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A novel method for the identification of seahorses (genus *Hippocampus*) using cross-species amplifiable microsatellites

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ABSTRACT

Seahorses (genus *Hippocampus*) are flagship species for marine conservation and evolutionary studies. Wild seahorse populations have declined sharply because of overfishing or other disrupting activities, which are attracting increasing global concern for their threatened status. For study of their population status, the identification of young seahorses by morphological examination has been controversial and remains a difficult task due to overlapping of meristic and morphological characters. Here we reported a simple and sensitive method to identify seahorse species from China based on cross-species microsatellites. The cross-species amplification success rate varied from 40.0% to 90.0% depending on the species, with a mean value of 59.6%. According to allele presence/absence of cross-species microsatellites, all the individuals representing 10 seahorse species could be accurately identified using one or more loci. In addition, we found both genome size and genetic distance between the source and target species negatively correlated with microsatellite cross-species amplification success. Moreover, a Neighbor-Joining dendrogram was constructed based on genetic similarity and suggested that the 10 seahorse species could be divided into four groups. These results demonstrate our method could improve the identification of seahorses, providing data to understand seahorse stock structure and recruitment dynamics, and therefore resources conservation.

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1. Introduction

Seahorses (genus *Hippocampus*) are in the family Syngnathidae, which also includes pipefish, pipehorses, and seadragons. Seahorses have a worldwide distribution inhabiting both temperate and tropical oceans, with 46 species identified to date (Lourie et al., 1999; Lourie and Kuiter, 2008). The global trade in seahorses is estimated to exceed 20 million animals per year (Vincent, 1996) to fulfill the strong demand for traditional Chinese medicine, aquaria and souvenir markets (Koldewey and Martin-Smith, 2010). However, due to the intense exploitation of seahorse populations, habitat fragmentation and pollution, over the last decades, wild populations have progressively declined and seahorse species are threatened (Vincent, 1996; Lourie et al., 1999; Martin-Smith et al., 2004; Lin et al., 2008). For this reason, all seahorse species have been included in Appendix II of the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) since 2002.

Analysis of species composition, abundance, dispersal and recruitment dynamics can help predict populations' demographic patterns and genetic structure of populations. These are important for developing appropriate, management strategies. Furthermore understanding the movement patterns of young seahorse is critical for recruitment dynamics, as well as spatial and temporal genetic structure. Traditionally, identification of young seahorses has relied on morphology. However, the morphological differences of seahorse species are small in their development (Teske et al., 2004; Kuiter, 2001). In addition, it is labor intensive and requires species-specific diagnostic characters, which may be absent, highly variable, or obscured by specimen damage during collection or preservation (Smith, 1977; Medeiros-Bergen et al., 1995).

With the rapid development of molecular markers, fish identification no longer depends on morphology alone. High-resolution molecular markers have been used for forensic studies, particularly microsatellites (Morgan and Rogers, 2001; Pampoulie and Daníelsdóttir, 2008; Wultsch et al., 2014). Microsatellites have high

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rates of mutation that occurs through slipped-strand mispairing and/or unequal crossover or gene conversion during recombination (Weber and Wong, 1993; Jeffreys et al., 1994; Pampoulie and Daníelsdóttir, 2008). Another characteristic of microsatellites is that the flanking sequences, where primers bind are conserved to a varying extent depending on the loci examined. Whereas some loci may be amplified over a range of taxa (Schlötterer et al., 1991), usually only closely related species share microsatellite loci and primer binding sites (Moore et al., 1991; D'Amato et al., 1999). Because of the specificity of microsatellite amplification, it is possible that these markers could be used as a simple method to identify seahorse species based on successful PCR reactions. In terms of species identification, compared with other DNA markers, the advantages of microsatellite marker for forensic science include (1) high conservation of flanking sequences including the priming sites ensures the PCR amplification much more reliable, repeatable and few null alleles are identified, as a consequence of higher accuracy of primer binding (Roques et al., 1999; Liu and Cordes, 2004; Pampoulie and Daníelsdóttir, 2008); (2) simple bands resulting from the microsatellite amplification are visualized by gel electrophoresis, and high species-specificity reduces the false positive rate (Liu et al., 2011).

Here we report a simple and sensitive method for young seahorse species identification using cross-species microsatellites and we also investigated how the amplification success relates to the genome size and the genetic distance between target and source species.

2. Materials and methods

2.1. Sample collection and DNA extraction

Hippocampus histrix (8 specimens), *Hippocampus mohnikei* (20), *Hippocampus trimaculatus* (20), *Hippocampus kelloggi* (20), *Hippocampus kuda* (20), *Hippocampus comes* (20), *Hippocampus barbouri* (8), *Hippocampus spinosissimuis* (15) and *Hippocampus ingens* (10) were collected along the coast of China between May 2010 and August 2014. The sampling sites include cities of Wenchang, Shanwei, Zhoushan, Qingdao and Qinhuangdao (Fig. 1). *Hippocamous erectus* (20), a western Atlantic species, were obtained from a seahorse-farming company (Shenzheng, China). Species identification was based on morphological characters described by Lourie et al. (1999). Then a non-lethal biopsy (dorsal fin) was taken from each specimen (Woodall et al., 2012) and preserved immediately in 95% ethanol for DNA extraction and subsequent cross-species analysis. Genomic DNA was extracted by using a salt-extraction method with slight modifications (Aljanabi and Martinez, 1997).

2.2. SSR mining and primer design

The microsatellites were mined from the scaffolds using the SSR Hunter software (http://www.bio-soft.net/dna/). The criterion was set for detection of tri-, tetra-, penta- and hexa-nucleotide motifs with a minimum of 5 repeats, respectively. 15, 10 and 10 loci were randomly selected from genome database of *H. trimaculatus*, *H. comes* and *H. erectus* genome database (unpublished) for primer design, respectively. Primers flanking of the microsatellites were designed using Primer 3 software (Rozen and Skaletsky, 2000). All SSR primer pairs were synthesized by Invitrogen Co. Ltd. (Shanghai, China).

2.3. PCR amplification and electrophoresis

The cross-species amplifications were tested for 35 newly developed SSR loci with 161 individuals from the 10 seahorse species. As a positive control, COI gene was amplified in each individual. The

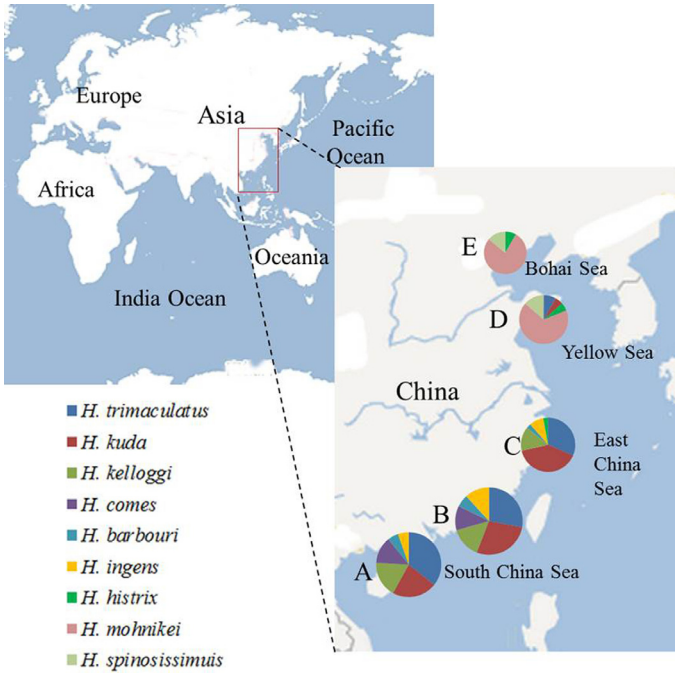

Fig. 1. Geographical location of sampling sites. Each color represents one seahorse species; each pie chart represents one sampling site ((A) Wenchang; (B) Shanwei; (C) Zhoushan; (D) Qingdao; (E) Qinhuangdao) and shows the proportion of each seahorse species.

reagents for PCR amplification were bought from Tiangen Biotechnology Co. Ltd. (Beijing, China). All the amplifications were carried out in a 10 μL volume containing 1 μL 10× buffer (with Mg^{2+}) for Taq DNA polymerase, 100 μM dNTP, 0.5 μL primer pairs, 1 U Taq DNA polymerase and 50 ng genomic DNA. The PCR thermal cycle profile was 5 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at optimized annealing temperature (Supplementary Table S1), 30 s at 72 °C and a final extension at 72 °C for 8 min; at last storing at 4 °C. The PCR products were separated by electrophoresis on 1.5% agarose gel with voltage of 90 V lasting for about 20 min.

2.4. Data analysis

To explore whether genome size (GS) affects the cross-amplification rate, the newly designed primers from *H. trimaculatus, H. comes and H. erectus* were cross-species amplified in 60 samples. Genome size were obtained from the genome data of seahorses, including *H. trimaculatus* (0.431 G), *H. comes* (0.688 G) and *H. erectus* (0.438 G). The cross-species amplification success of microsatellite loci was analyzed using the method as described in Hendrix et al. (2010). The amplification success was classified into four categories: (i) loci showing clear signals of amplification, (ii) loci with identifiable, but weak signals, (iii) loci without amplification success, and (iv) loci that showed multiple banding patterns (i.e., three or more alleles, which could not be scored unambiguously according to size and intensity). Amplified products that referred to categories (iii) and (iv) were considered as a negative result of cross-amplification. Subsequently, the proportion of cross-species amplification success was tested for a correlation with the genome size among *H. trimaculatus*, *H. comes* and *H. erectus* using Statistical Analysis System (SAS).

The pairwise genetic distance among the 10 seahorse species has been measured using the cytochrome *b* gene of mitochondrial DNA by Casey et al. (2004). The pairwise genetic distance is ranged from 0.07 (*H. comes* vs *H. barbouri*) to 0.186 (*H. trimaculatus* vs *H. mohnikei*). A correlation analysis between genetic

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