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Integrative Taxonomy of Amazon Reefs' *Arenosclera* spp.: A New Clade in the Haplosclerida (Demospongiae)

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Two new *Arenosclera* are described here on the basis of materials obtained from Amazon reefs in 2014, *A. amazonensis* sp. nov. and *A. klausi* sp. nov. Both are clearly distinct from all other *Arenosclera* by their erect, solid funnel to lamellate habit, larger oxeas, and ectosomal architecture bearing occasional multispicular tracts. An integrative approach to find the best classification for both new species failed to group them and *A. heroni*, the genus' type species. Nearly complete 28S rRNA sequences obtained from these species' metagenomes suggested instead a better placement for the new species and *A. brasiliensis* in clade C (*sensu* Redmond et al., 2013), while *A. heroni* fits best in clade A. We propose to name three clades according to the rules of the PhyloCode: *Arenospicula*^{p}, *Dactyclona*^{p}, and *Dactyspicula*^{p}, respectively for the clade originating with the most recent common ancestor of the three Brazilian *Arenosclera* spp.; the most inclusive clade containing *Dactylia varia* (Gray, 1843) and *Haliclona curacaoensis* (van Soest, 1980); and the least inclusive clade containing *Arenospicula*^{p} and *Dactyclona*^{p}. A Karlin dinucleotide dissimilarity analysis of metagenomes carried out on cryopreserved samples recognized *A. amazonensis* sp. nov. as the most dissimilar species, thus suggesting a more particular microbiota is present in this Amazon species, an open avenue for extended applied study of this holobiont.

Arenosclera amazonensis sp. nov. LSID: urn:lsid:zoobank.org:act:28C5BD00-0AA4-4903-82AC-BC1AAD666EA5
Arenosclera klausi sp. nov. LSID: urn:lsid:zoobank.org:act:16CBBD72-49FF-41A0-82E9-A2FA9D6C6930

Keywords: integrative taxonomy, sponges, biodiversity, Brazil, Atlantic Ocean, 28S phylgeny, Haplosclerida, Karlin's Signatures

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INTRODUCTION

"Integrative taxonomy" is defined as the science that aims to delimit the units of life's diversity from multiple and complementary perspectives (phylogeography, comparative morphology, population genetics, ecology, development, behavior; Dayrat, 2005). Although, traditional procedures remain useful in many cases, taxonomy has to be pluralistic and integrate new approaches for species delimitation, which appears to be the most reliable way to evaluate the specific status of specimens (Padial et al., 2010), thus permitting arrival at sounder diagnoses (Schlick-Steiner et al., 2010). It has been said that morphological data are frequently insufficient to resolve taxonomic questions in Porifera, as a consequence of their great simplicity coupled to great intraspecific variability.

Molecular, cytological, chemical, biogeographic, and ecologic characters are increasingly used in an integrative manner to help solve taxonomic dilemmas (Boury-Esnault et al., 2013). Today, this approach is generally accepted as the best way to answer the challenges of sponge systematics (Bergquist, 1994; Cárdenas et al., 2012).

The Haplosclerida Topsent, 1928 is a case in point. This is currently the third largest order in Porifera, with nearly 1,100 described species (Morrow and Cárdenas, 2015; van Soest, 2015). A growing body of evidence (McCormack et al., 2002; Raleigh et al., 2007; Redmond et al., 2007, 2011, 2013), highlights a staggering mismatch between the order's currently accepted morphology-based classification (van Soest, 2015) and its phylogenetic framework, as retrieved from 18S and 28S rRNA, and the COX I and NAD I gene sequences. The order is subdivided in five extant families, namely Callyspongiidae de Laubenfels, 1936; Chalinidae Gray, 1867; Niphatidae van Soest, 1980; Petrosiidae van Soest, 1980; and Phloeodictyidae Carter, 1882; and coincidently, in five main clades, viz. A–E, alas bearing no correspondence to the Linnean classification. The identification of haplosclerid sponges, thus carries a presently unavoidable ambiguity, where Linnean names are needed for practical purposes, and rank-free phylogenetic names (following the PhyloCode, www.ohio.edu/phylocode/preface.html) for narrowing the gap between names and clades.

In dealing with the haplosclerid sponges dredged off the mouth of the Amazon, we needed workable identifications, as well as an understanding of the phylogenetic affinities of these species. Among these, two *Arenosclera* spp. caught our attention, because the genus' type species, *A. heroni*, already integrates a molecular phylogeny based on nearly complete 28S rRNA, thus indicating a good marker to search for the affinities of the Amazon reef species. Furthermore, the detection of a particular class of tetracyclic alkylpiperidine alkaloids (the Arenosclerines and Haliclonaciclamines) in the Australian *Haliclona* sp. (Charana et al., 1996), and the Brazilian *A. brasiliensis* and *Pachychalina alcaloidifera* Pinheiro et al., 2005 (Torres et al., 2000; Oliveira et al., 2007), suggests that a deepened study of the taxonomy, phylogeny, metagenomics and metabolomics of *Arenosclera* spp. from the Amazon mouth may yield rewarding results, both in the natural products chemistry field, as well as possibly in chemosystematics. The distribution of these alkaloids cuts through three separate families, respectively Chalinidae, Callyspongiidae, and Niphatidae.

The objective of this paper is to identify and assess the phylogenetic relationships for the *Arenosclera* spp. from the reef systems off the mouth of the Amazon, using complete or nearly complete 28S sequences retrieved from their metagenomes, and to provide full descriptions of these sponges.

MATERIALS AND METHODS

Study Area and Sampling

The Amazon River mouth drains an enormous sedimentary basin carrying a massive load of siliciclastic sediment of Andean origin, suspended in 20% of the freshwater input in the oceans (Lentz and Limeburner, 1995). A large, low salinity plume (up to 2×10^6 km^2) moves northwest influencing the insular environment in the Southeast Caribbean (Lumpkin and Garzoli, 2005). The seabed in this area presents a regionalization. The central and southern areas are predominantly carbonate, while the northern area suffers more direct influence of the Amazon plume and its terrigenous sedimentation (Collette and Rützler, 1977; Moura et al., 2016). The confluence between the Amazon River and the Atlantic Ocean generates high fisheries production in this relatively shallow and wide shelf, including important demersal resources (fishes and crustaceans), associated to reefal environments (Moura et al., 2016).

An oceanographic expedition was carried in the Amazon mouth with the Brazilian Navy ship NHo "Cruzeiro do Sul" in 24–29 September 2014. A total of 90 specimens were collected by bottom trawls and dredges. The specimens were sorted, photographed and labeled on ship before fixation in 92% ethanol. After that, they were deposited in the Porifera Collection of Museu Nacional—UFRJ (MNRJ). A fragment of each species sorted on board was preserved in liquid N_2, but one of the *Arenosclera* spp. later recognized as new (namely *A. klausi* sp. nov. described below), was not cryopreserved because both were thought conspecific.

Taxonomy and Morphological Study

Samples were identified based on microscopic preparations of dissociated spicules, and thick anatomical sections obtained from fragments. These procedures are described in detail in Hajdu et al. (2011). For each specimen 100 oxeas were randomly selected and measured, unless stated otherwise. Dissociated spicules were also analyzed in a JEOL JSM6390LV Scanning Electron Microscope (SEM). Spicule dimensions are presented in micrometers as minimum–mean–maximum (±standard deviation). New species were compared with the specimens listed in **Table 1**.

Nomenclatural Acts

The new species described in this paper are registered online at ZooBank (http://zoobank.org/) as requested by the International Code of Zoological Nomenclature. Each species received a Life Science Identifier (LSID) discriminated above.

DNA Extraction, Sequencing, and Quality Control of the Metagenomes

Fragments of specimens were washed in filtered sea water to remove all microorganisms attached to the sponge's outer surface. Afterwards, samples were macerated and embedded in Solution A [CTBA—2% + 100 mM EDTA + 1.4 M NaCl + 100 mM Tris-HCL (pH 8.0) + 2.0 μL Beta-mercaptanol + 0.5 μL proteinase K a 20 mg/mL]. Three heat-shock cycles were made (65°C by 3 min and −80°C by 30 min). After that, 1 ml of phenol:chloroform:isoamilic Alcohol (25:24:1) was added, and centrifuged in 13,400 rpm for 10 min at 4°C. The supernatant was then transferred to a new tube, and the spin filters from PowerSoil DNA Isolation Kit (MoBio, USA) were used to further purify the solution containing nucleic acids (Garcia et al., 2013).

Next generation sequences were obtained through HiSeq (Illumina, USA) by the staff of Laboratório de Microbiologia

TABLE 1 | List of the studied specimens and comparative materials used to assess the phylogenetic relationships of Amazon *Arenosclera* spp.

Accession number	Species	Locality	Collector and date	Depth (m)
MNRJ 18798	*A. amazonenses* sp. nov. (Holotype)	Amazon Mouth, 00°45.359′N–046°38.49′W	F. Moraes and R. Moura 28 Sep 2014	51
MNRJ 18778	*A. amazonensis* sp. nov. (Paratype)	Amazon Mouth, 01°17.989′N–046°46.732′W	F. Moraes and R. Moura 27 Sep 2014	55
MNRJ 18757	*A. klausi* sp. nov. (Holotype)	Amazon Mouth, 00°14.742′S–044°54.089′W	F. Moraes and R. Moura 29 Sep 2014	23
UFRJPOR 4627	*A. brasiliensis* (Holotype)	João Fernandinho Beach, 22°44′20″S–41°51′28″W, Búzios, Rio de Janeiro	G. Muricy 30 Aug 1997	3
NCI 198	*A. heroni* sensu (Thacker et al., 2013)	Chuuk, N Quoi Cha, Micronesia, 07° 31.50N–151° 58.20E	19 Aug 1992	7
MNRJ 1839, 1859–1860	*Dactylia* sp. (*A. heroni* sensu Muricy and Ribeiro, 1999)	Heron Island, Great Barrier Reef, NE Australia	Heron Island Research Station staff, 1998	
MHNG 22920–22922	*A. heroni* sensu (Desqueyroux-Faúndez, 1984)	New Caledonia	1976–1978	25–35
ZMB 4272	*A. arabica* (Holotype)	Red Sea		

Each accession number comprises a single individual.

(at Departamento de Biologia Marinha/UFRJ). DNA libraries were prepared using Nextera XT DNA Sample Preparation Kit (Illumina, USA), that uses a tagmentation reaction for transposon cleaving and tagging of the double-stranded DNA with a universal adapter, followed by a limited cycle PCR to add primer sequences and indices. The size distribution of reads in each library was evaluated with a 2100 Bioanalyzer and its High Sensitivity DNA Kit (Agilent, USA). The accurate quantification of the libraries was accomplished using the 7500 Real Time PCR (Applied Biosystems, USA) and the KAPA Library Quantification Kit (Kapa Biosystems, USA). Paired-end sequencing (2 × 150 bp) was performed using the kits TruSeq® Rapid SBS Kit–HS (50 cycles and 200 cycles) and TruSeq® Rapid PE Cluster Kit–HS. Quality control of the metagenomes was undertaken with the PRINSEQ software (Standalone Lite Version 0.20.4; Schmieder and Edwards, 2011) by removal of sequences presenting Phred score lower than 30 ($Q < 30$), and with duplicate reads. Paired-end reads were merged using PEAR (Zhang et al., 2014) with a base Phred quality score of 20.

Besides Brazilian *Arenosclera*, we used the metagenomes of *Amphimedon compressa* Duchassaing and Michelotti, 1864 and *Callyspongia vaginalis* (Lamarck, 1814) collected in the same expedition, as controls to verify if there is influence of the environmental microbiome in this analysis. In addition, these species are important for the recovery of the phylogenetic affinities of *Arenosclera* as shown below. The biological materials available for each of these species are illustrated as a **Figure S1**. Sequences of the 28S rRNA from *Arenosclera* spp. nov., *Arenosclera brasiliensis*, *A. compressa*, and *C. vaginalis* were submitted to Genbank (**Table 2**). *Arenosclera brasiliensis* materials used here came from the Cabo Frio region (Rio de Janeiro, Brazil), and had been reported upon by Trindade-Silva et al. (2012). This metagenome originated from SANGER sequencing which generates larger reads (>500 bp). All other metagenomes were generated by HiSeq (Illumina, USA), and are thus composed of smaller reads.

Karlin's Signatures

Karlin's signatures compare the relative abundances of dinucleotides in different sequences (Karlin and Burge, 1995), and were used in the comparison among Amazon reef sponges' metagenomes and those obtained by Trindade-Silva et al. (2012) for *A. brasiliensis*. Frequency tabulation of the sequence data was performed using homemade Perl scripts according to Willner et al. (2009).

Phylogeny

We chose as genetic marker the 28S because it is a well-established gene for evolutionary studies with sponges, and this is the only sequence available from the type species of *Arenosclera*. The 28S sequences used were recovered from metagenomes compared to a referential database compiling all Genbank sequences showing over 80% similarity to the available nearly complete 28S sequence of *Arenosclera heroni* (2,488 bp; Thacker et al., 2013). Sequences compiled for this database were KC869461, KC869526, KC869599, KC869567, KC869609, KC869497, KC869562, KC869607, KC869455, AB511881, KC869527, KC869473, AY561893, KC869622, KC869553, KC869626, KC869626, KC869620, KC869460 from Genbank. Contigs were aligned against the 28S referential database created using BLASTN algorithm with at least 70% query coverage and an E-value cut-off of 10^{-5} (Altschul et al., 1990). We compared the metagenomes of *A. brasiliensis, C. vaginalis, A. compressa*, and the two new *Arenosclera* sponges from Amazon reefs. SPAdes software (v. 3.5; Bankevich et al., 2012) was used as a first approach to assemble the high quality short reads (<300 bp), followed by a second approach using CAP3 software (Huang and Madan, 1999).

Additional sequences reported in Thacker et al. (2013) were obtained from GenBank to settle our comparative nearly complete 28S rRNA dataset. We refrained from using further 28S GenBank sequences for the Haplosclerida because in general these were much smaller than the sequences we were working

TABLE 2 | List of specimens used in the phylogeny.

Species	References	Bases pairs	Accession number
Arenosclera amazonensis sp. nov. (Holotype)	This study	3,591	KY825182
Arenosclera amazonensis sp. nov. (Paratype)	This study	3,510	MF537184
Arenosclera klausi sp. nov.	This study	977	MF837183
Arenosclera brasiliensis	This study	2,172	KY825183
Amphimedon compressa	This study	3,553	KY825184
Callyspongia vaginalis	This study	2,385	KY825185
Arenosclera heroni	Thacker et al., 2013	2,488	KC869569
Chalinula molitba	Thacker et al., 2013	3,381	KC869463
Cladocroce sp.	Thacker et al., 2013	2,492	KC869567
Dactylia varia	Thacker et al., 2013	2,128	KC869581
Dasychalina melhor	Thacker et al., 2013	3,088	KC869455
Gelliodes callista	Thacker et al., 2013	3,341	KC869562
Haliclona curacaoensis	Thacker et al., 2013	2,052	KC869575
Haliclona facigera	Thacker et al., 2013	2,484	KC869611
Haliclona implexiformis	Thacker et al., 2013	3,367	KC869533
Haliclona manglaris	Thacker et al., 2013	3,377	KC869599
Haliclona sp.	Thacker et al., 2013	3,307	KC869487
Haliclona subtriangularis	Thacker et al., 2013	3,394	KC869691
Haliclona tubifera	Thacker et al., 2013	3,357	KC869461
Haliclona vansoesti	Thacker et al., 2013	3,282	KC869631
Neopetrosia carbonaria	Thacker et al., 2013	2,373	KC869628
Neopetrosia rosariensis	Thacker et al., 2013	3,409	KC869457
Oceanapia sp.	Thacker et al., 2013	2,461	KC869607
Petrosia lignose	Thacker et al., 2013	2,104	KC869595
Petrosia strogilata	Thacker et al., 2013	2,237	KC869619
Petrosia weinbergi	Thacker et al., 2013	3,383	KC869497
Siphonodictyon siphonum	Thacker et al., 2013	3,049	KC869626
Xestospongia sp.	Thacker et al., 2013	2,888	KC869593
*Haliclona oculata**	Morrow et al., 2012	824	HQ379251
*Haliclona oculata**	Morrow et al., 2012	698	HQ379326
*Haliclona oculata**	Morrow et al., 2012	729	HQ379392

**These sequences refer to different segments of the 28S (D1–D2; D3–D5; D6–D8, respectively) and were assembled in a single larger sequence.*

with, and spurious preliminary results suggested that extensive discussion of phylogenetic affinities within this order might be needed, going way beyond the scope of this contribution. A global alignment was performed using MAFFT software (version 7; Katoh and Standley, 2013) with FFT-NS-2 parameter. Gaps were only eliminated when occurring on 20% or more of analyzed sequences. Maximum Likelihood analysis was run in RAxML (Stamatakis, 2014) in CIPRES platform (Miller et al., 2010) using the substitutions model GTR+GAMMA+I selected by Model Generator (Keane et al., 2006) with 1,000 bootstrap.

RESULTS

We have found two new species of Haplosclerida, morphologically similar to known species of *Arenosclera* (see diagnosis below), and perfectly matching the genus definition proposed in the latest revised classification of the Callyspongiidae (Desqueyroux-Faúndez and Valentine, 2002). According to the 28S phylogeny obtained from our own and previously published sequences (**Figure 1**), the Amazon reefs' *A. amazonensis* sp. nov. and *A. klausi* sp. nov. (described below) cluster with *A. brasiliensis* with 100% bootstrap support. This clade of Brazilian *Arenosclera* sponges paired with *Dactylia varia* and *Haliclona curacaoensis* with 70% bootstrap support. A more inclusive clade with 69% bootstrap support comprises the latter, *Dasychalina melior* (Niphatidae), and the sister pair *Xestospongia* sp. and *Petrosia lignosa*. *Amphimedon compressa* (Niphatidae) comes next as sister to this large previous clade, with 100% bootstrap support.

Arenosclera heroni, the genus' type species, was retrieved in the same relationships obtained by Thacker et al. (2013), in a highly supported clade comprising several species of *Haliclona*. This clade integrated a more inclusive one, also highly supported, with additional *Haliclona* spp., *Chalinula molitba,* and *C. vaginalis*. This later clade appears only distantly related to the clade including Brazilian *Arenosclera* sponges, a fact we take as best coped with at the moment, through the parallel erection of a new group, *Arenospicula*^{p}, not yet recognizable on the basis of morphology alone, for which reason we refrain to erect a new higher taxon in the Linnean classification. Instead, we followed the PhyloCode rules and recommendations delineated in Cantino and de Queiroz (2010), which determine that the proposition of a branch-based definition, as done below for the clade containing the Brazilian *Arenosclera* spp., should be accompanied by proposition of another branch-based definition for the sister clade of the former (Recommendation 11E), and a node-based definition for the clade comprising both branch-based definitions (Recommendation 11F).

Table 3 lists the next-generation HiSeq (Illumina, USA) results. Karlin's signature of dinucleotides recognized a greater similarity in all six metagenomes of *A. brasiliensis* when compared to those of the other species analyzed. In addition, we clearly detected a divergence between *A. amazonensis* sp. nov. and *A. brasiliensis* (**Figure 2**). The former exhibited 20% dissimilarity toward all other species considered. The species pair *A. compressa* and *C. vaginalis* formed a separated group as well. Since *A. klausi* sp. nov. and the paratype of *A. amazonensis* sp. nov. were available only from ethanol preserved vouchers, it appeared to us inappropriate to compare their metagenomes to those of specimens which had gone through cryopreservation and permitted generation of considerably more data.

Systematics

Class Demospongiae Sollas, 1885

Sub-class Heteroscleromorpha Cárdenas, Pérez and Boury-Esnault, 2012

Order Haplosclerida Topsent, 1928

Family Callyspongiidae de Laubenfels, 1936

Genus *Arenosclera* Pulitzer-Finali, 1982

Diagnosis: Callyspongiidae with ectosomal skeleton of sand and foreign debris cemented by scarce spongin, rounded meshes. Choanosomal fibers irregular, discontinuous, with foreign debris, proper spicules or both (Desqueyroux-Faúndez and Valentine, 2002).

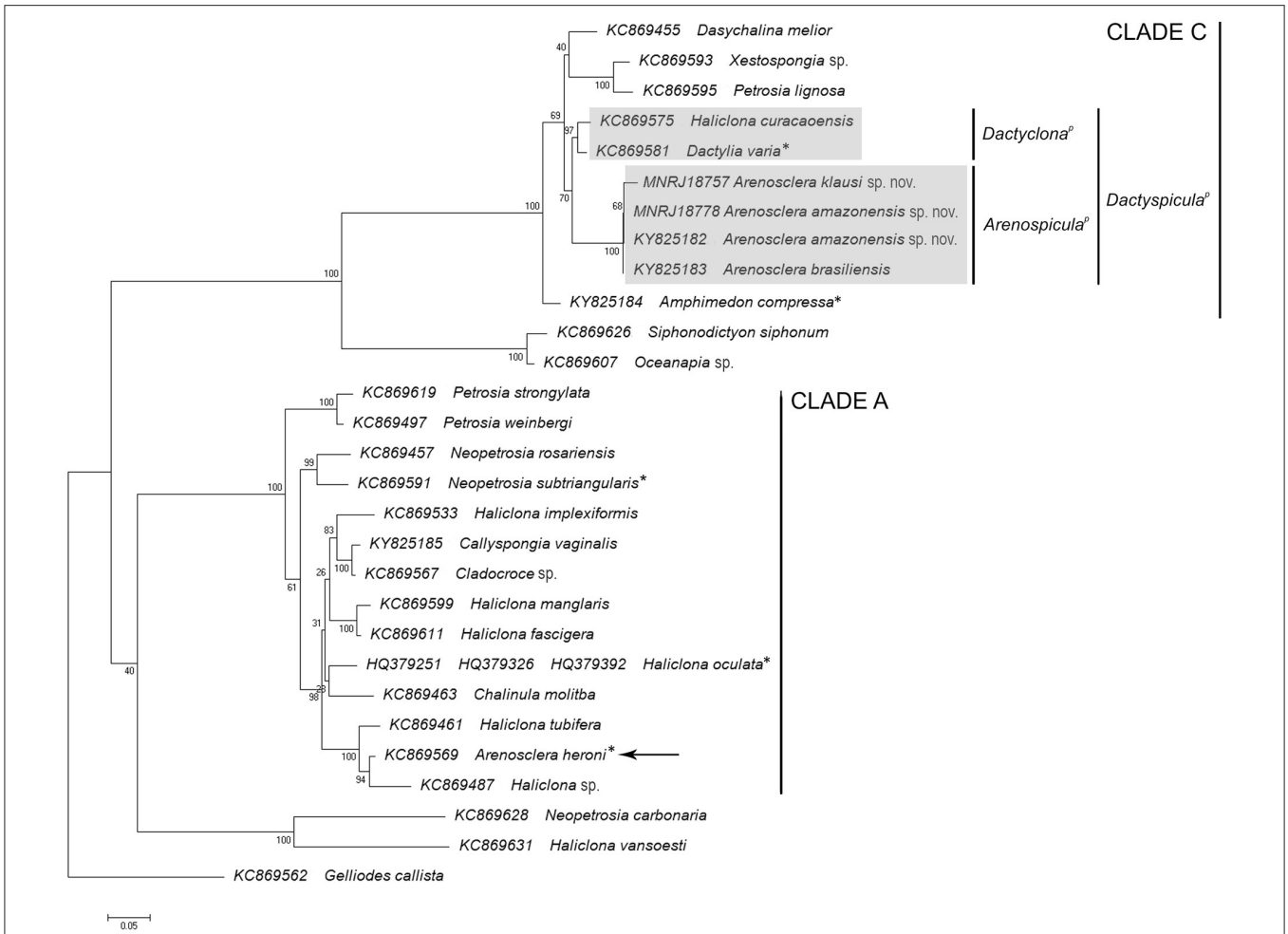

FIGURE 1 | ML phylogeny based on 28S rRNA including nearly complete sequences blasted from the metagenomes of *Arenosclera amazonensis* sp. nov., *Arenosclera klausi* sp. nov., *Amphimedon compressa, Callyspongia vaginalis*, all species belonging to Haplosclerida in Thacker et al. (2013), and *Haliclona oculata* from Morrow et al. (2012), for which complete or nearly complete 28S sequences are available in Genbank. *Type species of the genus.

Arenosclera amazonensis sp. nov. Leal, Moraes, Thompson, and Hajdu (Figure 3, Table 4)

LSID: urn:lsid:zoobank.org:act:28C5BD00-0AA4-4903-82AC-BC1AAD666EA5

Type Material

Holotype

MNRJ 18798, Station #8, off Marajó Bay, Amazon River mouth, Pará State, Brazil (00°45.359′N–046°38.49′W), 51 m depth, coll. F. Moraes and R. Moura/NHo Cruzeiro do Sul, 28 September 2014.

Paratype

MNRJ 18778, Station #6, off Marajó Bay, Amazon River mouth, Pará State, Brazil (01°17.989′N–046°46.732′W), 55 m depth, coll. F. Moraes and R. Moura/ NHo Cruzeiro do Sul, 27 September 2014.

Diagnosis

Erect, solid funnel to lamellate, stalked habit. Oscula clustered in slightly concave circular regions, which are randomly arranged. Ectosome with spongin fibers cored by uni- to multispicular tracts (9.6–46 μm wide), forming irregular to rounded meshes (diam. 97–493 μm). Choanosome with spongin fibers forming irregular to rounded meshes (diam. 116–1189 μm). Abundant sand grains, among and in the fibers, more so in the ectosome. Oxeas reach 130 μm in the holotype.

Description

External Morphology

Erect, solid funnel to lamellate habit, slightly flattened, pedunculate sponge, measuring 25 × 8 × 1 cm (larger specimen) with digitiform projections (9–12 cm long) showing wider base than top. Color light brown to beige *in vivo* and after fixation. Consistency soft and elastic. Oscula are grouped in slightly concave, round areas (diam. 1–2 mm), which are dispersed over the sponge surface, or aligned on the edges of the projections.

TABLE 3 | HiSeq (Illumina, USA) results.

Specimens	Number of sequences	Number of prunned sequences	Sequence mean length (bp)	Fixation method
Arenosclera amazonensis Holotype (MNRJ 18798)	14,373,435	13,160,844	146	N_2
A. amazonensis Paratype (MNRJ 18778)	1,149,731	963,004	n.r.	EtOH 92%
A. klausi Holotype (MNRJ 18757)	433,937	171,330	n.r.	EtOH 92%
Amphimedon compressa (MNRJ 18771)	11,561,906	10,252,945	148	N_2
Callyspongia vaginalis (MNRJ 18812)	17,049,106	15,434,325	146	N_2

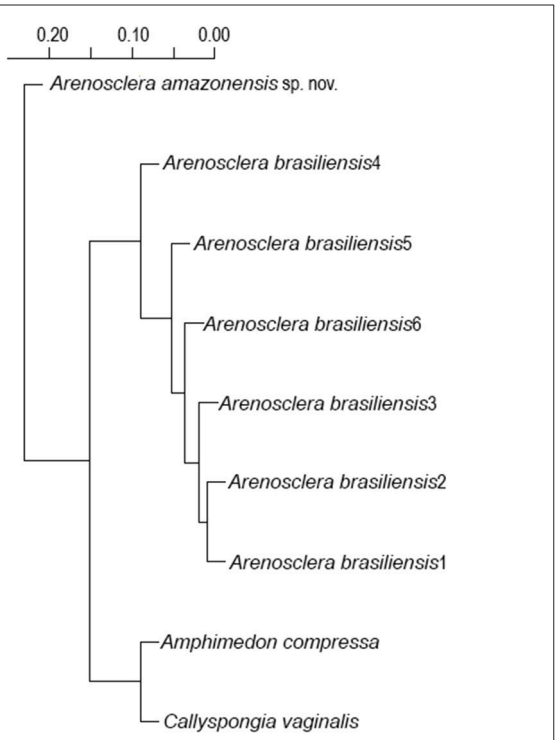

FIGURE 2 | Hierarchical clustering of metagenomes from dinucleotide relative abundances. Metagenomes are labeled according to their sponge source.

Surface regular, slightly rough to the touch, heavily cored by siliceous sand grains.

Skeleton

Ectosome formed by irregular to rounded meshes [diameter 97–274–493 (±143) μm] of spongin fibers cored by uni- to multispicular tracts [9.6–21.2–46 (±10) μm wide] and abundant sand grains, among and in the fibers. No clear distinction between primary and secondary fibers. Choanosome formed by irregular to rounded meshes [diameter 116–961–1189 (±296) μm] of spongin fibers cored by uni- to multispicular tracts [15–17.5–28 (±4.7) μm wide]. Sand and foreign debris, including exogenous spicules, dispersed among fibers in smaller quantity than the ectosome. Smallest oxeas dispersed in the free space in between meshes. No distinction between primary and secondary fibers.

Spicules

Oxeas straight to slightly curved (Holotype: 55–97.4–130 × 1–3–5 μm; Paratype: 44–79.1–112 × 2 μm).

Ecology

Specimens were rare and associated to rhodolith beds at 51–55 m depth. No organisms were recorded associated to this species.

Distribution

Known only from its type locality, the northern Brazilian continental shelf at the central sector off the Amazon River mouth (Pará, Brazil).

Etymology

In reference to the type locality, the Amazon River mouth.

Arenosclera klausi sp. nov. Leal, Moraes, Thompson, and Hajdu (Figure 4, Table 4)

LSID: urn:lsid:zoobank.org:act:16CBBD72-49FF-41A0-82E9-A2FA9D6C6930

Type Material

Holotype

MNRJ 18757, Station #10, off Mutuoca Bay, Maranhão State, Brazil (00°14.742′S-044°54.089′W), 23 m depth, coll. F. Moraes and R. Moura/NHo Cruzeiro do Sul, 29 September 2014.

Diagnosis

Erect, somewhat lamellate with a cylindrical stalk. Oscula flat on the surface, and concentrated on one edge of the sponge. Ectosome with spongin fibers cored by pauci- to multispicular tracts (7–22 μm wide), forming irregular to rounded meshes (diam. 116–793 μm). Choanosome with spongin fibers cored by pauci- to multispicular tracts (8–28 μm wide), forming irregular meshes (diam. 77–599 μm), becoming aspicular in the deeper parts of the sponge. Carbonate sand and foreign debris dispersed in large quantities among fibers all over the sponge. Only *Arenosclera* with larger oxeas attaining over 150 μm in length.

Description

Erect, somewhat lamellate sponge (7 × 3 × 1 cm), presenting a cylindrical stalk of 1 cm in diameter. Consistency soft and elastic, with softness increasing away from stalk. Oscula rounded (diameter 1–3 mm), flat on the surface, and concentrated on one edge of the sponge. Color beige *in vivo* (on deck) and after

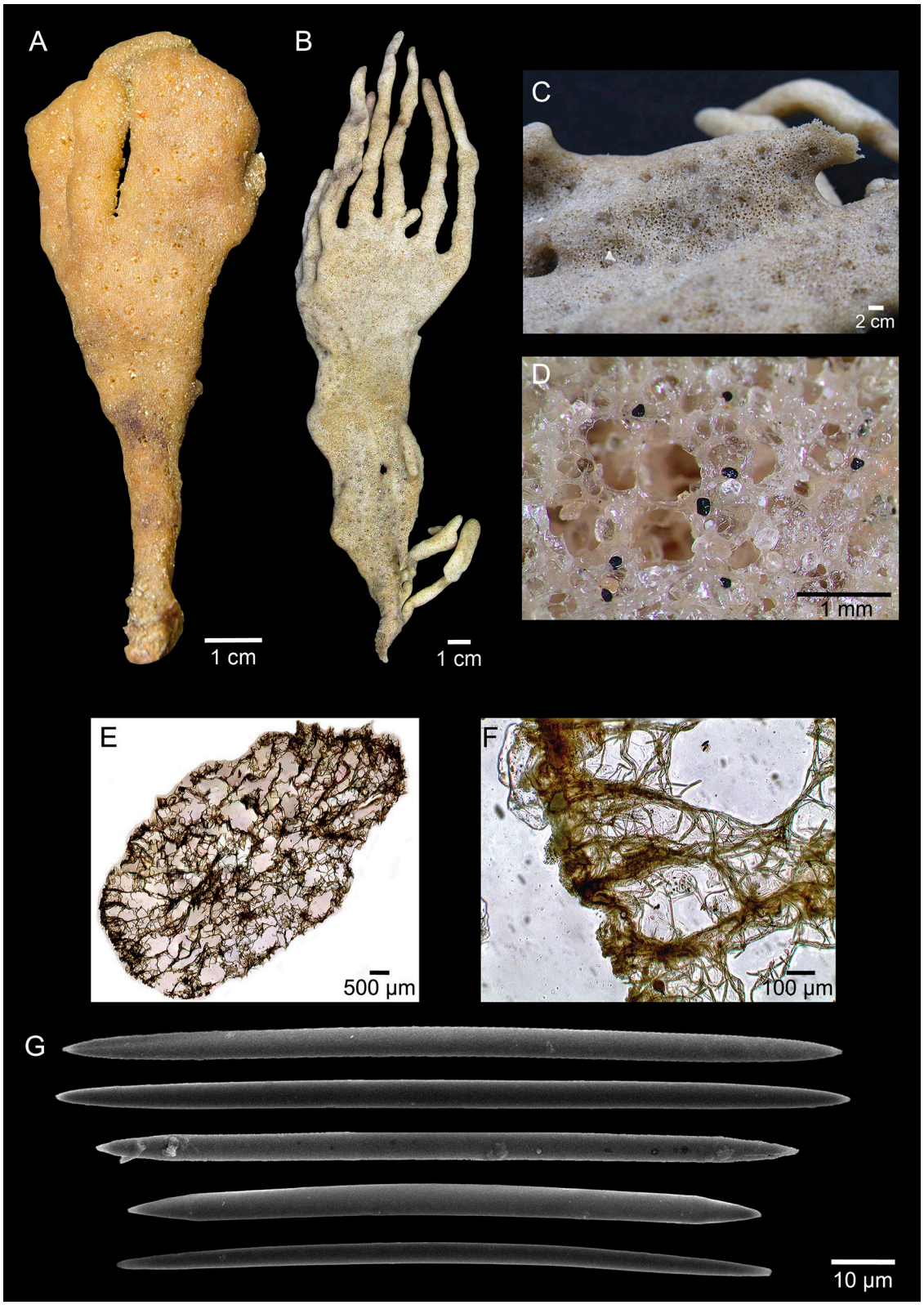

FIGURE 3 | *Arenosclera amazonensis* sp. nov. (holotype, **A**, MNRJ 18778; paratype, **B–F**, MNRJ 18798) morphological and anatomical traits. **(A–B)** Fixed specimens. **(C)** Sponge surface with several oscules. **(D)** Detail of surface and oscule rim showing rounded siliceous sand grains incorporated to spongin fibers. **(E)** ectosome and choanosome in transverse section showing reticulated spongin fibers. **(F)** Detail of ectosome and choanosome in transverse section showing spicule tracts inside spongin fibers. **(G)** Oxeas (both specimens).

TABLE 4 | Spicule micrometries and mesh diameter (μm) for the new species of *Arenosclera* reported here.

Specimens		Oxeas		Mesh diameter	
		Length	Width	Ectosome	Choanosome
***amazonensis* sp. nov.**					
MNRJ 18798 (Holotype)	Minimum	55	0.9	97	116
	Mean	97.4	2.8	274	461
	Maximum	130	5.2	493	1,189
	Stan. dev.	20.0	1.4	143	296
MNRJ 18778* (Paratype)	Minimum	44	–	–	164
	Mean	79.1	2	–	407
	Maximum	112	–	–	1,122
	Stan. dev.	13	–	–	295
***klausi* sp. nov.**					
MNRJ 18757 (Holotype)	Minimum	142	3.1	116	77
	Mean	166.0	5.4	304	307
	Maximum	182	7.2	793	599
	Stan. dev.	10.4	1.01	196	168

**Only 41 spicules measured.*

fixation. Surface irregular, slightly conulose, rough to the touch, heavily filled by foreign debris (mainly carbonate grains).

Skeleton

Ectosome formed by irregular to rounded meshes [diameter 116–304–793 (±196) μm] of spongin fibers cored by pauci- to multispicular tracts [7–13.7–22 (±4.2) μm wide]. Carbonate sand and foreign debris dispersed in large quantities among fibers. Primary and secondary fibers undistinguished. Choanosome formed by irregular meshes [diameter 77–307–599 (±168) μm] of spongin fibers cored by pauci- to multispicular tracts [8–14.9–28 (±6.8) μm wide] close to the ectosome, becoming aspicular in the deeper parts of the sponge. Sand and foreign debris, including exogenous spicules, are dispersed in large quantities among fibers. Primary and secondary fibers undistinguished.

Spicules

Oxeas thin, slightly curved, with irregular ends (142–166.0–182 × 3.1–5.4–7.2 μm).

Ecology

Rare species, with only one specimen collected, associated to coral-algal hard bottom at 23 m depth. No organisms were recorded associated to this species.

Distribution

Known only from its type locality, the northern Brazilian continental shelf at the south sector off the Amazon River mouth (Maranhão, Brazil).

Etymology

The species is named in honor of Dr. Klaus Rützler, a pioneer in the taxonomic study of sponges from off the Amazon River mouth.

PhyloCode

Arenospicula^{p} (*nomen cladi novum,* stem-based)

The most inclusive clade containing *A. brasiliensis, A. amazonensis* sp. nov., and *Arenosclera klausi* sp. nov. Etymology: from the included *Arenosclera* spp. (*Areno*, = sand in Latin; *spicula,* = diminutive of point, spear in Latin).

Dactyclona^{p} (*nomen cladi novum*, stem-based)

The most inclusive clade containing *D. varia* (Gray, 1843) and *H. curacaoensis* (van Soest, 1980). Etymology: from the included species *D. varia* (*Dacty*) and *H. curacaoensis* (*clona*).

Dactyspicula^{p} (*nomen cladi novum,* node-based)

The least inclusive clade containing *Arenospicula*^{p} and *Dactyclona*^{p}. Etymology: from the included groups *Dactyclona*^{p} (*Dacty*) and *Arenospicula*^{p} (*spicula).*

DISCUSSION

Identification of *Arenosclera* spp. nov

The state of uncertainty as regards higher taxa diagnoses in the Haplosclerida renders the assignment of new species to currently accepted generic names a taxonomic roulette. On the basis of the 18S marker, Redmond et al. (2013) retrieved not only polyphyletic families and genera, but also species such as *Callyspongia fallax, C. vaginalis, H. curacaoensis,* and *H. tubifera*. How can higher taxa boundaries in the Haplosclerida be discussed if species-limits remain ambiguous? Currently, one has to resort to morphology to recognize a new species' genus, regardless of the certainty of forthcoming major changes in higher taxa classification in the order; and to molecules for its clade A–E (Redmond et al., 2011, 2013), alas frequently not containing the genus' type species.

Accordingly, we observed the single available 28S sequence of *A. heroni* to cluster with two species of *Haliclona*, as originally retrieved by Thacker et al. (2013), instead of with the other *Arenosclera* spp. dealt with here. We had the opportunity to revise the voucher of Thacker et al.'s (Op. cit.) *A. heroni* (NCI 198), and found it to bear a clearly callyspongiid architecture, with a neat reticulation of spicule- and debris-cored fibers with abundant cementing spongin. However, our included *C. vaginalis* is inserted nowhere close to *A. heroni*. Rather, it groups with *Cladocroce* sp. with high support, and then, with moderate support to *Haliclona implexiformis*. Several clade A species reported in Redmond et al. (2013), namely *C. molitba, Haliclona fascigera, H. manglaris, H. oculata,* and *H. tubifera* integrate a larger clade including *A. heroni* with 87% bootstrap support. Still, the same two species of *Neopetrosia* (*N. rosariensis* and *N. subtriangularis*) and two species of *Petrosia* (*P. strongylata* and *P. weinbergi*), present in Redmond et al.'s (Op. cit.) clade A, are here placed in this same clade, and highly supported. Unfortunately,

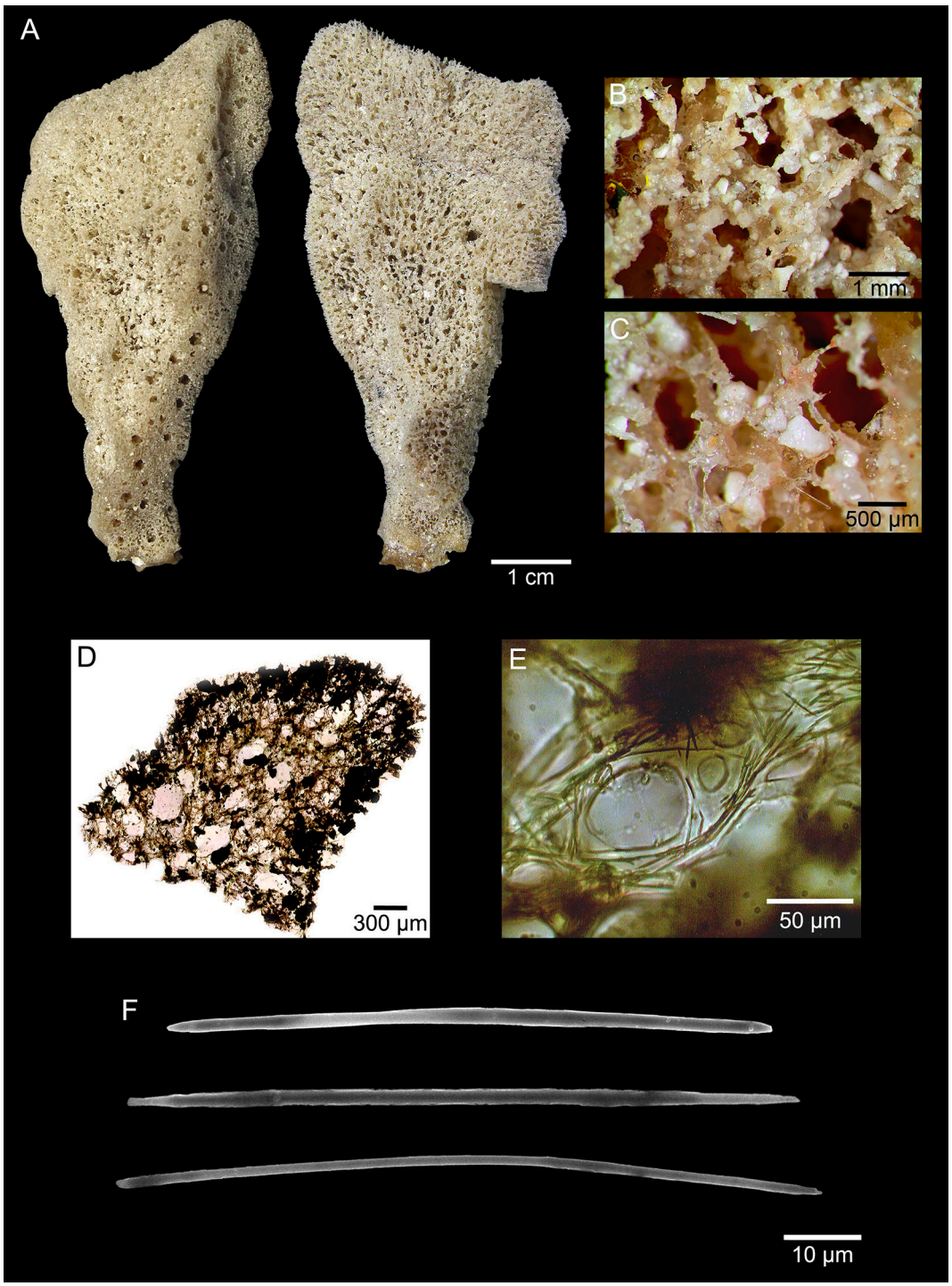

FIGURE 4 | *Arenosclera klausi* sp. nov. (holotype, MNRJ 18757) morphological and anatomical traits. **(A)** Both sides of the fixed specimen. **(B)** Detail of sponge surface. **(C)** Detail of oscule rim and calcareous debris incorporated to spongin fibers. **(D)** Ectosome and choanosome transverse section. **(E)** Detail of choanosome spongin fibers cored by oxeas. **(F)** Oxeas.

these authors were unsuccessful in retrieving an 18S sequence for *A. heroni*, which prevents further discussion on this genus' best phylogenetic assignment.

As pointed out above, all three Brazilian *Arenosclera* spp. clustered elsewhere, in a clade containing the type species of the niphatid genus *Amphimedon*. The same clade includes

TABLE 5 | *Arenosclera* species: comparative habit, anatomy, and distribution.

Species	Habit	Skeleton		Spicules	Distribution (Depth)
		Ectosomal	Choanosomal		
amazonensis sp. nov. (present study)	Palmate to pedunculate sponge reaching up to 25 × 8 × 1 cm. Color brown to beige *in vivo* and after fixation. Surface regular, filled mainly by siliciclastic sand grains. Oscula rounded and slightly concave	Rounded to irregular meshes with sand and foreign debris in large quantities dispersed among and into the fibers. Uni– to multispicular fibers with 10–21.2–46 (±10) μm wide cored by variable amount of oxeas	Uni- to multi-spicular rounded to irregular fibers with 15–17.5–28 (±4.7) μm wide cored by small oxeas. Sand and foreign debris include exogenous spics. dispersed between fibers in small quantities. Free and smallest oxeas dispersed in sparce spongin outside the fibers	Straight to slightly curved oxeas: 55–97.4–130 × 1–5 μm Exogenus spicules are common	Amazon River Mouth, Maranhão State, Brazil, Equatorial Western Atlantic (51–55 m)
klausi sp. nov. (present study)	Flabellate sponge with 7 × 3 × 1 cm, presenting a cylindrical stalk of 1 cm in diameter. Color beige *in vivo* and after fixation. Surface irregular, filled mainly by carbonate debris. Oscula at the surface, concentrated on one edge of the sponge	With sand and foreign debris in large quantities dispersed among fibers. Pauci– to multispicular fibers forming rounded to irregular meshes cored by thin and small oxeas with 7–22 μm wide. No distinction between prim. and second. fibers	Pauci– to multispicular, irregular fibers close to ectosome, which becomes aspicular toward the choan. with 8–28 μm wide. Sand and foreign debris, including exogenous spicules, in large quantities dispersed among fibers. No distinction between prim. and second. fibers	Thin, small and slightly curved oxeas with acerate ends: 142–166–182 × 3–7 μm	Amazon River Mouth, Maranhão State, Brazil, Equatorial Western Atlantic (23 m)
brasiliensis Muricy and Ribeiro, 1999 (orig. descr.)	Massive–lobate with terminal oscula at each lobe. Color whitish, drab or cream, alive and in spirit. Soft and compressible. Mucus present	Ectos. reticulated, formed by irregular, rectangular or rounded meshes (10–39 μm wide) of spongin fibers cored by 1–10 spicules and sand grains	Choan. reticulated, formed by irregular meshes (10–50 μm wide) of spongin fibers cored by 2–10 spicules and abundant sand grains	Oxeas slightly curved, hastate or fusiform, with acerate or blunt ends: 41–75–108 × 1.5–6.5 μm	Southeastern Brazil, Atlantic Ocean (2–10 m)
arabica (Keller, 1889) (adapted from orig. descr.)	White, cactoid, 5 cm high × 4 cm across, smooth surface with numerous, simple or furcated projections up to 1 ½ cm long. Oscula 1 cm diam., "pseudoscula" 1 mm diam.	Ectos. network with prim. fibers (diam. 40–60 μm) in regular roundish meshes (diam. 120–170 μm), with abundant sand, spongin barely visible, spicules missing; second. fibers form 3–4 sided *Reniera*–like meshes, with spicules but no sand	Not described	Oxeas slightly bent, pointed in both ends, 100 × 5 μm	Red Sea (depth not informed)
digitata (Carter, 1882) (orig. descr.)	Lobate, formed by knotted branched, or single hollow tubes: 25 × 17 cm. Oscula terminal, wider than tube bases: 2.5–5.0 cm diam.	Fibers resilient, cored by oxeas and sand grains		Oxeas	Western Australia, Indian Ocean
heroni Pulitzer-Finali, 1982 (orig. descr.)	Massive, irregular lobate, repent. Color light yellow *in vivo*, turning light yellowish–brown after fixation. Surface smooth with discontinuous low ridges. Oscula sparse, rounded (diam. 1.5–3 mm). Consistency firm and resilient	Ectos. formed by roundish meshes 130–160 μm wide, in a tangential network of strings of foreign debris (mainly sand grains) cemented by scarse spongin. Thin spongin fibers (6 μm thick) cored by unispicular tracts	Choan. in an irregular reticulation of undistinguished prim. and second. fibers, cored by foreign debris, proper spicules, or both	Oxeas straight, with well-developed axial canal: 80–90 × 1 μm	Great Barrier Reef, Northeastern Australia, Pacific Ocean (12 m)
heroni sensu Desqueyroux-Faúndez (1984)	Encrusting to massive base, with tubes 5–25 mm high and 5–15 mm diam., bearing apical pseudoscula 3–12 mm diam., oscula in the cavity (diam. 1–2 mm). Color is light to dark violet, turning gray or ocre in spirit. Rigid cosistency	Perpendicular network, with rounded to quadrang. meshes (diam. 100–250 μm), cored by sand, without spicules, diam. 60–70 μm. Second. unispicular fibers (diam. 10–15 μm), in isodictyal meshes	Network of polygonal meshes (diam. 200–600 μm), F1, with sand, spicules, or both (diam. 50–95 μm), F2 aspicular or unispicular, no sand (diam. 10–30 μm), F3 diam. 10–20 μm	Strongyloid oxeas, slightly curved, 80–90 × 1 μm	New Caledonia, Pacific Ocean

(Continued)

TABLE 5 | Continued

Species	Habit	Skeleton		Spicules	Distribution (Depth)
		Ectosomal	Choanosomal		
parca Pulitzer-Finali, 1982 (orig. descr.)	Irregular massive, with anast. folds and lobes. Color light brown. Consistency in spirit, firm, resilient	Ectos. with a regular reticulation of fibers (diam. 55–80 μm), with strings of debries cemented by variable amount of spongin, in polygonal or roundish meshes about 160 μm wide. Very few proper spicules and debris inside meshes	Choan. dense network of spongin fibers, forming meshes (270–540 μm wide). Prim. fibers (50–100 μm thick), cored by sand grains or spic. bundles, and second. ones (5–13 μm thick), free or cored by single oxeas	Oxeas straight or slightly curved, with long tapering points: 70–80/1 μm	Great Barrier Reef, Northeastern Australia, Pacific Ocean (10–13 m)
parca sensu Desqueyroux-Faúndez (1984)	Massive base with irregular lobules (10–20 × 5–10 mm), partially anast., bearing terminal oscula (diam. 4–6 mm). Color grayish to light brown/ ocre, fading after fixation. Surface reticulated, smooth, covered by abundant sand. Rigid consistency	Dense and regular network of rounded to quadrangular meshes (diam. 100–160 μm) of spongin fibers cored by sand (50–80 μm thick), F2 aspic. to unispicular, irregular (diam. 5 μm)	Dense irregular meshes (250–540 μm wide), of spongin fibers filled by multispicular tracts (F1 diam: 50–100 μm) or sand grains; joined by aspic. to paucispicular fibers (F2 diam: 5–13 μm). Fine F3 fibers (diam. 5 μm), loose spicules in the matrix	Strongyloid oxeas, irregular curved: 70–80 × 0.5–1.0 μm	New Caledonia, SW Pacific Ocean (12–45 m)
rosacea Desqueyroux-Faúndez, 1984 (orig. descr.)	Massive with digitiform tubes (10–35 × 8–17 mm) bearing terminal oscules (diam. 3.5–8 mm). Color pinkish–gray *in vivo*, faded away in alcohol. Surface smooth and velvety, flexible and fragile	Regular pentagonal reticulation (130–200 μm wide) of spongin fibers cored by sand and spicules, with an isodictyal fiber reticulation of unispicular tracts	Regular quadrangular to rectangular meshes (150–250 μm wide). F1 straight (diam. 40–60 μm), filled by multispicular tracts, heavily cored by sand grains closer to surface. F2 unispicular to paucispicular (diam. 10–20 μm), without sand, regular disposed in meshes, branching out as unispicular fibers (diam. 8–12 μm). Interstitial spicules abundant	Oxeas straight, some with truncate ends: 80 × 4 μm	New Caledonia, SW Pacific Ocean (0.6–15 m)

**anast., anastomozed; choan., choanossomal; diam., diameter; ectos., ectossomal; prim., primary; quadrang., quadrangular; second., secondary.*

H. curacaoensis, a sponge of uncertain phylogenetic affinity (see above), and *D, varia*, allegedly a callyspongiid. The latter, given its lack of spicules, is at best taken as a possible callyspongiid. However, it is interesting to note that Redmond et al.'s (2013) clade C, including both niphatid species clustering with Brazilian *Arenosclera* spp., also includes *Niphates erecta*, the type species of *Niphates*. Thus, albeit indirectly so, reinforcing the niphatid affinity of these *Arenosclera* spp. Obviously, *Arenosclera* needs a thorough revision, considering not only the species above, but also other additional little-known species from the Indo-Pacific.

Comparison with Additional *Arenosclera* spp.

Table 1 lists the comparative haplosclerid materials used here. Specimens from Heron Island referred to, as *A. heroni*, by Muricy and Ribeiro (1999) were found to be better assigned to *Dactylia*, as not a single spicule could be found in them. Thus, aside from Pulitzer-Finali's (1982) original description, our comprehension of *A. heroni*'s morphospace derives from hands-on study of Desqueyroux-Faúndez (1984) and Thacker et al.'s (2013) materials, alas, not topotypical. Both new species proposed appear quite distinct from this West Pacific species (**Table 5**) in overall habit (erect, somewhat lamellate), ectosomal architecture (commonly bearing uni- or pauci- to multispicular tracts), and usually considerably larger spicule dimensions (up to 112, 130, or 180 μm in the new species, against a maximum of 90 μm in *A. heroni*).

As simple as it is, these larger oxeas in the Amazon reefs' species set them apart from every other species previously assigned to *Arenosclera* (**Table 5**), where the maximum reported length for the oxeas was 108 μm in *A. brasiliensis*. The remaining *Arenosclera* spp. all possess oxeas smaller than 90 μm. Nevertheless, the paratype of *A. amazonensis* sp. nov. has comparatively smaller oxeas, thus coming quite close to the spectrum observable in *A. brasiliensis*. We are confident that both species are distinct as not a single (among hundreds observed by CVL and EH) *A. brasiliensis* possessed a lamellate-pedunculate body. Moreover, extensive surveys conducted along the Brazilian coast and its oceanic islands in the last couple decades, failed to spot any *A. brasiliensis* further north than 20°S, thus suggesting this species has a geographic range determined by the influence of SE Brazilian upwelling. This is in marked contrast to the Amazon environment were both new *Arenosclera* spp. were retrieved. While both new species proposed have flat, erect, somewhat lamellate morphologies, all other species considered bear massive to tubular habit (**Table 5**). In this way, we do not see risk of mistaken identifications, and the new species appear easy to recognize among other congeners.

Given the insertions of *A. heroni,* and the Brazilian *Arenosclera* spp. in our 28S phylogeny, monophyly of the remaining *Arenosclera* spp. is unlikely. This is an obvious assumption, consequence of the findings by McCormack et al. (2002), Raleigh et al. (2007), and Redmond et al. (2007, 2011, 2013), who have shown rampant polyphyletism of haplosclerid genera classified in every family. For instance, distribution of alkaloids such as arenosclerines and haliclonaciclamines cuts right through current genera and families. These compounds are present in *A. brasiliensis* (currently in the Callyspongiidae; Torres et al., 2000), *Haliclona* (Chalinidae; Charana et al., 1996) and *Pachychalina* (Niphatidae; Oliveira et al., 2007). It is thus clear that a major taxonomic revolution is necessary, unlikely to spare any such tool as detailed morphology, multi locus sponge genetics, metagenomics and metabolomics.

Preliminary Metagenome Data

The use of metagenomes as a data source for phylogenetic analyses in place of the traditional approach of isolation, amplification and sequencing of individual markers, proved a good strategy. Despite its currently larger cost, the advantages of producing a database for the genes of a given species, to be assessed at any time, without the need for further primers, isolation, amplification and sequencing, far outweighs any drawbacks. The characterization aimed at for the biome off the mouth of the Amazon river included metagenome analyses of the water column, sediments and biota (Moura et al., 2016), so that several sponge metagenomes had already been generated independently of any foreseen taxonomic necessity.

A curious outcome of our metagenome dinucleotide dissimilarity analyses is the observed 10% dissimilarity in the six available metagenomes of *A. brasiliensis* (Trindade-Silva et al., 2012), which is of the same magnitude as the value retrieved for *A. compressa* and *C. vaginalis*, species currently assigned to distinct families. These three species exhibit 15% dissimilarity, while *A. amazonensis* sp. nov. appears as most dissimilar. Given that all species, aside *A. brasiliensis*, were collected in the Amazon reefs area, it appears that neither a phylogenetic, nor an ecological signal were determinants for the observed dissimilarity.

One way to look at dissimilarity focuses on its translation into the uniqueness of the associated microbiota. As such, the higher the dissimilarity, the greater the potential for the finding of novelties, which suggests good avenues for the investigation of the chemo-pharmacological applicability of the associated microbiota in *A. amazonensis* sp. nov.

On the other hand, it is remarkable that despite originating from the same short stretch of SE Brazilian rocky coast, the six specimens of *A. brasiliensis* yielded 10% dissimilar metagenomes. This reminds of the importance to consider multiple samples when characterizing metagenomes, which has unfortunately not been possible here for many species considered, due to the limited number of duplicate and triplicate specimens obtained in the Amazon cruises.

AUTHOR CONTRIBUTIONS

FT designed and coordinated the fieldwork. FM, AM, and AS took part in fieldwork. CL, FM, AF, AS, Ld, AM, FT, and EH contributed with data and data analyses. CL, FM, FT, and EH wrote and revised the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2017.00291/full#supplementary-material

Figure S1 | (A) *Amphimedon compressa* Duchasssaing and Michelotti, 1864 (MNRJ 18771) and **(B)** *Callyspongia vaginalis* Lamarck, 1814 (MNRJ 18812) used to obtain HiSeq (Illumina, USA) metagenomes.

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