Geometric Morphometrics and Phylogeny of the Catfish genus *Mystus* Scopoli (Siluriformes:Bagridae) and North American Cyprinids (Cypriniformes)

by

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Abstract

Understanding the evolution of organismal form is a primary concern of comparative biology, and inferring the phylogenetic history of shape change is, therefore, a central concern. Shape is one of the most important and easily measured elements of phenotype, and shape is the result of the interaction of many, if not most, genes. The evolution of morphological traits may be tightly linked to the phylogeny of the group. Thus, it is important to test the phylogenetic dependence of traits to study the relationship between traits and phylogeny. My dissertation research has focused on the study of body shape evolution using geometric morphometrics and the ability of geometric morphometrics to infer or inform phylogeny. For this I have studied shape change in *Mystus* (Siluriformes: Bagridae) and North American cyprinids. *Mystus* Scopoli 1771 is a diverse catfish group within Bagridae with small- to medium-sized fishes. Out of the 44 nominal species worldwide, only 30 are considered to be part of *Mystus*. *Mystus* is distributed in Turkey, Syria, Iraq, Iran, Afghanistan, Pakistan, India, Nepal, Sri Lanka, Bangladesh, Myanmar, Thailand, Malay Peninsula, Vietnam, Sumatra, Java and Borneo. Species of *Mystus* are morphologically similar and diagnostic characteristics are usually subtle. The group is poorly diagnosed and is not likely monophyletic. Their classification has remained in disarray and there has been no phylogenetic study done on the genus *Mystus*. Among the 44 species of *Mystus, M. gulio* remains even a more problematic group. At least nine species have been named that are now all considered to be synonyms of *M. gulio*. *Mystus gulio* is morphologically distinct among *Mystus* species. So it is very important to resolve the taxonomy of the *M. gulio* species complex. For *Mystus*, I have first examined the molecular phylogeny using the mitochondrial cytochrome

b gene for about half of the recognized species of *Mystus* (Chapter 2). With few monophyletic clades, *M. gulio* came as a monophyletic clade in this analysis. In addition to phylogenetic relationship of *Mystus* species, I was also able estimate the timing of the divergence of *Mystus*. Using this molecular phylogeny I have tried to test if shape has evolved phylogenetically across the genus *Mystus* using geometric morphometrics (Chapter 3). A Principal Component Analysis (PCA) shows considerable dispersion between species and species groups within *Mystus*. Species were split between those with long adipose fins (adipose starts immediately after dorsal fin), medium dorsal fins (a small to relatively large gap is present between the dorsal and start of the adipose), and small adipose fins (adipose taller than long). Geometric Morphometrics show promise in being able to separate species within each of the adipose fin groupings. I also studied the taxonomy of *Mystus gulio* using traditional morphometrics (Chapter 4) which shows *M. gulio* as one single species. Lastly, I have combined these approaches and have added a test of a method to construct a phylogeny using geometric morphometric data on a much more morphologically and taxonomically diverse group, the North American cyprinids of the subfamily Leuciscinae. I used the cyprinids to study whether shape is evolving phylogenetically across cyprinids and if shape data can be used to elucidate phylogeny (Chapter 5).

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CHAPTER 1 – Introduction

There is great variation in morphology across the diversity of life, but this diversity of organismic form did not evolve uniformly (Foote 1997). Many groups of organisms, including Tanganyika Lake cichlids (Fryer and Iles 1972; Chakrabarty 2005), *Anolis* lizards (Warheit et al. 1999), and the lineage that includes Darwin's finches (Lack 1947), have evolved a wide range of morphologies with great differences between close relatives (Sidlauskas 2007). Shifts in rates of speciation, extinction and morphological change within evolving lineages can increase or decrease morphological diversity in descendant clades (Simpson 1944; Raup and Gould 1974; Foote 1996, 1997; Roopnarine 2003), and these shifts may help explain the unequal morphological diversification of closely related clades. However, the stochastic nature of evolution implies that evolutionary scenarios with unvarying rates of cladogenesis and morphological evolution can produce clades with widely varying morphological diversity (Gould et al. 1977; Raup 1977, 1985; Foote 1993). It can, therefore, prove difficult to determine whether observed differences in morphological diversity are best explained as outcomes of the same or different evolutionary scenarios (Sidlauskas 2007). Most existing tests identifying clades with evolutionary rates differing from their close relatives require extensive phylogenetic information (Collar et al. 2005; Sidlauskas 2007). As noted by Darwin (1859), "natural selection acts by either now adapting the varying parts of each being to its organic and inorganic conditions of life; or by having adapted them during past periods of time" (Chapter VI, 198pp). This implies that phylogenetic relationships must be taken into account to understand the phylogenetic history of shape change (Piras et al. 2010).

Shape analysis is fundamental to many biological studies, and it is one of the many approaches to understand the diverse causes of morphological variation (Zelditch et al. 2004).

Biologists have studied anatomical features and used shape analysis for centuries (Adams et al. 2004; Zelditch et al. 2004) and have classified organisms primarily on the basis of their form (Lele and Richtsmeier 2001; Macleod 2002). For early biologists, morphometrics and meristics served as primary methods of species discrimination. Eighteenth and early nineteenth century works frequently detailed differences in counts (Bloch 1794) and measured differences among species became part of standard practice by the mid 19th century (Müller and Troschel 1845; Muller and Troschel 1848; Cuvier and Valenciennes 1850; Gunther 1864). Differences among species were, and still are, explored commonly by comparing means and ranges of raw measures or ratios of these measures in relation to head or standard length e.g. (Hubbs and Bailey 1940).

Geometric morphometrics is a more recent approach that retains information on spatial covariation among landmarks (Rohlf and Marcus 1993). During the early twentieth century D'Arcy Thompson (1917) plotted specimens on Cartesian coordinates, and then produced transformation grids to show where organisms could change in shape either ontogenetically or phylogenetically (Macleod 2002). Although D'Arcy Thompson represents the start of the field, it took more complex methodologies and computers to fulfill his vision of looking at shape change across the whole of an organism. The landmark-based techniques pose no restrictions on the directions of the variation and the localization of shape changes, and they are effective in capturing meaningful information about the shapes of organisms.

Geometric morphometrics uses statistically comparable shape variables and can be used to test for significant correlations between body shape and ecological traits to evaluate the importance of phylogenetics on shape similarity (Clabaut et al. 2007). But the use of shape data to infer phylogeny has been long debated (Bookstein 1994; Zelditch et al. 1995; Monteiro 2000; Klingenberg and Gidaszewski 2010). One of the major issues in the controversy is the use of

shape as a character or set of characters. There is no debate about the definition of character; the disagreement is about how to use the multidimensional characters to obtain phylogenetic information (Bookstein 1994; Zelditch et al. 1995; Monteiro 2000; Klingenberg and Gidaszewski 2010).

Understanding the evolution of organismal form is a primary concern of comparative biology, and inferring the phylogenetic history of shape change is, therefore, a central concern (Klingenberg and Gidaszewski 2010). The evolution of morphological traits might be tightly linked to the phylogeny of the group. Shape is one of the most important and easily measured elements of phenotype, and shape expresses the interaction of many, if not most, genes. Thus, it is important to test the phylogenetic dependence of traits to study the relationship between traits and phylogeny (Ollier et al. 2006; Covain et al. 2008).

Understanding body shape evolution and how morphometrics could be used to infer or inform phylogeny has been the focus of my dissertation research. To answer these questions I have studied two diverse lineages of the Ostariophysi: Southeast Asian catfish of the genus *Mystus* (Siluriformes: Bagridae) and North American cyprinids (Cypriniformes).

DISSERTATION RESEARCH AND CHAPTERS

My dissertation research has focused on the study of body shape evolution using geometric morphometrics and the ability of geometric morphometrics to infer or inform phylogeny. To this end, I have approached shape change in *Mystus* and North American Cyprinids. For *Mystus*, I have first examined the molecular phylogeny using the mitochondrial cytochrome b gene for about half of the recognized species of *Mystus* (Chapter 2). Using this

molecular phylogeny I have used geometric morphometrics to test if shape has evolved phylogenetically across the genus *Mystus* (Chapter 3). Ancillary to these studies on *Mystus*, I have also studied the taxonomy of *Mystus gulio* using traditional morphometrics (Chapter 4) to determine if *M. gulio* is a single species or multiple species. Lastly, I have combined all of the approaches I used on *Mystus*, and have additionally added a test of a method to construct a phylogeny using geometric morphometric data on a much more morphologically and taxonomically diverse group, the North American cyprinids of the subfamily Leuciscinae. I use the cyprinids to study whether shape is evolving phylogenetically across cyprinids and if shape data can be used to elucidate phylogeny (Chapter 5).

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CHAPTER 2 – Molecular phylogeny of *Mystus* using Mitochondrial cytochrome *b* gene

Introduction

Catfishes (Order Siluriformes) are a diverse group of vertebrates with more than 3,000 valid living species in 37 families (Eschmeyer and Fong 2010; Armbruster 2011). Bagridae is the seventh most diverse catfish family currently recognized, and it includes more than 210 valid species in 17 genera (Armbruster 2011; Ng and Kottelat 2013). Bagrids are morphologically diverse, with sizes from 30 mm SL to 1500 mm SL, and they are widely distributed in fresh and brackish-water of Africa and Asia with two species entering marine habitats (Ng 2003). Only one genus, *Bagrus*, is endemic to Africa (Teugels 2003). All other bagrid genera are distributed in West Asia, Pakistan, India, Bangladesh, Sri Lanka, Myanmar, Malaysia, East Indies, Cambodia, Laos, Vietnam, China, Taiwan, Japan, Korea and Manchuria (Jayaram 2006). The members of this genus are abundant in most freshwater habitats.

Mystus Scopoli 1771 is a diverse catfish genus within Bagridae with 33 currently recognized small to medium-sized species (50 - 300 mm SL) (Chakrabarty and Ng 2005; Darshan et al. 2010). *Mystus* is distributed in Turkey, Syria, Iraq, Iran, Afghanistan, Pakistan, India, Nepal, Sri Lanka, Bangladesh, Myanmar, Thailand, Malay Peninsula, Vietnam, Sumatra, Java and Borneo (Jayaram 2006). The members of this genus are abundant in most freshwater habitats. Two species are found in brackish and marine habitats: *Mystus gulio* enters the sea and is found within the tidal limit (Jayaram and Sanyal 2003) and *M. wolffii* is found in estuaries (Ng 2012). Species of *Mystus* are morphologically similar and diagnostic characteristics are usually subtle. The genus is poorly diagnosed and is not believed to be monophyletic (Ng 2003).

The genus *Mystus* has undergone several nomenclatural changes and other taxonomic modifications. Even the use of the name *Mystus* has created much confusion as junior homonyms exist for bagrids, cyprinids, engraulids, notopterids (Jayaram and Singh 1984; Ng 2003). Taxonomic revisions within *Mystus* have been completed only at regional levels, and have included few species. These studies include nomenclature of the genus, fixation of type species, description of some new species and osteological comparisons of selected species (Jayaram and Sanyal 2003). No phylogenetic study has been done on the species of *Mystus*, but new species continue to be described (Ng and Kottelat 2013). Among the species of *Mystus, M. gulio* is a particularly problematic group. At least nine species have been named that are now considered to be synonyms of *M. gulio*.

A number of studies have attempted to resolve relationships among catfish families, but the phylogenetic position of the Bagridae is still equivocal (Ku et al. 2007). The most comprehensive work on bagrid phylogeny was done by Mo (1991). Mo's study focused on the relationships among bagrid catfishes as inferred from 126 morphological characters drawn from 214 species in 30 families. Mo (1991) proposed major changes to the taxonomy and his results strongly support the monophyly of the Bagridae. Mo (1991) used thirty-one species listed as *Mystus* in his study, but only 10 of these are now considered to be in *Mystus*: *M. armatus, M. cavasius, M. gulio, M. montanus, M. nigriceps, M. keletius, M. pelusius, M. tengara, M. vittatus* and *M. wolffii.* At the time of Mo's work some *Hemibagrus* were considered to be *Mystus* including *H. nemurus, H. guttatus, H. menoda, H. sabanus, H. peguensis, H. planiceps, H. punctatus, H. wychoides,* and *H. wyckii.* Other species of *Mystus* that Mo included are no longer considered valid or part of *Mystus*: *M. pahangensis* and *M. johorensis* are synonyms of *H. capitulum* and *M. corsula* is a synonym of *H. menoda.* In addition, *M. dayi* is now in *Batasio* and *M. baramenis* is in *Pseudobagrus.* In Mo's phylogenetic analysis *Mystus* was a composite taxon

including these species of *Hemibagrus*, *Batasio*, and *Pseudobagrus* and this composite taxon was sister to a clade of the remainder of *Hemibagrus*, *Sperata* (as *Aorichthys*), and *Bagrus* (Mo 1991). Ng (2003) re-analyzed Mo's data and the results were the same for *Mystus*.

The next detailed study on Bagridae was done by de Pinna (1993). In his unpublished PhD dissertation, he analyzed 239 morphological characters from 400 species representing 33 families of Siluriformes but compressed his data set into 79 representative terminals based on hypotheses of monophyly provided by previous phylogenetic studies and his preliminary analysis. He thereby enforced their monophyly in subsequent analyses and described the measure as one that fine-tuned the parsimony analysis among rather than within the families (Hardman 2005). De Pinna's study included four valid *Mystus* species (*M. gulio*, *M. malabaricus*, *M. nigriceps* and *M. vittatus*). He also included *H. nemurus* and *H. wyckii* in *Mystus*. As in Mo (1991), de Pinna's *Mystus* was a composite taxon of all of the above species, and he found that this composite was sister to a clade that included *Sperata*, *Bagrus* and the remainder of *Hemibagrus*. De Pinna's work has been criticized for very weak character support for *Mystus* and the *Sperata*, *Hemibagrus* and *Bagrus* clade (Ng 2003).

Hardman (2005) analyzed complete sequences of cytochrome b (cyt *b*) for 170 species of catfishes from 29 of 33 extant families, and focused on the relationships of the Ictaluridae to other catfishes. Hardman's results support a monophyletic Bagridae excluding *Rita*. Sullivan et al. (2006) used Recombination Activating Genes 1 and 2, and they described a clade they called 'Big Asia' including eight genera of the Bagridae along with other catfish families. Sullivan et al., also found Bagridae to be monophyletic if *Rita* is excluded. Hardman and Sullivan et al. focused on the deeper relationships of Bagridae. Sullivan et al., did not include any *Mystus* in

their studies. Hardman included six valid *Mystus* in his study and did not recover a monophyletic *Mystus*.

In this study, I have examined 70 individuals from 16 of the 33 valid *Mystus* species for the mitochondrial Cytochrome *b* (cyt *b*) gene. The taxonomy of *Mystus* follows Jayaram (2006) and Ferraris (2007). Mitochondrial DNA (mtDNA) sequence data provides several benefits for the study of intraspecific population structure (Avise 2000). Fish mtDNA all have a similar genomic organization (Lee et al. 2001; Kim et al. 2004). Many parts of mtDNA, such as those coding for protein genes or the regulatory part as the control region, are used as genetic markers for measurements of intraspecies and interspecies diversity. Cyt *b* is the most used gene in the elucidation of vertebrate relationship and has sections that evolve at different rates making it a good gene to examine relationships at different scales; however, it does have some resolution problems in catfishes as some well established taxa were found to be non-monophyletic in Hardman (2005).

With a very confusing taxonomy, poorly supported monophyly (Ng 2003) and no molecular phylogeny available for species level relationships, it is important to perform a species level phylogenetic study to clarify the taxonomic status of *Mystus*. A molecular phylogeny will also help to decide on the taxonomic status of populations of *M. gulio*. In addition, the phylogeny offers the possibility to examine the shape variation explored in the other chapters to determine if shape is congruent with phylogeny.

Materials and Methods

SPECIMEN AND TISSUE COLLECTION

Specimens of *Mystus* were collected from Bangladesh during field trips in 2005, 2007, 2010 and in Thailand in 2012 or obtained from other collections (Appendix 1). A small piece of

fin or muscle tissue was removed from each specimen and placed in separate vials of 95% ethanol for DNA analysis. Museum abbreviations follow Sabaj Perez (2013). A total of 70 individuals of 16 *Mystus* species were used in the phylogenetic analysis. Outgroup taxa were downloaded from Genbank and included *Hemibagrus*, *Pelteobagrus* and *Pseudobagrus*.

DNA SEQUENCING

Total genomic DNA was extracted from muscle or fin clips using the method described by Coffroth et al. (1992). The template was utilized to amplify ~1100bp fragment of the mitochondrial cytochrome *b* gene. Polymerase chain reaction (PCR) was conducted in 25 µL volumes containing ~10–30ng of template DNA, 10mM Tris-HCl (pH 8.3), 2.0mM KCl, 200 µM dNTPs and 0.4 µM each of primers Glu-2 (5'-AACCACCGTTGTTATTCAACTA-3') and Pro-R1(5'-TAGTTTAGTTTAGAATTCTGGCTTTGG-3') (Hardman 2005), and 1 U *Taq* DNA polymerase. PCRs were conducted in a PTC-100TM thermocycler (MJ Research) under the following conditions: initial denaturing step of 94ºC for 3 min, 34 cycles of 94ºC for 30s, 45ºC for 30s, 72ºC for 45s and a final extension of 72ºC for 5 min. Amplifications were visualized via electrophoresing 3µL of PCR product in a 1% agarose gel. Amplified products were sequenced by High-Throughput Genomics Unit (HTGU) at the University of Washington. All sequences were aligned using Geneious v. 5.6.4 with final alignments adjusted by eye.

Phylogenetic reconstructions were performed using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI) criteria. The MP reconstruction was conducted in Mega 5.0 (Tamura et al. 2011) with the branch-swapping algorithm. Clade support was evaluated with 1,000 pseudoreplicates of a non-parametric bootstrap analysis using random addition of sequences (10 replicates) and TBR.

The program Kakusan4 (Tanabe 2007) was used to determine appropriate models of molecular evolution for Maximum Likelihood and Bayesian Inference analyses, resulting in

GTR + Γ as the appropriate model. The Maximum Likelihood analysis was conducted using RAxML ver. 7.2.8 (Stamatakis 2006). BI analyses were performed using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) using GTR + Γ model and using four chains, one cold and three incrementally heated. Cytochrome *b* was partitioned by codon position. Models of DNA evolution were chosen for all partitions using the Bayesian Information Criterion (BIC) 4, as suggested by Tanabe (2007). Markov Chain Monte Carlo (MCMC) analyses were conducted to run for at least 10^6 generations, with trees sampled every 1000 generations. The first 25% of trees sampled in each MCMC run were discarded as burn-in. Trees remaining after burn-in were used to calculate posterior probabilities using the "sumt" command. The phylogenetic trees were visualized and edited with FigTree v1.1.2 (4.0) and Adobe Illustrator (6.0).

DIVERGENCE TIME ESTIMATION

To estimate timing of divergence events without assuming a strict molecular clock, estimations were performed using a Bayesian relaxed clock analysis as implemented in BEAST v. 1.6.1 (Drummond and Rambaut 2007). Watanabe and Uyeno (1999) mentioned *Pseudobagrus* fossils are present from the middle Miocene and other bagrid fossils are from the Pliocene (3–4 MY). Two fossil species of *Mystus* are reported from the Eocene of China (Chang and Zhou 1993). The analyses of Mo (1991) and Ng (2003) recovered the genus *Mystus* below *Hemibagrus*+*Bagrus*. Lundberg et al. (2007) in their phylogenetic analysis of the Chiapas Catfish (*Lacantunia enigmatica*), used an Eocene age constraint of 34–56 MYa to the common ancestral node of *Hemibagrus*+*Bagrus* and *Heterobagrus* (they did not include *Mystus* in their analysis). As there are no *Mystus* currently in the region of China where the fossils are from, *Mystus* and *Hemibagrus* (which is present in the region) are difficult to separate via osteology, and the specimens are not available to examine, the *Hemibagrus* + *Mystus* node was dated at 34-56 MY.

The BEAST XML file was generated in BEAUti v.1.5.7 (Drummond and Rambaut 2007). The substitution model was GTR + Γ (as selected by AIC) with base frequencies estimated empirically, using three partitions (separate codon positions). All parameters were unlinked and substitution rate unfixed utilizing a relaxed uncorrelated lognormal clock. The tree prior assigned was the species birth death incomplete sampling prior (Drummond et al. 2007). The calibration nodes were constrained using log normal distributions. Other priors and operators were set to their default settings.

A final method that employs a more general approach for estimating divergence was conducted by direct translation of genetic distances into time based on reported molecular clock rates in catfishes. For cyt *b*, it is estimated at 0.5-0.8%/MY/lineage in catfish (Hardman and Lundberg 2006) and for this study we used 0.8%/MY/lineage. Genetic distances were estimated under the uncorrected p model in MEGA 5 (Tamura et al. 2011).

Results and Discussion

DATA SEQUENCE CHARACTERISTICS AND PHYLOGENETIC INFERENCE

The final alignment of the cytochrome *b* dataset included 1154 bp from 75 taxa. There were no ambiguous positions within the chromatograms. Mean base composition for cyt *b* was A = 0.289, C = 0.229, G = 0.14, and T = 0.29. There were 447 parsimony informative sites. The nucleotide composition of the cyt *b* segment sequenced is G-deficient, whereas almost similar frequencies were observed among the other three nucleotides. This G-deficient pattern is reported from several other fish studies (Johns and Avise 1998). The Bayesian (Fig. 1) and Maximum Likelihood (Fig. 2) analyses differed only in the placement of *M. armatus* (sister to a clade of *M. malabaricus, M. tengara, M. vittatus*, and *M. pulcher* in BI and sister to *M. malabaricus* in ML); however, neither relationship is well supported. The MP

tree (Fig. 3) differed from ML and BI in several respects including the placement of *M. armatus* and *M. wolffii* and in the relationships of other clades.

Mystus is a monophyletic genus in ML and BI. The MP tree was not rooted so as to not bias the results, but there is no reason to reject the monophyly of *Mystus* in the MP analysis. The phylogenetic analyses (figs 1–3) strongly support some monophyletic clades within *Mystus* and these clades are largely concordant with groupings based on adipose-fin size (Chapter 3). However, given the differences between the MP, ML and BI analyses and given that morphological characters used to delineate groups within *Mystus* do not support monophyletic groups within *Mystus*, there is no reason to split *Mystus* into multiple genera as was suggested by Jayaram (2006).

The identification of *Mystus vittatus* and *M. tengara* has been problematic because the species are similar and sympatric (Jayaram 2006). Both species were found to be monophyletic in all analyses and sister taxa in MP supporting that they are separate species.

Mystus multiradiatus was inferred as paraphyletic in the analysis. The two individuals of *M. multiradiatus* came from two different localities in Indonesia. Using only one gene and two specimens, it is not informative enough to draw a conclusion on their specific status, and I conservatively consider them as one single species in this study.

In Chapter 3, *Mystus* species are divided based on the sizes of their adipose fins: extra large, large, medium, and small. In MP, the basal condition of *Mystus* is a small adipose fin, which is also found in the outgroup. Because a small adipose fin is the plesiomorphic condition, one might not expect the small adipose species to form a monophyletic group, and, indeed, *M. gulio* and *M. wolffii* did not form a clade in ML and BI; however, they did form a clade in MP. Medium adipose species + *M. multiradiatus* (a large adipose species) form a monophyletic

group. The large adipose group is monophyletic with the exception of *M. multiradiatus* (which is in the medium adipose group) and *M. montanus*, which is in a monophyletic extra large adipose clade with *M. bocourti, M. cavasius,* and *M. singaringan*. The remaining extra large adipose species (*M. bleekeri* and *M. falcarius*) are sister to the short adipose *M. gulio* in ML and BI and sister to all other *Mystus* except the short adipose species in MP.

Specimens of *Mystus gulio* were examined from near opposite ends of the range of the species (Bangladesh + India and Indonesia). A split between Bangladesh + India specimens and Indonesian specimens was found; however, the Indonesian specimens were paraphyletic in ML and BI. This basal split was poorly supported in all analyses, and the branch lengths are short.

DIVERGENCE TIME

Four major nodes, A, B, C and D in Figure 4 denote important clades: *Mystus vittatus*, *Mystus tengara* and *Mystus pulcher* (clade A), *M. cavasius*, *M. montanus*, *M. bocourti* and *M. singaringan* (Clade B); *M. wolffii, M. gulio, M. bleekeri, M. sp* 2 and *M. falcarius* (Clade C); and *M. gulio* (Clade D)**.**

The BEAST analysis resulted in high effective sample sizes (ESS) for all parameters, and the three runs converged on the posterior distributions and reached stationarity. Maximum clade credibility trees for the independent runs were identical in topology. It is not surprising that only one calibration point with a time span 34-56 MYa gave very wide divergence time ranges for the nodes (Fig. 4). Estimated mean divergence times for the basal split of *Mystus* species was 29.7 MYa (15.1 - 48.9 MYa, 95% high posterior density interval); clade A, 4.9 MYa (1.6 - 9.0 MYa, 95% high posterior density interval), Clade B, 16.6 MYa (7.4 - 28.7 MYa, 95% high posterior density interval), Clade C, 21.0 MYa (8.9 – 36.2 MYa, 95% high posterior density interval) and Clade D, 1.6 MYa (0.5 - 3.0 MYa, 95% high posterior density interval) respectively (Table 1).

Divergence ages were also estimated by a direct translation of genetic distances into time based on reported molecular clock rates in catfishes using cytochrome *b* (0.5-0.8%/MY/lineage) (Hardman and Lundberg 2006). A rate of 0.8%/MY/lineage was applied to the *p* genetic distance between clades and resulted in average estimates of 12-42 MYa, with most diversification occurring in the Miocene. The strict molecular clock estimated divergence time of 6.29 MYa for clade A, 26.9 MYa for clade B, 24.6 MYa for clade C and 1.69 MYa for clade D (Table 1), and generally suggested ages towards the higher side of the ranges in the Beast analysis.

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Divergence time estimated using relaxed and strict molecular clock have differences. For this study the average node-age estimates were roughly similar for *Mystus* suggesting that most diversification took place during Miocene. This result is not surprising given that there are two apparent *Mystus* fossils found from the Eocene of China (Chang and Zhou 1993). Bagrid catfishes are among those siluriform groups with an older fossil record (Ng 2003). Bagrids had most of the Cenozoic era to diversify, but for East Asian Bagrids most of the extant species may have resulted from rapid speciation within the last 10 MY (Ku et al. 2007).

Ages estimates within each of the extant *Mystus* species except *M. singaringan* is less than 2MY, an age that corresponds with Pleistocene glaciation 2.6–0.1 MYa. During glacial periods, the Sunda Shelf was exposed, connecting the islands of Sumatra and Borneo to the Southeast Asian mainland, which influenced the river courses and land mass configurations. Given the probable ages of the species, Sunda Shelf exposure likely influenced the dispersal, range expansion, and population structure of *Mystus* (Voris 2000; McConnell 2004). Deeper nodes within species groups may be related to earlier glacial cycles. The influence of shifting ancient river drainages on fish diversification has been well documented in North America (Burr and Page 1986; Hocutt et al. 1986; Mayden 1988).

Mystus singaringan has two distinct populations: one from Thailand and another from Indonesia. The average divergence time estimated between the Indonesian and Thailand population of *M. singaringan* is 6.7 MYa; this may have been linked to the periods of lowered sea level when Sunda Shelf was exposed and formed large lowland areas connecting the islands with each other and with the Malay Peninsula (Woodruff 2003). Many rivers that now drain into the Gulf of Thailand or the southern parts of the South China Sea were then united and enabled faunal exchanges of freshwater fishes between the presently isolated parts of SE Asia (Voris 2000; Bohlen et al. 2011).

We have found a divergence time of around 5.1 MYa between the two *M. singaringan* populations in Thailand from the Chi River (Haplotypes 5 and 6) and the Mun River (Haplotypes 7,8 and 9). The Chi River is a tributary of the Mun River, which is a tributary of the Mekong River. Though a high level of differentiation was evident between these populations, localities where these two clades of *M. singaringan* were collected are within 100 river km of one another. This could be due to the fact that upstream dispersal of *M. singaringan* in the Mekong River has been limited in recent history. The divergence between the two populations may best be explained by a historical separation of the two rivers. According to this view, the upper Mun River was once a separate river system west of the Mekong and it was probably not flowing from west to east to the Mekong River system as it does today (Rainboth 1996). Instead, it was flowing from east to west to the Chao Phraya River system and the Mun and Mekong Rivers did not combine until the lower and upper Mekong Rivers combined when the divide between them was breached at Khone Falls (Claude, 2011).

Tectonic activity of the Khorat Plateau caused significant rearrangement of the drainage system in this region during Tertiary-Quaternary boundary (Rainboth 1996). While there is little specific data to support the effects of this drainage shift (Rainboth 1996), drainage rearrangement

has often been used to explain deviations from genetic relationships that are built around a specific hierarchy of tributaries and distributaries (Bermingham et al. 1997; Hurwood and Hughes 1998; Burridge et al. 2006). There has been genetic structure found in populations of aquatic organisms in the Mun, Chi, and Mekong Rivers for the Mud snake (*Enhydris subtaeniata*), freshwater fishes (*Henicorhynchus siamensis*) and crocodilians (*Gavialis*) (Adamson et al. 2009; Claude et al. 2011; Lukoschek et al. 2011). Claude et al. (2011) found that upper and lower Mekong basin shows considerable genetic differentiation of *Gavialis* and the Middle Mekong has been isolated from the Lower Mekong and Chao Phraya populations for at least 1 million years and possibly much longer. These authors suggest that the Chi and Mun rivers west of their modern confluence flowed separately into the Chao Phrya, only combining near the Chao Phrya delta.

The genetic structure witnessed in the Mun River system today can be explained by the two clades representing populations restricted to either the Chi or Mun rivers when they flowed west or one clade representing this westward flowing Chi + upper Mun and an eastward flowing lower Mun. Drainage basins in northern Thailand have undergone a complex pattern of evolution that is yet to be fully documented and the current Mekong River is recently evolved (Rainboth 1996; Adamson et al. 2009). Because only two specimens from the Chi and Mun sites were sampled, we do not know if the two clades are found in the same localities, and further research is needed to determine the extent of these two clades and to examine the complex biogeography of fishes in the Mun River system specifically and the Mekong and Chao Phrya basins in general.

There is also a split between Indonesian populations of *Mystus singaringan*; *M. singaringan* haplotype 1 and 2 are from Borneo (South Kalimantan) and 3 and 4 from Sumatra. This separation between the Bornean and Sumatran populations of *M. singaringan* is dated

around 0.4 MYa. There have been few Pleistocene glacial spikes and two of them occurred at ~1.8Ma, (Eburonian), ~0.92 Ma (Menapian) (Chappell and Shackleton 1986; Chappell et al. 1996). During these glaciations, the Pleistocene land bridge connections acted as a great potential for the movement of freshwater fish between both regions (Kamaruddin and Esa 2009). Though the split of Bornean and Sumatran populations of *M. singaringan* occurred within these Pleistocene glaciations, most of Sumatra was elevated above sea level and emerged to its present size 5 MYa (Hall 2013). However, it may be difficult to correlate the separation between the species from Borneo and Sumatra with the geological history of these two islands. As Hall (2009) noted, these islands may have remained above or below sea level from time to time due to changes in sea level and glacial activity as well as overall tectonic movements. Because *M. singaringan* is distributed in Thailand, Borneo and Sumatra, the most likely explanation for the observed phylogenetic pattern is the result of a vicariance event separating the distribution of the common ancestor into Bornean + Sumatran and Thailand populations with a later split of Bornean and Sumatran populations. No diagnostic morphological differences are found across the range of *M. singaringan* (pers. observation). More detailed studies will be required to clarify the population structure of *M. singaringan*.

The average age of the split between Bangladesh + India *Mystus gulio* and those from Indonesia is 1.6 MYa. Probably Sunda Shelf uplifting (2.6 - 0.1 MYa) has influenced the dispersal of *Mystus gulio* in Southeast Asia like other fishes, primates and rodents (Voris 2000; Gorog et al. 2004; Harrison and Langdale 2006). Because *M. gulio* is found in brackish and marine habitats, exposure of the Sunda Shelf may actually have limited dispersal of the species, and the basal split may reflect this. Although this is a moderately deep split, no differences could be found across the range of *M. gulio* (Chapter 4), and further analysis of genetic samples in intervening areas of the range of *M. gulio* would be needed to determine if this 1.6 MY split is

significant enough to recognize cryptic species. The divergence time estimates should only be considered as a preliminary hypothesis and validated with rigorous fossil calibrations using more *Mystus* species and nuclear gene sequence data since divergence time analysis based on mitochondrial gene sequences are known to result in older age estimates (Near et al. 2012; Dahanukur et al. 2013).

Conclusion

This is the first study to elucidate a molecular phylogeny of *Mystus*. The taxonomy and systematics of *Mystus* are poorly resolved, but this study provides the framework for future revisions of the genus. The findings from the present study provide useful insights into the taxonomic status of all *Mystus* species, and set the stage for future investigations involving morphometrics, geometric morphometrics, taxonomic revision and the study of body shape evolution. The *Mystus gulio* species complex was supported as a monophyletic clade. The Indonesian *M. gulio* individuals are a different lineage than the India + Bangladesh origin *M. gulio*. The mean divergence time for *M. gulio* (1.6 MYa) is in line with some differences between species of catfishes, so it is possible that *M. gulio* represents multiple species, however, the taxon sampling is incomplete in this study (Fig. 1 - 4). Taxonomic revision of *M. gulio* is presented in Chapter 4 of this dissertation. Mitochondrial markers are suitable for more recent divergent times and not for deeper relationships. A combination of molecular phylogenetics, external characteristics, and morphometrics is likely to resolve relationships better than any by themselves, but this remains to be tested with a more robust phylogenetic analysis.

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Table 1. Divergence Time for few clades and also some nodes

Species	**Clade**	**Lognormal distribution** Mean (MYa)	95% HPD(MYa)	**Strict Clock** (MYa)
M. vittatus, M. tengara, M. pulcher	Clade A	4.9	1.6 - 9.0	6.29
M. singaringan, M. cavasius, M. montanus,M. bocourti	Clade B	16.6	7.4-28.7	26.98
M. wolffii, M. falcarius, Mystus sp.,M. bleekeri, M. gulio	Clade C	21.0	8.9 – 36.2	24.6
M. gulio	Clade D	1.6	0.5-3.0	1.69

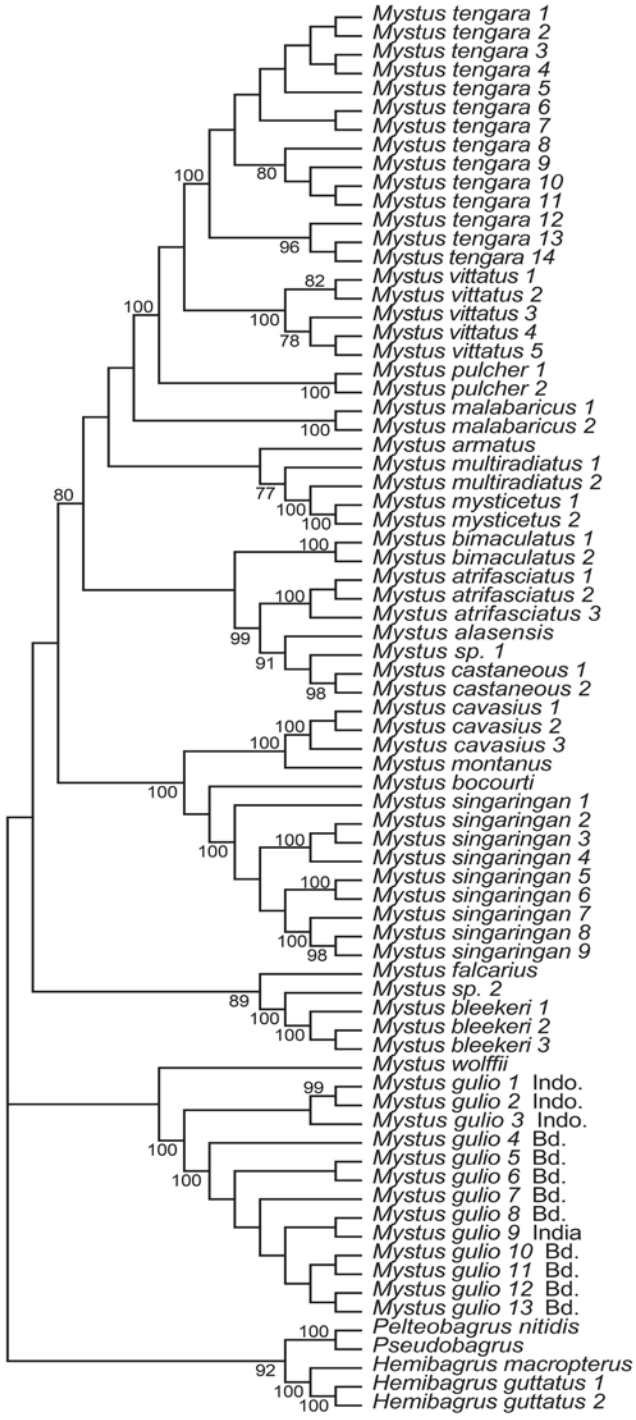

Figure 1. Phylogeny of *Mystus* inferred from Maximum Parsimony (MP). Number on nodes are bootstrap values. *Mystus gulio* specimens include country of origin Bangladesh (Bd.), India, or Indonesia (Indo.).

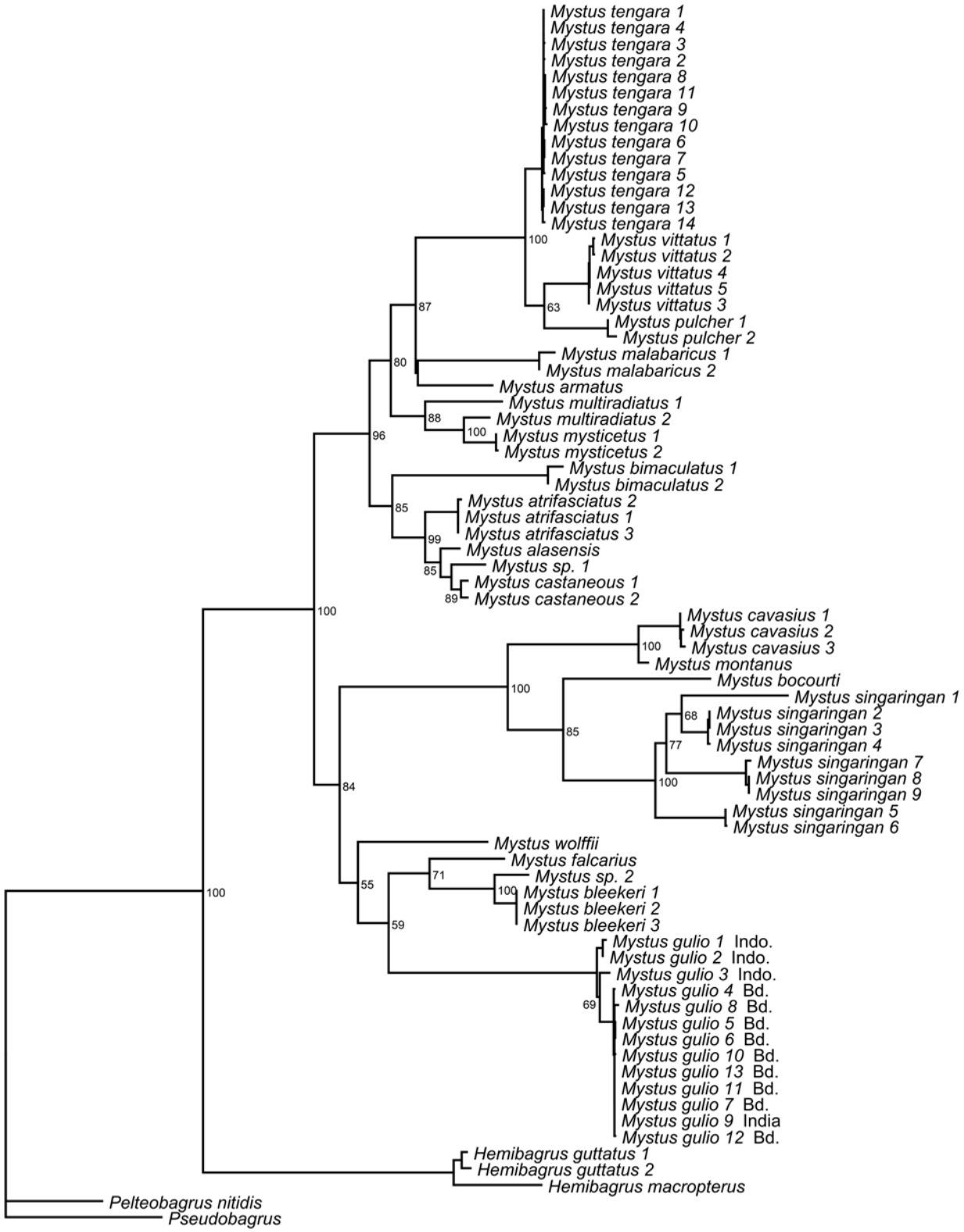

Figure 2. Phylogeny of *Mystus* as inferred from Maximum Likelihood (ML). Numbers on nodes are bootstrap values. *Mystus gulio* specimens include country of origin Bangladesh (Bd.), India, or Indonesia (Indo.).

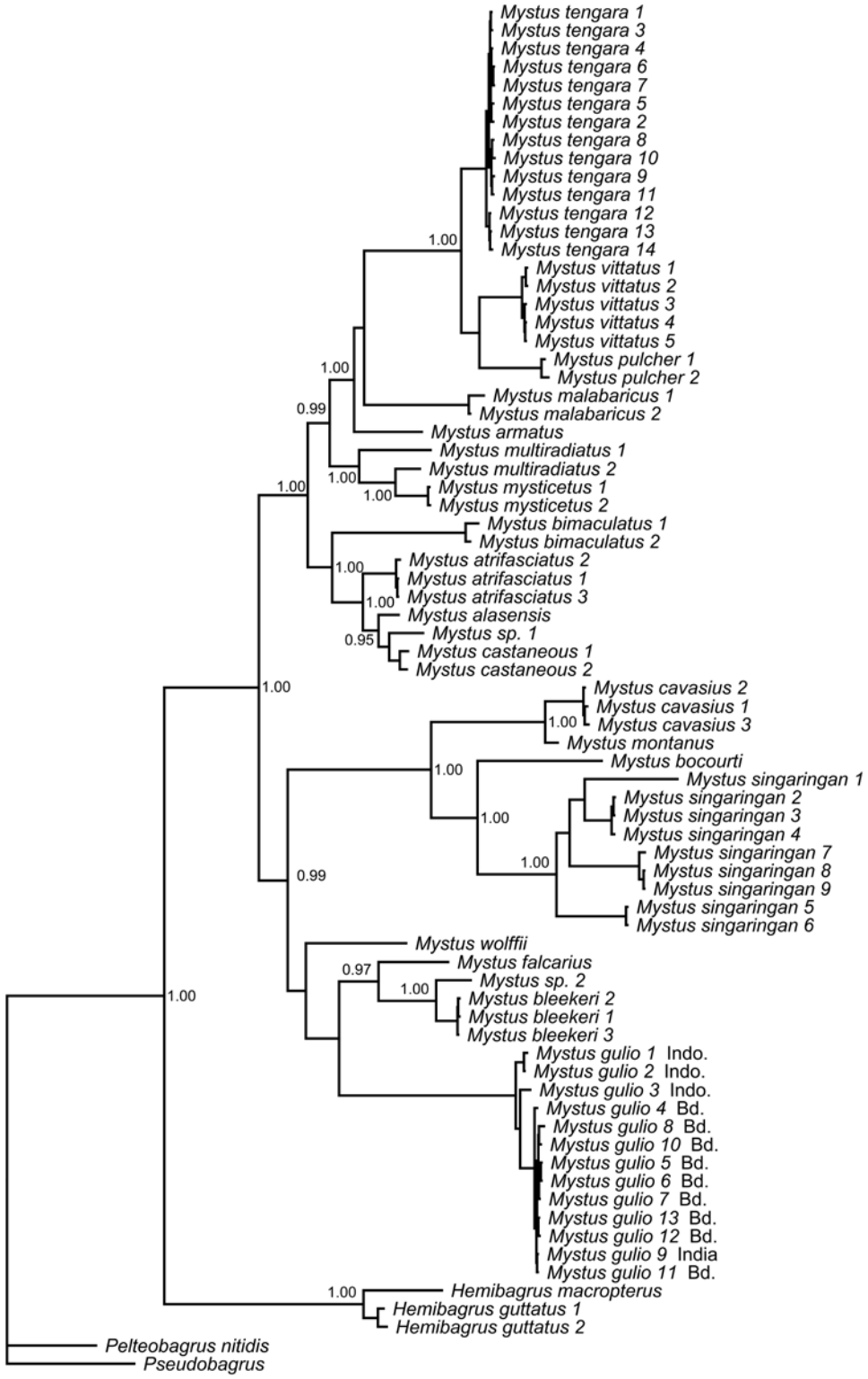

Figure 3. Phylogeny of *Mystus* as inferred from Bayesian Inference (BI). Numbers on nodes are posterior probabilities. *Mystus gulio* specimens include country of origin Bangladesh (Bd.), India, or Indonesia (Indo.).

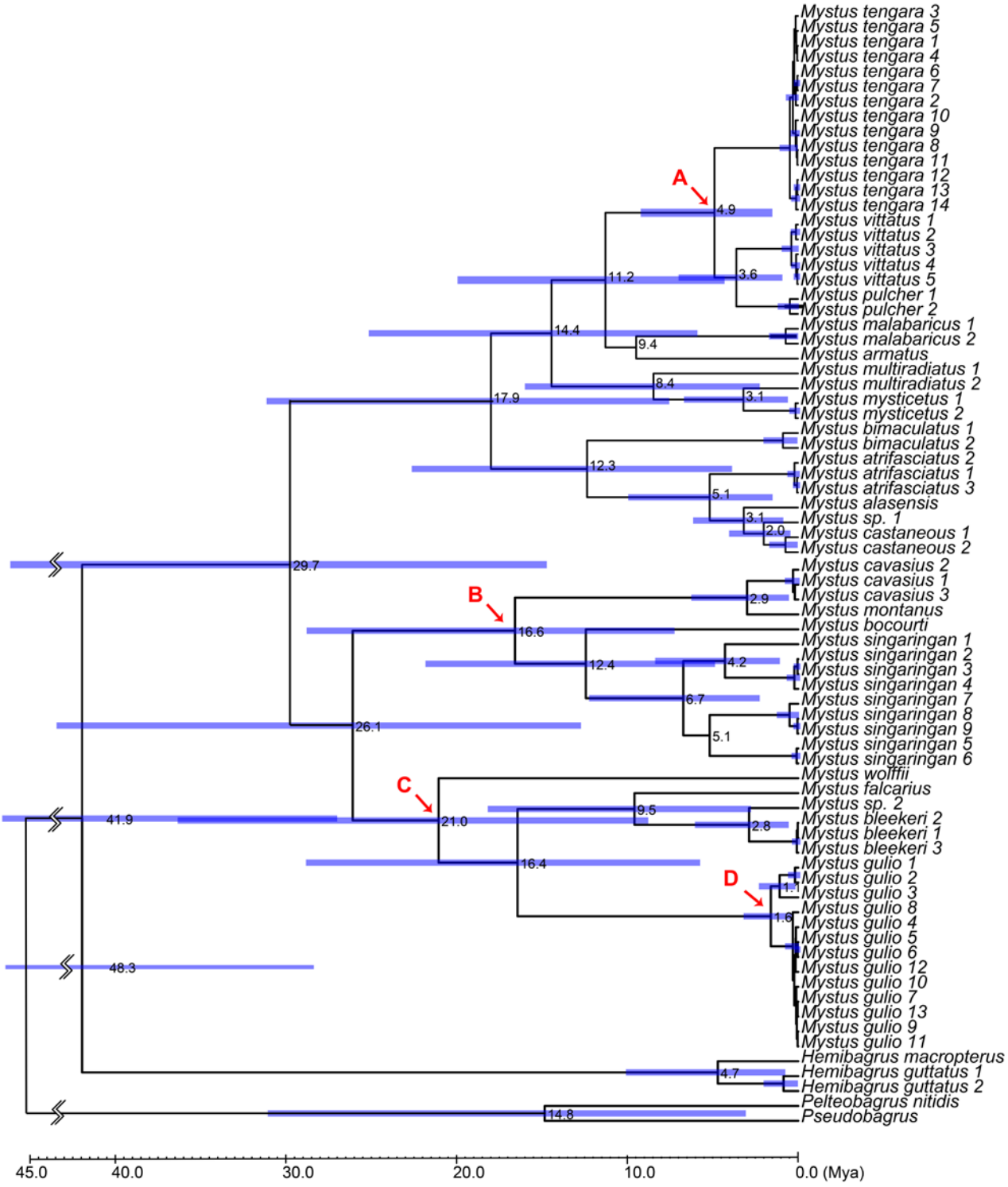

Figure 4. Bayesian consensus tree illustrating divergence time estimations of *Mystus*. Divergence times were estimated from a cytochrome *b* dataset with one calibration point. Labeled nodes (A-D) correspond to divergence dates of interest. Horizontal bars indicate 95% credibility intervals of joint prior and posterior estimate of divergence times.

CHAPTER 3 – Geometric Morphometrics of *Mystus* Scopoli (Siluriformes: Bagridae)

Introduction

Catfishes (Order Siluriformes) are a species rich and exceptionally diverse group of fishes among vertebrates. Catfishes have more than 3,000 valid living species in 37 recognized families (Eschmeyer and Fong 2010; Armbruster 2011). The Old World catfish family Bagridae, commonly found throughout fresh- and brackish-water bodies in Asia and Africa, includes more than 200 species in 17 genera and is one of the largest catfish families presently recognized (Armbruster 2011; Ng and Kottelat 2013). *Mystus* Scopoli 1771 is a diverse catfish genus within Bagridae with medium to small species (Chakrabarty and Ng 2005; Darshan et al. 2010). There are currently 33 valid species of *Mystus* distributed in Turkey, Syria, Iraq, Iran, Afghanistan, Pakistan, India, Nepal, Sri Lanka, Bangladesh, Myanmar, Thailand, Malay Peninsula, Vietnam, Sumatra, Java and Borneo (Jayaram 2006) (Figure 1). The members of this genus are abundant in most fresh-water habitats. Two species are found in brackish and salt water: *Mystus gulio* enter the sea and are found within the tidal limit (Jayaram and Sanyal 2003) and *M. wolffii* is found in estuaries (Ng 2012). Mystus is a poorly diagnosed group, species of *Mystus* are morphologically similar and diagnostic characteristics are usually subtle (Ng 2003).

The genus *Mystus* has undergone several nomenclatural changes and other taxonomic modifications. Taxonomic revisions of *Mystus* have been completed only at regional levels including few species and using traditional morphometrics. These studies include nomenclature of the genus, fixation of type species, description of some new species and osteological comparisons of selected species (Jayaram and Sanyal 2003).

Biologists have studied anatomical features and used shape analysis for centuries (Adams et al. 2004; Zelditch et al. 2004) and classified organisms primarily on the basis of their form (Lele and Richtsmeier 2001; Macleod 2002). During the early twentieth century, D'Arcy Thompson (1917) plotted specimens on Cartesian coordinates, and then produced transformation grids to show where organisms could change either ontogenetically or phylogenetically (Macleod 2002). In the first half of the twentieth century, development of statistical methods like analysis of variance (Fisher 1935), and principal components analysis (Pearson 1901; Hoetelling 1933) and advancement in quantitative description of morphological shape set off the modern field of morphometrics (Adams et al. 2004). Until recently, morphometric data were primarily based on linear measurements and therefore, dependent on size. Various methods to remove the size component from the data without affecting the shape failed one way or the other (Adams et al. 2004).

In the early eighties, developments in statistical shape analysis by David G. Kendall and Fred. L. Bookstein paved the way for a new approach on morphometrics based on images and geometric methods that analyze variation in coordinate systems (Bookstein 1991; Adams et al. 2004), thus introducing the new revolutionary field of geometric morphometrics (GM) (Bookstein 1985, 1991; Rohlf 1993; Rohlf and Marcus 1993; Adams et al. 2004; Gunz et al. 2004). Advocates of geometric morphometrics (GM) claim several benefits, including greater statistical power and improved ease of visualization. Landmark-based geometric morphometrics offer a means to evaluate variation in shape independent of size (Pierce et al. 2008). Landmarks are defined as homologous points which have information on the geometry of biological forms (Bookstein 1991; Gunz et al. 2004). GM is a powerful approach to study morphological variation and covariation because size and shape are considered independently.

Morphological and functional studies have begun to use some GM methods (Adams and Rohlf 2000; Gunz et al. 2004; Adams et al. 2011), but the full range of techniques (such as Procrustes methods, principal, partial and relative warps) has yet to be fully explored in fish taxonomy. GM has been used for the description of shape differences between fish species (Odhiambo et al. 2011), or within a fish species between females and males (Herler et al. 2010), between populations (Maderbacher et al. 2008), or between reared and wild individuals (Hard et al. 2000). The use of GM for fish species identification has not matured. So in this study, GM was used to analyze shape differences between the species of *Mystus* and to describe the major patterns of *Mystus* diversity within morphospace. Morphotypes are examined in light of current taxonomy, and the data is used to break *Mystus* into phenetic groupings to ease the revision of the genus. In addition, the analysis will provide a dendogram that is compared with a phylogenetic tree using landmark data and will be compared with a molecular phylogeny from Chapter 2. From this study, we can also infer if shape data has phylogenetic signal and if shape has evolved phylogenetically across the genus. Along with traditional morphological analysis, geometric morphometrics will also help to identify new species of *Mystus*.

Materials and Methods

SPECIMEN INFORMATION

The geometric morphometric analysis includes 486 specimens belonging to 22 of the 33 valid species of *Mystus* (Table 1). All the specimens were preserved in formalin and stored in 70% ethanol. Some of the specimens were collected from Bangladesh during December 2005-2010 field studies and euthanized using MS-222, and others came from different museums as loans (Appendix 1).

GEOMETRIC MORPHOMETRIC ANALYSIS

A digital image of the left lateral view of each specimen was taken with a Nikon D50 digital camera. Eighteen biologically homologous landmarks and eight sliding semi-landmarks (Figure 2) were digitized using the software TPSDIG 2 (Rohlf 2006). The semi-landmarks were chosen to represent the shape and outline of the head and adipose fin. All studied species were grouped depending on the approximate size of the adipose fin (Table 2).

To remove all information unrelated to shape, a generalized orthogonal least-squares Procrustes (GPA) superimposition (Figure 3A) (translation, scaling and rotation) described in Rohlf and Slice (1990) was conducted on the sets of landmarks using the software tpsRelw (Rohlf 2006), and a consensus configuration was computed (Figure 3B). Partial warps were used to compare each specimen to this consensus configuration, and variation in these shape variables was summarized by relative warp analysis, analogous to a principle component analysis of the partial warps (tpsRelw) (Rohlf 2006). Once the relative warp scores were calculated, the average scores for each species were calculated and used in all subsequent analyses. According to the software requirements, a separate sliding semi-landmark file was prepared for tpsRelw to distinguish landmarks from semi-landmarks. This way tpsRelw performs the relative warp using sliding-landmark information during computation (Rohlf 2010).

A principal components analysis (PCA) (Figure 4) was used to find the maximum amount of variation. A Canonical Variates Analysis (CVA) (Hotelling 1935) was not employed as this method can overfit the separation among groups and produce unreliable results due to inadequate degrees of freedom when sample sizes are smaller than the number of measured variables (Weinberg and Darlington 1976; Sidlauskas et al. 2011). MANOVA was computed using the shape variables (PWs). The principal components were used for SAHN (Sequential,

Agglomerative, Hierarchical, and Nested) clustering to obtain a UPGMA dendogram (Figure 6) by NTSYSpc, vers. 2.2 (Rohlf 2000). Thin plate spline deformation grids were used to visualize shape variation along PC axes (Bookstein 1991; Rohlf 1993). Images were edited using Adobe Illustrator (CS 6.0) for better visualization and enhancement of colors and contrasts.

GEOMETRIC MORPHOMETRIC AND PHYLOGENY COMPARISON

The molecular phylogeny of *Mystus* has been discussed in chapter two. The molecular dataset includes 70 individuals and 16 species of *Mystus*. 15 *Mystus* species are present in both the morphometric and molecular datasets. The species that are not present in both datasets were pruned from the Maximum Likelihood and Maximum Parsimony tree using APE (Paradis et al. 2004), which was also used to limit each species to one individual. The pruned topology (Figure 8.A and 8.B) was used in morphometric analyses.

Results and Discussion

PRINCIPAL COMPONENTS ANALYSIS

The PCA shows considerable dispersion across morphospace among species and species groups within *Mystus* (Figure 4). The first five principal components explained 91.6% of total variation with PC 1 explaining 72.6%, PC 2 explaining 10.2% and PC 3 explaining 4.24% of total variation. The wireframes in Figure 5 visualize the shape variation on each of these axes. PC1 described variation in size and shape of the adipose fin length and depth of the body. Specimens with high positive scores on PC1, such as *M. gulio*, have a deep body, short head, short caudal peduncle, short adipose fin and small eyes. Specimens with high negative scores on PC1, such as *M. singaringan*, have elongated, depressed and thin bodies, elongate adipose fins, compressed heads and elongate caudal peduncles. PC2 describes subtle variation in the eye

diameter and head shape, depth of the body, and position of the adipose-fin origin. PC3 shows variation toward a deeper body, short and deep caudal peduncle and curvature of the body. The remaining components each summarize 5.0% or less of total variance. A MANOVA of partial warps revealed significant phenotypic difference in body shape among species (Wilk's λ = 3.43e-6; F = 8.03; P<0.0001).

The distribution of *Mystus* in morphospace (Figure 7) shows that the short adipose *Mystus* (*M. carcio, M. gulio* and *M. wolffii*) have high PC1 scores. Medium adipose fin *Mystus* (i.e. *M. vittatus, M. tengara*) are in the middle position in the morphospace and show some overlap in morphospace with small adipose *M. wolffii* and large adipose *Mystus* species. *Mystus* species which have more elongated bodies, pointed heads, and extra large adipose fins are clustered together in morphospace and have overlap with large and medium adipose *Mystus* species.

UPGMA

The most common use of NTSYSpc is for performing various types of agglomerative cluster analysis of a similarity or dissimilarity matrix and then plotting the results in the form of a dendrogram. The resulting dendrogram from the cluster analysis (Figure 6) (UPGMA algorithm, Procrustes distances) shows 4 major clusters. The first major cluster groups together the extra large adipose species (*M. albolineatus, M. cavasius, M. singaringan, M. nigriceps, M. bleekeri, M. rufescens* and *M. bocourti*). The base of the next large cluster consists of all of the medium adipose species, except *M. armatus*, and the small adipose *M. wolffii;* the large adipose species (*M. atrifasciatus, M. castaneus, M. malabaricus*, and *M. micracanthus*) form a distinct cluster within this second cluster. The other two small adipose species, *M. carcio* and *M. gulio*, cluster together, but have very long branch lengths almost equal to that of all of the other species

suggesting that they are very different in morphology from one another. However, 95% confidence intervals show much overlap for adipose fin size (Figure 7).

The UPGMA tree successfully recovered a few clades from the molecular phylogenetic analysis in second chapter; for example, *M. tengara* + *M. vittatus* and *M. multiradiatus* + *M. mysticetus*. Topologies of trees between geometric morphometric and molecular analysis differ. The UPGMA tree shows extra large adipose groups clustered together, while in the molecular analysis *M. cavasius* + *M. bocourti* + *M. singaringan* forms a monophyletic clade along with other extra large adipose *Mystus*.

GEOMETRIC MORPHOMETRICS AND PHYLOGENY

When the MP and ML phylogenies (Chapter 2) are overlain on the plot of PC1 vs PC2 (Figure 9A, 9B), a permutation test for phylogenetic signal infers significant phylogenetic signal for the MP tree, but for the ML tree the null hypothesis of no phylogenetic signal cannot be rejected (Figure 2, Chapter 2). The major differences in the two tree topologies are the relationships of *M. wolffii, M. gulio,* and *M. bleekeri*. For MP analysis, *M. wolffii* is sister to *M. gulio*, whereas for ML analysis *M. bleekeri* is sister to *M. gulio*.

Although Geometric Morphometrics is not likely to provide enough signal to elucidate a phylogeny (see Chapter 5), the technique may be useful in deciding which phylogeny may have more support. There has been a lot of argument about which type of analysis is likely to have the most phylogenetic signal, the efficacy of utilizing mitochondrial genes in phylogenetics, and in the use of a single gene to elucidate phylogenetic relationships. The MP analysis differs significantly with ML and BI, and the Geometric Morphometric analysis suggests that the MP analysis is more likely. In this case, MP may be the better analysis as it conforms to the GM data

better and it also accounts for fewer steps in the evolution of adipose fin length (5 steps in MP and 6 in ML and BI).

The distribution of the PCA (Figure 4) is unusual as the points are in an arc while most PCA's result in a random scatter of points. This distribution suggests that the characters influencing PC1 have an opposite effect as those influencing PC2. The plots in figures 9A and 9B suggest two different scenarios. In MP analysis, high PC2 scores are the short adipose fin and deep and stout body *Mystus* primitive condition and low PC2 scores are derived, but the states are equivocal in ML. Both analyses suggest that median PC1 scores are primitive and that high and low values along PC1 are derived and convergent within several groups. MP posits less convergence than ML. What this explains is medium adipose fin size *Mystus* are primitive states and two very different body shape groups diverged from the primitive one. One being with a very large adipose fin and more elongated body and caudal peduncle (i.e. *M. singaringan*, *M. cavasius*) which are restricted to fresh water; compared with a very short adipose fin group with a short and stout body as in *M. gulio* and *M. wolffii* which are estuarine. Probably estuarine habitat and freshwater habitat might have played role in the body shape and adipose fin size evolution of these two major groups of *Mystus*. The evolution of body shape and adipose fin size is better explained using a MP tree than a ML tree.

Although single gene phylogenies are not state of the art in phylogenetic reconstruction, they often represent the only cost-effective method in many places around the world. If combined with a cheap Geometric Morphometric study that determines whether the resulting tree does follow morphological evolution, perhaps these single gene trees are sufficient to elucidate phylogeny. Morphological evolution can be very plastic, and the chances of convergence are great, so morphology will not always be indicative of phylogeny (Kocher et al. 1993). In this

case, a larger phylogenetic study with more species and more genes (particularly nuclear ones) would be needed to test the relationships of the species, but for now, it would appear that the MP tree holds the greater phylogenetic signal.

Potential limitations of this study are lack of ecological data for *Mystus*. *Mystus* species are nocturnal and insectivorous. A detailed ecology, food, prey, microhabitat and trophic level study for each species would have provided more insight into the body shape evolution of this group. Various ecological parameters are acting simultaneously on body shape evolution. The only available information about the feeding of *Mystus* is not enough to explain its influence on body shape evolution (Winemiller 1990; Langerhans et al. 2003; Clabaut et al. 2007). Species specific ecological parameters and geometric morphometric analysis more focused on the head shape would probably strengthen this result and enable a more explicit interpretation of the changes in shape in relation to ecology (Clabaut et al. 2007).

Conclusion

Geometric Morphometrics has been shown to be a highly effective method for discrimination between closely related fish species (Albertson and Kocher 2001; Costa and Cataudella 2006) and also between populations. GM methods can be broadly applied for a wide variety of evolutionary questions involving complex shape changes (Kerschbaumer et al. 2011). In this study, geometric morphometrics were used to analyze shape variation among the species of *Mystus* and to describe the major patterns of *Mystus* diversity within geometric morphospace. The present study also tested whether or not the morphotypes dispersed in the morphospace are consistent with the current *Mystu*s taxonomy, used the morphometric data to break *Mystus* into meaningful phenetic groupings to simplify taxonomic revision, and also to identify new species and species groups for taxonomic studies.

I started these studies with a focus on *Mystus gulio.* The distribution of *Mystus* in morphospace (Figure 7) shows that the short adipose *Mystus* (*M. carcio, M. gulio* and *M. wolffii*) have high PC1 scores; however, the small adipose species differ greatly in their coefficient scores in the UPGMA (Figure 6). In general, smaller distances in the morphospace are expected to be found between closely related organisms (Gatz 1979). Based on the UPGMA, *Mystus gulio* is far from all other *Mystus* in the morphospace. Being estuarine fish *M. gulio* also have a different ecology than the other species and face different and larger predators compared to freshwater congeners. *Mystus gulio* clustered with *M. carcio,* which has a different color pattern with stripes instead of plain in *M. gulio*. With its wide distribution, unique ecology and body shape, it is clear that *M. gulio* is in need of taxonomic revision (Chapter 4).

The morphotypes (short, medium, large and extra large adipose fins) within the morphospace and in the UPGMA show great overlap and thus do not display any distinct phenetic grouping (Figure 7); however, the UPGMA may provide some information as to which species need to be examined together. Other than perhaps the extra large adipose fin, there are no morphometric traits that can offer any evolutionary insight due to overlap. So the body shape of *Mystus* is likely a continuum (Nosil 2012). In addition, there is likely convergence within the genus.

Geometric Morphometrics are less constrained than the other morphometric methods and have proved capable of identifying shape differences in many systems (Berns and Adams 2010). Therefore, GM seems to be a promising tool for identifying fish species and shape variation within morphospace. A combination of several approaches – molecular phylogeny, traditional morphometrics and three dimensional geometric morphometrics – could be used to describe the phylogenetic relationships of *Mystus* and to infer appropriate interspecies relationships.

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Table 1. Numbers of specimens examined per species of *Mystus*.

Species	*N*	Adipose Size
M. albolineatus	7	Extra Large
M. armatus	1	Medium
M. atrifasciatus	18	Large
M. bleekeri	31	Extra Large
M. bocourti	9	Extra Large
M. carcio	9	Small
M. castaneus	16	Large
M. cavasius	49	Extra Large
M. gulio	83	Small
M. malabaricus	7	Large
M. micracanthus	6	Large
M. multiradiatus	7	Medium
M. mysticetus	26	Medium
M. nigriceps	5	Large
M. oculatus	2	Medium
M. pulcher	2	Medium
M. rhegma	19	Medium
M. rufescens	4	Extra Large
M. singaringan	92	Extra Large
M. tengara	38	Medium
M. vittatus	32	Medium
M. wolffii	23	Small

Table 2. *Mystus* groups based on the approximate size of the adipose fins.

Group	Distance between dorsal and adipose	Adipose base greater than dorsal base	Anterior edge of Adipose	Species
Extra Large	None	Yes	Steep	*M. albolineatus, M. bleekeri, M. bocourti, M. cavasius, M. rufescens, M. singaringan*
Large	None	Yes	Gradual increase	*M. atrifasciatus, M. castaneus, M. malabaricus* and *M. micracanthus*
Medium	>dorsal base	Yes	Gradual increase	*M. armatus, M. malabaricus, M. multiradiatus, M. oculatus, M. pulcher, M. rhegma, M. tengara, M. vittatus,*
Small	>dorsal base	No	Steep	*M. carcio, M. gulio, M. wolffii*

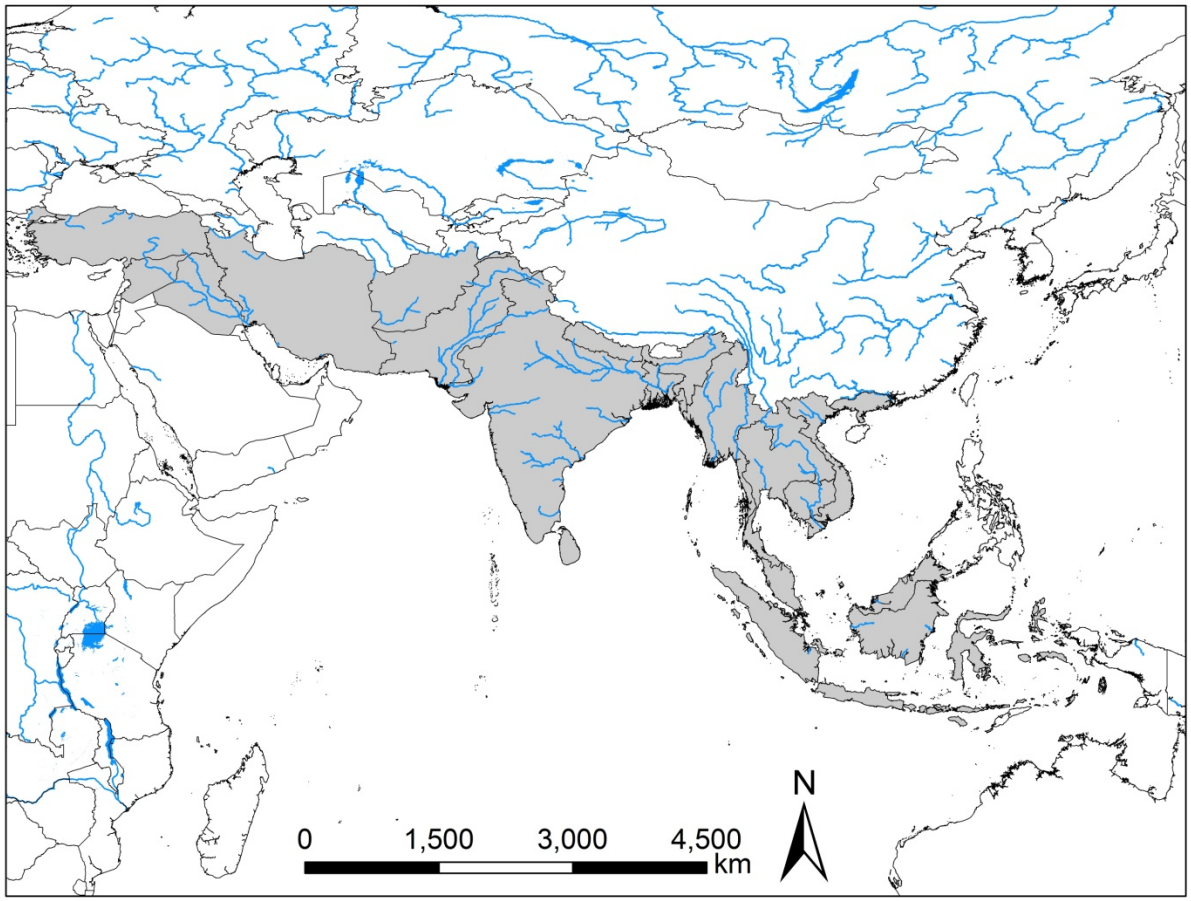


Figure 1. Approximate distribution of *Mystus* by country and major islands where they have been found with only the small portion of China indicated where they are found.

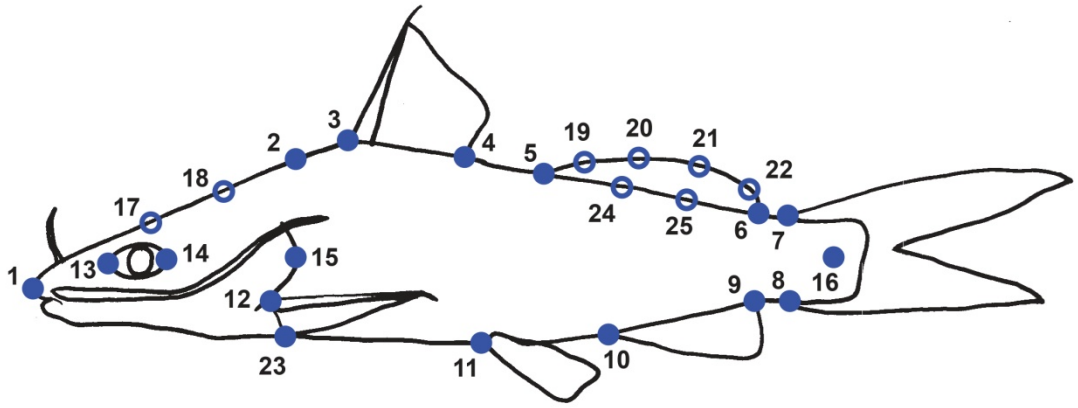
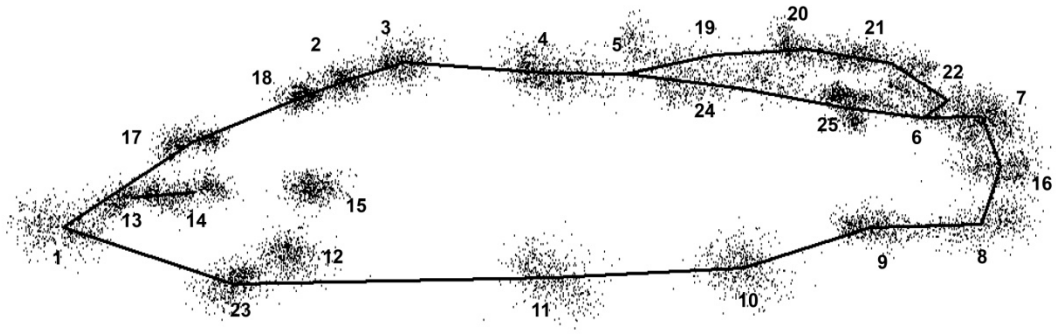


Figure 2. Landmark configuration of *Mystus*.

Description of the landmark s: (1) tip of snout; (2) length of head and parallel to the end of opercle straight on top of head; (3) anterior insertion of dorsal fin; (4) posterior insertion of dorsal fin; (5) anterior insertion of adipose fin; (6) posterior insertion of adipose fin; (7) base of the caudal fin, dorsal; (8) base of the caudal fin , ventral; (9) posterior insertion of anal fin; (10) anterior insertion of anal fin; (11) insertion of pelvic fin; (12) insertion of pectoral spine; (13) anterior margin of the longest axis of eye; (14) posterior margin of the longest axis of eye; (15) opercle margin at lateral line; (16) end of vertebral column; (17-18) curvature of snout to dorsal spine origin (19-22) curvature of upper margin of adipose fin surface-upper margin; (23) intersection of gill opening and ventral margin of body; (24-25) curvature of upper margin of adipose fin surface-lower margin.

A.



B.

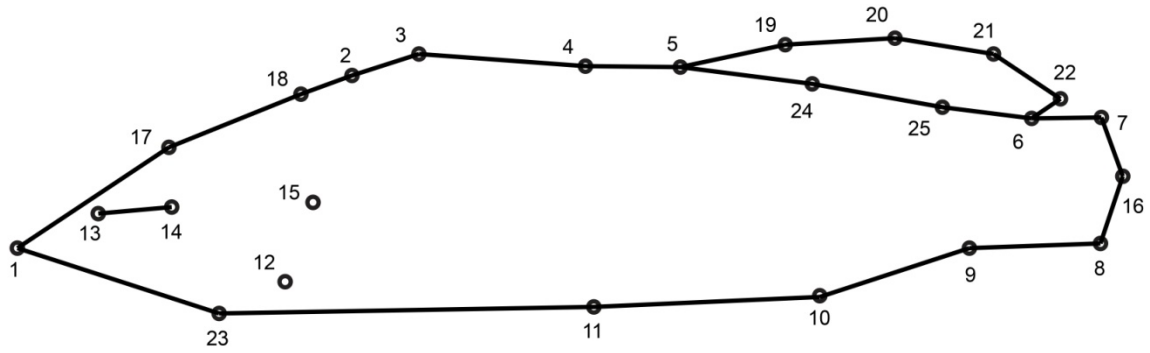


Figure 3. A. Procrustes Superimposition; B. Consensus configuration of all the specimens.

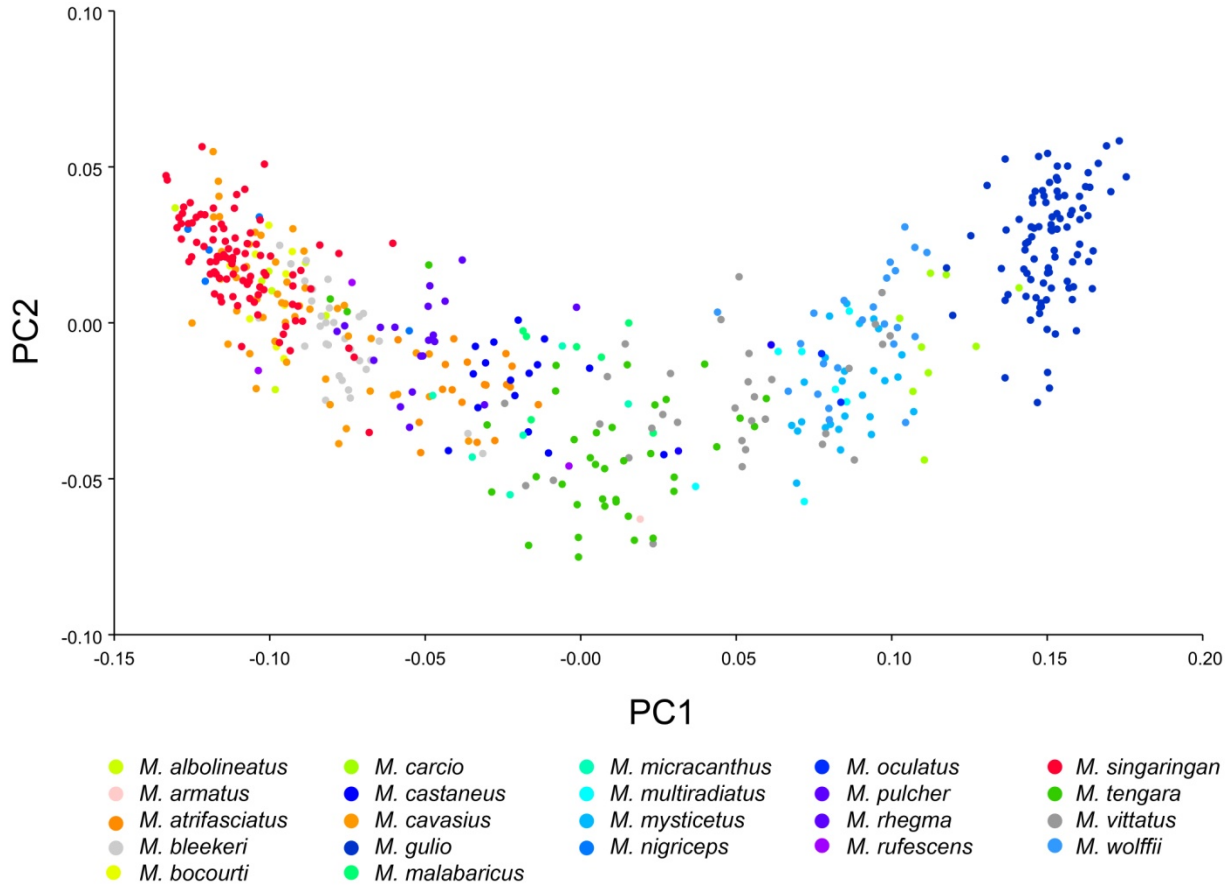


Figure 4. Results of Principal Components analysis of all specimens. PC1= 76.74%, PC2= 5.89%, accounting for 82.63% of total variation.

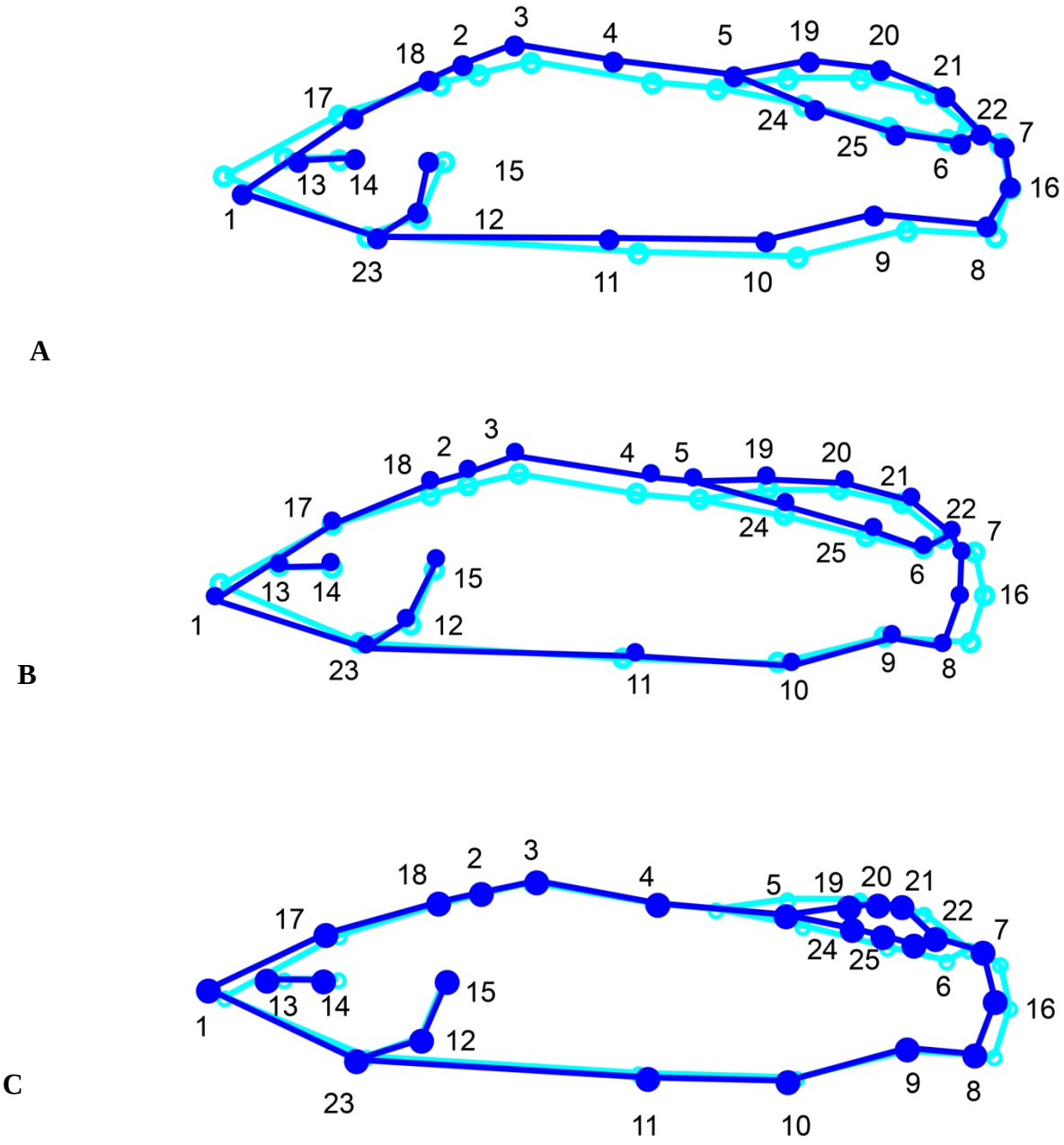


Figure 5. Wireframe visualization of shape variation along the principal components one, two and three from geometric morphometric analysis. Light blue landmarks represent the configuration of average specimen, dark blue landmarks represent one approximate extreme of the variation on that axis. Percentages indicate the proportion of total variance explained by each axis.

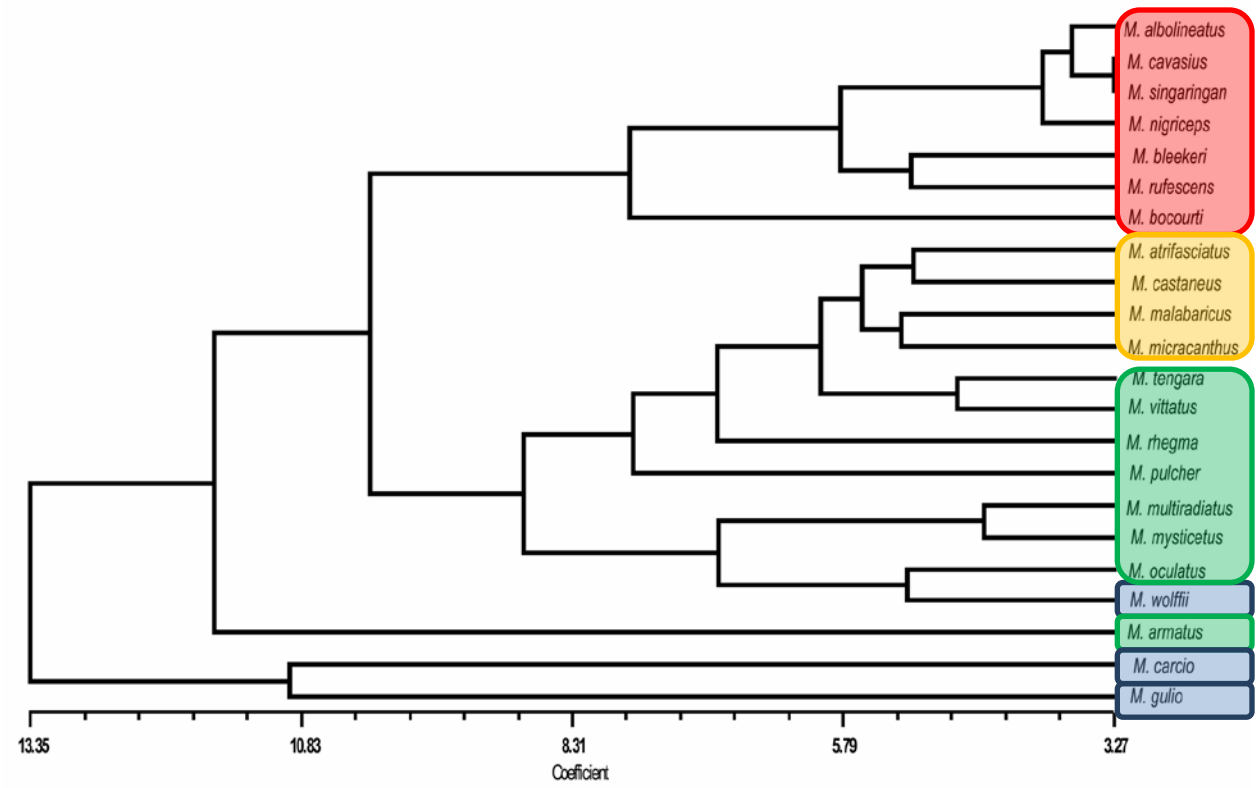


Figure 6. UPGMA Dendogram showing clustering of 22 *Mystus* taxa. Colored blocks represent size of the adipose fin for *Mystus*. X Large = ; Large = ; Medium = ; Small =

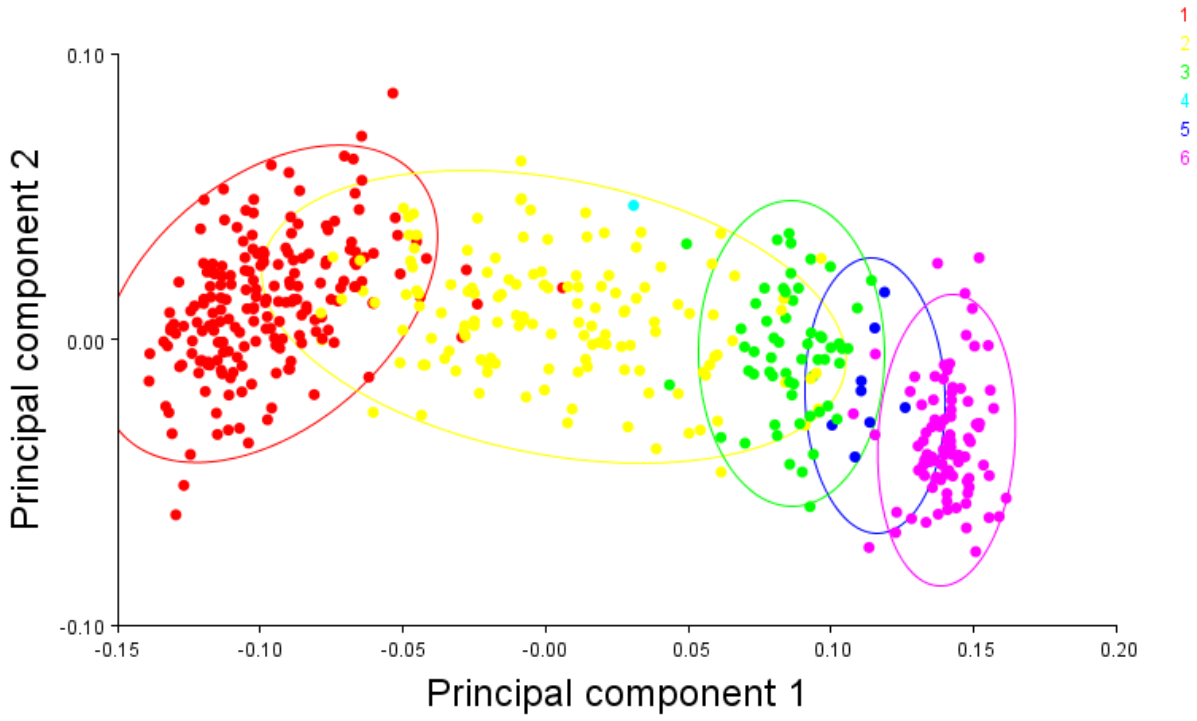


Figure 7. 95% confidence interval showing groups on the basis of UPGMA cluster analysis. Red = clade 1, Yellow= clade 2, green = clade 3, aqua= *M. armatus*, green = M. *wolffii*, blue = *M. carcio*, pink = *M. gulio*

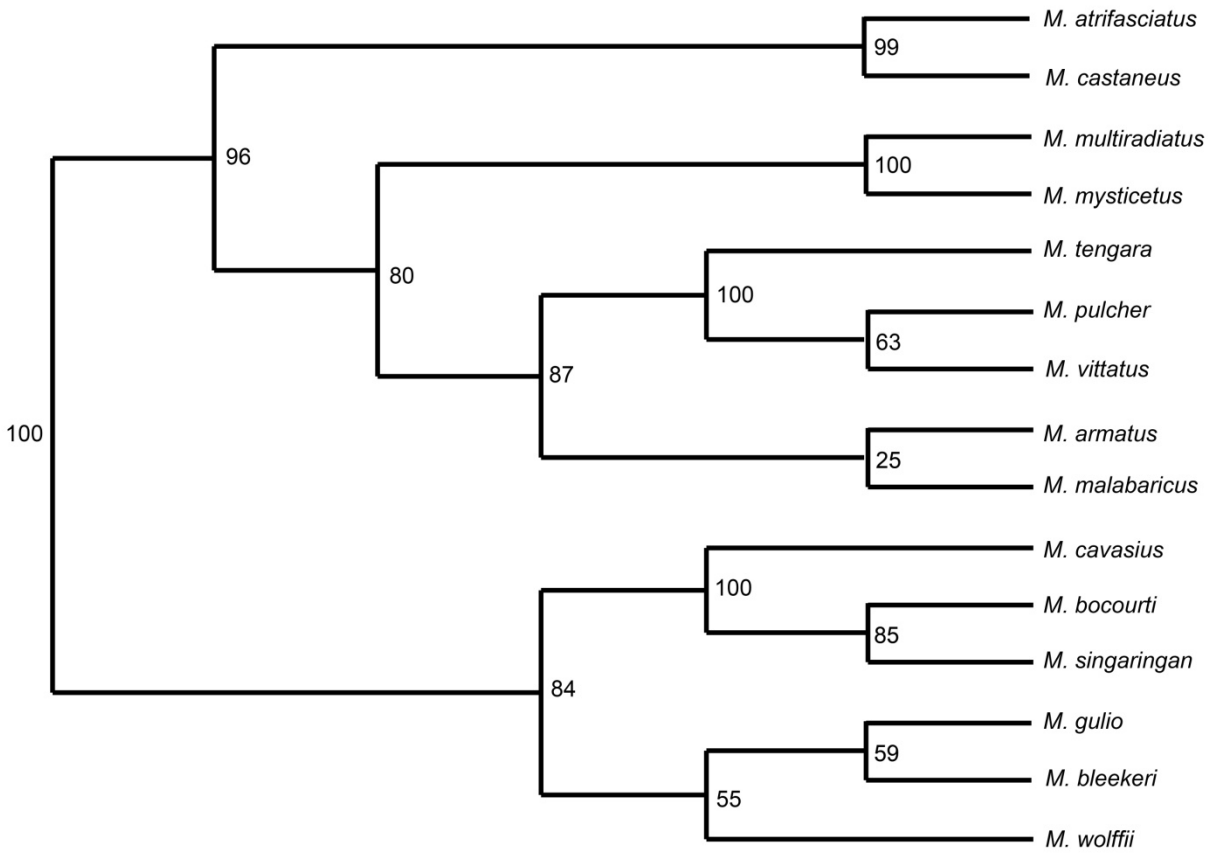


Figure 8A. Pruned Maximum Likelihood tree.

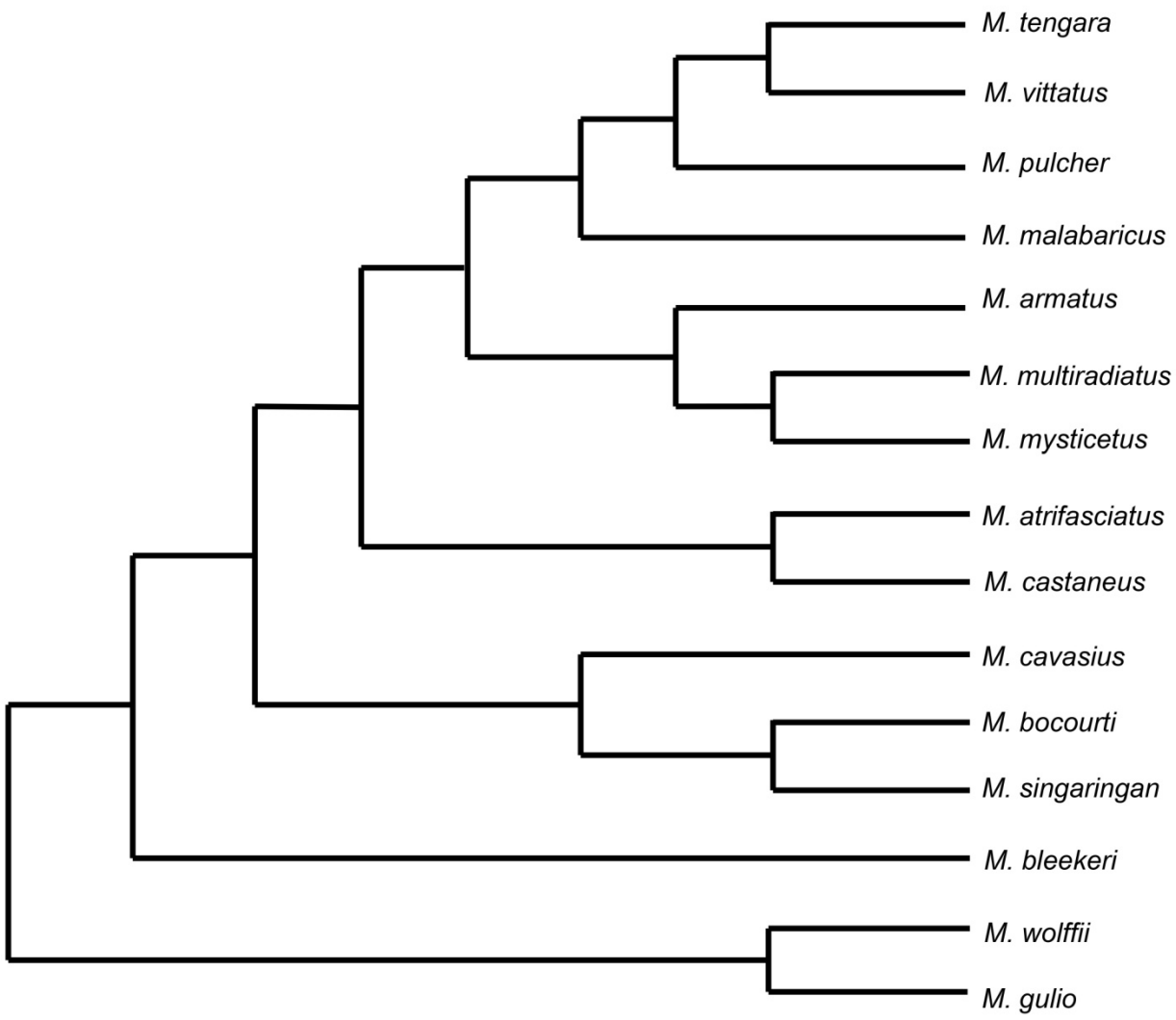


Figure 8B. Pruned Maximum Parsimony tree.

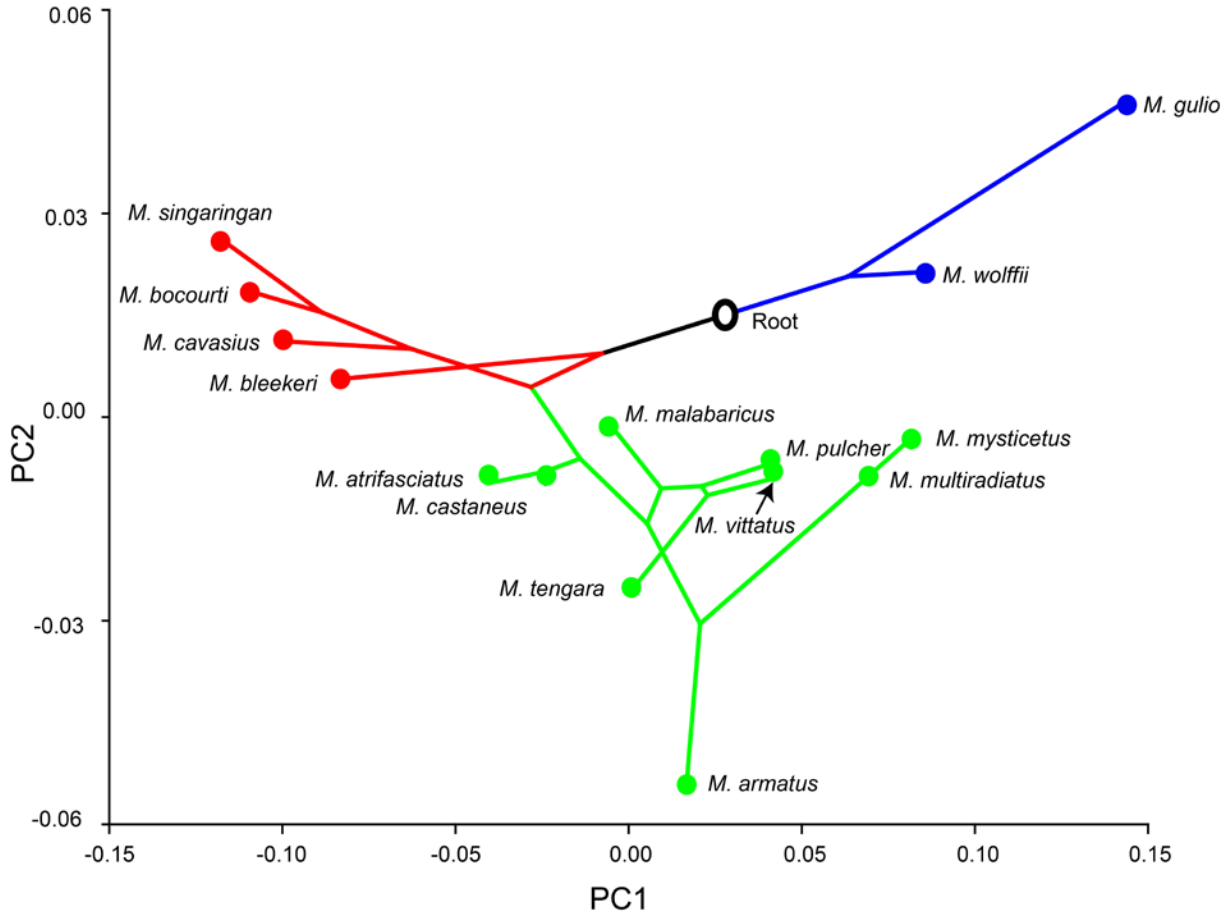


Figure 9A. Reconstruction of evolutionary changes in *Mystus* body shape. The phylogenetic tree (Maximum Parsimony) has been superimposed onto a plot of the first two principal components of the covariance matrix among species means. The tips of the terminal branches are at the locations of species means.

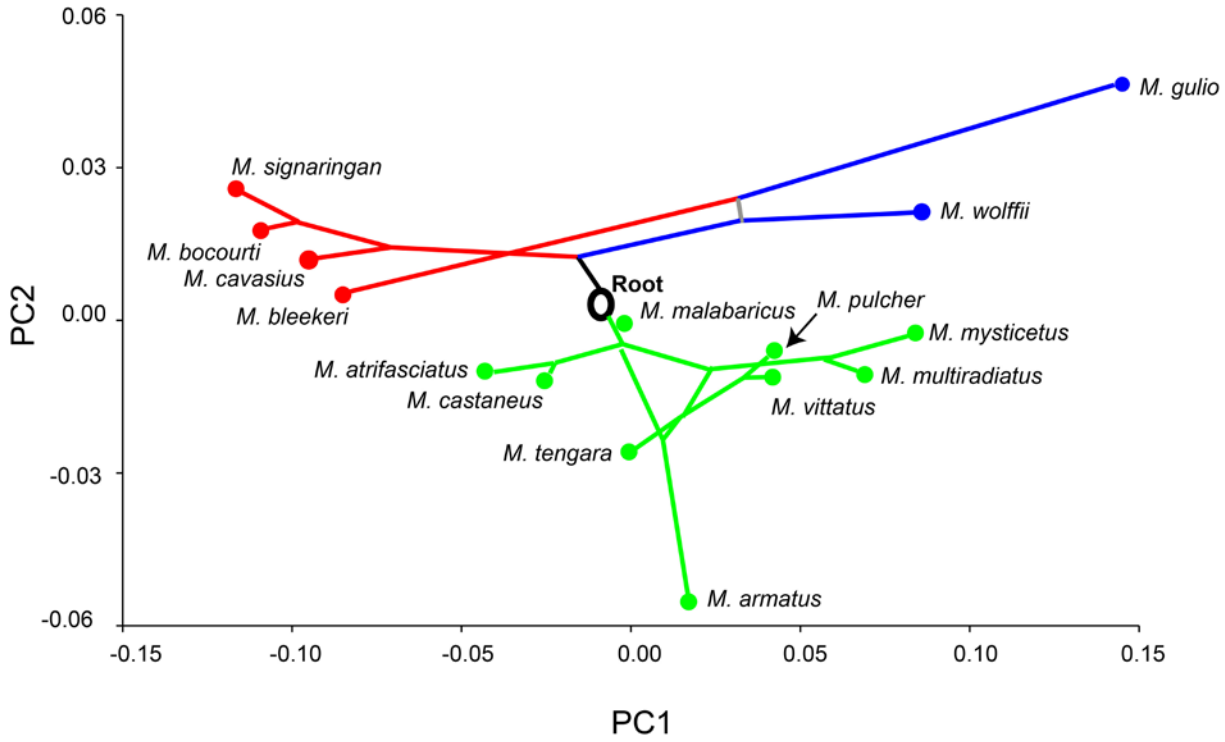


Figure 9B. Reconstruction of evolutionary changes in *Mystus* body shape. The phylogenetic tree (Maximum Likelihood) has been superimposed onto a plot of the first two principal components of the covariance matrix among species means. The tips of the terminal branches are at the locations of species means.

CHAPTER 4 – The identity of catfishes identified as *Mystus gulio* (Hamilton, 1822) (Teleostei: Bagridae), and designation of a neotype

Introduction

Catfishes (Order Siluriformes) constitute a large group of chiefly fresh water fishes distributed around the world. Africa, India and South America are rich in quantity and species diversity (Plamoottil and Abraham 2013). Bagridae is the seventh most species-rich catfish family currently recognized, and it includes 144 valid species in 18 genera (Jayaram 2006; Ku et al. 2007). *Mystus* Scopoli 1771 is a diverse catfish genus within Bagridae with small to medium species (50.0 mm SL- 300.0 mm SL) (Chakrabarty and Ng 2005; Darshan et al. 2010). Out of the 44 species originally described in *Mystus*, only 33 are currently considered to be in the genus. *Mystus* is distributed in Turkey, Syria, Iraq, Iran, Afghanistan, Pakistan, India, Nepal, Sri Lanka, Bangladesh, Myanmar, Thailand, Malay Peninsula, Vietnam, Sumatra, Java and Borneo (Jayaram 2006). The members of this genus are abundant in most fresh-water habitats. Two species, *Mystus gulio* and *Mystus vittatus*, enter the sea and are found within the tidal limit (Jayaram and Sanyal 2003). Species of *Mystus* are morphologically similar and diagnostic characteristics are usually subtle (Ng 2003).

There have been some previous studies on the taxonomy and distribution of *Mystus* (Roberts 1993, 1994; Jayaram and Sanyal 2003; Jayaram 2006). The use of the name *Mystus* is very confusing (Jayaram 1962). The genus has undergone several nomenclatural changes besides taxonomic modifications. *Bagrus halepensis* Valenciennes (1840) is designated as the type species (Kottelat and Ng 2007). Roberts (1994) recognized *Mystus* to have an elongate cranial fontanel reaching up to the base of the occipital process, a long maxillary barbel, a very long

adipose fin, 11–30 gill rakers on the first gill arch and 37–46 total vertebrae, about equally divided between abdominal and caudal regions. Roberts included only eight species under the genus. Mo (1991) studied three species of *Mystus* and diagnosed *Mystus* by having a thin, needle-like first infra-orbital and a twisted and thickened metapterygoid that is loosely attached to the quadrate by means of a ligament or small piece of cartilage. Jayaram and Sanyal (2003) and Ferraris (2007) respectively listed 44 and 33 species of *Mystus* as valid (Darshan et al. 2011).

Mystus gulio is one of the valid species in the genus *Mystus*. *Mystus gulio* looks different from other generalized *Mystus* species by having the dorsal profile slightly arched with a gentle rise from the tip of the snout to the base of occipital process (vs. some of the species with a steep dorsal profile), head flat, slightly depressed and blunt. The upper surface of the head is granulated, and the snout and upper jaw are rounded. *Mystus gulio* is also diagnosed by the presence of a shallow median longitudinal groove on the head, not reaching the base of the occipital process, occipital process short not reaching nuchal plate of dorsal fin, adipose fin short with a long interspace equaling about twice the length of its base from the rayed dorsal fin, body without stripes.

Pimelodus gulio was originally described by Hamilton-Buchanan (1822) from the upper part of the Gangetic estuaries, and it is the most widely distributed species of *Mystus* (found in Pakistan, India, Bangladesh, Myanmar, Thailand, Sir Lanka, Malaysia, and Indonesia and the East Indies) (Jayaram 2006). *Mystus gulio* is found in seas, estuaries and tidal waters. It is also known to occur in freshwater in the coastal plains (Jayaram and Sanyal 2003). For *Mystus*, the alpha level taxonomy is not well resolved and *M. gulio* is a species with particular problems. Eight species have been named that are now all considered to be synonyms of *M. gulio*: *Bagrus abbreviates* Valenciennes, in Cuvier & Valenciennes, 1840; *Bagrus albilabris* Valenciennes, in

Cuvier & Valenciennes, 1840; *Bagrus Birmannus* Valenciennes, in Cuvier & Valenciennes, 1840; *Bagrus fuscus* Valenciennes, in Cuvier & Valenciennes, 1840; *Bagrus gulioides* Bleeker, 1846; *Bagrus melas* Bleeker, 1846; *Bagrus rhodopterygius* Bleeker, 1846; and *Bagrus schlegelii* Bleeker, 1846. Valenciennes (1840) described *Bagrus albilabris*, *B. fuscus* and *B. birmannus*, which were placed into the synonymy of *M. gulio* by Bleeker (1863). Bleeker also placed five of his described species into *Mystus gulio*. Bleeker (1862, 1863) provided a new generic name *Aspidobagrus* for *M. gulio*, which was later synonymized into *Macrones gulio* by Gunther (1864). Jayaram and Sanyal (2003) found significant variation within individuals of the same population during his taxonomic study of *Mystus* and mentioned further investigation is needed to resolve the taxonomy for *M. gulio*. From the geometric morphometric analysis in Chapter 3, it is evident that *M. gulio* is separated from other *Mystus* in morphospace. The UPGMA dendrogram (Chapter 3) also shows *M. gulio* species is clustered with the small-adipose-fin species *M. carcio*, which has very distinct lateral stripes. A molecular phylogenetic analysis using Cytochrome b gene (Chapter 2) shows that the specimens of *Mystus gulio* form a clade. Specimens were examined from near opposite ends of the range of the species (Bangladesh + India and Indonesia). A split between Bangladesh + India specimens and Indonesian specimens was found, however the Indonesian specimens were paraphyletic in ML and BI, this basal split was poorly supported in all analyses, and the branch lengths are very short. The split between Bangladesh +India and Indonesia is approximately 60,000 years suggesting very recent movement of the species. So it is also important to study the morphological characteristics of *M. gulio* population across its distribution range.

The main objective of this study is a taxonomic reassessment of *Mystus gulio* to determine if the species represents multiple species as has been proposed by original describers of the synonymized species and by later authors (Jayaram 2006).

Materials and Methods

A modified morphometric truss (Bookstein 1985) was developed using 28 distances (Figure 1). Distances between each landmark are measured with digital calipers to the nearest 0.1 mm. Measurements are given in Table 1 as percentages of standard length (SL). Subunits of the head are presented as percentages of head length (HL). Head length and measurements of body parts are given as proportions of standard length (SL). Counts and measurements were made on the left side of specimens whenever possible following Roberts (Roberts 1994). Five individuals of *M. gulio* specimens (AUM 50570, CAS 55555, CAS 88628, CAS 200741, SU 32712) were cleared and stained according the procedure described in (Taylor and Van Dyke 1985). In addition, color pattern, counts of fin rays, position of mouth and relative length measurements were studied from museum specimens and from live specimens whenever possible. Over 300 museum specimens have been examined. Some live specimens have been studied and tissues have been collected during field trips to Bangladesh in 2005, 2007, 2010-11 (See Chapter 2). A few *M. gulio* from Bangladesh have been prepared for skeletal examination and one specimen (UF- 161553) has been scanned with the high resolution micro-CT scanner at University of Texas, Austin. Morphometric data were analyzed univariately by linear regression of each variable against standards like standard length (SL) and head length (HL) using JMP (ver. 10.0, SAS Institute, 2012). The data were also analyzed for the full dataset or subsets of all the individuals via Principal Components Analysis in JMP using a covariance matrix. Specimens were coded by region where they were collected. Institutional abbreviations are as listed at

http://www.asih.org/codons.pdf and the specimens are used in this study are deposited in these museums: AMNH, ANSP, AUM, CAS, KU, and UF.

Mystus gulio (Hamilton, 1822).

Pimelodus gulio Hamilton, 1822: 201, 379, pl. 23 (fig. 66). Type locality: Higher parts of the Gangetic estuaries, where the water is not very saline. No types known.

Bagrus abbreviatus Valenciennes, in Cuvier & Valenciennes, 1840: 420 (311 of Strasbourg deluxe edition). Type locality: Java. Holotype: RMNH 2942; previously unpublished illustration of holotype reproduced in Roberts (1993: 28, fig. 62).

Bagrus albilabris Valenciennes, in Cuvier & Valenciennes, 1840: 416 (308 of Strasbourg deluxe edition). Type locality: Calcutta. Syntypes: MNHN 0000-4172 (1), MNHN 0000-4336 (2), MNHN a-8967 (6), MNHN a-9009 (1).

Bagrus Birmannus Valenciennes, in Cuvier & Valenciennes, 1840: 419 (310 of Strasbourg deluxe edition). Type locality: dans l'Irawadi. Holotype: MNHN 0000-0577.

Bagrus fuscus Valenciennes, in Cuvier & Valenciennes, 1840: 417 (309 of Strasbourg deluxe edition). Type locality: Environs de Cananor. Holotype: MNHN 0000-0590.

Bagrus gulioides Bleeker, 1846: 152. Type locality: Batavia. Syntypes (size and number not stated): RMNH 6862 (some of 23).

Bagrus melas Bleeker, 1846: 152. Type locality: Java. Type(s) (size and number not stated): Whereabouts unknown.

Bagrus rhodopterygius Bleeker, 1846: 153. Type locality: Batavia. Type(s) (size and number not stated): Whereabouts unknown.

Bagrus Schlegelii Bleeker, 1846: 153. Type locality: Batavia. Type(s) (size and number not stated): Whereabouts unknown.

Neotype: AUM 55325, 952 mm SL; Bangladesh: Rupsha River, Khulna; S. Ferdous. (Figure 2).

MATERIALS EXAMINED

ANSP 122435 (3), 48.21-50.54 mm SL; India: Kashmir. ANSP 159315 (3), 52.88-71.87 mm SL; Thailand: Bangkok. ANSP 59402, 121.93- mm SL; Thailand: Bangkok, 30 mi up the Chao Phraya. ANSP 59410, 71.53- mm SL; Thailand. ANSP 77177, 70.55 mm SL; India: Bombay, Back Bay. ANSP 77246 (3), 57.66-85.16 mm SL; India: Bombay, Back Bay. ANSP 77248 (2), 31.36-59.39 mm SL; Burma. ANSP 87360, 84.29 mm SL; Thailand. ANSP 87856, 42.91 mm SL; Thailand. ANSP 89426 (6), 52.2-92.96 mm SL; Thailand: Tachin, Siam (town at mouth of the Tachin River, On N Shore of inner gulf; Dhachin, Samudh Sagorn. AUM 46297 (12), 72.57-130.83 mm SL; Java sea Basin. AUM 118 (2), 104.61-107.99 mm SL; Bangladesh. AUM 123 (10), 66.28-116.26 mm SL; Bangladesh. AUM 50359 (3), 56.06-162.14 mm SL; Bangladesh: Sundarbans River Dr. AUM 50414 (5), 78.99-118.76 mm SL; Bangladesh: Rupsha River Dr. AUM 50429 (2), 36.68-67.96 mm SL; Bangladesh: Pashur River Dr. AUM 50487 (6), 79.04-123.41 mm SL; Bangladesh: Cox's Bazaar. AUM 50570 (12), 70.86-106.13 mm SL; Bangladesh: Chittagong Fish landing. AUM 50636 (2), 88.3-89.69 mm SL; Bangladesh: Cox's Bazaar. AUM 55325 (12), 86.91-107.42 mm SL; Bangladesh: Padma Dr. BMNH 1898.4.2.167-168 (2), 88.5-92.97 mm SL; Thailand: River Menam. BMNH 1934.12.18.39-40 (2), 86.54-104.67 mm SL;

Thailand: River: Banghia River, Central Siam. CAS 55555 (19), 70.59-95.76 mm SL; Thailand: Gulf of Thailand. Brackish lagoon channel parallel to sand bar of Songkhla Channel (=Roads) near Gulf entrance. CAS 88628 (4), 86.75-99.57 mm SL; Myanmar: Bago Division, Bago (aka Pegu) Market. NMW 59628, 108.43 mm SL; India: Cochin. NRM 13689 (7), 53.36-64.57 mm SL; Sri Lanka: Southern Province, Malala Lewaya. NRM 13696 (6), 88.56-112.46 mm SL; Sri Lanka: Malala Lewaya. NRM 14506 (14), 61.59-118.47 mm SL; Sri Lanka: Malala Lewaya. NRM 27161 (2), 141.63-142.48 mm SL; Sri Lanka: Colombo River. NRM 40602 (5), 82.1-101.73 mm SL; India: West Bengal. SU 34855 (4), 76.3-95.92 mm SL; India: West Bengal, Calcutta. SU 40224, 46.03 mm SL; Myanmar: Rangoon. SU 41075, 86.7- mm SL; India: Kerala. Trivandrum, Travancore. SU 41076, 69.07 mm SL; India: Tamil Nadu. Ennur Fisheries Station, Madras. SU 41078, 95.07 mm SL; India: Goa. Samonia, Khadii. SU 61464 (4), 61.07-84.53 mm SL; Indonesia: Jawa Timur Prov. About 20 miles southeast of Surabaja, in fish ponds. UMMZ 186724 (9), 51.9-73.62 mm SL; Thailand: Prachuab Khirikhan. Lake just inland from Prachuab Khirikhan City, in city. UMMZ 208768, 108.13 mm SL; Bangladesh: Sylhet. UMMZ 227497 (12), 61.88-90.35 mm SL; Vietnam: Ba Xuyen Prov Giao, 3 km S. of Truong Binh at mouth of Bassac, Mekong River drainage. USNM 149732 (3), 114.14-125.34 mm SL; India: Travancore. USNM 298239, 83.11 mm SL; Sri Lanka, Just north of Kallu. USNM 317586, 44.14- mm SL; Sri Lanka: Jaffna. USNM 317606 (4), 85.57-98.29 mm SL; Sri Lanka: Jaffna. USNM 393629 (5), 45.41-57.84 mm SL; Indonesia: Province of Kalimantan Selatan. South East Borneo. USNM 393652 (4), 54.87-64.45 mm SL; Indonesia: Province of Kalimantan Selatan. South East Borneo. USNM 393749, 51.28 mm SL; Indonesia: Province of Kalimantan Selatan. South East Borneo.

Result and Discussion

Mystus gulio can be distinguished from other congeners except *M. wolffii* and *M. carcio* by a short-based adipose fin with a deeply incised posterior margin (Figure 4) (Ng 2012). *Mystus gulio* differs from the other estuarine species of *Mystus* (*M. wolffii*) by the length of maxillary barbel (reaches maximally to just beyond the anal fin origin in *M. gulio* vs. reaches the caudal fin in *M. wolffii*) and by the length of the cranial fontanel (does not reach the base of the supraoccipital process in *M. gulio* vs. reaches to the base of supraoccipital process in *M. wolffii*; Figure 4).

M. gulio differed from *M. carcio* in not having any stripes and *M. carcio* has a small adult size maturing at 44.0 mm SL. *Mystus carcio* also has a long posterior fontanel, which almost reaches the base of supraoccipital process vs. does not reach for *M. gulio*. *M gulio* does not have a coracoid shield (vs. presence) in *M. carcio*. *M. carcio* has black tympanic spot which is absent in *M. gulio*.

Francis Hamilton (1822) made all his drawing from fresh specimens and discarded them after drawing and, thus, did not preserve any type specimens (Darshan et al. 2010). The drawing and description of Hamilton (1822) do not match with one another. His description of head shape being short and flat, maxillary barbel beyond dorsal and caudal divided into two rounded lobes does not match with his drawing where the head is more pointed and longer than broad, the maxillary barbel goes almost to the end of adipose fin and the caudal fin is bifurcate into two pointed lobes (Jayaram and Sanyal 2003; Jayaram 2006). Given that the drawing and description of the species do not match, and there is no type specimen, it is important to designate a neotype.

I could find no consistent morphological differences between populations. Analyzing character bivariately showed no differences in individual measurements by geographic region.

No discreet differences were found between populations in the Principal Components Analysis, no populations formed a distinct cluster (Figure 5). In chapter 2, I show no significant geographical separation of populations using cytochrome *b* sequence data (0.5% sequence divergence per million years) (Ku et al. 2007). Although divergence times vary based on analysis, the strict clock puts the time of divergence around 60,000 years between India+Bangladesh and Indonesia, which is likely too short of a time to form species differences. Despite a large range, *M. gulio* live in estuaries and coastal marine waters. Such habitats in Southeastern Asia are extensively connected now, and were likely even more so when the Malaysian Archipelago was a peninsula during glacial periods, the last ending about 20,000 Ya (Voris 2000). Without any distinct biogeographic breaks in this habitat, there is nothing that would limit movement of *M. gulio* between regions. Despite authors noticing differences within *M. gulio*, this is just intraspecific variation.

DESCRIPTION

Biometric data is in Table 1. Head depressed, dorsal profile evenly sloping and slightly convex, ventral profile almost straight. Caudal peduncle deep. Bony elements of dorsal surface of head covered with thick skin and bones not always visible. Cranial fontanelle extends from behind snout to just posterior of orbit (Figure 4). Supraoccipital process elongate, slender. Eye ovoid, on dorsal half of head and cannot be seen from ventral side. Gill opening wide and extends from posttemporal to beyond isthmus. Gill membranes free from isthmus.

Mouth subterminal, fleshy upper lip extends anteriorly beyond lower lip. Teeth small and villiform in uninterrupted semi-lunar band across palate; about four rows on upper jaw and five to six mesially-interrupted bands on lower jaw (Jayaram and Sanyal 2003). Barbels in four pairs. Maxillary barbels long and slender, extending from beyond pelvic fins to anal-fin origin. Outer

mandibular barbel almost to end of pectoral fin. Inner mandibular barbel to origin of pectoral spine. Nasal to beyond orbit. Skin smooth. Lateral line complete and midlateral in position.

Dorsal fin spinelet, spine, and 6 - 7 (mode 7, *N*=66) rays; origin of dorsal fin anterior to mid-body; dorsal-fin margin slightly concave and first two fin rays longer than others. Dorsal-fin spine moderately long, smaller than longest fin rays; anterior edge of dorsal-fin spine smooth, posterior edge serrated. Nuchal plate triangular. Pectoral fin with a stout spine, sharply pointed at the tip, 7 - 8 (mode 6, *N*=65) principal rays; anterior margin of pectoral spine smooth and posterior with 12–13 serrae. Pelvic fin I, 5 (N=66) with slightly convex margin; origin slightly posterior to insertion of dorsal-fin. Tip of adpressed pelvic fin generally not reaching anal fin. Anus and urogenital opening located at vertical through middle of adpressed pelvic fin. Adipose fin very short with deeply incised posterior margin, adipose-fin base smaller than that of anal-fin base. Anal fin originates slightly forward of adipose-fin origin, anal finray 10-13(mode 11, N=66). Caudal fin deeply forked; with upper lobe (6-8, mode 8, *N*=43) and lower lobe (6-10, mode 9, *N*=43) principal rays, upper lobe slightly longer than the lower lobe. Both upper and lower lobe is pointed in smaller individuals and looks like lanceolate in larger individuals. Procurrent rays extending only slightly to anterior to caudal fin base. Gill rakers on first gill arch 28(2)-36 (4). Vertebrae 34(3)-36(3).

Coloration: In life, dark brown on dorsum, with silvery hue ventrally. Ventral side dull white. All fins grey with black margins and yellow hue at base. In 70% ethanol: dorsal surface dark brown with sliver, creamy white or dull white ventral surface. Maxillary barbel dark grey, matching dorsal body color. Mandibular barbels cream or light yellow. Adipose fin with black margins. No stripes or spots on body.

Sexual dimorphism: Urogenital papilla of males pointed and of females rounded.

Distribution: Distributed in Pakistan, India, Bangladesh, Myanmar, Thailand, Malaysia, Indonesia, Sri Lanka, the East Indies and the south China sea (Jayaram 2006).

Habitat and biology: *Mystus gulio* is primarily a brackish water fish, but is found in coastal marine waters, estuaries, and sometimes in freshwater near the coast. In freshwater, adults occur mainly in larger water bodies (rivers and streams) with mud or clay substrates, and are rarely found in smaller streams. They form schools of 10 to 25 individuals (http://eol.org/). They are diurnal and oviparous, (Breder and Rosen 1966).

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Table 1. Measurement of *Mystus gulio* (*N*=202)

Measure	NeoType	Range	Mean ± SD
SL (mm)	95.2	31.4 - 162.1	82.2±21.3
%SL			
Predorsal length	37.3	36.7 - 44.9	40.8±1.5
Prepectoral length	24.8	21. - 31.8	25.1±1.9
Thorax length	33	23.2 - 39.5	32.±2.8
Abdominal length	14.9	9.6 - 19.9	15.5±1.8
Postanal length	31.1	26.1 - 45.2	32.±2.3
Dorsal-pectoral distance	25.6	22.7 - 29.2	25.8±1.2
Dorsal-pelvic distance	28.8	20.3 - 38.2	27.7±2.6
Dorsal-anus distance	38.1	21.8 - 43.8	37.3±3.8
Caudal peduncle depth	12.3	8.7 - 14.	11.7±1.
Dorsal-spine length	16.2	11.5 - 21.2	15.8±1.7
Dorsal ray length	24.5	17.2 - 36.2	24.5±3.
Dorsal fin base length	10.7	7.2 - 13.8	10.4±1.1
Pectoral-spine length	17	13.1 - 23.7	18.1±2.1
Pectoral ray length	21.1	15.9 - 23.7	20.2±1.7
Pectoral fin base width	4.6	3.1 - 7.5	4.5±.7
Pelvic fin base width	4.4	2. - 4.9	3.1±.6
Adipose total length	14.9	5.4 - 22.1	8.9±2.7
Adipose height	3.5	1.8 - 7.7	3.9±.9
Anal width	15.8	8.4 - 21.6	12.8±1.6
Anal height	18.7	3. - 24.5	19.5±2.5
Head length	25.4	23.7 - 33.6	26.3±1.5
%HL			
Snout length	39	29. - 44.4	37.2±2.3
Mouth width	46.8	33.7 - 59.6	46.5±4.7
Interorbital distance	40.1	32. - 47.8	39.9±2.6
Orbit diameter	17.6	12.1 - 30.	20.9±2.8
Anterior internare	20.7	13.3 - 23.7	18.7±1.9
Nasal barbel inner	42.5	24. - 109.1	48.5±11.4
Maxillary barbel outer	202.3	157.2 - 406.1	228.8±31.5
Mandibular outer	111.4	53.2 - 185.	114.±18.1
Mandibular inner	75.3	33.4 - 95.5	61.5±10.6

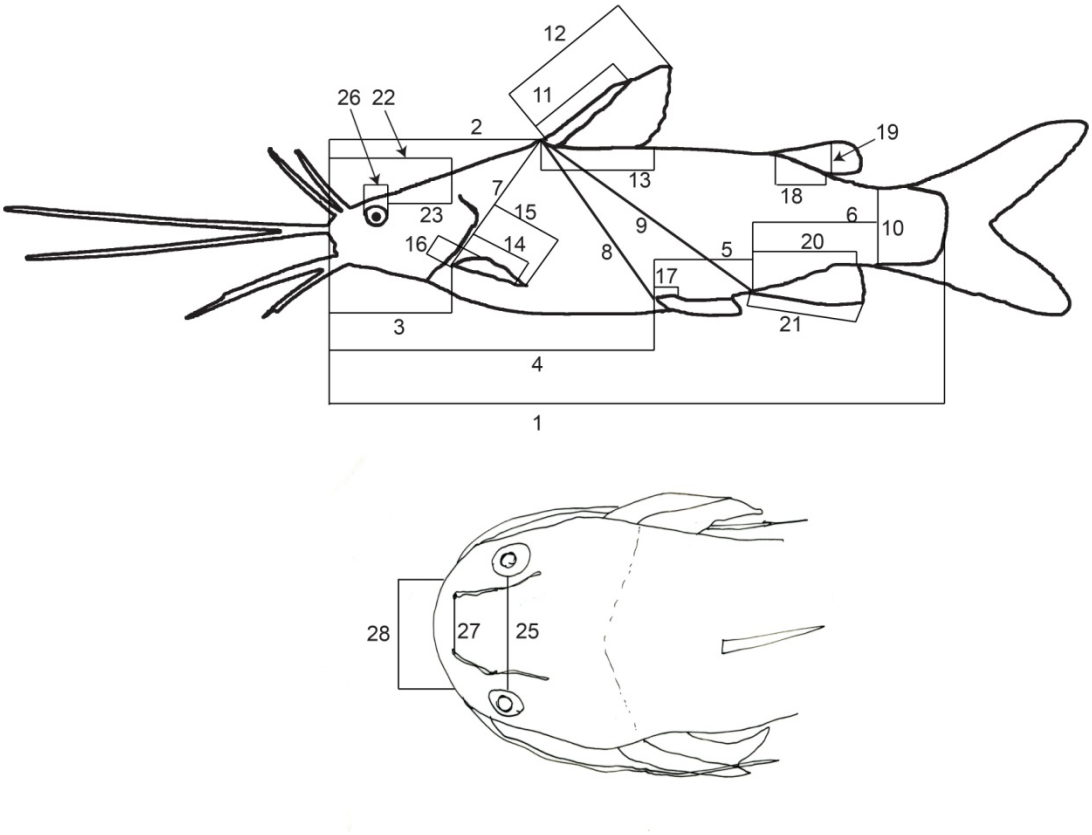

Figure 1. Morphometric truss and distances measured.
Description of the measurements: (1) standard length; (2) predorsal length; (3) prepectoral length; (4)thorax length ; (5) abdominal length; (6) postanal length; (7) dorsal to pectoral distance; (8) dorsal to pelvic distance; (9) dorsal to anal distance; (10) caudal peduncle depth; (11) dorsal spine length; (12) dorsal-fin ray length; (13) dorsal-fin base length; (14) pectoral spine length; (15) pectoral-fin ray length; (16) pectoral-fin base width; (17) pelvic-fin base width (18) adipose-fin base length; (19) adipose height; (20) anal-fin base length; (21) anal-fin height; (22) head length; (23) head-eye length; (26) orbit diameter; (25) interorbital distance; (24) mouth width; (27) anterior internares width; (28) nasal barbel width; (29) maxillary barbel; (30) mandibular outer barbel; (31) mandibular inner barbel. Lateral view based on a photo by A. Manimekalan and posted on the All Catfish Species Inventory website.

Figure 2. *Mystus gulio*, neotype, AUM 55325, 95.2 mm SL, dorsal, lateral and ventral views. Scale bar is 1 cm. Photo by J.W. Armbruster

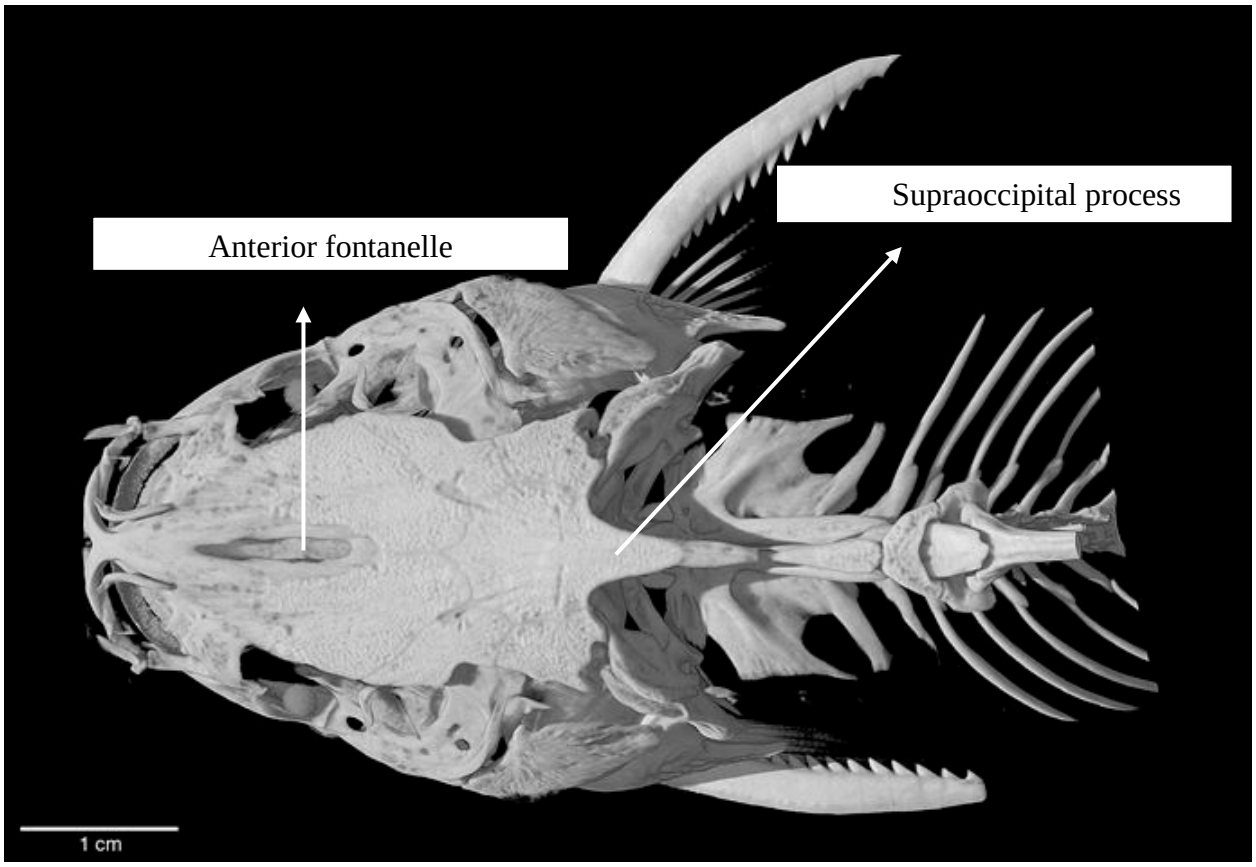

Figure 3. *Mystus gulio* (UF- 161553) dorsal view of head with High-Resolution X-ray CT, showing anterior fontanelle and supraoccipital process.

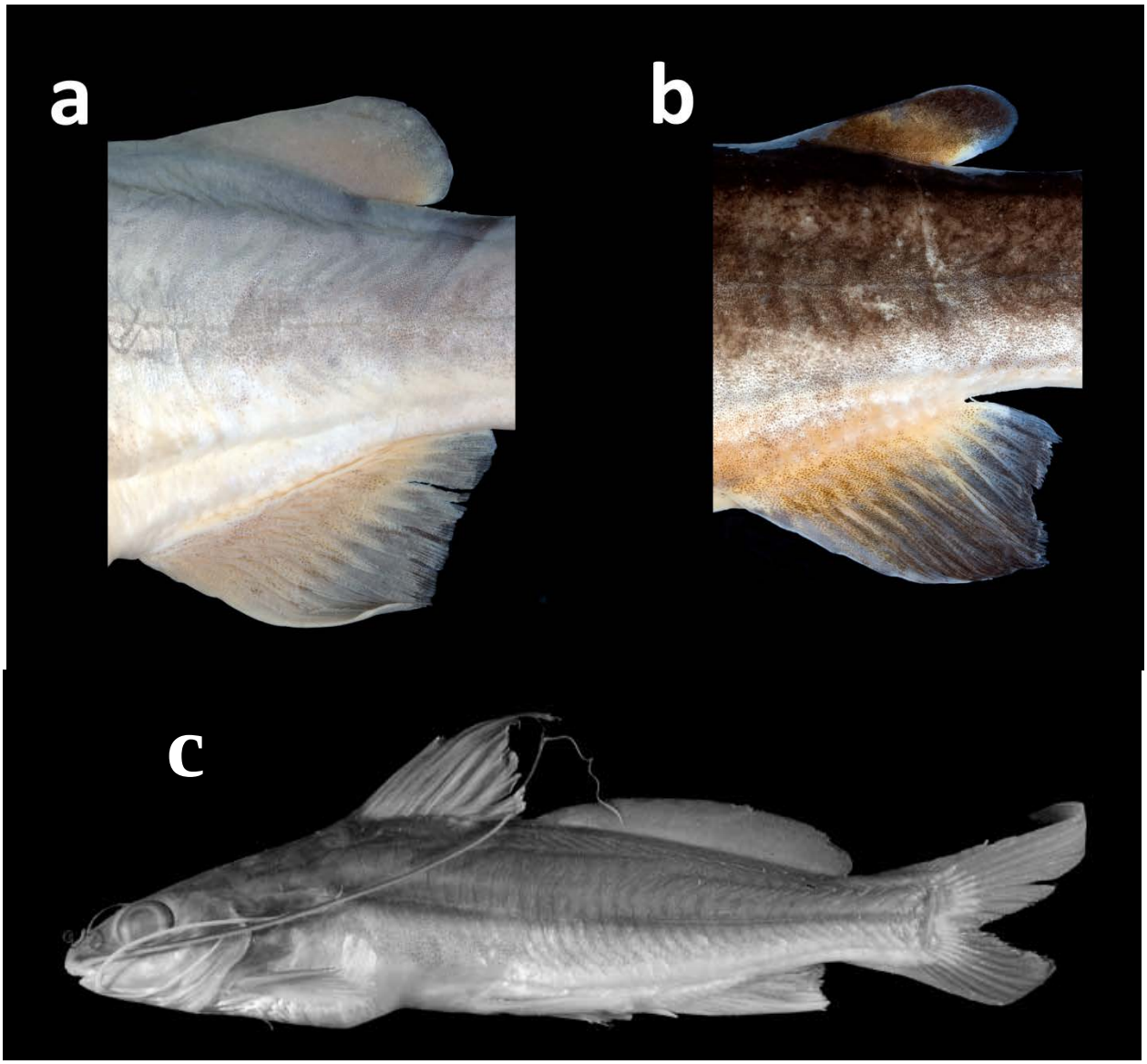

Figure 4. Adipose fins of: a) *Mystus wolffii*, ZRC 53387, 141.0 mm SL; b) *Mystus gulio*, ZRC 52087, 157.4 mm SL, shows differences in shape. Figures are not to scale (Ng, 2012) and c) Adipose fin of *Mystus cavasius* (Chakrabarty and Ng, 2005).

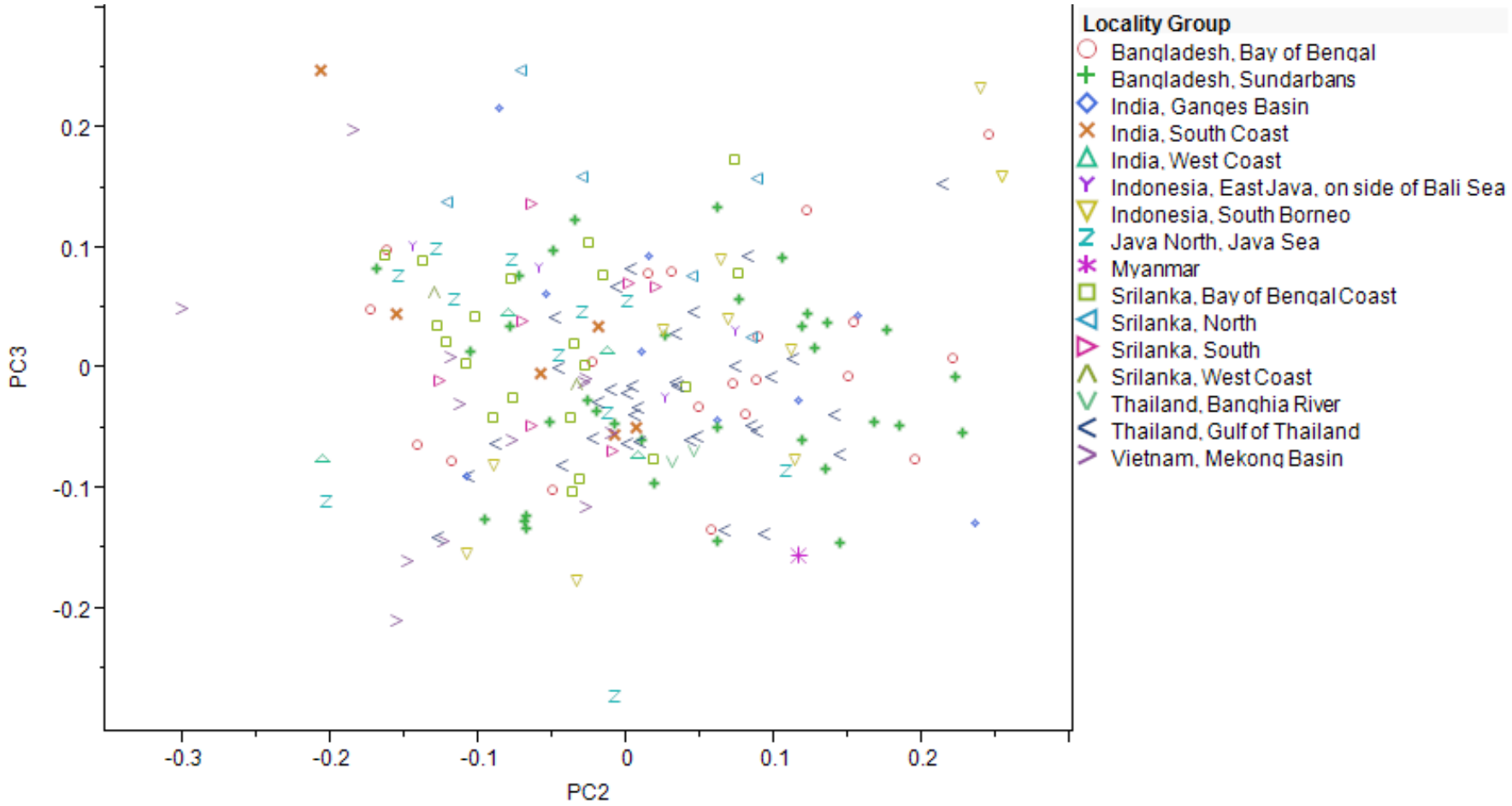

Figure 5. Principal Components Analysis of specimens of *M. gulio* coded by region.

CHAPTER 5 – Geometric Morphometrics as a tool to elucidate phylogenies: A review and test

Abstract

The use of morphometric data in phylogenetic analyses has long been debated. The disagreement primarily concerns the question of whether or not morphometric data could be used to estimate phylogeny. One of the major issues in the controversies over the use of geometric morphometrics in the context of phylogeny is the use of shape as a character or set of characters and how the partitioning of these separate characters should be done. The phylogenetics of the genera of the North American minnows, dace, chubs, and shiners of the Family Cyprinidae has been well-studied using morphological and molecular methods. The results of the nuclear and mitochondrial genes are almost perfectly consistent; meaning the phylogeny of the Cyprinidae is well established. We developed a database of representative Cyprinids from each of the major clades in order to test methods of phylogenetic construction and analysis based on geometric morphometrics. The main purpose of this study was to test whether any phylogenetic signal is present in the geometric morphometric data. In order to test this, we mapped morphometric data onto a known phylogeny for Cyprinid data set. We developed a set of 18 homologous landmarks for 30 genera, 40 species, and 352 specimens. We found significant phylogenetic signal in the geometric morphometrics data set. However, the tree computed from landmark data of Cyprinids is not consistent with the well-supported phylogenetic tree from molecular data. Analyzing geometric morphometric data phylogenetically offers great insight into the evolution of groups, but it is unlikely that geometric morphometrics can be used to infer phylogeny.

Introduction

Morphometrics is the study of variation and covariation of biological form (Bookstein 1991; Dryden and Mardia 1998; Adams et al. 2004). Morphometric methods are important for description and statistical analysis of the shape of an organism (Rohlf and Marcus 1993). In the 1980s and 1990s, significant advances were made in field of morphometrics with the introduction of coordinate-based methods, and advances in the necessary mathematical, statistical and computational techniques (Bookstein 1991; Rohlf 1993; Rohlf and Marcus 1993; Adams et al. 2004; Slice 2005; Sidlauskas et al. 2011). The term 'geometric morphometrics' (GM) was introduced to distinguish GM from the measurement-based techniques of 'traditional' morphometrics (Rohlf and Marcus 1993). Geometric morphometrics refers to the approach in morphometry where shapes are expressed as geometric coordinates and the representation and comparison of these shapes are subject to mathematical and statistical techniques (Zelditch et al. 2004). This allows visualization of shape independent of size (Rohlf and Marcus 1993; Adams et al. 2004) and proves useful in phylogenetic investigation (Monteiro 1999; Pierce et al. 2008).

With the advent of molecular techniques, genes are being more commonly used to construct phylogenies than morphology has been; but understanding morphological traits is important to understanding the evolution of a group (Covain et al. 2008). There are controversies about some of the methods and their usefulness in morphometrics. According to Zelditch et al. (2004), no good method has been developed to find characters in morphometric data, a statement that is likely true today.

The purpose of this study is to provide a review of the use of geometric morphometrics in phylogenetic analysis, and to provide a test of methods using the North American Cyprinidae (minnows, dace, shiners, and chubs) for which a well-supported phylogeny exists (Bufalino and

Mayden 2010b, c, a). We pay particular attention to recent advancements in different methods and approaches on the use of geometric morphometrics data to elucidate a phylogeny.

GEOMETRIC MORPHOMETRICS: BACKGROUND

Biologists have studied anatomical features and used shape analysis for centuries (Adams et al. 2004; Zelditch et al. 2004) and classified organisms primarily on the basis of their form (Lele and Richtsmeier 2001; Macleod 2002). During the early twentieth century, D'Arcy Thompson (1917) plotted specimens in Cartesian coordinates, and then produced transformation grids to show where organisms could change either ontogenetically or phylogenetically (Macleod 2002).

On the statistical front, significant advances resulted from the collaboration of Francis Galton (1822-1911), Karl Pearson (1857-1936) and W.F. R. Weldon (1860-1906) (Reyment 1996). In 1888, Galton introduced the correlation co-efficient in the analysis of human forms which was mathematically explained by Pearson (Mitteroecker and Gunz 2009; Reyment 2010); in 1890, Weldon used quantitative methods to study form of the shrimp, *Crangon vulgaris,* (Reyment 1996).

In the first half of the twentieth century, development of statistical methods, such as analysis of variance (Fisher 1935) and principal components analysis (Pearson 1901; Hoetelling 1933), and advances in quantitative description of morphological shape set off the modern field of morphometrics (Adams et al. 2004). Until recently, morphometric data were primarily based on linear measurements and therefore, dependent on size. But various efforts to remove the size component from the data without affecting the shape failed (Adams et al. 2004). In the early eighties, developments in statistical shape analysis by David Kendall and Bookstein's work on shape transformation paved the way for a new approach to morphometrics (Bookstein 1991;

Adams et al. 2004), thus introducing the new field of geometric morphometrics (Rohlf and Marcus 1993).

CURRENT GEOMETRIC MORPHOMETRICS

The most widely used methods in geometric morphometrics are based on landmarks (Adams et al. 2004). Landmarks are discrete, homologous anatomical locations that can be recognized as the same in all specimens under study (Zelditch et al. 2004). Each landmark is expressed as a set of two (e.g. X, Y) or three dimensional (e.g. X, Y, Z) coordinate positions. The complete set of landmarks chosen for an object describes its shape for the purpose of morphometric analysis.

In order to optimally represent shape, the three attributes that do not constitute shape, size, location and rotational effect, need to be identified and removed (Zelditch et al. 2004). Even if these variations are removed the shape of an organism is inherently multivariate – e.g., 20 landmarks in a two-dimensional system will still have 36 variables for each specimen.

Kendall's shape space addresses this problem by representing each shape as a point in multi-dimensional space (Zelditch et al. 2004). The mathematical foundation of this approach contributed to the development of General Procrustes Analysis (GPA) that removes translation and rotational variation (Adams et al. 2004) and lowers the number of dimensions associated with those attributes (Zelditch et al. 2004).

The resulting data can be further summarized by projecting the shape on a multi-dimensional plane that is tangent to its corresponding Kendall space (Adams et al. 2004; Zelditch et al. 2004). The information preserved in such a projection can be used to generate partial warp scores of landmarks and uniform component values (Adams et al. 2004). These measures of shape variation can be subject to multivariate analysis such as PCA. Figure 1 illustrates various

steps involved in this approach. Throughout these manipulations, information of shape is retained and the shape variation of any specimen can be visualized on a deformation grid.

In addition to the landmark method described above, there are several other methods that could be used to analyze geometric morphometric data. Outline methods were the first geometric morphometrics methods used, and they were based on digitized points along an outline. The sliding or semi-landmark method was proposed by Bookstein (1997). This procedure can capture outlines of structures and is also analyzed by GPA. The development of these methods has allowed addressing biological hypothesis (Adams et al. 2004).

MORPHOMETRICS AND PHYLOGENY

Phylogenies, or dendrograms of evolutionary relationships, are the basic structures necessary to visualize evolutionary relationships and differences between species and, to analyze those differences statistically. They have been around for over 140 years, but statistical, computational, and algorithmic work on phylogenies is barely 50 years old (Felsenstein 2004). The evolution of morphological traits might be tightly linked to the phylogeny of the group. Shape is one of the most important and easily measured elements of the phenotype, and shape expresses the interactions of many, if not most, genes. Thus, it is very important to test the phylogenetic dependence of traits to study the evolutionary relationships between traits and phylogeny (Ollier et al. 2006; Covain et al. 2008).

Despite the importance of phylogeny and morphometrics, one may find a lack of strong connection between systematics and morphometrics. Many systematists associate morphometrics with phenetics, though that is not correct (Bookstein 1994; Macleod 2002). Similarly, the morphometrics community has avoided taking phylogenetic pattern into consideration in their data analysis (Macleod 2002); however, this is changing.

GEOMETRIC MORPHOMETRIC DATA, PROBLEMS AND APPROACHES

The use of morphometric data in phylogenetic analyses has long been debated (Felsenstein 1988; Zelditch et al. 1995; Naylor 1996; Monteiro 2000; Polly 2001; Felsenstein 2002; Macleod 2002; Rohlf 2002; Lockwood et al. 2004; Cardini and Elton 2008; Gonzalez-Jose et al. 2008; Klingenberg and Gidaszewski 2010). The main disagreement is the question of whether or not morphometric data can be used to estimate phylogeny. Some of the proposed methods and major issues are briefly described below.

One of the major issues in the controversies for using geometric morphometrics to estimate phylogeny is the use of shape as a character or set of characters. There is no debate about the definition of character; however there are disagreements on how to use the multidimensional characters to obtain phylogenetic information, or how to explain what character states are and how the partitioning of these separate characters should be done (Bookstein 1994; Zelditch et al. 1995; Monteiro 2000; Klingenberg and Gidaszewski 2010). Phylogeneticists have seldom explicitly used continuous characters; however, many morphological phylogenies are implicitly based on morphometric characters (a short vs. long process on a bone, for example). This is due more to the lack of implementation than incompatibility. In fact, the first algorithm for character optimizations (Farris 1970) was described to work on continuous characters (Catalano et al. 2010). Cladistic procedures use discrete characters and can be interpreted separately (Adams et al. 2004).

One of the proposed solutions to combine morphometrics and phylogenetic analysis is to use shape as a cladistic character. One of the methods was to use partial warp scores from landmark data in this manner (Fink and Zelditch 1995; Zelditch et al. 1995; Klingenberg and Gidaszewski 2010). Zelditch et al. (1995) used partial warp scores to search for characters that

could be used in cladistic studies. They compared regressions of individual partial warps to obtain discrete characters and used linear (Wagner) parsimony to estimate a phylogeny. This approach is good as it treats shape variables as additional characters that can be combined with more conventional characters. The work of Zelditch et al. was criticized by Rohlf (1998) and Adams and Rosenberg (1998), who do not support using partial warps scores coded as separate characters because they are not independent and are not biologically meaningful data (Adams et al. 2004). According to Rohlf (1998), partial warp scores are influenced by the orientation of the reference. Zelditch et al. (1998) contends that partial warps are phylogenetically comparable by virtue of the homology of the landmarks. More recently, Zelditch et al. (2004) rejected partial warps as phylogenetic characters because although partial warps have spatial scales, an individual partial warp describes only part of the anatomical feature. Interpretation based on one variable violates the fundamental principles of geometric morphometric shape analysis – that results be invariant to the selection of variables. According to this view, a morphometric variable cannot be a character in its own right.

Another approach has been proposed by González-José et al. (2008). This approach subdivides the shape into smaller parts and derives shape variables as characters from them (Macleod 2002; Gonzalez-Jose et al. 2008). This approach, called the modular cladistic approach (MCL), could be used for modular development and evolution of complex phenotype where enough information is not available for phylogeny estimation. But this MCL approach has been challenged by Adams et al. (2011), who explained why MCL is not a reliable approach to address phylogenetic issues. According to Adams et al. there are several objections to the theoretical basis of MCL – the most important being its use of Manhattan distances, which makes the data sensitive to rotation. MCL has produced trees that were congruent with other

phylogenetic trees, but that does not prove that MCL reveals phylogenetic signal. Similar trees were generated using UPGMA methods. González-José et al. (2011) replies to Adams et al. (2011) that MCL should be tried rather than avoided. While accepting the fundamental objections presented by Adams et al. (2011) , Gonazalez-Jose et al. (2011) maintains that phylogenetic reconstruction should consider using MCL when modules are from all possible sources of approaches. MCL is not a complete solution; rather it is an improvement to the problem of using shape data for exploring phylogenetic relationships and phylogenetic reconstruction will benefit from the use of more realistic characters.

There are methods that incorporate geometric morphometric data in its original form into phylogeny, e.g., continuous maximum-likelihood (Felsenstein 1988, 2002), squared-change parsimony and neighbor joining methods. All of these methods can accommodate continuous data and do not depend on arbitrary rotations of the multivariate data space (Rohlf 2002; Adams et al. 2004). For example, squared-change parsimony minimizes the sum of squared distances in shape space between each node and the nodes to which it is connected by the branches of the phylogenetic tree. This is a very useful method as it estimates the ancestral shape in the phylogeny and readily integrates into the multivariate context of shape spaces (Adams et al. 2004). Square-changed parsimony is also widely used to determine if there is phylogenetic signal in shape data (Klingenberg and Gidaszewski 2010). These approaches, (i.e., squared change parsimony and continuous maximum likelihood), have been used to estimate phylogenetic signal in published literature where there is a well-supported phylogeny available.

Lockwood et al. (2004) used neighbor-joining and Fitch-Margoliash methods to study analyses of Procrustes distance for temporal bone shape and hominid phylogeny and found the results to be congruent with phylogeny. Several other works have found only partial congruence

or complete incongruence between phylogenetic trees obtained from simulated data and trees obtained from geometric morphometric data regardless of whether they were based on UPGMA clustering or maximum likelihood (Cardini and Elton 2008) or only maximum likelihood (Caumul and Polly 2005; Cardini and Elton 2008). These direct comparisons suggest that geometric morphometric data may not be a reliable indicator of phylogeny. Abundant homoplasy could be one of the reasons why morphometric traits often fail to estimate the correct phylogeny (Klingenberg and Gidaszewski 2010). Klingenberg and Gidaszewski (2010) suggested that even though there might be strong phylogenetic signal present in morphometric data, it might not be sufficient to reconstruct a phylogeny and a wide range of approaches should be used. They proposed the use of the permutation test and the use of consistency and retention indices to assess phylogenetic signals in morphometric data. There has been a strong trend in phylogenetic analyses to blame inconsistencies between morphological and molecular phylogenies on convergences in morphological data (Mooi and Gill 2008), but molecular datasets can also lead to inappropriate conclusions (Buhay 2009). Incongruence between morphometric phylogenies and traditional morphological phylogenies and molecular phylogenies is not a reason to reject outright the use of the morphometric phylogeny.

Catalano et al. (2010) discussed how to use geometric morphometric data directly to infer phylogeny. According to Catalano et al. (2010), a parsimony framework can accommodate certain types of geometric morphometric data well. Their approach is entirely equivalent to standard parsimony analysis which seeks the ancestral landmark configurations that minimize point displacements between ancestral/descendant nodes along all branches of the tree. The method is employed using the program TNT (Tree Analysis Using New Technology) (Goloboff

et al. 2008) which uses continuous data in phylogenetic analysis, and the methods are still under development (Catalano et al. 2010).

EXAMPLE: NORTH AMERICAN CYPRINIDAE DATA SET

The phylogenetics of the genera of the North American minnows, dace, chubs, and shiners of the family Cyprinidae (subfamily Leuciscinae) has been well-studied using morphological (Mayden 1989; Coburn and Cavender 1992) and molecular methods (Simons et al. 2003; Bufalino and Mayden 2010b, c, a). The results of analyses with morphology, nuclear genes, and mitochondrial genes are largely congruent; meaning the phylogeny of the Cyprinids is fairly well established. We developed a landmark-based database of representatives of Cyprinidae from each of the major clades using specimens at the Auburn University Museum Fish Collection (AUM) in order to test methods of phylogenetic construction based on geometric morphometrics.

It is possible to map the history of a clade's morphological diversification and understand the direction and magnitude of shape change along any branch of a phylogeny, by projecting the phylogeny into multivariate morphospace (Sidlauskas and Vari 2008). The main purpose of this study was to test whether there is phylogenetic signal present in geometric morphometric data, which is done by mapping morphometric data onto a phylogeny for the Cyprinidae. We also wanted to test whether geometric morphometric data could be used to infer phylogeny using UPGMA and TNT. We developed a set of 18 homologous landmarks for 30 genera, 40 species, and 352 specimens (usually at least 5-10 per species).

Materials and Methods

GEOMETRIC MORPHOMETRIC DATASET

A total of 352 specimens in 40 species in 30 genera were studied for the geometric morphometric analysis. We located eighteen landmarks in tpsdig2 (Figure 2) in digital photographs of the lateral view. Landmark configurations were subjected to Procrustes superimposition in MorphoJ v.1.02h (Klingenberg 2011), and a principal components analysis was performed on the covariance matrix. We analyzed patterns of body shape variation using multivariate analysis of variance (MANOVA) with genus. For all geometric morphometric analyses, group mean was used for each of the 30 genera. Phenetic relationship was analyzed using UPGMA cluster algorithm on the matrix of mean shape Procrustes distance. Data analysis was done using MorphoJ (Klingenberg 2011) programs of the tps series (Rohlf 2010) and NTSYSpc 2.2 (Rohlf 2007) software. In addition, the average coordinate data per genus were loaded into TNT and the Landsch script was used to run 100 replicates of a phylogenetic analysis of shape. Phylogenetic analyses in TNT were done on the entire dataset as well as on each clade with three to four members with *Acrocheilus* chosen as the outgroup for all clades except for the western clade which used *Semotilus* as the outgroup.

PHYLOGENETIC DATASET

This study used the phylogeny from Bufalino and Mayden (2010c) on the phylogenetics of North American Cyprinidae. The dataset includes 90 taxa of North American Cyprinidae, Leuciscinae (Appendix 2) and a combined dataset for nuclear DNA sequences from the RAG1 (exon 3) and S7 (intron 1) gene regions and mitochondrial 12S and 16S genes. The combined analysis resolves three major, well-supported lineages of North American Cyprinidae: western, creek chub-plagopterin (CC-P), and open posterior myodome (OPM) clades. For our analysis, the OPM clade was broken into nine smaller clades (Table 1). Fifty of the 90 species present in the phylogenies are not represented in the morphometric dataset. These 50 taxa were pruned

from the original tree, and a tree was constructed in MacClade 4.08 (Maddison and Maddison 2005) (Figure 3), transferred to PAUP* 4.0 b (Swofford 2002) and saved with the branch lengths. This tree was used in MorphoJ for other analyses. A morphometric tree was constructed using the TNT program (Goloboff et al. 2008).

Results

GEOMETRIC MORPHOMETRICS

In the PCA, representation of total shape variation, the first five principal components accounted for 24.8%, 16.8%, 12.8%, 11.7%, 11.4% variation among the taxa means. Therefore, plots (Figure 4) of these five principal components show 77.5 % of the total variation among taxa means in two dimensions. The wireframes in Figure 5 visualize the shape change on each of these axes. The first principal component primarily described variation in size and depth of the body, position of the dorsal fin and eye size. Specimens on the left side (negative scores) of this axis have a more elongated, compressed and thin body, larger and elongated head with the position of the dorsal fin slightly behind or opposite the pelvic fin insertion as in *Ptychocheilus*. The specimens on the positive end of this axis have smaller eyes and mouth with a ventral opening as in *Phenacobius*. PC2 appears to describe subtle variation in the position of the mouth, eye diameter, depth of the body, and position of the dorsal-fin origin. On the positive end of this axis are specimens with small eyes and at the negative extreme are specimens with a very deep body and terminal mouth and shorter caudal peduncle as in *Luxilus*. PC3 shows variation toward a deep body, upward mouth, short caudal peduncle and curvature of the body. PC4 describes variation in the anteroposterior elongation of the head, very thin and slender body and a larger eye. This finding is consistent with Coburn (1992) in that members of the OPM clade have larger eyes. The remaining components each summarize 5.7% or less of total variance. We found

significant phenotypic differentiation in body shape among genera (Wilk's $\lambda = 0.000000$, $F = 11.20$, $p < 0.0001$).

Morphometric data have been associated with phenetics and distance methods (Macleod 2002; De Bivort et al. 2010). A SAHN cluster analysis was done to construct a tree using UPGMA (Figure 6). The method successfully recovered some small clades; for example, *Campostoma* + *Nocomis* (*Campostoma* clade), *Rhinichthys* + *Tiaroga* (*Exoglossum* clade), and *Erimystax* + *Phenacobius* (*Phenacobius* clade). The phenogram shows that *Chrosomus* and *Agosia* (western clade) cluster with *Hemitremia, Margariscus*, and *Semotilus* (Creek Chub clade), which is similar to relationships based on the phylogenetic tree of Buffalino and Mayden (2010) (Figure 3). According to Coburn and Cavendar (1992), *Acrocheilus* is one of the basal taxa of the western clade. In our UPGMA tree, *Acrocheilus* was sister to *Meda* which is a member of the Creek Chub clade. *Agosia* was sister to *Hybopsis* which is consistent with the result of Mayden (1989) but is not congruent with the finding of Simons et al. (2003) or Coburn and Cavendar (1992). However, *Cyprinella* is sister to the notropin clade members which is consistent with Simons et al. (2003). The Creek Chub clade is more resolved compared with other basal clades such as the western clade. All other taxa are in the OPM clade.

PHYLOGENETIC SIGNAL IN MORPHOMETRIC DATA

The permutation test confirmed the hypothesis that there was phylogenetic structure in the data. For the combined tree topologies (tree length: 5759, Consistency Index: 0.493 and Retention Index 0.557) and for weighted squared change parsimony, the test found a phylogenetic signal that was statistically significant (Tree length: 0.0467, P value <0.0001). The permutation test is the first indication of the phylogenetic structure in the data. The null hypothesis of the test is the total absence of any phylogenetic signal. The rejection of the null

hypothesis implies that there is some degree of phylogenetic structure to the data (Klingenberg and Gidaszewski 2010). Thus, phylogeny was taken into account to test whether landmark data could infer phylogeny. For our study, the tree (Figure 3) using molecular data was graphed on a plot of the first two principal components (Figure 7). This plot suggests why geometric morphometrics may not be useful in reconstructing phylogeny for this group as it is clear that there is a lot of convergence in morphology.

TNT RESULTS

Given the results in Figure 7, it should not be surprising that shape was not able to recover the established phylogeny (Figure 8). The resultant phylogeny has almost no similarity to the established phylogeny (Figure 3). This is likely a result of shape evolving very quickly, and convergence in shape between species that share similar ecological conditions such as stream flow. It would be expected that shape could more accurately assess phylogeny of smaller groups of more closely related species; however, we found that shape only produced the established phylogeny in two of nine clades of three or four taxa (22.2%; *Agosia chrysogaster* + *Notropis harperi* + *Pteronotropis euryzonus* and *Pimephales* spp. + *Opsopoeodus emiliae* + *Erimonax monachus).*

Discussion

The present study tested whether or not geometric morphometric data could be used in the phylogenetic analysis of North American Cyprinidae. Our aim was not to find a method to use the GM data in phylogeny, but to test some of the methods proposed by other researchers and check the result with the established phylogeny. We used squared-change parsimony to map geometric morphometric data on phylogenetic trees derived from other sources and used

MorphoJ (Klingeneberg 2010) program. (Sidlauskas and Vari 2008; Astua 2009; Klingenberg and Gidaszewski 2010; Strauss 2010; Goloboff and Catalano 2011). We have also used the new permutation test proposed by Klingenberg and Gidaszewsky (2010) to test the presence or absence of phylogenetic signal in the morphometric data.

As Klingenberg and Gidaszewski (2010) stated, the presence of phylogenetic signal in a dataset does not mean a correct phylogeny can be constructed from the data. In our analysis of the Cyprinidae, we found that clustering algorithms and phylogenetic analysis of shape failed to recover most of the established phylogeny. Although some studies were successful at recovering a phylogeny from geometric morphometric data (David and Laurin 1996; Lockwood et al. 2004; Gonzalez-Jose et al. 2008), these are mostly small studies with few taxa. Once more complexity has been added, as in this analysis, GM data are not successful in recovering phylogeny because of multiple instances of convergence. When we looked at smaller clades, the established phylogeny was only recovered 22.2% of the time (two out of nine). With such a low percentage of success, GM data is not a good method for elucidating phylogeny; however, despite this, the data shows clear phylogenetic signal suggesting that shape is evolving in a phylogenetic manner. Many of the clades in Figure 8 are clustered in the analysis. So, if shape cannot elucidate phylogeny, what is the purpose of understanding shape phylogenetically?

Shape is important for animals as use of space is directly related to the functional morphology of organisms. One example is lateral compression; deep-body fish can swim better in the water column than at the surface of the water (Winemiller 1991). Patterns of morphological evolution are complex. Felsenstein (1985) assumed that evolution of the continuous characters could be modeled by a covarying Brownian motion on a scale proportional to the molecular branch lengths. A clade's morphological diversity correlates with the span of its

evolution (Felsenstein 1985; Collar et al. 2005; Sidlauskas 2007) with older clades likely to have more morphological diversity. The morphological evolution of the Cyprinidae is extremely interesting. Cyprinids have a great range of trophic morphologies, breeding behaviors and habitat preferences. Cyprinids have exploited many types of highly mobile and predatory methods of food capturing which includes benthic insectivores, drift feeders, piscivores and also filter feeders, and some are algae and biofilm scrapers (Cavender and Coburn 1992). If one examines the pattern found in Figure 7, the basal members (everything except the shiner clade, in blue) occupy almost the entire range of shape in the entire dataset. What this suggests is that the pattern of evolution of Cyprinids is for initial phenetic differentiation (the range of shape was explored early in the clade) followed by phenetic packing (the shiner clade simply filled in the gaps among the basal taxa and converged upon similar morphologies). This suggests limits to the Cyprinid body plan and perhaps increased competition once the shiners arose. The shiner clade has more species than all other clades in our analysis and occupy otherwise empty spaces in the morphospace which is consistent with (Winemiller 1991) findings where they studied relationships among species diversity, community structure and convergent evolution among divergent fish faunas. In this study they had Cyprinidae along with several other fish groups and concluded that ecomorphological divergence is prerequisite for ecomorphological convergence to happen (Winemiller 1991). In this study the basal clades of the Cyprinidae first had diverged and spread in the morphospace.

We used three methods: SAHN clustering algorithm, squared change parsimony method implemented in MorphoJ, and parsimony implemented in TNT software. Squared change parsimony, in the context of landmark data of two shapes x and y, is based on the cost function $f(x,y)$ which is the squared Procrustes distance between shapes x and y (Klingenberg 2010).

Catalano et al. (2010) criticizes the squared-change parsimony approach as it implies widespread homoplasy on the cladogram. According to Catalano et al. (2010), since shape is projected on a tangent space (from which we get the Procrustes distance), one cannot look for displacement (and hence, parsimony) between two multidimensional shape variables when change is computed from the difference of projections along each axis in the tangent space; the tangent space itself is an optimization of the landmark dataset.

Clouse et al. (2011) questions the way landmark data is used in TNT program to elucidate phylogeny. They criticized Macleod's (Macleod 2002) cartoon fish model used by TNT and questions its implication to assess the true phylogenetic tree. Re-analyzing Naylor's (Naylor 1996) data, they hypothesized that it was not the matter in which the data were used, but rather the study used the wrong algorithm, and there were lots of problems in the dataset. Though they used TNT for several analyses, they concluded that, morphometric data probably could not be used to determine phylogeny. Many biological questions, like the evolution of shape, may not be completely resolved by geometric morphometric methods. With our analysis and results, we agree with other authors about the findings that morphometric data contain useful phylogenetic signal, but that geometric morphometrics will generally fail to generate a robust phylogeny (Gonzalez-Jose et al. 2008; De Bivort et al. 2010; Klingenberg and Gidaszewski 2010).

We believe that the study of shape change phylogenetically is extremely important and interesting, but that the strength of geometric morphometrics is in analyzing shape on an established phylogeny and not to generate phylogenetic hypotheses.

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Table 1. List of the genera used in the current analysis and their grouping in different clades.

Clade Name	List of Genus
Western Clade	*Acrocheilus, Chrosomus, Ptychocheilus*
Creek Chub clade	*Hemitremia, Semotilus, Meda, Margariscus*
Open Posterior Myodome (OPM) clade	The following 6 clades
Exglossum clade	*Exglossum, Tiaroga, Rhinichthys*
Mylocheilus clade	*Mylocheilus, Clinostomus*
Campostoma clade	*Campostoma, Nocomis*
Phenacobius clade	*Erimystax, Phenacobius*
Platygobio clade	*Macrohybopsis*
Shiner clade	*Agosia, Erimonax, Notropis, Ericymba, Hybognathus, Luxilus, Lythrurus, Hybopsis, Opsopoeodus, Pimephales, Cyprinella, Pteronotropis.*

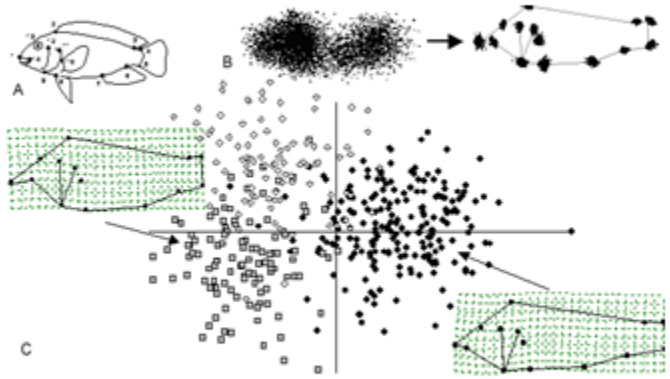


Figure 1. Graphical representation of the four-step morphometric protocol. A: quantify raw data (landmarks recorded on the bodies of cichlid fishes), B: remove non-shape variation (landmarks of 412 specimen before and after GPA), C: statistical analysis (Canonical Variates Analysis) and graphical representation of results. Deformation grids are for mean specimens for (right) *Eretmodus cyanostictus* and (left) *Spathodus erythrodon* (Adams et al., 2004).

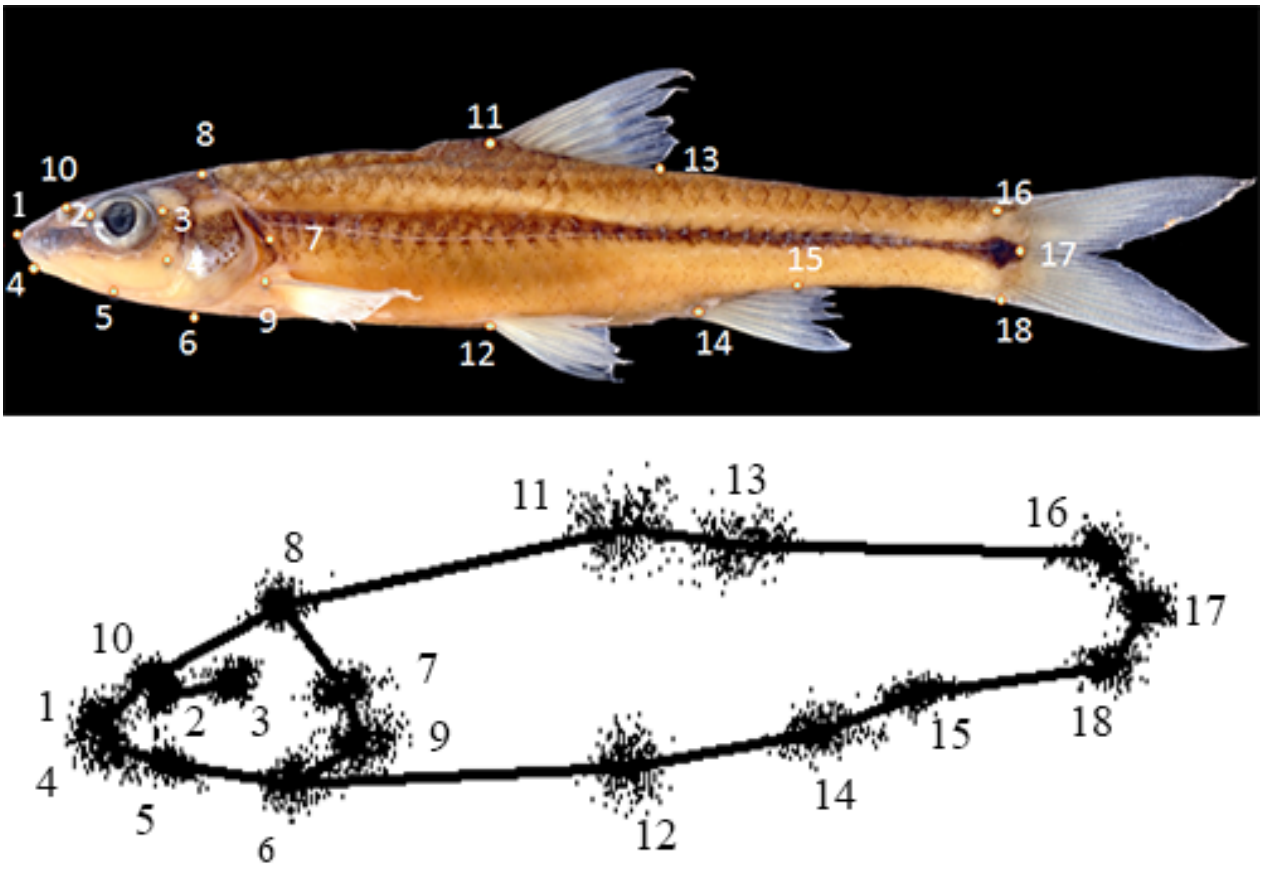


Figure 2. Landmark configuration. a) *Hybopsis lineapunctata*, showing positions of 18 landmarks used in geometric morphometric analysis. b). Scatterplot of all 18 landmark configurations after Procrustes superimposition. The diagram represents landmarks linked for better visualization, as used in some of the graphs. Landmarks represent: (1) tip of snout, (2) right orbit, anterior limit, (3) right orbit, posterior limit, (4) opening of mouth (5) posterior end of jaw, (6) intersection of gill opening and ventral margin of body, (7) posterior edge of opercle, (8) supraoccipital, posteromedial tip (visible as an indentation in dorsal surface), (9) insertion of pectoral fin, (10) posterior nare, posterior margin, (11) anterior origin of dorsal fin, (12) anterior insertion of pelvic fin, (13) posterior insertion of dorsal fin, (14) anterior insertion of anal fin, (15) posterior insertion of anal fin, (16) insertion of anterior dorsal procurrent caudal-fin ray, (17) end of vertebral column, (18) insertion of anterior ventral procurrent caudal-fin ray.

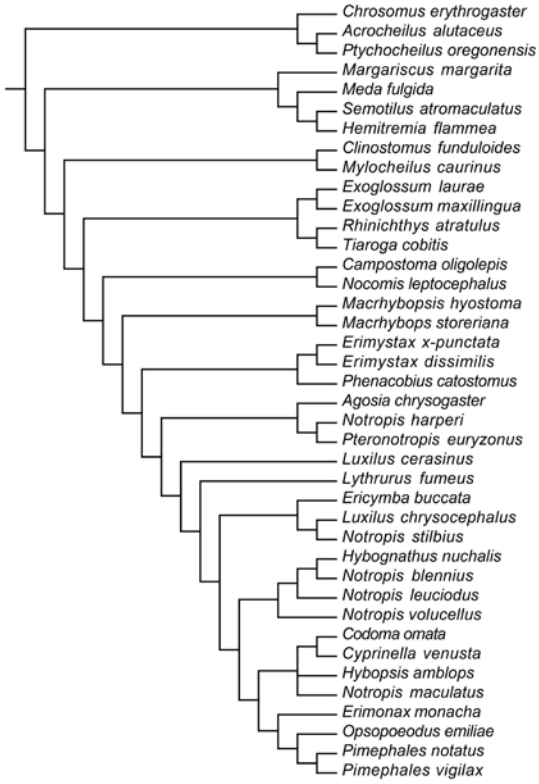
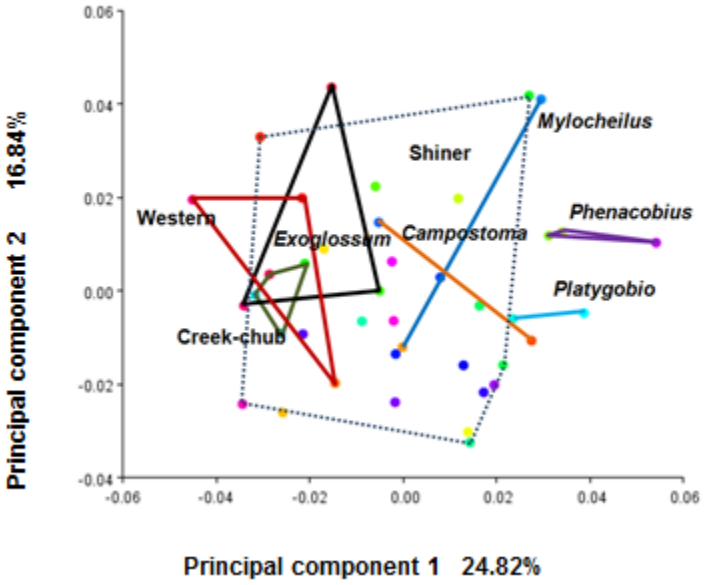


Figure 3. Phylogenetic Tree from Bufalino and Mayden (2010) using 40 taxa.



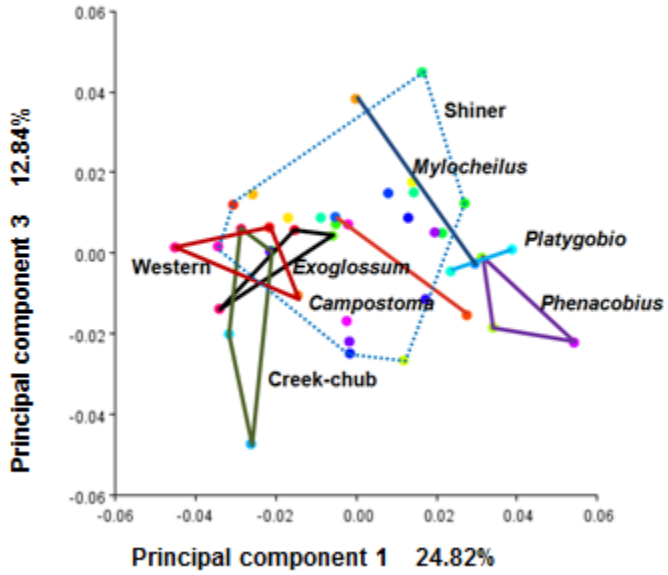


Figure 4. Results from principal components analysis. a) Scatterplot of principal components 1 and 2 from geometric morphometric analysis. Minimum polygons connect taxa means for defined clades and clades with only two members joined by a line. b) Principal components 1 and 3 from geometric morphometric analysis. Minimum polygons connect taxa means for defined clades and clades with only two members joined by a line.

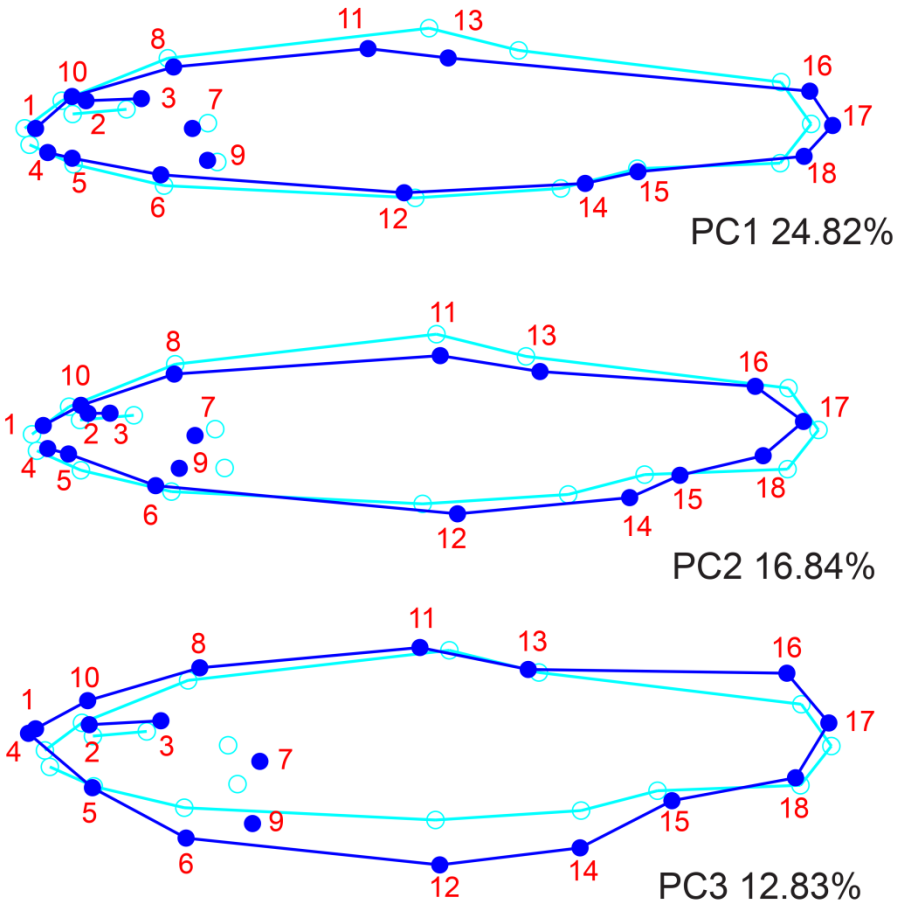


Figure 5. Wireframe visualization of variation among principal components 1, 2 and 3 of the geometric morphometric analysis. Light blue landmarks represent the configuration of the average specimen; dark blue landmarks represent one approximate extreme of variation on that axis. Percentages indicate the proportion of total explained by PC 1 and PC2.

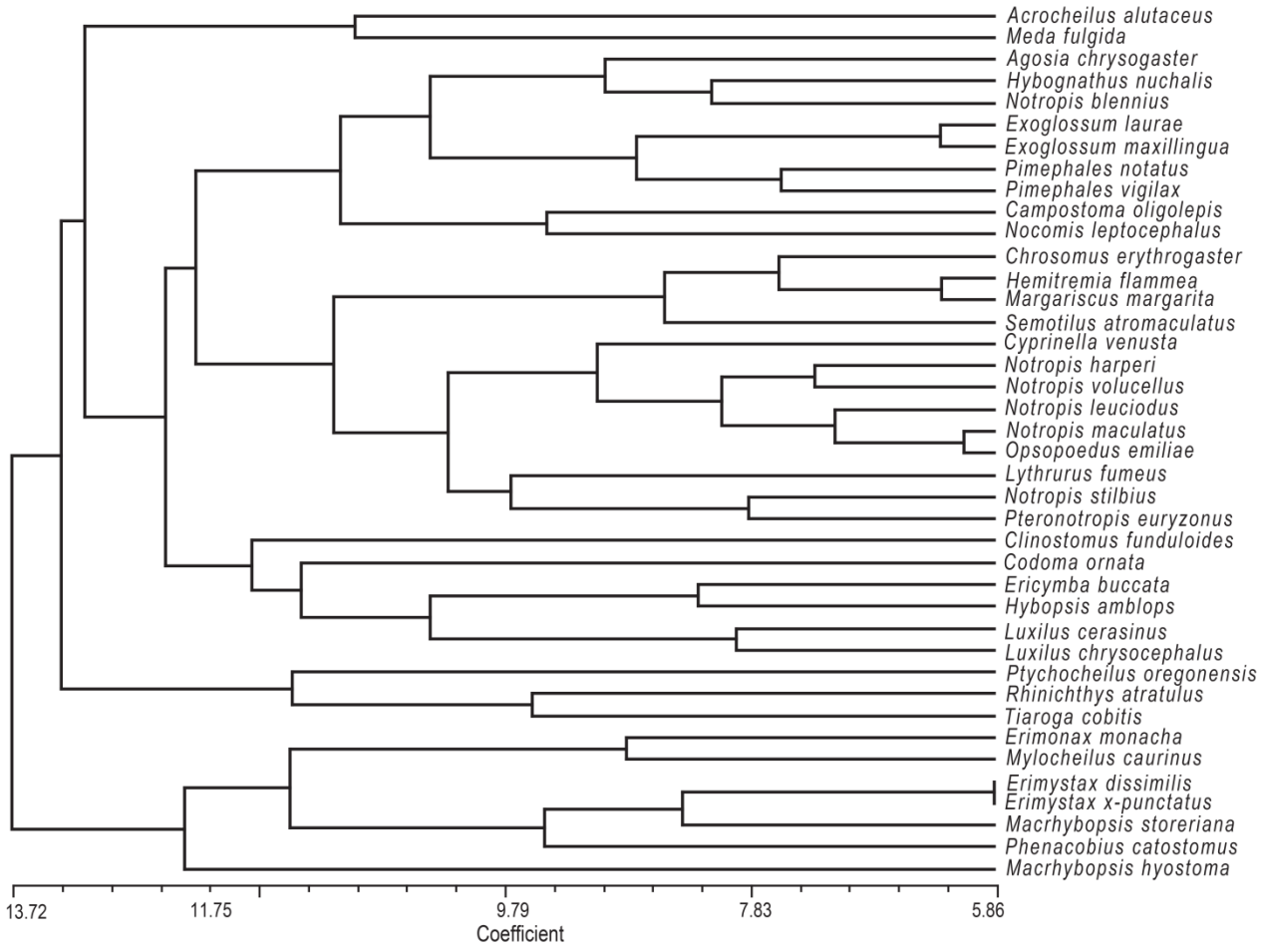


Figure 6. UPGMA phenogram showing clustering of 40 Cyprinidae taxa. Colored boxes represent some of the clades found to be congruent with the molecular phylogenetic analysis.

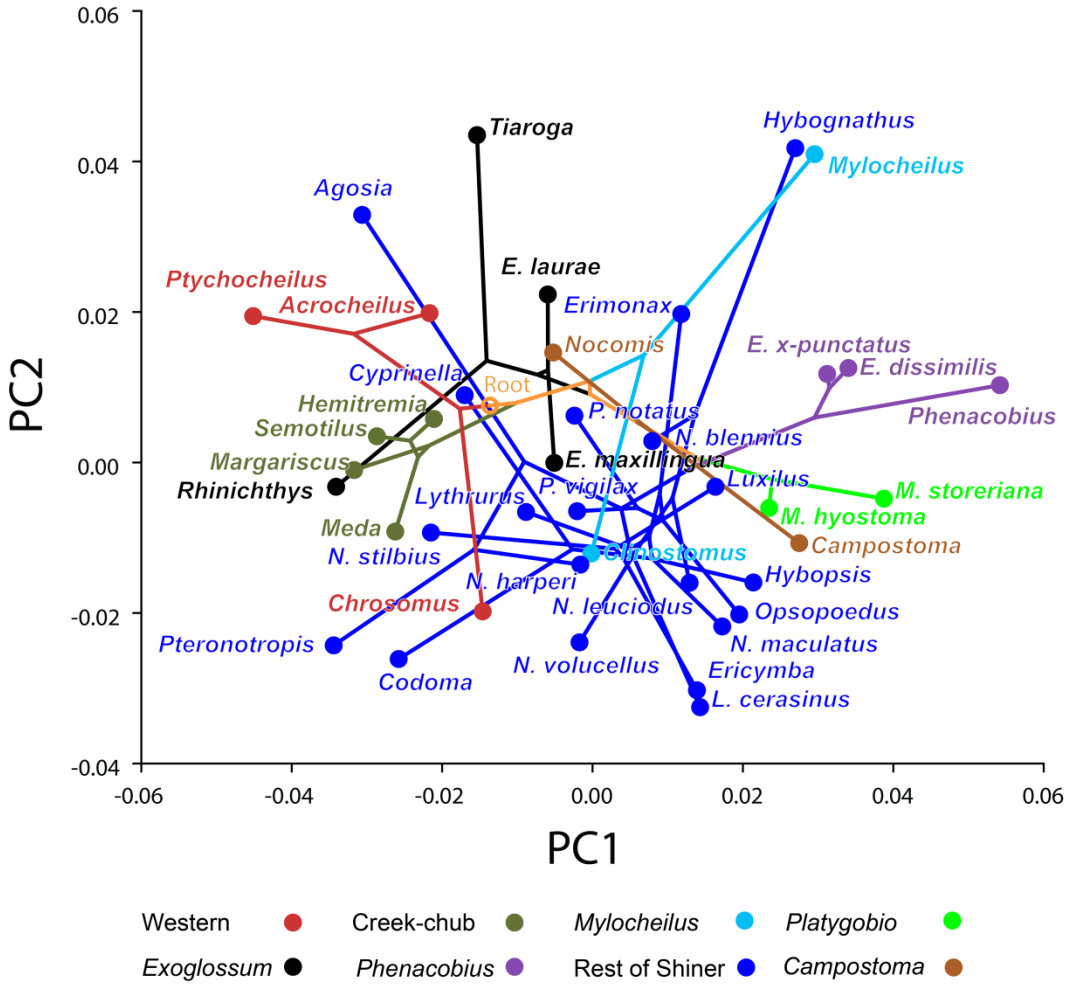


Figure 7. Reconstruction of evolutionary shape change for NA Cyprinidae clades. The phylogenetic tree has been superimposed onto a plot of the first 2 principal components of the covariance matrix among taxa means. The tips of the terminal branches are at the locations of taxa means. The positions of the internal nodes were reconstructed by squared-change parsimony. The backbone of the phylogeny and root are colored orange, and the remainder of the tree is colored by clade.

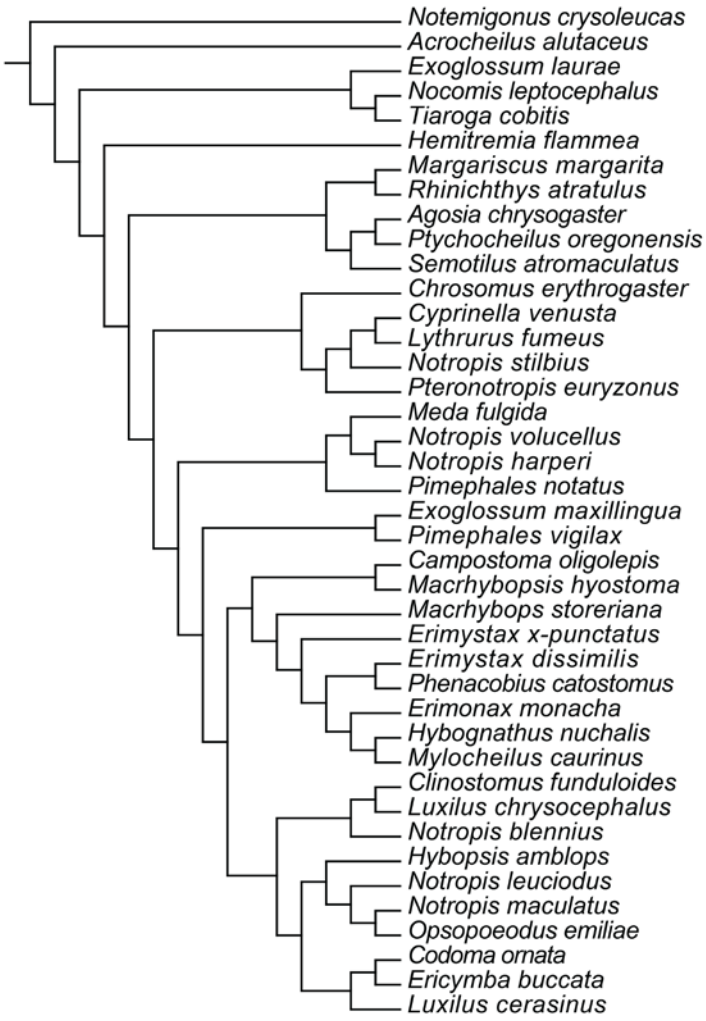


Figure 8. Resulting tree from the phylogenetic analysis in TNT.

Appendix 1. Taxonomic, voucher and sequence data for 75 specimens (70 *Mystus*, 5 outgroup) used in this study. Serial numbers are shown for taxa with more than one specimen. These serial numbers correspond to the numbers used in Figures 1-4 in Chapter 2.

Species	Tag No.	Serial No.	Museum Catalog Number	Locality
Mystus alasensis	MsB83B88	1	USNM 406510	Indonesia
Mystus armatus	Mm247452	1	UMMZ 247452	India, Kerala
Mystus atrifasciatus	Mi255	1	AUM 55361	Thailand
Mystus atrifasciatus	Mi315	2	AUM 55369	Thailand
Mystus atrifasciatus	Mn253	3	AUM 55368	Thailand
Mystus bimaculatus	M506	1	Aquarium	Srilanka
Mystus bimaculatus	Mb247115	2	UMMZ 247115	Indonesia (aquarium)
Mystus bleekeri	Mpng582	1	AUM 50374	Bangladesh
Mystus bleekeri	Mpn571	2	AUM 50518	Bangladesh
Mystus bleekeri	Mpng581	3	AUM 50374	Bangladesh
Mystus bocourti	mbocu286	1	AUM 55360	Thailand
Mystus castaneous	Mu162185	1	UF 162185	Indonesia
Mystus castaneous	Mc243272	2	UMMZ 243272	Indonesia, Sumatra
Mystus cavasius	Mcav1	1	AUM 55396	Bangladesh
Mystus cavasius	Mcav2	2	AUM 55396	Bangladesh
Mystus cavasius	Me	3	AUM 55305	Bangladesh
Mystus falcarius	Mf17101	1	DAN 171.10	Myanmar
Mystus gulio	Mg161937	1	UF 161937	Indonesia
Mystus gulio	Mge67	2	USNM 393749	Indonesia
Mystus gulio	MA27	3	USNM 406511	Indonesia
Mystus gulio	Mgsf0160	4	AUM 55325	Bangladesh, Rupsha Dr.
Mystus gulio	MgB1	5	AUM 55325	Bangladesh, Rupsha Dr.
Mystus gulio	Mg266	6	AUM 55325	Bangladesh
Mystus gulio	MgB2	7	AUM 55325	Bangladesh, Rupsha Dr.
Mystus gulio	MgB4	8	AUM 55325	Bangladesh, Rupsha Dr.
Mystus gulio	Mg247580	9	UMMZ 247580	India, West Bengal
Mystus gulio	MgB3	10	AUM 55325	Bangladesh, Rupsha Dr.
Mystus gulio	mg265	11	AUM 55325	Bangladesh
Mystus gulio	Mgsf0159	12	AUM 55325	Bangladesh
Mystus gulio	mg267	13	AUM 55325	Bangladesh
Mystus malabaricus	Ma246939	1	UMMZ 246939	India, Kerala
Mystus malabaricus	Ma247450	2	UMMZ 247450	India, Kerala
Mystus montanus	Mo247113	1	UMMZ 247113	India, Kerala
Mystus multiradiatus	Mr172615	1	UF 172615	Thailand
Mystus multiradiatus	Mr172613	2	UF 172613	Thailand
Mystus mysticetus	my172616	1	UF 172616	Thailand
Mystus mysticetus	my288	2	AUM 55359	Thailand

Appendix 1. Continued

Species	Tag No.	Serial No.	Museum Catalog Number	Locality
Mystus pulcher	ml172657	1	UF 172657	Thailand
Mystus pulcher	M247114	2	UMMZ 247114	Myanmar
Mystus singaringan	Mn162184	1	UF 162184	Indonesia
Mystus singaringan	Mnh70	2	USNM 393991	Indonesia
Mystus singaringan	msingi61	3	USNM 393937	Indonesia
Mystus singaringan	mn161483	4	UF 161483	Indonesia
Mystus singaringan	Mn312	5	AUM 55368	Thailand
Mystus singaringan	Mn313	6	AUM 55368	Thailand
Mystus singaringan	msing257	7	AUM 55362	Thailand
Mystus singaringan	mn172603	8	UF 172603	Thailand
Mystus singaringan	msing291	9	AUM 55362	Thailand
Mystus sp.	MspE37	1	USNM 393626	Indonesia
Mystus sp.	M516	2	Aquarium	Srilanka
Mystus tengara	Msf0165	1	AUM 55326	Bangladesh
Mystus tengara	Mstf0164	2	AUM 55326	Bangladesh
Mystus tengara	Mpng583	3	AUM 50375	Bangladesh
Mystus tengara	Msf0166	4	AUM 55326	Bangladesh
Mystus tengara	Mpng507	5	AUM 50375	Bangladesh
Mystus tengara	Mstf0161	6	AUM 55326	Bangladesh
Mystus tengara	Mntf0162	7	AUM 55326	Bangladesh
Mystus tengara	Pn560	8	AUM 50571	Bangladesh
Mystus tengara	Mpng504	9	AUM 50564	Bangladesh
Mystus tengara	Mpng508	10	AUM 50564	Bangladesh
Mystus tengara	Mpng503	11	AUM 50564	Bangladesh
Mystus tengara	Pn575	12	AUM 50517	Bangladesh
Mystus tengara	Mpng585	13	AUM 50375	Bangladesh
Mystus tengara	Mv246937	14	UMMZ 246937	India, Aquarium
Mystus vittatus	Mvsf517	1	Aquarium	Srilanka
Mystus vittatus	M518	2	Aquarium	Srilanka
Mystus vittatus	sf521	3	Aquarium	Srilanka
Mystus vittatus	Mvsf523	4	Aquarium	Srilanka
Mystus vittatus	mu514	5	Aquarium	Srilanka
Mystus wolffii	Mw161919	1	UF 161919	Indonesia

Appendix 1. Continued (Outgroups)

Species	**Tag No.**	**Serial No.**	**Museum Catalog Number**	**Locality**
Hemibagrus guttatus	AF416886	1	Genbank AF416886	China
Hemibagrus guttatus	EU439467	2	Genbank EU439467	China
Hemibagrus macropterus	AF416890	1	Genbank AF416890	China
Pelteobagrus nitidis	Pelnitid	1	Genbank AY912343	China
Pseudobagrus	Pseduobg	1	Genbank DQ321754.1	South Korea

Appendix 2. List of Specimens used in Geometric Morphometric analysis in Chapter 3.

Species	Museum Catalogue Number	Number of Individuals
Mystus albolineatus	ANSP 178849	1
Mystus albolineatus	AM 43487	1
Mystus albolineatus	INHS 93696	3
Mystus albolineatus	NRM 51030	2
Mystus armatus	AM 7573	1
Mystus atrifasciatus	UMMZ 214306	18
Mystus bleekeri	UMMZ 208768	20
Mystus bleekeri	UMMZ 208565	8
Mystus bleekeri	USNM 165113	2
Mystus bleekeri	USNM 274810	1
Mystus bocourti	AM 43486	1
Mystus bocourti	AM 43488	1
Mystus bocourti	INHS 93586	2
Mystus bocourti	UMMZ 2326966	5
Mystus carcio	UMMZ 208540	9
Mystus castaneus	UF 160963	2
Mystus castaneus	UF 162183	2
Mystus castaneus	UF 162185	12
Mystus cavasius	AUM 55327	2
Mystus cavasius	KU 12159	1
Mystus cavasius	KU 27891	1
Mystus cavasius	KU 28578	5
Mystus cavasius	NRM 15065	1
Mystus cavasius	NRM 24976	1
Mystus cavasius	UF 79593	1
Mystus cavasius	UMMZ 186739	19
Mystus cavasius	USNM 44748	1
Mystus cavasius	USNM 44753	1
Mystus cavasius	USNM 44981	1
Mystus cavasius	USNM 101256	1
Mystus cavasius	USNM 133099	3
Mystus cavasius	USNM 165124	2
Mystus cavasius	USNM 297277	4
Mystus cavasius	USNM 343550	1
Mystus cavasius	USNM 343638	2
Mystus cavasius	USNM 385266	3
Mystus cavasius	USNM 385267	1

Appendix 2. Continued

Species	Museum Catalogue Number	Number of Individuals
Mystus gulio	ANSP 59410	2
Mystus gulio	ANSP 60774	2
Mystus gulio	ANSP 89426	5
Mystus gulio	ANSP 89526	2
Mystus gulio	AUM 55325	7
Mystus gulio	CAS 55555	7
Mystus gulio	CAS 88628	3
Mystus gulio	SU 34856	5
Mystus gulio	UF 161397	4
Mystus gulio	UF 161550	1
Mystus gulio	UMMZ 186724	6
Mystus gulio	UMMZ 227497	10
Mystus gulio	USNM 44982	1
Mystus gulio	USNM 103175	3
Mystus gulio	USNM 149732	2
Mystus gulio	USNM 297155	1
Mystus gulio	USNM 298239	1
Mystus gulio	USNM 317573	3
Mystus gulio	USNM 317574	4
Mystus gulio	USNM 317575	1
Mystus gulio	USNM 317606	4
Mystus gulio	USNM 343552	4
Mystus gulio	USNM 372514	4
Mystus gulio	USNM 393629	7
Mystus gulio	USNM 393749	1
Mystus malabaricus	NRM 12057	1
Mystus malabaricus	NRM 12116	1
Mystus malabaricus	NRM 12146	3
Mystus malabaricus	NRM 12246	2
Mystus micracanthus	ANSP 60387	2
Mystus micracanthus	INHS 93518	4
Mystus multiradiatus	AM 43485	2
Mystus multiradiatus	AM 43486	1
Mystus multiradiatus	AM 43487	1
Mystus multiradiatus	UMMZ 232653	3
Mystus mysticetus	INHS 93697	2
Mystus mysticetus	UMMZ 186780	19
Mystus mysticetus	UMMZ 232633	5

Appendix 2. Continued

Species	Museum Catalogue Number	Number of Individuals
Mystus nigriceps	ANSP 20351	1
Mystus nigriceps	USNM 109576	1
Mystus nigriceps	USNM 230283	3
Mystus oculatus	NRM 12192	1
Mystus oculatus	NRM 12200	1
Mystus pulcher	USNM 385262	1
Mystus pulcher	USNM 385265	1
Mystus rhegma	UMMZ 186741	19
Mystus rufescens	USNM 346162	1
Mystus rufescens	USNM 372510	1
Mystus rufescens	USNM 385269	1
Mystus singaringan	INHS 93723	3
Mystus singaringan	UF 161463	10
Mystus singaringan	UF 161464	39
Mystus singaringan	UF 161483	1
Mystus singaringan	UF 161484	1
Mystus singaringan	UF 161554	13
Mystus singaringan	UF 162184	3
Mystus singaringan	UF 162464-71	1
Mystus singaringan	UMMZ 240635	10
Mystus singaringan	USNM 297274	9
Mystus singaringan	USNM 393937	1
Mystus singaringan	USNM 393963	2
Mystus tengara	AUM 55326	23
Mystus tengara	AUM 55394	1
Mystus tengara	AUM 55395	8
Mystus tengara	KU 12170	5
Mystus tengara	NRM 40303	2
Mystus tengara	NRM 40480	1
Mystus tengara	NRM 40492	1
Mystus vittatus	KU 28575	6
Mystus vittatus	KU 29576	1
Mystus vittatus	NRM 13700	1
Mystus vittatus	NRM 14500	1
Mystus vittatus	NRM 14558	2
Mystus vittatus	USNM 103198	1
Mystus vittatus	USNM 109571	3
Mystus vittatus	USNM 109572	1

Appendix 2. Continued

Species	Museum Catalogue Number	Number of Individuals
Mystus vittatus	USNM 109573	1
Mystus vittatus	USNM 118448	2
Mystus vittatus	USNM 165048	2
Mystus vittatus	USNM 317614	2
Mystus vittatus	USNM 317615	2
Mystus vittatus	USNM 317616	1
Mystus vittatus	USNM 317618	2
Mystus vittatus	USNM 317619	3
Mystus wolffii	ANSP 59426	1
Mystus wolffii	ANSP 59428	1
Mystus wolffii	ANSP 60779	3
Mystus wolffii	ANSP 61546	1
Mystus wolffii	ANSP 89526	6
Mystus wolffii	AM 43747	1
Mystus wolffii	NMW 92046	5
Mystus wolffii	UF 161919	1
Mystus wolffii	USNM 103173	1
Mystus wolffii	USNM 109584	1
Mystus wolfiii	ANSP 89427	2

Appendix 3. Fifty percent majority rule consensus tree from a total of 42 most parsimonious trees found in the MP analysis (A) and most likely tree from the Bayesian inference (BI) analysis (B) of the combined mt12S, 16S, S7 and RAG1 data sets used in Chapter 5. Bootstrap and partitioned Bremer support values, for selected branches, are provided for the MP analysis (A). Branches on the BI phylogram marked with an asterisk indicate posterior probability values ≥ 0.95 and ML bootstrap values are provided for branches shared between the BI and ML phylograms (B) copied from the original publication (Bufalino and Mayden, 2010b).