Molecular Phylogenetics and Evolution 94 (2016) 252–263

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Elucidating the evolutionary relationships of the Aiptasiidae, a widespread cnidarian–dinoflagellate model system (Cnidaria: Anthozoa: Actiniaria: Metridioidea) ☆

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ARTICLE INFO

Article history:
Received 6 August 2014
Revised 5 September 2015
Accepted 7 September 2015
Available online 12 September 2015

Keywords:
Aiptasia
Exaiptasia pallida
Exaiptasia brasiliensis sp. nov.
Nuclear rDNA
Sea anemone
Symbiodinium

ABSTRACT

Sea anemones of the family Aiptasiidae *sensu* Grajales and Rodríguez (2014) are conspicuous members of shallow-water environments, including several species widely used as model systems for the study of cni darian–dinoflagellate symbiosis and coral bleaching. Although previously published phylogenetic studies of sea anemones recovered Aiptasiidae as polyphyletic, they only included a sparse sample in terms of its taxonomic diversity and membership of the family had not been yet revised. This study explores the phylogenetic relationships of this family using five molecular markers and including newly collected material from the geographical distribution of most of the currently described genera and species. We find a monophyletic family Aiptasiidae. All the currently proposed genera were recovered as monophyletic units, a finding also supported by diagnostic morphological characters. Our results confirm *Bellactis* and *Laviactis* as members of Aiptasiidae, also in agreement with previous morphological studies. The monophyly of the group is congruent with the morphological homogeneity of the members of this family. The obtained results also allow discussing the evolution of morphological characters within the family. Furthermore, we find evidence for and describe a new cryptic species, *Exaiptasia brasiliensis* sp. nov., based on molecular data, geographical distribution, and the identity of its endosymbiotic dinoflagellate.

1. Introduction

Sea anemones of the family Aiptasiidae (Cnidaria: Anthozoa: Actiniaria: Metridioidea) are conspicuous components of tropical and subtropical shallow waters worldwide (Fig. 1). Members of the recently erected genus *Exaiptasia* (Grajales and Rodríguez, 2014) have been extensively used as a model organism for studies on dinoflagellate–cnidarian symbiosis, reproduction, and development (e.g. Dunn et al., 2002; Muller-Parker and Davy, 2001; Weis et al., 2008; LaJeunesse et al., 2010). Their rapid growth rate, facilitated by their symbiosis with dinoflagellates (*Symbiodinium* spp.), combined with asexual reproduction via pedal laceration (Clayton and Lasker, 1985; Lin et al., 2000) makes members of *Exaiptasia* excellent laboratory specimens; in other contexts, like the aquarium trade, these same traits lead to them being considered pests. Several clonal strains (e.g. CC7, H2, PLF3, PLF5) have been cultured and used in different studies for years; however, studies typically refer to this organism as *Aiptasia* spp. – now *Exaiptasia*, see Grajales and Rodríguez (2014) – (e.g. Dunn et al., 2002; Yokouchi et al., 2003) due to a lack of evidence about whether this "model organism" is actually one cosmopolitan species or a group of cryptic or insufficiently described species. Despite their ubiquity, symbiotic relationships, and ecological relevance the group has traditionally been neglected from a taxonomic and evolutionary point of view.

Few studies of sea anemones (Daly et al., 2002; Gusmão and Daly, 2010) have specifically focused on sampling efforts to test phylogenetic relationships at the genera and species levels. As a consequence, the formulation of hypotheses concerning the evolution of morphological characters, taxonomic diversity through time and across habitats, and reproduction at lower levels has not been feasible. Population-level studies are also hindered by the lack of clearly defined taxonomic units – as well as actiniarian taxonomists. Previous molecular phylogenetic studies of the group

☆ This paper was edited by the Associate Editor Bernd Schierwater.

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http://dx.doi.org/10.1016/j.ympev.2015.09.004
1055-7903/

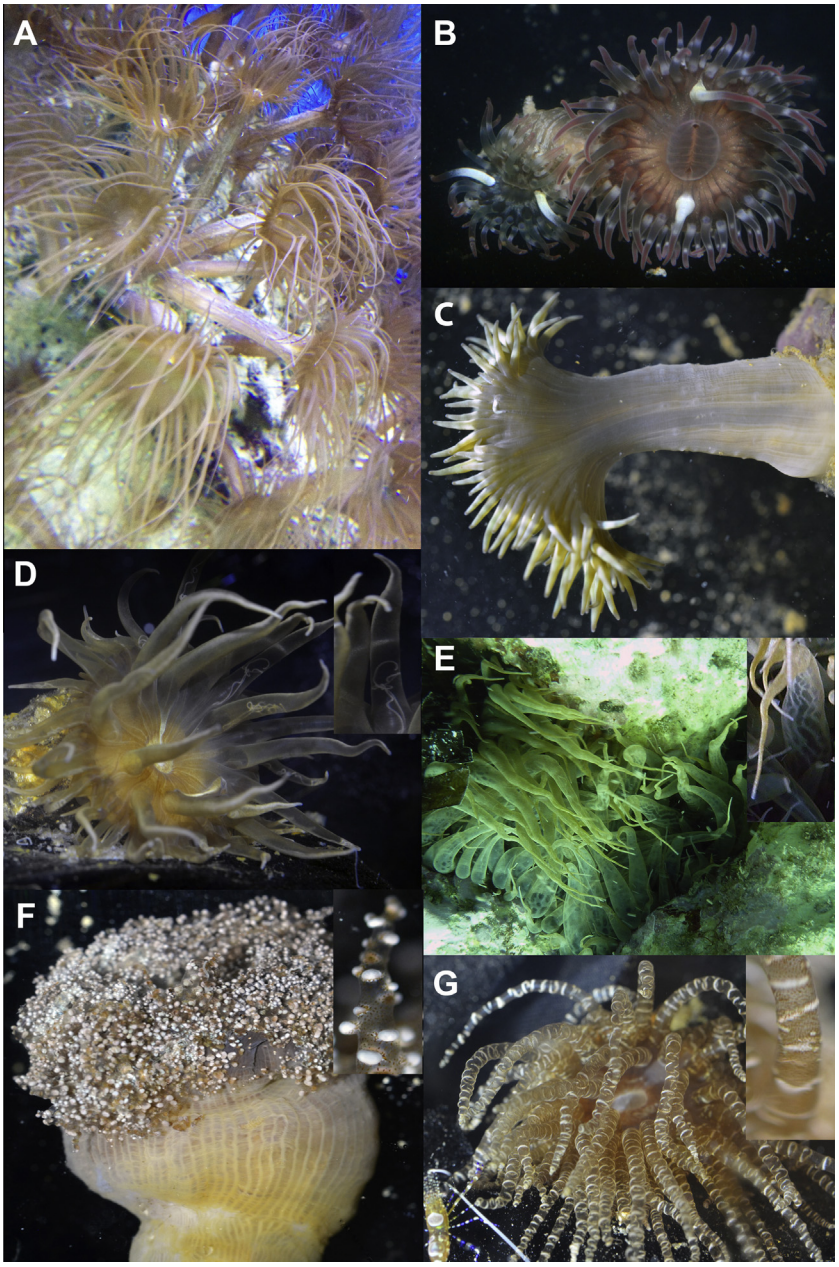

Fig. 1. Representative species of genera within Aiptasiidae. (A) *Exapitasia pallida*. (B) *Aiptasiogeton hyalinus*. (C) *Bellactis ilkalyseae*. (D) *Aiptasia couchii*. (E) *Aiptasia mutabilis*. (F) *Laviactis lucida*. (G) *Bartholomea annulata*. Top right rectangles in D, E, F, and G depict a detailed view of the tentacles, useful for field identification.

recovered the family Aiptasiidae as a non-monophyletic clade (because of *Neoaiptasia morbilla*) and showed that the genus *Aiptasia* was not monophyletic (Rodríguez et al., 2012, 2014); however, they only included four specimens representing three of six aiptasiid genera. Recently, Grajales and Rodríguez (2014) conducted a detailed taxonomic study of Aiptasiidae, which resulted in major membership and nomenclatural changes for the family based on a detailed revision of morphological characters; Aiptasiidae currently includes six genera and 12 species (see Grajales and Rodríguez, 2014). The genus *Aiptasia* was separated into two different genera, *Aiptasia* and *Exaiptasia*, based on differences in the cnidae and reproduction. Within *Aiptasia* two species were also recognized, *A. mutabilis* and *A. couchii*, due to differences in reproduction, number of mesentery and tentacle cycles, and their association with different genera of endosymbiotic algae (reviewed in Grajales et al., in press).

Within *Exaiptasia*, the authors examined specimens corresponding to most of the reported species but could not find morphological differences to separate described species, and thus synonymized all *Exaiptasia* species as a single widespread species, *Exaiptasia pallida* (Grajales and Rodríguez, 2014). Thornhill et al. (2013) provided independent evidence of the genetic homogeneity within *Exaiptasia* worldwide, with the exception of one locality. The authors conducted a comprehensive study on the identity of the endosymbionts in *Aiptasia* (currently *Exaiptasia*) and found that most individuals harbored a single endosymbiont species (*Symbiodinium minutum*), with the exception of populations in Florida, which harbored multiple *Symbiodinium* types. In addition, Thornhill et al. (2013) revealed a lack of population structure and allele sharing across different localities where the sea anemones were exclusively harboring *S. minutum*, while the specimens from Florida showed a distinctive genetic signature.

We sequenced and analyzed more than 8 kbp of mitochondrial and nuclear DNA of representatives of all currently described genera and most species within the family Aiptasiidae to test **1**: the monophyly, higher- and lower-level phylogenetic relationships of Aiptasiidae, **2**: the identity and phylogenetic position of the most commonly used clonal strains in the study of cnidarian–dinoflagel late symbiosis and coral bleaching (putatively *Aiptasia* spp.), **3**: discuss the evolution of morphological characters within the family. In addition, we describe a new cryptic species of *Exaiptasia* restricted to the Southern Caribbean Sea and the Southwestern Atlantic Ocean.

2. Material and methods

2.1. Taxonomic sampling

The studied material was collected by snorkeling or SCUBA diving during 2009–2012 from 45 different localities worldwide (Table 1). Ingroup sampling included 80 specimens (see Supplementary Table 1) representing the distributional range of all species and genera currently included in the family Aiptasiidae (i.e. *Aiptasia*, *Aiptasiogeton*, *Exaiptasia*, *Bartholomea*, *Bellactis*, and *Laviactis*) (Grajales and Rodríguez, 2014). Sampling effort was particularly exhaustive in terms of including representatives of *Exaiptasia*, including clonal strains used for cnidarian–dinoflagel late symbiosis studies (Supplementary Table 2). The newly collected material is from localities corresponding to populations previously considered as separate species but recently synonymized as *E. pallida* (see Grajales and Rodríguez, 2014), plus new records from localities in Brazil, Panama, and Australia (see Table 1). These efforts significantly increased the number of terminals from four representatives in previous studies to a total of 80 aiptasiid terminals. Outgroup sampling included representatives of previously identified clades Metridioidea, Actinioidea, Actinostoloidea, Actinernoidea, and Edwardsioidea (Rodríguez et al., 2014), plus three additional representatives of the family Aliciidae (*Lebrunia coralligens*, *L. danae*, and *Alicia mirabilis*, see Supplementary Table 1). Voucher specimens preserved in formalin and/or ethanol have been deposited at the American Museum of Natural History (AMNH) and the Museu de Zoologia da Universidade do São Paulo (MZUSP) (see Table 1). We have omitted authorship information of taxa within the text for readability; for this information refer to Fautin (2013), Grajales and Rodríguez (2014), and Rodríguez et al. (2014).

2.2. Data collection

Sea anemones were relaxed in seawater containing menthol crystals and photographed while alive. Small pieces of tissue from selected specimens were preserved in 100% ethanol for DNA analysis. Preliminary identifications were made examining external and internal anatomy (the later through histological sections) and the inspection of the cnidae of each specimen.

Genomic DNA was isolated from approximately 25 mg of tissue using the Qiagen DNeasy® kit. Whole genomic DNA was amplified using published primers and protocol detailed in Lauretta et al. (2014) for the mitochondrial markers 12S, 16S, and COIII, and the nuclear 28S. Sequences for nuclear 18S were amplified using newly designed primers specific to Actiniaria to avoid co-amplification of *Symbiodinium* nuclear ribosomal genes (see Table 2). Amplification of the 18S region was performed with the external primers 18S_NA (forward) and 18S_NB (reverse) and the following PCR conditions: 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 2 min and 25 s; PCR reactions terminated with 8 min at 72 °C. PCR products were cleaned using ExosapIT. Sequencing reactions used a total of 5 μL of cleaned PCR product, at a concentration of 25 ng of product for every 200 base pairs (bp) of marker length. Cycle sequencing products were sequenced using PCR amplification primers on an ABI 3770x at the in-house facilities of the AMNH; for the 18S region, a total of six primers (two external and four internal primers, see Table 2) were used as sequencing primers to obtain overlapping sequence fragments that were assembled into a single 18S sequence. Forward and reverse sequences were assembled in Geneious v. 6.16 (Biommaters) and blasted against the nucleotide database of GenBank to confirm that the obtained sequence corresponded to the target sequence/organism and not to their endosymbiotic algae. All sequences have been deposited in GenBank (Supplementary Table 1).

2.3. Data analysis

DNA sequences for each marker were combined and analyzed with sequences from Rodríguez et al. (2014) excluding anthozoan outgroups thus creating a single dataset comprised of 194 taxa. Sequences for each marker were separately aligned using MAFFT v.7.017 (Katoh et al., 2002) using the following settings: Strategy, L-INS-I; Scoring matrix for nucleotide sequences, 200PAM/k = 2; Gap open penalty, 1.53; Offset value, 0.05. Alignments for each marker were analyzed separately and as a concatenated dataset. Within the dataset, the COIII alignment was treated as two different partitions, one corresponding to the first two-codon positions and a second for the third position. Divergence estimates (based on the Kimura 2-parameter (K2P)) were obtained using Mega v.5.05 (Tamura et al., 2013).

2.3.1. Phylogenetic inference

Maximum Likelihood (ML) analyses were performed using RAxML v.7.6.3 (Stamatakis, 2006) as implemented on the CIPRES portal (Miller et al., 2010), using the GTR + Γ (GTRGAMMA) as the model of nucleotide substitution but allowing the estimation of different α shape, GTR rate, and base frequency for each partition in the combined alignment. The Majority Rule Criterion implemented in RAxML (-autoMRE) was used to assess clade support. Tree searches under Maximum Parsimony (MP) were conducted using random and constrained sectorial searches, tree drifting, and 100 rounds of tree fusing (command xmult = hits 10 rss drift css fuse 100) in TNT v.1.1 (Goloboff et al., 2008). Analyses were run with gaps treated as missing data. The concatenated dataset was subjected to 1000 bootstrap replicates to assess clade support on the obtained strict consensus tree.

2.3.2. Ancestral state reconstructions

Ancestral state reconstructions were performed under MP using Mesquite v.2.75 (Maddison and Maddison, 2011) counting only unequivocal states. A total of 13 morphological characters were obtained from a recent morphological revision of the family Aiptasiidae [see Grajales and Rodríguez (2014)]. From these, only twelve characters presented enough variability within the Aiptasiidae to allow analysis (Table 3). We used a representation of the phylogenetic relationships of Aiptasiidae derived from the ML topology obtained from the concatenated dataset; *Aiptasiogeton hyalinus* was used to polarize the character states.

3. Results

3.1. Phylogenetic position and membership of the family Aiptasiidae

Both ML and MP analyses of the concatenated dataset recovered the family Aiptasiidae *sensu* Grajales and Rodríguez (2014) as a

Table 1
Taxa included in this study, with voucher catalog numbers and location. Prior species concepts, plus changes proposed in this study and in Grajales and Rodríguez (2014) provided.

Species in this study	Previous concept	Museum voucher	Locality	Locality 2	Longitude	Latitude	Depth (m)
Aiptasia couchii Cocks, 1851	*A. mutabilis* (Gravenhorst, 1831)	AMNH5515.1	England	Plymouth	N 50 21 49.98	W 04 08 38.43	1
	A. mutabilis (Gravenhorst, 1831)	AMNH5516.2	England	Wembury	N 50 18 57.55	W 04 04 40.02	1
	A. mutabilis (Gravenhorst, 1831)	AMNH5542.1	France	Banyuls-sur-Mer	N 42 28 59.01	E 03 07 49.48	1
	A. mutabilis (Gravenhorst, 1831)	AMNH5501.1	Italy	Oristano	N 39 40 34.64	E 08 26 42.66	1
	A. mutabilis (Gravenhorst, 1831)	AMNH5500	Italy	La Caletta	N 39 40 34.63	E 08 26 04.65	1
		AMNH5502.1	Italy	Torre Vecchia	N 39 40 29.79	E 08 26 49.68	1
	A. mutabilis (Gravenhorst, 1831)	AMNH5531.1	Portugal	Madeira – Machico	N 32 42 52.84	W 16 45 47.85	1
	A. mutabilis (Gravenhorst, 1831)	AMNH5536.1	Spain	La Herradura	N 36 43 56.77	W 03 44 28.21	1
	A. mutabilis (Gravenhorst, 1831)	AMNH5521.1	Spain	Medes I.	N 42 02 51.25	E 03 13 08.91	1
	A. mutabilis (Gravenhorst, 1831)	AMNH5517.1	Spain	Cádiz – Tarifa	N 36 00 35.05	W 36 04 50.22	1
	A. mutabilis (Gravenhorst, 1831)	AMNH5482.3	Spain	Canary I. – Las Palmas de Gran Canaria	N 28 08 53.02	W 15 25 57.01	1
	A. mutabilis (Gravenhorst, 1831)	AMNH5520.1	Spain	Canary I. – Tenerife	N 28 33 50.35	W 16 19 55.33	1
Aiptasia mutabilis (Gravenhorst, 1831)	*A. mutabilis* (Gravenhorst, 1831)	AMNH5517.5	Greece	Crete – Agios Nikolaos	N 35 11 40.69	E 25 43 01.87	5
	A. mutabilis (Gravenhorst, 1831)	AMNH5503.1	Spain	Girona – L'Estartit	N 42 02 52.05	E 03 13 07.95	10
Exaiptasia brasiliensis sp. nov.	–	MZUSP002483	Brazil	Espírito Santo – Praia dos Padres	S 19 55 23.53	W 40 06 23.98	1
	–	MZUSP002493	Brazil	Espírito Santo – Guarapari	S 20 40 36.64	W 40 29 59.41	1
	–	MZUSP002512	Brazil	São Paulo – São Sebastião	S 23 49 40.36	W 45 25 19.78	1
	–	AMNH5504.1	Panama	Bocas del Toro	N 09 20 50.34	W 82 15 18.67	1
Exaiptasia pallida (Agassiz in Verrill, 1864)	*A. californica* (Carlgren, 1952)	N/A	Mexico	Baja California Sur – Bahía Concepción	N 24 15 46.46	W 110 36 52.12	1
	A. californica (Carlgren, 1952)	N/A	Mexico	Baja California Sur – Bahía Concepción	N 24 15 46.46	W 110 36 52.12	1
	A. californica (Carlgren, 1952)	N/A	Mexico	Baja California Sur – Bahía Concepción	N 24 16 17.03	W 110 20 0.22	1
	A. californica (Carlgren, 1952)	AMNH5541.2	Panama	Venado	N 07 25 50.46	W 80 11 36.24	1
	A. diaphana (Rapp, 1829)	N/A	Israel	Cesarea	N 32 29 37.62	E 34 53 22.57	1
	A. diaphana (Rapp, 1829)		Portugal	Madeira – Machico	N 32 42 52.84	W 16 45 47.85	1
	A. diaphana (Rapp, 1829)	AMNH5528.2	Spain	Canary I. – Las Palmas de Gran Canaria	N 28 08 53.02	W 15 25 57.01	1
	A. insignis (Carlgren, 1941)	N/A	St. Helena	Jamestown	S 15 55 33.00	W 05 43 24.00	1
	A. pallida (Agassiz in Verrill, 1864)	MZUSP002516	Brazil	Ceara – Fortaleza	S 03 42 54.25	W 38 32 27.26	1
	A. pallida (Agassiz in Verrill, 1864)	MZUSP002506	Brazil	São Paulo – São Sebastião	S 23 49 26.34	W45 25 06.28	1
	A. pallida (Agassiz in Verrill, 1864)	MZUSP002495	Brazil	Florianopolis	S 27 29 16.62	W 48 21 38.36	1
	A. pallida (Agassiz in Verrill, 1864)	AMNH5465.10	Bermuda	Ferry reach	N 32 22 12	W 64 41 47	1
	A. pallida (Agassiz in Verrill, 1864)	AMNH5466.1	Bermuda	Walsingham Ponds	N 32 21 01	W 64 42 36	1
	A. pallida (Agassiz in Verrill, 1864)	AMNH5452.1	Mexico	Puerto Morelos	N 20 50 18.57	W 86 53 02.86	1
	A. pallida (Agassiz in Verrill, 1864)	AMNH5507.2	Panama	Carenera Island	N 09 20 50.34	W 82 15 18.67	1
	A. pallida (Agassiz in Verrill, 1864)	AMNH5509.3	Panama	Bocas del Toro	N 09 20 50.34	W 82 15 18.67	1
	A. pallida (Agassiz in Verrill, 1864)	AMNH5450.5	USA	Florida Keys	N 25 03 36.78	W 80 25 22.92	2
	A. pulchella (Carlgren, 1943)	N/A	Australia	Townsville	S 19 14 46.89	E 146 49 44.02	1
	A. pulchella (Carlgren, 1943)	N/A	Israel	Eilat	N 29 30 45.21	E 34 55 38.44	1
	A. pulchella (Carlgren, 1943)	AMNH5464.3	Japan	Okinawa – Sesoko Island	N 26 38 10.70	E 127 51 55.03	1
	A. pulchella (Carlgren, 1943)	AMNH5464.4	Japan	Okinawa – Sesoko Island	N 26 38 10.70	E 127 51 55.03	1
	A. pulchella (Carlgren, 1943)	N/A	Taiwan	National Marine Aquarium – Pingtung	N 22 03 00.08	E 120 41 42.88	1
	A. pulchella (Carlgren, 1943)	AMNH5469.4	USA	Hawaii – Hilo	N 19 43 56.37	W 155 03 06.06	1
	A. pulchella (Carlgren, 1943)	AMNH5477.2	USA	Hawaii – Oahu	N 21 16 40.32	W 157 50 01.04	
	A. pulchella (Carlgren, 1943)	AMNH5477.5	USA	Hawaii – Oahu	N 21 24 43.67	W 157 46 36.61	1
	A. pulchella (Carlgren, 1943)	AMNH5479.4	USA	Hawaii – Kauai	N 22 03 38.67	W 159 19 06.61	1
Aiptasiogeton hyalinus (Delle Chiaje, 1822)	*Aiptasiogeton pellucidus* (Hollard, 1848)	AMNH6120.1	England	South Cornwall	N 50 20 31.11	W 04 10 41.12	1
	Aiptasiogeton pellucidus (Hollard, 1848)	AMNH6120.12	England	South Cornwall	N 50 20 31.11	W 04 10 41.12	1
	Aiptasiogeton pellucidus (Hollard, 1848)	AMNH5519.8	Spain	Huelva – El Portil	N 37 12 27.54	W 07 02 52.78	1
Bartholomea	*Bartholomea annulata* (Le Sueur, 1817)	N/A	Bermuda	Harrington Sound	N 32 19 38.77	W 64 42 53.89	1

(*continued on next page*)

Table 1 (*continued*)

Species in this study	Previous concept	Museum voucher	Locality	Locality 2	Longitude	Latitude	Depth (m)
annulata (Le Sueur, 1817)							
	Bartholomea annulata (Le Sueur, 1817)	N/A	Bermuda	Harrington Sound	N 32 19 38.77	W 64 42 53.89	2
	Bartholomea annulata (Le Sueur, 1817)	N/A	Bermuda	Harrington Sound	N 32 19 38.77	W 64 42 53.89	2
	Bartholomea annulata (Le Sueur, 1817)	N/A	Honduras	Cayos Cochinos	N 15 57 58.48	W 86 28 86.27	2
	Bartholomea annulata (Le Sueur, 1817)	N/A	Honduras	Cayos Cochinos	N 15 57 58.48	W 86 28 86.27	1
	Bartholomea annulata (Le Sueur, 1817)	AMNH	Mexico	Isla Contoy	N 21 30 09.20	W 86 48 07.06	1
	Bartholomea annulata (Le Sueur, 1817)	N/A	USA	Florida Keys	N 25 03 36.78	W 80 25 22.92	1
	Bartholomea annulata (Le Sueur, 1817)	N/A	USA	Florida Keys	N 25 03 36.78	W 80 25 22.92	1
	Bartholomea annulata (Le Sueur, 1817)	N/A	USA	Florida Keys	N 25 03 36.78	W 80 25 22.92	1
	Bartholomea annulata (Le Sueur, 1817)	N/A	US Virgin I.	St. Thomas	N 18 20 02.78	W 64 56 28.51	1
	Bartholomea annulata (Le Sueur, 1817)	N/A	US Virgin I.	St. Thomas	N 18 20 02.78	W 64 56 28.51	1
	Bartholomea annulata (Le Sueur, 1817)	N/A	US Virgin I.	St. Thomas	N 18 20 02.78	W 64 56 28.51	1
	Bartholomea annulata (Le Sueur, 1817)	N/A	US Virgin I.	St. Thomas	N/A	N/A	1
	Bartholomea annulata (Le Sueur, 1817)	AMNH	Panama	Bocas del Toro	N 09 20 50.34	W 82 15 18.67	1
Bellactis ilkalyseae	*Bellactis Ilkalyseae* (Dube, 1983)	MZUSP002484	Brazil	Espírito Santo – Praia dos Padres	S 19 55 23.53	W 40 06 23.98	1
	Bellactis Ilkalyseae (Dube, 1983)	MZUSP002510	Brazil	Espírito Santo – Praia dos Padres	S 19 55 23.53	W 40 06 23.98	1
	Bellactis Ilkalyseae (Dube, 1983)	MZUSP002511	Brazil	Espírito Santo – Praia dos Padres	S 19 55 23.53	W 40 06 23.98	1
Laviactis lucida (Duchassaing de Fombressin and Michelotti, 1860)	*Ragactis lucida* (Duchassaing de Fombressin and Michelotti, 1860)	N/A	Honduras	Cayos Cochinos	N 15 57 58.48	W 86 28 86.27	1
		AMNH5380	Mexico	Isla Contoy	N 21 30 09.20	W 86 48 07.06	1
	Ragactis lucida (Duchassaing de Fombressin and Michelotti, 1860)	AMNH5505.1	Panama	Carenera Island	N 09 20 50.34	W 82 15 18.67	1
	Ragactis lucida (Duchassaing de Fombressin and Michelotti, 1860)	AMNH5505.2	Panama	Carenera Island	N 09 20 50.34	W 82 15 18.67	1
	Ragactis lucida (Duchassaing de Fombressin and Michelotti, 1860)	AMNH5504.4	Panama	Carenera Island	N 09 20 50.34	W 82 15 18.67	1
	Ragactis lucida (Duchassaing de Fombressin and Michelotti, 1860)	AMNH5512.3	Panama	Carenera Island	N 09 20 50.34	W 82 15 18.67	1

monophyletic clade (Figs. 2 and 3, Supplementary Fig. 1) within the superfamily Metridioidea and within the Acuticulata clade (see Rodríguez et al., 2014). However, both methods of phylogenetic inference differed slightly in the sister relationship recovered for Aiptasiidae. In the ML analysis, Aiptasiidae was recovered as the sister group to two genera of the family Aliciidae (*Alicia* and *Lebrunia*) with low bootstrap support values (<50%). MP recovered Aiptasiidae as sister to the genus *Alicia* (bootstrap support values <50%) but did not recover members of *Lebrunia* as sister to *Alicia*; *Lebrunia* (the species with the longest branch in ML analysis, Fig. 2) was instead recovered as sister to a clade composed by (Gonactiniidae + Boloceroididae) + *Sagartia ornata* (Supplementary Fig. 1), a clade characterized by long branches and hypothesized to be highly derived within sea anemones (Rodríguez et al., 2014).

Our analyses (both ML and MP) recovered the genus *Neoaiptasia* (previously placed within Aiptasiidae) outside of Aiptasiidae, as shown in previous phylogenetic analyses (Rodríguez et al., 2012, 2014). In the ML analysis, *Neoaiptasia* was recovered as the sister to *Anthothoe* and *Actinothoe*, two genera belonging to the family Sagartiidae (Fig. 2). In the MP topology, *Neoaiptasia* had an unresolved position along with other taxa within the superfamily Metriodioidea (Supplementary Fig. 1).

Table 2
Details of the newly designed 18S primer specific to Actiniaria (Cnidaria: Anthozoa).

Primer	Length (bp)	Sequence (5′–3′)	Tm (°C)
18S_NA (External)	26	TAAGCACTTGT CTGTGAAACTGCGA	58.8
18S_NL	21	AACAGCCCGGTCAGTAACACG	58.5
18S_NC	27	AATAACAATACAGGGCTTTTCTAAGTC	53.5
18S_NY	21	GCCTTCCTGACTTTGGTTGAA	55.5
18S_NO	25	AGTGTTATTGGATGACCTCTTTGGC	57.2
18S_NB (External)	20	AGGAGTCCTCACTAAACCAT	58.2

Tm, melting temperature.

3.2. Phylogenetic relationships within Aiptasiidae

All aiptasiid genera, except *Aiptasia* (currently divided into *Aiptasia* and *Exaiptasia*), were recovered as monophyletic with strong support values from both ML and MP analyses (Fig. 3, Supplementary Fig. 1). The non-monophyly of *Aiptasia* was hinted in previous studies (Daly et al., 2008; Rodríguez et al., 2012, 2014); and our results further support this hypothesis, by including representatives from all described genera within the family *sensu* Grajales and Rodríguez (2014) and also *Neoaiptasia*.

Aiptasiogeton hyalinus was recovered as the sister group of all remaining members of Aiptasiidae; *Aiptasiogeton* is the only genus within the family lacking endosymbiontic algae (Fig. 3). The remaining members of Aiptasiidae comprised two well-supported clades, one corresponding to the genus *Exaiptasia*, and a second unresolved clade composed by all remaining aiptasiid genera and species (i.e. *Bellactis*, *Bartholomea*, and *Laviactis* + *Aiptasia*). *Exaiptasia* encompassed most of the species previously recognized as *Aiptasia* (see Table 1), except for the type species of the genus – *A. couchii* – and *A. mutabilis*, both of which are distributed in the Northwestern Atlantic Ocean and the Mediterranean Sea. The clonal strains commonly used as a model system for studies of dinoflagellate symbiosis, coral bleaching, and reproduction were recovered as belonging to *Exaiptasia pallida* (see Supplementary Table 2 for information of locality and previous taxonomic assignment). The subdivision between *Exaiptasia* and

Table 3
Matrix of morphological character states per species within the family Aiptasiidae.

Character/species	*Aiptasiogeton hyalinus*	*Aiptasia couchii*	*Aiptasia mutabilis*	*Bartholomea annulata*	*Bellactis ilkalyseae*	*Exaiptasia pallida*	*Exaiptasia brasiliensis* sp. nov.	*Laviactis lucida*
(A) No. of cycles of mesenteries proximally and distally	0	1	0	0	0	1	1	0
(B) No. of cycles of mesenteries proximally	0	0	0	0	1	0	0	0
(C) No. of cycles of mesenteries distally	0	1	0	0	2	1	1	0
(D) No. of tentacles	0	0	1	1	2	0	0	1
(E) Tentacle shape	0	0	0	1	0	0	0	1
(F) Distribution of fertility	0	0	0	0	1	1	1	0
(G) Endosymbionts	0	1	1	1	1	1	1	1
(H) Cinclides	0	1	1	1	0	1	1	1
(I) Pedal laceration	0	1	1	1	1	0	0	1
(J) Basilar musculature	0	0	0	1	0	0	0	1
(K) Scapus/Scapulus	0	0	1	1	0	0	0	1
(L) Length of microbasic *b*-mastigophores in column	0	1	1	1	1	0	0	1

Character states: (A) No. of cycles of mesenteries proximally and distally: Different, 0; same, 1; (B) no. of cycles of mesenteries proximally: Four, 0; ⩾four, 1; (C) no. of cycles of mesenteries distally: ⩾Four, 0; four, 1; three, 2; (D) no. of tentacles: to 96, 0; to 192, 1; >192, 2; (E) tentacle shape: Smooth, 0; not smooth, 1; (F) distribution of fertility: all cycles fertile, 0; 1st and 2nd cycle fertile, 1; (G) endosymbionts: Present, 0; absent, 1; (H) cinclides: scattered, 0; in rows, 1; (I) pedal laceration: Present, 0; absent, 1; (J) basilar musculature: Lobed, 0; flamed-like, 1; (K) scapus/scapulus: not differentiated, 0; differentiated, 1; (L) length of microbasic *b*-mastigophores in column: 11–15 μm, 0; 16–25 μm, 1.

the rest of the aiptasiid genera (except *Aiptasiogeton*) is further supported by two synapomorphies for the clade composed by *Aiptasia*, *Bartholomea*, *Bellactis*, and *Laviactis*. These four genera share an increase in the size of the microbasic *b*-mastigophores in the column (Figs. 3 and 4) and the lack of pedal laceration (present in *Aiptasiogeton* and *Exaiptasia*) as optimized in the ancestral state reconstruction (Fig. 4).

Despite the clear morphological differences (in size, number of mesenteries and tentacles, and endosymbiont type), as well as fixed differences in the mitochondrial gene 12S (one transition from C to T on position 616 tested in eight individuals belonging to *Aiptasia couchii* and two individuals from *A. mutabilis*—see alignment remarks below), the current markers did not provide enough resolution to further differentiate the two species within *Aiptasia*, *A. couchii* and *A. mutabilis*.

3.3. Morphological evolution within Aiptasiidae

The ancestral reconstruction analysis of morphological characters within Aiptasiidae showed convergence in characters such as the number of mesenteries proximally and distally in *Aiptasia couchii* and *Exaiptasia* (Fig. 4a and c), and the shape of the tentacles in *Bartholomea* and *Laviactis* (Fig. 4e); other characters, such as the presence of endosymbiotic algae, the loss of asexual reproduction by pedal laceration and the length of the microbasic *b*-mastigophores in the column have evolved once within the family (Fig. 4g and i). Results of this analysis are shown in Fig. 4.

3.4. A new cryptic species of Exaiptasia

Our results showed that *Exaiptasia* contains two distinctive and well-supported clades (Fig. 3, Supplementary Fig. 1). One clade included specimens corresponding to a cosmopolitan geographical distribution whereas the other clade corresponded to specimens restricted to the Southwestern Atlantic Ocean and Southwestern Caribbean Sea (coasts of Brazil and Panama, respectively). Although Grajales and Rodríguez (2014) could not find morphological or cnidae differences among specimens within *Exaiptasia*, we find fixed differences in the DNA between members in these two clades. Additionally, we find differences in geographical distribution and endosymbiont identity between these two clades. Thus, under the general lineage concept of species (de Queiroz, 1998), we consider members of the two clades of *Exaiptasia* as two distinct cryptic species that can live in sympatry but may be diagnosed based on independent lines of evidence. To address this situation we describe a new species for members of the clade restricted to the Southwestern Atlantic Ocean and the Southwestern Caribbean Sea (Fig. 3); we consider the cosmopolitan representatives in the *Exaiptasia* clade belonging to *E. pallida*.

3.4.1. *Exaiptasia brasiliensis* sp. nov.

3.4.1.1. Diagnosis. Aiptasiidae with wide, regularly shaped pedal disc to 10 mm in diameter. Column elongated, smooth, to 60 mm height and to 30 mm diameter in preserved specimens; with cinclides in 2–3 longitudinal rows in mid-column, ~12 cinclides per row; column not distinctly divisible into scapus and capitulum. Living specimens with column translucent proximally and greyish-brownish with scattered spots distally. Tentacles long, simple, to 96, always smooth, without projections; oral disc and tentacles greyish, the latter with scattered white transversal stripes. Whitish mouth and actinopharynx with yellowish circle around. Preserved specimens uniform tan in color. Mesogleal marginal sphincter muscle strong but short, diffuse, slightly reticulate. Strong longitudinal ectodermal muscles in distal column. Same number of mesenteries distally and proximally. Mesenteries hexamerously arranged in four cycles; only first cycle perfect, first two cycles fertile; gonochoric. Asexual reproduction by pedal laceration. Retractor muscles restricted, strong; parietobasilar muscles differentiated, weak. Acontia well developed. Symbiotic with *Symbiodinium* spp. Cnidom: spirocysts, basitrichs, microbasic *b*-mastigophores and *p*-amastigophores.

3.4.1.2. Differential diagnosis. *Exaiptasia* with three non-synonymous substitutions (W-M on position 117, P-A on position 118, and E-K on position 156) and one synonymous substitution (at position 126) in the COIII gene region of the mitochondrial rDNA; one transition (T to C, position 402) in the 16S alignment, and one transition and one transversion (T to C on position 905, and T to A on position 912, respectively) in the nuclear 18S rDNA. Geographical distribution restricted to the Southwestern Caribbean Sea (Panama) and the Southwestern Atlantic Ocean (Brazil). It associates with at least two different *Symbiodinium* species from clade A (but not with *S. minutum* subtype B1).

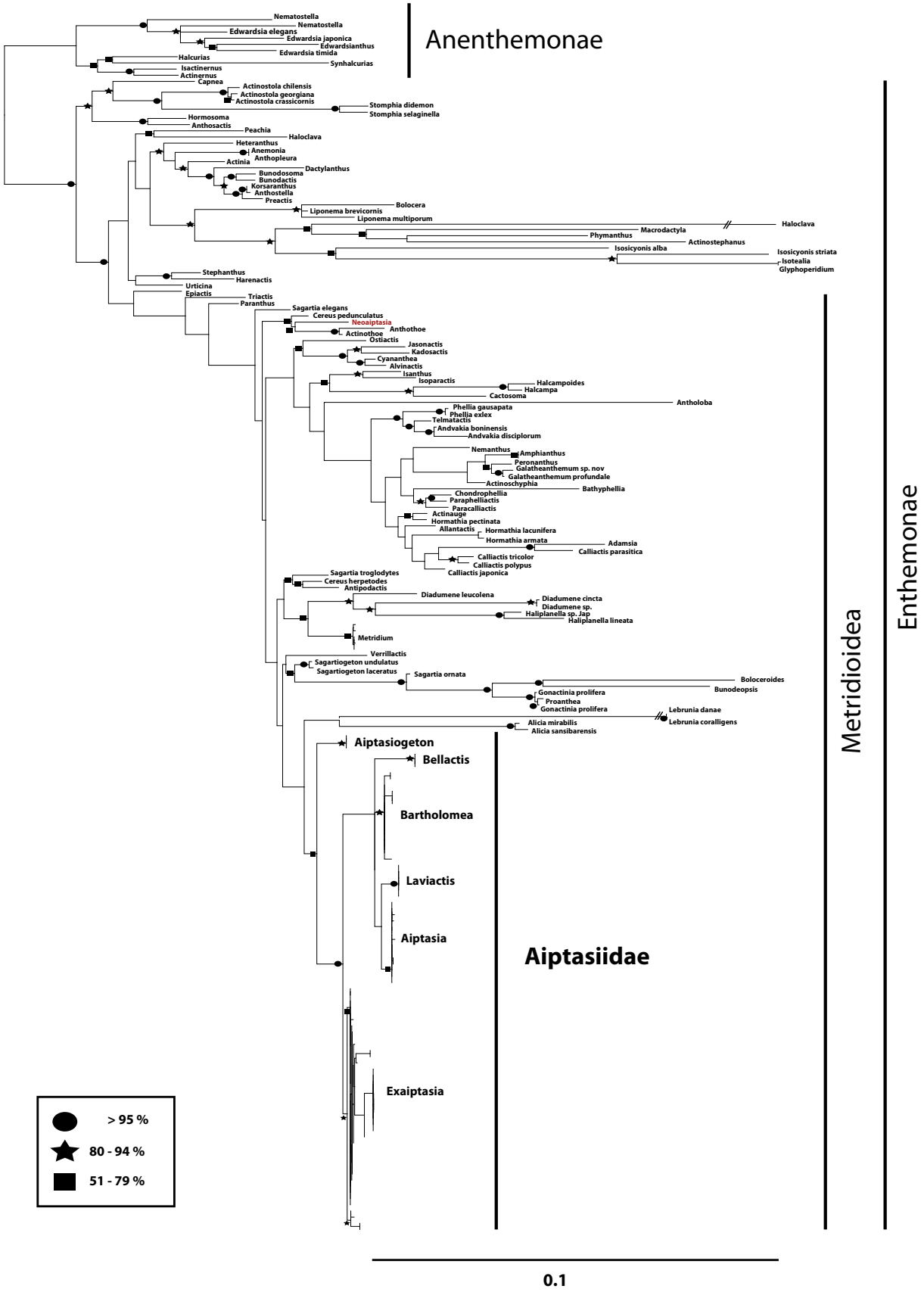

Fig. 2. Phylogenetic position of Aiptasiidae. Maximum Likelihood (ML) topology showing the hypothesized phylogenetic position of the family Aiptasiidae within Actiniaria. ML bootstrap support values (expressed as a percent) >50 are shown below the nodes.

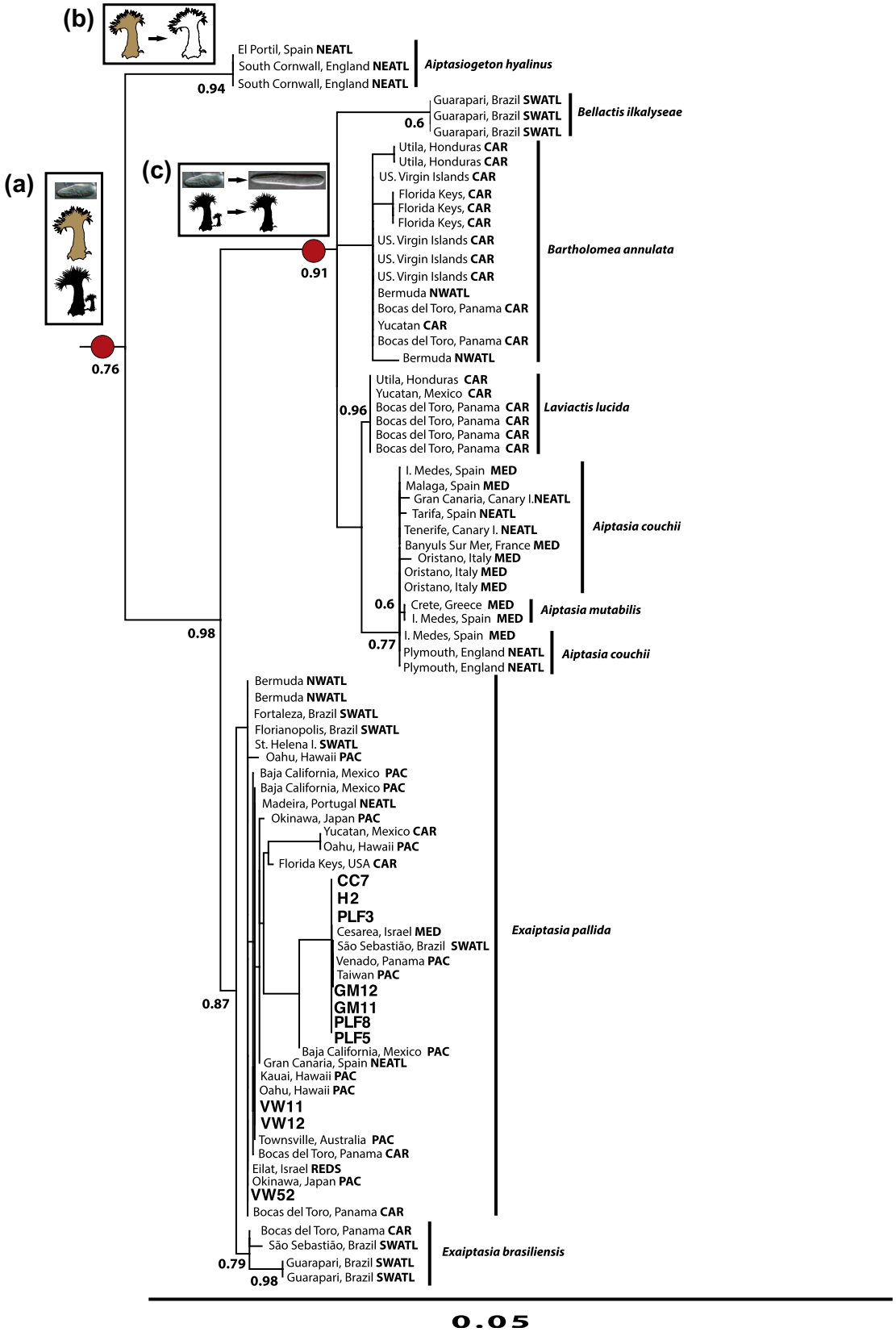

Fig. 3. Detailed phylogenetic relationships within Aiptasiidae extracted from the ML topology showing the phylogenetic relationships of genera and species within Aiptasiidae. Bootstrap support values (expressed as a percent) >50 are shown on top of each branch. Detailed locality is provided for species and genera with more than one sample, followed by their general location among the different Ocean basins. CAR = Caribbean Sea, MED = Mediterranean Sea, PAC = Pacific Ocean, RED = Red Sea, SWATL = South Western Atlantic Ocean, NEATL = North Eastern Atlantic Ocean, NWATL = North Western Atlantic Ocean. Boxes in the left represent character states inferred by ancestral state reconstruction. (a) Reduced size of microbasic *b*-mastigophores in the column, presence of endosymbiotic algae and pedal laceration, (b) loss of endosymbiotic algae in the genus *Aiptasiogeton*, (c) increase in the size of the microbasic *b*-mastigophores in the column and loss of pedal laceration in the clade composed by the genera *Bellactis*, *Bartholomea*, *Laviactis* and *Aiptasia*.

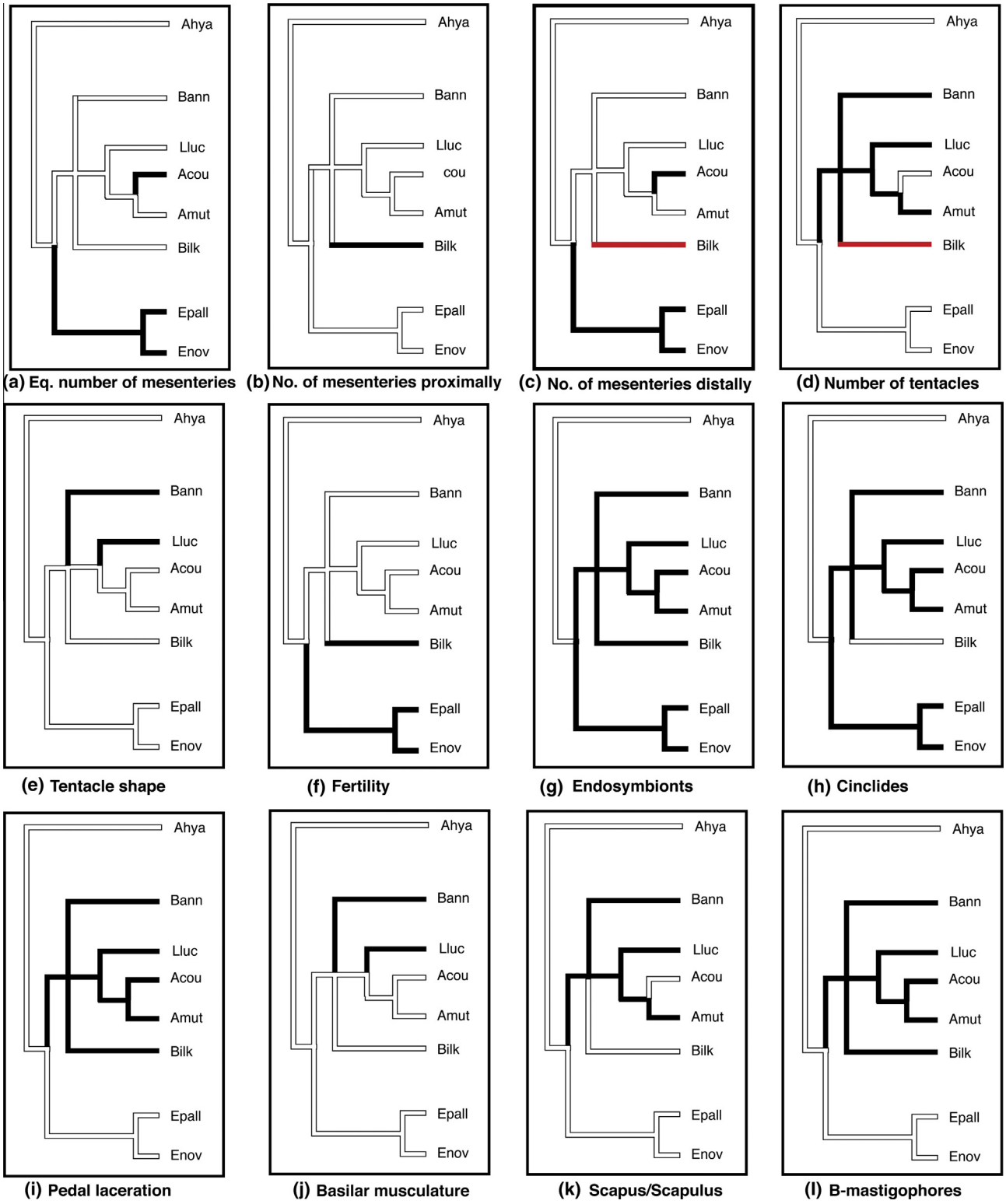

Fig. 4. Ancestral character reconstruction of morphological traits within Aiptasiidae. Representation of parsimony character state reconstructions of 12 morphological characters of the species within Aiptasiidae. Colors in the branches indicate different states (ancestral *vs.* derived); white indicates the state in *Aiptasiogeton hyalinus*, sister group to all other members of Aiptasiidae, black and red indicate derived, in the case of two or three states respectively. Ahya = *Aiptasiogeton hyalinus*, Bilk = *Bellactis ilkalyseae*, Lluc = *Laviactis lucida*, Acou = *Aiptasia couhcii*, Amut = *Aiptasia mutabilis*, Bann = *Bartholomea annulata*, Epall = *Exaiptasia pallida*, Ebra = *Exaiptasia brasiliensis* sp. nov. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Type material. Holotype: MZUSP-002505; São Sebastião, São Paulo, Brazil (23°49′40.36″S, 45°25′19.78″W); 29 November 2011; 1 m depth. Paratype: AMNH-5504.1 one specimen; Bocas del Toro, Panama (09°20′50.34″N, 82°15′18.78″W); 24 October 2012; 1 m depth.

Remarks. We provide a brief anatomical description of *Exaiptasia brasiliensis* sp. nov. to provide a complete understanding of the species anatomy within this study. Because *E. brasiliensis* sp. nov. is a morphologically cryptic species, its morphological description corresponds to that of *E. pallida* [see Grajales and Rodríguez (2014) for

more details]. The position of the mentioned diagnostic substitutions on the COIII gene (analyzed and found in two specimens of *E. brasiliensis* sp. nov., the holotype and paratype) are based on the COIII gene sequence from the species *Metridium senile* (GenBank accession No. NC_000933; 789 base pairs (bp) in length); the analyzed dataset begins at position 116 and ends at position 242. The reference for the diagnostic transition in the 16S gene (analyzed and found in three specimens of *E. brasiliensis* sp. nov.) is also *M. senile* (NC_000933; 2189 bp in length); 1352 bp into 16S (alignment length: 663 bp, shortest sequence: 169 bp, longest sequence: 651 bp). The reference for the transition and transversion in 18S gene (analyzed and found in three specimens of *E. brasiliensis* sp. nov.) is the species *Nematostella vectensis* (AF254382; 1723 bp length); alignment begins on first base of 18S and extends 20 bp into ITS1 (alignment length: 2164 bp, shortest seq: 697 bp, longest seq: 1901 bp). The reference for the transition found between *Aiptasia mutabilis* and *A. couchii* (two and eight specimens analyzed, respectively) is also *M. senile* 12S (NC_000933; 1082 bp); alignment begins 219 bp into 12S (alignment length: 982 bp, shortest sequence: 619 bp, longest sequence: 859 bp).

Table 4 summarizes divergences estimates (K2P) among sequences within all aiptasiid species studied. The highest range of intraspecific divergence was presented in the gene 12S, with percentages of at least one order of magnitude higher than the rest of the genes on the species *Bartholomea annulata* (0.052) and *Aiptasigeton hyalinus* (0.029). *Exaiptasia brasiliensis* sp. nov. and *Aiptasia couchii* showed no intraspecific divergence in any of the analyzed genes. COIII was the only gene that showed no divergence within any of the studied species.

4. Discussion

4.1. Phylogenetic position and membership of the family Aiptasiidae

The phylogenetic position of Aiptasiidae within Metridioidea has been shown to vary depending on the analyzed dataset (Daly et al., 2008; Rodríguez and Daly, 2010; Rodríguez et al., 2012, 2014). Our results concur with those of the most comprehensive molecular study to date (Rodríguez et al., 2014) recovering a monophyletic Aiptasiidae. The fact that *Neoaiptasia* was not recovered within Aiptasiidae is not surprising since previous works had showed similar results (Rodríguez et al., 2012, 2014), the original assignment of both species of this genus to the family Aiptasiidae was suspect and no morphological characters had supported the placement of the genus within this family (see Goodwill et al., 2009; Grajales and Rodríguez, 2014).

Although ML and MP recovered slightly different sister relationships for Aiptasiidae, both inferences suggest a close relationship with members of Aliciidae and a clade including highly derived acontiate anemones (members of families Boloceroididae and Gonactiniidae). Rodríguez et al. (2014) also recovered a close relationship between *Alicia*, and Boloceroididae and Gonactiniidae; however, these authors recovered members of Aiptasiidae as sister to the sagartiid genus *Verrillactis*, and these as sister to the rest of the members of Acuticulata. As discussed by Rodríguez et al. (2014), we find that the currently used molecular markers provide low nodal support values for some clades within the superfamily Metridioidea (e.g. Acuticulata); this together with the fact that the most species-rich acontiate family, Sagartiidae, and genera within (e.g. *Sagartia*, *Cereus*) are polyphyletic (see Rodríguez et al., 2012, 2014), renders relationships among most acontiate families far from resolved.

The present study represents a significant increase in taxon sampling within Aiptasiidae and Aliciidae, which is expected to positively impact resolution of phylogenetic trees and reduce errors (e.g. Zwickl and Hillis, 2002; Dunn et al., 2008; Rodríguez et al., 2012, 2014). The failure to recover the family Aliciidae as a monophyletic clade on the MP analysis (Supplementary Fig. 1) might be due to sampling (only sequences of 16S were available for *Lebrunia*) and long branch attraction artifacts (Bergsten, 2005). Long branches were observed for members of *Lebrunia*, as well as for members in the clade composed by *Sagartia ornata* + (Boloceroididae + Gonactiniidae). Members of Aliciidae are unique among sea anemones in sharing one type of nematocyst (*p*-rhabdoid C *sensu* Schmidt (1969) or macrobasic *p*-amastigophore *sensu* Mariscal (1974)). However, because of the position at the base of Metridioidea (in ML, MP, and in Rodríguez et al., 2014) of *Triactis* (the other representative of Aliciidae studied), the monophyly of Aliciidae needs to be addressed in further studies. Contra Carlgren (1949), a close relationship between Aiptasiidae and Aliciidae was suggested by Schmidt (1974) based on the presence of ectodermal longitudinal muscles on the column and in the cnidae; similarly, Schmidt (1974) considered Aliciidae, Boloceroididae, and Gonactiniidae closely related to each other. Only four families within Actiniaria present ectodermal longitudinal muscles in the column: Aiptasiidae, Aliciidae, Boloceroididae and Gonactiniidae (Carlgren, 1949). Our analysis supports, for the first time after Schmidt (1974), a close relationship among these four families, offering a different evolutionary scenario for some of the morphological characters analyzed by Rodríguez et al. (2014). Based in the present study, the ectodermal longitudinal muscles in the column evolved three times within Actiniaria (once in *Triactis* and twice within Acontiaria), as opposed to four separate events as showed in Rodríguez et al. (2014). The morphological reassessment of ectodermal longitudinal muscles in the column of an array of sagartiid species (in particular of *Sagartia ornata*) is necessary and might change our interpretation of the evolution of this character within Actiniaria.

4.2. Morphological evolution within Aiptasiidae

The family Aiptasiidae, as well as most families and genera within the order Actiniaria (see Carlgren, 1949), is defined by a unique combination of morphological characters rather than single synapomorphies. As in other cases (e.g. Daly and Gusmão, 2007; Rodríguez et al., 2009; Gusmão and Daly, 2010), the morphological features defining Aiptasiidae are not unique for the group; it is rather the combination of them that makes it possible to circumscribe taxonomic membership. Our results allowed evaluating if several morphological characters commonly used to define taxonomic groups within Actiniaria show any phylogenetic signal within Aiptasiidae or are the product of homoplasy. The reduced number of morphologically variable units is not uncommon for character-depauperate taxa such as sea anemones (e.g. Daly et al., 2002; Daly and Gusmão, 2007; Rodríguez et al., 2008).

Table 4
Intraspecific divergences estimates (K2P, expressed in percentage) of species included in this study based on sequence comparisons of the five molecular markers used.

	12S	16S	COIII	18S	28S
Aiptasia mutabilis	0–0.002	0.0003	0	–	–
Aiptasia couchii	0	0	0	0	–
Aiptasiogeton hyalinus	0–0.029	0	–	0–0.001	–
Bartholomea annulata	0–0.052	0–0.003	0	0	–
Bellactis ilkalyseae	0	0	–	0–0.002	–
Exaiptasia brasiliensis	0	0	0	0	–
Exaiptasia pallida	0.00–0.03	0	0	0–0.025	0–0.009
Laviactis lucida	0	0	–	0	–

12S: 913 base pairs (bp) compared; 16S: 663 bp compared; COIII: 771 bp compared; 18S: 2164 bp compared; 28S: 700 bp compared. (–) not available.

The comparison between the number of mesenteries proximally and distally, and the number of distal mesenteries and tentacles (Fig. 4a, c, and d, respectively) show the highest degree of homoplasy, with the most instances of independent origins within the family. These three characters are correlated (the number of tentacles in sea anemones is related to the number of mesenteries – see Stephenson, 1935). An equal number of mesenteries proximally and distally, the number of mesenteries distally, and number of tentacles (Fig. 4a, c, and d, respectively) are convergent characters between members of *Exaiptasia* and *Aiptasia couchii* (but not in *A. mutabilis*). An equal number of mesenteries proximally and distally was used to classify members of *Exaiptasia* within *Aiptasia* (Carlgren, 1949); this character was thought to the same for all species of *Aiptasia* until Schmidt (1972) noticed that *A. mutabilis* had more mesenteries distally than proximally. Based on this observation, England (1992) suggested that the number of aiptasiid genera might increase based on the relationship between the number of mesenteries proximally and distally. On the contrary, pedal laceration and differences in the size of microbasic *b*-mastigophores show no homoplasy (Fig. 4i and l); these two characters are not likely to be correlated. Traditionally, the presence or absence of a determined type of nematocyst is considered a strong character to define intermediate taxonomic ranks such as families (e.g. families within acontiate actiniarians, see Carlgren, 1949; Rodríguez et al., 2009, 2012). At the same time, differences in the size ranges of nematocyst capsules have been traditionally used as an indication of the presence of separate species (e.g. Allcock et al., 1998). In our particular case, a clear difference in the size of nematocysts (microbasic *b*-mastigophores in the column) corresponded to two well supported clades, and could be used as a diagnostic character above species level. However, Grajales and Rodríguez (2014) showed that this is rather an exception; all other nematocyst types did not show any indication of size differences across the entire family. Such results highlight the importance of relying on more than one character type in order to considered taxonomic delimitations in other actiniarian taxa (i.e. merging or splitting genera and families). Characters such as shape of the tentacles and shape of the basilar musculature (Fig. 4e and j, respectively) seem to be the product of convergence in aiptasiids as opposed to the general assumption of taxonomists of these features being the product of common ancestry in *Bartholomea* and *Laviactis* (e.g. Duerden, 1898; Dunn, 1981; England, 1992). However, the failure of the used markers to resolve the relationships among *Bellactis*, *Bartholomea* and *Laviactis* + *Aiptasia* render this question still open.

4.3. Species delimitation – *Exaiptasia*

Exapitasia pallida is considered a weedy, invasive species (Calado and Narciso, 2005), a circumstance that can be enhanced by the possibility of rapid spread via pedal laceration. Thornhill et al. (2013) suggested the presence of a single widespread species based on several population level (SCAR) genetic markers, as well as the genetic homogeneity of their endosymbionts based on microsatellite data; this finding is consistent with recent a introduction (Mito and Uesugi, 2004). Thornhill et al. (2013) discussed two possible explanations for such unusual pattern of genetic homogeneity, vectored introductions of specimens, via the aquarium trade, or ballast/fouling communities.

Our study confirms the presence of two genetically different species within *Exaiptasia* in Panama but also in Brazil. Both species were found to live in sympatry in a single locality (Fig. 3 – Caribbean Sea, Panama) and cannot be distinguished morphologically. *Exaiptasia pallida* have been suggested to be invasive (Mito and Uesugi, 2004; Thornhill et al., 2013), thus the presence of individual specimens from both species within a single locality is not completely unexpected, however alternatative hypothesis such as range expansion cannot be discarded.

Nevertheless, *Exaiptasia pallida* and *E. brasiliensis* sp. nov. differ in the species of endosymbionts that they associate with (Grajales et al., in press). The association of *E. pallida* with *Symbiodinium minutum* (ITS type B1) – a prevalent endosymbiont in the Caribbean Sea – has been reported previously (LaJeunesse, 2002) as well as its association with the endemic type A4 from Florida (Thornhill et al., 2013). However, *Exaiptasia brasiliensis* sp. nov. has been found to be associated with a divergent type of *Symbiodinium* from clade A that, to our knowledge, has only been found distributed in Brazil and the Southern Caribbean and in this species. The correlation between genetic differences found in both the host and the endosymbiont – we have not found *E. brasiliensis* sp. nov. associated with the B1 endosymbiont, which is present in *E. pallida* (Grajales et al., in press) as well as in other anthozoan hosts (Thornhill et al., 2014) – further supports the hypothesis of a new cryptic species of *Exaiptasia*.

5. Conclusions

Our results confirm the monophyly and membership of the family Aiptasiidae *sensu* Grajales and Rodríguez (2014). Molecular and morphological data suggest a close relationship of Aiptasiidae with a clade of highly derived acontiate families (Aliciidae, Boloceroididae and Gonactiniidae). However, the sister group to Aiptasiidae remains ambiguous because deep relationships within the superfamily Metridioidea are not well supported.

The genus *Exaiptasia* encompasses two cryptic species; one with a cosmopolitan distribution, *E. pallida*, typically associated with *Symbidinium* type B1, and one species restricted to the Southwestern Caribbean and Southwestern Atlantic Ocean, *Exaiptasia brasiliensis* sp. nov., typically associated with at least two different *Symbiodinium* species from clade A (but not with *Symbiodinium* type B1). These two species live in sympatry. We confirm that clonal strains widely used as a model system for studies of dinoflagellate-symbiosis, coral beaching, and reproduction belong to the widespread species *E. pallida.*

Acknowledgments

We are very grateful to colleagues who assisted us with fieldwork, particularly to C. Arvanitidis (Hellenic Centre for Marine Research, MRC, Greece), A. Baird (James Cook University, Townsville, Australia), J. Brown (Environment and Natural Resources Directorate St. Helena), J. Campbell (Smithsonian Marine Station at Fort Pierce, USA), R. Collin (Smithsonian Tropical Research Institute, STRI, Panama), V. Cumbo (James Cook University, Townsville, Australia), R. Durrant, J. Frommlet (Centro de Estudios do Ambiente e do Mar, CESAM, Portugal), R. González (Universidad Nacional Autónoma de Mexico, UNAM), L. Gusmão (Universidade de São Paulo, Brazil), K. Hiscock (Marine Biological Association Plymouth, U.K.), P.J. López González (Universidad de Sevilla, Spain), P. Manent (Instituto Canario de Ciencias Marinas, Spain), O. Nir (Haifa University, Israel), J. Reimer (Ryukyus University, Okinawa, Japan), M. Takabayashi (U. Hawaii, Hilo), B. Titus (Ohio State University) and P. Wirzt. John Pringle (Stanford University School of Medicine) kindly provide samples from clone strains of *Exaiptasia pallida*. Comments from M. Brugler (AMNH), M. Daly (Ohio State University), D. Thornhill (Defenders of Wildlife) and two anonymous reviewers improved this manuscript. This work was partially supported by the Lerner-Gray Fund for Marine Research (AMNH), a NSF Doctoral Dissertation Improvement Grant (NSF DEB 1110754) to AG and ER, the Bermuda Institute of Ocean Sciences (BIOS, Bermuda), and OSU Museum of Biological Diversity Trautman Fund and Operation Wallacea.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.09.004.

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