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Genetic Divergence between *Auxis thazard* and *A. rochei* based on PCR-RFLP Analysis of mtDNA D-loop Region

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Abstract

Tunas of genus *Auxis* are commercially and ecologically important fish of family Scombridae. There is currently no information concerning the genetic divergence between *A. thazard* and *A. rochei*. Therefore, a study was conducted to estimate the degree of genetic divergence between two species based on PCR-RFLP analysis of D-loop region (500 bp) of mitochondrial DNA in 100 individuals collected from Tuticorin (India). High level of genetic divergence (9.26%) was observed between *A. thazard* and *A. rochei*. Estimates of molecular diversity indices were higher in *A. rochei* (π = 3.8808; *hd* = 0.9641) compared to *A. thazard* (π = 2.2514; *hd* = 0.8441). Based on demographic parameters, population expansion of *A. thazard* estimated to begin about 67,167 years before present while of founding of *A. rochei* took place about 114,747 years before present in Indian Ocean. Thus, we conclude that *A. rochei* represents the ancestral species of genus *Auxis*.

Keywords: Tuna, Genetic variation, Conservation, Restriction sites, Haplotype.

Introduction

Fish of genus *Auxis* is important in commercial and artisanal fisheries with their widespread distribution in Atlantic, Indian, and Pacific Oceans (Collette and Nauen, 1983). It comprised of two species: *A. thazard* (Lacepede, 1800) and *A. rochei* (Risso, 1810) which are divided into four subspecies; *A. thazard thazard*, *A. thazard brachydorax*, *A. rochei rochei*, and *A. rochei eudorax* (Collette and Aadland, 1996). Although, these two species entered in the scientific literature more than two centuries ago; their detailed morphological studies to verify them at species level were done in a relatively recent time (Collette and Aadland, 1996). Juvenile *Auxis* are the most abundant of all the juvenile tunas. Because of their abundance, they are considered to be an important element of the food web, particularly as forage for other species of commercial interest (Collette and Nauen, 1983). Despite their commercial and ecological importance, little genetic investigations have been done on *A. thazard* and *A. rochei* (Robertson *et al.*, 2007; Catanese *et al.*, 2008; Melissa *et al.*, 2008; Kumar *et al.*, 2012a, b). The aforementioned studies have contributed to a better understanding of genetic identification, discrimination and assessment of stock structure, no attempt has been made to investigate the genetic divergence existing between *Auxis* spp. for the conservation and fishery management plans.

Currently, the most promising approach to estimate the divergence between species is mitochondrial DNA (mtDNA); because of its predominantly maternal inheritance, relatively rapid rate of base substitution, and lack of recombination (Avise *et al.*, 1987). MtDNA sequence differences can either be measured directly through restriction site analysis or by sequence analysis. Restriction site analysis is less expensive and allows rapid screening of large number of samples and hence has become a widely-used population genetics tool in fisheries (Menezes *et al.*, 2006; Kumar *et al.*, 2012b). In this study, we use PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) analysis of mtDNA D-loop markers to investigate the genetic divergence between *Auxis thazard* and *A. rochei* for the first time. Selection of the mtDNA D-loop for analysis was based on its documented hypervariability in many fish species (Menezes *et al.*, 2012; Kumar *et al.*, 2012a, Kunal *et al.*, 2013) and the availability of specific primers for amplification of this region (Menezes *et al.*, 2006).

Materials and Methods:

Sample Collection and DNA extraction

A total of 100 fin clip samples of *A. thazard* (*n* = 50) and *A. rochei* (*n* = 50) were collected from Tuticorin (8.49°N 78.08°E), India in September, 2009. Samples were preserved in 95% ethanol at 4°C until DNA extraction. Total genomic DNA for each sample was extracted by standard TNES-Urea-Phenol-Chloroform protocol (Asahida *et al.,* 1996). The quantity and quality of the extracted DNA was estimated using UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan).

DNA Amplification

The PCR was performed to amplify the *c.* 500 bp containing the first half of the control region (D-loop) using the primer set 5' CCGGACGTCGGAGGTTAAAAT 3' (forward) and 5' AGGAACCAAATGCCAGGAATA 3' (reverse) designed from GenBank sequences of *A. thazard* (accession number NC005318) (Menezes *et al.,* 2006). PCR was carried out in a final volume of 50 µl 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 (GeNei™) and milliQ water. PCR condition included an initial denaturation of 5 minutes at 94°C, followed by 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 carried out at 72°C for 5 minutes. The success of PCR reactions was verified after running 3 µl of PCR product through a 1% agarose gel using 100 bp DNA ladder.

RFLP

PCR products were subjected to restriction digestion with six endonucleases (*Rsa*I, *Alu*I, *Hinf*I, *Hha*I, *Msp*I, and *Hae*III). The selection of above mentioned endonucleases was based on documented polymorphism of their restriction sites in a genetic variation study (Menezes *et al.,* 2006) in skipjack tuna *Katsuwonus pelamis*. In present study, finally four enzymes (*Rsa*I, *Alu*I, *Hinf*I, *Hha*I) were found to be informative, producing variable banding patterns. Restriction digestion was carried out in a final volume of 10 µl containing 2 µl of PCR product, 2 units of restriction enzyme, 1 µl of the appropriate buffer and 7 µl of milliQ water. Reactants were incubated at 37°C for five hours. The digested mtDNA samples were resolved on a 2.5% agarose gel and stained with ethidium bromide. Gels were then viewed under UV transilluminator and photographed for further analysis.

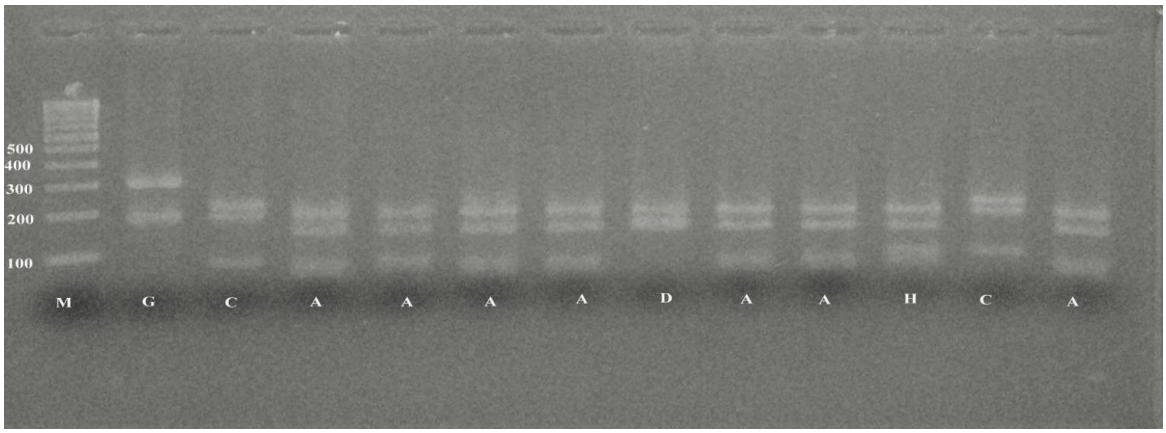
Data Analysis

Variable restriction patterns (haplotypes) generated by each endonuclease were designated by a capital letter in order of detection. Composite haplotypes were constructed from four letters corresponding to the haplotypes of *Rsa*I, *Alu*I, *Hinf*I, and *Hha*I respectively. Data analysis was initiated by preparing a matrix of data, which includes presence or absence of different restriction fragment patterns found with respect to each endonuclease. The presence of a restriction site was coded as "1" and its absence as "0". Matrix data were used to analyze the genetic divergence between *A. thazard* and *A. rochei* by Arlequin version 3.5 (Excoffier and Lischer, 2010). Genetic diversity in each species was measured as haplotype diversity (*hd*), and nucleotide diversity (π) (Nei, 1987). The extent of genetic differentiation between samples was estimated using the fixation index F_{ST} (Wright, 1951). Using *F* statistic, analysis of molecular variance (AMOVA) was performed to examine the amount of genetic variability partitioned between and within species (Excoffier *et al.,* 1992). The significance of variance components were tested using 1,000 permutations. The null hypothesis of neutral evolution was tested using Tajima's *D* test (Tajima, 1989) and Fu's *Fs* test (Fu, 1997) with 1,000 permutations. Arlequin was also used to calculate the historic demographic parameters θ_0 (population before expansion), θ_1 (population after expansion) and τ (relative time since population expansion). Tau (τ) value was transformed to estimate the actual time (*T*) since population expansion using formula $T = \tau/2\mu$ where µ is per generation probability of a mutation anywhere in the sequence. In present study, the mutation rate of 3.6×10^{-8} mutations per site and year was applied for the control region sequence of *A. thazard* and *A. rochei* as this rate has been reported for the mtDNA control region in teleosts (Donaldson and Wilson, 1999). The exact test of population differentiation based on haplotype frequencies was carried out in Arlequin. Statistical significance was estimated via 100,000 Markov Chain Monte Carlo simulations as proposed by Raymond and Rousset (1995). To examine phylogenetic relationships among haplotypes, a minimum spanning network was constructed using HapStar Version 0.5 (Teacher and Griffiths, 2011).

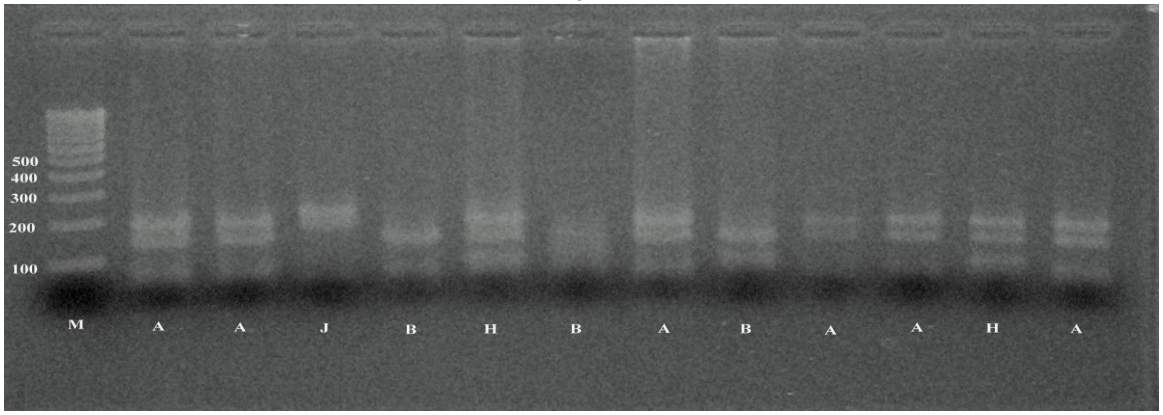
Results

Genetic Divergence

RFLP analysis of control-region DNA with restriction enzymes *Rsa*I and *Alu*I generated ten and seven haplotypes respectively while three haplotypes were produced by each *Hinf*I and *Hha*I (Figure 1 (a-i)). A total of 32 composite haplotypes was identified by combining haplotype designations for four



a



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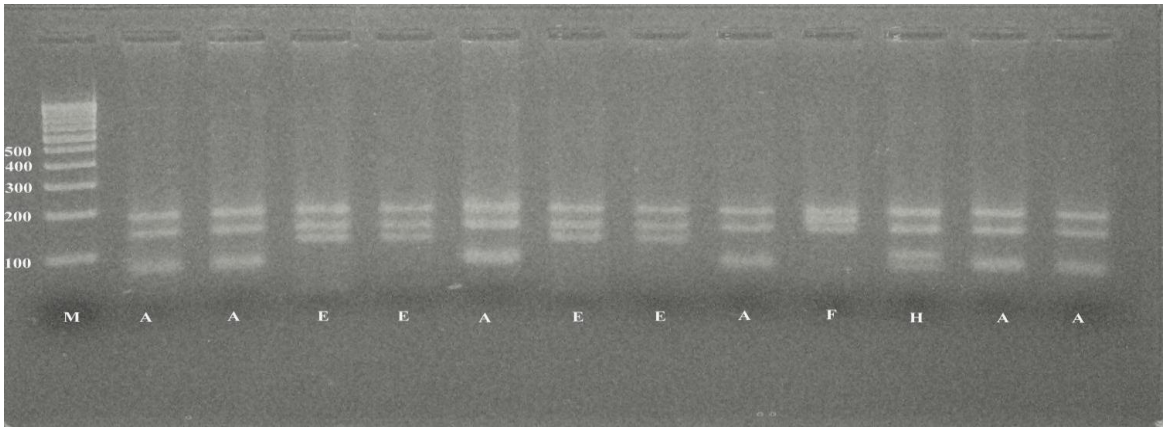
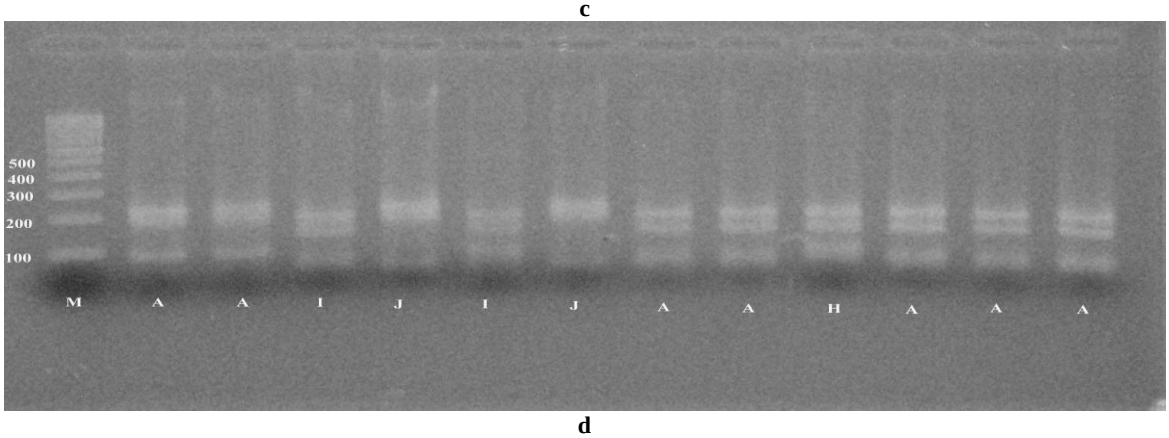
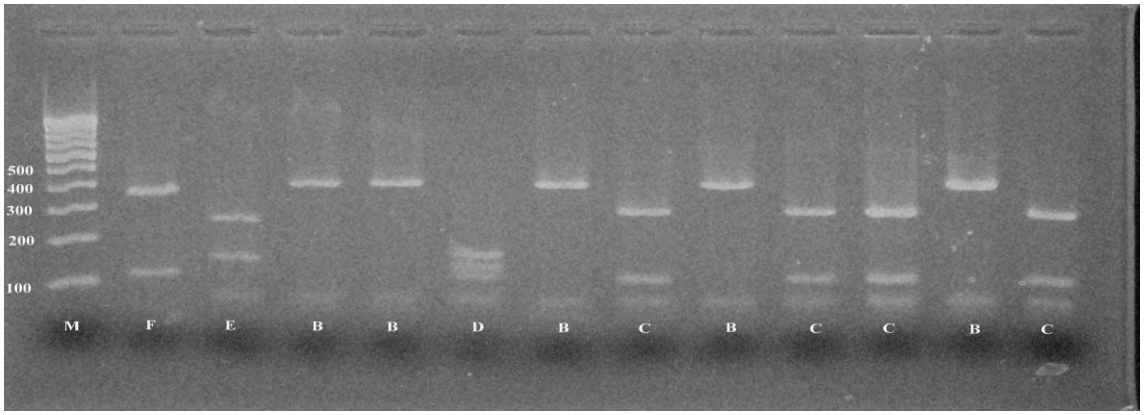


c

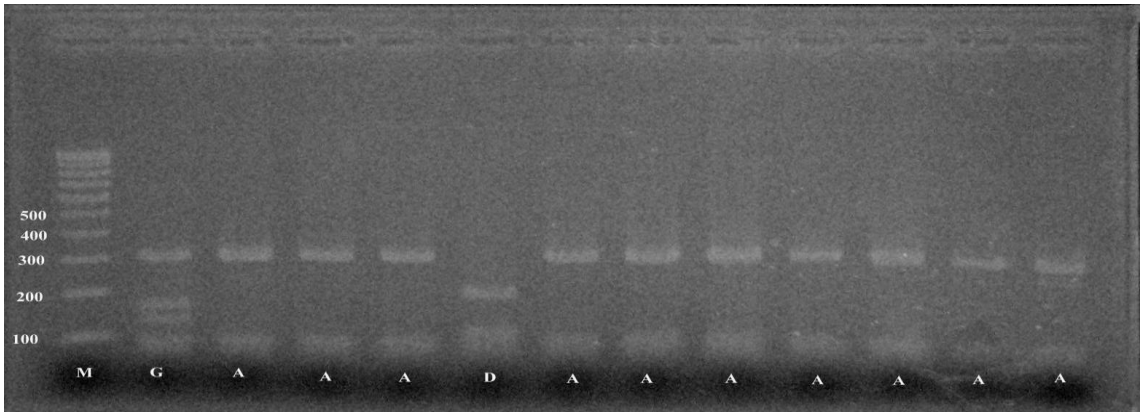


d

Figure 1 (a-d) shows the restriction digestion of mtDNA (D-loop) of *Auxis* spp. with enzyme *Rsa*I. Where M is the 100 bp DNA marker and A, B, C, D, E, F, G, H, I, and J are different haplotypes.



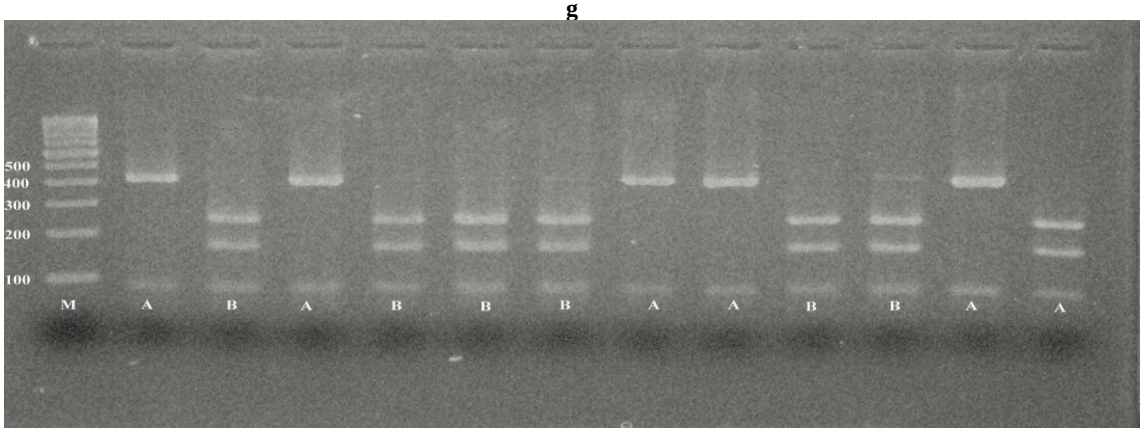
e



f

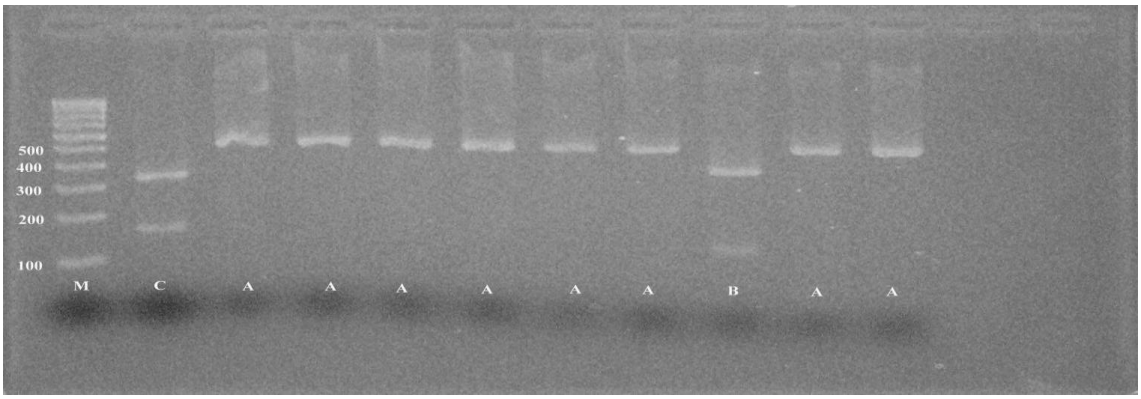
Figure 1 (e-f) shows the restriction digestion of mtDNA (D-loop) of Auxis spp. with enzyme AluI. Where M is the 100 bp DNA marker and A, B, C, D, E, F, and G are different haplotypes.

g



h

Figure 1 (g-h) shows the restriction digestion of mtDNA (D-loop) of *Auxis* spp. with enzyme *Hinf*I. Where M is the 100 bp DNA marker and A, B, and C are different haplotypes.



i

Figure 1 (i) shows the restriction digestion of mtDNA (D-loop) of *Auxis* spp. with enzyme *Hha*I. Where M is the 100 bp DNA marker and A, B, and C are different haplotypes.

polymorphic restriction enzymes surveyed among the 100 individuals (Table 1). Composite haplotypes AAAA and HAAA were the most common, occurring in 23% and 11% of individuals respectively. Seven haplotypes were common between the two species while 6 haplotypes of *A. thazard* and 19 haplotypes of *A. rochei* were unique to their respective individuals. Both nucleotide and haplotypes diversities were higher in *A. rochei* (π = 3.8808; *hd* = 0.9641) compared to *A. thazard* (π = 2.2514; *hd* = 0.8441) (Table 2). A total of 19 polymorphic sites were observed in *A. rochei* while 15 sites were polymorphic in *A. thazard*. The AMOVA analysis revealed significant genetic divergence (F_{ST} = 0.0926; P < 0.001) between the two species. The variation attributed to between species haplotype frequency differences was 9.26% while variation within species contributed 90.74% of the total variance (Table 3). The exact test of population differentiation gave P value of 0.00150 ± 0.00097, indicating that the composite haplotypes were not distributed randomly with respect to species. A minimum spanning network indicates that haplotypes are closely related, with multiple, rare haplotypes radiating from more common (higher frequency) haplotypes (Figure 2).

Historic Demography

Neutrality tests were used as an indication of recent population expansion. Strongly negative and significant values suggest recent population expansion or selection. Fu's *F*s statistics were negative and significant for *A. thazard* (-3.9991, P<0.05) as well as for *A. rochei* (-15.9287, P<0.001) (Table 4). Large differences were observed between θ_0 and θ_1 (Table 4). Although, Tajima's *D* values were negative they were not significant at *P*<0.05 for both species (Table 4). The tau (τ) value for *A. thazard* and *A. rochei* was 2.4180 and 4.1309 respectively, which corresponds to a time of founding of *Auxis* spp. in the Indian Ocean. Corresponding to tau value, founding of *A. thazard* in Indian Ocean took place about 67,167 years (40,083 to 87,250 years, 95% confidence interval) before present while *A. rochei* was founded about 114,747 years (81,750 to 140,889 years, 95% confidence interval) before present based on present study.

Discussion

The RFLP analysis of the mtDNA D-loop region revealed a high level of genetic divergence between the two species (*A. thazard* and *A. rochei*) examined. The level of divergence observed in present study (9.26%) is in general agreement with those proposed by Gonzalez-Villasenor and Powers (1990) between various fish species. Results of present study are also supported by inter-specific divergences observed for three species of *Scomber* (Scoles *et al.*, 1998) and *Aulostomus* (Bowen *et al.*, 2001). A higher degree of haplotype and nucleotide diversity is expected in an ancestral population (Nei, 1973; Nei and Tajima, 1981). In this study, we found greater degree of both haplotype and nucleotide diversity in *A. rochei*, suggesting that *A. rochei* is older ancestral species and *A. thazard* the more recent species.

To examine the evolutionary forces acting on the samples of *A. thazard* and *A. rochei* neutrality test statistics (Tajima's *D* and Fu's *Fs*) were used. Tajima's *D* test is more sensitive to selection while Fu's *Fs* test is more sensitive to population fluctuation. Negative and significant value observed for Fu's *Fs* test suggests a past population expansion for studied species. Past population expansion was also consistent with large differences observed between θ_0 and θ_1. Negative but non-significant values obtained for Tajima's *D* test suggest that control region (D-loop) sequences in studied samples are not under selection. The putative time of population expansion was estimated from the tau (τ) statistic. Corresponding to tau value of 2.4180, population expansion of *A. thazard* estimated to begin about 67,167 years before present while value of 4.1309 reflects 114,747 years of population history of *A. rochei* in Indian Ocean. The long evolutionary history of *A. rochei* further corroborates the hypothesis that *A. rochei* is older than *A. thazard*.

Based on the RFLP analysis, we can conclude that *A. rochei* represent the ancestral species of genus

Table 1. Haplotype frequency of *A. thazard* and *A. rochei* based on mtDNA D-loop region RFLP data

Number	Haplotypes	Frequency	
		A. thazard	*A. rochei*
m1	AAAA	0.17	0.06
m2	AAAB	0.01	0.01
m3	AAAC	0.0	0.01
m4	AABA	0.0	0.04
m5	ABAA	0.0	0.03
m6	ABBA	0.0	0.01
m7	ACAA	0.03	0.03
m8	ACAB	0.0	0.01
m9	ADAA	0.03	0.02
m10	ADBA	0.0	0.01
m11	ADCA	0.01	0.0
m12	AEAA	0.0	0.01
m13	AFBA	0.0	0.01
m14	AGAA	0.01	0.0
m15	BAAA	0.03	0.02
m16	CAAA	0.03	0.03
m17	CABA	0.01	0.0
m18	CCAA	0.0	0.01
m19	DBAA	0.0	0.02
m20	DBBA	0.0	0.02
m21	DCAA	0.0	0.01
m22	DCBA	0.0	0.03
m23	EAAA	0.0	0.01
m24	EABA	0.0	0.02
m25	ECAA	0.0	0.01
m26	FABA	0.0	0.01
m27	GBBA	0.0	0.01
m28	HAAA	0.07	0.04
m29	HABA	0.0	0.01
m30	HDAA	0.01	0.0
m31	IAAA	0.02	0.0
m32	JAAA	0.07	0.0

Table 2. Estimates of genetic diversity of *A. thazard* and *A. rochei* for RFLP data: number of samples (*n*); number of haplotypes (*nh*); haplotype diversity (*hd*); polymorphic sites (*ps*); and nucleotide diversity (π)

Variables	*A. thazard*	*A. rochei*
n	50	50
nh	13	26
hd	0.8441	0.9641
ps	15	19
π	2.2514	3.8808

Table 3. Results of analysis of molecular variance (AMOVA) testing genetic structure of *A. thazard* and *A. rochei* based on mtDNA D-loop region RFLP data

Source of variation	Variance	Percentage of variation	*F*- statistic	P-values
Among species	0.1565	9.26	F_{ST} = 0.0926	P = 0.0000
Within species	1.5331	90.74		

Auxis. However, this conclusion is based on only four restriction enzymes and 23 nucleotide sites. Therefore, the results should be complemented with more restriction enzymes as additional enzymes may detect additional variable cut sites. Since, RFLP is single nucleotide polymorphism assay of limited number of restriction nucleotide mutation sites; it has limited resolving power of genetic analysis. To increase the resolving power of genetic divergence, sequencing of mtDNA should be used in future study.

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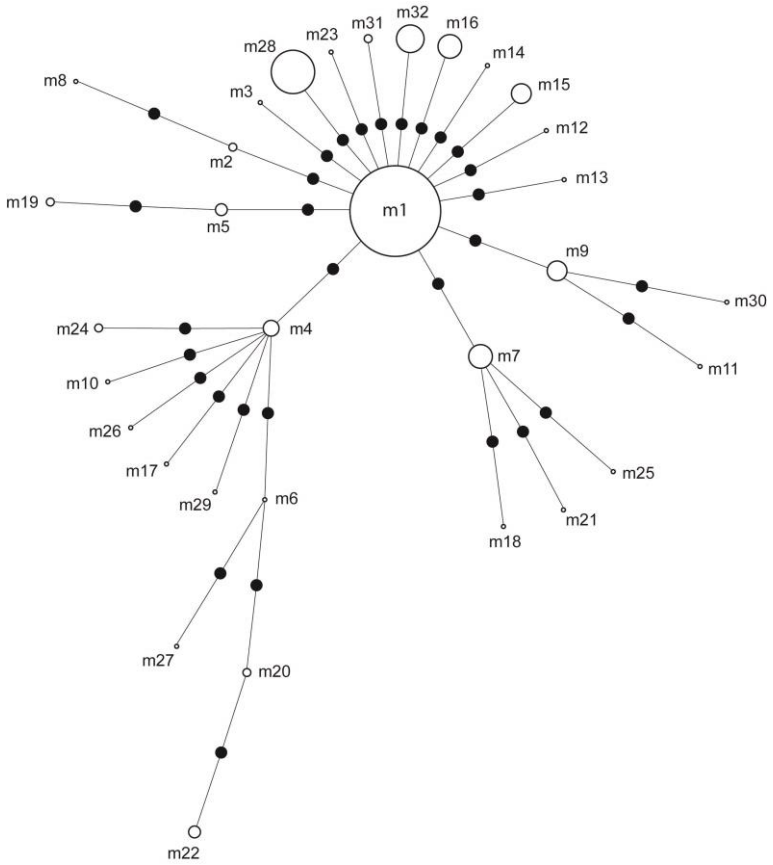

Figure 2. Minimum spanning network showing relationships among 32 mitochondrial DNA control region haplotypes. The diameters of circles are proportional to haplotype frequency.

Table 4. Historic demographic parameters of *A. thazard* and *A. rochei* based on mtDNA D-loop region RFLP data: relative time since population expansion (τ); population before expansion (θ_0); population after expansion (θ_1); Tajima's *D* test and Fu's *Fs* values

Demographic parameters	*A. thazard*	*A. rochei*
τ	2.4180	4.1309
θ_0	0.0018	0.0035
θ_1	99999	99999
Tajima's *D*	-1.0050	-0.2697
Fu's *Fs*	-3.9991*	-15.9287***

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

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