# NUCLEOTIDE SEQUENCE, MAPPING, AND RELATIVE EXPRESSION OF THE *Xiphophorus maculatus* CYCLOPHILIN 40 GENE (*PPID*).

THESIS

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# INTRODUCTION

## I. Cyclophilins

Cyclophilins are a family of conserved proteins and are also known as immunophilins (Yokoi *et al.*, 1996a; Marks, 1996). There are two classes of immunophilins: the cyclophilins, which bind the immunosuppressant drug cyclosporin A (CsA), and FK506 binding proteins (FKBP), which bind the immunosuppressant drugs FK506 and rapamycin. Cyclosporin A was first used in the 1980s for patients undergoing organ transplants, while FK506 and rapamycin are widely used in liver and renal transplants, respectively (Bierer, 1993).

### a) Cyclophilin Family Members

There are six known cyclophilin protein family members found in prokaryotes and eukaryotes. Each cyclophilin family member is thought to have distinct cellular functions.

Cyclophilin A (CypA), the first cyclophilin to be discovered and most abundant, is an 18 kDa cytosolic protein. It is found in the cytosol of cells from human, rat, mouse, bovine, *S. cerevisiae*, arabidopsis, and *E. coli* and serves as a cytosolic binding protein for the exogenous ligand, CsA (Marks, 1996). Cyclophilin B (CypB) is a 21 kDa protein that contains an N-terminal signal sequence for sorting to the endoplasmic reticulum (Marks, 1996). Recent studies have shown CypB co-localizes in the endoplasmic reticulum with the calcium binding protein calreticulin. This suggests that CypB may

play a role in mediating calcium signaling (Marks, 1996). Cyclophilin C (CypC), a 23 kDa protein, also has an N-terminal signal sequence for sorting to the endoplasmic reticulum and is predominately expressed in the kidney (Friedman and Weissman, 1991). Cyclophilin D (CypD) is an 18 kDa protein with a mitochondrial signal sequence at its N-terminus. Cyclophilin NK-TR (CypNK-TR), a 160 kDa protein, is the largest cyclophilin protein family member. It is thought to be part of the tumor recognition complex on the surface of natural killer cells (Anderson *et al.*, 1993). The N-terminus of CypNK-TR shares sequence similarity with Cyclophilin 40. Cyclophilin 40 (Cyp40) is a 40 kDa cytoplasmic protein and is thought to be part of the inactive steroid hormone receptor complex (Marks, 1996).

b) Plant Cyclophilins

Cyclophilin homologues are also found in higher plants. Gasser *et al.*, (1990) isolated plant cDNA clones encoding proteins homologous to mammalian cyclophilin. Clones from maize and tomato showed 83% identity with cyclophilin A (Gasser *et al.*, 1990). Northern analyses on tomato revealed cyclophilin genes are ubiquitously expressed at significant levels in all organs of higher plants tested (Gasser *et al.*, 1990). Luan *et al.*, (1994) reports a plant cyclophilin homologue that is closely related to mammalian cyclophilin B and cyclophilin C.

Plant cyclophilin genes have been isolated from tomato, maize, bean, *Arabidopsis thaliana*, and tobacco (Marivet *et al.*, 1992). These are CypA plant homologues that are

thought to have a similar function as the mammalian cyclophilins- protein folding, trafficking and complex formation (Luan *et al.*, 1994). The open reading frame of the maize cyclophilin mRNA consists of 516 bp encoding a protein of 172 amino acids with a calculated molecular weight of 18.2 kDa and an isoelectric point of 8.9 (Marivet *et al.*, 1995). Plant proteins are similar to each other, with 78% amino acid identity and 87% similarity allowing for conservative amino acids differences among maize and tomato (Gasser *et al.*, 1990).

## II. Cyclophilin Function

### a) Immunsuppression Mechanism

Cyclophilins and FKBPs mediate the immunosuppressive actions of CsA and FK506 by combining with these drugs and physically binding to calcineurin, a calcium-dependent serine-threonine phosphatase (Rutherford and Zuker, 1994). Cyclophilins and FKBPs when complexed with their respective immunosuppressive drugs, block intracellular signaling pathways important for the activation of T lymphocytes via inhibition of calcineurin function. Calcineurin is localized in T lymphocytes and is activated by a rise in calcium levels that occurs after stimulation of the T-cell receptor by antigen. Calcineurin dephosphorylates the cytoplasmic form of the (NFAT) nuclear transcription factor in activated T cells. Dephosphorylated NFAT is transported to the nucleus where it combines with *jun* or *fos*, transcription factors that are capable of initiating transcription of T-cell growth factor interleukin-2 (IL-2) and many other target

genes (Jain *et al.*, 1993; Perrino *et al.*, 1995). Consequently, the CsA:cyclophilin complex binds and inhibits calcineurin's phosphatase activity preventing transcriptional activation of target genes upon a rise in intracellular calcium levels. Thus, normal function of transcription factors that encode growth-promoting proteins (i.e. IL-2) is suppressed, resulting in immunosuppression (Marks, 1996;).

b) Cyclophilins are Chaperone Proteins

Due to their enzymatic properties, cellular localization, and role in protein folding, cyclophilins belong to the large class of proteins known as molecular chaperones (Andreeva *et al.*, 1999). Molecular chaperones are defined as proteins that participate in the correct folding, assembly, and transport of newly synthesized proteins (Freeman *et al.*, 1996). Rutherford and Zuker, (1994) suggest components of the protein folding machinery play crucial roles in maintaining correct functional conformation of signal transduction components in the cell. Several unifying features characterize both molecular chaperones and cyclophilins: They are both involved in protein folding and belong to large, highly conserved gene families. They are abundant, and constitutively expressed cytoplasmic isoforms and have isoforms that are induced by subjecting cells to treatments causing protein damage (i.e. heat shock proteins). Finally, members of both the chaperone and cyclophilin families reside in several subcellular compartments in all cells analyzed.

In addition, recent experiments suggest cyclophilins are heat stress inducible and thus involved in cellular responses to stress (Andreeva *et al.*, 1999). Andreeva *et al.*,

(1997) demonstrated, through western blot analyses, a significant increase in expression of CypB when eukaryotic (rat) myogenic cells are heat shocked. Thus, cyclophilins are thought to be involved in the response to cellular stress as are the well-studied heat shock proteins such as hsp60 and hsp70 (Andreeva *et al.*, 1997). Plant cyclophilins are also stress inducible. Marivet *et al.*, (1995) demonstrated abiotic stresses such as mercuric chloride treatment, heat shock, wounding, salt stress, and low temperature are stress-responsive in plant cyclophilins. In addition, northern blot analyses showed salicylic acid enhances transcription of maize cyclophilin genes (Marivet *et al.*, 1995).

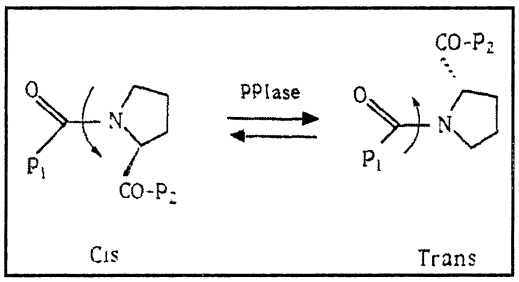
c) Enzymatic Active Sites and Peptidyl-Prolyl Isomerases (PPIases)

All cyclophilin family members are *cis-trans* peptidyl-prolyl isomerases (PPIases) (Marks, 1996). PPIases catalyze the interconversion of the *cis* and *trans* isomers of the peptidyl-prolyl bonds in peptide substrates (Figure 1-1) (Galat, 1993). PPIases are considered necessary for a number of cellular processes such as protein folding, protein stabilization, and protein transport in a similar manner to that mediated by heat shock proteins (Gething and Sambrook, 1992). Leverson and Ness, (1998) recently suggested PPIase activity has a role in the regulation of transcription and differentiation. In these studies, the DNA binding activity of the c-Myb proto-oncogene product was negatively regulated in vitro by the PPIase activity of cyclophilin 40.

In addition to PPIase activity, some cyclophilin members such as Cyclophilins A, B, and C contain a second active site, which imparts nuclease activity. This nuclease activity is capable of degradation of DNA in a calcium/magnesium dependent manner

(Montague *et al.*, 1994). The nuclease activity of Cyclophilin A, B, and C family members is capable of degrading single strand, double strand, and supercoiled DNA (Andreeva *et al.*, 1999) and is involved in the degradation of genomic DNA during apoptosis (Montague *et al.*, 1997).

**Figure 1-1 Schematic Presentation of *cis* to *trans* Isomerization** about a peptidyl-prolyl bond catalyzed by a proline isomerase (i.e. PPIase activity). Figure taken from Galat and Metcalf, (1995).




## III. Cyclophilin 40

The results reported here detail the cloning and characterization of a *Xiphophorus PPID* gene homologue that encodes Cyclophilin 40. Thus, some background regarding this particular cyclophilin gene family member is provided.

For clarity, the gene abbreviation for Cyclophilin 40 is *CYP40* while the protein abbreviation is cyp40. To differentiate among *CYP40* model systems, the following abbreviations are used: human *CYP40* (h*CYP40*), bovine *CYP40* (b*CYP40*), yeast (*S. cerevisae*) (s*CYP40*) and *X. maculatus CYP40* (x*CYP40*).

a) Structural Characteristics

Unlike other cyclophilin protein members cyp40 is comprised of a cyclophilin A-like domain at its N-terminus and an FKBP-like domain at its C-terminus. The FKBP-like domain is most similar to the C-terminal portion of FKBP52, a PPIase family member (Kieffer *et al.*, 1993) that harbors a C-terminal sub-region known as a tetratricopeptide repeat (TPR) motif. FKBP52 is identical to a 56-59 kDa protein that is also variously referred to as p56, hsp56, p59, and FKBP59 (Peattie *et al.*, 1992). Evidence indicates the TPR motifs are important to many cellular events such as chaperone activities, cell cycle control, transcription, and protein transport complexes (Blatch and Lassle, 1999). Ratajczak *et al.*, (1993) suggests the TPR domain in bcyp40 mediates protein-protein interactions through its hydrophobic regions at the N- and C-terminal ends of the TPR domain.

FKBP52 contains a potential calmodulin-binding site, suggesting calmodulin and intracellular calcium levels could affect FKBP52 function (Peattie *et al.*, 1992). When a steroid binds to its receptor, the receptor complex dissociates and the receptor dimerizes to become active (Pratt, 1987). Based on this, Lebeau *et al*, (1992) suggests the function of FKBP52 is involved in the formation, stabilization or dissociation of steroid receptor complexes.

Yokoi *et al.*, (1996a) suggests the function of hcyp40 may be similar to FKBP52 because hcyp40 shows similarity to FKBP52, contains TPR motifs, and a potential calmodulin-binding site.

b) Tetratricopeptide Repeat Motif (TPR)

The TPR motif consists of a tandemly reiterated 34 amino acid repeat. Examples of genes harboring TPR motifs include yeast gene products, STI1 and MAS70, which function as co-chaperones and assists in protein transport. Sikorski *et al.*, (1990) first identified and characterized the TPR repeat in *S. cerevisiae* while studying the cell division cycle (CDC) CDC23 gene product. In CDC23, nine tandemly arranged 34 amino acid repeat units were identified. Using quantitative matrix analysis and visual inspection, Sikorski *et al.,* (1990) identified TPR units in the products of four additional yeast genes; CDC16, SSN6, and SKI3 from *S. cerevisiae* and $nuc2^{+}$ from *Schizosaccharomyces pombe*. The existence of the TPR domain in several essential cell division cycle genes and in several genes involved in mRNA production, such as SSN6 and SKI3 from *S. cerevisiae,* suggests this domain may have a broadly defined role (Sikorski *et al.*, 1990). The TPR motif has also been found in a gene involved in *Drosophila* development, *crn (Drosophila crooked neck)* that contains sixteen 34 amino acid repeats (Goebl and Yanagida, 1991).

Many TPR proteins have cell cycle division associated functions. For instance, mutations in CDC16 and CDC23 cause cell cycle arrest before entry into M phase (i.e. the G2/M boundary) (Sikorski *et al.*, 1990) and the mitotic metaphase/anaphase progression is blocked in $nuc2^{+}$ mutants (Hirano *et al.*, 1988). *Drosophila, crn* mutants display nervous system defects late in embryogenesis (Goebl and Yanagida, 1991).

Based on these findings, Goebl and Yanagida, (1991) suggest the *crn* gene may be required for cell division.

Hirano *et al.*, (1990) determined the secondary structure of the TPR motif through circlular dichroism studies using a bacterial expression plasmid, which produced a 41 kDa polypeptide consisting of nine tandem repeats. Results from this study revealed the 34 amino acid repeating units as a helix-hinge configuration. The structure was 45% α-helical, 0% β sheets, and 55% remaining structures. A computer graphic image made specifically for the α-helical structure demonstrated the 34 amino acid consensus sequence forms a structure similar to a hole and knob (Hirano *et al.*, 1990).

Ratajczak *et al.*, (1993) has shown a domain containing 3 TPR units within a C-terminal 150 amino acid segment in bcyp40. Therefore, it was of interest to us to see if the fish *CYP40* gene contains similar TPR units.

c) Human Cyclophilin 40

A clone characterized by Yokoi *et al.*, (1996a) from human fetal brain cDNA library was designated as cyclophilin 40. The h*CYP40* gene is comprised of 10 exons and 9 introns encompassing 14.2 kb genomic DNA. This gene encodes a cDNA of 1.1 kb spanning an open reading frame of 370 amino acids. The hCYP40 gene was mapped to human chromosome 4 using somatic cell hybrid panels (Yokoi *et al.*, 1996a). As previously mentioned, the hcyp40 N-terminal region shows similarity with Cylcophilin A while the C-terminal region exhibits similarity with FKBP52. The 3'noncoding region of h*CYP40* gene revealed 9 Alu-repeats. Several putative control elements are located 506

base pairs (bp) 5' to exon 1 such as Ap1, E-box, Sp1, EF1A, NF-κB, and NF-μE3. This 5' flanking region lacked a TATA or a CAAT box, but was rich in GC bases and contained several SP1 sites that is considered characteristic of housekeeping genes (Yokoi *et al.*, 1996a) and consistent with the ubiquitous expression of h*CYP40* in widely divergent tissues as supported by northern blot analyses (Kieffer *et al.*, 1993).

Plant cyclophilins have an eight amino acid (GKMGKPLH) addition. Five of those eight amino acids (GKPLH) are conserved only in mammalian cyp40. This plant characteristic is seen in hcyp40 and bcyp40. Therefore it is of interest to us to see if xcyp40 also contains the five amino acid conservation.

## IV. Cyclophilin 40 Expression

Northern blot analyses indicate h*CYP40* is expressed in all tissues assayed including: heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Kieffer *et al.*, 1993). Similarly in the bovine system, b*CYP40* expression has been documented by northern blot analyses in brain, lung, kidney, heart, thymus, and spleen (Ratajczak *et al.*, 1993). In rodents, western blot analyses revealed cyp40 expression in brain, liver, kidney, spleen, thymus, heart, testes and lung (Kieffer *et al.*, 1992).

However, Ratajczak *et al.*, (1993) states (does not show results) higher expression levels are observed were in heart, thymus, and brain of bovine tissues, while not detected in bovine liver tissues. Kieffer *et al.*, (1993) compared h*CYP40* expression to β-actin expression and reports higher h*CYP40* expression relative to β-actin in heart, brain,

placenta, and skeletal muscle, while the lowest expression level was observed in lung tissue. Thus, careful expression analyses and correlation of expression patterns among diverse vertebrate species has not been performed.

## IV. *Xiphophorus maculatus* as a Genetic Model

### a) Background

*Xiphophorus* fishes of the Poeciliidae family are comprised of 22 species (Rosen, 1979). They are live bearing fishes that are found in the freshwaters of Mexico, Honduras, and Guatemala. Genetically characterized *Xiphophorus* lines are available and have been maintained since the 1920s. Selective interbreeding between *Xiphophorus* species produces fertile offspring, making them a valuable tool for genetic study. Certain genetic stocks have been inbred for over 100 generations making them essentially identical lines of value for backcross hybrid gene mapping of novel genetic loci (Morizot *et al.*, 1998).

Several *Xiphophorus* species fishes are characterized by large intensely black pigment cells (macromelanophores) that create bold markings or pigment patterns (Kallman, 1975). Previous studies discovered that certain hybrids of macromelanophore carrying fish (platyfish and swordtails) spontaneously develop malignant melanomas originating from the macromelanophore spots (Schartl, 1995).

### c) Gene Mapping

Gene mapping in *Xiphophorus* fishes initially began in the early 1970s as an effort to identify the genes regulating spontaneous malignant melanoma (Morizot *et al.*,

1998). Since then, more than 350 genetic markers and 100 mapped genes have been assigned to 24 mulitpoint linkage groups. Comparison of the *Xiphophorus* gene map to the gene maps from other vertebrates, including humans, has shown that some chromosome segments are conserved in gene content and order with other vertebrate species through ~450 million years of evolutionarily divergence (Morizot *et al.*, 1998). The study of evolutionary distant organisms may help in understanding the origins and divergence of gene families in vertebrates. Fishes in particular appear to retain many primitive vertebrate patterns of genome organization and genetic fine structure (Walter and Morizot, 1996). The continued assignment of loci to the *Xiphophorus* gene map is necessary to support research efforts using this powerful genetic system to study multi-factorial traits.

The *Xiphophorus* genetic system is able to clearly define genes involved in the neoplastic transformation (hereditary melanoma) by classical genetics (Schartl, 1995). For instance, it was discovered that certain hybrids of macromelanophore carrying platyfish and swordtails spontaneously develop melanomas linked to the spot dorsal (sd) pigment pattern locus (Gordon, 1927).

Gene mapping is accomplished through the use of reciprocal first backcross hybrids. Hybrids crossed from highly diverged *Xiphophorus* species attain numerous polymorphic markers in each genetic cross. For each genetic cross, joint segregation of each pair of markers is tested to detect a deviation from independent assortment due to genetic linkage. If two markers are not linked, Mendelian genetics would predict a 1:1

ratio of parental and recombinant markers in the backcross progeny. Linkage between two markers is determined by bias of this ratio from the 1:1 random segregation expectations and expressed statistically as a logarithm of the odds (LOD) score.

On the basis that the cyclophilin family of proteins is conserved among prokaryotes and eukaryotes and ubiquitously expressed, we hypothesize *Xiphophorus* fish harbors the *PPID* gene homologue that encodes cyp40 and is expressed in fish tissues. This study was initiated with two research objectives: 1) To provide nucleotide sequence data and characterization on the *X. maculatus PPID* gene and locus. 2) To determine a baseline *CYP40* expression in various *X. maculatus* tissues.

Here we present the cDNA sequence, predicted amino acid sequence, gene map assignment, and relative *CYP40* expression in *Xiphophorus* tissues.

## MATERIALS AND METHODS

### Fish Stocks

Fishes of the genus *Xiphophorus* (Teleostei: Poecilidae) used in these studies were obtained from the *Xiphophorus* Genetic Stock Center, Southwest Texas State University, San Marcos, IACUC approval 99-11. The fishes used are listed in Table 2-1.

**Table 2-1 *Xiphophorus* fishes used in this research**
The Designated Name is assigned for each genetic cross and is referred to herein.

| Designated Name | Genetic Backcross ($BC_1$) |
|---|---|
| ***X. maculatus* Jp 163 A** | *Xiphophorus maculatus* Jp 163 A |
| ***X. maculatus* Jp 163 B** | *Xiphophorus maculatus* Jp 163 B |
| ***X. helleri*** | *Xiphophorus helleri* |
| **$F_1$ Hybrids** | (1) *X. maculatus* Jp 163 A × *X. helleri*<br>(2) *X. maculatus* Jp 163 B × *X. helleri* |
| **Hybrid 1** | *X. helleri* (×) (*X. maculatus* Jp 163 B (×) *X. helleri*) |
| **Hybrid 5** | *X. helleri* (×) (*X. maculatus* Jp 163 A (×) *X. helleri*) |
| **Hybrid 7** | *X. couchianus* (×) (*X. maculatus* Jp 163 B (×) *X. couchianus*) |

### Isolation of the pWR3 Clone

In an effort to expand the *Xiphophorus* genetic map, several plasmid cDNA clones were isolated from a Lambda Zap II Phage library constructed from brain mRNA isolated from *X. maculatus* Jp 163 A. These clones were used as probes to establish RFLP polymorphisms in parental strains used for gene mapping following inter-species

hybridization. When the cloned genomic region of one of these clones, pWR3, was radiolabeled and used as a probe on Pst I cleaved *Xiphophorus* genomic DNA, two distinct polymorphic bands were observed between *X. maculatus* Jp 163 B and *X. helleri* parental strains. These bands were assigned to the *Xiphophorus* linkage map and the cDNA fragment producing the polymorphism was further characterized.

**Plasmid DNA Isolation**

For plasmid DNA isolation, the Concert™ High Purity Plasmid Purification System (Gibco BRL®; Rockville, MD) was used. One liter of TB broth (12 gms Bacto-tryptone, 24 gms Bacto-yeast extract, 4 ml glycerol, 2.31 g $KH_2PO_4$, 12.5 g $K_2HPO_4$), containing 25 µg/ml of ampicillin was inoculated with 500 µl from a fresh overnight culture of the appropriate bacterial strain and shaken vigorously overnight at 37° C. The cells were harvested by centrifugation at 11,000 x g (J2-21 Beckman Instruments; Fullerton, CA) for 10 minutes at 4° C. The supernatant was decanted and cells resuspended in 10 ml of Concert™ Cell Suspension buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA) containing 20 mg/ml RNase A by vortexing. The cells were lysed by addition of 10 ml of Concert™ Cell Lysis solution (200 mM NaOH, 1% SDS (w/v)) and mixed gently by inverting the tubes several times followed by incubation at room temperature for 5 minutes. Ten ml of Concert™ Neutralization buffer (3.1 M KOAc (pH 5.5)) was added and mixed thoroughly by inverting the tubes several times. To collect

the cell debris, the mixture was centrifuged at 11,000 x g at room temperature for 10 minutes. The supernatant was carefully transferred onto Concert™ columns equilibrated with Concert™ Equilibration buffer (600 mM NaCl, 100 mM sodium acetate (pH 5.0), 0.15% Triton® X-100 (v/v)). The columns were washed with 60 ml of Concert™ Wash Buffer (800 mM NaCl, 100 mM NaOAc (pH 5.0) and each eluate was discarded. The plasmid DNA was eluted with 15 ml of Concert™ Elution buffer (1.25 M NaCl, 100 mM Tris-HCl (pH 8.5)) into 50 ml polypropylene tubes. The plasmid DNA was precipitated with one volume of isopropanol and pelleted by centrifugation at 15,000 x g (J2-21 Beckman Instruments; Fullerton, Ca) at 4° C for 30 minutes. The supernatant was discarded and the pellet washed with 5 ml of 75% ethanol followed by centrifugation at 15,000 x g at 4° C for 5 minutes. The supernatant was discarded and the plasmid DNA pellet air-dried for 10 minutes. The dried DNA was dissolved in 500 µl of TE buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA). Yields from this protocol were generally 500 µg plasmid/liter cell cultured.

**Nucleotide Sequencing Determination of pWR3**

The pWR3 clone harbored a 1.6 kb cDNA insert in plasmid vector pBluescript (Stratagene, La Jolla, CA). The sequence of this cloned cDNA fragment was determined using a dideoxynucleotide chain termination protocol (Sanger, 1977), employing Sequenase® Version 2.0 T7 Polymerase kit (US Biochemical Corporation, Cleveland,

OH). Forty µl of DNA suspension at 0.1 µg/µl was denatured in 8 µl of 1N NaOH, 1.6 µl of 5 mM EDTA, in the presence of 1 µl of sequencing primer (Table 2-2). The suspension was incubated at 37°C for 30 minutes then 4 µl of 3 M NaOAc and 90 µl of 100% ice-cold ethanol were added and the DNA precipitated for 20 minutes at -80° C. Following centrifugation at 10,000 x g (Speedfuge HSC Savant Instruments; Holbrook NY) for 10 minutes, the supernatant was decanted and the pellet washed twice with 50 µl of 75% ethanol. The pellet was dried in a vacuum centrifuge (Savant Instruments; Holbrook, NY) and the DNA dissolved in 7 µl of distilled water. To each reaction, 2 µl Sequenase® reaction buffer (200 mM Tris HCl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl), and 1 µl of primer (30 µg/ml) was added. The reaction was incubated at 65°C for 5 minutes, room temperature for 5 minutes, then on ice for 5 minutes to promote annealing of primer and plasmid template. In individual tubes, 2.5 µl of each dideoxynucleotide mixture (8 µM of the appropriate ddNTP, 80 µM of the other dNTPs, 50 mM NaCl) was aliquoted and pre-warmed at 37°C. On ice, 1 µl of dithiothreeitol (DTT), 2 µl of Sequenase® diluted labeling mixture (diluted 1:4 with sterile water; 7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP), and 1 µl of [$\alpha$-$^{35}$S] dATP (12.5 mCi/ml, NEN Life Sciences, Boston, MA) was added to the annealed primer/template mixture. Two µl of T7 DNA Sequenase® enzyme (diluted 1 to 8 in T7 Dilution Buffer; 10 mM Tris-HCl (pH 7.5), 5 mM DTT) was added. Chain elongation occurred at room temperature for 5 minutes, then 3.5 µl of this elongation reaction was transferred to each of the pre-warmed

(37°) dideoxynucleotide aliquots and incubated at 37° C for 5 minutes. The reaction was terminated by the addition of 5 µl Stop Solution (95% formamide, 20 mM EDTA (pH 8.0), 0.05% bromophenol blue, and 0.05% xylene cyanol). Nucleotide sequence reactions were fractionated on 6.0% polyacrylamide 5 M urea denaturing gels with a model STS 45 electrophoresis unit (Kodak IBI; New Haven, CT). Gels were run at 2000 V/cm for 1.5 hours then transferred to 3 MM chromatography paper (Whatman; Maidstone, England) and dried on a gel dryer (Savant Instruments; Holbrook, NY) for 2 hours. The dried gels were used to expose to X-OMAT AR film (Eastman Kodak; Rochester, NY) for autoradiography. Sequencing reactions were performed with a forward T3 primer (5' AATTAACCCTCACTAAAGGG 3'), a reverse T7 primer (3' CGGGATATCACTCAGCATAATG 5'), and one of 7 synthetic oligonucleotide primers (Table 2-2). Synthetic oligonucleotide primers were designed from pWR3 cDNA to allow sequence extension distal to universal primer sequences and for PCR amplification. All oligonucleotides were custom synthesized by IDT® (Coraville, IA).

Sequence analysis was performed using Baylor College of Medicine (BCM) Search Launcher (http://www.hgsc.bcm.tmc.edu/SearchLauncher/) and GenBank Database. Nucleotide and amino acid alignments were performed using MacVector (Version 6.5, Eastman Kodak, NY, USA) Sequence Analysis Software.

**Table 2-2 Synthetic Oligonucleotide Primers used for Nucleotide Sequencing of pWR3.** Primers were designed from pWR3 cDNA nucleotide sequence.

| *Sequence Primer* | *Sequence (5' – 3')* |
|---|---|
| ***pWR3-S1*** | AGAGGACCACAGGAA |
| ***pWR3-S2*** | CACAGTGAAGTGTTA |
| ***SP-A*** | GCTGCAAGTGTAGCTGC |
| ***SP-1A*** | CCTACAGGCAAGGTGT |
| ***SP-2A*** | AGCCTTTGTTGTATTC |
| ***SP-3*** | TGTTCGTACTGTTTCA |
| ***SP-4*** | CCTGTCCACAATTTTC |

## RNA Isolation

Fourteen male *X. maculatus* Jp 163 A and four Hybrid 7 fish were sacrificed and dissected for RNA isolation. All supplies and reagents needed for the RNA isolation were treated with a 0.1% diethyl pyrocarbonate (DEPC) solution overnight at 37° C and autoclaved. Each animal was sacrificed by placing the fish in an ice-water bath followed by cranial pithing. Tissues from each fish were separated into dounce homogenizers submerged in a dry ice-ethanol bath. Tissues utilized from *X. maculatus* Jp 163 A included brain, eye, gill, liver, muscle, skin, and testes. Hybrid 7 tissues included spotted skin (partially pigmented), melanized skin (heavily pigmented), and tumor (heavily pigmented). Tissues were thawed and homogenized in Tri Reagent™ (Sigma, MO, USA; 1 ml Tri Reagent per 75 mg of tissue) and allowed to stand at room temperature for 5 minutes to allow complete dissociation of the nucleoprotein complexes. Next, 0.2 ml of chloroform per ml of Tri Reagent™ was added to each tissue sample followed by

incubation at room temperature for 15 minutes. The mixture was centrifuged at 12,000 x g (J2-21 Beckman Instruments; Fullerton, CA) for 15 minutes at 4° C to partition the aqueous, interphase, and organic phases. The aqueous phase, containing nucleic acids, was transferred to an RNase free tube (i.e. treated with RNase Zap Solution, Ambion; Austin, TX). To precipitate the RNA, 0.5 ml of isopropanol per ml of Tri Reagent™ was added. The RNA pellet was collected after centrifugation at 12,000 x g for 10 minutes at 4°C and washed twice with 75% ethanol then recollected by centrifugation at 7500 x g for 5 minutes at 4° C. The supernatant was decanted, the RNA pellet air dried for 10 minutes, and the pellet resuspended in 500 µl of DEPC treated water. To remove traces of DNA contamination, 2 U of DNase I (Ambion, (2 U/µl)) per 1 µg of RNA and DNASE Reaction buffer (100 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 1 mM $CaCl_2$ for a 10 X stock solution) was incubated at 37° C for 30 minutes. The RNA was extracted once with one volume Tris-saturated phenol and once with one volume Sevags solution (4% Iso-amyl alcohol, 96% chloroform), precipitated with 1/10 volume 3 M NaOAc and 2 volumes 100 % ice cold ethanol for 30 minutes at -80° C. The RNA was pelleted by centrifugation, dried in a vacuum centrifugation (Savant Instruments; Holbrook, NY), and resuspened in 500 µl of DEPC treated water. RNA concentrations were determined using a spectrophotometer (DU-64 Beckman Instruments, Fullerton, CA), using a value of 1 $A_{260}$ absorbance unit as equivalent to 40 µg/ml of RNA.

## Genomic DNA Isolation

Five *X. maculatus* Jp 163 A were sacrificed and dissected for genomic DNA isolation. Each animal was sacrificed and tissues isolated as previously described (see RNA Isolation). On ice, tissue samples were dounce homogenized in 3 ml of lysis buffer (1% SDS, 10 mM Tris/HCl (pH 7.5), 100 mM EDTA) per 1.0 gm of tissue then poured into a 15 ml polypropylene tube and incubated at 55° C with gentle mixing every 10 minutes for one hour. One volume of Tris-saturated phenol was added and the mixture was centrifuged at 2000 x g (TJ-6 Beckman Instruments; Fullerton, CA) for 20 minutes. The aqueous and interphase layers were removed and placed in a new polypropylene tube. The suspension was then extracted twice with phenol and Sevags solution (1:1). The aqueous layer was transferred to a new tube and 1/10 volume 3 M NaOAc and 2 volumes 100% ice-cold ethanol were added. The nucleic acids were precipitated at -80° C for 30 minutes. The DNA was pelleted by centrifugation at 4° C for 20 minutes at 12,000 x g (J2-21 Beckman Instruments; Fullerton, CA), washed twice with 70% ethanol, and dried in a vacuum centrifuge (Savant Instruments; Holbrook, NY). DNA was resuspended in an appropriate amount of TE buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA). To remove RNA, RNaseA (50 µg/ml,) was added and incubated at 37° C for 30 minutes. The DNA was then extracted once with phenol and once with Sevags solution, transferring the supernatant to a new tube. Then 1/10 volume 3 M NaOAc and 2 volumes 100% ice cold ethanol was added. The nucleic acids were again precipitated at -80° C for 15 minutes. The DNA was pelleted by centrifugation, washed, and dried as previously

described. The dried DNA was resuspended in an appropriate amount of TE buffer and DNA concentration determined using spectrophotometer (DU-64 Beckman Instruments, Fullerton, CA). One $A_{260}$ absorbance unit is considered equivalent to 50 µg/ml of double stranded DNA.

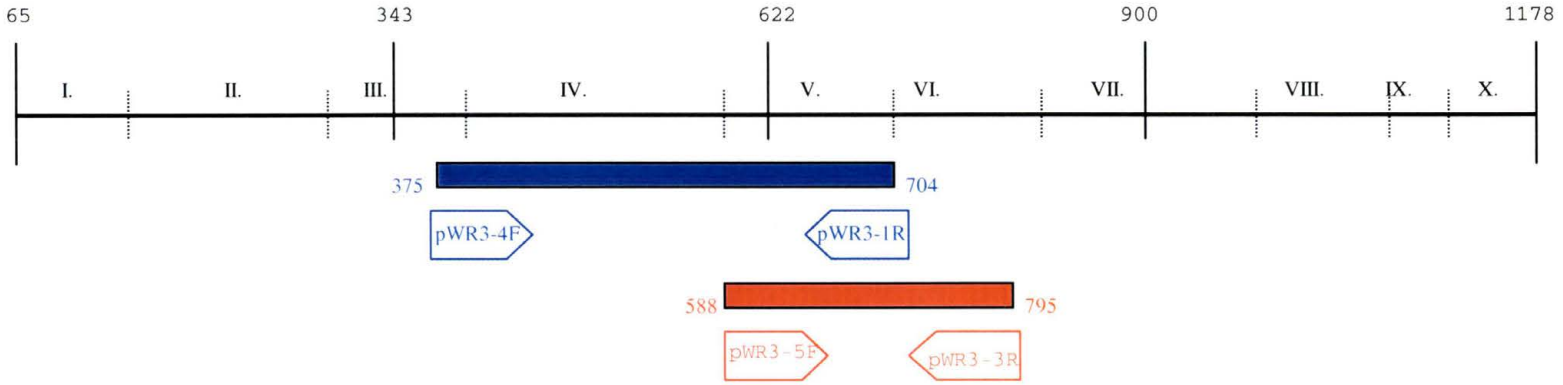
**PCR Polymorphisms**

To amplify a portion of the x*CYP40* gene by PCR, synthetic oligonucleotide primers, pWR3-5F (forward primer) and pWR3-3R (reverse primer) were designed using the deduced x*CYP40* nucleotide sequence (Figure 2-1). Restriction endonuclease digests were performed on amplified DNA to visualize a polymorphism between *Xiphophorus* parental strains *X. maculatus* and *X. helleri.*

A manual hot start PCR reaction in 50 µl final volume consisted of 1 µl genomic DNA (50 ng/µl), 5 µl of 10X Buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl), 3 µl of 25 mM $MgCl_2$, 0.5 µl of 25 µM dNTP mix (2.5 mM each dNTP), 0.4 µl of pWR3-5F and pWR3-3R primers (40 µM) (Table 2-3), 0.5 µl of Taq polymerase (5U/µl). The following parameters were used in a Perkin Elmer Gene Amp 2400 PCR System: (94° C 5 minutes for one cycle; 94° C, 30 seconds, 55° C, 1 minute, 72° C, 1 minute 45 seconds for 35 cycles; then 72° C for 7 minutes). Expected sizes of amplimers from the x*CYP40* cDNA for oligonucleotide primer pairs are listed in Table 2-4. Aliquots of the PCR amplified product (20 µl) were digested with the following restriction enzymes to

identify a polymorphism between *Xiphophorus* parentals: *Dde* I (10 U/µl), *Hpa* III (10 U/µl), *Xba* I (10 U/µl), *Rsa* I (10 U/µl), and *Taq* I (10 U/µl). Each enzyme was used according to supplier recommendations. The restriction digest products were fractionated on 1.0% agarose gels using 1 X TAE buffer (1 M Tris-acetate, 57.1 ml glacial acetic acid, 0.05 M EDTA (pH 8.0), per liter, for a 50 x stock solution) and stained with ethidium bromide. Fragment sizes were determined by comparison with a 1 kb Ladder (Gibco BRL; Rockville, MD).

**Figure 2-1 Synthetic Oligonucleotide Hybridization Sites.** Diagram of the pWR3 open reading frame is shown indicating size in basepairs with synthetic oligonucleotide primers used for PCR amplification. Arrows indicate location and direction of synthetic oligonucleotide primers for PCR Polymorphisms (in red) and Relative Q-RT-PCR (in blue) experiments. Dotted lines indicate putative exon boundaries and roman numerals indicate putative exon number. Expected cDNA product sizes for primer pairs are described in Table 2-3



**Relative Quantitative RT-PCR**

Relative expression of x*CYP40* mRNA was quantified with the aid of QuantumRNA Reverse Transcriptase (RT-PCR) kits (Ambion, TX, USA). Relative Quantitative RT-PCR measures the expression of the target gene amplicon relative to an endogenous 18S rRNA internal control. To determine the relative expression of x*CYP40* tissues, total cellular RNA is reverse transcribed to its complementary cDNA. Then the linear range of amplification for the target gene and the optimal ratio of 18S rRNA primer to competimer are determined.

a) Reverse Transcription (RT)

In a first-strand synthesis reaction, RNA is copied to its complementary DNA sequence (cDNA) by reverse transcriptase using a RETROscript Kit (Ambion, Austin, TX). In a 20 µl reaction, 2 µg of total RNA, 4 µl of dNTP mix (2.5 mM each dNTP), 2 µl of random decamers (50 µM first strand primers), and nuclease free water are heated to 85° C for 3 min. to eliminate secondary structure. On ice, 2 µl of Alternate 10 x First-Strand Buffer (500 mM Tris-HCl (pH 8.3), 750 mM KCl, 30 mM $MgCl_2$, 50 mM DTT), 1 µl Placental RNase Inhibitor (10 U/µl), and 1 µl Moloney-Murine Leukemia Virus (MMLV) Reverse Transcriptase (100 U/µl) was added to the RNA. The reaction was incubated at 42° C for 1 hour, then incubated at 92° C for 10 minutes to inactivate the reverse transcriptase, and stored at -20°C for further use.

b) Linear Range of Amplification

To obtain meaningful results, RT-PCR products are quantified when all samples are in the linear range of PCR amplification.

Five µl of the RT reaction from liver RNA was used to determine the linear range of amplification for x*CYP40*. For each sample, the following reagents were used in a 50 µl PCR reaction: 5 µl of 10 x Buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl), 2 µl of 25 mM $MgCl_2$, 0.5 µl of 25 µM dNTP mix (2.5 mM each dNTP), 0.4 µl of pWR3-4F and pWR3-1R primers (40 µM) (Tables 2-3 and 2-4), 0.5 µl of Taq polymerase (5 U/µl) and 5 µl of $\alpha$-$^{32}$P dCTP (3000 Ci/mmole, NEN Life Sciences, Boston, MA). PCR parameters were as follows: (94° C, 5 minutes for one cycle; 94° C, 30 seconds, 56° C, 1 minute, 72° C, 1 minute 45 seconds for 35 cycles), using 2400 Gene Amp PCR System (Perkin Elmer; Foster City, CA). Ten identical reactions were assembled and beginning with cycle 15, one tube was removed from the PCR machine after each cycle until cycle 31 was reached. Five µl of stop solution was added to each PCR reaction and the products denatured at 94° C for 3 minutes then fractionated on a 6.0% polyacrylamide, 5 M urea denaturing gel. Gel processing and autoradiography were as previously described. Results were analyzed using Packard Cyclone Storage Phosphor System Version 3.0, (Meriden, CT).

The Cyclone Storage Phosphor System is an imaging system comprised of a laser-scanning device. An image is obtained by exposing a sample to phosphor screens. The

screen's phosphorcrystals absorb energy emitted by sample radioactivity and re-emit that energy upon laser scanning. The intensity of the light from the stored energy is proportional to the amount of radioactivity in the sample over four orders of magnitude. To create a digitized image, the phosphor screens are scanned and analyzed using OptiQuant™ Software.

**Table 2-3 Synthetic Oligonucleotide Primers used for PCR Polymorphisms and Relative Q-RT-PCR**. These primers were used in PCR reactions for obtaining a PCR polymorphism between *Xiphophorus* parentals. Primers were also used in relative Q-RT-PCR experiments for *CYP*40 relative expression.

| *Polymorph and/or Rel Q-RT-PCR Primer* | *Sequence (5' – 3')* |
|---|---|
| ***pWR3-1F*** | CCTGTAAAGCCTTGCATCAT |
| ***pWR3-2F*** | CACTACAAGCATGACAAGT |
| ***pWR3-3F*** | ACAGAGGACACTCCTGTAAA |
| ***pWR3-4F*** | GGATGAAAACTTCCACTACA |
| ***pWR3-5F*** | GCATCATAGCGGACTGTGG |
| ***pWR3-1R*** | TTAAAGTCGATGTCGGAGTC |
| ***pWR3-2R*** | TCCAAAAACCACATGCTTTC |
| ***pWR3-3R*** | TTCACTGCAGCTGTCCAG |

**Table 2-4 Expected cDNA Product Sizes for Synthetic Oligonucleotide Primers** Synthetic oligonucleotide primers were designed from pWR3 cDNA. The expected cDNA product size is indicated for each pWR3 primer pair. For exact position and direction of primer pairs, refer to Figure 2-1.

| Primer Pair | *Expected cDNA Size* |
|---|---|
| **pWR3-4F/1R** | 331 |
| **pWR3-5F/3R** | 206 |

c) Determining the Optimal ratio of 18S Primers:Competimers

The majority of RNA is ribosomal (r) RNA and is considered to be essentially constant from sample to sample (Sambrook et al., 1989). In a multiplex reaction, the cDNA of interest is amplified with gene specific primers as well as an endogenous 18S rRNA internal control using 18S primers and 18S competimers. By mixing 18S primers with increasing amounts of 18S competimers, PCR amplification efficiency of 18S cDNA is reduced without the primers becoming limiting and without loss of relative quantitation (Ambion; Austin, TX).

To determine the optimal 18S rRNA primer to competimer ratio that would be roughly similar to the target gene amplicon, a series of reactions are assembled with varying 18S primer to competimer ratios (1:9 to 5:27). PCR products were analyzed using the Cyclone Phosphorimager and the primer/competimer ratio giving amplification of rRNA equal to the gene target determined.

Once the PCR conditions, the linear range of amplification for *X. maculatus* *CYP*40 specific primers, and optimal 18S primer to competimer ratios were established with liver cDNA, this information was used to perform relative quantitative RT-PCR experiments from RNA isolated from other tissues. Each experiment was repeated three times utilizing two different RT reactions from same total RNA source to produce template cDNA for Relative Q-RT-PCR reactions.

# RESULTS

## Isolation of the pWR3 Clone

As part of an ongoing collaborative gene mapping effort, with UT MD Anderson Cancer Center, Science Park, Smithville, TX the plasmid, pWR3 was isolated and used as a probe from a *X. maculatus* Lambda Zap II Phage cDNA library at UT MD Anderson Cancer Center. When radiolabeled and used as a probe on a panel of *X. maculatus* genomic DNA digested with Pst I, two distinct RFLP polymorphisms were visible (Figure 3-1). Backcross hybrid individuals were scored for each polymorphism, and map locations were assigned for each polymorphism designated as 3A1 and 3A2 (Figure 3-2).

The pWR3 clone was then sent to Southwest Texas State University for nucleotide sequencing and further characterization. The clone was digested with the restriction enzyme EcoR1 and agarose gel analysis of the restriction data indicated an insert size of approximately 1.5 kb (Figure 3-3).

**Figure 3-1 RFLP Polymorphism Panel**

Southern blot of DNA isolated from *X. maculatus* Jp 163 B, *X. helleri*, and individual backcross hybrid progeny (Hybrid 1 genetic cross). The DNA's were digested with Pst 1 and the blot probed with pWR3. Arrows indicate positions of initial polymorphisms and assigned locus name is in parenthesis. Each backcross hybrid individual is scored as heterozygous (indicated by red *) or homozygous (indicated by blue *). (6% acrylamide gel)

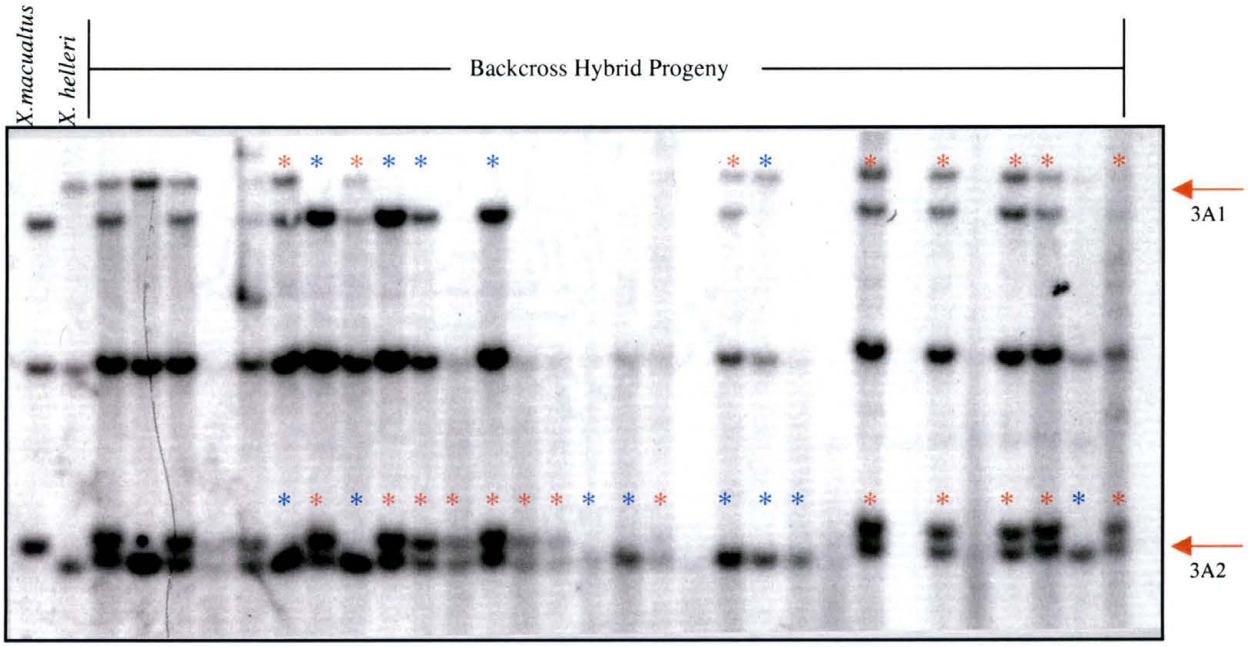


**Figure 3-2 Preliminary Markers Assigned on Linkage Group (LG) U16.** Loci 3A1 and 3A2, also known as anonymous markers XD0257 and XD0258, are shown at the distal end of LG U16. Recombination percentages are indicated in the left column.

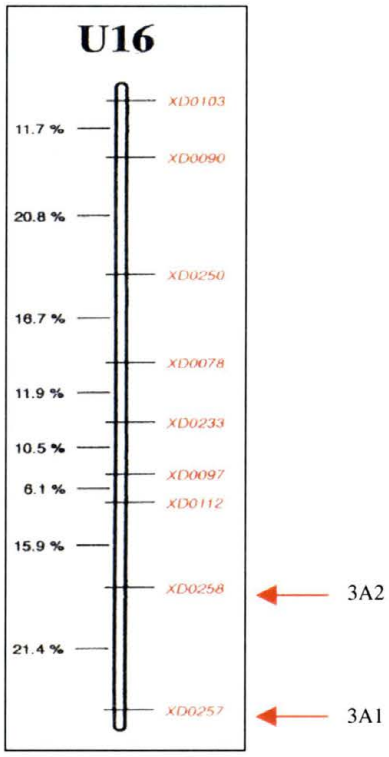
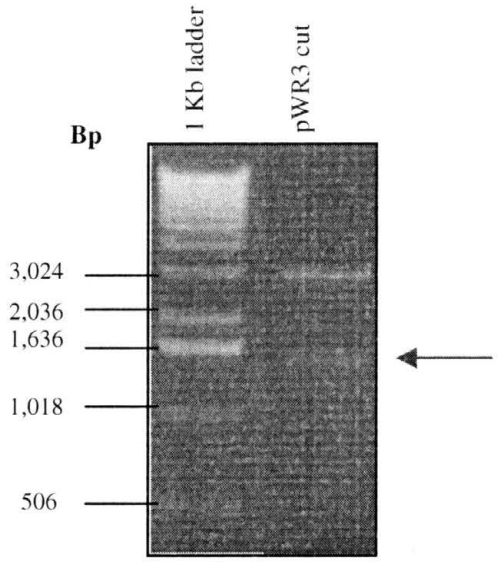


**Figure 3-3 Insert size of the pWR3 clone**. The cDNA clone, pWR3, was digested with EcoR1 restriction enzyme ("cut"). Restriction data analysis displays the plasmid (pBluescript) size of approximately 3.0 kb and an insert size of approximately 1.5 kb. Arrow indicates position of clone insert. 1 kb ladder sizes are indicated in base pairs (bp) on the left. (1% Agarose gel)




## Nucleotide Sequence Determination of pWR3

The plasmid subclone, pWR3, was subjected to complete dideoxy nucleotide sequence analysis using T3 and T7 primers in addition to synthetic oligonucleotide primers that were constructed to complete the sequence distal to the universal primer sequence runs (Figure 3-4). Figure 3-5 presents 1500 base pairs (bp) of cDNA sequence derived from the pWR3 clone. Translation of the cDNA sequence using MacVector Sequence Analysis software yielded a single open reading frame of 371 amino acids.

The start codon begins at position 65 and the stop codon is present at position 1178. There are 320 bp of trailer sequence after the stop codon (TGA) in which a putative polyadenylation signal has been identified at positions 1456-1460 (Figure 3-5).

**Figure 3-4 Oligonucleotide Primers Used to Sequence the pWR3 cDNA clone**

Diagram of the pWR3 cDNA open reading frame, indicating size in base pairs is shown with synthetic oligonucleotide primers (in gold) that were designed to complete the sequence of the insert. The primer's name, position and orientation are indicated in the respective boxes. T7, standard primer, is shown in grey. Dotted lines indicate putative exon boundaries and roman numerals indicated putative exon number.

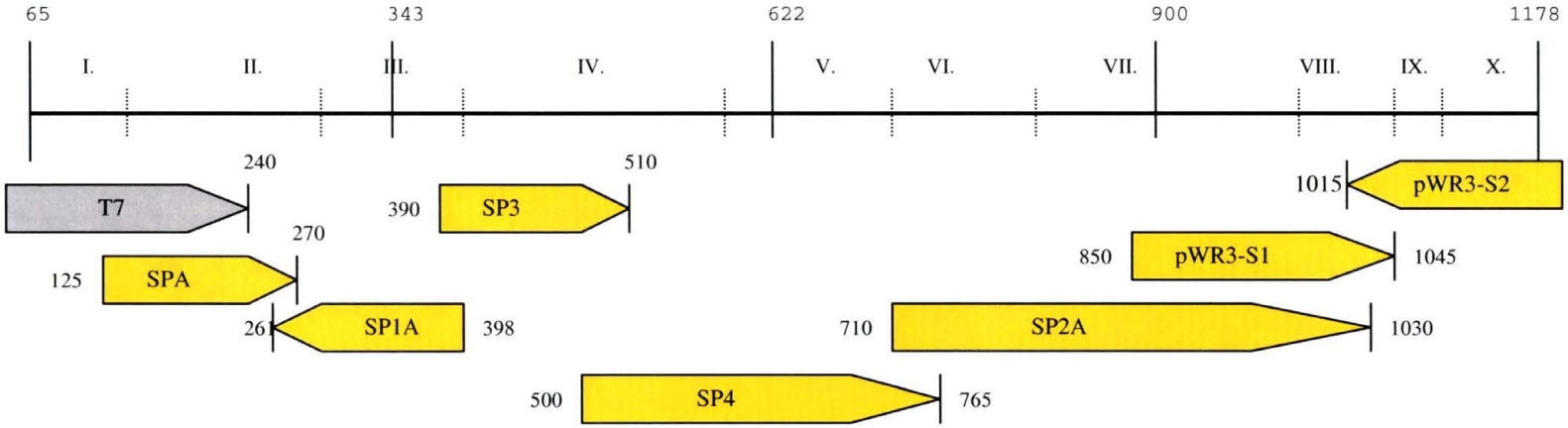


**Figure 3-5 Nucleotide Sequence of pWR3 cDNA and deduced amino acid sequence**
The stop codon (in red) is present at position 1178. Blue asterisks identify putative exon boundaries based on exon/intron organization of (h*CYP40*) (GenBank, accession number D63861; Yokoi *et al.*, 1996a). The green underlined region indicates a putative calmodulin-binding site (position 354-370) (Ratajczak *et al.*, 1993). A putative polyadenylation signal is indicated by the dotted underline at positions 1456-1460. Bold numbers on left side of sequence indicate amino acids

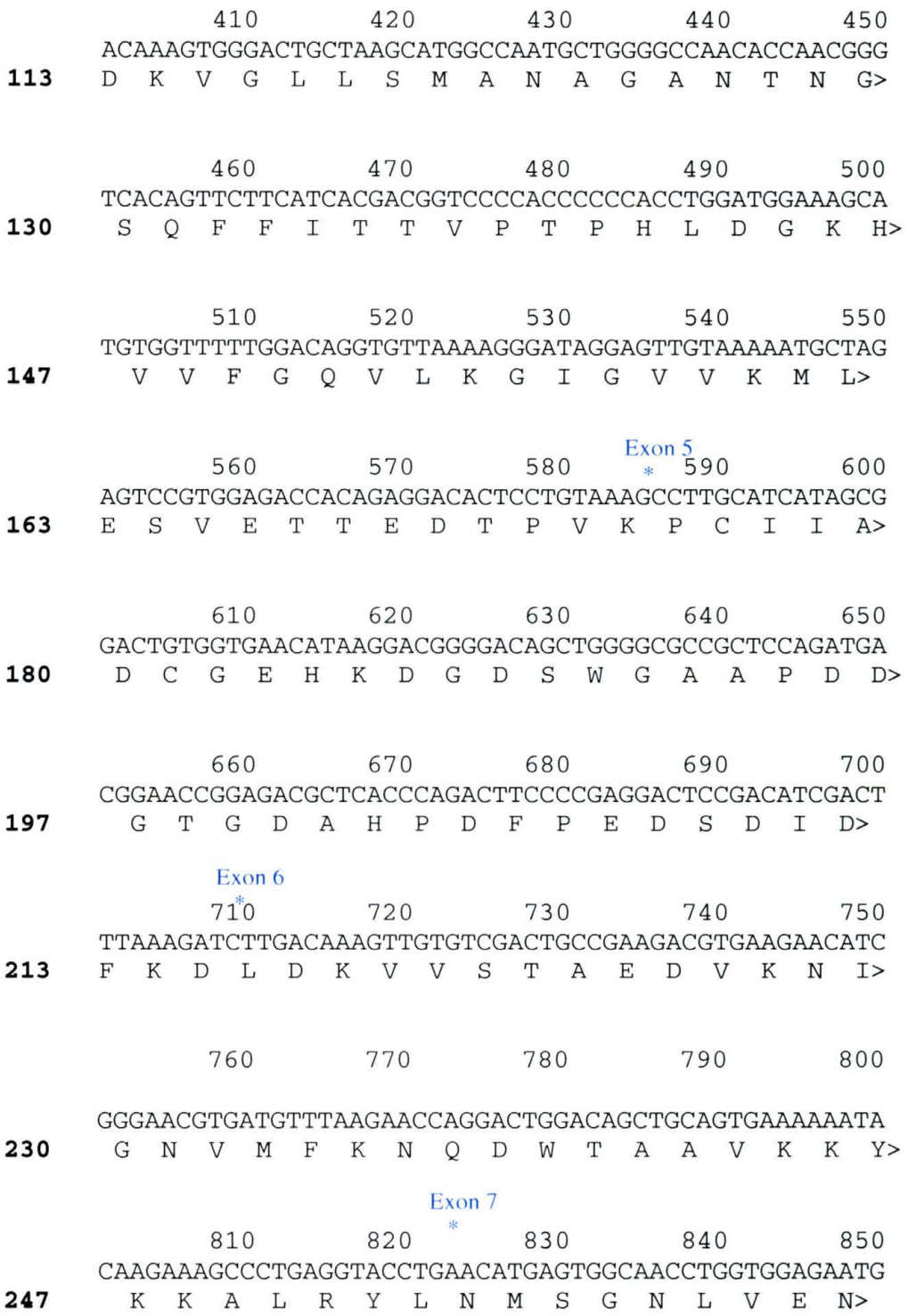
```
    10        20        30        40        50
     TTCGCGGCGTCGACCGCCGGTGTCGAGTTTTACAAGGTTTAGGTTCAACG

                    Exon 1
              60       *  70        80        90       100
     ATTGCTCCTTAAAATGTCTCACCCAATACCGTCGGAAAAGCCATCCAACC
1                  M  S  H  P  I  P  S  E  K  P  S  N>
                                                  Exon 2
                                                    *
            110       120       130       140       150
     CCGAGAATCCTCGCGTTTTTTTCGACGTTGACATCGACGGGGAAAAAGCC
13   P  E  N  P  R  V  F  F  D  V  D  I  D  G  E  K  A>

            160       170       180       190       200
     GGCCGCATTGTTCTGGAGCTGTTTGCCGACGTCACCCCTAAGACTGCTGA
30    G  R  I  V  L  E  L  F  A  D  V  T  P  K  T  A  E>

            210       220       230       240       250
     AAACTTCCGGGCACTCTGCACCGGAGAGAAAGGCGTCGGAAAATCGACGG
47     N  F  R  A  L  C  T  G  E  K  G  V  G  K  S  T>
                                                Exon 3
            260       270       280       290   *   300
     GGAAACCGCTGCACTTCAAGGGATGTCCGTTCCACAGAATCATCAAGAAG
63   G  K  P  L  H  F  K  G  C  P  F  H  R  I  I  K  K>

            310       320       330       340       350
     TTTATGATCCAGGGAGGTGATTTCTCTAACCACAACGGCACGGGGGGCGA
80    F  M  I  Q  G  G  D  F  S  N  H  N  G  T  G  G  E>
                                                Exon 4
                                                  *
            360       370       380       390       400
     GACGATCTACGGGGAGAAGTTTGAGGATGAAAACTTCCACTACAAGCATG
97     T  I  Y  G  E  K  F  E  D  E  N  F  H  Y  K  H>
```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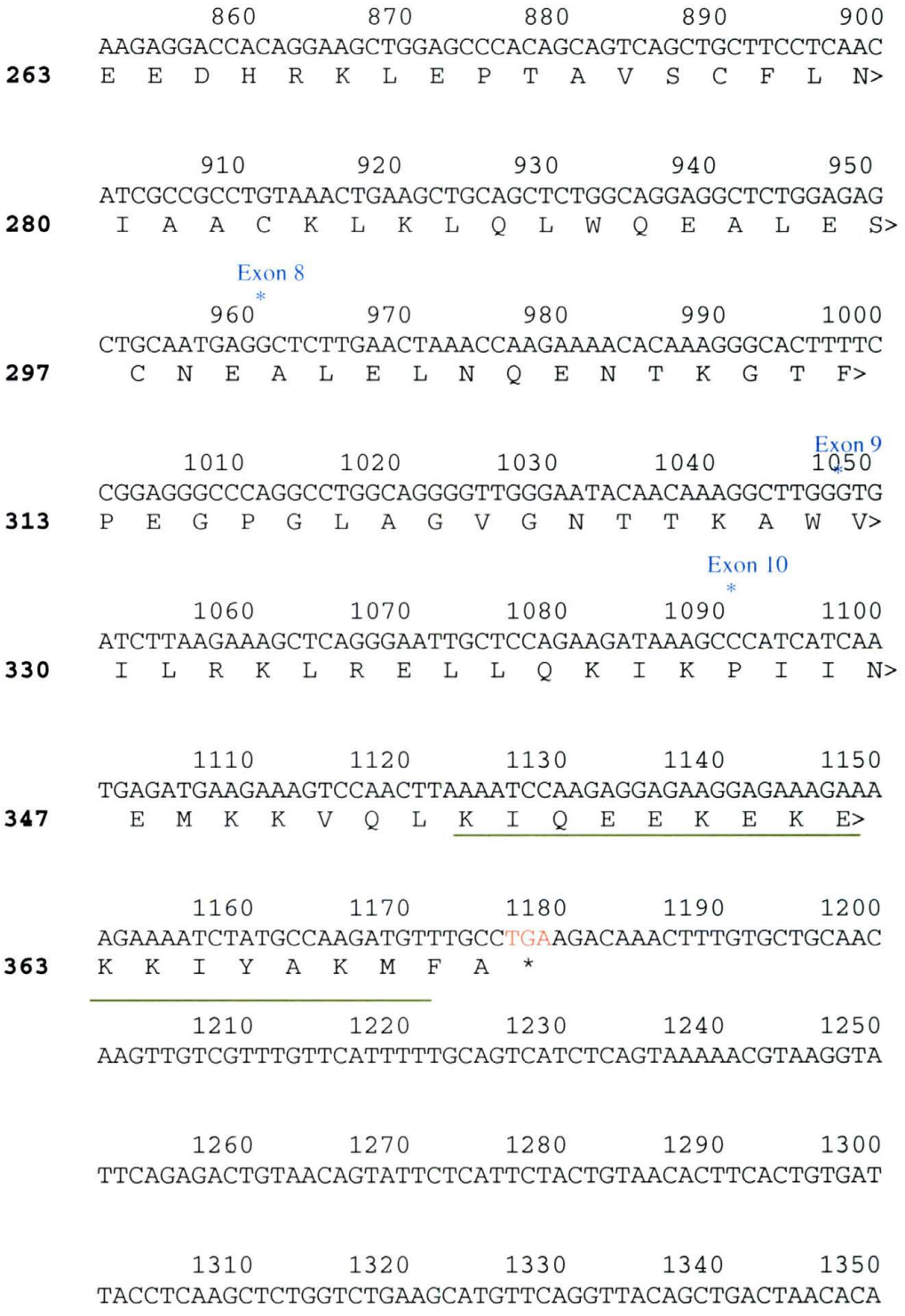
ACAAAGTGGGACTGCTAAGCATGGCCAATGCTGGGGCCAACACCAACGGG
113 D K V G L L S M A N A G A N T N G>
460 470 480 490 500
TCACAGTTCTTCATCACGACGGTCCCCACCCCCCACCTGGATGGAAAGCA
130 S Q F F I T T V P T P H L D G K H>
510 520 530 540 550
TGTGGTTTTTGGACAGGTGTTAAAAGGGATAGGAGTTGTAAAAATGCTAG
147 V V F G Q V L K G I G V V K M L>
Exon 5
560 570 580 590 600
AGTCCGTGGAGACCACAGAGGACACTCCTGTAAAGCCTTGCATCATAGCG
163 E S V E T T E D T P V K P C I I A>
610 620 630 640 650
GACTGTGGTGAACATAAGGACGGGGACAGCTGGGGCGCCGCTCCAGATGA
180 D C G E H K D G D S W G A A P D D>
660 670 680 690 700
CGGAACCGGAGACGCTCACCCAGACTTCCCCGAGGACTCCGACATCGACT
197 G T G D A H P D F P E D S D I D>
Exon 6
710 720 730 740 750
TTAAAGATCTTGACAAAGTTGTGTCGACTGCCGAAGACGTGAAGAACATC
213 F K D L D K V V S T A E D V K N I>
760 770 780 790 800
GGGAACGTGATGTTTAAGAACCAGGACTGGACAGCTGCAGTGAAAAAATA
230 G N V M F K N Q D W T A A V K K Y>
Exon 7
810 820 830 840 850
CAAGAAAGCCCTGAGGTACCTGAACATGAGTGGCAACCTGGTGGAGAATG
247 K K A L R Y L N M S G N L V E N>


860 870 880 890 900
AAGAGGACCACAGGAAGCTGGAGCCCACAGCAGTCAGCTGCTTCCTCAAC
263 E E D H R K L E P T A V S C F L N>
910 920 930 940 950
ATCGCCGCCTGTAAACTGAAGCTGCAGCTCTGGCAGGAGGCTCTGGAGAG
280 I A A C K L K L Q L W Q E A L E S>
Exon 8
960 970 980 990 1000
CTGCAATGAGGCTCTTGAACTAAACCAAGAAAACACAAAGGGCACTTTTC
297 C N E A L E L N Q E N T K G T F>
Exon 9
1010 1020 1030 1040 1050
CGGAGGGCCCAGGCCTGGCAGGGGTTGGGAATACAACAAAGGCTTGGGTG
313 P E G P G L A G V G N T T K A W V>
Exon 10
1060 1070 1080 1090 1100
ATCTTAAGAAAGCTCAGGGAATTGCTCCAGAAGATAAAGCCCATCATCAA
330 I L R K L R E L L Q K I K P I I N>
1110 1120 1130 1140 1150
TGAGATGAAGAAAGTCCAACTTAAAATCCAAGAGGAGAAGGAGAAAGAAA
347 E M K K V Q L K I Q E E K E K E>
1160 1170 1180 1190 1200
AGAAAATCTATGCCAAGATGTTTGCCTGAAGACAAACTTTGTGCTGCAAC
363 K K I Y A K M F A *
1210 1220 1230 1240 1250
AAGTTGTCGTTTGTTCATTTTTGCAGTCATCTCAGTAAAAACGTAAGGTA
1260 1270 1280 1290 1300
TTCAGAGACTGTAACAGTATTCTCATTCTACTGTAACACTTCACTGTGAT
1310 1320 1330 1340 1350
TACCTCAAGCTCTGGTCTGAAGCATGTTCAGGTTACAGCTGACTAACACA

```
      1360       1370       1380       1390       1400
GGCTTGTTGTGTCTTATTCTACATAATGTTGTGTATCTTGTATCATTTCA

      1410       1420       1430       1440       1450
TTGTTGACATTTTGATACAATACACCAGTTTGTTTTATTTATGTTTTTAC

      1460       1470       1480       1490       1500
TGGTCAATAAAACAAGGGATAACTCAAAAAAAAGTCGAGCGGCCGCGAAT
```

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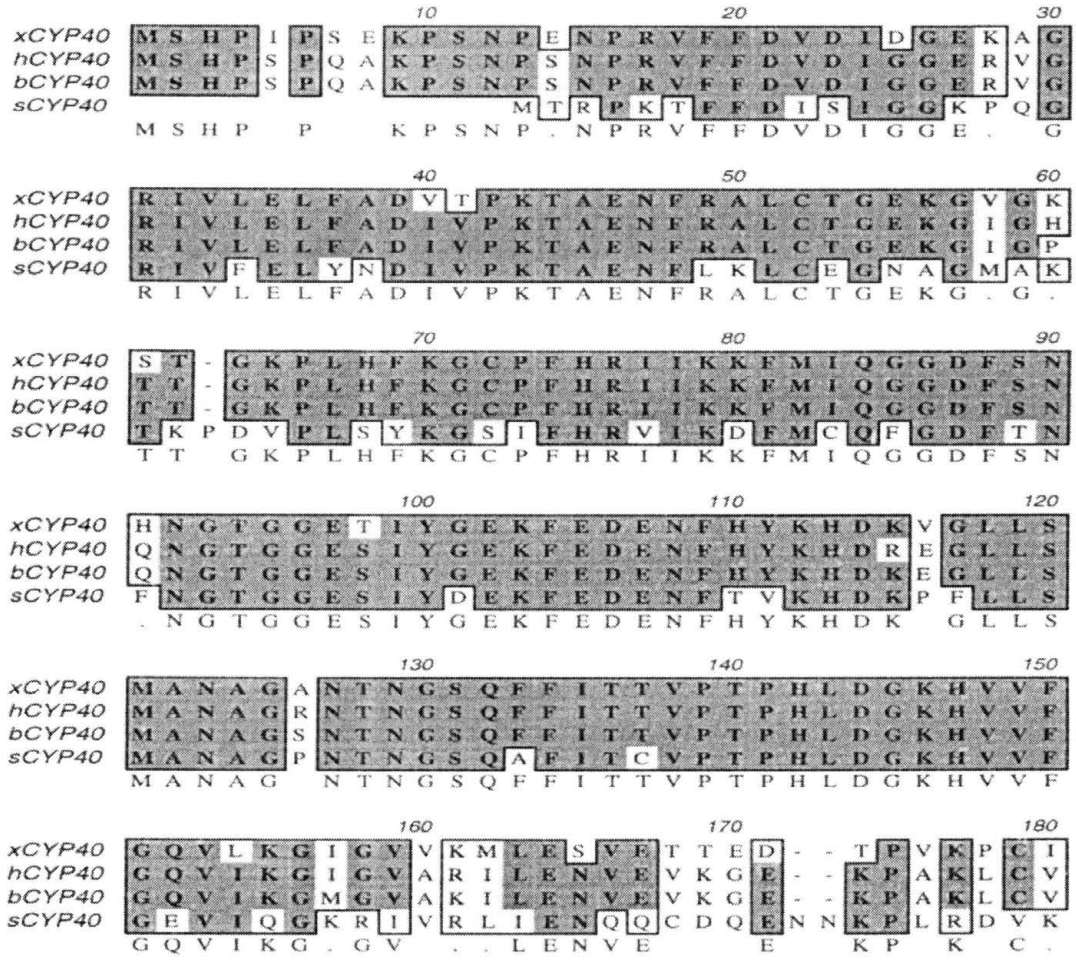
**Cyclophilin 40 Nucleotide and Amino Acid Identity**

To determine amino acid conservation and similarity of xcyp40 with other characterized cyp40 models, deduced amino acid sequence of pWR3 was aligned with other cylophilin 40 genes such as (h*CYP40*), (s*CYP40*), and (b*CYP40*) (Figure 3-6). Data analyses revealed high amino acid similarity (92%) when compared to hcyp40 within the first five exons (shaded areas in Figure 3-6). Overall nucleotide identity ranges from 66% to 62% and amino acid similarity ranges from 78% to 59%, for other *CYP40* sequences (Table 3-1).

**Figure 3-6 Amino Acid Alignment.** Amino acid alignments of *X. maculatus* Cyclophilin 40 (xCYP40) are shown with human (h*CYP40*) (GenBank accession number D63861; Kieffer *et al.*, 1993), bovine (b*CYP40*) (GenBank accession number D14074; Ratajczak *et al.*, 1993), and *S. cerevisae* (s*CYP40*) (GenBank accession number U48867; Duina *et al.*, 1996). Bolded and dark shaded regions indicate amino acid identity while lightly shaded areas indicated amino acid similarities. Consensus sequence is displayed at the bottom of the aligned amino acids. The period within the consensus sequence indicates similarity of residues boxed in light grey. Gaps (-) were introduced for optimal alignment, according to ClustalW Algorithm (Thompson *et al.*, 1994).



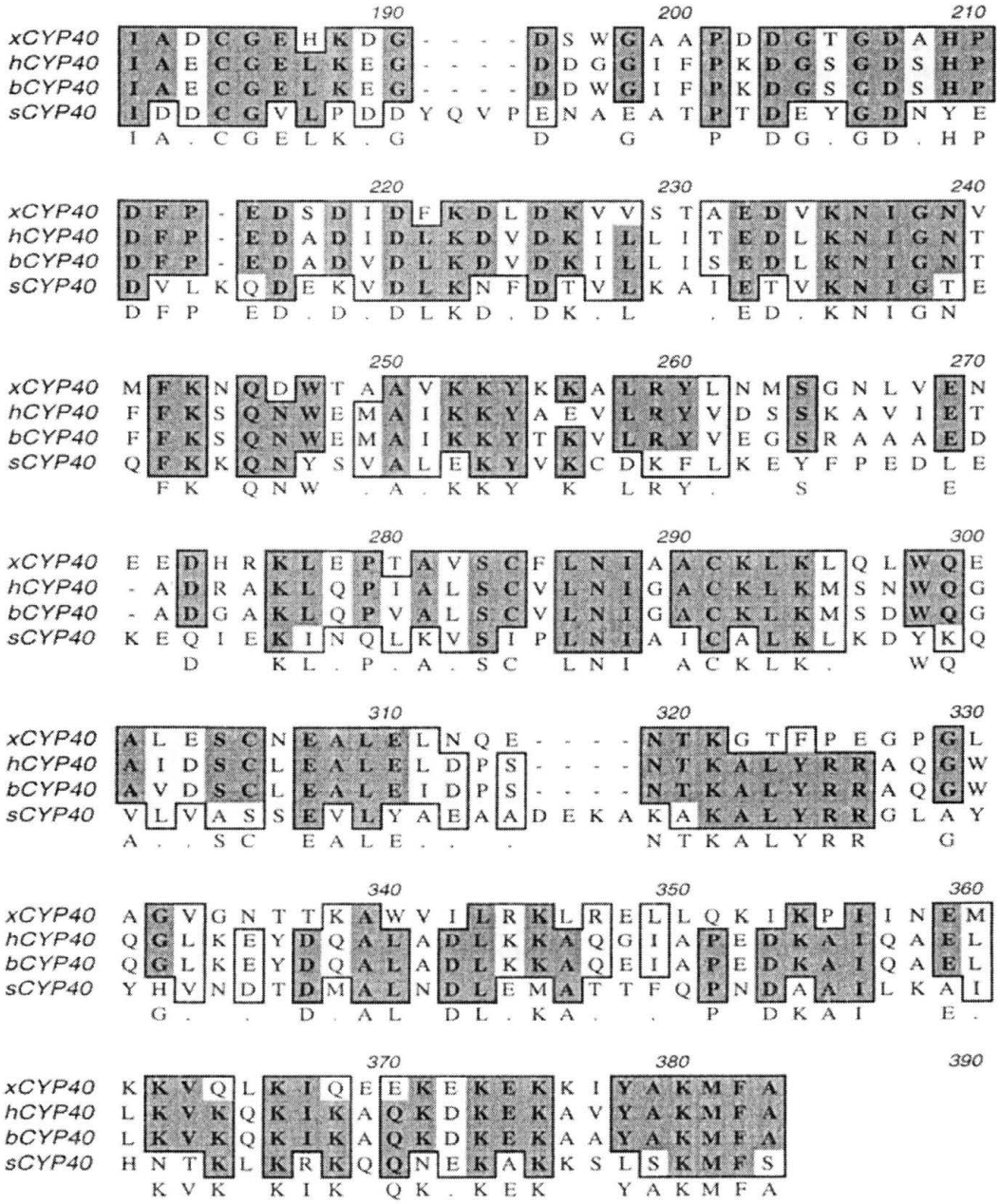

190 200 210
xCYP40 IADCGEHKDG----DSWGAAPDDGTGDAHP
hCYP40 IAECGELKEG----DDGGIFPKDGSGDSHP
bCYP40 IAECGELKEG----DDWGIFPKDGSGDSHP
sCYP40 IDDCGVLPDDYQVPENAEATPTDEYGDNYE
IA.CGELK.G D G P DG.GD.HP
220 230 240
xCYP40 DFP-EDSDIDFKDLDKVVSTAEDVKNIGNV
hCYP40 DFP-EDADIDLKDVDKILLITEDLKNIGNT
bCYP40 DFP-EDADVDLKDVDKILLISEDLKNIGNT
sCYP40 DVLKQDEKVDLKNFDTVLKAIETVKNIGTE
DFP ED.D.DLKD.DK.L .ED.KNIGN
250 260 270
xCYP40 MFKNQDWTAAVKKYKKALRYLNMSGNLVEN
hCYP40 FFKSQNWEMAIKKYAEVLRYVDSSKAVIET
bCYP40 FFKSQNWEMAIKKYTKVLRYVEGSRAAAED
sCYP40 QFKKQNYSVALEKYVKCDKFLKEYFPEDLE
FK QNW .A.KKY K LRY. S E
280 290 300
xCYP40 EEDHRKLEPTAVSCFLNIAACKLKLQLWQE
hCYP40 -ADRAKLQPIALSCVLNIGACKLKMSNWQG
bCYP40 -ADGAKLQPVALSCVLNIGACKLKMSDWQG
sCYP40 KEQIEKINQLKVSIPLNIAICALKLKDYKQ
D KL.P.A.SC LNI ACKLK. WQ
310 320 330
xCYP40 ALESCNEALELNQE----NTKGTFPEGPGL
hCYP40 AIDSCLEALELDPS----NTKALYRRAQGW
bCYP40 AVDSCLEALEIDPS----NTKALYRRAQGW
sCYP40 VLVASSEVLYAEAADEKAKALYRRGLAY
A..SC EALE.. NTKALYRR G
340 350 360
xCYP40 AGVGNTTKAWVILRKLRELLQKIKPIINEM
hCYP40 QGLKEYDQALADLKKAQGIAPEDKAIQAEL
bCYP40 QGLKEYDQALADLKKAQEIAPEDKAIQAEL
sCYP40 YHVNDTDMALNDLEMATTFQPNDAAILKAI
G..D.AL DL.KA..P DKAI E.
370 380 390
xCYP40 KKVQLKIQEEKEKEKKIYAKMFA
hCYP40 LKVKQKIKAQKDKEKAVYAKMFA
bCYP40 LKVKQKIKAQKDKEKAAYAKMFA
sCYP40 HNTKLKRKQQNEKAKKSLSKMFS
KVK KIK QK.KEK YAKMFA

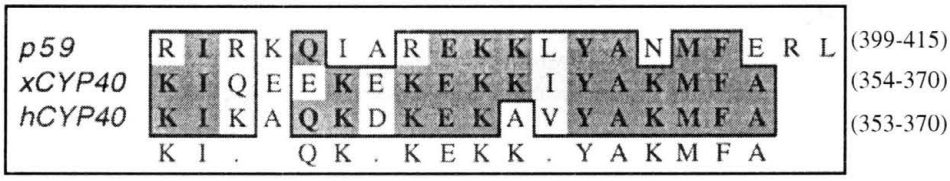
**Table 3-1 Cyclophilin 40 Identity Table**. Table indicating amino acid and nucleotide identities are listed for human (h*CYP40*) (GenBank accession number D63861; Kieffer *et al.*, 1993), bovine (b*CYP40*) (GenBank accession number D14074; Ratajczak *et al.*, 1993), and *S. cerevisae* (s*CYP40*) (GenBank accession number U48867; Duina *et al.*, 1996. Similarity was assessed using the BLOSUM 30 Similarity Matrix, MacVector 6.53 Sequence Analysis Software.

| | Human | Bovine | Yeast |
|---|---|---|---|
| Amino Acid Identity | 65% | 66% | 43% |
| Amino Acid Similarity | 78% | 78% | 59% |
| Nucleotide Identity | 66% | 51% | 51% |

a) Putative Calmodulin Binding Site

Calmodulin binding sites are identified within the C-terminus in p59 (Lebeau *et al.*, 1992) and h*CYP40* (Ratajczak *et al.*, 1993). To determine a putative calmodulin-binding site in *X. maculatus CYP40*, deduced amino acid sequence of pWR3 was aligned with the calmodulin binding sites in p59 (399-415) and hcyp40 (353-369) (Figure 3-7). Data analysis reveals 89% amino acid similarity (shaded areas in Figure 3-7) with the hcyp40 calmodulin binding site (aa 353-369).

**Figure 3-7 Putative Calmodulin Binding Site Alignment.** Partial amino acid alignment using MacVector Sequence Analysis Software is shown for *X. maculatus* (pWR3 translated) with calmodulin binding sites p59 (GenBank accession number M84474; Lebeau *et al.*, 1992) and human CYP40 (hCYP40) (GenBank accession number D63861; Kieffer *et al.*, 1993). Bold letters and dark shaded areas indicate amino acid identity while lightly shaded areas indicate amino acid similarity. Consensus sequence is indicated beneath the aligned amino acids with periods indicating similarity.




b) Putative Tetratricopeptide Repeat Motif

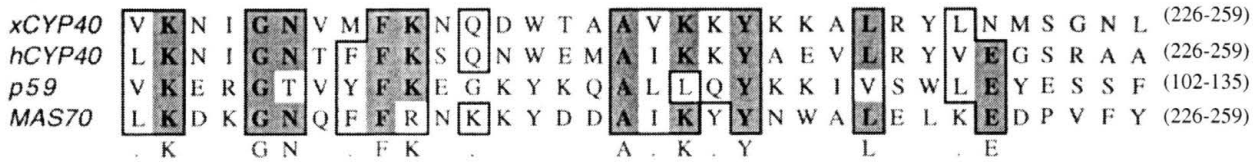
To determine a putative TPR motif(s) in x*CYP40*, deduced amino acid sequence of pWR3 was aligned with a 3-unit TPR repeat domain in p59 (Ratajczak *et al.*, 1993), MAS70 (Goebl and Yanagida, 1991), and hcyp40 (Blatch and Lassle, 1999) (Figure 3-8). Data analysis revealed amino acid similarity with hcyp40 TPR units (1-3) 50%, 71%, and 41% respectively.

The repeat units found in xcyp40 and p59 can be divided into two subdomains, A and B that are compatible with the formation of a 'hole' and 'knob' type structure (Hirano *et al.*, 1990). The amino acid residues surrounding the subdomains are hydrophobic and may function to stabilize the association of the two helices (Goebl and Yanagida, 1991).

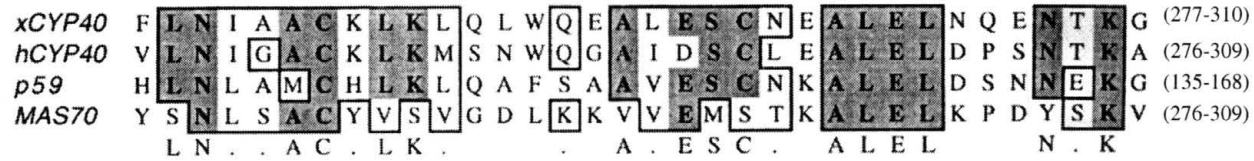
**Figure 3-8 Tetratricopeptide (TPR) Conserved Regions**

Three TPR units of MAS 70 (GenBank accession number 1709462; Hase *et al.*, 1983) human CYP40 (hCYP40) (GenBank accession number D63861; Kieffer *et al.*, 1993) and p59 (GenBank accession number M84474; Lebeau *et al.*, 1992) were aligned with deduced amino acid sequence of *X. maculatus CYP40* (xcyp40). Panels A, B, and C indicate the different TPR units. Amino acid positions are shown in parenthesis on right. Bolded letters and dark shaded areas indicate amino acid identity while lightly shaded areas indicate amino acid similarity. Consensus sequence is below the aligned sequences and periods indicate amino acid similarity.

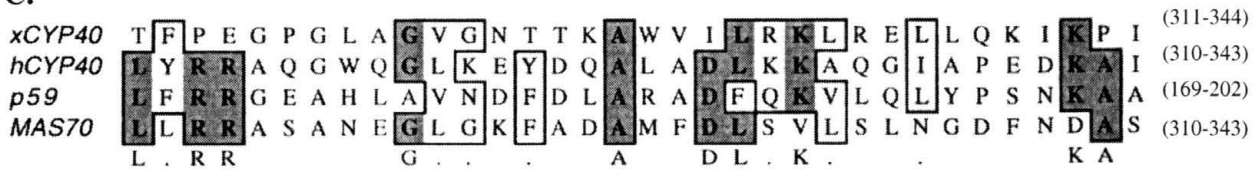
**A.**




**B.**




**C.**



## PCR Polymorphism and Mapping Data

A segment of genomic DNA from *X. maculatus* and *X. helleri* (Sarabia) was amplified using synthetic oligonucleotide primers pWR3-5F and pWR3-3R spanning exon 5 and half of exon 6 (Figure 2-1). The amplified product for both the *X. maculatus* and *X. helleri* parentals was approximately 900 bp (Figure 3-9). The amplified PCR product was digested with a battery of restriction enzymes to see if a polymorphism existed between the two parental species. The restriction enzyme Taq 1 produced a distinct polymorphism (Figure 3-10). Analysis of the restriction data produced an *X. maculatus* product size of approximately 800 bp while the *X. helleri* parent showed two products of approximately 500 bp and 275 bp (Figure 3-10). The PCR amplification was repeated using genomic DNA derived from 58-backcross hybrid progeny of a Hybrid 5 cross [*X. helleri* (×) (*X. maculatus* Jp 163 A (×) *X. helleri*)] (Figure 3-12). In addition, the PCR experiment was repeated using genomic DNA derived from 74 backcross hybrid progeny from a Hybrid 1 cross [*X. helleri* (×) (*X. maculatus* Jp 163 B (×) *X. helleri*)] (Figure 3-11). Each backcross hybrid individual was scored as either heterozygous or homozygous at the x*CYP40* locus based on the Taq 1 restriction polymorphism and entered into a database. Linkage of two loci was determined using MapMaker/QTL 1.1 Software. This software allows one to assign gene map locations to traits in backcrosses relative to a genetic linkage database using 2 x 2 chi square analyses (Paterson *et al.*, 1988). Pair-wise linkage analysis was performed on the data scored for x*CYP40* and approximately 350 markers in the backcross panels. To determine if two loci are linked

rather than unlinked, a logarithm of the odds (LOD) score is calculated. A LOD score of 3.0 or greater indicates the likelihood that two loci being compared are not independently assorting (unlinked) (Table 3-2).

Linkage analyses clearly show the x*CYP40* locus co-segregated with four loci on linkage group U16: XD0097, XD0262, XD0112, and XD0078 (Table 3-2). The order of loci for *X. maculatus* linkage group U16 was compiled using MapMaker/Exp 3.0 software. This software analyzes and calculates the maximum likelihood of loci order within linkage groups according to recombination estimates (Lander *et al.*, 1987). It performs multipoint linkage analysis for dominant, recessive and co-dominant markers while performing a simultaneous estimation of all recombination fractions from primary data. From this analysis it was indicated that x*CYP40* mapped to the distal end of linkage group U16 (Figure 3-13).

**Figure 3-9 PCR Products for pWR3-5F/3R Oligonucleotide Primers**. PCR sizes are indicated for *X. helleri* and *X. maculatus* Jp 163 A. (1% Agarose gel)

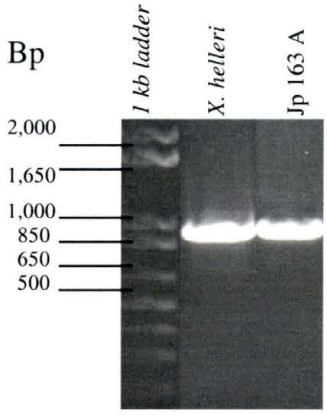



**Figure 3-10 PCR Products Digested with Taq I .** Polymorphism sizes are indicated for *X. helleri* and *X. maculatus* Jp 163 A. Pedigree numbers indicate individual Hybrid 1 $BC_1$ DNA's subjected to Taq I digestion. Yellow arrows denotes *X. helleri* products at ~500 bp and 275 bp, while red arrow denotes *X. maculatus* Jp 163 A product at ~800 bp. (1% Agarose gel)

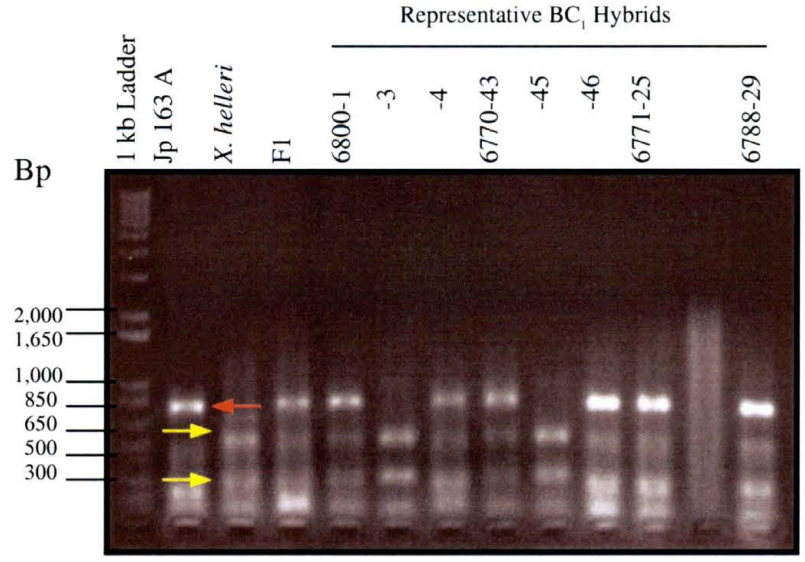


**Figure 3-11 Hybrid 1 Backcross.** *Xiphophorus* fishes used in the Hybrid 1 genetic cross. *X. maculatus* homozygote for spotted sided (Sp) pigment pattern locus is mated to an *X. helleri*. The heterozygous $F_1$ (Sp / +) is backcrossed to the *X. helleri* parent giving backcross hybrid progeny.

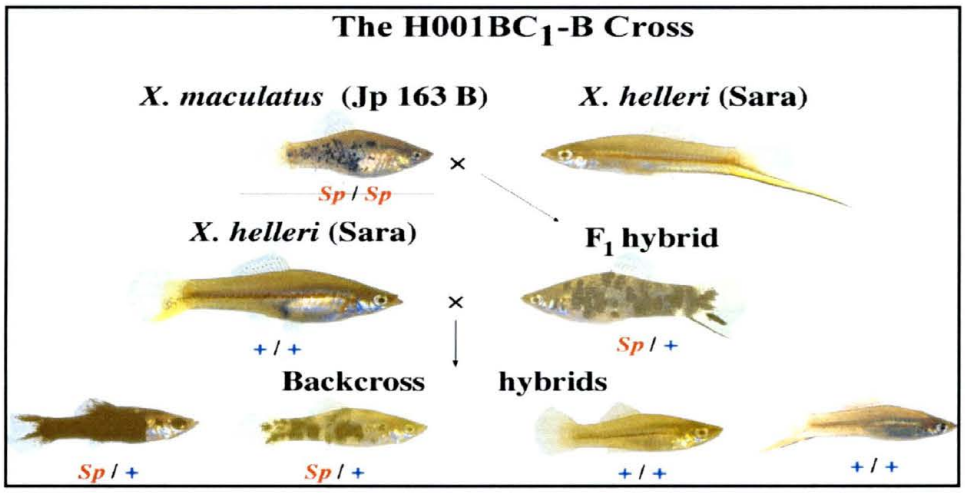



**Figure 3-12 Hybrid 5 backcross.** *Xiphophorus* fishes used in the Hybrid 5 genetic cross. *X. maculatus* homozygote for spotted dorsal (Sd) pigment pattern locus is mated to an *X. helleri*. The heterozygous $F_1$ (Sd / +) is backcrossed to the *X. helleri* parent giving backcross hybrid progeny.

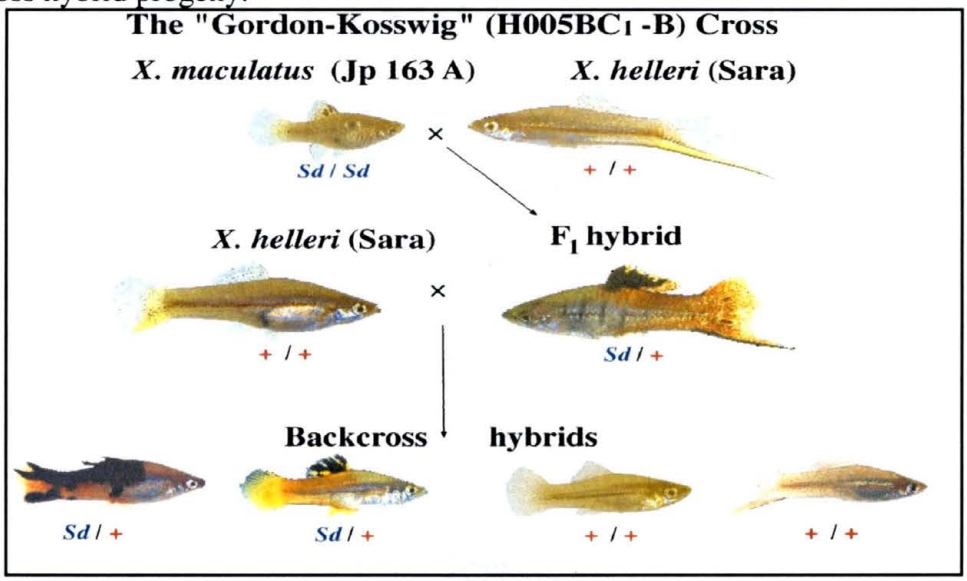
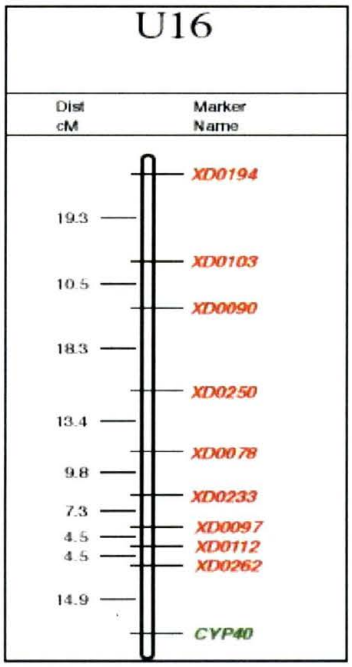


**Table 3-2 Linkage Analysis.** Results of joint linkage analysis of *X. maculatus CYP40* data compared to other polymorphic loci scored in Hybrid 5. Linkage group designations denote independently assorting segments of the *Xiphophorus* linkage map. LOD scores were calculated based on deviations from an expectation of 1 parental: 1 recombinant ratio in backcross hybrids resulting from independent assortment at meiosis. Loci shown in red have an LOD score greater than 3.0 and are on linkage group U16. XD denotes *Xiphophorus* anonymous DNA sequence.

| LOCUS | NAME | LINKAGE GROUP | RECOMBINANTS | TOTAL | % RECOMBINATION | LOD |
|---|---|---|---|---|---|---|
| ***GLNS*** | Glutamine synthetase | VI | 8 | 21 | 38.10 | 0.3 |
| ***ATP*** | Adenosine triphosphatase | III | 33 | 68 | 48.53 | 0 |
| ***GPI1*** | Glucosephosphate isomerase | IV | 23 | 50 | 46.00 | 0.1 |
| ***IDH2*** | Isocitrate dehydrogenase | VII | 46 | 94 | 48.94 | 0 |
| ***MPI*** | Mannose phosphate isomerase | II | 44 | 96 | 45.83 | 0.1 |
| ***PGAM1*** | Phosphoglycerate mutase | XI | 40 | 95 | 42.11 | 0.5 |
| ***XD0115*** | HOXL1 | XIII | 41 | 86 | 47.67 | 0 |
| ***XD0082*** | CP1.3 | XIV | 18 | 40 | 45.00 | 0.1 |
| ***XD0180*** | R1.11 | U19 | 20 | 45 | 44.44 | 0.1 |
| ***XD0182*** | R1.13 | X | 20 | 43 | 46.51 | 0 |
| ***XD0186*** | r22.3 | IX | 15 | 37 | 40.54 | 0.3 |
| ***XD0196*** | RP.283 | XII | 5 | 18 | 27.78 | 0.8 |
| ***XD0097*** | CP2.6 | U16 | 11 | 64 | 17.19 | 6.5 |
| ***XD0262*** | t39.2 | U16 | 15 | 87 | 17.24 | 8.8 |
| ***XD0112*** | g103 | U16 | 13 | 67 | 21.28 | 5.9 |
| ***XD0078*** | CP1.1 | U16 | 18 | 72 | 25 | 4.1 |
| ***XD0233*** | RTR1.8a | U16 | 16 | 59 | 27.12 | 2.97 |
| ***XD0225*** | RTR1.1 | U18 | 2 | 6 | 33.33 | .1 |
| ***XD0233*** | RTR1.8a | U16 | 16 | 59 | 37.00 | .3 |

**Figure 3-13 *X. maculatus* Linkage Group U16**
Graphic depiction of LG U16 is shown in which MapMaker/Exp 3.0 analysis determined a suggested loci order. The *X. maculatus* cyclophilin 40 (*CYP40*) gene mapped to the distal end of linkage group U16. The markers in red indicate anonymous markers. An estimated recombination distance in centimorgans (cM) is presented on the left. XD denotes *Xiphophorus* anonymous DNA sequence.




**Relative Quantitative RT-PCR**

Cyclophilin 40 synthetic oligonucleotide primers pWR3-4F and pWR3-1R (Tables 2-2 and 2-3) were designed from the deduced nucleotide sequence spanning part of exon 3 thru exon 5 (Figure 2-1) and used to amplify a 329 bp fragment of *Xiphophorus* cDNA (Figure 3-14). The relative x*CYP40* mRNA levels (in relation to endogenous 18S rRNA levels) were determined in a multiplex PCR reaction from several *Xiphoporus*

tissues. To develop the relative Q-RT-PCR assay, the linear range of amplification and the optimal 18S rRNA primer to competimer ratios were first determined using cDNA from pooled liver tissue of 14 male *X. maculatus* Jp 163 A fish. Total RNA, primed with random decamers, was reverse transcribed to produce cDNA. Using this cDNA, the linear range of amplification for the PCR product using pWR3-4F/1R primers was identified between cycles 19-25 (Figure 3-15). Using the same liver cDNA and a variable range of 18S primer to competimers ratios, the optimal 18S rRNA primer to competimer ratio was identified as 3:27 (18S primer:competimer) (Figure 3-16).

Four experiments using two different reverse transcription reactions from a total RNA stock determined the relative x*CYP40* mRNA levels in seven tissues of *X. maculatus* Jp 163 A (brain, eyes, gills, liver, testes, skin, and muscle tissues; Figure 3-18). In addition, relative x*CYP40* RNA levels for melanized skin, spotted skin and tumor tissue (Figure 3-18) were determined from four Hybrid 7 backcross fish [(*X. couchianus* (×) (*X. maculatus* Jp 163 B (×) *X. couchianus*; (Figure 3-17)]. Amplimers corresponding to mRNA transcripts were readily detected in all tissues tested (Figure 3-19). The relative x*CYP40* mRNA level for liver tissue was assigned a value of 1.0, and the other tissues were scored relative to the liver value (Table 3-3). Testes tissues consistently displayed the highest (23 fold higher) x*CYP40* mRNA levels, while muscle (0.87 fold) and skin (0.39 fold) tissues displayed the lowest x*CYP40* mRNA levels (Figure 3-20). Brain (1.96 fold) and eyes (2.34 fold) displayed relatively high mRNA levels (Figure 3-21). Gills showed a similar (0.95 fold) x*CYP40* mRNA level as the liver tissue in *X. maculatus* Jp

163 A. The relative x*CYP40* mRNA level for the unpigmented skin was assigned a value of 1.00, and the pigmented skins were scored relative to the unpigmented skin. Hybrid 7 heavily pigmented skin possessed 8.46 fold higher, partially pigmented skin possessed 3.95 fold higher, and tumor (from pigmented skin) possessed 7.64 fold higher x*CYP40* levels than unpigmented skin RNA isolated from *X. maculatus* JP 163 A (Figure 3-22).

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**Figure 3-14 PCR product size for Relative Expression Experiment.** PCR amplified products are shown from *X. maculatus* Jp 163 A cDNA using pWR3-4F/1R primers at different magnesium concentrations and annealing temperatures. PCR conditions are optimal at 56° C and final magnesium concentration at 1.0 μM. Red arrow displays 329 bp products. (2% Agarose gel)

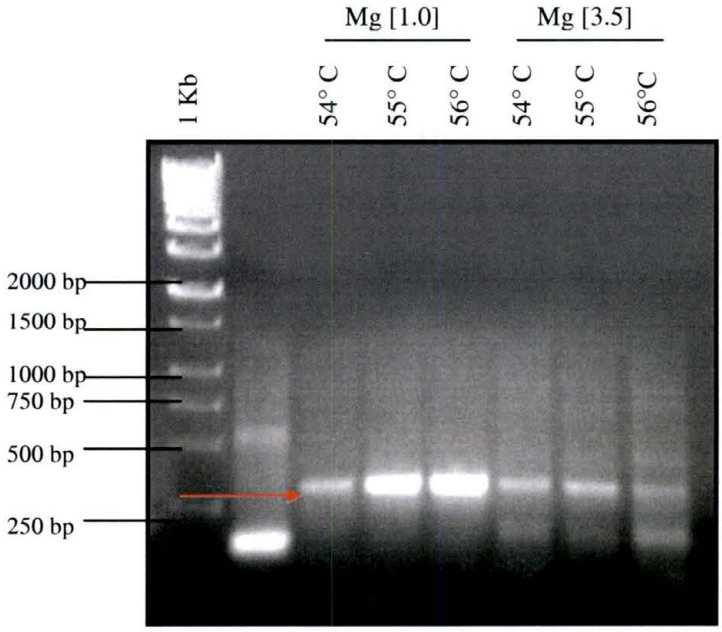
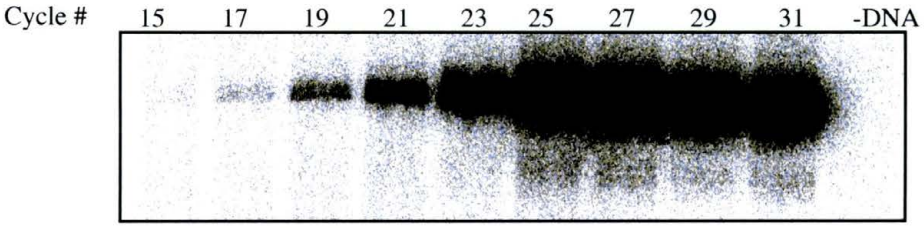


**Figure 3-15 Linear Range of Amplification of pWR3-4F/1R Primers.** (Panel A) Removing one aliquot every other PCR cycle beginning with cycle 15 determined linear range of amplification. To measure the products, radiolabeled dCTP was added to the PCR reaction and the amplified product was fractionated on a denaturing gel (6% Acrylamide) then quantified using a Packard Cyclone Storage Phosphor System Version 3.0, (Meriden, CT). This system measures the radioactivity of the sample and measures the distribution of the activity in selected areas (i.e. amplified PCR products) as Digital Light Units (DLU). PCR cycle numbers are indicated at the top. (Panel B). Graphical representation of the linear range of amplification is shown for increasing PCR cycles versus the radioactive product intensity. The linear range of amplification is identified between PCR cycles 19-25.

**A.**




**B.**

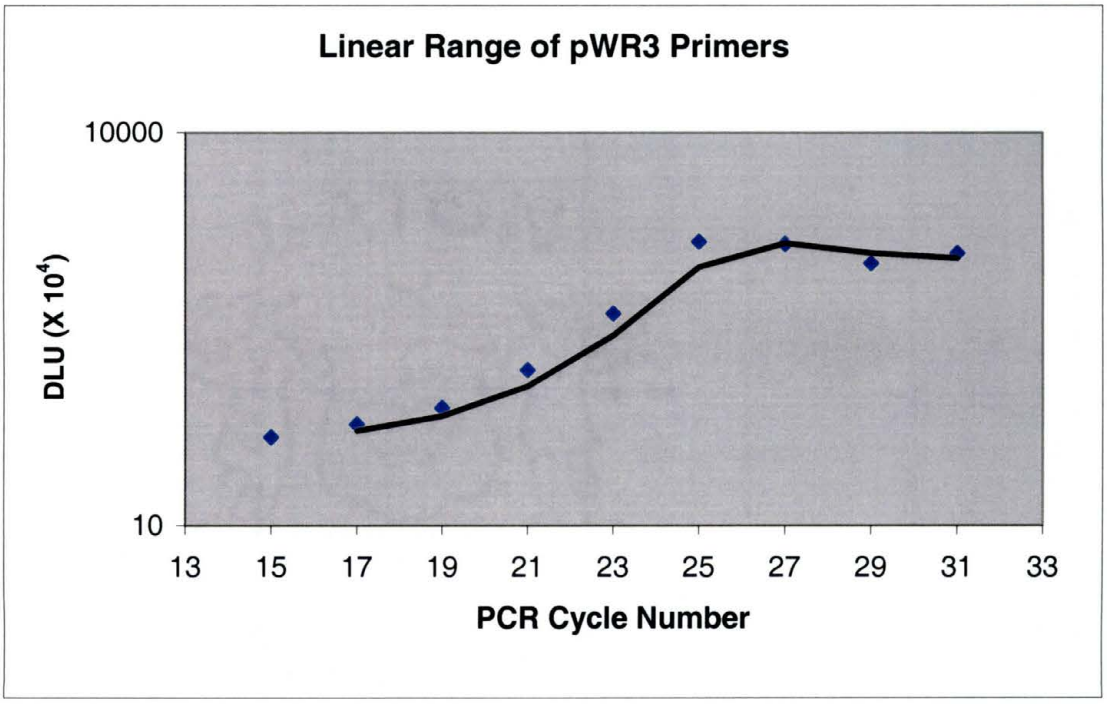


**Figure 3-16 18S rRNA Primer to Competimer Optimization.** A variable range of 18S Primer:Competimer ratios were used to determine the optimal ratio. Bands indicate multiplex reactions of varying 18S primer to competimer ratios. Red arrows indicate the optimal 18S rRNA primer to competimer ratio was identified as 3:27 (primer:competimer) showing bands of equal relative intensity (expressed in DLUs) between the 18S rRNA and gene product. (6% Acrylamide gel)

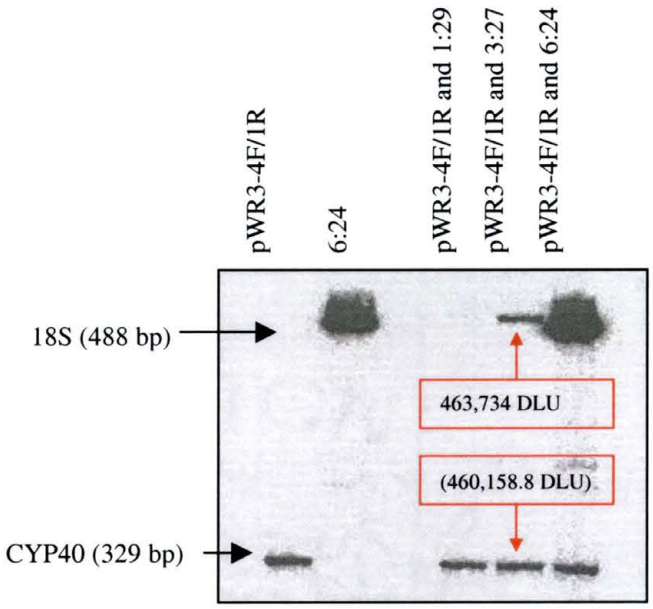
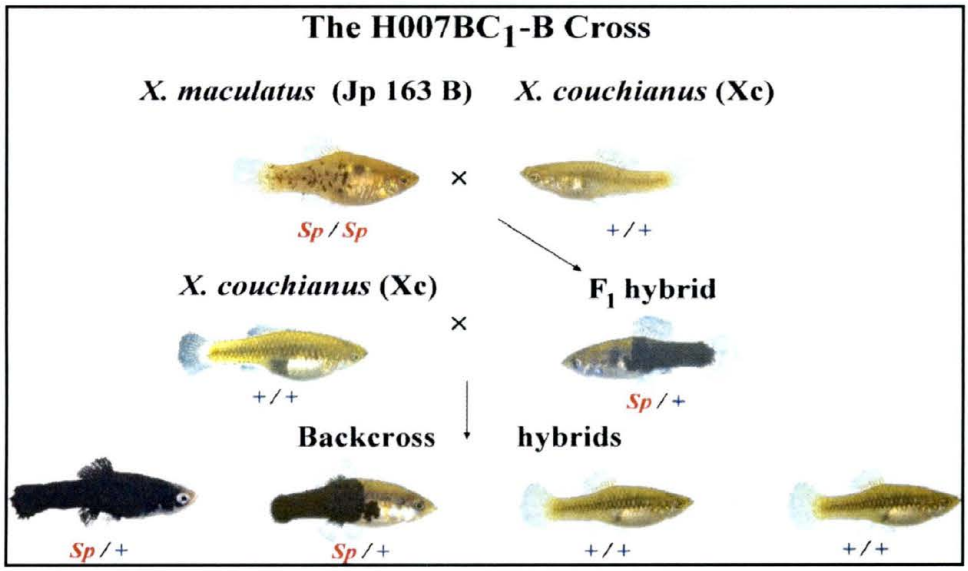
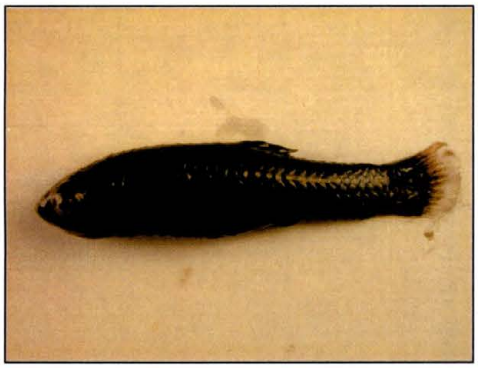


**Figure 3-17 Hybrid 7 Backcross**. (Panel A) *Xiphophorus* fishes used in the Hybrid 7 genetic cross. *X. maculatus* homozygote for spotted side (Sp) pigment pattern is mated to an *X. couchianus*. The heterozygous $F_1$ (Sp/+) is backcrossed to the *X. couchianus* parent giving backcross hybrid progeny. (Panel B) backcross hybrid progeny that are melanized (heavily pigmented) and (Panel C) partially pigmented (spotted skin).

**A.**




**B.**



**C.**

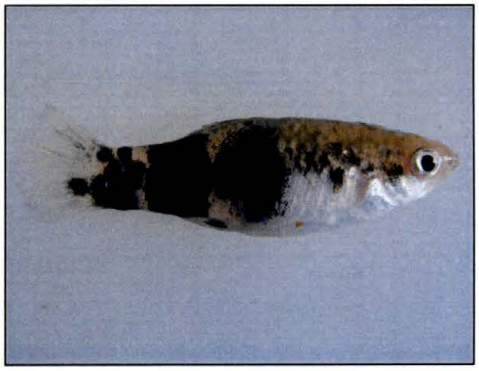
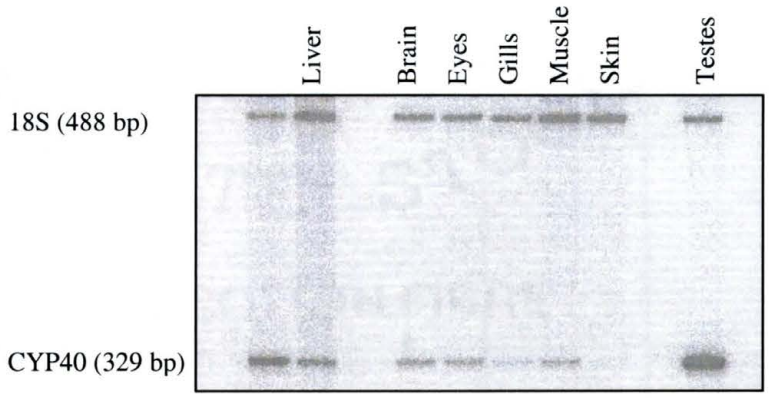


**Figure 3-18 Relative Q-RT-PCR Experiment.** (Panel A) Bands indicate *X. maculatus* Jp 163 A *CYP40* expression relative to 18S rRNA expression. Band intensities for each tissue were quantified in DLUs using Packard Cyclone Storage Phosphor System Version 3.0, (Meriden, CT). (Panel B) Bands indicate Hybrid 7 *CYP40* expression relative to 18S rRNA expression.

A.




B.

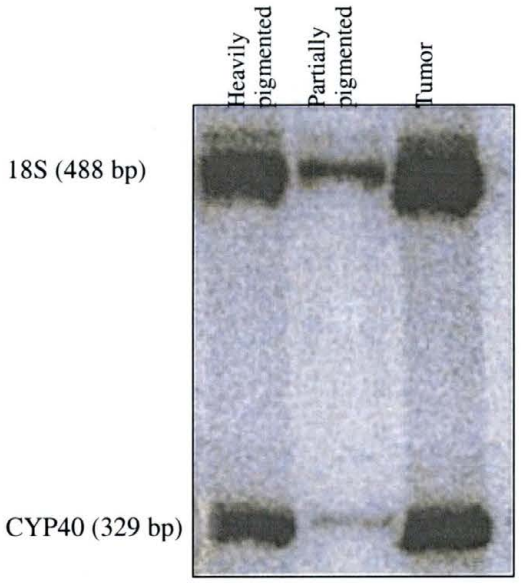


**Table 3-3 Relative and Scored Values for Relative Q-RT-PCR Experiments**
Relative values of *CYP40* levels (ratio of *CYP40* (DLU) versus 18S rRNA (DLU)) for *X. maculatus* Jp 163 A and Hybrid 7 (in red) tissues are shown for all Relative Q-RT-PCR experiments. Tissues are shown on the left column and relative values for experiments 1-4 are shown at the top. The Relative *CYP40* mRNA level for liver tissue was assigned a value of 1.0, and all other tissues were scored relative to the liver value (shaded column).

| ***Relative Values*** | ***Exp 1*** | ***Exp 2*** | ***Exp 3*** | ***Exp 4*** | ***Mean*** | ***Standard Deviation*** | ***Scaled Value*** |
|---|---|---|---|---|---|---|---|
| ***Tissues*** | | | | | | | |
| ***Brain*** | .476 | .188 | .360 | .292 | .319 | .145 | 1.96 |
| ***Eyes*** | .414 | .236 | 2.61 | .494 | .381 | .132 | 2.34 |
| ***Gills*** | .268 | .064 | .901 | .130 | .154 | .104 | 0.95 |
| ***Liver*** | .219 | .130 | .471 | .137 | .162 | .049 | 1.00 |
| ***Muscle*** | .154 | .142 | .456 | .128 | .141 | .012 | 0.87 |
| ***Skin*** | .018 | .023 | .706 | .146 | .062 | .072 | 0.39 |
| ***Testes*** | 5.60 | 4.05 | 7.89 | 1.96 | 3.87 | 1.82 | 23.8 |
| ***Melanized Skin*** | | | | .525 | | | 3.23 |
| ***Spotted Skin*** | | | | .245 | | | 1.51 |
| ***Tumor*** | | | | .474 | | | 2.91 |

**Figure 3-19 Graph of *X. maculatus* JP 163 A Relative Q-RT-PCR Experiments.** x*CYP40* levels are shown versus the ratio of *CYP40* (DLU) to 18s rRNA (DLU) for Relative Q-RT-PCR Experiments 1-4.

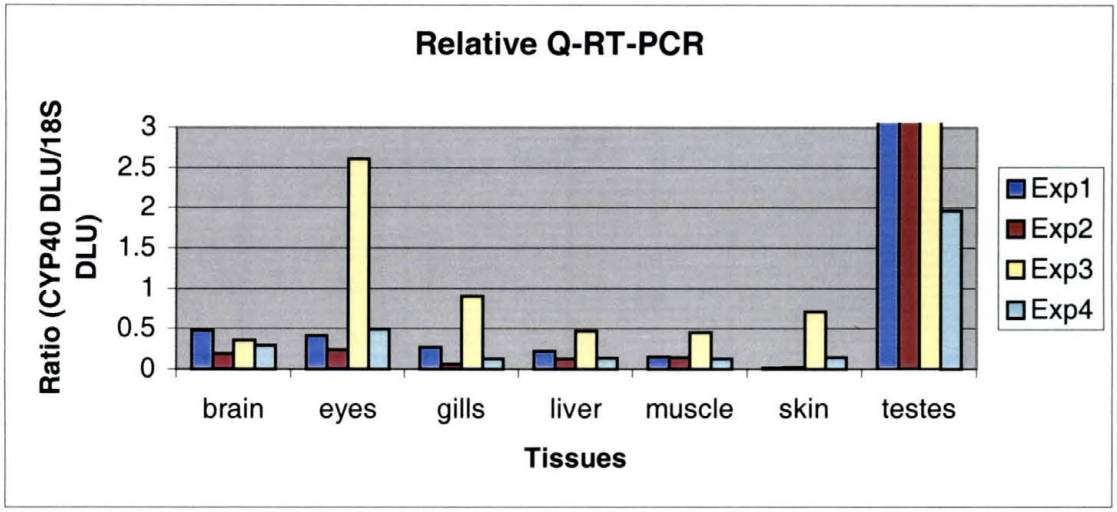



**Figure 3-20 Relative Q-RT-PCR Standard Error of the Mean (SEM).** Graphical representation of *CYP40* levels for *X. maculatus* Jp 163 A tissues are shown versus the ratio of *CYP40* (DLU) to 18S rRNA (DLU) for Relative Q-RT-PCR Experiments 1, 2, and 4. Error bars indicate standard error of the mean (SEM).

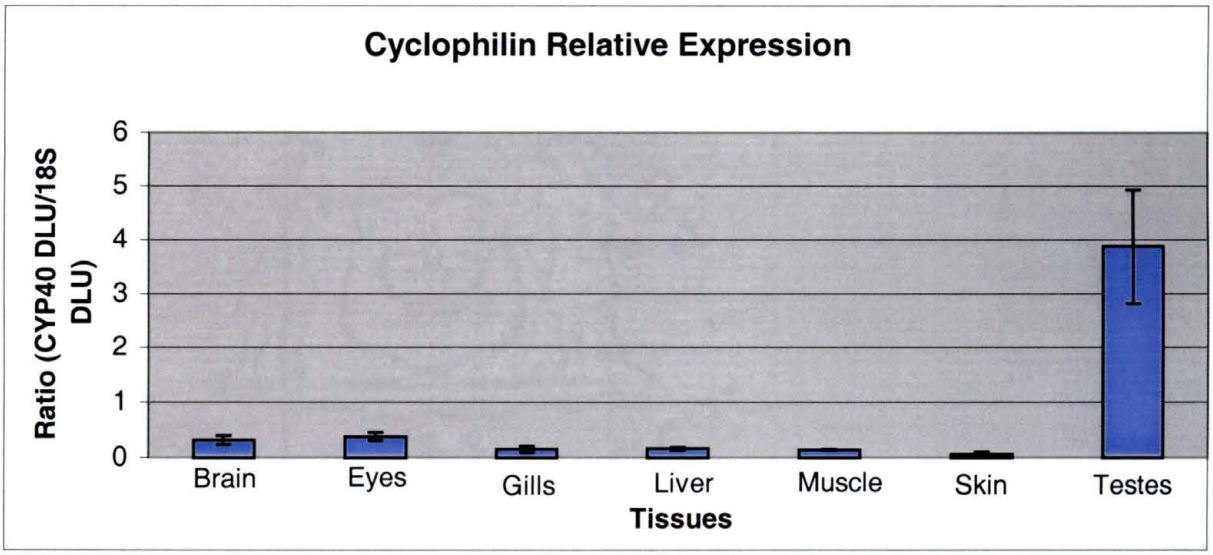


**Figure 3-21 Relative *CYP40* Expression minus testes tissue**
Relative *CYP40* levels of *X. maculatus* Jp 163 A tissues are shown without the testes tissue to determine statistically significant higher expression in other tissues (data is from Relative Q-RT-PCR Experiments 1, 2, and 4). Error bars indicate standard error of the mean.

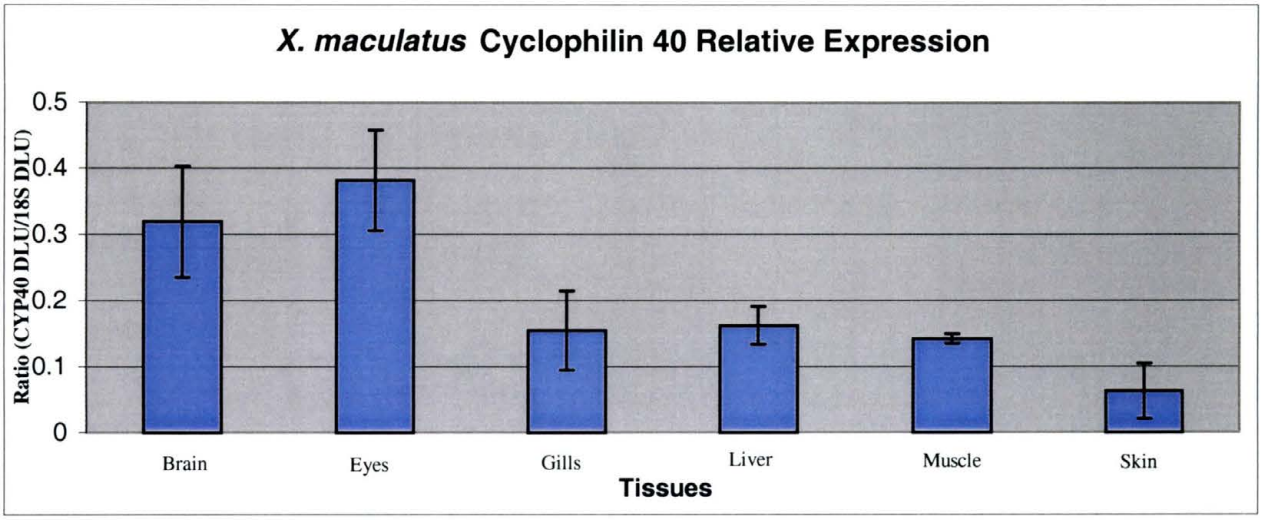
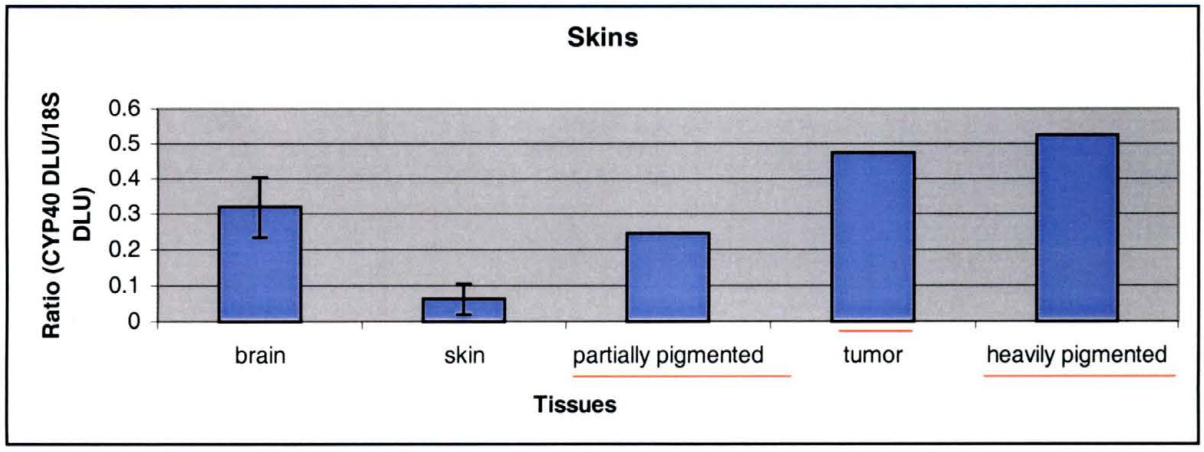



**Figure 3-22 Comparison of Relative *CYP40* levels between *X. maculatus* and Hybrid 7 tissues.** Graphical representation of *CYP40* levels for *X. maculatus* Jp 163 A skin (non-melanized) and brain and Hybrid 7 tissues (underlined in red- heavily pigmented, partially melanized skin, and tumor) are shown versus the ratio of *CYP40* (DLU) to 18S rRNA (DLU) for Relative Q-RT-PCR Experiments 1, 2, and 4. Error bars indicate standard error of the mean (SEM).



# DISCUSSION

## *X. maculatus CYP40* Organization

The *X. maculatus* plasmid subclone, pWR3, was found to harbor a cDNA that is likely to encode a Cyclophilin 40 (x*CYP40*) gene homologue. The nucleotide sequence exhibits a coding region consisting of 1113 bp, which translates to 371 amino acids (Figure 3-5). Putative exon boundaries were determined by an amino acid alignment with human Cyclophilin 40 cDNA (Kieffer *et al.*, 1993). A putative polyadenlyation signal (1456-1460 bp) was identified (Figure 3-5) within 320 bp of sequence distal to the stop codon.

## Comparison of the *X. maculatus CYP40* and other *CYP40* sequences

The x*CYP40* cDNA sequence was compared with cyclophilin 40 genes from other species. Computer searches of several current databases such as GenBank and Zebrafish database (ZFin; Zebrafish International Resource Center) did not reveal sequence analysis of any other *CYP40* gene from a fish species. When the x*CYP40* nucleotide sequence is compared to human, bovine, and yeast, 66%, 51%, and 51% identity is observed, respectively (Table 3-1). The deduced xcyp40 amino acid sequence, compared to human, bovine, and yeast cyp40s, exhibits 65%, 66%, 43% identity and 78%, 78%, 59% amino acid similarity, respectively (Table 3-1). The highest regional similarity is observed within the first five exons (positions 64-708) between xcyp40 and hcyp40,

where amino acid similarity, identity, and nucleotide identity increase to 92%, 86%, and 74%, respectively (Figure 3-6).

Comparison of xcyp40 with plant cyclophilin revealed regional conservation. First, the N-terminal region of xcyp40 is similar (63% identity) to plant cyclophilins (maize) due partly as a result of an 8-amino acid insert, 5 amino acids of which, GKPLH, are conserved in plant cyclophilins (Kieffer *et al.*, 1993). Amino acid alignments within this region (residues 63-67) indicate xcyp40 coincide with this 5 amino acid conservation (Figure 3-5). Second, xcyp40 has a critical amino acid substitution also observed in hcyp40, consisting of a tryptophan residue (position 120). In hCypA this tryptophan residue is postulated to be a crucial element for binding of Cyclosporin A with small cyclophilins (i.e. CypA) and is highly conserved in most cyclophilins (Galat, 1999). However, in hcyp40 the Try (W) residue is replaced by Histidine (H) (Kieffer *et al.*, 1993). Our results are consistent with this finding as amino acid alignments indicate a His residue in the corresponding xcyp40 position (position 142) (Figure 3-5).

a) Putative Calmodulin Binding Site

The xcyp40 C-terminus harbors a putative calmodulin binding site (Figure 3-7) as indicated by amino acid alignments with proposed calmodulin binding sites for human p59 and cyp40 proteins (Lebeau *et al.*, 1992). Amino acid similarity between the putative xcyp40 calmodulin binding site and the calmodulin binding sites of human p59 and hcyp40 are 76% and 94%, respectively. This suggests calmodulin and intracellular

calcium levels may affect xcyp40 through its C-terminal FKBP52 function (Peattie *et al.*, 1992).

b) Tetratricopeptide Repeat (TPR) Motif

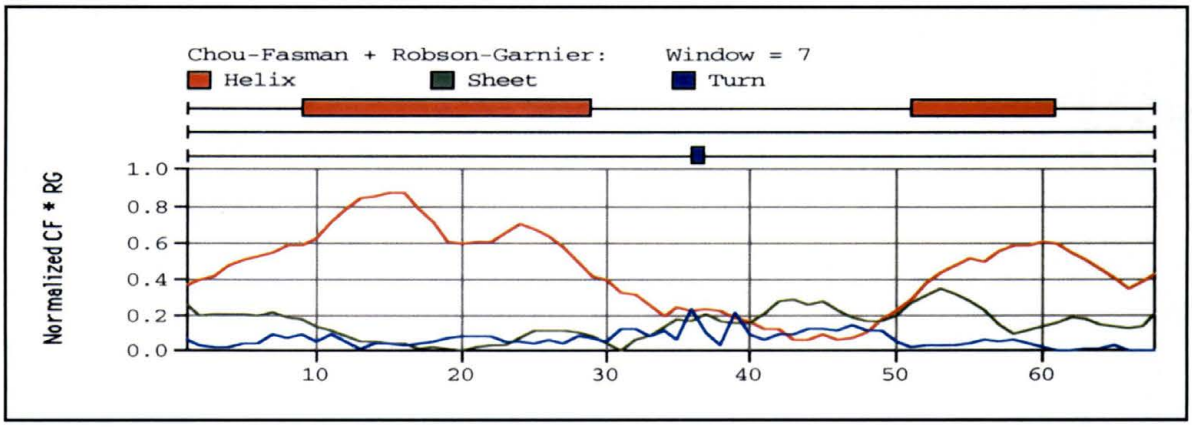
The C-terminal region of bcyp40 contains a 3-unit tetratricopeptide repeat (TPR) motif (Ratajczak *et al.*, 1993). Comparison of xcyp40 C-terminal region to that of known TPR motifs in p59 (Sikorski *et al.*, 1990), MAS 70 (Goebl and Yanagida, 1991), and hcyp40 (Ratajczak *et al.*, (1993) revealed 33% identity and 54% similarity to the aligned TPR regions (Figure 3-8). There is evidence that TPR motifs are important to cell cycle progression, transcription, and protein transport (Schouflor *et al.*, 1999). The TPR motif is arranged in tandem arrays in various proteins. There is a conserved pattern of amino acid similarity in terms of size, hydrophobicity, and spacing in the TPR motif (Blatch and Lassle, 1999). For instance, studies by Blatch and Lassle, (1999) show eight amino acids with a comparatively higher frequency of conservation between murine stress inducible protein (mST11), human protein phosphatase 5 (PP5), bovine Cyclophilin 40 (b*CYP40*), and rabbit FK506 binding protein (FKBP52). Figure 3-8 demonstrates an alignment of three TPR segments in p59, MAS 70, hcyp40, and xcyp40 where a consensus sequence is shown within the aligned TPR motifs. An amino acid comparison within these three segments with xcyp40 demonstrates 44%, 71%, and 50% similarity, respectively (Figure 3-8).

Sikorski *et al.*, (1990) predicted secondary structure for the *S. cerevisae* CDC23 TPR motif as a long (25-30 residue) $\alpha$ helix, punctuated by a 4-residue turn at the

carboxyl terminus and a central turn that divides the repeat into two subdomains (A and B) of shorter α helices. A secondary structure prediction for xcyp40 based on the Chou-Fasman (1978) and Robson-Garnier (1978) methods indicate the TPR units to be predominately α-helical (Figure 4-1). This is consistent with the joint consensus α-helix predicted for the TPR motif (Sikorski *et al.*, 1990). In addition, the C-terminal end of the predicted structure shows a central turn dividing the α-helixes, which is consistent with the 'snap' and 'hole' secondary structure for the yeast *nuc2*$^{+}$ protein of the C-terminal TPR motif (Hirano *et al.*, 1990). Hydrophobicity indexes for xcyp40 and hcyp40 is shown in Figure 4-2. Comparison between these two indexes does not show similarity.

**Figure 4-1** ***X. maculatus*** **cyp40 Tetratricopeptide Repeat (TPR) Motif Predicted Secondary Structure.** (Panel A) Predicted secondary structure is shown for the *X. maculatus* cyp40 TPR motif (277-344) (Figure 3-4) and human cyp40 (Panel B). Predicted α-helices and β-sheets were calculated using the methods of Chou and Fasman (1978) and Robson-Garnier (1978).

**A.**




**B.**

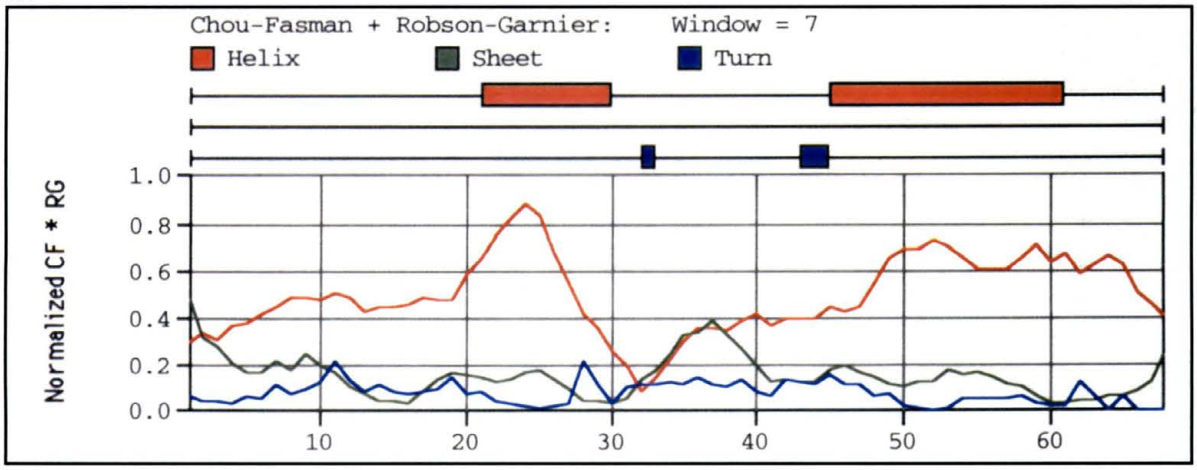
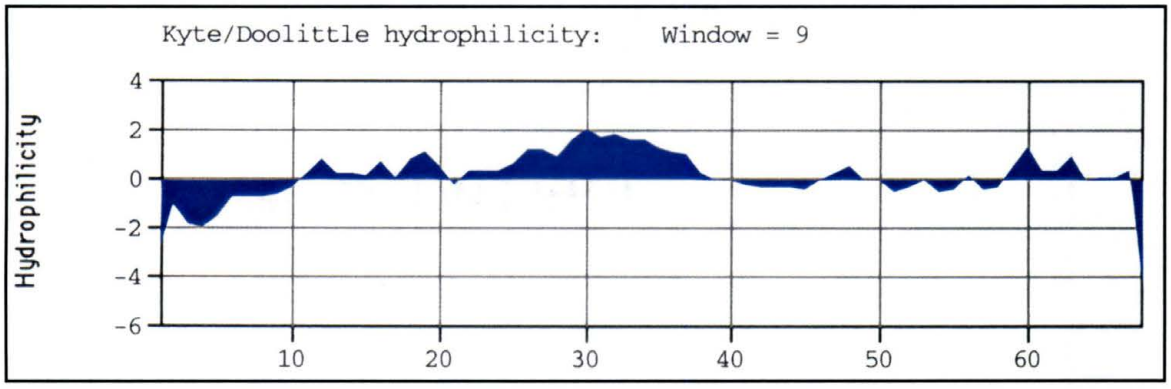
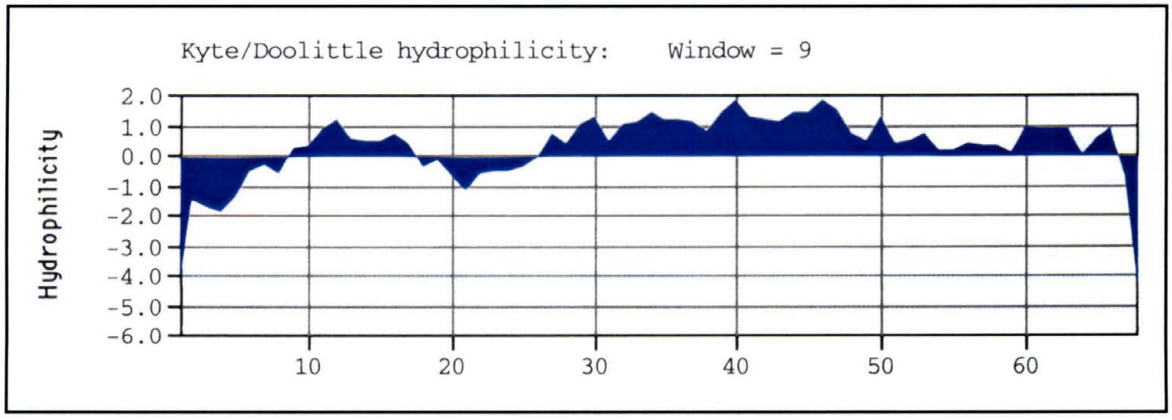


**Figure 4-2 *X. maculatus* cyp40 Tetratricopeptide Repeat (TPR) Motif Predicted Hydrophobicity.** (Panel A) Predicted hydrophobicity is shown for the *X. maculatus* cyp40 TPR motif (332-400) (Figure 3-4) and human cyp40 (Panel B).

**A.**




**B.**

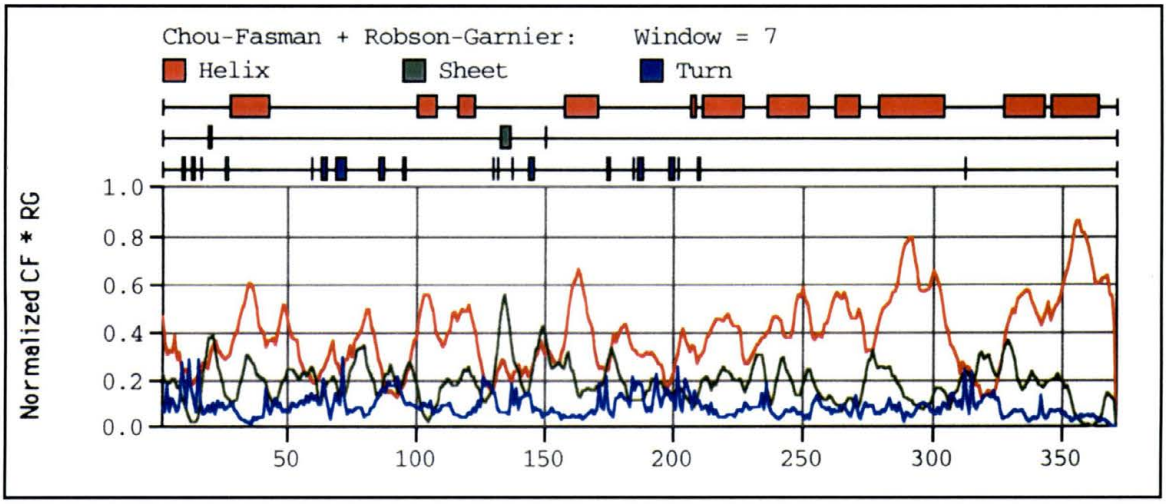


**Putative Cyclophilin 40 Secondary Structure**

The secondary structure of xcyp40 was predicted by the methods of Chou and Fasman (1978) and Robson-Garnier (1978). Figure 4-3 displays a comparison of the predicted secondary structures of the *X. maculatus* and human Cyclophilin 40. The predicted secondary structure indicates xcyp40 to be predominately α-helical, which is consistent with structure analysis of hcyp40 (Galat, 1999). Table 4-1 displays the predicted molecular mass, derived pI, and amino acid content, for xcyp40. Comparison with hcyp40 shows similarity between the two proteins. The predicted pI value for xcyp40 (5.9) is slightly acidic and is consistent with human (pI 6.8) and bcyp40 (pI 5.5) (Kieffer *et al.*, 1992).

**Figure 4-3 Comparison of Predicted Secondary Structure for *X. maculatus* cyp40 and Human cyp40 Secondary Structure.** Predicted α-helices and β-sheets calculated using the methods of Chou and Fasman (1978) and Robson-Garnier (1978). Panel A represents predicted xcyp40 secondary structure, while panel B represents hcyp40 secondary structure.

**A.**




**B.**

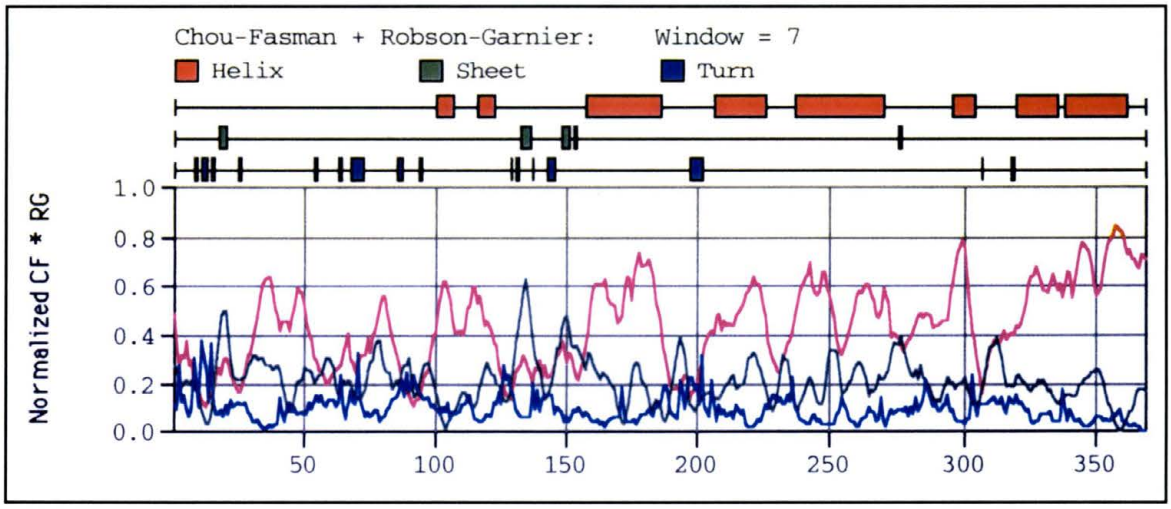


**Table 4-1 Putative *CYP40* Secondary Structure Comparison**

Predicted molecular mass, derived pI values, and amino acid content for *X. maculatus* cyp40 (xcyp40) is shown compared to human cyp40 (hcyp40). The individual amino acids are grouped as non-polar, polar, acidic, or basic amino acids. The frequency and percentage usage of each amino acid is also presented.

| | xcyp40 | | hcyp40 | |
|---|---|---|---|---|
| **Molecular Weight** | 41144.19 | | 40759.89 | |
| **Estimated pI** | 5.90 | | 6.88 | |
| **Non-polar:** | *No.* | *Percent* | *No.* | *Percent* |
| A | 24 | 6.45 | 31 | 8.38 |
| V | 24 | 6.45 | 20 | 5.41 |
| L | 27 | 7.26 | 28 | 7.57 |
| I | 20 | 5.38 | 25 | 7.03 |
| P | 19 | 5.11 | 17 | 4.59 |
| M | 8 | 2.15 | 6 | 1.62 |
| F | 19 | 5.11 | 18 | 4.86 |
| W | 4 | 1.08 | 3 | 0.81 |
| **Polar:** | 4 | | | |
| G | 32 | 8.60 | 34 | 9.19 |
| S | 14 | 3.76 | 17 | 4.59 |
| T | 21 | 5.65 | 13 | 3.51 |
| C | 7 | 1.88 | 7 | 1.89 |
| Y | 5 | 1.34 | 8 | 1.89 |
| N | 22 | 5.65 | 16 | 4.32 |
| Q | 10 | 2.69 | 15 | 4.05 |
| **Acidic:** | | | | |
| D | 26 | 6.45 | 26 | 7.03 |
| E | 32 | 8.60 | 26 | 7.03 |
| **Basic:** | | | | |
| K | 41 | 11.02 | 39 | 10.54 |
| R | 8 | 2.15 | 12 | 3.24 |
| H | 11 | 2.96 | 9 | 2.43 |

## Mapping of the *X. maculatus* Cyclophilin 40 Gene

a) RFLP Results

Initial work performed at UT MD Anderson Cancer Center, Science Park, Smithville, TX indicated the cloned cDNA in plasmid, pWR3, when radiolabeled and used as a probe, produced two distinct RFLP polymorphisms between *X. maculatus* Jp 163 A and *X. helleri*. These two polymorphisms were scored in genomic DNA of backcross Hybrid 1 individuals digested with the restriction enzyme Pst I (Figure 3-1) and the RFLPs applied to co-segregation analysis. Linkage analysis results indicated these two RFLPs (designated 3A1 and 3A2) mapped to *X. maculatus* Linkage Group (LG) U16, with 21.4% recombination between the two loci.

b) PCR Polymorphism

To confirm these mapping relationships, we generated a PCR polymorphism based on the x*CYP40* nucleotide sequence. Genomic DNA from parental, F1 hybrid, and over 100 backcross Hybrid 1 or Hybrid 5 progeny were amplified, digested with Taq 1, and visualized. Both Hybrid 1 and 5 backcrosses displayed an easily scorable polymorphism between two sets of parents (1) *X. maculatus* Jp 163 A and *X. helleri* (2) *X. maculatus* Jp 163 B and *X. helleri*) (Figure 3-11). Polymorphic data from each cross was subjected to pair-wise linkage analysis to determine the odds that two loci are independently assorting. Linkage results (Table 3-2) showed x*CYP40* is a single locus at the distal end of *Xiphophorus* linkage group U16 (Figure 3-14).

c) *X. maculatus CYP40* Loci Determination

Linkage data from the PCR polymorphism revealed the 3A2 locus corresponded to *CYP40*, while the 3A1 locus did not exist. Upon mapping x*CYP40* as a single locus on LG U16, we performed careful analyses of the original autoradiographs used to score the 3A1 and 3A2 RFLPs. The following conclusions were made that explains the discrepancies in RFLPs vs PCR mapping. First, we concluded the probe (pWR3) hybridized across one locus and two products were separated based on size since the probe contained two Pst I sites at positions 789 and 926. Second, the 3A1 locus was identical to the 3A2 locus. Re-scoring all five panels containing the 3A1 and 3A2 RFLP polymorphs from the original work showed the *X. helleri* allele was absent in pedigrees 6751, 6742, 6771, 6788, 6750, 6770, and 6800 (Hybrid 1 backcross) and was mistakenly scored as homozygote for the *X. maculatus* allele (Figure 3-1). The recombination percentage between 3A1 and 3A2 from the original mapping analysis was 21.4% (Morizot *et al.*, 1998). Linkage analysis after corrections were made to the 3A1 dataset now showed 0% recombination between the two loci. Third, the *X. helleri* parent used in the Hybrid 1 backcross was not monomorphic and thus it was impossible to distinguish which parental allele was inherited. Therefore, pedigrees originally designated as heterozygotes were eliminated and the linkage analysis recalculated. Fourth, the 3A2 locus corresponded to the x*CYP40* locus. Linkage analyses from the PCR polymorphism data of the same genetic backcross (Hybrid 1 backcross) revealed 0% recombination between 3A2 and x*CYP40* loci.

In summary, the 3A1 locus was eliminated and the original 3A2 locus was considered the same as *xCYP40.*

**Relative *X. maculatus* Cyclophilin 40 Expression**

Analyses of the x*CYP40* relative mRNA levels revealed ubiquitous expression in all the tissues tested (Figure 3-21). These ubiquitous mRNA levels of x*CYP40* are consistent with results obtained in human (Kieffer *et al.*, 1993), rat (Kieffer *et al.*, 1992), and bovine tissues (Ratajczak *et al.*, 1993).

Figure 3-22 shows a significantly higher (23.8 fold) x*CYP40* mRNA level for testes compared to the other tissues tested (Table 3-2). In addition, relatively high x*CYP40* mRNA levels were observed in brain (1.96 fold higher) and eyes (2.34 fold higher) (Figure 3-23). Our results are consistent with results from northern analyses by Kieffer *et al.*, (1993), where human brain tissue revealed higher expression compared to other tissues tested, however these results were not quantified. The lowest x*CYP40* mRNA levels observed were in muscle (0.87 fold) and skin (0.39 fold) (Figure 3-23).

Comparison of the non-melanized skin and melanized skin revealed a significant difference. When compared to non-pigmented *X. maculatus* Jp 163 A skin, the melanized skin, partially melanized skin, and tumor (from melanized tissue) showed 8.46, 3.95, and 7.64 fold higher levels, respectively.

A previous expression assay (Ribonuclease Protection Assay) measuring relative mRNA levels of ubiquitously expressed genes (i.e. β-Actin) has also demonstrated higher

mRNA levels in gonad tissues in comparison to other tissues tested as measured in the *X. maculatus* β-Actin Gene (Fossey, 1993).

Our findings contrast the expression levels determined in bovine liver (Kieffer *et al.*, 1992). Northern analyses of b*CYP40* expression in liver were undetectable. However, we observed intermediate and consistent x*CYP40* mRNA level in fish liver (Figure 3-23). The PCR based assay is more sensitive than northern hybridization analysis.

**Cyclophilin Evolutionary Conservation**

To determine the extent of evolutionary conservation of the h*CYP40* gene, Yokoi *et al.*, (1996a) used a portion of h*CYP40* cDNA sequence (within the ORF) as a hybridization probe. Southern blots containing *EcoRI* or *HindIII* –digested genomic DNA from mouse, chicken, frog, fish, lobster, mussel, fruit fly, nematode, yeast, and bacteria were probed and washed under low-stringency conditions. Yokoi *et al.*, (1996a) reported the h*CYP40* fragment hybridized to one sequence in each species, indicating these conditions did not allow for the detection of other cylcophilin family members. Positively hybridizing bands were observed in DNA of all vertebrates, lobster and mussel. Based on these results Yokoi *et al.*, (1996a) suggests the h*CYP40* gene is conserved throughout evolution. A similar study by Ratajczak *et al.*, (1993) reported multiple hybridizing bands of b*CYP40* in genomic DNA and suggested there maybe multiple copies of the b*CYP40* gene present.

Numerous proteins containing a TPR motif have been found in a number of different organisms such as in bacteria, cyanobacteria, fungi, insects, plants, animals, and humans (Schouflor *et al.*, 2000). This diverse spectrum of organisms containing TPR motif suggests the evolutionary conservation of the TPR motif is functionally and fundamentally important (Blatch and Lassle, 1999).

In summary, the *X. maculatus PPID* gene homologue encodes Cyclophilin 40. Comparison of the translated 1.5 kb nucleotide sequence to h*CYP40* cDNA shows 65% amino acid identity and 78% amino acid similarity. The, xcyp40 contained a putative calmodulin binding site, conserved five amino acids observed previously in plant cyclophilin, and a tetratricopeptide repeat motif. The *PPID* gene was assigned to the distal end of *Xiphophorus* linkage group U16. Relative Q-RT-PCR experiments revealed the *PPID* gene is ubiquitously expressed in all tissues tested with higher expression levels in testes and pigmented skin.

Future directions include further characterization of the *PPID* gene. Determining the genomic structure of this gene (i.e., intron sizes, splice junction, 3' UTR, etc.) will provide a comparison between fish gene structure and other vertebrates. Quantitative analysis on x*CYP40* expression will provide a more definitive analysis of x*CYP40* expression in different tissues compared to the relative analysis performed. Comparison of secondary structure analysis of xcyp40 and other cyp40 models will provide a better understanding of cyp40 function as an immunosuppressant and molecular chaperone. Finally, isolating, characterizing, and mapping additional cyclophilin members in the

*Xiphophorus* system will allow for an analysis of functional and evolutionary conservation among cyclophilin family members.

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