World travelers: DNA Barcoding unmasks the origin of cloning asteroid larvae from the Caribbean

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Abstract: The identity of wild cloning sea star larvae has been a mystery since they were first documented in the Caribbean. The most commonly collected cloning species was thought to belong to the Oreasteridae based on similarity with sequences from *Oreaster reticulatus* and *O. clavatus*. This larval form has recently been linked to a rare benthic juvenile. As part of two larger DNA barcoding projects we collected cloning asteroid larvae from the Caribbean coast of Panama and compared them to a large reference database of tropical echinoderms. Morphological and DNA barcode data from the cytochrome c oxidase subunit 1 (COI) gene demonstrated that Panamanian larvae belonged to the same operational taxonomic unit (OTU) as those recovered in previous studies of cloning larvae from the Caribbean. Much to our surprise, sequences from these larvae clearly identified them as belonging to *Valvaster striatus*, a species typically considered to be endemic to the Indo-West Pacific. A lineage of *Mithrodia clavigera* which occurs in both the Caribbean and Indo-west Pacific also has cloning larvae, suggesting that this unusual life history has allowed larvae to pass around the Cape of Good Hope and the Benguela upwelling region, which is a barrier to dispersal for most tropical marine invertebrates.

Introduction

Cloning can be induced in larvae of numerous echinoderm species under laboratory conditions, however natural asexual reproduction of larvae in the wild has been reported for only a few ophiuroid and asteroid larval morphotypes (Allen *et al*., 2018). The identities of these naturally cloning asteroid larvae have been a mystery since they were first documented to be cloning in the wild (Bosch *et al*., 1989; Rao *et al*., 1993; Jaeckle, 1994). They are often some of the most abundant asteroid larvae in plankton samples from the tropical Caribbean (Bosch *et al*., 1989; Knott *et al*., 2003), and yet their identities have remained unknown for over 30 years. Field-collected cloning larvae fall into at least 2 groups, several morphotypes appearing to be luidiids or other paxillosids (Bosch *et al*., 1989; Jaeckle, 1994) which always lack the brachiolaria stage. Others appear to be valvatids (Jaeckle, 1994, Knott, *et al*. 2003) possibly related to oreasterids or ophidiasterids (Balser, 2004; Janies *et al*., 2019).

Cloning larvae have been collected almost entirely from the western Atlantic Ocean (Balser, 2004; Allen *et al*., 2018) except for one report of these larvae from the coast of India (Rao *et al*., 1993). In the Florida Current and the Gulf Stream cloning valvatid larvae can reach densities of up to 300-400 larvae/m^3 (Balser, 2004). The larvae have also been observed in the southern Caribbean around Barbados, but their density was much lower with ~2 larvae/m^3 (Balser, 2004).

As they are so abundant, it seems likely that cloning valvatid larvae belong to species that are also abundant as adults in the Caribbean, unless they persist as clonal larval populations in the long term. Although the asteroid fauna of the Caribbean is fairly well known (Hendler *et al*., 1995), the identity of the most abundant cloning larvae has until now not been determined with any certainty. Knott *et al.* (2003) were the first to apply a molecular approach to attempt to identify them. They compared sequences of 5 mitochondrial tRNA genes from cloning larvae from Barbados and Florida to sequences from 44 species of identified adults. They recovered 5 operational taxonomic units (OTUs) of cloning larvae, none of which matched the adult sequences available at that time. They concluded that two OTUs were *Luidia* species, and two other, rare OTUs appeared to be related to *Linckia* (later identified as *Mithrodia clavigera* (Lamarck, 1816) by Galac *et al.* [2016]) and *Ophidiaster*. The most common cloning larva was considered to be an oreasterid based on the similarity with sequences from *Oreaster reticulatus* (Linnaeus, 1758), and it was suggested to be the larvae of *Oreaster clavatus* Müller & Troschel,

1842 transported from Africa (Knott *et al*., 2003). Subsequently Galac *et al*. (2016) demonstrated that these larvae are distinct from *O. clavatus* and suggested that the 16% divergence between this OTU and the two *Oreaster* species places them within Oreasteridae but not necessarily within *Oreaster*. While the adult form remained unknown, these larvae captured researchers' imaginations, as potentially being clonal species living only as a self-perpetuating larval population (Eaves and Palmer, 2003; Rogers-Bennett and Rogers, 2008; Ebert and Janies, 2019). Recently, however, Janies *et al.* (2019) reported the discovery of two unidentified juvenile sea stars in Mexico whose DNA sequences placed them in the same OTU as the cloning larval clade identified as an oreasterid.

As part of two larger DNA barcoding projects we collected cloning asteroid larvae from the Caribbean coast of Panama and compared them to a large DNA barcode reference database of tropical echinoderms. This approach showed that these cloning larvae can be clearly identified as belonging to *Valvaster striatus* (Lamarck, 1816), a species previously considered to be endemic to the Indo-west Pacific (IWP).

Materials and Methods

Larval Collection and Sequencing

Asteroid larvae were collected from Bahia Almirante in Bocas del Toro Province, Panama, with a 0.5 m diameter 125 µm mesh plankton net between 2013 and 2016, from 10-20 m depth. Most larvae were collected in 2015-2016 during 4 campaigns evenly spaced throughout a year (August 2015, November 2015, February-March 2016, and June 2016). Tows were conducted at a single location in the channel between Isla Colon and Isla Cristobal (latitude: 09° 20' 8.9" N to 09° 20' 36.3" N; longitude: 82° 15' 41.0" W to 82° 15' 50.0" W). Samples collected in 2013 were from throughout Bahia Almirante (for more details see Collin et al. [2019a,b,c; 2020]). Individual larvae were photographed alive under a dissecting microscope prior to preservation for DNA sequencing. Notes were taken on the overall appearance, morphological details, and approximate size of each larva before they were preserved.

Individual larvae were preserved in 150 µl of M2 extraction buffer (AutoGen) in 96-well plates, frozen and shipped to the Smithsonian's Laboratories of Analytical Biology (LAB) for extraction and sequencing. DNA was extracted from plates containing larval samples from a

variety of taxa using an AutoGenprep 965 extraction robot after overnight digestion in the AutoGen buffers with proteinase-K. The suspension volume of extracted DNA was 50 µl. The DNA barcode fragment of the cytochrome c oxidase subunit I (COI) was amplified using primer pair jgLCO1490/jgHCO2198 (Geller *et al*., 2013), dgLCO1490/dgHCO2198 (Meyer, 2003) and/or COIceF/COIceR (Hoareau and Boissin, 2010). The 10 µl PCR cocktail for COI included 5 µl GoTaq Hot Start Mix (Promega), 0.1 µl BSA, and 0.3 µl of each 10 mM primer. For amplification and sequencing of 16S, the primer pairs 16SAR/16SBR (Palumbi *et al*. 1991) or 16SL2/16SH2 (Arnedo *et al*., 2001; Schubart *et al*., 2002) were used. The cocktail for 16S used Biolase Taq (Bioline) with the addition of 0.5 µL 50 mM $MgCl_2$. The annealing temperature was 48°C for COI and 50°C for 16S. Both fragments were sequenced in both directions and resulting contigs were screened for quality, with only sequences of >90% the expected length and a Phred quality score of at least 30 for >85% of the bases retained for analysis.

Adult Reference Collection

Adult echinoderms have been collected and identified throughout the tropics by a research program focused on reef biodiversity. Numerous localities have been sampled across the IWP, with efforts ranging from incidental collecting to large, multi-year, team-based biodiversity surveys. Specimens were photographed alive, subsampled for tissues, fixed in alcohol, and vouchered in the Invertebrate Zoology collections of the Florida Museum of Natural History, University of Florida (UF; see http://specifyportal.flmnh.ufl.edu/iz/). The DNA barcode fragment of COI was sequenced for four adult *Valvaster striatus* following the methods of Boissin *et al*. (2017). These were UF 163 collected from Guam, Mariana Islands, UF5006 collected from Rarotonga Island, Cook Islands, and UF 12425 and UF 12426 from Moorea, Society Islands. In addition, the barcode fragment of COI was sequenced for 6 other species of valvatacean sea stars. These were *Dermasterias imbricata* (Grube, 1857) (UF8054), *Petricia vernicina* (Lamarck, 1816) (UF8877), *Protoreaster nodosus* (Linnaeus, 1758) (UF9625), *Culcita novaeguineae* Müller & Troschel, 1842 (UF1009), *Asteropsis carinifera* (Lamarck, 1816) (UF3917), and *Monachaster sanderi* (Meissner, 1892) (UF258A).

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Analysis

OTUs were identified following Collin et al. (2019a,b,c; 2020) and the single OTU that included cloning larvae was then compared to reference sequences from (Janies *et al*. 2019) to determine if our Panamanian cloning larvae were the same as their "*Oreaster* sp.", and with our global database of echinoderm sequences in an attempt to identify the larvae. The preliminary neighbor-joining analysis indicated that the cloning larvae belonged to *Valvaster striatus* (see below). We therefore conducted a haplotype network analysis including all of the COI sequences available from adult *V. striatus* and from the cloning larvae that fell into this clade from (Janies *et al*., 2019) and the present study. For this analysis, the alignment and guide tree (a maximum parsimony tree generated in MEGA) were labeled with four categories (Larvae from Bahia Almirante, larvae from (Janies *et al*., 2019), Juveniles from Parque Nacional Arrecife Alacranes and Triángulo Oeste, in the southern Gulf of Mexico (Janies *et al*., 2019), and adults from the Indo-west Pacific), loaded in Haplotype Viewer software (Ewing, 2020), and the output was edited to meet the figure requirements of the journal. The Genbank numbers were as follows: Larvae from Bahia Almirante, Panama: MN730656-MN730775; Larvae from "various points in the Florida Current-Gulf Stream system" (Janies *et al*., 2019): KP638309- KP638323 and MH319361; Juveniles from (Janies *et al*., 2019): MH319362 and MH319366; adults from the Indo-west Pacific: MT262516-MT262521.

To place our larvae and *V. striatus* into a broader phylogenetic context and to determine whether they are indeed close relatives to *Oreaster*, we performed a maximum likelihood analysis based on multiple markers (16S, 12S, H3 and COI) for all taxa falling inside clade "O" (Oreasteridae + Acanthasteridae + Asteropseidae) from the valvatacean tree of Mah and Foltz (2011). Their analysis did not include *Valvaster*, so our aim was to discover its placement within this unusual group of sea stars. We used the 16S, 12S and H3 sequences from [Mah and Foltz, 2011) with the addition of our COI sequences for the same taxa. For *V. striatus* we combined sequences from [Janies *et al*., 2019] with our sequences (note that 12S and H3 were not sequenced for this species). *Dermasterias imbricata* (GenBank MH319358) was used as the outgroup (Mah,and Foltz, 2011). Each marker was separately aligned with ClustalX [gap opening penalty: 15, gap extension penalty: 6.66, DNA weight matrix: IUB, transition weight: 0.5, delay divergent cutoff: 30%], followed by manual corrections. Then, each alignment was analyzed with JModelTest (Posada, 2008) and MEGA (Kumar *et al*., 2008) to find the best model of evolution associated with each marker plus their gamma and I parameters. The

alignments were concatenated and evaluated with PartitionFinder (Lanfear, 2017), which showed that no additional partitions were necessary, before the final analysis. This scheme was then incorporated in the subsequent ML analysis performed with RAxML (Stamatakis, 2014).

DNA sequences generated by this project have been deposited in GenBank (accession numbers: MN730656-MN730775 for larvae and MT260973-MT260976 for *Valvaster* adults and MT262516-MT262521 for other adults). In BOLD, all of our *Valvaster striatus* sequences are publicly available in the Barcode Index Number (BIN) BOLD:ABA3725.

Results and Discussion

We collected 7 cloning asteroid larvae that showed evidence of paratomous modification of the posterolateral arms (Figure 1C-E). These larvae fell into a single OTU that also contained 37 morphologically similar larvae that did not show definitive evidence of cloning (Figure 1A-B). Two larvae had developing juvenile rudiments (1E-F), one of which appeared to also be cloning. COI barcode sequences of these larvae place them in the same OTU as cloning larvae identified as "Oreasteridae sp." (Galac *et al*., 2016) and "*Oreaster* sp." (Janies *et al*., 2019). This OTU also included samples of *Valvaster striatus* collected from the Mariana Islands (Micronesia) and Cook and Society Islands (SE Polynesia) (Figure 2). This OTU was separated from *O. reticulatus* (the closest OTU in the analysis) by ~0.20 substitutions per site. Pairwise distances within this OTU did not exceed 0.025 substitutions per site. Placement of the adult Pacific *V. striatus* as sister to or paraphyletic with respect to the Caribbean cloning larvae could not be resolved as the outgroup available to root the network was too distant. However, the larval and adult sequences differed only by a single diagnostic base pair (Figure 2). We take this to indicate that these cloning larvae are the larval stages of *V. striatus*, which implies recent connectivity between Pacific and Atlantic populations. As there are no shared haplotypes these data cannot demonstrate current, active dispersal between the regions, but they do clearly indicate a recent connection. Lack of evidence for dispersal between the regions is not strong evidence that such dispersal does not occur, as only 3 animals have been sequenced from the Pacific and it is likely that much of the genetic diversity in that region remains unsampled.

Valvaster is a distinctive, monospecific genus currently assigned to the valvatid family Asteropseidae. Previously it was separated as the monospecific Valvasteridae Viguier, 1879 until transferred to the Asteropseidae by Blake (1980). Asteropseids currently include three other

monospecific genera, *Asteropsis, Dermasterias* and *Poraniella*, as well as *Petricia* with 2 species (Mah, 2019). The most extensive molecular phylogeny of valvataceans indicated that the Asteropseidae is polyphyletic, with *Asteropsis* within, and *Petricia* just outside, the Oreasteridae, and *Dermasterias* resolved as sister to the asterinid-solasterid clade (Mah and Foltz, 2011). *Valvaster* was not included in that study, but more limited comparisons place it as sister to *Oreaster* (Janies *et al*., 2019 Figure 2), while its morphology suggests affinity with *Asteropsis*. Our re-analysis of the Oreasteridae + Acanthasteridae + Asteropseidae clade from Mah and Foltz (2011) with the addition of *V. striatus* and COI for all taxa supports a monophyletic group containing *Protoreaster, Culcita* and *Valvaster* with *Oreaster* as the sister group to this clade (Figure 3), suggesting that *Valvaster* likely belongs to the Oreasteridae, although addition of sequences from more conservative genes and more extensive taxon sampling is necessary to generate a fully resolved phylogeny of the group.

Valvaster striatus (Figure 4) is a moderately large (5+ cm arm radius), distinctive sea star with conspicuous marginal spines and large, sessile, bivalved pedicellariae aligned aborally along the arm margins, which are the source of the generic name. It is widespread but uncommon across the IWP, recorded from the SW Indian Ocean (Comoros, Madagascar, Mascarenes; iNaturalist, UF collections and Conand *et al*., (2018**)**) to the remote central Pacific (Society Islands and Hawaii (UF collections and Fisher, (1906)). This species is rarely encountered as it lives within the reef framework, emerging only at night. Only 54 lots are represented in iDigBio, and 4 photos in iNaturalist (both checked 12/29/2019). Most of the specimens come from a few locations: Mascarene Islands, Ryukyus, Philippines, Guam and Hawaii, partially reflecting sampling intensity. It has been collected from 1-60+m depths, both in sheltered lagoons and fore reefs (UF collections). Even in areas where reefs have been frequently surveyed at night, *Valvaster* is rarely seen (Jangoux, 1986; Hoover, 2010; GP pers. obs.), and a large proportion of specimens have been taken from within the reef. This suggests that these animals do not routinely emerge at night and that their abundance may be higher than collections and observations suggest. Several other echinoderm species appear to spend most of their lives in the reef framework and rarely or never emerge (e.g. *Lissodiadema lorioli* Mortensen, 1903, *Chondrocidaris brevispina* Clark, 1925, numerous reef asterinids). Emergent specimens have been seen both on the reef and on sand adjacent to reefs, at night.

This is the first report of *V. striatus* from the Atlantic. The lack of benthic records of these animals from the tropical Atlantic until recently may be due to the challenge of encountering these cryptic animals. Janies *et al*. (2019) report a recent collection of juveniles from the SW Gulf of Mexico. The photographs of a live animal are clearly similar to *V. striatus*, with a conspicuous white madreporite, well-developed marginal spines, abundant spines covering the aboral surface and a coarsely granular aboral surface (compare their Figure 2 with Figure 4 below). These photographs are also consistent with the type material of *V. striatus* (Mah, 2020). These two animals that have been reported (but not identified) were encountered under rocks (Janies *et al*., 2019), thus within the reef framework, agreeing with the habits of *V. striatus* in the IWP. *Mithrodia clavigera*, another nocturnal IWP asteroid with a cloning larva, was only recently recorded from the tropical western Atlantic (Clark and Downey, 1992; Bribiesca-Contreras *et al*., 2013). This species, therefore appears to have a similar distribution to *V. striatus*, and also appears to be quite uncommonly encountered as an adult, although in some cases these may have been mistaken for *Linckia* species (see discussion in Galac *et al*., (2016)). As a larval clone could, theoretically, remain in the plankton indefinitely, researchers should be aware that adults of *V. striatus* and *M. clavigera* could be encountered in reef habitats throughout the Caribbean, and the tropical Brazilian and African coastlines.

Our surprising result has several important implications for understanding the biology of cloning larvae and global invertebrate biodiversity. The confirmation of what has previously been considered an IWP species in the Caribbean highlights the importance of using global rather than regional datasets to identify unknown species. It also changes our view of the biology of cloning larvae, raising new questions such as: (1) Are larvae extremely abundant and adults rare in the IWP, as they appear to be in the Caribbean? (2) Is there gene flow between the IWP and Caribbean, and if so, at what rate? (3) Are cloning larvae an innovation that allowed these species to break through the Benguela upwelling, a region often invoked as a barrier to the dispersal of tropical marine organisms? (4) Could adults or larvae dispersed between the oceans in ballast water, or on floating infrastructure like oil rigs or dry docks? Questions could be addressed with natural history observations and more molecular phylogeographic data. As pointed out by a recent attempt to model the life-histories of cloning larvae (Ebert and Janies, 2019), more life-table data are also desperately needed to help us better understand how cloning larvae contribute to populations dynamics. Their numerical model, which assumed only 2 buds

were produced by each larva, predicted that if a benthic stage exists adults must either be rare and extremely long-lived or they must be common (Ebert and Janies, 2019). We hope this note heightens awareness of the potential to encounter cloning asteroid larvae for those working in the IWP range of *V. striatus*, and of the potential to encounter adult *V. striatus* for those working in the Caribbean and tropical Eastern Atlantic.

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Data Accessibility

Aligned datasets from the analyses presented here are archived on FigShare with the following DOI: 10.6084/m9.figshare.12141213.

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Figures

Figure 1: Larvae identified by DNA barcoding as *Valvaster striatus* from the Caribbean of Panama. (A-B) Early and mid-stage bipinnaria, respectively, in ventral views; (C-D) Bipinnaria larvae in ventral view with larval clones (cl) developing on one of their posterolateral arms; (E-F) A late-stage bipinnaria/pre-brachiolaria larva in ventral (E) and right-lateral (F) views showing a developing juvenile rudiment with skeletal elements. ad, anterodorsal arm; af, anal field; an, anus; cl, clone of larva; es, esophagus; ff, frontal field; in, intestine; jr, juvenile rudiment; js, juvenile skeletal element; mo, mouth; pd, posterodorsal arm; pl, posterolateral arm; po, post-oral arm; pr, pre-oral arm; prl, pre-oral lobe; st, stomach. Scale bar = 500 μm.

Figure 2: Haplotype network of 65 COI sequences of *Valvaster striatus*. Each haplotype is represented by a circle whose size is proportional to its frequency in number of specimens. Larval sequences collected from Bahia Almirante are indicated with black, sequences from [6] are indicated with dark grey (larvae) and light grey (juveniles). Adults from the Indo-west Pacific are indicated with white. The distance between haplotypes, measured as number of substitutions, is proportional to the length of the branches separating them, with one line segment representing a single substitution. This analysis is based on a 357 bp fragment that was common for all the sequences evaluated.

Figure 3: Maximum likelihood phylogeny of Oreasteridae + Acanthasteridae + Asteropseidae taxa based on combined 16S, 12S, H3 and COI sequences obtained from [24], [6], and our own specimens. One *Valvaster striatus* adult and one larva were added to the analysis. Nodes with bootstrap values <60 were collapsed and bootstrap values ≥60 are shown below the branches. Branch lengths are proportional to the distance in substitutions per site. The aligned, concatenated dataset with associated GenBank numbers is archived in FigShare (see Data Accessibility section).

Figure 4: Photographs of two of adult specimens of *Valvaster striatus* from Moorea that were sequenced for this study. A. UF 12425 (6 cm diameter) B. UF 12426 (10 cm diameter).