

1 **Contrasting Responses to Selection in Class I and Class II $\alpha$**

2 **Major Histocompatibility Linked Markers in Salmon**

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23

24 †This paper is dedicated to the memory of Professor René Stet. Deceased,

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26

27 **Abstract**

28 Comparison of levels and patterns of genetic variation in natural populations  
29 either across loci or against neutral expectation can yield insight into locus-  
30 specific differences in the strength and direction of evolutionary forces. We  
31 used both approaches to test hypotheses on patterns of selection on major  
32 histocompatibility (MH)-linked markers. We performed temporal analyses  
33 of class I and class II $\alpha$  MH-linked markers and eight microsatellite loci in  
34 two Atlantic salmon populations in Ireland on two temporal scales: over six  
35 decades and nine years in the rivers Burrishoole and Delphi, respectively.  
36 We also compared contemporary Burrishoole and Delphi samples with  
37 nearby populations for the same loci. On comparing patterns of temporal  
38 and spatial differentiation among classes of loci, class II $\alpha$  MH-linked  
39 marker was consistently identified as outlier compared to patterns at other  
40 microsatellite loci or neutral expectation. We found higher levels of  
41 temporal and spatial heterogeneity in heterozygosity (but not in allelic  
42 richness) for the class II $\alpha$  MH-linked marker compared to microsatellites.  
43 Tests on both within and among population differentiation are consistent  
44 with directional selection acting on the class II $\alpha$ -linked marker in both  
45 temporal and spatial comparisons but only in temporal comparisons for the  
46 class I-linked marker. Our results indicate a complex pattern of selection on  
47 MH-linked markers in natural populations of Atlantic salmon. These  
48 findings highlight the importance of considering selection on MH-linked

49 markers when using these markers for management and conservation

50 purposes.

51

52 **Introduction**

53

54 Identifying loci under selection, and therefore potentially involved in  
55 adaptation, is a major aim in evolutionary ecology (Nielsen, 2005). Often,  
56 approaches to identifying selected loci entail comparing levels and patterns  
57 of variation in natural populations with an expectation under a neutral model  
58 of evolution (Beaumont and Balding, 2004; Ford, 2002; Goldringer and  
59 Bataillon, 2004; Kimura, 1985; Vitalis *et al*, 2001). Alternatively,  
60 contrasting variation among loci (or classes of loci) from the same  
61 individuals may reveal locus-specific selection (e.g. (Dhuyvetter *et al*, 2004;  
62 Dufresne *et al*, 2002; Jordan *et al*, 1997; Karl and Avise, 1992; McDonald,  
63 1991; Pogson *et al*, 1995; Vasemagi *et al*, 2005).

64

65 As a result of using both approaches, the genes of the major  
66 histocompatibility complex (MHC) are widely believed to be subject to  
67 strong balancing selection (Apanius *et al*, 1997; Hedrick, 1994; Hughes and  
68 Yeager, 1998). MHC molecules play a central role in the T-cell-mediated  
69 specific immune response (Klein, 1986; Parham and Ohta, 1996), encoding  
70 molecules that bind small self or non-self peptides within the cell and then  
71 present them on the cell-surface to T cells (Hedrick, 1994). Although MHC  
72 genes are among the most studied loci in vertebrates, the mechanisms that  
73 maintain their high levels of polymorphism remain vigorously debated

74 (Spurgin and Richardson, 2010). Sexual selection (Consuegra and García de  
75 Leániz, 2008; Jordan and Bruford, 1998; Landry *et al*, 2001; Potts and  
76 Wakeland, 1990; Potts and Wakeland, 1993; Reusch *et al*, 2001) and neutral  
77 forces (Landry and Bernatchez, 2001; Miller and Lambert, 2004; Seddon  
78 and Baverstock, 1999; Seddon and Ellegren, 2004) may have a role in  
79 determining levels of variability in MHC genes. However, pathogen-driven  
80 balancing selection (through overdominance, negative frequency  
81 dependence or temporal/spatial heterogeneity in pathogen phenotype) is  
82 thought by many to be the main force driving MHC evolution (Edwards and  
83 Hedrick, 1998; Hedrick and Kim, 2000; Jeffery and Bangham, 2000; Klein  
84 and O'HUigin, 1994; Parham and Ohta, 1996). Evidence of selection on  
85 MHC genes has traditionally come from four sources (Hughes and Yeager,  
86 1998): (a) long persistence times for MHC alleles compared to neutral  
87 expectation (often resulting in trans-specific polymorphism) (Figuroa *et al*,  
88 1988; Klein *et al*, 2007; Lawlor *et al*, 1988; McConnell *et al*, 1988), (b)  
89 frequency distributions of MHC alleles in natural populations that are more  
90 even than expected under a neutral model (Hedrick and Thompson, 1983;  
91 Markow *et al*, 1993), (c) high levels of non-synonymous versus  
92 synonymous substitutions in codons for peptide binding residues (PBRs)  
93 (Hughes and Nei, 1988; Hughes and Nei, 1989; Hughes and Nei, 1990) and  
94 (d) homogenisation of introns with concurrent diversification of exons at  
95 MHC loci (Cereb *et al*, 1997; Reusch and Langefors, 2005).

96 More recently, an increasing number of descriptions of the geographic  
97 distribution of MHC variation (or variation at markers tightly linked to  
98 MHC loci) - often in conjunction with analysis at other, (putatively) neutral  
99 loci - have provided further insight into the selective influences on MHC  
100 loci in a range of species (Aguilar and Garza, 2006; Alcaide *et al*, 2008;  
101 Bryja *et al*, 2007; Ekblom *et al*, 2007; Miller *et al*, 2001). In general, MHC  
102 heterozygosity within populations is higher than that for neutral loci  
103 (Aguilar *et al*, 2004; Huang and Yu, 2003); but see (Boyce *et al*, 1997).  
104 However, there appears to be a great deal of species-to-species variation in  
105 the relative levels of MHC and neutral variability among populations.  
106 Among-population differentiation at MHC loci has been observed to range  
107 from lower than (Aguilar *et al*, 2004; Sommer, 2003), similar to (Boyce *et*  
108 *al*, 1997; Hedrick *et al*, 2001; Huang and Yu, 2003; Parker *et al*, 1999) to  
109 higher than that at neutral loci (Beacham *et al*, 2004; Miller *et al*, 2001),  
110 reflecting differences in the relative strengths of natural selection, genetic  
111 drift and gene flow across species (Eizaguirre *et al*, 2010). Indeed, the  
112 relative levels of neutral and MHC variation within and among populations  
113 can vary across closely-related species (Hambuch and Lacey, 2002; Jarvi *et*  
114 *al*, 2004), and even within a species depending on the spatial scale of  
115 analysis (Landry and Bernatchez, 2001). In comparison to studies of  
116 geographic patterns in MHC variation, there have been relatively few  
117 studies on MHC which also include a temporal dimension, despite the

118 possibility that selective forces across time within a population may differ  
119 from those across populations (Beacham *et al*, 2004; Coughlan *et al*, 2006;  
120 Oliver *et al*, 2009; Seddon and Ellegren, 2004; Smulders *et al*, 2003;  
121 Sommer, 2003; Westerdahl *et al*, 2004). Moreover, most studies focus on a  
122 single class of MHC gene (most commonly class II  $\beta$ ), while selection can  
123 affect differentially the genetic diversity of both genes (Bryja *et al*, 2007).

124

125 The Atlantic salmon (*Salmo salar*) has proved to be an excellent model for  
126 the study of MHC evolution for a number of reasons. First, as in teleosts in  
127 general, MHC class I and class II loci are not physically linked in the  
128 species, allowing for independent evolution of these classes of genes  
129 (Grimholt *et al*, 2002). As MHC genes do not form a single complex they  
130 are therefore known simply as MH genes in teleosts (Stet *et al*, 2002).  
131 Second, while MH pseudogenes exist in the genome, and several non-  
132 classical class I genes have been described recently (Lukacs *et al*, 2010),  
133 the major classical MH genes expressed are class I (*Sasa-UBA*), class II $\alpha$   
134 (*Sasa-DAA*) and class II $\beta$  (*Sasa-DAB*) (Grimholt *et al*, 2000; Grimholt *et al*,  
135 1993; Stet *et al*, 2002), making analyses of functional MHC variation  
136 relatively simple. Third, the molecular structure of MH genes has been  
137 extensively studied in Atlantic salmon (Grimholt *et al*, 2002; Grimholt *et al*,  
138 2000; Grimholt *et al*, 1993; Hordvik *et al*, 1993; Stet *et al*, 2002) and there  
139 is evidence of balancing selection acting on potential peptide binding



140 residues (PBRs) in both class I and class II $\alpha$  loci (Consuegra *et al*, 2005a;  
141 Consuegra *et al*, 2005b). Fourth, a dinucleotide microsatellite repeat located  
142 in the 3' untranslated region (UTR) of the MH class I locus (termed here  
143 *Sasa-UBA-3UTR*) (Grimholt *et al*, 2002) and a 10-base pair minisatellite  
144 repeat in the 3' UTR of the MH class II  $\alpha$  locus (termed here *Sasa-DAA-*  
145 *3UTR*) (Stet *et al*, 2002) can be used to rapidly assay variation at these loci  
146 in Atlantic salmon. Fifth, the Atlantic salmon is one of the few species  
147 where MH polymorphism has been experimentally shown to be associated  
148 with resistance/susceptibility to specific pathogens (Grimholt *et al*, 2003).  
149 Furthermore, historical samples are widely available from Atlantic salmon  
150 populations, often as dried scales that are suitable for extraction of DNA,  
151 allowing analysis of temporal variation within populations (Ciborowski *et*  
152 *al*, 2007; Consuegra *et al*, 2002; Nielsen *et al*, 1999).

153

154 The aims of this study were to test the hypotheses that (a) MH-linked  
155 markers in natural Atlantic salmon populations show temporal and spatial  
156 levels of heterogeneity that differ from a sample of neutral microsatellite  
157 loci and fall outside the range expected from neutral models and (b) MH  
158 class I and class II-linked markers show differential responses to selective  
159 forces as expected from the different evolutionary rates of the MH genes  
160 (Consuegra *et al*, 2005a; Consuegra *et al*, 2005b). To test these hypotheses  
161 two case studies involving Atlantic salmon populations in western Ireland

162 were used: (1) based on the Burrishoole (BRH) river system for temporal  
163 analysis and the nearby rivers Moy (MOY), Owenmore (OWM) and  
164 Owenduff (OWD) for spatial analysis and (2) based on the Delphi (DPH)  
165 river system for temporal analysis and the nearby rivers Owenwee (OWW)  
166 and Carrowinskey (CAW) for spatial analysis.

167 **Materials and Methods**

168 *Samples*

169 Samples were obtained from both contemporary and historical sources. For  
170 contemporary analysis, juveniles were sampled from all study rivers by  
171 electro-fishing of different sections, each approximately 200 m in length, in  
172 2002 and fin clips stored in 95% ethanol for subsequent extraction of DNA.  
173 The maximum distance between the focal rivers and other spatial samples  
174 was 182km (Burrishoole-Moy) and 57km (Deplhi-Owenwee) (Figure 1).

175

176 Historical samples from the Burrishoole system were obtained from the  
177 Marine Institute (Newport) as dried scales from both angled fish and fish  
178 caught in a research fish trap in 1956, 1968, 1973, 1983 and 1995. All  
179 selected samples were from grilse salmon (adults returning to the river after  
180 a single winter in the sea) and, given the predominance of two year smolts  
181 in the Burrishoole, they mostly represented single cohorts. For the Delphi  
182 system, fin clips for DNA analyses were collected from juvenile salmon  
183 sampled by electro-fishing fish in 1993 and 2002, representing 4 different  
184 cohorts. Fish were assigned to either the 0+ or 1+ age class using length  
185 frequency analysis (cut off length was 7 cm).

186

187 *DNA isolation and microsatellite genotyping*

188 Genomic DNA was isolated from fin and scale samples (2 scales per  
189 individual) using the Wizard SV96 Genomic Purification kit (Promega),  
190 eluted in 500  $\mu$ l (fin samples) or 100  $\mu$ l (scale samples) elution buffer and  
191 stored at 4°C until use in PCR amplifications. All samples were amplified  
192 for eight microsatellite loci: *SsoSL 85* (Slettan *et al*, 1995), *Ssa 171*, *Ssa*  
193 *197*, *Ssa 202* (O'Reilly *et al*, 1996), *Ssa D144b*, *Ssa D170* (King *et al*,  
194 2005), *Ssa 2215SP* and *Ssa 1G7SP* (Paterson *et al*, 2004). These unlinked  
195 microsatellites were selected as they behaved in a neutral manner in  
196 previous studies in Atlantic salmon (de Eyto *et al*, 2007). Samples were also  
197 amplified for a microsatellite repeat located in the 3' untranslated region  
198 (UTR) of the MHC class I locus (*Sasa-UBA-3UTR*) and a minisatellite  
199 repeat located in the 3' UTR of the MHC class II  $\alpha$  locus (*Sasa-DAA-3UTR*)  
200 (Stet *et al*, 2002). Previous studies (e.g. (de Eyto *et al*, 2007; Grimholt *et al*,  
201 2002; Stet *et al*, 2002) have shown that the micro/minisatellite markers used  
202 in this study are uniquely- and tightly-linked to the expressed MH loci in  
203 Atlantic salmon.

204

205 All loci were amplified with the Qiagen Multiplex Kit (Qiagen) in three  
206 multiplex reactions with the following proportions of each 100 pM primer  
207 combined in a final volume of 500  $\mu$ l primer stock solution: (a) *SsoS 85* (20  
208  $\mu$ l), *Ssa2215SP* (5  $\mu$ l), *Ssa171* (10  $\mu$ l), *SsaD144b* (5  $\mu$ l); (b) *Ssa197* (10  $\mu$ l),

209 *SsaD170* (10 µl), *Ssa1G7SP* (10 µl), *Ssa202* (10 µl); and (c) *Sasa-UBA-*  
210 *3UTR* (10 µl); *Sasa-DAA-3UTR* (10 µl).

211

212 Each reaction consisted of 4 µl of the multiplex mix (containing hot-start  
213 *Taq* polymerase, buffer and dNTPs), 0.8 µl of the primer stock; 1.2 µl of  
214 nuclease-free water and 2-3 µl of template DNA. PCR conditions were as  
215 follows: denaturation step at 95°C for 15 min followed by 30/35 cycles  
216 (modern/historical samples): 94°C 30 sec; 58°C 90 sec; 72°C 60 sec; and a  
217 final extension at 60°C for 30 min. Fragment sizes were then analysed on an  
218 Applied Biosystems ABI377 automatic sequencer and estimated with the  
219 aid of GeneScan and Genotyper software (Applied Biosystems) using an  
220 internal molecular size marker (TAMRA 350/500) as a reference standard.

221

222 All historical samples and half of the modern samples were replicated at  
223 least once and only repeatable peaks were counted as real alleles. Error rates  
224 (allelic dropouts-ADO and false alleles-FA) were estimated using GIMLET  
225 v1.3.3 (Valière, 2002), which was also used to construct consensus  
226 genotypes from the PCR replicates of each sample.

227

228 *Statistical Analysis*

229 *Intra-population genetic diversity*

230 Concordance with Hardy-Weinberg expectation (significance of  $F_{IS}$  values)  
231 and linkage disequilibrium between pairs of loci were tested for each locus  
232 in all samples with GENEPOP 3.2 (Raymond and Rousset, 1995). Observed  
233 heterozygosity ( $H_O$ ) was also calculated in GENEPOP 3.2 while allelic  
234 richness ( $N_A$ ) was calculated using FSTAT (Goudet, 1995).

235

236 After testing for deviation from normality and heterogeneity of variances,  
237 temporal and spatial heterogeneity in mean  $H_O$  and  $N_A$  for microsatellites  
238 was tested using one-way ANOVA, with *post hoc* tests of differences  
239 between sample means where appropriate, using SYSTAT v.10. Spatial and  
240 temporal heterogeneity in frequency of heterozygotes and deviation from  
241 mean allelic richness at MH-linked loci was assessed using  $G$  tests.

242

243 For temporal and spatial samples from each case study Mantel tests were  
244 performed between matrices of genetic differentiation ( $F_{ST}$ ) at microsatellite  
245 and MH-linked markers and the significance of the test statistic was  
246 assessed by performing 10,000 permutations of the data using GENETIX  
247 (Belkhir *et al.*, 2001). Given that  $F_{ST}$  values can underestimate the  
248 differentiation between populations with highly polymorphic microsatellites  
249 and when variability differs between marker classes, we also estimated  
250 Hedrick's standardized  $G'_{ST}$  (Hedrick, 2005) that provide more robust  
251 estimates of population difference and the  $D_{est}$  estimate of differentiation

252 (Jost, 2008) based on allele identities, that also accounts for the fixation of  
253 alleles in different populations.  $G'_{ST}$  and  $D_{est}$  were estimated in SMOGD  
254 (Crawford, 2010).

255

256 Deviations of the distribution of genetic variability within populations from  
257 that expected under neutrality were tested using the Ewens-Watterson  
258 homozygosity test of neutrality (Ewens 1972; Watterson 1978) with  
259 significance assessed by the Slatkin exact  $P$ -test (Slatkin 1994, 1996)  
260 implemented in Arlequin v.3 (Excoffier *et al.* 2007). The Ewens-Watterson  
261 test compares the observed homozygosity ( $F_o$ ) with the equilibrium  
262 homozygosity under the neutral theory ( $F_e$ ) from a simulation of randomly  
263 generated populations (1000 in this case). Significant negative values are  
264 indicative of balancing selection while positive values indicate directional  
265 selection.

266

#### 267 *Inter-population genetic diversity*

268 Concordance to an isolation by distance spatial model of population  
269 structure between all contemporary samples was estimated with IBDWS  
270 (IBD Web Service at <http://ibdws.sdsu.edu/>) (Jensen *et al.*, 2005) for  
271 microsatellite loci and MH-linked markers separately using logarithm  
272 transformations of genetic ( $F_{ST}$ ,  $G'_{ST}$ ,  $D_{est}$ ) and geographic distance (Km)  
273 between rivers. We also performed partial Mantel tests using  $zt$  (Bonnet and

274 Van de Peer, 2002) to estimate the correlation between pairwise population  
275 differentiation ( $F_{ST}$ ,  $G'_{ST}$ ) at MH-linked markers and geographical distance  
276 while keeping differentiation at microsatellites constant, in order to test for  
277 significant positive correlation between geographical distance and MH  
278 differentiation independent of demographic and stochastic factors.

279 Further temporal and spatial analysis of evidence for selection at MH-linked  
280 loci was performed using BayeScan (Foll & Gaggiotti 2008). BayeScan uses  
281 an extension of the (Beaumont and Balding, 2004) method to detect outlier  
282 loci by employing a Bayesian likelihood method. To identify loci under  
283 selection we used 10 pilot runs of 5000 iterations to estimate the distribution  
284 of  $\alpha$  (the locus-specific component of  $F_{ST}$ ), followed by a burn-in period of  
285 50,000 iterations and 150,000 iterations, with sample size of 5,000 and a  
286 thinning interval of 20 between samples. The identification of loci under  
287 selection is based on the Bayes factor (BF), the ratio between the posterior  
288 probabilities of two models (i.e. with and without selection). According to  
289 the scale of evidence for BF, a BF above 100 ( $\log_{10} > 2$ ; posterior  
290 probabilities ranging between 0.99 and 1) is interpreted as decisive evidence  
291 for selection.

292

293



294 **Results**

295 *Intra-population variability*

296 Numbers of alleles in the two case studies were similar: mean number per  
297 locus for the BRH study was 23.9 (range 12-52) with that for the DPH study  
298 21.0 (range 9-33). Allele frequencies for each sample are given in  
299 Supporting Information.

300

301 There were a number of significant deviations from Hardy Weinberg  
302 equilibrium in the samples, even after correcting for the number of tests  
303 carried out using a Bonferroni procedure (Tables 1 and 2). These significant  
304 deviations were almost exclusively associated with a positive value for  $F_{IS}$ ,  
305 indicating an excess of homozygotes in the sample. In general, the  
306 significant deviations were not associated with any particular locus.  
307 However, the earlier sample (1956) from the BRH case study (Table 1) and  
308 the OWW sample from the DPH case study (Table 2) displayed relatively  
309 high levels of deviation from Hardy Weinberg equilibrium across many loci.  
310 Only thirty three of 184 tests for linkage disequilibrium gave significant  
311 results, fifteen of them in the DPH spatial study, mostly involving *Ssa171* (5  
312 tests) and *SasaDAA* (5 tests) (Supporting Information). The average value of  
313 ADO was 3.1% and of FA 0.1% suggesting genotyping error rate was low.

314

315 *Comparisons across loci*

316 Mean  $H_O$  values for microsatellite loci were high ( $>0.70$ ) in all samples, and  
317 while values of  $H_O$  for *Sasa-UBA-3UTR* tended to fall within the 95%  
318 confidence limits for the mean value for microsatellites, those for *Sasa-*  
319 *DAA-3UTR* were generally lower than the lower 95% bound for the  
320 distribution of  $H_O$  among microsatellites (Figure 2). Significant  
321 heterogeneity in  $H_O$  was found among temporal samples in the BRH and  
322 DPH case studies at the *Sasa-DAA-3UTR* locus (Table 3, Figure 2) and at  
323 spatial samples at the microsatellite loci in the DPH spatial comparison.  
324 *Post hoc* tests showed that most of the heterogeneity in mean  $H_O$  of  
325 microsatellites in the DPH case study was due to the OWW sample having a  
326 significantly lower value of  $H_O$  than the samples from the DPH and CAW.  
327 Conversely, there was no evidence for significant heterogeneity in  $H_O$   
328 among spatial or temporal samples in both case studies at *Sasa-UBA-3UTR*  
329 (Table 3, Figure 2).

330

331 There was relatively little fluctuation in values of  $N_A$  over space and time  
332 compared to that for  $H_O$  (Figure 3), with no significant heterogeneity  
333 detected at any locus or class of loci at any scale (Table 3). As for  $H_O$ ,  
334 values of  $N_A$  for *Sasa-DAA-3UTR* were generally lower than those for other  
335 loci (Figure 3).

336

337 Matrices of genetic distance measured as  $F_{ST}$ ,  $G'_{ST}$  and  $D_{est}$  among temporal  
338 and spatial samples in both case studies were not correlated with each other  
339 in the comparisons between microsatellites and *Sasa-DAA 3UTR* (BRH  
340 temporal  $F_{ST}$ :  $Z = 0.04$ ,  $P = 0.074$ ;  $G'_{ST}$ :  $Z = 0.649$ ,  $P = 0.056$ ;  $D_{est}$ :  $Z =$   
341  $0.080$ ,  $P = 0.074$ ; BRH spatial  $F_{ST}$ :  $Z = 0.019$ ,  $P = 0.432$ ;  $G'_{ST}$ :  $Z = 0.515$ ,  $P =$   
342  $= 0.450$ ;  $D_{est}$ :  $Z = 0.426$ ,  $P = 0.432$ ; DPH temporal  $F_{ST}$ :  $Z = 0.01$ ,  $P =$   
343  $0.074$ ;  $G'_{ST}$ :  $Z = 0.014$ ,  $P = 0.220$ ;  $D_{est}$ :  $Z = 0.014$ ,  $P = 0.208$ ; DPH spatial  
344  $F_{ST}$ :  $Z = 0.001$ ,  $P = 0.866$ ;  $G'_{ST}$ :  $Z = 0.012$ ,  $P = 0.886$ ;  $D_{est}$ :  $Z = 0.012$ ,  $P =$   
345  $0.867$ ). In contrast, most matrices of genetic distances measured with  
346 microsatellites and *Sasa-UBA-3UTR* were correlated (BRH temporal  $F_{ST}$ :  $Z$   
347  $= 0.010$ ,  $P = 0.043$ ;  $G'_{ST}$ :  $Z = 0.649$ ,  $P = 0.038$ ;  $D_{est}$ :  $Z = 0.584$ ,  $P = 0.016$ ;  
348 BRH spatial  $F_{ST}$ :  $Z = 0.024$ ,  $P = 0.141$ ;  $G'_{ST}$ :  $Z = 1.272$ ,  $P = 0.450$ ;  $D_{est}$ :  $Z$   
349  $= 1.160$ ,  $P = 0.383$ ; DPH temporal  $F_{ST}$ :  $Z = 0.012$ ,  $P = 0.039$ ;  $G'_{ST}$ :  $Z =$   
350  $0.292$ ,  $P = 0.049$ ;  $D_{est}$ :  $Z = 0.265$ ,  $P = 0.049$ ; DPH spatial  $F_{ST}$ :  $Z = 0.002$ ,  $P$   
351  $= 0.036$ ;  $G'_{ST}$ :  $Z = 0.882$ ,  $P = 0.025$ ;  $D_{est}$ :  $Z = 1.160$ ,  $P = 0.025$ ).

352

353 *Evidence for selection on MH-linked markers - comparison with neutral*  
354 *models*

355 The Ewens-Watterson test rejected the null hypothesis of neutrality for  
356 *Sasa-UBA-3UTR* in the OWD sample ( $F_o = 0.219$   $F_e = 0.122$  Slatkin Exact  
357  $P = 0.014$ ).

358 The geographical distribution of variation at microsatellite loci conformed  
359 to an isolation by distance model of population structure ( $F_{ST}$ :  $Z = -13.84$ ,  $P$   
360  $= 0.049$ ;  $G'_{ST}$ :  $Z = -18.99$ ,  $P = 0.032$ ;  $D_{est}$ :  $Z = -477.82$ ,  $P = 0.042$ ), whereas  
361 this was not true of either *Sasa-DAA-3UTR* ( $F_{ST}$ :  $Z = -50.05$ ,  $P = 0.382$ ;  
362  $G'_{ST}$ :  $Z = -50.66$ ,  $P = 0.331$ ;  $D_{est}$ :  $Z = -1327.82$ ,  $P = 0.355$ ) or *Sasa-UBA-*  
363 *3UTR* ( $F_{ST}$ :  $Z = -49.62$ ,  $P = 0.101$ ;  $G'_{ST}$ :  $Z = -25.69$ ,  $P = 0.098$ ;  $D_{est}$ :  $Z = -$   
364  $663.46$ ,  $P = 0.099$ ).

365

366 Partial Mantel tests controlling for differentiation in microsatellites did not  
367 reveal any significant correlation between differentiation at MH-linked  
368 markers between populations and geographical distance ( $F_{ST}$ : *Sasa-DAA-*  
369 *3UTR*:  $r = -0.0095$   $P = 0.5029$  (one tailed); *Sasa-UBA-3UTR*:  $r = 0.2406$   $P =$   
370  $0.1113$  (one tailed);  $G'_{ST}$ : *Sasa-DAA-3UTR*:  $r = 0.1627$   $P = 0.2610$  (one  
371 tailed); *Sasa-UBA-3UTR*:  $r = 0.3113$   $P = 0.0970$  (one tailed)).

372

373 *Sasa-DAA-3UTR* displayed evidence of selection with high  $\log_{10}BF$  with  
374 values above 2 in spatial and temporal samples in both BRH and DPH case  
375 studies (BRH temporal  $\alpha=1.42$ ; BRH spatial  $\alpha=0.98$ ; DPH temporal  
376  $\alpha=1.14$ ; DPH spatial  $\alpha=0.83$ ) (Figure 4). *Sasa-UBA-3UTR* also showed  
377 evidence of selection but only in the temporal samples of the BRH and DPH  
378 case studies, respectively (BRH temporal  $\alpha=0.76$ ; DPH temporal  $\alpha=0.83$ ).

379 *SasaD144b* showed evidence for balancing selection ( $\alpha=-0.72$ ) in the  
380 Burrishole spatial study (Figure 4).  
381

382 **Discussion**

383 Previous studies had shown that both neutral forces and natural selection  
384 can act in shaping variability of MHC genes (Charbonnel and Pemberton,  
385 2005; de Eyto *et al*, 2007; Landry and Bernatchez, 2001; Oliver *et al*, 2009;  
386 Seddon and Ellegren, 2004). To our knowledge this is the first study that  
387 compares the effects of selection and neutral forces at both class I and class  
388 II $\alpha$  MH-linked markers spatial and temporally. Here, using two case studies  
389 involving Atlantic salmon populations in western Ireland, we tested two  
390 hypotheses on patterns of selection on MH-linked markers. To do this we  
391 compared temporal and spatial heterogeneity at microsatellite loci, which  
392 were presumed to be neutral to the effects of natural selection, with  
393 variation at MHC-linked markers using three measures of genetic  
394 variability: heterozygosity, allelic richness and variance in allele frequency  
395 among samples. We also tested levels of temporal and spatial differentiation  
396 at MH-linked markers and microsatellites against neutral models of genetic  
397 differentiation. Finally, we compared patterns of variability between classes  
398 of MH-linked markers to look for evidence of differential selection.

399

400 *Comparison among loci*

401 Our first hypothesis was that MH-linked markers would show higher levels  
402 of heterogeneity over temporal and spatial scales than microsatellites. We  
403 found differences among loci in two out of three measures of variability.

404 Comparison of levels of heterozygosity across time suggested that the class  
405 II $\alpha$  MH-linked locus (*Sasa-DAA-3UTR*) showed more heterogeneity than  
406 microsatellite loci. However, this contention requires careful scrutiny and  
407 justification as heterogeneity in heterozygosity could arise as a technical or  
408 sampling artefact. Analysis of the pattern of deviation from Hardy-  
409 Weinberg equilibrium suggested that older samples from the Burrishoole  
410 and samples from the Owenwee were exceptional in that they displayed  
411 significantly higher levels of homozygosity across several loci (although not  
412 in *Sasa-DAA-3UTR*). In particular the Owenwee was identified by *post hoc*  
413 tests as significantly lower in microsatellite heterozygosity than other  
414 samples from the same population or case study. Given the known problems  
415 with PCR amplification of DNA extracted from old scale samples (Nielsen  
416 *et al*, 1997) and the potential for sampling a small number of families from  
417 salmonid populations (Hansen *et al*, 1997), it may be that allelic dropout (in  
418 older Burrishoole samples) or sampling effects (in the Owenwee sample)  
419 were responsible for some of the observed temporal and spatial  
420 heterogeneity at microsatellite and MH-linked loci. However, repeated  
421 genotyping (with low levels of genotyping error rate) and sampling across  
422 several kilometres of river were specifically employed in this study to avoid  
423 these potential problems. Moreover, there is no *a priori* reason to believe,  
424 and no evidence from our data, that MH-linked markers were more  
425 susceptible to these problems than other loci. On this basis, it appears that

426 heterogeneity in heterozygosity was indeed greater at *Sasa-DAA-3UTR* than  
427 at microsatellites in our case studies.

428

429 In contrast with heterozygosity, the lack of heterogeneity observed in allelic  
430 richness is perhaps surprising given that allelic richness is often assumed to  
431 be a more sensitive measure of demographic and selective effects than  
432 heterozygosity (Leberg, 1992). Correlations between allelic richness and  
433 heterozygosity are scale dependent, and tend to be higher in nearby  
434 populations that share similar environments and selective pressures (Comps  
435 *et al*, 2001) but in general allelic richness correlates better with demographic  
436 patterns as a consequence of the effect of genetic drift in rare alleles, that  
437 contribute little to heterozygosity values but are more easily lost by random  
438 mating (Leberg, 2002). In fact, demographic effects such as post-glacial  
439 history and bottlenecks can result in important population structuring at  
440 MHC genes (e.g. Miller *et al* (1997)) and selection on particular alleles also  
441 result in important allele differences between populations (e.g. MHC class II  
442 in *Fundulus heteroclitus*; Cohen (2002)). In our study, we did not observe  
443 allelic number reductions but we found some degree of population  
444 structuring at MH-linked markers suggesting that neutral forces are also  
445 important in shaping MH variability.

446



447 Analysis of  $F_{ST}$ ,  $G'_{ST}$  and  $D_{est}$  among samples showed no correlation  
448 between matrices of genetic differentiation based on microsatellites and the  
449 MH class II-linked locus (*Sasa-DAA-3UTR*). However, there was a  
450 correlation between matrices of genetic differentiation from microsatellites  
451 and the MH class I-linked locus (*Sasa-UBA-3UTR*) in the BRH temporal  
452 case study and in both DPH case studies (all the three distances). Results  
453 using the three estimates of genetic diversity were in general consistent.  
454 Only microsatellites conformed to an isolation by distance model of  
455 population structure, suggesting that neutral forces such as gene flow and  
456 migration played a more important role in shaping the variability of the  
457 neutral microsatellites than of the MH-linked markers (Dionne *et al*, 2007).  
458 Higher levels of population differentiation for MH-linked markers could be  
459 the result of directional selection and local adaptation (Cohen, 2002; Heath  
460 *et al*, 2006; Landry and Bernatchez, 2001).

461

#### 462 *Comparison with neutral models*

463 We also hypothesized that MH-linked markers would lie outside the  
464 expectation from neutral models of population structure. Indeed,  
465 comparisons with neutral models provided additional evidence for  
466 differences in response to selection of class I and class II $\alpha$ -linked markers.  
467 The Ewens-Watterson test confirmed that directional selection was acting  
468 on the *Sasa-UBA-3UTR* with higher homozygosity than expected under

469 neutrality, albeit in only one of the populations (Owenduff). However,  
470 variable results of this test are commonly found when several populations  
471 are compared, probably as a result of differences in the strength of selection  
472 in different environments (Bernatchez and Landry, 2003) and among  
473 different subpopulations (Garrigan and Hedrick, 2003). In addition, the  
474 power of the Ewens-Watterson test decreases rapidly when beneficial  
475 mutations reach fixation, the time to fixation for positively selected alleles  
476 being commonly short (Zhai *et al*, 2009).

477

478 Perhaps the most unequivocal evidence for selection on the MH-linked  
479 markers comes from the Bayesian analysis of deviation from a neutral  
480 model of genetic variation. In all tests *Sasa-DAA-3UTR* was a strong outlier  
481 from the neutral expectation, while *Sasa-UBA-3UTR* was an outlier in the  
482 two temporal tests. The strong evidence for selection on *Sasa-DAA-3UTR* in  
483 this analysis is perhaps surprising given its relatively low level of  
484 polymorphism which could reduce the power of the analysis to detect  
485 selection (Foll and Gaggiotti, 2008). In contrast, microsatellites conformed  
486 to the neutral model, with only one exception (*Sasa-DI44b* in the  
487 Burrishoole spatial analysis). Although assumed neutral, microsatellites can  
488 appear as outliers as a consequence of hitchhiking selection, and this has  
489 been seen previously in salmonids with microsatellites known to be  
490 associated to life history traits (Aguilar and Garza, 2006). However, we did

491 not find evidence in the literature suggesting that this could be the case for  
492 *Sasa-DI44b* and given that this only occurred in one case, while the  
493 evidence for selection at *Sasa-UBA-3UTR* and *Sasa-DAA-3UTR* was  
494 supported by results of several tests, we consider that this result does not  
495 invalidate the evidence found here for selection acting on the MHC linked  
496 markers.

497

498 Directional selection acting on MH class II has been previously observed in  
499 Atlantic salmon within a river drainage, and was attributed to local  
500 adaptation to environmental conditions or pathogen composition (Landry  
501 and Bernatchez, 2001). An extensive study in populations of sockeye  
502 salmon found evidence for directional and balancing selection acting on MH  
503 class II (Miller *et al*, 2001), with a high degree of population differentiation  
504 at the MH locus that may have resulted from directional selection spatial  
505 and / or temporal variability in pathogen composition. The directional  
506 selection we observed could also be the result of spatial and temporal  
507 variation in pathogen composition, if the selective advantage of MH alleles  
508 differed among environments (Bernatchez and Landry, 2003) and this was  
509 reflected in the linked markers studied here. In the Burrishoole system a  
510 previous study linking MH variability to survival and fitness in Atlantic  
511 salmon under natural conditions found evidence for disease-mediated  
512 selection acting on both the MH class II locus and the linked marker *Sasa-*

513 *DAA-3UTR* (de Eyto *et al*, 2007). Moreover, the results suggested that  
514 additive allelic effects were more important than heterozygote advantage for  
515 individual survival (de Eyto *et al*, 2007). Sexual selection could also favour  
516 individuals carrying specific resistance alleles against common parasites in  
517 different populations, and differences in MHC composition due to divergent  
518 parasite-mediated selection could be further maintained by assortative  
519 mating of females with locally adapted males (Eizaguirre *et al*, 2010;  
520 Eizaguirre *et al*, 2009).

521

#### 522 *Differences between MH-linked markers*

523 Our second hypothesis was that MH-linked markers would show different  
524 patterns of selection. To our knowledge, this is the first time that the  
525 response to selection has been compared between MHC class I and class II-  
526 linked markers at both temporal and spatial scales. Collectively, our results  
527 indicate that the diversity of both MH-linked markers may be affected by  
528 selection, while undoubtedly neutral forces (genetic drift) also play a role.  
529 We found strong evidence for directional selection acting on *Sasa-DAA-*  
530 *3UTR*; we also found some evidence for directional selection on *Sasa-UBA-*  
531 *3UTR* but at a different scale. While *Sasa-DAA-3UTR* showed evidence for  
532 directional selection at both temporal and spatial studies, we only found  
533 evidence of selection acting on *Sasa-UBA-3UTR* mostly in the temporal

534 comparisons and only in one of the populations for the Burrishoole spatial  
535 study.

536

537 Previous studies in salmonids found temporal stability at MHC class I  
538 (Hansen *et al*, 2007) and class II (Miller *et al*, 2001) genes and higher levels  
539 of population structuring at MHC class II (Heath *et al*, 2006; Landry and  
540 Bernatchez, 2001), while guppies seem to show low levels of differentiation  
541 on MH class II genes (Van Oosterhout *et al*, 2006) and evidence from  
542 mammals and birds suggests that there is temporal variation in selection  
543 acting at MHC class II and class I genes (Charbonnel and Pemberton, 2005;  
544 Westerdahl *et al*, 2004). Our finding of different responses to selection of an  
545 MH class II $\alpha$ -linked marker and a class I-linked marker might reflect an  
546 association between level of environmental heterogeneity (within and  
547 among rivers) and possibly differences in pathogen-driven selective  
548 pressures acting on variability at each class of MH locus (Paterson, 1998;  
549 Wegner *et al*, 2008; Wegner *et al*, 2003). Although we cannot automatically  
550 assume that the genetic variation at the MHC loci is reflected in the linked  
551 microsatellite loci, previous studies (e.g. (Grimholt *et al*, 2002; Stet *et al*,  
552 2002)) have shown that the micro/minisatellite markers used in our current  
553 study are uniquely- and tightly- linked to the expressed MH loci in Atlantic  
554 salmon, and can be used as good proxies for functional variation in MH  
555 genes. Class I and class II genes have different patterns of variability in

556 teleosts. For example, (Consuegra *et al*, 2005a) observed greater divergence  
557 of alleles in class I in contrast with an overlap of most of the allelic  
558 composition of class II in two isolated Atlantic salmon populations. In  
559 contrast, (Kruiswijk *et al*, 2005) found a complete divergence in class II but  
560 not in class I of a barbs species flock. Differences in evolutionary rates and  
561 the response to selection between both genes have been observed not only in  
562 fish but also in mammals and birds (Bryja *et al*, 2007; Go *et al*, 2003). Class  
563 I and class II molecules differ in the nature of the peptides that they bind, in  
564 how they bind and process them (Castellino *et al*, 1997; Go *et al*, 2003;  
565 Grommé and Neefjes, 2002; Kaufman *et al*, 1999) and in the T cells they  
566 react with (Housset and Malissen, 2003; Huseby *et al*, 2003). Our results  
567 suggest that differences in the response to selection of class I and class II  
568 markers could reflect structural and functional differences in the genes to  
569 which they are linked. Given the increasing importance of using MHC-  
570 linked markers for conservation and management purposes (Bos *et al*, 2008;  
571 Hedrick *et al*, 2001) our results highlight the need to consider both loci in  
572 population and evolutionary studies.

573

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583

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1064 **Figure Legends**

1065 **Fig. 1** Map showing the location of the study rivers for the Burrishoole and  
1066 Delphi case studies.

1067 **Fig. 2** Patterns of observed heterozygosity ( $H_o$ ) at microsatellite loci (mean  
1068  $\pm$  95% confidence intervals) and MH-linked markers across time in the (a)  
1069 Burrishoole and (b) Delphi river systems and across space for samples in the  
1070 (c) Burrishoole and (d) Delphi case studies.

1071 **Fig. 3** Patterns of allelic richness ( $N_A$ ) at microsatellite loci (mean  $\pm$  95%  
1072 confidence intervals) and MH-linked markers across time in the (a)  
1073 Burrishoole and (b) Delphi river systems and across space for samples in the  
1074 (c) Burrishoole and (d) Delphi case studies.

1075 **Fig. 4** Spatial and temporal analyses for identification of loci potentially  
1076 subject to differential selection. a) Burrishole temporal case, b) Delphi  
1077 temporal case c) Burrishole spatial case and d) Delphi spatial case. Vertical  
1078 lines mark the Log10 of the Bayes factors estimated using BAYESCAN,  
1079 Log10(BF)=2 corresponding to posterior probabilities of locus effects of  
1080 0.99 (decisive). Only loci identified as under selection are labelled.

**Table 1** Values of  $F_{IS}$  (Weir & Cockerham 1984) for samples from the Burrishoole case study and the significance of deviation of genotype frequencies from Hardy Weinberg equilibrium ( $P$ ). Values in bold are those that remained significant after Bonferroni correction for number of tests in this series ( $\alpha=0.05/90=0.0006$ ). River abbreviations: Burrishoole (BRH), Moy (MOY), Owenmore (OWM), Owenduff (OWD), Delphi (DPH), Owenwee (OWW) and Carrowinsky (CAW).

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		BRH						OWD	OWM	MOY
		1956	1968	1973	1983	1995	2002	(N=57)	(N=57)	(N=48)
		(N=50)	(N=44)	(N=48)	(N=47)	(N=40)	(N=57)			
<i>Ssa D144b</i>	$F_{IS}$	<b>+0.245</b>	+0.277	<b>+0.170</b>	<b>+0.183</b>	+0.160	+0.028	+0.023	+0.081	+0.150
	$P$	<b>&lt;0.0001</b>	0.0007	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	0.0179	0.0476	0.2994	0.0805	0.0417
<i>Ssa 171</i>	$F_{IS}$	+0.134	<b>+0.120</b>	-0.026	-0.117	+0.012	<b>+0.067</b>	+0.026	+0.089	<b>+0.299</b>
	$P$	0.1450	<b>&lt;0.0001</b>	0.8374	0.4654	0.2267	<b>&lt;0.0001</b>	0.2880	0.0512	<b>&lt;0.0001</b>
<i>Ssa 2215SP</i>	$F_{IS}$	-0.026	-0.009	+0.076	+0.116	<b>+0.326</b>	+0.205	-0.001	+0.092	+0.130
	$P$	0.1413	0.0520	0.0655	0.0119	<b>&lt;0.0001</b>	0.0014	0.1354	0.0621	0.0145
<i>SsoSL 85</i>	$F_{IS}$	<b>+0.275</b>	+0.189	+0.087	-0.049	+0.158	<b>+0.248</b>	+0.119	<b>+0.142</b>	<b>+0.407</b>
	$P$	<b>&lt;0.0001</b>	0.0094	0.0160	0.2018	0.0142	<b>&lt;0.0001</b>	0.1858	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>



<i>Ssa 197</i>	<i>F<sub>IS</sub></i>	+0.113	+0.085	+0.162	-0.067	+0.002	+0.034	+0.123	+0.099	+0.090
	<i>P</i>	0.0010	0.0025	0.0324	0.4183	0.3111	0.0034	0.1167	0.1488	0.5526
<i>Ssa 1G7SP</i>	<i>F<sub>IS</sub></i>	+0.079	+0.259	+0.292	-0.101	+0.075	+0.025	<b>+0.293</b>	+0.128	+0.156
	<i>P</i>	0.0018	0.0211	0.0113	0.559	0.5648	0.0789	<b>&lt;0.0001</b>	0.1692	0.0238
<i>Ssa 202</i>	<i>F<sub>IS</sub></i>	<b>+0.447</b>	+0.230	+0.251	+0.139	+0.158	+0.004	-0.027	+0.066	-0.025
	<i>P</i>	<b>&lt;0.0001</b>	0.0010	0.0234	0.0023	0.1124	0.0298	0.4732	0.5981	0.6107
<i>Ssa D170</i>	<i>F<sub>IS</sub></i>	<b>+0.319</b>	<b>+0.112</b>	<b>+0.189</b>	<b>+0.213</b>	+0.086	-0.031	+0.137	+0.098	-0.040
	<i>P</i>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	0.0334	0.3977	0.0243	0.3857	0.8574
<i>Sasa-DAA-3UTR</i>	<i>F<sub>IS</sub></i>	+0.082	+0.125	-0.044	+0.051	+0.148	-0.065	+0.203	+0.123	+0.062
	<i>P</i>	0.5158	0.4199	0.1343	0.8610	0.0873	0.3966	0.0013	0.4553	0.3670
<i>Sasa-UBA-3UTR</i>	<i>F<sub>IS</sub></i>	+0.052	-0.102	+0.161	<b>+0.297</b>	+0.028	-0.039	+0.111	<b>+0.182</b>	<b>+0.294</b>
	<i>P</i>	0.1460	0.2183	0.2725	<b>&lt;0.0001</b>	0.0746	0.1530	0.0078	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>

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**Table 2** Values of  $F_{IS}$  (Weir & Cockerham 1984) for samples from the Delphi case study and the significance of deviation of genotype frequencies from Hardy Weinberg equilibrium ( $P$ ). Values in bold are those that remained significant after Bonferroni correction for number of tests in this series ( $\alpha=0.05/60=0.0008$ ). River abbreviations: Burrishoole (BRH), Moy (MOY), Owenmore (OWM), Owenduff (OWD), Delphi (DPH), Owenwee (OWW) and Carrowinskey (CAW).

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		DPH				OWW (N=55)	CAW (N=48)
		1993 0+ (N=45)	1993 1+ (N=50)	2002 0+ (N=45)	2002 1+ (N=51)		
<i>Ssa D144b</i>	$F_{IS}$	-0.007	+0.114	+0.121	+0.157	<b>+0.031</b>	+0.029
	$P$	0.2089	0.0055	0.1098	0.0190	<b>&gt;0.0001</b>	0.0545
<i>Ssa 171</i>	$F_{IS}$	+0.013	-0.012	-0.147	+0.028	+0.028	+0.051
	$P$	0.0460	0.5265	0.9571	0.2574	0.0062	0.0357
<i>Ssa 2215SP</i>	$F_{IS}$	-0.009	+0.070	+0.113	+0.074	<b>+0.281</b>	-0.032
	$P$	0.8879	0.3332	0.0044	0.2369	<b>0.0002</b>	0.1871
<i>SsoSL 85</i>	$F_{IS}$	+0.025	+0.082	+0.004	-0.003	+0.063	+0.183
	$P$	0.1160	0.0158	0.5275	0.5162	0.0066	0.0037

<i>Ssa 197</i>	<i>F<sub>IS</sub></i>	+0.012	+0.036	-0.049	-0.002	<b>+0.575</b>	-0.028
	<i>P</i>	0.5630	0.2662	0.4270	0.6161	<b>&gt;0.0001</b>	0.7733
<i>Ssa 1G7SP</i>	<i>F<sub>IS</sub></i>	+0.069	+0.013	+0.067	<b>+0.172</b>	<b>+0.304</b>	+0.032
	<i>P</i>	0.7914	0.0505	0.3465	<b>&gt;0.0001</b>	<b>0.0001</b>	0.0592
<i>Ssa 202</i>	<i>F<sub>IS</sub></i>	+0.176	<b>+0.365</b>	-0.040	+0.012	+0.271	<b>+0.210</b>
	<i>P</i>	0.0013	<b>&gt;0.0001</b>	0.5938	0.1613	0.0033	<b>&gt;0.0001</b>
<i>Ssa D170</i>	<i>F<sub>IS</sub></i>	<b>+0.279</b>	+0.130	<b>+0.059</b>	+0.129	<b>+0.240</b>	<b>+0.007</b>
	<i>P</i>	<b>&gt;0.0001</b>	0.0036	<b>&gt;0.0001</b>	0.0011	<b>&gt;0.0001</b>	<b>&gt;0.0001</b>
<i>Sasa-DAA-3UTR</i>	<i>F<sub>IS</sub></i>	+0.132	+0.303	+0.104	-0.005	+0.118	-0.223
	<i>P</i>	0.0038	0.0029	0.0674	0.6681	0.6304	0.0013
<i>Sasa-UBA-3UTR</i>	<i>F<sub>IS</sub></i>	+0.113	+0.114	+0.115	<b>+0.262</b>	<b>+0.313</b>	+0.027
	<i>P</i>	0.3261	0.0055	0.1193	<b>&gt;0.0001</b>	<b>&gt;0.0001</b>	0.0390

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**Table 3** Results of tests of temporal and spatial heterogeneity in mean  $H_O$  and  $N_A$  at microsatellite loci, the frequency of heterozygotes at MH-linked makers and deviation from mean  $N_A$  at MH-linked makers. Numbers in subscript indicate degrees of freedom associated with each test. Values in bold were significant at the  $\alpha=0.05$  level.

		Temporal		Spatial	
		Burrishoole	Delphi	Burrishoole	Delphi
Heterozygosity ( $H_O$ )	microsatellites	$F_{5,42} = 1.24, P = 0.309$	$F_{3,28} = 0.292, P = 0.786$	$F_{3,28} = 0.98, P = 0.415$	$F_{2,21} = \mathbf{3.80}, P = \mathbf{0.039}$
	<i>Sasa-DAA-3UTR</i>	$G_5 = \mathbf{23.07}, P < \mathbf{0.001}$	$G_3 = \mathbf{8.17}, P = \mathbf{0.043}$	$G_3 = 1.35, P = 0.715$	$G_2 = 0.172, P = 0.917$
	<i>Sasa-UBA-3UTR</i>	$G_5 = 3.595, P = 0.606$	$G_3 = 0.719, P = 0.868$	$G_3 = 3.06, P = 0.380$	$G_2 = 3.07, P = 0.214$
Allelic Richness ( $N_A$ )	microsatellites	$F_{5,42} = 0.95, P = 0.461$	$F_{3,28} = 0.218, P = 0.883$	$F_{3,28} = 0.71, P = 0.553$	$F_{2,21} = 0.13, P = 0.876$
	<i>Sasa-DAA-3UTR</i>	$G_5 = 1.59, P = 0.896$	$G_3 = 0.99, P = 0.306$	$G_3 = 0.67, P = 0.879$	$G_2 = 0.29, P = 0.866$
	<i>Sasa-UBA-3UTR</i>	$G_5 = 1.61, P = 0.893$	$G_3 = 0.61, P = 0.892$	$G_3 = 4.48, P = 0.208$	$G_2 = 0.18, P = 0.913$

**Figure 1. Location of the study rivers.**

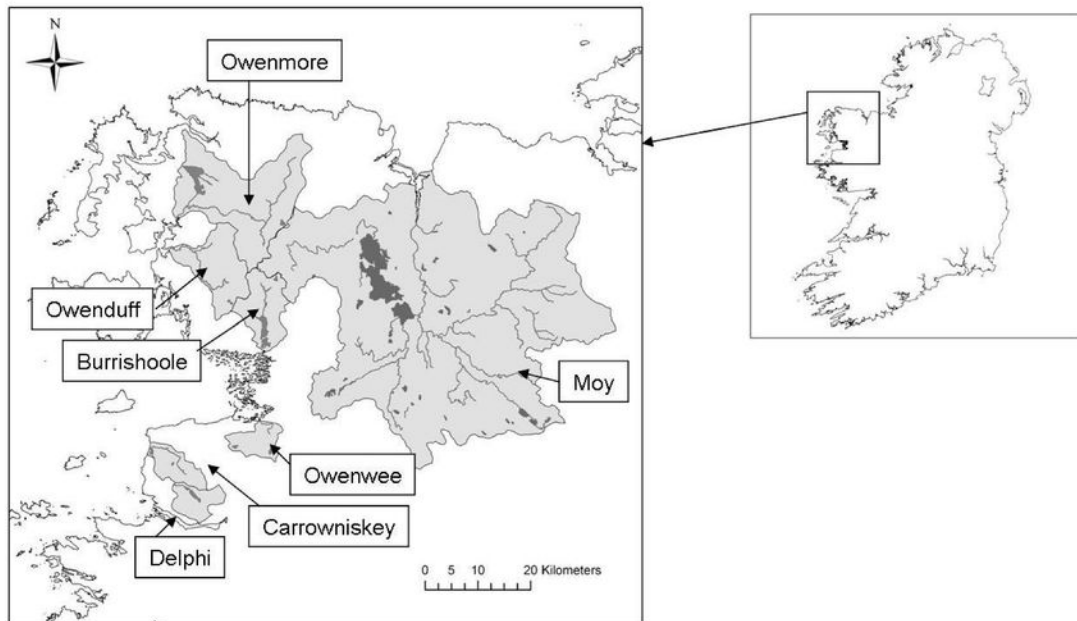
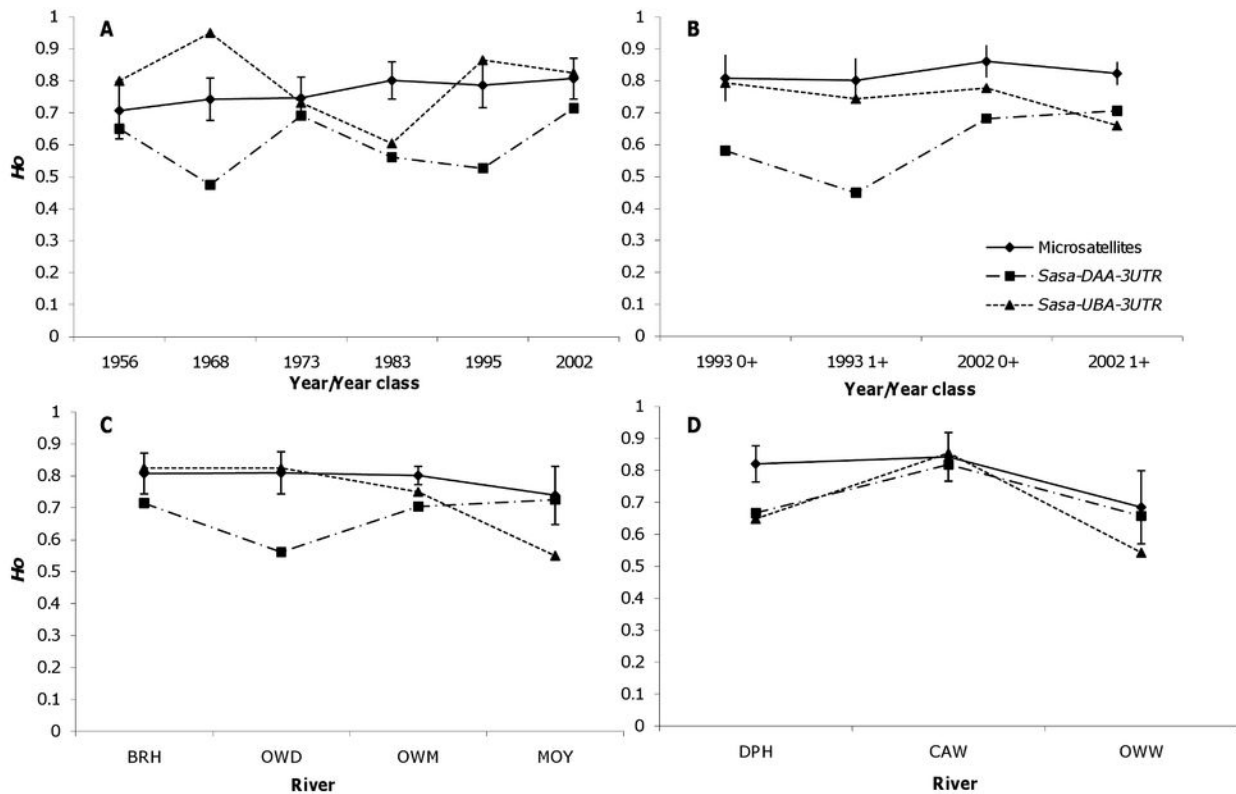


Figure 2. Heterozygosity at MHC and microsatellites.



**Figure 3. Allelic richness at MHC and microsatellites.**

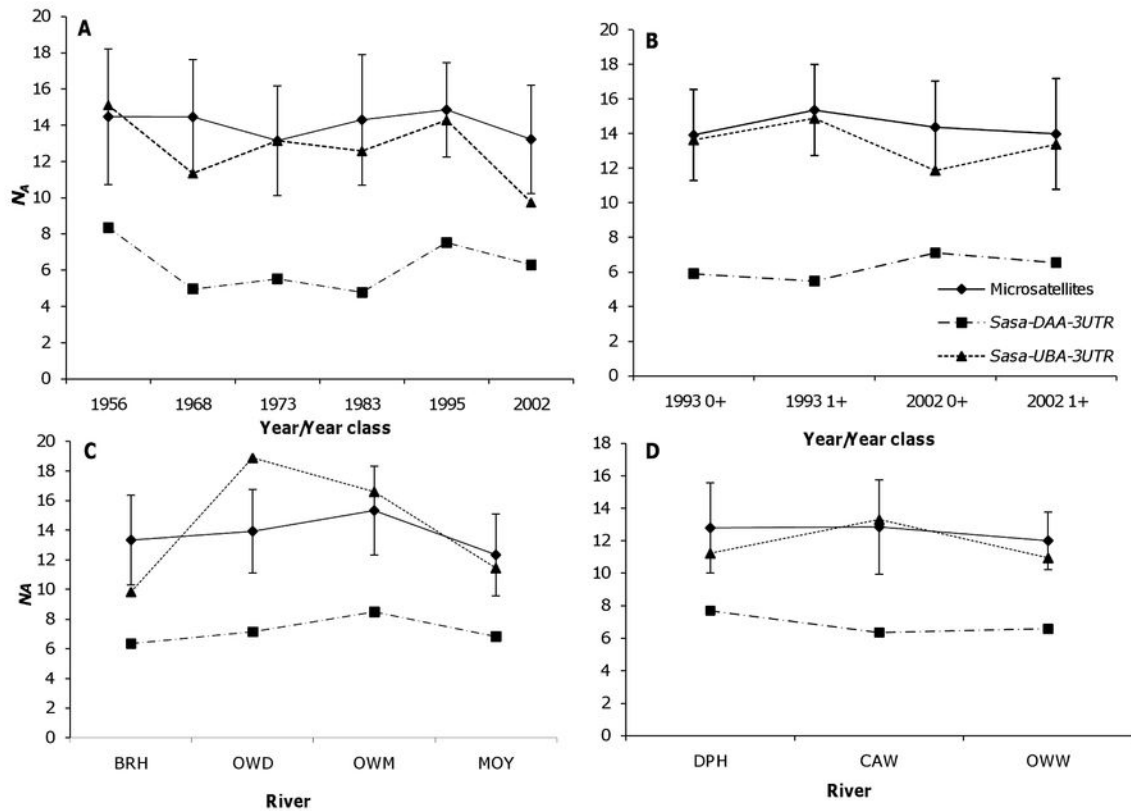


Figure 4. Results from bayesian analysis of selection.

