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Mitochondrial DNA analysis of the genetic relationships among populations of scad mackerel (*Decapterus macarellus*, *D. macrosoma*, and *D. russelli*) in South-East Asia

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Abstract The genetic relationships among South-East Asian populations of the scad mackerels *Decapterus macarellus*, *D. macrosoma* and *D. russelli* (Pisces: Carangidae) were investigated. In 1995 and 1996, 216 fish were sampled in seven localities spanning the seas of Indonesia and were examined for restriction-site polymorphisms using ten restriction enzymes for the mitochondrial (mt) DNA control region, amplified by the polymerase chain-reaction. The inferred phylogeny of haplotypes led to the recognition of three distinct mitochondrial lineages or phylads consistent with the distinctions of current taxonomy. All 15 mtDNA haplotypes found in *D. macarellus* and all 9 haplotypes found in *D. macrosoma* were arranged as star-like clusters, suggesting recent evolutionary history. In contrast, the phylad formed by 6 haplotypes in *D. russelli* from the Sulawesi Sea exhibited diffuse topology, suggesting that ancestral lineages of this species have been retained to the present. Average nucleotide-divergence estimates between haplotypes of different phylads were between 0.042 and 0.135, suggesting ancient separation, in consistency with published allozyme data. High levels of haplotype diversity, but no geographical heterogeneity, was detected within *D. macarellus* from the Molucca Sea and the Banda Sea. Populations of *D. macrosoma* exhibited both significant differences between adjacent regions (Sunda Strait and Java Sea), and broadscale genetic homogeneity from the South China Sea to the Sulawesi Sea via the Java Sea and Makassar Strait. The geographic isolation of the *D. macrosoma* population sampled in the Sunda Strait suggests that this region constitute a sharp transition zone between the Indian

Ocean and the Sunda Shelf. Near-monomorphism of haplotypes and low nucleotide diversity (d_X) were observed in the samples of *D. macrosoma* from the continental shelf (haplotype-diversity estimates, h , = 0.00 to 0.25 ± 0.08 and d_X = 0.000 to 0.002). This was in contrast to the comparatively high haplotype and nucleotide diversities observed in other pelagic fish species including *D. macarellus* (h = 0.82 ± 0.05 , d_X = 0.012 to 0.015) and *D. russelli* (h = 0.63 ± 0.12 , d_X = 0.016), and in the oceanic *D. macrosoma* population sampled in the Sunda Strait (h = 0.67 ± 0.31 , d_X = 0.005). We hypothesise that this may be the consequence of recent and perhaps repeated bottleneck events that have affected the *D. macrosoma* population sampled on the continental shelf.

Introduction

Mitochondrial DNA (mtDNA) yields genetic information that allows investigators to infer relationships between closely related taxa and the population history of a species (Avice 1994). Most studies of mtDNA polymorphism in pelagic marine fishes have shown low levels of molecular divergence within species compared to those among sister taxa (Grant and Bowen 1998), e.g. sardines (*Sardina pilchardus*, *Sardinops* spp.) and anchovies (*Engraulis* spp.). Genetic information from sardines and anchovies combined with paleoclimate data have revealed the effects of past environmental events on extant patterns of genetic variability and coalescence of mtDNA genealogies (Grant and Bowen 1998). These observations are of considerable evolutionary interest, since they contribute to our understanding of the processes of colonisation, extinction, population differentiation and allopatric speciation in the oceanic environment (Bowen and Grant 1997). However, the question remains as to whether these findings are specific to coastal clupeiforms such as sardines and anchovies, or are more generally applicable to pelagic fishes. Coastal clupeiforms are known to undergo dramatic population-

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size fluctuations over short time-scales in association with the climate-related productivity oscillations of their habitat, the coastal upwelling areas of the mid-latitudes (Cury 1988; Baumgartner et al. 1992). If habitat fluctuations were the main cause of the patterns of molecular divergence reported for sardines and anchovies, then such fluctuations are more likely to be reflected in coastal species, whose habitat has undergone dramatic fluctuation in the Pleistocene, than in oceanic species.

To test this, one must compare species from oceanic and coastal habitats that are genetically as closely related as possible. We investigated the phylogeography of three scad mackerel species (genus *Decapterus*, order Perciformes, family Carangidae). *Decapterus* species are pelagic, with a wide distribution in the tropical Indo-Pacific Ocean (Froese and Pauly 1995). *D. macrosoma* and *D. russelli* inhabit shallow seas, whereas *D. macarellus* prefers clear oceanic waters (Froese and Pauly 1995; Potier and Nurhakim 1995). According to our hypothesis, Pleistocene changes in the habitat should have markedly influenced the phylogenetic architecture of the coastal species *D. macrosoma* and *D. russelli*, but should have had less or no impact on the pelagic species *D. macarellus*.

Decapterus macrosoma and *D. russelli* are among the dozen pelagic finfish species subject to heavy exploitation by purse-seiners in South-East Asia (Widodo 1988; Potier and Boely 1990; Potier and Nurhakim 1995). In the South China Sea and in the Java Sea, catches are seasonal and their onset coincides with changes in monsoon-driven hydrological regimes (Hardenberg 1937; Widodo 1988; Sadhotomo 1998). Hardenberg raised the hypothesis of the seasonal occurrence of three distinct subpopulations or stocks of *D. macrosoma* in the western Indonesian archipelago. Potier and Boely (1990) and Sadhotomo and Potier (1995) subsequently considered that this region possibly harbours two seasonal *D. macrosoma* subpopulations, but in the absence of morphometric or genetic evidence this question remains open.

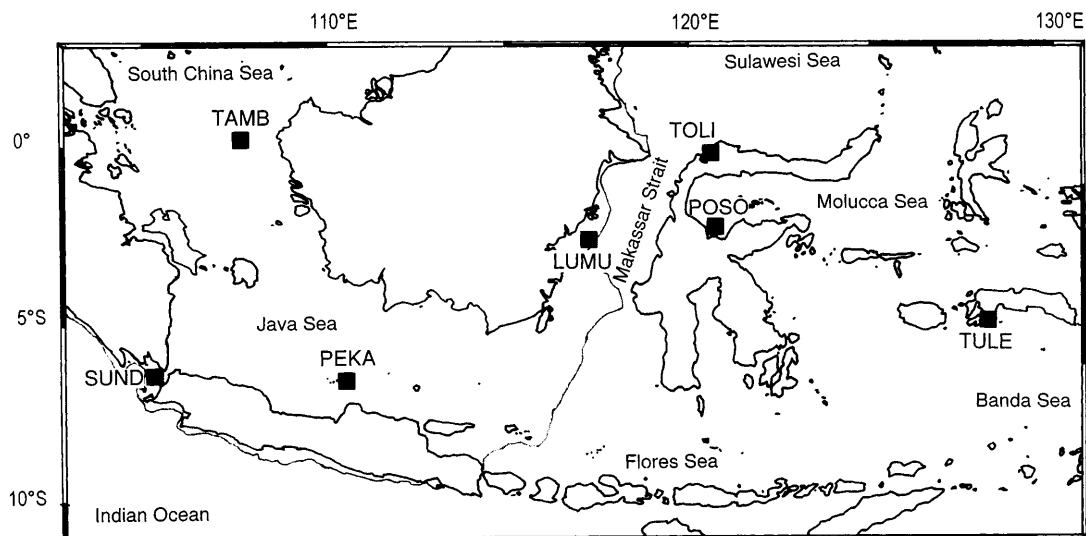
Decapterus macrosoma is often confounded in fisheries statistics with *D. macarellus* or *D. russelli* (e.g. SEAFDEC 1996). Fischer and Whitehead (1974) warned that the names *D. macrosoma* and *D. macarellus* have been interchanged in some instances, and Gushiken (1983) mentioned that they have even been considered synonyms. Confusion in the identification of the two species may reflect morphological overlap, possibly arising from some systematic confusion.

We determined both the genetic relationships among *Decapterus macarellus*, *D. macrosoma* and *D. russelli* and the level of genetic variability and genetic structure within *D. macarellus* and *D. macrosoma* in order to (1) test the impact of past global changes on phylogenetic architecture patterns in pelagic fishes, (2) validate the current taxonomy of the genus *Decapterus*, and (3) assess the possible occurrence of distinct subpopulations in the Indonesian archipelago. For this purpose, we analysed restriction-fragment length polymorphism (RFLP) on a variable region of the mitochondrial (mt) genome to characterise each of these species from samples collected in the Indonesian archipelago.

Materials and methods

Scad mackerels were sampled by Indonesian purse-seiners operating in the areas of the locations in Fig. 1, except at Location TOLI, where the fishes were sampled with a beach seine (sampling dates and sample sizes are given in legend to Fig. 1). The samples were identified as *Decapterus macarellus* Cuvier, 1833 (Samples POSO

Fig. 1 *Decapterus* spp. Collection sites in Indonesian archipelago [TAMB Tambelan, southern South China Sea, 12 April 1995 (sample size, $N = 6$); SUND Labuan, Sunda Strait, 26 May 1995 ($N = 3$); PEKA Pekalongan, Central Java, Java Sea, 12 April 1995 ($N = 37$); LUMU Lumu-Lumu, East Kalimantan, Makassar Strait, 10 February 1995 ($N = 51$); TOLI Toli-Toli, North Sulawesi, Sulawesi Sea, 26 May 1995 ($N = 46$); POSO Poso, Tomini Bay, Molucca Sea, 24 May 1995 ($N = 38$); TULE Tulehu, Ambon, Banda Sea, February 1996 ($N = 35$); thin line approximate 200 m depth contour delimitating Sunda Shelf] Map adapted from Anonymous (1989)



and TULE), *D. macrosoma* Bleeker, 1851 (Samples TAMB, SUND, PEKA, LUMU and TOLI), and *D. russelli* Rüppell, 1818 (Sample TOLI) on the basis of morphology (Gloerfelt-Tarp and Kailola 1984; Smith-Vaniz 1984). In TOLI, *D. macrosoma* and *D. russelli* occurred together in the same school (Sadhotomo personal communication).

A piece of muscle was dissected from each fish and stored in 95% ethanol until processed in the laboratory. Genomic DNA was obtained from ~5 mg of air-dried, macerated muscle tissue incubated for 5 to 10 h at 55 °C in an extraction buffer of 1 ml 0.02 M Tris-HCl, 0.02 M EDTA, 10% sodium dodecyl sulphate, pH 8.0, to which 1 mg proteinase K was added. This was followed by a two-cycle phenol-chloroform extraction procedure (Sambrook et al. 1989). The nucleic acid pellet was resuspended in deionised water and frozen at -20 °C for later use as template for the polymerase chain-reaction (PCR).

The entire replication control region of the mtDNA was PCR-amplified using Primers HN20 (5'-GTGTTATGCTTTAGT-TAAGC-3') and LN20 (5'-ACCACTAGCACCCAAAGCTA-3') designed from a sequence of European brown trout, *Salmo trutta* (Bernatchez et al. 1992; Bernatchez and Danzmann 1993). This pair of primers was also used to amplify the mtDNA control region in the Spanish sardines *Sardinella aurita* and *S. maderensis* (Chikhi 1996). A second pair of primers [DLEND (5'-GCGGATACTTGTCATGTGTAAG-3') and LN20Ms (5'-ACCACTCGCTCCCAAAGCCA-3')] was later designed from a sequence of the mtDNA control region of North-American seabass *Morone saxatilis* (J.E. Stabile and I.I. Wirgin unpublished data; GenBank Access No. L60529). PCRs using this new set of primers appeared to be more consistently positive and to yield larger quantities of DNA than the former. Hence, a new series of PCRs were run on the samples for an extension of the RFLP analysis using four new restriction enzymes, in addition to the six endonucleases of the first digestion series (listed in following paragraph). Thirty-five PCR cycles (94 °C for 1 min; 48 °C for 1 min; 72 °C for 1 min) with a final extension step of 72 °C for 5 min were run in a Crocodile III thermocycler (Appligène, Strasbourg, France) on a 62 ml reaction mixture containing ~5 to 20 ng genomic DNA, 0.65 mM primers, 2.5 mM MgCl₂, 1.3 mM dNTPs and 1 U *Taq* polymerase.

PCR products were aliquoted, and each aliquot was digested by one of the endonucleases *DdeI*, *HpaII*, *TaqI*, *MluI*, *XhoI* and *HinfI* (first digestion series on HN20-LN20 PCR products) and *HaeIII*, *RsaI*, *AluI* and *EcoRI* (new digestion series on DLEND-LN20Ms PCR products) according to the manufacturers' (Appligène, Strasbourg, France; Eurogentec, Liège, Belgium) instructions. All restriction enzymes were chosen without prior knowledge of site polymorphism in *Decapterus* spp. Digested DNA samples were electrophoresed on ethidium bromide (EtBr)-stained 2% agarose gel, and were photographed under ultraviolet light. The sizes of the DNA fragments were compared to the 100-base pair (bp) ladder of Pharmacia Biotech (Uppsala, Sweden) electrophoresed on the same gel.

Composite haplotypes were thus obtained from ten separate restriction profiles for each fish.

The presence or absence of restriction sites were deduced from the RFLP patterns obtained with each restriction enzyme. Maximum-likelihood (ML) and Wagner-parsimony analyses were conducted on the matrix of haplotypes × restriction sites using the RESTML and MIX procedures of the PHYLIP 3.57c computer package (Felsenstein 1995). While the Wagner-parsimony algorithm searches for the minimum total number of steps in the tree without any assumption on rates of evolution, the ML algorithm used here maximizes the likelihood of branch lengths (and chooses the topology which shows the highest likelihood value) under the probabilistic model that the expected rate of substitution is the same for each site. The level of homoplasy (reversion, parallelism, convergence) was assessed by comparing the number of steps required under the parsimony algorithm to the number of variable sites; these two values should be equal in a fully consistent set; i.e. without homoplasy. The robustness of the parsimony tree was checked by counting the frequency of occurrence of each node from

a set of 100 pseudotrees generated by bootstrap resampling (Felsenstein 1985) using the SEQBOOT and CONSENSE procedures of PHYLIP. The suboptimal ML tree topologies were examined with the help of the USERTREE option of PHYLIP, as advised by Felsenstein (1995).

Haplotype-frequency differences between populations were estimated using Weir and Cockerham's (1984) θ , the correlation of alleles at a locus within subpopulations relative to the total population. Here, haplotypes were treated as alleles at a single locus. Calculations were performed using the FSTAT procedure of GENETIX (Belkhir et al. 1996). Significance tests compared the θ -estimate of actual data with 1000 pseudo- θ generated by random permutations using GENETIX.

Haplotype (h) and nucleotide (d_x) diversities within samples, and nucleotide divergences between samples (d_{xy}), were estimated with the REAP package (McElroy et al. 1992), which uses the formulations of Nei and Tajima (1981) and Nei (1987).

Results

PCR amplification of the control region in all 216 mtDNA sampled in *Decapterus macarellus*, *D. macrosoma*, and *D. russelli* yielded a single DNA product of ~980 bp with Primers HN20 and LN20, or ~1080 bp with Primers DLEND and LN20Ms. Checks for PCR-amplification on EtBr-stained agarose gels revealed no size polymorphism. Restriction analysis of the 980 bp fragment using endonucleases *DdeI*, *HpaII*, *TaqI*, *MluI*, *XhoI* and *HinfI* and of the 1080 bp fragment using endonucleases *HaeIII*, *RsaI*, *AluI* and *EcoRI* resulted in the restriction patterns in Table 1. Composite haplotypes were then recorded for each individual fish (Table 2).

ML analysis was carried out on the presence/absence matrix of restriction sites deduced from the RFLP patterns observed in the samples (Tables 1 and 2). Eighteen variable characters were present, and the total number of mutational steps in the most-probable ML tree (not shown) was 38, indicating a high level of homoplasy. All *Decapterus macarellus* haplotypes were closely related: they clustered together in a star-like fashion at one end of the network, with Haplotype AABABABCBA (*POS03*) occupying a central position in the cluster. At the other end of the network, all *D. macrosoma* haplotypes, similarly arranged as a star-like cluster, diverged by 1 to 2 mutational steps from AAABAABBCB (*TAMB1*), the most common haplotype in all samples of *D. macrosoma* except SUND. The remainder, all consisting of *D. russelli* haplotypes, were arranged in a diffuse fashion and occupied an intermediate position in the network, being separated by 3 to 8 mutational steps from Haplotype AABA-BABCBA (*POS03*: *D. macarellus*) and by 4 to 9 mutational steps from Haplotype AAABAABBCB (*TAMB1*: *D. macrosoma*). Bootstrap resampling of restriction sites (100 pseudo-samples) indicated that this basic tree architecture was robust. The fact that the central group of *D. russelli* haplotypes did not form a monophyletic cluster, but instead was scattered along the longest central branch of the network (data not

Table 2 *Decapterus* spp. Distribution of composite haplotypes of mitochondrial DNA across samples. Restriction enzymes used to construct composite haplotypes were (left to right): *Dde*I, *Hpa*II, *Taq*I, *Mlu*I, *Xho*I, *Hin*fI, *Hae*III, *Rsa*I, *Alu*I and *Eco*RI [*Abbr.* haplotype abbreviation; $h \pm SD$ = haplotype diversity \pm SD (Nei and Tajima 1981); *sample abbreviations* as in legend to Fig. 1; (*N*) sample size]

Phylad, haplotype	Abbr.	Frequency per sample							Total	
		POSO	TULE	TAMB	SUND	PEKA	LUMU	TOLI		
<i>D. macarellus</i>										
CABABABCBA	POSO1	15	11							26
AABABBBBCBA	POSO2	6	5							11
AABABABCBA	POSO3	3	4							7
CABABBBBCBA	POSO4	3	9							12
AABABABCAA	POSO5	2								2
AABABBBCAA	POSO6	2								2
CABABABCAA	POSO7	2								2
BABABBBCAA	POSO8	1								1
CABABCACBA	POSO9	1								1
CABABCBCBA	POSO10	1								1
AACABABCBA	POSO11	1	1							2
CACABABCBA	POSO12	1	2							3
BABABBBBCBA	TULE1		1							1
AABABCBCBA	TULE2		1							1
ABBABBBBCBA	TULE3		1							1
<i>D. macrosoma</i>										
AAABAABBCB	TAMB1			6		35	44	25		110
AAABBABBCB	SUND1				2					2
AABBBABBCB	SUND2				1					1
AAABACBBCB	PEKA1					1		1		2
AACBAABBCB	PEKA2					1				1
ABABAABBCB	LUMU1						5			5
AAABABBBBCB	LUMU2						2			2
AAABAABBAB	TOLI4							1		1
AAABACBBCA	TOLI6							1		1
<i>D. russelli</i>										
ABBAAABABA	TOLI1								11	11
ABBAAABACA	TOLI2								3	3
AAABAABABA	TOLI3								1	1
ABCAAABABA	TOLI5								1	1
AAABAABACA	TOLI7								1	1
ABBBAABACA	TOLI8								1	1
(<i>N</i>)		(38)	(35)	(6)	(3)	(37)	(51)	(28)	(18)	(216)
<i>h</i>		0.82	0.82	0.00	0.67	0.11	0.25	0.20	0.63	
(\pm SD)		(\pm 0.05)	(\pm 0.04)	(\pm 0.00)	(\pm 0.31)	(\pm 0.07)	(\pm 0.08)	(\pm 0.10)	(\pm 0.12)	

Discussion

The phylogeny inferred from RFLP data on the mtDNA control region in *Decapterus macarellus*, *D. macrosoma* and *D. russelli* featured three clades in accordance with current taxonomy. In particular, the *D. macarellus* and *D. macrosoma* haplotypes both appeared to be organised as monophyletic, star-like clades, each with deep rooting. This erases doubts as to the systematic status of *D. macarellus* vs *D. macrosoma*, whose names in some instances have been interchanged and even been considered synonymous (Fischer and Whitehead 1974; Gushiken 1983). *D. russelli* appeared as a third entity, whose monophyly may however be questionable because of the looseness of the inferred coalescence pattern. This may be a result of homoplasy, but may also reflect the co-occurrence of ancient mitochondrial lineages within extant *D. russelli* populations. Since the number of *D. russelli* haplotypes

sampled so far has been limited ($N = 6$), it is premature to attempt further conclusions on the evolutionary relationships of this species.

The divergence among mtDNA clades (species) appeared to be high (with interclade nucleotide-divergence estimates ranging from 0.042 to 0.125). Correcting for within-clade variation according to the formula $d_{XY}(\text{net}) = d_{XY} - (d_X + d_Y)/2$ (Nei 1987) yielded only slightly lower nucleotide-divergence estimates between clades, which then ranged from 0.027 to 0.117. Conventional mtDNA clock calibrations assume a rate of 2% sequence divergence per million year (MY) between lineages (Avise 1994), but a several-fold slower value has been proposed for poikilotherms (Martin and Palumbi 1993; Avise et al. 1998). Also, one must take into account differences in the rate of molecular evolution between the whole mtDNA molecule (with which the above studies were concerned) and that of the control region. The control region of vertebrates generally

Table 4 *Decapterus* spp. Summary of genetic divergence estimates [d_{XY} , $d_{XY}(\text{net})$] uncorrected and corrected sequence divergence estimates (Nei 1987) between species, respectively, on basis of present results; π_{ij} , $\pi_{ij}(\text{net})$] average pairwise estimates of nucleotide differences (Nei 1987) between cytochrome *b* haplotypes (from unpublished data by C. Perrin and P. Borsa); $D(\text{Nei})$ Nei's (1971)

Species pair	Genetic divergence					Divergence time (MY)		
	d_{XY}	$d_{XY}(\text{net})$	π_{ij}	$\pi_{ij}(\text{net})$	$D(\text{Nei})$	Control R	Cyt <i>b</i>	Allozymes
<i>D. macarellus</i> / <i>D. macrosoma</i>	0.128	0.117	0.093	0.089	1.462	5.9–23.4	4.5–17.8	27.8
<i>D. macarellus</i> / <i>D. russelli</i>	0.042	0.027	0.100	0.086	0.919	1.4–5.4	4.3–17.2	17.5
<i>D. macrosoma</i> / <i>D. russelli</i>	0.096	0.084	0.096	0.086	1.255	4.2–16.8	4.3–17.2	23.8

or foundation event after which the mutation/drift equilibrium has still to be reached (see Nei 1987), or a combination of all three. Both haplotype- and nucleotide-diversity estimates were lowest in continental populations of *D. macrosoma*, suggesting that these were more affected by recent demographic events than oceanic *D. macrosoma* populations and *D. macarellus*. Paleoclimatic data indicate that the sea level rose and fell repeatedly throughout the Pleistocene, leaving the Sunda Shelf above sea level (Tjia 1980). We hypothesise, therefore, that the continental shelf population(s) of *D. macrosoma* have gone through recent and perhaps repeated bottleneck(s).

The geographical analysis of haplotype frequencies also allowed us to test the hypotheses on scad mackerel stock-structure.

The *Decapterus macarellus* samples, which were collected in the Molucca Sea (POSO) and the Banda Sea (TULE), displayed no heterogeneity in haplotype frequencies, implying that gene flow occurs over a broad scale and that a single *D. macarellus* population is present in this area.

Decapterus macrosoma is reported to inhabit high-salinity waters only (> 32‰S; Hardenberg 1937; Potier and Boely 1990). The salinity of the Java Sea is usually < 32‰, but intrusions of high-salinity waters occur seasonally (Hardenberg 1937; Wyrcki 1956; Durand and Petit 1995). During the wet monsoon from January to June, high-salinity waters may enter the north-western part of the Java Sea from the southern South China Sea (Wyrcki 1956). During the dry monsoon from June to September, a reverse phenomenon is observed, with high-salinity waters entering the eastern half of the Java Sea from the adjacent Flores Sea and Makassar Strait (Wyrcki 1956). From this hydrological model, one can hypothesise that two populations of *D. macrosoma* may occur in the area, one inhabiting the high-salinity waters of the South China Sea and perhaps migrating to the north-western part of the Java Sea during the wet monsoon season, the other inhabiting the oceanic waters of the Flores Sea and the Makassar Strait and entering the Java Sea from the east and north-east during the dry monsoon season (Hardenberg 1937; Potier and Boely 1990; Sadhotomo and Potier 1995). These two putative populations are thought to be permanently separated because of the salinity barrier in the Java Sea. Broad-

scale migration patterns in *D. macrosoma* independently inferred from the analysis of demographic data (Sadhotomo and Potier 1995; Sadhotomo 1998) agree with this hypothesis. However, Widodo (1988) noted that the geographical boundaries between the stocks were questionable, and emphasized the possibility of stock admixture in the exploited areas. Hardenberg (1937) also hypothesised that a proportion of the scad mackerels captured in the Java Sea during the wet monsoon originate from the Indian Ocean through the Sunda Strait, but the flow of high-salinity water through the Sunda Strait is likely to be negligible (Wyrcki 1961).

In fact, all three *Decapterus macrosoma* samples from the the Sunda Shelf (TAMB, PEKA, LUMU) and that from the Sulawesi Sea (TOLI) shared the same, common mtDNA haplotype. Haplotype frequencies did not vary significantly among these samples, possibly reflecting the presence of a single population in the whole area. The presence of several subpopulations cannot, however, be excluded, since the lack of genetic differences among samples does not necessarily imply homogeneity, especially when the level of polymorphism is low (as is the case here). Much larger sample sizes would be necessary for a more viable test. However, we expect that any haplotype-frequency differences will be weak.

To summarise, there was no evidence of the presence of two distinct *Decapterus macrosoma* populations on the Sunda Shelf. However, the three individuals comprising the SUND sample (Sunda Strait) were characterised by mtDNA haplotypes that were unique to this sample. Permutation tests demonstrated the probability that this sample were drawn from the same population as that sampled in the adjacent Sunda Shelf to be extremely low, pointing towards the presence of a separate, geographically isolated *D. macrosoma* population in the Sunda Strait. This was unexpected, considering the potential mobility of individuals, at both the egg/larva (Delsman 1926) and adult (Hardenberg 1937; Sadhotomo and Potier 1995) stages.

High levels of gene flow, a recurrent feature of marine fish populations (see Ward et al. 1994), probably account for the low levels of population structure evidenced here in *Decapterus macarellus* and, perhaps, among Sunda Shelf populations of *D. macrosoma*. In contrast, the distinctiveness of the population sampled in the Sunda Strait area indicates the presence of a

separate reproductive area, perhaps a result of past geographic isolation; it also reflects the present restriction to gene flow at the entrance of the Java Sea. Passive mechanisms (e.g. mortality of larvae due to hydrological variations) or active mechanisms (homing of adults) may be responsible for this restricted gene flow.

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