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Two *Pione* species (Hadromerida, Clionaidae) from the Red Sea: a taxonomical challenge

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Abstract Boring sponges of the genus *Pione* (Hadromerida, Clionaidae) are easily recognizable due to their spiculation. However, species identification is challenging, as the potentially diagnostic morphological character states of different species often overlap. For this reason, this group of species is frequently referred to as the '*Pione vastifica* complex', after the most well-studied species of the genus. Boring-sponge samples were collected in the Red Sea and identified as *P.* cf. *lampa* and *P.* cf. *vastifica*, respectively. So far, these two species names have usually been considered as valid, although some authors suggested them to be synonymous. Morphological analyses were performed on spicules and micro-erosion patterns by means of both light and scanning electron microscopy. Two apparent morphotypes can be distinguished, mainly by the growth form, but statistical analysis does not support a clear separation in two species. In addition, a DNA barcoding approach using sequences of CO1 has not identified any nucleotide sequence differences. These data support the hypothesis that *P.* cf. *lampa* and *P.* cf. *vastifica* from the Red Sea are conspecific.

Keywords *Pione vastifica* · *Pione lampa* · Boring sponges · Growth form · DNA barcoding

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Introduction

The genus *Pione* belongs to the family Clionaidae, which comprises the majority of excavating sponges. The genus was established by Gray in 1867, but since that time most species originally established in *Pione* have been classified in the genus *Cliona*. Rosell and Uriz (1997) revised the systematics of excavating sponges by means of a cladistic phylogenetic analysis, and resurrected *Pione* Gray. This genus is currently diagnosed as comprising Clionaidae with three different types of spicules: tylostyles, microspined oxeas and microrhabds (Rützler 2002a: 180).

However, although this spiculation makes it easy to assign members of *Pione* to the genus, it is more difficult to distinguish the species. This led Rützler and Stone (1986) to refer to a group of species in this genus as the "*Pione vastifica* complex". The latter currently contains 23 species (van Soest et al. 2008), which include *Pione carpenteri* (Hancock, 1867), *P. lampa* (de Laubenfels, 1950) and *P. vastifica* (Hancock, 1849) (Rützler and Stone 1986; Schönberg 2002).

Pione vastifica, described first from Scotland, was long considered a cosmopolitan species (Calcinai et al. 2000; Fromont et al. 2005; Rosell and Uriz 2002; Rützler and Stone 1986) growing exclusively in the alpha form (clionaid sponges in the adult stage "develop separate ostial and oscular papillae which puncture the substratum surface, the former bearing many ostia, the latter only one osculum. [...] Papillae may fuse and extend to encrusting habit (beta stage)" (Rützler 2002a: 174)). Recently, however, Carballo et al. (2004) pointed out that *P. vastifica* recorded from the Pacific Ocean may be considered as *P. mazatlanensis* (Hancock, 1867), while van Soest et al. (2008) reported an Atlanto-Mediterranean distribution for *P. vastifica*.

Pione lampa grows in both papillate and encrusting forms. It has been reported to have an Atlantic distribution mainly limited to the Carribean Sea (Pang 1973; Rützler 2002b; Schönberg 2002). Rützler (2002b) suggested that it may be an Indo-Pacific invader, in light of *P. lampa* material recorded in the Red Sea. Although the encrusting growth form has been considered a specific character of *P. lampa,* but not of *P. vastifica*, Zundelevich et al. (2007) identified specimens of an encrusting red boring sponge from the Red Sea as *Pione* cf. *vastifica*.

Morphological differences between *P. lampa* and *P. vastifica* are not pronounced; thus, synonymy of the two names was suggested by Desqueyroux-Faúndez (1990). Schönberg (2002) cited literature data on some ecological, reproductive and excavation features as evidence for that synonymy, but at the same time pointed out that characters such as growth form, oscular dimensions, excavation depth, current intensity requirements, and different spicule arrangement in the gemmules suggest *P. lampa* and *P. vastifica* as distinct species. Rützler (2002b) underlined a few morphological differences between the two papillate forms of *P. vastifica* and *P. lampa*, especially in the size, color and organisation of the papillae. In particular, *P. vastifica* has never been found in the beta stage (Rützler 1974; Rosell 1994; Rosell and Uriz 1997, 2002), whereas Rützler (2002b) recorded *P. lampa* specimens in the alpha stage in Bermuda and highlighted how the papillate growth stage of this species was different from the *P. vastifica* alpha stage. Currently, *Pione lampa* and *P. vastifica* are accepted as valid species (van Soest et al. 2008), despite earlier suggestions for revision of this classifications (Schönberg 2002).

During a recent study concerning the sponge diversity in the Ras Mohammed National Park, northern Red Sea (Sinai), samples of boring sponges were collected and provisionally identified as *Pione* cf. *vastifica* and *P.* cf. *lampa*, mainly on the basis of the observed papillate and encrusting growth forms, respectively. Besides these two supposed species, specimens of *Pione carpenteri* were recorded as well. *Cliona jullieni* Topsent, 1891 and a single specimen of a fourth species of *Pione* (*P. margaritifera* (Dendy, 1905)) were also recorded.

In this work we argue for conspecificity of Red Sea *Pione* cf. *vastifica* and *Pione* cf. *lampa* by means of both morphological and genetic approaches, and provide evidence that the debate over synonymy between these two *Pione* species names remains unresolved.

Material and methods

Morphological analysis

The study was conducted in the Ras Mohammed National Park, Egypt, in the southern part of the Sinai Peninsula (Fig. 1), with the permission of the Egyptian Environmental Affairs Agency. Specimens used for this work (Table 1) were collected in August 2006. Four fringing reef sampling stations in this area were chosen: Ras Nosrani, Camping Site, Marsa Bareika and Mangrove Island. Boring sponge samples were collected by SCUBA divers using a hammer and chisel, as part of a more comprehensive census project. The collected samples were photographed in situ using a digital camera, and fixed in 4% buffered formalin for morphological identification or in absolute ethanol for DNA barcoding.

Specimens were assigned to one of the two supposed species, *Pione* cf. *lampa*, *P.* cf. *vastifica*, or to *P. carpenteri* mainly on the basis of morphological features such as growth form, spicule shape and dimension. Spicule preparations were obtained as described by Rützler (1974).

The respective length and width of individual, complete spicules of each type (e.g. tylostyles, microxeas and michrorabds) were measured for 10 *P.* cf. *lampa*, 9 *P.* cf. *vastifica*, 3 *P. carpenteri*, and 1 *Cliona jullieni* specimens, with 40 replicate measurements taken for each spicule type (Table 1). Spicule measurements are reported as minimum-to-maximum ranges, including averages and standard deviations in brackets.

In order to determine whether spicules were mature, measurements of maximum width were plotted in histograms for each spicule type of each specimen (Schönberg and Beuck 2007).

Principal components analysis (PCA) was performed, considering as variables the size (length and width) of each spicule type in *P.* cf. *lampa* and *P.* cf. *vastifica*. In addition, the variables (tylostyle dimensions) were examined by discriminant analysis. The latter is a standard method for visually confirming whether two supposed species are morphologically distinct. An axis is constructed that maximizes the difference between the two data sets, which are then plotted along this axis using a histogram (Hammer et al. 2001).

Excavation chambers, papillae and oscula, where discernible, were measured using UTHSCSA Image Tool for Windows v.3 software calibrated using an additional lens grid or the scale bar in SEM pictures.

Excavation patterns, i.e. the ways the excavation chambers are organized, were observed using a stereomicroscope.

Excavation scars (pits) were studied in *Pione* cf. *lampa* and *P.* cf. *vastifica* using a TESCAN VegaTS 5136XM Scanning Electron Microscope (SEM). To observe pits, small rock fragments were taken (3 specimens per species, 1 rock fragment per specimen). Each fragment was cleaned by submersing it in hot sodium hypochlorite (maximum concentration 5%), then left overnight at room temperature. Cleaned samples were rinsed with water, submersed in acetone, and processed in an ultrasonic cleaner. After being glued onto the pin stubs, fragments were gold-coated. For each sample SEM pictures were taken. Because pits have an irregularly circular shape, the length of the longest axis (main axis) was taken into account in place of a diameter. Pit main axis length was measured (n=24) using UTHSCSA Image Tool for Windows v.3 software calibrated on the picture scale bar. Considering that excavation patterns could be affected by substratum characteristics (Calcinai et al. 2003), rock fragments were sampled from specimens boring the same kind of substrata (coral rock) and examined by SEM. Data were analysed by computing a two-way ANOVA.

All statistical analyses were performed with PaSt 1.88 (Hammer et al. 2001).

DNA barcoding

Total DNA was extracted from samples preserved in absolute ethanol. A commercial DNA extraction kit (Qiamp DNA Mini Kit, Qiagen) was used, following the manufacturer's instructions.

Fragments of the mitochondrial CO1 were sequenced using degenerate CO1 primers (Meyer et al. 2005) as described in Duran and Rützler (2006). Sequences were managed with MacClade v. 4.08 (Maddison and Maddison 1992). Alignment was done manually, and was unambiguous due to the protein-coding nature of the sequences. All sequences have been submitted to GenBank (www.ncbi.nlm.nih.gov; accession numbers: GU169290–GU169300) and to the Sponge Barcoding Database (www.spongebarcoding.org). Bayesian analyses on nucleotides were performed with MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003), with at least two simultaneous runs of eight Metropolis-coupled Markov chains under the default temperature parameter (0.2), and the GTR + G + I model (potential overparameterization of the model has been shown not to affect the results of Bayesian analyses (Huelsenbeck and Ranala 2004)). Analyses were terminated after the chains converged, as indicated by an average standard deviation of split frequencies <0.001. For comparison, maximum likelihood bootstrap analyses (1000 replicates) were conducted, using GARLI 0.951 (Zwickl 2006) under the GTR + G + I model as suggested by hLRT in Modeltest (Posada and Crandall 1998).

Results

Sponge identification and description

Initially in this study, the species *Pione* cf. *lampa* and *P.* cf. *vastifica* were recognised separately. Corresponding specimens were assigned on the basis of the two principal and common growth forms (Rützler 1974; Rosell 1994; Rosell and Uriz 1997, 2002) and on differences in excavation pattern, color and size of the papillae.

Cliona jullieni, *Pione carpenteri* and *P. margaritifera* were also recorded in the sampling area, but as their morphological identification was clear these species are not described here.

***Pione* cf. *lampa* (de Laubenfels, 1950)**

Cliona lampa de Laubenfels, 1950—de Laubenfels (1950: 110)

Pione lampa (de Laubenfels)—Rosell and Uriz (1997: 362)

Material studied

Marsa Bareka: specimens No. 6586, 6600, 6662, 10 m; 6870, 12 m. Ras Nosrani: 6927, 15 m; 6995, 27 m; 7018, 10 m. Mangrove Island: 6711, 15 m; 6769, 6809, 10 m. All specimens excavated biogenic calcareous rocks.

Description

Commonly growing in beta stage. Live specimens had a characteristic red vermillion color, which faded to yellowish in preserved samples. Oscula were easily recognizable, with diameters ranging from 2.7 to 5.4 mm (3.99±0.79 mm). Small inhalant papillae were densely distributed on the incrusted surfaces; diameters ranged from 0.26 to 1.49 mm (0.79±0.29 mm).

Excavation pattern

Excavations were visible down to 1 cm into the substrate. Chambers were subspherical, polygonal and well-spaced, with diameters ranging from 0.37 to 1.80 mm (1.03± 0.27 mm). Pits were circular with smooth surface (Fig. 2a); pit main axis length was 51.51 (mean) ± 15.72 μm.

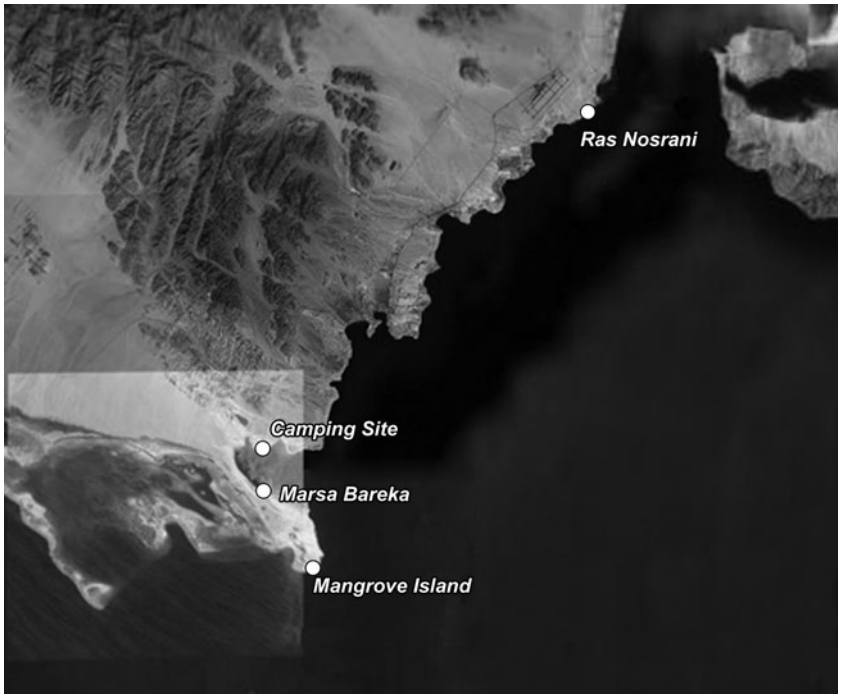

Fig. 1 Study area on the South Sinai peninsula; sampling sites: Ras Nosrani (27°58′3.4″N, 34° 25′15.7″E), Camping Site (27° 47′19.4″N, 34°13′29.3″E), Marsa Bareka (27°46′1.2″N, 34° 13′11.4″E), Mangrove Island (27°43′26.0″N, 34°15′0.5″E)

Spicules

Tylostyles straight with globular to ovoid heads (Fig. 3a), often irregular and in subterminal position; microxeas microspined, slightly bent in middle; microrhabds microspined, wavy with 2–3 bends, truncated tips (Fig. 3c). Measurement results are shown in Table 1.

Skeletal organization

Papillar skeleton characterized by palisade tylostyles with abundant microxeas and microrhabds. Disorganized microxeas predominant into choanosomal skeleton.

Distribution

Atlantic Ocean and Caribbean Sea, Red Sea (Rützler 2002b; Schönberg 2002).

Remarks

The typical colour in *P. lampa* is bright vermillion (Rützler 2002b). Spicules consist of straight tylostyles with spherical or ovoid heads, microspined microxeas, sometimes with a central swelling, and generally straight microrhabds, though undulated forms have also been reported by some authors (Pang 1973; Rosell and Uriz 2002; Rützler 2002b; Schönberg 2002). It is known that *P. lampa* is capable of encrusting the substratum, assuming a beta growth form and even invading living coral tissue (McKenna 1997; Pang 1973; Rützler 2002b; Schönberg 2002). Morphological features of the specimens identified by us as *P.* cf. *lampa* fit those reported in the literature (Table 2). The major differences concerned the shape of the microrhabds, which were straight in the specimens described by Rützler (1974) and Schönberg (2002), but curved in our specimens.

***Pione* cf. *vastifica* (Hancock, 1849)**

Cliona vastifica Hancock, 1849— Hancock (1849: 342)

Cliona northumbrica Hancock, 1849— Hancock (1849: 336)

Cliona lampa "forma occulta"—Rützler (1974: 23)

Studied material

Ras Nosrani: specimens No. 6472, 6489, 5 m, in biogenic calcareous rock. Camping Site: 6527, 15 m, in calcareous rock. Mangrove Island: 6722a, 6731, 6741, 6749, 5 m, in *Millepora* sp. rubble. Marsa Bareka: 6876, 6875, 5 m, in *Millepora* sp. rubble.

Description

Always found in alpha stage. Living tissue dull red, fading to light brown when preserved. Papilla diameter 0.40–1.30 mm (0.63±0.24 mm).

Excavation pattern

Chambers scattered into the rock; mainly spherical, but irregular shapes common. Superficial chambers may present a small papillar channel. Diameter ranged from 0.30 to 2.40 mm (1.11±0.44 mm). Pits regular and almost

Table 1 Information on the specimens studied; length values given in μm in the format: minimum–(average±standard deviation)–maximum

Sample Nr.	Species	Sampling site[a]	Depth [m]	Tylostyle length (n=40)	Microxea length (n=40)	Microrhabd length (n=40)	Spiraster length (n=40)	Pit main axis length (n=24)	Barcoded fragment
6586	*Pione* cf. *lampa*	MB	10	192.5–(232.0 ± 19.1)–270.0	57.5–(83.4± 9.3)–100.0	6.0–(8.3± 1.0)–10.0			–
6600	*Pione* cf. *lampa*	MB	10	197.5–(245.8± 27.1)–300.0	75.0–(94.5± 8.1)–110.0	6.0–(7.9± 0.9)–11.0		41.35–(64.87± 13.08)–92.17	–
6662	*Pione* cf. *lampa*	MB	10	192.5–(228.2± 22.7)–280.0	77.5–(97.9± 9.5)–117.5	6.0–(7.7± 1.1)–10.0			–
6995	*Pione* cf. *lampa*	RN	27	190.0–(242.0± 28.1)–300.0	65.0–(86.6± 8.5)–102.5	7.0–(9.0± 1.3)–12.0		18.68–(35.64± 9.19)–57.27	CO1
6711	*Pione* cf. *lampa*	MI	15	190.0–(232.4± 25.5)–325.0	40.0–(77.3± 12.4)–97.5	5.0–(7.5± 1.1)–10.0			CO1
6769	*Pione* cf. *lampa*	MI	10	180.0–(230.0± 18.9)–265.0	70.0–(86.8± 7.5)–100.0	6.0–(9.0± 1.5)–14.0			CO1
6809	*Pione* cf. *lampa*	MI	10	185.0–(237.5± 23.0)–280.0	60.0–(88.9± 8.9)–105.0	6.0–(7.3± 1.0)–9.0			CO1
6870	*Pione* cf. *lampa*	MB	12	225.0–(281.8± 23.2)–330.0	60.0–(93.9± 12.7)–127.5	5.0–(7.8± 1.3)–12.0		34.16–(54.97± 10.77)–83.82	CO1
6927	*Pione* cf. *lampa*	RN	15	210.0–(246.3± 20.4)–305.0	77.5–(87.5± 7.4)–102.5	5.0–(7.0± 1.0)–9.0			CO1
7018	*Pione* cf. *lampa*	RN	10	210.0–(252.8± 23.9)–300.0	87.5–(100.6± 7.6)–115.0	6.0–(7.3± 0.7)–9.0			CO1
6472	*Pione* cf. *vastifica*	RN	5	235.0–(336.3± 32.0)–390.0	92.5–(111.3± 9.6)–137.5	missing		50.86–(68.72± 11.56)–92.61	–
6489	*Pione* cf. *vastifica*	RN	5	200.0–(256.9± 24.3)–305.0	52.5–(82.4± 8.8)–100.0	6.0–(10.4± 2.1)–16.0			–
6527	*Pione* cf. *vastifica*	CS	15	170.0–(263.9± 30.3)–320.0	50.0–(80.3± 14.4)–117.5	7.0–(11.7± 2.6)–18.0		37.61–(61.89± 12.96)–86.84	–
6722a	*Pione* cf. *vastifica*	MI	5	185.0–(242.4± 22.9)–290.0	57.5–(77.8± 9.8)–92.5	7.0–(10.5± 1.7)–16.0			CO1
6731	*Pione* cf. *vastifica*	MI	5	210.0–(250.3± 22.3)–300.0	70.0–(82.0± 8.1)–105.0	7.0–(10.0± 1.1)–13.0			–
6741	*Pione* cf. *vastifica*	MI	5	195.0–(243.0± 17.5)–275.0	57.5–(79,2± 11,5)–100.0	6.0–(10.7± 1.9)–14.0		46.84–(68.33± 13.35)–91.56	–
6749	*Pione* cf. *vastifica*	MI	5	220.0–(311.3± 37.1)–355.0	52.5–(75.8± 15.4)–105.0	missing			–
6875	*Pione* cf. *vastifica*	MB	5	205.0–(264.7± 23.9)–310.0	70.0–(84.3± 7.6)–105.0	6.0–(12.0± 1.8)–15.0			–
6876	*Pione* cf. *vastifica*	MB	5	200.0–(268.7± 35.8)–330.0	55.0–(72.1± 10.8)–95.0	8.0–(11.8± 1.9)–15.0			CO1
6531a	*Pione carpenteri*	CS	15	255.0–(294.9± 23.0)–355.0	77.5–(102.7± 10.1)–122.5	1.7–(16.0± 12.0)–20.0			CO1
6628	*Pione carpenteri*	MB	5	255.0–(305.1± 20.7)–355.0	87.5–(102.4± 7.9)–120.0	10.0–(14.0± 1.7)–17.0			–
6724	*Pione carpenteri*	MI	5	155.0–(300.6± 31.5)–360.0	85.0–(99.8± 9.1)–115.0	10.0–(14.8± 2.3)–21.0			–
6685	*Cliona jullieni*	MI	15	285.0–(323.5± 20.8)–375.0			19.0–(26.3± 4.3)–34.0		CO1

[a] For geographical coordinates see Fig. 1; abbreviations: *CS* Camping Site, *MB* Marsa Bareka, *MI* Mangrove Island, *RN* Ras Nosrani

circular, with smooth surfaces (Fig. 2b); pit main axis length 65.34 (mean) ± 12.94 μm.

Spicules

Tylostyles straight with globular to ovoid heads, sometimes in subterminal position (Fig. 3b); microxeas microspined, slightly bent in middle; microrhabds microspined, with wavy, truncated tips (Fig. 3d). Measurement results are shown in Table 1.

Skeletal organization

Papillar skeleton characterized by palisade tylostyles with microxeas and microrhabds. Disorganized microxeas predominant into the choanosomal skeleton.

Distribution

Considered cosmopolitan (Calcinai et al. 2000; Rosell and Uriz 2002; Rützler and Stone 1986).

Remarks

Pione vastifica has been reported to have dull red to orange-red living tissue, which fades to pale greyish in preserved samples (Rützler 1973; Rosell and Uriz 2002). In this species the papillae are small, under 1 mm, and chambers measure up to 2 mm (Calcinai et al. 2000; Carballo et al. 1994; Rützler 1973, 2002a). Tylostyles are straight, with globular heads (Fig. 3); microxeas are microspined and bent in the middle. Microspined microrhabd shape is reported to vary from straight to S- or W-shaped, depending on the locality and depth (Calcinai et al. 2000; Carballo et al. 1994; Rosell and Uriz 2002; Rützler 1973, 2002a). Although some encrusting specimens from the Red Sea were tentatively identified as *P.* cf. *vastifica* (Zundelevich et al. 2007), only the alpha growth form has been recorded (Rosell and Uriz 2002; Rützler 2002a; Schönberg 2002). Records of *P. vastifica* from the Atlanto-Mediterranean area show great variation in this species; in particular, recorded measurement ranges for the rhabds are 9.6–19.2 μm (Rützler 1973); 3–9 μm, 10–22 μm, 10–25 μm (three detached morphotyps; Rosell and Uriz 2002); and 12 μm (holotype; Rützler and Stone 1986).

Morphological comparison

Spicule comparison

Mean tylostyle length was greater in *P.* cf. *vastifica* than in *P.* cf. *lampa* (269 μm versus 241 μm), but a two-way ANOVA did not show a significant difference ($F_{1,12}$=2.61, p=0.1318) between these means. Mean microxea length was smaller in *P.* cf. *vastifica* than in *P.* cf. *lampa* (86 μm vs. 89 μm). However, spicule length ranges overlapped extensively.

In the PCA analysis, 78.7% of the variation was accounted for by the first component, and variation was associated with tylostyle length (Fig. 4). However, the discriminant-analysis histogram did not show really good separation of the two suggested species (Fig. 5).

Excavation scar comparison

Excavation scar morphology observed in *Pione* cf. *lampa* and *P.* cf. *vastifica* showed high similarity: pits were regular and almost circular, with smooth surfaces.

Mean pit length (Table 1) was significantly different (p=5.273 E-14) between the two supposed species, but significant differences were also found among specimens within a single supposed species (p=3.61 E-9) (Table 3).

Molecular comparison

It was possible to obtain unambiguous CO1 fragments from eleven Red Sea sponge samples; seven specimens were identified as *Pione* cf. *lampa*, two as *P.* cf. *vastifica,* one as *P. carpenteri*, and one as *Cliona jullieni* Topsent, which also belongs to the family Clionaidae. The final dataset comprised 484 characters.

Pione cf. *lampa* and *P.* cf. *vastifica* showed no differences in their nucleotide sequences (haplotype); therefore, they were identified as a single taxon, which we called 'Haplotype 1'. *Pione carpenteri*, 'Haplotype 2', differs from Haplotype 1 by one missense mutation, resulting in a phenylalanine-leucine substitution in the protein sequence, and by 26 silent substitutions. All mutations involve the third-codon position. *Cliona jullieni*, 'Haplotype 3', differs from Haplotype 1 by a total of 47 nucleotide substitutions. There are two missense mutations (serine-alanine and valine-isoleucine) as a result of first-codon substitutions. Other mutations are silent (44 substitutions involving the third codon, one involving the first codon). For phylogenetic reconstructions, the dataset was extended by three additional *Pione* species sequences published in GenBank, two *P. vastifica* (Caribbean; Erpenbeck et al. 2007) and one *P. velans* (West-Australia); for accession numbers see Fig. 6. However, the morphological vouchers for these sequences were not re-examined by us. The resulting tree indicates a well-supported Red Sea (*P.* cf. *lampa* + *P.* cf. *vastifica*) clade, whereas the third Red-Sea specimen, *P. carpenteri*, forms a clade with the GenBank sequences. The two GenBank *P. vastifica,* which originated from the Caribbean, differ from Haplotype1 by five nucleotides, including one missense mutation (isoleucine-valine), and are non-monophyletic in the present CO1-based reconstruction.

Discussion

In the present work, Red Sea excavating sponge specimens were studied by means of both morphological and molecular approaches and assigned to three species belonging to the clionaid genus *Pione*: *P.* cf. *lampa*, *P.* cf. *vastifica*, and *P. carpenteri*. While attributing specimens to the third species was easy, assigning specimens to the first two was problematic. There had been confusion concerning the taxonomy and distribution of species in the '*Pione vastifica* complex'; particularly the synonymy between *P. lampa* and *P. vastifica* had been debated with no evident conclusion (Desqueyroux-Faúndez 1990; Rützler 2002b; Schönberg 2002). Thus, we decided to provisionally

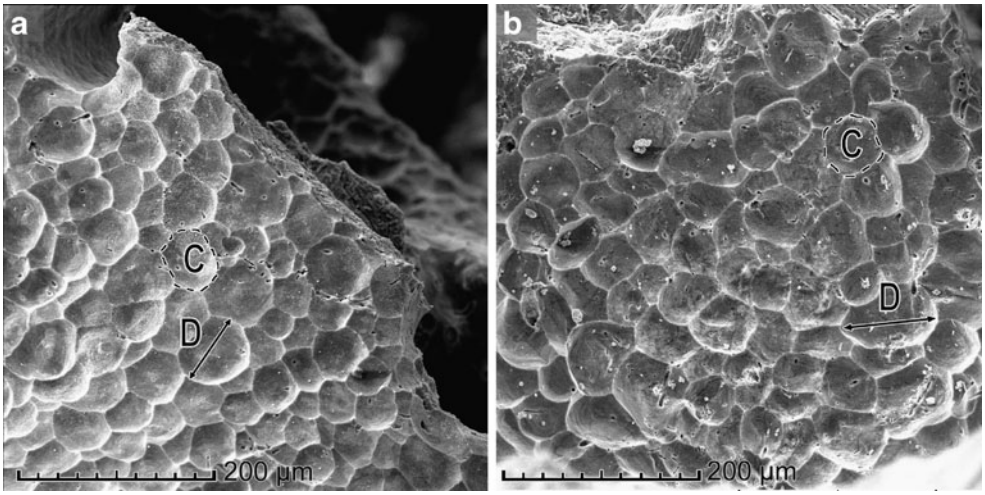

Fig. 2 Erosion scars by Scanning Electron Microscopy. **a** *Pione* cf. *lampa*. **b** *Pione* cf. *vastifica*. Dashed line around "C" marks pit outline; double-headed arrow at "D" indicates pit main axis

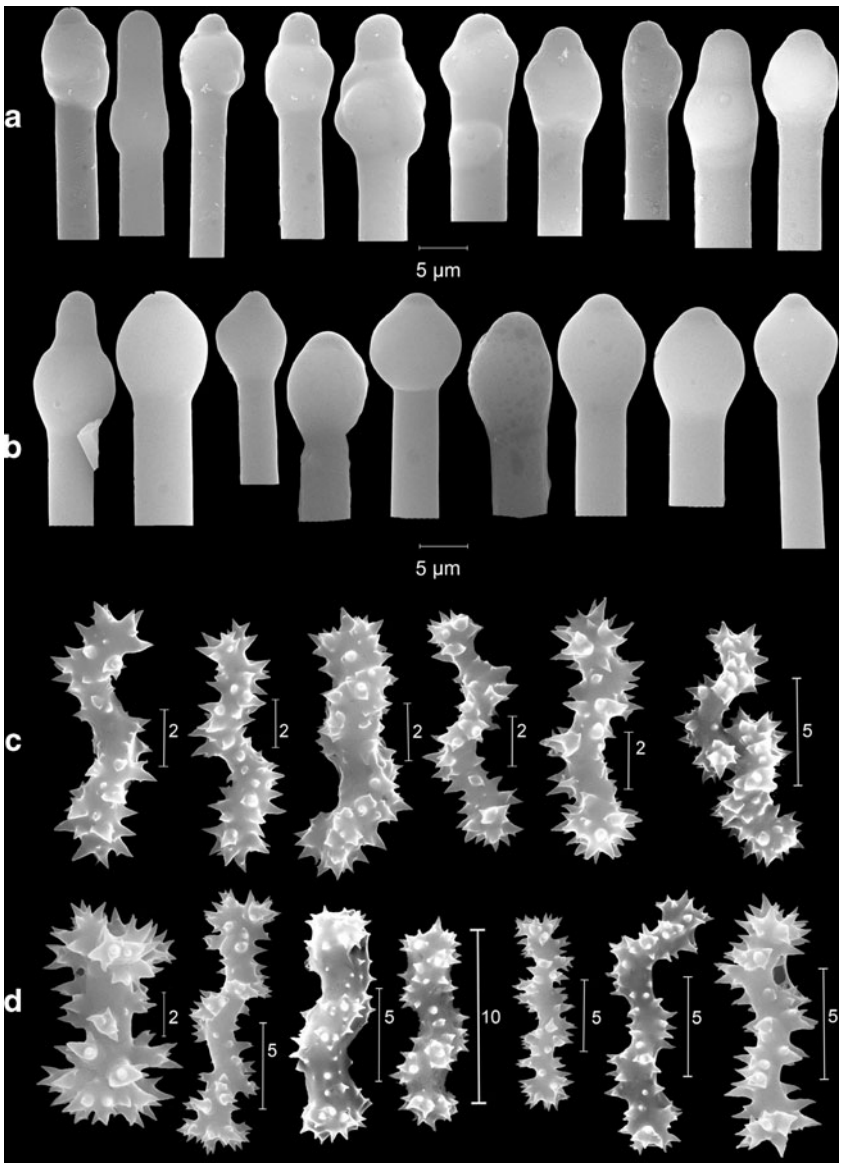

Fig. 3 **a** Tylostyle heads of *P.* cf. *lampa*. **b** Tylostyle heads of *P.* cf. *vastifica*. **c** Microrhabds of *P.* cf. *lampa*. **d** Microrhabds of *P.* cf. *vastifica*. All elements shown under either supposed species came from a single respective specimen. Numbers next to bars indicate scale lengths in µm

Table 2 Previously published spicule lengths (respective upper row) and widths (lower row, where applicable) in *Pione vastifica* and *P. lampa*; mean ± standard deviation shown between brackets, where available

Species	Reference	Tylostyles [μm]	Microxeas [μm]	Microrhabds [μm]
P. vastifica	Carballo et al. (1994)	(255.0)	(110.5)	(8.9)
P. vastifica	Rosell and Uriz (2002)[a]	140.0–(236.0)–340.0	48.0–(82.0)–150.0	Type I[a] 3.0–9.0
		2.0–(4.0)–7.0	1.0–(3.0)–6.0	0.5–5.0
				Type II[a] 10.0–22.0
				0.5–5.0
				Type III[a] 10.0–25.0
				0.5–5.0
P. vastifica	Rützler (1973)	197.5–(231.8)–277.5	48–(67)–92.8	9.6–(14.4)–19.2
		3.0–(3.5)–4.0	1.6–(2.2)–3.2	1.0–(1.1)–1.6
P. vastifica	Zundelevich et al. (2007)	155.0–(230.0±30.0)–241.0	77.0–(103.0±13.0)–125.0	6.3–(8.5±1.1)–10.0
		2.0–(3.6±0.6)–4.0	2.0–(2.8±0.4)–3.5	2.0–(2.5±0.5)–4.0
P. lampa	Pang (1973)	236–(280±4)–388	60.0–(79.1±2.1)–116.3	9.2–(15.2±0.3)–22.1
		4.0–(5.1±0.1)–7.1	2.1–(3.1±0.1)–4.3	1.1–(2.0±0.1)–2.6
P. lampa	de Laubenfels[b]	150–210[b]	70.0[b]	10.0[b]
		3.0[b]	2.0[b]	1.0[b]
P. lampa	Little[b]	153.0–(210.0)–240.0[b]	77.0–(92.0)–105.0[b]	5.0–(8.1)–13[b]
		2.0–(3.9)–6.0[b]	2.0–(2.4)–3.0[b]	1.0–(1.9)–2.2[b]
P. lampa	Schönberg (2002)	80.0–(170.2±21.4)–212.5	71.3–(93.5±11.7)–120.0	7.0–(13.4±2.1)–20.0
		2.5–(4.6±1.1)–7.5	2.5–(3.5±0.8)–5.0	1.5–(2.5±0.6)–4.0

[a] These authors identified three basic morphological types of microrhabds

[b] Data cited in Pang (1973)

separate specimens of the supposed species *P.* cf. *lampa* and *P.* cf. *vastifica*. Morphologically, specimens were assigned mainly on the basis of their two principal and common growth forms (Rosell 1994; Rosell and Uriz 1997, 2002; Rützler 1974), and on differences in excavation pattern, color and size of papillae. Even though tylostyle head shape and microrhabd size accounted for small differences between the two supposed species, spicules of *P.* cf. *vastifica* and *P.* cf. *lampa* were found to be similar in shape and dimension (length ranges greatly overlapped).

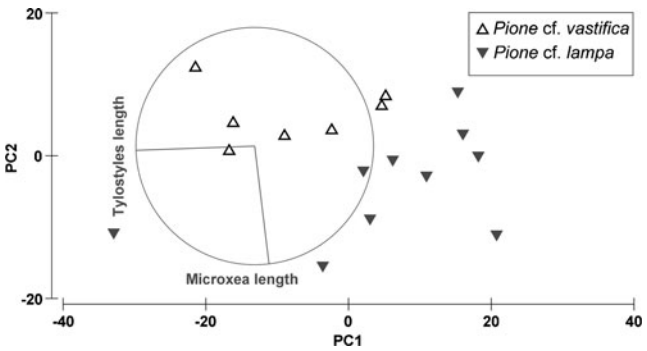

Fig. 4 Results of PCA analysis based on mean spicule lengths and widths of the two supposed species. Each triangle represents one specimen (only specimens having all three spicule types considered; see Table 1). First axis (PC1) explains 78.7% of variation among individuals

While PCA analysis resulted in tylostyle length best explaining the differences between *P.* cf. *vastifica* and *P.* cf. *lampa*, mean tylostyle length was not significantly different between the two sets of specimens. Thus, the results from ANOVA and discriminant analysis weaken those from the PCA, so that the two supposed species cannot be separated clearly.

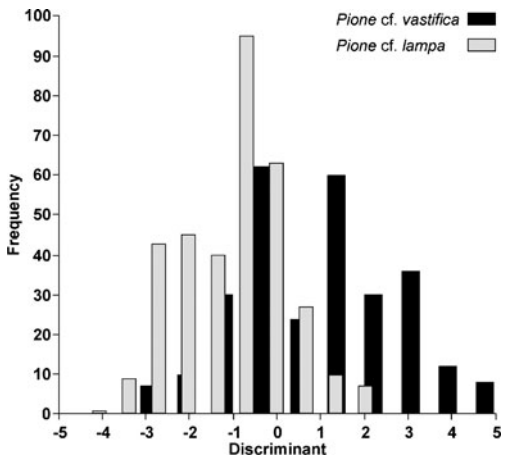

Fig. 5 Discriminant analysis based on tylostyle dimensions. On horizontal axis, "0" is midpoint between means of discriminant scores of the two groups

Table 3 Two-way ANOVA to evaluate differences in excavation scars between *P.* cf. *lampa* and *P.* cf. *vastifica*; performed on pit main axis length measures (n=24) collected from six specimens listed in Table 1

	Sum of squares	Degrees of freedom	Mean square	F-ratio	p-value
Species	9409	1	9409	70.31	5.273 E–14
Samples	6010	2	3005	22.45	3.610 E–9
Interaction	6621	2	3310	24.74	6.591 E–10
Within	1.847 E+4	138	133.8		
Total	4.051 E+4	143			

With regard to micro-erosion patterns, the two-way ANOVA showed a significant difference in scar size between the supposed species, but also large variation among the specimens of a single assumed species. Although the statistical test accounted for this high variability, its existence nevertheless weakened the diagnostic power of the character.

Indeed, morphometric parameters considered in this study could not be considered as truly conclusive evidence for species discrimination.

In addition, from a molecular point of view, there were no differences between the CO1 fragments sequenced from *P.* cf. *lampa* and *P.* cf. *vastifica* from the Red Sea, providing additional evidence that the corresponding sets of specimens actually represent a single taxon only (but see also Erpenbeck et al. 2006). This taxon was different from the other two identified in the present study: *Pione carpenteri* and *Cliona jullieni*.

In this context, the peculiar molecular split between the Red Sea *P.* cf. *vastifica* and their Caribbean counterparts must be explained (Fig. 6). Given that both populations are supposedly conspecific, the molecular distances would be expected to be comparatively small. Nucleotide diversities among *Crambe crambe* (Poecilosclerida) and *Astrosclera willeyana* (Agelasida) populations collected from locations several thousand kilometres apart were found to be very low (Duran et al. 2004; Wörheide 2006). However, the existence of cryptic species can cause unexpected molecular distances within a supposedly cosmopolitan species (Klautau et al. 1999; Lazoski et al. 2001; Nichols and Barnes 2005). In fact, the distribution of sponge species is frequently overestimated, for example as a result of difficulty in distinguishing taxa with few morphological characters, and the presence of sibling species could have been overlooked. In addition, the formation of species in marine organisms in general is higher than expected (Bierne et al. 2003; Palumbi et al. 1997). Another aspect to be considered is that traditional taxonomy has resulted in the assumption of some widely distributed cosmopolitan species, based on synonymisations actually due to the lack of discrimination tools with high resolution power. In particular, although *P. vastifica* is still considered a cosmopolitan, its actual distribution seems to be more limited, as some authors have shown large variation in diagnostic characters on a wide geographical scale, or have even revalidated species names previously synonymised with *P. vastifica* (Carballo et al. 1994; Rosell and Uriz 2002; Rützler 1973; Rützler and Stone 1986). For instance, Carballo et al. (2004) resurrected *Pione mazatlanensis*, rejecting the synonymy with *P. vastifica* for the Pacific records and proposing to consider all species records from the Pacific Ocean as *P. mazatlanensis.* For these authors, the main differences between the two species were the colour (red in *P. mazatlanensis*, orange in *P. vastifica*) and the size of the microrhabds (8.8–17.5 μm, average 10.8 μm in *P. mazatlanensis*) (Carballo et al. 2004). Therefore, the Caribbean *P. vastifica* might not be conspecific with the Red Sea *P.* cf. *vastifica*. The study of *P.* cf. *vastifica* awaits further molecular analysis among *Pione* spp. from the Caribbean, Atlanto-Mediterranean and Pacific areas.

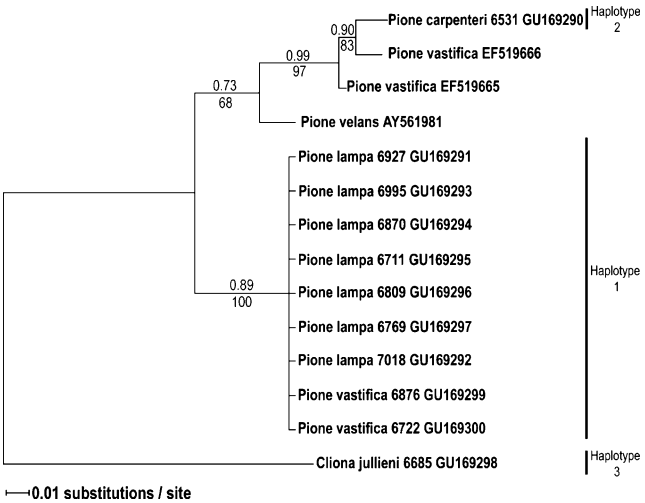

Fig. 6 Bayesian 50% majority rule phylogram based on CO1 sequences, reconstructed by MrBayes. Bayesian posterior probabilities shown above branches indicate, maximum-likelihood bootstrap values below branches. Haplotypes 1–3 from the Red Sea, other sequences from the Caribbean or Western Australia. For each Red Sea sequence, sample number and GenBank accession number are given. Tree has been submitted to TreeBASE (www.treebase.org) under accession number SN4780

Pione lampa, on the other hand, has been reported for 50 years only from Bermuda (where it was described originally), the Bahamas and Florida, yet recently this species has been suggested as an invader to those areas (Rützler 2002b). Consequently, *P. lampa*'s proper distribution area is still scarcely known. Speculation that *P. lampa* is an invasive species from the Red Sea transported in

ballast water is not likely to be confirmed, as such waters cannot be considered as conducive to carrying sponge larvae (Klautau et al. 1999; Lazoski et al. 2001). It could be hypothesized that boring sponges are transported via other invasion pathways, e.g. in calcareous substrates, as vessel fouling, or in organisms of commercial value imported for aquaculture (e.g. in live oyster shells). However, molluscan mariculture attempts in the Caribbean appear to have begun some years after the original description of *Pione lampa* was published (Jory and Iversen 1985). Moreover, the dispersal abilities of sponges in general (Klautau et al. 1999; Lazoski et al. 2001; Nichols and Barnes 2005), and of other members of the Clionaidae family in particular (Mariani et al. 2000), are rather limited. Thus, the records of *P. lampa* from various widely separated geographical areas, and the species' hypothesized cosmopolitanism, are unreliable as long as they have not been tested in molecular studies.

In conclusion, considering (1) that spicule dimension ranges greatly overlap among species of the '*Pione vastifica* complex', (2) the different shapes of the rhabds in Red Sea *P.* cf. *lampa* in comparison to those reported in literature, and (3) the molecular results of our study, we consider that the alpha and beta growth forms from the Red Sea are conspecific. Further studies on *Pione* species from Atlanto-Mediterranean, Carribean and Pacific areas will be necessary to definitely assign a species name to the growth forms of this study.

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