Fisheries Research 185 (2017) 137–144

Contents lists available at ScienceDirect

Fisheries Research

journal homepage: www.elsevier.com/locate/fishres

Full length article

DNA barcoding reflects the diversity and variety of brooding traits of fish species in the family Syngnathidae along China's coast

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ARTICLE INFO

Article history:
Received 22 April 2016
Received in revised form 9 September 2016
Accepted 15 September 2016
Handled by Prof. George A. Rose
Available online 20 September 2016

Keywords:
Seahorse
Pipefish
DNA barcoding
COI
Conservation

ABSTRACT

DNA barcoding offers a rapid and accurate assessment for species labelling and identification. Here, we investigated the performance of DNA barcodes in a sample of Syngnathidae, a unique group of fish with male pregnancy. A total of 1002 DNA barcodes using 649 base-pair fragments of the cytochrome c oxidase subunit I (COI) gene were generated. All species were associated with distinct DNA barcode and could be readily distinguished; seven of the COI barcode clusters represented the first species records submitted to the Barcode of Life Data Systems (BOLD) and GenBank databases. In the Neighbor-joining tree of COI sequences, two major clusters (Gastrophori and Urophori) were displayed, which could also be classified by their brood pouches. However, the tail-brooding *Hippichthys cyanospilus* and trunk-brooding *Syngnathoides biaculeatus* were reverse-clustered together with the Gastrophori and the Urophori, respectively. Both seahorse and pipefish sequences showed high frequencies of nucleotide substitutions. The probability of nucleotide substitutions, those in pipefish were higher than that of all seahorses. However, we identified no signal for positive selection based on the COI gene in any of the data sets. Our results supported DNA barcoding as an efficient molecular tool for achieving better monitoring, conservation, and management of fisheries.

1. Introduction

The family Syngnathidae (seahorses, pipefish, and seadragons) is the sole vertebrate group in which embryonic development occurs within a special pouch in males (Herald, 1959). Seamoths may be the primitive sister group of the seahorses, pipefish and seadragons (Pietsch, 1978). About 41valid seahorse species and over 400 pipefish species have been described along the majority inhabiting shallow seabed of the Indo-central western Pacific Oceans (below latitude 26°N) (Dawson, 1985; Koldewey and Martin-Smith, 2010; Lourie et al., 2016). Seahorses sold as traditional Chinese medicine, ornaments and aquaria presentation have shown increasing value in recent years (Koldewey and Martin-Smith, 2010; Lin et al., 2008). At least 77 countries trade seahorses in high volumes, meaning that various species of seahorse are being harvested on a large scale (McPherson and Vincent, 2004). Seahorses are frequently taken in as trawl by catch. This process makes their habitats vulnerable to degradation and destruction, which has led to a sharp decline in wild seahorse stocks. Unsustainable exploitation of seahorses, in addition to their unique reproductive system and morphology, have led researchers to pay increased attention to the large gaps in knowledge of wild seahorse biology and ecology (Woods, 2002).

There is taxonomic ambiguity for several genera and species. The seahorse and pipefish were originally placed in the same lineages based on morphological and biological characteristics (Wilson et al., 2001). More recently, the identification of fish in the family Syngnathidae has primarily been based on morphological recognition; however, the considerable degree of skill and taxonomic expertise required often complicate species identification (Gutiérrez et al., 2014). As an alternative, molecular methods like DNA barcoding were utilised to explore taxonomic issues. DNA barcoding is one potentially important technique used to identify species and determine biological diversity (Hebert et al., 2003a). The effectiveness of this technique relies on the relatively short universal molecular tag (approximately 650 bp from the 5′ region of the mitochondrial cytochrome oxidase I [COI] gen), which is substantially greater between than within species. Therefore, this approach has been widely applied across phylogenetically distant animal groups (Hajibabaei et al., 2006; Hebert et al., 2004). Notably, the development of DNA barcode libraries is based on community efforts, and the use of the Barcode of Life Data Systems (BOLD)

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http://dx.doi.org/10.1016/j.fishres.2016.09.015
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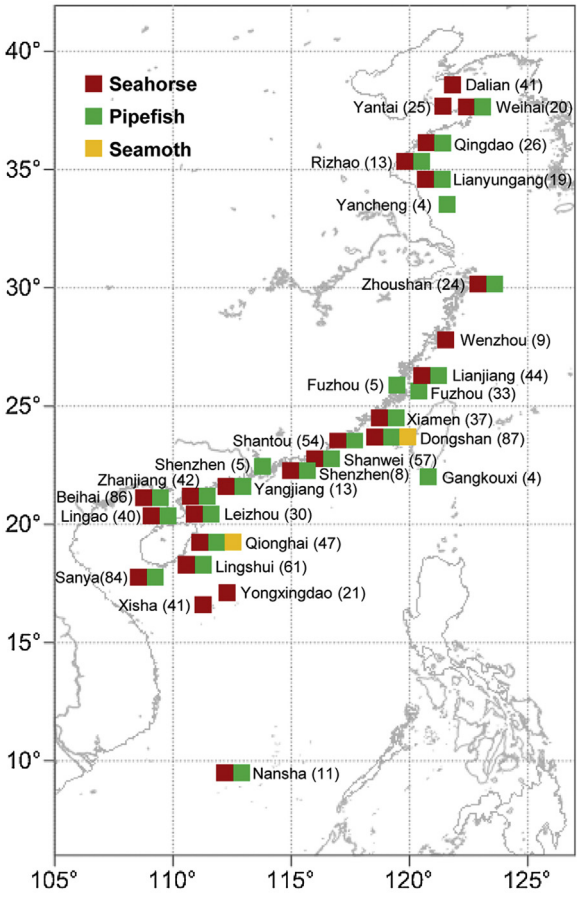

Fig. 1. Map generated using Surfer® for Windows showing the sampling locations for fishes along China's coast. Sample sizes per site are given in parentheses.

has resulted in DNA barcoding technology being commonly viewed as the gold standard in species identification. To date, many published papers have explicitly shown that COI barcoding can be used to effectively discriminate between fish species (Keskin and Atar, 2013; Knebelsberger et al., 2015, 2014; Ribeiro et al., 2012; Ward et al., 2005; Zhang and Hanner, 2012).

This data release presents the results of a DNA barcoding study of the seahorse and pipefish of China. China has a long distance coastline, and the constant ocean area along the mainland crosses an oriental zone, warm-temperature zone, and tropical zone (Briggs, 1995), which leads to wide distributions and abundances for some fish of the family Syngnathidae (Zhang et al., 2014). In the present study, we applied DNA barcoding for the identification of this group of fish along China's coast, which is regarded as an important branch of the marine biodiversity center of the Indo-West Pacific Ocean (Barber et al., 2000). The DNA barcoding data generated in this study will be used as an efficient molecular tool for achieving better monitoring, conservation, and management of the family Syngnathidae.

2. Material and methods

2.1. Sample collection

A total of 485 seahorses, 459 pipefish and 58 seamoths were collected from 31 locations along China's coast (Fig. 1) (collection information available at http://www.barcodinglife.org/). The sampling location map was created using Surfer® for Windows (Keckler, 1997). Most specimens were collected by researchers on board trawl boats, and a few were obtained with the help of local fishermen and buyers. A dorsal fin was removed from each specimen and was preserved in 95% ethanol until DNA isolation; then, all of the live seahorse specimens were released back into the sea. Vouchers were morphologically identified to identification reliability level two, as described by the Fish-BOL collaborators' protocol (Steinke and Hanner, 2011), namely, 'specimen identified by a trained identifier who had prior knowledge of the group in the region or used available literature to identify the specimen'. References in the literature used in our study are as follows: Cheng (1962), Lourie et al. (1999), Zheng (1987) and Zhu et al. (1963).

2.2. DNA extraction

A dorsal fin from each specimen was removed and macerated using phosphate-buffered saline (PBS) buffer for extraction. The macerating tissue from the specimens was frozen in liquid nitrogen and then ground into powder. Genomic DNA was extracted using an AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Biosciences, USA) following the manufacturer's protocol with minor modifications: the tissue homogenate was incubated at 56 °C for 2 h during cell lysis with Proteinase K. All DNA samples were stored at −80 °C until polymerase chain reaction (PCR) amplification.

2.3. PCR and DNA sequencing

Fragments of the mitochondrial COI gene were amplified using the following universal fish barcoding primers: forward Fish-F2 5′-TCGACTAATCATAAAGATATCGGGAC-3′ and reverse Fish-R2 5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′ (Ward et al., 2005). All PCR reactions were conducted in a total volume of 50 μl, utilising 3 μl (10–100 ng) DNA, 0.25 μl Taq DNA polymerase (5 U/μl, TaKaRa, China-Japan Joint Company, Dalian, China), 1 μl of each primer (10 μM), 4 μl dNTP Mixture (2.5 mM), 5 μl Ex Tag Buffer (10×), and 35.75 μl ddH_2O. The thermocycling sequence was performed with an initial step of 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, annealing at 52 °C for 30 s, 72 °C for 1 min, and a final step at 72 °C for 10 min. The amplified PCR products were checked for optimal fragment size on 1.5% agarose gels. The purification of PCR products for sequencing was conducted using an E.Z.N.A.® Gel Extraction Kit (Omega, USA). COI genes were commercially sequenced using PCR-purified products (BGI, China).

2.4. Data analyses

Nucleotide sequences were assembled and edited using BioEdit 7.0.9.0 (Hall, 1999) and then aligned using ClustalW (Larkin et al., 2007). The sequences were submitted to GenBank (accessions KP139900-KP140670 and KT355036 – KT355266), and the sequences and specimen details were submitted to BOLD (see Supplementary Table A online). The numbers of haplotypes for each species were estimated using the software DnaSP 5.10.00 (Librado and Rozas, 2009). Nucleotide compositions and nucleotide pair and codon usage frequencies were calculated using MEGA 6.0 software (Tamura et al., 2013). Sequence divergences were calculated using the Kimura 2-Parameter (K2P) distance (Kimura, 1980). The intraspecific distances, interspecific values within the same genus, and interspecific values between genera within the same family were so calculated, respectively. This system usually makes a suitable metric model when genetic distances are small (Nei and Kumar, 2000). An unrooted neighbor-joining (NJ) tree was created based on K2P distances using MEGA software (version 6.0) with bootstrap tests of 1000 replicates (Tamura et al., 2013). *Pegasus volitans* and *P. laternarius* were used as outgroups. To identify the variable selective pressures in COI, we employed the CODEML algorithm from the PAML package (Yang, 2007). The improved branch-site model (Yang and Nielsen, 2000; Zhang et al., 2005) was used to estimate the ratio (ω) of the rates of nonsynonymous