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SSCP analysis of *Mhc* class II*B* genes in the threespine stickleback

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Due to its universality, speed, sensitivity, precision and reproducibility, PCR followed by fluorescence SSCP analysis represents an attractive tool for the characterization of *Mhc* class II*B* genotypes and the estimation of DNA sequence variability of *Mhc* genes in natural stickleback *Gasterosteus aculeatus* populations.

Key words: *Mhc*; stickleback; *Gasterosteus aculeatus*; SSCP; capillary electrophoresis; polymorphism.

The threespine stickleback *Gasterosteus aculeatus* L. is a favoured model system in evolutionary biology due to its ecological versatility and highly complex mating behaviour (Bell & Foster, 1994). In the recent past, a number of molecular markers including microsatellites and major histocompatibility complex (*Mhc*) genes have been developed in this organism to facilitate evolutionary, behavioural and genetic studies (Sato *et al*., 1998; Largiadèr *et al*., 1999). The stickleback is an ideal model organism to test whether MHC-dependent mating preferences occur, favouring heterozygosity at *Mhc* loci and overall parasite resistance in the offspring as already postulated for house mice (Penn & Potts, 1999). A prerequisite for such studies is to characterize the *Mhc*-alleles of stickleback sibships or populations using a method which allows large amounts of samples to be assessed in a reasonable time span.

Single-strand conformation polymorphism (SSCP) analysis, first described by Orita *et al*. (1989), is based on the sequence-dependent electrophoretic mobility of single-stranded DNA fragments in nondenaturating polyacrylamide gels. The method is highly sensitive and fast, resolving single nucleotide polymorphisms (SNPs) without the need to sequence the gene. Thus, it is suited for the analysis of *Mhc* genes, especially in *G. aculeatus*, where a very high sequence polymorphism and the occurrence of many loci within the same individual have been described (Sato *et al*., 1998).

The first aim of the present study was to develop a molecular method, based on polymerase chain reaction (PCR) and followed by SSCP analysis, that allows the rapid typing of *Mhc* class II*B* genes in the stickleback. Then it was tested whether the SSCP analysis could be used to track inheritance of individual haplotypes.

The threespine sticklebacks used here originated from rivers and ponds near Bielefeld (Germany). Genomic DNA was extracted from the dorsal spine as described by Walsh *et al.* (1991) with the following modifications: one third of spine (0·5–1 mm in length) was placed in 50 µl Chelex (Biorad) solution (5% w/v in sterile water). The initial incubation

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step was omitted and replaced by a 15 min digest with 1 µl Proteinase K (20 mg ml^{-1}, Boehringer Mannheim). Consensus sequences within the exon 2 of *Mhc* class II*B* genes in the stickleback, based on the study of Sato *et al.* (1998), were used to design a primer pair amplifying most of the polymorphic region and yielding a DNA fragment of 124 bp (corresponding to amino acids 17 to 58). The resulting PCR primers were the following: GaIIExon2F: 5′-CAGCAGCTCAGTGGGGAAG-3′; GaIIExon2R: 5′-GTGGTTCAGACAGTAAACCTCCTTC-3′. These primers do not amplify all the alleles characterized by Sato *et al.* (1998) since the reverse primer is estimated to be specific for 40% of the reported sequences. PCR reactions (10 µl) were carried out in either a Geneamp 2400 thermal cycler (PE Biosystems) or a Biometra Thermocycler and employed: 1 µl of spine extract, 1X buffer (Amplitaq buffer II, PE Biosystems), 0·5 U of Amplitaq Gold polymerase (PE Biosystems), 2·5 mM $MgCl_2$, 200 µM dNTPs, 0·5 µM fluorescent labelled forward primer (5′ label: FAM), 0·5 mM labelled reverse primer (5′ label: HEX). Some PCR reactions were also run using Promega Taq polymerase and buffer system 'B' (PE Biosystems). PCR cycling parameters were the following: 10 min at 95° C (denaturation of the template and activation of the Amplitaq Gold polymerase), 40 cycles (33 when using the Promega Taq) of 95° C (denaturation) for 30 s, 15 s at 56° C (annealing) and 75 s at 72° C (primer extension) followed by a final step of 72° C for 7 min. 1 µl of PCR product was mixed with 9 µl loading mix (for 48 reactions: 396 µl formamide, 12·5 µl of commercial DNA standard ROX 350 (PE Biosystems), 12·5 µl ROX-labelled PCR products of particular alleles used as additional internal standards and 22 µl of 0·3 M NaOH. The mixture was denatured at 95° C for 5 min, snap cooled on ice and analysed by capillary electrophoresis on a ABI 310 Genetic Analyzer (PE Biosystems) using the following conditions: the polymer concentration was 5% (Genescan polymer, PE Biosystems) solubilized in 5% glycerol and 0·5 × TBE. The running buffer consisted of 0·9 × TBE and 10% glycerol. Negative controls were always included. Run conditions were 12 kV for 25 min at 30° C using the short capillary from PE Biosystems (green band, 34 cm). To determine the sequence of individual PCR fragments, unlabelled primers were used also to generate unlabelled products. Bands containing these DNA fragments were extracted from 2% low-melting-point agarose gel (Eurobio) and the DNA was purified using the QiaEXII Gel extraction kit (Qiagen). The eluted DNA was digested with the Klenov fragment of DNA polymerase I to remove nucleotide overhangs at the 3′ end, phosphorylated with T4 polynucleotide kinase and ligated to SmaI-digested pUC18 plasmid vector with the help of the SureClone Ligation Kit (Pharmacia Biotech). The plasmid containing the PCR fragment was then used to transform *Escherichia coli* DH5α competent cells (Gibco). Plasmid DNA (extracted using the Qiagen Plasmid Kit) from individual bacterial clones was sequenced using M13 universal forward and reverse primer on a ABI 377 DNA Sequencer (PE Biosystems). Plasmid DNA containing individual sequences also served as template for PCR with fluorescent labelled primers and subsequent SSCP analysis on the ABI 310 using the above PCR conditions.

Cloning and sequencing of a number of clones showed extensive homology to known stickleback *Mhc* class II*B* genes. Figure 1(a) shows the alignment of a published *Mhc* class II*B* sequence (GADE23,Genbank accession number AJ230199) with four newly characterized genes. SSCP analysis by capillary electrophoresis of these sequences using fluorescently labelled PCR products showed that all of them migrate differentially as compared with an internal standard [Fig. 1(b)]. This is true for both the blue labelled forward strand and the green labelled reverse strand. Therefore, it is possible to attribute a given nucleotide sequence to the particular migration pattern of the labelled PCR-product. In the case of non-synonymous substitutions, the migration pattern is also indicative of the resulting amino acid sequence. Using whole genomic DNA as a template resulted in the occurrence of many different PCR fragments per individual (Fig. 2). The parents and the offspring of two sibships were analysed and only four different genotypes were found per sibship. In the first sibship (n=41) the frequencies of genotypes were 13 : 12 : 10 : 6. In the second sibship (n=33), the frequencies were 8 : 8 : 9 : 8. This suggests that the loci amplified are localized in one linkage group, confirming an earlier observation reported by Sato *et al.* (2000).

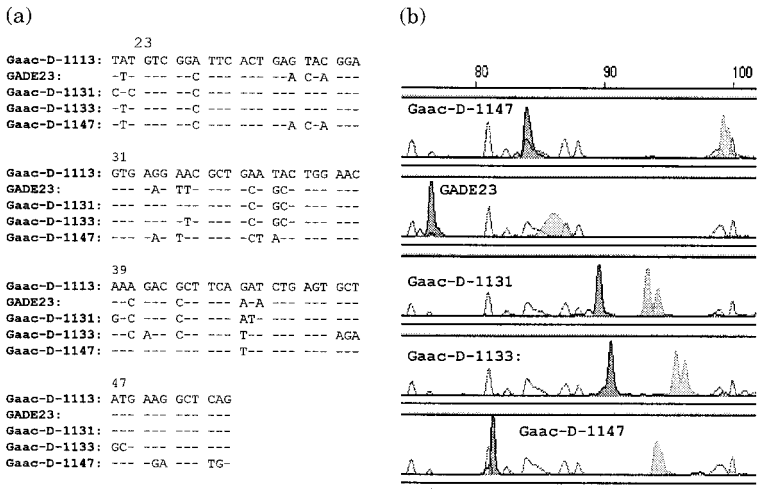

FIG. 1. Comparison of individual (cloned) *Mhc* class II*B* sequences from the threespine stickleback *Gasterosteus aculeatus* by SSCP employing capillary electrophoresis on an ABI 310 Genetic Analyzer and using fluorescently labelled PCR-products. (a) Nucleotide sequences of the PCR fragments amplified with the primers GaIIExon2F and GaIIExon2R (primer sequences excluded). Codons are numbered according to Sato *et al.* (1998). (b) Electropherograms of the sequences computed by the ABI 310. The x-axis gives the relative position of the DNA fragments as compared with the internal standard (in virtual base pairs) which is a combination of the commercial standard mix (ROX-350) combined with ROX-labelled particular PCR products amplified from stickleback DNA. The *y*-axis represents the fluorescent signal in arbitrary units. Open peaks: standard; dark filled peaks: coding strand of *Mhc* class II*B* sequences; light filled peaks: antisense strands of Mhc class II*B* sequences. Genbank accession numbers are: Gaac-D-1113 (AF249303), Ga-D-1131 (AF249304), Ga-D-1133 (AF249305), Ga-D-1147 (AF249306).

It is important to note that, due to the high sequence polymorphism of stickleback *Mhc* class II*B* exons, it was not possible to design one single reverse primer amplifying all the genes described by Sato *et al.* (1998). Therefore, the typing system does provide only a partial picture of the overall variation of *Mhc* genes in the stickleback. SSCP-analysis using fluorescent labelled PCR products and capillary electrophoresis has several advantages over conventional SSCP employing non-denaturing polyacrylamide gel electrophoresis (PAGE) followed by silver staining or radioactive detection. First, due to the high sensitivity of the detection method, only very small amounts of DNA (fmol-range) have to be obtained. Therefore, fewer PCR cycles may be performed, thus reducing nucleotide incorporation errors. Non-invasive sampling becomes feasible because only one third of a spine is needed for DNA extraction. Second, different haplotypes can be distinguished by this method, and the interpretation of the results obtained by capillary electrophoresis with the computer-assisted integration of the fluorescent signal and the use of internal standards is unambigous, easier and less time consuming than PAGE-based methods, especially when working with many similar alleles in one PCR reaction. Third, the method seems to be precise and highly reproducible with only $\pm 0{\cdot}2$ virtual base pair deviation in repeated measurements (virtual base pairs are a measure for electrophoretic retention and are abitrarily defined units calculated from the ROX-labelled internal standard peaks). Fourth, the procedure is essentially a double determination of the unique identity of a sequence since it is very unlikely that both single stranded fragments of two different sequences elute at the same position.

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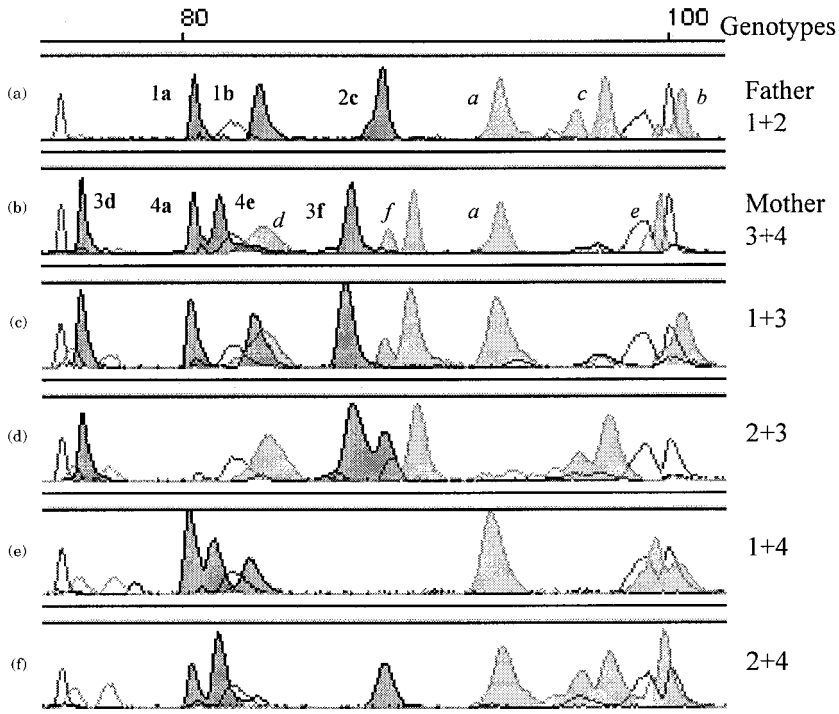

FIG. 2. Inheritance of *Mhc* class II*B* genes in one family of threespine stickleback as revealed by SSCP. (a) Genotype of the father, (b) genotype of the mother; (c)–(d) the four observed genotypes of the offspring (n=41). Open peaks: internal standard (contained only the commercial mix ROX-350); dark filled peaks: coding strand of *Mhc* class II*B* sequences; light filled peaks: antisense strands of *Mhc* class II*B* sequences. Numbers represent the homologous chromosomes. Letters in small caps indicate the individual sequences: bold, coding strand; italic, antisense strand.

References

Bell, M. A. & Foster, S. (1994). *Evolutionary Biology of the Threespine Stickleback*. Oxford: Oxford University Press.

Largiadèr, C. R., Fries, V., Kobler, B. & Bakker, T. C. M. (1999). Isolation and characterization of microsatellite loci from the three-spined stickleback (*Gasterosteus aculeatus* L.). *Molecular Ecology* **8,** 342–343.

Orita, M., Suzuki, Y., Sekiya, T. & Hayashi, K. (1989). Rapid and sensitive detection of point mutations and DNA polymorhisms using the polymerase chain reaction. *Genomics* **5,** 874–879.

Penn, J. D. & Potts, W. K. (1999). The evolution of mating preferences and major histocompatibility complex genes. *American Naturalist* **153,** 145–164.

Sato, A., Figueroa, F., O'hUigin, C., Steck, N. & Klein, J. (1998). Cloning of major histocompatibility complex (Mhc) genes from the threespine stickleback, *Gasterosteus aculeatus*. *Molecular Marine Biology and Biotechnology* **7,** 221–231.

Sato, A., Figueroa, F., Murray, B. W., Málaga-Trillo, E., Zaleska-Rutczynska, Z., Sültmann, 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.

Walsh, P. S., Metzger, D. A. & Higuchi, R. (1991). Chelex 100 as medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques* **10,** 506–513.