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TECHNICAL NOTE

Highly polymorphic microsatellite markers for the short-snouted seahorse (*Hippocampus hippocampus*), including markers from a closely related species the long-snouted seahorse (*Hippocampus guttulatus*)

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Abstract Species of the family Syngnathidae are increasingly being investigated for conservation purposes but also for mating system and genetic parentage studies. The availability of highly polymorphic markers will be of great importance to conduct these kinds of studies. This paper describes the development and characterization of 10 polymorphic microsatellite markers for *Hippocampus hippocampus* and the utility of nine markers which were developed for a closely related species *Hippocampus guttulatus*. The number of alleles per locus developed for *H. hippocampus* ranged from 14 to 30, with levels of heterozygosity between 0.90 and 0.95. Most of the markers were successfully cross-amplified in the sister species, giving clear and unambiguous peaks and maintaining high levels of polymorphism.

Keywords *Hippocampus hippocampus* · *H. guttulatus* · Microsatellites · Syngnathidae · Cross-species amplification · Conservation genetics

Seagrass habitat is declining worldwide (Hughes et al. 2009). Species of the family Syngnathidae, i.e. seahorses and pipefishes, depend largely on seagrass habitat (Erzini et al. 2002) and these dependent species show a severe decline in relation with seagrass reduction and habitat destruction (Hughes et al. 2009). The Ria Formosa lagoon in Southern Portugal is inhabited by two seahorse species, *Hippocampus hippocampus* and *H. guttulatus*. Both species can be found in high densities and it has been reported that the Ria Formosa harbors the highest densities of seahorses recorded in the world (Curtis and Vincent 2005). However, a 85% decline in seahorse numbers over the past 8 years was recently reported (PSnews 2009), which might be related to seagrass habitat fragmentation. There are no records in the literature about the effect of seagrass reduction on population genetic structure and connectivity in *Hippocampus* species. The primary goal of the microsatellite library was to develop genetic markers that could be used to address the genetic consequences of reduction in population size for both *Hippocampus* species. Markers for *H. guttulatus* were previously developed, and they were tested for cross-amplification success for the sister species. In addition, also for comparison purposes new highly polymorphic microsatellite markers were developed for *H. hippocampus*.

Genomic DNA was isolated from two adults of *H. hippocampus* collected in the Ria Formosa lagoon in Southern Portugal by using a standard phenol–chloroform extraction protocol (Sambrook et al. 1989). Two genomic libraries were constructed simultaneously by following mainly Gautschi et al. (2000) as described in more detail elsewhere (Van de Vliet et al. 2008). Oligonucleotide repeat probes were 5′-biotinylated by using biotin-16-ddUTP (Roche). For the first library DNA fragments were hybridized to a single biotinylated $(ATAG)_8$ probe; in the second library DNA fragments were hybridized to a mixture of biotinylated probes: $(CA)_{15}$, $(GA)_{15}$ and $(CAGA)_8$. Subsequently, probes were attached to streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, DYNAL biotech, Hamburg).

During the library construction for *Hippocampus hippocampus*, approximately 79 clones (55%) of the 144

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Table 1 The characteristics of polymorphic microsatellite loci isolated from the species *Hippocampus hippocampus* (*Hhip* , 10 loci) and *Hippocampus guttulatus* (*Hgut*, 9 loci)

Locus	Primer sequence (5′→3′)	Repeat structure	[a]No. of alleles; size range (bp); H_E	No. of alleles	size range (bp)	T_a (°C)	H_E	H_O
*Hhip*1	TGCAACAGGACACCCAAGGTAGCA ATGAAAAAGCCAAAGCGTGGCGG	$(ATCT)_{42}$		26	239–477	60	0.94	1.00
*Hhip*2	GACTCGGGGTTAAGGCAGAAGG AGATGGATGGACAGACGGACATAG	$(ATCT)_{19}$		22	140–336	Tdown	0.93	0.92
*Hhip*3	TGATGTTGCGTCTAAACAAGTGACA GCCACTCTAAAATGTGCCTCAGAAC	$(TCTA)_{22}TCCA(TCTA)_9$		18	158–304	55	0.92	0.96
*Hhip*4	CATGTAAACAAACTCCAGGCATCG AGGGTGATTCCATTTTATTGCGGG	$(AGAT)_{30}$		17	198–226	55	0.91	0.92
*Hhip*5	GTCGCCATCTATGAACCTTGCAGCC CGTGACCCCTTCAGACAAAGCGTG	$(CT)_{10}CC(CT)_{31}$		19	282–370	60	0.92	0.88
*Hhip*6	TGATTACAGCTTCCCTCACCTCCACC TGCAGTCTACCAGTGGTCCATAACAGC	$(TC)_{27}$		17	142–224	60	0.92	0.83
*Hhip*7	ACATGGGAGCCAGATGGGCAAAGG TGCAACAGGACACCCAAGGTAGCA	$(TAGA)_{42}$		30	251–527	Tdown	0.95	0.96
*Hhip*8	ACAGTTGTTGGTCAACTTCCTGTATG TCAGGGAGAGAAGGGAAAGTCACAG	$(GT)_{21}$		14	192–260	57	0.90	0.92
*Hhip*9	TGACACATGCTGGCTATGTCCGT GCCCCTGATTAACCTTGAACAACCTC	$(GA)_{20}TACAG(TAGC)_4(TAGA)_{22}$		22	259–385	Tdown	0.94	0.90
*Hhip*10	TCAAGAGAAGAGGATGACTGGTG AAACCAGCTTTATAGAAATATATGCCG	$(TC)_{16}GTCACTCTC(TCTA)_{28}$		22	200–270	Tdown	0.93	0.96
*Hgut*1	TGAGCCCAGGTCCTTTGTGAA AGGGATAAGGAATACAAGCGGACAAC	$(CT)_{19}$	5; 136–158; 0.67	1	134	Tdown		
*Hgut*2	CACTGTCCACACACAACCAACAGCA GCAGCAACTCACTCACAGGGAAAGG	$(CT)_{33}$	11; 218–292; 0.89	1	126	60		
*Hgut*3	TCACACCAAAACCCCATATCAGAAG ACATTGGACACATAACACACAGAGG	$(GT)_5(GCGT)_4GCCT(GT)_{23}$	8; 134–164; 0.81	1	216	Tdown		
*Hgut*4	GCCGCATACACTGGACCGCATC TGCGAGCTACGTGAGGGAGAACATC	$(GATA)_{25}$	11; 136–204; 0.90	16	176–344	60	0.89	0.92
*Hgut*5	TCGACCAGAGGCCAGGAAACTTG ACGTGCTAACCACTGGACTACCG	$(TATC)_{13}CATC(TATC)_{28}$	10; 193–269; 0.88	–	–	Tdown		
*Hgut*6	AGAGACGCCCAGTCCACACTGAA GAGGAGCAGAATGGTGGAAGGGACA	$(ATAG)_{28}$	11; 238–346; 0.90	17	284–342	60	0.91	0.83
*Hgut*7	AGCTCGAGAATGGCCAGATTCCCTC TGGAGGAGTGTGGAACGGGATGC	$(CAG)_6CAT(CCT)_{11}$	2; 123–126; 0.30	4	117–126	60	0.67	0.52

Table 1 continued

Locus	Primer sequence (5′→3′)	Repeat structure	[a]No. of alleles; size range (bp); H_E	No. of alleles	size range (bp)	T_a (°C)	H_E	H_O
Hgut8	TGCTAATACGTCAAACTGCCGC TCGAGGGCTACCTGTCTATCGA	$(ATCT)_{28}$	8; 177–209; 0.86	–	–	Tdown		
Hgut9	AATCACTTCTCAATCAACCAATCT ATGTGATCAATGAAGCCCAAAC	$(ATAG)_{17}$	10; 196–268; 0.86	17	200–384	Tdown	0.91	0.96

Allelic data and genetic variation were derived using 26 individuals of the species *H. hippocampus* collected from the Ria Formosa lagoon in Southern Portugal

– nonamplification, T_a locus-specific annealing temperature, H_O observed heterozygosity, H_E expected heterozygosity

Size range refers to the PCR product size at each locus. The characteristics of the repeat are based on the sequenced clones (GenBank accession numbers GQ148720–GQ148738)

[a] Previously calculated values of genetic diversity for the developed microsatellite markers isolated from *H. guttulatus*

totally screened clones enriched for $(ATAG)_n$ nucleotide repeats gave a strong positive hybridization signal. The enrichment for a combination of $(CA)_n$, $(GA)_n$ and $(CAGA)_n$ repeats was even more successful; 88% of the clones gave a strong positive signal. Of these, 75 and 36 inserts from ATAG and CA/GA/CAGA nucleotide repeat enriched libraries, respectively, were sequenced in an ABI prism 3130XL capillary sequencer. All inserts sequenced from both libraries contained microsatellites more than 10 nucleotide repeats, often even longer than 50 repeats. Microsatellites with a high number of repeats were also found in libraries developed for *H. guttulatus* as well for other species in the Syngnatidae family (unpublished data). High number of repeats found in inserts with sizes around 500 bp could make primer design difficult which can affect their specificity and might influence reliable genotyping of these loci (Pompanon et al. 2005).

The success of isolating microsatellites with high number of repeats provided us with the rare opportunity of selecting only microsatellite markers with more than 15 single repeat type units. In addition we could first select for loci containing a tetranucleotide repeat, which reduces the potential for genotyping errors. Primers were designed for inserts containing microsatellites (preferably with one unique repeat type in the fragment) and with flanking regions sufficiently large to allow primer development. Optimal primer conditions were obtained by performing PCRs using the DNA of two adult individuals. Following the optimal PCR conditions, polymorphism was tested in *H. hippocampus* using additional DNA of 24 juveniles obtained from 12 pregnant males of *H. hippocampus* (two juveniles from each male) collected in the same region as the two other adult individuals. This allows also further parentage analysis. Polymorphism levels in previously developed *H. guttulatus* loci were obtained using eight adult individuals. Subsequently, these same individuals were used to test for cross-species amplification with primers developed for *H. hippocampus*. PCR amplifications were performed in a 10 µl reaction volume containing approximately 20 ng DNA, 1 µM primer and 0.1 µM of fluorescently labelled reverse primer, 2.0 mM $MgCl_2$, 5× Go*Tag* Flexi buffer (Promega), 0.2 mM of each dNTP and 0.75 U Go*Tag* DNA Polymerase (Promega). The touchdown PCR program held two cycles of 30 s at 94°C, 30 s at 62°C, 30 s at 72°C, followed by 14 cycles of 0.5°C touchdown: 15 s at 94°C, 15 s at 62–55°C, 15 s at 72°C, followed by 24 cycles of 15 s at 94°C, 15 s at 55°C, 15 s at 72°C. For the other loci we used the following PCR program: 30 cycles with 95°C for 40 s, locus-specific annealing temperature (Table 1) for 40 s, and 72°C for 40 s. For all PCR we started with a denaturation step of 94°C for 5 min and the last cycle was followed by a 7-min extension at 72°C. Fragment size was determined on an

ABI prism 3130XL capillary sequencer using the GeneScan-500 LIZ size standard (Applied Biosystems). Allelic diversity, observed (H_O) and expected heterozygosities (H_E), deviation from Hardy–Weinberg equilibrium and linkage disequilibrium were calculated using GENETIX ver. 4.04 (Belkhir et al. 2000).

In order to prevent misinterpretation of allele banding pattern which would give an individual a wrong multilocus genotype, we selected loci which only give clear and consistent allele banding patterns with little 'stutter'. In particular, dinucleotide microsatellites holding high numbers of tandem repeats seemed to have the tendency to give more 'stutter'; microsatellites with this kind of pattern were discarded. This resulted in 10 microsatellites isolated from *H. hippocampus* and four extra microsatellites previously developed for *H. guttulatus* which successfully amplify DNA from *H. hippocampus* according to our criteria (Table 1). Five primer pairs developed for *H. guttulatus* were monomorphic for *H. hippocampus* or gave no amplification product. Table 1 summarizes the characteristics of the primer pairs of the developed microsatellites, including the primer pairs which were developed for the sister species. Number of alleles ranged from 14 to 30 and expected heterozygosities ranged from 0.90 to 0.95. Loci *Hhip*1 and *Hgut*7; our only locus holding a trinucleotide repeat, showed a significant deviation from Hardy–Weinberg expectations ($P < 0.05$). For locus *Hgut*7 this may have been due to null alleles (as determined using the software MICRO-CHECKER,Van Oosterhout et al. 2003). No linkage disequilibrium was detected among any pair of loci.

Cross-species amplification was attempted in eight adult individuals of *H. guttulatus* using optimal PCR conditions. High levels of genetic diversity were measured (Table 2) and all polymorphic loci gave a clear and unambiguous allele banding pattern mostly near the expected size range. Loci *Hhip*2 and *Hhip*6 showed to be monomorphic. No-namplification or monomorphic results occurred more frequently when *H. guttulatus* primers were used for *H. hippocampus* (55%), than when loci from *H. hippocampus* were cross-amplified with *H. guttulatus* (20%). Amplification products from primer pairs from the other species were not sequenced to determine homology or the presence of a repeat element. The levels of diversity given by our reported microsatellites were sufficient to produce unique multilocus genotypes for each individual, including those sharing the same father, which is very useful in future parentage studies or projects concerning conservation strategies for *H. hippocampus* as well for *H. guttulatus*.

Table 2 Cross-species amplification results for eight individuals of *Hippocampus guttulatus*

	Size range (bp)	No. of alleles
*Hhip*1	207–285	9
*Hhip*2	174	1 monomorphic
*Hhip*3	206–308	10
*Hhip*4	190–262	12
*Hhip*5	250–254	2
*Hhip*6	157	1 monomorphic
*Hhip*7	243–325	6
*Hhip*8	216–268	7
*Hhip*9	113–327	15
*Hhip*10	137–145	4

Size range refers to the PCR product size at each locus. Amplification conditions are similar to *H. hippocampus*

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