BULLETIN OF MARINE SCIENCE, 76(3): 691–698, 2005

PHYLOGENY OF SEA HARES IN THE *APLYSIA* CLADE BASED ON MITOCHONDRIAL DNA SEQUENCE DATA

Mónica Medina, Timothy Collins, and Patrick J. Walsh

ABSTRACT

Sea hare species within the *Aplysia* clade are distributed worldwide. Their phylogenetic and biogeographic relationships are, however, still poorly known. New molecular evidence is presented from a portion of the mitochondrial cytochrome oxidase c subunit 1 gene (cox1) that improves our understanding of the phylogeny of the group. Based on these data, a preliminary discussion of the present distribution of sea hares in a biogeographic context is put forward. Our findings are consistent with only some aspects of the current taxonomy, and nomenclatural changes are proposed. The first is the use of a rank free classification for the different *Aplysia* clades and subclades as opposed to previously used genus and subgenus affiliations. The second is the suggestion that *Aplysia brasiliana* Rang, 1828 is a junior synonym of *Aplysia fasciata* Poiret, 1789. The third is the elimination of *Neaplysia* since its only member is confirmed to be part of the large *Varria* clade.

Sea hares within the *Aplysia* clade are benthic herbivorous opisthobranch gastropods that reach large sizes compared to many other molluscs. They possess a planktonic larval stage, but have limited dispersal as adults, having only short-range crawling and swimming capabilities. Most species inhabit tidal and subtidal zones of most tropical seas, with some reaching subtropical ranges in their distributions, and one reaching polar waters (Table 1). The *Aplysia* clade has been the focus of research for many decades. Some key studies included thorough taxonomic examinations ranging from local geographic areas (Pruvot-Fol, 1929, 1934; Bebbington, 1974) to extensive worldwide revisions (Eales, 1960), while other studies involved a comprehensive appraisal of the ecology of several species (Carefoot, 1987; Johnson, 1999). A synopsis of the neurobiology of learning and behavior of several *Aplysia* species was another major contribution to the understanding of the biology of sea hares (Kandel, 1979). As Kandel (1979) pointed out, most of the research involving *Aplysia* as a model organism has been performed on *Aplysia californica*. As evolutionary neurophysiology studies include new species, however, the necessity of a phylogenetic framework for this clade has become evident. A recent attempt to use molecular data for resolving relationships among *Aplysia* species with myoglobin amino acid sequences included only three taxa (Rinaldi and Ohir, 1998). We (Medina et al., 2001) introduced the first explicit phylogenetic hypothesis for the genus (15 species included) by using partial mitochondrial DNA (mtDNA) sequence data for the small (12S) and large (16S) subunit ribosomal genes (rDNA). Although the rDNA genes clarified relationships between the early diverging lineages, relationships between species of more recent ancestry were not resolved. Particularly, the placement of the model organism *A. californica* remained unclear.

In this study we are opting for the use of a rank-free nomenclature (Cantino and De Queiroz, 2004), thus we will refer to monophyletic groups as clades as opposed to the genus or subgenus categories traditionally used in previous classifications (Fig. 1; Table 1). In her last taxonomic revision, Eales (1960) proposed a subdivision of 35 species of *Aplysia* into five subgenera. We have previously suggested that the

monotypic *Neaplysia* Cooper 1863 containing *A. californica* was nested within the paraphyletic *Varria* clade (Medina et al., 2001). We confirm this relationship in the present study. Eales (1960) clearly indicated that the *Pruvotaplysia* clade (subgenus in her paper) embraced the most basal species (*Aplysia parvula* and *Aplysia punctata*), and pointed out similarities between *Varria*, *Neaplysia*, and *Phycophila*. Thus, although she did not present a tree, a phylogenetic hypothesis was implicit in her classification.

At present there are 38 recognized valid species of *Aplysia* (Table 1). Although some are commonly found, some are rare and only known from one or a few specimens in museum collections. Three species are circumtropical: *A. parvula*, *Aplysia juliana* , and *Aplysia dactylomela*. The Indo-Pacific Ocean is the most diverse basin with 23 species, one of which is also found in the Mediterranean Sea. The Atlantic Ocean contains twelve species, four of which are also found in the Mediterranean Sea. The level of endemism is higher in the Indo-Pacific, which has 16 endemic species, compared to the Atlantic (three endemic species) and the southeastern Pacific (one endemic species, *Aplysia inca*). The opisthobranch fauna, however, has been poorly sampled in this region (reviewed by Eales, 1960).

In this study we increase phylogenetic resolution relative to our previously published topology by adding sequence data from an additional mitochondrial gene, a 658 bp portion of the cytochrome oxidase subunit 1 (cox1) gene. With an improved phylogeny we are able to address unresolved relationships within the *Varria* clade, and in particular, the placement of *A. californica*, to suggest changes in the classification of *Aplysia*, and to postulate possible biogeographic scenarios that can be tested with additional taxon sampling in future studies.

Methods

We obtained samples from opisthobranch specialists from around the world (listed in Medina et al., 2001). We performed cox1 amplifications in 25 µl volume of a solution containing approximately 50 ng of DNA, 1X PCR buffer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.5 µM of each primer, and 1.25 units of Taq polymerase (Perkin-Elmer/Cetus). After an initial denaturing step of 2 min at 94 °C, 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C, 30 sec at 72 °C were performed, followed by a final extension step of 5 min at 72 °C. We used the cox1 primers developed by (Folmer et. al., 1994): LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2198: (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). We cloned purified PCR fragments into a TA vector (Invitrogen) and sequenced individual clones using the Thermo Sequenase cycle-sequencing kit (Amersham) with dye-labeled universal M13 primers in a Licor automated sequencer. We were unable to obtain cox1 products from two species (*Aplysia depilans* and *A. parvula*) due to degraded DNA templates and unstable primer annealing. We deposited sequences in GenBank under accession numbers AF343425-AF343432, and AY275426.

We chose *Akera bullata* O. F. Muller, 1776, shown to be the most basal taxon in the anaspidean clade (Medina et al., 2001), as the outgroup taxon for this analysis. We performed all phylogenetic analysis using PAUP* 4.0 (Swofford, 1997). The ILD test (Farris et al., 1995) was used to determine if different data sets from the previous and present studies (rDNA and cox1) possessed significantly conflicting phylogenetic signals. Equal weights maximum parsimony (MP), minimum evolution (ME), and maximum likelihood (ML) analyses were conducted. To determine which model of sequence evolution best fit the data for ME and ML, we performed nested likelihood ratio tests (LRT) using Modeltest version 3.0 (Posada and Crandall, 1998). For ME and MP, we performed heuristic searches, with 1000 replicates of random

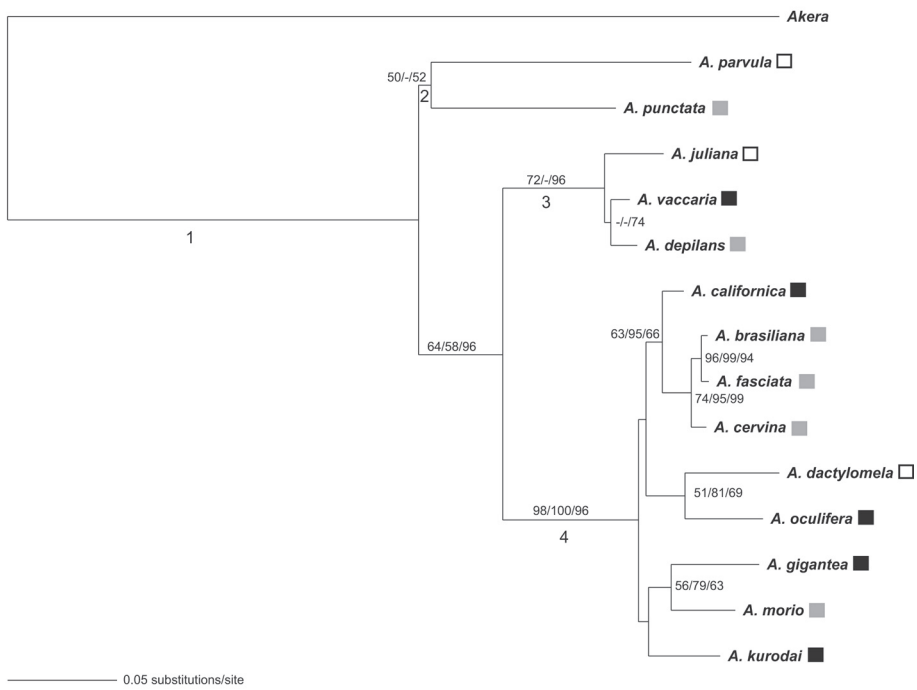

Figure 1. *Aplysia* phylogenetic analysis of rRNA and cox1 mitochondrial gene fragments. Best tree obtained by ML. Major clades are depicted by numbers below branches 1) *Aplysia*; 2) *Provotaplysia*; 3) *Subaplysia*; and 4) *Varria*. Values above branches correspond to bootstrap values for ML, ME, and MP, respectively. Symbols next to species names represent broad geographic distributions: White boxes = circumtropical, gray boxes = Atlantic, black boxes = Pacific.

stepwise addition and TBR branch swapping. For ML, we performed heuristic searches with five replicates of random stepwise addition and TBR branch swapping. Our bootstrap analyses included 1000 pseudoreplicates for MP and ME, and 100 pseudoreplicates for ML.

Results

All three genes exhibit base compositional bias, with A-T bias of 63.3% in cox1 (this study), 62.4% in 16S, and 65.5% in 12S (Medina et al., 2001). The nucleotide bias varied between codon positions of the cox1 gene, from 53.3% in the first-codon positions to 79% in the third-codon positions. The average nucleotide composition on the coding strand of the sequenced taxa for cox1 was 24% A, 40% T, 17% C, and 19% G. No species differed by more than 2% from the average values. Third positions were the most variable sites, and accounted for 84% of the variation, with first and second positions accounting for 14% and 2% of the variable sites, respectively, in this portion of cox1. Sequence divergence values among taxa for cox1 ranged from 1.1%–17.6% among *Aplysia* species, and from 17.5% to 20.1% for outgroup comparisons.

The ILD test (Farris et al., 1995) run with 10 random stepwise additions with TBR branch swapping and 1000 randomizations indicated no significantly conflicting phylogenetic signal between the ribosomal and cox1 data sets. *Aplysia depilans* and *A. parvula* have missing data for cox1, so their placement is based solely on sequences from the ribosomal genes. Results were congruent for the 13 and 15 taxon

Table 1. Classification and geographical distribution of species of *Aplysia* Linnaeus, 1767. Distribution ranges are based on Eales (1960), Bebbington (1974), and Marcus & Marcus (1960).

Clade (former subgenus)	Species	Distribution
Pruvotaplysia Engel, 1936	*A. parvula* Guilding Morch, 1863	Circumtropical
	A. punctata Cuvier, 1803	Northeast Atlantic, Mediterranean
Subaplysia nom. nov.	*A. cedroensis* Bartsch & Rehder, 1939	Northeast Pacific
	A. depilans Gmelin, 1791	Northeast Atlantic, Mediterranean
	A. dura Eales, 1960	Southeast Atlantic, southwest Pacific
	A. juliana Quoy and Gaymard, 1832	Circumtropical
	A. nigra d'Orbigny, 1837	Southwest Atlantic, South Pacific
	A. reticulopoda Beeman, 1960	Northeast Pacific
	A. vaccaria Winkler, 1955	Northeast Pacific
Varria Eales, 1960	*A. brasiliana* Rang, 1828	North Atlantic
	A. californica J. G. Cooper, 1863	Northeast Pacific
	A. cervina (Dall and Simpson, 1901)	West Atlantic
	A. cornigera Sowerby, 1869	Indian, West Pacific
	A. cronullae Eales, 1960	Southwest Pacific
	A. dactylomela Rang, 1828	Circumtropical
	A. denisoni Smith, 1884	Indian, West Pacific
	A. donca Ev. Marcus and Er. Marcus, 1960	West Atlantic
	A. extraordinaria Allan, 1932	Southwest Pacific
	A. fasciata Poiret, 1798	Southeast Atlantic, Mediterranean, Red Sea
	A. gigantea Sowerby, 1869	Indian, West Pacific
	A. gracilis Eales, 1960	Red Sea
	A. inca d'Orbigny, 1837	Southeast Pacific
	A. keraudreni Rang, 1828	South Pacific
	A. kurodai Baba, 1937	Northwest Pacific
	A. maculata Rang, 1828	Western Indian
	A. morio Verrill, 1901	Northwest Atlantic
	A. oculifera Adams & Reeve, 1850	Indian, West Pacific
	A. pulmonica Gould, 1852	West Pacific
	A. rehderi Eales, 1960	Northeast Pacific
	A. reticulata Eales, 1960	Southwest Pacific
	A. robertsi Pilsbry, 1895	Northeast Pacific
	A. rudmani Bebbington, 1974	Indian
	A. sagamiana Baba, 1949	Northwest Pacific
	A. sowerbyi Pilsbry, 1895	Southwest Pacific
	A. sydneyensis Sowerby, 1869	Southwest Pacific
	A. tanzanensis Bebbington, 1974	Indian
	A. winneba Eales, 1957	Tropical East Atlantic
Phycophila Adams, 1861	*A. euchlora* Adams in M.E. Gray, 1850	Northwest Pacific

data sets. We chose, therefore, to present the results from the most comprehensive data set (Fig. 1). We obtained three shortest trees (TL=756) in the MP search (trees not shown; MP bootstrap values are depicted in Figure 1). The LRT implemented in Modeltest 3.0 (Posada and Crandall, 1998) indicated that the model that best fit the data was a GTR + Γ + I. The assumed proportion of invariable sites was 0.4322, and the shape parameter (alpha) was 0.3460. Of the two model based approaches, ME was the most affected by the incomplete dataset. This is reflected in the lack of boot-

strap support for the nodes in the *Aplysia* clade, which are supported by the other optimality criteria. The ML tree (–Ln likelihood = 5363.12531) is depicted in Figure 1 with corresponding bootstrap values.

Discussion

Previous attempts to reconstruct the phylogenetic relationships of the *Aplysia* clade were those of Fiorito et al. (1990) and Rinaldi and Ophir (1998). Fiorito et al. (1990) attempted to test Eales's classification by means of a phenetic (UPGMA) analysis. They used 12 characters, several of which are redundant or of questionable utility. For example, four characters were redundant with respect to body size (i.e., body size, head size, foot size, and size of parapodia). The ink secretion character (purple vs. white) was ambiguously coded with respect to the purple and opaline glands, both ink-producing organs. Despite using characters from Eales (1960) taxonomic descriptions, Fiorito et al.'s results are incongruent with her classification. Rinaldi and Ophir (1998) used myoglobin amino acid sequence data to infer relationships among three *Aplysia* species. Their conclusions state that *A. juliana* and *Aplysia kurodai* are more closely related, and *A. fasciata* (junior synonym *Aplysia limacina* in Rinaldi and Ophir, 1998) is the most basal lineage of the three are not supported by our analysis.

The phylogeny recovered by the rRNA dataset was in agreement with the classification proposed by Eales (1960), except for the placement of *A. californica* (Medina et al., 2001). The phylogeny inferred from the cox1 data set was also congruent with both the rRNA results and Eales's inferred phylogenetic hypothesis. The cox1 fragment was more variable than the ribosomal genes, particularly at third positions. Thus, this gene was useful in recovering relationships within the *Varria* clade (Fig. 1). Our current results, despite limited taxonomic sampling, allow us to suggest that four clades should be recognized within *Aplysia* (Table 1): *Pruvotaplysia*, *Subaplysia* (nom. nov.), *Varria,* and *Phycophilia*. It should be noted, however, that 1) the monophyly of *Pruvotaplysia* is not well supported, and that 2) we were unable to obtain material for *A. euchlora*, and therefore could not determine if *Phycophilia* is a genuine clade.

The combined analysis in this study resulted in increased overall support for many nodes. The species placed in the *Pruvotaplysia* clade were confirmed as the most basal in the *Aplysia* clade. The monophyly of this clade, however, is still poorly supported. The molecular evidence also supported the divergence of the *Subaplysia* (nom. nov.) and *Varria* clades from a *Pruvotaplysia* ancestor as suggested by morphological data (Eales, 1960). The monophyly of *Varria* was supported by high bootstrap values with the inclusion of *A. californica*. Eales (1960) had already pointed out that except for the shape of the shell, which was the character she used to place this species in a different subgenus, *A. californica* resembled species of the subgenus *Varria*.

Within the *Varria* clade, *A. brasiliana*, *A. fasciata*, and *A. cervina* formed a monophyletic group. These three species not only have similar geographic ranges, but they also share similar morphological traits. Eales (1960) also pointed out the anatomical resemblance between *Aplysia brasiliana* and *Aplysia cervina*, which differ solely in average size and level of activity. In Eales's taxonomic descriptions, *A. fasciata* (found in the eastern Atlantic) resembles *A. brasiliana* (western Atlantic), in the similar opaline gland, penis, and swimming behavior (Eales, 1960). In addition, breeding ex-

periments by Susswein et al. (1993) have demonstrated that these two species freely interbreed with no apparent preferences for conspecifics. The sequence divergence between these two taxa was the lowest of all comparisons: 0.001 with the rRNA genes (Medina et al., 2001) and 0.011 with the cox1 gene (this study). This level of sequence divergence is more typical of intraspecific levels. Consequently, morphological (Eales, 1960), behavioral (Susswein et al., 1993), and molecular evidence (Medina et al. 2001; this study) suggest that *A. brasiliana* and *A. fasciata* represent different geographical populations of the same species rather than separate species. Based on the congruent evidence from morphological and mtDNA character data, in combination with reproductive evidence, the name *A. brasiliana* should be considered a synonym of *A. fasciata* which has nomenclatural priority.

Another group of interest is the *A. dactylomela–A. oculifera* clade. Eales (1960) and Gosliner (1987), noted that early taxonomic reports involving these two species should be taken with caution because they are morphologically quite similar. *Aplysia oculifera* specimens have been mistaken as *A. dactylomela* by taxonomists in the past, however, *A. oculifera* specimens are of smaller size and the black rings on the skin are not as large as in *A. dactylomela* (Gosliner, 1987). With the exception of the *A. brasiliana*, *A. cervina*, and *A. fasciata* clade, most sister taxon relationships within the genus are not of geographically adjacent populations. For example, within the *Subaplysia* clade, *A. vaccaria* (California coast) formed a well-supported clade with *A. depilans* (Mediterranean Sea). Within the *Varria* clade, *A. californica* (California coast) was closely related to the *A. cervina* clade (Atlantic), and *A. gigantea* (western Australian coast) formed a clade with *A. morio* (Texas coast).

Reconstructing the history of marine biogeographic events can be challenging (reviewed in Cunningham and Collins, 1998), and verifications of hypotheses from the fossil record are also fraught with difficulty. Although fossils of *Akera* have been found in Cretaceous-age rocks, the fossil record of *Aplysia* is sparse because of the extremely fragile shells, with the earliest record dating from the Miocene (discussed in Medina et al., 2001). Thus, the fossil record of this genus is not adequate for fossil tests of vicariance hypotheses. We observe, however, that the three circumtropical species in our topology are each a member of one of the three main clades (the three former subgenera), and the *Provotaplysia* lineage is the most basal, and sister group to the lineage that gave rise to the rest of the *Aplysia* sea hares. We hypothesize that the members of this clade were likely present before the closure of Tethys as suggested by Bebbington (1975). Another interesting result is that the divergence of *A. californica* from its sister clade is consistent with a transisthmian vicariance. Perhaps this relationship, in combination with more intensive taxon sampling, especially of the circumtropical species and *A. parvula*, would help us to date cladogenetic events, and postulate more precise historical biogeographic scenarios for the *Aplysia* clade.

Acknowledgments

We are grateful to J. Silberman and J. Fell for assistance in the laboratory. B. Dayrat, K. Rosen, and two anonymous reviewers made helpful comments on a previous draft of the manuscript. This paper was prepared in partial fulfillment of requirements for the PhD in Marine Biology and Fisheries at the University of Miami (M.M.). Support for this project was funded by NIH RR 10294. Part of this work was performed under the auspices of the U.S. Department

of Energy, Office of Biological and Environmental Research, by the University of California, Lawrence Berkeley National Laboratory under contract No. DE-AC03-76SF00098.

Literature Cited

Bebbington, A. 1974. Aplysiid species from East Africa with notes on the Indian Ocean Aplysiomorpha (Gastropoda: Opisthobranchia). Zool. J. Linn. Soc. 54: 63–99.

_____. 1975. On a collection of *Aplysia* species from Naples with a note on the distribution of *Aplysia parvula* (Gastropoda, Opisthobranchia) in the Mediterranean. Publ. Staz. Zool. Napoli 39: 121–128.

Cantino, P. and K. De Queiroz, 2004. Phlyocode [Internet]. Athens: University of Ohio; April 9, 2000 [modified March 29, 2005]. Available from: http://www.ohiou.edu/phylocode/.

Carefoot, T. 1987 *Aplysia*: its biology and ecology. Oceanogr. Mar. Biol. Am. Rev. 25: 285–351.

Cunningham, C. W. and T. M. Collins. 1998. Beyond area relationships: extinction and recolonization in molecular marine biogeography. Pages 297–321 *in* R. DeSalle and B. Schierwater B. eds. Molecular approaches to ecology and evolution. Birkhauser Verlag, Basel.

Cooper, J. G. 1863. On new or rare Mollusca inhabiting the coast of California. Proc. Calif. Acad. Sci. 3: 56–60.

Eales, N. B. 1960. Revision of the world species of *Aplysia* (Gastropoda, Opistobranchia). Bull. British Mus. (Nat. Hist.) Zool. 5: 266–404.

Farris, J. S., M. Kallersjo, A. G. Kuge, and C. Bult. 1995. Testing significance of incongruence. Cladistics 10: 315–319.

Fiorito, G., C. Capuano, and P. Bergamo. 1990. Analisi preliminare delle relazioni filogenetiche delle specie di *Aplysia* 1767 (Mollusca: Gastropoda, Opistobranchia). Lav. Sci. Ital. Malacol. 23: 261–270.

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotech. 3: 294–299.

Gosliner, T. 1987. Nudibranchs of Southern Africa. A guide to opisthobranch molluscs of Southern Africa. Sea Challengers & Jeff Haffman, Monterey. 137 p.

Johnson, P. M. 1999. Defense in sea hares (gastropoda, opisthobranchia, anaspidea): multiple layers of protection from egg to adult. Mar. Freshw. Beh. Physiol. 32: 147–180

Kandel, E. 1979. Behavioral biology of *Aplysia*. W.H. Freeman and Company, New York. 441 p.

Marcus, E. and E. Marcus. 1960. Some opisthobranchs from the northwestern Gulf of Mexico. Publ. Inst. Mar. Sci. Univ. Tex. 6: 251–264.

Medina, M., T. Collins, and P. J. Walsh. 2001. mtDNA ribosomal gene phylogeny of sea hares in the genus *Aplysia* (Gastropoda, Opisthobranchia, Anaspidea): implications for comparative neurobiology. Sys. Biol. 50: 676–688.

Posada, D. and K. A. Crandall 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817–818.

Pruvot-Fol, A. 1929. La symetrie fondamentale des invertebres et leurs symetries successives. Comp. Rend. l'Assoc. Anat. 24: 1–9.

_____. 1934. Les opisthobranches de Quoy et Gaimard. Arch. Mus. d'Hist. Natur. 11: 3–92.

Rinaldi, A. C. and R. Ohir. 1998. Phylogeny of anaspid taxa as inferred from amino acid sequences of monomeric myoglobins. Israel J. Zool. 44: 3–8.

Sowerby, G. B. 1869. *Aplysia in* Reeve A.L. ed. Conchologia Iconica 17: 1–10.

Susswein, A. J., S. Markovich, and Y. Achituv. 1993. Assymetry of male and female mating in mixed-species groups of *Aplysia fasciata* Poiret and *Aplysia brasiliana* Rang (Mollusca: Gastropoda). Israel J. Zool. 39: 213–229.

Swofford, D. L. 1997. PAUP*: Phylogenetic analysis using parsimony. Sinauer Associates, Sunderland. 140 p.

Date Submitted: 20 February, 2004. Date Accepted: 30 August, 2004.

Addresses: (M.M., P.J.W.) *Rosenstiel School of Marine and Atmospheric Science, Division of Marine Biology and Fisheries, University of Miami, 4600 Rickenbacker Causeway, Miami, Florida 33149.* (T.C.) *Department of Biological Sciences, Florida International University, University Park, Miami, Florida 33199.* Corresponding Author: (M.M.) Present Address: *School of Natural Sciences, University of California, Merced, P.O. Box 2039, Merced, California, 95344. E-mail: <mmedina@ucmerced.edu>.*