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Sex-Associated Mitochondrial Genes: Tools for Understanding Unionid Reproduction and Development

Amy Louise Maynard

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**SEX-ASSOCIATED MITOCHONDRIAL GENES: TOOLS FOR
UNDERSTANDING UNIONID REPRODUCTION AND DEVELOPMENT**

A Masters Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Amy Louise Maynard

December 2015

**SEX-ASSOCIATED MITOCHONDRIAL GENES: TOOLS FOR
UNDERSTANDING UNIONID REPRODUCTION AND DEVELOPMENT**
Biology

Missouri State University, December 2015

Master of Science

Amy Louise Maynard

ABSTRACT

Gonochoristic species of the bivalve order Unionida possess two types of mitochondria. The female-inherited mitochondria (F-mt) is found in the somatic cells of both sexes, while male-inherited mitochondria (M-mt) is found in the male gonad. These mitochondria possess different open reading frames (*M-ORF* or *F-ORF* respectively). Previous studies suggested that two types of sperm might exist, each carrying one or the other mitochondrial haplotype, and it was hypothesized that the presence of either F-mt or M-mt in sperm may be involved in determination of offspring sex. I investigated this hypothesis in Bleedingtooth (*Venustaconcha pleasii*). Sperm samples were obtained from field-collected males that spawned spontaneously in the lab. *M-ORF* and *F-ORF* mitochondrial DNA were cloned and sequenced, and PCR was used to test for the presence of *M-ORF* and *F-ORF* genes within spermatozoa. *F-ORF* was not detected within sperm of *V. pleasii* despite previous reports of the protein product of *F-ORF* within sperm of *V. ellipsiformis*. *M-ORF* was present within sperm and more than one haplotype of this gene appeared to exist within an individual mussel. *M-ORF* and *F-ORF* evolve rapidly and may be useful for phylogenetic comparisons. Comparisons with *V. ellipsiformis* showed 82.6% (*M-ORF*) and 76.1% (*F-ORF*) sequence homology. Comparison of *F-ORF* and *M-ORF* in *V. pleasii* display low sequence homology (20.6%). My results indicate that sperm only deliver the M-mt haplotype and therefore, that sex determination must involve mechanisms other than sperm mtDNA haplotype.

KEYWORDS: Unionida, mussel, *Venustaconcha pleasii*, mitochondria, sperm

This abstract is approved as to form and content

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Chairperson, Advisory Committee
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INTRODUCTION

Freshwater mussels (Bivalvia: Order Unionida) are a group of animals with ecological, economic, and conservation importance (Lydeard et al. 2004, Haag 2012). Mussels are suspension feeders of phytoplankton, bacteria, and organic detritus in rivers and lakes. When abundant, they provide significant ecosystem services including energy transfer, nutrient cycling and benthic habitat (Vaughn and Hakencamp 2001, Vaughn et al. 2008, Limm and Power 2011, Atkinson et al. 2013, Strayer 2014). Freshwater mussels are the basis of the worldwide cultured pearl industry (Dan and Ruobo 2002). Mussels are a rich source of environmental information. Individuals live for decades and their shells provide a record of past conditions of their aquatic habitat (McConnaughey and Gillikin 2008). Mussels are also increasingly important as indicators of water quality because they are sensitive to both sediment and water pollution (Angelo et al. 2007, Bringolf et al. 2007a,b,c, Wang et al. 2007a,b, Cope et al. 2008). Many freshwater mussels are of conservation concern. Seventy-four percent of North American species are federally classified as vulnerable, threatened, or extinct, leaving a mere twenty-six percent of species classified as stable (Williams et al. in press). Conservation efforts through habitat protection, research, propagation, and policy are all essential to the stability and recovery of endangered species.

Reproductive Biology

There is a particular need to understand the reproductive biology of mussels, which is complex and potentially vulnerable to disruption. Much of what is known about

the complex Unionoid life cycle was established 100 years ago, when mussels were studied intensively in efforts to enhance the pearl button industry (LeFevre and Curtis, 1912), but significant knowledge gaps remain. Mussels are typically dioecious, although completely hermaphroditic species are known to exist, and many dioecious species have been observed to have rare hermaphroditic individuals in their populations (van der Schalie 1970, Heard 1975, Bauer 1987, Ortmann 1919, Tepe 1943, Heard 1970, Cummings and Graf 2015). The mechanism for sex determination in Unionida is unknown, but a role for sperm and mitochondria in sex determination has been suggested and will be discussed in further detail (Breton et al. 2011, Shepardson et al. 2012).

Mussel spawning is seasonal in most North American species (Haag 2012). One cue for spawning appears to be water temperature (Hastie and Young 2003, Galbraith and Vaughn 2009). For example, the spawning of *Margaritifera* was denoted to be associated with rising temperature in the spring (Hastie and Young 2003). Although data are lacking for Unionoids, chemical signaling among individuals is involved in other bivalves. Chemical cues from algae trigger marine bivalves to spawn (*Ostrea gigas*, *Mytilus californianus*, *Mytilus edulis*), chemical cues from sperm trigger females to spawn (*M. edulis*, *Ostrea virginica*, *Dreissena polymorpha*), and cues from females cause males to continue spawning (*O. virginica*, *Crassostrea gigas*, *D. polymorpha*) (Ram and Nichols 1993). Osphradia, chemoreceptors within the mantle cavity of mollusks, are hypothesized to play a role in spawning coordination, but evidence for this has yet to be presented (Haszprunar 1987).

Male mussels fertilize downstream females by spermcasting, which is defined as the broadcasting of sperm by males to females that retain and brood their fertilized ova

(Bishop and Pemberton 2006). Male mussels release sperm in the form of spermatzeugmata (SZ), which are aggregations of sperm embedded in a spherical cupula (Figure 1) (Edgar 1965, Lynn 1994, Waller and Lasee 1997). The sperm head and midpiece are located inside the cupula and the flagella extend outside. The cupula is not cellular, but rather a gel-like matrix (Lynn, 1994). SZ range in diameter from 40 to 76 μ m and each contains thousands of sperm. SZ may limit sperm dilution in water, which may be important for successful fertilization of eggs (Cummings and Graf 2015).

Female mussels retain their eggs in spaces within their ctenidia (gills). The ctenidia are hollow structures adapted for suspension feeding. Water enters through microscopic openings and flows dorsally in spaces called water tubes. The water tubes converge on a dorsal passage at the top of the ctenidium, where the water flows posterior to the excurrent aperture (Silverman et al. 1997, Jørgensen et al. 1984). Female mussels also use the water tubes for incubation (brooding) of the eggs. It is believed that fertilization occurs in the dorsal passages of the ctenidia before the eggs are deposited into the water tubes (Matteson 1948), but the details of fertilization and deposition of the eggs has yet to be documented. During brooding, Unionoid embryos develop into a parasitic larva. In the family Unionidae the larva is a glochidium (plural glochidia). Glochidia typically attach to a fish host where they undergo metamorphosis to the juvenile stage (Carus 1832, Lefevre and Curtis 1912, Barnhart et al. 2008).

The duration of brooding varies among mussel species. Bradytictic mussels, or long-term brooders, spawn in the summer or fall, brood their developed larvae over the winter and release them in the spring or summer. Tachytictic mussels, or short-term

brooders, spawn in the spring, summer or fall, depending on species, and release their larvae immediately after development is complete (Ortmann 1911).

Doubly Uniparental Inheritance of Mitochondria

Yet another distinctive aspect of mussel reproductive biology is the sex-associated pattern of inheritance of mitochondria. In most organisms, the mitochondria (mt) of an individual are maternally derived (strict maternal inheritance, or SMI). In contrast, Unionida and at least two other bivalve clades exhibit doubly uniparental inheritance, or DUI, a system in which two mitochondrial lineages are maintained throughout a male individual's entire life (Skibinski et al. 1994a,b; Zouros et al. 1994a,b; Hoeh et al. 1996). A male-transmitted mt lineage, or M-mt, is inherited from the male parent and a female-transmitted mt lineage, or F-mt, is inherited from the female parent. Bivalve mollusks are the only animals known to exhibit such a system. Some plants and fungi exhibit biparental inheritance of mitochondria in the zygote, but paternal mitochondria do not persist as the embryo develops (Barr et al. 2005).

Among the bivalves, DUI has been documented only in the orders Mytiloida, Unionida, and one species of Veneroida (*Ruditapes philippinarum*) (Breton et al. 2007, Theologidis et al. 2008, Doucet-Beaupre et al. 2010, Zouros 2012). DUI has been sought, but not detected, in the oysters (Ostreoida) and scallops (Pectinoida) (Zouros 2012).

Most investigators have assumed that all sperm deliver M-mt to the embryos, while ova carry F-mt. Thus, both sexes of embryos would receive the paternal M-mt. However, the M-mt generally persist only in sons. *Mytilus* female embryos lose most or

all of their M-mt within 24 hours after fertilization (Sutherland et al. 1998) and are nearly homoplasmic when development is complete (Zouros 2012). Some evidence suggests that the mechanism for this difference involves different patterns of segregation of the sperm mt during embryonic development. Within male embryos, the five mitochondria from the fertilizing sperm evidently remain together in one cell during each of the early embryonic cell divisions, so that they are eventually segregated into the embryonic germ line cells (Cogswell et al. 2006). In contrast, it appears that in female zygotes the sperm mitochondria are dispersed in the cytoplasm before cell division, perhaps via microtubules (Cao et al. 2004, Obata and Komaru 2005, Cogswell et al. 2006).

In DUI bivalves, F-mt dominates somatic tissues while M-mt dominates the male gonad (Fisher and Skibinski 1990, Breton et al. 2009). In *R. philippinarum*, only F-mt was found in the ovaries and only M-mt was found in the testes (Passamonti 2003). However, in mytilids the tissues may not be purely homoplasmic. In *Mytilus*, F-mt can be detected within male gonads, and M-mt can sometimes be detected within male and female somatic tissues (Garrido-Ramos et al. 1998). Using real time PCR, Dalziel and Stewart (2002) found M-mt in somatic tissues of 15/30 of male *Mytilus* tested and in gonadal tissues of 1/6 females. They also found F-mt in 7/8 male gonads.

Masculinization is a phenomenon inferred to have occurred in some mytilids in which the M-mt lineage was lost and replaced by a new M-mt lineage derived from F-mt. When masculinization occurs, sequence divergence between F-mt and M-mt resets to zero. The new M-mt lineage then evolves and diverges over time, becoming more like the lost M-mt (Hoeh et al. 1997, Hoeh et al. 1996, Passamonti and Scali 2001). There appears to be no evidence that freshwater mussels have experienced masculinization

(Hoeh et al. 2002). The evidence against masculinization lies in the variable divergence between F-mt and M-mt sequences among species. In mytilid species this divergence is 2–20%, but the divergence is 28–34% in three species of Unionida (*Pyganodon grandis*, *Fusconaia flava*, and *Lampsilis fragilis*) (Hoeh 2002).

Sex-Associated Mitochondrial Genes

A typical Unionoid M-mt genome is about 17kb while the F-mt genome is about 16kb (Figure 2) (Breton et al. 2011). The size difference varies among bivalve taxa and even among congeners. The M-mt genome of *Mytilus galloprovincialis* is about 1kb larger than the F-mt (Mizi et al. 2005), while the M-mt and F-mt genomes of *M. edulis* differ by only about 100bp (Breton et al. 2006). A unique feature of the Unionoid M-mt is an extension of the *cox2* (cytochrome oxidase subunit 2, *coII*) gene. This extension is known as *cox2e*. It has been suggested that *cox2e* may facilitate the movement of the M-mt to the male gonad during development (Cao et al. 2004, Chakrabarti et al. 2006, Cogswell et al. 2006, Breton et al. 2007, Chakrabarti et al. 2007, Chapman et al. 2008).

Two other mt genes of particular interest are the female and male open reading frames (*F-ORF* and *M-ORF*). An open reading frame (ORF) is a sequence that begins with a start codon, ends with a stop codon, and has the potential to encode a polypeptide. Unassigned regions (UAR) of the Unionoid mt genome held candidates for protein coding genes that were designated as the *F-ORF* and the *M-ORF* (Breton et al. 2009). The ORF spanned most of the length of the sex-specific UAR in two Unionoid species (*Inversidens japonensis* and *Hyriopsis cumingii*). Interestingly, *F-ORF* and *M-ORF* are located on opposite strands of the mtDNA. *M-ORF* is on the heavy strand and *F-ORF* is

on the light strand (Breton et al. 2009). The heavy and light strands of mt DNA are distinguished by the ratios of purines and pyrimidines. Each strand is transcribed by its own promoter, has its own origin of replication, and their replication proceeds in opposite directions.

There are several methods by which to infer whether an ORF is a functional protein coding sequence (PCS). In one such method, named TESTCODE, values are assigned to a sequence as non-coding, no opinion, or coding based upon the regularity in which Thymine bases appear at the (2+3N) nucleotide position (i.e.: 2, 5, 8, 11...) (Fickett 1982). Another is the direct detection of homologous mRNA or proteins. Breton et al. (2009) conducted TESTCODE analysis indicating that *F-ORF* and *M-ORF* were PCS with 77% or greater likelihood for 5 species (*Cristaria plicata*, *H. cumingii*, *Lampsilis ornata*, *Quadrula quadrula*, and *V. ellipsiformis*). Two other species (*I. japonensis* and *P. grandis*) exhibited a 40% probability of being PCSs. The predicted M-ORF and F-ORF polypeptides also contain a single putative trans-membrane helix (TMH), giving further support to their role as PCSs. Proteins containing TMH are typically transmembrane proteins, weaving in and out of a cellular membrane and stably inserted therein. Finally, proteins apparently homologous with the ORFs were detected (see below).

F-Type Sperm and M-Type Sperm

In *V. ellipsiformis*, proteins apparently homologous with *F-ORF* were found in eggs and proteins apparently homologous with *M-ORF* were detected in male gonadal tissues using Western blots. M-mt specific proteins (F-ORF and COX2E) were observed

in sperm using immunoelectron microscopy (IEM) (Breton et al. 2009, Shepardson et al. 2012). The latter study revealed that individual sperm possessed proteins encoded by either the M-mt or F-mt (COX2E or F-ORF), but not both. These two sperm types will be referred to as F-type and M-type sperm. Researchers hypothesized that the ORF genes or their products might be involved in the transmission of the M-mt and F-mt to offspring or in other ‘gender-specific adaptive functions’ and possibly even determination of sex (Breton et al. 2009; Doucet-Beaupre et al. 2010; Breton et al. 2011).

It has been known for decades that freshwater mussels typically use two different cellular pathways for spermatogenesis, termed typical and atypical spermatogenesis. Both pathways apparently produce morphologically identical sperm (Heard 1975, Kotrla 1988). There is evidence that these two pathways involve different precursor cells and are spatially separated in the male gonad. Perhaps the two pathways produce the F-type and M-type sperm. The presence of an M-mt genome and presence of the atypical sperm production pathway are both derived character states relative to bivalves that lack DUI; for this reason Shepardson et al. (2012) suggested that sperm containing the F-mt genome are produced in the typical pathway and sperm containing the M-mt genome are produced in the atypical pathway. Interestingly, Heard (1975) noted that the atypical pathway, which may be linked to production of the male-determining sperm, was reduced in the hermaphrodite species *Utterbackia imbecillis* relative to gonochoristic species.

Sex Determination

Breton et al. (2011) presented further evidence that connects *F-ORF*, *M-ORF*, and dioecy. Firstly, they found that four hermaphroditic lineages of freshwater mussel each

possessed a modified F-mt genome (H-ORF) and lacked an M-mt genome. The nucleotide sequence of H-ORF is more similar to *F-ORF* than *M-ORF*, but differs from *F-ORF* by insertion of repeating units. These features appear to have evolved convergently 4 times. The loss of both dioecy and DUI seems to be linked to both the loss of *M-ORF* and the modification of *F-ORF*.

Another clue to ORF function was the detection by IEM of F-ORF protein within cytoplasm of the mitochondria, on the nuclear membrane and in the nucleoplasm of eggs. The extra-mitochondrial locations of the F-ORF protein suggest extra-mitochondrial functions. A 3-D structure prediction for the F-ORF protein using Iterative Threading ASSEmbly Refinement (I-TASSER) (Zhang 2008) suggested functions related to DNA replication and/or DNA binding (Breton et al. 2011). The *M-ORF* sequence of *V. ellipsiformis* may be involved in DNA or RNA binding based upon its degree of sequence similarity with the N-terminal nucleotide-binding domain of the seryl tRNA synthetase (Breton et al. 2009).

Thesis Objectives

I examined the possible role of DUI in determination of sex. The presence of two sperm types, distinguishable by proteins derived from F-mt and M-mt, respectively, led researchers to hypothesize that alternative gene products of the two mt haplotypes somehow determine offspring sex (Breton et al. 2011). Two alternative versions of this hypothesis can be proposed. In the first version, each sperm cell contains either F-mt or M-mt. According to this hypothesis, a zygote that receives M-mt becomes male, a zygote that receives F-mt becomes female, and mussels might control sex ratios by controlling

proportions of two sperm types (Shepardson et al. 2012). However, Venetis et al. (2006) were unable to demonstrate the presence of F-mtDNA in sperm of *Mytilus*. An alternative version of the sex determination hypothesis is that the sperm all deliver M-mt but differ in being “tagged” to colonize the gonad, or not, by the presence or absence of the protein products of *M-ORF*, *F-ORF*, or *cox2e* incorporated during gamete maturation.

Shepardson et al. (2012) presented evidence for F-ORF protein within sperm of *V. ellipsiformis*, but the presence of F-mtDNA in sperm has not been reported in any species of Unionida. In order to test this possibility, I obtained somatic and gonadal tissue and sperm samples from *Venustaconcha pleasii*, a gonochoristic species that is closely related to *V. ellipsiformis*. Second, I cloned and sequenced the *MORF* and *F-ORF* within *V. pleasii*. Thirdly, I developed primers homologous with *F-ORF* and *M-ORF* and used PCR to test for the presence of F-mt and M-mt in samples of sperm.

METHODS

Animal Collections and Spawning

I investigated sperm mt genetics in *V. pleasii*, a species common in headwater streams of the White River system in southern Missouri (McMurray et al. 2012). I chose this species because of its accessibility, small body size, and close phylogenetic relatedness to *V. ellipsiformis* (Zanatta and Harris 2013). All mussels were collected with appropriate permits. *Venustaconcha pleasii* were collected from Kinser Bridge and McGraw Ford of James River near Springfield, Swan Creek near Swan, and Finley River near Ozark, Missouri (Table 1, Figure 3). *Venustaconcha pleasii* were identified as male or female using morphological characteristics (female marsupial gill and sulcus of ventral margin of shell). To ensure that I would witness the spawning event I returned to one or more field sites each week during August to gather more animals, while returning the previously collected batch of animals.

I determined air and water temperature at the field sites using temperature loggers (DS1992L Maxim iButton®). The animals were housed within an incubator (Percival Scientific: Intellus Ultra) in aerated, 3.78-L jars containing about a 10-cm thick layer of large glass beads for substrate, or in 3-L tanks with no substrate. River water was collected weekly for culture purposes. Because of this, animals I housed in the laboratory were exposed to changes in river water chemistry weekly.

Temperature and photoperiod were adjusted to approximately match that of the study sites in order to encourage spawning. Captive animals were maintained at

temperatures between 24–25°C during the day and 20°C at night. During the final year of the study, animals were stored at a constant 22°C day and night.

Animals in the lab were checked daily for male spawning, which was detected visually by cloudy water (Figure 4). *Venustaconcha pleasii* males spawned readily after capture. To collect sperm I first attempted to filter spermatzeugmata (SZ) with a 35 micron nylon Nitex® mesh. This approach proved ineffective as SZ were disrupted by the mesh. Alternatively, sperm were collected from 30–350 mL of SZ-containing water. Sperm were pelleted by centrifugation at 3000 x g (Waterman-Storer 2001) and by decanting the supernatant. Sperm were stored in 15-mL BD Falcon® tubes containing 95% EtOH and held in a -80°C freezer.

Cloning and Sequencing

DNA was extracted from somatic and gonadal tissues using the QIAGEN DNeasy Blood and Tissue kit (QIAGEN 2006a). DNA samples were stored at -80°C in 95% EtOH. I designed primers for *M-ORF* and *F-ORF* based on orthologous genes in *V. ellipsiformis* mtDNA (NC_013659 and HM849530.1), using primer-BLAST software of GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) (Table 3). Because the *M-ORF* gene has not been previously delineated in the M-mt reference sequence for *V. ellipsiformis* on GenBank, I sequenced the entire span of the mt genome between *ND4L* and *ATP8*, including some overlap into the aforementioned genes (Figure 2).

I used touchdown PCR (Korbie and Mattick 2008) to amplify *M-ORF* within male gonadal tissue and *F-ORF* within female somatic tissue of *V. pleasii*. Touchdown PCRs were performed in a 19.7-μL reaction volume using the following cycling

parameters: 94°C for 2 min; 10 cycles: 94°C for 30 sec, 59°C (-1°C per cycle), and 72°C for 1 min; 28 cycles: 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 minute; 72°C for 15 min. Negative control PCR contained distilled water in place of DNA. Positive controls for *M-ORF* and *F-ORF* were gonadal and somatic tissue, respectively.

Using gel electrophoresis, I verified that the DNA was successfully amplified with the new primers I designed. The gels contained 2% agarose, 1 µg/mL ethidium bromide, and 0.5x Tris-borate-EDTA (TBE) buffer. Gel wells contained 5 µL DNA and 1 µL of 6x blue/orange loading dye.

I cloned mtDNA using Invitrogen's pCR™ 8/GW/TOPO® TA Cloning® kit (Life Technologies 2012) or Agilent's Strataclone™ PCR cloning kit (Agilent 2015). *Escherichia coli* vectors containing recombinant plasmids were selected on solidified Luria-Bertani (LB) medium containing agar (15%) and ampicillin (100 µg/µL), spectinomycin (100 µg/µL), or kanamycin (50 µg/µL). After approximately 12 hours of growth, colonies of *E. coli* vectors containing recombinant plasmids were transferred to a LB liquid medium supplemented with antibiotic and 3 mL of LB medium for another 12–16 hours. DNA was then extracted from the liquid culture using the GenElute™ Plasmid Miniprep kit (SIGMA-ALDRICH 2014); DNA was eluted and stored in sterile distilled water. The presence of the cloned DNA was verified using PCR and gel electrophoresis.

Mitochondrial DNA was sequenced at the Nevada Genomics Center at the University of Nevada, Reno for sequencing. Both strands of each fragment were sequenced to eliminate potential errors. The nucleotide sequences of the forward and reverse strands were inspected and were confirmed to be accurate. I then assembled sequences into contigs using CLC Genomics Workbench 8.0 (QIAGEN). Comparisons

were made between the newly constructed *V. pleasii* contigs and the entire published GenBank accessions for the *F-ORF* sequence in *V. ellipsiformis* (HM849529.1 (270bp), HM849530.1 (330bp)) and *M-ORF* (NC_013659). Predicted protein sequences were available on GenBank for *V. ellipsiformis* F-ORF (AEC14189.1 (long) and AEC14188.1 (short)).

I used CLC Genomics Workbench 8.0 for ORF detection, mtDNA translation, transmembrane helix prediction, phylogram production, alignments, and pairwise comparisons. Sequence translations were made using the invertebrate mitochondrial genetic code and were made to match *V. ellipsiformis* amino acid sequences from Breton et al. (2009). ORF detection used the invertebrate mt genetic code as well. The invertebrate mt genetic code utilizes several alternative start codons, AUA, AUU, AUC, GUG, and UUG, which have been utilized by invertebrates including Mollusca, Polyplacophora, and Athropoda. All of these start codons, have been found in *Lampsilis*, as well as one more, AUG (Serb and Lydeard 2003), which I incorporated into all necessary analyses. The invertebrate mt genetic code also incorporates alternative codon translations (Elzanowski et al. 2015).

F-ORF and *M-ORF* were analyzed with TESTCODE (<http://www.bioinformatics.org/sms/testcode.html>) to test the likelihood that they are protein-coding sequences (PCS). The CLC Genomics Workbench software uses Transmembrane Hidden Markov model (TMHMM) version 2.0, developed by Krogh et al. (2001) to predict transmembrane helices. Phylograms were constructed using the neighbor joining method with Jukes-Cantor nucleotide distances. GenBank accession

numbers of DNA sequences used in the construction of the phylogram can be found in appendix A.

Testing for *M-ORF* and *F-ORF* in Sperm

Sperm samples were washed three times with a PBS buffer to remove extracellular and environmental DNA. Prior to washing, the cells were pelleted in a 1.5-mL microcentrifuge tube at 3000 x g for 10 minutes and the supernatant was discarded. To wash the pellet, three times I added 200 μ L of PBS, resuspended the pellet, and re-centrifuged the sample. Following the resuspension and pelleting I sampled the supernatant to test for stray DNA before re-suspending the pellet. My goal was to obtain a sample free of extracellular and environmental DNA. I accomplished this by filtering 50 μ L of supernatant by centrifugation (18,407 x g for one minute) through a nested microcentrifuge filter (QIAGEN DNeasy Blood and Tissue Kit). This ensured that any un-pelleted sperm would be removed from the supernatant. I then added 50 μ L of water to elute DNA that was potentially retained in the filter and centrifuged the tube at 18,407 x g for one minute. The resulting sample was run as an additional negative control.

Mitochondrial DNA was extracted from sperm samples using a QIAGEN DNeasy Blood and Tissue Kit (QIAGEN 2006a) and a modified, user-developed protocol for sperm that incorporated a buffer containing TrisHCl, EDTA, NaCl, SDS, and DTT into the protocol (QIAGENb 2006). Alternatively, I used 5% Chelex (100 to 200 mesh, sodium form) from Carolina Biological Supply Company® to extract DNA from sperm. After washing sperm with PBS I centrifuged the pellet at 21,130 x g for five minutes. I then resuspended the pellet in 30 μ L of PBS, and added 100 μ L of well mixed Chelex to

the solution. Then, I incubated the samples at 99°C for 10 minutes, vortexed the sample for 10 seconds, and centrifuged them at 21,130 x g for two minutes. Lastly, I transferred the sample into a different tube.

I amplified *M-ORF* by standard PCR utilizing primers 3 and 14 (Tables 3, 4). I amplified *F-ORF* from sperm using nested PCR with other conditions unchanged. In the nested PCR, an inner and an outer pair of primers were used in successive amplifications to: (1) increase possibility of *F-ORF* amplification and (2) enhance product specificity. When I amplified *F-ORF* DNA by nested PCR, I used primers 9 and 12 followed by 17 and 18, whereas if the PCR was not nested only the latter two were used.

RESULTS

Animal Collections and Spawning

In 2013, 2014, and 2015 male *V. pleasii* spawned in the lab between August 21st and September 16th (Figure 5, Figure 6). These spawning events occurred at temperatures between 20 and 25°C (see Methods). The mussels spawned regardless of whether substrate was provided to allow a normal posture. Among years, 64–100% of the males spawned in the laboratory. Figure 6 displays the percentage of observed males that spawned on each date over three years.

Sperm samples were taken from 9 individuals in 2013, 11 individuals in 2014, and 13 individuals in 2015 (Table 2). The mean daily field water temperature on collection days that yielded spawning animals ranged 21.7°C–26.1°C. Field water temperature data were not available for two collection dates, 25 August 2014 and 2 September 2015. Water temperature was not measured in 2015. In 2013 all animals were collected from one site, McCraw Ford, and the spawning period spanned a range of 13 days. In 2014, individuals from Ozark Mill spawned over a range of 9 days. In 2015 the spawning period extended to at least 9 September (Kendell Lloyd, unpublished data), increasing the spawning period to 20 days. In 2013, 4 of 9 individuals spawned more than once and one of those four individuals spawned on a third occasion (Figure 5).

In 2014, 13% (1/8) of individuals spawned on more than one day (Figure 5); others may have as well. In 2015, 23% (3/13) of individuals spawned on more than one occasion. Between the first and last animals that spawned from successful collection trips, 64% (9/14) of animals collected from McGraw Ford yielded sperm in 2013. In

2014, 77% (10/13) of those collected from Ozark Mill and 40% (2/5) released sperm from Swan Creek. In 2015, 100% (13/13) animals released sperm.

Sequence Characteristics

CLC Workshop predicted one ORF on the positive strand within the *M-ORF* region. TESTCODE analysis suggested that the ORF ‘may or may not’ be a PCS, with a TESTCODE value of 0.805. The predicted ORF began with a TTG (Leucine) and ended with a typical termination codon, TAG. The predicted ORF spanned the base pairs 57–737, for a total of 680bp. The *M-ORF* region also contained one TMH (Figure 7). It should be noted that TESTCODE falsely categorizes 5% of sequences analyzed and makes no prediction in 20% of sequences (Fickett 1982).

CLC Workshop did not predict an ORF within the *F-ORF* region. The sequence contained a TTG (Leucine) codon early in the region, like the ORF within the *M-ORF* region. Therefore, I analyzed a 266 bp sequence (base 45–311) beginning with the Leucine, and ending with a typical termination codon, TAA, using TESTCODE. This range of nucleotides was given a TESTCODE value of 1.049, classifying the sequence, ‘YES’, a possible PCS. Just as Breton et al. (2009) found with *V. ellipsiformis*, *V. pleasii F-ORF* contains a single transmembrane helix (TMH) (Figure 8).

F-ORF* versus *M-ORF

F-ORF and *M-ORF* were highly divergent from one another. *V. pleasii F-ORF* was 311 bp while the entire *M-ORF* was 912 bp. Nucleotide sequence similarity was only 20.6% (Table 5). Sequence divergence between the *ORFs* are due primarily to 18

large indels and to numerous SNPs (Figure 9). Amino acid percent identity was lower at 11.8% (Figure 10). See Appendix B for amino acid single letter codes.

Interspecific Variation

Comparisons made between *V. ellipsiformis* and *V. pleasii* *FORF* reveal large indels (Figure 11). The *F-ORF* nucleotide sequences of the two species were 76.1% (HM849530.1) or 84.9% (HM849529.1) similar and amino acid sequences were 67.3% (AEC14189.1) or 69.2% (AEC 14188.1) similar (Table 5, Figure 11, 12). When compared with the shorter of the two *V. ellipsiformis* amino acid accessions (HM849529.1/AEC 14188.1), 1.1% (1/89) amino acids were lost from *V. pleasii* entirely, 16.9% (15/89) amino acids were nonsynonymous changes, and 5.6% (5/89) amino acids were synonymous changes. Table 6 presents a comparison between *V. pleasii* *F-ORF* nucleotide and amino acid similarities with other species (for GenBank accession numbers see Appendix A). Figure 13 presents a phylogram that shows inferred phylogenetic relationship of *F-ORF* of these two species and several more related species.

M-ORF sequences of *V. pleasii* and *V. ellipsiformis* were 82.6% similar in nucleotide sequence and 71.8% similar in amino acid sequence (Figure 14, 15). Attempts to determine whether amino acid changes were synonymous or nonsynonymous were unsuccessful using CLC workbench. Amino acids were not displayed as synonymous or nonsynonymous using different colors.

Polymorphism within *M-ORF*

Venustaconcha pleasii and *V. ellipsiformis* *M-ORF* sequences show evidence of being heteroplasmic (polymorphic) within individuals. Within one individual, the *M-ORF* from four cloned sequences differed among seven nucleotide positions; other nucleotide changes occurred at the ends of the sequences, where priming occurred (Table 7, Figure 16). Within the *M-ORF* each SNP caused a Thymine to Cytosine change, with the exception of one indel. This was not the case within the priming regions. Since the sequences were derived from four separate cloned contigs, and in turn, four separate mitochondria, the true number of mitotypes within the individual could not be ascertained, but the number could be between two and four. This polymorphism was not detectable when sequence data were obtained from PCR samples, as opposed to cloned mitochondrion that were subsequently amplified using PCR.

F-ORF sequences contained SNPs too, but all were contained within priming regions. These SNPs were discounted as probable artifacts.

Testing for *M-ORF* and *F-ORF* in Sperm

When an unwashed sperm sample was amplified, both F-mt and M-mt sequences were recovered (Figure 17). However, 15 washed samples yielded only M-mt sequences (Table 8, Figure 18). F-mt was absent from ten washed samples by standard PCR (Table 8, Figure 19). When I amplified *F-ORF* by nested PCR it was difficult to distinguish artificial products from the small products I amplified (Table 8, Figure 20).

DISCUSSION

Animal Collections and Spawning

Venustaconcha pleasii proved to be a convenient species to study. Males and females were readily distinguishable based on morphology. The small body size of individuals (typically 4–5 cm) made it practical to hold many individuals in a relatively compact space. I was able to collect dozens of mussels of both sexes from shallow water at multiple sites in the White River tributaries. I located the animals visually without having to excavate the substrate. Interestingly, the proportion of males and females at the surface appeared to vary seasonally. During summer 2015, I observed a disproportionate number of females versus males depending on when I collected the animals. Between 12 and 27 June, I found 20 females and only 4 males while searching for both sexes. These proportions differed significantly from 50:50 (chi-square $p = 0.001$). In contrast, between 27 July and 23 August, I collected 23 males with a similar effort. One possible interpretation is that the males move higher in the substrate during the spawning season (mid-August to mid-September). This might permit sperm to be released slightly higher in the water column for better dispersal.

Most males collected spawned each year. This suggests that most males spawn yearly. However, if males move to the surface prior to spawning, that behavior might have biased my sample toward reproductively active individuals.

Venustaconcha pleasii males spawned over a period of about 4 weeks in July and August (figure 4). This timing is characteristic of many mussel species in the tribe Lampsilini which are typically long-term brooders (Haag 2012). Other mussel species

that are tachytictic vary in the timing and the degree of synchronicity of spawning (Garner 1999, Haggerty 1995, Haggerty and Garner 2000, Haggerty et al. 2005). Both temperature and photoperiod probably affect the timing of spawning (Hastie and Young 2003; Galbraith and Vaughn 2009). *Venustaconcha pleasii* did not seem to require precise temperatures to spawn in the laboratory. In other species, temperature change can trigger spawning within a short period. For example, *Truncilla truncata* produced SZ when warmed in the spring (Waller and Lasee 1997). I was also able to reliably obtain sperm from this species by collecting males in February and March and holding them at 5°C. When warmed to room temperature, the mussels spawned within an average of ~5.5 hours (N = 5); one individual took three days to begin spawning (Maynard, unpublished data). A critical temperature near 10°C appeared to trigger spawning. Along similar lines, glochidia of *Margaritifera falcata* in Oregon were not detected until minimum and maximum water temperatures remained warmer than 5.5 and 8.0°C respectively, suggesting that they spawned in the spring in response to rising temperature (Allard et al. 2015). *Margaritifera margaritifera* in the Truckee River spawned when water temperatures were 7–10°C (Murphy 1942).

Other potential triggers of spawning include hydrology, and perhaps, photoperiod. Timing of brooding in *Quadrula cylindrica* is correlated with timing of the mean decrease in river discharge following spring rains, according to historical hydrograph data from four rivers (Fobian and Barnhart, article in press). The degree to which photoperiod is involved in timing of reproduction is unknown (Haag 2012). However, *Quadrula pustulosa* experiencing warm/dark (8h light/16h dark) treatments had higher sperm

concentrations than males experiencing warm/light (16h dark/8h light) treatments in the laboratory (Galbraith and Vaughn 2009).

For practical purposes, serotonin may be used to acquire sperm on-demand. For example, 0.4 mL of 2-mM crystalline serotonin (5-hydroxytryptamine, creatinine sulfate) administered through an adductor successfully triggered *T. truncata* to spawn within 30 minutes (N = 4) (Maynard unpublished data). The concentration and amount was partially determined using methods established by Gibbons and Castagna (1984).

The ability to visually detect brooding of female mussels has allowed more frequent observations regarding their reproductive timing than that of males. For example, a few studies have documented populations of mussels in which females produce more than one brood during a year. These species were short-term brooders in the genera *Elliptio*, *Unio*, and *Utterbackia* (Haag 2012). Comparable observations are very difficult to obtain for male mussels, although we can infer that both sexes must spawn for fertilization to occur. *Venustaconcha* are bradytictic (long term brooding). Species of Unionida that are bradytictic brood their young within their ctenidia over the winter. During the spring and summer bradytictic females release their young for subsequent infection of hosts by one manner or the other. This leaves no time for multiple brooding events, so populations of *Venustaconcha* and other bradytictic mussels only spawn once per year. During the spawning period, I observed more than one spat (of milt) to occur for some individuals. This seemingly novel observation is interesting in the context of spawning synchronization.

As discussed in the introduction, the cue for spawning synchronization between females and males is unknown. Spawning more than once, as I have observed, could be

advantageous for multiple reasons. First, it could give early spawning males a better opportunity to fertilize eggs throughout the spawning period. If the female's readiness depends on the presence of sperm in the water it would not be advantageous to be the first male delivering the sperm cue. Second, it could improve the likelihood of a synchronous spawning event between males and females. The first spat could prime females and the second one would allow fertilization. More studies should be conducted to test the degree to which multiple spat events occurs among the Unionoids and to see whether these observations hold true in situ.

I observed prolonged release of sperm in *V. pleasii* and *T. truncata*. Spawning individuals released for a period of time ranging 3.5–8 hours in *T. truncata* (N = 3). Sperm have a limited lifespan in water. Females need time to lay eggs and we presume that sperm must be present continuously, so a prolonged release along with multiple spawning events would be advantageous.

Sequence Characteristics

Start and stop codons within the *M-ORF* and *F-ORF* are consistent with those typical of invertebrates, other Lampsilini, and with other *Venustaconcha* (Wolstenholme 1992 Serb and Lydeard 2003, Breton et al. 2009, Huang et al. 2013). According to Serb and Lydeard (2003) *Lampsilis* species tested did not utilize Leucine as start codons as *V. ellipsiformis* (Breton et al. 2009) and *V. pleasii* do.

The complete M-mt has been sequenced in at least four species, *Utterbackia peninsularis*, *V. ellipsiformis* (Breton et al. 2011), *Solenia carinatus* (Huang et al. 2013), and *P. grandis* (Doucet-Beaupré et al. 2010), but the *M-ORF* has only been defined in *U.*

peninsularis. The *M-ORF* has been identified using PCR in at least eight species, which include *Lasmigona complanata*, *Lasmigona costata*, *Cumberlandia monodonta*, *Toxolasma glans*, *Toxolasma minor*, *Toxolasma paulus*, *Utterbackia peggyae*, and *U. peninsularis* (Breton et al. 2011). The *F-mt* has been sequenced for many more .

TESTCODE analysis predicted that the ORF within the *M-ORF* ‘may or may not’ be a PCS. TESTCODE analysis predicted that, ‘yes’, the 266 bp sequence within the *F-ORF* may be a PCS. Until actual polypeptides are identified from any of these ORFs, the true coding function of these predicted PCS remains unresolved. Western blot detected protein products homologous with *M-ORF* in male gonadal tissue, and IEM indicated that *cox2e* is present in sperm (Breton et al. 2009, Shepardson et al. 2012). Both sequences exhibited one TMH. Despite large intra- and interspecific sequence divergence between the *M-ORF* and *F-ORF*, this conserved secondary structure, suggests a transmembrane role for the ORF proteins (Breton et al. 2009).

F-ORF* versus *M-ORF

Considerable divergence between and among *M-ORF* and *F-ORF* sequences is typical. In fact, *F-ORF* and *M-ORF* of *V. pleasii* were less similar to each other than *F-ORF* of two different species or *M-ORF* of *V. pleasii* and *V. ellipsiformis*. Sex-associated genes tend to be highly divergent, because they have different selection pressures (Ellegren and Parsch 2007). Nucleotide (N) and amino acid (AA) sequence similarities (N, AA) reported between the *M-ORF* and *F-ORF* in Unionoids include: *Unio japonensis* (57%, 49%), *Q. quadrula* (58%, 48%), *P. grandis* (57%, 49%), and *V. ellipsiformis*

(59%, 50%) (Doucet-Beaupré et al. 2010, Huang et al. 2013). *Venustaconcha pleasii* *M-ORF* and *F-ORF*, in contrast, showed similarities of 20.6% and 11.8%.

Interspecific Variation

Venustaconcha ellipsiformis have a broad geographic range relative to *V. pleasii* and are found in Minnesota, Wisconsin, Iowa, Missouri, and Illinois. In Missouri, the distribution of *V. ellipsiformis* and *V. pleasii* is related to drainage basin. *Venustaconcha ellipsiformis* are found in streams north of the Ozark Plateau that include tributaries of the Missouri, Mississippi, and Arkansas rivers, whereas *V. pleasii* are found in streams south of the Ozark Plateau that are tributaries of the White River (Riusech and Barnhart 2000). Other aquatic taxa also show range boundaries and endemism in the White River Basin, including crayfish, fish and other mussels (Pflieger and Dryden 1996, Pflieger 1997, McMurray et al. 2012). Typically the closest relatives of these species are found in the northward tributaries of the Missouri River system.

Proteins that participate in reproduction frequently exhibit rapid rates of evolution (Clark et al. 2006, Metz et al. 1998, Swanson and Vacquier 2002). Researchers have observed this through sequence comparison between reproductive and non-reproductive genes across many taxa, which among other organisms include mammals, invertebrates, and plants. Rapid evolution of *F-ORF* is evident when compared to a more conserved mt gene like *cox1*. *Venustaconcha ellipsiformis* and *V. pleasii* *F-ORF* sequence similarities were 72.9 or 65.0% (nucleotide) and 58.5 or 59.7% (amino acid). In comparison, *cox1* sequence similarities between the two species are 95.6% (nucleotide) and 100% (amino

acid) (Appendix C), supporting the hypothesis that the *F-ORF* and *M-ORF* may have reproduction function (Breton et al. 2009).

Polymorphism within *M-ORF*

Between the four mt clones, 6 of 7 SNPs were cytosine to thymine mutations. Transition mutations occurred more often (transition bias) because the mutation occurred within the class of purines or pyrimidines instead of between the two structural classes of nucleotides (Wakeley 1996). It is possible that these transition mutations arose through deamination. During this process of mutation Cytosine loses an amino group, changing the nucleotide base to Uracil. When DNA replicates later, the Uracil is then replaced with a Thymine (Hartwell et al. 2008).

Previously, male and female freshwater mussels have not been known to possess more than one mitotype of F-mtDNA or M-mtDNA within an individual. I detected 7 polymorphic sites, resulting in clones with different sequences within the mitochondrial sequences of Unionida. The overlapping regions of the contigs represent a small portion of the 912 bp genome. Overlapping regions give coverage of only 285 bp, thus the true number SNPs within this region could be higher. Ladoukakis and Zouros (2001) and Passamonti et al. (2003) found similar results while studying the marine species *M. provincialis* and *T. phillipinarum*, respectively. Between 1 and 6 unique mtDNA sequences were found within gonadal samples in each of 10 *Mytilus* individuals. Given that I sequenced DNA from four mitochondria of one individual, the evidence for multiple mitotypes of mt within the individual is minimal, but worth pursuit.

Three hypotheses can be considered to try to gain an understanding of the mechanism by which two mitotypes are present within an individual. First, multiple mitotypes may arise as the egg is fertilized by more than one sperm. Polyspermy is blocked completely after 15 seconds in the marine clam *Spisula solidissima* and the egg experiences a 70% reduction in sperm receptivity after five seconds (Ziomek and Epel 1975). Fertilization occurs internally in Unionida, as in *S. solidissima*. Therefore, sperm concentrations are relatively high in contrast with bivalves experiencing external fertilization. Provided a similar length of time is available for polyspermy to occur in Unionida, would up to 15 seconds be enough time to allow multiple mitotypes of mitochondria to enter the egg? Also, would the resulting individual be viable? In the marine mussel *M. galloprovincialis*, a partial Na⁺-dependent block may be responsible for monospermic fertilization (Dufresne-Dubé et al. 1983). In Unionida such timing and mechanisms have not yet been reported.

Second, the sperm could contain more than one mitotype of mitochondria. Each sperm carries 5 mitochondria in Unionoid mussels (Franzén 1955, Popham 1979, Peredo et al. 1990, Shepardson et al. 2012). Is it physiologically possible for mitochondria of two different haplotypes to find themselves in one spermatozoa?

Third, the haplotype may have arisen within the life of the animal. Neutral mutations may arise during an individual's lifetime. In humans, for example, measures of average heteroplasmy increase over time at 75% of heteroplasmic sites that were evaluated for more than 2400 people (Sondheimer et al. 2011). Interestingly, an individual may also become more homoplasmic over time, which was observed in 10% of heteroplasmic sites in human mtDNA.

Venustaconcha pleasii are small mussels who reach reproductive maturity early and have short lives relative to our long lifespans. Workers have found that substitution rates vary among organisms. Therefore, the universal molecular clock may be less effective for understanding the rate of neutral mutation than previously believed until we understand the factors that cause differences in substitution rate. With some vertebrates for example, the substitution rate is correlated with body size. This trend does not follow for invertebrates, suggesting that something more complex underlying body size may be involved, such as metabolic rate, temperature, or speciation rate (Thomas et al. 2006).

Recombination of plant, fungus, and yeast mitochondria is known to occur (Barr et al. 2005). In animals, evidence supporting the existence of mt recombination is accumulating, but how widespread the occurrence is across the animal kingdom is not known (Lunt and Hyman 1997, Kraytsberg et al. 2004, Ladoukakis and Eyre-Walker 2004). Some evidence for mtDNA recombination has come from bivalves (Ladoukakis and Zouros 2001, Burzyński et al. 2003, Passamonti et al. 2003). Ladoukakis and Zouros (2001) also detected mt recombination within the unique clone lines of the 10 *Mytilus* that they observed multiple haplotypes of mitochondria in. My data does not provide evidence for recombination of mitochondrial DNA, but the observed SNPs provide a basis for investigating recombination.

Some polymorphic sites within *V. pleasii* *M-ORF* were contained within priming regions. The primers used to sequence *V. pleasii* were derived from *V. ellipsiformis* so it is possible that SNPs within priming regions were artifacts. SNPs outside priming regions, however, are probably real. This conclusion is supported by the fact that sequences of both the forward and reverse strands of mtDNA yielded the same results.

Cloning was conducted in a lab that had not previously been used for freshwater mussel genetics. Additionally, cloning was conducted prior to all other testing within this thesis, minimizing the chances of error of DNA contamination from other individuals.

Several researchers have sequenced the male mitochondrial genome of Unionoid mussels (Huang et al. 2013, Doucet-Beaupre et al. 2010, Breton et al. 2011, and Okazaki and Ueshima (direct submission to GenBank)) without reporting multiple haplotypes within an individual. Polymorphic sites are difficult to detect because the genomes in question only differ by a few base pairs. My study differed from others in that I amplified individual mitochondrial DNA sequences through cloning, rather than by sequencing the most common mitotype of mitochondria using PCR. This less often used method using cloning may reveal an anomaly that has otherwise been missed.

All polymorphic sites that were detected in the *F-ORF* were contained within priming regions. As with the *M-ORF*, the primers used to sequence *V. pleasii* were derived from *V. ellipsiformis* sequences, so it is possible that SNPs within these priming regions were artifacts. Ideally, primers would be constructed with *V. pleasii* sequences and the mtDNA sequence would be studied from several individuals to provide evidence to either support or reject the possibility of multiple mitotypes within the *F-ORF*. \

Lack of F-MtDNA in Sperm

Because we did not detect F-mtDNA in sperm we cannot provide evidence that sex is not determined by the delivery of F-mt or M-mt by sperm. It is curious that proteins analogous to the *F-ORF* and *M-ORF* are both present within sperm of *V. ellipsiformis* (Shephardson et al. 2012), but *F-ORF* mtDNA was absent within *V. pleasii*.

Future studies could address the mechanisms by which the protein arrives in the sperm given that it may not be produced within the sperm. Perhaps this is some difference that the two different spermatogenesis pathways possess. If I were to attempt to use nested PCR to detect *F-ORF* in the future I would design primers to produce a larger product.

Utility of Sex-Associated Mitochondrial Genes

Although the possible role of *F-ORF* and *M-ORF* in determining sex remains obscure, these genes clearly provide an opportunity to determine the sex of glochidia larvae and test the sex ratios of broods. Current estimates of sex ratio are based upon field sampling. It is difficult to achieve accurate measures of population structure due to the nature of the animal's lifestyle. Alternatively, the sex of glochidia can be determined using the M-specific region of the mtDNA.

Quite recently, Machordom et al. (2015) used sex-associated regions of mtDNA to discern that females of a Unionoid species, *Unio delphinus*, produce biased sex ratios of glochidia. Some females produced mostly daughters, others mostly sons, and some produced a ratio nearer to 50:50. If this is indeed an accurate measure of glochidia sex, the sex bias may be under female control, as in *M. edulis* and *R. phillipinarum* (Kenchington et al. 2002; Ghiselli et al. 2012), rather than male control through sperm. A study in which these sex ratios are confirmed using captive culture techniques would be of interest. Using genetic testing of larvae, other intra- and interspecific studies of sex ratio become possible. For example, do different fathers affect the sex of offspring with a common mother? Other population structure questions could be answered. For example, does sex ratio change along the length of a river?

Sex ratio may vary throughout the lifespan of a parent's brood. Primary sex ratio refers to sex ratio at fertilization, secondary sex ratio refers to sex ratio at birth, and tertiary sex ratio refers to sex ratio in adulthood (Mayr 1939). The life history of *Leptodea leptodon* provides an interesting example, where the primary and secondary sex ratios differ drastically from the organism's tertiary sex ratio. Adult females of this species are much less common than males. It has been suggested that the bias is the result of differential mortality of females, who may be eaten by the fish host, freshwater drum, as a strategy to infect the host. In captive culture we observe ratios closer to 50:50 in these organisms (Barnhart, unpublished data). Using molecular genetics we can study difference in sex ratio among different life stages and different taxa without requiring years of captive culture, which may be impractical for many facilities.

Rapid evolution of the M-mt make certain regions of the genome useful for taxonomic and eDNA applications. Both applications could benefit from the increased level of resolution that rapid evolution provides. For eDNA studies, species-specific markers could be found more easily using the F- and M-mtDNA. Already, the F-mt has been used to study mussel/host relationships. The species or genus of glochidia or juveniles coming from wild caught fish can be determined using molecular genetics (Katherine Bockrath, unpublished data).

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Table 1. Collection localities for *Venustaconcha pleasii* in Missouri.

Water Body	County	Coordinates (NAD83/WGS84)	Access
James River	Greene	UTM 15 481565.817E 4097912.429N	HWY 125 Bridge (McGraw Ford)
James River	Greene	UTM 15 481951.12E 4111512.70N	Kinser Bridge
Finley River	Christian	UTM 15 488600.601E 4116223.915N	Jim Turner Public Fishing Access
Swan Creek	Taney	UTM 15 494682.902E 4071261.854N	AA Bridge

Table 2. Collection information and spawning dates.

Animal ID	Collection Date	Locale	Spawn Date(s)
13-12	8/29/13	McGraw Ford, James River	9/7/13, 9/8/13
13-15	8/29/13	McGraw Ford, James River	9/6/2013
13-19	8/29/13	McGraw Ford, James River	9/4/2013, 9/7/2013
13-20	8/29/13	McGraw Ford, James River	9/5/2013
13-21	9/4/13	McGraw Ford, James River	9/16/2013
13-22	9/4/13	McGraw Ford, James River	9/10/2013, 9/12/13, 09/16/13
13-23	9/4/13	McGraw Ford, James River	9/16/2013
13-24	9/4/13	McGraw Ford, James River	9/7/2013
13-25	9/4/13	McGraw Ford, James River	9/6/2013, 9/11/13
14-13	08/18/14	Ozark Mill, Finley River	08/22/14
14-14	08/18/14	Ozark Mill, Finley River	08/22/14
14-15	08/18/14	Ozark Mill, Finley River	08/22/14
14-16	08/18/14	Ozark Mill, Finley River	08/21/14
14-26	08/25/14	Ozark Mill, Finley River	08/27/14, 09/01/14
14-27	08/25/14	Ozark Mill, Finley River	08/27/14
14-28	08/25/14	Ozark Mill, Finley River	08/27/14
14-29	08/25/14	Ozark Mill, Finley River	08/27/14
14-33	09/02/14	Ozark Mill, Finley River	09/05/14
14-40	08/20/14	AA bridge, Swan Creek	09/05/14
48-15	08/17/15	Kinser Bridge, James River	08/21/15
49-15	08/17/15	Kinser Bridge, James River	08/21/15
50-15	08/17/15	Kinser Bridge, James River	08/21/15
51-15	08/17/15	Kinser Bridge, James River	08/22/15
52-15	08/17/15	Kinser Bridge, James River	08/23/15
53-15	08/17/15	Kinser Bridge, James River	08/23/15, 08/29/15
54-15	08/23/15	McGraw Ford, James River	08/26/15, 09/01/15
55-15	08/23/15	McGraw Ford, James River	08/26/15

Table 3. Primers used for cloning and sequencing of *M-ORF* and *F-ORF*.

Ref. no.	Primer Name ^{1,2,3}	Sequence
1	V-M-For-1	ACA CAA ACC AGA CCC AAT ACA AGT
2	V-M-Rev-1	ATG AGG TTG GGG TGG AAG CC
3	V-M-For-2	TAT CCA CAC CTG CCT TGT TAC
4	VE-M-Rev-2	GTG GAT AAG TTG AAG GAG GAG GAG
5	V-M-For-3	TCT CCC TCA ATT TCT TTG TCA GC
6	V-M-Rev-3	GGG TTT GTT CGC GGT ATT GTG
7	V-M-For-4	CCT ACC CAA CGC AAC TTC CT
8	VE-M-Rev-4	TCG TTT GTT TGC GTG AAA GTC A
9	V-F-For-1	ACG TCA AAG GGG CAC ACA G
10	V-F-Rev-1	GTG ACA GTT CGG GAT AAG TGA T
11	VE-F-For-2	TCA TTT TAC CAA GCC CCT TCT
12	V-F-Rev-2	GTT GAT CCT AGT GCT TGG GGT

¹ Venustaconcha-specific (V) or *Venustaconcha ellipsiformis*-specific (VE)

² *M-ORF* (M) or *F-ORF* (F)

³ Forward (For) or Reverse (Rev) Primer

Table 4. Primers used for detection of *M-ORF* and *F-ORF* in sperm.

Ref. No.	Primer Name ^{1,2,3}	Sequence
13	V-M-Rev 14519	GAG TGG CTT AGA CTT GAT GTA AAG A
14	V-M-Rev-14385-14748	GCT GAC AAA GAA ATT GAG GGA GA
15	VP-F-pt1 Nest For	TTG GCT ACC AAC TAC TTA CCT TG
16	VP-F-pt1 Nest Rev	TTG CAC TGT TTT GTG TTG AAG T
17	VP-F-pt2 Nest For	CAA CCA ACT AGC ACA TCC ACA GC
18	VP-F-pt2 Nest Rev	CAG AGA TGT TGG TTG AAG CAG GA

¹ *Venustaconcha*-specific (V) or *Venustaconcha pleasii*-specific (VP)

² *M-ORF* (M) or *F-ORF* (F)

³ Forward (For) or Reverse (Rev) Primer

Table 5. Nucleotide and amino acid sequence similarity within *V. pleasii* and between *V. pleasii* and *V. ellipsiformis*.

Species 1	Species 2	Region 1	Region 2	Sequence Type	Percent Sequence Similarity
VP	VP	FORF	MORF	Nucleotide	20.6
VP	VP	FORF	MORF	Amino Acid	11.8
VE	VP	FORF	FORF	Nucleotide	84.9
VE	VP	FORF	FORF	Amino Acid	69.2
VE	VP	FORF	FORF	Nucleotide	76.1
VE	VP	FORF	FORF	Amino Acid	67.3
VE	VP	MORF	MORF	Nucleotide	82.6
VE	VP	MORF	MORF	Amino Acid	71.8

Table 6. F-ORF pairwise comparison of 17 Unionid species. Percent nucleotide (lower left corner) and protein sequence divergence (upper right corner); darker shades indicate sequence dissimilarity. All sequences except *V. pleasii* were derived from GenBank (Appendix A).

Reference No.	<i>V. iris</i>	<i>T. lividus</i>	<i>E. lineolata</i>	<i>L. siliquioidea</i>	<i>L. powelii</i>	<i>F. flava</i>	<i>V. ellipsiformis</i>	<i>V. pleasii</i>	<i>T. macrodon</i>	<i>P. metnecktayi</i>	<i>M. nervosa</i>	<i>C. tuberculata</i>	<i>P. grandis</i>	<i>complanata</i>	<i>U. peninsularis</i>	<i>A. marginata</i>	<i>S. undulatus</i>
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		58.6	52.6	52.2	50.4	41.4	48.7	57.8	39.7	41.9	32.5	36.2	11.7	11.7	12.5	12.5	14.1
2	73.3		50.9	55.8	55.8	42.6	50.4	53.5	45.3	41.9	30.5	32.2	12.7	13.6	16.2	15.3	14.3
3	71.4	71.3		43.5	43.5	44.8	43.5	51.7	48.7	50.0	32.2	38.8	12.6	15.3	15.3	15.3	15.0
4	59.8	61.4	54.8		91.0	43.1	74.2	60.6	47.0	53.1	27.4	35.9	12.5	16.7	15.8	14.6	15.5
5	60.3	61.1	55.9	90.7		41.3	71.9	56.7	47.0	51.0	26.6	35.9	14.6	15.6	17.9	15.6	13.4
6	63.9	60.6	64.7	53.5	53.8		39.5	42.7	36.2	43.2	36.2	38.9	15.0	15.0	12.5	15.0	13.2
7	59.0	58.5	56.1	78.2	76.4	53.7		69.2	46.0	44.8	29.2	41.3	11.5	14.6	15.8	15.6	14.4
8	70.5	69.5	65.0	66.4	64.8	63.1	76.1		42.9	44.0	28.0	34.9	11.9	14.7	15.7	14.7	12.7
9	57.9	60.6	63.0	58.9	59.3	56.9	59.7	65.2		55.5	33.6	40.0	14.4	15.4	15.5	18.3	18.1
10	54.3	52.7	57.0	62.6	61.9	56.9	63.4	56.8	64.7		29.2	40.6	14.0	15.0	14.1	15.0	14.0
11	55.7	58.3	56.4	44.3	44.0	59.1	45.1	52.3	47.1	53.7		47.3	15.4	16.2	13.8	16.2	18.0
12	42.1	40.8	42.7	49.8	50.5	43.8	53.6	43.8	47.4	47.9	49.3		16.9	17.4	14.1	19.8	17.9
13	29.9	26.2	29.2	32.1	34.5	26.7	34.1	29.6	31.6	29.6	26.7	35.1		45.9	49.4	48.2	39.6
14	27.4	28.5	27.2	32.4	33.5	27.0	32.8	29.3	33.4	34.2	26.7	37.5	57.8		44.2	71.4	41.8
15	24.9	24.2	25.3	28.1	30.6	25.7	31.9	27.4	27.9	28.5	22.8	30.7	56.2	56.0		52.0	35.2
16	28.0	30.1	28.9	34.8	35.9	27.8	35.2	30.8	34.7	32.2	27.5	36.7	62.0	73.1	60.7		46.2
17	29.9	29.5	31.2	34.3	35.4	28.2	35.0	31.4	33.3	32.8	28.4	30.3	59.2	58.8	48.7	57.4	

Table 7. Polymorphic sites within the *M-ORF* of *V. pleasii*.

Position	Nucleotide Bases	Contig Overlap Zone
163	T/D	1-2
299	C/T	2-3
313	C/T	2-3
620	C/T	3-4
639	C/T	3-4
665	C/T	3-4
679	C/T	3-4

Table 8. Presence and absence (+ or -) of *M-ORF* and *F-ORF* by individual. Spaces with no mark have not been tested for the presence or absence of the *F-ORF*.

Individual	<i>M-ORF</i>	<i>F-ORF</i>
9	+	-
11	+	
14	+	-
26	+	-
27	+	-
28	+	-
29	+	-
48	+	-
49	+	-
50	+	
51	+	-
52	+	
53	+	-
56	+	
58	+	

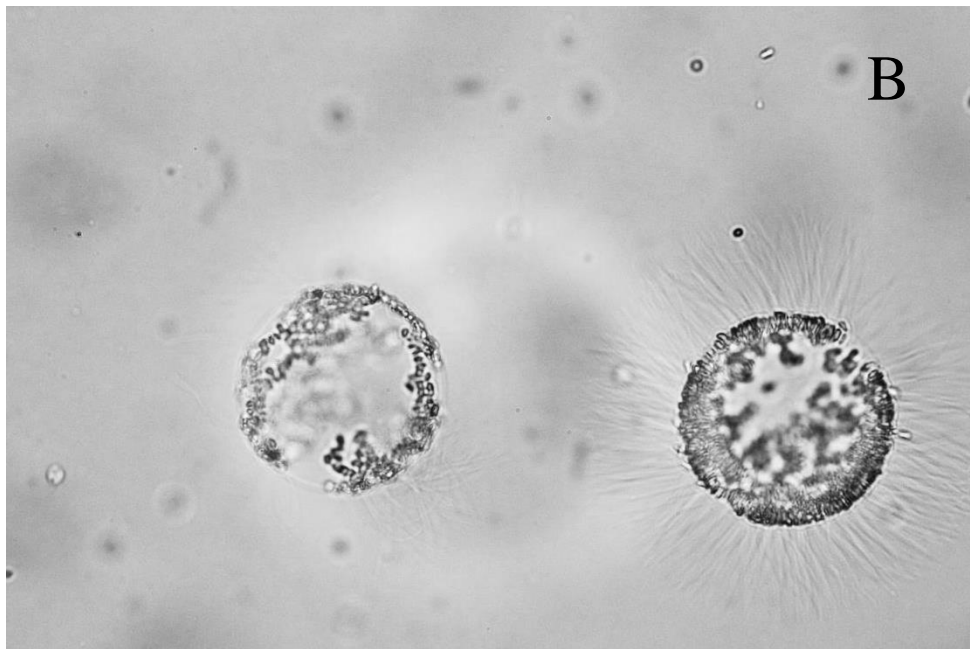


Figure 1. Spermatozeugmata from *Truncilla truncata*. Freshly released (A) and 48 hour old (B) spermatozeugmata. SZ are variable in size and number of embedded sperm (A).

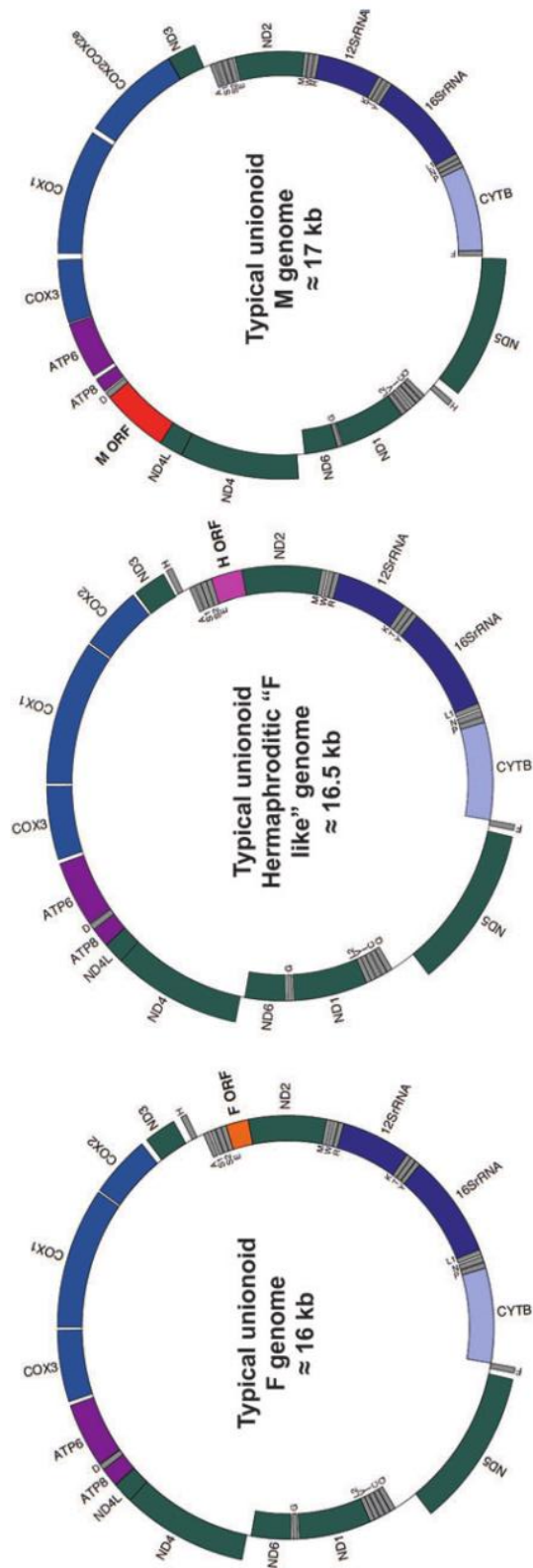


Figure 2. Gene maps of the typical male, female, and hermaphrodite mitochondrial genome of Unionida. Reproduced from Breton et al. (2011).



Figure 3. Collection localities for *Venustaconcha pleasii*.



Figure 4. System for holding mussels and observing spawning. Male mussels were kept individually in aerated gallon jars in a temperature-controlled incubator. The water in the jar at right is clouded by spermatozeugmata.

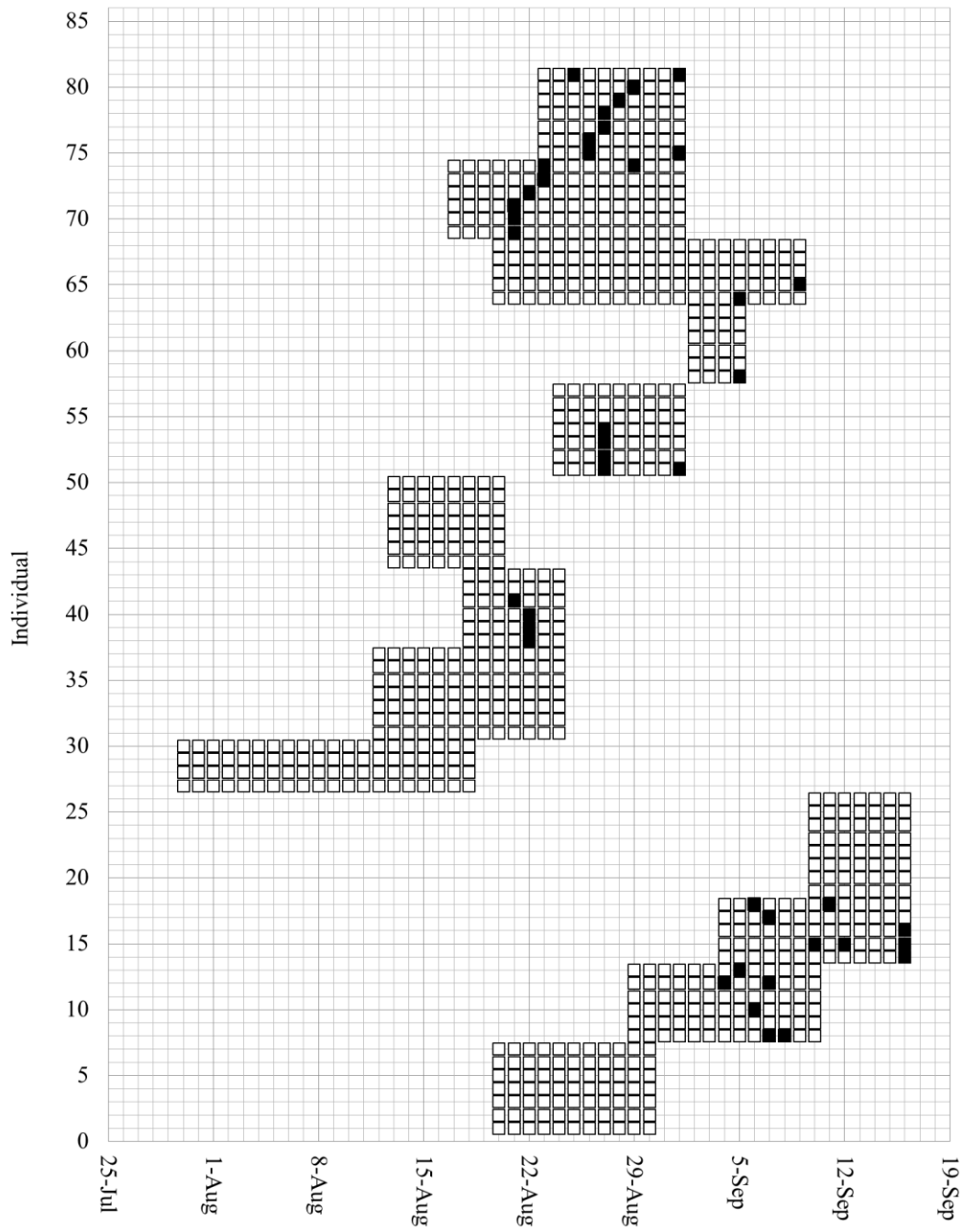


Figure 5. Spawning events in male *V. pleasii* during three years. Each row represents an individual male. Boxes indicate the dates on which each individual was observed in the laboratory. Individuals numbered 1-26 were observed in 2013, 27-68 in 2014, and 69-81 in 2015. Individuals 9 and 11 were collected in 2013 and again in 2015, where they are represented as 80 and 81.

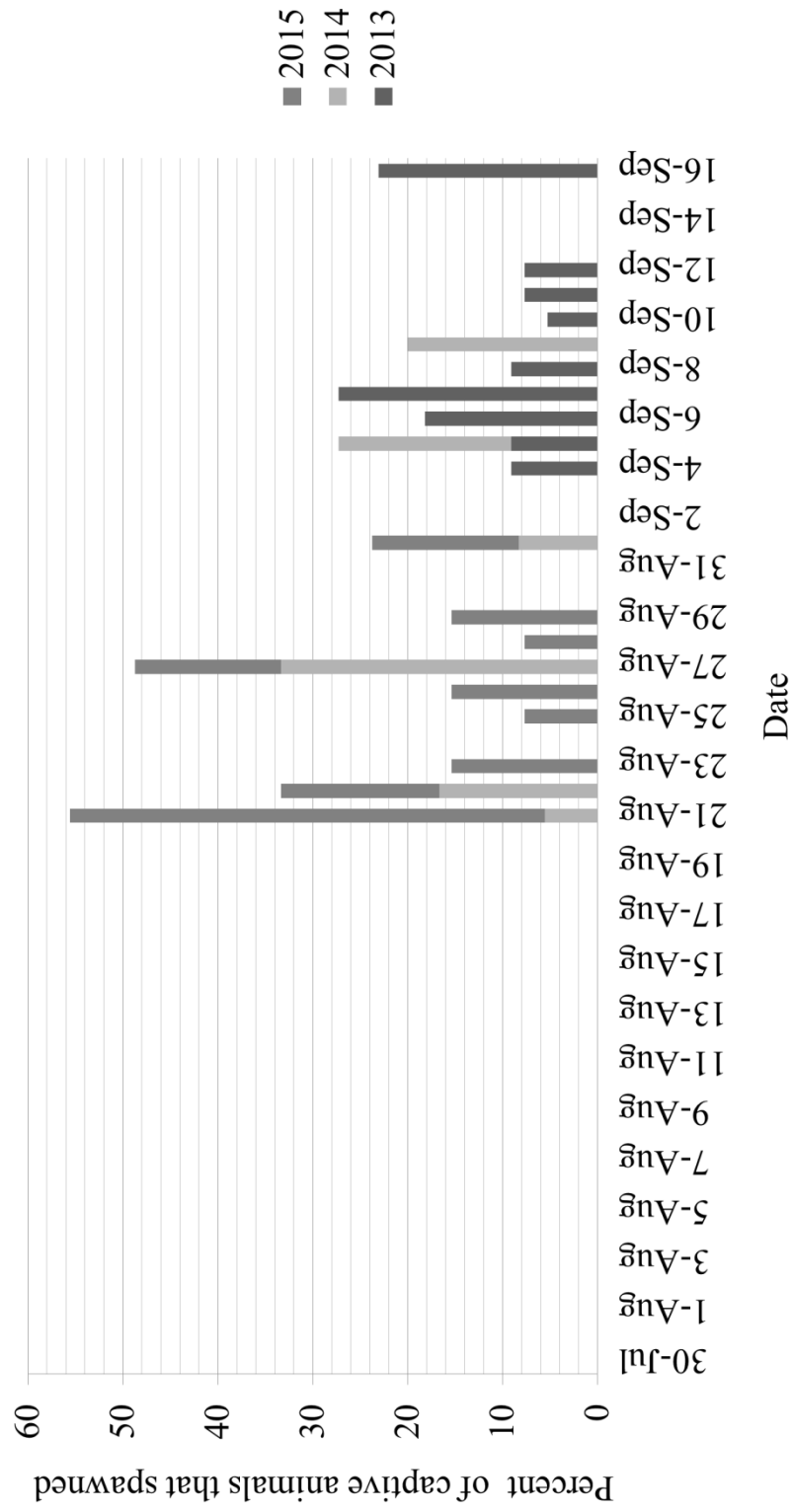


Figure 6. Percent of captive animals that spawned during holding as arranged by date.

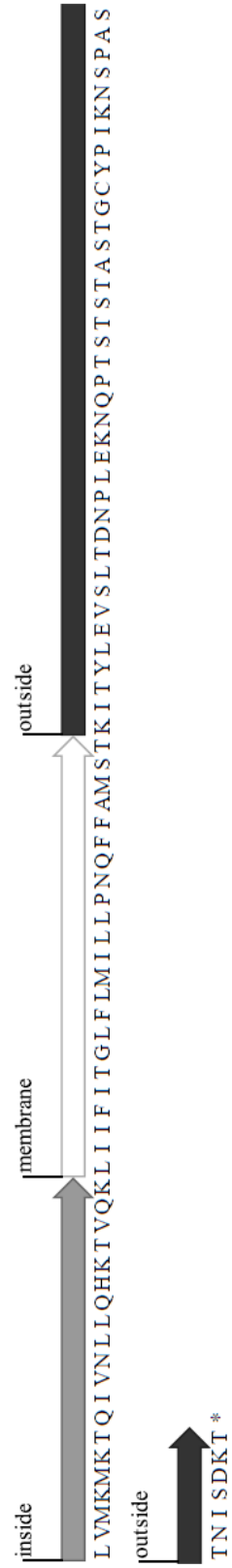


Figure 8. Predicted transmembrane helix of *V. pleasii* F-ORF.

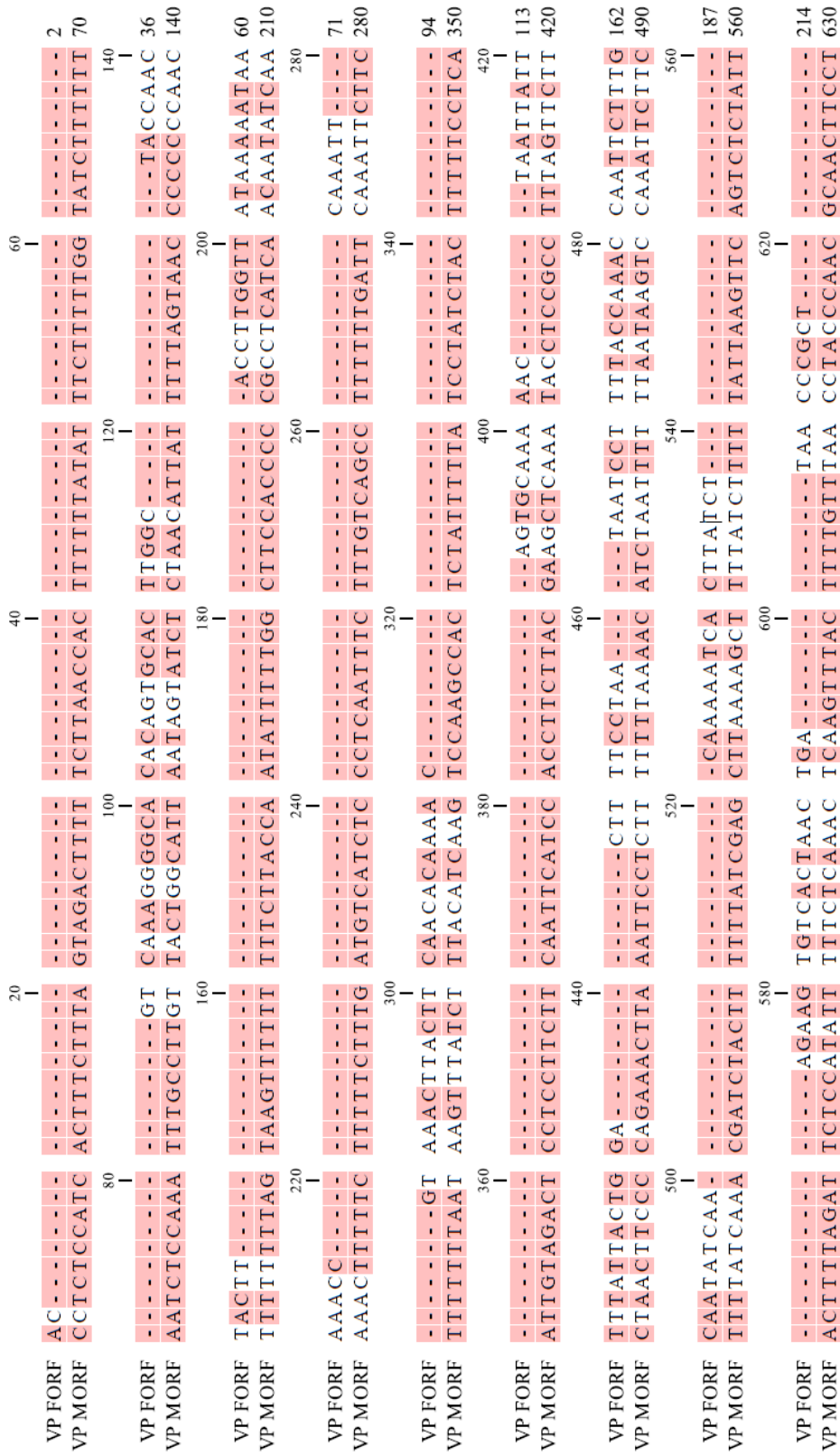


Figure 9a. *V. pleasii* F-ORF versus M-ORF nucleotide sequence alignment. Shading indicates sequence divergence or lack of homology.

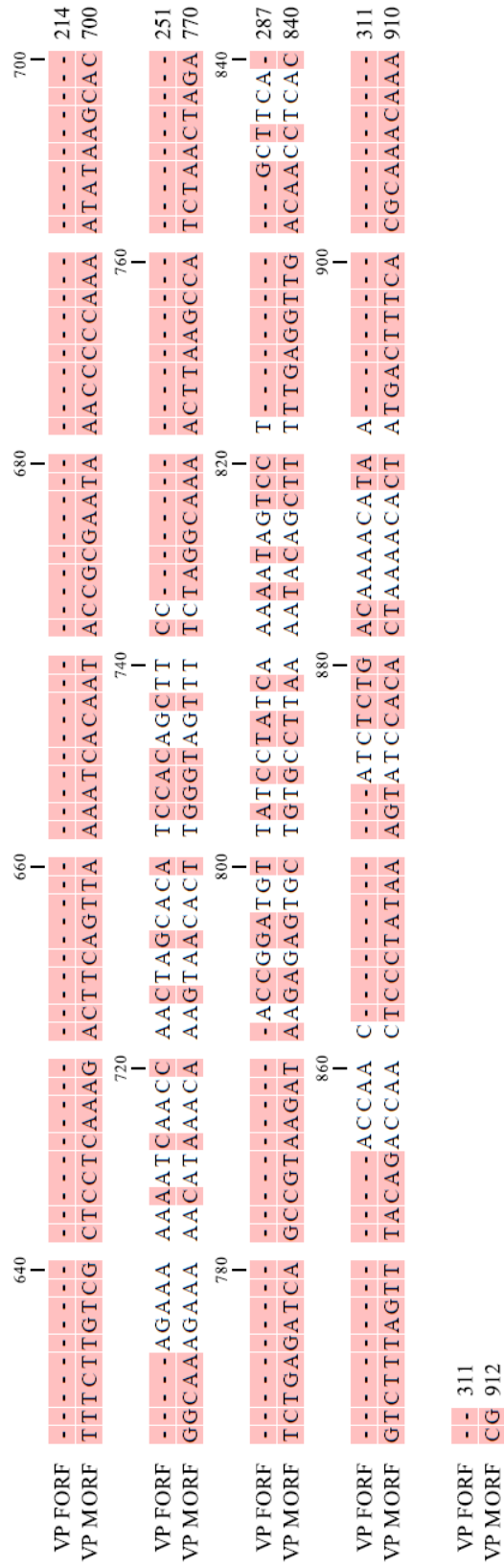


Figure 9b. *V. pleasii* F-ORF versus M-ORF nucleotide sequence alignment. Shading indicates sequence divergence or lack of homology.

VP FORF	- - - VKGAHSA	20	LG YQL L L T L V M	40	- - - - L Q H K T	60	- - - - - - - -	37
VP MORF	F V C V K V M V F *		C G Y F M G S W S V		K L Y F K A Q H S L		W L K F C L E N Y P	70
		80		100				140
VP FORF	- - - - - F I T G		L F L M I - - - - -		- - - - - - - -		- - - - - - - -	49
VP MORF	V L M F W G F I R G		I V I L T E V F E E		L N K S K L E F E K		E L N K K M K A F K	140
		160		180				200
VP FORF	N Q F - - - - -		- - - - - - - -		F A M S T K I T Y L		- - - - - - - -	62
VP MORF	E E F G L I K K L D		V L K K E E F K F L		F E L R K K V D E L		V D S M K M E W L G	210
		220		240				280
VP FORF	- - - E V S L T D N		P L E - - - - -		- - - - - - - -		- - K N Q P T S T S	89
VP MORF	E E F E S K K A D K		E I E G D D I K E K		G V E A K N I G K K		V T K N N V S D T I	280
		300		320				360
VP FORF	K N S P A - - - -		- - - - S T N I S		D K T *			103
VP MORF	K K D T K K K Y K K		V V K K K S T K E S		D G - E			303

Figure 10. *V. pleasii* *F-ORF* versus *M-ORF* amino acid sequence alignment. Shading indicates sequence divergence or lack of homology.

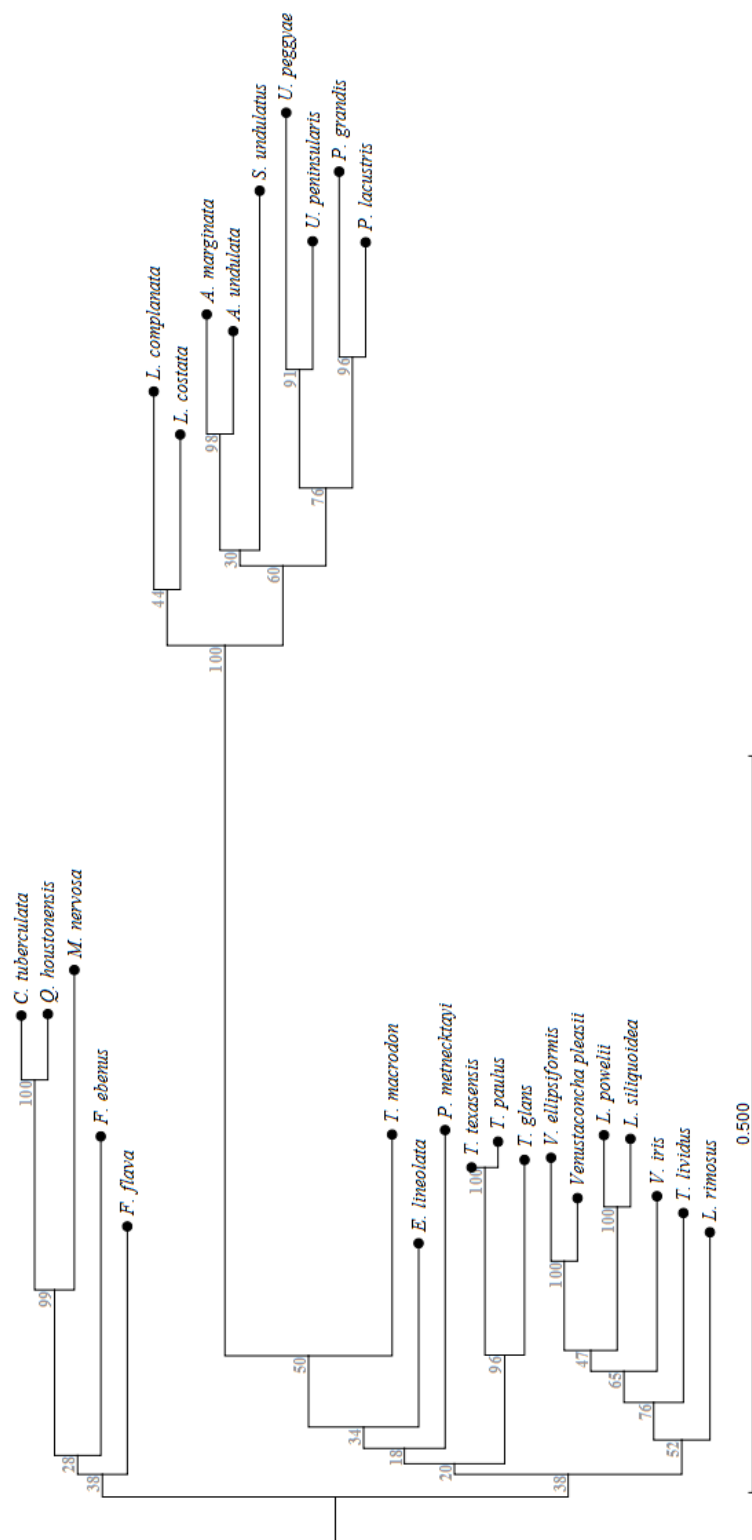


Figure 13. *F-ORF* based phylogram using 27 Unionid species. Tree constructed by neighbor joining and using Jukes-Cantor nucleotide distances.

VE MORF	CCTC	CCCATC	ACCT	TCCTTCA	GTGG	TTTTTT	TCTTAAT	CAC	TTTCT	TTATAT	TTCTTTT	TAA	TACCT	TTTTT	70
VP MORF	CCTC	TCATC	ACTT	TCCTTIA	GTAG	ACTTTT	TCTTAAC	CAC	TTTT	TTATAT	TTCTTTT	TTGG	TATCT	TTTTT	70
VE MORF	GAT	ATCCAA	CCTG	CCCTTGT	TACCA	AGCACT	AGTA	ATAGCA	TCCCC	CCCCCTT	CAATG	ATATG	TTTAA	TTGTA	140
VP MORF	AAT	CTCCAA	TTTG	CCCTTGT	TACTG	GCAT	AATAG	TA	-----	-----	-----	-----	-----	-----	107
VE MORF	GTG	TTTTTCT	TTTT	TAGT	TATTT	TTAGT	AACCC	CCCCCA	A--	CTTTTT	TAGCA	AGTTT	TTTT	-CTTA	207
VP MORF	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	TTTT	CTTA	167
VE MORF	TCA	ATATCT	TGGC	TTCCAC	CCCA	ACCTCA	TCA	TCAACAA	TATCA	AAAAAC	TTTCT	CTTTT	TC	TTGATGT	277
VP MORF	CCA	ATATTT	TGGC	TTCCAC	CCCG	CCCTCA	TCA	-----	-----	-----	TTTCT	CTTTT	TC	TTGATGT	234
VE MORF	CATC	TCCTC	AATTT	CTTTG	TCAG	CCCTTTT	TTGAT	TCATA	TTCT	CTTTT	TTAGT	GTAGT	TTATC	TTTAC	347
VP MORF	CATC	TCCTC	AATTT	CTTTG	TCAG	CCCTTTT	TTGAT	TCATA	TTCT	CTTTT	TTAA	ATAAGT	TTATC	TTTAC	304
VE MORF	ATCA	AGTCTA	AGCC	ACTCTA	TTTT	TATCAT	ATCTAC	CTTT	TCCTC	GATTA	TAGAC	TCCTC	CT	CTTCAAC	417
VP MORF	ATCA	AGTCCA	AGCC	ACTCTA	TTTT	TATCCT	ATCTAC	CTTT	TCCTC	AATTG	TAGAC	TCCTC	CT	CTTCAAT	374
VE MORF	TTAT	CCACCT	TC	TTACGAAG	CTCA	AAATACC	TC	TGCTTTA	ACTC	TTTCAA	CTTTC	CTTGA	AACTT	AAAT	487
VP MORF	TCAT	CCACCT	TC	TTACGAAG	CTCA	AAATACC	TC	TGCTTTA	GTTC	TTCTAA	CTTC	CCCCAGA	AACTT	AAAT	444
VE MORF	CCTC	CTTTT	TAAAG	GCATCT	ACC	TTTTTAA	TAAG	TCCAAA	TTCT	TTCTTTT	TTCAA	ACGAT	CC	ACTTTTTT	557
VP MORF	CCTC	CTTTT	TAAAG	GCATCT	AAT	TTTTTAA	TAAG	TCCAAA	TTCT	TTCTTTT	ATCAA	ACGAT	CT	ACTTTTTT	514
VE MORF	ATCA	AAGCTCA	AACG	CTTTA	TC	TTTTTGT	AAGT	TCAT	TTAT	TTT	TTAGG	TTCCC	CATAT	TTTTTC	627
VP MORF	ATCG	AGCTTA	AAAG	CTTTA	TC	TTTTTAT	AAGT	TCAG	TTAT	TTT	TTAG	ATTCT	CATAT	TTTTTC	584

Figure 14a. *V. pleisii* versus *V. ellipsiformis* nucleotide sequence alignment for the *M-ORF*. Shading indicates sequence divergence or lack of homology.

VE MORF	TCAA	AACTCAA	GTTTATCTTT	ATTTAACCTA	CCCAACGCAA	CTTCCTTCTC	TTGTTGCTCC	TCAA	AGACCT	697		
VP MORF	TCAA	AACTCAA	GTTTACTTTT	GTTTAAACCTA	CCCAACGCAA	CTTCCTTTTC	TTGTCGCTCC	TCAA	AGACTT	654		
VE MORF	CAGT	TAAAGT	CACAATACCG	CGAACAAACC	CCCAAAATAT	AAGCACGGTA	AAGAA	CAACA	TAA	CAAAAGT	767	
VP MORF	CAGT	TAAAGT	CACAATACCG	CGAATAAACC	CCCAAAATAT	AAGCACGGCA	AAGAA	CAACA	TAA	CAAAAGT	724	
VE MORF	AAGA	ATTGGG	TAGTTTTCTA	GGCAAAACCC	AAGCCATCTA	ACTAGTCTG	AGATCAGCCG	TAA	GATAAGA	837		
VP MORF	AAC	ACTTGGG	TAGTTTTCTA	GGCAAAACCTT	AAGCCATCTA	ACTAGTCTG	AGATCAGCCG	TAA	GATAAGA	794		
VE MORF	GAGT	GCTGTG	TCTTAAATA	CAGCTTTT	AGGTTGACAA	CCTCAGTCT	TTA	ACTT	CA	GAC	CAACTCC	905
VP MORF	GAGT	GCTGTG	CCTTAAATA	CAGCTTTT	AGGTTGACAA	CCTCAGTCT	TTA	GTTT	ACA	GAC	CAACTCC	864
VE MORF	CTAT	AAAATA	TCCATACTAA	AATA	-----	-----	-----	-----	-----	929		
VP MORF	CTAT	AAAATA	TCCACACTAA	AACACTATGA	CTTTCACGCA	AA	CAAA	ACG	912			

Figure 14b. *V. pleasii* versus *V. ellipsiformis* amino acid sequence alignment for the *M-ORF*. Shading indicates sequence divergence or lack of homology.

VE MORF	YFSMDIL*GV	20	S	40	60	I	140	62
VP MORF	FVCVKVMVF*	- - - - - GL	SREVVNK	KLYFKTQHS	ILRLISDLVS	WLGFCLENYP	WLGFCLENYP	SVTLFMLFFT
	CGYFMGWSV	- - - - - GL	N	KLYFKAQHS	ILRLISDLVS	WLGFCLENYP	WLGFCLENYP	SVTLFMFFFA
VE MORF	VLFWGFVRG	IVTLTEVFEE	QKEKEVALGS	LNKDKLEFEK	NMGNLKMMEI	ELNKKMKAFE	ELNKKMKAFE	LKKKVDRLKK
VP MORF	VLFWGFIRG	IVILTEVFEE	RQKEKEVALGS	LNSKLEFEK	NMENLKVMEI	ELNKKMKAFK	ELNKKMKAFK	LKKKVDRLMK
VE MORF	EEFGLIKKVD	160	GKLEELKAEV	180	KKEEESMIEEK	VDMKMEWLS	VDMKMEWLS	LKKKVDRLKK
VP MORF	EEFGLIKKLD	VLKKKEEFKFL	GKLEELKAEV	FELRKKVDKL	KKEESTIEEK	VDSMKMEWLG	VDSMKMEWLG	LKKKVDRLKK
VE MORF	EEYESKKADK	EIEGDDIKEK	EKVFDIVDDE	240	VGVEAKNIDK	KKNLKK-VG	GVTKNSD*KE	KHYN*TYHWS
VP MORF	EEFESKKADK	EIEGDDIKEK	EKVFDIVD-E	260	AGVEAKNIGK	KKKLTKKKL	GVTKNN--	-VSDTINAS
VE MORF	GGCYY*CW*Q	300	GSCGYQKSY*	KEM*-ESD*E	KTHWS	SWWG	309	
VP MORF	NKANLEIKKD	TKKKYKKVVK	KKSTKESDGE	- - - - -	- - - - -	- - - - -	303	

Figure 15. *V. pleasii* versus *V. ellipsiformis* amino acid sequence alignment for the *M-ORF*. Shading indicates sequence divergence or lack of homology.

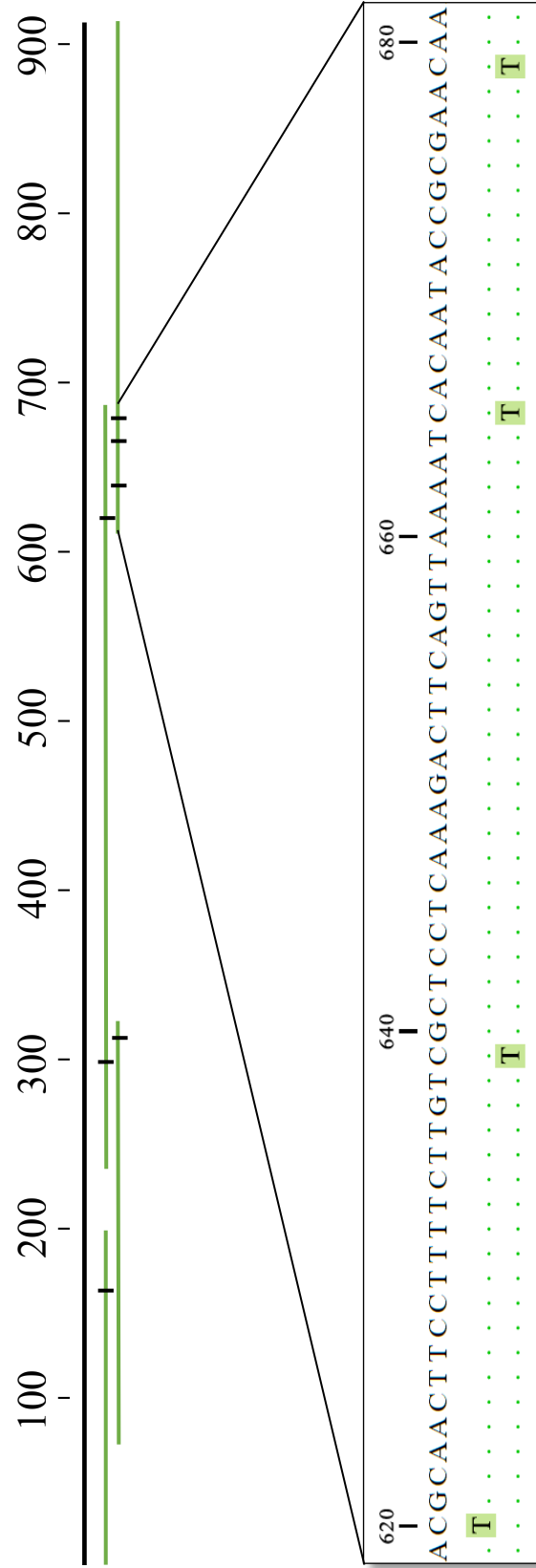


Figure 16. Heteroplasmic sites within the *M-ORF* of *V. pleasii*.

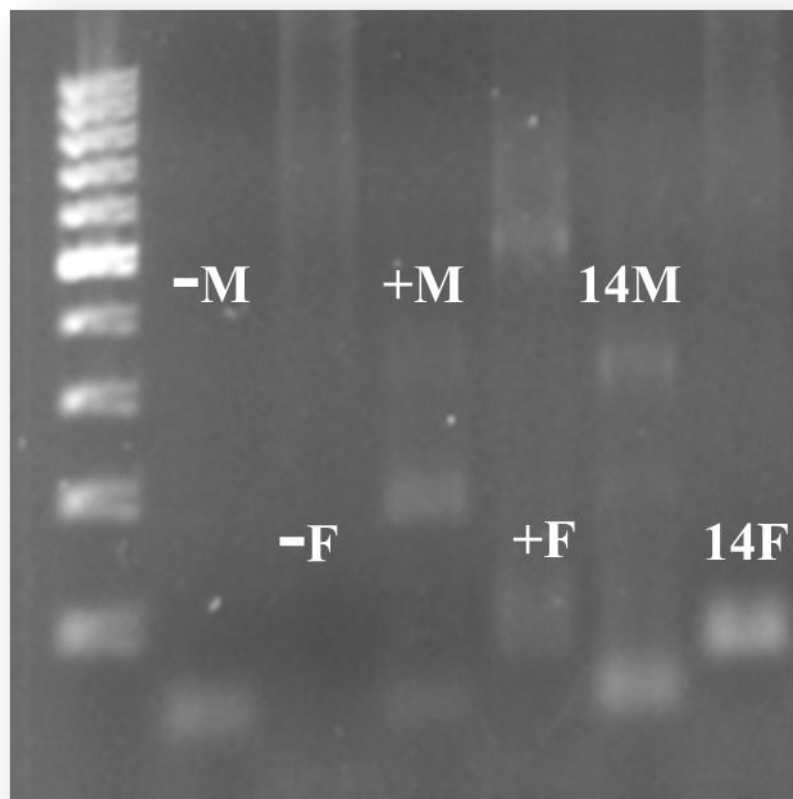


Figure 17. PCR of unwashed sperm showing *F-ORF*. *F-ORF* mtDNA appeared in sperm samples within individual 14 when the sample was not washed. Negative and positive signs indicate the negative or positive controls. M and F represent DNA amplified from *M*- or *F-ORF* respectively. The number indicates the individual tested. [D1]

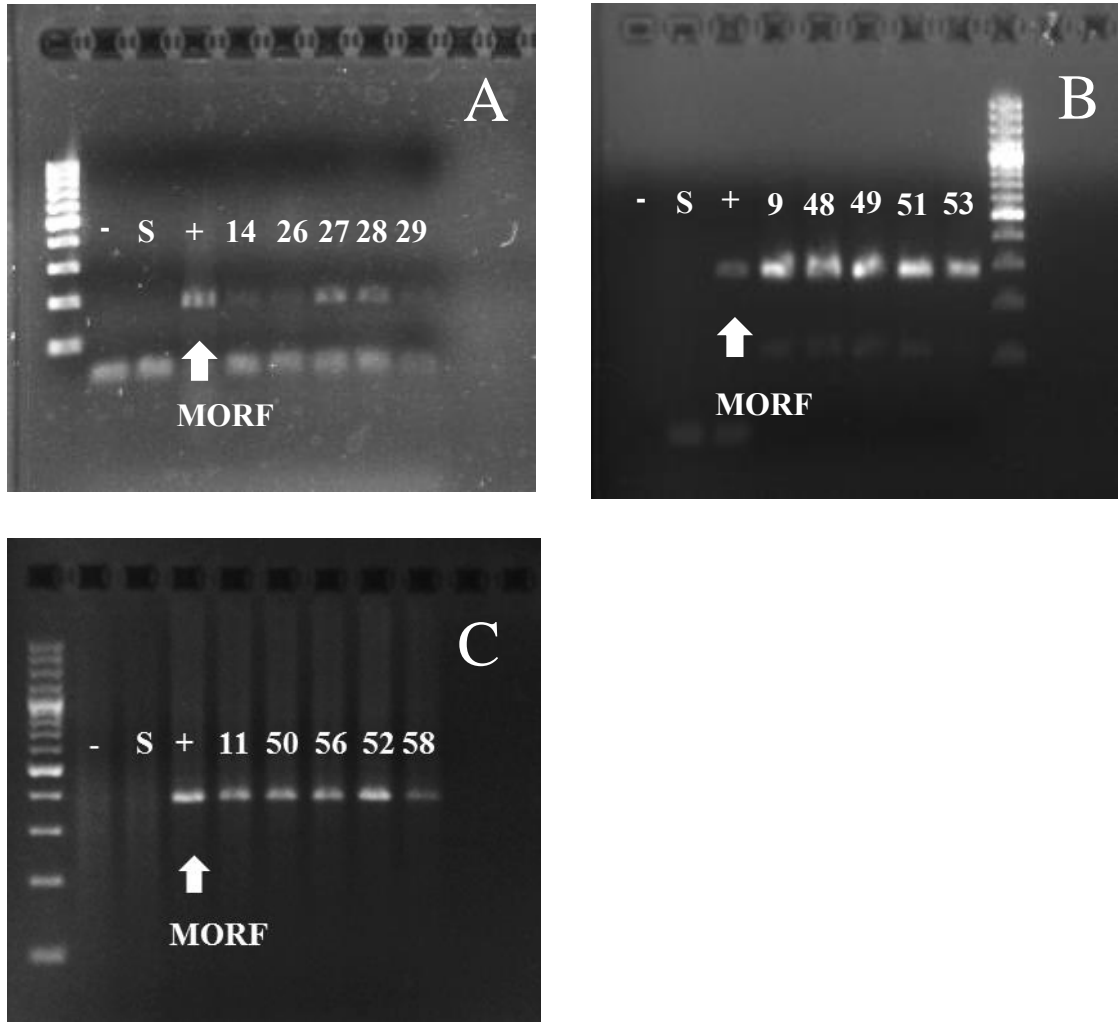


Figure 18. Non-nested PCR of washed sperm using *M-ORF* primers. Negative and positive signs indicate negative and positive controls. Supernatant (S) samples are present as a negative control to ensure stray DNA did not contaminate the samples. Each number represents an individual. Panels A, B, and C display individuals tested in different PCR reactions.

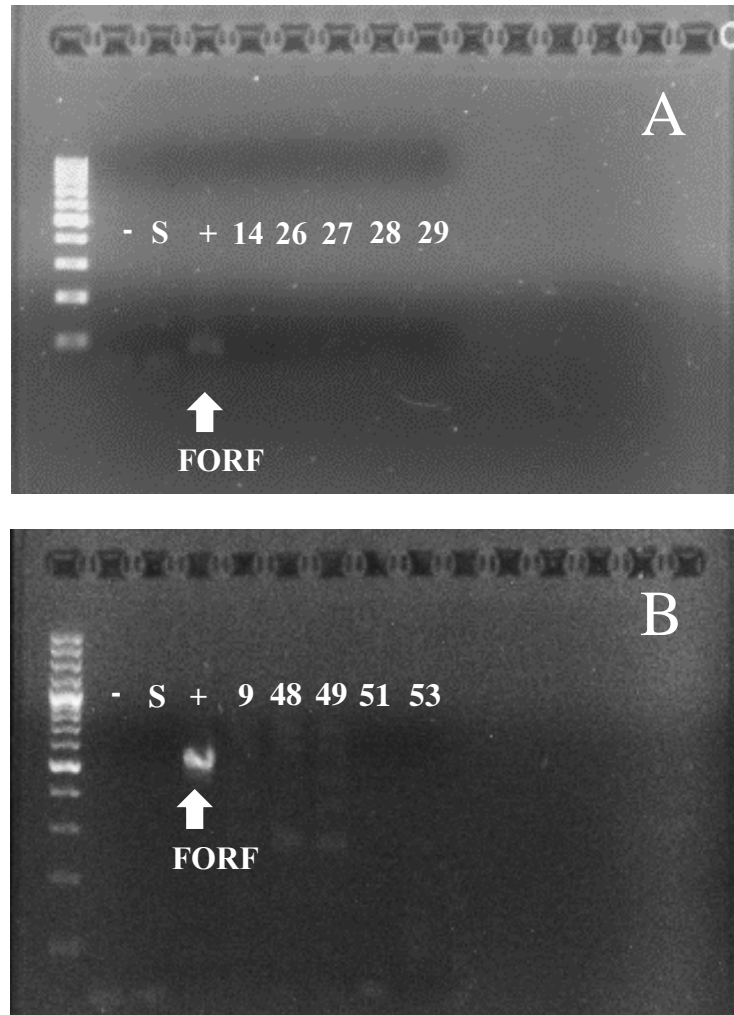


Figure 19. Non-nested PCR of washed sperm using *F-ORF*-specific primers within sperm. *F-ORF* was not detected within sperm using nested PCR. Negative and positive signs indicate negative and positive controls. Supernatant (S) samples are present as a negative control to ensure stray DNA did not contaminate the samples. Each number represents an individual. [D2] Panels A and B display individuals tested in different PCR reactions.

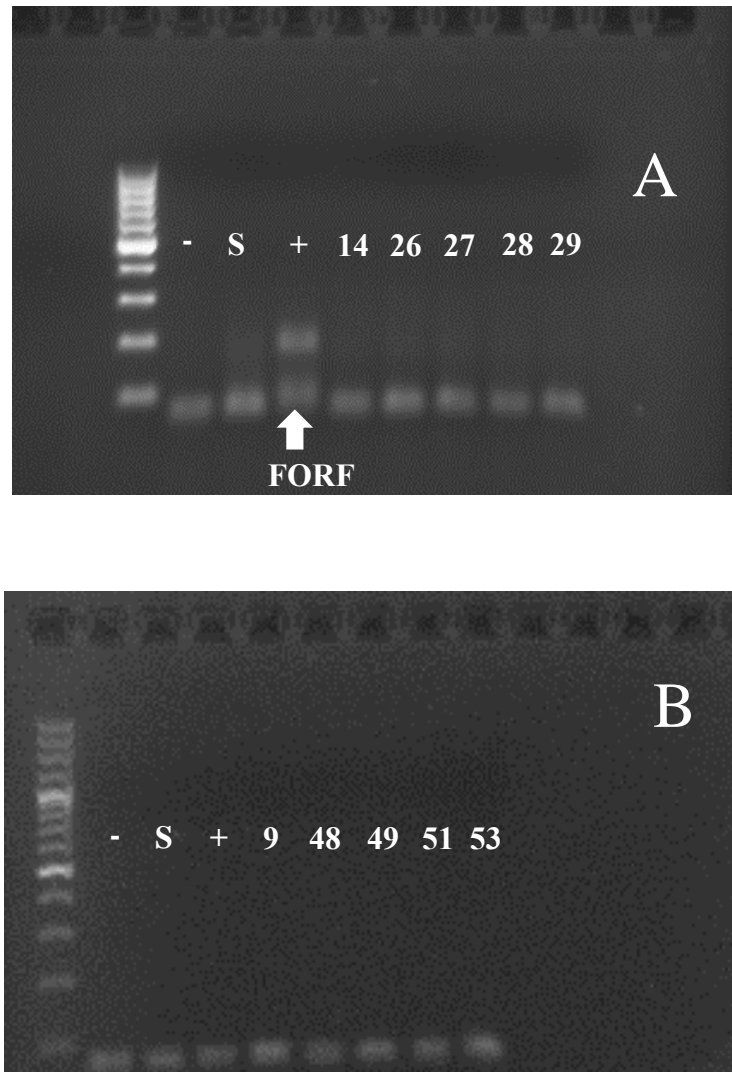


Figure 20. Nested PCR of washed sperm using *F-ORF*-specific primers within sperm. Contamination of negative control usually appeared. Negative and positive signs indicate negative and positive controls. Supernatant (S) samples are present as a negative control to ensure stray DNA did not contaminate the samples. Each number represents an individual. Panels A and B display individuals tested in different PCR reactions.

[D3]

APPENDICES

Appendix A. *F-ORF* and *M-ORF* GenBank accession numbers.

Species	Mt region	Nucleotide accession	Amino acid accession	Figure
<i>V. iris</i>	<i>F-ORF</i>	HM849533.1	AEC14192.1	Table 7
<i>T. lividus</i>	<i>F-ORF</i>	HM849457.1	AEC14116.1	Table 7
<i>E. lineolata</i>	<i>F-ORF</i>	HM849378.1	AEC14037.1	Table 7
<i>L. siliquoidea</i>	<i>F-ORF</i>	HM849385.1	AEC14044.1	Table 7
<i>L. powelii</i>	<i>F-ORF</i>	HM849384.1	AEC14043.1	Table 7
<i>F. flava</i>	<i>F-ORF</i>	HM849382.1	AEC 14041.1	Table 7
<i>V. ellipsiformis</i>	<i>F-ORF</i>	HM849529.1	AEC 14188.1	T7, F12,13,14
<i>V. ellipsiformis</i>	<i>F-ORF</i>	HM849530.1	AEC14189.1	T7, F12,13,14
<i>V. ellipsiformis</i>	<i>M-ORF</i>	NC_013659	N/A	Figure 15
<i>T. macrodon</i>	<i>F-ORF</i>	HM849478.1	AEC 14137.1	Table 7
<i>P. metnecktayii</i>	<i>F-ORF</i>	HM849406.1	AEC 14065.1	Table 7
<i>C. tuberculata</i>	<i>F-ORF</i>	HM849377.1	AEC 14036.1	Table 7
<i>M. nervosa</i>	<i>F-ORF</i>	HM849404.1	AEC 14063.1	Table 7
<i>P. grandis</i>	<i>F-ORF</i>	HM849429.1	AEC 14086.1	Table 7
<i>L. complanata</i>	<i>F-ORF</i>	HM849393.1	AEC 14052.1	Table 7
<i>U. peninsularis</i>	<i>F-ORF</i>	HM849528.1	AEC 14187.1	Table 7
<i>A. marginata</i>	<i>F-ORF</i>	HM849366.1	AEC 14025.1	Table 7
<i>S. undulatus</i>	<i>F-ORF</i>	HM849450.1	AEC 14109.1	Table 7
<i>L. rimosus</i>	<i>F-ORF</i>	HM849398.1	N/A	T7, F14
<i>T. texasensis</i>	<i>F-ORF</i>	HM849477.1	N/A	T7, F14
<i>T. Paulus</i>	<i>F-ORF</i>	HM849474.1	N/A	T7, F14
<i>T. glans</i>	<i>F-ORF</i>	HM849456.1	N/A	T7, F14
<i>F. ebenus</i>	<i>F-ORF</i>	HM849379.1	N/A	T7, F14
<i>Q. houstonensis</i>	<i>F-ORF</i>	HM849441.1	N/A	T7, F14
<i>U. peggyae</i>	<i>F-ORF</i>	HM849506.1	N/A	T7, F14
<i>P. lacustris</i>	<i>F-ORF</i>	HM849439.1	N/A	T7, F14
<i>L. costata</i>	<i>F-ORF</i>	HM849397.1	N/A	T7, F14
<i>A. undulata</i>	<i>F-ORF</i>	HM849369.1	N/A	T7, F14

Appendix B. Amino acid single letter codes.

Amino acid	Single letter codes	Charge
Isoleucine	I	N/A
Leucine	L	N/A
Valine	V	N/A
Phenylalanine	F	N/A
Methionine	M	N/A
Cysteine	C	N/A
Alanine	A	N/A
Glycine	G	N/A
Proline	P	N/A
Threonine	T	N/A
Serine	S	N/A
Tyrosine	Y	N/A
Tryptophan	W	N/A
Glutamine	Q	N/A
Asparagine	N	N/A
Histidine	H	N/A
Glutamic acid	E	-
Aspartic acid	D	-
Lysine	K	+
Arginine	R	+
Stop codon	*	

Appendix C. *CoxI* GenBank accession numbers.

Species	Nucleotide accession	Amino acid accession
<i>Venustaconcha pleasii</i>	KC537311.1	AGK83434.1
<i>Venustaconcha ellipsiformis</i>	KC537304.1	AGK83427.1