International Journal of Agriculture, Environment and Biotechnology
Citation: IJAEB: 8(3): 511-519 September 2015
DOI Number: 10.5958/2230-732X.2015.00058.3

Generation of DNA barcodes in Indian mottled EEL (*Anguilla Bengalensis*): A threatened ichthyofauna of Assam, India

Shamim Rahman[1*], Ellora Barua[2], Jitendra K Choudhury[3], Amalesh Dut a[4] and Mohan C Kalita[1]

[1]Department of Biotechnology, Gauhat University, Guwahat 781014, Assam, India.
[2,3]Department of Zoology, North Gauhat College, Guwahat 781031, Assam, India.
[4]Department of Zoology, Gauhat University, Guwahat 781014, Assam, India.

*Corresponding author: shamim.piku85@gmail.com

Paper No. 342 Received: 8 August 2014 Accepted: 21 August 2015

Abstract

Eels have always been a source of fascination because of their charismatic shape and size. They are good source of animal protein and bear high food values. Dwindling population of eels has meanwhile led certain species to IUCN threatened categories. In spite of these, scientific investigations on the only species of this genus, *Anguilla bengalensis*, in this region have not been reported much. Many biological questions of the catadromous fish are still unanswered. Quick but authentic identification of threatened species is vital to unveil such query and frame out conservation and management strategies. DNA barcodes utilising partial mitochondrial cytochrome c oxidase I gene and nuclear rhodopsin gene were developed in this current study. Conventional taxonomic information has also been included contemplating inevitable role of it in unambiguous species level discrimination. The study has generated novel barcode of the species from this region to decipher implications on congeneric and conspecific divergence.

Highlights

- Generation of mt-COI DNA barcode
- Generation of nuclear rhodopsin gene DNA barcode
- Taxonomic review of *Anguilla bengalensis*

Keywords: DNA-Barcoding, threatened fish, eel, *Anguiila bengalensis*

Fish is an integral part of socio economic fabric of the state of Assam which is part of biodiversity hot-spot. Anguillids and other eel shaped fish species of Assam bear high market price and many a times more compatible than carps. A few *Anguilla* species are introduced in aquaculture in countries like Japan (Leander *et al.* 2012). Ege (1939), Castle and Williamson (1974) and Watanabe *et al.* (2009) mentioned sixteen species of the genus *Anguilla* (Aoyama 2009). Eels are found in the tropical, subtropical and temperate parts of the globe and not available at South Atlantic and the west coasts of North and South America (Aoyama 2009). At present FISH-BOL has enlisted eighteen species of genus *Anguilla*, viz., *Anguilla anguilla, Anguilla australis, Anguilla bengalensis, Anguilla bicolor, Anguilla borneensis,*

Anguilla celebesensis, Anguilla dieffenbachii, Anguilla interioris, Anguilla japonica, Anguilla luzonensis, Anguilla malgumora, Anguilla marmorata, Anguilla megastoma, Anguilla mossambica, Anguilla nebulosa, Anguilla obscura, Anguilla reinhardtii and Anguilla rostrata. Among these, *Anguilla bengalensis* is the only species of genus *Anguilla* in Assam. In addition to its high market price, the species plays role in traditional therapeutics in Assam and neighbouring states in treatment of burn injury (Rahman *et al.* 2014). The mucous of *Anguilla bengalensis* is reported to be used in arthritis in combination of rice or wheat flour (Arunachalam, M. and Sankaranarayanan, A. 2000). Observation of intraspecific variation creates two sub species of *Anguilla bengalensis*. The native in this region is the *Anguilla bengalensis bengalensis* (Indian Mot led Eel) and *Anguilla bengalensis labiata* (Peter 1852) is the African Mot led Eel (IUCN 2015). The first systematic comprehensive study on genus *Anguilla* was carried out by Ege (1939), followed by Watanabe (2001).

Eels have always been a source for human interest for their interesting shape and size (Leander *et al.* 2012). They are the source of good food values in different part of the globe. Moreover, there are many unanswered facts to be resolved on eels (Leander *et al.* 2012). Tropical eels migrate shorter distances, in contrast to European eels those show long distance migration. Tropical eels with short migration habits are considered to be the basal group that gave rise to long distance migration of European eels (Aoyama 2009). Facultative catadromy, *i.e.* eels remain in estuary or move back and forth between freshwater and estuary has also been observed in many eels (Tsukamoto and Arai 2001). The only species of this genus, *Anguilla bengalensis* in this region has not obtained much at ention towards scientific exploration. In most of the classical works on Anguillids in the world, position of *Anguilla bengalensis* seems to be ambiguous due to either non inclusion in the investigation or use of doubtful synonyms. In addition mere use of morphology will be insufficient in identification of eels at different life stages (Aoyama 2009). Identification through protein profiling in closely related species is difficult as their proteins contain similar kinds of constituents (Bartlet and Davidson 1991), (Smith *et al.* 1996). DNA based techniques independent of cell type and age has grown as choice for researchers in fish identification (Davidson 1998), (Bossier 1999), (Lockley and Bardsley 2000).

DNA barcoding has been proved to be a successful mode in error free identification specially in case of animals utilising mitochondrial partial cytochrome c oxidase subunit I gene (COI), nearer to its 5' end (Hebert *et al.* 2003). In popular term 'barcode' is known as some codes in the form of bars to give a unique identity to a product. The cardinal objective of barcode is the easy and quick investigation of any product in the midst of lot. In simple term, it is the nucleotide differences between sequences, whereby, a gap of difference is always found functional between individuals of different species in comparison to individuals of the same species. The gap is referred as 'barcode gap' and is considered as 'threshold value' for species discrimination. DNA barcoding is not to wipe out traditional methods. On the contrary, DNA barcoding is a complementary approach to conventional taxonomy for providing indications towards phylogenetics and population genetics (Hajibabaei *et al.* 2007). Mitochondrial genes are considered as good choice (Kochzius 2009), (Teletchea 2009) for fish identification due to high copy numbers of mtDNA in cells and consequently high probability of DNA recovery (Hubert *et al.* 2008), maternally inherited nature giving rise to rare or no recombination (Sangthong and Jondeung 2003). The advantage over selecting COI gene is the universal primers for this gene are very robust, enabling recovery of its 5'end from the representatives, mostly in case of animals. Moreover, COI appears to possess a greater range of phylogenetic signal than any other mitochondrial genes. Its third position nucleotide shows a high incidence of base substitutions, leading to a rate of molecular evolution that is about three times greater than that of 12S or 16 rDNA (Ghosh 2012). However, simultaneous use of two genes has also been advocated by researchers to make good use of DNA barcoding in fish (Sevilla *et al.* 2007).

Fig. 1: *Anguilla bengalensis*

Materials and Methods

Fish samples were collected from various locations of Assam and Assam-Meghalaya border zones with the aid of fishermen. The sampling stations with their coordinates are given in Table: 1. Diagnostic keys (Vishwanath *et al.* 2007) were used in identifying the fish and morphometric measurements were considered following Ege (1939). Radiographic image of the fish were taken for vertebrae reckoning. Before preservation of the specimen, small amount of tissue (white muscle) was kept in -20°C for short period of time or in 100% ethanol for long term preservation.

Table 1. Sampling stations with coordinates

Sl. No.	Sampling Stations	Coordinates
1	Near Palashbari, Kamrup, Assam	26°12′17″ North 91°53′58″ East
2	Near Rani, Kamrup, Assam	26°01′44″ North 91°35′46″ East
3	North Guwahati, Assam	25°50’34”North 91°20’41”E East
4	Ukium, Assam-Megha-laya transition zone	26°12′17″ North 91°53′58″ East
5	Maligaon, Guwahati	26°10′18″ North 91°41′12″ East

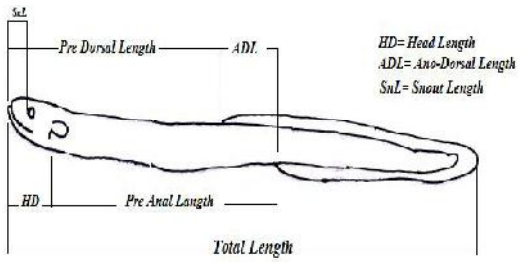


Fig. 2: Schematic representation of important body measurements used in *Anguilla bengalensis*

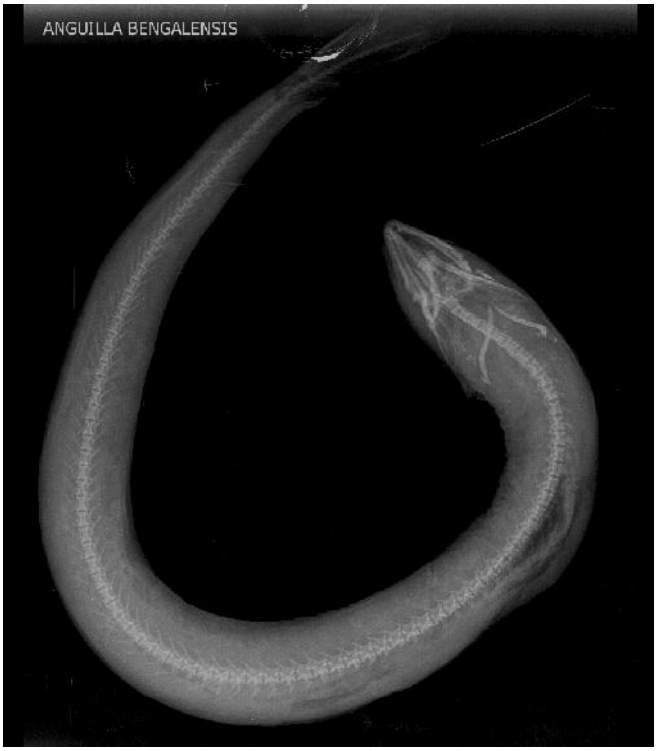


Fig. 3: Radiographic image of *Anguilla bengalensis*

Total genomic DNA was isolated from white muscle tissue following the procedure developed by Miller *et al.* (1988) with minor alterations. Genomic DNA, so obtained, was passed through quantitative and qualitative checking using 0.8% agarose gel in horizontal electrophoresis. Partial mitochondrial cytochrome c oxidase I (COI) and nuclear rhodopsin gene were amplified using genomic DNA as template in polymerase chain reactions (PCR). Sets of universal primers, *viz.* Fish F1: 5′-TCAACCAACCACAAAGACATTGGCAC-3′ and FishR1: 5′-TAGACTTCTGGGTGGCCAAAGAATCA-

3′, were used in amplification of 669bp COI gene (Ward *et al.* 2004) and RodF2W: 5′-AGCAACTTCCGC TTCGGTGAGAA-3′ and Rod4Rn: 5′-GGAACTGCTTGT TCATGCAGATGTAGAT-3′ were used in amplification of 447 bp nuclear rhodopsin gene (Sevilla *et al.* 2007). Both the genes were amplified in 25 µl reaction mixture containing 1X PCR buffer, 2mM $MgCl_2$, 10 pmol each primer, 1mM dNTPs and 1U Taq polymerase (New England Biolabs) in case of COI amplification and 1X PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs and 1 U Taq DNA polymerase. 1µl DNA was used as template with concentration at 50-100 ng/µl for COI and 20-30 ng/µl for rhodopsin. The thermal conditions for polymerase chain reactions were as follows: an initial denaturation at 95°C for 2 minutes, 35 cycles at denaturation temperature of 95°C for 30 seconds, annealing temperature of 55°C for 30 seconds and extension temperature of 68°C for 1 minute and concluded with a final extension step at 68°C for 10 minutes followed by a hold at 4°C for COI and an initial denaturation at 95°C for 4 minutes, 40 cycles at denaturation temperature of 95°C for 30 seconds, annealing temperature of 60°C for 30 seconds and extension temperature of 72°C for 45 seconds and concluded with a final extension step at 72°C for 8 minutes for rhodopsin.

The amplified PCR products were analysed in 1% agarose gel containing ethidium bromide (10mg/ml) and single band was obtained without non specific amplification. PCR products were purified using HiMedia PCR product purification kit and sequenced commercially on both direction.

All the COI PCR products and their subsequent sequences are more than 600 bp and this indicates that there is no NUMT sequence; because vertebrates NUMTs are typically smaller than 600 bp. Raw sequences were edited by trimming noisy terminals and contig was obtained from bi directional sequences in soft ware BIOEDIT (Hall, 1999). No insertion/deletion (indel) was observed either in sequence replications or in BLASTN use of National Centre for Biotechnology Information (NCBI).

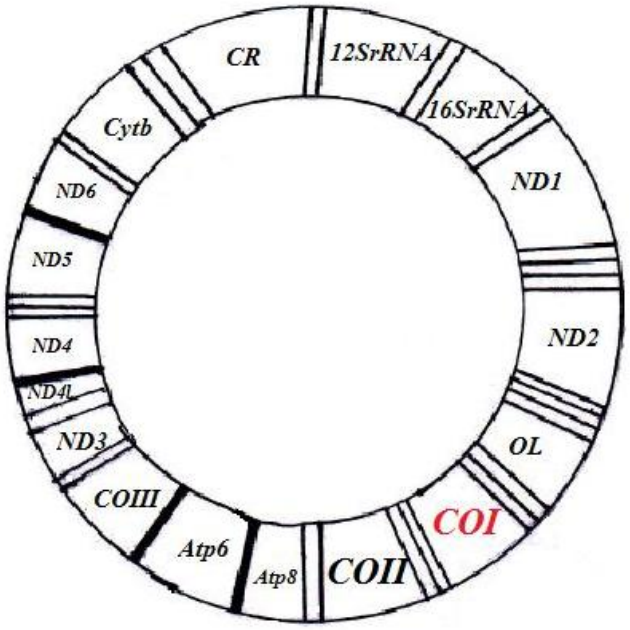


Fig. 4: Schematic map of mtDNA in fish showing COI in corresponding to Hrbek and Farias, 2008

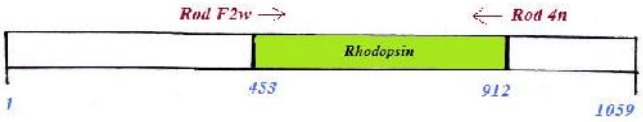


Fig. 5: Schematic representation of targeted portion of rhodopsin gene with primer location in corresponding to Yokoyama *et al.* 1995

NCBI ORF (open reading frame) finder was utilised in translating the sequences and sequences were aligned in BLASTP (Altschul *et al.* 1990) and these were in consonance with the functional partial amino acid codes of fish mitochondrial COI gene and nuclear rhodopsin gene. COI barcodes of another Anguilliformes fish (Family Ophichthidae) *Pisodonophis boro* (BIN: BOLD: ACP1605) and four Synbranchiformes fishes found in Assam and often regarded as eel shaped fish species, *Macrognathus aral* (NCBI accession: KJ946382), *Macrognathus pancalus, Mastacembelus armatus* (NCBI accession: KJ946383) and *Monopterus chuchia* (NCBI accession: KJ946384) were also developed to get an indication of best suited interrelation. Furthermore, secondary data available on COI/ Rhodopsin sequences were

obtained from National Centre for Biotechnological Information (NCBI) and Barcode of Life Database (BOLD). These primary and secondary information have been utilised in determination of congeneric and conspecific genetic divergences using Kimura 2-parameter distance model with *Anguilla bengalensis* (NCBI accession: KP982886) and Neighbor Joining trees were created using sof ware MEGA version 6 (Tamura *et al.* 2013)

Results and Discussion

Taxonomic Review

Muraena bengalensis Gray, 1831

Anguilla nebulosa nebulosa McClelland, 1844: unclear synonym (Ege, 1939)

Anguilla anguilla Kulkarni and Ranade, 1974

Anguilla bengalensis Day, 1878

Current Valid Scientific Name: *Anguilla bengalensis* Gray, 1831

Vernacular: Nadal Bami, in Assamese

Fin formula: D 250-305; A 220-250; P 18

Phylum: Chordata

Class: Actinopterygii

Order: Anguilliformes

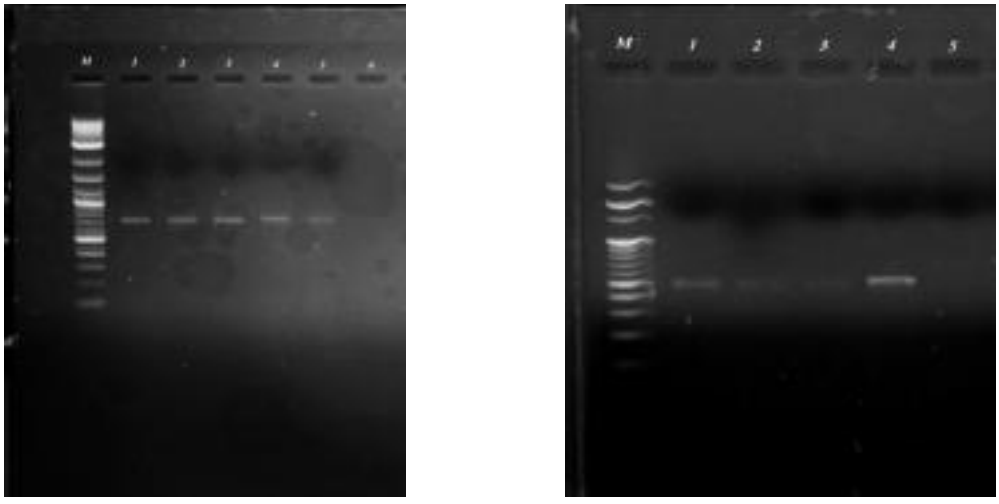
Family: Anguillidae

Genus: *Anguilla*

Species: *bengalensis*

Diagnosis (Vishwanath, 2007) (Jayaram, 2010)

Body elongated, cylindrical, abdomen rounded, head long and compressed, dorsal and anal fin continuous around tail, lips thick, villiform teeth on jaws and palate, dorsal fin inserted nearer anus than gill opening, body colour variegated yellow-olive over brown markings, vertebrae 106-112.



(A) (B)

Fig. 6. 1% Agarose gel image of PCR products. A: M-100bp DNA ladder, 1-5 COI, 6 negative control. B: M-100 bp DNA ladder, 1-4 Rhodopsin, 5 negative control.

Table 2: Average body measurements in terms of percentage of Total Length with standard deviations

Measurements	Mean (% of Total Length) with SD
Standard Length	97.25 ±0.77
Head Length	12.92 ±0.53
Body Depth	7.64±1.39
Pre Dorsal Length	31.05 ±1.39
Pre Anal Length	44.19 ±2.58
Ano-dorsal length	13.11 ±1.48

Genetic divergence analysis using Kimura2-parameter model based on COI gene sequence showed less conspecific divergence (0.024) in comparison to other Anguillliformes (0.164) and Synbranchiformes (0.212 with *Macrognathus pancalus*, 0.144 with *Monopterus cuchia*, 0.247 with *Mastacembelus armatus* and 0.252 with *Macrognathus aral*). The neighbour joining tree prepared on the basis of developed COI barcodes and barcodes available on BOLD database revealed close conspecific cluster of *Anguilla bengalensis* with congeners *viz. Anguilla nebulosa* (0.012 K2P genetic divergence).

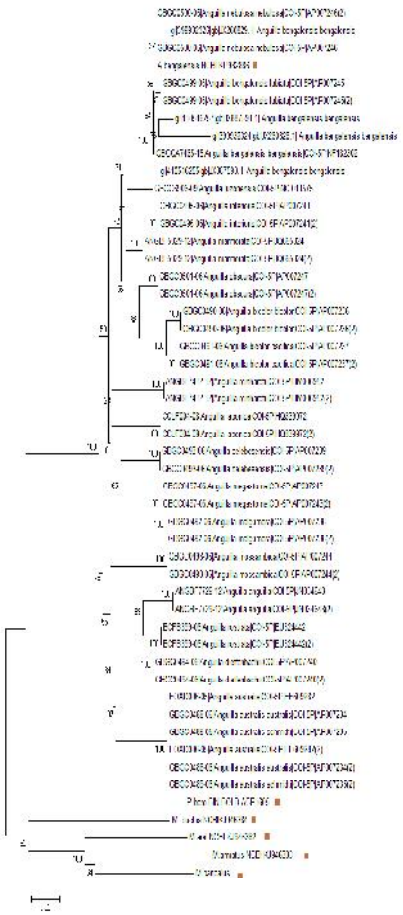


Fig. 7: Neighbour-joining (NJ) tree developed using K2P distance among primary (marked) and secondary COI gene sequences

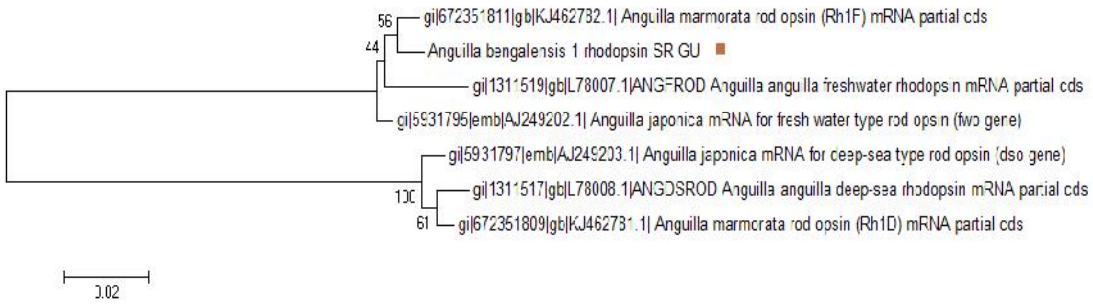


Fig. 8: Neighbour-joining (NJ) tree developed using K2P distance among primary (marked) and secondary Rodopsin gene sequences

On the other hand distinct grouping has been observed with other cogeners. The genetic divergence analysis on the basis of rhodopsin gene sequence was found limited because of the paucity of sequence information in public databases.

There is plethora of fish species from India's Northeast declining from Earth even before their proper scientific documentation. In case of *Anguilla bengalensis*, being no exception, the research scenario is in nascent stage, specially in terms of molecular characterization. More number of sequence information from this region would reveal newer information to the scientific community. In this study, novel DNA barcodes from this region are developed in *Anguilla bengalensis* in complementary to conventional taxonomy realising inevitable role of traditional taxonomy in resolving species level discrimination. Ano-dorasal length in relation to total length (ADL%TL) had played an important role in species differentiation across the genus *Anguilla*. ADL%TL obtained in this study (13.11%) showed obvious distinction with congeners; 10.03% in *Anguilla japonica,* 0.43% in *Anguilla bicolour pacifica,* 15.57% in *Anguilla marmorata* and 10.30% in *Anguilla celebesensis* (Leander *et al.* 2012).

COI barcode of *Anguilla bengalensis* from this part of the world would complement earlier mitochondria based studies on genus *Anguilla* (Tagliavini *et al.* 1995). Close cluster with *A. nebulosa* provides an indication of use of synonyms in consonance with *A. bengalensis* in past records (Aoyama 2009). Lesser genetic divergence between *A. bengalensis* and *A. nebulosa* in comparison to conspecific genetic divergence of *A. bengalensis* asserts the said statement. Moreover, less genetic divergence between *A. bengalensis bengalensis* and *A. bengalensis labiata* (0.016) and between *A. bengalensis labiata* and *A. nebulosa nebulosa* (0.004) advocates need of taxonomic review of these Anguillids incorporating newly generated sequences. Authentic identification of freshwater eels and their phylogenetic analysis would remain inconclusive till morphological and molecular characterisations are put together across the Anguillids all over the globe. Generation of DNA barcodes through this study would plug the voids of database to hammer out interrelations among the species. Development of rhodopsin gene based barcode in *Anguilla bengalensis* would broaden the use of multigene to make results more conclusive. This also generates information such as nucleotide frequencies (A= 25.9%, G= 18.1%, C= 26.9%, T= 29.1% for COI) and (A= 18.6%, 23.7%, C= 32&, T= 25.7% for rhodopsin). Different organisms show different pat ern of nucleotide composition, GC content and substitutions bias. Mitochondrial genomes show profound shif in nucleotide usage and GC content and can have serious impact on phylogenetic analysis. Therefore, it is indeed important to analyse GC content and substitutional bias for reference in evolutionary history (Tamura *et al.* 2013). Through sequence characterisation, it would be possible to follow gene frequency changes in course of time using both old museum specimens and current representatives of a population (Kocher *et al.* 1989)**.** Besides the inherent benefit of DNA barcoding in tagging species, this would create baseline information on molecular characterization to decipher some indication on phylogenetic significance of this species. The outcome of the study may be of good use for conservationists and field biologists as a whole.

Acknowledgment

We do sincerely acknowledge Department of Biotechnology, Government of India sponsored Institutional Biotech Hub of Gauhati University and North Gauhati College and Department of Biotechnology and Department of Zoology, Gauhati University for providing infrastructure facility to carry out the research.

References

Leander, N.J., Shen, K.N., Chen, R.T. and Tzeng, W.N. 2012. Species Composition and Seasonal Occurrence of Recruiting Glass Eels (Anguilla spp.) in the Hsiukuluan River, Eastern Taiwan. *Zoological Studies* **51**(1): 59-71.

Aoyama, Jun. 2009. Life History and Evolution of Migration in Catadromous Eels (Genus Anguilla). *Aqua-BioScience Monographs* **2**(1): 1–42. ht p://dx.doi.org/10.5047/absm. 2009.00201.0001

Fish barcode of life. 2015. www.fishbol.org

Rahman, S., Choudhury, J.K., Dut a, A. and Kalita, M.C. 2014. Eel Ichthyofauna of Assam in folklore therapeutic practices. *International Journal of Interdisciplinary and Multidisciplinary Studies*. **1**(5): 273-276.

Arunachalam, M. and Sankaranarayanan, A. 2000. Some economically important and cultivable in Gadana river, western Ghats, In A.G. Ponniah and A. Gopalakrishnan (eds.) Endemic fish diversity of Western Ghats. *NBFGR-NATP Publication. National Bureau of Fish Genetic Resources, Lucknow.* 244-246.

The IUCN Red List of Threatened Species. 2015. ht p://www.iucnredlist.org

Ege, V. 1939. A revision of the genus Anguilla Shaw. *Dana Rep*. **16**: 8–256.

Watanabe, S. 2001. Taxonomic study of the freshwater eel, the genus Anguilla, Schrank 1798. Thesis Ph.D. University of Tokyo.

Tsukamoto, K. and Arai, T. 2001. Facultative catadromy of the eel Anguilla japonica between freshwater and seawater habitats. *Marine Ecology Progress Series* **220**: 265–276. ht p://dx.doi.org/10.3354/meps220265

Bartlet , S.E. and Davidson, W.S. 1991. Identification of Thunnus tuna species by the polymerase chain reaction and direct sequence analysis of their mitochondrial cytochrome b genes. *Canadian Journal of Fisheries and Aquatic Sciences*. **48**: 309-317. ht p://dx.doi.org/10.1139/f91-043

Smith, P. J., Roberts, C.D., McVeagh, S.M. and Benson, P.G. 1996. Genetic evidence for two species of tarakihi. Teleostei: Cheilodactylidae: Nemadactylus in New Zealand waters. *New Zealand Journal of Marine and Freshwater Research*. **30**: 209-220. ht p://dx.doi.org/10.1080/00288330.1996.9516709

Davidson, W.S. 1998. PCR techniques. In PR Ashurst and MJ Dennis (eds.). Analytical methods of food authentication. Blackie Academic and Professional, London. 182-203.

Bossier, P. 1999. Authentication of seafood products by DNA pat erns. *Journal of Food Science*. **64**: 189-193. ht p://dx.doi.org/10.1111/j.1365-2621.1999.tb15862.x

Lockley, A.K. and Bardsley, R.G. 2000. DNA-based methods for food authentication. *Trends Food Science and Technology* 11: 67-77. ht p://dx.doi.org/10.1016/s0924-2244(00)00049-2

Hebert, P.D.N., Cywinska, A., Ball, S.L. and deWaard, J.R. 2003. Biological identification through DNA barcodes. *Proc. R.Soc.Lond.B*. **270**: 313–321. ht p://dx.doi.org/10.1098/rspb.2002.2218

Hajibabaei, M., Singer, G.A.C., Hebert, P.D.N. and Hickey, D.A. 2007. DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in genetics*. **23**(4): 167–172. ht p://dx.doi.org/10.1016/j.tig.2007.02.001

Kochzius, M. 2009. Trends in fishery genetics. In Beamish RJ and BJ Rothschild (eds). The future of fisheries science in North America. The Netherlands: Fish and Fisheries. *Springer* **31**: 453-493. ht p://dx.doi.org/10.1007/978-1-4020-9210-7_24

Teletchea, F. 2009. Molecular identification methods of fish species: reassessment and possible applications. *Review in Fish Biology and Fisheries* **19**: 265-293. ht p://dx.doi.org/10.1007/s11160-009-9107-4

Hubert, N., Torrico, J.P., Bonhomme, F. and Renno, J.F. 2008. Species polyphyly and mtDNA introgression among three Serrasalmus sister-species. *Molecular Phylogenetics and Evolution* **46**: 375-381. ht p://dx.doi.org/10.1016/j.ympev.2007.04.002

Sangthong, P. and Jondeung, A. 2003. Cloning and nucleotide sequence of four tRNA genes in mitochondrial genome of Thai walking catfish (Clarias macrocephalus) Kasetsart *Journal Natural Science* **37**: 33-40.

Ghosh, S.K. 2012. A text book on DNA barcoding. Books space, Kolkata.

Sevilla, R.G., Diez, A., Norén, M., Mouchel, O., Jérôme, M., Verrez-Bagnis, V., Pelt, H.V., Favre-Krey, L. and Krey, G. 2007. The FishTrace Consortium, Bautista, J.M. Primers and polymerase chain reaction conditions for DNA barcoding teleost fish based on the mitochondrial cytochrome b and nuclear rhodopsin genes. *Molecular Ecology Notes*. **7**(5): 730-734. ht p://dx.doi.org/10.1111/j.1471-8286.2007.01863.x

Vishwanath, W., Lakra, W.S. and Sarkar, U.K. 2007. Fishes of North East India. National Bureau of Fish Genetic Resoures, Lucknow.

Miller, S.A., Dykes, D.D. and Polesky, H.F. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acid Research*. **16**(3):1215. ht p://dx.doi.org/10.1093/nar/16.3.1215

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R. and Hebert, P.D.N. 2004. DNA barcoding Australian fish species. *Philosophical Transactions Royal Society* B. **360**: 1847–1857. ht p://dx.doi.org/10.1098/rstb.2005.1716

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

ht p://blast.ncbi.nlm.nih.gov/Blast.

Hrbek, T. and Farias, I.P. 2008. The complete mitochondrial genome of the pirarucu (Arapaima gigas, Arapaimidae, Osteoglossiformes). *Genetics and Molecular Biology* **31**(1): 293-302. ht p://dx.doi.org/10.1590/s1415-47572008000200024

Yokoyama, R., Knox, B.E. and Yokoyama, S. 1995. Rhodopsin from the fish, Astyanax: role of tyrosine 261 in the red shif . Invest. *Ophthalmology and Visual Science* **36**: 939–945.

NCBI ORF 2015. ht p://www.ncbi.nlm.nih.gov/gorf/gorf.html

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403–410. ht p://dx.doi.org/10.1016/s0022-2836(05)80360-2

National Centre for Biotechnological Information. 2015. ht p://ncbi.nlm.nih.gov/

Ratnasingham, S. and Hebert, P.D.N. 2007. The Barcode of Life Data System (www.barcodinglife.org). *Molecular Ecology Notes* **7**: 355-364. ht p://dx.doi.org/10.1111/j.1471-8286.2007.01678.x

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. Molecular Evolutionary Genetics Analysis version 6. 2013. *Molecular Biology and Evolution* **30**: 2725-2729. ht p://dx.doi.org/10.1093/molbev/mst197

Jayaram, K.C. 2010. The freshwater fishes of Indian region. Narendra Publishing House, Delhi.

Tagliavini, J., Gandolfi, G., Cau, A., Salvadori, S., Deiana, A.M. and Gandolfi, G. 1995. Mitochondrial DNA variability in Anguilla anguilla and phylogenetical relationships with congeneric species. *Bollet ino di Zoologia* **62**: 147–151. ht p://dx.doi.org/10.1080/11250009509356062

Kocher, T.D., Thomas, W.K., Meyer, T.T., Edwards, S.V., Paabo, S., Villablanca, F.X. and Wilson, A.C. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the United States of America USA* **86**: 6196-6200. ht p://dx.doi.org/10.1073/pnas.86.16.6196