

Department of Biotechnology Hargobind Khurana School of Life Sciences Assam University, Silchar (A Central University constituted under Act XIII of 1989) Silchar- 788 011, Assam, India

DECLARATION

I, Khomdram Bijoya Devi, bearing Registration No. PhD/808 dated 17/08/2009, hereby declare that the subject matter of the thesis entitled "Studies on DNA barcoding of Ornamental fishes of Manipur" is the record of work done by me and that the contents of this thesis did not form the basis for award of any degree to me or to anybody else to the best of my knowledge. The thesis has not been submitted in any other University/ Institute.

Place: Assam University, Silchar

Date: February, 2014

Department of Biotechnology

Assam University, Silchar

Khandram Bijaja Du

KHOMDRAM BIJOYA DEVI

ACKNOWLEDGEMENT

I would like to utilize this privilege while documenting my research findings in the present

form of a thesis; to express my deep sense of gratitude to my revered supervisor Prof. (Dr.)

Sankar Kumar Ghosh, Department of Biotechnology, Assam University, for his constant

guidance, frequent encouragement and resourceful comments towards fulfillment of this

thesis being submitted for achieving the degree of Doctor of Philosophy.

I also would like to thank the honourable Vice Chancellor for his kind support by providing

various facilities in our department.

I extend my heartiest gratitude to Boni da, Shantanu and Joyraj for helping me in my whole

research work and finally preparing the thesis. I am indebted to many of my colleagues to

support me namely Miranda, Monika, Shilpi di, Mohua, Pradosh, Jagadish and Bishal for

supporting me always and encouraging me.

I owe my deepest gratitude to Prof. W. Vishwanath and his team, Department of Life Sciences,

Manipur University for helping me in identifying my fish samples and my thanks are also to

our departmental staffs and official staffs.

Last but not the least I would like to thank my Family members and close friends for their

immense support all the time.

Khandram Bijoya Dwi.

KHOMDRAM BIJOYA DEVI

CONTENTS

Sl No.			Description	Page No.
1			Introduction	1-15
1.1			Fishes	2
1.2			Ornamental Fishes	2
1.3			Importance of ornamental fishes	3
1.4			Distribution of ornamental fishes-World	4
	1.4.1		India	5
	1.4.2		Manipur	6
1.5			Problem in species identification	7
1.6			Mitochondrial DNA, special references to COI genome	8
	1.6.1		DNA barcoding	9
1.7			Applications of Barcoding	10
	1.7.1		Integration of Taxonomy and Molecular approaches	11
1.8			Success of DNA barcoding	12
1.9			State of Art of the problem	14
1.10			Aim and Objective of the current research	15
2			Review of literature	16-31
2.1			Ornamental fish inventory in India and Northeast India	18
2.2			perspective (morphology related work)	1.0
2.2			Progress of molecular taxonomy in ichthyodiversity	19
2.3			Application of DNA barcoding for decoding of	25
3			ornamental fish	32-49
3.1			Materials and Methods	
3.1	211		Materials	33-39
	3.1.1		Selection of sampling site	33
	3.1.2		Sampling, documentation of experimental taxa and accessing the database	34
	3.1.3		Chemicals	34
	3.1.4		Primers	38
	3.1.5		Additional data acquired from databases	38
3.2			Methods	40-49
	3.2.1		Isolation and quantification of DNA	40
		3.2.1.1	Isolation of DNA from different samples	40
		3.2.1.2	Spectrophotometric determination	42
		3.2.1.3	Agarose gel electrophoresis	42
	3.2.2		PCR amplification of <i>COI</i> barcode segment	43
	00202	3.2.2.1	PCR reaction settings	43
		3.2.2.2	PCR cycling condition	44
		3.2.2.3	Purification of PCR products	44
	3.2.3		Sequencing of PCR amplicons	45
	3.2.4		Sequence checking and formatting	45
	3.2.5		Sequence submission in GenBank and BOLD	47
	3.2.6		Similarity search	44
	3.2.7		Sequence alignment	47
	3.2.8		Model selection and sequence analysis	48
	3.4.0		wioder selection and sequence analysis	40

4		Results	50-85
4.1.		Morphology based identification of the ornamental fishes	51-59
	4.1.1	Cyprinidae	51
	4.1.2	Siluridae	52
	4.1.3	Sisoridae	53
	4.1.4	Bagridae	53
	4.1.5	Gobiidae	56
	4.1.6	Badidae	56
	4.1.7	Amblycipitidae	56
	4.1.8	Nandidae	56
	4.1.9	Cobitidae	56
	4.1.10	Channidae	58
4.2.		Molecular identification and characterization of the different species of ornamental fishes found in Manipur	60-66
	4.2.1.	Genomic DNA isolation from blood and tissue	60
	4.2.2.	PCR amplification of COI DNA barcode and purification	60
	4.2.3.	Final sequences	61
	4.2.4.	Dataset characteristics	63
	4.2.5.	Submitted sequences in GenBank and BOLD	63
	4.2.6.	DNA barcode based characterization of the ornamental fishes of Manipur	63
	4.2.6.1.	Species identification based on similarity match with database	63
4.3.		Sequence analysis of the DNA barcode region of the ornamental fishes	67-76
	4.3.1.	Disparity index test of pattern homogeneity	67
	4.3.2.	Genetic divergence	67
	4.3.3.	Maximum Likelihood estimate of substitution matrix	68
	4.3.4.	Maximum Likelihood estimate of Transition transversion bias	69
	4.3.5.	Maximum Composite Likelihood estimate of the pattern of Nucleotide substitution	69
	4.3.6.	Nucleotide composition analysis	71
	4.3.7.	Amino acid composition and Codon usage	74
4.4.		Development of Phylogenetic relationship between the different species of ornamental fishes identified	76-83
	4.4.1.	Neighbor Joining tree	76
	4.4.2.	Model selection for analysis of Maximum likelihood based approach	77
	4.4.3.	Phylogenetic analysis based on Maximum Likelihood and Maximum Parsimony approach	79
4.5.		Development of reference barcode library of the ornamental fishes from Manipur	78
5		Discussion	86-93
6		Summary	94-95
7		Bibliography	86-108

LIST OF FIGURES

Figure No.	Description	Page No.
1	Global distribution of ornamental fishes	05
2	Mitochondrial genome showing the different arrangements of genes	08
3	Map of Manipur, India showing the different locations of sample collection	33
4	Few representatives of genomic DNA extracted pictures from blood and tissue samples	60
5	COI DNA barcode PCR amplicons of ornamental fish samples	61
6	Example of sequence editing	62
7	Chart showing genetic divergence within and between species	68
8	Overall nucleotide composition of the 27 sequences under study	71
9	Nucleotide composition in 1 st codon position of the 27 sequences generated	72
10	Nucleotide composition in 2 nd codon position of the 27 sequences generated	72
11	Nucleotide composition in 3 rd codon position of the 27 sequences generated	73
12	Chart showing average amino acid composition for the ornamental fishes under study	75
13	Neighbor Joining tree developed by using K2P distances generated by our generated sequences of ornamental fishes with sequences from GenBank	81
14	Maximum Likelihood tree generated from the ornamental fish sequences by adopting GTR+G+I model	82
15	Maximum Parsimony tree developed from the sequences under study	83
16	DNA barcode of the ornamental fish, SGBK-AUFO35 which represents <i>Cirhinus mrigala</i> .	85

LIST OF TABLES

Table No.	Description	Page No.
1	Details of GenBank DNA barcode sequences retrieved in the present study for analysis	38
2	Checklist of ornamental fish species collected with their local names in Manipuri and location	59
3	Accession number of the sequences submitted in GenBank with their sample codes and species name	64
4	Sample identification results (homology comparison) based on GenBank and BOLD	65
5	Estimation of substitution index by Maximum Likelihood approach	69
6	Estimation of pattern of nucleotide substitution by maximum Composite Likelihood approach	70
7	Frequency of codons in COI sequences of ornamental fishes under study	76
8	Model test for phylogenetic analysis by Maximum Likelihood, generated by putting the sequences used in the study using MEGA 5.0	78
9	The ornamental fishes under study mentioning their ornamental characters	88

LIST OF PLATES

Plate No.	Description	Page No.
1	Specimens of the ornamental fish species- Labeo bata, Labeo gonius, Labeo calbasu, Puntius sophore, Puntius chola, Cirrhinus mrigala.	54
2	Specimens of the ornamental fish species- Amblyceps mangois, Rasbora daniconius, Channa orientalis, Channa punctatus, Badis badis, Esomus danricus.	55
3	Specimens of the ornamental fish species- Barbonymus gonionotus, Mystus vittatus, Wallagu attu, Sisor rabdophorus, Glossogobisus giuris, Channa sriata.	57

LIST OF ABBREVIATIONS

μg	:	Microgram
μl	:	Microliter
⁰ C	:	Degree Celsius
A	:	Adenine
AG	:	Adenine- Guanine
AT	:	Adenine- Thymine
BLAST	:	Basic Local Alignment Search Tool
BLASTn	:	Basic Local Alignment Search Tool Identification
BOLD	:	Barcode of Life Data System
BOLD- IDs	:	Barcode of Life Data System Identification
Вр	:	Base pair
С	:	Cytosine
CBOL	:	Consortium of Barcode of Life
COI/ COX1	:	Cytochrome oxidase subunit I
LRnt	:	Near Threatened
LC	:	Least Concern

VU	:	Vulnerable
LRlc	:	Least Concern
EN	:	Endangered
СТ	:	Cytosine- Thymine
DNA	:	Deoxy Ribonucleic acid
EDTA	:	Ethylene diamine tetra acetate
EN	:	Endangered
EtBr	:	Ethidium Bromide
FASTA	:	Fast Align Sequences Tag Assembly
G	:	Guanine
GC	:	Guanine- Cytosine
Gm	:	Gram
IUCN	:	International Union for Conservation of Nature and Natural Resources
K2P	:	Kimura 2 Parameter
Lb	:	Pound
MCL	:	Maximum Composite Likelihood
MEGA	:	Molecular Evolutionary Genomic Analysis
mg	:	Milligram
ml	:	Milliliter
ML	:	Maximum Likelihood
Mm	:	Milli molar
mtDNA	:	Mitochondrial DNA
NCBI	:	National Centre for Biotechnological Information
NE	:	North East
Ng	:	Nanogram
NJ	:	Neighbor- Joining
NN	:	Nearest Neighbor
O.D	:	Optical Density
ORF	:	Open Reading Frame
PCR	:	Polymerase Chain Reaction

N	:	North
GPS	:	Global Positioning System