

Original Article

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Morphological and molecular taxonomy of *Timea* (Porifera: Timeidae) from the Gulf of Mexico with the description of a new species and re-description of *T. hechteli*

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Abstract

We present the description of a new species of genus *Timea* and the re-description of *T. hechteli* from cryptic habitats in the Gulf of Mexico. *Timea alcoladoi* sp. nov. is an encrusting, pale orange sponge, with megascleres ranging from styles to subtylostyles (185–990 µm long) and a single category of microscleres, spherostrongilaster characterized by a prominent rounded centre (5–15 µm in diameter). *Timea hechteli* was established by Lehnert & Heimler, based on the description of a very small specimen, previously recorded as *Halicometes stellata* by Little, and transferred to the genus *Timea* by Hechtel. However, because the name ‘*stellata*’ was preoccupied by the type species, *T. stellata*, the species was renamed *T. hechteli*, to honour George Hechtel. After the revision of Little’s material and new material, we present the re-description of *T. hechteli*. For both species, partial sequences of COI mtDNA and ITS1-5.8S-ITS2 rDNA region were obtained in order to complement our morphological taxonomy and establish their molecular systematic position among tethyids. Tree topologies for both loci were congruent by grouping together our species as a sister group of the main Tethyida clade, which is according to the current systematics of the group. The COI fragment showed *Timea* as polyphyletic due to two sequences of *Timea* (from GenBank) that were clustered with other sponge groups. The species described here are the first record of the genus *Timea* from the Mexican coast of the Gulf of Mexico. A taxonomic key for West Atlantic *Timea* species is also presented.

Introduction

Sponges belonging to the genus *Timea* Gray, 1867 (Tethyida: Timeidae) are usually small and thinly encrusting species living in marine cryptic habitats. They are characterized by megascleres that are styles, with transitions to tylo- and subtylostyles, and microscleres that are euasters (Rützler, 2002). Originally, *Timea* was erected by Gray (1867) in the family Tethyidae, but after the *Systema Porifera* revision (Hooper & Van Soest, 2002), it was the only genus that remained in the family Timeidae, belonging to the order Hadromerida (see Rützler, 2002). However, in the most current sponge systematic restructuring, the order Hadromerida was dissolved, and the family Timeidae was relocated in the order Tethyida containing two more families, Tethyidae and Hemiasterellidae (Morrow & Cárdenas, 2015).

Currently, *Timea* contains about 57 valid species distributed around the world (Van Soest *et al.*, 2019). This number could be an underestimation, owing to the fact that cryptic habitats of the species are underexplored and that there is the potential of cryptic species complexes, such as *Timea unistellata* (Topsent, 1892) which is recorded with extremely wide distribution. The morphological taxonomy of *Timea* is mainly based on the size and shape of euasters (including small details in ornamentation). However, it is not easy to determine whether the degree of euaster morphological variation is enough to discriminate between species, or if it is the result of a high intraspecific phenotypic plasticity (Carballo & Cruz-Barraza, 2006). In most cases, the simplicity of skeletal structure does not provide enough diagnostic characters for species discrimination, but some external characteristics, such as colour and surface ornamentation, may contribute suitable information for separating species.

In the last years, molecular markers have significantly contributed to sponge systematics (see Morrow & Cárdenas, 2015) and taxonomy (e.g. Blanquer & Uriz, 2008; Cruz-Barraza *et al.*, 2017; among others). However, to date, there is only a small number of sequences for *Timea* species, making it difficult to understand its diversity and phylogenetic relationships (see Kober & Nichols, 2007; Redmond *et al.*, 2013; Lim *et al.*, 2017).

Currently, 21 species of *Timea* are known from the Western Atlantic (Macola & Menegola, 2018; see discussion), of which only one species has been recorded in the Gulf of Mexico (Van Soest *et al.*, 2019) (USA coast). Here we report the first records of the genus *Timea* from



the Mexican coast of the Gulf of Mexico, including the description of a new species and the re-description of *T. hechteli* Lehnert & Heimler, 2001, based on old and new material. Additionally, we obtained partial sequences of mitochondrial COI mtDNA ('DNA Barcoding') and ITS's ribosomal region rDNA of these species, aimed to complement the morphological interpretation and taxonomic assignation of our species into *Timea* (integrative taxonomy). Also, we give information about their phylogenetic relationships and systematics position with respect to other Tethyida. Finally, we include a taxonomic key, following Lehnert & Heimler's (2001) and Van Soest's (2009) efforts for the Central West Atlantic, which are based mainly on euaster morphology.

Materials and methods

Specimen collection and morphological analyses

Six *Timea* specimens were collected from shallow-water rocky-sedimentary substrates by snorkelling at two locations in Campeche State (south-eastern Gulf of Mexico) (Figure 1). Specimens and spicule slides analysed have been deposited in the 'Colección de Esponjas' (LEB-ICML-UNAM), of the Instituto de Ciencias del Mar y Limnología, UNAM, in Mazatlán (Mexico). The type material of *T. hechteli* is located in the Smithsonian's National Museum of Natural History (USNM).

Collected specimens were labelled and small fragments of fresh samples were preserved in 100% ethanol for molecular analysis. Then, specimens were fixed in 4% formaldehyde for 24 h, and transferred to 70% ethanol for storage. External morphology and skeletal elements and their arrangement were recorded for each individual. Spicule preparation followed the techniques described by Carballo & Cruz-Barraza (2008) for light and scanning electron microscopy (SEM).

Twenty-five or more spicules chosen at random were measured for each specimen studied. Megasclere measurements were taken to include total length widths of shaft and head; microsclere (euaster) measurements are of the total diameter. In each description and in the tables, the number in parentheses is the mean of all measurements. Sponge-specific terms are used according to Boury-Esnault & Rützler (1997), but also from the most current literature related to genus *Timea*.

DNA purification, amplification and sequencing

Total genomic DNA was extracted from four specimens of both species, following the steps described in detail in Cruz-Barraza *et al.* (2017). Originally, we tried to obtain different loci fragments from mitochondrial and ribosomal DNA using several sets of primers, but after numerous assays, we only could amplify the COI standard barcoding fragment for the holotype of *Timea alcoladoi* sp. nov. and two specimens of *T. hechteli*, and the ITS1-5.8S-ITS2 rDNA for the holotype of *Timea alcoladoi* sp. nov., and three specimens of *T. hechteli*. For COI mtDNA we used primers PLAKLCOdegF (modified from Folmer *et al.*, 1994 by Cruz-Barraza *et al.*, 2014): 5'-TCW ACD AAY CAT AAA GAY ATW GG-3'; and C1J2165-R (from Misof *et al.*, 2000 modified as reverse): 5'-CCN GGT AAAATT AAAATA TAA ACT TC-3'. For the ITS1-5.8S-ITS2 gene ribosomal region, we used primers from White *et al.* (1990), universal primers ITS4 5'-TCC TCC GCT TAT TGA TAT GC-3' and ITS5 5'-GGA AGT AAA AGT CGT AAC AAG G-3'. PCR reactions were carried out in a volume of 12.5 µl and consisted of 6.0 µl distilled H₂O (sterile MilliQ), 0.75 µl deoxyribonucleotide triphosphates (0.2 mM), 0.75 µl MgCl₂ (8 mM), 0.70 µl of each primer (10 µM), 2.50 µl 5× PCR buffer (Promega), 0.2 µl Taq DNA polymerase and 1 µl genomic DNA (c. 50–100 ng). Thermal cycling conditions were: initial denaturation step at 94°C for 2 min, and 35 cycles of 94°C for

30 s, 50°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 5 min. PCR-products were run in a 1.5% agarose gel to corroborate the positive amplification. Products were purified using the Wizard purification kit (Promega) and sequenced in both directions using Applied Biosystems 3730xl DNA analysers by Macrogen, Korea.

Sequence analyses

Sequences were edited with the program Codon Code Aligner 7.1.2 (CodonCode Corporation), and verified with the bioinformatics tool BLAST (Search National Centre for Biotechnology Information/Blast) to confirm the species identity. Phylogenetic reconstructions (COI mtDNA and ITS rDNA) were made separately and included all available sequences from order Tethyida in GenBank. For COI analysis, additional sequences from other Heteroscleromorpha groups were included, because two sequences of *Timea* (obtained from GenBank) showed a closer relationship with Poecilosclerida than Tethyida. Here, we used the freshwater sponge *Racekiela montemflumina* Carballo, Cruz-Barraza, Yáñez & Gómez, 2018 to root the tree, because recent studies have suggested Haplosclerida as the sister group to the remaining Heteroscleromorpha (Lavrov *et al.*, 2008; Morrow & Cárdenas, 2015). For the ribosomal region, a few sequences of species of Order Clionaida were used as outgroups. Owing to the low number of available sequences of *Timea*, we included original sequences (COI and ITSs) of a specimen of *Timea* cf. *authia* from Eastern Pacific Ocean, only to strengthen the Timeidae clade. The specimen is characterized by small tylasters from 12.5–22.5 µm in diameter, and tylostyles from 345–750 µm length.

Sequences were initially aligned in Mega 7 (Kumar *et al.*, 2016), using the CLUSTALW alignment under the default opening-gap extension parameters (15.0–6.66). For analysis of ribosomal region, we also used the program Gblocks 0.91b (Castresana, 2000) in order to determine and exclude ambiguously aligned regions under the following parameters: minimum number of sequences for a conserved position: seven; minimum number of sequences for a flanking position: 11; maximum number of contiguous non-conserved positions: eight; minimum length of a block: 10; allowed gap positions: with half; use similarity matrices: yes. For both loci, Bayesian inference analysis was performed with MrBayes 3.2.1 (Ronquist & Huelsenbeck, 2003) using the Hasegawa–Kishino–Yano plus Invariant sites plus Gamma distributed model of sequence evolution, which was selected through the JModelTest 2.0.1 program (Posada, 2008) using the Bayesian information criterion. The program was run with four simultaneous Monte Carlo Markov chains for 10,000,000 generations, which were sampled every 200 trees with a burn-in of 20%. In addition, maximum likelihood (ML) analyses were generated with RAxML 8.1.11 (Stamatakis, 2014) on the CIPRES science gateway v.3.3 portal (<http://www.phylo.org>) (Miller *et al.*, 2010).

Sequences of the species presented here are available in the database of GenBank with the codes: *Timea alcoladoi* sp. nov. holotype LEB-ICML-UNAM-3140 (COI: MN562235, ITS: MN556959) and *T. hechteli*, specimens: LEB-ICML-UNAM-2232 (COI: MN562233, ITS: MN556962), LEB-ICML-UNAM-2573 (COI: MN562234), LEB-ICML-UNAM-2878 (ITS: MN556961) and LEB-ICML-UNAM-3131 (ITS: MN556963). Additional sequences of *Timea* cf. *authia* LEB-ICML-UNAM-1950 also are available (COI: MN562236, ITS: MN556960).

Results

SYSTEMATICS

Order TETHYIDA Morrow & Cárdenas, 2015
Family TIMEIDAE Topsent, 1928

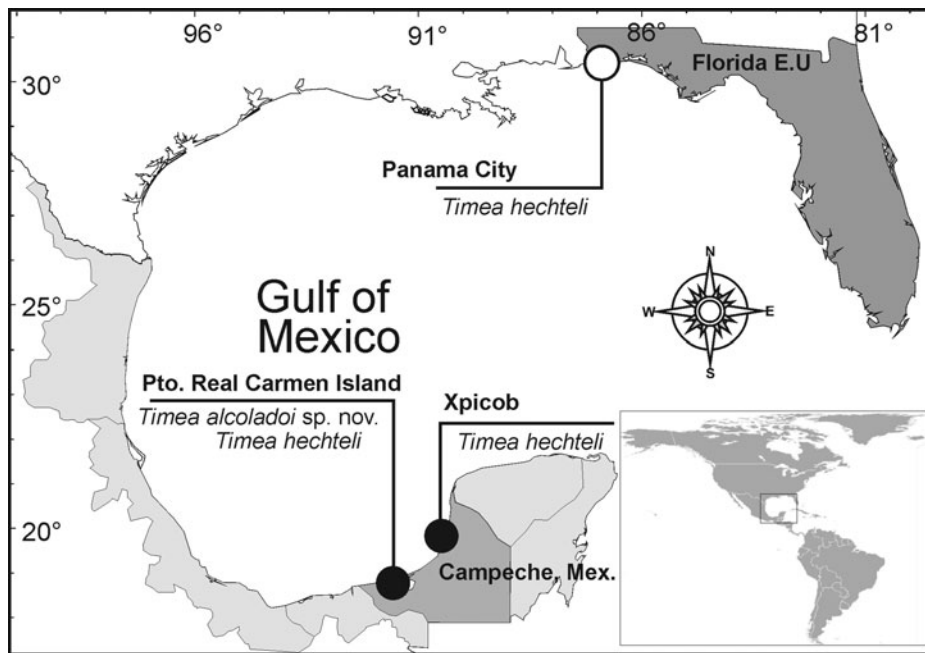


Fig. 1. Sampling localities and distribution of *Timea alcaladoi* sp. nov. and *T. hechteli* along the coast of the Gulf of Mexico. The black circles show sample localities for this study, and the white circle shows the type locality of *T. hechteli*.

Diagnosis: Tethyida presenting encrusting growth form with tylostyles arranged in tracts ending as bouquets, which generally pierce the surface (hispid). Tracts may be absent, in which case tylostyles are organized as bouquets. Additional tylostyles may occur in criss-cross fashion between the tracts or sparsely disposed tangentially to the surface or to the substrate. Microscleres are euasters dispersed in the choanosome, denser at the substrate and towards the surface, and forming a crust at the surface (Leite *et al.*, 2015).

Genus *Timea* Gray, 1867

Diagnosis: Same as family.

Timea alcaladoi sp. nov.
(Figures 2 & 3)

ZooBank LSID for the new species: urn:lsid:zoobank.org:act:2D31E3DB-B780-4435-BFBD-0E67D4ED6F87

MATERIAL EXAMINED

Holotype: LEB-ICML-UNAM-3140, Puerto Real, Isla del Carmen (Campeche, Mexico), 18°46'44.1"N 91°31'51"W, 11.4.2015, 0.5–1 m depth. Paratype: LEB-ICML-UNAM-3141, Puerto Real, Isla del Carmen (Campeche, Mexico), 18°46'44.1"N 91°31'51"W, 11.4.2015, 0.5–1 m depth.

MATERIAL EXAMINED FOR COMPARISON

Paratypes of *Timea chiasterina* Carballo & Cruz-Barraza, 2006, LEB-ICML-UNAM-244, Isla Tunosa (Topolobampo, Sinaloa, Mexico), 25°34'58"N 109°00'51"W, 22.6.2000, 1.5 m depth, under rocks. LEB-ICML-UNAM-1174 and ICML-UNAM-1175, Antiguo Corral del Risco (Punta Mita, Nayarit, Mexico), 20°46'20"N 105°32'49"W, 18.7.2005, 2 m depth, on rocks.

Diagnosis. Small and thinly encrusting orange sponge. Megascleres are styles straight or slightly curved, sometimes with very incipient heads 'subtylostyles' (from 185–1010 µm length). Microscleres are spherostrogylasters (type chiaster) (from 5–15 µm diameter), with a well-marked rounded centre and typically six rays (although spicules with four and seven rays also are present), which end in small bouquets of diverging conical spines.

Description. Thickly encrusting specimens, from 1–3.5 mm in thickness, covering a surface of 3 × 5 cm (holotype) and 2 × 3 cm

(paratype) on rocks (Figure 2A). The specimens broke up into several fragments, 1–2 cm, owing to the high content of foreign material embedded in the choanosome (Figure 2B, C). The surface is slightly micro-conulose and hispid, with some small sub-ectosomal channels (<1 mm in diameter). Conules are 300–550 µm in diameter and are characterized by the tips of the choanosomal megasclere tracts protruding in bouquets, from 100–350 µm. The consistency is firm to hard but fragile and brittle. Colour in life is orange and light brown in alcohol (Figure 2A, B).

Skeleton. It is difficult to observe due to a dense concentration of foreign material (Figure 2C–E), but it is possible to distinguish a very thin sub-ectosomal layer from 0.3–1 mm in thickness (Figure 2D), with styles arranged in tracts (from 80–150 µm in diameter) ending as bouquets at the top of superficial conules, giving a hispid aspect to sponge surface. Megascleres are also present free or forming vague tracts between foreign material in the choanosome. Spherostrogylasters are distributed throughout the entire sponge body but more densely towards the surface and around the foreign material (Figure 2E).

Spicules. Megascleres are mostly styles with a straight to slightly curved or even flexuous shaft and a sharp point, although some of them show a small annular rim in the upper end, turning them to incipient subtylostyles (Figures 2F & 3A, Table 1). Length: 185–(571)–1010 µm; shaft diameter: 4–(10)–20 µm. Microscleres are small strongylasters (type chiaster), very homogenous in size. They are characterized by a spherical, well-marked centrum 'spherostrogylaster' commonly with six rays (Figure 3B), although there also are with seven (Figure 3C) and four (Figure 3D) rays, in less proportion. The rays are typically short and robust, ending in bouquets with diverging conical spines (Figure 3B, Table 1). Rarely, one of the rays ends in a sharp point. Measurements: 5–(10.3)–15 µm.

Etymology. The species is named after Dr Pedro Alcolado, from La Habana, Cuba, for his substantial contributions to our knowledge of the Caribbean sponge fauna.

Distribution and ecology. Specimens of this species are difficult to observe *in vivo* because they are commonly almost completely covered by sediments. A large number of sites were sampled

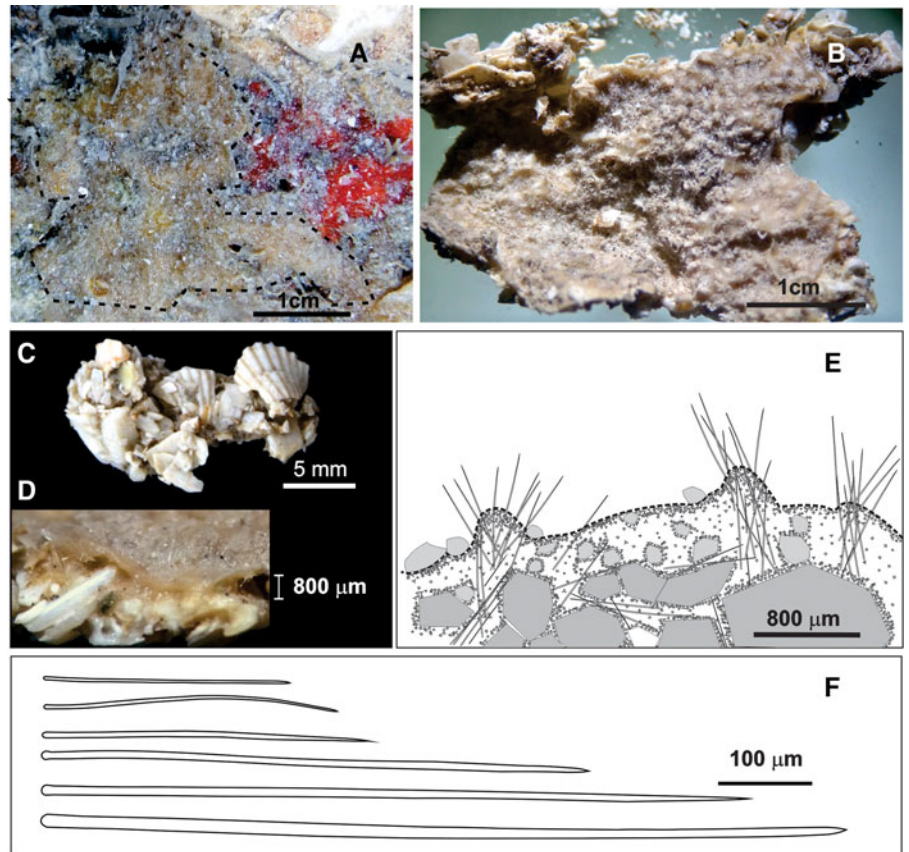


Fig. 2. External morphology and skeletal characteristics of *Timea alcoladoi* sp. nov.: (A, B) alive and preserved holotype (LEB-ICML-UNAM-3140); (C, D) View of the bases and cross-section of a fragment of holotype, showing the dense proportion of sand and shell fragments in the sponge's body; (E) Drawings of cross-section of the skeletal structure; (F) Drawings of spicule styles and subtylostyles morphologies.

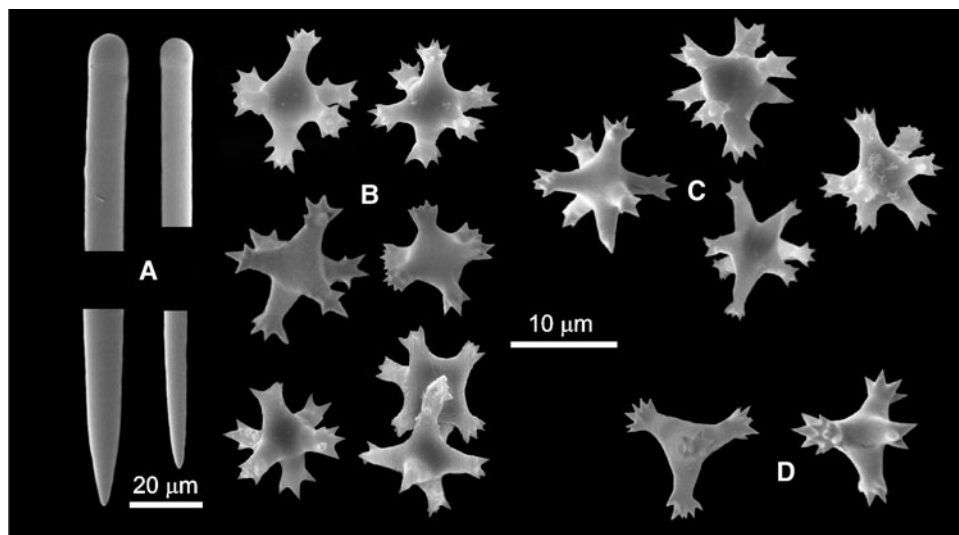


Fig. 3. Scanning electron microscopy images of spicules of *Timea alcoladoi* sp. nov.: (A) head of the style subtylostyles; (B–D) spherostrogylasters; (B) typical with 6 rays; (C) with 7 rays; (D) with 4 rays.

along the coast of the Gulf of Mexico and Mexican Caribbean, but the species was found only in shallow waters, about 1.5 m depth, encrusting the lower rock surfaces on sand-muddy bottoms at Puerto Real, Campeche (Figure 1).

Remarks. *Timea alcoladoi* sp. nov. is characterized by the presence of megascleres in the form of styles, some of them with a transition to incipient subtylostyles, and by a single category of microscleres, spherostrogylasters, which are very homogenous in size and shape (see Table 1).

The morphologically closest species to *Timea alcoladoi* sp. nov. is *Timea chiasterina*, described from the Gulf of California

(Mexican Pacific). Both species possess a single category of microscleres euasters similar in morphology. However, they differ in external morphology: *Timea alcoladoi* sp. nov. presents a granular surface (Figure 2A, B), while *T. chiasterina* is characterized by a relatively smooth surface (Carballo & Cruz-Barraza, 2006). Megascleres are also different between both species, *T. alcoladoi* sp. nov. presents mostly styles (sometimes with a small annular rim), which are wider and with a larger range of size than those of *T. chiasterina* which are thinner tylostyles (with well-rounded heads) with smaller range of size ($185\text{--}1010 \times 4\text{--}20 \mu\text{m}$ vs $92\text{--}750 \times 2\text{--}13$; heads from $3\text{--}15 \mu\text{m}$, respectively, Table 1). Microscleres in both species are also similar in size and general

Table 1. Comparative data for the dimensions of the spicules (in μm) and distribution of *Timea alcoladoi* sp. nov. and the morphologically closest species *Timea chiasterina* Carballo & Cruz Barraza, 2006. Shaft length \times width; head diameter is given for megascleres and diameter for euasters

<i>Timea alcoladoi</i> sp. nov.	Reference	Colour	Styles	Tylostyles	Strongylaster	Distribution
Holotype LEB-ICML-UNAM-3140	Present study	Orange	185(591)1010 \times 4(9.2)20		5(10)15	Mexican coast from Gulf of Mexico
Paratype LEB-ICML-UNAM-3141	Present study	Orange	210(612)1000 \times 4(11)20		6.5(10.5)14	Mexican coast from Gulf of Mexico
Type material of <i>T. chiasterina</i> for comparison to <i>T. alcoladoi</i> sp. nov.						
Holotype: MNCN 1.01/348	Carballo & Cruz-Barraza (2006)	Dark yellowish to orange		92.5(426)750 \times 5(8.5)13; 6(10)15	8.5(11.3)15	Topolobampo, Sinaloa, Mexico
Paratype: LEB-ICML- UNAM-244	Carballo & Cruz-Barraza (2006)	Dark yellowish to orange		100(360)615 \times 3(6.2)10; 5(8)12.5	8(12)15	Topolobampo, Sinaloa, Mexico
	Present study			92(234)390 \times 3.8(5.6)7.5; 5(7.9)10	7.5(9.8)12.5	
Paratype: LEB-ICML- UNAM-1174	Carballo & Cruz-Barraza (2006)	Dark yellowish to orange		152(246)350 \times 2.5(2.7)4.5; 3.8(5)8	10(12.5)15	Punta Mita, Nayarit, Mexico
	Present study			115(241)410 \times 2(3.4)7; 2(5)9	5(10.5)15	
Paratype: LEB-ICML- UNAM-1175	Carballo & Cruz-Barraza (2006)	Dark yellowish to orange		155(313)545 \times 2.5(4)5.5; 3(6.4)10	8(12)15	Punta Mita, Nayarit, Mexico
	Present study			125(295)525 \times 1(3.8)–6; 3(6.1)10	5.8(9.8)12.5	

Values in parentheses are means.

morphology, but in *T. chiasterina* the strongylasters possess commonly 7 and 8 (in a range of 6–9) large and thin rays diverging from a small centrum, while in the new species, the strongylaster's centre is commonly stout and wide, typically with 6 (in a range of 4–7) short and wide rays. After a detailed revision of slides of both species, we never found strongylasters of four rays in *T. chiasterina*, whereas those of 8 or 9 rays were absent in the new species.

The geographic distance and continental barrier that separates both species are other important factors to consider in species differentiation, especially considering that following sampling along the Mexican Pacific and Atlantic coasts, species present a very restricted distribution (*T. chiasterina* in two localities in the Gulf of California vs *T. alcoladoi* sp. nov., in one locality from the south of Gulf of Mexico). Contemporary authors also have discussed the importance of the Isthmus of Panama (raised about 3.5 MA ago) for Atlantic and Pacific sponge fauna, and the possible absence of connectivity of putative conspecific sponge populations from both sides of the Isthmus (Boury-Esnault *et al.*, 1999), preferring to keep separated names for related species (see Hajdu & Rützler, 1998; Carballo & Cruz-Barraza, 2010).

Another *Timea* species morphologically close to *Timea alcoladoi* sp. nov. is *T. tylasterina* Van Soest, 2017 from the Guyana Shelf. This species is characterized by the presence of tylostyles and tylasters. It differs from *Timea alcoladoi* sp. nov. by its tylostyles (characterized by a rather elongate tyle), which are very much smaller than the styles of *T. alcoladoi* sp. nov. (204–388 \times 2–(3.2)–4 μm vs 140–1000 \times 4–(11)–20 μm , respectively). *Timea tylasterina* also possesses two categories of tylasters, the larger one (13–15.5 μm) 8–10 rays ending in a crown with 3–7 spines, and the smaller one (7–10 μm) with 7–11 short rays (Table 2), each ending in a profusely spined crown, often with double circles of spines. *Timea alcoladoi* sp. nov. possesses a single category, which is very consistent in morphology, with stout centre and commonly 6 (less commonly 4 and 7) divergent rays.

Other *Timea*-species bearing a variety of tylasters to strongylasters (9 spp.) have been described from the Western Atlantic, but they differ from *Timea alcoladoi* sp. nov. by possessing at least one other complementary euaster category (see also Table 2):

T. hechteli Lehnert & Heimler, 2001, also has oxyasters (see next description); *T. bioxyasterina* Mothes, Santos & Campos, 2004, additionally has two oxyasters categories; *T. berlincki* Leite, Fonseca, Leal & Hajdu, 2015, has acanthose spheroxyasters, with conical rays ending in sharp or blunt extremities; *T. clandestina* Leite, Fonseca, Leal & Hajdu, 2015 has spherostylasters (with smooth conical rays) to tylasters (rays ending in extremities with distally oriented spines); *T. curacaoensis* Van Soest, 2009, has a large and not truly asymmetrical euaster category, with branching rays, proliferated at their endings;

Timea perastra de Laubenfels, 1936, possesses a combination of tylasters of 12 rays and 12 μm in diameter, and armed asters from 8 μm in diameter; *T. secirm* Moraes, 2011 possesses microspined oxyspherasters, and finally, *T. parasitica* (Higgin, 1877) has spheroxyasters. The other species of *Timea* known from the Western Atlantic (12 spp.) are easy to differentiate from *Timea alcoladoi* sp. nov. because they present a very different variety of euasters morphology.

Timea hechteli Lehnert & Heimler, 2001 (Figures 4 & 5)

Synonymy.

Halicometes stellata sensu Little, 1963, p. 56.

Halicometes stellata sensu de Laubenfels, 1950, p. 99.

Non *Halicometes stellata* (Schmidt, 1870)

MATERIAL EXAMINED

Holotype: USNM 23571. Panama City (Florida, USA), 1958 collected by Dr Meredith Jones.

New material: LEB-ICML-UNAM-2232, Puerto Real, Isla del Carmen (Campeche), 18°46'38"N 91°31'47.4"W, 6.3.2013, 1.5 m depth. LEB-ICML-UNAM-2573, XPICOB (Campeche), 19°43'15.89"N 90°40'9.11"W, 7.10.2013, 0.5 m depth. LEB-ICML-UNAM-3131, Puerto Real, Isla del Carmen (Campeche), 18°46'44.1"N 91°31'51"W 11.4.2014, 1.5 m depth. LEB-ICML-

Table 2. Comparative data for the dimensions of the spicules (in μm) and distribution of *Timea hechteli* Lehnert & Heimler, 2001 re-described here, in addition to some species of *Timea* for comparison with *T. alcoladoi* sp. nov. Shaft length \times width; head diameter is given for megascleres and diameter for euasters

<i>Timea</i> species	Reference	Colour	Tylostyles	Tylasters	Oxyasters	Strongylasters	Distribution
<i>T. hechteli</i> Lehnert & Heimler, 2001	Little (1963)		211(569)1014 \times 3(7.1)12		18(26.4)33	7(12.8)22	Florida
<i>T. hechteli</i> LEB-ICML-UNAM-2232	Present study	Ochre light yellow	430(702)950 \times 10(12.6)18 \times 12(14.8)20		10(21.5)30	5(11)20	Mexican coast from Gulf of Mexico
<i>T. hechteli</i> LEB-ICML-UNAM-2573	Present study	Ochre light yellow	410(818)1100 \times 8(15.7)20 \times 10(18.1)25		12.5(25.1)35	5(11)22.5	Mexican coast from Gulf of Mexico
<i>T. hechteli</i> LEB-ICML-UNAM-3131	Present study	Ochre light yellow	450(818.5)1150 \times 8(13)20 \times 10(16)22		7.5(20.2)30	3.7(11)20	Mexican coast from Gulf of Mexico
<i>T. tylasterina</i> Soest, 2017	Van Soest (2017)	Whitish to greyish in alcohol	204(292)388 \times 2(3.2)4	I: 13(14.1)15.5 II: 7(8.4)10			Guyana
<i>T. bioxyasterina</i> Mothes et al., 2004	Leite et al. (2015)	Light-brown in alcohol	304(597.5)769.5 \times 2.5(12.3)17.5; 6.3(10.5)15	2.4(3.6)5	I: 32(62)85.8 II: 16.1(20.7)27.6		North Brazil
<i>T. berlincki</i> Leite et al., 2015,	Leite et al. (2015)	Orange-brown	204(454)630 \times 2.4(7)9.6; 4.8(8)9.6	8.5(12.3)15.3	9.4(11)13.4		South-east Brazil
<i>T. clandestina</i> Leite et al., 2015	Leite et al. (2015)	Greyish beige in alcohol	310(382)436 \times 2.4(4.8)7.2; 4.8(6.9)9.6			6(12.3)20.4	South-east Brazil
<i>T. curacaoensis</i> Van Soest, 2009	Van Soest, 2009	White in alcohol	299(834.2)1357 \times 2(8.2)14	5.5–6.0–7.5	Branched asters: 14–19.2–23		Curaçao
<i>T. perastra</i> de Laubenfels, 1936	Leite et al. (2015)	Yellow	540(690)1888 \times 7(9)13	12	Armed Asters: 8		Bahamas
<i>T. secirm</i> Moraes, 2011	Leite et al. (2015)	Yellow	200(429)715 \times 1(4)7		12(18)28	4–7–11	North Brazil
<i>T. parasitica</i> (Higgin, 1877)	Leite et al. (2015)		580 \times 7.3	13	:>26		Bahamas

Values in parentheses are means.

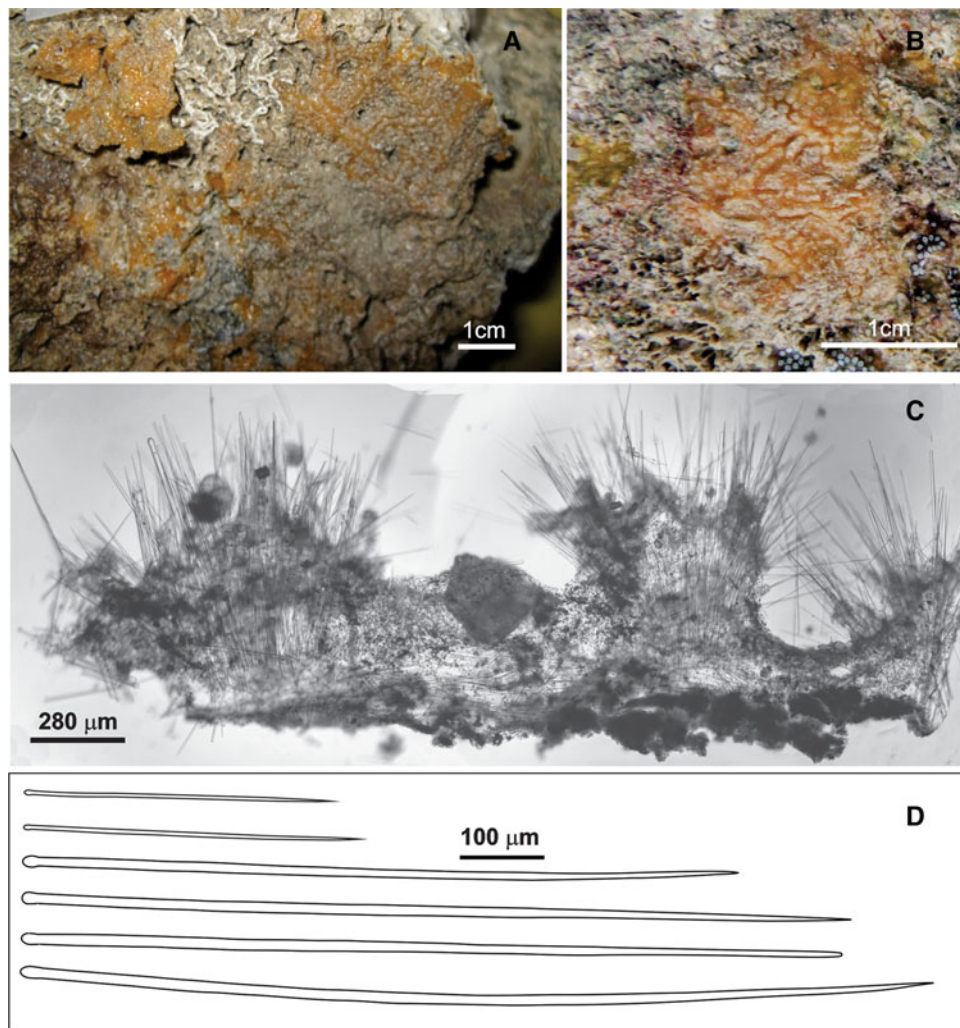


Fig. 4. External morphology and skeletal characteristics of *Timea hechteli*: (A, B) alive specimens LEB-ICML-UNAM-2232 and LEB-ICML-UNAM-2573, respectively; (C) light microscopy (LM) image of cross section of the skeletal structure; (D) Drawings of spicule tylostyles morphologies.

UNAM-3147, Puerto Real, Isla del Carmen (Campeche), 18°46'38"N 91°31'47.4"W, 2.2.2017, 1.5 m depth.

Description. Encrusting sponge, 8 mm thick, covering areas of 8 × 5 cm, over rocks. Specimens are almost completely covered by sediment, and they are visible mainly by orange meandering ectosomal channels, which ornament the surface (Figure 4A, B). The surface is smooth to slightly conulose to the naked eye, but under the microscope is unevenly hispid with the tips of the tylostyles protruding from the surface as small bouquets. Oscula are oval-circular shaped from 1–3 mm in diameter. Consistency is firm but easy to tear. The specimens are ochre to light yellow in life and pale ochre when preserved.

Skeleton. The skeleton is of the typical *Timea* structure, with tylostyle tracts (from 150–300 µm) distributed perpendicularly to the sponge surface, where they diverge into brushes, giving the surface a hispid appearance; length: 150–600 µm. Tylostyles free or forming tracts also are distributed parallel to the substrate surface. Euasters are randomly distributed throughout the sponge body but mainly concentrated in the sponge surface and near the substrate (Figure 4C).

Spicules. Tylostyles are mostly slightly curved or straight (Figure 5B), with a generally well-formed spherical or oval head (Figures 4D, 5A, D, G). They measure 410–(79)–1150 µm length,

8–(13.7)–20 µm shaft width, 10–(16.3)–25 µm head width (Table 2). Microscleres are euasters in two morphological categories. (I) Oxyaster, with about 10 large and thick, straight or slightly curved rays ending in sharp point (Figure 5B, E, H). In some stage of development (less common), euasters are oxysphaerasters, with a large centrum and short and stout, and sometimes mucronate rays, which end in sharp or rounded points (Figure 4E). Measurements: 7.5–(22.3)–35 µm diameter. (II) Strongylaster (type chiaster), very variable in size, but only one size category, with 8–10 stout rays diverging from a slender to moderated centre, and ending in small bouquets of 3–5 diverging, short conical spines (Figure 5C, F, I). Measurements: 3.7–(11)–22.5 µm diameter.

Ecology and distribution. *Timea hechteli* has never been recorded since its original description (as *Halicometes stellata*) by Little (1963) in Panama City, Florida, USA, in the northern Gulf of Mexico (Figure 1). Specimens in this study were found in Puerto Real (Isla del Carmen) and XPICOB, at Campeche State (Gulf of Mexico). The species was found on rocks almost completely covered by sediment, living in shallow waters from 0.5 to 1 m depth, in sedimentary environments with strong wave action.

Remarks. Little (1963) recorded *Halicometes stellata* from Panama City, Florida (northern Gulf of Mexico) based on some

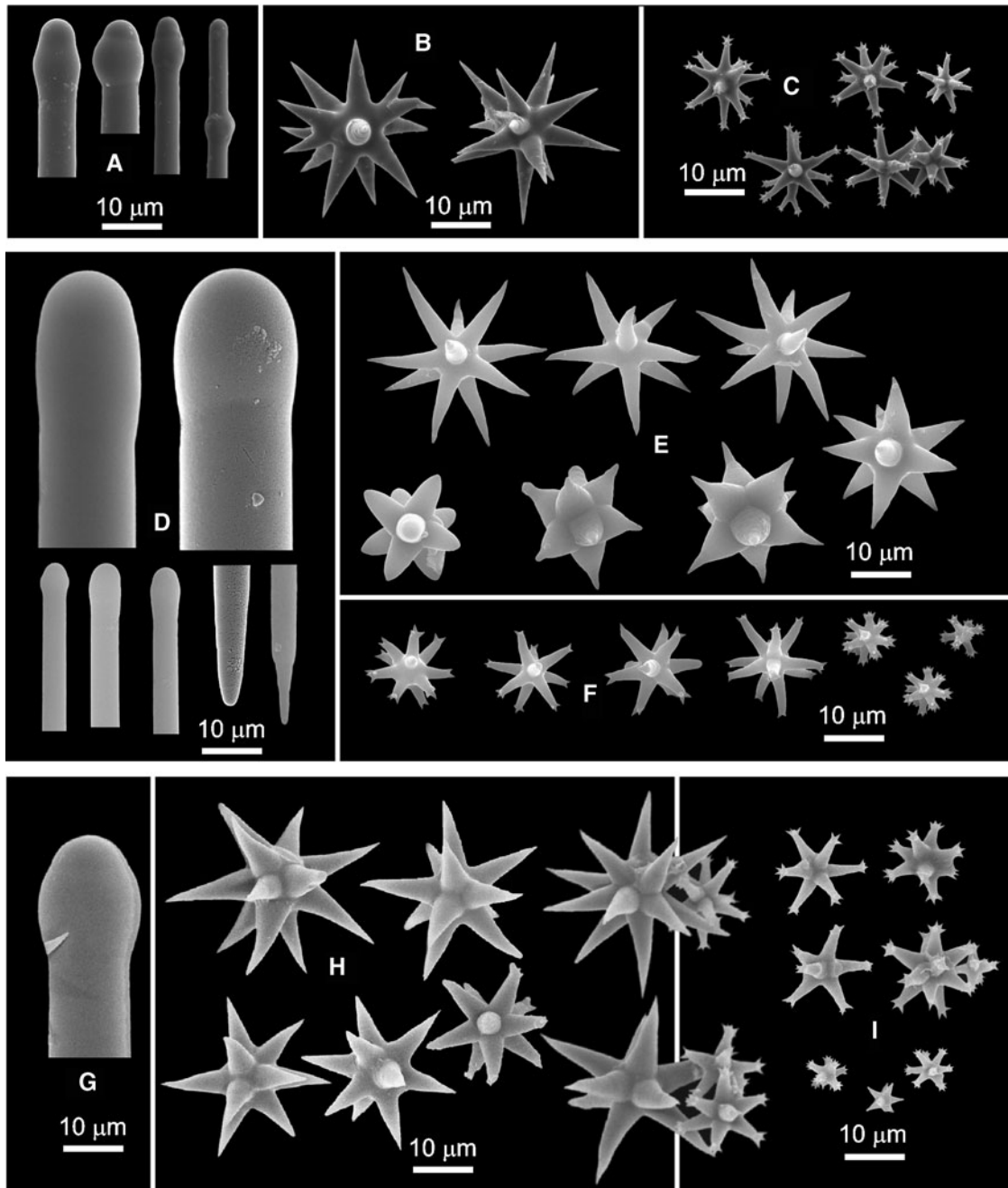


Fig. 5. Scanning electron microscopy images of spicules of *Timea hechteli*: (A–C) spicules of the Holotype (recorded as *Halicometes stellata* USNM 23571); (D–F) spicules from specimen LEB-ICML-UNAM-2232; (G–I) spicules from specimen LEB-ICML-UNAM-2573. (A, D, G) details of heads and ends of the subtylostyles; (B, E, H) smooth oxyasters; (C, F, I) spiny strongylasters.

very small encrusting specimens which, according to the author, agree very well with the description of specimens from the Bermudas (de Laubenfels, 1950). Later on, Hechtel (1969) transferred the records by Little (1963) and de Laubenfels (1950) to the genus *Timea*, arguing that these represent a species with two euaster categories, but he did not propose a species assignment. In *Timea*, the specific name 'stellata' was already preoccupied by the type species *T. stellata* (Bowerbank, 1866), then Lehnert & Heimler (2001) assigned a new name, *Timea hechteli*, to Little's (1963) specimens. By giving this name, they erected a new species, but without a formal description of the type specimen (holotype). Due to the fact that Lehnert & Heimler (2001) only referred to the record of *Halicometes stellata sensu* Little, it became the type specimen of *T. hechteli*.

Little (1963) described a small specimen, 0.5–1.5 cm in diameter and 2–5 mm in height, which was used up for the

spicule slide preparation. However, a very small portion of a specimen (about 3 mm in diameter) was found in the Invertebrate Zoology Collections in the Smithsonian National Museum of Natural History (Catalogue Number USNM 23571), which was used here to make a spicule slide and SEM photomicrographs, corroborating that the specimen corresponds to Little's original description. Little (1963) stated that the species possesses tylostyles (211–(569.1)–1015 µm length), a few of which approach the shape of stylotes, which was confirmed by review of the spicules slide (original specimen), and in our own specimens (see Table 2 and Figure 5). The euasters were separated by Little into two types, I, oxyspherasters (18–33 µm, 26.4 µm average diameter) and II, chiasters (7 to 22 µm, 12.8 µm on average). Measurements were confirmed and complemented during the review of type material and agree with our specimens (see Table 2 and Figure 5). The

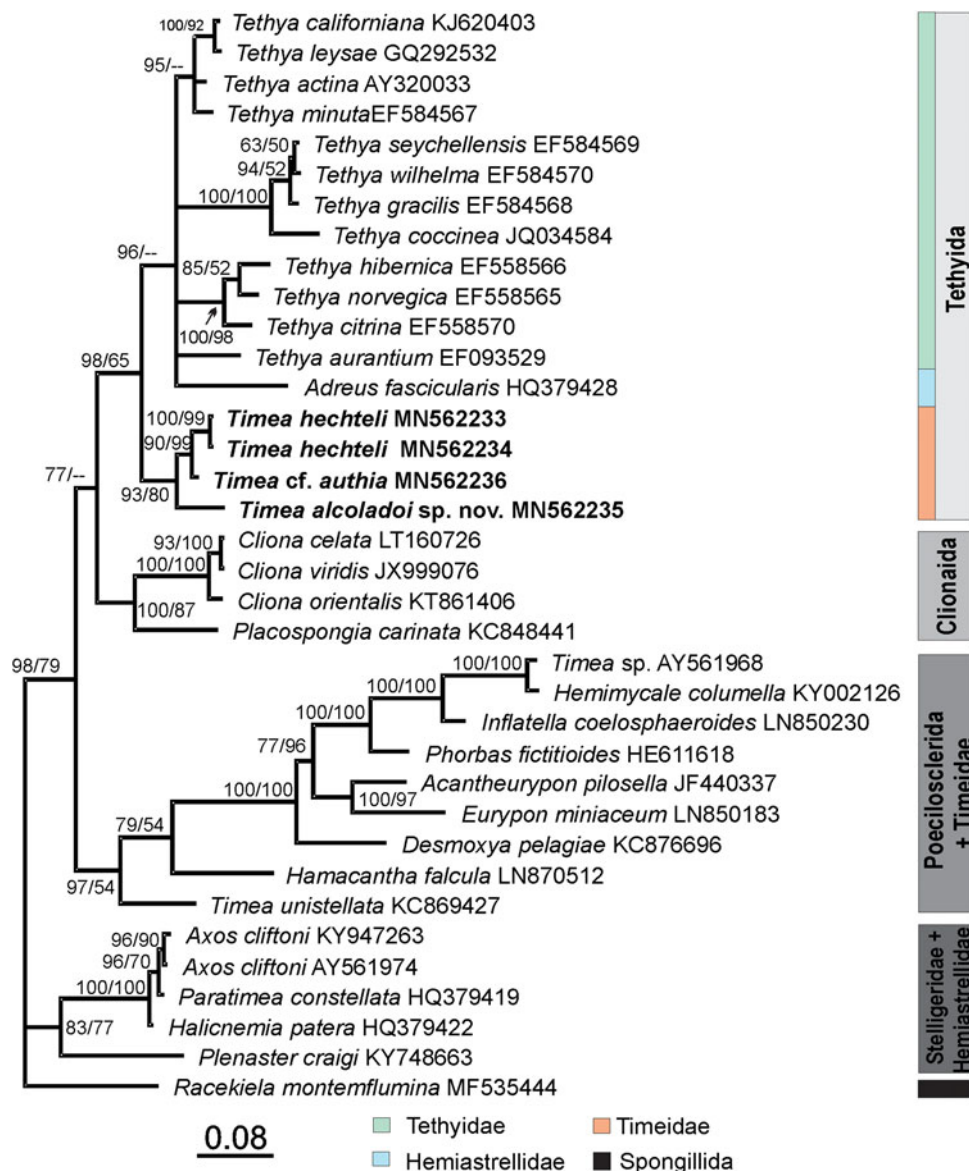


Fig. 6. Tree topology of COI mtDNA fragment. The tree presented was obtained by Bayesian inference (MrBayes) and includes Maximum likelihood (RA × ML) values. The number at each node represents the posterior probability (%) (BI), followed by the Bootstrap proportion (ML); a (-) indicates that a particular analysis supported the node at less than 50%, or supported an alternative phylogenetic arrangement in ML tree. The GenBank sequence accession code is included after each species name.

species is well-differentiated from other *Timea*-species from the Western Atlantic by its typical spicular characteristics, specifically in size and shape (see Table 2).

Molecular taxonomy

In this study, we provide original DNA sequences for the new species *Timea alcoladoi* sp. nov. as well as for *T. hechteli* aiming to establish their taxonomic position among the Tethyida group. The COI fragment provided 579 pb (after clipping low-quality end-reads) for *Timea alcoladoi* sp. nov. (one sequence) and 709 pb for *Timea hechteli* (two sequences). The ribosomal region, ITS1 of *T. alcoladoi* sp. nov. (one sequence) was smaller with 323 pb than *T. hechteli* (three sequences) with 343 pb, while ITS2 provided 125 pb and 127 pb respectively. After a BLAST search (NCBI/Blast) comparison sequences of both species showed a high similarity to Tethyid species. For COI, both species showed major similarities with *Tethya californiana* de Laubenfels, 1932 (KJ620403, AY561978), *Tethya leysae* Heim & Nickel, 2010 (GQ292532) and *Tethya minuta* Sarà, Sarà, Nickel & Brümmer, 2001 (EF584567)

with 90% for *Timea alcoladoi* sp. nov. and 91% for *T. hechteli*. For the ribosomal region (ITS1-5.8S-ITS2), both species showed major similarities with *Timea* sp. (AJ633880), *T. hechteli* with 82% and *T. alcoladoi* sp. nov. with 83%.

The COI data set was larger (36 sequences) than the ITS data set (12 sequences) owing to the inclusion of other sponge groups by their proximity with sequences of *Timea* from GenBank; the alignment consisted of 483 pb with 206 variable sites, 181 of which were parsimony-informative. The rDNA region data set was considerably smaller due to the fewer sequences available from GenBank. After excluding ambiguous regions (through Gblocks), the alignment was 526 pb, where 262 were variable sites, with 189 parsimony-informative.

The Bayesian inference (BI) and Maximum likelihood (ML) trees for both data sets (COI and ITS) were mostly congruent in topology. For both loci, tree topologies of BI are presented in Figures 6 and 7. The number at each node represents the Bayesian posterior probability (PP) followed by the Bootstrap proportion (BP) from ML analysis. All our analyses were congruent in the allocation of *Timea hechteli* and *Timea alcoladoi* sp. nov. as a

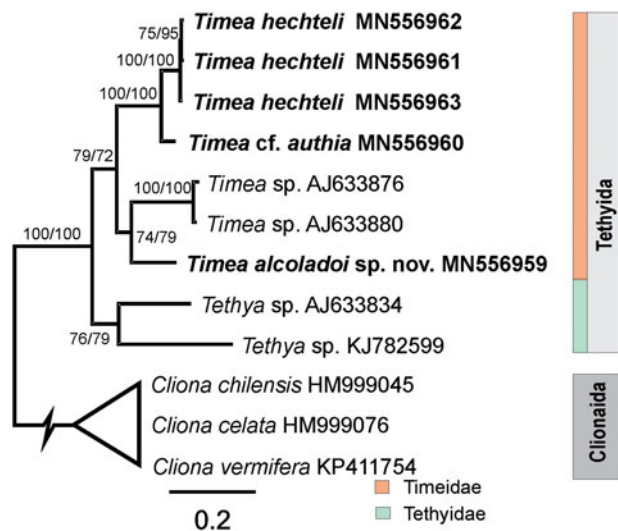


Fig. 7. Tree topology of rDNA region ITS1-5.8-ITS2. The tree presented was obtained by Bayesian inference and includes Maximum likelihood (RA \times ML) values. The number at each node represents the posterior probability (%) (BI), followed by the Bootstrap proportion (ML); a (-) indicates that a particular analysis supported the node at less than 50%, or supported an alternative phylogenetic arrangement in ML tree. The GenBank sequence accession code is included after each species name.

sister group of the principal clade of Tethyida with acceptable values (COI: 98PP/65BP; ITSs 100PP/100BP). They also were consistent in the relationship among species in this clade, where *T. hechteli* was clustered together to *Timea* cf. *authia* in a well-supported clade (COI: 90PP/99PB; ITSs 100PP/100PB), and forming a sister group with *Timea alcoladoi* sp. nov. (COI: 93PP/80BP; ITSs 79PP, 72BP). Additionally, in rDNA region (ITS1-5.8-ITS2) other two *Timea* sequences (GenBank) were grouped to *Timea alcoladoi* sp. nov. strengthening the *Timea* clade (Figure 7).

Regarding COI tree topology, genus *Timea* and the order Tethyida were not recovered as monophyletic. Although *Timea*-species sequenced here were clustered beside other tethyid species (including genera *Tethya* and *Adreus*), two sequences from GenBank *Timea* sp. (AY561968) and *Timea unistellata* (KC869427) were clustered in a Poecilosclerida clade (Figure 6). Sequences of *Axos cliftoni* Gray, 1867 (Hemiasterellidae: Tethyida) also were clustered far from the main Tethyida group, in an Axinellida basal clade (including genera of family Stelligeridae: *Paratimea*, *Halicnemia* and *Plenaster*). For rDNA region *Timea* was monophyletic, but it is probably due to the scarce numbers of available sequences. Even so, tree topology was congruent with the COI hypothesis about the phylogenetic relationships of our species as a sister clade of all other Tethyida.

Discussion

Diversity and distribution of *Timea*

Including our new species, 58 species of *Timea* are currently recognized around the world (Van Soest *et al.*, 2019). The West Atlantic is the richest area in *Timea*-species biodiversity, with about 22 species recorded, from which 16 remain clearly described today, but the rest are problematic: *Timea agnani* (Boury-Esnault, 1973) was reallocated to *Cyamom* (see Van Soest *et al.*, 2012); the specimen from south-eastern Brazil of *Timea* cf. *authia* (de Laubenfels, 1956) needs to be described; *Timea innocens* Schmidt, 1870 and *Timea stelligera* (Carter, 1882) even though they are accepted as valid, the first is unrecognizable and the second one is probably not a proper *Timea* (see Van Soest, 2009; Van Soest *et al.*, 2019). Records of the

Mediterranean species *Timea stellifasciata sensu* Boury-Esnault (1973) and *Timea mixta sensu* Wiedenmayer (1977) are considered inaccurate (see Van Soest, 2009; Van Soest *et al.*, 2019), but we prefer including them in the taxonomic key for future confirmation or specimen reallocation.

The Mediterranean Sea, Eastern Atlantic and Indian Ocean possess about 13 species each. The Western Pacific has 10 species recorded, while the Eastern Pacific only has six species (Van Soest *et al.*, 2019). For the Gulf of Mexico area, the only previous record of *Timea* corresponds to *T. hechteli* (as *Halicometes stellata sensu* Little, 1963) from the USA coast. Although there is a record of *Hymedesmia stellata* (type species of genus *Timea*) by Topsent (1889) from Campeche Bank, it is considered to be a misapplication (Van Soest *et al.*, 2019). So, the species presented here correspond to the first record of the genus *Timea* for the Mexican coast of the Gulf of Mexico.

Morphology and molecular markers in *Timea*

The traditional taxonomy of *Timea* has been mostly based on spicules morphology and size, and especially the euasters ornamentation (Rützler, 2002; Carballo & Cruz-Barraza, 2006). However, the lack of detailed euaster descriptions (mostly in old descriptions) associated with very complex terminology, make the species interpretation difficult (Carballo & Cruz-Barraza, 2006). The problem increases when a few species have shown an intraspecific variety of euaster morphology (Carballo & Cruz-Barraza, 2006), which could affect their utility as main diagnostic characters. The challenge in *Timea* taxonomy is not only at the intra- or interspecific level, sponges with quite similar morphology have been included in four different families from two orders: Timeidae, Tethyidae and Hemiasterellidae (order Tethyida), and Stelligeridae (order Axinellida) (Cruz-Barraza *et al.*, 2017; Lim *et al.*, 2017). Authors have discussed the narrow gap between some genera of Tethyidae and a few species of *Timea* (e.g. *Timea bioxyasterina*, *T. clippertoni* and *T. tethyoides*) due to the presence of ‘megaster-like’ asters that are rather larger than their ‘micraster-like’ counterparts (Leite *et al.*, 2015). However, the absence of a more detailed diagnosis for *Timea* hinders a correct species allocation.

Molecular markers have contributed to understanding the relationships of some *Timea* species and species with similar *Timea* morphology (see Redmond *et al.*, 2013; Thacker *et al.*, 2013; Lim *et al.*, 2017). Nevertheless, there are still a low number of available sequences dispersed in different gene fragments (e.g. COI, 18S, ITS and 28S) hindering their use in a robust systematic approximation. Our COI tree was congruent with previous molecular approximations, showing *Timea* as polyphyletic (Redmond *et al.*, 2013; Thacker *et al.*, 2013; Lim *et al.*, 2017) due to sequences of *Timea* sp. and *T. unistellata* (from GenBank) that were clustered outside the Tethyida clade. In the future, it would be interesting to review the morphology of these samples to establish their taxonomic status. The tree topology of the rDNA region showed *Timea* as monophyletic, probably due to the low number of available sequences. Even so, the tree topologies of both fragments consistently show a robust clade of *Timea* (including our specimens) clustered as a sister group of the main Tethyida group. This clade, formed at the first time for COI, is not only congruent with previous 28S analyses (Thacker *et al.*, 2013; Cruz-Barraza *et al.*, 2017), but also is according to the current systematics of Porifera (see Morrow & Cárdenas, 2015) where Timeidae is one of the three families of Tethyida. So, this result together with the morphological analysis of our specimens supports our taxonomic decision to allocate our species into the genus *Timea*.

The *Timea* systematics is still difficult to resolve today. The low number of available sequences of three families of Tethyida; the non monophyly suggested by a few loci and the controversial similar morphology with species of other Porifera groups, involves the necessity to sequence the type species *T. stellata* as

the first step to establishing their phylogeny. Meanwhile, for this morphologically difficult group, molecular markers with high resolution such as COI or ITS could be helpful to improve the interpretation of morphological characteristics for the taxonomic identification and genus assignation of the species.

Identification key for West Atlantic *Timea* species

This is to supplement the key provided by Lehnert & Heimler (2001) and Van Soest (2009).

1. *Timea* with diplasters..... *T. diplasterina*
Timea without diplasters 2
2. Single size category of euaster 3
Two or more categories of euasters 7
3. Single form (morphology) of euasters 4
Variation of euaster morphology, from spherostongylasters to tylasters..... *T. clandestina*
4. Oxyspherasters only 5
Small tylasters or strongylasters 6
5. Oxyspherasters with conical, microspined rays (<25 µm) *T. unistellata**
Oxyspherasters with cylindrical, microspined rays (>25 µm) *T. oxiasperina*
6. Tylasters of about 12 rays (up to 12 µm) *T. perastra*
Spherostongylasters (chiasters) with 6 rays (7.5–12.5 µm) *T. alcoladoi* sp. nov.
7. Two categories (size and/or shape) of asters 8
Three categories of asters: two oxyasters and one tylaster *T. bioxyasterina*
8. Large euaster category with proliferated rays 9
Large euaster category with simple (non-proliferated) rays 11
9. Large euaster category with unbranched proliferated rays 10
Large euaster category with branching rays, proliferated at their endings, small category tylaster *T. curacaensis*
10. Small euaster category are oxyasters..... *T. stellifasciata* sensu Boury-Esnault (1973)
Small euaster category are spheroxyasters (20–38 µm) and strongylasters (5–8 µm) *T. cumana* sensu Muricy & Santos (2007)
11. Larger asters are spherasters, small asters are tiny (2–3 µm) with proliferated rays *T. micraster*
Small aster category with simple rays, larger asters are oxyasters 12
12. Asters in small and large categories with similar morphology 15
Asters in small and large categories but with different morphology 13
13. Large spheroxyasters (9–43.9 µm) and small oxyasters (4.5–11 µm) *T. kamasary*
Asters a combination of small tylasters or strongylasters (chiaster) and large oxyasters 14
14. Two spheroxyasters categories *T. stenoscлера*
Two tylasters categories *T. tylasterina*
15. Strongylasters (chiasters) all less than 11 µm 16
Strongylasters or tylasters >11 µm 17
16. Strongylasters (chiasters) *T. mixta* sensu Wiedenmayer (1977)
Spiny strongylasters (4–11 µm) *T. secirum*
17. Small asters are strongylasters chiasters (3.7–22.5 µm) *T. hechteli*
Small asters are tylasters 18
18. Tylasters >25 µm in diameter *T. parasitica*
Tylasters <20 µm in diameter *T. berlincki*

* *Sensu* Pulitzer-Finali (1986) oxyspherasters 20–25 µm; Rützler *et al.* (2014) oxyspherasters 9–18 µm.

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