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Research

Phylogenetic Relationship of *Diadema*: Emphasis on The Two Distinct Clades of *D. Setosum* With The Inclusion of Long Spine Black Sea Urchin From Malaysian Borneo

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ABSTRAK

Diadema urchins (family Diadematidae) are ecologically important bioindicators of coral reef ecosystems and seagrass beds. *Diadema* urchins which are widely distributed and broadcast spawners, have been frequently utilized as model invertebrate species for zoogeography research of the Indo-West Pacific region. So far, Malaysian Borneo, located at the geographic center of Maritime Southeast Asia, has been under-sampled. This study aims to fill this sampling gap and provide the first record of *Diadema setosum* from Malaysian Borneo using genetic diagnostics to conclusively establish the clade-level identity of the species. According to Cytochrome Oxidase I gene analysis, *Diadema* is monophyletic. Seven species of *Diadema* namely *Diadema palmeri*, *Diadema clarki*, *Diadema mexicanum*, *Diadema antillarum*, *Diadema paucispinum*, *Diadema africanum*, and *Diadema savignyi*, formed their subclades with strong bootstrap values, demonstrating interspecific variation. The findings of this study provide further evidence for the presence of two distinct monophyletic clades, with all *D. setosum* individuals forming a monophyletic clade that later split into two distinct subclades, dividing Red Sea population (*D. setosum-b*) and Indo-West Pacific populations (*D. setosum-a*), supported by a significant genetic divergence value ranging from 6.3% to 9.1%. This study also revealed notable levels of nucleotide and population subdivision between the *D. setosum* from the Indo-West Pacific and the Red Sea populations (Nst = 0.891; Fst = 0.886) with a low number of migrants per generation (Nm = 0.065). This may suggest geographic isolation due to ecological factors preventing each other from surviving in the territory of the other, or that the two clades of *D. setosum* were a separate species. Additional morphological and molecular analysis is required in the future to ascertain the level of divergence and further resolve the taxonomic confusion within the genus *Diadema.*

Key words: COI gene, *D. setosum-a*, *D. setosum-b*, Indo-West Pacific, monophyletic clades

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INTRODUCTION

The *Diadema* urchins play important roles in the restoration of coral reef ecosystems throughout the globe by effectively reducing the population of benthic algae. Some of the *Diadema* species are considered the keystone herbivore throughout the Caribbean basin and the most severe die-offs ever documented for marine invertebrates between 1983 to 1984, were caused by the mass mortality of these urchins (Williams, 2022). The global reduction in coral reefs highlights the need to understand coral resilience elements and the importance of *Diadema* urchins in maintaining healthy ecosystems (Do *et al*., 2020). Eight well-known contemporary species make up the *Diadema* (family Diadematidae, order Diadematoida), including *Diadema setosum*, *Diadema savignyi*, *Diadema palmeri*, *Diadema africanum*, *Diadema paucispinum*, *Diadema antillarum*, *Diadema mexicanum* and *Diadema clarki*. Additionally, *Diadema ascensionis*, has been recognized as a subspecies of *D. antillarum*, although further study is necessary for species validation (Moore *et al*., 2019).

The benthopelagic life cycle of the *Diadema* species allows its pelagic planktotrophic larva to metamorphose and settle into benthic juveniles with a maximum stay of 40 days

(varies depending on species) making it possible for drifting over vast geographic distances, which promotes genetic homogeneity. Lessios *et al*. (2012) documented the widespread occurrence of *D. palmeri* on the northern and southern coasts of New Zealand and Australia, respectively. It has been determined that *D. setosum*, the long-spine black sea urchin, and *D. savignyi* have congruent ranges that go from the middle of the Pacific to the eastern coast of Africa. Several Atlantic Islands, including Cape Verde, Canary, and Madeira, as well as the western coast of Africa, are home to *D. africanum* (Rodriguez *et al*., 2013). *D. antillarum* can be found along the tropical Atlantic coast from Bermuda and Florida to Brazil, as well as from Madeira to the Gulf of Guinea, whereas *D. paucispinum* is primarily found in Hawaii. On the tropical eastern Pacific, including the islands of Galapagos, Clipperton, Isla del Coco, and Revillagigedos, *D. mexicanum* can be found from the Sea of Cortez to Ecuador. Due to sea urchin's high capacity for dispersal provided by planktonic larvae and the difficulty of resolving studies on the barriers to gene flow in the vast ocean, research on the process of speciation and evolutionary path of the sea urchin has been mostly complex (Mongiardino *et al*., 2018).

When it comes to identifying and defining species, molecular methods, particularly those involving mitochondrial DNA, are accurate and helpful in estimating the degree of divergence, which enables us to confirm the existence of monophyletic entities and determine the magnitude of divergence (Bronstein *et al*., 2017). *Diadema* urchins, which are widely distributed and broadcast spawners, have been utilized as model invertebrate species for research on the zoogeography of the tropical Indo-Pacific. As for the Borneo island, especially in Sabah and Sarawak, has been under-sampled. This study aims to fill this sampling gap and uncover the phylogenetic groupings of the long-spined sea urchin *D. setosum* in the Indo-West Pacific. To learn more about the origins of *Diadema* in Malaysian Borneo and its relationships with global *Diadema* urchins, we use Cytochrome Oxidase I gene sequences to provide a better phylogenetic resolution of the genus. The rapid increase in population studies to identify the expansion of the two distinct clades of *D. setosum* across the globe has also been observed (Bronstein *et al*., 2018; Artüz & Artüz, 2019; Vafidis *et al*., 2021; Vimono *et al*., 2023). In this article, we provide the first record of *D. setosum* from Malaysian Borneo using genetic diagnostics to conclusively establish the clade-level identity of the species.

MATERIALS AND METHODS

Sample collection

A total of 16 samples of *D. setosum* were collected from various locations to represent *D. setosum* samples from Malaysian Borneo (Table 1). Muscle tissues (brown to red) were collected from the lateral line inside the carapace of the *D. setosum* samples and the tissues were preserved in labeled tubes containing 70% ethanol and 5% EDTA. The samples were then kept in a cooler box and transported back to the Molecular Aquatic Laboratory, UNIMAS, stored in an ultra-low freezer at -80 °C until further DNA analysis. Gene sequences of other seven species of *Diadema* urchins (*D. palmeri*, *D. africanum*, *D. mexicanum*, *D. antillarum, D. savignyi, D. clarki,* and *D. paucispinum*) and available sequences of *D. setosum* were obtained from Genbank (Table 2).

DNA extraction and amplification

The genomic DNA was extracted from the muscle tissue of each *D. setosum* individual by using the modified CTAB protocol (Doyle & Doyle, 1987). The ethanol-preserved tissue samples were initially washed three times in wash solution (10 mM Tris-HCl and 0.1 mM EDTA) for 1 min each, which was then rinsed with ddH_20 twice to reduce the ethanol residue on samples as suggested by Littlewood and Smith (1995). All DNA extraction products were then subjected to 1% agarose gel electrophoresis. Optical density readings were taken at 230, 260, and 280 nm using a UV spectrophotometer (Ultraspec® 100 Pro). A total length of 550 bp of COI gene was amplified using the universal pair of primer, COI*f* as the forward primer (5'-AGT ATA AGC GTC TGG GTA GTC -3') and COI*e* as the reverse primers (5'- CCA CAG ATT AGA GGG AAT CAG TG-3'), designed by Palumbi *et al*. (1991). Amplifications of the COI gene were performed in a total reaction volume of 25 µL containing 100 ng DNA template, 5x PCR buffer, 25 mM MgCl, 10 mM deoxynucleotide triphosphates (dNTPs), 0.2 µM each COI*f* and COI*e* primers and 5 U/µL *Taq* DNA polymerase (Invitrogen). PCR thermal cycling profile for the amplification of the COI gene involved a pre-denaturation temperature of 95 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, 45 to 48 °C annealing temperature for 35 s, and extension at 72 °C for 30 s, and a final extension at 72 °C for 2 min.

Sequencing and data analysis

DNA sequences of *Diadema* urchins were extracted from GenBank and combined with sequences of *D. setosum* from Malaysian Borneo to illustrate the phylogenetic relationships. Sequences of *Echinothrix diadema and Echinothrix calamari* collected from GenBank have been utilized as the outgroup. *Echinotrix* and *Diadema* are members of the same class in the phylum Echinodermata, namely the class Echinoidea, which is why this outgroup was selected. This study makes sure that the comparison is pertinent to the evolutionary relationships within this particular group of sea urchins by choosing a taxonomically closed group. Multiple sequence alignments and stop codon removal were carried out via BioEdit v7.2.6 (Tom Hall, 2013, http://www.mbio.ncsu.edu/BioEdit). The aligned data were then transformed into a distance matrix using Kimura's Two Parameter model (Kimura, 1980) based on unequal base frequencies and unequal ratio of transitions to transversions (Ti:Tv) which in this case, transition generally occurs more frequently than transversion. Maximum likelihood (ML) was conducted based on the substitution model of the Akaike information criterion (TrN+I+G) as selected by Modeltest 3.7 (Posada & Crandall, 1998). The confidence level of the maximum likelihood was assessed using PAUP version 4.0b10 (Swofford, 2000), with only bootstrap values of more than 80% shown and regarded as sufficiently resolved topologies (Huelsenback & Hillis, 1993). Bayesian approach was conducted in MrBayes version 3.1.2, (Ronquist & Huelsenback, 2003) using the same model of evolution (TrN+I+G) with two simultaneous metropolis-coupled Monte-Carlo Markov chains that were run for 4,000,000 generations. A consensus topology was calculated for 2,500 trees by omitting the first 10,000 trees as burn-in and the confidence level of tree nodes was determined by posterior probabilities. This study has selected *D. setosum* populations (with more than four individuals per population) to conduct the measure of nucleotide diversity (π), net nucleotide divergence (Da), nucleotide subdivision among populations (Nst), estimate of population subdivision (Fst) and number of migrants per generation (Nm) using DNASP 5.0 (Librado & Rozas, 2009). Three localities were involved (Malaysian Borneo, Japan & Red Sea) to further illustrate the distinction between *D. setosum-a* and *D. setosum-b*.

RESULTS

Genetic divergence analysis

Approximately, 284 variables consisting of 54 singleton sites, and 230 (80.9%) parsimonious informative sites have been found, indicating that the COI gene is a reliable marker to be employed for the genetic variation analysis at the genus level. The average genetic distance between *Diadema* species and the outgroups, *E. diadema* and *E. calamaris*, was found to be 28.2% and 23.1%, respectively (Table 3), suggesting distinct taxonomic identity because the outgroup is from the genus *Echinothrix* while the remaining sequence data is from the genus *Diadema*. When comparing *D. setosum* to all other *Diadema* species, the genetic divergence study showed that the average value of ingroup taxa was 17.4%, while the average value of genetic divergence within *D. setosum* was 4.6%. Given that the Red Sea population reported a range of 6.3% to 9.1% divergence compared to other *D. setosum* populations in this study (Malaysian Borneo, Japan & Australia), the high value of divergences within species of *D. setosum* may be caused by the influence of Red Sea populations. There was a 1.8% observed difference within Malaysian Borneo groups, while 1.6% and 0.8% average divergence were detected when compared to populations in Australia and Japan, respectively. The average genetic difference between all other *Diadema* samples and *D. palmeri* was found to be about 15.2%, whereas the divergence between *D. clarki* and *D. mexicanum* and other *Diadema* samples was 12.1% and 10.1%, respectively. The average genetic divergence among the remaining four *Diadema* species (*D. paucispinum*, *D. antillarum*, *D. africanum*, and *D. savignyi*) was lower, recorded at 3.0% divergence, indicating a high degree of genetic similarity. An average genetic distance of 0.3% divergence was recorded between *D. paucispinum* from Reunion and USA, while 0.5% divergence was observed between *D. savignyi* from Japan and Guam, 0.9% between *D. savignyi* from Japan and Samoa and 1.2% between *D. savignyi* from Samoa and Guam. The genetic distance values between the samples of *Diadema* (Table 3) showed some degree of differentiation within this genus, with values ranging from 2.3% to 17.4%. This is consistent with the phylogenetic analyses of sea urchins previously conducted by Rodriguez *et al*. (2013), Chow *et al*. (2016), and Bronstein *et al*. (2017).

Table 1. *D. setosum* samples collected from Malaysian Borneo were examined for DNA sequence variation

Locality	*n*	GPS Reading	Field Voucher	GenBank Accession No.
Satang Island, Sarawak	6	1°46'44.8"N 110°9'54"E	STG02W; STG03W; STG04W; STG09B; STG10B; STG14B	OR463047 – OR463052
Sampadi Island, Sarawak	2	1°43'51.6"N 110°5'14"E	SM01; SM02	OR463053; OR463054
Tanjung Datu, Sarawak	2	2°4'27.9"N 109°38'56.9"E	TD01; TD02	OR463055; OR463056
Mantanani Island, Sabah	2	6°42'48"N 116°18'18"E	M01; M02	OR463057; OR463058
Sapi Island, Sabah	2	6°0'30.9"N 116°0'32.7"E	PS01; PS02	OR463059; OR463060
Larapan Tengah, Sabah	2	4°33'33.5"N 118°36'22.7"E	LT01; LT02	OR463061; OR463062

Table 2. Summary of DNA sequences obtained from GenBank used in this study. Sources were from Lessios *et al*. (2001); Rodriguez *et al*. (2013); Chow *et al*. (2014;2016); and Bronstein *et al*. (2018)

Species	*n*	Sample Id	Sampling Location	GenBank Accession No.
D. setosum	1	Dok15	Okinawa, Japan	AY012747
(*n*=13)	1	WAW14	Australia	AY012746
	4	DEL1	Red Sea	AY012732
		DEL2		AY012733
		JoMSS180214_B		KY817840
		JoMSS180214_C		KY817841
	5	DST4 – DST8	Arasaki, Japan	AB909925 – AB909929
	2	DST9	Okinawa, Japan	AB909930
		DST10		AB909931
D. clarki	1	Seto16	Japan	AY012745
(*n*=6)	1	DSM6	Marshall Island	AY012744
	4	AT1; AT3; AR54; AR59	Arasaki, Japan	LC037355 – LC037359
D. mexicanum	2	DM1	Panama: Taboguilla Island	AY012734
(*n*=2)		DM3		AY012735
D. *palmeri*	2	DPNZ1.5	New Zealand	AY012736
(*n*=2)		DPZE2		AY012737
D. savignyi	1	GSA1	Guam	AY012743
(*n*=4)	1	SA10	Samoa	AY012742
	2	DSV23	Japan	AB909954
		DSV26		AB909957
D. paucispinum	2	REU6	Reunion: Ebang Sal, Fringing Reef	AY012741
(*n*=4)		REU5		AY012740
	2	HI19	USA: Oahu Island	AY012739
		HI15		AY012738
D. africanum	1	TFMCBMEQ	Canary Island, Spain	KC622343
(*n*=1)				
D. antillarum	2	DCA3	Canary Island, Spain	AY012731
(*n*=5)		DCA2		AY012730
	2	DA9441	Panama: Fort Randolph	AY012729
		DA9414		AY012728
	1	TFMCBMEQ/00237	Cuba: Juventud Island	KC622344

Table 3. Genetic divergences average values (%) based on COI gene sequences analysis. Bold numbers refer to divergence values within species

		1	2	3	4	5	6	7	8
1	*D. setosum*	**4.6**							
2	*D. palmeri*	17.4	**0.4**						
3	*D. clarki*	14.2	13.0	**0.3**					
4	*D. mexicanum*	15.8	14.7	10.1	**0.2**				
5	*D. paucispinum*	15.7	15.5	10.5	5.9	**1.1**			
6	*D. antillarum*	16.1	14.7	10.0	4.4	3.1	**0.3**		
7	*D. africanum*	17.4	15.4	10.2	4.4	2.9	2.3	**0.0**	
8	*D. savignyi*	17.1	15.5	10.6	4.9	3.7	3.2	2.5	**0.7**
9	*E. diadema*	24.3	25.9	28.3	30.5	29.3	28.5	28.8	29.9
10	*E. calamaris*	22.2	23.6	22.1	21.6	23.7	22.5	24.0	25.2

Phylogenetic analysis

Phylogenetic analyses of the genus *Diadema* produced fundamentally similar tree topologies of maximum parsimony , neighbor-joining , maximum likelihood, and Bayesian inference (Figure 1) which revealed a clear separation of all species in genus *Diadema* concerning the outgroup *E. diadema* and *E. calamaris* (Family Diadematidae) with significant bootstrap support of 100% (ML) and Bayesian posterior probability of 1.00 (BPP). *D. setosum* split from the outgroup and other species of *Diadema*, subsequently creating a distinct group. This divergence is supported by substantial bootstrap values of 100% (ML) and a Bayesian posterior probability (BPP) of 1.00. The monophyletic group consisting of *D. setosum* further formed two distinct clades. One of these clades, referred to as *D. setosum-b*, is comprised of the Red Sea population which is genetically separated from the remaining *D. setosum* samples. This separation is supported by strong bootstrap values of 100% (ML), and BPP 1.00, as depicted in Figure 1. Lessios *et al*. (2001) and the latest study by Bronstein *et al*. (2017) had previously described the *D. setosum-b* clade as a separate group of *D. setosum*, and two more samples (KY817840 and KY817841) from the Red Sea are included in this work to further clarify its relationship with other *D. setosum* samples from around the world.

Numerous studies have been carried out to establish and define the existence of two genetic clades (*D. setosum-a* & *D. setosum-b*) such as by Lessios *et al*. (2012), Rodriguez *et al*. (2013), Chow *et al*. (2014; 2016) and Bronstein *et al*. (2017). The findings of this study indicate that the *D. setosum* samples collected from Malaysian Borneo belonged to clade *D. setosum-a*, alongside samples from Japan (Okinawa & Arasaki) and Northwest Australia supported by significant bootstrap support of 99% (ML), and BPP 1.00. No distinct clade of *D. setosum* has been observed to be grouped based on geographical locations, except for the Red Sea populations. The remaining *D. setosum* in the Indo-West Pacific region are clustered together forming clade *D. setosum-a*, with relatively low genetic divergence (ranging from 0.2% to 2.9%). Apart from the *D. setosum* clade, in the clade which included all other extant species of *Diadema*, *D. palmeri* was the first to diverge with significant bootstrap support of 100% (ML), and 1.00 BPP, followed by *D. clarki* with significant bootstrap support of 99% (ML), and 1.00 BPP, and *D. mexicanum* with substantial bootstrap support of 100% (ML), and 1.00 BPP (Figure 1). The sister clade to *D. mexicanum* comprises four distinct species, namely *D. savignyi*, *D. africanum*, *D. antillarum,* and *D. paucispinum* supported by significant bootstrap values of 100% (ML), and 1.00 BPP, with a relatively low average value of genetic divergences (3.0%) among the four species, indicating a close phylogenetic relationship between the four species.

When compared to other *Diadema* species, *D. setosum* exhibited a notably high genetic divergence value, as indicated in Table 3. This finding suggests that *D. setosum* is distinct from other species of *Diadema*. In addition, a significant genetic divergence value ranging from 6.3% to 9.1% was evident between *D. setosum-a* (Indo-West Pacific region) and *D. setosum-b* (Red Sea). This finding provides further evidence for the presence of two distinct monophyletic clades, labeled as clade a and clade b, within the *D. setosum* samples. This finding is supported by previous and current genetic studies conducted by Rodriguez *et al*. (2013), Chow *et al*. (2016), Bronstein *et al*. (2018); Artüz and Artüz (2019); Vafidis *et al*. (2021); Vimono *et al*. (2023) on *D. setosum*. Nevertheless, further investigation is necessary to confirm the distinct genetic identity of the two monophyletic clades of *D. setosum* through a more detailed analysis of their morphology and molecular characteristics. The present study has chosen populations of *D. setosum* that possess more than four individuals per population, as the subject of investigation to assess various genetic parameters, including nucleotide diversity (π),

net nucleotide divergence (Da), among-population nucleotide subdivision (Nst), estimate of population subdivision (Fst), and number of migrants per generation (Nm).

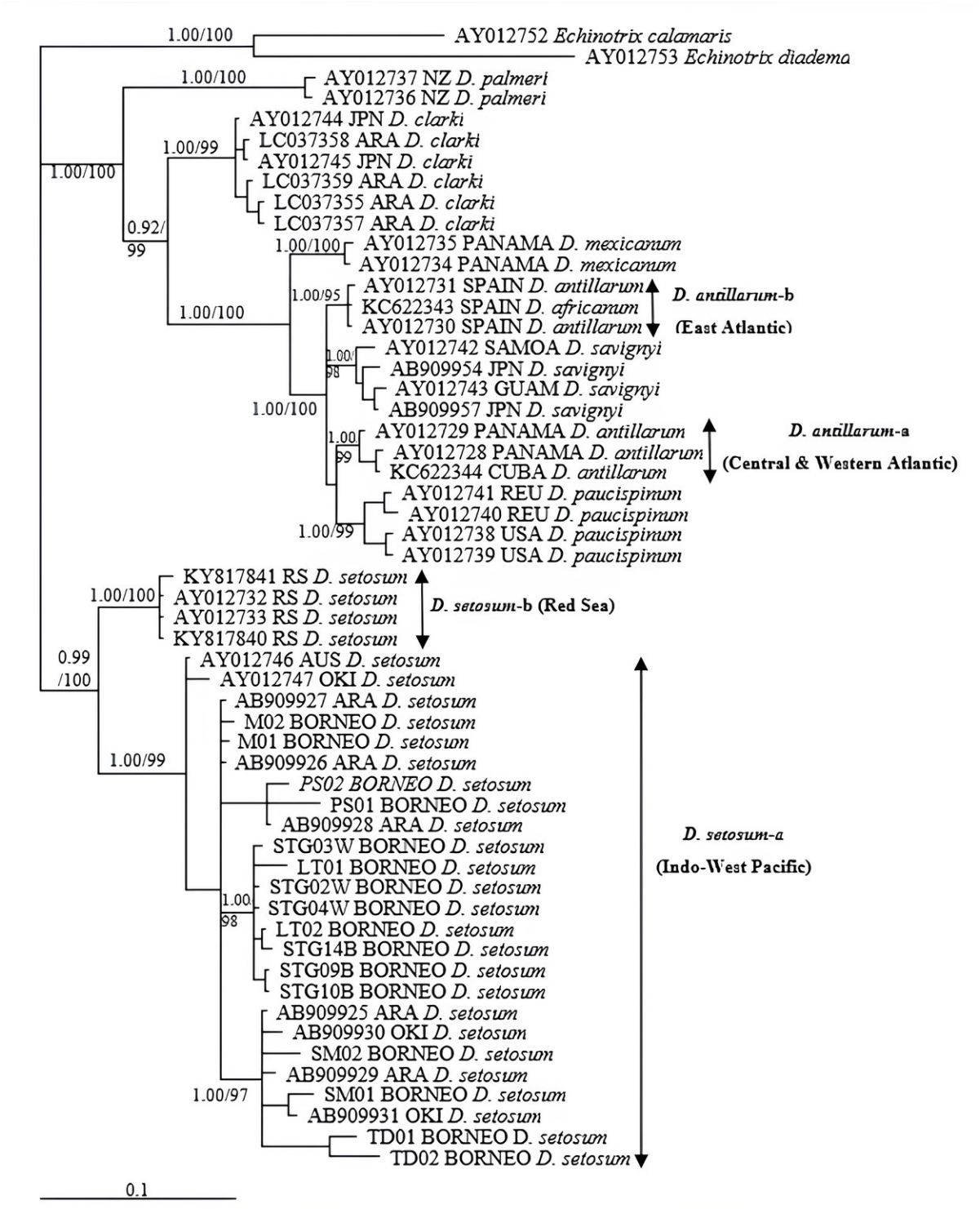

Fig. 1. Bayesian inference of the 50% majority rule consensus tree of COI gene sequences of *Diadema* urchins with *E. diadema* and *E. calamaris* as the outgroup. Bootstrap values of Bayesian posterior probabilities (BPPs) and Maximum Likelihood are accordingly indicated above the branch nodes.

According to the findings presented in Table 4, a total of 20 haplotypes were identified through the analysis of COI gene sequences of 28 *D. setosum* samples from three different geographical locations. The highest level of nucleotide diversity (π), indicating genetic variation, was observed within the Malaysian Borneo population, with a value of 0.0112. Conversely, the Red Sea population exhibited the lowest nucleotide diversity (π) at a value of 0.0029. The nucleotide diversity (π) value between the

Malaysian Borneo and Japan populations was measured as 1.04%, whereas the value between the Malaysian Borneo and Red Sea populations was 3.2%, and 3.6% between the Japan and Red Sea populations. The net nucleotide divergences (Da) exhibited a lower value of 0.04% across Indo-West Pacific populations, in contrast to the range of 5.7% to 5.9% observed between the Red Sea and Indo-West Pacific populations (Table 5).

Furthermore, the lowest level of nucleotide subdivision (Nst = 0.035) and population subdivision (Fst = 0.035) were observed between the populations of Malaysian Borneo and Japan (Arasaki & Okinawa) with the highest number of migrants per generation (Nm = 8.64) (Table 6). This finding supports the tree topologies, low divergence values, and low net nucleotide divergence data between Malaysian Borneo and Japanese populations and suggests that there is evidence of gene flow between populations, which may be attributed to either recent population expansions or historical connections. This study also reveals notable levels of nucleotide and population subdivision between the *D. setosum* populations of Japan and the Red Sea (Nst = 0.913; Fst = 0.909) with the lowest number of migrants per generation (Nm = 0.05). Similarly, the Malaysian Borneo population displayed significant divergence from the Red Sea population (Nst = 0.868; Fst = 0.863) with a low number of migrants per generation (Nm = 0.08). These findings provide further support for the substantial divergence (ranging from 6.3% to 9.1%) between the Indo-West Pacific region and the Red Sea population of *D. setosum*. This divergence is also evident in the formation of separate clades in the phylogenetic trees (Figure 1).

Table 4. Measures of haplotypes, percent pairwise divergence, gene diversity, and nucleotide diversity (π) within three populations of *D. setosum* analyzed by location using COI gene sequences

Locality	N	H	Percent Pairwise Divergence[a]	Nucleotide Diversity (π)[a,b]
Malaysian Borneo	16	12	0% - 3.6%	0.0112
Japan	8	6	0% - 1.7%	0.0085
Red Sea	4	2	0% - 0.6%	0.0029

N, number of individuals. H, number of haplotypes. [a]Estimated using Kimura two-parameter model (Kimura, 1980). [b]Sites with gaps were completely excluded

Table 5. Measures of nucleotide diversity (π) and net nucleotide divergence (Da) among three populations of *D. setosum* analyzed by location using COI gene sequences

Locality	Nucleotide Diversity (π)[a,b]	Net Nucleotide Divergence (Da)
Malaysian Borneo – Japan	0.01048	0.00035
Malaysian Borneo – Red Sea	0.03200	0.05695
Japan – Red Sea	0.03646	0.05864

[a]Estimated using Kimura two-parameter model (Kimura, 1980). [b]Sites with gaps were completely excluded

Table 6. Measures of nucleotide subdivision (N_{st}), estimate of population subdivision (F_{st}), and number of migrants per generation (N_m) based on COI gene sequences for three populations

Locality	Nucleotide Subdivision (N_{st})[a]	Estimate of Population Subdivision (F_{st})[b]	Number of Migrants per Generation (N_m)[b]
Malaysian Borneo – Japan	0.868	0.863	0.08
Malaysian Borneo – Red Sea	0.035	0.035	8.64
Japan – Red Sea	0.913	0.909	0.05

[a]Estimated using Lynch and Crease (1990). [b]Estimated using Hudson *et al*. (1992)

DISCUSSION

The identification of species formation patterns can be achieved through the evaluation of recently diverged species, whose primary differentiation may align with many postulated speciation pathways. The utilization of molecular tools and methodologies has facilitated the study of the geography, pace, and mechanisms of marine speciation. Additionally, these breakthroughs have enabled researchers to gain a better understanding of the closely related species that are undergoing divergence within a diverse range of taxa (Vafidis *et al*., 2021). Phylogenetic analysis revealed that *D. palmeri* diverged from the other *Diadema* species and formed its subclade with a high bootstrap value which implies that Baker's (1967) judgments to incorporate *D. palmeri* within the genus *Diadema* is appropriate. *D. clarki* formed a subclade comprising six samples collected from Arasaki and various other regions in Japan, separated from other *Diadema* species. The present study has combined the COI gene sequences of *D. clarki* obtained from GenBank, which were previously designated as *Diadema-sp*. The species in question has been recently confirmed as *D. clarki* in the study conducted by Chow *et al*. (2016) and this species was previously identified as *D. savignyi*-like individuals and *D. setosum*-like individuals due to

similar characteristics. There has been some ambiguity in the past regarding the correlation between *D. setosum*, *D. savingyi*, and *D. clarki* (formerly identified as *Diadema* sp.). According to Chow *et al*. (2016), *D. clarki* was known as *D. savignyi*-like individuals and regarded as its subspecies for more than 50 years because of the geographic distributions of all three species in Japanese waters, but current morphological and molecular studies have confirmed *D. clarki* as a distinct species. The present study utilized all accessible sequences of *D. clarki* to establish a more thorough phylogenetic structure for *Diadema* urchins. This study provides evidence for the separation of *D. clarki* from *D. setosum* and *D. savignyi*, as indicated by the significant genetic divergence observed (Table 3). Furthermore, the establishment of a unique subclade is supported by a high bootstrap value (Figure 1). Chow *et al*. (2016) have noted that the misidentification of *D. clarki* as *D. savignyi* has occurred in many locations, including Iki Island, Sagami Bay, and the coastal areas of Okinawa. This misidentification is attributed to the physical similarities between these two species. Chow *et al*. (2014) subsequently verified the high abundance of *D. clarki* in the mainland waters of Japan, while noting that *D. savignyi* is primarily confined to the southern island and exhibits lower abundance in the mainland waters of Japan. This further highlights the necessity of integrating molecular and morphological analyses as a means of confirming the classification of *Diadema* species.

D. savignyi exhibited greater divergence from its sister clade, resulting in the formation of a distinct subclade consisting only of *D. savignyi* samples from Guam, Samoa, and Japan (Figure 1). Notably, no discernible clade formation was observed depending on geographical locales. Although the differences in their pedicellariae make *D. savignyi* and *D. setosum* easy to differentiate by morphological assessment, there has been much uncertainty over their regional distributions and specific status. Nevertheless, this matter was successfully addressed through the utilization of isozyme data and mtDNA data (Lessios *et al*., 2001), which provided validation for the distinct classification of these two species. The findings of this study also revealed high genetic divergence (17.1%) between *D. setosum* and *D. savignyi* with the two species forming different subclades in the phylogenetic trees (Figure 1). Besides, *D. mexicanum* later formed a distinct subclade (Figure 1) supported with high bootstrap values. Recently, *D. mexicanum* and *D. antillarum* have been identified as geminate species with geographical distributions that are shared between the two species (Lessios *et al*., 2012). However, biogeographic and geological data support the separation of the eastern Pacific population from the central Pacific population due to the existence of the eastern Pacific barrier before the rise of the Isthmus of Panama. *D. antillarum*, *D. paucispinum*, and *D. savignyi* continued to be connected after the Isthmus of Panama was completed, with the single connecting path encircling the southern tip of Africa, thereby separating *D. africanum* from these three species (Precht & Precht, 2015).

Phylogenetic analysis also revealed a close relationship between *D. africanum* and *D. antillarum* (Figure 1). *D. antillarum*-b is closely related to *D. africanum* with zero (0%) divergence and supported by significant bootstrap values of 95% (ML) and BPP 1.00 (Figure 1) which are not previously illustrated by earlier studies. *D. antillarum-b* was formerly identified as *D. aff. antillarum* in which a polytomy was observed in phylogenetic reconstruction, based on subsequent studies on *D. antillarum* by Hernandez *et al*. (2006) and Clemente *et al*. (2007). In a recent study, Rodriguez *et al*. (2013) performed a comprehensive analysis of both morphological and molecular characteristics to confirm the distinctiveness of *D. antillarum-b* from *D. antillarum*. As a result, this species has now been officially recognized and designated as *D. africanum*. By the findings of Rodriguez *et al*. (2013), phylogenetic analysis (Figure 1) in this study also showed that *D. africanum* was a genetically different entity, forming a separate clade from *D. antillarum* (AY012729, AY012728 & KC622344). Furthermore, it was observed that *D. africanum* exhibited a close genetic relationship with *D. antillarum-b*, with no apparent genetic divergence shown among the analyzed samples and corrections on species status for *D. antillarum* AY012731 and AY012730 (obtained from GenBank) must be noted as it is now recognized as *D. africanum*, a separate genetic entity from *D. antillarum*. In this study, two other samples of *D. antillarum* were used to illustrate the phylogenetic relationship with *D. africanum,* and clearly, the two species are distinct genetic entities from *D. antillarum*, forming different sister taxa with the inclusion of *D. africanum* sample, supported by significant bootstrap value (Figure 1).

The findings of this study demonstrate that *D. setosum* exhibits a distinct separation from all other species within the *Diadema* genus, which aligns with previous research conducted on *Diadema* (Lessios *et al*., 2012; Chow *et al*., 2016; Bronstein *et al*., 2017). The divergence of *D. setosum* from other *Diadema* species (Figure 1) could perhaps be attributed to significant fluctuations in global sea levels that occurred during the Miocene, as reported in prior studies (Bronstein *et al*., 2016). Lessios *et al*. (2001) reported that *D. setosum* underwent additional divergence approximately 3 to 5 million years ago, resulting in the formation of two distinct clades. One of these clades is predominantly distributed in

the Indo-West Pacific region (referred to as *D. setosum-a*), while the other clade is primarily found near the Arabian Peninsula (referred to as *D. setosum-b*). Similarly, this study also found two monophyletic clades in *D. setosum*, where samples from Malaysian Borneo, Australia, and Arasaki and Okinawa, Japan, formed the Indo-West Pacific clade, separating the Red Sea populations into another clade (Figure 1). Similar to these findings, several studies such as Chow *et al*. (2016), Bronstein *et al*. (2018), Artüz and Artüz (2019), Vafidis *et al*. (2021), and Vimono *et al*. (2023) have documented the presence of divergence within *D. setosum*, leading to the isolation of populations in the Arabian Peninsula from those in the Indo-West Pacific region.

It is postulated by researchers that *D. setosum-b* has its origins in the Red Sea and afterward underwent allopatric speciation, leading to its dispersion into the Persian Gulf and the surrounding regions of the Arabian Peninsula. However, other than the physical distance between the Indo-West Pacific populations and the Red Sea of *D. setosum*, there does not appear to be any current barrier that could still exist between these two clades. However, a low number of migrants per generation is reported in this study (Nm = 0.05 to 0.08) between Malaysian Borneo and Japanese populations with the Red Sea populations supported by high divergence values and the formation of distinct monophyletic clade. The value of divergence between *D. setosum-a* and *D. setosum-b* was even higher than interspecific variation of species within *Diadema* urchins. This may suggest that geographic isolation occurs due to ecological factors preventing each other from surviving in the territory of the other, or that interbreeding is restricted causing the two clades of *D. setosum* to be separate species thus *D. setosum* from the Indo-West Pacific to remain isolated from those of the Arabian Peninsula (Bronstein *et al*., 2018; Artüz & Artüz, 2019; Vafidis *et al*., 2021). More studies should be conducted to validate this species to determine whether chaotic taxonomic occurs or cryptic species are possible in *D. setosum*.

CONCLUSION

In conclusion, COI gene analysis of the genus *Diadema* revealed that each *Diadema* species formed its subclades supported by a high bootstrap value, thereby demonstrating interspecific variation. *D. setosum* formed a monophyletic clade, which was further subdivided into two subclades, isolating the Red Sea population from those of the Indo-West Pacific. This study also supports the notion that *D. setosum* possesses a high degree of intraspecific variation that is capable of grouping the species based on locality. Findings revealed that *D. setosum* samples from Malaysian Borneo belong to clade *D. setosum-a* alongside *D. setosum* samples from Japan and Northwest Australia, isolating the Arabian Peninsula and the Red Sea into clade *D. setosum-b* supported by high genetic divergence values, high nucleotide diversity, high population subdivision, and very low number of migrants per generation. The phylogenetic trees also revealed a high level of genetic diversity between these two clades. The difficulty of finding reliable morphological characters for distinguishing *Diadema* urchin points to the need to combine molecular and morphological assessments to clearly illustrate the relationship and phylogeny of the genus *Diadema*. Future research should focus on the existence of morphotypes within Genus *Diadema* and enhance efforts for species validation. To validate that *D. setosum-b* is a distinct species from *D. setosum*, additional morphological and molecular analysis is required in the future, to further resolve the taxonomic confusion within genus *Diadema*.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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