Molecular, Osteological and Morphological evolution in freshwater bagrid catfish (Siluriformes: Bagridae) from Northern Western Ghats of India

5th year BS-MS Thesis

By

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Certificate

This is to certify that this dissertation entitled “Molecular, Osteological and Morphological evolution in fresh water Bagrid catfish (Siluriformes: Bagridae) from Northern Western Ghats of India” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by “Aniket Mokashi at Indian Institute of Science Education and Research (IISER), Pune” under the supervision of “Neelesh Dahanukar, INSPIRE Faculty Fellow, Department of Biology, IISER Pune” during the academic year 2013-2014.

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Declaration

I hereby declare that the matter embodied in the report entitled “Molecular, Osteological and Morphological evolution in fresh water Bagrid catfish (Siluriformes: Bagridae) from Northern Western Ghats of India” are the results of the investigations carried out by me at the Department of Biology, Indian Institute of Science Education and Research (IISER), Pune, under the supervision of Neelesh Dahanukar and the same has not been submitted elsewhere for any other degree.

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Abstract

Freshwater catfishes are major group of aquatic organisms. Lack of comprehensive work on Indian catfishes of family Bagridae has rendered the true diversity being obscure. Further, for the species which are already know, there is relatively less studies on the systematics and evolution. In this study we have tried to understand the diversity of bagrid catfishes and their evolutionary relationships using fish barcode gene cytochrome oxidase subunit I (Cox1) and cytochrome b (Cytb) along with the morphometric and osteological analysis. The Phylogenetic analysis was done using Maximum likelihood for cox 1 and cyt b gene and phylogenetic tree was constructed and the reliability of clustering was checked for 1000 bootstrap iterations. The morphometric study for 121 samples of bagridae was done and the morphometric data was used for multivariate statistical analysis. Further clearing and staining procedure was used to understand the osteology of selected species of bagrids with respect to their head structure, gill rakers and number of vertebra. Our result show a presence of several species complexes among the known species of bagrids, indicating that there are many new species which are yet to be discovered and described from Western Ghats. Further we show based on molecular, osteological and morphological studies that the two nominal species *Mystus cavasius* and *M. seengtee* are one and the same and the species *M. cavasius* is distributed throughout India. Our results have implications in understanding the bio geographical patterns in species distribution.

Key words: Molecular phylogeny, Morphometry, Osteology, Bagridae, *Mystus*, *Sperata*, *Rita*, *Hemibagrus*, Western Ghats of India.

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INTRODUCTION

The Western Ghats of India or the great Escarpment of India which runs along a range of total 1600 Km Starting from the border of Gujarat and Maharashtra down the way till southern coast of Kerala. Western Ghats is one of the 34 bio diversity hotspots in the world (Mittermeier et al., 2005) and only one among 2 in south Asia. (Mayers et al., 2000). The Western Ghats is rich in its freshwater fish diversity with 290 species out of which 189 species are endemic to this region (Dahanukar et al., 2011).The Western Ghats of India is so much rich in its diversity but a major part of this diversity is threatened by a various anthropogenic activities (Dahanukar et al., 2004). From Western Ghats of India 38 east-flowing and 27 west-flowing major rivers originates. The West flowing rivers originates in Western Ghats and drain in Arabian Sea and East flowing rivers merge into one of 3 rivers system Krishna Cauvery or Godavari before they drain into Bay of Bengal (Dahanukar et al., 2004).

Catfishes belong to order Siluriformes. The catfishes group consist of an approximately 35 families, 437 genera and 2734 species across the globe starting from South America, Africa, Europe and Asia to Japan. Globally, the population of catfishes makes up to the 1/3 of freshwater fish fauna (Jayaram 2009). Evolution of catfishes date as back as Eocene times. Indian catfishes come under 13 families, 52genera and 197species. About 50 species of order Siluriformes are found in the Western Ghats of India and associated river system, out of which 21 species come under family bagridae. Catfishes are mostly consumed all over the world and hence they hold a pretty important market value. Catfishes of the family Bagridae are highly threaten because of variety of threats including pollution, biological resources use and habitat modification and it is suggested that more than 50% of the species are threatened with extinction (Dahanukar et al., 2011). Catfishes of the family Bagridae are extensively studies all over the world however in Indian context they are relatively less studies. Even though some taxonomic literature is available (Jayram 2009) osteology and molecular phylogeny is very less studied in India and much of the classification were based on morphometric analysis. Due to adherent impediments with traditional taxonomy (Dahanukar et al., 2011) a few species claimed to be new are not valid. Instead there have been cases of synonym species so there is need for taxonomical revision. This is especially true for the Western Ghats of India. Recently Identification and Re-evolution of fresh water catfishes

through DNA barcoding (Bhattacarjee et al., 2012) of North east India was done but catfishes from Western Ghats of India is not that clearly understood.

Our attempt through this study is to have look at Molecular, Osteological and Morphological evolution in fresh water Bagrid catfish (Siluriformes: Bagridae) of Western Ghats of India. Currently we have done an extensive studies on the catfishes of Western Ghats of India at osteology, morphometric, and molecular level. The cytochrome oxidase 1 (Cox1) and cytochrome b (Cytb) gene were used to draw an evolutionary tree of the Catfishes. The 26 morphometric character were used to study the morphometry of catfishes. Principle component analysis was used on the size corrected values to understand the morphological patterns in various species of bagrids. The clearing and staining method was used for osteological study of bagrid. Therefore, in the current study we attempt to study the molecular, osteological and morphological characters of bagrid catfishes focusing mainly on genus *Mystus*, followed by *Sperata, Rita* and *Hemibagrus*.

MATERIALS AND METHODS

Study site and sample collection

Four genera in family Bagridae were collected during the present study including *Mystus*, *Hemibagrus*, *Rita* and *Sperata*. The species included *Mystus cavasius*, *M. seengtee*, *M. cf. gulio*, *M. bleekeri*, *M. cf. bleekeri*, *M. malabaricus*, *Sperata aor*, *S. seenghala*, *Hemibagrus punctata* and *Rita gogra*. A list of all the samples used in the study is provided in Table 1, while photographs of some of the bagrids are provided in Figure 1.

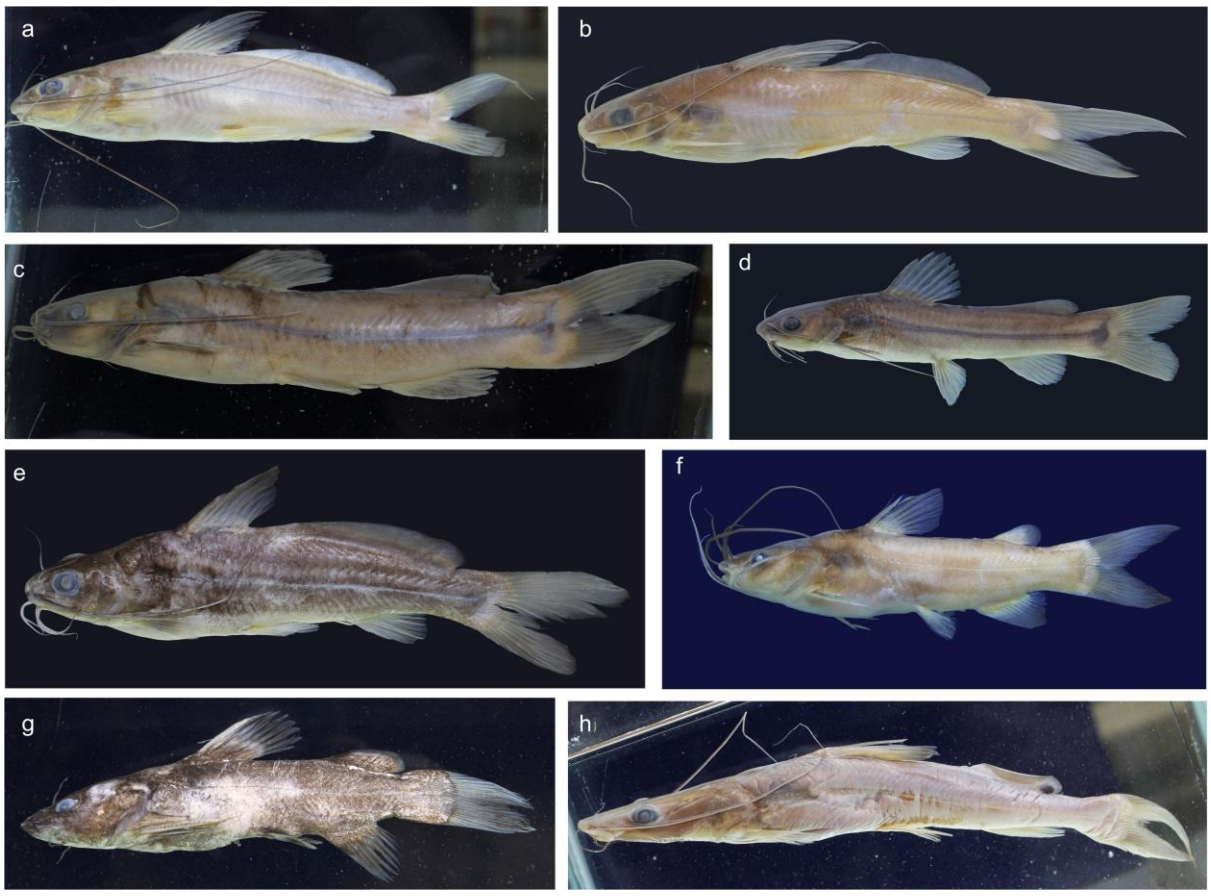

Figure 1: Bagrids used in the present study. (a) *Mystus seengtee* from Yerawada,(b) *Mystus cavasius* from Godavari river (c) *Mystus malabaricus* from Satara (d) *Mystus cf. malabaricus* from Amboli Azara (e) *Mystus bleekeri* from Bhima (f) *Mystus Guilo* from Amboli azara (g)*Rita gogra* from Bhima (h)*Sperata seenghala* from Koyna.

Table 1: Bagrid fish species collected from various locations.

Location	Species	No of specimen used for	
		Morphometic analysis	Molecular analysis
Gangapur dam	*Mystus cavasius*	6	0
Solapur	*Sperata seenghala*	1	1
	Mystus seengtee	3	2
Ghod taleghar	*Mystus malabaricus*	1	0
Veer	*Mystus seengtee*	2	2
	Sperata seenghala	1	0
Kerala	*Mystus seengtee*	3	2
Bhor	*Mystus seengtee*	3	1
Neera	*Mystus bleekeri*	1	0
Karjat	*Mystus malabaricus*	3	1
Lonavala	*Mystus malabaricus*	1	1
Bhigwan	*Mystus seengtee*	2	2
Ujjani dam	*Mystus seengtee*	4	2
Yerwada	*Mystus seengtee*	9	5
	Mystus bleekeri	2	2
Ganga	*Mystus cavasius*	1	1
Wai	*Mystus malabaricus*	3	3
	Mystus cf. bleekeri	3	2
Satara	*Mystus malabaricus*	4	4
Chiplun	*Mytus cf. gulio*	1	1
Aundh	*Mystus seengtee*	17	0
Mangaon	*Mystus malabaricus*	3	3
	Mystus seengtee	1	0
Ratanagiri	*Mystus malabaricus*	3	3
	Mystus cf. gulio	3	3
Raigad	*Mystus seengtee*	3	0
	Mystus malabaricus	3	0
Patan	*Mystus malabaricus*	6	0
	Mystus seengtee	1	0
Kudal	*Mystus malabaricus*	1	0
Amboli-azara	*Mystus malabaricus*	1	2
	Mystus cf. bleekeri	0	1
Panvel	*Mystus seengtee*	1	1
Hali village	*Mytus cf. gulio*	1	0
Orissa	*Mystus cavasius*	1	1
Panjim Goa	*Mystus cf. gulio*	3	3
Kudal	*Mystus malabaricus*	1	0
Paud market	*Mystus seengtee*	1	0
Bhavani River	*Hemibagrus punctatus*	0	1
Phansad	*Mystus malabaricus*	1	0
Bhima	*Mystus seengtee*	13	8
	Rita gogra	1	2
	Mystus bleekeri	4	2
	Mystus malabaricus	2	2

Samples were collected mainly from the northern Western Ghats of India except for some which were collected from Kerala and northeast India for comparison (Figure 2). The sample collection were directly from the rivers with the help of net while others were brought from the local fish market. The samples collected were stored in 4% formalin and some part of the samples were kept in absolute alcohol for DNA extraction.

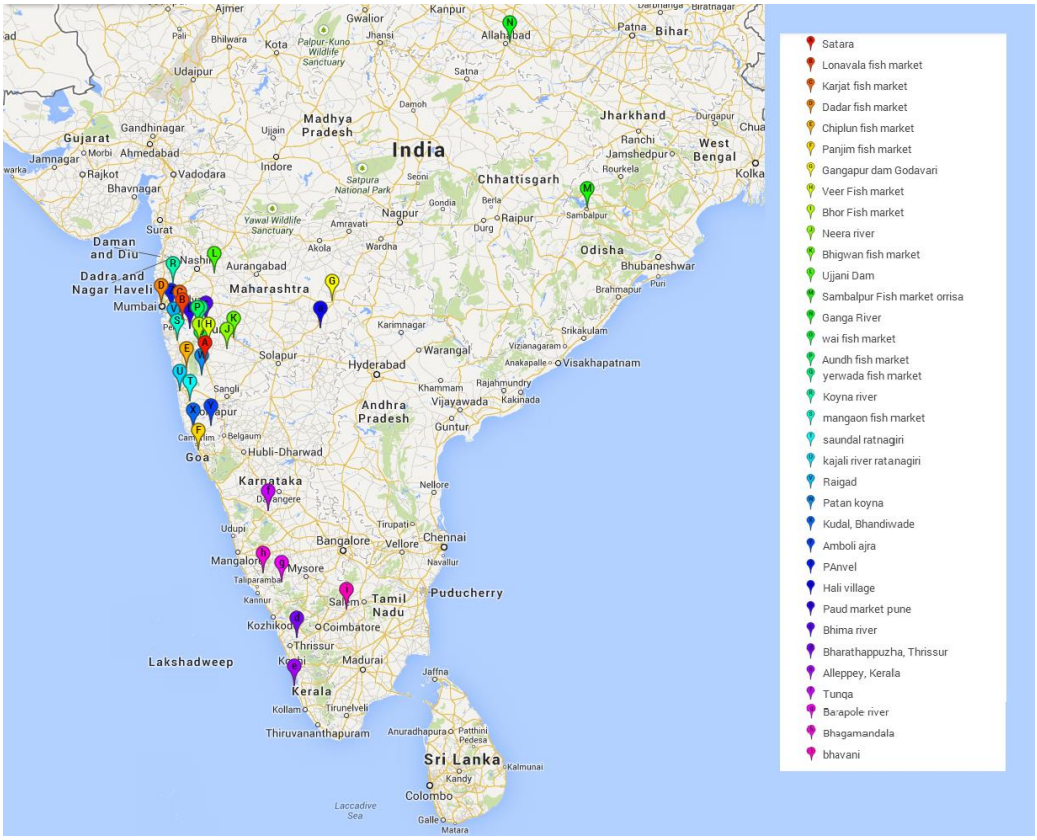

Figure 2: Map of peninsular India showing the places of collection of Specimen.

The samples were collected from the different places such as: Ujjani dam, Lonavala, Bhigwan, Chiplun, Karjat, Satara, Yerwada, Wai, Mangaon, Kerala, Shirur, Amboli-Ajara, Solapur, Paud, Patan, Godavari, Bhor, Paingin (Goa), Mula-Mutha, Raigad, Ghod at Taleghar, Ratnagiri, Veer, Panvel, Indrayani, Phansad, Koyna, Kudal, Hali Village and Neera river (Figure 2). Samples were also obtained from Ganga and Orissa for comparision. Some of the samples were collected by myself but for better comparision samples collected by Neelesh Dahanukar, Mandar

Paingankar, Unmesh katwate, Ashwini Keskar, Pradeep Kumkar, S.S.Jadhav, Rajeev Raghavan and Anvar Ali were also considered.

Genetic analysis

A total of 121 samples were brought out of which 64 samples were used for DNA extraction, cytochrome b and cox1 gene were amplified and sequenced. The DNA was extracted from the gills which were kept in 100% ethanol following protocols by Ali et al. (2013) and Dahanukar et al. (2011). The tissue was digested at 60°C for 2 hours using STE buffer (0.1M Nacl, 0.05 M Tris-HCL, 0.01M EDTA, 1% SDS) with 15μl Proteinase K(20mg/ml) per 500μl of STE buffer. DNA was extracted using conventional phenol-chloroform method and re-suspended in nuclease free water. Extracted DNA was checked with 1% Agarose gel electrophoresis and nanodrop. The DNA was stored at -20°C. The PCR were done for Cytochrome b and Cytochrome Oxidase subunit 1 gene (Table 2) and the PCR product was checked for its purity and length in 1% Agarose Gel and the gel was observed under UV Tran illuminator EP-04. The PCR product was purified using 'Promega Wizard Gel and PCR clean up' and then sent for sequencing.

Table 2: Primers used for the study.

Gene	Primer	Sequence(5'→3')	Tm	Approximate size of Amplification (bp)	Reference
Cyt b	L14724	GACTTGAAAAACCACCGTTG	50	1118	Chen et al. (2007)
	H15915	CTCCGATCTCCGGATTACAAGAC	57		
Cox1	FishF1	TCAACCAACCACAAAGACATTGGCAC	58	655	Sharina and Kartavtsev (2010)
	FishR1	TAGACTTCTGGGTGGCCAAAGAATCA	58		

The sequences received were analyzed using BLAST (Altschul et al. 1990) and DAMBE (Xia 2013) and the phylogenetic Trees were generated using MEGA 6 (Tamura et al. 2013). Additional 273 sequences of species under family Bagridae were downloaded from NCBI GenBank. Sequences were aligned using MUSCLE (Edgar 2004). The sequences then analyzed and the phylogenetic tree were constructed. For cox 1 gene a Maximum Likelihood (ML) tree was constructed using HKY+G+I model (AICc=13070.017, lnL=-5946.481, +G=0.99, +I=0.44). For cytb gene ML tree was constructed using GTR+G+I model (AICc=32089.087, lnL=-

15754.698, +G=1.00, +I=0.45). Reliability of clustering was checked for 1000 bootstrap iterations. *Glyptothorax poonaensis* (Family: Sisoridae) was used as out group for both the genes.

Morphological analysis

Morphometry was done using a digital vernier caliper (Mitutoyo: 500-197) with the least count of 0.01mm. Morphometric analysis was done for a total of 26 Morphometric characters and 4 meristematic characters following Ng and Kottelat (2013). Morphometrical characters includes: 1, Standard length; 2, Predorsal length; 3, Preanal length; 4, Prepelvic length; 5, Prepectorial length; 6, Dorsal-spine length; 7, Dorsal-fin length; 8, Length of dorsal fin base; 9, Length of anal-fin base; 10, Pelvic-fin length;11, Pectorial –fin length; 12, Pectorial-spine length; 13, Caudal-fin length; 14, Length of adipose-fin base; 15, Maximum height of adipose fin; 16 Dorsal to adipose distance; 17, Post-adipose distance; 18, Length of caudal peduncle; 19, Depth of caudal peduncle; 20, Body depth at anus; 21, Head length; 22, Head width; 23, head depth; 24, Snout length; 25, Interorbital distance; 26, Eye diameter. Meristematic characters include: 1, Dorsal fin ray count; 2, Pectoral fin ray count; 3, Pelvic fin ray count; 4, anal fin ray count.

The predorsal, preanal, prepelvic and pectorial lengths were measured from the snout till the anterior base of dorsal, anal, pelvic and pectorial fins. Pelvic and pectorial fins were measured from base till tip of the longest fin. The dorsal fin is measured from base of dorsal spine to longest fin. The length of adipose fin base is measured from the anterior most point of origin to the posterior most point of adipose fin base. Dorsal and pectorial spine were measured from the tip base of spine till the tip. The dorsal to adipose distance is measured from the base of last dorsal-fin ray to the origin of adipose fin. The maximum height of adipose fin is the maximum vertical distance between the base and upper edge of fin. The length of the caudal-fin length is the length of longest ray of lower lobe measured from the middle of the base of caudal fin. The length of caudal peduncle is measured from the base of the last anal-fin ray to the start of caudal fin. The post adipose distance is measured from the posterior most point of the adipose-fin base to the middle of the base of the caudal fin. The depth of the caudal peduncle is the least vertical distance from the midline of the dorsal surface to the midline of the ventral surface. The depth of the body at anus is measured as vertical distance from the midline of the dorsal surface to the

midline of ventral surface at anus. The length of head is measured from the tip of the snout to hard bony flap covering the gills. The width of the head is measured at its widest point. The Interorbital distance is measured as the longest distance between two eyes from the center. The diameter of eye is measured as the longest horizontal distance of the eye. The meristematic characters were measured under a microscope manually. The fin rays were measured under light microscope.

Morphometric data was used for statistical analysis. The subunits of body were considered as percentage of standard length and subunits of head as percentage of head length to remove the size effect. Principle component analysis was used on the size corrected values. Statistical analysis was done in the freeware PAST (Hammer et al. 2001).

Osteological analysis

The clearing of the tissue and staining of cartilage and bone (Potthoff 1984) were done in our osteological study. The specimen were preserved in 10% formalin for 2-3 days after that the specimen were transferred to Absolute ethanol for dehydration so even small amount of water will be removed so that water should not interfere with the staining of cartilage. The specimen were kept for week in the alcohol followed by staining the cartilage. The specimen were kept in an acidified alcohol solution of alcian blue stain (60 ml absolute ethanol, 40ml acetic acid, and 30 mg alcian blue.) the cartilage is permanently stained such as stain cannot be removed by any solution in the process of osteology, the specimen were kept in staining solution for two days. The next step neutralization the specimen is kept in saturated sodium borate solution for 2 days to increase the pH within the specimen because higher the pH there is less calcium loss from the bone so the staining will be better. The next step is Bleaching in this step the specimen is kept in bleaching solution which contains (15ml of 3% H_2O_2, 85 ml 1% KOH), the specimen is kept in the bleaching solution for 2 hours and later the specimen were transferred to trypsin digestion stage. In trypsin digestion the specimen were kept in the trypsin digestion solution which contains (100 ml solution: 35ml saturated sodium borate, 65 ml distilled H_2O and 0.2 g trypsin powder) till the specimen is 60% cleared. The specimen were kept at 37°C and the trypsin solution were changed after 7-10 days. After the specimen were cleared the staining of bone were done using the Staining solution 1% KOH

with alizarin red stain until the solution turns deep purple. The specimen were kept in the staining solution for overnight. Staining of the specimen were followed by the destaining, the solution were kept in destaining solution (100 ml solution: 35 ml saturated sodium borate, 65 ml distilled H_2O, 0.2 g) until the specimen were cleared and the stained bone were clearly visible and then the photograph were taken and analysis was done.

Osteology was done with the help of drawing the fish head structure and fish pectoral spine. The head and spine were observed under a microscope and the drawing of different specimen were drawn. The specimen head and spine was observed under a Leica S8AP0 microscope and the images were taken under the microscope. c. The terminology of osteological structures follow Anganthoibi (2012).

RESULTS AND DISCUSSION

Molecular analysis

Maximum Likely hood analysis of cytochrome oxidase 1 (cox1) sequenced in the present study and downloaded from NCBI is shown in Figure 3.

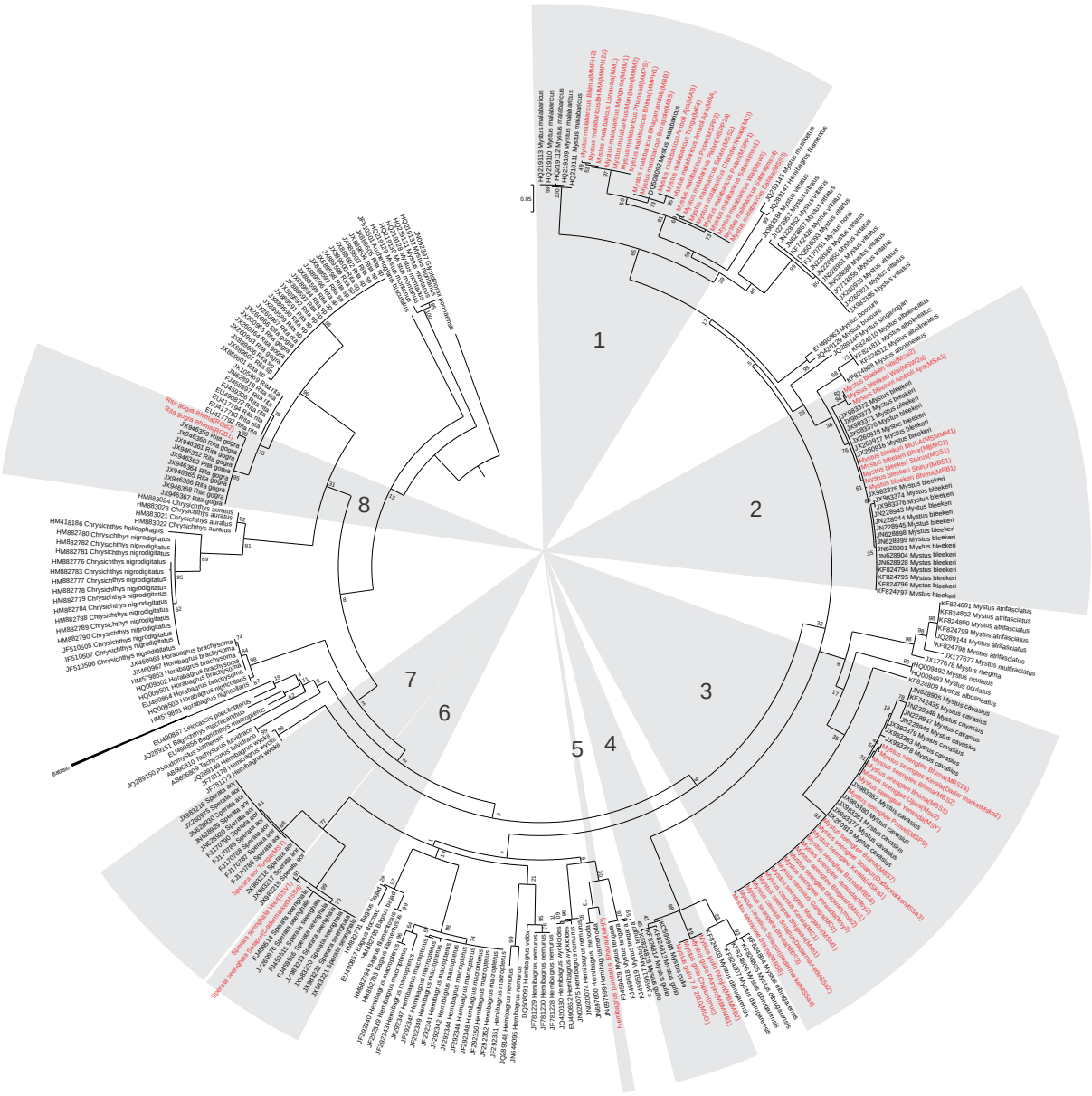

Figure 3: Maximum likelihood tree for cox1 gene of Bagrid fishes. The sequences highlighted in red were generated in the current study. Values along the nodes are percent bootstrap values.

The 8 species were studied are highlighted in grey areas. The grey area labelled as “1” consists of *Mystus* malabaricus sp complex which shows presence of at least 4 clusters along the stretch of entire Western Ghats of India. The grey area “2” comprises if *Mystus* bleekeri and a new species which is similar to *Mystus* bleekeri from satara and amboli area. The grey area “3” comprises of *Mystus* cavasius and *Mystus seengtee* from all over the peninsular India which consists of Krishna, Godavari, Ganges and Mahanadi river system. The grey area “4” comprises of *M.cf guilo* which is likely to be a new species from Konkan region. The grey area “5” states the phylogenetic position of *Hemibagrus punctatus* collected from Bhavani River. The grey area “6” comprises of *sperata*.seenghala collected from its type locality in Bhima River as compared to sequences from NCBI submitted from north east India. Grey area “7” shows the phylogenetic position of *Sperata* aor from Tunga River. Grey area “8” shows the *Rita* gogra collected from its type locality from Bhima River and compared with the sequences from NCBI which were uploaded from North east India. Separate result and discussion for Important Sub clusters are provided below.

Figure 4 depicts the Sub cluster containing *Mystus Malabaricus* Species complex. We could identify 4 major cluster separated by high bootstrap values. 4 Clusters which were separated from each other with difference of more than 3% M1, M2, M3, and M4. The average distance within cluster M1 is 0.1%, M2 is 0.6%, M3 is 1% and M4 is 0.2%. The difference between Cluster M1 and M2 was 14.6% while difference between clusters M2 and M4 = 4.7%, M3 and M4 = 2.8%, M1 and M4 = 13.3%, M1 and M3 = 12.9% M3 and M2 = 5%. Because cox1 gene is a barcoding gene 3% variation has been suggested as a cut off for a designated species (Hebert et.al 2003). We therefore suggest that *Mystus malabaricus* is likely to be a species complex with around 4 Putative species distinct throughout the Western Ghats of India however further genetic analysis and morphological analysis is essential to identify this species. The original *Mystus malabaricus* is from Malabar region of Kerala. Cluster M1 could only be true *Mystus malabaricus.*

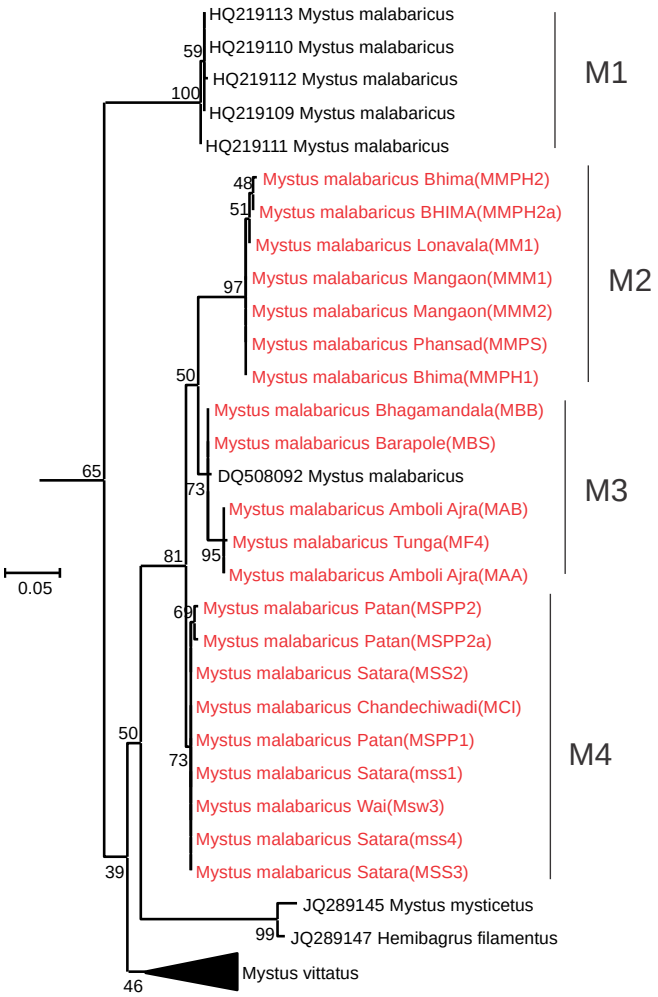

Figure 4: Subcluster of *Mystus malabaricus* species complex based on cox1 gene analysis depicting four clusters M1 to M4.

Mystus bleekeri showed 2 distinct cluster comprises of specimen from Bhima river system [Mula, Bhor, Bhima and Shirur] which sowed genetic similarities with true *Mystus bleekeri* from gangetic samples sequences (Figure 5) which were downloaded from NCBI however specimen from Wai and Amboli which superficial resembled *bleekeri* from a distinct cluster supported by high bootstrap value (94%) we suspect this is a distinct species which is different from *Mystus bleekeri.*

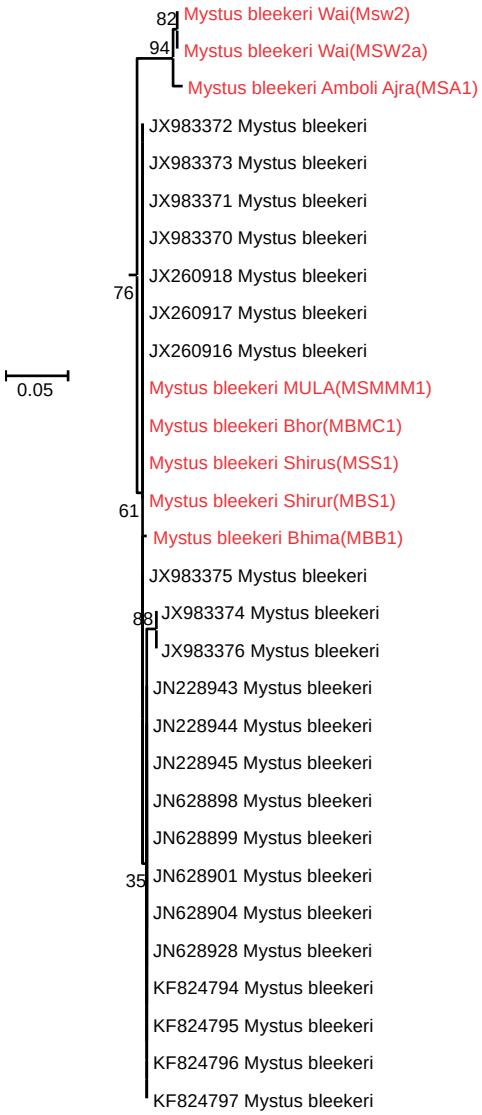

Figure 5: Subcluster of Mystus bleekeri species complex based on cox1 gene analysis.

Chakrabarty and NG [2005] suggested that *Mystus cavasius* and *Mystus seengtee* are 2 different species. They limited the *Mystus cavasius* to Godavari, Ganges and other river system between these 2 species and suggested that Species in Krishna river and rest of peninsular India as *Mystus seengtee*. Our genetic analysis [Figure 6] suggested that *Mystus cavasius* and *Mystus seengtee* are not genetically distinct. We considered samples of *Mystus seengtee* from its type locality in pune and from a variety of river system in entire peninsular India. Similarly we sampled *Mystus cavasius* from Godavari, Mahanadi and Ganges. 100% similarity in Cytochrome Oxidase 1 gene of both *seengtee* and *cavasius*. Suggested that both are same species.

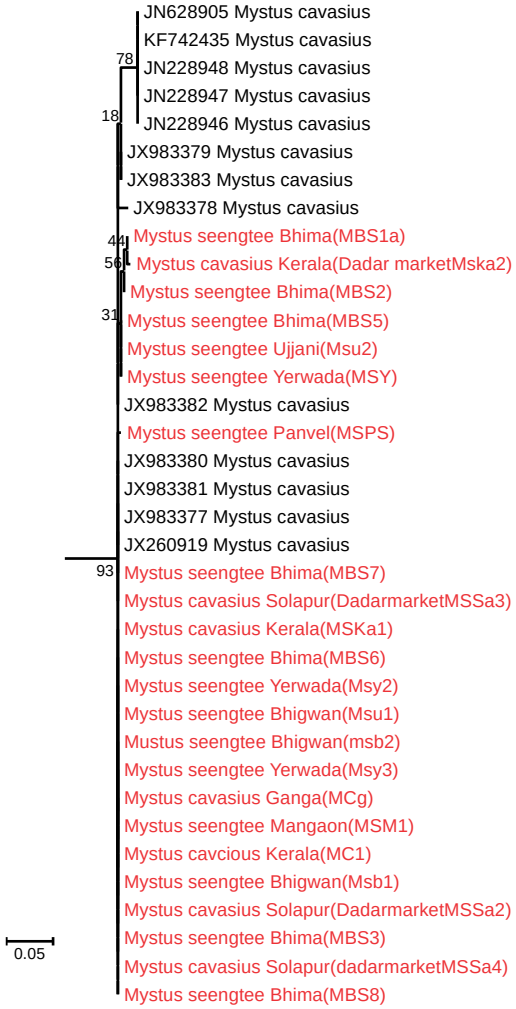

Figure 6: Subcluster of Mystus cavasius and M. sengtee based on cox1 gene analysis suggesting that both the species are one and the same.

Mystus guilo Collected from west flowing river of Western Ghats of India. The difference between *Mystus guilo* from Ganges and M.cf *guilo* from Western Ghats is 5.4% which suggest that M.cf *guilo* is likely to be a distinctly different species (Figure 7).

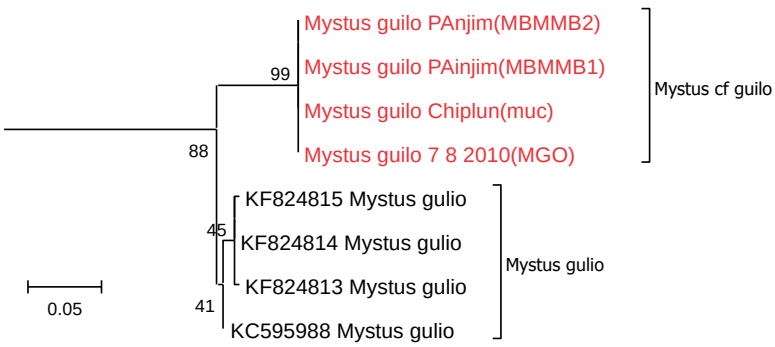

Figure 7: Subcluster of *Mystus gulio* species complex based on cox1 gene analysis.

Sperata Aor, *Sperata seenghala*, *Rita gogra* showed very high similarities with available sequences [Figure 8] from Ganges and north east India indicating *Sperata Aor*, *Sperata seenghala*, *Rita gogra* are wide spread in entire peninsular India.

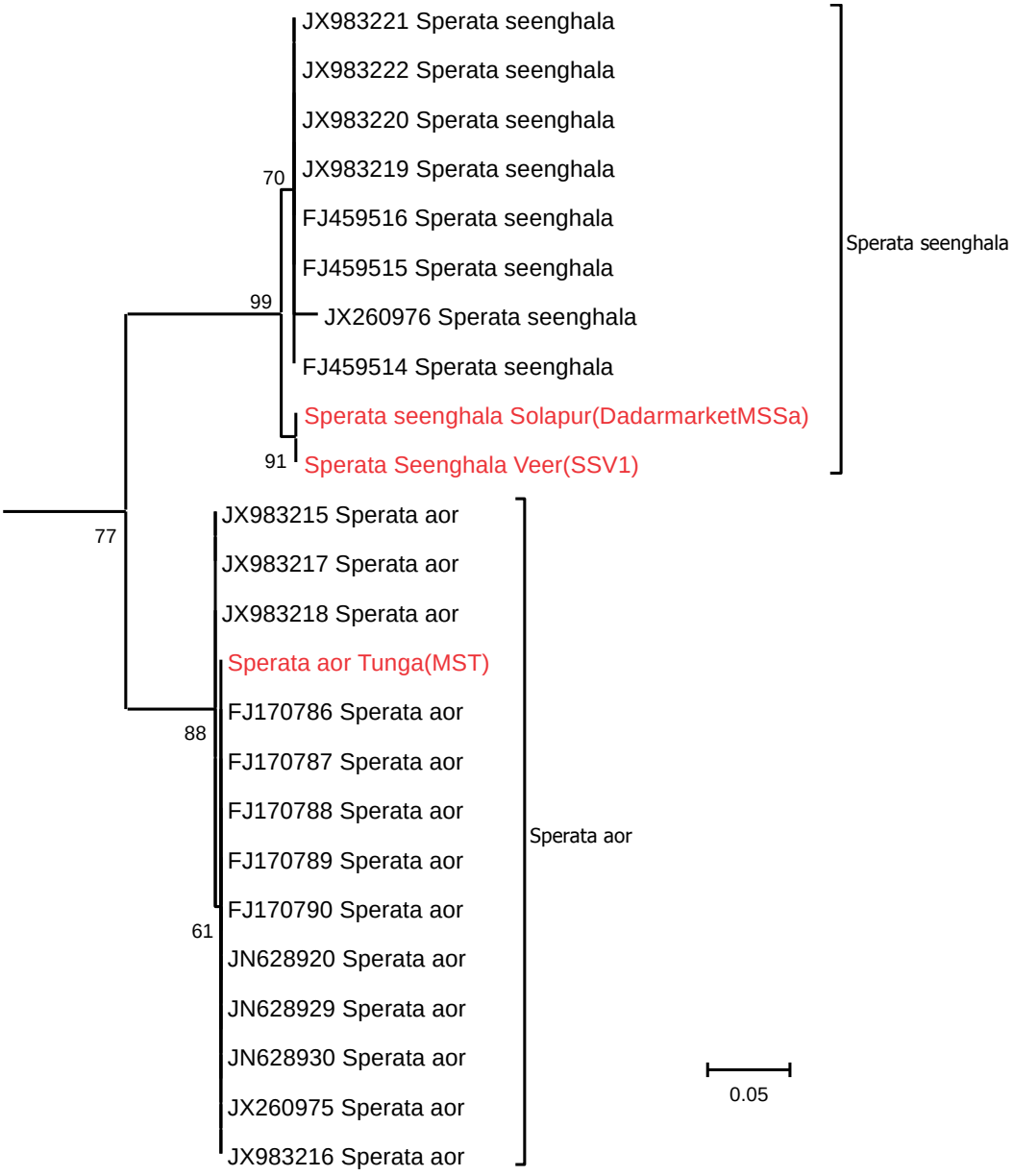

Figure 8: Subcluster of species belonging to genus *Sperata* based on cox1 gene analysis.

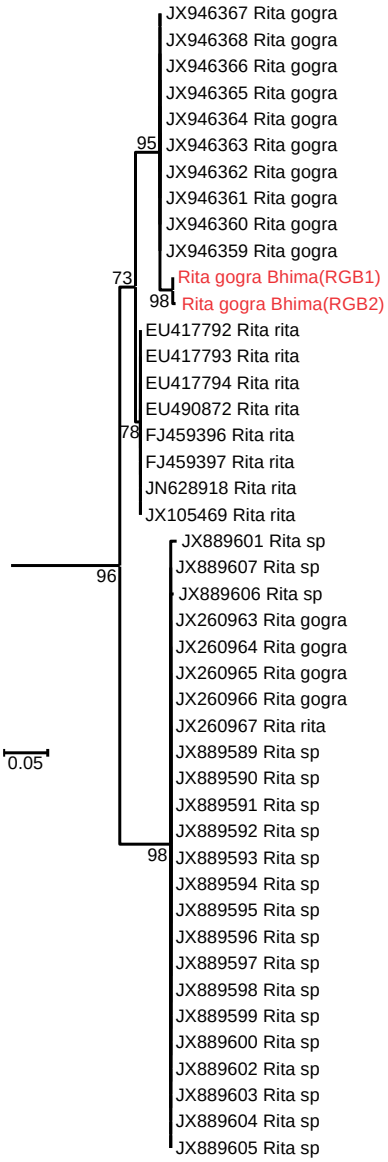

Figure 9: Subcluster of species belonging to genus *Rita* based on cox1 gene analysis.

Cytochrome b sequences of bagrid are represented by very less species as compared to cox1 gene in NCBI data base. Because of logistic reason we could not sequence all the sequences from Cytb however the sequences that we could generate from various species of *Mystus.* Showed similar clustering patterns as that of cox 1 (Figure 10).

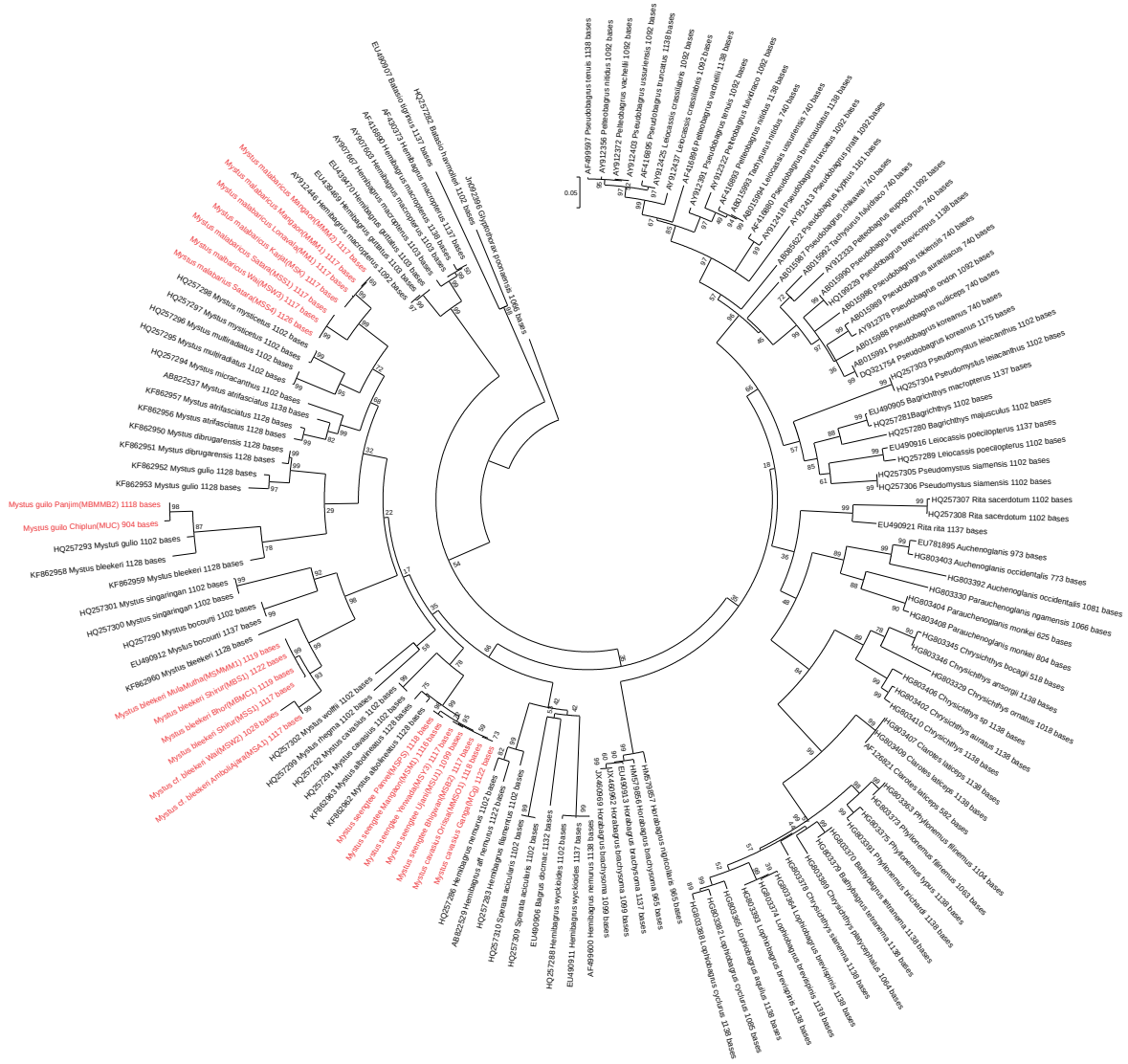

Figure 10: Maximum likelihood tree for cytb gene of Bagrid fishes. The sequences highlighted in red were generated in the current study. Values along the nodes are percent bootstrap values.

Analysis of cytb also suggested that *Mystus malabaricus* from Lonavala, Karjat, Mangaon is genetically different from Satara and Wai [Figure 11]. Because no information is available on *Mystus malabaricus* from Kerala we cannot comment on the affinity of *Mystus* from Northern Western Ghats with true *Mystus malbaricus* similar to cox 1. Cyt b also suggest that *Mystus bleekeri* from Bhima River [Mula mutha, Shirur, bhor] is genetically different from *Mystus cf bleekeri* from wai and azara. *Mystus cavasius* and *Mystus seengtee* were genetically similar in cyt b further supporting our claim both species are one and same. *Mystus cf guilo* from Western Ghats shows high genetic distance from *Mystus guilo* of Gangetic provinces.

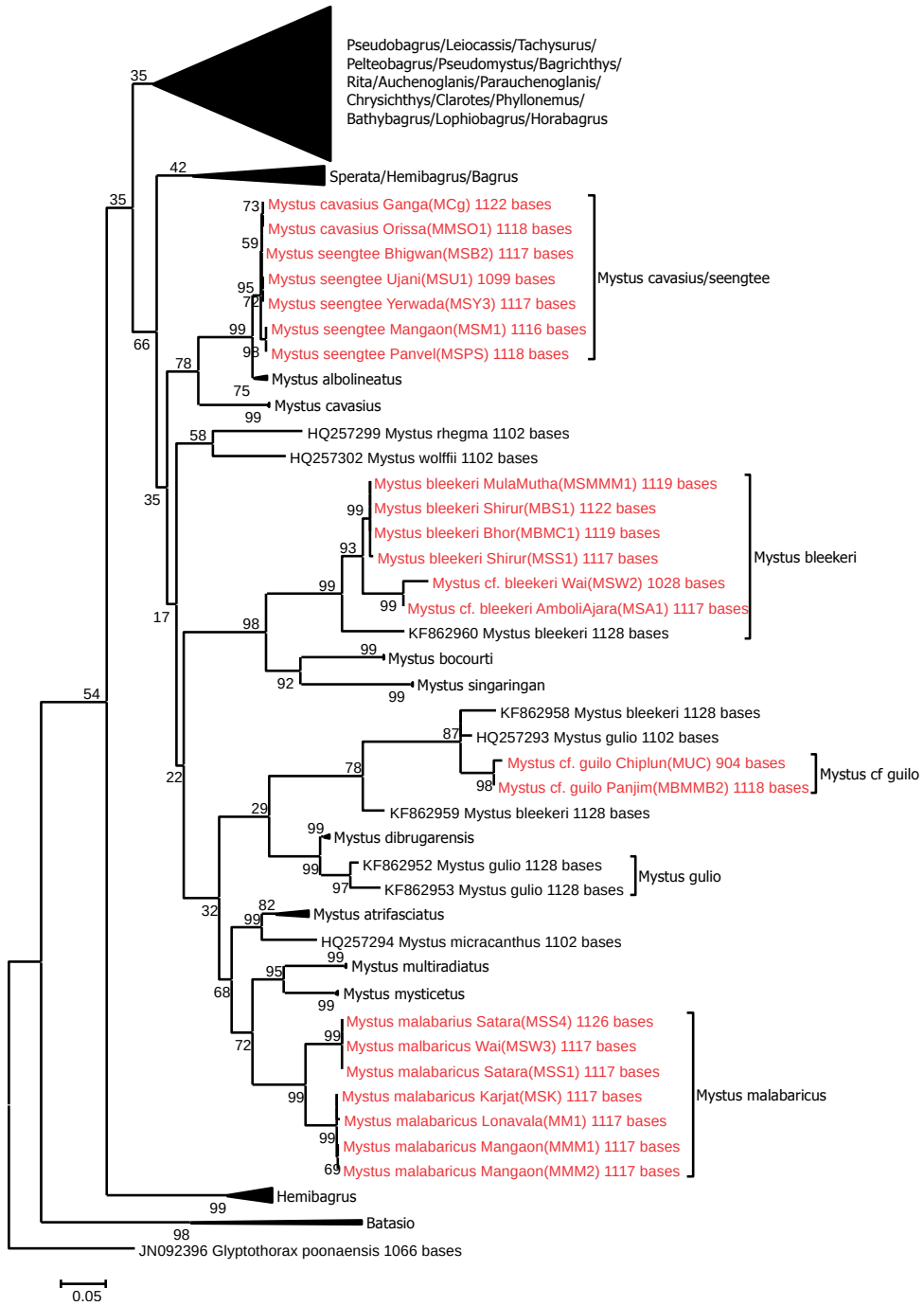

Figure 11: Maximum likelihood tree for cytb gene of Bagrid fishes under genus *Mystus*. The sequences highlighted in red were generated in the current study. Values along the nodes are percent bootstrap values.

Morphological analysis

In all 121 specimen were used for morphometric study. Principle component analysis (PCA) of different species used for morphometric analysis is shown in (Figure 12). Size corrected morphometric data and correlation matrix were used for analysis. PCA analysis extracted 6 axis with eigenvalues more than unity (Table 3) however because it was difficult for us to plot graph in multidimensional space we plotted the graph in 2 axis. First axis explained around 34% of total variation, second axis explained 15 % of remaining variance. All different species of *Mystus* and *Rita Gogra* were separated on first axis while *Sperata seenghala* were separated on second principle axis. Separation on first axis is based on character such as pre pelvic length, pre pectoral length, length of anal fin base, Pelvic fin length, length of adipose fin base, dorsal to adipose distance, post adipose distance, length of caudal peduncle, depth of caudal peduncle, head length and eye diameter. While separation on second axis was based on head width, head depth, snout length and interorbital distances.

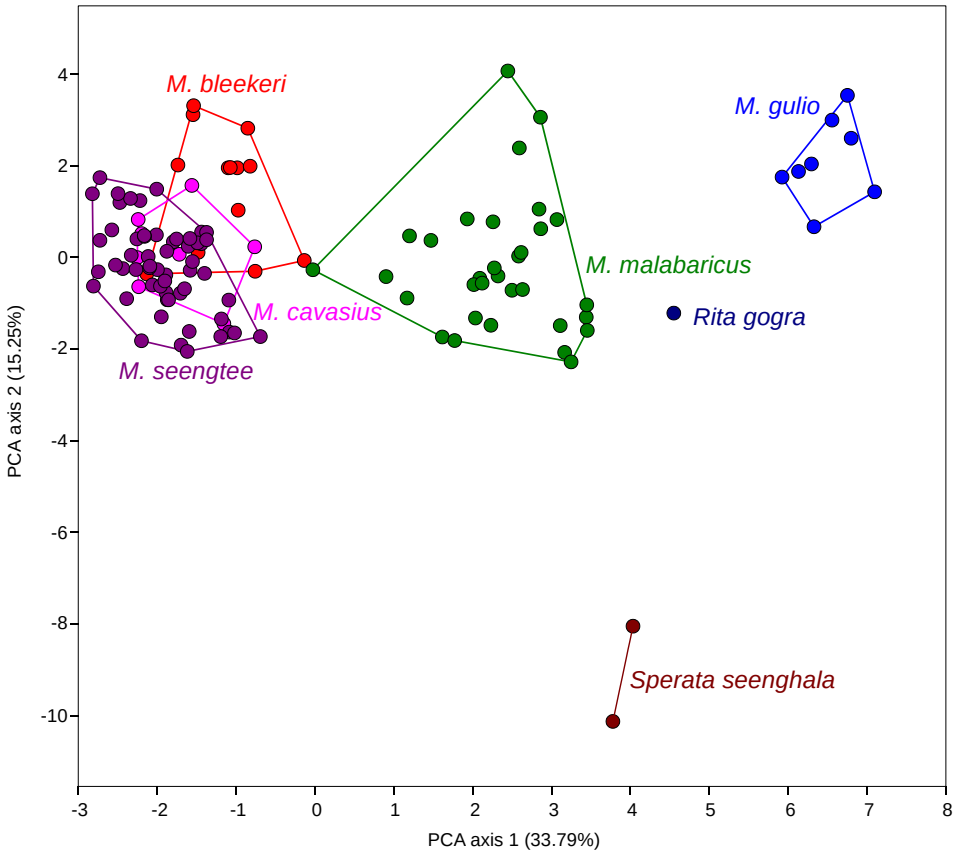

Figure 12: Principle component analysis of the specimens studied for morphometric analysis.

Table 3: Descriptive statistics and factor loading for the first six PCA factors.

	Axis 1	Axis 2	Axis 3	Axis 4	Axis 5	Axis 6
Eigenvalue	7.10	3.20	2.35	1.50	1.09	1.01
% variance	33.79	15.25	11.17	7.14	5.21	4.82
pre_dorsal_length	0.16	-0.07	0.08	0.24	-0.37	0.70
pre_anal_length	-0.04	0.00	0.29	0.43	-0.52	-0.28
pre_pelvic_length	0.26	0.02	0.20	0.14	0.04	-0.47
pre_pectorial_length	0.23	-0.02	0.23	0.08	0.22	-0.22
dorsal_fin_lenth	-0.16	0.03	0.38	-0.35	0.15	0.11
length_of_dorsal_fin_base	-0.07	-0.02	-0.20	0.55	0.47	0.11
length_of_anal_fin_base	0.29	0.07	-0.20	-0.01	0.26	0.17
pelvic_fin_length	-0.24	0.11	0.23	0.06	0.09	0.19
length_of_adipose_fin_base_	-0.36	0.01	-0.06	0.13	0.05	-0.06
MAX_height_of_adipose_fin	0.03	0.24	0.42	0.16	0.25	0.08
dorsal_to_adipose_distance_	0.34	0.08	0.09	-0.16	-0.12	0.05
post_adipose_distance	0.29	0.04	0.09	-0.35	0.05	0.08
length_of_caudal_pendancle	-0.24	0.19	-0.09	-0.12	0.07	0.05
depth_of_caudal_pendancle	0.25	0.25	-0.26	0.20	0.05	0.05
body_depth_and_anus	-0.01	0.42	0.22	0.12	0.01	0.14
head_length	0.23	-0.12	0.40	0.14	0.21	0.12
head_width	0.10	0.42	-0.18	0.10	-0.02	-0.09
head_depth	-0.12	0.44	0.07	-0.11	0.00	0.01
snout_length	-0.13	0.35	-0.01	0.01	-0.25	-0.01
inter_orbital_distance	0.21	0.35	-0.14	-0.09	-0.08	-0.09
eye_diameter	-0.31	0.08	0.08	-0.01	0.13	-0.04

Morphometric analysis of *Mystus malabaricus* clusters identified in genetic analysis showed high overlap (Figure 13) suggesting that if *Mystus malabaricus* is a species complex then the species are likely to be cryptic in nature with very less morphological differences.

Principle component analysis (PCA) of *Mystus bleekeri* and *Mystus cf bleekeri* (Figure 14). Showed 2 clusters separated on first principle axis *Mystus cf bleekeri* differed from *Mystus bleekeri* in having higher head length and pre pectorial length, And lesser head depth, length of anal fin base, body depth at anus, depth of caudal peduncle and length of caudal peduncle.

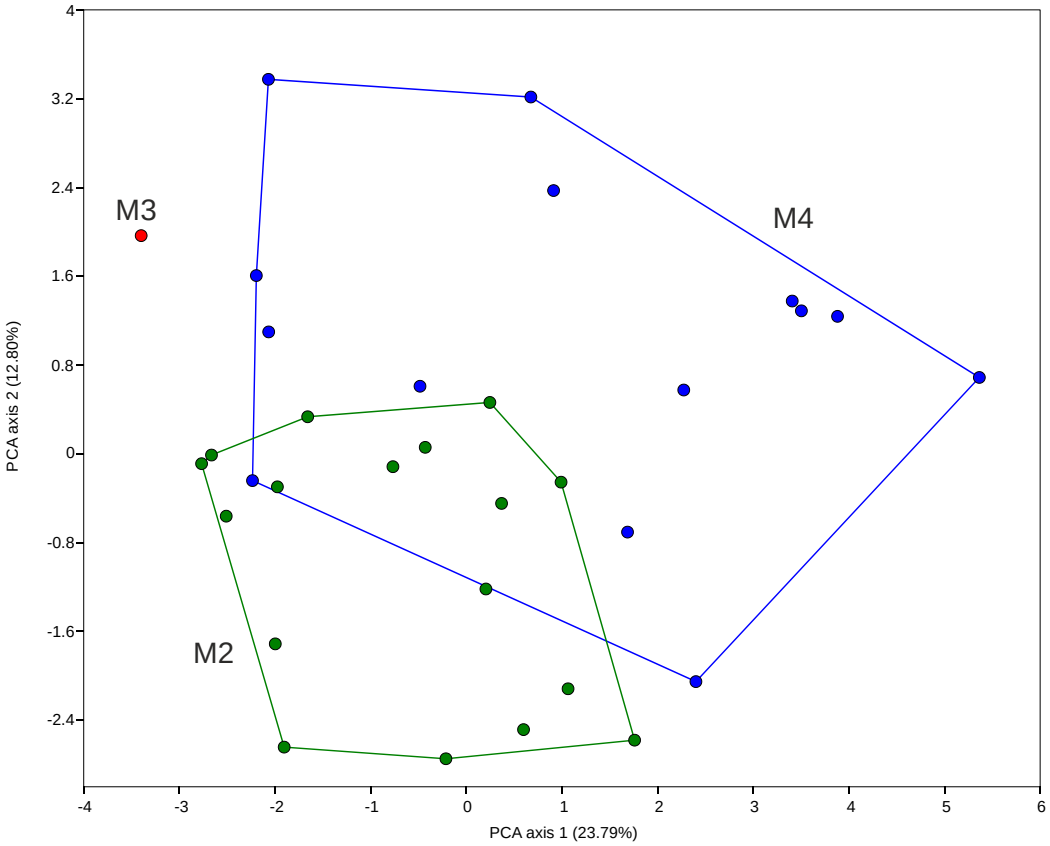

Figure 13: Principle component analysis of the three clusters of *Mystus malabaricus* as identified in genetic analysis (Figure 3).

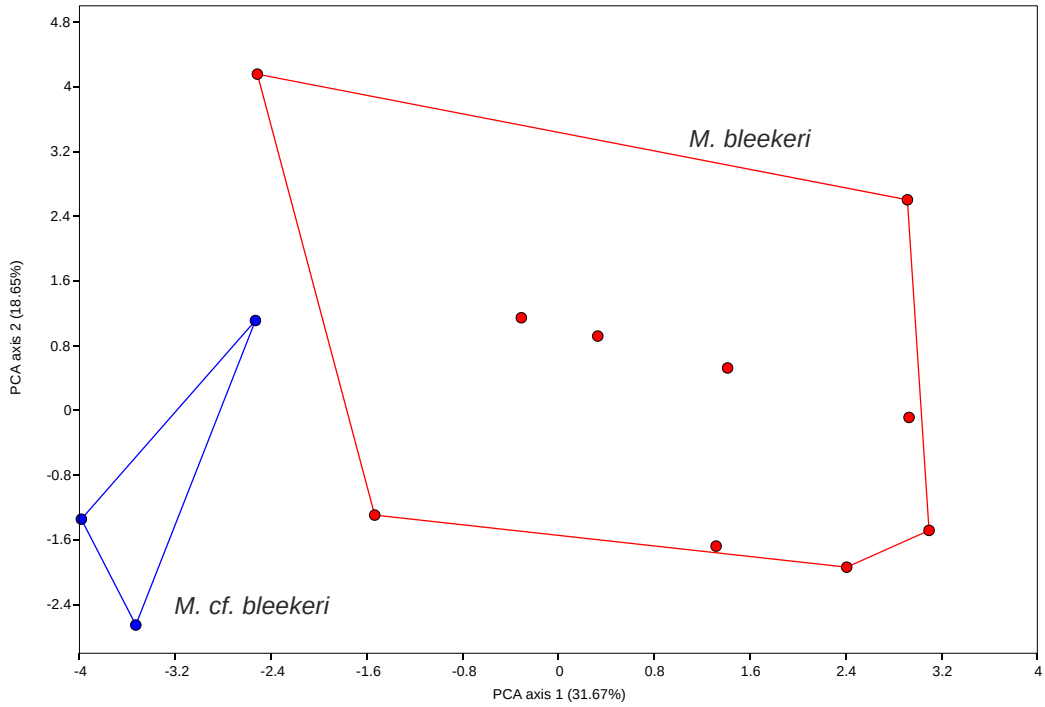

Figure 14: Principle component analysis of *Mystus bleekeri* and *M. cf. bleekeri*.

Principle component analysis (PCA) of *Mystus seengtee* and *Mystus cacasius* showed overlap (Figure 15) bolstering overview that both the species are one and the same based on Cox1 and Cytb gene sequence.

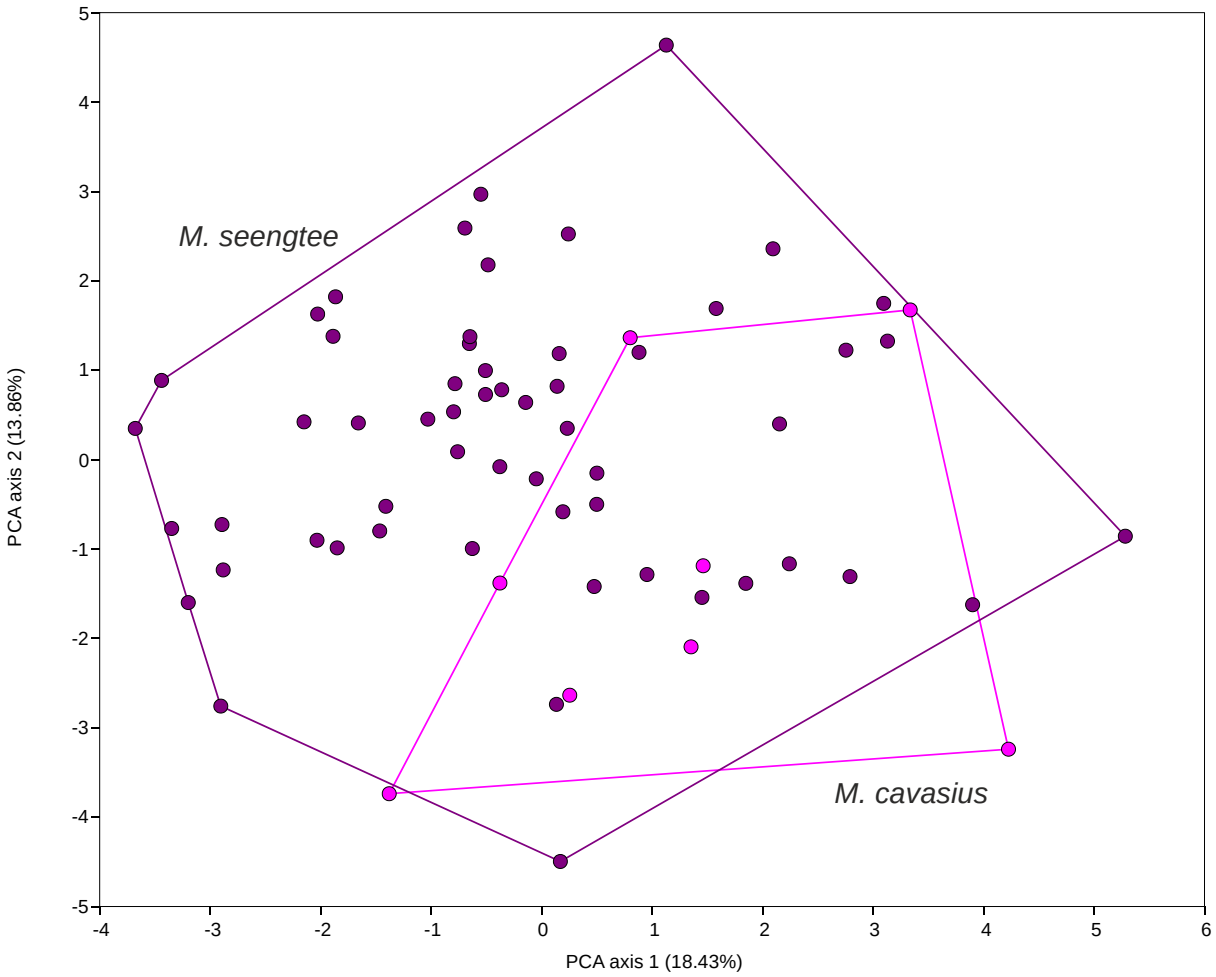

Figure 15: Principle component analysis *Mystus seengtee and M. cavasius*.

Our morphological analysis is still incomplete because even though we could download genetic information of comparative material from Ganges and Kerala we could not get more sequences of the specimen for morphological analysis.

Osteological analysis

The clearing and staining protocol was used for 6 species of bagride however despite of 7 month of incubation the clearing is not yet complete. Nevertheless based on partial clearing of 3 species we provide the basic analysis. Partially cleared whole specimen of *Mystus seengtee, Mystus cavasius* and *mystus malabaricus* are shown in Figure 16. *Mystus seengtee* has 19 preanal and 18 postanal vertebra with a single supraneural spine. *Mystus cavasius* from Godavari has 20 preanal and 18 postanal vertebra with single supra neural spine. *Mystus malabaricus* has 21 preanal and 15 postanal vertebra with single supraneural spine.

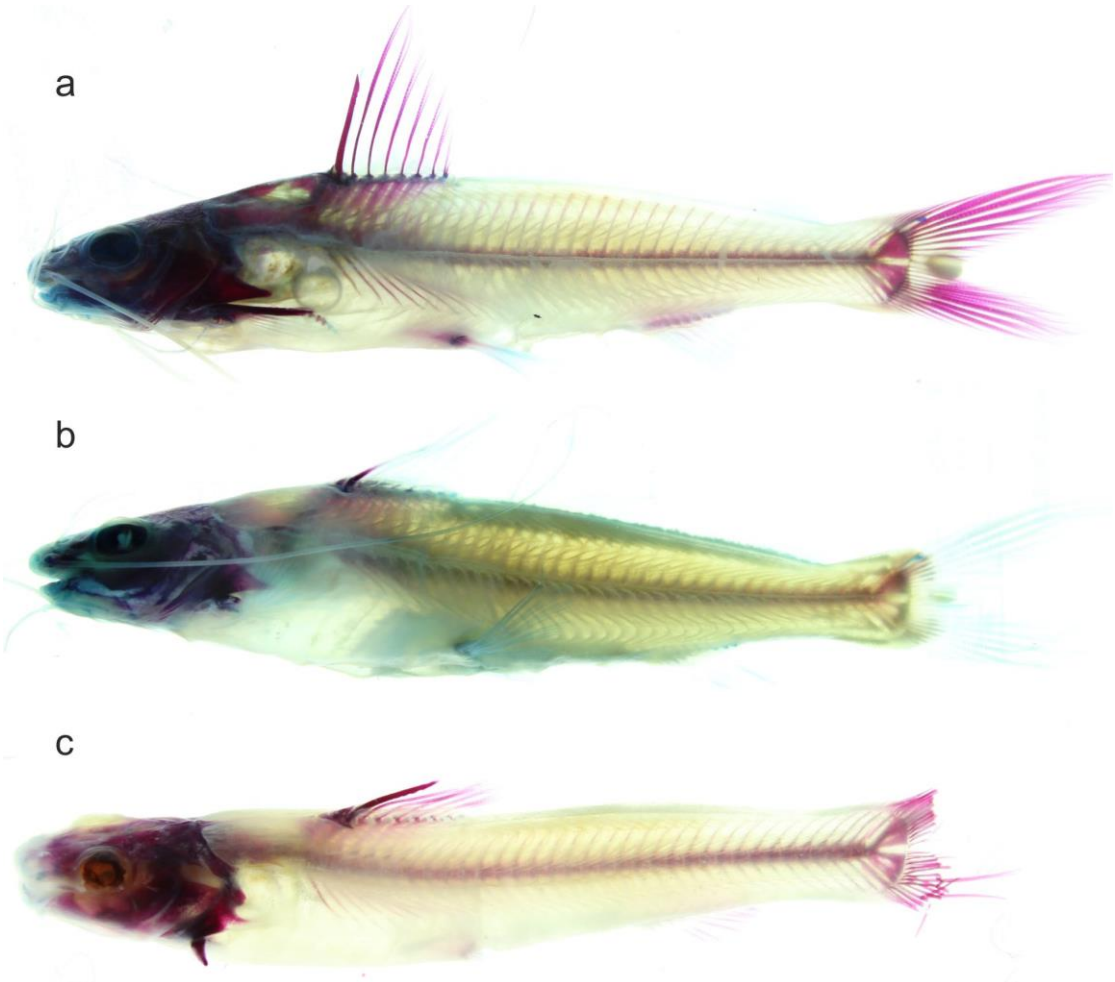

Figure 16: Cleared and stained specimens used for osteological studies. (a) *Mystus seengtee*, (b) *M. cavasius* and *M. malabaricus*.

Osteology of head structure of three species is given in Figure 16. Even though there were minor differences in anterior frontanelle and posterior frontanelle structure of *Mystus seengtee* and *Mystus cavasius* (Figure 17 b and d) the difference was not sufficient to suggest that two species are different. The structure of supra occipital was also remarkably similar. For the two nominal species where supra occipital process almost reached basal bone of dorsal fin, on the contrary osteology of *Mystus malabaricus* showed a remarkable difference (Figure 17f) the differences include much reduced anterior and posterior frontanelle and supra occipital process. The supra occipital process did not reach the basal bone of dorsal fin and both the structure were separated by a very large distance. Unfortunately because osteological study are relatively rare in India and our analysis is only preliminary it is difficult to trace the evolution in variation of head osteology further studies with more specimen and different species of bagrid will be able to shed more better light on the osteology of Bagrid.

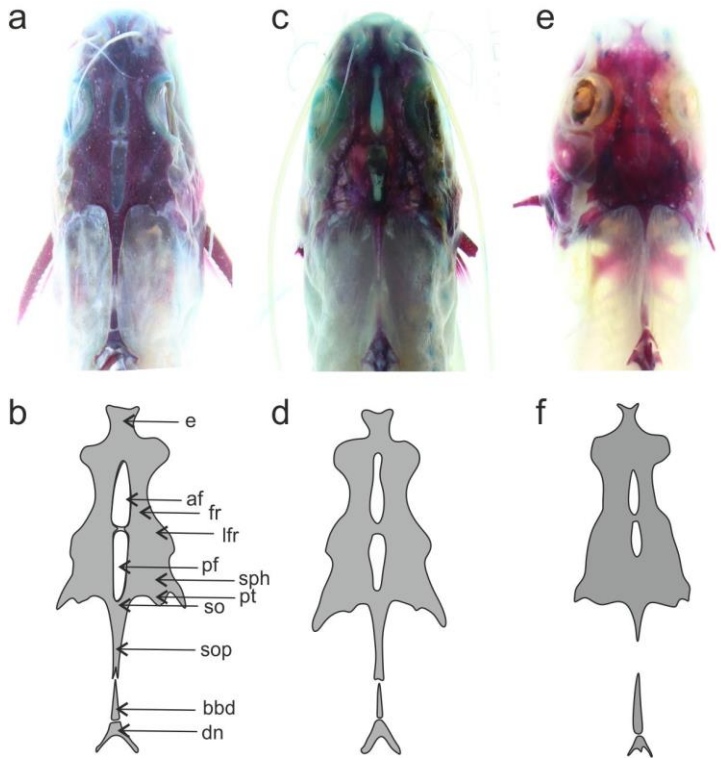

Figure 17: Osteological details of the head. (a, b) *M. seengtee*, (c, d) *M. cavasius* and (e, f) *M. malabaricus*. Key: e = mesoethmoid, af = anterior frontanelle, fr = frontal, lfr = lateral frontal, pf = posterior frontal, sph = phenotic, pt = pterotic, so = supra occipital, sop = supra occipital process, bbd = basal bone of dorsal fin base, dn = dorsal notch.

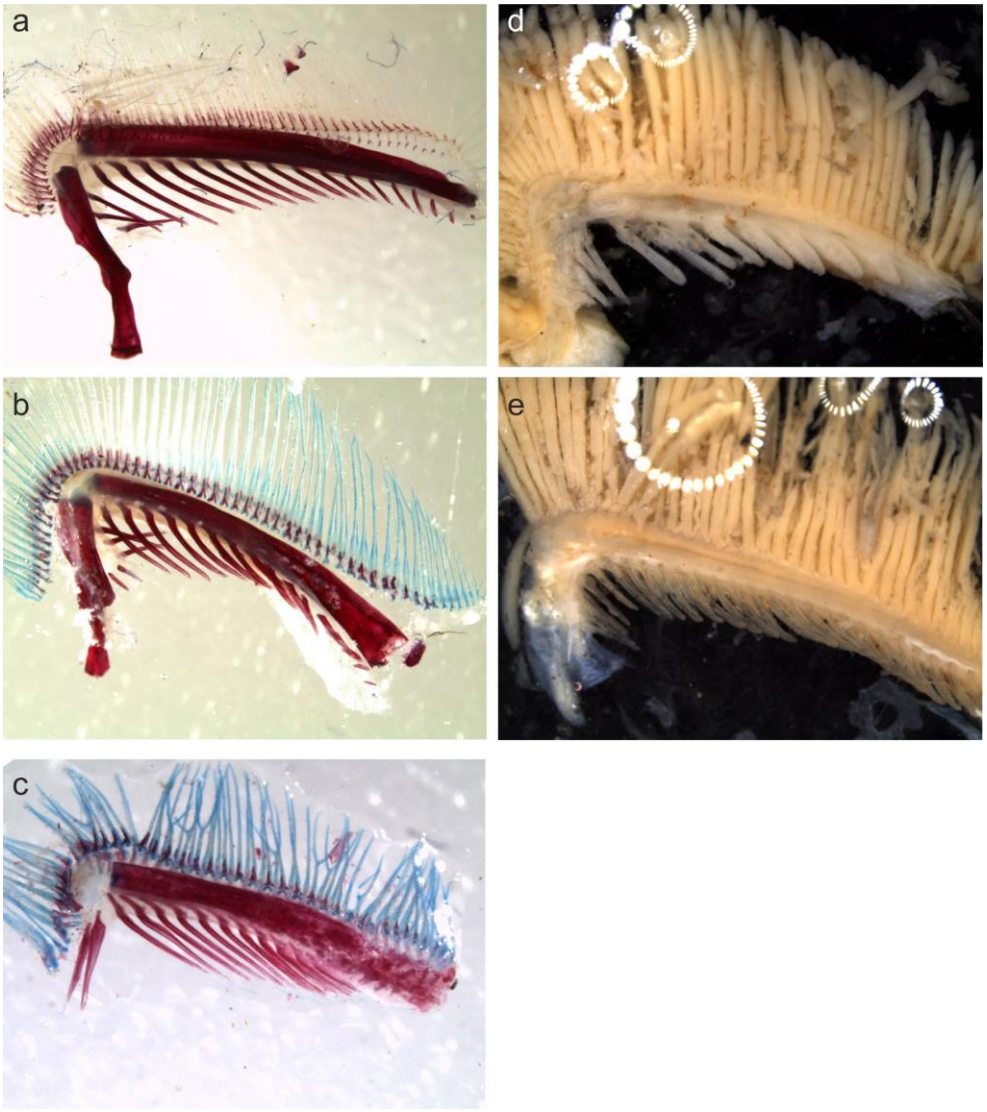

Figure 18: Gill rakers on the first gill arch. (a) *M. seengtee*, (b) *M. cavasius*, (c) *M. malabaricus*, (d) *M. bleekeri* and (e) *M. cf. gulio*.

Gill rakers on the first gill arch has been considered as an important taxonomical characters for *Mystus Species.* Gill rakers on the first gill arch of five *Mystus species* is shown of Figure 18 both *Mystus seengtee* and *Mystus cavasius* had around (20-21) gill rakers suggesting that this character used by chakrabarty and NG (2005) for separating the two nominal species is invalid. *Mystus malabaricus* also has around (19-20) gill rakers around first arch while *Mystus bleekeri* had the least number of gill rakers (13-14) and *Mystus guilo* has highest number of gill rakers (28-30) Both the structure and number of gill raker did not show fixed pattern that could co-inside with evolutionary tree (Figure 3). Gill raker is likely to be a complex phenomenon.

CONCLUSIONS

Following conclusions can be drawn from our analysis.

1. There is a hidden diversity in the Western Ghats of India. From our experiment we found that there are several species complex of *Mystus* is present in Western Ghats of India.
2. *Mystus malabaricus* is likely to be a species complex based on our Cox1 and Cytb gene sequence. *Mystus malabaricus* consists of 2 distinct species in Kerala and two other distinct species in northern Western Ghats of Maharashtra. However Morphometric analysis of *Mystus malabaricus* clusters identified in genetic analysis showed high overlap (Figure 13) suggesting that if *Mystus malabaricus* is a species complex then the species are likely to be cryptic in nature.
3. *Mystus bleekeri* Both Cox1 and Cyt b showed the presence of undescribed species of *Mystus bleekeri* supported by morphometric analysis.
4. *Mystus seengtee* and *Mystus cavasius* which are considered as two different species, which are likely to be the same species distributed throughout India.
5. *Mystus guilo* from Western Ghats of India have a distinctly different evolutionary linage different from Gangetic *M. guilo*.
6. *Sperata seenghala* from its type locality in Pune is different from sequence deposited from northeast India, indicating presence of a different species from northeast India.
7. *Rita gogra* which is thought to be distributed all over the India is likely to be comprises of 3 distinct species.
8. We have provided osteological details for *Mystus* species from Western Ghats for the first time. We show that the osteological structures of the head region, gill rakers on the first gill arch and number of vertebra could be used in combination as differentiating characters for separating the species of *Mystus* but with caution. For instance, the gill rakers was used as separating character for *M. seengtee* and *M. cavasius*, but we show that both of them have overlapping ranges. The number of gill rakers of *M. malabaricus* are also similar to the gill rakers of *M. seengtee* and *M. cavasius* but the head structure, especially the supraoscipital process differed substantiated between these species.

9. Our analysis also suggests that there are some widespread species present throughout India, such as *Mystus cavasius* (as M. seengtee is likely to be the synonym of *M. cavasius*), while certain widespread species such as *M. bleekeri* also has some undescribed species. Further the presumably widespread species such as *M. gulio* is actually made up of different species in Ganges and Western Ghats. Our study therefore sheds light on the biogeography of bagrid species of the Western Ghats.
10. In our future studies we will try to tie up all 3 molecular, morphometric, osteological analysis and look at their evolutionary pattern.

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