Genetic Stock Structure and Phylogenetic Relationship of Kawakawa *Euthynnus affinis* – Cantor (1849) in the Northern Coastal Waters of Tanzania Using Mitochondrial DNA Control Region

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Abstract: Accurate identification of population genetic subdivision is crucial when planning for management-conservation strategies of highly migratory marine fishes like Kawakawa *Euthynnus affinis* Cantor (1849). Although the species is of commercial importance in the Indo-Pacific Ocean, its management and conservation strategies are hindered partly by the lack of information on the population genetic structure. The present study investigated the genetic structure and phylogenetic relationship of the species at 500bp of the mitochondrial D-loop region for the 46 samples collected at two localities in Tanzania coastal waters. The study indicated higher overall haplotype (0.969) and nucleotide (0.108) diversities indicating stable habitats, absence of strong directional or stabilizing selection. However, the study indicated the presence of both sharing and locality specific haplotypes. Further analysis using hierarchical AMOVA tests indicated a single genetic structure of the Kawakawa ($P>0.05$) in the northern Tanzania coastal waters. Therefore, a null hypothesis that Kawakawa in the northern Tanzanian coastal waters are composed of single genetic stock can not be rejected. In addition, the study revealed the existence of a single evolutionary clade demonstrating that Kawakawa caught in the northern coastal waters of Tanzania share the same gene pool. Another study covering large geographical areas by applying more than one genetic marker is recommended to precisely the genetic structure and possible sex biased migration in the Indian Ocean.

Keywords: ***Euthynnus affinis*, mitochondrial D-loop region, genetic structure, phylogenetic relationship and Tanzania**

1.0 Introduction

Accurate identification of population subdivision is crucial when planning for management-conservation strategies of highly migratory marine fishes. Although Kawakawa *Euthynnus affinis* C. (1849) is one of the large pelagic and highly migratory tuna species, lack of information on genetic studies hinders management and conservation strategies. Genetic methods are useful in estimating a number of population parameters relevant to fishery management such as effective population size, gene flow and natural level of genetic diversity (Galarza 2007). In the Indian Ocean, Kawakawa is exploited by commercial and artisanal drifting longline, pole and line, purse seining and ring seining. The average landings of Kawakawa in India coastal waters during 2011-2012 were estimated at 41271 tones, accounting for 12.03% of the total neritic tuna catches from the Indian Ocean countries (Babu and Anrose 2013). It should, however, be noted that loss of genetic diversity due to fishing pressure can lower the species ability to withstand changes in the environment (Conover and Munch 2002).

A long held view of '*panmixia*' population in marine fishes is suspected in Kawakawa (Santos *et al*., 2010). This could probably be due to its capability for long distance migration, the cosmopolitan geographic distribution and absence of physical barriers to dispersal (Palumbi 1994). However, recent genetic studies on tunas and other scombroid species (Carlsson *et al*., 2004; Vinas *et al*., 2004a) revealed significant genetic structuring, a factor that accelerate collapse of stocks if not properly taken into account by fishery management policies. Some studies cite ecological, self-recruitment (Viñas *et al*., 2004b; Zardoya *et al*., 2004) and climatic variations associated with Pleistocene glacial cycles (Chow *et al*., 2000; Graves and McDowell 2003; Lecomte *et al*., 2004; Viñas *et al*., 2004a) as mechanisms underlying genetic differentiation. Genetic structure and historical demography are of primary importance when planning management and conservation strategies (Slatikin and Hudson 1991; Excoffier *et al*., 1992) as they can

provide complementary information on morphometric and meristic data. However, it is acknowledged that morphometric and meristic data if used alone can create a room for over-fishing and subsequent resource collapse.

Non-genetic methods on the other hand can only infer different fish breeding units, whereas a population genetics approach can directly test the hypothesis that genetically different breeding units do exist (Ward 2000). Lack of detailed population genetic studies on Indian Ocean Kawakawa stocks has hindered development of scientific management strategies for the species. Currently the Indian Ocean Tuna Commission (IOTC 2014) manages the specie as a single stock with limited knowledge on scientific aspect.

Information on genetic structure of Kawakawa from other regions beyond the Western Indian Ocean (WIO) region is relatively well documented. In Philippines and Southeast Asia for instance, genetic data on Kawakawa indicated a '*panmixia*' or mixing population (Santos *et al*., 2010). Lack of genetic heterogeneity among samples was reported from the populations of Kawakawa collected in Taiwan waters (Chiou and Lee 2004) and straits of Malacca (Masazurah *et al*., 2012). On the other hand, the same information was reported in Australia (Robertson *et al*., 2007), Indian coast (Kumar *et al*., 2012a) and in the Northern and Eastern Indian Ocean waters (*e.g*. Robertson *et al*., 2007; Masazurah *et al*., 2012).

Mitochondrial DNA (mtDNA) control region has been shown to be particularly sensitive in detecting population genetic structure of marine migrating and predatory fishes (Buonnacorsi *et al*., 2001). The current study employed mtDNA control region sequence data to determine (i) spatial and temporal genetic variability, (ii) population stock structure and (iii) phylogenic/genealogical relationship and historical demography of Kawakawa in the northern coastal waters of Tanzania. A null

hypothesis of a 'single panmictic population' of Kawakawa across the geographical cline along the northern Tanzania coastal waters was tested.

2.0 Methods and Materials

2.1 Study Areas

The present study was conducted in the two coastal sites; Dar es Salaam and Pangani in the northern coastal waters of Tanzania (Figure 1). The coastal waters of Tanzania are characterized with seasonal variations in water circulation connected with the periods of northeast monsoon (NEM) and southeast monsoon (SEM) seasons. Along this coast, the NEM season occurs from November to March and the SEM season commences from April to October (Mahongo *et al*., 2012). The NEM season is dominated by lighter and predominantly northerly (blowing from north to south) winds, while the latter are usually strong and predominantly southerly (blowing from south to north). The SEM season is usually associated with strong current speeds of up to 2 m/s whereas NEM season is associated with weaker current speed that is less than 0.5 m/s. March and October are the transition periods and there is reversal from the NEM to the SEM monsoon seasons and vice versa, respectively (Iversen *et al*., 1984; Mahongo *et al*., 2012). However, despite the presence of the two nearly opposing monsoon wind systems, the dominant current prevailing in the Tanzanian coastal waters is the East African Coastal Current (EACC), which flows northwards throughout the year.

2.2 DNA Extraction

Tissue samples (white muscle) were obtained and preserved in 95% ethanol before genomic extraction at the Molecular Biology Laboratory of the Mikocheni Agricultural Institute Dar es Salaam Tanzania. High molecular weight genomic DNA was extracted from the muscle samples using the standard TNES-Phenol-Chloroform protocol as modified from Miller *et al*. (1988) and Asahida *et al*. (1996).

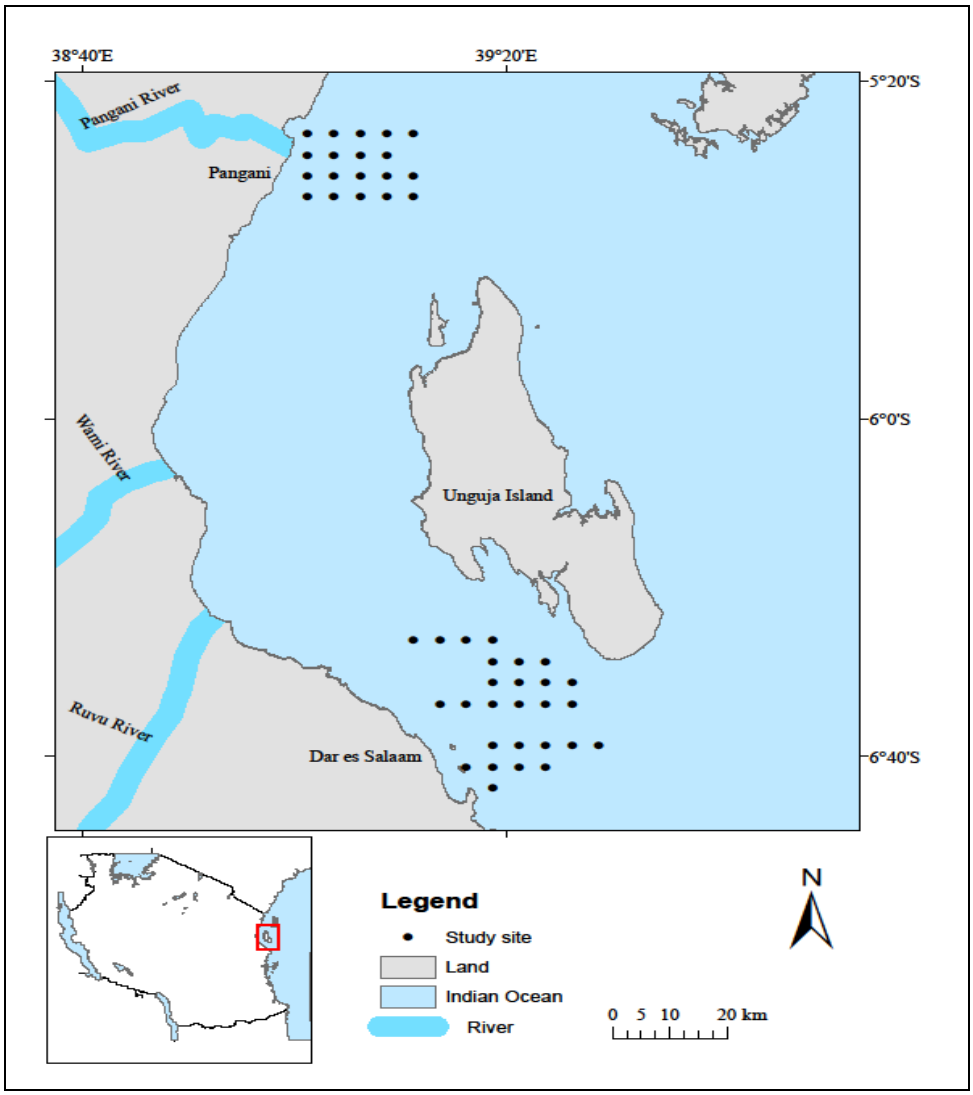

Figure 1: Map of Tanzania coastal waters showing the study sites (Source: IMS 2015)

2.3 DNA Amplification and Sequencing

A fragment of 500bp containing the first half of the mtDNA control region was amplified using the primer set designed by Menezes *et al.* (2006). Primers sequences used in study of Kawakawa were: 5′-CCGGACGTCGGAGGTTAAAAT-3′ and 5′-AGGAACCAAATGCCAGGAATA-3′. Amplification was conducted in 50 µl reaction mixture. The PCR products were visualized on 1% agarose gels and image obtained (Plate 1). The amplified products were purified and the mtDNA control region gene

were sequenced at Macrogen Laboratory South Korea using 6 Applied Biosystems 3730×l sequencer following manufacturer protocol.

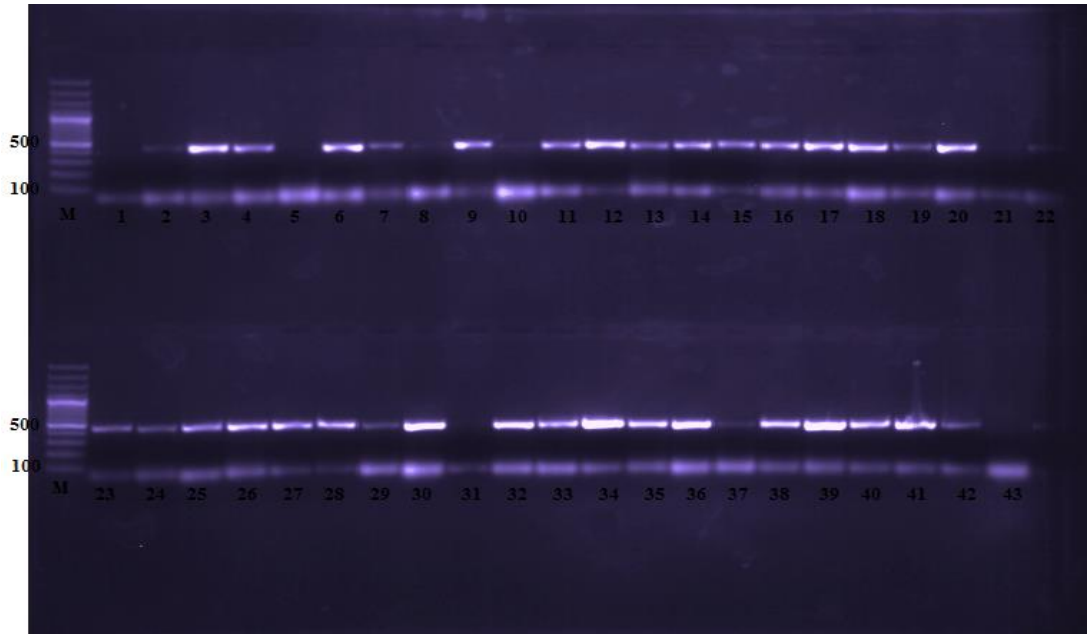

Plate 1: 500bp PCR amplified product of Kawakawa. M is the 100bp DNA marker and 1-43 is the amplified DNA product

2.4 Data Analysis

DNA sequences were assembled and edited with Genious version 5 and adjusted manually as needed. Alignment was carried out using the ClustalW algorithm (Thompson *et al.*, 1994) implemented in MEGA 6.0.6 (Tamura *et al.*, 2013). Genetic variations were determined using Arlequin 3.11 (Excoffier *et al.*, 2005). The extent of population differentiation between and within sampling sites was analyzed by calculating the inbreeding coefficient (F_{ST}) using Arlequin 3.11 (Excoffier *et al.*, 2005). F_{ST} significance (5% level) was tested by 10000 permutations for each pairwise comparison. Analysis of molecular variance (AMOVA) was used to examine genetic variability partitioned within and between populations. Hierarchical AMOVA was used to assess variation between sample populations (F_{ST}); variation between sample populations

within regions (F_{SC}) and at a higher level of hierarchy describes variation between regions for sample populations (F_{CT}) according to Wright (1969) and Nei (1987). F_{ST} is the proportion of genetic variation that exists between populations (subpopulations or samples): F_{ST} ranges from 0 to 1, whereas 0 implies no differences among samples and 1 represent a completely differentiated population. Evolutionary relationships between haplotype variants were obtained by constructing a haplotype network using Network 4.5.1 software which applies the Median- Joining algorithm (Bandelt *et al*., 1999). A maximum likelihood (ML) phylogenic tree of the sequences was constructed in MEGA 6.0.6 to further assess the genealogical relationship between my samples and those collected elsewhere. The robustness of ML phylogenetic hypothesis was tested by 10,000 bootstrap replicates.

3.0 Results

3.1 Genetic Variations

Genetic variation indices of Kawakawa between and within studied populations are presented in Table 1. A total of 85 polymorphic sites and 185 genetically conserved sites were defined. In addition, 29 haplotypes, 69 parsimony informative sites and 16 singleton sites were described. Overall haplotype diversity was high (0.969) and of the same level of magnitude across the studied populations. Nucleotide diversities (Pi) were almost similar within and between localities (Table 1).

Six haplotypes were shared between localities, while only one haplotype was shared by specific individuals within the Dar es Salaam site. This is an indicative of the existence of large number of haplotypes that are specific and/or endemic to Dar es Salaam and Pangani populations. The Dar es Salaam samples comprised of seven specific haplotypes that were not shared within the population and only one haplotype (Hap_8) which was shared by two individuals within the geographical edge. By contrast, Pangani samples formed six specific haplotypes both of which indicated no sharing within the locality.

Table 1: Genetic diversity of Kawakawa from northern Tanzania coastal waters

Parameters	Within populations		Between populations
	Dar es Salaam (1)	Pangani (2)	
Number of sequences	25	21	46
Number of haplotypes (h)	17	17	29
Haplotype diversity (Hd)	0.96	0.97619	0.96908
Number of polymorphic sites	72	75	85
Average number of nucleotide differences (k)	26.77	30.371	28.217
Nucleotide diversity, Pi(1)	0.10416	0.11818	0.10986
Nucleotide diversity with Jukes and Cantor, Pi(1)JC	0.121±0.018	0.137±0.012	0.12732
Average number of nuc. subs. per site between populations (Dxy)			10.97%
Number of net nuc. subs. per site between populations (Da)			-1.38%
Number of observed transitions	54	53	
Number of usable loci	78	78	
Number of observed indels	0	0	
Number of observed sites with transitions	44	46	
Number of observed transversions	32	32	
Number of observed sites with transversions	32	32	
Number of substitutions	86	85	
Number of observed sites with substitutions	69	72	

3.2 Genetic Structure and Differentiation

The summary of the results on AMOVA analysis are presented in Table 2. The results show that the inbreeding coefficient F_{ST} was equal to 0.0035 (p = 0.331), suggesting lack of genetic differentiation between populations. On the other hand, the spatial genetic differentiation between sites irrespective of time (F_{CT}) indicated a non significant genetic differentiation (F_{CT} = 0.00367, p >0.05) in Dar es Salaam and in Pangani populations (F_{CT} = 0.00329, p >0.05). When the samples were treated on seasonal basis (NEM vs SEM seasons), a non significant genetic differentiation, F_{SC} (p >0.05) between and within the samples was evident. This is an indicative of a stable population

structure over at least a duration of 16 months sampling period of the present study. AMOVA analyses indicated higher genetic variation within (99.65%) populations than between (0.35%) populations (Table 2). A higher variation within than between localities is also evident from the haplotype network (Fig 2 below). Furthermore, some of squares (SS) were moderately high within population than between populations (Table 2).

Table 2: AMOVA of Kawakawa genetic differentiation inferred from haplotype frequencies

Source of variation	**d.f**	**Sum of squares (SS)**	**Variance components**	**% variation**
Among populations	1	0.522	0.001Va	0.35
Within populations	44	21.822	0.48Vb	99.65
Total	45	21.804	0.485	
Fixation index (between populations) FST: 0.0035*				
Population specific F_{CT} indices (Dar es Salaam Tanzania): 0.00367*				
Population specific F_{CT} indices (Pangani Tanzania: 0.00329*				
ns, not significant;				
*,0.01<P<0.05				

3.3 Phylogenetic Relationship

The analysis of the genealogical relationship among haplotypes was analyzed using a median joining network cladogram (Figure 2) with haplotype Hap_1 at the centre of radiation. The analysis indicated that most haplotypes were closely related to the most ancestral haplotype (Hap_1). Under coalescent theory; this haplotype was the most common, widespread and central to the network. Haplotypes differ by one to four mutational steps from Hap_1. The star-like median network suggests existence of recent historic population expansion (Figure 2). From mismatch distribution, the expansion in this case is demographic expansion.

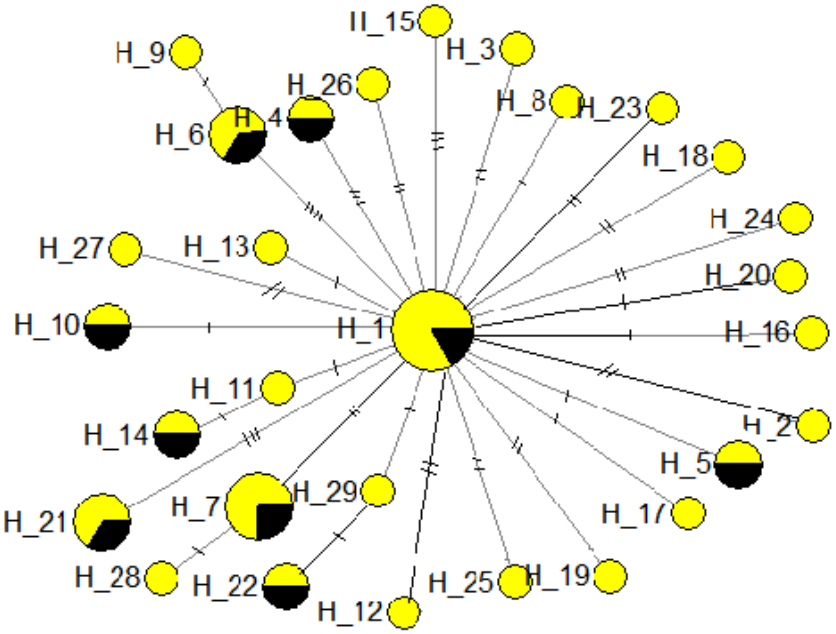

Figure 2: Haplotype network cladogram showing the evolutionary relationship between Kawakawa haplotypes. Each circle represents a unique haplotype in the sample, and the size of each circle represents the relative frequency of each haplotype

The ML tree (Figure 3a) modeled using ML statistics indicated a single mixing clade of Kawakawa in the northern Tanzania coastal waters. This is an indication that individual samples did not cluster according to the geographical regions (Figure 3.3a). The ML phylogeny analysis agrees well with AMOVA which indicated a single mixing population in the study areas. Long and wide genealogical branches in Figure 3a suggest population expansion. On the other hand, the phylogenic relationship analyses of the sequences from Indian coastal waters and Southeast Asia region within the Indian Ocean did not cluster with the one collected from northern Tanzania coastal waters and subsequently formed two distinct clades (Figure 3b). The sequences from the northern Tanzania mixed together to form clade 1 while those sequencies from Southeast Asia and Indian coastal waters grouped to form a single separate regional mixing group (clade 2). This is an indication that individuals from Southeast Asia and

Indian coastal waters do not have the same evolutionary history to that from the northern Tanzania coastal waters.

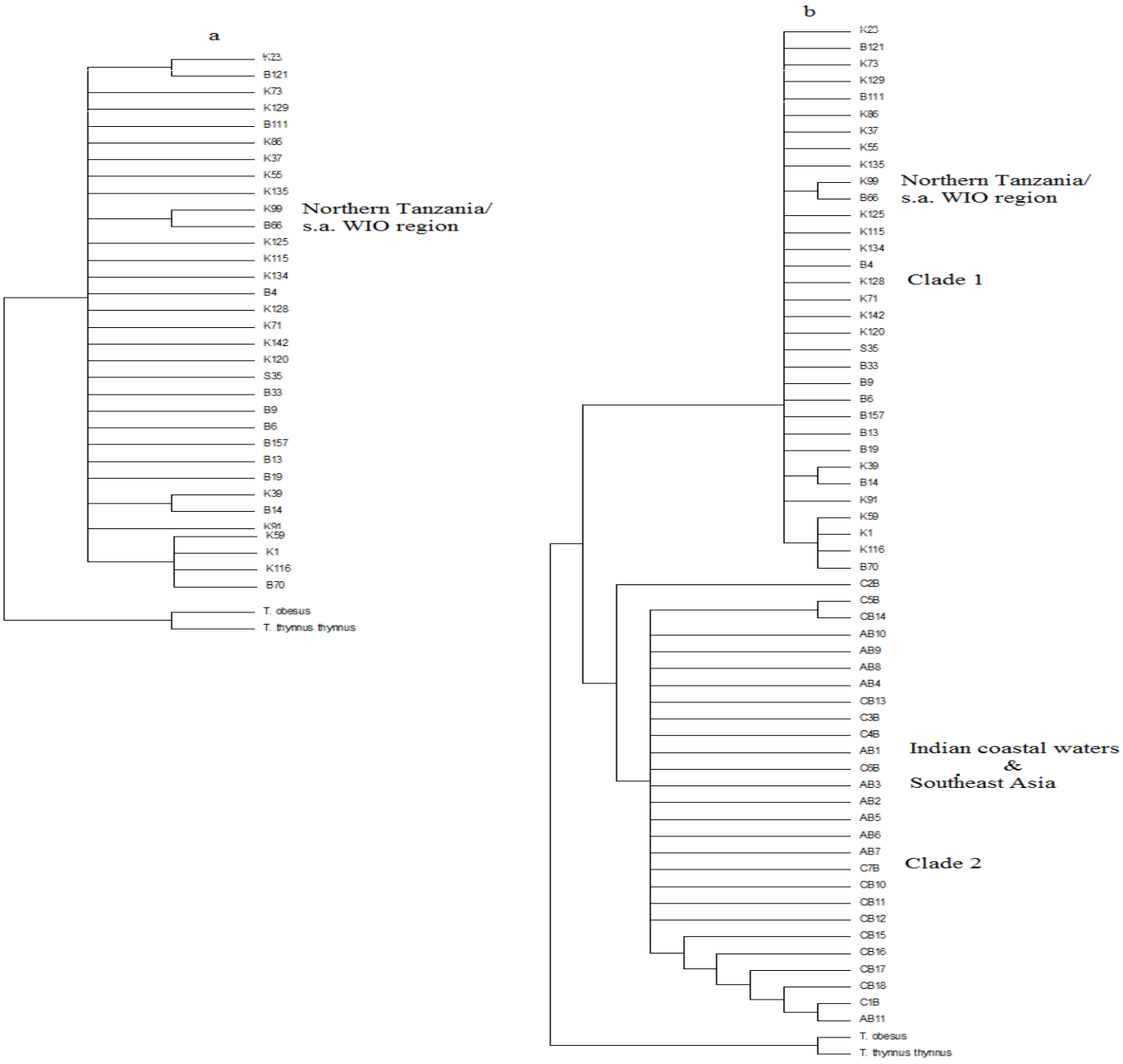

Figure 1: Maximum likelihood (ML) phylogenetic relationship of Kawakawa. Nodal bootstrap support is displayed where the nodal support is ≥70%. Scale represents the proportion of polymorphic sites between sequences a) Samples from northern Tanzania coastal waters

Discussion

The analysis of the mtDNA control region sequences of Kawakawa revealed high level of mitochondrial polymorphism, haplotype and nucleotide diversities. Present study observed that haplotype diversity within and between populations was consistently higher than those found in the coastal waters of India by Kumar *et al*., (2012). This is an indication that Kawakawa population along the northern Tanzania coastal waters is stable population and not affected by fishing pressure. Present findings on the other hand are comparable with the pattern observed by Ward *et al*. (1994) on other large pelagic marine fishes. Higher levels of nucleotide diversity (Pi) were in agreement with that of Kumar *et al*. (2012a) and Santos *et al*. (2010) in Southeast Asia (Table 3.8). Likewise, haplotype diversity were within the range for other tuna populations at 0.60 (Chow and Ushiama 1995) and billfishes at 0.922 (Chow *et al*., 1997). In the earlier studies by Grant and Bowen (1998) that attempted to infer population history, marine fishes were classified into four categories based on different combinations of small and large values for haplotype and nucleotide diversity of mitochondrial DNA sequences. A high level of haplotype and nucleotide diversities in this study belongs to the fourth category. This might be attributed to secondary contact between previously differentiated allopatric lineages or to a long evolutionary history in large stable population (Grant and Bowen 1998). The existence of higher genetic diversity in this study can be explained the presence of genetic processes such as mutation and migration which have the tendency to increase genetic diversity and counteract the effect of inbreeding and genetic drift.

In the present study, AMOVA test between and within localities failed to reveal a significant genetic differentiation in the two studied geographical areas in the northern Tanzania coastal waters. Hence, a null hypothesis of a single demographic unit of Kawakawa population in the northern Tanzania coastal waters could not be rejected, however given the fact that few migrant females are enough to reduce heterogeneity,

'*panmixia*' was not confirmed. Low genetic variation in the present study indicates genetic connectivity and suggests stock homogeneity in the northern coastal waters of Tanzania. The genetic connectivity between Dar es Salaam and Pangani populations is revealed by the presence of haplotype sharing. In addition, haplotypes sharing signifies that Kawakawa individuals migrate and have large dispersal over long distances between the study sites. This result further suggests long pelagic larval distance of Kawakawa. Haplotype sharing indicates a gene flow between populations, hence genetic connectivity and low genetic differentiation. Gene flow among populations increases genetic homozygosity and reduce heterozygosity. Although the study found haplotypes sharing between populations, the presence of the haplotypes restricted to locality indicates the existence of unique characters that are specific to Dar es Salaam and Pangani populations.

The ML tree indicated a single mixing clade along the northern Tanzania waters while formed a separate clade with haplotypes from Southeast Asia and Indian waters. Lack of genealogical signals between these regions may probably have resulted from either lack of gene flow, adult homing behavior to the spawning ground and geographical distances. Marjoram' and Donnelly (1994) reported that the amount of migration plays a crucial role in the behavior of the sample genealogy. This study further emphasized that in a population with a high migration probability of greater than 0.01, the distribution of pairwise differences is very close to that for a randomly mating populations.

Conclusion and Recommendations

i. The current study revealed higher haplotype and nucleotide diversities at spatial and temporal scale in the northern Tanzania waters which is an indicative of the areas ecological potential for providing local retention or larval accumulation.
ii. Genetic differentiation was not documented from the mtDNA control region and hence Kawakawa in the northern Tanzania waters forms a single mixing

population. However, given that few migrating females are enough to reduce heterogeneity between populations, '*panmixia*' could not be confirmed. Given the fact that 'panmixia' could not be confirmed and due to the migratory nature of the studied species, the present study suggest the use of a single model and co-management among coastal nations to ensure stock sustainability.

iii. A single evolutionary clade was revealed for the Kawakawa samples collected from the two geographical sites along the northern coastal waters of Tanzania signifying similar genealogical history.

iv. A study covering Indo-Pacific Ocean by using both mtDNA and nDNA is suggested to further provide information on the genetic structure and phylogenic information of Kawakawa.

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