MATE CHOICE, GENETIC VARIATION, AND POPULATION STRUCTURE IN HYBRID ZONES

A Dissertation

by

ZACHARY WYATT CULUMBER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2011

Major Subject: Biology

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Approved by:

Committee Chair,	Gil Rosenthal
Committee Members,	Charles Criscione
	Adam Jones
	Kirk Winemiller
Department Head,	Jack McMahan

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ABSTRACT

Mate Choice, Genetic Variation, and Population Structure

in Hybrid Zones. (December 2011)

Zachary Wyatt Culumber, B.S., University of Illinois Urbana-Champaign;

Chair of Advisory Committee: Dr. Gil Rosenthal

Natural hybrid zones provide opportunities to study a range of evolutionary phenomena from speciation to the genetic basis of fitness-related traits. Additionally, investing the structure of hybrid zones can provide valuable insight in the ecology and evolution of species. The present dissertation approaches the investigation of natural hybrid zones between *Xiphophorus birchmanni* and *X. malinche* from a population genetics perspective. The goal of the chapters herein are to investigate the genetic structure of these natural hybrid zones overall and the genetic structure of the populations within them in an effort to better understand the factors producing and maintaining spatial genetic patterns among this species pair and their hybrids.

Using informative single nucleotide polymorphisms (SNPs) in one mitochondrial and three nuclear intron loci, I show that hybrid zones occur in replicated fashion in multiple stream reaches along a gradient from high to low elevation. Tests of F_{IS} and linkage disequilibrium (LD) revealed significant genetic

structure within a small subset of populations. Specifically, parentals and hybrids all three occur in some locations while other locations appear to be hybrid swarms.

I then investigated a behavioral mechanism of reproductive isolation – social association, which might affect population structure. In clean water, individuals shoaled significantly more closely with conspecifics. Additionally, genotyping of females and their embryos revealed signatures of non-random mating in structured populations. Taken together, assortative social grouping, which may translate to assortative female mate choice, likely plays a role in maintaining population structure. Finally, I show that fluctuating asymmetry is significantly higher in unstructured than structure populations. This is a further indication that some form of non-random mating occurs in structured populations and has effects on male phenotypes.

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Rowdy Tobler, Heidi Fisher, Mariana Mateos, and Don Shepard. I owe a debt of gratitude to The Applied Biodiversity Science core students and particularly the ABS co-directors, Lee Fitzgerald and Amanda Stronza and coordinators Liz Shapiro and Leslie Ruyle, for all their help and guidance during my dissertation studies.

I have to say thank you to my family for their continual support during my time in Texas and Mexico, and especially for their encouragement of my pursuit of a doctorate in something that – let's face it – does not make much sense to them most of the time! And last but certainly not least, to my fiancé, Liz Bautista-Hernandez, for her un-yielding patience, willingness to listen to me rave or rant in good times and bad, respectively, and encouragement to persevere during the toughest times. Gracias por estar a mi lado!

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CHAPTER I

INTRODUCTION

Natural hybridization is an important evolutionary mechanism in the diversification of both plants and animals (Arnold 1992; Rieseberg 1997; Barton 2001). More specifically, hybridization is at once an agent of speciation (Arnold 2006; Melo et al. 2009) and a unique model for mechanisms of reproductive isolation (Rieseberg and Carney 1998; Mallet 2007). Mating decisions – pre-zygotic mechanisms that bias the frequency of gametic pairings –play a critical role in determining the evolutionary fate of natural hybrids. Mate choice determines whether hybridization occurs at all (Ryan and Rand 1993; Fisher et al. 2006) and whether males of one species mate with females of the other more often than the reverse (Wirtz 1999). Further, mate choice influences hybrid fitness, trait introgression, and hybrid speciation (Burke and Arnold 2001; Meyer et al. 2006; Stein and Uy 2006). Specifically, if hybrids and parentals mate assortatively, hybrids with hybrids and parentals with con-specific parentals, this can lead to hybrid speciation (Melo et al. 2009). If females exhibit no mate preference this should lead to hybrid swarms, with frequent hybrid backcrossing.

This dissertation follows the style of *Molecular Ecology*.

Disruption of female mate choice can lead to the collapse of species boundaries and hybridization (Seehausen et al. 1997; Taylor et al. 2006). Disruption of female choice has also been documented in swordtail fish, the study system in this proposal (Fisher et al. 2006).

Most studies of mate choice and hybridization have focused on preferences in parent species in the context of reinforcement or reproductive character displacement, in cases where there is a viability cost to hybridization (Doherty and Gerhardt 1984; Hobel and Gerhardt 2003; Smadja and Ganem 2005; Arnold 2006). Few studies have addressed the role of mate choice in maintaining natural hybrid zones, and most studies have focused on natural or artificial F_1 hybrids (von Helversen and von Helversen 1975; Doherty and Gerhardt 1984; Kyriacou and Hall 1986; Christophe and Baudoin 1998; Pfennig and Simovich 2002). More importantly there is a paucity of empirical evidence demonstrating the role of pre- and post-zygotic mechanisms of isolation in hybrid speciation (Figure I-1).

Pre-zygotic isolation can be either pre-copulatory or post-copulatory. Pre-copulatory would be behavior such as spatial use of the habitat (with whom do parentals and hybrids associate) or female mate choice. Additionally, post-copulatory mechanisms such as cryptic female choice (Eberhard 1996) and sperm competition can result in differential fertilization and result in isolation. Con-specific sperm precedence is one mechanism of fertilization bias that has been observed in a variety of taxa (Price 1997; Fricke and Arnqvist 2004; Geyer and Palumbi 2005). Finally, **post-zygotic** differential survival of offspring can also be an

isolating mechanism. Despite the various possible forms of reproductive isolation, the breadth of these mechanisms and their roles in reproductive isolation is not commonly tested (but see (Mendelson et al. 2007). Together, these factors determine the evolutionary fate hybrids and whether speciation occurs (reviewed in (Bolnick 2009)).

Pre-zygotic	**Pre-mating**	**· Social grouping/habitat use** **· Mate choice**
	Post-mating	**Differential fertilization via sperm competition or cryptic female choice**
Post-zygotic	**Survival differences**	**Early embryo, late embryo, post-parturition**

Figure I-1. Mechanisms of reproductive isolation. Pre- and post-zygotic isolation that could lead to population structuring and speciation in viviparous or ovoviviparous species. Each of these areas will be tested empirically by the proposed research.

The following experiments address the role of these reproductive isolating mechanisms in naturally-hybridizing populations of swordtails, *Xiphophorus*. Swordtails are ovoviviparous and are among the unique group of fishes with internal fertilization. *Xiphophorus* have a gestation period of approximately 30 days on average (Bailey 1933; Turner 1937); but the length of gestation can vary within and among females (Turner 1937) and females can retain viable sperm from previous matings (reviewed in(Constantz 1989). Given these characteristics, pre- or post-

zygotic isolation could be occurring at any stage of reproduction in hybrid swordtail populations.

In my dissertation research, I focused on testing each of the above mechanisms in hybrid populations of *X. birchmanni* and *X. malinche*. I first use a population-genetic approach to characterize geographic variation in population structure. Analyses of initial data indicated that some populations conform to a model of hybrid panmixia, while others show clear separation between parental and hybrid subpopulations. To follow this line of investigation I used behavioral experiments in the field and parentage analysis of wild-caught females and their progeny to characterize pre-mating isolation, mating patterns, and post-zygotic selection.

CHAPTER II

REPLICATED HYBRID ZONES*

Introduction

Natural hybridization is an important evolutionary mechanism in the diversification of both plants and animals (Arnold 1997; Dowling and Secor 1997; Rieseberg et al. 2003). The mixing of divergent genomes from different parent taxa can generate new genetic combinations leading to novel, transgressive phenotypes upon which selection can act (Rieseberg et al. 1999; Bell and Travis 2005). As a result of the variation generated by hybridization, hybrid populations are subject to a broader range of possible evolutionary trajectories (Guillaume and Whitlock 2007; Kalisz and Kramer 2008). The relative fitness of this broad variety of new phenotypes ultimately determines the stability and fate of hybrid zones (Barton and Hewitt 1985; Barton 2001; Burke and Arnold 2001).

Much research on hybridization focuses on effects of intrinsic postzygotic reproductive isolation, where incompatibilities between genes from both parents affect hybrid fitness reducing survivability or fertility (for review see Burke and Arnold 2001). In such cases with reduced hybrid fitness, hybrid zones can be stably maintained by continual immigration of parental forms to the center of the zone (Barton and Hewitt 1985).

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*Reprinted with permission from "Replicated hybrid zones of Xiphophorus swordtails along an elevational gradient" by ZW Culumber, HS Fisher, M Tolber, M Mateos, PH Barber, MD Sorenson and GG Rosenthal, 2011. Molecular Ecology, 20, 342-356, Copyright 2011 by John Wiley & Sons Inc.

More recent work, however, suggests that extrinsic postzygotic isolation, in which hybrid fitness is determined by an interaction of hybrid genotype and the environment, may be more common than previously thought (Schluter 2009; Schluter & Conte 2009; Johannesson et al. 2010). In those cases, a hybrid zone can be maintained with negligible immigration of parental forms, and hybrids are expected to be at least as fit as the parentals in intermediate ecotonal habitats ("bounded hybrid superiority" model; Moore 1977). Since intrinsic isolating mechanisms can be relatively easily assessed in the laboratory, there is a wealth of data on genetic incompatibilities, while environmentally dependent hybrid fitness remains much poorer understood (Wolf et al. 2010).

Environmental effects of hybridization can be studied where different species adapt to local environmental conditions (Kawecki & Ebert 2004), and closely related species occupy proximate but environmentally distinct habitats (Fuller et al. 2007; Tobler et al. 2008). Environmentally intermediate zones, where locally adapted species may come in contact, may facilitate hybridization if there is a breakdown in reproductive isolation. Accordingly, natural hybrid zones commonly occur along gradients of climatic and ecological variables (Yanchukov et al. 2006; Nikula et al. 2008; Ruegg 2008). If selection along environmental gradients is driving hybrid zone formation and maintaining hybrid zone structure, as in the model of "bounded hybrid superiority", hybrid zones with replicated structure should occur in independent tributaries across the landscape wherever the selective

gradient occurs. Swordtail fish of the genus *Xiphophorus* allow us to test this hypothesis in a natural setting.

Xiphophorus birchmanni and *X. malinche* form natural hybrids in the Sierra Madre Oriental of eastern Mexico (Rosenthal et al. 2003). *X. birchmanni* and *X. malinche* are members of the monophyletic, northern or Río Pánuco clade of swordtails (Figure II-1). Kallman and Kazianis (2006) suggested that the northern swordtails diversified as a result of the formation of the Sierra Madre Oriental of eastern Mexico. Uplifting and subsequent folding of the landscape produced isolated species endemic to small geographic areas, like *X. malinche, X. continens*, *X. montezumae*, *X. multilineatus* and *X. nigrensis* (Rauchenberger et al. 1990; Kallman and Kazianis 2006). Phylogenetic hypotheses have differed somewhat in the placement of *X. birchmanni* and *X. malinche*. Most phylogenetic analyses including behavioral, morphological and randomly amplified polymorphic DNA (RAPD) data have placed them as sister species (Rauchenberger et al. 1990; Borowsky et al. 1995; Marcus and McCune 1999; Morris et al. 2001), while Meyer et al.'s (1994) phylogenetic hypothesis based on sequence data from three loci placed them as distant relatives within the clade.

Xiphophorus birchmanni and *X. malinche* differ in a number of morphological traits (Rauchenberger et al. 1990; Rosenthal et al. 2003), most notably the lack of the sexually-dimorphic sword on the caudal fin in *X. birchmanni*, which has been secondarily lost most likely due to a reversal of female sexual preferences (Wong and Rosenthal 2006). The distributions of *X. birchmanni* and *X. malinche* also differ.

Xiphophorus malinche is typically found in headwaters and highland streams, while *X. birchmanni* are found at lower elevations (Rauchenberger et al. 1990; Rosenthal et al. 2003; Gutiérrez-Rodríguez et al. 2008). *Xiphophorus birchmanni* has a wider distribution overall but borders the distribution of *X. malinche* in our study area, and the two were previously documented in sympatry without mention of hybrids (Rauchenberger et al. 1990). Natural hybrids between these species have been abundant in populations at least since the late 1990's (Rosenthal et al. 2003). Morphological and electrophoretic studies of specimens collected in 1997 revealed extensive hybridization at intermediate elevations of the Río Calnali with an upstream-to-downstream gradient from *malinche*-type to *birchmanni*-type morphological and isozyme traits (Rosenthal et al. 2003). The breakdown in reproductive isolation in areas of parapatry may be facilitated by recent increases in organic pollution that resulted in impaired ability to distinguish between species-typical olfactory cues (Fisher et al. 2006).

In the present study I investigated the *X. birchmanni-X. malinche* hybrid zones with three primary objectives: (1) To test whether the parental populations at either end of the hybrid zone are indeed phylogenetically distinct *X. birchmanni* and *X. malinche*. (2) Develop informative SNP markers from DNA sequence data. (3) Test for an upstream to downstream gradient in genetic markers in multiple stream reaches.

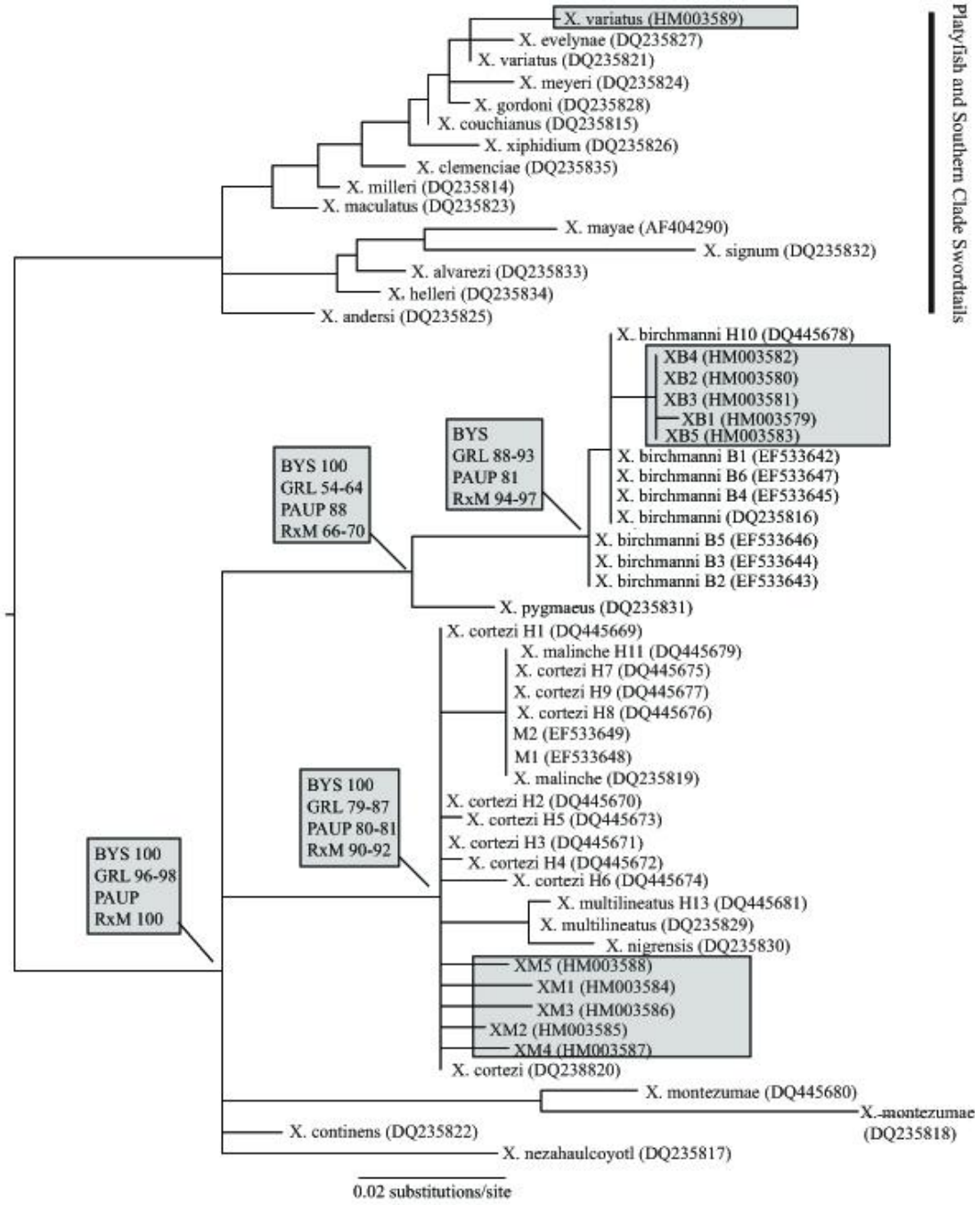

Figure II-1. Strict consensus tree. Tree based on analyses using three maximum-likelihood (ML) models implemented in PAUP. Bootstrap support values from ML analyses (GRL-Garli, PAUP, and RxM-RAxML) and Bayesian posterior probabilities (BYS-MRBAYES) from separate analyses are shown for the nodes of interest. Values not shown were below 50. Sequences from the present study are highlighted in gray. XM2, the only mitochondrial sequence observed in *X. cortezi*, is shared with *X. malinche.*

I use phylogenetic analysis of mitochondrial sequence data to demonstrate that *X. birchmanni* and *X. malinche* form two distinct lineages found at opposite ends of an elevational gradient. I further show that hybrids are present in at least seven distinct stream reaches separated by mountain ridges and that each hybrid zone is characterized by elevational gradients in allele frequencies. By analyzing patterns of co-segregation in single nucleotide polymorphism (SNP) markers designed from mitochondrial and unlinked nuclear DNA loci, I show substantial variation in population structure both within and among stream reaches.

Methods

Sampling

I collected whole specimens or fin clips of *X. malinche, X. birchmanni* and their hybrids from 39 sites in the states of Hidalgo, San Luis Potosi and Veracruz, Mexico between 2003 and 2007 (Figure II-2, Table II-1). Though collecting occurred between 2003 and 2007, for each population, samples from only one point in time were used for SNP genotyping. At a few sites, samples were collected in both 2003 and 2007, with the first sample used for DNA sequencing and the second for SNP genotyping. As previous mark-recapture experiments found no recaptures after three years (GGR and HSF unpublished data), it is unlikely I re-sampled any of the same individuals. Another northern swordtail, *X. cortezi,* was collected from the Río Axtla due to past uncertainties of its relationship with *X. malinche* (see sources above on placement of *X. birchmanni* and *X. malinche*). For outgroup comparison, the more distantly related variable platyfish, *Xiphophorus variatus*, was collected from the Río

Garces and the Río Venado. Tricaine methylsulfonate (MS-222) was used to anesthetize fish for photographing or euthanize fish prior to preservation. For genotyping, I either removed a small piece of the upper portion of the caudal fin, or preserved the whole fish. Tissues were stored in 70-95% ethanol.

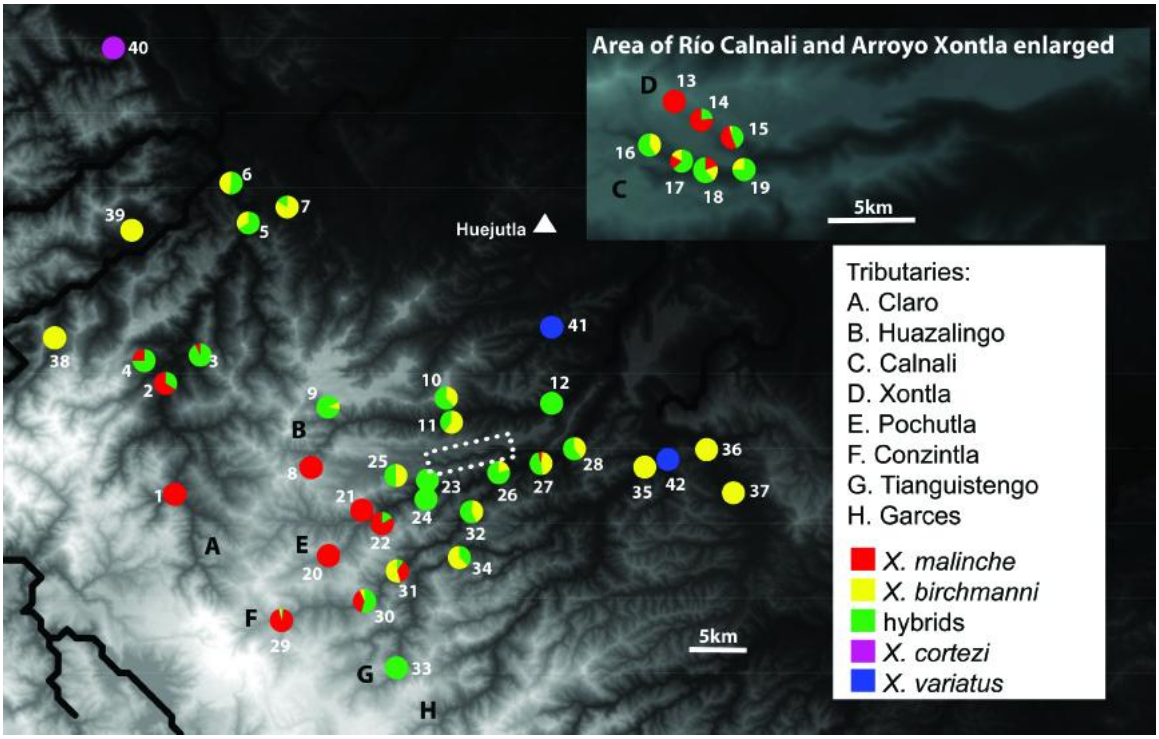

Figure II-2. Sampling localities, frequencies, and geographic distribution. Map of parental species and hybrids. All localities used for initial DNA sequencing and SNP genotyping are shown with *X. malinche* (red), *X. birchmanni*, (yellow) and hybrids (green) based on multilocus genotypes of four SNP markers. Two tributaries (C & D) highlighted by a box at the center of the figure are enlarged in the inset image.

Table II-1. Collecting localities for DNA sequencing and SNP genotyping. Superscript M and B following population names indicate pure, distal populations used for SNP discovery. Indicated for each population are: elevation in meters (E) and sample size for sequencing of mtDNA and nuclear introns (SEQ) and SNP genotyping (SNP). For populations with SNP data, the following measures are shown: the proportion of hybrid multilocus genotypes (HYB), deviations from HWE for three nuclear markers (F_{IS}), linkage disequilibrium (LD) corrected for allele frequency [$D/(p_i q_i p_j q_j)^{1/2}$], frequency of *X. malinche* mtDNA (mtDNA), and a hybrid index showing number of individuals in each population with 0-8 *X. malinche* nuclear alleles. F_{IS} and LD values where $p < 0.05$ after correction for multiple comparisons are indicated by an asterisk (*) and where $p < 0.05$ before but not after correction (†).

No.	Locality	Drainage	E (m)	N		HYB	F_{ST}			LD			mtDNA	Hybrid Index - No. of *X. malinche* nuclear alleles								
				SEQ	SNP		LIG1	POLB	TP53	LIG1/POLB	LIG/TP53	POLB/TP53		0	1	2	3	4	5	6	7	8
1	Tlatzintla[M]	Rio Claro	658	12	22	0	-	-	-	-	-	-	1	0	0	0	0	0	0	0	0	22
2	Tamala	Rio Claro	320	6										0	0	0	0	0	1	0	0	4
3	Tlatemaco	Tributary of Rio Claro	480		30	0.93	0.016	0.102	0.183	0.040	-0.082	-0.204	1	0	0	0	0	2	4	10	12	2
4	Apantla	Trib. of Rio Claro	352		23	0.74	-0.180	-	-0.007	0.350	0.042	0.177	1	0	0	0	0	1	0	7	9	6
5	Xuchipantla	Rio Claro	193		24	0.71	0.785†	0.184	-0.211	0.246	-0.164	-0.198	0.042	8	13	2	0	0	0	1	0	0
6	Tenexco	Rio Claro	122		19	0.53	1†	-0.161	-0.091	-0.102	-0.081	-0.092	0	9	8	2	0	0	0	0	0	0
7	Huitzitzilingo	Arroyo Tultitlán	161	6										5	0	0	0	0	0	0	0	0
8	Chiquitla[M]	Rio Huazalingo	1499	6										0	0	0	0	0	0	0	0	6
9	Totonicapa	Rio Huazalingo	720		30	0.91	-0.335	0.039	0.202	-0.414†	0.298	-0.173	0.414	4	6	6	2	5	4	3	0	0
10	Cocalaco	Trib. of Rio Huazalingo	450		30	0.63	-	-0.025	-0.063	-	-	-0.019	0	11	14	4	1	0	0	0	0	0
11	San Pedro	Rio Huazalingo	384	6	39	0.41	-	0.646†	0.063	-	-	-0.102	0	25	9	5	0	0	0	0	0	0
12	Achiquihuixtla	Rio Huazalingo	186	6										1	3	2	0	0	0	0	0	0
13	Chicayotla[M]	Arroyo Xontla	1003	6										0	0	0	0	0	0	0	0	6
14	T-Dubs	Arroyo Xontla	986		30	0.27	-	0.478	-0.055	-0.034	-0.033	-0.070	1	0	0	0	0	0	0	1	7	22
15	Spider	Arroyo Xontla	921	6	19	0.53	0.234	0.463	0.495	0.322	0.167	0.28	0.947	1	0	0	0	0	0	1	9	8
16	Calnali-High	Rio Calnali	1168		30	0.6	0.057	0.604*	0.609*	0.416†	0.478*	0.798*	0.433	12	3	2	0	0	2	4	7	0
17	Calnali-Mid	Rio Calnali	1007	6	30	0.63	0.414†	0.216	0.861*	0.546*	0.510*	0.484*	0.333	5	8	4	3	0	0	0	4	6
18	Aguazarca	Rio Calnali	981		41	0.61	0.371	0.425†	0.799*	0.428†	0.535*	0.466*	0.39	8	10	6	1	0	0	2	6	8
19	Calnali-Low	Rio Calnali	920		28	0.71	0.424	-0.256	-0.174	0.141	0.163	0.373	0.1	9	10	4	0	2	2	1	0	0
20	Cuilhuacán[M]	Arroyo Pochutla	1272	6										0	0	0	0	0	0	0	0	6
21	Pochutla-High	Arroyo Pochutla	877	5										0	0	0	0	0	0	0	1	4
22	Pochutla-Trib	Arroyo Pochutla	916	6										0	0	0	0	0	0	0	0	6
23	Nicolasia	Trib. of Arroyo Pochutla	440		14	1	0.217	-0.040	-0.110	-0.404	0.269	0.191	0.429	0	3	2	1	2	2	4	0	0
24	Tula	Rio Tula	422	5	30	1	0.016	0.084	0.223	-0.427†	-0.417†	0.142	0.033	0	0	4	12	11	3	0	0	0
25	Ahuamole	Trib. of Arroyo Pochutla	869	6										2	2	0	0	1	0	0	0	0
26	Papatlata	Rio Atlapexco	272	8	19	0.79	0.077	0.384	-0.333	0.032	0.523†	-0.381	0	4	5	7	2	1	0	0	0	0
27	Huitzmopala	Rio Atlapexco	244	5	30	0.6	-0.094	0.133	-0.160	0.315	0.253	0.386†	0	16	5	6	3	0	0	0	0	0
28	El Arenal	Rio Atlapexco	219	5										2	3	0	0	0	0	0	0	0
29	Malila[M]	Rio Conzintla	1364	6	30	0.03	1*	0.659†	1*	0.856*	0.996*	0.856*	0.93	1	1	0	0	0	0	0	0	28
30	Xochicoatlán	Rio Conzintla	1012		29	0.24	0.711*	0.833*	0.738*	0.785*	0.656*	0.570*	0.759	6	0	2	0	0	0	1	4	17
31	Mixtla	Rio Conzintla	941		10	0.1	0.816†	1*	1*	0.903*	0.903*	1*	0.4	5	1	0	0	0	0	0	0	4
32	Comala	Rio Conzintla	383		29	0.59	-0.120	-0.167	1	-0.153	0.184	0.166	0	12	14	2	1	0	0	0	0	0
33	Soyatla	Rio Tianguistengo	1287	6										0	0	0	0	1	0	4	1	0
34	Tlacolula	Rio Tianguistengo	469		30	0.37	-0.160	-	-	-0.054	-0.054	-0.017	0	19	11	0	0	0	0	0	0	0
35	Garces[R]	Rio Garces	229	7										7	0	0	0	0	0	0	0	0
36	Agua Fria[R]	Rio Zontecomatlán	241	3										3	0	0	0	0	0	0	0	0
37	Benito Juárez[R]	Rio Zontecomatlán	272	4										4	0	0	0	0	0	0	0	0
38	Tenango	Arroyo Tenango	274	6										6	0	0	0	0	0	0	0	0
39	Mamey	Arroyo Mamey	292	6										6	0	0	0	0	0	0	0	0
40	Xilitla	Rio Axtla	1889	5										*X. cortezi*								
41	Atlatipa	Rio Venado	172	6										*X. variatus*								
42	Garces	Rio Garces	229	5										*X. variatus*								

DNA extraction, mtDNA and intron amplification and sequencing

Whole genomic DNA was extracted from fin clips with a DNeasy tissue kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. The mitochondrial control region d-loop (CR) was amplified with polymerase chain reaction (PCR) using primers CR-A and CR-E (Lee et al. 1995). Reaction conditions for 15μL PCRs were as follows: 10X PCR Buffer, 2.5mM $MgCl_2$, 0.5μM each F and R primers, 1.5μL dNTPs, 0.625U Taq polymerase, 1μL genomic DNA (gDNA) at 20ng/μL and ddH_2O to 15μL. A hot-start PCR was used for mtDNA. Briefly, samples containing all of the above components except Taq polymerase were placed in a thermocycler. A touchdown thermocycler protocol was initiated but the program was paused when it reached 80C at which time Taq polymerase was added to each sample. The program was resumed and consisted of the following: 94°C for 7min, 12 cycles of 94°C for 15s, 66-60°C for 15s decreasing by 0.5°C each cycle, 72°C for 1min followed by 28 cycles at 60°C with a final extension step at 72°C for 7min.

I amplified three nuclear introns that map to distinct genetic linkage groups in crosses of *X. maculatus* and *X. hellerii* (Walter et al. 2004). DNA ligase 1 (LIG1; linkage group VI), DNA polymerase beta (POLB; linkage group XII), tumor protein 53 (TP53; linkage group XIV). The same PCR conditions and touchdown protocol as above were used for introns except a hot-start was not used. Following all PCRs of introns and mtDNA, 2μL of each sample was scored on a 2% agarose gel to confirm amplification success and the remaining volume was then sent for

sequencing in both forward and reverse directions at either High Throughput Sequencing Solutions (University of Washington) or the Nevada Genomics Center (University of Nevada – Reno). All sequences were aligned in Sequencher 4.2 or higher (Gene Codes Corp.) with further manual adjustments made by eye.

Phylogenetic analyses

To reconstruct phylogenetic relationships among taxa, I used all mtDNA haplotypes of *X. birchmanni*, *X. malinche*, *X. cortezi* and *X. variatus* sequenced in this study, as well as other homologous *Xiphophorus* sequences downloaded from Genbank (accession numbers: AF404290, DQ235814-DQ235835, DQ445669-DQ445681, and EF533642-EF533649). The monophyly of the northern swordtails, including *X. birchmanni* and *X. malinche*, is well supported (Meyer et al. 1994; Borowsky et al. 1995; Marcus and McCune 1999; Morris et al. 2001), so I used all other *Xiphophorus*, the platyfish and the southern clade swordtails, collectively as an outgroup. Sequences downloaded from Genbank were trimmed such that only the portion of sequence that overlapped with our control region d-loop sequences was used. This resulted in sequences that ranged from 417 to 446bp in length and 55 total sequences for phylogenetic reconstructions, as there were multiple haplotypes for some species (alignment submitted to TreeBase).

Phylogenetic analyses were conducted using maximum-likelihood (ML) and Bayesian inference. To determine the most appropriate model of DNA substitution I used jModeltest V0.1.1 (Posada 2008), to evaluate 88 substitution models, under the Akaike Information Criterion (AIC), corrected AIC(c), and Bayesian

Information Criterion (BIC). Models that accounted for 95% of the weight for all three criteria (AIC, AICc, and BIC) included either a proportion of invariable sites (I), a Gamma distribution for rate variation among sites (G), or both, and several relative rates in the substitution matrix (TIM2 = 4; TrN = 3; TIM3 = 4 , GTR = 6). I used these models or the closest more complex model available in ML analyses, including bootstrap, using three different programs: (a) PAUP*4b10 (Swofford 2001), in which parameters values were fixed to those obtained with jModeltest (models: TIM2+G, TIM2+I+G, TIM2+I); (b) RaxML 7.0.4 (Stamatakis 2006b, a; Stamatakis et al. 2008) with two different models (GTR+G and GTR+G+I) and number of bootstrap replicates determined automatically, as implemented in the CIPRES portal (http://www.phylo.org/); and (c) GARLI v.0.96beta8 (Zwickl 2006) with three different models (GTR+G, GTR+I and GTR+G+I) and 100 bootstrap replicates, also implemented in CIPRES. In addition, I conducted Bayesian analyses (models: GTR+G, GTR+I and GTR+G+I) using the Parallel version of MrBayes v 3.1.2 (Ronquist and Huelsenbeck 2003) implementing four runs, with four chains each, for 50,000,000 generations sampled every 1000 generations (all other parameters were default). The appropriate "burnin" length (i.e., samples discarded prior to reaching stationarity) was determined based on small and stable average standard deviation of the split frequencies, potential scale reduction factor close to 1, and stable posterior probability values (see MrBayes manual).

Single nucleotide polymorphism markers

Sequences from specimens collected in 2004 and earlier from pure *X. birchmanni* (N=14 individuals from 3 populations) and pure *X. malinche* (N=36 individuals from 5 populations) were compared by manual alignment in Sequencher. Populations and sample sizes within populations for SNP development are given in Table II-1. In order to identify informative differences between the two species, I sequenced *X. birchmanni* and *X. malinche* from distal populations that matched the documented distribution and morphological traits of the two species (Rauchenberger et al. 1990; Gutiérrez-Rodriguez et al. 2008, Rosenthal et al. 2003). Furthermore, in all distal populations used to determine SNPs, all mature males matched the diagnostic morphological characteristics of only one species (GGR unpublished data). Thus, I avoided using any populations in which hybrids existed.

I used sequence alignments of all alleles for mtDNA (CR) and the three nuclear loci (LIG1, POLB and TP53) to identify SNPs separating *X. birchmanni* and *X. malinche*. These SNPs were used to design bi-allelic Custom TaqMan SNP Genotyping Assays (Applied Biosystems). Briefly, each species-typical TaqMan SNP probe has its own dye color and fluorescence of that color is given off when a copy of that probe is incorporated during PCR. Following PCR on a real-time PCR system, an end-plate reading of fluorescence levels allows assignment of individual genotypes as heterozygous or homozygous for one probe or the other. All SNP PCRs were carried out with 8ng of genomic DNA in 25µL reactions. The 25µL reactions contained 12.5µL TaqMan genotyping Master Mix (Applied Biosystems),

1.25μL custom SNP assay mix (20x), 2μL of 4ng/μL genomic DNA and 9.25μL ddH_20. Samples were run and analyzed on a 7500 Fast Real-Time PCR System (Applied Biosystems) by performing a post-run read of fluorescence after amplification at 95C for 10 minutes followed by 40 cycles of 92°C for 15s and 60°C for 1 minute. All mtDNA and nuclear intron sequences have been deposited on Genbank under accession numbers HM003579-HM003607.

Geometric morphometric analysis

To further confirm that our SNP discovery method produced informative markers, I conducted a geometric morphometric analysis of mature males collected from a population in the Rio Calnali, Calnali-Mid, where *X. malinche*, *X. birchmanni* and hybrid multilocus SNP genotypes are all found together. For the analysis, lateral photographs were taken of all mature males for which I had SNP genotypes. I digitized landmark points on each image using the software program tpsDig (Rohlf 2004). Landmarks included the tip of the upper jaw (1); the anterior (2) and posterior (3) junction of the dorsal fin with the dorsal midline; the junction of the caudal fin with the dorsal (4) and ventral midline (5); the anterior junction of the anal fin with the ventral midline (6); the bottom of the head where the operculum breaks away from the body outline (7); the upper edge of the operculum (8), the upper (9) and lower (10) insertion of the pectoral fin; and the center of the eye (11). I also included four semi-landmarks to estimate the dimensions of the dorsal and caudal fin (including the sword - an extension of the lower caudal fin ray) by digitizing the length of the first and last fin rays in both fins. To remove potential

effects of differential fin expansion, x and y coordinates were standardized for the dorsal and caudal fin (i.e., the location of semi-landmarks only varied in the distance but not the angle from other landmarks).

Based on the coordinates of the digitized landmarks, a geometric morphometric analysis was performed (e.g., see Zelditch et al. 2004 for an introduction to geometric morphometric analyses). Landmark coordinates were aligned using least-squares superimposition as implemented in the program tpsRelw (Rohlf 2007) to remove effects of translation, rotation, and scale. Based on the aligned coordinates, I calculated centroid size and partial warp scores with uniform components for each individual (weight matrix). The weight matrix was subjected to a principal component analysis based on a covariance matrix to reduce the data to true dimensionality. Dimensions with eigenvalues >1 were retained as shape variables. Unless otherwise stated, all statistical analyses were performed using SPSS 17 (SPSS, Inc., Chicago, IL, USA).

To test for phenotypic differentiation among parental species, backcrosses, and F2 and later hybrid individuals, I used multivariate analyses of covariance (MANCOVA) to analyze body shape variation (7 principal components accounting for over 77% of the total variation). Putative F1s are not included as none were observed in this sample of individuals. Assumptions of multivariate normal error and homogeneity of variances and covariances were met for all analyses performed. *F*-values were approximated using Wilks' lambda and effect strengths by use of partial eta squared (η_p^2). I tested for effects of "centroid size" to control for

multivariate allometry and included genotype (parental *X. birchmanni*, backcross *X. birchmanni*, F2 and later hybrid, backcross *X. malinche*, or parental *X. malinche*) as a factor. The interaction term was not significant, so only main effects were analyzed ($F_{28,300}$=1.345, P=0.119). Shape variation along the first two principal component axes was visualized with thin-plate spline transformation grids using tpsRegr (Rohlf 2005).

To test for congruence between genotype categories and phenotypes, I conducted a discriminant function analysis (DFA) to determine the percentage of specimens that could be correctly classified to the correct genotype solely based on body shape. To facilitate the DFA, I first removed the effect of allometry by using the residuals of a preparatory MANCOVA. In this MANCOVA, the partial warp scores with uniform components were used as dependent variables and centroid size as a covariate.

Geographic structure of hybrid zones

To characterize elevational gradients in the geographic distribution of each parental species and their hybrids, I mapped the frequency of each species and hybrids for each locality sampled. Individuals with any combination of both *X. birchmanni*- and *X. malinche*-typical SNP markers (including those inferred from original sequence data) were classified as hybrids. Estimates of hybrid frequencies could be biased upwards, if SNPs are not completely fixed within the two parental species. However, estimates could also be biased downwards because some hybrid

individuals will be homozygous at all loci and erroneously classified as a parental. I then conducted MANCOVA on allele frequencies of all four markers. In the MANCOVA, I tested for drainage and elevation effects on allele frequency and tested the interaction terms (drainage*marker and elevation*marker). I further included a repeated measure to test for differences in allele frequencies among the four markers. The analysis included all 23 populations sampled for SNP genotyping (Table II-1).

Population genetic analyses

Deviations from Hardy-Weinberg equilibrium (HWE) in a population can occur if individuals do not mate at random, if there is gene flow into the population, or if there is strong selection (Hartl and Clark 2007). The inbreeding coefficient, F_{IS}, was used to measure deviation from HWE proportions resulting from heterozygote deficiency (*f*, Weir and Cockerham 1984) for each nuclear SNP locus for all populations using GENEPOP V4 (Raymond and Rousset 1995). If there is sufficient gene flow among populations, connectivity is maintained and prevents genetic differentiation among populations. To test for genetic differentiation among populations, I calculated F_{ST} (ϑ, Weir and Cockerham 1994) across the data set and assessed significance using the log-likelihood G-statistic in FSTAT (Goudet 1995; Goudet et al. 1996). The estimate of ϑ as implemented in FSTAT was also used to calculate pairwise-F_{ST} values between neighboring localities within stream reaches.

In hybrid zones, high LD can represent recent or ongoing hybridization, but can also be a sign of population structure (Jiggins and Mallet 2000). Interspecific crosses generate perfect linkage disequilibrium (LD) in F_1 offspring. If F_1 offspring backcross and/or mate with each other, this LD will then erode in each generation. Selection, assortative mating, or continual immigration of parental genotypes could maintain LD in a population. I tested for LD among all pairwise comparisons of nuclear SNP markers in each population according to Hill (1974) which provides a method to estimate of disequilibrium, *D*, and a test statistic that has an approximate $\chi 2$ distribution. Values of *D* were divided by allele frequency $[D/(p_i q_i p_j q_j)^{1/2}]$ to standardize LD estimates across populations. I also calculated genotypic disequilibrium for cases when phase is not known using GENEPOP. All *p*-values were adjusted for multiple comparisons within populations.

Table II-2. Sequence consensus between *X. birchmanni* and *X. malinche.* Interspecific base substitutions are denoted in brackets [*X. birchmanni* base/*X. malinche* base] and the underlined portion is the reporter sequence used in multilocus genotyping with TaqMan SNP chemistry. Brackets containing a colon represent and insertion/deletion event rather than base substitution.

mtDNA Control Region d-loop

TTTCCACCTCTAACTCCCAAAGCTAGGGTTCTAATTTAAACTATTCTTTGACCGGACTCTGCCC
CTCCT**[T/A]**AGTACATGTATGTATTATCCCCATTAATAGATTTTAACCATTTAAAGT**[G/A]**ATGT
AATTCTACATTAATGAAAAATCAAAA**[G/:]**TTATA**[A/G]**GAACTTAAATACATTA**[T/C]**ATCATC
AAATAAATATGAAGGTAGACATAAACCA**[C/T]**T**[A/G]**AA**[C/T]**TT**[C/T]**AAA**[C/T]**T**[C/T]**CATT
AAA**[T/C]**ATGTTA**[T/A]**AAAAATGACGATATTGAATTG**[T/C]**CCTATCA**[T/C]**AACTCTCATCAG
TCTAGATATACCAGGACTCACCAC<u>CTCTGCAAGT**[C/A]**AGAGTC</u>AAATG**[T/C]**AGTAAGAGAC
CACCATCAGTTGATTTCTTAATGTACACGTTTATTGATGGTCA**[A/C]**GGACAAAAATCGTGGG
GGT**[A/C]**GCACACAGTGAACTATTCCTGGCATCTGGTTCCTACTTCAGG

DNA Ligase 1

GCCCTCGCAGGAGTCTGCACAAACAAACACATTAATGCACCAAAACACGAGTCTGGATGTGC
AGAAAGGAAATTAAGAGATTATCTGTTGGATATACGGAGTGTCTTTGTGTTGGTGGGTCTCA
AAGGGAAACAAACGGGTTTTGGGAAACAAAGAGGCCGTGGACAAGGACGGGGACATAACT
CATATGCACAAGGAAAACACATCCTTTATACGTCAGAATCACACACACACACAACACAAAAT
AAATCCAT**[T/C]**ATACAACCCACAAGTTCTGGCGCTGCTTTGGATACACTCACACAGACAGTT
TACACGTTAAGGCAGCTGAGAAAGACACAAATAAAATGAAACTTTTACTCAAAGCACCC<u>ACA
CACG**[C/A]**AGAGATT</u>CAGGGGAGCCAACAAACAGGAA**[G/A]**ATGACACAAACTAGAAGATG
ACACAAAC**[:/T]**AG**[G/A]**A**[:/G]**ATGACACAAAGGAAGGAAAACGAGAAGAAAAAGTTTGCCT
CTGTTTCACAGACCTCGGACG

DNA Polymerase Beta

CTTTCCTATCCACA**[G/T]**TACAGAATAAAAACATTATCGAATGGCTAAAAGAAGTATTTATAA
AGAAAG<u>TACTTTAAAG**[C/T]**TTT</u>AGTAATATGATATGAACAAGCAAGCTAGTTTACAATACCT
TGATCTCATGTGCCATACATTATACAATGAATATATGATTTTGTTATTGTAAAAGGATTTTAAA
ACGTCATTGTCAGAGTATACTCTAAATATCTGACCGGAAGTGAGTGAAAAATGAAGCAGAAA
TAAAACCTTTTGAGCAGATAAATTAAGTCTATCCAAAATAACCACTGAATATGTGGATTCTAC
TCGTATCTGCAAATAAAAGCAAAGGTTTTTAGGAACTCACCTGTATGCATTGTACTTAT

Tumor Protein 53

AAACCTGGAAAAAAGTGGGAC<u>TAAGCAGAC**[G/A]**AAGAAA</u>AGAAGTATGCTTTTAAATTGT
TTTATATGTATGTATAGTAGACTAAAGTTTTATTCTATCTGTCACTTATCATGAACATTCTTCA
TTTTAGAGAGTGCTCCTGCTCCAGATACCTCCACCGCAAAAAAGTCCAAGTCTGCCTCTAGTG
GAGAGGATGAGGACAAGGAGATTTACACTCTCTCTGTAAGGCCTGTTTCTGCAACTGGATGA
CATCACAATATTAGGATGAATGAAGTAACTTTTTTACTTTCTAACTTTGTTGCAGATCCGGGG
CCGTAATCGTTATCTGTGGTTCAAGAGC

Results

Phylogeny and SNP markers

Sequencing of 160 individuals (Table II-1) for the mitochondrial control region d-loop produced 10 distinct haplotypes among *X. birchmanni*, *X. malinche*, their putative hybrids and *X. cortezi*. The only haplotype observed in *X. cortezi* (haplotype XM2; Figure II-1) was also observed in putative *X. birchmanni*/*X. malinche* hybrids and is mutationally intermediate among other *X. malinche* haplotypes. As such, it was considered and designated as a haplotype shared with *X. malinche* (incomplete lineage sorting. There was one haplotype observed for *X. variatus.* The optimal trees from all ML searches were compared to one another and to the consensus tree from two Bayesian analyses. The consensus tree from ML analyses in PAUP implementing the three best-fit models of sequence evolution is reported here along with bootstrap support values from all ML analyses and posterior probabilities from MrBayes shown for nodes of interest (Figure II-1). Here I are primarily concerned with the relationships among *X. birchmanni*, *X. malinche* and *X. cortezi*, thus support values for relevant nodes are given. All runs gave strong support for monophyly of the five *X. birchmanni* haplotypes sequenced in this study along with all other previously sequenced *X. birchmanni* haplotypes. All runs also placed *X. malinche* together with *X. cortezi, X. multilineatus* and *X. nigrensis*.

There were five mitochondrial control region d-loop haplotypes for *X. birchmanni* and four for *X. malinche.* *X. cortezi* and *X. malinche* shared one haplotype, accounting for the total of five haplotypes that clustered with other *X. malinche* on

the phylogenetic tree. The majority of populations (19 of 27) contained only one mtDNA control region haplotype. Both the mtDNA control region d-loop and DNA ligase 1 exhibited an accumulation of substitutions between the species, consistent with historical divergence and more recent secondary contact and introgression (Table II-2). Both the polymerase beta and tumor protein 53 gene showed fewer interspecific differences but contained informative SNPs nonetheless. The complete consensus sequences between *X. birchmanni* and *X. malinche* are shown in Table II-2 with interspecific SNPs in brackets and reporter sequences for TaqMan SNP assays underlined.

Table II-3. Classification results of the discriminant function analysis.

			Predicted Group Membership				
			X. birchmanni	*X. birchmanni* backcross	F2	*X. malinche* backcross	*X. malinche*
Original	Count	*X. birchmanni*	17	3	1	0	0
		X. birchmanni backcross	4	17	1	0	1
		F2	1	2	9	1	0
		X. malinche backcross	0	0	0	19	0
		X. malinche	2	1	0	5	15
	%	*X. birchmanni*	81.0	14.3	4.8	0.0	0.0
		X. birchmanni backcross	17.4	73.9	4.3	0.0	4.3
		F2	7.7	15.4	69.2	7.7	0.0
		X. malinche backcross	0.0	0.0	0.0	100.0	0.0
		X. malinche	8.7	4.3	0.0	21.7	65.2

Geometric morphometric analysis

Among 99 males, body shape was significantly influenced by centroid size, indicating allometric effects ($F_{7,87}$=12.461, P<0.001, η_p^2=0.501), and genotype

($F_{28,315.1}$=4.045, P<0.001, η_p^2=0.242). Visualizing the first two axes of shape variation indicates that backcrosses cluster with the respective parental species, with F2s intermediate between the two parental species (Figure II-3). This is also indicated by the discriminant function analysis that assigned over 77% of the specimens (compared to the expected 17% under a null hypothesis of no pattern) to the correct genotype solely based on geometric morphometric data (Table II-3); misclassifications were relatively common between parentals and backcrosses. Over 30% of F2 or later hybrids were misclassified, indicating overlap in morphospace occupation with other groups.

Replicated geographic structure

There were elevational clines in the distribution of each species and hybrids, with *X. malinche* at high elevations, *X. birchmanni* at low elevations and hybrids at intermediate elevations. These gradients were replicated across stream reaches as demonstrated in the distribution of multilocus genotypes (Figure II-2).

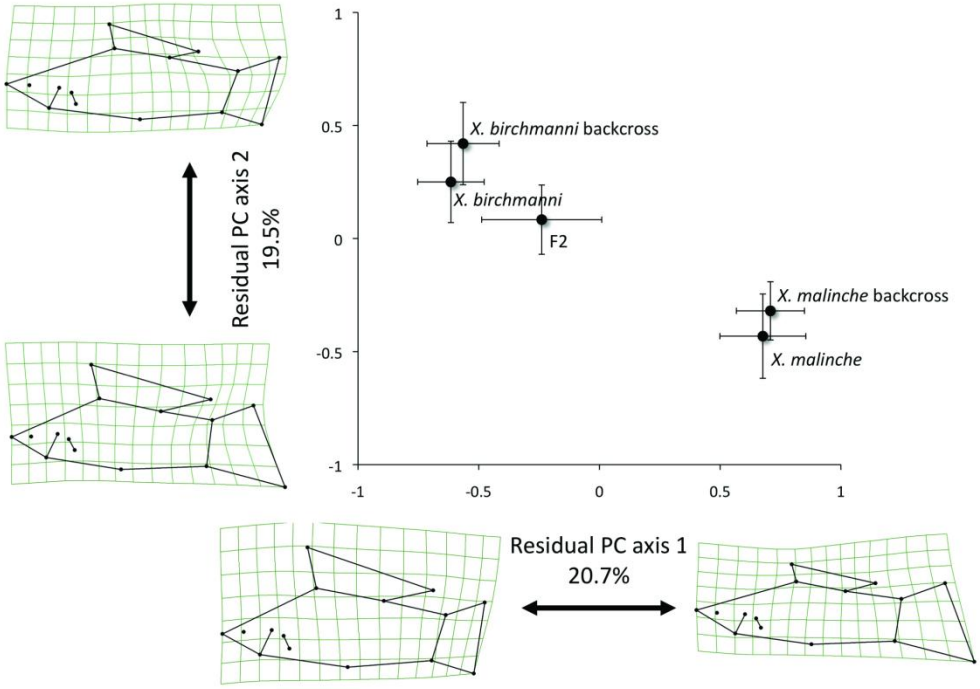

Figure II-3. Body shape variation in male *Xiphophorus.* Depicted are mean residual principal component scores (corrected for allometric effects) and standard errors of measurement for parental species, backcrosses, and F2s. Note that backcrosses cluster with parental species. The thin-plate spline transformation grids show shape variation along each principal component axis.

MANCOVA on SNP marker frequency revealed significant effects of both elevation (F=7.655, d.f. = 6, p=0.001) and drainage (F=36.852, d.f. = 1, p<0.001). There was a significant interaction between drainage and marker (F=2.278, d.f. = 18, p=0.013) but not between elevation and marker (F=2.199, d.f. = 3, p=0.101). There was no difference in patterns of allele frequency change among the four markers (Wilk's λ=0.635, F=2.487, d.f. = 3, p=0.106). Figure II-4 shows allele frequency clines for all SNP markers in seven stream reaches.

SNP population genetics

The frequency of hybrids varied widely among localities, ranging from 0 to 0.93. The frequency of hybrid multilocus genotypes for all populations (i.e. localities) is given in Table II-1 and depicted in Figure II-3. Hybrids were found in 22 of the 23 localities sampled for SNP genotyping. In these 22 localities population structure falls along a continuum. Two populations were composed entirely of hybrids. In 14 localities, one parent species coexists with hybrids. In the remaining six populations, both parent species and hybrids are found together. It is in these populations where tests for deviations in the distribution of SNP markers from Hardy-Weinberg expectations produced significant results (Table II-1).

Linkage disequilibrium (*D*) was highly significant in several populations (Table II-1) and estimates of genotypic disequilibrium from GENEPOP produced the same results as calculating Hill's (1974) metric *D* (not shown). In these populations, significant heterozygote deficiency and linkage disequilibrium, potentially indicate some degree of reproductive isolation between *X. malinche, X. birchmanni*, and hybrids. I observed highly significant genetic differentiation among populations (ϑ=0.407; p=0.001). I further tested for differentiation among populations within stream reaches along the upstream-to-downstream gradients. F_{ST} in pairwise comparisons of adjacent populations varied from 0-0.6335 (Table II-4). Values reported as negative are effectively zero, because F_{ST} can only be greater than or equal to zero.

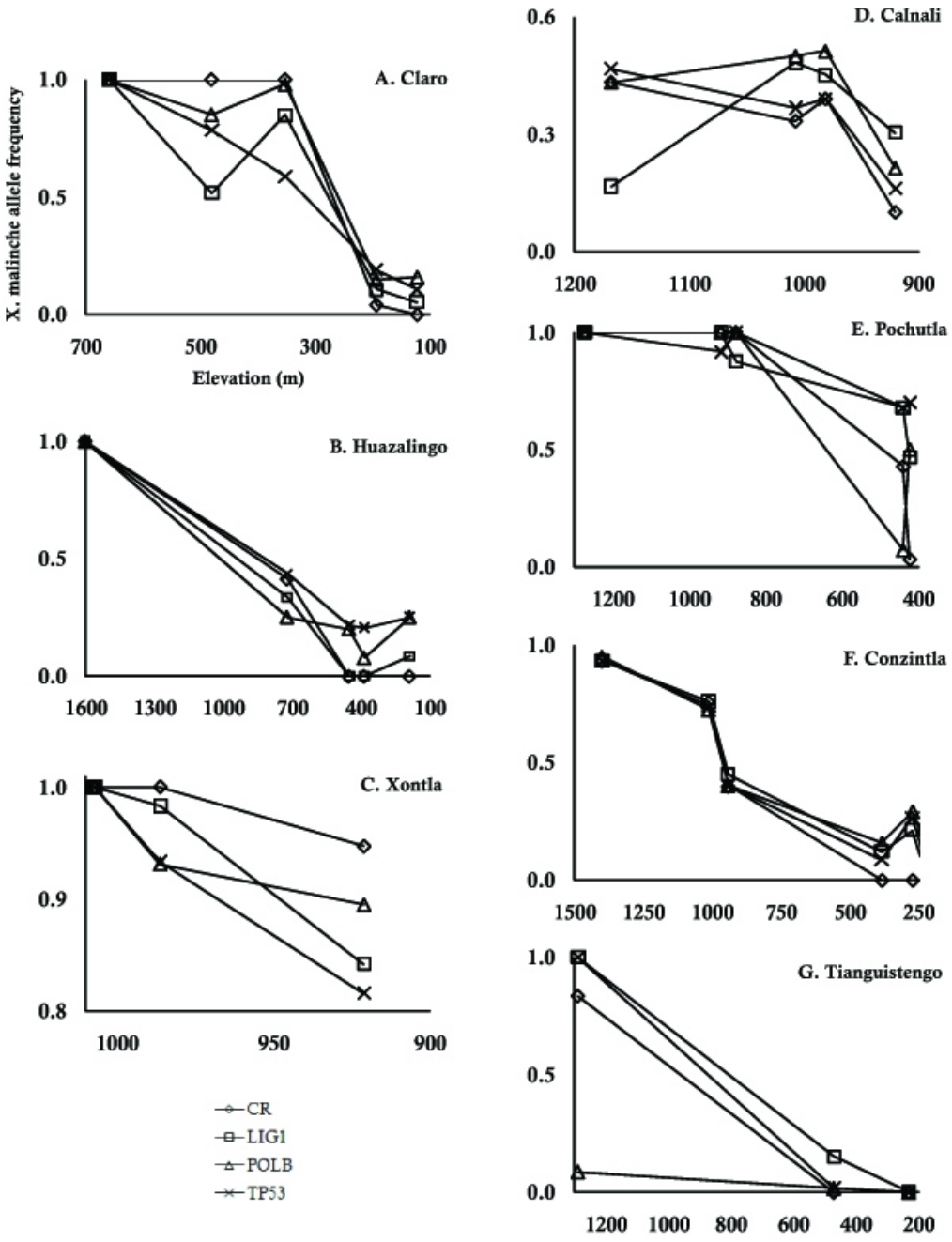

Figure II-4. Allele frequency clines. Clines along each of the seven tributaries as a function of elevation.

Table II-4. Pairwise F_{ST} values. Values shown for each population and the next closest sampling location within stream reaches. In many cases low F_{ST} is likely correlated to the distance between sampling sites and local geography (i.e. barriers to dispersal) as sampling locations are not spaced evenly along stream reaches.

Drainage/Comparison		F_{ST}
Río Calnali		
Calnali-High	Calnali-Mid	0.0541†
Calnali-Mid	Aguazarca	-0.0211
Aguazarca	Calnali-low	0.0936†
Río Claro		
Tlatzintla	Tlatemaco	0.3086*
Tlatemaco	Apantla	0.1298*
Apantla	Xuchipantla	0.6335*
Xuchipantla	Tenexco	-0.0129
Arroyo Pochutla		
Nicolasia	Tula	0.1227*
Tula	Papatlatla	0.1723*
Río Conzintla		
Malila	Xochicoatlán	0.1064†
Xochicoatlán	Mixtla	0.1641
Mixtla	Comala	0.2064†
Xochicoatlan	Comala	0.562*
Comala	Papatlatla	0.0447†
Papatlatla	Huitznopala	0.0106
Río Huazalingo		
Totonicapa	Cocalaco	0.1261*
Cocalaco	San Pedro	0.0095
Arroyo Xontla		
T-Dubs	Spider	0.0435

*$p<0.05$

† significant before, but not after, correction for multiple tests

Discussion

Extensive hybridization was detected in seven separate stream reaches with two main patterns emerging throughout the hybrid zone. First, in each stream reach, there was a distinct elevational gradient between *X. malinche* upstream and *X. birchmanni* downstream. Second, population structure varied widely among sites. At some localities, there were low levels of introgressive hybridization and others were composed completely of hybrid individuals. By contrast, six of the twenty-three sampled populations were highly structured into three groups: hybrids and each of the two parent species. These population genetic patterns likely indicate sharp geographic differences in the dynamics of hybridization and selection.

Phylogeny and SNP markers

Phylogenetic analysis of mitochondrial control region d-loop sequences showed that *X. birchmanni* and *X. malinche* are in distinct and well-supported clades. *X. malinche* haplotypes were more closely related to those in *X. cortezi*, *X. multilineatus* and *X. nigrensis*, but due to limited data and/or incomplete lineage sorting, relationships among these species were not well-resolved. Our results were consistent with previous mtDNA analyses, in which these four species form a well-supported clade that is distinct from a more distantly related *X. birchmanni* (Meyer et al. 1994; Marcus and McCune 1999). Earlier analyses based on nuclear allozymes and RAPDs but on only one or a few individuals or populations per taxon (Borowsky et al. 1995; Morris et al. 2001) suggest a closer relationship between *X. birchmanni* and *X. malinche*, perhaps reflecting the effects of hybridization between

these species. Our results, however, indicated fixed differences between pure populations of these species at several nuclear loci. Sequencing and alignment of these nuclear loci and mtDNA allowed us to identify informative SNPs that separate the two species. In addition to our conservative approach selecting only distal populations matching historical species distributions, and only those populations where 100% of the observed males matched diagnostic morphological traits of only one species, geometric morphometric analyses supported the informative nature of the SNP markers. In a large hybrid population containing *X. birchmanni* parentals, *X. malinche* parentals and hybrids, parental genotypes had parental (pure species) morphology as did their respective backcrosses while hybrid (F2 and later individuals) had intermediate, hybrid morphology.

Replicated geographic structure

One of the strengths of this system is the replication in multiple stream reaches. Hybrid zones between these species occur in at least seven separate streams along similar ecological gradients. Gene flow among hybrid zones and among *X. malinche* populations in stream headwaters is impeded by high mountain ridges, long upstream to downstream distances and geographic barriers in some places. Thus, each hybrid zone likely represents an independent outcome of secondary contact due to a shift towards higher elevation in *X. birchmanni*, perhaps associated with Pleistocene climate change or more recent anthropogenic effects, pushing *X. malinche* into multiple high elevation refugia. Alternatively, as Kallman and Kazianis (2006) suggest, an ancestral form may have been uplifted and populations

isolated by folding of mountain ridges resulting in the current distribution of *X. malinche*. Though commonly touted as natural laboratories, replicating studies of hybridization phenomena is difficult. Nolte et al. (2009) demonstrated the utility of such natural replication, testing for loci contributing to reproductive isolation between two species of sculpin (*Cottus*), in two independent hybrid zones. Even more recently, a study of two independent hybrid zones of cyprinid fish demonstrated the heterogeneity of outcomes of hybridization within a species pair (Aboim et al. 2010). Taking advantage of these and other replicated hybrid zones - like the *birchmanni-malinche* hybrid zones - should continue to provide even greater power in studying evolutionary processes and patterns.

In the *birchmanni-malinche* system, each hybrid zone is characterized by an elevational gradient in the frequency of parental and hybrid individuals and of individual, species-diagnostic SNP allele frequencies. In each stream reach, hybrid zones had clear geographic structure, with *X. malinche* alleles being replaced along an upstream-to-downstream gradient by *X. birchmanni* alleles and with hybrids prevalent at intermediate elevations. Analysis of SNP marker frequencies showed significant effects of drainage and elevation. Mid-elevation hybrid populations contained a preponderance of backcross, F2 or later generation hybrid individuals, whereas F_1s were rare. Out of 760 individuals genotyped for one mtDNA and three nuclear loci, 13 had genotypes consistent with first-generation hybrids. However, only a few of those occurred in populations where both parental forms are present, suggesting that the majority of "F_1" fish were erroneously classified backcross fish.

The factors that maintain hybrid zone structure in this system are not yet known as the data here do not address fitness differences or allow for explicit tests of among hybrid zone models. However, the consistency of overall structure across all stream reaches along the same elevation gradient suggests that selection by the environment could play a role.

The hybrid zones in different stream reaches all exhibited consistent upstream-to-downstream structure, but there were differences among the zones. In the Río Claro, *X. malinche* were found at much lower elevation than observed in other stream reaches. This may reflect differences in historical distributions of species or differences in environmental conditions among stream reaches. Most of the hybrid zones were bounded on each end by pure populations of parental species, but no pure *X. malinche* population is observed in the Río Calnali and no pure populations of *X. birchmanni* occur before the 60m waterfall at Chahuaco. Hybrid populations were composed of hybrids and one parental (or only hybrids) in some hybrid zones, while hybrids and both parentals occur in sympatry in populations in other hybrid zones. This heterogeneity could be a consequence of any number of biotic or abiotic variables such as differences in migration, historical frequency of parentals, connectivity of populations and variation in natural or sexual selection.

SNP population genetics

Heterozygote deficiency and LD were generally low in most sampled populations. Low F_{IS} and LD suggest that hybrids are both viable and fertile and have backcrossed extensively with the parental species; thus, there is little evidence

of reproductive isolation in most locations. This is supported by a low frequency of F_1s and high frequencies of F2 (or later) and backcross individuals in almost all populations. By contrast, when heterozygote deficiency and LD were observed, they co-occurred in the same populations. Both parent species coexist with hybrids in those populations that deviate from HWE and have LD, with hybrids accounting for 10-61% of the population with the exception of Calnali-high where only *X. birchmanni* and hybrids were observed (Table II-1). Genetic differentiation across all populations was significant as expected, and F_{st} between populations within stream reaches was large and significant in some but not all cases. However, pairwise comparisons should be interpreted with caution because distance between populations is not constant and may account for the large variation in pairwise F_{st}. It should be noted, however, that even over distances as short as 200-300 m (22 meters elevation) there was significant differentiation in some cases even in the absence of apparent physical barriers to migration.

As with overall geographic patterns, the extent to which migration and selection from the biotic and abiotic environment play in shaping population-level patterns is unknown. Deviations from HWE and LD could be maintained by migration-selection balance such as in a tension zone model (Reugg 2007; Gay et al. 2008) or if hybrids are relatively fit compared to parentals, with some degree of assortative mating in some populations, as in hybrid superiority (Good et al. 2000). Studies of mate choice have shown that *X. birchmanni* has strong preferences for both visual and olfactory signals of their own males over *X. malinche* males (Wong

and Rosenthal 2006; Fisher et al. 2006). This may play a role in mating patterns in structured populations with high F_{IS} and LD. Additionally, hybrids may have an advantage due to sexual selection. Certain hybrid males are likely preferred by females because they have recombinant phenotypes not possible in pure species males (Fisher et al. 2009). Future work will be able to build on our current understanding of the hybrid zones and the evolutionary processes within them. Persistence of substantial frequencies of parental fish in populations with no source of pure parentals and a tendency for assortative mating in *X. birchmanni* suggest that mating preferences could play a role in population structure. However, measurements of gene flow and fitness-related traits are necessary to begin to weigh the relative contributions of environmental selection to overall geographic structure and the role behavior, migration and selection in both stream and population-level patterns in the hybrid zones.

The mechanisms by which population structure arises and potentially leads to speciation in hybrid populations are not well understood. The breadth of pre- and post-zygotic factors contributing to these processes is rarely tested (but see Mendelson et al. 2007). However, it is the combination and interaction of these various factors that determine the fate of hybrids, population structure and, consequently, whether or not speciation occurs (Bolnick 2009), making hybrid zones useful study systems. The *malinche-birchmanni* hybrid zones and molecular markers described here provide an opportunity to test the effects of selection at different points along the pre- to post-zygotic continuum. Together, behavioral and

molecular approaches in this and other systems can better clarify the processes of hybridization and hybrid speciation.

CHAPTER III

SOCIAL ASSOCIATION

Introduction

Many animals form social groups that are nonrandom with respect to factors like kinship (Garza et al. 1997), size (Croft et al. 2005), or agonistic interactions (Arnold 1997). Such social groupings can play a critical role in reproductive isolation and population structure (Hochberg et al. 2003), particularly if social structure ultimately leads to assortative mating (e.g. Behrmann-Godel et al. 2006; Plath and Strecker 2008). For example, brown-headed cowbirds of different social cultures that exhibit different behavioral patterns preferentially pair and copulate with birds of their own cultural background (Freeberg 1996). Such observations demonstrate how powerful social and behavioral interactions can be, even between populations of the same species, and lead to reproductive barriers that produce subpopulation structure or even speciation (Mendelson et al. 2007; Melo et al. 2009).

The structure of natural hybrid zones, and population structure within them, are commonly studied to provide new understanding of mechanisms of reproductive isolation (Rieseberg and Carney 1998; Mallet 2007). While the overall structure of hybrid zones is commonly the focus of studies (Barton and Hewitt 1985), reproductive isolation, or lack thereof, takes place at the level of populations. Hybrid populations typically take the form of hybrid swarms, where parentals mate

at random resulting in populations of only hybrids, or populations with more structure where one or both parentals are maintained at some frequency.

Hybrid swarms form when no pre-mating mechanisms such as female mate choice (Rosenfield and Kodric-Brown 2003) nor post-mating selection (Burke and Arnold 2001) exist to maintain some degree of reproductive isolation between species. Additionally, even when females are choosy, females may prefer heterospecifics (Shapiro 2001; Stein and Uy 2006) or environmental disturbance can interfere with signaling modalities and lead to hybrid swarms or species replacement (Seehausen et al. 1997). On the other hand, any form of pre- or post-mating selection can be enough to generate population structure and maintain some level of reproductive isolation. For example, female parentals of both species involved in a *Chorthippus* grasshopper hybrid zone strongly prefer the songs of conspecifics males over hybrids (Bridle et al. 2006). Mechanisms of post-mating selection are also important in determining the structure of populations as environmental factors commonly influence survival and determine whether hybrids or parentals are more fit under local environmental conditions (Milne et al. 2003; Pfennig and Simovich 2002; Culumber et al. in prep).

Breakdown of pre-mating, behavioral isolation appears to be an important factor in the origin of hybrid zones between the swordtails *Xiphophorus birchmanni* and *X. malinche* (Rosenthal et al. 2003). Male swordtails produce urine-borne pheromones during courtship (Rosenthal et al. 2011), which are critical to conspecific mate recognition (Derapona and Ryan 1990; Hankison and Morris

2003; Fisher et al. 2006). In *X. birchmanni*, altering water chemistry impairs olfactory communication, eliminating the strong female preferences for conspecific pheromones. Females continue to exhibit reduced preferences in clean water after exposure to a pulse of humic acid, indicating an effect on signal reception (Fisher et al. 2006). This effect of organic pollution on olfactory reception may be responsible for a breakdown in reproductive barriers that has led to widespread hybridization among multiple, independent streams (Culumber et al. 2011).

Population structure varies considerably among hybrid populations. At most sites, population structure is largely absent, with no linkage disequilibrium among genetic loci and no deviation from Hardy-Weinberg equilibrium. In a small group of populations, however, there is significant population structure, with *X. birchmanni, X. malinche,* and hybrids occurring together. While environmental conditions, particularly temperature, allow for the coexistence of all three groups at intermediate elevations (Culumber et al. in prep.), this does not explain the maintenance of distinct subpopulations given that natural hybridization is widespread. Variation in levels of contaminants that disrupt the olfactory environment may result in variation in population structure within and among hybrid zones.

In the present study, I used open field trials to test shoaling preferences of *X. birchmanni*, *X. malinche* and hybrids, all three collected from the same population, before and during disruption of the olfactory environment. Based on the presence of population structure and the findings of Fisher et al. (2006) I predicted that social

groupings would be assortative by species in clean water, but that addition of humic acid to disrupt the chemical signaling environment would lead to a breakdown of assortativity.

Methods

Collection and genotyping

Adult *X. birchmanni*, *X. malinche* and their hybrids were collected in early January 2010 at Calnali-Mid on the Río Calnali (Culumber et al. 2010; Table II-1 and Figure II-2). All individuals were collected with baited minnow traps and transported to the Centro de Investigaciones Científicas de las Huastecas "Aguazarca" (CICHAZ). In order to identify individuals, all fish were given a series of three small marks by injecting colored elastomer just beneath the scales near the caudal peduncle. Colors and positions were varied systematically among species. At the same time, a small fin clip was taken from the upper portion of the caudal fin and stored in 100% ethanol for genetic analyses. They were then maintained at CICHAZ in three 200 liter aquaria until the time of the experiment. Fish were fed TetraMin flake food twice daily and decapsulated brine shrimp once every 2-3 days.

Genotyping followed protocols described in Culumber et al. (2011). Briefly, DNA was extracted from fin clips and each sample was genotyped for one mitochondrial (CR) and three nuclear (LIG1, POLB, TP53) bi-allelic, single nucleotide polymorphisms (SNPs) with custom TaqMan SNP probes (Applied

Biosystems). Individuals were typed as *X. birchmanni* or *X. malinche* if they contained alleles of a single species at all loci, and as hybrids otherwise.

Social grouping experiments

Fish were assigned to one of three experimental replicates. Each replicate had three males and three females each of *X. birchmanni*, *X. malinche* and their hybrids for a total of 18 fish. One replicate had only two *X. malinche* males (total N=17 fish) due to the fact that parentals are at relatively low frequency (15-20%) at Calnali-mid. As a result, the sample of fish collected in January did not contain enough pure *X. malinche* males to have three in the final replicate. In order to enable observation and data collection by eye from above, all experimental fish were marked once more with a series of two colors on the upper body near the dorsal fin. In each replicate, one male and one female of each species had the same color combination and all other male-female pairs, both conspecific and heterospecific, had novel color combinations. Marking was done in this manner to control for any systematic color preference that may exist in any sex or species and to make species identity anonymous to the human observer. Color combinations were randomized among the species across replicates.

Each replicate was placed outside in identical 2 m x1 m concrete tanks filled to a depth of 1 m.). Since *Xiphophorus* occur in streams rocky substrates, , two size-matched rock piles were placed in opposite corners of each tank in order to more accurately imitate natural habitat and promote natural behavior. Fish were acclimatized to the experimental tanks for five days and food was administered once

daily at 9AM. Observations of shoaling were completed on two consecutive days following the five day acclimatization period. The first replicate tank was observed and the position of all visible individuals was recorded four times at 90 second intervals and this was repeated for the second and third replicates. The position of each fish was recorded by writing their color combination and sex on a grid proportional to the bottom of the experimental tanks. These observations were repeated for all three tanks three times each day at 90 minute intervals such that position of each fish was recorded 12 times each day. Observations were made between 11AM and 4PM each day when swordtails are most active and the order of replicate observation was reversed on the second day.

Following the above trials, experimental animals were returned to aquaria and maintained in their replicate groupings. Due to post-experiment mortality in one of the three original replicates, the effects of humic acid were only tested on two replicates. Because there was no day effect on assortative shoaling, data across days were combined and day was not further included in statistical models. An outdoor, experimental tank (described above) was treated with 20mg/L humic acid. The first replicate group of fish was introduced to the tank immediately after adding humic acid and was allowed to acclimatize approximately 18 hours before observations began. After observations ended, the first replicate was removed and the second was placed in the tank to acclimate for 18 hours before testing began. During these two days of testing, the flow through of water was turned off to avoid lowering the concentration of humic acid.

Data analysis

The grids containing the position of individuals for all observations were digitized and the Cartesian coordinates of all individuals for each observation were recorded using ImageJ software. I calculated the pairwise distance between all fish in each observation Using pairwise distances prevented pseudoreplication by counting the distance between any two fish in an observation only once. This provided us with pairwise distance matrices for all tanks, trials and observations, and a table of the distance of each fish to the nearest structure in each observation.

I conducted repeated measures ANOVAs for effects of pairing-type, sex and tank on pairwise distances between individuals. Pairwise distances were classified according to pairing type (*birchmanni-birchmanni* (BB), *birchmanni-malinche* (BM), *birchmanni*-hybrid (BH), and so forth) and sex (female-female, male-male, and male-female). First, the effects of pairing type, sex, and tank were tested in the control treatment with the six control trials as the repeated measure. When significant differences were observed, Fisher LSD post-hoc tests were performed. Second, trials within each treatment were collapsed into treatment means and treatment (control or humic acid) was used as the repeated measure to test for effects of treatment, pairing type, sexual type, tank on pairwise distance. Based on a significant species effect between the control and humic treatments, I did post-hoc, paired t-tests on control versus humic treatments for each pairing type and adjusted the significance level to $p = 0.0083$ to control for multiple comparisons. For reasons of test subject mortality described above, data for all three replicate tanks were used for analyses

on the control treatment alone. In analyses where humic acid treatment data were used, the third tank that underwent only the control treatment was excluded.

Results

Grouping patterns in clean water

The ANOVA for pairwise distances in the control treatment alone revealed significant effects of all factors including the repeated measure 'trial' ($F_{3.7} = 3.139$, $p = 0.016$). There were significant effects pairing-type ($F_5 = 6.768$, $p < 0.001$), sex ($F_2 = 6.557$, $p = 0.002$) and tank ($F_2 = 26.251$, $p < 0.001$). The interaction term pairing-type*sex was not significant ($F_{10} = 0.292$, $p = 0.983$). Post-hoc tests further showed which pairing-type comparisons were significantly different and that all sex comparisons were different (Figure III-1).

Humic acid versus clean water

There was no effect of treatment on pairwise distances ($F_1 = 0.033$, $p = 0.856$) meaning that, irrespective of pairing-type, sex or tank, subjects were no closer nor farther apart in clean or humic acid water overall. There were also overall experiment-wide effects of pairing-type ($F_5 = 7.166$, $p < 0.001$), sex ($F_2 = 5.280$, $p = 0.006$) and tank ($F_1 = 16.772$, $p < 0.001$) irrespective of treatment, but no pairing-type*sex effect across all control and humic trials ($F_{10} = 0.220$, $p = 0.991$).

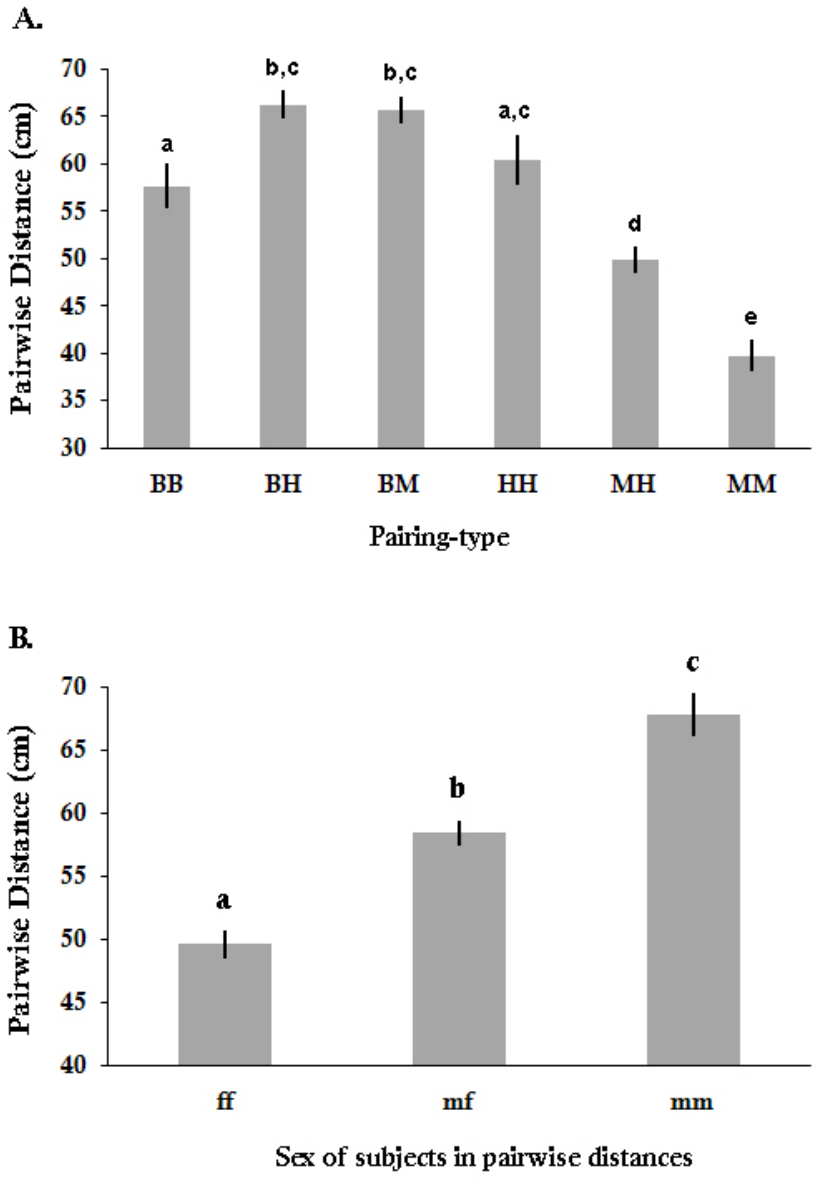

Figure III-1. Pairwise distances between individuals. A) Distances (+/- SE) in clean water where pairing type indicates *birchmanni-birchmanni* (BB), *birchmanni*-Hybrid, (BH), etc. B) Pairwise distances (+/- SE) between sexes (ff: female-female, mf: male-female, mm: male-male) in clean water. In A and B, letters above the bars indicate pairing-types that are significantly different from one another in post-hoc Fisher's LSD tests ($p < 0.05$).

The interaction of treatment*pairing-type, which is the term that indicates whether humic acid affected social grouping was significant indicating that humic acid did impact the distance between some pairing-types (treatment*pairing-type: $F_5 = 2.29$, $p = 0.047$; Figure III-2). However, further break-down of the treatment*pairing-type term with post-hoc, paired t-tests revealed only one significant pairwise comparison. The distance between *X. malinche* and hybrids was significantly reduced in the humic acid treatment ($t = 2.00$, d.f. = 54 $p < 0.001$). All other comparisons were non-significant (data not shown – maybe show in supplemental table). The effect of treatment on sex was non-significant (treatment*sex: $F_2 = 0.033$, $p = 0.968$) but treatment did change the tank effect (treatment*tank: $F_1 = 15.251$, $p < 0.001$).

Discussion

Pure parental *X. birchmanni*, *X. malinche* and their hybrids grouped assortatively by species when allowed to associate freely in tanks of clean water. The use of humic acid to mask chemical communication affected grouping but not in the manner predicted: the interspecific, pairwise distances between *X. birchmanni* and *X. malinche* individuals did not decrease as expected. This is further confirmed by the fact that the species term by itself in the control and humic combined ANOVA was still significant suggesting the assortativity of grouping observed in the control treatment was still largely intact in the humic acid treatment. Nonetheless, the fact that assortative grouping was observed suggests that social preferences may be an important factor in maintaining population structure in these hybrid zones,

particularly if assortative social preferences go hand-in-hand with sexual selection (i.e. female mating patterns).

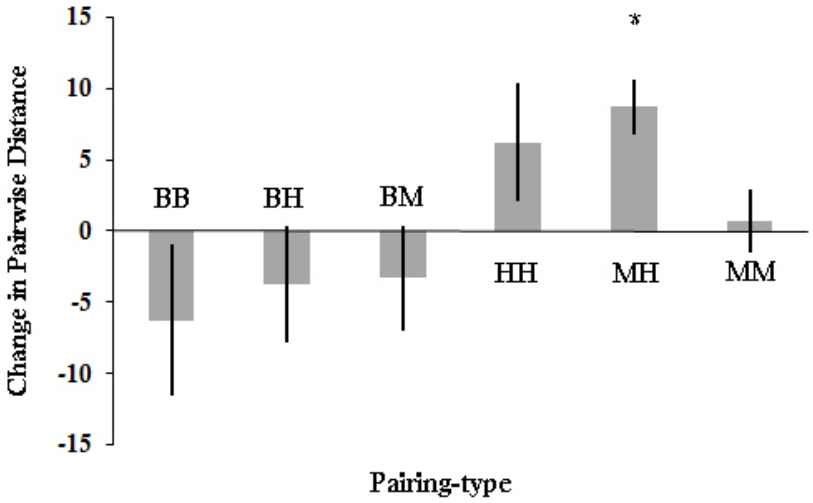

Figure III-2. Change in pairwise distances (+/- SE) between species pairs. Change in clean and humic acid water. Positive values indicate that the distance between that species pair was greater in humic acid while negative values indicate that the species pair was closer disturbed water. Asterisks denote comparisons that were significant after adjusting the p-value for multiple post-hoc t-tests (adjusted $p < 0.0083$).

In sticklebacks, pre-mating isolation between benthic and limnetic forms found in sympatry results in a preference for conspecifics ecotypes (Rundle and Schluter 1998). Thus, spatial segregation within a habitat is sufficiently strong to have effects on female mate choice. Similarly, Mendelson (2003) found that behavioral isolation based on sexual characters resulted in faster reproductive isolation than genetic incompatibilities within the genus *Etheostoma*. Reproductive

behaviors have also long been known to be powerful pre-mating isolating mechanisms in *Drosophila* (Coyne and Orr 1989; Coyne and Orr 1997; Kaneshiro 1976). Social grouping is one of a variety of pre-mating mechanisms that can influence mating patterns and in turn lead to reproductive isolation.

Such pre-mating isolation appears to play an important role in population structure in the birchmanni-malinche hybrid zones, as well. In clean water, individuals were significantly closer to conspecifics than to heterospecifics. The general pattern observed in the hybrid zones is one of panmixia in most hybrid populations resulting from random mating and a lack of pre-mating isolation. Rather, individuals in the present study, collected from a structured population, associated preferentially with conspecifics, which could likely translate into assortative mating. I cannot, however, rule out the possibility that post-mating selection plays a role in maintaining population structure and further study is be needed to determine the degree to which assortative mating or post-mating selection contribute to population structure.

Disruption of the chemical signaling environment alone was not sufficient to break down assortativity between parental species. One plausible explanation for this is that visual cues may have also played a role in grouping. While chemical cues generally result in stronger species-specific preferences in *Xiphophorus* than do visual cues (Rosenthal and Garcia de Leon in press and references therein), I cannot rule out that individuals in this study may have been using visual cues in combination with chemical cues. Previous analysis of morphology in this

population, Calnali-mid, shows that parentals and backcross hybrids occupy significantly different clusters of morphometric space, suggesting that there is a basis for visual discrimination between the pure parental species even when chemical signaling is gone (Culumber et al. 2011). Female *X. birchmanni* do exhibit a preference for visual stimuli of conspecific over heterospecific males (Wong et al. 2005), which suggests that in the absence of chemical cues females may still exhibit some degree of assortative preference based on male morphology.

Population structure can result from a variety of mechanisms along a pre- to post-mating spectrum. Our study indicates that pre-mating isolation among subpopulations via social grouping may work together with mate choice (Fisher et al. 2006; Wong et al. Rosenthal 2005) and post-mating mechanisms to maintain reproductive isolation and population structure among the birchmanni-malinche hybrid zones. Studies of female preference (Fisher et al. 2006, Wong and Rosenthal 2006), selection from the abiotic environment (Culumber et al. in prep), and now social grouping are beginning to elucidate the evolutionary mechanisms that structure and maintain these hybrid zones both within and across populations.

CHAPTER IV

MATING PATTERNS

Introduction

The evolutionary creative force that is natural hybridization has received newfound recognition in the literature over the past 10-15 years (Rieseberg et al. 1997; Arnold 2006; Green et al. 2010; Reich et al. 2010). However, the conservation implications of the loss of species through hybridization have also been increasingly recognized (Olden et al. 2004; Rhymer and Simberloff 1996). Environmental disturbance can act to break down reproductive barriers between species, producing interspecific hybrids (Mecham 1960; Lamont et al. 2003; Taylor et al. 2006). Ample evidence has demonstrated the effect anthropogenic disturbance can have on reproductive barriers, particularly in situations where disturbance is ongoing (Taylor et al. 2006; Behm et al. 2010; Seehausen 1997; Mercader et al. 2009). However, both natural and anthropogenic disturbance is often episodic.

Theoretical studies are beginning to project the potential fate of species boundaries following anthropogenic disturbance (Candolin 2009; Gilman and Behm 2011). Using computer simulations, Gilman and Behm (2011) recently showed that the resurrection of species following even temporary disturbance and periodic hybridization is rarely observed. Few empirical studies have investigated the resiliency of reproductive barriers to episodic disturbance. If hybrids have equal or higher fitness to parentals (Burke and Arnold 2001), and if hybrids continue to backcross to parentals (Ma et al. 2010), introgressive hybridization can continue to

occur even after disturbance has stopped. Hybridization and introgression as a result of human interference can thus have a big reach in time and space and important evolutionary consequences (Palumbi 2001; Seehausen 2006; Olden et al. 2004) even if isolating mechanisms are maintained between the two species.

The swordtails *X. birchmanni* and *X. malinche* form natural hybrids in sympatry (Rosenthal et al. 2003; Culumber et al. 2011). Hybridization is a recent phenomenon that is likely due to the disruption via organic pollution of chemical signals used in conspecific mate recognition (Fisher et al. 2006). As recently as the late 1980's, in-depth field exploration and studies found *X. birchmanni* and *X. malinche* in sympatry in at least the Rio Calnali, but no hybrids were reported (Rauchenberger et al. 1990). By 1997, specimens of intermediate morphology and genetic background were being discovered (Rosenthal et al. 2003). Despite an apparent recovery of pre-mating isolation via assortative social interaction (Culumber and Rosenthal in prep), hybrids continue to exist at high frequencies throughout the range of both parental species. Previous evaluation of population structure revealed primarily panmictic populations, but several populations have substantial structure where both parental species and hybrids coexist (Culumber et al. 2011).

In the present study, I compared the genetic structure of adults to that of embryos dissected from gravid females of the same populations. These comparisons allowed us to test for selection that might favor or disfavor hybrids and result in the observed variation in population structure. Embryo genotyping also allowed us to

simultaneously test mating patterns and backcrossing rates. Finally, evaluating deviations from equilibrium expectations in the population genetics of the embryos provided us with an avenue to test for random or non-random mating in both structured and unstructured populations.

Methods

DNA extraction and genotyping

Tissue from females and embryos or newly born fry from the structured populations Calnali-mid and Aguazarca (n = 59 females) and the unstructured population Apantla (n = 16 females) were collected in one of two ways. When possible, females were euthanized by overdose of tricaine methanesulfonate (1 mg/L) and embryos were dissected out and placed in 100% ethanol. To ensure that I had embryo DNA and to avoid contamination with maternal DNA, embryos were only used if they were at least 50-60% developed - eyes and body had begun to develop and could be separated from the yolk (Figure 5 & 6 from Plate III of Bailey 1933). Following dissection, the females were also preserved in 100% ethanol. In instances where female fish were needed alive for other experiments, they were housed individually in 12.5 L aquaria that were monitored periodically on a daily basis for new fry. When fry were discovered, they were removed, euthanized and preserved in ethanol.

Genomic DNA was extracted from females and 6 embryos or fry from each female using a protinase K digestion followed by extraction with NH_4A_0C and isopropyl alcohol precipitation. Samples were then washed once with 70% ethyl

alcohol. This DNA was then used to genotype all samples for the same three nuclear single nucleotide polymorphism markers as described in Culumber et al. (2010). Five females did not have at least 6 embryos, so only 5 (N=4), 4 (N=1), and 3 embryos (N=1) were genotyped for those females. Six embryos were genotyped for each of the remaining 54 females. All fish were genotyped using three single nucleotide polymorphism (SNP) markers in three different nuclear introns (LIG1, POLB and TP53) as described in Culumber et al. (2011).

Test of selection

I tested for selection against parentals or hybrids that would occur between the embryo stage and adulthood. This was done by comparing the embryo and adult distributions to observe any changes in frequency of genotypes between embryo and adulthood. These comparisons were done in two ways. First, embryos were classified according to their multilocus genotype as being *X. birchmanni*, *X. malinche*, or hybrid if they had alleles of both species. Next, embryos were given a hybrid index score in which each *X. birchmanni* allele (of six possible alleles across three nuclear loci) adds one to an individual's score. The index ranges from pure *X. birchmanni* (index score = 6) down to pure *X. malinche* (index score = 0) with hybrids from 1-5. This second method gives us a finer classification scheme than a broad "species"-level analysis.

For these tests of selection and for tests of non-random mating in the following section, I wrote code in MATLAB (The Mathworks Inc.) that allowed us to randomly select one embryo genotype from each adult female and then generate a

distribution of those randomly drawn embryos, creating a simulated embryo population. This random sampling process was iterated to generate 1000 random embryo populations. Since the frequency of females dissected for embryos from the structured populations did not exactly match the observed frequency of adults in the wild, the code was written to randomly select embryos from only 14 of the 20 *X. malinche* females. This adjustment resulted in proportions of each species' female in the sample that did not differ significantly from proportions observed in the wild population ($\chi^2_2 = 3.87$, $p = 0.14$; 19% *X. birchmanni*, 21% *X. malinche*, and 60% hybrid). The proportion of embryos in each category (Test 1 by "species": B, M, or H; Test 2 by hybrid index: 0-6) were calculated and tested against the proportion of each category observed in adults collected in the wild using a likelihood ratio test, generating a p-value for each of the 1000 distributions. A binomial test was then conducted on the 1000 p-values to assess overall significance.

Test of nonrandom mating

In order to identify effects of nonrandom mating, I tested for deviation from Hardy-Weinberg equilibrium proportions (HWE) in the embryos at each of the three genotyped loci. As above, code written in MATLAB created embryo distributions, equal in size to the number of females sampled, by randomly selecting one embryo from each female. The two structured populations used in this analysis are separated by only 21 vertical meters do not differ significantly in the frequency of each parental species and hybrids nor is F_{ST} significant between the two (Culumber et al. 2011), thus samples from these populations were pooled for analyses. As

above, the code was written to sample a random subset of fourteen *X. malinche* females to achieve sample proportions equal to that of adult frequencies in the wild. I then tested for deviation from HWE using a likelihood ratio test on the number of BB (*X. birchmanni*), MM (*X. malinche*), and BM (heterozygote) genotypes in each of the 1000 distributions with a final binomial test as above. The same was done for the unstructured population Apantla. Finally, following the results of the HWE tests in the structured populations, I used a χ^2 test to evaluate the frequencies of matings among pure parentals and hybrids.

Results

Tests of post-zygotic selection

I tested for selection between embryo and adult stage by comparing genotype distributions of embryos and adults in two ways. When classifying embryos and adults as *X. birchmanni*, *X. malinche* or hybrid, there was no difference between embryo and adult distributions in the structured populations ($p > 0.99$) nor the unstructured population ($p > 0.99$). This suggests that there is no selection against parentals nor hybrids as a group in either population type. I further broke our classification down to a finer scale using a hybrid index as a more sensitive test of selection against particular classes of individuals. Again, there was no evidence of selection against parentals or any group of hybrids regardless of their genetic background in either the structured ($p > 0.99$) or the unstructured population ($p > 0.99$).

Tests of nonrandom mating

Likelihood ratio tests for HWE revealed significant deviation from HWE proportions at all three loci in the structured populations (LIG: $p < 0.0001$; POLB: $p < 0.0001$; TP53: $p < 0.0001$). In all cases, significantly fewer heterozygotes were observed in the embryos than in the adult distribution. In the unstructured population, there were no deviations from HWE proportions in any of the markers (LIG: $p > 0.99$; POLB: $p > 0.99$; TP53: $p > 0.99$); embryo frequencies matched adult frequencies.

The χ^2 test of mating frequencies in the structured population showed that there was a significant deviation from random expectations ($\chi^2 = 9.08$; $p = 0.011$). There were no F_1s observed in the embryos indicating that there were no interspecific hybridization events between parental species in our data set and, consequently, an excess of assortative matings within parental species (Figure IV-1). However, parentals backcrossed to hybrids with considerable frequency – 47% of the time versus the expected 52%.

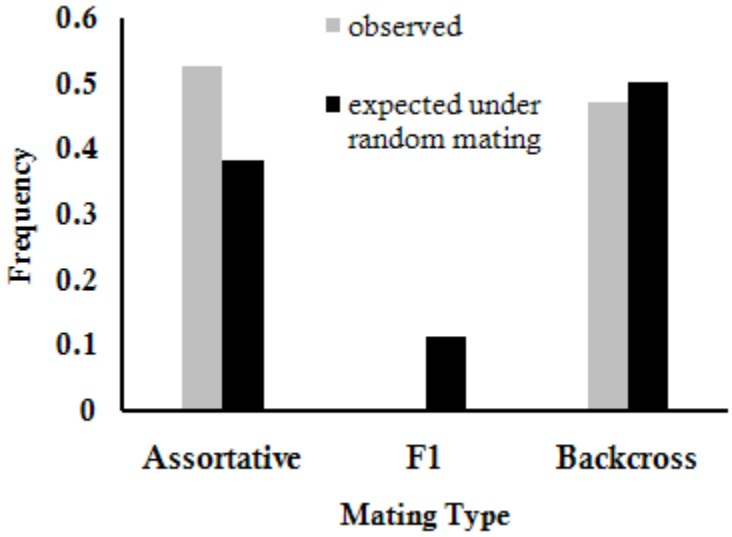

Figure IV-1. Frequency of mating types. Frequencies inferred from female and embryo genotypes. Assortative matings is any mating with a conspecific, F_1 is an interspecific mating between *X. birchmanni* and *X. malinche* and backcross is any mating between a hybrid and a pure parental. The lack of interspecific matings that would result in F_1 embryos and the associated increase in assortative matings within each parental species resulted in a significant deviation from random expectation of mating frequencies ($\chi^2 = 9.08$; $p = 0.011$).

Discussion

A lack of interspecific matings coupled with frequent backcrossing to parentals, demonstrates that mating decisions involving hybrids can generate irreversible evolutionary effects even after disturbance has occurred. Gilman and Behm (2011) demonstrated that even temporary disturbance is sufficient to permanently erode species barriers in theoretical simulations. As suggested by their findings, despite a recovery of pre-mating isolation between a species pair following temporary disturbance, pure parentals may still be subject to a high degree of gene

exchange via backcrossing as in the natural hybrid zones between *X. birchmanni* and *X. malinche*. There was no evidence of selection against hybrids in any populations, and significant deviation from HWE for all markers in the structured but not in the unstructured population suggests that non-random mating may be the critical mechanism generating variation in population structure throughout the hybrid zones.

As stated before, hybridization can have long-lasting effects even after disturbance is removed if hybrids are at least as fit as parentals and if parentals continue to backcross to hybrids. In many hybrid zones, particularly the classic 'tension zone' scenario, immigration and mating between pure parentals regenerates new hybrids which are less fit than parentals (Barton and Hewitt 1985). Hybrids commonly suffer from either pre- or post-zygotic selection due to genetic incompatibilities (Burke and Arnold 2001), female discrimination against hybrid males (Bridle et al. 2006), or environmental selection (Milne et al. 2003) meaning that hybrids are often either experience negative ecological selection or do not backcross to parentals.

I first tested for evidence of selection against hybrids by comparing genotyping frequencies at two different life stages. Gow et al. (2007) previously reported ecological selection against hybrids as they observed a decline in the frequency of stickleback hybrids across life stages. I observed so such discrepancy between embryos and adults in our populations. Hybrid embryos occurred at the same frequency as hybrid adults in both structured and unstructured populations.

Since I sampled embryos at mid to late stages or used newly born fry, I can take this to mean that post-zygotic selection against hybrids is inconsequential. This is consistent with the prevalence of hybrids at intermediate elevations and their apparent adaptation to the thermal environment at these elevations where they are just as fit as parentals (Culumber et al. in prep). Thus, hybrids are at least as fit as pure *X. birchmanni* and *X. malinche* in the hybrid zones.

However, hybrids must also be fertile and, for introgression to occur, must backcross with parentals. Even when hybrids are viable, they may not be fertile (Burke and Arnold 2001) or may be highly disfavored by parental females (Vamosi and Schluter 1999; Bridle et al. 2006; Lancaster et al. 2007). Comparing mating types in the structured populations revealed two interesting results. First, there were abundant backcrosses between both parentals and hybrids at the frequency expected based on their proportions in the populations. Second, the lack of interspecific matings between pure parental species not only demonstrates that hybridization is likely not ongoing but that hybrids are fertile. The result is that both the requirements for ongoing introgression into the pure parental species are met despite the fact that the original disturbance and hybridization occurred in the past.

Finally, significant deviations from HWE were observed in the structured populations but not in the unstructured population. This is an important result as these tests not only further support the fact that interspecific matings (i.e. new hybridization events) are fleeting or non-existent at this point, but also provides new insight into the mechanisms maintaining population structure. A previous

investigation of pre-mating associative interactions in the structured population Calnali-mid, revealed that pure parentals and hybrids associate more closely with conspecifics than heterospecifics (Culumber and Rosenthal in prep). This could have important implications for mating patterns if that social behavior translates into assortative mating. These tests of HWE suggest that this assortative, pre-mating interaction may very well result in assortative mating in these structured populations. I cannot entirely rule out some effect of genetic incompatibility or sperm competition that may have generated these deviations. Only further experiments such as in-vitro fertilization will be able to disentangle any such effects.

Theory and now empirical evidence both highlight the sensitive nature of the relationship between temporary disturbance and reproductive barriers. As a by-product of evolutionary potential, fit hybrids can act as a conduit for gene exchange, despite the recovery of reproductive isolation between parental species. Subtle disruptions to the communication environment may therefore pose a greater challenge to biodiversity conservation than has been anticipated.

CHAPTER V

FLUCTUATING ASYMMETRY

Introduction

Fluctuating asymmetry (FA), a measure of deviation from perfect bilateral symmetry, is often considered an indicator of an individual's ability to buffer against environmental stress (Parsons 1992) or against random 'noise' during the developmental process (Van Valen 1962). Asymmetry is often negatively associated with fitness metrics such as fighting ability, growth and survival (Møller 1997). Overwhelmingly, studies of FA have shown that females prefer to mate with symmetric males, and that FA is negatively correlated with male reproductive success (reviewed in Møller and Thornhill 1998; Polak 2008). Thus, given this correlation, even when trait symmetry is not directly under sexual selection, strong female choice, could generate indirect selection for reduced FA.

Males of many species of swordtails (Teleostei: *Xiphophorus*), express dark, pigmented vertical bars on each side of the body. Within the genus, there are some species in which no males express bars, while in other species, males are polymorphic (Rauchenberger et al. 1990). These are intensified during courtship and male-male aggression, and the presence and number of vertical bars is subject to both inter- (Zimmerer and Kallman 1998; Moretz 2005) and intrasexual (Morris et al. 1995; Fisher et al. 2009) selection. As in many other systems, empirical evidence has shown that females prefer males with symmetrical vertical bars (Morris 1998; Morris and Casey 1998). However, the largest *X. cortezi* and *X. malinche* females

may prefer males with some degree of asymmetry, which may help to maintain FA observed in natural populations (Morris et al. 2006).

In tributaries of the Rio Pánuco basin of Mexico, *Xiphophorus birchmanni* and *X. malinche* meet and produce fertile, viable hybrids. *X. malinche* are restricted to highland sites; *X. birchmanni* occur in lowlands, and hybrid or mixed hybrid/parental populations are found at intermediate elevations (Rosenthal et al. 2003; Culumber et al. 2011). Hybrid sites vary considerably in population-genetic structure, ranging from apparently random-mating, unstructured hybrid swarms, to populations with distinct subpopulations of hybrids and both parental species (Culumber et al. 2011). Immigration of parentals is unlikely to maintain these large deviations from HWE and LD and selection against hybrids is weak or absent (Culumber et al. 2011; Culumber et al. in preparation). This suggests that nonrandom mating could be maintaining population structure by promoting reproductive isolation among subpopulations (Culumber et al. 2011).

Here I evaluated the relationship between the degree of vertical bar FA and population-genetic structure. Whether FA is under direct or indirect sexual selection in this system, I should expect non-random mating to result in lower FA, either via inter- or intrasexual selection acting directly on symmetry (Morris 1998) or indirectly via selection on viability (Fisher and Rosenthal 2006) or performance traits (Møller 1997) which might be correlated with FA. I first investigated whether hybridization has affected FA by comparing among FA between the two parental species and hybrids. Having ruled out an effect of hybridization, I tested two

hypotheses regarding the influence of nonrandom mating on the vertical bar trait. If nonrandom mating generates stronger sexual selection in structured populations, then 1) I should observe a greater occurrence of vertical bars and 2) FA should be lower in males bearing the trait.

Methods

Collecting, trait measurements, and genotyping

Adult males were collected, using baited minnow traps, from two *X. malinche* populations, two *X. birchmanni* populations and twelve hybrid populations (Figure V-1). The total number of bars on each side of the fish was counted and recorded either directly from live specimens or from digital photographs of live specimens. Asymmetry was calculated as the difference in bar number between the left and right sides of the body. The standard length of each individual was measured from the tip of the snout to the caudal peduncle. I also measured standard length on females collected from the same locality. Individuals from hybrid populations were genotyped for four single nucleotide polymorphisms (SNPs) in one mitochondrial and three nuclear intron loci. An individual was classified as a parental if it contained alleles from only one species and as a hybrid otherwise.

Statistical analysis

Populations were classified based on data in Culumber et al. (2011). LD and F_{IS} values (a measure of deviation from HWE) for these populations are presented in Appendix A. Populations with significant LD, deviations from HWE, and where subpopulation of each of the three types of fish are found together are

classified as 'structured' populations. Those without LD nor deviations from HWE are classified as 'unstructured'. Those populations where all individuals genotyped as pure parentals were classified as 'parental'.

To test for an effect of structure (pure parental, unstructured and structured) and species (*X. birchmanni*, *X. malinche* and hybrids) I performed separate analyses of covariance (ANCOVA) with absolute bar difference as the dependent variable and standard length as the covariate. I could not run a combined (structure X species) ANCOVA because hybrid individuals do not occur in parental populations. Fisher's LSD post-hoc tests were used to make pairwise comparisons when a significant relationship was observed in an ANCOVA. Individuals with no bars were excluded from all analyses. An ANOVA was used to test for an effect of population type on female size to determine whether female size could explain variation in FA. I used Pearson's χ^2 to compare bar prevalence (proportion of individuals with bars) among population types and species.

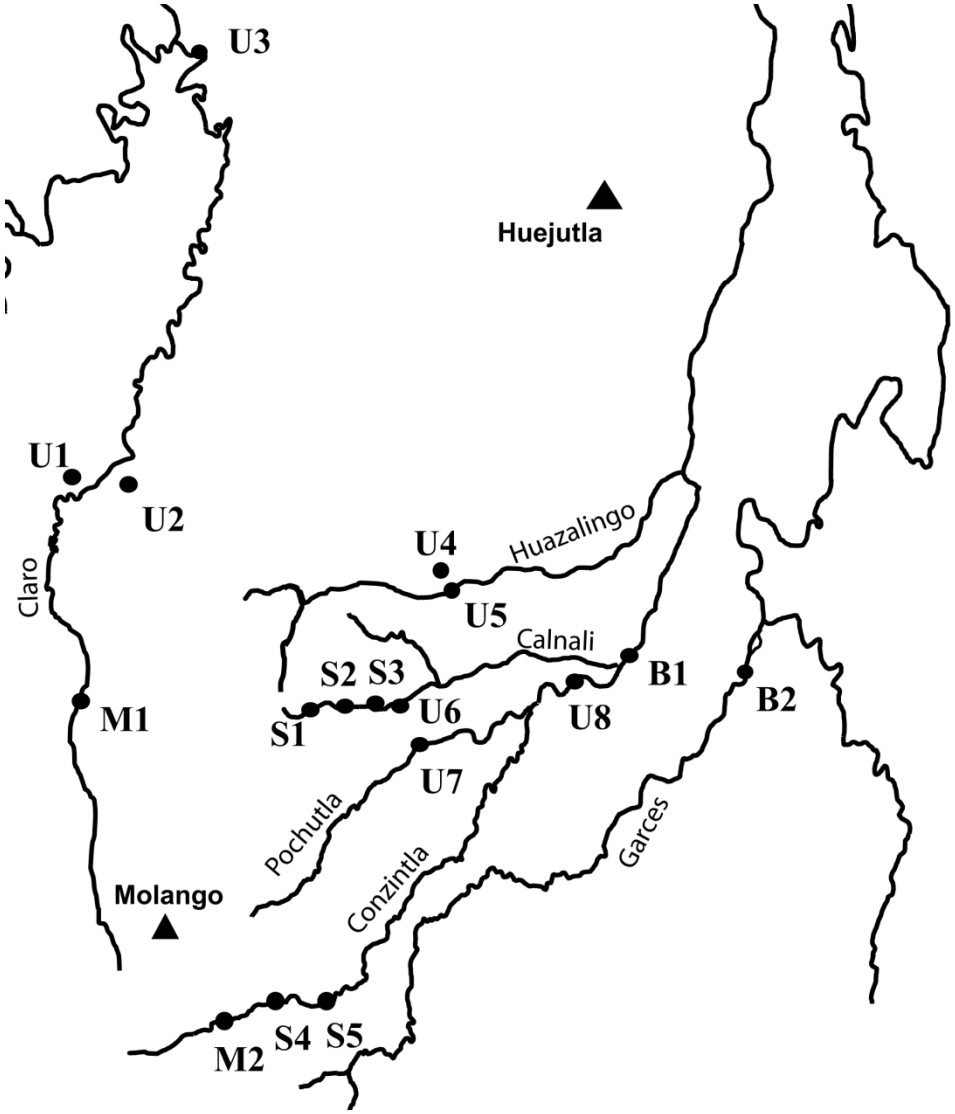

Figure V-1. Collecting localities. Map of hybrid zones with cities (triangles) as reference. Population type is designated by the letter in each name: B, *X. birchmanni*; M, *X. malinche*; S, structured; and U, unstructured. See Culumber et al. (2011) for locality details.

Results

Bar prevalence

There was no difference in the proportion of barred males among species (χ_2^2 = 3.645, p = 0.162; *X. malinche*: 84% barred, n = 109; *X. birchmanni,* 92% barred, n = 103; hybrids: 84% barred, n = 240. There was, however, a significant difference in the proportion of barred and barless individuals among population types (χ_2^2 = 18.973, $p < 0.001$; Figure V-2). The proportion of individuals with bars was significantly higher in structured compared to unstructured populations (χ_1^2 = 19.171, $p < 0.001$).

Fluctuating asymmetry

Across the entire data set, there was no difference in FA among *X. birchmanni*, *X. malinche* and hybrids ($F_{2,403}$ = 0.717, p = 0.489). There was a significant effect of population type on FA ($F_{2,404}$ = 3.096, p = 0.037; Figure V-2) and post-hoc tests revealed that structured populations had significantly lower FA than unstructured (p = 0.021) and parental populations (p = 0.039). Unstructured and parental populations did not differ (p = 0.781). Hybrid individuals from structured populations had significantly lower FA than those from unstructured populations ($F_{1,199}$ = 8.235, p = 0.005).

Female standard length varied by population type ($F_{2,294}$ = 25.962, $p < 0.001$). Females from structured populations were significantly larger (N = 79, 47.5 ± 0.78 mm) than those from both parental (N = 74, 41.8 mm ± 0.68) and unstructured (N

= 144, 42.0 mm ± 0.47) hybrid populations ($p < 0.001$ for both). There was no difference between females from parental and unstructured populations ($p = 0.483$).

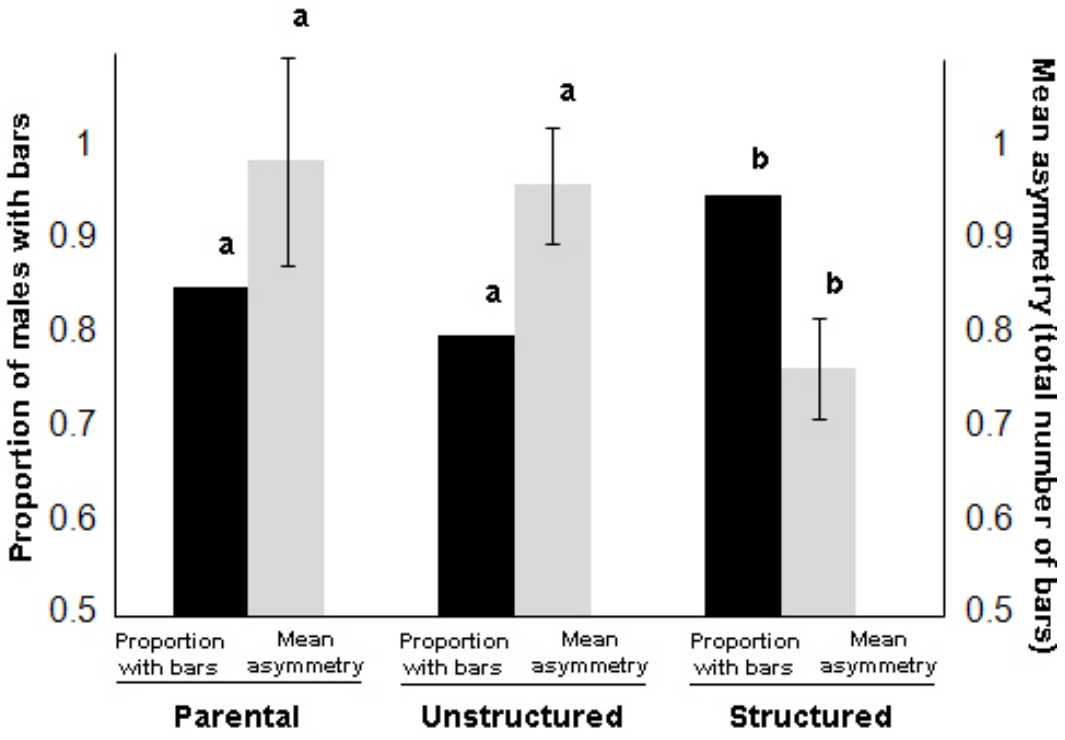

Figure V-2. Proportion of males with bars and asymmetry. Asymmetry (mean ± SE) was measured as absolute difference in bar number between left and right side. Bars with different letters indicate significant differences in contingency tables ($p < 0.05$).

Discussion

Fluctuating asymmetry in vertical bars covaried with population structure: males from structured populations, where mating is non-random, were significantly

more symmetrical than males from unstructured and parental populations. Further, males from these structured populations were more likely to bear vertical bars. Since preference forvertical bars, and particularly bar symmetry, have been found in closely related species (Morris et al. 1995; Morris 1998; Morris et al. 1998), our findings suggest that among-population variation in FA may be driven by variation in the strength of sexual selection among populations.

Our results concur with previous studies investigating the relationship between selection and FA. In Drosophila melanogaster, artificial selection on symmetry of laboratory lines led to reduce FA, which subsequently returned to natural levels when selection was relaxed (Mather 1953). Similarly in the Pecos pupfish, *Cyprinodon pecosensis*, population characteristics associated with increased sexual selection correlated positively with FA in breeding males (Kodric-Brown 1997).

Morris et al. (2006) showed that female preference for bar symmetry in *X. malinche* varied with body size, with largest females preferring asymmetrical bars. Greater FA in parental and unstructured populations could therefore be a consequence of differences in female size distributions. However, female size was in fact greatest in structured populations where FA was the lowest. Any size-associated preference variation therefore does not appear to be driving population-level FA in the *birchmanni-malinche* hybrid zones.

Vertical bar symmetry in swordtail males was unaffected by hybridization. Hybridization has long been thought to either positively or negatively affect

developmental stability (Soule 1967; Soule 1979), but empirical data support both hypotheses. In naturally occurring hybrid zones of both ground beetle and oak trees, FA was found to be higher in hybrids Garnier et al. 2006; Albarrán-Lara et al. 2010). While in the *Mus musculus* hybrid zone, hybrids exhibited lower FA than parentals (Alibert et al. 1994). Still other studies found no effects of hybridization on FA such as in lake white fish and *Dalechampia* flowers (Lu and Bernatchez 1999; Pelabon et al. 2003). Similarly, our findings suggest that hybrids have similar FA to that of parentals in the *birchmanni-malinche* hybrid zones.

In *Xiphophorus,* mate choice and male-male competition are both likely to favor males with more bars, and more symmetric bars. Population-genetic data suggest that mating patterns vary considerably among populations. This raises the question, of course, of what factors drive variation in population structure. This could be the result of differences in conditions for sexual communication; Fisher et al. 2006 showed that female *X. birchmanni* fail to discriminate conspecifics from *X. malinche* in contaminated water. Alternatively, populations could vary in recency of contact or historical frequencies of each parent species. Nonetheless, assortative shoaling and non-random mating in structured populations (Culumber and Rosenthal in prep.; Culumber et al. in prep.), together with observations of FA in populations throughout the hybrid zones, highlight the importance of sexual selection in determining both population structure and levels of fluctuating asymmetry.

CHAPTER VI

CONCLUSIONS

The mechanisms by which population structure arise and potentially lead to speciation in hybrid populations are not well understood. I used the *Xiphophorus* hybrid zones to empirically test how behavioral patterns shape hybrid population structure. Genetic data demonstrated that populations varied from unstructured hybrid swarms, to distinctly structured subpopulations of both parental species and hybrids.

In order to better understand mechanisms maintaining population structure, behavioral and genetic tests were used. Experiments of social behavior in clean water demonstrated that pure parentals and hybrids associated with conspecifics to a significantly greater extent than with heterospecifics. Furthermore, surprisingly, these associations still existed in water contaminated with humic acid to disrupt chemical signaling. This suggests that masking chemical cues alone may not be enough to break reproductive barriers and that visual cues or other behavioral traits may also be important. Thus, more specific conditions than just chemical signaling interference are likely needed to get a breakdown in reproductive isolation. However, those results were important in that, such behavioral assortativity could lead to assortative mating patterns and result in population structure. Genotyping females and embryos confirmed that non-random mating patterns likely occur in structured populations and this non-random mating appears to have important morphological effects as vertical bar symmetry was related to population structure.

Finally, genotyping embryos and comparing those genotypes with adults revealed that there is no selection against hybrids in structured nor unstructured populations. When taken altogether, the results of the proceeding chapters and the work therein, represents a step forward in our understanding of the selective pressures that underlie both spatial and temporal scales from the ecological selection that drives overall hybrid zone structure to the sexual selection that generates variation in population structure within individual streams. Future studies can hopefully address even finer-scale selection such as sperm competition or selection at the early embryo stage in order to pinpoint the mechanism(s) that result in population structure and understand the architecture of reproductive barriers between these two species.

REFERENCES

Aboim MA, Mavarez J, Bernatchez L, Coelho MM (2010) Introgressive hybridization between two Iberian endemic cyprinid fish: a comparison between two independent hybrid zones. *Journal of Evolutionary Biology* **23**, 817-828.

Albarran-Lara AL, Mendoza-Cuenca L, Valencia-Avalos S, Gonzalez-Rodriguez A, Oyama K (2010) Leaf fluctuating asymmetry increases with hybridization and introgression between *Quercus magnifolia* and *Quercus resinosa* (Fagaceae) through an altitudinal gradient in Mexico. *International Journal of Plant Sciences* **171**, 310-322.

Alibert P, Renaud S, Dod B, Bonhomme F, Auffray JC (1994) Fluctuating asymmetry in the *Mus musculus* hybrid zone - A heterotic effect in disrupted co-adapted genomes. *Proceedings of the Royal Society of London Series B-Biological Sciences* **258**, 53-59.

Arnold ML (1992) Natural hybridization as an evolutionary process. *Annual Review of Ecology and Systematics* **23**, 237-261.

Arnold ML (1997) *Natural Hybridization and Evolution.* Oxford University Press, New York.

Arnold ML (2006) *Evolution Through Genetic Exchange.* Oxford University Press.

Bailey RJ (1933) The ovarian cycle in the viviparous teleost *Xiphophorus helleri*. *Biological Bulletin* **64**, 206-225.

Barton NH (2001) The role of hybridization in evolution. *Molecular Ecology* **10,** 551-568.

Barton NH, Hewitt GM (1985) Analysis of hybrid zones. *Annual Review of Ecology and Systematics* **16**, 113-148.

Behm JE, Ives AR, Boughman JW (2010) Breakdown in postmating isolation and the collapse of a species pair through hybridization. *American Naturalist* **175**, 11-26.

Behrmann-Godel J, Gerlach G, Eckmann R (2006) Kin and population recognition in sympatric Lake Constance perch (*Perca fluviatilis* L.): can assortative shoaling drive population divergence? *Behavioral Ecology and Sociobiology* **59**, 461-468.

Bell MA, Travis MP (2005) Hybridization, transgressive segregation, genetic covariation, and adaptive radiation. *Trends in Ecology & Evolution* **20**, 358-361.

Bolnick DI (2009) Hybridization and speciation in Centrarchids. In: *Biology of the Centrarchids*, pp. 39-69. Oxford University Press, Oxford.

Borowsky RL, McClelland M, Cheng R, Welsh J (1995) Arbitrarily primed DNA-Fingerprinting for phylogenetic reconstruction in vertebrates - the *Xiphophorus* model. *Molecular Biology and Evolution* **12**, 1022-1032.

Bridle JR, Saldamando CI, Koning W, Butlin RK (2006) Assortative preferences and discrimination by females against hybrid male song in the grasshoppers *Chorthippus brunneus* and *Chorthippus jacobsi* (Orthoptera : Acrididae). *Journal of Evolutionary Biology* **19**, 1248-1256.

Burke JM, Arnold ML (2001) Genetics and the fitness of hybrids. *Annual Review of Genetics* **35**, 31-52.

Candolin U (2009) Population responses to anthropogenic disturbance: lessons from three-spined sticklebacks *Gasterosteus aculeatus* in eutrophic habitats. *Journal of Fish Biology* **75**, 2108-2121.

Christophe N, Baudoin C (1998) Olfactory preferences in two strains of wild mice, *Mus musculus musculus* and *Mus musculus domesticus*, and their hybrids. *Animal Behaviour* **56**, 365-369.

Constantz GD (1989) Reproductive biology of Poeciliid fishes. In: *Ecology and evolution of livebearing fishes (Poeciliedae)* (eds. Meffe GK, Snelson FF), pp. 33-50. Prentice Hall, Englewood Cliffs, NJ, 07632.

Coyne JA, Orr HA (1989) Patterns of speciation in *Drosophila*. *Evolution* **43**, 362-381.

Coyne JA, Orr HA (1997) "Patterns of speciation in *Drosophila"* revisited. *Evolution* **51**, 295-303.

Croft DP, James R, Ward AJW, Botham MS, Mawdsley D, *et al.* (2005) Assortative interactions and social networks in fish. *Oecologia* **143**, 211-219.

Culumber ZW, Fisher HS, Tobler M, Mateos M, Barber PH, *et al.* (2011) Replicated hybrid zones of *Xiphophorus* swordtails along an elevational gradient. *Molecular Ecology* **20**, 342-356.

Culumber ZW, Tobler M, Shepard DB, Coleman SW, Rosenthal GG (In preparation) Physiological adaptation along environmental gradients and replicated hybrid zone structure in swordtails (Teleostei: *Xiphophorus*).

Decaprona MDC, Ryan MJ (1990) Conspecific mate recognition in swordtails, *Xiphophorus nigrensis* and *pygmaeus* (Poeciliidae) - olfactory and visual cues. *Animal Behaviour* **39**, 290-296.

Doherty JA, Gerhardt HC (1984) Acoustic communication in hybrid treefrogs - sound production by males and selective phonotaxis by females. *Journal of Comparative Physiology* **154**, 319-330.

Dowling TE, Secor CL (1997) The role of hybridization and introgression in the diversification of animals. *Annual Review of Ecology and Systematics* **28**, 593-619.

Eberhard WG (1996) *Female Control: Sexual Selection by Cryptic Female Choice* Princeton University Press, Princeton, NJ.

Fisher HS, Mascuch SJ, Rosenthal GG (2009) Multivariate male traits misalign with multivariate female preferences in the swordtail fish, *Xiphophorus birchmanni*. *Animal Behaviour* **78**, 265-269.

Fisher HS, Wong BBM, Rosenthal GG (2006) Alteration of the chemical environment disrupts communication in a freshwater fish. *Proceedings of the Royal Society B-Biological Sciences* **273**, 1187-1193.

Freeberg TM (1996) Assortative mating in captive cowbirds is predicted by social experience. *Animal Behaviour* **52**, 1129-1142.

Fricke C, Arnqvist G (2004) Conspecific sperm precedence in flour beetles. *Animal Behaviour* **67**, 729-732.

Fuller RC, McGhee KE, Schrader M (2007) Speciation in killifish and the role of salt tolerance. *Journal of Evolutionary Biology* **20**, 1962-1975.

Garnier S, Gidaszewski N, Charlot M, Rasplus JY, Alibert P (2006) Hybridization, developmental stability, and functionality of morphological traits in the ground beetle Carabus solieri (Coleoptera, Carabidae). *Biological Journal of the Linnean Society* **89**, 151-158.

Garza JC, Dallas J, Duryadi D*,* Gerasimov S, Croset H, *et al.* (1997) Social structure of the mound-building mouse *Mus spicilegus* revealed by genetic analysis with microsatellites. *Molecular Ecology* **6**, 1009-1017.

Gay L, Crochet PA, Bell DA, Lenormand T (2008) Comparing clines on molecular and phenotypic traits in hybrid zones: a window on tension zone models. *Evolution* **62**, 2789-2806.

Geyer LB, Palumbi SR (2005) Conspecific sperm precedence in two species of tropical sea urchins. *Evolution* **59**, 97-105.

Gilman RT, Behm JE (2011) Hybridization, species collapse, and species reemergence after disturbance to premating mechanisms of reproductive isolation. *Evolution* **65**, 2592-2605.

Good TP, Ellis JC, Annett CA, Pierotti R (2000) Bounded hybrid superiority in an avian hybrid zone: effects of mate, diet, and habitat choice. *Evolution* **54**, 1774-1783.

Goudet J (1995) FSTAT (Version 1.2): A computer program to calculate F-statistics. *Journal of Heredity* **86**, 485-486.

Goudet J, Raymond M, de-Meeus T, Rousset F (1996) Testing differentiation in diploid populations. *Genetics* **144**, 1933-1940.

Gow JL, Peichel CL, Taylor EB (2007) Ecological selection against hybrids in natural populations of sympatric threespine sticklebacks. *Journal of Evolutionary Biology* **20**, 2173-2180.

Green RE, Krause J, Briggs AW, Maricic T, Stenzel U, *et al.* (2010) A draft sequence of the neandertal genome. *Science* **328**, 710-722.

Guillaume F, Whitlock MC (2007) Effects of migration on the genetic covariance matrix. *Evolution* **61**, 2398-2409.

Gutierrez-Rodriguez C, Shearer AE, Morris MR, de Queiroz K (2008) Phylogeography and monophyly of the swordtail fish species *Xiphophorus birchmanni* (Cyprinodontiformes, Poeciliidae). *Zoologica Scripta* **37**, 129-139.

Hankison SJ, Morris MR (2003) Sexual selection and species recognition in the pygmy swordtail, *Xiphophorus pygmaeus*: conflicting preferences. *Behavioral Ecology and Sociobiology* **51**, 140-145.

Hartl DL, Clark AG (2007) *Principles of Population Genetics*, 4th edn. Sinauer Associates, Sunderland, MA.

Hill WG (1974) Estimation of linkage disequilibrium in random mating populations. *Heredity* **33**, 229-239.

Hobel G, Gerhardt HC (2003) Reproductive character displacement in the acoustic communication system of green tree frogs (*Hyla cinerea*). *Evolution* **57**, 894-904.

Hochberg ME, Sinervo B, Brown SP (2003) Socially mediated speciation. *Evolution* **57**, 154-158.

Jiggins CD, Mallet A (2000) Bimodal hybrid zones and speciation. *Trends in Ecology & Evolution* **15**, 250-255.

Johannesson K, Panova M, Kemppainen P*,* André C, Rolán-Alvarez E, *et al.* (2010) Repeated evolution of reproductive isolation in a marine snail: unveiling mechanisms of speciation. *Philosophical Transactions of the Royal Society B-Biological Sciences* **365**, 1735-1747.

Kalisz S, Kramer EM (2008) Variation and constraint in plant evolution and development. *Heredity* **100**, 171-177.

Kallman KD, Kazianis S (2006) The genus *Xiphophorus* in Mexico and Central America. *Zebrafish* **3**, 271-285.

Kaneshiro KY (1976) Ethological isolation and phylogeny in Plantibia subgroup of Hawaiian *Drosophila*. *Evolution* **30**, 740-745.

Kawecki TJ, Ebert D (2004) Conceptual issues in local adaptation. *Ecology Letters* **7**, 1225-1241.

Kodric-Brown A (1997) Sexual selection, stabilizing selection and fluctuating asymmetry in two populations of pupfish (*Cyprinodon pecosensis*). *Biological Journal of the Linnean Society* **62**, 553-566.

Kyriacou CP, Hall JC (1986) Interspecific genetic-control of courtship song production and reception in *Drosophila*. *Science* **232**, 494-497.

Lamont BB, He T, Enright NJ, Krauss SL, Miller BP (2003) Anthropogenic disturbance promotes hybridization between Banksia species by altering their biology. *Journal of Evolutionary Biology* **16**, 551-557.

Lancaster ML, Bradshaw CJA, Goldsworthy SD, Sunnucks P (2007) Lower reproductive success in hybrid fur seal males indicates fitness costs to hybridization. *Molecular Ecology* **16**, 3187-3197.

Lee W-J, Conroy J, Hunting Howell W, Kocher TD (1995) Structure and evolution of teleost mitochondrial control regions. *Journal of Molecular Evolution* **41**, 54-66.

Lu GQ, Bernatchez L (1999) A study of fluctuating asymmetry in hybrids of dwarf and normal lake whitefish ecotypes (*Coregonus clupeaformis*) from different glacial races. *Heredity* **83**, 742-747.

Ma JX, Li YN, Vogl C, Ehrendorfer F, Guo YP (2010) Allopolyploid speciation and ongoing backcrossing between diploid progenitor and tetraploid progeny lineages in the *Achillea millefolium* species complex: analyses of single-copy nuclear genes and genomic AFLP. *Bmc Evolutionary Biology* **10**.

Mallet J (2007) Hybrid speciation. *Nature* **446**, 279-283.

Marcus JM, McCune AR (1999) Ontogeny and phylogeny in the northern swordtail clade of *Xiphophorus*. *Systematic Biology* **48**, 491-522.

Mather K (1953) Genetical control of stability in development. *Heredity* **7**, 297-336.

Mecham JS (1960) Introgressive hybridization between two southeastern treefrogs. *Evolution* **14**, 445-457.

Melo MC, Salazar C, Jiggins CD, Linares M (2009) Assortative mating preferences among hybrids offers a route to hybrid speciation. *Evolution* **63**, 1660-1665.

Mendelson TC (2003) Evidence of intermediate and asymmetrical behavioral isolation between orangethroat and orangebelly darters (Teleostei: Percidae). *American Midland Naturalist* **150**, 343-347.

Mendelson TC, Imhoff VE, Venditti JJ (2007) The accumulation of reproductive barriers during speciation: postmating barriers in two behaviorally isolated species of darters (Percidae : *Etheostoma*). *Evolution* **61**, 2596-2606.

Mercader RJ, Aardema ML, Scriber JM (2009) Hybridization leads to host-use divergence in a polyphagous butterfly sibling species pair. *Oecologia* **158**, 651-662.

Meyer A, Morrissey JM, Schartl M (1994) Recurrent origin of a sexually selected trait in *Xiphophorus* fishes inferred from a molecular phylogeny. *Nature* **368**, 539-542.

Meyer A, Salzburger W, Schartl M (2006) Hybrid origin of a swordtail species (Teleostei: *Xiphophorus clemenciae*) driven by sexual selection. *Molecular Ecology* **15**, 721-730.

Milne RI, Terzioglu S, Abbott RJ (2003) A hybrid zone dominated by fertile F(1)s: maintenance of species barriers in *Rhododendron*. *Molecular Ecology* **12**, 2719-2729.

Moller AP (1997) Developmental stability and fitness: a review. *American Naturalist* **149**, 916-932.

Moller AP, Thornhill R (1998) Bilateral symmetry and sexual selection: a meta-analysis. *American Naturalist* **151**, 174-192.

Moore WS (1977) Evaluation of narrow hybrid zones in vertebrates. *Quarterly Review of Biology* **52**, 263-277.

Moretz J (2005) Aggression and fighting ability are correlated in the swordtail fish *Xiphophorus cortezi*: the advantage of being barless. *Behavioral Ecology and Sociobiology* **59**, 51-57.

Morris MR (1998) Female preference for trait symmetry in addition to trait size in swordtail fish. *Proceedings of the Royal Society of London Series B-Biological Sciences* **265**, 907-911.

Morris MR, Casey K (1998) Female swordtail fish prefer symmetrical sexual signal. *Animal Behaviour* **55**, 33-39.

Morris MR, de Queiroz K, Morizot DC (2001) Phylogenetic relationships among populations of northern swordtails (*Xiphophorus*) as inferred from allozyme data. *Copeia*, 65-81.

Morris MR, Mussel M, Ryan MJ (1995) Vertical body bars on male *X. multilineatus*: a signal that deters rival males and attracts females. *Behavioral Ecology* **6**, 274-279.

Morris MR, Rios-Cardenas O, Tudor MS (2006) Larger swordtail females prefer asymmetrical males. *Biology Letters* **2**, 8-11.

Nikula R, Strelkov P, Vainola R (2008) A broad transition zone between an inner Baltic hybrid swarm and a pure North Sea subspecies of *Macoma balthica* (Mollusca, Bivalvia). *Molecular Ecology* **17**, 1505-1522.

Nolte AW, Gompert Z, Buerkle CA (2009) Variable patterns of introgression in two sculpin hybrid zones suggest that genomic isolation differs among populations. *Molecular Ecology* **18**, 2615-2627.

Olden JD, Poff NL, Douglas MR, Douglas ME, Fausch KD (2004) Ecological and evolutionary consequences of biotic homogenization. *Trends in Ecology & Evolution* **19**, 18-24.

Palumbi SR (2001) Evolution - humans as the world's greatest evolutionary force. *Science* **293**, 1786-1790.

Parsons PA (1992) Fluctuating asymmetry - a biological monitor of environmental and genomic stress. *Heredity* **68**, 361-364.

Pelabon C, Carlson ML, Hansen TF, Yoccoz NG, Armbruster WS (2004) Consequences of inter-population crosses on developmental stability and canalization of floral traits in *Dalechampia scandens* (Euphorbiaceae). *Journal of Evolutionary Biology* **17**, 19-32.

Pfennig KS, Simovich MA (2002) Differential selection to avoid hybridization in two toad species. *Evolution* **56**, 1840-1848.

Plath M, Strecker U (2008) Behavioral diversification in a young species flock of pupfish (*Cyprionodon spp*.): shoaling and aggressive behavior. *Behavioral Ecology and Sociobiology* **62**, 1727-1737.

Polak M (2008) The developmental instability - sexual selection hypothesis: a general evaluation and case study. *Evolutionary Biology* **35**, 208-230.

Posada D (2008) jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* **25**, 1253-1256.

Price CSC (1997) Conspecific sperm precedence in *Drosophila*. *Nature* **388**, 663-666.

Rauchenberger M, Kallman KD, Moritzot DC (1990) Monophyly and geography of the Rio Pánuco basin swordtails (genus *Xiphophorus*) with descriptions of four new species. *American Museum Novitates*.

Raymond M, Rousset F (1995) GENEPOP (Version1.2) - population-genetics software for exact tests and ecumenicism. *Journal of Heredity* **86**, 248-249.

Reich D, Green RE, Kircher M, Krause J, Patterson N, *et al.* (2010) Genetic history of an archaic hominin group from Denisova Cave in Siberia. *Nature* **468**, 1053-1060.

Rhymer JM, Simberloff D (1996) Extinction by hybridization and introgression. *Annual Review of Ecology and Systematics* **27**, 83-109.

Rieseberg LH (1997) Hybrid origins of plant species. *Annual Review of Ecology and Systematics* **28**, 359-389.

Rieseberg LH, Archer MA, Wayne RK (1999) Transgressive segregation, adaptation and speciation. *Heredity* **83**, 363-372.

Rieseberg LH, Carney SE (1998) Plant hybridization. *New Phytologist* **140**, 599-624.

Rieseberg LH, Raymond O, Rosenthal DM, Lai Z, Livingstone K, *et al.* (2003) Major ecological transitions in wild sunflowers facilitated by hybridization. *Science* **301**, 1211-1216.

Rohlf F (2004) tpsDig (ed. http://life.bio.sunysb.edu/morph/).

Rohlf F (2005) tpsRegr (ed. http://life.bio.sunysb.edu/morph/).

Rohlf F (2007) tpsRelw (ed. http://life.bio.sunysb.edu/morph/).

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572-1574.

Rosenfield JA, Kodric-Brown A (2003) Sexual selection promotes hybridization between pecos pupfish, *Cyprinodon pecosensis* and sheepshead minnow, *C.variegatus*. *Journal of Evolutionary Biology* **16**, 595-606.

Rosenthal GG, De La Rosa Reyna XF, Kazianis S, Stephens MJ, Morizot DC, *et al.* (2003) Dissolution of sexual signal complexes in a hybrid zone between the swordtails *Xiphophorus birchmanni* and *Xiphophorus malinche* (Poeciliidae). *Copeia*, 299-307.

Rosenthal GG, Fitzsimmons JN, Woods KU, Gerlach G, Fisher HS (2011) Tactical release of a sexually-selected pheromone in a swordtail fish. *Plos One* **6**, e16994.

Rosenthal GG, Garcia de Leon FJ (in press) Speciation and hybridization. In: *Ecology and Evolution of Poeciliid Fishes* (eds. Schlupp I, Pilastro A, Evans J). University of Chicago Press, Chicago.

Ruegg K (2008) Genetic, morphological, and ecological characterization of a hybrid zone that spans a migratory divide. *Evolution* **62**, 452-466.

Rundle HD, Schluter D (1998) Reinforcement of stickleback mate preferences: sympatry breeds contempt. *Evolution* **52**, 200-208.

Ryan MJ, Rand AS (1993) Species recognition and sexual selection as a unitary problem in animal communication. *Evolution* **47**, 647-657.

Schluter D (2009) Evidence for ecological speciation and its alternative. *Science* **323**, 737-741.

Schluter D, Conte GL (2009) Genetics and ecological speciation. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 9955-9962.

Seehausen O (2006) Conservation: losing biodiversity by reverse speciation. *Current Biology* **16**, R334-R337.

Seehausen O, vanAlphen JJM, Witte F (1997) Cichlid fish diversity threatened by eutrophication that curbs sexual selection. *Science* **277**, 1808-1811.

Shapiro LH (2001) Asymmetric assortative mating between two hybridizing *Orchelimum* katydids (Orthoptera : Tettigoniidae). *American Midland Naturalist* **145**, 423-427.

Smadja C, Ganem G (2005) Asymmetrical reproductive character displacement in the house mouse. *Journal of Evolutionary Biology* **18**, 1485-1493.

Stamatakis A (2006a) Phylogenetic models of rate heterogeneity: a high performance computing perspective.

Stamatakis A (2006b) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688-2690.

Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML web servers. *Systematic Biology* **57**, 758-771.

Stein AC, Uy JAC (2006) Unidirectional introgression of a sexually selected trait across an avian hybrid zone: a role for female choice? *Evolution* **60**, 1476-1485.

Swofford DL (2001) *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.* Sinauer Associates, Sunderland, MA.

Taylor EB, Boughman JW, Groenenboom M,Sniatynski M, Schluter D, *et al.* (2006) Speciation in reverse: morphological and genetic evidence of the collapse of a three-spined stickleback (*Gasterosteus aculeatus*) species pair. *Molecular Ecology* **15**, 343-355.

Tobler M, Schlupp I, Plath M (2008) Does divergence in female mate choice affect male size distributions in two cave fish populations? *Biology Letters* **4**, 452-454.

Turner CL (1937) Reproductive cycles and superfetation in poeciliid fishes. *The Biological Bulletin* **LXXII**, 145-164.

Vamosi SM, Schluter D (1999) Sexual selection against hybrids between sympatric stickleback species: evidence from a field experiment. *Evolution* **53**, 874-879.

Van valen L (1962) A study of fluctuating asymmetry. *Evolution* **16**, 125-142.

von Helversen D, von Helversen O (1975) Genetic approach to the acoutsic communication of grasshoppers. *Journal of Comparative Physiology* **104**, 301-333.

Walter RB, Rains JD, Russell JE, Guerra TM, Daniels C, *et al.* (2004) A microsatellite genetic linkage map for *Xiphophorus*. *Genetics* **168**, 363-372.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population-structure. *Evolution* **38**, 1358-1370.

Wirtz P (1999) Mother species-father species: unidirectional hybridization in animals with female choice. *Animal Behaviour* **58**, 1-12.

Wolf JBW, Lindell J, Backstrom N (2010) Speciation genetics: current status and evolving approaches introduction. *Philosophical Transactions of the Royal Society B-Biological Sciences* **365**, 1717-1733.

Wong BBM, Fisher HS, Rosenthal GG (2005) Species recognition by male swordtails via chemical cues. *Behavioral Ecology* **16**, 818-822.

Wong BBM, Rosenthal GG (2006) Female disdain for swords in a swordtail fish. *American Naturalist* **167**, 136-140.

Yanchukov A, Hofman S, Szymura JM, Mezhzherin SV (2006) Hybridization of *Bombina bombina* and *B-variegata* (Anura: Discoglossidae) at a sharp ecotone in western Ukraine: comparisons across transects and over time. *Evolution* **60**, 583-600.

Zelditch M, Swiderski D, Sheets H, Fink W (2004) *Geometric Morphometrics for Biologists* Elsevier Academic Press, Amsterdam.

Zimmerer EJ, Kallman KD (1988) The inheritance of vertical barring (aggression and appeasement signals) in the pygmy swordtail, *Xiphophorus Nigrensis* (Poeciliidae, Teleostei). *Copeia*, 299-307.

Zwickl DJ (2006) *Genetic Algorithm Approaches for the Phylogenetic Analysis of Large Biological Sequence Datasets Under the Maximum Likelihood Criterion*, The University of Texas, Austin, TX.

APPENDIX A

No.	Locality	Drainage	E (m)	N		HYB	F_{IS}			LD			mtDNA	Hybrid Index - No. of *X. malinche* nuclear alleles								
				SEQ	SNP		LIG1	POLB	TP53	LIG1/POLB	LIG/TP53	POLB/TP53		0	1	2	3	4	5	6	7	8
M1	Tlatzintla[P]	Río Claro	658	12	22	0	-	-	-	-	-	-	1	0	0	0	0	0	0	0	0	22
	Tamala	Río Claro	320	6										0	0	0	0	0	1	0	0	4
U2	Tlatemaco	Tributary of Río Claro	480		30	0.93	0.016	0.102	0.183	0.040	-0.082	-0.204	1	0	0	0	0	2	4	10	12	2
U1	Apantla	Trib. of Río Claro	352		23	0.74	-0.180	-	-0.007	0.350	0.042	0.177	1	0	0	0	0	1	0	7	9	6
	Xuchipantla	Río Claro	193		24	0.71	0.783†	0.184	-0.211	0.246	-0.164	-0.198	0.042	8	13	2	0	0	0	1	0	0
U3	Tenexco	Río Claro	122		19	0.53	1†	-0.161	-0.091	-0.102	-0.081	-0.092	0	9	8	2	0	0	0	0	0	0
	Huitzitzilingo	Arroyo Tultitlan	161	6										5	0	0	0	0	0	0	0	0
	Chiquitla	Río Huazalingo	1499	6										0	0	0	0	0	0	0	0	6
	Totonicapa	Río Huazalingo	720		30	0.91	-0.335	0.039	0.202	-0.414+	0.298	-0.173	0.414	4	6	6	2	5	4	3	0	0
U4	Cocalaco	Trib. of Río Huazalingo	450		30	0.63	-	-0.025	-0.063	-	-	-0.019	0	11	14	4	1	0	0	0	0	0
H5	San Pedro	Río Huazalingo	384	6	39	0.41	-	0.646†	0.063	-	-	-0.102	0	25	9	5	0	0	0	0	0	0
	Achiquihuixtla	Río Huazalingo	186	6										1	3	2	0	0	0	0	0	0
	Chicayotla	Arroyo Xontla	1003	6										0	0	0	0	0	0	0	0	6
	T-Dubs	Arroyo Xontla	986		30	0.27	-	0.478	-0.055	-0.034	-0.033	-0.070	1	0	0	0	0	0	0	1	7	22
	Spider	Arroyo Xontla	921	6	19	0.53	0.234	0.463	0.495	0.322	0.167	0.28	0.947	1	0	0	0	0	0	1	9	8
S1	Calnali-High	Río Calnali	1168		30	0.6	0.057	0.604*	0.609*	0.416+	0.478*	0.798*	0.433	12	3	2	0	0	2	4	7	0
S2	Calnali-Mid	Río Calnali	1007	6	30	0.63	0.414†	0.216	0.861*	0.546*	0.510*	0.484*	0.333	5	8	4	3	0	0	0	4	6
S3	Aguazarca	Río Calnali	981		41	0.61	0.371	0.425†	0.799*	0.428+	0.535*	0.466*	0.39	8	10	6	1	0	0	2	6	8
U6	Calnali-Low	Río Calnali	920		28	0.71	0.424	-0.256	-0.174	0.141	0.163	0.373	0.1	9	10	4	0	2	2	1	0	0
	Culhuacán	Arroyo Pochutla	1272	6										0	0	0	0	0	0	0	0	6
	Pochutla-High	Arroyo Pochutla	877	5										0	0	0	0	0	0	0	1	4
	Pochutla-Trib	Arroyo Pochutla	916	6										0	0	0	0	0	0	0	0	6
	Nicolasia	Trib. of Arroyo Pochutla	440		14	1	0.217	-0.040	-0.110	-0.404	0.269	0.191	0.429	0	3	2	1	2	2	4	0	0
U7	Tula	Río Tula	422	5	30	1	0.016	0.084	0.223	-0.427+	-0.417+	0.142	0.033	0	0	4	12	11	3	0	0	0
	Ahuamole	Trib. of Arroyo Pochutla	869	6										2	2	0	0	1	0	0	0	0
	Papatlata	Río Atlapexco	272	8	19	0.79	0.077	0.384	-0.333	0.032	0.523+	-0.381	0	4	5	7	2	1	0	0	0	0
U8	Huitzmopala	Río Atlapexco	244	5	30	0.6	-0.094	0.133	-0.160	0.315	0.253	0.386+	0	16	5	6	3	0	0	0	0	0
B1	El Arenal[P]	Río Atlapexco	219	5										2	3	0	0	0	0	0	0	0
M2	Malila[P]	Río Conzintla	1364	6	30	0.03	1*	0.659†	1*	0.856*	0.996*	0.856*	0.93	1	1	0	0	0	0	0	0	28
S4	Xochicoatlan	Río Conzintla	1012		29	0.24	0.711*	0.833*	0.738*	0.785*	0.656*	0.570*	0.759	6	0	2	0	0	0	1	4	17
S5	Mixtla[S5]	Río Conzintla	941		10	0.1	0.816†	1*	1*	0.903*	0.903*	1*	0.4	5	1	0	0	0	0	0	0	4

	Comala	Rio Coxintla	383		29	0.59	-0.120	-0.167	1	-0.153	0.184	0.166	0	12	14	2	1	0	0	0	0	0
	Soyatla	Rio Tianguistengo	1287	6										0	0	0	0	1	0	4	1	0
	Tlacolula	Rio Tianguistengo	469		30	0.37	-0.160	-	-	-0.054	-0.054	-0.017	0	19	11	0	0	0	0	0	0	0
B2	Garces[P]	Rio Garces	229	7										7	0	0	0	0	0	0	0	0
	Agua Fria	Rio Zontecomatlan	241	3										3	0	0	0	0	0	0	0	0
	Benito Juarez	Rio Zontecomatlan	272	4										4	0	0	0	0	0	0	0	0
	Tenango	Arroyo Tenango	274	6										6	0	0	0	0	0	0	0	0
	Mamey	Arroyo Mamey	292	6										6	0	0	0	0	0	0	0	0
	Xilitla	Rio Axtla	1889	5																		
	Atlatipa	Rio Venado	172	6																		
	Garces	Rio Garces	229	5																		

X. cortezi

X. variatus

X. variatus

A. Modified Table II-1 from Culumber et al. (2011). Populations used in the present study are designated with numbers corresponding to Figure VI-1. Those without numbers are for comparative purposes. Indicated for each population are: elevation in meters (E) and sample size for sequencing of mtDNA and nuclear introns (SEQ) and SNP genotyping (SNP). For populations with SNP data, the following measures are shown: the proportion of hybrid multilocus genotypes (HYB), deviations from HWE for three nuclear markers (F_{IS}), linkage disequilibrium (LD) corrected for allele frequency $[D/(p_i q_i p_j q_j)^{1/2}]$, frequency of *X. malinche* mtDNA (mtDNA), and a hybrid index showing number of individuals in each population with 0-8 *X. malinche* nuclear alleles. F_{IS} and LD values where $p < 0.05$ after correction for multiple comparisons are indicated by an asterisk (*) and where $p < 0.05$ before but not after correction (†).

VITA

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