Integrative Taxonomy and Molecular Phylogeny of Genus *Aplysina* (Demospongiae: Verongida) from Mexican Pacific

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**Abstract**

Integrative taxonomy provides a major approximation to species delimitation based on integration of different perspectives (e.g. morphology, biochemistry and DNA sequences). The aim of this study was to assess the relationships and boundaries among Eastern Pacific *Aplysina* species using morphological, biochemical and molecular data. For this, a collection of sponges of the genus *Aplysina* from the Mexican Pacific was studied on the basis of their morphological, chemical (chitin composition), and molecular markers (mitochondrial COI and nuclear ribosomal rDNA: ITS1-5.8-ITS2). Three morphological species were identified, two of which are new to science. *A. clathrata* sp. nov. is a yellow to yellow-reddish or brownish sponge, characterized by external clathrate-like morphology; *A. revillagigedi* sp. nov. is a lemon yellow to green, cushion-shaped sometimes lobate sponge, characterized by conspicuous oscules, which are slightly elevated and usually linearly distributed on rims; and *A. gerardogreens* a known species distributed along the Mexican Pacific coast. Chitin was identified as the main structural component within skeletons of the three species using FTIR, confirming that it is shared among Verongida sponges. Morphological differences were confirmed by DNA sequences from nuclear ITS1-5.8-ITS2. Mitochondrial COI sequences showed extremely low but diagnostic variability for *Aplysina revillagigedi* sp. nov., thus our results corroborate that COI has limited power for DNA-barcoding of sponges and should be complemented with other markers (e.g. rDNA). Phylogenetic analyses of *Aplysina* sequences from the Eastern Pacific and Caribbean, resolved two allopatric and reciprocally monophyletic groups for each region. Eastern Pacific species were grouped in general accordance with the taxonomic hypothesis based on morphological characters. An identification key of Eastern Pacific *Aplysina* species is presented. Our results constitute one of the first approximations to integrative taxonomy, phylogeny and evolutionary biogeography of Eastern Pacific marine sponges; an approach that will significantly contribute to our better understanding of their diversity and evolutionary history.

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**Introduction**

Taxonomy and species recognition are a fundamental basis for all theoretical and applied biological research. For centuries, traditional taxonomy has been based on comparative morphology, and even today most species descriptions are mainly based on morphology. However, this traditional approach can bear some subjectivity in the interpretation of characters, making the identification of species difficult, and causing unstable systematics across entire taxa (e.g. Porifera) [1,2]. Technological advances have provided new tools that facilitate obtaining different kinds of biological data (e.g. micro morphology, biochemistry, ecology, genetics etc.), which are confronted in taxonomy and evolutionary studies of the species [3]. DNA barcoding [4] has become a particularly efficient tool in the identification and delimitation of new and known species from various groups [5–7]. Although it has demonstrated limited resolution power in some cases [8–10].

In the last decade, an “integrative taxonomy” combining multiple kinds of data and complementary perspectives (i.e., phylogeography, comparative morphology, population genetics, ecology, development, behavior, etc.) has been recognized as the most objective means to delimit the units of life’s diversity (see [3,11]). Several operational approaches have emerged to implement an integrative taxonomy, such as the “taxonomic circle”, among others [3,12]. The taxonomic circle seeks to reconcile different types of characters in order to determine species boundaries through a process of hypothesis testing, corroboracion,
reciprocal illumination and revision. These approaches have been well received and adopted in several recent studies, increasing the use of combined lines of evidence to test taxonomic hypothesis of species status [3,12,13].

The Phylum Porifera is a very diverse and abundant group in the marine benthic ecosystem [14]. Currently, sponges have attracted a growing interest due to their evolutionary, ecological and economical importance (particularly in the pharmaceutical, biomaterial and biotechnology fields) (e.g. [14–16]). However, their taxonomic complexity makes species identification difficult and consequently hinders their potential application. The problem increases in groups lacking a mineral skeleton (e.g. horny sponges), where morphological characteristics hardly vary, making it difficult to establish the boundaries between species and challenging their phylogenetic interpretation. However, the combined use of several markers such as morphology and DNA sequences has generated major comprehensive information about species determination, this is particularly relevant when morphological characters used for taxonomic interpretation are unstable (e.g. [17,18]) and there is no molecular standard fragment for species discrimination.

Horny sponges (Demospongiae; orders Verongida, Dictyoceratida, and Dendroceratida) have a skeleton formed by spongins, a protein resulting from a super-compaction of collagen fibrils and filaments [19,20]. However, phylogenetic and embryological studies have shown that Verongida, Dictyoceratida, and Dendroceratida – although characterized by fibrous spongins skeletons – do not make up a cohesive phylogenetic unit [21,22]. Recent re-examination showed that chitin, rather than spongins, forms the main organic component of verongid skeletal fibres [23]. The presence of chitinous skeletons in different representatives of Verongida sponges [23–27] seems to be a characteristic property of this group, which has apparently evolved independently from Dictyoceratida and Dendroceratida [21]. Because sponges are often regarded as the most ancient metazoans, the finding of chitin in their skeleton is of major evolutionary significance.

Species of Aplysina (Order Verongida) are common inhabitants of shallow tropical and subtropical marine waters [28,29]. They are usually large sponges, variable in form (massive, tubular, ramose, pedunculate, etc.), and with live colors, usually ranging from yellow to green [28,30]. The genus is characterized by having a skeleton of pithed fibers in a single category, forming a regular reticulum of polygonal meshes without an ectosomal component. The fibers form a regular reticulum with large polygonal meshes and no specialized surface arrangement [29]. In contrast to other keratose sponges, Aplysina skeletons are mostly made not of spongins, but of alpha-chitin [23–25], which gives this group an interesting biomedical potential in tissue engineering [24,25].

Aplysina-species are also recognized by the possession of brominated alkaloid compounds with cytotoxic activities [31,32], and microbial symbionts producing compounds with antibiotic activity [33].

The systematic history of the group is quite complex [29,34–36]. The name Aplysina was established by Nardo (1834) and was commonly used, during the early 19th and 20th centuries, although some authors (e.g. [37–39]) used Verongia Bowerbank (1845). De Laubenfels (1948) presented an extensive discussion in order to establish Verongia instead of Aplysina. He proposed to synonymize Aplysina with Spongia, and to establish Verongia as the valid name for species described as Aplysina. Finally, Wiedenmayer (1977) resolved the status of the name Aplysina in relation to Verongia under the international code of zoological nomenclature, stabilizing the validity of genus Aplysina as a senior name over Verongia (see [35]).

The classification of Aplysina was first based on external morphology and skeleton, and was later complemented with reproductive, histological and biochemical characteristics [29,34]. However, the absence of a mineral skeleton has limited species identification and the analysis of phylogenetic relationships in the group, particularly in the face of potential cryptic species and extreme phenotypic plasticity, which remain largely unapprised in these sponges.

Molecular markers have been increasingly used to resolve sponge relationships [24,40]. DNA sequences from the mitochondrial (mtDNA) gene coding for the cytochrome oxidase subunit I (COI) have shown low resolution for the discrimination of Aplysina species [41,42]. Partial sequences of the nuclear ribosomal DNA (rDNA), which may include the 18S, ITS1, 5.8S, ITS2, and 28S genes, have been successfully used to infer phylogenies from the ordinal- [42,43] to species-level in Verongida [29]. More recently, analyses of single-stand conformational polymorphisms (SSCP) of PCR-amplified ITS have also been used to elucidate speciation patterns of Aplysina species, showing concordance between morphological and genetic data for species discrimination [44].

To date, only two valid species of genus are known in the Eastern Pacific: Aplysina gerardogomei Gómez and Bakus 1992 (from Mexico) and A. chinuensis Díaz et al. 2005 (from Panama to Galapagos Islands). Even though other congeneric species have been cited, most of them may be invalid due to the absence of detailed morphological descriptions (see discussion).

Here we analyze the status of the genus Aplysina in the Eastern Pacific using an integrative inferential approach based on morphological, chemical and molecular data to establish species boundaries and to unravel their evolutionary patterns. We describe two new species as well as new material of A. gerardogomei based on morphological and molecular data. We also analyze the phylogenetic relationships of the Mexican Aplysina with other congeneric species from the Caribbean. Finally, a morphological identification key is provided for the species of Aplysina from the Eastern Pacific.

Results

Systematic descriptions

Order Verongida Bergquist, 1978
Family Aplysiniidae Carter, 1875
Genus Aplysina Nardo, 1834
Diagnosis. Aplysiniidae characterized by possession of fibers of only one kind with no foreign detritus and having a thick pith component. The fibers form a regular reticulum with large polygonal meshes and no specialized surface arrangement [29].

Aplysina clathrata sp. nov.
(Figs. 1 A, B; 2 A–E)

Material examined. Holotype: MNCN 1.01/637, Cerro Pelón, isla Isabel (Nayarit) 21°51′21″N, 105°53′33″W, 15 m depth, 01/30/2003. Paratypes: BMNH 2010.11.01.7, Cerro Pelón, isla Isabel (Nayarit) 21°51′21″N, 105°53′33″W, 15 m depth, 01/27/2003. LEB-ICML-UNAM-57, bahía Tiburones, isla Isabel (Nayarit), 21°50′33″N, 105°53′10″W, 12 m depth, 11/20/1999. LEB-ICML-UNAM-2072, Los Islotes, Isla Espíritu Santo (Baja California Sur), 24°35′57″N, 110°24′04″W, 6 m depth, 08/10/2009.

GenBank accessions rDNA: JN596957 and : JN596956; mDNA-COI: JQ437579.

The ZooBank LSID: urn:lsid:zoobank.org:act:0BB11475-FRAD-4188-BC96-FEED8B09D865.

Description. Semi-spherical to massive reticulated or clathrate sponge, up to 40 cm in diameter, and from 3 to 10 cm high. The branches are from 2 to 8 mm in thickness, which form meshes from 0.3 to 1.5 cm in opening. Reticulum is more open in young
specimens and becomes narrow in larger specimens (Fig. 1 A, B). Surface with lobules rounded, from 3 to 9 mm high and from 2 to 5 mm in diameter, slightly widened in the distal part. Occasionally they are fused forming lobes up to 1.5 cm long. The surface of the lobules is smooth, sometimes minute-conulose due to the tip of the fibers. Conula (from 100 to 300 μm) are more evident in preserved specimens. Ostial apertures are from 15 to 85 μm in diameter, and they are regularly distributed on surface. Oscules are relatively large, conspicuous, circular to oval-shaped, 2–4 mm in diameter, and distributed regularly around the surface, mainly between lobes. They are surrounded by a lightly elevated diaphragm-like membrane without skeletal fibers. The consistency is soft and flexible, harder in preserved specimens. Ectosome is a thin layer 66 μm thick, easily detachable in some parts of the body. The choanosome is cavernous, with circular to oval shaped channels from 0.6 mm to 1 mm of opening. The structure of the fibers varies considerably from fibers with a thick core and a nearly imperceptible bark, to a regular core with a thick strongly striated bark (Fig. 2 B–E). Some fibers present short protuberances (Fig. 2 D). Some fibers present short protuberances (Fig. 2 D).

Etymology. Clathrum (Greek) = lattice or grate, referring to the shape of an open latticework of anastomosed tubes.

Figure 1. External morphology of Mexican Pacific Aplysina species. A,B) Aplysina clathrata sp. nov.; C,D) Aplysina revillagigedi sp. nov.; E,F) Aplysina gerardogreeni. doi:10.1371/journal.pone.0042049.g001

Distribution and habitat. The species is distributed in tropical and subtropical coastal areas from the Mexican Pacific: Isla Espiritu Santo (La Paz, Baja California Sur), Isla San Pedro Nolasco (Sonora) and Isla Isabel (Nayarit) (Fig. 3). Specimens are common between 5 to 15 m deep, but the largest one was found at 20 m at the Isla Isabel. They are typical on rocks, in areas with high water flow and scarce sediment deposition.

Remarks. The combination of morphological characters and the presence of a regular tridimensional polygonal network of spongin fiber in a single category is typical of genus *Aplysina*. This is supported by molecular data of fragments from both genomes (see discussion). *A. clathrata* sp. nov. is clearly different from other *Aplysina* species by their typical latticework of anastomosed tubes, unique in the genus (see Table 1), which is very consistent and facilitates their identification. The only intraspecific variation seemingly ontogenetical is the more open clathrate structure in small specimens, and narrower to almost closed in the largest one (Fig. 1 A, B). The color varies from yellow in specimens from shallow water, to yellow and darker brown colorations in specimens living deeper.

*Aplysina revillagigedi* sp. nov.

(Figs. 1 B, C; 2 F–I)

Material examined. Holotype: MNCN 1.01/638 Punta Tosca, Isla Socorro (Revillagigedo), 18°47’01"N, 111°02’42"W, 3 m depth, 05/08/2008. Paratypes: BMNH 2010.11.01.8 Punta Tosca, Isla Socorro (Revillagigedo), 18°47’01"N, 111°02’42"W, 3 m depth, 05/08/2008. LEB-ICML-UNAM-1236, Isla Clarion, Roca Norte (Revillagigedo), 18°47’44"N, 110°35’42"W, 4 m depth, 03/12/2003.

GenBank accessions: rDNA: JN596955; COI: JQ437580.

Figure 2. Skeletal characteristics of Mexican Pacific Aplysina species. A–E) A. clathrata sp. nov.; A) Regular tridimensional skeletal reticulation of a fistular projection; B,C,D) Detail of skeleton sponging fibers with nodular pith and short protuberances (showing by arrows); E) Transversal view of fibers showing by arrows; F–H) A. revillagigedi sp. nov.; F) Tridimensional skeletal reticulation at deep choanosome; G) Dendritic-like terminal skeletal fibers; H) Transversal view of fibers showing by arrows; I) A. gerardogreeni; I) Regular tridimensional skeletal reticulation; J) Transversal view of fibers showing by arrows. doi:10.1371/journal.pone.0042049.g002
The Zoobank LSID: urn:lsid:zoobank.org:act:E7BEC0FA-038D-4C14-BA46-88A19AD9498D.

Description. Cushion shaped to massive sponge (from 0.5 to 3 cm high), sometimes with rounded lobes from 0.8 to 1.2 cm high, covering area from 2 to 30 cm in diameter (Fig. 1 B, C). Surface is smooth; oscules are evident only in preserved specimens, soft to the touch. Ostial pores from 16 to 67 μm in diameter. Oscules are very conspicuous, from 1 to 5 mm in diameter, circular to oval-shaped, regularly distributed on the surface, or linearly distributed on rims. They are slightly elevated from the surface. Consistence is flexible and firm. Ectosome is a thin membrane from 110 to 250 μm in thickness. Choanosome is dense with scarce canals from 86 to 170 μm in diameter, and little foreign debris. Color in life is commonly green, sometimes lemon yellow. Preserved specimens turn to the typical dark coloration of verongid sponges.

Skeleton. The skeletal structure is somewhat confuse but it is possible to distinguish an irregular polygonal tridimensional reticulation of sponging fibers in a single category (Fig. 2 F). Near the surface, fibers usually become ramified, and end in rounded tips (Fig. 2 G). Mesures from 0.630 to 3.5 mm of opening. Fiber color varies in specimens from dark brown to amber; they are from 40 to 130 μm in thickness, with pith from 25 a 110 μm wide, which cover between 55 and 84% of the fiber diameter (Fig. 2 H).

Distribution and habitat. Aplysina revillagigedi sp. nov. is only known from Socorro and Clarion islands, and several rock pinnacles from Revillagigedo Archipelago (Mexican Pacific Ocean) (Fig. 3). The species is common in shallow clear waters, occasionally forming a cushion-shaped sponge on rocky substrates exposed to high movement. It was also found at 38 m.

Etymology. The specific epithet refers to the Revillagigedo Archipelago where species is distributed.

Remarks. The only relatively similar species to A. revillagigedi sp. nov. in the Eastern Pacific area is Aplysina gerardogreeni which is characterized by having tabular lobules typically with an apical oscule, while in A. revillagigedi sp. nov. the tubes and lobes are uncommon, and usually have oscules organized on rims. In addition, the characteristic green color of A. revillagigedi sp. nov. has never been seen in A. gerardogreeni. Differences are also present at the skeletal level; A. gerardogreeni has a regular reticular spongin skeleton, while in A. revillagigedi sp. nov. skeletal structure is irregular with large meshes and fibers ending in a dendritic pattern. The species described as Verongia thiona de Laubenfels, 1930 from California (USA), is another close species to A. revillagigedi sp. nov. This species is an incrusting sponge with a few scattered oscules; skeletal structure is formed by large and irregular meshes some similar to A. revillagigedi sp. nov. However, A. revillagigedi sp. nov. is a cushion-shaped sponge with lobular formations, and oscula typically distributed on rims. In V. thiona the skeletal structure is composed by a reticulation of scattered fibers, whereas in A. revillagigedi sp. nov. the sponging structure is proportionally more abundant despite the presence of irregular meshes. In addition, the fibers in A. revillagigedi sp. nov. generally become dendritic near the surface, which was not described for V. thiona. Nevertheless, although there is not a formal decision, it has been suggested that V. thiona should be transferred to the genus Asiochelia Wiedenmayer [35].

Figure 3. Sampling localities and distribution of Aplysina species along the Mexican Pacific Ocean. Numbers correspond to different species: (1) Aplysina clathrata sp. nov.; (2) Aplysina revillagigedi sp. nov.; (3) Aplysina gerardogreeni. doi:10.1371/journal.pone.0042049.g003
Table 1. Comparative data of external morphology, skeletal characteristics and distribution of *Aplysina* species from Eastern Pacific and Atlantic Oceans and the Mediterranean Sea.

| **Aplysina** species | **Eastern Pacific species** | **Western Atlantic species** | **Mediterranean species** |
|---------------------|----------------------------|-----------------------------|--------------------------|
|                     | **External Characteristics (Color/form/oscula diameter)** | **Skeletal structure/Fibers/pith (diameter)** | **Distribution** |
| A. clathrata sp. nov. holotype MNCN 1.01/637 | Yellow to yellow-brown/Sub-spherical clathrate-like/2–4 mm | Regular polygonal reticulation/50–100/25–58 | Mexico |
| A. revillagigedi sp. nov. holotype MNCN 1.01/638 | Green to green yellow/Cushion-shaped with oscula commonly organized on rims/1–5 mm | Irregular polygonal reticulation/70–130/50–110 | Revillagigedo archipelago, Mexico |
| A. gerardogreeni Gomez & Bakus, 1992 | Yellow and slightly pink, red or brown/ Massive with oscular, lobular to tubular projections/3–5 mm | Regular polygonal reticulation/60–150/50–120 | Mexico to Panama |
| A. chiriensis Diaz et al., 2005 | Pinkish-red or purple to bright yellow/ Ramose departing from a stalk | Polygonal to oval reticulation/30–210/11–70 | Panama to Galapagos Islands |
| *Verongia thione* de Laubenfels, 1930 | Lemon yellow with greenish tins/ Encrusting from 4 cm thick/2–7 mm | Irregular, size average more than 1 mm/80–150/50–110 | California EU. |
| A. cristagallus Pinheiro et al., 2007 | Bright yellow/Digitiform with oscula at the top of digits/1.5–4 mm | Delicate and irregular network/46–232/13–50 | Brazil |
| *A. insularis* (Duchassaing & Michelotti, 1863) | Delicate and irregular network/50–142.5/15–82.5 | Delicate and irregular network/35–125/12–37 | Tropical Western Atlantic |
| A. lactuca Pinheiro et al., 2007 | Delicate and irregular network/37–155/7–115 | Delicate and irregular network/37–192/10–35 | Brazil |
| *A. insularis* (Duchassaing & Michelotti, 1863) | Delicate and irregular network/37–196/7–37 | Delicate and irregular network/7–196/7–37 | Tropical Western Atlantic |
| A. lingua Pinheiro et al., 2007 | Delicate and irregular network/37–126/8–50 | Delicate and irregular network/100–307/10–40 | Brazil |
| A. muriyana Pinheiro et al., 2007 | Delicate and irregular network/38–126/8–50 | Delicate and irregular network/38–126/8–50 | Brazil |
| A. uncisata Pinheiro et al., 2007 | Delicate and irregular network/100–307/10–40 | Delicate and irregular network/100–307/10–40 | Brazil |
| A. pseudolacunosa Pinheiro et al., 2007 | Delicate and irregular network/22–167/8–47 | Delicate and irregular network/22–167/8–47 | Brazil |
| A. solangensis Pinheiro et al., 2007 | Delicate and irregular network/37–158/11–55 | Delicate and irregular network/37–158/11–55 | Northeastern Brazil |

**Reference**

doi:10.1371/journal.pone.0042049.t001

**Integrative Taxonomy of Mexican Pacific Aplysina**

LEB-ICML-UNAM-1002, cerro Peñón, isla Isabel (Nayarit), 21°1’51”W, 10°56’33”S, 21 m depth, 12/10/2003. LEB-ICML-UNAM-2028, Isla Maria Cleofas (Islas Marias), 21°17’59”N, 106°16’24”W, 3 m depth, 06/21/2008. LEB-ICML-UNAM-2042, Isla Maria Cleofas (Islas Marias), 21°17’59”N, 106°16’24”W, 8 m depth, 06/21/2008. LEB-ICML-UNAM-2026, Isla Maria Cleofas (Islas Marias), 21°17’59”N, 106°16’24”W, 3 m depth, 06/21/2008.
ICML-UNAM-2073, Los Islotes, Isla Espíritu Santo (Baja California Sur), 24°35'57 N, 110°24'04 W, 6 m depth, 08/10/2009.

GenBank accessions rDNA: JN596958; mDNA-COI: JQ437578.

Description. Cushion shaped to massive lobulated sponge (1 to 5 cm high), characterized by having several tubular lobules (from 1 to 2.5 cm high and 1 cm wide), each one with an apical oscular aperture (Fig. 1 E, F). Specimens cover areas from 2 to 10 cm in diameter. The surface is smooth to minute conulose, with conula from 250–750 \( \mu \text{m} \) high. Surface is perforated by small ostial apertures from 40 to 150 \( \mu \text{m} \) in diameter. Oscules are circular or oval-shaped from less of 1 mm in smaller specimens to 3–5 mm in the largest one. The consistency is firm and slightly compressible. The ectosome membrane is an easily detachable dermis. The choanosome is cavernous, with channels 40 to 160 \( \mu \text{m} \) in diameter. Color in life is very variable, from bright to dull yellow and some parts are slightly pink, red or brown; it turns dark brown or purple in contact with the air.

Skeleton. Skeletal structure consists of a single class of fibers, which form a regular tridimensional polygonal network with meshes from 1.1 to 1.9 mm wide (Fig. 2 I). The fibers are smooth; color varies in specimens from amber or dark. They are from 60 to 150 \( \mu \text{m} \) in diameter, with darker pith from 50 to 120 \( \mu \text{m} \) diameter, covering between 76 and 95% of the fiber (Fig. 2 J). Near the surface the fibers usually bifurcate and form the conular surface ending in rounded tips.

Distribution and habitat. The species was described in the Mexican Pacific Ocean [45], and later reported in Panama [46]. *A. gerardogreeni* is the most common verongid species along the Mexican Pacific coast. Specimens were found in Baja California Sur, Sonora, Sinaloa, Nayarit, Jalisco, Michoacán, Guerrero and Oaxaca (Fig. 3, see [45]). The species is found attached to hard substrates such as rocks, coral rubble or artificial substrates from the intertidal to 30 m deep.

Identification key of the Eastern Tropical Pacific Aplysina -species

| Pedunculate sponge | A. chiriquensis |
|--------------------|---------------|
| Clathrare like sponge | A. clathrata sp. nov. |
| Cushion- shaped to massive species | \( \Rightarrow \) A. gerardogreeni |
| A. clathrata sp. nov. |
| A. revillagigedi sp. nov. |
| A. archeri |
| A. insularis |
| A. crassa |
| V. gigantea |

Chitin analyses

Chitin as main structural component of the *Aplysina* spp. has been unambiguously identified using FTIR (Fig. 5) and specific Calcofluor White staining [23] (Fig. 6). The Morgan-Elson assay for the determination of N-acetyl-D-glucosamine (NAG) in chitin-based scaffolds indicated some variability among the species studied. The mean amounts of NAG have been estimated as 350 (±10 S.D.) \( \mu \text{g/mg} \), 285 (±10 S.D.) \( \mu \text{g/mg} \) and 375 (±10 S.D.) \( \mu \text{g/mg} \) of dry skeleton for *A. revillagigedi* sp. nov., *A. gerardogreeni*, and *A. clathrata* sp. nov., respectively.

Molecular analyses

Genetic variation and inter-specific divergence. The origin of our sequences as Porifera was confirmed by BLAST searches, thereby discarding the possibility of contamination. Comparisons with other *Aplysina* sequences from GenBank revealed high similarities (≥90%) for both genes (COI mtDNA and ITS1-5.8-ITS2 rDNA). New sequences were deposited in GenBank (Table 2).
We aligned 523 bp of the mtDNA COI gene of *A. gerardogreeni*, *A. clathrata* sp. nov. and *A. revillagigedi* sp. nov. (Table 2). COI sequences were extremely conserved showing only one segregating site among species (Fig. 7). The variable site was diagnostic for *A. revillagigedi* sp. nov. Hence, no subsequent analyses were carried out on these data.

After clipping low quality end-reads, the multiple alignments spanning the ITS1-5.8S-ITS2 genes of the rDNA encompassed 705 bp from 11 specimens of the three species of *Aplysina* in the Mexican Pacific (Table 2).

Based on the identical result obtained with different amplification strategies from the same specimens, i.e. entire fragment and ITS1 and 2 separately, we inferred the absence of intragenomic rDNA polymorphisms in the analyzed organisms.

We detected intraspecific ITS-1 rDNA polymorphisms only in *A. clathrata* sp. nov., in which two haplotypes were found (*h* = 0.67 and *π* = 0 -nucleotide differences correspond to indels-). *A. revillagigedi* sp. nov. and *A. gerardogreeni* were monomorphic.

Given the absence of intraspecific nucleotide substitutions, we treat interspecific polymorphic sites as provisionally diagnostic. Following Davis and Nixon (1992) [48] character-based diagnosis, we found four diagnostic nucleotides for *A. gerardogreeni* (ITS1-2), whereas *A. clathrata* could be diagnosed by two nucleotides (ITS1-2, COI) and an additional diagnostic combination of five nucleotides (ITS1-2). *A. clathrata* could be diagnosed by a single
nucleotide and a diagnostic combination of four nucleotides (ITS1-2) (Fig. 7).

Interspecific polymorphisms of the nuclear ITS regions, including additional Aplysina species from GenBank, revealed a total of 35 variable sites, 12 of which were parsimony-informative. Pair-wise sequence divergence (uncorrected p-distance) between A. clathrata sp. nov. and A. revillagigedi sp. nov., was 0.288%. In contrast, the divergence between A. clathrata sp. nov. and A. gerardogreeni and between A. revillagigedi sp. nov. and A. gerardogreeni was 0.719% (Table 3). The Eastern Pacific individual identified as A. fistularis by Schmitt et al. (2005) is more similar to A. clathrata sp. nov.

Table 2. Accession numbers of the specimens sequences, vouchers and DNA sequences analyzed.

| Species                  | Collection/museum accession number | Locality         | GenBank accession (rDNA/COI) |
|--------------------------|------------------------------------|------------------|-------------------------------|
| **Eastern Pacific**      |                                     |                  |                               |
| Aplysina clathrata sp. nov. | MNCN 1.01/637                       | Isla Isabel, México | JN596956/JQ437579             |
|                          | BMNH 2010.11.01.7                   | Isla Isabel, México | JN596957/JQ437579             |
|                          | LEB-ICML-UNAM-2022                  | Isla Isabel, México | JN596956/JQ437579             |
|                          | LEB-ICML-UNAM-2023                  | Isla Isabel, México | JN596956/JQ437579             |
|                          | LEB-ICML-UNAM-2072                  | Isla Espíritu Santo, México | JN596956/JQ437579 |
| Aplysina revillagigedi sp. nov. | MNCN 1.01/638                       | Isla Socorro, México | JN596955/JQ437580             |
|                          | BMNH 2010.11.01.8                   | Isla Socorro, México | JN596955/JQ437580             |
|                          | LEB-ICML-UNAM-2024                  | Isla Socorro, México | JN596955/JQ437580             |
|                          | LEB-ICML-UNAM-2025                  | Isla Socorro, México | JN596955/JQ437580             |
| Aplysina gerardogreeni    | LEB-ICML-UNAM-429                   | Isla Pájaros México | JN596958/JQ437578             |
|                          | LEB-ICML-UNAM-1002                  | Isla Isabel, México | JN596958/JQ437578             |
|                          | LEB-ICML-UNAM-2027                  | Isla María Cleofas, México | JN596958/JQ437578 |
|                          | LEB-ICML-UNAM-2028                  | Isla María Cleofas, México | JN596958/JQ437578             |
|                          | LEB-ICML-UNAM-2042                  | Isla María Cleofas, México | JN596958/JQ437578             |
|                          | LEB-ICML-UNAM-2073                  | Isla Espíritu Santo, México | JN596958/JQ437578 |
| Aplysina fistularis       |                                     |                  |                               |
|                          | California EU                       |                  | AY591792.1                    |
| **Caribbean**            |                                     |                  |                               |
| Aplysina insularis        | Bahamas                             |                  | AY591794.1                    |
| Aplysina fulva            | Bahamas                             |                  | AY591793.1                    |
| Aplysina fistularis       | Bermuda                             |                  | AJ621545.1                    |
| Aplysina fistularis       | Bahamas                             |                  | AY591791.1                    |
| Aplysina archeri          | Bahamas                             |                  | AY591788.1                    |
| Aiolochroia crassa        | Bahamas                             |                  | AY591798.1                    |
| Verongula gigantea        | Bahamas                             |                  | AY591797.1                    |

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Figure 7. Diagnostic nucleotides and groups of nucleotides following Davis and Nixon [48] for three species of Aplysina from the Mexican Pacific. Single-nucleotide pure diagnostic characters are individually color-coded for each species (green: A. gerardogreeni, blue: A. revillagigedi, and yellow: A. clathrata); additional composite diagnostic combinations are indicated for A. revillagigedi (orange) and A. clathrata (gray). ITS1-5.8S-ITS2, nuclear ribosomal DNA; COI, mitochondrial cytochrome oxidase subunit I. Nucleotide residues refer to the individual alignments of ITS1-5.8S-ITS2 and COI sequences (GenBank accessions: ITS1-5.8S-ITS2 rDNA JN596955–58 and COI mtDNA JQ437578–80). Nucleotide 101 is ITS1 and the rest are ITS2.

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nov. (p-distance = 1.007%) than to the rest of the species (p-distance > 1.29%).

Phylogenetic analyses. All methods of phylogenetic reconstruction strongly supported the monophyly of Eastern Pacific and Caribbean Aplysina species relative to Aiolochroia cauca and Verongula gigantea (Fig. 4). The rDNA phylogeny of Aplysina revealed a phylogeographic pattern. A well-supported (91% NJ, 85% MP bootstrap, 100% posterior probability) monophyletic clade grouped all Eastern Pacific sequences from this and previous studies, including a sequence from a putative A. fistularis specimen collected in California (Schmitt et al. 2005) [42] (see discussion). A. cauliformis from the Caribbean was consistently placed as sister taxon to the Pacific species. In all trees, A. gerardogreeni appears as sister taxon to A. revillagigedii sp. nov. and A. clathrata sp. nov., strong support for this relationship is mostly present in the NJ tree (Fig. 4). A second set of Caribbean sequences from previous studies (excluding A. cauliformis) was paraphyletic and very poorly resolved. In the single most parsimonious tree (length = 165, not shown) A. fulva and the two Caribbean A. fistularis sequences grouped as a sister clade to [A. cauliformis, Pacific species]; whereas A. archeri and A. insularis formed a basal sister clade to the rest of Aplysina. However, these relationships were not supported by high bootstrap levels.

Discussion

Taxonomy of Eastern Pacific Aplysina-species

Although 84 species of Aplysina have been described so far, only 44 are considered valid (Pforzera Data Base [47]), and from these, only 23 remain clearly valid today [28,30]. The rest are considered unrecognized, due to the lack of good descriptions and the poor preservation of type material [28].

Aplysina has a circumtropical distribution, but the status of the species recorded in the Indo-Pacific remains unclear and needs to be revised [28]. The Western Atlantic is the region with the highest diversity (16 species), whereas the Mediterranean Sea has only two known species. In the Eastern Pacific only two species; Aplysina gerardogreeni and A. chiriquensis should be hitherto considered valid. Others, such as A. aurea (by [48,49]), A. lendenfeldi and A. fulva (by [50]), and A. fistularis (by [51]), must be considered invalid due to absence of detailed morphological description and nonspecialized identification. The latter should not be confused with A. fistularis sensu Green 1977 [52], which is a valid record from Veracruz, Gulf of Mexico (Atlantic), but not from the Pacific as was considered by Diaz et al. (2005) [28].

Other Eastern Pacific species related to Aplysina are Suberea azteca (Gomez & Bakus 1992) described in Mexico, and Verongia thiona de Laubenfels, 1932 described in California (USA). Suberea azteca was originally described as Aplysina, but was later transferred to the genus Suberea based on its skeletal fiber structure [53]. V. thiona should be attributed to the genus Aplysina due to the synonymy of Verongia with Aplysina [35]. However, the lack of a detailed original description hinders the clarification of its taxonomic status, which has been suggested to correspond to the genus Aiolochroia Wiedenmayer, 1977 (see [47]), but no formal amendment has been published. According to de Laubenfels (1932) specimens of V. thiona are abundant in Laguna Beach California USA (type locality), and moderately common in the intertidal areas of southern California. A specimen of Aplysina (identified as A. fistularis) was collected in La Jolla, Southern of California, near V. thiona’s type locality (less than 100 km away), and was sequenced by Schmitt et al. (2005) [42]. Our phylogenetic analyses place this sequence within the Eastern Pacific clade of Aplysina (Fig. 4). Therefore, we hypothesize that the specimen sequenced by

Table 3. Pairwise (TS1-5S-ITS2 rDNA genetic divergence (% uncorrected p-distance) between Aplysina species from Eastern Pacific and Caribbean.

| Species | A. revillagigedii sp. nov. | A. clathrata sp. nov. | A. fulva | A. archeri | A. cauca | A. clathrata (CAL) | A. fulva (CAL) | A. archeri (BER) | A. cauca (BER) | A. fulva (BAH) | A. archeri (BAH) | A. cauca (BAH) |
|---------|--------------------------|----------------------|---------|-----------|---------|-----------------|---------------|----------------|---------------|---------------|----------------|---------------|
| A. revillagigedii sp. nov. | 0.719                   | 0.279                | 1.125   | 1.727     | 1.777   | 1.727           | 1.727         | 1.727          | 1.727         | 1.727         | 1.727         | 1.727         |
| A. clathrata sp. nov. | 0.719                   | 0.279                | 1.125   | 1.727     | 1.777   | 1.727           | 1.727         | 1.727          | 1.727         | 1.727         | 1.727         | 1.727         |
| A. fulva | 1.125                   | 0.279                | 1.125   | 1.727     | 1.777   | 1.727           | 1.727         | 1.727          | 1.727         | 1.727         | 1.727         | 1.727         |
| A. archeri | 1.727                   | 1.727                | 1.727   | 1.727     | 1.727   | 1.727           | 1.727         | 1.727          | 1.727         | 1.727         | 1.727         | 1.727         |
| A. cauca | 1.727                   | 1.727                | 1.727   | 1.727     | 1.727   | 1.727           | 1.727         | 1.727          | 1.727         | 1.727         | 1.727         | 1.727         |
| A. clathrata (CAL) | 0.719                   | 0.279                | 1.125   | 1.727     | 1.777   | 1.727           | 1.727         | 1.727          | 1.727         | 1.727         | 1.727         | 1.727         |
| A. fulva (CAL) | 0.719                   | 0.279                | 1.125   | 1.727     | 1.777   | 1.727           | 1.727         | 1.727          | 1.727         | 1.727         | 1.727         | 1.727         |
| A. archeri (BER) | 1.727                   | 1.727                | 1.727   | 1.727     | 1.727   | 1.727           | 1.727         | 1.727          | 1.727         | 1.727         | 1.727         | 1.727         |
| A. cauca (BER) | 1.727                   | 1.727                | 1.727   | 1.727     | 1.727   | 1.727           | 1.727         | 1.727          | 1.727         | 1.727         | 1.727         | 1.727         |
| A. fulva (BAH) | 1.727                   | 1.727                | 1.727   | 1.727     | 1.727   | 1.727           | 1.727         | 1.727          | 1.727         | 1.727         | 1.727         | 1.727         |
| A. archeri (BAH) | 1.727                   | 1.727                | 1.727   | 1.727     | 1.727   | 1.727           | 1.727         | 1.727          | 1.727         | 1.727         | 1.727         | 1.727         |
| A. cauca (BAH) | 1.727                   | 1.727                | 1.727   | 1.727     | 1.727   | 1.727           | 1.727         | 1.727          | 1.727         | 1.727         | 1.727         | 1.727         |

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Schmitt et al. (2005) may correspond to *V. thiona* and not to *A. fistularis*, (possibly endemic to the Caribbean). If the identity of this specimen can be verified as such, then our molecular phylogenetic analyses support the synonymy of *Verongia* and *Aplysina*.

The Eastern Pacific *Aplysina* species are easily distinguishable from each other. *A. chitiquensis* is characterized by having a pedunculate shape. *Aplysina clathrata* sp. nov. is also well differentiated by its typical clathrate-like morphology, which has never been described for a species in the genus. *Aplysina gerardogreeni* may be more similar to *Aplysina revillagigedi* sp. nov. because both may have cushion-shaped form. However, *A. gerardogreeni* is characterized by a completely lobular surface with an apical oscule on lobes and tubes, whereas lobules are uncommon in *A. revillagigedi* sp. nov. and oscula are usually organized on rims.

Molecular Systematics of Aplysina

The order Verongida is a cohesive group of marine sponges whose systematics has been traditionally based on morphology, and more recently on biochemical and genetic data [43]. Even though morphological characters have not been useful to resolve its phylogeny [43], molecular data have supported the monophyly of the group in several gene trees (see [1,2,43,54]), and have shown to be useful to address relationships down to the species level [43].

Our phylogenetic analyses have also supported the monophyly of *Aplysina* (Fig. 4), as shown in analyses of 18S and ITS-2 in species of Verongida [42,43]. ITS spacers have also been useful for species-level discrimination in other sponges (e.g. in genera *Dysidea*, *Axinella,* among others) [55,56]; and in the genus *Aplysina* this gene region produced a robust phylogeny at the species level [43]. Being a multicyclic gene cluster, rDNA has shown elsewhere evidence of intragenomic variation in *Aplysina* [41,57]. However, our genetic analyses of Mexican sponges did not reveal this kind of variation. Nevertheless, the presence of intragenomic polymorphisms does not preclude the usefulness of the analyzed rDNA fragment as a suitable genetic marker; but it calls for caution in the interpretation of the patterns of diversification [10,57,58].

The phylogenetic relationships of Caribbean and Pacific *Aplysina* species had already been mentioned by Schmitt et al. (2005) [42] who have also included sequences from the Mediterranean in their analyses. They suggest a biogeographical hypothesis where *Aplysina* could have a Tethyan origin resulting in a Mediterranean and a Caribbean-Pacific clade. The subsequent and recent adaptive radiation of all extant Caribbean and Eastern Pacific *Aplysina*, explain their high degree of genetic similarity [42]. Our results clearly show a monophyletic Pacific clade vis-à-vis a paraphyletic group of poorly resolved Caribbean sequences, suggesting a single Pacific ancestor and that speciation may have ensued following the rising of the Isthmus of Panama, when amphio-american faunas were separated. Our gene tree also reveals that *A. cauliformis* (a Caribbean species) represents a lineage more closely related with the Eastern Pacific clade, which may be interpreted either as an extant representative of the lineage that gave rise to the Pacific clade or a possible invasion of the Caribbean by an early Pacific branch.

Eastern Pacific species were grouped in general accordance with the taxonomic hypothesis based on morphological characters. *A. revillagigedi* is an insular species separated from the rest and is also a divergent lineage among the Eastern Pacific *Aplysina*. On the other hand, *A. clathrata* and *A. gerardogreeni* are sometimes sympatric and their genetic divergence is less pronounced.

DNA Barcoding

Although the mtDNA COI gene has been a valuable marker to differentiate many metazoan species and is the marker of choice in the Barcoding of Life initiative [4], in general it has shown very low levels of variation in diploblastic organisms such as sponges and corals [8,9,10]. The virtual lack of variation in our COI sequence supports the shared ancestry of the mitochondrial lineages. On the other hand, it is not entirely devoid of power for species discrimination, given that the only polymorphic nucleotide resulted in a mutation that appears to be diagnostic for *A. revillagigedi* sp. nov. vis-à-vis the other two sequenced species (*A. clathrata* sp. nov. and *A. gerardogreeni*) (Fig. 7). Previous analyses of COI sequences failed to resolve seven species of *Aplysina* from the Mediterranean and Caribbean. The Caribbean *A. insularis*, *A. cauliformis*, *A. fulva* and *A. fistularis* possessed identical COI sequences whereas the Mediterranean *A. aerophoba* and *A. caveniola* differed by one base pair [41,42]. The presence of a single polymorphic site in the COI gene was also found to be congruent with morphological differences observed among species, as it is the case in this study [41,59]. However, our results and others confirm that in some instances COI-DNA barcoding is an efficient method for the discrimination of sponge taxa at least to the generic level [60,61].

In addition, COI-DNA barcoding for sponges may be complemented by other molecular markers for a finer resolution. Non-coding intergenic transcribed spacers ITS-1 and ITS-2 from rDNA have been successfully used in micro- and macro-evolutionary studies due to their faster rate of evolution relative to functional genes [57,62]. They have been used in population genetic studies, but among sponge taxa mainly to infer phylogenies [42,43,56,63–66]. Intra-genomic variation in the ITS region has been found in *Aplysina* from the Caribbean and Mediterranean, suggesting that this region may not be entirely appropriate for fine-scale population studies [41]. However, we found no evidence of intra-individual polymorphisms in the specimens analyzed in this study. In our analyses, the ITS region provided enough variation for Eastern Pacific species differentiation (Figs. 4, 7) and, even though it has shown intra genomic polymorphisms elsewhere, it remains a good candidate for DNA barcodes of plants and animals [67].

The skeletons: chemical chitin composition

The absence of a mineral skeleton in *Aplysina* spp. has represented a challenge for species identification, and for the estimation of the phylogenetic relationships in the group. The chemical analysis showed that alpha-chitin is present in all the species studied, which is highly relevant and strengthens the hypothesis that all species of *Aplysina* share with other members of the order Verongida the presence of chitin-based scaffolds [24] as a synapomorphy supporting the monophyly of Verongida.

The chemical analyses of the fibers indicated some variability of N-acetyl-D-glucosamine concentration among the Eastern Pacific species of *Aplysina*, which were also similar to the one found in Atlantic and Mediterranean *Aplysina*-species [24]. Spectra were also very similar between the Mexican *Aplysina*, and the one obtained previously from the skeleton of *A. fulva* [24], but each species revealed differences in absorbance at different wave numbers (Fig. 5), which could have taxonomical importance. Nevertheless, the significance of these differences for species distinction is still unclear and more research may be needed to evaluate their possible use in *Aplysina* taxonomy.

Integrative taxonomy in Aplysina

The systematics of Porifera is traditionally based on skeletal characteristics (spicules and fibers, and their arrangement on sponge body), but the paucity, simplicity and plasticity of these characters are the principal limitation in species determination.
and phylogenetic interpretation [68]. Alternatives to traditional taxonomy have been created as a result of technological advances (e. g. chemotaxonomy and molecular taxonomy). However, in several cases they act independently and sometimes differing to traditional conceptions. Currently the major approximation to species identification could be the combined use of morphological and molecular data, both characteristics could give an efficient way to evaluate the taxonomic status of species [17,18].

The importance of the integrative approach used in this study is emphasized by the difficulty in the interpretation of phenotypic variation sometimes encountered in sponge systematics. Although the analyzed species can be readily recognized as *Aplysina*, they also show some characteristics that could confuse the taxonomic determination. For instance, *A. clathrata* sp. nov. has an external morphology previously undescribed in *Aplysina*. The external surface is perhaps more similar to species of the genus *Aiolochroia*, which is characterized by rounded tubercles surrounding depressions giving it an overall polygonal appearance. In addition, *A. clathrata* sp. nov. also possesses fibers with nodules and short protuberances characteristic of *Aiolochroia* (see [29]). However, our genetic results are emphatic that these species are not close to *Aiolochroia*, which was used as an outgroup to root our phylogenetic reconstruction (Fig. 4). *A. revillagigedi* sp. nov. has an irregular skeletal structure which becomes dendritic towards the surface, resembling the genus *Suberea* (Verongida: Aplysinellidae) which is characterized by a coarse irregular dendritic fiber skeleton (see [29]). However, in both cases, the molecular analyses support that they are well assigned to the genus *Aplysina* and therefore shed light on the plasticity of the morphological characters in the group.

The use of an “Integrative taxonomy” for species delimitation was pointed out separately by several authors [11,68,69]. The proposal was quickly adopted and several approaches have been presented for how the integration of different kinds of characters should be addressed (e. g., [3,11,13,17,18]). Among these, authors have argued about the importance of congruence between morphological and molecular characters (i.e., “integrative taxonomy by congruence”, [3]), which is well exemplified by the taxonomic circle [12]. This procedure illustrated that congruence of two or more taxonomic characters is an important factor in species identification, assuming that increased support for distinction arising from independent data sets decreases the likelihood of erroneous delineations of novel taxa. Operationally, the circle represents the experimental routes (e. g., DNA, morphology, reproduction, ecology and geography) involved in taxonomic inference to objectively corroborate taxonomic hypotheses. The only way to delineate a new taxon is to break out of the circle following an initial hypothesis after visiting independent lines of evidence placed on the perimeter of the circle [12].

Following this approach, the delineation and distinction of both *A. clathrata* sp. nov. and *A. revillagigedi* sp. nov. from *A. gerardogreeni* comes from morphology, which provides the initial hypothesis and the most conspicuous evidence to discriminate the three *Aplysina* species (Fig. 8). However, the difficulty in morphological taxonomy of *Aplysina* is mainly due to the lack of diagnostic characters and high phenotypic plasticity of the individuals, making it hard to establish the boundaries between species. So it is very important to incorporate other characters, and corroborate whether they are congruent with morphology in species discrimination. In addressing the distinction of the new species from the widespread and valid *A. gerardogreeni*, geographical information could help “break out of the taxonomic circle” for *A. revillagigedi* sp. nov., by geographical isolation (Fig. 8 A). This species of *Aplysina* is endemic to the Revillagigedo Archipelago, where the other two species are absent. Unlike chitin spectroscopic profiles, which remain inconclusive at the moment and more research is needed to evaluate their possible use in *Aplysina* taxonomy, nuclear ITS1-2 rDNA sequences provide diagnostic characters as well as quantitative divergence and phylogenetic analyses congruent with the other evidence allowing to break out of the circle for the distinction of the new species from *A. gerardogreeni* (Fig. 8 A). In a second hypothesis, the distinction between the two new species, *A. clathrata* sp. nov. and *A. revillagigedi* sp. nov., is also prompted by morphological differences (Fig. 8 B). Following the circle, the distinction is clearly supported by geographical distribution (continental vs. insular), which could help to break out of the circle. However, genetic distinction at the DNA level and diagnostic nucleotides in nuclear (ITS1-2) and mitochondrial (COI) genomes provide unequivocal evidence of reproductive isolation, also allowing to break out of the circle (Fig. 8 B).

Hence, our research supports that the best approach for species identification of Porifera is through of integrative use of taxonomic characters that can reciprocally shed light in the evolution of each other. In the case of Eastern Pacific *Aplysina*, integrated information of molecular and morphological characters, in addition to geographic and, to a lesser extent, chemical information provided congruent support to the taxonomic distinctions.

To date, there are several studies emphasizing the importance of combined traditional morphological taxonomy with molecular markers in species identification [40,70–72]. They have been useful in clarifying the systematics of several sponge groups [59,73–75]. In fact the recent initiative Sponges Barcoding Project http://www.spongebarcoding.org (see [40]) not only makes available DNA-barcoding of sponges but it also includes detailed morphological characteristics of analyzed species. However in spite of these important approaches favoring an integrative taxonomy in Porifera, a protocol facilitating the unification of criteria is missing. The proposed taxonomic circle is a good example that could be adopted in the taxonomy of Porifera.

Our results constitute one of the first approximations to integrative taxonomy, phylogeny an evolutionary biogeography of Eastern Pacific marine sponges (see also [63]). Subsequent information about distribution patterns of sponges will help clarify the evolutionary process and speciation mechanisms of the Eastern Pacific sponge faunas.

**Materials and Methods**

**Specimens**

Ninety-six specimens were collected by scuba diving and snorkeling in 43 localities along the Mexican Pacific Ocean (Fig. 3). Some were used for both morphological and molecular analyses (Table 2). For morphological analyses specimens were fixed in 4% formalin for 24 h and later transferred to 70% ethanol for storage whereas tissue samples for molecular analyses were preserved in 96% ethanol and stored at −10°C. Biological material was deposited in the Colección de Esponjas del Pacífico Mexicano (LEB-ICML-UNAM), of the Instituto de Ciencias del Mar y Limnología, UNAM, in Mazatlán (México). Type material has been deposited in the Museo Nacional de Ciencias Naturales in Madrid (Spain) (MNCN), and in the British Museum of Natural History (BMNH) (London).

**Morphological analyses**

External morphology and fiber characteristics were recorded for each species. Fiber preparation for light microscopy involved the chemical digestion of surrounding tissue by incubation in a clearing solution (1/3 of distilled water, 1/3 hydrogen peroxide
A. clathrata

Figure 8. Delineation of new *Aplysina* species from the Mexican Pacific under the framework of the taxonomic circle [12]. A) The first hypothesis consists in differentiating the new species *A. clathrata* sp. nov. and *A. revillagigedi* sp. nov. from *A. gerardogreeni*, based on an initial morphological distinction. For this, ecological and structural biochemical evidence per se are not conclusive to break-out of the circle. Geography could allow breaking-out the circle for *A. revillagigedi* sp. nov. (dashed arrow), an insular endemic, but not for both. However the integration of the molecular data in the form of diagnostic characters as well as quantitative divergence and phylogenetic analyses provide congruent information with unequivocal evidence of reproductive isolation, allowing breaking out of the circle.

B) The second hypothesis consists in differentiating the two new species. For this geographical distribution (continental vs. insular) could help to break out of the circle (dashed arrows) but structural biochemistry remains inconclusive. However, the addition could allow breaking-out the circle for morphological distinction. For this, ecological and structural biochemical evidence per se are not conclusive to break-out of the circle. Geography

H$_2$O$_2$, and 1/3 of ammonia). Fragments were agitated daily, and the solution replaced every 24 hours until the fibers were cleared of tissue [76]. Fiber measurements were obtained from a minimum of 25 portions chosen randomly for each specimen. They are given in fiber and pith width, and percentage of pith with respect to the entire fiber. Fibers, pith and skeletal measurements are given in micrometers or millimeters and mean values are presented in brackets. Anatomical terms follow Boury-Esnault & Rützler [77].

Chemical analyses

Chitin has been extracted from ca. 2×3 cm fragments of the sponges (*A. revillagigedi* sp. nov. *n* = 3, *A. gerardogreeni* *n* = 3, *A. clathrata* sp. nov. *n* = 3), by subjecting them to chemical treatment fully described previously [23,24]. To remove other compounds from the chitin, the sample underwent a series of extraction steps to remove impurities. These extractions included step-by-step treatment as follows: an acidic extraction, an alkali-based extraction, an optional hydrogen peroxide treatment, and washing steps using distilled water before and after each treatment step.

In order to estimate N-acetyl-d-glucosamine contents (NAG), 6 mg of each dried skeleton were pulverized to a fine powder in an agate mortar [24]. Preparation of colloidal chitin from a crab alpha-chitin standard (Sigma) was performed according to Boden et al. (1983) [78]. The Morgan-Elson assay was used to quantify the N-acetyl-d-glucosamine released after chitinase treatment as described previously [78].

To elucidate the particular location of chitin in investigated samples, we used Calcoflour White (Fluorescent Brightener M2R, Sigma). Samples were placed in 0.1 M Tris-HCl at pH 8.5 for 30 min, then stained using 0.1% Calcoflour White solution for 30 min in darkness, rinsed five times with deionized water, dried at room temperature, and finally observed using fluorescence microscopy.

Fourier Transform Infrared spectroscopy was carried out using FTIR Bruker IFS 66/s with the following parameters: spectral resolution: 2 cm$^{-1}$; scans: 500 scans; spectrum: 3900-1000 cm$^{-1}$; aperture: 1 mm; MCT Detector; mirror rate: 40 KHz.

Light and fluorescence microscopy observations were carried out using Digital fluorescence microscope BZ-8000 (Keyence).

Molecular analyses

DNA purification, amplification and sequencing. Total genomic DNA was extracted using standard protease K digestion in CTAB extraction buffer and purified with a LiCl salting-out protocol, followed by organic extraction using chloroform–isoamyl alcohol, and subsequent ethanol precipitation [79]. Subsequently, the mtDNA COI gene and the nuclear rDNA encompassing the ITS1-5.8S-ITS2 gene region were PCR-amplified and sequenced from 11 specimens of the different species (Table 2).

The mtDNA gene was initially amplified using invertebrate universal primers [80] yielding inconsistent results. Therefore, we designed nested primers (COXI-*Aplysina*F 5′-TTG CTG GTA TGA TAG GAA CAG-3′ and COXI-*Aplysina*R 5′-TGA TAT AAA ATT GGG TCC-3′), which were used to amplify around 523 base pairs (bp) of the COI gene. PCR reactions (25 μl) consisted of 8.30 μl dH$_2$O (sterile MilliQ), 9 μl dNTPs (0.5 mM each), 0.50 μl each primer (10 μM), 2.50 μl 10× PCR buffer (20 mM MgCl$_2$), 1 μl Tag DNA polymerase, 1 μl BSA and 3 μl of genomic DNA (ca. 50–100 ng). Thermal cycling conditions were: initial denaturation of 94°C for 2 min, and 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final extension of 72°C for 5 min.

The rDNA gene region was amplified using universal primers (ITS4 5′-TCC TGC TAT GAT TGA TAG GAA CAG-3′ and ITS5 5′-GGA AGT AAA AGT CGT AAC AAG G-3′) [81]. PCR reactions (25 μl) consisted of 9.30 μl dH$_2$O (sterile MilliQ), 9 μl of dNTPs (0.5 mM each), 1 μl of each primer (10 μM), 2.50 μl of 10× PCR buffer (20 mM MgCl$_2$), 1 U Tag DNA polymerase, and 2 μl of genomic DNA (ca. 40–67 ng). Thermal cycling conditions were: initial denaturation of 94°C for 2 min, and 36 cycles of 94°C for 60 sec, and 36 cycles of 94°C for 60 sec, 55°C for 60 sec, 72°C for 2 min, and a final extension of 72°C for 8 min. The rDNA was PCR-amplified and sequenced twice for each organism, in order to screen for intragenomic polymorphisms (IGP). The first time the entire
region (ITS1-5.8S-ITS2) was amplified and directly sequenced. In a second set of reactions we used internal primers (ITS2 5'-GGTCGTCCCTTATGATGGC-3', ITS3 5'-GCA TCG ATG AAG AAC GCA GC-3') to amplify and sequence the internal transcribed spacers separately: ITS1 (using primers ITS5 and ITS2) and ITS2 (using primers ITS3 and ITS4).

PCR products were purified using ExoSAP-IT® (USB, Cleveland, OH), and sequenced using BigDye Terminator version 3.1 following manufacturer protocols. Products were analyzed in an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems, Foster City, CA). *Aplysina*-sequences were deposited in GenBank (Table 2).

**Sequence analyses.** Sequences were verified and edited with Codon Code Aligner 2.0.1 (CodonCode Corporation). BLAST (NCBI/Blast) searches were used to verify the identity of sequences and check for possible contamination. Phylogenetic analyses included previously published rDNA sequences of *Aplysina* from the Caribbean and sequences of *Aiolochroia crassa* and *Verongula gigantea*, the latter were used as outgroup (Table 2). We reconstructed a Neighbor-Joining (NJ) tree, using a matrix of maximum composite likelihood distances, and a maximum parsimony (MP) tree, using an exact branch-and-bound search, as implemented in Mega 5.04 [82]. For these, non-parametric bootstrap (10,000 pseudo-replicates) was used to assess branch support. In addition, a Bayesian inference analysis was performed with MrBayes 3.1.2 [83] using the HKY model of sequence evolution as obtained with jModelTest 0.1 [84]. The program was run with four Markov chains (one cold and three heated) each 1,000,000-generation long, which were sampled every 100th trees and a burn-in of 2,500. Posterior probabilities were computed from the remaining trees.

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**Author Contributions**

Conceived and designed the experiments: JACB ARO JLC. Performed the experiments: JACB MH. Analyzed the data: JACB JLC ARO HE MH. Contributed reagents/materials/analysis tools: JACB JLC ARO HE. Wrote the paper: JACB JLC ARO HE.

**References**

1. Nichols SA (2005) An evaluation of support for order-level monophyly and interrelationships within the class Demospongiae using partial data from the large subunit rDNA and cytochrome oxidase subunit I. Mol Phylo Evol 34: 81–96.
2. Borchiellini G, Chombard C, Manuel M, Alivon E, Vacelet J, et al. (2004) Molecular phylogeny of Demospongiae: implications for classification and scenarios of character evolution. Mol Phylo Evol 32: 823–837.
3. Pfenninger M, Nowak C, Kley C, Stinke D, Striet B (2007) Utility of DNA taxonomy in foraminiferal biodiversity studies. Mar Biol 151: 427–437.
4. Hebert PDN, Cywinska A, Ball SL, de Waard JR (2003) Biological identifications through DNA barcodes. Proc R Soc B 270: 313–322.
5. Clare EL, Lim BK, Engstrom MD, Eger JL, Hebert PDN (2007) DNA barcoding of Neotropical bats: species identification and discovery within Guayana. Mol Ecol Not 7: 184–190.
6. Pfenninger M, Nowak C, Kley C, Stinke D, Striet B (2007) Utility of DNA taxonomy and barcoding for the inference of larval community structure in morphological cryptic *Chironomus* (Diptera) species. Mol Ecol 16: 1957–1968.
7. Vences M, Thomas M, Bonett RM, Vieites DR (2010) The integrative future of taxonomy. Front Zool 7: 16.
8. Bell JJ (2008) The functional roles of marine sponges. Estuar Coast Shelf Sci 79: 341–353.
9. Ehrlich H (2010) Chitin and collagen as universal and alternative templates in bio-mineralization. Int Geol Rev 52: 661–699.
10. Wo¨rheide G (2005) Low variation in partial cytochrome oxidase subunit I (COI) mitochondrial sequences in the coralline demosponge *Astrosclera willeyana* (Poecilosclerida). Mar Biol 144: 31–35.
11. Bell JJ (2008) The functional roles of marine sponges. Estuar Coast Shelf Sci 79: 341–353.
12. Desalle RM, Egan G, Siddall M (2005) The unholy trinity: taxonomy, species delimitation, and DNA barcoding. Phil Trans R Soc B 360: 1859–1868.
13. Hellberg ME (2006) No variation and low synonymous substitution rates in coral mDNA despite high nuclear variation. BMC Evol Biol 6: 24.
14. Bell JJ (2008) The functional roles of marine sponges. Estuar Coast Shelf Sci 79: 341–353.
15. Ehrlich H (2010) Chitin and collagen as universal and alternative templates in bio-mineralization. Int Geol Rev 52: 661–699.
16. Wulff J (2006) Ecological interactions of marine sponges. Can J Zool 84(2): 146–166.
17. Vences M, Thomas M, Bonett RM, Vieites DR (2005) Deciphering amphibian diversity through DNA barcoding: a new approach that challenges the debates questioning both methodologies. Biota Neotrop 10(2): 339–366.
18. Pires A, Marinoni L (2010) DNA barcoding and traditional taxonomy unified through Integrative Taxonomy: a view that challenges the debate questioning both methodologies. Biota Neotrop 10(2): 339–366.
19. Pires A, Marinoni L (2010) DNA barcoding and traditional taxonomy unified through Integrative Taxonomy: a view that challenges the debate questioning both methodologies. Biota Neotrop 10(2): 339–366.
20. Pires A, Marinoni L (2010) DNA barcoding and traditional taxonomy unified through Integrative Taxonomy: a view that challenges the debate questioning both methodologies. Biota Neotrop 10(2): 339–366.
21. Maldonado M (2009) Embryonic development of verongid demosponges. J Exp Zool B Mol Dev Evol 308: 347–356.
22. Bell JJ (2008) The functional roles of marine sponges. Estuar Coast Shelf Sci 79: 341–353.
23. Maldonado M (2009) Embryonic development of verongid demosponges supports the independent acquisition of spongian skeletons as an alternative to the silicious skeleton of sponges. Biol J Linn Soc 97(2): 447–447.
24. Borchiellini G, Chombard C, Manuel M, Alivon E, Eger JL, Hebert PDN (2007) DNA barcoding of Neotropical bats: species identification and discovery within Guayana. Mol Ecol Not 7: 184–190.
25. Pfenninger M, Nowak C, Kley C, Stinke D, Striet B (2007) Utility of DNA taxonomy in foraminiferal biodiversity studies. Mar Biol 151: 427–437.
27. Brummer E, Ehlich H, Schupp P, Hohrich R, Hunold S, et al. (2009) Chitin-based scaffolds are an integral part of the skeleton of the marine demosponge Isosthela hysta. J Struct Biol 168: 539-547.

28. Diaz MC, Soest RWMM Van, Rutkier K, Guzman HM (2003) Aplysina chrysipina, a new pedunculate sponge from the gulf of Chiriqui, Panama, Eastern Pacific (Aplysinae, Verongida). Zootaxa 1012: 1–12.

29. Bergquist PR, Cook SC (2002) Order Verongida Bergquist, 1978. In: Hooper JNA, Soest RWMM Van, editors. Systema Porifera: A guide to the classification of sponges. Kluwer, Dordrecht, Plenum Publishing Vol. (1), 1011–1085.

30. Pinheiro US, Hajdu E, Custódio MR (2007) Aplysina Nardo (Porifera, Verongida, Aplysinae) from the Brazilian coast with description of eight new species. Zootaxa 1609: 1–51.

31. Weiss B, Ebel R, Elbracht M, Kirchner M, Proksch P (1996) Defense metabolites from marine sponges. Biochem Syst Ecol 24(1): 1–12.

32. Kreuter M, Robitzki A, Chang S, Steffen R, Michaelis M, et al. (1992) Production of the cytostatic agent aphelopin from the sponge Verongia aerophoba in vitro cultures. Comp Biochem Physiol C Comp Pharmacol 101(1): 183-187.

33. Hentschel U, Schmid M, Wagner M, Fieseler L, Gernert C, et al. (2001) Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges Aplysina aerophoba and Aplysina cauliformis. FEMS Microbial Ecol 35(3): 303-312.

34. Bergquist PR (1980) A revision of the supraspecific classification of the orders Dityectozoaria, Dendroceratoida and Verongia (Class Demospongiae). N Z J Zool 7: 445–503.

35. Weydmann F (1977) Shallow-water sponges of the western Bahamas. Experimentia (Basel) 23: 1–287.

36. Laubenfels MW (1948) The order Keratosa of the phylum Porifera. A monographic study. Allan Hancock Found Publ Occ Pap 3: 217 p.

37. Laubenfels MW (1939). The sponges of California. Stanford Univ Bull 59(6): 24–29.

38. Hyatt A (1875) Revision of the North American Porifera with remarks upon foreign species. Part I. Mem Boston Soc Nat Hist 2: 399–408.

39. Bowerbank JS (1845) Observations on the Spongiadae, with descriptions of some foreign species. Part I. Mem Boston Soc Nat Hist 2: 399–408.

40. Heim I, Nickel M, Brümmer F (2007) Rio de Janeiro molecular markers for the order Verongida: a comparison of morphological and molecular data. J Mar Biol Assoc UK 90: 845–850.

41. Schmitt S, Hentschel U, Zea D, Dandekar T, Wolf M (2005) ITS–2 and 18S rRNA gene phylogeny of Aplysinidae (Verongida, Demospongiae). J Mol Evol 60: 327–336.

42. Davis JI, Nixon KC (1992) Populations, genetic variation, and the delimitation of phylogenetic species. Syst Biol 41: 421–435.

43. Duran S, Giribet G, Turon X (2004) Phylogeographical history of the sponge Chondrilla nucula (Porifera, Demospongiae) new species from the Mediterranean Sea and a redescription of Pachymatisma verrucosa (Porifera, Demospongiae) biogeography of the marine sponge genus Pachymatisma (Porifera, Demospongiae) indicate low dispersal capabilities and widespread crypts. J Mar Biol Assoc UK 84: 1511–1525.

44. van Oppen MJH, Wörheide G, Takabayashi M (2002) Nuclear markers in evolutionary and population genetic studies of scleractinian corals and sponges. In: Moosa KM, Soemodihardjo S, Soeijarto A, Romimohtar K, Nontji A, Soekarno, Suharsono, editors. Proceedings of the 9th International Coral Reef Symposium, Bali, Ministry for Environment, Indonesian Institute of Sciences, International Society for Reef Studies, 2001. Pp. 131–139.

45. Green G (1977) Sinopsis taxonomica de trece especies de esponjas del arrecife de Isla Mujeres. Auton Mex 15(1): 73–96.

46. van Soest RWM (2011) Aplysinidae. In: Soest RWM Van, Boury-Esnault N, Ružička K, editors. Systema Porifera. A guide to the classification of sponges. Experientia (Basel) 28: 1–287.

47. Rubinoff D, Holland BS (2005) Mitochondrial DNA is neither the panacea nor the nemesis of phylogenetic and taxonomic inference. Syst Biol 54: 311–333.

48. Nichols SA, Barnes PAG (2005) A molecular phylogeny and historical biogeography of the marine sponge genus Pachymatisma (Porifera) indicate low dispersal capabilities and widespread crypts. J Mar Biol Assoc UK 85: 1311–1321.

49. Davis JI, Nixon KC (2005) Evaluation of phylogenetic species. Syst Biol 54: 844–851.

50. Schuster RW, Starner S (2003) Host specificity of the symbiotic cytonbacterium Oscillatoria sp? in marine sponges, Dysidea sp. Mar Biol 142: 643–648.

51. Lopez JV, Peterson CL, Willoughby R, Wright AE, Enright E, et al. (2002) Characterization of genetic markers for in vitro cell line identification of the marine sponge Aspidella coruscans. J Hered 93(1): 27–36.

52. Bevere G, Nichols SA, Goldberg J (2004) Intragenomic variation of the rDNA internal transcribed spacers in sponges (Phylum Porifera): implications for phylogenetic studies. Mol Phylogenet Evol 33: 816–830.

53. Redmond NE, van Soest RWM (2002) Urraveling host and symbiont phylogenies of halichondrinid sponges (Demospongiae, Porifera) using mitochondrial marker. Mar Biol 141: 377–386.

54. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for the amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mar Mol Biol Technol 3: 299–299.

55. Chace MV (1996) Amplification of cytochrome oxidase subunit II from single ciliated protozoa. In: Innes M, Gralland J, Smulski J, White T, editors. PCR protocol: a guide to methods and applications. Academic Press Inc., New York, N.Y. Pp. 315–322.

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82. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596–1599.
83. Ronquist FR, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.
84. Posada D (2008) jModelTest: Phylogenetic model averaging. Mol Biol Evol 25: 1253–1256.
85. Carballo JL (1994) Taxonomı́a, zoogeografı́a y autoecologı́a de los Porı́feros del Estrecho de Gibraltar. Unpublished D. Phil. Tesis Doctoral, Universidad de Sevilla, España, 316 pp.