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ORIGINAL ARTICLE

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# A comparative study of sperm morphology, cytology and activation in *Caenorhabditis elegans*, *Caenorhabditis remanei* and *Caenorhabditis briggsae*

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**Abstract** Studies of sterile mutants in *Caenorhabditis elegans* have uncovered new insights into fundamental aspects of gamete cell biology, development, and function at fertilization. The genome sequences of *C. elegans*, *Caenorhabditis briggsae* and *Caenorhabditis remanei* allow for informative comparative studies among these three species. Towards that end, we have examined wild-type sperm morphology and activation (spermiogenesis) in each. Light and electron microscopy studies reveal that general sperm morphology, organization, and ultrastructure are similar in all three species, and activation techniques developed for *C. elegans* were found to work well in both *C. briggsae* and *C. remanei*. Despite important differences in the reproductive mode between *C. remanei* and the other two species, most genes required for spermiogenesis are conserved in all three. Finally, we have also examined the subcellular distribution of sperm epitopes in *C. briggsae* and *C. remanei* that cross-react with anti-sera directed against *C. elegans* sperm proteins. The baseline data in this study will prove useful for the future analysis and interpretation of sperm gene function across nematode species.

**Keywords** Sperm · Spermiogenesis · *C. elegans* · Activation

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## Introduction

Fertilization is a process of prime importance to sexually reproducing species that accomplishes two critical things: the development of a new and unique (genetically distinct) individual and continuity of genetic information between generations. The importance of fertility research can hardly be overestimated, especially given current concerns of plummeting birth rates in developed countries, high incidences of unplanned pregnancies, and the risks of environmental threats to our reproductive health (Aitken et al. 2004; Butler 2004; Strauss and Kafrissen 2004). Exciting progress has been made in our understanding of fertilization via the genetic, molecular, and cell-biological study of sterile mutants in the model system *Caenorhabditis elegans* (Shakes and Ward 1989a,b; L'Hernault 1997; Singson 2001; Geldziler et al. 2004). A greater understanding of the processes/events required for successful fertilization can also provide insight into a wide variety of somatic cellular interactions.

To further our understanding of new molecule functions and evolutionary relationships, it is both important and necessary to expand studies to other organisms. Toward that end, eight more nematode draft genomes will soon be available, for example (Mitreva et al. 2005). To maximally profit from such studies, however, it is necessary to understand the normal processes in these species and to establish baseline phenotypic data.

We have examined and compared the structure/morphology of the sperm of *C. elegans*, *Caenorhabditis remanei*, and *Caenorhabditis briggsae* animals using both light and electron microscopy and have performed in vitro sperm activation assays on each. We have also looked at the localization of the cell surface protein SPE-9 (which is required for fertilization in *C. elegans*) and 1CB4 (a marker for internal sperm vesicles) in both *C. briggsae* and *C. remanei*. These results will help establish a baseline for expected sperm morphology in these related species and also serve to illustrate that techniques developed for *C. elegans* manipulation/study can also be used for similar studies in these organisms.

Recent accomplishments in genome sequencing projects have now made possible comparative, homology-based genetic studies in closely related species. With their emerging wealth of genetic information and the ease with which they can be studied, nematodes can be exploited as a useful tool in delineating homologies and the phylogenetic relationships within species. The three nematode species *C. elegans*, *C. remanei* and *C. briggsae* are closely related and show significant similarity (Sudhaus and Kiontke 1996). Despite their nearly identical morphology, phylogenetic evidence based on molecular data shows genetic divergences in these three species (Fitch and Thomas 1997). Contributing towards this divergence and playing a key role in establishing species boundaries are genes involved in reproduction (Baird and Yen 2000; Hill and L'Hernault 2001). In the genus *Caenorhabditis*, *C. elegans* and *C. briggsae* exist as self-fertilizing hermaphrodites with occasional males arising via non-disjunction during meiosis. *C. remanei*, however, is dioecious with males and females occurring in equal numbers (Brenner 1974; Baird et al. 1992). Fertilization in each species is internal and takes place within the spermatheca. Sperm activation is an essential step in gamete differentiation and is required for fertilization competence. Although a few genes involved in sperm activation and fertilization (gamete recognition and fusion) have been well characterized (Singson et al. 1998; Nance et al. 1999, 2000; Muhlrad and Ward 2002), there has been little information available to date regarding the evolutionary relationships in the pathways in which these genes participate.

## Materials and methods

### Worm culture and strains

Wild-type *C. elegans* (strain N2), *C. remanei* (strain EM 464) and *C. briggsae* (strain AF16) were used in this study. All strains used were maintained on *Escherichia coli OP50*-seeded NGM plates and manipulated as previously described (Brenner 1974), and were provided by the Caenorhabditis Genetics Center (CGC). The *C. elegans* genes studied include F26E4.5, *spe-4*, *spe-6*, *spe-8*, *spe-9*, *spe-10*, *spe-11*, *spe-12*, *spe-15*, *spe-17*, *spe-19*, *spe-26*, *spe-27*, *spe-29*, *spe-38*, *spe-39*, *spe-41/trp-3*, *spe-42*, and *fer-1*.

### Bioinformatics

Nucleotide BLAST searches (Altschul et al. 1997) were performed against the *C. briggsae* (http://genome.wustl.edu/blast/briggsae_client.cgi) and *C. remanei* (http://genome.wustl.edu/blast/client.pl) genome sequences using the Wormbase-annotated spliced and unspliced sequences for each. In each case where a homolog was found, the spliced and unspliced sequences of the *C. elegans* genes matched identical regions of the genomes from *C. briggsae* or *C. remanei*, increasing the confidence of our homology prediction. The genome sequence around the sequence thus identified was used as an input for the gene prediction algorithms FGeneSH (http://www.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind) and GeneMark (Besemer and Borodovsky 2005). Translations of the predictions from these programs were then used for protein BLAST searches against the NIH non-redundant protein database. The presence of a homolog in the other species was confirmed by observing the original *C. elegans* protein in the results of this BLAST.

For each gene compared, the predicted protein sequences from all three species were aligned using the ClustalW algorithm (http://clustalw.genome.jp/). The output from this multiple alignment was used to generate a phylogenetic tree using the MEGA (Kumar et al. 2004) program (neighbor-joining method). For both the ClustalW and the MEGA analysis, the default parameters were used.

Three-way alignments for the predicted protein sequences were performed using Vector NTI Advanced Suite (Invitrogen).

### Light microscopy/sperm activation

Sperm development was assessed as previously described (Nelson and Ward 1980; Shakes and Ward 1989b) by dissecting the testis or spermatheca in pH 7.8 sperm medium (SM) both with and without the known in vitro activators Pronase (a non-specific collection of proteases) (Garner et al. 1974) at 200 ug/ml or triethanolamine (TEA—a weak base that induces sperm activation in vitro via pH change) at 120 mM, pH 7.8. All specimens were viewed using a Zeiss Axioplan microscope and images were captured using an Optronics CCD camera.

### Electron microscopy

Animal preparation, fixation and sectioning procedures were performed essentially as previously described (White et al. 1986). All TEM were taken from sperm in the spermatheca that were activated in vivo. Thus, they are from either selfing hermaphrodites (in the case of *C. elegans* and *C. briggsae*) or mated females (in the case of *C. remanei*). Briefly, worms were anesthetized with 0.5% propylene phenoxytol in M9 buffer and then fixed in a dish well with 2.5% gluteraldehyde in 0.1 M HEPES buffer for 3–4 h. The worms were then post-fixed in 1% osmium tetroxide for 1 h, after which they were aligned on a thin layer of 1% agar. They were next covered with a drop of molten 1% agar and worm-containing blocks of agar cut out. These were dehydrated in a graded series of acetone and infiltrated and imbedded in Epon–Spurr resin. Thin sections (90–100 nm) were cut using a Reichert Ultracut E microtome with a diamond knife. The sections were lifted to copper grids and double-stained using ethanoic uranyl acetate (10 min) and lead citrate (2 min). Sections were examined and photographed at 80 kV with a JEOL 1200 electron microscope.

### Immunofluorescence

Males from all three species were dissected in sperm spmedia (SM) containing dextrose (Nelson and Ward 1980; Machaca et al. 1996). Spermatids were isolated from virgin *C. elegans*, *C. remanei*, or *C. briggsae* males. For in vitro activation experiments, virgin males were dissected directly into sperm media containing Pronase E (200 mg/ml). Sperm were fixed in 4% paraformaldehyde in SM for 20 min at room temperature. They were then permeabilized with 0.2% Triton X-100 and stained with anti-SPE-9-EX (KCN conjugated) polyclonal antibody (Zannoni et al. 2003) or the mouse monoclonal antibody 1CB4 (Okamoto and Thomson 1985; Zannoni et al. 2003). SPE-9 and 1CB4 staining was detected using Alexa Fluor 488 goat anti-rabbit and FITC-conjugated goat anti-mouse secondary antibodies. Slides were dip-washed in PBS and mounted with GelMount (Biomedia) containing DAPI.

## Results

### Light microscopy

Viewed as whole-worm mounts under the light microscope, *C. elegans*, *C. briggsae*, and *C. remanei* worms are practically indistinguishable from each other due to their similarities in size (approximately 1.5 mm adult length) and general morphology at this level (Fig. 1). The spermatids themselves from males of these species are also morphologically similar (Fig. 2a,d,g) as round non-motile cells, although spermatids from the nematode *C. remanei* are an average of ×1.8 larger than those of *C. briggsae* and *C. elegans* (see below).

*C. elegans* spermatids can be induced to undergo post-meiotic differentiation/maturation (spermiogenesis/sperm activation) via treatment with Pronase, a collection of non-specific proteases (Nelson and Ward 1980; Shakes and Ward 1989b). Sperm activation in *C. elegans* is a dramatic event during which much cellular morphogenesis and re-arrangement occurs. Plasma membrane flow to the site of the newly developing pseudopod is seen (unlike flagellated mammalian sperm, *C. elegans* sperm are amoeboid), as is fusion of novel so-called membranous organelles (MO) to the cell body plasma membrane (for glycoprotein delivery and cytoplasmic partitioning) (Arduengo et al. 1998; Chatterjee et al. 2005) and the formation of a dynamic cytoskeleton in the pseudopod (L'Hernault 1997). Based on such general morphological similarity as noted above, we wondered whether any of the tools developed for studying *C. elegans* sperm activation would prove useful for examining the sperm of these other species and to what extent sperm activation differs among them. To address these questions, we used a standard *C. elegans* Pronase activation protocol (see Materials and methods) on the male-derived spermatids of *C. remanei* and *C.*

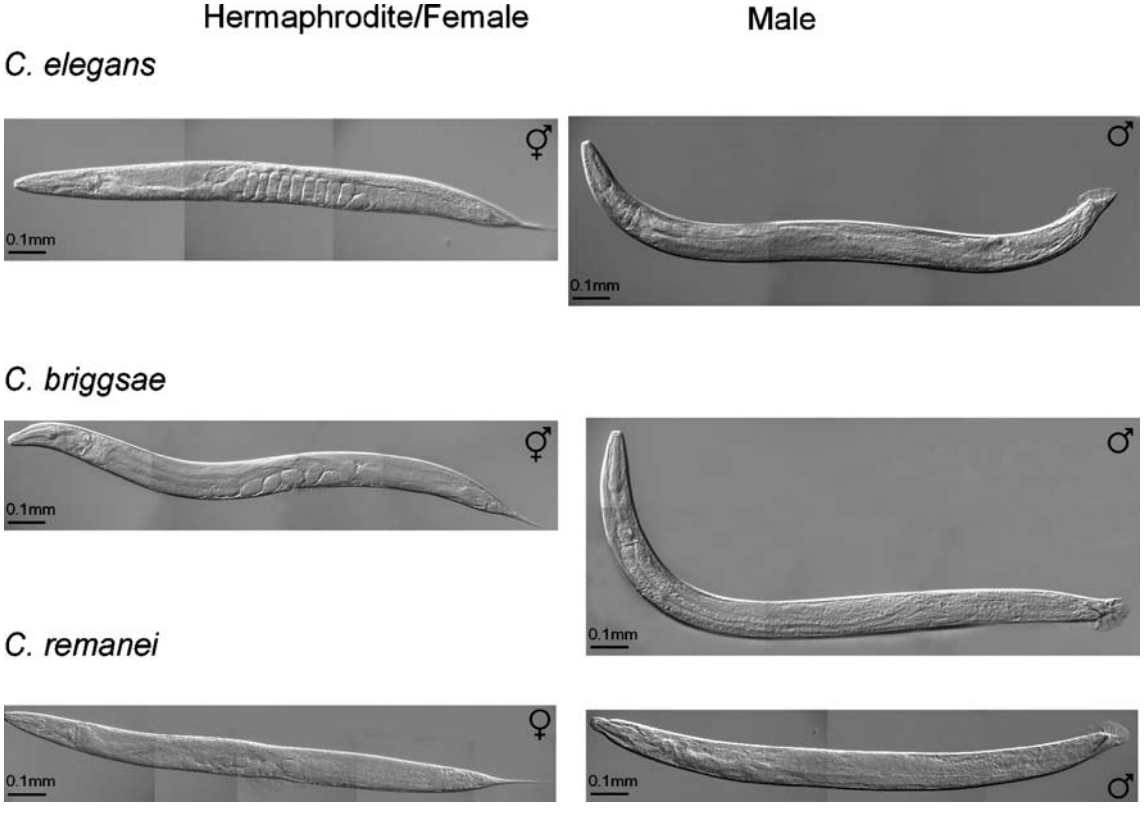


**Fig. 1** Whole-worm mounts of *C. elegans*, *C. briggsae*, and *C. remanei*. Sex and species are as indicated. Scale is as indicated

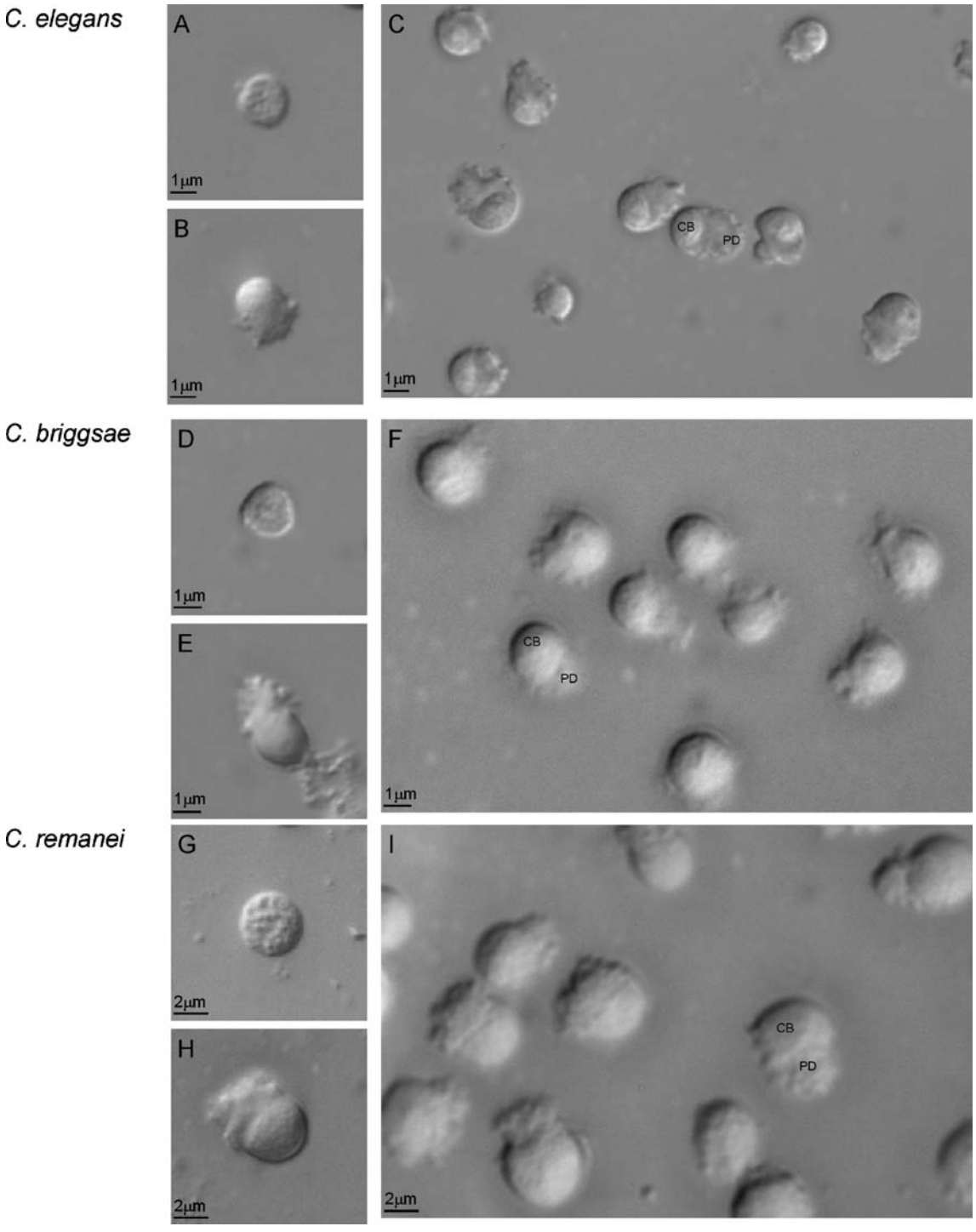


**Fig. 2** Spermatid morphology and activation in three *Caenorhabditis* species. Both unactivated spermatids (**a**, **d**, **g**) and activated spermatozoa (**b**, **c**, **e**, **f**, **h**, **i**) are shown. **a**–**c** *C. elegans*, **d**–**f** *C. briggsae*, and **g**–**i** *C. remanei*. Examples of the cell body (*CB*) and pseudopod (*PD*) are marked. Scale is as indicated

*briggsae*. We find that both *C. remanei* and *C. briggsae* spermatids activate to form mature spermatozoa similarly to *C. elegans* (Fig. 2b,c,e,f,h,i). In each species, almost all (>95%) spermatids activate to form amoeboid, motile spermatozoa within 5 min of Pronase treatment, clearly establishing the utility of the protocol in these species. Treatment with TEA using another standard *C. elegans* activation protocol (see Materials and methods) yielded similar results (data not shown). The mature spermatozoa of *C. remanei* (male) worms are significantly larger than their counterparts in *C. elegans* and *C. briggsae*. Average sperm cell body diameters are 2.03 μm for *C. elegans*, 2.05 μm for *C. briggsae*, and 3.68 μm for *C. remanei* ($n$=98, 26, and 19, respectively).

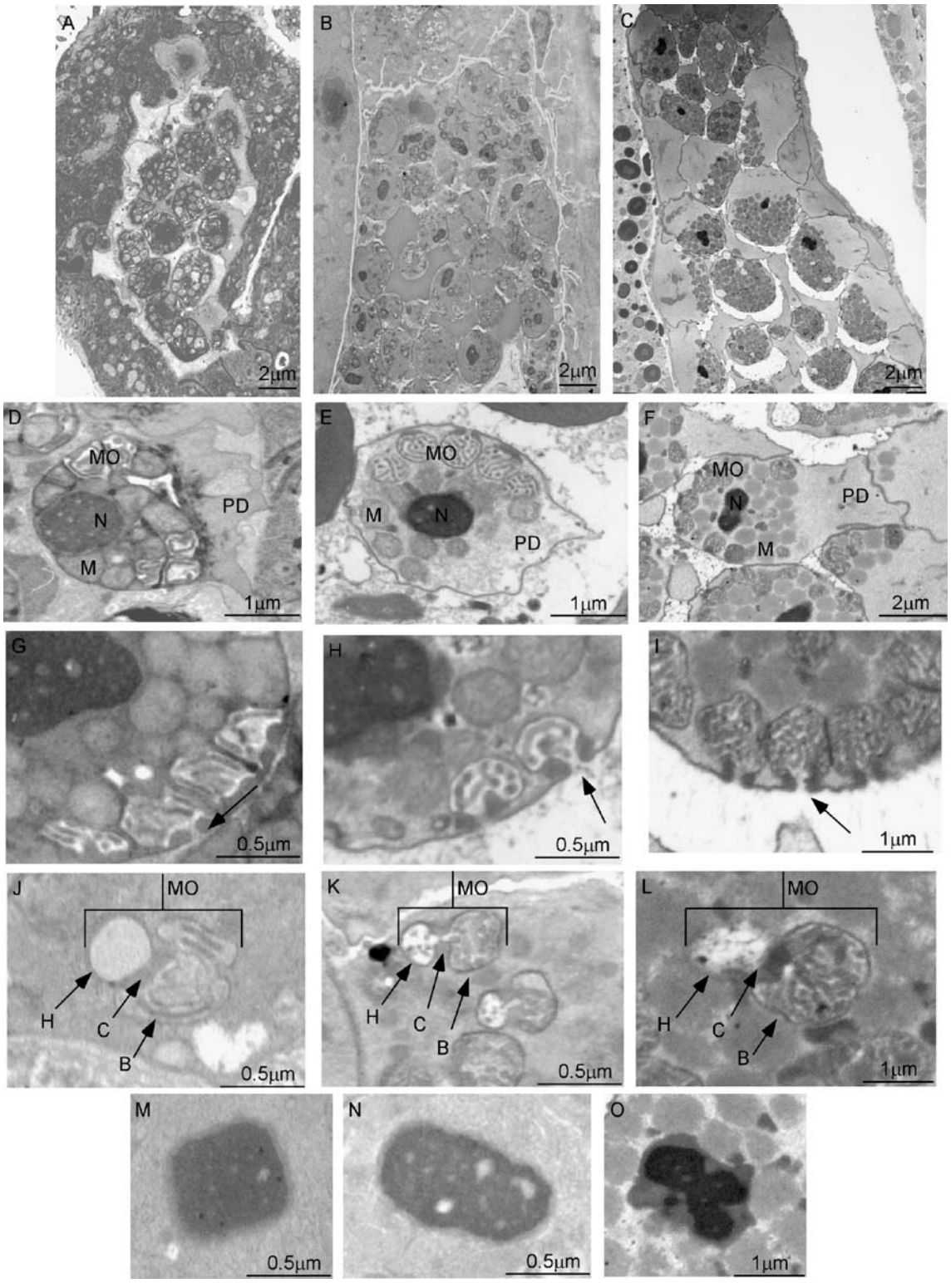


**Fig. 3** Transmission electron micrographs showing spermatheca and spermatozoa ultrastructure in three *Caenorhabditis* species. **a**–**c** Sperm-filled spermathecae: **a** *C. elegans*, **b** *C. briggsae*, and **c** *C. remanei*. **d**–**f** Individual spermatozoa with major components labelled. **d** *C. elegans*, **e** *C. briggsae*, and **f** *C. remanei*. **g**–**i** Membranous organelle fusion at the plasma membrane: **G** *C. elegans*, **h** *C. briggsae*, and **i** *C. remanei*. *Arrows* point to fusion pores. **j**–**l** Unfused membranous organelles: **j** *C. elegans*, **k** *C. briggsae*, and **l** *C. remanei*. *Arrows* denote the head (*H*), collar (*C*) and body (*B*) regions. **m**–**o** Nuclear morphology: **m** *C. elegans*, **n** *C. briggsae* and **o** *C. remanei*. *N* Nucleus, *MO* membranous organelles, *M* mitochondria, *PD* pseudopod. Scale is as indicated

## Electron microscopy

Although ultrastructural studies of nematode sperm have been previously published (Jamuar 1966; Clark et al. 1967; Lee and Anya 1967; Foor 1968, 1970), we know of none that has compared these three closely related species directly. Ultrastructural examination using TEM again shows *C. remanei* and *C. briggsae* spermatozoa to be very similar to *C. elegans* in appearance. Representative micrographs (Fig. 3a–c) clearly show spermatozoa packed in the spermatheca (the site of both sperm storage and fertilization in each species). A closer look at individual sperm reveals the extent of the similarities; in each species we note a clearly visible organelle-containing cell body as well as a pseudopodal region devoid of any cellular organelles. The sperm nucleus, MOs, and mitochondria can be seen in the cell body, clearly separated from the pseudopod (Fig. 3d–f). As in *C. elegans*, membranous organelles can be seen both fused (Fig. 3g–i) and unfused (Fig. 3j–l) to the plasma membrane (depending on activation state), and the nuclear material is easily visible as a dark, condensed electron-dense mass (Fig. 3m–o) in each species. Qualitative differences in organelle number and nuclear shape are summarized in Table 1.

## Bioinformatics

While *C. remanei* is a dioecious (male/female) species, both *C. briggsae* and *C. elegans* are androdioecious (hermaphrodite/male) species. Given the physiological similarities of the spermiogenesis pathway in these species, we were interested in examining components of the sperm activation pathway to see if there exist mechanistic differences based on reproductive mode. We find that with the exception of *spe-12*, homologs of all spermiogenesis mutants identified in *C. elegans* exist in both *C. briggsae* and *C. remanei* (Fig. 4). Thus, although *C. remanei* differs in its reproductive mode, the sperm activation pathway appears conserved, both at the physiological and the molecular levels. Interestingly, we note that *spe-8* has an additional paralog (Fig. 4) in the *C. elegans* genome (F2 6E4.5) that is also conserved in both *C. briggsae* and *C. remanei* (Muhlrad and Ward 2002). Further, the expression of F26E4.5 is predicted to be highly enriched during spermatogenesis (Reinke et al. 2004), suggesting a role during the development and/or maturation of sperm. The phylogenetic tree for *spe-8* and F26E4.5 (Fig. 4) indicates that these genes most probably arose from a gene duplication event that occurred in the last common ancestor shared by the three species. The two genes are subsequently evolving independently and, despite their similarity, *spe-8* and F26E4.5 appear to have non-redundant roles in spermiogenesis, as null mutations in *spe-8* give rise to hermaphrodites that are profoundly sterile (Shakes and Ward 1989b).

A number of *spe* genes have been identified in *C. elegans* that play integral roles in various aspects of sperm development and function. We identified homologs for all these genes in both *C. briggsae* and *C. remanei*, pointing to a conservation of sperm development and function, both at the morphologic as well as the molecular level. Further, unlike sex-determination genes in *C. elegans* and other organisms (Stothard and Pilgrim 2003), all of the *spe* genes cloned to date show high conservation—all genes studied show >60% conservation across the three species (Table 2).

The phylogenetic trees for most of the genes that we studied correspond to the established phylogeny of the three species (Fig. 4), i.e., *C. briggsae* and *C. remanei* share a common ancestor after they branch off from *C. elegans* (Kiontke et al. 2004). While the phylogenetic trees for some *spe* genes are different from the others (*spe-6*, *spe-15*, *spe-19*, *spe-27*, *spe-29*, and *spe-42*; Fig. 4), we can observe no pattern for this difference and believe that such differences do not tell us anything meaningful about the evolution of sperm development or function in these species.

**Table 1** Sperm ultrastructure

| | MO # | Mito # | Nuclear lobing |
|---|---|---|---|
| *C. elegans* | + | + | + |
| *C. briggsae* | + | + | ++ |
| *C. remanei* | ++ | ++ | +++ |

A qualitative comparison of sperm ultrastucture in *C. elegans*, *C. briggsae*, and *C. remanei*
+ Relative amount

## Immunofluorescence

To look for useful cytological markers, we examined our collection of anti-sera raised against *C. elegans* proteins. The *spe-9* gene in *C. elegans* encodes a transmembrane protein with ten EGF-like repeats in the extracellular domain (Singson et al. 1998). Specific antiserum (EX) raised to a 13-amino-acid peptide region between EGF motifs 4 and 5 of SPE-9 was chosen for immuno-localization studies (Fig. 5a,b). It has been shown that in spermatids of wild-type males, SPE-9 localizes to the plasma membrane (Fig. 6a,b; Zannoni et al. 2003). Upon activation, spherical, immobile spermatids undergo differentiation to become polar, motile amoeboid cells. Concomitantly, there occurs a dynamic redistribution of cellular surfaces and molecules in the cell membrane. SPE-9 is one such protein that undergoes redistribution to accumulate in the pseudopod of the mature sperm cell (Fig. 7a,b; Zannoni et al. 2003). We performed a study of the cross-reactivity of the polyclonal anti-SPE-9-EX antiserum in both *C. briggsae* and *C. remanei* spermatids and spermatozoa. In *C. briggsae* spermatids, the EX serum binds to the cell surface in a manner similar to that seen in *C. elegans* (compare Fig. 6b–d).

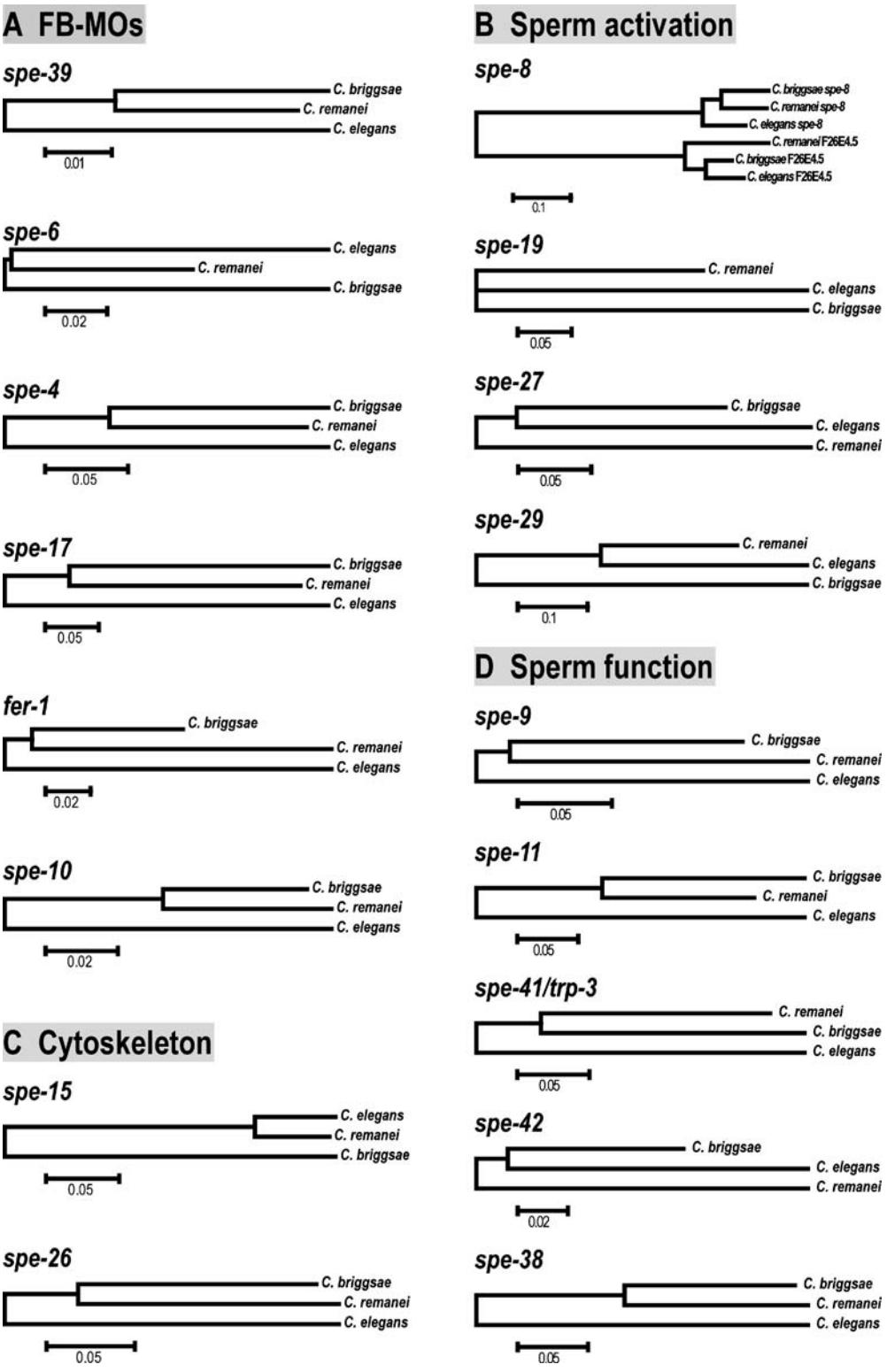


**Fig. 4** Phylogenetic trees derived from the predicted amino acid sequences of the *fer* and *spe* genes. The genes have been grouped according to their role in sperm development and function. Genes involved in fibrous body and membranous organelle (FB-MOs) fusion and function during spermatogenesis are grouped into the "FB-MO" category (**a**). Molecules that play a role in spermiogenesis are in the "sperm activation" group (**b**). Genes related to cytoskeletal elements are the "cytoskeleton" group (**c**). Molecules present in the sperm that are required for sperm function during fertilization or development of the embryo are classified into the "sperm function" group (**d**)

Upon Pronase activation to mature spermatozoa, the stain localizes to the pseudopod also in a pattern similar to that seen in *C. elegans* sperm (compare Fig. 7b–d; Zannoni et al. 2003). Spermatids from *C. remanei* worms also exhibit cell surface staining (Fig. 6f,h); however, mature spermatozoa show no obvious pseudopod staining enrichment as do *C. elegans* and *C. briggsae*. Instead, staining remains on the entire cell surface (Fig. 7f). *C. remanei* spermatids were also stained with 1CB4, a marker for MOs (Okamoto and Thomson 1985; Fig. 6f) and DAPI,

**Table 2** Percent identity and similarity for 3-way comparisons of the various *spe* and *fer* genes for which phylogenetic analysis was performed

| Gene | 3-way similarity | 3-way identity |
|---|---|---|
| *spe-39* | 98 | 83 |
| *spe-6* | 90 | 54 |
| *spe-4* | 90 | 56 |
| *spe-17* | 84 | 41 |
| *fer-1* | 96 | 69 |
| *spe-10* | 98 | 81 |
| *spe-15* | 94 | 55 |
| *spe-26* | 92 | 59 |
| *spe-8* | 92 | 70 |
| *spe-19* | 77 | 26 |
| *spe-27* | 80 | 32 |
| *spe-29* | 86 | 21 |
| *spe-9* | 82 | 50 |
| *spe-11* | 89 | 51 |
| *trp-3* | 62 | 26 |
| *spe-42* | 67 | 21 |
| *spe-38* | 83 | 46 |

The genes are grouped in the same functional clusters as the phylogenetic trees in Fig. 4

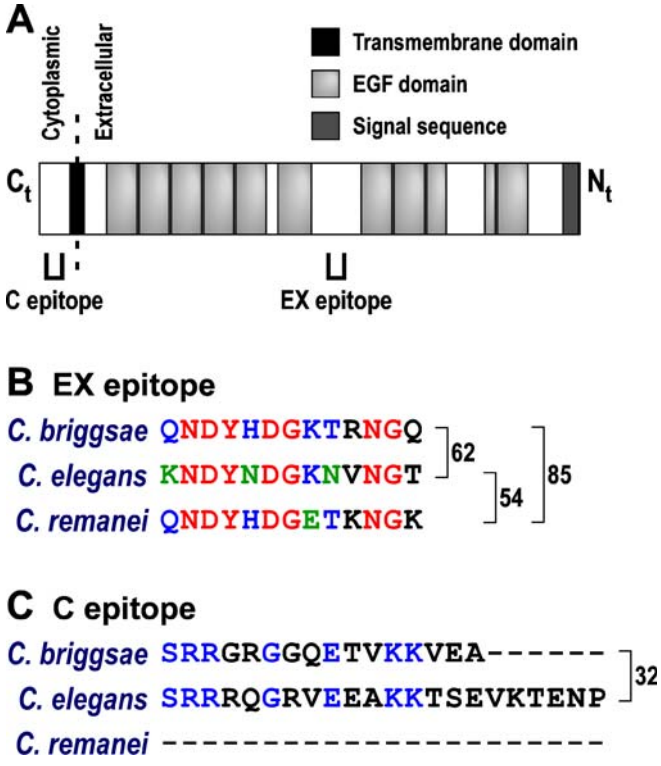


**Fig. 5** Alignment of the peptides to which SPE-9 anti-sera was generated. **a** The regions of *spe-9* against which the EX and C anti-sera were generated. **b** The alignment for the EX peptide and **c** the alignment for the C peptide. *Numbers* correspond to the percent conservation for the respective comparison

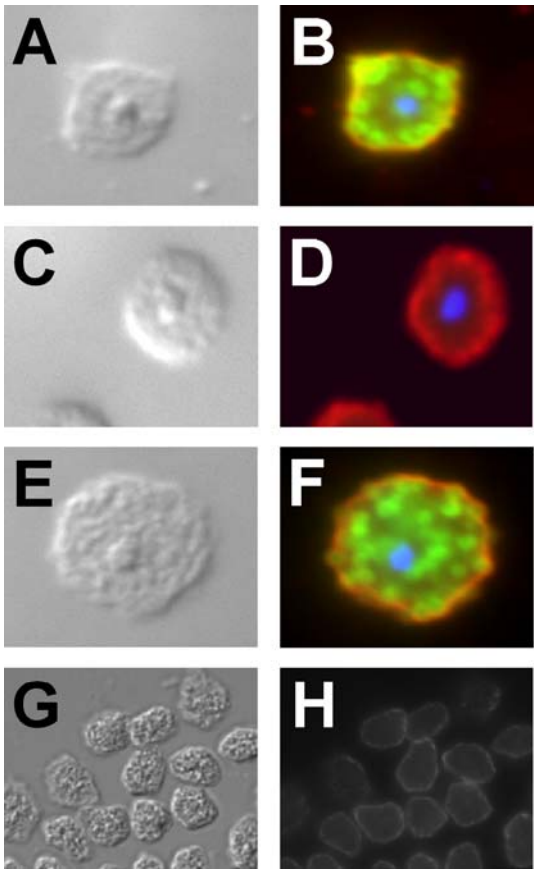


**Fig. 6** Cross-reactivity of anti-SPE-9-EX and 1CB4 sera on spermatids from *C. briggsae* and *C. remanei*. **a**–**b** Anti-SPE-9-EX stains the plasma membrane and 1CB4 stains MOs of *C. elegans* spermatids (Zannoni et al. 2003; Okamoto and Thomson 1985). **c**–**d** Anti-SPE-9-EX staining of *C. briggsae* spermantids. Anti-SPE-9-EX (**e**–**h**) and 1CB4 (**e**–**f**) staining of *C. remanei* spermatids. Nomarski DIC images of spermatids are shown in (**a**), (**c**), (**e**), and (**g**). Corresponding images of merged immunofluorescence staining are shown in (**b**), (**d**), (**f**), and (**h**). Anti-SPE-9-EX staining is in *red* (**b**, **d**, **f**) or in *white* (**h**). 1CB4 staining is in *green* (**b**, **f**). DAPI staining is in *blue* (**b**, **d**, **f**)

which stains nuclei (Fig. 6b,d,f). No staining was seen in *C. briggsae* spermatids for 1CB4, indicating that the 1CB4 antiserum does not cross-react with any *C. briggsae* epitope. Furthermore, we found that our anti-SPE-9-C antibody (directed to the less-conserved-across-species C-terminal end of SPE-9 [Fig. 5a-c]) did not cross-react to SPE-9-like epitopes (Fig.5a) on *C. briggsae* or *C. remanei* sperm (Chatterjee and Singson, unpublished observations). Negative controls performed without primary antibody showed no staining (data not shown).

## Discussion

The general sperm morphology of *C. briggsae* and *C. remanei* is similar to that of *C. elegans* at the level of the

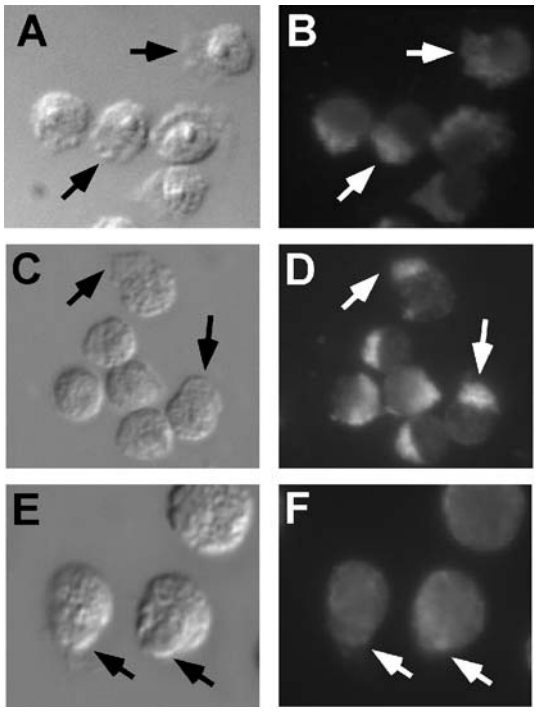


**Fig. 7** Cross-reactivity of anti-SPE-9-EX sera on spermatozoa from *C. briggsae* and *C. remanei*. **a**–**b** Anti-SPE-9-EX staining concentrates on the pseudopod of *C. elegans* spermatozoa (Zannoni et al. 2003). **c**–**d** Anti-SPE-9-EX staining of *C. briggsae* spermatozoa. The epitope recognized by this sera concentrates on the pseudopod. **e**–**f** Anti-SPE-9-EX staining of *C. remanei* spermatozoa. Staining is diffuse and does not appear to concentrate significantly on the pseudopod. Nomarski DIC images are shown in **a**, **c**, and **e**. Corresponding immunofluorescence images are shown in **b**, **d**, and **f**

light microscope, as is sperm ultrastructure as evidenced by transmission electron microscopy. Although it is possible that there exist ultrastructural differences between the sexes in *C. briggsae*, we believe it unlikely as no such differences have ever been observed from *C. elegans* (for *C. remanei*, this is obviously not an issue as sperm are derived only from males). The significantly larger spermatids/spermatozoa of *C. remanei* reflect its reproductive mode, as sperm are generally smaller in hermaphroditic species than in gonochoristic ones as a consequence of reduced polyandry and sperm competition (LaMunyon and Ward 1999). Qualitatively, we note an increase in the number of both membranous organelles and mitochrondria in *C. remanei* spermatozoa, as well as a lobed nuclear morphology, the significance of which is unclear. Additionally, in vitro sperm activation occurs similarly in all three species and can be induced in each using techniques developed for *C. elegans*.

We also find that with the exception of *spe-12*, all the molecules established to have roles in *C. elegans* spermatogenesis, spermiogenesis, and sperm function have strongly conserved homologs in *C. briggsae* and *C. remanei*. Thus, it would appear that in these species there is a close correlation between physiology, morphology, function and molecular mechanism. By establishing normal sperm, sperm activation, and sperm function phenotypes, this baseline data should prove useful for the study of sterile mutants and their homologs in each species, helping to increase our understanding not only of sperm activation/spermiogenesis but of fertilization in general.

We find that most, but not all, of the phylogenetic trees for the genes studied here correspond to the established phylogeny of the three species. Those that do not have various roles in sperm development and function, and more work (and more genes) will be necessary to allow for meaningful assessments of the evolution of sperm function and development in these species.

From the available genomic data, we find that the peptide sequence for *C. briggsae* corresponding to the EX epitope of SPE-9 is 62% identical and that of *C. remanei* is 54% (Fig. 5b). This indicates that the EX epitope is fairly well conserved across species and that the antibody recognizes epitopes most similar to SPE-9 in other species.

The affinity of the SPE-9 antibody to other epitopes depends on the extent of sequence, structural, and chemical similarity. Nevertheless, these cross-reacting antigens can serve as useful cytological markers in sperm of other species. Sperm activation pathways seem to be conserved across the three species of nematodes. Molecules such as SPE-9 play a critical role in sperm function after spermatogenesis for gamete interaction and fertilization (Singson et al. 1998). Depending on function, each species may have a unique way of representing molecules such as SPE-9 within the cell. In *C. elegans*, for example, very little SPE-9 is required for full fertility (Putiri et al. 2004). Perhaps *C. remanei* has dispensed the need for pseudopod concentration of this molecule. If function is conserved, however, it is likely that these molecules will have a similar pattern of sub-cellular localization. This was seen in our immunolocalization experiments for *C. elegans* and *C. briggsae*. No cross-reactivity would be expected where epitopes are not conserved, as was observed with anti-SPE-9-C (Chatterjee and Singson, unpublished; see above).

The *C. elegans* sperm activation pathway is believed to require either a male-specific or a hermaphrodite-specific activator (Shakes and Ward 1989b; Minniti et al. 1996; Nance et al. 1999, 2000; Geldziler et al. 2005). In this species, mutations in many of the genes required for sperm activation lead to hermaphrodite-specific sterility, suggesting that these genes do not have an obligate role in male sperm activation (Minniti et al. 1996; Nance et al. 1999, 2000; Geldziler et al. 2005). We therefore looked for homologs of such genes in the dioecious species, *C. remanei*. Although *C. remanei* has only a male–female mode of reproduction, these genes [except for *spe-12*, a novel peripheral membrane protein of unknown biochemical function (Nance et al. 1999) are all conserved, suggesting that in *C. remanei* males, the pathway for sperm activation has been conserved at the molecular level.

This begs an interesting question—if these genes are required only for hermaphrodite sperm activation, why are they conserved in a species without hermaphrodites? Several possibilities exist. Perhaps these molecules occur vestigially and have no real function in *C. remanei*. We consider this to be unlikey, however, as hermaphrodism is thought to be derived from an ancestral gonochoristic state (Cho et al. 2004; Kiontke et al. 2004). Perhaps, instead, these molecules do function in males in *C. remanei* (and possibly in *C. elegans* and *C. briggsae* as well) and have been co-opted to function in *C. elegans* and *C. briggsae* hermaphrodites. Functional studies involving knocking out these genes in *C. remanei* would be useful to address this question. Also, once the molecules that trigger the activation of sperm in males and hermaphrodites have been identified in *C. elegans*, it would be of great interest to see whether these molecules are also conserved in *C. remanei* spermiogenesis. Such studies might also shed light on the evolution of the male–female paradigm of sexual reproduction and help us better understand the evolution of molecules involved in reproduction.

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