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Discovery of Adults Linked to Cloning Oceanic Starfish Larvae (*Oreaster*, Asteroidea: Echinodermata)

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Abstract. Two juvenile specimens of a new species of *Oreaster* were collected at Parque Nacional Arrecife Alacranes and Triángulos Oeste in the southern Gulf of Mexico. DNA of mitochondrial loci identifies them as members of the same clade as cloning larvae of *Oreaster* found abundantly in waters of the Florida Current-Gulf Stream system, and distinct from *Oreaster clavatus* and *Oreaster reticulatus*, the two known Oreasteridae species in the North Atlantic. Larvae from the new species of *Oreaster* persist as clones but also metamorphose and settle to the benthos with typical asteroid morphology.

Introduction

Several clades of echinoderms clone as larvae (reviewed in Allen *et al.*, 2018). Bosch *et al.* (1989) discovered cloning asteroid larvae in the Gulf Stream and in the Sargasso Sea (Fig. 1). Neither Knott *et al.* (2003) nor Galac *et al.* (2016) identified the clonal larvae as Oreasteridae, but they could associate the larvae with known species of *Oreaster*. Galac *et al.* (2016) found that clonal larvae belonged to clades distinct from but closely related to *Oreaster reticulatus* and *Oreaster clavatus* in the western and eastern Atlantic, respectively. A question is whether the unidentified clonal larvae might actually be self-sustaining, as suggested as a possibility (Eaves and Palmer, 2003), or whether such larvae have a benthic stage. Here we present evidence of a link between the unidentified clonal larvae and a previously unknown benthic stage collected from 10–15-m depths at Parque Nacional Arrecife Alacranes and Triángulos Oeste in the southern Gulf of Mexico.

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Abbreviations: BLAST, Basic Local Alignment Search Tool; *COI, cytochrome c oxidase I*; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.

Materials and Methods

In 2016 and 2017, Hernández-Díaz and Solís-Marín discovered specimens of a putative new species of *Oreaster* Müller & Troschel, 1842, vouchers ICML-UNAM-18240-QH411 and ICML-UNAM-18241-FM066 (hereafter QH411 and FM066), at Parque Nacional Arrecife Alacranes and Triángulos Oeste, in the southern Gulf of Mexico (Fig. 2). The small sea stars were attached to the underside of boulders at 10–15-m depths in a coral reef habitat. Their morphology was so distinct from that of small *Oreaster reticulatus* (Linnaeus, 1758) that they were recognized as a new form.

We sequenced the DNA coding for mitochondrial loci (Table 1) from *Oreaster* QH411 and FM066, *O. reticulatus* from the Gulf of Mexico off of Florida, and cloning larvae of *Oreaster* from various points in the Florida Current-Gulf Stream system. All voucher data were deposited in GenBank (Table 2). We sampled tube feet from benthic stages and

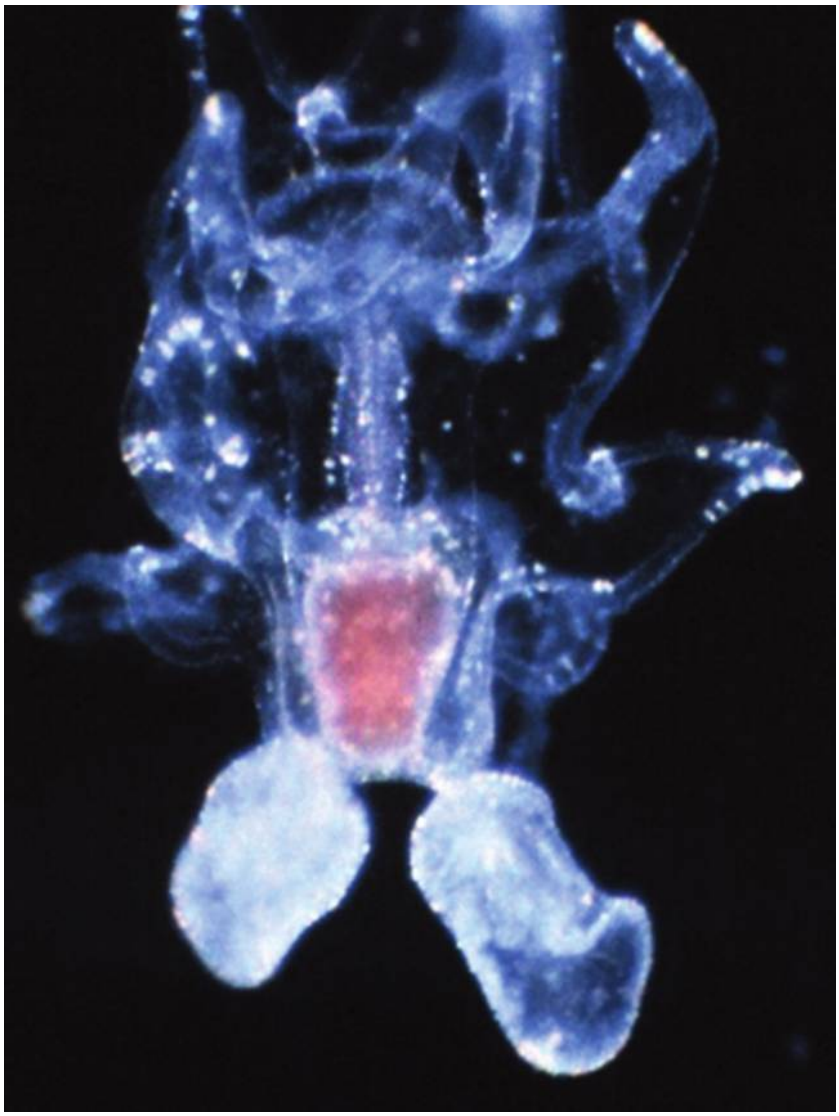

Figure 1. Bipinnaria of *Oreaster* sp. nov. in the process of cloning.

whole larvae for DNA extractions with Qiagen (Venlo, The Netherlands) DNeasy Blood and Tissue Kit, following the manufacturer's Spin-Column protocol for animal tissue. We measured DNA concentration and quality on a NanoDrop (Thermo Fisher Scientific, Waltham, MA) ND-1000 spectrophotometer to ensure that the amplicons were adequate for sequencing. Using the primers referenced in Table 1, we targeted 2 amplicons for polymerase chain reaction (PCR) and direct sequencing, including (1) a 1650-bp fragment consisting of the mitochondrial markers *cytochrome c oxidase I* (*COI*) and *tRNA-Ala*, *tRNA-Leu*, *tRNA-Asn*, *tRNA-Gln*, and *tRNA-Pro*, and (2) a 1250-bp fragment consisting of the mitochondrial markers *12S rDNA*, *tRNA-Glu*, *tRNA-Thr*, and *16S rDNA*.

We completed PCR using PuReTaq Ready-to-Go PCR beads (GE Healthcare Life Sciences, Marlborough, MA). We set PCR conditions for the ~1600-bp fragment at (1) 95 °C for 5 min, (2) 95 °C for 50 s, (3) 58 °C for 30 s, (4) 72 °C for 1 min, (5) repeat steps 2–4 for 34 more cycles, and (6) 72 °C for 10 min. We set PCR conditions for the 1200-bp fragment at (1) 94 °C for 5 min, (2) 94 °C for 50 s, (3) 52 °C for 30 s, (4) 72 °C for 1 min, (5) repeat steps 2–4 for 34 more cycles, and (6) 72 °C for 10 min. We cleaned PCR products with 10 units of Exonuclease I (Thermo Fisher Scientific) and 0.5 units of shrimp alkaline phosphatase (Affymetrix; Thermo Fisher Scientific) at 37 °C for 1 h, followed by a 15-min incubation at 65 °C to deactivate the enzymes. We sequenced PCR products with Applied Biosystem's BigDye Terminator (Thermo Fisher Scientific), following the manufacturer's protocol. We used the sequencing instrument and software as follows: Applied Biosystem's 3130/3130xl Series Data Collection Software 4 and Sequencing Analysis Software 6 running under a Microsoft (Redmond, WA) Windows 7 operating system.

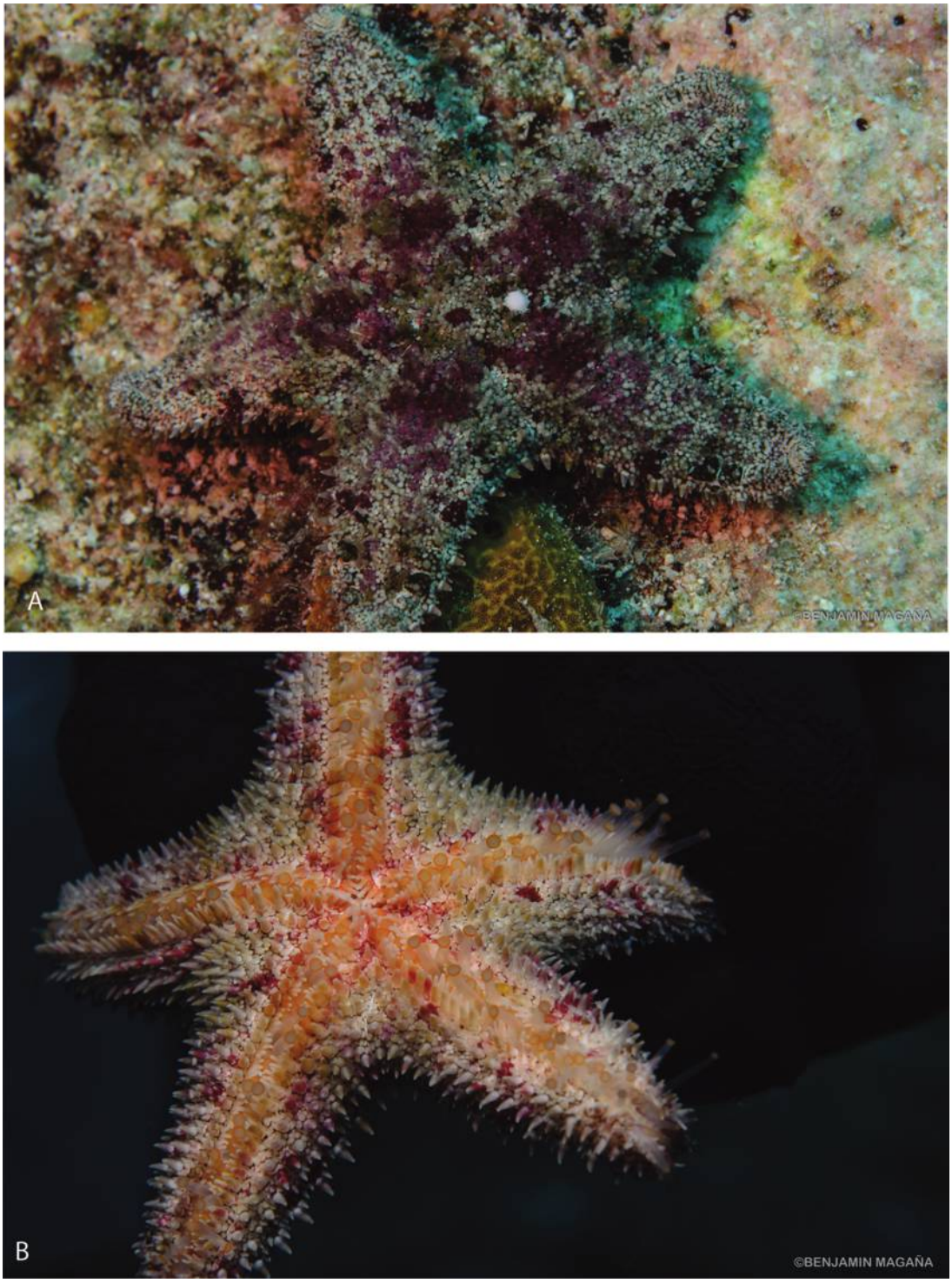

Figure 2. (A) Aboral and (B) oral views of QH411, now *Oreaster* sp. nov. (Photo credit Benjamin Magaña.)

We edited and trimmed the sequencing trace files, using CodonCode Aligner (CodonCode, Centerville, MA). We obtained comparable asteroid sequences that covered the same genetic regions as the novel sequence data produced herein by using the novel sequence data to search GenBank's core nucleotide database with BLAST (Altschul *et al.*, 1990) under default conditions. Among those data retrieved were *COI* and *tRNA-Ala*, *tRNA-Leu*, *tRNA-Asn*, *tRNA-Gln*, and *tRNA-Pro* for several larvae that Knott *et al.* (2003) sequenced and recorded in GenBank as "valvatids" and in their publication as "larval

Table 1

Primers, references, and parameters used in polymerase chain reaction (PCR) and Sanger sequencing

Amplicon	Primer	Sequence 5′ to 3′	Source	PCR parameters
tRNAs + COI	PAT-2	CTTTGAAGGCTTTTAGTTTAGATTAAC	Knott and Wray, 2000	95 °C for 5 min 35 cycles: 95 °C for 30 s 48 °C for 30 s 72 °C for 1 min, 42 s 72 °C for 10 min
	ECOIB	GGTAGTCTGAGTATCGTCG(A/T)G	Knott and Wray, 2000	
	COIceF	ACTGCCCACGCCCTAGTAATGATATTTTTTATGGTNATGCC	Hoareau and Boissin, 2010	95 °C for 5 min 35 cycles: 95 °C for 50 s 58 °C for 30 s 72 °C for 1 min 72 °C for 10 min
	COIceR	TCGTGTGTCTACGTCCATTCCTACTGTRAACATRTG	Hoareau and Boissin, 2010	
12S, tRNAs, 16S	12Sa	ACACATCGCCCGTCACTCTC	Smith *et al.*, 1993	95 °C for 5 min 35 cycles: 95 °C for 30 s 60 °C for 30 s 72 °C for 1 min, 16 s 72 °C for 10 min
	16Sb	GACGAGAAGACCCTATCGAGC	Smith *et al.*, 1993	
	16SAN-R	GCTTACGCCGGTCTGAACTCAG	Zanol *et al.*, 2010	94 °C for 5 min 35 cycles: 94 °C for 50 s 52 °C for 30 s 72 °C for 1 min 72 °C for 10 min
	16SarL	CGCCTGTTTATCAAAAACAT	Palumbi *et al.*, 1991	

group 1" and recorded in their table 2. We aligned GenBank data with novel sequence data by using MAFFT (Katoh and Standley, 2013). Due to different procedures in various labs over time, alignments contained partial sequences. We trimmed the ragged edges of these alignments and used question marks to replace leading and trailing gaps.

To optimize the completeness of data matrices, we developed four independent nucleotide alignments: (1) *16S rDNA*; (2) *12S rDNA*, *tRNA-Glu*, *tRNA-Thr*, and a portion of *16S rDNA*; (3) *COI*; and (4) *tRNA-Ala*, *tRNA-Leu*, *tRNA-Asn*, *tRNA-Gln*, and *tRNA-Pro*. Because each data set had slightly different taxonomic coverage, we analyzed each independently. We used 100 replicates of the rapid bootstrapping algorithm followed by a thorough maximum likelihood tree search with RAxML (ver. 7.2.8, Stamatakis, 2006) under a GTR+G (general time reversible gamma) model of substitution. We rooted trees for all data sets with the outgroups *Parvulastra vivipara* and *Parvulastra exigua*. We used Mesquite (Maddison and Maddison, 2018) to visualize the trees.

In order to discover a region suitable for genetic distance analyses and population diagnostic single nucleotide polymorphisms (SNPs), we isolated regions of the alignment to produce a 48-bp fragment of *COI* that minimized missing data while optimizing taxonomic coverage. We conducted *p*-distance analyses in MEGA X (Kumar *et al.*, 2018). To analyze the 48-bp fragment of *COI* for diagnostic SNPs, we used 100 replicates of tree search in Tree analysis using New Technology (TNT; level 1) with a strict consensus calculation to identify main clades that can be consistently recovered across tree space (Goloboff *et al.*, 2008). We used visualization of the aligned positions on the consensus tree in Mesquite (Maddison and Maddison, 2018) to search for SNPs with patterns of variation that could be used to diagnose clades.

Results

In Table 3, we use the index of aligned columns based on the corresponding bases in the *Patiria pectinifera* mitochondrial genome (GenBank reference sequence record NC 001627). We present specimen and tissue voucher data and sequence accession numbers for GenBank in Table 2.

16S rDNA

This alignment contains 42 taxa and 832 positions. Knott *et al.* (2003) did not sample this locus. The resulting tree (Fig. 3) includes three major ingroup clades that are subtended by long branches. One clade contains QH411 and

Table 2

GenBank accession numbers, voucher information, comments from collector, and final species name for specimens used in this study

GenBank accession no.	Voucher	Comments from collector	Species
MH319344	IB 14-0102	Sid tube feet	*O. clavatus*
MH319345	IB 14-0105	Sid tube feet	*O. clavatus*
MH319346	IB 14-0104	Sid tube feet	*O. clavatus*
MH319363	IB 14-0102	Sid tube feet	*O. clavatus*
MH319364	IB 14-0105	Sid tube feet	*O. clavatus*
MH319365	IB 14-0104	Sid tube feet	*O. clavatus*
KP638238	DAJ01-14	*O. clavatus* voucher DAJ01 14	*O. clavatus*
KP638324	DAJ01-14	*O. clavatus* voucher DAJ01 14	*O. clavatus*
MH319339	FSBC I 092405	FWRI-I-00001 092405	*O. reticulatus*
MH319340	FSBC I 109312	FWRI-I-00002 109312	*O. reticulatus*
MH319341	FSBC I 115738	FWRI-I-00003 115738	*O. reticulatus*
MH319358	FSBC I 092405	FWRI-I-00001 092405	*O. reticulatus*
MH319359	FSBC I 109312	FWRI-I-00002 109312	*O. reticulatus*
MH319360	FSBC I 115738	FWRI-I-00003 115738	*O. reticulatus*
MH319348	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319349	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319350	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319351	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319352	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319353	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319354	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319355	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319356	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319357	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319367	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319368	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319369	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319370	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319371	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319372	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319373	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319374	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
MH319375	FSBC I 135398	FWRI I 00567 Vladimir DNA	*O. reticulatus*
KP638237	TF-H1	*O. reticulatus* voucher TFH1	*O. reticulatus*
KP638240	TF-F1	*O. reticulatus* voucher TFF1	*O. reticulatus*
KP638235	TF-G1	*O. reticulatus* voucher TFG1	*O. reticulatus*
KP638239	N237-5	*O. reticulatus* voucher N2375	*O. reticulatus*
KP638236	TF-L1	*O. reticulatus* voucher TFL1	*O. reticulatus*
KP638326	N237-5	*O. reticulatus* voucher N237 5	*O. reticulatus*
U50060		*O. reticulatus*	*O. reticulatus*
MH319342	IB 2012-05-15 01	Sid larva	*Oreaster* sp. nov.
MH319343	ICML-UNAM-18240	Quetzalli QH411	*Oreaster* sp. nov.
MH319361	IB 2012-05-15 01	Sid larva	*Oreaster* sp. nov.
MH319362	ICML-UNAM-18240	Quetzalli QH411	*Oreaster* sp. nov.
MH319347	ICML-UNAM-18241	Kirk FM066	*Oreaster* sp. nov.
MH319366	ICML-UNAM-18241	Kirk FM066	*Oreaster* sp. nov.
KP638252	C6	Oreasteridae sp. C6 12S	*Oreaster* sp. nov.
KP638255	C1	Oreasteridae sp. C1 12S	*Oreaster* sp. nov.
KP638244	NCY2	Oreasteridae sp. NCY2 12S	*Oreaster* sp. nov.
KP638250	NCY3	Oreasteridae sp. NCY3 12S	*Oreaster* sp. nov.
KP638256	C2	Oreasteridae sp. C2 12S	*Oreaster* sp. nov.
KP638245	C10	Oreasteridae sp. C10 12S	*Oreaster* sp. nov.
KP638248	C8	Oreasteridae sp. C8 12S	*Oreaster* sp. nov.
KP638246	C7	Oreasteridae sp. C7 12S	*Oreaster* sp. nov.
KP638243	C11	Oreasteridae sp. C11 12S	*Oreaster* sp. nov.
KP638242	C4	Oreasteridae sp. C4 12S	*Oreaster* sp. nov.
KP638249	NCY4	Oreasteridae sp. NCY4 12S	*Oreaster* sp. nov.
KP638251	NCY1	Oreasteridae sp. NCY1 12S	*Oreaster* sp. nov.
KP638247	C3	Oreasteridae sp. C3 12S	*Oreaster* sp. nov.

Table 2 (*Continued*)

GenBank accession no.	Voucher	Comments from collector	Species
KP638254	C5	Oreasteridae sp. C5 12S	*Oreaster* sp. nov.
KP638253	C9	Oreasteridae sp. C9 12S	*Oreaster* sp. nov.
KP638320	NCY1	Oreasteridae sp. NCY1	*Oreaster* sp. nov.
KP638319	C9	Oreasteridae sp. C9	*Oreaster* sp. nov.
KP638321	NCY2	Oreasteridae sp. NCY2	*Oreaster* sp. nov.
KP638318	C8	Oreasteridae sp. C8	*Oreaster* sp. nov.
KP638309	C1	Oreasteridae sp. C1	*Oreaster* sp. nov.
KP638311	C11	Oreasteridae sp. C11	*Oreaster* sp. nov.
KP638317	C7	Oreasteridae sp. C7	*Oreaster* sp. nov.
KP638310	C10	Oreasteridae sp. C10	*Oreaster* sp. nov.
KP638322	NCY3	Oreasteridae sp. NCY3	*Oreaster* sp. nov.
KP638313	C3	Oreasteridae sp. C3	*Oreaster* sp. nov.
KP638316	C6	Oreasteridae sp. C6	*Oreaster* sp. nov.
KP638314	C4	Oreasteridae sp. C4	*Oreaster* sp. nov.
AY249948		Valvatida sp. 1 EK 2003 isolate 12	*Oreaster* sp. nov.
AY249950		Valvatida sp. 1 EK 2003 isolate 28	*Oreaster* sp. nov.
AY249972		Valvatida sp. 1 EK 2003 isolate 34	*Oreaster* sp. nov.
AY249967		Valvatida sp. 1 EK 2003 isolate 60	*Oreaster* sp. nov.
AY249969		Valvatida sp. 1 EK 2003 isolate 43	*Oreaster* sp. nov.
AY249949		Valvatida sp. 1 EK 2003 isolate 68	*Oreaster* sp. nov.
AY249968		Valvatida sp. 1 EK 2003 isolate 56	*Oreaster* sp. nov.
AY249965		Valvatida sp. 1 EK 2003 isolate 24	*Oreaster* sp. nov.
AY249966		Valvatida sp. 1 EK 2003 isolate 1	*Oreaster* sp. nov.
AY249974		Valvatida sp. 1 EK 2003 isolate 11	*Oreaster* sp. nov.
AY249971		Valvatida sp. 1 EK 2003 isolate 4	*Oreaster* sp. nov.
AY249970		Valvatida sp. 1 EK 2003 isolate 18	*Oreaster* sp. nov.
AY249973		Valvatida sp. 1 EK 2003 isolate 16	*Oreaster* sp. nov.
KP638323	NCY4	Oreasteridae sp. NCY4	*Oreaster* sp. nov.
KP638315	C5	Oreasteridae sp. C5	*Oreaster* sp. nov.
KP638312	C2	Oreasteridae sp. C2	*Oreaster* sp. nov.
AY396074		*Patiriella* (*sic*) *exigua* strain AM2	*P. exigua*
U50053		*P. exigua*	*P. exigua*
AY396050		*Patiriella* (*sic*) *vivipara* strain TAS	*P. vivipara*
U50054		*P. vivipara*	*P. vivipara*

Table 3

Diagnostic single nucleotide polymorphisms (SNPs) in cytochrome oxidase c subunit I *(*COI*) for various clades*

Position in 48-bp fragment of *COI* sequence	Position against reference sequence NC_001627 for *Patiria pectinifera*	Species for which allele is diagnostic	Note	Allele for valvatid larvae	Allele for QH411- and FM066-cloning larvae	Allele for *Oreaster clavatus*	Allele for *Oreaster reticulatus*
4	12157	*Oreaster* sp. nov.	SNP for QH411- and FM066-cloning larvae, valvatid larvae	A	A	C	C
7	12160	*O. reticulatus*	SNP for *O. reticulatus*	C	C	C	A
30	12183	All species	SNPs for each clade	T	T	A	G
36	12189	*O. clavatus*	SNP for *O. clavatus*	C	C	T	C
42	12195	*Oreaster* sp. nov.	SNP for valvatid larvae	T	C	C	C
45	12198	*O. reticulatus*	SNP for *O. reticulatus*	A	A	A	C
48	12201	*Oreaster* sp. nov.	SNP for QH411- and FM066-cloning larvae	A	G	A	A

Figure 3. Phylogeny based on *16S rDNA* with bootstrap values at key nodes.

FM066 and cloning *Oreaster* larvae. Another clade contains *Oreaster clavatus*. The third clade contains *Oreaster reticulatus*. Bootstrap support is strong for all of these clades (99%–100%). The tree score is log likelihood = −2337.878043.

12S rDNA, *tRNA-Glu*, *tRNA-Thr, and a portion of 16S rDNA*

This alignment contains 41 taxa and 937 positions. This locus was not sampled from valvatid larvae by Knott *et al.* (2003). The resulting tree (Fig. 4) contains the same three major clades as 16S rDNA. Bootstrap support is strong for all of these clades (100%). The likelihood score is log likelihood = −4930.081070.

Cytochrome C oxidase subunit I

The alignment of *COI* sequence data contains 51 taxa and 1273 positions. The main difference in these data and the two previous data sets is inclusion of the sequences for cloning larvae labeled as valvatids from Knott *et al.* (2003), which were sampled for a small 48-bp fragment of *COI*. The resulting tree (Fig. 5) contains the same main clades as described for the previous two data sets, with one difference. These cloning valvatid larvae form a clade that is sister to the clade comprising QH411, FM066, and cloning *Oreaster* larvae. The bootstrap support for the clade including just the cloning valvatid larvae is low, at 53%. The bootstrap support for the clade including QH411, FM066, and cloning *Oreaster* larvae is marginal, at 73%. The bootstrap support for the more inclusive clade including valvatid larvae, QH411, FM066, and cloning *Oreaster* and those labeled as valvatid larvae is high, at 88%. This more inclusive clade is well supported and is similar to clades in the results from other data sets. For *COI* clades, as in all data sets, the separate clades containing *O. clavatus* or *O. reticulatus* have bootstrap support at 100%. The tree score is log likelihood = −4434.747.

The number of base differences per site between taxa based on the *COI* sequences (Table 4) is summarized as follows. QH411, FM066, and cloning larvae of *Oreaster* have no genetic distance in this marker. The intraspecific genetic distance in *Oreaster* is, at most, half or less than half of the interspecific differences. The genetic distance from the clade formed by *Oreaster* QH411 and FM066 and cloning larvae of *Oreaster* to cloning valvatid larvae is 0.0455. This is half the distance among species and reflects that the valvatid larvae represent an earlier sampling (about nine years earlier) and perhaps a distinct population but not a distinct species. The low bootstrap support for the clade of cloning valvatid larvae also contributes to the argument that the cloning valvatid larvae are earlier samples of the same species of *Oreaster* QH411 and FM066 and cloning larvae of *Oreaster*. The intraspecific differences among species within *Oreaster* range from 0.0909 to

Figure 4. Phylogeny based on *12S rDNA*, *tRNA-Glu*, *tRNA-Thr*, and a portion of *16S rDNA*.

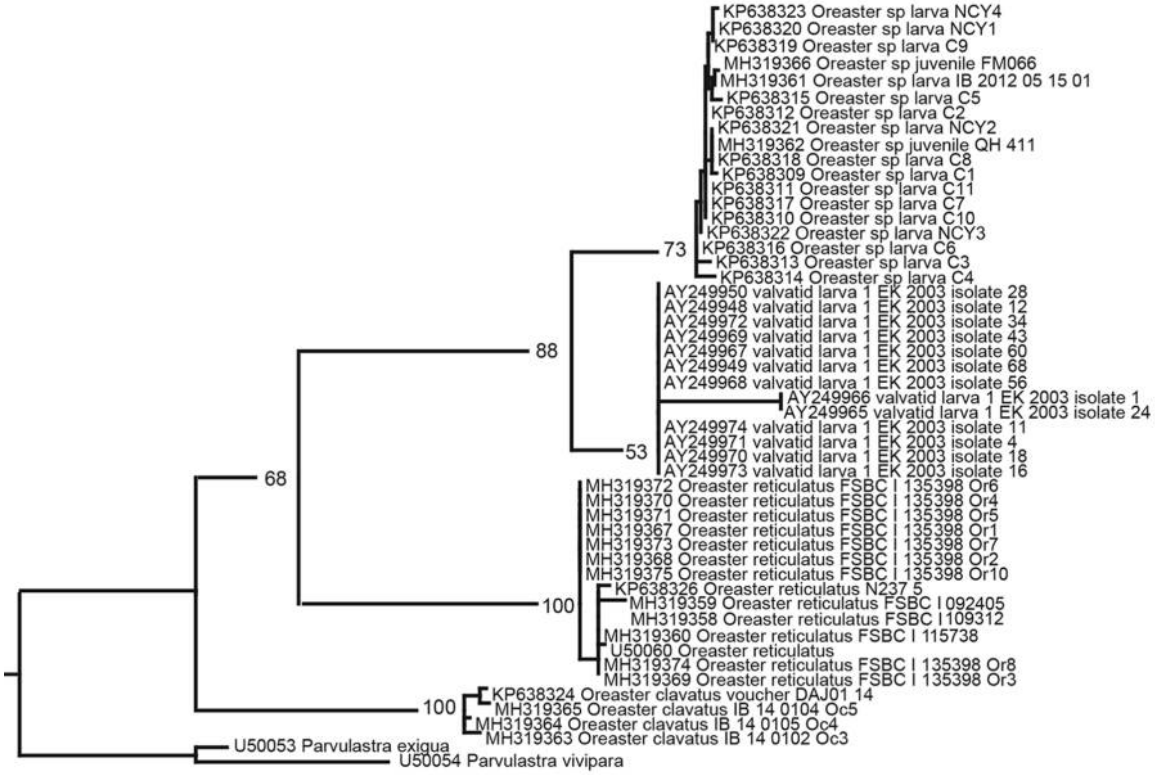

Figure 5. Phylogeny based on *cytochrome oxidase c subunit I* with bootstrap values at key nodes.

Table 4

*Genetic distance (*p*-distance) between taxon sets of interest in a region of* COI

Taxa compared	Genetic distance
Parvulastra vivipara to *Parvulastra exigua*	0.0000
P. vivipara to *Oreaster reticulatus*	0.0909
P. vivipara to QH411 and FM066 and larval *Oreaster*	0.1591
P. vivipara to *Oreaster clavatus*	0.1364
P. vivipara to valvatid larvae	0.1591
O. reticulatus to QH411 and FM066 and larval *Oreaster*	0.1591
O. reticulatus to *O. clavatus*	0.1364
O. reticulatus to valvatid larvae	0.1591
QH411 and FM066 and larval *Oreaster* to *O. clavatus*	0.0909
QH411 and FM066 and larval *Oreaster* to valvatid larvae	0.0455
QH411 and FM066 to larval *Oreaster*	0.0
O. clavatus to valvatid larvae	0.1364
Within valvatid larvae ranges	0.0–0.0227
QH411 to FM066	0.0

0.1591. *Parvulastra vivipara* and *Parvulastra exigua* have no genetic distance in this marker. Analysis of the 48-bp fragment of *COI* revealed SNPs that contain alleles that are diagnostic for each clade of interest (Table 3).

tRNAs (tRNA-Ala, tRNA-Leu, tRNA-Asn, tRNA-Gln, and tRNA-Pro)

The alignment of these *tRNA* sequence data contains 42 taxa and 388 positions. These data for the *tRNAs* are similar to those for *COI* and include sequences from cloning valvatid larvae from Knott *et al.* (2003). However, unlike the partial *COI* data for valvatid larvae, the coverage for the valvatid larvae in the *tRNAs* includes the entire 388 positions of the alignment. The phylogenetic tree resulting from analyses of *tRNAs* (Fig. 6) is somewhat different from that of *COI*. The tree based on *tRNAs* includes a mix of cloning larvae of *Oreaster*, QH411 and FM066, and valvatid larvae in one large clade. This large clade has short internal branches with bootstrap support of 88%. Sister to the large clade is a clade (86% bootstrap) with two long internal branches that lead to distinct clades of *O. reticulatus* (87% bootstrap) and *O. clavatus* (100% bootstrap). The tree score is log likelihood = −1190.099.

We created a subalignment of *tRNA* data with 319 aligned positions and 43 taxa to minimize missing data for genetic distance analyses. The number of base differences per site between taxa based on the subalignment (Table 5) is summarized as follows. The intraspecific genetic distance in *Ore-*

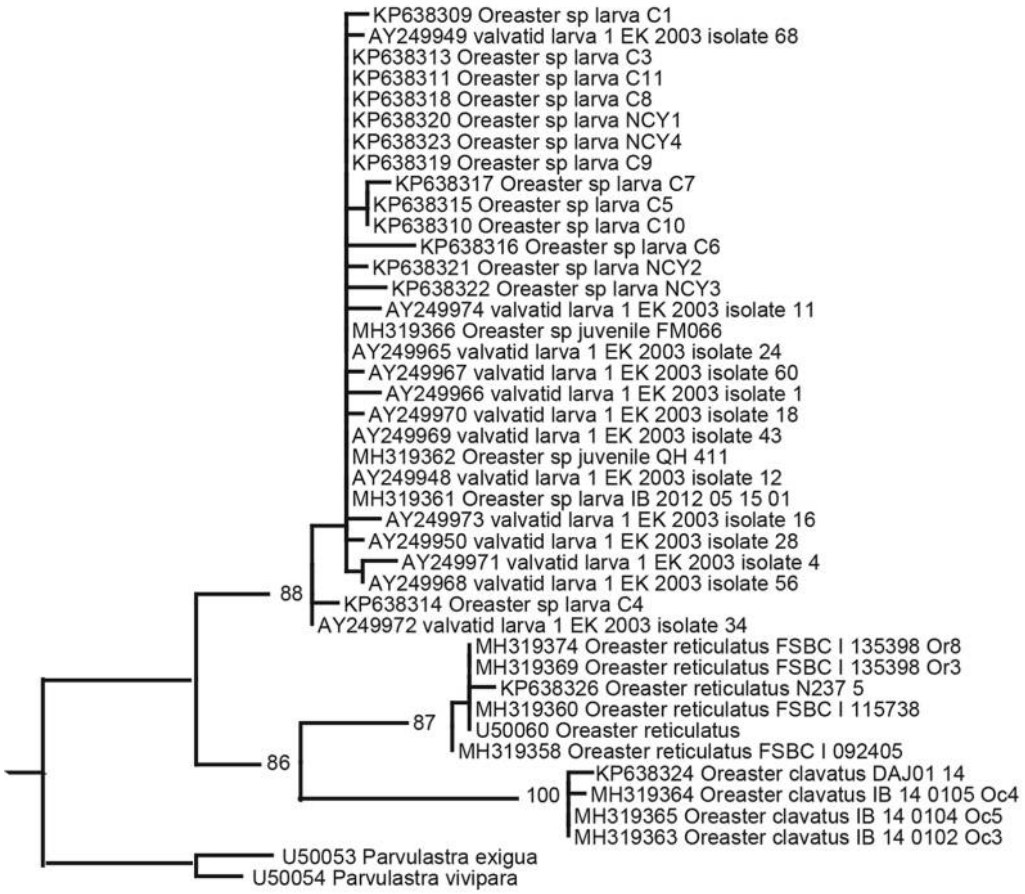

Figure 6. Phylogeny based on *tRNAs* (*tRNA-Ala*, *tRNA-Leu*, *tRNA-Asn*, *tRNA-Gln*, and *tRNA-Pro*) with bootstrap values at key nodes.

Table 5

*Genetic distance (*p*-distance) between taxon sets of interest in a region of the* tRNAs

Taxa compared	Genetic distance
Parvulastra vivipara to *Parvulastra exigua*	0.0305
P. vivipara to *Oreaster reticulatus* ranges	0.1424–0.1458
P. vivipara to QH411 and FM066 and larval *Oreaster* ranges	0.1390–0.1492
P. vivipara to *Oreaster clavatus* ranges	0.1458–0.1492
P. vivipara to valvatid larvae ranges	0.1390–0.1424
O. reticulatus to QH411 and FM066 and larval *Oreaster* ranges	0.0712–0.0814
O. reticulatus to *O. clavatus* ranges	0.0644–0.0712
O. reticulatus to valvatid larvae ranges	0.0712–0.0780
QH411 and FM066 and larval *Oreaster* to *O. clavatus*	0.0814–0.0915
QH411 and FM066 and larval *Oreaster* to valvatid larvae ranges	0.0–0.0068
QH411 and FM066 to larval *Oreaster* ranges	0.0000–0.0102
O. clavatus to valvatid larvae ranges	0.0814–0.0847
Within valvatid larvae ranges	0.0–0.0068
QH411 to FM066	0.0

aster is, at most, half of the interspecific difference and often less. The genetic distance between *O. reticulatus* and *O. clavatus* ranges from 0.0644 to 0.0712. The genetic distance from QH411 and FM066 to cloning larval *Oreaster* ranges from 0 to 0.0102. The distance from QH411 and FM066 and cloning larval *Oreaster* to cloning valvatid larvae ranges from 0 to 0.0068. These values are about one-sixth to one-tenth or less the interspecific differences in *Oreaster*. Thus, for this marker, the data indicate a vanishingly small genetic distance between valvatid larvae and the group that includes cloning larval *Oreaster* and QH411 and FM066. Comparison of *P. vivipara* and *P. exigua* indicates that they have moderate genetic distance in this marker (0.0305). *Oreaster* QH411 and FM066, cloning larvae of *Oreaster*, and cloning larvae of valvatids group with long branch lengths that lead to separate clades of *O. reticulatus* and *O. clavatus* in Figures 3–6. Also, there are zero to short genetic distances within *Oreaster* QH411 and FM066, cloning larvae of valvatids, and cloning larvae of *Oreaster*; and there are long genetic distances from these taxa to other established species (Tables 4, 5). Our methods and results for the distance analyses are similar to those of Knott *et al.* (2003) when they compared valvatid larvae to *O. reticulatus*. There is genetic distance in the *COI* data between valvatid larvae and the clade formed by *Oreaster* QH411 and FM066 and *Oreaster* larvae, but this distance is very small compared to interspecific distances. Similarly, the genetic distances within members of the clade formed by *Oreaster* QH411 and FM066, cloning *Oreaster*, or valvatid larvae for tRNA are very small compared to interspecies differences. Taken together, these results provide strong evidence that *Oreaster* QH411 and FM066 larvae identified as *Oreaster* larvae or cloning valvatid larvae are the same species. Thus, we have linked a population of previously unidentified oceanic larvae that clone to juveniles of a new cryptic species, *Oreaster* sp. (YQH-D and C. M. Cao-Romero, Universidad Nacional Autónoma de México, unpubl. data). In addition, the cloning larvae referred to as valvatids throughout the article, in order to preserve data provenance of the specimens with their GenBank records, should now be considered of the same species as *Oreaster* QH411 and FM066 and their larvae. More thoroughly, the labels cloning valvatid larvae, cloning *Oreaster* larvae, and *Oreaster* QH411 and FM066 can all be synonymized as *Oreaster* sp. nov. (Tables 2, 3).

Discussion

The clade structure, branch lengths, and genetic distance between the three taxa *Oreaster clavatus*, *Oreaster reticulatus*, and *Oreaster* sp. nov. indicate that these clades are genetically distinct. The interrelationship of the clades is not consistent among loci. However, this does not change the clade membership needed to support the hypothesis. We conclude that a larger survey of taxa and loci is necessary for further analysis of the interrelationships of *Oreaster* species and related taxa.

Our knowledge of the reproduction and dispersal of *Oreaster* species is largely based on data from *O. reticulatus*. *Oreaster reticulatus* spawns in July–October in the Caribbean (Scheibling, 1982) and has planktotrophic larvae (Metaxas *et al.*, 2008). A spawning season for *Oreaster* sp. nov. has not yet been observed. Larvae of *Oreaster* sp. nov. occur most of the year in open ocean regions, such as the Florida Current-Gulf Stream system off of the Florida Keys, Central Florida, the Sargasso Sea, the Caribbean, and the Yucatan Current. *Oreaster clavatus* occurs off of West Africa and is genetically equidistant from both *O. reticulatus* and *Oreaster* sp. nov. This discordance between genetic distance and geographic distance merits further study. It is also an intriguing question as to whether cloning larval genets of *Oreaster* sp. nov. could be very long-lived, enter the North Atlantic gyre, and disperse to the coast of Europe or Africa through the north Atlantic gyre.

By connecting larvae and juveniles with molecular data, we infer that larvae of *Oreaster* sp. nov. are not all cloning perpetually—at least some metamorphose. The frequency and periodicity of metamorphosis in cloning *Oreaster* sp. nov. are not yet understood. Galac *et al.* (2016) studied non-clonal brachiolaria that were identified as older stages of the cloning larvae. These later-stage larvae do not usually clone (IB, pers. obs.); and it may be that, at some stage, cloning activity ceases, and development proceeds toward metamorphosis. Larvae of *Oreaster* sp. nov. disperse from the Caribbean current northward to the Yucatan current that travels past Belize and Mexico. The Yucatan current feeds into the Gulf of Mexico current, passing over reef systems such as Alacranes and Triángulos Oeste (Gyory *et al.*, 2004), where QH411 and FM066 were

first discovered. The Yucatan current also connects with the Florida Current, where larvae are abundant most of the year. We have surveyed populations of *Oreaster* in coastal Yucatan Peninsula, Lake Worth Inlet in South Florida, and the Bahamian Island of San Salvador. We examined more than 75 adults and identified all as *O. reticulatus* (IB, pers. obs.). If the larvae are entrained in the North Atlantic gyre, as surmised, it is possible that the bulk of the adult populations of *Oreaster* sp. nov. are located off of the coast of Africa.

Based on the genetic distances and the sister group relationships between *Oreaster* sp. nov., *O. reticulatus*, and *O. clavatus*, *Oreaster* sp. nov. is as old as the split between *O. clavatus* and *O. reticulatus*. With this inference, the question remains: Why were *Oreaster* sp. nov. in benthic form not discovered earlier? The benthic stages of *Oreaster* sp. nov. are well camouflaged against the flora and fauna of the benthos (Fig. 2). Moreover, Hernández-Díaz and Solís-Marín found both specimens of *Oreaster* sp. nov. living under rocks in hard-to-access areas of reef that are not regularly sampled by echinoderm researchers in scuba equipment. These areas have been sampled previously by dredges, which could have sampled only over rocks.

Previous studies could not place cloning *Oreaster* larvae in a known clade because the juveniles of *Oreaster* sp. nov. had not yet been discovered. Going forward, much more information should be accessible because (1) *Oreaster* sp. nov. are readily distinguishable from *O. reticulatus* on the basis of anatomy and color patterns, and (2) we have developed molecular diagnostic tools for larval identification within *Oreaster* spp. (Table 3).

Questions that remain surrounding *Oreaster* sp. nov. include:

1. When does the benthic stage reproduce and how often? The number of individuals found is small, and the individuals are immature; thus, this process remains to be understood. With this information in hand, it will be interesting to study the heterogeneity of nuclear loci and haplotypes to address whether *Oreaster* sp. nov. is occasionally undergoing meiosis and sexual recombination.

2. Why is the benthic stage apparently so rare? *Oreaster* sp. nov. is well camouflaged (Fig. 2), and specimens were found in structurally complex and remote reef areas.

3. What trade-off allows long-term planktonic persistence of larvae of *Oreaster* sp. nov. despite the potential danger of predation in the plankton?

4. Are the cyanobacteria that live in the larvae of *Oreaster* sp. nov. (Bosch, 1992; Galac *et al.*, 2016) an important source of nourishment that might facilitate cloning in the phytoplankton-poor open ocean?

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Data Accessibility

The raw sequence and voucher data listed in Table 2 are available from GenBank (http://ncbi.nlm.nih.gov). The multiple sequence alignments, log files for phylogenetic tree searches, and resulting trees are available at https://doi.org/10.5281/zenodo.2792837.

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