

# Polymorphic MHC loci in an asexual fish, the amazon molly (*Poecilia formosa*; Poeciliidae)

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## Abstract

Genes of the major histocompatibility complex (MHC) encode molecules that control immune recognition and are highly polymorphic in most vertebrates. The remarkable polymorphisms at MHC loci may be maintained by selection from parasites, sexual selection, or both. If asexual species show equal (or higher) levels of polymorphisms at MHC loci as sexual ones, this would mean that sexual selection is not necessary to explain the high levels of diversity at MHC loci. In this study, we surveyed the MHC diversity of the asexual amazon molly (*Poecilia formosa*) and one of its sexual ancestors, the sailfin molly (*P. latipinna*), which lives in the same habitat. We found that the asexual molly has polymorphic MHC loci despite its clonal reproduction, yet not as polymorphic as the sexual species. Although the nucleotide diversity was similar between the asexual and sexual species, the sexual species exhibited a greater genotypic diversity compared to the asexual one from the same habitats. Within-genome diversity was similar for MHC class I loci, but for class IIB, the sexual species had higher diversity compared to the asexual – despite the hybrid origins and higher levels of heterozygosity at microsatellite loci in the asexual species. The level of positive selection appears to be similar between the two species, which suggests that these polymorphisms are maintained by selection. Thus, our findings do not allow us to rule out the sexual selection hypothesis for the evolution of MHC diversity, and although the sexual fish has higher levels of MHC-diversity compared to the asexual species, this may be due to differences in demography, parasites, or other factors, rather than sexual selection.

**Keywords:** clonal, gynogenesis, MHC, *Poecilia formosa*

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## Introduction

The genes of the major histocompatibility complex (MHC) are highly polymorphic loci in most vertebrates, and it has been a considerable challenge to understand how such diversity can be maintained by natural selection (Apanius *et al.* 1997; Wegner *et al.* 2004; Van Oosterhout *et al.* 2006). MHC genes encode class I and class II molecules that control immune recognition of pathogens and parasites by binding

self- and non-self peptides and presenting them to T-cells. Subsequently, T-cells discriminate between self and foreign by recognizing the specificity of the peptide-MHC-complex, triggering an appropriate immune response (Janeway *et al.* 2001). MHC class I and class II loci are usually closely linked and organized in a gene dense cluster; however, in teleost fishes, they are unlinked and distributed over two or more chromosomes (Sato *et al.* 2000; Stet *et al.* 2003) and can therefore recombine independently. Several lines of evidence indicate that MHC polymorphisms are maintained by some form of balancing selection, either from pathogens and parasites (Apanius *et al.* 1997; Lohm *et al.* 2002; Wegner *et al.* 2003; Prugnolle *et al.* 2005), disassortative mating preferences (Yamazaki *et al.* 1978), or both (Penn & Potts 1999; Penn 2002). We suggest that if the asexual species

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show high levels of MHC polymorphisms, or at least comparable to their sexual congeners, then sexual selection is unnecessary to explain the unusual diversity of MHC genes. We examined this hypothesis in two species, an asexual fish and its sympatric congener.

The amazon molly (*Poecilia formosa*), a clonal, all-female, teleost (family Poeciliidae) originated between 40 000 and 100 000 years ago through a single natural hybridization event between the two closely related sexual species, *P. latipinna* (sailfin molly) and *P. mexicana* (Atlantic molly; Avise *et al.* 1991; Schartl *et al.* 1995a; Loewe & Lamatsch 2008). The amazon molly occurs along the eastern coast of northern Mexico into south and central Texas and overlaps in distribution with both *P. mexicana* and *P. latipinna* (Schlupp *et al.* 2002). Although inheritance is clonal, amazon females must mate with heterospecific males, either *P. latipinna* or *P. mexicana*, because they require sperm to trigger embryogenesis (gynogenesis; Schlupp 2005). Male genes may enter the amazon molly's genome, but apparently this is rare (Schartl *et al.* 1995b, 1998; Lamatsch *et al.* 2004; Schlupp 2005).

Amazon molly populations have a lower genotypic diversity compared to their sexual relatives (Lampert *et al.* 2005; M. Plath *et al.*, unpublished data). Recent population genetic studies using microsatellite analyses showed that two dominant clones are accompanied by a set of rare clones in each amazon molly population (Lampert *et al.* 2005; M. Plath *et al.*, unpublished data). Although genotypic diversity in amazon mollies is low, individuals exhibit higher heterozygosity at most microsatellite loci than sexual sailfin mollies, which is likely because of the hybrid origin of asexuals (Tiedemann *et al.* 2005; M. Plath *et al.*, unpublished data). Some microsatellite loci apparently encompass only alleles of one ancestral species, suggesting genome homogenization through mitotic gene conversion (Tiedemann *et al.* 2005; M., Plath *et al.*, unpublished data). Because the amazon molly's overall genotypic diversity is low, we expect within-population MHC diversity to be reduced compared to its sexual congeners as well. On the other hand, within-genome diversity might be high due to the hybrid origin. Virtually nothing is known about MHC diversity in this asexual fish, and to our knowledge, only one study has ever examined MHC variation in an asexual species (Radtkey *et al.* 1996). Our aim was to survey the MHC class I and class IIB diversity of the asexual amazon molly (*P. formosa*) and one of its sexual relatives, the sailfin molly (*P. latipinna*). This study is a first step towards determining whether pathogens drive MHC diversity in these species, and it is part of a larger project aimed to test the hypothesis that sexual reproduction functions to provide a 'moving target' against rapidly evolving pathogens and parasites (Red Queen hypothesis; Jaenike 1978; Hamilton 1980; Bell 1982; Hamilton *et al.* 1990; Tobler & Schlupp 2008).

## Materials and methods

### *Samples and DNA isolation*

In our study, we included a total of 113 wild-caught amazon mollies (*Poecilia formosa*) and 45 wild-caught sailfin mollies (*P. latipinna*) from Texas (USA). The amazon molly sample encompassed individuals from two populations: south Texas (two sampling sites: Weslaco ( $n = 14$ ) and Lincoln Park ( $n = 4$ )) and central Texas (two sampling sites: Martindale ( $n = 76$ ) and County 101 ( $n = 19$ )). The sailfin molly individuals were obtained from the same two populations and sampling sites (except County 101) as the amazon mollies [south Texas: Weslaco ( $n = 14$ ) and Lincoln Park ( $n = 5$ ); central Texas: Martindale ( $n = 26$ )]. Genomic DNA was extracted from tissue samples with a DNA extraction kit DNeasy Tissue Kit (QIAGEN GmbH) according to the manufacturer's protocol.

### *PCR amplification, primers, and cloning of MHC class I genes*

The exon 2 (encoding the  $\alpha$ -1 domain) of the MHC class I genes [alleles were named in this study as UA (teleost MHC class I genes) with serial numbers attached] was amplified using the published primer pair Tu1372/Tu1373 of the guppy (*Poecilia reticulata*; Sato *et al.* 2000). The guppy primer pair successfully amplified the MHC class I exon 2 in all molly samples. The 25  $\mu$ l polymerase chain reaction (PCR) mixture contained 1 $\times$  PCR buffer, 200 mM dNTPs mix, 1.5 mM MgCl<sub>2</sub>, 15 pM/ $\mu$ l of each primer, 1–2  $\mu$ l of genomic DNA sample, and 1 U *Taq* Polymerase (QIAGEN GmbH). The thermal cycling profile for the PCR consisted of 5 min preheating at 95 °C, followed by either 23 or 26 cycles (depending on the PCR yield) of 35 s at 94 °C, 35 s at 54 °C and 60 s at 72 °C with a final extension of 10 min. The PCR products were checked by electrophoresis on a 1.5% agarose gel and then immediately cloned into the PCR 2.1-TOPO plasmid (TOPO TA cloning kit, Invitrogen Ltd). Subsequently, the cloning reactions were transformed into *Escherichia coli* (TOP10) competent cells (Invitrogen Ltd). From each individual, between 10 and 15 positive (blue/white selection) clones were picked and grown overnight in 3 mL of LB-Medium (containing 50  $\mu$ g/mL ampicillin). Plasmids were isolated by the QIAprep spin miniprep kit (QIAGEN GmbH) and checked for the correct inserts with *Eco*RI digestion or by running a second PCR with M13 sequencing primers before sequencing.

### *PCR amplification, primers, and cloning of MHC class IIB genes*

We focused on the exon 2 that encode the PBR ( $\beta$ 1-domain) of the MHC class IIB genes. We first tried to amplify the

exon 2 by using the published MHC class IIB exon 2 primer pair TU1292/TU1293 of the guppy (Sato *et al.* 2000), but this primer pair did not amplify the exon 2 in the first molly samples. Subsequently, we used the three-spined stickleback (*Gasterosteus aculeatus*) specific MHC class IIB forward primer GA11 (Sato *et al.* 2000), which binds in a rather conserved region of the exon 2 5'-region in teleosts, together with the guppy-specific reverse primer TU1293. With this primer combination, we amplified the exon 2 in seven out of 11 molly samples we tested. To increase the specificity and performance of the primers, we designed a new molly-specific reverse primer in accordance to a sequence alignment of MHC class IIB sequences of various bony fish species and of molly MHC class IIB sequences obtained previously with the guppy/stickleback primer combination. The GA11 forward primer and the newly designed reverse primer (PoeR1: 5'-TTGGTAGTCGATACCAACGTTG-3') successfully amplified part of the exon 2 of the MHC class IIB genes in all samples. The PCR conditions as well as the cloning procedure were as described for the MHC class I analysis (see above).

#### Sequencing analyses and detecting positive selection

Sequences were determined using the CEQ DTCS quick start kit (Beckman Coulter Inc.), dye-labeled dideoxy-terminator cycle sequencing kit, and the CEQ 8000 capillary DNA Sequencer (Beckman Coulter Inc.). Preliminary sequence processing and analysis was performed with BioEdit (Hall 1999). Single nucleotide substitutions between sequences were possible PCR/cloning artefacts and therefore were used only if found at the minimum in three clones from the same specimen or in clones of another sample. Thus, each MHC allele described in the present study was confirmed by a minimum of three sequenced clones. All nucleotide and amino acid sequences were aligned with the program Clustal\_X (Thompson *et al.* 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al.* 2004). The estimates for the nucleotide diversity  $\pi$  (Nei 1987) were calculated using DnaSP version 4.10.3 (Rozas *et al.* 2003). The selective pressure at the amino acid level was measured by the nonsynonymous/synonymous rate ratio ( $\omega = d_N/d_S$ ), whereby  $\omega > 1$  indicates positive selection (e.g. Yang & Bielawski 2000). We used the maximum-likelihood method (Yang *et al.* 2000) implemented in the program CODEML of the PAML 4.1 software package (Yang 2007) to test whether codon sites were affected by positive selection (Yang *et al.* 2005). The models considered in this study were M7 (beta), and M8 (beta and  $\omega$ ; for details, see Yang *et al.* 2000; Yang & Swanson 2002). While recombination can potentially generate false-positives in the detection of positive selection, these two models are much more robust against the occurrence of recombination than the other models implemented in CODEML (Anisimova *et al.* 2003). Under

the model M7 (beta) the  $\omega$  ratio varies according to the beta distribution (with parameters  $p$  and  $q$ ) and does allow for positive selected sites ( $0 < \omega < 1$ ), and thus serves as the null model for comparison with model M8 (beta &  $\omega$ ). The models M7 and M8 can be compared in pairs using the likelihood-ratio test (LRT; Nielsen & Yang 1998). The LRT statistics calculate twice the log-likelihood difference compared with a chi-squared ( $\chi^2$ ) distribution with degrees of freedom equal to the difference in the number of parameters between the two compared models. To provide phylogenetic information required for the analysis (Yang 2007), the best tree for MHC sequences was identified by maximum likelihood under the one-ratio model (M0) in CODEML. Positively selected codons ( $\omega > 1$  with  $P > 95\%$ ) were identified through an empirical Bayes approach implemented in CODEML (Yang *et al.* 2005).

#### CE-SSCP analysis of MHC class I and class IIB genes

To screen and identify the allelic variants of the MHC genes in a larger number of samples, we used capillary electrophoresis single-strand conformation polymorphism (CE-SSCP). This method can be automated and is characterized by high throughput, high sensitivity, and good reproducibility (Binz *et al.* 2001; Bryja *et al.* 2005; Schaschl & Wegner 2006). We refer here to MHC sequence variants detected by CE-SSCP for simplicity as 'alleles', although we were unable to assign any sequence variants to a certain MHC locus. For all PCRs, the QIAGEN Multiplex PCR Kit with fluorescent-labeled primers (forward primer by 6'-FAM and reverse primer by HEX), was used to amplify the MHC genes in the molly samples. The thermal cycling profile for the PCRs consisted of initial heating at 95 °C for 15 min (hot-start polymerase activation), followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 35 s, extension at 72 °C for 90 s, and ending with a 10-min extension step at 72 °C. For the CE-SSCP analyses, the fluorescent-labeled PCR samples were prepared for electrophoresis by combining 1  $\mu$ L PCR product with 14  $\mu$ L loading mix [13.75  $\mu$ L Hi-DI formamide, 0.25  $\mu$ L GeneScan ROX 350 standard (Applied Biosystems)]. The mixture was heated for 3 min at 95 °C to separate the complementary DNA strands, chilled on ice for 4 min and analysed by capillary electrophoresis on an ABI PRISM 3130xl automated DNA Sequencer (Applied Biosystems). The CE-SSCP polymer consisted of 5% GeneScan polymer (Applied Biosystems), 10% glycerol, 1xTBE, and HPLC-water. The running buffer mixture contained 10% glycerol, 1xTBE and HPLC-water. The separation of the allelic variants was achieved by run conditions at 12 kV for 36 min and by a run temperature at 22 °C. The retention times of the allelic variants were identified relative to the ROX 350 standard. The GeneMapper software packages 4.05 from Applied Biosystems were used to process the obtained SSCP data.



**Table 1** Comparison of the number of obtained MHC sequences and nucleotide diversity calculated for the MHC class I exon 2 ( $\alpha 1$ -domain) and MHC class IIB exon 2 ( $\beta 1$ -domain) sequences in the two *Poecilia* species investigated

		<i>P. formosa</i>	<i>P. latipinna</i>
MHC class I	No. of sequenced individuals	29	14
	No. of sequence variants	24	23
	Nucleotide diversity ( $\pi$ )	0.229	0.220
MHC class IIB	No. of sequenced individuals	29	14
	No. of sequence variants	9	12
	Nucleotide diversity ( $\pi$ )	0.214	0.206

**Table 2** Log-likelihood values and parameter estimates for the *Poecilia* MHC class I and MHC class IIB sequences.

Model	ln L		Estimates of parameters		Positively selected sites	
	<i>P. formosa</i>	<i>P. latipinna</i>	<i>P. formosa</i>	<i>P. latipinna</i>	<i>P. formosa</i>	<i>P. latipinna</i>
MHC class I						
M7 (beta)	-2044.14	-1732.82	$p = 0.28680$ $q = 0.16085$	$p = 0.25042$ $q = 0.12695$	Not allowed	Not allowed
M8 (beta & $\omega$ )	-2023.98	-1755.84	$p_0 = 0.66396$ ( $p_1 = 0.33604$ ) $p = 0.38136$ $q = 0.31118$ $\omega = 2.7$	$p_0 = 0.64836$ ( $p_1 = 0.35164$ ) $p = 0.28525$ $q = 0.17919$ $\omega = 3.1$	35** 78* 38** 79** 45** 80** 52** 82** 63* 88** 64* 92** 65** 93** 74*	23* 78** 35* 79** 38** 82* 45** 85* 52** 88* 54** 92** 64** 93* 77*
MHC class IIB						
M7 (beta)	-880.19	-880.19	$p = 0.22129$ $q = 0.29375$	$p = 0.21660$ $q = 0.34060$	Not allowed	Not allowed
M8 (beta & $\omega$ )	-868.60	-868.60	$p_0 = 0.82550$ ( $p_1 = 0.17450$ ) $p = 0.34611$ $q = 0.60563$ $\omega = 3.3$	$p_0 = 0.79418$ ( $p_1 = 0.20582$ ) $p = 0.32889$ $q = 0.67685$ $\omega = 3.6$	34** 27** 35** 50** 56* 64**	25** 68** 35** 74** 50** 62* 64** 67**

ln L is the log-likelihood value,  $\omega$  is the selection parameter and  $p_n$  is the proportion of sites that fall into  $\omega_n$  site class. Sites inferred to be under positive selection are given at the 95% (\*) and 99% (\*\*) confidence interval level.

MHC class I sequence (Pofu-UA\*22) was probably generated by a point mutation, since the amazon molly class I sequence Pofu-UA\*22 and the sailfin molly sequence Pola-UA\*07 differ only at one nucleotide position by a nonsynonymous substitution. This single nucleotide difference between the two sequences may not be the result of a sequencing artefact because we obtained each sequence separately in each studied species and by sequencing the forward and the reverse strand.

We tested for positive selection using maximum-likelihood analysis (Yang *et al.* 2000). The LRT, comparing the two

models M7 and M8, demonstrated that M8 [the model that accounts for sites under positive selection ( $\omega > 1$ )] showed a significantly better fit for both molly species ( $P < 0.001$ ) than the model M7 [that does not allow for positive selection ( $0 < \omega < 1$ )]. In both species, the  $\omega$  ratio is more than 1 (Table 2), indicating positive selection in the MHC class I sequences. The estimates from M8 suggested that about 33% of the sites were under strong positive selection in the amazon molly class I sequences ( $\omega = 2.7$ ) and 35% in the sailfin molly sequences ( $\omega = 3.1$ ; Table 2). Bayes identification of sites under positive selection is listed in Table 2.

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Pofo-class_IIB*01 EYIRSYYYNK LETNRFTSSL GKFVGYTAAG VRQAEIWNKD PSLIAGLKAT KETICL
Pofo-class_IIB*02 .F.Y..... .YA..D.NV .....EY. .KN..R... T.Q..A...Q ..AY..
Pofo-class_IIB*03 .....MF... .FA..... .R.....EW. .K..NY..NN ..Y.....Q ...Y..
Pofo-class_IIB*04 ...Y.M.... .PL..D.NV ..Y....EL. .KN..RA... ..E..MRM.Q ...Y..
Pofo-class_IIB*05 QF...SI... .WL..D... .R.....EF. .KE..R..N. ..YL.AM..Q R..Y..
Pofo-class_IIB*06 QF.S..I... I.YV..D.NV ...I...EF. .KN..Q.... ..R..MM..Q ...Y..
Pofo-class_IIB*07 .F.....Y... .LL..D.NV ..Y....EL. .KN..Q.... ..E..M..Q ..AY..
Pofo-class_IIB*08 .....DF... .IM..S... .....EF. .K..KYF.NN ..Y.....Q ..AY..
Pofo-class_IIB*09 .F...QF... .IF..... .R.....EY. .K...YR.NN ..F..QM..E ..RF..

Pola-class_IIB*01 .F.Y..... .YA..D.NV .....EY. .KN..R... T.Q..A...Q ..AY..
Pola-class_IIB*02 QF...SI... .WL..D... .R.....EF. .KE..R..N. ..YL.AM..Q R..Y..
Pola-class_IIB*03 Q..N..... .YA..D.NV .....Y. .KN..RL... ..V..VR..E ..RY..
Pola-class_IIB*04 .....EF... .QA..S... .R.....EW. .K..NY..N. ..Y.....S ...Y..
Pola-class_IIB*05 .....DF... .IM..S... .....EF. .K..KYF.NN ..Y.....Q ..AY..
Pola-class_IIB*06 .F...QF... .IF..... .R.....EY. .K...YR.NN ..F..QM..E ..RF..
Pola-class_IIB*07 ...Y.EF... .QA..S... .R.....EW. .K..NY..N. ..Y..QM..E ..RY..
Pola-class_IIB*08 ...Y..... .FI..D.NV .R.....EL. .KN..Q.... ..K..AM..Q ...Y..
Pola-class_IIB*09 .MY.QF... .IY..S... .R.....EY. .K...YF... T.V..RRM.Q ..RF..Q
Pola-class_IIB*10 ...Y..... .FI..D.NV .RY....EL. .KN..Q.... Q.Q..VQ..Q ..AY..
Pola-class_IIB*11 ...Y..... .....T.Y..Q...D ..RY..
Pola-class_IIB*12 ...Q..... .YA..D.NV .RY.....Y. .MN..RF... ..V...R..E ..RY..

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**Fig. 2** Sequence alignment of the predicted amino acid of the MHC class IIB ( $\beta 1$  domain) sequences of *Poecilia formosa* (Pofo-class IIB) and *P. latipinna* (Pola-class IIB). Dots indicate identity in the amino-acid sequence to the sequence of the *P. formosa* Pofo-class IIB\*01. The number above the sequence indicates codon position.

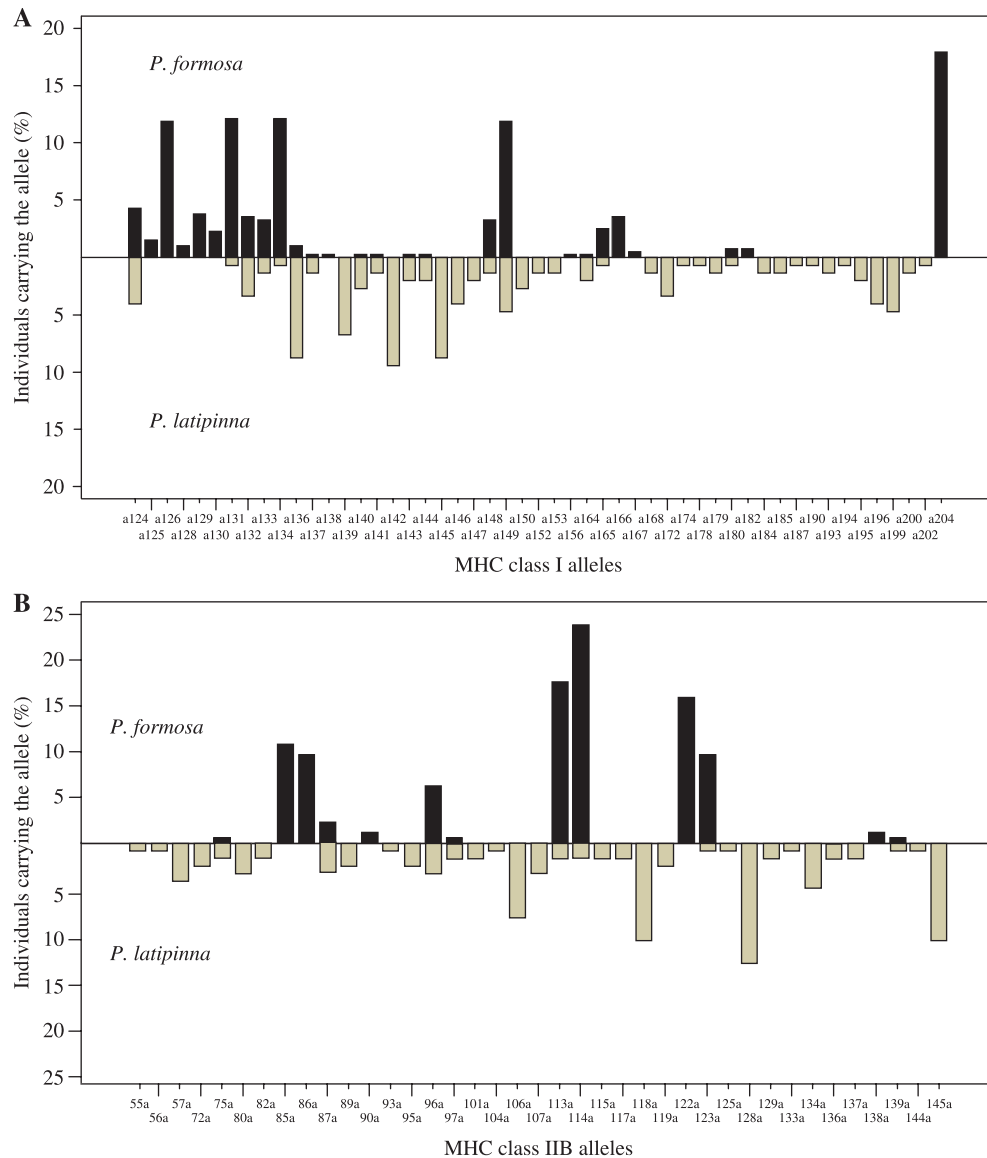
### Sequence analyses of the MHC class IIB genes

The same individuals were sequenced for MHC class IIB as for MHC class I. The molly MHC class IIB exon 2 sequences we obtained were 216 bp in length (including primer sequences). All sequences have been deposited in GenBank (*P. formosa* Accession nos EU790517–EU790525; *P. latipinna* Accession nos EU790549–EU790560) and the sequences showed no deletions, insertions, or stop codons, indicating that all sequences probably encode functional MHC class IIB molecules (Fig. 2). Between one and four class IIB sequences were found per individual in both species, suggesting that the primer pair we used may amplify from one to four loci in these species. We found nine distinct MHC class IIB sequences in the asexual amazon molly and 12 class IIB sequences in the sailfin molly (Fig. 3). Nucleotide sequence variation among all pairwise comparisons of amazon molly and sailfin class IIB sequences, corrected for multiple substitutions, ranged from 12.1 to 34.7% (mean  $25.5\% \pm 2.8\%$ ) and from 6.1 to 41.4% (mean  $25.3\% \pm 2.8\%$ ), respectively. Amino acid replacement among all pairwise comparison ranged from 9 to 23 (mean  $17.4 \pm 2.4$ ) in the amazon molly class IIB sequences and from 6 to 24 (mean  $17.3 \pm 2.3$ ) in the sailfin molly. The nucleotide diversity ( $\pi$ ) of the MHC class IIB sequences for both species are given in Table 1. Similar to the finding for the MHC class I sequences, the LRT statistics demonstrated also for the MHC class IIB sequences that M8 fitted the data from both molly species significantly ( $P < 0.001$ ) better than the model M7. In both species, the  $\omega$  ratio was more than 1 (Table 2), indicating positive selection also in the MHC class IIB sequences. The estimates from M8 suggested that about 17% of the sites were under strong positive selection in the amazon molly class IIB sequences ( $\omega = 3.3$ ) and 20% in the sailfin molly class IIB sequences ( $\omega = 3.6$ ) (Table 2). Sites in the MHC class IIB sequences identified as being under positive selection in the Bayesian analysis are listed in Table 2.

### CE-SSCP analysis of MHC class I and class IIB diversity

We carried out CE-SSCP analysis to investigate the MHC diversity in several populations of these two species. The CE-SSCP analysis revealed that the MHC class I diversity was reduced in the asexual amazon molly populations compared to the sexual sailfin molly. We obtained only 27 MHC class I sequence variants (alleles) in the asexual species whereas we found 40 in the sexual species (Fig. 3). Consequently, genotypic diversity is significantly reduced in asexual mollies as we found 34 distinct MHC class I genotypes in 40 sailfin molly, but only 20 distinct genotypes in 113 amazon mollies ( $\chi^2 = 58.593$ ,  $P < 0.001$ ; Table S1, Supporting Information). Similarly to the sequence analysis, we also obtained between one and six alleles per individual in the CE-SSCP analysis. The mean within-genome MHC class I diversity did not differ significantly between amazon mollies (mean  $\pm$  SD:  $3.57 \pm 1.75$ ) and sailfin mollies ( $3.82 \pm 1.34$ ; independent sample  $t$ -test corrected for unequal variances:  $t_{89,01} = 0.966$ ,  $P = 0.337$ ) alleles per individual.

As in the CE-SSCP analysis of the MHC class I diversity, we obtained fewer sequence variants from the MHC class IIB genes in the asexual amazon molly as compared to the sexual molly species. The CE-SSCP analysis revealed 13 MHC class IIB alleles in the Amazon molly samples and 34 in the sexual sailfin molly (Fig. 3), and genotypic diversity was significantly lower in asexuals (14 distinct genotypes in 112 samples) than sexuals (25 genotypes in 44 samples;  $\chi^2 = 33.091$ ,  $P < 0.001$ ; Table S2, Supporting Information). In the amazon molly, the number of sequence variants obtained by CE-SSCP analysis varied from one to three per individual. In most individuals, however, we detected only two MHC class IIB sequences with a mean of  $1.59 (\pm 0.53)$  per individual. In the sailfin molly, the MHC class IIB sequences varied from one to four with a mean of  $2.23 (\pm 0.89)$  per individual. Hence, asexual mollies had a significantly lower within-genome diversity in MHC IIB alleles



**Fig. 3** MHC class I (A) and class IIB (B) allele frequency distribution (%) detected by CE-SSCP analysis in the *Poecilia formosa* (dark bars) and in *P. latipinna* (light bars) populations from Texas (USA).

compared to sexuals (independent sample *t*-test corrected for unequal variances:  $t_{55,50} = 4.475$ ,  $P < 0.001$ ).

Some of the MHC sequences found in the amazon molly were not detected in the sailfin molly samples. These MHC sequences may either (i) represent Sailfin-molly-derived sequences that have not been detected in our Sailfin molly samples, (ii) have been derived from the second sexual ancestor (*P. mexicana*), (iii) be shared sequences between the two sexual species, or (iv) represent novel MHC class I sequences generated by point mutations after the hybridization event. Notably, the MHC alleles shared between the asexual and sexual molly species occurred at very different frequencies in each species (Fig. 3).

## Discussion

We examined the diversity at class I and class IIB MHC loci in an asexual fish and its sexual congener and found that the asexual amazon mollies were polymorphic, but not nearly as diverse as the sexual sailfin mollies. Therefore, our results do not allow us to rule out the sexual selection hypothesis for MHC diversity in sailfin mollies. We first sequenced the exon 2 of both the MHC class I and class IIB genes in both species. The exons encode part of the peptide (antigen) binding region in the two types of MHC molecules, which is likely the most polymorphic region in the MHC sequences. The number of sequences in the molly samples

ranged from one to six sequences per individual for MHC class I, and one to four sequences for MHC class IIB genes. If the number of MHC loci varies among individuals (copy number variation), then the number of class I loci could range from one to six and the number of class IIB loci from one to four. Alternatively, the number of loci may be fixed (e.g. three MHC class I and two class IIB loci), and only the level of heterozygosity per locus varies. Similar results have been described in other studies where an MHC primer pair simultaneously amplified sequences from multiple MHC genes (Bonneaud *et al.* 2004; Westerdahl *et al.* 2004; Schaschl & Wegner 2006; Jäger *et al.* 2007). Similar to the MHC class I sequences of another poeciliid (the guppy, *Poecilia reticulata*), a six-nucleotide indel position occurs in the molly MHC class I sequences (Sato *et al.* 1995). The indel position is probably located in the loop region of the MHC class I molecules, and it has been argued that these indels may reflect permissibility of loop-length variation without functional consequences (Sato *et al.* 1995). This possibility could be tested using crystallography and functional studies of fish MHC class I molecules.

#### *MHC diversity in asexual and sexual mollies*

The nucleotide diversity and amino replacements in the MHC class I and class IIB sequences were very similar in the asexual and sexual species; however, the CE-SSCP analysis, which is based on a larger samples size, indicated that the population-wide MHC diversity (i.e. number of MHC class I and class IIB alleles present in the populations) is reduced in the asexual amazon molly compared to its sexual ancestor. The sailfin molly had about 1.5 times more alleles from the MHC class I genes and about 2.6 times more alleles from the MHC class IIB genes compared to the amazon molly. Similarly, a lower diversity in MHC class I genes compared to closely related sexual species was found in a parthenogenetic vs. a sexual gecko (*Hemidactylus* and *Lepidodactylus*, respectively; Radtkey *et al.* 1996). Furthermore, in mangrove killifish (*Rivulus marmoratus*) – an obligatorily selfing, synchronous hermaphroditic fish species – up to 44% of the fish were heterozygous at the MHC class I loci as compared to near homozygosity at non-MHC loci (Sato *et al.* 2002).

Using microsatellite markers, genotypic diversity within asexual populations has been shown to be much lower as compared to the sexual populations, although the allelic richness does not differ significantly between sexuals and asexuals (Lampert *et al.* 2005; M. Plath *et al.*, unpublished data). Similarly, we found that the asexual amazon mollies exhibit a smaller genotypic diversity at MHC class I and IIB loci compared to sexual sailfin mollies in the same populations. The reduction of genotypic diversity is likely due to the lack of recombination in asexually reproducing organisms. In the central Texas population, where amazon mollies

were introduced in the 1950s, one MHC class I genotype occurred at a frequency of 49%. This eminently low MHC diversity may reflect a less diverse clonal population due to a bottleneck effect after introduction (Turner 1982). In the south Texas population, no dominant MHC genotype has been found in this study, but sample sizes were not sufficient to accurately estimate the genotype diversity in this population.

Due to their hybrid origin, amazon mollies are expected to exhibit high within-genome diversity, and indeed previous studies report that they exhibiting a higher degree of heterozygosity compared to sexual mollies (Lampert *et al.* 2005; Tiedemann *et al.* 2005; M. Plath *et al.*, unpublished data). Despite the hybrid origin, within-genome diversity (measured as the number of alleles per individual) did not differ among asexual and sexual species at MHC class I loci, and was even significantly lower in asexuals at MHC IIB loci.

#### *Evolution of MHC diversity in asexual mollies*

Unlike other asexual vertebrates, which originated as a result of multiple hybridization events, amazon mollies appear to have a single origin and – being ameiotic – the current clonal diversity is thought to be resulting from mutations rather than from multiple hybridization events (Turner *et al.* 1990; Lampert *et al.* 2005). In sexual vertebrates, several studies suggested that point mutations and extraordinarily high rates of meiotic recombination coupled with strong positive selection can rapidly generate and maintain new MHC alleles in populations (e.g. Richman *et al.* 2003; Reusch *et al.* 2004; Consuegra *et al.* 2005; Schaschl *et al.* 2006). In an asexual species lacking meiotic recombination, however, only point mutations can generate novel MHC alleles. For example, the amazon molly MHC class I sequence Pofa-UA\*22 differs at one nonsynonymous position from the sailfin molly class I sequences Pola-UA\*07 and could therefore represent such a novel allele. However, we cannot exclude the possibility that this sequence is a sailfin molly sequence that has not yet been sampled or is a sequence derived from its second sexual ancestor, the Atlantic molly (*P. mexicana*). In addition, due to its sperm dependence, the inclusion of paternal genes may elevate the individual MHC diversity in the amazon molly. Inclusion of paternal genes has been documented and leads to stable triploid lineages (Lampert *et al.* 2005) and lineages containing micro-chromosomes (Schartl *et al.* 1995b).

The genotypic diversity in asexual populations may have been reduced due to drift and/or selection since the original hybridization event (Loewe & Lamatsch 2008). Furthermore, some amazon mollies exhibited MHC alleles from only one of the sexual ancestors. For example, the MHC class IIB alleles a113 and a114, which both were the

most frequent alleles in amazon mollies, have probably been derived from the sailfin molly only. This finding is in agreement with a recent microsatellite study in amazon mollies, where at some loci, only alleles from one ancestral species are present in the asexual hybrid (Tiedemann *et al.* 2005; M. Plath *et al.*, unpublished data). This result was interpreted as genome homogenization through mitotic gene conversion (Tiedemann *et al.* 2005).

Interestingly, the two dominant MHC class IIB alleles (a113 and a114) as well as some MHC class I alleles (e.g. a131, a134, and a149) were not only found in amazon mollies, but also in the sexual sailfin molly; however, these alleles occurred at vastly different frequencies in the two species (Fig. 3). This suggests that either these alleles have been in high frequency during the time of hybridization and were subsequently maintained in this frequency in the asexual amazon molly populations, or these alleles confer a recent advantage in immune defence for the asexual molly species and increased their frequency over time. Since asexual mollies must occur in sympatry with their sexual relatives and are exposed to the same environmental conditions and parasite fauna (Tobler *et al.* 2005), it is unclear how specific alleles could confer an advantage for one, but not the other molly species (although some evidence indicates that the genetic background influence how MHC alleles affect a hosts' resistance to pathogens).

We examined evidence for positive selection for both MHC class I and class IIB in both molly species, and found that the  $\omega$ -ratios were very similar between the two species for both MHC class I and class IIB sequences (Table 2). This finding suggests a similar level of positive selection acting on the MHC genes and in both species. Bayesian analysis identified largely consistent individual positively selected sites in MHC class I and class IIB across both species, which indicates that probably both species are exposed to the same selective pressure. Indeed, a previous study has shown that both species harbour similar levels of parasites (Tobler *et al.* 2005).

It is surprising that we found such diversity at MHC loci in the asexual amazons, as they are a 'clonal' species, and future studies on the evolution of MHC diversity could potentially benefit by more work on such asexual species (for focusing on pathogen-mediated selection). It is not surprising that the amazon mollies have lower levels of diversity than the sexual molly, but this difference may be due to selection from pathogens, genetic drift, or other factors, and not necessarily mate choice. Other studies that have examined parasitism in amazon and sexual mollies found, contrary to the expectations of the Red Queen hypothesis, that amazon mollies do not suffer from higher parasite loads as compared to their sexual relatives (Tobler & Schlupp 2005; Tobler *et al.* 2005; M. Tobler *et al.*, unpublished data). Our findings therefore suggest that asexual species may not have more parasites despite having significantly lower

within-genome diversity at MHC class IIB loci. Clearly, these findings need to be addressed in future studies.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1** Allelic composition of different MHC class I genotypes identified in asexual *Poecilia formosa* and sexual *P. latipinna*. The number of alleles as well as the number of individuals of the respective genotype are also given.

**Table S2** Allelic composition of different MHC class IIB genotypes identified in asexual *Poecilia formosa* and sexual *P. latipinna*. The number of alleles as well as the number of individuals of the respective genotype are also given.

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## Online supplementary materials

Table S1: Allelic composition of different MHC class I genotypes identified in asexual *Poecilia formosa* and sexual *P. latipinna*. The number of alleles as well as the number of individuals of the respective genotype are also given.

Species	Genotype ID	No alleles	No individuals	Allele-1	Allele-2	Allele-3	Allele-4	Allele-5	Allele-6
<i>P. formosa</i>	1	1	1	130					
<i>P. formosa</i>	2	1	8	164					
<i>P. formosa</i>	3	1	7	165					
<i>P. formosa</i>	4	1	14	166					
<i>P. formosa</i>	6	2	3	165	180				
<i>P. formosa</i>	7	2	2	167	182				
<i>P. formosa</i>	8	2	1	204	220				
<i>P. formosa</i>	14	3	1	124	129	133			
<i>P. formosa</i>	15	3	3	125	130	133			
<i>P. formosa</i>	16	3	1	137	140	143			
<i>P. formosa</i>	17	3	1	138	141	144	156		
<i>P. formosa</i>	18	3	1	131	134	204			
<i>P. formosa</i>	30	4	1	124	129	132	148		
<i>P. formosa</i>	31	4	1	124	129	132	204		
<i>P. formosa</i>	32	4	2	124	130	133	204		
<i>P. formosa</i>	33	4	3	125	130	133	204		
<i>P. formosa</i>	34	4	4	128	133	136	204		
<i>P. formosa</i>	44	5	11	124	129	132	148	204	
<i>P. formosa</i>	45	5	47	126	131	134	149	204	
<i>P. formosa</i>	50	6	1	124	129	132	148	182	204
<i>P. latipinna</i>	5	1	1	199					
<i>P. latipinna</i>	9	2	1	141	144				
<i>P. latipinna</i>	10	2	1	143	146				
<i>P. latipinna</i>	11	2	1	148	165				
<i>P. latipinna</i>	12	2	1	133	172				
<i>P. latipinna</i>	13	2	1	164	186				
<i>P. latipinna</i>	19	3	2	140	144	147			
<i>P. latipinna</i>	20	3	1	142	145	149			
<i>P. latipinna</i>	21	3	1	143	146	152			
<i>P. latipinna</i>	22	3	1	168	172	174			
<i>P. latipinna</i>	23	3	1	142	145	196			

<i>P. latipinna</i>	24	3	1	134	178	196			
<i>P. latipinna</i>	25	3	1	172	187	196			
<i>P. latipinna</i>	26	3	1	142	145	199			
<i>P. latipinna</i>	27	3	1	145	150	199			
<i>P. latipinna</i>	28	3	1	142	146	200			
<i>P. latipinna</i>	29	3	1	143	146	200			
<i>P. latipinna</i>	35	4	1	136	139	141	199		
<i>P. latipinna</i>	36	4	1	136	139	142	145		
<i>P. latipinna</i>	37	4	1	136	139	145	149		
<i>P. latipinna</i>	38	4	1	140	142	145	179		
<i>P. latipinna</i>	39	4	1	140	142	145	195		
<i>P. latipinna</i>	40	4	1	137	146	148	196		
<i>P. latipinna</i>	41	4	1	137	145	149	199		
<i>P. latipinna</i>	42	4	1	168	172	179	190		
<i>P. latipinna</i>	43	4	2	196	199	210	214		
<i>P. latipinna</i>	46	5	1	124	132	136	172	194	
<i>P. latipinna</i>	47	5	1	133	136	139	142	195	
<i>P. latipinna</i>	48	5	3	136	139	142	145	149	
<i>P. latipinna</i>	49	5	1	149	152	164	186	202	
<i>P. latipinna</i>	51	6	1	124	131	136	146	150	195
<i>P. latipinna</i>	52	6	2	124	132	136	150	153	184
<i>P. latipinna</i>	53	6	2	124	132	136	139	142	193
<i>P. latipinna</i>	54	6	1	139	142	145	147	164	180

Table S2: Allelic composition of different MHC class IIB genotypes identified in asexual *Poecilia formosa* and sexual *P. latipinna*. The number of alleles as well as the number of individuals of the respective genotype are also given.

Species	Genotype ID	No alleles	No individuals	Allele-1	Allele-2	Allele-3	Allele-4
<i>P. formosa</i>	1	1	1	75			
<i>P. formosa</i>	2	1	2	85			
<i>P. formosa</i>	3	1	2	86			
<i>P. formosa</i>	4	1	10	96			
<i>P. formosa</i>	6	1	5	113			
<i>P. formosa</i>	7	1	16	114			
<i>P. formosa</i>	9	1	11	122			
<i>P. formosa</i>	10	1	1	139			
<i>P. formosa</i>	13	2	15	85	86		
<i>P. formosa</i>	14	2	2	85	87		
<i>P. formosa</i>	16	2	1	96	97		
<i>P. formosa</i>	18	2	27	113	114		
<i>P. formosa</i>	24	2	17	122	123		
<i>P. formosa</i>	30	3	2	87	90	138	
<i>P. latipinna</i>	5	1	1	104			
<i>P. latipinna</i>	8	1	1	118			
<i>P. latipinna</i>	10	1	1	139			
<i>P. latipinna</i>	11	1	6	145			
<i>P. latipinna</i>	12	2	4	57	120		
<i>P. latipinna</i>	15	2	2	95	97		
<i>P. latipinna</i>	17	2	4	106	128		
<i>P. latipinna</i>	19	2	1	114	128		
<i>P. latipinna</i>	20	2	1	115	128		
<i>P. latipinna</i>	21	2	1	115	129		
<i>P. latipinna</i>	22	2	1	117	118		
<i>P. latipinna</i>	23	2	3	118	119		
<i>P. latipinna</i>	25	2	1	136	137		
<i>P. latipinna</i>	26	2	2	145	146		
<i>P. latipinna</i>	27	3	1	55	123	128	
<i>P. latipinna</i>	28	3	1	72	80	87	
<i>P. latipinna</i>	29	3	2	75	82	89	
<i>P. latipinna</i>	31	3	1	95	101	107	

<i>P. latipinna</i>	32	3	2	106	118	128	
<i>P. latipinna</i>	33	3	2	106	128	145	
<i>P. latipinna</i>	34	3	1	113	125	134	
<i>P. latipinna</i>	35	3	1	114	129	137	
<i>P. latipinna</i>	36	4	2	57	96	107	120
<i>P. latipinna</i>	37	4	1	93	96	101	107
<i>P. latipinna</i>	38	4	1	93	96	101	136