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Isolation and characterization of 13 new nine-spined stickleback, *Pungitius pungitius*, microsatellites located nearby candidate genes for behavioural variation

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Nine-spined stickleback (*Pungitius pungitius*) is a good model species for studying personality traits such as boldness and aggression as large variation in these behaviours has been observed in populations from different habitats. Here we used genomic information from three-spined sticklebacks to identify and characterise 13 new nine-spined stickleback microsatellite loci which are located close to candidate genes for behaviour. All loci were polymorphic with 3–31 alleles per locus and expected heterozygosity varied from 0 to 0.97 and observed heterozygosity from 0 to 1. These markers should provide a useful resource for better understanding the genetic basis of behaviour in stickleback fishes.

Individual and population-level differences in behavioural traits such as aggression and boldness are suggested to be widespread in the animal kingdom and to have important fitness consequences (e.g. Sih *et al*. 2004a, 2004b, Reale *et al*. 2007). Despite their potential ecological and evolutionary significance, the underlying genetic bases of such behaviours have rarely been studied in free-living populations. However, recent studies have demonstrated how knowledge of genes that have been demonstrated to be associated with personality traits in "model" organisms (including humans) can be utilized for studying the genetic basis of behavioural variation in wild animal populations through the use of a 'candidate gene approach' (Fidler *et al*. 2007).

Nine-spined sticklebacks (*Pungitius pungitius*) are a good model species for behavioural ecology studies because fish from different habitats in Fennoscandia exhibit differing behavioural averages, with fish from isolated pond populations being quicker in feeding, bolder and more aggressive than individuals from marine environments (Herczeg *et al*. 2009, Herczeg and Välimäki 2011). In addition to these features, nine-spined sticklebacks are amenable to controlled experiments and there is access to detailed genome sequence information (http://

www.ensembl.org/Gasterosteus_aculeatus/Info/Index) from a related species, the three-spined stickleback (*Gasterosteus aculeatus*). These two lineages have diverged more than 10 million years ago which is equivalent to 5–10 millions of generations (Baker 1994, Bell 1994).

Earlier studies have demonstrated that molecular markers located within or nearby target candidate genes can be a useful resource for the identification of genes associated with adaptive phenotypic divergence especially when there is no other genomic resources available (Shikano *et al*. 2010a, Tonteri *et al*. 2010). In addition, markers closely linked to functionally important genes are useful in construction of comparative genetic maps, in which they can be used as comparative anchor-tagged sequence loci (Lyons *et al*. 1997). Here we report 13 new polymorphic microsatellite loci for nine-spined sticklebacks. These loci are located near genes that have earlier been shown to be associated with behavioural variation in other species.

Microsatellite markers were designed using a candidate-gene approach (Fitzpatrick *et al*. 2005, Shikano *et al*. 2010b). Genes earlier shown to be associated with aggression and boldness were identified using data available from literature on human, mouse and domestic-animal genetics (Table 1). Altogether 51 candidate genes associated with aggression and boldness variation were identified and homologues were then identified by using the gene name or the homologue sequence for all these genes in the three-spined stickleback genome obtained from Ensembl (Genebuild January 2009, database version 56.1j/57.1j).

Candidate genes and genomic regions closely linked to the genes (< 10 kb) were screened for microsatellite repeats using the Phobos 3.3.11 software (http://www.ruhr-uni-bochum.de/spezzoo/cm/cm_phobos.htm) included in Geneious ver. 5.4 (http://www.geneious.com/) where repeat unit length was set from one to four. Homologous gene sequences from additional teleost fish species such as medaka (*Oryzias latipes*), fugu (*Takifugu rubripes*) and zebrafish (*Danio rerio*) were aligned with three-spined stickleback gene sequences in order to identify conserved sequence regions flanking the microsatellite repeat region. Primers were designed manually so that GC content was 45%–60%, the primer melting temperature was approximately 60 °C and the length of the primers was 20–26 bp. The aim was for the primers to amplify the microsatellite region with a fragment size of approximately 800 bp. In the three-spined stickleback genome sequence, suitable microsatellite regions were found in a total of 30 genes and in some cases multiple primers were designed for the microsatellite region, or for different microsatellites nearby the same gene. Genomic DNA from nine-spined stickleback individuals from Pyöreälampi, Finland (66°15´N, 29°26´E) were used for testing of primers by amplifying and sequencing the target regions while DNA from one three-spined stickleback individual caught in the Baltic Sea near Helsinki (60°10´N, 25°00´E) was used as a positive control. DNA was extracted from fin samples by using a modified salt extraction protocol of Aljnabi and Martinez (1997).

PCR reactions were carried out in a 20 μl reaction volume consisting of 1× PCR buffer (Bioline, London, UK), 1.5 mM $MgCl_2$, 0.25 mM dNTP (Finnzymes, Espoo, Finland), 0.1 U BIOTAQ DNA polymerase (Bioline, London, UK), 10 pmol of each primer and approx. 30 ng of genomic DNA. The initial touchdown PCR profile was as follows: initial degeneration step at 95 °C for 3 minutes (min), followed by 20 cycles of denaturation for 30 seconds (s) at 95 °C, annealing starting at 60 °C for 30 s and dropping by 0.5 °C per cycle and extension at 72 °C for 1 min, followed by 20 cycles of 30 s denaturation at 94 °C and 30 s annealing at 50 °C and extension at 72 °C for 1 min with a final extension at 72 °C for 6 min. The PCR product profiles for specific loci were then optimised by either increasing or decreasing the annealing temperature. The success of PCR amplification and the size of the amplicons were determined by electrophoresis on 1.5% agarose gel with a DNA ladder (GeneRuler™ DNA Ladder Mix, Fermentas, Helsinki, Finland). PCR products deemed suitable for sequencing were prepared using exonuclease I (Fermentas, Helsinki, Finland) and shrimp alkaline phosphatase (Fermentas, Helsinki, Finland) and sequenced directly in both forward and reverse directions with the primers used in the PCRs. The sequencing reactions were performed in 10 μl volumes using a

Table 1. Microsatellite loci for the nine-spined stickleback, including adjacent gene name (abbreviation) and a citation where association with a personality trait has been reported, Genbank accession number, primer sequences, the dye used, repeat motif, total number of alleles (Total A), size range (base-pairs) and amplification quality rating.

Locus	Gene name	Prior behaviour association	Genbank	Primer sequence[1] (5´–3´)	Dye	Repeat	Total A	Size (bp)	Quality rating[2]
Ppbig1	Androgen receptor (*AR*)	Dominance in fish (Burmeister *et al.* 2007)	JQ012804	F- AGTGAGGATTCGGAGGGCCA R- GTTTCTGAGCAGGGAGAGGCACGA	NED	$(AC)_{12}$	5	172–182	little stutter
Ppbig2	Cholecystokinin B receptor (*CCK-B*)	Anxiety in rats (Wang *et al.* 2005)	JQ012805	F- CCCATCGCAACGGCAGAGCA R- GTTTGGTGCGCGTGCCCAGATTT	FAM	$(AT)_{11}$	5	279–293	little stutter
Ppbig3	Corticotropin-releasing hormone (*CRH*)	Aggression in fish (Filby *et al.* 2010)	JQ012806	F- TCCCCTGCGTGCATTGTTTGG R- GTTTCGTTAGTGCAACCGGGCTGT	VIC	$(C)_9$	5	276–282	moderate stutter
Ppbig4	Dopamine transporter (*DAT1*)	Aggression in humans (Chen *et al.* 2005)	JQ012807	F- ACCTGGATCCTCCGTTTGGGC R- GTTTCGCACTCTGTACCCGTGGCT	VIC	$(C)_7ACA(C)_4$	3	345–348	no stutter
Ppbig5	Dopamine receptor D_1 (*DRD1*)	Aggression in dogs (Våge *et al.* 2010)	JQ012808	F- CACAAACACAGGAGGACGGA R- GTTTCCGAGGCACGATGAGGCCAT	PET	$(AC)_{43}$	28	280–384	little stutter
Ppbig6	Dopamine receptor D_4 (*DRD4*)	Novelty seeking in birds (Fidler *et al.* 2007)	JQ012809	F- CTGCGGACCACCTTGACACC R- GTTTCGCCGCGGAAGCATTCCTCC	FAM	$(A)_{23}$	15	335–354	moderate stutter
Ppbig7	Estrogen receptor beta (*ESR2*)	Anxiety in rats (Lund *et al.* 2005)	JQ012810	F- GCCGCCGGAGATCCTCCTCA R- GTTTGTGTCTGGCGTCGCATCGG	NED	$(A)_7$	4	281–301	no stutter
Ppbig8	5 hydroxytryptamine receptor 3B (*HTR3B*)	Antisociality in humans (Ducci *et al.* 2009)	JQ012811	F- GGACCATGCAATCCACACAAACAA R- GTTTCCTCCACAGCGCCAGGTCTA	VIC	$(AC)_{11}$	10	148–170	no stutter
Ppbig9	Monoamine oxidase A (*MAOA*)	Aggression in humans (Brunner *et al.* 1993)	JQ012812	F- TAAACCGCGTGATTGTGTGC R- GTTTAAGAAACGGGGAGGTGAGG	FAM	$(C)_{15}$	6	94–107	moderate stutter
Ppbig10	Progesterone receptor (*PGR*)	Panic disorder in humans (Ho et a. 2004)	JQ012813	F- TCCGGATGACTCACTCTTCAC R- GTTTCAAAGTTCTCCTGTCGGTGTACCA	PET	$(AC)_{16}$	31	139–255	little stutter
Ppbig11	Protein Kinase G (*PKG*)	Food related behaviour (Osborne *et al.* 1997)	JQ012814	F- CGGCTGTCTATGGCGCATCT R- GTTTCTGTCCCGCTCTTTGACCCTT	NED	$(AC)_{18}$	13	395–419	little stutter
Ppbig12	Excitatory amino acid transporter 2 (*SLC1A2*)	Activity in dogs (Takeuchi *et al.* 2009)	JQ012815	F- TCCCAGAGAACTTGGTGCAGGCT R- GTTTGCCACTCACTTGACGATTGTATGTC	FAM	$(C)_{16}$	9	152–162	little stutter
Ppbig13	Serotonin transporter (*SLC6A4*)	Aggression in rats (Holmes *et al.* 2003)	JQ012816	F- CAGGACGTGGGTGCTGTGGC R- GTTTACCAGGGTGCAATTGTTGTGAGC	NED	$(AAC)_6$	3	104–110	no stutter

[1] the GTTT 'tail' (underlined) was added to the 5´ end of each non-labelled primer to enhance 3´OH A addition to the forward strand (Brownstein *et al.* 1996).
[2] *see* Primmer & Merilä 2002.

BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Espoo, Finland) according to manufacturer's instructions. Obtained sequencing products were cleaned with a Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore, Espoo, Finland). Sequencing products were visualised with an ABI 3130xl DNA Analyzer (Applied Biosystems, Espoo, Finland).

Sequences were aligned and edited in the Geneious analysis package using both forward and reverse sequences. BLASTN searches against the three-spined stickleback genome were used to ensure that the sequences obtained were homologous to the expected regions. In all cases, BLASTN also revealed similarity with homologous gene regions in more distantly related fish species such as zebrafish and therefore it is likely that the markers are linked to similar gene regions in nine-spined sticklebacks. Primer3 (Rozen 2000: source code available from http://fokker.wi.mit.edu/primer3/) included in Geneious was used to design primers generating a product of 100–500 bp. Fragment lengths were selected so as to optimise the number of markers with non-overlapping size ranges. Subsequent PCR optimisation and polymorphism detection was conducted using genomic DNA from eight individuals from two different populations. The final panel of 13 polymorphic markers was then optimised to enable amplification in a single multiplex PCR reaction that included 1× Qiagen Multiplex PCR Master Mix, 0.08 μM fluorescent labelled forward primer, 0.08 μM reverse primer (for *ppbig11* the concentration for forward and reverse was 0.1 μM), approx. 30 ng of genomic DNA, and dH_2O to a final volume of 7 μl. The thermal cycling program for the multiplex was: 95 °C for 15 min; 35 cycles of 94 °C for 30 s, 61 °C for 1 min 30 s, 72 °C for 1 min 30 sec, followed by 60 °C for 30 min. PCR products were subsequently diluted by adding 5 μl of water to the PCR wells and then diluting 1:200 and electrophoresed using an ABI3130xl Genetic Analyzer with GeneScan-600 LIZ size standard (Applied Biosystems, Foster City, CA). Genotypes were scored using GeneMapper 4.0 (Applied Biosystems, Foster City, CA). Genetic diversity levels were determined by genotyping 10 putatively unrelated individuals from each of four populations: two sampled from small ponds, Bynästjärnen (64°27´N, 19°26´E, BYN) and Pyöreälampi (PYÖ) and two sampled from the marine environment, Helsinki (HKI) and Levin Navolok, White Sea (66°18´N, 33°25´E, LEV). A map indicating the population locations can be found in Herczeg *et al*. (2009).

Genetic diversity indices were calculated using the MICROSATELLITE TOOLKIT EXCEL add-in (Park 2001) and tests for Hardy-Weinberg and linkage equilibrium were conducted using GENEPOP ver. 4.0 (Raymond & Rousset 1995, Rousset 2008). Sequences for all loci reported have been submitted to Genbank (Accession numbers JQ012804-JQ012816).

Mono-, di- or trinucleotide repeat sequences were identified in 22 of the 30 regions amplified and one to three primer pairs were designed in efforts to amplify these 22 loci from nine-spined sticklebacks genomic DNA. Fourteen of the 22 loci revealed length polymorphisms in nine-spined stickleback's DNA. However one of these loci proved to PCR-amplify unreliably and a total of 13 loci were optimised to be PCR amplified in a single PCR reaction (Table 1).

After Bonferroni correction (Rice 1989), all loci conformed to Hardy-Weinberg expectations in every population. None of the 13 loci was in linkage equilibrium with each other. Across all four populations, the number of alleles observed per locus ranged from 3 to 31. At the population level, observed heterozygosities ranged from 0 to 0.90 (6 loci > 0.5) in BYN, from 0.50 to 1.0 (13 loci > 0.5) in HKI, from 0.20 to 1.0 (11 loci > 0.5) in LEV and from 0 to 0.60 (1 locus > 0.5) in PYÖ (Table 2). The lower levels of observed heterozygosities in populations from pond habitats (BYN, PYÖ) when compared with those from marine habitats (HKI, LEV) were also found in earlier studies (Shikano *et al*. 2010c). Some cross-species testing was conducted using three-spined stickleback genomic DNA and at least two of the markers (*Ppbig1* and *Ppbig5*) revealed polymorphisms (data not shown). These markers add to the anonymous markers and are linked to physiologically important genes currently available for nine-spined sticklebacks (Koizumi *et al*. 2007, Meguro *et al*. 2009, Shapiro *et al*. 2009, Shikano *et al*. 2010b). Overall, these markers should provide a useful resource for better understanding the genetic basis of behaviour in sticklebacks.

Table 2. Population specific diversity indices for four nine-spined stickleback populations (n = 10 per population): number of alleles (A), observed heterozygosity (HO), expected heterozygosity (HE), P-value for deviation from Hardy-Weinberg equilibrium (H-W) (prior to Bonferroni correction).

	BYN				HKI				LEV				PYÖ			
Locus	A	HO	HE	H-W*	A	HO	HE	H-W	A	HO	HE	H-W	A	HO	HE	H-W*
Ppbig1	2	0.1	0.27	0.16	3	0.5	0.58	0.45	4	0.5	0.65	0.01	2	0.4	0.34	1
Ppbig2	2	0.2	0.19	1	4	0.6	0.77	0.9	3	0.4	0.64	0.21	2	0.2	0.19	1
Ppbig3	2	0.6	0.51	1	5	0.9	0.77	0.51	2	0.5	0.39	1	1	0	0	–
Ppbig4	1	0	0	–	3	0.7	0.56	0.74	2	0.2	0.19	1	1	0	0	–
Ppbig5	4	0.6	0.68	0.74	14	1	0.95	1	15	1	0.97	1	3	0.4	0.56	0.31
Ppbig6	2	0.6	0.44	0.48	12	1	0.95	1	9	1	0.83	0.67	3	0.4	0.54	0.68
Ppbig7	3	0.5	0.59	0.24	4	0.6	0.6	0.84	3	0.9	0.69	0.51	1	0	0	–
Ppbig8	1	0	0	–	7	0.9	0.82	0.9	7	0.6	0.77	0.23	2	0.3	0.27	1
Ppbig9	2	0.3	0.27	1	6	0.9	0.8	0.8	3	1	0.57	0.01	2	0.2	0.19	1
Ppbig10	6	0.9	0.78	0.99	15	1	0.97	1	15	0.9	0.96	0.37	5	0.6	0.62	0.57
Ppbig11	3	0.6	0.53	1	6	0.7	0.66	0.62	9	0.7	0.79	0.12	2	0.1	0.1	–
Ppbig12	1	0	0	–	6	0.7	0.62	0.82	7	0.9	0.85	0.95	1	0	0	–
Ppbig13	1	0	0	–	3	0.6	0.57	0.03	3	0.7	0.65	0.11	1	0	0	–
Average	2	0.34	0.33	0.73	7	0.78	0.74	0.74	6	0.72	0.69	0.48	2	0.20	0.22	0.79

* results indicated with '–' are either monomorphic or loci with very low heterozygosity, hence the H-W value could not be calculated.

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