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Phylogeny-based species delimitations and the evolution of host associations in symbiotic zoanthids (Anthozoa, Zoanthidea) of the wider Caribbean region

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Zoanthids are marine cnidarians with simple morphologies that challenge our ability to delineate species. Phylogenetic analyses of internal transcribed spacer (ITS) sequences are consistent with six morphologically described species from the wider Caribbean region, and reveal four additional species that were not previously recognized. Histological examinations of unidentified species reveal cryptic *Isozoanthus* and Edwardsiidae (Actiniaria) species. Observations of zoanthids *in situ* reveal geographic distributions that range from regional to trans-Atlantic. ITS and 16S data are consistent with hypotheses of paraphyly in some higher taxa of zoanthids; however, the clades of zoanthids recovered in both analyses can largely be defined by their host associations, thereby supporting phylogenetic conservatism in zoanthid–host association evolution. The single clear example of a zoanthid switching hosts was accompanied by a compensatory loss of endosymbiosis, which maintained the match in photosynthetic symbioses between zoanthids and sponge hosts. *Zoological Journal of the Linnean Society*, 2009, **156**, 223–238.

ADDITIONAL KEYWORDS: biogeography – cryptic species – Demospongia – Hydrozoa – ITS ribosomal – RNA – specificity – symbiosis – Zoanthidea.

INTRODUCTION

The accurate and repeatable identification of species is the prelude to the study of any biological system. Our ability to recognize species as independent units of evolution will directly affect our assessment of how biological systems are structured, function, and evolve; especially in symbiotic systems, where particular interspecific interactions are linked to the fitness of associated species.

Although there are at least 22 different species concepts (Mayden, 1997), the rise of molecular genetic techniques has led to phylogenetic species concepts gaining prominence in addressing the species problem (Knowlton, 2000). Genetic studies of species delimitations have led to the synonymization of taxa that had been separated because of minor morphological differences, and to the splitting of other taxa where apparently minor variation has been demonstrated to be taxonomically important (reviewed in Knowlton, 2000). Recent molecular phylogenetic analyses of zoanthids (phylum Cnidaria, class Anthozoa, order Zoanthidea, also referred to as Zoantharia or Zoanthiniaria) suggest similar conclusions, and provide data to support the synonymization of morphologically distinct species (e.g. Reimer *et al.*, 2004) or the separation of previously unrecognized species (e.g. Reimer *et al.*, 2006), as well as supporting (or invalidating) other taxa at higher levels of the Linnean hierarchy (Reimer *et al.*, 2007).

Because of their simple morphology and variable coloration, delineating zoanthid species is a challenge that may require genetic techniques. The examination of genetic species delimitations has begun in Zoanthidea, with the revision of the free-living (suborder Brachycnemina) zoanthids of Japan (e.g. Reimer *et al.*, 2006). Phylogenetic analyses of Zoanthidea (Sinniger *et al.*, 2005) have suggested that similar revisions may be necessary among symbiotic

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zoanthids (suborder Macrocnemina). Sinniger *et al.* (2005) have shown that there is a detectable genetic difference between light- and dark-coloured zoanthids that are symbiotic with Caribbean hydroids. The original description (Duerden, 1900), and a subsequent redescription (West, 1979), of this hydroid symbiont disagree regarding morphology and photoendosymbionts; however, they do agree about colour. Intraspecific colour variation is apparently common in both macrocnemic (e.g. Herberts, 1972) and brachycnemic (e.g. Duerden, 1898) zoanthids; therefore, knowing when colour variation is informative for distinguishing between species may be useful, particularly in symbiotic associations that rely on aposematism (West, 1976).

Conservatism of ecological niches between species through evolutionary time is predicted by theory (Peterson, Soberón & Sánchez-Cordero, 1999), and should include phylogenetic conservatism of specificity for hosts in symbiotic species (Mouillot *et al.*, 2006), because hosts represent the niches of symbionts (Price, 1990). Macrocnemic zoanthids associate with (among other invertebrates) gorgonians (e.g. Cutress & Pequegnat, 1960), antipatharians (e.g. Ocaña & Brito, 2003), hydroids (e.g. West, 1979), demosponges (e.g. Swain & Wulff, 2007), hexactinellid sponges (e.g. Beaulieu, 2001), and pagurid crabs (e.g. Ates, 2003); examples of similar associations are partitioned among different genera and families (e.g. Swain & Wulff, 2007). The extraordinary diversity of host associations among closely related zoanthids seems to be a direct challenge to phylogenetic conservatism in symbiosis evolution; however, initial analyses suggest that some higher taxa within Zoanthidea may not represent natural evolutionary clades. A phylogenetic analysis by Sinniger *et al.* (2005) found some genera, families, and suborders of zoanthids to be paraphyletic, but zoanthids with similar symbiotic associations to be closely related. An analysis of similarity among symbiotic zoanthid associations by Swain & Wulff (2007) concluded that some heterogeneric zoanthids had greater similarity than congeneric zoanthids, suggesting further paraphyly in Zoanthidea systematics.

The analyses presented here use the ribosomal RNA (rRNA) internal transcribed spacer (ITS) nuclear gene sequence from individual colonies, representing the morphologic and chromatic range of taxa observed throughout the wider Caribbean, to reconstruct a regional phylogeny for symbiotic zoanthids. Phylogenetic analyses of DNA from multiple specimens collected across most of the natural distribution of each taxon are used to expose the diversity of species in the region, to clarify inconsistencies about intraspecific morphologic and chromatic variability, and to elucidate the geographic distribution of taxa or morphotypes. Phylogenetic relationships inferred from ITS nuclear gene and 16S rRNA mitochondrial gene sequences are used to evaluate phylogenetic conservatism in the evolution of host associations in symbiotic zoanthids, and to assess the morphology-based taxonomy of Zoanthidea.

MATERIAL AND METHODS

Sampling strategy

DNA sequences of rRNA genes were analysed from symbiotic zoanthids collected throughout the wider Caribbean region. The zoanthid species sampled included: *Epizoanthus cutressi* West, 1979 (*E.c.*); '*Epizoanthus*' sp. nov. *sensu* Crocker & Reiswig, 1981; *Parazoanthus catenularis* (Duchassaing & Michelotti, 1860) (*P.c.*); *Parazoanthus parasiticus* (Duchassaing & Michelotti, 1860) (*P.pa.*); *Parazoanthus puertoricense* West, 1979 (*P.pu.*); *Parazoanthus swiftii* (Duchassaing & Michelotti, 1860) (*P.s.*); and *Parazoanthus tunicans* Duerden, 1900 (*P.t.*), where the abbreviations given in parentheses are used in the figures. Between five and fifteen whole polyps from each morphologically and chromatically distinct colony were collected from the following locations: near Búzios, Brazil (22°44′S, 41°51′W); Curaçao (12°03′N, 68°51′W); Flower Garden Banks National Marine Sanctuary, Galveston, TX, USA (28°09′N, 94°17′W); St. John, US Virgin Islands (18°18′N, 64°49′W); and at field sites described by Swain & Wulff (2007) (Table 1). Ancillary samples of *Parazoanthus axinellae* (Schmidt, 1862) (*P.a.*) were collected from Mediterranean locations near the Medes Islands, Spain (42°02′N, 3°13′W), Banyuls-sur-Mer, France (42°29′N, 3°08′W), and from Omiš (43°26′N, 16°39′W), Vis Island (43°01′N, 16°12′W), and Fraškerić Island (44°49′N, 13°50′W), Croatia. Additional sequences culled from GenBank were included in the 16S analysis to provide the appropriate context for evaluating species groups. Four nonsymbiotic zoanthids from the genus *Zoanthus* were used to represent the suborder Brachycnemina, two anemones (order Actiniaria) were used to represent the family Edwardsiidae, and a black coral (order Antipatharia) was used as the outgroup (Table 1), because independent evidence indicates that antipatharians are an appropriate outgroup (Berntson, France & Mullineaux, 1999; Daly, Fautin & Cappola, 2003).

Amplification and sequencing

Polyps were preserved in absolute ethanol following collection, and, after several substitutions of ethanol to counter dilution, were stored at –80 °C. Total nucleic acid was extracted from individual polyps

Table 1. Genus and species, colour, collection locality, host taxon, Genbank accession numbers, and individual identifier of individual zoanthids, actiniarians, and antipatharians used in this study. Individuals with identical sequences not included in the final internal transcribed spacer (ITS) analyses are indicated by a superscript of the individual identifier of the identical sequence that was included

Genus and species	Colour	Collection locality	Host	ITS accession #	16S accession #	Individual identifier
Epizoanthus cutressi[TOB 44]	Golden	Barbados	*Cribrochalina vasculum* (Lamark, 1814)	EU418264		BAR 123
Epizoanthus cutressi	Golden	Dominica	*Cribrochalina dura* (Wilson, 1902)	EU418265		DOM 27
Epizoanthus cutressi	Golden	Navassa, USA	*Cribrochalina vasculum* (Lamark, 1814)	EU418266		NAV 61
Epizoanthus cutressi	Golden	Tobago	*Cribrochalina vasculum* (Lamark, 1814)	EU418267	EU828759	TOB 44
***Isozoanthus* sp. nov.**	Brown	Curaçao	*Dentitheca dendritica* (Nutting, 1900)	EU418275		CUR 203
***Isozoanthus* sp. nov.**	Brown	Dominica	*Dentitheca dendritica* (Nutting, 1900)	EU418276		DOM 31
***Isozoanthus* sp. nov.**	Brown	Bocas del Toro, Panamá	*Dentitheca dendritica* (Nutting, 1900)	EU418277	EU828761	PAN 21
Parazoanthus axinellae[FLG 1]	Yellow	Fraškerić Island, Croatia		EU418278		CRO F11
Parazoanthus axinellae	Yellow	Omiš, Croatia		EU418279		CRO V1
Parazoanthus axinellae[FLG 1]	Yellow	Vis Island, Croatia		EU418280		CRO R1
Parazoanthus axinellae	Yellow	Florida (gulf), USA	Yellow Halichondrida	EU418281		FLG 1
Parazoanthus axinellae	Yellow	Banyuls-sur-Mer, France		EU418282		FRA 64
Parazoanthus axinellae	Yellow	Medes Islands, Spain		EU418283	EU828754	SPA M1
Parazoanthus catenularis	Brown	Barbados	*Cribrochalina vasculum* (Lamark, 1814)	EU418284		BAR 124
Parazoanthus catenularis	Brown	Curaçao	*Xestospongia* sp.	EU418285		CUR 206
Parazoanthus catenularis	Brown	Dominica	*Neopetrosia proxima* (Duchassaing & Michelloti, 1864)	EU418286		DOM 14
Parazoanthus catenularis	Brown	Dominica	*Xestospongia muta* (Schmidt, 1870)	EU418287		DOM 16
Parazoanthus catenularis	Brown	Dominica	*Xestospongia muta* (Schmidt, 1870)	EU418288		DOM 25
Parazoanthus catenularis[NAV 60]	Brown	Navassa, USA	Purple encrusting Haplosclerida	EU418289		NAV 59
Parazoanthus catenularis	Brown	Navassa, USA	*Cribrochalina vasculum* (Lamark, 1814)	EU418290		NAV 60
Parazoanthus catenularis	Brown	Bocas del Toro, Panamá	*Neopetrosia proxima* (Duchassaing & Michelloti, 1864)	EU418291		PAN 17
Parazoanthus catenularis	Brown	Tobago	*Xestospongia muta* (Schmidt, 1870)	EU418292	EU828757	TOB 37
Parazoanthus catenularis	Brown	Tobago	*Cribrochalina vasculum* (Lamark, 1814)	EU418293		TOB 38
Parazoanthus catenularis[DOM 25]	Brown	Tobago	*Cribrochalina vasculum* (Lamark, 1814)	EU418294		TOB 46
Parazoanthus parasiticus	Brown	Barbados	*Niphates erecta* Duchassaing & Michelloti, 1864	EU418295		BAR 122
Parazoanthus parasiticus	Brown	Curaçao	*Callyspongia (Cladochalina) vaginalis* (Lamark, 1814)	EU418296		CUR 214
Parazoanthus parasiticus	Brown	Dominica	*Callyspongia (Cladochalina) vaginalis* (Lamark, 1814)	EU418297		DOM 1
Parazoanthus parasiticus	Brown	Dominica	*Spirastrella* sp.	EU418298		DOM 5
Parazoanthus parasiticus	Brown	Dominica	*Niphates erecta* Duchassaing & Michelloti, 1864	EU418299		DOM 9
Parazoanthus parasiticus	Brown	Dominica	*Spirastrella cf. coccinea* (Duchassaing. & Michelotti, 1874)	EU418300		DOM 23
Parazoanthus parasiticus	Brown	Florida (gulf), USA	Tan Haplosclerida	EU418301		FLG 11
Parazoanthus parasiticus	Brown	Florida (gulf), USA	*Callyspongia (Cladochalina) vaginalis* (Lamark, 1814)	EU418302		FLG 63
Parazoanthus parasiticus	Brown	Navassa, USA	*Callyspongia (Cladochalina) vaginalis* (Lamark, 1814)	EU418305		NAV 57
Parazoanthus parasiticus	Brown	Bocas del Toro, Panamá	*Niphates erecta* Duchassaing & Michelloti, 1864	EU418303		PAN 13
Parazoanthus parasiticus	Brown	Bocas del Toro, Panamá	*Niphates erecta* Duchassaing & Michelloti, 1864	EU418304		PAN 15
Parazoanthus parasiticus	Brown	Tobago	*Niphates erecta* Duchassaing & Michelloti, 1864	EU418306	EU828756	TOB 47

Table 1. *Continued*

Genus and species	Colour	Collection locality	Host	ITS accession #	16S accession #	Individual identifier
Parazoanthus parasiticus	Brown	US Virgin Islands, USA	*Callyspongia (Cladochalina) vaginalis* (Lamark, 1814)	EU418307		USVI 148
Parazoanthus puertoricense	Maroon	Barbados	*Agelas* sp.	EU418308		BAR 120
Parazoanthus puertoricense	Maroon	Curaçao	*Svenzea zeai* (Alzerez, van Soest, & Rützler, 1998)	EU418309		CUR 212
Parazoanthus puertoricense	Maroon	Dominica	*Svenzea zeai* (Alzerez, van Soest, & Rützler, 1998)	EU418310		DOM 7
Parazoanthus puertoricense	Maroon	Dominica	*Agelas conifera* (Schmidt, 1870)	EU418311		DOM 12
Parazoanthus puertoricense	Maroon	Navassa, USA	*Agelas sceptrum* (Lamark, 1815)	EU418312	EU828758	NAV 58
Parazoanthus puertoricense$^{\text{TOB 36}}$	Maroon	Tobago	*Agelas conifera* (Schmidt, 1870)	EU418313		TOB 35
Parazoanthus puertoricense	Maroon	Tobago	*Svenzea zeai* (Alzerez, van Soest, & Rützler, 1998)	EU418314		TOB 36
Parazoanthus swifti$^{\text{TOB 42}}$	Yellow	Barbados	*Iotrochota birotulata* (Higgin, 1877)	EU418315		BAR 121
Parazoanthus swifti$^{\text{BRA 165}}$	Salmon	Búzios, Brazil	Red encrusting Poecilosclerida	EU418316		BRA 163
Parazoanthus swifti	White	Búzios, Brazil	Red encrusting Poecilosclerida	EU418317		BRA 165
Parazoanthus swifti	Salmon	Georgia, USA	*Clathria (Clathria) prolifera* (Ellis & Solander, 1786)	EU418318		C&G 129
Parazoanthus swifti	Salmon	Georgia, USA	*Clathria* sp.	EU418319		C&G 131
Parazoanthus swifti	Yellow	Curaçao	Orange encrusting Poecilosclerida	EU418321		CUR 200
Parazoanthus swifti	Yellow	Curaçao	*Iotrochota birotulata* (Higgin, 1877)	EU418320		CUR 204
Parazoanthus swifti	Orange	Dominica	*Agelas* sp.	EU418322		DOM 11
Parazoanthus swifti	Salmon	Florida (gulf), USA	Poecilosclerida	EU418323		FLG 5
Parazoanthus swifti	White	Florida (gulf), USA	Poecilosclerida	EU418324		FLG 7
Parazoanthus swifti$^{\text{FLG 54}}$	White	Florida (gulf), USA	*Clathria* sp.	EU418325		FLG 9
Parazoanthus swifti	Salmon	Florida (gulf), USA	Orange Poecilosclerida	EU418326		FLG 13
Parazoanthus swifti	White	Florida (gulf), USA	Orange encrusting Poecilosclerida	EU418327		FLG 50
Parazoanthus swifti	White	Florida (gulf), USA	Yellow branching Poecilosclerida	EU418328		FLG 53
Parazoanthus swifti	Salmon	Florida (gulf), USA	Black branching Poecilosclerida	EU418329		FLG 54
Parazoanthus swifti	White	Florida (gulf), USA	Orange Poecilosclerida	EU418330		FLG 55
Parazoanthus swifti	Yellow	Navassa, USA	*Agelas* sp.	EU418331		NAV 56
Parazoanthus swifti	Yellow	Bocas del Toro, Panamá	*Iotrochota birotulata* (Higgin, 1877)	EU418332	EU828755	PAN 9
Parazoanthus swifti	Orange	Bocas del Toro, Panamá	*Clathria (Thalysias) schoenus* (de Laubenfels, 1936)	EU418333		PAN 11
Parazoanthus swifti	Orange	Tobago	*Iotrochota birotulata* (Higgin, 1877)	EU418334		TOB 39
Parazoanthus swifti	Orange	Tobago	*Topsentia ophiraphidites* (de Laubenfels, 1954)	EU418335		TOB 41
Parazoanthus swifti$^{\text{CUR 200}}$	Orange	Tobago	*Agelas clathrodes* (Schmidt, 1870)	EU418336		TOB 42
Parazoanthus swifti$^{\text{TOB 42}}$	Yellow	Tobago	*Topsentia* sp.	EU418337		TOB 45
Parazoanthus swifti$^{\text{CUR 200}}$	Yellow	US Virgin Islands, USA	*Clathria (Thalysias) juniperina* (Lamark, 1814)	EU418338		USVI 151
Parazoanthus tunicans	White	Curaçao	*Dentitheca dendritica* (Nutting, 1900)	EU418339		CUR 71
Parazoanthus tunicans	White	Dominica	*Dentitheca dendritica* (Nutting, 1900)	EU418340		DOM 30
Parazoanthus tunicans	White	Tobago	*Dentitheca dendritica* (Nutting, 1900)	EU418341	EU828760	TOB 40
Zoanthus pulchellus		Bocas del Toro, Panamá			EU828762	PAN 7
Zoanthus sansibaricus		Japan			AB235412	
Zoanthus kuroshio		Japan			AB235410	
Zoanthus gigantus		Japan			AB235411	
Edwardsiidae sp. (BAR)	Transparent	Barbados	*Plakortis* sp.	EU418268		BAR 05A
Edwardsiidae sp. (BAR)	Transparent	Barbados	*Plakortis* sp.	EU418269	EU828764	BAR 06W
Edwardsiidae sp. (BAR)	Transparent	Barbados	*Plakortis* sp.	EU418270		BAR 06Y
Edwardsiidae sp. (CUR)	Transparent	Curaçao	*Plakortis* sp.	EU418271		CUR 213

Table 1. *Continued*

Genus and species	Colour	Collection locality	Host	ITS accession #	16S accession #	Individual identifier
Edwardsiidae sp. (CUR)	Transparent	Curaçao	*Plakortis* sp.	EU418272	EU828763	CUR E1
Edwardsiidae sp. (CUR)	Transparent	Curaçao	*Plakortis* sp.	EU418273		CUR E2
Edwardsiidae sp. (CUR)	Transparent	Curaçao	*Plakortis* sp.	EU418274		CUR E3
Nematostella vectensis					AY169370	
Nematostella sp.					DQ643835	
Chrysopathes formosa		NE Pacific			NC_008411	

using a cetyl-trimethyl-ammonium bromide extraction technique (Doyle & Doyle, 1987). Polymerase chain reaction (PCR) amplification was performed using the Platinum® PCR Supermix (Invitrogen) and the following primers: novel primers designed for anthozoan complete ITS (ITSf, 5′-CTAGTAAGCGCGAGTCATCAGC-3′; ITSr, 5′-GGTAGCCTTGCCTGATCTGA-3′), novel primers designed for anthozoan 16S (16Sf 2824, 5′-TCGACTGTTTACCAAAAACATAGC-3′; 16Sr 3554, 5′-CAATTCAACATCGAGGTCGCAAAC-3′), and the 16S primers of Sinniger *et al.* (2005). The thermal protocol used for all primers consisted of 94 °C for 3 min, 32 cycles of 94 °C for 30 s, 50 °C for 60 s, 72 °C for 90 s, with a final extension step of 72 °C for 10 min. The PCR products were purified by enzymatic digestion (ExoSAP-IT®; USB Corporation), and were directly sequenced in both the forward and reverse directions using the amplification primers and Big-Dye® Terminator (Applied Biosystems) chemistry at the Florida State University Sequencing Facility.

Phylogenetic analyses

Forward and reverse sequences were edited and assembled using SEQUENCHER 4.0.5 (Gene Codes Co.), and an initial alignment of all sequences was made using CLUSTAL X 1.81 (Thompson *et al.*, 1997) with the default settings. The CLUSTAL X-derived alignment was adequate for 16S, 5.8S, the 3′ end of 18S, and the 5′ end of 28S for all sequences; however, the ITS1 and ITS2 regions could only be reasonably aligned by CLUSTAL X within groups of individuals that represented species or closely related species. Phylogenetic analyses of ITS regions often exclude large portions of ITS1 and ITS2 because of alignment difficulties (e.g. Reimer *et al.*, 2007). In order to include all nucleotides of the ITS genes in the phylogenetic analyses, blocks of unambiguously aligned sequences were shifted to create non-overlapping character sets in the alignment, and the resulting gaps were coded as missing characters using BIOEDIT 7.0.5.2 (Hall, 1999). The final ITS alignment contains the complete sequence of each individual, but regions that aligned among subsets of individuals were staggered throughout the alignment, in an organization analogous to a concatenated multigene alignment, with incomplete taxon sampling for each gene (see Fig. S1 for a schematic of ITS alignment). Exact duplicate haplotypes were removed from the ITS alignment (indicated by superscript notations in Table 1), and were not included in further analyses.

Model selection and parameter estimation were performed using the Akaike information criterion in MODELTEST 3.7 (Posada & Crandall, 1998). The Tamura–Nei model (Tamura & Nei, 1993) with invariable sites and gamma parameter (TrN + I + G) gave the best fit to the ITS data, with the following parameters: base frequencies, A = 0.2270, C = 0.2626, and G = 0.2704; substitution-rate matrix, rAC = 1.0000, rAG = 2.1157, rAT = 1.0000, rCG = 1.0000, and rCT = 2.8980; gamma shape parameter, 0.4557; proportion of invariable sites, 0.3616. The Tamura–Nei model (Tamura & Nei, 1993) with gamma parameter (TrN + G) gave the best fit to the 16S data, with the following parameters: base frequencies, A = 0.3112, C = 0.1900, and G = 0.2566; substitution-rate matrix, rAC = 1.0000, rAG = 4.5496, rAT = 1.0000, rCG = 1.0000, and rCT = 8.6916; gamma shape parameter, 0.3976. Phylogenetic analyses were conducted using PAUP 4.0 b10 (Swofford, 2000) and MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2001). Maximum likelihood (ML) searches were performed using a heuristic search algorithm with tree-bisection-reconnection branch swapping and five random-sequence taxon additions. Estimates of support were obtained by ML bootstrapping using the same likelihood parameters as the topology search, with 100 pseudoreplicates, and a Bayesian statistical approach using Markov-chain Monte Carlo simulations (Huelsenbeck & Ronquist, 2001). Bayesian analyses of the ITS data were performed on an alignment partitioned into three data subsets (ITS1; ITS2; and a concatenated 18S, 5.8S, and 28S), using models of molecular evolution empirically determined for each partition by MRBAYES. Every five-hundredth tree was sampled during a 5 million iteration chain, and, after inspection for convergence using AWTY (Wilgenbusch,

Warren & Swofford, 2004), the first two million iterations were discarded as 'burn-in'. A 50% majority rule consensus tree was calculated from the remaining Bayesian iterations using PAUP.

Species delimitations and biogeography

Species delimitations were determined from the ITS phylogeny using a history-based phylogenetic species concept (Baum & Donoghue, 1995) by identifying reciprocally monophyletic crown clades, which were then assessed by concordance with published descriptions of gross morphology (colour, number of tentacles, number of scapular ridges, and size of polyps). Individual zoanthids were initially identified *in situ* by field observations and macroscopic photography of zoanthid–host holobionts, using a combination of polyp and colony morphology, and host specificity, as described by Duerden (1900), Pax & Müller (1962), West (1979), Crocker & Reiswig (1981), and Swain & Wulff (2007).

Species that did not match published morphological descriptions of Caribbean zoanthids were subjected to further microscopic examination of internal morphological structures. Individual polyps were decalcified in a formic acid fixative decalcifier (Formical-4™; Decal Chemical Corporation) for 4 h, and were then desilicified in 10% hydrofluoric acid for 4 h, before being stored in 70% ethanol. Polyps were dehydrated, stained with Harris' hematoxylin and eosin, imbedded in paraffin, and sectioned at the Florida State University Histology Facility. Longitudinal and cross-sectional serial sections were made from several different polyps from each colony sampled for histology, at a thickness of 8–10 μm.

The colour of individual colonies was mapped onto the ITS phylogeny to assess whether colour could be used to distinguish species. The collection locations for zoanthid specimens included in the phylogenetic analyses were mapped on the resulting ITS phylogeny to assess the effect of geography on the estimation of species delimitations.

The geographic distributions of species were determined by compiling genetically verified species occurrence data from field collections, supplemented with occurrence data published in the sponge and zoanthid literature, and occurrence data transcribed from the labels of specimens in the Porifera and Cnidaria collections of the United States National Museum of Natural History (USNM).

Phylogenetic relationships and the evolution of host associations

The ITS phylogeny, constructed to analyse the delimitations of species, also reveals the evolutionary relationships between species, and is therefore useful in forming hypotheses about the the evolution of symbioses in zoanthids and the validity of current zoanthid systematics. The host species of individual zoanthids were mapped onto the ITS phylogeny to assess the effects of particular host associations on zoanthid species clade topology.

The 16S phylogeny was constructed to provide an independent assessment of the clades of species inferred in the ITS analysis. The host associations of zoanthid species (as defined by Pax & Müller, 1962; Herberts, 1972; West, 1979; Swain &Wulff, 2007) were mapped onto the ITS and 16S phylogenies to assess phylogenetic conservatism in the evolution of zoanthid–host associations, and also to detect host switches. The occurrence of zoanthid photoendosymbionts (*Symbiodinium*; as defined by West, 1979) was also mapped onto the ITS and 16S phylogenies to assess phylogenetic conservatism in the evolution of zoanthid–*Symbiodinium* associations, and to detect changes in zoanthid associations with *Symbiodinium*.

RESULTS

Phylogenetic analyses

Electrophoresis of ITS PCR products produced single compact bands of approximately 900 nucleotides in length, and direct sequencing produced forward and reverse sequences with no indication of prominent intragenomic nucleotide variation (with minimal background noise and ambiguities in chromatographs, and minimal or no variation between genomes of the same species; Fig. 1) or length variation, except in haplotypes of *P. swiftii*. There is evidence of isolated intragenomic length variation in all haplotypes of *P. swiftii*, which is apparently caused by a microsatellite (multiple peaks downstream of a repeated sequence in the forward and reverse direction chromatographs) composed of between one and four repetitions of AGGG, located 36 nucleotides downstream from the 5′ end of ITS2 in all of the *P. swiftii* individuals examined. This microsatellite is excluded from further analyses because of uncertainty about the number of repeats within a genome. The sequences of the ITS region (ITS1, 5.8S, and ITS2) ranged from 656 to 930 nucleotides in length; however, the complete alignment (that also contained segments of 18S and 28S) consisted of 2266 characters because of the additional alignment length introduced by staggering hypervariable regions within ITS1 and ITS2.

A search for the optimal ML tree (Fig. 1) resulted in three best trees (each with a score = –9854.54) that differed only in the relationships among individuals

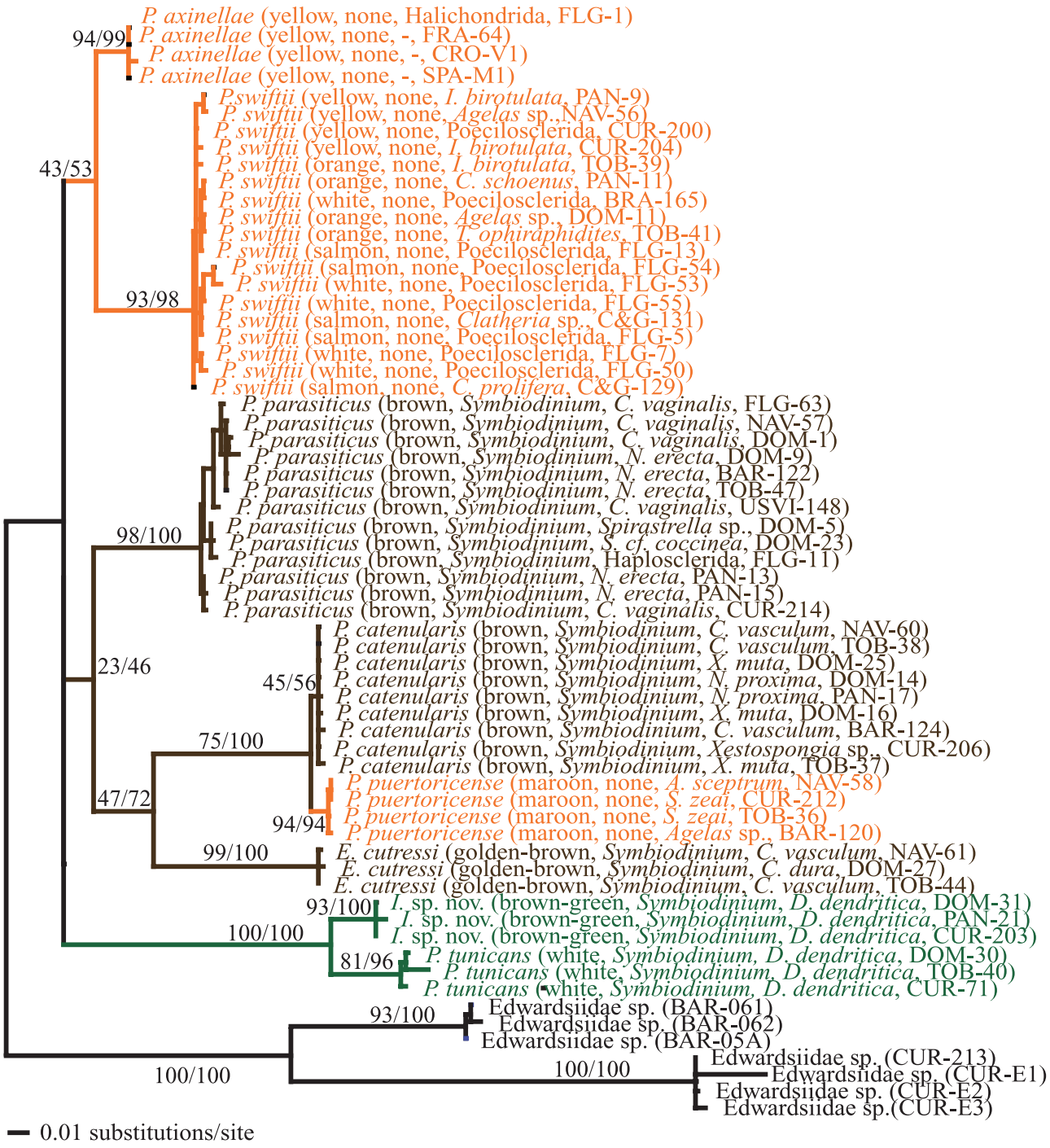


Figure 1. Phylogeny of Caribbean symbiotic zoanthids based on the internal transcribed spacer (ITS) region of the rRNA nuclear gene. Support values are 100 pseudoreplicate maximum likelihood (ML) bootstrap values followed by three million iteration Bayesian posterior probabilities. The clades of symbiotic species are colour coded according to their host associations. The information presented in parentheses after the specimens collected for this study includes: the colour of the zoanthid, presence of *Symbiodinium*, host taxa, and individual identifier (which includes the collection location).

Figure 2. Phylogeny of Caribbean symbiotic zoanthids based on the 16S region of the rRNA mitochondrial gene. Support values are 100 pseudoreplicate maximum likelihood (ML) bootstrap values followed by three million iteration Bayesian posterior probabilities. The clades of symbiotic species are colour coded according to their host associations. The information presented in parentheses after the specimens collected for this study includes: presence of *Symbiodinium* and individual identifier (which includes the collection location). Sequences culled from GenBank only use the accession number.

within crown clades, and therefore the differences between the trees are not relevant to the questions posed here.

Electrophoresis of 16S PCR products produced single compact bands of approximately 900 nucleotides in length. The sequences of the 16S region ranged from 884 to 941 nucleotides in length, using the primers of Sinniger *et al.* (2005), and were 623–655 nucleotides in length using newly designed primers. The complete 16S alignment consisted of 1118 characters. A search for the optimal ML tree (Fig. 2) resulted in a single best tree (score = –4058.72).

Species delimitations

The ML and Bayesian analyses of the ITS data found ten crown clades, and each clade is well supported by bootstrapping (> 70) and Bayesian posterior probabilities (> 80), except for the *P. catenularis* clade (Fig. 1). Crown clades of symbiotic species resolved in this analysis are congruent with the published descriptions of the gross morphology and host associations of named species (*P. axinellae*, *P. catenularis*, *P. parasiticus*, *P. puertoricense*, *P. tunicans*, and *E. cutressi*), except for three clades of individuals. Histological examination of the three unidentified species reveal an *Isozoanthus* species [the fifth septa is complete (suborder Macrocnemina), the marginal sphincter muscle is entodermal (family Parazoanthidae), and there is no conspicuous mesogloeal ring sinus (genus *Isozoanthus*)], and two species with affinity to the actiniarian family Edwardsiidae (eight coupled mesenteries, basilar and sphincter muscles absent, and no pedal disc). These unidentified species are both genetically and morphologically distinguishable from their nearest relatives on the ITS phylogeny. *Isozoanthus* sp. nov. has larger polyps, darker coloured tissues, and significantly (Student's *t*-test: $t = 23.4$, $\mathrm{df} = 190$, $P = 8.2 \times 10^{-58}$) more tentacles or scapular ridges in comparison with *P. tunicans* (30–38 tentacles and 22–30 tentacles, respectively). The polyps of Edwardsiidae sp. (BAR) have significantly (Student's *t*-test: $t = 18.6$, $\mathrm{df} = 56$, $P = 1.2 \times 10^{-25}$) fewer tentacles (10–12 rather than 13–16) compared with Edwardsiidae sp. (CUR).

The colour of individuals only indicated species-level differences when there were other morphological differences that were correlated with colour. For example, white-, salmon-, yellow-, and orange-coloured polyps were all genetically indistinguishable *P. swiftii* individuals of similar size and number of tentacles, whereas white *P. tunicans* (smaller, with a mode of 28 tentacles) and seal-brown *Isozoanthus* sp. nov. (larger, with a mode of 32 tentacles) were genetically differentiated (Fig. 1).

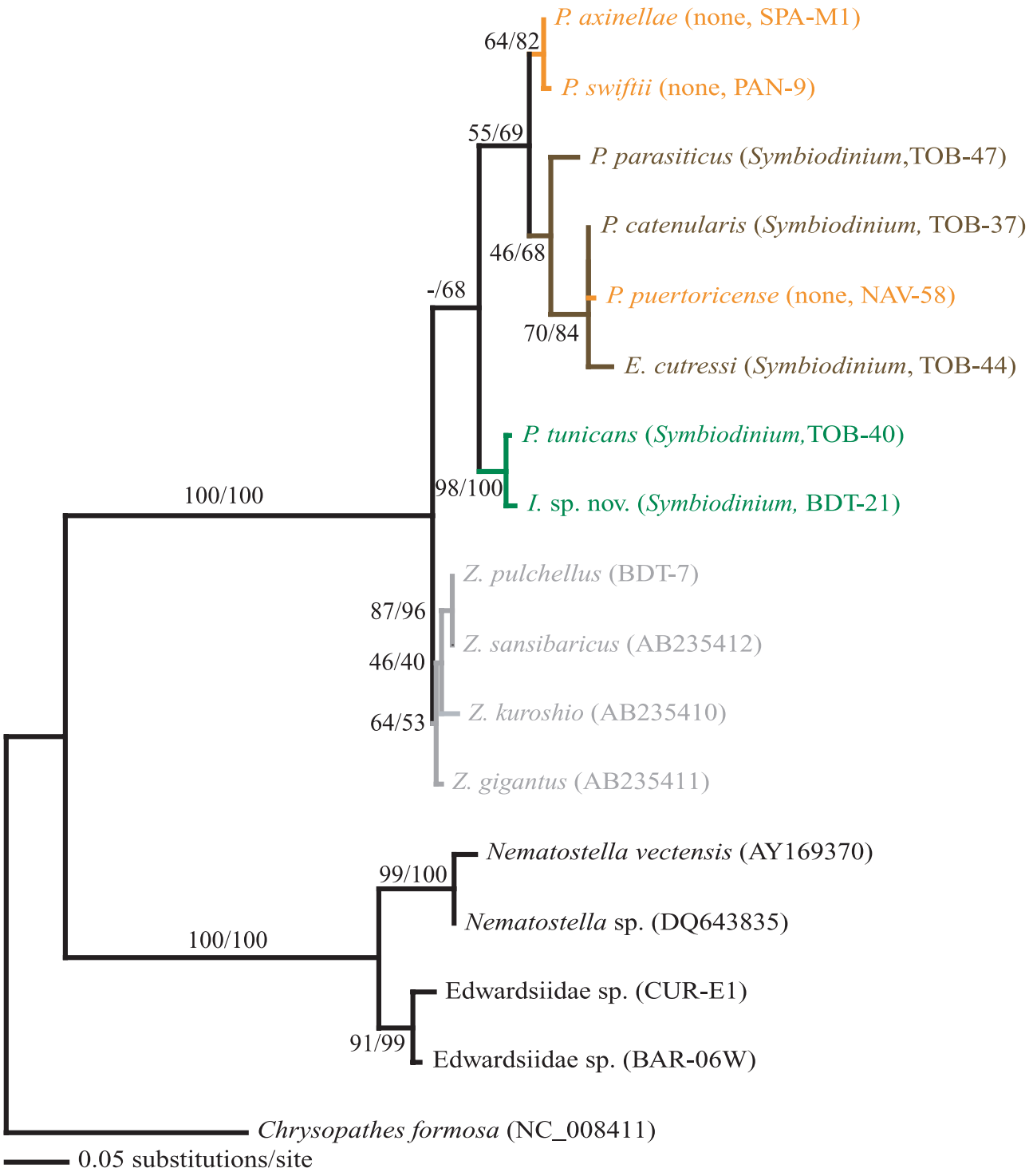
Biogeography of symbiotic zoanthids

Within the crown clades of the ITS phylogeny, the ML and Bayesian analyses cannot detect any phylogenetic structure that can be attributed to geographic location (Fig. 1 and Table 1). Individuals collected throughout the wider Caribbean region and across the Atlantic Ocean, separated by thousands of kilometres, share identical ITS haplotypes (Table 1). There is a geographic- and habitat-specific pattern to the colour morphs of *P. swiftii*; which are exclusively white- to salmon-coloured in the subtropical regions and (potentially) marginal tropical habitats (wave-swept reef crests and rocky overhangs), and pale yellow to bright orange on tropical coral reefs. However, this geographic pattern did not correspond to any phylogenetic pattern within the *P. swiftii* clade (Fig. 1).

The distribution of symbiotic zoanthids observed (or reported) in the wider Caribbean region thus far is characterized by relatively low species diversity in the subtropical regions (four species observed on the Gulf and Atlantic coasts of the south-eastern USA, and two species from Brazil), and relatively high species diversity in the tropical Caribbean (six species in the eastern Caribbean – Belize, Honduras, and Panama – and seven species in the western Caribbean – Barbados, Curaçao, Dominica, and Tobago; Fig. 3). Although some species are nearly ubiquitous throughout the region (*P. swiftii* and *P. parasiticus*), the composition of species changes geographically, and some species have only been observed in the northern-most regions of the wider Caribbean (*P. axinellae*), or in the eastern Caribbean (*E. cutressi*; Fig. 3).

Phylogeny of Zoanthidea

The interpretation of the Zoanthidea ITS and 16S phylogenies must be tempered by regional taxonomic sampling, and by weak bootstrap (< 70) and Bayesian



(< 80) support values at some of the internal nodes. The phylogenetic analyses of ITS and 16S data recovered the same clades of symbiotic species with similar host associations (Figs 1, 2). *Parazoanthus axinellae* and *P. swiftii* form a clade of symbionts of sponges representing the order Halichondrida (and orders Poecilosclerida and Agelasida), *P. parasiticus*, *P. catenularis*, and *E. cutressi* form a clade of symbionts of sponges representing the order Haplosclerida (and order Hadromerida), and *P. tunicans* and *Isozoanthus* sp. nov. form a clade of symbionts of a hydroid representing the genus *Dentitheca*. The ITS and 16S data

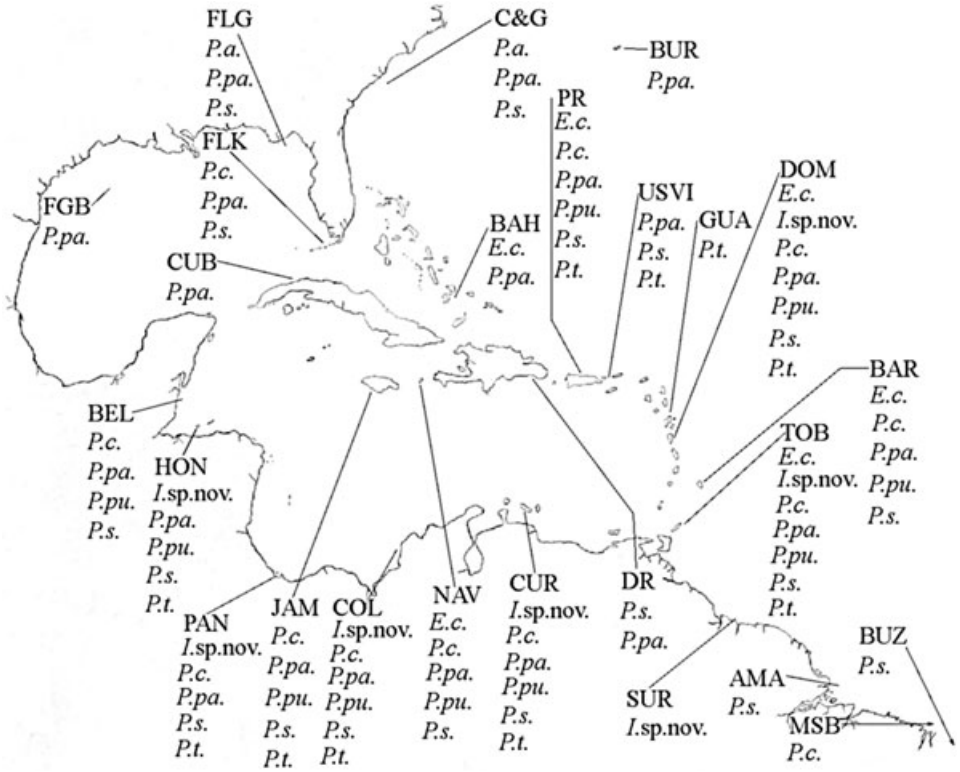


Figure 3. Map of the wider Caribbean region showing a compilation of observed symbiotic zoanthid species in each location. The following list defines the location abbreviations, and credits the source of observations. Species observations without citations are from the current study. Abbreviations: PR, La Parguera, Puerto Rico, West 1979; USVI, US Virgin Islands, Duchassaing & Michelotti, 1860, this study, and (*P.t.*) Pax, 1910; GUA, Guadeloupe, Pax & Müller, 1956; DOM, Dominica; BAR, Barbados, Crocker & Reiswig, 1981 and this study; TOB, Tobago; SUR, Suriname, USNM 50878; AMA, Amazon River outfall, Brazil, USNM 1084839; MSB, Maranhão State, Brazil, Campos *et al.*, 2005; BUZ, Búzios, Brazil; CUR, Curaçao; COL, Colombia, (Santa Marta, *P. pu.*) Alvarez, Van Soest & Rützler, 1998, (Cartagena) J. Sanchez pers. comm.; PAN, Bocas del Toro, Panama; HON, Utila, Honduras, Sinniger *et al.*, 2005; BEL, Carrie Bow Cay, Belize, (*P.c.*) USNM 32338, (*P.pa.*) Lewis, 1982, (*P.pu*) USNM 32345, (*P.s.*) J. Wulff pers. comm.; CUB, Havana, Cuba, Varela, Ortiz & Lalana, 2003; FGB, Flower Garden Banks, USA; FLG, Gulf coast of Florida, USA; FLK, Florida Keys, USA, (*P.c.*) USNM 41535; JAM, Jamaica, Duchassaing & Michelotti, 1860, (*P.pu.* and *P.t.*) West, 1979; NAV, Navassa Island, USA; BAH, Bahamas, Duchassaing & Michelotti 1860, (*E.c.*) Willenz & Hartman, 1994; DR, Dominican Republic, Williams *et al.*, 1983; C&G, Carolinas and Georgia, USA, (*P.a.*) USNM 16870, (*P.pa.*) USNM 51535, (*P.s.*) this study; BUR, Bermuda, Ryland & Westphlen, 2004.

both support conservatism in the evolution of zoanthid host associations, with host switching an apparently rare event. A single host switch was detected in *P. puertoricense*, which is a symbiont of sponges representing the orders Agelasida and Halichondrida (similar to the host species of the *P. axinellae* and *P. swiftii* clade), whereas the other members of this clade (*P. parasiticus*, *P. catenularis*, and *E. cutressi*) are symbionts of sponges representing the order Haplosclerida (and order Hadromerida). The host switch of *P. puertoricense* appears to be linked with a loss of *Symbiodinium* symbiosis, as *P. parasiticus*, *P. catenularis*, and *E. cutressi* all maintain symbioses with *Symbiodinium*, but *P. puertoricense* does not.

The four zoanthid genera represented in these analyses (*Epizoanthus*, *Parazoanthus*, *Isozoanthus*, and *Zoanthus*) represent three different families (Epizoanthidae, Parazoanthidae, and Zoanthidae) and two different suborders (Macrocnemina, which contains Epizoanthidae and Parazoanthidae, and Brachycnemina, which contains Zoanthidae) within the order Zoanthidea. Whereas some higher taxa (orders, suborders, families, and genera) were found to be monophyletic (Fig. 2), *Parazoanthus* and Parazoanthidae are paraphyletic in the ITS (Fig. 1) and 16S (Fig. 2) phylogenies, and *Epizoanthus* (Epizoanthidae) and *Isozoanthus* were nested within clades of *Parazoanthus*.

DISCUSSION

Species delimitations

The ITS phylogeny-based species delimitations were congruent with the gross morphology of species descriptions for *P. axinellae*, *P. catenularis*, *P. parasiticus*, *P. puertoricense*, *P. tunicans*, and *E. cutressi*, and detected three unidentified species: *Isozoanthus* sp. nov., Edwardsiidae sp. (BAR), and Edwardsiidae sp. (CUR). The presence (in the Caribbean) of *P. axinellae* and three unidentified species seems to have been previously overlooked, because of similarity with other species (*Isozoanthus* sp. nov. and *P. axinellae*), or because they are extremely inconspicuous [transparent tissues, and small size of Edwardsiidae sp. (BAR) and Edwardsiidae sp. (CUR)].

The morphological and host similarities (Pax & Müller, 1962) of *P. axinellae* may result in mistakenly identifying *P. swiftii* when observing *P. axinellae* (a possibility we were aware of, and which we avoided in Swain & Wulff 2007). In the field, these two species may be particularly hard to distinguish: they are approximately the same size, the same colour (and range of colour variation), associate with the same groups of sponges, and occur sympatrically in the temperate northern Caribbean. The morphological similarity is so great that *P. swiftii* and *P. axinellae* were briefly synonymized (Pax, 1910). However, the genetic differences between *P. axinellae* and *P. swiftii* are large (Fig. 1), and tentacle counts can be used to distinguish between these two species (*P. swiftii* has a maximum of 26 tentacles, whereas *P. axinellae* has a maximum of 38 tentacles). Furthermore, the ITS DNA sequences from specimens collected across the geographic distribution of both species (from Florida to Croatia for *P. axinellae*, and from Panamá to Barbados, and from Georgia to Brazil, for *P. swiftii*) are nearly indistinguishable within species (Fig. 1 and Table 1), thereby providing a mechanism for reliable genetic verification of field identifications.

The host similarities of *P. tunicans* and *Isozoanthus* sp. nov., along with inconsistent descriptions in the literature, may have resulted in mistakenly identifying *P. tunicans* when observing *Isozoanthus* sp. nov. The only known hydroid host of both *P. tunicans* and *Isozoanthus* sp. is *Dentitheca dendritica* (Nutting, 1900). The accepted diversity of morphology within *P. tunicans* has been in question since a redescription by West (1979) contained inconsistencies with the original Duerden (1900) description, and with the subsequent redescription by Pax (1910). Most notably, Duerden (1900) and Pax (1910) describe a species with 28–32 or 28–30 (respectively) tentacles that are colonized by *Symbiodinium*, whereas West (1979) describes a species with a maximum of 36 tentacles and no *Symbiodinium*. The inconsistencies between descriptions may have led to the broad acceptance of variation in morphology and coloration within *P. tunicans* in popular field guides (e.g. Humann & DeLoach, 2002) and scientific publications (e.g. Sinniger *et al.*, 2005), which assign a dark and a light colour morph to *P. tunicans*. The ITS phylogeny suggests that the light- and dark-coloured hydroid symbionts are separate species, thereby confirming the results obtained with mitochondrial data (Sinniger *et al.*, 2005) that were first used to detect a genetic difference between the putative colour morphs. *In situ* macro photographs that included examples of visibly 'bleached' zoanthid colonies, taken while collecting specimens for this study, reveal that the light-coloured species is congruent (22–30 tentacles, coloured brown by *Symbiodinium* colonizations, with white polyp columns, and coenenchyme) with the original species description (Duerden, 1900) of *P. tunicans*, and that the dark-coloured species is not congruent (30–38 tentacles, with seal-brown polyps, and coenenchyme) with the descriptions of Duerden (1900), Pax (1910), or West (1979). Histological examinations indicate that the microscopic internal morphology of the dark-coloured hydroid symbiont more closely matches the description of *Isozoanthus* than *Parazoanthus*, confirming that the dark-coloured hydroid symbiont is not *P. tunicans*.

The only reports (Lewis, 1965; Acosta *et al.*, 2005) of a Caribbean hydroid-symbiotic zoanthid (other than *P. tunicans*) are referred to as '*Isozoanthus mirabilis* (Verrill)'. However, a published description of '*I. mirabilis*' has not been found, and therefore (under article 11 of the International Code of Zoological Nomenclature), the name is a *nomen nudum*. The museum specimens of '*I. mirabilis*' (USNM 17218, 50354, 50777, 50778, 50878, and 52526) include a specimen collected by Verrill in 1880 (USNM 17218), labelled as '*Synackis mirabilis*' and 'name change by Carlgren 1930'. '*Synackis mirabilis*' seems to be a misspelling of *Synathis mirabilis* Verrill, a junior synonym of the actiniarian *Amphianthus mirabilis* (Verrill, 1879). No Carlgren publication from 1930 discusses a species with the specific epithet '*mirabilis*' (Carlgren, 1930a, b), although Carlgren (1949) establishes *A. mirabilis* as the senior synonym of *S. mirabilis*. Histological preparations of USNM 50878 are indistinguishable from *Isozoanthus* sp. nov., and were collected from the same hydroid host species as *Isozoanthus* sp. nov., indicating that '*I. mirabilis*' may (in part) be conspecific with *Isozoanthus* sp. nov.

The macroscopic size, transparent tissues, and ability to retract completely beneath the surface of host sponges is likely to have kept Edwardsiidae sp. (BAR) and Edwardsiidae sp. (CUR) from being noticed. The polyps of both species are difficult to observe in the field; however, their presence can be

detected by the pores or volcano-shaped protuberances on the surface of host *Plakortis* spp. sponges (see '*Epizoanthus* sp. nov.' in Swain & Wulff, 2007: fig. 1) that are otherwise absent. The first specimens of Edwardsiidae sp. (BAR) were reported (as an unidentified *Epizoanthus* sp.) by Crocker & Reiswig (1981) from Barbados, and (with the generous guidance of H. Reiswig, University of Victoria) the specimens reported here are from the same reef. Histological slides and *in situ* photographs loaned by H. Reiswig are indistinguishable from the material reviewed in this study. The two whorls of alternating tentacles (typical of Zoanthidea), symbioses with sponges (typical of *Epizoanthus* and *Parazoanthus*), macroscopic size, and notoriously simple morphology of the Edwardsiidae (Daly, 2002) make the original identification of this species as *Epizoanthus* understandable. A second species, extremely similar to Edwardsiidae sp. (BAR), was collected in Curaçao, and is genetically and morphologically (16 tentacles compared with 12) distinct from the Barbados species.

Biogeography of symbiotic zoanthids

The ITS phylogeny did not detect any phylogenetic structure that can be attributed to geographic location (Fig. 1; Table 1), although undetected intragenomic polymorphisms may distort the signal of population-level structure (e.g. Wörheide *et al.*, 2004).

The geographic distribution of symbionts are limited by the availability of suitable hosts; however, sponge distributions do not seem to be able to fully explain the distribution of symbiotic zoanthids (e.g. *P. puertoricense* and *E. cutressi* associate with sponge species in the genera *Agelas* and *Xestospongia*, respectively, which are common in Bocas del Toro, Panama, but these zoanthid species have not been observed there; Fig. 3). *Parazoanthus swiftii* and *P. parasiticus* are present and conspicuously common in nearly all of the locations examined, whereas the other zoanthid species are usually rarer locally, and geographically less widespread (Fig. 3).

This is the first report of *P. axinellae* in the western Atlantic, which has been known from the northeastern Atlantic and Mediterranean for more than a century. A sponge (USNM 16870) collected from North Carolina, USA, in 1860 (two years before *P. axinellae* was first described by Schmidt in the Mediterranean), is colonized with zoanthids that are apparently *P. axinellae* (identified by the gross morphology of the colony and polyps, and by the host sponge association), thereby indicating that the current distribution is not the result of a recent invasion.

Parazoanthus axinellae may be particularly capable of obtaining large geographic distributions because it can flourish in the absence of hosts (Haddon & Shackleton, 1891), produce thread-like asexual propagules, which have the potential to be dispersed by water currents (Ryland, 1997), and because several representatives of its host sponge genera are found on both sides of the Atlantic (e.g. sponges representing the genus *Axinella*). Other pan-Atlantic macrocnemic zoanthids include the deep-sea sponge symbionts *Parazoanthus anguicomus* (Norman, 1868), reported by Verrill (1882) as '*Epizoanthus americanus*' *n.n.* (Haddon & Shackleton, 1891; Carlgren, 1913), and *Epizoanthus norvegicus* (Koren & Danielssen, 1877), which are found on both the North American (USNM 22495) and European coasts. The deep-sea pagurid crab symbionts *Epizoanthus incrustatus* (Düeben & Koren, 1847), *Epizoanthus paguriphilus* Verrill, 1882, and *Epizoanthus abyssorum* Verrill, 1885 are also known from both sides of the north Atlantic (Haddon & Shackleton, 1891; Muirhead, Tyler & Thurston, 1986), although the mobility of the crab and relative continuity of their habitat may be an additional advantage for distant dispersal. Zoanthids from the sister suborder Brachycnemia also have pan-Atlantic distributions (e.g. *Isaurus tuberculatus*, Muirhead & Ryland, 1985), but their dispersal abilities are thought to stem from long-lived larvae (Ryland *et al.*, 2000). The larvae of macrocnemic zoanthids have never been observed; however, they may share some of the same characteristics as their brachycnemic relatives (Ryland & Westphalen, 2004) that may aid in long-distance dispersal.

Both *P. axinellae* and *P. swiftii* show extensive colour variation over their distributions. In the Mediterranean, *P. axinellae* is reported to range in colour from 'pale grayish-yellow to the brightest orange' (Herberts, 1972), and to match the colour of host sponges (Pax & Müller, 1962) independent of habitat (Herberts, 1972). I have observed similar colour matching between *P. axinellae* and sponge hosts in the Gulf of Mexico, suggesting that colour may serve to conceal *P. axinellae* in both populations. In temperate regions (and apparently marginal tropical habitats like wave-swept reef crests and walls), I have observed that *P. swiftii* is usually pale salmon or drab white. Whereas on tropical reefs, *P. swiftii* is usually bright yellow or orange, and often contrasts with the colour of host sponges so strikingly that the colour difference is thought to be aposematic (West, 1976). The golden colour of both species is likely to be created by parazoanthoxanthins: a fluorescent-yellow nitrogenous pigment that has been isolated from *P. axinellae* and several other zoanthids (Cariello *et al.*, 1979), and is thought to serve as a chemical defence against predators (Sepčić, Turk & Maček, 1998; Pašić *et al.*, 2001). Therefore, difference in colour variation between *P. axinellae* and *P. swiftii* may reflect an

adaptive response to differences in predation pressure in the two regions. In the temperate region where sponge predation is predominately by invertebrates (which have not been shown to influence the distribution of sponges; Wulff 2006), symbiotic zoanthids seem to disguise their presence with matching or dull coloration. In the tropical region, where predation is predominately by vertebrates (which have been shown to influence the distribution of sponges; Wulff 2006), symbiotic zoanthids seem to advertise their presence with contrasting yellow/orange coloration. The predators of the symbiotic zoanthids themselves include both fishes of the genus *Chaetodon* and fireworms of the genus *Hermodice*; however, no experiments on the effect of predation on symbiotic zoanthid populations or distributions have yet been performed.

Phylogeny of Zoanthidea

The ITS phylogeny was constructed to examine species delimitations of Caribbean symbiotic zoanthids in a phylogenetic context, and any interpretation of the broader interspecific relationships of the Zoanthidea is limited by regional taxonomic sampling. The 16S phylogeny was included to independently assess the interspecific relationships hypothesized in the ITS phylogeny. The clades of symbiotic zoanthid species recovered by both the ITS and 16S analyses are distinguishable by the symbioses that they form, rather than by the morphological characters (briefly reviewed in Walsh, 1967) that have traditionally defined the zoanthid genera and families. With the exception of *P. puertoricense*, zoanthid symbionts of sponges representing the order Halichondrida (and orders Poecilosclerida and Agelasida), symbionts of sponges representing the order Haplosclerida (and order Hadromerida), and symbionts of hydroids representing the genus *Dentitheca*, are each monophyletic (Figs 1, 2). A previous mitochondrial-based phylogenetic analysis (Sinniger *et al.*, 2005) found clades of symbiotic zoanthid species that had similar host associations within the genus *Parazoanthus*. The repeated finding of monophyletic host associations suggests phylogenetic conservatism in the evolution of zoanthid host associations. The analyses reported here further suggest that there may be unrecognized phylogenetic structure within the order Zoanthidea that could provide a more parsimonious organization of the large diversity of associations currently observed within *Epizoanthus*, *Isozoanthus*, and *Parazoanthus*; and new taxon names may be required to clarify the phylogenetic relationships.

Although most symbiotic zoanthid species are members of phylogenetic clades that have similar host associations, *P. puertoricense* is conspicuously embedded in a clade with different host associations. The hosts of *P. puertoricense* are sponges representing the order Halichondrida (similar to the hosts of zoanthids in the *P. axinellae* and *P. swiftii* clade), whereas *P. parasiticus*, *P. catenularis*, and *E. cutressi* all form associations with sponges representing the order Haplosclerida (Figs 1, 2). Furthermore, *P. puertoricense* is the only species in this clade that does not host *Symbiodinuim*. The most parsimonious explanation for the differences between *P. puertoricense* and other members of this clade is that during the evolution of *P. puertoricense*, it switched its associations from sponges representing Haplosclerida to sponges representing Halichondrida, and lost its symbiosis with *Symbiodinuim*. An analyses of the specificity of Caribbean sponge–zoanthid symbioses demonstrated that if a sponge had photo-endosymbionts (either cyanobacteria or *Symbiodinuim*), then the associations that it formed were with zoanthids that also hosted photo-endosymbionts (*Symbiodinuim*) at a ratio of 13 : 1. If a sponge did not have photo-endosymbionts, then the associations that it formed were with zoanthids that also did not host photo-endosymbionts at a ratio of 2.2 : 1. These findings suggest that matching symbioses with photo-endosymbionts between sponges and zoanthids are important to the symbiosis (Swain & Wulff, 2007). In support of this hypothesis, *Symbiodinuim*-hosting *P. parasiticus*, *P. catenularis*, and *E. cutressi* associate with sponges hosting photo-endosymbionts at a ratio of 1.2 : 1, whereas *Symbiodinuim*-free *P. puertoricense* associates with sponges free of photo-endosymbionts at a ratio of 5 : 1, suggesting that the loss of *Symbiodinuim* or the shift in host use of *P. puertoricense* may have been a compensatory shift in symbiotic state that maintained the match between sponge and zoanthid photo-endosymbionts.

The ITS and 16S phylogenies recovered congruent symbiotic zoanthid species groups, and found the zoanthid genus *Parazoanthus* and family Parazoanthidae to be paraphyletic, a result largely congruent with hypotheses presented in previous analyses based on symbiosis similarity (with the exception of host switching *P. puertoricense*; Swain & Wulff 2007), and combined 12S and 16S mitochondrial DNA (Sinniger *et al.*, 2005). The 16S analysis found all other multi-species orders, suborders, families, and genera to be consistent with classical taxonomy, but to be inconsistent with the previous combined 12S and 16S analysis of Sinniger *et al.* (2005), which recovered clades of zoanthids representing the suborder Brachycnemina within the suborder Macrocnemina in a clade with *P. tunicans*. With regional sampling, and weak support values at some of the internal nodes in both the ITS and 16S gene trees, a better estimation of the higher-order relation-

ships awaits more extensive taxonomic sampling, which is beyond the scope of this study.

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Note added in proof

Isozoanthus sp. nov. has now been described as *Isozoanthus antumbrosus* Swain, 2009 (*Zootaxa* **2051:** 41–48).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Internal transcribed spacer region of the ribosomal RNA nuclear gene.

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