

**MITOCHONDRIAL DNA SEQUENCE ANALYSIS ON ALBACORE, *THUNNUS ALALUNGA*,
MEAT SAMPLES COLLECTED FROM THE WATERS OFF WESTERN SOUTH AFRICA
AND THE EASTERN INDIAN OCEAN**

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SUMMARY

Meat samples removed from (1) five Atlantic albacore (caught in early 1996 in waters off Cap Town by a South African sport vessel using rod and reel); and (2) five Indian albacore (caught in late 1990 in waters off western Australia by the R/V "Haikung" using longline) were used for mtDNA sequence analyses in this study.

The isolation of mtDNA is performed basically according to the Hills and Moritz (1990) procedure. The double-stranded PCR amplification technique was adopted in this study. Two primers, viz. P3 and PB, were used to latch the segment of mtDNA for sequence analyses. Nucleotide sequencing was performed by the di-deoxy chain-termination method.

The results of the aligned sequences of 295 nucleotides of the control D-loop region showed that: (1) the between-group variation of nucleotide sequence is significantly higher than those of the within-group; and (2) that the positions of the 23rd, 85th, and 97th nucleotides are distinctive between those albacore meat samples of south Atlantic origin and those of eastern Indian Ocean origin.

RESUME

On a utilisé dans la présente analyse de la séquence de l'ANDmt des échantillons de chair prélevés sur: 1) 5 germons atlantiques (capturés début 1996 à la canne/moulinet par un pêcheur sportif sud-africain au large du Cap) ; et 2) 5 germons de l'Océan Indien (capturés fin 1990 à la palangre par le N/O Haikung au large de l'Australie occidentale).

On a essentiellement isolé l'ADNmt selon la méthode de Hills et Moritz (1990). Une technique d'amplification du PCR à double tresse a été retenue. Deux primers, P3 et PB, ont servi à relier le segment d'ANDmt pour analyser la séquence. La séquence des nucléotides a été effectuée par la méthode de terminaison de la chaîne di-deoxy.

Les résultats de l'alignement des séquences de 295 nucléotides de la région de contrôle du D-loop ont montré que : 1) la variation de la séquence des nucléotides entre les groupes est bien plus forte qu'au sein des groupes ; et 2) la position des 23ème, 85ème et 97ème nucléotides diffère entre les échantillons de chair de germon en provenance de l'Atlantique Sud et ceux qui proviennent de l'est de l'Océan Indien.

RESUMEN

Para el análisis secuencial de ADN mitocondrial en este estudio, se usaron muestras de carne de (1) cinco atunes blancos del Atlántico (capturados a principios de 1996 en aguas frente a Ciudad del Cabo por un barco sudafricano con caña y carrete) y (2) cinco atunes blancos del Índico (capturados a finales de 1990 en aguas frente a la costa oeste de Australia por el R/V "Haikung" con palangre).

El aislamiento del ADN mitocondrial se hace básicamente de acuerdo con el procedimiento de Hills y Moritz (1990). En este estudio se adoptó la técnica de amplificaciones PCR doble. Se usaron dos *primers*, P3 y PB para unir el fragmento de ADN mitocondrial para el análisis secuencial. La secuenciación nucleotídica se termina por la introducción de un nucleótido.

Los resultados de los análisis de secuencias alineadas de 295 bases en el D-Loop mostraron que (1) entre grupos, la variación de la secuencia nucleotídica es muy superior a la que existe dentro de un grupo y (2) las posiciones de las bases 23, 85 y 97 son distintas en las muestras de carne de atún blanco procedente del Atlántico sur de las procedentes del este del Océano Índico.

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INTRODUCTION

The commencement of Taiwanese longline fishery into the Indo-Atlantic region began in mid 1960s and has swiftly developed into one of the major fishing fleets targetting on albacore since late 1960s. In the past decade, for instance, the total albacore catch by Taiwanese vessels ranged 18,400-28,800 mt per year in the South Atlantic region and 10,100-28,900 mt per year in the Indian Ocean. The total catch of albacore by Taiwanese fleet ranged 30,000-56,300 mt per year in the Indo-Atlantic region.

As one of the major fishing nations harvesting albacore from the Indo-Atlantic region, much fisheries biological research efforts in company with the compilation of catch statistics have been devoting for a better understanding the various aspects of this species. Studies as: (1) the age and growth (Kuo 1990, Lee 1990, Lee and Yeh 1992) and (2) the stock assessment (Huang *et al.* 1986, Lee and Liu 1988, Yang and Sung 1983, Yeh *et al.* 1993) of this species were carried out by Taiwanese fishery biological scientists in addition to those previous studies (Koto 1969, Nishikawa 1985, Shiohama 1985).

To utilize a fishery resource, particularly for those highly migratory species, can only be sustainable if not to jepordize its renewable capability through a proper management scheme has been a worldwidely accepted rationale. As the first step toward a meaningful fishery resource management, however, accurate delimitation of a unit stock is essential. Although the South Atlantic albacore and the Indian albacore are assumed for many years to be two independant stocks, there is still no concrete evidence yet to fully support such postulation.

Based on geographical distribution pattern of monthly albacore catch rate compiled from Taiwanese longline fishery in the Indo-Atlantic region (Fig. 1), a continuation of albacore catch rate distribution from the Atlantic crossover to the Indian Ocean can be observed in certain period of the year. It implies that the stock structure of Indo-Atlantic albacore

nearby South Africa, where physical oceanographic boundary of the two Oceans often exits yet situation varies from year to year, is complicated and perhaps a contact or exchange individuals between albacores originated from the two sources can not be ruled out.

Main purpose of this study is thus to analyze and to compare the mtDNA sequence patterns of albacore meat samples collected from (1) South Atlantic Ocean nearby Cape Town and (2) Eastern Indian Ocean for throwing lights on albacore stock structure in the Indo-Atlantic region.

MATERIALS AND METHODS

Meat samples removed from (1) five Atlantic albacores (caught in early 1996 in the waters off Cape Town by a South African sport vessel using rod and line); and (2) five Indian albacore (caught in late 1990 in the waters off western Australia by the R/V Haikung using longline) were the source of materials used for mtDNA sequence analyses in this study.

A fisheries research survey of using longline in the Eastern Indian Ocean region by the R/V Haikung, which belonged to the Taiwan Fisheries Research Institute, was conducted in the late 1990. Soon after a hooked albacore was landed and morphometrically measured on the deck, 9 square cm skin at the central area between dosal fin base and lateral line was removed to expose the underneath muscular tissue, a cut of about 4 cubic cm muscle tissue was made. This piece of mussel tissue was quickly transferred to the biological laboratory (which is one floor below deck) for further cleaning, trimming, and packing into a little bottle of 2 cubic cm for the storage in liquid nitrogen. These samples were then transferred to -75 degree C refrigerator when R/V Haikung completed the cruise.

In early 1996, a South African sport rod and line vessel were arranged for catching South Atlantic albacore.

Ten albacores were captured at 34.34 degree S and 18.03 degree E. These captured albacores were killed immediately, measured to the nearest mm using a standard measure board and flesh samples transferred into 96% ethanol within ten minutes of capture at room temperature.

Meat samples, one for each albacore specimen, collected from (1) five albacore individuals caught in the Eastern Indian Ocean by the R/V Haikung; and (2) five albacore individuals caught in the waters off western South Africa by a South African sport rod and line vessel were used for mtDNA sequence analyses in this study. Detailed information of these captured albacores are shown in Table 1 and Figure 2.

The isolation of albacore meat sample mtDNA is basically following Hillis and Moritz (1990) procedure with minor modifications. About 100 mg of muscle was incubated in 1 ml of Digestion buffer (10 mM EDTA, 100 mM Tris-HCl pH 8, 100 mM NaCl, 0.1% SDS, 50 mM Dithiothreitol, 0.5 mg/ml Proteinase K) for 2-4 hours at 50 degree C. DNA was purified with standard phenol chloroform extraction method, precipitated with ethanol, and washed with 70% ethanol. DNA pellet was dried by vacuum and resuspended in 50ul TE buffer (10 mM Tris-HCl pH 8, 1mM EDTA pH 8) and stored at 4 degree C.

Double-stranded PCR amplifications were performed adapting the protocols of Innis *et al.* (1989). Amplifications were performed in 50 ul solution containing 50mM KCl, 10mM Tris-HCl pH 8.3, 1.5mM MgCl₂, 0.1% gelatin, 0.4 mM dNTP, 10 to 25 ng of template DNA, 2 units of SuperTaq polymerase (HT Bio-technology LTD), and 20 pmole of each primer. A total of 35 cycles of 1 minute at 94 degree C for denaturation, 1 minute at 50 degree C for annealing, 1.5 minutes 72 degree C for extension were carried out with a thermal cycler (PTC-100, MJR Inc.). The respective locations in mtDNA of the two primers (P3

and PB), which were used for PCR process in this study, are shown in Figure 3. The nucleotide sequences of P3 and PB are as follow (Tzeng *et al.* 1992):

P3: 5'-AACTTCCATCCTCAACTCCCAAAGC-3'

PB: 5'-AGTGGGGTATCTAATCCCAG-3'

A 1.35 kb DNA fragment was amplified. The amplification product was separated by agarose gel electrophoresis, visualized by staining with ethidium bromide, and the amplified fragment of DNA was excised from the gel. JETsorb Gel

Kit (GENOMED inc.) was used to extract DNA from excised gel. Finally the purified DNA was resuspended in 25 ul double distilled water and used as template for DNA sequencing.

Nucleotide sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the Amplicycle sequencing Kit. [α 35S-dATP] was used as label. Taq DNA polymerase (Perkin Elmer Cetus), two units for each reaction, was added to the labeling mixture. Only one primer, i.e., P-Phe (Pla *et al.* 1994) was prepared and used in sequencing reaction. The corresponding location of P-Phe is shown in Figure 3, and its sequence is 5'-GCTTTAGTTAAGCTACG-3'. A total of 30 cycles of 1 minute at 94 degree C, 1 minute at 50 degree C, 1.5 minutes at 72 degree C were carried out. After this sequencing reaction, 4ul Stop-dye solution were added to each tube. The resulting single strand DNA products were then through electrophoretic process in a 6% Polyacrylamide gel. The resultant gel was fixed, transferred to a 3mm filter, dried, and visualized by exposure to X-Omat film (Kodak) for 12-72 hours (Sambrook *et al.* 1989).

After the sequences of this particular mitochondrial D-loop portion were obtained, sequence alignments were performed using Pileup program, which is provided by the GCG software package (Genetic Computer Group, version 8.01,

University of Wisconsin). The Plotsimilarity program in the GCG software was also used to compare between-sequence similarity or between-specimen distances. These informations were then used to construct genealogical relations by unweighted pair-group method with arithmetic mean (UPGMA) algorithm.

RESULTS

The aligned 10 sequences of 295 nucleotides, which is a part of the control region (D-loop), of the sampled 10 albacores are shown in Figure 4. A total of 295 nucleotides obtained in this study represent approximately 30% of the complete nucleotides contained in its D-loop region, if albacore mtDNA is comparable to the Genus *Thunnus* provided by Pla *et al.* (1994).

A total of 10 (out of 295) variable nucleotide positions were identified among the 10 albacore sequences, in which five samples were collected from the South Atlantic Ocean nearby Cape Town and the rest five were from the Eastern Indian Ocean. Four variable nucleotides positions were identified from South Atlantic group, whereas six were identified for the Eastern Indian group (Fig. 4). The segment of sequences from 51st to 100th appears to be the most variable ones, which comprised of 70% (7 out of 10) of the total variations. Sequence segments which contains 1 (out of 50) variable nucleotide position are: (1) 1st to 50th; (2) 101st to 150th; and (3) 201st to 250th. No variable nucleotide position was observed in sequence segments 151st to 200th; and 251st to 295th.

Further analyses of these 10 variable nucleotide positions reveal that three of them are areal distinctive: (1) sequence 23rd is nucleotide G (100%) in South Atlantic, whereas in Eastern Indian is 80% A and 20% G; (2) sequence 85th is 100% G in South Atlantic and 100% A in Eastern Indian; (3) sequence 97th is 100% A in the Eastern Indian, while in the South Atlantic is 80% G and 20% A.

Plot of similarity score (Figure 5) shows there are two segments in the whole sequence appears to have higher variability (score about 0.92): (1) segment sequence of 20th to 30th; and (2) segment sequence of 50th to 100th. While most of the rest locations its similarity score is 1. Table 2 shows the paired distance matrix between two nucleotide sequences and this matrix was then used in the construction of genealogical tree. The genealogical tree thus constructed by UPGMA algorithm is shown in Figure 6.

As indicated by the molecular genealogical tree (Figure 6), there are two distinctive genealogical branches: (1) one branch its components were all from the South Atlantic Ocean and (2) the other branch were all from the Eastern Indian Ocean. Secondly, the within group heterogeneity in the Eastern Indian samples appears to be higher than that in the South Atlantic samples.

SUMMARY AND DISCUSSION

The results so far obtained in this study show that (1) sequence mtDNA, especially in D-loop region, is a feasible technique to assist identification of Indo-Atlantic albacore stock structure; (2) nucleotide obtained in the positions of 23rd, 85th, and 97th sequence of the albacore mtDNA samples collected in this study appeared to be areal distinctive; (3) heterogeneity between albacore samples collected from South Atlantic Ocean nearby Cape Town is much higher than that of within group variation; (4) albacore sampled from South Atlantic is genealogically distinctive from those from the Eastern Indian Ocean by UPGMA algorithm.

Because that characters such as: (1) maternal inheritance (2) no crossing over and (3) faster rate of mutation attracted many biological scientists nowadays to use mtDNA in various aspects. Fisheries scientists have to deal with population at unit stock level, which is generally under the taxum of species and thus generally difficult to detect only by means of

morphologic characters. The introduction of mtDNA sequence techquic perhaps can improve stock identification more precise and confident.

In addition to the mtDNA sequence analyses, other relevant means such as morphological analyses (Yeh *et al.* 1995; Penney *et al.* 1996) were also conducted concurrently to elucidate the Indo-Atlantic albacore stock structure. Based on various information thus obtained, particularly enlightened by the mtDNA sequence results, the authors believed that the South Atlantic albacore and the Indian albacore are two distinctive stocks (during the last glacial period) and became interchangeable (perhaps only a small portion though) between the two since the rising of the sea level some 10 thousand years ago.

Nishikawa (1985) indicated that dense larval distribution areas can be found both in the waters off northern Australia on the east side of the Indian Ocean and the areas nearby Madagasca, which is on the west side of the Ocean. It is unfortunate that there is virtually no larval distribution information between the two ends, thus it is very difficult to judge whether the spawning of Indian albacore is continuously distributed from east to west or there are two different spawning stocks at both ends of the eastwest bounds of the Ocean. The whole picture of larval distribution pattern certainly will provide very important information for the albacore stock structure in the Indian Ocean. Further information related to this respect is needed.

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Table 1. Localities, sampling dates, and fork length of captured albacore samples used in this study.

Sample Number	Species	Date of Capture	Locality (degree)		Fork Length (mm)
			S. Latitude	E. Longitude	
SA1	Albacore	Jan.-24-1996	34.34	18.03	739
SA2	Albacore	Jan.-24-1996	34.34	18.03	773
SA3	Albacore	Jan.-24-1996	34.34	18.03	674
SA4	Albacore	Jan.-24-1996	34.34	18.03	608
SA5	Albacore	Jan.-24-1996	34.34	18.03	772
EI1	Albacore	Dec.-30-1990	29.56	99.30	1110
EI2	Albacore	Dec.-30-1990	29.56	99.00	1040
EI3	Albacore	Dec.-20-1990	37.30	106.29	786
EI4	Albacore	Nov.-30-1990	20.09	107.30	1061
EI5	Albacore	Nov.-26-1990	19.23	102.23	1125

Table 2. Pairwise Tamura-Nei Distance between nucleotide sequence among the ten albacore samples by using the Distance program in the CGC software.

	SA1	SA2	SA3	SA4	SA5	EI1	EI2	EI3	EI4	EI5
SA1	.0000	.0000	.0069	.0034	.0068	.0104	.0104	.0173	.0173	.0140
SA2		.0000	.0069	.0034	.0068	.0104	.0140	.0173	.0173	.0140
SA3			.0000	.0103	.0138	.0104	.0140	.0173	.0173	.0140
SA4				.0000	.0102	.0138	.0174	.0208	.0208	.0173
SA5					.0000	.0173	.0209	.0242	.0242	.0209
EI1						.0000	.0034	.0068	.0069	.0103
EI2							.0000	.0103	.0103	.0140
EI3								.0000	.0138	.0138
EI4									.0000	.0173
EI5										.0000

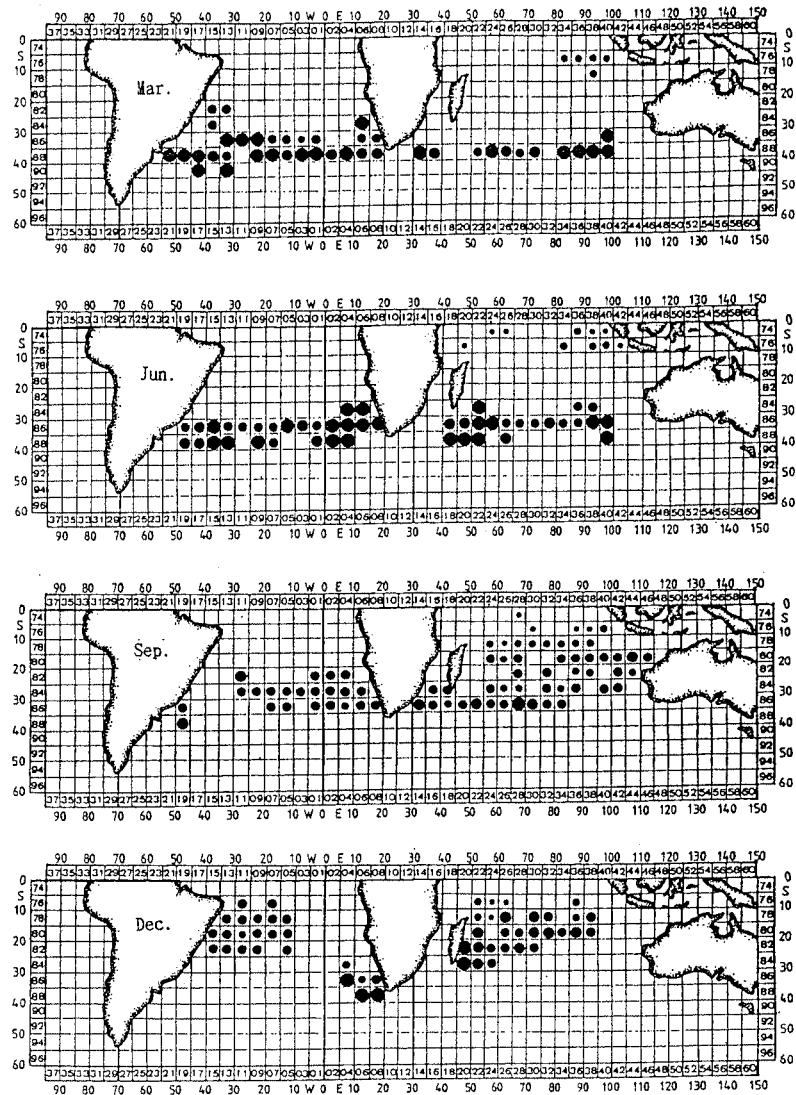


Fig. 1 Geographical distribution of monthly nominal CPUE (fish / 100 hooks) of albacore caught by Taiwanese longline fishery in 1987 (· : <0.03 ; • : 0.04~0.75 ; ◐ : 0.76~2.19 ; ◑ : 2.20~2.91 ; ● : >2.92) .

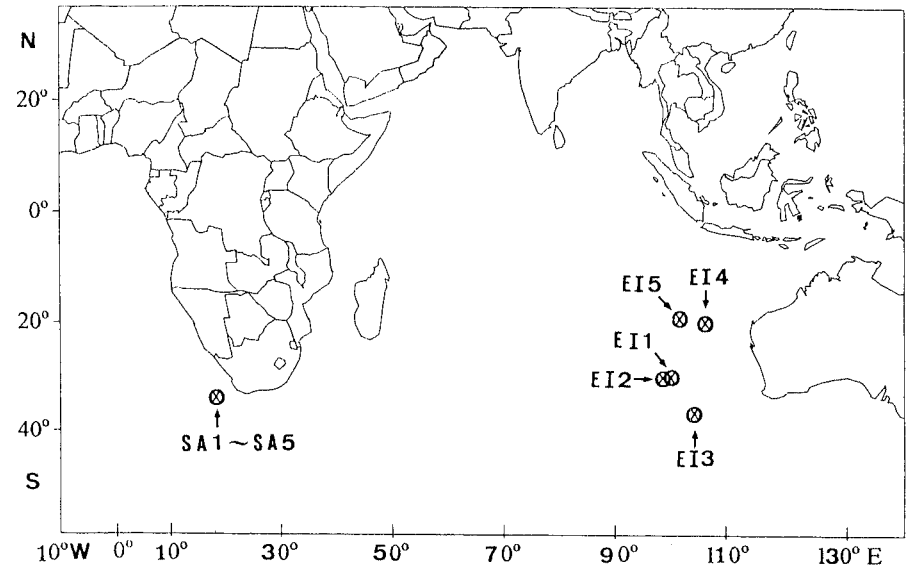


Fig. 2 Map shows the capture locations of albacores used in this study.

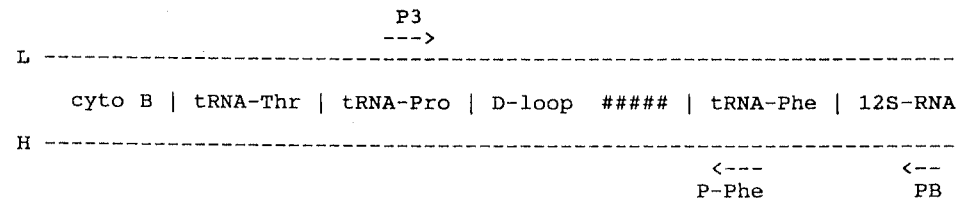


Fig. 3. Schematic diagram of the amplified region of the albacore mtDNA between P3 and PB. The locations and orientations of the three primers on the amplified region are indicated by arrows. Designated P3 and PB are PCR primers and P-Phe is sequencing primer and ##### area is the sequenced region.

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1
SA1 tttggcatct cacagtycaa atgcaacaat gatcagcaag gtagaacatt 51 100
SA2 .....
SA3 .....
SA4 .....
SA5 .....
E11 .....
E12 .....
E13 .....
E14 .....
E15 .....

101
SA1 taaccacata ttgggatata atgagcataa tgataatatt acccgtaaaa tatctaagac accccctctc ggcttttggc cgttaaacc ccoataccccc 200
SA2 .....
SA3 .....
SA4 .....
SA5 .....
E11 .....
E12 .....
E13 .....
E14 .....
E15 .....

201
SA1 ctaaactcgt gatatacatta acactcctgt aaaccccccg taacaggaa 251 295
SA2 .....
SA3 .....
SA4 .....
SA5 .....
E11 .....
E12 .....
E13 .....
E14 .....
E15 .....
  
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Fig. 4. Sequences of a 295 nucleotide region of the mitochondrial D-loop from ten albacores. Dots represent matching sequences.

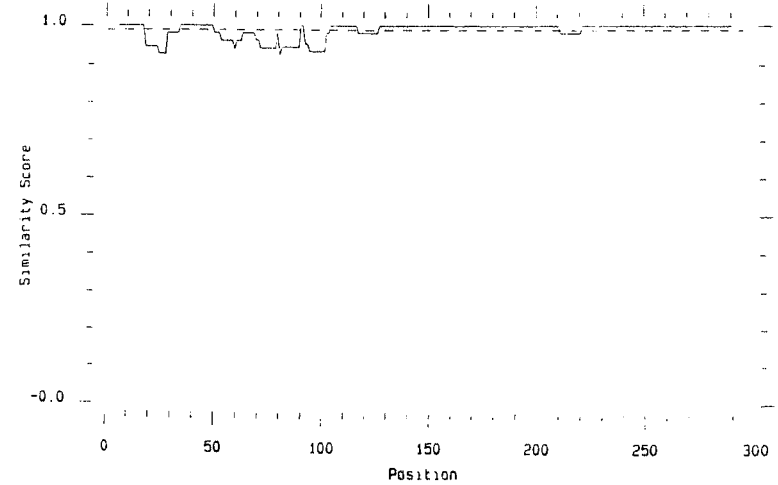
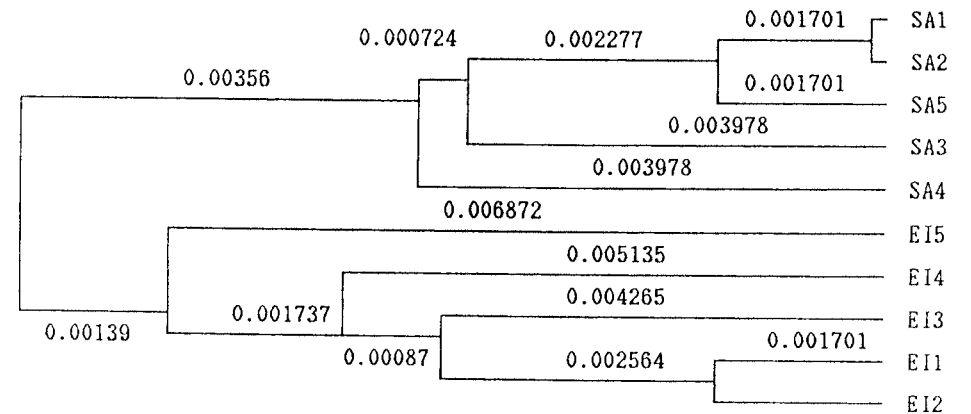


Fig. 5. Plot of similarity score versus sequence of nucleotide.



Scale: each - is approximately equal to the distance of 0.00012

Fig. 6. Plot of genealogical tree constructed by UPGMA.