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Genetic analysis reveals two stocks of skipjack tuna (*Katsuwonus pelamis*) in the northwestern Indian Ocean

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Abstract: Skipjack (SJT) (*Katsuwonus pelamis*) is a medium-sized, pelagic, highly dispersive tuna species that occurs widely across tropical and subtropical waters. SJT constitute the largest tuna fishery in the Indian Ocean and are currently managed as a single stock. Patterns of genetic variation in a mtDNA gene and six microsatellite loci were examined to test for stock structure in the northwestern Indian Ocean. 324 individuals were sampled from five major fishing grounds around Sri Lanka and from single sites in the Maldive Islands and the Laccadive Islands. Phylogenetic reconstruction of mtDNA revealed two coexisting divergent clades in the region. Analysis of molecular variance (AMOVA) of mtDNA data revealed significant genetic differentiation among sites (Φ_{ST} = 0.2029, $P < 0.0001$), also supported by spatial AMOVA results. AMOVA of microsatellite data also showed significant differentiation among most sampled sites (F_{ST} = 0.0256, $P < 0.001$), consistent with the mtDNA pattern. STRUCTURE analysis of the microsatellite data revealed two differentiated stocks. While both marker types examined identified two genetic groups, microsatellite analysis indicates that the sampled SJT are likely to represent individuals sourced from discrete breeding grounds that are mixed in feeding grounds in Sri Lankan waters.

Résumé : La thonine à ventre rayé (SJT) (*Katsuwonus pelamis*) est une espèce de thon de taille moyenne, à vie pélagique et à grand pouvoir de dispersion qui est largement répandue dans les eaux tropicales et subtropicales. Les SJT représentent la pêche commerciale de thons la plus importante dans l'Océan Indien et elles sont gérées actuellement comme un seul stock. Nous avons examiné les patrons de variation génétique dans un gène de l'ADNmt et dans six locus microsatellites afin de déterminer la structure des stocks dans le nord-ouest de l'Océan Indien. Nous avons prélevé 324 individus dans cinq importantes zones de pêche au large du Sri Lanka ainsi que dans des sites isolés aux îles Maldives et aux îles Laquedives. La reconstruction phylogénétique de l'ADNmt révèle la coexistence de deux clades divergents dans la région. Une analyse de variance moléculaire (AMOVA) des données d'ADNmt montre une différentiation génétique significative entre les sites (Φ_{ST} = 0,2029, $P < 0,0001$), ce qui est aussi confirmé par les résultats d'une AMOVA spatiale. Une AMOVA des données des microsatellites montre aussi une différentiation significative entre la plupart des sites échantillonnés (F_{ST} = 0,0256, $P < 0,001$) qui est compatible avec la structure basée sur l'ADNmt. Une analyse STRUCTURE des données des microsatellites indique deux stocks différentiés. Bien que les deux types de marqueurs utilisés identifient deux groupes génétiques, l'analyse des microsatellites indique que les SJT échantillonnés représentent des individus provenant d'aires de fraie discrètes qui se mêlent sur les aires d'alimentation dans les eaux du Sri Lanka.

[Traduit par la Rédaction]

Introduction

A widely accepted view among many fisheries managers is that most tuna species are wide-ranging, dispersive species that given their cosmopolitan distributions, large population sizes, high fecundity, and production of pelagic eggs and larvae, most probably constitute essentially homogeneous populations over oceanic spatial geographical scales. General characteristics of the open ocean environment combined with tuna life history traits have led to the expectation that most tuna species will show little, if any, population structure even at very large spatial scales (interoceanic) (Ward et al. 1994; Chow and Ushiama 1995; Ely et al. 2005). In this context, populations of most tuna species are currently managed as single stocks (e.g., yellowfin tuna (*Thunnus albacares*) and bigeye tuna (*Thunnus obesus*) in the Pacific, Atlantic, and Indian oceans). Given this background, skipjack tuna (*Katsuwonus pelamis*) (SJT) are currently considered, like most tuna species, to constitute a single panmictic population and hence are managed as single stocks at an ocean-wide scale in all oceans by the respective international commissions (i.e., IOTC, Indian Ocean Tuna Commission; WCPFC, Western and Central Pacific Fisheries Commission; IATTC, Inter-American Tropical Tuna Commission; and ICCAT, International Commission for the Conservation of Atlantic Tuna). This approach is not based, however, on experimental stock delineation studies but largely on preconceptions about general tuna life history traits and fishery data.

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Stock delineation studies of large tuna species have most often been based on sampling designs at oceanic scales because of an expectation that populations are unlikely to show structure at finer spatial scales. Indeed, a population genetic study of SJT carried out at an oceanic scale in the Atlantic and Pacific oceans that employed mitochondrial DNA (mtDNA) restriction fragment length polymorphism data could not detect any SJT differentiation between these water bodies (Graves et al. 1984). A more recent oceanic-scale study of SJT from the Atlantic and Indian oceans that employed mtDNA D-loop sequence data also could not differentiate SJT from the two oceans (Ely et al. 2005). However, a recent study of SJT from the east coast of India and from the Pacific Ocean near Japan could differentiate the two samples using mtDNA restriction fragment length polymorphism markers (Menezes et al. 2005). However, it failed to differentiate the Japanese sample and an SJT sample from the west coast of India using microsatellite markers (Menezes et al. 2008). The scale of these studies, perhaps combined with a lack of sensitivity in the molecular markers used, may have hindered potential for detecting real population differentiation, if it was present. In contrast, earlier allozyme studies, which employed relatively fine-scale sampling, detected population structure at both inter- and intra-ocean spatial scales (e.g., Sharp 1978; Fujino et al. 1981).

Evidence for heterogeneous intraspecific stocks of tuna species and other large marine pelagic fishes have increased in recent times (Atlantic cod (*Gadus morhua*): Ruzzante et al. 1998; Knutsen et al. 2003; Atlantic bluefin tuna (*Thunnus thynnus*): Carlsson et al. 2004, 2006; Boustany et al. 2008; yellowfin tuna: Dammannagoda et al. 2008; swordfish (*Xiphias gladius*): Kotoulas et al. 1995; Reeb et al. 2000; European flounder (*Platichthys flesus*): Florin and Höglund 2008; wahoo (*Acanthocybium solandri*): Garber et al. 2005; sailfish (*Istiophorus platypterus*): Hoolihan et al. 2004). Most have employed sampling at relatively fine geographical scales and have considered ocean current patterns, historical geomorphological factors, bathymetry of the ocean basin, salinity gradients, and specific life history trait characteristics of individual species. Thus, simplistic assumptions about a species' general life history traits (e.g., large, fast-swimming, highly mobile, pelagic larvae, etc.) used to define the scale at which sampling is conducted may inadvertently miss real population subdivision and hence potentially mislead management decisions.

SJT constitute the largest tuna fishery in the world, and this is also the case in the Indian Ocean (Food and Agriculture Organization of the United Nations 2008). Substantial increases, however, in the fishing effort employed by purse seiners over the last decade in the western Indian Ocean have suggested that the relative abundance of SJT in these areas may have declined. As the western Indian Ocean is considered to be an important SJT recruitment area, authorities are concerned about the health of the fishery there (Indian Ocean Tuna Commission 2007). Importantly, unlike other industrial tuna fisheries around the world, the SJT fishery is the principal animal protein resource for many coastal communities in developing nations in the Indian Ocean and provides important employment and livelihood opportunities for many poor communities. A collapse of SJT stocks in the region could compromise not only food security of many poor people, but also important employment opportunities for coastal populations.

The IOTC currently considers that the Indian Ocean SJT stock is resilient. In addition, IOTC tagging studies have shown rapid, large-scale movements of SJT in the western Indian Ocean, and they have used these data to argue that SJT is a single stock for management purposes in that region (Indian Ocean Tuna Commission 2007), and as such SJT are currently managed as a single stock in the Indian Ocean. In reality, however, little is known about SJT stock delineation or maximum sustainable yield in this region (Indian Ocean Tuna Commission 2007).

Managing any fishery does not simply mean managing total fish numbers. While nongenetic methods of stock assessment including morphological–meristic or fishery-based methods can only infer fish breeding units, a population genetics approach can directly test the hypothesis that genetically different breeding units may be present (Ward 2000). If one or more of the genetically distinct breeding units is at low abundance, equal fishing pressure on both groups can lead to extinction of one or more of the low abundance groups. Furthermore, a number of marine fisheries around the world consist of mixtures of genetically discrete stocks in commercial fishing grounds, where individuals have originated from discrete spawning sites (e.g., salmon fisheries in the northwest Pacific; Shaklee et al. 1999). Evidence has also increased that some tuna fisheries consist of mixed stocks in fishing grounds (e.g., Atlantic bluefin tuna in the Mediterranean Sea (Carlsson et al. 2006) and the Gulf of Mexico (Boustany et al. 2008)).

Uncontrolled harvesting of wild populations can perturb natural genetic subdivision among populations and reduce overall stock productivity. This may not be recognized, however, unless subpopulations have been identified independently and individuals from population mixtures are assigned to their correct subpopulations (Allendorf et al. 2008). Consequently, to manage populations in a sustainable way, we need to understand "real" stock structure (Waples and Gaggiotti 2006; Palsbøll et al. 2007). It is likely, therefore, that a lack of detailed population genetic studies of Indian Ocean SJT stocks has constrained development of appropriate and effective scientific management strategies for this species.

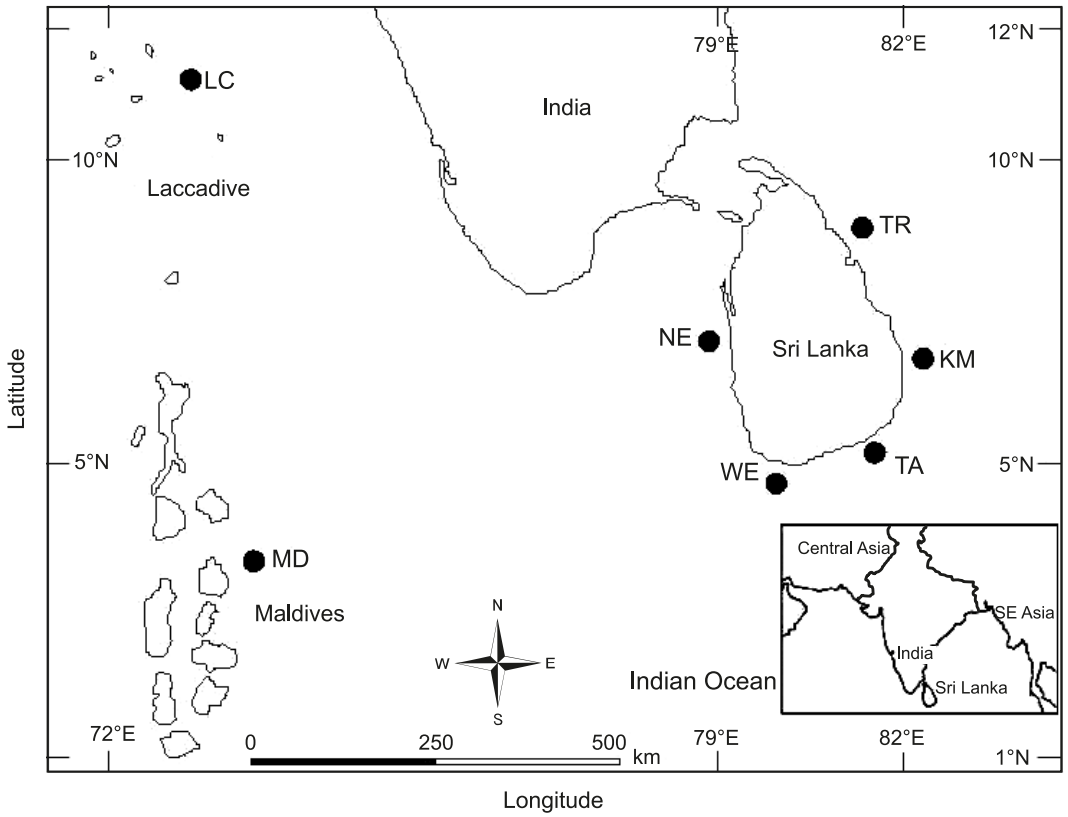
Here we examine the extent of genetic differentiation in both mtDNA and microsatellite markers among SJT samples collected between 2001 and 2004 in waters of the northwestern Indian Ocean around Sri Lanka, including the Maldive Islands and Laccadive Islands. Our aim was to test for population structure among major fishing grounds around Sri Lanka and to evaluate whether SJT catches in this region could represent heterogeneous stocks.

Materials and methods

Sampling

SJT samples were collected from commercial fishing operations from five sites around Sri Lanka and from single sites in the Maldive Islands and the Laccadive Islands (Fig. 1). Sampling sites were selected to represent major fishing grounds in Sri Lankan waters and two collections

Fig. 1. Skipjack tuna sampling sites. Abbreviations correspond to sampling localities: NE, Negombo; WE, Weligama; TA, Tangalle; KM, Kalmunei; TR, Trincomalee; LC, Laccadive Islands; MD, Maldive Islands.



(Maldive Islands and the Laccadive Islands) as outgroups to compare the levels of genetic differentiation in SJT populations from geographically remote regions in the northwestern Indian Ocean. Samples were collected between 2001 and 2004, with white muscle tissue collected from approximately 50 individuals per site (Table 1). Muscle tissue samples were removed from fish and stored in 95% ethanol for later genetic analyses.

DNA extraction, PCR, and screening for variation

Total genomic DNA was extracted from each sample using either a phenol–chloroform (Sambrook et al. 1989) or a modified salt extraction method (Miller et al. 1988). The whole mtDNA ATP synthase subunits 6 and 8 region (ATPase 6, 8) was targeted using the ATP 8.2 L (5′AAAGCRTYRGCCTTTTAAGC3′) and COIII.2H (5′GTTAGTGGTCAKGGGCTTGGRTC3′) primers (http://striweb.si.edu/bermingham/research/primers/index.html), resulting in an ~950 base pair (bp) fragment. Subsequently, two ATPase internal primers were designed to produce a 540 bp fragment, a size more appropriate for temperature gradient gel electrophoresis (TGGE) analysis (forward primer: 5′CCTAGTGCTAATGGTGCGATAAA3′; reverse primer: 5′TTCCTCCAAAAGTTATAGCCCAC3′) that was used in further analyses. mtDNA polymerase chain reactions (PCRs) consisted of 2.5 μL Roche 10× buffer, 0.5 μL 25 mmol·L^{-1} Fisher $MgCl_2$, 1 μL Roche deoxynucleotide triphosphate (dNTP), 1 μL 10 mmol·L^{-1} primer, 0.2 μL Roche *Taq* DNA polymerase, 1 μL DNA template (~200 ng) adjusted to a final volume of 25 μL with 16 μL ddH_2O. mtDNA PCR conditions were as follows: 5 min at 94 °C for initial denaturation, then 30 cycles of 40 s at 94 °C, 40 s at 52 °C, 40 s at 72 °C, with the final extension step 8 min at 72 °C. mtDNA variation was assayed using TGGE with outgroup heteroduplex analysis (TGGE–OGHA) as described in Lessa and Applebaum (1993) and Campbell et al. (1995). Each unique haplotype identified using TGGE–OGHA was sequenced. A few unique haplotypes were sequenced in both directions, and as forward and reverse sequences were perfectly complementary, remaining haplotypes were then sequenced only in the forward direction. All sequencing was performed at the Australian Genome Research Facility using an ABI 3730xl sequencing platform. PCR products from each unique haplotype were purified using ethanol precipitation and sequenced using the forward primer and the BigDye terminator Sequencing Ready Reaction v3.1 kit (Applied Biosystems, California, USA) following manufacturer's specifications. Sequences were uploaded to GenBank under accession Nos. FJ481378–FJ481426.

SJT genomic libraries were developed specifically for the current study using a radioisotopic method as described in Chand et al. (2005). Microsatellite markers were targeted, isolated, and trialed to produce polymorphic loci that were optimized to screen for variation in the sampled populations. Two tri- (UTD328, UTD203) and four tetra-nucleotide microsatellite loci (UTD73, UTD535, UTD523, and UTD172) were amplified and analysed here (see Supplemental

Table 1. Sampling details and descriptive mtDNA statistics for skipjack tuna samples.

Population	Location	*n*	*h*	*S*	H_d	*k*	π	θ_S
Negombo (NE)	79°18′N, 6°057′E	53	11	22	0.706	2.506	0.005	1.646
Weligama (WE)	80°18′N, 5°034′E	52	12	18	0.800	3.165	0.006	1.409
Tangalle (TA)	81°14′N, 5°042′E	41	10	20	0.431	1.404	0.002	1.667
Kalmunei (KM)	82°29′N, 7°008′E	54	15	20	0.797	5.420	0.011	1.513
Trincomalee (TR)	81°51′N, 8°058′E	49	7	14	0.669	2.622	0.005	1.181
Laccadive (LC)	72°31′N, 11°01′E	48	7	17	0.733	3.531	0.007	1.383
Maldives (MD)	73°09′N, 4°20′E	27	9	23	0.649	5.096	0.010	2.207
Clade I		281	37	38	0.959	2.639	0.005	6.115
Clade II		43	12	28	0.773	4.686	0.009	6.471
Total collection		324	49	52	0.965	3.855	0.007	8.180

Note: Variables are represented by the following: *n*, sample size; *h*, number of haplotypes; *S*, number of polymorphic sites; H_d, haplotype diversity; *k*, mean pairwise nucleotide difference; π, nucleotide diversity; θ_S, equilibrium heterozygosity per site estimated from *S*.

Appendix S3[2]). Microsatellite PCR reaction mix consisted of 1 μL DNA (~50 ng·μL^{-1}), 1.25 μL of 10× PCR buffer (Roche), 0.25 μL of 25 mmol·L^{-1} $MgCl_2$, 0.5 μL of 10 mmol·L^{-1} dNTP (Roche), 0.5 μL of each 10 mmol·L^{-1} forward and reverse primers, 0.1 μL of *Taq* (Roche), and ddH_2O to a final volume of 10 μL. PCR conditions were 5 min at 94 °C for initial denaturation, then 30 cycles of 30 s at 95 °C, 30 s at relevant annealing temperature (see Supplemental Appendix S3), 30 s at 72 °C, and a final extension step at 72 °C for 8 min. Microsatellite polymorphisms were analysed on a Gelscan2000 System (Corbett Research) in 5% acrylamide gels run according to the manufacturer's instructions. A 50–350 bp size standard (Tamra 350) was run at both ends of each gel and in two additional lanes to confirm allele size. In addition to the size standard, an allele reference standard was generated for known SJT allele sizes, and this standard was run in two additional lanes to ensure consistent allele scoring across all gel runs. Microsatellite allele sizes were scored using One D-scan 2.05 (Scanalytics Inc.).

Statistical analysis

mtDNA variation

mtDNA haplotype sequences were edited and aligned in BioEdit version 7.0.1 (Hall 1999) and sequence data then tested for deviation from neutral expectations using both Tajima's *D* (Tajima 1989) and Fu's F_S (Fu 1997) tests implemented in Arlequin version 2.00 (Schneider et al. 2005). Significance for both tests was determined using the coalescent simulation process in DnaSP 4.10 (Rozas et al. 2003) with 1000 replicates. *P* values of multiple neutrality tests were adjusted using the Bonferroni correction (Rice 1989).

An mtDNA parsimony cladogram of haplotypes was constructed (at 95% level connectivity) using TCS version 1.18 (Clement et al. 2000). Haplotype networks reconstruct the genealogical history of haplotypic variation and illustrate the evolutionary relationship among unique haplotypes. Under coalescent principles, internal haplotypes in a network are assumed to be ancestral, while tip haplotypes are considered younger, more recently derived types (Templeton et al. 1987; Templeton and Sing 1993; Crandall 1996). Frequency and site information were incorporated into the SJT network here to illustrate the distribution of haplotypes among locations.

Genetic variation was examined using several standard diversity indices, including the number of segregating (polymorphic) sites (*S*), haplotype diversity (H_d), molecular diversity indices including nucleotide diversity (π) (Nei 1987), the average number of pairwise nucleotide differences (*k*) (Tajima 1983), and expected heterozygosity based on number of segregating sites (θ_S) (Watterson 1975) implemented in Arlequin for the total SJT sample collection and for individual samples.

Population genetic analyses were performed using Arlequin and DnaSP. Spatial differentiation was estimated using the fixation index (Φ_{ST}; Excoffier et al. 1992) that includes information on mitochondrial haplotype frequency (Weir and Cockerham 1984) and genetic divergence among unique haplotypes. For all Φ_{ST} analyses, the Tamura and Nei (1993) distance method was used, as it accounts for mutational rate heterogeneity among nucleotides within the gene fragment. Using Φ statistics, analysis of molecular variance (AMOVA) was performed to examine the amount of genetic variability partitioned within and among SJT populations (Excoffier et al. 1992). Hierarchical AMOVA was used to investigate the effects of temporal and spatial sampling. Hierarchical AMOVA partitions total genetic differentiation (Φ_{ST}) among predefined groups and yields two measures of genetic differentiation: Φ_{SC}, which describes variation among sample populations within groups, and at a higher level in the hierarchy, Φ_{CT}, which describes differentiation among groups (Excoffier et al. 1992). In the current study, the entire sample collection was tested for genetic differentiation to test the null hypothesis of panmixia among collections. Secondly, hierarchical AMOVA analysis was undertaken to determine if any observed structure was partitioned temporally or spatially. Hierarchical groups were organized as year groups. For year groups, sites were grouped based on year of sampling (i.e., four groups: 2001, 2002, 2003, and 2004). Genetic variation was therefore partitioned among year (Φ_{CT}), among sites within years (Φ_{SC}), and within sites. Using this hierarchical grouping, we tested the stability of any inferred structure among sampling sites that remained stable over time and simultaneously assessed ge-

[2] Supplementary data for this article are available on the journal Web site (http://cjfas.nrc.ca).

netic differentiation among sites within years. All other mtDNA analyses were carried out for pooled temporal samples from each site, as there was no temporal variation. The significances of variance components for each hierarchical comparison were tested using a nonparametric permutation procedure incorporating 1000 iterations.

Spatial population structure was also examined by estimating genetic differentiation between all pairs of sites (pairwise Φ_{ST} analysis). Significance of pair site comparisons was tested using the permutation process, as above. In all instances with multiple tests, *P* values were adjusted using the Bonferroni correction (Rice 1989).

Spatial structure was investigated further via spatial analysis of molecular variance (SAMOVA) (Dupanloup et al. 2002). This analysis identifies groups of sample sites that are most similar and that are geographically meaningful. SAMOVA uses the statistics derived from an AMOVA and incorporates geographical information on sampling sites (i.e., geographical distances among sites) with a simulated annealing approach to maximize the Φ_{CT} among groups of populations as well as identifying possible genetic barriers between them, without predefining populations (Dupanloup et al. 2002). Thus, SAMOVA defines groups of samples that are geographically homogeneous and also maximally differentiated from each other (Dupanloup et al. 2002).

Microsatellite variation

Microsatellite data were checked for presence of null alleles, large allele dropout, and errors in scoring due to stutter bands using MICRO-CHECKER software version 2.2.3 (Oosterhout et al. 2004). Each locus and each site was also tested for deviation from Hardy–Weinberg equilibrium (HWE) in Arlequin with significance of deviations in observed vs. expected heterozygosity tested using Exact tests (Guo and Thompson 1992). Inbreeding coefficient (F_{IS}) for each locus and each site were tested using FSTAT version 2.9.3.2 (Goudet 1995). The possibility of linkage disequilibrium among loci was investigated using the method of Slatkin and Excoffier (1996) in Arlequin with 1000 permutations ($\alpha = 0.05$). *P* values were adjusted using the Bonferroni correction. Measures of genetic variation including number of alleles and expected and observed heterozygosities were calculated in Arlequin. AMOVA was used to examine the amount of genetic variation partitioned within and among populations (Excoffier et al. 1992). AMOVA and hierarchical AMOVA tests for the microsatellite data set were organized in a similar way to the mtDNA analysis described above. Further analyses of microsatellite data were carried out for the pooled temporal samples from each site, as there was also no temporal variation for microsatellite data.

We assessed the microsatellite data set for sufficient statistical power to detect genetic heterogeneity (i.e., estimating the potential for type II error) at various true levels of divergence using POWSIM software as described by Ryman et al. (2006). This program uses sample sizes, number of loci, and allele frequencies and simulates genetic sampling from multiple populations that have drifted apart to a predefined expected degree of divergence defined as F_{ST}. Samples from these populations are used to test the hypothesis of genetic homogeneity and to estimate the α error at each locus separately, using both Fisher's exact test and traditional chi-square approaches, respectively.

Presence of multiple SJT breeding units was first tested using the multilocus microsatellite data set in STRUCTURE version 2.2 (Pritchard et al. 2000). STRUCTURE uses a model-based full Bayesian Markov chain Monte Carlo (MCMC) approach that clusters individuals to minimize Hardy–Weinberg disequilibrium and gametic phase disequilibrium between loci within groups. For each value of *K*, (i.e., the number of genetically distinct populations), the MCMC scheme was run with a burn-in period of 100 000 steps and a chain length of 1 000 000 replicates following the admixture model. Multiple runs were performed to evaluate the reliability of the results, and the number of populations was then determined from posterior probabilities of *K* estimated, assuming that they originated from one to seven populations (i.e., $K = 1$ to 7). In the case of migration rates between populations not being equal, *K* values may be incorrectly estimated, so values of ΔK following Evanno et al. (2005) were also calculated for each value of *K*. Individuals were assumed to have been correctly assigned to a population when their *q* value (i.e., their posterior probability of belonging to an original population) was at least 80% for the population (Pritchard et al. 2000).

Results

Mitochondrial DNA variation

Genetic variation

Genetic analyses were conducted on 324 individuals sampled from five fishing grounds around Sri Lanka (Negombo, NE; Weligama, WE; Tangalle, TA; Kalmunei, KM; Trincomalee, TR) and from single sites from the Maldive Islands (MD) and Laccadive Islands (LC) (Fig. 1 and Table 1). mtDNA haplotype sequence data produced alignment of a 488 bp fragment that included partial ATPase 6 and the ATPase 8 gene regions. A total of 52 nucleotide sites were variable (segregating sites) (see Supplemental Table S1). Statistical tests of neutrality, Fu's F_S and Tajima's *D* tests for each sample population, showed that sample populations did not deviate from the expected neutral model of evolution (data not shown).

Polymorphic sites defined a total of 49 unique haplotypes (Supplemental Tables S1 and S2). Analysis of variable sites showed that all mutations were synonymous. 29 haplotypes were singletons, and the most abundant haplotype (haplotype 6, frequency 23.46%) was present at six out of seven sample sites (absent from site MD) (Supplemental Table S2). The second most abundant haplotype (haplotype 37, frequency 8.33%) was present at five sites. Overall nucleotide diversity and the average number of pairwise nucleotide differences were 0.007 and 3.855, respectively. Population genetic summary statistics are presented in Table 1.

Phylogenetic relationships

Haplotypes grouped into two distinct, divergent clades (Tamura and Nei corrected mean divergence among clades was 0.015). Haplotypes belonging to both clades were found at all sample sites. Clade I contained 37 of the 49 haplotypes identified, while only 12 haplotypes were present in

Clade II. The parsimony network (Fig. 2) shows that the most common haplotype (Ht6) was ancestral in Clade I (occurs in the centre of network), while Ht37 was ancestral in Clade II. Uncorrected pairwise divergence among haplotypes in the parsimony cladogram ranged from 0% to 3.7%. Collections at all sample sites represent mixtures of haplotypes from both clades that are independent of year of collection or relative sample size at a site.

Population differentiation

The pattern of SJT mtDNA haplotype diversity observed at all sites (Supplementary Table S2 and Fig. 2) consisted of a single haplotype (sites TA and MD) or two haplotypes (sites NE, WE, KM, TR, and LC) present at high frequency. Common haplotype frequencies, however, varied widely among sites. A large number of singleton haplotypes were also present at individual sites (29 of 49 possible haplotypes).

Hierarchical analysis that incorporates Tamura and Nei's genetic distance in AMOVA is summarized (Table 2). Across the total sample collection, there was significant genetic differentiation among sites (global $\Phi_{ST} = 0.2029$, $P < 0.001$) when collections over years within sites were pooled. The entire sample collection, when grouped into year classes to assess the impact of temporal collections, showed no significant genetic differentiation among groups for years (among year collections in 2001, 2002, 2003, and 2004) ($\Phi_{CT} = 0.0002$, $P = 0.8739$). As no significant genetic variation was evident among year total collections (i.e., among 2001, 2002, 2003, and 2004 collections, irrespective of sampling site), this implied that overall genetic composition of sampled SJT populations remained temporally stable across the study period. Significant genetic differentiation was evident, however, among sites within all sample years, indicating that sampled populations showed consistent spatial heterogeneity for genetic variation ($\Phi_{SC} = 0.2030$, $P < 0.001$) over years. Cladewise hierarchical AMOVA, while predictably showing significant differentiation among clades ($\Phi_{CT} = 0.563$, $P < 0.001$), also detected significant structure among samples within clades ($\Phi_{SC} = 0.295$, $P < 0.001$).

Further analysis of individual clades revealed that among site genetic differentiation was limited only to Clade I individuals ($\Phi_{SC} = 0.2136$, $P < 0.001$; data not shown), while no significant genetic variation was evident for Clade II individuals. Thus, overall genetic differentiation among sites resulted essentially from spatial variation in the distribution of Clade I individuals among sites. This may have been influenced, however, by the relatively low numbers of Clade II ($n = 43$) individuals in the total sample; hence, there may have been insufficient power to reject the null hypothesis of panmixia for this clade.

Spatial genetic variation among SJT sampling sites was tested at higher resolution using pairwise Φ_{ST} analysis. Pairwise Φ_{ST} estimates for the entire collection are shown in Table 3. Highly significant genetic differentiation was evident between the majority of pairs of sites. After Bonferroni correction for multiple comparisons, 16 pairs of sites were significantly differentiated. AMOVA analyses showed that while significant spatial genetic differentiation was evident among sampled SJT populations, in general, the entire SJT collection remained genetically stable over time (i.e., the same haplotypes remained in similar frequencies within sites across years).

Genetic differentiation among sampled sites not only resulted from relative admixture of the two clades at individual sites, but was also influenced by divergent frequencies of Clade I haplotypes among sites. As a consequence, SAMOVA analysis was employed for the total SJT sample and Clade I to assess whether genetically homogeneous SJT groups were present across the study area.

As the best significant homogeneous grouping, SAMOVA indicated three genetically differentiated SJT population groups (Table 4). Specifically, the analysis recognized KM and MD as discrete populations and all remaining sites (NE, WE, TA, TR, LC) together as a third discrete population. Similarly, SAMOVA analysis of Clade I individuals also showed additional structuring within the clade, with three genetically differentiated groups identified as (*i*) TR, (*ii*) MD, and (*iii*) all other sites (NE, WE, TA, KM, and LC), which appear to lack any strong geographical pattern.

Microsatellite variation

Genetic variability estimates and Hardy–Weinberg and linkage equilibria

No null alleles, large allele drop out, or error scoring were detected using MICRO-CHECKER (95% confidence interval) for the five of the six SJT microsatellite loci (UTD73, UTD203, UTD535, UTD523, and UTD172). The exception was at locus UTD328. The analysis showed that locus UTD328 results could have been affected by null alleles. Subsequent analyses in AMOVA both including, then excluding locus UTD328 data, however, produced similar outcomes. All six loci were therefore included in all further analyses. Descriptive statistics for the six loci are summarized (Table 5). A small number of individuals could not be scored at specific loci because of amplification problems.

Sample populations were then tested for conformation to HWE. Significant heterozygote deficiencies ($P < 0.001$) were observed in some comparisons, particularly at UTD328 (in all seven collections), supporting the hypothesis of the presence of null alleles at this locus. Other loci (except for locus UTD203) also showed significant heterozygote deficiencies at two to six sites (Table 5).

In addition to heterozygote deficiencies, some loci showed linkage disequilibrium, but after Bonferroni correction this constituted only 2 out of 15 comparisons (data not shown). Heterozygote deficiencies with evidence for linkage disequilibrium, in combination, is a pattern consistent with admixture of genetically heterogeneous SJT groups among the sampled populations (e.g., So et al. 2006).

Population structure

Hierarchical AMOVA analysis results for SJT microsatellite data are summarized in Table 6. The result for the entire data set showed significant genetic differentiation among sites ($F_{ST} = 0.025$, $P < 0.001$). Pairwise F_{ST} analysis of microsatellite data identified that NE, TR, KM, and LC populations were significantly genetically differentiated from most other populations, a result in concord with mtDNA pairwise Φ_{ST} data (Table 3 and Table 7).

Hierarchical AMOVA was conducted to determine tempo-

Fig. 2. Parsimony cladogram of skipjack tuna haplotypes (Ht) showing the evolutionary relationships among haplotypes. Each circle represents a unique haplotype in the sample, and the size of each circle represents the relative frequency of each haplotype. Patterns and their percentage in each circle represent the presence of each haplotype at individual sites and their relative abundance, respectively. Black dots between haplotypes represent haplotypes that were not sampled in the study. See Fig. 1 for abbreviation codes.

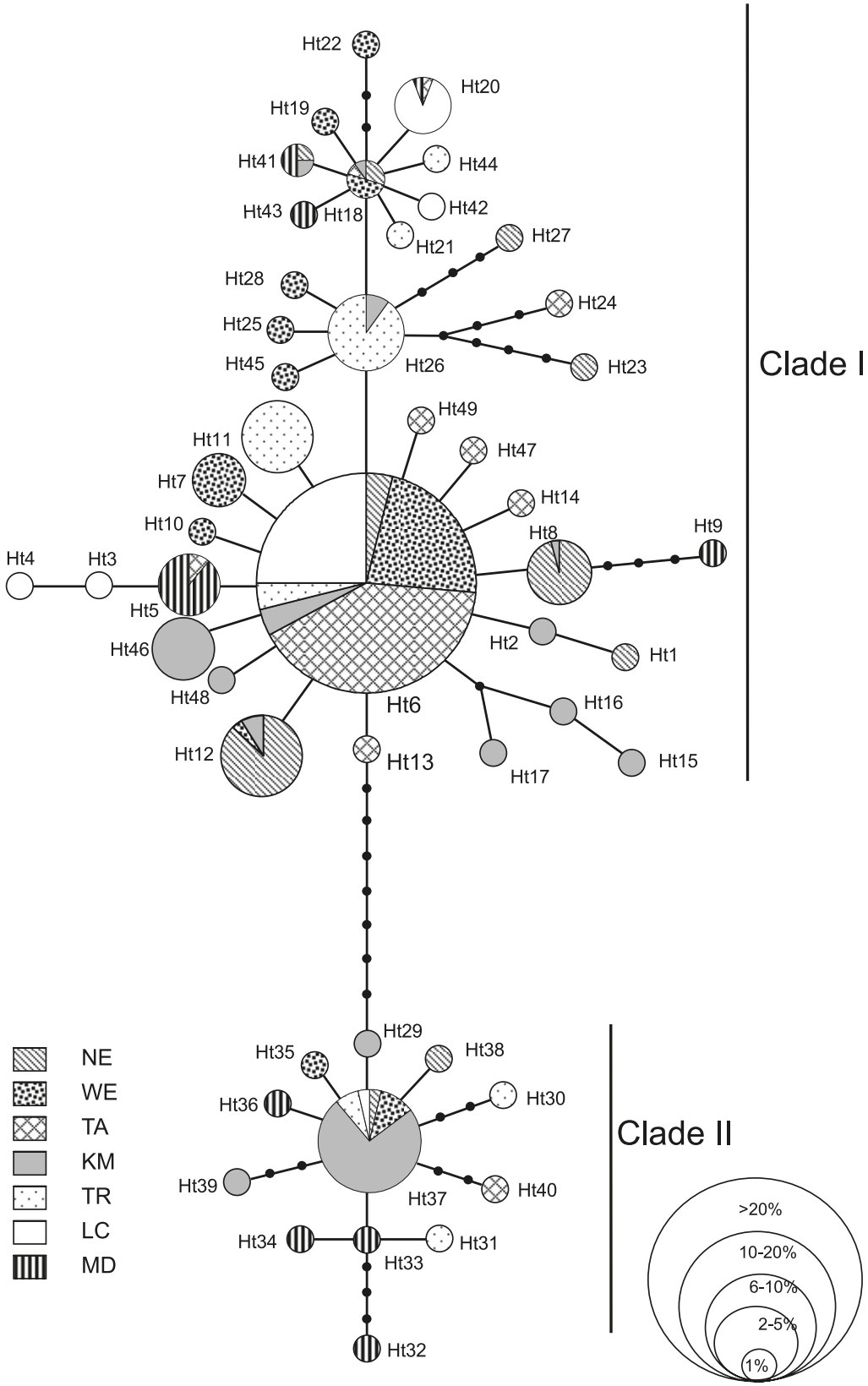


Table 2. Results of analysis of molecular variance (AMOVA) testing genetic structuring of skipjack tuna samples based on mitochondrial ATPase region sequence data.

	Observed partition		
Structure tested	Variance	% of total	Φ statistic
Total collection (2001, 2002, 2003, 2004) — one gene pool			
Among populations	0.6286	20.29	Φ_{ST} = 0.2029*
Within populations	2.4689	79.71	
Among years			
Among years (TEMPORAL)	0.0007	0.02	Φ_{CT} = 0.0002
Among populations within years (SPATIAL)	0.6280	20.30	Φ_{SC} = 0.2030*
Within populations	2.4647	79.68	Φ_{ST} = 0.2032
Cladewise			
Between two clades	1.9336	56.35	Φ_{CT} = 0.5635*
Among samples within clades	0.4457	12.86	Φ_{SC} = 0.2945*
Within populations	1.0677	30.80	Φ_{ST} = 0.6920

Note: *, $P < 0.001$.

Table 3. mtDNA pairwise Φ_{ST} among skipjack tuna sampling sites after Bonferroni correction for entire collection.

	NE	WE	TA	KM	TR	LC	MD
NE							
WE	0.116**						
TA	0.081**	0.059					
KM	0.271**	0.186**	0.237**				
TR	0.171**	0.069*	0.117*	0.229**			
LC	0.187**	0.070	0.142**	0.267**	0.111**		
MD	0.142**	0.065	0.071	0.116	0.112**	0.116**	

Note: See Fig. 1 for abbreviation codes. $\alpha = 0.05/21 = 0.002$. *, $P < 0.002$; **, $P < 0.0001$.

Table 4. Population structure based on mtDNA differentiation of skipjack tuna (in spatial analysis of molecular variance, SAMOVA).

No. of groups	Structure	Variation among groups	Variation %	Φ_{CT}	*P*
2	(KM); (NE,WE,TA,TR,LC,MD)	0.328	14.95	0.149	0.135
3	**(KM); (MD); (NE,WE,TA,TR,LC)**	**0.269**	**12.68**	**0.126**	**0.048**
4	(KM); (MD); (NE,TA); (WE,TR,LC)	0.234	11.54	0.115	0.003
5	(KM); (MD); (TR); (NE,TA); (WE, LC)	0.230	11.52	0.115	0.006
6	(KM); (MD); (TR); (NE); (LC); (WE,TA)	0.261	13.13	0.131	0.046

Note: The row in bold type indicates the details of geographically meaningful groups with maximum genetic differentiation. See Fig. 1 for abbreviation codes.

ral and spatial variation among sites. No significant temporal genetic differentiation was evident among year groups sampled from 2001 to 2004 (F_{CT} = –0.0096, P = 0.861). Significant spatial genetic variation was detected, however, among sites within years (F_{SC} = 0.0326, $P < 0.001$) (Table 6). These results were also consistent with mtDNA hierarchical AMOVA results.

As two divergent clades were identified in the mtDNA analysis, microsatellite data were tested to assess the pattern of differentiation for nuclear DNA (nDNA) among clades. No significant differentiation was observed when individuals were grouped by their mtDNA clade type, although significant differentiation was evident among populations within clades (Table 6). The very high within population variation (>97%) and moderate among population within clade variation (2%) could have contributed to a lack of significant differentiation among clades. Therefore, we carried out analysis of the microsatellite data using POWSIM to test whether the microsatellite data set possessed sufficient statistical power to detect true genetic differentiation, if it were present, that may have gone undetected in hierarchical AMOVA analysis.

Separate POWSIM analyses of the seven sampled sites and two mtDNA clades showed clearly that our nDNA data set had high statistical power for detecting true genetic differentiation among populations, but insufficient power to differentiate the two (microsatellite) clades. The microsatellite data set from seven sample sites was capable of detecting a true F_{ST} estimate of as low as 0.0025 with a probability of 98% and a true differentiation of 0.005 with a probability of 100%. However, the probability of detecting a true F_{ST} estimate of as low as 0.0005 (from AMOVA analysis) of micro-

Table 5. Descriptive statistics for six microsatellite loci among skipjack tuna collections.

		Locus						
Sample		UTD535	UTD523	UTD172	UTD328	UTD203	UTD73	Average across loci
NE	*n*	36	36	40	45	43	37	39
	a	14	21	7	22	5	7	12.67
	H_e	0.916	0.949	0.725	0.935	0.344	0.727	0.77
	F_{IS}	0.122	0.124	–0.139	0.140*	0.343	–0.041	0.09
WE	*n*	44	52	43	52	39	41	45
	a	26	25	9	16	8	12	16.00
	H_e	0.962	0.951	0.725	0.904	0.423	0.809	0.80
	F_{IS}	0.175*	0.028*	–0.287*	0.189*	–0.049	0.143	0.03
TA	*n*	33	33	38	44	35	24	34
	a	15	23	11	18	8	6	13.50
	H_e	0.914	0.951	0.845	0.899	0.459	0.738	0.80
	F_{IS}	0.107	0.045	–0.027*	0.101*	0.05	0.291*	0.09
KM	*n*	45	36	42	47	47	45	43
	a	14	21	8	16	5	13	12.83
	H_e	0.912	0.931	0.681	0.861	0.464	0.845	0.78
	F_{IS}	0.075	0.046	–0.37*	0.263*	–0.245	0.145*	–0.01
TR	*n*	35	32	32	46	46	33	37
	a	15	20	6	20	3	15	13.17
	H_e	0.913	0.938	0.558	0.903	0.231	0.881	0.74
	F_{IS}	0.094	0.43*	0.088	0.420*	0.291	0.000*	0.22
LC	*n*	33	35	44	48	48	47	42
	a	16	22	7	24	7	8	14.00
	H_e	0.916	0.946	0.672	0.932	0.409	0.751	0.77
	F_{IS}	0.176*	0.157*	–0.323*	0.213*	0.072	–0.02	0.05
MD	*n*	41	43	45	49	51	51	46
	a	16	27	10	13	7	11	14.00
	H_e	0.914	0.961	0.826	0.853	0.463	0.72	0.79
	F_{IS}	0.175	0.422*	–0.131*	0.224*	–0.006	0.047	0.12

Note: Significant probability values after Bonferroni correction ($\alpha = 0.05/42 = 0.0011$). Data is represented by number of samples (*n*), number of alleles (*a*), and H_e (expected heterozygosity). See Fig. 1 for abbreviation codes. *, $P < 0.0001$.

Table 6. Analysis of molecular variance (AMOVA) results testing spatial and temporal genetic structuring of skipjack tuna samples based on microsatellite data.

	Observed partition		
Structure tested	Variance	% total	*F* statistic
Total collection (2001, 2002, 2003, 2004)			
Among populations	0.045	2.56	$F_{ST} = 0.0256$*
Within populations	1.728	97.44	
Yearwise collection (2001, 2002, 2003, 2004)			
Among groups	–0.0171	–0.97	$F_{CT} = -0.0096$
Among populations (of the same year) within groups	0.0583	3.30	$F_{SC} = 0.0326$*
Within populations	1.7278	97.67	$F_{ST} = 0.0233$
Cladewise			
Between two clades	0.0009	0.05	
Among samples within clades	0.0404	2.28	$F_{CT} = 0.0005$
Within populations	1.7321	97.66	$F_{SC} = 0.0228$*

Note: *, $P < 0.001$.

satellite data among mtDNA clades was <42.0%. The α error (corresponding with the probability of obtaining false significance when the true $F_{ST} = 0$) was close to 5% in all cases.

We further tested the nDNA data for admixture using the program STRUCTURE (Pritchard et al. 2000). STRUCTURE analysis recognized two distinct groups among the samples using only the "Log probability of data [$L(K)$]" approach (as described in Pritchard et al. 2000). The number of clusters that fitted our data best was not clear because the $L(K)$ did not reach a plateau after $K = 1$ (Table 8). The ΔK statistic described by Evanno et al. (2005), however, clearly showed presence of two distinct groups (the highest ΔK was obtained with $K = 2$). All seven sample sites were mixtures of the two identified groups in different propor-

Table 7. Microsatellite pairwise F_{ST} among skipjack tuna collections after Bonferroni correction (initial $\alpha = 0.05/21 = 0.002$).

	NE	WE	TA	KM	TR	LC	MD
NE							
WE	0.0080*						
TA	0.0193*	0.0062					
KM	0.0266*	0.0018	0.0048				
TR	0.0722*	0.0605*	0.0618*	0.0744*			
LC	0.0101	0.0003	0.0127*	0.0132*	0.0778*		
MD	0.0218*	0.0046	–0.0034	0.0174*	0.0559*	0.0159*	

Note: See Fig. 1 for abbreviation codes. *, $P < 0.001$.

Table 8. Log probability and ΔK for clusters using Bayesian assignment test in STRUCTURE.

K	$L(K)$	SD	$L'(K)$	$\lvert L''(K)\rvert$	ΔK
1	–8412.7	8.94			
2	–8246.5	21.20	166.2	161.2	7.60
3	–8085.3	25.25	161.2	102.2	4.05
4	–7983.1	28.79	102.2	22	0.76
5	–8005.1	33.82	–22	135.7	4.01
6	–7869.4	33.83	135.7	30.3	0.90
7	–7899.7	37.64			

Note: K, number of clusters; $L(K)$, average of log probability; SD, standard deviation.

tions (Fig. 3*a*), with the vast majority of individuals assigned with 80% or greater probability to one microsatellite group or the other.

The fact that two well-supported and genetically identifiable groups were detected using both mtDNA and nDNA markers would strongly suggest that the samples from each site represented a mixture of two separate breeding units. However, for this to be true, cytonuclear disequilibrium between the two marker systems should be evident. We therefore tested this hypothesis by repeating the microsatellite assignment analysis in STRUCTURE for $K = 2$ but separating samples into groups based on their respective mtDNA clades. In this analysis, Clade I individuals had an approximate 50/50 split of confidently assigned individuals from both microsatellite groups. The pattern was different for Clade II individuals, where the split was approximately 75/25 (Fig. 3*b*). From this result, it appears that there is little evidence to suggest that the two groups identified by both marker systems have resulted from the same evolutionary–demographic processes.

Discussion

Harvesting fish populations has the potential to cause three types of genetic change: alter population subdivision, cause genetic variation levels to decline, and produce selective genetic changes (Allendorf et al. 2008). To sustain the productivity of any harvested fish population, it is crucial to incorporate genetic considerations into their management. Management plans should be developed by applying basic genetic principles combined with molecular genetic monitoring to minimize any harmful genetic change (Allendorf et al. 2008). Understanding the genetic changes and evolutionary responses of exploited populations is also crucial to design management strategies aimed at sustainable exploitation of natural biological resources (Walsh et al. 2006). SJT in the Indian Ocean are currently managed as a single stock; yet results presented in this study strongly suggest that mixed stocks exist in waters surrounding Sri Lanka.

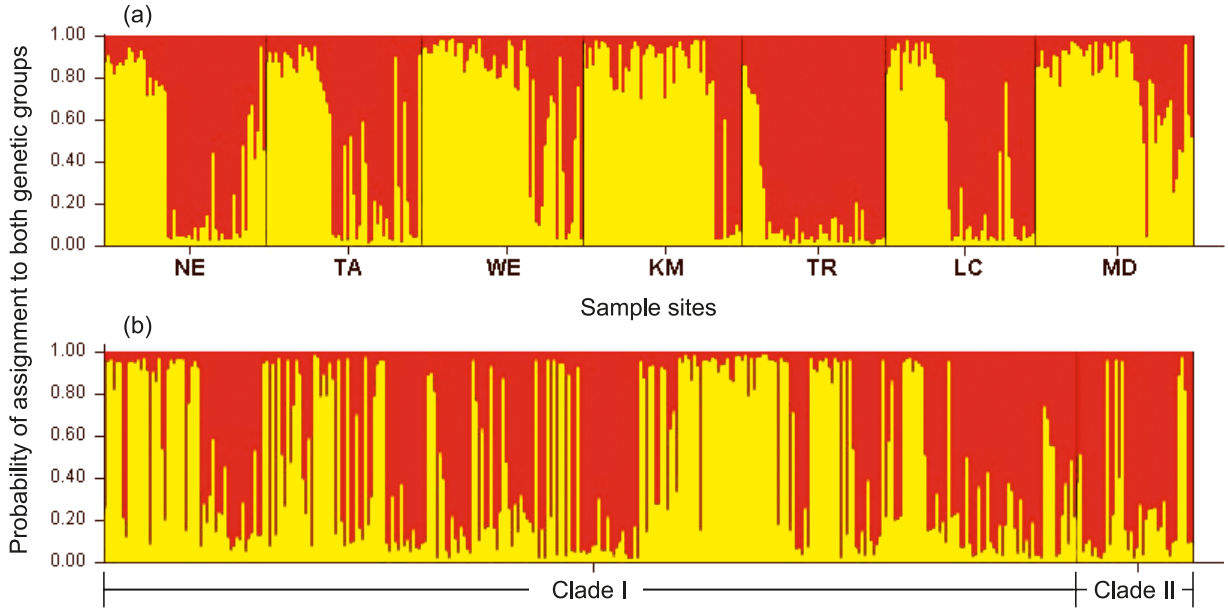
Phylogenetic relationships

Phylogenetic analyses provide strong support for presence of two divergent mtDNA clades in the northwestern region of the Indian Ocean. Pairwise divergence among haplotypes in the parsimony cladogram (based on the ATPase region) ranged from 0% to 3.7%, while Tamura and Nei corrected mean divergence among clades was 1.5%. Recent studies of other tuna and billfish species have also reported multiple highly divergent intraspecific mtDNA lineages both within or among ocean basins, notably for Atlantic bigeye tuna (Martínez et al. 2006), Atlantic bonito (*Sarda sarda*; Viñas et al. 2004), and several billfishes (Scombroidei: Xiphidae), including blue marlin (*Makaira nigricans*; Buonaccorsi et al. 2001), sailfish (Graves and McDowell 2003), and swordfish (Buonaccorsi et al. 2001; Graves and McDowell 2003; Alvarado Bremer et al. 2005). Two sympatric clades have also been recognized for Atlantic bonito in the Mediterranean Sea (Viñas et al. 2004), while for some billfishes (Buonaccorsi et al. 2001; Graves and McDowell 2003; Alvarado Bremer et al. 2005) and bigeye tuna (Alvarado Bremer et al. 1998; Martínez et al. 2006), multiple sympatric clades were reported in the Atlantic Ocean. mtDNA heterogeneity observed in these studies and in the present study may have been caused by common vicariance events during Pleistocence glacial maxima that resulted in isolation of populations by reduction in availability of tropical marine habitats due to decreases in ocean water temperature (e.g., Graves and McDowell 2003; Viñas et al. 2004; Alvarado Bremer et al. 2005). Further, potential unidirectional gene flow of formerly allopatric populations during interglacial periods providing secondary contacts may have resulted in asymmetrical distribution of clades (Alvarado Bremer et al. 2005). While few studies have focused their attention on SJT, two divergent mtDNA lineages of SJT were detected in the Atlantic and Pacific oceans (Ely et al. 2005), although not specifically reported at the time. The level of divergence shown by the Ely et al. (2005) study for mtDNA control region sequence is consistent with that of ATPase divergence reported here.

Population structure

Both mtDNA and nDNA microsatellite data show strong evidence for spatial genetic heterogeneity among the SJT

Fig. 3. (*a*) A bar plot of Bayesian clustering analysis ($K = 2$) for skipjack tuna microsatellite data set (364 individuals; 6 loci) performed using STRUCTURE. Each bar represents proportional probability of assignment to each genetic group. See Fig. 1 for abbreviation codes. (*b*) A bar plot of Bayesian clustering analysis ($K = 2$) for skipjack tuna two mtDNA clades (364 individuals; 6 loci) performed using STRUCTURE.



sample sites around Sri Lanka and adjacent areas in the northwestern Indian Ocean. In addition, evidence for fine geographical scale genetic heterogeneity was confirmed by pairwise analysis of both mtDNA and microsatellite data. It appears, however, that fine-scale structuring was influenced primarily by differing frequencies of Clade I and II individuals and nDNA genetic group individuals identified in the mtDNA and microsatellite analyses, respectively. It is possible that this is a random sampling effect.

From a fisheries management perspective, it was important to determine whether individuals belonging to the two mtDNA clades interbreed. This, however, requires analysis of nDNA because information is only available from the maternal parent for mtDNA. STRUCTURE analysis of nDNA microsatellite data identified the existence of two genetically distinct groups; hence, this suggests that individuals belonging to the two mtDNA clades may not interbreed. This depends, however, on there being a relatively close association between an individual's mtDNA clade membership and their nDNA group identified in the cluster analysis. If we assume that the divergent mtDNA clades have arisen in allopatry (i.e., there are two geographically distinct breeding grounds and there is a high degree of natal site philopatry over a long evolutionary period) and that individuals of two distinct breeding units mix only after reproduction, then there should be a strong correlation between their individual mtDNA clade and the alleles they carry at nDNA loci. This was not the case here. A simple explanation for this pattern is that there was some historical secondary contact, thereby admixing the mtDNA clades. More recently, however, there has been a constraint on gene flow between breeding grounds resulting in nuclear allele frequencies diverging because of genetic drift, which has provided the signature of differentiated stocks seen in the microsatellite analysis.

The two different stocks may spawn in distant areas of the Indian Ocean, following which juveniles may disperse towards Sri Lanka, because this area is a highly fertile feeding ground. Satellite images have shown very high primary productivity – chlorophyll *a* concentrations around Sri Lanka during the southwest monsoon period (Wiggert et al. 2006). High primary productivity will lead to high zooplankton levels and large schools of bait fish on which SJT feed. As evidence for this relationship, the Wadge Bank near Sri Lanka is recognized as an important tuna fishing ground. Another explanation for physical mixture of the two SJT clades at sampling sites includes differential passive transport of larvae belonging to the different clades to fishing grounds in Sri Lanka by monsoonal currents in the Indian Ocean.

It should be recognized, however, that there was no strong pattern in the geographical distribution of the groups to provide insight into the regions for respective breeding grounds (i.e., there is no discernable genetic group frequency cline for either group from east to west). Therefore, while we can be confident that all samples in this study are a mixture of two discrete breeding units, there is no evidence to suggest that the fishing grounds around Sri Lanka represent a zone of admixture per se. Furthermore, as the present study only analysed data on SJT samples from the northwestern Indian Ocean, the results do not preclude presence of additional SJT spawning stocks and spawning areas in other regions of the Indian Ocean. More work is warranted, therefore, to confirm the natal homing of the two SJT stocks to identify potential spawning sites in the Indian Ocean, and to analyse more extensively the genetic stock structure of SJT populations across the region.

It is obvious from the results presented here that SJT stock structure is more complex than had originally been thought, i.e., that SJT populations within ocean basins are not necessarily homogeneous. Given the importance of SJT as a regional food resource to millions of people in the In-

dian Ocean, effective management of this resource in the future will require developing a better understanding of the scale at which populations in the region should be managed and conserving unique areas where discrete stocks may reproduce (spawning grounds).

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