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Cytochrome b gene sequence divergence of seven sisorid species of catfish genus *Glyptothorax* (siluriformes, sisoridae) from India

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Abstract Samples of seven Sisorid catfish species of the genus *Glyptothorax* (*G. garhwali*, *G. dakpathari*, *G. brevipinnis*, *G. ngapang*, *G. granulus*, *G. ventrolineatus,* and *G. davissinghi*) were collected from the Himalayan region and the Western Ghats of India. They were analyzed for the mitochondrial cytochrome *b* gene (*Cyt b*). Out of 1152 nucleotide positions analyzed, 269 (23.3%) were found to be variable and 235 (20.3%) were parsimoniously informative. The sequences showed 111 (9.6%) fourfold degenerate sites. The overall transition/transversion bias was $R = 3.457$. The average proportion of base substitutions measured as *P*-distance for all sequences of seven *Glyptothorax* species and other five species comprising four comparison groups was intraspecies, $P = 0.17 \pm 0.05\%$, intragenus, $P = 10.75 \pm 0.48\%$, intrafamily, $P = 20.07 \pm 1.43\%$, and intraorder, $P = 21.10 \pm 0.45\%$. Within the *Glyptothorax* genus maximum divergence was obtained among *G. brevipinnis* sequences, whereas the least divergence was obtained within *G. davissinghi*. The phylogenetic trees for 193 and 47 sequences of Sisorid catfishes together were developed using the *Cyt b* gene and four different analytical approaches: Bayesian (BA), neighbour-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML). The analysis revealed a monophyletic origin for the all investigated representatives of the genus *Glyptothorax* (99, 100, 99, and 73% support level in our BA, NJ, MP, and ML analyses respectively) and with some reservations for Sisoridae, which is the principal family investigated. The monophyletic origin of the two subfamilies of Sisorid catfish defined in the literature was partly also supported by molecular phylogenetic data.

Keywords Cytochrome b · Evolutionary distance · Sequence divergence · Phylogenetic tree

Introduction

The order Siluriformes includes 37 recognized families of catfishes widely distributed and diverse in freshwaters [1]. The family Sisoridae, established by Regan [2], is an exclusively Asian family of bottom dwelling catfishes. Based on morphological characters and meristic counts de Pinna [3] classified the family Sisoridae into two subfamilies, Sisorinae with genera *Bagarius*, *Sisor*, *Nangra, Gagata* and *Ayarnangra,* and Glyptosterninae ("glyptosternoids") with genera *Glyptothorax, Pseudecheneis*, *Caraglanis, Euchiloglanis* and others. *Glyptothorax* Blyth [4] with 89 species (29 species from India alone) is a widely distributed genus with plenty of taxonomic ambiguity [5]. In earlier descriptions, the adhesive apparatus, barbel length and fin formula were diagnostic characters for species' resolution, but later studies also included osteological characters [6, 7]. Despite the marvellous work by morphological taxonomists, overlap and ambiguity in

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many of morphological characters, especially in catfishes, requires robust DNA markers for species identification and phylogenetic studies.

Mitochondrial DNA (mtDNA) can be very useful in resolving phylogenetic relationships between closely related taxa [8]. Different regions of the mitochondrial genome evolve at different rates allowing suitable regions to be selected for inferring evolutionary relationship among higher taxa, species, recently divergent groups, populations, and even individuals [9]. Complete mitochondrial DNA sequencing gives the combined inference for fish phylogenetics and species identification [10, 11], but involve more cost and effort with present DNA sequencing techniques. Hence single markers like 16S rRNA and cytochrome *c* oxidase I (*COI*) have been used [12, 13]. The cytochrome *b* (*Cyt b*) gene is suitable from intraspecies to order level [14–16] and has been proven very useful for resolving the evolutionary relationships in a variety of fish families viz. Pleuronectidae, Gobiidae, Ictaluridae, Sisoridae, and Amblycipididae [17–21]. *Cyt b* was also used to establish phylogenetic relationships among closely related species of subfamily Schizothoracinae [22], and to raise distinct clades to subspecies level in *Glyptothorax fokiensis* [23]. Although molecular phylogenies of *Glyptothorax* have been studied in China [20, 24, 25], but there was no record of molecular phylogeny of this genus in India. The current paper addresses the sequence diversity and molecular phylogeny of seven species of the genus *Glyptothorax* and analyse the place of this genus in Sisoridae family.

Materials and methods

Sample collection

In total 193 specimens of seven species of the genus *Glyptothorax* were collected from across India (Table 1). Species were identified based on morphometric and meristic data, following 'Catfishes of India' by K.C Jayaram [26], 'Inland Fishes of India & Adjacent Countries' by Talwar & Jhingran [27] and descriptions given in journals [6, 7, 28]. Fish specimens and the adhesive apparatus were photographed and meristic counts of all specimens were recorded. As the adhesive apparatus is key character, it was photographed in mature specimens of all seven species (Fig. ESM1, Online Resource 1). Fin formula (D/P/V/A/C) included Dorsal fin (D), Pectoral fin (P), Pelvic fin (V), Anal fin (A) and Caudal fin (C). Fin formulae were I,6/I,8–9/i,5/i–iii 8–9/17–18 (*G. Garhwali*); I,6/I,8–9/i,5/i,9–11/17 (*G. dakpathari*); I,6/I,7–8/i,5/iii,7–8/17–18 (*G. brevipinnis*); I,6/I,9/i,6/ii,9–10/17–18 (*G. ngapang*); I,6/I,8–9/i,6 / ii,8/17 (*G. granulus*); I,6/I,8/i,5/ii 8/17 (*G. ventrolineatus*) and I,6/I,9/i,5/iii,8/17 (*G. davissinghi*). Muscle and fin tissues were preserved in 95% v/v ethanol and the vouchers were kept in 10% v/v formaldehyde. Specific and unambiguous code was given to tissue and voucher of each fish specimen (Table 1) and all voucher specimens were photographed. All these materials are kept under the supervision of the first author, at the Centre for DNA Barcoding at National Bureau of Fish Genetic Resources (NBFGR), Lucknow.

Table 1 Details of the fish sample collection and accession numbers from various parts of India

Species name	Site of collection (river, place)	Geographical position (lat/long)	Sample size	GenBank accession numbers	No. of haplotypes
G. garhwali	Alkananda (tributry of Ganga), Srinagar Garhwal, Uttrakhand.	30.13/078.47	34	EU637404–EU637437	7
G. dakpathri	Sutlej River, Tatapani, Shimla, Himachal Pradesh.	31.14/077.05	38	EU637438–EU637450, FJ357213– FJ357237	10
G. brevipinnis	Song stream (tributry of Ganga), Maldevta, Dehradun, Uttrakhand	30.20/078.07	23	EU637451–EU637471, FJ357238, FJ423584	7
	Yamuna Barrage, Dakpathra, Dehradun, Uttrakhand	30.30/077.47	7	FJ208934–FJ208940	
	Khanda stream (tributry of Ganga), Srinagar Garhwal, Uttrakhand	30.12/078.44	6	FJ349103–FJ349108	
G. ngapang	Iril River, Serou, Bishenpur, Manipur.	24.16/093.52	36	FJ349109–FJ349144	15
G. granulus	Iril River, Serou, Bishenpur, Manipur.	24.16/093.52	32	FJ349145–FJ349176	8
G. ventrolineatus	Iril River, Serou, Bishenpur, Manipur.	24.16/093.52	9	FJ349177–FJ349185	4
G. davissinghi	Challakudy river, Poringal Kuthu Dam, Kerala.	10.16/076.26	8	FJ423576–FJ423583	4
Total			193		55

DNA extraction, PCR amplification and DNA sequencing

Approximately 50 mg of tissue was used for DNA isolation following standard phenol/chloroform method [29] with partial modifications in the initial step of homogenisation. The complete *Cyt b* gene was amplified using the universal primers LI4724 (TGACTTGAARAACCAYCGTTG) & H15915 (CTCCGATCTCCGGATTACAAGAC) [30]. The 50 µl PCR reaction volume for *Cyt b* region included 5.0 µl PCR buffer (10×), 1.0 µl dNTPs (2.5 mM each), 2.0 µl $MgCl_2$ (25 mM), each primer 0.5 µl (10 µM), *Taq* polymerase 0.5 µl (3 U/µl) and template DNA 2.0 µl (50 ng/µl). Amplifications were performed in MJ PTC-200 (BIORAD). The thermal regime for *Cyt b* consisted of initial denaturation of 3 min at 94°C, 35 cycles of 45 s at 94°C, 50 s at 50°C, 80 s at 72°C followed by final extension of 10 min at 72°C and held at 4°C. PCR products were visualized on 1.2% agarose gels and documented using a gel documentation system (Biovis). DNA sequencing was performed following the dideoxynucleotide chain termination method [31], using an automated ABI 3730 sequencer. Products were labelled using the BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc). Reagent quantities for one sequencing PCR reaction cocktail were: Terminator Ready Reaction Mix (2.5×) 8.0 µl, BigDye Sequencing buffer (5×) 4.0 µl, PCR product (50 ng/µl) 1 µl, primer (3 µM) 1.0 µl, deionised water 6.0 µl, total volume 20 µl. PCR cycle sequencing conditions were 96°C for 1 min and then 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min.

DNA sequence analysis

Reverse strand sequences were inverted (reversed and complimented) and aligned with the forward strand sequence. Ambiguities were referred against the sequencing electrophoregrams and corrected accordingly. Full length consensus sequences were made from forward and reverse strands for all samples of a species and aligned using ClustalW [32]. The consensus sequences were blasted in National Center for Biotechnolgy Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov) for the nearest similar sequence matches [33] and submitted to GenBank. Prior to tree building all sequences were aligned with ClustalW, an integrated tool in MEGA-4 [34].

The phylogenetic trees were constructed by neighbor-joining (NJ) method using K2P distance, by maximum parsimony (MP) criteria both in MEGA-4, by Bayesian algorithm (BA) using MrBayes and by maximum likelihood (ML) approach in PAUP 4.0 10b [35–39]. In MP the CNI (Close-Neighbor-Interchange) algorithm [40] was used for tree search and in ML branch and bound search algorithm was applied as given in PAUP 4.0 10b. Bootstrap values were included to test the reliability of inferred trees [41] ($k = 1{,}000$ replicates) and all codon positions were included. Analyses and tree building were made in two steps, in the first step all 193 *Glyptothorax* sequences were used together with one GenBank sequence, of *Bagarius yarrelli* (Accession number AF416897), or with five GenBank sequences (see below). In the second step, initial six sequences of each of *Glyptothorax* species (in *G. brevipinnis*, sequences with accession numbers EU637458, EU637460, EU637463, EU637464, FJ208934, and FJ208939) and five from GenBank were included in tree building using BA, NJ, MP, and ML techniques. The following five sequences were retrieved from GeneBank for tree rooting and comparative phylogenetic analysis: *Ameiurus platicephalus* (Ictaluridae; NCBI accession No. AY184261, specimen from USA), *Walago attu* (Siluridae; AF477828), *Gagata cenia* (Sisoridae; AF499599), *Euchiloglanis davidi* (Sisoridae; AF416883), and *Glyptothorax cavia* (Sisoridae; AF477830).

The GTR+G substitution model was estimated as the best fit for the dataset by the maximum likelihood test in MODELTEST 3.7 [42]. The GTR+G model ($-\ln L = 4944.12$, $K = 9$, A:C:G:T = 0.2848, 0.3206, 0.1278, 0.2667, G = 0.2127) was used in BA and ML tree building. Therefore, BA ($n = 10^6$ generations) and ML ($k = 1{,}000$ bootstrap replicates) approaches were based on the described model parameters. BA tree calculations performed with parameters: MCMCP ngen = 1,000,000, printfreq = 1,000, samplefreq = 1,000, nchains = 4, SUMP burnin = 100; default program options were used for the remaining parameters. The NJ tree with bootstrapping ($k = 1{,}000$ replicates) was based on the K2P distance with the uniform rate substitution among sequences and with G parameter estimate as defined above. Starting trees were obtained via a step-wise addition, and branch lengths at start-up were obtained using the Rogers-Swofford approximation method as implemented in PAUP* 4.0 10b. A tree bisection-reconnection technique was used for branch swapping under a heuristic search (with two repetitions). The main calculations were performed by MrBayes 3.1.1 [38, 43], MEGA-4.0 [34] and PAUP* 4.0 10b [39] program packages. The phylogenetic trees were visualized and edited where necessary with TreeView software [44], and a general statistical analysis was performed using STATISTICA 6.0 [45].

Results

Analysis of diversity

Out of 193 specimens of seven *Glyptothorax* species that were analyzed for the *Cyt b* gene, 55 haplotypes were identified (Table 1). Out of 1,152 positions in the *Cyt b* gene sequences analyzed in 193 specimens, 269 positions

Table 2 Average percent nucleotide frequency of all the three codon positions and percent nucleotide frequency at third position of codons in cytochrome *b* gene sequences of seven *Glyptothorax* species

S.No.	Nucleotide → species ↓	Average nucleotide frequency (%) of three codon positions				Nucleotide frequency (%) at third position of codons			
		T	C	A	G	T	C	A	G
1.	*G. garhwali*	29.7	28.9	27.5	14.0	23.2	35.4	36.7	4.7
2.	*G. dakpathri*	27.8	30.9	27.5	13.9	17.2	41.4	38.2	3.2
3.	*G. brevipinnis*	28.2	30.5	27.4	14.0	17.8	40.5	38.1	3.6
4.	*G. ngapang*	28.6	29.3	28.4	13.7	18.6	37.7	41.2	2.4
5.	*G. granulus*	29.9	28.1	28.4	13.6	21.9	34.7	40.6	2.9
6.	*G. ventrolineatus*	29.1	29.2	27.9	13.8	20.3	37.1	39.7	2.9
7.	*G. davissinghi*	28.2	29.8	27.8	14.2	17.7	39.3	38.8	4.3
	Average (seven species of Glyptothorax)	28.8	29.6	27.8	13.8	19.6	38.1	39.0	3.4

(23.3%) were variable, and 235 (20.3%) were parsimoniously informative. The average base composition [thymine/uracil (T/U); cytosine (C); adenine (A) and guanine (G)] over all the three codon positions and third codon position is given in Table 2. The number of identical pairs (ii), transitional pairs (ti) and transversional pairs (tv) were calculated as 1074, 62 and 17, respectively and nucleotide substitutions were biased toward transitions. The average K2P distance between conspecific individuals was highest (0.0069) for *G. brevipinnis* and lowest (0.0012) for *G. davissinghi*. The interspecies K2P distance was highest (0.1293) between *G. ventrolineatus* and *G. garhwali* and lowest (0.0417) between *G. brevipinnis* and *G. dakpathri* (Table 3). The p distance and standard error at intraspecies, intragenus, intrafamily and intraorder level is 0.17 ± 0.05, 10.75 ± 0.48, 20.07 ± 1.43, and $21.10 \pm 0.45\%$ (Fig. ESM2, Online Resource 1).

Phylogenetic analysis

As the NJ tree built for the whole set of 198 sequences had topology similar to the tree based on 47 sequences, so for illustration tree based on 47 sequence is shown in Fig. 1(support levels are shown for four tree kinds in the order BA, NJ, MP, and ML). The topologies of the BA and NJ trees were nearly identical, differing basically in support values of nodes, while MP and ML trees gave slightly different clustering with the Sisoridae representatives of GenBank sequences, which gave monophyletic clades in the BA, but formed paraphyletic clades joining first with Siluridae in MP, ML, and partly in NJ trees. The MP and ML trees differed from the above two trees as well in somewhat lower resolution of some branches. All four types of trees showed five major properties: (1) there is a separate cluster for outgroup species (Ictaluridae) that joins closely with Siluridae representative and along with Sisoridae form three branches, in the tree of the order Siluriformes (2) there is a cluster representing the Sisoridae family and its different genera (3) there is a sharp cluster of *Glyptothorax* genus that includes seven Indian and one Chinese species from GenGank (4) there is a set of well supported clusters representing eight *Glyptothorax* species, and (5) there exists a set of the nearest clusters for all single individuals that are classified as specimens of the same species. Repetition frequencies or posterior probabilities for the BA tree (%) and bootstrap support levels for NJ, MP, and ML (%) at the clusters 2–5 were within the limits: (2) from no support up to 57–99% (3) 73–100%

Table 3 Kimura two parameter distance (K2P) for seven species of genus *Glyptothorax* based on *Cyt b* gene

	G. garhwali	*G. dakpathri*	*G. brevipinnis*	*G. ngapang*	*G. granulus*	*G. ventrolineatus*	*G. davissinghi*
G. garhwali	0.001905						
G. dakpathri	0.112636	0.003625					
G. brevipinnis	0.121684	0.041705	0.006917				
G. ngapang	0.109046	0.073580	0.083269	0.005955			
G. granulus	0.114286	0.088954	0.104469	0.061424	0.002268		
G. ventrolineatus	0.129256	0.076143	0.084923	0.069283	0.049807	0.001884	
G. davissinghi	0.126753	0.072556	0.080727	0.083928	0.092768	0.077889	0.001159

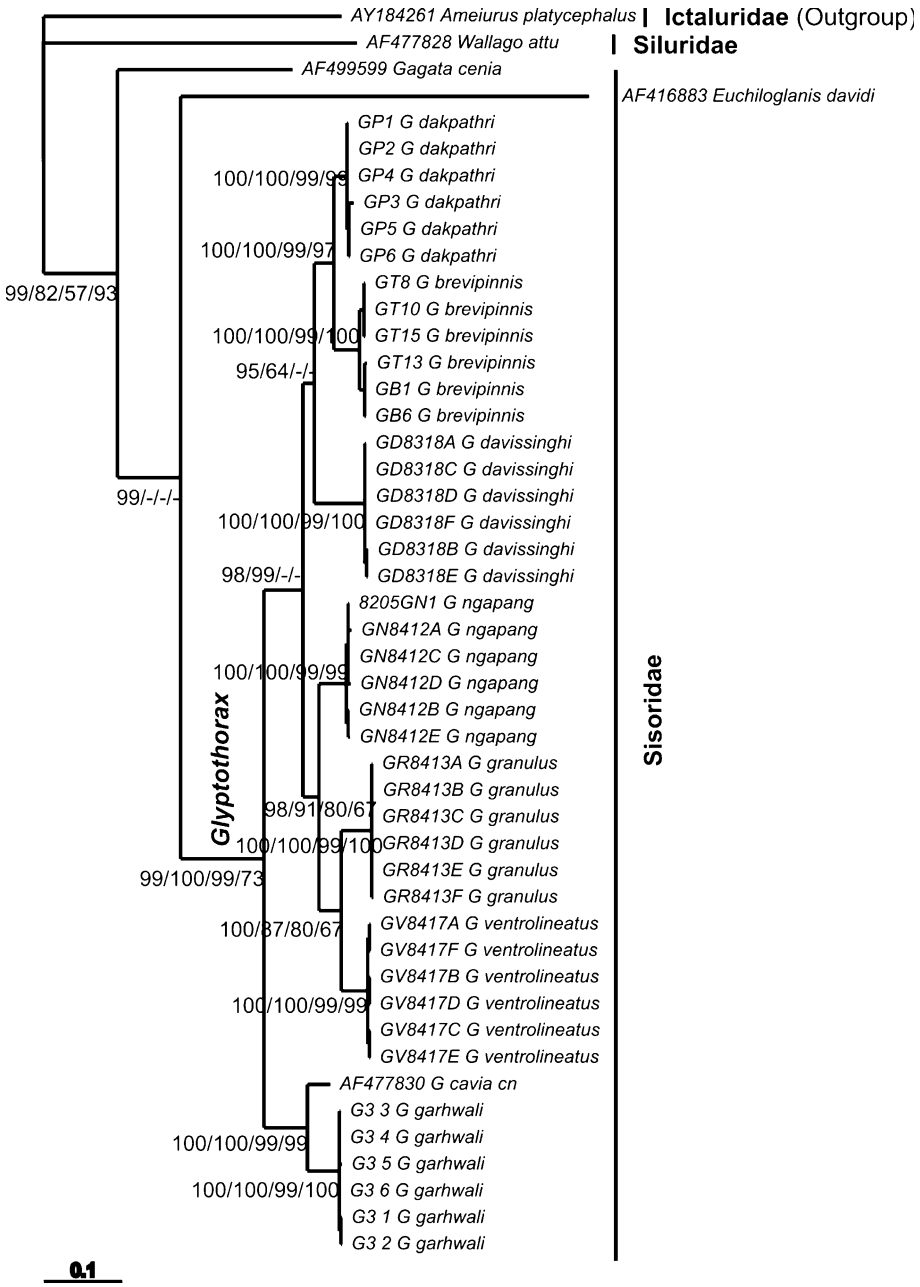

Fig. 1 Cyt-b gene tree of Indian and close genera of Sisorid catfish built by BA, NJ, MP and ML algorithms. *Numbers* beside nodes show support levels (BA/NJ/MP/ML). *Dash* denotes different clustering in others, not the BA base-tree for few branches. *Scale* in left bottom corner gives representation on the branch lengths for BA-based phylogram

(4) 64–100%, and (5) 99–100%. Some intraspecies clusters were detected as in *G. brevipinnis*, where two sub-groups were clearly separated. Although support values are not shown in the Fig. 1 for clusters of level (5), all of them were well defined by four phylogenetic methods. Comparing average intra and interspecies sequence divergence and other data above, the pattern of clustering for these 1 and 2 categorical levels in Fig. ESM2 (Online Resource 1) and cluster types (4) and (5) in Fig. 1 (see above, tree properties), it can be concluded that interspecies differentiation in the *Glyptothorax* genus is an order of magnitude higher than intraspecies differentiation. This difference allows easy species discrimination based on *Cyt b* sequence data. The phylogenetic signal for clusters (1) and (2) is not so strong as for lower level clusters. That is why there are differences in topology of BA and NJ trees for these cluster levels, i.e. the monophyly of Sisoridae family is supported by the BA tree but not supported by the NJ, MP, and ML trees. This same conclusion is applicable to subfamily level.

Discussion

Analysis of Diversity

The equal base composition results of *Cyt b* sequences calculated in this article are comparable with Chinese *Glyptothorax* species (T 30.3%, C 27.5%, A 28.4% and G 13.8%) [23] and slightly different from *Schizothorax* species (T 30.7%, C 26.7%, A 25.9% and G 16.7%) [22]. All these studies show strong bias against G, a result consistent with most vertebrate mitochondrial genomes [46] and with fish *Cyt b* sequences in particular [47, 48]. This anti G bias in nucleotide composition was further magnified at third codon positions, where the average *G* value for *Glyptothorax* was 3.4%. This *G* value was unique for five species (*G. garhwali,* 4.7%; *G. dakpathri* 3.2%; *G. brevipinnis,* 3.6%; *G. ngapang,* 2.4% and *G. davissinghi,* 4.3%) and shared by two species (*G. granulus* and *G. ventrolineatus,* both 2.9%), which is in concordance with their very close phylogenetic relationship (Fig. 1). This *G* value at third codon position needs to be further tested in wider range for its potential as a taxa specific parameter. The overall transition/transversion bias (3.457) is close to the biases reported for *Glyptothorax* species [23] and vertebrates in general [49, 50]. The graph of a one-factor ANOVA and mean *P*-distance values at four levels of differentiation in Sisorid and other catfish species for *Cyt b* gene sequence data represents an obvious increase of mean *P*-distance score with rank of comparison taxa (Fig. ESM2, Online Resource 1). This is statistically significant: $F = 123.30$, $df = 3; 30$, $P < 0.0001$. A similar pattern was obtained for both *Cyt b* and *CO1* gene sequence divergence for a vast number of animal species [15, 51]. It is accepted generally that taxa of higher ranks exist longer than those of lower rank [52, 53]. Thus, taking into consideration significant differences between intraspecies and interspecies levels within genera, it might be possible to attribute these data to a certain mode of speciation. More specifically, together with an increase of *P*-distances at two stages (subspecies and species), these data lead to the conclusion that speciation in the compared representatives of the order Siluriformes followed a geographic mode [54], with the accumulation of numerous per site base changes (mutations) over a long period of time since separation of gene pools of local populations and/or subspecies. Finally, this mode forecasts the species formation and later independent evolution of species themselves and consequent divergence of representatives in higher lineages or phyletic evolution [54–59].The geographic mode of speciation has been substantiated for fish and other animals (24,000 species) and for a wider list of rank categories viz. subspecies, semispecies, sibling species, morphologically distinct species, genera etc. [15, 17, 21, 47, 51, 60, 61]. A wider set of descriptors [51, 61, 62] in Sisoridae and other Siluriformes are required to draw a more precise conclusion on the speciation mode. The average scores of *P*-distances at the *Cyt b* gene (Fig. ESM2, Online Resource 1) showed that intraspecies diversity is lower in *Glyptothorax* than in other animal groups but intrageneric and intrafamily diversities exhibited a similar pattern [15, 51]. The *P*-distances of other animal taxa at comparable categorical levels were estimated as: (1) 1.46 ± 0.34% (3) 10.46 ± 0.96% and (4) 17.99 ± 1.33% [15]. *G. brevipinnis* showed the maximum intraspecific divergence among seven species. This is because of wide distribution of this species. Samples collected from steep and fast flowing rivers in hills like *G. dakpathri* from Sutlej River and *G. ngapang* from Iril River also showed more intraspecific genetic diversity. During peak monsoon very fast flowing water, renders *Glyptothorax* unable to attach to boulders, consequently these fishes are brought to low altitude plains. Comparatively low intraspecies diversity seems due to their habits of living in small groups firmly attached to rock. At the time of peak summer in May/June, most of the rivers in mountains are left with small and shallow patches of discontinuous water, where a few adults are left, decreasing the population size for breeding. This low level of diversity may lower the adaptability of the species to biotic and abiotic stresses. During sample collection it was pointed out by fishermen that abundance of *Glyptothorax* has been decreasing and we also noticed their absence in earlier reported area of Himachal, Kerala and Tamilnadu. *Glyptothorax* is not commercially important, so its decline is not taken seriously. As it has adhesive apparatus, it is sticked to stones and can withstand water flow, consequently these fishes are an important component (carnivore) in food chains and aquatic ecosystems.

Phylogenetic analysis

Sisorid catfish species within a single genus are differentiated clearly and within species compact clusters are formed (Fig. 1). The investigated representatives of the family Sisoridae join as a monophyletic branch with 99% probability for the BA tree only. Paraphyly with bootstrap support of 82, 57 and 93% was obtained for NJ, MP, and ML trees respectively. Unlike our data for Sisoridae monophyly, having 99% support for BA tree only, the resolution of the family level branches was good in two other studies with *Cyt b* [20, 24]. Possible explanation may be, the information capacity in our 1,152 bp *Cyt b* sequences is not enough to cover available nucleotide sequence diversity. Also, it is true that the real tree topology may still remain obscure, despite statistical support, if the analyzed taxa are not representative of natural diversity [63], which is especially true for such species-rich groups

such as catfishes. The BA tree supported the monophyly of two Sisorid subfamilies [3], but the subfamily division by de Pinna is neither supported by recent molecular phylogenetic study [20] nor by the present study.

Monophyly for the genus *Glyptothorax,* as defined by osteological morphology and molecular data [25], is strongly supported by this study (Fig. 1, support levels 99, 100, 99, and 73% for BA, NJ, MP, and ML trees respectively). The inclusion of two other genera *Gagata* and *Euchiloglanis* in Sisoridae, is well supported by this study and there is sharp difference among genera and congeneric species (Fig. 1). The difference between intraspecies and congeneric interspecies levels of genetic diversity, is an indicator of the barcoding value of a gene sequence for species discrimination and may be used as a tool for comparative evolutionary genetics. These kinds of data are very reliable as they are supported both by the tree topology (Fig. 1), and *P*-distance scores (Fig. ESM2, Online Resource 1).

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