Do genes lie? Mitochondrial capture masks the Red Sea collector urchin's true identity (Echinodermata: Echinoidea: *Tripneustes*)

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Abstract

Novel *COI* and *bindin* sequences of the Red Sea collector echinoid *Tripneustes gratilla elatensis* are used to show that 1) discordance between mitochondrial and nuclear loci exists in this echinoid genus, 2) *Tripneustes gratilla* as currently defined possibly comprises a complex of cryptic species, and 3) Red Sea *Tripneustes* form a genetically distinct clade in the *bindin* tree, which diverged from other *Tripneustes* clades at least 2-4 million years ago. Morphological reassessment of *T. gratilla elatensis* shows perfect congruence between identification based on skeletal features and genetic data based on a nuclear marker sequence. Hence the Red Sea *Tripneustes* subspecies established by Dafni in 1983 is a distinct biological unit.

All *T. g. elatensis* samples analyzed are highly similar to or share mtDNA haplotypes with Philippine *T. g. gratilla*, as do representatives from other edge-of-range occurrences. This lack of genetic structure in Indo-Pacific *Tripneustes* is interpreted as a result of wide-spread mitochondrial introgression. New fossil specimens from the Red Sea area confirm the sympatric occurrence of *T. g. elatensis* and *T. g. gratilla* in the northern Red Sea during Late Pleistocene, identifying a possible timing for the introgression. In addition, present-day distribution shows a contact zone in the Southern Red Sea (in the Dahlak Archipelago).

T. g. elatensis, is yet another example of a Red Sea taxon historically identified as conspecific with its Indo-Pacific relatives, but which turned out to be a morphologically and genetically distinct endemic taxon, suggesting that the level of endemism in the Red Sea may still be underestimated.

Keywords

Molecular phylogeny

Biogeography

Mitochondrial capture

Integrative taxonomy

Toxopneustidae

Red Sea

1. Introduction

Understanding the forces that drive speciation in marine environments remains a challenging goal despite significant progress in recent years. High dispersal potential of organisms with planktonic larvae and lack of clear geographic barriers to gene flow between populations, greatly complicate our ability to reconstruct the chain of events leading to the formation of new species (McCartney et al., 2000; Palumbi, 1994). Owing to their relatively low number of extant species and to the existence of detailed alpha taxonomical studies, sea urchins had long been used as model organisms for the study of marine speciation (Mayr, 1954; Palumbi, 1996). Mayr (1954) extracted taxonomic and distributional information from Mortensen's (1928-

51) “Monograph of the Echinoidea”, and concluded that speciation in shallow water echinoids occurs allopatrically. However, since Mayr’s pioneering work, several studies demonstrated the occurrence of species diversification even without clear geographic isolation (e.g., Coppard et al., 2013; Metz and Palumbi, 1996). Nonetheless, with time, geographic isolation is prone to give rise to highly diverged populations, and, owing to ecological speciation, this process may accelerate in areas of markedly different environmental conditions, such as those reported for the Red Sea (RS) during glacial cycles (DiBattista et al., 2016a).

The RS is a deep and narrow semi-enclosed body of water and is considered the world’s northernmost tropical sea. It spans more than 2,200 km from the northern gulfs of Suez and Aqaba to the Strait of Bab-el-Mandeb in the south. The RS is a biogeographical region of exceptional high taxonomic diversity and high levels of endemism (see review by DiBattista et al., 2016b) and as such is referred to as a biodiversity hotspot. The latter authors report that 12.9% of fishes, 12.6% of polychaete, 8.1% of echinoderms, 16.5% of ascidians and 5.8% of scleractinian corals in the RS are endemic, making this region a hot spot for endemism with some of the highest levels of endemism recorded in the entire Indo-West Pacific. Such highly diverged peripheral regions as the RS, are also referred to as “evolutionary incubators” recognizing their important contribution of unique genetic lineages (Bowen et al., 2013; DiBattista et al., 2013; Malay and Paulay, 2010; Skillings et al., 2011). The geological history of the RS and its potential isolation barriers have been discussed by DiBattista et al. (2016a). The history of the RS dates back to the Late Cretaceous, but it was not until 5 to 4 million years ago (Ma) that conditions in the RS stabilized from the early unfavorable, highly saline environment, to facilitate the proliferation of marine fauna during the Pliocene and Pleistocene (4 to 3 Ma) when also a more permanent connection to the Indian Ocean (IO) was consolidated (Bailey et al., 2007). Except for the man-made Suez Canal completed in 1869, connecting the RS with the Mediterranean Sea at the northern tip of the Gulf of Suez, the narrow and shallow Strait of Bab-el-Mandeb (29 km and 137 m, respectively), constitute the only connection of the RS to the IO (Bailey, 2009). This topographical setup renders the RS susceptible for isolations during periods of low sea level that are often associated with glacial maxima (Ludt and Rocha, 2015; Rohling et al., 2009). The latest such occasion is believed to have occurred during the Last Glacial Maximum, ca. 20 to 15 ka ago when sea level dropped by 115 m (Ludt and Rocha, 2015; Siddall et al., 2003). Moreover, the monsoonal-driven cold-water upwelling off the coasts of Somalia and Oman (reaching temperatures as low as 13 °C; Currie et al.,

1973; Smeed, 1997) is believed to act as yet another isolating barrier for the RS fauna (Kemp, 1998, 2000). Regardless of the mechanism of isolation, different basins within the RS experienced significantly different environmental conditions during periods of isolation. Of these conditions, hyper-salinity is acknowledged as the major driver of extinction (Kinsman, 1964; Por, 1972, 2008). Interestingly, estimations based on planktonic foraminiferans suggest that salinity in the Gulf of Aqaba (GOA) remained within tolerable levels of below 45‰, at times when the rest of the RS Basin experienced hyper-saline conditions of ca. 55-57‰ (Fenton et al., 2000; Thunell et al., 1988), suggesting the GOA may have served as refugium for RS fauna (DiBattista et al., 2016a).

Sea urchins of the genus *Tripneustes* L. Agassiz, 1841 are some of the most common and widespread shallow-water echinoids (Lawrence and Agatsuma, 2013). Within this genus three extant species are currently recognized: the Indo-Pacific *Tripneustes gratilla* (Linnaeus, 1758), the eastern Pacific *Tripneustes depressus* (A. Agassiz, 1863), and the Atlantic *Tripneustes ventricosus* (Lamarck, 1816) (Mortensen, 1928-51). Due to their wide circum-tropical distribution and high abundance at the sites where they occur, these species received much attention in the scientific literature including detailed studies of their ecology (Lawrence and Agatsuma, 2013; Mortensen, 1928-51; Vonk et al., 2008), growth (Dafni, 1992; McPherson, 1965), behavior (Amato et al., 2008; Kehas et al., 2005), and reproduction (Chen and Chang, 1981; Muthiga, 2005; Pearse, 1974; Vařtilingon et al., 2005). Furthermore, due to their fast growth rate and high market value, *Tripneustes* are also highly valued by the fisheries and aquaculture industry (Mos et al., 2012).

High interspecific similarity in this genus, with only minor morphological characteristics to distinguish the species (Mortensen, 1928-51), led some workers to suggest that they are probably but a single species (Clark, 1912). These claims are in part supported by later studies incorporating molecular genetic evidence that, while ratifying the taxonomic status of *T. ventricosus*, question the validity of treating *T. gratilla* and *T. depressus* as two separate species (Lessios et al., 2003). Of all *Tripneustes* species, *T. gratilla* is the most common and widespread species, ranging from the East-Pacific to East Africa and the RS and from the Kermadec Islands (New Zealand) to Japan (Lawrence and Agatsuma, 2013). However, across its vast range, *T. gratilla* shows considerable morphological variability and representatives from the RS are morphologically distinct from other *T. gratilla* populations. These differences include sparse inter-ambulacral tuberculation, compressed shape of the corona

(lower height), larger peristome, fewer inter-ambulacral plates and distinct coloration (Dafni, 1983). Consequently, a new subspecies was proposed for the RS population: *T. gratilla elatensis* Dafni, 1983. Phylogeographic studies of *Tripneustes*, targeting populations across their range, demonstrated a lack of phylogenetic structure in mitochondrial (mt) DNA (Lessios et al., 2003; Liggins et al., 2014). Zigler and Lessios (2003) studied variation of the nuclear *bindin* gene (coding for a gamete recognition protein) in *Tripneustes* from the Pacific, Atlantic, and Indian oceans. They found that the Atlantic *T. ventricosus* differed from the eastern Pacific *T. depressus* and the Indo-West Pacific *T. gratilla*, but found no differences between the *bindins* of *T. depressus* and *T. gratilla*, nor any geographic structure in the distribution of *bindin* alleles. Lack of phylogeographic structure and haplotype sharing between widely distant localities as the Philippines, Kermadec Island and the Galapagos Islands is suggestive of recent radiation and high dispersal potential. However, *Tripneustes* from the RS and Gulf of Aqaba have never been studied and illustrated in a modern taxonomical manner and were not included in any of the phylogeographic studies of the genus (e.g., Lessios et al., 2003; Liggins et al., 2014; Zigler and Lessios, 2003). Thus, the taxonomic status of the northern RS subspecies *T. gratilla elatensis* and its relation to the two mt clades of *Tripneustes* are currently unclear.

In discussing phylogenies and species delimitation it is important to discern two fundamental concepts: phylogenetic (species) trees and gene trees. Phylogenetic trees are defined as the branching pattern of species lineages via the process of speciation, while gene trees are contained within species trees and represent the branching pattern of a single locus in the genome (Maddison, 1997). Consequently within a species, many gene trees can be found and a subset of those can potentially disagree with their underlying species trees. To mitigate the potential risk of drawing erroneous phylogenetic conclusions, multiple independent genetic markers including loci both of the mt genome (mtDNA) and the nuclear genome (nuDNA) are commonly used (Avice, 1994). Of all genetic markers, mitochondrial genes have long served as the markers of choice for phylogeographic and species-level phylogenetic analyses (Avice, 2000). Analyses based on mitochondrial data often provide robust phylogenetic and phylogeographic estimates, however, under certain circumstances, a mitochondrial gene tree may fail to reflect phylogenetic descent properly (Doyle, 1997; Nichols, 2001), but rather reveal other evolutionary processes such as introgressive hybridization (Funk and Omland, 2003; Maddison, 1997; Ruedi et al., 1997). If left undetected, such processes may confound phylogenetic inference (Leaché and McGuire, 2006), however, if the signatures of

these processes are recognized, their occurrence can provide insights into the evolutionary process that would otherwise remain undetected (McGuire et al., 2007).

Here we combine genetic (mt and nc), morphological, paleontological and ecological data to resolve the taxonomic status and reconstruct the phylogeny of RS *Tripneustes*. We provide a taxonomic re-evaluation of RS *Tripneustes*, and present the first genetic data for *Tripneustes* from this region. We then use these data to explore the forces shaping the evolutionary history of RS *Tripneustes*, which also provides new insights relevant to the phylogeny and taxonomy of *Tripneustes* as a whole.

2. Materials and methods

2.1. Study area and sample collection

Specimens of *Tripneustes* were collected around the world at depths of 0.5 to 10 meters (Fig. 1). All tissue samples were fixed and preserved in 96% molecular grade EtOH. Table S1 gives an overview of all analyzed samples and lists sample names, collection locations, GenBank accession numbers and the source of the data. Specimens have been collected under permit numbers: (1) 2014/40622 issued on October 20th 2014 by Israel Nature and National Parks Protection Authority, (2) TAFIRI HQ/BIW/Vol.III/103 issued on August 31st 2014 by The Permanent Secretary, Ministry of Livestock and Fisheries Development, Dar Es Salaam, Tanzania, and (3) Gratuitous Permit numbers GP-0077-14 and GP-0085-15, made available in accordance with a material transfer agreement between the Natural History Museum Vienna and the California Academy of Sciences (CAS).

2.2. DNA extraction, PCR and sequencing

Total genomic DNA was extracted from spine muscle or tube feet tissue using Promega's Tissue and Hair Extraction Kit used in conjunction with the DNA IQ™ System (Promega Corporation, WI, USA) following manufacturer's instructions. Two molecular markers were analyzed: the mt *cytochrome c oxidase subunit I* gene (*COI*) and the nu *bindin* gene (partial sequences of two exons and the intervening intron).

Three primer pairs were used to amplify up to three overlapping fragments of the *COI* gene (Table 2), covering and adding on the existing published *COI*

sequence data for *Tripneustes* (Liggins et al., 2014; Zigler and Lessios, 2003). The largest *COI* amplicon (primers TripCOI1+ / TripCOI5-) had a length of 1168 bp. For details on the other fragments see Supplementary Material. In total, *Tripneustes* sequences from 23 RS, 6 Western Indian Ocean (WIO) and 9 Western Pacific individuals have been obtained. Polymerase chain reactions (PCR) were performed in 25 µl using 1 µl DNA (10-15 ng), 2.5 µl 10X AmpliTaq Gold® 360 Buffer (Applied Biosystems), 2 mM Magnesium Chloride, 1 µl 360 GC Enhancer, 0.2 mM dNTPs, 0.1 µM of each primer, and 1.25 units AmpliTaq Gold® 360 DNA Polymerase. PCR conditions consisted of an initial denaturation step at 95 °C for 5 min followed by two cycles of [95 °C for 15 s, 60 °C for 30 s and 72 °C for 70 s], additional 38 cycles of [95 °C for 15 s, 56 °C for 30 s and 72 °C for 70 s], and a final extension step at 72 °C for 7 min. For amplifications using the primer pair TripCOI1+/TripCOI4- or TripCOI3+/TripCOI5-, 40 cycles of single step PCRs were applied differing only in the annealing temperatures (62 °C and 54 °C, respectively). Amplified fragments were visualized on 1% agarose gels and purified using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's protocol. Direct sequencing (in both directions) was performed at LGC Genomics (Berlin, Germany) using PCR primers. *COI* sequences are deposited in GenBank under the accession numbers KU314827 to KU314864.

Bindin sequences were obtained from 28 specimens: 11 from RS, 5 from the WIO, and 12 from the Western Pacific. The complete target sequence (~1613 bp) was amplified with primers Tripbin1a+ / Tripbin3a- (Table 2). PCR reactions were performed in 25 µl containing 2 µl DNA (10-15 ng), 2.5 µl 10X High Fidelity PCR Buffer, 2 mM MgSO₄, 0.2 mM dNTPs, 0.2 µM of each forward and reverse primer, and 0.5 units Platinum® Taq High Fidelity polymerase. PCR conditions were 2 min at 94 °C, 2 cycles [94 °C for 15 s, 62 °C for 30 s, 68 °C for 90 s] followed by 35 cycles [94 °C for 15 s, 53 °C for 30 s, 68 °C for 90 s]. PCR products were sequenced directly at LGC Genomics (Berlin, Germany) using the primers Tripbin1a+, Tripbin3a-, Tripbin5+ and Tripbin4-. Sequences with double peaks in chromatograms were cloned prior to sequencing (for details see Supporting Information). *Bindin* sequences are deposited in GenBank under the accession numbers KU355756 to KU355783.

Table 1

Primer sequences for amplification and sequencing of the *COI* and *bindin* fragments.

Primer sets		
Region	Primer name	Sequence (5' to 3')
<i>COI</i> (fwd)	TripCOI1+	GCCTGAGCCGGAATGGTAGG
<i>COI</i> (rev)	TripCOI5-	ACCTTTCCTCATAGAGGGTG
<i>COI</i> (internal fwd)	TripCOI3+	TCTTTACCAGTTCTAGCAGG
<i>COI</i> (internal rev)	TripCOI4-	TCTCCTCCGCCTGCTGGGTC
<i>Bindin</i> (fwd)	Tripbin1a+	CCCAGAAATTTCCGACGATT
<i>Bindin</i> (rev)	Tripbin3a-	CCTTGCCGATAACCCTGATTGT
<i>Bindin</i> (internal rev)	Tripbin2-	TGGTAGGTCAATCTTCGTGG
<i>Bindin</i> (internal rev)	Tripbin4-	TACCTATTTACAAAAGTGGC
<i>Bindin</i> (internal fwd)	Tripbin5+	AATACAGTTCAGCTAGCACCG

2.3. Sequence statistics and phylogenetic tree reconstruction

Phylogenetic analyses were based on the *COI* and *bindin* datasets. In order to facilitate the integration of our sequences with previously published data, three *COI* datasets were created: the first contained 37 full length *COI* sequence (1126 bp after sequence editing) with *Strongylocentrotus purpuratus* as outgroup. The second (531 bp long) containing 23 *T. g. elatensis* and 34 *T. g. gratilla* sequences plus an additional 14 *T. ventricosus*, 6 *T. depressus* and *Lytechinus variegatus* serving as outgroup (see tables S1 and S2 for details), and a third (449 bp long) comprising our data plus an additional 289 comparable *Tripneustes* *COI* sequences from the literature yielding 328 sequences in total: 23 *T. g. elatensis*, 35 *T. depressus*, 201 *T. g. gratilla*, 22 *Tripneustes* of uncertain specific identity, and 47 *T. ventricosus*, Table S2). Similarly, our full length *bindin* alignment (comprising two partial exons and an intron; 1613 bp) was used to compile two datasets; one ("*bin-compI*"; 1186 bp), contained parts of both exons and the intron after removal of gaps and ambiguous sites. The second dataset ("*bin-exons*"; 539 bp) was prepared without the intron (857-881 bp) which could not be unambiguously aligned with sequences of outgroup taxa. When applicable, *Lytechinus variegatus* was used as outgroup (i.e., for the *Bin-*

exons and for the *COI* subset datasets) since this genus has been identified as a sister taxon of *Tripneustes* (Smith and Kroh, 2013; Zigler and Lessios, 2003).

Raw sequences were aligned automatically using ClustalX v2.1 (Larkin et al., 2007) and edited manually using Bioedit v.7.1.3 (Hall, 1999). Median-Joining networks were calculated for the 449 bp *COI* dataset with PopArt (Leigh and Bryant, 2015) applying the default settings. For the other datasets positions with gaps were removed using TrimAl v.1.4 (Capella-Gutiérrez et al., 2009) and identical sequences identified using DAMBE v.6.0.1 (Xia, 2013). Our 39 *de-novo COI* 531 bp sequences collapsed into 16 haplotypes to which an additional 17 unique haplotypes from the other *Tripneustes* species were added and *L. variegatus* served as outgroup. Our 28 sequences of the full length *bindin* collapsed into 27 haplotypes and an additional 15 unique sequences of the other *Tripneustes* species were added. Phylogenetic reconstructions were performed using both the maximum likelihood (ML) method and Bayesian inference (BI). ML analyses were all performed with RAxML v.7.2.6 (Stamatakis, 2006) while BI analyses were conducted with MrBayes v.3.2.2 (Ronquist and Huelsenbeck, 2003). Partitions and optimal substitution models were determined for all individual data sets using the Bayesian information criterion (BIC) metric for model selection (Schwarz, 1978) in PartitionFinder v.1.1.1 (Lanfear et al., 2012). *COI* datasets were partitioned according to codon positions. The best-fitting substitution models for the 531 bp *COI* datasets were GTR+G and HKY+I (on all codon positions) for ML and BI, respectively. The *bindin* gene was partitioned by 1st and 2nd exons, the intron, and codon positions. For both *bindin* datasets (*bin-compl* and *bin-exons*) the best fitting model for the ML analyses was the GTR+G model. For the BI analyses the HKY+G and K80 substitution models were found best fitting for the *bin-compl* and *bin-exons* datasets, respectively.

RAxML tree reconstructions were carried out using 100 random starting trees. Branch support was computed based on 1000 bootstrap replications. Analyses in MrBayes were calculated for each of the datasets according to the respective data partitions. Applying the respective model parameters, the analyses were run for 10 million generations each (2 runs with 4 chains, one of which was heated), sampling every 100th tree. Convergence was assessed according to the average standard deviation of split frequencies <0.01. The runs were also visually checked by plotting generations vs. likelihood scores and in Tracer v.1.6 (Rambaut et al., 2013) to assess whether the two runs had converged and when the stationary phase was reached. In a conservative approach, the first 25% of trees were discarded as burn-in

and a 50% majority rule consensus tree was calculated from the remaining 75,000 trees.

Based on the *bin-compl* dataset, uncorrected mean p-distances and Kimura two-parameter (K2P; Kimura, 1980) genetic distances were calculated within and between species / clades using MEGA v.6.0.6 (Tamura et al., 2013). BEAST v.1.8.2 (Drummond and Rambaut, 2007) was used to estimate the timing of divergence of the different *bindin* clades based on both the *bin-compl* and the *bin-exons* datasets. The relative rate variation among lineages was tested separately for each dataset with the molecular-clock test implemented in MEGA v.6.0.6 (Tamura et al., 2013) applying the best fitting substitution models for each of the datasets (which are the same as used in the respective BI analyses). Since the null hypothesis of equal evolutionary rates throughout the trees were rejected at a 5% significance level ($P=0$), divergence times were estimated under a relaxed molecular-clock. Following previous studies investigating trans-Isthmian speciation in echinoids (Lessios et al., 2012; Coppard et al., 2013) the divergence times of the *bindin* lineages were estimated based on the split between the Atlantic and the Pacific *Tripneustes* set to 2.95 ± 0.15 Ma, assuming their separation at the latest at the time of the closure of the Isthmus of Panama (Coates and Obando, 1996; Coates et al., 2003). While the exact timing of the closure and establishment of an effective barrier to gene flow has recently been questioned (see Bacon et al. 2015a, b; Lessios, 2015; Marko et al., 2015), the date used herein represents the final closure and thus will result in minimum divergence ages. Settings in BEAUti v.1.8.2 (part of the BEAST package) included the best fitting substitution models for each dataset, implementing the Yule Process (Gernhard, 2008) speciation model. The BEAST analyses were each performed independently four times for 10^7 generations sampling every 1000th tree. Convergence of the runs was checked using Tracer v.1.6 (Rambaut et al., 2013), and combined with LogCombiner v.1.8.2 (part of the BEAST package). Subsequently, 25% of the trees were discarded as burnin and maximum clade credibility trees were calculated each from the remaining 30,000 trees. The secondary structure of the *bindin* intron was predicted using RNAstructure v.5.8 (Reuter and Mathews, 2010).

2.4. Morphological analysis

In order to account for true interspecific morphological differences rather than ontogenetic variation, RS *Tripneustes* specimens ranging from very young, newly

settled individuals (2.6 mm in diameter) to fully mature specimens (72.8 mm in diameter) were studied and compared to *T. gratilla* specimens from Tanzania and the Philippines. External appearance and coloration patterns (epidermis, pedicellariae, spines, and tube-feet) were noted for each specimen. The skeletal components (corona, plates, tuberculation, etc.) of selected specimens were assessed following removal of the soft tissue. This was done using enzymatic digestion of the soft tissue with Enzyrim (Bauer Handels GmbH, Germany) following the manufacturers protocol. Measurements of both the corona (height and diameter) and peristome were performed using digital Vernier calipers to the nearest 0.1 mm. Skeletal elements of the jaws were observed and measured under a Scanning Electron Microscope (SEM) (JEOL, JSM-6610LV) at the Central Research Laboratories of the Natural History Museum in Vienna, Austria. The type material of *T. g. elatensis*, deposited in the Hebrew University of Jerusalem (voucher number: HUJEC1) was examined and compared to the newly collected material. In addition, several public and private collections were screened for fossil *Tripneustes* specimens from RS Late Pleistocene raised reef terraces (Mansour et al., 2015). Fossil specimens used in the present study were deposited at the NHMW (collection of the Department of Geology and Paleontology).

3. Results

3.1. Phylogeny

The phylogenetic estimates obtained here were generally in agreement with respect to previously published assessments. However, as RS *Tripneustes* were never investigated using molecular tools its placement within *Tripneustes* is discussed here for the first time. Below we present the results of both ML and BI analyses for the mt and nc markers, respectively.

3.1.1. mtDNA

BI and ML analyses produced congruent phylogenies for all clades and subclades and thus only BI inferred topologies are presented (Fig. 3 and Fig. S1). Phylogenetic reconstructions based on the mt *COI* datasets failed to provide a clear structure in compliance with either the geographical distribution or morphological attribution of known *Tripneustes*. *T. g. elatensis* from the RS appeared polyphyletic

and nested within clades or shared haplotypes with the IWP *T. g. gratilla* (Fig. 3 and Fig. S1). The 531 bp *COI* dataset was constructed in an attempt to gain further insights on the phylogenetic framework of *Tripneustes*, accommodating comparisons of our newly generated sequences with the other known species of *Tripneustes* as well as to facilitate the rooting of the subsequent phylogenies. Rooting with *Lytechinus variegatus*, only the monophyly of *T. ventricosus* was strongly supported (Fig. 3, clade 2). *T. depressus* (Fig. 3, clades 4 and 6) as well as the RS *Tripneustes* (Fig. 3, clades 3 and 5) appear to be polyphyletic and nested within the widely distributed *T. g. gratilla* (Fig. 3, clade 1). The mt diversity of *Tripneustes* was further explored by displaying the distributional patterns of the *COI* haplotypes through a Median joining haplotype network (Fig. 4). Similarly to the topologies of the trees, the *COI* network is clearly divided into two clusters of haplotypes: the first includes all Atlantic haplotypes. The second cluster is formed by Pacific *Tripneustes*. Haplotypes of both *T. depressus* and *T. g. elatensis* are embedded within the large variety of *T. g. gratilla* haplotypes, and both share haplotypes with *T. g. gratilla*. Thus, neither *T. depressus* nor *T. g. elatensis* could be delimited from *T. g. gratilla* based on their mt *COI* sequences.

3.1.2. nuDNA

With *bindin* sequences, both BI and ML analyses yielded compatible tree topologies for all clades and subclades, and thus we again present the BI inferred topologies only. Similarly to the topologies inferred by *COI*, the resulting trees recovered *T. ventricosus* as a strongly supported clade (Fig. 5 and Fig. S2, clade E). The analyses also recovered the RS *T. g. elatensis* as a well-supported monophylum (Fig. 5 and Fig. S2, clade C). However, the *bin-compl* dataset also revealed that *Tripneustes* is split into two distant lineages comprising of *T. ventricosus* + a subset of *T. g. gratilla* specimens and *T. g. elatensis* + another subset of *T. g. gratilla* specimens (including all *T. depressus*) (Fig. 5, clades E+D_{1,2} and clades A_{1,2}+C, respectively). The RS *Tripneustes* clade is the sister group of the *T. g. gratilla* + *T. depressus* clade (Fig. 5 clades A₁+A₂ and Fig. S2, clade A), the later once again nested within *T. g. gratilla* (Fig. 5 and Fig. S2, clade B). Surprisingly, in all analyses, *T. g. gratilla* was not recovered as a monophylum, but is rather polyphyletic, as it occurs in several (partially highly supported) clades. One of these clades represents the sister group of *T. ventricosus* (Fig. 5, clade D_{1,2}), while the other (Fig. 5, clades A₁+A₂) clustered to form a sister group to *T. g. elatensis*. The later cluster was further

diverged into a branch containing *T. g. gratilla* taxa only (Fig. 5, clade A₂), and a branch containing *T. g. gratilla* and *T. depressus* specimens (Fig. 5, clade A₁). Divergence within the *T. g. elatensis* clade was low (0.4% mean K2P divergence) based on the *bin-compl* dataset, similar only to the divergence within *T. ventricosus* (Table 2). The intraspecific divergence within the clades of *T. g. gratilla* were 3-4 times higher than those of *T. g. elatensis* (1.1% and 1.5% vs. 0.4%, respectively). Interspecific divergence between *T. g. elatensis* and the other *Tripneustes* clades ranged from 3.4% to 3.6% and was comparable to the divergence recorded between *T. ventricosus* and *T. g. gratilla* clade A₁₊₂ (3.6%). *T. ventricosus* and the basal *T. g. gratilla* clade D₁₊₂ (Fig. 5) were the least diverged, showing only 2.4% genetic divergence.

The molecular clock estimations of minimum divergence dates based on the *bin-compl* dataset (Fig. S4) date the branching of *T. g. elatensis* at 3.74 Ma (2.04-5.6 Ma., 95% highest posterior density [HPD] interval). This split predates the divergence between *T. ventricosus* and the Pacific clades of *T. g. gratilla* (Fig. 5). The most basal split separating the *T. ventricosus* + *T. g. gratilla* clade D from the remaining clades was estimated to 4.63 Ma (2.99-6.6 Ma, 95% HPD).

Table 2

Intraspecific and interspecific distances (in percent) within *Tripneustes* based on the *bin-compl* dataset. Ranges and means (in brackets) of p-distances between (above diagonal) and within (diagonal, after slash) groups as well as K2P distances between (below diagonal) and within (diagonal before slash) groups are shown. Letters in brackets correspond to the clades in Fig. 5.

	<i>T. g. elatensis</i> (C)	<i>T. g. gratilla</i> + <i>T. depressus</i> (A ₁ +A ₂)	<i>T. g. gratilla</i> (D ₁ +D ₂)	<i>T. ventricosus</i> (E)
<i>T. g. elatensis</i> (C)	0.1-0.7 (0.4)/ 0.1-0.7 (0.4)	3.0-3.8 (3.4)	3.2-3.8 (3.5)	3.2-3.9 (3.6)
<i>T. g. gratilla</i> + <i>T. depressus</i> (A ₁ +A ₂)	3.1-3.8 (3.5)	0.1-2.4 (1.1)/ 0.1-2.6 (1.2)	2.6-4.4 (3.4)	3.2-4.4 (3.7)
<i>T. g. gratilla</i> (D ₁ +D ₂)	3.1-3.6 (3.4)	2.3-4.4 (3.2)	0.1-3.0 (1.5) /0.2-3.1 (1.6)	1.7-3.4 (2.4)
<i>T. ventricosus</i> (E)	3.3-3.9 (3.6)	3.0-4.3 (3.6)	1.7-3.4 (2.4)	0.2-0.7 (0.4) /0.1-0.7 (0.4)

3.2. Unique sequence traits

Alignments of the *bindin* sequences of RS *T. g. elatensis* with the other *Tripneustes* species revealed a unique 30 bp long insertion in the first exon (Table S3). This sequence feature, coding to amino acids 5'-GPVGGGGNMG-3', is part of a glycine-rich repeat G(P/Q/S)(V/G)GGG(G/S)(N/G)(M/R/E/G)(G/E). Similar glycine-rich motifs have also been reported from *bindin* genes of other camarodont (e.g., *Echinometra* and *Strongylocentrotus*, see discussion in Zigler and Lessios, 2003). Two copies of this repeat are present in *T. ventricosus*, three in all IWP *T. g. gratilla* (including *T. depressus*) except for RS *Tripneustes*, which have four copies. The region was excluded in the ML and BI analyses.

In addition, the 5'-end of the *bindin* intron appears highly divergent between RS *Tripneustes* and other members of the genus. In *T. ventricosus* and *T. g. gratilla* we identified [using RepeatMasker and Censor (Kohany et al., 2006)] this part of the intron consistently as a 148-bp-long transposon named *DNA-1-2_SP*, which was originally discovered by Bao and Jurka (2008) in *Strongylocentrotus purpuratus*. Interestingly, it is reverse in *T. ventricosus* and *T. g. gratilla* clades D₁ and D₂ (Fig. 5; see also Table S3), but normal in the two other *T. g. gratilla* clades (Fig. 5, clades A₁ and A₂). In RS *Tripneustes*, in contrast, we identified a highly divergent sequence comprising an inverted 169 bp-repeat at the same position (426 to 594, following the positions on the supplementary alignment, S6). This inverted repeat may form a secondary hairpin structure (Fig. S3). As with the unique insertion in the first exon, this inverted repeat was present in all RS specimens but never appeared in any of the other *Tripneustes* populations. This portion of the intron was excluded from all further analyses.

3.3. Morphology

3.3.1. Skeletal features

Dafni (1983) established *T. g. elatensis* for northern RS *Tripneustes* populations that showed A) a more compressed corona, B) a larger peristome, C) fewer interambulacral plates, D) sparser interambulacral tuberculation and E) more vivid coloration than Indo-Pacific *T. g. gratilla*. Based on our comparison of the surviving type material and new RS samples with *Tripneustes* from Tanzania and the Philippines we can confirm Dafni's observations. In addition, several further

differences were observed: 1) missing primary tubercles also on aboral ambulacral plates (very regular occurrence of primary tubercles are on every 3rd plate in *T. g. gratilla*); 2) peristomal margin level with oral surface (sunken in *T. g. gratilla*); 3) periproctal opening larger at same test diameter (10.3-12.0% of test diameter in *T. g. elatensis* vs. 7.1-10.1% in Tanzanian *T. g. gratilla*); 4) ambital poriferous zones slightly wider (31.0-36.0% of ambulacrum width in *T. g. elatensis* vs. 25.8-31.7% in Tanzanian *T. g. gratilla*); 5) distal compass elements more robust, flattened in cross section and less strongly bifurcated vs. delicate, round cross-section and deep bifurcation in *T. g. gratilla*; Fig. 6I-L vs. 6M-P). Together with Dafni's original features these characters allow confident recognition of *T. g. elatensis*, even for fragmented and/or fossil material (e.g., such as those illustrated by Nebelsick, 1992: pl. 3, fig. 4 or Ragaini, 1997: pl. 1, fig. 4).

3.3.2. Identification in the field

Distinction of *T. g. gratilla* and *T. g. elatensis* in the field or based on images of live specimens is often easier than recognition of preserved specimens. While color in general is highly variable in both forms (see below and Dafni, 1983), distribution of spines and pedicellariae is not. Representatives of *T. g. gratilla* from Eastern Africa and the Philippines appear very spiny and typically have numerous spines of similar length that are held erect; only the midline of the interambulacra is free of large spines. This area is densely covered with globiferous pedicellariae, as is the midline of the ambulacra, while the pore zones are largely free of pedicellariae. This, together with epidermis color, causes a striking pattern of dark and bright bands in most specimens. In *T. g. elatensis*, in contrast, this pattern never is developed and pedicellariae form a dense cover all over the aboral corona, including the pore zones, apical disc and peristomal membrane. The spine cover appears much reduced, and the few large spines present typically are held oblique, protecting adjacent pore zones and the apical disc. The interambulacral areas thus look almost naked (except for the pedicellariae). This often causes a distinct subpentagonal appearance in aboral view. When disturbed (e.g., by removal of the camouflaging debris) *T. g. elatensis* tends to extend all of its tube feet in all directions – up to half the corona diameter, a behavior we have rarely seen in *T. g. gratilla*.

3.3.3. Color

All *Tripneustes* populations studied so far show distinct color polymorphism (e.g., Toha et al., 2015). In *T. g. elatensis* three main epidermis colors are common: “white” (not pigmented), red and black. Typical spines colors are white, white with red distal

half, fully red and black; often primary and secondary spines show different coloration. Pedicellariae are usually white or light grey (in specimens with black epidermis). Tube feet either are uniformly clear/whitish or black, less commonly red (in the distal half). Almost any combination of epidermis, spine, pedicellarial and tube feet color is possible. Some combinations, however, seem to be more common than others: almost completely white and completely black specimens, as well as greenish individuals with red spines and dark individuals with red spines were particularly abundant at the study sites in the Gulf of Aqaba and Egypt. Juveniles, in contrast, (up to ca. 20 mm diameter) typically all are more or less uniformly light grayish green, sometimes with a brown tinge, which makes them hard to find in the field, where they usually cling to the undersides of rocks or dead corals. Upon preservation most of the coloration is lost and alcohol preserved specimens usually are pale cream or grayish black.

T. g. gratilla from the Philippines and Zanzibar usually have either white or black epidermis. Their spines are white, orange (rarely red) or black, often showing different coloration in ambulacral and interambulacral areas. Again, secondary spines may differ from primary ones in color. Pedicellariae usually are black, but can appear fluorescent dark blue in photographs. Tube feet, like in *T. g. elatensis*, are mostly white or black, but in *T. g. gratilla* white tubefeet often show a striking black band ca. halfway along their length (when extended) and lack pigment in the proximal half, creating a very distinct appearance. As in the former species, almost any combination of these colors is possible, but dark specimens with bright vertical bands and whitish specimens with dark vertical bands are most common, as are dark specimens with orange interambulacral primary spines. In *T. g. elatensis* we never observed banded tubefeet.

3.4. Fossil occurrences

Apart from a single exceptional site in the Early Miocene of France (Bongrain, 2013) members of the genus *Tripneustes* are usually found in form of isolated plates and spines, owing to their rapid disarticulation after death. Greenstein (1991) reports that under field conditions *T. ventricosus* is reduced to bleached skeletons devoid of tissue within six days. Unless rapidly embedded by sediment such bleached coronas are particularly susceptible to further decay and usually disintegrate upon transport (Kier, 1977; Greenstein, 1993). Consequently, remains of *Tripneustes* are typically rare in sediments, even in areas where large populations exist in adjacent modern habitats (Gordon and Donovan, 1992; Nebelsick, 1992; Greenstein, 1993).

Sometimes, however, fossil reefs provide abundant fissures and cavities in which fragile echinoid coronas may be trapped and preserved. Screening public and private collections of material from the Pleistocene fringing reefs exposed along the Egyptian RS coast we were able to locate several largely complete coronas of *Tripneustes* (Fig. 6). To our great surprise these contained examples of both *T. g. gratilla* and *T. g. elatensis*, both of which agreed perfectly in shape and tuberculation with extant material of the respective subspecies.

4. Discussion

The main aim of the current study was to clarify the taxonomic status of RS *Tripneustes* and elucidate its phylogenetic relationships within the genus. Here we employed two independent strategies for species delimitation, a morphological taxonomic approach together with ecological data to outline morpho-species, and molecular phylogenies to assert phylogenetic species. Using both approaches concurrently allowed us to draw robust conclusions and to check for discrepancies between the two. Below we discuss each methodology independently and conclude with a synthesis of both.

4.1. The taxonomy of RS *Tripneustes*

As previously noted by former workers, the high interspecific morphological similarity of *Tripneustes* renders species delimitation in this genus elusive and most likely incomplete (Clark, 1912; Mortensen, 1928-51). Nonetheless, differences in the gross external appearance between the RS *Tripneustes* and those from the IWP are pronounced. The compressed test morphology, larger relative diameter of the peristome, reduced number of interambulacral plates and the patchy tuberculation of the aboral interambulacra, manifest significant morphological differences between RS and IWP *Tripneustes*. These differences were the basis for nominating RS *Tripneustes* as a sub-species: *Tripneustes gratilla elatensis* Dafni, 1983. Our findings ratify Dafni's observations and demonstrate additional morphological differences in the distal part of the lantern's compass ossicles and periproct size. These five rod-like ossicles are believed to be involved in a rhythmic upwards and downwards motion that primarily serves as a respiratory pump whose role is to oxygenate the lantern muscles (Wilkie et al., 2015). While in *T. gratilla* these skeletal elements have a slender, deeply bifurcated structure, in RS *Tripneustes* they take a flattened robust appearance (Fig. 6). The observed differences might be related to differences in the

feeding and respiration physiology between the RS and IWP *Tripneustes* owing to habitat or food preference differences between the two groups of sea urchins. While IWP *T. g. gratilla* seems to have a strong preference for shallow sandy sea grass beds, RS *Tripneustes* are more common on rocky and coral covered substrates (Lawrence and Agatsuma, 2013). Our own field observations also demonstrate that while the Zanzibarian *Tripneustes* sampled in this study did exclusively occur in sea grass meadows, *T. g. elatensis*, inhabited back reef area, reef flats and lagoons, often perching directly on coral heads (Fig. 6A-C). A recent biodiversity study of the sea grass beds in the Gulf of Aqaba reported a large, 9.7 km stretch of sea grass beds parallel and adjacent to the coral reef zone (Winters et al., 2015). Very few *Tripneustes* have been observed during that survey (only two *Tripneustes* individuals in more than 2,800 data points collected at the northern and southern ends of the sea grass beds; Winters pers. comm.). In contrast, *T. g. elatensis* was very common along the same stretch of coastline on adjacent reef flats. Although RS *Tripneustes* have occasionally also been reported occurring on sea grass beds (Dafni and Tobol, 1986; Jafari and Mahasneh, 1987; Nebelsick, 1992), this seems to be the exception rather than the rule. The substantially higher abundance of RS *Tripneustes* on hard substrate environments (Dafni, 1992; James and Pearse, 1969; Schuhmacher, 1974), despite the spatial proximity of sandy sea grass beds, implies habitat preference.

An important aspect of our morphological dataset is the size range of the individuals used to generate the data. Each of the morphological features we examined was followed and compared between similarly sized individuals ranging from 40 mm to 80 mm (Fig. 6). Following the morphological attributes across the entire size range of *Tripneustes*, allowed us to confirm that the observed differences are not an artifact of ontogeny. Ontogenetic differences, where morphological traits appear, disappear or change over the life course of an individual, may pose a fundamental pitfall in many morphological taxonomic studies (Kroh et al., 2012; Martynov et al., 2015), as exemplified by extreme instances of new genera established by juveniles of other species (e.g., Mooi et al., 2014). A possible solution for this problem is to utilize a wide size range of individuals rather than targeting only the dominant or 'average' size individuals of the studied taxa and to compare specimens of similar size whenever possible.

Inspection of fossil specimens sheds some light on the history of occurrence of the different forms of *Tripneustes* in the RS. The fossil material clearly demonstrates the occurrence of two forms of *Tripneustes*, corresponding to the recent *T. g.*

elatensis and *T. g. gratilla*, in the RS during the Late Pleistocene (Fig. 6). Except for pedicellariae distribution and compass ossicles that are unaccounted for in the fossil material, all other morphological features demonstrate complete concordance with the recent material. The pronounced compressed test appearance and missing interambulacral tuberculation of one fossil type in contrast to the elevated, completely tuberculated second type is in complete agreement with recent RS and non RS *Tripneustes*. The presence of these two well-differentiated *Tripneustes* morphotypes at at-least 120,000 years ago indicates that RS *Tripneustes* is not a recent offshoot of IWP *Tripneustes* that formed during the isolation of the RS during the Last Glacial. Instead, it must have evolved well before and presumably survived the Last Glacial hypersaline period in one of the known refugia within the Red Sea Basin (e.g., the Gulf of Aqaba; see DiBattista et al., 2016a), as RS-type *Tripneustes* are unknown outside the RS, both in modern and fossil faunas. The RS holds 210 echinoderm species, 8.1% of which are endemic (DiBattista et al., 2016b). Our findings demonstrate that the true extent of RS echinoderm biodiversity and endemism may still be underestimated. The minimum age of the RS *Tripneustes* clade, however, is in stark conflict with the observed mtDNA data, which show haplotype sharing with as widely distant locations as the Philippines (more than 9,000 km away). A possible explanation for the observed *COI* pattern could be complete replacement of RS *Tripneustes* mtDNA through recent introgression by hybridization with *T. g. gratilla*. Albeit data on southern RS benthic faunas are scarce and field-work impossible due to the current political situation in the area, there is evidence of a contact zone between *T. g. gratilla* and *T. g. elatensis* in the southern-most part of the RS (Fig. 1). Dafni (1983) reported specimens corresponding to *T. g. gratilla* from the Dahlak Archipelago and the Austrian Red Sea Expedition of the vessel “Pola” in 1897/98 collected specimens (NHMW-ZoolIII 12639a-d) in nearby “Abajil” (= Jebel Abayil), between Edd and Beylul in Eritrea. Although none of these are suitable for DNA-extraction, their morphological characters are fully consistent with IWP *Tripneustes* and do not at all resemble the depressed RS-form with patchy spines.

4.2. The phylogeny of *Tripneustes*

The present inconclusive taxonomic status of *Tripneustes* is also reflected by previous molecular studies suggesting that two of the currently recognized species, *T. gratilla* and *T. depressus*, should in fact be treated as a single species (Lessios et al., 2003). This notion was based on both mtDNA (Lessios et al., 2003) and nuDNA

data (Zigler and Lessios, 2003), and is also reflected in our data, where *T. depressus* was recovered nesting within or sharing haplotypes within *T. gratilla* (Fig. 3 and 5). It is clear that an in-depth re-evaluation of the taxonomic status of *T. depressus* is needed to clarify the current morpho-molecular discrepancies that exist in this species, specifically so as there are claims that “a recent global survey of *Tripneustes* using microsatellite markers revealed that *T. gratilla* can be genetically distinguished from the *T. depressus* at nuclear loci” (Carlson, pers. comm. in Casilagan et al., 2013).

Similar to previous phylogenetic studies of *Tripneustes*, our results show strong support for the monophyly of *T. ventricosus*. This notion is confirmed by both mt- and nuDNA data and reflects the morphological and geographic separation of this Atlantic species from the Indo-Pacific *Tripneustes* species. However, in contrast to earlier phylogenetic reconstructions (Lessios et al., 2003; Zigler and Lessios, 2003), we found well supported genetic clades within *T. gratilla*. Moreover, although the current study focused on RS *Tripneustes*, we have examined a large number of *T. gratilla* specimens from various locations throughout their extensive geographic range and also noted several potentially morphologically differentiated populations (Bronstein and Kroh, in prep). Thus, we are compelled to refer to *T. gratilla* as a species complex rather than a single species. Incorporating new *Tripneustes* material, preferably from remote and potentially isolated populations throughout the IWP, may shed new light on their evolutionary history and elucidate their true species boundaries.

The RS *Tripneustes* are presented here in a phylogenetic context for the first time. Our analyses of nuDNA demonstrate their highly supported monophyly (Fig. 5 and S2), which is in line with our morphological taxonomic assessment. *T. g. elatensis* seems to have branched off from *T. g. gratilla* clade A₁₊₂ (including *T. depressus*) long before *T. ventricosus* diverged from the Indo-Pacific stock (ca. 2.8-3.8 Ma ago). However, these phylogenetic relationships were observed only in the nc (*bindin*) dataset while the mt data failed to reveal the clustering of *T. ventricosus* with a subset of *T. g. gratilla* (clade D₁₊₂), nor did it depict the monophyly of *T. g. elatensis*. The mechanisms leading to the observed discrepancies between the mt and nc markers are discussed below.

4.3. Mitochondrial and nuclear discordance

COI sequences analyzed in previous studies (Lessios et al., 2003; Liggins et al., 2014) do not show geographic structure within the IWP *Tripneustes* (Fig. 4), nor do our new data (Fig. 3), which are based on much longer *COI* sequences and for the first time include RS representatives of the genus. The latter group appears polyphyletic based on mtDNA data only. In contrast, phylogenies based on the *nc bindin* gene produced highly resolved, well supported trees not just between *T. ventricosus* and the rest of *Tripneustes*, but also between the RS and the other IWP *Tripneustes* (Figs. 5 and S2). The conflicting results in respect to the RS *Tripneustes* are intriguing as the phylogenetic resolution of *bindin*, a *nc* gene not under positive selection (Zigler and Lessios, 2003), was expected to be lower than that of the mt *COI*, as nuclear genes are anticipated to evolve slower (Moore and McPherson, 1965; Palumbi et al., 2001).

4.4. Unique sequence elements

The different *Tripneustes* clades discovered herein show several unique features in their *bindin* sequences. All of them possess a glycine-rich repeat composed of up to four 30 bp repeat sequences in exon 1. Apart from the RS clade, all Indo-Pacific *Tripneustes* possess three imperfect repeats. The RS clade shows a fourth repeat, inserted between repeat two and three of Indo-Pacific *Tripneustes*. *T. ventricosus*, in contrast, possesses only two repeats (Table S3). These patterns lend independent support to the monophyly of *T. ventricosus* and the RS *Tripneustes* clade.

Mapping of the transposon discovered in the intron of *T. g. gratilla*, and the unique inverted repeat replacing this transposon in *T. g. elatensis* shows perfect match with the tree topology as recovered by BI under exclusion of this region (Fig. 5, Table S3), again providing independent support for the relationships among clades, the polyphyly of *T. g. gratilla*, and the distinctiveness of the RS clade.

4.5. Conclusions

Based on the evidence put forward above, we can confirm Dafni's (1983) conclusions that the northern RS population of *Tripneustes* is a distinct taxon from IWP *Tripneustes*. It A) can be straightforwardly differentiated morphologically, both in the field and in preserved specimens, B) represents a distinct genetic clade, C) exists as morphologically well-differentiated form already in the Late Pleistocene (fossil

evidence), D) has diverged from other *Tripneustes* clades more than 2 million years ago (based on the molecular clock analysis presented above), and E) show different habitat preference than IWP *Tripneustes*. Dafni, therefore, was correct to establish a novel taxon for the northern RS population of *Tripneustes* – *T. gratilla elatensis*. While the morphological data show that it is very different from IWP *T. g. gratilla* and possible is, in fact, a distinct species, we currently lack sufficient molecular data to judge if the differentiation seen in *bindin* is reflected in other nuclear markers as well.

We assume that recent mt introgression due to hybridization in the contact zone between RS *T. g. elatensis* and *T. g. gratilla* is the most parsimonious explanation for the mt/nc discordance observed. In addition, disconnection of the RS from the Gulf of Aden during the Pleistocene may have temporarily separated these two clades, promoting speciation. Similar introgression must have happened (or might still be ongoing) between members of the sympatric *T. g. gratilla* clades identified by *bindin* data. For these, more detailed studies involving additional loci, material from the complete range of *T. g. gratilla* and specimens of different ontogenetic stages will be necessary to resolve their true distribution, relationship and morphological distinction (if any). This in turn could provide fundamental insights onto the evolutionary history of this genus and advance our understanding of marine speciation.

Acknowledgements

We wish to thank Rich Mooi and Christina Piotrowski (California Academy of Sciences), Gustav Paulay (Florida Museum of Natural History), Emmanuel Antig (Cebu City), Baraka Kuguru (Tanzania Fisheries Research Institute) for facilitating access to specimens and Heidi Friedhoff for loan and donation of fossil *Tripneustes* specimens. Josef Harl (NHM Vienna) as well as Tamar Feldstein and Michal Hadas-Sason (SMNH, Tel Aviv University) are acknowledged for assistance in the laboratory and discussion of results. We particularly want to thank Barbara Däubel (NHM Vienna) for her help in adapting the molecular protocols to *Tripneustes* and continuous support during laboratory work. We gratefully acknowledge Harilaos Lessios (Smithsonian Tropical Research Institute) for providing assistance during the early stages of the study, and the National Natural History Collections at the Hebrew University of Jerusalem for providing access to their collections.

We gratefully acknowledge the Department of Agriculture of the Philippines, particularly the Bureau of Fisheries and Aquatic Resources (BFAR) and the National

Fisheries Research and Development Institute (NFDRI) for support of the 2014-2016 Verde Island Passage Expeditions of the California Academy of Sciences (CAS). Tissue samples used herein were collected by Rich Mooi (CAS) under funding from NSF grant 12-57630 “Collaborative Proposal: Documenting Diversity in the Apex of the Coral Triangle: Inventory of Philippine Marine Biodiversity”.

Finally we thank Gustav Paulay and an anonymous reviewer for their constructive comments that helped to improve the manuscript.

This research received support from the SYNTHESYS Project (<http://www.synthesys.info/>), which is financed by European Community Research Infrastructure Action under the FP7 “Capacities” Programme.

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Figure Captions

Fig. 1. Maps of the Red Sea and Indian Ocean indicating distribution (according to Mortensen, 1943) and sample collection localities of *Tripneustes*. Colored areas indicate species ranges being green for *Tripneustes g. gratilla* and red for *Tripneustes elatensis*. Stars indicate sampling sites of the current study. Red and open circles indicate confirmed occurrences of recent and fossil *T. g. elatensis* in the Red Sea, respectively. Green and open triangles indicate confirmed occurrences of recent and fossil *T. g. gratilla* in the Red Sea, respectively. The maps were compiled based on data generated in the current study and the data obtained from: Dollfus and Roman (1981), Dafni (1983), Ragaini (1997), and specimens deposited at The Steinhardt Museum of Natural History, Tel-Aviv, Israel and specimens collected during the “Pola” Red Sea expedition (deposited at the Natural History Museum, Vienna, Austria).

Fig. 2. Schematic illustrations of gene regions and primer binding sites of the mitochondrial *cytochrome c oxidase* subunit 1 gene (*COI*) (A), and the nuclear *bindin* gene (B), used in the current study. The *COI* fragments generated in the current study are illustrated alongside the PCR fragments generated by Lessios et al. (2003) and Liggins et al. (2014). Position of the exons and the intron is outlined for the *bindin* gene. Corresponding fragment lengths are indicated in parentheses.

Fig. 3. Phylogenetic BI tree of 531 bp long *Tripneustes COI* sequences. The tree reconstruction is based on 33 unique haplotypes representing the three extant species of *Tripneustes* and the RS *T. g. elatensis*; rooted on *Lytechinus variegatus*. Supporting values (> 0.9 posterior probabilities and > 50% ML bootstrap values) are shown above the nodes. The colors of the specimen labels correspond to: Dark green – *T. g. gratilla*, Red – *T. g. elatensis*, Light green – *T. depressus*, and Blue – *T. ventricosus*. Clades 1-5 are discussed in the text. Details on the sequences used for this tree are given in Table S2.

Fig. 4. Median-joining haplotype network with *COI* sequences of *Tripneustes*. The network comprises 328 sequences, 449 bp long, of sequences generated in the current study and most comparable *Tripneustes COI* sequences in the literature (for details see Table S2). Color code as in Fig. 3. Bars indicate the number of substitutions between nodes. The frequency of each haplotype is indicated by size (see key, bottom left).

Fig. 5. Phylogenetic BI tree of 1226 bp long *Tripneustes bindin* sequences including exons 1 (without glycine-rich repeat) and 2 and parts of the intron (see Fig. 2; *bin-*

compl dataset). The tree reconstruction is based on 42 unique haplotypes representing the three extant species of *Tripneustes* and the RS *T. g. elatensis*; applying midpoint rooting. Supporting values (> 0.9 posterior probabilities and > 50% ML bootstrap values) are shown above the nodes. Color code as in Fig. 3. Clades A-E are discussed in the text. Details on the sequences used for this tree are given in Table S2.

Fig. 6. Morphological features of *Tripneustes g. elatensis* in comparison with *T. g. gratilla*. A-D: Life images of *T. g. elatensis* color morphs (A-C: El Quesir, Egypt) in comparison to *T. g. gratilla* (D: Luzon, Philippines; specimen CASIZ 187156). E-F: Fossil *Tripneustes* specimens from Late Pleistocene raised reef terraces in Egypt (MIS 5e, ca. 120,000 yrs old); E: *T. g. elatensis*, El Quesir, Egypt, NHMW 2013/0280/0012; F: *T. g. gratilla*, Hurghada, Egypt, NHMW 2016/0013/0001). G-H: Distribution of primary tubercles in the aboral interambulacra of *Tripneustes* (G: *T. g. elatensis*, specimen NHMW 2016/0012/0003; B: *T. g. gratilla*, specimen ZnTg100). I-P: Comparison of distal compass shape at different specimen sizes; this a part of the Aristotle's lantern, which is much more robust in *T. g. elatensis* {I-L: *T. g. gratilla* [I: 40.8 mm test diameter (TD), Cebu, Philippines, NHMW 2014/0208/0001; J: 48.2 mm TD, Cebu, Philippines, NHMW 2014/0208/0004; K: 63.1 mm TD, Cebu, Philippines, NHMW 2016/0025/0007; L: 71.38 mm TD, Cebu, Philippines, NHMW 2012/0193/0006]; M-P: *T. g. elatensis* [M: 45.1 mm TD, Aqaba, Jordan, NHMW 2016/0012/0001; N: 49.3 mm TD, Eilat, Israel, NHMW 2016/0012/0004; O: 63.5 mm TD, Aqaba, Jordan, NHMW 2016/0012/0002; P: 71.4 mm TD, Aqaba, Jordan, NHMW 2016/0012/0003]}.

Figure 1

Figure 2

(A) Cytochrome c oxidase (COI)

(B) Bindin

Figure 3

Tripneustes COI 531bp

Bayesian inference tree

Nucleotide substitution model:

HKY+I

Figure 4

Figure 5

Tripneustes bindin exons + intron

Bayesian inference tree

Nucleotide substitution model:

HKY+G

Figure 6