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Nicola gen. nov. with redescription of *Nicola tetela* (Borojevic & Peixinho, 1976) (Porifera: Calcarea: Calcinea: Clathrinida)

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Abstract

Guancha tetela was originally described as a species having a peduncle and a skeleton exclusively composed of sagittal triactines. Therefore, according to the most recent phylogeny of Clathrinida, it should be placed in the genus *Clathrina*. This species was collected on the Northeastern Brazilian coast in 1968 and it was not collected again until 2011 in Curaçao. In this study, we reanalyzed the type material and the new specimens from Curaçao under a morphological-molecular approach. Morphological analysis revealed the presence of tetractines in the skeleton of all the studied specimens, including a slide of the holotype. In the molecular phylogeny *G. tetela* grouped with genera containing tetractines, but as an independent new lineage, different from all the other genera of Clathrinida. Based on these results, we propose the erection of a new genus, *Nicola* gen. nov., to include species whose body is composed of tubes without anastomosis nor branches but that run in parallel and coalesce at the apical and basal regions. Moreover, the skeleton is exclusively composed of sagittal triactines and tetractines.

Key words: Atlantic Ocean, Northeastern Brazilian Coast, Caribbean Sea, Curaçao, molecular systematics, new genus, *Guancha tetela*

Introduction

The genus *Guancha* was originally proposed by Miklucho-Maclay (1868) in order to describe *Guancha blanca*, a species from the Canary Islands (Lanzarote) that presented a clathroid cormus with a stalk. Although Miklucho-Maclay (1868) considered that the presence of a stalk was sufficient to separate this species in a new genus, subsequent authors did not agree with this point of view and placed *G. blanca* in different genera: *Ascetta* (Haeckel 1872; Vosmaer 1881; Lendenfeld 1891), *Leucosolenia* (Lackschewitsch 1886; Vosmaer 1887; Breitfuss 1896, 1898; Dendy & Row 1913; Arndt 1928; Hôzawa 1929; Burton 1930; Brøndsted 1931; Breitfuss 1932, 1935; Topsent 1936; Arndt 1941; Tanita 1943), and *Clathrina* (Minchin 1896; Jenkin 1908). Only in 1976 the genus *Guancha* became accepted, with the description of *G. tetela* by Borojevic and Peixinho (1976), and had its first diagnosis:

"*Clathrinidae à cormus constitué d'un pédoncule et d'un corps clathroïde. Spicules réguliers et parassagittaux, ou uniquement parasagittaux, orientés parallèlement dans les parois des tubes, au moins dans la partie basale de l'éponge avec l'actine impaire basipète*" (Borojevic & Peixinho 1976) (Translation: Clathrinidae with a cormus composed of a stalk and a clathroid body. Spicules are regular and parasagittal or only parasagittal, organised in parallel in the tubes wall, at least at the basal part of the sponge with the unpaired actine basipetally oriented).

Later on, in the Systema Porifera (Borojevic *et al*. 2002), the diagnosis proposed was:

"Clathrinidae with a cormus composed of a peduncle and a clathroid body. The peduncle may be formed by true tubes with a normal choanoderm, or may be solid with a special skeleton. The skeleton is composed of regular (equiangular and equiradiate) spicules to which parasagittal spicules are added, at least in the peduncle. In some

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species only parasagittal spicules are present. The unpaired actine of parasagittal spicules is always basipetally oriented."

Since the publication of the Systema Porifera and this new diagnosis of *Guancha*, four new species were described within this genus: *Guancha arnesenae* Rapp, 2006, *Guancha camura* Rapp, 2006, *Guancha pellucida* Rapp, 2006, and *Guancha ramosa* Azevedo *et al*., 2009. More recently, however, molecular studies showed that *Guancha* was not a monophyletic genus, and the authors proposed its synonymisation with *Clathrina* (Rossi *et al*. 2011; Klautau *et al*. 2013). This synonymisation was confirmed when the type species of the genus (*G. blanca*) was included in a molecular tree (Imešek *et al*. 2014). Currently, we follow what was proposed by Klautau *et al*. (2013), that all *Guancha* species with only triactine spicules should be transferred to *Clathrina*, and that *Guancha* species with triactines and tetractines should be assigned to any of the other genera proposed or rediagnosed in the same article (namely *Arthuria*, *Ascandra*, *Borojevia*, *Brattegardia*, and *Ernstia*).

According to this proposal, the species *Guancha tetela*, originally described as having a skeleton exclusively composed of sagittal triactines, should be placed in *Clathrina*. However, a more detailed revision of this species revealed the presence of tetractines in its skeleton, which precludes its classification as a *Clathrina*. As *G. tetela* presents more triactines than tetractines, it should then be considered *Arthuria*, however, the organisation of the cormus of *G. tetela* is different of that of other *Arthuria*. Hence, molecular and detailed morphological analyses were performed in the present work to verify the correct classification of *G. tetela*.

Material and methods

The holotype of *Guancha tetela* is deposited in the Muséum Nationale d'Histoire Naturelle de Paris under the number MNHN-LBIM-C-1975-1 and there are also two slides from the holotype (one spicule slide and one section slide) deposited in the collection of the Museu Nacional do Rio de Janeiro (MNRJ 40). In the present work we analysed the slides MNRJ 40. The holotype of *G. tetela* was collected by dredging during a survey of the oceanographic vessel "*Almirante Saldanha*" along the Northeastern Brazilian Continental Shelf. Recently (2011), five specimens were collected from Curaçao by SCUBA diving and were deposited in the Porifera Collection of the Biology Institute of the Universidade Federal do Rio de Janeiro (UFRJPOR 6714, UFRJPOR 6723, UFRJPOR 6724, UFRJPOR 6746, and UFRJPOR 6767). Species names, voucher numbers, and GenBank accession numbers of the DNA sequences used for a phylogenetic analysis are listed in Table 1.

Morphological analyses. For the preparation of spicule slides, fragments of the sponge were dissolved in sodium hypochlorite (commercial bleach) in a test tube. After digestion, the spicules were washed five times in distilled water and three times in absolute ethanol. They were then transferred to slides and the ethanol was heat-evaporated. The mounting medium used was Entellan (Merck).

For the scanning electron microscopy (SEM), the spicules were placed on a cover-slip mounted on a stub with double-sided carbon tape and sputter-coated with gold. The analysis was performed in a JSM-6510 scanning electron microscope at the Institute of Biology of the Universidade Federal de Rio de Janeiro.

For the preparation of the slides sections, small fragments of the sponge were stained with a 5% acid Fuchsin alcoholic solution for 10 min. The excess of Fuchsin was removed with absolute ethanol for 5 min and the fragments were transferred to the slides, covered with some drops of xylene and mounted with Entellan.

Spicules measurements were made using an ocular micrometer. The length and the width at the base of each actine were measured for every spicule category. The results are presented in tabular form, featuring length and width (minimum, mean, standard deviation [s], and maximum), and sample size (n). Photographs were taken with a Zeiss AxioCam ERc5s coupled to a ZEISS Stemi 2000C stereoscope and with a digital camera connected to a Zeiss Axioscop microscope.

Molecular phylogenetic analyses. Genomic DNA guanidine/phenol-chloroform protocol (Sambrook *et al*. 1989) and stored at –20°C until amplification. The region comprising the partial 18S and 28S, the spacers ITS1 and ITS2 and the 5.8S ribosomal DNA was amplified by PCR with the following primers: 18S (5`-TCATTTAGAGGAAGTAAAAGTCG-3`) and 28S (5`-GTTAGTTTCTTTTCCTCCGCTT -3`) (Lobo-Hajdu *et al*. 2004). Each PCR amplification reaction mixture contained: 1X buffer (5X GoTaq R Green Reaction Buffer Flexi, PROMEGA), 0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.5 µg/µL bovine serum albumin (BSA), 0.33 µM of each primer, one unit of Taq DNA polymerase (Fermentas) and 1 µL of DNA, summing up to 15 µL with Milli-Q water. PCR

steps included one first cycle of 4 min at 94°C, 1 min at 50°C and 1 min at 72°C, 35 cycles of 1 min at 92°C, 1 min at 50°C and one minute at 72°C, and a final cycle of 6 min at 72°C. Forward and reverse strands were automatically sequenced in an ABI 3500 sequencer (Applied Biosystems). Sequences were aligned through the online version of the program MAFFT v.7 (Katoh & Standley 2013) using the strategy Q-INS-i (Katoh & Toh 2008).

Phylogenetic analyses were performed under maximum likelihood (ML) and Bayesian inference (BI). The ML analysis was conducted online on PhyML 3.0 (Guindon *et al*. 2010; available at http://www.atgc-montpellier.fr/phyml). The model for the ML analysis was selected using Modeltest 3.7 and the Akaike information criterion (AIC) (Posada & Crandall, 1998), which indicated GTR (General Time Reversible). One thousand bootstrap pseudo-replicates were performed (Felsenstein 1985). Bayesian inference reconstructions were obtained with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) under 10^6 generations and a burn-in of 1000 sampled trees, yielding a consensus tree of majority.

TABLE 1. Analyzed specimens with voucher numbers and GenBank accession numbers.

Species	Voucher number	Genbank accession number
Ascaltis reticulum	UFRJPOR6258	HQ588973
Ascandra falcata	UFRJPOR5856	HQ588962
Ascandra contorta	UFRJPOR6327	HQ588970
Arthuria hirsuta	ZMAPOR07061	KC843431
Arthuria spiralatta	MNRJ12860	KC985139
Borojevia cerebrum	UFRJPOR6322	HQ588964
Borojevia brasiliensis	UFRJPOR5214	HQ588978
Brattegardia nanseni	UFRJPOR6332	HQ588982
Clathrina antofagastensis	MNRJ 9289	HQ588985
Clathrina blanca	PMR-14307	KC479087
Clathrina clathrus	UFRJPOR6315	HQ588974
Clathrina lacunosa	UFRJPOR6334	HQ588991
Clathrina ramosa	MNRJ 10313	HQ588990
Ernstia tetractina	UFRJPOR5183	HQ589000
Ernstia sp.	UFRJPOR6621	KC843433
Guancha tetela	UFRJPOR 6723	KU568492
Leucaltis clathria	UFRJPOR 6944	KU568493
Leucaltis nodusgordii	QMG316050	AJ633857
Leucettusa nuda	MNRJ 10804	KC843453
Leucascus simplex	BMOO16283	KC843454
Leucetta floridana	PTL09.P100	KC843456
Leucetta potiguar	UFPEPOR547	EU781986

Results

Class Calcarea Bowerbank, 1864

Subclass Calcinea Bidder, 1898

Order Clathrinida Hartman, 1958 emend.

Nicola gen. nov.

Etymology. For Nicole Boury-Esnault in recognition of her dedicated work on the taxonomy of sponges, including calcareous sponges.

Type species. *Nicola tetela* (Borojevic & Peixinho, 1976)

Diagnosis. Clathrinida with a globular to ovoid body composed of parallel tubes that coalesce at the apical and basal regions. They do not anastomose nor ramify. The skeleton exclusively contains sagittal spicules: triactines and tetractines. The paired actines are rudimentary and they form a straight angle (180°s). The unpaired actine is always the longest actine. Diactines including trichoxeas may be added. The aquiferous system is asconoid.

Nicola tetela comb. nov.

Synonyms: *Guancha tetela*, Borojevic & Peixinho 1976: 998

Material examined. Slide of the holotype (MNRJ 40), Station 1966, Northeastern Brazilian Continental Shelf (Southern coast of Bahia State) (17°55'S, 37°30'W), collected by dredging by the "Almirante Saldanha" (SAL) vessel, 17th August 1968, 47 m deep; UFRJPOR 6714, UFRJPOR 6723, UFRJPOR 6724, Playa Kalki, Westpunt, Curaçao (12°22'29.86″N, 69°09'30.63″W), collected by E. Hajdu and B. Cóndor-Luján, 21st August 2011, 5.6 m deep; UFRJPOR 6746, Sunset Waters, Soto, Curaçao (12°16'01.58″N, 69°07'44.85″W), collected by E. Hajdu, 20th August 2011, 8.9 m deep; UFRJPOR 6767, Sunset Waters, Soto, Curaçao (12°16'01.58″N, 69°07'44.85″W); collected by B. Cóndor-Luján; 22nd August 2011, 9–12 m deep.

Colour. Bright orange in life and white in ethanol.

Description. The specimens have a globular (Figure 1A) to ovoid body (Figure 1B), with apical osculum and a peduncle at the base. The peduncle is formed by coalescent tubes with choanoderm. Above the stalk, each tube is divided into two tubes which do not anastomose nor ramify; instead, they run in parallel and then converge to form the osculum (Figure 1C). The aquiferous system is asconoid, with choanocytes, intercalated by porocytes, covering the interior of the tubes (Figure 1D). The surface is smooth and bright and the consistency is fragile.

The skeleton is composed of triactines and tetractines arranged in parallel, the triactines being more abundant than the tetractines. The unpaired actine of the spicules is always basipetally oriented (Figure 1E). The apical actine of the tetractines protudes into the lumen of the tubes (Figure 1F).

Triactines are equally distributed all over the sponge body, whereas tetractines seem to be more concentrated in the apical region, near the osculum (at least in UFRJPOR 6723). The size of the spicules is very variable and although the unpaired actine is frequently much longer than the paired ones (Figures 1G, H), it sometimes can be only a little longer (Figure 1I).

Spicules (Table 2, Figures 1G–K).

Triactines. Sagittal. Actines are straight, conical, with sharp tips. The unpaired actine presents a constriction near its base. They present very variable size and are the most abundant spicules (Figures 1G-I). Size: 75.0-440.0/5.0-10.0 µm (unpaired actine); 17.5-60.0/5.0-7.5 µm (paired actine).

Tetractines. Sagittal. Actines are straight, conical with sharp tips. The unpaired actine has a constriction near its base (Figures 1G, J). The apical actine is smooth and can be straight or curved. It is longer and, generally, narrower than the paired actines (Figure 1K). They present very variable size. Size: 80.0-370.0/5.0-10.0 µm (unpaired actine); 12.5-45.0/5.0-8.7 µm (paired actine); 17.5-75.0/2.5-6.2 µm (apical actine).

Ecology. The Brazilian specimen was collected at 47 m deep in a calcareous-algae bottom, whereas the specimens from Curaçao were found underneath broken corals in shallow waters down to 12 m. No organisms were found on the surface or among the tubes of the studied specimens.

Remarks. Borojevic & Peixinho (1976) originally described the skeleton of this species as being exclusively composed of triactines. Reanalysing the type material, we also found tetractines, although those spicules were outnumbered by triactines (Figures 2A, B). Because of the scarce quantity of tetractines in the holotype slide, they might have neglected them. Moreover, Borojevic & Peixinho (1976) characterized the spicules as parasagittal, pointing out the straight angle (90°s) formed by the unpaired and paired actines. We do recognize the referred angle, however, we consider that it would be more correct to characterize these spicules as sagittal, according to Boury-Esnault & Rützler (1997).

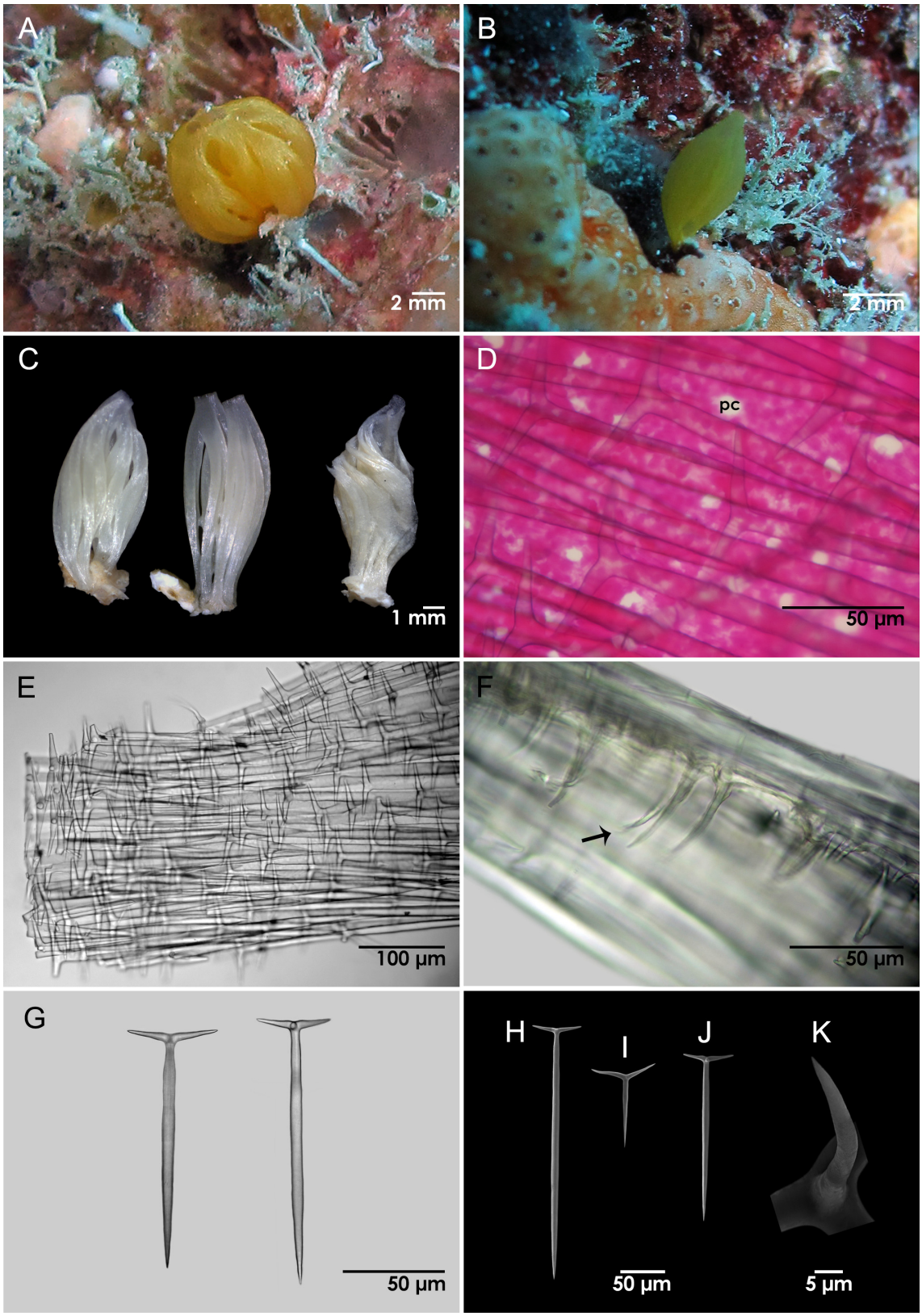

FIGURE 1. *Nicola tetela* comb. nov. A-B: Live specimens: UFRJPOR 6714 (A) and UFRJPOR 6746 (B) (photos taken by E. Hajdu). C. Specimens after fixation (UFRJPOR 6714, 6723, 6724). D. Tangential section of the skeleton showing choanocytes and porocytes (pc). E. Detail of the apical region of the body. F. Apical actines protruding into the lumen of a tube (arrow pointing to one apical actine). G. Spicules: Triactine (left) and tetractine (right). H – K: SEM images of spicules: H. Large triactine. I. Small triactine. J. Tetractine. I. Apical actine of a tetractine. All skeleton and spicule images were taken from the specimen UFRJPOR 6723.

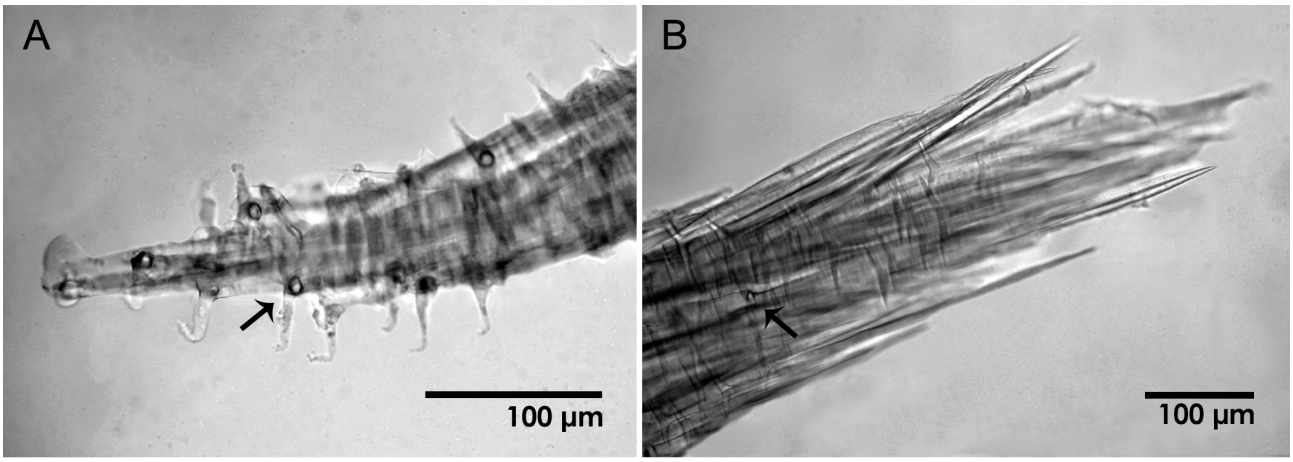

FIGURE 2. Photographs taken from the original slide of the holotype (MNRJ 40). A. Apical region. B. Basal region. In each photo a tetractine is indicated by an arrow.

TABLE 2. Spicule measurements of *Nicola tetela* comb. nov., including the original measurements of the holotype (MNRJ 40) by Borojevic & Peixinho (1976).

Specimen	Spicule	Actine	Length (µm)				Width (µm)				N
			Min	Mean	SD	Max	Min	Mean	SD	Max	
UFRJPOR	Triactine	Unpaired	75.0	223.6	91.7	440.0	5	7.5	0.9	10	30
6723		Paired	27.5	31.3	4.3	47.5	5	6.4	1.0	7.5	30
	Tetractine	Unpaired	102.5	202.2	54.9	305.0	5	8.0	1.3	10	30
		Paired	25.0	31.4	3.2	37.5	5	6.1	1.2	8.7	30
		Apical	30.0	50.4	10.4	62.5	2.5	3.9	1.0	5	20
UFRJPOR	Triactine	Unpaired	75.0	231.6	95.3	435.0	5	6.5	1.2	8.7	30
6746		Paired	17.5	29.6	5.0	40.0	5	5.3	0.7	7.5	30
	Tetractine	Unpaired	80.0	215.0	110.5	370.0	5	6.7	1.0	7.5	6
		Paired	25.0	32.5	8.1	45.0	5	5.2	0.5	6.3	6
		Apical	35.0	58.5	15.5	75.0	2.5	3.3	1.1	5	5
UFRJPOR	Triactine	Unpaired	102.5	213.5	69.2	375.0	5	7.1	1.1	8.7	30
6767		Paired	20.0	33.4	6.7	50.0	5	6.3	1.2	7.5	30
	Tetractine	Unpaired	100.0	173.7	40.4	255.0	5	7.0	1.1	8.7	30
		Paired	12.5	29.8	7.1	40.0	5	5.6	1.0	7.5	30
		Apical	17.5	37.5	8.9	47.5	2.5	2.7	0.7	5	12
MNRJ 40	Triactine	Unpaired	150	-	-	400	7	-	-	10	-
(original)		Paired	30	-	-	60	-	-	-	-	-
MNRJ 40	Triactine	Unpaired	100.0	229.3	64.2	375.0	6.3	7.4	0.4	7.5	20
(present work)		Paired	32.5	36.9	3.0	42.5	5	6.4	1.0	7.5	20
	Tetractine	Unpaired	145.0	222.3	51.3	315.0	5	7.4	0.7	8.7	20
		Paired	30.0	35.0	5.0	45.0	5	7.1	0.8	8.7	20
		Apical	32.5	32.5	0.0	32.5	5	5.6	0.9	6.2	2

The molecular analysis produced the same tree topology with both phylogenetic methods (ML and BI) and recovered the lineages found by Klautau *et al*. (2013) (Figure 3). *Nicola tetela* comb. nov. did not cluster with any of the already known genera, not even *Arthuria*, confirming that it is a new genus.

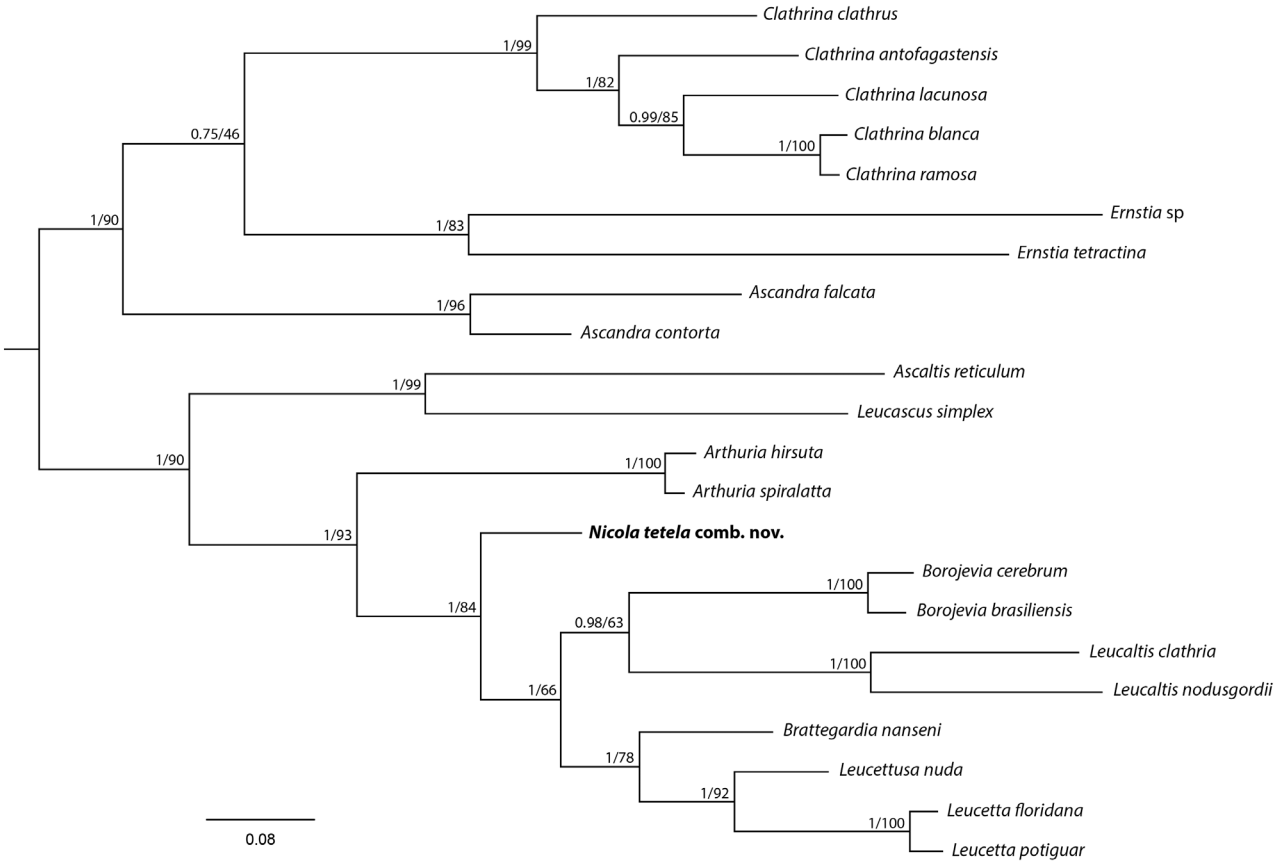

FIGURE 3. Bayesian 50% majority rule consensus tree (10^6 trees sampled; burn-in =1000 trees) inferred from the ITS rDNA sequences under the GTR model. Bayesian posterior probabilities (BI) and bootstrap (ML) are given on the branches.

Discussion

The anastomosis of the cormus is an important taxonomic character in the order Clathrinida. For example, all species of the genus *Ascandra* show a different anastomosis, with tubes free at least at the apical region, while *Arthuria*, *Borojevia*, *Brattegardia*, *Clathrina*, and *Ernstia* have well anastomosed tubes. In the new genus *Nicola*, tubes are not anastomosed but run in parallel and are reconnected at the base and at the osculum. Another remarkable morphological character of *Nicola* gen. nov. is its skeleton composed exclusively of sagittal spicules, which has not been found in any other Calcinean genus. The triactines with reduced paired actines present in *Nicola* gen. nov. are even similar to the "*nail-spicules*" found in the Calcaronean genera *Kebira* and *Grantiopsis* (Calcaronea: Lelapiidae), most probably as a result of convergence. Our results point that spicule shape and composition together with the organisation of the body are very important characters for the taxonomy of Calcinea. Considering only spicule composition we would expect to find a closer proximity of *Nicola* gen. nov. with *Arthuria*, however, we observed in our phylogenetic tree that they are not sister genera. This means that the presence of only sagittal spicules and the differentiated organisation of the cormus in *Nicola* gen. nov. are strong characters that define well this new genus as separated from all the others known up to date.

Although yet a monospecific genus, the distribution of *Nicola tetela* comb. nov. is not restricted to a unique locality nor depth. This species occurs in the South of Bahia (Brazil) (Borojevic & Peixinho 1976) and in Curaçao, and from 5.6 m to 47 m of depth, which suggests that it may be present at least from the Northeast of Brazil up to the Caribbean Sea. New studies will probably widen the distribution of *Nicola tetela* comb. nov. and perhaps result in the discovery of other species of this genus.

Our results emphasize the relevance of taxonomic studies integrating morphological and molecular approaches to improve the understanding of distribution patterns and phylogenetic relationships.

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