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NOTE

Christmas tree worms of Indo-Pacific coral reefs: untangling the *Spirobranchus corniculatus* (Grube, 1862) complex

Demian A. Willette[1] · Abril R. Iñiguez[1] · Elena K. Kupriyanova[2] · Craig J. Starger[3] · Tristan Varman[4] · Abdul Hamid Toha[5] · Benedict A. Maralit[5,6] · Paul H. Barber[1]

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Abstract Christmas tree worm is the common name of a group of colorful serpulid polychaetes from the genus *Spirobranchus* that are symbionts of hermatypic corals. As is increasingly common with reef-associated organisms, *Spirobranchus* is arranged as a complex of species with overlapping geographic ranges. Current species delimitations based largely on opercular morphology are problematic because of high intraspecific variation. Here, a multi-gene phylogeny of the *Spirobranchus corniculatus* complex, which tentatively includes *S. corniculatus*, *S. cruciger,* and *S. gaymardi*, sampled from the Coral Triangle, Australia, and Fiji, was reconstructed to test whether the complex includes three genetically distinct lineages identifiable by their opercula. Maximum-likelihood analyses of nuclear and mitochondrial markers revealed a single, monophyletic clade for the *S. corniculatus* complex. Furthermore, the genetic and morphological variation observed is not geographically based, indicating that the former *S. corniculatus* complex of three morphospecies is a single, morphologically variable species across the Central Indo-Pacific. Resolving the taxonomy of *S. corniculatus* presents novel opportunities to utilize this tentative bio-indicator species for monitoring reef health.

Keywords Annelida · Mitochondrial DNA · Nuclear DNA · Polychaeta

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✉ Demian A. Willette
demianwillette@hotmail.com

[1] Department of Ecology and Evolutionary Biology, University of California, Los Angeles, CA 90095, USA

[2] Australian Museum Research Institute, 6 College Street, Sydney, NSW 2010, Australia

[3] Science and Technology Policy Fellowships, Center of Science, Policy and Society Programs, American Association for the Advancement of Science, Washington, DC 20005, USA

[4] Department of Environmental Science and Management, University of Newcastle, Ourimbah, NSW 2258, Australia

[5] Fisheries Department, State University of Papua, Manokwari, Papua Barat 98314, Indonesia

[6] Genetic Fingerprinting Laboratory, National Fisheries Research and Development Institute, Mo. Ignacia Avenue, 1103 Quezon City, Philippines

Introduction

Coral reefs are complex and highly productive marine ecosystems that encompass the highest biodiversity dominated by invertebrates. Estimates of the number of species found on coral reefs range from 172,000 to over 9 million (Stella et al. 2011). Some of the most conspicuous invertebrates of shallow coral reefs are Christmas tree worms of the genus *Spirobranchus* (Polychaeta: Serpulidae). This festive common name refers to their brightly colored cones of spiralling radioles (Fig. 1), and is reserved for species of the genus *Spirobranchus* (Blainville, 1818) that are obligatory associates of hermatypic corals. However, reliable biodiversity estimates of Christmas tree worms on corals are still not available.

Previously, many broadly distributed coral reef species were believed to be circumtropical because the marine environment was seen as an open system that promoted the broad dispersal of larvae. However, with the application of genetics, these circumtropical species are increasingly found to be species complexes or even cryptic species

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Fig. 1 Color morphs of Christmas tree worms on corals reefs of Lizard Island, Australia (**a**, **b**) and Leyte, Philippines (**c**). **a**, **b**—photo A. Semenov, **c**—photo O. Paderanga

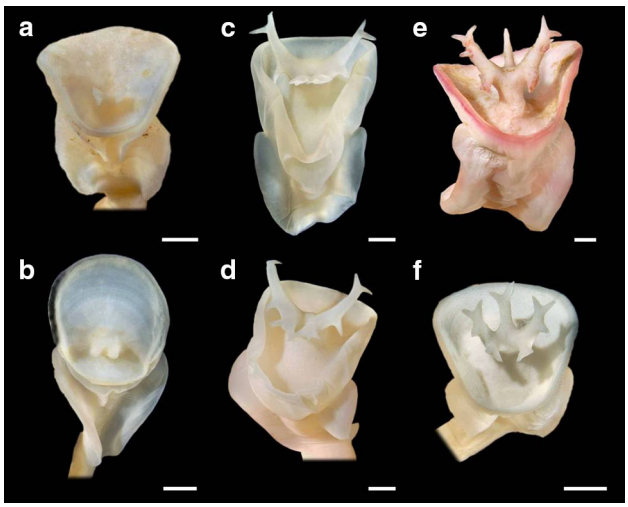

Fig. 2 Typical opercula of *Spirobranchus corniculatus* complex: **a**, **b** *S. gaymardii*, **c**, **d** *S. corniculatus*, and **e**, **f** *S. cruciger*. *Scale bar* is 1 mm. Photos: E. Wong

(Dawson and Jacobs 2001; Landry et al. 2003; Barber and Boyce 2006; Sienes et al. 2014). Polychaete taxa have been no exception with broadly distributed species being subsequently partitioned into cryptic lineages (Nygren 2014). The iconic Christmas tree worms had been indiscriminately treated as a single circumtropical species *Spirobranchus giganteus* (Pallas, 1766) until ten Hove (1970) proposed a sub-specific status, *S. giganteus corniculatus* (Grube, 1862) for the Indo-Pacific specimens. Later, ten Hove (1994) concluded that the Indo-Pacific subspecies is a complex of at least three full species, 'types A, B, and C' (sensu Smith 1985). Fiege and ten Hove (1999) re-described 'type B' as *Spirobranchus gaymardi* (Quatrefages, 1866) and stated that the *S. corniculatus* complex was comprised of *S. corniculatus*, *S. gaymardi,* and *S.* sp. 'type C', probably identical with *S. cruciger* (Grube, 1862). Currently, these three putative species are distinguished morphologically by the number and the shape of spines on their opercula (i.e., the calcified structures used as tube plugs; Smith 1985; Fig. 1). Figure 2 shows characteristic opercular types, but variability is significant, and intermediate forms are common. As a result, attributing individual specimens to one of these species based on morphology alone is not always possible.

The use of DNA sequence data over the past few decades has led to significant changes in our understanding of species diversity. Here, multi-locus genetic data were used to determine whether the *S. corniculatus* complex of Indo-Pacific reefs is a single, widely distributed species or includes three full species.

Materials and methods

Specimens were collected from the Central Indo-Pacific in Australia, Fiji, Indonesia, and the Philippines. Particular attention was given to sampling in Indonesia and the Philippines, which lie within the Coral Triangle at the epicenter of marine biodiversity (Sanciangco et al. 2013). Ninety *Spirobranchus* specimens were collected by SCUBA or snorkeling at eight sites (Table 1) and preserved in 95 % ethanol. After analysis, tissue samples were registered in specimen collections at the University of the Philippines Marine Science Institute (M. A. Juinio-Menez Lab), the University of Los Angeles (P. H. Barber Lab), and the Australian Museum.

Specimens were qualitatively assigned to one of three morphospecies according to the arrangement and shape of the opercular spines (Fig. 2; Smith 1985; Fiege and ten Hove 1999). Identification to morphospecies was reached by consensus after independent examination of the operculum by E. Kupriyanova, T. Varman, and H. A. ten Hove. All specimens had two dorso-lateral antler-like spines originating from a stem, often each spine with 2–3

Table 1 Sampling locations, approximate coordinates, total number of specimens (*N*), and number of specimens per morphospecies based on morphological features

Locality	Coordinates	*N*	*S. corniculatus*	*S. gaymardi*	*S. cruciger*
Australia—Heron Island	23°26′S, 151°54′E	7	1	0	6
Fiji—Naselesele/Navatu/Yasawas	16°59′S, 170°00′E	23	1	22	0
Indonesia—Cenderawasih	0°52′S, 134°04′E	10	0	3	7
Indonesia—Halmahera	01°40′N, 127°34′E	13	3	8	2
Indonesia—Pulau Weh	05°53′N, 95°19′E	10	2	8	0
Indonesia—Raja Ampat	00°53′N, 131°15′E	8	1	3	4
Philippines—Guimaras	10°30′N, 122°30′E	4	1	3	0
Philippines—Quezon	14°10′N, 121°55′E	15	10	4	1

secondary spinules and with a short basal dorsal tine. *Spirobranchus gaymardi* was recognized by two short, broad, dorsal tines that have blunt, abraded tips and meet above the midline. The medio-ventral spine was absent or present only as a knob (Fig. 2a, b). In *S. corniculatus,* all opercular spines were small, and the tines, if present, were narrow (Fig. 2c, d). In *S. cruciger*, dorso-lateral spines were long, unabraded tines terminated in sharp spinules, the thin dorsal tines were not abraded, with pointed tips and spaced widely apart, and the medio-ventral spine was long and branching (Fig. 2d, e).

A total of 90 *Spirobranchus* specimens were sampled from the eight locations (Table 1). Approximately 57 % of specimens were identified as *S. gaymardii*, 22 % as *S. cruciger*, and 21 % as *S. corniculatus* with at least two of the three morphospecies represented at each location (Table 1). Additionally, the geographic distribution of morphospecies was not partitioned based on locality (Fig. 3).

Total DNA was extracted in 300 µL of 5–10 % Chelex® (Bio-Rad, Hercules, CA) using a modified extraction protocol (Walsh et al. 1991). A 339 bp fragment of the cytochrome oxidase *b* (Cyt-*b*) gene was amplified using primers Cytb424F: 5′-GGWTAYGTWYTWCCWTGRGG WCARAT-3′ (Boore and Brown 2000) and cobr825: 5′-GC RTAWGCRAAWARRAARTAYCAYTCWGG-3′ (Burnette et al. 2005) and PCR conditions described by Burnette et al. (2005), except for a 42 °C annealing temperature during 15 amplification cycles and a 37 °C annealing temperature for 20 cycles. A 1670 bp fragment of the 18S nuclear rDNA region was amplified using primers F19: 5′-ACCTGGTTGATCCTGCCA-3′ (Tuberville et al. 1994) and R1843: 5′-GGATCCAAGCTTGATCCTTCTGCAG GTTCACCTAC-3′ (Elwood et al. 1985) and PCR conditions described by Lehrke et al. (2007), except for an initial 80 °C hold when the polymerase was added and a 54.4 °C annealing temperature. Similarly, a 628 bp fragment of the ITS2 nuclear rDNA region was amplified using primers ITS3: 5′-GCATCGATGAAGAACGCAGC-3′ and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ (White et al. 1990) and PCR parameters described by Halt et al. (2009), with an additional initial hold of 80 °C when the polymerase was added and an annealing temperature of 53.7 °C.

Amplification was verified via gel electrophoresis in 1 % agarose, and PCR products were sent for dye terminator sequencing at the University of California, Berkeley DNA Sequencing Facility. Sequences were assembled in Sequencher v4.8 (GeneCode, Ann Arbor, MI), aligned in Se-AlV2.0a11 (Rambaut 2002) and MUSCLE v3.8 (Edgar 2004), and deposited in GenBank (KP892762–KP892882). Both the ITS2 and 18S rDNA regions demonstrated minimal heterogeneity between alleles within a given

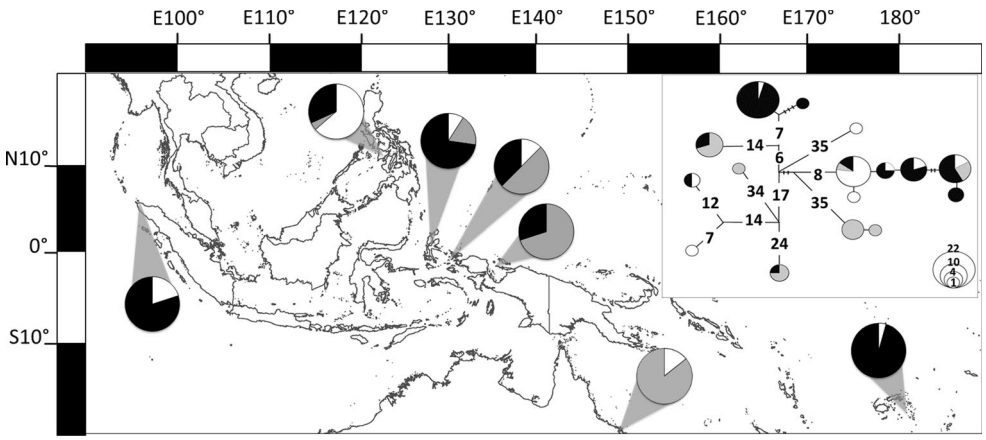

Fig. 3 Geographic distribution and number of mutational steps (haplotype network) between haplotypes of *Spirobranchus corniculatus* (*white*), *S. gaymardii* (*black*), and *S. cruciger* (*gray*)

individual and were treated as haploid sequences in subsequent analyses.

Phylogenies of *S. corniculatus* complex were inferred using maximum-likelihood analysis for the mtDNA Cyt-*b*, nDNA 18S, and rRNA ITS gene regions. The Kimura 2-Parameter distance model (Kimura 1980) identified as the best-fit model was used to estimate divergence with node support using 1000 bootstrap replicates in MEGA v5 (Tamura et al. 2011). Sequences from GenBank and unpublished Cyt-*b* region sequences were included as outgroups.

Observed variability in the mtDNA Cyt-*b* sequence data was used to explore genetic diversity among the *Spirobranchus* sequences. Sequences were collapsed to unique haplotypes then pooled by morphospecies to identify haplotype frequencies in DnaSP v5.1 (Librado and Rozas 2009). Pairwise genetic distance using the Kimura 2-Parameter distance model was calculated among pooled morphospecies and outgroups in MEGA v5. To estimate the relationship between the Cyt-*b* haplotypes, an unrooted median-joining parsimony network was constructed in NETWORK v4.6.1.1 (Fluxus Technology).

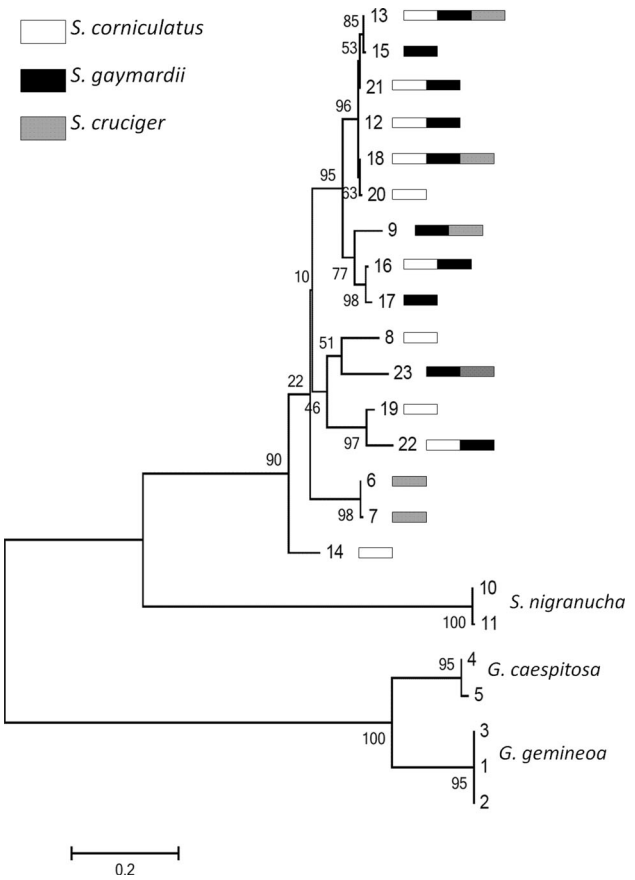

Fig. 5 Maximum-likelihood tree inferring phylogenetic relationship of three *Spirobranchus* morphospecies using Cyt-*b* sequence data. Bootstrap probability (%, 1000 replicates) shown for major clades

Results and discussion

Maximum-likelihood analyses of the nDNA ITS2 and 18S data both support a single, monophyletic clade with high bootstrap support (Fig. 4). The single clade was inclusive of all sampled specimens. Pairwise genetic distance between the three morphospecies was extremely low, ranging between 0.2 and 1.2 % (SE ± 0.2–0.3 %) for ITS2 and between 0.0 and 0.1 % (SE ± 0.0–0.1 %) for 18S sequence data. Similarly, mtDNA Cyt-*b* sequences infer a single, highly supported, monophyletic clade containing all three morphospecies (Fig. 5).

The 90 Cyt-*b* sequences collapsed to 16 haplotypes, including six singletons. No haplotypes were shared between sampling locations, except a single haplotype between Raja Ampat and Halmahera, Indonesia. Overall, samplings from three of the eight locations were composed of a single, unique haplotype. Additionally, haplotypes

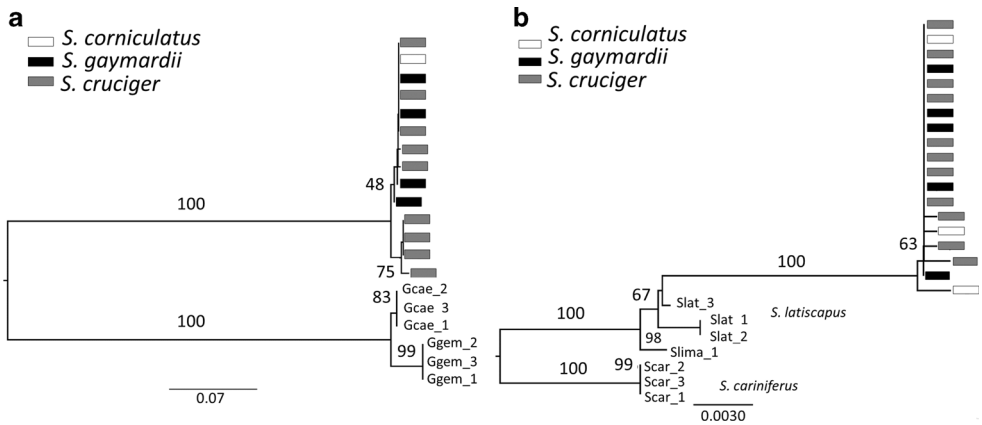

Fig. 4 Maximumlikelihood trees inferring phylogenetic relationship of three *Spirobranchus* morphospecies using **a** ITS2 and **b** 18S sequence data. Bootstrap probability (%, 1000 replicates) shown for major clades

were not partitioned by worm morphology. Rather, 56 % of haplotypes were identified from two of the distinct morphospecies, and 12 % of the haplotypes were found in representatives from all three putative taxa. This is supported by the median-joining haplotype network in which haplotype nodes containing multiple representatives of each morphospecies are separated by both few and numerous mutational steps (Fig. 3).

Mean genetic distance among the three pooled morphospecies was low (6.3–11.4 %, SE ± 0.8–1.2 %), indicating a high degree of genetic similarity. By comparison, mean genetic distance between any morphospecies and the outgroup *S. nigranucha* (Fischli, 1903) was 47.0–48.3 %, SE ± 4.9–5.1 %. These divergence data are corroborated by the high bootstrap support values between *S. nigranucha* and the single clade containing the three morphospecies (Fig. 5).

The genetic uniformity of *S. corniculatus* across the sampled Central Indo-Pacific range has implications for biodiversity of the complex. Given that genetic distance measures among morphospecies revealed minimal differentiation among samples, there is no genetic support for the hypothesis that the *S. corniculatus* complex consists of three species that can be distinguished by opercular morphology. Although sampling was limited to the Central Indo-Pacific and exclusive of the West and East Indo-Pacific regions, the observed genetic and morphological variation does not appear to be geographically based, and thus, we conclude that the former *S. corniculatus* complex of three morphospecies is in fact a single, morphologically variable species across the Central Indo-Pacific.

Previous studies on a range of polychaete taxa have shown that morphologically uniform species are often composed of a number of genetically distinct cryptic lineages (Nygren 2014). However, reports of distinct morphospecies being attributed to a single, genetically homogeneous species are less common (Meyer et al. 2008; Nygren et al. 2011; Ahrens et al. 2013). In marine systems in general, occurrences of cryptic speciation based on geographic distribution (Barroso et al. 2010; Carr et al. 2011; Thomas et al. 2014) and ecological factors (Nygren et al. 2011) are far more common. Our results do not support the morphological distinction among three species of *S. corniculatus* complex in the Indo-Pacific; instead, they re-affirm the older notion that *S. corniculatus* is a cohesive species broadly distributed across the Pacific Ocean.

A large portion of coral reef biodiversity is composed of scleractinian-associated invertebrates (Stella et al. 2011). Climate change and anthropogenic factors that threaten coral reefs also threaten these invertebrates, including symbiotic serpulid polychaetes, which could further exacerbate the loss to reef form and function (Stella et al. 2011). Assessing and monitoring reef health is important in reef recovery, and *Spirobranchus* worms have been proposed as bioindicator species for reef health (Scaps and Denis 2008; Harty 2011; Lance 2012). Further, *Spirobranchus* is an easy-to-recognize, colorful, charismatic species that would be ideal to use in volunteer-based reef monitoring programs. Our capacity to utilize *Spirobranchus* as a bioindicator of reef health must be rooted in a clear understanding of its taxonomy and biodiversity, a task now more resolved with this study's conclusion of a single, broadly distributed *Spirobranchus corniculatus* species.

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