Genotyping MHC classIIb in non-model species by reference strand-mediated conformational analysis (RSCA)

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Abstract Major histocompatibility complex (MHC) diversity is described in many species and, in particular, is germane to the conservation of endangered species. However, studies addressing the functional link between individual MHC-variation and, for example, disease susceptibility are underrepresented. This is most likely due to the complex nature of MHC in most species, which hampers reliable individual typing. To date MHC-genotyping by reference strand-mediated conformational analysis (RSCA) has been used in only a few model species where extensive prior information on MHC-gene sequences was available. In those cases, RSCA was found to be a highly accurate and reliable method. Here we adapt this method to two non-model species, Eurasian perch (*Perca fluviatilis*) and Alpine whitefish (*Coregonus* spp.). We show that RSCA can be quickly applied as a flexible genotyping method to any species, even with only limited prior information about its MHC-genes.

Keywords RSCA · Eurasian perch · Whitefish · MHC · Capillary electrophoresis · Heteroduplex analysis

The exceptionally polymorphic major histocompatibility complex (MHC) genes are among the most important candidate genes for adaptive genetic variation. The proteins coded by these genes trigger and control the adaptive immune response in vertebrates and are involved in disease and parasite susceptibility, kin recognition and mate choice (Apanius et al. 1997; Milinski 2003). Thus, methods for

genotyping individuals for MHC are widely applicable for researchers working in the fields of evolution, ecology and conservation (Sommer 2005). However, genotyping of individuals at MHC is more difficult than genotyping at other genes or neutral markers. This is mostly due to the multi-genic appearance of the classical MHC-genes in most species: a general primer set will amplify many different alleles and lead to exhaustive cloning and sequencing. Next generation sequencing (NGS) has recently been praised as a way to solve this problem (Babik 2009), and will most probably replace any two step methods (as e.g. cloning and sequencing) in the near future. However NGS still is not practicable for smaller studies, such as e.g. transcription analyses. Thus, electrophoretic separation of alleles, for example by reference strand-mediated conformational analysis (RSCA), provides a valuable alternative (Lenz et al. 2009; Alcaide et al. 2010). Sequence information can be complemented via single strand conformation polymorphism (SSCP) analysis or directed cloning of specific genotypes. In RSCA, every allele in the PCR amplicon of an individual forms a heteroduplex with a single fluorescently marked allele. The different mobility characteristics of the heteroduplexes in electrophoresis are dependent on the sequence motif of the alleles. RSCA has been successfully applied in MHC-genotyping of several species including fish (e.g. Arguello et al. 2003; Noakes et al. 2003; Worley et al. 2008; Lenz et al. 2009; Cloutier et al. 2011; Strand and Höglund 2011) but has not yet found broad acceptance.

Here we promote RSCA as a genotyping method for the analysis of MHC-genes and display its flexibility and applicability using two non-model species as examples, Eurasian perch (*Perca fluviatilis*) and Alpine whitefish (*Coregonus* spp.). MHC classII-genes of both species are very polymorphic. Whereas at least 3 loci are described for

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whitefish (Binz et al. 2001), we found a minimum of 8 loci in perch (Michel et al. 2009) thus a high number of MHC classII gene alleles may be expressed in a single individual. We show that reference strands can be obtained easily by different ways and that the optimization process is straightforward and time effective. Additional sequence information can be obtained by classical SSCP and sequencing of pre-selected alleles.

We extracted DNA from fin clips of both species by standard salt extraction (Aljanabi and Martinez 1997). For perch, MHC-class-II genes were PCR-amplified with the primers pfluco1 (5' TTG TGT GTT TAA CTC GTC TGA TCT 3') and StviMH5R (Michel et al. 2009). The new forward primer pfluco1 was designed based on a sequence alignment with all MHC classII sequences from perch of our former study (Michel et al. 2009). It was situated in a conservative region downstream of the formerly used forward primer StviMH5F and prevents additional amplification of the first intron, which would prohibit genotyping by RSCA due to its length variability (see Michel et al. 2009 for further details). For reverse primer we decided to restrict to StviMH5R. A preliminary gene expression experiment showed that all alleles amplified with the other two published reverse primers (PfluMH3R and PfluMH4R, Michel et al. 2009) could be amplified only from genomic but not from complementary DNA, and thus may display sets of non-functional genes (data unpublished). This restriction however still allows amplification of MHC classII genes from at least 5 different loci in perch (see Michel et al. 2009). To test the new primer system, 39 perch from our former study (Michel et al. 2009) were reamplified, analysed with RSCA and the results were compared between both studies. For whitefish, PCR amplification was done using the general primers B1RA and B1FA (Binz et al. 2001). PCR was carried out in 20 µl reaction volumes with 1 µl DNA, 1.5 mM MgCl₂, 0.28 µM of each primer (Eurofins MWG), 140 µM of each dNTP (Peqlab) and 0.5U Taq polymerase (Peqlab). Denaturation of 5 min at 94 °C was followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, 1.5 min at 72 °C, with a final elongation of 7 min at 72 °C. PCR-products were purified by ethanol/ammonium acetate precipitation and dissolved in 20 µl water. We used two different methods to obtain MHC-alleles as templates for single fluorescently labelled reference strands (FLRs). Templates were obtained (1) for perch by PCR from minipreps of clones with known sequences. (2) for whitefish by SSCP and reamplification of single bands (Oppelt et al. 2010). Products were sequenced in both directions using the ABI 3130 BigDye Terminator chemistry 3.1. From the single allele sequences gained via one of the two described methods, several were chosen as reference strands and fluorescently labelled by reamplification with a FAM-labelled forward primer (MWG Eurofins). Afterwards, PCR-products were cleaned (Innuprep PCRpure, Analytic

Jena), diluted 1:10, aliquoted and frozen at -20 °C. These can be used for at least 1 year without decline in performance.

For genotyping of individual fish, MHC-genes were amplified with the general primer sets as described above. A mastermix was made of 0.7 µl purified PCR-product, 6.3 µl highly deionised formamide (Applied Biosystems), 0.7 µl of one diluted FLR and 0.07 µl size standard (ABI GeneScan LIZ600). The mixture was heated to 95 °C for 10 min, cooled to 55 °C with 2 °C/s, held at 55 °C for 20 min and immediately placed on ice water for at least 10 min.

RSCA runs were conducted on an ABI 3130 Genetic analyser (4 capillaries) with a 36 cm capillary array with 1× running buffer (ABI). The running conditions were 18 °C, pre-run 180 s at 15 kV, injection 15 s at 15 kV, run voltage at 10 kV for 1,800 s after 650 s delay time. According to Lenz et al. (2009) we used a 5 % conformational analysis polymer (CAP, Applied Biosystems). After each plate (24 runs per capillary) the array was filled with polymer three times at 65 °C, because the low run temperature promotes array contamination.

We analysed several PCR-products with all previously obtained FLRs. Suitable FLRs for further genotyping were chosen based on the following criteria: The peaks in the chromatograms should be of the same magnitude and they should be homogeneously distributed (see e.g. Fig. 1). Chromatograms were analysed in the Genemapper software with the size standard as artificial reference for heteroduplex running properties. Additionally, single allele amplicons were tested with each of the reference strands to identify peaks. In whitefish, individuals with unknown peak sizes were analysed by SSCP analysis, bands were excised and sequenced to identify new alleles and genotype all individuals at the sequence level.

In both species we used two FLRs: in perch pefuDXB01 and pefuDXB08 (accession numbers: FN293126; FN293147), in whitefish cospDXB01 and cospDXB10 (accession numbers: JN029749, JN029758). The run distances measured by the use of the size marker between the FLRs and the different heteroduplexes correlated well with the p-distance between allele sequences calculated in MEGA4, (Tamura et al. 2007) (Fig. 2). This may help to assign peaks to specific sequences when RSCA is initiated in a new species. The absolute number of different MHC classII alleles found for perch was comparable between RSCA and cloning (Michel et al. 2009). We found 15 alleles with cloning and 15 (with pefuDXB08) respective 19 (with pefuDXB01) with RSCA. However, discrepancies in the number of alleles per individual were found. In 34 of the 39 individuals analysed, the number of alleles found was higher, in 3 the same and in 2 lower with RSCA as compared to cloning. This discrepancy can be easily explained by the very low number of clones (on average 5 per individual) that we had analysed in our former study. Thus the majority of the individual variability was not detected with cloning and many more

Fig. 1 MHC classII RSCA peak patterns for individuals of **a** two perch, amplified with both reference strands pefuDXB01 and pefuDXB08 **b** four whitefish, amplified with cospDXB01. The *leftmost* peak (weaker in whitefish) represents the homoduplex (hs). The *rightmost* peak (out of range in whitefish) represents the single stranded fluorescent reference allele (ssFLR). All other peaks

represent alleles that form a heteroduplex with the fluorescently labelled reference (FLR). In perch, peaks are labelled by the artificial size values, because many of the peaks are not assigned to specific sequences yet. In whitefish, peaks are assigned to specific sequences (accession numbers JN029749 JN029765)

clones must be sequenced per individual to use cloning as an individual genotyping method (see also: Strand and Höglund 2011).

We have shown that RSCA is a straightforward, and cost effective method for MHC genotyping of non-model species. It requires only very limited prior information: MHC

Fig. 2 Correlation between electrophoresis run distance (in artificial base pairs) and sequence p distance between target alleles and the FLRs. *Filled dots*: perch samples with FLR pefuDXB01, *open dots*: perch samples with FLR pefuDXB08, *filled triangles*: whitefish samples with FLR cospDXB01, *open triangles*: whitefish samples with FLR cospDXB08 (Spearman rank correlations, perch: $R_s = 0.73$, $p < 0.01$; whitefish: $R_s = 0.88$, $p < 0.01$)

sequences of isolated alleles, e.g. from preliminary cloning and sequencing studies, and general primers. Many species, including non-model species, would meet these requirements. Thus we are convinced that RSCA allows for a growth of studies that combine MHC analysis with, for example, conservation issues, mate choice or parasite host interaction.

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