Preliminary Analysis of Molecular Biological Methods for Stock Identification of Small Yellow Croaker (*Pseudosciaena polyactis*) in the Yellow Sea

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The stock identification of small yellow croaker, *Pseudosciaena polyactis* from Mokpo area was carried out using molecular biological methods such as mt-DNA restriction fragment length polymorphism(RFLP) and the N-terminal fragment polymorphism of muscle actin obtained after protease digestion.

The entire mt-DNA genomic size from the small yellow croaker at Mokpo area was estimated to be about 16 ± 0.2 Kb. Furthermore, fourteen restriction endonucleases revealed a total of 37 restriction sites to the mt-DNA molecule, however, eight of the fourteen enzymes showed a significant restriction site variation. Six of the enzymes examined produced a single restriction profile for all individuals surveyed, indicating that they don't react on the same mt-DNA obtained from small yellow croaker. The *Staphylococcus aureus* V_8 protease is able to cleave the muscle actin of small yellow croaker and to yield a N-terminal peptide of 26 and 16 KDa, respectively.

Introduction

It is well known that the Yellow Sea containing a multispecies and a multinational fishery is one of the most intensively exploited area in the world. The number of species commercially harvested is about 100 including Cephalopods and Crustacea (Zhang *et al.*, 1988).

The populations of the Yellow Sea can be divided into two categories according to the habitats of resources, that is, nearshore and migratory groups. Small yellow croaker, known as a remarkable migratory species, have distinct seasonal movements and migrate out of the Yellow Sea to the East China Sea in winter season(Zhang *et al.*, 1988).

The habitat of spawning and migration of this species can be divided into four groups(NFRDA, 1979; Lee *et al.*, 1983); 1) migration and spawning population in the Yellow Sea including western part of Korean Peninsula, 2) the population from the central part of the Yellow Sea to the bay of Pohai, 3) the population in the southern part of Shandong Peninsula of the China, and 4) the population in the estuary of Yangtze River of the China, respectively(Fig. 1).

Small yellow croaker is one of the most commercially important demersal species not only in the Yellow Sea but also in the western part of the East China Sea. In Korea, the annual catch reached the highest in 1940s and early of 1950s but present annual catch is only about 20,000 to 30,000 MT which are mainly composed of young fish aged 0 to 1 year(Zhang *et al.*, 1988; 1992b). At present, however, the annual catch declined drastically year after year(Fig. 2), although the exploitation of spawning stock in the western Yellow Sea has been prohibited since 1980 and there has been no identified recovery of this species until now(Zhang *et al.*, 1988).

Mt-DNA and muscle actin analyses were employed to identify the small yellow croaker from Mokpo area. This study has an important implication for both political and economical aspects because

this is the first trial to examine the genetic relationships among the small yellow croaker stocks in the Yellow Sea including western part of Korea.

Currently, more precise techniques are urgently needed to delineate target stocks because traditional approaches such as tagging experiments, morphometric or meristic indices and electrophoretic techniques often produce inconsitent results. This approach to stock identification is often more sensitive than conventional allozyme studies and thus may expose additional differences among populations(Avise *et al.*, 1986; Kornfield and Bogdanowicz, 1987; Mulligan and Chapman, 1989).

In the present paper, we described the use of biochemical approaches to clarify the general pattern of mt-DNA variation and structural differences and similarities of muscle actin in small yellow croaker, *Pseudosciaena polyactis* caught from Mokpo area was done for preliminary experiment on stock identification among small yellow croaker with different site in the Yellow Sea.

One of the goals is to provide new insights into small yellow croaker population biology and to continue the process of understanding the zoogeography of important species with economic value in the Yellow Sea.

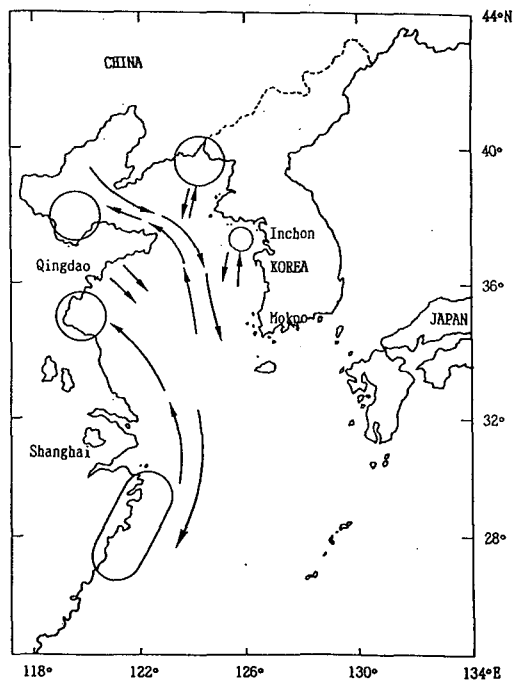

Fig. 1. Migration and spawning patterns of small yellow croaker, *Pseudosciaena polyactis* (from NFRDA, 1979; Lee *et al.*, 1983).

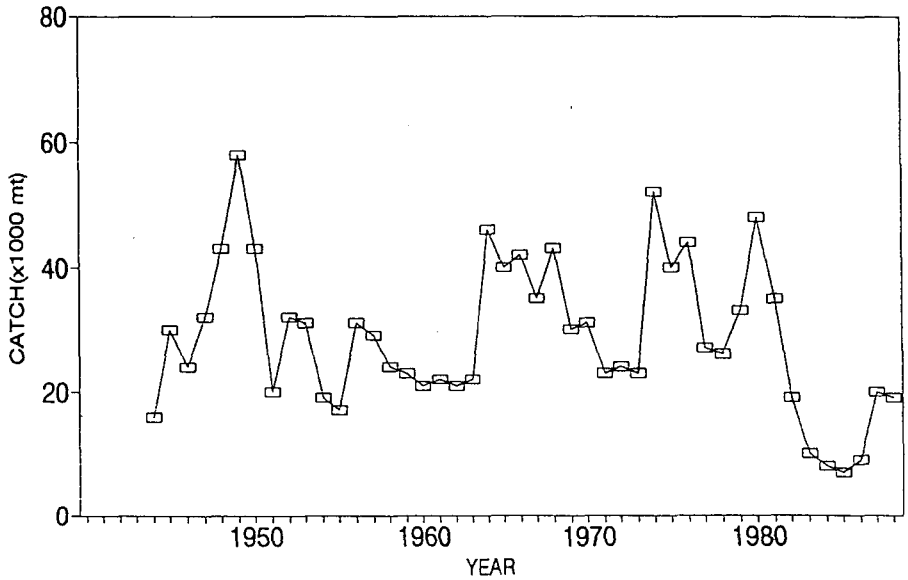

Fig. 2. Annual catches of small yellow croaker, *Pseudosciaena polyactis* (source Zhang *et al.*, 1992b).

Materials and Methods

Small yellow croaker, *Pseudosciaena polyactis* were collected by 150*m* stow net or bottom pair trawls from March to November, 1991 at Mokpo and Inchon area. The sample sizes were 37 individuals from above mentioned areas with average length of 15~20*cm*, respectively(Table 1, Fig. 3).

1) Mt-DNA preparation and electrophoresis

For sample preparations, fresh eggs were removed and immediately frozen in dry-ice on board. In laboratory, approximately 20*g* of each ovarian tissue sample was thawed gradually at 7℃, homogenized with 30*ml* of cold TEK buffer(50mM Tris, 10mM EDTA, 1.5% KCl, pH 7.5) and centrifuged(1,000×*g*) for 20 min at 4℃. After centrifugation, the resulting pellet was discarded, and this procedure was repeated twice for the supernatant. The resulting supernatant was centrifuged again at 12,000×*g* for 50 min. The supernatant from this spin was discarded, while the mitochondrial pellet was resuspended in the TEK buffer and centrifuged at 12,000×*g* for 50 min. The mitochondrial pellet was then resuspended in a 1% non-idet P-40 TEK solution and centrifuged at 12,000×*g* for 10 min to remove cell debris. The supernatant from this spin was decanted and mixed with an equal volume of water-saturated redistilled phenol to precipitate proteins. After through shaking, the sample was allowed to stand for 5 min before centrifugation at 12,000×*g* for 15 min. The aqueous layer was then drawn off and re-extracted with phenol to ensure the complete removal of proteins. The transparent aqueous phase was extracted with a chloroform: iso-amyl alcohol solution(24:1, V/V) to remove any trace of phenol. The aqueous phase was then mixed with 2 volume of cold 95% ethanol(4℃) and stored at -20℃ for further experiments. Nucleic acids were collected by centrifugation at 12,000×*g* for 15 min (Mulligan and Chapman, 1989).

Samples of mt-DNA were digested with six base recognition restriction endonucleases such as Apa I, Bam HI, Bcl I, Bgl I, Bgl II, Cla I, Dra I, EcoR I, Hha I, Kpn I, Nci I, Nco I, Pst I, Pvu II, Sac I, Sma I, Sst I, Sst II, Xba I and Xho I. After digestion with above enzymes, the resulting fragments were separated via electrophoresis on 0.8% agarose gels containing 40mM sodium acetate and 20mM EDTA(pH 8.05). The gels were stained with ethidium bromide and photographed by using the U. V. illuminator(Mulligan and Chapman, 1989).

Table 1. Location and dates of samples, small yellow croaker, *Pseudosciaena polyactis* used in this study.

Location	Date	Sample size
Mokpo area	15 Mar. 1991	11
〃	24 Sep. 1991	8
Inchon area	17 May 1991	9
〃	25 Nov. 1991	9

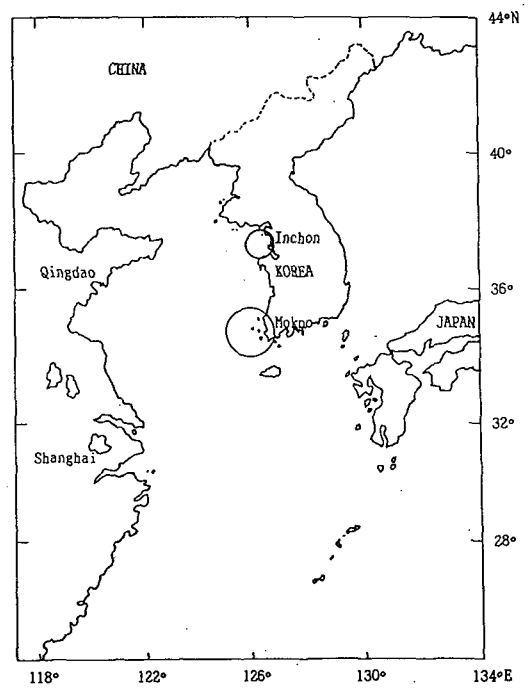

Fig. 3. Map of the sampling sites. Open circles indicate the Mokpo and Inchon area.

2) Muscle actin preparation and fragmentation

The muscle actin of small yellow croaker was obtained from acetone powder as described by Spudich and Watt(1971). For the purification of ac-

tin, an acetone powder of the muscle was homogenized at 0℃ for 20 min with 10 volume of Buffer A(Tris 2mM, $CaCl_2$ 0.1mM, ATP 0.2mM, DTT 2 mM, pH 7.8) and cleared by centrifugation at 22, 000×*g* for 15 minutes. The actin was allowed to polymerize for 2 hours by adding KCl(to 2.5M) and $MgCl_2$(to 0.05mM) to the resulting supernatant (dilution 1/25). The solution was stirred gently for 5 minutes and then was centrifuged at 22,000×*g* for 1.5 hours. The pellet was resuspended at 5 volumes of Buffer A and dialyzed at 4℃ against same buffer.

Actin was digested with *Staphylococcus aureus* V_8 protease(Roustan *et al.*, 1985). The standard procedure chosen was as follows; 2*mg* actin was incubated with 1*ml* of 50mM Tris buffer(pH 7.8) containing of 1% SDS and 2mM $MgCl_2$ with an enzyme/substrate ratio of 1/50(W/W) at 25℃. After digestion, sodium dodecyl sulphate(0.1%)/polyacrylamide-slab-gel electrophoresis(SDS-PAGE) was run according to the procedure of Laemmli(1970).

Results

Restriction endonuclease digest of mt-DNA with twenty endonucleases on the eggs of small yellow croaker, *Pseudosciaena polyactis* at Mokpo area revealed a total of 37 unique restriction sites. Among them, six restriction enzymes such as Sst I, Xho I, Bam HI, Sac I, Cla I and Hha I were not produced any restriction fragments(Table 2).

As a result, among all of the used enzymes, fourteen endonucleases produced significant restriction patterns in the case of mt-DNA from the Mokpo area(Table 2). Especially, eight restriction endonucleases(EcoR I, Kpn I, Sst II, Xba I, Bgl I, Bgl II, Nci I and Sma I) produced twenty two restriction sites on the tested mt-DNA which comprised more than 60% total restriction sites surveyed(Table 3).

Molecular weight estimates for these fragments are presented in Table 3. The estimated molecular weight for the entire mt-DNA genome size was 16 ± 0.2 Kb.

The agarose gel electrophoretic pattern of mt-DNA fragments digested with indicated restriction endonucleases are presented in Figs. 4, 5 and 6, respectively.

Actin from small yellow croaker at Mokpo and Inchon areas purified as described earlier was homogeneous as judged by electrophoresis on SDS-PAGE(Fig. 7). Its molecular weight calculated from the relative mobility was about 42 KDa.

Selective digestion of actin at glutarmyl residues

Table 2. Molecular weight estimates for restriction fragments of mt-DNA of small yellow croaker at Mokpo area produced by the twenty endonucleases listed. (unit: Kb)

EcoR I	Kpn I	Sst II	Pst I	Pvu II	Xba I
7.0	10.0	10.0	9.7	7.0	12.0
6.0	3.0	5.0	5.0	5.5	4.2
4.0	3.0	3.0		2.1	
Bcl I	Bgl I	Bgl II	Nci I	Nco I	Apa I
9.7	8.0	10.0	9.5	9.7	6.8
3.0	4.0	3.0	3.0	5.0	5.0
1.5	4.0	3.0	3.0		2.3
Sma I	Dra I	Sst I	Xho I	Bam HI	Sac I
12.0	9.7	--	--	--	--
3.0	2.0				
Cla I	Hha I				
--	--				

Table 3. Number of fragment and average molecular size of mt-DNA of small yellow croaker at Mokpo area that showed significant results among twenty enzymes surveyed.

Enzyme	Number of Fragment	Average Mol. Size
EcoR I	3	17 Kb
Kpn I	3	16
Sst II	3	18
Xba I	2	16
Bgl I	3	16
Bgl II	3	16
Nci I	3	15.5
Sma I	2	15.0
	Total 22	Average 16.2

by *Staphylococcus aureus* V_8 protease was carried out under unfolding condition. The presence of 1% SDS, in the reaction mixture increases the yield of actin cleavage products(Fig. 8). However, at high concentration of SDS(>2%) led to a rapid inactivation of the protease. The *Staphylococcus aureus* V_8 protease first release two major fragments of 26 KDa and 16 KDa, respectively, and they were degraded further along with the incubation time. Thus, another fragments of 19 KDa appears more slowly (Fig. 8).

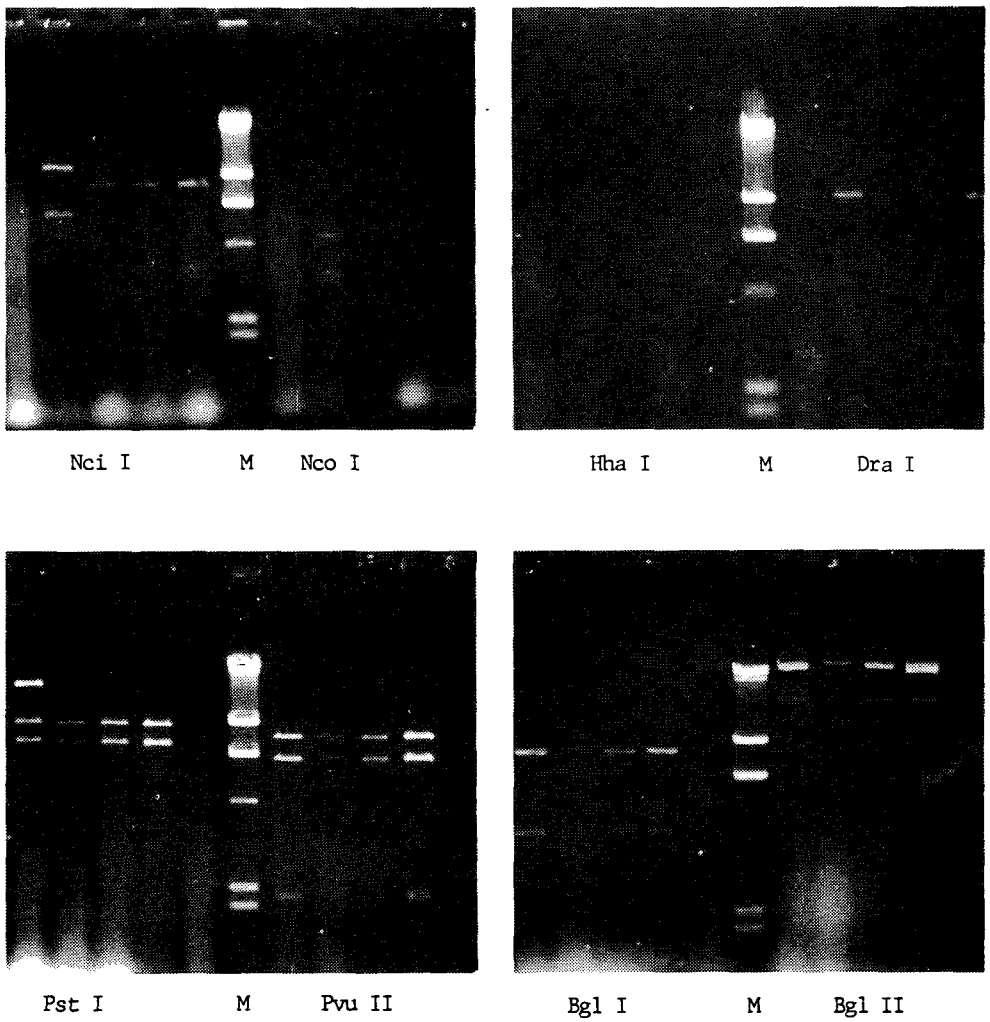

Fig. 4. Agarose gel electrophoretic patterns of mt-DNA fragment of small yellow croaker at Mokpo area digested with indicated restriction endonucleases(M: marker DNA).

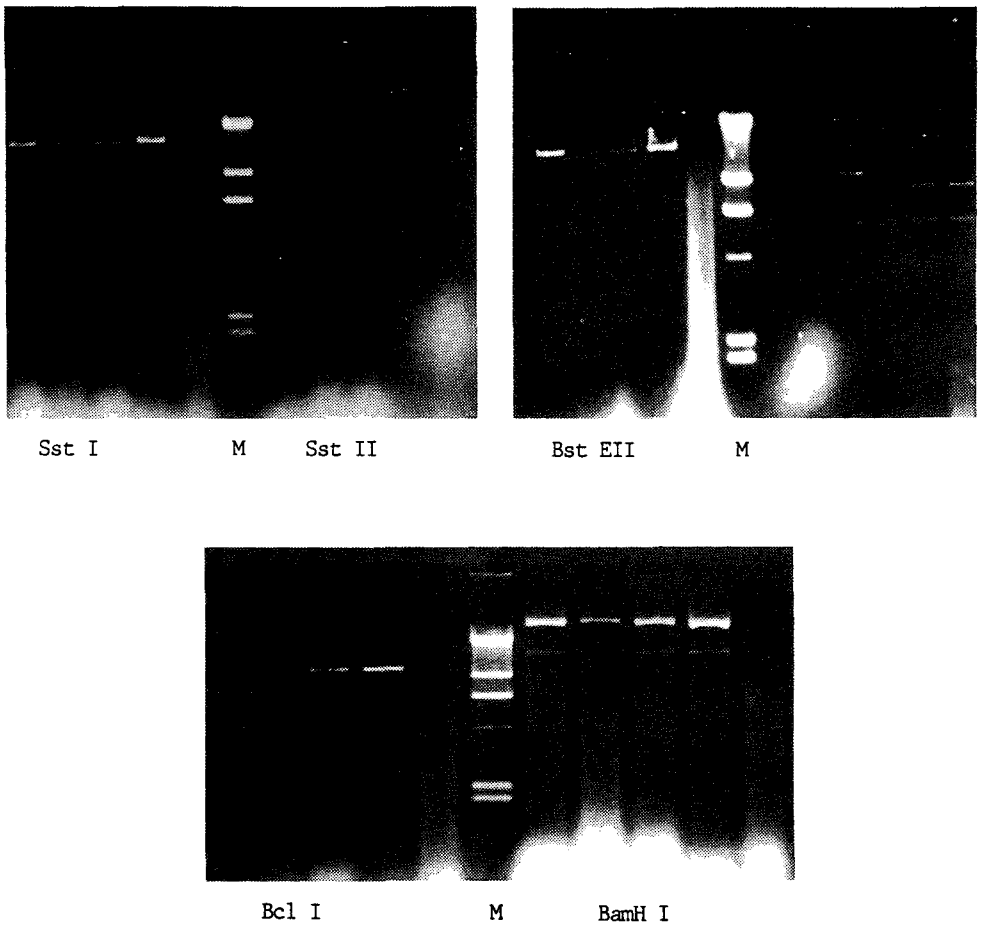

Fig. 5. Agarose gel electrophoretic patterns of mt-DNA fragment of small yellow croaker at Mokpo area digested with indicated restriction endonucleases (M: marker DNA).

Discussion

It is generally accepted that stock definition is critical for an effective fisheries management, because in theory, each stock must be identified and management separately to optimize its yield.

In this respect, the studies on mt-DNA variation have focused on evolutionary and systematic problems(Berg and Ferris, 1984; Avise, 1986; Avise *et al.*, 1987) and upon more applied problems in fisheries management(Graves *et al.*, 1984; Chapman, 1987; 1989). Intraspecific mt-DNA sequence divergence has recently been reported in a number of fish species. Graves *et al.*(1984) noted that no mt-DNA sequence divergence observed between Atlantic and Pacific skipjack tuna, *Katsuwonus pelamis* but considerable intraspecific divergence has been reported in sunfishes, genus *Lepomis*(Avise *et al.*, 1984; Kessler and Avise, 1985; Bermingham and Avise, 1986), trout *Salmo clarki, S. gairdneri* and *Salvelinus fontinalis*(Gyllensten *et al.*, 1985; Wilson *et al.*, 1985), Pacific salmon, genus *Oncorhynchus* (Thomas *et al.*, 1986), American shad, *Alosa sapidissima*(Bentzen *et al.*, 1988) and striped bass, *Morone saxatilis*(Chapman, 1987; 1989), respectively.

To investigate the stock identification of fish populations with economic value in the Yellow Sea, small yellow croaker, *Pseudosciaena polyactis* was selected as a target sample. Firstly, as a preliminary survey of the stock identification of small yel-

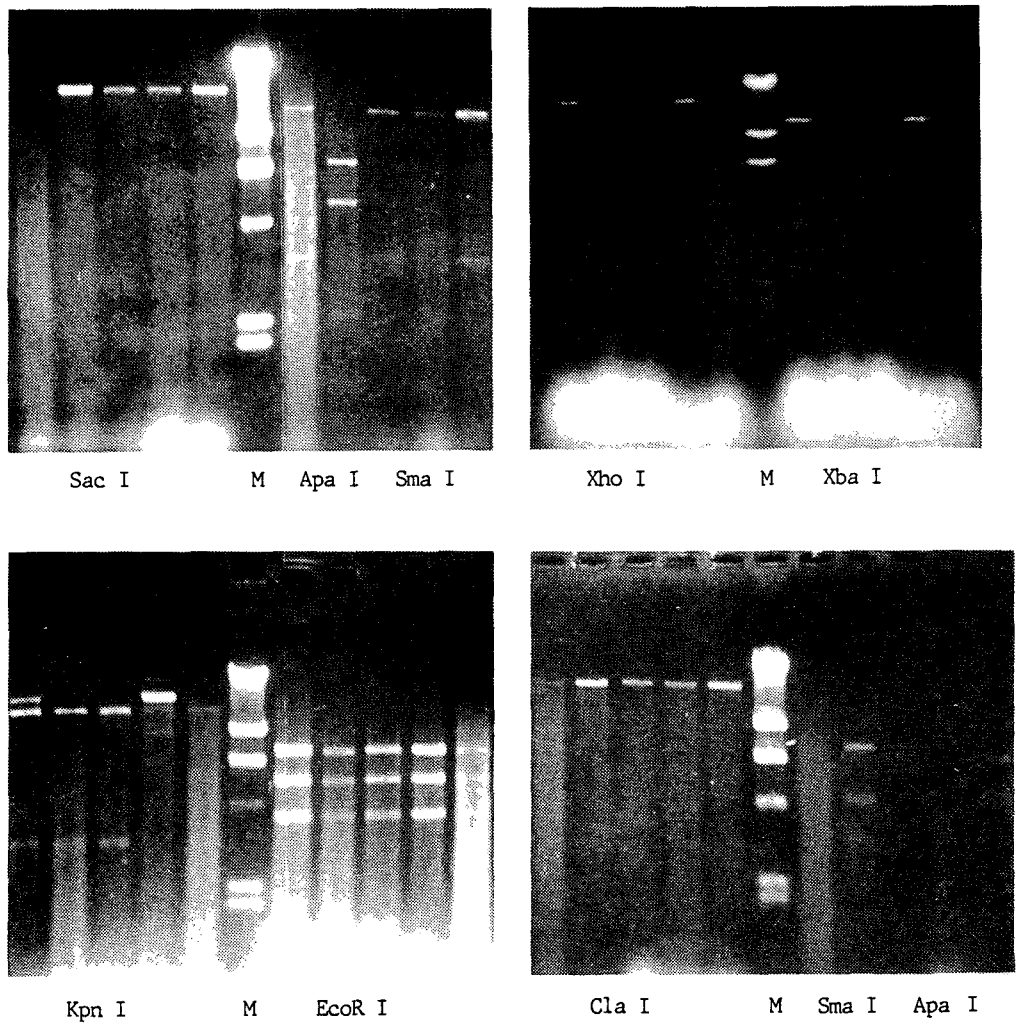

Fig. 6. Agarose gel electrophoretic patterns of mt-DNA fragment of small yellow croaker at Mokpo area digested with indicated restriction endonucleases (M: marker DNA).

low croaker from Mokpo area, the purified mt-DNA was digested with various restriction endonucleases to know the specificity of enzymes on the target mt-DNA.

Among twenty endonucleases used in this study, fourteen endonucleases produced two or three restriction fragments. However, six endonucleases (Xho I, Bam HI, Sst I, Cla I and Hha I) did not cleaved the mt-DNA at all(Table 2), while eight endonucleases such as EcoR I, Kpn I, Sst II, Xba I, Nci I, Bgl I, Bgl II and Sma I were digests of mt-DNA from the eggs(Table 3), indicating the above eight endonucleases were specific in the case of small yellow croaker from Mokpo area.

Actin, which is one of the major elements of the contractile system in muscle, is known to be a highly conserved protein(Vandekerckove and Weber, 1984). Specifically, six isoactins has been described in higher vertebrates, and only 6% mutation occurs between actins from invertebrate to vertebrate skeletal muscles(Vandekerckove *et al.*, 1983; Vandekerckove and Weber, 1984). These mutation sites are mainly located in the precise regions of the

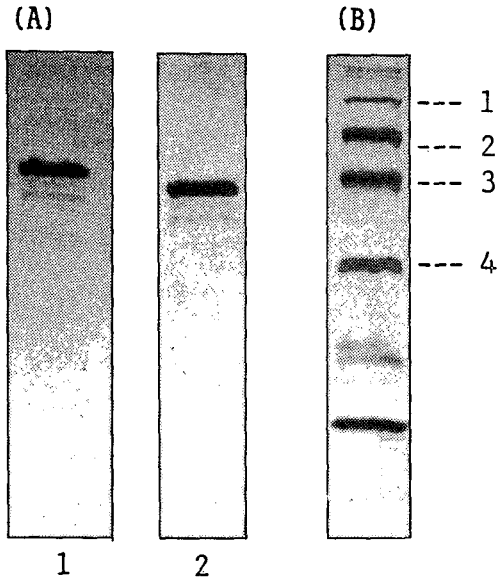

Fig. 7. (A) Electrophoretic analysis on SDS-PAGE(15 %) from the muscle of small yellow croaker.
1. muscle actin from Inchon(0.47 *mg/ml*)
2. muscle actin from Mokpo(0.40 *mg/ml*)

(B) Molecular weight of marker proteins(KDa)
1. phophorylase b(94 KDa)
2. albumin(67 KDa)
3. ovalbumin(43 KDa)
4. carbonic anhydrase(30 KDa)

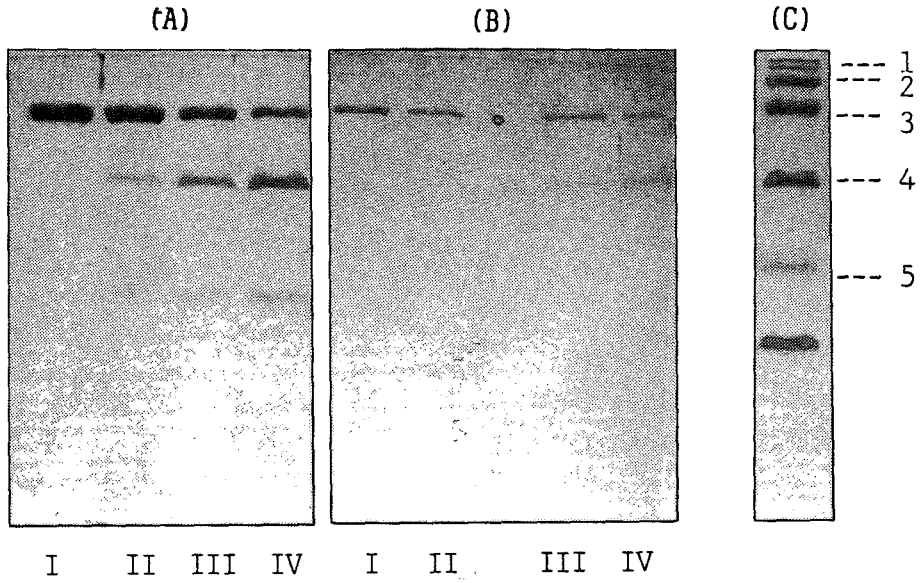

Fig. 8. Cleavage of muscle actin of small yellow croaker by *Staphylococcus aureus* V_8 protease. Actin was incubated in 50 mM Tris, 2 mM $MgCl_2$ buffer in the presence of 1% SDS for 0 min(Ⅰ), 1 min(Ⅱ), 5 min (Ⅲ) and 10 min(Ⅳ).
(A) muscle actin from Inchon(0.47 *mg/ml*)
(B) muscle actin from Mokpo(0.40 *mg/ml*)
(C) Molecular weight of marker proteins(KDa)
1. phophorylase b(94 KDa)
2. albumin(67 KDa)
3. ovalbumin(43 KDa)
4. carbonic anhydrase(30 KDa)
5. trypsin inhibitor(20 KDa)

sequence. The N-terminal regions are known to be the most variable region and is characteristic to each isoform(Hue *et al.*, 1989).

In this study, it has been studied that the *Staphylococcus aureus* V_8 protease was able to cleave the actin, and to yield the N-termal peptide of 26 KDa and 16 KDa(Fig. 8). According to these results, there was no significant difference observed in cleavage pattern between two types actins(from Mokpo and Inchon area), suggesting the actin N-terminal sequence of small yellow croaker from Mokpo is closely related from that of Inchon area.

Consequently, for better understanding on the stock structure of the small yellow croaker in the Yellow Sea, additional samples from different area must be added, providing more information in the relatedness of population stocks based on the analysis of restriction fragment length polymorphism (RFLP) and muscle actin isoform.

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황해산 참조기(*Pseudosciaena polyactis*)의 계군 분석을 위한 분자생물학적 방법 검정

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황해서식 참조기(*Pseudosciaena polyactis*)에 대한 계군을 분석하기 위한 연구의 일환으로 우선 황해의 목포 연근해에서 채집된 참조기의 난자와 근육으로 부터 mt-DNA와 근육 actin을 추출하였다. 난자의 경우 mt-DNA를 추출한 뒤 제한효소로 처리하여 절편다형현상을 관찰하였으며 근육 actin의 경우 단백질 분해효소(*Staphylococcus aureus* V_8 protease)로 처리하여 N-terminal 절편의 다형현상을 각각 관찰하였다. Mt-DNA의 genome 크기는 약 16 ± 0.2 Kb였고 전체 절편수는 37개였으며 사용된 20개의 제한효소중 8개의 제한효소만이 3개 정도의 절편을 보이므로써 유의성을 관찰할 수 있었다. 한편 근육 actin을 *Staphylococcus aureus* V_8 단백질 분해효소로 처리하였을 때 N-terminal 부위에서 26 KDa 및 16 KDa의 분자량을 갖는 특이절편을 관찰할 수 있었다.