with slender seminal tubules and rete apparatus and there were no germ cells (Plate II, 7, 8).

3. Reproductive ability

Although it was presumed that reciprocal hybrids between *brevipoda* and *esculenta* were quite sterile, some experiments were made to confirm this presumption by making use of eleven male hybrids which were two or three years old and revealed secondary sexual characters.

In the breeding season of the year 1962, 19 *brevipoda* ♀ × *esculenta* ♂ and four *esculenta* ♀ × *brevipoda* ♂ hybrids were produced. The latter four were killed in 1965 for autopsy after they had been raised for three years. They were all males; the size of testes are presented in Table 18, together with their body

TABLE 18

Size of the testes of 3-year-old hybrids, *R. esculenta* ♀ × *R. brevipoda* ♂

Individual no.	Body length (mm.)	Size of testes (mm.)	
		Left	Right
62EBI	47.0	1.0 × 0.5	1.5 × 1.5
62EBII	47.5	2.5 × 2.0	2.5 × 2.0
62EBIII	50.5	4.5 × 2.5	2.5 × 2.0
62EBIV	50.0	Irregular shape	2.5 × 1.5

length. Three males which had comparatively large testes were examined in their reproductive ability. Out of the 19 hybrids produced from the reciprocal crosses five females and two males were reared for three years, although there were twelve females and seven males among the hybrids. Only the two 3-year-old males were used to examine their reproductive ability, as it was quite clear that the females were perfectly sterile; there were no ova in their ovaries.

In the season of 1963, 31 *brevipoda* ♀ × *esculenta* ♂ and 24 *esculenta* ♀ × *brevipoda* ♂ hybrids were produced. While there were 20 females and 11 males among the former and 14 females and 10 males among the latter, 13 females and 5 males of the former and 3 females and five males of the latter were reared for two years. Out of these 2-year-old males and females, three *brevipoda* ♀ × *esculenta* ♂ and three *esculenta* ♀ × *brevipoda* ♂ males with secondary sexual characters were examined in their reproductive ability.

Eleven male hybrids in all, three 2-year-old and two 3-year-old *brevipoda* ♀ × *esculenta* ♂ and three 2-year-old and three 3-year-old *esculenta* ♀ × *brevipoda* ♂ hybrids, were mated with two female *brevipoda* collected from the field by the artificial fertilization method in the breeding season of 1965. As the result, fertilized eggs were obtained from none of these male hybrids. Accordingly, it was clear that both male and female hybrids produced from reciprocal crcsses between *brevipoda* and *esculenta* were completely sterile.

DISCUSSION

If we call pond frogs similar to *Rana esculenta* distributed in Europe as the *esculenta* group, this group consists of two species in each of Japan, Korea and eastern China. They are *Rana nigromaculata* HALLOWELL and *Rana brevipoda* ITO in Japan (KAWAMURA, 1962), *Rana nigromaculata* and *Rana plancyi chosenica* OKADA in Korea (SHANNON, 1956), and *Rana nigromaculata* and *Rana plancyi plancyi* LATASTE in eastern China (LIU and HU, 1961). In Europe there seem to be two species, *Rana esculenta* L. and *Rana ridibunda* PALLAS (MERTENS and WERMUTH, 1960; NIKOL'SKII, 1962), although BERGER (1967) recently reported that the *esculenta* group collected from Poznań, Poland consisted of three morphological forms, *Rana lessonae* CAMERANO, *Rana esculenta* and *Rana ridibunda,* and that the *esculenta* specimens were probably the hybrids between the other two forms.

It has been reported by MORIYA (1955) that there is neither gametic isolation nor hybrid inviability between *Rana nigromaculata* and *Rana brevipoda.* However, males produced from reciprocal crosses of these two species are nearly perfectly sterile, while females are quite fertile (MORIYA, 1960). The sterility of male hybrids between these two species has been confirmed by KAWAMURA and NISHIOKA (1963).

Nearly the same relationship as found between *Rana nigromaculata* and *Rana brevipoda* is found between the two species, *Rana nigromaculata* and *Rana plancyi chosenica,* distributed in Korea, as well as between *Rana nigromaculata* from Korea and *Rana brevipoda* from Japan (NISHIOKA, KURAMOTO, unpublished). It seems necessary to add here the fact that *Rana brevipoda* are almost completely isolated from *Rana plancyi chosenica* by hybrid sterility (NISHIOKA, 1971).

TING (1939) made crossing experiments between *Rana nigromaculata* and *Rana plancyi plancyi* and obtained many reciprocal hybrids. A part of these hybrids grew normally and completed their metamorphosis. Although half of 30 hybrid frogs reared continuously died at intervals, the remaining survived in a good condition (TING, 1948). Afterwards, a two-year-old male hybrid produced many healthy tadpoles by mating with *Rana nigromaculata* by the method of artificial fertilization. By histological observation of one testis of this male, it was found that there were numerous spermatozoa in the seminal tubules. According to TING's reports, the Chinese *Rana plancyi* seems to be physiologically very different from the Korean *Rana plancyi*, in spite of the fact that both species are very similar to each other in shape and color pattern. However, it seems possible that the single matured hybrid obtained by TING was an exceptional individual. As there was so high mortality both in the hybrids and in the controls, it was impossible to know about gametic isolation or hybrid inviability, even if there were such isolating mechanisms between the two species.

In Europe, MANDEVILLE and SPURWAY (1949) reported on reciprocal hybrids between *Rana esculenta* and *Rana ridibunda*. The *ridibunda*, used as material, were the offspring of those which had been imported from Hungary to England. The *esculenta* came from the colonies distributed in some districts of Surrey, England, although their origin was unknown. The crosses were performed in outdoor ponds, by leaving several males of one species together with several females of the other species, in a basin. According to them, there seemed to be little mortality between the complicated crises of hatching and beginning to feed, and of metamorphosis and leaving the water. There was no evidence of a difference between the hybrids and wild forms. However, mortalities of the former during the second summer were much more than those of the latter, although their absolute number was very small. One *ridibunda* ♀ × *esculenta* ♂ hybrid survived for a little more than one year, while five of the reciprocal hybrids did for about one year and a half or a little longer. BERGER's opinion that *Rana esculenta* are the hybrids of *lessonae* and *ridibunda* is principally based on the results of his mating experiments by making use of these three forms. When female *esculenta* were mated with males of the same form, there were usually very high mortalities of tadpoles, as compared with those of the other kinds of matings. He considered the lower viability of the progeny of *esculenta* as the manifestation of genetic incompatibility. Differing from his experiments, one of the present writers has made clear that in crossing experiments between *esculenta* from Luxemburg and *ridibunda* from France, the control *esculenta* as well as the control *ridibunda* are quite normal in development and viability. Moreover, in contrast with the results obtained by MANDEVILLE and SPURWAY, reciprocal hybrids between these two species are completely sterile, although there is scarcely mortalities of the hybrids (NISHIOKA, unpublished).

The *esculenta* used as material in the crosses performed by the present writers in the years 1961 ~ 1963 came from Luxemburg. When female *esculenta* were mated with males of the same species in the control series, the fertilization rate of eggs, the development and viability of embryos and tadpoles, and the metamorphosis of the latter were quite normal. There were no remarkable differences in these respects between the control *esculenta* and the control *nigromaculata* or *brevipoda*. Accordingly, it seems clear that the *esculenta* from Luxemburg are pure in nature and different from Polish *esculenta* used by BERGER.

The *esculenta* used in 1959 and 1960 came from Dresden, East Germany. Regret to say, there were no control *esculenta* accompanying the crosses, since there were no matured females in 1959, while no matured males in the next year. However, the results of crosses between *esculenta* and *nigromaculata* or *brevipoda* were nearly the same as those obtained by the use of *esculenta* from Luxemburg. In 1961, 1962, 1963 and 1965, the *brevipoda* ♀ × *esculenta* ♂ tadpoles were 34.4%, 28.3%, 26.4% and 17.5% of the total eggs, respectively, and the *nigromaculata* ♀ × *esculenta* ♂ hybrids in 1962, 1963 and 1965 were 4.1%, none and none, respectively, while in 1959 the *brevipoda* ♀ × *esculenta* ♂ tadpoles were 33.6% and the *nigromaculata* ♀ × *esculenta* ♂ were 0.2%. On the other hand, the *esculenta* ♀ × *brevipoda* ♂ tadpoles in 1961, 1962 and 1963 were 15.0%, 8.4% and 13.9% of the total eggs, respectively, and no hatched tadpoles were produced from among the *esculenta* ♀ × *nigromaculata* ♂ hybrids in 1962 and 1963. In contrast with these, the *esculenta* ♀ × *brevipoda* ♂ and the *esculenta* ♀ × *nigromaculata* ♂ tadpoles in 1960 were 9.9% and none, respectively. From these data, it is believed that the *esculenta* used in 1959 and 1960 are nearly the same in nature as those used in the years 1961 ~ 1965, that is to say, the *esculenta* from Dresden, if this origin is correct, are of nearly the same nature as those of Luxemburg.

Differing from the relationship between *nigromaculata* and *brevipoda*, there are slight gametic isolation and severe hybrid inviability between *esculenta* and *nigromaculata* or *brevipoda*. Especially, it has been ascertained that nearly all the reciprocal hybrids between *esculenta* and *nigromaculata* become abnormal and die by the hatching stage. Although the hybrid inviability is incomplete between *esculenta* and *brevipoda*, there is complete hybrid sterility between them. In other words, the two species of Japanese pond frogs are completely isolated from the European *esculenta* by reproductively isolating mechanisms.

It seems interesting to compare the two kinds of isolating mechanisms, hybrid inviability and hybrid sterility, with each other. The hybrid inviability has not only been found between remotely related species, such as between the *esculenta* group and the others, but also found between closely related species or even between geographic races of the same species. MOORE (1946, '47, '50) has reported that the northern and southern races of *Rana pipiens* are isolated from each other by severe hybrid inviability. These races differ from each other in a number of embryonic characters such as temperature tolerance, rate of development and temperature coefficient, as the northern and southern species of amphibians do. He considers such hybrid inviability as a result of incompatibility in the interaction of a low temperature genome and a high temperature genome in the same zygote. The same embryonic abnormalities were found by VOLPE (1954) and RUIBAL (1955) in reciprocal hybrids between geographically extreme members of *Rana pipiens.* KAWAMURA (1953) has reported that there is a similar phenomenon between high and low temperature adapted races of *Hynobius nebulosus.*

It can not be known whether the alliance of *nigromaculata* with *esculenta* is closer than that of *brevipoda* with *esculenta* or not. However, it is noticeable that *nigromaculata* embryos are slightly delayed in development until the neural tube or tail-bud stage, as compared with *esculenta,* while the latter develop synchronously with *brevipoda.* The hybrid inviability between *nigromaculata* and *esculenta* seems to occur owing to their asynchronism in the rate of development.

The complete sterility of the reciprocal hybrids between *brevipoda* and *esculenta* seems to indicate that the relationship of these two species is more remoter than that of *nigromaculata* and *brevipoda* or *plancyi chosenica,* in which the hybrid sterility is incomplete. This is more emphasized by the fact that there is hybrid inviability, though incomplete, between *brevipoda* and *esculenta,* while none between *nigromaculata* and *brevipoda* or *plancyi chosenica.*

A similar complete sterility has been observed in the hybrids between different species related closely to *Rana temporaria,* too (DÜRKEN, 1935, '38; KAWAMURA, 1943, '50; KAWAMURA and KOBAYASHI, 1959, '60; KOBAYASHI, 1962a, '62b). However, all these hybrids are males, differing from the case of the *esculenta* group, in which there are sterile males and females. Such a difference seems to show that the sex determining mechanism is very stable in the *esculenta* group, in contrast to the situation in the *temporaria* group, in which the sex reversal from a female to a male occurs very easily.

SUMMARY

1. Reciprocal crosses were made between Japanese and European pond frogs to clarify their biological differences. Two Japanese species, *Rana nigromaculata* and *Rana brevipoda,* and a European species, *Rana esculenta,* were used as material.

2. There are slight gametic isolation and severe hybrid inviability between *esculenta* and *nigromaculata* or *brevipoda*; nearly all the reciprocal hybrids between *esculenta* and *nigromaculata* become abnormal and die by the hatching stage, while a small number of reciprocal hybrids between *esculenta* and *brevipoda* grow into frogs. The hybrids between female *brevipoda* and male *esculenta* seem to be superior in viability during the embryonal stages to the reciprocal hybrids.

3. All the four kinds of hybrids, *brevipoda* ♀ × *esculenta* ♂, *esculenta* ♀ × *brevipoda* ♂, *esculenta* ♀ × *nigromaculata* ♂ and *nigromaculata* ♀ × *esculenta* ♂, are a little delayed in development during the embryonal stages, as compared with the three kinds of controls, *brevipoda, esculenta* and *nigromaculata.* The latter two kinds of hybrids are more retarded than the former two and become abnormal in shape after the stage of gastrulation.

4. As for the time of metamorphosis, reciprocal hybrids between *brevipoda* and *esculenta,* roughly speaking, are similar to or a little later than the control

esculenta, which are distinctly earlier than the *brevipoda*. Immediately after metamorphosis, *brevipoda* ♀ × *esculenta* ♂ frogs are nearly equal to the control *brevipoda* in body length. One year after metamorphosis these hybrids are nearly equal or inferior to the latter.

5. The dorsal green color of *esculenta* is inherited by a single dominant gene to the hybrids between this species and *brevipoda*.

6. There are nearly an equal number of males and females in the hybrids, *brevipoda* ♀ × *esculenta* ♂, immediately after metamorphosis. The ovaries of these females are ill-developed or degenerative, while the testes of the males are nearly normal or ill-developed. More than one year after metamorphosis, the ovaries and testes are all ill-developed or very degenerative. In the reciprocal hybrids, *esculenta* ♀ × *brevipoda* ♂, the sex ratio and the structures of gonads are nearly the same as those of the above hybrids.

7. Both male and female hybrids between *brevipoda* and *esculenta* are quite sterile, although the males reveal their secondary sexual characters.

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EXPLANATION OF PLATES

PLATE I

Cross-sections of the gonads of matured frogs killed ca. 12 or 21 months after metamorphosis. ×300

1. Type 1a (ill-developed) ovary of the *brevipoda* ♀ × *esculenta* ♂ hybrid, No. BVIIIE185, 21 months after metamorphosis.
2. Type 1b (degenerative) ovary of the *brevipoda* ♀ × *esculenta* ♂ hybrid, No. BVIIE173, 21 months after metamorphosis.
3. Type 2a (nearly typical) testis of the *brevipoda* ♀ × *esculenta* ♂ hybrid, No. BVIIIE85, 12 months after metamorphosis.
4. Type 2b (ill-developed) testis of the *brevipoda* ♀ × *esculenta* ♂ hybrid, No. BVIIE71, 12 months after metamorphosis.

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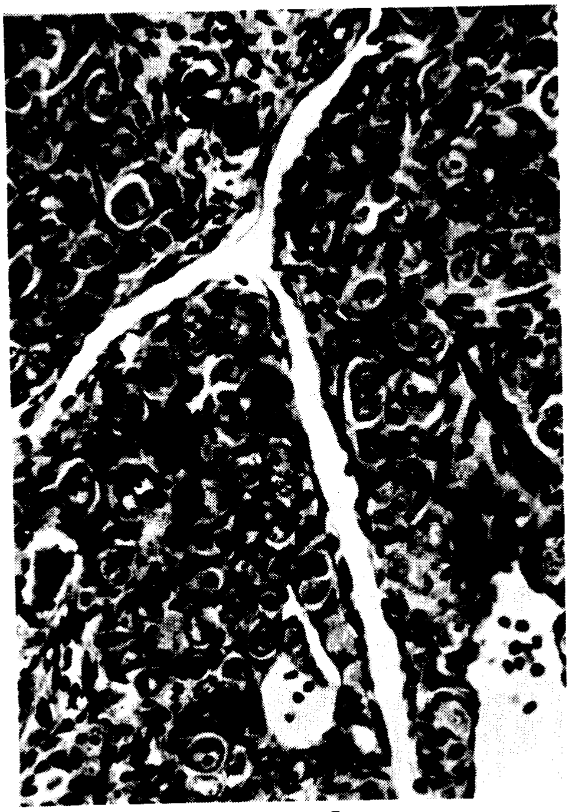

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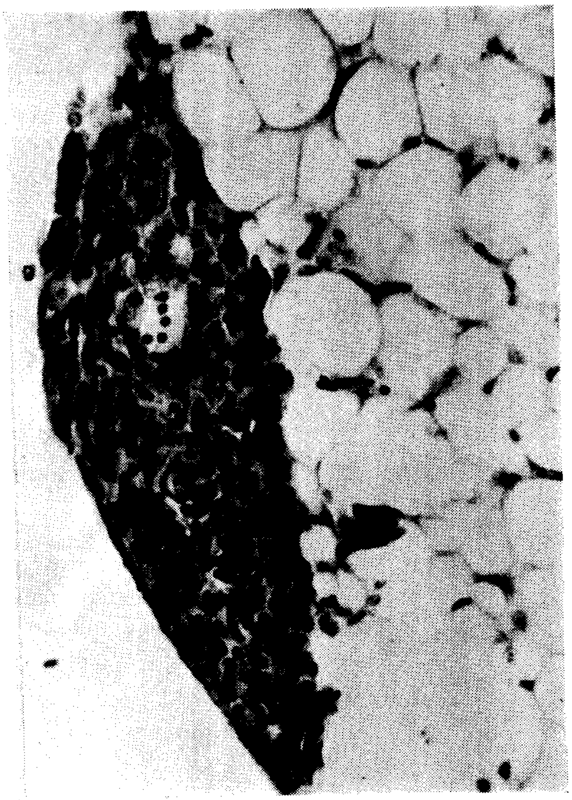

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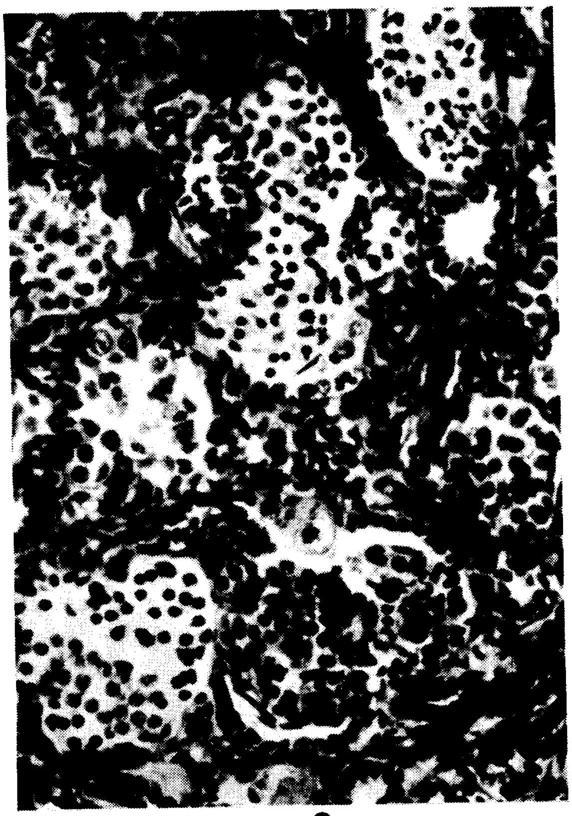

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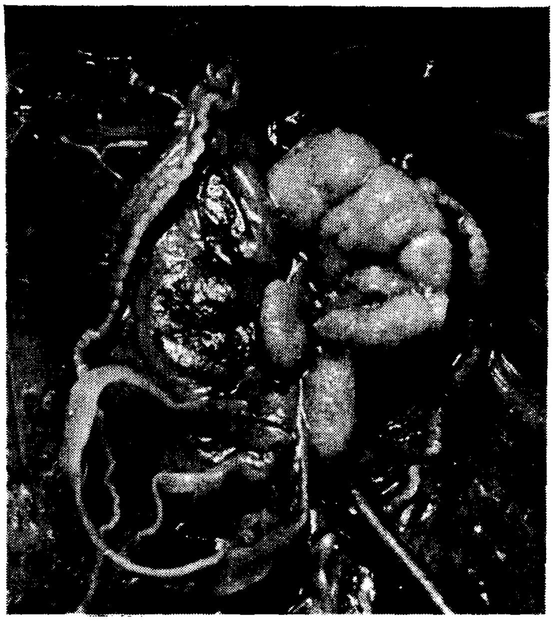
PLATE II

External appearances and cross-sections of the gonads of two *esculenta* ♀ × *brevipoda* ♂ hybrids, three years old.

5. The ovaries of a female hybrid. ×5.
6. Cross-section of the left ovary of the same frog as (5). ×300.
7. The testes of a male hybrid. ×5.
8. Cross-section of the left testis of the same frog as (7). ×300.



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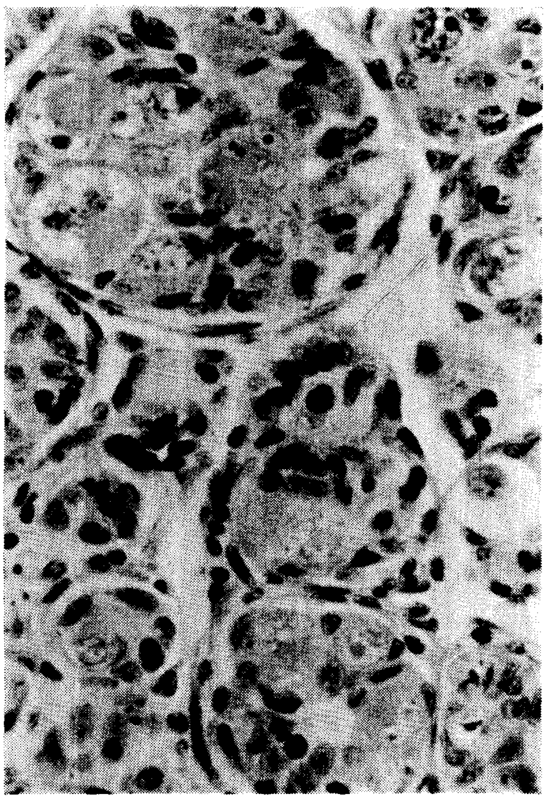

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