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Low Genetic Variation Suggest Single Stock of Kawakawa *Euthynnus affinis* (Cantor, 1849) along the Indian Coast

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Abstract

Kawakawa *Euthynnus affinis* is an epipelagic migratory tuna species, widely distributed in the tropical and subtropical waters of the Indo-Pacific region. It constitutes the largest tuna fishery in Indian waters. In present study, restriction fragment length polymorphism (RFLP) analysis of the mitochondrial D-loop region was employed to examine the levels of genetic diversity among kawakawa samples collected from eight main fishing zones (Veraval (VE), Ratnagiri (RA), Kochi (KO), Kavaratti (KA), Port-Blair (PB), Tuticorin (TU), Pondicherry (PO) and Vizag (VI)) along the Indian coast. A 500 bp segment of mitochondrial D-loop region was screened in 400 samples using six restriction enzymes (*Rsa* I, *Alu* I, *Hinf* I, *Hha* I, *Msp* I and *Hae* III), resulting in 13 composite morphs. Analysis of molecular variance (AMOVA) of mtDNA data revealed no significant genetic differentiation among sites (F_{ST} = -0.00446, P = 0.84946). Results of the genetic analyses of present study suggest the single stock of kawakawa along the Indian coast.

Keywords: PCR-RFLP**,** mtDNA, D-loop, Indian waters.

Introduction

Kawakawa is migratory, coastal pelagic species inhabiting waters temperatures ranging from 18 to 29° C (Collette and Nauen, 1983). It is widely distributed in the tropical and subtropical waters of the Indo-Pacific region. Kawakawa occurs in open waters but always remains close to the shoreline (Collette and Nauen, 1983) and are rarely captured beyond the edge of the continental shelf (Yesaki, 1994). *Euthynnus affinis* is the most abundant tuna species occurring in coastal waters of India and contributed 35.25% of total tuna landing in 2009 (Vijayakumaran and Varghese, 2010). It is highly commercial and generally marketed as canned and frozen; also utilized dried, salted, smoked and fresh (Collette, 2001). In spite of high commercial value and abundance there is very little information about the stock structure of this species in Indian waters.

The first attempt to estimate the state of the tuna species and exploitation rate from Indian seas was made by Silas *et al.* (1986) followed by James *et al.* (1987). Central marine fisheries research institute (CMFRI) has collated the tuna fishery and biological data collected from nine different centers along west coast, east coast and Lakshadweep and made study on stock assessment of coastal tuna species *(Euthynnus affinis, Auxis thazard, Auxis rochei, and Thunnus tonggol).* Based on the population parameters, exploitation rate of *E. affinis* has been found to be above 0.75 along the coastal waters of India whereas the optimum exploitation rate of *E. affinis* was estimated at 0.4 only (Pillai and Gopakumar, 2003). This indicates that there is intense fishing pressure on this species in the current fishing grounds. It is estimated that about 25% of marine fish stocks are overexploited, depleted, or recovering from depletion, while 50% are fully exploited and, therefore, producing catches that are close to their maximum sustainable limits (FAO, 2007). Conservation of these genetic resources is thus especially critical in the context of species or populations under intensive exploitation.

A number of methods have been used in analyzing population structure of marine species. This includes morphometrics, growth rates, age composition, and genetic analysis. Molecular genetic techniques have become increasingly important for determining fish stock structure (Menezes *et al.*, 2008; Reiss *et al.*, 2009). In particular, analysis of restriction-site polymorphisms of mitochondrial DNA (mtDNA), which because of its maternal mode of

inheritance and rapid evolution has become a widely used population genetics tool in fisheries (Ravago-Gotanco *et al*., 2004; Quinta *et al*., 2004; Hoolihan *et al*., 2006; Menezes *et al*., 2006; Turan *et al*., 2009). Selection of the mtDNA D-loop for analysis was based on its documented hypervariability in many fish species (Ovenden, 1990; Cronin *et al*., 1993; Alvarado Bremer, 1994; Menezes *et al*., 2006) and the availability of specific primers for amplification of this region.

In this study, we use PCR-RFLP of mtDNA D-loop region to investigate the genetic variation among samples of *E. affinis* along Indian coast while testing the null hypothesis of panmixia.

Materials and Methods

DNA Isolation

In order to determine the genetic structure of kawakawa, fin clip samples were collected from the eight main fishing zones all along the Indian coast (Table 1; Figure 1) and were preserved in absolute alcohol until DNA isolation. High molecular weight (HMW) genomic DNA was isolated from the fin clip using the standard TNES-Urea-Phenol-Chloroform protocol (Asahida *et al*., 1996). The DNA pellet was suspended in 50 µl of Tris-EDTA buffer (pH 8.0). Quality and quantity of DNA present in each sample was determined using UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan). Each sample was estimated to have 20-30 ng of DNA per micro litre of solution. The DNA samples were stored at 4°C prior to PCR analysis.

DNA Amplification

We amplified a fragment of 500 bp (Figure 2a) containing the first half of the control region (D-loop) using the primer set designed by Menezes *et al.* (2006) from GenBank sequences of *Auxis thazard* (accession number NC005318). The primer sequences

Table 1. Estimates of genetic diversity within populations for RFLP data: number of samples (*n*); number of composite morphs (*nh*); composite morph diversity (*h*); and nucleotide diversity (π)

Samples	Date of collection	Location	(*n*)	(*nh*)	(*h*)	(π)
Veraval (VE)	October, 2007	20.54°N 70.22°E	50	5	0.1910	0.5388
Ratnagiri (RA)	January, 2008	16.59°N 73.18°E	50	4	0.1902	0.3935
Kochi (KO)	February, 2008	9.58°N 76.16°E	50	4	0.1176	0.2384
Kavaratti (KA)	November, 2008	10.34°N 72.37°E	50	4	0.1543	0.3935
Port-Blair (PB)	July, 2008	11.40°N 92.46°E	50	2	0.0400	0.0800
Tuticorin (TU)	September, 2009	8.49°N 78.08°E	50	6	0.2269	0.6237
Pondicherry (PO)	July, 2008	11.56°N 79.50°E	50	3	0.1167	0.2367
Vizag (VI)	February, 2009	17.42°N 83.15°E	50	7	0.2620	0.6971
Total			400	13	0.1617	0.3986

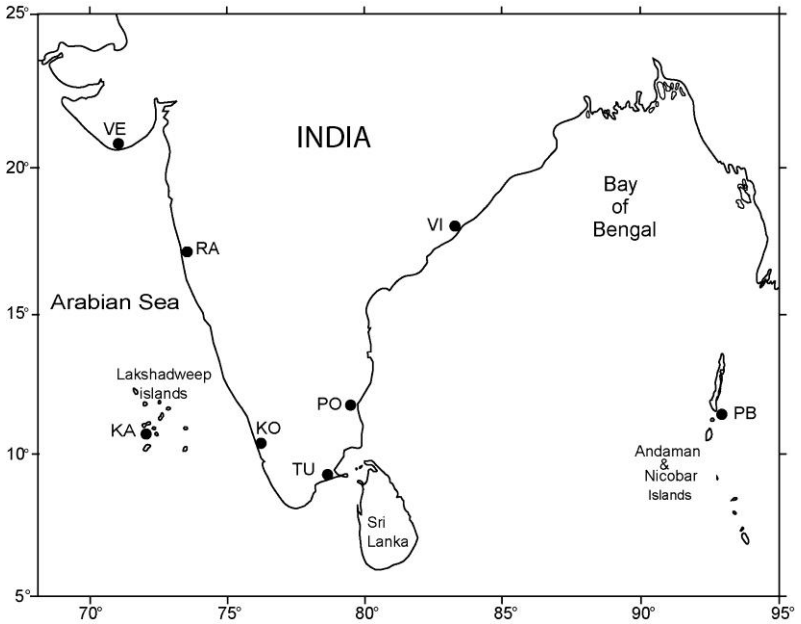

Figure 1. Map showing the sampling sites of kawakawa along Indian coast. VE, Veraval; RA, Ratnagiri; KO, Kochi; KA, Kavaratti; PB, Port-Blair; TU, Tuticorin; PO, Pondicherry; VI, Vizag.

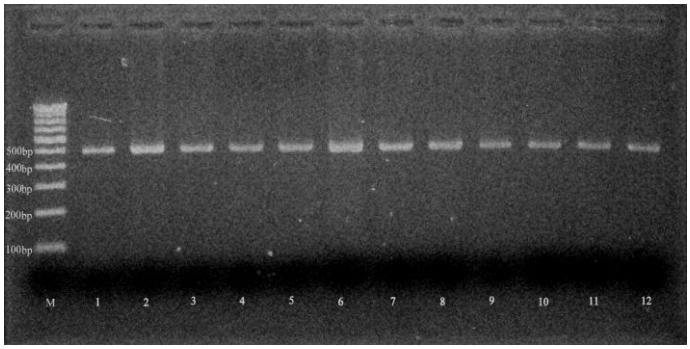

Figure 2a. 500bp PCR amplified product of kawakawa. Where M is the 100bp DNA marker and 1-12 is the amplified DNA product.

were as follows: $^{5'}$CCGGACGTCGGAGGTTAAAAT$^{3'}$ (forward) and $^{5'}$AGGAACCAAATGCCAGGAATA $^{3'}$ (reverse). DNA samples were amplified in Eppendorf Thermocycler (ep gradient S). Amplification was carried out in 50 µl reaction mixture containing 2 µl of template DNA; 5 µl of 10X buffer (100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 500 mM KCl); 1.0 µl of each primer (100 pmol); 5 µl of a 2.5 mM solution of each deoxyribonucleoside triphosphate (dNTP); 2.5 units of Taq DNA polymerase and milliQ water. Polymerase chain reaction (PCR) parameters consisted of 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. Final extension was done at 72°C for 5 minutes.

RFLP

PCR products were digested with six restriction enzymes (*Rsa* I, *Alu* I, *Hinf* I, *Hha* I, *Msp* I and *Hae* III). Restriction digestion was carried out in a 10 µl volume containing 2 µl of PCR product, 2 units of restriction enzyme, 1 µl of the appropriate buffer, and 7 µl of ultrapure water, at 37°C for overnight. Restriction fragments were resolved on a 2.5% agarose gel buffered with 1X Tris-Borate-EDTA (TBE), and stained with ethidium bromide. Bands on the gels were visualized by UV transilluminator and photographed for further analysis.

Data Analyses

Data analysis was initiated by preparing a matrix data, which includes presence or absence of different restriction fragment pattern with respect to each endonuclease. The presence of a given fragment was indicated by "1" while absence was indicated by "0". For each enzyme, variable restriction patterns were alphabetically designated as they were encountered and composite morphs were assigned to each individual. Since *Msp* I and *Hae* III did not show polymorphism, restriction pattern of these two enzymes were not used for constructing composite morphs. The restriction site matrix was used to calculate nucleotide diversity (π) and composite morph diversity (h) using program ARLEQUIN version 3.11 (Excoffier *et al*., 2005). The extent of genetic differentiation between samples was estimated using the fixation index F_{ST} (Wright, 1951). Estimates of expected number of migrant females between populations per generation (N_fm) were calculated using the formula $2\ N_fm = ((1/F_{ST})-1)$ (Takahata and Palumbi, 1985). The significance of F_{ST} was tested by 1,000 permutations for each pairwise comparison. Hierarchical analysis of molecular variance was performed to partition variance components attributable to (1) variation among groups; (2) variation among populations within groups; and (3) variation within populations, to evaluate hypothesized patterns of spatial genetic structure. The null hypothesis of population panmixia was also tested using an exact test of composite morph homogeneity among samples. The exact test of population differentiation of composite morphs tests the hypothesis that the observed distribution of frequencies is less likely than the distribution expected under panmixia. Statistical significance was estimated via 1,000 Markov Chain Monte Carlo simulations as proposed by Raymond and Rousset (1995). The population parameters θ and τ were also estimated for the kawakawa samples. Tau (τ) is a relative measure of time since population expansion, but can be transformed to estimate the actual time (T) since a population expansion using formula $T = \tau/2\mu$ where μ is the mutation rate per site per generation. In the present study, the mutation rate of 3.6×10^{-8} mutations per site and year was applied for the control region sequence of kawakawa as this rate has been reported for the mtDNA control region in teleosts (Donaldson and Wilson, 1999). Historical demographic/spatial expansions were investigated using Tajima's D test (Tajima, 1989) and Fu's F_S test (Fu, 1997) with 1,000 permutations. ARLEQUIN was also used to estimate both Harpending's raggedness index (*Hri*; Harpending, 1994) and mismatch distributions (*SDD*), to test the goodness-of-fit of observed mismatch distributions to the theoretical distribution under a sudden expansion model (Rogers and Harpending, 1992). To examine genealogical relationships among composite morphs, a minimum spanning network was constructed using HapStar Version 0.5 (Teacher and Griffiths, 2011).

Results

Genetic Diversity

RFLP analysis of 400 samples of kawakawa exhibited 16 restriction sites from six enzymes, resulting in 13 composite morphs (Table 1). The polymorphic band patterns of restriction enzymes (Rsa I, Alu I, Hinf I, Hha I) are presented in Figure 2b-2l. The composite morph AAAA was most commonly observed in all the samples followed by CAAA. Composite morphs (h3 and h5) were unique to VE while h8, h9, and h13 were present only in KO, KA, and VI respectively (Table 1). Composite morph diversity (h) and nucleotide diversity (π) were low, ranged from 0.0400 to 0.2620 and 0.0800 to 0.6971 respectively (Table 2). All the pairwise comparisons were insignificant and most of the pairwise F_{ST} values were negative (Table 3).

Population Genetic Structure

Analysis of molecular variance (AMOVA) performed on mtDNA RFLP data set revealed no significant genetic heterogeneity among the eight sampling sites (F_{ST} = -0.45%, P = 0.84946) (Table 4).

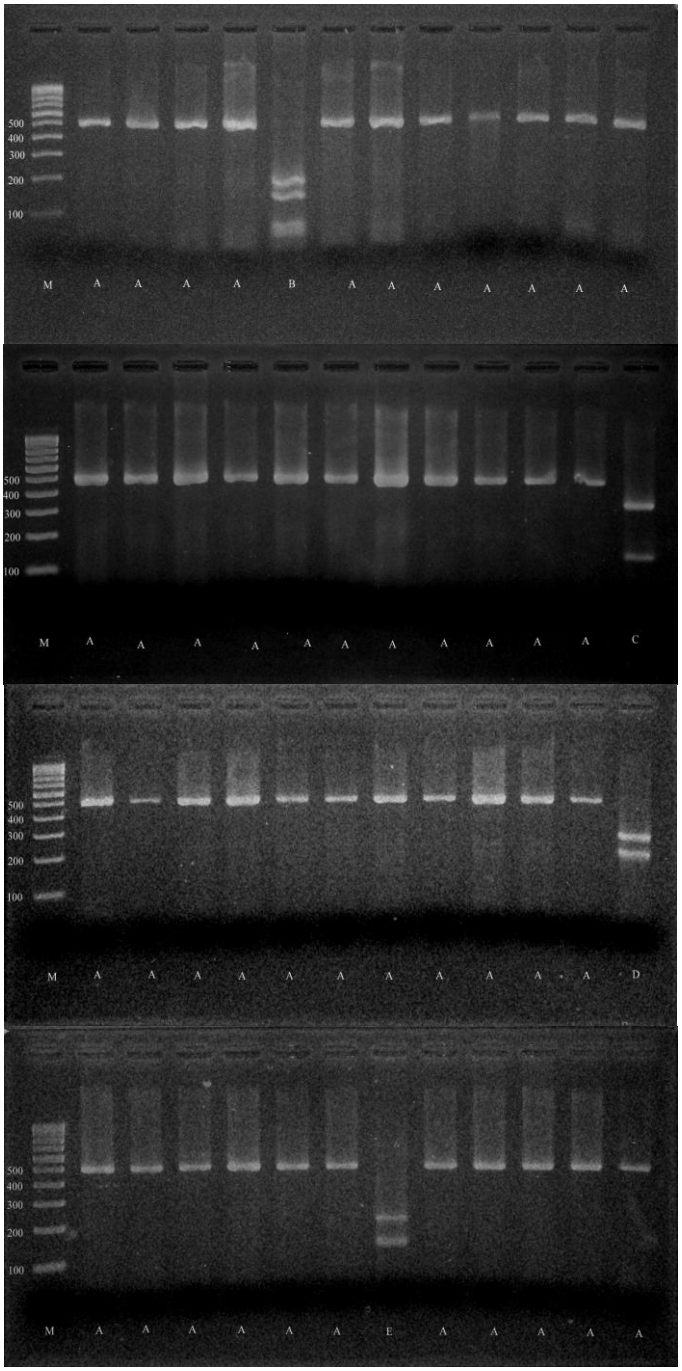

Figure 2 (b, c, d, e). The restriction digestion of mtDNA (D-loop) of kawakawa with enzyme *Rsa* I. Where M is the 100 bp DNA marker and A, B, C, D, and E are different restriction patterns.

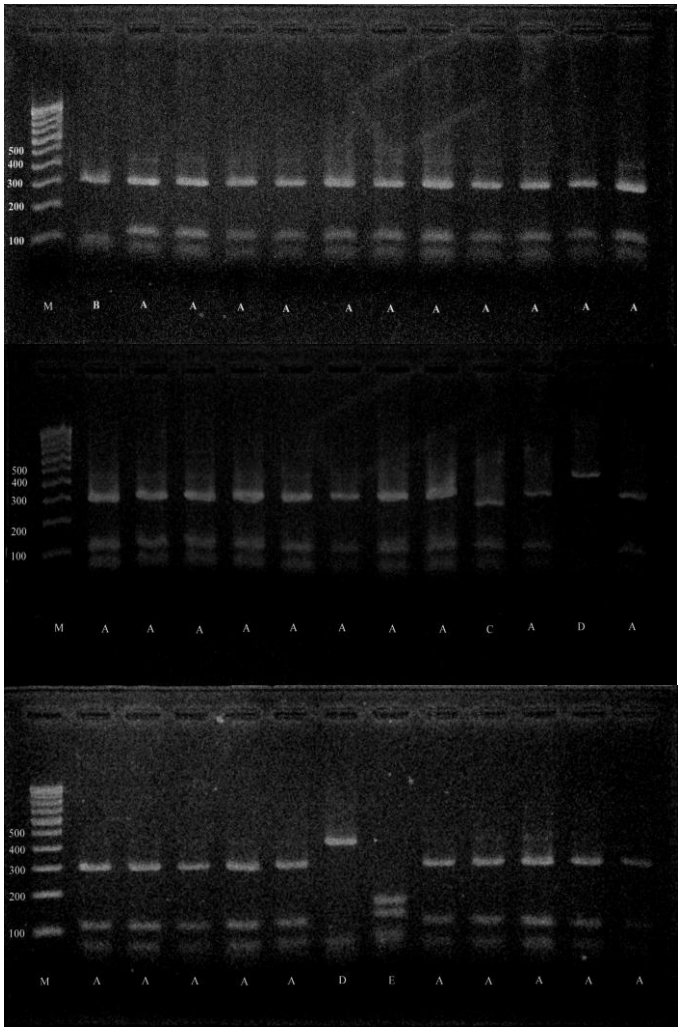

Figure 2 f, g, h. The restriction digestion of mtDNA (D-loop) of kawakawa with enzyme *Alu* I. Where M is the 100bp DNA marker and A, B, C, D, and E are different restriction patterns.

The estimated value of female migrants per generation (using F_{ST} = -0.00446; Table 4) was 112 among the eight samples. Hierarchical AMOVA was performed to test the significance of the partitioning of genetic variance resulting from different groupings of the populations into geographical groups. Results revealed that variation attributed to among groups composite morph frequency differences was very low (<1%), with almost all of the variation (100.33%) found in samples within populations (Table 4). The exact test of population differentiation (non-differentiation exact P values) showed no significant differences among eight samples (P = 0.94910). A minimum spanning network (Figure 3) indicates that composite morphs are closely related and there is no clear pattern of composite morph and geographic location among samples (Menezes *et al*. 2008).

Historic Demography

The ARLEQUIN analyses of mtDNA showed large differences in θ_0 (population before expansion) and θ_1 (population after expansion) within all collections, suggesting rapid population expansion (Table 5). In addition, all Harpending's raggedness indices and mismatch distributions were non-significant. Tajima's *D* (-2.011) and Fu's *Fs* (-12.676) values were negative and highly significant (P=0.000). The overall tau (τ) value was estimated to be 3.000 (95% confidence interval), which reflects the time of founding of kawakawa in Indian waters of 83,333 years ago.

Discussion

The analyses of mtDNA RFLP data exhibited very little divergence among the eight samples analyzed, suggesting the existence of single panmictic population of kawakawa in Indian waters. Thus, null hypothesis of single stock structure for all samples cannot be rejected. This outcome is supported by non-significant value of pairwise F_{ST} (Table 3) as well as AMOVA analyses, which did not show any geographically meaningful group along Indian coast (Table 4). The null hypothesis is also corroborated by exact test of population differentiation which gave non-significant P value (P = 0.94910), indicating that the composite morphs were distributed randomly with

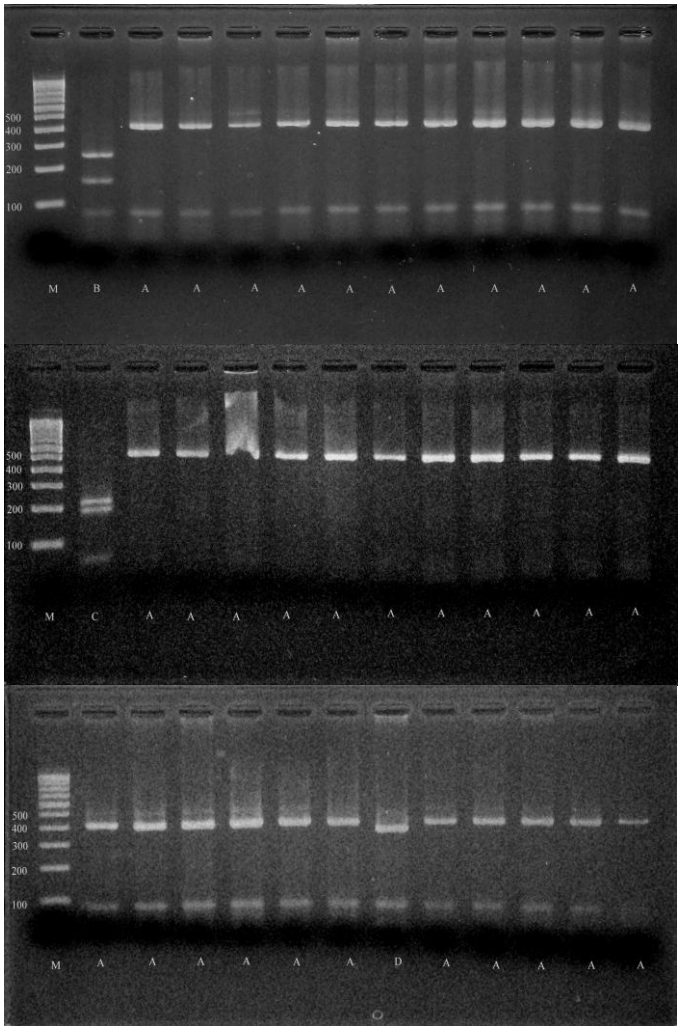

Figure 2 (i, j, k). The restriction digestion of mtDNA (D-loop) of kawakawa with enzyme *Hinf* I. Where M is the 100 bp DNA marker and A, B, C and D are different restriction patterns.

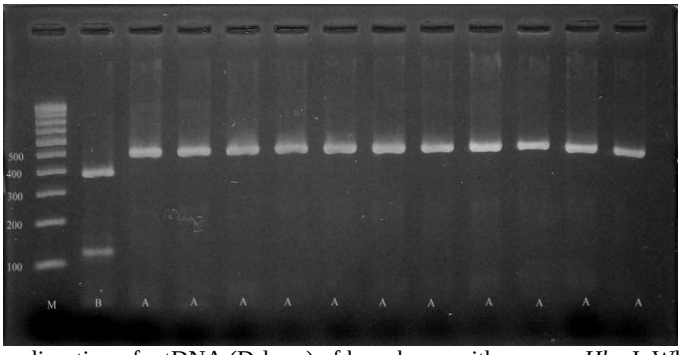

Figure 2 (l). The restriction digestion of mtDNA (D-loop) of kawakawa with enzyme *Hha* I. Where M is the 100 bp DNA marker and A, B are different restriction patterns.

respect to locality.

Marine species are generally characterized by large population sizes, high dispersion capacity during pelagic larvae stages, and wide biogeographical distribution. The apparent lack of barriers to dispersal in the marine environment effectively reduces heterogeneity among populations, often making it difficult to differentiate discrete populations (Palumbi, 1992; Menezes *et al*., 2008). Tested samples for D-loop by PCR-RFLP analyses of kawakawa conform to this pattern. The results of AMOVA and pairwise comparison of F_{ST} statistics were not significant, indicating that no significant population genetic structure exist along the Indian coast. Results of present study are supported by mtDNA sequence analyses of five populations of kawakawa in waters of Taiwan which showed no genetic heterogeneity among samples and population

Table 2. Distribution of 13 composite morphs from RFLP data. Letters reflects individual composite morph for restriction enzymes *Rsa*I, *Alu*I, *Hinf*I, and *Hha*I (left to right)

Number	Composite Morphs	VE	RA	KO	KA	PB	TU	PO	VI
h1	AAAA	0.9	0.9	0.94	0.92	0.98	0.88	0.94	0.86
h2	CAAA	0.04	0.04	0.02	0.04	0.02	0.02	0.04	0.04
h3	CCAA	0.02	-	-	-	-	-	-	-
h4	DAAA	0.02	-	0.02	-	-	0.02	-	0.02
h5	BABA	0.02	-	-	-	-	-	-	-
h6	ACAA	-	0.04	-	-	-	0.02	0.02	0.02
h7	AAAB	-	0.02	-	-	-	-	-	-
h8	ABAA	-	-	0.02	-	-	-	-	-
h9	AABA	-	-	-	0.02	-	-	-	-
h10	BBAA	-	-	-	0.02	-	-	-	0.02
h11	ADAA	-	-	-	-	-	0.04	-	0.02
h12	EEDA	-	-	-	-	-	0.02	-	-
h13	ABCA	-	-	-	-	-	-	-	0.02

Table 3. Demographic parameters of kawakawa based on mitochondrial D-loop region RFLP data: τ, θ_0, θ_1, Tajima's *D* test and Fu's *Fs* values, Harpending's Raggedness index (*Hri*), and sum of squared differences (*SDD*)

Population	τ	θ_0	θ_1	Tajima's *D*	Fu's *Fs*	*Hri*	*SSD*
VE	3.000	0.00000	99999	-1.906**	-1.794	0.6919	0.0321
RA	3.000	0.00000	99999	-1.798*	-1.385	0.7233	0.0449*
KO	3.000	0.00000	99999	-1.906**	-2.567**	0.8056	0.0189
KA	3.000	0.00000	99999	-1.979**	-1.385	0.7439	0.0234
PB	3.000	0.00000	99999	-1.464*	-0.858	0.9248	0.0023*
TU	3.500	0.00176	99999	-2.060**	-2.516	0.6671	0.0497
PO	3.000	0.00000	99999	-1.667*	-1.064	0.8066	0.0186*
VI	3.500	0.00176	99999	-1.967**	-3.312*	0.6212	0.0571*
Total	3.000	0.00000	99999	-2.011***	-12.676***	0.7378	0.0271

*, **, *** Significant at P<0.05, P<0.01, and P<0.001 respectively.

Table 4. Pairwise F_{ST} (below) and P (above) values among populations of kawakawa

Sample	VE	RA	KO	KA	PB	TU	PO	VI
VE		0.62695	0.50098	0.95703	0.21387	0.58203	0.75000	0.81250
RA	-0.00457		0.81836	0.81641	0.63281	0.72559	0.99902	0.64844
KO	-0.00147	-0.00739		0.99902	0.99902	0.61523	0.99902	0.55957
KA	-0.01331	-0.00786	-0.01256		0.59668	0.57812	0.99902	0.79102
PB	0.01343	0.00028	-0.01005	-0.00311		0.16309	0.99902	0.10449
TU	-0.00211	-0.00588	-0.00144	-0.00270	0.01391		0.58398	0.99121
PO	-0.00559	-0.01515	-0.01344	-0.01254	-0.01000	-0.00141		0.49316
VI	-0.00842	-0.00536	-0.00291	-0.00833	0.01677	-0.01290	-0.00116	

pairwise F_{ST} among the five populations were <0.02 (Chiou and Lee, 2004). A pilot study carried out by Santos *et al.* (2010), to investigate the genetic stock structure of kawakawa in Southeast Asia, suggested that kawakawa in the Philippines and in Southeast Asia is near "panmixia" or mixing. Results of present study are also supported by previous studies of many highly vagile *Scomberomorus* spp. For example, Hoolihan *et al.* (2006) reported a single genetic stock of *S. commerson* in ROPME sea area based on PCR-RFLP and sequence analyses of mtDNA D-loop region. Mitochondrial and nuclear DNA analyses of *S. maculate* have shown homogenous distribution throughout the western Atlantic and Gulf of Mexico (Buonaccorsi *et al*., 1999). Based on sequence analyses of mtDNA Shui *et al.* (2009) reported a single population genetic structure of *S. niphonius* in East China Sea and Yellow sea.

It has been reported that marine fishes have greater intraspecific gene flow and reduced population structure compared to freshwater fishes, likely due to fewer barriers to dispersal in the marine environment (Ward *et al*., 1994). In particular, genetic differentiation is low between tuna populations within and between oceans (Alvarado Bremer *et al*., 1998; Gerwe and Hampton, 1998; Chow *et al*., 2000; Appleyard *et al*., 2002; Durand *et al*., 2005) because of the occurrence of continuous, circumtropical pelagic environment and a wide range of suitable spawning grounds. Kawakawa is widely distributed in the tropical and subtropical waters of the Indo-Pacific region and was observed to spawn in waters of Hong Kong, India, Thailand, and Philippines (Yesaki, 1994). The continuous continental shelf connecting

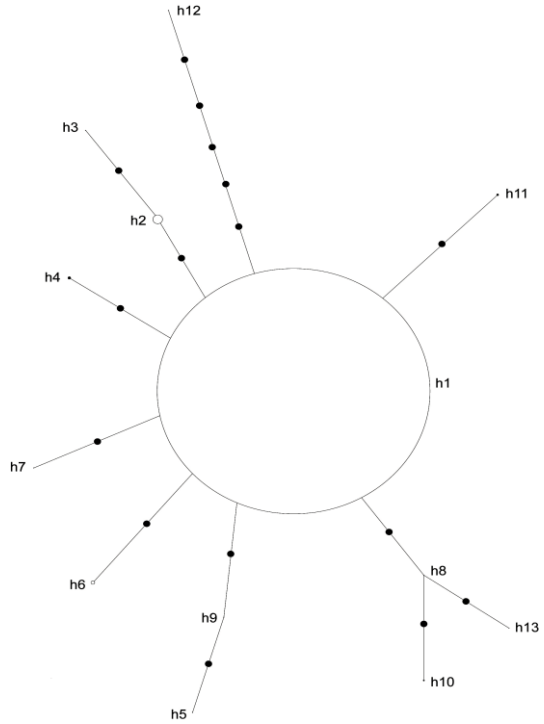

Figure 3. Minimum spanning network showing relationships among 13 mitochondrial DNA D- loop region composite morphs. Each circle represents a unique composite morph in the sample, and size of each circle represents relative frequency of each composite morph.

Table 5. Results of analysis of molecular variance (AMOVA) testing genetic structure of kawakawa based of mitochondrial D-loop region

Source of variation	Variance	Percentage of variation	*F* statistic	P-values
One group- (VE, RA, KO, PB, TU, PO, VI)				
Among populations	-0.00089	-0.45	F_{ST} = -0.00446	P = 0.84946
Within populations	0.20010	100.45		
Five groups- (VE, KA); (RA, KO, PB); (TU); (PO) and (VI)				
Among groups	0.00166	0.83	F_{CT} = 0.00834	P = 0.05083
Among populations within groups	-0.00231	-1.16	F_{SC} = -0.01170	P = 0.95015
Within populations	0.20010	100.33	F_{ST} = - 0.00326	P = 0.84555

the countries in Southeast Asia could also facilitate migration of the kawakawa since it is essentially confined to the continental shelf and rarely captured beyond the edge of the continental shelf (Yesaki, 1994; Santos *et al*., 2010). Low F_{ST} value and high rates of gene flow ($N_f m$ =112) observed in present study supports the low genetic differentiation and presence of single genetic stock among eight kawakawa samples.

Lack of population subdivision observed in the kawakawa samples could also be related to seasonal variation in water circulation associated with the monsoon currents in Indian Ocean. During northeast monsoon, the flow of the upper ocean is directed westward from near the Indonesian Archipelago to the Arabian Sea. During southwest monsoon, the direction reverses, with eastward flow extending from Somalia into the Bay of Bengal (Schott *et al*., 2001). The seasonally reversing monsoon currents causing the mixing of the kawakawa populations from different spawning grounds in Indian waters.

The low levels of composite morph and nucleotide diversity were observed for kawakawa samples along the Indian coast (Table 2). The low genetic diversity at both composite morphs and nucleotide levels may be attributed to recent population bottleneck or founder event by single or few mtDNA lineages (Grant and Bowen, 1998). The above statement is corroborated by large differences observed in θ_0 and θ_1 within all collections (Table 5), which is expected during rapid population expansion. Past population expansion is also supported by the Tajima's *D* and Fu's *F* statistics (Table 5) which were both negative and significant as well as non-significant deviations for the sum of squared deviations (*SSD*) and Harpending's raggedness index

(*Hri*) (Table 5). Furthermore, tau (τ) values for mtDNA data set were all same (τ = 3.000), indicating that all the samples originated from a single colonization event about 83,333 years before present (95% confidence interval) in Indian waters. Thus, recent founding and insufficient time to attain the migration-drift equilibrium among populations of kawakawa could be the possible reason for lack of genetic structure observed in present study.

The importance of knowing stock structure in a managed fishery is the implicit assumption that the fish being managed belong to a single stock (Gulland, 1965; Ricker, 1975). This assumption is essential to management decisions because reproductive potential, pattern of recruitment, mortality and adaptation to exploitation differ significantly for non-mixing populations of a single species. Results of the genetic analyses of present study fail to reject the null hypothesis. However, failure to reject the null hypothesis of sample homogeneity does not necessarily mean that the samples are homogeneous. Increasing sample size or using a different class of marker with different population dynamics may reveal heterogeneity and restrictions on gene flow among regions once classed as homogeneous (Ward, 2000). MtDNA has inherent limitations as a molecular marker to detect the genetic stocks, as it is a non-recombining genome and best treated as a single character. Also, because mtDNA is maternally inherited, genetic consequences of male dispersal will not be detected. These limitations may be addressed by analyzing regions of nDNA which is based on many independent characters. Therefore, it is necessary to employ more genetic data, such as microsatellite DNA analysis, in further studies.

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