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VERIFICATION OF FOUR SPECIES OF THE MUD LOBSTER GENUS *THALASSINA* (CRUSTACEA: DECAPODA: GEBIIDEA: THALASSINIDAE) USING MOLECULAR AND MORPHOLOGICAL CHARACTERS

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ABSTRACT. — Population and species distinctness among three sympatric species of mud lobsters from Malaysia (*Thalasssina kelanang* Moh & Chong, 2009, *T. anomala* (Herbst, 1804) and *T. gracilis* Dana, 1852) and *T. squamifera* De Man, 1915 from Australia, are verified by discriminant analysis of their morphological traits and molecular gene markers (PEPCK, NaK, and COI). Both methods agree that *T. anomala* and *T. gracilis* are the most distant pair among the four species. Molecular analysis of the combined markers shows that the four species of *Thalassina* belong to a monophyletic clade, and that *T. squamifera* and *T. kelanang* are two closely similar, but distinct, species. Based on the molecular evidence, their distinct movable scaphocerite and existing records of collections, it is hypothesized that *T. squamifera* and *T. kelanang* are two basal sister species separated by Wallace's line to the east and west, respectively.

KEY WORDS. — Malaysia, Central Indo-Pacific, *Thalassina*, molecular gene markers, morphology, distribution

INTRODUCTION

The family Thalassinidae Latreille, 1831, contains one genus *Thalassina* Latreille, 1806, with members, commonly called mud lobsters, widely distributed across the Indo-West Pacific region. This genus had, for some time, been considered monotypic (Glaessner, 1969), but a recent work and review by Ngoc-Ho & de Saint Laurent (2009) recognised seven species. In the latter's study, *Thalassina gracilis* Dana, 1852, collected from Telegraph Island near Singapore and synonymised with *Thalassina anomala* (Herbst, 1804) by De Man (1928), was redescribed and a neotype, collected from Lim Chu Kang mangroves, Singapore, was designated. Ngoc-Ho & de Saint Laurent (2009) further described three new species—*Thalassina spinirostris* (type locality: Lim Chu Kang mangroves, Singapore), *T. spinosa* (type locality: Mentawi Island, Indonesia), and *T. krempfi* (type locality: Saigon, Vietnam). At almost the same time, another new species, *T. kelanang*, collected from Kelanang Beach, Selangor, western Peninsular Malaysia, was described by Moh & Chong (2009).

De Man (1915) described certain unusual specimens of *T. anomala*, collected off Beo, Karakelong Island (northern Sulawesi) during the Siboga Expedition, which had some notable morphological differences, in particular, a distinct, movable scaphocerite, and recognised them as a distinct variety, *T. anomala* var. *squamifera*. Later, Poore & Griffin (1979) redescribed and formally elevated the Australian specimens of *T. anomala* var. *squamifera* De Man, 1915, as a valid species. The extant Australian mud lobsters may bear another species, *T. emerii* Bell, 1844, a fossil species considered extant by Ngoc-Ho & de Saint Laurent (2009) based on their examination of recent specimens (MNHN

Th 1524, MNHN Th 1523, and RMNH D 51758) collected from Australia and Indonesia. However, in the most recent review of the genus, Sakai & Türkay (2012) argue that *T. emerii* is a nomen dubium based on their examination of the recent specimens which, instead, comprised of two new species: *T. australiensis* Sakai & Türkay, 2012 (RMNH D 51758, type locality: Aru Islands, Indonesia; MNHN Th 1523, type locality: NE of Port Hedland, N.W. Australia) and *T. saetichelis* Sakai & Türkay, 2012; NHN-IU-2011-5615 (=MNHN Th1524), type locality: Roebourne, N.W. Australia). Hence, these authors have updated the present number of mud lobster species to nine.

The identification keys by Ngoc-Ho & de Saint Laurent (2009) and Sakai & Türkay (2012) both largely rest on the adult morphologies of the carapace, rostrum, cheliped and the abdominal sternites, which nonetheless overlap among species or are variable within species to some degree. Moh & Chong (2009), however, distinguished four outwardly similar species, *Thalassina kelanang*, *T. anomala*, *T. gracilis*, and *T. squamifera*, based on their distinct male gonopods. The importance of this character for species diagnosis is also recognised by Sakai & Türkay (2012) in their figures.

The work of Ngoc-Ho & de Saint Laurent (2009) cast doubt on the geographical distribution of *T. squamifera* which, according to them, was found from Australia to Thailand, including the Solomon Islands, Vanuatu, Fiji, New Caledonia, Papua New Guinea, the Philippines, Indonesia, Singapore and Malaysia. They rested their argument on purported *T. squamifera* specimens collected from these countries (except Malaysia). This wide distribution could be an oversight because of the closely similar morphological characters shared by *T. squamifera* and *T. kelanang*. There is also some uncertainty in the species distinction between *T. squamifera* and *T. gracilis*. Ngoc-Ho & de Saint Laurent (2009) reported that the type of *T. gracilis* was very likely to be lost and selected a neotype (male, TL = 91.5 mm; ZRC 2007.0511) from Singapore to stabilise the taxonomy of this species, citing the unique morphology of the rostrum. Sakai & Türkay (2012) argued, however, that the selection of this neotype was inappropriate because the original type specimen was a small female (TL = 63.5 mm) which they believed was morphologically different from the neotype. They pointed out that the number of spinules/denticles on the dorsal margin of the cheliped, as figured by Dana (1855: pl. 32, fig. 5a, d), did not match that of the neotype. Based on similar cheliped armature, they argue that *T. gracilis* Dana, 1852 in the original sense should be synonymous with *T. squamifera*.

It is apparent that population and species distinctness of the outwardly similar *Thalassina anomala*, *T. kelanang* and *T. gracilis* from the Malay Peninsula, and *T. squamifera* from Australia, need verification. In this paper, we report on (1) the population distinctiveness among the three sympatric species, and between *T. kelanang* and *T. squamifera* based on their meristic and morphometric features, and (2) species differentiation among *T. kelanang*, *T. squamifera*, *T. anomala* and *T. gracilis* based on three molecular markers comprising two nuclear protein-coding genes, phosphoenolpyruvate carboxykinase (PEPCK) and sodium–potassium ATPase a-subunit (NaK), and one mitrochondrial-coding gene, cytochrome *c* oxidase subunit I (COI).

MATERIAL AND METHODS

Material examined. — Mud lobsters were sampled from two nearby sites at Kelanang Beach and Carey Island (<50 km apart) in Selangor, Malaysia. A total of 58 mud lobsters comprising *T. anomala* (n = 24), *T. kelanang* (n = 25) and *T. gracilis* (n = 9) were collected inside or near to the mangrove forest for meristic and morphological studies. All specimens were deposited in the Zoological Museum University of Malaya (ZMUM). Eleven specimens of *T. squamifera* were loaned out from the Museum and Art Gallery of the Northern Territory (MAGNT), Darwin, Australia. A further two specimens of each species were collected and prepared for molecular analysis. Tissues from the chelae were removed from fresh specimens killed by freezing and immediately preserved in absolute ethanol (99.9%).

Meristics and morphometrics. — A total of 13 morphometric and six meristic characters were used for discriminant analysis (Table 1). The morphometric measurements were made using a pair of digimatic calipers, with a precision of 0.01mm. The meristic characters were counted under a dissecting binocular microscope.

Statistical analysis. — Data on 11 morphometric (ABL, CW, ABW, ATUL, LPL, LPW, LPH, SPL, SPW, SPH, RL) and six meristic (LMDS, LGP, LMLS, SMDS, SGP, SMLS) characters were used for discriminant analysis. To approximate multivariate normality and linear relationships, all data were first transformed to base 10 logarithms (Pimentel, 1979). Because of the variation in size of mud lobsters, all body part measurements were corrected for differences in body size. Carapace length (CL) was used to indicate body size and as the covariate. Analysis of covariance was used to adjust each morphometric character to the overall mean total length (Misra & Ni, 1983). This adjustment used the following formula: $M'_{ij} = \log M_{ij} - [RC_{ij} (\log CL_i - \log \overline{CL})]$ where M'_{ij} is the measurement adjusted for character *j* of individual *i*, M_{ij} is the original value, RC_{ij} is the pooled regression coefficient of logM on logCL, CL_i is the carapace length of individual *i*, and $\overline{CL}$ is the overall mean carapace length.

The meristic and morphometric variations among species were analysed using forward stepwise discriminant function analysis (SDFA). All statistical analyses were performed using the software Statistica Version 10. Default settings were retained as the following:-tolerance level at 0.010, F to enter at 3 and F to remove at 2.

MOLECULAR ANALYSIS

DNA extraction, polymerase chain reaction (PCR) and sequencing. — The genomic DNA were isolated from approximately 100 mg of *Thalassina* cheliped tissues preserved in absolute ethanol (99.9%) using i-genomic CTB DNA Extraction Mini Kit (iNtRON Biotechnology, Inc, Korea).

Table 1. Definitions of 13 morphometric and six meristic characters of four species of *Thalassina* (*T. anomala*, *T. gracilis*, *T. kelanang*, and *T. squamifera*).

	Abbreviation	Character	Description
Morphometric Measurements	TL	Total length	Tip of the rostrum to the end of the telson
	CL	Carapace length	Tip of the rostrum to the posterior edge of the carapace
	ABL	Abdomen length	Anterior edge of the first tergite to the tip of the telson
	CW	Carapace width	Straight line measurement between lateral surfaces across the linea thalassinica
	ABW	Abdomen width	Straight line measurement between lateral surfaces of third abdominal segment at midregion
	ATUL	Antennule length	Base to tip of the antennule
	LPL	Propodus length, large chela	Proximal to distal edge of propodus along the mesial dorsal carina
	LPW	Propodus width, large chela	Straight line measurement between lateral surfaces at the midregion of propodus
	LPH	Propodus height, large chela	Dorsal to ventral edge measured at the midregion of propodus
	SPL	Propodus length, small chela	Proximal to distal edge of propodus along the mesial dorsal carina
	SPW	Propodus width, small chela	Straight line measurement between lateral surfaces at the midregion of propodus
	SPH	Propodus height, small chela	Dorsal to ventral edge measured at the midregion of propodus.
	RL	Rostral length	Tip of rostrum to postorbital edge of carapace
Meristic Counts	LMDS	No. of dorsal spines on the merus of large chela	
	SMDS	No. of dorsal spines on the merus of small chela	
	LGP	No. of spines/tubercles on the mesial dorsal carina of propodus of large chela	
	SGP	No. of spines/ tubercles on the mesial dorsal carina of propodus of small chela	
	LMLS	No. of large dorsal spines on anteriormost margin of the merus of large chela	
	SMLS	No. of large dorsal spines on anteriormost margin of the merus of small chela	

The sequences of nuclear encoded phosphoenolpyruvate carboxykinase (PEPCK) and sodium–potassium ATPase a-subunit (NaK) were amplified using the following primer sets: (1) PEPCK for2: 5'-GCA AGA CCA ACC TGG CCA TGA TGA C-3' and PEPCK rev3: 5'- CGG GYC TCC ATG CTS AGC CAR TG-3' and (2) NaK for-a: '5- GTG TTC CTC ATT GGT ATC ATT GT-3' and NaK rev2: 5'- ATG ACA GTT GCT CAT ATG TGG TT-3' (Tsang et al., 2008). The partial sequences of mitochondrial encoded markers, namely, cytochrome *c* oxidase subunit I (COI) were amplified using primer sets LCO1490: 5'- GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (Folmer et al., 1994).

PCR amplification of all the molecular markers was carried out using MultiGene Gradient Thermal Cycler (Labnet, USA). The PCR amplification was carried out as in Lim et al. (2012) except that the annealing temperature was varied for PECK and NaK at 60°C and for COI at 50°C. PCR products were assayed by electrophoresis on 1.0% agarose mini gel stained with SYBR®Safe DNA gel stain (Invitrogen, USA) and visualised under UV light. The target DNA fragments were isolated and purified by the LaboPass™ PCR purification kit (Cosmo Genetech, South Korea). The purified PCR products were sent to a commercial company, Lucigen (Taiwan) for sequencing. The same primers set for PCR amplifications were used for DNA sequencing. Two decapod species, a penaeid, *Metapenaeus brevicornis* (H. Milne Edwards, 1837) and a palaemonid, *Exopalaemon styliferus* (H. Milne Edwards, 1840), were used as outgroups in this study. The palaemonid belongs to the Caridea, a sister group to the Gebiidea within the Pleocyemata, while the penaeid represents the Dendrobranchiata, sister group to the Pleocyemata (Bracken et al., 2009; Lin et al., 2012).

Sequence alignment and molecular analysis. — The generated sequences were initially aligned using the CLUSTAL X program (Thompson et al., 1997) and subsequently aligned manually. Additional *T. anomala* (from Singapore) sequences, for PEPCK (EU427241) and NaK (EU427172), from GenBank were used in the analysis. The aligned sequences were subjected to maximum-parsimony (MP). The MP tree was constructed using the heuristic search option, 100 random sequence additions, tree bisection reconnection (TBR) branch swapping, and unordered and unweighted characters. Bootstrap percentage (BP) was computed with 1000 replications. Maximum Likelihood (ML) analysis was performed by Treefinder version October 2008 (Jobb et al., 2004). Bayesian inference (BI) analysis was performed using MrBayes 3.1.1 (Huelsenbeck & Ronquist, 2001). Best-fit nucleotide substitution model was determined using KAKUSAN v.3 (Tanabe, 2007), which also generated the input files for ML and BI.

Best-fit models were evaluated using the corrected Akaike Information Criterion (AICc) (Akaike, 1973; Shono, 2000) for

Table 2. GenBank accession numbers for the four species of thalassinids and outgroups.

Species	Voucher Number	GenBank Accession Number		
		PEPCK	NaK	COI
Thalassina anomala 1	ZMUMCTA19	JX100440	JX100454	JX100447
Thalassina anomala 2	ZMUMCTA20	JX100441	JX100455	JX100448
Thalassina gracilis 1	ZMUMCTG02	JX100442	JX100456	JX100449
Thalassina gracilis 2	ZMUMCTG03	JX512419	JX512423	JX512421
Thalassina kelanang 1	ZMUMCTK17	JX100443	JX100457	JX100450
Thalassina kelanang 2	ZMUMCTK18	JX512420	JX512424	JX512422
Thalassina squamifera 1	Cr014928	JX100444	JX100458	JX100451
Metapenaeus brevicornis	ZMUMCMB01	JX100445	JX100459	JX100452
Exopalaemon styliferus	ZMUMCES01	JX100446	JX100460	JX100453

Table 3. Standardised coefficients for canonical variables derived by SFDA of morphometric and meristic characters.

Variable	Standardised Coefficient for Canonical Variables		
	Root 1	Root 2	Root 3
ABL	0.6827	–0.2218	0.1755
SMLS	–0.1709	0.6094	–0.5403
SGP	0.0618	–0.3607	–0.3778
CW	0.4518	0.2088	–0.4275
RL	0.5026	0.2392	0.1471
LMLS	–0.1244	0.4241	–0.5837
LGP	–0.1483	–0.1739	–0.5837
Eigen	67.3980	14.9089	3.1199
Cum. P	0.7890	0.9635	1.0000

Eigen = eigenvalue, Cum. P = cumulative proportion of total variance. For variables, see Table 1 for explanation.

ML and the Bayesian Information Criterion (BIC) for BI. ML analysis was performed with 1,000 bootstrap replicates. Two parallel runs were performed in MrBayes, each consisting of four chains, two "cold" and two incrementally heated. Four million Markov chain Monte Carlo (MCMC) generations were run, with convergence diagnostics calculated every 1000th generation for monitoring the stabilisation of log-likelihood scores. Trees in each chain were sampled every 100th generation. A 50% majority rule consensus tree was generated from the sampled trees after discarding the first 20%. The likelihood scores stabilised before 800,000 generations (20%) for all three individual molecular marker analyses and also the combined markers analysis.

To assess the level of variation in PEPCK, NaK and COI among the selected samples of different taxa, uncorrected "p" pairwise genetic distances were estimated using PAUP* 4.0b10 software (Swofford, 2002). The DNA sequences used in this study were deposited in GenBank and their accession numbers are given in Table 2.

RESULTS

Analysis of morphometric and meristic data. — Of the 17 characters used in the discriminant analysis (SDFA), seven characters (ABL, SMLS, SGP, CW, RL, LMLS, LGP) were adopted by the SDFA model that best distinguished the four species of *Thalassina*, while the remaining 10 characters were removed (Wilks' $\lambda = 0.00022$; F value (21, 169) = 143.20, $P < 0.001$). The classification matrix which compares the known membership with the predicted membership, based on the model's classification functions, showed 100% correctly predicted membership for all species.

The SDFA generated three canonical functions (roots), with the first root contributing to 79% and the second root 17% of the total variance. Hence, the first two roots captured most of the discriminatory power of the SDFA model and were used to interpret the contribution of the measured characters to discrimination of the four species.

The first root was loaded highest by the abdominal length, ABL (0.683), rostral length, RL (0.503) and carapace width, CW (0.452) (Table 3). The second root was loaded highest by the number of large dorsal spines on the anteriormost margin of the merus of the small chela, SMLS (0.609), number of large dorsal spines on the anteriormost margin of the merus of the large chela, LMLS (0.424) and number of spines or tubercles on the inner ridge of the propodus of the small chela, SGP (–0.361).

Plots of the canonical scores of all specimens show clear separation of the four population samples of *Thalassina* species (Fig. 1). On the first root, *T. anomala* with the longest ABL, RL and CW was farthest from *T. gracilis* which had the shortest measurements of these characters. The second root separates *T. kelanang* with the highest SMLS (3–5) from *T.*

anomala (2) and *T. gracilis* (2–3). On the other hand, SGP was highest in *T. gracilis* (17–22) and *T. anomala* (12–17) as compared to *T. squamifera* (8–15) and *T. kelanang* (8–14).

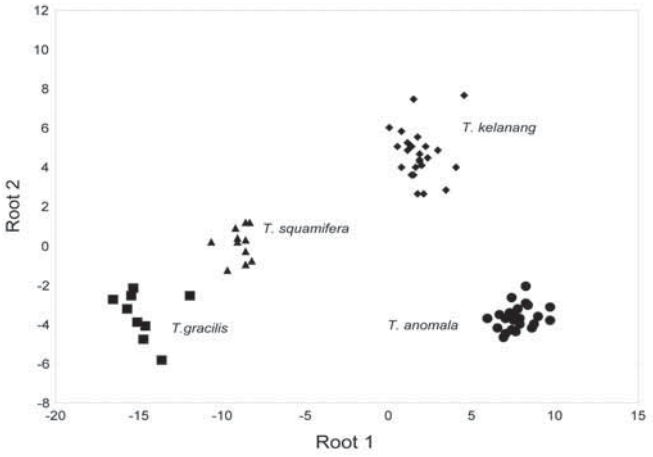

Fig. 1. SFDA ordination diagram of morphological and meristic characters for four species of *Thalassina*.

The squared Mahalanobis distance between the group centroids shows the farthest distance between *T. anomala* and *T. gracilis* (516.8) and the shortest distance between *T. squamifera* and *T. gracilis* (84.3). *Thalassina kelanang* was not the closest to *T. squamifera* (154.0), as it was with *T. anomala* (103.9).

DNA sequences. — The aligned sequences of PEPCK consisted of 607 sites, of which 425 characters were constant, 79 characters were parsimony informative and 103 characters were parsimony uninformative. The aligned sequences of NaK consisted of 770 sites, of which 565 characters were constant, 73 characters were parsimony informative and 132 characters were parsimony uninformative. The aligned sequences of COI consisted of 710 sites, of which 459 characters were constant, 184 characters were parsimony informative and 67 characters were parsimony uninformative.

Molecular analysis. — The phylogenetic trees constructed using the three methods (ML, MP and BI) for molecular markers PEPCK, NaK and COI, and combined markers, had

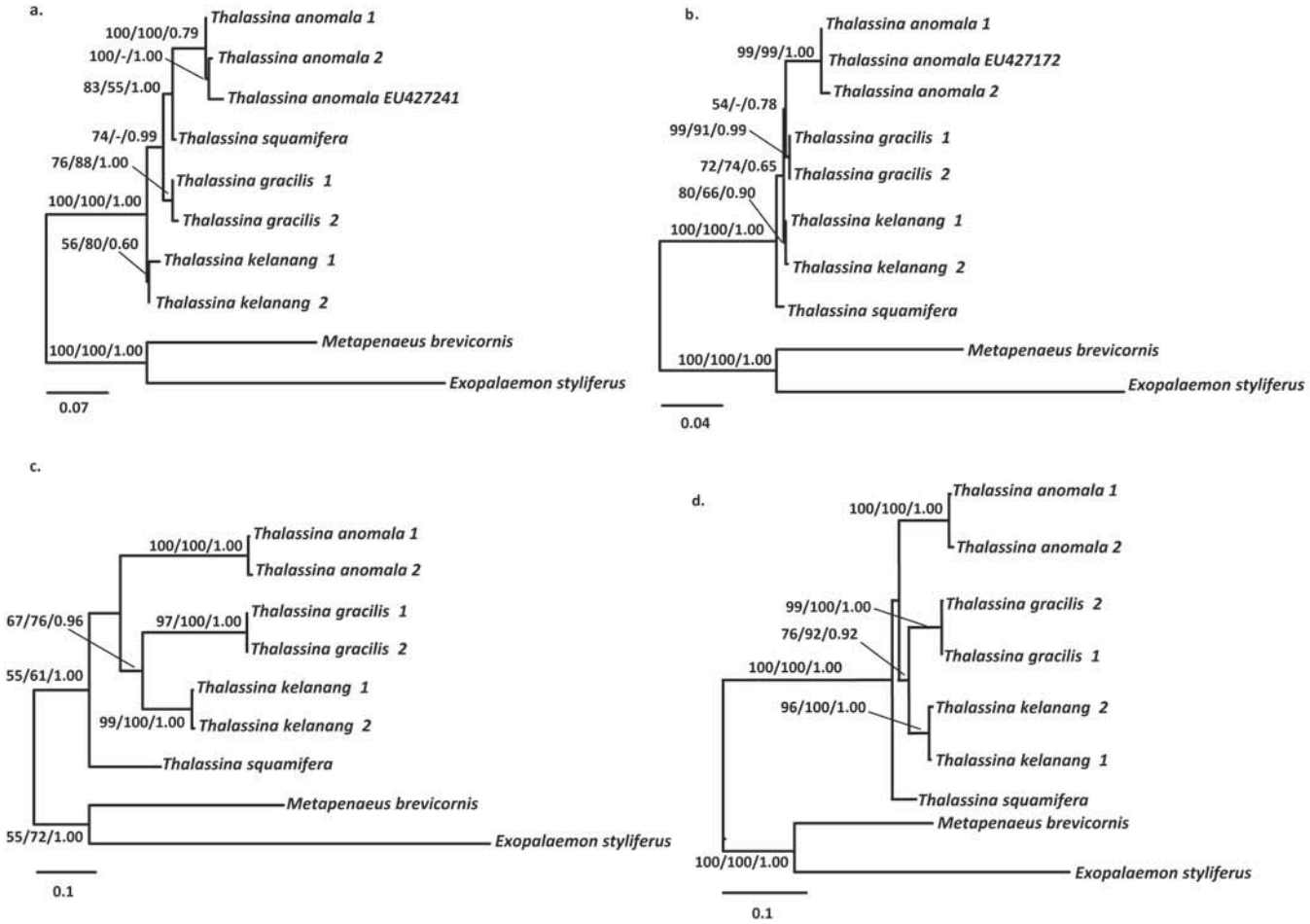

Fig. 2. The 50% majority-rule consensus tree resulting from maximum likelihood analysis of (a) partial PEPCK sequences (substitution rate parameters: TC = 0.5206, TA = 0.1254, TG = 0.0125, CA = 0.1254, CG = 0.0125, AG = 0.2036), - Ln likelihood 1935.877; (b) partial NaK sequences (TC = 0.5183, TA = 0.1061, TG = 0.0727, CA = 0.0972, CG = 0.0223, AG = 0.1834), - Ln likelihood 2117.352; (c) partial COI sequences (TC = 0.7231, TA = 0.1315, TG = 1.4301e–5, CA = 0.0153, CG = 0.0311, AG = 0.0991), - Ln likelihood 2729.365; (d) combined PEPCK, NaK and COI DNA sequences (TC = 0.5687, TA = 0.1190, TG = 0.0211, CA = 0.1190, CG = 0.0211, AG = 0.1511), - Ln likelihood 6914.207.
The bootstrap values (ML/MP/BI) are shown at the branches. Bar indicates substitutions per site.

similar topology except for variation in the bootstrap support values (Fig. 2a–d). Hence only ML trees are presented here with support from all the analyses. Only results from one specimen of *T. squamifera* were usable because the DNA of the second sample was not able to be extracted due to its poor condition.

PEPCK. — The phylogenetic tree of PEPCK (Fig. 2a) showed that all the four species *T. anomala*, *T. squamifera*, *T. gracilis*, and *T. kelanang* are grouped in a monophyletic clade with full support for all analyses. *Thalassina kelanang* (*T. kelanang* 1 and *T. kelanang* 2) was the most basal species among the four species. The three *T. anomala* (*T. anomala* 1, *T. anomala* 2, and *T. anomala* EU427241) were grouped in a monophyletic clade with strong to moderate support values (ML = 100%, MP = 100%, BI = 0.79). *Thalassina squamifera* was included in the same clade with *T. anomala* and *T. gracilis.*

NaK. — The phylogenetic tree of NaK (Fig. 2b) showed all four species grouped in a monophyletic clade with full support for all analyses. *Thalassina squamifera* was the most basal species and showed a sister relationship with the clade containing the other three species (*T. anomala*, *T. gracilis*, and *T. kelanang*) which has moderate support from the various analyses (ML = 72%, MP = 74%, BI = 0.65).

COI. — The phylogenetic tree of COI (Fig. 2c) showed the four species grouped in a monophyletic clade, with bootstrap support values varying from low to high (ML = 55%, MP = 61%, BI = 1.00). *Thalassina squamifera* was the most basal species. *Thalassina kelanang* and *T. gracilis* were in the same clade, supported by variable bootstrap values (ML = 67%, MP = 76%, BI = 0.96).

Combined markers. — The combined phylogenetic tree of PEPCK, NaK, and COI (Fig. 2d) also grouped the four species *T. anomala*, *T. squamifera*, *T. gracilis*, and *T. kelanang* in a monophyletic clade with full support from all analyses. *Thalassina squamifera* was shown to be the most basal species among the four species. *Thalassina squamifera* showed a sister relationship to the other three species.

Uncorrected "p" distance. — The uncorrected "p" distances of the three markers (PEPCK, NaK, COI) and their combined markers are shown in Table 4. The uncorrected "p" distances between *T. anomala* 1 and *T. anomala* 2 were close, which ranged from 0.5% for NaK to 0.7% for PEPCK; the combined markers gave 0.6%.

The uncorrected "p" distance between *T. gracilis* and *T. kelanang* ranged from 0.5% for NaK to 13.1% for COI; the combined markers gave 5.2–5.4%. The distance between *T. gracilis* and *T. squamifera* ranged from 1.3% for NaK to 16.5% for COI, with 6.6–6.7% for combined markers. Between *T. kelanang* and *T. squamifera*, the distance ranged from 2.5% for PEPCK to 14.1% for COI, with the combined markers giving 5.9–6.0%. In summary, the uncorrected "p" distance among species varied according to the type of molecular markers used. The COI gave the highest distance among species ranging from 13.1% (*T. kelanang* 1 and *T. gracilis* 1) to 17.1% (*T. anomala* 1 and *T. gracilis* 2), while NaK gave the lowest distance among species of 0.5% (*T. kelanang* 2 and *T. gracilis* 1 & 2) to 3.3% (*T. anomala* 2 and *T. squamifera*). Overall, based on the combined markers, *T. anomala* 2 was the most distant from *T. gracilis* 2 (8.2%), while the closest pair was *T. kelanang* 1 and *T. gracilis* 1 (5.2%).

DISCUSSION

Meristic, morphometric and molecular evidence has verified that the sampled populations of *Thalassina* belong to four distinct species, which form a monophyletic clade. The molecular evidence justifies the recognition of *T. squamifera* and *T. anomala* as distinct species. Also, *T. kelanang* (from Malaysia) is a distinct species from *T. squamifera* (from Australia), supporting the assertion of Moh & Chong (2009) which was based on its distinctive rostrum and male gonopod. On the basis of the figures and descriptions of Ngoc-Ho & de Saint Laurent (2009: 149, fig. 12A, B), the identity of their *T. squamifera* specimen from Thailand (MNHN Th 438) is doubtful since it very closely resembles *T. kelanang* (cf. Moh & Chong, 2009: 466, 468, figs. 1, 3A). Moh & Chong (2009) described *T. kelanang* as having a waisted rostrum, adrostral carina extending ½ the distance of the gastro-orbital carina and median sulcus extending behind the adrostrals (clearly seen in MNHN Th 438, fig. 12A in Ngoc-Ho & de Saint Laurent, 2009). Moh & Chong (2009) also described the chela as having a dorso-lateral carina extending ¾ the propodal length and merus bearing 3–5 large dorsal spines (clearly seen in MNHN Th 438, fig. 12B in Ngoc-Ho & de Saint Laurent, 2009). However, the Australian material of Ngoc-Ho & de Saint Laurent (2009: 149, fig. 12C, D) is clearly *T. squamifera* (MNHN Th 1518, bearing 2 or 3 large dorsal spines on the merus). Therefore, it is more likely that *T. kelanang* has a widespread distribution in the Southeast Asian region, rather than *T. squamifera* spreading out from Australia to Thailand. Our recent examination of one Indonesian specimen of '*T. squamifera*' (MZB.Cru.211, coll. C. Boden Kloss, 1924), collected from Sipora, W. Sumatra, now in the Wet Biological Collection, Indonesian Institute of Sciences (LIPI) (Citra Dewi, LIPI, pers. comm.), revealed that it was actually a female *T. kelanang*. Another specimen from LIPI (MZB.Cru. 2252, coll. W. T. Laksono & D. C. Murniati, 2008), a male collected from Legon Cibariang, Panaitan Island, Java, is also identified as *T. kelanang*. Two other confirmed records of *T. kelanang* from locations outside Peninsular Malaysia, Semakau Island, Singapore (Ron Yeo, Raffles Museum of Biodiversity Research, Singapore, pers. comm., 2009), and Lahat Datu, Sabah (Tungku Beach Resort, pers. comm., 2011), have been made based on specimen photographs which included the rostrum and male gonopod.

Results from morphometric and molecular data concur in that *T. anomala* and *T. gracilis* form the most distant pair in terms of morphology and genetics, respectively, while

Table 4. Uncorrected "p" distance measures (%) among four species of *Thalassina* based on PEPCK, NaK, COI and combined molecular markers.

Species (site)		Species					
		1	**2**	**3**	**4**	**5**	**6**
1. *T. anomala* 1							
2. *T. anomala* 2	PEPCK	0.7					
	NAK	0.5					
	COI	0.8					
	COMBINED	0.7					
3. *T. gracilis* 1	PEPCK	5.0	4.6				
	NAK	2.5	3.0				
	COI	16.8	16.9				
	COMBINED	7.9	8.0				
4. *T. gracilis* 2	PEPCK	5.1	4.8	0.5			
	NAK	2.5	3.0	0.0			
	COI	17.1	16.9	0.0			
	COMBINED	8.0	8.2	0.2			
5. *T. kelanang* 1	PEPCK	4.6	4.5	2.3	2.3		
	NAK	2.6	2.9	0.7	0.7		
	COI	15.2	14.7	13.1	13.1		
	COMBINED	7.3	7.4	5.2	5.3		
6. *T. kelanang* 2	PEPCK	4.9	5.1	3.2	3.2	1.0	
	NAK	2.5	3.0	0.5	0.5	0.1	
	COI	15.5	15.1	12.8	12.7	0.6	
	COMBINED	7.4	7.7	5.3	5.4	0.5	
7. *T. squamifera*	PEPCK	3.6	3.3	2.7	2.0	2.5	2.7
	NAK	2.7	3.3	1.3	1.3	1.2	1.0
	COI	15.8	15.1	16.4	16.5	13.8	14.1
	COMBINED	7.3	7.3	6.7	6.6	5.9	6.0

1, *T. anomala* 1 (Kelanang Beach); 2, *T. anomala* 2 (Kelanang Beach); 3, *T. gracilis* 1 (Carey Island); 4, *T. gracilis* 2 (Carey Island); 5, *T. kelanang* 1 (Kelanang Beach); 6, *T. kelanang* 2 (Kelanang Beach); 7, *T. squamifera* (northern Australia).

T. anomala and *T. squamifera* form the third most distant pair (Table 4). Except for these agreements, the conclusions regarding the affinities among other species pairs did not match. For instance, *T. kelanang* and *T. gracilis* were considered to be the closest pair (uncorrected "p" distance = 5.2) based on molecular evidence, but were the second most distant pair based on meristics and morphometrics (Mahalanobis distance = 348.67). In fact, meristics and morphometrics placed *T. gracilis* as morphologically closest to *T. squamifera*. The incongruence is not unexpected since the meristic and morphometric characters used were selected while the molecular gene markers do not necessarily reflect these expressions.

The molecular evidence suggests that *T. kelanang* or *T. squamifera* are basal species depending on the molecular marker used. In fact, NaK, COI, and combined markers indicate the affinity between the two species based on their uncorrected 'p' distance (Table 4). The combined gene markers, however, indicate *T. squamifera* to be the most basal species (Fig. 2d). Also, only these two species consistently retain a distinct, movable, and setose scaphocerite: one of the caridoid facies of primitive eumalacostracans. Thus, *T. squamifera* and *T. kelanang* are likely two basal sister species retaining most of their ancestral characters, and the other species are possibly derived from their common or shared ancestor. One such species, *T. anomala*, the most distant from all other species, is likely derived from a shared ancestor with *T. kelanang*. *Thalassina anomala* is reported to have a wide distribution from west India to Fiji, and as far north as southwest Japan but it is not known in Australia (Davie, 2002; Ngo-Ho & de Saint Laurent, 2009). Some of the derived traits of *T. anomala* may include the distinctively long hooked spine on the posterior dorsomedian margin of the carapace, absence of (or rudimentary) movable scaphocerite on the antennal peduncle, and sexually dimorphic 3rd maxilliped (dactylus bearing stiff setae in males).

Since the molecular gene markers have also conclusively resolved the distinction between the species *T. squamifera* and *T. kelanang*, we hypothesize that these species represent two sister groups in their present biogeographical regions. The records of their collections, especially *T. squamifera* (see Ngoc-Ho & de Saint Laurent, 2009: 148; Sakai & Türkay, 2012: 1371–1373), attest to their distribution east and west of Wallace's line, respectively (Fig. 3). This hypothesis of two sister groups is also supported by the molecular evidence and by the retention of closely similar morphological traits in the two 'basal' sister species (see Moh & Chong, 2009). These morphological traits have caused confusion in the

work of Ngoc-Ho & de Saint Laurent (2009) regarding the biogeographical occurrence of *T. squamifera*. The identity of the lone specimen of *T. squamifera* from Ranong, Thailand, in Sakai & Türkay (2012) is similarly doubtful. Like Ngoc Ho & de Saint Laurent (2009), they did not examine material of *T. kelanang*. It is not likely that *T. squamifera* is found in Singapore and Thailand, but escaped detection in Peninsular Malaysia. On the basis of the available evidence, *T. squamifera* cannot be regarded as synonymous to *T. gracilis* Dana, 1852, as suggested by Sakai & Türkay (2012). The evidence also supports the identities of these species from the perspective of the the neotype designation of *T. gracilis* by Ngoc Ho & de Saint Laurent (2009). Sakai & Türkay's (2012) assertion that the wrong specimen was selected may not be valid. From an ecological point of view, it is unlikely that two very similar species are found together (co-exist) in a similar habitat. For instance, in the Langat estuary, Selangor, either *T. kelanang* or *T. gracilis* live sympatrically on the lower intertidal shore with *T. anomala* on the upper and supratidal shore of mangrove forests (Moh, unpublished data). From the molecular data, *T. kelanang* is the species closest to *T. gracilis*, but the indication from extensive samplings in Selangor is that they are spatially separated—the latter occupying the upper estuary while the former is on the coast.

As for *T. gracilis* Dana, 1852 being a nomen dubium, we have examined further our material of *T. gracilis*, in particular two small females slightly larger than Dana's type specimen: 1) TL = 77.50 mm/ CL = 28.15 mm, (ZMUM CTG010), coll. H. H. Moh, 10 Nov.2010, number of dorsomesial denticles on cheliped: right = 15, left = 16; and 2) TL=99.19 mm/ CL=32.34 mm (ZMUM CTG011), coll. H. H. Moh, 10 Nov.2010, number of dorsomesial denticles on cheliped: right = 18, left = 17. These results appear to support Sakai & Türkay's (2012) argument that this is a case of nomen dubium, on the assumption that the small and schematic drawing provided by Dana (1855: pl. 32, fig. 5d) accurately portrays the spination of the cheliped. Nonetheless, his description

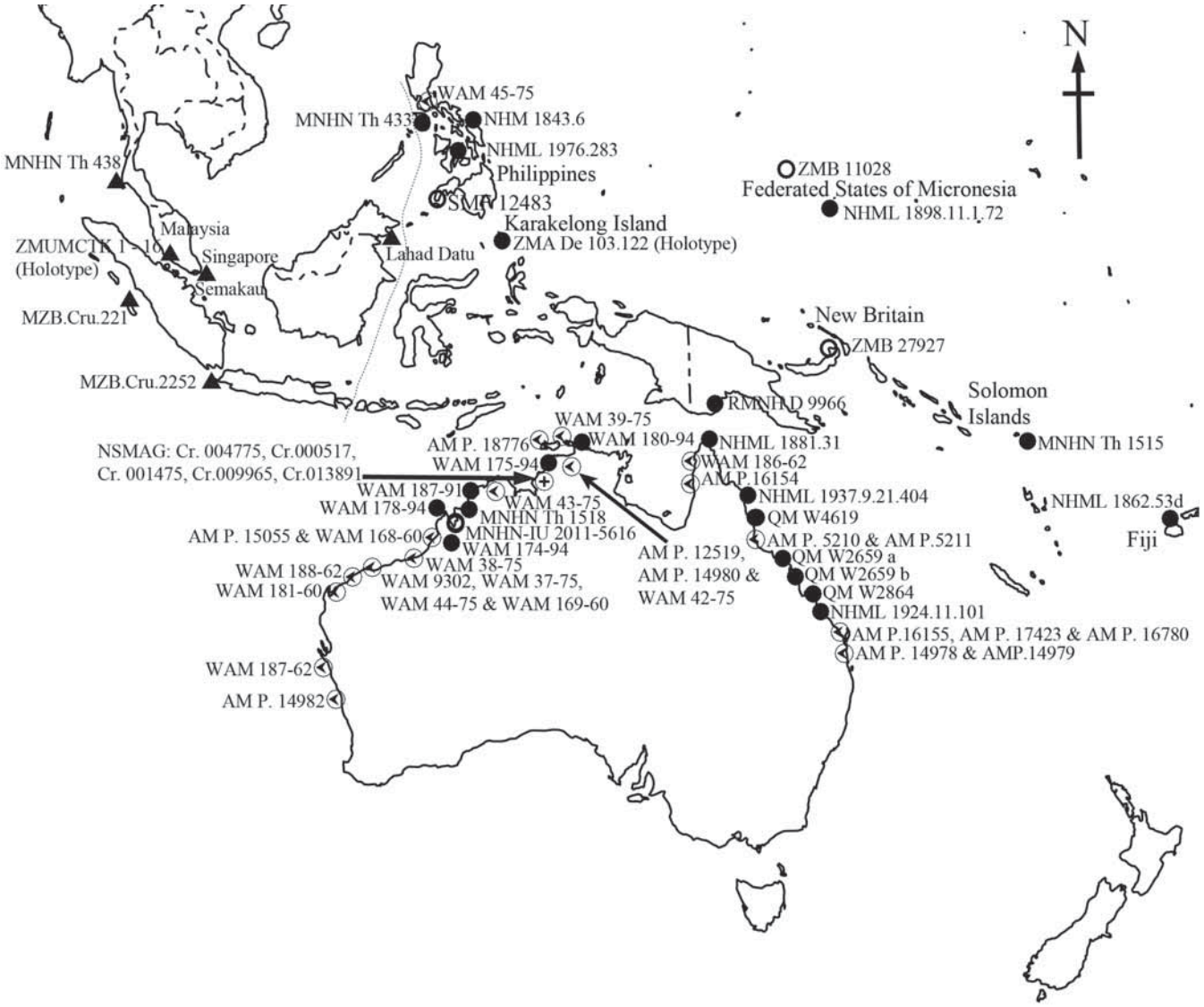

Fig. 3. Locations of examined specimens of *T. squamifera* (circles) and *T. kelanang* (triangles) by various authors. Museum catalogue numbers at each site are indicated. ⊙ = Poore & Griffin, 1979; ● = Ngoc-Ho & de Saint Laurent, 2009; ⊕ = Moh & Chong, 2009; ○ = Sakai & Turkay, 2012; ▲ = Moh & Chong, 2009, this study. Dotted line indicates Wallace's Line. Modified base map from http://commons.wikimedia.org.

and illustration of a short acute rostrum (Dana, 1852: 515; 1855: pl. 32, fig. 5c) are clearly not in agreement with *T. anomala*, *T. squamifera* or *T. kelanang* as we understand these species. This is inclusive of three young individuals (TL 37.80, 47.14, and 61.63 mm) of *T. kelanang* in our collection. Dana's description that on "either side of the beak there is a slight ridge running longitudinally for a short distance from the front edge" clearly meant the rostrum of *T. gracilis*. None of the other species show this feature since the ridge on their rostrum extends anteriorly to the rostral tip. Even if the description meant extending posteriorly, the ridge in *T. gracilis* only extends about the same length as its short rostrum, whereas in the others it extends a distance farther than the length of their (longer) rostrum (see Moh & Chong, 2009: 464, fig. 3). On this basis, we believe that it is likely that Dana's drawings of the denticles on the cheliped of *T. gracilis* were done somewhat schematically and he had missed some of the less obvious denticles. It appears, therefore, that Ngoc-Ho & de Saint Laurent's (2009) use of rostral characters to decide on the choice of a neotype was warranted. Needless to say, their taxonomic action of formally selecting a neotype is valid under the Code (ICZN, 1999) and, therefore, binding on all subsequent workers. *Thalassina gracilis* Dana, 1852, therefore cannot be treated as a nomen dubium.

Fossil specimens of *Thalassina emerii* Bell, 1844 are known from Australia, but living specimens with rudimentary scaphocerites, thought to be of this species, have been redescribed by Ngo-Hoc & de Saint Laurent (2009). Notwithstanding the assertion of Sakai & Türkay (2012) that *T. emerii* is a species inquirenda without further status, the living specimens of two new species (Sakai & Türkay, 2012) instead may suggest a similarly derived condition from *T. squamifera*. Three further thalassinid species not treated in the present study (*Thalassina spinosa*, *T. krempfi*, and *T. spinirostris*) are unlikely to be basal species based on their described morphological traits. Unlike the basal species with movable developed scaphocerite, *Thalassina spinosa*, *T. krempfi*, and *T. spinirostris* have either a rudimentary scaphocerite or none at all. *Thalassina spinosa* and *T. krempfi* are described as morphologically similar to *T. anomala*, while *T. spinirostris* is similar to *T. gracilis* (Sakai & Türkay, 2012).

The molecular evidence based on NaK, COI and combined markers suggests a close phylogenetic relationship between *T. gracilis* and *T. kelanang*, probably reflecting a more recent speciation of the former due to its small scaphocerite, short dorsomedian process, and a third maxilliped that is not sexually dimorphic. Nonetheless, the depressed, spiny-tipped rostrum of *T. gracilis* is likely a derived feature not observed in the basal species. On the other hand, the examined morphological traits suggest a close relationship between *T. gracilis* and *T. squamifera*, which is supported by their similar morphologies with *T. kelanang*. The *T. kelanang* + *T. gracilis* pairing is however more plausible based on the molecular evidence (Fig. 2b–d), and the fact that more specimens of *T. gracilis* have been collected in the Asian region including Thailand, Malaysia, Singapore and Indonesia (Sumatra), whereas there was only one broken specimen of dubious identity recorded from northwest Australia (Ngoc-Ho & de Saint Laurent, 2009). Our hypotheses and speculations, however, require further substantiation from molecular work on the remaining extant species, *T. krempfi*, *T. spinosa*, *T. spinirostris*, *T. australiensis*, and *T. saetichelis*, in order to validate their species identities and to fully elucidate the phylogenetic relationships of the thalassinid mud lobsters.

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