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Descriptions of new sponge species and genus, including aspiculate Plakinidae, overturn the Homoscleromorpha classification

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Among the Porifera, the taxonomy and systematics of Homoscleromorpha is one of the most challenging. Over the last two decades, this class and its single order Homosclerophorida have seen a high rate of new descriptions and phylogenetic investigations which have led to the resurrection of two well-supported families, defined as the 'spiculate' Plakinidae and the 'aspiculate' Oscarellidae. In recent years, the development of an integrative taxonomic approach and the exploration of new marine ecosystems have revealed an even higher diversity of Homoscleromorpha and highlighted the importance of complementary datasets (cytological, biochemical and molecular) to better explain the phylogenetic classification. Using this integrative approach, we here describe two new species of Plakinidae from submarine caves in the Caribbean Sea: ***Plakina arletensis* sp. nov.** and ***Aspiculophora madinina* gen. nov. sp. nov.,** the latter being aspiculate with an unusually well-developed collagen layer in its mesohyl and an abundant and diverse prokaryote community. The recently described *Oscarella nathaliae* was reassigned to *Plakina* on the basis of new molecular data and a careful re-examination of morphological characters. Thus, ***Plakina nathaliae* comb. nov.** and *A. madinina* are the first aspiculate representatives of Plakinidae and indicate the need for a detailed re-examination of Homoscleromorpha using this integrative taxonomic approach.

ADDITIONAL KEYWORDS: Homoscleromorpha – integrative taxonomy – phylogeny – Porifera – submarine caves – systematics.

INTRODUCTION

Sponges often dominate in terms of both diversity and biomass in semi-dark and dark rocky marine habitats (Hart, Manning & Iliffe, 1985; Vacelet, 1999). In the last four decades, studies of submarine caves have revealed many new species which often exhibit original biological (adaptive) traits (Hart *et al.*, 1985; Vacelet, Boury-Esnault & Harmelin, 1994; Vacelet, 1999). These studies were mainly conducted in the Mediterranean Sea and focused on habitat, community and species descriptions (Pouliquen, 1972; Harmelin, Vacelet & Vasseur, 1985; Fichez, 1990; Harmelin, 2003; Pérez *et al.*, 2004; Gerovasileiou *et al.*, 2015). Among submarine cave-dwellers, homoscleromorph sponges represent an abundant and diverse taxonomic group; 82% of the Mediterranean homoscleromorph species can be found in submarine caves, and 41% of them are cave-exclusive (Ereskovsky, Ivanišević & Pérez, 2009; Gerovasileiou & Voultsiadou, 2012). It is likely that surveys of as yet unexplored submarine caves throughout the oceans will lead to the discovery of

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new representatives of this poorly known group of sponges.

Homoscleromorpha was recently elevated to the rank of class (Gazave *et al.*, 2012), and is currently represented by one order, Homosclerophorida Dendy, 1905, and two families, Plakinidae Schulze, 1880 and Oscarellidae Lendenfeld, 1887. Plakinidae comprises the following six genera: *Corticium* Schmidt, 1862; *Plakina* Schulze, 1880; *Plakortis* Schulze, 1880; *Plakinastrella* Schulze, 1880; *Placinolopha* Topsent, 1897; *Tetralophophora* Rützler *et al.*, 2014. Plakinidae is currently defined as 'Homosclerophorida with siliceous skeletal components represented by calthrops, diods and triods' (Gazave *et al.*, 2010: 11). This family is also characterized by a sylleibid-like or leuconoid aquiferous system harbouring eurypylous or aphodal choanocyte chambers (Gazave *et al.*, 2010). Oscarellidae is defined as 'Homosclerophorida without spicules, with a sylleibid-like or leuconoid aquiferous system made up of spherical, eurypylous or diplodal choanocyte chambers' (Gazave *et al.*, 2010: 11). Within Oscarellidae, only two genera are recognized as valid in the World Porifera Database (Van Soest *et al.*, 2016): *Oscarella* Vosmaer, 1884 and *Pseudocorticium* Boury-Esnault, Muricy, Gallissian & Vacelet, 1995.

The lack of diagnostic morphological characters combined with phenotypic plasticity has resulted in much debate regarding the systematics of Homoscleromorpha, and its challenging taxonomy has led to several erroneous identifications, cases of alleged cosmopolitanism and unresolved species-complexes (Boury-Esnault *et al.*, 2013). Clearly there was a need for the exploration of other characters and datasets which may have the potential to shed light on the taxonomy and systematics of this group. Allozymes were first used to distinguish sibling species in *Oscarella* and *Corticium* (Boury-Esnault, Solé-Cava & Thorpe, 1992; Solé-Cava *et al.*, 1992). Cytological investigations also provided complementary characters for species delimitation among Oscarellidae (Boury-Esnault *et al.*, 1992; Muricy *et al.*, 1996; Muricy, 1999) and to a lesser extent in the genus *Plakina* (Muricy *et al.*, 1999). More recently, metabolomics was presented as a promising method to be included in an integrative approach to solve the new challenges in sponge systematics (Cárdenas, Pérez & Boury-Esnault, 2012). This integrative approach led to new insights into Homoscleromorpha interspecific relationships (Ivanišević *et al.*, 2011; Boury-Esnault *et al.*, 2013; Ruiz *et al.*, 2015) but maintained the distinction between the aspiculate family Oscarellidae and the spiculate Plakinidae.

In this paper, we use morphological, cytological and molecular datasets to describe one new genus and two new species of Plakinidae from submarine caves in the Caribbean Sea (Lesser Antilles, eastern Caribbean). We also present original molecular data which indicate that *Oscarella nathaliae* Ereskovsky, Lavrov & Willenz, 2013 should be reassigned to the genus *Plakina*, and consequently to the family Plakinidae. Together, these new data demonstrate that the present definitions of the two Homosclerophorida families are too simplistic and must be revised, especially because our new species descriptions provide new evidence that characters (synapomorphies) can be lost during evolution. Although this is not new for the Porifera, it was a matter of debate for Homoscleromorpha.

MATERIAL AND METHODS

Sampling

Specimens were collected by SCUBA diving in June 2011, March 2012, December 2013 and March 2014 from various sites around La Martinique Island, in December 2014 from the Caribbean coast of Colombia and then during the PACOTILLES cruise across the Lesser Antilles in May and June 2015 (Fig. 1). The specimens were all found on vertical walls, under horizontal rocks or on the ceiling of submarine caves at 7–25 m depth. Underwater photos and general information (size, shape, colour and consistency) were registered for each individual. Each sample was fixed in 95% ethanol and in 2.5% glutaraldehyde for molecular and morphological analyses, respectively.

Skeletal analysis

A piece of sponge was embedded in Araldite, at least three sections of about 5 mm thick were cut with a low-speed saw and wet-ground with abrasive paper or polishing discs to obtain thinner sections (< 1 mm thick), which were then mounted on glass slides and observed under a light microscope. For spicule preparations, a small fragment of each specimen was boiled with 5 mL nitric acid for organic degradation, and then washed several times with distilled water. The resulting solution was placed on slides for light microscopy. A minimum of 20 measurements were carried out for each spicule type. Spicule measurements are expressed as minimal and maximal length in micrometres. For scanning electron microscopy (SEM), slides containing spicules were coated with gold–palladium and observed under a Hitachi S-570 microscope.

Cytology

A small fragment of each specimen was fixed in 2.5% glutaraldehyde in 2 M phosphate buffer and filtered

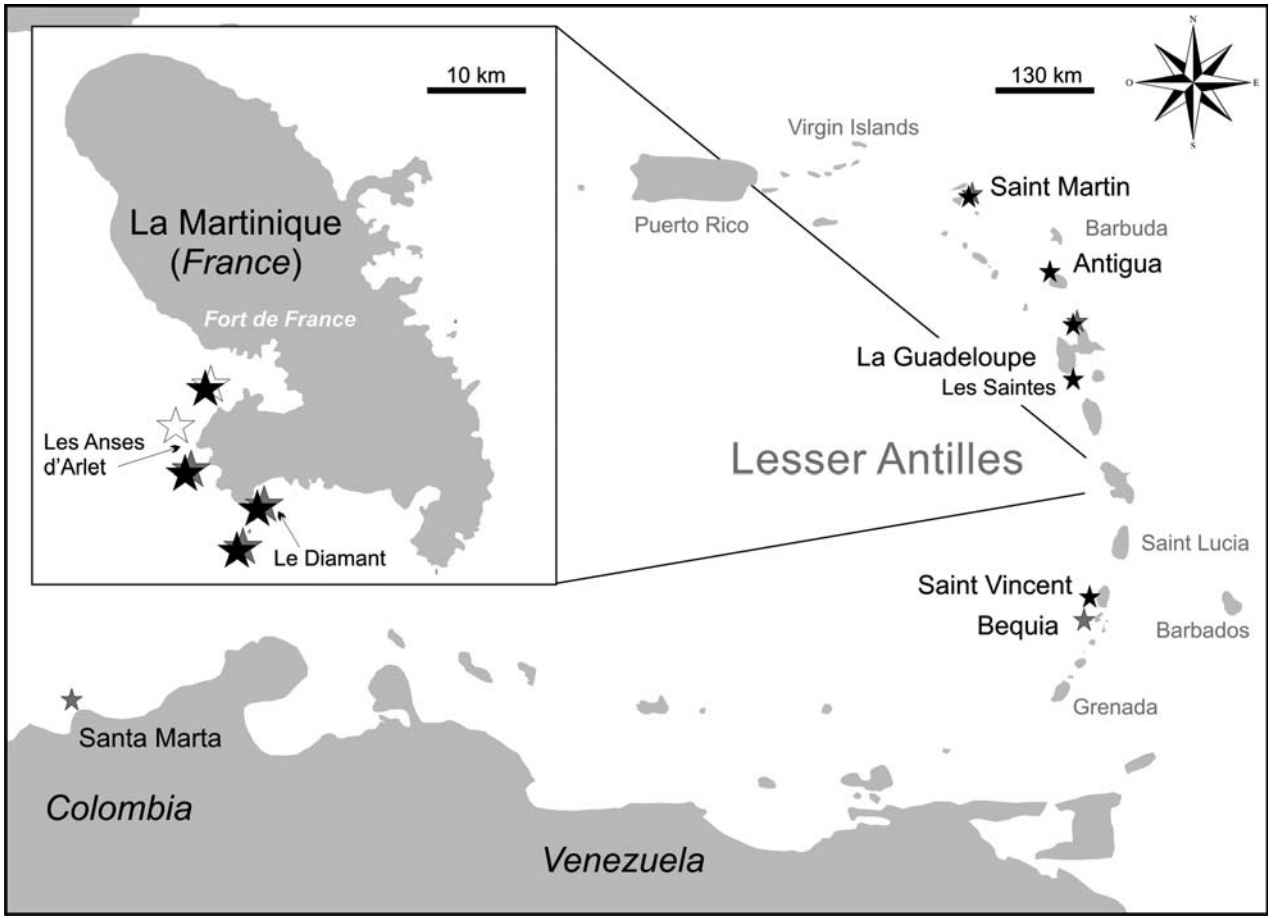
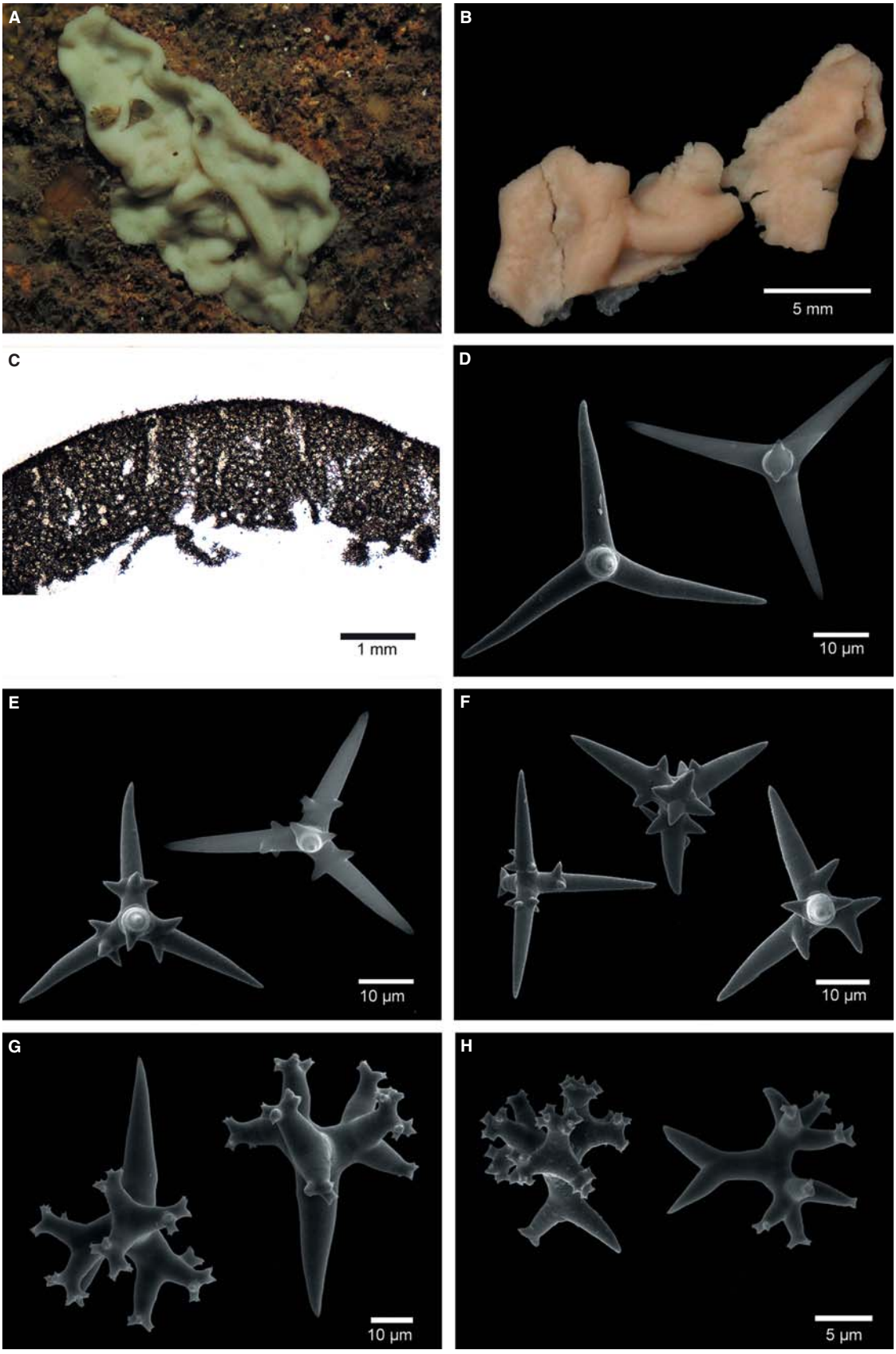


Figure 1. Known distribution in the Caribbean Sea of ***Plakina arletensis* sp. nov.** (white stars), ***Plakina nathaliae* comb. nov.** (grey stars) and ***Aspiculophora madinina* gen. nov., sp. nov.** (black stars).

seawater (1:4:5, by vol.), then post-fixed in 2% OsO_4 in seawater (Boury-Esnault, De Vos & Vacelet, 1984). Siliceous spicules were dissolved with 5% hydrofluoric acid for 2 h. For semi-thin and ultra-thin sections, each fragment was embedded in Araldite. Semi-thin sections were stained with toluidine blue and observed under a Leica DMBL light microscope. Ultra-thin sections were made using an RMC ultramicrotome PTXL. The cuts were placed on a copper grid (3.05 mm in diameter, 300 meshes) and stained with 2% uranyl acetate for 15 min. Observations were carried out with a JEOL JEM-1400 (Peabody, MA, USA) transmission electron microscope. The cytological and prokaryotic composition of each sample was analysed, taking into account the cells' morphology, dimensions and cytoplasm characteristics.

DNA analysis

For our molecular systematics study, we chose cytochrome oxidase 1 (CO1) markers because of their known value in differentiating closely related species among Homoscleromorpha (see, for instance, Pérez *et al.*, 2011; Boury-Esnault *et al.*, 2013; Ruiz *et al.*, 2015). DNA extractions were performed from small sponge fragments (2 cm^3) using a QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France). Two sets of primers were used: the universal primers LCO1490 and HCO2198 to amplify a 584-bp portion of the CO1 mitochondrial gene (Folmer *et al.*, 1994), and C1-Npor2760 and C1-J2165 to amplify a 507-bp fragment (I3-M11) of the CO1 mitochondrial gene (Misof, Erpenbeck & Sauer, 2000; Erpenbeck *et al.*, 2002). Amplifications were done in a 40μL total reaction volume with: 4 μL of each primer (10 μM), 6.4 μL dNTPs (10 mM), 8 μL polymerase buffer, 5 μL $MgCl_2$ (25 mM), 0.2 μL *Taq* polymerase (5 U μL^{-1}), 2.4 μL extracted DNA and 10 μL of ultrapure (Milli-Q) water. PCRs were performed on a Mastercycler gradient PCR-S Eppendorf thermocycler with an initial step of 5 min at 94 °C followed by 40 amplification cycles (denaturation at 94 °C for 1 min; annealing at 42 °C for 1 min; and extension at 72 °C for 1 min),


A
B
5 mm
C
1 mm
D
10 µm
E
10 µm
F
10 µm
G
10 µm
H
5 µm

and a final extension step at 72 °C for 5 min. PCR products were directly sequenced in each primer direction by Eurofins laboratory. For the phylogenetic tree using the Folmer fragment, 14 sequences were downloaded from GenBank and compared with five sequences obtained during this study. For the I3-M11 fragment, we used 14 sequences downloaded from GenBank with eight new sequences from the present study. A concatenated tree was also constructed using all new and downloaded sequences obtained from both fragments (total of 1078 bp). At least one species of each homoscleromorph genus was included in the analysis with the exception of *Placinolopha*.

Sequences were aligned using BIOEDIT 7.0.5.3 (Hall, 1999). Phylogenetic trees were constructed using both the neighbour-joining (NJ) method (1000 bootstrap replicates) with CLUSTAL X 2.0 (Larkin *et al.*, 2007) and maximum-likelihood (ML) method, under the GTR + I + G model (previously tested using Jmodeltest software; Darriba *et al.*, 2012) with a non-parametric bootstrap resampling of 100 replicates using PhyML algorithms (Guindon *et al.*, 2010). The concatenated tree was made with RAxML (Stamatakis, 2014) on XSEDE with the online platform CIPRES (http://www.phylo.org). In all analyses, the Demospongiae *Xestospongia muta* (Schmidt, 1870) was used as the out-group.

RESULTS

Systematics

Phylum Porifera Grant, 1836
Class Homoscleromorpha Bergquist, 1978
Order Homosclerophorida Dendy, 1905
Family Plakinidae Schulze, 1880

Definition: Homosclerophorida with siliceous spicules represented by diods, triods and/or calthrops. Aquiferous system sylleibid-like or leuconoid, made up by eurypylous or aphodal choanocyte chambers.

Genus *Plakina* Schulze, 1880

Type species
Plakina monolopha Schulze, 1880

Definition
Plakinidae with spicules diods, triods and calthrops in a single size class, and with homolophose calthrops with one to four lophate rays (Muricy & Díaz, 2002).

Plakina arletensis sp. nov.
Figs 2, 3

Material examined
Holotype: MNHN DJV177, Grotte Chauve-Souris at 12 m depth, Anse Noire, La Martinique (14°32.024′N, 61°05.278′W). Collector: T. Pérez, 10 December 2013. Slides and a fragment of the holotype were deposited in the sponge collection of the Museu Nacional of Universidade Federal do Rio de Janeiro, Brazil (MNRJ 18460).

Paratype: MNHN DJV178, Grotte Chauve-Souris at 11 m depth, Anse Noire, La Martinique. Collector: T. Pérez, 6 March 2014.

Other specimens examined
150530-GU6-TP04, Grotte Amédien at 12 m depth, La Guadeloupe (16°30.033′N, 061°28.774′W). Collector: T. Pérez, 30 May 2015.

150516-MT8-CR03, Anse Fortune at 7 m depth, La Martinique (14°30.377′N, 61°05.850′W). Collector: C. Ruiz, 16 May 2015.

Comparative material examined
Plakina jamaicensis Lenhert & van Soest, 1998. RBINS POR 70, Chalet Caribe caves, West of Montego Bay, Jamaica (18°27.246′N, 77°58.287′W). Collector P. Willenz and A. Ereskovsky.

Etymology: Plakina arletensis refers to the little village 'Les Anses d'Arlet', close to the cave where this sponge was first found, which is one of the most beautiful places in La Martinique. This is also a special dedication to the kind and warm inhabitants of this village.

Diagnosis: Thin encrusting and white *Plakina*, with a rugose folded surface, a skeleton made of monolophose, trilophose and tetralophose calthrops, actines with and without terminal spines. Diods and triods are absent. Well-developed mesohyl with a high abundance of prokaryotic symbionts.

Description
Small crust, about 2–15 cm in size, 0.2–0.5 cm thick. White colour *in vivo*, becoming slightly cream or light brown in alcohol (Fig. 2A, B). The consistency

Figure 2. ***Plakina arletensis*** sp. nov. A, *in situ* photo. B, same specimen after fixation in alcohol. C, transversal cut of skeleton. D, calthrops without spines. E, calthrops with spines. F, monolophose calthrops. G, trilophose calthrops. H, tetralophose calthrops.

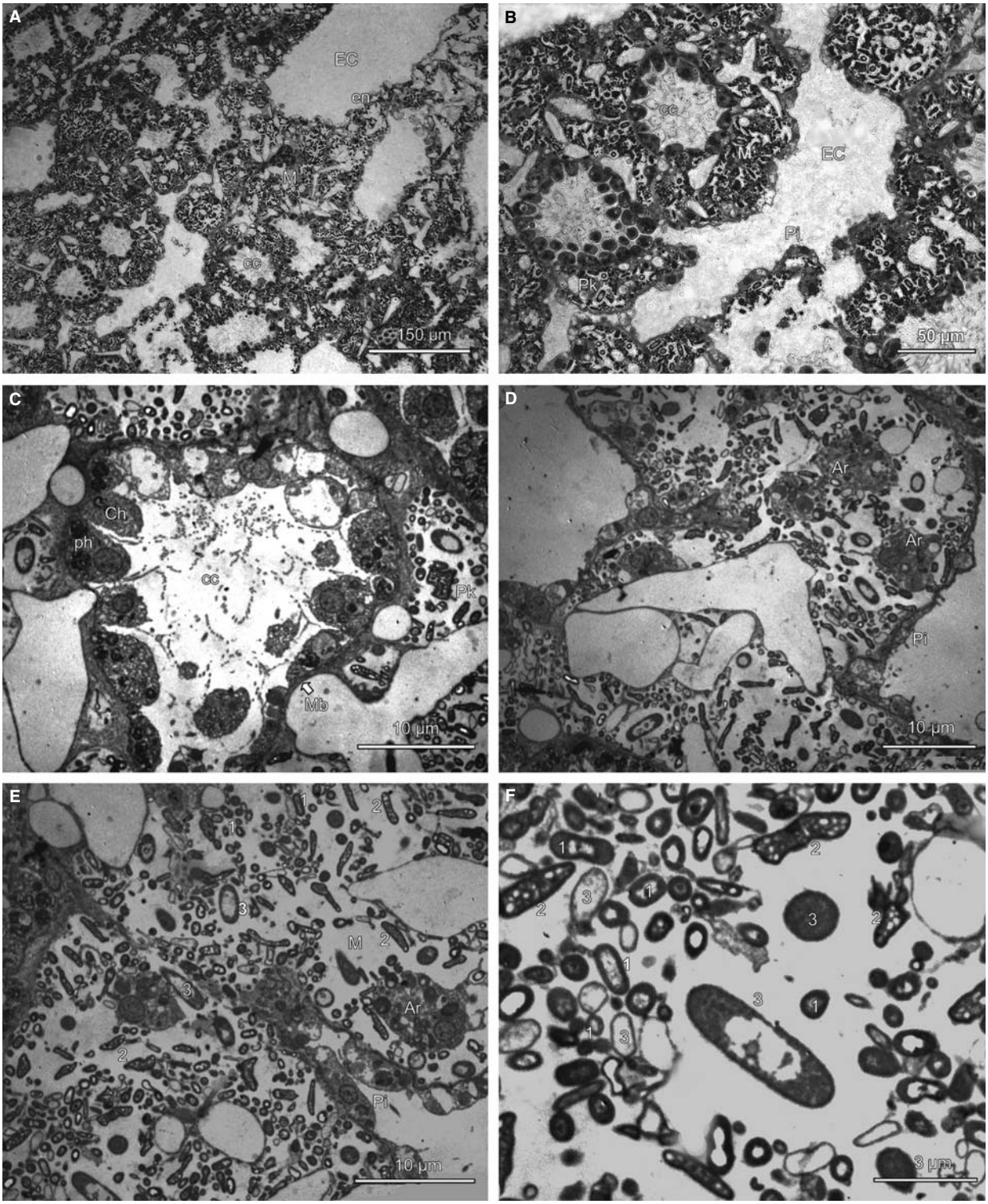


Figure 3. A, general soft tissue organization of ***Plakina arletensis* sp. nov.** B, detailed view of the mesohyl, showing the aquiferous system. C, transmission electron micrograph of a choanocyte chamber. D transmission electron micrograph with archeocytes and pinacocytes. E, transmission electron micrograph showing the abundance of prokaryotic cells inside the mesohyl and the three main morphotypes (1–3). F, transmission electron micrograph with a detailed view of the prokaryote symbionts (1–3). Ar, archeocytes; cc, choanocyte chamber; Ch, choanocyte; en, endosome; EC, exhalant canal; Mb, basement membrane; M, mesohyl; ph, phagosome; Pi, pinacocytes; Pk, prokaryotes.

is cartilaginous and the surface is rugose and irregularly folded. Oscules are circular, 1–2 mm in diameter, with a slightly elevated rim.

Skeleton: The skeleton is dense, especially at the surface which is pierced principally by trilophose calthrops. The choanosomal skeleton has an alveolar arrangement formed by all spicule types (Fig. 2C).

Spicules: Diods and triods are absent. Calthrops are irregular, abundant, their actines (20–30 μm long) being with or without spines. Spines (1–4) are short and conical, usually at the basis of each actine (Fig. 2D, E). Monolophose calthrops are abundant (13–28 μm actines long). The lophose actine is distally ramified by 2–4 short conical rays with sharp endings (Fig. 2F). Trilophose calthrops are common; lophose actines ramify close the base in 2–3 rays, distally in 2–4 smaller rays with terminal spines (ramification pattern 1 proximal, 2 distal, terminal spines; for the terminology of ramification patterns, see Muricy & Díaz, 2002). The non-lophose actines are 10–30 μm long (Fig. 2G). Tetralophose calthrops are heterolophose and the less abundant spicule type. The three apical actines ramify distally in 3–4 rays, then distally in 2–5 short rays with terminal spines. The basal actine ramifies distally in two rays, and then distally again in 2–3 short rays with terminal spines (ramification pattern 1 distal, 2 distal, terminal spines). The actine length is 5 μm on average (Fig. 2H; Table 1).

Tissue general organization: The ectosome is 15–30 μm thick, separated from the choanosome by subectosomal cavities (15–40 μm) with a well-developed system of inhalant/exhalant canals (25–140 μm wide). The aquiferous system is leuconoid (Fig. 3A, B). Choanocyte chambers, 47–78 μm in diameter, are spherical and diplodal (Fig. 3B, C).

Cytology: Choanocytes are 3–7 μm wide and 4–6 μm high, their collar, 40–50 μm in diameter, is composed of about 50 microvilli. The choanocyte nucleus is spherical and located on the apical area of the cell (4–5 μm in diameter). Their cytoplasm is dense, often with one to five phagosomes of about 1.0–1.5 μm (Fig. 3B, C). The exo- and endopinacocytes are flat or ovoid (15–27 μm long and 7–11 μm large), with several vacuoles observed in their cytoplasm (Fig. 3B–D). Their nucleus is also flattened (4–5 μm in diameter). Choanocytes and pinacocytes are flagellated, underlined by a basement membrane (Fig. 3C, D). Few archaeocytes were observed. They have an irregular form with a spherical nucleus 2 μm in diameter (Fig. 3D, E).

Symbiotic prokaryotes: Plakina arletensis sp. nov. can be considered as a high microbial abundance (HMA) sponge. Three main morphotypes were easily detected, all three being extracellular and randomly dispersed in most of the mesohyl (Fig. 3E, F). The first morphotype corresponds to an abundant ovoid cell (1.5–1.8 μm long; 0.4–1 μm high), with a more or less dense periplasm. We consider most of the small spherical forms as a perpendicular view of morphotype 1 (Fig. 3E, F). The second morphotype, less abundant, has a rod-like to irregular shape (2–3 × 0.3–0.8 μm) with several translucent vacuoles. The third morphotype, less abundant, has the same ovoid form and dense periplasm as the first morphotype, but is bigger (3–5 × 1–1.7 μm) (Fig. 3E, F).

Ecology: Plakina arletensis sp. nov. is found only in shallow water caves, where it forms a white crust with a patchy distribution on vertical walls. No indications of epibiosis or predation were observed.

Taxonomic remarks

Plakina arletensis sp. nov. has a growth-form typical of the 'true' *Plakina* species. It has mono-, tri- and tetra-lophose calthrops like the Mediterranean species *P. trilopha* Schulze, 1880, *P. jani* Muricy, Boury-Esnault, Bézac & Vacelet, 1998, *P. endoumensis*

Table 1. Comparison of spicule size and abundance between *Plakina* species from the Caribbean, the Mediterranean Sea and the north-east of Brazil

Spicule characters (size in μm)	*P. trilopha*	*P. jani*	*P. jamaicensis*	*P. coerulea*	*P. arletensis*
Diods (length)	A, 40–88	A, 43–100	A, 61–91	63–90	–
Triods (actine length)	A, 12–33	A, 16–37	A, 22–36	60–75	–
Calthrops (actine length)	C, 10–35	C, 16–38	A, 6–33	41–55	A, 20–30
Monolophose calthrops (actine length)	R, 20–30	R, 22–38	R, 20–32	25–36	A, 13–28
Dilophose calthrops (actine length)	R, 20–35	R, 30–35	R, 25–36	28–35	–
Trilophose calthrops (actine length)	A, 16–27	C, 19-32	R, 22–29	15–21	C, 10–25
Tetralophose calthrops (actine length)	C, 10–25	C, 19–25	C, 17–26	12–21	R, 5–7
Ramification pattern of lophose calthrops	1 md, ts	1 m, 2d, ts	1 m, 2d, ts	1m, 2d, ts	1d, 2d, ts

A, abundant; C, common; R, rare (see Muricy *et al.*, 1998 for a description of the ramification patterns).

Muricy, Boury-Esnault, Bézac & Vacelet, 1998 and *P. weinbergi* Muricy, Boury-Esnault, Bézac & Vacelet, 1998 or like *P. jamaicensis* from the Caribbean Sea or *P. coerulea* Cedro, Hajdu & Correia, 2013 from north-eastern Brazil. In contrast to these two sponges, the new species does not possess diods or triods (Table 1). The tetralophose calthrops are heterolophose, but not candelabra-like as in *Corticium*. The basal actin is best described as bifurcated, with different ramification patterns in comparison with the apical actines. This type of heterolophose calthrops can be observed in other *Plakina* species such as *P. jani* and *P. endoumensis* (Muricy *et al.*, 1998).

Plakina arletensis sp. nov. has a well-developed mesohyl, with subectosomal cavities similar to *P. trilopha*, *P. jani* and *P. kanaky* Ruiz & Pérez, 2015 (Table 2). As with most *Plakina* species, the new species does not have vacuolar cells in the mesohyl, but prokaryote symbionts are abundant (Muricy *et al.*, 1999). In addition to the similarities in morphological traits, the assignment of the new species to *Plakina* is also highly supported by phylogenetic analyses. The CO1 sequences for our new species show it clustering with other *Plakina* species and also show it to be a distinct species (see below).

PLAKINA NATHALIAE (ERESKOVSKY, WILLENZ & LAVROV, 2013) COMB. NOV.
FIG. 4

Synonymy
Oscarella nathaliae Ereskovsky, Willenz & Lavrov, 2013

Holotype: RBINS POR 90, 22 m depth. Le Diamant, Grotte du Fer à Cheval, La Martinique (14°28.067′N, 61° 00.983′W). Collector: P. Willenz and A. Ereskovsky. 7 June 2003.

Paratype: MNHN DJV179, 17 m depth. Rocher du Diamant, Grotte de Zeb, La Martinique (14°26.5′N, 61°03.083′W). Collector: T. Pérez. 13 June 2011.

Other material examined
250312-MT4b-TP2. Grotte du Fer à Cheval at 20 m depth, le Diamant, La Martinique (14°28.067′N, 61°00.983′W). Collector: T. Pérez. 25 March 2012.

131203-MT3-AE4. Grotte Couleur, Pointe Burgos at 12 m depth, Anse d′Arlet, La Martinique (14°29.752′N, 61°05.407′W). Collector: A. Ereskovsky. 3 December 2013.

150529-GU3-CR6. Grotte Cathédrale at 15 m depth, Anse Bertrand, La Guadeloupe (16°27.740′N, 061°31.837′W). Collector: C. Ruiz. 29 May 2015.

GR34HOM30. Grotte Cathédrale at 15 m depth, Anse Bertrand, La Guadeloupe (16°27.740′N, 061°31.837′W). Collector: A. Ereskovsky. 18 May 2012.

150527-SN5-TP1. Basses Espagnoles at 12 m depth, Saint Martin (18°07.821′N, 63°00.270′W). Collector: T. Pérez. 27 May 2015.

141211-SM5-CR3. Punta Gaira at 17 m depth, El Rodadero, Santa Marta, Colombia (11°13.133′N, 74°14.45′W). Collector: C. Ruiz. 12 December 2014.

141212-SM7-CR2. Punta Venado at 20 m depth, Taganga, Santa Marta, Colombia (11°16.217′N, 74°12.367′W). Collector: C. Ruiz. 12 December 2014.

Diagnosis (modified from Ereskovsky *et al.*, 2013): Cave-dwelling thin and translucent aspiculate Plakinidae. Colour milky beige, sometimes pale orange, forming pendulous thin layers with a perforated surface and a clearer membrane in the periphery. Consistency soft, slimy and very fragile. Two mesohylar cells with inclusions: large, abundant vacuolar cells and small, rare granular cells.

Distribution (modified from Ereskovsky et al., 2013): Caribbean: Greater Antilles, Jamaica, and Lesser Antilles, Saint Martin, La Guadeloupe, La Martinique, Les Grenadines (Bequia); south-western Caribbean: Colombia (Santa Marta). This species is always found in semi-dark caves, from 10 to 50 m depth.

Taxonomic remarks
Eight specimens of *P. nathaliae* were identified by comparing morphological, cytological and/or genetic characters. All individuals share the features of the species described by Ereskovsky *et al.* (2013): cave-dwelling species, thin layer yellow to beige in colour (Fig. 4A–D), thin ectosome (3–50 μm high) with syllebeid aquiferous system and spherical choanocyte chambers (Fig. 4E). The two mesohyl cells originally described were also found in the studied specimens.

ASPICULOPHORA GEN. NOV.
ASPICULOPHORA MADININA SP. NOV.
FIGS 5, 6

Diagnosis: Plakinidae without spicules, well-developed mesohyl with a thick collagen layer and without subectosomal cavities. Generally brown, but also cream to white. Smooth and 'grooved' surface. Jelly-like consistency. Leuconoid aquiferous system and aphodal choanocyte chambers. High abundance of prokaryotic symbionts.

Holotype: MNHN DJV180, Rocher du Diamant at 12 m depth, La Martinique (14°26.5′N, 61°03.083′W). Collector: T. Pérez, 13 June 2011.

Table 2. Comparison of principal cytological characters among *Plakina* species

Character	*Plakina trilopha*	*Plakina jani*	*Plakina crypta*	*Plakina endoumensis*	*Plakina kanaky*	*Plakina jamaicensis*	*Plakina arletensis*	*Plakina nathaliae*	*Aspiculophora madinina*
Well-developed mesohyl	+	+	–	–	+	NA	+	–	+
Sub-ectosomal cavities	+	+	–	–	+	–	+	–	–
Collagen density	Moderate	Moderate	Low	Low	Moderate	Moderate	Moderate	Low	High
Aquiferous system	Leuconoid	Leuconoid	Leuconoid	Leuconoid	Leuconoid	Leuconoid	Leuconoid	Leuconoid	Leuconoid
Mesohyl/chambers ratio	1.5:1	1.6:1	0.7:1	0.9:1	1.8:1	1.8:1	1.5:1	0.7:1	1.8:1
Choanocyte chambers	Eurypylous	Eurypylous	Eurypylous	Eurypylous	Eurypylous	Eurypylous	Eurypylous	Eurypylous	Eurypylous
Choanocytes	Pyramidal	Pyramidal	Pyramidal	Cylindrical	Ovoid–pyramidal	Ovoid–spherical	Ovoid–spherical	Ovoid–spherical	Ovoid–spherical
Archaeocytes	+	+	+	+	+	+	+	–	+
Vacoular cells	–	+	–	–	–	–	–	+	–
Number of prokaryote morphotypes	>8	>8	6	1	>8	NA	3	2	5
Morphotypes shared by two or more species	A, B, H, J	B, D	B		3, 6	NA	3, 1	B2	1, 2
Bacterial distribution	Patchy	Patchy	Random	Random	Patchy	NA	Patchy	Random	Patchy
Abundance of prokaryote symbionts	HMA	HMA	LMA	LMA	HMA	NA	HMA	LMA	HMA

+, Presence; –, absence; HMA, high microbial abundance; LMA, low microbial abundance; NA, data not available (modified from Muricy *et al.*, 1999).

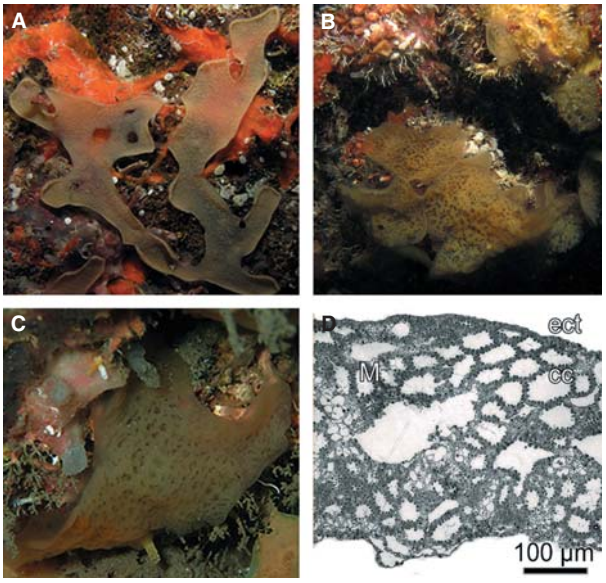


Figure 4. ***Plakina nathaliae*** **comb. nov.** A, *in situ* picture of a specimen from Rocher du Diamant, La Martinique. B, *in situ* picture of a specimen from Grotte Cathédrale, La Guadeloupe. C, *in situ* picture of a specimen from Punta Gaira, Colombia. D, light micrograph of a transversal section through the ectosome and the choanosome of *P. nathaliae*. cc, choanocyte chamber; ect, ectosome; M, mesohyl.

Paratype 1: MNHN DJV181, Tintamare, Les Arches at 10 m depth, Saint Martin (18°07.588′N, 62°58.248′W). Collector: C. Ruiz, 26 May 2015.

Paratype 2: MNHN DJV182, Grotte Cathédrale at 16 m depth, Anse Bertrand, La Guadeloupe (16°27.740′N, 061°31.837′W). Collector: C. Ruiz, 29 May 2015.

Other specimens examined

150514-MT6-TP1, Grotte Chauve-Souris at 7 m depth, Anse Noire, La Martinique (14°32.024′N, 61°05.278′W). Collector: T. Pérez, 14 May 2015.

150517-SV1-CR14, Bat Cave at 8 m depth, Bucament Bay, Saint Vincent (13°11.275′N, 61°16.174′W). Collector C. Ruiz, 17 May 2015.

150523-GU1-CR7, Cave north-west of Les Saintes at 8 m depth, Les Saintes, La Guadeloupe (15°52.984′N, 61°34.25′W). Collector: C. Ruiz & T. Pérez, 23 May 2015.

131206-MT3-AE2, Grotte Couleur, Pointe Burgos at 12 m depth, Anse d'Arlet, La Martinique (14°29.752′N, 61°05.407′W). Collector: A. Ereskovsky, 6 December 2013.

150527-SN5-TP5, Basses Espagnoles at 10 m depth, Saint Martin (18°07.821′N, 63°00.270′W). Collector: T. Pérez, 27 May 2015.

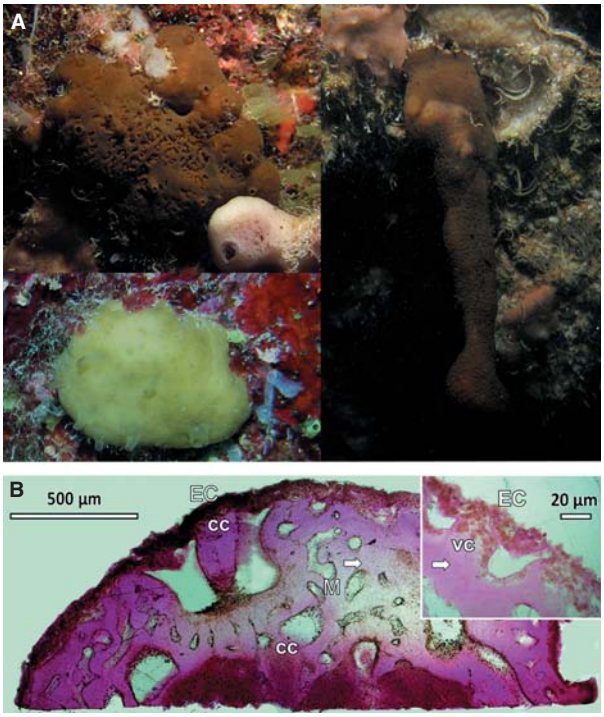


Figure 5. ***Aspiculophora madinina*** **gen. nov. sp. nov.** A, *in situ* pictures of three specimens collected in La Martinique. B, general histological organization, with a detailed view of the mesohyl and the collagen layer (arrow). cc, choanocyte chamber; EC exhalant canal; M, mesohyl; vc, vacuolar cells.

150528-AG3-CR1, Little Scrub at 15 m depth, Anguilla (18°17.903′N, 62°57.294′W). Collector C. Ruiz, 28 May 2015.

150530-GU6-TP7, Grotte Amédien at 12 m depth, La Guadeloupe (16°30.033′N, 061°28.774′W). Collector: T. Pérez, 30 May 2015.

Etymology: The genus name reflects the absence of skeleton: from Latin *a* (= without) *spiculum* (= spearhead, arrowhead) *phora* (= bearing). The species name, *madinina*, refers to the amerindian name, 'Flower Island', of La Martinique Island, which is the first island of the Caribbean Sea where this aspiculate Plakinidae was found.

Description: Aspiculophora madinina has mainly a cushion shape, measuring 2–15 cm in diameter by 2–5 cm high, but sometimes it can be found hanging down, with prominent oscules surrounded by a thin membranous collar (Fig. 5A). The colour *in vivo* is mainly brown, occasionally yellow to cream (Fig. 5A). Brown specimens produce a dark exudate in contact with alcohol. The consistency is soft, almost gelatinous. The surface is smooth and grooved. Oscules are circular, 1–2 mm in diameter with an elevated rim.

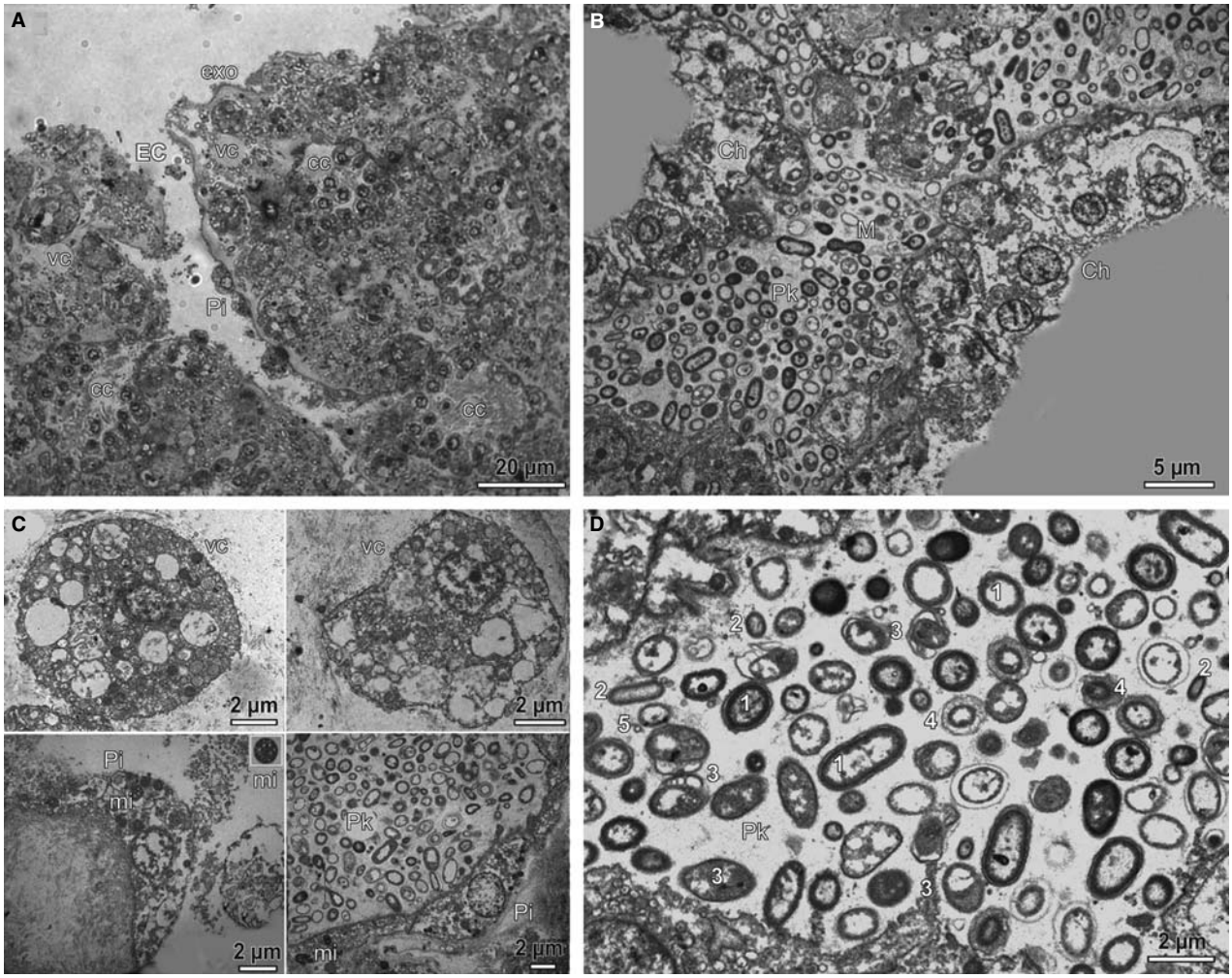


Figure 6. A, light micrograph of the ectosome and choanosome of ***Aspiculophora madinina* gen. nov., sp. nov.** B, transmission electron micrograph showing the high prokaryotic density in a part of the mesohyl located between two choanocyte chambers. C, transmission electron micrographs of vacuolar cells and pinacocytes. D, prokaryote morphotypes associated with the mesohyl of *Aspiculophora madinina*. Ch, choanocyte; cc, choanocyte chamber; EC, exhalant canal; exo, exopinacoderm; M, mesohyl; Pk, prokaryotes.

Soft tissue organization: The ectosome is 6–15 µm. The aquiferous system is leuconoid with inhalant/exhalant canals about 30–100 µm wide. A dense collagen fibril layer, about 8 µm in thickness, surrounds the exhalant canals. Some specimens also exhibit a thicker collagenous layer, about 1 mm, between the ectosome and the basal part of the sponge, where very few cells or prokaryotes are found (Fig. 5B). Choanocyte chambers (18–36 µm in diameter) are spherical and aphodal.

Cytology: The choanocytes are cylindrical to spherical, 4–7 µm wide and 6–7 µm high. Their nucleus, 2 µm in diameter, is spherical and in apical position (Fig. 6A, B). Their cytoplasm is not dense, often with one to three phagosomes and microgranular inclusions of about 1.5 µm in diameter. These inclusions are also observed in endopinacocytes (Fig. 6C). The exo- and endopinacocytes are flattened, 15–20 µm long and 7–11 µm wide. Only one type of vacuolar cell is present and restricted to the ectosomal region, sometimes in aggregates, always close to exhalant canals (Fig. 6C). This spherical cell, 9–13 µm in diameter, has a nucleus of 3–4 µm and harbours between two and ten vacuoles. Few archaeocytes were observed, with an irregular form and a nucleus of about 2 µm in diameter.

Symbiotic prokaryotes: This sponge can be considered as an HMA sponge. Five morphotypes were distinguished based on their morphological traits. All prokaryotes are randomly dispersed and

Table 3. Accession numbers of the specimens sequenced in the present study using the Folmer (Folmer *et al.*, 1994) and the I3-M11 fragment (Misof *et al.*, 2000; Erpenbeck *et al.*, 2002) of the CO1 gene

	Sample code	CO1 fragment	Accession no.
Plakina arletensis	110613-MT6-TP1	I3-M11	KU674369.1
	140306-MT6-TP1	I3-M11	KU674370.1
	150516-MT8-CR3	I3-M11	KU674371.1
Plakina nathaliae	110613-MT4-TP2	Folmer	KU674377.1
	141211 SM5-CR03	Folmer	KU674378.1
	141212 SM7-CR02	Folmer	KU674379.1
Aspiculophora madinina	110613-MT4-TP11	I3-M11	KU674367.1
	131206 MT3-AE2	I3-M11	KU674368.1
Corticium diamantense	110613MT4-TP5	Folmer	KU674376.1
Plakinastrella onkodes	141209SM1CR01	I3-M11	KU674372.1
Plakinastrella sp.	GR26H7	I3-M11	KU674373.1
	150514-MT6-CR5	I3-M11	KU674374.1
	150515-MT4-CR15	I3-M11	KU674375.1
	130205NC10-01	Folmer	KU674380.1

occupy most of the mesohyl (Fig. 6B–D). The first morphotype corresponds to an abundant ovoid cell (2–3 μm long; 1–1.5 μm wide), with a clear membrane about 0.1 μm wide. The second morphotype, also abundant in the sponge mesohyl, has a rod form (1.5 μm long; 0.5 μm wide). The third morphotype has an irregular ovoid form (1.5–2.0 μm long; 1 μm wide), with a dense cytoplasm and one to three external vacuoles in contact with the cells' outer membrane. The fourth morphotype is a spherical cell, with a dense periplasm, of about 1 μm in diameter. The fifth morphotype is a small spherical cell of about 0.1 μm in diameter. This morphotype was sometimes observed in groups of three to five cells in a row (Fig. 6D).

Ecology: Aspiculophora madinina sp. nov. is a sciaphilous species, living on vertical walls of semi-dark and dark caves or under overhangs. It was recorded between 5 and 50 m depth.

Taxonomic remarks

Aspiculophora madinina sp. nov. is a homoscleromorph sponge without skeleton and with a high content of collagen, providing a jelly-like consistency. As yet, massive forms such as this have not been reported in the aspiculate Oscarellidae. On the other hand, this species could be a *Plakortis* without spicules because most of its representatives occur as massive or globular forms. There is one report of such a *Plakortis* without spicules in the Caribbean Sponge Guide (Zea, Henkel & Pawlik, 2014), but the taxonomic affiliation to *Plakortis* was based only on the external morphology and the pungent smell of some specimens. It is difficult to compare the cytological characteristics of a putative new species with other Plakinidae, because of the lack of data at this level of biological organization. Indeed, traditional taxonomy has placed most attention on the skeletal description of this family, even if skeletal differences are poorly detectable in many species. Some other characters such as a leuconoid aquiferous system, eurypylous choanocyte chambers, well-developed mesohyl and subectosomal cavities are not sufficiently diagnostic, as they can be present or absent among *Plakina*, *Plakortis* and *Plakinastrella* species. We thus believe that more thorough cytological investigations of *Plakortis*, *Plakinastrella* and *Corticium* might help in resolving a good number of taxonomic questions in this family.

The new genus is proposed because of the singularity of its CO1 sequences positioning the new species among the Plakinidae, near the base of this clade, but outside all previously known genera.

Moreover, we believe that its internal organization, with a thick collagen layer surrounding the inhalant canals and within the mesohyl, is unique among Homoscleromorpha.

DNA analysis of studied species

GenBank accession numbers for all sequences in this study are presented in Table 3. The two families of Homoscleromorpha are well supported in the phylogenetic reconstructions (Figs 7–9). With the Folmer partition (Fig. 7), *Corticium* appears as a monophyletic taxon of Plakinidae. *Plakortis* seems to be paraphyletic, a first clade being composed of *Plakortis angulospiculatus* (Carter, 1879) and the type species *P. simplex* Schulze, 1880, while a second clade clusters *P. albicans* Cruz-Barraza & Carballo, 2005 with two *Plakinastrella* species. In this

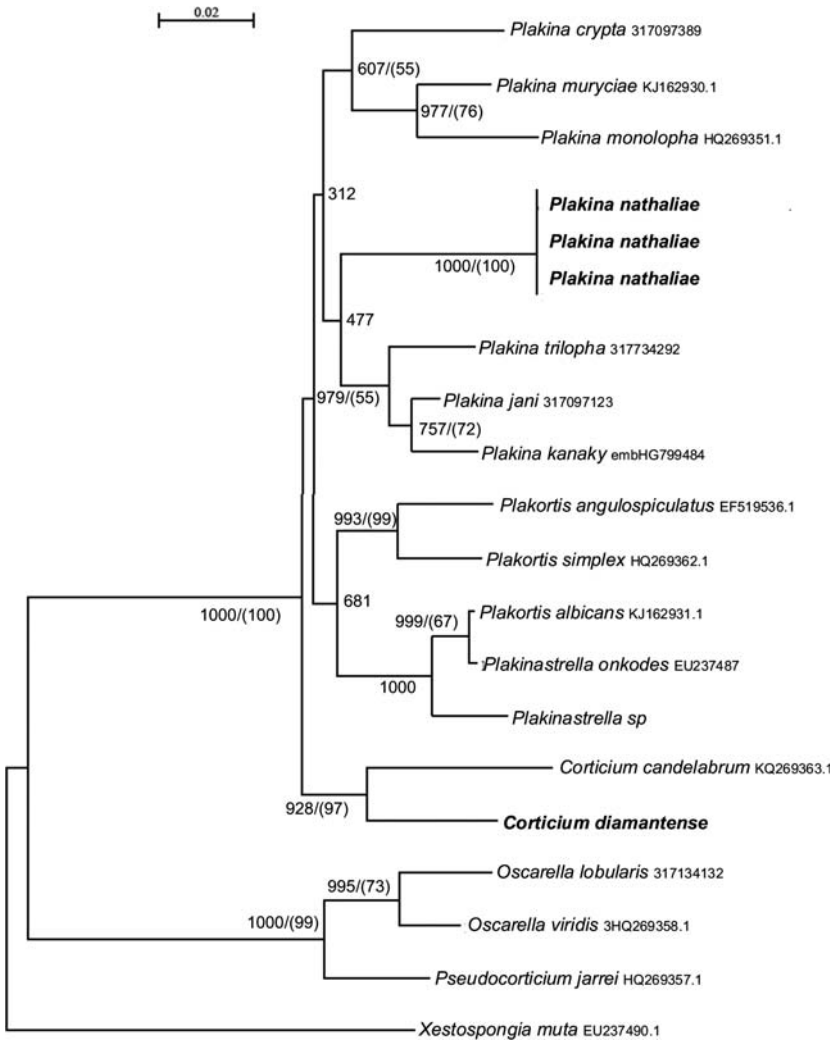


Figure 7. Phylogenetic reconstruction of the universal Folmer portion of the CO1 gene using neighbour-joining analysis and indicating maximum-likelihood (ML) values. Bootstrap values are given for both analyses; ML values are in parenthesis. The accession numbers of each download sequence are next to the species name.

representation lacking a sequence of the type species, *Plakina* appears as a poorly supported monophyletic group (Fig. 7), with two clades (bootstrap values of 312/47), one with *P. crypta* Muricy, Boury-Esnault, Bézac & Vacelet, 1998, *P. monolopha* and *P. muricyae* Cruz-Barraza, Vega & Carballo, 2014 closely related, and the other comprising three specimens of *P. nathaliae*, *P. trilopha*, *P. jani* and *P. kanaky*.

The second phylogenetic tree using the I3-M11 CO1 fragment (Fig. 8) supports three monophyletic genera among Plakinidae, *Aspiculophora*, *Plakinastrella* and *Plakortis*. In this analysis *Plakina* is paraphyletic, *P. monolopha* (type species) forming a highly supported clade with *C. candelabrum* Schmidt, 1862, a second group containing *P. arletensis* sp. nov. and *P. jani*, and a third with *P. crypta* and *P. trilopha*.

The third phylogenetic tree was made by concatenating all available sequences (Fig. 9). Because of missing data for one of the two fragments, the alignment contains several gaps and the concatenated tree appears less robust than the two-first representations of the inter-specific relationships among Homoscleromorpha. However, the topology of this third tree is quite congruent with the previous ones. In this representation, *Aspiculophora* forms a separate clade within the Plakinidae. Also, *Plakina* seems paraphyletic with two different clades, the first one grouping *P. arletensis* sp. nov. with *P. kanaky*, *P. jani*, *P. trilopha* and *P. nathaliae*, whereas the second one, containing the type species

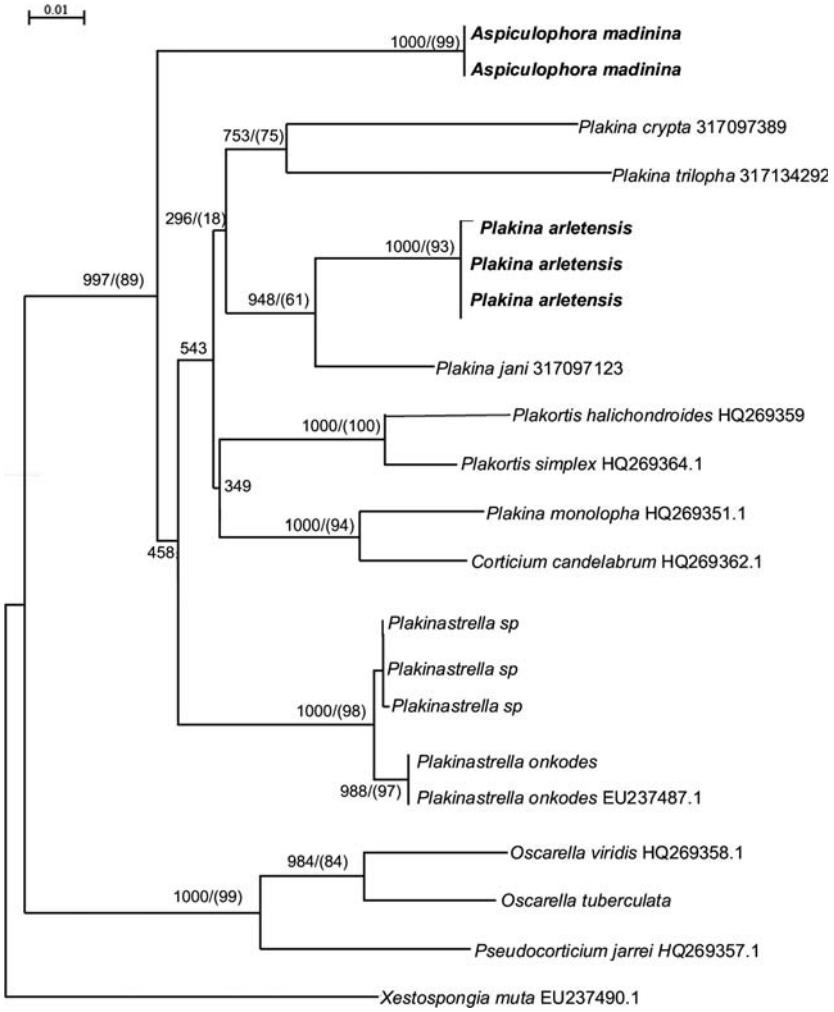


Figure 8. Phylogenetic reconstruction of the I3-M11 portion of the CO1 gene using neighbour-joining (NJ) analysis and indicating maximum-likelihood (ML) values. Bootstrap values are given for both analyses; ML values are in parenthesis. The accession numbers of each download sequence are next to the species name.

P. monolopha, *P. muryciae* and *P. crypta*, is related to *Corticium*, which seems monophyletic. *Plakortis* also appears paraphyletic, a first clade grouping the type species *P. simplex*, *P. halichondroides* and *P. angulospiculatus*, and a second clade containing *P. albicans*, *Plakinastrella onkodes* and an undetermined *Plakinastrella*. Finally, a separate clade containing five specimens of *Plakinastrella* also makes this genus paraphyletic in this phylogenetic representation.

DISCUSSION

Eighty species of Plakinidae are recognized as valid in the World Porifera Database (Van Soest *et al.*, 2016), to which the present study adds another three. Plakinidae is found throughout the world's oceans except in polar regions, and appears to be often found in shady rocky habitats. In the Caribbean Sea, several new species and a new genus have recently been described (Ereskovsky *et al.*, 2013; Rützler *et al.*, 2014), although it is likely that the diversity of this family remains underestimated, mainly because of the lack of exploration of cryptic habitats and submarine caves.

Twenty-seven species of *Plakina* are now described, mainly on the basis of the composition of their spicules. The presence of lophose calthrops is considered a diagnostic character that separates *Plakina* species from other Plakinidae. Different genetic markers have been used to shed light on Homoscleromorpha systematics, and the phylogenetic trees using 18S and 28S (Gazave *et al.*, 2010, 2013), cytochrome oxidase b and CO1 (Ereskovsky *et al.*, 2013; Cruz-Barraza *et al.*, 2014; Ruiz *et al.*, 2015) are largely congruent. In the present study, CO1 has

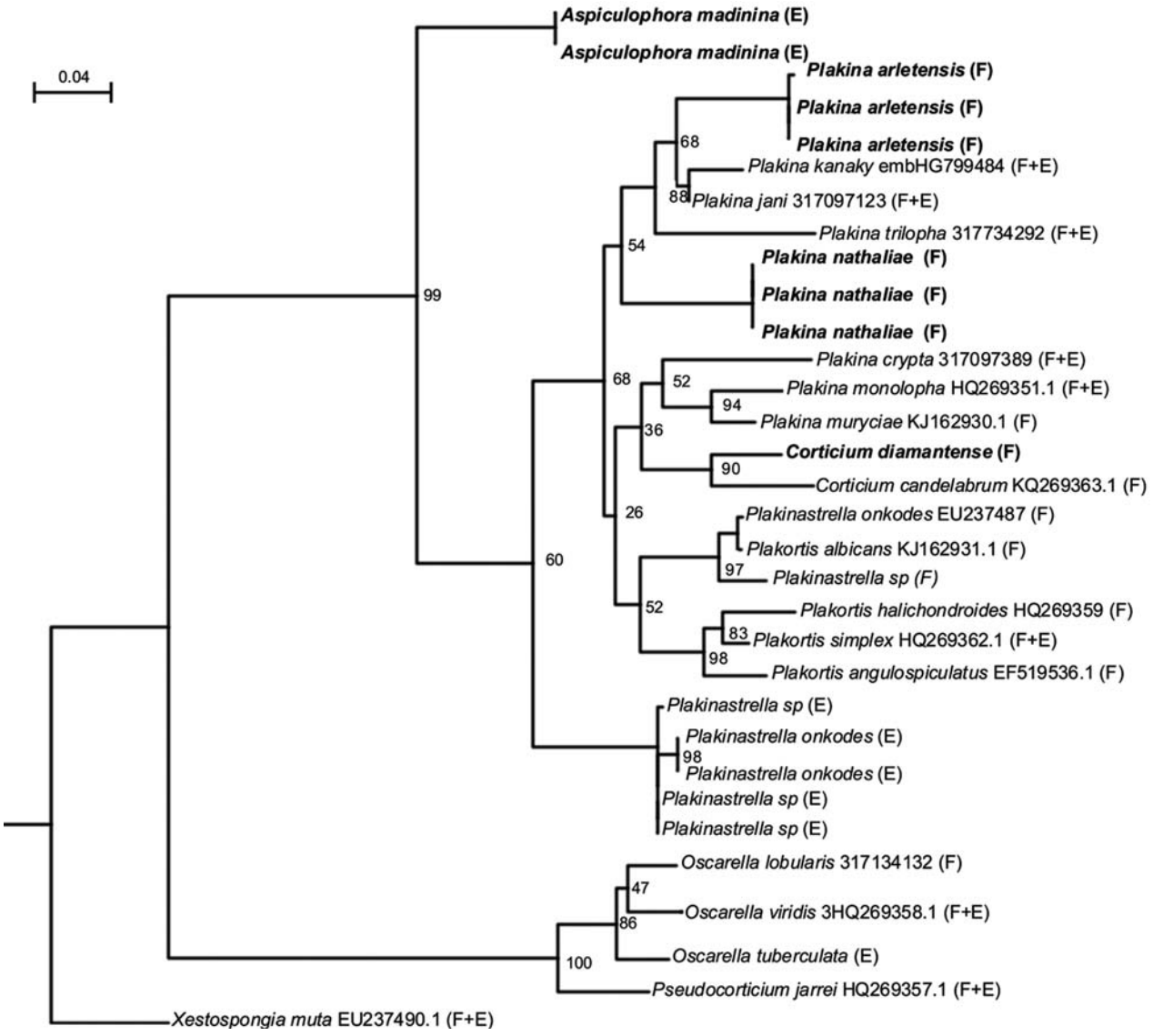


Figure 9. Phylogenetic reconstruction concatenating the Folmer and I3-M11 fragments of the CO1 using maximum-likelihood analysis. All sequences from this study were aligned with both fragments (F+E), Folmer (F) and I3-M11 (E). The accession number of each downloaded sequence is next to the species name.

been used in an integrative systematics approach, combining the analyses of morphological and cytological characters, to place our specimens among the different genera and families of Homoscleromorpha. Our phylogenetic analysis supports the allocation of *Aspiculophora madinina* gen. nov., sp. nov. to Plakinidae, and clearly differentiates this new representative of the family from all other taxa. Taking into account these results and an original internal bodyplan, we propose the creation of the first genus of aspiculate Homoscleromorpha outside of Oscarellidae.

Previous classifications of Homoscleromorpha recognized the strength and weakness of the present genus composition in both families, but until now no one has clearly established the phylogenetic interspecific relationships among the different genera. *Plakina arletensis* sp. nov. and *P. jani* form a well-supported clade (bootstrap values 967/76), which is in accordance with their morphological traits. We conclude that *P. arlentensis* is a new species and must be included in clade B3 proposed by Gazave *et al.* (2010) grouping *P. trilopha*, *P. jani* and *P. kanaky*. We add new data showing the non-monophyly of *Plakina*, but before proposing any changes to the status of this genus, we consider it necessary to obtain more DNA sequences and a revision of the morphological descriptions of the

type species *Plakina monolopha* and *Corticium candelabrum*. Indeed, the I3-M11 sequences of these two species form a single, well-supported clade. We are convinced that the addition of other datasets such as metabolomic fingerprints or meta-barcoding of the symbiotic prokaryote communities will help to better understand the evolutionary mechanisms at play in the origin of this group of sponges.

We decided to rename *Oscarella nathaliae* as analyses of the CO1 sequences derived from the first specimen collected in La Martinique in 2011 showed it was clearly affiliated with Plakinidae. Due to technical difficulties, this specimen remained the only one that could be sequenced. In the meantime, Ereskovsky *et al.* (2013) described this new species without any molecular data. Our genetic analysis of the CO1 of numerous new records of this species, from the south-western Caribbean (Colombia) to the northernmost part of the eastern Caribbean, showed clear affiliations with Plakinidae. Considering its position among most representatives of the *Plakina* (Fig. 7), even given the sometimes low bootstrap support and the recognized non-monophyletic status of the genus, we considered the renaming of this species as *Plakina nathaliae* as the most parsimonious option. This sponge was originally described as having a unique external morphology within *Oscarella*: 'a thin and leaf-like sponge, with a surface perforated by abundant pores' (Ereskovsky et al., 2013: 289). Another characteristic noted by authors was the 'irregular and punctual attachment to the substratum' (Ereskovsky et al., 2013: 302). These morphological traits are actually shared with *Plakina crypta*, a sciaphilous, exclusively Mediterranean species. *Plakina nathaliae* also has an internal organization that is similar to *Plakina crypta* and *P. endoumensis*. These three species share the absence of subectosomal cavities, a similar proportion mesohyl/choanocyte chambers, their leuconoid aquiferous system and their eurypylous choanocyte chambers. Finally, all three *Plakina* species can be considered as LMA sponges (Table 2), another anatomical trait which separates them from several other *Plakina* species such as *P. jani* or *P. kanaky* (Muricy *et al.*, 1999; Ruiz *et al.*, 2015). Meanwhile the report of two species without skeleton within Plakinidae again demonstrates that spicules can be secondarily lost in sponges and the heavy reliance on a classification based on spicule composition can often lead to homoplasious results. Recent sampling cruises that focused on submarine caves from the Pacific Ocean and the Caribbean Sea identified numerous new representatives of Homoscleromorpha which have as yet not been described. It is likely that the inclusion of new taxa in combination with additional complementary molecular and cytological investigations will permit a thorough revision of *Plakina*. For now, on the basis of these new descriptions and revision, we propose to update the taxonomic keys for Homoscleromorpha taking into account that Plakinidae contains 'Homosclerophorida with inorganic (spicular) skeletal complements', which can be lost in some cases. The absence of spicules is no longer exclusive to Oscarellidae.

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