



<https://doi.org/10.11646/zootaxa.4497.4.9>

<http://zoobank.org/urn:lsid:zoobank.org:pub:268716E0-82E6-47CA-BDB2-1016CE202A93>

## Needle in a haystack—genetic evidence confirms the expansion of the alien echinoid *Diadema setosum* (Echinoidea: Diadematidae) to the Mediterranean coast of Israel

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### Abstract

*Diadema setosum* (Leske, 1778), a widespread tropical echinoid and key herbivore in shallow water environments is currently expanding in the Mediterranean Sea. It was introduced by unknown means and first observed in southern Turkey in 2006. From there it spread eastwards to Lebanon (2009) and westwards to the Aegean Sea (2014). Since late 2016 sporadic sightings of black, long-spined sea urchins were reported by recreational divers from rock reefs off the Israeli coast. Numerous attempts to verify these records failed; neither did the BioBlitz Israel task force encounter any *D. setosum* in their campaigns. Finally, a single adult specimen was observed on June 17, 2017 in a deep rock crevice at 3.5 m depth at Gordon Beach, Tel Aviv. Although the specimen could not be recovered, spine fragments sampled were enough to genetically verify the visual underwater identification based on morphology. Sequences of *COI*, *ATP8-Lysine*, and the mitochondrial Control Region of the Israel specimen are identical to those of the specimen collected in 2006 in Turkey, unambiguously assigning the specimen to *D. setosum* clade b. This lends support to the hypothesis of a single introduction event and fits well with the habitat suitability and distribution model for *D. setosum* published recently. The more rapid and larger range extension along a south-eastern, counter-current trajectory may reflect a strong habitat preference for *D. setosum* in this area of the Levantine Basin as predicted by the model.

**Key words:** Alien species, molecular diagnostics, Mediterranean Sea, range expansion, Marine invasions

### Introduction

Invasive species have the capacity to dramatically transform marine habitats and obliterate indigenous communities (Byrnes *et al.* 2007, Sala *et al.* 2011, Edelist *et al.* 2013). As such, alien species pose an eminent threat to marine biodiversity (Molnar *et al.* 2008, Walther *et al.* 2009, Belmaker *et al.* 2013). One of the most heavily invaded marine regions to date is the Mediterranean Sea, and in particular its eastern basin (Belmaker *et al.* 2013, Edelist *et al.* 2013). Most of the alien species invading this basin are of Indo-Pacific origin and are thought to have infiltrated the Mediterranean through the Suez Canal (Golani 2010, Galil & Goren 2014) in a process referred to as Lessepsian Migration (Por 1971). Yet, despite the unparalleled magnitude of species introductions into the eastern Mediterranean (Galil 2012, Galil & Goren 2014), only seven alien echinoderms have so far been reported from this region (Bronstein *et al.* 2017), among them only one echinoid species, *Diadema setosum* (Leske 1778) (Yokes & Galil 2006).

*Diadema* urchins are prominent and abundant members of benthic communities throughout their vast range (Lawrence & Sammarco 1982, Birkeland 1989, Bronstein & Loya 2014, Bronstein *et al.* 2016) and typically inhabit shallow-water hard substrates (Bauer 1980). *Diadema setosum* was first observed in the Mediterranean off the Kaş Peninsula (36.140833°N, 29.655000°E), Turkey, on August 12, 2006 (Yokes & Galil 2006). This species has latter been reported from various other localities throughout the Mediterranean including four other localities in Turkey, as well as Greece and as far south as Lebanon (see Bronstein *et al.* 2017; fig. 7). Nevertheless, the only specimen collected to date is that of the original report by Yokes and Galil 2006 (deposited at The Steinhardt Museum of Natural History, Tel Aviv University under specimen voucher number SMNH\_EC\_25437).

Molecular studies show that *D. setosum* diverge from the other extant *Diadema* sometime during the Miocene, and that the *D. setosum* lineage split into two clades ca. 3-5 million years ago: a widespread Indo-West Pacific clade (*D. setosum* clade a) and a clade confined to the areas surrounding the Arabian Peninsula (*D. setosum* clade b) (Lessios *et al.* 2001, Bronstein *et al.* 2017). Recently, Bronstein *et al.* (2017) investigated the distribution of the two *D. setosum* clades and modelled their expansion in the Mediterranean. Their model predicts high probability for the expansion of clade b throughout the eastern Mediterranean while predicting only low probability for the expansion of clade a in this region (Bronstein *et al.* 2017; figures 6 and 7).

Here we provide the first confirmed record of *D. setosum* from the coast of Israel utilising molecular diagnostics to unambiguously assert the identification of this species to the clade level. We apply novel genetic markers, designed to provide higher phylogenetic resolution, to gain new insights on the origin of *Diadema* in the Mediterranean. Our findings conform to the suggested model of Bronstein *et al.* (2017) and call for attention from management and conservation agencies.

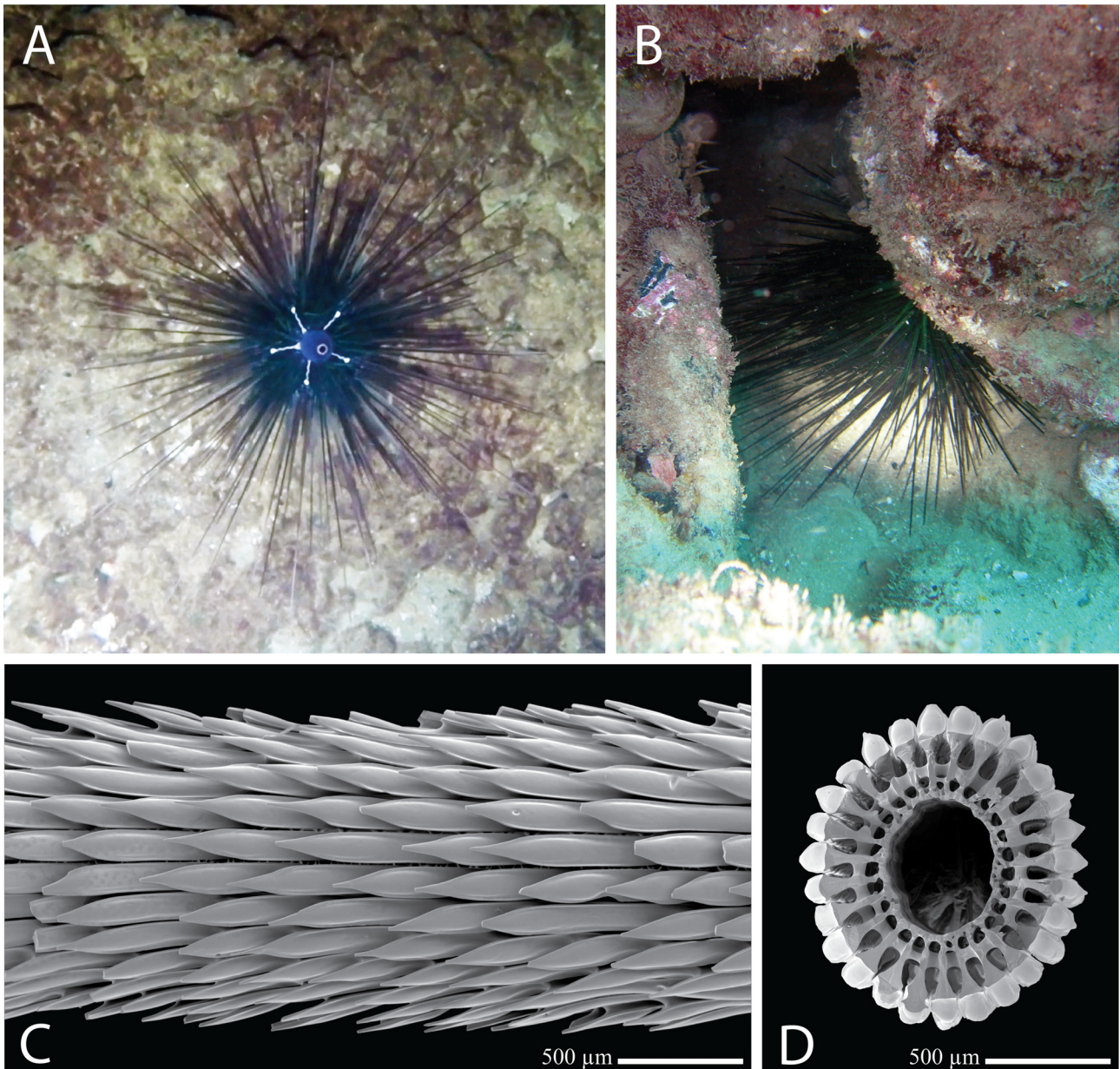
## Methods

**Material.** Evidence suggesting the presence of *D. setosum* in Israeli waters started accumulating over the past 18 months and was mostly based on reports from recreational divers. Photographic documentation of several encounters unfortunately yielded very poor results that failed to provide enough data to even assert the presence of a diadematoid species, let alone *D. setosum*. The first convincing evidence appeared on November 10, 2016 during a night dive at the Gordon Beach site, Tel Aviv, Israel, when a group of recreational divers recorded a video of an apparent *D. setosum* at a depth of 6–8 meters (Fig. 1A and Supplementary materials Video S1). Following this observation, frequent attempts were made to locate and sample a specimen for use of molecular identification and assertion of molecular lineage. These included efforts within the framework of the marine BioBlitz task force, a comprehensive monitoring program of Israel's marine protected areas (Yahel & Fried 2018), as well as hours of underwater surveys at the Gordon Beach site, where the most promising field observation was made. Finally, on June 17, 2017, a single adult specimen (ca. 60 mm test diameter) of *D. setosum* was observed at a depth of 3.5 m (Fig. 1B) at a nearby site (32.083633°N, 34.762836°E). The specimen was situated in a deep rock crevice at a site known as the 'Gordon Caves'. Due to its rareness, non-lethal sampling was applied, and several spine fragments including a single intact spine with the attached spine muscles was retrieved (by OB) from this particular specimen for morphological and molecular analyses.

**Molecular diagnostics.** In total, we targeted three mitochondrial regions: (1) a fragment of the Lysine-tRNA, ATPase-6 and ATPase-8 regions (*LYS*) and (2) a fragment of the *cytochrome c oxidase subunit I (COI)* gene (following Lessios *et al.* 2001). Laboratory procedures for these markers including DNA extractions, PCR reaction conditions and sequencing are as in Bronstein *et al.* 2017. In order to further investigate genetic variation within *D. setosum*, an additional (3) highly variable area in and around the echinoid control region (*CR*) (suggested as a target for inferring the phylogeny of closely related species, Bronstein *et al.* 2018) was amplified and compared among members of both clades of *D. setosum*. The new sequences have been deposited in GenBank under the accession numbers: MG764560 (*COI*), MG764561 (*LYS*), and MG764562 (*CR*).

63 additional *COI* sequences and 399 additional *LYS* sequences comprising all extant species of *Diadema* were recovered from GenBank (Supplementary materials Tab. S2) and used to compile a comprehensive phylogenetic tree of *Diadema*. Sequences were treated as in Bronstein *et al.* 2017 yielding a final *COI* dataset contained 526 bp long, a *LYS* dataset 524 bp long, and a *CR* dataset 405 bp long. *Astropyga radiata* was used as outgroups for the *COI* dataset and *Echinothrix calamaris*, *A. pulvinata* and *A. radiata* served as outgroups for the *LYS* dataset (Supplementary materials Tab. S2).

Phylogenetic analyses were conducted using both Maximum Likelihood (ML) and Bayesian Inference (BI) approaches as described in Bronstein *et al.* 2017. The best fitting models for the ML and BI analyses were selected using PartitionFinder2 (Lanfear *et al.* 2017) based on the Bayesian Information Criteria (BIC) (Schwarz 1978). The best fit models for the *COI* dataset were GTR+G and HKY+G for the ML and BI analyses, respectively. For the *LYS* dataset, the best fit model for both ML and BI analyses was GTR+G+I. Sequence similarity for the *CR* dataset was evaluated using the BLAST Global Alignment module on the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

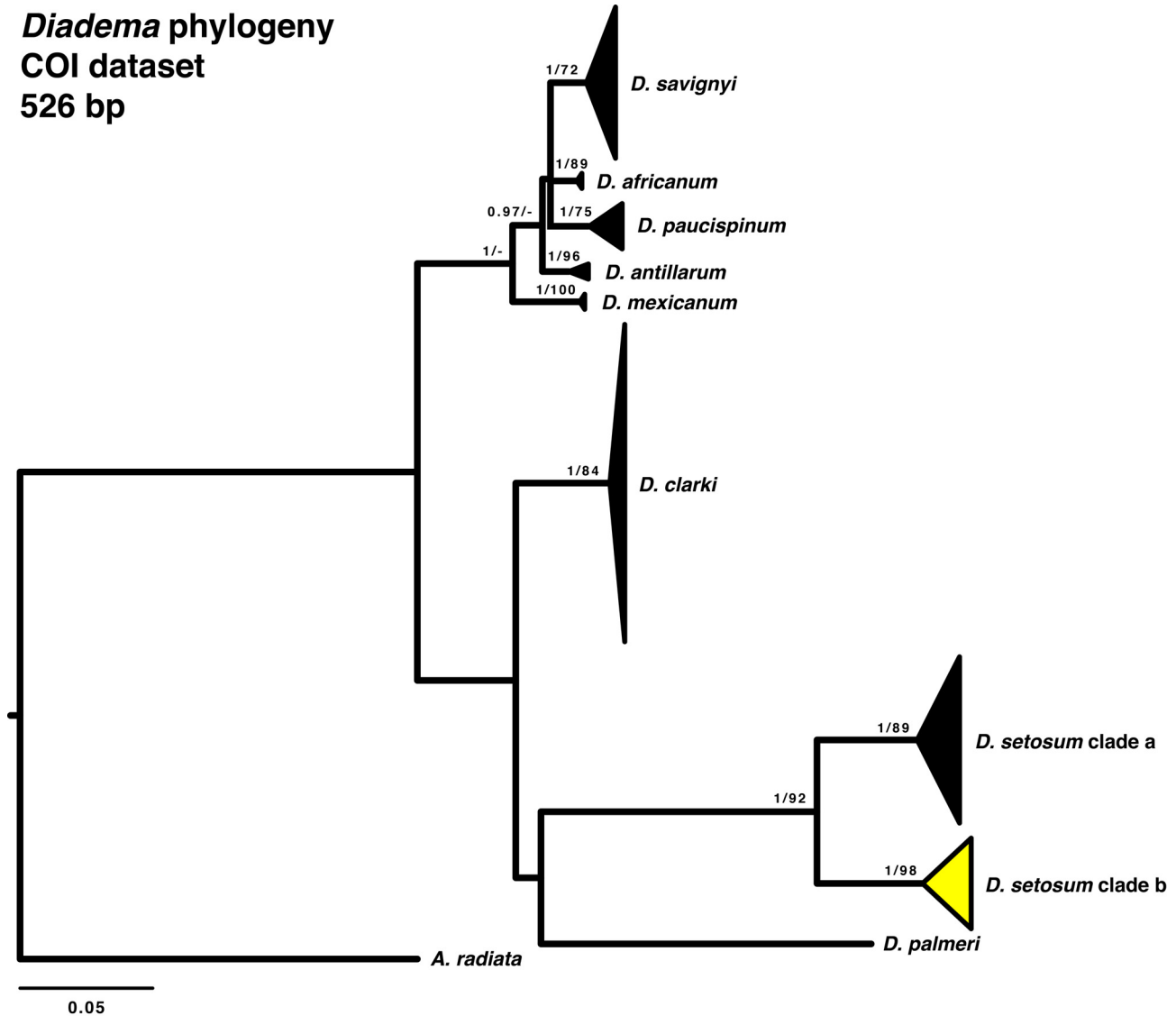


**FIGURE 1** Images of live *Diadema setosum* in the field: A) from Tel Aviv, Israel (32.086358°N, 34.763918°E), on 10.11.2016, at a depth of 6–8 m (Photograph: Adi Gvir; specimen not collected). B) from Tel Aviv, Israel (32.083633°N, 34.762836°E), on 17.06.2017, at depth of 3.5 m (a single spine was retrieved from specimen, deposited at the Steinhardt Museum of Natural History, Tel Aviv University under voucher number SMNH\_Ec\_25635). Scanning electron microscope images of *D. setosum* spines from specimen shown in B. C) longitudinal view showing the characteristic diadematoid verticillation; D) transverse section showing the axial cavity and solid wedges of the spine. See also Supplementary materials Fig. S3.

## Results

**Morphological characteristics.** The general appearance of the two specimens (Fig. 1A and B) reported here shows the typical characteristics of *D. setosum*. The largely spherical, black test is slightly dorso-ventrally compressed. The presence of the five white spots, as well as a bright orange ring surrounding the distal margins of the periproctal cone, a distinguishing character for this species, were clearly visible in both specimens (Fig. 1A). White interradial lines connecting the apical disc to the white spots were very prominent in one of the two specimens observed (Fig. 1A), but are well within the variability observed by us in the native habitat (Red Sea and Zanzibar; OB unpubl. data).

Length of slender black spines is ca. 1.5–2.5 times the test diameter (TD) in both specimens (Fig. 1A and B). Typical for Diadematae, the spines are characterised by a large, hollow central cavity formed by a foraminated cylinder to which 27 solid wedges are attached in the present specimen (Fig. 1C and D). The outer surface of the spine is characterised by verticillation (pointed solid barbs pointing distally), arranged in a spiral manner (Fig. 1C). Specimens of *Diadema paucispinum* and *D. setosum* have been reported to have distinct spine structures in comparison to the other species of this genus (Coppard & Campbell 2004). In particular, the spines of *D. setosum* are characterized by their length in relation to test size and reduced flaring of their verticillations (see Coppard & Campbell 2004 for detailed description).



**FIGURE 2** Phylogenetic relationships of the genus *Diadema*. Superimposed Maximum Likelihood and Bayesian Inference phylogenetic trees based on *COI* sequences representing all extant species of *Diadema*, rooted by *Astropyga radiata*. Major clades were collapsed. The clades including Mediterranean *Diadema* sequences are highlighted in yellow (*D. setosum* clade b). Supporting values ( $> 0.9$  posterior probabilities and  $> 70\%$  ML bootstrap values) are shown above the nodes (before and after the slash, respectively). ML bootstrap support was calculated from 1000 replications and BI posterior probabilities from 7.5 million generations (after burn-in). GenBank accession numbers of the corresponding sequences used in the analysis are given in Supplementary materials Tab. S2.

**Molecular diagnostics.** Bayesian Inference (BI) and Maximum Likelihood (ML) analyses for each marker (i.e., *LYS* and *COI*) produced congruent topologies for all major clades and subclades, consequently only the BI inferred topologies are presented showing both posterior probabilities and bootstrap support for each clade. Furthermore, sequence similarity of the specimen analyzed in the current study is identical to the Mediterranean *D.*

*setosum* sequenced in Bronstein *et al.* (2017). Here only the *COI* tree is presented, as currently this marker is the only to include all extant species of *Diadema* (*LYS* sequences are currently not available for *D. clarki*, and *CR* sequences only exist for *D. setosum* clades a and b).

Our novel sequence data confirms that the specimen (Fig. 1B) encountered on June 17, 2017 in the waters of Tel Aviv, Israel (32.083633°N, 34.762836°E), is in fact *D. setosum* clade b (i.e., belonging to the Red Sea/Arabian Peninsula clade) (Fig. 2). Comparisons of the highly variable *CR* marker, to further explore molecular diversity within *D. setosum*, revealed remarkable similarity within this clade as both Mediterranean *Diadema* (the current specimen sampled in Israel and the 2006 specimen sampled in Turkey) showed 100% sequence similarity. Additional *CR* sequences were used to compare sequence similarity between the two Mediterranean *D. setosum* clade b (GenBank Accession numbers: MG198159 and MG764562), Red Sea representatives of *D. setosum* clade b (GenBank Accession numbers: MG198160–MG198162) and Indo-Pacific *D. setosum* clade a (GenBank Accession number: KX385835). These comparisons showed 99–100% similarity between members of clade b of both Red Sea and Mediterranean origin and 97% similarity between clade a and the members of clade b.

## Discussion

The present data provides the first verified record of *D. setosum* in Israel. This finding is supported by both morphological and molecular evidence. Only the latter enables distinguishing the two mitochondrial clades of *D. setosum*, and the present study unambiguously demonstrate that *D. setosum* from the Mediterranean coast of Israel belongs to clade b of this species, native to the Red Sea, Arabian Peninsula and the Persian Gulf. These findings serve as a vivid example for the advantages of utilising molecular genetic tools to complement morphological examinations. Here, a single spine obtained with negligible impact on the live specimen, facilitated molecular analysis. In the case of *Diadema*, such molecular diagnostics provide insights beyond the reach of morphological traits as the two *D. setosum* mitochondrial clades are currently only detectable through genetic tools. Remarkably, the recent specimen sequenced from Israel showed 100% sequence similarity to the 2006 specimen from Turkey in all three markers utilised. This may imply limited genetic diversity within the Mediterranean *Diadema* population as might be expected in the case of a single introduction event (e.g., *ATPase-6* and *ATPase-8* sequences of clade b show ten haplotypes globally, only one of which is represented in all Mediterranean samples analysed so far, see Bronstein *et al.* 2017: fig. 4). However, more samples and potentially specific population level markers (such as microsatellites) are needed to test this hypothesis.

The current report lends support to the habitat suitability and distribution model presented by Bronstein *et al.* 2017 for clade b along the eastern Mediterranean shoreline. *Diadema setosum* clade b has now expanded over most of the Levantine Sea, covering more than 2000 km of the Levantine coast, with its present location in Israel more than 1500 km shoreline distance from the site of the initial report in 2006, off the Kaş Peninsula in Turkey. *Diadema setosum* has now been repeatedly collected for more than a decade in the eastern Mediterranean and thus seem to have established a stable population. This population seems to have expanded its range since the first record near the north-western margin of the Levantine Basin. The relatively slow south-bound progress may be attributed to the general counter-current direction of this expansion. Nevertheless, the range expansion of this species advanced more than three times further on a south-eastern trajectory than it had in a north-western direction from the point of first sighting in Kaş (ca. 1500 vs. 500 km of shoreline distance). This prominent bias is even more pronounced due to the counter-current direction of this expansion, and may reflect a strong habitat preference for *D. setosum* in the south-eastern boundary of this basin as predicted by Bronstein *et al.* (2017). Interestingly, *D. setosum* is currently spreading throughout the Eastern Mediterranean at comparatively low local population densities and, therefore, is hard to detect. This may indicate that *D. setosum* is still in an early stage of the invasion process, identified by some as “establishment phase” (Arim *et al.* 2006). Its spread may thus have progressed much further than currently documented, considering the difficulty in verifying its existence even in a highly surveyed area such as the Israeli coast line. Yet, without further sampling, the possibility of reoccurring introductions, seeding *D. setosum* populations at various localities along this route, cannot be excluded at this stage.

Finally, despite the significant improvement in habitat monitoring along the Israeli coast (Yahel & Fried 2018), identifying *Diadema* would not have been made possible without the valuable data generated by recreational divers. Equipped with high-resolution cameras and intimately familiar with the dive sites they regularly visit, these

individuals are gathering large amounts of essential geo-referenced data and spend much more time in the field than ever possible for most active scientists. Eager to contribute and support science these people constitute a valuable asset that deserves to be acknowledged and adopted by the scientific community.

## Acknowledgments

We acknowledge the Steinhardt Museum of Natural History (SMNH), Tel-Aviv University for facilitating access to the collections. The Central Research Laboratories at the Natural History Museum Vienna are acknowledged for facilitating the molecular analysis and SEM imaging. We gratefully acknowledge Shevy Rotman (SMNH), Amos and Ben Horesh, Adi Gvir, Yaron Hershkovici, Dudu Nachmias, and Dror Gilat for assistance in the field and for providing invaluable field observations and documentation that greatly contributed for this study. The constructive comments by Marc Eléaume (MNHN Paris) and John Pearse (UC Santa Cruz) are gratefully acknowledged.

## Supplementary materials

Supplementary data accompanying this paper is available from: <https://doi.org/10.11646/zootaxa.4497.4.9>

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