Cloning, Characterization, and Expression Analysis of the *Vasa* Gene from *Kryptolebias marmoratus*

A Thesis submitted
to the Graduate School
Valdosta State University

in fulfillment of requirements
for the degree of

MASTER OF SCIENCE

in Biology

in the Department of Biology
of the College of Arts and Sciences

December 2012

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BS, Valdosta State University, 2008

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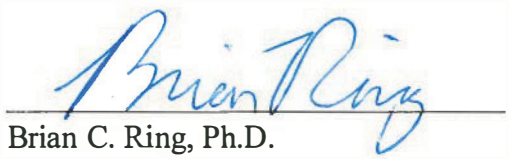

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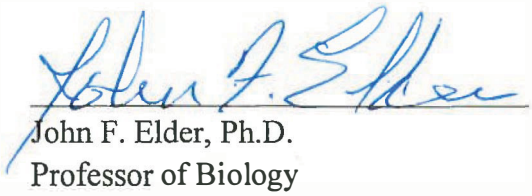

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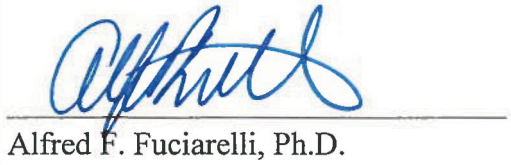

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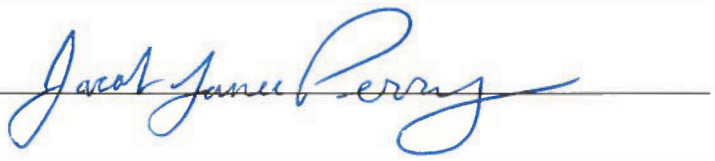
Signature 

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ABSTRACT

Kryptolebias marmoratus, formerly known as the Mangrove Rivulus, has been studied in the laboratory setting since the late 1950s. *K. marmoratus* is a selfing hermaphrodite with a unique gonad, termed the ovotestis, consisting of both ovarian and sperm tissue. One particular gene of interest to the development of this gonad is the *vasa* gene. *Vasa* is an ATP-dependent RNA helicase, which has been studied in many different organisms such as: *Drosophila melanogaster* (fruit fly), *Homo sapiens* (humans), *Ambystoma mexicanum* (salamander), *Xenopus laevis* (frog), *Danio rerio* (zebrafish), *Oryzias latipes* (medaka), and *Strongylocentrotus purpuratus* (sea urchin). The *vasa* gene was cloned and sequenced from *K. marmoratus*. The gene consists of 2,273 nucleotides, a predicted amino acid sequence of 641 base pairs, and a 3' untranslated region (UTR) of 234 base pairs in length. BLASTp and phylogenetic analysis revealed that the *kmvasa* gene is closely related to *T. oreintalis* (Pacific blue fin tuna) and has a close relationship to *O. latipes* (medaka). The ATP-dependent RNA helicase *vasa* protein was detected in only the gonad region of *K. marmoratus* hermaphrodites and males. In other species, the *vasa* protein is detected throughout embryogenesis. It was successfully shown that the *K. marmoratus vasa* gene is also expressed at similar levels throughout embryogenesis.

Key words: (ovotestis, *vasa* ATP-dependent RNA helicase, UTR, phylogenetic analysis, and QPCR)

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ACKNOWLEDGEMENTS

I cannot express enough thanks to my committee who has helped me through every step of the way through this process of learning how to become a better scientist. I would also like to thank my family, friends, and especially Landis International, Inc. for their continued support through these years of continuing my education.

Chapter I

INTRODUCTION

The mangrove killifish, *Kryptolebias marmoratus* (Teleost: cyprinodontiformes) was first discovered in 1956 (Harrington & Rivas, 1958; Harrington 1961, 1963). Populations of *K. marmoratus* are distributed from the Gulf of Mexico to the Caribbean Sea, with Vero Beach, Florida, as the northern limit of its range, and Brazil as the southern limit (Harrington & Rivas, 1958; Taylor et al., 1995). Its habitat mainly consists of mangrove estuaries containing brackish water, but it can occasionally inhabit freshwater as well (Harrington, 1961). Harrington (1961) was the first to report that *K. marmoratus* could self-fertilize and produce viable offspring, and hence was a selfing hermaphrodite. Harrington (1961) also noted that hermaphrodites are pale in color with a well-developed black spot on the caudal-fin base, whereas males were colorful. Rigorous analysis showed that some hermaphroditic individuals converted into males through a process that reduced the ovarian tissue and enlarged the sperm producing tissue (Harrington, 1967, 1971; Soto et al., 1992). Eggs incubated at 18-20°C, or treated with 17-methyl-testosterone (androgen hormone), produce primary males that develop only a testis (Harrington, 1967; Kanamori et al., 2006). Wild-type populations are composed of 2-25% primary males as estimated by the levels of heterozygosity within populations due to presumed intermittent outcrossing (androdiecy) of hermaphrodites to males (Harrington, 1967, 1968, 1975; Mackiewicz et al., 2006a, b, c; Turner et al., 2006; Tatarenkov et al., 2007).

K. marmoratus hermaphrodites have a unique gonad or "ovotestis" that contains both functional ovarian and sperm tissue. Simultaneous internal fertilization occurs within the ovotestis (Harrington, 1963; Sakakura et al., 2006). *K. marmoratus* is unique when compared to other well-established teleost models such as medaka and zebrafish which are gonochoristic (existing as either male or female individuals only). Over the past 40 years, *K. marmoratus* has been used as a model to study toxicology, morphology, ecology, pollution, carcinogenesis, phylogeny, developmental biology, and genetics (Park & Kim, 1984; Park et al., 1990; Mackiewicz et al., 2006; Lee et al., 2007; Mourabit et al., 2012; Lee et al., 2008; Moore et al., 2012; Tatarenkov et al., 2010). In spite of the varied interest in its unique biology, little has been done to investigate the development of the ovotestis that is unique to this vertebrate fish. One experimental approach to better understand this important developmental anomaly is through investigation of genetic determinants known to be involved in gonadogenesis.

One potential and valuable gene for the study of the highly dynamic ovotestis ontogeny within *K. marmoratus* is the *vasa* gene. The *vasa* gene has been detected in organisms such as: *Drosophila melanogaster* (fruit fly), *Homo sapiens* (humans), *Ambystoma mexicanum* (salamander), *Xenopus laevis* (frog), *Danio rerio* (zebrafish), *Oryzias latipes* (medaka), and *Strongylocentrotus purpuratus* (sea urchin). The earliest discovery of the *vasa* gene was through classic genetic grandchild-less screens during early embryogenesis of the fruit fly (Schüpbach & Wieschaus, 1986). The *vasa* protein is required for primordial germ cell development, and mutant analysis of fruit flies lacking this gene has shown that they do not form germ cells (Schüpbach & Wieschaus, 1986). The *vasa* gene transcript is localized to the polar region of the fertilized egg during pre-

gastrulation. There it directs the cytoplasmic zone formation during primordial germ cell (PGC) development (Raz 2000). During this period *vasa* is expressed, and is detectable as both messenger ribonucleic acid (mRNA) and protein. The active *vasa* protein unwinds bound ribonucleic acid (RNA) that is specific for cell fate determination (Cordin et al., 2006). After development of the cytoplasmic zone, *vasa* activity is still detected in specific cells, and *vasa* specific cells will migrate with its other constituents of the PGCs to the gonadal ridge (Castrillon et al., 2000). Eventually protein activity in this specialized area of the organism will form the tissue which produces gametes for sexual reproduction in the adult, which can be autonomous or regulative in cell specification (Raz 2000). Among vertebrate (*i.e.,* zebrafish) *vasa* orthologous genes, autonomous specification of *vasa* has been observed (Knaut et al., 2002). However, in medaka *vasa* specification of the PGCs is not predetermined autonomously during early development, but becomes localized gradually through a regulative mechanism (Knaut et al., 2002).

The *vasa* gene is an ATP-dependent RNA helicase within the superfamily two of RNA helicase proteins (Lasko & Ashburner, 1988; Linder et al., 1989; Cordin et al., 2006). The *vasa* DEAD-box family of proteins is highly homologous to other RNA helicases families like DEAH, DExH, and DExD proteins described in (Cordin et al., 2006). These particular helicase proteins have similar regions of amino acid homology. Cordin et al. (2006) described nine highly conserved regions of the DEAD-box protein that are homologous to other RNA helicase proteins across these domains. The conserved regions implicated in protein activity are: Q-motif, motif-I (Walker A motif), motif-Ia, motif-Ib, motif II (DEAD-box region), motif III (SAT region of helicase activity), motif-IV, motif-V, and motif-VI (Cordin et al., 2006).

Until now, the *vasa* gene has not been studied from *K. marmoratus*. To begin to understand the role of *vasa* in directing the unique gonadogenesis of *K. marmoratus*, the cloning and sequencing of the gene from a single clonal laboratory line of *K. marmoratus* (*Hon 9* from Valdosta State University; Tatarenkov et al., 2010) is described. A phylogeny of the *K. marmoratus vasa* (*kmvasa*) gene to other similar teleost fish and selected higher vertebrate *vasa* sequences is proposed. The expression of *kmvasa* by Quantitative Polymerase Chain Reaction (QPCR) from *K. marmoratus* adult tissues is characterized to establish its specific gonadal expression in the ovotestis and testis. Lastly, QPCR was used to quantify the relative expression levels of *kmvasa* gene mRNA across 11 key embryonic developmental stages to characterize the expression pattern of *vasa* over time.

Chapter II

MATERIALS AND METHODS

Fish Husbandy and Embryo Collection

Test animals were maintained according to Institutional Animal Care and Use Committee (IACUC) regulations. The IACUC protocol approval number is AUP-00045-2012 (see Appendix B).

K. marmoratus hermaphrodites were maintained at 28°C on a 14 h light:10 h dark photoperiod at the Valdosta State University Aquatic Laboratory. Single fecund fish were reared in 2-L breeder tanks (Aquatic Habitats, Apopka, FL) in a brackish water solution (17 parts per thousand Instant Ocean, Madison, WI) with weekly water changes. The fish were fed freshly hatched *Artemia* brine shrimp (2 g/L of cysts; Artemac, Ogden, UT) collected from a commercial hatchery funnel (Aquatic Ecosystems, Apopka, FL) in brackish water supplemented with 20 g of Instant Ocean daily. *Artemia* cysts were weighed into two-gram portions each time the funnel was setup for the next feeding period. Cysts were grown for 18 to 24 h before purifying the hatched shrimp from the egg shells. Each fish was given between 5-9 mL of shrimp per day to increase egg laying production as described by Moore et al. (2012). Embryos were collected from the breeder tanks by first removing the hermaphroditic parent using the basket insert from the breeder tank. Following the removal of the parent fish, embryos were collected using a 3-mL disposable pipette. Embryos were observed in plastic petri dishes (100 x 15 mm) containing brackish water solution for specific stages of development ranging from

32-cell through pectoral fin movement as described in Mourabit et al. (2011). The embryos were then placed into sample tubes containing RNAlater (Life Technologies, Carlsbad, CA) solution, and stored frozen at -70°C.

Cloning of kmvasa Gene

For cloning of *kmvasa*, total RNA was purified from fresh adult *K. marmoratus* ovotestis using the Qiagen RNeasy mini kit (Qiagen, Germantown, MD). First strand complimentary DNA (cDNA) was synthesized from total RNA using the Superscript III Reverse Transcriptase kit according to the manufacturer's protocol (Life Technologies, Carlsbad, CA). Specific primers were designed based on comparison of *O. latipes* (*olvas*) sequence (National Center for Biotechnology Information, NCBI # NM_001104676; Shinomiya et al., 2000) to a *K. marmoratus* draft genome (personal collaboration via e-mail, Joanna Kelly, Stanford University).

Initially, a partial sequence of k*mvasa* was developed from a fragment obtained using 5 PRIME HotMasterMix protocol (Fisher Scientific, Pittsburg, PA). A 50-µL polymerase chain reaction (PCR) using the gene specific primers F2 (5'-TGGAGGCCAGGAAGTTTGC-3'), R1 (5'-CTCCCGATACGGTGAACATA-3') was used to amplify the fragment. PCR was carried out in a thermocycler (Bio-Rad, Hercules, CA) using 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and finally 1 cycle of 72°C for 10 min. PCR products were electrophoresed on a 1X Tris-Acetate-EDTA (TAE) diagnostic agarose gel, where a single 700-bp fragment was observed. This fragment was subsequently ligated into a pCR 2.1-TOPO plasmid vector and transformed into One Shot Top10 Chemically Competent *Escherichia coli* cells (Life Technologies, Carlsbad, CA). White colonies, positive for antibiotic resistance and

fragment inserts, were sampled by using a sterile wooden probe to sample each colony, and then placed directly in 5 mL of lauria broth (LB) (Difco, Sparks, MD) containing 500 μg/mL of ampicillin. Cultures were incubated for 16 h at 37°C, and shaken at 200 rpm. Plasmids were purified from 4 mL of the bacterial cultures utilizing a Qiagen quick plasmid mini-prep kit (Qiagen, Germantown, MD). A 1-mL aliquot of each culture was frozen in 50% glycerol at -70°C for archival storage. An aliquot of purified plasmids were used for restriction digestion with *EcoRI* to confirm the presence of recombinant cloned inserts of appropriate size on 1X TAE gels. All plasmid samples were then packaged and shipped to the Florida State University for DNA sequencing (Steve Miller, King Life Science Building). The initial *kmvasa* partial cloned sequence was queried against the NCBI database by BLASTn and the conserved DEAD-box of *vasa* was confirmed by sequence similarity to other species.

In order to isolate the full-length k*mvasa* gene, 3' and 5' Rapid Amplification of cDNA Ends (RACE) was performed utilizing the SMART RACE cDNA Amplification Kit according to the manufacturer's protocol (Clontech Laboratories, Inc., Mountain View, CA). RACE-ready cDNA for the 3' region of the *kmvasa* was synthesized by PCR amplification of the cDNA using the forward primers: 3R1 (5'-AGCAGGCCCTTACAGAC-3') and 3R2 (5'- TATGTTCACCGTATCGGGAG-3'). The reverse primer was a 10X Universal Primer A Mix (Clontech Laboratories, Inc., Mountain View, CA). RACE-ready cDNA for the 5' region of *kmvasa* was synthesized by PCR amplification of the cDNA with a forward 10X Universal Primer A Mix and the reverse primer 5R1 (5'- GGATCTCACTGAAACGACTG-3'). Nested PCR with the reverse primer 5R2 (5'-TGGGAATAGCATGCTTCTGG-3') was performed on the

previous PCR sample to amplify more of the 5' end of *kmvasa*. PCR parameters for all RACE reactions were: 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 3 min. PCR products were electrophoresed on a 1X TAE diagnostic agarose gel and cloned into a pCR 2.1-TOPO vector.

A complete 2X consensus sequence for *kmvasa* gene was subsequently generated from PCR by amplifying the entire gene with primer UTR-F4 (5'-CAGAGAGCCGATAGGACAG-3'), and reverse primer UTR-R4 (5'-TTCTGTAACACTTCCTGGATC-3'). BigDye® Terminator chemistry was performed on this fragment using a Applied Biosystems 3730 Genetic Analyzer with Capillary Electrophoresis (Life Technologies, Carlsbad, CA) at Florida State University with the primers: *Kmvasa* F1, F2, F3-Gap, UTRF4, R1, R2, R3-Gap, UTR-R4, 5R1, 5R2, 3R1, and 3R2 (see Table 1). The final sequence of *kmvasa* was deposited on NCBI Genbank, accession number JX880071.

Phylogenetic Analysis

The full length open reading frame of *kmvasa* was translated into a predicted protein sequence to search the NCBI database using BLASTp (Altschul et al., 1997). Closely related sequences of teleost fish were ranked and selected based on max score (e-value) and percent identity (see Table 2 for NCBI accession numbers). Nucleotide sequence data was retrieved for each organism selected and placed into a text file in FASTA format including the full length *kmvasa* nucleotide sequence. The FASTA text file was then loaded into the SEAVIEW program (Galtier et al., 1996) to edit sequences by deleting the 5' UTR and the 3' UTR so that only the *vasa* open reading frames were compared among organisms. A multiple sequence alignment was performed on the protein sequence data utilizing the MAFFT algorithm option EINSI (Katoh et al., 2005)

located on the SEAVIEW program. The aligned protein sequence was converted back to the nucleotide level and the newly manipulated sequence was saved as a FASTA file. The aligned sequence was loaded into the program MACCLADE 4.08 (Maddison, 2005) and converted into a NEXUS-format file (see Appendix A). The file was subjected to phylogenetic analysis using PAUP 4.0 by neighbor-joining analysis (Swafford, 2001). The gene tree was saved as a TREE viewable format and further edited utilizing MACCLADE 4.08. *Pantodon buchholzi*, better known as the African butterfly fish, was selected as the out-group for initial comparison to other fish species. For phylogenetic comparison across the animal kingdom, *kmvasa* was compared to other organisms as described above. The invertebrate *D. melanogaster vasa* sequence was used as the out-group.

QPCR Analysis of kmvasa Expression in Adult Organs and Embryos

Kmvasa detection from tissues was analyzed from three different adult fish. Total RNA was extracted from seven tissues [brain, heart, liver, gall bladder, intestine, ovotestis (hermaphrodite), and testes (primary male)] of individual adult fish from *Hon9* strain (Tatarenkov et al., 2010), and purified with the RNeasy® Plus Universal Mini Kit (Qiagen, Germantown, MD). RNA was quantified using a Nanodrop™ 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Approximately 100-130 ng of total RNA from adult organs was converted to cDNA using an Applied Biosystems™ High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA). *Kmvasa* expression levels in these tissues was measured by real-time RT-PCR with Power SYBR® Green (Applied Biosystems, Foster City, CA) using a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Detection was performed using primer pair real-F and real-R (see Table 1). PCR parameters were: 1 cycle of 95°C

for 10 min, 40 cycles of 95°C for 30 s, and 60°C for 1 min followed by a melt curve. The *km β-actin* gene was used as a housekeeping reference standard to normalize expression levels between samples (Lee et al., 2008). All experimental samples were performed in triplicate utilizing the *km β-actin* gene to normalize for expression detection in tissue samples against *kmvasa*. Upon completion of QPCR, tissue-specific samples were electrophoresed on a 5% acrylamide gel for semi-quantitative purposes. Gel images were taken with an ImageQuant LAS 4000 from GE™ (GE Healthcare Biosciences, Pittsburg, PA) (see Figure 6).

Similarly, total RNA was extracted from 11 different stages of embryos from *Hon9* hermaphrodites using an RNeasy® Plus Universal Mini Kit (Qiagen, Germantown, MD) (see Table 3). RNA was quantified and 100 – 130 ng of total RNA was used from each sample to produce cDNA as previously described. *Kmvasa* expression levels in embryos were measured by real-time RT-PCR with Power SYBR® Green (Applied Biosystems, Foster City, CA) using a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA). The *km β-actin* gene was used as a housekeeping reference standard, and all experimental samples were performed in triplicate as previously described.

Expression data were transformed by collecting cycle threshold (C_T) values for three subsamples within each embryo stage. The mean C_T value with in each embryo stage was then calculated. This process was repeated three separate times (*i.e.*, three "biological replicates"). Using the following equation (Giulietti et al., 2001):

$$\Delta\Delta C_T = \Delta C_T \text{ (Sample)} - \Delta C_T \text{(Calibrator)},$$

a $\Delta\Delta C_T$ value was calculated for each embryo stage within each biological replicate. Each value was then transformed (using $2^{-\Delta\Delta Ct}$) and a mean (n=3) value was calculated for each stage. These values represent the normalized expression levels of *kmvasa* within each stage of development relative to the normalized expression of the calibrator (*km β-actin*) (see Figure 8).

Chapter III

RESULTS

Results from this study present a summary of the full length DNA sequence of the *vasa* gene from hermaphroditic *K. marmoratus* (*kmvasa*). This *kmvasa* sequence was compared to other *vasa* sequences from similar fish species, higher order vertebrates, and invertebrates through phylogenetic analysis. Semi and quantitative polymerase chain QPCR data revealed that *kmvasa* gene expression is localized to the gonads. Varying temporal expression of *kmvasa* was detected during 11 stages of embryogenesis by QPCR. Tables and figures are found at the end of this chapter.

Cloning of Kmvasa

Total RNA was isolated from ovotestis to make cDNA. Sets of specific primers were designed to amplify regions of the *kmvasa* gene (see Table 1). Primer set *kmvasa* F2 and R1 produced a 700-bp PCR product across the middle of the gene (see Figure 1). Using *kmvasa* primer sets 5R1, 5R2, 3R1, and 3R2, the 5' and 3' ends of the gene were extended by RACE methods. *Kmvasa* F3-Gap and R3-Gap were used to fill in a sequence gap in the 5' region upstream of the 700-bp interior of the gene in order to fully connect the 5' region to the 700-bp region of the gene. Finally, *kmvasa* primer sets UTR-F4 and UTR-R4 were used to amplify the full length *kmvasa* gene (see Figure 2). The *kmvasa* mRNA is 2,273 nucleotides long and is predicted to code for a 641 amino acid long protein. The protein coding region or open reading frame (ORF) of the *kmvasa* gene contains a number of regions that are homologous to other members of

RNA helicase gene families. The first region is in the N-terminus of the ORF, consisting of five arginine-glycine-glycine (RGG) repeats (see Table 2). Secondly, a number of homologous motif regions can be identified, such as the Q-motif, motif-I, motif-Ia, motif-Ib, motif-II (DEAD-box), motif-III (SAT helicase region), motif-IV, motif-V, and motif-VI. The carboxyl terminus at nucleotide position 1894 - 1917 contains a short GAG repeat, which is not present in other vertebrates/invertebrates included in this study (see Figure 2).

Phylogenetic Analysis

By utilizing the NCBI database search engine setting BLASTp, *kmvasa* was compared to other database sequences with high scoring values of homology (see Table 2). A master list of fish (as well as other organisms) was produced in order to sort organisms according to taxonomic order (data not shown). Some organisms did not produce a max score, but were used in the phylogenetic analysis because they are frequently studied model organisms.

A phylogenetic analysis of the full length *kmvasa* gene sequence in relation to the *vasa* sequence of other teleost fish species revealed that *K. marmoratus* is a sister group to the euteleost, and its most recent common ancestor is most closely related to *O. latipes* (see Figure 3). The gene tree is rooted to *Pantadon buchholzi* (African butterfly fish) because it is the most ancestral fish within the order. Most bootstrap values for fish were between 98 and 100. The branch at *Osphronemus goramy* had a value of 77, and the *K. marmoratus* branch had a bootstrap value of 52. Two of the remaining branches in the analysis produced a value that was below 50.

A rooted tree analysis of the *kmvasa* gene in relation to other teleost fish species

using only the most highly conserved region of the *vasa* gene (nucleotide position 1057 – 2148, see Appendix A) was performed (see Figure 4). The out-group for this analysis was *P. buchholzi* and bootstrap values were significantly higher at four of the branches: *K. marmoratus* 89, *O. goramy* 97, *S. schlegelii* 50 and 61, respectively. Also, two of the branches recorded lower values in comparison to the first analysis, *Seriloa quinqueradiata* decreased from 100 to 76, and *O. niloticus* decreased from 98 to 54.

Phylogenetic analysis of full length *vasa* DNA sequences from specific vertebrates and invertebrates that are widely used as model organisms were also compared to *kmvasa*. The gene tree was produced by analyzing the full length *vasa* gene reported for each organism. *Drosophila melanogaster* was designated as the representative protostome out-group. The other organisms are representative deuterostomes (*i.e.,* fish, mammals, amphibians, and echinoderms). Bootstrap values between all vertebrate species were between 84 and 100, whereas two of the branches for the two invertebrate species show a bootstrap value below 50. The gene tree analysis of only the *vasa* gene reveals that the *K. marmoratus vasa* sequence is most closely related to the *T. orientalis* (tuna) with a bootstrap value of 99. The *K. marmoratus vasa* gene is also similar to the *vasa* gene of *O. latipes* showing a bootstrap value of 100 (see Figure 5).

Highly conserved regions of the *vasa* gene were used to perform a rooted tree analysis of the *K. marmoratus vasa* gene in comparison to specific vertebrate and invertebrate *vasa* genes. Using *D. melanogaster* as the out-group, bootstrap values were the same across all species except for *A. mexicanum* 80 and *K. marmoratus* 78. Using nucleotide position 1081 – 2424 (see Appendix A) in the analysis instead of the whole

protein decreases two of the branch points; however, bootstrap values were still high enough for confidence in positioning of the species (see Figure 6).

Kmvasa Expression in Adult Tissues and Embryos

Expression of *vasa* mRNA at the tissue level, and temporally through early to late stages of embryogenesis, were assessed. Tissue specific presence or absence of *kmvasa* mRNA was assayed by semi-quantitative PCR. QPCR was performed to detect and quantify mRNA expression levels. Protocol stringency was developed for embryo analysis so that *vasa* expression during embryogenesis could be analyzed with rigor.

Organs for tissue analysis were collected from adult hermaphrodites as well as males. Semi-quantitative PCR and QPCR were performed on *K. marmoratus* tissue cDNA's such as: liver, brain, lower intestine, gall bladder, heart, ovotestis, and testis of males. The mean amount of total RNA used to reverse transcribe first strand cDNA was 126 ng (+/- 6.12 ng) across six of the seven tissues analyzed. The testis total RNA input was 1000 ng, and was not subjected to normalization to 126 ng, (data not included in the mean). *Kmvasa* was expressed only in the gonad region (ovotestis and testis) of the fish (see Figure 7). The *kmvasa* primer pair real-F and real-R (Table 1) for QPCR flanks a 1.9 kilo-base intron (data not shown). The *vasa* plasmid construct was used as a positive control. *K. marmoratus β-actin* was used as a normalizing gene in parallel with *kmvasa* (see Figure 7). The *β-actin* control was used to determine whether genomic DNA (gDNA) was contaminating the sample of freshly transcribed total mRNA (see Figure 7, lane 3). The shift in the band in the control lane clearly shows that gDNA was used as a template, whereas in the tissue sample lanes only *K. marmoratus β-actin* cDNA was detected. By calculating the mean of the *kmvasa* C_T values, and dividing by the *kmβ-*

actin C_T mean values, ovotestis and testis *vasa* expression were found to be 0.93 : 0.89, respectively.

Ovotestis total RNA was normalized to 125.4 ng and the testis total RNA was normalized to 1000 ng. Even though the starting template amount was not the same, QPCR data revealed that *vasa* mRNA expression was similar.

Temporal vasa expression was analyzed in developing *K. marmoratus* embryos at 11 different stages of development. Embryos were collected (n = 47) at each embryonic stage, and individuals were pooled to isolate total RNA (see Table 3). The average total RNA input for each cDNA reaction was 134.23 ng (+/- 28.6 ng) per reaction (see Table 3). *Kmvasa* expression was relatively high in stage 1 embryos but decreased by stage 2 and 3 (see Figure 8). Expression of *kmvasa* in stage 4 and 5 embryos was similar but lower than that found in stage 1 embryos. Stages 6 through 9 embryos revealed a comparatively low *kmvasa* expression level, whereas an increase was observed in stages 10 and 11 embryos (see Figure 8). The decrease observed in *kmvasa* expression in stage 10 and 11 embryos may have been due, in part, to the increase in the embryo size which would have produced a larger ovotestis organ with an increased overall *vasa* expression.

Table 1. Primers used to amplify the vasa gene from *K. marmoratus* as well as RT-PCR primers for quantitative analysis.

Gene	Oligo name	Sequences (5' ⟶ 3')	Purpose
Kmvasa	F1	CCAGAAGCATGCTATTCCCA	cDNA amplification
	F2	TGGAGGCCAGGAAGTTTGC	cDNA amplification
	F3-Gap	GTGAACGACCCAGAGTTACAT	cDNA amplification
	UTR-F4	CAGAGAGCCGATAGGACAG	cDNA amplification
	R1	CTCCCGATACGGTGAACATA	cDNA amplification
	R2	TGAGGACATCCAGAGGATGGC	cDNA amplification
	R3-Gap	GATCTGGTATCCGGTGCTGA	cDNA amplification
	UTR-R4	TTCTGTAACACTTCCTGGATC	cDNA amplification
	5R1	GGATCTCACTGAAACGACTG	PCR for 5'-RACE
	5R2	TGGGAATAGCATGCTTCTGG	Nested PCR
	3R1	AGCAGGCCCTTACAGAC	PCR for 3'-RACE
	3R2	TATGTTCACCGTATCGGGAG	PCR for 3'-RACE
	real-F	TGTGGAAACACTGGTCGGG	Real-time RT-PCR
	real-R	GTGTTGAATGTGGTGCTGGA	Real-time RT-PCR
β-actin [1]	real-F	GATCCAAACAAACGAAGAAGCG	Real-time RT-PCR
	real-R	TCGAACGTGAAGGGTGAACACC	Real-time RT-PCR

[1] β-actin primer set was retrieved from Lee et al. (2008).

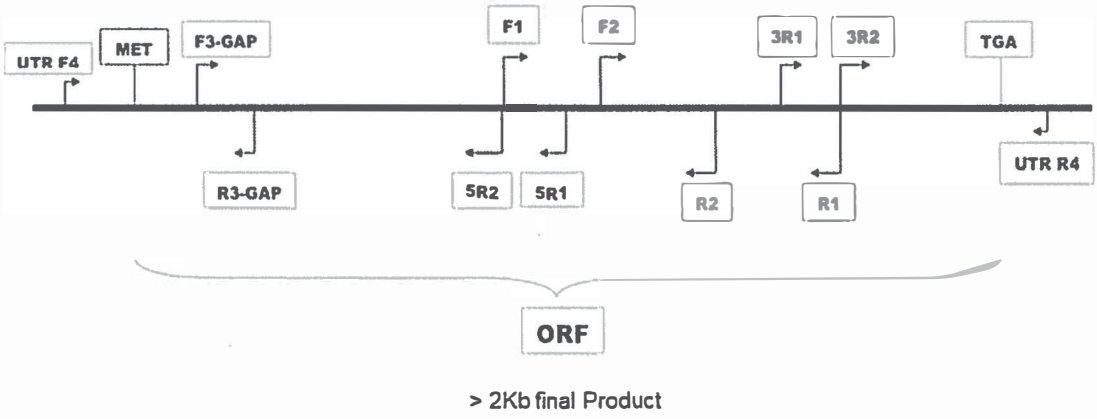

Figure 1. *Kmvasa* primer map in conjunction with gene orientation from 5' to 3', MET start site to TGA stop site, respectively.

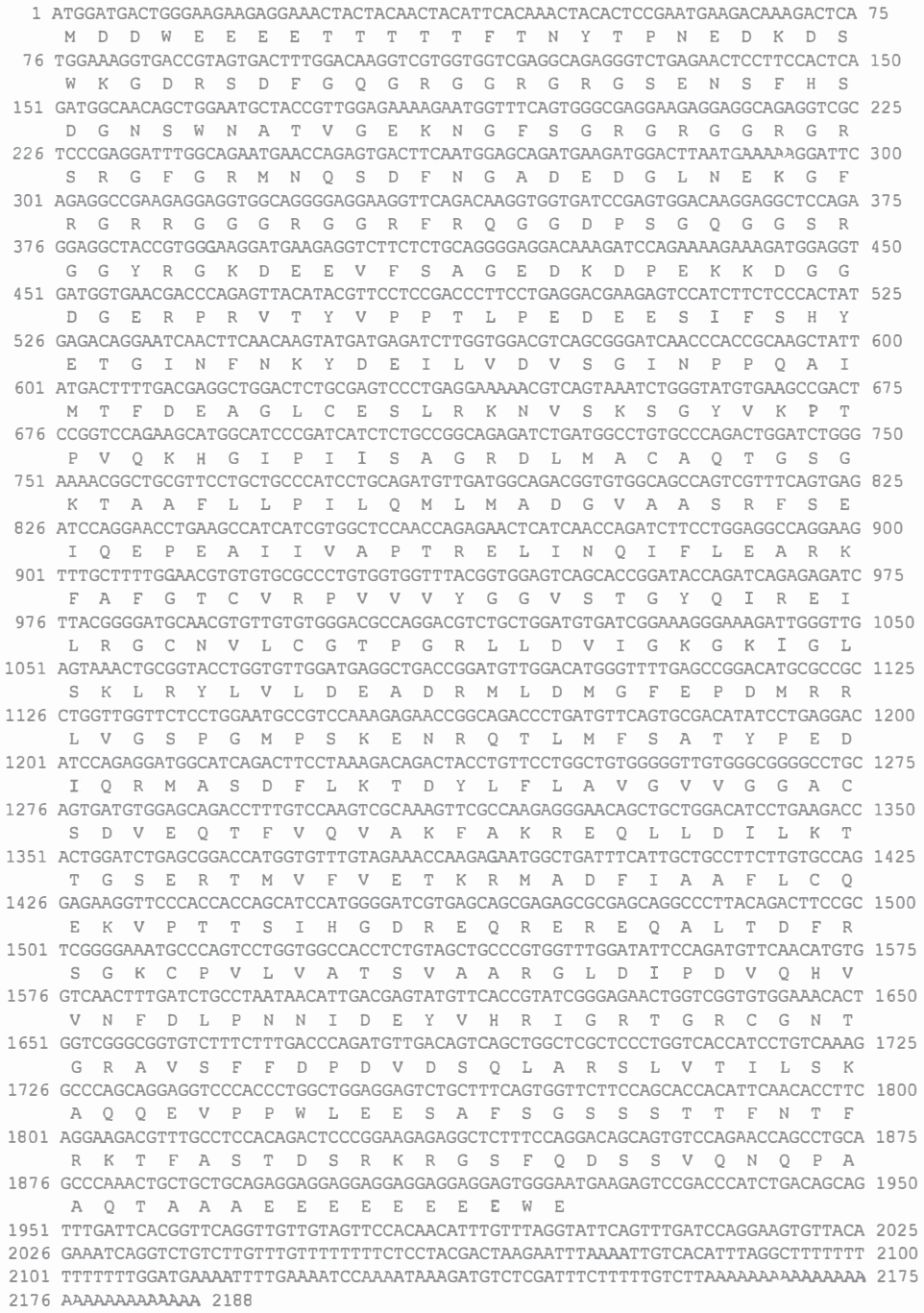

Figure 2. *K. marmoratus* full length *vasa* gene sequence and deduced amino acid sequence.

Table 2. List of *vasa* gene information[1] used in sequence and phylogenetic analysis of vertebrates and invertebrates.

		Nucleotide	A.A.	Full length	A.A.	NT	NT
Common Name	**Genus species**	**Accession No.**	**Max Score**	**Max Identity (%)**	**RGG repeats**	**GAG repeats**	**3' UTR Length**
K. marmoratus	*Kryptolebias marmoratus*	**JX880071**	N/A	100	5	8	234
Pacific blue fin tuna	*Thunnus orientalis*	**EU253482**	950	79	8	1	366
Giant Gourami	*Osphronemus goramy*	**GQ422440**	936	83	8	1	258
European Seabass	*Dicentrarchus labrax*	**GU987023**	922	81	7	0	303
Chub mackeral	*Scomber japonicus*	**GQ404693**	913	79	8	0	380
Stripetail Rockfish	*Sebastes schlegelii*	**JN634874**	912	77	9	0	352
Japanese Amberjack	*Seriola quinqueradiata*	**GU596411**	902	86	7	1	506
Nile Tilapia	*Oreochromis niloticus*	**AB032467**	894	74	4	1	124
Medaka	*Oryzias latipes*	**NM_001104676**	864	83	6	1	240
Japanese Trout	*Salvelinus leucomaenis*	**EU448254**	816	79	4	2	658
Goldfish	*Carassius auratus*	**AY821684**	809	73	11	1	620
Zebrafish	*Danio rerio*	**NM_131057**	796	77	11	1	644
African Butterfly Fish	*Pantodon buchholzi*	**AF479823**	N/A	N/A	N/A	0	313
Human	*Homo sapiens*	**NM_024415.2**	711	64	4	1	606
Mouse	*Mus musculus*	**NM_010029.2**	711	63	5	1	613
Fruit Fly	*Drosophila melanogaster*	**NM_165103.2**	N/A	N/A	10	2	148
Salamander	*Ambystoma mexicanum*	**AY542375.1**	N/A	N/A	5	1	202
African Clawed Frog	*Xenopus laevis*	**AF046043.1**	N/A	N/A	7	1	319
Sea Urchin	*Strongylocentrotus purpuratus*	**NM_001146193.1**	N/A	N/A	4	2	N/A

[1] N.T. – nucleotide, A.A. – amino acid.

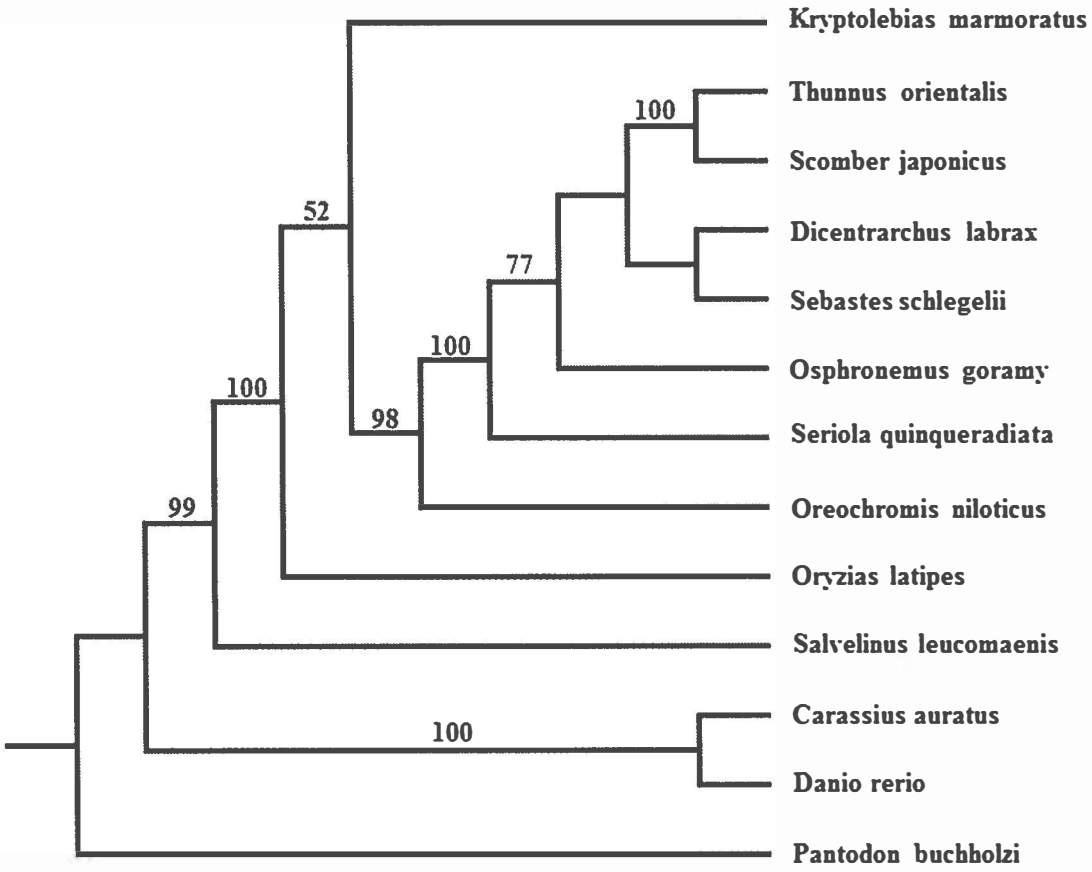

Figure 3. Gene tree analysis of the full length *kmvasa* gene along with other teleost fish *vasa* gene sequences.

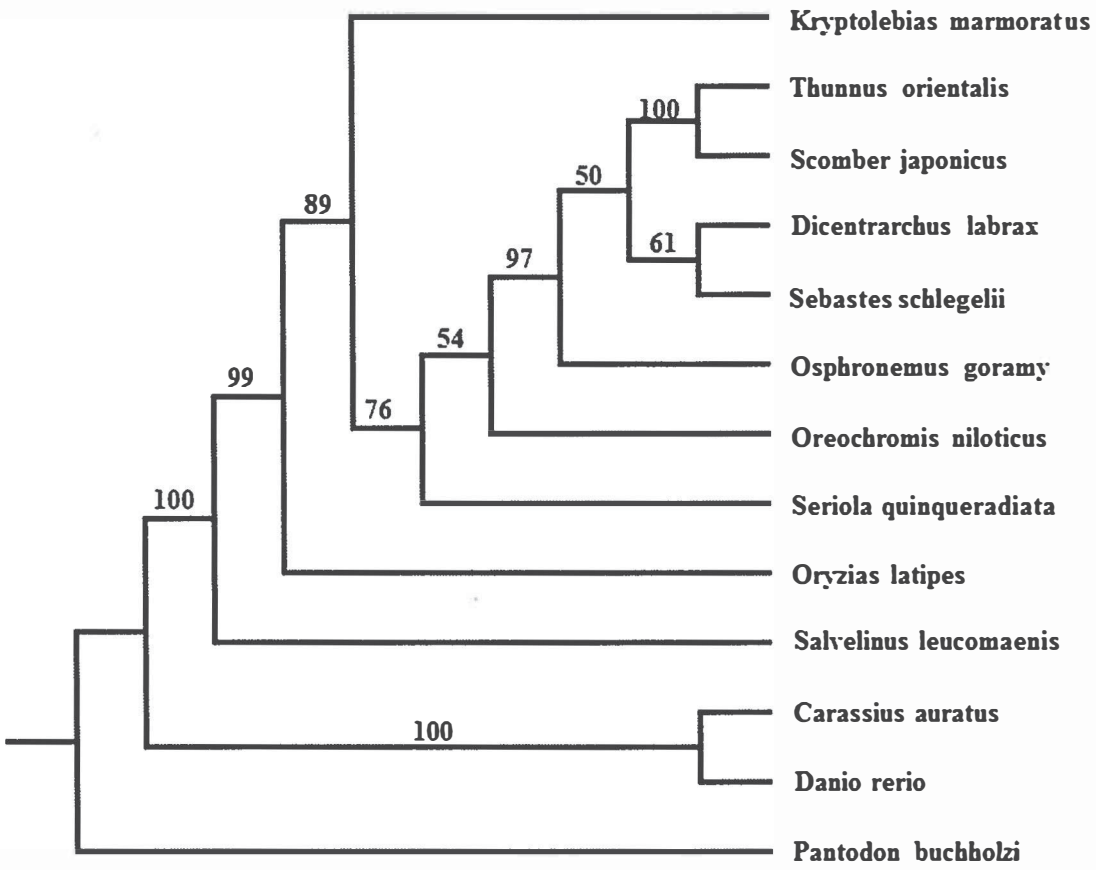

Figure 4. Gene tree analysis of the most highly conserved regions of *kmvasa* compared to other teleost fish *vasa* sequences utilizing nucleotide sequence 1057-2148.

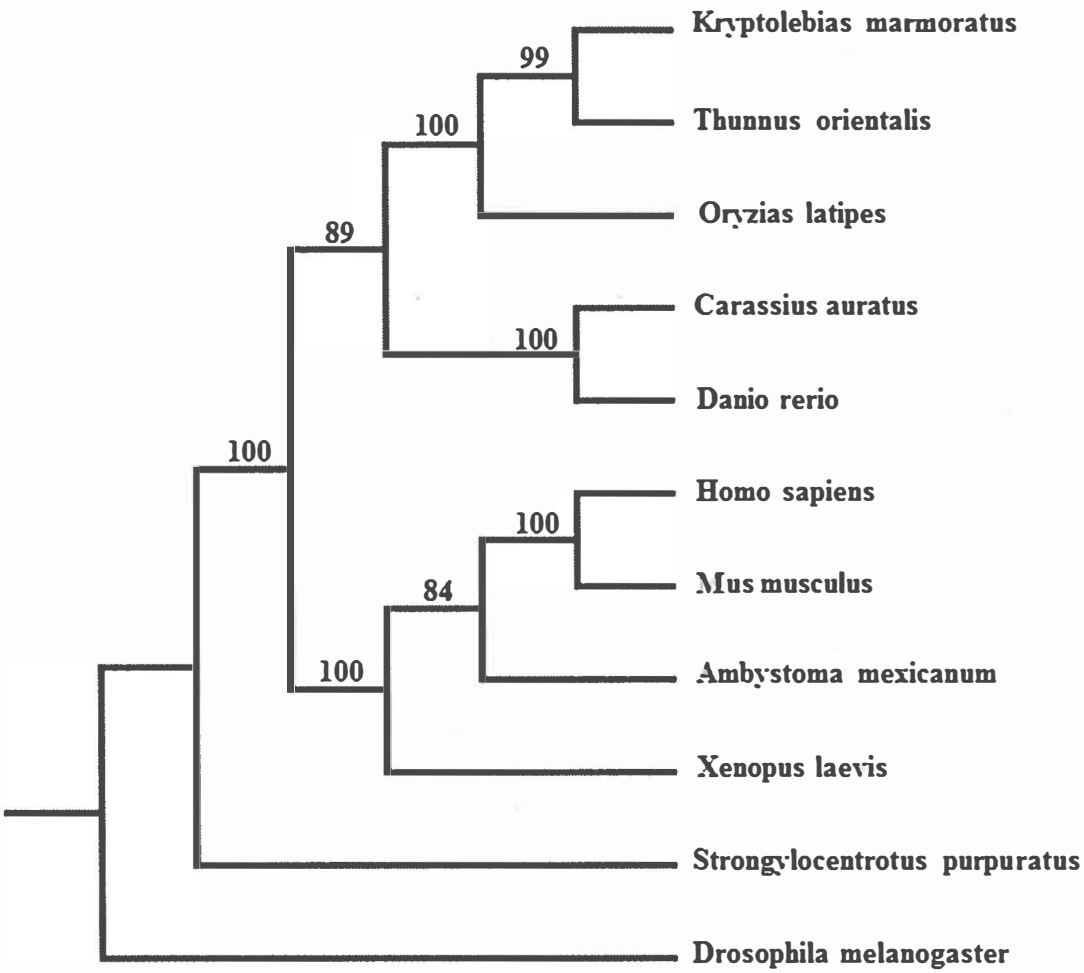

Figure 5. Gene tree analysis of the full length *kmvasa* gene along with other vertebrates, and invertebrates which are used as model organisms in the molecular genetic field.

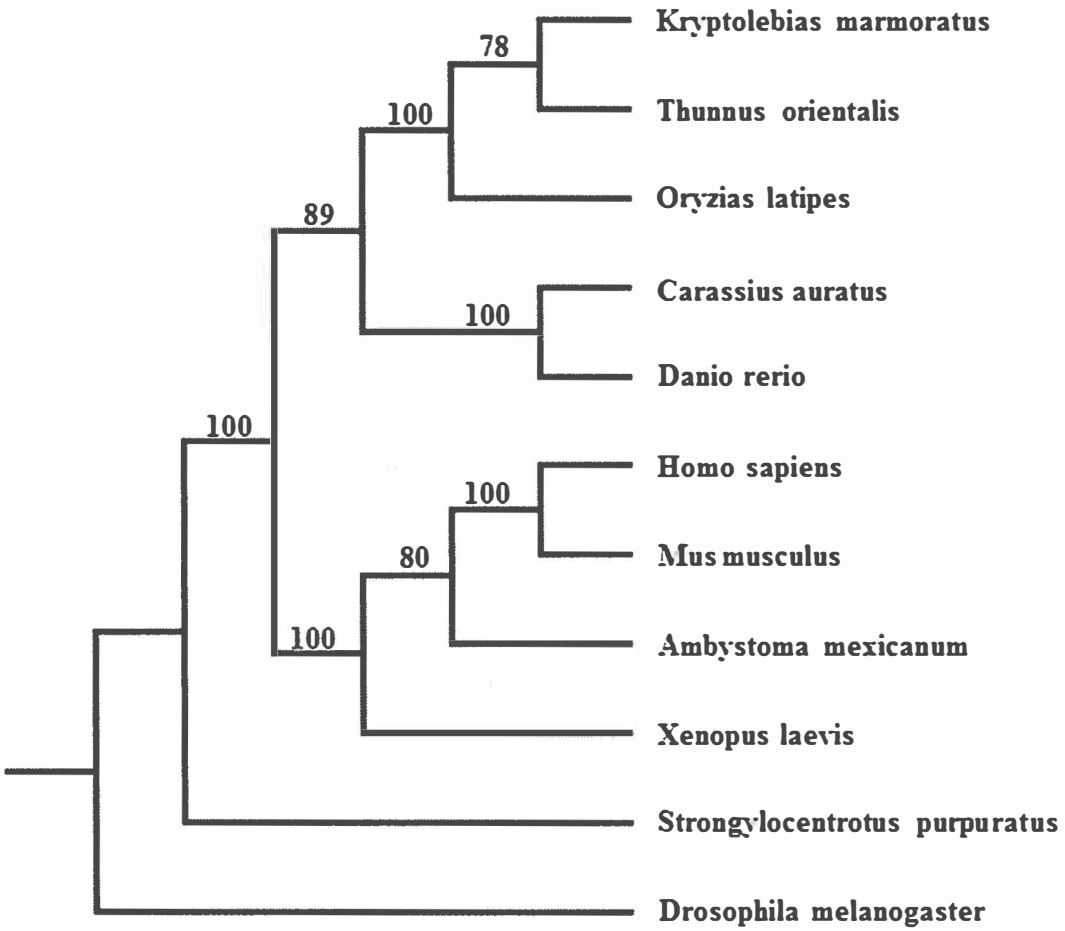

Figure 6. Gene tree analysis of the most highly conserved regions of *kmvasa* along with other vertebrates and invertebrates which are used as model organisms in the molecular genetic field utilizing nucleotide sequence 1081-2424.

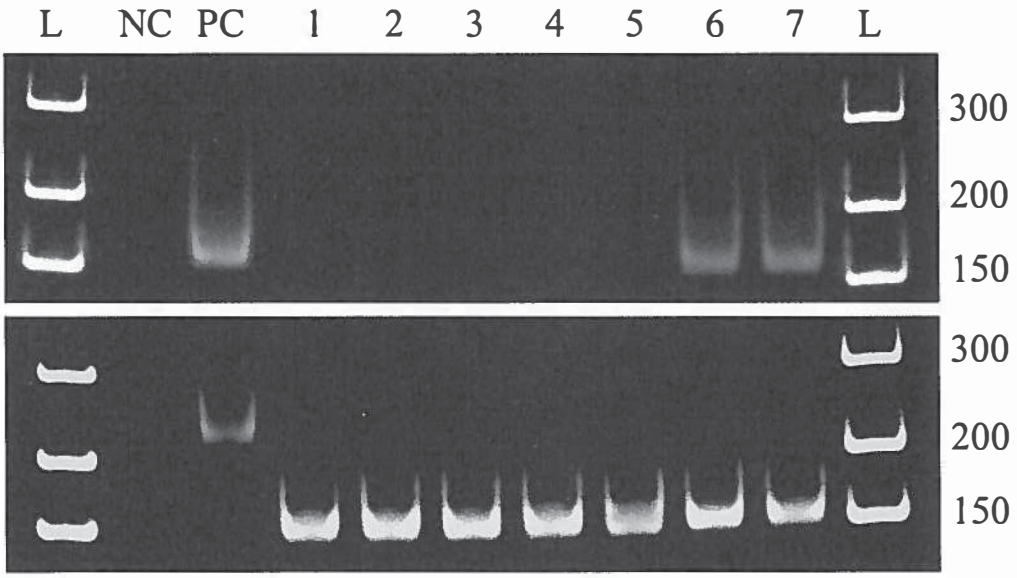

Figure 7. Gel photograph of a 5% acrylamide gel of *kmvasa* gene expression from tissues of adult *K. marmoratus*. L) O'generuler ultra low range DNA ladder, NC) Negative control, PC) Positive control, 1) Liver, 2) Brain, 3 Lower intestine, 4) Gall bladder, 5) Heart, 6) Ovotestis (hermaphrodite), 7) Testis (male), L) O'generuler ultra low range DNA ladder.

Table 3. *K. marmoratus* embryo collection summary and total RNA input summary.

			Egg count				*ng* of total RNA used			
Stage	Mourabit et al., 2012	Time (hrs)	Biological reps				Biological reps			
			I	II	III	total	I	II	III	Mean
6/7	32-cell - Early blastula	6.5/8	30	20	25	75	139.8	127.5	115.5	127.6
9	Late blastula	10.5	30	31	22	83	127.17	131	132	130.05
12	Mid-gastrulation	22	38	20	23	81	127.5	145.5	129	134
14	Late gastrulation	25.5	21	21	21	63	126	85.5	135	115.5
17/18	Optic vesicle/Otic vesicle	36/43.5	20	20	18	58	136.5	125	126	129.16
21/23	Body movements	58/71	12	10	10	32	110	116	127.2	117.73
25/26	Pectoral fin/Liver formation	84/90	10	10	10	30	119.4	130	405	218.13
27a/b	Increased coloration	105/120	10	10	10	30	121.1	114	112.5	115.86
28	Caudal fin formation	140	5	10	10	25	124.5	129	122	125.16
29	Anal fin formation	180	5	6	7	18	130.4	137	130.2	132.53
31	Pectoral fin movement	240	10	5	5	20	128.75	133	130.8	130.85
					Mean	46.81			Mean	134.23
					Stdev	25.43			Stdev	28.60

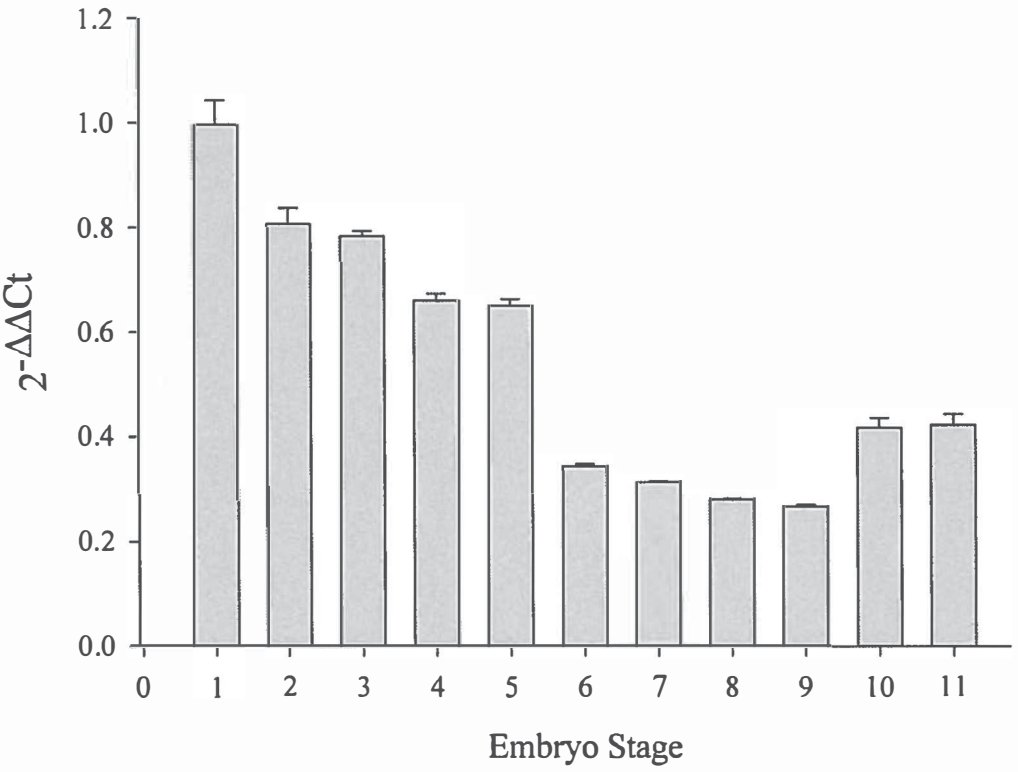

Figure 8. Relative levels of *K. marmoratus* embryo *vasa* gene expression (mean ± standard error). 1) 32-cell/early blastula – stage 6/7, 2) Late blastula – stage 9, 3) Mid-gastrulation – stage 12, 4) Late gastrulation – stage 14, 5) Optic vesicle and somite formation/Otic vesicle formation – stage 17/18, 6) Body movements/Increased vitelline circulation – stage 21/23, 7) Pectoral fin development, Erythrophore formation/ Liver formation – stage 25/26, 8) Increased pigmentation and body movement – stage 27a/b, 9) Caudal fin formation – stage 28, 10) Air bladder and anal fin formation – stage 29, 11) Pectoral fin movement – stage 31. For stage description see Mourabit et al., 2011.

Chapter IV

DISCUSSION

Since the initial discovery of the *vasa* gene from *Drosophila* (Schüpbach & Wieschaus, 1986), it has been observed that many species use this gene for development of the germ line (Raz, 2000). Because *vasa* is a member of the RNA helicase gene family of proteins, this research directly adds to what is already known about RNA helicase proteins. These findings are important since cells utilize RNA helicases for developmental and physiological functions. Now that *vasa* has been cloned and sequenced from *K. marmoratus*, more research can be performed on this particular fish model to better understand how its specialized gonad, the ovotestis, is formed. The use of the *kmvasa* gene marker for PGC development may be useful to future researchers to determine how this unique ovotestis forms. Understanding this unique developmental pathway may be directly applicable to research on human diseases of sexual development, particularly those resulting in sterility, in which an ovotestis-like structure is known to develop (Baig et al., 2005).

Initially, when comparing the *kmvasa* DNA sequence to several different species such as *D. melanogaster*, *H. sapiens*, *A. mexicanum*, *X. laevis*, *D. rerio*, *O. latipes*, and *S. purpuratus*, certain regions within the ORF show high sequence homology across the protein. Those highly conserved regions of homology are also consistent with that of other RNA helicase proteins described by Cordin et al., (2006). Multiple *vasa* phylogenies were constructed utilizing these species to compare similarities to *K.*

marmoratus. Max score results derived from BLASTp analysis show that *kmvasa* is most closely related to *T. orientalis* (Pacific blue fin tuna), which is consistent with the DNA sequence phylogenies that were developed in this study. The phylogenetic analyses of the *kmvasa* sequence yields a gene tree that identifies relationships among these organisms with high confidence levels for the teleost order of fishes, the invertebrates, and the vertebrates. The *kmvasa* sequence is also similar to that of *O. latipes* (*olvas*) at the protein coding level. Further comparison of *kmvasa* 3' UTR demonstrates both *K. marmoratus* and *O. latipes* fish species lack localizing signals for *vasa*. Knaut et al., (2002) empirically demonstrated that the *vasa* 3'UTR contains conserved motifs among ostariophysan fish for cell specific localization via a localizing signal in the 3' region. Whereas euteleost fish, including *O. latipes*, do not use this mode for localization, and show a concomitant loss of the 3'UTR motif sequences. The 3'UTR of the *kmvasa* gene when compared to *olvas* is 234 bp versus 240 bp, respectively. Zebrafish *vasa* 3' UTR is 644 nucleotides and contains the localizing signal motif for cell localization (Knaut et al., 2002).

In a separate collaboration with Tetsuhiro Kudoh and Sulay Mourabit (Exeter University, UK), we have shown by whole mount *in situ* hybridization that *kmvasa* follows a regulative mode of specification similar to that of *O. latipes* (unpublished). More research to determine how *kmvasa* is localized in early stage embryos is needed to understand specifically how it functions, and whether it progresses in an autonomous or regulative manner.

Another region of *kmvasa* that is of importance is the N-terminus region of the predicted protein that contains five RGG repeats. Gustafson and Wessel (2011)

confirmed through *vasa* green fluorescent protein (GFP) fusion analysis in sea urchins that the *vasa* gene protein is subjected to cytoplasmic degradation by an ubiquitin protease at the site of the RGG repeats, which limits the *vasa* proteins functional lifetime. These repeating RGG units were found in all *vasa* sequences that were analyzed in this study (see Table 2). The number of RGG repeats is variable among all organisms observed, which might also be indicative of whether or not *vasa* expression is maintained in a regulative or autonomous manner. This may suggest that there could be some convergent evolution of the *vasa* gene occurring at the post-translational level through RGG targeted destruction.

Kmvasa expression was detected in the ovotestis and the testis of hermaphrodites and males, which is similar to other gonochoristic species which have been studied. This makes *vasa* a highly important protein for germ-cell development, because without *vasa* gene expression, germ cells would not exist (Schüpbach & Wieschaus, 1986). Semi-quantitative PCR data showed that only the gonad regions expressed the *vasa* mRNA, which confirmed that *kmvasa* was restricted to the gonad tissue of *K. marmoratus* (see Figure 7). However, only seven tissues were assayed, so there is a possibility that some *vasa*-like protein could be detected in other tissues that were not assayed in this study. Considering that *vasa* is a RNA helicase protein and has many highly conserved regions of homology to that of eIF4A protein, it is arguable that there could be some cross amplification if proper primer development is not taken into consideration (Lasko & Ashburner, 1988; Linder et al., 1989).

Expression in developing embryos was analyzed from early to late stages of *K. marmoratus* development (32-cell/early blastula through stage 31; see Mourabit et al.,

2011 for stage definitions). In this study *kmvasa* expression was detected and measured at relative rates using the *K. marmoratus β-actin* gene as a normalizing standard. Specific stages of embryonic development were analyzed for temporal expression patterns because *vasa* is such an important protein for keeping embryonic cells totipotent during embryogenesis (see Figure 8). Not only is *vasa* expressed at these times during embryogenesis, but expression of *kmvasa* is definitively higher at early stages of development. As the *K. marmoratus* embryo develops from early to later stages, *vasa* is slowly degraded and remains confined locally to only a small number of specific embryonic cells devoted to gonadogenesis.

Here the discovery and rigorous identification of the RNA helicase encoding *K. marmoratus vasa* gene has been presented. An examination into the *vasa* gene similarities between organisms from similar versus different taxonomic groups has been provided. Tissue analysis of this gene shows that it is only expressed in gonadal tissue of the *K. marmoratus* fish and relative gene expression over time during embryogenesis was also confirmed. *Kmvasa* appears to follow a pattern of regulative specification of the PGCs. With this first and foremost analysis of the *kmvasa* gene, tools have been developed for future studies in the area of developmental biology, specifically the study of the specialized gonad of the *K. marmoratus*. The *kmvasa* gene will serve as a molecular marker for the study of gonadogenesis in reproductive biology using the unique vertebrate model *K. marmoratus*.

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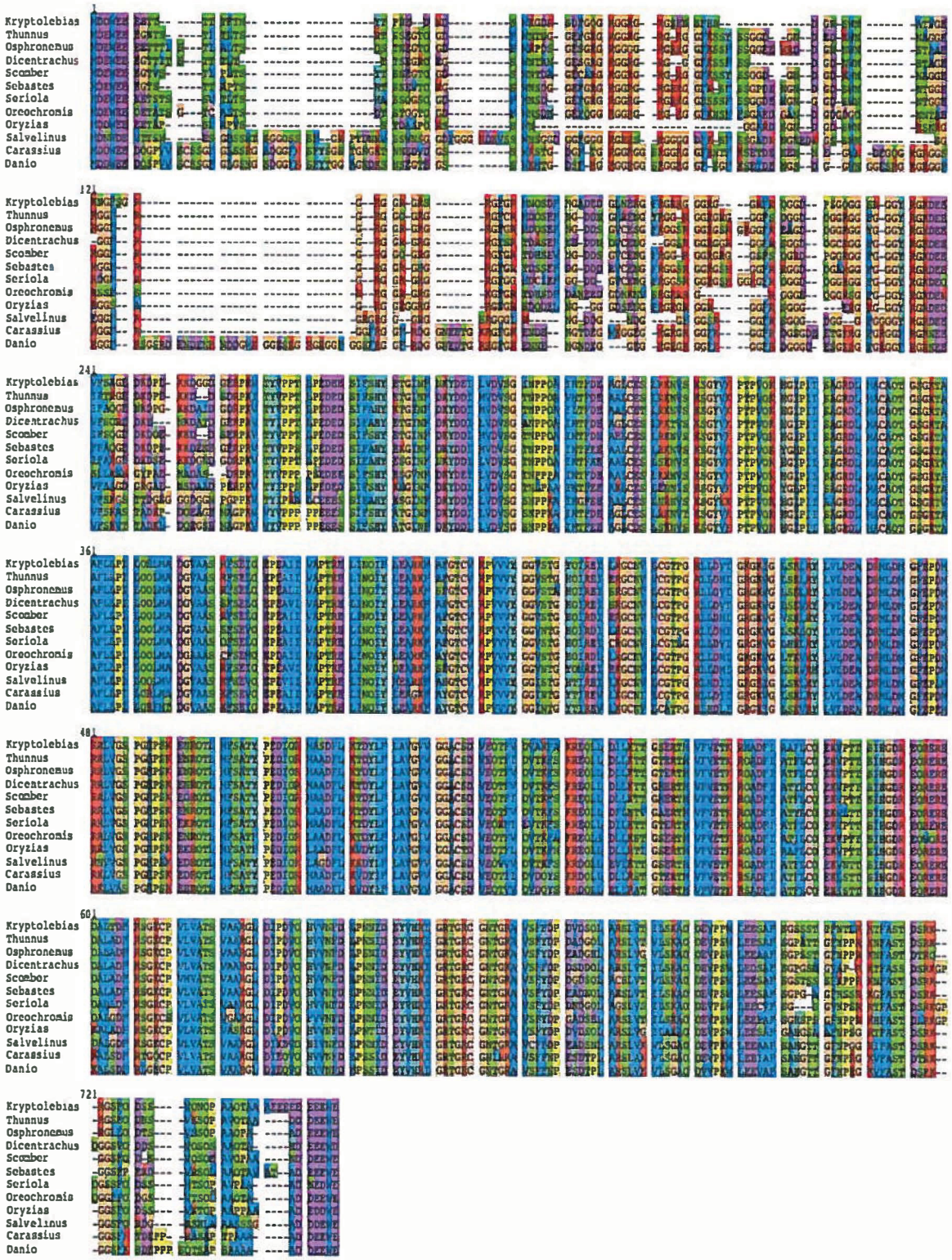
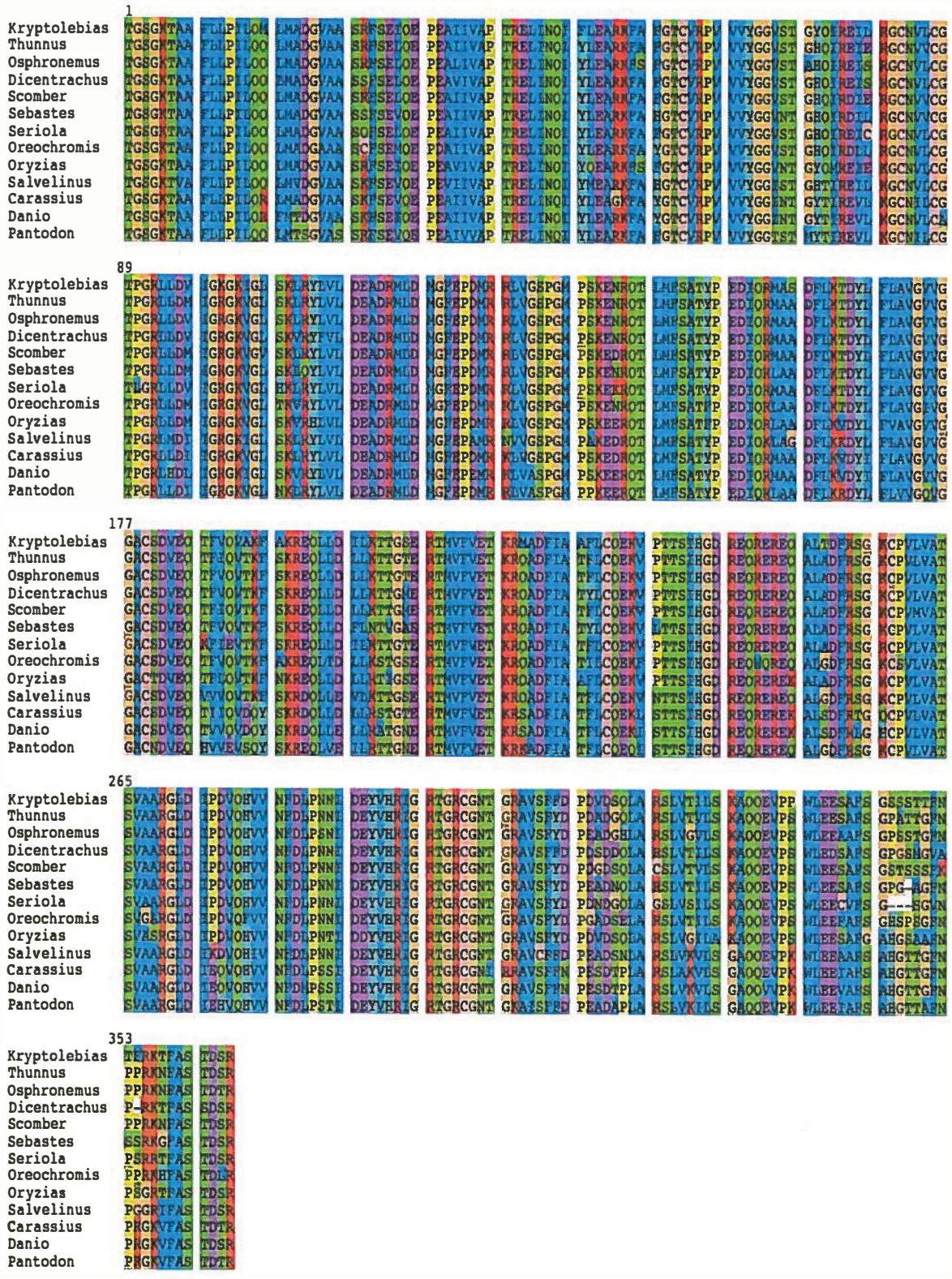
APPENDIX A

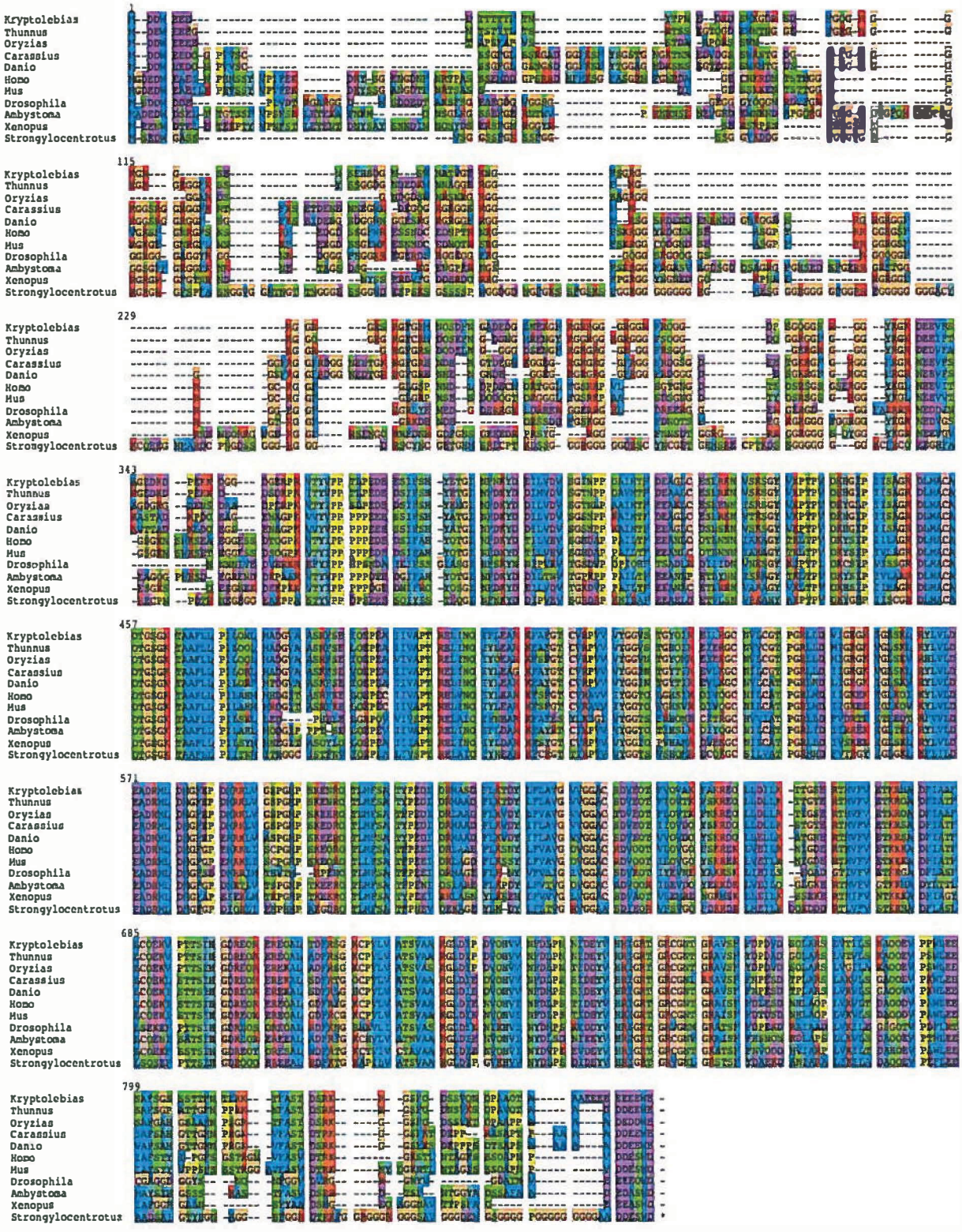
Phylogenetic Alignments Used for Gene Trees



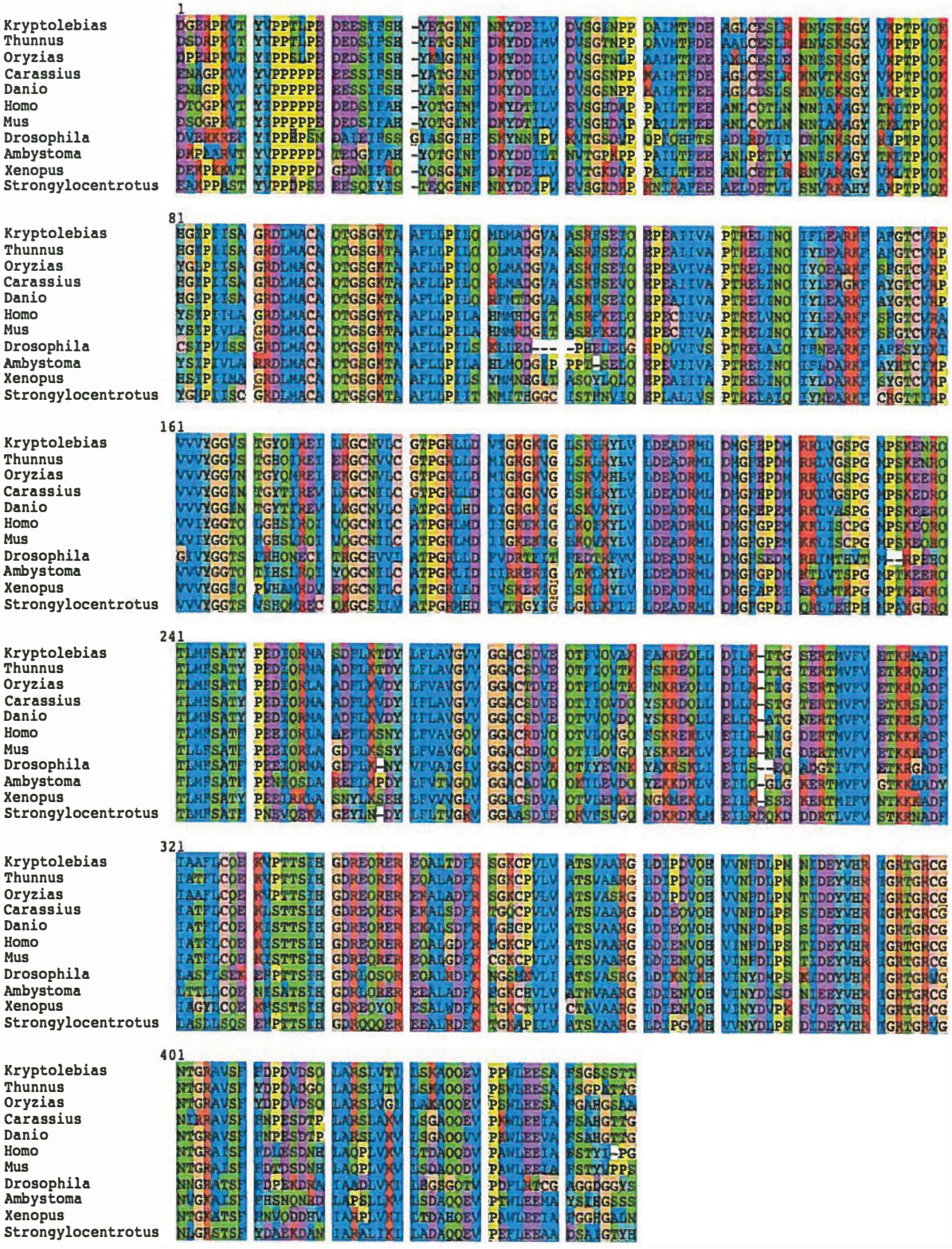
Alignment used for Figure 3.



Alignment used for Figure 4.



Alignment used for Figure 5.



Alignment used for Figure 6.

APPENDIX B

Valdosta State University Institutional Animal Care and Use Committee Approval Form

VALDOSTA STATE UNIVERSITY
Building for Our Next Century

June 12, 2012

Dr. Brian Ring
Department of Biology
Valdosta State University

Dear Dr. Ring:

Your Animal Use Protocol, "*Krptolebias marmoratus* (mangrove killifish) research" (AUP-00045-2012) has been approved by the Institutional Animal Care and Use Committee (IACUC). This approval is for the period of June 12, 2012, through June 11, 2015. Each year, an annual review and report must be submitted to the IACUC to keep your protocol active. You will be contacted by the Office of Sponsored Programs and Research Administration approximately two months before the annual review and report is due.

Please remember that you must obtain IACUC approval before amending or altering the scope or procedures of the protocol. You are also required to report to the Attending Veterinarian, the IACUC Chair, and the IACUC Administrator any unanticipated problems with the animals which become apparent during the course, or as a result, of the research or teaching activity.

If you have any questions, please contact the IACUC at iacuc@valdosta.edu.

Sincerely,

Barbara Gray

Barbara Gray
IACUC Administrator

cc: Dr. Karla Hull, Institutional Official
Dr. Theresa Grove, IACUC Chair
Dr. Teresa Doscher, Attending Veterinarian
Dr. Robert Gannon, Biology Department Head

Office of Sponsored Programs & Research Administration
Location Regional Center for Continuing Education • 903 N. Patterson St. • **Address** 1500 N. Patterson St. • Valdosta, GA 31698-0429
Phone 229.259.5045 • **Fax** 229.245.3853 • **Web** www.valdosta.edu/ospra/

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