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GENETIC IDENTIFICATION AND DIFFERENTIATION OF THE FORMOSAN LANDLOCKED SALMON, *ONCORHYNCHUS MASOU FORMOSANUS*, BY RESTRICTION ANALYSIS OF MITOCHONDRIAL DNA

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Ken-Ichi Numachi, Takanori Kobayashi, Kun-Hsiung Chang and Yao-Sung Lin (1990) Genetic identification and differentiation of the Formosan landlocked salmon, *Oncorhynchus masou formosanus* by restriction analysis of mitochondrial DNA. *Inst. Zool., Academia Sinica* 29(3, Supplement): 61-72. Totally 29 specimens of the Formosan salmon consisted of 8 natural fishes caught at the Chichiawan Stream of Tachia River system, and 21 fishes of the first generation artificially propagated from the fishes collected at the same stream were studied by restriction endonuclease fragment pattern analyses of mitochondria DNA (mtDNA) using 10 kinds of 6 base-pair restriction endonuclease. All the 29 specimens of the Formosan salmonid showed the same single clonal genotype of mtDNA. This clonal genotype was much similar with one clonal genotype found in populations of the masu salmon, *Oncorhynchus masou masou* distributed in Japan. The base pair-substitution (σ) calculated between the above two types by the approach of Nei and Li (1979) was 0.202%. The heighest value of the base pair-substituon among the Formosan and Japanese masu salmon was 1.59%. These value corresponded to 101 and 795 thousands years in differentiation time. The classification, nomenclature and distribution range of the masu salmon including the Formosan salmon were reviewed, and concluded that the Formosan salmonid originated from the masu salmon distributed in the Sea of Japan through the Tsushima channel at 100~800 thousands years ago.

Unusual homogeneity of mtDNA type in the population of the Formosan salmon was considered to be accounted by contracted stage of the population.

Key words: Formosan salmon, Genetic differentiation, mtDNA, *Oncorhynchus masou*, Restriction fragmental pattern.

The Formosan landlocked slamon (*Oncorhynchus masou formosanus*) was first reported by Aoki (1917), and Jordan and Oshima (1919). This landlocked salmon distributed at the southern limit in the distribution range of salmonids has attracted much attention from taxonomi-

cal, morphological and ecological points of view (Behnke *et al.*, 1962; Oshima, 1934, 1936; Teng, 1959; Watanabe and Lin, 1985). This species was first considered as identical with a southern local form or subspecies of masu salmon (*O. masou ishikawae*), native at the Pacific side of southern part of Japan, known as Amago on the basis of the description of the presence of red spots on the back. However, Oshima (1936) revised this description, and considered that the Formosan landlocked salmon is identical with the northern form of masu salmon (*O. masou masou*), called Sakuramasu or Yamame, widely distributed in the Sea of Japan and Sea of Okhotsk, and streams down to these waters. He suggested that the masu salmon distributed in the Sea of Japan extended the distribution rage to the old East China Sea in the pleistocene, and a part of the population persisted at the restrict regions of upper streams of the Tachia (Taiko) River.

We interested in estimating the amount of genetic differentiation of the Formosan landlocked salmon from the major population of masu salmon distributed in Japan. We expected that this would suggest us the differentiation time of the Formosan salmon. In this study we examined mitochondrial DNA (mtDNA) divergence of the Formosan salmon from the masu salmon distributed in Japan by analyzing restriction fragment patterns. River populations and artifically sustained population of masu salmon in Japan so far examined contained several clonal genotypes of mtDNA, but 29 specimens of the Formosan landlocked salmon showed only one clonal genotype, and this type much resembled to one clonal genotype found in population samples of masu salmon collected in Japan.

MATERIALS AND METHODS

Samples: Totally 29 fishes of the Formosan landlocked salmon consisted of 8 fishes caught at the Chichiawan Stream of the area of Wuling Farm in the Tachia (Taiko or Daiko) River on 26, July 1989, and 21 specimens of artifically propagated stock cultured at the hatchery of the stream were used for this study. The artificially propagated stock is the first generation raised by artificial insemination of gametes from the natural fishes caught at the Chichiawan Stream. The number of parents used for propagation was not exactly known, but estimated less than 20. Body length, weight and sex of the specimens are listed in Table 1.

Tissue disected from the specimens were packed in plastic bags and kept in a insulated container with crashed ice, and sent to the laboratory for mtDNA extraction.

mtDNA purification: Extraction of mtDNA was started within 48 hrs after sacrifice the fishes by using liver. Livers from each specimens were homogenized in a 3 volumes of 0.25 M sucrose solution containing 1 M Tris / HCl, pH 7.4 and 0.5 M EDTA, with a motor driven glass-teflon Potter-Elvehjem type homogenizer. These were centrifuged at 1,000 X g for 10 mins. at 2°C, and supernatant were centrifuged at 10,000 X g for 10 min. Mitochondria pellets thus obtained was washed with EST solution (0.1 M EDTA, 0.15 M NaCl, 10 mM tris / HCl, pH 8.0). Pellets were lyzed by adding sodium dodecyl sulfate (SDS) solution at room temperature. Then, 5 M NaCl solution was added to the lyzates at a final con-

Table 1. Records of the specimens studied. The specimens No. 1~21 are artificially propagated fish and No. 22~29 are wild fish collected at the Chichiawan Stream of Taiko River system on July 26, 1989.

Specimens No.	Sex	Total length	Fork length	Body Weight
		cm	cm	g
1	♂	24.6	23.5	191.2
2	♀	22.6	21.5	139.8
3	♀	22.4	21.4	128.6
4	♂	25.2	23.3	203.2
5	♂	23.4	22.9	166.4
6	♂	22.8	21.8	138.8
7	♂	25.0	23.9	188.2
8	♀	21.1	20.3	113.8
9	♀	19.7	19.0	102.4
10	♂	28.6	27.5	296.6
11	♂	28.6	27.5	296.6
12	♂	29.5	28.4	315.7
13	♂	25.3	24.3	195.8
14	♀	25.5	24.4	197.4
15	♂	28.1	27.2	247.8
16	♀	22.7	21.8	154.2
17	♀	20.3	19.5	92.4
18	♂	25.2	24.0	183.6
19	♂	22.1	21.1	142.4
20	♀	25.2	24.3	182.4
21	♀	22.7	22.5	148.8
22	♂	32.0	30.6	358.6
23	♀	26.8	25.9	192.4
24	♂	24.2	23.6	143.4
25	♀	24.8	24.0	150.8
26	♂	23.2	21.9	134.2
27	♀	19.2	18.5	71.8
28	♀	21.5	20.6	96.4
29	♀	22.8	21.9	127.4

centration of 1 M, to precipitate nuclear DNA according to the method described by Hirt (1976) originally developed for purification of plasmid DNA. After immersing the sample tube in ice water for 1 hr to precipitate SDS, the samples were centrifuged 10,000 X g at 2°C for 10 min. Proteins were removed by phenol, phenol-chloroform. After removing traces of phenol by mixing with an equal volume of chloroform-isoamyl alcohol, DNA was precipitated by adding chilled ethanol. The precipitated DNA was dried by vacuum, and was solved in 1 ml TE buffer (10 mM Tris / HCl, 1 mM EDTA, pH 8.0. After adding 6 μ 1 RNase A solution (10 mg/ml), samples were incubated for 20 min. to 1 hr at room temperature. Dialyses against 0.1 mM EDTA, pH 8.0 were made overnight at 2°C.

Crude mtDNA thus obtained could be directly used for digestion. Whenever necessary, a new rapid and simple ultracentrifugal purification (Numachi and Kobayashi) were applied. To 0.75 ml of the crude mtDNA solution, 1.25 ml of cesium chloride solution (1.2 g/ml TE solution) and 20 μl ethidium bromide (10 mg/ml) were added, and centrifuged 399,000 X g for 3 hrs in a 2 ml quick-seal tube, at 20°C in a vertical rotor TLV-100 of Beckman TL-100, applying deceleration and acceration profile 5. The two bands, closed circular mtDNA and open circular DNA containg linear nuclear DNA could be clearly separated, the distance between the two bands is about 7 mm. Both the fractions were collected by a bottom puncture device, Beckman Biobox. Ethidium bromide (EtBr) was removed by water saturated isopropanol, and dialyses against 0.5 mM EDTA solution were made.

mtDNA digestion: Ten kinds of following 6 base-pair sequence recognition endonucleases obtained from Takara Shuzo Co, were used for digestion of mtDNA purified from 29 specimens of the Formosan landlocked salmon; *Acc* I, *Ava* I, *Bam*H I, *Bgl* I, *Bgl* II, *Eco*R I, *Hin*c II, *Hin*d III, *Pst* I, *Xba* I. Digestion was completed within 4 hours at 37°C using 10~20 units of restriction endonuclease for 20 μl of mtDNA solution.

Gel electrophoresis: Electrophoresis was made in 1% agarose gel (Seakem LE or Takara H 14) in TPE buffer (36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA2Na) at a constant current of 12 mA/1 cm^2.

Restriction fragment patterns obtained of each enzyme digestions were stained by EtBr solution (2 mg/1), and recorded on Polaloid 667 film using transmitted 310 nm UV light.

RESULTS

We have demonstrated 22 clonal genotypes of mtDNA in masu salmon consisted of 86 specimens of 5 population samples collected at the northern part of Japan, by examining restriction fragmental patterns of ten kinds of restriction enzymes mentioned above (Kobayashi, 1988; Kobayashi and Numachi). Retraction sites were totally 49, and variant patterns were observed in 7 of 10 enzymes digestion as in Fig. 1. The number of morphs were 4 in *Bgl* II and *Hin*d III digestion, 3 in *Acc* I, *Ava* I, and *Hin*c II, and 2 in *Bam*H I, and *Pst* I.

All of the 29 specimens of the Formosan landlocked salmon showed the same morph in all the 10 enzyme digestions. Table 1 shows the length of fragment produced by 10 restriction endonuclease. The fragments less than

Table 1. The number and length of fractions formed by restriction endonuclease digestion of mtDNA from the Formosan masu salmon. The length is shown in K base-pairs. The total length of mtDNA calculated in each digestion is shown at the bottom. The morph name for each enzyme digestions used commonly throughout *Oncorhynchus masou* is shown in parentheses.

Fraction	*Acc* I (B)	*Ava* I (A)	*Bam*H I (B)	*Bgl* I	*Bgl* II (B)
1	7.00	7.80		12.00	
2.			12.00	4.90	12.00
3	3.80	3.15	5.40		
4		2.60			
5	3.20				3.90
6		2.15			
7	2.10	1.20			
8	1.90				1.10
9	0.80	0.48			
Σ	18.80	17.38	17.40	16.90	17.00

Fractions	*Eco*R I	*Hinc* II (C)	*Hind* III (B)	*Pst* I	*Xba* I
1	12.00	5.00		17.00	5.90
2	4.40				
3		4.00	4.10		4.10
4			3.90		3.70
5		3.10	3.10		2.70
6		2.70			1.65
7		1.85	2.40		
8		1.10	2.30		
9		0.80			
10		0.25	1.75		
11			0.46		
Σ	16.40	18.80	18.01	17.00	18.05

300 base pairs (bp) could not be detected by ETBr staining due to the lowered staining intensity and fluorescent background in this position. The mean length of mtDNA calculated by averaging the sums of all the digestions was 17.5 ± 0.815

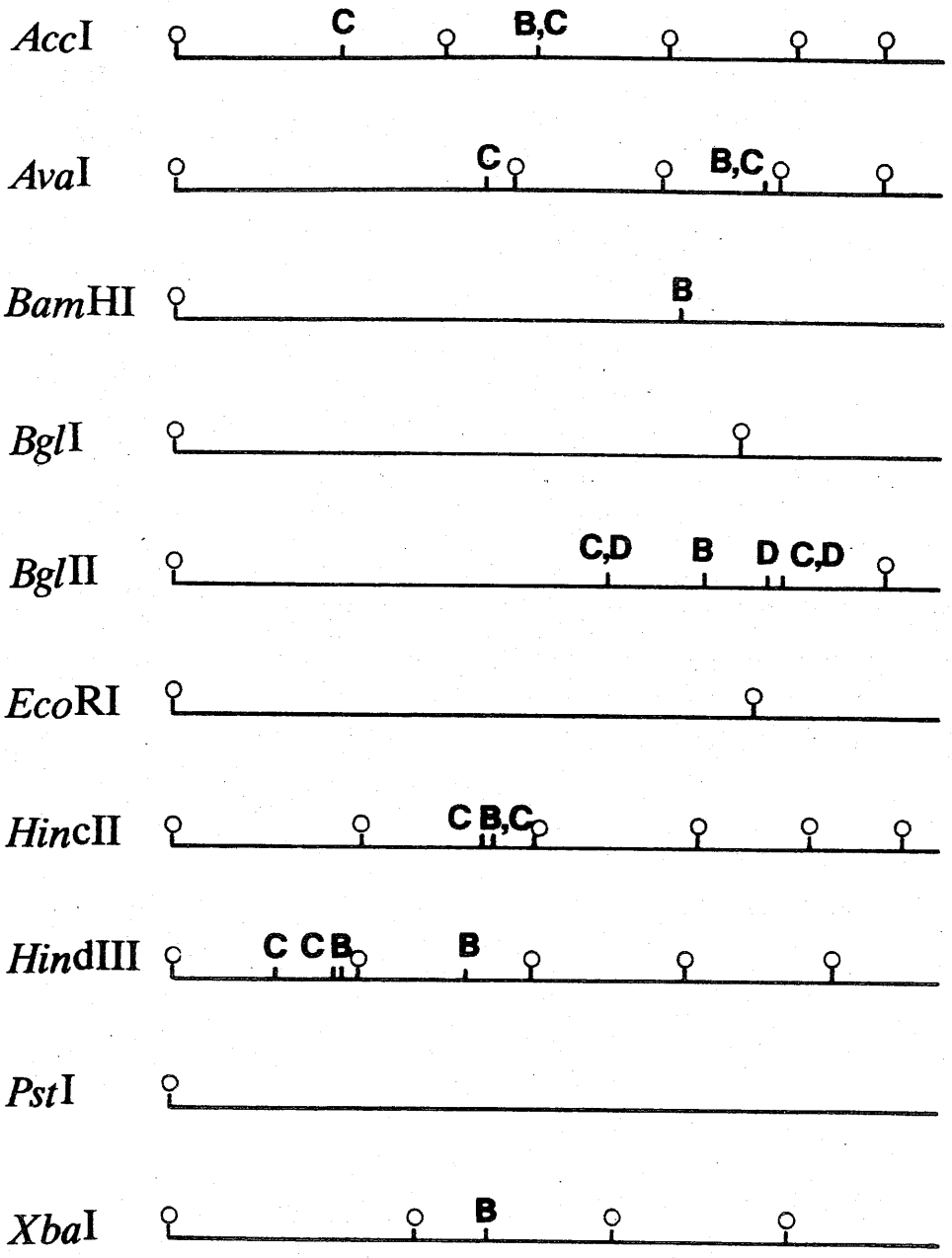

Fig. 1. Restriction maps of mtDNA from the Formosan masu salmon for 10 kinds of restriction endonuclease. Restriction sites are shown by vertical lines topped with the following symbols: ○, common sites to all the morphs; B~D, the specific sites of each morph.

Kbp. This is very same with the value so far reported of mtDNA in a variety of organisms.

The clonal genotype of mtDNA found in the Formosam salmon was unique of this species, and termed as clonal genotype 23. This clonal genotype 23 was very similar with the clonal genotype 7

which was predominant (74%) in an articially sustained population, Okutama strain. The clonal genotype 23 showed the same morph with the clonal type 7 for 9 of 10 restriction enzymes, but only the *Ava* I morph was different from type 7, showing the morph A instead of the B in the type 7 (Fig. 2). The clonal genotype 7 was also found in Hokkaido strain in 25% of incidence. Estimated number of nucleotide substitutions per nucleotide site (σ) among the clonal genotypes were calculated by the approach of Nei and Lie (1979). The base pair-substitution (σ) between the type 23 and type 7 was 0.202%. Assuming the rate 2% substitution per base pair per million years of differentiation time according to Brown *et al.* (1979), this substitution corresponds to 101,000 years. The differentiation time between the clonal genotype 23 and 18 on the basis of nucleotide substitution (σ= 0.0159) in which the most big difference was observed among all the combination of clonal genotypes, was 795 thousands years. According to Gotoh *et al.* (1979), nucleotide divergence and divergence time between the type 23 and 7 were 0.213 and 144,489 years respectively, and nucleotide diversity and divergence time between the formosan salmon (No. 23) and Okutama population of *O. masou masou* were 0.529 and a 360 thousands years.

DISCUSSION

The masu salmon, *Oncorhynchus masou* is distributed in a rather limited area of only the western side of the North Pacific (Fig. 3), in contrast with the other 5 species of *Oncorhynchus*. This species shows a remarkable variability in morphological, physiological, and ecologiccal features. A part of fish spends all the life in fresh water and can come to mature. The others even of the fish inhabited in the same stream go to sea. There are dis-

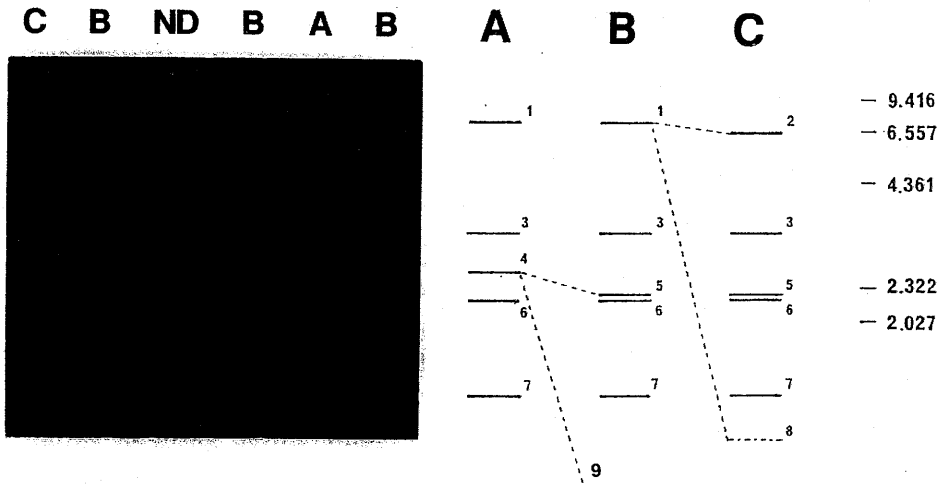

Fig. 2. Comparison of restriction fragment patterns of morphs of mtDNA in *Oncorhynchus masou* observed after digestion with *Ava* I. At the top of pattern, the name of morph is shown, ND means control sample of no digestion. All the Formosan salmon showed the morph A for *Ava* I.

Fig. 3. Distribution range of *Oncorhynchus masou.*

tinct differences in the body length and coloration between the anadromous and fresh water fishes. Even among the anadromous, some fishes called Taiko-masu are very high in body height, and some are much slender, and a big differences in the body length can be also observed at the same year class. Thus, there has been many confusions between taxonomists in the classification and nomenclature. At present, such variable forms become to regard as a single species *O. masou*, including *O. masou masou* of anadromous called Sakuramasu, and juveniles and freshwater forms known as Yamame, distributed in the north of the major zone of the distribution range: *O. masou ishikawae* called Amago, red spotted and distributed in a part of the southwest of the Main Island of Japan, Shikoku and northeastern part of Kyushu, Japan; and *O. masou subsp.* or *rhodurus* known as Biwamasu native in the lake Biwa. In genetic distance (D) calculated on the basis of the isozyme analysis according to Nei (1971), these forms mentioned above showed much similarity ($D < 0.03$), which corresponds to the differences usually observed among population level (Numachi, 1982, 1984). Thus, the interests are focused on the Formosan masu salmon, especially of the identity

and origin of this species geographically isolated distinctly from the major distribution range of masu salmon.

The Formosan landlocked masu salmon first named as *Salmo formosanus* by Jordan and Oshima (1919) have been regarded as *Oncorhynchus masou* by Oshima 1936, Behnke *et al.* (1962) from morphological view points, and designated as *O. masou formosanus* by Watanabe and Lin (1985). Behnke *et al.* (1962) speculated the possibilities of existence of plural endemic salmonids persisted in Formosa, concerning to the presence or absence of basibranchial teeth and red spots. The presence of red spots, as in the Amago first described in the original description (Jordan and Oshima, 1919) was denied by one of the author (Oshima, 1936) and Behnke (1959), and no one has demonstrated the red spots in the Formosan masu salmon so far. Thus, the Formosan masu salmon has been regarded to resemble to *O. masou masou*, Sakuramasu or Yamame, in outer coloration. The presence of the basibranchial teeth in the specimens examined in present paper are now studying. However, nevertheless the presence or absence of the basibranchial teeth, there are many reason that only one species of the masu salmon is existing in Formosa at present.

The distribution range of the Formasan masu salmon was reported to be extended over six streams of the Tachia (Taiko or Daiko) River system (Kano, 1940), but the present distribution area is restricted only at a single stream of the Chichiawan Stream in the Tachia River system (Lin *et al.*, 1989). The carrying capacity of the stream for the landlocked masu salmon in this range should be 5,000 at most. In fact, the population size of the fish is estimated 650 to 1,800 in the survey of 1986 to 1987 (Lin *et al.*, 1988).

In the present report, we demonstrated only one clonal genotype in mtDNA after examining all the specimens of the masu salmon consisting of 8 wild from the Chichiawan Stream and 21 fish artificially propagated from the fish captured at the same stream. This indicates that all the specimens examined in this report are the same species, and it seems very probable that this only one species exists in this stream at present. Furthermore, remarkable genetic homogenity demonstrated in mtDNA should be accounted by bottleneck effect in the population size. The small population size mentioned above or a more contracted stage of the population size in the Formosan salmon probably caused such marked genetic homogeneity of individuals in this population or species. This homogeneity is unusual. In the survey of mtDNA variation of the natural populations and artificially sustained populations of masu salmon in Japan, more than four clonal genotypes were demonstrated in a single population (Kobayashi, 1989; Kobayashi and Numachi, 1990), and this level of polymorphism or variability is common in a variety of fish species (Bermingham and Avise, 1986; Hanzawa *et al.*, 1987). The genetic homogeneity observed in the Formosan masu salmon is to be especially noted. We are now examining variability in genetic loci coding to various enzymes.

Neave (1958) suggested that *Oncorhynchus* diverged from *Salmo* at or near the beginning of the pleistocene, that is about 2 million ago, and that *O. masou* is most closely related to *Salmo* of all existing *Oncorhynchus* showing unspecialized or primitive features in anatomi-

cal, ecological and physiological traits. In this assumption, it should be considered that the ancestral species of *Oncorhynchus* should be much similar to *O. masou*. This was strongly supported in our isozyme analysis of salmonid species (Numachi, 1982, 1984). In the dendrogram obtain on the basis of genetic distance (D) between the species calculated from the results of isozyme analysis, salmonids largely divided into two groups, *Oncorhynchus-Salmo* and *Salvelinus* group, and the former consisted of two groups, *Oncorhynchus* and *salmo*. The *Oncorhynchus* group was composed of five species of *Oncorhynchus*. *O. masou* was not included in this group, but was comprised in the *Salmo* group. Differentiation time (t) of the species in the *Oncorhynchus-Salmo* group were estimated 428 (2,895) thousands years, and of species in *Oncorhynchus* and of *Salmo* group 200 (1,350) and 290 (1,960) thousands years respcectively, assuming $t = 7.4 \times 10^6 D$ ($t = 5 \times 10^6 D$) according to Nei 1971 (1975). Thus, the view that the differentiation of *Oncorhynchus* and *Salmo* took place after the pleistocene was supported from the genetical data.

The minimum and maximum of divergence time on the basis of base pair-substitution (δ) between the clonal genotypes in mtDNA from the Formosan and Japanese masu salmon corresponded to the differentiation time 100 and 800 thousands years. The exact estimation of differentiation time should be difficult, because of the substitute position at the codon, transition or transversion type change, and kinds of animals etc. are known to make change of the speed in molecular clock. Monomorphism of mtDNA in the Formosan masu salmon tends to increase the deviation in estimation the time of differentiation. However, it should be very important to note that the formosan masu salmon is much similar in the base pair arrangement of mtDNA with some of masu salmon distributed in the north part of Japan. We suppose that the masu salmon distributed in Formosa came from the Sea of Japan through the Tsushima channel (Fig. 3), but not from the Pacific coast, at 100~800 thousands to no more than one million years ago, when the cold current came from the Sea of Japan to the Formosa channel, and a part of population persisted at the cold upper streams of the Tachia River.

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以粒腺體去氧核醣核酸限制分析探討臺灣陸封性櫻鮭之遺傳鑑別及其分化

沼知健一　小林敬典　張崑雄　林曜松

利用 10 種限制內切酶分析 8 尾捕自大甲溪系七家灣溪及 21 尾經人工繁殖 第一代 臺灣櫻鮭的粒腺體 DNA 之被限制酶所切下來的片段型式。研究上述野生及人工繁殖之臺灣櫻鮭其粒腺體 DNA 均呈現相同的基因型。此基因型與日本櫻鮭之族羣非常相似。由 Nei 及 Li 的公式計算出上述兩基因型間之鹼基對取代率爲 0.202%。而臺灣櫻鮭 與日本櫻鮭間最高爲 1.59%。由這個值 來換算相當於 101,000～795,000 年之前臺灣櫻鮭與日本櫻鮭就分開來。回顧包括臺灣櫻鮭之分類，命名及分佈來討論臺灣櫻鮭係經由日本海之津島海峽在 100,000～800,000 年移棲過來的。臺灣櫻鮭族羣的粒腺體 DNA 呈現均質現象，我們可以認爲是族羣的萎縮時期。