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PO Box 117  
221 00 Lund  
+46 46-222 00 00



# Experimental evidence for major histocompatibility complex-allele-specific resistance to a bacterial infection

Jakob Lohm<sup>1\*</sup>, Mats Grahn<sup>2</sup>, Åsa Langefors<sup>1</sup>, Øivind Andersen<sup>3</sup>,  
Arne Storset<sup>4</sup> and Torbjörn von Schantz<sup>1</sup>

<sup>1</sup>Department of Animal Ecology, Ecology Building, Lund University, SE-223 62 Lund, Sweden

<sup>2</sup>Södertörn University College and Department of Bioscience, Karolinska Institute, Box 4101, SE 141 04 Huddinge, Sweden

<sup>3</sup>AKVAFORSK, The Institute of Aquaculture Research, PO Box 5010, N-1432 Ås, Norway

<sup>4</sup>Aqua Gen AS, N-6600 Sunndalsøra, Norway

The extreme polymorphism found at some major histocompatibility complex (MHC) loci is believed to be maintained by balancing selection caused by infectious pathogens. Experimental support for this is inconclusive. We have studied the interaction between certain MHC alleles and the bacterium *Aeromonas salmonicida*, which causes the severe disease furunculosis, in Atlantic salmon (*Salmo salar* L.). We designed full-sibling broods consisting of combinations of homozygote and heterozygote genotypes with respect to resistance or susceptibility alleles. The juveniles were experimentally infected with *A. salmonicida* and their individual survival was monitored. By comparing full siblings carrying different MHC genotypes the effects on survival due to other segregating genes were minimized. We show that a pathogen has the potential to cause very intense selection pressure on particular MHC alleles; the relative fitness difference between individuals carrying different MHC alleles was as high as 0.5. A co-dominant pattern of disease resistance/susceptibility was found, indicative of qualitative difference in the immune response between individuals carrying the high- and low-resistance alleles. Rather unexpectedly, survival was not higher among heterozygous individuals as compared with homozygous ones.

**Keywords:** major histocompatibility complex class II; disease resistance; alleles; bacterial infection

## 1. INTRODUCTION

Major histocompatibility complex (MHC) molecules have a central role in the adaptive immune system by presenting foreign peptides to T cells. The evolution and maintenance of the extraordinary polymorphism of the genes encoding MHC molecules are generally attributed to balancing selection caused by infectious pathogens (Clarke & Kirby 1966; Takahata *et al.* 1992; Apanius *et al.* 1997). So far, experimental infections in congenic mice and other inbred animal models provide compelling evidence that MHC genes affect host resistance to infectious agents (Apanius *et al.* 1997). However, background genes (including non-immune system genes) appear to have an important role in MHC-dependent parasite resistance where a particular MHC haplotype may be associated with either resistance or susceptibility to a given pathogen depending on the genetic background of the host (Apanius *et al.* 1997; Medina & North 1998). Hence, it is still a contentious issue to what extent the results from inbred laboratory strains can be extrapolated to outbred populations (Apanius *et al.* 1997), where evidence is solely based on correlative data with no control for effects of non-MHC genes (Briles *et al.* 1983; Hill *et al.* 1991; Thursz *et al.* 1997; Paterson *et al.* 1998; Carrington *et al.* 1999; Langefors *et al.* 2001).

Unlike the situation in other vertebrates, in bony fishes the two classical MHC regions, class I and class II, are not linked (Bingulac-Popovic *et al.* 1997; Flajnik *et al.* 1999; Sato *et al.* 2000). Furthermore, studies in the Atlantic salmon (*Salmo salar* L.) have shown that only a single MHC class II B locus exists in this species (Langefors *et al.* 2000). These two features of the Atlantic salmon MHC make it an ideal species to test interactions between infectious pathogens and disease resistance caused by specific MHC class II B alleles.

Furunculosis in salmonids is caused by the bacterium *Aeromonas salmonicida*. It is an infectious disease that has a strong impact on survival in salmonids and in Atlantic salmon. Macrophage-like cells have been shown to regulate the expression of MHC class II mRNA in response to lipopolysaccharide antigen from *A. salmonicida* (Koppang *et al.* 1999). Furthermore, selection experiments have revealed a substantial genetic component to resistance against furunculosis in fishes (Gjedrem 2000; Marsden *et al.* 1996).

In our previous survey (Langefors *et al.* 2001) conducted on juveniles from an outbred hatchery population of Atlantic salmon (Gjedrem 2000), correlations were found between survival probability and three different MHC class II B alleles (out of nine) following controlled infection with *A. salmonicida*. One of these three alleles was associated with reduced survival probability and the other two with increased survival (Langefors *et al.* 2001). Three years later, in the autumn of 1998, when

\* Author for correspondence (jakob.lohm@zooekol.lu.se).

the uninfected siblings of these fishes from our previous survey became sexually mature, their MHC class II B genotype was determined and they were selectively mated in order to create different MHC genic groups combining high- and low-resistance alleles within full-sibling families. The offspring from these matings, carrying controlled MHC genotypes and on average the same genetic background within each group because they consist of full-sibling families, were then infected with furunculosis and their survival was monitored. Through this experiment, we were able to study the effect of specific MHC alleles on disease resistance while excluding potential effects of family. We describe here the outcome of the experiment.

## 2. MATERIAL AND METHODS

### (a) *Experimental fishes*

Fishes belonging to a large population originating from a number of wild Norwegian populations maintained under farming conditions for six generations (since 1974) where selective breeding has mainly focused on increased growth rate and delayed sexual maturation (Gjedrem 2000) were reared by Aqua Gen AS, Norway. Norway is not considered to be a furunculosis endemic area (Johnsen & Jensen 1994). Pedigree analysis (data not shown) of individuals used to create the full-sibling families in this experiment shows that, in five generations (starting with wild fishes caught in 1974), there have been no matings between known relatives within their parental lineages (except for one case; a sibling mating in the second generation in one of the families), minimizing the likelihood that any non-random associations between the studied MHC gene and other potentially disease controlling loci were generated by inbreeding. To exclude the possibility that our captive population was inbred due to non-random sampling in the founding generation, the genetic variation within the captive population was compared with that of two Baltic salmon populations, the Kalixälven and Dalälven populations. This was done by the use of the amplified fragment length polymorphism (AFLP) technique (Vos *et al.* 1995), which is a DNA fingerprinting method based on the PCR. The Dalälven population is a non-captive cultured population that has been maintained by culturing since the 1950s to compensate for the loss of natural spawning sites caused by dams built for hydropower production. The Kalixälven population is one of the few remaining unmanaged naturally reproducing Swedish salmon populations.

AFLP analyses were performed as described elsewhere (Vos *et al.* 1995), with the modification that the *EcoRI* selective primers were labelled with fluorescein (Life Technologies), on eight randomly selected individuals from each of the three populations using five sets of selective amplification primers (*EcoRI* + TAG/*MseI* + CAC, *EcoRI* + TAG/*MseI* + CAG, *EcoRI* + TCT/*MseI* + CGT, *EcoRI* + TCT/*MseI* + CGG and *EcoRI* + TGA/*MseI* + CGC). Fragments were analysed in a Vistra Fluorimager and generated a total of 255 markers. There was no significant difference in the number of polymorphic markers between the three populations ( $\chi^2_{2d.f.} = 0.36$ ,  $p = 0.83$ ). Marker polymorphism was 11.4% in Norway (28 out of 245 markers), 13.2% in Kalixälven (33 out of 250 markers) and 12.5% in Dalälven (31 out of 248 markers) populations.

### (b) *Artificial mating and furunculosis challenge test*

To arrange the preferred matings 321 individually tagged (PIT-tag, Trovan) adults were screened for their MHC class II

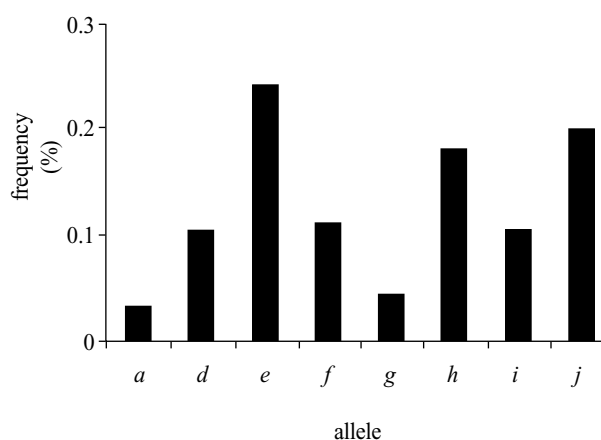


Figure 1. MHC class II B allele frequencies in 321 adult Atlantic salmon analysed with DGGE (Langefors *et al.* 2000) from the parental generation of which 10 were used to create desired family groups. The *h* bar includes alleles *b* and *c*; these alleles were not separated in this particular study (Langefors *et al.* 2000).

B gene (in May 1998) prior to the time of the artificial matings. DNA was recovered from a small tissue sample from the adipose fin without causing any harm to the fishes. The complete exon 2, encoding the putative antigen-binding region, was analysed by a combination of PCR and denaturing gradient gel electrophoresis (DGGE) on parallel gels with a denaturant gradient of 20–45% (Langefors *et al.* 2000). In the experimental matings, we focused on the most common high-resistance allele (*e*) and the low-resistance allele (*j*) identified in the previous study (Langefors *et al.* 2001; figure 1). Five full-sibling families were designed consisting of combinations of homozygote and heterozygote siblings with respect to the high- and low-resistance alleles (*e/e*, *j/j* and *e/j*), or the high- and low-resistance alleles combined with other alleles (+) or together (*e/+*, *j/+* and *e/j*; table 1). By constructing large full-sibling families with known MHC genotypes, we were able to control for the effect of randomly segregating genes while retaining an outbred genetic background within each sibling group. Desired mating combinations were artificially performed in November 1998 from single mating pairs and the five full-sibling families ( $r = 0.5$ ) were established. On 10 September 1999 (day 0; figure 2*a–c*), a sample, ranging from 17 to 40 siblings (a total of 150 individuals) from the five experimental full-sibling families was subjected to the annual furunculosis challenge test at Viken Aqua Vet, Viken, Norway (Gjedrem *et al.* 1991). The five experimental full-sibling families were exposed to furunculosis, together with *ca.* 9000 other juveniles in a 6 m<sup>3</sup> experimental tank, by putting 300 previously infected fishes of the same origin as the experimental fishes into the tank. These infected fishes had been inoculated by an intraperitoneal injection of  $5 \times 10^4$  viable cells of the Gram-negative proteobacteria *A. salmonicida* *salmonicida* from the specific strain as used in the previous study (stored at  $-79^\circ\text{C}$  between the two infection events). Dead individuals were collected, recorded and frozen until analyses (survivors were collected when the experiment was terminated). To identify the allelic variants the complete exon 2 of the MHC class II B gene was analysed by DGGE (Langefors *et al.* 2000) in all individuals from the five families. The experimental dataset was then analysed with a Cox proportional hazards regression, stratified for family groups (SPSS 1998).

Table 1. Survival distribution and genotypes of Atlantic salmon from five families 18 days after infection with *A. salmonicida*. *salmonicida*.

(The numbers of *e/e* and *j/j* homozygous individuals are given within parentheses. Two matings were *e/j* × *e/j*, *n* = 40 and *n* = 17 offspring, respectively. Two matings were *e/j* × *+/+*, *n* = 38 and *n* = 15. One mating was *e/j* × *j/+*, *n* = 40.)

	<i>e/+</i>	<i>e/j</i>	<i>j/+</i>
before selection	44 (11)	35	71 (28)
dead	26 (7)	23	55 (22)
surviving	18 (4)	12	16 (6)
proportional survival	0.41	0.34	0.23

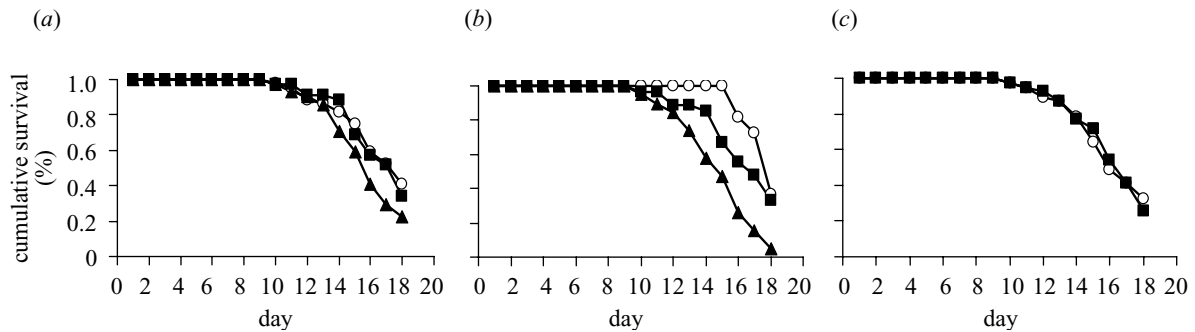


Figure 2. Kaplan-Meier plots (SPSS 1998) of the survival of different MHC class II B genotypes in Atlantic salmon from five different families after infection (day 0) with *A. salmonicida*. (a) Kaplan-Meier plots of the survival of *e/+* (open circles), *e/j* (filled squares) and *j/+* (filled triangles) fishes. The survival probability for the *j/+* individuals was significantly different from the *e/+* and the *e/j* individuals (see § 3). (b) Kaplan-Meier plots of the survival of *e/e* (open circles), *e/j* (filled squares) and *j/j* (filled triangles) fishes from the two *e/j* × *e/j* matings. The survival probability for the *j/j* individuals was significantly different from the *e/e* and the *e/j* individuals (see § 3). (c) Kaplan-Meier plots of the survival of MHC class II B heterozygous (open circles) and homozygous (filled squares) fishes.

### 3. RESULTS AND DISCUSSION

The mortality peaked at day 16 and the challenge test was terminated two days later when the overall accumulated mortality was 69.7%. The allele frequencies within the five families did not differ from Mendelian expectations ( $\chi^2_{2d.f.} = 2.78$ ,  $p = 0.25$ ,  $n = 300$  alleles; table 1) indicating that there was no selection against any particular allele (*e*, *j* or *+*) from fertilization until the experiment started 10 months later. During the experiment (figure 2a–c), 104 individuals from our experimental broods died and the remaining 46 were scored as survivors (table 1). Ten per cent of all infected fishes dying during the furunculosis outbreak between day 10 and 18 were examined bacteriologically and *A. salmonicida* was re-isolated in all cases. We predicted, based on our previous results (Langefors *et al.* 2001), that individuals carrying allele *e* would have a higher survival probability than individuals carrying allele *j*. The risk of dying of infection was significantly higher for *j/+* compared with *e/+* individuals (Relative Hazard (RH) = 1.95,  $p = 0.008$ ,  $n = 115$ ; figure 2a) and with *e/j* individuals (RH = 1.81,  $p = 0.043$ ,  $n = 106$ ; figure 2a). However, the survival rate for the heterozygotes (*e/j*) was not significantly different from that for the *e/+* genotype (RH = 1.57,  $p = 0.257$ ,  $n = 79$ ; figure 2a). Separate analyses of the two families that only carried the two specific alleles (sibling genotypes: *e/e*, *e/j* and *j/j*) showed the same result, with even more pronounced differences. The survival probability of the *j/j* fishes was significantly different from that of the *e/e* (RH = 3.35,  $p = 0.008$ ,  $n = 30$ ; figure 2b) and *e/j* (RH = 2.11,  $p = 0.029$ ,  $n = 46$ ; figure 2b) genotypes but there was no difference

in survival between *e/e* and *e/j* individuals (RH = 1.49,  $p = 0.379$ ,  $n = 38$ ; figure 2b). These phenotypic differences are congruent with a dominant effect on disease resistance linked to allele *e* that is stronger than the dominant susceptibility linked to allele *j*. The *j/+* fishes did not survive better than the *j/j* fishes (RH = 0.41,  $p = 0.123$ ,  $n = 71$ ; figure 2a,b), indicating that the increased susceptibility of the *j* allele is not due to a deficiency of antigen presentation in homozygous *j/j* individuals that could have been compensated for by the presence of a neutral allele in the heterozygotes. Instead, this co-dominant pattern of disease resistance/susceptibility is indicative of a qualitative difference in the immune response between individuals carrying the *j* and the *e* alleles, perhaps mediated by a difference in how the alleles regulate the T-cell response. Because there was no significant difference in survival between homozygotes and heterozygotes (RH = 0.95,  $p = 0.822$ ,  $n = 150$ ; figure 2c) and the difference in survival between genotypes was due to the *j/+* group compared with the *e/j* and *e/+* groups, the results demonstrate that resistance or susceptibility to furunculosis infection in Atlantic salmon is associated with specific MHC class II B alleles rather than MHC heterozygosity.

Interactions between MHC and other genes is generally believed to decrease the relative importance of the MHC for disease resistance and the effect of genetic background is often suggested as an explanation to the difficulties in demonstrating MHC effects in outbred populations (Apanius *et al.* 1997). While this experiment was done in an outbred genetic background, where the genetic background within each group is on average constant because

the groups consist of full-sibling families, there was a strong effect of the MHC. The difference between the MHC class II B *e* and *j* alleles can be expressed in terms of relative fitness (*w*) calculated from the proportion surviving *j*/+ individuals divided by the proportion surviving *e*/+ individuals (table 1), giving  $0.23/0.41 = 0.55$ . The corresponding selection coefficient ( $s = 1 - w$ ) against the *j* allele would then become 0.45. This calculation however only uses fishes surviving to the end of the experiment and a more accurate estimate of *s* can be computed from the relative hazard value from the Cox regression analysis,  $s = 1 - (1/1.95) = 0.49$ . This value of *s* is very high compared with previous estimates of the selection coefficient at MHC genes (Edwards & Hedrick 1998). This can be explained by the fact that we do not account for differences over the whole lifespan. Yet, our experiment demonstrates that a pathogen has the potential to cause intense selection pressure on particular MHC alleles. A strong directional selection, if it persists over several generations, is incompatible with the high levels of MHC polymorphism observed in this and other species (Clarke & Kirby 1966; Takahata *et al.* 1992; Apanius *et al.* 1997; Langefors *et al.* 2001; figure 1). Thus, it is probable that the fitness of different MHC alleles differs for different pathogens (Penn & Potts 1999) and shifts over time.

This study clearly shows a strong survival advantage for individuals carrying a high-resistance allele when exposed to a bacterial infection. The design of our experiment permitted us to compare the different MHC class II alleles while holding the effects on survival due to other segregating genes constant by comparing full-sibling families. It also allowed for genetic variation at other unlinked loci making it a more realistic model of a wild population than the inbred congenic lines most often used to establish phenotypic effects of the MHC (Apanius *et al.* 1997; Edwards & Hedrick 1998). This kind of experimental design, allowing direct comparison within families, is only possible in organisms with very large family sizes such as fishes. It should be pointed out that the net strength of selection on the locus is dependent of the net effects of all other pathogens and selective forces that may be operating. Nevertheless our finding of a directional selection acting on the MHC despite its high polymorphism stresses the importance of renewal of genetic variation at these kinds of loci, either from mutation, recombination or immigration from other populations, when combating new or coevolving virulent pathogens (Hamilton *et al.* 1990; Eshel & Hamilton 1984).

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## REFERENCES

- Apanius, V., Penn, D., Slev, P. R., Ruff, L. R. & Potts, W. K. 1997 The nature of selection on the major histocompatibility complex. *Crit. Rev. Immunol.* **17**, 179–224.
- Bingulac-Popovic, J., Figueroa, F., Sato, A., Talbot, W. S., Johnson, S. L., Gates, M., Postlethwait, J. H. & Klein, J. 1997 Mapping of MHC class I and class II regions to different linkage groups in the zebrafish, *Danio rerio*. *Immunogenetics* **46**, 129–134.
- Briles, W. E., Briles, R. W., Taffs, R. E. & Stone, H. A. 1983 Resistance to a malignant lymphoma in chickens is mapped to subregion of major histocompatibility (B) complex. *Science* **219**, 977–979.
- Carrington, M., Nelson, G. W., Martin, M. P., Kissner, T., Vlahov, D., Goedert, J. J., Kaslow, R., Buchbinder, S., Hoots, K. & O'Brien, S. J. 1999 HLA and HIV-1: heterozygote advantage and B\*35-Cw\*04 disadvantage. *Science* **283**, 1748–1752.
- Clarke, B. & Kirby, D. R. 1966 Maintenance of histocompatibility polymorphisms. *Nature* **211**, 999–1000.
- Edwards, S. V. & Hedrick, P. W. 1998 Evolution and ecology of MHC molecules: from genomics to sexual selection. *Trends Ecol. Evol.* **13**, 305–311.
- Eshel, I. & Hamilton, W. D. 1984 Parent–offspring correlation in fitness under fluctuating selection. *Proc. R. Soc. Lond. B* **222**, 1–14.
- Flajnik, M. F., Ohta, Y., Namikawa-Yamada, C. & Nonaka, M. 1999 Insight into the primordial MHC from studies in ectothermic vertebrates. *Immunol. Rev.* **167**, 59–67.
- Gjedrem, T., Salte, R. & Gjoen, H. M. 1991 Genetic variation in susceptibility of Atlantic salmon to furunculosis. *Aquaculture* **97**, 1–6.
- Gjedrem, T. 2000 Genetic improvement of cold water fish species. *Aquacult. Res.* **31**, 25–33.
- Hamilton, W. D., Axelrod, R. & Tanese, R. 1990 Sexual reproduction as an adaptation to resist parasites (a review). *Proc. Natl Acad. Sci. USA* **87**, 3566–3573.
- Hill, A. V., Allsopp, C. E., Kwiatkowski, D., Anstey, N. M., Twumasi, P., Rowe, P. A., Bennett, S., Brewster, D., McMichael, A. J. & Greenwood, B. M. 1991 Common west African HLA antigens are associated with protection from severe malaria. *Nature* **352**, 595–600.
- Johnsen, B. O. & Jensen, A. J. 1994 The spread of furunculosis in salmonids in Norwegian rivers. *J. Fish Biol.* **45**, 47–55.
- Koppang, E. O., Dannevig, B. H., Lie, O., Ronningen, K. & Press, C. M. 1999 Expression of MHC class I and II mRNA in a macrophage-like cell line (SHK-1) derived from Atlantic salmon, *Salmo salar* L., head kidney. *Fish Shellfish Immunol.* **9**, 473–489.
- Langefors, Å., Lohm, J., von Schantz, T. & Grahn, M. 2000 Screening of MHC variation in Atlantic salmon (*Salmo salar*): a comparison of restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE) and sequencing. *Mol. Ecol.* **9**, 215–219.
- Langefors, Å., Lohm, J., Grahn, M., Andersen, O. & von Schantz, T. 2001 Association between major histocompatibility complex class II B alleles and resistance to *Aeromonas salmonicida* in Atlantic salmon. *Proc. R. Soc. Lond. B* **268**, 479–485. (DOI 10.1098/rspb.2000.1378.)
- Marsden, M. J., Freeman, L. C., Cox, D. & Secombes, C. J. 1996 Non-specific immune responses in families of Atlantic salmon, *Salmo salar*, exhibiting differential resistance to furunculosis. *Aquaculture* **146**, 1–16.
- Medina, E. & North, R. J. 1998 Resistance ranking of some common inbred mouse strains to *Mycobacterium tuberculosis* and relationship to major histocompatibility complex haplotype and Nramp1 genotype. *Immunology* **93**, 270–274.
- Paterson, S., Wilson, K. & Pemberton, J. M. 1998 Major histocompatibility complex variation associated with juvenile survival and parasite resistance in a large unmanaged ungulate population. *Proc. Natl Acad. Sci. USA* **95**, 3714–3719.
- Penn, D. J. & Potts, W. K. 1999 The evolution of mating pref-

- erences and major histocompatibility complex genes. *Am. Nat.* **153**, 145–164.
- Sato, A., Figueroa, F., Murray, B. W., Malaga-Trillo, E., Zaleska-Rutczynska, Z., Sultmann, H., Toyosawa, S., Wedekind, C., Steck, N. & Klein, J. 2000 Nonlinkage of major histocompatibility complex class I and class II loci in bony fishes. *Immunogenetics* **51**, 108–116.
- SPSS 1998 *SPSS*, v. 8.0.0. Chicago, IL: SPSS Inc.
- Takahata, N., Satta, Y. & Klein, J. 1992 Polymorphism and balancing selection at major histocompatibility complex loci. *Genetics* **130**, 925–938.
- Thursz, M. R., Thomas, H. C., Greenwood, B. M. & Hill, A. V. 1997 Heterozygote advantage for HLA class-II type in hepatitis B virus infection. *Nat. Genet.* **17**, 11–12.
- Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J. & Kuiper, M. 1995 AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**, 4407–4414.