International Research Journal of Natural and Applied Sciences
Volume-1, Issue-7 (December 2014) ISSN: (2349-4077)

DNA BARCODE OF TROPICAL LOACH , LEPIDOCEPHALUS THERMALIS (VALENCIENNES) OF TAMIL NADU

[1.]Jaculine Pereira. J*, [2] R. Jeya Shakila [3.]B. Ahilan,[4.] and K.Karal Marx,

[5.]C.B.T. Rajagopalsamy

1. Assistant Professor

2. Professor, Department of Fish Quality Assurance and Management,

3. Professor, Department of Aquaculture ,

4. Professor and Head, Department of Fisheries Biotechnology,

5. Professor and Head, Department of Aquaculture

Fisheries College and Research Institute

Thoothukudi-628 008

Tamil Nadu Fisheries University

Tamil Nadu

ABSTRACT

*The Tropical loach, **Lepidocephalus thermalis** was collected from various regions of Tamil Nadu. Morphometric and meristic characterization were carried out for L. thermalis and DNA was isolated from L .thermalis using phenol chloroform rapid non-invasive technique. Purity of DNA was checked with UV spectrophotometer at 260 nm and 280nm and DNA was confirmed with 1% Agarose gel Electrophoresis. DNA barcoding was carried out for the DNA*

isolates using Cytochrome C Oxidase subunit 1(COI) gene. Polymerase Chain Reaction (PCR) was carried with COI gene primer and the PCR product was confirmed with 2 % Agarose gel electrophoresis using 100bp DNA ladder and DNA content was analysed with Biophotometer. PCR products were sequenced and submitted to Genbank (NCBI).

Keywords: Tropical Loach, DNA barcode, COI and DNA signature

Introduction

DNA barcoding is designed to provide accurate and automated species identification through molecular species identification based on standard gene region. Research using cytochrome *c* oxidase barcoding techniques on zoological specimens was initiated by Hebert et al. (2003). By March 2004, the Consortium for the Barcode of Life started to promote the use of a standardized DNA barcoding approach, consisting of identifying a specimen as belonging to a certain animal species based on a single universal marker: the DNA barcode sequence. Over the last nine years, this approach has become increasingly popular and advances as well as limitations have clearly emerged as increasing amounts of organisms have been studied. Our purpose is to briefly expose DNA Barcode of Life principles, pros and cons, relevance and universality. The initially proposed Barcode of life framework has greatly evolved, giving rise to a flexible description of DNA barcoding and a larger range of applications.

Species identification and classification have traditionally been the specialist domain of taxonomists, providing a nomenclatural backbone and a key prerequisite for numerous biological studies. Indeed, today's society has to resolve many crucial biological issues, among which are the need to maintain biodiversity, to ensure bio-security, to protect species and to avoid pandemics. The achievement of such goals and the success of subsequent action programs require efficient global networks and rely on our capacity to identify any described species. As Dayrat (2005) clearly expressed, 'delineating species boundaries correctly – and also identifying species – are crucial to the discovery of life's diversity because it determines whether different individual organisms are members of the same entity or not'. The identification of species depends on the knowledge held by taxonomists whose work cannot cover all taxon identification requested by non specialists. To deal with these difficulties, the 'DNA Barcode of Life' project

aims to develop a standardized, rapid and inexpensive species identification method accessible to non-specialists (i.e. non-taxonomists). The idea of a standardized molecular identification system emerged progressively during the 1990s with the development of PCR-based approaches for species identification.

Scientists are now facing problems of erosion of earth's biodiversity due to human activity. Nowadays one of the most popular technique for molecular phylogenic is Cytochrome C Oxidase subunit 1 (COI) gene sequences which is usually utilized for species and family level analysis (Hebert *et al.*,2003 and Hebert *et al.*,2005). This technique provides an effective genetic characterization of the species identification by using COI gene. The primary goal of DNA barcoding focus on the assembly of reference literature of barcode sequence of known species to develop reliable genetic tool for species identification in the nature. In nature, intraspecific phenotypic variation often overlaps with that of its taxa and can lead to wrong identification which is based on phenotype only. DNA barcodes are most effective for various life stages of species identification and undetectable taxonomic diversity have been reported by various scientists (Ward *et al*, 2005 and Smith *et al*., 2007). Finally DNA barcode libraries are fully available as they are deposited in a major sequence database. Once the library is available recent studies with wide range of application can be applied to use them into genetic engineering ecology of IUCN Red listed communities, tracking of invasive species and for the identification of ecologically important species and genetically improved varieties. With the help of assigning species to know species based on the DNA tags, a 652 bp segment of the 5' region of the mitochondrial COI gene forms the library of primary barcodes for the fishes (Hebert *et al*.,2003). Mitochondrial DNA have several advantage that make it well suited for large scale DNA (molecular) tagging because it has large number of copies and also have advantages of high mutation rate and small effective population size which make an informative genome for evolutionary patterns and processes (Brown *et al.*, 1979 and Birky *et al.*, 1989).

The economic importance and identification challenges associated with fishes made to launch an international fish barcoding of life (FISH –BOL) with the aim of barcoding of both marine and freshwater fishes. In the context of FISH- BOL, this study was conducted and allows species identification of native fishes of Indian spiny loach *L. thermalis*. This paper emphasis

on DNA barcode study of Indian spiny loach, *Lepidocephalus thermalis*. *L. thermalis* is endemic to the Western Ghats. This species is coming under threatened in the IUCN Red list, it requires research and conservation as its habitat is in declining stage. The major threats to the species are tourism, wild catch, construction of dams and pollution.

Materials and method

Morphometric and Meristic characterization

Ninety numbers of *L. thermalis* were collected from various places of Tamil Nadu (Tirunelveli, Tenkasi and Thanjavur) and were subjected to morphometric and meristic characterization by following standard method.(Menon, 1992 and Jayaram, 2013). Then the fishes were preserved in 10% formalin for further analysis.

DNA Analysis

L. thermalis were preserved in fresh condition in absolute alcohol for molecular study. DNA was extracted from muscle tissue by following the method of Kumar *et al.* (2007) with modifications. Approximately, 50 mg of muscle tissues were taken from each individuals and dried on a tissue paper. The tissues were cut into small pieces and placed in a 2 ml-Eppendorf tube containing 940µl lysis buffer (200mM Tris-HCl, pH 8.0; 100mM EDTA, pH 8.0; 250mM NaCl), 30 µl Proteinase K (20mg/ml) and 30 µl of 20% SDS. The contents in the tubes were incubated at 50ºC for 45-50 min in a water bath. After incubation, an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1) was added to the tube containing lysed cells. The contents were mixed properly by gently inverting the Eppendorf tube for 10 min to precipitate the proteins and other part of the nucleic acids. The tube was centrifuged at 9,200 rpm for 10min. The top aqueous layer was transferred to a new 2 ml-Eppendorf tube. The DNA was then precipitated by adding equal volume of isopropanol and 0.2 volumes of 10 M ammonium acetate and inverting the tubes gently for 10 min. The precipitated- DNA was pelleted by centrifugation at 13,200 rpm for 10min. The supernatant was removed by pouring out carefully,

without loss of DNA pellet. Then the pellet was washed in 500 µl chilled 70% ehanol, air dried and resuspended in 200 µl TE buffer. Finally the DNA was confirmed with 1% agarose gel electrophoresis using ethidium bromide (Fig.1)and the purity was checked with UV spectrophotometer.

Polymerase Chain reaction (PCR) and Sequencing

The DNA isolates were amplified with gradient PCR thermal cycler (Eppendorf). A 652-bp segment was amplified from the mitochondrial COI gene using primers FP 5'TCA ACC AAC CAC AAA GAC ATT GGC AC3' and RP 5' TAG ACT TCT GGG TGG CCA AAG AAT CA 3' (Hubert *et al*.,2008 and Collins *et al*.,2013). PCR amplifications were performed in 25µl volume including 21 µl of PCR master mix 1 µl of forward primer 1 µl of backward primer and 2 µl of template DNA. The PCR conditions consisted of 94^0c for 2mn, 35 cycles of 94^0c for 30s, 52^0 for 40s and 72^0c for 1 min with a final extension of 72^0c for 10min and 4^0c for ever (Hubert *et al*.,2008). The PCR products were confirmed with 2% agarose gel electrophoresis using ethidium bromide. The molecular weight of the PCR products (652 bp) were determined with 100bp DNA ladder. The DNA content in the PCR product was analysed with Biophotometer (Eppendorf) (Table 3).

The sequences of the PCR products (5 replicates) were analysed by Eurofins Genomics India Ltd.(AB Applied Biosystems). The sequences were aligned and compared with NCBI BLAST nucleotide sequences and finally one sequence was submitted to Genbank (NCBI).

Results and discussion

Morphometric and meristic characters of *L. thermalis* were analysed (Table 1). Mercy *et al.* (2007) reported the morphometric and meristic characters of *L. thermalis* and her findings are closely similar to the present study (Table1). *L. thermalis* were observed as grey to delicate grey-green with somewhat dark 8 to 10 irregular blotches along flanks, a small black spot on the upper half of base of caudal fin dorsal and anal fins with rows of spots. In addition, very little is known about its feeding ecology, reproductive biology, mortality rates and longevity,

therefore,needs to focus on filling these knowledge gaps. It is need of extensive microgeographic surveys and improved taxonomic research (Dahanukar *et al.* 2011, Emmanuel *et al*,.2013 and Raghavan *et al*.2012,2013)

The isolation of high quality DNA for genetic analysis has become one of the major concerns for DNA based techniques especially when a large number of samples must be analysed. In the present study a very rapid (around 2hr), simple, reproducible and less expensive method for DNA extraction from fresh and ethanol preserved fish muscle tissue has been described. This protocol is faster even than the ultra fast method of DNA extraction proposed by Kumar *et al.* (2007). However, there are some difficulties due to consistency and small size of these tissues which can led to a low amount and poor quality of total recovered DNA (Wasko *et al* .,2003) (Table2).The DNA isolated from fish muscle from different parts of Tamil Nadu have been confirmed in agarose gel electrophoresis is presented in Figure 1.

As per Wasko *et al*.(2003) and Kumar *et al*.(2007) the purity of DNA observed with UV spectrophotometer (A_{260}/A_{280}nm) was 1.60-2.1, this study the result showed was1.65-1.84.so it is very close to the result of Wasko *et al*.(2003) and Kumar *et al*.(2007) (indicating a good quality DNA) (Table 2).In this technique, DNA isolation and PCR amplification can be perfomed on the same day Kumar *et al*.(2007). This study has shown the efficacy of COI barcodes for diagnosting the loach, *L. thermalis* of Tamil Nadu. The success of the barcoding approach depends on the genetic distances between conspecific individuals and heterospecific individuals.

As per Hubert *et al.* (2008) COI barcodes of different isolates of different species recorded was 652bp, In the present study, the different isolates of *L. thermalis* of Tamil Nadu recorded also was 652bp. (Figure 2). The DNA content in the PCR products also analysed (Table 3). Sequencing was carried by Eurofins Genomics of India, Bangalore and the Sequences were submitted to Genbank (NCBI). Although barcode analyses primary seek to delineate species boundaries at the COI locus for the assignment of unknown individuals to known species, unsuspected diversity and overlooked species are often detected through barcode analysis (Hebert *et al.*,2004; Pons *et al*.,2006; and Hubert *et al*., 2008).

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Table 1. Morphometric and meristic characters of Tropical loach, *Lepidocephalus thermalis*

Morphometric	Mean± S.E
Total length	6.5cm±0.5
Standard length	5.0cm±0.5
Head length	0.9cm±0.05
Snout length	0.4cm±0.02
Eye dia	0.2cm±0.01
Dorsal fin length	0.9cm±0.02
Anal fin length	0.7cm±0.02
Pectoral length	0.85cm±0.02
Pro pelvic length	3.0cm±0.01
Pre dorsal length	3.0cm±0.01
Pre anal length	4.2cm±0.2
Pre pectoral length	1.4±0.02
Post dorsal length	2.8cm±0.2
Pelvic axilary scale length	0.4cm±0.02
Heed depth	0.75cm±0.02
Body depth	0.1cm±0.01
Meristic	

Dorsal fin spines	2-3
Dorsal fin rays	5
Anal fin spines	2-3
Anal fin rays	5
Pectoral fin spines	1
Pectoral fin rays	6-7
Ventral fin spines	1
Ventral fin rays	6

Table 2. Purity of DNA analysis with UV spectrophotometer - Indian Spiny loach,

Lepidocephalus thermalis

Sl.No.	260nm	280nm	A_{260nm}/A_{280nm}
1	0.100	0.059	1.69
2	0.102	0.057	1.79
3	0.105	0.059	1.78
4	0.112	0.065	1.72
5	0.110	0.062	1.77
6	0.101	0.056	1.8
7	0.115	0.066	1.74
8	0.103	0.056	1.84
9	0.102	0.060	1.70
10	0.102	0.062	1.65

Table 3. Analysis of DNA concentration in the PCR products with Biophotometer - - Indian Spiny loach, *Lepidocephalus thermalis*

Sl.No.	A_{260nm}/A_{280nm}	DNA conc. (µg/ml)
1	1.61	5.8
2	1.62	7.3
3	1.66	5.1
4	1.5	7
5	1.59	3.2
6	1.57	6.9

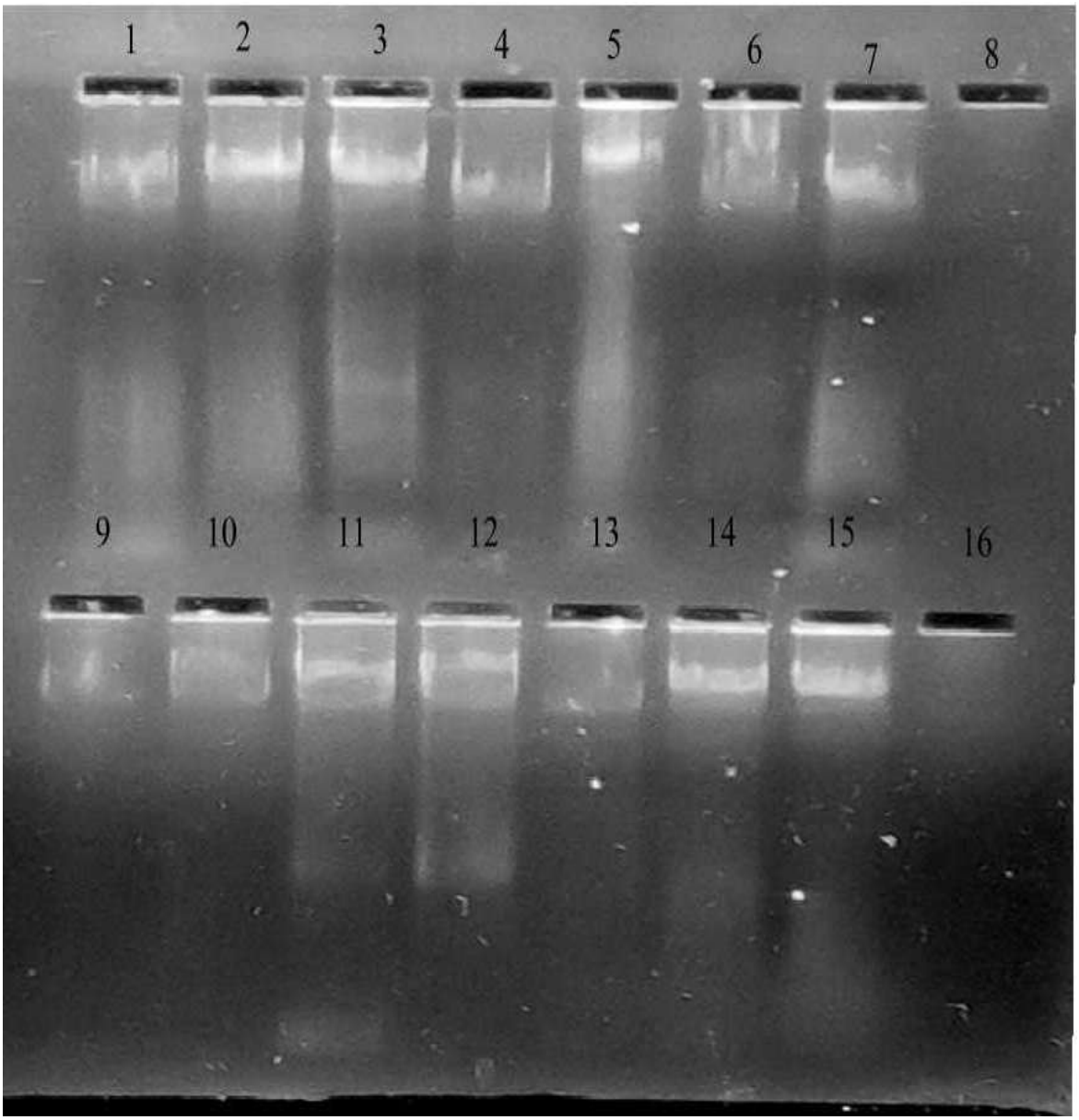

Figure 1. 0.8% Agarose Gel Electrophoresis of DNA samples obtained from muscle tissue. Lane 1-7 and Lane 9-15 are total DNA isolated from muscle tissue of Tropical loach, *Lepidocephalus thermalis*

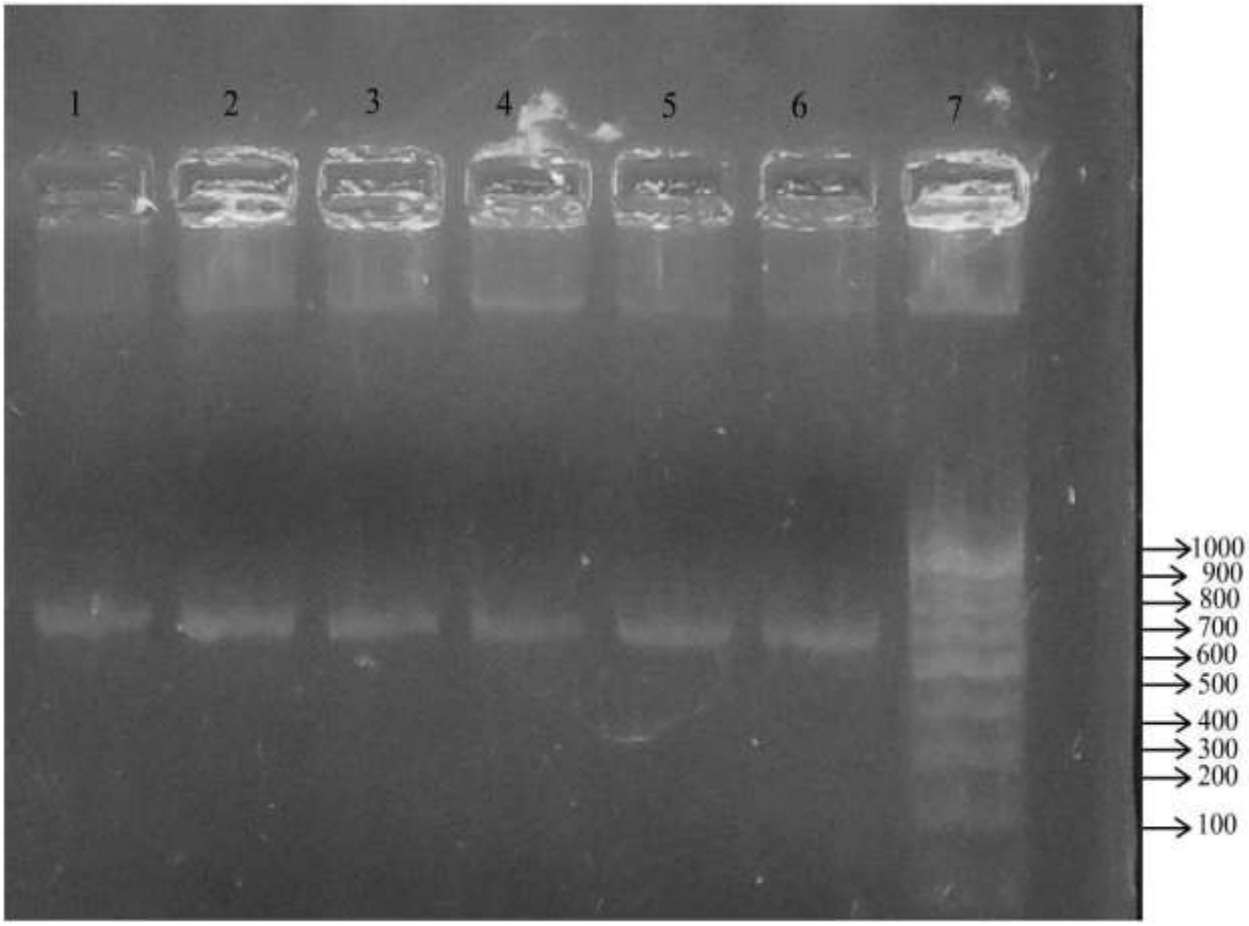

Figure 2. 1.5 % Agarose Gel Electrophoresis of PCR products using total DNA of *Lepidocephalus thermalis.* Lanes (1-6) are PCR of COI gene primer and Lane (7) is molecular weight marker