Ann. Zool. Fennici 44: 218–224 ISSN 0003-455X
Helsinki 21 June 2007

Cross-species amplification of microsatellite loci for nine-spined stickleback *Pungitius pungitius*

Hannu. S. Mäkinen, Kaisa Välimäki & Juha Merilä*

*Ecological Genetics Research Unit, Department of Biological and Environmental Sciences, P.O. Box 65, FI-00014 University of Helsinki, Finland (*corresponding author's e-mail: juha.merila@helsinki.fi)*

Received 12 Jan. 2007, revised version received 21 Feb. 2007, accepted 1 Mar. 2007

Mäkinen, H. S., Välimäki, K. & Merilä, J. 2007: Cross-species amplification of microsatellite loci for nine-spined stickleback *Pungitius pungitius*. — *Ann. Zool. Fennici* 44: 218–224.

Microsatellite loci of nine-spined stickleback (*Pungitius pungitius*) were optimized using primers originally designed for the three-spined stickleback (*Gasterosteus aculeatus*). Of the 57 three-spined stickleback loci tested, only 18 loci (32%) amplified a specific PCR product in the nine-spined stickleback. Further analysis for two Fennoscandian populations revealed 11 polymorphic and six monomorphic loci. The eleven polymorphic markers were optimized into two ready-to-go genotyping panels to facilitate genotyping applications, and these markers should prove useful for population genetic studies in the nine-spined stickleback. Comparison of polymorphism in the 11 loci between three- and nine-spined sticklebacks collected from the same two (lake or sea) localities revealed significantly lower polymorphism in the nine- than in the three-spined sticklebacks, and in the lake than in the sea populations of both species. Moreover, loss of polymorphism in the lake population was especially pronounced for the nine-spined stickleback (target species) as compared to the three-spined stickleback (source species). This suggests that the success of cross-species amplification may, in addition to well-known effects of e.g. species evolutionary divergence, depend on population history.

The nine-spined stickleback (*Pungitius pungitius*) is a sister species to the three-spined stickleback (*Gasterosteus aculeatus*), having diverged approximately 10 million years ago (Bell & Foster 1994, *see* also Mattern 2004, Mattern & McLennan 2004). Both species are popular models in ecological, evolutionary and behavioural studies (Bell & Foster 1994). However, the lack of microsatellite markers have been hindering population genetic and parentage studies in the nine-spined stickleback (but *see* Shapiro *et al*. 2006, Tsuruta & Goto 2006). In this note, we report the results of a cross-species amplification study that aimed to optimize microsatellite markers to the nine-spined stickleback using primers developed for three-spine sticklebacks.

Total genomic DNA was extracted from pectoral fins using a salt extraction method (Aljanabi & Martinez 1997). The initial testing — carried out with fish from Lake Pulmanki (*see* below) — started with 57 loci originally developed for the three-spined stickleback (Largiader *et al*. 1999, Peichel *et al*. 2001). PCR amplification was conducted in a total volume of 10 μl consisting of 1 × PCR buffer (160 mM $(NH_4)_2SO_4$, 670 mM, Tris-HCl (pH 8.8), 0.1% Tween-20) (Bio-

line), 1.5 mM $MgCl_2$ (Bioline), 0.2 mM dNTPs (Finnzymes), 0.25 U Biotaq DNA-polymerase (Bioline), 5 pmol of each primer and approximately 20 ng of template DNA. The PCR cycle started with a denaturation step of 3 min at 95 °C which was followed by 30 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s plus a final extension of 5 min at 72 °C. The PCR products were resolved on 1.5% agarose gels and visualized with ethidium bromide. The microsatellite loci that produced a specific band at a size range comparable to the three-spined stickleback were chosen for further analysis.

Based on the results of the initial optimization procedure two genotyping panels were designed with non-overlapping size ranges (Table 1). For the PCR amplifications a commercial multiplex PCR kit (Qiagen) was used to speed up the multiplex optimization and genotyping procedures. The PCR was carried out in a 10 μl total volume (manufacturer recommends 50 μl) containing 1 × Qiagen multiplex PCR Master Mix, 0.5 × Q-Solution, 2 pmol of each primer and approximately 20 ng of template DNA. The initial PCR cycling was modified according to the manufacturer's instructions starting with an activation step of 15 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 90 s at 53 °C and 60 s at 72 °C and a final extension at 72 °C for 10 min. Fluorescently labelled forward primers (FAM, HEX or TET) were used for visualization of PCR products and the 5´-end of reverse primer was modified with a GTTT-tail to improve the 3´-adenylation (Brownstein *et al*. 1996). The PCR products were resolved with MegaBACE 1000 automated sequencer (Amersham Biosciences) and the allele peak data was processed with FRAGMENT PROFILER 1.2 (Amersham Biosciences). The final screening for polymorphism was conducted using two Scandinavian populations: Kotka in the Baltic Sea (ca. 60°27´N, 26°15´E) and Lake Pulmanki (ca. 69°58´N, 27°58´E) draining to the Barents Sea. Basic gene diversity indices (number of alleles, expected heterozygosity) and deviations from Hardy-Weinberg and linkage equilibrium were estimated using FSTAT 2.9.3 (Goudet 2001).

From the 57 tested loci 18 amplified a specific PCR product visible on the agarose gels (*see* Appendix 1 for the complete list of the analyzed loci). This rather low cross-species amplification success is line with some earlier studies of teleostei fish (Holmen *et al*. 2005, *see* also Primmer *et al*. 2005), but in stark contrast with results of studies showing high (94%–100%) cross-species amplification success among species that diverged up to several hundreds of millions of years ago (Rico *et al*. 1996, Maes *et al*. 2006).

Further analysis revealed 11 polymorphic loci in the Kotka population and nine in Lake Pulmanki population (Table 2). Six loci were monomorphic in both study populations and one locus (STN195) showed complex stuttering patterns and was omitted from the further consideration. In general, the polymorphic loci showed easily interpretable allele profiles (*see* Appendix 2 for further information about stuttering). However, further testing of the six monomorphic loci in other populations might reveal some additional polymorphisms. For example, the locus 7033PBBE was found to be polymorphic in a population (Lake Myrdalsvannet 60°19´N, 5°22´E) not included into this note.

No deviations from the Hardy-Weinberg equilibrium were detected, although STN49 and STN148 showed an excess of homozygosity in the Kotka population. A further analysis with MICRO-CHECKER (Van Osterhout *et al*. 2004) did not reveal indications of the presence of null alleles in these loci. Similarly, no linkage disequilibrium was observed between the eleven loci in either of the populations when corrected for multiple tests (table-wide sequential Bonferroni correction, $\alpha = 0.05/11$).

In general, the 11 loci were less polymorphic in the nine-spined stickleback as compared to the three-spined stickleback (Tables 2 and 3), and the loci appeared to have more alleles and higher expected heterozygosities in the Baltic Sea population (Kotka) than in the land-locked Lake Pulmanki population (Tables 2 and 3). Interestingly, a significant species–population interaction revealed that the reduction in expected heterozygosity in lake population was significantly more pronounced in the nine- as compared with that in three-spined sticklebacks (Table 3).

The patterns described above are consistent with the findings of earlier studies showing lowered polymorphism in target as compared with that in source species in cross-species amplifi-

Table 1. Characteristics of the 11 dinucleotide microsatellite loci in the nine-spined stickleback. *A* = number of alleles, H_E = expected heterozygosity; F_{IS} = deviation from the Hardy-Weinberg expectations. Panel refers to a set of non-overlapping markers that can run in same lane/capillary. 'Chromosomal position' (= chromosome no.) refers to position of the locus in the three-spine stickleback genome (http://www.ensembl.org/Gasterosteus_aculeatus/index.html).

Locus[4]	Size range	Primer sequences (5´–3´)	Label	Panel	*A*	H_E	F_{IS}	Genebank accession no.	Chromosomal position
[1]1125 PBBE	130–146	F: CATCACACCCAGCCTCTC R: CCTCCCTCCAACTCTTATCA	TET	1	5	0.65	0.015	AJ010354	XX
[2]STN100	176–188	F: GGAGTCCGAGTGGTTCAGC R: GAACTCAGGCAGAGGATTGC	TET	1	4	0.43	0.054	G72177	IX
[2]STN130	196–210	F: TCAATATTCTGGGATTGCCG R: CGAGGACAGGATGTAAGACCC	HEX	1	6	0.64	–0.07	G72286	XI
[2]STN163	136–148	F: GAGAAGACAACAGGGAAGCG R: CGCCTGCAGTCAACCTACC	HEX	1	6	0.40	–0.10	G72304	XIV
[2]STN173	112–120	F: ACCACTTTGATTGGAATGGG R: GACGAAATGTACAGCACATCC	HEX	2	4	0.59	–0.03	G72309	XV
[2]STN19	158–176	F: ACAGGCATGAATGACACTGG R: GATGAGCACAACACCTGAGC	HEX	2	4	0.65	–0.15	G72135	XII
[2]STN196	192–196	F: GAGATGGCAATGAAGATGCC R: ACACATACACAGTCGTGGGC	FAM	2	3	0.61	–0.21	G72320	XVIII
[2,3]STN198	207–212	F: AGAAGGCCATCCCTTTGG R: AAAGAACGAACGAGTGAGCG	TET	1	5	0.49	0.01	G72222	IV
[2]STN49	165–173	F: GCAGACAGGTCTTGGAAAGG R: GGTTAAAGGAGCGCAGTAGG	FAM	1	4	0.56	0.16	G72153	IV
[2]STN96	221–245	F: ACACCTTCGGCTCCATATCC R: CGCAGCTCTCTGCTTTGC	HEX	2	8	0.77	–0.02	G72176	VIII
[2]STN148	191–199	F: AACCCTTACTCAACTCAGCCC R: GAGGAACTTCATTTGGCAGC	FAM	1	5	0.51	0.24	G72198	XIII

[1] PBBE loci originally described in Largiader *et al*. 1999

[2] STN loci originally described in Peichel *et al*. 2001

[3] Contains 1 bp variation

[4]Following loci appeared to be monomorphic in the two study populations (Genebank accession numbers in brackets); 7033PBBE (AJO10360), STN180 (G72313), STN185 (G72214), STN167 (G72208), STN178 (G72312), STN110 (G72182).

Table 2. Comparison of genetic diversity in 11 microsatellite loci of 20 nine-spined (*P. pungitius*) and three-spined (*G. aculeatus*) sticklebacks from two Finnish populations. $H_E(A)$ = expected heterozygosity (number of alleles), F_{IS} = deviation from the Hardy-Weinberg expectations. "Size" indicates the size range (in bp) of observed alleles.

	Kotka (Baltic Sea)						Lake Pulmanki					
	P. pungitius			*G. aculeatus*			*P. pungitius*			*G. aculeatus*		
Locus	Size	$H_E(A)$	F_{IS}	Size	$H_E(A)$	F_{IS}	Size	$H_E(A)$	F_{IS}	Size	$H_E(A)$	F_{IS}
1125 PBBE	130–146	0.65(5)	0.02	157–217	0.93(17)	–0.03	136–140	0.05(2)	0.00	163–195	0.70(7)	0.05
STN100	176–188	0.58(4)	0.05	183–299	0.98(27)	0.19	180	0.00(1)	NA	183–291	0.75(8)	0.06
STN130	196–204	0.51(4)	–0.01	166–204	0.63(10)	0.13	206–210	0.05(2)	0.00	184–204	0.73(7)	–0.02
STN163	136–148	0.64(6)	–0.10	141–183	0.86(11)	0.07	138	0.00(1)	NA	143–159	0.65(6)	–0.21
STN173	112–120	0.49(4)	–0.13	108–128	0.74(7)	–0.08	112–120	0.53(3)	0.06	118–124	0.50(3)	0.02
STN19	158–176	0.60(4)	–0.08	174–228	0.93(16)	–0.08	158–176	0.36(2)	–0.27	166–234	0.87(10)	–0.03
STN196	192–196	0.58(3)	–0.21	164–168	0.48(3)	–0.44	196	0.00(1)	NA	162–168	0.52(3)	–0.05
STN198	207–212	0.70(5)	–0.00	191–207	0.64(4)	–0.04	207–208	0.10(2)	–0.03	191–203	0.62(3)	–0.33
STN49	165–173	0.46(4)	0.35	155–167	0.81(7)	–0.03	169–171	0.45(2)	0.00	145–163	0.79(6)	–0.11
STN96	221–245	0.67(8)	0.11	227–249	0.66(7)	0.24	235–245	0.65(3)	–0.16	151–255	0.78(8)	–0.03
STN148	193–199	0.56(4)	0.35	201–239	0.83(11)	0.15	191–197	0.20(3)	–0.07	205–229	0.68(6)	–0.10
Mean		0.59(4.6)	0.017		0.77(10.1)	0.022		0.22 (2)	–0.07		0.69(6.1)	–0.06

Table 3. General linear model of expected heterozygosity (H_E) and allele number (A log-transformed) in three-spined and nine-spined sticklebacks collected from same two locations (lake *vs.* sea population). r^2 = proportion of variation explained by model.

		H_E (r^2 = 0.66)		A (r^2 = 0.66)	
Source	df	F	P	F	P
Species	1	47.80	< 0.001	43.07	< 0.001
Population	1	22.28	< 0.001	24.18	< 0.001
Species × Population	1	9.02	0.005	2.11	0.15
Error	40				
Model	3	26.36	< 0.001	11.05	< 0.001

cation studies (e.g. Primmer *et al.* 2005), and studies showing lower polymorphism in land-locked as compared with that in sea populations of the same species (e.g. Mäkinen *et al.* 2006). However, the significantly more pronounced reduction in polymorphism in land-locked nine-spined sticklebacks as compared with that in land-locked three-spined sticklebacks suggests that the success of cross-species amplification efforts may in addition to well-known effects of evolutionary divergence and annealing temperatures (e.g. Primmer *et al.* 2005) depend on ecological factors.

In conclusion, despite of the fact that the optimized loci are less polymorphic in the nine-spined stickleback as compared to the three-spined stickleback, and especially so in land-locked populations, they should be useful in population genetic, parentage and kinship analyses of nine-spined sticklebacks.

Acknowledgements

We thank Tuomas Leinonen and Jose Manuel Cano Arias for help with collecting the samples, and two anonymous reviewers for useful suggestions on earlier version of this manuscript. Our research was supported by grants from the Academy of Finland.

References

Aljanabi, S. M. & Martinez, I. 1997: Universal and rapid salt-extraction of high quality genomic DNA for PCR-based

techniques. — *Nucleic Acid Research* 25: 4692–4693.

Bell, M. A. & Foster, S. A. 1994: Introduction to the evolutionary biology of the threespine stickleback. — In: Bell, M. A. & Foster, S. A. (eds.), *The evolutionary biology of the threespine stickleback*: 1–26. Oxford University Press, Oxford.

Brownstein, M. J., Carpten, J. D. & Smith, J. R. 1996: Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. — *Biotechniques* 20: 1004–1010.

Colosimo, P. F., Hosemann, K. E., Balabhadra, S., Villarreal, G. Jr., Dickson, M., Grimwood, J., Schmutz, J., Myers, R. M., Schluter, D. & Kingsley, D. M. 2005: Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. — *Science* 307: 1928–1933.

Goudet, J. 2001: *FSTAT: a computer program to estimate and test gene diversities and fixation indices* (ver. 2.9.3). — Available at http://www2.unil.ch./izea/softwares/fstat.html.

Holmen, J., Vollestadt, L. A., Jakobsen, K. S. & Primmer, C. R. 2005: Cross-species amplification of zebrafish and central stoneroller microsatellite loci in six other cyprinids. — *Journal of Fish Biology* 66: 851–859

Largiader, C. R., Fries, V., Kobler, B. & Bakker, T. C. 1999: Isolation and characterization of microsatellite loci from the three-spined stickleback (*Gasterosteus aculeatus* L.). — *Molecular Ecology* 8: 342–344.

Maes, G. E., Pujolar, J. M., Raeymaekers, J. A. M., Dannewitz, J. & Volckaert, F. A. M. 2006: Microsatellite conservation and Bayesian individual assignment in four *Anguilla* species. — *Marine Ecology Progress Series* 319: 251–261.

Mattern, M. Y. 2004: Molecular phylogeny of the Gasterosteidae: the importance of using multiple genes. — *Molecular Phylogenetics and Evolution* 30: 366–377.

Mattern, M. Y. & McLennan, D. A. 2004: Total evidence phylogeny of *Gasterosteidae*: combining molecular, morphological and behavioral data. — *Cladistics* 20: 14–22.

Mäkinen, H. S., Cano Arias J. M. & Merilä. J. 2006: Genetic relationships among marine and freshwater populations of the European three-spined sticklebacks (*Gasterosteus aculeatus*) revealed by microsatellites. — *Molecular Ecology* 15: 1519–1534.

Peichel, C. L., Nereng, K. S., Ohgi, K. A., Cole, B. L. E., Colosimo, P. F., Buerkle, C. A., Schluter, D. & Kingsley, D. M. 2001: The genetic architecture of divergence between threespine stickleback species. — *Nature* 414: 901–905.

Primmer, C. R., Painter, J. N., Koskinen, M. T., Palo, J. U., Merilä, J. & Piironen, J. 2005: Factors affecting avian cross-species microsatellite amplification. — *Journal of Avian Biology* 36: 348–360.

Rico, C., Rico, I. & Hewitt, G. 1996: 470 million years of conservation of microsatellite loci among fish species. — *Proceedings of the Royal Society of London B* 263: 549–557.

Shapiro, M. D., Bell, M. A. & Kingsley, D. M. 2006: Parallel genetic origins of pelvic reduction in vertebrates. — *Proceedings of the National Academy of Sciences* 103: 13753–13758.

Tsuruta, T. & Goto, A. 2006: Fine scale genetic population structure of the freshwater and Omono types of ninespined stickleback *Pungitius pungitius* (L.) within the Omono River system, Japan. — *Journal of Fish Biology* 69: 155–176.

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. & Shipley, P. 2004: MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. — *Molecular Ecology Notes* 4: 535–538.

Appendix 1. A complete list of the three-spined stickleback loci tested for cross-species amplification in the nine-spined stickleback. YES = successful amplification of a given locus in nine-spined stickleback, NO = no amplification was observed, ? = chromosomal position of the locus is unknown.

Locus	Amplification	Genebank accession no.	Chromosomal position	Locus	Amplification	Genebank accession no.	Chromosomal position
1125Pbbe	YES	AJ010354	XX	STN15	NO	G72236	I
STN100	YES	G72177	IX	STN12	NO	G72132	I
STN130	YES	G72286	XI	STN21	NO	G72136	II
STN163	YES	G72304	XIV	STN38	NO	G72145	IV
STN173	YES	G72309	XV	STN174	NO	G72310	XVI
STN19	YES	G72135	XII	STN26	NO	G72240	II
STN196	YES	G72320	XVIII	STN9	NO	G72131	I
STN198	YES	G72222	IV	STN219	NO	BV102497	XXI
STN49	YES	G72153	IV	STN61	NO	G72158	VI
STN96	YES	G72176	VIII	STN82	NO	G72168	VII
STN148	YES	G72198	XIII	STN1	NO	G72126	I
7033Pbbe	YES	AJ010360	XI	STN23	NO	G72137	X
STN180	YES	G72313	?	STN381*	NO	*see* Colosimo	
STN185	YES	G72214	XIX			*et al*. 2005	
STN167	YES	G72208	XIV	STN30	NO	G72241	III
STN178	YES	G72312	XVI	STN37	NO	G72144	VII
STN110	YES	G72182	IX	STN59	NO	G72156	V
STN195	YES	G72221	XVIII	STN64	NO	G72160	VI
1STN365	YES	*see* Colosimo		STN67	NO	G72161	VI
		et al. 2005		STN118	NO	G72186	IX
1STN380	YES	*see* Colosimo		STN125	NO	G72189	X
		et al. 2005		STN158	NO	G72300	XIII
STN52	YES	G72154	V	STN 83	NO	G72263	VII
STN70	NO	G72164	VII	STN 134	NO	G72287	XII
STN81	NO	G72262	VII	STN 146	NO	G72296	XII
STN160	NO	G72301	XIV	STN 159	NO	G72206	XIII
4147Pbbe	NO	AJ010358	IV	STN 200	NO	G72224	XVII
STN3	NO	G72128	I	STN 201	NO	G72225	XVII
STN 90	NO	G72173	VIII	STN 205	NO	G72324	?
STN57	NO	G72155	X	STN 208	NO	G72229	XXI

* Linked to Eda-gene.

Appendix 2. Representative electrophoregrams of loci 1125Pbbe and STN100 produced with FRAGMENT PROFILER 1.2 (Amershamn Biosciences) showing the degree of stutter for two successfully amplified loci. In the rest of the polymorphic loci the allele profiles are comparable to the examples shown here. STN195 was rejected from further analysis, because it showed more complex stuttering/unspecific amplification.