Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ. 7: 157–179 (1985)

Two Kinds of Black-eyed Variants in *Hyla arborea japonica*

By

Midori NISHIOKA and Hiroaki UEDA

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

(With 10 Text-figures and 1 Plate)

INTRODUCTION

Melanoid variants due to a single recessive gene have been reported in the axolotl (HUMPHREY and BAGNARA, 1967; BENJAMIN, 1970; DUNSON, 1974), *Pleurodeles waltl* (LACROIX and CAPURON, 1970) and *Rana pipiens* (RICHARDS, TARTOF and NACE, 1969). NISHIOKA (1977) obtained three stocks of variants similar to the melanoids in *Rana nigromaculata* by exposing gametes to X-rays or neutrons. These variants were named black-eyed and confirmed to be due to a single recessive gene by the author. Black-eyed mutants of *Rhacophorus schlegelii* were reported by the present authors (1985b).

In 1971, a kind of black-eyed variants of *Hyla arborea japonica* was discovered in gynogenetic diploids produced from eggs of a female. In 1980, another kind of black-eyed variants of the same species was found in a place located about 30 km from the place where the above variants were obtained. These two kinds of black-eyed variants fairly differed from each other in external characters as well as in ultramicroscopic structures of dermal chromatophores. It was evident that these variants are due to a single recessive gene, as those of the melanoids and black-eyed variants stated above.

In this paper, the genetics and morphological characters of the two kinds of black-eyed variants of *Hyla arborea japonica* will be reported.

MATERIALS AND METHODS

One of two kinds of black-eyed variants in *Hyla arborea japonica* GÜNTHER was discovered in gynogenetic diploids which had been produced by Mr. K. NISHIGUCHI in 1971 from a female collected from Hesaka (Hs), Hiroshima City. This female was also mated with a normal male and produced 97 wild-type offspring. Four black-eyed variants were obtained from one pair of brother and sister matings conducted among these offspring. The dermal chromatophores of the black-eyed variants were examined under an electron microscope.

The other kind of black-eyed variants in the same species was found at a green house of TAKAGI farm in Shimohaji, Yachiyo-cho (Yc), Takata-gun, Hiroshima Prefecture; Yachiyo-cho is about 30 km north of Hesaka. There were three

black-eyed variants, one of which was discovered in August of 1980 as a juvenile, while the other two were caught in May of 1981 as mature tree-frogs. Of these three variants, the one caught in 1980 and one of the two caught in 1981 were females, and the remainder was a male. These color variants were mated with normal tree-frogs collected from the field to obtain heterozygous offspring. The hereditary mode of the black-eyed variation was examined by matings between female and male offspring and by the method of diploid gynogenesis applied to the eggs of a female variant.

The method of diploid gynogenesis is as follows. Sperm suspension of a male *Hyla arborea japonica* is exposed for 2 minutes to UV emitted from a mercury lamp, GUL-5·J Type of Toshiba Co. make, 2537 Å in the main wave length, at a distance of 20 cm (2400 erg/cm^2/sec). By this exposure the nuclei become incompetent, although the spermatozoa are motile and enter into eggs. After the inseminated eggs are left for 20 minutes at room temperature (20~25°C), they are exposed to low temperature (1~2°C) for about one hour. By this treatment, the nucleus of the second polar body is retained in the egg and fused with the egg pronucleus. Then the egg develops as a gynogenetic diploid.

Matings were always made by the method of artificial fertilization. Ovulation was accelerated by injection of pituitaries of *Rana nigromaculata* or of a mixture of gonadotropin and pituitaries of *Rana catesbeiana*.

The two kinds of black-eyed variants and the normal tree-frogs were adapted for 24 hours to a light environment under a fluorescent lamp. Pieces of skin, about 5 mm square in size, were cut off from the dorsal and ventral surfaces of each of these tree-frogs, cut into minute pieces in cold 0.1 M phosphate buffer (pH 7.4) containing 4% glutaraldehyde and then kept in the same solution for 2 hours after renewal of the fluid. The minute pieces were washed and then postfixed in 0.1 M phosphate buffer (pH 7.4) containing 2% osmic acid for 2 hours. These fixing procedures were performed at 2~4°C. The fixed pieces were dehydrated in an ethanol series and embedded in Epon 812. Sections were made on a Porter-Blum MT-1 ultramicrotome at the thickness of silver or silver-gold and double stained with saturated uranyl acetate and alkaline lead citrate. Observation was made under a Hitachi Hs-8 electron microscope.

The developmental stages reported in this paper follow those of *Rana pipiens* established by SHUMWAY (1940) and TAYLOR and KOLLROS (1946) for convenience sake.

OBSERVATION

I. Normal tree-frogs

The dorsal surface of normal tree-frogs is green or yellowish green in most cases and becomes gray, grayish brown, or dark brown in accordance with changes of the environment (Plate I, 1, 2). When tree-frogs are adapted for 24 hours to a light environment under a fluorescent lamp, their dorsal surfaces

usually become bright yellowish-green. When the dermal chromatophores of the dorsal skin are observed by lighting with transmitted light under an optic microscope, both dull-purple iridophores and brown melanophores are widely expanded. The xanthophores are recognized as expanded yellow flecks above the iridophores, although the outlines of xanthophores are not clear-cut. When observed by lighting with reflected light, the flat iridophores covered with yellow xanthophores appear green with metallic luster, while the parts which are not covered with xanthophores appear sky blue with metallic luster.

The dermal chromatophores of six normal tree-frogs adapted to a light environment were observed under an electron microscope (Fig. 1). The three kinds of dermal chromatophores, upper xanthophores, middle iridophores and under melanophores, form the chromatophore unit coined by BAGNARA, TAYLOR and HADLEY (1968).

a. Xanthophore

Xanthophores are convex lens-shaped cells which are expanded horizontally and have short dendritic processes. They are isolated from one another and are scarcely contacted or overlapped with their neighbors. When the median sections of 20 xanthophores were measured, they were 15.6～30.5 μ, 20.17 μ on the average, in width and 4.5～8.5 μ, 6.0 μ on the average, in thickness, respectively. The convex undersurface of each xanthophore is usually suited close to the concave surface of an underlaid cup-like iridophore. A single xanthophore is rarely extended over two or more iridophores.

The cytoplasm of xanthophores is filled with two kinds of granules, pterinosomes with concentric lamellar structure and carotenoid vesicles like oil droplets. The carotenoid vesicles are nearly spherical. Fifty of them were 0.15～0.35 μ, 0.26 μ on the average, in diameter. However, a few large carotenoid vesicles, being 0.45～0.55 μ in diameter, were found here and there. Pterinosomes are ellipsoids in shape. Fifty of them were 0.75～1.06 μ, 0.93 μ on the average, in major axis and 0.50～0.85 μ, 0.68 μ on the average, in minor axis.

In expanded xanthophores, pterinosomes are mingled with carotenoid vesicles and evenly distributed in the cytoplasm, although the most part of the cytoplasm is occupied by carotenoid vesicles. In contracted xanthophores, when the dorsal surfaces of tree-frogs are grayish brown or dark brown, the two kinds of granules are separately distributed, that is, carotenoid vesicles are gathered in the center of the cytoplasm and surrounded by pterinosomes (Fig. 2a).

b. Iridophore

Cup-shaped iridophores are closely attached to the convex undersurfaces of xanthophores and arranged in a single layer together with the xanthophores. These two kinds of chromatophores have many short processes at the contact area. When the median sections of 20 iridophores having the nucleus in the center of the cytoplasm were measured, they were 17.5～24.0 μ, 19.96 μ on the average, in width and 4.5～7.2 μ, 5.84 μ on the average, in thickness. Adjacent

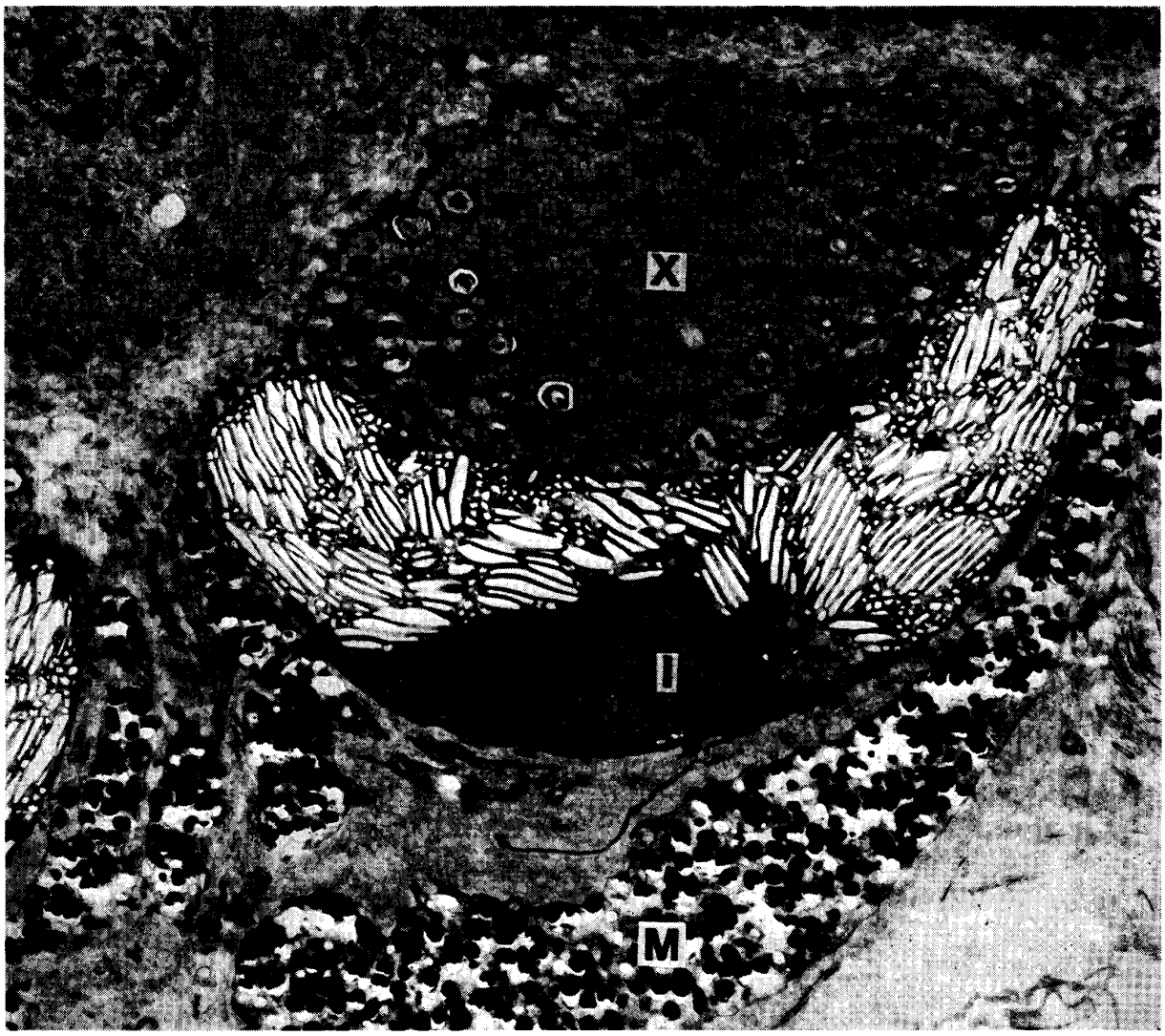


Fig. 1. Electron microphotograph of dermal chromatophores in the dorsal skin of a wild-type *Hyla arborea japonica*. ×4600

X, xanthophore I, iridophore M, melanophore

iridophores are usually separated from each other at an interval of about 1 μ on the average, 3.3 μ at the widest. A narrow area surrounding the nucleus contains GOLGI complex, endoplasmic reticula, a centrosome and mitochondria, while the remaining large portion of the cell is filled with reflecting platelets. In electron microphotographs, only traces of reflecting platelets are observable, as the crystalline structures of reflecting platelets have fallen out. About 10 reflecting platelets are arranged in parallel to one another and form a group. The groups of reflecting platelets are mostly arranged in almost parallel to the cell surface. When 42 traces of reflecting platelets of four groups arranged in parallel to the cell wall were measured, it was found that they were 1.0~2.5 μ, 1.45 μ on the average, in length and 0.05~0.12 μ, 0.09 μ on the average, in thickness. The spaces between the limiting membranes of reflecting platelets arranged in a group were 0.03~0.06 μ wide, while the intervals between the holes were 0.09

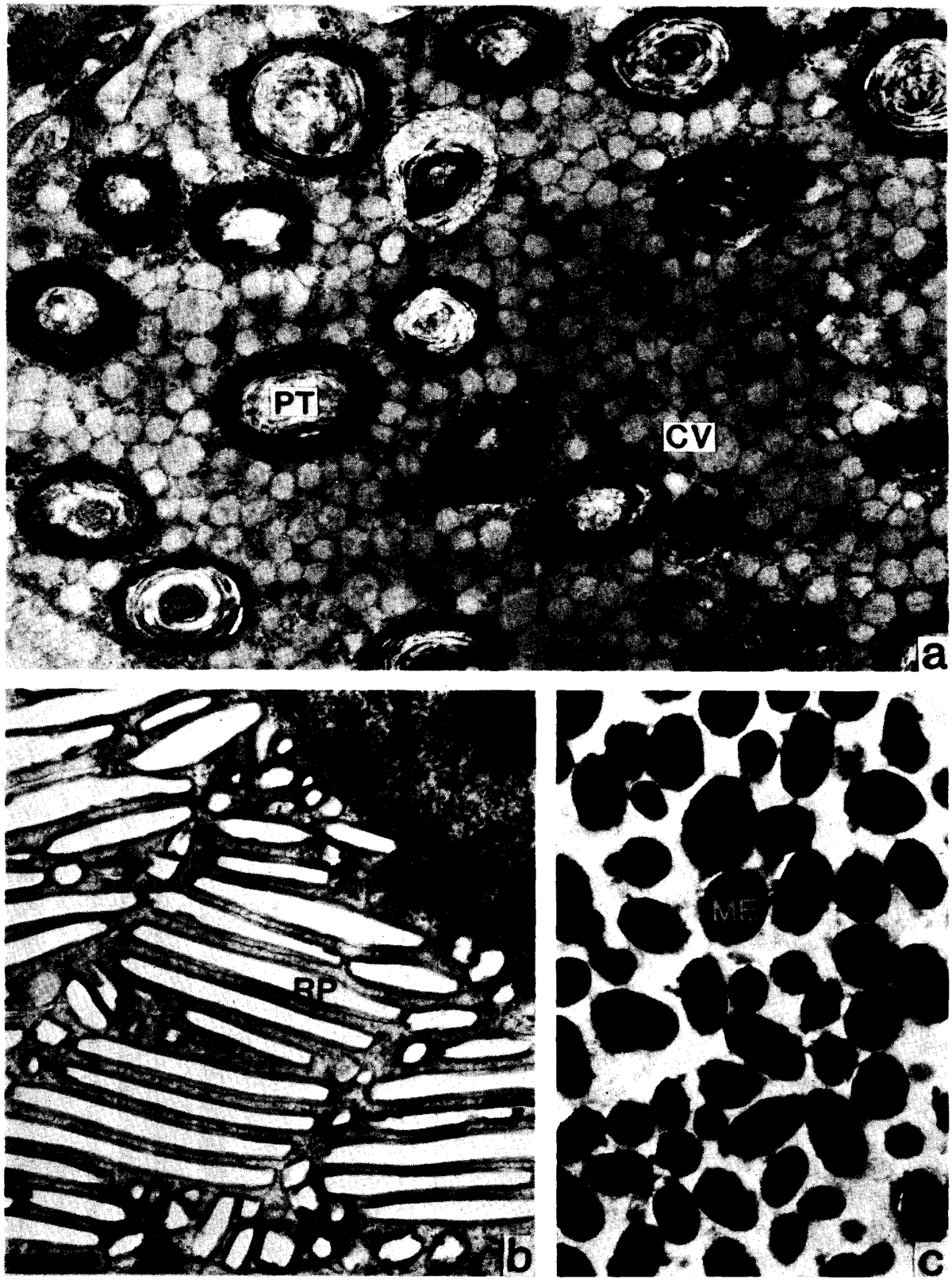


Fig. 2. Electron microphotographs of three kinds of dermal chromatophores in the dorsal skin of a wild-type *Hyla arborea japonica*. ×22000

a. Xanthophore. b. Iridophore. c. Melanophore.
PT, pterinosomes. CV, carotenoid vesicles RP, reflecting platelets ME, melanosomes

$\sim$0.11 μ wide (Fig. 2b).

There are some iridophores which include a few carotenoid vesicles in the cytoplasm.

c. Melanophore

Melanophores with many long dendritic processes constitute a thick layer under the iridophores. The processes of adjacent melanophores are complicatedly overlapped with one another. Each melanophore and its processes are filled with spherical or ellipsoidal melanosomes. When 20 ellipsoidal melanosomes were measured, they were 0.35$\sim$0.77 μ, 0.68 μ on the average, in major axis and 0.23$\sim$0.55 μ, 0.35 μ on the average, in minor axis. When 10 nearly spherical melanosomes were measured, they were 0.35$\sim$0.70 μ, 0.49 μ on the average, in diameter (Fig. 2c).

II. Black-eyed variants of the Hs stock

1. Genetics

In the breeding season of 1971, 42 female tree-frogs collected from Hesaka (Hs), Hiroshima City were injected with frog pituitaries in order to accelerate ovulation. Although 23 females laid eggs, 13 of them were used in the experiments of diploid gynogenesis and in matings with normal males, as their eggs were more numerous and appeared to be more normal than the others.

The results of these experiments showed that 26 black-eyed tree-frogs were produced together with 32 normal ones from a female, W. 71♀, No. 1, by diploid gynogenesis. On the other hand, the offspring of the same female mated with the normal male (W. 71♂, No. 1) were all of the wild-type. In 1972, as the males and females of these offspring were sexually matured, they were mated to produce the next-generation offspring. It was found that one of the matings produced four black-eyed variants and 21 normal tree-frogs.

These findings evidently indicate that the black-eyed variants of the Hs stock are mutants whose character is controlled by a single recessive gene (b^h) and that the female, W. 71♀, No. 1, used in the experiment of diploid gynogenesis conducted in 1971 as well as the male and female used in a mating experiment which produced four black-eyed variants were heterozygous (Bb^h) for the black-eyed gene.

2. Dermal chromatophores

The dorsal surface of the Hs black-eyed mutants was always dark grayish-brown. The ventral body wall was semitransparent so that several visceral organs like the liver, heart, stomach, small and large intestines, fat bodies and ovaries were observable to some extent. The iris had no metallic luster and appeared as black as the pupil (Plate I, 3). The name of the black-eyed variant was given from this distinguishing feature. When the dermal chromatophores

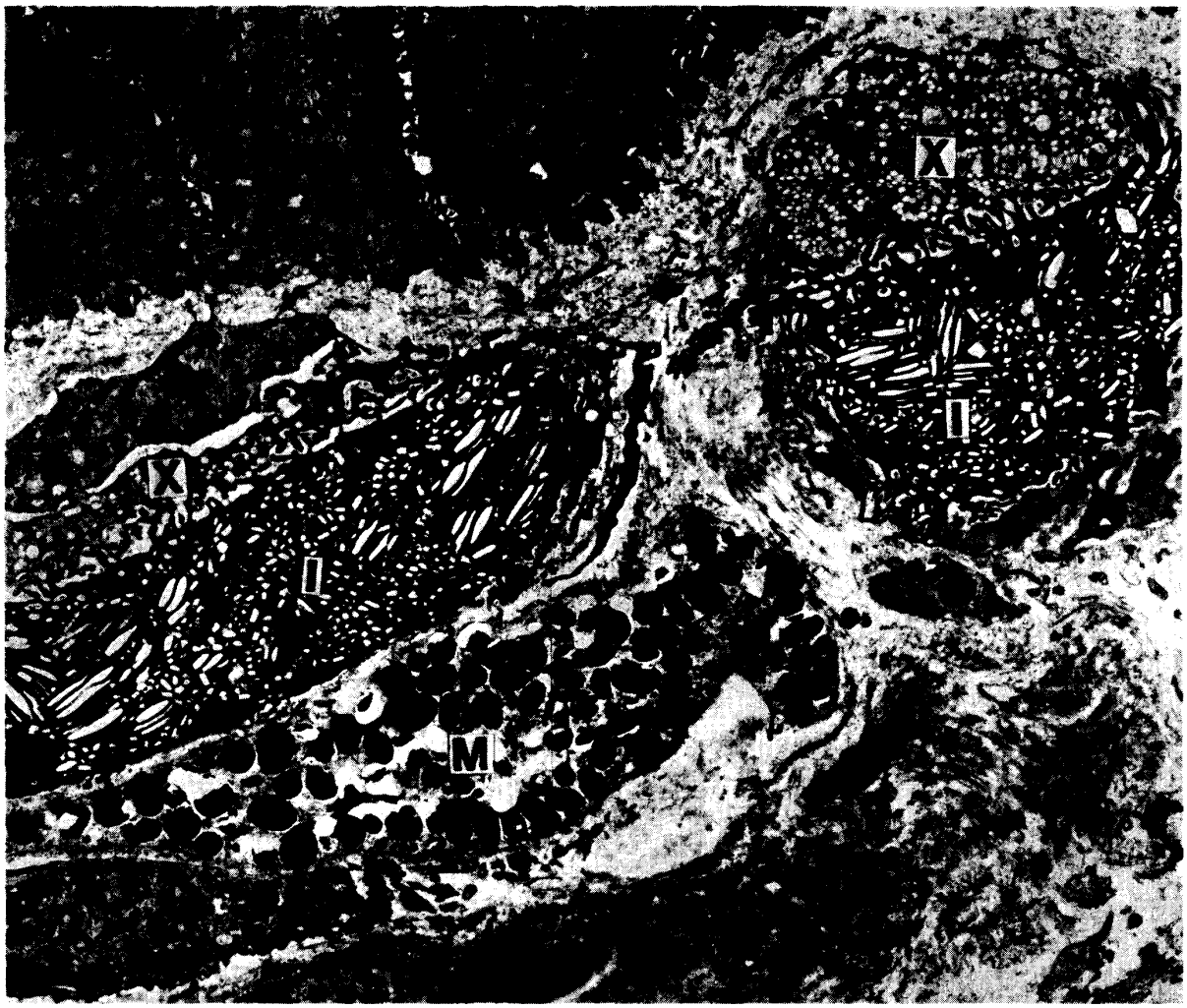


Fig. 3. Electron microphotograph of dermal chromatophores in the dorsal skin of a black-eyed mutant belonging to the Hs stock. ×4600

X, xanthophore I, iridophore M, melanophore

in the dorsal skin of two black-eyed mutants were examined, it was found that they were smaller and fewer than those of the wild-type tree-frogs, although there were chromatophore units consisting of three kinds of chromatophores. A considerably wide space was always found between adjacent chromatophore units. Each kind of chromatophores remarkably differed from that of the wild-type tree-frogs in inner structure, too (Fig. 3).

a. Xanthophore

The median sections of 10 xanthophores were 11.6～14.0 μ, 12.32 μ on the average, in width and 1.7～5.1 μ, 2.74 μ on the average, in thickness. As compared with the xanthophores of the normal tree-frogs, those of the black-eyed mutants were about two-thirds in width and about half in thickness. Thus, the xanthophores were always separated from one another at wide intervals.

The pigment granules contained in the xanthophores were distinctly inferior in development as compared with those of the wild-type tree-frogs. Xanthophores

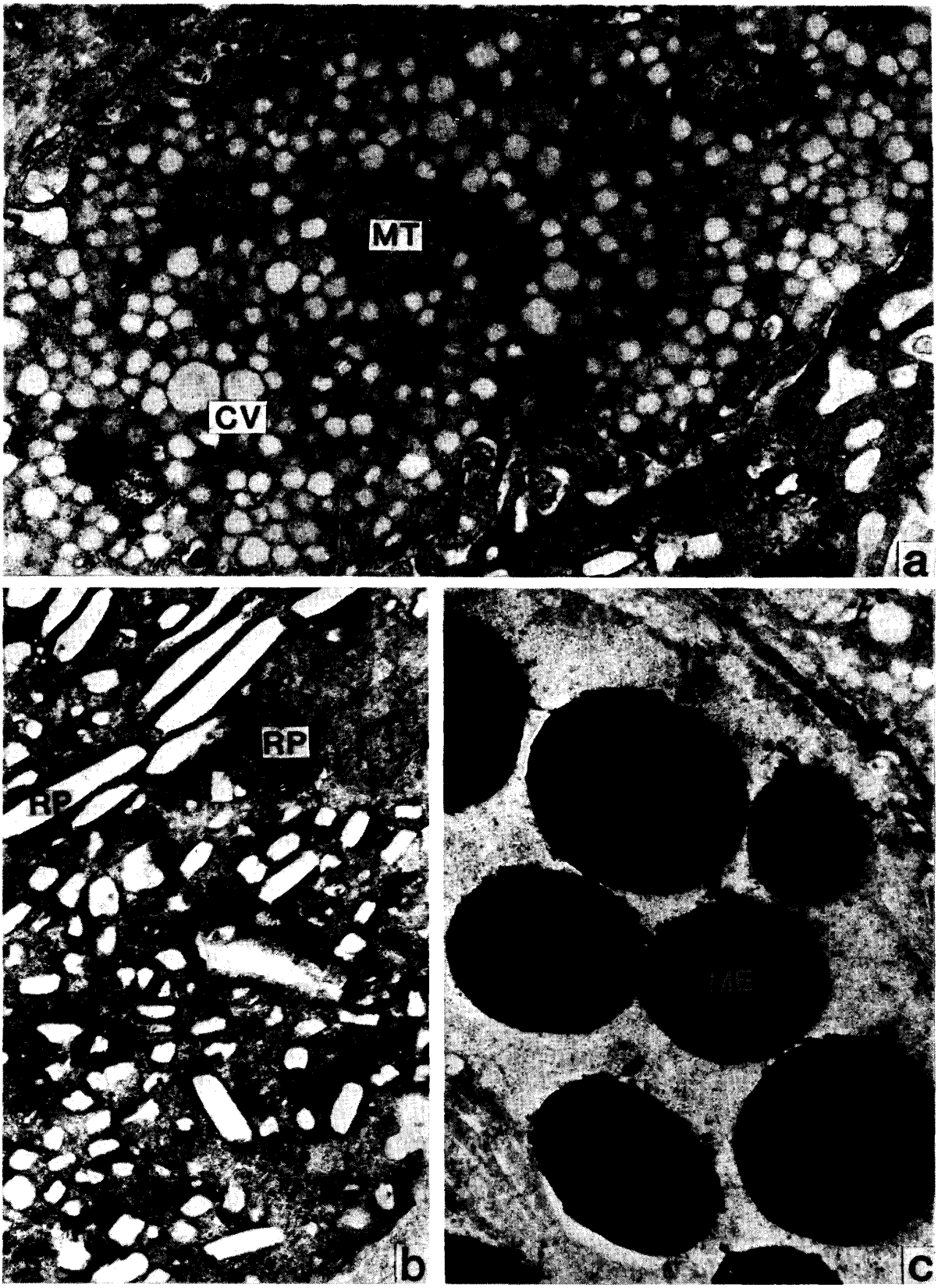


Fig. 4. Electron microphotographs of three kinds of dermal chromatophores in the dorsal skin of a black-eyed mutant belonging to the Hs stock. ×22000

a. Xanthophore. b. Iridophore. c. Melanophore.

CV, carotenoid vesicles MT, mitochondria RP, reflecting platelets ME, melanosomes

were almost filled with carotenoid vesicles and scarcely contained pterinosomes. The carotenoid vesicles were considerably smaller than those of the normal tree-frogs. When 25 of them were measured, they were 0.14～0.32 μ, 0.19 μ on the average, in diameter (Fig. 4a).

b. Iridophore

As found in the normal tree-frogs, the iridophores were in close contact with the overlaid xanthophores. These two kinds of chromatophores had many short processes which were intertwined each other at the contact area.

The iridophores were remarkably smaller than those of the wild-type tree-frogs. The median sections of 10 iridophores were 9.7～16.3 μ, 12.61 μ on the average, in width and 2.9～7.8 μ, 4.60 μ on the average, in thickness. As compared with those of the normal tree-frogs, they were about half in width and two-thirds in thickness. The iridophores were isolated from one another at considerably wide intervals, as found in xanthophores.

The reflecting platelets contained in the iridophores were very abnormal in size and shape. The cytoplasm of each iridophore was mostly filled with small granular reflecting platelets, although the latter were not so crowded as found in the wild-type tree-frogs. Among these small reflecting platelets, there were a small number of nearly normal-shaped ones which were 0.3～1.75 μ, 0.50 μ on the average, in length and 0.03～0.11 μ, 0.05 μ on the average, in thickness. These reflecting platelets were remarkably smaller in length and width than those of the normal tree-frogs (Fig. 4b).

c. Melanophore

Although measurement of melanophores was difficult, as they radially extended their long and slender processes, they seemed to be fewer and smaller than those of the normal tree-frogs. They were somewhat isolated from one another, as the xanthophores and iridophores of this mutant were.

The melanosomes contained in melanophores were mostly spheroids and distinctly large in contrast to those of the wild-type tree-frogs. When 25 melanosomes were measured, they were 0.7～1.4 μ, 1.12 μ on the average, in major axis and 0.6～1.3 μ, 0.94 μ on the average, in minor axis. They were nearly 14 times larger in volume than those of the normal tree-frogs (Fig. 4c).

3. Iridophores in the ventral body wall

In the ventral body wall of the normal tree-frogs, there were one or two layers of iridophores underneath the basal lamina of the epidermis. Besides, there were thick layers of iridophores in loose connective tissues under the layers of

---

Fig. 5. Electron microphotographs of dermal chromatophores in the ventral skin of a wild-type *Hyla arborea japonica*.

a. Cross-section of the ventral skin. ×6000

b. A part of an iridophore. ×22000

EP, epidermal cell I, iridophore RP, reflecting platelets

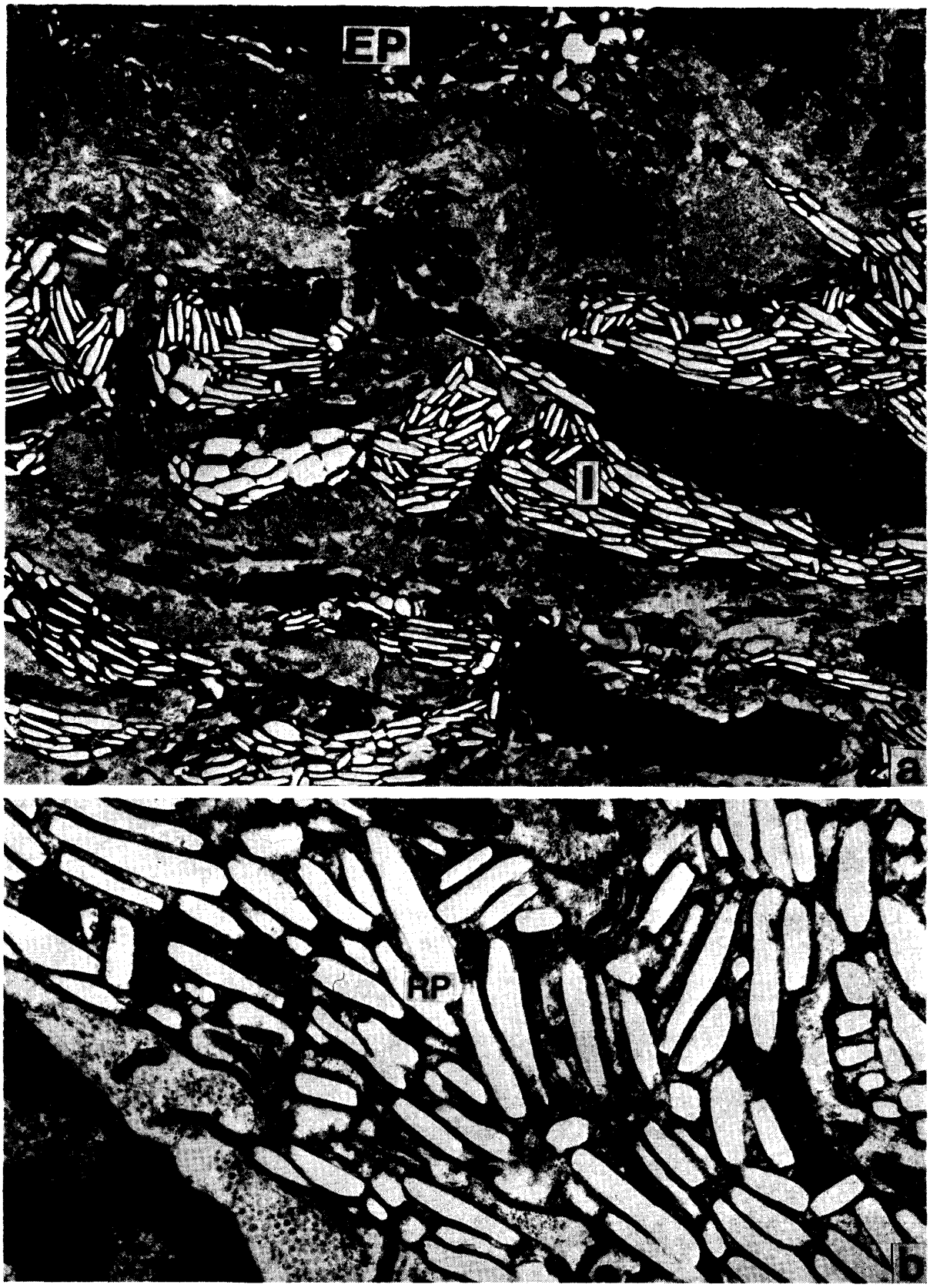


Fig. 5

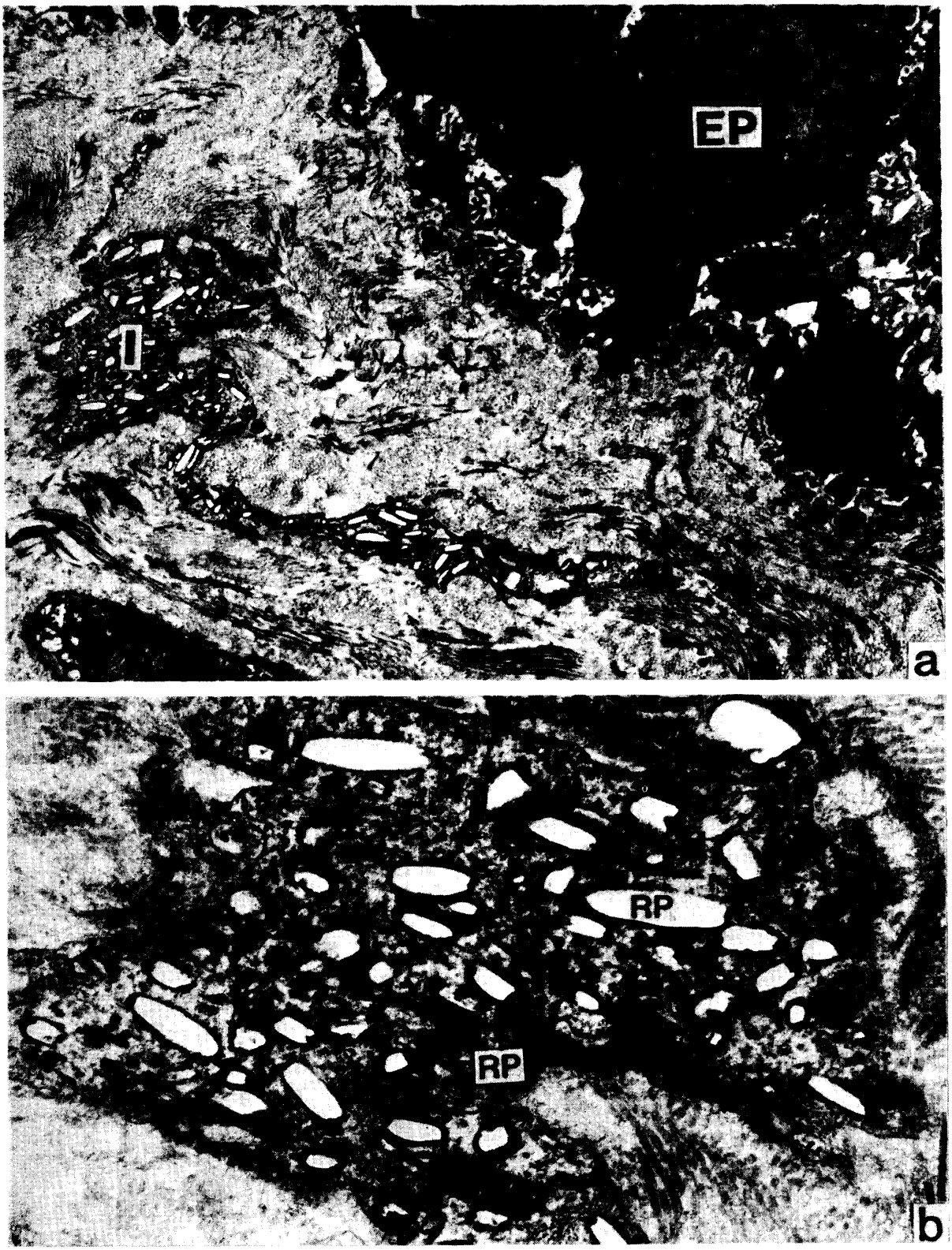


Fig. 6. Electron microphotographs of dermal chromatophores in the ventral skin of a black-eyed mutant belonging to the Hs stock.

a. Cross-section of the ventral skin. ×6000

b. A part of an abnormal iridophore. ×22000

EP, epidermal cell I, iridophore RP, reflecting platelets

collagenous fibers. The iridophores situated in these two different sites of the ventral skin were the same in inner structure as the dermal iridophores in the dorsal skin (Fig. 5).

The iridophores in the ventral skin of the black-eyed mutants remarkably differed from those of the wild-type tree-frogs in inner structure. The reflecting platelets contained in these iridophores were very similar to those of the dorsal skin in number, size and shape. They were distinctly fewer and smaller than those of the wild-type tree-frogs. Most of them were granular in shape (Fig. 6).

III. Black-eyed variants of the Yc stock

1. Genetics

A black-eyed variant at the juvenile stage discovered in August of 1980 at a green house in Yachiyo-cho (Yc) was given before long to our laboratory. As this variant became a mature female in the breeding season of 1981, a mating experiment was performed between this female (Yc. Blk. 80♀, No. 1) and a normal male (W. 80♂, No. 1) which had been reared in our laboratory (Table 1). It was found that 124 (94.7%) of 131 eggs cleaved normally, 118 (90.1%) hatched normally, 116 (88.5%) became feeding tadpoles at stage III and 72 (55.0%) attained completion of metamorphosis. All these juveniles were of the wild-type On the other hand, 94 eggs of the black-eyed female (Yc. Blk. 80♀, No. 1) were

TABLE 1

Offspring of a female black-eyed mutant of the Yc stock in *Hyla arborea japonica*

Year	Parents		No. of eggs	No. of normal cleavages	No. of normally hatched tadpoles	No. of feeding tadpoles (st. III)		
	Female	Male				Total	Wild	Mutant
1981	Yc. Blk. 80, No. 1	W. 81, No. 1	131	124 (94.7%)	118 (90.1%)	116 (88.5%)	116	0
		GD	94	80 (85.1%)	42 (44.7%)	39 (41.5%)	0	39
1982	Yc. Het. 81, No. 1	Yc. Het. 81, No. 1	273	270 (98.9%)	256 (93.8%)	248 (90.8%)	191	57
	Yc. Het. 81, No. 2	Yc. Het. 81, No. 2	417	403 (96.6%)	392 (94.0%)	370 (88.7%)	281	89
	Yc. Het. 81, No. 3	Yc. Het. 81, No. 3	426	415 (97.4%)	403 (94.6%)	399 (93.7%)	302	97
	Total		1116	1088 (97.5%)	1051 (94.2%)	1017 (91.1%)	774	243
1984	Yc. Blk. 82, No. 1	Yc. Blk. 82, No. 1	200	169 (84.5%)	152 (76.0%)	150 (75.0%)	0	150
	Yc. Blk. 82, No. 2	Yc. Blk. 82, No. 2	200	123 (61.5%)	92 (46.0%)	81 (40.5%)	0	81
	Total		400	292 (73.0%)	244 (61.0%)	231 (57.8%)	0	231

Blk, black-eyed mutant W, field-caught GD, diploid gynogenesis Het, heterozygote

refrigerated after pseudofertilization with UV-irradiated sperm of a male *Hyla arborea japonica*. The results showed that 80 (85.1%) eggs cleaved normally, 42 (44.7%) hatched normally and 39 (41.5%) became feeding tadpoles at stage III. All these tadpoles were black-eyed variants. Eleven (11.7%) of them attained completion of metamorphosis.

As the tree-frogs produced in 1981 from a mating, Yc. Blk. 80♀, No. 1 × W. 80♂, No. 1, were sexually matured, brother and sister matings were made in the breeding season of 1982 between three females (Yc. Het. 81♀, Nos. 1～3) and three males (Yc. Het. 81♂, Nos. 1～3). It was found that 1088 (97.5%) of 1116 eggs cleaved normally, 1051 (94.2%) hatched normally and 1017 (91.1%) became feeding tadpoles at stage III. Of these tadpoles, 774 were of the wild-type and 243 were black-eyed variants.

In the breeding season of 1984, mating experiments were made between two females, Yc. Blk. 82♀, Nos. 1 and 2, and two males, Yc. Blk. 82♂, Nos. 1 and 2, which had been produced in 1982 from matings, Yc. Het. 81♀, Nos. 1～3 × Yc. Het. 81♂, Nos. 1～3, and were black-eyed variants having no traces of iridophores. It was found that 292 (73.0%) of 400 eggs cleaved normally, 244 (61.0%) hatched normally and 231 (57.8%) became feeding tadpoles at stage III. All these tadpoles were black-eyed variants (Table 1).

These findings show that the black-eyed variants of the Yc stock are mutants controlled by a single recessive gene (b^y).

2. Dermal chromatophores

The dorsal surface of the black-eyed mutant collected in 1980 from Yachiyo-cho was always dark grayish-olive and did not change their body color. As the ventral body wall was semitransparent, a part of visceral organs such as the liver, heart, stomach, fat bodies and ovaries was seen from the outside. Although this black-eyed mutant from Yachiyo-cho (Yc) resembled the same mutant from Hesaka (Hs) in this respect, the ventral body wall of the former was higher in transparency than that of the latter. The iris was deep black and indistinguishable from the black pupil (Plate I, 4, 5).

When 50 black-eyed mutants of the second-generation offspring obtained in 1982 from matings between three female and three male heterozygous tree-frogs were continuously reared, 49 of them attained sexual maturity in April of 1984. The dorsal color of these black-eyed mutants changed from grayish olive to dark grayish-brown in accordance with the environment. As the ventral body wall was semitransparent, a part of visceral organs was seen from the outside. In some of the mutants, a part of the iris, which was various in size from a needle-point to nearly the whole area, had golden luster owing to existence of iridophores, in contrast to those of the three black-eyed mutants collected in 1980 and 1981. Twelve (24.5%) of the 49 two-year-old black-eyed mutants had irises with a minute area of golden luster (Plate I, 6, 7). Of these mutants, nine had one or more dull-yellow spots on the dorsal surface of the body. The dull-yellow spots did not occupy a wide area on the back even in the mutants having the most

numerous spots. The remaining three black-eyed mutants had no dull-yellow spots on the back. The ventral body wall of all the black-eyed mutants of the second-generation offspring was highly semitransparent by lack of iridophores.

In 1984, the third-generation offspring of black-eyed mutants were produced from matings between two females, Yc. Blk. 82♀, Nos. 1 and 2, and two males, Yc. Blk. 82♂, Nos. 1 and 2, which were completely black-eyed mutants having no iridophores in the irises as well as in the dorsal and ventral skins. The results showed that they were all black-eyed mutants.

Of 150 black-eyed tadpoles produced from a mating, Yc. Blk. 82♀, No. 1 × Yc. Blk. 82♂, No. 1, 145 normally completed metamorphosis and about 2.5 months later 142 frogs, 20～30 mm in body length, were living. Of these frogs, 64 had some dull-yellow spots on the back. In 11 of them, more than half area of the dorsal surface appeared dull-yellow owing to accumulation of dull-yellow spots and flecks (Plate I, 8). Four of these 11 frogs had irises with golden luster. The ventral surface of all the black-eyed mutants was semitransparent.

Of 81 black-eyed tadpoles produced from another mating, Yc. Blk. 82♀, No. 2 × Yc. Blk. 82♂, No. 2, 75 metamorphosed normally and 61 were living about 2.5 months after completion of metamorphosis. They were 20～30 mm in body length and 21 of them had dull-yellow spots on the back. The irises were always black. The ventral body wall was semitransparent.

Dermal chromatophores of the dorsal and the ventral skin were examined

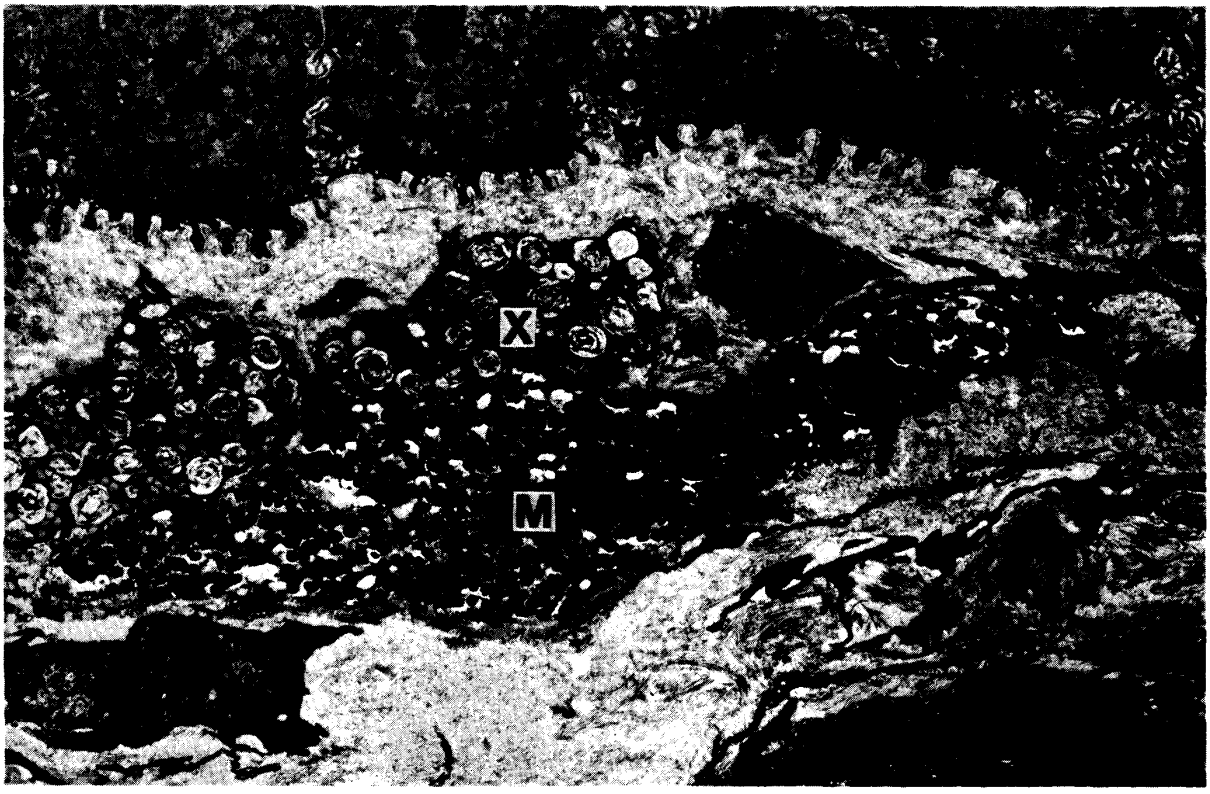


Fig. 7. Electron microphotograph of dermal chromatophores in the dorsal skin of a black-eyed mutant belonging to the Yc stock. ×4600

X, xanthophore M, melanophore

under an electron microscope in five black-eyed mutants including a female discovered in 1980, Yc. Blk. 80♀, No. 1, and four individuals of the second-generation offspring obtained in 1982. Of the latter four black-eyed mutants, two (Yc. Blk. 82♀, Nos. 3 and 4) had no iridophores in the dorsal skin and the irises, one (Yc. Blk. 82♂, No. 3) had a very small dull-yellow spot on the dorsal surface of the body and the remainder (Yc. Blk. 82♀, No. 5) had two dull-yellow flecks, each of which was about 1.5 mm in diameter, on the back.

a. Xanthophore

When 17 xanthophores of these black-eyed mutants were measured, they were 13.0～28.7 μ, 19.3 μ on the average, in width and 3.7～10.5 μ, 6.3 μ on the average, in thickness. They were nearly the same as those of the wild-type tree-frogs. Both pterinosomes and carotenoid vesicles contained in the xanthophores were well developed. When 20 pterinosomes were measured, they were 0.51～1.09 μ, 0.77 μ on the average, in major axis and 0.45～0.82 μ, 0.63 μ on the average, in minor axis. When 30 carotenoid vesicles were measured, they were 0.20～0.65 μ, 0.31 μ on the average, in diameter. It was found that the xanthophores of the black-eyed mutants were much the same as those of the wild-type tree-frogs in sizes of cells, pterinosomes and carotenoid vesicles (Figs. 7, 9a).

b. Iridophore

i) In cross-sections of the dorsal skin from the five black-eyed mutants except the area of a dull-yellow spot, there were no iridophores containing reflecting platelets or their precursory organelles in any place. While iridophores are situated between xanthophores and melanophores in the dorsal skin of the normal tree-frogs, the site of iridophores was usually occupied by a narrow interspace in the black-eyed mutants. In this interspace, slender dendritic processes having no contents were often observable, although the derivation of the processes was not evident. In a few cases, there was no interspace between xanthophores and melanophores (Fig. 7).

ii) In the area of a small dull-yellow spot on the back of a black-eyed mutant (Yc. Blk. 82♂, No. 3), there were iridophores including almost normal reflecting platelets. When four iridophores were measured, they were 6.5～15.2 μ, 10.5 μ on the average, in width and 3.0～4.5 μ, 3.8 μ on the average, in thickness. It was found that they were remarkably smaller than those of the normal tree-frogs. However, the reflecting platelets contained in these iridophores did not distinctly differ in size and shape from those of the wild-type tree-frogs. When 20 reflecting platelets were measured, they were 0.32～2.09 μ in length and 0.09～0.14 μ in width. They were various in shape between rods and dice, although most of them were more than 1 μ in length and 0.09 μ in width.

iii) In contrast to the iridophores in the area of the small dull-yellow spot on the back of the black-eyed mutant (Yc. Blk. 82♂, No. 3), those in the areas of

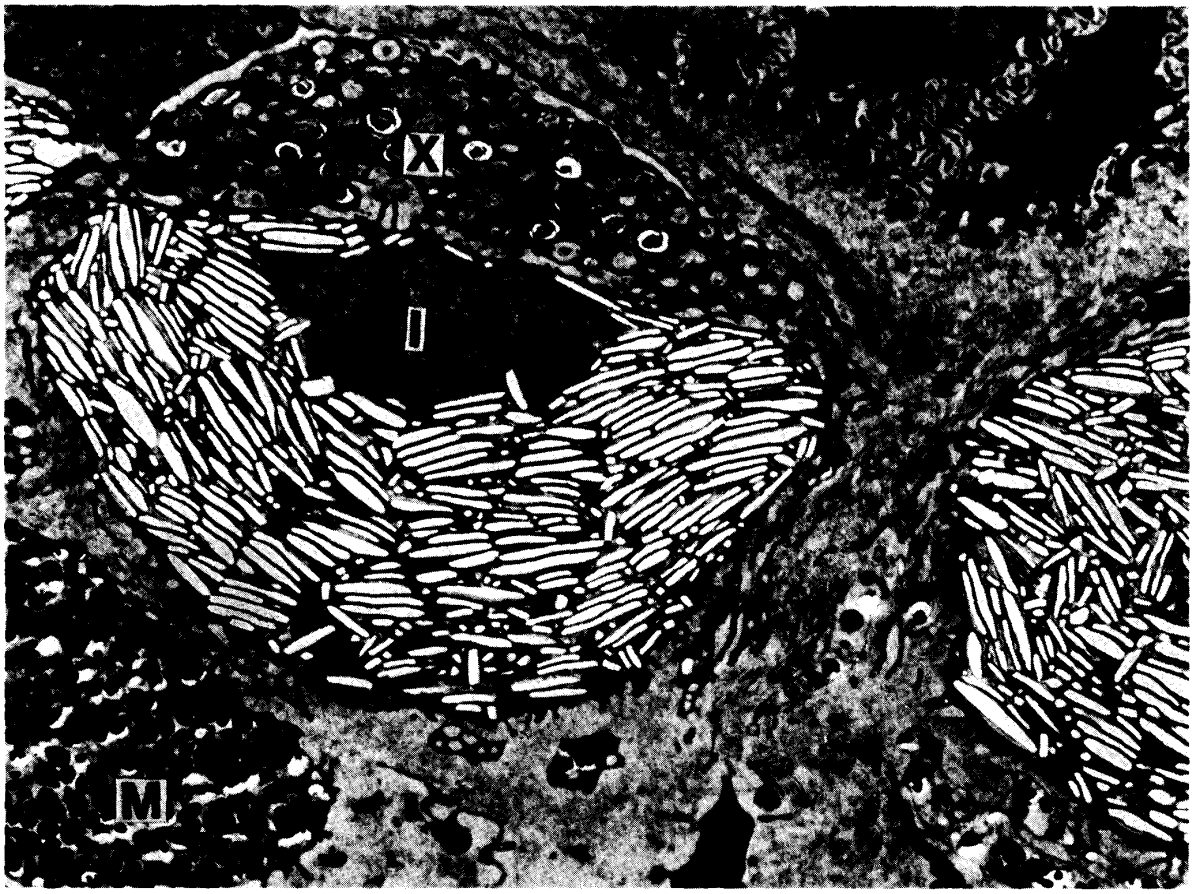


Fig. 8. Electron microphotograph of dermal chromatophores in a dull-yellow spot of the dorsal skin of a black-eyed mutant belonging to the Yc stock. ×5000
X, xanthophore I, iridophore M, melanophore

two dull-yellow spots, about 1.5 mm in diameter, found on the back of the other black-eyed mutant (Yc. Blk. 82♀, No. 5) were almost completely the same as the iridophores of wild-type tree-frogs in number, size and shape (Fig. 8). When 10 iridophores were measured, they were 12.5～25.5 μ, 17.07 μ on the average, in width and 6.0～12.0 μ, 9.01 μ on the average, in thickness. When 20 reflecting platelets were measured, they were 0.20～2.20 μ, 1.25 μ on the average, in length and 0.09～0.18 μ, 0.12 μ on the average, in width (Fig. 9b).

c. Melanophore

The melanophores of the black-eyed mutants were nearly the same as those of the wild-type tree-frogs in the state of distribution. They extended their dendritic processes in every direction. The processes were various in length and thickness. The melanosomes were ellipsoidal or almost spherical. When 20 ellipsoidal melanosomes were measured, they were 0.47～0.86 μ, 0.59 μ on the average,

Fig. 9. Electron microphotographs of three kinds of dermal chromatophores in a dull-yellow spot of the dorsal skin of a black-eyed mutant belonging to the Yc stock. ×22000
a. Xanthophore. b. Iridophore. c. Melanophore.
CV, carotenoid vesicles RP, reflecting platelets PT, pterinosomes ME, melanosomes

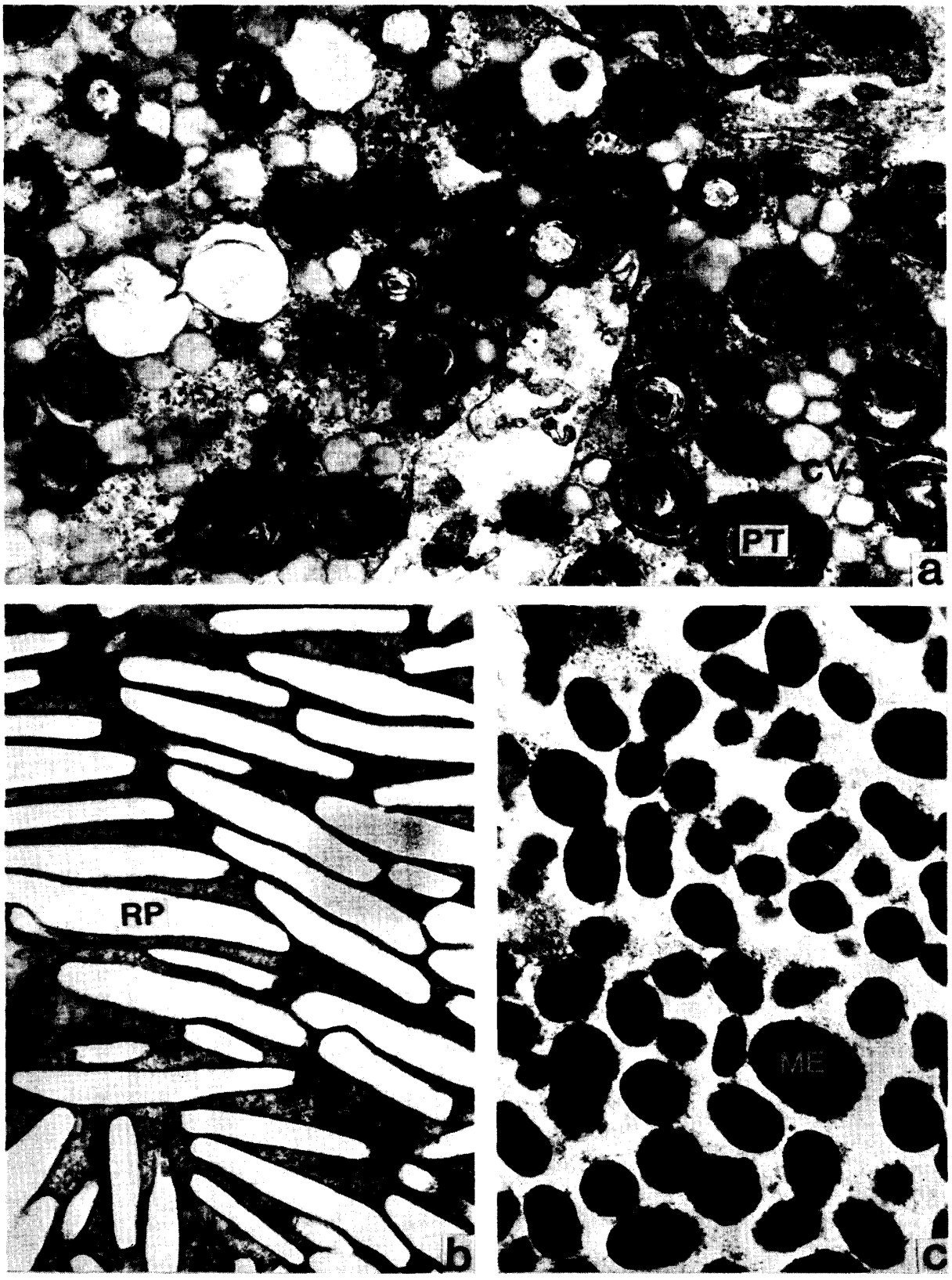


Fig. 9

in major axis and 0.24~0.47 μ, 0.37 μ on the average, in minor axis. When 10 spherical melanosomes were measured, they were 0.31~0.90 μ, 0.47 μ on the average, in diameter. These melanosomes did not distinctly differ from those of the wild-type tree-frogs in size and shape (Fig. 9c).

3. Iridophores in the ventral body wall

In the ventral body wall of the wild-type tree-frogs, there were one or two layers of iridophores just below the basal lamina. The dendritic processes of iridophores which were various in length and thickness were extended in every direction and overlapped one another. Thick layers of iridophores were also found in loose connective tissues under the layers of collagenous fibers.

In the black-eyed mutant, no iridophores were found anywhere in the ventral body wall (Fig. 10).

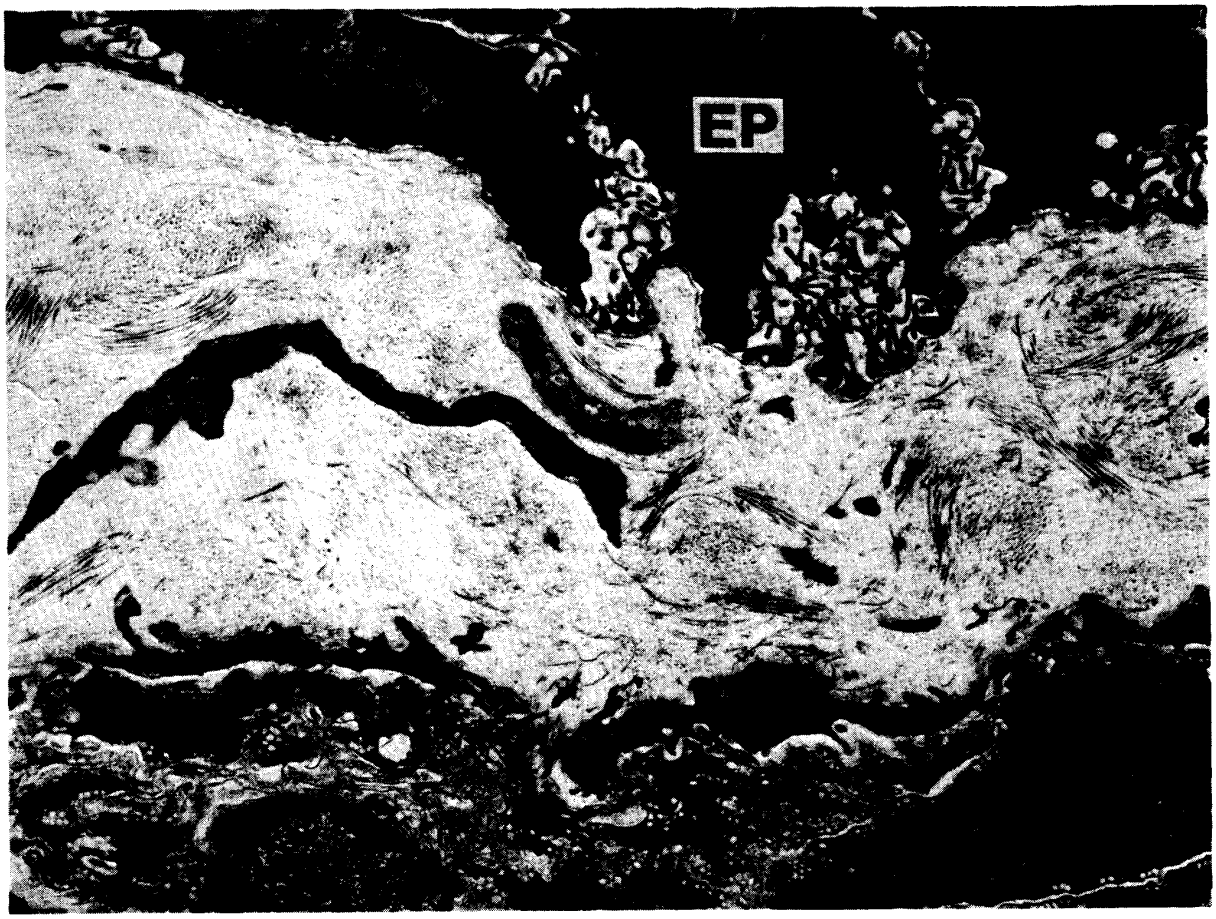


Fig. 10. Electron microphotograph of a cross-section of the ventral skin of a black-eyed mutant belonging to the Yc stock. ×6000

EP, epidermal cell

DISCUSSION

The melanoid mutant was first described by HUMPHREY and BAGNARA (1967) in the Mexican axolotl, *Ambystoma mexicanum*. The melanoid animals are overall

dark charcoal-gray or dull black and have solid black irises, owing to a relatively small number of xanthophores, an increased number of melanophores and complete lack of iridophores. HUMPHREY and BAGNARA confirmed that the melanoid is due to a recessive gene (*m*) in the homozygous condition. As gene *m* is not an allele of *D*, which determines the ordinary dark color pattern, the effect of homozygosity for *m* is limited to reduction in number of xanthophores and to elimination of iridophores in white axolotls (*d*/*d*). No effect on melanophores is evident. BENJAMIN (1970) reported on the biochemical effects of gene *m* together with genes *d* and *a* (for albino) on pigment cell differentiation in the axolotl. Gene *m* has primarily an effect on the production of propigment cells by the neural crest. It was indicated by transplantation experiments that in axolotls homozygous for *m*, more melanophores and fewer xanthophores than in the wild-type and no iridophores are produced. In correlation with these observations, a 35% increase in tyrosinase activity and a 40% decrease in pteridine synthesis are measured in melanoid animals. DUNSON (1974) reported complete lack of iridophores in melanoid mutants of the axolotl. LACROIX and CAPURON (1970) discovered melanoid mutants in *Pleurodeles waltl* and confirmed that they are due to a recessive gene (*m*) in the homozygous condition, as in those of the axolotl.

In anurans, RICHARDS, TARTOF and NACE (1969) obtained melanoid variants in *Rana pipiens*. These variants were produced from two females by the method of diploid gynogenesis and considered to be mutants due to a recessive gene. Of the three kinds of dermal chromatophores, only melanophores are pigmented, while the other xanthophores and iridophores are not detectable. The dorsal surface of the body is black at the tadpole stage and dull gray at the adult frog stage, instead of the usual olive and green or brown at the respective stage. The iris is black and the ventral body wall is semitransparent through life.

NISHIOKA (1977) obtained three stocks of black-eyed variants from gametes exposed to X-rays or neutrons in *Rana nigromaculata*. These variants are apparently similar to the melanoid mutants of *Rana pipiens*. Their whole body is distinctly blackish and semitransparent. The irises of tadpoles and frogs are black. The name of black-eyed variant was given by the author from this character. NISHIOKA elucidated that the black-eyed variants are mutants due to a recessive gene (*b*) in the homozygous condition and that the three stocks of black-eyed mutants have the same gene. NISHIOKA and UEDA (1985b) have reported black-eyed variants in *Rhacophorus schlegelii*. In contrast to that the dorsal surface of the wild-type frog is yellowish green, those of the variants are dark reddish-brown. The irises are black and the ventral body wall is semitransparent. Of the dermal chromatophores, iridophores are abnormal and have no reflecting platelets, while the other chromatophores are almost normal. These variants are confirmed to be mutants due to a single recessive gene.

In the olive mutants of *Rana nigromaculata* obtained by NISHIOKA (1977) from gametes irradiated with X-rays or neutrons, the dorsal surface is of olive color and the ventral body wall is semitransparent. These traits are attributable to an abnormality of iridophores and due to a single recessive gene in the homozygous

condition. By observing the dermal chromatophores under an electron microscope, the present authors (1977a) elucidated that the reflecting platelets contained in the iridophores are remarkably fewer and smaller than those of the wild-type frogs, while the xanthophores and melanophores are completely normal.

In the present study, two stocks of black-eyed variants of *Hyla arborea japonica* were genetically and morphologically examined. These two stocks, Hs and Yc, were collected from two different places, Hesaka and Yachiyo-cho, which are only about 30 km from each other. The variants of the Hs stock somewhat differ from those of the Yc stock in various morphological characters. While the iridophores are lacking in the Yc variants, those of the Hs variants contain reflecting platelets which are very abnormal in size and shape as those of the black-eyed *Rana nigromaculata*. While the xanthophores and melanophores of the Yc variants are nearly the same as those of the wild-type tree-frogs in size, shape and inner structure, those of the Hs variants are smaller and fewer than those of the wild-type tree-frogs and abnormal in inner structure. Pterinosomes are scarcely found in the xanthophores, and melanosomes contained in the melanophores are distinctly large. Moreover, in some of the black-eyed variants of the Yc stock, a part of the iris has golden luster owing to existence of iridophores. The dorsal surfaces of some variants have dull-yellow spots or flecks in which iridophores containing nearly normal reflecting platelets are found. These unstable phenomena of the black-eyed variants of the Yc stock seem to be attributable to incomplete function of the gene for the black-eyed phenotypes. The existence of similar dull-yellow areas in the dorsal surfaces of black-eyed variants has been reported in *Rhacophorus schlegelii* (NISHIOKA and UEDA, 1985b).

On the basis of the results of mating experiments and diploid gynogenesis, it is evident that the black-eyed variants of the Hs and Yc stocks are mutants due to a single recessive gene (b^h or b^y) in the homozygous condition. Gene b^h for the black-eyed phenotype of the Hs stock and gene b^y for that of the Yc stock are provisionally treated as alleles of the same locus at the present time, as mating experiments between these two stocks have not yet been performed. However, in *Hyla arborea japonica*, NISHIOKA and UEDA (1977b) confirmed the existence of three loci of albino genes. The present authors (1985a) have also reported five loci of albino genes in *Rana nigromaculata*. Thus, it seems possible that there are two loci of black-eyed genes in *Hyla arborea japonica*.

SUMMARY

1. Two kinds of black-eyed variants were discovered in *Hyla arborea japonica*. One of them appeared in gynogenetic diploids produced from eggs of a female collected in 1971 from Hesaka (Hs), Hiroshima City, and included four variants, while the other was detected in 1980 and 1981 at Yachiyo-cho (Yc), about 30 km north of Hesaka, and included two females and one male.

2. It was evident that these two kinds of variants are controlled by a single recessive gene (b^h or b^y).

3. The two kinds of black-eyed mutants, Hs stock (b^hb^h) from Hesaka and Yc stock (b^yb^y) from Yachiyo-cho, somewhat differ from each other in various morphological characters.

The dorsal surfaces of the black-eyed mutants of the Hs stock are dark grayish-brown. The iris has no metallic luster. The three kinds of dermal chromatophores are smaller and fewer than those of the wild-type tree-frogs. The xanthophores are almost filled with carotenoid vesicles and scarcely contain pterinosomes. The reflecting platelets contained in the iridophores are very abnormal in size and shape. The melanosomes contained in melanophores are mostly spheroids and distinctly large.

The ventral wall is semitransparent and contains iridophores whose reflecting platelets are remarkably smaller than those of the wild-type tree-frogs.

4. The dorsal surfaces of the black-eyed mutants of the Yc stock are dark grayish-olive. The iris is usually deep black and indistinguishable from the pupil. However, in some of the black-eyed mutants, a part of the iris has golden luster owing to existence of iridophores. While the dermal xanthophores and melanophores of the dorsal skin are nearly the same as those of the wild-type tree-frogs in size, shape and inner structure, there are no iridophores containing reflecting platelets or their precursory organelles in any place other than the dull-yellow spots or flecks found in some black-eyed mutants. In these spots or flecks, there are iridophores containing almost normal reflecting platelets. The ventral body wall has no iridophores everywhere.

ACKNOWLEDGMENTS

Sincere thanks are extended to Emeritus Professor Toshijiro KAWAMURA under whose encouragement and guidance this study was carried out. The authors are especially grateful to him for his critical review of the manuscript. They are also grateful to Mr. S. TAKAGI and Mr. K. NISHIGUCHI for their gift of mutants.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture.

LITERATURE

BAGNARA, J. T., J. D. TAYLOR and M. E. HADLEY 1968. The dermal chromatophore unit. J. Cell Biol., **38**: 67–79.

BENJAMIN, C. P. 1970. The biochemical effects of the *d*, *m* and *a* genes on pigment cell differentiation in the axolotl. Develop. Biol., **23**: 62–85.

DUNSON, M. K. 1974. Ultrastructure of pigment cells in wild type and color mutants of the Mexican axolotl. Cell Tiss. Res., **151**: 259–268.

HUMPHREY, R. R. and J. T. BAGNARA 1967. A color variant in the Mexican axolotl. J. Hered., **58**: 251–256.

LACROIX, J.-C. et A. CAPURON 1970. Sur un facteur récessif, modifiant le phénotype pigmentaire de la larve, chez l'Amphibien Urodèle *Pleurodeles waltlii* MICHAHELLES. C. R. Acad. Sc. Paris, Série D, **270**: 2122–2123.

NISHIOKA, M. 1977. Color variants induced by radiation and their inheritance in *Rana nigromaculata*. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **2**: 25–89.

NISHIOKA, M. and H. UEDA 1977a. An electron-microscopic study on six kinds of color variants induced by radiation in *Rana nigromaculata*. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **2**: 91–102.

——— 1977b. Genetic and morphologic studies on ten albino stocks in *Hyla arborea japonica*. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **2**: 103–163.

——— 1985a. Genetics and morphology of 13 albino stocks in the *Rana nigromaculata* group. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **7**: 1–121.

——— 1985b. Electron-microscopic observation on the dermal chromatophores of normal frogs and three kinds of color variants in *Rhacophorus schlegelii*. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **7**: 123–155.

RICHARDS, C. M., D. T. TARTOF and G. W. NACE 1969. A melanoid variant in *Rana pipiens*. Copeia, 1969: 850–852.

SHUMWAY, W. 1940. Stages in the normal development of *Rana pipiens*. I. External form. Anat. Rec., **78**: 139–147.

TAYLOR, A. C. and J. J. KOLLROS 1946. Stages in the normal development of *Rana pipiens* larvae. Anat. Rec., **94**: 7–24.

EXPLANATION OF PLATE

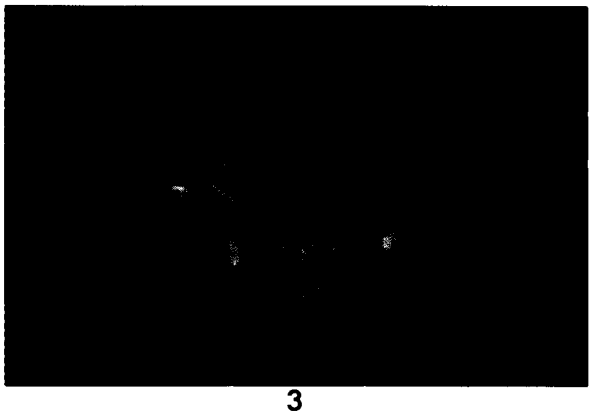
PLATE I

Two kinds of black-eyed mutants and wild-type tree-frogs.

1. Green wild-type tree-frog. ×1
2. Brown wild-type tree-frog. ×1
3. Black-eyed mutant of the Hs stock. ×1
4. Black-eyed mutant, Yc. Blk. 80♀, No. 1, of the Yc stock. ×1
5. The head of black-eyed mutant, Yc. Blk. 80♀, No. 1. ×2.5
6. Black-eyed mutant,Yc. Blk. 82♀, No. 5, of the Yc. stock. Iridophores are found in the iris. ×1
7. The head of black-eyed mutant, Yc. Blk. 82♀, No. 5. ×2.5
8. Black-eyed mutant, Yc. Blk, 84♂, No. 1, of the Yc stock, having dull-yellow spots and flecks on the dorsal surface. ×1.5

1

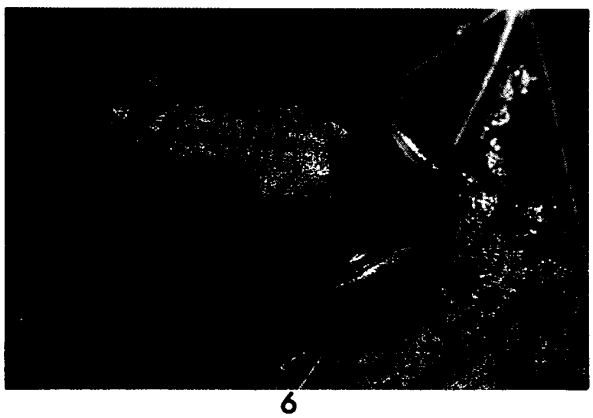
2



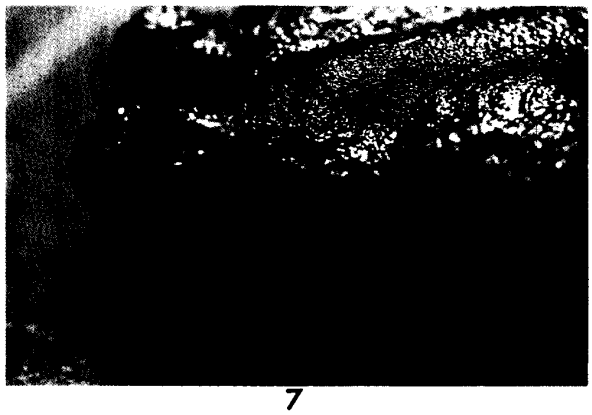
3

4

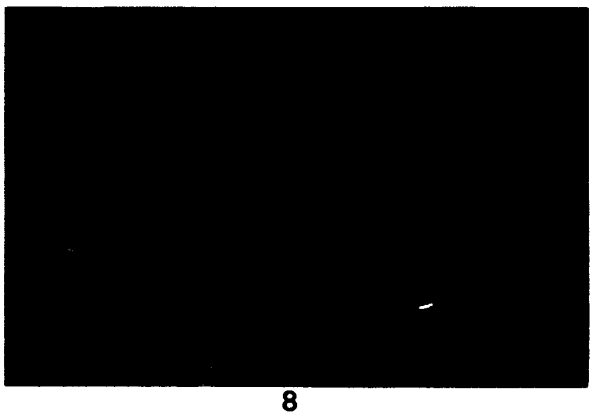
5



6



7



8