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Characterization of nine microsatellite markers and development of multiplex PCRs for the Chinese huge mussel *Anodonta* (*Sinanodonta*) *woodiana* Lea, 1834 (Mollusca, Bivalvia)

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1. Introduction

The freshwater mussel *Anodonta* (*Sinanodonta*) *woodiana* (Lea, 1834) (Chinese Huge Mussel or Swan Mussel) (Bivalvia: Unionidae) is the largest unionid species present in the European fauna. Its native range is in East Asia (South-Eastern Russia to Malaysia), but it has spread rapidly across Europe over the last few decades and the species is invasive also in other parts of the world (Bogan et al., 2011; Demayo et al., 2012).

The initial establishment of *A. woodiana* in Europe was enabled by introduction of East Asian cyprinids species (*Ctenopharyngodon idella*, *Hypophthalmichthys molitrix*) for aquaculture in 1963–1965. Stocked fish possessed glochidia, the parasitic larval stage of the unionids. Further spread of the species is believed to be correlated with translocations of their native hosts across Europe (Sarkany-Kiss, 1986). Trade with the host fish species between different countries and regions enabled *A. woodiana* to penetrate to new regions. Physiological, ecological and biological peculiarities of the species offered it some advantages compared with the native unionid species and can explain its invasive success (Corsi et al., 2007).

Studies of the population genetics of *A. woodiana* in Europe used slowly evolving markers such as allozymes and mitochondrial COI DNA sequences for the analysis of several isolated populations (Nagel et al., 1996; Soroka, 2005; Soroka et al.,

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2014). However, for the recent and rapid spread of the species across Europe, fast evolving markers, such as DNA microsatellites, are needed to understand important aspects of the population genetics of this invasive species: the route(s) of invasion, the time and number of colonization events, and other details. The first eight microsatellite markers for the species have been described in 2011 (Popa et al., 2011). This number is rather low to infer aspects of the evolutionary history of populations and additional microsatellite loci are needed to increase to power of future genetic studies of this species (Koskinen et al., 2004).

In this paper we describe the development of nine new polymorphic microsatellite loci for *A. woodiana*. We also combined new and previously described loci into three multiplex sets allowing reducing the time and money costs of genotyping, as well as decreasing the risk for samples mishandling.

2. Materials and methods

2.1. Analyzed samples and DNA extraction

Specimens of *A. woodiana* (N = 27 individuals) were collected from the Kyjovka River (Czech Republic) and a tissue sample was preserved in ethanol from each specimen. DNA was isolated using the NucleoSpin® Tissue kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany), according to the producer's specifications.

2.2. 454 pyrosequencing

The microsatellite markers were isolated using the 454 FLX Titanium pyrosequencing technologies. Genomic DNA was isolated as previously described. Eight probes (TG, TC, AAC, AAG, AGG, ACG, ACAT, ACTC) were used for the production of a highly enriched DNA library which was sequenced by the 454-GsFLX Titanium chemistry according to the protocol developed by Malausa et al. (2011). All data obtained have been pooled and analyzed with QDD software, which ended in bioinformatic validated pairs of primers (Meglécz et al., 2010).

2.3. Primer validation in simplex PCR

The first step in the process of new microsatellite description is the validation of primer pairs in simplex PCR reactions. In this step, loci exhibiting excessive stuttering will be eliminated. This will also minimize, in the following multiplexing step, the risk to subsequently discover new alleles differing widely in size and overlapping with the allelic range of other loci labeled with the same fluorochrome (Guichoux et al., 2011).

The PCR genotyping reaction for the simplex step was performed in a 10 μL total volume containing about 50 ng of DNA template, 10 mM Tris-HCl (pH 8.8 at 25 °C), 50 mM KCl, 0.08% (v/v) Nonidet P40, 2.5 mM $MgCl_2$, each dNTP at 0.1 mM, each primer at 0.1 μM (one of the primers was M13 tailed), 0.02 μM of IRD700 or IRD800 labeled M13 primer and 0.5 units of Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania). The temperature profile of the PCR reaction consisted of an initial denaturation step at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at a specific temperature for each locus (see Table 1 for details for each locus) for 30 s and extension at 72 °C for 45 s, followed by a final extension step

Table 1
Nine new polymorphic microsatellites for *A. woodiana* and their genetic diversity, estimated in the non-native population in the Kyjovka River (Czech Republic).

Locus	Primer sequence (5′–3′)	GenBank Accession no.	Repeat motif	Annealing temp. °C	Kyjovka River (N = 27 individuals)			
					NA	H_O/H_E	pHWE	F_{IS}
AW 28	F: ttggttgtagctaaggctgga R: tagcagtggaacagcgtttg	KM242679	$[tg]_8$	59	3	0.333/0.374	0.852	0.108
AW 238	F: ccaccataaatactggtcgca R: ttcatggtcgaaaacgttca	KM242680	$[aca]_9$	55	6	0.680/0.770	0.051	0.116
AW 292	F: ttcatctcttaattgttgactgaa R: gtttgacagtcgcgttttga	KM242681	$[gta]_{19}$	56	6	0.731/0.536	0.953	−0.363
AW 324	F: cataggtctccacagccaca R:gatgaatggataaacgtgatcata	KM242682	$[atg]_{20}$	56.5	4	0.360/0.458	0.479	0.213
AW 378	F: tgggaaaactaggagacaatca R: tcgtgtgctaacaaattgcc	KM242683	$[tgt]_8$	55	3	0.308/0.297	0.430	−0.037
AW 514	F: cgccatgtatagcctagcgt R: ttgataggtgcttgctgtgc	KM242684	$[aca]_{10}$	58	5	0.800/0.718	0.173	−0.114
AW 521	F: tcggattcttcatccatcatt R: tgatgatcaggaagttgaagagg	KM242685	$[caa]_{17}$	56	6	0.920/0.770	0.037	−0.195
AW 536	F: ccacgttcagtcatgtcagtg R: ttagagaaaacgagaatgtccg	KM242686	$[at]_7$	57	4	0.625/0.709	0.182	0.119
AW 570	F: acgctgatgtacaaatctaacga R: ggaaagacgtagtctgattcattat	KM242687	$[gt]_{15}$	56	8	0.920/0.846	0.172	−0.087

N number of individuals, N_a number of alleles, H_O and H_E observed and expected heterozygosity, pHWE −p values for Hardy–Weinberg equilibrium test, F_{IS} inbreeding coefficient.

at 72 °C for 5 min. The amplified SSR (simple sequence repeats) fragments were genotyped on a LICOR 4300L genetic analyzer and SagaGT ver. 3.1 software package was used for alleles scoring.

2.4. Development of multiplex sets

We labeled the forward primer of the loci selected in the previous step, as well as the loci described by Popa et al. (2011) (17 loci in total) with fluorescent dyes (6-FAM, VIC, PET or NED; Applied Biosystems). We designed three multiplex panels for the 17 microsatellite loci by optimizing the annealing temperature for multiplexing (T_a), the concentrations of primers and maximizing the spacing between markers, to reduce unbalance of signal intensity among markers and to avoid marker overlap (Table 2). Finally, we confirmed the consistency of genotyping results between single-marker PCR and multiplex PCR reactions. PCR amplifications were conducted with the Qiagen Multiplex PCR kit (QiagenTM) in a 10 μl reaction volume containing 5 μl of Multiplex PCR Master Mix (2X) (including HotStarTaq Plus DNA Polymerase, Multiplex PCR Buffer with 3 mM $MgCl_2$), between 0.05 and 0.4 μM primer (Table 2), 1 μl genomic DNA (10 ng) and RNase-free water. A touchdown PCR was performed with the following thermocycling regime: 95 °C (15 min) followed by 5 cycles of 94 °C (30 s); annealing temperature stepdowns every 1 cycle of 1 °C (from 60 °C to 55 °C for sets 1 and 2, from 64 °C to 59 °C for set 3, 90 s); 72 °C (90 s). The annealing temperature for the final 30 cycles was 55 °C (alternatively 59 °C for set 3) with denaturation and extension phases as above.

PCR products were sized by capillary electrophoresis using an ABI Prism® 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA) and the GeneScan™ 500 LIZ® Size Standard (Applied Biosystems, Foster City, USA). Alleles were scored in the software GENEMAPPER v. 5.0 (Applied Biosystems, Foster City, USA) and double-checked manually.

2.5. Data analysis

GenAlEx 6.501 was used to test for Hardy–Weinberg equilibrium at each locus and to estimate the number of alleles (N_A), observed (H_O) and expected heterozygosity (H_E) and fixation index (F_{IS}) (Peakall and Smouse, 2006). The presence of null

Table 2
Characteristics of the three microsatellite PCR multiplexes in *A. woodiana*.

Locus	Size range	Label	Primer sequence (5′–3′)	Annealing temp. (T_a) °C	Conc. (μM)
PCR multiplex set 1					
SW 2	132–174	FAM	F: caaaaatgaaccggacacct	55 °C	0.2
			R: cccaaactcgttttcattgg		0.2
SW4	195–209	VIC	F: agcgcaattaccagtggttt		0.2
			R: ttgattgcgatgactggaaa		0.2
SW7	193–213	PET	F: ctgccacactgcagatattgt		0.35
			R: aacacgttcgaaatccgagt		0.35
SW13	217–263	FAM	F: cgccatgtcaaaaatcaaag		0.45
			R: gccacaagcatgagatgtgt		0.45
AW 521	89–128	VIC	F: tcggattcttcatccatcatt		0.05
			R: tgatgatcaggaagttgaagagg		0.05
AW 536	187–195	NED	F: ccacgttcagtcatgtcagtg		0.1
			R: ttagagaaaacgagaatgtccg		0.1
PCR multiplex set 2					
SW3	134–164	FAM	F: tgaaactggtgtccaatcca	55 °C	0.25
			R: ctcccgaaagagcacaacat		0.25
SW14	158–202	VIC	F: cccgtgttgtcaaaggaaat		0.35
			R: tttttctggcgactttccac		0.35
SW15	287–325	VIC	F: aaccgacaagtccttgcaata		0.3
			R:cagctgagtcgattaggacaga		0.3
SW18	122–200	PET	F: gtaacgtctcgtcgggtcat		0.1
			R: gcctcggtgctagacatcac		0.1
AW 238	171–195	NED	F: ccaccataaatactggtcgca		0.2
			R: ttcatggtcgaaaacgttca		0.2
AW 292	243–282	NED	F: ttcatctcttaattgttgactgaa		0.1
			R: gtttgacagtcgcgttttga		0.1
PCR multiplex set 3					
AW 28	125–131	PET	F: ttggttgtagctaaggctgga	59 °C	0.1
			R: tagcagtggaacagcgtttg		0.1
AW 324	179–266	FAM	F: cataggtctccacagccaca		0.1
			R:gatgaatggataaacgtgatcata		0.1
AW 378	111–123	NED	F: tgggaaaactaggagacaatca		0.1
			R: tcgtgtgctaacaaattgcc		0.1
AW 514	161–182	VIC	F: cgccatgtatagcctagcgt		0.05
			R: ttgataggtgcttgctgtgc		0.05
AW 570	180–222	NED	F: acgctgatgtacaaatctaacga		0.4
			R: ggaaagacgtagtctgattcattat		0.4

T_a anneling temperature.

alleles, large alleles dropout and scoring errors by stuttering, was tested using Micro-Checker ver. 2.2.3 (Van Oosterhout et al., 2004), while linkage disequilibrium tests were carried out using Genepop 4.2 (Raymond and Rousset, 1995).

3. Results and discussion

A total of 7091 sequences containing microsatellites motifs were obtained, among which 103 pair of primers were validated by bioinformatics tools. We tested 94 primer pairs in simplex PCR reactions and selected 54 primer pairs which gave constant amplification with a PCR product of expected size. A total of 9 polymorphic loci were successfully genotyped in all samples of *A. woodiana*.

The microsatellite loci are polymorphic (Table 1), with a number of alleles per locus ranging from 3 to 8. The observed and expected heterozygosity values ranged from 0.308 to 0.920 for H_O and between 0.297 and 0.846 for H_E. The F_{IS} values ranged from −0.363 to 0.213. Departure from the Hardy–Weinberg equilibrium was found in one locus AW 521 ($p = 0.037$), but no departure was observed after Bonferonni correction. After Bonferonni correction linkage disequilibrium was detected in 6 out of 136 loci comparisons (AW521 vs. AW536; AW 521 vs. AW18; SW3 vs. AW292; AW238 vs. AW514; AW238 vs. AW570; AW514 vs. AW570). The results of the Micro-Checker testing showed no evidence for homozygotes excess or presence of null alleles.

The three multiplex panels (Table 2) were designed manually. We tested the amplification success with the 17 polymorphic loci grouped in 3 multiplex panels: Multiplex 1 (6 loci), Multiplex 2 (6 loci) and Multiplex 3 (5 loci). The adjusted primers concentration after the optimization step is presented in Table 2.

Anodonta woodiana is one of the most invasive bivalves in European waters. Phenomena of freshwater biological invasions are becoming increasingly common, but our understanding of the process from biological and genetic point of view, is still largely unknown (Douda et al., 2012). The development of new DNA markers is the first step to study important aspects of the invasion process of invasive species such as the route(s) of invasion and the time and number of colonization events.

The used protocol with highly enriched DNA library allowed us to identified 9 new microsatellite loci for *A. woodiana*. The number of the polymorphic loci from the total number of loci tested in *A woodiana* is 9.57% (94 loci tested only 9 polymorphic). The low number of polymorphic loci could be explained by intrinsic factors, such as a particular microsatellite frequency within the genome of these species, the structure of microsatellite sequences and their flanking region and also by extrinsic factors such as the well known difficulties occurring in the development of molecular markers in mollusk species (McInerney et al., 2011). The polymorphic microsatelite loci described in this paper exhibit a number of alleles that ranged from 3 (loci AW 378 and AW28) to 8 (AW 570) in the sample of 27 genotyped individuals. These values are in the same range with the previously published study of this mussel species (Popa et al., 2011).

The three PCR multiplex reactions were optimized to facilitate large-scale populations studies for the invasive Chinese huge mussel. Multiplex PCR amplification often requires an extensive optimization in order to minimize the excessive stuttering, the primer dimmer formation which can complicate the genotyping process. In our study, the characteristics of the loci (Na, Ho and He) were not affected by multiplex PCR amplification.

In conclusion, the set of newly isolated markers for *Anodonta* (*Sinanodonta*) *woodiana*, combined with those previously described, provides a powerful tool for population genetic studies of this invasive species.

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