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Pathogen-mediated selection for MHC variability in wild zebrafish

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ABSTRACT

Hypothesis: Genes of the major histocompatibility complex (MHC) show high variability, which is believed to arise through pathogen-mediated selection.

Organism: Zebrafish (*Danio rerio*) from seven independent natural populations.

Location: Bangladesh: two sites in Khulna District in the River Ganges drainage, and five sites in Mymensingh District in the River Brahmaputra drainage.

Methods: We investigated the relationship of parasite abundance and diversity with allelic variability of the MHC class II *B* gene in natural populations of zebrafish. A sample of 120 zebrafish was screened for metazoan parasites, and a subsample of 77 was assessed for immunogenetic variation. In total, 2475 metazoan parasites from 23 taxa were identified and 13 unique MHC class IIB alleles were isolated.

Conclusion: We found no evidence for a heterozygote advantage, and overall there was lower than expected heterozygosity of MHC alleles. We identified lower parasite abundance and diversity with specific MHC alleles, and this pattern varied between geographic regions. Results suggest that MHC variability in zebrafish is maintained by frequency-dependent and fluctuating selection.

Keywords: digenean, frequency-dependent selection, heterozygote advantage, major histocompatibility complex, metazoan parasite, pathogen-driven selection.

INTRODUCTION

The major histocompatibility complex (MHC) is a vertebrate multigene family that mediates the link between detection of invading pathogens and the activation of the immune system. The variability of MHC alleles is unusually high among characterized genetic systems (Potts and Wakeland, 1993). Several lines of evidence support the idea that selection

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acts on MHC genes to drive the evolution of variation (Piertney and Oliver, 2006). Understanding how this variation arises and is maintained has been considered a key goal in evolutionary biology, with parasite-mediated selection and female mating preferences central concepts (Potts and Slev, 1995; Jordan and Bruford, 1998; Jeffery and Bangham, 2000), and evidence for a role of parasites in MHC polymorphism demonstrated in a range of taxa, including in fish (Šimková *et al.*, 2006), birds (Spurgin *et al.*, 2011), reptiles (Olsson *et al.*, 2005), amphibians (Teacher *et al.*, 2009), and mammals (Paterson *et al.*, 1998; Oliver *et al.*, 2009). Parasite-mediated selection against common host genotypes has also been demonstrated in field studies of invertebrates (Dybdahl and Lively, 1998; Lively and Dybdahl, 2000; Jokela *et al.*, 2009; Wolinska and Spaak, 2009). Experimental studies have shown rapid evolution by parasites to over-infect common host genotypes, resulting in an increase in frequency of rare genotypes (Koskella and Lively, 2009).

There are three principal hypotheses that have been invoked to explain parasite-driven MHC polymorphism. The *heterozygote advantage* hypothesis postulates that heterozygotes at MHC loci will have higher average fitness than homozygotes, either because of dominance effects or, if an increased number of MHC alleles provides protection against a larger spectrum of parasites, through overdominance (Doherty and Zinkernagel, 1975). The *negative frequency-dependent selection* (rare allele advantage) hypothesis proposes that new or rare MHC alleles will have a selective advantage over common alleles, since it is less likely that the majority of parasites will have evolved to counteract them. As rare alleles become more common, they lose this advantage because of the increasing selection pressure on the parasite fauna to adapt to them, leading to a continuous cycling of alleles (Van Valen, 1973; Jaenike, 1978; Hamilton, 1980). Finally, MHC variability might be maintained through what has been termed *fluctuating selection*. In this case, fluctuations in the spatial and temporal abundance and distribution of pathogens will result in selection for different MHC alleles at different times and locations, irrespective of allele frequency, thereby generating a fluctuating mosaic of selection pressures on MHC alleles. Under this hypothesis no state of equilibrium (stable or cyclical) is achieved between host MHC and pathogens, with external forces responsible for driving changes in pathogen abundance, and MHC allele polymorphism maintained via a repeatedly changing selective landscape (Hedrick, 2002; Bernatchez and Landry, 2003; Loisel *et al.*, 2008; Spurgin and Richardson, 2010). The term 'fluctuating selection' is somewhat disingenuous, since it implies that the direction of selection on individual strategies or genotypes oscillates over time in response to changes in pathogen abundance, which is not a fundamental feature of the hypothesis. Whether this essentially haphazard mechanism could maintain MHC allele polymorphism in the long term remains to be demonstrated.

Our aim was to examine the role of parasite-mediated MHC polymorphism in natural populations of zebrafish (*Danio rerio*), a widely distributed freshwater fish that is also an important laboratory model, to understand the selective mechanisms that act on MHC in response to parasitism. We tested for an association between: (1) individual parasite load and diversity and MHC heterozygosity (indicating heterozygote advantage); (2) individual pathogen load and specific MHC alleles (negative frequency-dependent selection and fluctuating selection); and (3) spatial variation in individual pathogen load and specific MHC alleles (negative frequency-dependent and fluctuating selection) (Spurgin and Richardson, 2010). Analysis was focused on exon 2 of the MHC Class II DAB gene, which encodes the biologically significant protein-binding region (Ono *et al.*, 1992) and shows the greatest allelic variation (Sültmann *et al.*, 1994). Recently, a number of studies have addressed the role of MHC on the parasite loads of wild vertebrates (Froeschke and Sommer, 2005; Oliver *et al.*, 2009;

Fraser *et al.*, 2010), but none using the zebrafish model (Spence *et al.*, 2008). We examined the full range of parasites in zebrafish to overcome a weakness of many MHC studies, which tend to focus on just a single pathogen and thereby ignore the full suite of pathogens to which a population is exposed (Langefors *et al.*, 2001).

The zebrafish is a small (~30 mm length) cyprinid fish that is one of the most important vertebrate model organisms in developmental biology, genetics, and neurophysiology (for a review, see Spence *et al.*, 2008). This fish has emerged as a key model in biomedical research (Dooley and Zon, 2000; Shin and Fishman, 2002), particularly as a model of human disease (Berghmans *et al.*, 2005; Amsterdam and Hopkins, 2006; Guyon *et al.*, 2007) and for the screening of therapeutic drugs (Rubinstein, 2006). The zebrafish also lends itself to behavioural, ecological, and evolutionary research (Spence and Smith, 2005, 2007; Gerlach and Lysiak, 2006; Engeszer *et al.*, 2007). The natural range of the zebrafish is centred on the River Ganges and Brahmaputra basins in north-eastern India, Bangladesh, and Nepal (Laale, 1977; Barman, 1991; Spence *et al.*, 2008). This region has a monsoon climate, with wide seasonal variation in the extent of freshwater habitats where the fish inhabits shallow, slow-moving or standing water bodies (McClure *et al.*, 2006; Spence *et al.*, 2006; Engeszer *et al.*, 2007). The parasite fauna of wild zebrafish has hitherto never been described (Spence *et al.*, 2008).

METHODS

Fish sampling

We based the study on an analysis of 120 zebrafish collected in January 2005 from seven sites in two regions of Bangladesh. The habitat, diet, growth rates, and fish assemblage of these zebrafish populations has been characterized (Spence *et al.*, 2006, 2007). Zebrafish were analysed from two sites in Khulna District in the River Ganges drainage [GPS reading 22°N, 90°E; sites 1 and 9 in Spence *et al.* (2006)], and five sites in Mymensingh District in the River Brahmaputra drainage [GPS reading 24°N, 90°E; sites 15, 16, 17, 22, and 23 in Spence *et al.* (2006)] (Table 1). [See Spence *et al.* (2006) for full details of collection sites.] Sampling was

Table 1. Zebrafish sampling sites

Site no.	Location	Region	No. fish screened for parasites	No. fish screened for MHC
1	Khulna University Campus: ditch surrounding a series of ponds	Khulna	8	6
9	Isolated channel of River Golamari	Khulna	25	15
15	Sutiakhali: shallow cultivated pond with connection to paddy field	Mymensingh	15	8
16	Sutiakhali: first isolated pond	Mymensingh	16	14
17	Sutiakhali: second isolated pond	Mymensingh	16	11
22	Mymensingh University Campus: semi-natural pond	Mymensingh	21	11
23	Mymensingh University Campus: ditch with connection to paddy field	Mymensingh	19	12

Note: Site numbers refer to sites in Spence *et al.* (2006).

conducted using a fine mesh seine net measuring 5.0 × 1.3 m, with a mesh size varying from 2 to 7 mm. Within regions, populations were isolated but could potentially mix during flood events, which occur routinely in Bangladesh (Brammer, 1990). Sampling regions, in contrast, were discrete, since they were located in different river drainages.

Parasitological examination

Zebrafish were transported live to a field laboratory, individually killed by cutting the spine at the base of the skull, and examined under a binocular microscope for the presence of metazoan parasites. Parasites were preserved in 4% formaldehyde (acanthocephalans, digeneans, cestodes, bivalves, crustaceans), in a mixture of ammonium picrate and glycerine (monogeneans, larval digeneans, larval cestodes), and in a mixture of glycerine and ethanol (nematodes). Before identification, digeneans and cestodes were stained in ferric aceto-carmine (IAC), dehydrated in a gradual alcohol series, and mounted in 'Canada' balsam. Identification was carried out using a light microscope equipped with phase-contrast, differential interference contrast, and digital image analysis. In the majority of cases, parasites were identified to genus or higher taxonomic level, as most of the parasites collected were larval stages. A total of 120 zebrafish from all sites were examined for parasites. The body length (measured from the tip of the snout to the base of the caudal fin) of each fish was measured to the nearest millimetre using calipers, and a fin clip collected and stored in 95% ethanol for subsequent identification of MHC polymorphism.

MHC analysis

We assessed immunogenetic variation in a subsample of 77 fish from all seven study sites. Previous studies have established that the MHC Class IIB DAB gene is the only one of the six class IIB loci to be expressed in zebrafish and demonstrates the only significant polymorphism (Sültmann *et al.*, 1994). The most polymorphic segment of the coding region of MHC class IIB genes is exon 2, which encodes for the β_1 domain (Ono *et al.*, 1992). Based on the polymorphisms reported in Ono *et al.* (1992) and in Genbank, we designed a primer set (Forward: AGCACCAGTGATTACAGTGATATGG, Reverse: AGCTGAGTCCCAGA-TCTGAGC) to isolate a 219 bp fragment containing almost the complete exon 2 of the *mhc2dab* gene. Total genomic DNA was isolated from fin clips using the Promega Wizard extraction kit in accordance with the manufacturer's instructions. A 20 μL reaction was used in which 1 μL genomic DNA was added to the reaction mixtures containing 10 μL PCR reaction mix (YorkBio), 1 μL (10 μM) of each primer, and 7 μL of H_2O. The PCR was performed with a T1 Thermocycler (Biometra, Goettingen, Germany). The PCR conditions were 24 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, elongation at 72°C for 1 min, and a final extension at 72°C for 30 min.

Denaturing high-pressure liquid chromatography (dHPLC) is an efficient method for the identification of unknown polymorphisms by heteroduplex analysis of PCR fragments (Wulfert *et al.*, 2006). It requires that a homozygote of known sequence be used as a comparison. To create this we amplified the exon 2 fragment from a wild-type WIK strain fish obtained from the Tübingen stock centre and cloned into the pDrive cloning vector according to the manufacturer's instructions (Qiagen, UK). Ten clones were sequenced by the John Innes Genome laboratory using an ABI3700 capillary sequencer. This individual was homozygous – that is, all sequences were the same. The PCR fragments (5 μL) from all

wild fish were compared with this WIK 'wild-type' (5 μL) using dHPLC on our WAVE-System (Transgenomic, Crewe, UK). Each fish was assigned to a type based on a common chromatogram produced by dHPLC. A PCR fragment from a representative fish from each type was cloned as above and ten clones sequenced to assess the heterozygosity and sequence of the type.

Data analysis

Parasite abundance was defined as the total number of metazoan parasites per individual, and infracommunity diversity ('diversity') was estimated using the Brillouin index (H') (Holmes and Price, 1986). Analysis of covariance (ANCOVA) was used to compare parasite abundance and diversity in each sampling region, using fish body length as a covariate; body length, however, was not a significant covariate and was excluded from subsequent analyses. An unpaired *t*-test was used to compare mean fish body length between regions and a Pearson's correlation to test for a relationship between body length and parasite abundance and diversity in each region. An unpaired *t*-test was used to compare parasite abundance and diversity with respect to zygosity in each sampling region, as a test of the heterozygote advantage hypothesis. One-way analysis of variance (ANOVA), with Tukey's test for pairwise *post hoc* comparisons, was used to test for a difference among MHC alleles in each region as support for (but not a definitive test of) the negative frequency-dependent selection hypothesis. Calculations were two-tailed and based on an alpha of 0.05. Bonferroni-corrected significance levels were used for multiple comparisons. A Fligner-Killeen test was used to measure homoscedasticity. Diagnostic plots of residuals against fitted values, and QQ plots of residuals were used to assess assumptions of normality. Statistical analyses were performed using the R statistical package 2.12.1 (R Development Core Team, 2006).

RESULTS

Parasite load and diversity

In total, 2475 metazoan parasites from 23 taxa were collected and identified. Protozoan infection by trichodinid ciliates and myxozoan infection by *Myxobolus* spp. and *Thelohanellus* sp. were also observed. Zebrafish showed high levels of infection by larval endoparasites, comprising 96.6–100% of the metazoan parasite community among all host populations (Table 2). The majority (92.8%) of parasites were larval digeneans, while cestodes were the second most frequently represented group (6.3%). Ectoparasite infections were rare, with infrequent occurrence of monogeneans, bivalves, and crustaceans. Maximum prevalence (100%) was observed for the metacercariae of *Acanthostomum* sp. in one population and *Centrocestus formosanus* in two populations. These two digeneans, together with one undetermined strigeid, parasitized zebrafish in all populations sampled (Table 2).

Only 13 of the 120 zebrafish screened for parasites were found to be uninfected. There was a significant difference between regions in parasite abundance (ANCOVA, data $\log_{10}+1$ transformed, $F_{1,117}=80.08$, $P<0.001$; fish body length as covariate, $F_{1,117}=2.29$, $P=0.133$) and diversity ($F_{1,117}=17.61$, $P<0.001$; body length, $F_{1,117}=0.23$, $P=0.633$). Mean (± S.E.) parasite abundance in Khulna region was 6.3 ± 1.99 parasites per fish and in

Table 2. Infection parameters of zebrafish among sample sites

	Sampling sites																				
	Khulna						Mymensingh														
	1			9			15			16			17			22			23		
Parasite taxa	*Prv*	*Abn*	*Int*	*Prv*	*Abn*	*Int*	*Prv*	*Abn*	*Int*	*Prv*	*Abn*	*Int*	*Prv*	*Abn*	*Int*	*Prv*	*Abn*	*Int*	*Prv*	*Abn*	*Int*
Ciliata																					
Trichodinidae fam. spp.	—	—	—	—	—	—	7	—	—	38	—	—	25	—	—	5	—	—	26	—	—
Myxozoa																					
Thelohanellus spp.[a]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—				11	0.1	1
Myxobolus spp. [a]	—	—	—	—	—	—	7	0.1	1	44	0.4	1	6	0.1	1	10	0.1	1	42	1.6	1–7
Monogenea																					
Gyrodactylus sp. 1	—	—	—	—	—	—	—	—	—	6	0.2	3	—	—	—	—	—	—	5	0.1	2
Gyrodactylus sp. 2	—	—	—	—	—	—	—	—	—	6	0.1	1	—	—	—	5	0.1	1	5	0.1	1
Dactylogyrus sp.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	0.1	1
Digenea (metacercariae)																					
Acanthostomum sp.	100	6.9	3–16	44	0.8	1–5	33	0.7	1–4	69	1.3	1–6	13	0.2	1–2	95	7.6	1–23	84	6.3	1–19
Centrocestus formosanus	38	0.8	1–4	20	0.6	1–6	27	0.5	1–3	44	1.3	1–10	13	0.2	1–2	100	23	1–55	100	39	6–87
Heterophyidae fam. sp.	63	4.8	4–13	36	1.3	1–20	—	—	—	50	1.9	1–13	50	2.4	1–15	81	4.9	1–20	90	6.4	1–27
Haplorchis sp.	38	0.4	1	—	—	—	—	—	—	6	0.1	1	6	0.1	1	43	0.9	1–5	63	4.7	1–18
Strigeida sp. 1	40	1.3	1–7	8	0.1	1–2	13	0.1	1	94	3.5	1–11	69	1.8	1–4	10	0.2	1–4	21	0.3	1–3
Strigeida sp. 2	25	0.9	3–4	—	—	—	7	0.2	3	13	0.1	1	6	0.1	1	29	0.4	1–2	21	0.5	1–6
Strigeida sp. 3	—	—	—	—	—	—	13	0.1	1	6	0.2	3	—	—	—	10	0.1	1	16	0.2	1
Strigeida sp. 4	—	—	—	—	—	—	13	0.2	1–2	—	—	—	—	—	—	—	—	—	—	—	—
Digenea sp. 1	13	0.1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Digenea sp. 2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	16	0.2	1
Digenea sp. 3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	0.1	1	—	—	—

Cestoda (larva)																				
Proteocephalida sp.	—	—	—	—	—	—	7	0.2	3	88	3.9	1–17	63	0.8	1–3	76	1.3	1–6	84	2.6	1–9
Nematoda (larva)																				
Capillariidae fam. spp.	13	0.1	1	4	0.1	2	7	0.1	1	—	—	—	—	—	—	—	—	—	—	—	—
Acanthocephala																				
Pallisentis sp.	13	0.3	2	4	>0.1	1	—	—	—	—	—	—	—	—	—	—	—	—	5	0.1	1
Bivalvia (glochidia)																				
Unionidae fam. sp.	—	—	—	—	—	—	—	—	—	13	0.1	1	—	—	—	—	—	—	—	—	—
Crustacea																				
Argulus sp.	—	—	—	—	—	—	—	—	—	6	0.1	1	—	—	—	—	—	—	—	—	—
Ergasilidae fam. sp.	13	0.4	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Note: Site numbers refer to Spence *et al.* (2006). *Prv* = prevalence (in %), defined as the percentage of fish infected by a given parasite species in a sample. *Abn* = mean abundance, defined as the mean number of parasites per host (infected and non-infected) in a sample. *Int* = range in intensity of infection, minimum – maximum. Estimates of mean abundance and mean intensity of infection were not obtained for protozoa.

[a] Epidemiology calculated for number of plasmodia.

Table 3. Distribution of MHC alleles among sampled populations

	Site number						
	Khulna		Mymensingh				
MHC allele	1	9	15	16	17	22	23
mhc2dab-GU987086				*	*	*	*
mhc2dab-GU987087	*	*		*	*	*	*
mhc2dab-GU987088	*	*	*		*	*	
mhc2dab-GU987089		*	*			*	
mhc2dab-GU987090	*	*	*		*	*	
mhc2dab-GU987091		*					
mhc2dab-GU987092	*	*					
mhc2dab-GU987093		*					
mhc2dab-GU987094		*		*		*	*
mhc2dab-GU987095		*	*			*	
mhc2dab-GU987096		*	*	*		*	
mhc2dab-GU987097				*	*	*	*
mhc2dab-GU987098	*	*					

Note: Site numbers refer to sites in Spence *et al.* (2006).

Mymensingh 26.9 ± 3.60 per fish. Mean ($\pm$ S.E.) parasite diversity in Khulna was 0.32 ± 0.064 per fish and in Mymensingh 0.72 ± 0.42 per fish. The mean body length of fish did not vary between regions (unpaired t-test; $t_{118} = 1.07$, $P = 0.290$). Mean ($\pm$ S.E.) body length was 18.9 ± 0.27 mm. There was no correlation between fish size and parasite abundance or diversity in either the Khulna (Pearson's correlation, abundance: $r_{15} = -0.096$, $P = 0.723$; diversity: $r_{11} = -0.386$, $P = 0.216$) or Mymensingh region (abundance: $r_{55} = -0.762$, $P = 0.577$; diversity; $r_{54} = -0.158$, $P = 0.250$), and fish length was not considered in subsequent analyses.

MHC variability

A total of 13 unique MHC class IIB alleles were detected in 77 individuals from seven populations (Table 3). These were submitted to GenBank under the accession numbers GU987086-98 and designated as alleles *mhc2dab*-GU987086-98. There was no significant difference between fish that were screened for parasites and genotyped and those that were only screened for parasites in mean parasite abundance (unpaired t-test; data $\log_{10} + 1$ transformed, $t_{118} = 0.01$, $P = 0.992$) or diversity ($t_{117} = 1.45$, $P = 0.149$).

The mean number of alleles in each population was 6.3 and ranged from 4 to 11. The average observed heterozygosity was 0.56 and ranged from 0.36 to 0.67. Estimates of observed heterozygosity were consistently lower than the expected heterozygosity in every population (paired t-test, $t_6 = 1.45$, $P = 0.011$). The mean number of alleles per individual was 1.6 and ranged from 1.4 to 1.7 (Table 4). The phylogenetic relationship of the alleles

Table 4. Summary of the MHC class IIB locus sampled in seven zebrafish (*Danio rerio*) populations

	Population						
Parameter	1	9	15	16	17	22	23
Sample size	6	15	8	14	11	11	12
Total alleles	5	11	5	5	5	9	4
Mean alleles	1.7	1.5	1.5	1.6	1.6	1.4	1.7
Expected heterozygosity	0.76	0.88	0.67	0.77	0.75	0.80	0.71
Observed heterozygosity	0.67	0.53	0.50	0.57	0.64	0.36	0.67

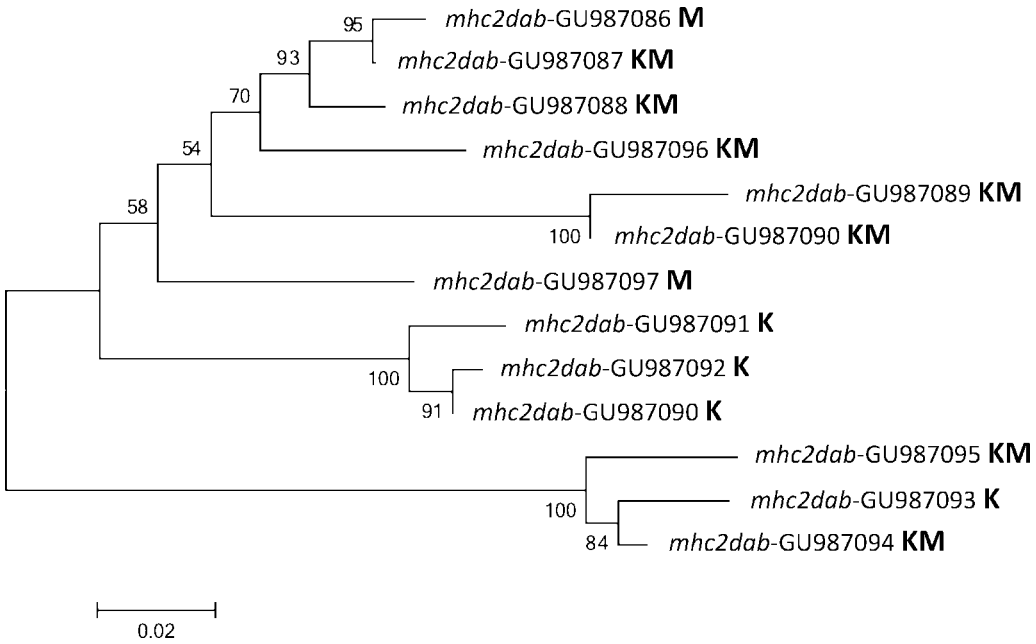

Fig. 1. Minimum evolution tree of the 13 MHC class IIB sequences, based on nucleotide sequences (Kimura 2-parameter). Bootstrap values >50 are displayed (1000 replications). The scale bar indicates genetic distance in units of nucleotide substitutions per site. Alleles found only in Khulna region are indicated with K, those only in Mymensingh with M, and in both populations by KM.

is displayed in Fig. 1. No allele was present in all study populations, but three alleles (*mhc2dab*-GU987086, *mhc2dab*-GU987087, and *mhc2dab*-GU987097) were relatively common with a frequency exceeding 10%. Four alleles (*mhc2dab*-GU987091, *mhc2dab*-GU987092, *mhc2dab*-GU987093, and *mhc2dab*-GU987098) were detected in fewer than five individuals and were excluded from statistical analyses. Four alleles were exclusively detected in Khulna region and two in Mymensingh, with the remaining seven common to both regions (Table 3; Fig. 2). In all 13 alleles, 73 (33.3%) of 219 nucleotide positions were variable. Alleles differed between 3 and 37 nucleotide positions (mean = 22.5, S.E. = 3.08).

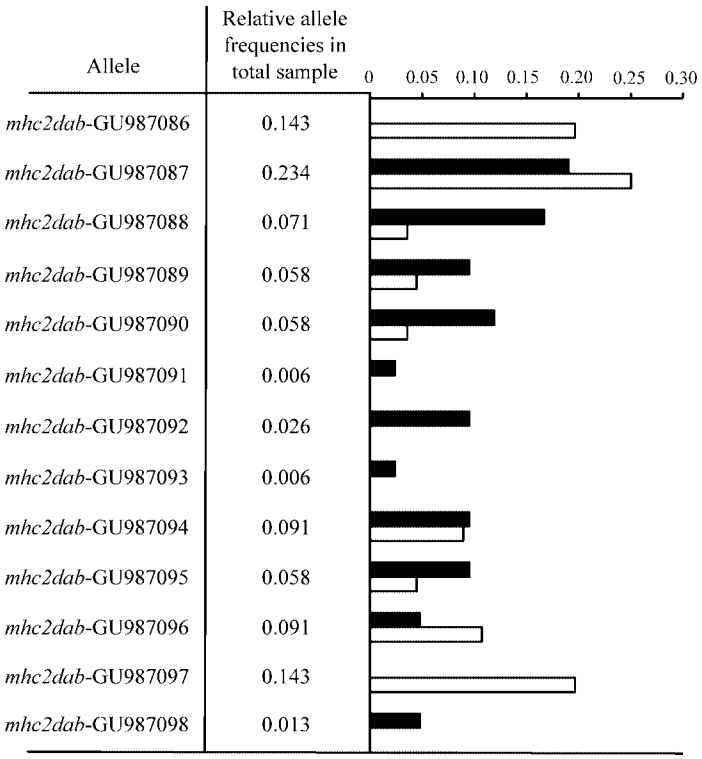

Fig. 2. Frequencies of all identified MHC class IIB alleles in the entire sample ($N = 77$), as well as for Khulna region (black bars) and Mymensingh region (white bars) separately.

Prediction 1: Association between parasite infection and MHC heterozygosity

In Khulna region, there was no significant difference in parasite abundance with respect to zygosity (unpaired *t*-test, data $\log_{10} + 1$ transformed, $t_{14} = 0.978$, $P = 0.345$; Fig. 3), although there was for parasite diversity ($t_{10} = 2.88$, $P = 0.017$; Fig. 3), with the mean diversity of parasites infecting homozygotes lower than heterozygotes. In Mymensingh, there was no significant difference in either abundance or diversity with respect to zygosity (abundance: data $\log_{10} + 1$ transformed, $t_{54} = 1.37$, $P = 0.178$; diversity: $t_{53} = 1.062$, $P = 0.293$; Fig. 3).

Prediction 2: Association between parasite infection and specific MHC alleles

There was no significant difference in parasite abundance among specific MHC alleles in Khulna region (one-way ANOVA, data $\log_{10} + 1$ transformed, $F_{6,25} = 0.48$, $P = 0.816$), although there was a difference in parasite diversity ($F_{6,25} = 3.13$, $P = 0.020$; Fig. 4). There was a significant difference in parasite abundance and diversity among MHC alleles in Mymensingh (abundance: $F_{8,103} = 2.31$, $P = 0.026$; parasite diversity: $F_{8,103} = 3.55$, $P = 0.001$; Fig. 4). There was no correlation in Khulna region between allele frequency and parasite abundance (Pearson correlation, $r_{1,8} = 0.182$, $P = 0.696$) or parasite diversity ($r_{1,8} = 0.302$, $P = 0.511$), or in Mymensingh (parasite abundance: $r_{1,6} = 0.517$, $P = 0.154$; diversity: $r_{1,6} = 0.384$, $P = 0.308$).

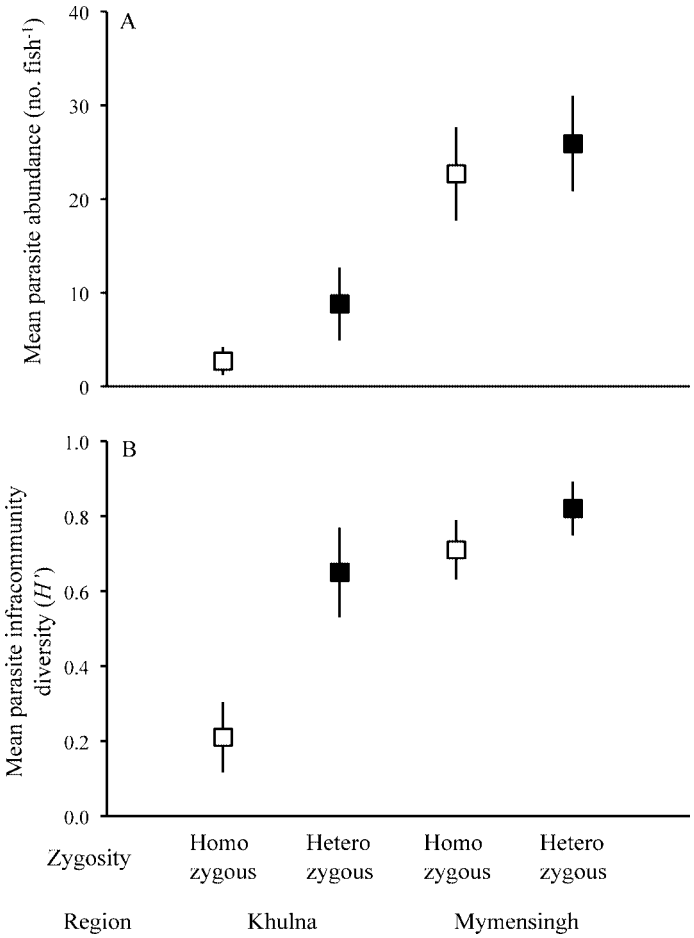

Fig. 3. Association of MHC class IIB alleles with mean (± 1 S.E.) parasite abundance (A), and parasite infracommunity diversity (H') (B), with respect to zygosity in Khulna and Mymensingh regions.

Prediction 3: Spatial variation in parasite infection and specific MHC alleles

The alleles associated with high and low parasite diversity differed between sampling regions. In Khulna, alleles *mhc2dab*-GU987088 and *mhc2dab*-GU987090 were associated with high parasite diversity, and *mhc2dab*-GU987087 with low diversity (Tukey's test: $P < 0.05$; Fig. 4). In Mymensingh, in contrast, alleles *mhc2dab*-GU987086, *mhc2dab*-GU987087, *mhc2dab*-GU987094, and *mhc2dab*-GU987097 were associated with high parasite abundance and diversity, and *mhc2dab*-GU987096 with low abundance and diversity (Tukey's test: $P < 0.05$; Fig. 4).

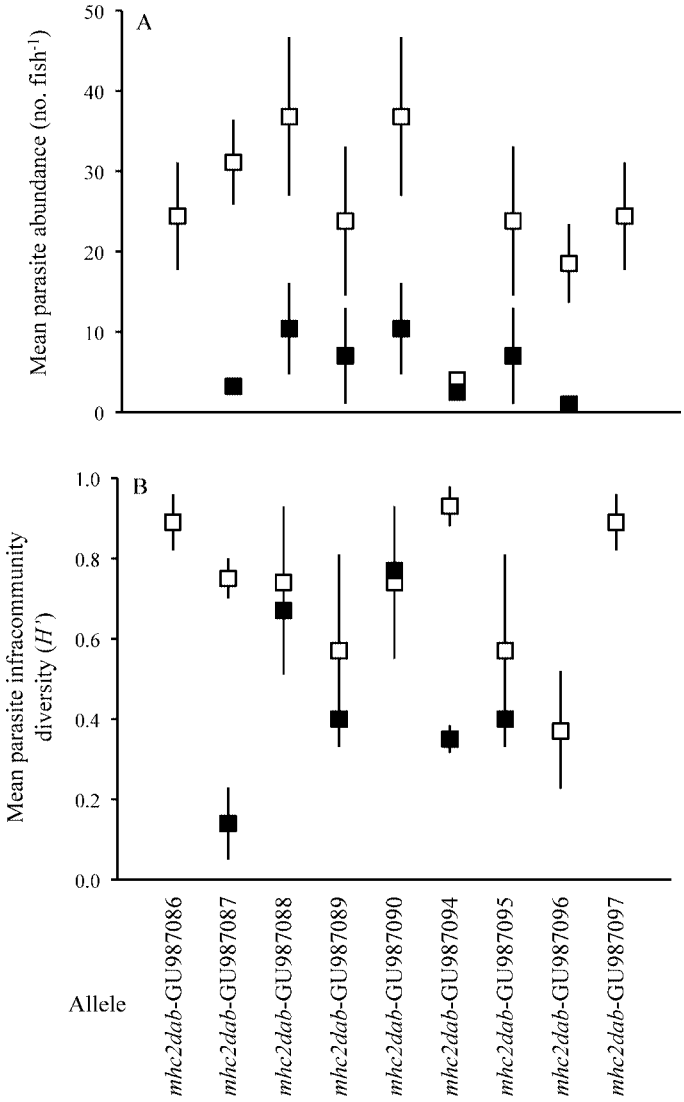

Fig. 4. Association of MHC class IIB alleles with mean (± 1 S.E.) parasite abundance (A), and parasite infracommunity diversity (H') (B), with respect to specific alleles in Khulna (black squares) and Mymensingh regions (white squares).

DISCUSSION

The aim of this study was to determine whether parasite-mediated selection could explain MHC variability in zebrafish. Parasite abundance and diversity and *mhc2dab* polymorphism were described for wild populations for the first time. We did not find evidence for a heterozygote advantage, and overall there was lower than expected heterozygosity of MHC alleles in all study populations. There was evidence for lower parasite abundance and diversity with specific MHC alleles, and this pattern varied between study regions.

The presence of heterozygote advantage can be demonstrated by contrasting parasite load with MHC variation, and several studies have done so (e.g. Penn *et al.*, 2002; Froeschke and Sommer, 2005; Oliver *et al.*, 2009). We did not find evidence of heterozygote advantage, and observed MHC heterozygosity to be lower than expected in all study populations. These findings suggest little or no direct selection for MHC heterozygosity in zebrafish at our study sites, at least for the generation of fish we sampled. Consistent low MHC heterozygosity may have arisen from underdominance. Alternatively, it might have been because MHC diversity was not fully characterized as a result of methodological errors, such as null alleles. Overall, MHC variability in the present study was low, with only 13 unique alleles detected. A comparable study by Fraser *et al.* (2010) on wild populations of the guppy (*Poecilia reticulata*), another small-bodied, short-lived freshwater fish, yielded 43 MHC alleles, substantially more than we detected in zebrafish. Underdominance effects can lead to the erosion of MHC polymorphism (Pitcher and Neff, 2006), and could explain why MHC polymorphism in the populations we studied was relatively limited.

We detected an association between individual parasite abundance and diversity and specific MHC alleles, which suggested either a rare-allele advantage or fluctuating selection. In Khulna, alleles *mhc2dab*-GU987088 and *mhc2dab*-GU987090, and in Mymensingh, alleles *mhc2dab*-GU987086, *mhc2dab*-GU987087, *mhc2dab*-GU987094, and *mhc2dab*-GU987097 were associated with high parasite abundance and diversity. Low parasite abundance and diversity were associated with alleles *mhc2dab*-GU987087 in Khulna region and *mhc2dab*-GU987096 in Mymensingh. Differences in fish susceptibility to parasitism were not explained by differences in the geographic distribution of alleles; all alleles were detected in at least four populations, in most cases in both sampling regions. Alleles associated with low abundance and diversity also differed between sampling regions; *mhc2dab*-GU987087 was associated with low parasite diversity in Khulna, but high diversity in Mymensingh. The association between certain alleles and susceptibility or resistance to certain parasites, and spatial variation in resistance, matches the predictions for frequency-dependent selection, but also for fluctuating selection. While the mechanisms by which frequency-dependent and fluctuating selection differ, separating the two is difficult, requiring long-term studies of multiple populations to examine temporal changes in parasite resistance to alleles, and spatio-temporal variation in forces driving parasite abundance (Spurgin and Richardson, 2010).

Some aspects of the biology of zebrafish suggest that stable equilibria between these fish and their parasites, which is a prerequisite for frequency-dependent selection, may rarely be achieved. Zebrafish occur primarily in the catchments of the River Ganges and Brahmaputra. Both are large rivers and flood extensive areas of eastern India and Bangladesh annually; it is not unusual for over 50% of the land area of Bangladesh to be submerged during flood events (Brammer, 1990). Mixing of zebrafish and parasite populations over an extensive area is possible (Gratton *et al.*, 2004) and the dynamic nature of this environment may mitigate against the evolution of a stable equilibrium between host MHC polymorphism and parasite abundance and diversity. In addition, most of the parasites hosted by zebrafish (Table 2) do not have direct life-cycles, and variability among populations may be dependent on the abundance of intermediate or final hosts of these parasites. In the case of digeneans, which were found to be the most abundant and widespread parasites of zebrafish, the abundance of mollusc intermediate hosts can affect population-level differences in parasite abundance (Halmetoja *et al.*, 2000). Irregular drying of water bodies and the presence of molluscivorous fishes could affect mollusc population

dynamics, with consequences for parasite populations. Thus, a significant association between parasite infection and specific MHC alleles, marked spatial variation in parasite infection and an association with specific MHC alleles, and the dynamic nature of the environment in which zebrafish are found, all implicate a role for fluctuating selection in maintaining MHC polymorphism in zebrafish.

A potential criticism of the present study is that *mhc2dab* genes function primarily in response to extracellular antigens (mainly bacterial), although our current understanding of the relationship between different MHC haplotypes and disease resistance is incomplete (Bernatchez and Landry, 2003; Jensen, 2007). Despite these uncertainties, there is circumstantial evidence that MHC variability has also evolved in response to metazoan parasites in fishes. For example, Šimková *et al.* (2006) demonstrated a significant correlation between parasite diversity and MHC variability in European cyprinid fishes, and Wegner *et al.* (2003) showed an effect of MHC variation on parasite abundance in the three-spined stickleback (*Gasterosteus aculeatus*). A further deficiency of the study was that we failed to measure neutral variation, which would have enabled us to control for confounding effects of demographic processes, such as migration and genetic drift, on MHC variability, which can mask the effect of selection on MHC genes (Spurgin and Richardson, 2010). Finally, our analysis assumes that MHC sequence differences represent functional differences at the molecular level, an assumption that we have not tested.

An additional explanation for the evolution and maintenance of MHC variability in zebrafish, which does not require pathogen-mediated selection to be invoked, is through inbreeding avoidance associated with female mate choice (Pusey and Wolf, 1996). Here the prediction is that females avoid inbreeding as this enriches the genome-wide variation of their offspring. MHC genes may serve as inbreeding avoidance markers, since their complex architecture and high levels of polymorphism provide the variability necessary for a genetically based recognition system (Grafen, 1990). There is evidence for MHC-based mate choice, often linked to odour cues, in a range of vertebrate taxa (Wedekind and Füri, 1997; Roberts and Gosling, 2003; Neff and Pitcher, 2005). Olfactory cues are known to play a key role in female mating decisions in zebrafish (Spence *et al.*, 2008), and female mate preferences in zebrafish are not congruent, which would be expected if mate choice is based on MHC dissimilarity (Spence and Smith, 2006; Agbali *et al.*, 2010). Consequently, there may also be a role for mate choice in maintaining MHC variability in zebrafish, although this has yet to be formally tested.

In conclusion, our results are consistent with pathogen-mediated selection operating through frequency-dependent and fluctuating selection, although these two mechanisms could not be differentiated. Our results failed to show any effect of heterozygote advantage, and MHC polymorphism in wild zebrafish may be constrained through underdominance.

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