Analysis of major sperm proteins in two nematode species from two classes, Enoplus brevis (Enoplea, Enoplida) and Panagrellus redivivus (Chromadorea, Rhabditida), reveal similar localization, but less homology of protein sequences than expected for Nematoda phylum

Julia K. Zograf
A.V. Zhirmunsky National Scientific Center of Marine Biology FEB RAS
Yulia A. Trebukhova
A.V. Zhirmunsky National Scientific Center of Marine Biology FEB RAS
Vladimir V. Yushin
A.V. Zhirmunsky National Scientific Center of Marine Biology FEB RAS
Konstantin V. Yakovlev (konstantin.yakov@gmail.com)
A.V. Zhirmunsky National Scientific Center of Marine Biology FEB RAS https://orcid.org/0000-0002-0718-0775

Original Article

Keywords: Chromadorea, Enoplea, evolution, MSP, Nematoda, spermatozoa

Posted Date: February 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-172088/v1

Version of Record: A version of this preprint was published at Organisms Diversity & Evolution on September 20th, 2021. See the published version at https://doi.org/10.1007/s13127-021-00522-y.

Analysis of major sperm proteins in two nematode species from two classes, *Enoplus brevis* (Enoplea, Enoplida) and *Panagrellus redivivus* (Chromadorea, Rhabditida) reveal similar localization, but less homology of protein sequences than expected for Nematoda phylum

Julia K. Zograf[1], Yulia A. Trebukhova[2], Vladimir V. Yushin[1], Konstantin V. Yakovlev[3*]

[1] Laboratory of Embryology, A.V. Zhirmunsky National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, Russia.

[2] Laboratory of Systematics and Morphology, A.V. Zhirmunsky National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, Russia.

[3] Laboratory of Cytotechnology, A.V. Zhirmunsky National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, Russia.

Vladimir V. Yushin and Konstantin V. Yakovlev contributed equally to this work

*Corresponding author. Email: konstantin.yakov@gmail.com

ORCID IDs

Julia Zograf https://orcid.org/0000-0002-4723-7078

Yulia Trebukhova https://orcid.org/0000-0003-0538-6005

Vladimir Yushin https://orcid.org/0000-0002-0622-3112

Konstantin Yakovlev https://orcid.org/0000-0002-0718-0775

Abstract

Major sperm proteins (MSP) are a nematode-specific system of motor proteins required for amoeboid sperm movement. A number of MSP genes vary in different nematode species, but encoded protein sequences reveal high homology between these proteins. In fact, all studies of MSPs localization and functions are based exclusively on the representatives of the order Rhabditida belonging to the nematode class Chromadorea, while MSP-driven sperm movement in Enoplea, another major clade of the phylum Nematoda is still unconfirmed. In this study, we found out the presence of MSPs in the enoplean nematode *Enoplus brevis* (Enoplida) and compared MSP localization in sperm of this species with the chromadorean nematode *Panagrellus redivivus* (Rhabditida). Then, we analyzed the putative MSP sequences of both species. Our results indicate that MSPs are presented in *E. brevis* spermatozoa and form filamentous structures after sperm activation, which may be considered as the evidence of their motor functions similar to those in the spermatozoa of chromadorean nematodes. We found that *E. brevis* MSPs show lower homology to known proteins of rhabditids which species reveal hyper-conservatism in MSP protein sequences. It reflects evidently more distant evolutionary relationships of Enoplea and Chromadorea than exist within Rhabditida order. Our data denote necessity of reconsideration of view on MSP evolution within Nematoda.

Keywords Chromadorea · Enoplea · evolution · MSP · Nematoda · spermatozoa

Acknowledgments The authors are grateful to staff of the Far Eastern Center of Electron Microscopy (NSCMB FEB RAS) for their assistance in imaging. The authors are also grateful to staff of White Sea Biological Station for the help in *E. brevis* collection.

Funding information This study was supported by Russian Foundation for Basic Research (Grants No. 17-04-00719-a and 14-04-00334-a).

Data availability statement Nucleotide sequences coding *E. brevis* MSP124-1, MSP124-2 and MSP124-3 were deposited to the Third Party Annotation Section of the DDBJ/ENA/GenBank databases under the accession numbers TPA: BK014294-BK014296..

Conflict of interest The authors declare that they have no conflict of interest.

Introduction

Nematodes are one of several animal groups which spermatozoa are amoeboid cells devoid of flagella (Morrow 2004). Nematode spermatozoa accumulated in the seminal vesicles of males are non-motile cells and may be considered as *immature spermatozoa* because the final step of spermiogenesis proceeds only after insemination and activation in the female gonoduct where spermatozoa are drastically transformed into pseudopod bearing motile cells termed as *mature spermatozoa* (Shepherd 1981).

The amoeboid mature spermatozoa move by crawling which resembles amoeboid motility in other eukaryotic cells including some aflagellate spermatozoa. Instead of actin, motility of nematode sperm is driven by unique motor system based on the major sperm protein (MSP) filaments (Roberts and Stewart 2012). Molecular machinery of nematode sperm crawling well-studied in *Ascaris suum* and *Caenorhabditis elegans* (Smith 2014) is similar to actin-based amoeboid movement (Ryan et al. 2012). A basis of this movement is assembly-disassembly of MSP filaments in pseudopod, where MSP is accumulated after spermatozoon activation and forms long multi-filament fibers (King et al. 1994). MSP filaments assemble preferentially at the leading edge of the sperm pseudopod and are organized into long, multi-filament fiber complexes. These filamentous arrays are linked to the pseudopod plasma membrane and extend back to the junction between the cell body and pseudopod. As sperm crawl forward, these complexes flow back towards the cell body due to filaments assembly at the leading edge and disassembly at the rear of the pseudopod (King et al. 1994; Sepsenwol et al. 1989). Both processes are tightly regulated by set of cytosolic, membrane and MSP filament-associated proteins (Ellis and Stanfield 2014; Roberts and Stewart 2000; Singaravelu and Singson 2011; Smith 2014). Although many proteins with MSP domain persist in plants, fungi and other animals (Tarr and Scott 2005) motor functions of MSPs have been found only in nematode sperm. Motor (cytoskeletal) MSPs conserved between nematode species consist of 126-127 amino acids and detected as small 14-17-kDa proteins (Höglund et al. 2008; King et al. 1992; Klass and Hirsh 1981; Strube et al. 2009).

The classification based on morphological and molecular data subdivides the phylum Nematoda into two classes Enoplea and Chromadorea (Fig. 1) (De Ley and Blaxter 2002). The latter comprises seven orders where the movement of spermatozoa have been observed and studied in details in the most diverse order Rhabditida due to the free-living soil nematode *C. elegans* and several parasitic taxa including *Ascaris*. The MSPs as the nematode-specific cytoskeleton proteins or MSP-coding genes have also been found in all tested species of the order Rhabditida (Fig. 1). Within this order, MSP identity of amino acid sequences is over 80% between species (Kasimatis and Phillips 2018; Scott et al. 1989). In representatives of other orders of Chromadorea amoeboid movement of spermatozoa confirmed by development of prominent pseudopods filled with cytoskeleton fibers reminding well cytoskeleton of pseudopods in rhabditids (Justine 2002; Yushin and Malakhov 2014). We assume that the amoeboid movement based on MSP fibers is characteristic for entire class Chromadorea.

Another nematode class Enoplea includes two well defined subclasses Dorylaimia and Enoplia (De Ley and Blaxter 2002). In the representatives of Dorylaimia amoeboid spermatozoa with pseudopods filled with cytoskeleton fibers have been described in many taxa (Justine 2002; Yushin and Malakhov 2014). This specific morphology of motile sperm is confirmed by direct observation of sperm movement in the dorylaimian *Gastromermis* sp (order Mermithida) (Poinar and Hess-Poinar 1993). However, analysis of genome of the dorylaimian *Trichinella spiralis* (Trichinellida) did not show the presence of presumptive amino acid sequences with great similarity to spermatozoan MSPs of rhabditids (Kasimatis and Phillips 2018). So, proteins driving ameboid movement of sperm in species of Dorylaimia are still undetermined.

The representatives of Enoplida have plesiomorphic features such as nuclear envelope in spermatozoan nucleus, early indeterminate cleavage, late establishment of bilateral symmetry, late separation of the germ line, absence of eutely (cell constancy), capability to limited regeneration, absence of distal tip cell in gonads (Felix 2004; Joshi and Rothman 2005; Malakhov 1994, 1998; Rusin and Malakhov 1998; Schulze and Schierenberg 2011; Voronov 1999; Voronov and Panchin

1998; Yushin et al. 2014; Yushin and Malakhov 2004). Basal position of Enoplida is confirmed also by latest molecular phylogenetic analyses of nematode relationships (Bik et al. 2010; Blaxter and Koutsovoulos 2015; Holterman et al. 2006; Smythe et al. 2019; van Megen et al. 2009).

The subclass Enoplia includes taxa where characteristic pseudopod bearing spermatozoa also have been described (Justine 2002; Lak et al. 2015; Yushin and Malakhov 2014). The spermatozoa of nematodes of the genus *Enoplus* (order Enoplida) are amoeboid cells moving by crawling; as in many other nematodes they are subdivided into posterior main cell body and anterior pseudopod filled with cytoskeleton fibers (Yushin and Malakhov 1994). Similar morphology and behavior of *Enoplus* sperm may points to the presence of similar motor proteins, like MSPs. Nevertheless, as in the case of Dorylaimia, it is still unclear whether MSPs underlie sperm crawling in Enoplia.

The goal of this study is to define the presence of MSP in Enoplia, and estimate possible motor functions of MSP in this taxon. In this issue, we analyzed the presence of MSP and its localization in spermatozoa of the marine enoplean species *Enoplus brevis* Bastian, 1865 (Enoplida) and compared results with MSP protein sequences and localization in the chromadorean nematode *Panagrellus redivivus* Linnaeus, 1767 (Rhabditida), the species which has typical spermatozoan morphology (Zograf 2014) and genome of which contains several MSP genes (Scott et al., 1989; Srinivasan et al., 2013). Our choice of this representative of Chromadorea as reference species is also based on the ability of *P. redivivus* mature spermatozoa for conjugation in female gonoduct with formation of characteristic sperm chains (Zograf 2014). These chains can be easily isolated from inseminated females for convenient observations of mature spermatozoa.

Next, to estimate phylogenetic relationships of both *P. redivivus* and *E. brevis* MSPs we carried out phylogenetic analysis using MSP protein sequences of both taxa and available sequences in databases of several Rhabditida and Dorylimia MSPs. By using anti-MSP antibodies, we revealed that MSPs in cases of *E. brevis* and *P. redivivus* are cytoskeletal proteins that undergo reorganization during maturation that led to formation of MSP fibers in mature sperm. Then, based

on antigen sequence used for antibody generation we found mRNA possibly encoding cytoskeletal MSP in *E. brevis* transcriptome. We conclude that *E. brevis* spermatozoa have MSP and its amino acid sequence is similar to *P. redivivus* and *C. elegans* MSPs (75% and 72%, respectively). Here, our results demonstrate that sperm of nematode *E. brevis* have MSPs, but their amino acid sequences are less similar than between Rhabditida species. Thus, we conclude that within the Nematoda phylum MSPs may more rapidly evolve in their protein sequences than expected, but it does not lead to loss of their motor function.

Materials and methods

Animals

P. redivivus cultures were kept in oatmeal and bread-based medium with addition bakery yeasts. Adult males and females of *E. brevis* were obtained from sand collected in the intertidal zone at White Sea Biological Station of Lomonosov Moscow State University (Kandalaksha Bay, White Sea).

Production of anti-MSP antibodies

Polyclonal anti-MSP antibodies were raised in rabbits against synthetic peptide IKTTNMKRLGVDPPCGVLDPKE, which corresponds to part of the MSP domain of several Rhabditida species, like *C. elegans* and *Onchocerca volvulus* (GenBank accession numbers CCD73220.1 and AAA29421.1, respectively). For immunization synthetized peptide was conjugated with keyhole limpet hemocyanin (KLH). Then antibodies were purified using antigenic peptide by affinity chromatography. All procedures of antibody production were made in Cytokine company (St. Petersburg, Russia).

SDS-electrophoresis and Western blot analysis

Animals were frozen at -80° C in minimal volume of 20 mM potassium phosphate buffer with 100 mM NaCl (pH 5.7) (*P. redivivus*) or Ca^{2+}, Mg^{2+}-free salt solution (CMFSS) (*E. brevis*). Samples were thawed out, briefly homogenized in Sample buffer and boiled. Samples were resolved with 10% (tubulin detection), 12% (*E. brevis*, MSP detection) and 14% (*P. redivivus*, MSP detection) SDS-PAGE and transferred to PVDF membrane. Membranes were incubated with 4% milk in PBST (PBS with 0.05% Tween 20) for 1 h and then with primary antibodies diluted with 1% milk overnight at 4° C. Monoclonal mouse anti-α-tubulin antibody (clone DM1A, Sigma-Aldrich, USA) was used at 1:10000 dilution. Rabbit anti-MSP antibodies were used at concentrations 0.24 ug/ml for *P. redivivus* and 1.6 ug/ml for *E. brevis*. After washing membranes were incubated with HRP-conjugated goat anti-rabbit (1:5000 dilution, PI-1000, Vector Laboratories, USA) and anti-mouse antibodies (1:5000 dilution, PI-2000, Vector Laboratories, USA) for 1 h. Then, membranes were washed with PBST and PBS and then developed with Clarity Western ECL Substrate (Bio-Rad, USA). To perform peptide competition assay, 2.25 ug of anti-MSP antibodies were incubated with 10 ug of the synthetic peptide overnight at 4° C, centrifuged at 10000xg for 15 min and then used with *E. brevis* samples.

Immunofluorescence and imaging

Immunostaining procedure was performed on sperm isolated by dissection from males and mated females. Adult *P. redivivus* were directly dissected on poly-L-lysine-coated slides in PBS and then incubated for 10 min for binding extracted sperm to slides. *E. brevis* were dissected on poly-L-lysine-coated slides in filtered sea water. All samples were fixed with 4% PFA in PBS for 30 min. Slides were rinsed with PBS, permeabilized 0.1% Triton X-100 for 20 min and then blocked with 1% normal goat serum and 1% BSA in washing buffer (0.01% Tween 20, PBS) for 2 h. Samples were incubated with anti-MSP antibodies (2.4 ug/ml for *P. redivivus*, 12ug/ml for *E. brevis*) overnight at 4° C. After washing slides were incubated with secondary goat anti-rabbit antibodies labeled with Alexa Fluor 488 and Alexa Fluor 546 (1:500) diluted in 0.1% BSA for 1 h. Nuclei

were stained with DAPI (2 µg/ml) for 20 min. Slides were rinsed with PBS and mounted in Vectashield medium (Vector Laboratories, Burlingame, CA, USA). Fluorescent images were taken using LSM 510 Meta and LSM 780 confocal microscopes (Carl Zeiss, Jena, Germany) and processed using ImageJ software (National Institutes of Health, USA).

Transcriptome assembly and phylogenetic analysis

E. brevis transcriptome was assembled using RNA-seq library available in GenBank (BioProject PRJEB7588, SRA experiment accession number ERX616982) in Galaxy web-based platform (https://usegalaxy.org) (Afgan et al. 2018). Initially, reads were filtered by quality (Filter by quality option, quality cut-off: 20, minimum percentage: 90) and then by Trimmomatic (Bolger et al. 2014). Filtered RNA-seq reads were used for *de novo* transcriptome assembly with Trinity with default parameters (exceptions, minimum contig length: 250). Protein coding sequences were extracted using TransDecoder (https://github.com/TransDecoder). MSP-domain proteins were identified by Pfam search (El-Gebali et al. 2019).

Nucleotide sequences were aligned using MUSCLE (Edgar 2004). Protein sequences were aligned using ProbCons (Do et al. 2005) and visualized in Jalview (Waterhouse et al. 2009). Phylogenetic tree was created using maximum likelihood method with WAG substitution model and branch support with SH-like aLRT (Shimodaira–Hasegawa-like approximate likelihood ratio test). Multiple alignments and phylogenetic tree construction were done in Phylogeny.fr resource (Dereeper et al. 2008).

Results

Localization of MSP in sperm

Firstly, we give data obtained on *P. redivivus* because this species has conserved MSPs that can be detected by generated anti-MSP antibodies. In *P. redivivus* samples, Western Blot analysis using generated anti-MSP antibodies showed two bands with approximate weight of 15 and 16

kDa in both males and females, which is corresponds to mobility of nematode MSPs (Fig. 2). The presence of MSP in female samples confirms that they include mated females bearing deposited spermatozoa. Immunostaining of isolated spermatozoa revealed cytoplasmic localization of MSP. In immature spermatozoa extracted from males, MSP localization is granular with highest signal in cell periphery (Fig. 3a). Mature spermatozoa extracted from mated females were found as conjugated into the chains (Fig. 3b). These chains supporting by tight contacts between spermatozoa were earlier observed in the female gonoduct of *P. redivivus* and described by transmission electron microscopy (Zograf 2014). Our results show that chains retain their organization in PBS after dissection procedure. In activated spermatozoa, MSP localize predominantly in well-defined pseudopodia, where MSP have granulo-fibrillar pattern (Fig. 3b).

In *E. brevis*, anti-MSP antibodies detected a band with approximate size of 36-38 kDa (Fig. 4a), which is higher than known for MSPs in Rhabditida. Subsequent peptide competition assay using peptide antigen showed significant reduction of the signal that confirm specificity of anti-MSP reactivity in the case of the *E. brevis* samples (Fig. 4b). The presence of MSP in female samples point to the presence of mature spermatozoa in uteri. Immunostaining of isolated spermatozoa revealed different localizations. In immature spermatozoa, MSP was detected in granules (Fig. 5a). Notably, incubation of spermatozoa in the sea water for 5-10 minutes led to re-distribution of MSP toward more diffuse and filamentous manner in peripheral cytoplasm though pseudopodia did not appear (Fig. 5b). Mature spermatozoa extracted from females had pseudopodia where MSP now is localized (Fig. 5c).

Analysis of MSP sequences and phylogeny

For reasons given that the peptide antigen sequence is identical or highly homological to MSPs of Rhabditida species, we decided to use its amino acid sequence to find MSP sequences of both *P. redivivus* and *E. brevis* that potentially recognized by the generated antibodies. *P. redivivus* putative protein sequences of MSPs are available and we used them in our analysis. Blast search

using peptide antigen as a query in WormBase Parasite detected six most homologous putative MSPs of *P. redivivus* consisting of 127 amino acids (Fig. 6a). These proteins are highly homologous to each other with identity between them 94-99%. Alignment of the found *P. redivivus* MSPs with *C. elegans* one with accession number P53017 showed identity 87-90%. In our research we used generated transcriptome of *E. brevis* from available SRA data (ERX616982). Blast search among putative proteins generated from the transcriptome with peptide antigen sequence as query detected three 124-amino acid proteins called MSP124-1, MSP124-2 and MSP124-3.

Independent Pfam search among putative protein sequences generated from whole transcriptome attributed MSP124 proteins to MSP family. The presence of MSP124 transcripts was validated by RT-PCR (Fig. S1). Primers and PCR conditions are shown in Table S1. Multiple alignment of their coding mRNAs showed that these transcripts are highly homologous in their coding regions and variable in untranslated regions (Fig. S2). Amino acid sequences of MSP124-1, MSP124-2 and MSP124-3 are almost identical to each other (98-99%) (Fig. 6b). Among proteins with MSP domain of *E. brevis* MSP124 is a group, which most homologous to sperm MSPs of Rhabditida. ExPASy calculations showed that all MSP124 are basic proteins with predicted weight 13.7 kDa (https://web.expasy.org/compute_pi/) (Gasteiger et al. 2005). Predicted molecular weight of MSP124 proteins did not conform with results of Western Blot, when molecular weight of *E. brevis* MSP was much higher than expected. Though, peptide competition assay showed specific binding of anti-MSP antibodies to this protein band (Fig. 4). So, we concluded that MSP124 proteins may have unusual mobility in SDS-PAGE conditions due to post-translation modifications or some unknown features of these proteins.

To evaluate phylogenetic relationships of *P. redivivus* and *E. brevis* MSPs, we analyzed these proteins by multiple alignment and created phylogenetic tree. In this alignment, we used one of six found *P. redivivus* MSPs (Pan_g9068.t1), all three MSP124 proteins of *E. brevis* and available sequences of Rhabditida and Dorylaimia species. The latter were chosen as most

homological proteins to *C. elegans* and *P. redivivus* (*Trichuris trichiura*, *Trichinella nativa*, *T. pseudospiralis* and *T. papuae*) and subsequently selected by pl value (pl>7) by ExPASy analysis, because MSPs found in rhabditids are basic proteins. Multiple alignment, which has been done in ProbCons, is given in Fig. 7a. MSP of *P. redivivus* related to that of Rhabditida with 88% identity to *C. elegans* one. *E. brevis* MSP124 proteins have the same values of similarity to MSPs of *C. elegans* (52% identity and 72% similarity) and *P. redivivus* (55% identity and 75% similarity). Also, similarity between MSPs of Dorylaimia and Rhabditida taxa is 48-62%, and similarity between Dorylaimia taxa and *E. brevis* is 48-53%. Between Dorylaimia species there is high divergence in MSP sequences. Similar protein sequences can be found only within the same genus, for example, in *Trichinella* (Fig. 7a, *T. pseudospiralis* and *T. papuae*). Unlike Rhabditida, different genera of Dorylaimia, as *Trichuris* and *Trichinella*, do not have great homology among submitted MSPs. Brief screening of available genomes of at least fourteen Dorylaimia species (WormBase Parasite) showed that these species have also relatively distant MSPs to both Rhabditida and *E. brevis* proteins (these sequences are not included in alignment). The presented alignment was subsequently used as input for generation of Maximum likelihood phylogenetic tree (Fig. 7b). As expected, *P. redivius* MSP is related to the Rhabditida proteins. The most interestingly that Rhabditida and *E. brevis* MSPs form sister groups with significant SH-aLRT branch support (0.84). Dorylaimia MSPs are located on different branches, and more careful phylogeny requires more MSP sequences of this group.

Discussion

Aflagellate spermatozoa appeared independently during evolution in different metazoan taxa many times. Nematoda and its sister group, Nematomorpha (horsehair worms), both produce aflagellate spermatozoa (Schmidt-Rhaesa 1997/98). It is known that spermatozoa of nematodes locomote by amoeboid movement, while spermatozoan motility in horsehair worms has not been described to date. In the most cases, amoeboid motility is driven by actin polymerization or cortical actin-

myosin contraction (Miyata et al. 2020). Numerous studies on Rhabditida, an order of the nematode class Chromadorea, showed an existence of unique MSP-based sperm locomotion. MSP protein sequences among Rhabditida, as previously noted, are highly conserved and many researchers conclude that it is the case for the whole phylum Nematoda (Höglund et al. 2008; Hojas and Post 2000; Scott et al. 1989). Kasimatis and Phillips (2018) proposed that conservation of MSP sequences should be tightly evolutionary regulated, as nonsynonimous mutations lead to lack or incorrect MSP filament assembly (del Castillo-Olivares and Smith 2008).

Nematodes can be found everywhere; they inhabit different ecological niches and comprise both free-living and parasitic taxa. In spite of the great ecological and taxonomical diversity of nematodes, it has been postulated that sequences of MSPs to be highly conserved. This suggestion originates from the fact that all tested species, both free-living and parasitic, have highly similar MSP protein sequences. The exception may be some parthenogenetic nematodes which MSPs have not been found at protein level, though their genomes contain functional MSP genes (Heger et al. 2010). Nevertheless, a hypothesis of MSP conservatism is based only on studies of species belonging to one chromadorean order Rhabditida. Evidences that MSPs in another class of nematodes, Enoplea, are identical over 80 % to those of Rhabditida, have not been published to date.

Important questions originate from the well-known MSP-based locomotion of spermatozoa in rhabditids and the lack of direct evidences, whether Enoplea also use MSP machinery for sperm movement. Does the origin of the MSP-based locomotion correlate with appearance of amoeboid-moving sperm of nematodes? To answer to this question, it is necessary ascertain, whether MSP-based sperm locomotion exists in species of the enoplean clades Enoplia and Dorylaimia. To search for MSPs in *E. brevis*, we applied a comparative approach using *P. redivivus*, the Rhabditida species, which sperm have MSP. This approach is a combination of the detection of MSPs by antibodies and the subsequent search of genome- or transcriptome-encoded MSP sequences using the antigenic peptide sequence as a query.

Firstly, we tested the chosen approach on *P. redivivus*, which sperm cells is of typical morphology for Rhabditida (Zograf 2014). MSP localization in *P. redivivus* showed that in the round immature spermatozoa MSPs were found throughout cytoplasm with maximal signal in cell periphery, while in the amoeboid mature spermatozoa MSPs mainly localized in pseudopodia. These changes of MSP localization before and after sperm activation are typical for *C. elegans* (Chu and Shakes 2013) and correlate with appearance of MSP fibers required for sperm movement (Marcello et al. 2012). These data and finding presumptive encoded MSPs in genome, which are highly homologous to those of other Rhabditida species, suggest that the *P. redivivus* spermatozoa use MSP-based movement.

Secondly, the used approach allowed us to detect MSPs in the spermatozoa of *E. brevis* and find out three MSP-coding sequences in transcriptome that we called MSP124-1, 2 and 3. Despite the lack of functional analysis, we suggest that *E. brevis* MSPs are motor proteins because their intracellular localization changed after sperm activation toward formation of MSP fibers in a pseudopod, as it was demonstrated in spermatozoa of *P. redivivus* and other rhabditids (Yushin et al. 2016).

Our data show that the *E. brevis* MSP124 proteins are less homologous to MSPs of Rhabditida. All three MSP124 proteins showed less similarity (identity and positive substitutions) to those of *C. elegans* (72%) and *P. redivivus* (75%), than it is known for Rhabditida (83.5-97.7% identity between species) (Kasimatis and Phillips 2018). Our results showed that MSPs of the representatives of all three subclasses, Enoplia, Dorylaimia and Rhabditida, are moderately similar. The lack of highly homologous MSPs between different genera of Dorylaimia does not allow discussing MSP phylogeny in this group and requires detailed phylogenetic study using additional MSP samples from diverse taxa of this subclass. The fact that the similarity of MSP sequences between representatives of three nematode subclasses is ranged from 48 to 75% show that MSPs in Nematoda are less conserved proteins by sequences than it has been expected earlier. MSP sequences retained high identity during approximate 500 million-year evolution withing

order Rhabditida (Blaxter 2009) revealing protein sequence hyper-conservation (Kasimatis and Phillips 2018). Nevertheless, MSP sequence hyper-conservation is not the case for phylum Nematoda as a whole. This difference in MSPs variability between two major clades of nematodes correlates well with sperm diversity which is very wide in Enoplea but relatively low in Chormadorea, especially in the order Rhabditida where sperm patterns are enormously uniform (Justine and Jamieson 1999; Slos et al. 2020; Yushin and Malakhov 2014).

In summary, we found the first evidences that *E. brevis* spermatozoa use MSP-based locomotion and suggest that it may be the case for other species of Enoplia. Though, the early evolution of nematodes is still unresolved due to controversy in different phylogenetic analyses (Smythe et al. 2019), it is known that Enoplia is one of early-branching group, which reveals presumably ancestral features among nematodes (Bik et al. 2010; Blaxter and Koutsovoulos 2015; Felix 2004; Holterman et al. 2006; Joshi and Rothman 2005; Malakhov 1994, 1998; Rusin and Malakhov 1998; Schulze and Schierenberg 2011; Smythe et al. 2019; van Megen et al. 2009; Voronov 1999; Voronov and Panchin 1998; Yushin and Malakhov 2004). More basal phylogenetic position of Enoplia in relation to Chromadorea should give a contribution to understanding of origin and evolution of nematode sperm motility based of MSP function.

References

Afgan, E., Baker, D., Batut, B., van den Beek, M., Bouvier, D., Cech, M., et al. (2018). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Research, 46*(W1), W537-W544, doi:10.1093/nar/gky379.

Bik, H. M., Lambshead, P. J. D., Thomas, W. K., & Lunt, D. H. (2010). Moving towards a complete molecular framework of the Nematoda: a focus on the Enoplida and early-branching clades. *BMC Evolutionary Biology, 10*, 353, doi:10.1186/1471-2148-10-353.

Blaxter, M. (2009). Nematodes (Nematoda). In S. B. Hedges, & S. Kumar (Eds.), *The Timetree of Life* (pp. 247-250). New York: Oxford University Press.

Blaxter, M., & Koutsovoulos, G. (2015). The evolution of parasitism in Nematoda. *Parasitology, 142*, S26-S39, doi:10.1017/s0031182014000791.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics, 30*(15), 2114-2120, doi:10.1093/bioinformatics/btu170.

Chu, D. S., & Shakes, D. C. (2013). Spermatogenesis. *Advances in Experimental Medicine and Biology, 757*, 171-203, doi:10.1007/978-1-4614-4015-4_7.

De Ley, P., & Blaxter, M. (2002). Systematic position and phylogeny. In D. L. Lee (Ed.), *The Biology of Nematodes* (pp. 1-30). London and New York: Taylor & Francis.

del Castillo-Olivares, A., & Smith, H. E. (2008). Critical contact residues that mediate polymerization of nematode major sperm protein. *Journal of Cellular Biochemistry, 104*(2), 477-487, doi:10.1002/jcb.21636.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., et al. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research, 36*(Web Server issue), W465-W469, doi:10.1093/nar/gkn180.

Do, C. B., Mahabhashyam, M. S., Brudno, M., & Batzoglou, S. (2005). ProbCons: Probabilistic consistency-based multiple sequence alignment. *Genome Research, 15*(2), 330-340, doi:10.1101/gr.2821705.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research, 32*(5), 1792-1797, doi:10.1093/nar/gkh340.

El-Gebali, S., Mistry, J., Bateman, A., Eddy, S. R., Luciani, A., Potter, S. C., et al. (2019). The Pfam protein families database in 2019. *Nucleic Acids Research, 47*(D1), D427-D432, doi:10.1093/nar/gky995.

Ellis, R. E., & Stanfield, G. M. (2014). The regulation of spermatogenesis and sperm function in nematodes. *Seminars in Cell & Developmental Biology, 29*, 17-30, doi:10.1016/j.semcdb.2014.04.005.

Felix, M.-A. (2004). Developmental biology of nematodes–what we learn from Caenorhabditis elegans. In Z. X. Chen, S. Y. Chen, & D. W. Dickson (Eds.), *Nematology–Advances and Perspectives* (Vol. 1, pp. 71-174). Wallingford, UK: CABI Publishing.

Gasteiger, E., Hoogland, C., Gattiker, A., Wilkins, M. R., Appel, R. D., & Bairoch, A. (2005). Protein identification and analysis tools on the ExPASy server. In J. M. Walker (Ed.), *The Proteomics Protocols Handbook* (pp. 571-607): Humana Press.

Heger, P., Kroiher, M., Ndifon, N., & Schierenberg, E. (2010). Conservation of MAP kinase activity and MSP genes in parthenogenetic nematodes. *BMC Developmental Biology, 10*, 51, doi:10.1186/1471-213X-10-51.

Höglund, J., Engstrom, A., Morrison, D. A., Mineur, A., & Mattsson, J. G. (2008). Limited sequence variation in the major sperm protein 1 (MSP) gene within populations and species of the genus Dictyocaulus (Nematoda). *Parasitology Research, 103*(1), 11-20, doi:10.1007/s00436-008-0877-8.

Hojas, R. M., & Post, R. J. (2000). Regional genetic variation in the major sperm protein genes of Onchocerca volvulus and Mansonella ozzardi (Nematoda: Filarioidea). *International Journal of Parasitology, 30*(14), 1459-1465.

Holterman, M., van der Wurff, A., van den Elsen, S., van Megen, H., Bongers, T., Holovachov, O., et al. (2006). Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown Clades. *Molecular Biology and Evolution, 23*(9), 1792-1800, doi:10.1093/molbev/msl044.

Joshi, P. M., & Rothman, J. H. (2005). Nematode gastrulation: Having a BLASTocoel! *Current Biology, 15*(13), R495-R498, doi:10.1016/j.cub.2005.06.030.

Justine, J.-L. (2002). Male and female gametes and fertilisation. In D. L. Lee (Ed.), *The Biology of Nematodes.* (pp. 73-120). London and New York: Taylor & Francis.

Justine, J.-L., & Jamieson, B. G. M. (1999). Nematoda. In B. G. M. Jamieson (Ed.), *Reproductive Biology of Invertebrates* (Vol. 9, Part B, pp. 183-266). New Delhi & Calcutta: Oxford & IBH.

Kasimatis, K. R., & Phillips, P. C. (2018). Rapid gene family evolution of a nematode sperm protein despite sequence hyper-conservation. *G3: Genes, Genomes, Genetics, 8*(1), 353-362, doi:10.1534/g3.117.300281.

King, K. L., Stewart, M., & Roberts, T. M. (1994). Supramolecular assemblies of the Ascaris suum major sperm protein (MSP) associated with amoeboid cell motility. *Journal of Cell Science, 107 (Pt 10)*, 2941-2949.

King, K. L., Stewart, M., Roberts, T. M., & Seavy, M. (1992). Structure and macromolecular assembly of two isoforms of the major sperm protein (MSP) from the amoeboid sperm of the nematode, Ascaris suum. *Journal of Cell Science, 101 (Pt 4)*, 847-857.

Klass, M. R., & Hirsh, D. (1981). Sperm isolation and biochemical analysis of the major sperm protein from Caenorhabditis elegans. *Developmental Biology, 84*(2), 299-312, doi:10.1016/0012-1606(81)90398-5.

Lak, B., Yushin, V. V., Slos, D., Claeys, M., Decraemer, W., & Bert, W. (2015). High-pressure freezing and freeze-substitution fixation reveal the ultrastructure of immature and mature spermatozoa of the plant-parasitic nematode Trichodorus similis (Nematoda; Triplonchida; Trichodoridae). *Micron, 77*, 25-31, doi:10.1016/j.micron.2015.05.012.

Malakhov, V. V. (1994). *Nematodes. Structure, development, classification and phylogeny*. Washington, USA: Smithsonian Institution Press.

Malakhov, V. V. (1998). Embryological and histological peculiarities of the order Enoplida, a primitive group of nematodes. *Russian Journal of Nematology, 6*(1), 41-46.

Marcello, M. R., Singaravelu, G., & Singson, A. (2012). Fertilization. *Advances in Experimental Medicine and Biology, 757*, 321-350, doi:10.1007/978-1-4614-4015-4_11.

Miyata, M., Robinson, R. C., Uyeda, T. Q. P., Fukumori, Y., Fukushima, S. I., Haruta, S., et al. (2020). Tree of motility - A proposed history of motility systems in the tree of life. *Genes to Cells, 25*(1), 6-21, doi:10.1111/gtc.12737.

Morrow, E. H. (2004). How the sperm lost its tail: the evolution of aflagellate sperm. *Biological Reviews of the Cambridge Philosophical Society, 79*(4), 795-814, doi:10.1017/s1464793104006451.

Poinar, G. O., & Hess-Poinar, R. T. (1993). The fine-structure of Gastromermis sp (Nematoda, Mermithidae) sperm. *Journal of Submicroscopic Cytology and Pathology, 25*(3), 417-431.

Roberts, T. M., & Stewart, M. (2000). Acting like actin. The dynamics of the nematode major sperm protein (msp) cytoskeleton indicate a push-pull mechanism for amoeboid cell motility. *Journal of Cell Biology, 149*(1), 7-12, doi:10.1083/jcb.149.1.7.

Roberts, T. M., & Stewart, M. (2012). Role of major sperm protein (MSP) in the protrusion and retraction of Ascaris sperm. *International Review of Cell and Molecular Biology, 297*, 265-293, doi:10.1016/b978-0-12-394308-8.00007-8.

Rusin, L. Y., & Malakhov, V. V. (1998). Free-living marine nematodes possess no eutely. *Doklady Biological Sciences, 361*, 331-333.

Ryan, G. L., Petroccia, H. M., Watanabe, N., & Vavylonis, D. (2012). Excitable actin dynamics in lamellipodial protrusion and retraction. *Biophysical Journal, 102*(7), 1493-1502, doi:10.1016/j.bpj.2012.03.005.

Schmidt-Rhaesa, A. (1997/98). Phylogenetic relationships of the Nematomorpha - a discussion of current hypotheses. *Zoologischer Anzeiger, 236*, 203-216.

Schulze, J., & Schierenberg, E. (2011). Evolution of embryonic development in nematodes. *EvoDevo, 2*(1), 18, doi:10.1186/2041-9139-2-18.

Scott, A. L., Dinman, J., Sussman, D. J., & Ward, S. (1989). Major sperm protein and actin genes in free-living and parasitic nematodes. *Parasitology, 98 Pt 3*, 471-478, doi:10.1017/s0031182000061564.

Sepsenwol, S., Ris, H., & Roberts, T. M. (1989). A unique cytoskeleton associated with crawling in the amoeboid sperm of the nematode, Ascaris suum. *Journal of Cell Biology, 108*(1), 55-66, doi:10.1083/jcb.108.1.55.

Shepherd, A. M. (1981). Interpretation of sperm development in nematodes. *Nematologica, 27*(1), 122, doi:10.1163/187529281X00151.

Singaravelu, G., & Singson, A. (2011). New insights into the mechanism of fertilization in nematodes. *International Review of Cell and Molecular Biology, 289*, 211-238, doi:10.1016/b978-0-12-386039-2.00006-7.

Slos, D., Yushin, V. V., Claeys, M., Ivanova, E. S., Kosaka, H., & Bert, W. (2020). Structure, development, and evolutive patterns of spermatozoa in rhabditid nematodes (Nematoda: Rhabditida). *Journal of Morphology, 281*(11), 1411-1435, doi:10.1002/jmor.21255.

Smith, H. E. (2014). Nematode sperm motility. *WormBook*, 1-15, doi:10.1895/wormbook.1.68.2.

Smythe, A. B., Holovachov, O., & Kocot, K. M. (2019). Improved phylogenomic sampling of free-living nematodes enhances resolution of higher-level nematode phylogeny. *BMC Evolutionary Biology, 19*(1), 121, doi:10.1186/s12862-019-1444-x.

Strube, C., Buschbaum, S., & Schnieder, T. (2009). Molecular characterization and real-time PCR transcriptional analysis of Dictyocaulus viviparus major sperm proteins. *Parasitology Research, 104*(3), 543-551, doi:10.1007/s00436-008-1228-5.

Tarr, D. E. K., & Scott, A. L. (2005). MSP domain proteins. *Trends in Parasitology, 21*(5), 224-231, doi:10.1016/j.pt.2005.03.009.

van Megen, H., van den Elsen, S., Holterman, M., Karssen, G., Mooyman, P., Bongers, T., et al. (2009). A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences. *Nematology, 11*, 927-S927, doi:10.1163/156854109x456862.

Voronov, D. A. (1999). The embryonic development of Pontonema vulgare (Enoplida : Oncholaimidae) with a discussion of nematode phylogeny. *Russian Journal of Nematology, 7*(2), 105-114.

Voronov, D. A., & Panchin, Y. V. (1998). Cell lineage in marine nematode Enoplus brevis. *Development, 125*(1), 143-150.

Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M., & Barton, G. J. (2009). Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics, 25*(9), 1189-1191, doi:10.1093/bioinformatics/btp033.

Yushin, V. V., Afanasiev-Grigoriev, A. G., & Malakhov, V. V. (2014). The male gonad of the marine nematode Enoplus: no single distal tip cell but multiple uniform epithelial cells. *Invertebrate Zoology, 11*(2), 361-372, doi:10.15298/invertzool.11.2.07.

Yushin, V. V., Claeys, M., & Bert, W. (2016). Ultrastructural immunogold localization of major sperm protein (MSP) in spermatogenic cells of the nematode Acrobeles complexus (Nematoda, Rhabditida). [Article]. *Micron, 89*, 43-55, doi:10.1016/j.micron.2016.07.004.

Yushin, V. V., & Malakhov, V. V. (1994). Ultrastructure of sperm cells in the female gonoduct of free-living marine nematodes from genus Enoplus (Nematoda: Enoplida). *Fundamental and Applied Nematology, 17*(6), 513-520.

Yushin, V. V., & Malakhov, V. V. (2004). Spermatogenesis and nematode phylogeny. In R. C. Cook, & D. J. Hunt (Eds.), *Proceeding of the Fourth International Congress of Nematology* (Vol. 2, pp. 655-665, Nematology Monographs and Perspectives).

Yushin, V. V., & Malakhov, V. V. (2014). The origin of nematode sperm: Progenesis at the cellular level. *Russian Journal of Marine Biology, 40*(2), 71-81, doi:10.1134/S1063074014020114.

Zograf, J. K. (2014). Ultrastructure of spermatogenesis and sperm of the free-living soil nematode Panagrellus redivivus (Rhabditida: Panagrolaimidae). *Russian Journal of Nematology, 22*(1), 39-48.

Figure legends

Fig. 1 Phylogeny of nematodes and MSP-based sperm motility. Phylogenetic relationships within phylum Nematoda derived primarily from SSU rDNA sequence data are given according to De Ley and Blaxter (De Ley and Blaxter 2002). Suborders of Rhabditida order, in which representatives highly homologous MSPs are found at DNA, RNA or protein levels, are marked by underlining. Taxa whose species used in this study are marked with asterisks. Orders Trefusiida, Isolaimida, Dioctophymatida, Muspiceida, Marimermithida and Desmoscolecida are not shown in this tree.

Fig. 2 Western blot analysis of MSP in *P. redivivus*. In adult animals, MSP is detected as double band with approximate weight 15 and 16 kDa. Both male and female samples reveal MSP signal, because the latter include mated females. α-Tubulin was used as a loading control (approximate weight 55 kDa).

Fig. 3 Immunolocalization of MSP in *P. redivivus* sperm. **a** Immature spermatozoa extracted from male. MSP localizes in granules. In some cells, MSP has strongest signals in the periphery (arrowheads) (scale bar 10 µm). **b** Chain of activated spermatozoa extracted from female. MSP has punctate and fibrillar pattern of distribution in pseudopodia that marked by arrows. Selected area is given in higher magnification (scale bar 10 µm, magnified area 2 µm).

Fig. 4 Western blot analysis of MSP in *E. brevis*. **a** MSP has unusual mobility in gel and is found as protein with weight 36-38 kDa. Both male and female samples reveal MSP signal, because the latter include inseminated females. α-Tubulin was used as a loading control (approximate weight 55 kDa). **b** Peptide competition assay confirms reactivity of anti-MSP antibodies with protein band of 36-38 kDa.

Fig. 5 Immunolocalization of MSP in *E. brevis* sperm. **a** Immature spermatozoon from male. MSP concentrates in large granules (scale bar 10 µm). **b** Spermatozoon recovered from male and partially activated by 10 min incubation in sea water. MSP revealed more diffuse pattern with appearance of longitudinal fibrillar structures. Selected area is given in higher magnification (scale bar 10 µm, magnified area 2 µm). **c** Mature spermatozoon from female. Most of MSP signal is found in pseudopod (scale bar 10 µm).

Fig. 6 Putative MSPs that are most similar to peptide antigen. **a** *P. redivivus* MSPs aligned with peptide antigen. Protein sequences (Pan_g61.t1, Pan_g6018.t1, Pan_g6424.t1, Pan_g9068.t1, Pan_g19433.t1 and Pan_g21178.t1) were found by Blast using peptide antigen as query in WormBase Parasite (https://parasite.wormbase.org). **b** *E. brevis* MSPs aligned with peptide antigen. Multiple alignment was performed using ProbCons and visualized in Jalview with BLOSUM62 color scheme.

Fig. 7 Multiple alignment and phylogenetic relationships of MSPs. **a** Multiple alignment of MSPs from 13 taxa of three subclasses of Nematoda. MSPs of six Rhabditida, four Dorylaimia and one Enoplia species were used. Multiple alignment was performed using ProbCons and visualized in Jalview with BLOSUM62 color scheme. MSP sequences of nematodes and MSP domains of *Saccharomyces cerevisiae* and *Homo sapiens* used in multiple alignment were downloaded from following databases, GenBank (https://www.ncbi.nlm.nih.gov) (*A. suum*: CAA63933.1, *Dictyocaulus viviparus*: AAB27962.2, *Strongyloides ratti*: XP_024503659.1, *Trichuris trichiura*: CDW57515.1, *Trichinella nativa*: OUC40810.1, *Trichinella pseudospiralis*: KRX99722.1, *Trichinella papuae*: KRZ74366.1, *S. cerevisiae* MSP domain of Scs22p: AJP97989.1), WormBase Parasite (https://parasite.wormbase.org) (*P. redivivus*: Pan_g9068.t1) and UniProt (https://www.uniprot.org) (*C. elegans*: P53017, *Onchocerca volvulus*: P13262.3, *H. sapiens* MSP

domain of VAPA: Q9P0L0). **b** Maximum likelihood tree with SH-aLRT branch support. Only significant values (≥0.8) are shown. MSP domains of *S. cerevisiae* (Scs22p) and *H. sapiens* (VAPA) proteins were chosen as an outgroup.

Figure 1

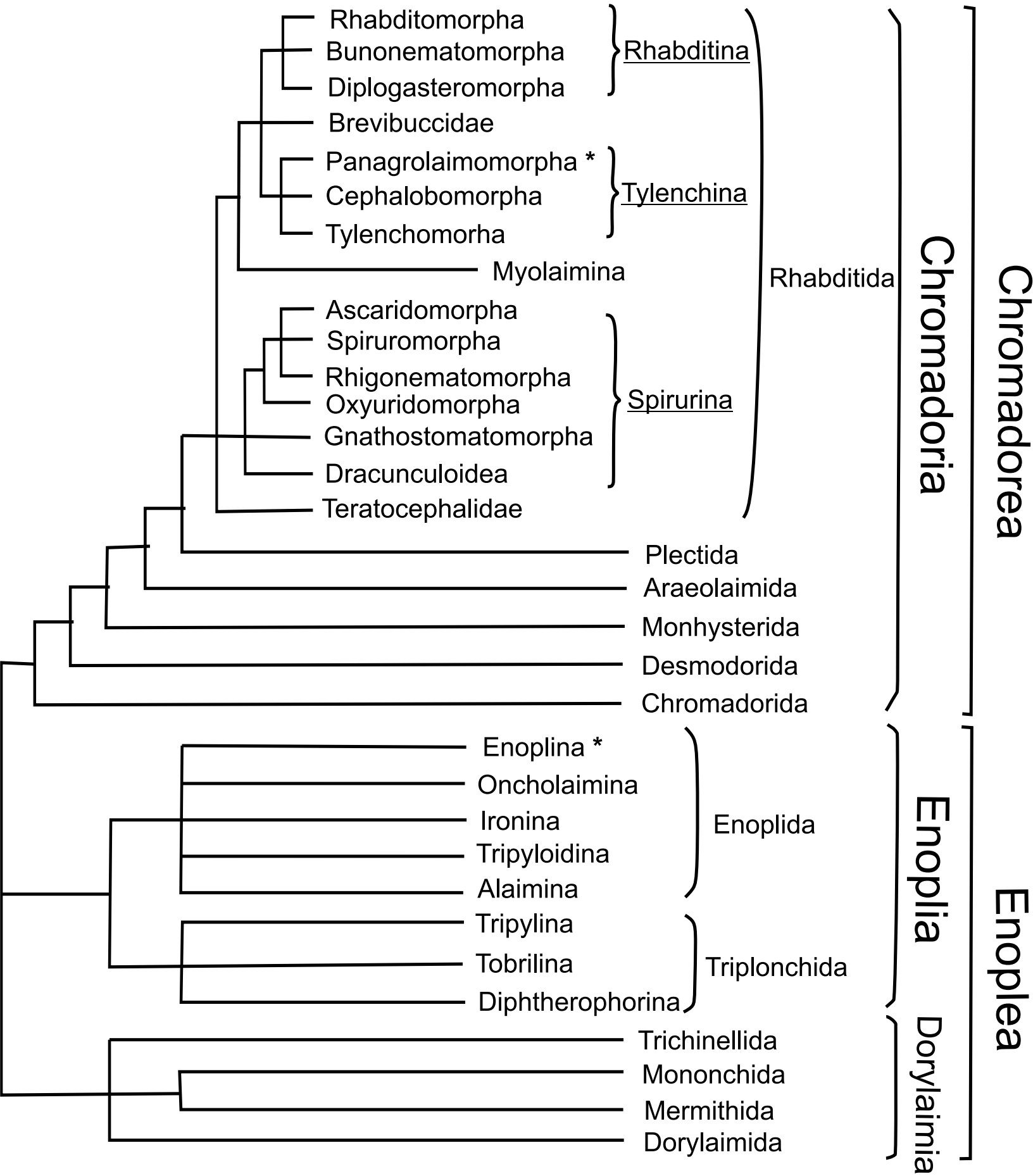

Rhabditomorpha
Bunonematomorpha
Diplogasteromorpha
Rhabditina
Brevibuccidae
Panagrolaimomorpha *
Cephalobomorpha
Tylenchomorha
Tylenchina
Myolaimina
Ascaridomorpha
Spiruromorpha
Rhigonematomorpha
Oxyuridomorpha
Gnathostomatomorpha
Dracunculoidea
Spirurina
Teratocephalidae
Rhabditida
Plectida
Araeolaimida
Monhysterida
Desmodorida
Chromadorida
Chromadoria
Enoplina *
Oncholaimina
Ironina
Tripyloidina
Alaimina
Enoplida
Tripylina
Tobrilina
Diphtherophorina
Triplonchida
Enoplia
Trichinellida
Mononchida
Mermithida
Dorylaimida
Dorylaimia
Chromadorea
Enoplea

Figure 2

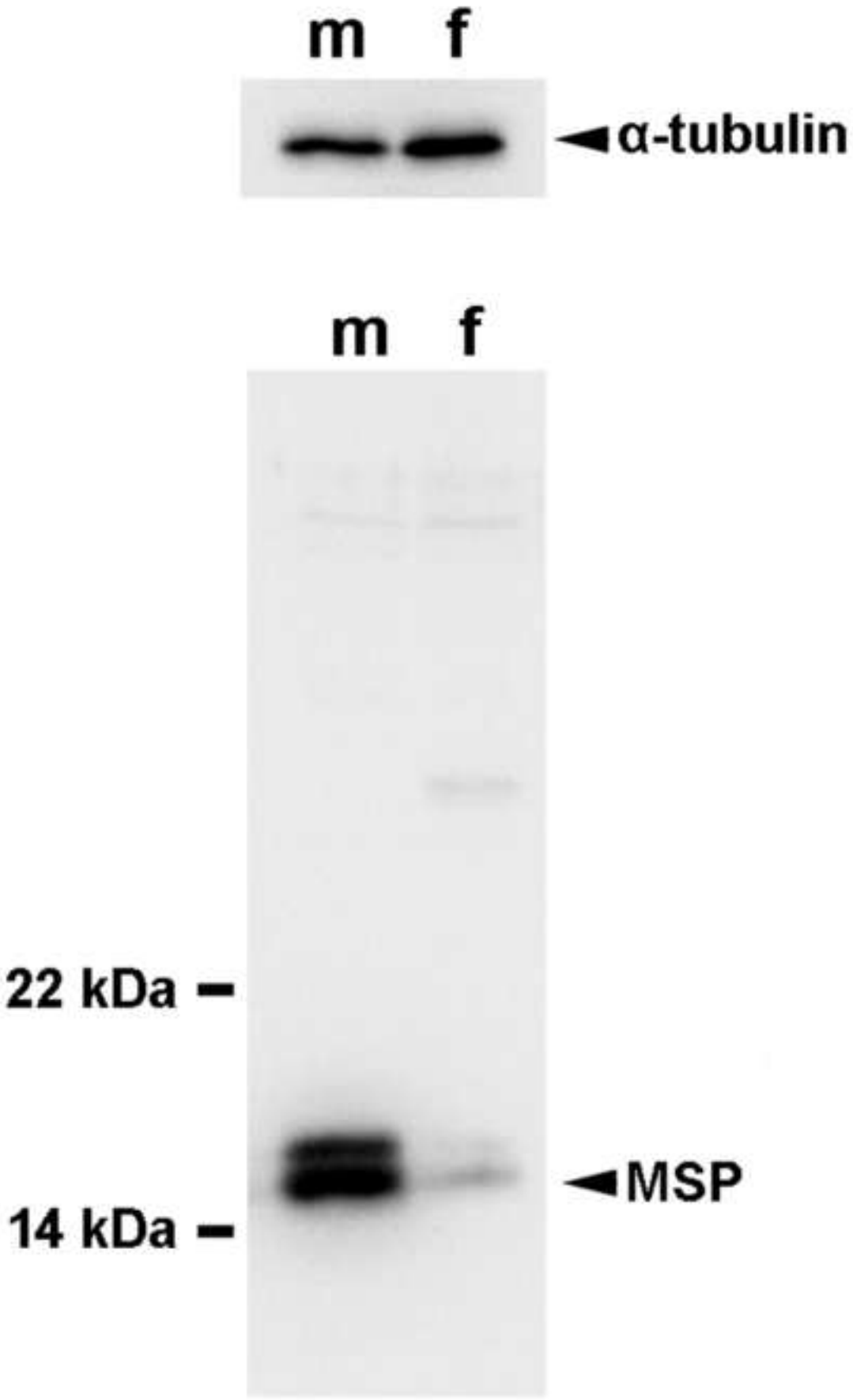


Figure 3

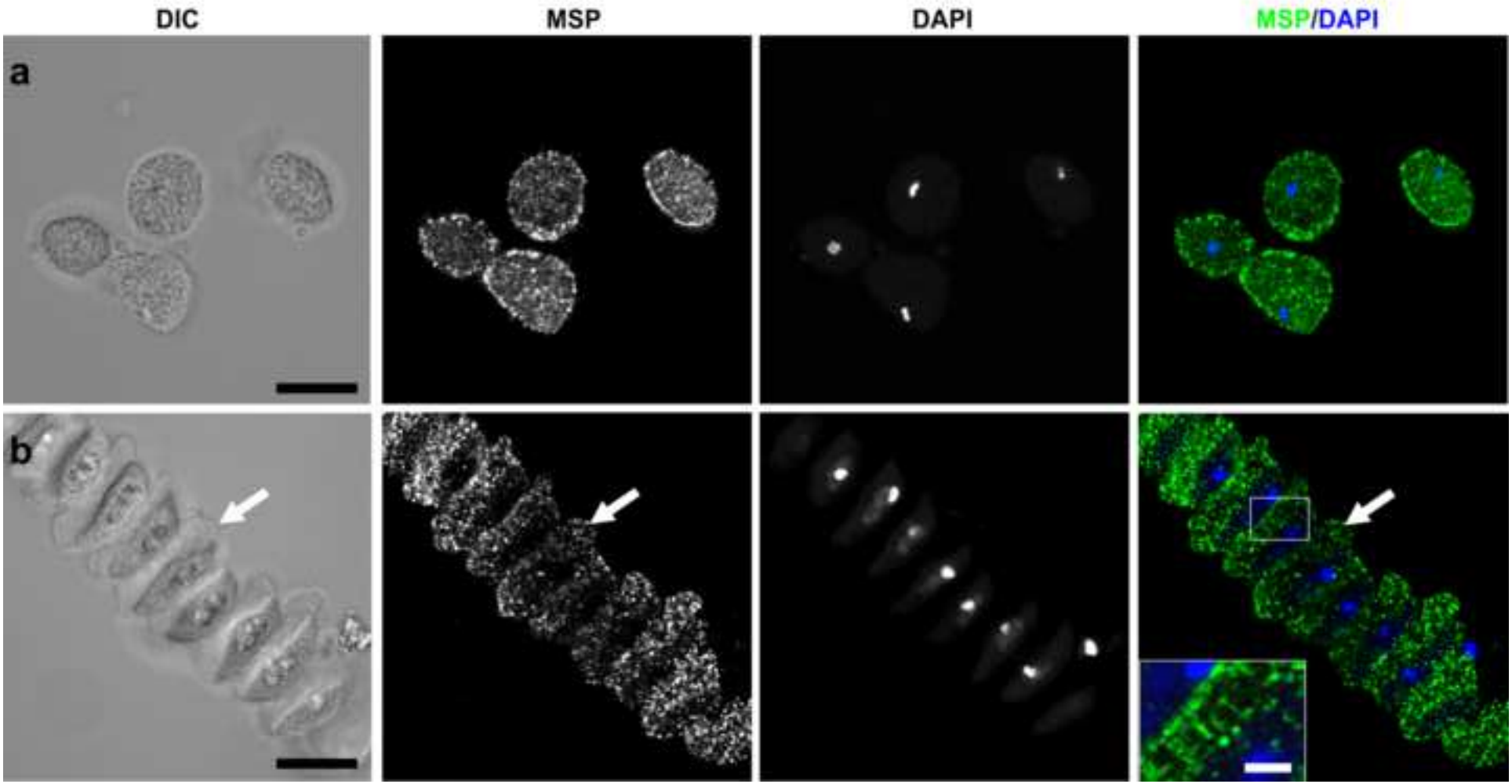


Figure 4

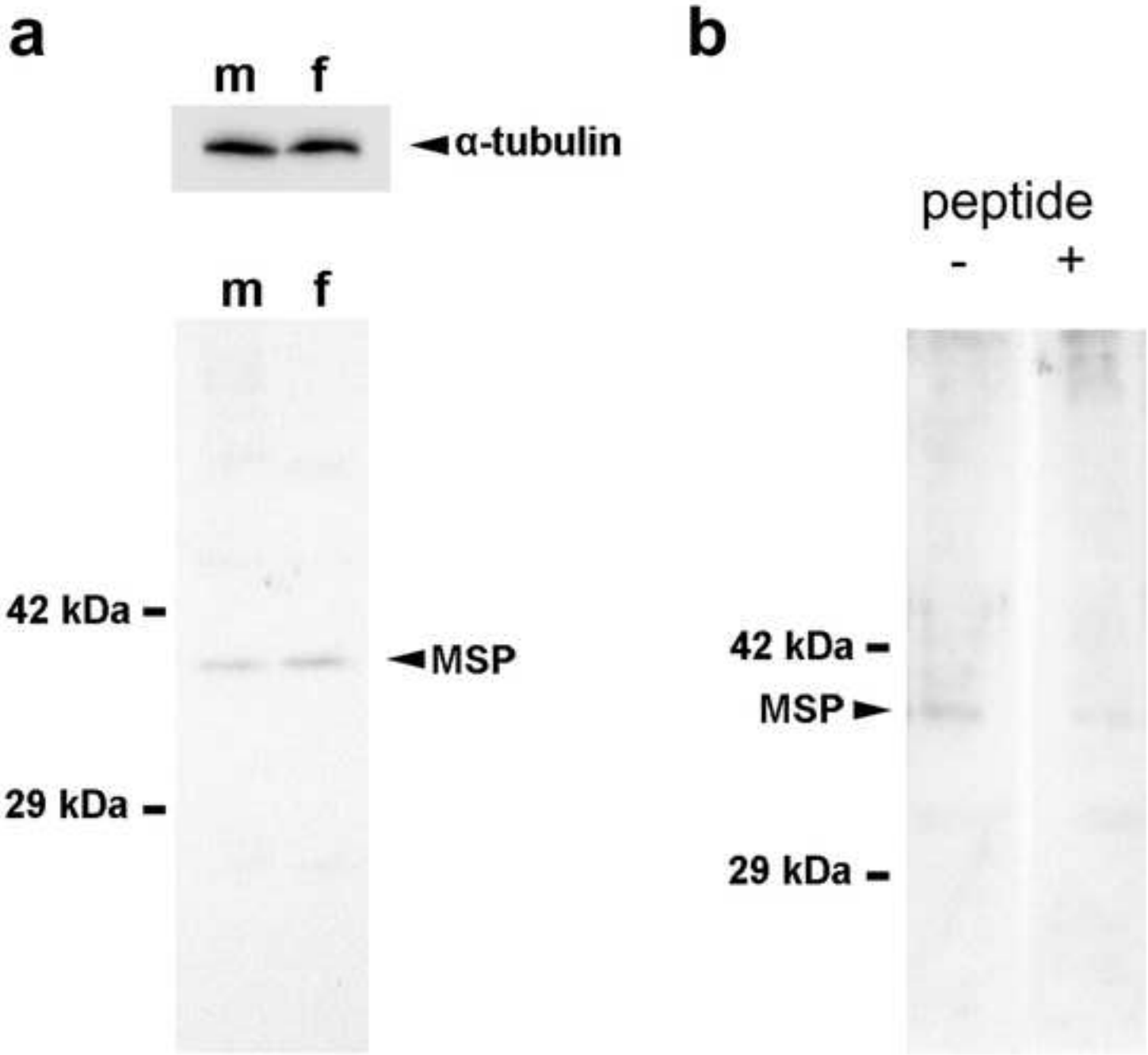


Figure 5

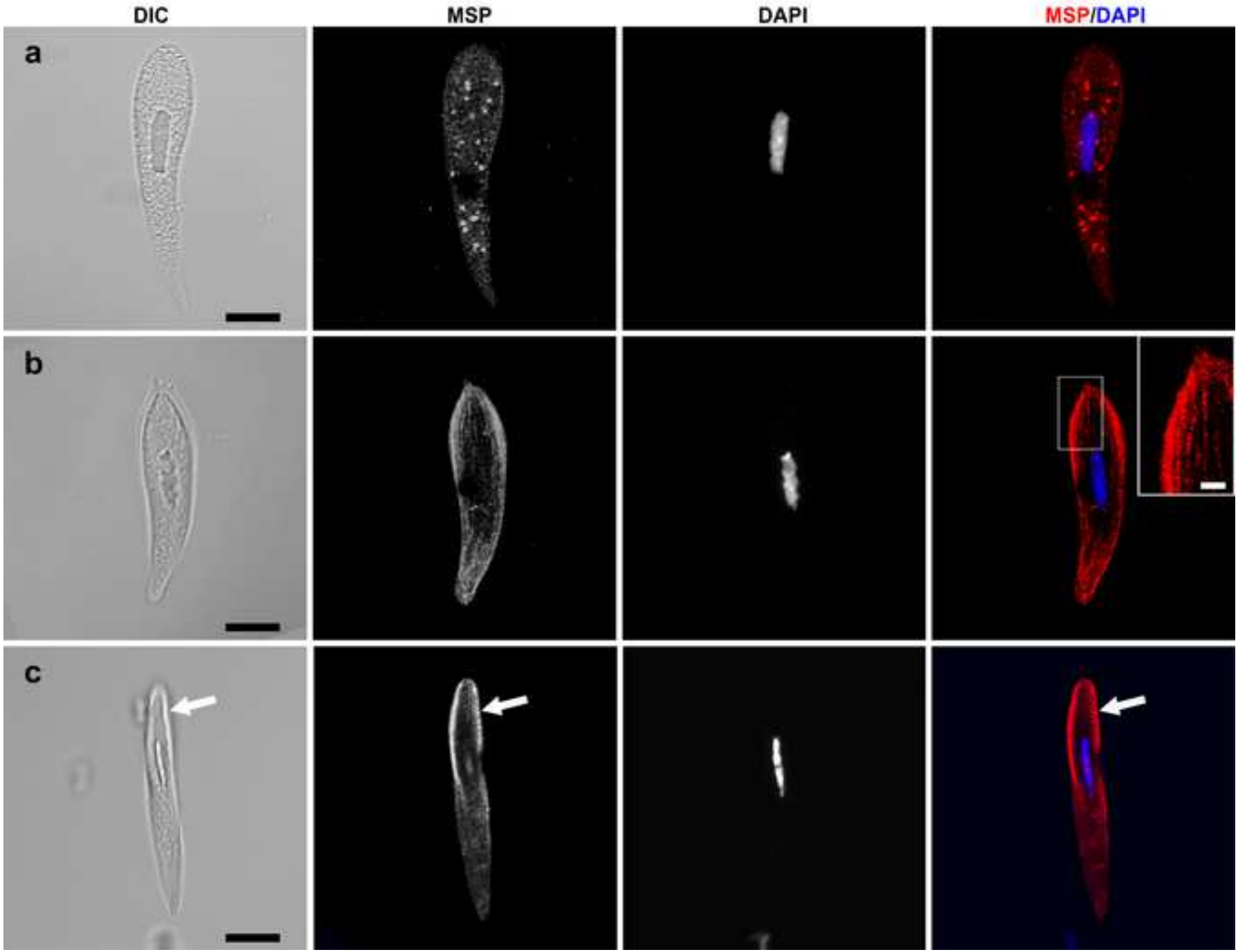


Figure 6

a

```
peptide antigen   1  ---------------------------------------------IKTTNMKRLGVDPPCGVLDP  20
Pan_g61.t1        1  MAQSVPPGDIGTQPGTKIVFNAPYDDKHTYHIKITNSGGRRIGWAIKTTNMKRLGVDPPCGVLDP  65
Pan_g6018.t1      1  MAQSVPPGDIGTQPGTKIVFNAPYDDKHTYHIKITNAGGRRIGWAIKTTNMKRLGVDPPCGVLDP  65
Pan_g6424.t1      1  MAQSVPPGDIGTQPGTKIVFNAPYDDKHTYHIKITNSGGRRIGWAIKTTNMKRLGVDPPCGVLDP  65
Pan_g9068.t1      1  MAQSVPPGDIGTQPGTKIVFNAPYDDKHTYHIKITNSGGRRIGWAIKTTNMKRLGVDPPCGVLDP  65
Pan_g19433.t1     1  MAQSIPPGDIGTQPGTKIVFNAPYDDKHTYHIKIINSGGRRIGWAIKTTNMKRLGVDPPSGVLDP  65
Pan_g21178.t1     1  MAQSVPPGDIGTQPGTKIVFNAPYDDKHTYHIKITNSGGRRIGWAIKTTNMKRLGVDPPCGVLDP  65

peptide antigen  21  KE------------------------------------------------------------  22
Pan_g61.t1       66  KENVLMAVSCDAFDAAAEDTNNDRITIEWTNTPDGAAKTFRREWFQGDGMVRRKNLPIEYNP  127
Pan_g6018.t1     66  KENVLMAVSCDSFKYGEEDTNNDRITIEWTNTPDGAAKTFRREWFQGDGMVRRKNLPIEYNP  127
Pan_g6424.t1     66  KENVLMAVSCDAFDPAAEDTNNDRITIEWTNTPDGAAKTFRREWFQGDGMVRRKNLPIEYNP  127
Pan_g9068.t1     66  KENVLMAVSCDAFKFGEEDTNNDRITIEWTNTPDGAAKTFRREWFQGDGMVRRKNLPIEYNP  127
Pan_g19433.t1    66  KENVLMAVSCDAFQFGQEDTNNDRITIEWTNTPDGAAKTFRREWFQGDGMVRRKNLPIEYNP  127
Pan_g21178.t1    66  KENVLMAVSCDAFKFGEEDTNNDRITIEWTNTPDGAAKTFRREWFQGDGMVRRKNLPIEYNP  127
```

b

```
peptide antigen   1  ------------------------------------------IKTTNMKRLGVDPPCGVLDPKE-  22
MSP124-1          1  MTMPGEIKTQPENKLIFGAPFDAPVTVSLRATNAGGKKIGWAIKTTNMRRFSVEPGMGTMEPKAH  65
MSP124-2          1  MTMPGDVKTQPENKLIFGAPFDAPVTVSLRATNAGGKKIGWAIKTTNMRRFSVEPGMGTMEPKAH  65
MSP124-3          1  MTMPGEIKTQPENKLIFGAPFDAPVTVSLRATNAGGKKIGWAIKTTNMRRFSVEPGMGTMEPKAH  65

peptide antigen      -----------------------------------------------------------
MSP124-1         66  VNLSVTCNPFDIGNEDISNDRITIEWTDTPAGAGDKFQREWFQGSGIIRRKVINCEYNV  124
MSP124-2         66  VNLSVTCNPFDIGNEDISNDRITIEWTDTPAGAGDKFQREWFQGSGIIRRKVINCEYNV  124
MSP124-3         66  VNLSVTCNPFDIGNEDISNDRITIEWTDTPAGAGNKFQREWFQGSGIIRRKVINCEYNV  124
```

Figure 1

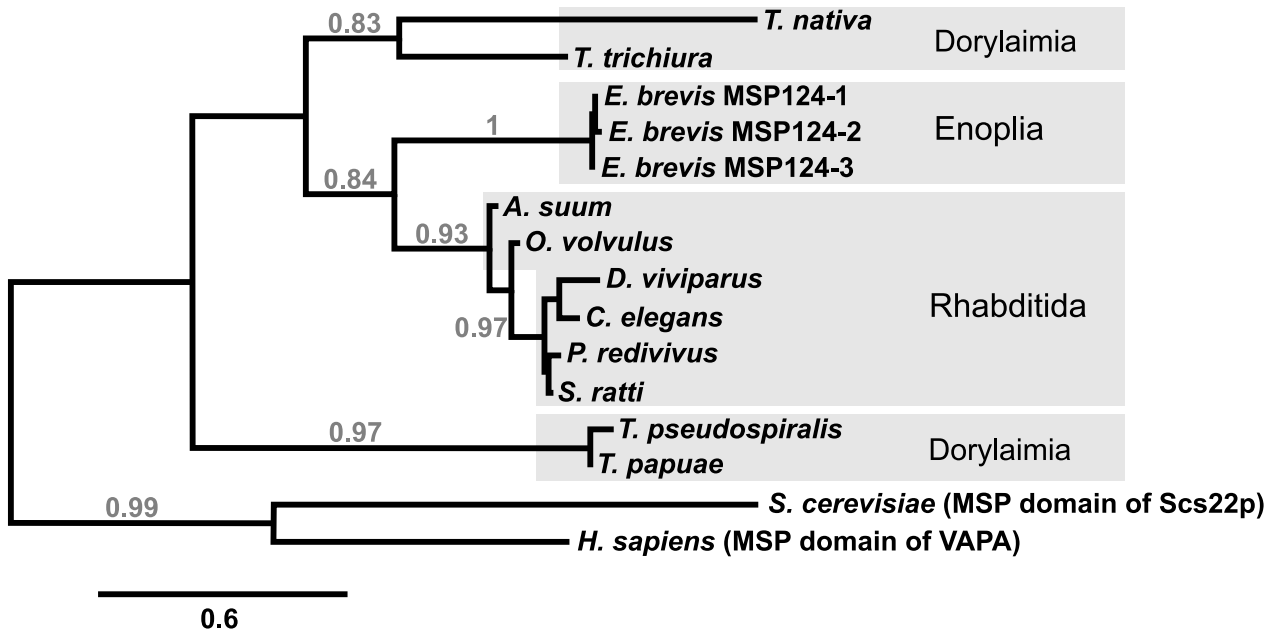
a

```
S. cerevisiae MSP domain of Scs22p  1 MR----------IVP-EKLLFKAPLNKQSTEYIKLENDGEKRVIFKVRTS  39
H. sapiens MSP domain of VAPA       1 IL---------VLDPPTDLKFKGPFTDVVTTNLKLRNPSDRKVCFKVKTT  41
T. pseudospiralis                   1 MRNE-IPHDIIIEPSTCLFFNGPFDEAKSQSVRMRNPGGQAVAWAIKTN  48
T. papuae                           1 MRNE-IPHDITIEPSTCLFFNGPFDETKSQAVRMRNPGGQAIAWAIKTN  48
T. nativa                           1 MPKP-IPSELKTSPAERIVFNAPFEEKKNYPFSIINNGKEKIAYMIKLS  48
T. trichiura                        1 MAKQ-MPADIKTEPLDKLYFNAPFKEKKNYKIAVTNTGAKPLAWCVKST  48
E. brevis MSP124-1                  1 MT---MPGEIKTQPENKLIFGAPFDAPVTVSLRATNAGGKKIGWAIKTT  46
E. brevis MSP124-2                  1 MT---MPGDVKTQPENKLIFGAPFDAPVTVSLRATNAGGKKIGWAIKTT  46
E. brevis MSP124-3                  1 MT---MPGEIKTQPENKLIFGAPFDAPVTVSLRATNAGGKKIGWAIKTT  46
C. elegans                          1 MAQSVPPGDIQTQPGTKIVFNAPYDDKHTYHIKVINSSARRIGYGIKTT  49
D. viviparus                        1 MAS-VPPGDINTQPNSKIVFNAPYDDKHTYHIKIINASGRRIGWAIKTT  48
P. redivivus                        1 MAQSVPPGDIGTQPGTKIVFNAPYDDKHTYHIKITNSGGRRIGWAIKTT  49
S. ratti                            1 MAQSVPPGDIQTQPGTKIVFNAPYDDKHTYHIKITNSGGRRIGWAIKTT  49
O. volvulus                         1 MAQSVPPGDIHTQPGSKIVFNAPYDDKHTYHINITNAGGRRIGWAIKTT  49
A. suum                             1 MAQSVPPGDINTQPSQKIVFNAPYDDKHTYHIKITNAGGRRIGWAIKTT  49

S. cerevisiae MSP domain of Scs22p 40 APTKYCVRPNVAIIGAHESVNVQIVFLGLPKSTADDEMDQKRDKFLIVT  88
H. sapiens MSP domain of VAPA      42 APRRYCVRPNSGIIDPGSTVTVSVMLQPFDYDPN----EKSKHKFMVQT  86
T. pseudospiralis                  49 NRARLNAEPPGGILQAGTQIAVNIISAPVRRAHQVG--KQESDSIIFEW  95
T. papuae                          49 NRARLNAEPPGGILQAGTQIVVNIISAPVRRAHQVG--KQENDSIIFEW  95
T. nativa                          49 NEMRTMCEPSHGVLNPGENIWIRVHLEEFKPTVE----NTQPNTLTIEY  93
T. trichiura                       49 NVSRISFDPSAGVLDANETFMFTAVTEVFEPTPE----NLKQDQITIEW  93
E. brevis MSP124-1                 47 NMRRFSVEPGMGTMEPKAHVNLSVTCNPFDIGNE----DISNDRITIEW  91
E. brevis MSP124-2                 47 NMRRFSVEPGMGTMEPKAHVNLSVTCNPFDIGNE----DISNDRITIEW  91
E. brevis MSP124-3                 47 NMRRFSVEPGMGTMEPKAHVNLSVTCNPFDIGNE----DISNDRITIEW  91
C. elegans                         50 NMKRLGVDPPCGVLDPKEAVLLAVSCDAFAFGQE----DTNNDRITVEW  94
D. viviparus                       49 NMKRLGVDPACGVLDPKEATLMAVSCDTFEYGRE----DTNNDRITVEW  93
P. redivivus                       50 NMKRLGVDPPCGVLDPKENVLMAVSCDAFKFGEE----DTNNDRITIEW  94
S. ratti                           50 NMKRLGVDPPSGVLDPKENVLMAVSCDAFAYGQE----DTNNDRITIEW  94
O. volvulus                        50 NMKRLGVDPPCGVLDPKENVLMAVSCDTFDATRE----DINNDRITIEW  94
A. suum                            50 NMRRLSVDPPCGVLDPKEKVLMAVSCDTFNAATE----DLNNDRITIEW  94

S. cerevisiae MSP domain of Scs22p 89 LPIPAAYQNV---------------EDGELLSDW 107
H. sapiens MSP domain of VAPA      87 IFAPPNTSDM---------------EAVWKEAKP 105
T. pseudospiralis                  96 CQVES--DIPFSIELLKGDALLRRRKIKIIYNP 126
T. papuae                          96 CQVES--DIPFSIDLLKGDALLRRRKIKIIYNP 126
T. nativa                          94 CFPPEGSDKNFNPSWFRLNVIIRRKHVALEFNA 126
T. trichiura                       94 ILAPDGEGRKFNREWMQRDVIVRRKHITVFYNP 126
E. brevis MSP124-1                 92 TDTPAGAGDKFQREWFQGSGIIRRKVINCEYNV 124
E. brevis MSP124-2                 92 TDTPAGAGDKFQREWFQGSGIIRRKVINCEYNV 124
E. brevis MSP124-3                 92 TDTPAGAGNKFQREWFQGSGIIRRKVINCEYNV 124
C. elegans                         95 TNTPDGAAKQFRREWFQGDGMVRRKNLPIEYNP 127
D. viviparus                       94 CNTPDGAAKQFRREWFQGDGMVRRKNLPIEYNP 126
P. redivivus                       95 TNTPDGAAKTFRREWFQGDGMVRRKNLPIEYNP 127
S. ratti                           95 TNTPDGAAKTFRREWFQGDGMVRRKNLPIEYNP 127
O. volvulus                        95 TNTPDGAAKQFRREWFQGDGMVRRKNLPIEYNL 127
A. suum                            95 TNTPDGAAKQFRREWFQGDGMVRRKNLPIEYNL 127
```

b



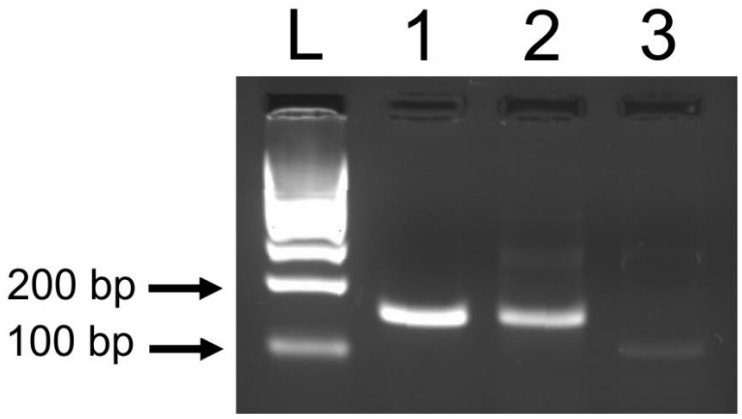


FIGURE S2 RT-PCR analysis of the presence of putative MSP-coding transcripts found in *E. brevis* transcriptome. Fragments corresponding to MSP mRNAs were amplified and then resolved in 3% SB gel, MSP124-1 (line 1, 162 bp), MSP124-2 (line 2, 166 bp) and MSP124-3 (line 3, 118 bp). Ladder is marked by L.

TABLE S1 Primers and conditions of amplification of MSP124 fragments by RT-PCR

transcript	Primer	Ta	cycles	size, bp
Msp124-1	F: AACTTGTCCGTCACCTGCAAC R: GCAGTTGATCACCTTCCGG	58	25	162
Msp124-2	F: AACTTGTCCGTCACCTGCAAC R: ATTCGCAGTTGATGACTTTCCTC	55	35	166
Msp124-3	F: AACTTGTCCGTCACCTGCAAC R: ATTCCCGCTGGAACTTGTTG	55	35	118

Note: to amplify fragments of all fragments the same forward primer was used.

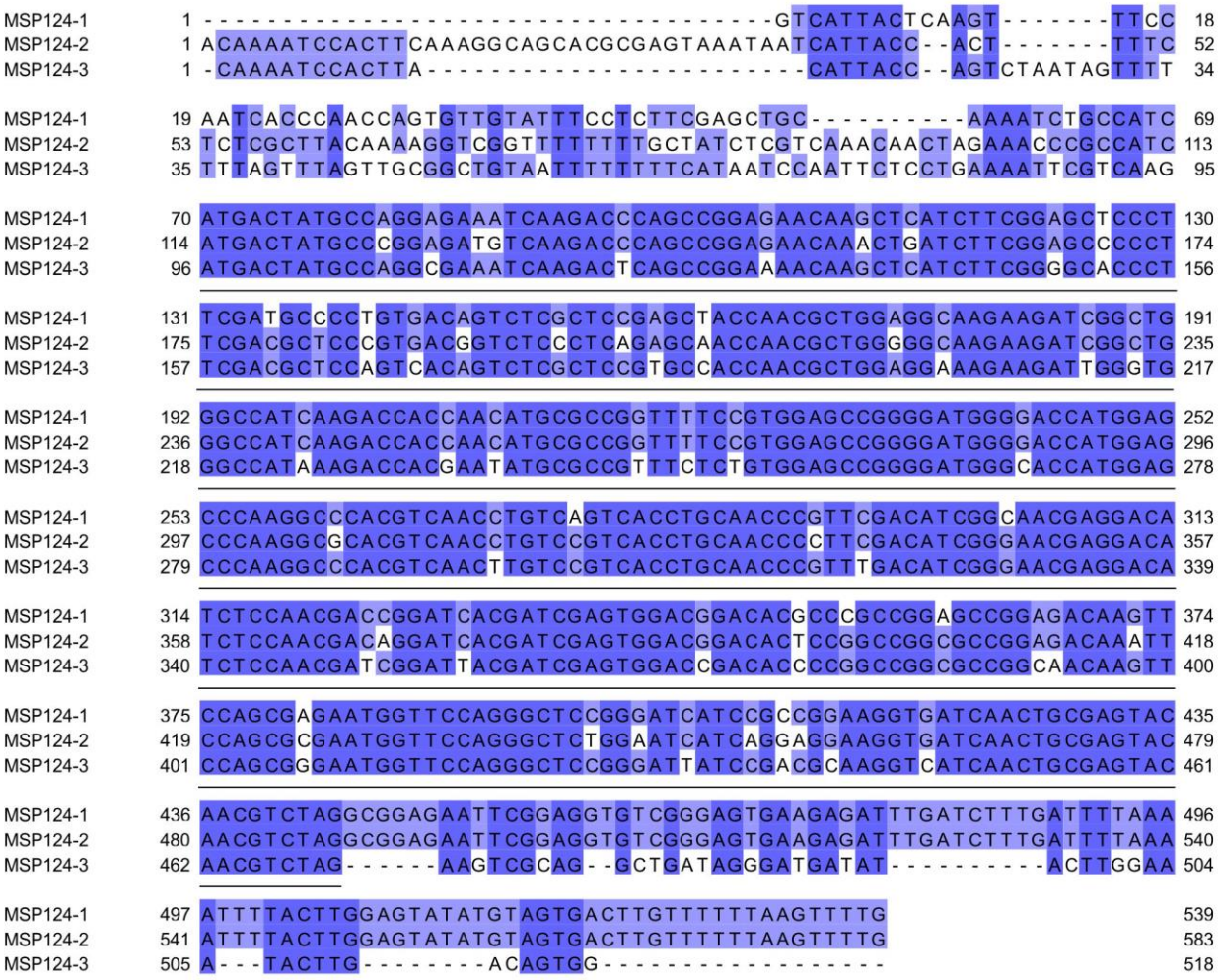


FIGURE S1 A group of *E. brevis* MSP transcripts called MSP124 that encode putative MSPs. Multiple alignment was done using MUSCLE and visualized in Jalview with Percentage Identity color scheme. Coding regions of the transcripts are underlined.

Figures

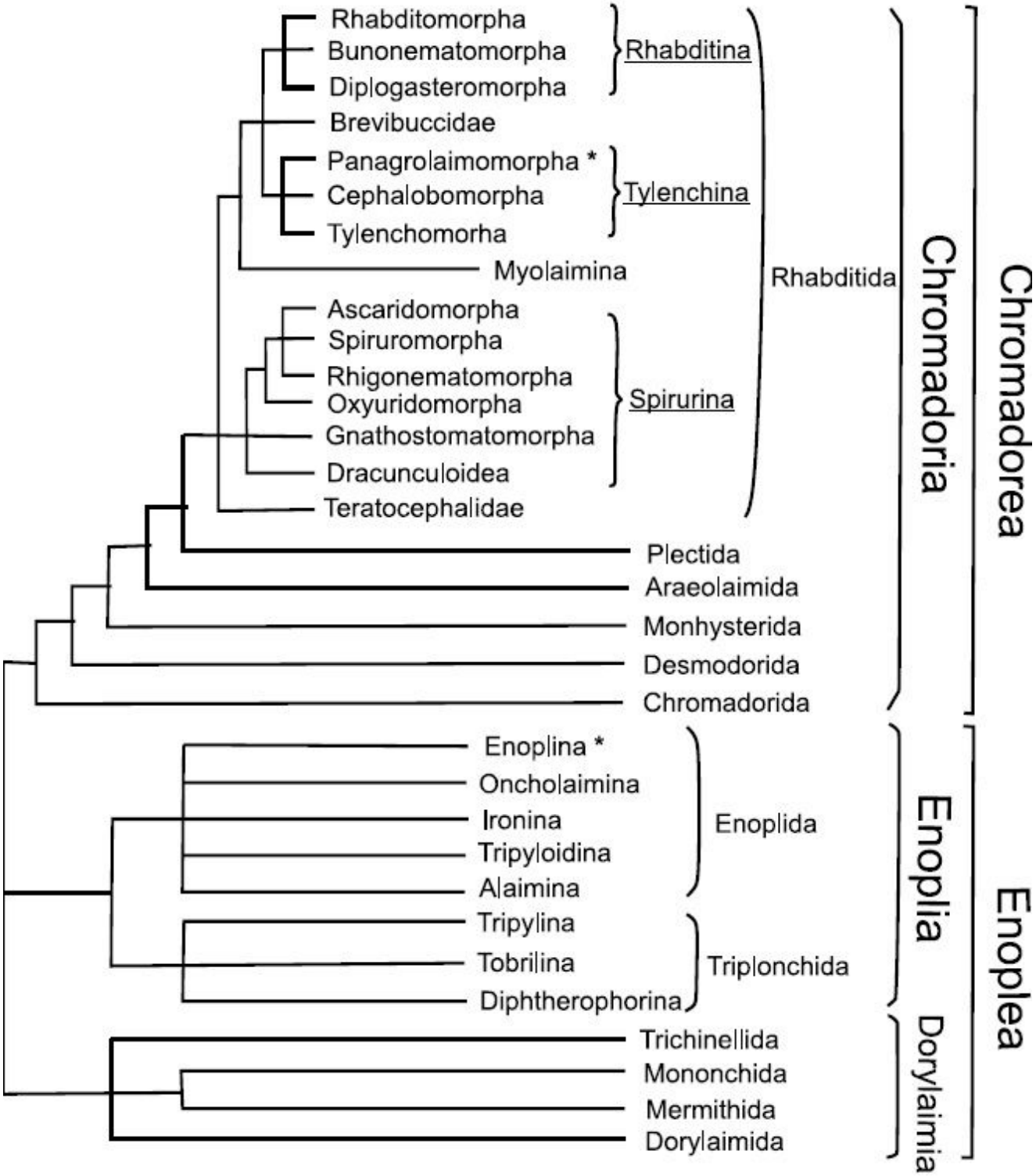


Figure 1

Phylogeny of nematodes and MSP-based sperm motility. Phylogenetic relationships within phylum Nematoda derived primarily from SSU rDNA sequence data are given according to De Ley and Blaxter (De Ley and Blaxter 2002). Suborders of Rhabditida order, in which representatives highly homologous MSPs

are found at DNA, RNA or protein levels, are marked by underlining. Taxa whose species used in this study are marked with asterisks. Orders Trefusiida, Isolaimida, Dioctophymatida, Muspiceida, Marimermithida and Desmoscolecida are not shown in this tree.

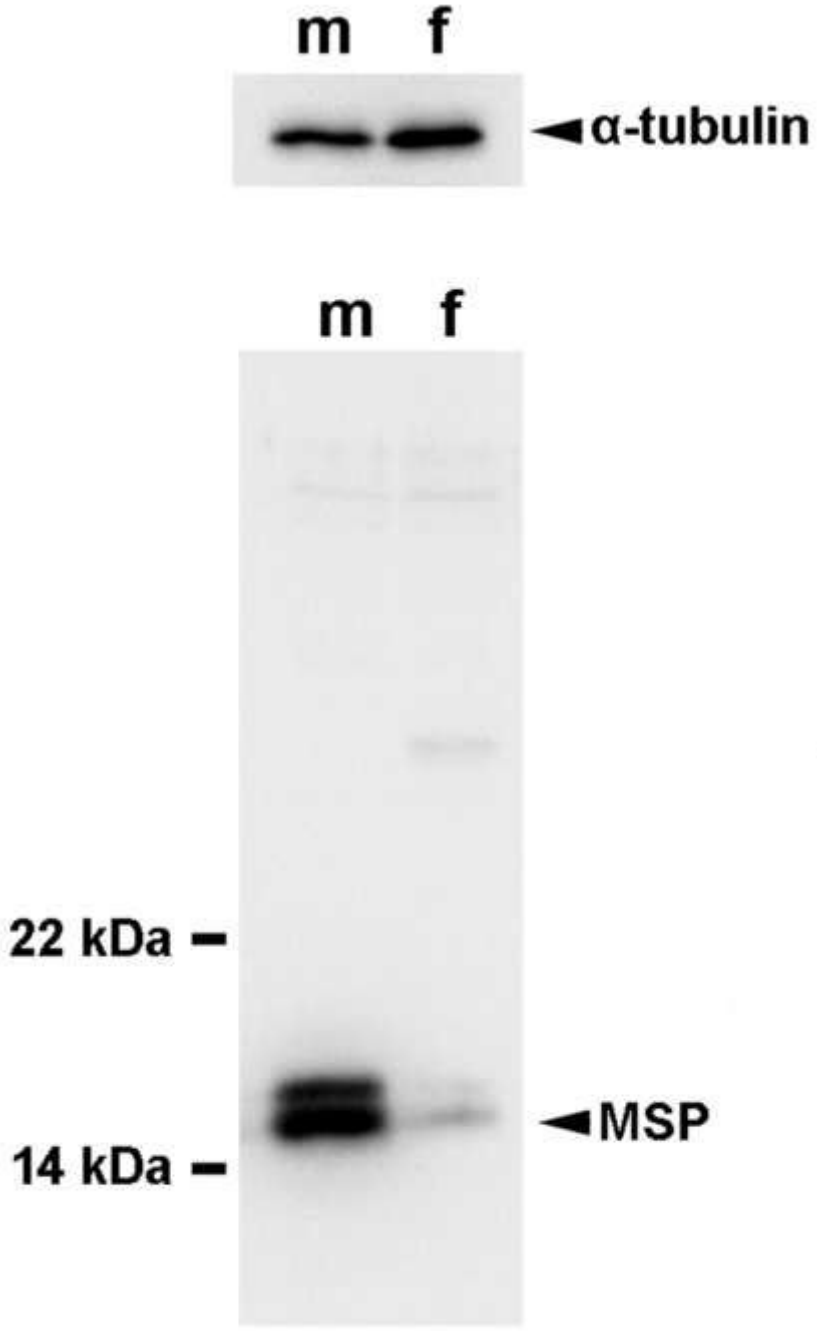


Figure 2

Western blot analysis of MSP in P. redivivus. In adult animals, MSP is detected as double band with approximate weight 15 and 16 kDa. Both male and female samples reveal MSP signal, because the latter include mated females. α-Tubulin was used as a loading control (approximate weight 55 kDa).

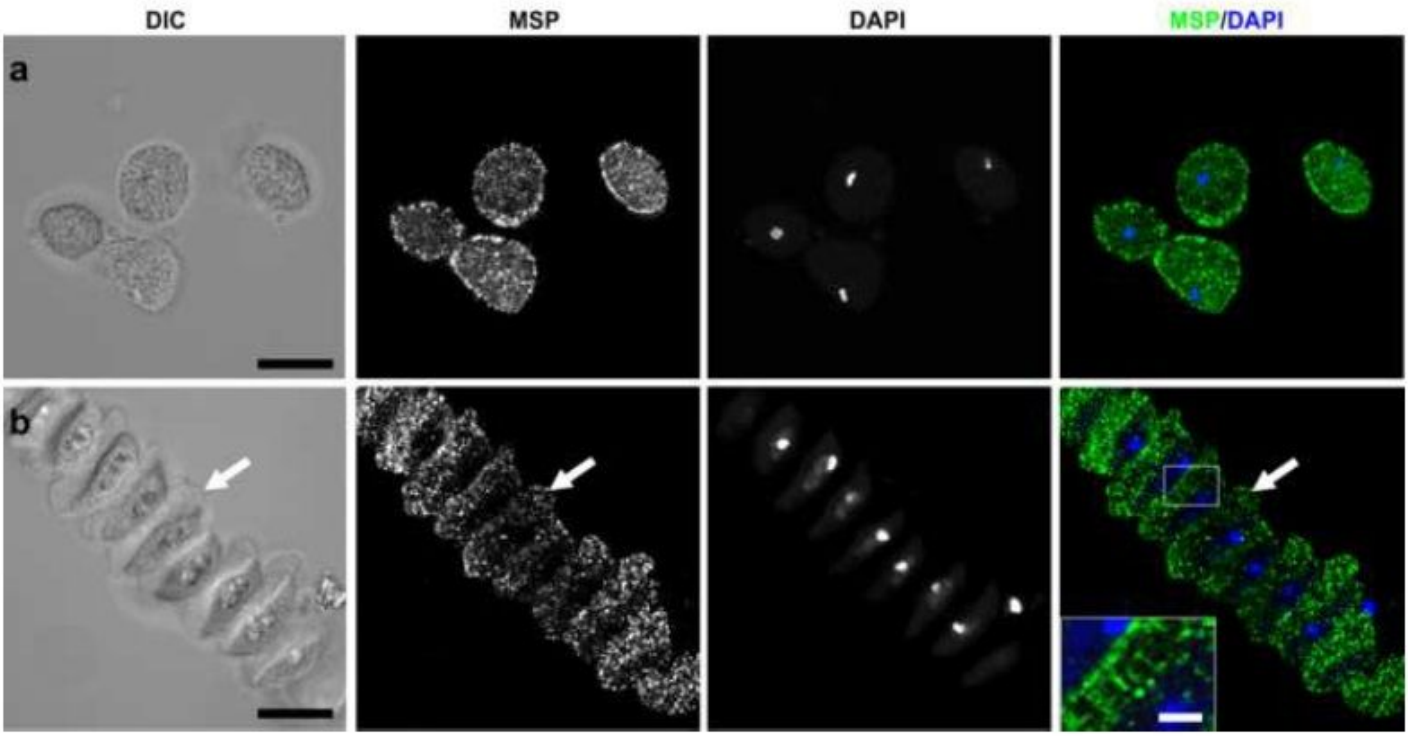


Figure 3

Immunolocalization of MSP in P. redivivus sperm. a Immature spermatozoa extracted from male. MSP localizes in granules. In some cells, MSP has strongest signals in the periphery (arrowheads) (scale bar 10 μm). b Chain of activated spermatozoa extracted from female. MSP has punctate and fibrillar pattern of distribution in pseudopodia that marked by arrows. Selected area is given in higher magnification (scale bar 10 μm, magnified area 2 μm).

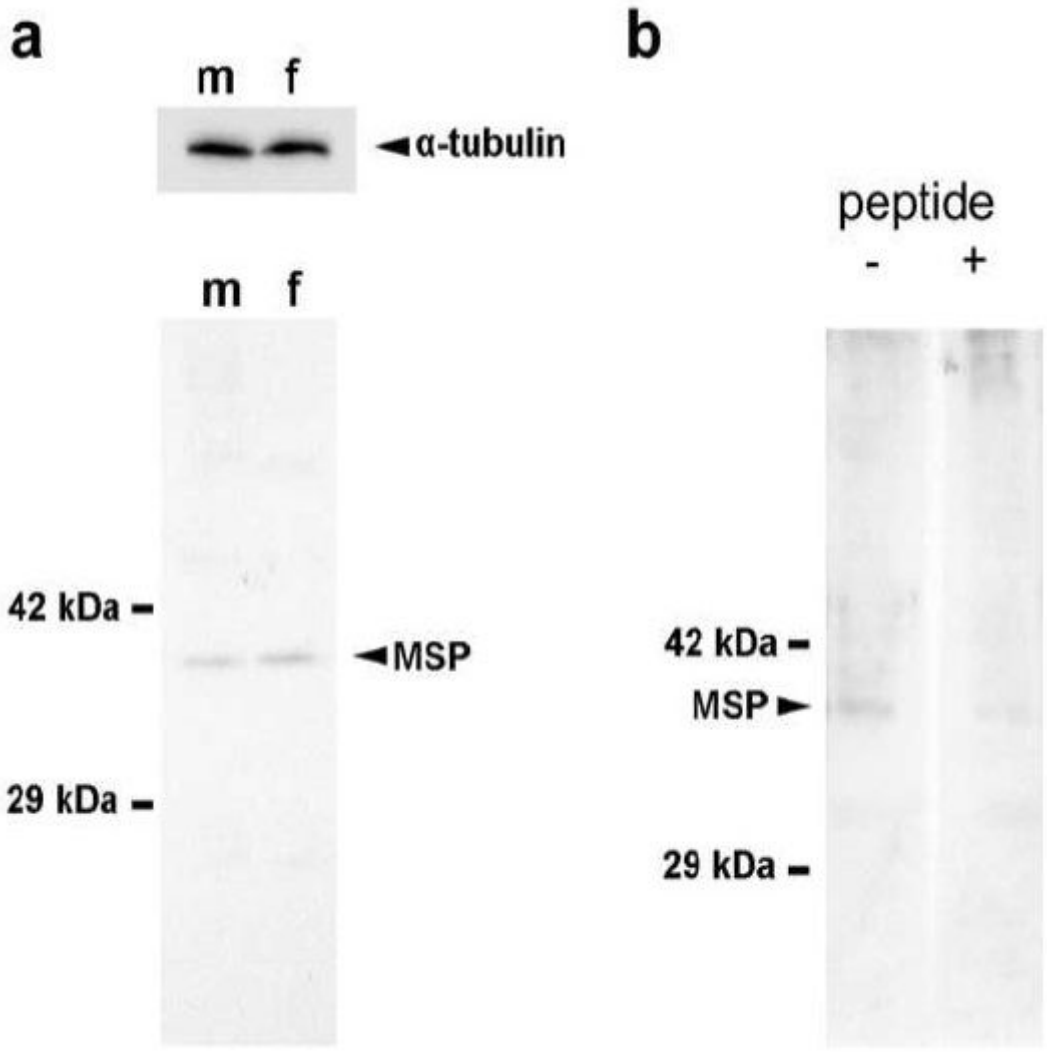


Figure 4

Western blot analysis of MSP in E. brevis. a MSP has unusual mobility in gel and is found as protein with weight 36-38 kDa. Both male and female samples reveal MSP signal, because the latter include inseminated females. α-Tubulin was used as a loading control (approximate weight 55 kDa). b Peptide competition assay confirms reactivity of anti-MSP antibodies with protein bandof 36-38 kDa.

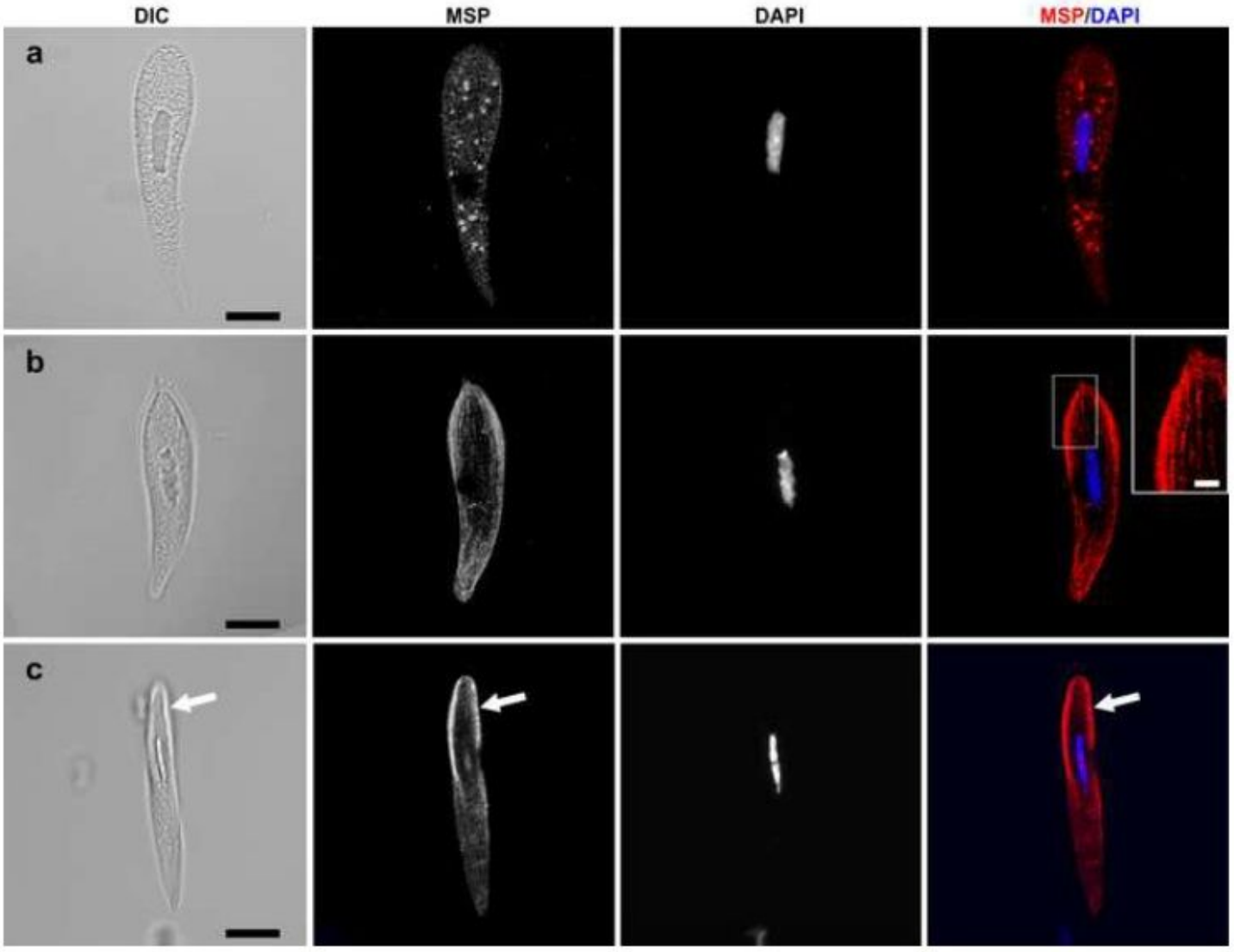


Figure 5

Immunolocalization of MSP in E. brevis sperm. a Immature spermatozoon from male. MSP concentrates in large granules (scale bar 10 μm). b Spermatozoon recovered from male and partially activated by 10 min incubation in sea water. MSP revealed more diffuse pattern with appearance of longitudinal fibrillar structures. Selected area is given in higher magnification (scale bar 10 μm, magnified area 2 μm). c Mature spermatozoon from female. Most of MSP signal is found in pseudopod (scale bar 10 μm).

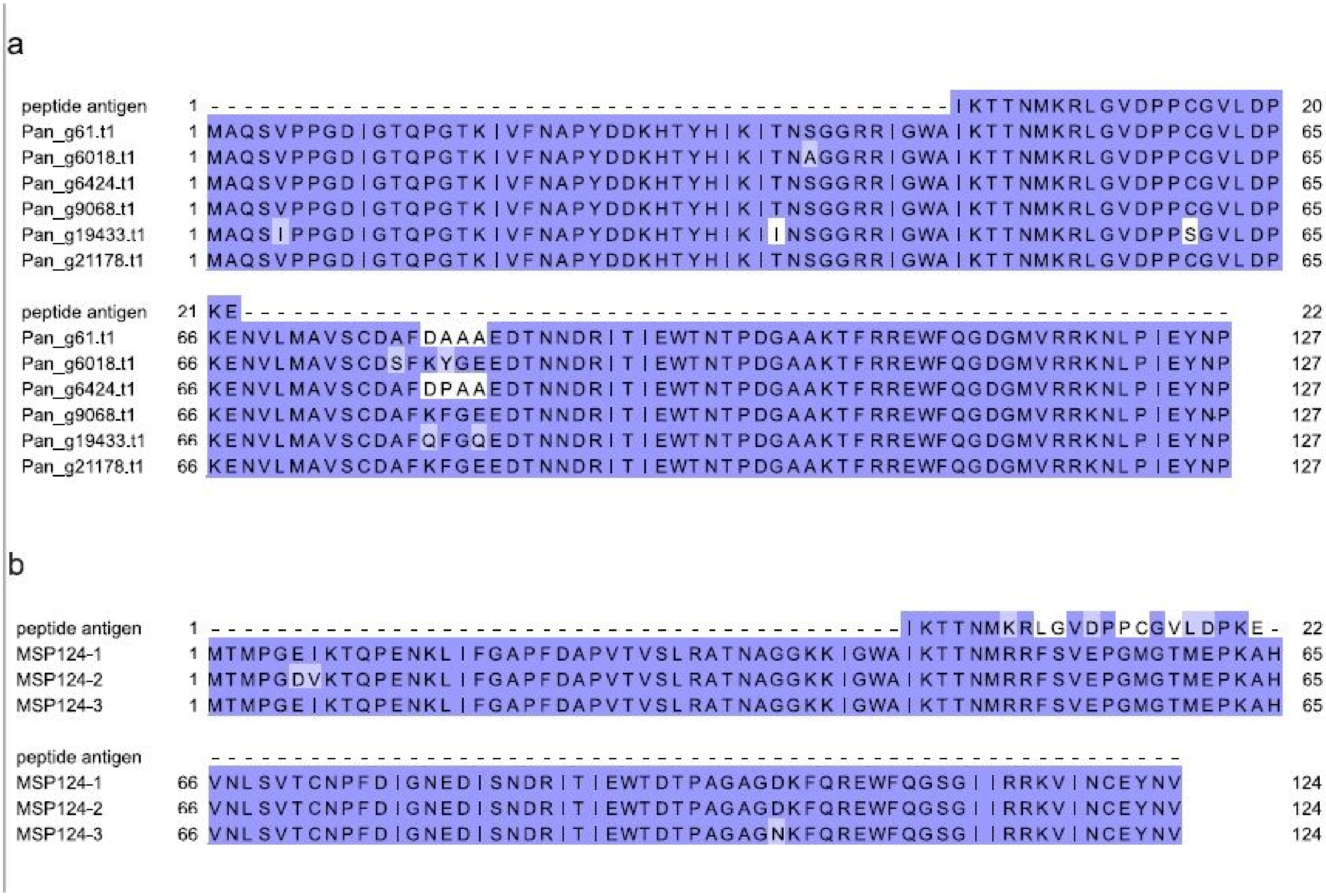


Figure 6

Putative MSPs that are most similar to peptide antigen. a P. redivivus MSPs aligned with peptide antigen. Protein sequences (Pan_g61.t1, Pan_g6018.t1, Pan_g6424.t1, Pan_g9068.t1, Pan_g19433.t1 and Pan_g21178.t1) were found by Blast using peptide antigen as query in WormBase Parasite (https://parasite.wormbase.org). b E. brevis MSPs aligned with peptide antigen. Multiple alignment was performed using ProbCons and visualized in Jalview with BLOSUM62 color scheme.

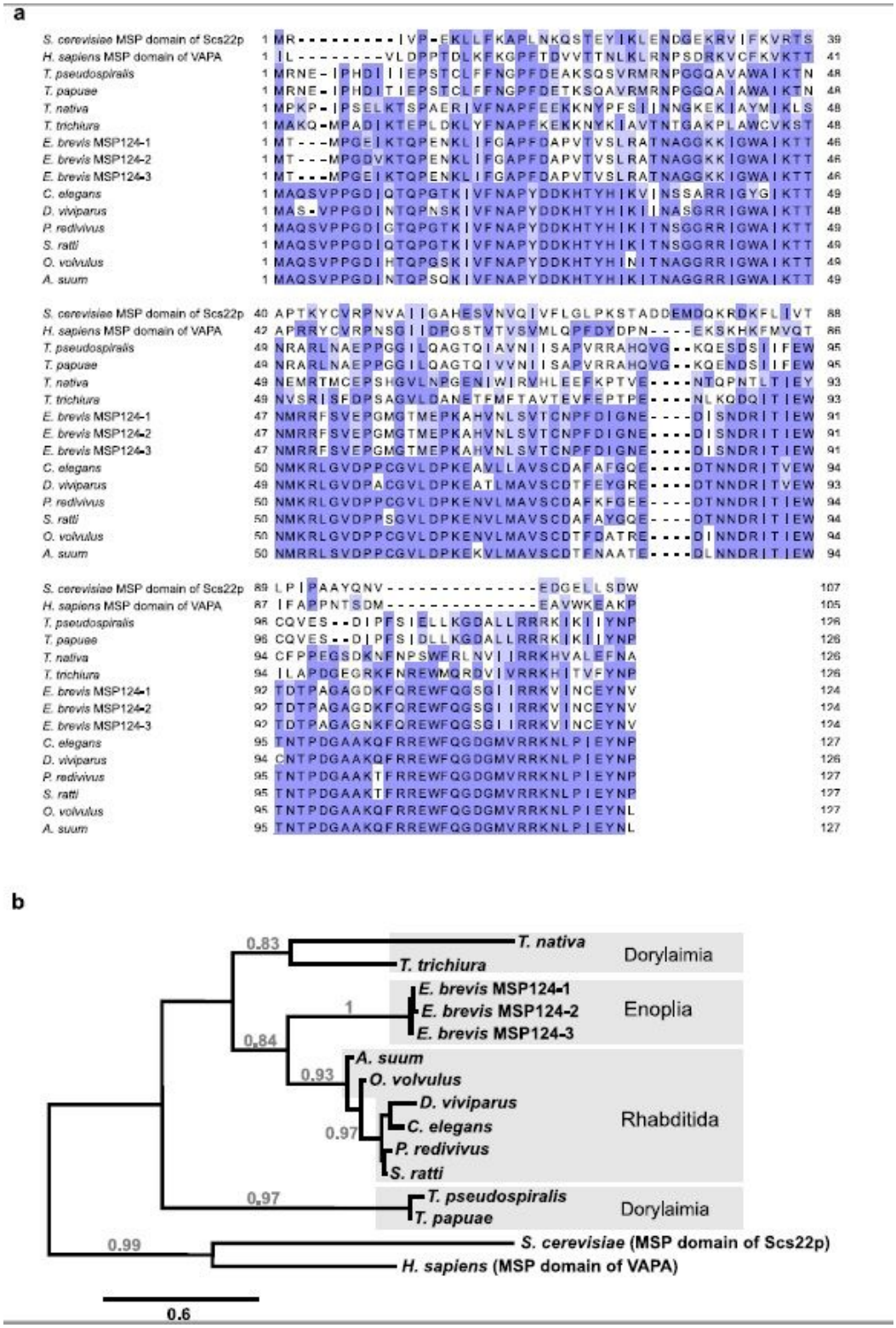


Figure 7

Multiple alignment and phylogenetic relationships of MSPs. a Multiple alignment of MSPs from 13 taxa of three subclasses of Nematoda. MSPs of six Rhabditida, four Dorylaimia and one Enoplia species were used. Multiple alignment was performed using ProbCons and visualized in Jalview with BLOSUM62 color scheme. MSP sequences of nematodes and MSP domains of Saccharomyces cerevisiae and Homo sapiens used in multiple alignment were downloaded from following databases, GenBank

(https://www.ncbi.nlm.nih.gov) (A. suum: CAA63933.1, Dictyocaulus viviparus: AAB27962.2, Strongyloides ratti: XP_024503659.1, Trichuris trichiura: CDW57515.1, Trichinella nativa: OUC40810.1, Trichinella pseudospiralis: KRX99722.1, Trichinella papuae: KRZ74366.1, S. cerevisiae MSP domain of Scs22p: AJP97989.1), WormBase Parasite (https://parasite.wormbase.org) (P. redivivus: Pan_g9068.t1) and UniProt (https://www.uniprot.org) (C. elegans: P53017, Onchocerca volvulus: P13262.3, H. sapiens MSP domain of VAPA: Q9P0L0). b Maximum likelihood 571 tree with SH-aLRT branch support. Only significant values (≥0.8) are shown. MSP domains of S. cerevisiae (Scs22p) and H. sapiens (VAPA) proteins were chosen as an outgroup.