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# MITOCHONDRIAL DNA IDENTITY OF *CROSSOSTOMA* (HOMALOPTERIDAE, PISCES) FROM TWO RIVER SYSTEMS OF THE SAME GEOGRAPHICAL ORIGIN

CHYNG-SHYAN TZENG[1], SHIH-CHIEH SHEN[1+]
and P. C. HUANG[2]

*Institute of Zoology*
*National Taiwan University*
*Taipei, Taiwan 10764, Republic of China*[1]
*and*
*Institute of Molecular Biology*
*Academia Sinica*
*Nankang, Taipei, Taiwan 11529, Republic of China*[2]

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**Chyng-Shyan Tzng, Shih-Chieh Shen and P. C. Huang** (1980) Mitochondrial DNA identity of *Crossostoma* (Homalopteridae, Pisces) from two river systems of the same geographical origin. *Bull. Inst. Zool., Academia Sinica* **29**(1): 11-19. Two previously named species, *Crossostoma lacustre* and *C. tengi* of Taiwan, were reevaluated for their identity using mitochondrial DNA organization as a fingerprinting index. With over 30 samples from each species, collected at three different localities, all individuals examined display identical patterns for parallel restriction endonucleases: *Acc*I, *Ava*I, *Ava*II, *Bam*HI, *Bgl*II, *Eco*RI, *Hae*II, *Hind*III, *Hpa*I, *Kpn*I, *Pvu*II, *Pst*I, *Sma*I and *Xba*I. Estimating the mitochondrial genome to be 16.86 kilobases, the homogeneity detected represents P=0.02 level of confidence for total similarity. We speculate that these species are indeed of the same genetic origin, as their spawning habitats share a common geological river system. Morphological differences on which earlier taxonomical studies were based could have arisen by mutations in the nuclear genes and are maintained throughout by geographical separation. We propose that these species be henceforth recognized as one valid species and placed under: *Crossostoma lacustre*.

**Key word**: Mitochondrial DNA, *Crossostoma*, Taiwan.

Freshwater fishes in Taiwan, first collected by Swinhoe during his travel to the Far East in 1857 were classified as 16 species by Günther (Günther, 1859-1870). Since then, additional species were recorded by Boulenger (1894), Jordan and Richardson (1908), Regan (1908), Pellegrin (1908) and Steindachner (1908). Concerted efforts were subsequently made by Oshima, who extended the number of species to 76 (Oshima, 1919). Ten new species, which had not been previously reported, were later added (Oshima, 1920). Except for the work of Fowler and Bean (1922), further studies on the systematics of Taiwan freshwater fishes have not been seen in the literature until the 1960s and even then they appeared intermittently. Major work concerns cataloguing and

+ To whom to address correspondence

recording of new species (e. g. Tzeng and Shen, 1982).

New species of fishes have often been named on the basis of distinct morphological and anatomical features. Although species are more aptly defined by genetics through outcrosses, difficulty in breeding for certain fish has necessitated a more descriptive approach to taxonomy. Recent advent of recombinant DNA technology, however, has resulted in methods with which genetic components, especially the organelle DNA, can be analyzed in great detail. Mitochondrial DNA, being maternally inherited and readily characterized, has been shown to be useful as a marker for phylogenetic relatedness between individuals (Nei and Li, 1981; Lansman *et al.*, 1981).

Among the freshwater fishes thus far identified in Taiwan, we note that there are instances in which different species may be genetically closely related, since they can be traced to a limited ecological niche. Moreover, their phenotypic distinction is often trivial. It appears that some of these phenotypic characteristics may well be encoded by simple or complex Mendelian determinants and it is entirely possible that the variations observed simply reflect segregation of progeny from the same species in the wild. To test this notion, we have chosen two species of *Crossostoma*: *C. lacustre* and *C. tengi* in two rivers, which by geological evidence diverged from the same prehistorical Northern Water System of Taiwan. The former is found exclusively in the Tadu River, while the latter is distributed throughout the Tachia River. These two species differ mainly in color, with *C. lacustre* being spotty and *C. tengi* uniformly blackish brown. In this communication we shall present evidence to show that these two previously named species from these rivers share common restriction enzyme cleavage patterns for their mitochondrial DNA. It is thus likely that they are not, genetically, two distinct species.

## MATERIALS AND METHODS

Specimens of *Crossostoma lacustre* and *C. tengi* for this study were collected from the Tadu River and the Tachia River, respectively, which run parallel east to west across the central area of Taiwan (Fig. 1). Samples were identified on site, brought back to the laboratory

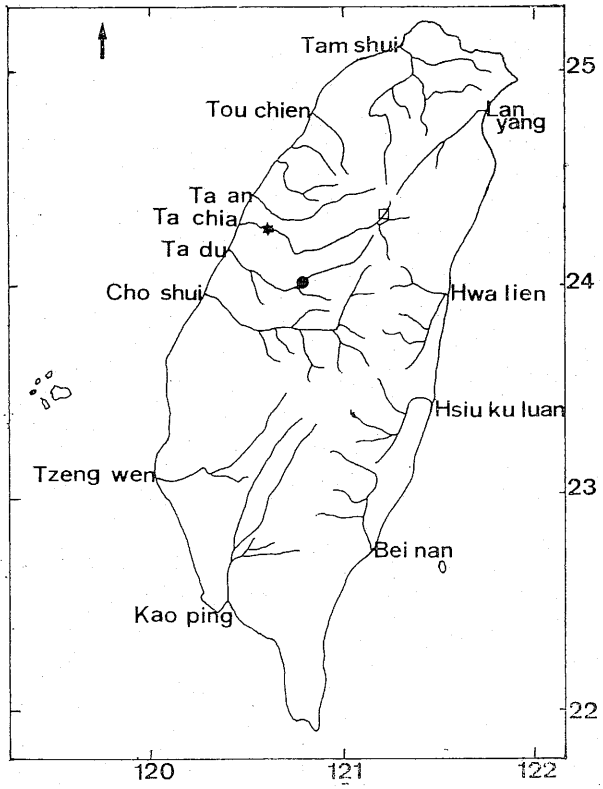


Fig. 1. Map of major river systems of Taiwan. Symbols denotes sites for sampling in this study. Individuals are classified based on the original nomenclatures: *Crossostoma lacustre* (●) and *C. tengi* (★) and (□). The exact collection sites and sample sizes for the former is Village Puli, Nantou County (N=52) and for the latter Village Tongshih, Taichung County (N=47) and Wulien Farm of Village Hopin, Taichung County (N=32), respectively.

and kept alive until sacrificed. Both the ovaries and livers were used for the extraction of mitochondrial DNA following the procedures developed by Chapman and Powers (1984) for fish. Restriction enzymes were purchased from Boehringer-Mannheim and used per supplier's specifications. Electrophoretic analysis of the digestion products was carried out on 1% agarose gel by standard procedure (Sambrook *et al.*, 1989). Cloning of the mitochondrial genome, determination of its sequences and deduction of physical and genetic map will be described in detail elsewhere.

## RESULTS

All of the *Crossostoma* fishes collected can be classified into two previously named species on the basis of criteria established by Steindachner (1908) for *C. lacustre* and Watanabe (1983) for *C. tengi*, respectively (Fig. 2). We note that there is little difference in their skeleton, average size and fin strips. The anal fin (2, 5), pectoral fin (1, 13-14) and ventral fin (1, 7-8) characteristics are alike. With a limited sample size, there is no obvious disparity detectable in the number of vertebrate and dorsal rays (Table 1).

MtDNAs from individuals of *C. lacustre* obtained from the Tadu, as well as other Northern rivers, share the same restriction endonuclease digestion patterns. The results, showh in Table 2, indicate that no detectable variation could be observed for fourteen enzymes, which include *Acc*I, *Ava*I, *Ava*II, *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Hae*II, *Hpa*I, *Kpn*I, *Pst*I, *Xba*I, *Sma*I and

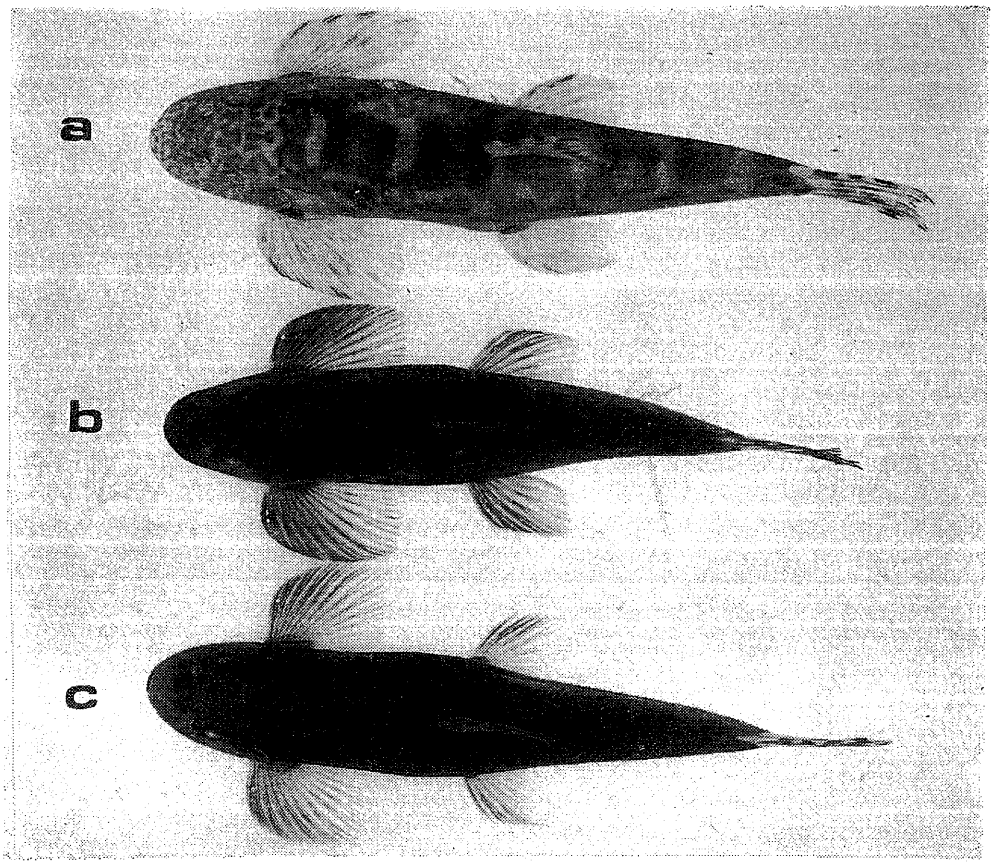


Fig. 2. Dorsal view of *Crossostoma lacustre* (a, Puli) and *Crossostoma tengi* (b, Wulien and c, Tongshih), identified according to their distinct coloration and as previously named.

Table 1
Morphological comparisons among *Crossostoma* fishes

| | Locality | No. of specimens | Vertebrae | | | Dorsal ray | |
|---|---|---|---|---|---|---|---|
| | | | 34 | 35 | 36 | 3.7 | 3.8 |
| *Crossostoma lacustre* | Puli | 27 | 3 | 17 | 7 | 0 | 27 |
| *C. tengi* | Tongshih | 3 | 0 | 3 | 0 | 1 | 2 |
| *C. tengi* | Wulien | 2 | 1 | 1 | 0 | 0 | 2 |

The number of vertebrae includes that of second, curving neck complex vertebrae and the dorsal end. Dorsal ray includes both the branching and unbranching ray. No significant morphological differences could be observed. Neither can differences be observed in anal fin (2, 5), pectoral fin (1, 13-14) and ventral fin (1, 7-8).

Table 2
Summary of data on restriction endonuclease digestion of *Crossostoma* mitochondrial DNA

| Enzymes digested | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Acc*I | *Ava*I | *Ava*II | *Bam*HI | *Eco*RI | *Hae*II | *Hind*III | *Hpa*I | *Pst*I | *Sma*I | *Xba*I |
| 4.50* | 6.25 | 4.0 | 8.25* | 8.43* | 6.55* | 5.42 | 9.70* | 6.40 | 9.00 | 12.4* |
| 3.90 | 4.10 | 3.15 | 8.25 | 8.43 | 4.05 | 2.85* | 5.80 | 6.10* | 5.80 | 4.5 |
| 1.90 | 2.05 | 1.6* | 0.36 | | 3.68 | 2.10 | 0.80 | 4.38 | 1.20 | |
| 1.40 | 1.54* | 1.14 | | | 2.02 | 1.80 | 0.63 | | | |
| 1.28 | 1.20 | 0.98 | | | 0.77 | 1.62 | | | | |
| 1.24 | 0.86 | 0.9 | | | | 1.44 | | | | |
| 0.76 | 0.53 | 0.71 | | | | 0.96 | | | | |
| 0.53 | | 0.62 | | | | 0.67 | | | | |
| 0.49 | | 0.53 | | | | | | | | |
| 0.42 | | 0.49 | | | | | | | | |
| 0.34 | | 0.41 | | | | | | | | |
| | | 0.35 | | | | | | | | |
| 16.76 | 16.53 | 14.88 | 16.86 | 16.86 | 17.07 | 16.86 | 16.93 | 16.88 | 16.00 | 16.90 |

A total of fourteen restriction endonucleases were used to digest mitochondrial DNA from *Crossostoma*, both *C. lacustre* and *C. tengi*. Results from eleven digestions were summarized. The resultant fragment sizes in Kb, listed under the enzymes respectively, were computed from mobility with calibrating standards run parallel electrophoretically on 1% agarose gels.

Not listed are *Bgl*II, *Kpn*I and *Pvu*II, which find no recognition and cutting sites in *Crossostoma* mtDNA.

Additional fragments smaller than 200 bp may be present in *Ava*II digests, but were not readily detected in the gel system used here, hence a lower total length when compared to the other digestions.

*C. lacustre* and *C. tengi* mtDNA restriction patterns are identical, except in fragments noted with a*, which are 0.22 Kb larger in the case of *C. tengi* from downstream Tachia and *C. lacustre* of Tadu Rivers.

*Pvu*II. Its size, 16.86 Kb, is identical to that of *C. tengi* obtained from Wulien, upstream of the Tachia. *C. tengi* from upstream or downstream of the Tachia River are similar in their mtDNA, having identical restriction patterns except for an extra sequence of 220 bp in those obtained downstream (Fig. 3). Figure 4 is a physical map of *C. lacustre* based on Southern hybridization (Sambrook *et al.*, 1989), using cloned restriction fragments (*C. lacustre*/*Hind*III, 2.85 Kb) as probes. These results show that *Crossostoma* has a well-defined mitochondrial genome,

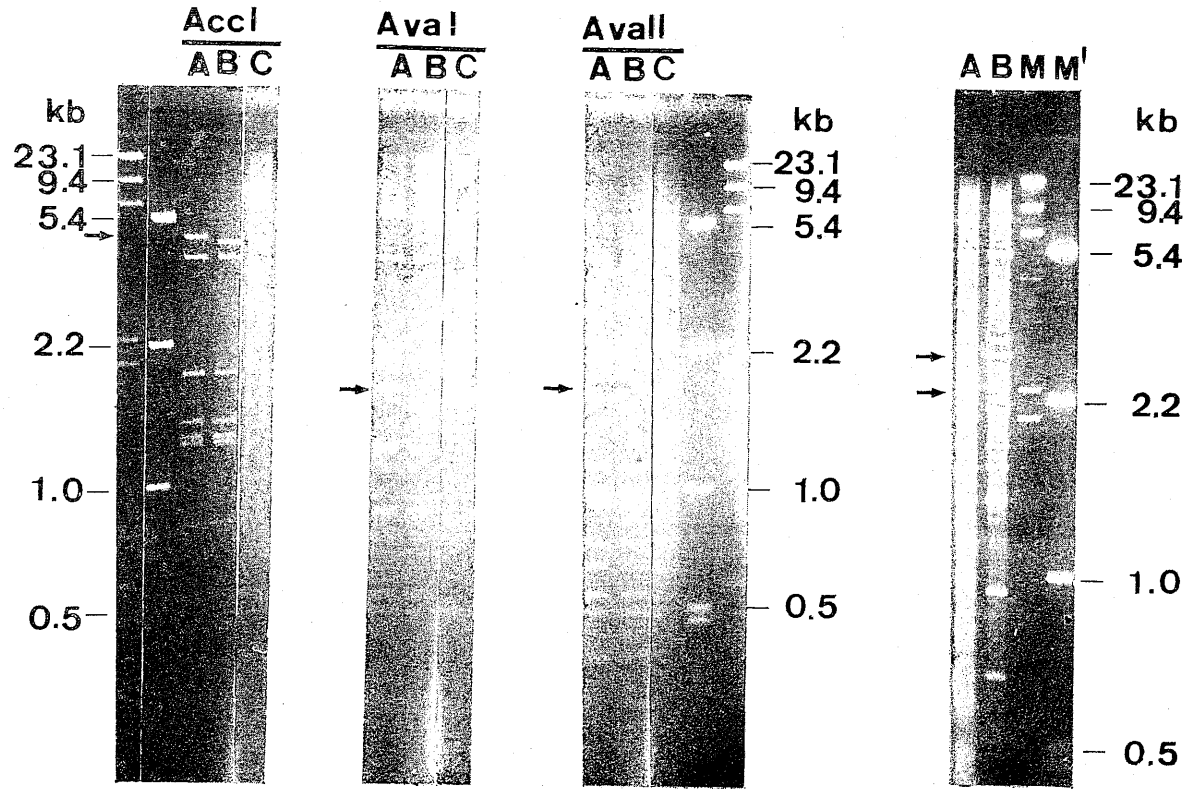


Fig. 3. Polymorphism in mtDNA from *Crossostoma.*

Left three panel—mtDNA from *C. tengi* obtained from downstream (A) and upstream (B) were compared, along with *Noemacheilus toni* (Dybowski) (Homalopteridae) of Hokkaido (C). All three restriction endonucleases *Acc*I, *Ava*I and *Ava*II yielded identical digestion patterns for *C. tengi*, except the extra length of 220 bp (pointed by arrow) for mtDNA of *C. tengi* downstream (A). Markers shown are *Hind*III fragments of lambda and PM2, respectively.

Right panel—Restriction enzyme fragment length polymorphism is evident in *C. tengi* from upstream Tachia (B). Arrows indicate the *Hind*III digestion fragment missing in *C. lacustre* from Tadu (A). M and M′ are *Hind*III digested lambda and PM2 markers as in the left three panel.

which is apparently rather stable in contrast to other heteroplastic species. Details of the sequence data and deduced genetic map will be presented elsewhere (in preparation).

## DISCUSSION

We show in this study that two previously named species of fishes within the genus of *Crossostoma* share identical sites for restriction enzyme recognition in their mitochondrial DNA. Since as many as fourteen restriction endonucleases were used, each of which would recognize five or six-base sites, a total of over 63 sites have been surveyed. By both of the equations of Nei and Li (1979) and Nei and Tajima (1981) one can estimate that P nucleotide sequence divergence between these two previously classified species of *Crossostoma* can be estimated to be equal to or smaller than 0.02 with a standard deviation less than 0.014. This estimation is made by assuming that the molecular weight of *Crossostoma* mtDNA is about

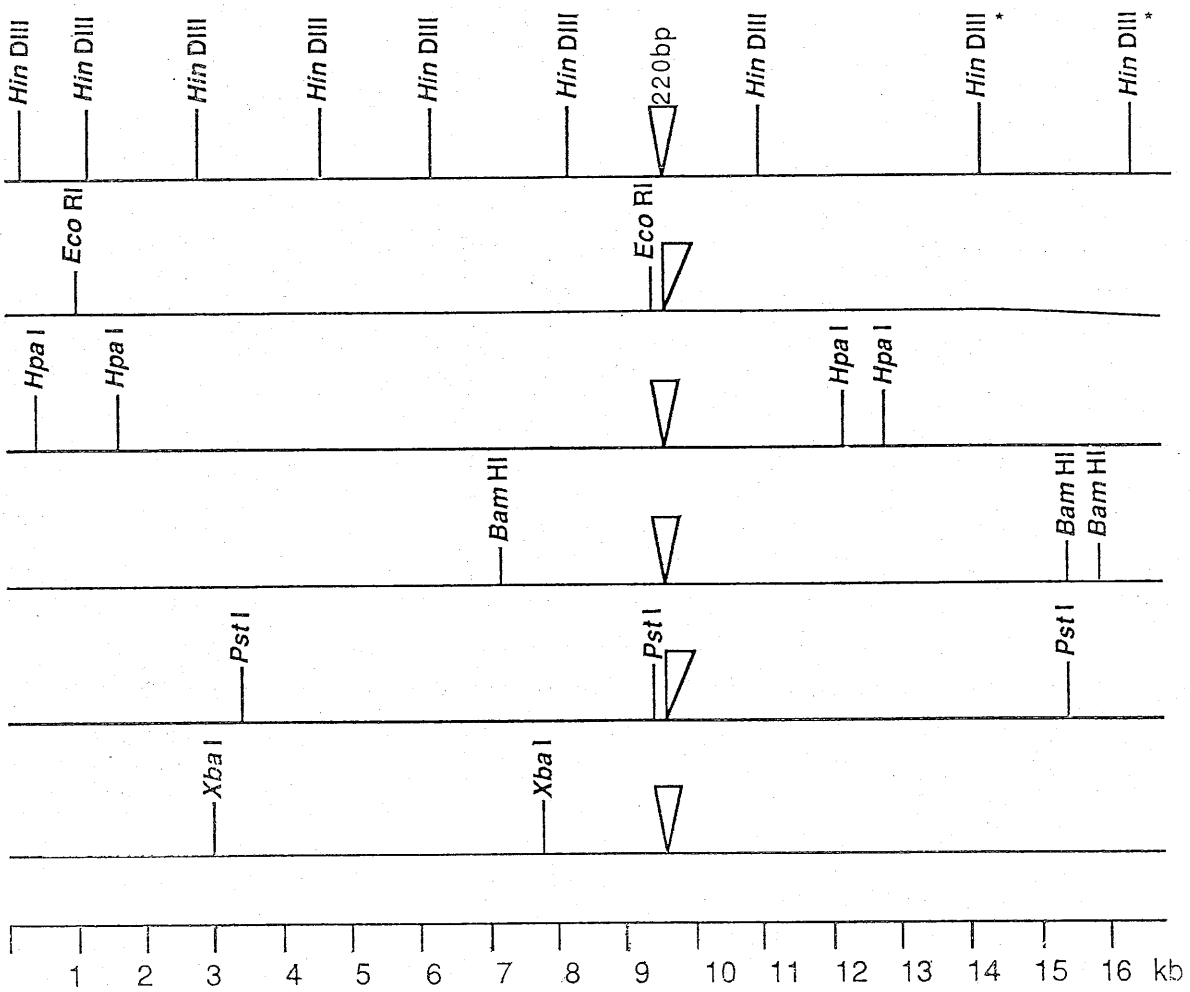


Fig. 4. Physical map of *Crossostoma*. Individual restriction fragments from *Hind*III are cloned into Bluescript plasmid and radiolabelled as probes for hybridization with DNA fragments obtained with other restriction endonucleases, following the standard Southern protocol (Sambrook *et al.*, 1989). The map was constructed by overlapping and oriented by matching with homologous DNA sequences with GCG algorithm, with both the EMBO and GenBank™ nucleic acid data stored on microvax II at IMB, Academia Sinica.

The extra length of 220 bp of mtDNA of *C. tengi* downstream of Tachia River and *C. lacustre* of Tadu River are located at the ▽ marker site.

Two *Hind*III sites (*) are absent in the mt*DNA* of *C. lacustre* of Tadu River (see also in Fig. 3, right panel).

16,860 and that all nucleotides are randomly distributed in the genome and for each site the expected frequency is about 0.0003 (Nei and Li, 1979). Thus the probability that major substitution exist is limited. This finding would suggest that these species are the same and the more recent naming of *C. tengi* is unwarranted.

The use of mitochondrial DNA as a marker for taxonomy has generated several instances in which genetic identity must be reconsidered. On the other hand, new species may be so recognized. The American eels, *Anguilla rostrata* and *A. anguilla*, were considered the same panmictic population, yet differences in

mitochondrial DNA set them apart genetically (Avise *et al.*, 1986). Others have shown that species of fishes from the same genus have between 2 and 10% variation in their mitochondrial DNA sequences (Avise *et al.*, 1987; Sakaizumi, 1987; Bentzen *et al.*, 1987; Bermingham *et al.*, 1986; Hanzawa *et al.*, 1987; Kornfield *et al.*, 1987). A specific example is the interspecies divergence of 2 to 3.5% between *Salmo clarki* and *S. gairdneri*. In constrast, intraspecies divergence of *Salmo* seldom exceeds 1.5% and is often below 1% (Wilson *et al.*, 1984). Heteroplasticity of mitochondrial DNA between geographically separated populations of the same species has also been noted among anadromous fish (Chapman *et al.*, 1982). While distinct restriction patterns can be used to characterize a variety of marine fishes in Taiwan (Huang, 1986), we have observed extensive variations of restriction enzyme fragment length polymorphism (RFLP) within a school hagfish (*Paramyxine* sp.) collected from the Southeastern and Southwestern seas surrounding Taiwan (K.F. Huang, H.K. Mok and P.C. Huang, in preparation).

On the other hand, progenies showing cryptic morphological differences may be genetically identified as one species. Several examples exist in fresh water fishes of Taiwan that may be analyzed. *Spinibarbus elongatus*, (Cyprinidae) (Oshima, 1920) for instance, is distinguished from *S. hollandi* (Oshima, 1919) on the basis of additional lateral line scales (28-29 vs. 26-27). Similarly, *Acrossocheilus formosanus* (Cyprinidae) differs from *A. invirgatus* in the presence of color strips in the former (Oshima, 1923). *Lissocheilichthys matsudai* (Cyprinidae) and *L. paradoxus* are considered separate species due to cryptic variation in the structure of their mouths. Other minute morphological differences have also been used to classify *Hemiculter akoensis* (Cyprinidae), *H. macrolepsis* and *H. leucisculus*; to distinguish *Erythroculter aokii* (Cyprinidae) from *E. oxycephalus*; and to generate four species of *Liobagrus* (Bagridae): *L. formosanus*, *L. taiwanensis*, *L. brevianalis* and *L. adiposalis* (Chen, 1969).

We show in this study that individuals of *C. tengi* from two localities may differ by polymorphism and heteroplasmy. Their basic restriction patterns are, however, identical to *C. lacustre*. These observations enforce our notion that *C. tengi* and *C. lacustre* are genetically the same. While the phylogeny of heteroplasmy in this species is not known, genetic diversity may reflect organismal responses to environmental changes such as the increasing pollution of the downstream Tachia in recent years.

It is of interest to note that, geologically, the Tadu and Tachia rivers in which the *Crossostoma* species for this study thrive are both tributaries of the Northern Water System of Taiwan. Taiwan was postulated to have been formed from the coastal plain of China; the island remained connected to the mainland about one million years ago (Lin, 1957, 1966). Repetitive separation by water and rejoining by land of present day Taiwan with the Asian continent and gradual uplifting of the central mountain range during the Pleistocene epoch generated a series of lesser ranges, plateaus and valleys. It is further posturated that the well-watered valleys evolved only 10,000 years ago into the 14 major rivers running east-west across the island today. These rivers form two major water systems, Northern and Southern, which have been isolated by the land rise named Formosan Bank since the middle and late Pleistocene. Yet rivers within each of these systems were at one time communicable, as at least ten distributional types of freshwater fishes and their origin could be recognized (Tzeng, 1986). The rivers Tadu and Tachia, being completely separated

today, lie in the Central West sub-district of the Northern Water System where waters for the Southern System also converged in the early days. As noted by Lin (1957a), the upper Tachia we see today could well be where the Tadu originated. It is not unreasonable to expect that segregation of a given fish species under such a geographical setting would result in faunal diversity (Bermingham *et al.*, 1986).

It remains an enigma how each of two phenotypically distinct populations of the same species from *Crossostoma* are found in separate rivers of identical age, length (110 vs. 120 kms) and latitude (24.3°N Tachia vs. 24.1°N Tadu). Neither is there an obvious difference in the water or environmental quality that can have contributed to the development of their specific coloration, although fishes in general are known to adapt by developing unique skin color. Further studies into the genetics of these and other fishes in question for taxonomical identity may shed light into these phylogenetic uncertainties.

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# 以粒腺體去氧核醣核酸 (DNA) 鑑定同源水系之纓口鰍

曾晴賢　沈世傑　黃秉乾

本研究以粒腺體去氧核醣核酸（DNA）構造的分析，重新檢討臺灣以往所發表的臺灣纓口鰍 *Crossostoma lacustre* 和鄧氏纓口鰍 *C. tengi*。在兩條不同河流（大甲溪上、下游及大肚溪中游）採集到每種 30 尾以上的標本，分別以 14 種限制內切酶進行分析鑑定。發現該兩種魚類的粒腺體去氧核醣核酸 (DNA) 約長 16,860 個鹼基，而且在大甲溪下游和大肚溪中游的族羣裏，具有較長一段 220 個鹼基之構造。除了在大甲溪上游的族羣內可以檢視到一個內切酶切位的變化之外，其餘不論是種間和種內的相異度均小於 P=0.02。因此認爲原來的這兩種仍應歸屬於同一種臺灣纓口鰍 *C. lacustre* 較爲恰當。

本研究同時建立了本種魚類的粒腺體去氧核醣核酸 (DNA) 之物理圖 (physical map)。