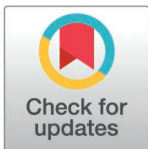


RESEARCH ARTICLE

Cryptic biodiversity of freshwater fish species in Bangladesh

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Data availability statement: All DNA sequences files are available from the GenBank

Abstract

Unrecognized cryptic species impede conservation planning and biodiversity assessments. DNA barcoding has tremendously expanded the number of novel and cryptic species in biological science. Despite few sporadic studies, the exact number of freshwater species found in Bangladesh is not known. To assess this biodiversity, we sequenced the COI gene of 124 freshwater specimens, which were gathered from various localities around Bangladesh. Seven cryptic species hidden among the currently studied specimens were identified based on the findings of phylogenetic and species delimitation analyses. The preliminary assessment also encompassed a restricted morphological examination of these cryptic taxa. The appearance of cryptic species, some of them possibly endemic, has been hypothesized. This raises concerns regarding the true diversity and evolutionary history of freshwater species in Bangladesh, which are significantly underrepresented in the current systematic frameworks that do not account for DNA data. Our current study provides baseline data that might aid local ichthyologists in their quest to identify additional new species by combining several variables (morphology and ecology). Further research is warranted to protect the priceless freshwater species in Bangladesh.

Introduction

Bangladesh is situated in the oriental region of the Indo-Himalayan and Indo-Chinese sub-regions [1]. This country is home to the Ganges-Brahmaputra river delta, which is the largest delta in the world. Approximately 230 rivers crisscross through the lands of Bangladesh. Among these rivers, 57 are transboundary rivers (54 rivers are shared with India and three are shared with Myanmar) [2,3]. The downstream region consisting of many different rivers, canals, lakes with both flowing as well as static water (beels), and harbors are home to several freshwater fish. Approximately 265 species of freshwater fish are found in this lowland nation [4]. Freshwater fish are found in Bangladesh's biggest river systems, the Ganges, Brahmaputra, and Meghna, which are collectively equal to 4,339,694 hectares of inland water [5]. Approximately 30.2% of all fish species found worldwide are freshwater fish [6] and these are very valuable both nutritionally and economically [7]. The fishing industry is unquestionably important for Bangladesh's economy, culture, and nutrition [8,9].

database (accession numbers: LC823243 and LC823366). Other relevant data are within the paper and its supporting files.

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According to Shelley et al. [10], cryptic fish species present one of the main obstacles to the proper management, conservation, and planning of fish biodiversity. Cryptic species, of which are genetic variants, however, physically identical are a common occurrence and they are concealed as nominal species. Although morphology-based investigations have been conducted in Bangladesh [11–13], accurate identification of a species macroscopically is difficult for ichthyologists as well, leading to errors in lists of species included in the literature. It is evident from several publications and review papers that, Bangladesh's taxonomy is out-of-date and not harmonized, when compared with that of the neighboring countries [14]. Hence, an integrative taxonomy method incorporating molecular and morphological data is required to overcome this obstacle and provide a seamless and widely recognized list of species inhabiting this nation. Researchers describe additional new species of fish each year, but it is not clear as to how many species are endemic to this nation.

In the current study, the authors have conducted phylogenetic analyses on fish specimens available in fish markets and natural resources nationwide to examine freshwater fish biodiversity in Bangladesh. Whenever necessary, morphological data has been used to identify cryptic or candidate species. The DNA-based barcoding method has proven to be an important molecular tool for non-specialists for species identification [15], and the mitochondrial cytochrome *c* subunit I (COI) sequence is a dependable barcode marker for measuring and assessing the taxonomic status of fish species [14,16–18]. This study attempts to review the biodiversity of Bangladesh's freshwater fishes, and explore the possible presence of cryptic species using the DNA barcoding technique.

Materials and methods

Study area, duration and identification of sample

A total of 124 fish specimens were collected from all regions of Bangladesh (see Table 1), photographed and their sex was recorded. All specimens were transferred at the “Evolution and Diversity Research Laboratory” at Jamalpur Science and Technology University, located in Jamalpur, Bangladesh. A voucher number was assigned to each specimen (please refer to Table 1). For subsequent molecular research, the species were maintained in either saturated DMSO/NaCl solution or 95% ethanol. Table 1 lists the study sites in detail. Initial identification of the collected fish species was based on current taxonomy knowledge and literature dealing with the fisheries science [4]. If necessary, the names of the species that have been accepted by the Catalog of Fishes online portal have been adhered to [6].

DNA extraction and sequencing

Genomic DNA of all fish specimens was extracted and purified from conserved fin tissues using Chelex 100 [19]. Using the primers Fish-F1, Fish-F2, Fish-R1, and Fish-R2, the partial COI fragment was amplified by polymerase chain reaction (PCR) using EmeraldAmp PCR Master Mix (Takara Bio) [20]. Cycle conditions described in a previously conducted study [14] were followed for performing PCR. The amplified PCR products were purified using polyethylene glycol precipitation. Cycle sequencing procedures were then performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Bioscience). Sequencing was performed using ABI 3500 automated sequencers. The newly discovered sequences were incorporated in the International Nucleotide Sequence Databases (INSD) through the DNA Data Bank of Japan (Accession numbers LC823243–LC823366).

Table 1. Specimens used and identified COI haplotypes found in this study.

Voucher Number	Scientific Name	Family	Local Name	Location	Latitude and Longitude	Sample Collection Date	Accession number
MHBSFMSTU Fish 1	<i>Cabdio morar</i>	Cyprinidae	Pieli	Sariakandi, Bogra	24°55'08.4"N 89°38'26.0"E	01-07-2018	LC823243
MHBSFMSTU Fish 2	<i>Tenualosa ilisha</i>	Clupeidae	Ilish	Sariakandi, Bogra	24°55'08.4"N 89°38'26.0"E	01-07-2018	LC823244
MHBSFMSTU Fish 3	<i>Ailia</i> sp.	Ailiidae	Bash patari/ Kajuli	Sariakandi, Bogra	24°55'08.4"N 89°38'26.0"E	01-07-2018	LC823245
MHBSFMSTU Fish 5	<i>Pisodonophisboro</i>	Ophichthidae	Bamush/ Kharu	Sariakandi, Bogra	24°55'08.4"N 89°38'26.0"E	01-07-2018	LC823246
MHBSFMSTU Fish 6	<i>Awaous</i> sp.	Gobiidae	Bochabele	Sariakandi, Bogra	24°55'08.4"N 89°38'26.0"E	01-07-2018	LC823247
MHBSFMSTU Fish 7	<i>Mastacembelusarmatus</i>	Mastacembelidae	ShalBaim	Sariakandi, Bogra	24°55'08.4"N 89°38'26.0"E	01-07-2018	LC823248
MHBSFMSTU Fish 8	<i>Botiadarior</i>	Cobitidae	Boumach	Sariakandi, Bogra	24°55'08.4"N 89°38'26.0"E	01-07-2018	LC823249
MHBSFMSTU Fish 9	<i>Channaorientalis</i>	Channidae	Raga	Jaliarhaor, Netrokona	24°41'31.3"N 90°51'46.5"E	25-08-2018	LC823250
MHBSFMSTU Fish 10	<i>Aplocheiluspanchax</i>	Aplocheilidae	Kanpona	DangarBeel, Jamalpur	24°55'06.5"N 89°51'03.6"E	02-02-2019	LC823251
MHBSFMSTU Fish 11	<i>Trichogaster lalius</i>	Osphronemidae	Khoilsha	DangarBeel, Jamalpur	24°55'06.5"N 89°51'03.6"E	02-02-2019	LC823252
MHBSFMSTU Fish 12	<i>Trichogaster lalius</i>	Osphronemidae	Borokhoilsha	DangarBeel, Jamalpur	24°55'06.5"N 89°51'03.6"E	02-02-2019	LC823253
MHBSFMSTU Fish 13	<i>Trichogaster chuna</i>	Osphronemidae	Lalkholisha	DangarBeel, Jamalpur	24°55'06.5"N 89°51'03.6"E	02-02-2019	LC823254
MHBSFMSTU Fish 14	<i>Colisachuna</i>	Osphronemidae	ChunaKholisha	DangarBeel, Jamalpur	24°55'06.5"N 89°51'03.6"E	02-02-2019	LC823255
MHBSFMSTU Fish 15	<i>Trichogaster chuna</i>	Osphronemidae	Lal Kholisa	DangarBeel, Jamalpur	24°55'06.5"N 89°51'03.6"E	02-02-2019	LC823256
MHBSFMSTU Fish 16	<i>Macrognathusaral</i>	Mastacembelidae	Tara Baim	Jaliarhaor, Netrokona	24°41'31.3"N 90°51'46.5"E	25-08-2018	LC823257
MHBSFMSTU Fish 17	<i>Lepidocephalichthyssp.</i>	Cobitidae	Morichapuiya/ Gutum	Jaliarhaor, Netrokona	24°41'31.3"N 90°51'46.5"E	25-08-2018	LC823258
MHBSFMSTU Fish 18	<i>Ompokpabda</i>	Siluridae	Pabda	Gaglajur bazar, Netrokona	24°52'36.2"N 91°31'38.4"E	25-08-2018	LC823259
MHBSFMSTU Fish 19	<i>Mystus cavasius</i>	Bagridae	Gulsha	Jaliarhaor, Netrokona	24°41'31.3"N 90°51'46.5"E	25-08-2018	LC823260
MHBSFMSTU Fish 20	<i>Mystus tengara</i>	Bagridae	Tengra	Jaliarhaor, Netrokona	24°41'31.3"N 90°51'46.5"E	25-08-2018	LC823261
MHBSFMSTU Fish 22	<i>Mystus carcio</i>	Bagridae	Bujuri	Jaliarhaor, Netrokona	24°41'31.3"N 90°51'46.5"E	25-08-2018	LC823262
MHBSFMSTU Fish 23	<i>Channamarulius</i>	Channidae	Gojar	Ghaglajur, Netrokona	24°52'36.2"N 91°31'38.4"E	15-12-2018	LC823263
MHBSFMSTU Fish 25	<i>Nandus nandus</i>	Nandidae	Bheda	Jaliarhaor, Netrokona	24°41'31.3"N 90°51'46.5"E	25-08-2018	LC823264
MHBSFMSTU Fish 26	<i>Channapunctata</i>	Channidae	Taki	Jaliarhaor, Netrokona	24°41'31.3"N 90°51'46.5"E	25-08-2018	LC823265
MHBSFMSTU Fish 27	<i>Mystus cavasius</i>	Bagridae	Kabashi Tengra	Mawaghat Munshigonj	23°27'59.3"N 90°17'22.1"E	25-08-2018	LC823266
MHBSFMSTU Fish 28	<i>Bagariusbagarius</i>	Sisoridae	Bagha air	Kongso, Netrokona	24°52'50.2"N 90°44'03.2"E	25-08-2018	LC823267
MHBSFMSTU Fish 30	<i>Sperata aorella</i>	Bagridae	Air	Kongso, Netrokona	24°52'50.2"N 90°44'03.2"E	25-08-2018	LC823268

(Continued)

Table 1. (Continued)

Voucher Number	Scientific Name	Family	Local Name	Location	Latitude and Longitude	Sample Collection Date	Accession number
MHBSFMSTU Fish 31	<i>Trichopsis vittata</i>	Osphronemidae	PokaKholisha	MawaghatMunshigonj	23°27'59.3"N 90°17'22.1"E	25-08-2018	LC823269
MHBSFMSTU Fish 32	<i>Parambassis lala</i>	Ambassidae	Lalchanda	Jaliarhaor, Netrokona	24°41'31.3"N 90°51'46.5"E	25-08-2018	LC823270
MHBSFMSTU Fish 34	<i>Xenentodon cancila</i>	Belontiidae	Kakila	Jaliarhaor, Netrokona	24°41'31.3"N 90°51'46.5"E	25-08-2018	LC823271
MHBSFMSTU Fish 36	<i>Macrognathus pancalus</i>	Mastacembelidae	Chikra/ Gujibaim	Jaliarhaor, Netrokona	24°41'31.3"N 90°51'46.5"E	25-08-2018	LC823272
MHBSFMSTU Fish 37	<i>Badis badis</i>	Badidae	Napit koi	Jaliarhaor, Netrokona	24°41'31.3"N 90°51'46.5"E	25-08-2018	LC823273
MHBSFMSTU Fish 38	<i>Chachaca</i>	Chacidae	Bengachaca	Gaglajur Bazar, Netrokona	24°52'36.2"N 91°31'38.4"E	25-08-2018	LC823274
MHBSFMSTU Fish 39	<i>Notopterus notopterus</i>	Notopteridae	Foli	Balikhola, Kishorganj	24°13'27.8"N 91°06'32.2"E	25-08-2018	LC823275
MHBSFMSTU Fish 40	<i>Labeorohita</i>	Cyprinidae	Rui	Sokal bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	17-04-2019	LC823276
MHBSFMSTU Fish 41	<i>Labeocalbasu</i>	Cyprinidae	Kali baush	Sokal bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	17-04-2019	LC823277
MHBSFMSTU Fish 46	<i>Esomus danrica</i>	Cyprinidae	Darkina	Sokal bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	25-08-2018	LC823278
MHBSFMSTU Fish 47	<i>Setipinnaphasa</i>	Engraulidae	Chibuk chela/ Phasha	Guthail, Jamalpur	25°05'13.3"N 89°42'49.0"E	25-08-2018	LC823279
MHBSFMSTU Fish 48	<i>Catlacatla</i>	Cyprinidae	Katol	Sokal bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	17-04-2019	LC823280
MHBSFMSTU Fish 49	<i>Cirrhinus mrigala</i>	Cyprinidae	Mrigal	Sanondobari, Jamalpur	25°22'51.3"N 89°44'40.8"E	17-04-2019	LC823281
MHBSFMSTU Fish 50	<i>Daniorerio</i>	Cyprinidae	Zebra anju/ Zebra	Kongsho, Sherpur	24°59'52.2"N 90°00'29.6"E	17-04-2019	LC823282
MHBSFMSTU Fish 51	<i>Coricasoborna</i>	Clupeidae	Subornakaski	Kongso, Sherpur	24°59'52.2"N 90°00'29.6"E	15-03-2019	LC823283
MHBSFMSTU Fish 52	<i>Salmostoma phulo</i>	Cyprinidae	Narikeli Chela/ Katari	Sokal bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	02-02-2019	LC823284
MHBSFMSTU Fish 53	<i>Hyporhamphus limbatus</i>	Hemiramphidae	Ekthota	Kendua, Netrokona	24°39'49.9"N 90°50'28.7"E	15-03-2019	LC823285
MHBSFMSTU Fish 54	<i>Channa striata</i>	Channidae	Shol	Kendua, Netrokona	24°39'49.9"N 90°50'28.7"E	02-02-2019	LC823286
MHBSFMSTU Fish 55	<i>Pseudeutropius the-rinoides</i>	Schilbeidae	Batai	Kendua, Netrokona	24°39'49.9"N 90°50'28.7"E	22-04-2019	LC823287
MHBSFMSTU Fish 56	<i>Ophichthys</i> sp.	Synbranchidae	kuchia	Sokal bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	02-02-2019	LC823288
MHBSFMSTU Fish 57	<i>Wallago attu</i>	Siluridae	Boal	Kongso, Netrokona	24°52'50.2"N 90°44'03.2"E	25-08-2018	LC823289
MHBSFMSTU Fish 59	<i>Heteropneustes fossilis</i>	Heteropneustidae	Shing	Dangarbeel, Jamalpur	24°55'06.5"N 89°51'03.6"E	02-02-2019	LC823290
MHBSFMSTU Fish 61	<i>Puntius sophore</i>	Cyprinidae	Jatpunti/ Bhadiputi	JaliarHaor, Netrokona	24°41'31.3"N 90°51'46.5"E	25-08-2018	LC823291
MHBSFMSTU Fish 63	<i>Pethia conchoniis</i>	Cyprinidae	Tit punti	Sokal bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	17-04-2019	LC823292
MHBSFMSTU Fish 64	<i>Chandanama</i>	Ambassidae	Chanda (lomba)	Balikhula, Kishorgonj	24°13'27.8"N 91°06'32.2"E	17-04-2019	LC823293

(Continued)

Table 1. (Continued)

Voucher Number	Scientific Name	Family	Local Name	Location	Latitude and Longitude	Sample Collection Date	Accession number
MHBSFMSTU Fish 65	<i>Parambassis ranga</i>	Ambassidae	Chanda (Gol)/ Phopa	Kendua, Netrokona	24°39'49.9"N 90°50'28.7"E	17-04-2019	LC823294
MHBSFMSTU Fish 66	<i>Macrogathus pancalus</i>	Mastacembelidae	Guchibaim	Mawaghat, Munshigonj	23°27'59.3"N 90°17'22.1"E	17-04-2019	LC823295
MHBSFMSTU Fish 67	<i>Amblypharyngodon mola</i>	Cyprinidae	Fakasemola	JaliarHaor, Netrokona	24°41'31.3"N 90°51'46.5"E	17-04-2019	LC823296
MHBSFMSTU Fish 69	<i>Channagachua</i>	Channidae	Cheng	Dangarbeel, Jamalpur	24°55'06.5"N 89°51'03.6"E	17-04-2019	LC823297
MHBSFMSTU Fish 70	<i>Salmostoma bacalia</i>	Cyprinidae	Ful chela	Sokal bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	17-04-2019	LC823298
MHBSFMSTU Fish 71	<i>Eutropiichthysvacha</i>	Ailiidae	Bacha	Ghaglajur, Netrokona	24°52'36.2"N 91°31'38.4"E	25-08-2018	LC823299
MHBSFMSTU Fish 72	<i>Gudusia chapra</i>	Clupeidae	GoniChapila	Sariakandi, Bogra	24°55'08.4"N 89°38'26.0"E	01-07-2018	LC823300
MHBSFMSTU Fish 73	<i>Clupisomagarua</i>	Ailiidae	Ghaura	Mawaghat, Munshigonj	23°27'59.3"N 90°17'22.1"E	17-04-2019	LC823301
MHBSFMSTU Fish 74	<i>Rita rita</i>	Bagridae	Rita	Balikhola, Kishorgonj	24°13'27.8"N 91°06'32.2"E	01-07-2018	LC823302
MHBSFMSTU Fish 75	<i>Hemibagrusmenoda</i>	Bagridae	Hugli/ Arwari/ Ghagla	Ghaglajur bazaar, Netrokona	24°52'36.2"N 91°31'38.4"E	01-07-2018	LC823303
MHBSFMSTU Fish 77	<i>Leiodon cutcutia</i>	Tetraodontidae	Patipotka	Bramaputra river, Jamalpur	24°55'37.0"N 89°57'42.6"E	10-11-2018	LC823304
MHBSFMSTU Fish 78	<i>Devariodevario</i>	Cyprinidae	Hingra kata punti/ Debari/ Chapchela	Ghaglajur Bazar, Netrokona	24°52'36.2"N 91°31'38.4"E	02-02-2019	LC823305
MHBSFMSTU Fish 79	<i>Osteobramacotio</i>	Cyprinidae	Dhela	Gaglajur Bazar, Netrokona	24°52'36.2"N 91°31'38.4"E	02-02-2019	LC823306
MHBSFMSTU Fish 80	<i>Mylopharyngodon piceus</i>	Cyprinidae	Mohashol	Sokal Bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	25-08-2019	LC823307
MHBSFMSTU Fish 81	<i>Labeo gonius</i>	Cyprinidae	Gonia	Sokal Bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	25-08-2019	LC823308
MHBSFMSTU Fish 82	<i>Labeo gonius</i>	Cyprinidae	Tatkini/ Bhagna/ Raik/ Lacho/bata	Balikhula, Kishorgonj	24°13'27.8"N 91°06'32.2"E	25-08-2019	LC823309
MHBSFMSTU Fish 83	<i>Erethistes</i> sp.	Sisoridae	Kutakanti	Ghaglajur Bazar, Netrokona	24°52'36.2"N 91°31'38.4"E	25-08-2019	LC823310
MHBSFMSTU Fish 84	<i>Gagatayoussoufi</i>	Sisoridae	Gum/ Gagata	Ghaglajur, Netrokona	24°52'36.2"N 91°31'38.4"E	25-08-2019	LC823311
MHBSFMSTU Fish 85	<i>Pethia gelius</i>	Cyprinidae	Gilipunti/ puti	Ghaglajur Bazar, Netrokona	24°52'36.2"N 91°31'38.4"E	07-01-2020	LC823312
MHBSFMSTU Fish 86	<i>Rhinomugil corsula</i>	Mugilidae	Folla, khalla, halla, khorsula	Balikhula, Kishorgonj	24°13'27.8"N 91°06'32.2"E	01-01-2020	LC823313
MHBSFMSTU Fish 87	<i>Chandramara chandramara</i>	Bagridae	Jolbujuri	Ghaglajur Bazar, Netrokona	24°52'36.2"N 91°31'38.4"E	07-01-2020	LC823314
MHBSFMSTU Fish 90	<i>Silonia silondia</i>	Ailiidae	Shilong	Kawniar char, Dewangonj	25°23'59.3"N 89°47'49.0"E	15-01-2020	LC823315
MHBSFMSTU Fish 91	<i>Glyptothorax telchitta</i>	Sisoridae	Lalkutakanti	Kawniar char, Dewangonj	25°23'59.3"N 89°47'49.0"E	15-01-2020	LC823316
MHBSFMSTU Fish 92	<i>Gagatacenia</i>	Sisoridae	Kutakanti, Cenia, Jangla, Kaowa, Gang tengra	Sanondabar, Jamalpur	25°22'51.3"N 89°44'40.8"E	25-01-2020	LC823317
MHBSFMSTU Fish 93	<i>Gogangra laevis</i>	Sisoridae	Gang Tengra	Dewangonj bazar, Jamalpur	25°10'01.7"N 89°45'57.5"E	15-01-2020	LC823318

(Continued)

Table 1. (Continued)

Voucher Number	Scientific Name	Family	Local Name	Location	Latitude and Longitude	Sample Collection Date	Accession number
MHBSFMSTU Fish 96	<i>Rasboradaniconius</i>	Cyprinidae	Darkina	Kendua, Netrokna	24°39'49.9"N 90°50'28.7"E	22-01-2020	LC823319
MHBSFMSTU Fish 97	<i>Botialohachata</i>	Cobitidae	Bou/Rani	Sokal bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	22-01-2020	LC823320
MHBSFMSTU Fish 98	<i>Labeo boggut</i>	Cyprinidae	Vangon/ Gonari	Sokal bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	22-01-2020	LC823321
MHBSFMSTU Fish 99	<i>Paracanthocobitis abutwebi</i>	Nemacheilidae	Balichata/ Puiya	Sanondabari, Jamalpur	25°22'51.3"N 89°44'40.8"E	25-01-2020	LC823322
MHBSFMSTU Fish 100	<i>Schistura</i> sp.	Nemacheilidae	Balikhura/ Puiya	Sanondabari, Jamalpur	25°22'51.3"N 89°44'40.8"E	25-01-2020	LC823323
MHBSFMSTU Fish 101	<i>Anodontostomachacunda</i>	Clupeidae	Chacunda	Defla Bazar, Jamalpur	25°01'17.1"N 89°50'16.3"E	25-01-2020	LC823324
MHBSFMSTU Fish 102	<i>Salmostoma bacalia</i>	Cyprinidae	Chela	Soal bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	21-12-2020	LC823325
MHBSFMSTU Fish 103	<i>Badis badis</i>	Badidae	Napit koi	Sokal bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	01-02-2020	LC823326
MHBSFMSTU Fish 104	<i>Rhinomugil corsula</i>	Mugilidae	Flathead mullet	Sokal Bazar, Jamalpur	24°55'47.4"N 89°56'45.2"E	01-02-2020	LC823327
MHBSFMSTU Fish 105	<i>Pethia conchoni</i>	Cyprinidae	Kanchonputi/ Taka puti	Guthail, Jamalpur	25°05'13.3"N 89°42'49.0"E	05-02-2020	LC823328
MHBSFMSTU Fish 106	<i>Neoeucirrhichthys maydelli</i>	Cobitidae	GualParapuiya	Guthail, Jamalpur	25°05'13.3"N 89°42'49.0"E	05-02-2020	LC823329
MHBSFMSTU Fish 107	<i>Chela laubuca</i>	Cyprinidae	Chep chela	Guthail, Jamalpur	25°05'13.3"N 89°42'49.0"E	05-02-2020	LC823330
MHBSFMSTU Fish 108	<i>Tariqilabeo latius</i>	Cyprinidae	Matikhora/kalabata	Guthail, Jamalpur	25°05'13.3"N 89°42'49.0"E	05-02-2020	LC823331
MHBSFMSTU Fish 109	<i>Pangio Pangia</i>	Cobitidae	Kuttapuiya/Panga/ Kolipuiya	Guthail, Jamalpur	25°05'13.3"N 89°42'49.0"E	05-02-2020	LC823332
MHBSFMSTU Fish 110	<i>Odontamblyopus rubicundus</i>	Gobiidae	Lalchewa	Mawaghat, Munshigonj	23°27'59.3"N 90°17'22.1"E	08-01-2020	LC823333
MHBSFMSTU Fish 111	<i>Apocryptes bato</i>	Gobiidae	Chewa/ Chiring	Mawaghat, Munshigonj	23°27'59.3"N 90°17'22.1"E	08-01-2020	LC823334
MHBSFMSTU Fish 112	<i>Sperata aor</i>	Bagridae	Guizza air	Mawaghat, Munshigonj	23°27'59.3"N 90°17'22.1"E	08-01-2020	LC823335
MHBSFMSTU Fish 118	<i>Ompok bimaculatus</i>	Siluridae	Kala pabda	Rangamati	22°58'00.8"N 92°12'05.6"E	11-01-2020	LC823336
MHBSFMSTU Fish 121	<i>Latescalcarifer</i>	Centropomidae	Deshivetki	PaikariMotshoArot, Barishal	22°42'13.4"N 90°22'32.4"E	27-01-2021	LC823337
MHBSFMSTU Fish 126	<i>Cynoglossus</i> sp.	Cynoglossidae	Kukurjib	Fishery Ghat, Chittagong	22°19'43.9"N 91°50'49.6"E	11-02-2021	LC823338
MHBSFMSTU Fish 130	<i>Polynemusparadiseus</i>	Polynemidae	Taposhi	PaikariMotshoArot, Barishal	22°42'13.4"N 90°22'32.4"E	27-01-2021	LC823339
MHBSFMSTU Fish 134	<i>Tariqilabeo latius</i>	Cyprinidae	Kalobata	Shampurnagarghat, Rajshahi	24°20'55.7"N 88°39'26.5"E	22-12-2020	LC823340
MHBSFMSTU Fish 135	<i>Psilorhynchus sucatio</i>	Psilorhynchidae	Titari	CharlokkhipurShibgonj, Chapainawabgonj	24°35'56.2"N 88°05'20.1"E	21-12-2020	LC823341
MHBSFMSTU Fish 136	<i>Glossogobius giuris</i>	Gobiidae	Bele	Narayanpur, Shibgonj, Chapainawabgonj	24°34'05.7"N 88°05'48.8"E	21-12-2020	LC823342

(Continued)

Table 1. (Continued)

Voucher Number	Scientific Name	Family	Local Name	Location	Latitude and Longitude	Sample Collection Date	Accession number
MHBSFMSTU Fish 137	<i>Setipinna phasa</i>	Engraulidae	Pasha	Charlokkhipur, Shibgonj, Chapai Nawabgonj	24°35'56.2"N 88°05'20.1"E	21-12-2020	LC823343
MHBSFMSTU Fish 138	<i>Terapon jarbua</i>	Terapontidae	Rekha	Shondhya bazar, Moylaputa, Khulna	22°48'46.7"N 89°33'25.6"E	28-01-2021	LC823344
MHBSFMSTU Fish 139	<i>Cynoglossus</i> sp.	Cynoglossidae	Kukurjib	RupshaGhat, Khulna	22°48'05.5"N 89°34'52.1"E	28-01-2021	LC823345
MHBSFMSTU Fish 140	<i>Maculabatis pastinacoides</i>	Dasyatidae	Shaplapata	PaikarimothsoArot, Barishal	22°42'13.4"N 90°22'32.4"E	27-01-2021	LC823346
MHBSFMSTU Fish 141	<i>Stigmatogobius sadanandio</i>	Gobiidae	Nondibaila	RupshaGhat, Khulna	22°48'26.0"N 89°34'49.5"E	28-01-2021	LC823347
MHBSFMSTU Fish 142	<i>Acentrogobius viridipunctatus</i>	Gobiidae	Shobujfutkibaila	Sondha bazar, Rupsha, Khulna	22°48'47.1"N 89°33'25.0"E	28-01-2021	LC823348
MHBSFMSTU Fish 143	<i>Apocryptes bato</i>	Gobiidae	Chiring	Sondha bazar, Rupsha, Khulna	22°48'47.1"N 89°33'25.0"E	28-01-2021	LC823349
MHBSFMSTU Fish 145	<i>Trypauchen vagina</i>	Gobiidae	Raja chewa	RupshaGhat, Khulna	22°48'05.5"N 89°34'52.1"E	28-01-2021	LC823350
MHBSFMSTU Fish 146	<i>Platycephalusindicus</i>	Platycephalidae	Mur baila	RupshaGhat, Khulna	22°48'05.5"N 89°34'52.1"E	28-01-2021	LC823351
MHBSFMSTU Fish 147	<i>Eleutheronematetradactylum</i>	Polynemidae	Tailla	RupshaGhat, Khulna	22°48'05.5"N 89°34'52.1"E	28-01-2021	LC823352
MHBSFMSTU Fish 148	<i>Mystusgulio</i>	Bagridae	Nunatengra	PaikarimothsoArot, Barishal	22°42'13.4"N 90°22'32.4"E	27-01-2021	LC823353
MHBSFMSTU Fish 149	<i>Sillaginopsis domina</i>	Sillaginidae	Kulardati/ Tulardanti	PaikarimothsoArot, Barishal	22°42'13.4"N 90°22'32.4"E	27-01-2021	LC823354
MHBSFMSTU Fish 150	<i>Toxoteschatareus</i>	Toxotidae	Deha	RupshaGhat, Khulna	22°48'05.5"N 89°34'52.1"E	25-01-2021	LC823355
MHBSFMSTU Fish 151	<i>Arius gagora</i>	Ariidae	Jonglitengra	RupshaGhat, Khulna	22°48'05.5"N 89°34'52.1"E	28-01-2021	LC823356
MHF154	<i>Puntius sophore</i>	Cyprinidae	Chela puti	HaziShariotullah Market, Faridpur	23°36'04.4"N 89°49'51.1"E	26-01-2021	LC823357
MHBSFMSTU Fish 156	<i>Gagatagagata</i>	Sisoridae	Gang tengra/ Deshitengra	PaikariMotshoArot, Barishal	22°42'13.4"N 90°22'32.4"E	27-01-2021	LC823358
MHBSFMSTU Fish 157	<i>Awaous</i> sp.	Gobiidae	Chewa/Bele	Tepakhula Bazar, Faidpur	23°36'48.0"N 89°51'22.8"E	26-01-2021	LC823359
MHBSFMSTU Fish 158	<i>Glossogobius giuris</i>	Gobiidae	Lalbele	Tepakhula Bazar, Faidpur	23°36'48.0"N 89°51'22.8"E	26-01-2021	LC823360
MHBSFMSTU Fish 159	<i>Glossogobius giuris</i>	Gobiidae	Bele	Tepakhula Bazar, Faidpur	23°36'48.0"N 89°51'22.8"E	26-01-2021	LC823361
MHBSFMSTU Fish 161	<i>Acanthopagruslatus</i>	Sparidae	Datina	Chakaria, Chittagong	21°45'04.2"N 92°01'11.0"E	26-01-2021	LC823362
MHBSFMSTU Fish 163	<i>Coiliaramcarati</i>	Engraulidae	Holudolua	Chakaria, Chittagong	21°45'04.2"N 92°01'11.0"E	27-01-2021	LC823363
MHBSFMSTU Fish 164	<i>Nandus nandus</i>	Nandidae	Meni	Ilisharot, Rangamati	22°38'54.8"N 92°11'05.0"E	30-01-2021	LC823364
MHBSFMSTU Fish 301	<i>Amblypharyngodon mola</i>	Cyprinidae	Lalmola	Mawaghat, Munshigonj	23°27'59.3"N 90°17'22.1"E	08-01-2020	LC823365
MHBSFMSTU Fish 302	<i>Awaous</i> sp.	Gobiidae	Bele Mach	Tepakhula Bazar, Faidpur	23°36'48.0"N 89°51'22.8"E	26-01-2021	LC823366

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Phylogenetical analysis

The resulting COI gene nucleotide sequences were matched with additional COI sequences of Bangladeshi fish from INSD [14] and the MAFFT (Multiple Alignment using Fast Fourier Transform) v. 7.427 with the L-INS-I option [21]. Phylogenetic trees were inferred using maximum likelihood (ML) and Bayesian inference (BI) techniques. IQ-TREE v. 1.6.12 was used to estimate nucleotide substitution models for ML and BI analyses using AICc [22]. The IQ-TREE was used to determine the ML phylogeny, and 1000 pseudo replicates were used for an ultra-fast bootstrapping (BS). MrBayes v. 3.2.6 was used to obtain the Bayesian posterior probabilities and BI tree [23]. A tree sample was obtained after every 1000 generations, throughout two separate runs of four Markov chains totaling 100 million generations. Tracer v. 1.7.1 [24] was used to examine the parameter estimates and convergence, and the first 10% trees were eliminated in light of the findings. MEGA11's pairwise-deletion option was used for computing pairwise comparisons of the uncorrected p-distance [25] (S1 Table).

Species delimitation analyses and morphology measurement

Species delimitation investigations were conducted using Assemble Species by Automatic Partitioning (ASAP) [26] based on 712 COI sequences used in phylogenetic analysis (S2 Table). The ASAP analysis was conducted using the online ASAP version (<https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html>) using the uncorrected pairwise distances with the default parameters. We looked at morphological parameters under both a microscope and a magnifying glass. We only took morphometric measurements of the chosen cryptic fish using a digital vernier caliper (Mitutoyo, accuracy ± 0.02 mm).

Ethics statement

The specimens were obtained from either from markets or fishermen, or they were collected from the wild using a beach seine or hand net. The fish were then euthanized by immersion in buffered tricaine-methanesulphonate (MS 222) for thirty minutes after the animals stopped moving, in accordance with the guidelines outlined in the permits issued by Jamalpur Science and Technology University's Ethics Committee (Letter no. 37.01.0044.064.05.001.24.553).

Results

Fish specimens were gathered nationwide from more than 28 locations (Table 1), and analyzed the mitochondrial COI sequences of 124 individuals representing 121 species, 94 genera, 39 families, and 19 orders. Sequences with ≥ 1 nucleotide change were considered distinct haplotypes. Our COI alignment matrix consisted of 655 bp representing 119 haplotypes. Table 1 provides a summary of all haplotypes. Based on COI sequences, nearly identical topologies were observed in the ML ($\ln L = -59788.54$) and BI ($\ln L = -60808.92$) trees (S1 Fig).

Uncorrected p-distance and species delimitation analysis demonstrated 243 groups with the best score. Most of the OTUs (operational taxonomy units) sequenced in this study were assigned to groups with known species; however, some specimens formed their own unique groups. In particular, seven OTUs had to be assigned to different groups, despite the presence of the same species identified morphologically, indicating that these specimens were cryptic species (Table 2).

Specifically, the specimen of *Ailia* sp. (Ailiidae) had been morphologically identified as *Ailia coila*, but genetically it was found to belong to a different species (*Ailia* sp.) compared to its nearest congener (COI divergence 8.2%) (Fig 1). This specimen was collected from the area adjacent to the Jamuna River (Sariakandi, Bogura). Our examined specimens of genus *Awaous*

Table 2. Possible cryptic species found in this study.

Voucher Number	Scientific Name (Family)	Morphological Characteristics	Total length (mm)	Ratios	Accession Number
MHBSFMSTU Fish 3	<i>Ailia</i> sp. (Ailiidae)	1. Body elongate, deeply compressed and silvery in color 2. Four pairs of barbells present. 3. Adipose fin present. 4. Lateral line extends up to tip of the caudal fin.	78	TL:SL=7.8:7.2 TL:HB=7.8:1.3 TL:LBD=7.8:0.4 TL:HL=7.8:1.1 TL:ED=7.8:0.3 TL:PROL=7.8:0.2 TL:PTOL=7.8:0.6	LC823245
MHBSFMSTU Fish 6	<i>Awaous</i> sp. (Gobiidae)	1. Elongate, sub-cylindrical anteriorly, compressed posteriorly 2. Head obtusely convex and mouth little oblique 3. Spiny dorsal fin. 4. Opercle scaled.	99	TL:SL=9.9:8.2 TL:HB=9.9:1.9 TL:LBD=9.9:0.8 TL:HL=9.9:2.1 TL:ED=9.9:0.4 TL:PROL=9.9:0.7 TL:PTOL=9.9:1.0	LC823247
MHBSFMSTU Fish 17	<i>Lepidocephalichthys</i> sp. (Cobitidae)	1. Caudal fin rounded; a light band extends from snout to caudal 2. Deep black spots present pectoral region and these spots become irregular at the base of caudal peduncle. 3. Small pelvic fin present.	69	TL:HB=6.9:1.2 TL:LBD=6.9:0.8 TL:HL=6.9:1.1 TL:ED=6.9:0.2 TL:PROL=6.9:0.4 TL:PTOL=6.9:1.2	LC823258
MHBSFMSTU Fish 56	<i>Ophichthys</i> sp. (Synbranchidae)	1. Body cylindrical, elongated with rounded abdomen. 2. Small eye and head not conspicuous. 3. Tail tapering and compressed. 4. Upper jaw longer, fleshy lips.	256	TL:LBD=25.5:1.3 TL:HL=25.5:0.5 TL:ED=25.5:0.1 TL:PROL=25:0.3	LC823288
MHBSFMSTU Fish 83	<i>Erethistes</i> sp. (Sisoridae)	1. Head depressed and flattened ventrally. 2. Dorsal profile arched and ventral profile nearly horizontal. 3. Mouth small inferior and upper jaw little longer. 4. Four pairs of barbells and pectoral girdle present	57	TL:SL=5.7:4.7 TL:HB=5.7:1.1 TL:LBD=5.7:0.3 TL:HL=5.7:1.5 TL:ED=5.7:0.1 TL:PROL=5.7:0.8 TL:PTOL=5.7:0.6	LC823309
MHBSFMSTU Fish 100	<i>Schisturasp.</i> (Nemacheilidae)	1. Snout conical and abdomen rounded. 2. Eyes dorso-lateral. 3. Nostril in front of eye separated by valve. 4. Caudal fin weakly forked.	38	TL:SL=3.8:3.3 TL:HB=3.8:0.5 TL:LBD=3.8:0.1 TL:HL=3.8:0.6 TL:ED3.8:0.1 TL:PROL=3.8:0.2 TL:PTOL=3.8:0.3	LC823323
MHBSFMSTU Fish 126	<i>Cynoglossus</i> sp. (Cynoglossidae)	1. Body elongated, flat and tapering posteriorly. 2. Eyes close together, on the left side of the body, upper one is advanced compared to lower one 3. Snout obtusely pointed. 4. Dorsal and anal fins confluent with caudal.	291	TL:SL=29.1:27.2 TL:HB=29.1:6.1 TL:LBD=29.1:0.2 TL:HL=3.8:4.8 TL:ED=29.1:0.5 TL:PROL=29.1:0.9 TL:PTOL=29.1:3.4	LC823337

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formed clades with other *Awaous* sp. (Gobiidae) and demonstrated poor degrees of divergence (COI divergence 0.3%) (Fig 2). These specimens were collected from the Bogura and Faridpur districts which are linked with the rivers Jamuna and Padma. *Lepidocephalichthys* sp. (Cobitidae) collected from Netrokona (24°41'31.3"N 90°51'46.5"E), formed clades with other known sequences (MK572291, MK572294) and demonstrated 15.50% COI divergence from its nearest congeners (Fig 1). *Ophichthys* sp. (Synbranchidae) demonstrated 7.7% genetic divergence from its near congeners (Fig 1). *Erethistes* sp. (Sisoridae), belonging to the genus

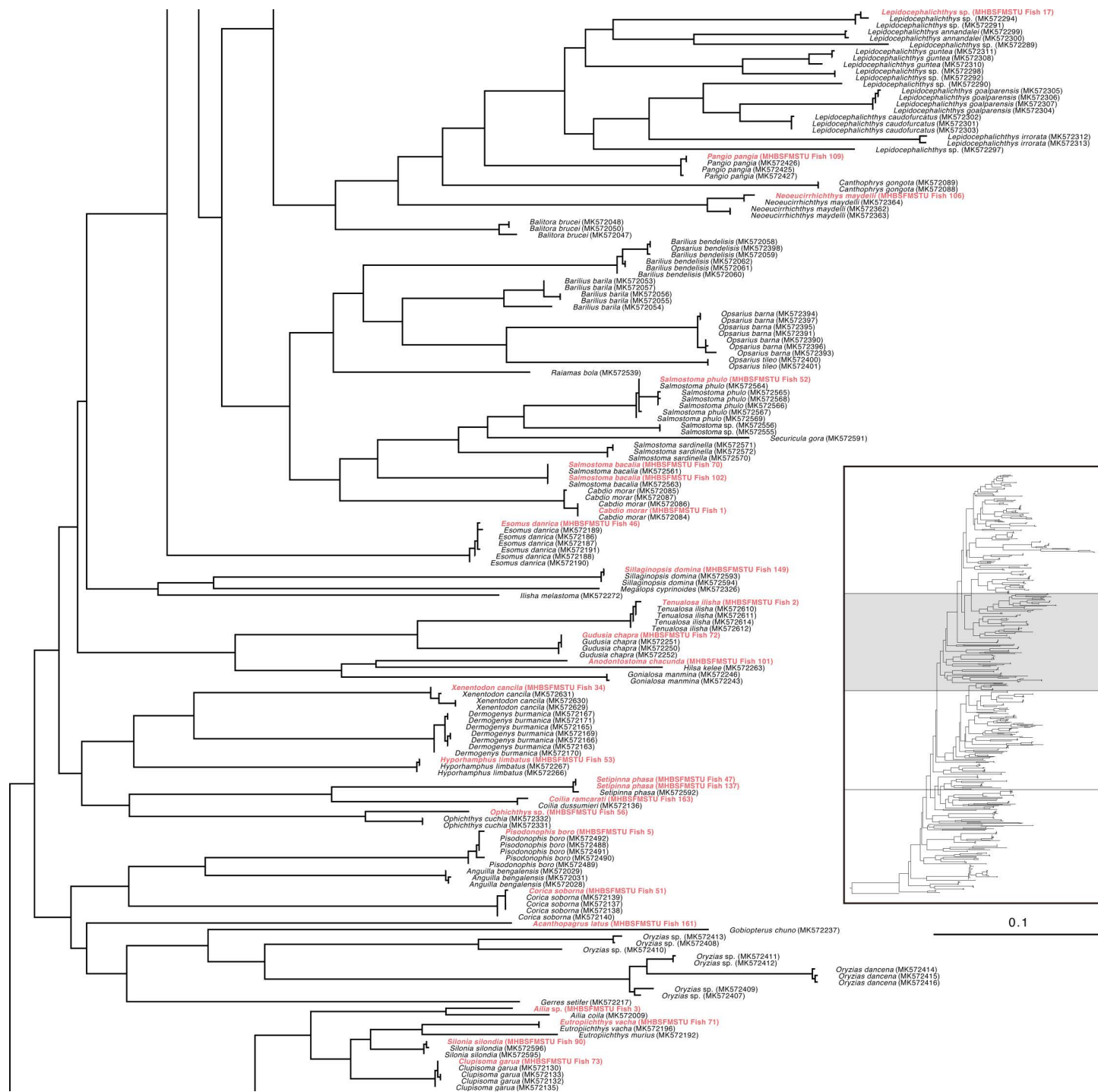


Fig 1. Maximum Likelihood (ML) tree of freshwater species of Bangladesh based on nucleotide sequences of COI gene (partial portion of S1 Fig).

<https://doi.org/10.1371/journal.pone.0318982.g001>

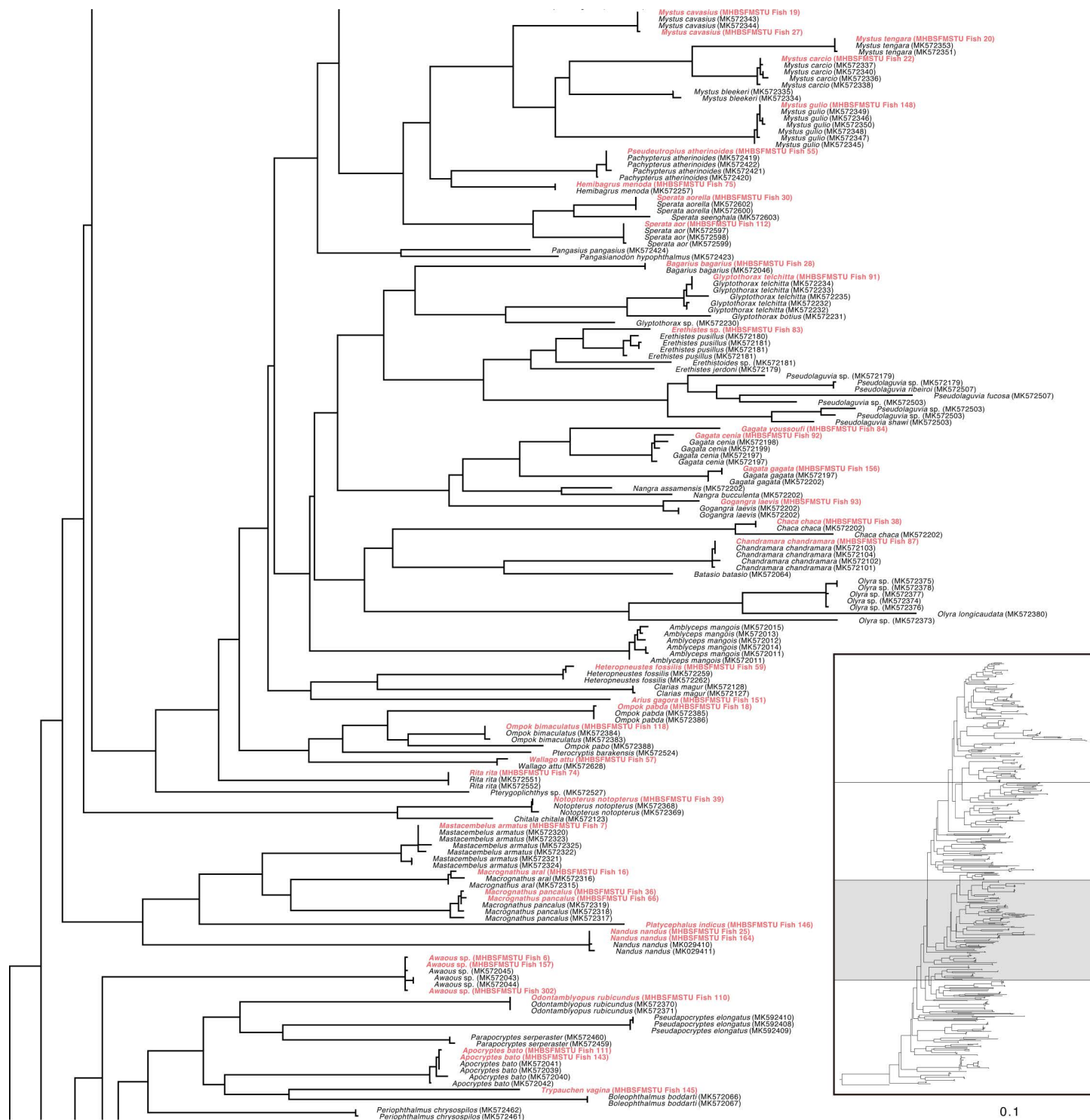


Fig 2. Maximum Likelihood (ML) tree of freshwater species of Bangladesh based on nucleotide sequences of COI gene (partial portion of S1 Fig).

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Erethistes, exhibited a genetic variation of more than 5.9% from the most closely related *E. pusilus* (Fig 2). This specimen was collected from the Netrokona region. Similarly, *Schistura* sp. under the family Nemacheilidae (collected from Sakal Bazar, Jamalpur), belonging to the genus *Schistura* demonstrated a genetic variation of more than 9.2% from its closest related species *Schistura corica* (Fig 3). Two specimens of genus *Cynoglossus*, collected from Khulna and Chittagong close to the coastal belt in Bangladesh, identified as *Cynoglossus* sp. (Cynoglossidae) were found to be distantly related (COI gene divergence 17.52%) with their congeners *Cynoglossus* (MK572284) (Fig 4). The graphic quality of seven cryptic species is subpar, yet we have included images here for enhanced clarification and comprehension (see Fig 5).

Discussion

Bangladesh is home to 265 [4] or 293 freshwater species, including coastal fishes, since no physiological alterations transpire during their journey [27]. Rahman et al. [14] collected 694 specimens nationwide, representing 243 species-level Operational Barcode Units (OBUs), although our sampled specimens are fewer than their findings. The absence of anticipated species in our aquatic environments was one of the problems we faced throughout the sampling procedure. The current sample size is extensive and includes the majority of available species; nonetheless, it is likely incomplete.

Our research identified seven cryptic fish species in the northeastern areas of Bangladesh and has contributed to a more thorough method of clarifying species delimitation based on COI gene. Although Rahman et al. [14] developed the DNA barcoding system, our work largely differs from their work with few overlapping aspects due to the study's inadequate coverage of Bangladesh's western and southwestern regions, primarily the Sundarbans and a fig of river Ganga and its tributaries. We attempted to cover the Sherpur region and some selected areas of the Mymensingh region as well as the exclusive region of the Old Brahmaputra River, which is included in the Ganga tributaries. Although specimens from the Khulna (Sundarban) area are limited, we have included samples from this location (see Table 1). Despite the several investigation based on molecular data [14,28,29], the presence of overlooked species of freshwater fishes in this study indicates that the hidden diversity among well-known and widely distributed fish species in Bangladesh might be more prevalent than previously believed. Despite being a low-lying country, Bangladesh's robust river systems impede gene flow and affect the population structure of *Hoplobatrachus tigerinus* frogs [30]. Further, both intrinsic species characteristics, such as dispersal capability and habitat specialization, and extrinsic environmental factors, such as physical obstructions and geographic separation, often determine the level of population connectivity and gene flow in the freshwater fishes [31].

The occurrence of cryptic, spatially dictated variety in fish species in Bangladesh, as indicated in this study, is not a recent revelation. Hasan et al. [2] examined the significant cryptic anuran biodiversity in Bangladesh utilizing 16S mitochondrial DNA (mtDNA); however, Raman et al. [14] contended the taxonomic ambiguity and advocated for the use of DNA barcoding (COI sequence) for the taxonomic revision of Bangladeshi freshwater fish species. We employed the COI gene in conjunction with restricted morphological data to identify the cryptic species. Identifying species merely based on physical features may prove challenging, especially when their phenotypes exhibit considerable variation [32]. Moreover, precise taxonomic identification is sometimes unattainable due to the reliance on species identification keys, which are often effective only at specific life stages. Researchers have used DNA as an alternative diagnostic tool for species, regardless of the integrated taxonomic approach [14,33]. Generally closely related vertebrate species consistently demonstrate over



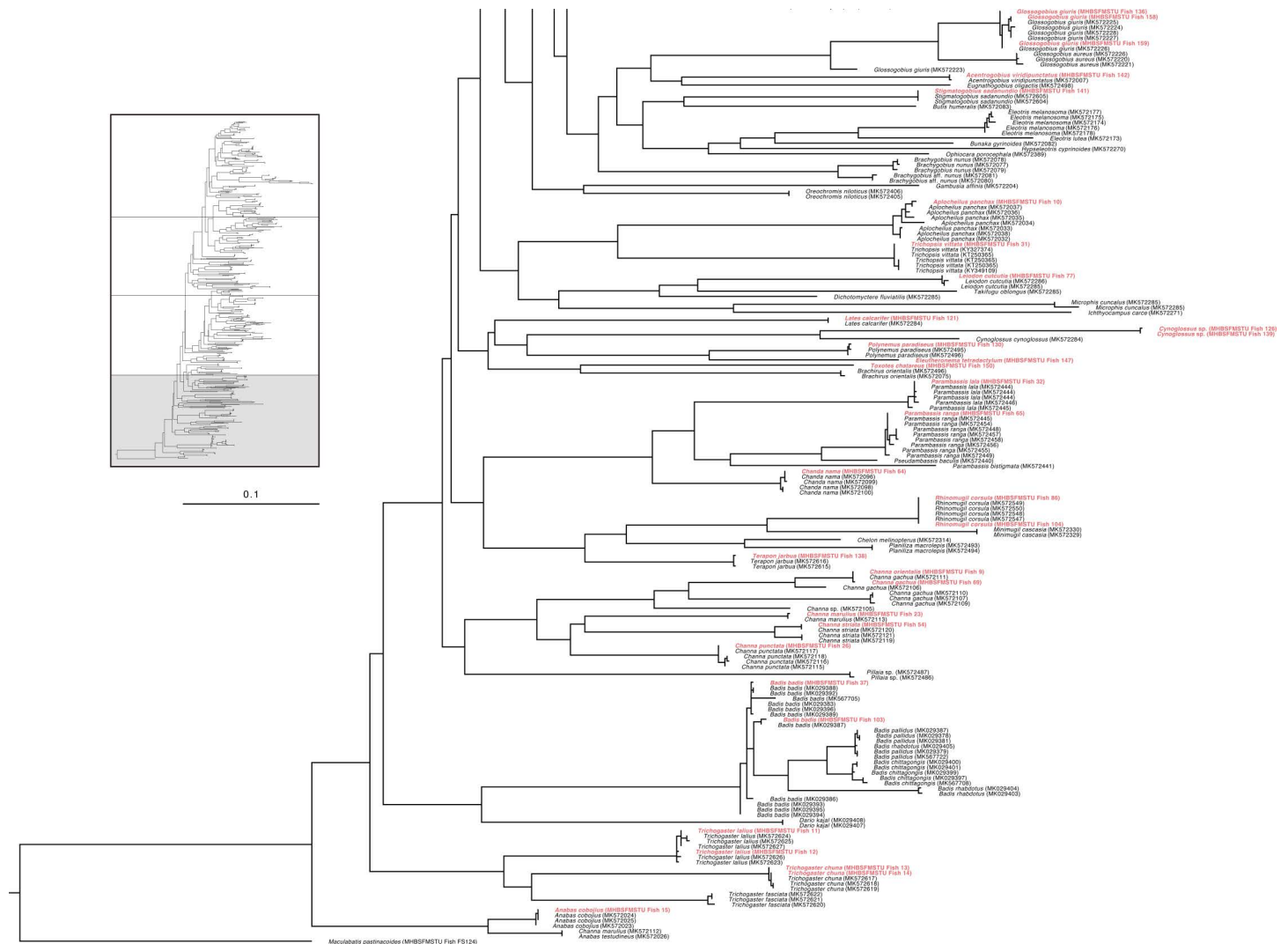


Fig 4. Maximum Likelihood (ML) tree of freshwater species of Bangladesh based on nucleotide sequences of COI gene (partial portion of S1 Fig).

<https://doi.org/10.1371/journal.pone.0318982.g004>

2% divergence in the mitochondrial cytochrome b gene [34] and COX1 gene for conspecific genetic divergence in fish [35]. Fouquet et al. [36] demonstrated that a 3% cutoff value for 16S mtDNA yielded a greater number of potential neotropical frog species. In this work, intra-specific genetic divergence exceeds the minimum intraspecific distance of 3% (the COI gene), which is higher than the threshold value of 2% for fish species identification via DNA barcoding [32,37].

Based on the results of this biodiversity study, it is tempting to make conjectures about the extent of unexplored freshwater fish diversity that still exists in Bangladesh. There might still be several undiscovered cryptic species in Bangladesh. Our investigation involving the analysis of the COI gene has revealed the presence of seven likely cryptic species. These species are genetically distinct from their closely related counterparts. *Ailia* sp. is often wrongly identified as its closely related species, *Ailia coila*. Our preliminary morphological (Table 2) and genetic findings indicate that *Ailia* sp. is cryptic and necessitates further investigation, utilizing multiple datasets, to establish its classification as a new species. Other genera, such as *Awaous*, *Lepidocephalichthys*, *Ophichthys*, *Erethistes*, *Schistura*, and *Cynoglossus*, contain cryptic species

as well. Furthermore, the specimens we studied are poorly preserved, with some having lost their scales and/or fins. The key morphological features of each proposed cryptic species need further check and investigation with the type locality specimen whether our speculated cryptic species have any diagnosable character or not. Because, in this study we just focused cryptic species based on molecular data along with limited morphological feature which does not give any guarantee that they are each a new species. Additionally, digital photos must be captured to accurately verify the identity.

In this study, we identified several COI sequences and connected them to previously identified species, but they have recommended that the most significant aspect of the DNA barcoding is that the end users can access it and can alter taxonomies to help with identification, nomenclature, species delimitation, introgression, and multiple mitochondrial lineages [14]. However, it appears that this was only the beginning of the barcoding effort. As a result, this study is an extra effort to produce more DNA barcode data and facilitate specimen identification. A major portion of Bangladesh's small indigenous species is a primary source of protein. Together with the recent developed deep learning-based techniques [38] and molecular-morphology based species/genus identification [39] our DNA barcode library can assist the accurate species identification and contribute to their proper management for conservation. To prevent a potential wave of extinctions, we must quickly curtail anthropological activities that are detrimental to freshwater fish populations [40]. Immediate habitat restoration and species-specific conservation efforts are essential to save our valuable fish species and fisheries biodiversity in Bangladesh.

Conclusion

Annotated species lists are essential for creating appropriate conservation policies. Our findings of seven cryptic species (Table 2, Fig 5) provide additional information regarding the freshwater species check list in Bangladesh. To figure out the taxonomic status of the

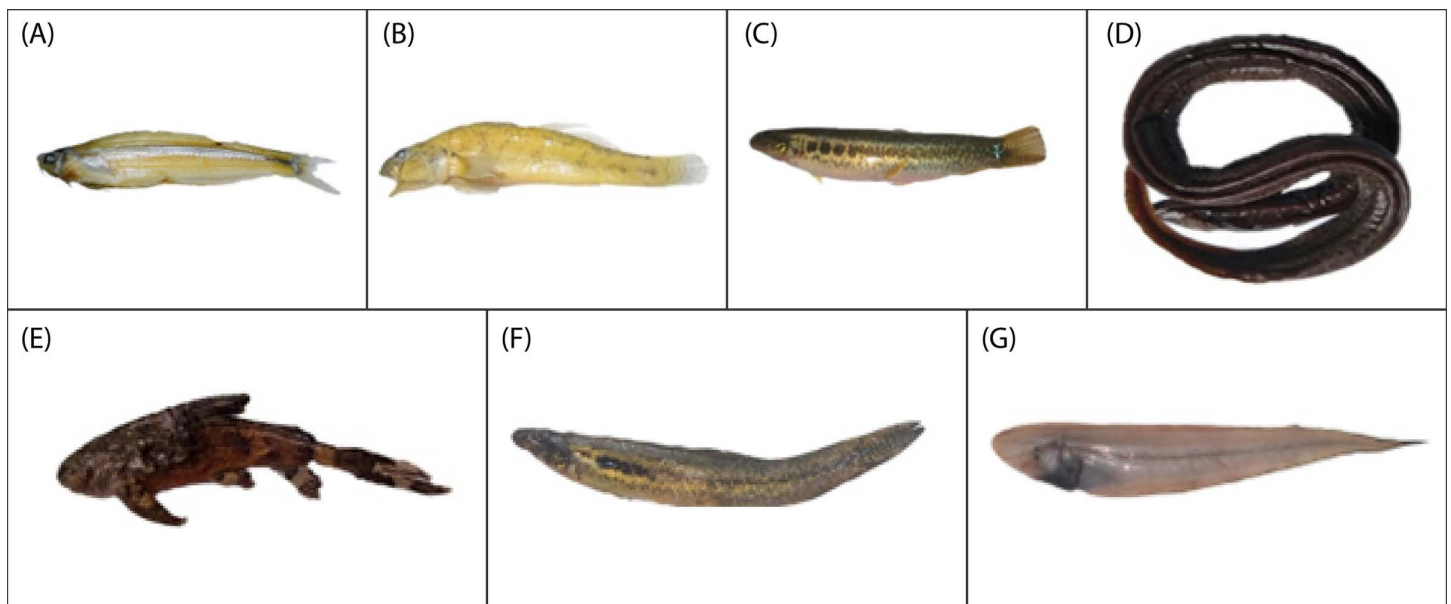


Fig 5. Seven possible cryptic species found in this study. (A) *Ailia* sp. (Ailiidae), (B) *Awaous* sp. (Gobiidae), (C) *Lepidocephalichthys* sp. (Cobitidae), (D) *Ophichthys* sp. (Synbranchidae), (E) *Erethistes* sp. (Sisoridae), (F) *Schistura* sp. (Nemacheilidae) and (G) *Cynoglossus* sp. (Cynoglossidae). Not in scale.

<https://doi.org/10.1371/journal.pone.0318982.g005>

seven species that were found in this study but haven't been named yet, more comprehensive sampling that combines morphological and DNA data would need to be done. Researchers and policymakers can utilize the results of our study, which reveals that Bangladesh is home to many cryptic species, as baseline data.

Supporting information

S1 Fig. Maximum Likelihood (ML) tree of freshwater species of Bangladesh based on nucleotide sequences of COI gene.

(TRE)

S1 Table. Uncorrected *P* distance among the analyzed taxa.

(CSV)

S2 Table. Result of species delimitation analyses.

(TXT)

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