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Reproductive Function for a C-terminus Extended, Male-Transmitted Cytochrome *c* Oxidase Subunit II Protein Expressed in Both Spermatozoa and Eggs

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Abstract

Our previous study documented expression of a male-transmitted cytochrome *c* oxidase subunit II protein (M*COX2*), with a C-terminus extension (M*COX2*e), in unionoidean bivalve testes and sperm mitochondria. Here, we present evidence demonstrating that M*COX2* is seasonally expressed in testis, with a peak shortly before fertilization that is independent of sperm density. M*COX2* is localized to the inner and outer sperm mitochondrial membranes and the M*COX2* antibody's epitope is conserved across >65 million years of evolution. We also demonstrate the presence of male-transmitted mtDNA and season-specific M*COX2* spatial variation in ovaries. We hypothesize that M*COX2* plays a role in reproduction through gamete maturation, fertilization and/or embryogenesis.

Keywords

cytochrome c oxidase subunit II; COX2; DUI; C-terminus extension; mtDNA; male-transmitted mitochondria; Unionoidea

1. Introduction

Mitochondrial DNA (mtDNA) inheritance in some bivalves involves distinct maternal and paternal transmission routes and divergent gender-associated mtDNA genomes. Therefore, doubly uniparental inheritance (DUI) of mtDNA is a genetic transmission system that violates the rule of standard maternal inheritance (SMI) of organelles [1]. DUI was discovered in *Mytilus* and our brief overview is based principally on that genus. In lineages with DUI, mothers

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transmit mitochondria containing female-transmitted mtDNA (F-type) to both sons and daughters (identical to mothers in SMI systems). Unlike SMI, fathers also transmit mitochondria, containing paternally inherited mtDNA (M-type), to their sons and daughters. However, shortly after fertilization, embryos destined to become female either lose this M-type mtDNA (true homoplasmy) or the amount of M-type mtDNA declines to low levels (functional homoplasmy) [2,3]. Relatively low levels of M-type are often present in the somatic tissues of males, but it is abundant in testicular tissue [4,5]. The observed lack of deletions and premature stop codons in M genomes is inconsistent with the hypothesis that M genomes are selfish genetic elements [6]. Specialization for selectively advantageous male reproductive functionality has been offered as an ultimate explanation for the maintenance of male-transmitted mt genomes, with sperm competition hypothesized as the proximate driving force [7,8]. An alternative model postulates a causal relationship between the retention of paternally transmitted mitochondria and sex determination with the latter being controlled by nuclear genes in the mother [9].

In animals, cytochrome *c* oxidase subunit II (*COX2*) is a mitochondrially encoded protein functioning in the inner mt membrane. It belongs to the respiratory chain terminal enzymatic complex (IV) and facilitates transfer of electrons from cytochrome *c* to molecular oxygen [10]. The M genomes of unionoidean bivalves possess a unique, rapidly evolving coding 3′ extension of *cox2*, resulting in an ~80% increase in gene length over that in the corresponding F genomes [11,12]. Using *Venustaconcha ellipsiformis* as a model system, we demonstrated that the M*COX2e* coding extension is expressed as multiple transmembrane helices (TMHs) [13]. Based on the taxonomic conservation of the M*cox2*e gene region and the tissue distribution of the extended M*COX2* protein in *V. ellipsiformis*, we hypothesized functional significance for M*COX2*e.

In the present study, we (1) investigated in detail the tissue distribution of M*COX2* in both male and female *V. ellipsiformis*, (2) studied the seasonal aspects of M*COX2* expression in *V. ellipsiformis*, (3) utilized immunohistochemistry (IHC) to localize M*COX2* in male and female gonads, (4) identified the sub-cellular location of M*COX2* in sperm, using immuno electron microscopy (IEM) and (5) analyzed testes protein extracts from representatives of six additional genera to determine if M*COX2*e is expressed in unionoidean bivalve species other than *V. ellipsiformis*. Our results indicate, for the first time, a role for a mitochondrion-encoded protein, M*COX2*, in male and female reproduction.

2. Methods

2.1. DNA extraction and PCR analyses

DNA extraction and general PCR protocols were performed as described in [14]. The M genome-specific primer pair MCOI 22F and HCO-700dy2, which amplifies the 5′ half of M*cox1* in *V. ellipsiformis* (716 bp amplicon) [14], was used to assess M-type mtDNA presence/absence in *V. ellipsiformis* DNA extracts. Amplicons were detected in 2% NuSieve GTG (Cambrex Bio Science Rockland, Inc., Rockland, ME) agarose gels post-stained with SYBR Green® (Invitrogen Corp., Carlsbad, CA).

2.2. Protein sample preparation and Western blots

Male and female tissues stored at −70°C were homogenized in RIPA lysis buffer containing proteolytic inhibitors. Tissue preparations were separated by 12% SDS-PAGE and Western blotting was performed as described in [13].

2.3. Immunohistochemistry

Bivalve tissues were fixed in 1.5% formaldehyde in phosphate-buffered saline (PBS) at 4°C overnight. Immunostaining was performed as described in [13].

2.4. Electron microscopy

IEM (Immunoelectron Microscopy)—Bivalve tissues were fixed in 1.0% glutaraldehyde and 4.0% paraformaldehyde in 0.1M phosphate buffer, pH 7.2, for 4 h and washed 3 times for 10 min in buffer. Tissues were sectioned (70nm), placed on nickel grids and incubated for 15 min in a low molecular weight blocking solution (0.05% glycine in PBS), followed by 30 min in a high molecular weight blocking solution (goat block solution, Electron Microscopy Sciences, Fort Washington, PA) and a 5 min wash in incubation buffer (10mM phosphate buffer, 150 mM NaCl, pH 7.4 with 0.2% BSA-c and 15 mM NaN_3). Sections were then incubated with the M*COX2*primary antibody (1: 200; [13]) in incubation buffer for 1 h. After 3, 5 min incubation buffer washes, grids were placed in gold solution (10 nm gold in goat anti rabbit serum, diluted 1: 20 in incubation buffer, Electron Microscopy Sciences, Fort Washington, PA) for 2 h. Six, 5 min incubation buffer washes were followed by 3, 5 min. PBS washes. Sections were post fixed in 2.0% glutaraldehyde in PBS for 5 min, washed in PBS for 5 min and 5 times in dH_20 for 2 min each. Sections were post stained in uranyl acetate for 15 min and lead citrate for 1 min and viewed with a JEOL 100 CX TEM.

SEM (Scanning Electron Microscopy)—Bivalve tissues were prepared for SEM in 2.5% glutaraldehyde in mussel buffer [15] followed by four phosphate buffer washes and post-fixation in 1.0% OsO_4 in the latter buffer. After four phosphate buffer washes, the tissue was dehydrated in a graded series of acetone, critical point dried, mounted on aluminum stubs, sputter coated with gold, and viewed in a JEOL JEM5400 SEM.

3. Results

3.1 Presence of M-type mtDNA in Venustaconcha ellipsiformis ovaries

Using the M genome-specific primer pair, we tested for the presence of M-type genomes in *V. ellipsiformis* gonadal tissues. The 716 bp M*cox1* amplicons obtained using template DNA from female gonads indicate the presence of M genomes in this tissue (Fig. 1). M*cox1* amplicons derived from testicular DNA were detectable using ethidium bromide staining but the amplicons derived from ovarian DNA necessitated visualization with the more sensitive SYBR Green staining technique.

3.2 Seasonal expression of MCOX2 in testes and ovaries

To examine the functional aspects of M*COX2*, we investigated its expression in a year-long study of *V. ellipsiformis*, which produces one brood of young per year. Testes M*COX2* expression was higher during the summer months, with maximum expression observed just prior to fertilization (determined by the appearance of brooded embryos in females [Fig. 2A]). Testicular M*COX2* expression decreased following the appearance of brooded embryos and little to no expression was observed in all non-summer months (Fig. 2A, D). Male somatic tissues (*e.g.*, mantle) and female ovary showed no seasonal pattern in M*COX2* expression (Fig. 2C and 2E, respectively) suggesting that the pre-fertilization spike in M*COX2* expression occurs only in male gonads. Testicular sperm densities, estimated for 18 individuals from 7 μm paraffin sections stained with hematoxylin and eosin, varied little (Fig. 2F) over four sampling dates (covering the 8 July 2006 to 25 July 2006 time period). Additionally, all testicular (N=20) and ovarian (N=8) tissues analyzed *via* western blotting tested positive for the M*COX2* protein.

3.3 Immunohistochemistry of testes and ovaries demonstrates unique spatial distribution of MCOX2

To better understand the spatial expression of M*COX2*, we examined M*COX2* in testes and ovaries using immunohistochemistry. Testicular M*COX2* expression showed distinct spatial and temporal patterns (Fig. 3A, B, C). Approximately five weeks prior to fertilization, M*COX2* expression was weak in the testicular acini (arrow pointing to acinar wall) despite the presence of numerous sperm in the acinar lumen (Figs. 3A, D). Two weeks prior to fertilization, M*COX2* expression was strong (Fig. 3B, E) in sperm and acinar walls (arrow), which is in agreement with western blot data (Fig. 2A) and localization of M*COX2* to sperm mitochondria [13]. Approximately 10 days post-fertilization, testicular M*COX2* expression was reduced, despite the presence of sperm in the acini (Figs. 3C, F), and principally appeared in the acinar walls (arrow).

In ovaries, M*COX2* expression remained relatively constant (Fig. 2E) but the spatial distribution appeared to change over the three IHC time points (Fig. 3G, H, I). At the earliest time point, M*COX2* expression was uniform across the ovarian tissue sections (Fig. 3G) with the protein found in eggs, acinar walls (arrow) and muscle fibers (double arrow). Just prior to fertilization, M*COX2* displayed a more heterogeneous localization and was expressed in ovarian acinar walls (arrow) and in some eggs (Fig. 3H). Other eggs displayed low or undetectable levels of M*COX2* expression. After fertilization, high levels of M*COX2* expression were localized in acinar walls (arrow) and in a small number of eggs (Fig. 3I). In eggs, M*COX2* was found in either the cytoplasm, nucleoplasm or in both locations but never in the nucleolus.

3.4 Electron microscopy indicates multiple sub-cellular locations for MCOX2 in sperm

We identified the sub-cellular location of M*COX2* in mature sperm from three unionoidean bivalve species: *V. ellipsiformis*, *Fusconaia subrotunda* and *Plethobasus cyphyus*. Unionoidean spermatozoa contain five large, spherical, radially arrayed mitochondria, which are not covered by a sheath (Fig. 4A). Spermatozoan mitochondria consistently displayed identical M*COX2* antibody-mediated immunogold labeling in our IEM experiments (Fig. 4B). Staining was observed in both the outer and the inner mitochondrial membranes (Fig. 4B), but no signal was detected with the secondary antibody alone (Fig. 4C).

3.5 The MCOX2 antibody epitope is conserved across nine unionoidean bivalve genera

To examine if M*COX*2 expression is present in other unionoidean bivalve genera, we performed Western blot analysis on testicular extracts from representatives of six additional genera (Fig. 5). The M*COX2* protein was observed around the 36 kDa region in the six additional genera surveyed (Fig. 5). The observed unequal band intensities are likely due to interspecifically variable antibody-epitope affinities and that most tissue samples were collected irrespective of reproductive season.

4. Discussion

Our seasonal study of M*COX2* expression and histological localization in *V. ellipsiformis* provides insights into the functional aspects of M*COX2* (Figures 2 & 3). The seasonal peak in testicular M*COX2* expression (both WB and IHC data) immediately prior to fertilization and independent of sperm density, its low expression in testes during the winter months (= non-reproductive season) and the low, uniform expression in male somatic tissues suggests that M*COX2* functions in male reproduction (e.g., sperm maturation, fertilization and/or embryogenesis). The apparent over-expression of *COX2* during spermatogenesis in rats and humans [16, 17], as well as in bivalves, suggests that *COX2*'s involvement in male reproduction may be a relatively ancient and general phenomenon.

The presence of M-type mtDNA and M*COX2* in *V. ellipsiformis* ovaries, as well as the former in *Mytilus galloprovincialis* ovaries [3], suggests that adult females in DUI systems are heteroplasmic for the F- and M-type mt genomes. Thus, M genomes derived from their fathers' sperm are not completely lost in adult females, but rather persist and are expressed in ovarian tissue. Whether the "native" M-type mtDNA located in ovaries is typically transmitted to progeny [3] or represents an evolutionary dead-end is unclear. Our previous failure [13] to detect the presence of M*COX2* in female gonadal tissues was likely due to the small number of samples analyzed and the spatial heterogeneity of M*COX2* localization in ovarian tissue. Although the overall level of M*COX2* expression in female gonad does not fluctuate substantially over the oogenenic cycle, M*COX2* spatial distribution does vary with the timing of reproduction. The localization of M*COX2* in eggs occurs immediately prior to fertilization and is consistent with a reproductive role for M*COX2* in females.

As expected, our IEM findings support an inner mt membrane function for M*COX2*. These observations are consistent with the hypothesis that unionoidean bivalve M*COX2* participates in electron transport chain (ETC) processes. Surprisingly, our IEM experiments also indicate an outer mt membrane location for M*COX2* (Fig. 4), consistent with a novel, non-ETC function for the protein in sperm mitochondria. Given the exposed, outer membrane location of M*COX2* (Fig. 4) and the TMH structure of the M*COX2*e region [13], the protein may serve to "tag" sperm-derived mitochondria to enable gender-specific mt localizations similar to that observed in *Mytilus* [18,19]. The conservation of the M*COX2* antibody's epitope (located in the M*COX2*e C-terminus tail) over >65 myr of evolutionary history (Fig. 5) is consistent with the hypothesis that the mt surface tagging involves the C-terminus tail of M*COX2*e.

Given our evidence for spatial and temporal heterogeneity of M*COX2* expression, we can address the hypotheses regarding the maintenance of male-transmitted genomes under DUI. The "specialization for selectively advantageous male reproductive functionality" hypothesis is unlikely given the biparental maximal just before fertilization gamete expression phenotypes of M*COX2*. An obligate functionality for M*COX2* in eggs would oppose any selective pressure on the M genomes to increase male fitness at the expense of female fitness. Alternatively, the "participates in sex determination" hypothesis for the maintenance of male-transmitted genomes is still viable.

Overall, these findings suggest opportunities for new insights into the evolutionary processes responsible for the origin and maintenance of organellar genetic transmission systems. The novel expression and localization patterns of M*COX2* and the over-expression of *COX2* [16, 17] are consistent with the ability of mtDNA-encoded gene products to participate in non-ETC processes [20–22], including germ line formation [23]. Experimental studies, in conjunction with evolutionary genetic analyses, are needed to further clarify the functions of the M*COX2* protein and the mt transmission genetics of DUI-containing species.

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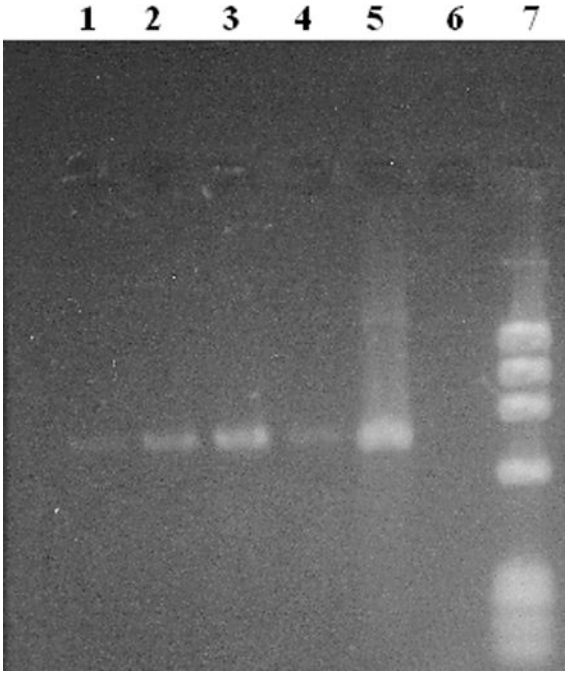

Figure 1. Presence of M-type mtDNA in *Venustaconcha ellipsiformis* female gonad

A SYBR Green stained, 2% agarose gel displaying M*cox1* amplicons (716 bp) obtained from *V. ellipsiformis* female (lanes 1–4) and male (positive control, lane 5) gonad DNA extractions. Twenty microliters (of 50 μl total reaction volumes) was loaded in lanes 1–4 & 6 with 5 μl loaded in lane 5. A negative control is in lane 6 and a phiX174/HaeIII size marker in lane 7.

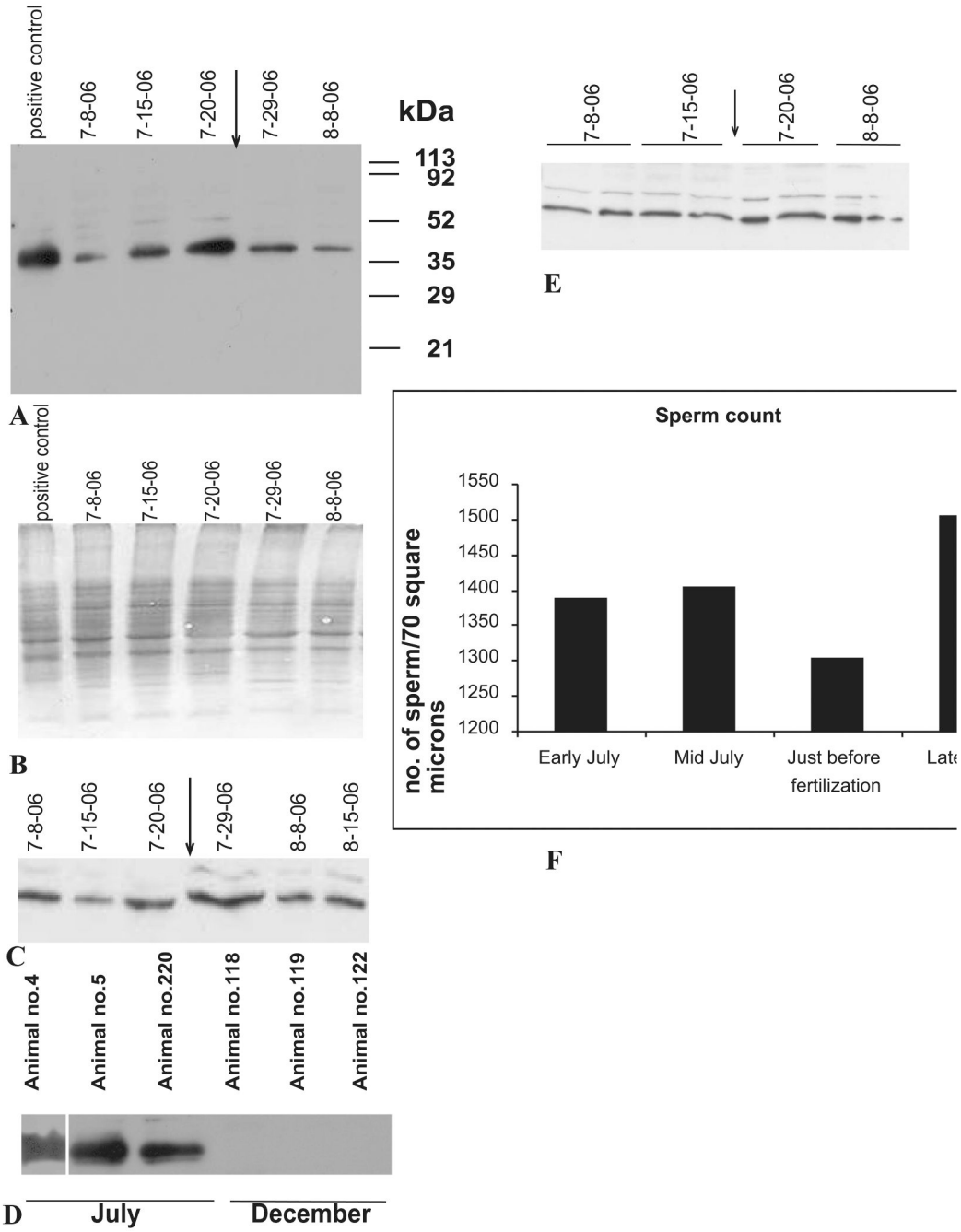

Figure 2. Western blots demonstrating the seasonal aspects of M*COX2* expression in *Venustaconcha ellipsiformis* gonadal and somatic tissues

(A) Illustrates the testicular M*COX2* expression phenotype in *V. ellipsiformis* tissues collected on multiple dates before and after the initiation (indicated by arrow) of female brooding of embryos in 2006. The positions of marker proteins are shown (in kDa). (B) SDS gel electrophoresis image of (A). (C) Expression of M*COX2* in mantle tissue from male *V. ellipsiformis* from the same time points as given in (A). (D) M*COX2* expression phenotypes from three mid-July 2006- *vs.* three December 2005-collected testes samples. (E) Expression of M*COX2* in *V. ellipsiformis* ovaries from the same time points (two animals per time point) as in (A). All lanes in (A), (B), (C), (D) and (E) were loaded with an equivalent amount of

protein. (F) Graphical representation of mean testicular sperm counts from a total of 18 individuals over four sampling dates.

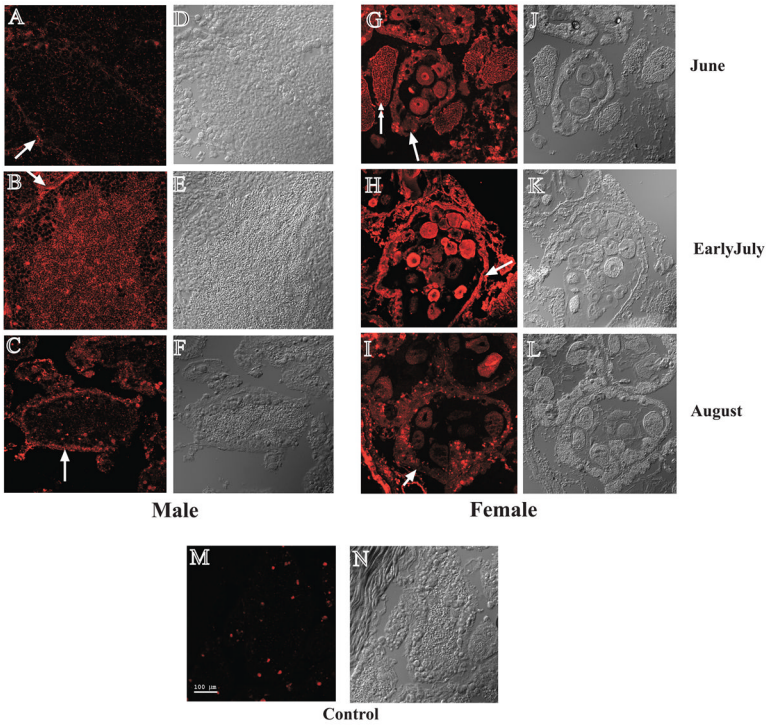

Figure 3. Spatial distribution of M*COX2* in male and female gonads

(A-C) M*COX2* expression in male gonads at three time points. Note: distribution of M*COX2* changes from before to after fertilization period with its expression peaking just before fertilization (see Fig. 2 for fertilization timing). Images on the right (D-F) are the corresponding brightfield DIC images of the confocal immunofluorescence images on the left. (G-I) Spatial distribution of M*COX2* in female gonads, at three time points showing maximum expression of M*COX2* in the cytoplasm and nucleoplasm of eggs just prior to fertilization. Images on the right (J-L) are the corresponding brightfield DIC images of the confocal immunofluorescence images on the left. (M-N) Only relatively small, localized areas demonstrated immunoreactivity in male gonads using only secondary antibody. Bar (A-N) = 100 μm

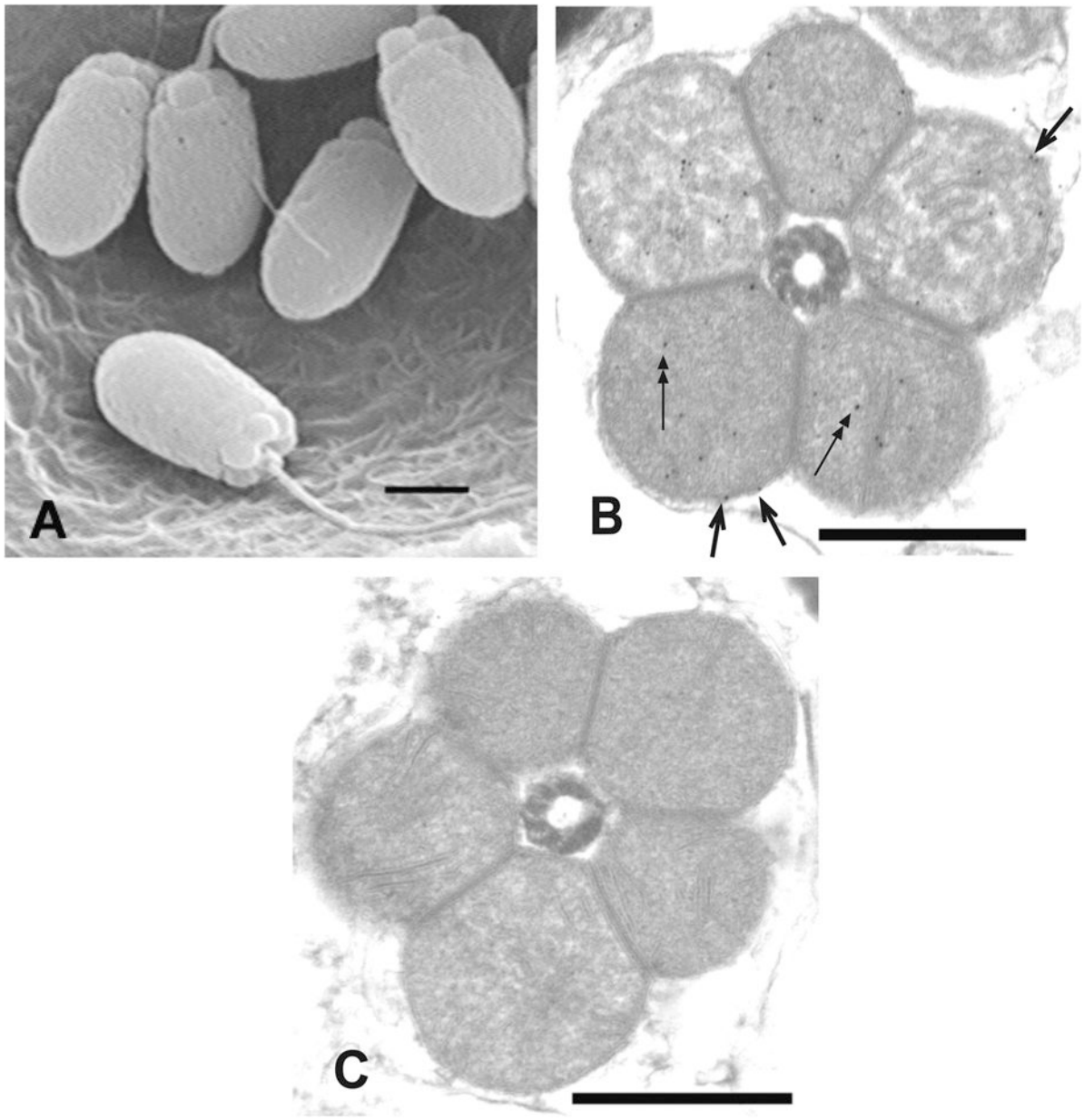

Figure 4. EM photomicrographs demonstrating unionoidean bivalve sperm morphology and the sub-cellular location of M*COX2* in sperm mitochondria

(A) The general morphology of unionoidean bivalve spermatozoa (SEM of *Plethobasus cyphyus* sperm; scale bar = 1μm). (B) experimental IEM photomicrograph which displays immunogold labeling, using the M*COX2* primary antibody, in both the inner (two headed arrows) and outer (single headed arrows) mt membranes in a cross-section of the mitochondrial "ring" from *Fusconaia subrotunda* sperm, and (C) a negative control IEM photomicrograph from *F. subrotunda* sperm (both IEM scale bars = 0.5μm).

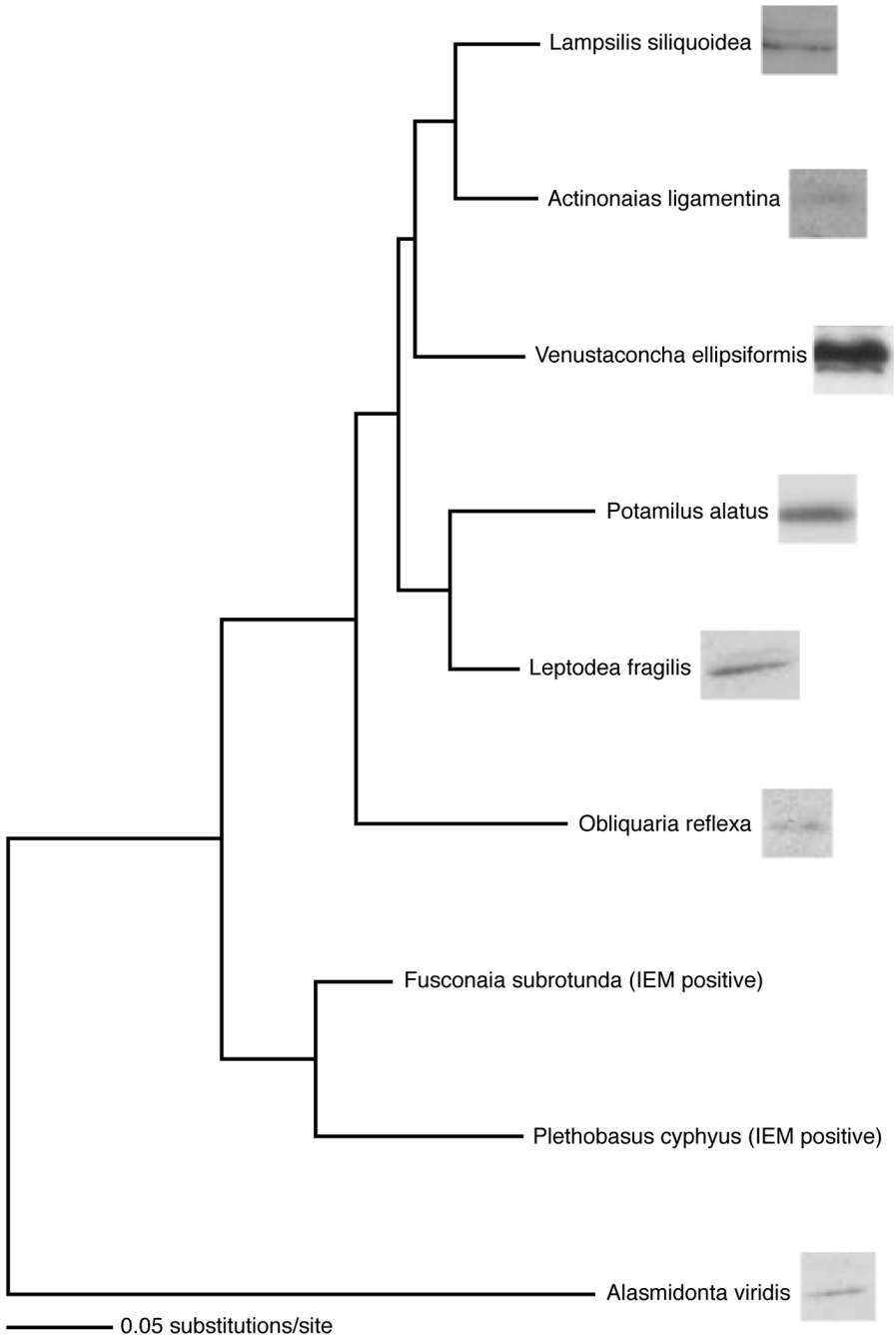

Figure 5. Evidence for expression of M*COX2* in male gonads from nine unionoidean bivalve genera

Evolutionary tree depicting species relationships and maximum likelihood-based patristic distances, using F*cox1* DNA sequences, among the nine genera represented in this study (tree after Campbell *et al.* [24]). Western blot analysis lanes are presented after the species names and show M*COX2* expression in male gonads representing seven genera. In *Fusconaia* and *Plethobasus*, M*COX2* expression is evidenced by IEM results. The divergence of these genera occurred over an evolutionary history >65 myr. Equal amounts of protein were loaded in all lanes.