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Unexpected discovery of *Diadema clarki* in the Coral Triangle

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Abstract

Sea urchins of the genus *Diadema*, key herbivores in coral reef ecosystems, also provide habitat for other organisms. Our research extended *Diadema* biogeography in seaways east and west of Sulawesi and identified *Diadema* species associated with the endemic Banggai cardinalfish (*Pterapogon kauderni*) using field surveys and molecular DNA barcoding methods. Field observations (20 sites, $n = 11{,}223$) found urchins with morphological phenotypes typical of *Diadema setosum* (≈ 74%, all sites), *D. savignyi* (≈ 24%, 19 sites) and atypical or mixed traits (≈ 2%, 19 sites). Distribution of these phenotype groups across the three main habitat types (i.e. coral reef, reef flat and seagrass beds) differed significantly ($\chi^2 = 533.03$, $p < 2.2e^{-16}$), indicating overlapping but non-equivalent ecological niches. *Pterapogon kauderni* associated with all urchin morphological phenotypes present. *Diadema* mtDNA CO1 sequences were obtained from tissue samples collected (4 sites, $n = 62$) from specimens with typical *D. savignyi* and *D. setosum* phenotypes. Phylogenetic tree analysis resolved the sequences into four clades. Three clades from our analysis were identified as *D. savignyi*, *D. setosum* and *D. clarki* based on additional sequences obtained from GenBank. This unexpected first record of *D. clarki* mtDNA in the Coral Triangle implies a substantial extension of the known range of this recently resurrected species. Our findings indicate the occurrence and/or introgression of *D. clarki* may be widespread, and misidentification of *Diadema* urchins based on external morphology may be relatively common. Further research is required to determine the distribution and functional roles of Indo-Pacific *Diadema* species, contributing to our understanding of processes underpinning biodiversity.

Keywords Diadematidae · Cryptic species · Range extension · Biogeographic phylogeny · mtDNA barcode · Congeneric hybridisation

Introduction

The mechanism by which marine biodiversity is generated and persists is a key question for biogeography and biodiversity conservation (Carpenter et al. 2011; von der Heyden et al. 2014). This issue is of particular relevance in the Coral Triangle region (Barber 2009), widely acknowledged as a globally important marine biodiversity 'hotspot' (Allen 2008; Veron et al. 2009). Carpenter et al. (2011) group the mechanisms put forward to explain the concentration of marine biodiversity in the Coral Triangle under four headings: (i) accumulation (inwards dispersal from speciation in peripheral areas), (ii) origin (vicariant speciation driven by the interactions of marine currents, sea level rise and fall and complex island geography), (iii) overlap (at the interface of Indian Ocean and Pacific Ocean biota) and (iv) refuge (survival of relict populations extinct elsewhere). These mechanisms may operate independently or in synergy and at multiple scales.

In the context of marine biodiversity and biogeography, molecular biology tools are providing new insights as well as raising new questions (Palumbi 1994; Bucklin et al. 2011; Bowen et al. 2013). The exponential increase in cryptic or sibling species detection across marine taxa as diverse as vertebrates including reef-associated fishes (Victor 2015) and diadromous fishes (Watanabe et al. 2009), Cnidaria (Holland

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et al. 2004; Ladner and Palumbi 2012), Porifera (Erpenbeck et al. 2017), Mollusca (Huelsken et al. 2013) and Echinodermata (Uthicke et al. 2010; Mah and Blake 2012; Bribiesca-Contreras et al. 2013; Chow et al. 2016; Egea et al. 2016; Addison and Kim 2018) presents a particular challenge with implications for our knowledge of biodiversity and its management (Bickford et al. 2007; Carpenter et al. 2011).

The phylum Echinodermata is one taxon within which contrasting evolutionary and distribution patterns and processes have been proposed at global and regional scales (Lessios et al. 2001; Mah and Blake 2012; Stöhr et al. 2012). Many echinoderms, and in particular the class Echinoidea (sea urchins), are ecologically important members of the biodiverse biotic communities living in tropical coastal ecosystems (Birkeland 1989; Lawrence 2006, 2013). The predominantly herbivorous urchins of the genus *Diadema* Gray, 1825, Diadematidae, are thought to play a role in coral reef resilience under anthropogenic stresses, in particular through regulating algal biomass and thereby providing substrate for coral recruitment (Birkeland 1989; Aronson and Precht 2006; Carpenter and Edmunds 2006; Maciá et al. 2007; Mumby et al. 2007; Lessios 2016). The slow and uneven recovery to date of *Diadema antillarum* Philippi, 1845 (Lessios 2016; Rogers and Lorenzen 2016) indicates that, once depleted beyond a certain point, *Diadema* populations may not easily return to their former abundance and distribution, entailing long-term loss of ecosystem services. Conversely, at high densities, diadematid urchin grazing can result in damaging levels of bioerosion (Carreiro-Silva and McClanahan 2001). As productive and highly biodiverse tropical coastal ecosystems (in particular coral reefs and seagrass meadows) come under increasing threat from global change and local anthropogenic impacts (Hoegh-Guldberg et al. 2017; Unsworth et al. 2018), maintaining healthy, balanced urchin populations should promote ecosystem resilience and thus support the maintenance of biodiversity.

The long-spined urchins of the genus *Diadema* also provide habitat for a variety of associated species (Magnus 1967; Coppard and Campbell 2004). One such association with implications for biodiversity conservation is the relationship between the Banggai cardinalfish *Pterapogon kauderni* Koumans, 1933 and these urchins (Kolm and Berglund 2003; Moore et al. 2012; Ndobe et al. 2013a). Traded as a marine ornamental fish since the 1990s (Lunn and Moreau 2004), the International Union for Conservation of Nature (IUCN) Red List evaluation (Allen and Donaldson 2007) places *P. kauderni* in the Endangered category, with overexploitation and habitat loss or degradation as major threats to the species. First described from two specimens collected from the waters around Banggai Island (Koumans 1933), the known native (endemic) distribution of this species is limited to around 34 small islands in or adjacent to the Banggai Archipelago, Indonesia (Vagelli 2011). The exceptionally fine-scale genetic population structure of *P. kauderni* (Hoffman et al. 2005; Vagelli et al. 2009) indicates a minimum of 21 evolutionarily significant units (sensu Moritz 1994) within this endemic distribution (Moore et al. 2017a). Several introduced *P. kauderni* populations have become established outside the native (endemic) range of this species, mostly along the aquarium trade routes from the Banggai Archipelago to major ornamental fish trading and export centres in Indonesia (Moore and Ndobe 2007; Lilley 2008; Vagelli 2011; Ndobe et al. 2013b, 2018a).

A sharp increase in *Diadema* urchin exploitation (mainly for human consumption, also as feed for carnivorous fish grow-out) has greatly reduced the abundance of *Diadema* populations at several sites in the Banggai Archipelago, Indonesia (Moore et al. 2012, 2019; Ndobe et al. 2018b, 2019). The resultant decline in microhabitat provided by these urchins is considered a major threat to endemic *P. kauderni* populations in the Banggai Archipelago, Indonesia (Moore et al. 2012, 2017a, 2019; Ndobe et al. 2013b, 2017, 2018a, b, 2019).

Knowledge of *Diadema* biogeography is thus important in the context of biodiversity conservation, at ecosystem and species levels. In contrast with the Caribbean, where the genus *Diadema* is represented by one species (*Diadema antillarum* Philippi, 1845), at least 3 species are found in the Coral Triangle region of the Indo-Pacific: *Diadema setosum* Leske, 1778; *D. savignyi* Audouin, 1809; and *D. paucispinum* Agassiz, 1863 (Lessios et al. 2001). Furthermore, some diadematid species found within this region are known to hybridise in the laboratory and in the wild, in particular *D. savignyi* and *D. setosum* (Uehara et al. 1990; Lessios and Pearse 1996). Ecological and lifestyle traits may differ between these congeneric species, for example grazing and erosion rates (Carreiro-Silva and McClanahan 2001; Bronstein and Loya 2014), microspatial preferences (McClanahan 1988) and response to environmental conditions such as temperature (Chow et al. 2016).

Perhaps inevitably in view of the vast extent of the Indo-Pacific, sampling has often been patchy in large-scale biogeographic studies (von der Heyden et al. 2014). Still the most comprehensive study on the biogeography and biodiversity of the genus *Diadema* using molecular biology methods, the seminal global phylogeography of Lessios et al. (2001) did not include any Indonesian specimens. Despite (non-molecular) survey data from the Spermonde Archipelago, Makassar Strait (de Beer 1990) and certain areas of North Sulawesi (Pearse 1998), there is a dearth of data on *Diadema* species diversity and biogeography in Indonesia.

The goal of our research was to examine the biogeography of urchins within the genus *Diadema* around Sulawesi, a poorly studied region of the Coral Triangle marine biodiversity hotspot, in the context of the association between these species and the Banggai cardinalfish *Pterapogon kauderni*. Specific

objectives were to elucidate the biodiversity and distribution of *Diadema* sea urchins east (Gulf of Tolo/Banggai Archipelago) and west (Makassar Strait/Palu Bay and Spermonde Archipelago) of Sulawesi Island, and to identify the *Diadema* urchin species associated with the restricted-range endemic Banggai cardinalfish *P. kauderni*.

Material and methods

Field survey

Surveys were carried out at 20 sites in the Banggai Archipelago and Makassar Strait (Table 1; Fig. 1). A more detailed map showing the Banggai Archipelago (survey sites 1–14) and surrounding area (survey site 15) is provided in Online resources (ESM 2). Site location was recorded using Garmin GPS units (WGS 84 datum). At each site, data were collected within a minimum of 10 transects, each approximately 100 m^2 in area. These transects comprised belt transects (20 m × 5 m, comprising 2.5 m either side of a 20-m-reel tape measure) and/or swim survey transects (approximately 40 × 2.5 m, estimated with calibrated fin kicks).

For the 14 sites in the *P. kauderni* native distribution, the dominant habitat in each transect was noted as belonging to one of three types: coral reef (CR), dominated by hard corals, typically furthest from shore; reef flat (RF), substrate dominated by sand and rubble with a variety of sessile benthic organisms including hard coral colonies and some seagrasses, typically intermediate distance from shore; and seagrass bed (SG), with seagrasses as the visually dominant benthic cover, typically closest to shore. At most sites, all three habitat types were present; however, at Toado (site 14), extensive seagrass meadows extended seawards from underneath the prop roots of dense mangrove stands (genus *Rhizophora* Linnaeus, 1753).

Field identification of *Diadema* urchins as *Diadema setosum* (Leske, 1778) or *D. savignyi* (Audouin, 1809) followed Chow et al. (2014, 2016), based on three phenotypic characteristics: anal cone colour pattern, interambulacral spots and iridophore line pattern (Fig. 2). Individuals were recorded as follows: (i) *Diadema setosum*: orange ring on the anal cone, with five white interambulacral spots and dotted blue iridophore lines in the naked space of the interambulacral areas (Fig. 2a); (ii) *Diadema savignyi*: Y-shaped continuous (partially double) blue iridophore lines running along the naked space of the interambulacral areas, interambulacral spots generally absent (occasionally very small white or pink spots in the crux of the Y formed by the iridophores), and no orange ring on the anal cone (Fig. 2b); (iii) undetermined: any phenotype other than (i) or (ii). For the 14 native sites, the distribution of *Diadema* morphological types (*D. savignyi*, *D. setosum*, undetermined) by habitat (CR, RF, SG) was calculated. Pearson's Chi-square test (chisq.test in R version 3.4.2 (R Core Team 2017), implemented in R.Studio version 1.1.456 (RStudio Team 2016)) was applied to the resultant contingency table in order to test the null hypothesis of similar habitat use by each species or morphological type.

At introduced *P. kauderni* sites (15–18, Table 1), the presence/absence of associated *P. kauderni* was noted. At native population sites (1–14, Table 1), *P. kauderni* present in each transect were counted and microhabitat association was noted as *Diadema* or other. For these 14 native population sites, the total number of *P. kauderni* observed and the proportion of *P. kauderni* associated with *Diadema* urchins were calculated.

Molecular phylogeny

Tissue samples (spine attachment muscle) from specimens classified as *Diadema savignyi* and *D. setosum* based on external morphology (Fig. 2) were collected (96% alcohol) during field surveys at sites in each seaway (Fig. 1; Table 2, Online resources ESM 1 and ESM 2). DNA was extracted from 0.05–0.10 g of tissue taken from each sample using QIAGEN DNeasy blood and tissue kits, following the manufacturer's protocol. Cytochrome oxidase 1 (CO1) mitochondrial DNA (mtDNA) from 62 samples classified as *Diadema savignyi* ($n = 48$) and *D. setosum* ($n = 14$) (Table 2) was amplified through hotstart PCR using a standard protocol in use at the Bionesia Laboratory, Denpasar. Each PCR reaction contained 14.5 μl ddH_20, 2.5 μl 10× PCR buffer (PEII), 2.5 μl dNTPs (8 mM), 2 μl $MgCL_2$, 1.25 μl each of forward and reverse primers, 0.125 μl Amplitaq (5 units/μl) and 1 μl DNA template, making 25 μl per sample. The reaction master mix was prepared in two parts: one (MM2) containing 0.125 μl PE Amplitaq together with 9 μl ddH_2O and 1 μl PE-II buffer per sample, the other (MM1) with the remaining components. The following primer pair from Geller et al. (2013) was used: forward jgLCO: 5′-TITCIACIAAYCAYAARGAYATTGG-3′; reverse jgHCO: 5′-TAIACYTCIGGRTGICCRAARAAYCA-3′. Aliquots of 14 μl MM1 mix were placed in each reaction tube and DNA template added. The following PCR profile was used: pre-denaturation at 80 °C for 10 s; pause to add an aliquot of 10.125 μl MM2 to each reaction tube; denaturation at 94 °C for 15 s; 35 cycles (denaturation at 94 °C for 30 s; annealing at 50 °C for 30 s; extension at 72 °C for 40 s); extension at 72 °C for 5 min; final extension at 24 °C for 1 min. PCR product quality was checked through electrophoresis of the PCR product on 1% agarose gel.

Nucleotide sequences obtained through Sanger sequencing of the PCR product were aligned using the ClustalW algorithm (Higgins et al. 1994) implemented in MEGA7 (Kumar et al. 2016). Firstly, the AB1 files for forward (F) and reverse (R) sequences for each sample were imported, taking the reverse complement of the R sequence. Unidentified nucleotides (N) in

Table 1 *Diadema* survey sites in the Gulf of Tolo (1–14, Banggai Archipelago; 15, Banggai District, Sulawesi) and Makassar Strait (16–17, Palu Bay; 18–20, Spermonde Archipelago)

Survey/collection site		Location[a]		Survey month (2017)	Survey method transect type	*Pterapogon kauderni* population
No.	Name	Latitude (S)	Longitude (E)			
1	Liang	01° 33′ 03″	123° 14′ 26″	June	Belt	Native
2	Popisi	01° 30′ 27″	123° 31′ 13″	July	Belt	Native
3	Bone Baru	01° 31′ 26″	123° 29′ 33″	June	Belt	Native
4	Tinakin Laut	01° 36′ 11″	123° 29′ 16″	June	Belt	Native
5	Monsongan	01° 38′ 15″	123° 28′ 58″	October	Swim survey	Native
6	Tolokibit	01° 42′ 48″	123° 31′ 36″	October	Belt	Native
7	Kapela	01° 42′ 52″	123° 34′ 45″	June	Belt	Native
8	Toropot	01° 56′ 34″	123° 38′ 04″	October	Swim survey	Native
9	Kombongan	01° 52′ 47″	123° 41′ 23″	October	Swim survey	Native
10	Nggasuang	02° 00′ 42″	123° 46′ 22″	October	Swim survey	Native
11	Mandel	01° 59′ 52″	123° 50′ 33″	October	Swim survey	Native
12	Mbuang-Mbuang	02° 04′ 19″	123° 52′ 10″	October	Swim survey	Native
13	Melilis	02° 04′ 38″	123° 52′ 20″	October	Swim survey	Native
14	Toado	02° 04′ 52″	123° 54′ 29″	October	Belt	Native
15	Luwuk kilo 5	00° 57′ 08″	122° 47′ 42″	July	Belt and swim surveys	Introduced
16	Mamboro	00° 47′ 53″	119° 52′ 20″	July	Belt	Introduced
17	Kadongo	00° 47′ 01″	119° 51′ 32″	July	Belt	Introduced[b]
18	Langkai	05° 01′ 49″	119° 05′ 48″	May	Swim survey	None
19	Bone Batang	05° 01′ 05″	119° 19′ 46″	May	Swim survey	None
20	Barrang Lompo	05° 02′ 54″	119° 19′ 35″	August	Swim survey	None

[a] Approximate coordinates, using GPS (Garmin, WGS 84)

[b] Threatened with extirpation due to severe environmental degradation caused by a reclamation project

forward and reverse sequences were checked manually, and replaced with the dominant base (A, C, T, G) when clearly visible. Both sequences were then trimmed to remove poor quality or unclear nucleotide positions from each end. To compile a dataset comprising one nucleotide sequence for each of the 62 *Diadema* samples, the sequence (F or R) with overall highest quality (based on visual observation of the AB1 file in MEGA7) was selected. Nucleotide positions from the unselected sequence extending beyond the overlap were added to the appropriate end of the selected sequence. The nucleotide sequences (partial CO1) produced were deposited as GenBank accessions under accession numbers MG988406–MG988411, MH051847–MH051888 and MK296413–MK296426 (Table 2).

Prior to analysis, the sequences were renamed according to the codes in Table 2. Further manual trimming was carried out in MEGA7, to obtain 62 fully overlapping sequences of equal length (653 nucleotide positions). The on-line GenBank BLAST function (implemented in MEGA7) and taxonomic searches of the NCBI GenBank database yielded a total of 48 nucleotide sequences selected for further analysis, each having a 50% or higher overlap with our 653 nucleotide position dataset (Table 3). These comprised 46 mtDNA nucleotide sequences isolated from six species of the genus *Diadema*, and two 'out–group' sequences from the genus *Astropyga* Gray, 1825, also in the family Diadematidae. Three *Diadema* species were not included in the phylogenetic analysis, as the mtDNA sequences deposited in GenBank do not cover the CO1 region used in this study: *Diadema paucispinum* Agassiz, 1863; *D. palmeri* Baker, 1967; and *D. africanum* Rodríguez, Hernández, Clemente and Coppard, 2013.

Phylogenetic trees were constructed for two datasets: our 62 *Diadema* specimen 653 nucleotide position dataset (Table 2; see Fig. 3a and Online resource ESM 3) and the 110 nucleotide sequence dataset in which our 62 nucleotide sequence dataset was combined with the 48 GenBank accessions in Table 3 (see Fig. 4). Three approaches were implemented and the results compared: Neighbour-Joining (NJ), Maximum Likelihood (ML) and Bayesian Inference (BI). Representative phylogenetic trees (Figs. 3 and 4; Online resource ESM 3) were edited using the on-line interactive Tree Of Life (iTOL) (Letunic and Bork (2019).

The NJ method (Saitou and Nei 1987) was implemented in MEGA7 (Kumar et al. 2016) with bootstrapping (1000 replicates) (Felsenstein 1985). Evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) in units of the number of base substitutions per site.

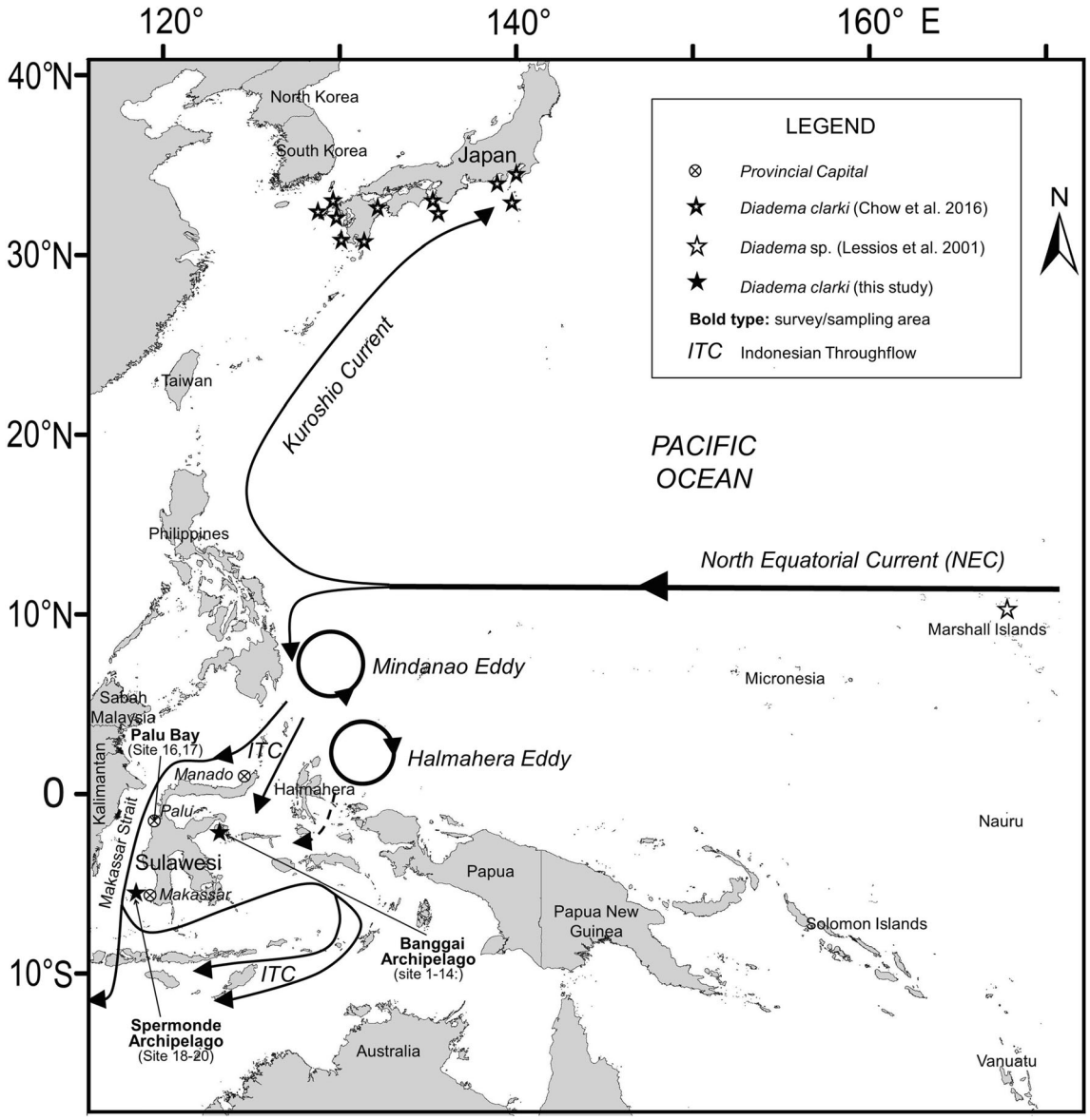
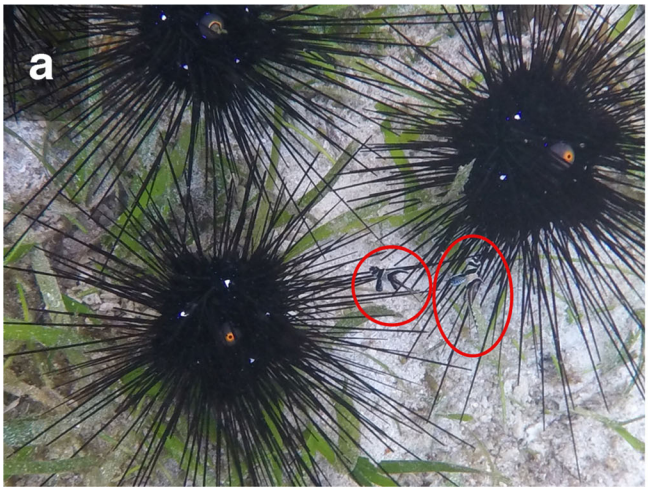


Fig. 1 Map showing the western Indo-Pacific area including survey sites (see Table 1 for geographical coordinates) and major ocean currents. Stars indicate the sites of published and new (this study) *Diadema clarki*/*Diadema* sp. records

BI was implemented in TOPALi version 2.5 (Milne et al. 2004), enabling remote running of MrBayes v3.1.1 (Ronquist and Huelsenbeck 2003) at the James Hutton Institute. For both the 62 and 110 nucleotide sequence datasets described above, the best fitting model was the Hasegawa, Kishino and Yano model (Hasegawa et al. 1985) with gamma rate heterogeneity (HKY+G). As a test of tree structure robustness, tree structures generated by the five next highest scoring models were compared with the HKY+G model output. Analysis parameter settings were optimised based on values of the convergence diagnostic potential scale reduction factor (PSRF) equal to or very close to 1 (Gelman and Rubin 1992). For the HKY+G model, the optimised parameters were number of runs = 2; number of generations = 100,000; sample frequency = 20; and burn-in = 30%.

The ML method was implemented using several models and programs, and the phylogenetic tree structures generated were compared. The Tamura-Nei model (Tamura et al. 2004) was implemented in MEGA7, with Neighbour-Join and BioNJ algorithms applied to a matrix of pairwise distances estimated using a Maximum Composite Likelihood approach and selection of the topology with superior log likelihood value. The generalised time-reversible (GTR) nucleotide substitution model with gamma rate heterogeneity (GTR+G) in RAxML version 2.2.3 (Stamatakis 2006) was implemented in TOPALi v2.5 (Milne et al. 2004) with bootstrapping (100 replicates). The HKY+G model in PhyML-aLRT version 2.4.5 (Anisimova and Gascuel 2006; Guindon and Gascuel 2003) was implemented in TOPALi v2.5 with default settings.



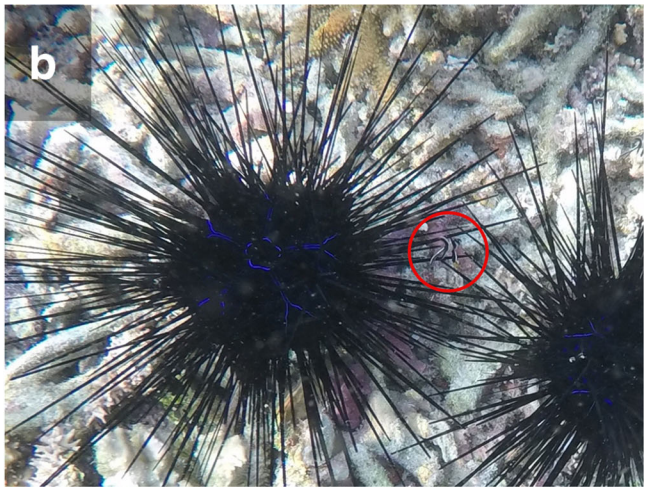


Fig. 2 Typical specimens of: **a** *Diadema setosum* from Bone Baru (site 3) with associated juvenile (red circle) and sub-adult (red oval) Banggai cardinalfish (*Pterapogon kauderni*); **b** *Diadema savignyi* from Nggasuang (site 10), with associated recently released *P. kauderni* juvenile (red circle). The key diagnostic features used for field identification of *D. setosum* were usually highly visible; however, the wide dotted blue iridophore lines were difficult to capture on camera. In typical *D. savignyi* and atypical specimens, the fine unbroken Y-shaped iridophore lines were clearly visible to the naked eye though often much less visible or indistinct when recorded on camera

Results

Field observations

Based on external morphological characters, approximately 74% ($n = 8279$) of *Diadema* urchins observed ($n = 11{,}723$) were identified as *D. setosum* and 24% ($n = 2696$) as *D. savignyi*, and just over 2% ($n = 248$) exhibited atypical or mixed traits and were recorded as undetermined (Table 4). All three *Diadema* morphological phenotype categories were present at 19 out of the 20 (95%) survey sites. At these 19 sites, groups of *Diadema* frequently comprised individuals of two or more morphological phenotypes. At Toado (site 14), all *Diadema* observed conformed to the typical *D. setosum* morphological phenotype. At both endemic (1–14) and introduced (15–17) population sites, *P. kauderni* groups were observed associated with all *Diadema* morphological phenotypes present (Table 4). Within mixed *Diadema* flocks, *P. kauderni* individuals and groups frequently moved between individual urchins without making any apparent distinction between *Diadema* species or morphological

Table 2 *Diadema* tissue (DNA) sample origin and GenBank accession numbers of mtDNA CO1 sequences produced under this study

Collection site				*Diadema* samples	
No.	Name/prefix code	Coordinates	Location	*n*	GenBank accession numbers
Gulf of Tolo/East Sulawesi				33	
1a	Liang/LSAV	01° 33′ 03″ S; 123° 14′ 26″ E	Peleng Island Banggai Archipelago	12	MG988409–MG988411 MH051880–MH051888
1b	Liang/LSET			6	MK296413, MK296414, MK296416, MK296417, MK296420, MK296421
2a	Tinakin Laut/TSAV	01° 36′ 11″ S; 123° 29′ 16″ E	Banggai Island Banggai Archipelago	12	MH051847–MH051858
2b	Tinakin Laut/TSET			3	MK296415, MK296418, MK296419
Makassar Strait/West Sulawesi				29	
3a	Mamboro/PSAV	00° 47′ 53″ S; 119° 52′ 20″ E	Palu Bay	12	MH051868–MH051879
3b	Mamboro/PSET			2	MK296422, MK296423
3c	Kadongo/PSET	00° 47′ 01″ S; 119° 51′ 32″ E		1	MK296424
4a	Bone Batang/SSAV	05° 01′ 05″ S; 119° 19′ 46″ E	Spermonde Archipelago	5	MG988406–MG988408 MH051859–MH051860
4b	Langkai/SSAV	05° 01′ 49″ S; 119° 05′ 48″ E		7	MH051861–MH051867
4c	Barranglompo/SSET	05° 02′ 45″ S; 119° 19′ 48″ E		2	MK296425, MK296426

Prefix codes denote location (Liang (*L*); Tinakin Laut (*T*); Palu Bay (*P*); Spermonde Archipelago (*S*)) and morphological phenotype (*D. savignyi* (*SAV*); *D. setosum* (*SET*))

Table 3 Additional GenBank sequences used in the phylogenetic analysis

No.	Geographic origin	GenBank accession metadata		GenBank accession ID No.
		Taxon	Reference	
1	Japan	*Diadema savignyi*	Chow et al. (2014)	AB909949
				AB909951–AB909957
		Diadema setosum		AB909922–AB909926
				AB909928–AB909931
		Diadema sp./*D. clarki*[a]	Chow et al. (2014)	AB909932–AB909937
				AB909939–AB909943
				AB909945; AB909947
				AB909948
	Japan	*Diadema setosum*/*D. clarki*[b]	Chow et al. (2016)	LC037357
2	South China Sea	*Diadema setosum*	Li et al. (2016)	KX385835
3	Korea, *D. setosum*	*Diadema setosum*	Lee (2011)	JQ341146–JQ341147
4	Red Sea, *D. setosum*	*Diadema setosum*	Al-Rshaidat et al. (2016)	KU496324–KU496327
5	Bahamas	*Diadema antillarum*	iBOL (2010)	GU670185; GU670186
6	Mexico	*Diadema antillarum*	Bribiesca-Contreras et al. (2013)	KC626158–KC626160
				KC626162
		Diadema mexicanum		KC626163
7	Unknown	*Astropyga* sp.1	Hoareau and Boissin (2010)	GU480569
		Astropyga sp.2		GU480570

[a] *Diadema* sp. in GenBank accession data (Chow et al. 2014); identified as *D. clarki* in Chow et al. (2016)

[b] *Diadema setosum* in GenBank accession data; identified as *D. clarki* in Chow et al. (2016)

phenotypes present. At the native *P. kauderni* population sites, the proportion of *P. kauderni* associated with *Diadema* urchins varied considerably; however, cumulatively they accounted for over half of all *P. kauderni* observed. At two of the introduced sites (15, 17), all (100%) of the *P. kauderni* observed were associated with *Diadema* urchins. At the third site (Mamboro, site 16), most juvenile *P. kauderni* were associated with sea anemones, while the remainder tended to move between *Diadema* and surrounding predominantly acroporid corals. Typically, approximately half of the larger *P. kauderni* were associated with *Diadema* microhabitat at any one time.

The aggregated data on distribution of *Diadema* by habitat (Table 5) indicate different patterns of habitat use by each of the three morphological phenotype groups. All phenotypes were relatively common in the intermediate reef flat habitat, including over 60% of the individuals classified as undetermined. Of the urchins classified as *D. savignyi* and *D. setosum*, the smallest proportions (around 20%) were found in seagrass habitat and coral reef habitat, respectively. The Pearson's Chi-squared test (df = 4) produced $\chi^2 = 533.03$, with p value $< 2.2e^{-16}$. Pearson's Chi-squared test with simulated p value (based on 1000 replicates) returned $p < 0.001$. The results indicate that the null hypothesis of similar habitat use by these three groups should be rejected.

All *Diadema* specimens observed had some pattern of iridescent blue lines on the test, although the precise colour tone varied considerably between individuals, ranging from turquoise or greenish-blue to almost purple. Most individuals had fully black spines on the aboral face and sides of the test. However, individuals with some proportion of white spines (highly variable but never 100%) were observed in all variants of iridescent line pattern and anal sac colouration.

The most common iridescent line pattern exhibited in urchins recorded as undetermined (Table 4) comprised continuous blue lines forming the 'Y' shape typical of *D. savignyi*. However, the lines were wider than usual in this species, though not as wide as the dashed lines typical of *D. setosum*. The much fainter looping lines around the circumference of the test (present in most individuals recorded as *D. savignyi*) were not present in these specimens. Red or pink dots (sometimes with a faint white line around the circumference) were nearly always apparent in the crux where the Y branches meet. These specimens did not have the orange anal ring typically seen in *D. setosum*. The colour of the anal sac varied between individuals, with dominant tones of either brown or grey. The colouration could be relatively uniform or graded vertically, with the paler hues most commonly around the widest circumference, and the darkest hues near the base. In specimens with predominantly brown anal sacs, paler or orangey hues were sometimes seen. In specimens with predominantly grey anal sacs, the shades observed ranged from mid-grey to almost black.

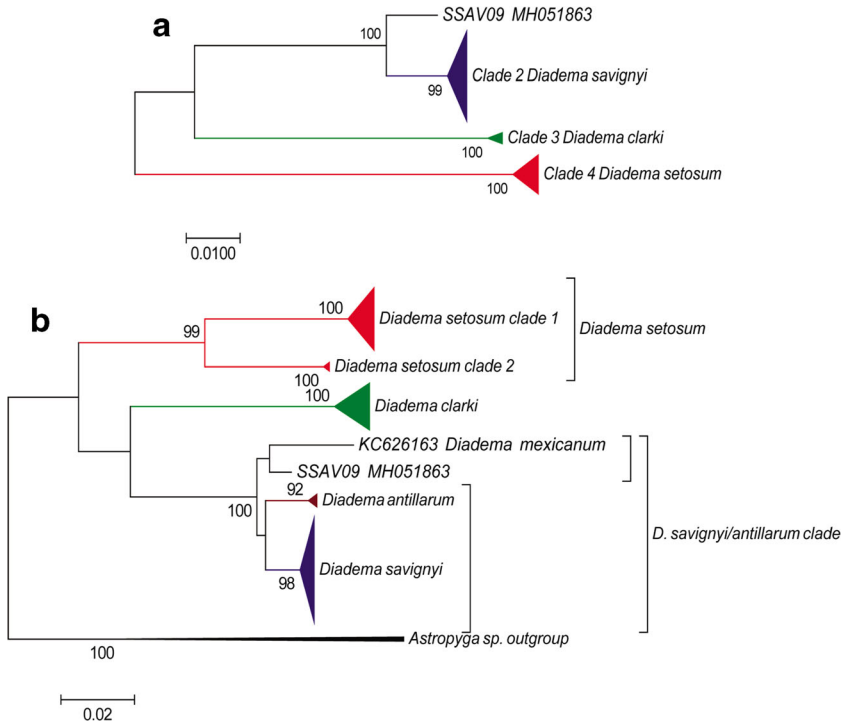


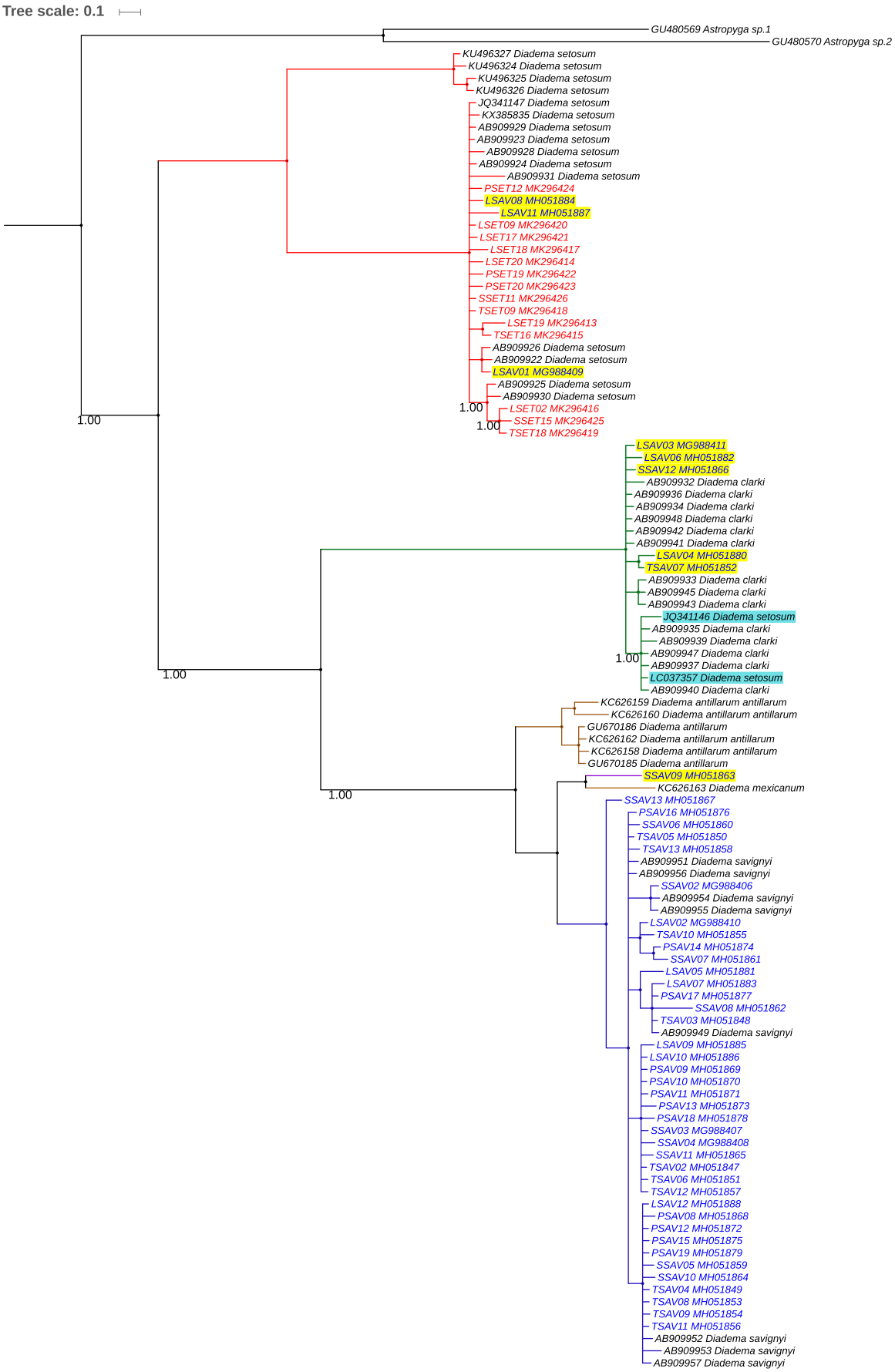
Fig. 3 Condensed phylogenetic trees based on CO1 mtDNA nucleotide sequences representative of all trees produced (using BI, NJ and ML methods) for two datasets: **a** the 62 sequence (653 nucleotide positions) dataset obtained from *Diadema* specimens collected during this study (see Table 2); **b** the 110 sequence dataset combining these 62 sequences with publicly available GenBank accessions (see Table 3) of *Diadema* ($n = 46$), with *Astropyga* sequences ($n = 2$) as outgroup to root the tree. The trees shown were produced in MEGA7 (Kumar et al. 2016) and show the evolutionary relationships inferred using the Neighbour-Joining method (Saitou and Mei 1987). All ambiguous positions were removed for each sequence pair. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The trees are drawn to scale, with branch lengths computed using the Kimura 2-parameter method (Kimura 1980), in the unit of evolutionary distance (base substitutions per site) used to infer the phylogenetic trees. The trees were edited (condensed, colour-coded and annotated) using the on-line interactive Tree Of Life (iTOL) (Letunic and Bork 2019) and combined in Adobe Photoshop CS4 v.11

Phylogenetic analysis

All phylogenetic analysis methods (NJ, ML and BI, all programs, models and parameter setting selections used) produced the major clade level structure displayed in the representative condensed trees shown in Fig. 3, albeit with some variation (e.g. branch length, node order, bootstrap values). The same specimens resolved into each major clade under all analyses, although within these major clades internal structure (e.g. sub-clade nodes and groupings, branch lengths, and, where applicable, bootstrap values) did vary between methods, models and parameter settings.

The 62 *Diadema* sequence set consistently resolved into the same three major clades, with one specimen in a fourth clade (Fig. 3a, Online resource ESM 3). This tree configuration was strongly supported by all analyses, with NJ and BI bootstrap values of 100% (Fig. 3a and Online resource ESM 3). All 14 specimens with typical *D. setosum* morphological phenotype were assigned to the same main clade (clade 4, Fig. 3a). The 48 specimens with morphological characteristics typical of *Diadema savignyi* were not recovered as a monophylum. The majority (39) formed a well-defined clade (clade 2, Fig. 3a), while five formed a second well-defined clade (clade 3, Fig. 3a), three resolved within clade 4 and one specimen from Langkai in the Spermonde Archipelago,

Fig. 4 Representative phylogenetic tree for 110 CO1 mtDNA nucleotide sequences: 62 *Diadema* specimens collected under this study and GenBank accessions of *Diadema* ($n = 46$), with *Astropyga* ($n = 2$) as outgroup to root the tree. The tree was produced through remote running of MrBayes v3.1.1 (Ronquist and Huelsenbeck 2003) in TOPALi version 2.5 (Milne et al. 2004) using the best-fitting (HKY+G) model (Hasegawa et al. 1985). Optimised parameter settings were number of runs = 2; number of generations = 100,000; sample frequency = 20; and burn-in = 30%. Bootstrap values of 0.99–1.00 are shown. The tree was edited (colour-coded and annotated) using the on-line interactive Tree Of Life (iTOL) (Letunic and Bork (2019). Text colour: blue, *D. savignyi* morphological phenotype; red, *D. setosum* morphological phenotype; black, GenBank accession sequence. Highlighting colour: yellow, samples with *D. savignyi* morphological phenotype outside the *D. savignyi* clade; turquoise, GenBank accessions deposited as *D. setosum* resolved within the *D. clarki* clade. Branch/node colour: red, *D. setosum* clade; blue, *D. savignyi* clade; green, *D. clarki* clade; purple, unidentified (SSAV09_ MH051863); brown, other clades within the high-level *D. savignyi/D. antillarum/D. mexicanum* clade; black, *Astropyga* outgroup and top-level branches/nodes


Tree scale: 0.1
GU480569 Astropyga sp.1
GU480570 Astropyga sp.2
KU496327 Diadema setosum
KU496324 Diadema setosum
KU496325 Diadema setosum
KU496326 Diadema setosum
JQ341147 Diadema setosum
KX385835 Diadema setosum
AB909929 Diadema setosum
AB909923 Diadema setosum
AB909928 Diadema setosum
AB909924 Diadema setosum
AB909931 Diadema setosum
PSET12 MK296424
LSAV08 MH051884
LSAV11 MH051887
LSET09 MK296420
LSET17 MK296421
LSET18 MK296417
LSET20 MK296414
PSET19 MK296422
PSET20 MK296423
SSET11 MK296426
TSET09 MK296418
LSET19 MK296413
TSET16 MK296415
AB909926 Diadema setosum
AB909922 Diadema setosum
LSAV01 MG988409
AB909925 Diadema setosum
AB909930 Diadema setosum
LSET02 MK296416
SSET15 MK296425
TSET18 MK296419
1.00
1.00
1.00
LSAV03 MG988411
LSAV06 MH051882
SSAV12 MH051866
AB909932 Diadema clarki
AB909936 Diadema clarki
AB909934 Diadema clarki
AB909948 Diadema clarki
AB909942 Diadema clarki
AB909941 Diadema clarki
LSAV04 MH051880
TSAV07 MH051852
AB909933 Diadema clarki
AB909945 Diadema clarki
AB909943 Diadema clarki
JQ341146 Diadema setosum
AB909935 Diadema clarki
AB909939 Diadema clarki
AB909947 Diadema clarki
AB909937 Diadema clarki
LC037357 Diadema setosum
AB909940 Diadema clarki
1.00
KC626159 Diadema antillarum antillarum
KC626160 Diadema antillarum antillarum
GU670186 Diadema antillarum
KC626162 Diadema antillarum antillarum
KC626158 Diadema antillarum antillarum
GU670185 Diadema antillarum
SSAV09 MH051863
KC626163 Diadema mexicanum
1.00
SSAV13 MH051867
PSAV16 MH051876
SSAV06 MH051860
TSAV05 MH051850
TSAV13 MH051858
AB909951 Diadema savignyi
AB909956 Diadema savignyi
SSAV02 MG988406
AB909954 Diadema savignyi
AB909955 Diadema savignyi
LSAV02 MG988410
TSAV10 MH051855
PSAV14 MH051874
SSAV07 MH051861
LSAV05 MH051881
LSAV07 MH051883
PSAV17 MH051877
SSAV08 MH051862
TSAV03 MH051848
AB909949 Diadema savignyi
LSAV09 MH051885
LSAV10 MH051886
PSAV09 MH051869
PSAV10 MH051870
PSAV11 MH051871
PSAV13 MH051873
PSAV18 MH051878
SSAV03 MG988407
SSAV04 MG988408
SSAV11 MH051865
TSAV02 MH051847
TSAV06 MH051851
TSAV12 MH051857
LSAV12 MH051888
PSAV08 MH051868
PSAV12 MH051872
PSAV15 MH051875
PSAV19 MH051879
SSAV05 MH051859
SSAV10 MH051864
TSAV04 MH051849
TSAV08 MH051853
TSAV09 MH051854
TSAV11 MH051856
AB909952 Diadema savignyi
AB909953 Diadema savignyi
AB909957 Diadema savignyi

Makassar Strait (sample code SSAV09, GenBank accession MH051863) formed a separate monophyletic clade (clade 1, Fig. 3a).

The inclusion of GenBank data (Table 3) consistently produced trees similar to Fig. 3b. The expanded BI tree in Fig. 4 has a structure representative of all phylogenetic trees produced from NJ, ML and BI analyses, regardless of the models and parameters/settings selected. While the GenBank *D. savignyi* accessions in Table 3 formed a single clade, accessions deposited as *D. setosum* consistently resolved into two well-defined clades (clades 1 and 2, Fig. 3b), with two sequences nested in the clade containing all *Diadema* sp. accessions, identified by Chow et al. (2016) as *Diadema clarki* Ikeda, 1939. These were as follows: accession JQ341146, one of two sequences recorded as *D. setosum* by Lee (2011); and accession LC037357, also recorded in GenBank as *Diadema setosum* but identified as *D. clarki* in Chow et al. (2016). Each of the three main clades shown in Fig. 3a (and Online resource ESM 3) resolved within the clade of a different species (clade 2: *D. savignyi*; clade 3: *D. clarki* (formerly *Diadema* sp.); clade 4: *D. setosum* clade 1).

Table 5 *Diadema* distribution by habitat type at 14 native *Pterapogon kauderni* population sites in the Banggai Archipelago

Diadema urchins		% urchins by dominant habitat type		
Morphological phenotype	*n*	Coral reef	Reef flat	Seagrass
Diadema savignyi	2204	42.15	36.25	21.60
Diadema setosum	6456	19.47	46.31	34.22
Undetermined	199	27.64	63.82	8.54
Total	8859	25.30	44.20	30.50

The greatest variation in tree structures produced by the 110 nucleotide sequence dataset analyses occurred within the higher level clade containing *D. savignyi*, *D. antillarum* and *D. mexicanum* Agassiz, 1863. Node configuration within this clade varied, in particular regarding the placement of the specimen comprising clade 1 in Fig. 3a (sample code SSAV09, GenBank accession MH051863), which formed a separate branch in all trees produced. Phylogenetic trees output from BI analyses, most NJ and some ML models and settings resolved this monophyletic clade as the closest sister

Table 4 Censused *Diadema* population based on external characters and associated *Pterapogon kauderni* (numbers and proportions of *P. kauderni* recorded) at the survey sites in the Gulf of Tolo (1–14, Banggai Archipelago; 15, Banggai District, Sulawesi) and Makassar Strait (16–17, Palu Bay; 18–20, Spermonde Archipelago)

Survey site			*Diadema* morphological phenotype (%)			*P. kauderni*	
No.	Name	*n*	*D. savignyi*	*D. setosum*	Undetermined	*n*	% in *Diadema*
1	Liang	222	5.41	92.34	2.25	265	46.42
2	Popisi	514	22.96	74.90	2.14	1184	77.70
3	Bone Baru	308	20.45	74.35	5.19	1128	16.22
4	Tinakin Laut	986	14.20	84.48	1.32	1017	75.02
5	Monsongan	733	18.28	78.44	3.27	695	44.17
6	Tolokibit	1469	52.83	44.59	2.59	1562	79.77
7	Kapela	1047	9.07	89.59	1.34	824	8.86
8	Toropot	540	57.96	37.04	5.00	2890	93.53
9	Kombongan	93	10.75	87.10	2.15	381	20.21
10	Nggasuang	1046	43.40	52.87	3.73	1002	92.81
11	Mandel	991	1.61	98.08	0.30	75	66.67
12	Mbuang-Mbuang	306	2.61	96.73	0.65	479	93.95
13	Melilis	586	11.09	88.05	0.85	542	90.96
14	Toado	18	0.00	100.00	0.00	4423	10.58
15	Luwuk Kilo 5	688	7.99	89.39	2.62	introduced	100
16	Mamboro[a]	462	63.64	33.12	3.25	introduced	≈50
17	Kadongo	55	16.36	81.82	1.82	introduced	100
18	Langkai	331	38.07	58.31	3.63	none	
19	Bone Batang	458	1.09	98.69	0.22	none	
20	Barranglompo	370	0.81	98.65	0.54	none	
Total		11,223	24.02	73.77	2.21	16,467	53.36

[a] Similar large flocks of *Diadema* in and around hard corals, especially stands of *Acropora* sp., observed in July 2018, prior to the tsunami on 28 September 2018. Post-tsunami survey (census) in March 2019: zero *Diadema* urchins; less than 1% live coral cover with no branching corals; 10 *P. kauderni*, all juveniles and associated with sea anemones (Moore, unpublished data)

clade to *D. mexicanum* (as shown in Fig. 3b and Fig. 4), while in some NJ and most ML analyses, this clade was closest to *D. savignyi*.

Discussion

First record and potential range extension of *Diadema clarki*

The most surprising finding from our study is the resolving of the CO1 sequences from five of our 62 *Diadema* specimens into a well-differentiated clade consonant with *Diadema clarki*. These five sequences consistently nested within the clade containing 14 sequences submitted by Chow et al. (2014) as *Diadema* sp. and later identified as *Diadema clarki* by Chow et al. (2016). This result provides the first record of *D. clarki* mtDNA from Indonesia, the Coral Triangle and the southern hemisphere. Four of these specimens were collected in the Banggai Archipelago, to the east of Sulawesi; one was collected in the Spermonde Archipelago, in the Makassar Strait, off the west coast of Sulawesi Island.

The presence of both *Diadema setosum* and *D. savignyi* at a majority of sites within the study area was to be expected based on distribution patterns inferred from Lessios et al. (2001) and previous records within the Coral Triangle. Both species have been reported from sites around Sulawesi, including the Spermonde Archipelago (de Beer 1990), northern Sulawesi (Pearse 1998) and the Banggai Archipelago (Moore et al. 2017b; Ndobe et al. 2018b). The nesting of all specimens in clade 4 (Fig. 3a) within *D. setosum* clade 1 (Fig. 3b) is consonant with known distribution patterns. *D. setosum* clade 1 in Fig. 3b corresponds to *D. setosum*-a clade in Lessios et al. (2001) and Bronstein et al. (2017), with a known Indo-Pacific distribution including Indonesia. *D. setosum* clade 2 (Fig. 3b) corresponds to the *D. setosum-b* clade, considered native to the Arab Peninsula (Bronstein et al. 2017), represented in the 110 sequence dataset by four Red Sea *D. setosum* sequences (Al-Rshaidat et al. 2016). However, the presence of *Diadema clarki* (or at least *D. clarki* mtDNA) in both the Banggai and Spermonde Archipelagos was unexpected.

Diadema clarki was originally described from specimens collected in Japanese waters by Ikeda (1939) and was reported as having an orange anal ring. Mortensen (1940) placed *D. clarki* in synonymy with *D. setosum* based on the presence of this anal ring as well as skeletal and other characteristics. Chow et al. (2016) resurrected *D. clarki* as a valid species based primarily on molecular analysis of mtDNA. They successfully amplified a fragment of mtDNA from a preserved specimen collected by Ikeda in Japan. The sequence was sufficiently similar to the mtDNA of specimens referred to as *Diadema* sp. in Lessios et al. (2001) from Japan and the Marshall Islands, as well as *Diadema* sp. specimens more recently collected in Japanese waters (Chow et al. 2014, 2016), to warrant grouping these specimens in the same species. Based on this similarity, Chow et al. (2016) resurrected *Diadema clarki* (Ikeda 1939) as a valid species, distinct from any of the other currently recognised *Diadema* species. Based on an extensive survey in Japanese waters, Chow et al. (2016) also concluded that the distribution of *D. clarki* in Japan appears to be limited to a narrow latitudinal band of 31–35° N.

The discovery of *D. clarki* mtDNA in seaways both east (Banggai Archipelago) and west (Spermonde Archipelago) of Sulawesi potentially extends the reported distribution of *D. clarki* around 3800 km southwards, from Japan to Indonesia, as well as over 5300 km to the west and south of the Marshall Islands (Fig. 1). Interestingly, although Chow et al. (2016) specifically state that "no *D. clarki* were observed in Indonesia", they do suggest that *D. clarki* might be distributed much more widely, and in particular might be found in Papua New Guinea and Indonesia. Like the recent range extension of *D. mexicanum* by 600 km to the northernmost island in the Gulf of California (Paz-García et al. 2016), the discovery of *Diadema africanum* in the eastern Atlantic (Rodriguez et al. 2013) and the delineation of two clades within *D. setosum* (Bronstein et al. 2017), our results underline the fact that there is still much to be discovered about *Diadema* urchin biogeography.

Hybridisation and introgression

Processes promoting the persistence of biodiversity, especially in closely related taxa with overlapping geographical distributions and ecological niches, seem at least as complex and arguably less well understood than those which can lead to speciation. Lessios et al. (2001) found indications of hybridisation resulting in gene transfer between *D. paucispinum* and *D. savignyi*. The study by Uehara et al. (1990) demonstrated that *D. savignyi* and *D. setosum* are completely inter-fertile in both directions. Genetic studies and laboratory experiments indicate that three congeneric species (*D. setosum*, *D. savignyi* and *D. paucispinum*) can produce F1 hybrid progeny viable to sexual maturity and capable of reproduction, producing F2 or later-generation backcrosses (Lessios and Pearse 1996).

It is worth noting that mtDNA molecular markers have been the most widely used in studies on the genus *Diadema*, in particular in the molecular biology studies to date on *D. clarki*. Mitochondrial DNA of the female parent is passed down to all her descendants, and thus all first-generation hybrid offspring receive maternal mtDNA. In subsequent generations (including backcrosses), this mtDNA from the maternal parent of the first-generation hybrid will continue to be passed on through the matrilineal line of descent. This means that mtDNA cannot distinguish between a pure-strain individual and a hybrid with the same matrilineal ancestry.

Morphological traits of specimens with *Diadema* sp. mtDNA in Lessios et al. (2001) included *D. setosum*, *D. savignyi* and intermediate phenotypes (JS Pearse, personal communication 2018). The morphological traits of specimens identified as *Diadema* sp. in Chow et al. (2014) and as *D. clarki* in Chow et al. (2016) were extremely diverse, with some phenotypes closely resembling those typical of *D. setosum* or *D. savignyi*. As recently reported for another echinoderm genus, *Tripneustes* (Bronstein et al. 2016), it is possible that some, or perhaps even all, of the specimens identified as *D. clarki* in both our study and other studies, including Chow et al. (2016), might not be true (pure-strain) *D. clarki*, but in fact recent hybrids or individuals with mtDNA signatures resulting from historical introgression.

Mitochondrial introgression as a result of historical hybridisation with species no longer present has been reported in fish (Wilson and Bernatchez 1998) and echinoids (Bronstein et al. 2016). It should be remembered that Lessios et al. (2001) proposed *Diadema* sp. (*D. clarki* according to Chow et al. 2016) as one of two most basal species (the other being *D. palmeri*). It is possible that *D. clarki* is indeed an extant species with a wide Indo-Pacific distribution. It is also possible that a once-distinct *D. clarki* is now mainly present as mtDNA introgressed into both *D. savignyi* and *D. setosum*, over a wide area or in a number of widely separated regions. Indeed, while it may be a widespread cryptic species, it is not impossible that *D. clarki* might in fact be a 'ghost species', present only through continued introgression, but leaving a clear signal in the mitochondrial DNA of its descendants through the female line. This is one possible explanation for the specimen identified as *D. setosum* by Lee (2011) but with mtDNA assigned to the *D. clarki* clade (JQ341146 in Fig. 4).With a growing number of studies proving that hybridisation is possible within or even between closely related genera, Lessios (2007) stressed that there are still many unanswered questions regarding the apparent rarity of urchin hybridisation in nature. In urchins, mechanisms promoting reproductive isolation include differences in spawning times of sympatric species at some sites (Coppard and Campbell 2005; Muthiga 2003), and divergent evolution of bindin, a reproductive molecule involved in gamete incompatibility (Lessios 2007). Within the genus *Diadema*, as it is apparent that several species are capable of forming viable hybrids, spawning times are likely to be important in maintaining species integrity (Muthiga 2003). No data were found on the lunar spawning rhythms of *D. clarki*; however, the lunar phase of spawning in *D. savignyi* reportedly tends to peak just after full moon throughout the tropical Pacific, while in *D. setosum*, the spawning periodicity varies between sites, and in some places can overlap with that of *D. savignyi* (Pearse 1990; JS Pearse, personal communication 2018). Occasional field observations of *D. setosum* spawning in the study areas east and west of Sulawesi Island (Moore, unpublished data 2004–2018) indicate that in both seaways this species tends to spawn around the full moon, sometimes over several consecutive days, during several months of the year. Spawning of *D. savignyi* in the field has not yet been observed by the authors. However, if at least some *D. savignyi* populations in the seaways around Sulawesi do in fact follow the spawning pattern reported from other regions, it would seem reasonable to expect that overlaps between the spawning periods of the two species could occur.

Hybridisation is a likely but as yet untested explanation for the three specimens with *D. savignyi* morphological phenotypes resolved within the *D. setosum* clade in our study, which could be hybrids or backcrossed descendants of a *D. setosum* maternal ancestor. The mature hybrid with gametes reported by Uehara et al. (1990) was the offspring of a *D. savignyi* female and a *D. setosum* male. No *D. setosum* phenotype resolved into clades other than the *D. setosum* clade. Despite low sample numbers, this result might indicate that hybridisation is more common in one direction, i.e. *D. savignyi* eggs fertilised by *D. setosum* sperm, rather than the reverse. Some proportion of the undetermined specimens observed in the field might have been *D. clarki* but might also have been hybrids or back-crosses between any of the *Diadema* species present. Research using genomic nuclear DNA markers (e.g. the use of single nucleotide polymorphisms (SNPs) or the echinoid nuclear marker bindin) may shed more light on this question, as might more detailed morphological and morphometric studies.

Whether or not *D. clarki* is indeed a valid extant species, the question arises as to how many full-strain, hybrid or introgressed urchins with *D. clarki* mtDNA have been misidentified based on morphological phenotypic traits. This question is now of relevance over a geographical area extending at the very least from Japan to Indonesia and the Marshall Islands. The variety of *D. clarki* phenotypes described in Chow et al. (2014, 2016) is much greater than that reported for any other species within the genus *Diadema*. The specimens photographed include an individual resembling *D. setosum* (phenotype V) and another resembling *D. savignyi* (phenotype II). Phenotypes II, IV and V present broken iridophore lines reminiscent of *D. setosum* (albeit considerably narrower), while phenotypes I and II have unbroken iridophore lines like *D. savignyi*, though the authors state that their shape 'was substantially different from that of *D. savignyi*'. In this context, it is worth noting that accession LC037357, subsequently identified as *D. clarki* (phenotype III) in Chow et al. (2016), was identified and deposited in GenBank as *Diadema setosum*.

The undetermined specimens observed in our field surveys did not conform completely to any of the four *D. clarki* phenotypes described by Chow et al. (2016). Some resembled phenotype I, albeit with wider iridophore lines, while others presented a mix of features shown in two or more of the 5 phenotypes. While it is possible that some of these individuals were indeed true-strain *D. clarki* specimens, as noted

previously, it is also possible that some might have been hybrids or back-crosses between any of the *Diadema* species currently or historically present in the area.

In our study, all specimens assigned to the *D. clarki* clade had a *D. savignyi* phenotype. However, one explanation for the assignment of accession JQ341146 (Lee 2011) from Korean waters (deposited in GenBank as *Diadema setosum*) into the *D. clarki* clade is the possibility of phenotypic confusion between *D. clarki* and *D. setosum*. Such confusion does not seem unlikely based on the original description of *D. clarki* by Ikeda (1939), the placing in synonymy with *D. setosum* by Mortensen (1940) and phenotype descriptions (III, IV and V) in Chow et al. (2016). The assignment of accession JQ341146 to the *D. clarki* mtDNA clade would appear to add Korea to the range of *D. clarki* or at the very least to the regions where *D. clarki* mtDNA is present in the population. It would appear that *D. clarki*, or at the very least hybrids with *D. clarki* maternal ancestry, might be present across a wide latitudinal gradient in urchin populations comprising *D. savignyi* and/or *D. setosum* phenotypes.

A fourth *Diadema* species?

The single specimen from the Spermonde Archipelago (SSAV09_MH051863 in Fig. 3) which did not resolve within any species clade was consistently nested within the *Diadema savignyi-mexicanum-antillarum* clade. While other explanations cannot be ruled out (including the possibility of human error in the processes prior to, during and post-sequencing), the consistent branch architecture and strong bootstrap support (100% in Fig. 3a; similar or higher to that for the *D. antillarum–D. savignyi–D. mexicanum* nodes in all analyses of the 110 sequence dataset) would seem to indicate the possible presence of a fourth species. Two Indo-Pacific *Diadema* species, *Diadema paucispinum* and *D. palmeri* were not included in the phylogenetic analysis due to the absence of published sequences covering the CO1 mtDNA region sequenced in our study.

Further research would be necessary to assign specimen SSAV09_MH051863 to any known extant species. However, compared with *D. palmeri*, it does seem more likely that *D. paucispinum* (or its mitochondrial DNA) might be present in Indonesia. Once considered endemic to Hawaii (Agassiz 1863; Mortensen 1940), *D. paucispinum* mtDNA has been reported from tropical seas in the western Pacific and the Indian Ocean (Lessios et al. 2001). Although to date no morphological specimens of *D. paucispinum* have been described from much of its putative range based on mtDNA (JS Pearse, personal communication 2018), mtDNA might be present as a result of recent or past hybridisation or introgression. With reports from sites to the north (Philippines), east (Easter Island, Papua New Guinea) and west (East Africa) of Indonesia, finding *D. paucispinum* or its mtDNA in the Makassar Strait does not seem unlikely. In contrast, *D. palmeri* appears to be a subtropical to temperate species, with a known distribution limited to waters around New Zealand and southeast coast of Australia (Baker 1967; Lessios et al. 2001). Furthermore, the phylogenetic relationship of SSAV09_MH051863 relative to the other *Diadema* species in Fig. 4 is similar to that of *D. paucispinum* to these species in Lessios et al. (2001) and Rodriguez et al. (2013) but does not resemble that of *D. palmeri*.

Biogeographic processes of origin and dispersal

The origin and persistence of the Coral Triangle marine biodiversity hotspot have been attributed to several processes or combinations thereof. For the genus *Diadema*, Lessios et al. (2001) proposed a model of allopatric speciation and subsequent range extension, broadly consonant with current distributions of extant species. Recent research has further clarified aspects of the phylogeny, distribution and ecology of some taxa, for example the two *D. setosum* clades (Bronstein et al. 2017). Nonetheless, the origins, distribution and evolutionary relationships of several taxa within the genus remain unclear. In particular, the deep division between the *D. clarki* clade and other clades prompts speculation with regard to possible speciation and dispersal scenarios, taking into account recent and past geology and oceanography, and various mechanisms described by, inter alia, Carpenter et al. (2011) and von der Heyden et al. (2014).

While many factors and processes can influence species distributions, the position of known and suspected *D. clarki* records with respect to the major ocean currents in the Indo-Pacific region (Fig. 1) indicates one possible explanation for the same species occurring in three widely separated areas: Japan, the Marshall Islands and Indonesia. The easternmost distribution of the currently known distribution of *D. clarki*, the Marshall Islands might be a place of origin or a stepping stone along a dispersal route from an area or point of origin, most likely further east. Under a scenario of allopatric speciation such as that proposed by Lessios et al. (2001), speciation could have been followed by widespread but uneven radiation. Unpredictable large and small-scale three-dimensional variations in the Equatorial Current and other ocean currents, followed by localised spread and/or extirpation, could have led to isolated colonisation or introgression events. Research on the dispersal of Plutonium isotopes from nuclear tests in the Pacific indicates that material transported from Marshall Islands via the North Equatorial Current (NEC) can exit the Mindanao Eddy to enter both the Kuroshio Current flowing northwards to Japan (Lee et al. 2004) and the south-bound Indonesian Throughflow (Pittauer et al. 2017). The dispersal of *D. clarki* larvae (or larvae bearing *D. clarki* mtDNA) might thus include routes similar to those proposed for other taxa

including polychaete worms (Imajima and Hove 1984) and anguillid eel larvae (Miller et al. 2002). Such a scenario could explain the presence in Korea of a specimen with *D. clarki* mtDNA (accession JQ341146, Lee 2011). In-depth studies of *Diadema* mitochondrial and nucleic genomes, making use of classic and advanced molecular methods as advocated by Willette et al. (2014), should help to resolve many of the still unanswered questions regarding the evolution, diversity and biogeography of diadematid urchins.

Ecological niches and inter-species associations

Closely related species tend to occupy overlapping but not entirely equivalent ecological niches (Tornabene et al. 2013). An example within the genus *Diadema* is provided by the habitat modelling for the two clades of *Diadema setosum* in Bronstein et al. (2017). The data on habitat use (Table 5) strongly indicate that *Diadema* urchin species present in the Banggai Archipelago have overlapping but significantly different patterns of habitat use. Several studies (Pearse 1998; de Beer 1990; Bronstein and Loya 2014) report a higher proportion of *D. savignyi* at sites characterised as exposed to the open sea or outer fringing reefs, with *D. setosum* tending to dominate in relatively sheltered environments, including sites with quite high levels of anthropogenic impacts. Similar trends noticed in this study include the higher prevalence of *D. savignyi* in coral reef habitat, typically more exposed, while conversely *D. setosum* had a higher prevalence in seagrass beds, generally closer to shore and/or more sheltered. With respect to anthropogenic impact levels, *D. setosum* was generally more abundant close to villages and port areas, where *D. savignyi* tended to be rare or absent. For example, although *D. savignyi* was present at the relatively exposed Luwuk Kilo 5 site (site 15 in Table 1; see Online resource ESM 2), *D. setosum* was the only species observed in the nearby Luwuk harbour, a site which is sheltered and visibly polluted.

As pointed out by Crandall et al. (2008, 2012), different species, even within the same genus, can have very different responses to environmental cues, larval dispersal and connectivity patterns, overall and at finer scales. Community structure in tropical marine ecosystems can be determined by regional, local and evolutionary factors and may be consistent with niche and/or neutral theory at different life stages or under different conditions (Ahmadia et al. 2018). Trophic ecology and food habits could be among the factors affecting fine-scale distribution. Coppard and Campbell (2007) found significant differences in grazing preference between diadematid genera and species in Fiji, in particular *D. setosum* and *D. savignyi*. Little is known about the ecology of *D. clarki*; however, Chow et al. (2016) inferred possible differences in temperature tolerance between *D. savignyi*, *D. setosum* and *D. clarki*, suggesting that *D. clarki* might be the most cold-adapted and *D. savignyi* the least tolerant to lower temperatures. It is possible that thermal preferences might also play a role in *Diadema* habitat use and fine-scale distribution patterns, with typically greater fluctuations in temperature nearer to shore than closer to the reef crest.

While data on *Diadema*-associated faunal communities are limited, they indicate that *Diadema* urchin community composition and diversity could have implications for ecosystem-level biodiversity. Coppard and Campbell (2004) found considerable overlap in the organisms associated with sympatric *Diadema* species in Fiji. Species associated with both *D. savignyi* and *D. setosum* included three cardinalfishes (Apogonidae) as well as the clingfish *Diademichthys lineatus* Sauvage, 1883. However, at least one invertebrate (a copepod of the genus *Echinosocius*) was only found associated with *D. savignyi*, while the fish *Dascyllus trimaculatus* Rüppell, 1829 was observed to associate with *D. savignyi* (and *Echinothrix* sp.), but not *D. setosum*. Conversely, invertebrate taxa reported to date solely from *D. setosum* include the echinoid-associated stalked barnacle *Rugilepus pearsei* (Grygier and Newman 1991), while *D. setosum* appears to be the main host of some gastropod molluscs of the genus *Pulicicochlea* (Ponder and Gooding 1978). Differential inter-species associations mean that the distribution patterns of different *Diadema* species could influence biodiversity at various levels, including site and even finer (within-site) scales.

With respect to the Banggai cardinalfish *Pterapogon kauderni*, this study confirms the importance of *Diadema* urchins as a microhabitat for this species. The overall proportion of native *P. kauderni* associated with *Diadema* rises from around 53 to 69% if Toado (site 17, Tables 1 and 4) is excluded. The rarity of *Diadema* urchins and other typical microhabitats combined with the high abundance of *P. kauderni* make this ecologically atypical site an outlier which strongly skews aggregate microhabitat data. A majority of *P. kauderni* observed at Toado were swimming between the prop roots of mangroves belonging to the genus *Rhizophora*. This makes them very hard to catch, and is most likely one reason for the unusually high abundance of *P. kauderni* at this site.

Individual fish or groups of all size classes were observed to associate with and move between all *Diadema* morphological phenotypes present at both native/endemic (sites 1–14) and introduced (sites 15–18) *P. kauderni* population sites. Ndobe et al. (2018b) reported a similar pattern at five of the sites surveyed in this study (Liang, Popisi, Bone Baru, Tinakin and Kapela; sites 1–4 and 7 in Table 1; Online resource ESM 2) and presented experimental evidence for a lack of significant preference by *P. kauderni* between *D. setosum* and *D. savignyi.* Despite this apparent equivalence, multiple *Diadema* urchin species (and hybrids) may interact directly or indirectly with *P. kauderni* in overlapping but non-redundant ways. In particular, the differential distribution observed in this study (Table 5) indicates they may well play

such overlapping but non-equivalent roles in providing microhabitat opportunities for *P. kauderni*.

Conclusion

This study presents the first record of *Diadema clarki* mtDNA in the Coral Triangle, from sites both east and west of Sulawesi Island. This unexpected discovery could significantly expand the known range of *D. clarki*, a cryptic species recently resurrected based principally on mtDNA sequence data from Japan and the Marshall Islands. However, it remains unclear whether *D clarki* is extant in this expanded range, or whether the currently detected mtDNA signature of this species is merely a relict of its maternal lineage passed through time by introgression.

It seems increasingly likely that some proportion of individuals exhibiting morphologic phenotypes apparently typical of one *Diadema* species may in fact be cryptic hybrids, back-crosses or carriers of introgressed mtDNA. Such a situation would entail considerable likelihood of misidentifying *Diadema* urchins when relying on field observations of readily visible external morphologic characters (e.g. the colour patterns typical of species including *D. savignyi* and *D. setosum*) and/or mtDNA molecular markers such as CO1. Research combining mtDNA and genomic DNA markers should enable the testing of hypotheses regarding the prevalence of recent hybrids and historical introgression, as well as the number and identity of species and their respective distributions. Furthermore, multi-disciplinary research could contribute to elucidating the evolutionary patterns of *Diadema* urchins and the factors affecting *Diadema* species biogeography at multiple scales, from ocean basins (e.g. Japan to Sulawesi) to individual islands and even bays (e.g. in the context of *P. kauderni* conservation).

The ecological niches and roles of each *Diadema* species are also of interest, in particular with respect to associated organisms. Our results indicating non-equivalent habitat use between *Diadema* species indicates that the services they provide as microhabitats for associated organisms may also be non-equivalent, even in the case where associated organisms, such as the Banggai cardinalfish *Pterapogon kauderni*, do not appear to exhibit a preference between species within the genus *Diadema*. A better understanding of these niches and roles would inform biodiversity conservation at multiple scales, from ecosystems to species-level genetic diversity, as well as helping to predict trends under local and global change.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed by the authors.

Sampling and field study All necessary permitting and procedures were followed for the observational field study and the collection of samples, and the appropriate documents were provided to and/or obtained by the authors from the competent authorities.

Data availability The datasets generated during the current study have been deposited in the GenBank repository under MG988406–MG988411, MH051847–MH051888 and MK296413–MK296426.

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