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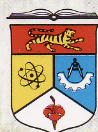

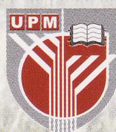

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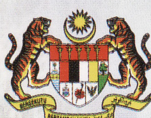

Genetic identification of four Malaysian mackerel species off Coast of Peninsular Malaysia based on molecular marker

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Abstract

Random Amplified Polymorphic DNA (RAPD) markers and cytochrome *b* (Cyt-*b*) gene sequences were utilized to fingerprint and construct phylogenetic relationships among four species of mackerel commonly found in the Straits of Malacca namely *Rastrelliger kanagurta, R. brachysoma, Decapterus maruadsi* and *D. russelli.* The UPGMA dendogram and genetic distance clearly showed that the individuals clustered into their own genus and species except for the *Decapterus*. These results were also supported by partial mtDNA cytochrome *b* gene sequences (279 bp) which found monotypic sequence for all *Decapterus* studied. Cytochrome b sequence phylogeny generated through Neighbor Joining (NJ) method was congruent with RAPD data. Results showed clear discrimination between both genera with average nucleotide divergence about 25.43%. This marker also demonstrated *R. brachysoma* and *R. kanagurta* as distinct species separated with average nucleotide divergence about 2.76%. However, based on BLAST analysis, this study indicated that the fish initially identified as *D. maruadsi* was actually *D. russelli*. The results highlighted the importance of genetic analysis for taxonomic validation, in addition to morphological traits.

Introduction

Carangidae and scombridae are two closely related families (Mansor *et al*., 1998). Among members of these families, *Decapterus* (carangidae) and *Rastrelliger* (scombridae), two genera of mackerels are some of the most exploited pelagic fish as it is a low priced source of animal protein and commonly used as fish live bait (Froese & Pauly, 2009). The chub mackerel, *Rastrelliger* is considered to be highly variable with a reported number of ten species but which have now been reduced to synonyms of three species; *R. kanagurta*, *R. brachysoma* and *R. faughni* (Chee, 2000). These species are found in the Indo-West Pacific with *R. kanagurta* introduced into Mediterranean waters through the Suez Canal. However *R. brachysoma* and *R. faughni* distribution are restricted to the central region (FAO, 2008, Froese & Pauly, 2009). All three species can be found in the Malacca straits with *R. kanagurta* and *R. brachysoma* the predominant species. *Rastrelliger faughni* has been reported in the Malacca Straits but not highly commercially caught (FAO, 1987; Khoo, pers. Comm.). *Rastrelliger brachysoma* can be identified and separated from other members of its genus by its deep body and absence of dusky stripes along the sides of the body. *Rastrelliger kanagurta* can be identified by its dusky stripes, slender body with more than 30 gill rakers present (Mansor *et al.,* 1998; FAO, 2008). Based on recent published literature, studies of *Rastrelliger* are only based on population, ecological, morphological characters and biological features but to date little or no genetic information exists on inferences between species. *Decapterus* generally referred as scads, round scads and mackerel scads can be divided into *c.* twelve species; *Decapterus akaadsi, D. koheru, D. kurroides, D. lajang, D. macarellus, D. macrosoma, D. maruadsi, D. muroadsi, D. punctatus, D. russelli, D. scombrinus* and *D. tabl* (Froese & Pauly, 2009). The distribution of this genus is circumglobal of deep water, temperate, tropical and subtropical seas (Atlantic and Pacific water) (Froese & Pauly, 2009, FAO, 2008). *Decapterus maruadsi* and *D. russelli* are two interesting species to study because according to an FAO report (1987) report, the status of its occurrence and identification in the Malacca Straits is still controversial. The report indicated that *D. maruadsi* may not occur in the Malacca Straits but the obtained species are *D. russelli* morphotypes. Traditionally, both species can be morphologically separated by body depth, and predorsal scales (Mansor *et al.,* 1998). The aims of the study were twofold: to clarify taxonomic status of the sampled species (*R. kanagurta, R. brachysoma, D. maruadsi,* and *D. russelli*) and to assess the phylogenetic relationships among the species using two molecular markers.

Materials and Methods

Fifty three individuals from *Rastrelliger kanagurta,* twenty four from *R. brachysoma,* thirteen from *Decapaterus maruadsi* and four from *D. russelli* were collected along the West Coast of Peninsular Malaysia. Genomic DNA was extracted using Genispin™ Tissue DNA Kit (BST Tech Laboratory). Of twenty decamer oligonucleotides from Operon Technology Kit C (OPC), four, namely OPC 05, OPC 06, OPC 08 and OPC 15 were selected for the subsequent analysis. Amplification reactions were performed in a total volume of 25.0µl using 0.8X PCR Buffer, 4.0mM $MgCl_2$, 0.2mM dNTP, 0.02µM primer, 0.08U *Taq* polymerase and 20-25ng of DNA template. Amplification involved 35 cycles at 94^0C for 30 seconds, 36^0C for 30 seconds, 72^0C for 60 seconds and finally 120 seconds of final extension at 72^0C. DNA amplification products were separated in 2.0% agarose gels at 100V with TBE buffer. For all primers, presence (1) or

absence (0) of a fragment was scored and the species/genus-specific diagnostic markers were defined. Data analysis was performed with RAPDistance Package version 1.04 software. Twenty two specimens (seven from *R. kanagurta*, five from *R. brachysoma*, six from *D. maruadsi* and four from *D. russelli*) were selected for mtDNA analysis. The primers L14841 and H15149 (Kocher *et al.*, 1989) was used to amplify partial cyt-*b* gene by PCR. Amplification was carried out in 25µl reaction mixture containing 1.25µl template DNA, 1.0X PCR buffer, 3.5mM $MgCl_2$, 0.2mM dNTPs, 0.02 µM each primer and 0.08U *Taq* DNA polymerase. PCR was performed with the following profile: initial denaturation at 98°C for 1 minute followed by 35 cycles consisting of 95°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes and finally final extension at 72°C for another 2 minutes. PCR products were purified using QIAquick PCR purification (Qiagen) and sequenced on an ABI3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). *Siganus canaliculatus (*DQ274055), a sister taxon to both families was selected as an outgroup. All sequences were viewed, edited and aligned using MEGA version 4.0 (Tamura *et al*., 2007). Phylogenetic was carried out using neighbour-joining (NJ) (MEGA ver. 4.0) with confidence limits assessed using bootstrap procedure with 1000 replicates. Nucleotide pairwise differences were estimated using Kimura 2-parameter distance model implemented in the same software. All haplotypes have been deposited in GenBank under accession numbers EU170507-EU170522 and FJ375335-FJ375340.

Results and discussion

The four primers tested gave unique visible RAPD fragment patterns (Figure 1a). Each genus or species except *D. maruadsi* and *D. russelli* presented distinct RAPD patterns. Depending on the primer used, 9 to 12 loci were selected with a total of 42 loci ranging in size, from approximately 200bp to 1350bp. Four diagnostic markers consisting of three genus-specific markers as well as a single species-specific marker were observed. However, only OPC-08 can be used in discriminating among the species. The UPGMA dendogram (Figure 1b) generated from RAPD data showed the separation of the two genera, the first main cluster contained seventy seven individuals of *Rastrelliger* and the other main cluster was of *Decapterus* only. The first cluster branched into to two smaller clusters, divided between individuals of *R. kanagurta* and *R. brachysoma*. However, the second main cluster collacated the two species of *Decapterus* ; *D. maruadsi* and *D. russelli,* which showed that the individuals of each presumed species did not cluster together.

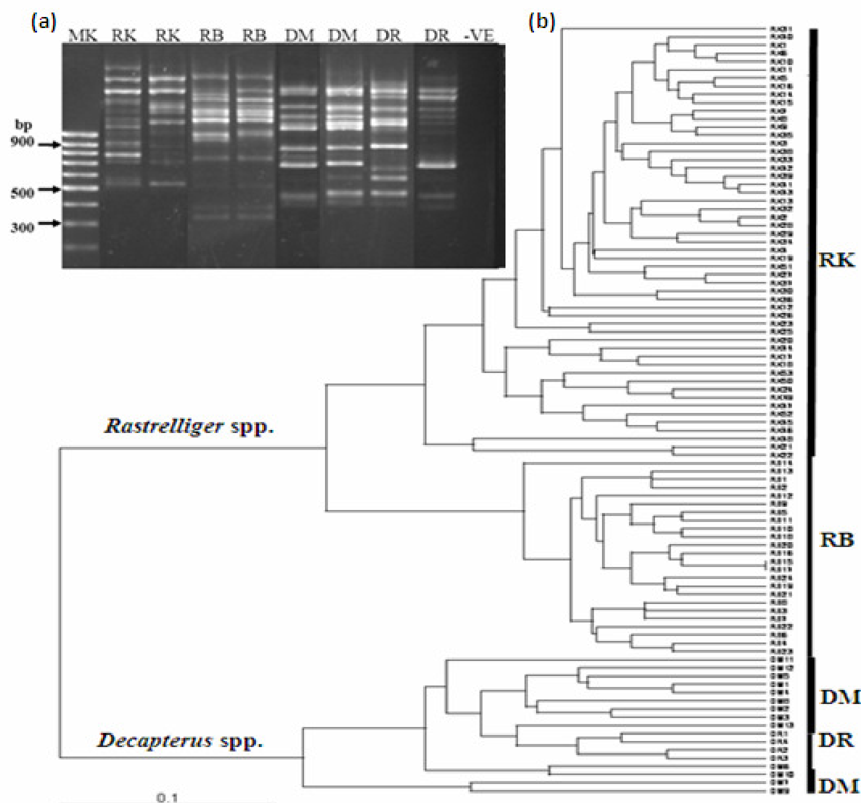

Figure 1a. UPGMA dendrogram generated from RAPD data showing the genetic relationships of 94 individuals from four species studied based on Nei & Li's (1979) coefficient.

b. RAPD banding pattern amplified by OPC-05. RK (*R. kanagurta)*; RB (*R. brachysoma),* DM (*D. maruadsi)*, DR (*D. russelli)*; (MK) GeneRuler 100bp DNA ladder; (-VE) negative control.

The average genetic distance (GD) value was calculated based on the combined four primers (Table 1). For intraspecies comparison, *D. maruadsi* showed the highest GD at 0.3011 while *D. russelli* had the lowest with an average of 0.1531. For interspecies comparison, the GD between *Rastrelliger – Decapterus* ranged from 0.6278 to 0.7213. The GD between *R. kanagurta* - *R. brachysoma* was 0.4064. Interestingly, *D. maruadsi – D. russelli* showed GD with an average of 0.3037 similar to values of intraspecies comparison in *D. maruadsi* (0.3011).

Table 1 Average of genetic distance (interspecies and intraspecies). (RK) *R. kanagurta*; (RB) *R. brachysoma;* (DM) *D. maruadsi*; (DR) *D. russelli*.

Species	RK	RB	DM	DR
RK	0.2129			
RB	0.406	0.165		
DM	0.6435	0.627	0.3011	
DR	0.677	0.721	0.3037	0.153

Of the 279 bp Cyt *b* sequences and 22 samples available for analysis, a total of seven haplotypes were revealed (five for *R. kanagurta*, one for *R. brachysoma*, one from the putative species – *D. maruadsi/D. russelli*). All sequences have been deposited and available in GenBank with accession numbers EU170507-EU170522 and FJ375335-FJ375340. It is commonly known that the nucleotide composition of the cyt-*b* gene is G-deficient (17.2%) in contrast with the other three nucleotides (24.2% to 29.0%). This study found monotypic sequence for *Decapterus*. Excluding the outgroups, of all four species revealed 61 polymorphic nucleotides with 11 polymorphic sites within *Rastrelliger* and none in *Decapterus*. Pair-wise distance (Table 2) between the two *Rastrelliger* varied from 0.0260 to 0.0299. Genetic distances between these two mackerel families were from 0.2503 to 0.2563. Dendogram (Figure 2) generated from neighbor joining showed two distinct groups. The first clade (A), *R. brachysoma* and *R. kanagurta* formed a sister taxon relationship and were then joined to the *D. maruadsi/D. russelli* cluster (clade B). This critical node received 100% unequivocal bootstrap support.

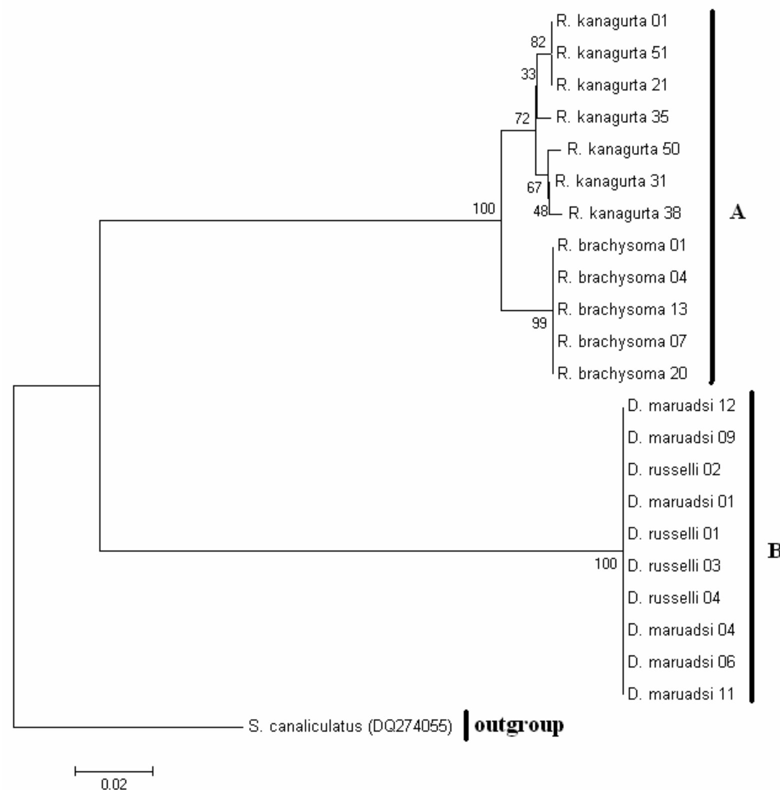

Figure 2 Genetic distannces between individual species; *R. kanagurta, R. brachysoma, D. maruadsi,* and *D. russelli* based on partial cytochrome b gene generated through NJ analysis. Values at nodes represent bootstrap confident level (1000 replicates). Values below 50% are not shown.

	1	2	3	4	5	6
[1] D. Maruadsi / D. russelli						
[2] R. Brachysoma	0.2563					
[3] R. Kanagurta 01	0.2563	0.0260				
[4] R. Kanagurta 31	0.2563	0.0260	0.0073			
[5] R. Kanagurta 35	0.2563	0.0260	0.0073	0.0073		
[6] R. Kanagurta 38	0.2503	0.0299	0.0110	0.0036	0.0110	
[7] R. Kanagurta 50	0.2503	0.0299	0.0110	0.0036	0.0110	0.0073

Table 2 Pair-wise nucleotide distances matrix (K-2-P model) derived from cytochrome *b* gene sequence data

Both genetic analyses supported the classification of *Rastrelliger* and *Decapterus* and the classification of *R. kanagurta* and *R. brachysoma* as two distinct species. Given the lack of support for the identification of *D. maruadsi* and *D. russelli*, a BLAST analysis was conducted to assign to the known species sequences. All presumed *Decapterus* sequences (including those identified as *D. maruadsi*) were found to align to *D. russelli* with 99% homology.

Conclusion

In this study, placement and identification of the presumed *D. maruadsi* and *D. russelli* were unresolved using both markers. It is clear that determination of putative collections of *D. maruadsi* found in the Malacca Straits requires validation. This study indicated that all the *Decapterus* captured in this study was *D. russelli*. However, the presence of *D. maruadsi* cannot be ruled out until further study and more samples can be obtained. Thus, further studies should be carried out to support this preliminary study involving additional species, increasing the sample size and increased sequencing effort will be necessary for a better understanding of the interrelationships within the species. This study give further support for both molecular approaches as efficient markers for taxonomic identification such as in clarifying the status of organisms of controversial systematic status.

Acknowledgement

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