Allele-Specific Marker Generation and Linkage Mapping on the *Xiphophorus* Sex Chromosomes

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ABSTRACT

There is great interest in the sex chromosomes of *Xiphophorus* fishes because both WY/YY and XX/XY sex-determining mechanisms function in these species, with at least one taxon possessing all three types of sex chromosomes, and because in certain interspecific hybrids melanoma arises as a consequence of inheritance of the sex-linked macromelanophore determining locus (MDL). Representational difference analysis (RDA) has been used to clone two sequences from the sex-determining region of *X. maculatus*, including a cholinergic receptor, nicotinic, delta polypeptide (CHRND) orthologue. Allele-specific assays for these sequences, as well as for the sex-linked XMRK1 and XMRK2 genes, were developed to distinguish W, X, and Y chromosomes derived from a *X. maculatus* (XX/XY) strain and a *X. helleri* (WY/YY) strain. Linkage mapping localized these markers to linkage group (LG) 24. No recombinants were observed between XMRK2 and MDL, confirming a role for XMRK2 in macromelanophore development. Although the master sex-determining (SD) locus certainly resides on *Xiphophorus* LG 24, autosomal loci are probably involved in sex determination as well, as indicated by the abnormal sex ratios in the backcross hybrids that contrast theoretical predictions based on LG 24 genotyping. Marker development and allelic discrimination on the *Xiphophorus* sex chromosomes should prove highly useful for studies that utilize this genus as an animal model.

INTRODUCTION

*Xiphophorus* are freshwater poeciliid fishes that inhabit eastern Mexico, Guatemala, Belize, and Honduras. There are presently 26 described species and they are distributed within three broad taxonomic groups, the northern and southern swordtails and the platyfishes.1 These fishes are utilized as animal models for the study of behavioral ecology,2,3 cancer research,4 and other topics. Interest in these fish as a model organism has lead to a great deal of genetic marker development that has been greatly facilitated by the use of interspecific hybrids. This has resulted in one of the most extensive teleost genetic linkage maps and consists of isoenzyme, microsatellite, restriction fragment length polymorphisms (RFLPs) and other markers.5–7 Special interest lies in the genetic map of the *Xiphophorus* sex chromosomes because inheritance of the sex-linked complex macromelanophore determining locus (MDL, previously referred to as pigment pattern locus) can lead to melanoma in certain backcross hybrids.4,8 Also these species have been extensively used for the study of vertebrate sex determination, with the

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earliest reports having been written more than seven decades ago, yet the sex-determining gene(s) are still unknown. Cloning of sex-linked sequenced tagged sites (STS) will be necessary to provide a framework for physical map generation and large scale sequencing of this important genomic region.

In *Xiphophorus* species sexes can be determined by chromosomal (genetic) systems which may include male heterogamety, female heterogamety or multiple sex chromosomes. In the genetically sex-determined southern platyfish, *X. maculatus*, male heterogamety and female heterogamety can coexist in a single breeding population; thus XY and YY males and XX, WY, and WX females are possible. The master sex-determining locus (SD) is tightly linked to MDL and a locus (RY) regulating the formation of red (erythrophore) and yellow (xanthophore) pigment patterns. This genomic region also harbors a locus PIT (for Pituitary Gonadotropin; also known as the “maturation” locus) that is involved in regulating the time of maturation within individual fish. The pigment pattern loci, in particular, are highly polymorphic within populations. This fact has enabled convenient tracking of sex chromosome inheritance through pigment pattern phenotypes but few molecular markers have been described for the *Xiphophorus* sex chromosomes. The best characterized of these markers are the epidermal growth family homologue XMRK1 (also called EGFRB or Xmrk-INV) and a gene that arose via duplication of XMRK1 named XMRK2 (or Xmrrk). XMRK2 has a well documented role in macromelanophore and melanoma development and is a candidate to be a constituent of MDL.

Representational difference analysis (RDA) is a powerful technique utilizing subtractive hybridization and kinetic enrichment to isolate DNA restriction fragments present in one genome but not in another nearly identical genome. RDA does not require availability of a pre-existing genetic map but can target polymorphisms linked to a trait of interest. We have performed RDA using female and male *X. maculatus* DNA to generate marker candidate sequences in the region of the sex chromosomes that harbors the SD and MDL loci. These markers have been tested for sex linkage and specific assays were developed to distinguish *X. maculatus* and *X. helleri* chromosome specific alleles. We have also developed PCR-based tests to distinguish between two cloned gene loci, XMRK1 and XMRK2, both of which have been shown to reside in this genomic area.

Linkage analysis of these markers was accomplished using first generation backcross (BC1) hybrids of these species (Fig. 1). Using these data we have constructed a genetic map of the *Xiphophorus* sex chromosomes.

**RESULTS**

**RDA**

To perform RDA, “Tester” and “Driver” amplicons or “Representations” are prepared by restriction digestion of genomic DNA and subsequent amplification after ligation to a universal adapter. Several cycles of subtraction and amplification will highly enrich DNA fragments from the Tester Representation if they are either completely absent or highly underrepresented in the Driver, or if the restriction fragment is small enough to be amplified in the Tester but a restriction fragment length polymorphism results in a fragment that is too large to be efficiently amplified in the Driver. We used *X. maculatus* female (WX) DNA as Tester and male (YY) DNA as Driver to isolate sex chromosome DNA restriction fragments; two distinct sequences were cloned. BLASTN and BLASTX searches against the GenBank database revealed that an approximately 900 bp RDA product had highly significant similarity to the last three exons of the cholinergic receptor, nicotinic, delta polypeptide (CHRND) gene (also known as acetylcholine receptor delta subunit) derived from human, mouse, rat, cow, chicken, African clawed-frog, pufferfish, and zebrafish. This is most clearly shown as an alignment of the predicted *Xiphophorus* amino acid sequence to some other vertebrate protein sequences (Fig. 2). In contrast, BLAST searches with an approximately 800 bp RDA product showed no significant sequence similarity to any known coding or noncoding loci. We have designated this anonymous sequence XD0266.
FIG. 1. Mating schemes for generating backcross hybrids. (A) A female *X. maculatus* Jp 163 A, homozygous for an X-chromosome carrying the spotted dorsal (*Sd*) MDL, is mated to a male *X. helleri* Sarabia (Sara) homozygous for a Y-chromosome. F1 hybrids are then crossed back to a female *X. helleri* heterozygous for W and Y sex chromosomes. The presence of the pigment patterns in the first backcross generation indicate the inheritance of the *X. maculatus* X-chromosome. (B) Same as A except *X. maculatus* Jp 163 B, which is homozygous for an X-chromosome carrying the spotted side (*Sp*) MDL, is used.
To further characterize and verify sex chromosome specificity of these sequences, we designed primers based on the RDA products to amplify Sequence Tagged Sites (STSs). First, PCR with these primer sets confirmed that the RDA-derived sequences were indeed present in the female (WX) tester amplicons and absent from the male (YY) driver amplicons (data not shown). Next, PCR amplifications using each of these primer sets were performed on various male and female total genomic DNAs of *X. maculatus* with sex chromosomes marked by different alleles of MDL. Also included were fish from the *X. helleri* Sarabia strain which has WY/YY sex determination; Y-chromosomes are marked by alleles of the sword coloration locus. Results are shown in Figure 3A. As expected, XD0266 primers amplified a 650 bp fragment from the *X. maculatus* WX female but not the YY male DNA. In fact, the fragment was amplified from all twelve WX female DNAs that contributed to the tester of the RDA experiment and did not amplify from any of the twelve YY male DNAs that were pooled for the driver (data not shown). Since these animals were siblings, this demonstrates a highly significant association of XD0266 with sex ($\chi^2 p = 0.000001$). XD0266 was also amplified from *X. maculatus* WY DNA but did not amplify from fish homozygous for the X-chromosome that was present in the RDA tester DNA. These results indicate that the difference-product in the RDA experiment must have been amplified from the W-chromosome. On the other hand, XD0266 did amplify from *X. maculatus* strain Jp 163 B homozygous for the Xsp chromosome showing that this DNA is not limited to the W-chromosome. No primer sets were found that could generate fragments from *X. maculatus* Y chromosomes or from the X-chromosome of the strain Jp 163 A suggesting that these particular chromosomes have deletion polymorphisms.

XD0266 primers also amplified a product from *X. helleri* (Sarabia) WY females and YY males similar in size to the *X. maculatus* fragment suggesting that the sequence is shared between these distinct taxa.

The CHRNND primers amplified a fragment from all genomic DNAs tested, regardless of the genotype, including the total genomic WX and YY DNAs used to create both the tester and driver representations of the RDA experiment (Fig. 3A). However, Southern blot analysis (Fig. 3B) reveals that CHRNND polymorphisms are sex-linked. A CHRNND RDA fragment probe hybridized to multiple bands in all 12 WX female DNAs but only a single band in the 12 YY male DNAs demonstrating, as for XD0266, a highly significant association with sex ($\chi^2 p = 0.000001$). Only a single band hybridized in

**FIG. 2.** Alignment of CHRNND proteins. The *X. maculatus* protein sequence was predicted through a combination of TBLAST alignments and predictions of mRNA donor and acceptor sites in genomic DNA sequence identified by RDA. The partial *Xiphophorus* protein aligns with the carboxyl terminus of vertebrate CHRNND sequences, including zebrafish (GenBank NP_996947), *Xenopus* (Swiss Prot. P09628), and human (GenBank NP_000742). Conserved amino acid residues are boxed in black and conservative substitutions are in gray.
DNA from fish homozygous for the X-chromosome indicating that the polymorphism is linked to the W-chromosome. To determine if multiple copies of CHRND are always associated with W-chromosomes, we performed Southern blot analysis of other X. maculatus strains and species. Multiple copies of CHRND were only unequivocally detected in X. maculatus Usumacinta carrying a W-chromosome but not in other fish, including X. helleri, regardless of whether they carried a W-chromosome (data not shown). Thus, while the presence of multiple copies of CHRND may mark some W-chromosomes, it is not definitive for a W-chromosome.

**STS marker development**

To apply RDA-derived DNA fragments as STS markers for the purpose of linkage mapping in BC1 hybrids we first established polymorphisms between X. maculatus and X. helleri Sarabia for CHRND and XD0266. These fragments were amplified by PCR and cloned from a female X. maculatus Jp 163 B homozygous for the XSp chromosome and three X. helleri Sarabia genotypes: a WYGr female, a YGrYGr male which exhibits a green sword phenotype and a YGrYOr male which exhibits an orange sword phenotype (Or is phenotypically dominant to Gr). Sequence analysis of several clones from each individual fish was obtained. As expected, only one CHRND sequence was cloned from the X. maculatus female. Three different CHRND sequences, uniquely identifiable by small insertions or deletions and substitutions, were cloned from the X. helleri fish. One of these was cloned from all three genotypes but was the only sequence cloned from the YGrYGr male. A second sequence was found only in the WYGr female and the third was found only in the YGrYOr male. Thus, it was possible to identify XSp, W, YGr, and YOr CHRND alleles because we could infer the chromosomal assignment for each sequence from the phenotypes and sex chromosome complements of the fish. Similar analysis of the XD0266 clones revealed X, YGr, YOr alleles. Since the only sequence found in the X. helleri WYGr female was identical to that found in the YGrYGr X. helleri male,
**FIG. 4.** DNA sequence alignment of CHRNDR STS marker fragments from the *X. maculatus* X-chromosome and *X. helleri* W, YGr and YOr chromosomes. PCR primer sequences are underlined, identical nucleotide bases are indicated by an asterix (*) and deletions are indicated by a dash (--). Polymorphic Taq I and Pst I restriction enzyme sites are in bold. The X-chromosome specific primer sequence is overlined. Putative donor splice sites are marked by an open arrow head (△) and putative acceptor splice sites by a filled arrowhead (▲).
it is assumed there are no allelic differences between the W and YGr copies of XD0266.

Although DNA sequence alignment of CHRND alleles (Fig. 4) as well as XD0266 alleles (Fig. 5) revealed few substitutions or deletions it was possible to design primers that could be used to specifically amplify the X. maculatus X-chromosome alleles or to amplify both X. maculatus and X. helleri DNA and then use RFLPs to identify specific alleles (summarized in Table 1; Figs. 6A and B). The RFLPs are described briefly: the X. maculatus X-chromosome allele of CHRND could be identified because a 16 bp deletion results in loss of a Taq I site found in the X. helleri alleles. The W CHRND allele was distinguishable because a base pair substitution and insertion result in loss of a Pst I recognition sequence found in the X-chromosome and YGr-chromosome alleles. However, since the YOr-chromosome allele also lacks this Pst I site only pedigrees that do not have the YOr-chromosome are informative. The X. maculatus X-chromosome allele of XD0266 could be identified by a unique Hph I recognition sequence, and the X. helleri YOr linked XD0266 allele could be identified because a base substitution removes a Rsa I recognition sequence found in all other alleles.

To complement the PCR-based assays, the CHRND fragment was also used to probe HindIII-restricted DNA and generated polymorphisms as indicated in Table 1C. The highly inbred X. maculatus fish reveal a 4.3 Kb band. In the inbred X. helleri, the females show 3.6 and 2.4 Kb bands whereas the males only show a 2.4 Kb band confirming a WY/YY sex-determining mechanism in these fish. A total of 57 individual BC1 hybrids were genotyped by Southern blot analysis. This assay distinguished WX (3.6 and 4.3 Kb), WY (3.6 and 2.4 Kb), XY (4.3 and 2.4 Kb), and YY (2.4 Kb) individuals. Individuals typed in this manner either showed a single 2.4 Kb band or two bands; no individuals showed three bands. These results provide additional confidence that the CHRND genes in the two species reside on the same chromosome in Xiphophorus.

In addition to the polymorphisms we established for the RDA products, we also identified polymorphisms for two genes that have been previously mapped to LG 24, XMRK1 and XMRK2 (Table 1; Figs. 6C and D). XMRK2 originated by duplication of the ancestral XMRK1 and shares greater than 95% nucleotide identity with it. X. helleri lacks XMRK2 and the allele of XMRK2 that is found on the X-chromosomes of both X. maculatus Jp163 A and Jp163 B has a 1398 bp deletion compared to XMRK1. Therefore, primers flanking the XMRK2 deletion amplify a 791 bp XMRK2 fragment but only poorly amplify a 2.1 Kb XMRK1 fragment. XMRK1 amplification alone was obtained by using a primer to the sequence that has been deleted in XMRK2. The X. maculatus allele can be distinguished by a unique Sst I site or by also amplifying with a primer specific for X. maculatus.

Mapping of LG 24 sequences

BC1 hybrid progeny of X. maculatus (Jp 163 A and Jp 163 B) and X. helleri (Rio Sarabia strain) as the recurrent parent (Fig. 1) have previously been used to create a linkage map with
multi-point linkage group representation of all 24 *Xiphophorus* chromosome sets.\textsuperscript{5,7} The SD and MDL loci have been localized to LG 24 which also harbors a *Xiphophorus* homologue of the *CMYC* gene, in addition to an unsequenced marker locus (Prm3.9) derived from Arbitrarily Primed-PCR (AP-PCR) and a microsatellite marker (*Msa040*) derived from a sub-genomic library.\textsuperscript{5,7} A total of 394 additional markers have been placed on LGs 1–23 and were available for the linkage studies.\textsuperscript{5,7} To position *CHRND* and *XD0266* on the *Xiphophorus* linkage map BC\textsubscript{1} hybrid individuals were tallied for heterozygosity or homozygosity at these loci as well as *XMRK1* and *XMRK2*. Presence or absence of macromelanophore cells derived from the *X. maculatus* MDL locus also established zygosity at the LG 24 master sex-determining region.

\begin{table}
\centering
\begin{tabular}{ll}
\hline
Gene & Sequence Alignment & Base Count \\
\hline
*X. maculatus* & GGA\ldots GAC & 60 \\
*X. helleri* & CAG\ldots C & 60 \\
\hline
*X. maculatus* & TA\ldots G & 120 \\
*X. helleri* & AG\ldots A & 120 \\
\hline
*X. maculatus* & CT\ldots T & 180 \\
*X. helleri* & C\ldots C & 180 \\
\hline
*X. maculatus* & CA\ldots C & 240 \\
*X. helleri* & C\ldots C & 240 \\
\hline
*X. maculatus* & C\ldots C & 300 \\
*X. helleri* & C\ldots C & 300 \\
\hline
*X. maculatus* & G\ldots G & 360 \\
*X. helleri* & C\ldots C & 360 \\
\hline
*X. maculatus* & T\ldots T & 420 \\
*X. helleri* & A\ldots A & 420 \\
\hline
*X. maculatus* & G\ldots G & 480 \\
*X. helleri* & C\ldots C & 480 \\
\hline
*X. maculatus* & A\ldots A & 540 \\
*X. helleri* & G\ldots G & 540 \\
\hline
*X. maculatus* & C\ldots C & 600 \\
*X. helleri* & A\ldots A & 600 \\
\hline
*X. maculatus* & T\ldots T & 659 \\
*X. helleri* & C\ldots C & 659 \\
\hline
\end{tabular}
\caption{DNA sequence alignment of *XD0226* STS marker fragments from the *X. maculatus* X-chromosome and *X. helleri* Y\textsuperscript{Gr} and Y\textsuperscript{Or} chromosomes. The *X. helleri* W-linked sequence is presumed to be identical to the Y\textsuperscript{Gr} sequence. PCR primer sequences are underlined, identical nucleotide bases are indicated by an asterisk (*) and deletions are indicated by a dash (—). Polymorphic Hph I and Rsa I restriction enzyme sites are in bold. The site of the X-chromosome specific primer is overlined.}

FIG. 5. DNA sequence alignment of *XD0226* STS marker fragments from the *X. maculatus* X-chromosome and *X. helleri* Y\textsuperscript{Gr} and Y\textsuperscript{Or} chromosomes. The *X. helleri* W-linked sequence is presumed to be identical to the Y\textsuperscript{Gr} sequence. PCR primer sequences are underlined, identical nucleotide bases are indicated by an asterisk (*) and deletions are indicated by a dash (—). Polymorphic Hph I and Rsa I restriction enzyme sites are in bold. The site of the X-chromosome specific primer is overlined.
ALLELE-SPECIFIC MARKERS AND LINKAGE MAPPING

**Table 1A. Allele-Specific PCR Amplification Assays**

<table>
<thead>
<tr>
<th>STS</th>
<th>Species</th>
<th>Allele specificity</th>
<th>Upstream primer</th>
<th>Downstream primer</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XMRK1</td>
<td><em>X. maculatus</em></td>
<td>X chromosome</td>
<td>tatgccgctactcttg</td>
<td>gttctggctggctggga</td>
<td>278</td>
</tr>
<tr>
<td>XMRK2</td>
<td><em>X. maculatus</em></td>
<td>X chromosome</td>
<td>gctgagctgtatgcagcag</td>
<td>ggcacgggtgtcagcgcg</td>
<td>791</td>
</tr>
<tr>
<td>CHRND</td>
<td><em>X. maculatus</em></td>
<td>X chromosome</td>
<td>gccgcgccagcactagag</td>
<td>ggtctacccgagcgcgcag</td>
<td>236</td>
</tr>
<tr>
<td>XD0226</td>
<td><em>X. maculatus</em></td>
<td>X chromosome</td>
<td>ggatggatgtgcgtagacaga</td>
<td>aggtcctgtacacgctga</td>
<td>466</td>
</tr>
</tbody>
</table>

**Table 1B. Allele-Specific PCR-RFLP Assays**

<table>
<thead>
<tr>
<th>STS</th>
<th>Species</th>
<th>Allele specificity</th>
<th>Upstream primer</th>
<th>Downstream primer</th>
<th>Polymorphic enzyme site</th>
</tr>
</thead>
<tbody>
<tr>
<td>XMRK1</td>
<td><em>X. maculatus</em></td>
<td>X chromosome</td>
<td>gctgagctgtatgcagcag</td>
<td>gttctggctggctggga</td>
<td>Contains SstI</td>
</tr>
<tr>
<td>CHRND</td>
<td><em>X. maculatus</em></td>
<td>W chromosome</td>
<td>agctgatgtgccagcgcag</td>
<td>ggtctacccgagcgcgcag</td>
<td>Lacks TaqI</td>
</tr>
<tr>
<td>CHRND</td>
<td><em>X. helleri</em></td>
<td>W chromosome</td>
<td>agctgatgtgccagcgcag</td>
<td>ggtctacccgagcgcgcag</td>
<td>Lacks PstI</td>
</tr>
<tr>
<td>XD0226</td>
<td><em>X. maculatus</em></td>
<td>X chromosome</td>
<td>ggtctgatgtgcgtacaga</td>
<td>gtcagctgtgcacattagagaa</td>
<td>Contains HphI</td>
</tr>
<tr>
<td>XD0226</td>
<td><em>X. helleri</em></td>
<td>Y chromosome</td>
<td>ggtctgatgtgcgtacaga</td>
<td>gtcagctgtgcacattagagaa</td>
<td>Lacks Rsal</td>
</tr>
</tbody>
</table>

**Table 1C. Allele-Specific CHRND RFLP/Southern Blot Assay**

<table>
<thead>
<tr>
<th>STS</th>
<th>Species</th>
<th>Allele specificity</th>
<th>Polymorphic enzyme site</th>
<th>RFLP (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHRND</td>
<td><em>X. maculatus</em></td>
<td>X chromosome</td>
<td>HindIII</td>
<td>4.3</td>
</tr>
<tr>
<td>CHRND</td>
<td><em>X. helleri</em></td>
<td>Y chromosome</td>
<td>HindIII</td>
<td>2.4</td>
</tr>
</tbody>
</table>

*PCR were performed with Taq DNA polymerase or Elongase mix and optimal [Mg++] and annealing temperatures chosen for each primer set. Detailed PCR protocols for each primer set is available on request.*

The derived map of the region that harbors MDL is shown in Figure 7. Among 378 animals no recombinants were discovered between the XMRK2 and MDL loci, the latter of which is tallied phenotypically, suggesting that the XMRK2 locus is a component of MDL. CHRND and XD0266 were positioned between XMRK1 and XMRK2 loci within LG 24, also as indicated in Figure 7. The region between the ancestral XMRK1 and XMRK2 is known to harbor the master sex-determining locus for the platyfish *X. maculatus*. Therefore, we attempted to assess potential associations between LG 24 loci genotypes and sex determination in hybrid fishes between *X. maculatus* and *X. helleri*.

**Hybrid crossing and sex determination**

Interspecific hybrid crossing was conducted between two *Xiphophorus* species that harbor alternate sex determination mechanisms. *X. maculatus* Jp 163 A and B harbor XX/XY mechanisms, while the *X. helleri* (Sarabia) harbors a WY/YY mechanism (Kazianis et al., in press). The predicted outcome from the depicted crosses in Figure 1 would be that all F1 offspring would be males, assuming there is compatibility of sex determination mechanisms and chromosomal homology of the primary sex-determining loci. The experimental results suggest that this is indeed the case, as F1 offspring were nearly all males (1 female: 124 males in Fig. 1A, 0 female: 78 males in Fig. 1B). A male F1 hybrid harboring an X (from *X. maculatus*) and Y (from *X. helleri*) mated with a female X. helleri (WY) should produce WX and WY females, and YY or XY males, with an overall ratio of male to female approaching 1:1. Examination of BC1 hybrids did not reveal such a relationship and a significant excess of male fish was discovered (269 females, 349 males, $\mu^2 p < 0.01$). Such significant biases of sex ratio have been reported numerous times in the *Xiphophorus* lit-
erature and have been observed in both non-hybrid and hybrid fish.\textsuperscript{15,25}

The developed allele-specific \textit{CHRND} PCR marker, PCR-RFLP markers and RFLP/Southern blot markers (Table 1) were utilized to distinguish between all possible genetic constitutions at the sex chromosomes within BC\textsubscript{1} hybrids. Individuals that were WY and WX would be predicted to be females, while YY and XY fishes would be males. Since \textit{CHRND} is a marker for \textit{SD}, and not predicted to be involved in the sex-determination cascade, some recombinants could be expected between these loci.

The predicted sex chromosomal constitution of 341 BC\textsubscript{1} individuals is depicted in Table 2. Individuals predicted to be YY based on \textit{CHRND} allele determinations, strongly tended to be males (72/81), suggesting close linkage between \textit{SD} and \textit{CHRND}. However, the other classes of sex-chromosomal combinations yielded dramatically different results. XY fish, which strongly tended to be males in the F\textsubscript{1} generation, showed much-reduced ratio of male: female offspring. In addition, WX fish, predicted to be females, showed large numbers of male offspring (34/81). In addition, WY fish, exclusively harboring \textit{CHRND} alleles from \textit{X}.
helleri, and theoretically the SD alleles from the same taxon, showed a 1:1 ratio of male to female.

The results suggest that specific alleles of one or more non LG 24 loci could act as modifiers of the Xiphophorus master sex-determining locus within LG 24 to determine the sexual phenotype of the hybrid fish. In these crosses it was not possible to localize a single autosomal modifier that could be involved in the sex-determination cascade, even after controlling for the genetic constitution of LG 24. Analysis of the XY genotyped individuals is illustrative. Although F1 hybrids with XY were predominantly males with only one exceptional animal, BC1 hybrids show 30 females among 119 total genotyped fishes. If a single autosomal locus was involved, such as that homozygosity in X. helleri alleles would lead to male or female individuals, a predicted 50:50 ratio of males: females would be observed, but was not. Therefore, it is inferred that multiple autosomal loci are implicated in sex determination in these Xiphophorus hybrid fish.

### DISCUSSION

RDA using female X. maculatus (WX) DNA as tester and male X. maculatus (YY) DNA as driver resulted in cloning of two sex-linked sequences, CHRND and an anonymous sequence, XD0266. Multiple CHRND-related sequences were associated with the W-chromosome of the X. maculatus sub-strain used for RDA but were not definitive of the W-chromosome in this species or in the other species tested.

Using CHRND and XD0266 as well as XMRK1 and XMRK2 polymorphisms we constructed a genetic map of the Xiphophorus sex chromosomes. No recombination between XMRK2 and MDL was found. The X. maculatus MDL is a complex locus that codes for the development of the very large, melanin-containing cell, the macromelanophore, for the location or pattern these cells form on the body, and the propensity for melanoma formation in certain hybrids. Whether all of these functions are encoded by a single gene or by several tightly linked genes is currently unknown but there is some evidence suggesting that these functions can be separated by genetic recombination.

The lack of recombination between XMRK2 and MDL suggests that XMRK2 is a component of MDL and is involved in macromelanophore development. This is in agreement with several previous studies that show higher expression of this gene in melanized versus nonmelanized tissue sources and highly elevated expression within melanomas. In addition, mutants of XMRK2 have shown pigmentation phenotypes that are abnormal.

Linkage analysis of backcross hybrids clearly positions CHRND and XD0266 between XMRK2 and XMRK1. Since the master sex-determining locus in X. maculatus is known to lie between the latter two markers, which may be separated by as little as 300 kb, CHRND and XD0266 must be physically very near SD. The

<table>
<thead>
<tr>
<th>Table 2</th>
<th>LG 24 Genetic Constitutiona vs. Sex in BC1 Hybrids</th>
</tr>
</thead>
<tbody>
<tr>
<td>WX</td>
<td>XY</td>
</tr>
<tr>
<td>Number of males</td>
<td>34</td>
</tr>
<tr>
<td>Number of females</td>
<td>47</td>
</tr>
</tbody>
</table>

*aAs determined by genotypic assessment of CHRND allele inheritance.
segregation of these makers in the backcross hybrids clearly shows that CHRND and XD0266 are on homologous chromosomes in the two species so it is assumed that SD of X. helleri Sarabia also lies very near these markers. However, the segregation of sexual phenotype of BC1 hybrids deviated substantially from that expected from the inheritance of CHRND. This deviation appears to be too great to be explained by crossovers between CHRND and SD or by misclassifications of the phenotype; we hypothesize that sexual phenotype in these backcross hybrids is determined by the combination of alleles of the SD locus within LG 24 and modifier alleles at non LG 24 loci. The existence of an autosomal locus that regulates or modulates the primary sex-determining locus has been hypothesized for some time. Recent data strongly implicates a peptidase S (PEPS) linked autosomal locus, termed Atypical Sex Determination 1 (ASD1), as a dominantly acting locus overriding the SD inherited from both parental species in certain Xiphophorus crosses (Kazianis et al. in press). No association with PEPS or any other single autosomal marker could be established in the X. maculatus and X. helleri cross reported here. Although this could be due a lack of genetic markers in the region of such a modifier locus, we assume that multiple autosomal loci are probably interacting in complex ways with SD to determine phenotypic sex in these hybrids.

The genetic mechanism of sex determination involves a cascade of sex-determining and differentiation genes. In mammals sry is at the top of this genetic hierarchy but arose as the master sex-determining gene only recently. DMRT/Y may play a similar role in the teleost Medaka, but does not appear to be the primary sex-determining gene in Xiphophorus or most other fish. The identity of the Xiphophorus SD is currently unknown but it is likely to have been evolutionarily conserved as one of the genes, many of which are still unknown, that are involved in mammalian sex determination or differentiation. One interesting possibility is that the Xiphophorus SD gene may be orthologous to a locus involved in mammalian intersexes. A locus mapping to human chromosome 2p is involved in XY female intersexes. Similarly, XY females are also found in mouse and some pedigrees have shown linkage to a region of chromosome 17, which is syntenic to human chromosome 2p. The implicated locus is not yet identified in mammals. Current work in Xiphophorus, suggests that at least two recently cloned loci (MSH2, and CRIPT-ps) within the SD genomic region have mammalian orthologues that map to the same chromosomes implicated above. The human CHRND receptor also maps to human chromosome 2. When these data are considered, it is possible that the terminal region of Xiphophorus LG 24 harbors a conserved vertebrate syntenic group of loci in which the Xiphophorus SD gene is evolutionarily conserved as the locus involved in mammalian intersexes. Further experimentation and implementation of newer technologies and methods will almost certainly lead in the near future to the discovery of SD, as well as autosomal loci that obviously influence sex-determination in Xiphophorus.

**MATERIALS AND METHODS**

**RDA**

All fish used for RDA were bred and maintained in the aquarium of the British Columbia Cancer Research Centre. Genomic DNA was extracted from X. maculatus brain using a QIAgen (Qiagen Operon, Alameda, CA, USA) genomic DNA purification kit following the manufacturer’s protocol. To specifically target polymorphisms that would map to the sex chromosome region near the SD and MDL loci, DNAs from 12 females of the genotype WX-Dr/H9004 were pooled for Tester DNA and 12 DNAs from male siblings with the genotype YArSr/YArSr/H11032 were pooled for Driver DNA. RDA was performed using BamHI restriction endonuclease and anchor primers as described. After three rounds of RDA two bands of approximately 900 bp and 800 bp were distinctly enriched from the female amplicons. These difference products were digested with BamHI and cloned into pBluescript (SK+; Invitrogen Corporation, Carlsbad, CA, USA). The inserts of these clones were used as probes for Southern blot analysis and partially sequenced in order to design oligonucleotide primer pairs for sequenced-tagged sites (STTs).
PCR, cloning, and sequencing of sex-linked STSs

RDA-derived primers were used with a DNA polymerase mix containing a proofreading enzyme (Elongase Enzyme mix, Invitrogen) to amplify STSs from 10 ng genomic DNA from the following animals: a female *X. maculatus* Jp163 B (XX), a female *X. helleri* Sarabia (WYGr), a male *X. helleri* Sarabia (YGrYGr) with a green sword phenotype (Gr) and a male *X. helleri* Sarabia (YGrYOr) with an orange sword phenotype (Or). Polymerase chain reactions (PCRs) were performed in a 50 μL total volume with 60 mM Tris- SO4 (pH 9.1), 18 mM (NH4)2SO4, 1.5 mM MgSO4, 0.2 mM each dNTP, 0.4 μM each primer and 1 μL Elongase enzyme mix. Reactions were incubated at 95°C for 4 min before 30 cycles of 95°C, 15 sec and 68°C, 3 min followed by a final extension of 68°C for 27 min. Primer sequences for the 900 bp RDA product were 5'-tccacatggcccagcagcggac-3' and 5'-ggttctaccccagcaggcgcag-3'. For the 800 bp RDA product primers were 5'-ggatggatct-gcgctgagacaga-3' and 5'-gtctcagctgctcaccatcagaa-3'.

A 1.6 kb fragment of the *XMRK1* gene was amplified from 10 ng of *X. helleri* Sarabia female DNA as described above except after the initial 4 min incubation at 95°C, 30 cycles of 95°C, 15 sec and 68°C, 3 min followed by a final extension of 68°C for 27 min were performed. Primer sequences derived from the sequence of the *X. maculatus* XMRK1 (GB Accession X56317) were 5'-gctgagctcttgagacg-3' and 5'-gttctgggtcgggtcgga-3'.

Amplification products were cloned by taking 0.5–1.0 μL of the above PCR reactions for ligation into pCR2.1TOPO (Invitrogen) following the manufacturer’s recommendations. For each RDA-derived STS the entire insert of at least two clones of each sex-linked allele were sequenced. Only partial sequences of the *XMRK1* clones were determined. Fluorescent DNA sequencing was performed on the ABI Prism 310 Genetic Analyzer. Nucleotide sequence data for the *X. maculatus* and *X. helleri* W, YGr, and YOr CHRND alleles have been deposited in GenBank under accession numbers DQ333447, DQ333448, DQ333449, DQ333450, and the *X. maculatus* and *X. helleri* YGr and YOr XD0266 alleles under DQ333451, DQ333452, DQ333453.

PCR allotyping of sex-linked STSs

STS alleles were detected by PCR of 5–10 ng genomic DNA with either allele specific oligonucleotide primers or by PCR followed by restriction enzyme digestion. Because there were few sequence differences between alleles the selection of primers was limited and the optimal amplification parameters for each set of primers did not allow for multiplexed reactions. The primers and restriction enzymes used are listed in Table 1. PCR products were separated on 1.5% agarose gels and visualized by UV light after ethidium bromide staining. For each allele-specific primed PCR assay at least 15% of the samples and all putative recombinants were re-assayed by the PCR-RFLP method to ensure accuracy of the allele designation.

Mapping panels and map construction

Parental stocks and hybrids used in this project were derived from the *Xiphophorus* Genetic Stock Center (Texas State University, San Marcos, TX). BC1 hybrids were created between the southern platyfish, *X. maculatus* (strains Jp 163 A and Jp 163 B), and the green swordtail, *X. helleri* (Rio Sarabia strain) as the recurrent parent (Fig. 1). Artificial insemination was only employed for the production of F1 hybrids. All first generation backcross (BC1) hybrids were derived using F1 hybrid male parents. A total of 646 BC1 hybrids were used in the analysis (109 using the Jp 163 A strain as a progenitor). All BC1 hybrids were phenotypically scored for macromelanophore (Sd, Sp or W) and micromelanophore (Dot or Gr) patterning, sex (M or F), sword coloration (Gr or Or, when applicable) and other phenotypes.

Genotypic data were entered in Excel (Microsoft Corporation) in an expanded MAP-MAKER format that includes supplemental information. Exported data were analyzed with the aid of MAPMAKER (version 3.0b, DOS version, used in Windows XP), and Map Manager QT. This program was also used to create custom sorted datasets. Graphic map files were generated using MAPMAKER for Macintosh version 2.0 (obtained from S. Tingey, DuPont Co., Wilmington, DE).
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REFERENCES


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