Chromosomal location: The porcine *IGF2* gene has been mapped to chromosome 2p1.7¹

Acknowledgements: The authors are grateful to Professor Dr. H. Geldermann for providing DNA samples from the pedigree material generated by the University of Hohenheim (Stuttgart, Germany). This work was supported by Grant Agency of the Czech Republic (523/99/0842, 523/99/P043) and EC (ERBIC15T960902).

References

- 1 Nezer, C. *et al.* (1999) *Nat Genet* **21**, 155–7.
 - 2 Jeon, J.T. *et al.* (1999) *Nat Genet* **21**, 157–8.
 - 3 Catchpole, I.R. *et al.* (1990) *Nucleic Acids Res* **18**, 6430.
 - 4 Dull, T.J. *et al.* 1984 *Nature* **310**, 777–81.
 - 5 Geldermann, H. *et al.* (1999) *Arch Tierzucht, Dummerstorf* **42**, 67–81.
- Correspondence: S. Čepica (e-mail: cepica@iapp. cas. cz).

Broad taxonomic applicability of microsatellites developed for the highly polymorphic neotropical cichlid, *Amphilophus citrinellum*

K Noack¹, A B Wilson², A Meyer^{1,2}

¹Department of Ecology and Evolution, State University of New York, Stony, Brook, New York, 11794–5245, U.S.A.; ²Department of Biology, University of Konstanz, D-78457, Konstanz, Germany

Accepted 10 November 1999

Neotropical cichlids are some of the most important food fishes of Central America¹. In addition to its important economic role as part of the freshwater fishery, the Midas cichlid, *Amphilophus citrinellum*, exhibits a high level of intraspecific variation in both coloration¹ and pharyngeal jaw morphology², which has made it a model species for the study of incipient, possibly, sympatric speciation^{2,3}. While behavioural and ecological studies of *A. citrinellum* have been numerous and thorough, investigations of the genetic variation of this species are almost completely missing. We have previously found exceedingly low levels of variation in the mitochondrial control region and cytochrome *b* gene which suggests a recent origin of the species (Meyer *et al.* unpublished data).

While a suite of microsatellite loci have been developed for African cichlids, these microsatellite primer sets have proven largely ineffective in amplifying Neotropical species^{4,5,6}. In this study, we describe the identification of six di- and tri-nucleotide microsatellite loci in *A. citrinellum* that can also be amplified in many species of cichlids both from the Neotropics and the Old World.

Genomic DNA was extracted from a single *A. citrinellum* specimen collected from Lake Nicaragua using a previously published

extraction protocol⁷. *Eco*R1-digested DNA fragments were ligated to pUC18 (Gibco BRL) and transformed into SURE cells (Stratagene). The resulting library was screened with a [γ -³²P]-ATP end-labeled (GT)₁₀ oligonucleotide using standard hybridization techniques. Twenty-five positive clones of 300–1000 bp were sequenced using a *Taq* Dye-Deoxy Termination Cycle Kit (ABI – following manufacturer's recommendations) and analyzed with an ABI 373 Stretch DNA automated sequencer. Primer sets were developed for six of the 25 clones containing GT-microsatellites and adequate single-copy flanking DNA for primer design.

Amplification of the six microsatellite loci was carried out in a GeneAmp 9700 Thermocycler (ABI) using 25 μ l reaction volumes (Tris 67 mM, pH. 8.8; 1.5 mM MgCl₂; 0.4 mM of each dNTP; 75 ng of each primer and one unit of AmpliTaq DNA Polymerase (Perkin-Elmer Cetus)). Forward primers were labelled with tetrachloro-6-carboxyfluorescein (TET). Amplification reaction conditions consisted of an initial denaturing step of 3 min at 94 °C followed by 30 cycles of 94 °C for 1 min, an optimised annealing temperature (see Table 1) for 30 s, and 72 °C for 1 min. PCR products were visualised on agarose gels stained with ethidium bromide and diluted according to their strength. One microlitre of each sample was then mixed with 2 μ l formamide and 0.5 μ l each of size standard (GeneScan TAMRA-500, Applied Biosystems) and TAMRA buffer. The samples were denatured at 98 °C for two minutes, loaded on a 5% denaturing 19 : 1 acrylamide:bisacrylamide gel and analysed using an ABI 373 A Stretch Automated Sequencer. Allele sizes were determined by the GeneScan software (Perkin Elmer) based on comparison of migration distances with the TAMRA ladder fragments of known size within each lane.

The six microsatellite loci were amplified in a total of 140 *A. citrinellum* individuals from four lacustrine populations in Nicaragua. Levels of intraspecific variation varied considerably amongst loci, with allele numbers at each locus ranging from 1 to 26 and observed heterozygosity ranging between 0.000 and 0.664.

In addition to a high overall level of intraspecific variation, these six microsatellite loci have also proven useful in a broad taxonomic array of cichlid species (Table 2). In contrast to the majority of microsatellites identified in African species that fail to amplify Neotropical cichlids⁵, the present six microsatellite loci amplify both Neotropical and Old World species. These markers may prove effective in a further taxonomic clarification of relationships between New World and Old World cichlids.

The high intraspecific variation of these microsatellites makes them ideally suited to a detailed molecular investigation of observed anatomical and behavioural polymorphism in *A. citrinellum* and to molecular characterisation of wild stocks of the species. At the same time, the broad taxonomic applicability of these markers offers an opportunity to further examine evolutionary questions related to the rapid speciation of cichlid fishes.

Table 1 Primer sequences and core repeat structure for *Amphilophus cichlasoma* microsatellites. All loci were tested on 140 individuals from four lacustrine populations in Nicaragua

Locus	No. of alleles	H _O	H _E	Primer sequence (5'–3')	Cloned repeat motif	Size of sequenced product (bp)	Annealing temperature (°C)
<i>Acit1</i>	6	0.101	0.487	F AAA TGA GTT CAG CGA TGG CTG AG R TGC ACA TCA TGT CCG CCG AAC A	(AG) ₁₁	168–174	49
<i>Acit2</i>	26	0.593	0.926	F GGC ACT GAG GAT TTA TAT TAC AGG R GAG GTC CAG CTG AGA ACA GGG	(GT) ₃₅	184–232	52
<i>Acit3</i>	18	0.664	0.905	F CTT AAG GTG TAC CTG CTT AGC R GAG TGG GAA GAC AGA TGT TGA GG	(GT) ₃₂	161–195	51
<i>Acit4</i>	15	0.593	0.829	F CCT TCC TAC TAG TTA GTC TTT CAC R CAC ATA GCA CAG TGC ATT CAC CC	(GT) ₂₂	347–375	49
<i>Acit5</i>	1	0.000	0.000	F GCC GCA CCC TCA TTA TCC TCA C R GTG ACT CCA ACG TGT AGC TTC C	(AGC) ₈	157	52
<i>Acit6</i>	1	0.000	0.000	F GAA TTC ACA AAG GCC AAT CCT AC R GGA TAC TGA GCA TGA CAA TAA GC	(CA) ₉ (TA) ₁₆ (TG) ₁₈ (TTA) ₃	268	50

Genbank accession numbers: AF237713–AF237718.

H_O = observed heterozygosity; H_E = expected heterozygosity.

Acknowledgements: We thank Rafael Zardoya for technical assistance with the preparation of the genomic library. This work was partially supported by grants from the U.S. National Science Foundation (BSR-9107838, BSR-9119867, DEB-9615178) the University of Konstanz, the DFG, the Fond der Chemischen Industrie to AM and a NSERC PGSB scholarship to ABW.

Table 2 Success of cross-species amplification of *Amphilophus citrinellum* microsatellite loci

	<i>Acit1</i>	<i>Acit2</i>	<i>Acit3</i>	<i>Acit4</i>	<i>Acit5</i>	<i>Acit6</i>
Neotropics						
<i>Amphilophus citrinellum</i>	+	+	+	+	+	+
<i>Cichla cichla</i>	+	+	+	+	+	+
<i>Crenicichla saxatilis</i>	+	+	+	+	?	+
East Africa						
<i>Astatoreochromis alluaudi</i>	+	–	+	+	+	+
West Africa						
<i>Hemichromis bimaculatus</i>	+	–	+	–	–	+
Madagascar and India						
<i>Etroplus maculatus</i>	–	+	–	+	?	–

(+) product; (–) no obvious product (?) product of questionable size.

References

- Barlow, G.W. (1976) The Midas cichlid in Nicaragua. In: *Investigations of the Ichthyofauna of Nicaraguan Lakes* (ed. Thorson, T.B.), pp. 333–58 University of Nebraska Press, Lincoln, NB.
- Meyer, A. (1990) *Biol J Linnean Soc* **39**, 279–99.
- McKaye, K.R. (1980) *Environ Biol Fishes* **5**, 75–8.
- Kellogg, K.A. *et al.* (1995) *Proc R Soc Lond Series B* **260**, 79–84.
- Zardoya, R. *et al.* (1996) *Proc R Soc Lond Series B* **263**, 1589–98.
- Van Oppen, M.J.H. *et al.* (1997) *Mol Ecol* **6**, 387–8.
- Karl, S.A. & Avise, J.C. (1993) *Mol Biol Evolut* **10**, 342–61.

Correspondence: Axel Meyer (e-mail: axel.meyer@uni-konstanz.de)

Two polymorphic markers for the horse *SLC11A1* (*NRAMP1*) gene

P Hořín, J Matiašovic

Institute of Animal Breeding and Genetics, Faculty of Veterinary Medicine, Palackého 1/3, 612 42 Brno, Czech Republic

Accepted: 15 November 1999

Source/description: Primers (EqNRAMPF and EqNRAMPR), based on two conserved sites within the coding regions of the exons 5 and 6 of the human¹, bovine², sheep³, and porcine⁴ *NRAMP1* gene, now known as *SLC11A1*, were designed and used to amplify a product of expected size of 547 bp (PCR I). Individual samples of genomic DNA from 8 different animals of 4 different breeds (Warmblood, Old Kladruher, Arab, and Shetland Pony) were used for the PCR. The PCR products were cloned into the pCR TOPO vector (Topo TA Cloning Kit, Invitrogen, USA), and sequenced with an ABI310 Automatic Sequencer. Analysis of 11 PCR I clones obtained from 8 different animals of 4 different breeds (Warmblood, Old Kladruher, Arab, and Shetland Pony) revealed the existence of a DNA sequence homologous to exons 5 and 6 of the *SLC11 A1* gene of other species. Based on alignments with Genbank sequences reported for man, cattle, sheep and pig (Genbank accession numbers L32185, U12862, AF005380, and U55068, respectively)^{1–4}, the intron boundaries and the corresponding parts of exons 5 and 6 could be identified. The horse sequence showing a high level of sequence identity with other species was therefore considered part of the putative horse *SLC11 A1* gene. In all 11 clones analysed, the partial horse exon sequences were identical. The amino acid position corresponding to the nucleotide 169 substitution in intracellular parasite resistant or susceptible mouse inbred strains⁵ is occupied by a glycine in the horse, identical to the resistant mouse allele and all other species examined so far. Two allelic exon 5–intron–exon 6 sequences,

confirmed from at least two independent PCRs, were identified in related animals (Genbank AF163297, and AF163298). Therefore, another pair of oligonucleotides (I5ENRF and I5ENRR), amplifying the polymorphic region was designed (PCR II). The PCR II product of expected size (250 bp) was obtained.

Primer sequence:

PCR I

Primer EqNRAMPF: 5'–GCATTCTCTCTGGCTGACC–3'

Primer EqNRAMPR: 5'–CGAGGAAGAGGAAGAAGAARGTGT–3'

PCR II

Primer I5ENRF: 5'–CAATCTGCTCTCAGCTGGACGGTAC–3'

Primer I5ENRR: 5'–GATGGCTGCGTGACTGACTT–3'

PCR conditions: Both PCRs were performed in a total volume of 12.5 µL. The PCR I mixture consisted of: 100 ng genomic DNA, 50 pmol of each primer (EqNRAMPF, EqNRAMPR), 0.2 mM of dNTPs, 1.5 mM MgCl₂, 0.5 U of *Taq* polymerase with 10× reaction buffer, and acetamide added to a final concentration of 5%. Reactions were run on an MJ Research DNA thermocycler (Watertown, MA) with a hot start of 2 min at 94 °C (Promega *Taq* Polymerase). The conditions for amplification were 30 cycles of 30 s at 94 °C, 1 min at 62 °C, and 90 s at 72 °C. The program was completed by a final extension of 30 min at 72 °C followed by cooling the reaction mixture to 4 °C. The long extension time was needed for subsequent TA cloning of the PCR product.

The PCR II mixture contained: 50 ng DNA, 20 pmol of each primer (I5ENRF and I5ENRR), 0.2 mM of dNTPs, 1.5 mM MgCl₂, 0.5 U of *Taq* polymerase. The temperature cycling was carried out by an initial denaturation of 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 65 °C, then 30 s at 72 °C, with a final extension of 10 min at 72 °C and cooling to 4 °C.

Polymorphisms: The 250 bp PCR II product was digested with two restriction enzymes, *MnII* and *MspI*, selected according to the nucleotide sequence polymorphisms previously identified. The gels were run at 300 V for 30 min on a 6% polyacrylamide gel (37.5 : 1), and silver stained. Two bi-allelic RFLPs were identified by analysis of sequenced clones as well as of genomic DNA. We designated the allelic variants *MnII.1*, *MnII.2*, *MspI.1*, and *MspI.2*, respectively. The *MnII.1* and *MnII.2* alleles differ by absence and/or presence of a T within a polyC array, while the *MspI.1* and *MspI.2* alleles differ by presence and/or absence of an A within the same array, respectively. Informative RFLP fragments for individual alleles were 48, 95 and 137 bp for *MnII*, and 89, 112, and 201 bp for *MspI*, respectively. Additional small fragments were omitted. The polymorphisms detected were accompanied by a length variation of the polyC region which allowed, nevertheless, a reliable identification of the informative fragments. Homozygous and heterozygous animals were detected, and Mendelian inheritance in 2 families was confirmed (data not shown). In 27 unrelated animals of the Old Kladruher breed allele frequencies for *MnII.1* and *MnII.2* were 0.83 and 0.17, respectively, while the frequencies of alleles *MspI.1*, and *MspI.2* in the same animals were 0.48 and 0.52, respectively. Family RFLP analysis confirmed existence of the two haplotypes (*MnII.1-MspI.2* and *MnII.2-MspI.1*) identified by sequencing, and revealed existence of another haplotype, *MnII.1-MspI.1*.

Acknowledgements: The authors thank Dr A. Stratil for his helpful comments. Technical assistance of Mrs. E. Hantáková and Mrs. V. Nováková is acknowledged. This work was supported by the grants of the Grant Agency of the Czech Republic 523/99/1152 and of the Internal Agency of the UVPS Brno 22/98.

References

- Cellier, M.F. *et al.* (1994) *J Exp Med* **180**, 1741–52.
- Feng, J. *et al.* (1996) *Genome Res* **6**, 956–64.
- Matthews, G.D. & Crawford, A.M. (1998) *Anim Genet* **29**, 1–6.
- Tuggle, C.K. *et al.* (1997) *J Anim Sci* **75**, 277.
- Vidal, S.M. *et al.* (1993) *Cell* **73**, 469–85.

Correspondence: P Hořín.