

## Experimental Hybridization among *Oryzias* Species

### I. *O. celebensis*, *O. javanicus*, *O. latipes*, *O. luzonensis* and *O. melastigma*

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#### ABSTRACT

As a step to determining the affiliation of each *Oryzias* species, experimental interspecific hybridization among *Oryzias celebensis*, *O. javanicus* (Jakarta and Singapore), *O. latipes*, *O. luzonensis*, and *O. melastigma* was carried out and six kinds of adult F<sub>1</sub> hybrids were obtained. The proteins of their muscles and eyes were electrophoretically analyzed. The developmental ability, morphological and meristic characteristics of the hybrids seemed to depend on the phenotypic differences between the parent species and to be closely related to the geographical distances between their habitats. The electrophoretic data on the proteins in the hybrids may also indicate a close relationship to the geographical distribution of the parental species.

#### INTRODUCTION

The habitat of the medakas (*Oryzias*) is found in the East and South of Asia. So far, fourteen species have been reported (see Iwamatsu, 1993). In order to clarify the phylogenetic relationships among the fishes of the genus *Oryzias*, several recently collected species have been investigated by means of embryological, morphological, cytogenetic and biochemical analyses by several investigators (Iwamatsu, 1986; Sakaizumi, 1985; Uwa, 1986). The systematic relationships of some species have also been examined by interspecific hybridizations (Iwamatsu *et al.*, 1984, 1986; Magtoon and Uwa, 1985; Sakaizumi *et al.*, 1992; Uwa, 1991).

We have examined the electrophoretic mobilities of proteins from interspecific hybrids in addition to the developmental ability of the hybrids to analyze the genetic relationships. The present paper presents morphological and electrophoretic data on interspecific hybrids of several species, with special reference to the classification of genetic characteristics.

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## MATERIALS AND METHODS

*Oryzias celebensis* were collected from Ujun Pandan of the South Celebes island, *O. javanicus* from Singapore and Jakarta by K. Hirata and T. Iwamatsu, *O. latipes* (Nara Prefect.), *O. luzonensis* from the Luzon island of the Phillipines. Specimens of *O. luzonensis* were provided by Dr. H. Uwa (Shinshu Univ.). These *Oryzias* as well as the Japanese *O. latipes* were maintained in glass quaria (60×35×30cm) equipped with a circulator to percolate water through a filter. The illumination (150 lux, 14 hr) and temperature (26–28°C) were controlled. Only a single pair of *O. luzonensis* was available in our laboratory.

Unfertilized eggs were obtained from the ovarian cavity of females shortly after ovulation and artificially inseminated by squeezing spermatozoa ( $0.1-1 \times 10^7$  spermatozoa/ml) out of a few testes from mature males into 0.5 ml of saline. Fertilization was indicated by cortical reactions followed by formation of the perivitelline space and by cleavage. After hatching, the fry were raised in glasswares (30 cm in diameter, water depth ca. 15 cm) under continuous illumination by a tungsten lamp (60 w). When they had grown to more than 10 mm in standard length, they were transferred to the glass aquaria as described above. Sex was determined by dissecting the gonads to ascertain the existence of oocytes or spermatozoa.

To allow observations of the skeleton, fishes were treated with 1–2 N NaOH (room temp.) for 6–12 hr and stained with 0.1% Alizarin S overnight. Some of the fertilized eggs were fixed with Bouin's fixative and examined for formation and syngamy of male and female pronuclei by staining with Hoechst dye.

For examination by scanning electron microscopy, samples were prefixed with 4% glutaraldehyde and post-fixed with 1% OsO<sub>4</sub> solution (Iwamatsu *et al.*, 1984).

In order to electrophoretically examine adult tissue specificity, eyes and skeletal muscle were obtained from the parental fishes of the hybrids. In some cases, samples stored at –20°C were used.

Eggs and adult tissues were homogenized for 3 min (1,500 rpm, 0–4°C) in 10 volumes of extraction buffer (1% SDS, 1% β-mercapto-ethanol, 20% glycerol and 10 mM Tris-HCl, pH 6.8), and proteins were extracted for 30 min at 0–4°C. Each homogenate was centrifuged at 10,000 g for 20 min (4°C), and the supernatant was subjected to electrophoretic analysis. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1 mm in thickness) was conducted on 15% polyacrylamide-slab gels, which were prepared and run according to the procedure of Laemmli (1970). Focused protein bands were stained with 0.05% Coomassie brilliant blue R 250. Molecular weight standards (Kit 200, Sigma Chemicals Co., St. Louis, MO. USA) were run concurrently for determination of molecular weights.

Electrophoretic patterns of lactate dehydrogenase (LDH) were examined according to the modified procedure (7.5% separation gel, 3% stacking gel) of Davis (1964).

**Table 1** Development of interspecific hybrids between *O. celebensis*, *O. javanicus*, *O. latipes*, *O. luzonensis* and *O. melastigma*

Parents Female × Male	No. of eggs used (females)	% of eggs undergoing		
		activation	morula	embryonic body
cel. × cel.	75 (5)	97.2	97.2	69.5 (52.8)*
lat. × lat.	168 (5)	91.2	91.2	91.2 (87.7)*
mel. × mel.	85 (5)	96.5	96.5	96.5 (94.2)*
cel. × jav.**	49 (2)	61.2	59.2	57.1 (57.1)
cel. × jav.	27 (1)	100	100	81.5 (0)
cel. × lat.	53 (5)	79.2	66.7	56.0 (46.0)
cel. × mel.	19 (1)	100	47.4	36.8 (36.8)
jav. × cel.	69 (3)	75.2	75.2	64.5 (44.0)*
jav. × lat.	49 (3)	90.0	90.0	83.7 (0)
jav. × mel.	57 (3)	100	100	29.8 (0)
lat. × cel.	970 (24)	94.6	94.6	72.2 (62.6)*
lat. × jav.**	114 (4)	79.8	79.8	77.8 (0)
lat. × mel.	682 (28)	97.3	95.7	84.2 (66.0)
lat. × luz.***	23 (2)	100	100	100 (90.0)*
mel. × cel.	194 (13)	93.5	93.5	92.5 (91.8)*
mel. × jav.	136 (6)	97.1	97.1	84.0 (0.7)*
mel. × lat.	160 (12)	81.3	74.2	72.1 (71.4)*

\*Fry were obtained: \*\*Collected from Singapore: \*\*\*Natural mating. Numbers in parentheses indicate the percentage of eggs with normal embryonic body. cel.: *O. celebensis*, jav.: *O. javanicus*, lat.: *O. latipes*, luz.: *O. luzonensis*, mel.: *O. melastigma*.

## RESULTS

### *Development of interspecific hybrids*

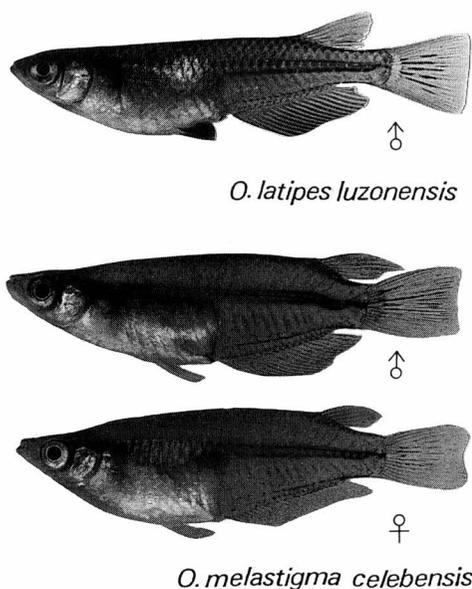
The size of mature eggs varied among different species as described in a previous report (Iwamatsu, 1986). Unfertilized eggs except for those of *O. luzonensis* were inseminated by a sperm suspension that was prepared by incising the testes of mature males in a saline solution. No eggs of *O. javanicus* (Singapore) could be obtained because this species was so nervous in the aquarium. The results of combinations of artificial insemination among 5 species are shown in Table 1. The fertilizability was comparatively high, except for the *O. celebensis* eggs inseminated with sperm of *O. javanicus* (Singapore). A low developmental capacity was also observed in hybrids resulting from *O. celebensis* eggs inseminated with heterogenous sperm. Most of the fertilized eggs, as shown in Table 1, developed to the stage of embryonic body formation, irrespective of the species providing sperm. *O. celebensis* eggs inseminated with sperm from *O. javanicus* (Jakarta), *O. latipes*, or *O. melastigma* exhibited a low rate of development while those inseminated with sperm of *O. javanicus* (Singapore). Eggs of *O. javanicus* (Jakarta) when stimulated with sperm from *O. celebensis*, *O.*

**Table 2** Morphology of interspecific hybrids of *Oryzias*

Hybrids	<i>O. melastigma latipes</i>	<i>O. latipes melastigma</i>	<i>O. latipes celebensis</i>	<i>O. melastigma celebensis</i>	<i>O. javanicus celebensis</i>	<i>O. latipes luzonensis</i>
No. of hybrids examined	5	3	8	15	2	12
TL (mm)	27.4±2.5	—	—	34.4±1.0	—	33.7±0.8
SL (mm)	22.8±2.1	—	—	27.9±1.0	—	27.4±0.7
HL*	25.1	—	—	22.2	—	24.0
Tail*	67.0	—	—	67.8	—	65.0
Hight*	20.0	—	—	28.4	—	22.0
A.F.base*	31.3	—	—	32.4	—	27.9
D.F.base*	8.1	—	—	9.2	—	8.7
(Number)						
Vertebrae	28.2±0.5 (27–30)	29.7±0.5 (29–31)	30.5±0.3 (29–32)	28.5±0.3 (28–29)	28.5 (28–29)	28.5±0.2 (28–29)
Rib	10.2±0.3 ( 9–11)	10.3±0.3 (10–11)	11.8±0.3 (11–13)	10.0±0 (10)	10.0 (11)	9.8±0.1 ( 9–10)
Pterygium	4.0±0 (4)	2.7±0.3 (2–3)	3.0±0.4 (1–4)	3.8±0.1 (3–4)	4.0 (4)	4.0±0 (4)
Fan-shaped neural spine	6.4±1.2 (6–8)	7.0±0.5 (6–8)	8.1±0.5 (7–9)	7.5±0.1 (7–8)	6.5 (6–7)	6.0±0 (6)
Branchial s.c.	14.0±3.3 (11–18)	—	13.1±0.7 (11–18)	12.2±0.3 (11–14)	10.0 ( 9–11)	16.2±0.3 (15–17)
Branchial f.	32.5±1.3 (31–43)	—	43.1±1.5 (39–51)	43.0±0.5 (42–46)	25.5 (23–28)	38.6±0.7 (36–43)
Branchiostegal r.	5.0±0.4 (4–6)	—	5.5±0.2 (5–6)	5.3±0.2 (5–6)	5.5 (5–6)	5.7±0.1 (5–6)
Pectral f.r.	10.0±0.5 (9–11)	10.0±0 (10)	10.2±0.1 ( 9–11)	10.7±0.3 ( 8–12)	11.0 (11)	10.8±0.1 (10–11)
Dorsal f.r.	6.0±0 (6)	6.0±0.5 (5–7)	7.8±0.1 (7–9)	7.3±0.1 (6–8)	7.5 (7–8)	6.1±0.1 (6–7)
Caudal f.r.	20.0±0.4 (19–20)	18.3±0.7 (17–20)	22.5±0.1 (21–15)	19.2±0.1 (18–20)	21.0 (21)	21.8±0 (21–23)
Anal f.r.	19.5±0.4 (18–20)	19.0±0.5 (18–20)	21.4±0.2 (20–23)	21.2±0.3 (20–22)	19.0 (19)	17.0±0.2 (16–18)
Ventral f.r.	5.8±0.2 (5–6)	6.0±0 (6)	6.3±0.1 (6–8)	6.1±0 (6–7)	6.5 (6–7)	6.1±0.1 (6–7)

\*Expressed as a percentage of standard length. f.r.; fin rays. The numbers in parentheses indicate the range of each value.

*latipes* or *O. melastigma*, developed to the morula stage. But, normal embryos between *O. javanicus* and *O. latipes* or *O. melastigma* could not be obtained in spite of the high fertilization rate. When *O. melastigma* eggs were inseminated with sperm from *O. celebensis*, *O. javanicus* (Jakarta) or *O. latipes*, they developed to the stage of embryonic body formation. Of these embryos, some hatched out and grew into swimming



**Fig. 1** Photographs of interspecific hybrids, *Oryzias latipes-luzonensis* and *O. melastigma-celebensis*.  $\times 2.7$

fry.

**Morphology and reproductive ability of interspecific hybrids**

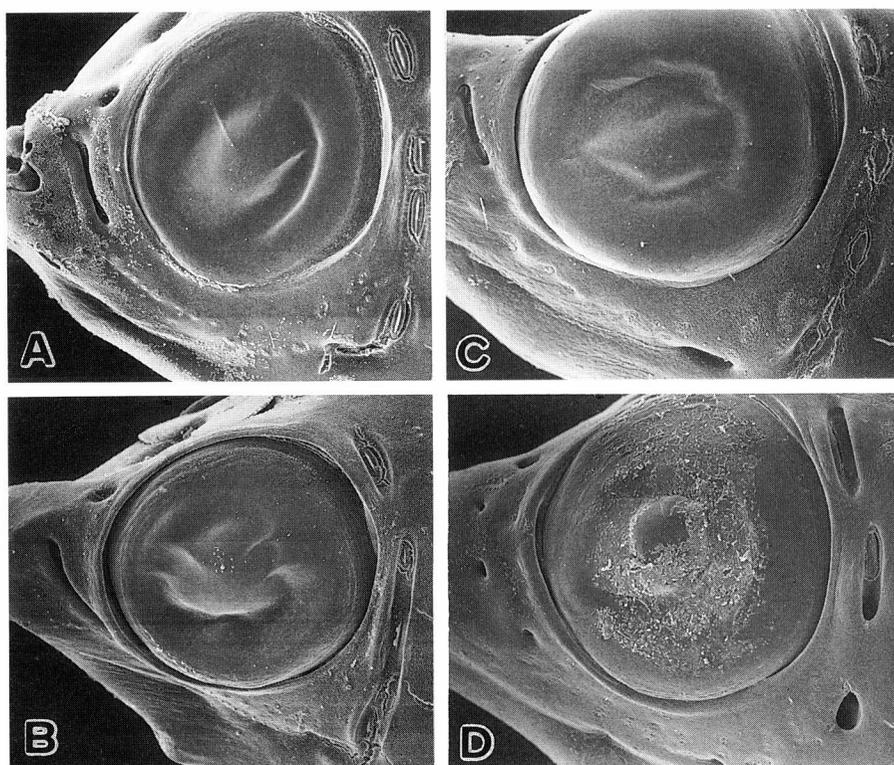
Of the interspecific hybrids obtained, the morphology of *O. melastigma-latipes*, *O. latipes-melastigma*, *O. latipes-celebensis*, *O. melastigma-celebensis*, *O. javanicus-celebensis*, and *O. latipes-luzonensis* was examined. The results are summarized in Table 2. The numbers of vertebrae with ribs, the fan-shaped neural spines, dorsal fin rays and anal fin rays tended to be smaller in the hybrids than in the parental species. Typical profiles of the body form of the interspecific hybrids *O. latipes-luzonensis* and *O. melastigma-celebensis* are shown in Fig. 1. The former had the fin type of the maternal

*O. latipes* type, while the latter had that of the paternal *O. celebensis*. Two types of large pit organs were observed, an open type and a sunken (or tunnel) type (Fig. 2). The open type was observed in *O. javanicus* (Jakarta) and *O. luzonensis*. Although *O. melastigma* had the sunken type of large pit organ, the hybrids of *O. latipes* had the open type, and the hybrids with *O. celebensis* retained the sunken type. The individuals with the waved vertebrae were frequently observed in *O. javanicus-melastigma*, as reported in a previous paper (Iwamatsu *et al.*, 1984).

The reproductive ability of *O. latipes-celebensis* was ascertained and reported previously (Iwamatsu *et al.*, 1984). Reproductive capability was observed in *O. melastigma-latipes*, but not in any of the other hybrids studied.

**Protein patterns in electrophoresis**

When extracts of the muscle and the eye tissues were electrophoresed using the buffer system of Davis (1964), LDH isozymes were dissociated into 5 bands. The electrophoretic patterns of LDH isozymes were uniform within each species. The identified isozyme bands from the eye tissue reveal a striking similarity between the pattern for *O. javanicus* (Singapore) and that for *O. melastigma* (Fig. 3). The pattern of the eye LDH in *O. celebensis* was in conspicuous contrast to that in *O. luzonensis* (Fig. 3). The pattern of eye LDH differed between geographically separate groups of *O. javanicus* (Jakarta and Singapore), although the muscle LDH pattern was the same. The eye LDH pattern of *O. javanicus* (Singapore) was the same as that of *O.*

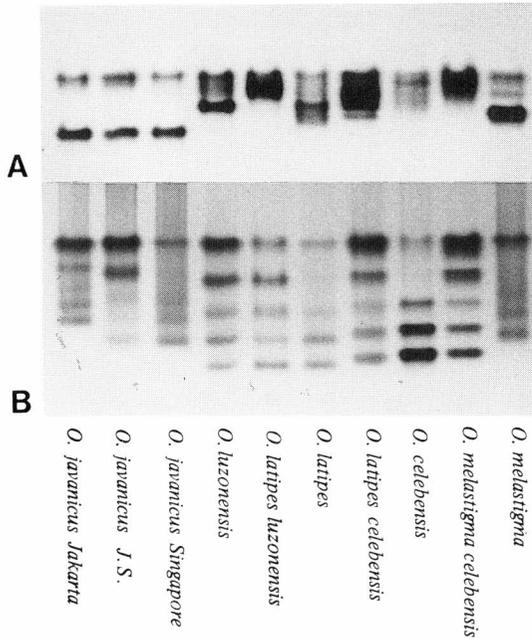


**Fig. 2** Large pit organs of *Oryzias javanicus*, *O. luzonensis*, and the interspecific hybrids, *O. melastigma-celebensis* and *O. melastigma-latipes*. Large pit organs in *O. luzonensis* (A), *O. javanicus* (B) and *O. melastigma-latipes* (C) are of the open type, and that in *O. melastigma-celebensis* (D) is of the sunken type. x 35

*melastigma* but the patterns of their muscle LDH were different.

In the hybrids, *O. latipes-celebensis*, and *O. melastigma-celebensis*, the eye LDH pattern was the same as that of *O. luzonensis*, in spite of the different patterns in their parental species. In the two hybrids of *O. latipes-luzonensis* and *O. melastigma-celebensis* the muscle LDH had the same electrophoretic pattern. Band 2 appeared in all hybrids, although it was lacking in the eye LDH pattern of *O. celebensis* and *O. latipes*. It was very interesting that the eye LDH of hybrids between *O. javanicus* from Jakarta and Singapore formed patterns different from those of the parents. Judging from these electrophoretic patterns, hybrids seem to express the LDH isozyme patterns from the parents. The electrophoretic data on the proteins in the hybrids may also indicate a close relationship to the geographical distribution of the parental species.

Muscle extracts of each species were subjected to SDS-PAGE. The electrophoretic pattern of the proteins in each species is seen in Fig. 4. As for the LDH patterns, the SDS-PAGE pattern of low molecular weight muscle proteins (parvalbumin) in *O.*



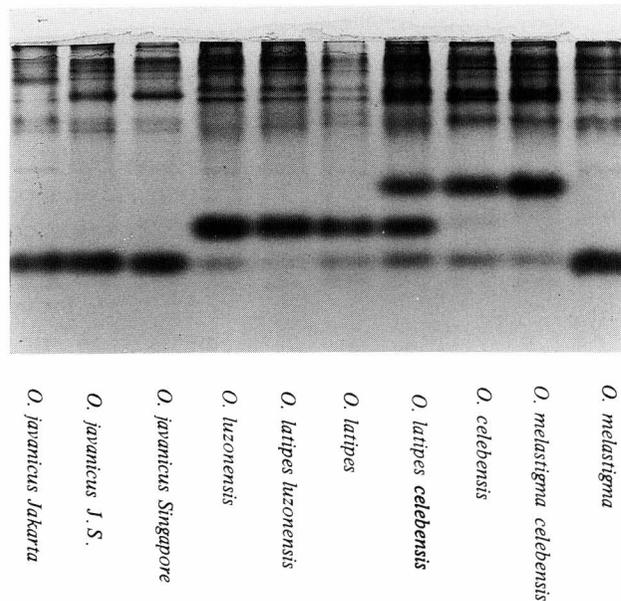
**Fig. 3** Electrophoretic patterns of LDH obtained from five *Oryzias* species and their interspecific hybrids.  
 A: Muscle homogenates, B: Eye homogenates.  
 There are interspecific variations among the five species.

*javanicus* was very similar to that of *O. melastigma*. There was also similarity between *O. latipes* and *O. luzonensis*. The patterns of high molecular weight proteins differed in all species. The pattern of *O. javanicus* collected from Jakarta and Singapore, respectively. Each hybrid formed patterns intermediate to its two parental species.

### DISCUSSION

The teleostean egg usually has one micropyle, through which the spermatozoon reaches the egg surface. In order for the spermatozoon to go through the micropyle, its head diameter should be smaller than the inner diameter of the micropylar canal. According to the previous note (Iwamatsu, 1981), the diameter of the sperm head was not very different among

different fishes including *Oryzias* and was smaller than the inner diameter of the micropylar canal of *O. latipes*, although the inner diameter of the micropylar canal has not been measured. The present experiments in which 17 artificial hybrid combinations were carried out among five species of *Oryzias* revealed generally high percentages of interspecific fertilization, i.e. non-specific fertilization. This indicates that the guidance factor for spermatozoa to enter the micropylar canal and the sperm-binding sites on the egg surface may be non-specific. This observation is also consistent with the previous note (Iwamatsu, 1981). The fact that most eggs exhibiting cortical reaction following activation by heterogenous sperm undergo cleavage infers penetration of the sperm centrioles, which was involved in formation of the mitotic apparatus. This shows a good agreement with the experimental results in which sperm fragments of the same species (Iwamatsu and Ohta, 1972) or different animals such as the loach, the frog and the rat (Iwamatsu and Ohta, 1974), or sperm centrioles of the same species (Ohta and Iwamatsu, 1981) were directly microinjected. Thus, these results indicate that the processes of fertilization and early development are non-species specific.



**Fig. 4** Electrophoretic patterns of muscle proteins of five *Oryzias* species and their interspecific hybrids. A potential of 100V was applied to a 15% acrylamide gel for 6 hr at room temperature.

The meristic characteristics of *Oncorhynchus* hybrids, such as the number of dorsal fin rays, anal fin rays and gill-rakers are intermediate between those of the parent species (Inaba, 1953). Although these characteristics in *Oryzias* hybrids also tend to be intermediate, the dorsal fin rays and vertebrae are likely to be either meristically intermediate or to have counts similar to the parental species with the higher count. The number of anal fin rays, on the other hand, may match the type of parental species with the lower count. In some cases, the morphological characteristics depend on the combination of parental species hybridized. It is of great interest whether or not these phenotypes in interspecific hybrids reveal a direction of evolution or species differentiation in each morphological characteristic. Notably, the secondary sexual dimorphism in hybrids is manifested in conspicuous characteristics such as elongated fin rays or notches in the dorsal and anal fins and the papillar process in the anal fin, as seen in *Oryzias* males. These sexual characteristics are dependent on androgens (Egami, 1975). The elongated fin rays and the notches are also observed in hormone-treated fish, but the papillar processes in the anal fin are not detected in the hybrids between *O. latipes* and *O. celebensis* (Iwamatsu *et al.*, 1984). The papillar process may be genetically recessive, so that it is not manifested even following the administration of testosterone at concentrations high enough to manifest male sex

characteristics in *O. latipes*. These processes are distributed on both the nodes and internodes of the anal fin ray in *O. luzonensis*, while in *O. latipes* they are observed only in the internodes. Their hybrids show the *O. luzonensis* type.

There are two types of large pit organs present around the eye, i. e. the open type (*O. celebensis*, *O. latipes*, *O. luzonensis*) and the sunken type (*O. melastigma*, *O. javanicus*). In the hybrids, only *O. melastigma-celebensis* had the sunken type of large pit organ. However, hybrids between *O. latipes* and *O. melastigma* had the open type. Therefore, the phenotype of the large pit organ does not seem to be controlled by a single gene. Reciprocal crosses between these species are required for the analysis of gene expression controlling the large pit organ.

In a previous study (Iwamatsu *et al.*, 1986), it was reported that *O. javanicus* from Singapore were not meristically distinct from those from Jakarta and West Kalimantan, except for their standard length. The present electrophoretic data showed that the pattern of eye LDH in *O. javanicus* from Singapore was distinct from that of fish from Jakarta, although the electrophoretic patterns of the muscle LDH were the same. Variations in electrophoretic patterns within a species, *O. latipes*, have been reported for many different isozymes (Sakaizumi *et al.*, 1983). The data for *O. javanicus* may indicate such an intraspecific variation in the electrophoretic pattern of LDH. However, the length and shape of the body of *O. javanicus* from Singapore are very similar to those of *O. melastigma* as well, and they have similar patterns of eye LDH but not the muscle LDH (Fig. 3). Furthermore, *O. javanicus* from Singapore are able to produce hybrids with *O. melastigma* but those from Jakarta do not or it is rare. The karyotypes of *O. javanicus* from Jakarta (Iwamatsu and Uwa, unpublished data) and from Singapore (Uwa and Iwata, 1981) differ. These facts cast doubt as to whether or not *O. javanicus* from Jakarta and Singapore should be treated as the same species.

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