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Genetic Analysis of Two Sympatric Sea Urchins from Genus *Diadema* (Echinodermata: Echinoidea: Diadematidae) from Malaysian Borneo

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ABSTRACT

Genus *Diadema* (Echinoidea: Diadematidae) has been reported to be the most widespread and ecologically important shallow water tropical sea urchins. Morphological variations and species distributions are complicated to elucidate due to complexity in making reliable identifications. Genus *Diadema* had involved in many debates, particularly on the mode of speciation and the specific status of *Diadema setosum* and *Diadema savignyi*. Therefore, relationships among *Diadema* species found in Malaysian Borneo have been examined using 16S rRNA gene analysis. Monophyletic clade of genus *Diadema* with respect to the outgroup was obtained with high bootstrap values of 100% (MP), 100% (NJ), 100% (ML) and Bayesian Posterior Probability is equal to 1.00. Two monophyletic clades were apparent separating *D. setosum* (Clade I) and *D. savignyi* (Clade II), with strong support of 100% (MP), 100% (NJ), 80% (ML) and Bayesian Posterior Probability is equal to 1.00. In addition, high genetic variation among species had been recorded (9.85%), suggesting that *D. setosum* and *D. savignyi* are two distinct entities. Furthermore, *D. setosum* and *D. savignyi* are sympatric species based on their distribution and overlapping ranges in Malaysian Borneo.

Keywords: *Diadema*, sympatric species, phylogenetic, 16S rRNA gene

INTRODUCTION

Genus *Diadema* had been reported to be the most widespread and ecologically important shallow water genera of tropical sea urchins (Lessios, 2001) by controlling the population of algae and maintaining the balance of food chain relationship. Distinctions in their distributions among species of genus *Diadema*, are complicated to elucidate due to complexity in making reliable identifications (Pearse, 1998). Their systematics and biogeography are enmeshed in uncertainty until study conducted by Mortensen (1940) appeared to stabilize the systematics of *Diadema*. There are eight species in genus *Diadema*: *Diadema-sp, D. mexicanum*, *D. antillarum*, *D. ascensionis*, *D. palmeri*, *D. paucispinum*, *D. setosum* and *D. savignyi* (Lessios, 2001). However, only two species *D. setosum* and *D. savignyi* (Figure 1) could be found in Malaysian Borneo (Rahim & Nurhassan, 2012).

D. setosum Leske 1778, is a long spine black sea urchin characterized by the unique morphology of orange anal ring, green bands of iridophores down the midlines of interambulacra and five blue or white spots on the anal tube (Coppard & Campbell, 2006). This species possesses three to five tubercles along the inner edge of the genital plates and at larval stage, it has several long arms to funnel food particles and facilitate their movement (Yokota *et al*., 2002).

On the other hand, *D. savignyi* Michelin 1845 is known as a black sea urchin, has shorter spine compared to *D. setosum* with bold pattern of iridophores on the aboral surface forming connecting lines rather than spots. It possesses black anal tube, distinct arch-shaped depression on the genital plate in adult form and the black test was distinctively horizontally circular with white line radiated out from the genital plates down the midlines of inter ambulacra (Coppard & Campbell, 2006). The naked median areas can be observed with small red or brown spot during the day and the spots will be seen larger in size at night as the chromatophores retracted and revealed the test (Coppard & Campbell, 2006).

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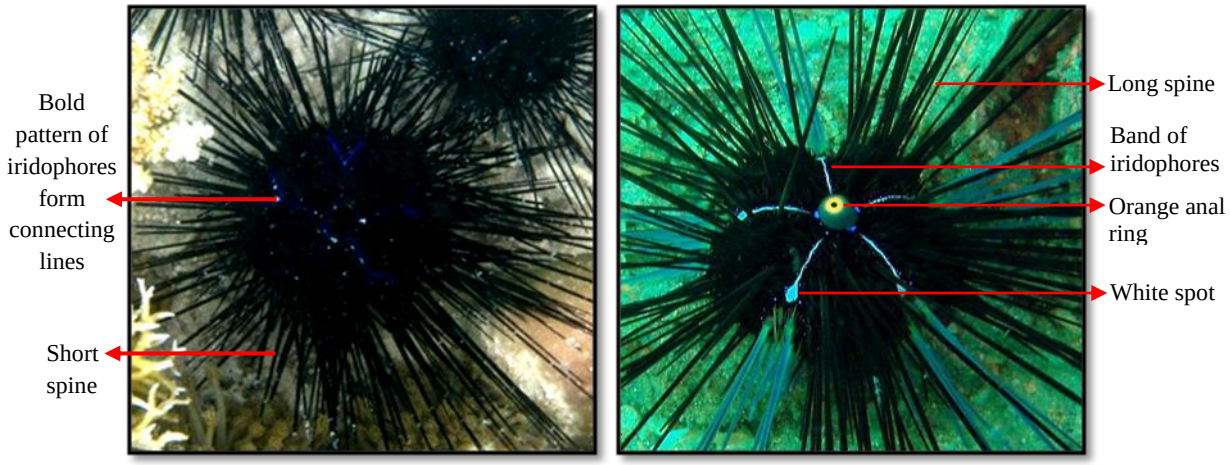

Figure 1. Photographs showing morphological characteristics of *D. savignyi* (left) and *D. setosum* (right).

According to Lessios *et al*. (2001), one of the earliest study on species distributions of tropical sea urchin was conducted by Mayr (1954) in which the study found that the model of speciation for sea urchin was allopatric due to the distribution of the organisms on either side of a land or the open sea barrier. However, problem arise as to determine the model of speciation in genus *Diadema* as two morphologically similar species namely *D. setosum* and *D. savignyi* with overlapping ranges in the Indo-Pacific were thought to be identical and that the two species were speculated to be the result of 'double invasion' in the Indo West Pacific (Lessios *et al*., 2001).

There has been great confusion regarding the specific status and geographical distributions of *D. setosum* and *D. savignyi*. Debates on either they are sympatric species have been discussed by many researchers for example hypothesis on overlapping populations (Mortenson, 1940; Mayr, 1954), distribution pattern in the wide ocean (Pearse, 1998; Lessios, 2001), morphological similarities (Clark, 1966; Coppard & Campbell, 2006), theories on natural hybrids (Lessios & Pearse, 1996) and annual rhythm of active spawning (Adel & Khalid, 2000). Thus, phylogenetic study was suggested to reveal whether the two morphologically similar urchins have distinct genetic identity.

At the very beginning of this study, hypothesis null stated that both *D. setosum* and *D. savignyi* have the same genetic composition and could be a subspecies. Whereas hypothesis alternative is *D. setosum* and *D. savignyi* have substantial genetic variation and are reciprocally monophyletic. In the study, 16S rRNA gene information was utilized as the molecular marker to illustrate phylogenetic relationships and the specific status of genus *Diadema* in Malaysian Borneo involving two species, *D. setosum* and *D. savignyi*.

MATERIALS & METHODS

A total of 20 samples consisted of eight samples of *D.setosum*, three samples of *D. savignyi*, five samples of *E. calamaris* and four samples of *T. gratilla* were collected from various locations in Malaysian Borneo (Figure 2; Table 1). Altogether, two species from genus *Diadema* were found in Malaysian Borneo with *E. calamaris*, member of family Diadematidae and one species from family Toxopneustidae were used as the outgroups.

The specimens were collected using SCUBA diving, snorkeling or manual collection depending on the tide level and beach profile involved (Figure 2). An amount of 1 g of soft tissues from the lateral line inside the carapace (red to brown in color) have been collected using sterile scalpel blade and placed into labeled tubes based on localities. The tissue samples were preserved in 70% ethanol and 5% EDTA, later kept in Aquatic Molecular Laboratory, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak during this study.

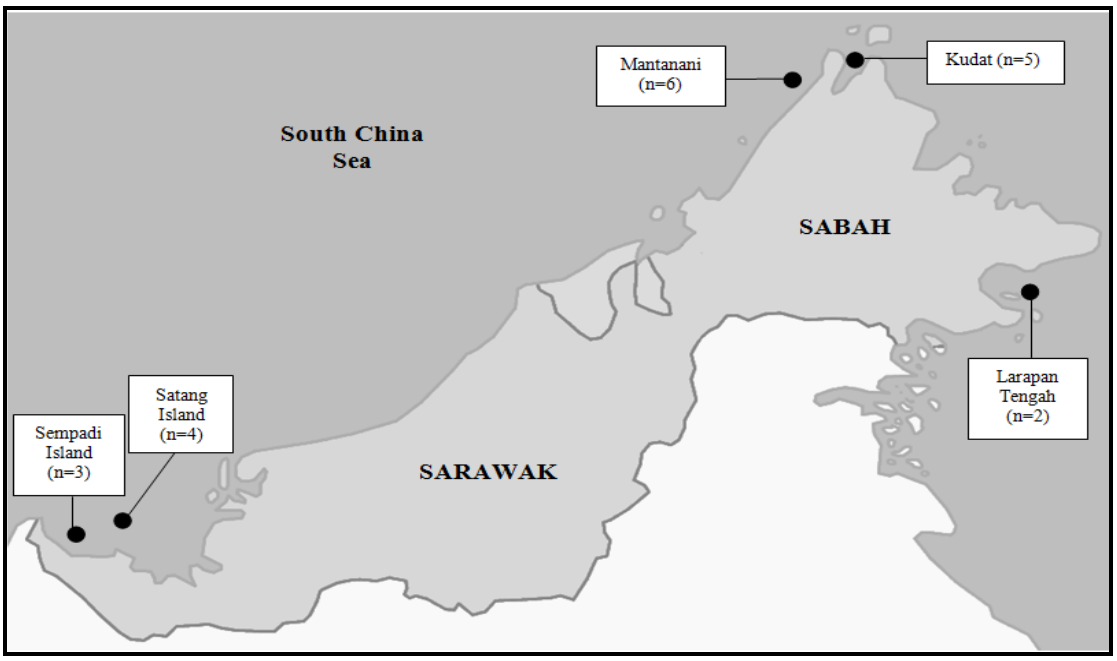

Figure 2. Map of Malaysian Borneo showing study locations and sample size (n) of the sea urchins collected for this study.

Table 1. Samples of *D. setosum*, *D. savignyi*, *E. calamaris* and *T. gratilla* analysed for 16S rRNA gene sequences variation with locality, GPS reading and field voucher.

Species	Locality	GPS reading	Field voucher	Accession number
D. setosum	Satang Island, Sarawak	1°46'44.8"N 110°9'54"E	STG02B	KJ874195
D. setosum	Satang Island, Sarawak	1°46'44.8"N 110°9'54"E	STG07B	KJ874199
D. setosum	Kudat, Sabah	6°56'684"N 116°50'387"E	K08	KJ874192
D. setosum	Larapan Tengah, Sabah	4°33'275"N 118°36'696"E	LT07	KJ874185
D. setosum	Larapan Tengah, Sabah	4°33'275"N 118°36'696"E	LT08	KJ874186
D. setosum	Mantanani Island, Sabah	6°42.8'N 116°18.3'E	M06	KJ857078
D. setosum	Mantanani Island, Sabah	6°42.8'N 116°18.3'E	M07	KJ857079
D. setosum	Mantanani Island, Sabah	6°42.8'N 116°18.3'E	M10	KJ857082
D. savignyi	Mantanani Island, Sabah	6°42.8'N 116°18.3'E	SV01	KJ908847
D. savignyi	Mantanani Island, Sabah	6°42.8'N 116°18.3'E	SV03	KJ908848
D. savignyi	Mantanani Island, Sabah	6°42.8'N 116°18.3'E	SV04	KJ908849
E. calamaris	Satang Island, Sarawak	1°46'44.8"N 110°9'54"E	TG01	KJ908852
E. calamaris	Satang Island, Sarawak	1°46'44.8"N 110°9'54"E	TG02	KJ908853
E. calamaris	Sampadi Island, Sarawak	1°43'59.988"N 110°4'59.988"E	TG03	KJ908854
E. calamaris	Sampadi Island, Sarawak	1°43'59.988"N 110°4'59.988"E	TG04	KJ908855
E.calamaris	Sampadi Island, Sarawak	1°43'59.988"N 110°4'59.988"E	TG05	KJ908856
T. gratilla	Kudat, Sabah	6°56'684"N 116°50'387"E	GR01	KJ908857
T. gratilla	Kudat, Sabah	6°56'684"N 116°50'387"E	GR05	KJ908858
T. gratilla	Kudat, Sabah	6°56'684"N 116°50'387"E	GR06	KJ908859
T. gratilla	Kudat, Sabah	6°56'684"N 116°50'387"E	GR10	KJ908860

Total genomic DNA extraction was conducted using modified CTAB protocol (Doyle and Doyle, 1987) with addition of Proteinase-K. Optical Density reading at 260 nm and 280 nm were conducted using UV spectrophotometer model Ultraspec® 100 Pro. Amplification of approximately 600 base pair of 16S rRNA gene used a pair of primers, 16SAR forward primer (5'- CGC CTG TTT ATC AAA AAC AT-3') and 16SBR reverse primer (5'- CCG GTC TGA ACT CAG ATC ACG -3') designed by Palumbi (1996). PCR was performed in a total reaction volume of 25 µl containing 100 ng of DNA template, 5X PCR buffer, 25 mM of MgCl, 10

mM of deoxynucleotide triphosphate (dNTP), 0.2 µM of primer and 5u/µl *Taq* DNA polymerase. PCR thermal cycling profile for amplification of 18S rRNA gene started with pre-denaturation temperature of 95°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, 55°C annealing temperature for 30 seconds, and extension temperature of 72°C for 30 seconds, and a final extension at 72°C for 2 minutes.

All DNA extraction products and PCR products were later subjected to 1% agarose gel electrophoresis (AGE). The amplification products were purified using DNA purification kit (Vivantis, Kuala Lumpur, FT, Malaysia) according to the manufacturer's instructions. Then, the purified PCR products were sent to FirstBASE Sdn.Bhd. (Selangor) for the sequencing of forward and reverse strands.

Multiple sequence alignments were carried out *via* CLUSTALX program (vers. 1.81, Thompson *et al*., 1997). The aligned data were then transformed into a distance matrix using Kimura's Two Parameter Model (Kimura, 1980). Maximum likelihood (ML) was conducted based on the substitution model of Akaike information criterion (TrN + G) as selected by Modeltest 3.7 (Posada and Crandall, 1998). Confidence level of the Neighbor-Joining (NJ), Maximum Parsimony (MP) and ML were assessed using PAUP version 4.0b10 (Swofford, 2000), with only bootstrap values more than 80% were shown and regarded as sufficiently resolved topologies as Heulsenback and Hillis (1993) stated that only bootstrap values of 70% or greater indicate sufficiently resolved topologies. Bayesian approach (BA) was conducted in MrBayes version 3.1.2 (Ronquist & Huelsenback, 2003) using the same model of evolution (TrN + G) with two simultaneous metropolis-coupled Monte-Carlo Markov chains that were run for 110 000 generations. A tree was sampled every 100 generations and a consensus topology was calculated for 825 trees by omitting the first 275 trees as burn-in and the confidence level of tree nodes was indicated by posterior probabilities which they represented the true probabilities of the clades (Rannala & Young, 1996) and that probability equal or greater than 95% were considered significant (Leache & Reeder, 2002).

All sequences of *D. setosum* obtained were deposited in the Genbank with accession number of KJ874185 to KJ857082, sequences of *D. savignyi* can be obtained by the accession number of KJ908847 to KJ908849, sequences of *Echinothrix calamaris* can be obtained by the accession number KJ908852 to KJ908856 while sequences of *Tripneustes gratilla* can be obtained by the accession number of KJ908857 to KJ908860.

RESULTS & DISCUSSION

Out of the 542 bp of 16S rRNA gene obtained from *D. setosum* and *D. savignyi* samples, a total amount of 56 variables sites have been observed with 5 singleton sites and 51 (91%) parsimonious informative sites which indicates that 16S rRNA gene is a reliable marker to infer genetic variation and phylogenetic relationship among *Diadema* species. Genetic divergence analysis of *Diadema* species showed more than 30% genetic distance value with respect to the outgroup *T. gratilla* (Table 2), indicate specific recognition as the outgroup belongs to family Toxopneustidae while *D. setosum* and *D. savignyi* belong to family Diadematidae. This finding is congruent with the study conducted on echinoderm by McCartney *et al*. (2000), Sponer *et al*. (2001) and Stockley *et al*. (2005) in which more than 30% genetic distance value was recorded between ingroup and outgroup taxa using 16S rRNA gene analysis.

Moreover, genetic divergence analysis revealed genetic distance value of more than 13% recorded between species of *Diadema* and the outgroup, *E. calamaris* (Table 2). Higher genetic similarity can be observed between species of genus *Diadema* and *E. calamaris* as they are members of the same family, Diadematidae. An average value of 9.85% genetic distance was recorded among *D. setosum* and *D. savignyi* samples from Malaysian Borneo (Table 2) indicating certain degree of distinctions and also similarities which is congruent with Lessios (2001). Low number of samples involved in this study may lead to underestimation of the actual genetic variation among samples of Malaysian Borneo *Diadema*. Thus, the relationship between *D. setosum* and *D. savignyi* from Malaysian Borneo was conducted to clarify relationships among the two species.

Table 2. Average genetic distance (%) values based on 16S rRNA gene sequences analysis using Kimura Two Parameter Model (Kimura, 1980).

	D. setosum (n = 8)	*D. savignyi* (n = 3)	*E. calamaris* (n = 5)	*T. gratilla* (n = 4)
D. setosum	0.45	-		
D. savignyi	9.85	0.75	-	
E. calamaris	13.85	13.55	0.20	-
T. gratilla	31.50	32.30	33.0	2.3

Phylogenetic analyses of Malaysian Borneo Diadematidae produced essentially the same trees topologies for MP (Figure 3), NJ (not shown), BA (Figure 4) and ML (not shown) which revealed a monophyly of the Malaysian Borneo Diadematidae (including *E. calamaris* as ingroup) with respect to the outgrop *T. gratilla* (family Toxopneustidae) with bootstrap values of 100% (MP), 100% (NJ), 100% (ML) and bayesian posterior probability value of 1.00. Moreover, *E. calamaris* were later diverged from the Diadematidae forming Clade III (Figure 3 and Figure 4), excluding the *Diadema* species into another clade, with strong support as the bootstrap values are 100% (MP), 100% (NJ), 96% (ML) whereas the Bayesian Posterior Probability (BPP) value is 1.00.

Monophyly of Malaysian Borneo *Diadema* was revealed with high bootstrap values of 99% (MP), 94% (NJ), 84% (ML) and the BPP value of 98, with respect to the outgroup *E. calamaris* and *T. gratilla* (Figure 3 and Figure 4). The monophyletic grouping of Malaysian Borneo *Diadema* was further divided into two distinct monophyletic clades comprised all *D. setosum* samples into Clade I and all *D. savignyi* samples into Clade II (Figure 3 and Figure 4).

Monophyletic grouping of *D. setosum* (Clade I) has strong support with high bootstrap values of 100% (MP), 100% (NJ), 80% (ML) and BPP value of 100. Similarly, monophyletic grouping of *D. savignyi* also recorded strong support with bootstrap values of 100% (MP), 100% (NJ), 100% (ML) and BPP value of 100 (Figure 3 and Figure 4), thus one may say both species are reciprocally monophyletic.

In this study, phylogenetic relationship of genus *Diadema* from Malaysian Borneo revealed that both *D. setosum* and *D. savignyi* have distinct genetic identity showing deep split between *D. setosum* and *D. savignyi* (Figure 3 and Figure 4) which would have been sufficient to reject the suggestion by Clark (1966) that these two species are subspecies. This finding is congruent with other studies conducted by Mortenson (1940), Lessios (2001) and Lessios *et al*. (2001) in which *D. setosum* and *D. savignyi* formed two distinct monophyletic clades.

Clark (1966) claimed that due to morphological similarities of species in genus *Diadema*, identification of each species was usually based on locality. However, identification based on locality for *D. setosum* and *D. savignyi* has been chaotic until Pearse (1998) established that *D. savignyi* populations extended from the central Pacific Ocean to the Indian Ocean, while *D. setosum* populations only limited to the continental margins. Incongruent with Clark (1966) and Pearse (1998) on the geographical distributions of *D. setosum* and *D. savignyi*, sample of *D. setosum* (M06, M07 and M10) and *D. savignyi* involved in this study were collected from the same location (Mantanani Island, Sabah; 6º42.8'N 116º18.3'E) at the continental shelf of South China Sea suggesting that theory to identify species based on locality (Clark, 1966) and that *D. setosum* only existed at the continental margins (Pearse, 1998), should be nullified. In Malaysian Borneo, *D. setosum* and *D. savignyi* co-exist in the same area and have overlapping ranges, similar to the findings by Mortenson (1940) and Lessios *et al*. (2001) who reported that these two species were found to be overlapped at the western margins of the Pacific Ocean and at the Australian and African shores of the Indian Ocean.

Moreover, this study also supports the theory on *D. setosum* and *D. savignyi* as the sympatric species, based on their overlapping

ranges; thus the theory that sea urchin undergo allopatric speciation by Mayr (1954) is no longer valid. Debates on the specific status of sympatric species *D. setosum* and *D. savignyi* have been documented by many researchers for example Mortenson (1940); Mayr (1954); Clark (1966); Pearse (1998); Adel and Khalid (2000); Lessios (2001); Lessios *et al*. (2001) and Coppard and Campbell (2006). Most studies agreed that *D. savignyi* was believed to be the result of *D. setosum* annual rhythm of active spawning activities and over certain period of time, the annual rhythm of active spawning had led to the formation of sympatric species *D. savignyi* (Adel & Khalid, 2000).

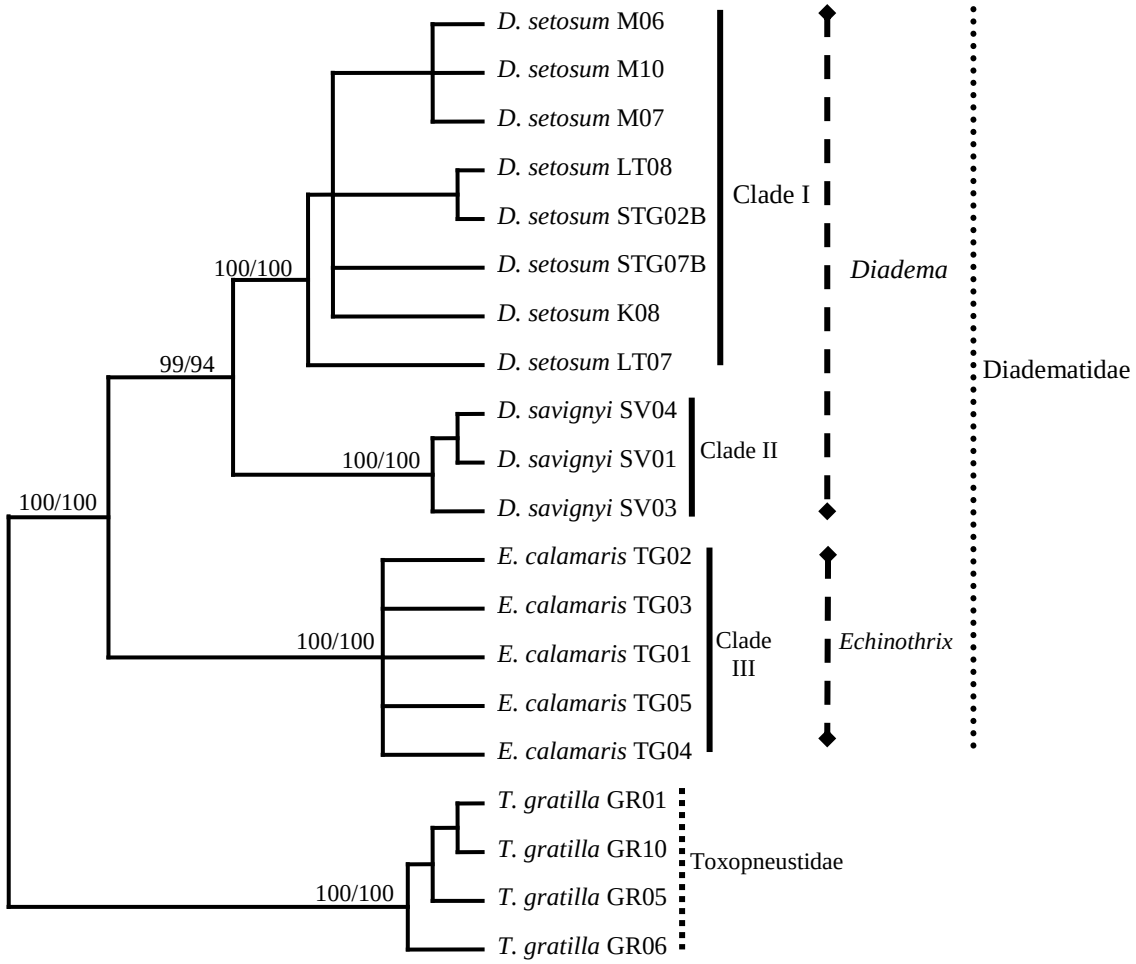

Figure 3. A maximum parsimony 50% majority rule consensus tree constructed of 16S rRNA gene sequences of *D. setosum, D. savignyi*, *E. calamaris* with *T. gratilla* as the outgroup. Bootstrap values above 90% are indicated above branch and correspond to Maximum Parsimony and Neighbour joining, respectively. Tree length is 240 with consistency index (CI) = 0.9292 and retention index (RI) = 0.9779.

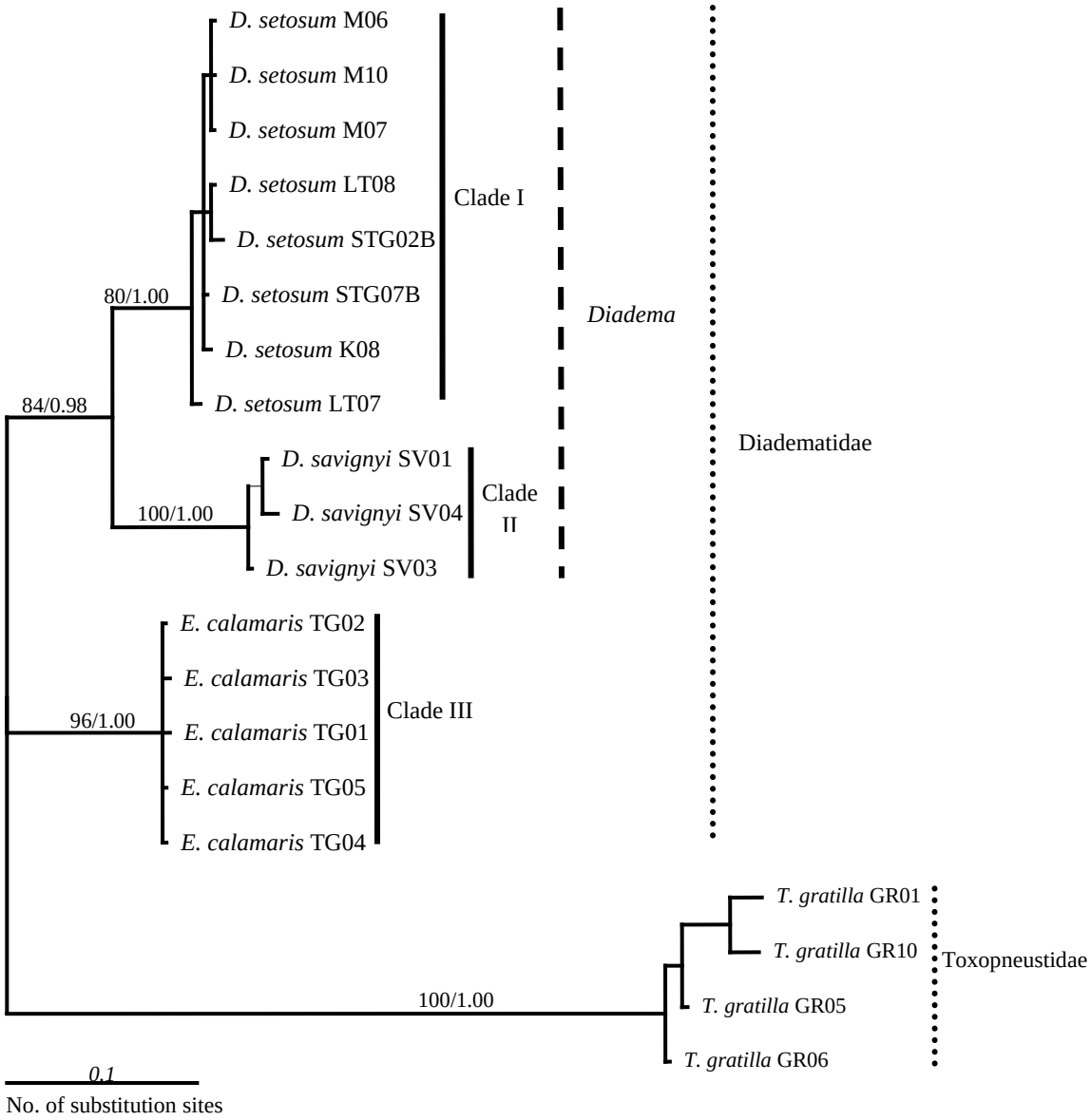

Figure 4. Bayesian inference of the 50% majority rule consensus tree of 16S rRNA gene sequences of *D. setosum*, *D. savignyi* and *E. calamaris* with *T. gratilla* as the outgroup. Bootstrap values of maximum likelihood and Bayesian posterior probabilities (BPPs) are accordingly indicated above the branch nodes.

CONCLUSION

In conclusion, based on 16S rRNA gene analysis, genus *Diadema* from Malaysian Borneo is monophyletic with respect to the outgroup, *E. calamaris* and *T. gratilla*. *D. setosum* and *D. savignyi* are reciprocally monophyletic, therefore hypothesis null is rejected. This study supports the theory on the sympatric species of *D. setosum* and *D. savignyi* based on their geographical distributions and overlapping ranges in Malaysian Borneo. Due to limited samples involved in this study, it is likely that the actual genetic variations within and among sea urchin species in Malaysian Borneo is not yet fully clarified, thus future studies should involve larger sample size collected from various localities.

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REFERENCES

Adel, H.A. & Khalid, P.L. (2000). Reproductive cycles of *Diadema setosum* and *Echinometra mathaei* (Echinoidea: Echinodermata) from Kuwait (Northern Arabian Gulf). *Bulletin of Marine Science*, 67(2): 845-856.

Clark, A.M. (1966). Echinoderms from the Red Sea, Part 2. *Bulletin of Sea Fisheries Resources Israel*, 41: 26-58.

Coppard, S.E. & Campbell, A.C. (2006). Taxonomic significance of test morphology in the echinoid genera *Diadema* Gray, 1825 and *Echinothrix* Peters, 1853 (Echinodermata). *Zoosystema*, 28: 93-112.

Doyle, J.J. & Doyle, J.L. (1987). A rapid DNA isolation procedure from small quantities of fresh leaf tissue. *Phytochemical Bulletin*, 1: 11-15.

Huelsenbeck, J.P. & Hillis, D.M. (1993). Success of phylogenetic methods in the four-taxon case. *Systematic Biology*, 42: 247-264.

Kimura, M. (1980). Kimura's two-parameter model of Models of DNA Evolution. In J. Felsenstein (Eds.), *Inferring Phylogenies.* Sunderland, Massachusetts: Sinauer Associates, Inc.

Leache, A.D. & Reeder, T.W. (2002). Molecular systematics of the eastern fence lizard (*Sceloporus undulatus*): a comparison of parsimony, likelihood and bayesian approaches. *Systematic Biology*, 51: 44-68.

Lessios, H.A. (2001). Molecular phylogeny of *Diadema*: Systematic implications. In M. Barker (Eds.), *Echinoderms 2000*. Lisse: CRC Press. Pp 487-495.

Lessios, H.A. & Pearse, J.S. (1996). Hybridization and introgression between Indo-Pacific species of *Diadema*. *Marine Biology*, 126: 715-723.

Lessios, H.A., Kessing, B.D., & Pearse, J.S. (2001). Population structure and speciation in tropical seas: global phylogeography of the sea urchin *Diadema*. *Evolution*, 55: 955-975.

Mayr, E. (1954). Geographic speciation in tropical Echinoids. *Evolution*, 8: 1-18.

McCartney, M.A., Keller, G., & Lessios, H.A. (2000). Dispersal barriers in tropical oceans and speciation in Atlantic and eastern Pacific sea urchins of the genus *Echinometra*. *Molecular Ecology*, 9: 1391-1400.

Mortenson, T. (1940). *A monograph of the Echinoidea. III(i)*. *Aulodonta*. Denmark: Reitzel. Pp 369-379.

Palumbi, S.R. (1996). PCR and molecular systematic. In D. Hillis, C. Moritz, & B. Mable (Eds.), *Molecular Systematic*, 2nd edition. Sunderland: Sinauer Press.

Pearse, J.S. (1998). Distributions of *Diadema savignyi* and *Diadema setosum* in the tropical Pacific. In R. Mooi & M. Telford (Eds.), *Echinoderms: San Francisco*. Rotterdam: Balkema. Pp 777-782.

Posada, D. & Crandall, K.A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics*, 14(9): 817-818.

Rahim, S.A.K.A., & Nurhassan, R. (2012). Edible sea urchin species in Sabah waters. *Faculty of Resource Science and Technology Bulletin*, 1: 2-3.

Rannala, B. & Yang, Z. (1996). Probability distribution of molecular evolution trees: a new method of phylogenetic inference. *Journal of Molecular Evolution*, 43: 304-311.

Ronquist, F. & Huelsenback, J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Version 3.0b4. *Bioinformatics*, 19: 1572-1574.

Sponer, R., Deheyn, D., & Roy, M.S. (2001). Large genetic distances within a population of *Amphipholis squamata* (Echinodermata: Ophiuroidea) do not support color varieties as sibling species. *Marine Ecological Progress Series*, 219: 169-175.

Stockley, B., Smith, A.B., Littlewood, D.T.J., Lessios, H.A., & MacKenzie-dodds, J.A. (2005). Phylogenetic relationships of spatangoid sea urchins (Echinoidea): taxon sampling density and congruence between morphological and molecular estimates. *Zoological Science*, 34: 447-468.

Swofford, D.L. (2000). PAUP. *Phylogenetic Analysis Using Parsimony*. Version 4.0b10. Sunderland, Massachusetts: Sinauer Associates.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., & Higgins, D.G. (1997). The ClustalX Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tool. *Nucleic Acids Research*, 24: 4876-4882.

Yokota, Y., Matranga, V., & Smolenicka, Z. (2002). *The sea urchin: From basic biology to aquaculture*. Netherlands: A.A Balkema Publication.

APPENDIX

Aligned partial of 16S rRNA of mitochondrial DNA (excluded sites with missing/ambiguous data and gaps) for Malaysian Borneo D. setosum and D. savignyi. Sequence differences are indicated with respect to the uppermost sequence dot (.) indicates base similarity and shaded colors indicate potential sites for species DNA barcoding.

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D.savignyi_SV01  TGCGAAGTTA AAACGCCGCG GTATCTTGAC CGTGCGAAGG TAGCATAATT ATTTGTCTCC TAAATAGAGA CTGGCATGAA TGGCAAGACG  [ 90]
D.savignyi_SV04  ..TTT..... .......... .......... .......... .......... .......... .......... .......... ..........  [ 90]
D.savignyi_SV03  ...A...... ..CG...... .......... .......... .......... .......... .......... .......... ..........  [ 90]
D.setosum_STG07B ..AA...... ..CG...... .......... .......... .......... .......... .......... .......... ..........  [ 90]
D.setosum_K08    ..AA...... ..CG...... .......... .......... .......... .......... .......... .......... ..........  [ 90]
D.setosum_LT08   ..AA...... ..CG...... .......... .......... .......... .......... .......... .......... ..........  [ 90]
D.setosum_STG02B ..AA...... ..CG...... .......... .......... .......... .......... .......... .......... ..........  [ 90]
D.setosum_M06    ..AA...... ..CG...... .......... .......... .......... .......... .......... .......... ..........  [ 90]
D.setosum_M07    ..AA...... ..CG...... .......... .......... .......... .......... .......... .......... ..........  [ 90]
D.setosum_M10    ..AA...... ..CG...... .......... .......... .......... .......... .......... .......... ..........  [ 90]
D.setosum_LT07   ..AA...... ..CG...... .......... .......... .......... .......... .......... .......... ..........  [ 90]

D.savignyi_SV01  GGGCCCTACT GTCTCCTTAT CTCCTACCTG AAATTCACAT CTTTGTGAAG AGGCAAAGAT AAAATCGTTA GACGAGAAGA CCCTATCGAG  [180]
D.savignyi_SV04  .......... .......... .......... .......... .......... .......... .......... .......... ..........  [180]
D.savignyi_SV03  .......... .......... .......... .......... .......... .......... .......... .......... ..........  [180]
D.setosum_STG07B ....T..... .......... ....C..... .......... .......... .......... .......C.. .......... ..........  [180]
D.setosum_K08    ....T..... .......... ....C..... .......... .......... .......... .......C.. .......... ..........  [180]
D.setosum_LT08   ....T..... .......... ....C..... .......... .......... .......... .......C.. .......... ..........  [180]
D.setosum_STG02B ....T..... .......... ....C..... .......... .......... .......... .......C.. .......... ..........  [180]
D.setosum_M06    ....T..... .......... ....C..... .......... .......... .......... .......C.. .......... ..........  [180]
D.setosum_M07    ....T..... .......... ....C..... .......... .......... .......... .......C.. .......... ..........  [180]
D.setosum_M10    ....T..... .......... ....C..... .......... .......... .......... .......C.. .......... ..........  [180]
D.setosum_LT07   ....T..... .......... ....C..... .......... .......... .......... .......C.. .......... ..........  [180]

D.savignyi_SV01  CTTAAACTAA AGCAAAAGCC TAAATCCCCC TCACCATTTA CCTAATTAAG TTCATTTAGT AAAGGCCCGA GAGATGGTTT TTTGCAGTGG  [270]
D.savignyi_SV04  .......... .......... .......... .......... .......... .......... .......... .......... ..........  [270]
D.savignyi_SV03  .......... .......... .......... .......... .......... .......... .......... .......... ..........  [270]
D.setosum_STG07B .......... ....G....T .....T.... ........C. T.....C..A ....C...A. G...AA.T.. ..A..C.... ....TG.GA.  [270]
D.setosum_K08    .......... ....G....T .....T.... ........C. T.....C..A ....C...A. G...AA.T.. ..A..C.... ....TG.GA.  [270]
D.setosum_LT08   .......... ....G....T .....T.... ........C. T.....C..A ....C...A. G...AA.T.. ..A..C.... ....TG.GA.  [270]
D.setosum_STG02B .......... ....G....T .....T.... ........C. T.....C..A ...GC...A. G...AA.T.. ..A..C.... ....TG.GA.  [270]
D.setosum_M06    .......... ....G....T .....TT... ........C. T.....C..A ....C...A. G...AA.T.. ..A..C.... ....TG.GA.  [270]
D.setosum_M07    .......... ....G....T .....TT... ........C. T.....C..A ....C...A. G...AA.T.. ..A..C.... ....TG.GA.  [270]
D.setosum_M10    .......... ....G....T .....TT... ........C. T.....C..A ....C...A. G...AA.T.. ..A..C.... ....TG.GA.  [270]
D.setosum_LT07   .......... ....G....T .....T.... ........C. T.....C..A ....C...A. G...AG.T.. ..A..C.... ....TG.AA.  [270]
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D.savignyi_SV01   TTTTAGTTGG GGCAACTGCG GAGAAGAAGA CCCTCCGCTA CAATTTAAGT TTCTAGGAAG ACTGTCCTGG AACAGTACTG AAGAGTGATC  [362]
D.savignyi_SV04   .......... .......... .......... .......... .......... .......... .......... .......... ..........  [362]
D.savignyi_SV03   .......... .......... .......... .......... .......... .......... .......... .......... ..........  [362]
D.setosum_STG07B  .......... .......... ........A. T......... TC..C....C ..T......A ...A....AA .G..AG.ACA GCA.......  [362]
D.setosum_K08     .......... .......... ........A. T......... TC..C....C ..T......A ...A....AA .G..AG.ACA GCA.......  [362]
D.setosum_LT08    .......... .......... ........A. T......... TC..C....C ..T......A ...AC...AA .G..AG.ACA GCA.......  [362]
D.setosum_STG02B  .......... .......... ........A. T......... TC..C....C ..T......A ...AC...AA .G..AG.ACA GCA.......  [362]
D.setosum_M06     .......... .......... ........A. T......... TC..C....C ..T......A ...A....AA .G..AG.ACA GCA.......  [362]
D.setosum_M07     .......... .......... ........A. T......... TC..C....C ..T......A ...A....AA .G..AG.ACA GCA.......  [362]
D.setosum_M10     .......... .......... ........A. T......... TC..C....C ..T......A ...A....AA .G..AG.ACA GCA.......  [362]
D.setosum_LT07    .......... .......... ........A. T......... TC..C....C ..T......A ...A....AA .G..AG.ACA .CA.......  [362]

D.savignyi_SV01   CACTTAGGTG ATCAAAGGAA CAAGTTACCG TAGGGATAAC AGCGTAATCT TTTCCGAGAG TTCATATTGA CAAAAAGGTT TGCGACCTCG  [452]
D.savignyi_SV04   .......... .......... .......... .......... .......... .......... .......... .......... ..........  [452]
D.savignyi_SV03   .......... .......... .......... .......... .......... .......... .......... .......... ..........  [452]
D.setosum_STG07B  ...CCT.... .......... .......... .......... .......... ...T...... .......... .......... ..........  [452]
D.setosum_K08     ...CCT.... .......... .......... .......... .......... ...T...... .......... .......... ..........  [452]
D.setosum_LT08    ...CCT.... .......... .......... .......... .......... ...T...... .......... .......... ..........  [452]
D.setosum_STG02B  ...CCT.... .......... .......... .......... .......... ...T...... .......... .......... ..........  [452]
D.setosum_M06     ...CCT.... .......... .......... .......... .......... ...T...... .......... .......... ..........  [452]
D.setosum_M07     ...CCT.... .......... .......... .......... .......... ...T...... .......... .......... ..........  [452]
D.setosum_M10     ...CCT.... .......... .......... .......... .......... ...T...... .......... .......... ..........  [452]
D.setosum_LT07    ...CCT.... .......... .......... .......... .......... ...T...... .......... .......... ..........  [452]

D.savignyi_SV01   ATGTTGGATC GGGACATCCT AATAGTGCAG AAGCTTTTAA GGGTTGGTCT GTTCGACCAT TAAAGTCCTA CGTGATCTGA GTTCAGACCG  [542]
D.savignyi_SV04   .......... .......... .......... .......... .......... .......... .......... .......... ..........  [542]
D.savignyi_SV03   .......... .......... .......... .......... .......... .......... .......... .......... ..........  [542]
D.setosum_STG07B  .......... .......... .......... .......... .......... .......... .......... .......... ..........  [542]
D.setosum_K08     .......... .......... .......... .......... .......... .......... .......... .......... .....T....  [542]
D.setosum_LT08    .......... .......... .......... .......... .......... .......... .......... .......... ..........  [542]
D.setosum_STG02B  .......... .......... .......... .......... .......... .....G.... .......... .......... ..........  [542]
D.setosum_M06     .......... .......... .......... .......... .......... .......... .......... .......... ..........  [542]
D.setosum_M07     .......... .......... .......... .......... .......... .......... .......... .......... ..........  [542]
D.setosum_M10     .......... .......... .......... .......... .......... .......... .......... .......... ..........  [542]
D.setosum_LT07    .......... .......... .......... .......... .......... .......... .......... .......... ..........  [542]
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