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Research on Barcoding of the Indian Freshwater Pipefish and Intra-Species Variations within the Barcode Region

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Abstract
Rapid loss of marine and terrestrial biodiversity is a result of the global biodiversity crisis. Managing species diversity becomes crucial. DNA barcoding is a new technique for identifying biological items and managing species diversity. In our research, we used the freshwater pipefish *Microphis brachyurus*. We analyzed intra-species variations discovered in the barcode regions of samples from Tamilnadu, India, and Chiba, Japan. Multiple sequence alignment with CLUSTAL 2.0.10 was used to analyze intra-species variations. SNPs have been found in freshwater pipefish collected from Indian seas, according to the results.

Keywords: freshwater pipefish, COI, DNA barcoding, mitochondrial DNA, PCR, clustal W

Introduction

Despite the fact that marine species have evolved to live in the ocean, it's possible that the changing climate is shifting their environment more quickly than they can adjust to it. (Jones *et al*. 2013; Munday *et al*. 2013). Marine biologists have progressively started to grow more concerned about the survival of marine species and their offspring due to the way that the warming climate is changing the ecosystem of the ocean. (Komyakova *et al*. 2013; Wu Y *et al*. 2013).

The more extreme viewpoint, sometimes referred to as "molecular taxonomy," aims to replace conventional (morphology-based) nomenclature with pure molecular classification. It will be more effective to understand marine life if a DNA-based tool is created to validate identification and compare sequences in order to investigate species diversity.

DNA Barcoding

The limitations of recent advancements in biodiversity analysis and the realization that our knowledge of the world's biodiversity is insufficient are the primary factors influencing new developments in species identification. (Mostofa *et al*. 2013; Sutherland *et al*. 2013; Martín *et al*. 2013). According to current estimations, there are a lot of marine animals. Even with the lower estimations, we were only able to identify a small portion of the vast diversity of life (Qin *et al*. 2013). The systematic frontline for tackling concerns of life's diversity is made up of taxonomists and systematists (Mat Jaafar *et al*. 2012). The entire scientific community as well as society at large are in fact affected by this taxonomic barrier, which frequently makes it impossible to obtain taxonomic knowledge (Wang *et al*. 2013). With the help of DNA barcodes, laypeople can now recognize species on their own, even from small, damaged, or chemically changed materials (Viswambharan *et al*. 2013). A "DNA barcode" is an uncommon pattern of DNA sequence that identifies each living item, just like the distinctive pattern of bars in a Universal Merchandise Code (UPC) categorises each buyer's product. Computer algorithms could quickly read short DNA barcodes, roughly 700 nucleotides in length, from thousands of specimens and specifically examine them (Weigt *et al*. 2013a, b, Ribeiro *et al*. 2013). DNA barcoding is different from traditional taxonomic detection methods and instruments in a number of ways, and it has many advantages over them. (Ward 2009). The standardization of a global master key in a format that removes ambiguity and makes it simpler to directly compare specimens to a global reference database allows for species identification from remains and at any point of their life cycles. (Scherner *et al*. 2013; Silveira *et al*. 2013).

A stream or river pipefish from the Syngnathidae family, *Microphis brachyurus* is primarily found in fresh and brackish waters (Mabraga *et al*. 2011; Brown *et al*. 2013). Most of this fish's distribution is in the Indo-Pacific region (Lakra *et al*. 2011). The family Syngnathidae, which has roughly 52 genera, includes pipefishes. The two subfamilies of the family Syngnathidae are Syngnathinae and Hippocampinae. Pipefishes and pipehorses belong to the Syngnathinae family, whereas seahorses of the Hippocampinae family belong to a single genus. Duncker (1908) asserts that the primary body ridges formed the basis for the early classification of pipefishes.

The Promise of DNA barcoding

DNA barcoding offers to open doors to a wide range of scientific and social applications and for a variety of end-users, from the scientific expertise to the individual citizen. This is because it helps to remove the "taxonomic impediment," which will in turn enable a variety of scientific and social applications. Our improved ability to recognize existing and cryptic species will be of benefit to environmental sciences, forensics, pharmaceutics, agriculture, conservation, biological and molecular evolution, to countermeasures to biological warfare, to name but a few (V.Savolainen et al. 2005) . One of the most direct beneficiaries of DNA barcoding may well be the scientific discipline of taxonomy. With this new and powerful tool,

taxonomists can be freed from maintenance and routine tasks, and focus instead on the description and investigation of newly discovered species, (Ibid) thus greatly accelerating the rate of new entries in the encyclopedia of life. However, crucially for addition of any species' DNA barcode to BOLD, it will remain necessary to deposit a voucher specimen, (C. Schander and E.Willassen, 2005) a requirement that emphasizes the intended integration of DNA barcoding with the Linnaean system.

Benefits will likely extend to more than purely technical aspects, and many view DNA barcoding as a key opportunity to revitalize the scientific discipline of taxonomy, (CBOL homepage : http://barcoding.si.edu/) which has progressively become one of the most underfunded within biological sciences. In reality, differing perspectives on the potential effects of DNA barcoding in taxonomy have been a topic of lively debate: (Smith, 2005) some critics argue that it will signal the end of a vital but dormant discipline. (Will,K.W., B.D,Mishler, and Q.D. Wheeler, 2005) while for others it is a valuable opportunity to revolutionize and revitalize the subject (Herbert and Gregory, 2005, D.E.Schindel and S.E.Miller, 2005 and T.R.Gregory, 2005).

Such a debate might soon become a redundant one, since the prime concept and current practice of DNA barcoding is built upon establishing a match between a known vouchered species and a DNA sequences. The development of taxonomy and biodiversity inventories follows naturally from the success of DNA barcoding. By highlighting the importance of taxonomists and the value of taxonomy, the Barcode of Life Initiative has already begun to increase funding for the field. Unprecedented thorough biodiversity inventories have been sparked by DNA barcoding. The barcode of Life Initiative has already started to draw attention to the value of taxonomy and the key role of taxonomists, and has attracted new sources of funding for the discipline. DNA barcoding has prompted unprecedented large-scale biodiversity inventories, which will provide new raw materials for taxonomy and systematics. It is raising standards for incorporating taxonomic information into genomic data repositories. (Hanner et al, 2007). Moreover, it is establishing a new and valuable type of genetic bank (by means of archiving tissue samples or DNA extracts) from which the genome of each species can be accessed in the future. (P.R.Becker et al. 2006 and The Ocean Genomic Legacy) Hence, benefits start to emerge, not only for taxonomy, but also for other disciplines within biological sciences and related scientific fields.

The impacts of Barcode of Life Initiative are expected to extend beyond the scientific arena and ultimately influence society as a whole. Societies will be able to manage biological resources in a way that is more sustainable and ethical as we gain more information about the biodiversity of the planet. Ironically, the taxonomic impediments is most acute in developing countries, where biodiversity is highest (Schindel and Miller, 2005). DNA barcoding has the potential to democratize access to taxonomic information worldwide and open the doors of biodiversity information to the ordinary non-expert individual. These features of DNA barcoding include rapid, precise, and cost-effective specimen identification.

The ultimate development of a handheld device that could be used to identify any life form at anytime, anywhere, and at little to no cost is one of the most iconic goals of the Barcode of Life Initiative. (Savolainen et al, 2005 and D.H.Janzen, 2004). Such a "Bio-pod" would not only help identify the species, but it would also make it possible to link to the entry for that species in the Encyclopedia of Life on the Internet, which includes images and related data. The commentary on a DNA barcoding article that was published on a free website can be found below. It synthesizes in a rather spontaneous fashion the type of reaction that the average citizen may have to the concept of a Bio-pod.

Materials and Methods

DNA Sampling

The most up-to-date standard in genetic resource collection uses a system of redundancy: in an ideal scenario, two arcival quality tissue samples were immediately taken from each specimen, one frozen to preserve the widest range of molecular character possible, and one was placed into a preservation fluid, like ethanol, to serve as a back up in case of a meltdown or loss of the frozen specimen. Fish DNA can be extracted from a variety of tissues. The following are among them.

Musculature: From the right side of the specimen, one or more lateral culex (5–7mm) were cut out.

Gill tissue: From the right side, one or more gill arches with associated filaments were taken off.

Eye: From incredibly tiny specimens, such as larvae, the right eye was removed.

Instead of sub-sampling in species with small bodies, whole specimens can be preserved. Unless a group of conspecifics is available for normal morphological study, this should be avoided.

For DNA extraction, tissue samples were either frozen or preserved in fresh 95% ethanol and kept in a cool environment, preferably a freezer. To allow for appropriate fluid penetration, large tissue fragments were divided into smaller pieces (<5 – 7mm).

Extraction of mtDNA

Day I

A blood sample that had been kept at 4°C in 95% ethanol was gently shaken before 50 ml of blood or 5 x 5mm fin clips/20 mg of muscle were taken in eppendorf tubes. Following a 20-minute, 10,000 rpm centrifugation at 40°C, ethanol was decanted. For the correct suspension of the cell, 1 ml of high TE was added to the pellet. TE was then discarded after it was centrifuged one more for 10 minutes at 10,000 rpm. After centrifuging, the previous process was repeated with a high TE. Each tube received 0.5 ml of the incubation buffer before the sample was heated in a water bath to between 55 and 60 °C and incubated at 37 °C overnight.

Day II

From the waterbath, a sample containing buffer was obtained. Each tube first received 1.5 millilitres of lysis buffer and 2 millilitres of Tris-saturated phenol. Then it was slowly rocked for 10 minutes, followed by 10 minutes of centrifugation at 12,000 rpm and 40C.

This was mixed with 2ml of chloroform: isoamyl alcohol (24:1 V/V), carefully shaken for 10 minutes, and then centrifuged at 10,000 rpm at 20 ^{0}C. Supernatant was removed using a pipette with a 1 ml cut tip while avoiding the interphase layer. The supernatant in each tube was then dissolved in 2ml of chloroform and isoamyl alcohol, which

were mixed in a 24:1 ratio. After being shook gently for 10 minutes, it was centrifuged at 10,000 rpm for 10 minutes at 20^0 C.

Air-filled supernatant was carefully pipetted out to avoid mixing with the bottom layer. 1/10 (150 l vol) of 3M sodium acetate (PH 5.2) and 2.5 times (3.5 ml) ice-cold absolute ethanol were added to the aforementioned solution. After obtaining the DNA, the solution was slowly and completely mixed. All of the tubes were then placed on ice for an hour. The solution was then centrifuged once more for 15 minutes at 12,000 rpm at 4^0C. Finally, ethanol was carefully poured out. The pellet's location was marked, and 2 ml of 70% ethanol was added before it was centrifuged once more at 10,000 rpm for 10 min at 4^0C. Once more, ethanol was dumped with care. The tubes were left inverted to empty out any remaining ethanol and were let to dry naturally for five to ten minutes. Then, at P^H 8, TE was added. By gently shaking the tube, the pellet was loosened from its walls. At 4 ^{0}C, a DNA sample was kept.

Day III

Samples were taken out from the 4^0C environment and tested to see if the pellet had dissolved or not. 1 l of RNAase was added to the table above, and the sample was heated to 37°C in a water bath for two hours before being once more stored at 4°C.

Procedure

The master mixture was made, and each PCR tube was organised by labelling. Then, 24µl of it was added to each PCR tube. Following the addition of 1µl of genomic DNA to each tube, the contents were gently shaken to mix, and 25µl of autoclaved PCR oil was then added. After that, the PCR machine was turned on and the programme ran for at least 15 minutes before the sample was placed inside. The PCR was then carried out under ideal circumstances while the tubes were still in the thermal cycler.

The DNA was isolated and kept at 200°C until it was shipped in a light-proof container to a sequencing facility.

Results

The data representing different geography of barcode sequences of *Microphis brachyurus* used for intra- species variation analysis is mentioned below (Table I).

Table 1: Representing different geography of barcode sequences of *Microphis brachyurus* used for intra-species variation analysis

S.No.	Sequence name	Location	Country	Latitude/Longitude
1	C1	Chiba	Japan	35º36'N/140º05'E
2	C2	Chiba	Japan	35º36'N/140º05'E
3	IOBML 201	Parangipettai	India	11º30' N/ 79 º 47'E

Barcoding *Microphis brachyurus*

After sequencing of COI, around 570 nt were recovered from the sequence chromatogram following COI sequencing. The NCBI (National Center for Biotechnological Information) database was searched for the COI sequence of Microphis brachyurus from international seas. Only two sequences from Japan's Chiba seas (latitude: 35°36'N, longitude: 140°05'E) could be found and used for the analysis (Acc. Nos. NC 010273 & AP005986).

Microphis brachyurus' COI sequence was uploaded to the Barcoding of Life Database (BOLD), where it may be accessed using the accession number IOBML 201.

Intra-species variations within *Microphis brachyurus*

To detect the intra-species variations within the barcode region of *Microphis brachyurus* Clustal W was employed. Clustal W could be worked through online via http://www.ebi.ac.uk/Tools/clustalw2/index.html.

Discussion

When compared to Chiba species, a single nucleotide polymorphism (SNP) was found in the barcode area of *Microphis brachyurus* from Parangipettai waters. Sites of 61nt, 467nt, and 484nt show SNPs. It is evident that the *Microphis brachyurus* COI gene contains phylogeographic information.

In the barcode region of *M. brachyurus* from Parangipettai waters, Thymine was discovered to have a greater molar content than the other nucleotides, although Guanine was on the lower end.

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