RESEARCH

Genetic Mapping in *Xiphophorus* Hybrid Fish: Assignment of 43 AP-PCR/RAPD and Isozyme Markers to Multipoint Linkage Groups

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The combined use of the arbitrarily primed polymerase chain reaction [AP-PCR, also known as random amplification of polymorphic DNA (RAPD)] and isozyme mapping resulted in the production of 87 potential marker loci, enabling an overall expansion within the genetic map of the fish genus *Xiphophorus*. Use of DNA sequencing-style acrylamide gels and carefully controlled conditions of amplification and silver staining allowed exceptional resolution and reproducibility of AP-PCR/RAPD generated markers. Linkage analysis of AP-PCR/RAPD and isozyme markers resulted in the addition of 16 new markers to *Xiphophorus* linkage groups (LGs) I, II, III, V, IX, X, XII, and XIV. Addition of 5 AP-PCR/RAPD markers to linkage group U6 containing the Tailspot pigment pattern locus (*P*) and designation of eight new unassigned linkage groups with 22 markers was also accomplished. Genetic linkage data allowed inference of the existence of a novel pigment pattern modifier locus. Expansion of the *Xiphophorus* gene map by linkage analysis of AP-PCR/RAPD markers in conjunction with isozyme polymorphisms should lead to the rapid saturation of genetic linkage groups such as LG V, which will probably be instrumental to cloning the *Diff* tumor suppressor gene locus.

The study of cancer can be greatly benefited by the use of animal models that simplify the search for implicated genes and/or environmental factors (see Friend 1993). Models that employ organisms with relatively short generation times and robust genetics afford the opportunity to study the process of neoplastic transformation and malignant progression and to identify both primary and secondary agents of carcinogenesis (for discussion, see Mechler 1990).

Melanoma is a somewhat difficult disease to study genetically in humans, and therefore, fish have long been used as experimental models for research. Specifically, the genus *Xiphophorus* (presently comprising 22 species; Rauchenberger et al. 1990) has been studied extensively. Of critical relevance is the fact that genetic hybrids can be created between species that often exhibit phenotypic anomalies. Enhanced phenotypic modification of macromelanophore pigment patterns (Gordon 1927) frequently results in the formation of neoplastic melanin-containing cells. In some cases, this situation leads to the spontaneous formation of melanomas (for review, see Anders 1991). In other cases, *Xiphophorus* melanomas can be induced by chemical or UV exposures (Anders et al. 1985; Setlow et al. 1989).

With 22 almost universally interfertile species and many macromelanophore pigment patterns available for hybrid creation, numerous investigators have performed many different genetic crosses with dozens of distinct combinations of species and pigment patterns. These studies have addressed questions of universality in pigment pattern modification and melanoma formation by documenting a range of expression, from the absence of macromelanophore pattern formation to malignant melanoma production, among different cross types (for examples and discussion, see Gordon and Smith 1938; Atz

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1962; Zander 1969; Anders et al. 1973; Vielkind et al. 1989). One particular cross has become known as the "classical" hybrid melanoma model, studied by many *Xiphophorus* melanoma researchers. This cross involves hybridization of *Xiphophorus maculatus* with a spotted dorsal (Sd) macromelanophore pattern with *Xiphophorus helleri;* most genetic analyses of melanoma formation utilize first backcrosses of this hybrid to *X. helleri*. The use of *Xiphophorus* for melanoma studies has been popularized in the scientific community largely because of this cross (e.g., see Mechler 1990; Kefford 1992; Friend 1993). The widespread use of the classical melanoma cross stems from its simple two-gene inheritance that suggests the existence of an oncogene and an apparent tumor suppressor and to the reproducibility and relatively quick production of melanomas. The oncogene has been cloned and is referred to as the *Xiphophorus* melanoma receptor tyrosine kinase [*Xmrk,* although alternative naming schemes exist in the literature (Zechel et al. 1988, 1989; Wittbrodt et al. 1989; Woolcock et al. 1994)]. The tumor suppressor gene *Diff* (Anders 1967; Vielkind 1976) remains molecularly uncharacterized although it has been mapped to a linkage group (LG) defined by isozyme loci [*Xiphophorus* linkage group V (LG V); Siciliano et al. 1976; Ahuja et al. 1980; Morizot and Siciliano 1983; Förnzler et al. 1991; Morizot et al. 1991]. Unfortunately, mapping studies have to date failed to define a closely linked marker locus that could be used for the eventual cloning of *Diff*. In addition, no DNA markers have yet been assigned to this linkage group.

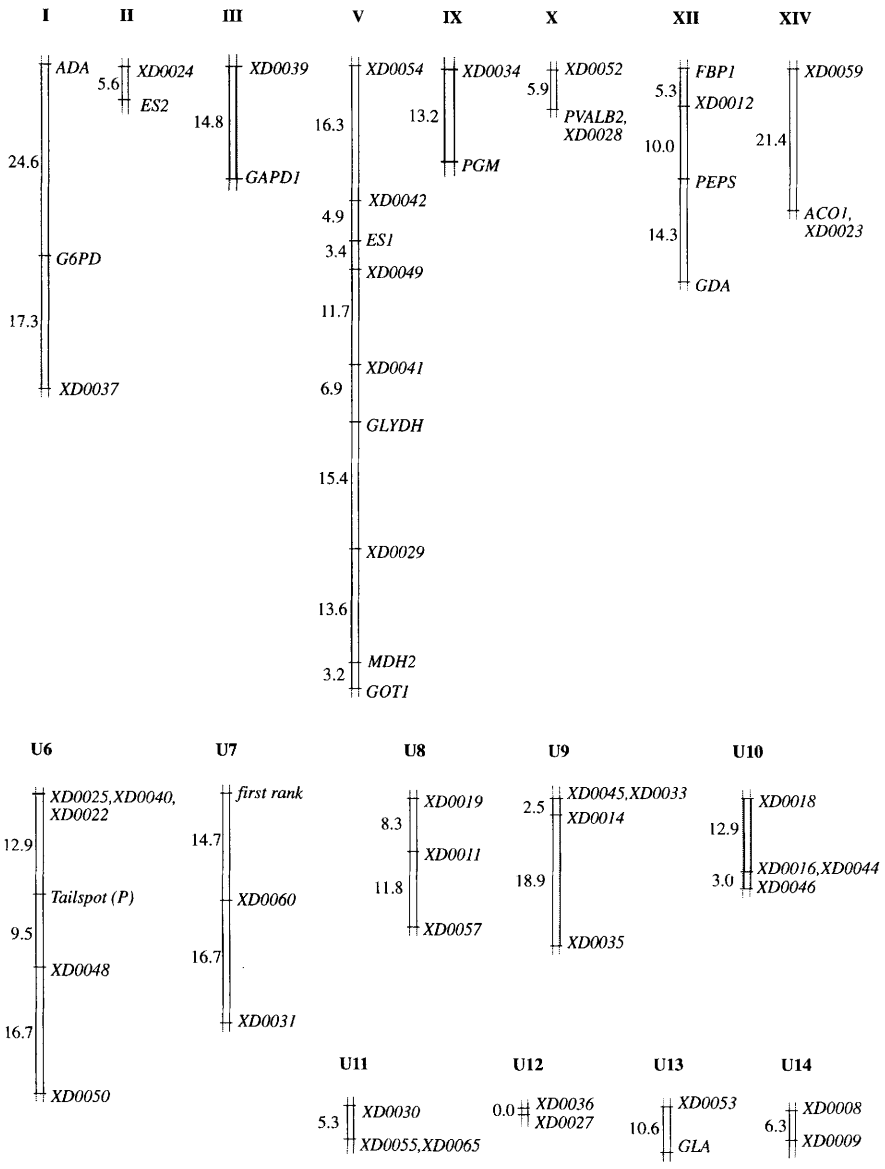

Figure 1 Graphic depiction of linkage assignments within backcross hybrid progeny. Distances represent direct recombination percentage between adjacent loci. "*Tailspot (P)*" and "first rank" represent morphological traits, whereas the rest are isozyme and AP-PCR/RAPD marker loci.

Ongoing genetic research on *Xiphophorus* hybrid melanomas has provided impetus for assembly of a detailed genetic map. To date, >100 isozyme, DNA RFLP, and pigment pattern loci have been assigned to multipoint linkage groups that may reside on as many as 22 of the 24 chromosome pairs (Morizot et al. 1991, 1993; D. Morizot, unpubl.). Recent advances in genetic mapping methodologies should greatly facilitate expansion of this already extensive map. The speed and ease of the production of DNA markers generated by arbitrarily primed poly-

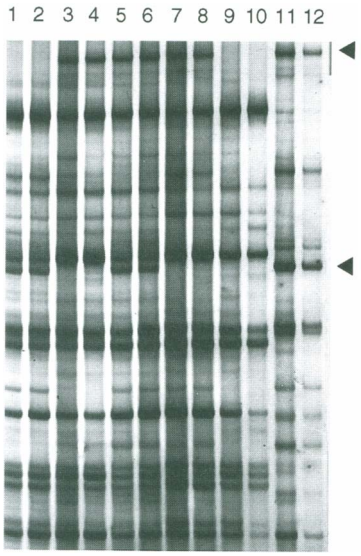

Figure 2 Section of an exemplary silver stained AP-PCR/RAPD gel (origin is at *top* and not shown). Each sample is repeated using two dilutions of genomic DNA: 19.5 ng (lanes *1,3,5,7,9,11*) and 3.9 ng (lanes *2,4,6,8,10,12*). (Lanes *1–8*) Four backcross hybrids examined using two different dilutions of genomic DNA. Lanes *1* and *2* thus represent one individual as do lanes *3,4*, etc. (Lanes *9,10*) *X. helleri* (effectively, a negative control); (lanes *11,12*) *X. variatus* (positive control). The arrowheads delineate the two most obvious AP-PCR/RAPD markers (*XD0022*, above, and *XD0016*, below).

merase chain reaction [AP-PCR, also known as random amplification of polymorphic DNA (RAPD)] makes AP-PCR one such method of great promise (Welsh and McClelland 1990; Williams et al. 1990). As an example of the usefulness of the technique, a genetic map was generated for the zebrafish *Danio rerio* composed of 414 markers, of which 401 were AP-PCR/RAPD polymorphisms (Postlethwait et al. 1994). An obvious advantage over isozyme and DNA restriction fragment length polymorphism (RFLP) mapping techniques is that many more polymorphic loci can be detected quickly in most genetic crosses with AP-PCR/RAPD.

We report here the first extensive application of AP-PCR/RAPD techniques in *Xiphophorus* genetic mapping, together with isozyme marker analysis. Sixty AP-PCR/RAPD polymorphisms and 27 isozyme loci polymorphic between *Xiphophorus variatus* and *X. helleri* were assessed for proper segregation and linkage in backcross hybrids of the cross type *X. helleri* × (*X. helleri* × *X. variatus*). This cross effectively parallels the classical cross with a substitution of *X. maculatus* by *X. variatus* and the use of different pigment pattern loci (using the P^2 pigment pattern as opposed to Sd). As it appears that this exact cross has never been performed before, one can for the first time compare how the P^2 pigment pattern (of *X. variatus*) differs from the Sd pigment pattern (of *X. maculatus*) under a hybrid context, examining phenotypic modification and underlying genetic control. The P^2 pattern is especially interesting because it has been shown that *X. variatus* nonhybrid animals can develop age-related melanomas stemming from it, whereas such a phenomenon has never been reported for the Sd pigment pattern of *X. maculatus* (Kazianis and Borowsky 1995; Schartl et al. 1995).

Linkage analysis of AP-PCR/RAPD and isozyme polymorphisms allowed the addition of 14 AP-PCR/RAPD and 2 new isozyme markers to *Xiphophorus* LGs I, II, III, V, IX, X, XII, and XIV and the addition of 5 AP-PCR/RAPD markers to linkage group U6 containing the Tailspot pigment pattern locus (*P*). Eight new unassigned linkage groups with 22 markers were also defined. In addition, the existence of a novel pigment pattern modifier locus was inferred from genetic linkage results.

RESULTS

In total, 60 AP-PCR/RAPD genetic markers were generated. Because 2 (*XD0015* and *XD0020*) of the 60 markers failed to meet the χ^2 test criterion for a 1:1 segregation ratio, they were excluded from subsequent analyses. Twenty-seven isozyme loci proved to be polymorphic. *CKM* and *PK2* did not satisfy a χ^2 criterion for a 1:1 segregation ratio and were excluded from subsequent analyses.

Expansion of the *Xiphophorus* Gene Map

Linkage analyses resulted in assignment of 40 AP-PCR/RAPD markers, 15 isozyme loci, and the Tailspot micromelanophore pattern gene (*P*) to 17 linkage groups (Fig. 1). Fourteen AP-PCR/RAPD markers mapped to previously designated linkage groups I–XIV (Morizot et al. 1993; Nairn et al. 1996), as well as two new isozyme locus assignments, *GOT1* to LG V and *FBP1* to LG XII. Five AP-PCR/RAPD markers were linked to the Tailspot micromelanophore locus, previously assigned to LG U6 (Morizot et al. 1993). Following the convention of Morizot et al. (1993) to desig-

nate linkage groups incompletely tested for independent assortment as "unassigned" (U), significant linkages that were identified among the remaining 21 AP-PCR/RAPD markers and *GLA* are assigned here to multipoint linkage groups U7–U14. No AP-PCR/RAPD or isozyme loci were found to be linked to the P^2 macromelanophore pigment pattern gene that is assigned to LG XXIV. Linkage between P^2 and both the platyfish sex-determining region and the *Xmrk* gene has been established in previous studies (Kazianis and Borowsky 1995; Schartl et al. 1995). A typical silver-stained acrylamide gel displaying AP-PCR/RAPD markers is shown in Figure 2.

Association of P^2 Pigment Pattern Expression with LG U7

The P^2 macromelanophore pigment pattern was enhanced in both F_1 and backcross hybrids, most profoundly in the latter. Three aspects of enhancement were manifested: (1) The pigment pattern appeared temporally early in all hybrids; (2) the pattern development progressed more rapidly in hybrids; and (3) tumors stemming from melanin-containing cells within the pigment pattern developed sooner in hybrid fish (S. Kazianis and R. Borowsky, in prep.). Figure 3 compares a typical *X. variatus* with P^2 to an *X. helleri* × *X. variatus* F_1 hybrid and to a backcross hybrid (to *X. helleri*) with a "2nd rank/severe" phenotype (see Methods for definitions of "rank" and "severity" in this context).

The phenotypic enhancement of P^2 upon hybridization with *X. helleri* parallels the condition in the classical *Xiphophorus* melanoma model that involves the Sd pigment pattern of *X. maculatus*. Although two distinct phenotyping systems were used to categorize P^2 pigment pattern expression in the hybrids of this study, no evidence for association with any LG V locus was found, in contrast to the well documented LG V control of Sd phenotypes in the classical melanoma cross. With the assignments of six additional marker loci to LG V and adequate sample sizes in the present study, evidence for a *Diff* gene effect could have been easily observed but was not.

Although considerable variability in P^2 expression was observed throughout much of the life of hybrids, at 2 months of age approximately equal numbers of backcross individuals either resembled F_1 hybrids or exhibited more enhanced expression of P^2, categorized as "1st rank/less severe" and "1st rank/severe" phenotypes, respectively. Figure 4 depicts two backcross siblings that represent the 1st rank/less severe and 1st rank/severe phenotypes. AP-PCR/RAPD marker *XD0060* showed significant association with the first ranking system in the hybrids, suggesting the existence of a locus (labeled "*first rank*" in Fig. 1) associated with this early phenotypic expression. Another marker locus, *XD0031* was also assigned to this linkage group, designated U7, owing to its linkage with *XD0060*.

DISCUSSION

In this study we have shown that generation of AP-PCR/RAPD markers and their use in genetic

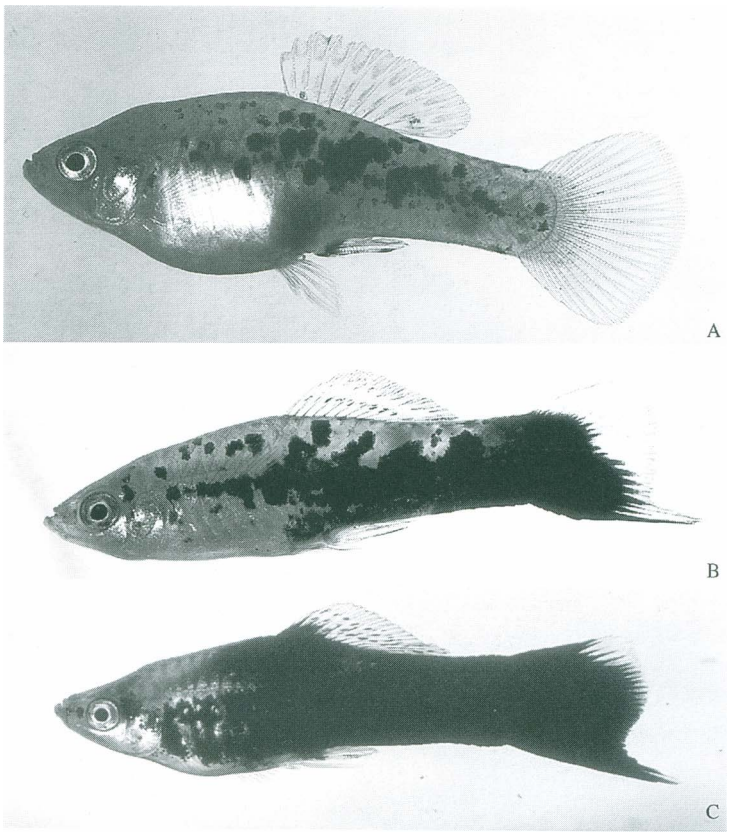

Figure 3 Photographic representation of phenotypic enhancement of the P^2 pigment pattern of *X. variatus*. (*A*) A *X. variatus* at 12 months of age; (*B*) an F_1 hybrid between *X. helleri* and *X. variatus* at 10 months of age; (*C*) HHV-BC_1 individual at 10 months of age.

linkage analysis together with isozyme polymorphisms establish a means for rapid expansion and localization of the number of informative markers from the available isozyme and DNA RFLP loci for any cross within *Xiphophorus*. Two factors particularly expedited marker production. Because the hybrids used for mapping involved two different species of the genus *Xiphophorus*, the considerable genetic distance between species facilitates AP-PCR/RAPD mapping by providing more marker loci per available primer. In addition, coupling of DNA sequencing-style acrylamide gels with silver staining resulted in the resolution and detection of numerous bands that were unscorable on ethidium-stained agarose gels. In some cases, >100 bands could be identified in a lane. In our opinion, silver staining of acrylamide gels represents a viable (and reproducible) alternative for researchers who require the resolution capabilities offered by long gels but prefer not to use radioisotopic detection methods.

With the newly developed degree of coverage within *Xiphophorus* LG V, attempts at linking severity of melanotic phenotype in the backcross hybrids with one of the nine LG V loci should have been straightforward, if such a phenomenon existed. However, no significant linkage was associated with LG V regardless of which phenotypic ranking system (first or second) was used in analysis. However, the existence of a gene locus involved in temporal control of the P^2 macromelanophore pattern in hybrids is strongly indicated by our linkage analyses. Phenotypic enhancement was evident by ~10 days of age in some F_1 and backcross hybrids, whereas the pigment pattern in *X. variatus* appeared much later, usually at 2–3 months of age. In the backcross hybrid animals, there was a pronounced phenotypic range of P^2 expression; this was not the case in the F_1 or nonhybrid animals. Whether this phenomenon represents a progressive replacement of *X. variatus* pigment pattern suppressors or zygosity effects of *X. helleri* enhancers cannot be addressed from our data.

The apparent lack of involvement of a LG V locus in pigment pattern phenotypic expression may be an indicator of wholesale differences between the Sd pigment pattern of *X. maculatus* (of the classical melanoma model) and the P^2 pigment pattern of *X. variatus* (used here). Previous studies have shown that *X. variatus* nonhybrid animals can develop age-related melanomas stemming from their P^2 pigment pattern, whereas such a phenomenon has never been reported for the Sd pigment pattern of *X. maculatus* (Kazianis and Borowsky 1995; Schartl et al. 1995).

Because numerous other *Xiphophorus* genetic crosses are possible and several have been generated already, further use of the AP-PCR/RAPD markers (or their cloned products as probes for DNA RFLPs), together with use of isozyme and other DNA polymorphisms, will test the generality of involvement of LG V and/or U7 loci in pigment pattern expression.

In summary, incorporation of AP-PCR/RAPD, pigment pattern, and isozyme loci in the present study enabled an overall expansion of the *Xiphophorus* genetic map. Linkage analyses resulted in addition of 16 markers to *Xiphophorus* LGs I, II, III, V, IX, X, XII, and XIV. Addition of 5 AP-PCR/RAPD markers to linkage group U6 containing the Tailspot locus (*P*) and designation of eight new unassigned linkage groups with 22 markers were also accomplished. With the 87 AP-PCR/RAPD and isozyme markers used, only 2 of the

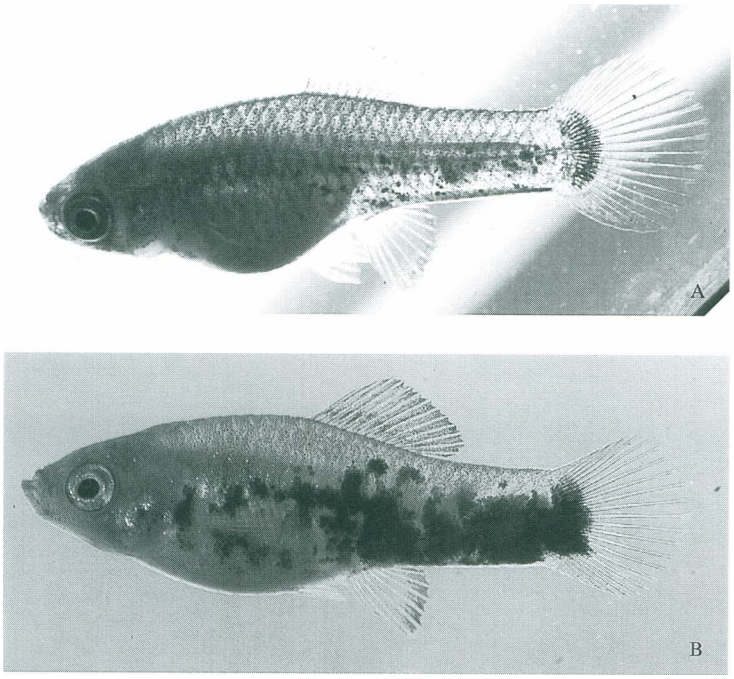

Figure 4 Photographic depiction of temporally early phenotypic modification of the P^2 pigment pattern of *X. variatus*. (*A*) A 1st rank/less severe fish; (*B*) a sibling of identical age that was categorized as 1st rank/severe.

14 known independently assorting linkage groups (IV and VI) were not represented. Analyses of independent assortment of markers in linkage groups U7–U14 described here versus markers within the 21 previously described linkage groups (I–XIV, the *X. maculatus* sex chromosome group XXIV, and U1–U6) in additional genetic crosses should coalesce the 29 linkage groups now comprising ~150 loci into 24 groups residing on each of the 24 acrocentric or telocentric chromosome pairs (Ohno and Atkin 1966). Thus, genetic mapping approaches using AP-PCR/RAPD markers combined with conventional linkage analysis of DNA and protein polymorphisms have the potential to be an invaluable tool in the genetic analysis of fishes of the genus *Xiphophorus*. This technique allows for the creation of a large amount of marker loci and their association with established anchor loci in a short amount of time by a limited number of workers. Cloning of genes such as the *Diff* tumor suppressor gene will probably be accomplished only after extensive mapping has been performed.

METHODS

Experimental Animals and Genetic Crosses

Male *X. variatus* carrying the P^2 macromelanophore pattern and either the crescent (C) or cut-crescent (Ct) micromelanophore tail spot patterns were descended from individuals collected in the Arroyo Zarco, Tamaulipas, Mexico (Borowsky 1984). *X. helleri* (Sarabia strain originally collected from the Rio Sarabia, Rio Coatzacoalcos drainage, Oaxaca, Mexico) was obtained from Dr. Klaus D. Kallman of the *Xiphophorus* Genetic Stock Center and the New York Zoological Society.

Hybrids were created without the use of artificial insemination. Interspecific hybrids were backcrossed to *X. helleri* to produce first backcross (HHV-BC_1) individuals. In all, 170 individuals were used in the mapping endeavors. Fishes were kept in 6, 17, and 40 liter aquaria under conditions very similar to those described previously (Gordon 1950). Laboratory temperature was usually 22°C (±2°). The diet of fish fry was supplemented by the use of *Artemia salina* (brine shrimp) nauplii.

Ranking of P^2 Pigment Pattern Expression

First-generation hybrid backcross animals to *X. helleri* were phenotypically ranked in two ways. The first ranking relied on the fact that siblings differed in P^2 pigment pattern coverage when they were examined at 2 months of age. Fish were examined and ranked in tanks with white surroundings, then segregated into 1st rank/severe and 1st rank/less severe melanotic hyperplasia classes. Fish were ranked a second time when sacrificed. In this case, amount of P^2 body coverage was used and relative rankings were made within broods with separate rankings for males and females. This second ranking was performed independently of the first ranking, and broods were ranked according to their sex classes. As male hybrids (F_1 or HHV-BC_1) generally showed a greater phenotypic expression of P^2 (data not shown), a "2nd rank/less severe" male could have P^2 coverage equal to a 2nd rank/severe female sibling. Increased phenotypic expression in males has also been documented for the Sd macromelanophore pattern in classical cross hybrids (Siciliano et al. 1971).

Isozyme Analyses

Tissue preparation, vertical starch gel electrophoresis, histochemical staining, and genotypic assignment of allozyme phenotypes follow previously published conventions and protocols (Morizot and Schmidt 1990; Morizot et al. 1991). Polymorphic loci within backcross hybrids were *ACO1, ADA, CKM, ES1, ES2, ES3, FBP1, G6PD, GAPD1, GDA, GDH, GLA, GLYDH, GOT1, GPI1, IDH2, MDH2, MP5, PEPA, PEPS, PGAM1, PGAM2, PGK, PGM, PK2, PVALB2,* and *UMPH1*. Locus abbreviations are those of Morizot et al. (1991), with the exception of *FBP1*, which stands for fructose-1,6-diphosphatase-1 or fructose bisphosphatase-1.

DNA Extraction, Quantitation, and PCR

DNA extractions were performed according to published protocols (Kazianis and Borowsky 1995). Aside from standard quantitation by spectrophotometry, additional quantitation was necessary for AP-PCR/RAPD analysis. This was performed by analyzing dilutions of genomic DNA using 0.8% agarose gels with λ *Hin*dIII size standards of known concentration. AP-PCR/RAPD was performed with two different DNA concentrations for each individual. Oligonucleotide primer names and sequences are located in Table 1.

Ten-microliter PCR reactions contained either 19.5 or 3.9 ng of fish DNA, 200 μM each nucleotide, 0.025 U/μl of AmpliTaq (Perkin-Elmer, Branchburg, NJ), 10 mM Tris-HCl (pH 8.3) and 50 mM KCl. In addition, each reaction con-

Table 1. Oligonucleotide Names and Sequence Composition

Name	Sequence composition
3138	GTCTTGTTGG AGATGCACGT GCCCCTTGC
3139	GGAGAAATTA TGGAGGGAAA T
3140	GTTCCTCAGG ATCAAAGTAT GTAC
3906	CCTCGGTGCT GGAGAA
CP1	GATGAGTTCG TGTCCGTACA ACTGG
CP2	GGTTATCGAA ATCAGCCACA GCGCC
D1	CCCCAGACCT GTTTGTGTTG G
PROM3	AATGACTGGG CAGTGCTAAG G
VARXM1	GAAACTGGAG CAGAATGACG GGTCAGA

Table 2. AP-PCR/RAPD Marker Information

Locus	Primer used	Approximate size (bp)	Linkage group assignment
XD0008	CP1	243	U14
XD0009	3138	1123	U14
XD0010	D1	347	
XD0011	CP1	282	U8
XD0012	3138	1151	XII
XD0013	D1	381	
XD0014	CP1	304	U9
XD0015	CP1	409	
XD0016	CP1	353	U10
XD0017	D1	541	
XD0018	CP1	368	U10
XD0019	3139	587	U8
XD0020	D1	671	
XD0021	D1	681	
XD0022	CP1	513	U6
XD0023	D1	1111	XIV
XD0024	3906	355	II
XD0025	D1	1134	U6
XD0026	3906	405	
XD0027	VARXM1	443	U12
XD0028	3906	420	X
XD0029	3139	664	V
XD0030	3906	603	U11
XD0031	CP2	278	U7
XD0032	CP2	870	
XD0033	3906	678	U9
XD0034	CP2	301	IX
XD0035	CP1	546	U9
XD0036	3906	745	U12
XD0037	CP2	325	I
XD0038	3140	452	
XD0039	CP2	397	III
XD0040	3140	610	U6
XD0041	3139	580	V
XD0042	CP2	424	V
XD0043	3140	732	
XD0044	3140	768	U10
XD0045	3139	709	U9
XD0046	3140	813	U10
XD0047	CP2	291	
XD0048	CP2	667	U6
XD0049	PROM3	334	V
XD0050	CP2	323	U6
XD0051	PROM3	357	
XD0052	CP2	435	X
XD0053	CP2	852	U13
XD0054	PROM3	495	V
XD0055	CP2	456	U11
XD0056	3138	407	
XD0057	PROM3	563	U8
XD0058	CP2	493	
XD0059	PROM3	583	XIV
XD0060	CP2	772	U7

Table 2. *(Continued)*

Locus	Primer used	Approximate size (bp)	Linkage group assignment
XD0061	PROM3	705	
XD0062	CP2	470	
XD0063	3139	245	
XD0064	3138	585	
XD0065	CP2	459	U11
XD0066	3138	1046	
XD0067	PROM3	620	

tained a final concentration of 5.0 mM $MgCl_2$ and 2.0 μM single primer. Each tube was overlaid with 30 μl of mineral oil. An initial denaturation at 95°C was set for 2 min, followed by five cycles of 94°C for 70 sec, 40°C for 3 min, and 72°C for 3 min. Immediately following, 35 cycles of 94°C for 70 sec, 50°C for 1 min, and 72°C for 1.5 min were performed along with a 10 min elongation at 72°C.

The post-PCR samples were made 1× with respect to gel-loading buffer type II (Maniatis et al. 1982). Ficoll within the gel buffer was critical as it seemed to yield sharper DNA bands after polyacrylamide gel electrophoresis (as opposed to use of buffers with glycerol; data not shown).

Amplified DNA samples were first examined using 2.5% agarose gels by employing approximately one-fourth of the above reaction products. These agarose gels only served to assess the overall quantity of amplification and were generally not used to determine genotypic information. Only reactions with robust amplification were employed for subsequent electrophoresis on polyacrylamide gels.

Polyacrylamide Gel Electrophoresis

Nondenaturing 4.0% acrylamide gels were prepared according to standard protocols (Maniatis et al. 1982). Long "sequencing-style" (20 × 50 × 0.15 cm) gels were used with the aid of vertical gel stands (Dan-Kar Plastic Products, Reading, MA). Combs of 25 wells were employed, and gels were prerun for 1 hr before sample loading. ΦX174 *Hae*III marker was used as a molecular weight standard and positive DNA control. Electrophoresis was performed for 12 hr at 400 V.

Aside from the DNA size standard used, each gel had specific control reactions. Because backcross hybrids were usually being examined, both *X. helleri* and *X. variatus* post-PCR samples were loaded stemming from multiple DNA template concentrations.

Silver Staining of Polyacrylamide Gels

To obtain a maximum amount of data per primer, the sequencing style gels were silver stained. An important component was the formation of a clear plastic box (23 × 56 × 7 cm, with a valve in one bottom corner and a removable top). This enabled gentle handling and quick changes of solutions, both critical issues for consistent silver staining.

Our silver staining methodology combined elements of previously published protocols (Merril 1981; Bassam et al. 1991). One liter of fixative was composed of 500 ml of absolute methanol, 100 ml of glacial acetic acid, 25 ml of glycerol, and 375 ml of water. Oxidizer solution contained 1 gram of potassium dichromate and 0.2 ml of nitric acid per liter. One liter of silver stain "solution 1" contained 1 gram of silver nitrate and 1.5 ml of 40% formaldehyde. Silver stain "solution 2" contained 50 grams of sodium bicarbonate, 0.02 gram of sodium thiosulfate, and 1.5 ml of 40% formaldehyde per liter. All water used in the staining procedure was deionized to 13 megohms/cm resistance (at 25°C) or greater, a critical prerequisite for uniform staining.

Gels were treated in 1 liter of fixative for 30 min, oxidizer solution for 30 min, then rinsed six times with water (6 liters for 30 min total). Gels were then placed in silver solution 1 for 30 min and then rerinsed twice with water. They were subsequently immersed in silver solution 2 until adequately stained (usually 1 min). The reaction was then immediately stopped by adding 1 liter of 10% acetic acid. Gels were photographed and stored in heat-sealed plastic bags in 5% acetic acid.

Mapping Analysis

For an AP-PCR/RAPD band to be considered a marker, it had to be present in *X. variatus* (positive control) and not in *X. helleri* (negative control). Bands were sized by manually measuring and comparing mobilities to ΦX174 *Hae*III size standards. Band sizes were subsequently estimated using the Dnafrag computer program (Schaffer and Sederoff 1981). Names of generated AP-PCR/RAPD markers, specific primers used, and approximate band sizes are listed in Table 2.

Phenotypic, isozyme, and AP-PCR/RAPD marker locus data were treated identically. All data were entered into a computer spreadsheet and tested for segregation with a Yates corrected χ^2 analysis ($P > 0.01$). If deviation from the expected 1:1 homozygote/heterozygote ratio was significant, a locus was excluded from subsequent analysis.

Marker loci were analyzed for linkage using both Mapmaker (version 3.0b; Lander and Green 1987) and

JoinMap (version 1.4; Stam 1993) computer programs. Markers were not considered linked unless a LOD score of 3.0 or higher was obtained. Map orders were estimated by Mapmaker using a maximum-likelihood algorithm through exhaustive searches (Lander and Green 1987). Only the most likely map orders are depicted here, although in many cases alternative orders could not be excluded. Marker name information, recombination percentages along with standard errors, and pertinent LOD values have been submitted to the LODSOURCE data base.

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