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Short communication

Population structure of bigeye tuna (*Thunnus obesus*) in the South China Sea, Philippine Sea and western Pacific Ocean inferred from mitochondrial DNA

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Abstract

The population structure of bigeye tuna (*Thunnus obesus*) in the South China Sea, Philippine Sea and western Pacific Ocean was investigated using sequence data of the first hypervariable region (HVR-1) of the mitochondrial control region. Nucleotide diversities, ranging from 3.8% in the Philippine Sea to 4.3% in the western Pacific Ocean, and haplotypic diversities, ranging from 0.998 in the South China Sea to 1.000 both in the Philippine Sea and western Pacific Ocean, are high in all sampling regions. Both Tajima's *D* and Fu and Li's *D* statistics are non-significant, indicating the scenario of an effective large and stable population size. Neighbor-joining tree and minimum spanning network showed that the haplotypes from all three regions can be grouped into two lineages. Lineage I is the major lineage which consists of more than 88% specimens in each regional population, and no differences are found in the frequencies of these two lineages among the three regional populations. Analyses of the HVR-1 sequences revealed a very high gene flow between the three sampling regions ($Nm = 71.91$–2479.68). Furthermore, all analyses indicate that bigeye tuna over the sampling area constitute a single panmictic population.
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Keywords: Bigeye tuna (*Thunnus obesus*); Mitochondrial DNA; Control region; Population structure; Western Pacific Ocean

1. Introduction

The bigeye tuna (*Thunnus obesus* Lowe, 1839) is a large pelagic fish widely distributed in tropical and subtropical waters (45°N to 43°S). It is an important commercial species of the world fisheries, and its resources appear to be decreasing due to overfishing in recent years (ICCAT, 2003). A better understanding of fish population structure is important to an effective fisheries management. However, our knowledge of the bigeye tuna population structure is still limited (Suzuki, 1962; Alvarado-Bremer et al., 1998; Chow et al., 2000; Appleyard et al., 2002; Durand et al., 2005).

In 1962, Suzuki published the first molecular genetic analysis on bigeye tuna populations. He studied the Tg blood type in the samples from eastern Pacific and Indian Oceans and found no significant differences between the two oceans (Suzuki, 1962). Thirty-six years later, Alvarado-Bremer et al. (1998) examined the mitochondria DNA (mtDNA) control region of this species from Atlantic, Indian and Pacific Oceans using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay to investigate its population structure. Analyses of frequency distribution of the RFLP haplotypes indicate that while no significant differences were found between the Pacific and Indian Ocean samples, bigeye tuna samples of these waters are genetically distinct from those of the Atlantic Ocean. The existence of two Clades (I and II) of bigeye tuna was found; Clade

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I exists both in the Atlantic and Indo-Pacific Oceans, and Clade II is mainly limited to the Atlantic Ocean (Alvarado-Bremer et al., 1998). These results were later corroborated by those of PCR-RFLP analyses of two mtDNA segments, the control region and a segment (ATCO) flanking the ATPase and cytochrome oxidase III genes (Chow et al., 2000). It is interesting to note that only two genotypes (α and β) were detected following *RsaI* digestion of the ATCO segment. The α type predominates in the Atlantic samples (i.e., 178 of 244), and all but one of the 195 Indo-Pacific samples are β type. Moreover, bigeye tuna samples of the Indian Ocean were examined for variations at seven microsatellite loci and at the ATCO region by PCR-RFLP analyses (Appleyard et al., 2002). The results indicate that genetic differentiation is non-significant between samples collected from eastern and western Indian Ocean. Recently, the genetic differentiation between the Atlantic and Indo-Pacific mitochondrial lineages was further confirmed by characterization of four nuclear DNA loci (Durand et al., 2005). The results also indicate unidirectional gene flow from Indo-Pacific to Atlantic bigeye tuna populations and their admixture off southern Africa.

Despite the fact that many studies have been performed on the bigeye tuna population structures, none was undertaken regarding the bigeye tuna population in the South China Sea, Philippine Sea and western Pacific Ocean. Based on the fact that these regions are important fishing grounds, studies of the bigeye tuna population structure in these areas will help enhance management and conservation of the fishery. In this study, 100 bigeye tuna specimens were sampled from the Western Pacific Ocean, including the South China Sea, Philippine Sea and western Pacific Ocean. Their first hypervariable region (HVR-1) of the mitochondrial control region was amplified, sequenced, and analyzed for population structure and phylogenetic information.

2. Materials and methods

2.1. Sampling

Bigeye tuna samples were collected from commercial fishing vessels during May 2000–January 2003. They were sampled from 15 locations representing three regions: South China Sea ($n = 50$), Philippine Sea ($n = 34$) and western Pacific Ocean ($n = 16$) (Fig. 1 and Table 1). Muscle tissue specimens were fixed in 95% ethanol and stored at −20 °C until DNA extraction.

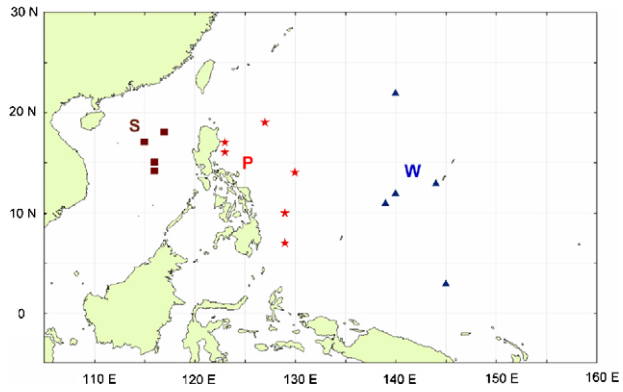

Fig. 1. Map of the bigeye tuna (*Thunnus obesus*) sampling locations and regions. S: South China Sea, P: Philippine Sea, and W: western Pacific Ocean.

2.2. DNA extraction, amplification and sequencing

Total DNA was extracted from 50 mg of muscle tissue samples using a standard proteinase K digestion/phenol–chloroform extraction procedure (Sambrook et al., 1989). Amplifications were carried out in a 50 μl final volume of a PCR mix containing Combizyme DNA Polymerase Mix (Invitek, Berlin, Germany). Two primers, L15998-PRO (5′-TAC CCC AAA CTC CCA AAG CTA-3′) and CSBDH (5′-TGA ATT AGG AAC CAG ATG CCA G-3′), were used to amplify mtDNA fragment containing a small portion of the $tRNA^{Pro}$ gene and the 5′-end of the control region (Alvarado-Bremer et al., 1998). The PCR reaction was performed as following: an initial incubation at 94 °C for 5 min, followed by 40 cycles of PCR (denaturing at 94 °C for 45 s, annealing at 54 °C for 45 s, and extension at 72 °C for 1 min), and a final extension at 72 °C for 5 min. The reactions were carried out in a GeneAmp PCR system 2700 thermocycler (Applied Biosystems, Foster City, CA). PCR products were sequenced directly using Beckman Coulter CEQ2000 SL automated DNA sequencer, applying the PCR-primers as sequencing primers.

2.3. Data analyses

DNA sequences were aligned using CLUSTAL X 1.4 (Thompson et al., 1994, 1997). Nucleotide composition and numbers of variable sites were assessed with the MEGA Version 2.1 (Kumar et al., 1993). Genetic diversity in each sampling region was measured as haplotypic diversity (h) (Nei, 1987) and nucleotide diversity (π) (Lynch and Crease, 1990).

Table 1
Sampling regions, codes, dates, numbers of fish (N) and latitude/longitude for collections of bigeye tuna (*Thunnus obesus*)

Sampling region	Region code	Latitude; longitude	N	Sampling date
South China Sea	S	115–119°E; 9–18°N	50	May 2000–January 2003
Philippine Sea	P	123–130°E; 7–19°N	34	June 2001–December 2001
Western Pacific Ocean	W	140–144°E; 11–22°N	16	May 2001–January 2003

An analysis of molecular variance (AMOVA; Excoffier et al., 1992) as implement in Arlequin 2.000 (Schneider et al., 2000) was used to test temporal and spatial genetic variation within the three areas. Tajima's *D* statistical test (Tajima, 1989a, 1989b) and Fu and Li's *D* statistical test (Fu and Li, 1993) were carried out to examine whether samples from different regions are at genetic equilibrium, considering that a significant deviation from mtDNA genetic equilibrium is presumably a result of recent population expansion or bottleneck in situations where no selective advantage between haplotypes exists (Rand, 1996). Tajima's test compares the number of segregating sites with nucleotide diversity (defined as the average number of nucleotide differences per site between any two sequences), while Fu and Li's test takes into account the polarity of mutations and estimates θ based on the number of derived unique mutations (singletons).

Neighbor-joining tree (Satiou and Nei, 1987) based on the Kimura two-parameter model (Kimura, 1980) was constructed using MEGA Version 2.1 (Kumar et al., 1993). The robustness of statistical support for the tree branch was determined by 1000 bootstrap replicates (Felsenstein, 1985). A statistical parsimony algorithm was employed to construct the minimum haplotype spanning tree (Excoffier, 1993) using Arlequin Version 2.000 (Schneider et al., 2000). Such network uses a coalescence model to calculate the probable mutation steps between haplotypes and is suitable to examine the intra-specific relationship. Estimates of the expected number of migrant females between populations per generation ($N_e m_f$) and Wrights *F*-statistics (F_{ST}) (Hudson et al., 1992) were calculated using the software DnaSP 4.0 (Rozas et al., 2003).

3. Results

3.1. Molecular characteristics

A 380-bp fragment including the 5′-end of the mtDNA control region immediately flanking the *tRNA*^{Pro} gene was sequenced in 100 bigeye tuna (*T. obesus*). In total, 118 variable sites were observed, and 96 haplotypes were defined. Most of the haplotypes were unique to particular individuals. The exceptions were four haplotypes in two individuals (P38 = S05, S02 = S38, S14 = S35, S17 = S23). Representative sequences have been deposited in Genbank, with Accession nos. AY640289–AY640303. Among the sequences examined, A/T base content are obviously higher than C/G base content (mean: A = 38%, T = 28.2%, C = 20.2%, G = 13.6%), which is consistent with previous findings that the D-loop is an A–T rich region of the mitochondrial genome (Brown et al., 1986; Saccone et al., 1987). Both nucleotide diversities (π) (ranging from 0.03797 in the Philippine Sea to 0.0433 in the western Pacific Ocean) and haplotypic diversities (*h*) (ranging from 0.998 in the South China Sea to 1.000 in the western Pacific Ocean and Philippine Sea) are high in the three sampling regions (Table 2).

3.2. Patterns of population structure

The phylogenetic analyses using neighbor-joining method revealed two lineages (Fig. 2). Lineage I is the major lineage which contains most specimens in all three sampling regions (South China Sea = 98%, Philippine Sea = 94% and western Pacific Ocean = 88%) and is weakly supported with a bootstrap value smaller than 50%. In contrast, lineage II is a minor lineage which is strongly supported with a bootstrap value of 99%. It contains only four specimens which consist of four haplotypes (W16, W08, P02 and S45), and its frequency distribution is similar in all three regional populations.

To unravel and delineate the evolutionary relationships between the haplotypes of bigeye tuna, a minimum spanning network was constructed. Two haplotype groups separated by 22 mutation steps were observed and were nested into two different lineages (Fig. 3). Lineage I is a complex network. Its haplotypes exhibit several 'star-like' patterns (e.g., haplotypes W06, 07, S01, S36, P37 and P24 connected to the central haplotype S14 with 1, 1, 4, 4, 7, and 11 mutations, respectively) and extend widely to South China Sea, Philippine Sea and western Pacific Ocean. Lineage II contains four haplotypes that are the same as those clustered in lineage II by neighbor-joining analysis. Thus, nested clade analysis also revealed no geographical isolation among haplotypes within the two lineages.

AMOVA analyses revealed no temporal genetic variations, so we pooled samples collected from different time periods within South China Sea and western Pacific Ocean (all *P*'s > 0.10). Subsequently, AMOVA analyses also revealed no spatial genetic variations within all three areas (Fixation index = 0.001, $P > 0.10$). Tajima's *D* and Fu and Li's *D* statistical tests were performed to determine departures from neutrality in sequence data. Both non-significant values of these two tests were obtained in all sampling regions ($P > 0.10$ in the populations of Philippine sea and western Pacific Ocean, and $0.10 > P > 0.05$ in South China Sea; Table 2). In addition, gene flow values (*Nm*) were very high between all sampling regions (Table 3).

Table 2
Haplotypic diversity (*h*) and nucleotide diversity (π), as well as the Tajima's *D* and Fu and Li's *D* statistical tests of South China Sea, Philippine Sea and western Pacific Ocean

Sampling region	$h \pm$ S.E.	π	Tajima's *D*	Fu and Li's *D*	*P*-values
South China Sea	0.998 ± 0.005	0.03803	−1.39622	−1.90680	$0.10 > P > 0.05$
Philippine Sea	1.00 ± 0.007	0.03797	−1.14099	−1.49018	>0.1
Western Pacific Ocean	1.00 ± 0.022	0.04330	−0.80351	−0.62279	>0.1

Fig. 2. Neighbor-joining tree estimated from Kimura two-parameter distances among mtDNA lineages of bigeye tuna. Haplotypes collected from the South China Sea, Philippine Sea, and western Pacific Ocean are shown as black circles, gray triangles and white squares, respectively. Numbers at nodes indicate the bootstrap values, and only values >50% are shown.

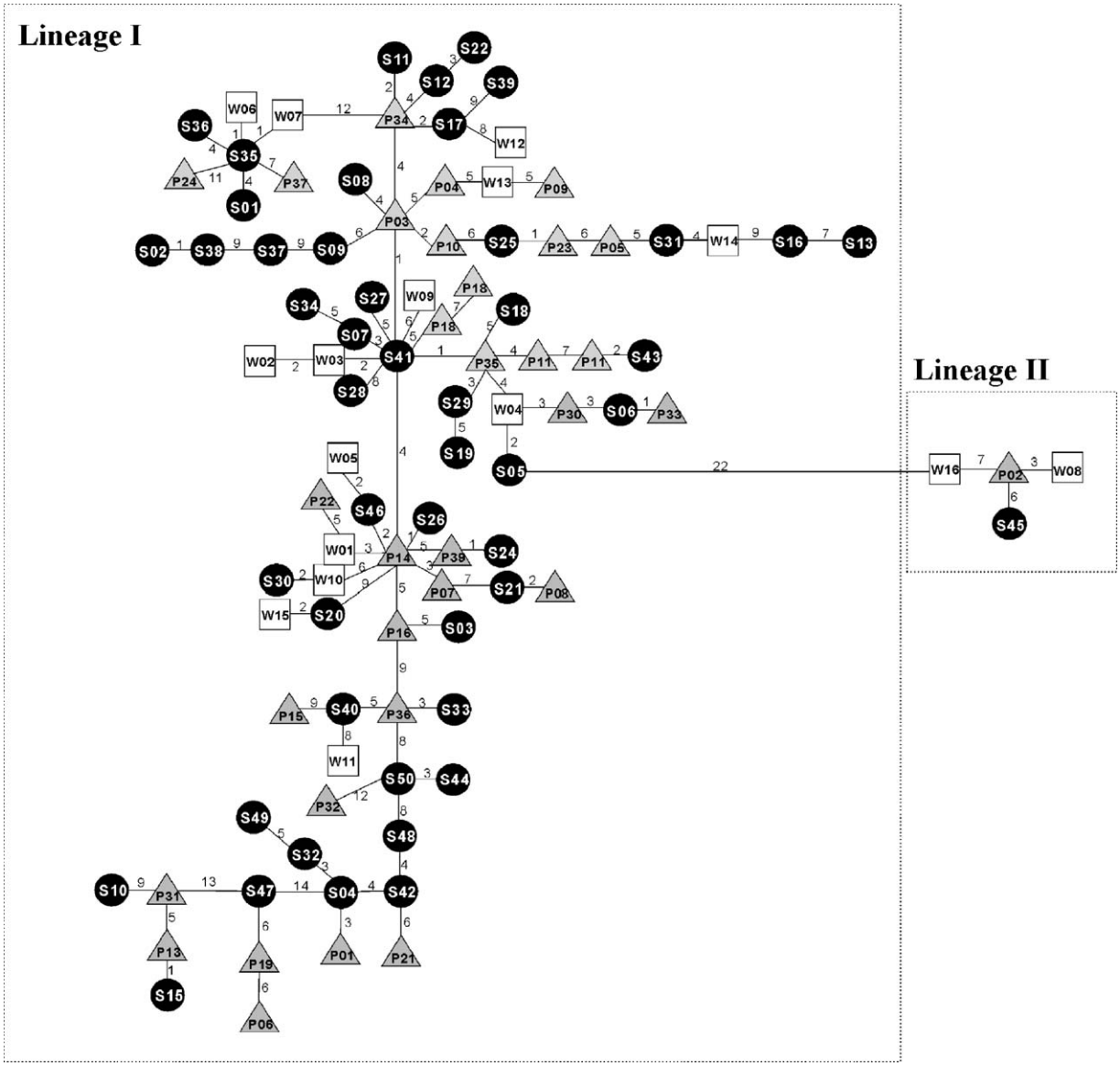

Fig. 3. Minimum spanning network among haplotypes of bigeye tuna. Each circle represents a unique haplotype except the circles S35, S17, S05 and S32, which are composed of two specimens. Numbers at nodes indicate expected mutation steps between haplotypes. Haplotypes collected from the South China Sea, Philippine Sea, and western Pacific Ocean are shown as black circles, gray triangles and white squares, respectively.

Table 3
F_{ST} (above the diagonal) and $N_e m_f$ values (below the diagonal) between sampling regions

	South China Sea	Philippine Sea	Western Pacific Ocean
South China Sea	–	0.00700	0.00020
Philippine Sea	35.96	–	0.00588
Western Pacific Ocean	1239.84	42.77	–

The F_{ST} values are non-significant (P's > 0.10).

4. Discussion

The DNA sequence analysis assay is particularly useful for population studies of animal species (e.g., bigeye tuna) with a worldwide geographic distribution that makes complete sampling very difficult. Prior to the present study, there have been only four mtDNA genetic analyses on the bigeye tuna population structure (Alvarado-Bremer et al., 1998; Chow et al., 2000; Appleyard et al., 2002; Durand et al., 2005). They were carried out by PCR-RFLP method which is a single nucleotide polymorphism assay of a limited number of restriction nucleotide mutation sites. To increase the resolution power of the molecular genetic analysis, in this study we sequenced and analyzed the HVR-1 region of the mitochondrial control region to further investigate phylogenetic relationship and population structure of bigeye tuna in the Western Pacific Ocean.

This study showed high levels of haplotype and nucleotide diversity in the HVR-1 region (Table 2), which were similar to those reported for other highly migratory pelagic fishes

(Alvarado-Bremer et al., 1997, 2005; Grant and Bowen, 1998; Carlsson et al., 2004). Several explanations have been proposed for the maintenance of high haplotypic diversity within populations, including large population sizes, environmental heterogeneity, and life-history traits that favor rapid population increase (Nei, 1987). It has been generally thought that large population sizes are responsible for extraordinarily high levels of genetic diversity in marine fishes (reviewed in Avise, 1998). Bigeye tuna is widely distributed in the world, indicating that large population sizes may account for the high levels of haplotypic diversity observed in this study.

It has been reported that genetic sub-population differentiation was greater in freshwater species than in marine ones. For example, population genetic studies on mtDNA of Pacific yellowfin revealed very low level of genetic differentiation (Scoles and Graves, 1993; Ward et al., 1994, 1997; Appleyard et al., 2001). Particularly, it has been demonstrated that genetic differentiation is generally low between tuna populations within and between oceans (Alvarado-Bremer et al., 1998; Grewe and Hampton, 1998; Chow et al., 2000; Appleyard et al., 2002; Durand et al., 2005). The lack of genetic structure within an ocean demonstrates extensive gene flow at intra-oceanic scales. Consistent with previous studies, low F_{ST} values and high $N_e m_f$ values from this investigation indicate low genetic differentiation and high rates of gene flow between these three bigeye tuna populations.

Previous PCR-RFLP studies have shown no genetic differentiation of bigeye tuna haplotypes in Pacific Ocean (Alvarado-Bremer et al., 1998; Chow et al., 2000; Durand et al., 2005). However, in this sequence analysis study, both the neighbor-joining tree and the minimum spanning network constructed from bigeye tuna haplotypes suggest the existence of two lineages (Figs. 2 and 3). Lineage I is a loose construction, weakly supported with a bootstrap value smaller than 50%. In contrast, lineage II is strongly supported with a bootstrap value of 99%. Both lineages consist of the haplotypes of all three sampling regions. Lineage I is the major lineage that consist of 98%, 94% and 88% of bigeye tuna specimens in the South China Sea, Philippine Sea and western Pacific Ocean, respectively. No differences are found in the frequencies of these two lineages between the three regional populations.

It has been proposed that marine fishes can be classified into four categories based on different combinations of small and large values for haplotypes diversity (h) and nucleotide diversity (π) of mtDNA sequences (Grant and Bowen, 1998). Our study of western Pacific bigeye tuna revealed large values of both h and π (Table 2), which are used to characterize the fourth category of marine fishes by Grant and Bowen (1998). The high level of divergence is attributed to a long evolutionary history in a large stable population or to a secondary contact between previously differentiated allopatric lineages. To decide which explanation fits the Western Pacific bigeye tuna, the sequence data were further tested by Tajima's and Fu and Li's statistical tests. The results of both tests are non-significant, indicating the scenario of an effective large and stable population size. In addition, the results of neighbor-joining tree and minimum spanning network reveal two divergent lineages that are evenly distributed in all three sampling regions. In summary, these genetic data indicate a single panmictic bigeye tuna population in the Western Pacific Ocean and that this panmictic population appears to contain two divergent lineages. Previous population genetic studies revealed that a single stock of bigeye tuna, which consists of only one clade (Clade I), exists in the Indo-Pacific Ocean (Alvarado-Bremer et al., 1998; Chow et al., 2000; Durand et al., 2005; Martinez et al., 2005). In agreement, our results showed that western Pacific Ocean consists of a single panmictic population of bigeye tuna. In addition, these data suggested that bigeye tuna in this region may have two divergent lineages. It is of interest to note that in a recent study Martinez et al. (2005) also found that several samples, particularly those in the Indian and Pacific Oceans, could not be confidently placed within the two well-established clades.

Western Pacific Ocean is an important fishing ground for surrounding southeastern and eastern Asian countries. For reef fishes, such as six bar wrasse and damselfish, four major regional management units in the South China Sea and adjacent areas has been suggested based on recent population genetic studies (Ablan et al., 2002; Chen et al., 2004). However, it is uncertain whether these regional management units are adequate for bigeye tuna, a large, highly migratory pelagic fish. This study revealed a single panmictic stock of bigeye tuna in Western Pacific Ocean and may help its future fishery management policies.

After this manuscript was submitted, Martinez et al. (2005) reported a study of genetic diversity and historical demography of Atlantic bigeye tuna, which examined the mitochondria DNA control region sequences. The results indicated that two divergent mitochondrial lineages existed in the Atlantic bigeye tuna and suggested present uni-directional gene flow from the Indo-Pacific into the Atlantic Ocean.

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