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Integrative analysis of sperm ultrastructure and molecular genetics supports the phylogenetic positioning of the sympatric rock shrimps Sicyonia dorsalis and Sicyonia typica (Decapoda, Sicyoniidae) — Source link

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ORIGINAL PAPER

Integrative analysis of sperm ultrastructure and molecular genetics supports the phylogenetic positioning of the sympatric rock shrimps *Sicyonia dorsalis* and *Sicyonia typica* (Decapoda, Sicyoniidae)

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Abstract We describe the sperm ultrastructure of two sympatric rock shrimps, *Sicyonia dorsalis* and *Sicyonia typica*, and compare them in a molecular context to provide new insight into the sperm morphology of the Sicyoniidae. To accomplish this, the vasa deferentia of males of both species were fixed and prepared for transmission electron microscopy and light microscopy cytochemistry. Sperm of *S. typica* exhibit an oval main body and a short subacrosomal complex region with convoluted membrane pouches, a crystalline lattice and a well-developed large granule anterior to the spike, as previously described for *Sicyonia carinata*. In contrast, the ultrastructure of *S. dorsalis* sperm features an elongated complex subacrosomal region above the main body and a flat nucleus, and small cytoplasmic membrane vesicles are not present. Although *S. dorsalis* sperm are similar to those of *Sicyonia ingentis*, the spike of *S. dorsalis* is shorter than S. ingentis. A phylogenetic tree was built by Bayesian inference based on 19 sequences of 16S rDNA of some representatives of the genus. Our findings show that sperm analysis of these shrimps reflects their phylogenetic history and that such analysis is very useful for taxonomic studies. Species with corresponding sperm ultrastructure are in the same clade of the proposed phylogeny. Moreover, it is possible to detect different groups within the genus based on the presence of at least two distinct morphological patterns of ultrastructure in the spermatozoa of *Sicyonia*.

Keywords Bayesian inference · Phylogeny · Spermatozoa · Transmission electron microscopy · 16S gene

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Introduction

The Sicyoniidae are a monogeneric family that includes 52 species of rock shrimps of the genus *Sicyonia* H. Milne Edwards 1830 (De Grave and Fransen 2011). Six of these species have been reported on the Brazilian coast: *Sicyonia burkenroadi* Cobb 1971, *Sicyonia dorsalis* (Kingsley 1878), *Sicyonia laevigata* Stimpson 1871, *Sicyonia olgae* Pérez Farfante 1980, *Sicyonia parri* (Burkenroad 1934) and *Sicyonia typica* (Boeck 1864) (D'Incao 1995; Costa et al. 2000). In general, these species are found in the bycatch of non-selective commercial shrimp trawling (Castilho et al.

2008). In southeastern Brazil, both *S. dorsalis* and *S. typica* are relatively easy to collect, whereas the other species have a very low frequency of occurrence (for review, see Pires 1992; Furlan et al. 2013).

Although these shrimps are diverse and frequently reported, studies of sperm ultrastructure have been published only for *Sicyonia carinata* (Brünnich 1768) (Medina et al. 1994a) and *Sicyonia ingentis* (Burkenroad 1938) (Kleve et al. 1980). For the latter, detailed descriptions of the acrosome reaction (Clark et al. 1981; Griffin et al. 1987; Clark and Griffin 1988; Wikramanayake et al. 1992; Chen et al. 1994; Baldwin et al. 1998), vas deferens morphology (Subramoniam 1995), polar body formation in oocytes (Hertzler 2002), and spermiogenesis (Shigekawa and Clark 1986) have been published. Spermiotaxonomy has become a very efficient tool in the resolution of taxonomic issues involving phylogenetic relationships among decapod crustaceans (Jamieson 1991, 1994; Medina et al. 1994a, b; Medina 1995a; Tudge 2009). Some earlier phylogenetic proposals were based only on the morphology of sperm (Brown et al. 1977; Felgenhauer and Abele 1991; Jamieson 1991, 1994; Bauer and Holt 1998; Jamieson and Tudge 2000; Tudge et al. 2001; Benetti et al. 2008; Tudge 2009; Klaus et al. 2009; Terossi et al. 2012). Advances in the phylogenetic classification of decapods can be obtained by matching sperm analysis with molecular data. In particular, the 16S rDNA gene has provided a useful molecular marker for delimiting boundaries between lineages and/or species and can contribute to the interpretation of biodiversity patterns (Baker et al. 2008; Bracken et al. 2010).

Decapod sperm have been classified into two main categories based on their morphological features: unistellate spermatozoa (found in Dendrobranchiata and Caridea) and multistellate spermatozoa (found in Brachyura, Anomura and Pleocyemata). However, most previous studies have focused on multistellate sperm taxa. Because there are many gaps in the taxonomy of species with unistellate sperm, it is extremely difficult to perform phylogenetic analyses of these species based on their sperm morphology, as discussed by Medina et al. (2006a) and Braga et al. (2013b).

Dendrobranchiata crustaceans show a wide range of sperm morphologies (Medina et al. 2006a). In general, "unistellate" sperm possess a spherical main body, a long spike and an acrosomal cap that forms an acrosomal vesicle (Jamieson 1991; Medina et al. 1994a, b; Medina 1995a; Jamieson and Tudge 2000; Tudge 2009). Studies that have described sperm ultrastructure in Dendrobranchiata include only a few species of the superfamily Penaeoidea, and the phylogenetic relationships within this superfamily are poorly known, mainly among the families Aristeidae, Solenoceridae, Penaeidae and Sicyoniidae (De Grave and Fransen 2011). The Aristeidae are currently considered an exception to the typical morphology of Penaeoidea due to their lack of an extension of the acrosome that forms the spike, which is considered the most basal morphology of the group (Medina et al. 2006a). In contrast, some species of Sicyoniidae exhibit ultrastructural traits intermediate between those of Aristeidae and Penaeidae, showing a complex subacrosomal region formed by several distinct elements found in other Dendrobranchiata (Kleve et al. 1980; Scelzo and Medina 2003, 2004; Medina et al. 2006b).

Despite the advent of new tools and methodologies such as molecular data and reproductive morphology that can be used to infer phylogeny, studies of shrimps of the Sicyoniidae are still incipient. Sperm morphology in the genus *Sicyonia* remains poorly known, and the available information does not permit substantial inferences about phylogenetic relationships based on spermiotaxonomy. Therefore, in this work, the sperm ultrastructure of two Atlantic species, *S. typica* and *S. dorsalis*, is described and compared with the sperm ultrastructure of other species of the genus reported in the literature. The results are contextualized with a phylogenetic hypothesis from molecular data (16S rDNA) with the goal of exploring the biological diversity and improving the understanding of the evolutionary relationships of this group.

Materials and methods

Animals

Sicyonia dorsalis and *Sicyonia typica* specimens used in the morphological analysis were collected from July 2011 to April 2014 in the region of Cananeia and Ubatuba, São Paulo, Brazil, by double-rig shrimp trawling. Additional specimens of the *Sicyonia* [*S. brevirostris*; *S. disedwardsi* (Burkenroad 1934); *S. dorsalis*; *S. martini* Pérez Farfante and Boothe 1981; *S. mixta* Burkenroad 1946; *S. picta* Faxon 1893, *S. ingentis* and *S. typica*] from the American coast and *S. carinata* from the European coast were used in the molecular analysis (Table 1). Most of the individual specimens used in the study have been deposited in the Crustacean Collection of the Department of Biology (CCDB), Laboratory of Bioecology and Crustacean Systematics (LBSC) of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto (FFCLRP) at the University of São Paulo (USP), Brazil. Others were loaned from the Universidad de Costa Rica (UCR). All individuals were identified according to Perez-Farfante (1980) and Costa et al. (2003).

Sperm ultrastructure and cytochemistry

A total of 46 mature males of *S. dorsalis* and *S. typica* were transported alive to the laboratory and were cold-

Table 1 Species of *Sicyonia* and the outgroup (*Xiphopenaeus kroyeri*) that were used for the phylogenetic analysis, with the respective collected locality, geographic distribution of the species, catalogue number, and the accession code at GenBank

Species	Locality	Geographic distribution	Catalogue no.	Accession no. GenBank
1—*S. brevirostris*	Florida, USA	Western Atlantic	UCR 933-001	KT935437
2—*S. burkenroadi*	na	Western Atlantic	SGM6 012	AY601741[1]
3—*S. carinata*	Cadiz, ESP	Adriatic Sea	CCDB 5370	KT935442
4—*S. disedwardsi*	Puntarenas, CRI	Eastern Pacific	UCR 2915-01	KT935439
5—*S. dorsalis*	Guanacaste, CRI	Eastern Pacific	CCDB 2206-1	KT935428
6—*S. dorsalis*	Guanacaste, CRI	Eastern Pacific	CCDB 2206-2	KT935429
7—*S. dorsalis*	Rio de Janeiro, BRA	Western Atlantic	CCDB 4245	KT935426
8—*S. dorsalis*	Sao Paulo, BRA	Western Atlantic	CCDB 3648	KT935427
9—*S. ingentis*	na	Eastern Pacific	KC4282	GQ487492[2]
10—*S. martini*	Guanacaste, CRI	Eastern Pacific	UCR 2463-07-1	KT935440
11—*S. martini*	Guanacaste, CRI	Eastern Pacific	UCR 2463-07-2	KT935441
12—*S. mixta*	Puntarenas, CRI	Eastern Pacific	UCR 2212-02	KT935438
13—*S. picta*	Guanacaste, CRI	Eastern Pacific	UCR 2460-04	KT935430
14—*S. picta*	Puntarenas, CRI	Eastern Pacific	UCR 1982-03-1	KT935431
15—*S. picta*	Puntarenas, CRI	Eastern Pacific	UCR 1982-03-2	KT935432
16—*S. typica*	São Paulo, BRA	Western Atlantic	CCDB 341-1	KT935434
17—*S. typica*	São Paulo, BRA	Western Atlantic	CCDB 341-2	KT935435
18—*S. typica*	São Paulo, BRA	Western Atlantic	CCDB 341-3	KT935436
19—*S. typica*	São Paulo, BRA	Western Atlantic	CCDB 3951	KT935433
Xiphopenaeus kroyeri	Gulf of Mexico, USA	Western Atlantic	na	AY622217[3]

Superscript numbers indicate the original reference at GenBank files: [1] Bracken et al. (2010); [2] Voloch et al. (2009); [3] Chan et al. (2008)
na not available

anesthetized and dissected to access the reproductive system. The distal vas deferens was fixed for 3–4 h in fixative solution for marine shrimps [2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.6) containing 5 % sucrose (Ro et al. 1990)], washed three times in the same buffer (pH 7.4) and post-fixed in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.6) containing 5 % sucrose. After washing in the same buffer, the samples were stained "en bloc" with 1 % aqueous uranyl acetate and dehydrated in a graded acetone series. The tissues were embedded in Epon–Araldite resin. Thin and ultrathin sections were obtained with a Leica UC7 ultramicrotome and contrasted with 2 % uranyl acetate in water for 45 min and 0.4 % lead citrate in 0.1 N NaOH for 10 min (Reynolds 1963; Venable and Coggeshall 1965). Images were obtained with a JEOL—JEM 1010 transmission electron microscope at 80 kV. The terminology used in the description of the sperm followed Kleve et al. (1980) and Medina et al. (1994a).

For the histological and cytochemical analysis, vasa deferentia of both species of *Sicyonia* were fixed in 4 % paraformaldehyde and 0.2 M sodium phosphate buffer (pH 7.2) for 24 h. After fixation, the samples were dehydrated and embedded in methacrylate Historesin Leica® according to routine procedures. Serial sections 5–7 μm in thickness were obtained with a rotary microtome. The sections were stained with hematoxylin and eosin (H&E) according to Sant'Anna et al. (2010), with toluidine blue (pH 4.0) for nucleic acids and acidic substrates, the periodic acid–Schiff (PAS) method for neutral polysaccharides (Junqueira and Junqueira 1983) and acid fast green for proteins (Gabe 1976).

DNA extraction and analysis

DNA extraction and partial amplification of the 16S ribosomal gene by polymerase chain reaction (PCR) (Saki et al. 1988) followed the protocols of Mantelatto et al. (2009) with some modifications. The extracted tissues were macerated and incubated for 12–24 h in 600 ml of lysis buffer at 65 °C. Proteins were separated by the addition of 200 ml of ammonium acetate (7.5 M) followed by centrifugation. Then, 600 ml of isopropanol was used for DNA precipitation, also followed by centrifugation. The pellet was washed with ethanol (70 %), lyophilized and suspended in 20 ml TE buffer.

The 16S rDNA gene was sequenced using the previously described primers (Porter et al. 2005; Page et al. 2008) H2 (AGA TAG AAA CCA ACC TGG) and L2 (TGC CTG TTT ATC AAA AAC AT) (Crandall and Fitzpatrick Jr.

Table 2 Comparative sperm ultrastructural traits of four species of the genus *Sicyonia*

Sperm character	*Sicyonia carinata*[1]	*Sicyonia dorsalis*[2]	*Sicyonia ingentis*[3]	*Sicyonia typica*[2]
Sperm morphology	Round[a]	Elongated[b]	Elongated[b]	Round[a]
Spike (length)	Long[a]	Short[b]	Long[a]	Long[a]
Spike (surface)	Smooth[a]	Smooth[a]	Spiralled[b]	Smooth[a]
Main body	Round[a]	Elongated[b]	Elongated[b]	Round[a]
Acrosomal vesicle	Thin[a]	Thick[b]	Thick[b]	Thick[b]
Acrosomal cap	Short[a]	Elongated[b]	Elongated[b]	Short[a]
Acrosomal cap surface	Wrinkled[a]	Wrinkled[a]	Wrinkled[a]	Wrinkled[a]
Subacrosomal region	Short[a]	Large[b]	Large[b]	Short[a]
Anterior granule	Small[a]	Large[b]	Large[b]	Small[a]
Anterior granule	Granular[a]	Fibrous[b]	Granular[a]	Granular[a]
Saucer-shaped plate	Flat[a]	Cross-shaped[b]	Flat[a]	Flat[a]
Central core	Thick[a]	Thick[a]	Thick[a]	Thin[b]
Peripheral ring around central core	Present[a]	Present[a]	Present[a]	Absent[b]
Crystalline lattice	Thin[a]	Thin[a]	Thin[a]	Thin[a]
Crystalline lattice striation	Present[a]	Present[a]	Present[a]	Present[a]
Nucleus	Round[a]	Slit[b]	Round[a]	Round[a]
Nuclear plate	Present[a]	Present[a]	Present[a]	Present[a]
Chromatin	Fibrous[a]	Granular[b]	Fibrous[a]	Granular[b]
Membrane pouches	Present[a]	Absent[b]	Present[a]	Present[a]
Membrane pouches	Thin[a]	Absent[c]	Thick[b]	Thin[a]
Small vesicles associated to plasma membrane	Present[a]	Absent[b]	Present[a]	Present[a]
Large vesicles (mitochondria)	Few[a]	Many[b]	Few[a]	Few[a]

Different letters indicate variation among species

[1] Medina et al. (1994b); [2] present study; [3] Kleve et al. (1980)

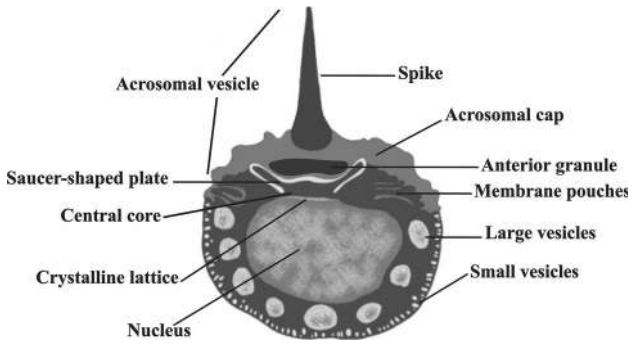


Fig. 1 General scheme of *Sicyonia* sperm in longitudinal section. This diagram is based on the simplest sperm found, that of *S. typica*, and presents the nomenclature used in this work according to Kleve et al. (1980) and Medina et al. (1994a)

1996). Reactions were performed in a 25-µl volume containing 6.5 µl of distilled water, 3 µl of 10× PCR buffer II, 3 µl of $MgCl_2$ (25 mM), 5 µl of betaine (5 M), 1 µl of each primer (10 mM), 4 µl of dNTP (10 mM), 0.5 µl of AmpliTaq DNA polymerase and 1 µl of DNA. The PCR results were verified by electrophoresis of the reaction products in agarose gels (1 %).

PCR products were purified using the SureClean Plus kit and sequenced using ABI Big Dye® Terminator Mix in an ABI Prism 3100 Genetic Analyzer® following the protocols provided by Applied Biosystems. The sequencing was conducted in an automated sequencer, the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems automated sequencer) at the Department of Technology of FCAV/UNESP using the reaction kit ABI Mix—Big Dye Terminator® (Biosystems).

Sequence and phylogenetic analysis

The obtained sequences were reconciled and confirmed by sequencing both DNA strands (sense and antisense) using BioEdit 7.0.9 software (Hall 1999). Primers and indeterminate regions at the beginnings of the sequences were removed from the data. The consensus sequences were blasted in GenBank and compared with previous sequences to ensure that the original samples were not contaminated. The partial sequences of the 16S ribosomal gene of *S. burkenroadi* Cobb 1971, *S. ingentis* and *Xiphopenaeus kroyeri* (Heller 1862) (accession code: AY622217) were

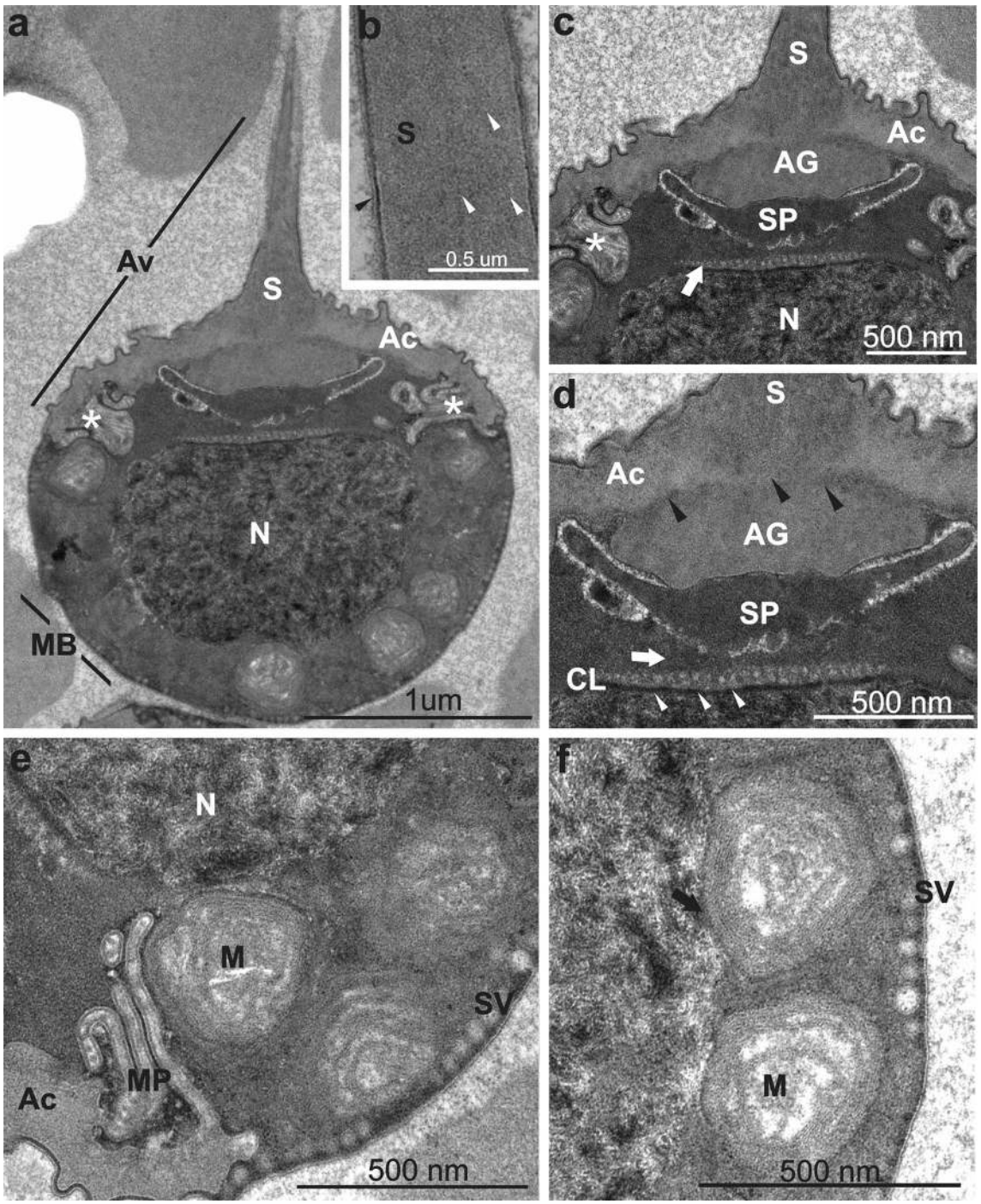


Fig. 2 Sperm ultrastructure in *Sicyonia typica.* **a** Longitudinal section of a sperm showing the acrosomal vesicle (*Av*) formed by the long spike (*S*) and the acrosomal cap (*Ac*). The acrosomal cap (*Ac*) is short and is formed by the complex subacrosomal cytoplasm on the main body (*MB*) (*asterisk* membrane pouches; *N* nucleus). **b** Detail of the smooth surface (*black arrowheads*) and bundles of fibrils (*white arrowheads*); long spike (*S*). **c** Insertion of the spike (*S*) in the short acrosomal cap (*Ac*). The anterior granular region (*AG*) of the subacrosomal cytoplasm lies beneath the acrosomal cap (*Ac*). In the subacrosomal cytoplasm of the cap, an electron-dense saucer-shaped plate (*SP*) can be seen between the anterior granular region (*AG*) and the crystalline lattice (*white arrow*). The nucleus (*N*) is filled with granular and homogeneous chromatin (*asterisk* membrane pouches). **d** Detail of the crystalline lattice (*CL*) with electron-dense borders forming the nuclear plate (*arrowheads*). In this species, the region of the central core just below the saucer-shaped plate is thin (*white arrow*). Above the plate, the anterior granule (*AG*) fills the subacrosomal space bordered by the membrane (*black arrowheads*) of the acrosome cap (*Ac*) (*S* spike; *SP* saucer-shaped plate). **e** Detail of the edge of the acrosomal cap (*Ac*), which forms convoluted membrane pouches (*MP*) (*M* mitochondria; *N* nucleus; *SV* small cytoplasmic vesicles). **f** Detail of small vesicles (*SV*) associated with the plasma membrane and mitochondria (*M*) of the main body. Note the presence of mitochondria (*M*) with two membrane units (*arrow*)

retrieved from GenBank and included in the analysis (Table 1). The last was used as an outgroup, following Ma et al. (2009) and Tavares and Martin (2009). The specimens' DNA-extract vouchers were deposited in the CCDB/FFCLRP/USP or returned with appropriate labels to the original collections. The nucleotide sequences were edited in BioEdit and aligned in ClustalW (Thompson et al. 1994, implemented in BioEdit).

Genetic distance and phylogenetic analyses were performed for the partial sequences of the 16S genes. The genetic distances were calculated in BioEdit using uncorrected p distance. A phylogenetic hypothesis was generated based on this gene and estimated by Bayesian inference (BI) (Huelsenbeck et al. 2001). The BI was performed in the MrBayes 3.1 program (Huelsenbeck and Ronquist 2001) and configured to use the following parameters: sampling frequency 500, four-chain heating (three heated and one cold), a value of "stop heating chains" of <0.01 after at least 2 million generations; the GTR model was applied. Data were subsequently collected from the stationary phase and chain, and the initial states were discarded (burn in = 15 %). The levels of branch support were obtained by the method of posterior probability. A 50 % majority-rule consensus tree was obtained from the remaining saved trees. The saved trees were edited using Mega 4 (Tamura et al. 2007).

Results

Sicyonia sperm consist of a main body with a nucleus and a region that forms the acrosomal vesicle; the latter is composed of the acrosomal cap and spike and forms the acrosomal complex, as observed in other members of Sicyoniidae described in the literature (Table 2). The general structure of the sperm regions is illustrated in Fig. 1, which is based on *S. typica*.

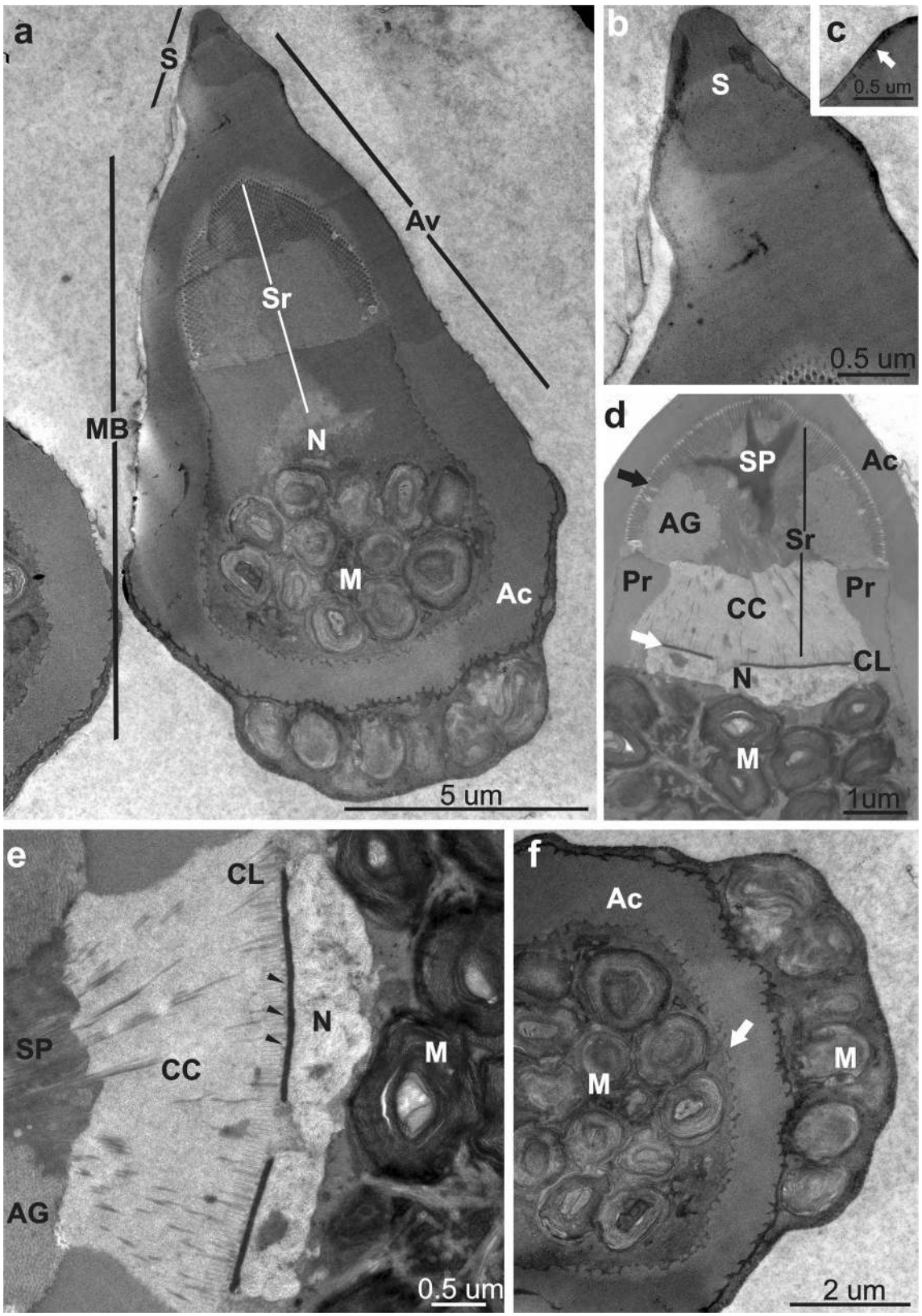
Sicyonia typica

The spermatozoa of *S. typica* are 5.10 ± 1.09 μm ($n = 21$) in length and are dispersed in the posterior vas deferens, where they are immersed in secretory fluid. The sperm exhibit the typical morphology of those of other Penaeoidea shrimps, consisting of a spherical main body surrounded partially by the acrosomal vesicle, which is formed by the anterior spike and the acrosomal cap (Fig. 2a). The anterior spike has a smooth, flat surface and is internally composed of bundles of fibrils (Fig. 2b). The acrosomal cap on the main body is short, producing a round sperm that presents an irregular surface (Fig. 2a, c, d). In the subacrosomal region, underneath the acrosomal cap, is the anterior granule and the electron-dense saucer-shaped plate surrounded by a thin electron-lucent layer (Fig. 2c, d). Subjacent to the saucer-shaped plate, there is a homogeneously electron-dense region that forms a thin central core (Fig. 2d). The central core extends laterally to the posterior margin of the acrosomal cap, which is differentiated into membrane pouches (Fig. 2a, c, e). Beneath the central core, there is a thin crystalline lattice that is associated with the nuclear envelope through a thin and electron-dense lining of the nuclear plate (Fig. 2c, d). In the thick cytoplasm of the main body, the centrally located nucleus is filled with granular chromatin (Fig. 2a, c, e). Several small cortical vesicles are associated with the plasma membrane from the membrane pouches surrounding the whole main body at the opposite pole from the acrosomal cap (Fig. 2a, e, f). The medullary cytoplasm of the sperm body contains many mitochondria (sensu Medina et al. 1994a; Medina 1995a) that exhibit irregular and almost concentric cristae (Fig. 2f).

Fig. 3 Sperm ultrastructure in *Sicyonia dorsalis*. **a** Longitudinal section of a sperm showing the main body (*MB*) and the acrosomal vesicle (*Av*). The acrosomal vesicle (*Av*) is formed by the reduced spike (*S*), which shows two posterior projections that end in two posterior acrosomal pouches. The acrosomal cap (*Ac*) extends from the base of the spike (*S*) to the nucleus (*N*) (*M* mitochondria; *Sr* subacrosomal region). **b** Detail of the apex of the spike (*S*), showing a slightly irregular surface. Note the anterior projections on the spike, which are filled with material of the same electron density. **c** Detail of the short anterior spike surrounded by a membrane (*arrow*). **d** Subacrosomal region (*Sr*) near the cap, showing the anterior granular region (*AG*) with a central core (*CC*). The wide central core is delimited by the crystalline lattice (*CL*), the nuclear plate (*white arrow*) and surrounded by the peripheral ring (*Pr*). The saucer-shaped plate (*SP*) is electron-dense and modified into a cross shape. Note the presence of fibrils at the edge of the granular region (*black arrow*) (*Ac* acrosomal cap; *M* mitochondria; *N* nucleus). **e** Detail of the thick crystalline lattice (*CL*) forming the nuclear plate (*arrowheads*). Note the narrow nucleus (*N*) with granular chromatin (*AG* anterior granule, *CC* central core, *M* mitochondria, *SP* saucer-shaped plate). **f** Mitochondria (*M*) and the posterior acrosomal pouch. Note the portion of the cytoplasm external to the edge of the acrosomal cap (*Ac*) on the opposite pole from the spike (*arrow*)

Sicyonia dorsalis

Most of the main body of the sperm of *S. dorsalis* is covered by the acrosomal cap (Fig. 3a). The cell measures 15.50 ± 1.37 μm ($n = 25$) in length. The acrosomal vesicle comprises a short anterior spike, which extends anteriorly on the main body, plus the acrosomal cap (Fig. 3a–c), forming a single acrosomal vesicle that appears to be equivalent to that of other Sicyoniidae. The acrosomal cap begins at the posterior margin of the spike base in a region that is less electron-dense than the surrounding material and almost completely surrounds the main body (Fig. 3a). The subacrosomal complex region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lies beneath the acrosomal vesicle and consists of the anterior granule, a saucer-shaped plate, a central core, a peripheral ring, a crystalline lattice and the nuclear plate (Fig. 3d, e). The anterior granule region is wide and is formed by several electron-dense cores that are associated with the spike (Fig. 3d). In the center of the granular region lies the electron-dense saucer-shaped plate, which is modified into a cross shape and surrounded by material that is more electron-dense that the remainder of the granular region (Fig. 3d). Underneath the anterior granular region is the central core, which is delimited by a dense peripheral ring (Fig. 3d, e). The central core exhibits thin fibrils anchored in the crystalline lattice and nuclear plate (Fig. 3e). The nucleus is narrow in the region beneath the crystalline lattice, and the nuclear plate shows granular chromatin (Fig. 3d, e). The perinuclear cytoplasm is homogeneously electron-dense and is in contact with the acrosomal cap (Fig. 3f). The cytoplasm at the pole opposite to the spike contains many degenerated mitochondria with concentric myelin-like cristae (Fig. 3a–f).

Sperm cytochemistry

Light microscopy confirmed the observed differences in sperm size in the two studied species of *Sicyonia*. In *S. typica*, the round nucleus and spike are basophilic (Fig. 4a); toluidine blue revealed the presence of nucleic acids, confirming the nuclear morphology observed under transmission electron microscopy (Fig. 4b). The acrosomal cap, the subacrosomal region and the spike were reactive to PAS, demonstrating the presence of neutral polysaccharides in the anterior granule, whereas the saucer-shaped plate, cytoplasmic vesicles and nucleus showed no PAS staining (Fig. 4c). The spike and the acrosomal cap were strongly stained by fast green protein stain; the subacrosomal region, nucleus and large vesicles were also positive for protein, and the saucer-shaped plate was less reactive than the other regions (Fig. 4d). The cytochemistry results obtained with *S. dorsalis* were similar to those obtained with *S. typica*. The nucleus and acrosomal cap appeared basophilic when stained with H&E (Fig. 4e). The acrosomal vesicle showed differential reaction; the apex of the short spike and the acrosomal cap was strongly positive for acidic substrates and neutral polysaccharides (Fig. 4f–h), whereas the spike was intensely stained for proteins and the acrosomal cap was less intensely stained but was also positive to acid fast green (Fig. 4h). The base of the spike, which extends into the main body of the sperm cell, was positive for acidic substances and neutral polysaccharides and strongly positive for proteins (Fig. 4f–h). A narrow and compact nucleus located beneath the central core was strongly stained with toluidine blue and negative to PAS (Fig. 4f–g). The anterior granule and the peripheral ring were stained with toluidine blue, PAS and acid fast green, but the central core was negative to neutral polysaccharides (Fig. 4f–h). The modified saucer-shaped plate was eosinophilic and stained intensely for protein (Fig. 4e–h). Table 3 shows a summary of the cytochemical results obtained in this study and compares them with data available in the literature.

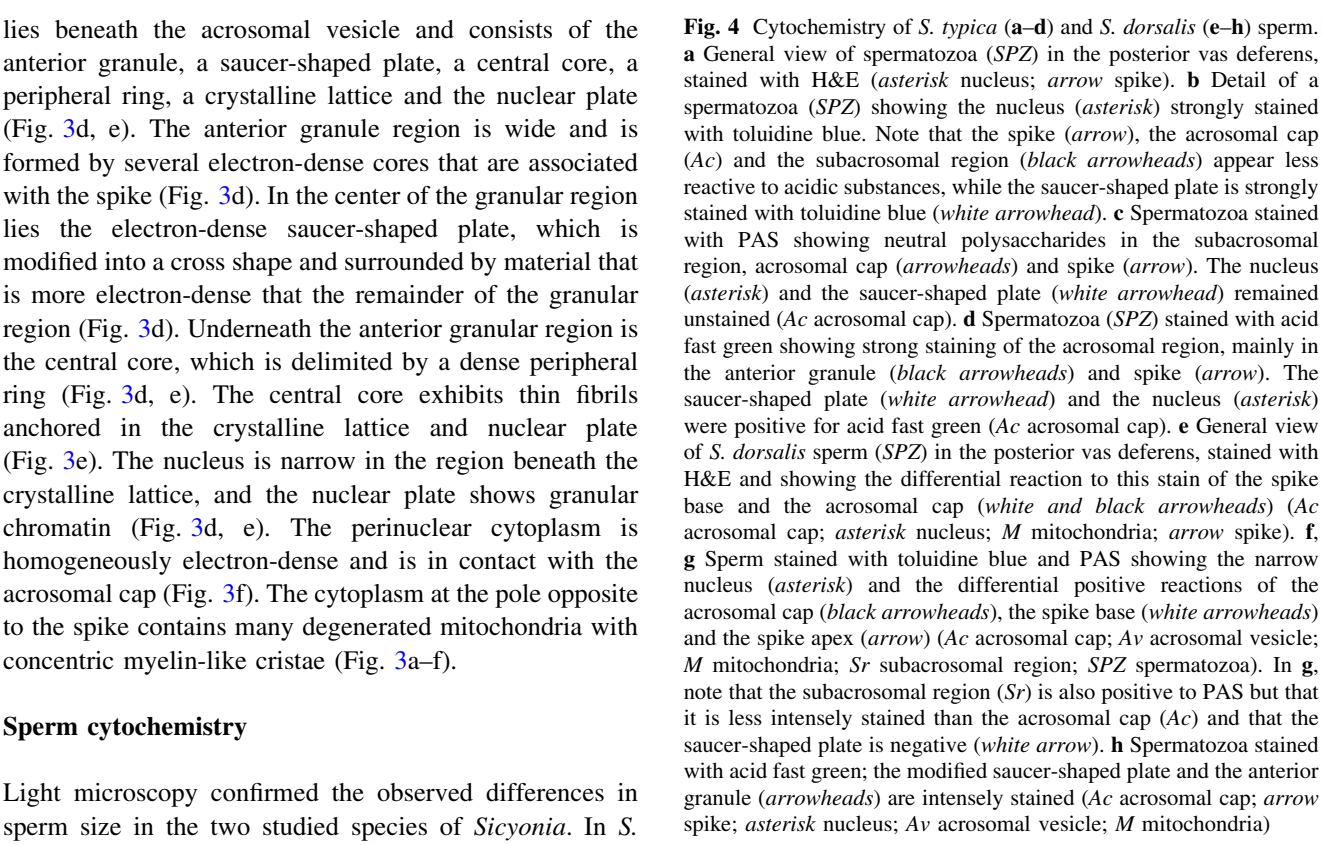
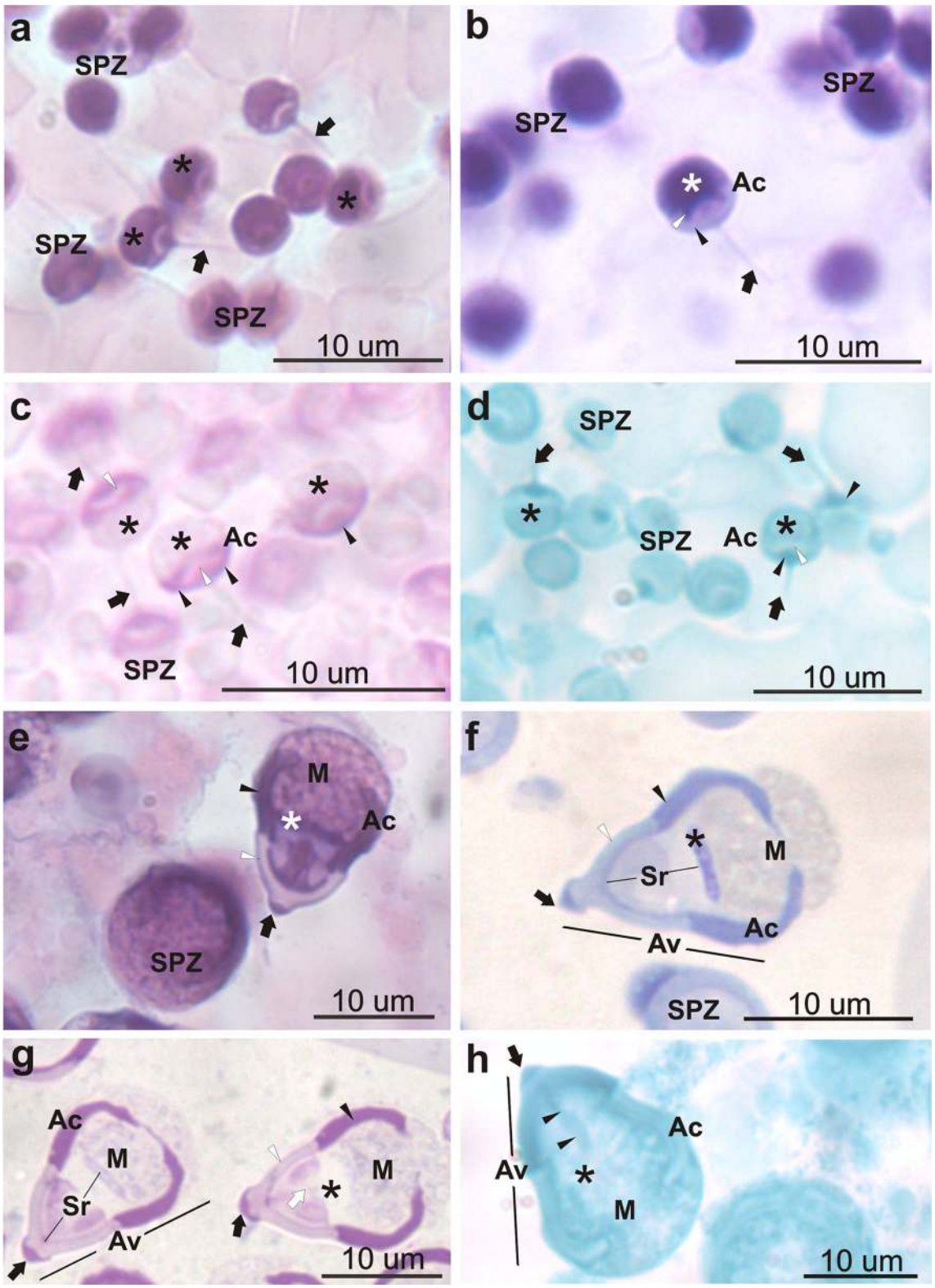


Fig. 4 Cytochemistry of *S. typica* (**a–d**) and *S. dorsalis* (**e–h**) sperm. **a** General view of spermatozoa (*SPZ*) in the posterior vas deferens, stained with H&E (*asterisk* nucleus; *arrow* spike). **b** Detail of a spermatozoa (*SPZ*) showing the nucleus (*asterisk*) strongly stained with toluidine blue. Note that the spike (*arrow*), the acrosomal cap (*Ac*) and the subacrosomal region (*black arrowheads*) appear less reactive to acidic substances, while the saucer-shaped plate is strongly stained with toluidine blue (*white arrowhead*). **c** Spermatozoa stained with PAS showing neutral polysaccharides in the subacrosomal region, acrosomal cap (*arrowheads*) and spike (*arrow*). The nucleus (*asterisk*) and the saucer-shaped plate (*white arrowhead*) remained unstained (*Ac* acrosomal cap). **d** Spermatozoa (*SPZ*) stained with acid fast green showing strong staining of the acrosomal region, mainly in the anterior granule (*black arrowheads*) and spike (*arrow*). The saucer-shaped plate (*white arrowhead*) and the nucleus (*asterisk*) were positive for acid fast green (*Ac* acrosomal cap). **e** General view of *S. dorsalis* sperm (*SPZ*) in the posterior vas deferens, stained with H&E and showing the differential reaction to this stain of the spike base and the acrosomal cap (*white and black arrowheads*) (*Ac* acrosomal cap; *asterisk* nucleus; *M* mitochondria; *arrow* spike). **f**, **g** Sperm stained with toluidine blue and PAS showing the narrow nucleus (*asterisk*) and the differential positive reactions of the acrosomal cap (*black arrowheads*), the spike base (*white arrowheads*) and the spike apex (*arrow*) (*Ac* acrosomal cap; *Av* acrosomal vesicle; *M* mitochondria; *Sr* subacrosomal region; *SPZ* spermatozoa). In **g**, note that the subacrosomal region (*Sr*) is also positive to PAS but that it is less intensely stained than the acrosomal cap (*Ac*) and that the saucer-shaped plate is negative (*white arrow*). **h** Spermatozoa stained with acid fast green; the modified saucer-shaped plate and the anterior granule (*arrowheads*) are intensely stained (*Ac* acrosomal cap; *arrow* spike; *asterisk* nucleus; *Av* acrosomal vesicle; *M* mitochondria)

Molecular data

Fragments of the mitochondrial 16S rDNA gene from 10 (11 including the outgroup *X. kroyeri*) species of shrimps were used in this study. We generated 17 new 16S rRNA partial sequences from 8 species of *Sicyonia*. All of these sequences were deposited in GenBank; the accession numbers are listed in Table 1. The phylogenetic analysis included an alignment with 20 sequences with 568 base pairs. In general, distance analyses revealed that the amount of interspecific variation (3.16–12.27 %) is superior than the amount of intraspecific variation (up to 0.36 %) for each group (Table 4). The values between *S. dorsalis* and *S. typica* were 6.52 %; that between *S. dorsalis* and *S. ingentis* was 3.16 %; that between *S. typica* and *S. carinata* was 11.63 % and that between *S. ingentis* and *S. carinata* was 9.56 %.

The topology resulting from the BI approach received high support in most nodes (Fig. 5). *Sicyonia martini* is separated from the congener species, and the other species


a
SPZ
SPZ
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10 um
b
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Ac
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c
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d
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Table 3 Sperm cytochemistry in three species of *Sicyonia*

Species	*Sicyonia dorsalis*[1]			*Sicyonia ingentis*[2]			*Sicyonia typica*[1]		
Sperm structure/stain	PAS	TB	FG	PAS	AO	FG	PAS	TB	FG
Spike apex	+++	+++	+++	N	N	N	++	++	+++
Spike base[a]	++	++	+++						
Acrosomal cap	+++	+++	++	++	N	++	+++	++	+++
Anterior granule	+++	−	+++	++	N	++	+++	++	++
Saucer-shaped plate	−	+++	+++	−	N	N	−	++	++
Nucleus	−	+++	++	N	++	N	−	+++	++
Subacrosomal region	++	++	++	++	++	++	+++	++	+++
Spike	+++	+++	+++	N	N	N	++	++	+++

AO, acridine orange and DNA digestion; FG, acid fast green for proteins; PAS, periodic acid–Schiff for neutral polysaccharides; TB, toluidine blue for DNA and acid substrates; N, not informed

+++, strongly positive; ++, positive; − negative

[a] Spike base is the portion running over the main body only found in *S. dorsalis*

[1] Present study; [2] Kleve et al. (1980)

Table 4 Genetic divergence matrix of the 16S mitochondrial gene among selected species of the *Sicyonia* obtained by distance *p*

	1	2	3	4	5	6	7	8	9	10
1—*S. brevirostris*	**0.00**									
2—*S. burkenroadi*	6.79	**0.00**								
3—*S. carinata*	9.59	11.57	**0.00**							
4—*S. disedwardsi*	4.04	7.41	10.83	**0.00**						
5—*S. dorsalis*	6.01	3.26	11.48	5.21	**−0.18**					
6—*S. ingentis*	4.70	4.21	9.56	5.44	3.16	**0.00**				
7—*S. martini*	5.58	7.74	10.65	4.41	5.39	6.20	**−0.36**			
8—*S. mixta*	6.54	7.67	12.27	5.93	6.32	6.43	6.69	**0.00**		
9—*S. picta*	6.00	6.11	11.74	5.60	4.80	4.93	5.97	5.55	**0.00**	
10—*S. typica*	6.12	8.71	11.63	4.38	6.52	6.47	5.73	6.49	4.81	**0.00**

Intraspecific (bold numbers) and interspecific values

are organized into two main clades with almost 60 % of posterior probabilities. One clade includes *S. typica*, *S. disedwardsi*, *S. brevirostris* and *S. carinata*, and the other includes *S. mixta*, *S. picta*, *S. ingentis*, *S. burkenroadi* and *S. dorsalis.*

Discussion

Analysis of sperm ultrastructure in the Sicyoniidae indicates that the sperm of species in this family exhibit at least two morphological patterns that can be characterized as featuring round or elongated main bodies. *Sicyonia typica* shares the round-shaped main body sperm morphology with *S. carinata* (Medina et al. 1994a; Medina 1995b), and sperm with elongated main bodies were observed in *S. dorsalis* and *S. ingentis* (Kleve et al. 1980) (Table 2). Each of these pairs of species was grouped into a separate clade in our phylogeny based on molecular data (Fig. 5). Two different clades were previously proposed for the family Sicyoniidae by Ma et al. (2009) based on the analysis of DNA sequences of two protein-coding nuclear genes. The Bayesian inference tree proposed by those authors shows that *Sicyonia lancifer* Balss 1914 is in a branch separate from that of *Sicyonia curvirostris* Balss 1913 and *Sicyonia fallax* De Man 1907.

Our phylogenetic tree shows that *S. typica* is a sister taxon of *S. disedwardsi* and that *S. brevirostris* is a sister taxon of *S. carinata*; all form a monophyletic group. Although there is little information on the ultrastructure of *S. disedwardsi* and *S. brevirostris* sperm, we expected that all species of this clade should produce sperm that are round in shape. Within the other clade, *S. dorsalis* is closer to *S. burkenroadi* and *S. ingentis*, with high support of nodes (84.2 %). As previously stated, the sperm of *S. dorsalis* and *S. ingentis* share morphological features,

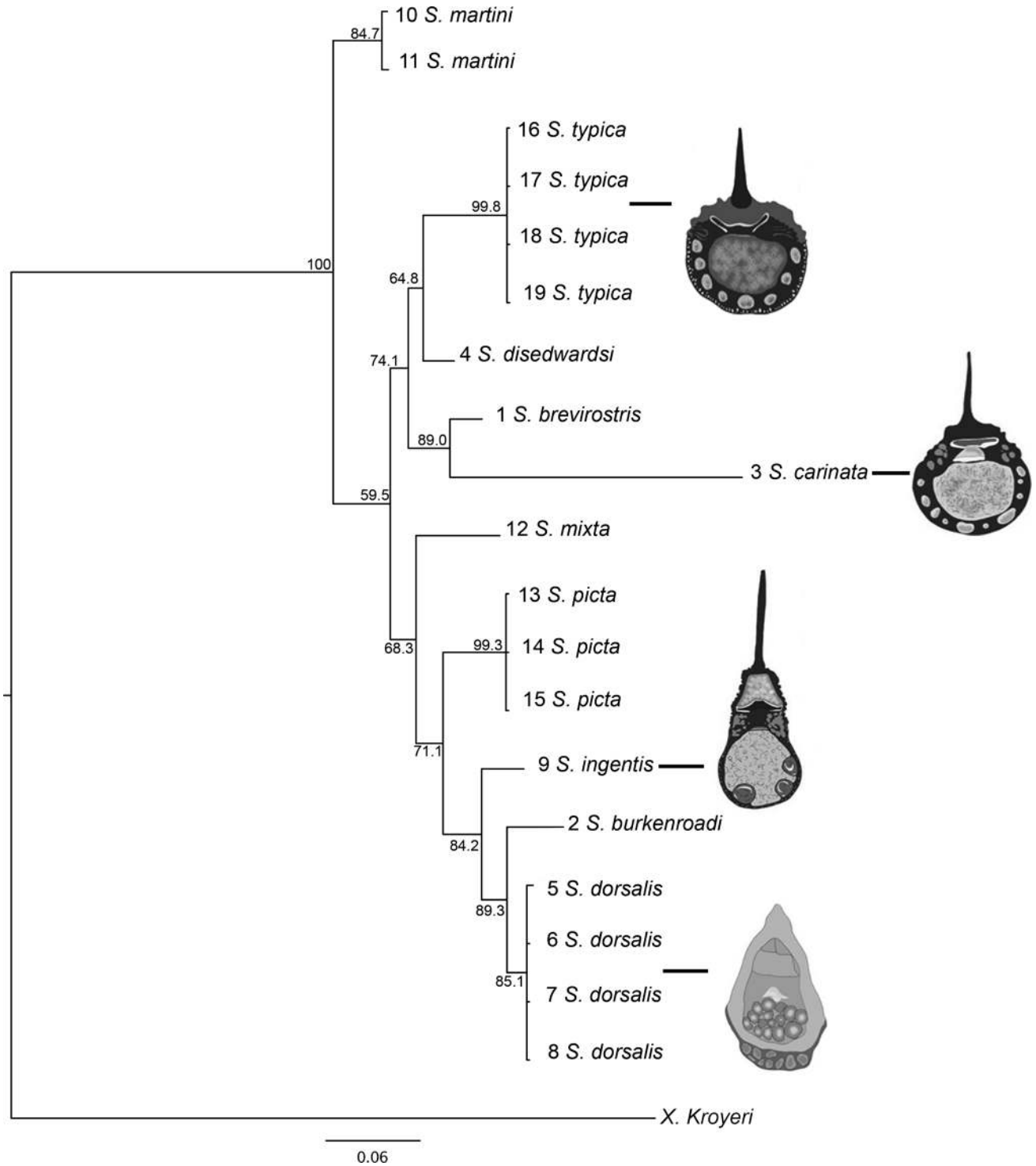

Fig. 5 Phylogenetic tree of several species of *Sicyonia* based on Bayesian inference analysis of 16S rRNA data sets. The numbers in the nodes are the posterior probabilities. The sperm of *S. carinata* and *S. ingentis* were redrawn from Medina et al. (1994b) and Kleve et al. (1980), respectively

whereas the ultrastructure of *S. burkenroadi* sperm has not been described. Although *Sicyonia picta* appears to be a sister taxon of these three species, few inferences about patterns within this group are possible due to the paucity of available data on the spermatozoal configuration of these species.

According to Braga et al. (2013a), a round-shaped main sperm body is considered a plesiomorphic character in

Penaeidae (Dendrobranchiata). Species of the Trachypeneini tribe (Ma et al. 2009) such as *Xiphopenaeus kroyeri* (Heller 1862) and *Rimapenaeus similis* (Smith 1885) exhibit an elongated body pattern by scanning electron microscopy (Bauer and Min 1993; Alfaro et al. 2003; Ma et al. 2009), whereas the sperm of *Farfantepenaeus paulensis* (Pérez Farfante 1967), *Litopenaeus vannamei* (Boone 1931) and *Litopenaues occidentalis* (Streets 1871), species that belong to the Peneini tribe (Ma et al. 2009), are round-shaped (Dougherty and Dougherty 1989; Braga et al. 2013a, b). Like the family Penaeidae, Sicyoniidae exhibit great morphological divergence in sperm main body shape among subdivided clades in the phylogenetic tree. Based on these facts, it is possible that *Sicyonia* includes more than one evolutionary lineage.

In *Sicyonia*, the sperm nucleus is separated from the cap-like acrosomal complex by a dense plate and a highly organized crystalline lattice (Jamieson 1991). In all species of *Sicyonia* examined, the acrosomal complex includes the cap region and the spike. Underneath is the complex subacrosomal region, which is composed of several structures including the granular central core, a saucer-shaped plate, a crystalline lattice and membrane pouches (Medina et al. 1994b). This configuration of the spermatozoal cap can be considered a synapomorphy of the group (Braga et al. 2013b). Nevertheless, some modifications of structures comprising the complex region were observed. The granular region, for example, is relatively small in *S. carinata* and *S. typica*, whereas it is elongated and fibrous in *S. ingentis* and *S. dorsalis*, thereby modifying the subacrosomal region. This elongated granular region is acidic and is composed of neutral polysaccharides and proteins in both species examined, as well as in *S. ingentis* (Kleve et al. 1980). Thus, in this study we show that these important compounds are present in sperm of both lineages: those with round and those with elongated main bodies. Another modification involves the central core. In *S. ingentis*, the central core is a region that separates the saucer-shaped plate from the crystalline lattice; in *S. dorsalis*, it is adjacent to the anterior granular region but remains in contact with the modified saucer-shaped plate. In *S. typica*, the central core is very thin in the region beneath the saucer-shaped plate, and it extends laterally with the same electron density to both sides of the subacrosomal region without forming a peripheral ring. In contrast, in *S. dorsalis* as well as in *S. ingentis* and *S. carinata* (Kleve et al. 1980; Medina et al. 1994b), a peripheral ring that can be identified by differences in electron density surrounds the central core. Therefore, the presence of a thin central core and the lack of a marked peripheral ring characterize *S. typica* sperm. The presence of a peripheral ring with differences in electron density is typical of lineages with elongated sperm but also occurs in *S. carinata*.

In Sicyoniidae, the acrosomal vesicle is filled with glycoproteins, as observed in the species examined here and in *S. ingentis* (Kleve et al. 1980). The vesicle is very thin, and it extends from the base of the spike beneath the cap region in *S. typica* and as observed in *S. carinata* and *S. ingentis* (Kleve et al. 1980; Medina et al. 1994a; Medina 1995b). In *S. dorsalis*, the acrosomal vesicle is thick and is located closest to the opposite pole of the spike. The presence of membrane pouches at the ends of the nuclear pole of the acrosomal vesicle and of small vesicles in the cytoplasmic cortex along the plasma membrane are traits that are only observed in *S. carinata*, *S. typica* and *S. ingentis* and are absent in *S. dorsalis.* However, the membrane pouches are thin in lineages with round-shaped main bodies and thick in *S. ingentis* (observed in Fig. 10, page 35 in Kleve et al. 1980). Therefore, both membrane pouches and small vesicles can be considered basal traits because they occur in lineages with round as well as in lineages with elongated main bodies. In *S. dorsalis*, these structures have been lost, and the acrosomal vesicle is displaced and thick. Another type of organelle found in the main body cytoplasm is the mitochondrion. The mitochondria are degenerate (or nearly so) in both lineages, but the general morphology of the mitochondria in *S. typica* is quite similar to that of *S. carinata* (Medina et al. 1994a), whereas the mitochondria in *S. dorsalis* sperm resemble those found in the sperm of *S. ingentis* (Kleve et al. 1980). This organelle was first described as a large vesicle in *S. ingentis* (Kleve et al. 1980) and was later identified as a mitochondrion in *S. carinata* (Medina et al. 1994a). The mitochondria usually appear to be degenerate and are considered a unique decapod sperm character (Tudge 2009). Despite the lack of cytochrome C testing in this work, our ultrastructural results are generally in agreement with the proposal of Medina et al. (1994a) and Tudge (2009) that the large vesicles described in *S. ingentis* are actually mitochondria in Sicyoniidae shrimps.

The traits described above demonstrate that, among species with elongated sperm, *S. dorsalis* shows the greatest number of modifications, including differences in several elements of the subacrosomal region. For example, the subacrosomal region, considered a synapomorphy of the group, is modified in *S. dorsalis* that it has become simpler than in other members of the family. The saucer-shaped plate is found in *S. carinata*, *S. typica*, and *S. ingentis* but is highly modified in *S. dorsalis*, indicating the diversification of this species. The modification of the saucer-shaped plate into a cross-shaped plate has resulted in the elongation of the central core and the peripheral ring, both of which are smaller in *S. ingentis* than in other species. Consequently, this saucer-shaped plate can be considered an autapomorphy in *S. dorsalis*; it might have contributed to the modification of the shape of the nucleus,

which is narrow, unlike those of other species of *Sicyonia*. The narrow and elongated nucleus, which was only observed after acid nucleic staining in *S. dorsalis*, is another exclusive trait associated with divergence from the round-shaped pattern observed in *S. typica* and in other species described in the literature (Brown et al. 1977; Kleve et al. 1980; Medina et al. 1994b; Medina 1995a).

The spike in *S. typica* and *S. dorsalis* consists of neutral glycoproteins in the form of fibrils, intensely staining to proteins, and following the same pattern observed in *S. ingentis* (Kleve et al. 1980). Given that *S. dorsalis* sperm possesses a short spike and that its subacrosomal region is modified compared to those of other species of the family Sicyoniidae, some steps of the acrosomal reaction may differ from those of *S. ingentis*. In this species, the acrosomal reaction is divided into two phases: the first involves reduction in the spike, along with swelling and exocytosis of the vesicle, exposing the subacrosomal region; the second is characterized by the formation of a long filament projecting the saucer-shaped plate forward. The apical granule is also projected forward due to the presence of the crystalline lattice (Clark et al. 1981; Clark and Griffin 1988; Baldwin et al. 1998). The presence of a short spike in *S. dorsalis* suggests that the initial phase of fertilization observed in *S. ingentis*, in which the reduction in the spike occurs, might have regressed in this species. The second phase of the acrosomal reaction has also not been detected in some Penaeidae, including *Farfantepenaeus aztecus* (Ives 1891), *Penaeus monodon* Fabricius 1798, *Litopenaeus setiferus* Olivier 1811 and *Litopenaeus stylirostris* (Stimpson 1874) (Clark and Griffin 1993). The absence of this second phase in Penaeidae might be associated with the presence of a less complex subacrosomal region than is found in the Sicyoniidae (Wikramanayake et al. 1992; Braga et al. 2013b). Another difference in the acrosomal reaction is its velocity. This process occurs more rapidly in Penaeidae, requiring only a few minutes in *P. monodon* (Kruevaisayawan et al. 2008), while in *S. ingentis* it lasts between 45 and 60 min (Griffin et al. 1987). Comparative studies of the acrosomal reaction in the two lineages of Sicyoniidae using *S. dorsalis* and *S. typica* as models, as well as in the various lineages of Penaeidae, should be conducted to elucidate the stages of this process in the different families, as also suggested by Braga et al. (2013b).

Based on our compilation of the evolutionary history of currently known sperm morphology for the species of this genus (Fig. 5), we note that *S. carinata*, *S. brevirostris*, and *S. typica* occupy a basal position, with strong evidence suggesting that they are the ancestors of the Sicyoniidae family. Similarly, *S. ingentis* and *S. dorsalis* may be closely related and derived species, forming another branch, as suggested by the elongated sperm main body of these species and their more complex acrosomal and subacrosomal structures.

In conclusion, the species of *Sicyonia* evaluated here exhibit at least two distinct patterns of sperm morphology. We distinguished a more primitive pattern found in *S. typica*, *S. carinata*, and *S. brevirostris* sperm, which are characterized by a round-shaped main body, and a more derivative pattern observed in *S. ingentis* and *S. dorsalis* that is characterized by a more elongated main body. The sperm of *S. typica* share more morphological traits with those of the European *S. carinata* and the Northern Atlantic *S. brevirostris* than with those of the sympatric *S. dorsalis* (Southern Atlantic). The latter seems to be the species that has diverged most within the Sicyoniidae; most of its modified traits are associated with its spike, saucer-shaped plate, and narrow nucleus. The morphological modifications observed in *S. dorsalis* may reflect successive stages of reduction in the acrosomal reaction. Notwithstanding the evidence presented here for the existence of complex sperm morphology in the genus *Sicyonia*, complementary analyses, such as analysis of sperm transfer mechanisms, are needed. Study of additional specimens of other members of this genus is essential to complete the phylogenetic hypothesis and the sperm morphological analysis, to confirm the proposed relationships and to determine whether the sperm traits observed in this study are phylogenetically consistent.

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