再刊編號：12
Reprint No. 12

抽印自　中央研究院　動物研究所集刊
第22卷　第2期
Reprinted from
Bulletin of the
Institute of Zoology, Academia Sinica
Vol. 22(2), pp. 133-140, 1983

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吳郭魚品系體表黏液之酯酶類酵素電泳差異性研究

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73農建-4. 1-產-128(1)

Bull. Inst. Zool., Academia Sinica 22(2): 133-140 (1983)

ELECTROPHORETIC DIFFERENCES OF ESTERASE ISOZYMES FROM THE SURFACE MUCUS OF *OREOCHROMIS* FISHES

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(Received March 11, 1983)

Jen-Leih Wu and Shaw-Yun Wu (1983) Electrophoretic differences of esterase isozymes from the surface mucus of *Oreochromis* fishes. *Bull Inst. Zool., Academia Sinica* 22(2): 133-140. There were distinct identification characters in *Oreochromis* species, *O. niloticus, O. aureus, O. mossambicus* and red tilapia, which were based on their esterase pattern of surface mucus by using polyacrylamide gel electrophoresis. The Es-1^c allele and sometimes with Es-1^b allele could serve as species markers for *O. niloticus*, the Es-1^b allele for *O. aureus*, while *O. mossambicus* possessed both Es-1^a and Es-1^c alleles could serve as its species markes. The red tilapia had similar species esterase markers as *O. niloticus*. The Es-1 locus belonged to carboxylesterase because of its inhibition by diisopropylfluorophosphate. On studying the phylogenetic relationship among these tilapia species by the genetic distance and genetic similarity analysis, it showed that the red tilapia was closely related to *O. niloticus*. Since the accuracy of surface mucus esterase isozyme pattern were species-specific, the unnecessary of sacrifice the stocking fish for species identification was a valuable application in tilapia aquaculture.

The first tilapia species introduced to Taiwan for aquaculture was *Oreochromis mossambicus* in 1946 (Chen, 1957). The introduction of *O. aureus* and *O. niloticus* was followed for the advancement of tilapia culture (Liao and Huang, 1976; Tseng, 1976; Yuo, 1975). The tilapia annual production was increased from 13,000 tons in 1974 (Chen, 1976) to 48,500 tons in 1981 (Taiwan Fisheries Bureau, 1982). The rapid growth of tilapia culture was due to the application of all-male hybrids breeding between female *O. niloticus* and male *O. aureus* (Pruginin *et al.*, 1975; Hepher and Pruginin, 1982), and the discovery of red tilapia. The pure stock maintenance and hybrid breeding are the foundation of tilapia culture. The success of these techniques are depend on the correct identification of each tilapia species. The traditional method of species identification is solely relied on morphological examination. However, it is always confused when the hybridization event occurred between different tilapia species. For accurate species identification and phylogenetic study, the biochemical approach by isozyme and protein electrophoresis techniques has been widely applied in fish (Morgan and Ulanowicz, 1976; Yardley and Hubbs, 1976), amphibian (Case, 1978), reptile (Lou and Lin, 1983), insect (Ayala *et al.*, 1970; McReynold, 1967) and mammals (Aquadro and Patton, 1980). The specific characters of tilapia species from different organs have fully established by esterase isozymes (Wu *et al.*, 1983; Herzberg, 1978) for further genetic study of tilapia. But for the

1. Paper No. 241 of the Journal series of the Institute of Zoology, Academia Sinica.

sampling propose, we always encountered the sacrifice and blood drawing the breeder fish. Due to the sacrifice or stress on the breeder fish, these methods are not suitable for the hatchery.

In this paper, we studied the esterase isozyme of surface mucus of *Oreochromis* by polyacrylamide slab gel electrophoresis. By this approach, a rapid and reliable method is set up for the species identification. The genetic relationship between different *Oreochromis* species are explored by the surface mucus esterase isozymes.

MATERIALS AND METHODS

Fish lines

O. mossambicus: The first species was introduced to Taiwan from Indonesia in 1946, and was stocked in pond at Tainan Fishery Station, Taiwan Fisheries Research Institute (Chen, 1957). Twenty-six samples were examined.

O. niloticus: The species was obtained via Japan, stocked in pond at Chupei Fishery Station, Taiwan Fisheries Research Institute since 1966 (Yuo, 1975). Twenty-nine samples were examined.

O. aureus: The species was introduced to Taiwan from Israel, stocked in pond at Chupei Fishery Station, Taiwan Fisheries Research Institute since 1974 (Liao and Huang, 1976). Twenty-nine samples were examined.

Red tilapia: The fish was brought from the Full High Cooperative Farm and thirty samples were examined.

Preparation of mucus extract

A modified procedure of Herzberg (1978) was used to prepare the surface mucus. The sample was taken with a sterile cotton swab from fish and was extracted with 10 ml of 1 mM Tris-HCl buffer (pH 8.0). The extract was centrifuged at 17,300 xg for 20 minutes at 4°C and the supernatant was concentrated by lyopholization. The mucus powder was redissolved in 1 ml of 1 mM Tris-HCl buffer as mucus extract.

Gel electrophoresis

Polyacrylamide slab gel electrophoresis method of Wu *et al.* (1983) was used in this study. A 7.5% separating gel served for the electrophoretic migration of the mucus samples from the tilapias. A 3% stacking gel was poured on the polymerized separating gel. Following polymerization, the gel was used immediately for electrophoresis. Thirty μl of mucus extract was applied into each slot of the polyacrylamide gel and was separated under a constant current of 12.5 mA per gel at 4°C. The electrode buffer system was Tris-glycine buffer (pH 8.3) (Gabriel, 1971).

Esterase staining

After electrophoresis, the gel was removed from the glass plate and immediately incubated in 50 ml phosphate buffer (pH 6.5) which contained 4 ml of 1% β-naphthyl acetate in 50% acetone for 20 minutes at room temperature, then added 5 ml of 0.1% fast garnet GBC salt solution and incubated for another 10 minutes (Wu *et al.*, 1983). The gel was destained with 7% acetic acid and dried on Whatman 3 MM filter paper by suction and IR heating.

Calculation of genetic similarity and genetic distance

Genetic similarity and genetic distance between the species were estimated by using Nei's (1972) method. Let X_i and Y_i be the frequencies of the ith alleles in species X and Y, respectively. The probability of identity of two randomly chosen genes is $j_x=\sum X_i^2$ in species X, while it is $j_y=\sum Y_i^2$ in species Y. The probability of identity of a gene from X and a gene from Y is $j_{xy}=\sum X_i Y_i$. The normalized similarity of genes between X and Y of a given locus is defined as

$$I_j=j_{xy}/\sqrt{j_x j_y}$$

The normalized similarity of genes between X and Y of all loci is defined as

$$I=J_{xy}/\sqrt{J_x J_y}$$

where J_x, J_y and J_{xy} are the arithmetic means of j_x, j_y and j_{xy}, respectively, over all loci, including monomorphic loci. The genetic distance between X and Y is given by

$$D = -\log_e I$$

The values of I range from 0 to 1; the value 1 indicates the identical distribution of allelic frequencies in the two species, and the value 0 indicates the non-overlapping distribution of allelic frequencies. The values of D range from 0 to infinity; the value 0 indicates the identical distribution of allelic frequencies in the two species.

RESULTS

From the esterase pattern of the tilapia species, twelve discrete bands were observed after gel electrophoretic analysis. However, only three bands belonged to Es-1 locus could be routinely and easily demonstrated. The other bands were very faint and might be disappeared if the mucus extract was stored too long. The locus was determined according to its electrophoretic mobility and the clustering position under defined condition. The alleles were designated alphabetically as Es-1^a, Es-1^b and Es-1^c in order of decreasing distance from the anode end of the gel (Fig. 1).

Both the Es-1^b and Es-1^c alleles were found in *O. niloticus* and the Es-1^c allels was existed in each individual (Fig. 2). In *O. aureus* mucus extract, only the Es-1^b allele was found in each sample (Fig. 3). Es-1^a and Es-1^c alleles were appeared in *O. mossambicus* and each individual possessed these two esterase bands in the mucus extract (Fig. 4). The esterase pattern in the red tilapia consisted of Es-1^b and Es-1^c alleles (Fig. 5). In most cases, both bands were existed simultaneously as the *O. niloticus*. However, single band of either Es-1^b or Es-1^c could be observed occasionally in the mucus extract of red tilapia.

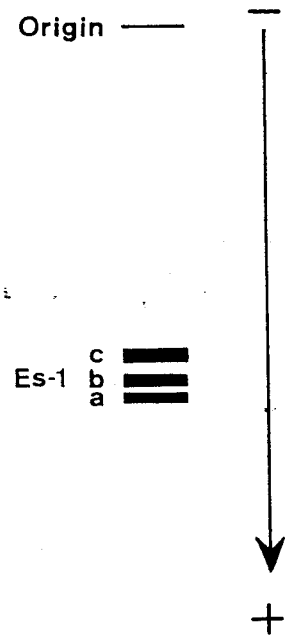

Fig. 1. Composition diagram of the esterase electrophenotype from the surface mucus of *O. niloticus*, *O. aureus*, *O. mossambicus* and red tilapia.

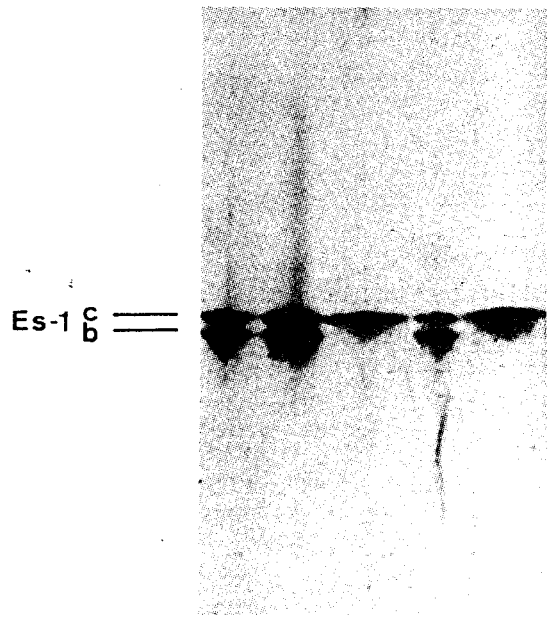

Fig. 2. Esterase electropherogram of surface mucus in *O. niloticus*. Both Es-1^b and Es-1^c alleles were appeared in the species, but only Es-1^c allele was common to each individual.

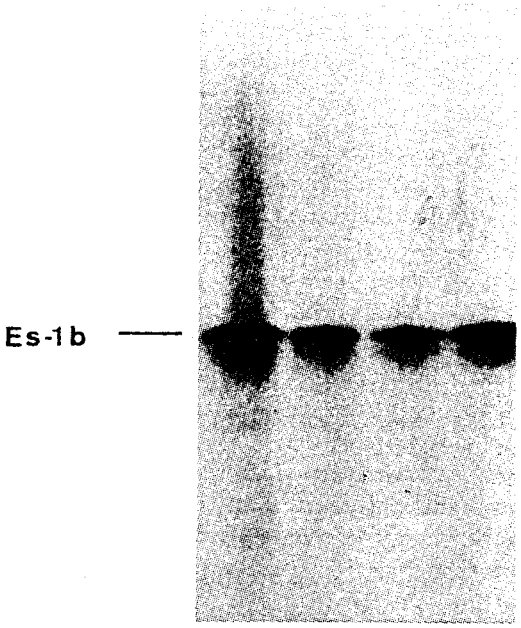

Fig. 3. Esterase electropherogram of surface mucus in *O. aureus*. Only Es-1^{b} allele was appeared and the allele was appeared in each individual.

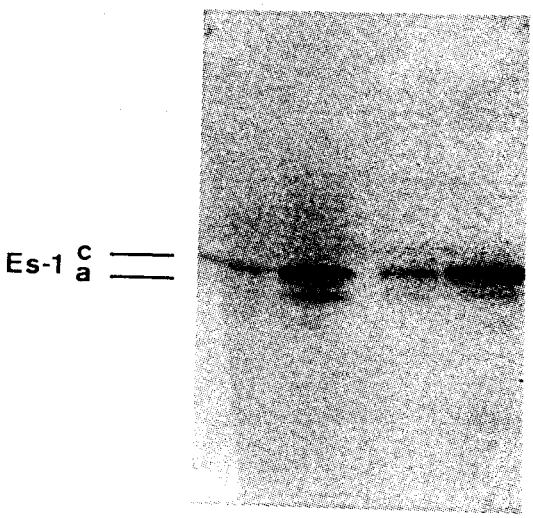

Fig. 4. Esterase electropherogram of surface mucus in *O. mossambicus*. Both Es-1^{a} and Es-1^{c} alleles were appeared in the species, and the two alleles were common to each individual.

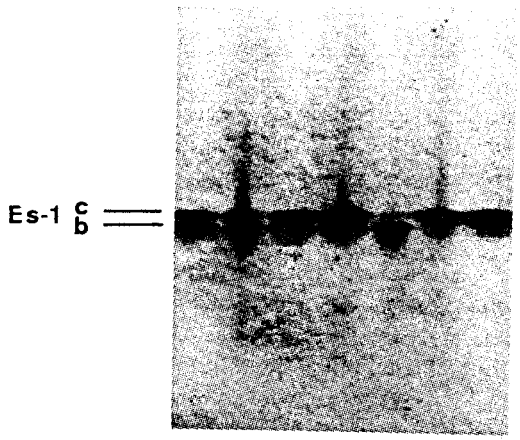

Fig. 5. Esterase electropherogram of surface mucus in the red tilapia species. The Es-1^{b} and Es-1^{c} alleles were appeared, but there wasn't any common allele appeared.

The electrophoretic results of mucus esterase were summerized in Table 1. The allelic frequencies could be calculated by dividing the number of a given specific allele by the total number of alleles in the Es-1 locus in each species.

According to the method proposed by Nei (1972), the normalized genetic similarity (I) and genetic distance (D) are shown in Table 2. The genetic similarities between *O. aureus* and *O. niloticus* or red tilapia were 0.5480 and 0.7515, respectively. However, the genetic similarities between *O. mossambicus* and *O. niloticus*, red tilapia and *O. aureus* were 0.5915, 0.5126 and 0. Thus, we can derive the phylogenetic dendrogram of these tilapia species from the genetic similarity by using the UPGMA method (Sneath and Sokal, 1973) (Fig. 6). The genetic similarity between the four taxa showed that the red tilapia and *O. niloticus* was closely related by I=0.9839. *O. aureus* was located in the middle by showing I=0.6498 to *O. niloticus* and red tilapia. *O. mossambicus* was farest in this phylogenetic relationship since the genetic similarity was lowest among these tilapia species.

The Es-1 locus of tilapia mucus esterase was further compared to its muscle esterase

TABLE 1
Allelic frequency at Es-1 locus of tilapia species

Tilapia	Number of individuals	Number of allele			Allele frequency		
		Es-1^a	Es-1^b	Es-1^c	Es-1^a	Es-1^b	Es-1^c
O. niloticus	29	—	19	29	—	0.3958	0.6024
O. aureus	29	—	29	—	—	1.0000	—
O. mossambicus	26	26	—	26	0.5000	—	0.5000
Red tilapia	30	—	19	20	—	0.4872	0.5128

TABLE 2
The genetic distance (D) and genetic similarity (I) between the tilapia species

D \ I	*O. niloticus*	*O. aureus*	*O. mossambicus*	Red tilapia
O. niloticus	—	0.5480	0.5915	0.9839
O. aureus	0.6015	—	0.0000	0.7515
O. mossambicus	0.5251	∞	—	0.5126
Red tilapia	0.0162	0.2857	0.6682	—

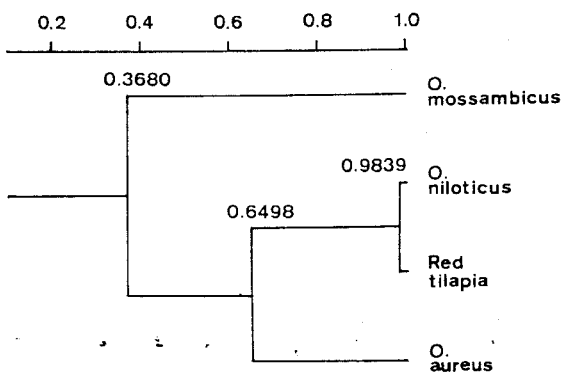

Fig. 6. Phylogenetic dendrogram of *O. niloticus*, *O. aureus*, *O. mossambicus* and red tilapia, derived from the genetic similarity (I) in Table 2.

pattern by using electrophoresis. As shown in Fig. 7, the Es-1 locus of mucus esterase was correspondent to Es-5 locus of muscle esterase pattern (Wu *et al.*, 1983) in tilapias. The esterase activity of Es-5 locus were completely inhibited by diisopropylfluorophosphate (DFP) but not inhibited by p-chloromercuribenzoic acid (PCMB) and eserine sulfate (ES) treatments. Therefore, the mucus Es-1 locus as well as muscle Es-5 locus were classified as carboxylesterase (Metcalf *et al.*, 1972; Wu *et al.*, 1983).

DISCUSSION

The protein phenotypes are treated as genotypes, although this commonly accepted transformation has not been tested from breeding tests. Lewontin and Hubby (1966) and Selander *et al.* (1971) gave evidence supporting that the electrophoretic patterns for most proteins were similar to those of their homologues in studies that have included breeding tests. Therefore, those individuals possess the same protein phenotype may be regarded as the same genotype. The hybridization test of Wu *et al.* (1983) revealed that these alleles were autosomally codominant inheritance.

There was clear intraspecific difference among *O. niloticus*, *O. aureus* and *O. mossambicus* on the mucus esterase pattern. The esterase pattern differed markedly between species. This suggested that the differences could be used to identify species and study genetic variations within a species (Latner and Skillen, 1968). The electropherogram revealed that Es-1^c allele

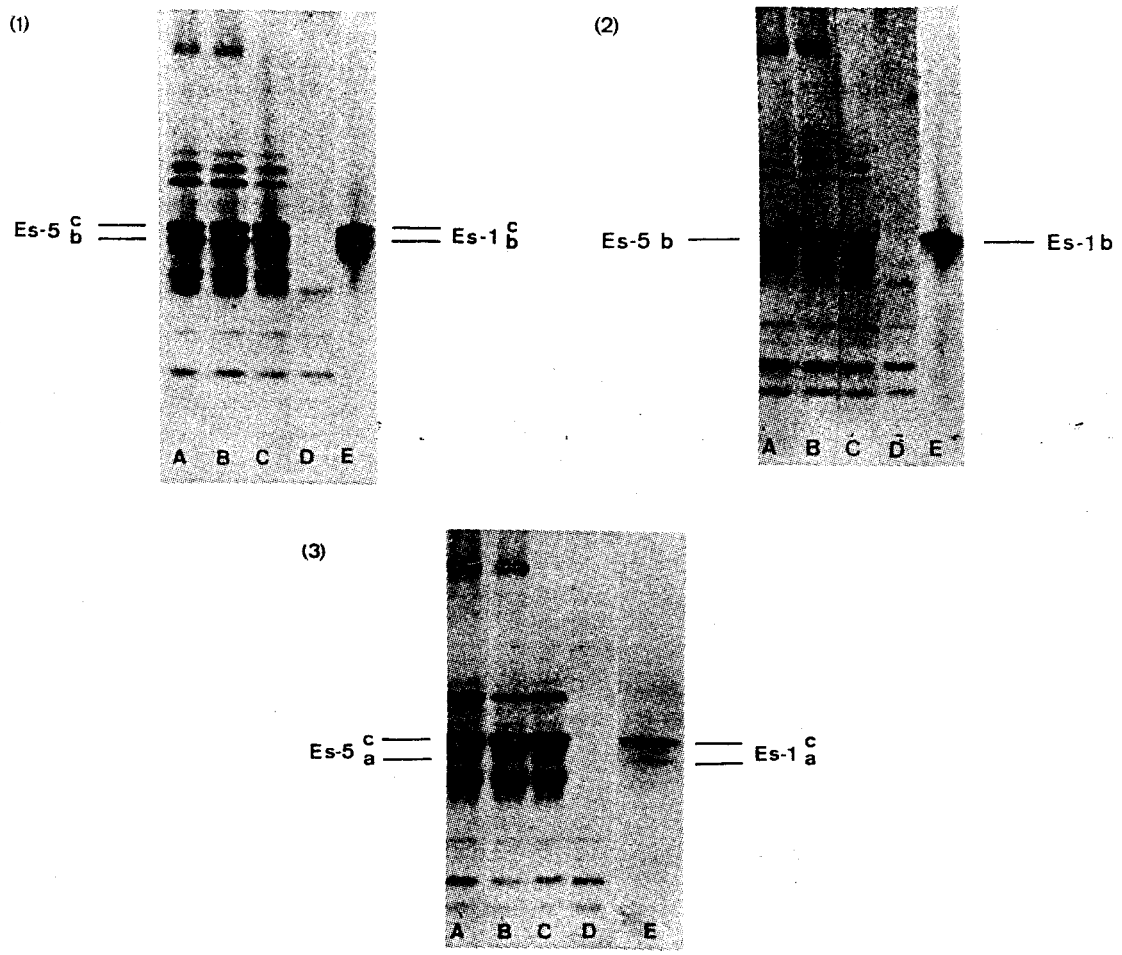

Fig. 7. The comparison of mucus esterase and muscle esterase pattern in *Oreochromis* species. The Es-1 locus of mucus esterase was consistent with the Es-5 locus of muscle esterase. Muscle esterase were demonstrated with *p*-chloromercuribenzoic acid (PCMB) in B lane, eserine sulfate (ES) in C lane, and diisopropylfluorophosphate (DFP) in D lane as inhibition tests and control in A lane, while the mucus esterase was demonstrated in E lane. The species of *Oreochromis* were (1) *O. niloticus*; (2) *O. aureus*; (3) *O. mossambicus*.

and sometimes with Es-1^b allele were the species markers for *O. niloticus*, Es-1^b allele was the marker for *O. aureus*, Es-1^a and Es-1^c alleles were markers for *O. mossambicus*. The red tilapia had very similar esterase marker as that of *O. niloticus*. Based on these marker alleles, it could be easy to distinguish the four tilapia taxa without ambiguity.

Among the genetic similarity, the genetic similarity between *O. niloticus* and the red tilapia was the closest one, in other words, there was the least genetic differentiation between the two species (Ayala *et al.*, 1974). A dendrogram derived from the genetic similarity of the four taxa was shown that the red tilapia and *O. niloticus* was extremely close. The resemblance of esterase electropherogram between the red tilapia and *O. niloticus* was proved by the minute genetic distance between them (D=0.0162), and might qualified them into population level

of a species (Adest, 1977). The results of genetic relationship of mucus esterase study correspond with the genetic study of muscle esterase (Wu *et al.*, 1983). Kuo and Neal (1982) proposed that the red tilapia was presumably the hybrid of *O. niloticus*×*O. mossambicus*. From our survey, based on the minute genetic distance and similar esterase pattern, we propose that the red tilapia is a variant of *O. niloticus*.

Gorman and Renzi (1979) suggested that the heterozygosity estimates and the genetic distance estimates were far more severely affected by the number of loci sampled than by the number of individuals sampled. The infinity value (∞) of genetic distance between *O. mossambicus* and *O. aureus* could be the result of the above defects. However, the unnecessary of sacrifice fish and the highly species-specific markers found in mucus esterase isozyme pattern, which make it possible to establish a standard when applying in species identification, especially in *Oreochromis* species. As for discussing the phylogeny of the red tilapia with the other *Oreochromis* species, we suggest that more enzymes and proteins system should be surveyed in order to confirm the phylogeny of the tilapias.

Acknowledgements: This investigation was supported by the Committee of Agricultural Planning and Development, Republic of China. We thank to Dr. I. C. Liao, Mr. C. K. Liu, Mr. H. Kou, Mr. T. C. Yu, and Mr. Y. Y. Ting for their generous supply of the tilapia.

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吳郭魚 (*Oreochromis*) 品系體表黏液之酯酶類酵素電泳差異性研究

吳 金 洌　　吳 孝 芸

利用魚體體表黏液進行膠體電泳，所得之酯酶電泳圖，可作爲吳郭魚 (*Oreochromis*) 屬的分類標記。Es-1^c 和 Es-1^b 對偶基因可作爲尼羅魚 (*O. niloticus*) 種的標記，但 Es-1^b 對偶基因並不時常出現於電泳圖上。奧利亞吳郭魚 (*O. aureus*) 則以 Es-1^b 對偶基因當作種的標記。南洋鯽魚 (*O. mossambicus*) 則以 Es-1^a 和 Es-1^c 二對偶基因作爲種的標記，至於紅色吳郭魚，其酯酶電泳圖則與尼羅魚相似。利用抑制劑實驗，可得知 Es-1 基因座乃屬於羧基酯酶 (carboxylesterase)。計算此四種魚之間的遺傳距離值 (genetic distance) 和遺傳相似值 (genetic similarity)，發現紅色吳郭魚和尼羅魚之間的遺傳距離值最爲相近，顯示二者之間有極相近的親緣關係。利用體表黏液酯酶電泳圖非但可確切地作爲本屬各種魚的鑑定，並且由於此法不需犧牲魚體，因此對養殖上而言，具有實際應用的價值。