Eastern little tuna or kawakawa (*Euthynnus affinis*) is one of the commercially important tunas in the Philippines and in the Southeast Asian region. Therefore, its sustainability needs to be ensured by effective management. Unfortunately, knowledge on the stock structure of this migratory species in the region, needed for management, is unclear. Here, we studied its genetic population structure using a 300 bp mitochondrial DNA control region (D-loop) marker. Thirty-five samples collected in five areas around the Philippines (Aparri, Palawan, Cagayan de Oro, Tawi-Tawi, and Davao) and thirteen from Peninsular Malaysia (Pangkor Island, Penang) were sequenced and analyzed. Forty five haplotype sequences were homologous (99%) to each other while three (all from Malaysia) were divergent (80%) sequences indicating misidentification. Haplotype distribution, low overall $F_{ST}$ value and non-concordant phylogeographic grouping of the haplotypes indicated that eastern little tuna is panmixing in Southeast Asia.

**A Pilot Study on the Genetic Variation of Eastern Little Tuna (*Euthynnus affinis*) in Southeast Asia**

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**INTRODUCTION**

Eastern little tuna or kawakawa, *Euthynnus affinis* (Cantor 1849), a small epipelagic, migratory, neritic tuna is one of the major commercial tuna species being caught worldwide. Its production has steadily increased from 20,000 tons in 1950 to about 280,000 tons in 2006 (FAO 2009). In the Philippines, eastern little tuna remains an important commercial fish species being landed in the country in terms of volume (BAS 2006). Majority of eastern little tuna caught by the commercial fisheries of the country is exported in processed/ canned form because there is no existing fresh or dried export market. On the other hand, catches from municipal fisheries goes directly to the domestic market for local consumption. The eastern little tuna population is relatively healthy in spite of the fact that many of the country’s fishery resources have already shown signs of overexploitation and decline (Barut et al. 2003; Barut et al. 1997).

Because of its commercial importance and migratory nature, eastern little tuna should be properly studied and managed to ensure its sustainability. To do this, the structure of its stock in the Philippines and in Southeast Asia needs to be elucidated. It is extremely important to know whether 2 or more local communities or, on a regional scale, countries, share one stock of eastern little tuna do that collaborative type of management strategy has to be initiated. A stock consists of randomly interbreeding members whose genetic integrity persists whether they remain spatially and temporally isolated as a group or whether they alternately segregate breeding and otherwise mix freely with members of other unit stocks of the same species. Population or stock structuring could therefore refer to whether or not the total stock or population exhibit subpopulations or sub-set breeding.
groups where migration and gene flow between the groups is restricted to a significant degree.

The stock structure of eastern little tuna in the Philippines and in Southeast Asia is conjecture at best and unclear (Devaraj and Vivekanandan 1997). Differential spawning patterns of the species at different geographical location suggests the presence of distinct subpopulations across its geographical range. Two recruitment pulses i.e. during the first and the second half of the year was noted for the Northern Hemisphere e.g. Thailand and Philippines and a single spawning activity towards the middle of the year was observed in higher altitudes of the tropical zone e.g. Hongkong (Yesaki 1994). Other reports observed varying seasonal spawning peaks according to regions: i.e. March to May in Philippine waters; October- November to April-May (during the NW Monsoon) around the Seychelles; January to July (from the middle of the NW monsoon period to the beginning of the SE monsoon) off East Africa; and probably from August to October off Indonesia (FAO date here).

Stock structures in tunas have been determined previously using different methods including biochemical studies, tagging experiments, morphometrics, meristics, length frequencies and genetic markers (allozyme, RFLP, RAPD, mitochondrial DNA and microsatellites) (Fujino 1976; Fujino et al. 1981; Ward 1995; Waples 1990). Among the genetic markers, mitochondrial DNA (MtDNA) has been employed a number of times because it can be easily extracted, purified and sequenced (if required), it has a relatively fast rate of evolution i.e. base substitution compared to genomic DNA (5-10X faster) and it is maternally inherited. It is therefore not surprising that the MtDNA control region has been successfully used in previous studies on tunas and tuna-like species (Chow et al. 1997; Ward et al. 1997; Ward 1995).

In this study, we present a first report on the genetic variation of eastern little tuna population in the Philippines and Southeast Asia using mitochondrial DNA control region (D-loop) analysis.

**MATERIALS AND METHODS**

**Sample collection and preservation**

Eastern little tuna samples were sampled from 1997-1999 in landing areas or markets around the Philippines that were adjacent to the following seas/ channels- South China Sea (Macarascas, Palawan - M); Balintang Channel (Aparri, Cagayan- AC); Sulu Sea (Tawi-Tawi, Sulu- TS); Celebes Sea- Davao Gulf (Davao- DG); Bohol Sea (Cagayan de Oro- CO) (Figur 1). Identity of sampled fish were confirmed by the distinct morphologica; characteristic of eastern little tuna i.e. having finlets, keel at the caudal peduncle, presence of broken oblique stripes in the dorsal part of the body and dark spots below the pectoral fin (Collete and Nauen 1983). Fish samples were bought whole and fresh from landing centers. Approximately 300 g of tissues was dissected out from the left posterior side of each individual fish. These were then frozen overnight and/or stored in ethanol before processing. On the other hand, tissue samples in absolute ethanol from Penang, Malaysia were kindly provided by Dr. Abhu Thalib of the Fisheries Research Institute of Malaysia.

**Mitochondrial DNA extraction and sequencing**

The ethanol preserved tissues were rinsed with de-ionized H2O. After which approximately 150 mg were sliced off with an uncontaminated disposable razor. The tissues were then be minced and placed in a properly marked 1.5 mL Eppendorf tubes containing Cetyl Trimethyl Ammonium Bromide (CTAB) Extraction buffer (600 uL 2% CTAB pH 8.5, 30 uL of 1% Proteinase K) incubated overnight in a water bath at 55°C with occasional shaking. After incubation, 600 uL of chloroform: isoamyl (3:1) solution was added to each of the sample, shaken by hand for about 3 min. and then centrifuged for 5 min. at 8,000 rpm. The upper aqueous supernatant was then transferred in new, marked 1.5 mL tubes, avoiding inclusion of the organic phase. Described steps of clean-up will be done twice. DNA precipitation was then be carried out by mixing 50 uL of 3M Sodium Acetate (NaOAc) and 900 uL 95 % Ethanol to the tubes containing the supernate. These were then hand shaken for 3 min. and placed overnight in a –20°C freezer. After precipitation, the tubes are then spun in a microcentrifuge at 13,000 rpm for 30 min and then the aqueous phase was carefully pipetted out leaving the DNA pellet at the bottom of the tube. The pellet was then rinsed by adding 500 uL 70% ethanol and spun for 13,000 rpm for 3 min. before removal of ethanol. Rinsing was done twice. Drying was carried out by using a vacuum dryer (Speed-Vac) for 10 minutes. DNA pellets were then rehydrated in 100 uL of 1X TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). Resulting stock DNA extracts were stored in cryo vials at -80°C.

A 300 bp mitochondrial DNA fragment covering a portion of the variable 5’ end of the control region (D-loop) was amplified through PCR machine (Perkin-Elmer Cetus 480). Forward primer CB3R420 (5’ - CCCCTAACTCCCAAAATCAGg – 3’) and reverse primer 12Sar430 (5’ – gCCTgGgggCTTTCTAggCC – 3’) designed by the author and manufactured by GENSET, USA were used in the amplification. A 25 uL
reaction mixture was prepared containing the following reagents; 11.4 uL of milliQ H2O, 2.5 uL Kocher’s Buffer, 5 uL dNTP’s, 2.5 uL each of 10 uM primers, 0.4 uL taq and 1 uL of DNA template. Two (2) drops of mineral oil will be added to cover the reaction mixture. After the reaction, amplicons were electrophoresed through a 1 % agarose gel in TBE buffer (90mM Tris-Boric acid, 2mM EDTA). The gel was stained with Ethidium Bromide and bands were visualized by ultraviolet (UV) illumination. A Polaroid camera was utilized in documenting the resultant bands immediately after electrophoresis. Size of DNA fragments were estimated by comparing with 100 bp DNA ladder (GIBCO BRL).

Both sense and antisense part of the 300 bp fragment were sequenced. Cycle sequencing was done by preparing a 12 uL cycle sequence reaction using, 2 uL of dRhodamine dye, 1 uL of 10uM of the primer, 4 uL of clean DNA and 5 uL of mQ H2O. These were then ran in a PCR machine (Perkin-Elmer GeneAmp PCR System 9600) at the following profile: 25 cycles, 96°C 0:10, 55°C 0:10, 60°C 4:00 and 4°C. Ethanol precipitation was used to clean the cycle sequence reactions. Entire contents of the extension product was pipetted out and placed into a tube containing 1.2 uL of sodium acetate (NaOAc) pH 5.2 and 26 uL of 95% ethanol (ETOH). The tubes were then vortexed and were place on ice for 15 minutes. Afterwhich, these were spun for 30 min. at 13,000 rpm on an eppendorf benchtop microcentrifuge 5415C or 5417C. Supernatant was then carefully aspirated and discarded. Resulting pellet was then rinsed with 250 uL of 70% ethanol, vortexed briefly and then spun again for 2 minutes at 13,000 rpm. The supernatant was aspirated and discarded again and the pellet was dried in a vacuum centrifuge (Speed-vac Savant) for 10 to 15 min. Amplicons were sequenced using ABI PRISM Model 377 ver. 3.3 Automated Sequencer.

Data Analysis

Raw sense and antisense sequence data were assembled, analyzed and aligned using Sequencher Ver. 3.0 (GeneCodes) and GENETYX Ver. 8.0. (Japan). Haplotype frequency, genetic diversity, and global fixation index (Fst) of the 45 confirmed samples were computed using Arlequin ver. 3.11 package (Excoffier et al. 2005). Percent identities between haplotypes were computed using Megablast (for highly similar sequences) under the BLAST 2 sequence (bl2seq). A 99% homology was set to confirm species identity. Stock structuring was analyzed using the Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992), plotted in Minimum Spanning Network (MSN) in Arlequin, and the tree drawn using the Phylogenetic Analysis Using Parsimony or PAUP ver. 4.
Figure 1. Map of Southeast Asia showing the sampling sites in the Philippines and Malaysia: Aparri, Cagayan (AC), Macarascas, Palawan (M), Opol, Cagayan (OC), Tawi-Tawi, Sulu (TS), Davao Gulf (DG) and Pangkor Island, Penang, Malaysia (PM). Distribution and frequency of eastern little tuna mitochondrial DNA haplotypes across sampling sites as well as the existing Pacific Ocean currents in the Eastern side of the Philippines; Pacific North Equatorial Current, Kuroshio Current and the Mindanao Current are likewise shown.

Table 1. Eastern little tuna mitochondrial DNA control region haplotypes and its genetic and nucleotide diversity of in each of the 6 sampling sites in Southeast Asia. The 3 divergent haplotypes from Malaysia are not included.

<table>
<thead>
<tr>
<th>Sampling Site</th>
<th>No. of Samples</th>
<th>Sampling Date</th>
<th>Average Fork Length (cm)</th>
<th>Average Weight (grams)</th>
<th>Hap A</th>
<th>Hap B</th>
<th>Unique Hap</th>
<th>Expected Heterozygosity</th>
<th>Nucleotide Diversity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aparri, Cagayan (AC)</td>
<td>7</td>
<td>06/20/1998</td>
<td>26</td>
<td>730</td>
<td>4</td>
<td>2</td>
<td>1 (C)</td>
<td>0.67 +/- 0.16</td>
<td>1.24</td>
</tr>
<tr>
<td>Macarascas, Palawan (M)</td>
<td>6</td>
<td>10/23/1997</td>
<td>26</td>
<td>290</td>
<td>3</td>
<td>1</td>
<td>2 (D,E)</td>
<td>0.80 +/- 0.18</td>
<td>2.13</td>
</tr>
<tr>
<td>Opol, Cagayan (OC)</td>
<td>8</td>
<td>04/02/1997</td>
<td>36</td>
<td>723</td>
<td>3</td>
<td>1</td>
<td>4 (F,G,H,I)</td>
<td>0.89 +/- 0.11</td>
<td>2.61</td>
</tr>
<tr>
<td>Tawi-Tawi, Sulu (TS)</td>
<td>7</td>
<td>05/18/1997</td>
<td>27</td>
<td>342</td>
<td>3</td>
<td>1</td>
<td>3 (J,K,L)</td>
<td>0.86 +/- 0.14</td>
<td>2.14</td>
</tr>
<tr>
<td>Davao Gulf (DG)</td>
<td>7</td>
<td>06/30/1997</td>
<td>28</td>
<td>386</td>
<td>-</td>
<td>-</td>
<td>7 (M,N,O,P,Q,R,S)</td>
<td>1.00 +/- 0.08</td>
<td>4.19</td>
</tr>
<tr>
<td>Pangkor Is., Penang, Malaysia (PM)</td>
<td>10</td>
<td>01/14/1999</td>
<td>32</td>
<td>573</td>
<td>3</td>
<td>-</td>
<td>7(T,U,V,W,X,Y,Z)</td>
<td>0.93 +/- 0.08</td>
<td>5.17</td>
</tr>
</tbody>
</table>

Hap: haplotypes, (-): absent
Figure 2. Nucleotide sequences of the identified D-loop region of the mitochondrial DNA haplotypes of eastern little tuna. Number per haplotype are shown in parenthesis. The 45 haplotype sequences with 99% homology among them were deposited in the DNA Data Bank of Japan (DDBJ) with accession numbers from AB519197 to AB519294.

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found to be of different species. In the absence of detailed information on the collection process for the contributed tissue, we speculate that some of the source of the samples was from juvenile tunas, which are difficult to differentiate because their morphological characteristics are very similar. Such mis-identification is not uncommon for tuna species. For example, yellowfin tuna that have been identified manually in the field were found to consist of bigeye tuna using mtDNA at frequencies of 0% to 30% (Grewe and Hampton 1998). Miyabe et al. (2005) likewise observed that eight (1.6%) of the 502 tunas identified in the field were misidentified. This discovery actually underscores the utility of the 300 bp mtDNA control region fragment, as well as the primers used, for further taxonomic and assessment studies.

The high genetic diversity of the 300 bp mtDNA marker in this study (about 86%) was similar to the genetic diversity of mtDNA markers used in previous studies on tuna population at 0.60 (Chow and Ushima 1995) and billfishes at 0.922 (Chow et al. 1997), suggesting that this region is a useful genetic marker for this particular species for population studies. Interestingly, based on genetic and nucleotide diversity and geographic location, the haplotypes could be divided into four regions: the mid-level diversity Balintang Channel, the high-level diversity central Philippines, the very high-level diversity Southeastern Philippines and the very high-level diversity Western Peninsular Malaysia. This observation could have implications to the population or the biogeography of the region and is worth studying in the future.

Varying recruitment pulses of eastern little tuna in the general Southeast Asian region (Yesaki 1994), indicated the presence of sub-set breeding groups for the species. However, our present data suggest otherwise, albeit our sample size is relatively small. We observed that 2 dominant mtDNA haplotypes are dispersed all throughout the general area of Southeast Asian region. Moreover, the haplotypes exhibited a very low $F_{ST}$ value and there is no apparent structuring in the Minimum Spanning Network. All of this suggests that the eastern little tuna in the Philippines and in Southeast Asia is near "panmixia" or mixing. The star-like appearance of the MSN though
indicates a recent population expansion of the species (a common inference for species in the Indo-West Pacific (Crandall E. pers. comm.).

The observed homogeneity in the population of eastern little tuna in Southeast Asia is similar to other tunas and billfishes caught from different oceans (Chow et al. 1997; Rosel and Block 1996; Ward et al. 1997). “Panmixing” of the species in the region is very possible because of the high mobility of the species, the play of the prevailing currents in the region (Wyrtki 1961) and accompanying larval dispersal. This small tuna is widely distributed in the tropical and subtropical waters of the Indo-Pacific Region and its larvae are found to be widely dispersed throughout the Philippines (Abuso 1988; Wade 1950) and was observed to spawn in Hongkong, India, Thailand and the South China Sea (Yesaki 1994). The continuous continental shelf connecting the countries in Southeast Asia could also facilitate migration of the said species since they were found to have strong affinities to continental shelves (Yesaki 1994). Graves (1996) suggested that several pelagic fishes including skipjacks, albacore, yellowfin and dolphin fishes have exhibited little spatial partitioning within and between ocean basins because of the occurrence of continuous, circumtropical pelagic environment and a wide range of suitable spawning grounds. Tunas in particular have also been suggested by Ward (1995) to exhibit a low population divergence and that migration of just a few individuals per generation could produce near-genetic homogeneity. Very recently, Magsino and Juninio-Ménez (2008) found no significant genetic stock structuring in the pelagic rabbitfish, *Siganus argenteus* sampled from Northern to Southern Philippines even though the geographic location of the fish is bifurcated by the Northern Equatorial Current (NEC).

While the evidence for panmixia is strong, we could not ignore the absence of haplotypes A and/or B in the Davao Gulf samples. Is it possible that we could be seeing some evidence of a subpopulation in this area, which is not significantly detectable with the present number of samples in the study? This is similar to the observation of Arnaud et al. (1999) where they detected genetic homogeneity in scad mackerel *Decapterus macarellus* from Molucca Strait to Banda Sea and for *D. macrosoma* from South China Sea to Java Sea and Makassar Strait but found distinct populations of *D. macrosoma* in Sunda Strait, a sharp transition between the Indian Ocean and Sunda Shelf. Genetic homogeneity does not also mean that there is no population subdivision as has been observed in the stock identity of horse mackerel (*Trachurus trachurus*) in Northeast Atlantic and Mediterranean Sea where stocks were identified using morphometrics, parasites and life history but not with genetic markers (Abanuza et al. 2008). It is possible that the Davao Gulf population could be affected by the prevailing current system (Mindanao Current) in the Southeastern Philippines (Figure 1) (Lukas et al. 1991), which in some studies was implicated to the limited larval dispersal and gene flow in mantis shrimp found in Northern Indonesia and Papua (Barber et al. 2006). Arnaud et al. (1999) also implicated the geographic isolation, mortality of larvae due to hydrological variations and homing of adults as probable factors that brought about distinctiveness of the population of *D. macrosoma* in Sunda Strait, Indonesia. It is thus, interesting to pursue studies on this in the future using a larger sample size and additional sampling sites in the area e.g. Sulawesi and Papua New Guinea.
Indeed the number of samples analyzed in the study needs to be increased to attain more significant results. Furthermore, there are limitations to the use of mtDNA in analyzing populations because it does not account for paternal inheritance. Nevertheless, this study provides important initial evidence of the sharing of eastern little tuna among the countries in Southeast Asia, which would warrant co-management strategies among the member countries to ensure the sustainability of this important fishery resource. It also provides some impetus for looking further into a possible subpopulation in Southern Philippines and adjacent areas that has also implications to the management of the stocks separate from the greater Southeast Asian region.

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