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TECHNICAL NOTE

Novel microsatellite loci in the threatened European long-snouted seahorse (*Hippocampus guttulatus*) for genetic diversity and parentage analysis

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Abstract The long-snouted seahorse *Hippocampus guttulatus* is one of the two European seahorse species. We describe the isolation of the first 12 microsatellite loci in this threatened species. These new markers were tested in non-invasive samples of 32 seahorses from NW Spain. The number of alleles ranged from 2 to 15 (mean: 6.3) and expected heterozygosity from 0.031 to 0.912 (mean: 0.500). All loci conformed to Hardy–Weinberg expectations and no genotypic disequilibrium was observed between any pair of loci. The theoretical exclusion probabilities for this set of loci, when no parental information exists or when one parent is known, were 0.973 and 0.998, respectively. This study indicates the usefulness of these novel loci for population analysis and kinship studies in *Hippocampus guttulatus*. Their potential application is extended to the other European seahorse species, since all loci were successfully cross-amplified in *H. hippocampus*.

Keywords Seahorse · *Hippocampus guttulatus* · Microsatellites · Enriched library · Polymorphism · Parentage

Seahorses (Syngnathidae, Gasterosteiformes) are emblematic and threatened fish with remarkable morphology and biology, including male pregnancy (Avise et al. 2002; CITES 2002). Only two of the 32 species in the world live in the Northeast Atlantic: *Hippocampus guttulatus* and *H. hippocampus*. No accurate biological and distribution data are available for these species, further research being needed to assess their conservation status (IUCN 2006). In order to address population genetic analysis of the long-snouted seahorse *H. guttulatus*, we have developed primers for 12 polymorphic microsatellite loci from enriched genomic libraries in this species. Microsatellites have proven to be powerful markers for population genetics and its application to conservation biology (Ellegren 2004). These loci are also useful for parentage analysis to support reproduction in captivity (Castro et al. 2004) and to study genetic mating systems (Avise et al. 2002).

The genetic data presented here represent the first population analysis in this European seahorse. *H. guttulatus* was collected in Galician coasts (NW Spain), using non-invasive sampling of dorsal fin in live seahorses, and tissues stored in 100% ethanol. Genomic DNA was isolated by Chelex resin (Estoup et al. 1996) and standard phenol–chloroform methods.

Microsatellites were isolated using a partial enriched genomic library from muscle of a single dead seahorse according to the FIASCO protocol (Fast Isolation by AFLP of Sequences Containing repeats; Zane et al. 2002). Linked genomic fragments were enriched using a $(AC)_{17}$ biotinylated probe as described by Pardo et al. (2006).

Polymorphism was preliminarily evaluated in four individuals using 2.5% agarose gels dyed with ethidium bromide using unlabelled primers. Allelic variation of putative polymorphic markers was confirmed in 32 wild seahorses using an ABI 3100 automated sequencer (Applied Biosystems). The forward primer of each pair was 5′ fluorescently labelled (Applied Biosystems). Genotyping was carried out using the

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GENEMAPPER 3.7 software (Applied Biosystems), with GenScan 500LIZ as internal size standard.

One hundred and fifty six clones out of 263 sequenced, revealed characteristic short tandem repeats of microsatellite loci. Primers pairs were designed for 52 sequences, where enough large flanking regions existed. Nineteen of these were successfully amplified and 12 of them result polymorphic (Table 1). Ten out of the variable loci contained dinucleotide repeats and the remaining ones presented tetranucleotide motifs. Eight microsatellites were perfect, three imperfect and one compound. The final yield of variable microsatellite markers rendered by this enrichment protocol was 4.6%, higher than in other fish studies using the same procedure (2.3%, Zane et al. 2002; 0.6%, Carreras-Carbonell et al. 2004).

Population genetic parameters were estimated using the program CERVUS 3.0 (Marshall et al. 1998) in 32 seahorses from NW Spain (Table 2). Number of alleles ranged from 2 (Hgu-USC10 and Hgu-USC11) to 15 (Hgu-USC7). Expected heterozygosity and PIC ranged from 0.031 to 0.912 and from 0.030 to 0.889, respectively, at loci Hgu-USC11 and Hgu-USC7. All loci conformed to Hardy–Weinberg and genotype linkage equilibrium expectations (GENEPOP 3.1 program; Raymond and Rousset 1995), after sequential Bonferroni correction. These data are compatible with random mating, and suggest no close linkage between loci. However, null alleles might be present at two loci (Hgu-USC1 and Hgu-USC7; Table 2) as revealed by MICRO-CHECKER 2.2.3 program (van Oosterhout et al. 2004), suggesting caution in their application for kinship analysis. The probability of exclusion of a false parent for this set of loci when the other parent is unknown or known was 0.973 and 0.998, respectively, using CERVUS program (Table 2). All these data support the usefulness of this set of loci for population genetic studies and parentage analysis. They will permit to evaluate wild and cultured genetic resources, to address genetic mating system in this species and to support the development of its reproduction in captivity.

We also evaluated the cross-amplification of these loci in two individuals of the short-snouted seahorse *H. hippocampus*, using a range of $MgCl_2$ (1.5–2 mM) and temperature (48–60°C) PCR conditions. All loci

Table 1 Characteristics of isolated microsatellite loci in the long-snouted seahorse *H. guttulatus* and cross-amplification in *H. hippocampus*

Locus	GenBank no.	Primer sequence (5′–3′)	Repeat unit	$MgCl_2$	T_a	*H. hippocampus*
Hgu-USC1	DQ986275	GGATTTGACCCATTTCGATG GTGTATTTGGCGCTGTTTGA	$(TG)_9$	1	58	+[b]
Hgu-USC2	DQ986276	TTGCGCTGATCCTTCTCTTT GCAGGAACCACCAAGTAACC	$(GT)_{10}$	1	58	+[a]
Hgu-USC4	DQ986278	CCGACAGGAAGTAGCTGGAA GTGGCAGTTGCACAGAGGTA	$(TG)_{13}$	1	60	+[a]
Hgu-USC5	DQ986279	GTGTGTTGGATTGCTGGATG ATGACAAGTGCCTGAGCGTA	$(TGCG)_4(TG)_6$ $C(GT)_4A(TG)_3$	1	60	+[a]
Hgu-USC6	DQ986280	CAGTCCCTGAAGCTATTCCTGT AAGGACTTTGTGTTCACTTGC	$(GT)_{12}$	1,5	55	+[a]
Hgu-USC7	DQ986281	CAGAGCAGTGTACCCATTCG TTTCACCGTCCATCTTCCTC	$(GA)_8A(AG)_{10}$	1,5	58	+[c]
Hgu-USC8	DQ986282	CGCCGGAAGATGTGT GGCCTGGTCAAGAAGATCAA	$(AT)_{18}$	1	60	+[a]
Hgu-USC9	DQ986283	TTGCAGAATGTGGCTGGATA AGTGGAGGCTGACAGGGTAA	$(TG)_{14}$	1	58	+[a]
Hgu-USC10	DQ986284	AAGGACGCTTGTTGTCCATC ATTGTGCTGCAATGATACCG	$(GT)_8T(GT)_5$ $T(GT)_{48}$	1.5	58	+[a]
Hgu-USC11	DQ986285	TTACACTTAGACGGCCCTGTT CGGAATTGGACACAAGGTTC	$(GT)_3C(TG)_7$	1.5	58	+[a]
Hgu-USC12	DQ986286	CGCATCTACTCACCCATTCA TGCTGGAACAGAGAGTGTGG	$(TCCA)_4$	1	60	+[a]
Hgu-USC13	DQ986287	AAATTAGCCATCGGAAAGCA GGACTGAAGCCATGAACCAA	$(TG)_{11}$	1	58	+[a]

+successful cross-amplification without further optimization

[a] band of expected size

[b] band larger than expected

[c] band smaller than expected

Table 2 Genetic diversity estimates of isolated microsatellite loci in the long-snouted seahorse *H. guttulatus*

Locus	Size range	*A*	H_o	H_e	PIC	Excl1	Excl2
Hgu-USC1	303–329	10	0.613	0.672	0.637	0.278	0.467
Hgu-USC2	140–150	3	0.531	0.523	0.398	0.133	0.207
Hgu-USC4	130–136	4	0.469	0.508	0.415	0.126	0.231
Hgu-USC5	240–274	9	0.781	0.818	0.779	0.445	0.621
Hgu-USC6	282–314	7	0.500	0.564	0.512	0.169	0.329
Hgu-USC7	373–413	15	0.719	0.912	0.889	0.658	0.794
Hgu-USC8	146–180	11	0.844	0.896	0.870	0.610	0.760
Hgu-USC9	300–338	6	0.484	0.438	0.410	0.102	0.255
Hgu-USC10	411–437	2	0.034	0.034	0.033	0.001	0.017
Hgu-USC11	100–102	2	0.031	0.031	0.030	0.000	0.015
Hgu-USC12	139–151	3	0.250	0.254	0.234	0.031	0.127
Hgu-USC13	327–339	3	0.367	0.352	0.297	0.060	0.155
Mean/Total		6.3		0.500	0.459	0.973	0.998
SE		1.2		0.086	0.084		

Allelic size range is given in base pairs. A: number of alleles per locus; H_o and H_e: observed and expected heterozygosity; PIC: Polymorphic Informative Content; Excl1 and Excl2: probability of exclusion of a false parent unknowing and knowing the other parent, respectively estimated using CERVUS. Mean/Total: Mean genetic diversity over loci with their respective standard errors (SE), and total exclusionary power estimates (Excl1 and Excl2)

were successfully amplified, some of them showing interspecific size differences (Table 1). This set of markers are potentially useful to obtain appropriate population data for conservation and management of genetic resources of the two threatened European seahorses.

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