

Department of Biotechnology Hargobind Khurana School of Life Sciences ASSAM UNIVERSITY, SILCHAR (A central university constituted under act XIII of 1989) Silchar- 788011, Assam, India

DECLARATION

I, Mohua Chakraborty, bearing Registration Number, Ph.D. 1711/2011 dated 15.09.11, and Assam University Registration Number 16-100010544 of 2010-11, do hereby declare that the subject matter of the thesis entitled "DNA Barcode based calibration of Indian freshwater fishes for analyzing species diversity and shared nucleotide traits" 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 or Institute.

Place: Silchar Mohua Chakṛaborty

Date:

Acknowledgement

I am using this opportunity to express my gratitude to everyone who supported me throughout the course of this program.

Foremost, I would like to express my sincere gratitude to my supervisor Dr. Sankar Kumar Ghosh, Professor, Department of Biotechnology, for the continuous support of my Ph.D. study and research. I was constantly motivated by his patience, motivation, enthusiasm, and immense knowledge. I am thankful to him for encouraging my research and for allowing me to grow as a research scientist. His advice on both research as well as on my career have been invaluable. I could not have imagined having a better advisor and mentor for my Ph.D. study.

I thank Honourable Vice Chancellor, Dean School of Life Sciences, Head of the Department of Biotechnology and Board of Research Studies, Assam University for their support for various phases of the programme.

I gratefully acknowledge the funding sources that made my Ph.D. work possible. In this regard I extend my gratitude to DBT for providing me financial aid during the first two years of my research.

I am extremely thankful to Dr. Amalesh Choudhury for helping me in sample collection and identification. Working with him was a great learning experience.

All the faculty members and non-teaching staffs of the Department of Biotechnology, Assam University have been very kind to extend their help at various phases of this research. I would especially like to thank Dr. Yashmin Choudhury and Dr. Mohua Sengupta for their support and encouragement.

I thank my fellow labmates and seniors Boni Amin Laskar, M. Joyraj Bhattacharjee, Bishal Dhar, Ruhina Shirin Laskar, Fazlur Rehman, Shantanu Kundu, Ksh Miranda Devi, Monika Ahanthem, Shilpi Srivastava, Pradosh Mahadani, Jagadish Hansa, Rosy Mondal, Sharbadeb Kundu, Moonmi Chetia, Anil Seram, Joyobrata Nath, Shailendra Yadav, Susweta Mitra, Mridul Das, Dr. Bishwadeep Choudhury and my friends Saradia Kar, Aditi Bhowmik, Sarmistha Dey. It was their wonderful

companionship that helped me enjoy and survive through these four years of my life. The discussions in the lab, sharing of knowledge, cumulative effort in solving various technical and theoretical problems have enriched me in numerous ways.

I cannot but give full credit to my family members for having encouraged me and driving me forward to this point. I am thankful to my parents Haran Chakraborty and Uttara Chakraborty and my in-laws Bijon Kanti Choudhury and Nabanita Choudhury for their moral support and guidance throughout this period. I am grateful to my husband Budhaditya Choudhury for his unparalleled sacrifice and understanding. Without his constant support in all my pursuits, nonstop motivation and constructive criticism, it was impossible for me to complete my journey. I am extremely thankful to my brother Hillol Chakraborty for his much needed input in the entire coding related work of my study. Finally, I extend my gratitude to all other family members and friends especially to my brother in law Bappaditya Choudhury for their good wishes and encouragement.

Contents

Chapter 1.	INTRODUCTION	1
1.1 Fresh	nwater fishes: biodiversity and taxonomy	1
1.1.1	Ichthyology	1
1.1.2	Global geographical distribution of freshwater fishes	2
1.1.3	Taxonomy of fishes	3
1.1.4	Impediments in freshwater fish taxonomy	4
1.2 India	nn freshwater fish diversity and problem in taxonomy	5
1.2.1	Fish biodiversity in India	5
1.2.2	Taxonomic impediments in Indian freshwater fishes	7
1.3 Mole	ecular methods to resolve taxonomic impediments	8
1.3.1	Molecular vs. morphological methods	8
1.3.2	Mitochondrial DNA in species identification	8
1.3.3	The advent of DNA barcoding	9
1.3.4	Fish DNA barcoding	11
1.4 DNA	barcode for higher taxon assignment	13
1.5 Facto	ors effecting efficacy of DNA barcodes	15
1.5.1	Reference barcode library	15
1.5.2	Sample condition	16
1.5.3	Algorithms for DNA barcode based identification	18
1.6 Prosp	pect of the study	21
1.7 Obje	ctive of the study	22
Chapter 2.	REVIEW OF LITERATURE	23
2.1 Cata	loging and classification of global fish species	23
2.2 Cata	loging and classification of Indian freshwater fishes	25
2.3 Mole	ecular approaches and use of DNA barcoding in fish systematics	27
2.3.1	Early molecular studies	27
232	Mitochondrial DNA in taxonomy studies	28

2.3.	.3	Intr	oduction of DNA Barcoding	29
2.3.	.4	Lau	nch of iBOL and next phase of DNA Barcoding	31
2.4	Appli	catio	ons of fish DNA barcoding	35
2.5 I	Evolu	ition	of DNA barcoding as a technique	40
2.5.	.1	Dist	tance and monophyly based methods	40
2.5.	.2	Adv	vent of Character based methods	41
2.5.	.3	Con	nparison of distance based and character based methods	42
2.5.	.4	Nev	v methods of barcoding	43
Chapter	3.	MA	TERIALS AND METHODOLOGY	45
3.1	Mat	erial	S	45
3.1.	.1	DN.	A barcode sequence	45
3	3.1.1.	1	Retrieved DNA barcode sequences	45
3	3.1.1.	2	Generated barcode sequences from eastern and Northeastern India.	.46
3.1.	.2	Che	micals	47
3	3.1.2.	1	Reagents	47
3	3.1.2.	2	Buffers	48
3	3.1.2.	3	Primers	49
3.2	Met	hodo	ology	50
3.2.	.1	Gen	nerating COI DNA barcodes from fish samples	50
3	3.2.1.	1	Isolation from tissue	50
3	3.2.1.	2	Purity check and yield of the extracted DNA	51
3	3.2.1.	3	Spectrophotometric determination	51
3	3.2.1.	4	Agarose gel electrophoresis for genomic DNA quantification and	
C	qualit	y an	alysis	52
3	3.2.1.	5	PCR amplification of barcode segment of COI gene	53
3	3.2.1.	6	Gel-electrophoresis and purification of PCR product	53
3	3.2.1.	7	Sequencing of PCR amplicons	55
3	3.2.1.	8	Sequence submission	55
3.2.	.2	In s	ilico sequence analysis	55
3	3.2.2.	1	Sequence formats used for sequence analysis	55

3.2.2	2.2	Data analysis	56
3.2.2	2.3	Rseq calculation for sequence information analysis	58
3.2.2	2.4	Selecting high-informative segment within the <i>COI</i> barcode	58
3.2.2	2.5	In silico primer designing of minibarcode segment	59
3.2.2	2.6	Neighbor Joining tree construction	59
3.2.2	2.7	Developing consensus barcode motifs using MOTIF-BUILD	59
3.2.2	2.8	Specificity check of the barcode motifs using MEGA	60
3.2.2	2.9	Specificity check using MOTIF-MATCH	60
Chapter 4.	RES	SULTS	63
Chapter 4	.1	Assessment of freshwater fishes of India	64
4.1.1	Ass	essment of the recorded status	64
4.1.2	Ass	essment of the barcoded status	66
4.1.3	Spe	cies authentication using DNA barcoding	70
4.1.3	3.1	Detecting erroneous identification of fillet.	70
4.1.3	3.2	Detecting erroneous identification of juvenile market specimen	75
Chapter 4	.2	Compositional analysis of COI DNA barcodes.	77
4.2.1	Nuc	eleotide composition analysis	77
4.2.2	Nuc	cleotide pair frequencies	84
4.2.3	Coc	lon usage	85
4.2.4	Seq	uence information in the <i>COI</i> barcode using R _{seq} value	87
Chapter 4		DNA barcode based taxonomic rank assignment using distance	02
	_	cies level identification	
4.3.2	•	cial case of the unusual genetic diversity in Clarias batrachus	
4.3.3		nus level identification	
4.3.4		nily level identification	
4.3.5		er level identification	
Chapter 4 taxon		Development of character profiles for different hierarchical level	
4.4.1		racter profiles for species	
4.4.2		racter profiles for genus	
1.7.2		autor promes for Sends	113

4.4.3	3	Character profiles for family	18
Chapte	er 4.5	5 Development of species specific barcode motif	21
4.5.	1	Retrieval of highly informative region	21
4.5.2	2	Primer design and validation	21
4.5.3	3	Validation of the proposed segment for use in species delimitation 1	24
4.5.4	4	Design of barcode-motifs	24
4.5.5	5	Motif specificity validation	25
4.5.0	6	Validation of the barcode motifs with global Cypriniformes data 1	28
Chapter	5.	DISCUSSION1	31
5.1	Stat	us of DNA barcoding of freshwater fishes in India	31
5.2	Sequ	uence composition of COI barcode and taxonomic rank assignment 1	32
5.3	Spec	cies level identification using distance based DNA barcode method 1	35
5.4	Cha	racter based assessment and development of Barcode Motif	40
5.5	Con	clusion1	43
Chapter	6.	SUMMARY1	44
BIBLIO	GRA	NPHY	46
APPENI	DICE	ES	66
PUBLIC	ATI	ONS1	90

List of Tables

No	Table Title	Page No
4.1	Summary of occurrence status of species belonging to different orders of Indian freshwater fishes.	65
4.2	Species recorded versus Species barcoded in different orders of Indian freshwater fishes.	69
4.3	Similarity search table of specimens barcoded in the study using BLAST search and BOLD identification engine.	73
4.4	Details of specimen sequenced as submitted in GenBank.	74
4.5	Nucleotide compositional analysis of 1307 species belonging to 10 orders of Indian freshwater fishes.	78
4.6	Nucleotide pair frequencies of 1383 sequences of 10 orders of Indian freshwater fishes across 510bp region of <i>COI</i> gene.	84
4.7	Codon Usage for 1383 sequences of Indian freshwater fishes across 510bp region of <i>COI</i> gene.	86
4.8	Frequency distribution of R_{seq} value of variable nucleotides and amino acids in COI gene of different taxonomic ranks of Indian freshwater fishes.	91
4.9	Summary of genetic divergences (K2P model) for each taxonomic level of comparison.	92
4.10	Same named species with divergence above minimum congeneric value and formed two or more distinct sub-cluster.	95
4.11	Mean divergence within and between clusters of Barilius genus.	100
4.12	Different named species that clustered cohesively and showed divergence below maximum conspecific divergence.	102
4.13	Genetic divergence of different species of Clarias genus across various geographical location calculated using K2P model.	105
4.14	Summary of genera forming distinct cluster in NJ tree.	107
4.15	Summary of families forming distinct cluster in NJ tree.	108
4.16	Summary of orders forming distinct cluster in NJ tree.	109
4.17	Character profiles of <i>COI</i> barcode for species of Cyprinidae family at 30 nucleotide positions	113
4.18	Character Profiles of <i>COI</i> barcode for genera of Cyprinidae family at 20 nucleotide positions	117
4.19	Character profiles of <i>COI</i> barcode for families of Cypriniformes order at 22 nucleotide positions.	120
4.20	Motif verification of Cypriniformes species developed from Indian sequences against representative barcodes from other parts of the world present in BOLD.	129
5.1	Correlation of Nucleotide and Amino acid Rseq values between different orders of Indian freshwater fishes.	133
5.2	Number of indistinguishable species and the number of species with UCS.	137

List of Figures

No.	Figure Title	Page No
1.1	Map showing drainage and river system of India.	6
3.1	Flowchart describing the categorization of the species into three groups based on clustering pattern in NJ tree.	57
3.2	Flow chart describing the algorithm of MOTIF-MATCH Program.	61
3.3	Flowchart describing the Match-String Module of the MOTIF-MATCH Program.	62
4.1	Diagram showing a) species recorded b) species barcoded c) species recorded vs species barcoded in 20 recorded orders of Indian freshwater fishes.	68
4.2	Representative image of DNA sequencing process showing: a) Gel image of PCR amplicons of COI barcode region of fish samples. b) DNA sequencing Chromatogram data.	71
4.3	Representative image of collected specimen of Labeo bata.	43
4.4	Nucleotide compositions in 10 orders of Indian freshwater fishes.	79
4.5	AT-GC Bias in 10 orders of Indian freshwater fishes.	79
4.6	Correlation of average GC content with GC content at each codon position, in 10 orders of Indian freshwater fishes.	80
4.7	Correlation between GC content at each codon position and average GC content in 1307 species of the orders Cypriniformes, Siluriformes and Perciformes.	80
4.8	Plot of AT-GC SKEW of 1307 species of Indian freshwater fishes a) Overall b) 1st codon position c) 2nd codon position d) 3rd codon position.	82
4.9	Correlation between GC skew at each codon position and average GC skew in species of different orders of Indian freshwater fishes.	83
4.10	Correlation between AT skew at each codon position and average AT skew in species of different orders of Indian freshwater fishes.	83
4.11	Pattern of distribution of sequence conservation across various taxonomic ranks of Indian freshwater fishes.	88

4.12	Distribution on conspecific and congeneric K2P mean divergence among 175 species of Indian freshwater fishes (arranged in ascending order).	94
4.13	(i)Sections of Neighbor–Joining (from Appendix 3) tree showing the problematic groups of (a) <i>Clarias batrachus</i> (b) Heteropneustes genus.	97
	(ii) Sections of Neighbor–Joining tree (from Appendix 3) showing the problematic groups of (c) Labeo genus (d) Channa genus (e) Macrognathus genus.	98
4.14	Neighbor joining tree showing clustering of different species of Barilius genus.	101
4.15	Neighbor joining tree showing genetic divergence of <i>Clarias batrachus</i> from different geographic location.	105
4.16	Pairwise interspecies difference in character attributes between 137 species.	111
4.17	Average intraspecies differences in character attributes within 116 species.	111
4.18	Pairwise intergenus difference in character attributes between 64 genera.	116
4.19	Average congeneric differences in character attributes within 64 genera.	116
4.20	Distribution of number of diagnostic characters within families.	119
4.21	Distribution patterns of transitions and transversion across full-length barcode (654bp) of 1307 sequences.	122
4.22	Comparison of interspecies distance between full length <i>COI</i> barcode (513bp) and minibarcode (154bp).	126
4.23	Comparison of intraspecies distance between full length <i>COI</i> barcode (513bp) and minibarcode (154bp).	127
5.1	Divergence summary (1:1, computed with K2P model) of 90 <i>COI</i> barcode sequences of the genus Barilius and Opsarius.	139

List of Appendices

NO	APPENDIX	PAGE No.
Appendix 1	List of recorded freshwater fish species of India along with representative number of barcoded sequence present in the database.	166
Appendix 2	List of Indian freshwater fish <i>COI</i> barcode sequences used in the study.	175
Appendix 3	Neighbor joining tree using full length <i>COI</i> barcode of 175 species of Indian freshwater fishes.	180
Appendix 4	Neighbor joining tree of 160 species of Indian freshwater fishes using 154bp length <i>COI</i> barcode fragment.	183
Appendix 5	154bp length <i>COI</i> Barcode motifs designed in this study for 109 species.	186

Abbreviations

μl microliter
° C Degree Celsius

A Adenine

BOLD Barcode of Life Data System

bp base pair C Cytosine

CBOL Consortium of Barcode of Life COI Cytochrome c oxidase subunit I

DNA Deoxy Ribonucleic acid

EMBL European Molecular Biology Laboratory

G Guanine gm gram

IUPAC International Union of Pure and Applied Chemistry

IUB International Union of Biochemistry

K2P Kimura 2 Parameter

lb pound

MEGA Molecular Evolutionary Genomic Analysis

mg milligram
ml millilitre
mM millimolar

mtDNA Mitochondrial DNA

NCBI National Centre for Biotechnological Information

NJ Neighbor Joining
ORF Open Reading Frame
PCR Polymerase Chain Reaction

pmole picomole

rpm Revolution per minute

RSCU Relative Synonymous Codon Usage

T Thymine