MICROSATELLITE-BASED TOOL FOR GENETIC MATING SYSTEM ANALYSIS AND BROODSTOCK MANAGEMENT OF LONG-SNOUTED SEAHORSE *HIPPOCAMPUS GUTTULATUS* UNDER CAPTIVE BREEDING CONDITIONS

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Abstract

A suitable microsatellite-based tool for parentage and relatedness analysis in *Hippocampus guttulatus* under captive conditions was characterized. A total of 401 offspring obtained by natural spawning from 49 potential founder breeders distributed in different aquaria were genotyped for a selected subset of six loci. The high polymorphism of these six microsatellite loci ensured a theoretical exclusion power for parentage inference close to 1. This high potential, together with the simple mating scenario managed, enabled to trace back all offspring to a single mating pair with high confidence. The *H. guttulatus* families identified were then used to analyze genotyping errors at these loci and thus to check the performance of these loci for parentage assignment. The percentage of false exclusions was very low (0.75%) indicating the high genotyping confidence for these loci, being the presence of low-frequency null alleles at a specific locus (*Hgu*-USC7) the main source of false exclusions (72% of mismatches). Those mismatches that were not explained by genotyping errors or null alleles were considered mutations, yielding an average rate of 4.16×10^{-4} estimated over 4,812 gametes, within the range for microsatellites in fish. All loci analyzed conformed to Mendelian segregation. The potential of the microsatellite tool was confirmed in a more complex scenario, using all breeders as parental candidates, above 95% of offspring being assigned to the same single couple either with maximum likelihood or exclusion methods. Parentage data revealed a genetic monogamous mating system of *H. guttulatus* in captivity. The *H. guttulatus* family data were also used to evaluate the performance of the microsatellite loci for pairwise relatedness estimation. High kinship classification success was achieved (above 80% global and close to 90% for unrelated individuals), supporting further estimation of relationships among broodstock founders.

In summary, these microsatellite loci provide a helpful tool for genealogical traceability and kinship classification to assist captive breeding strategies of *H. guttulatus.*

1. Introduction

Seahorses (*Hippocampus* spp.) are endangered fish species due to the progressive regression of wild populations (Foster and Vincent 2004; Martin-Smith et al. 2004). All seahorse species are listed as threatened by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2010). Aquaculture has been proposed as one solution to address unsustainable trade of seahorses (Koldewey and Martin-Smith 2010). In the last decade, research, conservation and commercial aquaculture operations for different species have been suffered a considerable expansion. However, the rearing in captivity is still in its infancy for many species (Olivotto et al. 2011), and the scarcity of information available on biology, behavior and life history for most species has delayed the success of seahorse aquaculture (Koldewey and Martin-Smith 2010).

The long-snouted seahorse *Hippocampus guttulatus* (Cuvier 1829) is one of the two seahorse species found in the northeast Atlantic Ocean and Mediterranean Sea. It is considered as a data deficient species, with insufficient available information to assess risk of extinction based on its distribution and/or population status (IUCN 2011). Such biological knowledge would be of major importance for conservation actions, development of captive breeding programs, and recovery of wild populations. In NW Spain, a *H. guttulatus* captive broodstock has been recently founded for research and conservation purposes, in response to the decline of wild populations of this species in Spanish coasts (Planas et al. 2008). The development of techniques for reproduction and

management of this broodstock has been basic requirements for its successful captive breeding (Planas et al. 2008).

Preservation of genetic diversity and avoidance of inbreeding are primary issues in conservation breeding programs for threatened species (Caballero et al. 2010). Thereby, broodstock foundation and management should be monitored and fish progeny traced back to their family groups. However, pedigree records are often unavailable or difficult to obtain in fish aquaculture, especially for species only viable under natural spawning, such as seahorses, with complex reproductive and social behaviour (Foster and Vincent 2004). In addition, social promiscuity exhibited by seahorses makes hard tracing back the contribution of broodstock individuals to the offspring (Planas et al. 2008; Wilson and Martin-Smith 2007).

Microsatellite DNA markers permit to solve most issues related with the family analysis to support broodstock management and reproduction in captive conditions. They allow retrospective assignment of individuals to family groups reared communally, estimation of relatedness between individuals, and evaluation of genetic mating systems in fish (Avise et al. 2002; Castro et al. 2004, 2006, 2007; Jackson et al. 2003; Kozfkay et al. 2008; Pino-Querido et al. 2010; Sekino et al. 2003, 2004; Sriphairoj et al. 2007). Paternity inference, using exclusion (EXC) or maximum likelihood (ML) approaches, is the best method to identify familiar relationships when all candidate parents are available (Jones and Ardren 2003; Jones et al. 2010). Pairwise relatedness estimators also provide useful tools in aquaculture, supporting kinship classification for broodstock management when no information exists on consecutive generations (Pino-Querido et al. 2010). A previous step to use a microsatellite-based strategy for assisting conservation programs is to check for conformance to the theoretical assumptions of the statistical methods used to assess parentage relationships (Castro et al. 2004, 2006,

2007; Wilson and Ferguson 2002). Mendelian segregation, Hardy-Weinberg and linkage equilibrium assumptions, as well as genotyping errors, null allele frequency and mutation rate of microsatellites, are essential issues to assess the accuracy of parentage relationships inferred from these markers. The evaluation of relatedness estimators based on true family data using microsatellites under actual aquaculture scenarios has also been recommended (Castro et al. 2004; Pino-Querido et al. 2010; Wilson and Ferguson 2002).

Microsatellite markers have demonstrated their ability to perform parentage analysis in wild and captive populations of different seahorse species, mostly devoted to evaluate social and genetic mating systems (Jones et al. 1998; Kvarnemo et al. 2000; Wilson and Martin-Smith 2007). Recently, several microsatellites have been isolated in the long-snouted seahorse suitable for genetic diversity and kinship analyses in *H. guttulatus* (Pardo et al. 2007; van de Vliet et al. 2009). To our knowledge, testing of basic microsatellite parameters to develop a consistent parentage tool based on family data has not been tackled in *H. guttulatus*. In the present work, we characterized 401 *H. guttulatus* offspring obtained by natural spawning from the captive broodstock maintained at Instituto de Investigaciones Marinas (IIM-Vigo, NW Spain), using six highly polymorphic *H. guttulatus* microsatellites. The study was focused on evaluating the potential and properties of these microsatellites for parentage assignment in order to obtain a useful tool to assist captive breeding programs in this species. Results obtained also enabled to obtain relevant information on the genetic mating system of *H. guttulatus*, a recurrent topic on the biology of this species, and to provide genetic support for broodstock reproduction in captivity.

2. Materials and Methods

120 2.1. Sampling and DNA extraction

121 Samples from all adult *H. guttulatus* specimens comprising the whole broodstock
122 founded at the Instituto de Investigaciones Marinas (IIM) in Vigo (Spain) were
123 collected by means of non-invasive sampling of dorsal fin and skin filaments (Planas et
124 al. 2008). The IIM-broodstock was founded with wild individuals from NW Spain and it
125 comprised 21 males and 28 females distributed in different aquaria (3-8 breeders per
126 aquarium) under variable sex-ratio criteria (male-biased, female-biased and unbiased
127 sex ratios; Planas et al. 2008). Since 2006, a total of 401 offspring from 19 batches of
128 newborn seahorses were sampled for molecular parentage analysis. Genomic DNA from
129 offspring (died embryos or young individuals) and most adult samples was isolated
130 using Chelex® 100 resin (Estoup et al. 1996). For very small pieces of adult skin
131 filaments and dorsal fin, DNA was purified using NucleoSpin® Tissue XS kit
132 (Macherey-Nagel) and, when necessary, amplified using GenomiPhi V2 kit (GE
133 Healthcare).

134 2.2. Microsatellite amplification

135 Four out of twelve previously isolated *H. guttulatus* microsatellites (Pardo et al. 2007)
136 were selected for parentage analyses in this study, attending to polymorphism and
137 technical criteria (*Hgu*-USC5, *Hgu*-USC6, *Hgu*-USC7 and *Hgu*-USC8). In addition, two
138 highly variable loci recently reported in this species (*Hgut*4, *Hgut*6; van de Vliet et al.
139 2009) were included in the study. PCR amplification was performed as described
140 previously (Pardo et al. 2007; van de Vliet et al. 2009). All candidate parents and
141 offspring obtained in each aquarium were genotyped with the six selected loci.
142 Genotyping was performed on an ABI 3730xl DNA Analyzer using GENEMAPPER v3.7
143 (Applied Biosystems).

144 2.3. Genetic diversity and parentage analyses

145 Genotyping accuracy was tested in the IIM-broodstock using MICRO-CHECKER v2.2.3
146 (van Oosterhout et al. 2004). Conformance to HW and genotypic equilibrium was
147 checked using exact tests implemented in GENEPOP v4.0 (Rousset 2008). Allele number
148 per locus (A), observed and expected heterozygosity (H_o and H_e), polymorphic
149 information content (PIC), the two theoretical exclusion probabilities (Excl1 and Excl2,
150 when genetic information from the other parent is unknown and known, respectively),
151 and the exclusion probability for identity of two siblings (SI) were estimated for each
152 locus and over loci using CERVUS v3.0 (Kalinowski et al. 2007).
153 Microsatellite profiles of the six loci were used to identify the most likely parents for all
154 offspring sampled in each aquarium (actual scenario). In addition, the parentage
155 assignment of each offspring was performed considering the whole IIM-broodstock to
156 test the potential of microsatellite tool in a more demanding unstructured scenario. In
157 the actual scenario, paternity or maternity inference was studied in aquaria under male-
158 biased (1 female (F):≥2 males (M)) or female-biased (1 M:≥2 F) sex-ratios,
159 respectively. Biparental parentage analysis was required for aquaria under unbiased sex-
160 ratio (≥2 M:≥2 F). Parentage analysis was performed using both EXC- and ML-based
161 methods implemented in FAP v3.6 (Taggart 2007) and CERVUS (default genotyping
162 error rate: 1%; all parents sampled), respectively.
163 Conformance to Mendelian inheritance was tested by χ^2 tests in the six largest full-sib
164 families inferred by parentage analysis (mean family size: 62 offspring), applying
165 sequential Bonferroni correction for multiple tests.
166 Presence of null alleles was checked in the IIM-broodstock using MICRO-CHECKER.
167 Null alleles were also identified by evaluating homozygote-homozygote mismatches
168 between parents and offspring using family data (Castro et al. 2006). Single parent-
169 offspring mismatches not attributed to null alleles or genotyping errors were considered

mutational events after new DNA extraction and regenotyping of parents and offspring involved.

2.4. Relatedness analysis

Relatedness between all pairs of the *H. guttulatus* founders was estimated for aiding in the organization of the captive broodstock, lacking of ancestry records (Pino-Querido et al. 2010). Wang's (2002; W) pairwise relatedness (*r*) estimator was used, since it has been reported as the best-performing one according to the scale of this study: wild origin of founders, family-structured scenario, and absence of an appropriate reference population for estimating allele frequencies (Pino-Querido et al. 2010; Wang 2002). Relatedness between all individual pairs was estimated using SPAGEDI v1.2 (Hardy and Vekemans 2002). The microsatellite tool was first evaluated for relatedness estimation using the actual family groups obtained from the IIM-broodstock in captivity, with known kinship relationships (full-sib, FS; half-sib, HS; unrelated, UR) assuming most wild breeders were unrelated. For this, two reference populations were considered to obtain allele frequencies (the offspring sample itself and the IIM-broodstock). The midpoints 0.125 and 0.375 between the expected *r*-values (UR=0, HS=0.25 and FS=0.5) were used as thresholds to classify individuals using molecular parentages ($UR \leq 0.125 < HS < 0.375 \leq FS$; Martínez and Fernández 2009). The estimated classification was contrasted against the true family data as reported in previous studies (Pino-Querido et al. 2010; Rodríguez-Ramilo et al. 2007).

3. Results

3.1. Genetic diversity and theoretical exclusion probabilities

All loci conformed to HW expectations and genotypic equilibrium in the *Hippocampus guttulatus* IIM-broodstock. MICRO-CHECKER showed genotyping accuracy at most loci,

only suggesting the presence of null alleles at *Hgu*-USC7 (frequency: 6%). The six microsatellites showed high variability (mean estimates: A=18.7; H_e=0.861; PIC=0.835; Table 1). High combined theoretical exclusion probabilities of a false parent (Excl1=0.9980; Excl2=0.9999) and exclusion probability for identity of two siblings (SI=0.9987) were obtained. According to the predictive analysis by FAP, it was expected to achieve 100% assignment at single family with these six microsatellites under the actual scenario of the IIM-broodstock.

3.2. Parentage analysis

Under the actual IIM-broodstock scenario and using the CERVUS ML method, 100% of offspring were assigned to a single couple of parents per aquarium as the most likely explanation. The FAP EXC-based approach assigned 95.8% of the offspring to a single couple within each aquarium. Fully congruent parentage assignment was observed with both approaches. The proportion of unassigned individuals with the EXC method (4.2%) was due to a few mismatches (usually a single and very occasionally two) between these offspring and their putative parents. These incongruities were considered as false exclusions because all putative parents had been genotyped, the next couple to which the offspring was assigned always showed higher number of mismatches, and the couple identified was the same as with the ML method. Therefore, microsatellite profiles were reanalyzed under this assumption, obtaining a real exclusion power of 100%. The mismatches identified (18 over 2,406 genotypes: 0.75%) were ascribed to null alleles (72.2%) at *Hgu*-USC7 and to genotyping errors by allele dropout (16.7%) at *Hgu*-USC8. The remaining two mismatches (11.1%) observed at the locus *Hgut*6 in two different families were considered as mutation events involving the gain of a single repeat unit. Overall, mutation could be considered as a minor factor of false exclusion,

since only two over the 4,812 gametes analyzed were identified as mutations (average mutation rate: 4.16×10^{-4}).

The parentage analysis of the 19 batches of newborn seahorses revealed nine full-sib families (FS1 to FS9; Table 2). Only two families of half-sibs (HS) were inferred from parentage analysis and they were related to reorganizations of the IIM-broodstock (M. Planas, personal communication): i) FS1 and FS2 families shared the same father by the death of the first female and the subsequent mating with a second female within the same aquarium; ii) FS7 and FS8 families shared the same mother because of the temporal removal and the replacement of the first male.

When all IIM-broodstock individuals were considered as candidate parents, irrespective of their organization in different aquaria, the predictive exclusion power estimated with FAP for the six microsatellites decreased to 99.7% (*vs*. 100% in the actual scenario). Using the ML method and the EXC approach in this unstructured scenario, 99.8% and 95.8% of the offspring, respectively, were assigned to a single couple. Both methods agreed with the parentage assignment obtained under the actual scenario, the same mismatches being identified and considered as false exclusions. This provided a correct assignment of 99.8% of the offspring to the same couple previously identified in the actual scenario. Only a single discrepancy was detected, likely due to the high relatedness estimate observed between the two candidate females involved (r=0.44; see section 3.3).

None of the six microsatellite loci assayed showed significant deviations from Mendelian expectations in the six largest full-sib families tested, excluding *Hgu*-USC6 in a single family.

3.3. Relatedness estimation: Family analysis and founder relationships

Wang's (W) relatedness coefficient (*r*) was estimated between all pairs of offspring obtained in captivity with known kinship (80,200 dyads; 64,185 UR; 3,020 HS; 12,995 FS). W estimator was downward biased for the three kinships studied when the offspring sample was used to estimate allele frequencies (0.480 *vs*. 0.5 expected for FS; 0.142 *vs*. 0.25 for HS; and -0.096 *vs*. 0 for UR; Table 3). The particularly high bias for HS could be related to the low number of half-sib families available in this study. The bias across kinships was lower when the IIM-broodstock was used to estimate allele frequencies (0.025 *vs*. 0.075). The standard deviation, which represents the error for a single pairwise estimation, was higher for FS and HS (>0.18) than for UR (0.12). The error across kinships was very similar irrespective of the reference population for estimating allele frequencies (0.16 *vs*. 0.17).

The classification success of the W estimator was also evaluated by comparing estimated and true kinships for each dyad. Global classification success across the three kinships studied slightly increased when allele frequencies were estimated from the offspring sample itself (89.2%) than from the IIM broodstock (83.4%). This was mainly due to the highest classification success at UR (95% *vs*. 88%, using the sample itself and the IIM-broodstock, respectively), largely the most frequent class, whereas the classification was quite similar irrespective of the reference population considered for HS (~45%) and FS (~70%). The W relatedness coefficients between founders within the *H. guttulatus* broodstock population ranged from -0.175 to 0.743, with a mean figure of -0.009. Using conservative cutoffs, most pairwise dyads showed low relatedness estimates ($r \leq 0.125$), with only 13 dyads revealing high relatedness estimates ($r \geq 0.375$) between founder breeders.

4. Discussion

In this work the genetic and technical properties of six microsatellite loci isolated in the long-snouted seahorse *Hippocampus guttulatus* (*Hgu*-USC5, *Hgu*-USC6, *Hgu*-USC7 and *Hgu*-USC8 by Pardo et al. (2007); *Hgut*4 and *Hgut*6 by van de Vliet et al. (2009)) were analyzed to develop a suitable tool for parentage and relatedness analysis to assist breeding in captivity of this threatened species.

4.1. Microsatellite-based tool for parentage assignment

Microsatellite-based traceability methods have demonstrated to be very useful for accurate pedigree analysis in fish including seahorses (Castro et al. 2004, 2006; Jones et al. 1998; Kvarnemo et al. 2000; Wilson and Martin-Smith 2007). The six loci assayed in this work showed high genetic variation according to previous data (Pardo et al. 2007; van de Vliet et al. 2009), yielding high combined figures of exclusion potential suitable for parentage analysis in *H guttulatus.* Assignment success with microsatellites also relies on accurate genotyping, low frequency of null alleles and not too high mutation rate (Jones and Ardren 2003; Marshall et al. 1998; Slate et al. 2000). Parentage estimation can be compromised even with few genotyping errors, especially if candidate parents are excluded on the basis of a single mismatch (Glaubitz et al. 2001; Hoffman and Amos 2005; Marshall et al. 1998). Familiar analysis represents the best approach to identify the different sources of error (Castro et al. 2004). Given the high exclusion potential of the six loci and the simplicity of the parentage scenario in the actual *H. guttulatus* IIM-broodstock, all offspring were assigned with full confidence to a single couple of parents. These data revealed a low mismatching-frequency (0.8%) regarding other studies in fish (around 2%; Bonin et al. 2004; Castro et al. 2007; Hoffman and Amos 2005). The main source of mismatches was attributable to null alleles (72%), as usually reported in parentage studies in fish (Banks et al. 1999; Castro et al. 2004, 2006,

293 2007; McGoldrick et al. 2000). Null allele frequencies below 5% have been suggested
294 for pedigree inference accuracy when using microsatellites (Dakin and Avise 2004;
295 Marshall et al. 1998). In this study, only one locus showed evidence of null alleles at
296 low frequency in the IIM-broodstock (*Hgu*-USC7; frequency between 3% and 6%
297 estimated either from family or population analysis, respectively), which suggests
298 caution for its use in parentage analysis. However, it could be still helpful given its high
299 polymorphism and exclusionary potential (A: 16; H_e: 0.892; Excl1: 0.620; Excl2:
300 0.766), especially if null allele frequency can be estimated in the candidate parents
301 using population or family data, as suggested (Dakin and Avise 2004). Genotyping
302 errors were only due to allele dropout at *Hgu*-USC8, accounting for 17% of parent-
303 offspring mismatches, a value slightly higher than that reported in other studies (Castro
304 et al. 2006; Hoffman and Amos 2005). Mutation was associated to 11% of mismatches,
305 providing an estimate of mutation rate of 4.16×10^{-4} averaged over the six loci tested.
306 This figure is within the range previously reported for microsatellite loci, including fish
307 (Banks et al. 1999; Castro et al. 2004, 2006, 2007; Ellegren 2004; Jones et al. 1999; Yue
308 et al. 2007).
309 Theoretical power for exclusion of false parents relies on several assumptions, which if
310 unfulfilled, could determine a deviation of real parentage assignment from expectations.
311 In our study, the six selected microsatellites did depart neither from Mendelian
312 segregation nor from independent transmission at all pairs of loci. In agreement with the
313 high combined theoretical exclusion potential over loci, high assignment success was
314 achieved, both in the actual scenario, as well as when all breeders were considered as
315 candidate parents (unstructured scenario) (100% and 99.8% assignation success,
316 respectively). These values indicate the potential of this microsatellite set for parentage
317 analysis in even more complex captive or wild scenarios. The EXC and ML approaches

318 performed quite similar in the context of this study. The selection of microsatellites with
319 low null allele frequency and high technical accuracy favoured the performances of the
320 EXC approach. Additionally, the low relatedness in the broodstock of wild origin (see
321 section 4.3) diminished the existence of ambiguities with the EXC approach or incorrect
322 assignments with the ML method (Jones and Ardren 2003; Marshall et al. 1998). The
323 actual assignment success in this study seems to be enough for the main purposes
324 related with *H. guttulatus* populations under captive conditions, such as evaluating
325 reproductive success of breeders and genetic mating system (see section 4.2). Also, to
326 obtain genealogical records needed for further translocation practices to other aquarium
327 facilities or wild populations.

328 4.2. Genetic mating system of *Hippocampus guttulatus* in captivity

329 Genealogical traceability of the offspring obtained under different sex ratio treatments
330 (Planas et al. 2008) allowed to assess the genetic mating system in the *H. guttulatus*
331 captive IIM-broodstock. Most batches (73.7%) were obtained in female-biased aquaria
332 and the remaining ones under unbiased treatment. Each batch from female-biased
333 aquaria was compatible with a single female, suggesting genetic monogamy within
334 single male broods. Similarly, each batch from unbiased sex-ratio treatment was
335 compatible with a single couple, and no simultaneous broods for the same female was
336 observed, indicating female monogamous genetic mating in the captive *H. guttulatus*
337 population under study. Single-brood monogamy in seahorses has been supported by
338 biological and behavioural characteristics (Stölting and Wilson 2007; Wilson et al.
339 2003), assuming the transference and incubation of all eggs from a single female into a
340 single male batch. However, repeated egg-transfer process over a few days has been
341 recently reported in *H. erectus* (Lin et al. 2008), which might suggest possible mate
342 change between successive egg-transfers. In this study, no signs of multiple maternity

within a single male clutch were found, supported by the high probability of genetic identity of the microsatellite set assayed (P>0.99) and the offspring sample size genetically analyzed, similarly to the previous genetic data reported in other seahorse species to date (Jones and Ardren 2003; Jones et al. 1998; Kvarnemo et al. 2000, 2007; Wilson and Martin-Smith 2007).

4.3. Microsatellite-based tool for relatedness estimation

Accurate estimation of relatedness between pairs of individuals is necessary to organize aquaculture broodstocks of unknown or wild origin without ancestry records (Pino-Querido et al. 2010). In absence of a complete pedigree, molecular estimates of relatedness can provide information about the relationships existing among individuals in a population and serve as a reasonable reference to organize seahorse breeders in aquaculture, avoiding inbred crosses. Given the complex reproductive behavior in seahorses (Foster and Vincent 2004), which precludes the design of targeted crosses, the detection of breeders with high kinship (first-order relatives) is essential to avoid their distribution within the same aquaria.

Actual family data from *H. guttulatus* in this work enabled to contrast this information with molecular relatedness estimated using the Wang's (2002) estimator. Its global accuracy was high irrespective of the reference population used to estimate allele frequencies, as previously reported (Pino-Querido et al. 2010; Wang 2002). The error, measured as the standard deviation (SD) for each kinship tested, was even lower for UR than reported in previous studies (Pino-Querido et al. 2010). However, higher errors were observed for FS, HS and the average over kinships, probably influenced by the limited number of HS and FS families available for genetic analyses from the captive breeding program. Regarding bias, higher figures were obtained when allele frequencies were estimated from the sample itself, as reported (Pino-Querido et al. 2010; Wang

2002). On the other hand, the W estimator provided good success in correctly classifying dyads in the main kinship categories occurring after one generation (close to 90% on average), using both the sample itself or the broodstock as reference populations. Particularly, actual family data yielded very high classification success of UR dyads (95%), quite high percentages of FS dyads correctly assigned (~70%), and low incorrect classification of UR (<1%) and HS (10%) dyads as first-order relatives. These values are within acceptable ranges described for minimizing inbreed mating and also to retain genetic diversity in captive breeding programs with small population sizes for threatened species (Ivy et al. 2009). Accordingly, relatedness coefficients using the W estimator were obtained between all pairs of *H. guttulatus* founders of the captive broodstock, of wild origin and unknown ancestry. In agreement with conformance to HW of the wild founder population, small global kinship within the broodstock was observed, 85% of total dyads showed low relatedness estimates, 77% of them even below a stricter cutoff (r<0). High relatedness figures were only observed for a very small fraction of dyads (1%), some of these could be suggesting putative pairs of first-degree relatives according to additional sampling data (same age and location) under a population decline scenario for this threatened seahorse species (Planas et al. 2008). The kinship classification has been considered for broodstock organization under minimum inbreeding criteria.

5. Conclusions

Results from this work are relevant for the genetic management of *Hippocampus guttulatus* in captivity. The set of six highly informative microsatellite loci proved to be helpful for solving unknown parentage in captive progenies obtained from wild long-snouted seahorses. The resolution of these contemporary pedigrees by parentage

assignment provided: i) evidence of monogamous genetic mating system under captive conditions for the first time in this species, relevant to understand and improve breeding success in captivity; ii) improvement of relatedness calculations across filial generations, thus, contributing to the short-term genetic management of long-snouted seahorse population; and iii) a useful set of real family data to evaluate the theoretical and actual power of the microsatellite tool for parentage and relatedness analysis in *H. guttulatus*. Accordingly, the same set of microsatellite markers could be used for exploratory relatedness estimation among broodstock individuals with unknown relationships for stock organization.

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Table 1. Genetic diversity and exclusion probabilities for six microsatellite loci in the founder population of a captive *Hippocampus guttulatus* broodstock.

Locus	A	H_o	H_e	PIC	Excl1	Excl2	SI
Hgu-USC5	9	0.755	0.796	0.757	0.411	0.588	0.625
Hgu-USC6	10	0.583	0.670	0.621	0.259	0.434	0.543
Hgu-USC7	16	0.776	0.892	0.872	0.620	0.766	0.685
Hgu-USC8	13	0.833	0.882	0.861	0.595	0.748	0.678
*Hgut*4	35	1.000	0.972	0.960	0.856	0.923	0.730
*Hgut*6	29	0.938	0.952	0.939	0.791	0.883	0.719
Mean/Global	18.7	0.814	0.861	0.835	0.9980	0.9999	0.9987
SD	10.8	0.147	0.112	0.127			

A: Number of alleles per locus; H_o, H_e: Observed and expected heterozygosity, respectively; PIC: Polymorphic information content; Excl1: Exclusion probability when no parent is known; Excl2: Exclusion probability when one parent is known; SI: Exclusion probability for identity of two siblings.

Table 2. Parentage analysis of 19 batches of newborn *Hippocampus guttulatus* seahorses obtained in captivity from IIM-broodstock.

Batches	N	Parents assigned Male-Female	Family
1	8	G1-G13†	FS 1
2	26	G1-G16	FS 2
3	11	G5-G33	FS 3
4	18	G3-G18	FS 4
5	20	G3-G18	
6	22	G3-G18	
7	26	G3-G18	
8	9	G24-G28	FS 5
9	25	G24-G28	
10	35	G24-G28	
11	14	G24-G28	
12	53	G47-G38	FS 6
13	12	G47-G38	
14	26	G42-G36	FS 7
15	15	G42-G36	
16	20	G42-G36	
17	13	G42-G36	
18	38	G45-G36	FS 8
19	10	G0-G6	FS 9

N: genotyped offspring samples

† Dead specimen

Table 3. Family analysis of relatedness estimation in *Hippocampus guttulatus*.

	Reference population	
	Broodstock	Offspring sample
UR	-0.017 (0.114)	-0.096 (0.122)
HS	0.198 (0.174)	0.142 (0.179)
FS	0.505 (0.212)	0.480 (0.214)
Bias	0.025 (0.024)	0.075 (0.048)
Error	0.167 (0.049)	0.172 (0.046)

Mean and standard deviation (SD) of the relatedness coefficient (*r*) for three kinship categories (full-sibs (FS), half-sibs (HS) and unrelated (UR)) were estimated using Wang's (2002) pairwise relatedness estimator and two reference populations for obtaining allele frequencies (IIM-broodstock and offspring sample itself). The mean and SD of the bias and error across kinships is also shown.