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SHORT COMMUNICATION

Development and multiplexing of microsatellite loci for the near threatened freshwater mussel Potomida littoralis *(Cuvier, 1798) using 454 sequencing*

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ABSTRACT

1. The Unionidae are among the most endangered fauna in the world and globally in decline. They are particularly vulnerable to habitat loss and fragmentation, susceptible to flow, pollution and climatic disturbances and introduction of invasive species. Despite their well-recognized ecological and conservation importance, there is a surprising lack of genomic resources currently available for European species.

2. The aim of this study was to develop and characterize microsatellites for the near threatened freshwater mussel, *Potomida littoralis* using 454 sequencing.

3. In order to improve genotyping throughput as well as cost-effectiveness, two multiplex-PCR reactions were designed to amplify 16 new loci. All the new 16 microsatellites were successfully combined in two multiplexed PCR with the number of alleles ranging from 2 to 25 per locus (with a mean of 10), confirming the utility of the new markers.

4. The new genetic markers can therefore be used for studying the population genetic structure and evolution of this species, e.g. to examine current levels of genetic variability within and between populations and thus to contribute to conservation and management.

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KEY WORDS: river; conservation evaluation; new techniques; genetics; invertebrates; molluscs

INTRODUCTION

Microsatellites or single sequence repeats (SSRs) are arrays of short repetitive motifs of 2–6 bp, that are distributed throughout the genome, co-dominantly expressed (Goldstein and Schlötterer, 1999). The number of times the unit is repeated in a given microsatellite can be highly variable, a characteristic that makes them one of the most common types of genetic markers. These markers are particularly suitable for studies at the population and individual levels (Duran *et al.*, 2009) and are of great utility in conservation and management plans (Bouza *et al.*, 2007; Geist *et al.*, 2010a) since they are very effective for examining current levels of genetic

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variability within and between populations. Genetic variability is important for population persistence, especially in species that have become fragmented or bottlenecked. The distribution of microsatellite allele frequencies can be used as analytical tools in identifying populations that have suffered recent bottlenecks, making it possible to quantify the severity of the size reduction, and by allowing the assessment of effective population size, inbreeding, and migration (Allendorf and Luikart, 2007). Traditional cloning methods of developing microsatellites involve significant trial and error (Queller *et al.*, 1993), are very time consuming and as a result can be cost-prohibitive. Recent advances in sequencing technology, such as 454 (Roche), designed for whole-genome sequencing, are valuable and cost-effective means of searching for microsatellites on non-model organisms. This new sequencing scheme represents a significant gain in time and cost, by producing millions of base pairs of short fragment reads, which may be screened using bioinformatics toolsets to identify primers that amplify polymorphic microsatellite loci (Duran *et al.*, 2009).

The aim of this study was to develop and characterize microsatellites for the near threatened (Cuttelod *et al.*, 2011) freshwater mussel, *Potomida littoralis* (Cuvier, 1798), (Bivalvia: Unionoida). This species occurs in a patchy circum-Mediterranean distribution, from Portugal to France, Greece, Middle East (Turkey and Syria) and Northern Africa. On the other hand, the species has been declining in recent decades in all Iberian Peninsula populations (Pérez-Quintero, 2007; Barea-Azcón *et al.*, 2008) and should be considered for full protection by the European Habitats Directive (Bouchet *et al.*, 1999). The Unionidae (Mollusca) are among the most endangered fauna in the world and globally in decline (Lydeard *et al.*, 2004). They are particularly vulnerable to habitat loss and fragmentation, susceptible to flow, pollution and climatic disturbances and introduction of invasive species (Strayer *et al.*, 2004). Moreover, and despite their well-recognized ecological importance, there is a surprising lack of genomic resources currently available for European species (Skidmore *et al.*, 2010). To the best of our knowledge, existing microsatellite primers are only available for two of the native European unionoids *A. cygnea* (Geist *et al.*, 2010b) and *M. margaritifera* (Geist *et al.*, 2003). The knowledge and measurement of genetic variation within and among populations is important for many unionoid species (Bogan and Roe, 2008). Thus questions about population structure, effective population size, the impact of habitat modification, reproductive ecology and contemporary phylogeography (which still remain virtually unknown for the majority of European unionoid species) will greatly benefit from the availability of microsatellites isolated using modern pyrosequencing of enriched DNA libraries. Such research can in turn be used in developing future conservation strategies designed to maintain the variability of the species, essential for its evolution and survival, by identifying conservation units, i.e., evolutionary significant units and management units.

METHODS

A small tissue sample from the foot of 75 *P. littoralis* was collected (following Naimo *et al.*, 1998) from three distinct populations, each corresponding to three major Iberian river basins: Douro, Tejo and Guadiana. Total genomic DNA was extracted from a single individual using a standard high-salt protocol (Sambrook *et al.*, 1989) and sent to Genoscreen (Lille, France) for microsatellite-enriched library preparation and sequencing by 454 Genome Sequencer FLX Titanium (454, Roche Applied Science) using the method described in Malausa *et al.* (2011) The sequences were received as multiple fasta files with the corresponding quality files.

To improve genotyping throughput as well as cost-effectiveness, two multiplex-PCR reactions were designed to amplify the new loci. Multiplexing consists of the amplification of several markers in a single PCR and can, therefore, significantly improve genotyping throughput as well as cost-effectiveness. For the first choice of loci to take on to further testing, two sets of 10 primers pairs each were chosen, taking into account several criteria such as the size of the resulting amplicons (small and large loci to maximize their capillary separation), the different motif classes (the three perfect motifs were chosen with simple repeats only) and, evidently, the compatibility of the primers from the different loci for PCR multiplexing.

A GTTT-sequence 'pig-tail' tag was added to the 5' end of all reverse primers (reduces stutter bands; Brownstein *et al.*, 1996) and a combination of the NED, VIC, PET, or 6FAM (Applied Biosystems, CA) dyes to the forward primers according to the multiplex essay (Table 1). Allelic variation was initially tested on nine individuals (three per

Table 1. Characteristics of 16 polymorphic microsatellite loci in *Potomida littoralis* including primer sequences (F = forward primer sequence; R = reverse primer sequence); Fluorescent dyes; Primer concentration (μmol L^{-1}); GenBank Accession numbers; Repeat type; N_a = number of observed alleles per locus; sizes of amplified fragments; H_e = expected heterozygosity; H_o = observed heterozygosity; and Hardy–Weinberg Equilibrium exact test *P* value

Locus	Primer sequences (5′-3′)	Fluorescent dye	Primer concentration	GenBank Accession no.	Repeat	N_a	Allele size range (bp)	H_e	H_o	HWE
MixA										
PL2	F: TGCTGTGATGAACTTTGATGC R: TATGCCCAACACTTCCAATG	FAM	0.8 μM	BankIt1594175 PL2 KC432760	$(taca)_{12}$	16	175-243	0.735	0.724	0.060
PL4	F: TGAATTTGATATGGACAAAGGG R: TGCATTCAAAGCTAAGACAGGA	VIC	0.8 μM	BankIt1594175 PL4 KC432761	$(ac)_{10}$	3	123-127	0.339	0.312	0.194
PL22	F: TCAAGGATTGTCGATTCACTTC R: TCCCACTTTAAATACAAGTAAGACAGA	VIC	2.8 μM	BankIt1594175 PL22 KC432773	$(ca)_{9}$	2	185-187	0.312	0.300	0.502
PL7	F: CCCGTTTTATAAATTACTTG AATATGG R: CCTCGTTGTTTACTACTTATGCG	NED	1.2 μM	BankIt1594175 PL7 KC432762	$(ct)_{13}$	8	141-185	0.485	0.442	0.056
PL24	F:AGATAAAATCGCCGGTGTCA R: CGTTTAGCTTTGTCTATGTG TTGA	NED	1.2 μM	BankIt1594175 PL24 KC432774	$(ag)_{13}$	16	217-255	0.689	0.769	0.982
PL10	F: TCACTTTTCAAATGAAGCTGGA R: CAGGTCAGGGCTTTATTCAGA	PET	1.2 μM	BankIt1594175 PL10 KC432763	$(gtat)_{15}$	8	182-230	0.555	0.562	0.667
PL25	F:TTGTGACAGGTGTGAAATAGCC R: TTTCTGAAACATTGTTAA AGCTACTTA	PET	4 μM	BankIt1594175 PL25 KC432775	$(gt)_{12}$	9	113-137	0.723	0.711	0.430
MixB										
PL11	F: AAGGATTGATTACAGTTGCGCT R: AGCCGGGTTTATTACCATGC	FAM	0.6 μM	BankIt1594175 PL11 KC432764	$(tc)_{10}$	3	110-114	0.353	0.248	0.001
PL12	F: CAGTAACCCCACCAACATCC R: TTGTCACTCCCATGGAGATAAT	FAM	1.6 μM	BankIt1594175 PL12 KC432765	$(taca)_{12}$	11	181-225	0.720	0.797	0.918
PL13	F: GTCATTTAGGAGTCATCGCTTT R: GAATCTAATTCACAGCA AACGG	FAM	0.8 μM	BankIt1594175 PL13 KC432766	$(atgt)_{11}$	8	258-298	0.704	0.769	0.877
PL14	F: TCATCCATATCCTTCTTCTGTGT R: GCTTTAAACAACGACAATTTGC	VIC	1.6 μM	BankIt1594175 PL14 KC432767	$(tcta)_{10}$	7	134-158	0.602	0.370	0.001
PL15	F: GTTCCTGATTCGTGCCAAAC R: TCTTAGGTTGTTCAAGGTGCAA	VIC	0.8 μM	BankIt1594175 PL15 KC432768	$(ga)_{15}$	12	193-241	0.677	0.704	0.725
PL17	F: TGTTTCATCTGGATGGGATG R: CTGCTTCTGCACACTCTGCT	NED	0.8 μM	BankIt1594175 PL17 KC432769	$(ac)_{18}$	13	136-174	0.728	0.699	0.086
PL18	F: TCTTTAGGGATGAAGCCTGC R: TGGTTCCATTTAAGCCATATCC	NED	1.4 μM	BankIt1594175 PL18 KC432770	$(ga)_{15}$	25	271-339	0.802	0.877	0.617
PL19	F: TGCCATTTTGTATTTAGGTCCA R: TCATAGATGTAATGGGCTGTCA	PET	1.2 μM	BankIt1594175 PL19 KC432771	$(atac)_{12}$	12	142-194	0.641	0.534	0.005
PL20	F: TGGTGGAGTTGTAAAGAGGGA R: ATACTGCATATGGTCGGCCT	PET	1 μM	BankIt1594175 PL20 KC432772	$(ac)_{15}$	7	216-236	0.620	0.643	0.723

population) with PCR reactions in simplex to validate selected loci and ascertain optimal annealing temperatures. After this trial, 16 loci were selected, combined in two multiplex-PCR reactions (seven in MixA and nine in MixB) and tested for polymorphism in all the 75 individuals. PCR amplifications were performed in 10 μL reactions containing 1 μL of 25 ng of template DNA, 5 μL of Qiagen multiplex PCR mastermix, 1 μL of primer-mix (variable concentrations of primer pairs and fluorescent dyes according to prior optimization of multiplex reactions; see Table 1) and 3 μL of H_2O. PCR reactions were performed on a DNA Engine Dyad® Peltier Thermal Cycler (Bio-Rad Laboratories), consisting of a denaturing step at 95°C for 15 min followed by 11 cycles of denaturation at 95°C for 30 s, 60 s annealing at 60°C where the annealing temperature was lowered by 0.5°C with each consecutive cycle, and 30 s elongation at 72°C; 21 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 30 s; eight cycles at 95°C for 30 s, 53°C for 30 s, 72°C for 30 s, and a final extension at 60°C for 30 min. Labelled PCR amplicons were resuspended in 10 μL Hi-Di™ Formamide and their sizes determined in an Applied Biosystems 3100 DNA analyser, with LIZ 500 size standard as an internal size standard.

Alleles were scored using GeneMapper® v 4.0 (Applied Biosystems). GENEPOP v 4.0 (Raymond and Rousset, 1995) and Arlequin v 3.5.1.2 (Excoffier and Lischer, 2010) were used to estimate diversities and expected and observed heterozygosities (H_e and H_o, respectively) and to test Hardy–Weinberg equilibrium (HWE).

RESULTS

Of the 12561 reads obtained, 3566 contained microsatellite inserts, of which 1558 contained microsatellite inserts with perfect motifs (only one motif) and simple repeats only, from di- to tetranucleotide, suitable for primer design. The amplicon sizes varied from 90 bp to 320 bp. Of these, 58.4% were dinucleotides, 20.0% trinucleotides and 21.6% tetranucleotides. GA and AC repeats were the most common dinucleotide repeats, TTG and ACA the most common trinucleotide repeats, and GTAT and CATA the most common tetranucleotide repeats.

All 16 loci were successfully combined in two multiplexed PCRs (Table 1) and yielded very clear and balanced electrophoresis profiles that matched the peaks obtained from single-locus PCRs.

Number of alleles ranged from 2 to 25 per locus with a mean of 10 (Table 1). H_e and H_o as well as the *P* values for the Hardy–Weinberg Equilibrium are also shown in Table 1.

DISCUSSION

Overall, this study shows the usefulness of second-generation sequencing of microsatellite-enriched library to develop new microsatellites in non-model organisms in a shorter time and at relatively low expense compared with traditional methods. In addition, the strategy used here, i.e. the amplification of several markers in a single PCR, significantly improved genotyping throughput as well as cost-effectiveness. This technique is still underused when scoring microsatellites, and given the excellent results shown in this study it appears promising for future and wider applications in conservation genetics.

The new 16 loci characterized here confirm their utility and great potential for fine-scale geographic studies in *P. littoralis*, since there are several and they are polymorphic. Microsatellites are good indicators of genetic diversity, measuring between-population connectivity at a scale equal to or smaller than the dispersal range of the species under study (Allendorf and Luikart, 2007). They can also allow the identification of bottlenecked populations, consequently holding promise for effective conservation measures for this species (Allendorf and Luikart, 2007). By examining the current levels of genetic variability within and between populations, and thus improving the understanding of this species' life history, they can be used in future conservation management actions (Geist *et al.*, 2010a).

Preventing the extinction of populations is a basic goal in conservation, and maintaining genetic diversity will tend to reduce the probability of extinction. Besides identifying populations that have suffered recent bottlenecks, analytical tests using the microsatellite results can help quantify the severity of the size reduction (Luikart *et al.*, 1998). Therefore, even before a substantial loss of genetic variability occurs, a population might have a bottleneck signature that is detectable using microsatellites in bottleneck detection tests (Piry *et al.*, 1999). Genetic considerations are probably most useful when incorporated early in a species' conservation plan, when the existence of some robust populations across a species' geographical

range offers the possibility of a variety of creative solutions to conservation problems (Hedrick, 2001). Many native European unionoid species have suffered serious declines but are not yet exposed to an imminent risk of extinction, rendering molecular studies particularly timely. Freshwater mussels depend on aquatic habitats, and for protection and management plans their local population dynamics as well as the degree of population connectivity must be considered. Microsatellites are particularly suitable for inferring recent population history and contemporary gene flow between fragmented subpopulations. These new markers may also be tested and used for related species.

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