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Longtail tuna *Thunnus tonggol* (Bleeker, 1851) shows genetic partitioning across, but not within, basins of the Indo-Pacific based on mitochondrial DNA

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Summary

Genetic stock structure is atypical in tuna species, with most species demonstrating geographically-broad, panmictic populations. Here, genetic data suggest a distinct pattern for *Thunnus tonggol* across the Indo-Pacific region. The genetic variation in the coastal tuna *T. tonggol* sampled from across the South China Sea was examined using the highly variable mitochondrial DNA displacement loop (D-loop) gene region. One hundred and thirty-nine specimens were sampled from four locations in Indonesia, Vietnam and the Philippines. Phylogenetic reconstruction of genetic relationships revealed no significant ϕ_{ST} statistics and hence no population structure within the South China Sea. However, subsequent analysis with sequence data from coastal northwest India infers discrete genetic stocks between the Indian Ocean and the South China Sea. Consistent with previous genetic analyses of tuna species in the Indo-Pacific, the findings in this study infer no population structure within each basin, but rather show a significant partitioning across the wider region. Furthermore, these results have implications for the management of the commercially valuable *Thunnus tonggol* across national boundaries, and thus requiring collaboration among countries to ensure its sustainable use.

Introduction

An important early first step in any fisheries management is identifying the extent of the stocks to be managed as well as any distinct sub-populations or stocks within a species (Stephenson, 1999). Longtail tuna (*Thunnus tonggol*) are a neritic species widely distributed in the inshore tropical waters of the northern Indian and the western Pacific oceans. Reported catches have grown within the past 50 years to about 250 000 tonnes (FishStat, 2013), with signs of overexploitation (Sharma et al., 2012). The landings data may not reflect the true take due to under- and misreporting (Pauly and Froese, 2012). Despite the important contributions of this species to local food supplies and commerce, there is very little research on *T. tonggol* in either ocean. Indeed, there is little information on some basic parameters, such as the number and distribution of the stocks.

This lack of information is complicated by the transitory nature of these fish across political boundaries where they are exposed to a mosaic of regulatory and management policies. An 8–10 October 2013 agreement by the South East Asian Fisheries Development Centre (SEAFDEC) to prepare a Regional Plan of Action for Neritic Tunas prioritized the research and management needs of *T. tonggol*. Resolving questions on the number of stocks has important implications for the design of research and the nature of management agreements, whereby monitoring of the catch and DNA work has been called for in the past (Griffiths et al., 2010a).

Population genetics is a tool applied to marine fisheries in recent decades and which has led to improved knowledge of stock delineation and population dynamics of exploited fishes (Hauser and Seeb, 2008). For example, investigation into the population structure of the bigeye tuna *Thunnus obesus* inferred a single, intermixing population among localities in the South China Sea, Philippine Sea and western Pacific Ocean (Chiang et al., 2006). Similarly, yellowfin tuna *T. albacores* also exhibited little genetic differentiation between localities in the western Pacific and western Indian Oceans (Wu et al., 2010); however there are some instances of multiple geographically distinct stocks along the Indian coast (Kunal et al., 2013). Genetic data often, but not always, reveals intermixing or panmictic populations of highly migratory fishes. For example, skipjack tuna *Katsuwonus pelamis* sampled from two distant localities in the Indo-Pacific region maintained discrete genetic lineages, suggesting that the two populations should be managed separately (Menezes et al., 2006). Unlike the aforementioned studies that revealed panmictic tuna stocks, Menezes et al. (2006) utilized the highly polymorphic mtDNA D-loop and identified geographic population differentiation. Such results support the appropriateness for using the D-loop for population studies of species.

In molecular genetic studies, neritic tuna species occurring in the shallower, coastal waters of Southeast Asia have received less attention than their pelagic counterparts. Further, population patterns may be distinct from pelagic tuna species due to their tendency to be associated with shelf habitats and the potential influence of localized ocean currents. Santos et al. (2010) noted the large growth in catches and the lack of knowledge in basic, management oriented parameters for the eastern little tuna (*Euthynnus affinis*), another neritic tuna that shares the same habitat as *T. tonggol* and is

often caught in the same fishing gear; in their DNA study of fish from the Philippines and Malaysia, they inferred a panmictic stock and suggested this was due to the migratory nature of the species, larval dispersal, and the contiguous nature of the continental shelf between Malaysia and the Philippines. In contrast, hyper-variable mtDNA sequence data from frigate tuna (*Auxis thazard*) and eastern little tuna sampled from across the Indonesian archipelago did suggest a population structure of these tuna into eastern, central, and western clades (Jackson et al., 2014).

The status of longtail stocks is unknown, with conflicting evidence over whether *T. tonggol* is a long-lived and slow growing species making it vulnerable to fishing pressure, or is a fast growing species and robust to fishing pressure (Yesaki, 1994; Griffiths et al., 2010a,b). Whichever the case, there can be little doubt that catches cannot continue to grow indefinitely. Improving an understanding of the status of *T. tonggol* is timely as there is a growing interest in this species in some export markets. Fishing is an important source of income and food for many coastal peoples in Thailand, Indonesia, Malaysia, and the Philippines. Good resource management is vital if continued economic progress and poverty alleviation is to be achieved. In many parts of Southeast Asian fisheries the resources have become depleted due to inadequate management; however, there is a current opportunity to attain sustainable use for *T. tonggol*.

Understanding of longtail tuna populations is based at present largely on tagging (Griffiths et al., 2011) and morphometric data (Gibbs and Collette, 1967; Yesaki, 1987). Gene based population studies have been limited and provided only inconclusive evidence of population differentiation of *T. tonggol* populations in the Indo-Pacific region (Lewis, 1981; Kunal et al., 2014).

Here we examine the genetic patterns of the longtail tuna *T. tonggol* from four locations in the South China Sea region, specifically Vung Tau, Vietnam; Pemangkat, Indonesia (Borneo); Pekalongan, Indonesia; and Palawan, Philippines (Fig. 1). By utilizing the highly variable and non-coding D-loop region of the mtDNA, the genetic variation within and between sampled localities in Indonesia, Vietnam,

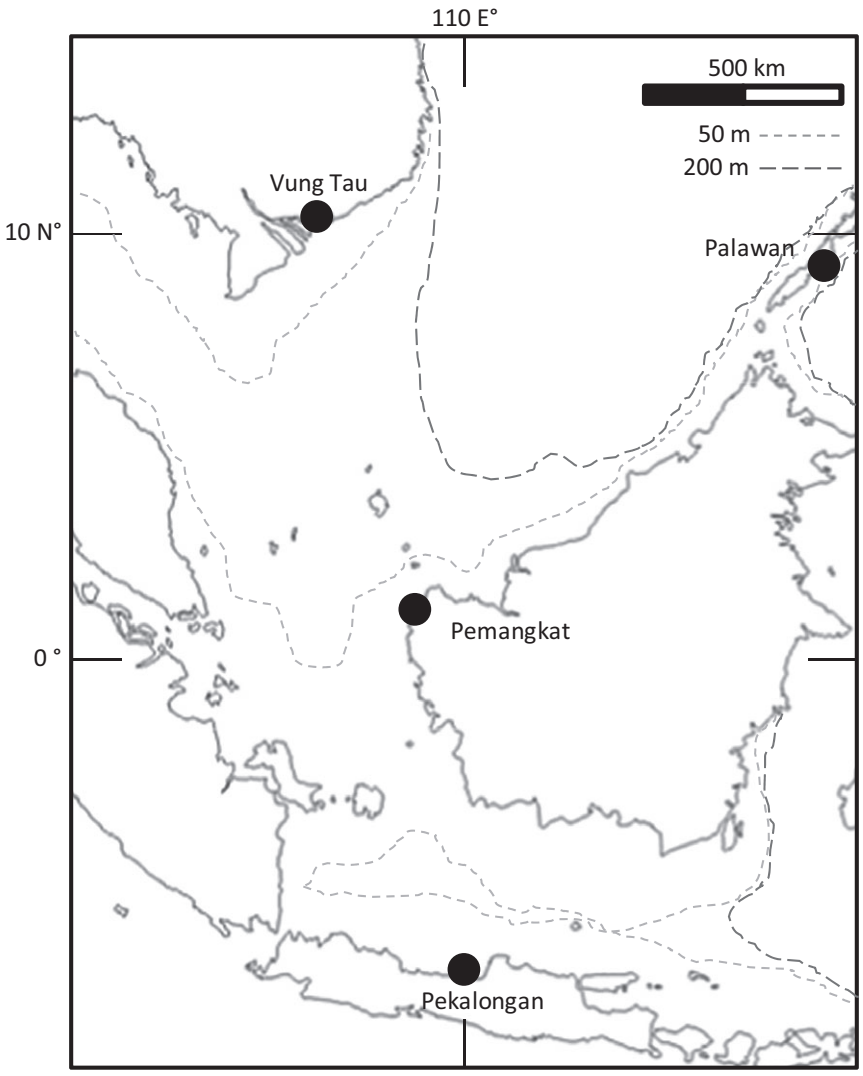

Fig. 1. Sampling locations of *Thunnus tonggol* from Indonesia, Vietnam and the Philippines. Dotted lines = bathymetry at 50 m depth contour and 200 m depth contour

and the Philippines infer the phylogeographic pattern of *T. tonggol* across the South China Sea. Further, we utilize recently published sequence data of *T. tonggol* from two additional locations in northwest India (Kunal et al., 2014) to broaden the geographic range of our conclusions.

Materials and methods

Tissue samples of *T. tonggol* were collected from whole specimens landed at local fish ports in Pemangkat and Pekalongan, Indonesia, Vung Tau, Vietnam, and Puerto Princesa, Palawan, Philippines (Fig. 1; Table 1). To aid in determining the geographic source of the fish, vendors were asked if the fish were harvested from local waters. Tissue samples were taken from the flank of the fish and preserved in 95% ethanol until molecular analysis. Partial mitochondrial DNA sequences (885 bp) of the D-loop region were used to examine phylogeographic relationships among the four sampled locations. Whole genomic DNA was isolated from tissues using the CTAB extraction method or the commercially available DNA extraction kits (Qiagen, Valencia, CA).

Amplification was carried out using Polymerase Chain Reaction (PCR) with the primers CB3R420 (5′- CCCCC TAACTCCCAAAGCTAGG -3′) and 12Sar430 (5′- GCCTG CGGGGCTTTCTAGGGCC -3′). PCR was performed with a final volume of 25 μl containing 2.5 μl of 10× PCR Buffer and dNTPs (8 mM), 2 μl of $MgCl_2$, 1.25 μl of both primers, 0.125 μl of *Taq* DNA polymerase, and 1 μl of DNA template. Thermal cycle was set as follows: initial denaturation at 95°C for 10 min, then 38 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 45 s, and a final extension of 10 min at 72°C. All PCR products were checked for positive amplification by visualizing them in a 1% agarose gel stained with ethidium bromide. A successfully amplified PCR product was purified and sequenced by commercial sequencing facilities (Macrogen, Korea). Sequences were assembled, proofread, and aligned in Sequencher v4.8 (GeneCode, Ann Arbor, MI) and MUSCLE v3.8 (Edgar, 2004).

Genetic relatedness was inferred between mtDNA D-loop sequences via maximum-likelihood tree in MEGA v5 (Tamura et al., 2004) using the Tamura-Nei G+I substitution model (identified as the best-fit model per the MEGA v5 subroutine), inclusive of several tuna out-groups from the public domain database GenBank (*T. orientalis* [JN631250.1, JN631208.1], and *K. pelamis* [AB101290.1]). To broaden the analysis and strengthen the assertion that all samples were *T. tonggol*, a D-loop sequence of *T. tonggol* available through GenBank [HQ630771.1] was included. Next, sequences from each sampling location were pooled by site and calculated for nucleotide diversity (π), haplotype diversity (*Hd*), and number of haplotypes in DNASP v5.1 (Librado and Rozas, 2009) to assess the population level variation.

Divergence between sampling sites was estimated using the Tamura-Nei distance model with each sampling site treated as a putative discrete population. Population structure was explored in ARLEQUIN v3.5 (Excoffier et al., 2005) using Phi-statistics calculated from pairwise differences of each site, again treating sampling locations as separate putative populations. Sequential Bonferroni Correction was applied to the data to account for multiple pairwise analyses. The Tajima's *D* and Fu and Li's (1993) *D* statistical tests of neutrality were calculated in DNASP v5.1. Seventy-three D-loop sequences from Veraval and Ratnagiri, India [KC313300-KC313393] were included to broaden the scope of the analysis. The shorter Indian sequences (467 bp) required the sequences (885 bp) in the present study be trimmed to the same length; the Phi-statistics were then estimated among all six localities in ARLEQUIN v3.5, as described above.

Results

Twenty-two to forty-seven mtDNA D-loop sequences (885 bp) were obtained from specimens from each of the four sampling sites with 132 of 139 haplotypes unique to a single site (Table 1). Five haplotypes were shared between 2 and 3 locations; two between Palawan and Pekalongan, one between Vung Tau and Palawan, one between Pekalongan and Pemangkat, and one between Palawan and both Indonesian sites. All sites had high haplotype diversity (0.99–1.00) and moderate nucleotide diversity (1.2–1.6%; Table 1). A total of 112 polymorphic sites were identified, including 42 singleton variable sites and 70 parsimony informative sites. The phylogenetic reconstruction inferred a single, highly-supported (>90%) clade containing all *T. tonggol* sequences, including the reference *T. tonggol* data from GenBank (Fig. 2).

Intraspecific genetic distance between *T. tonggol* sampling locations was low (1.3–1.9% sequence divergence) and within the range expected intraspecific divergence. All putative *T. tonggol* populations were more similar to each other (1.4–1.6% sequence divergence) than to any of the three out-groups (4.7–26.7% sequence divergence), and only 1.8% divergent from the reference *T. tonggol* sequence from GenBank.

Table 1
Population information, sequence, diversity, and neutrality test metrics per sampling site

Population	Location	N	H	S	*Hd*	π%	Tajima's *D*	Fu and Li's *D**
Palawan, Philippines	09°44′N, 118°44′E	22	21	50	0.996	1.2	−1.10	−0.94
Vung Tau, Vietnam	10°20′N, 107°04′E	26	24	68	0.994	1.6	−0.86	−0.24
Pekalongan, Indonesia	06°51′S, 109°41′E	44	41	78	0.997	1.4	−1.26	−1.10
Pemangkat, Indonesia	01°10′N, 108°58′E	47	46	72	0.999	1.3	−1.20	−0.90

Sampling site; coordinates of sampling sites; N, sample size; H, number of haplotypes; S, number of polymorphic sites; *Hd*, gene diversity; π, nucleotide diversity; Tajima's *D* neutrality test; and Fu and Li's *D** neutrality test. No neutrality test was significant.

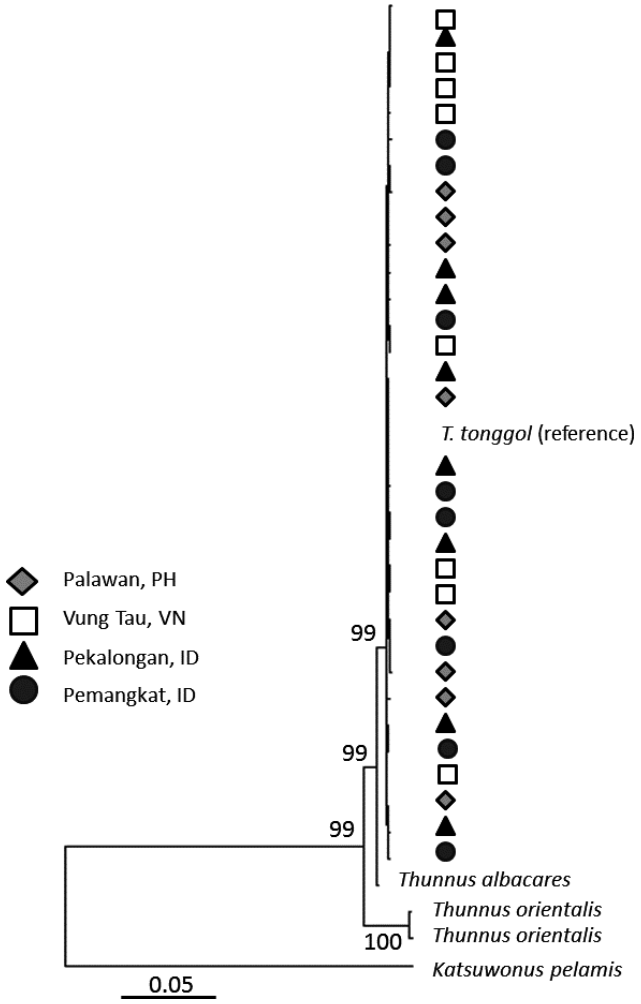

Fig. 2. Neighbor-joining phylogenetic tree of three *T. tonggol* populations (eight representative sequences per site) with 1000 BP node support based and Tajima-Nei substitution model, with one reference *T. tonggol* sequence (GenBank) and three out-group species

Global population structure analysis of the SCS localities revealed a statistically non-significant fixation value (F_{ST} 0.006, $P > 0.10$). Examination of the population structure among the Indonesian, Vietnamese, and Philippine localities using Phi-statistics also did not reveal significant genetic differentiation between Vung Tau, Vietnam and the two localities of Palawan, Philippines and Pekalongan, Indonesia (Table 2). Genetic data infers a single *T. tonggol* stock within the South China Sea. Both Tajima's *D* and Fu and Li's *D** tests of neutrality for all sites were negative, suggesting recent population expansion (Grant and Bowen, 1998); however, none were significant.

Re-analysis of trimmed *T. tonggol* sequences (467 bp) to match 73 sequences from northwest India resulted in a statistically significant global fixation value (F_{ST} 0.062, $P < 0.01$). A lack of genetic partitioning within the South China Sea remained unchanged when utilizing the shorter sequence data, while all South China Sea locations were statistically divergent from the Indian Ocean locations (Table 3). Consistent with Kunal et al. (2014), the Indian sites of Veraval and Ratnagiri were genetically similar.

Discussion

Phylogenetic reconstruction of molecular variation between *T. tonggol* sampled from Indonesia, Vietnam, and the Philippines reveal high levels of genetic diversity with no clear pattern of haplotype-partitioning associated with a specific geographic location (Fig. 2), nor significant population structure based on site-by-site pairwise comparisons. This suggests a single, intermixing population of *T. tonggol* across the South China Sea sample sites and gene flow across distances of more than 2000 km, but all situated on a continuous stretch of shallow shelf habitat in the Vietnam, Borneo, Java and the Philippine archipelago (Fig. 1). Significant population structure was, however, inferred when including sequence data from locations within the Indian Ocean, hence suggesting genetic partition across greater distances and/or in relation to the Sunda Shelf Barrier (Carpenter et al., 2011).

Genetic population structure is uncommon in mtDNA mitochondrial studies of migratory pelagic fishes; instead, patterns of panmixia are often revealed, including pelagic cosmopolitan *Thunnus* congeners such as the bigeye tuna *T. obesus* (Durand et al., 2005; Chiang et al., 2006) and yellowfin tuna *T. albacores* (Wu et al., 2010). This panmictic pattern was also reported previously in *T. tonggol* (Kunal et al., 2014) and in other neritic tuna species, including *E. affinis* (Santos et al., 2010). The limitation, however, of their previous neritic tuna studies was the geographic range, with sampling sites for both of the aforementioned studies

Table 2
Summary of analysis of molecular variance (AMOVA) showing ϕ_{ST} statistics among South China Sea localities (below diagonal) and P-value (above diagonal). No P-values were statistically significant at a 5% significance level after application of Sequential Bonferroni Correction

Location	1	2	3	4
1. Vung Tau, VN	–	0.01	0.05	0.07
2. Palawan, PH	0.0328	–	0.49	0.42
3. Pekalongan, ID	0.0158	0.0013	–	0.82
4. Pemangkat, ID	0.0141	0.0005	0.0055	–

Table 3
Summary of analysis of molecular variance (AMOVA) showing ϕ_{ST} statistics among South China Sea and Indian Ocean localities (below diagonal) and P-value (above diagonal). The ϕ_{ST} values in bold are statistically significant based on Sequential Bonferroni Correction

Location	1	2	3	4	5	6
1. Vung Tau, VN	–	0.02	0.08	0.05	**0.01**	**0.01**
2. Palawan, PH	0.033	–	0.70	0.50	**0.01**	**0.01**
3. Pekalongan, ID	0.013	0.006	–	0.79	**0.01**	**0.01**
4. Pemangkat, ID	0.018	0.002	0.006	–		0.13
5. Veraval, IN	**0.089**	**0.092**	**0.088**	**0.094**	–	
6. Ratnagiri, IN	**0.113**	**0.114**	**0.107**	**0.113**	0.006	–

confined to the same shelf region. In contrast, studies encompassing multiple shelf regions have inferred significant divergence of stocks of *A. thazard* and *E. affinis* (Menezes et al., 2006, 2012; Dammannagoda et al., 2011; Jackson et al., 2014). These results may have direct management implications in recommending that *K. pelamis* and *A. thazard* be managed as discrete, genetically differentiated stocks. Results of our study would also encourage a multi-stock management strategy for *T. tonggol* between the Indian Ocean and the South China Sea.

To our knowledge, this is the first study to explore the genetic variation and genetic population structure of *T. tonggol* across multiple shelf habitats to reveal multiple stocks. Further, our data indicate *T. tonggol* at each sampling location have likely undergone recent population expansion based on neutrality tests. The lack of a dominant or commonly shared haplotype across all four South China Sea localities further supports recent population expansion or purifying selection (Grant and Bowen, 1998). Geological changes in the region may provide an explanation. During the Pleistocene, a lower sea level and cooler sea temperatures could have limited population size and distribution of tuna across a smaller marine habitat in the Indo-Pacific basins, namely, due to the exposed Sunda Shelf (Shackleton, 1994; Voris, 2000). Subsequent warming and submergence of the Sunda Shelf in the early Holocene may have fueled a population expansion in *T. tonggol*, a putative signature still present in the mtDNA sequence data. By including sampling localities around the periphery of the South China Sea and being inclusive of published sequence data from the Indian Ocean, this study is a critical first step in providing insight for stock management of this neritic species. We caution, however, that although the mtDNA D-loop is an appropriate and frequently employed molecular marker to assess the genetic population structure, it does have limitations in only illustrating maternal genetic exchange (Avise et al., 1987). Hence, it is possible that a more complete investigation of longtail tuna populations in the region that include nuclear DNA markers or single nucleotide polymorphisms could reveal a finer structure. At this time, we cannot fully rule out such a possibility and hence we identify each basin's population as putative.

Implications for management

Understanding the distribution of fish stocks is an important component for understanding stock status and the design and implementation of management regimes. The importance of *T. tonggol*, the existing pressures on *T. tonggol* resources and the need for a more solid basis for understanding its stock distribution have all been highlighted by previous authors and justify studies like the present for unraveling the population structure of target fisheries via molecular genetic tools.

The importance of understanding the stock structure is exemplified by a number of questions mentioned or alluded to in previous studies, and essential contributions for organizations such as the Indian Ocean Tuna Commission and others working to manage regional tuna stocks.

In identifying two geographically adjacent yet genetically differentiated stocks between the South China Sea and the Indian Ocean stocks, this study makes a case for an approach to management that involves all key regional nations in whose waters longtail tuna are to be found and fished. As these stocks are obviously important for many countries, this requires collaboration in the areas of stock status assessment, catch monitoring and reporting, catch and effort controls and surveillance/enforcement.

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