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1 # Fish diversity in a doubly landlocked country - a description of the
2 # fish fauna of Uzbekistan using DNA barcoding

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18 **Abstract**

19 Uzbekistan is one of two doubly landlocked countries in the world, where all rivers are endorheic
20 basins. Although fish diversity is relatively poor in Uzbekistan compared to other regions, the
21 fish fauna of the region has not yet been fully studied. The aim of this study was to establish a
22 reliable barcoding reference database for fish in Uzbekistan. A total of 666 specimens, belonging
23 to 59 species within 39 genera, 16 families, and 9 orders, were subjected to polymerase chain
24 reaction amplification in the barcode region and sequenced. The length of the 666 barcodes was
25 682 bp. The average K2P distances within species, genera, and families were 0.22%, 6.33%, and
26 16.46%, respectively. The average interspecific distance was approximately 28.8 times higher
27 than the mean intraspecific distance. The Barcode Index Number (BIN) discordance report
28 showed that 666 specimens represented 55 BINs, of which five were singletons, 45 were
29 taxonomically concordant, and five were taxonomically discordant. The barcode gap analysis
30 demonstrated that 89.3% of the fish species examined could be discriminated by DNA
31 barcoding. These results provide new insights into fish diversity in the inland waters of
32 Uzbekistan and can provide a basis for the development of further studies on fish fauna.

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33 **Introduction**

34 Spanning more than 35,800 species [1], fish account for half of all extant vertebrate species and
35 are well known for their uneven distribution of species diversity [2]. Consequently, fish
36 constitute a significant component of biodiversity in the composition of animal taxa [3, 4].
37 Additionally, they have direct economic value and are important sources of animal protein for
38 humans [5, 6]. However, every year the richness and abundance of fish biodiversity in aquatic
39 ecosystems become more vulnerable, owing to human disturbances [7, 8]. Although
40 approximately 400 new fish species have been described annually over the past 20 years [1],
41 anthropogenic impacts, such as water pollution from plastic and other household waste, river
42 dams, water withdrawal, overfishing, poaching, and habitat degradation have resulted in a
43 catastrophic loss of fish diversity [9-11]. In-depth taxonomic studies of species are key to
44 conserving biodiversity.

45 Generally, fish species identification and taxonomy rely on morphometric and meristic
46 characteristics, such as body shape, the number of fin rays or lateral line scales, allometric
47 features, and colour patterns. However, morphological characters are not always stable during
48 various developmental stages and often cannot be assessed in incomplete samples or rare and
49 cryptic species. Moreover, fish identification can be challenging, owing to the similar
50 morphology of congeners during their early life histories as well as due to contradictions in the
51 existing literature and taxonomic history; this is true even if experienced taxonomists work with
52 whole intact adults. In addition, different taxonomists may have different identification abilities
53 and skills, thus even the same specimen may be identified inconsistently, thereby resulting in
54 confusion when summarising and comparing data [12-14]. However, environmental and
55 conservation studies call for a high level of accuracy, requiring specimens to be identified

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56 entirely at the species level [15]. The inherent limitations of morphology-based taxonomy and
57 the decreased number of taxonomists require molecular approaches for fish species identification
58 [16].

59 Molecular identification, which identifies species using molecular markers, is widely used
60 today. Among the various molecular approaches used for species molecular identification, DNA
61 barcoding based on mitochondrial DNA (mtDNA) is one of the most suitable tools for species-
62 level identification [17, 18]. In addition, mtDNA-based molecular identification has several
63 advantages over morphological approaches. First, species identification does not require
64 complete specimens; however, a tiny piece of tissue such as muscle, skin, fin, or teeth is
65 acceptable for DNA extraction [18-20]. Second, DNA is more stable than morphological
66 characters and is more resistant to degradation. For example, DNA can be extracted from water
67 and soil previously occupied by an organism, or from samples that have been processed or
68 digested [21-24]. Third, it is difficult to distinguish some species with similar morphological
69 characteristics, such as cryptic or sibling species. Molecular identification can help accurately
70 distinguish among such species [25, 26]. Fourth, DNA is invariable throughout the
71 developmental stages of an organism. In contrast, morphological characters can change during a
72 life cycle, thereby leading to species misidentification [12]. Therefore, molecular approaches can
73 be applied in the identification of fish eggs, larvae, juveniles, and adults [13, 27]. Fifth,
74 becoming a professional traditional taxonomist requires a lot of time, work, and resources [28,
75 29]. Advances in technology make it fairly easy to replicate and read DNA sequences, while
76 bioinformatic software can automatically compare the resulting sequences; therefore, the training
77 required to approach molecular identification is much less than that required for morphological
78 identification. Molecular identification is widely used in a number of other fields besides species

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79 identification, including illegal species trade, food fraud, biological invasions, and biodiversity
80 monitoring [30-33].
81 If mitochondrial DNA contains 37 genes, a number of mitochondrial genes, such as 12S
82 ribosomal RNA (12S), 16S rRNA (16S), cytochrome b (CYTB), and control region (D-loop
83 region), have been used as genetic markers for molecular identification [34-36]. Hebert et al. [17]
84 pioneered the use of cytochrome c oxidase subunit I (COI) for molecular species identification,
85 showing that this genetic marker can serve as a DNA barcode for biological identification in both
86 invertebrates and vertebrates [18, 25, 37-39]. The Fish Barcode of Life Initiative (FISH-BOL) is
87 an international research collaboration aimed at creating a standardised reference library of DNA
88 barcodes for all fish species [40, 41]. The main goal of this project is to enable the identification
89 of fish species by comparing the sequence of queries against the database of reference sequences
90 in the Barcode of Life Data Systems (BOLD) [42]. To date, many studies have been carried out
91 worldwide on fish DNA barcoding dedicated to FISH-BOL [3, 4, 18, 43, 44]. Compared to other
92 regions of the world, studies devoted to fish barcoding are almost absent in Central Asia.
93 Uzbekistan is one of two doubly landlocked countries in the world, where all rivers are
94 endorheic basins; therefore, fish biodiversity is poor. According to Mirabdullaev and Mullabaev
95 [45], the total number of fish species in Uzbekistan exceeds 71, including 26 fish species
96 introduced into the inland waters of the country. At the same time, the drying up of the Aral Sea,
97 which is the largest water basin in the region, global climate change, population growth, river
98 damming, water pollution, water withdrawals for agriculture, poaching, overfishing, and habitat
99 destruction, all affect the fish species in the region [46, 47]. To date, studies on piscifauna have
100 been based mainly on traditional morphological criteria and have not been comprehensively

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101 barcoded, except in our recent studies [48-50]. Recently, molecular identification has been
102 applied to identify mainly nematodes among animal species [51].
103 Consequently, the main aim of the present study was to provide the first inventory of
104 freshwater fish species in Uzbekistan based on DNA barcoding. This inventory could serve as a
105 reference for screening DNA sequences in future studies. Additionally, we assessed the genetic
106 diversity of freshwater fish species. The DNA barcode records generated in this study will be
107 available to researchers for the monitoring and conservation of fish diversity in Uzbekistan.
108
109 **Results**
110 **Morphology-based species identification** First, all collected specimens were identified using
111 morphological approaches. Morphological identification classified all samples into 59 species
112 belonging to 39 genera and 16 families that represented nine orders (Table 1). The identified
113 specimens included 50 (84.75%) species identified to the species level and nine (15.25%) species
114 that could not be identified to the species level (Tables 1 and S2). Approximately three-quarters
115 of the species (44 species, 74.58%) belonged to the order Cypriniformes. The remaining eight
116 orders included one or two species.
117 Of the 59 fish species collected from the inland waters of Uzbekistan,
118 *Pseudoscaphirhynchus hermanni* and *P. kaufmanni* were classified as critically endangered
119 (CR), *Acipenser baerii* and *Capoetobrama kuschakewitschi* were classified as endangered (EN),
120 and *Cyprinus carpio* and *Luciobarbus brachycephalus* were classified as vulnerable (VU)
121 according to International Union for Conservation of Nature's (IUCN) Red List of Threatened
122 Species. The remaining species were grouped into the least concern (LC) and data deficient (DD)
123 categories (Table 1).

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126 **Table 1.** List of the fish species of Uzbekistan using in this study

Species No.	Order	Family	Genus	Species	Sample size	IUCN status
1	Acipenseriformes	Acipenseridae	*Acipenser*	*baerii*	2	EN
3			*Pseudoscaphirhynchus*	*hermanni*	3	CR
5				*kaufmanni*	2	CR
2	Anabantiformes	Channidae	*Channa*	*argus*	6	-
4	Cypriniformes	Acheilognathidae	*Rhodeus*	*ocellatus*	21	DD
8				sp.	1	-
6		Cobitidae	*Sabanejewia*	*aurata*	27	LC
7		Cyprinidae	*Capoeta*	*heratensis*	25	-
9			*Carassius*	*auratus*	7	LC
10				*gibelio*	28	-
11			*Cyprinus*	*carpio*	9	VU
12			*Luciobarbus*	*brachycephalus*	8	VU
13				*conocephalus*	15	-
14			*Schizothorax*	*eurystomus*	38	LC
15				*fedtschenkoi*	5	LC
16				sp.	5	-
17		Gobionidae	*Abbottina*	*rivularis*	11	-
18			*Gobio*	*lepidolaemus*	18	LC
19				*nigrescens*	8	-
20				*sibiricus*	2	LC
21			*Pseudorasbora*	*parva*	11	LC
22		Leuciscidae	*Abramis*	*brama*	2	LC
23			*Alburnoides*	*holciki*	41	-
24			*Alburnus*	*chalcoides*	12	-
25				*oblongus*	8	-
26				*taeniatus*	3	DD
27			*Capoetobrama*	*kuschakewitschi*	8	EN
28			*Leuciscus*	*aspius*	1	LC
29				*lehmanni*	10	LC
30			*Pelecus*	*cultratus*	4	LC
31			*Petroleuciscus*	*squaliusculus*	10	LC
32			*Rutilus*	*lacustris*	11	-
33		Nemacheilidae	*Dzihunia*	*amudarjensis*	11	LC
34				sp. 1	11	-
35				sp. 2	3	-
36				sp. 3	8	-
37			*Paracobitis*	*longicauda*	25	-
38			*Triplophysa*	*ferganaensis*	20	-
39				*strauchii*	29	LC
40				sp. 1	4	-
41				sp. 2	4	-
42		Xenocyprididae	*Ctenopharyngodon*	*idella*	8	-
43			*Hemiculter*	*leucisculus*	25	LC
44			*Hypophthalmichthys*	*molitrix*	8	NT
45				*nobilis*	5	DD
46			*Mylopharyngodon*	*piceus*	2	DD
47			*Opsariichthys*	*bidens*	7	LC
48			*Parabramis*	*pekinensis*	13	-
49	Cyprinodontiformes	Poeciliidae	*Gambusia*	*holbrooki*	44	LC
50	Esociformes	Esocidae	*Esox*	*lucius*	3	LC
51	Gobiiformes	Gobiidae	*Neogobius*	*melanostomus*	1	LC
52				*pallasi*	1	LC
53			*Rhinogobius*	sp.	37	-
54	Perciformes	Cottidae	*Cottus*	*spinulosus*	6	LC
55		Percidae	*Sander*	*lucioperca*	10	LC
56	Salmoniformes	Salmonidae	*Oncorhynchus*	*mykiss*	2	-
57	Siluriformes	Siluridae	*Silurus*	*glanis*	8	LC

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58	Sisoridae	*Glyptosternon*	*oschanini*	1	LC
59			sp.	8	-

127 IUCN, International Union for Conservation of Nature; CR, critically endangered; EN,
128 endangered; VU, vulnerable; NT, near threatened; LC, least concern; DD, data deficient, -, no
129 assessment.

130 **Identification of fish species using DNA barcodes** A total of 666 fish samples were
131 successfully amplified using three primers and PCR. After editing, all COI barcode sequences
132 were 682 for each sample and the mean nucleotide frequencies of the entire dataset were A
133 (24.49%), T (29.01%), G (18.50%), and C (28.00%). The genetic distance within species ranged
134 from 0.000 to 0.0149.

135 For species identification at the species level, a total of 666 COI barcode sequences
136 representing 59 different species were employed (mean of 11.3 samples per species). The
137 GenBank and BOLD databases were used for species identification (Table S2). The GenBank-
138 based identification of all species ranged from 98.58% to 100.00%. The COI sequences of 22
139 fish species had not been reported in the GenBank database. Among them, *P. hermanni* was
140 identified as *P. kaufmanni, Cottus spinulosus* as *C. ricei*, *L. conocephalus* as *L. capito*, *Alburnus*
141 *oblongus* and *A. taeniatus* as *A. escherichii*, *Leuciscus lehmanni* and *Petroleuciscus*
142 *squaliusculus* as *L. baicalensis*, and *Triplophysa* sp. 1 as *T. aliensis* with 99.71%, 98.47%,
143 98.83–100%, 98.39–98.82%, 99.71–99.85%, and 98.37% similarity, respectively.

144 The BOLD-based identification of 46 fish species ranged from 98.36% to 100%. No
145 matches were found for 13 species. *Pseudoscaphirhynchus. hermanni* was identified as *P.*
146 *kaufmanni*, *Cottus spinulosus* as *C. ricei*, *A. oblongus* and *A. taeniatus* as *A. escherichii*, *L.*
147 *lehmanni* and *P. squaliusculus* as *L. baicalensis*, and *Triplophysa* sp. 1 as *T. aliensis* with 99.85–
148 100%, 98.48%, 98.62–98.92%, 99.8%–100%, and 98.36% similarity, respectively. Despite the

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149 GenBank databases, *L. conocephalus*, *Neogobius pallasi,* and *Rhinogobius* sp. were identified
150 with high similarities (> 99.4%).

151 The Taxon ID tree shows that the specimens formed phylogenetic clusters that reflected
152 previous taxonomic results based on morphology (Fig. S1). In turn, the barcode gap analysis
153 revealed that five species lacked a barcode gap (intraspecific K2P distance ≥ interspecific one),
154 and four species had a low K2P distance to another species (≤2%), which indicates that the
155 majority of the investigated species could be identified by the DNA barcode approach (Table
156 S3). Generally, the mean K2P distance of a species to its nearest neighbour (NN) was 8.04%
157 (SD: 0.11%).

158 The mean K2P distances within species, within genera, and within families were 0.22%,
159 6.33%, and 16.46%, respectively (Table 2; Fig. 1). The largest intraspecific K2P distance was
160 observed in *Opsariichthys bidens* (five specimens; Fig. 2; Table S3). The specimens obtained
161 from several species, such as *Abramis brama* (two specimens), *Capoetobrama kuschakewitschi*
162 (eight specimens), *Gobio nigrescens* (eight specimens), and *Rhinogobius* sp. (37 specimens),
163 carried the same haplotype (Table S3). The average congeneric distance was approximately 28.8
164 times higher than the mean conspecific distance, but approximately 2.6 times less than the
165 average genetic distance between families, thus the average genetic distance grew based on the
166 taxonomic level.

167 The Barcode Index Number (BIN) discordance report showed that 666 specimens
168 represented 55 BINs; among them, 45 BINs were taxonomically concordant, five BINs were
169 taxonomically discordant, and five BINs were singletons. For the best match (BM), best close
170 match (BCM), and all species barcodes (ASB) analyses of the 666 sequence data set with
171 singletons, the percentages of correct identification were 94.74%, 94.74%, and 89.03%,

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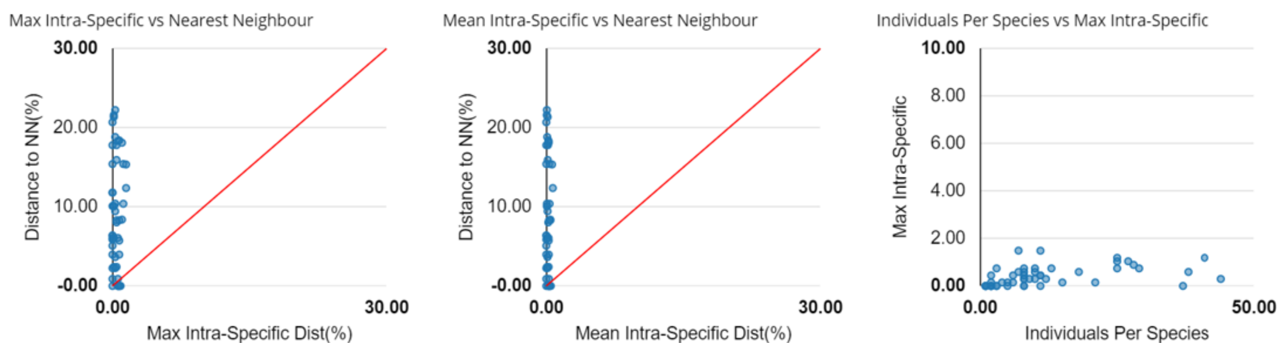
172 respectively; those of ambiguous identification were 4.05%, 4.05%, and 10.51%, respectively;
173 those of incorrect identification were 1.2%, 1.2%, and 0.44%, respectively. Moreover, for the
174 same three analyses of the dataset without singletons (661 sequences), the percentages of correct
175 identification were 95.46%, 95.46%, and 89.71%, respectively; those of ambiguous identification
176 were 3.93%, 3.93%, and 10.13%, respectively; those of incorrect identification were 0.6%, 0.6%,
177 and 0.15%, respectively (Table 3).

178 **Table 2.** Summary of K2P genetic distances (%) calculated for different taxonomic levels

				K2P genetic distance (%)		
	N	Taxa	Comparisons	Minimum	Maximum	Mean and SD
Within species	661	54	6608	0.00	1.49	0.22 ± 0.00
Within genus	309	13	2534	0.00	11.78	6.33 ± 0.00
Within family	512	3	75196	0.00	22.19	16.46 ± 0.00

179

180



181

182 **Figure 1.** Barcoding gap: Maximum intraspecific Kimura 2-parameter (K2P) distances
183 compared with the minimum interspecific K2P distances recorded in fish from Uzbekistan. The
184 graphs show the overlap of the maximum and mean intra-specific distances with the inter-
185 specific (NN = nearest neighbor) distances.

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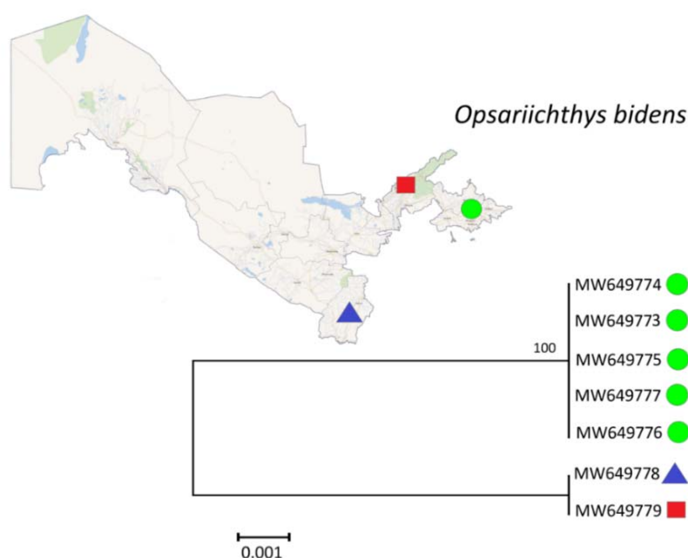

186

187 **Figure 2.** Neighbour-joining tree of *Opsariichthys bidens* from DNA barcode sequences with
188 100 000 bootstrapping replicates. Sampling localities: Syr Darya (green circle), Chirchik River
189 (red square), and Surkhan Darya (blue triangle).

190

191 **Table 3.** Results of identification success analysis for the criteria: best match, best close match
192 and all species barcodes

	Best match (%)	Best close match (%)	All species barcodes (%)
With singletons			
Correct identifications	631 (94.74%)	631 (94.74%)	593 (89.03%)
Ambiguous identifications	27 (4.05%)	27 (4.05%)	70 (10.51%)
Incorrect identifications	8 (1.2%)	8 (1.2%)	3 (0.44%)
Sequences without any match closer than threshold	NA	NA	NA
Without singletons			
Correct identifications	631 (95.46%)	631 (95.46%)	593 (89.71%)
Ambiguous identifications	26 (3.93%)	26 (3.93%)	67 (10.13%)
Incorrect identifications	4 (0.6%)	4 (0.6%)	1 (0.15%)
Sequences without any match closer than threshold	NA	NA	NA

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195 **Automated barcode gap discovery (ABGD) analyses of species delimitation** The ABGD tool
196 was used for species delimitation. A partition with prior maximal distance P = 0.0359 and 0.0046

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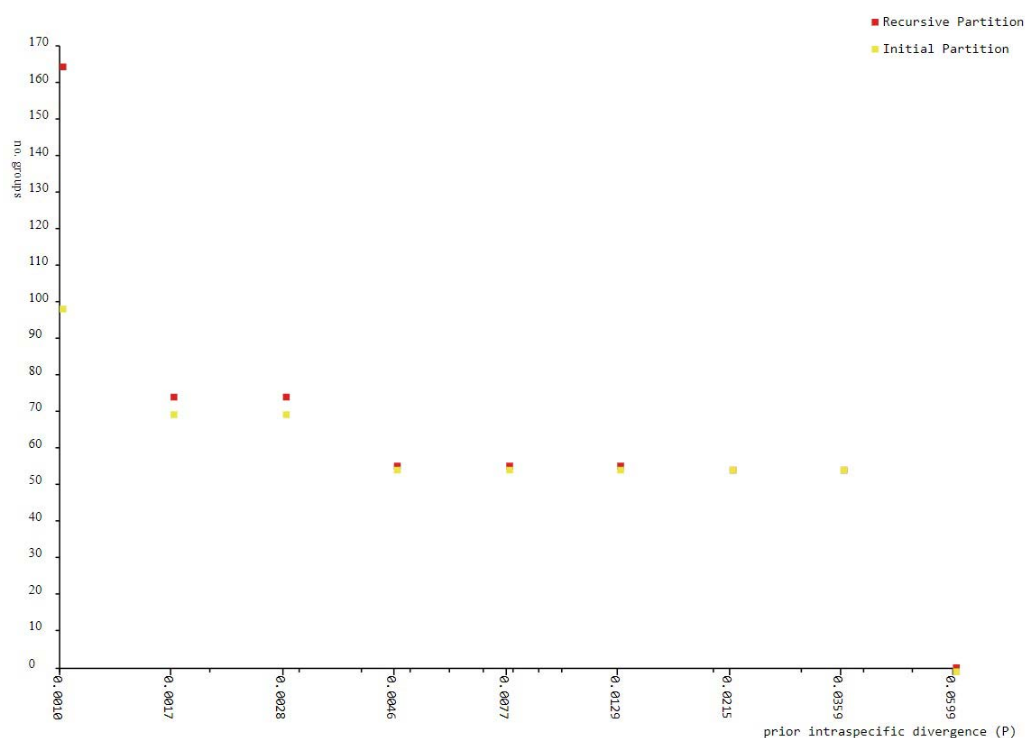
197 delimited the entire dataset into 55 putative species (Fig. 3). Of the 59 morphological-based
198 identified species, 55 (93.22%) were delimited clearly through the ABGD at a prior maximal
199 distance of 0.0359, which was consistent with the observations of genetic distance and
200 neighbour-joining (NJ) and Bayesian inference (BI) analyses (Figs. S1 and 4). Furthermore, at a
201 prior maximal distance of 0.0359, few species, such as *Carassius auratus*, *C. gibelio*, *Gobio*
202 *lepidolaemus*, *G. sibiricus*, *L. lehmanni*, *P. squaliusculus*, *P. hermanni*, and *P. kaufmanni* could
203 not be delimited into different putative species. No clear divergence between these
204 morphologically distinct species was observed in the NJ and BI analyses, with the exception of
205 *Gobio* species.
206



207
208 **Figure 3.** The number of groups inferred from ABGD analysis according to prior intraspecific
209 divergence (*P*)

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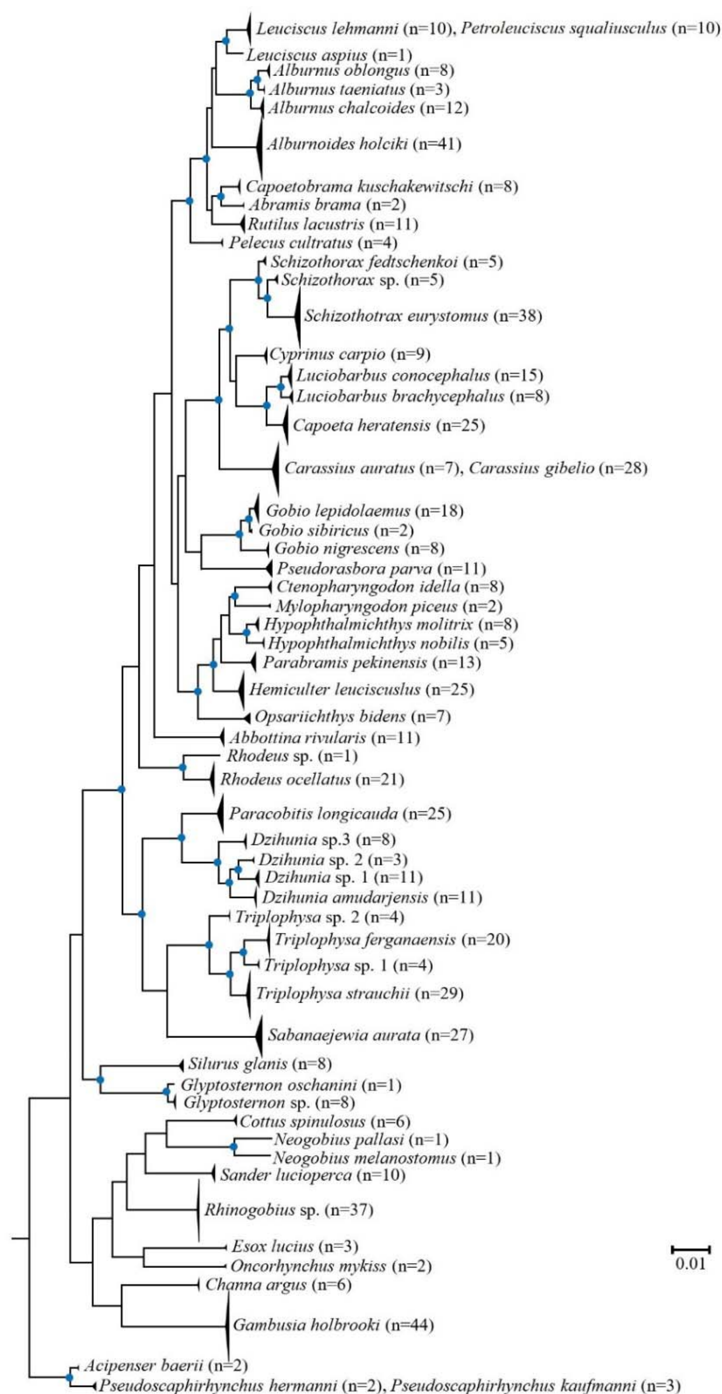

217

218 **Figure 4.** Bayesian inference (BI) consensus tree based on the COI partial gene sequences. The
219 blue circle at nodes represents BI posterior probabilities values >50%. Posterior probability
220 values for all species are >95%.

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221 **Discussion**

222 This study of the fish fauna of the inland waters of Uzbekistan is the first to compile the data in a
223 sequence library, which contributes to the FISH-BOL in the BOLD system. This study included
224 the molecular identification of 59 species. These 59 species included 83.1% of the reported fish
225 fauna of the region [45]. Relationships among species are shown in the topology of the BI tree
226 (Fig. 4).

227 The gap between COI intraspecific and interspecific diversity is called the 'barcode gap',
228 which is decisive for the discriminatory ability of DNA barcoding [52]. The barcode gap can be
229 seen in our study (Table 2), as well as in many other previous studies [3, 44, 53], thereby further
230 confirming that this approach is an effective way to distinguish between fish species.

231 This study clarified the taxonomic status of a number of taxa, such as *A. oblongus* and *A.*
232 *taeniatus*, which belong to *Alburnus*, which is consistent with the results of Matveyev et al. [54]
233 and Jouladeh-Roudbar et al. [55]; *Schizothorax fedtschenkoi* is a valid species; another
234 *Schizothorax* sp. from the southern part of the country is an undescribed species; the *Alburnoides*
235 population (previously considered as *A. eichwaldii*) from the inland waters of Uzbekistan, is de
236 facto *A. holciki* [49]; three *Gobio* species occur in the inland waters of the country [50];
237 *Glyptosternon* and *Rhodeus* each consist of two species and not just one, as previously believed;
238 thus, additional taxonomic research is required; two species of the genus *Neogobius* (*N.*
239 *melanostomus* and *N. pallasi*) (previously believed to belong to *N. melanostomus* and *N.*
240 *fluviatilis* [56]) occurred in the lower reaches of the Amu Darya; the population of *Opsariichthys*
241 in Uzbekistan belongs to the same species, and *O. bidens* is not *O. unirostis* as previously
242 believed [56]; the entire *Rhinogobius* population in Uzbekistan belongs to the same species
243 (*Rhinogobius* sp.), which is neither *R. brunneus* nor *R. similis* as previously thought [56, 57];

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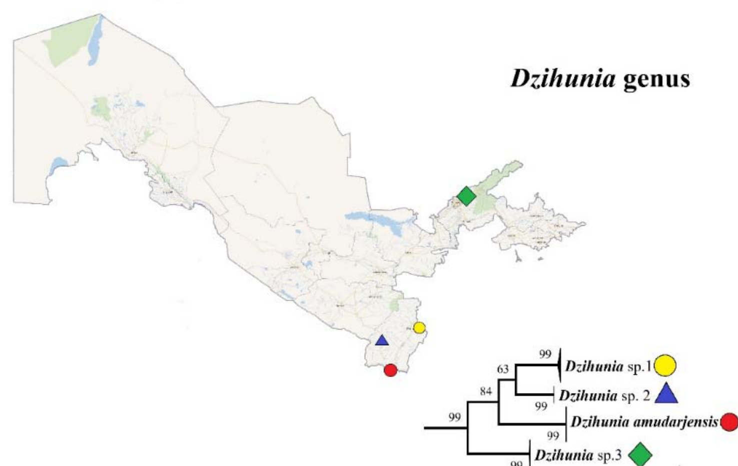
244 thus, taxonomic clarification is required; moreover, there is only one species of *Gambusia* (*G.*
245 *holbrooki*) occurring in the waters of Uzbekistan, while previously it was believed that both *G.*
246 *affinis* and *G. holbrooki* were found in the waters of the country [56, 58] (Figs S1, 4; Table S2).

247 Only a single species of *Petroleuciscus* in Central Asia from the upper reaches of the Syr
248 Darya, joined with *L. lehmanni* from the Zeravshan River in our phylogenetic analysis based on
249 the *COI* barcode marker. However, our unpublished work (nuclear molecular and morphology)
250 showed that they are two separate valid species, and *P. squaliusculus* belongs to *Leuciscus*.

251 Currently, three *Dzihunia* Prokofiev, 2001 species are found in the Amu Darya (*D.*
252 *amudarjensis*), Zeravshan (*D. ilan*), and Talas (*D. turdakovi*, outside Uzbekistan) rivers [59, 60].
253 Apparently, the species diversity of *Dzihunia* seems to be much higher than previously thought
254 (Fig. 4). In addition to *D. amudarjensis*, two more undescribed species were found in the upper
255 reaches of Amu Darya. Another undescribed species was found in the Chirchik River; however,
256 members of *Dzihunia* had not previously been found in this river (Fig. 5). On the other hand, *D.*
257 *ilan* was not found in two of our expeditions to the Zeravshan River; moreover, it is believed that
258 this species may have become extinct [59].



259

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260 **Figure 5.** Neighbour-joining tree of *Dzihunia* spp. from DNA barcode sequences with 100
261 000 bootstrapping replicates. Sampling localities: lower Surkhan Darya (red circle) upper
262 Surkhan Darya (yellow circle), Sherabad River (blue triangle) and Chirchik River (green square).
263

264 The inability of DNA barcodes to identify species may be due to incomplete sorting by
265 lineage associated with recent speciation and haplotype sharing as a result of hybridisation. In
266 our study, DNA barcodes of two *Leuciscus* and *Petroleuciscus* (*L. lehmanni* and *P.*
267 *squaliusculus*), two *Carassius* (*C. auratus* and *C. gibelio*), and two *Pseudoscaphirhynchus* (*P.*
268 *hermanni* and *P. kaufmanni*) species were sequenced, and the BIN discordance report illustrated
269 that these six species could not be distinguished by the COI barcode gene (Figs. S1 and 4). In
270 this case, a more rapidly evolving DNA fragment, such as the mitochondrial control region
271 (mtCR) or the first internal transcribed ribosomal DNA spacer (ITS1), may be better for
272 identification [3].

273 A similar situation occurred with *Carassius* species collected in the Mediterranean basin
274 [61]. In addition, among the four *Leuciscus* (*L. baicalensis*, *L. bergi*, *L. dzungaricus*, and *L.*
275 *lindbergi*) species from Central Asia, Russia, and Mongolia, no interspecific differences were
276 found based on the COI gene (J. Freyhof, personal communication). However, in
277 *Pseudoscaphirhynchus* species, no interspecies differences were found either when using other
278 rapidly evolving mtDNA markers [62], the entire mtDNA genome [63], or nDNA markers (our
279 unpublished data). In fact, these two sturgeon species are morphologically easy to distinguish
280 from each other [64]. Thus, the complete genome sequencing of *Pseudoscaphirhynchus* may be
281 important for their molecular authentication.

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282 Unexpectedly, *Abbottina rivularis* from Gobionidae is nested with members of the genus
283 *Rhodeus* from Acheilognathidae in our NJ phylogenetic tree (Fig. S1). Despite the sharp
284 differences in morphology, the fact that these two genera are sister taxa has also been observed in
285 previous studies [65, 66].

286 The global fish diversity is currently a serious threat. Along with natural limiting factors to
287 native species, the negative impact of introduced species is also increasing [67-70]. At the same
288 time, the negative impact of anthropogenic factors on the biodiversity of freshwater basins is also
289 growing [71]. The number of biological species is declining annually; therefore, DNA barcoding
290 is becoming a versatile approach that can be used to assess fish biodiversity, monitor fish
291 conservation, and manage fishery resources [72-75]. While our DNA barcoding study is
292 beneficial for the taxonomy and phylogenetics of fishes in the Amu Darya and Syr Darya basins,
293 it is also important to clarify the taxonomy of misidentified invasive species acclimatised to
294 Central Asian watersheds [58].

295 Unfortunately, fish diversity in Uzbekistan has decreased in recent years. A rare sturgeon
296 fish, *Acipenser nudiventris*, is completely extinct in the Aral Sea basin [76]. Another sturgeon
297 species endemic to the Syr Darya, *Pseudoscaphirhynchus fedtschenkoi*, has been possibly extinct
298 since the 1990s [63]. The Syr Darya population of *Capoetobrama kuschakewitschi* has not been
299 recorded in recent decades, and so far, this species has survived only in the lower reaches of the
300 Amu Darya [77]. *Gymnocephalus cernuus* and *Perca fluviatilis* have not been recorded in water
301 bodies in the country since the late 1990s [45]. Monitoring the existing populations of other rare
302 native fish species and studying the negative impact of invasive species on them is advisable.
303 The traditional monitoring of fish diversity is usually time-consuming, expensive, and labour
304 intensive. However, with an ever-expanding barcode database and advances in biotechnology

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305 (such as environmental DNA analysis), the assessment of fish diversity is becoming more
306 efficient [78-80]. As our molecular study of fishes develops in Uzbekistan, data on fish species in
307 this region will become more readily available than ever.
308

309 **Methods**

310 **Ethical Statement.** Fish sampling for this research has complied with the Law of the Republic
311 of Uzbekistan 'On the protection and use of wildlife' (No. 545-I 26.12.1997). No
312 experimentation was conducted on live specimens in the laboratory, and the work performed in
313 the laboratory followed the rules in the Guide for the Use and Care of Laboratory Animals of
314 Southwest University.
315

316 **Sample collection and morphological identification.** A total of 666 fish samples were collected
317 from February 2016 to August 2020 using gill nets or cast nets from 53 distant locations in
318 different rivers, tributaries, canals, springs, and lakes. Information about the sampling stations,
319 along with geographical coordinates and sampling dates, is given in Table S1.
320 Initially, all specimens were identified to the species level based on morphological
321 characteristics following the identification keys of Berg [64, 81] and Mirabdullaev et al. [82]. If
322 identification was not correctly assigned to a specific species, the 'sp.' and 'cf. abbreviations
323 were applied [83]. Two pieces of right pectoral fin tissue and muscle tissue were dissected from
324 each fish specimen and stored in 99% ethanol at -20 °C. Fin-clipped whole specimens and excess
325 specimens for further morphological analyses were fixed in 10% formalin. After 5–7 days they
326 were transferred to 70% ethanol for long-term storage and deposited in the Key Laboratory of
327 Freshwater Fish Reproduction and Development at the Southwest University, School of Life

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328 Sciences (China), respectively, with the exception of sturgeon species, which were deposited in
329 the Department of Biology at the Fergana State University, Faculty of Life Sciences
330 (Uzbekistan).
331
332 **DNA extraction, COI amplification, and DNA sequencing** Genomic DNA was extracted from
333 muscle or fin tissues by proteinase K digestion followed by a standard phenol-chloroform
334 method. The DNA concentration was estimated using a nano-volume spectrophotometer
335 (NanoDrop 2000; Thermo Fisher Scientific Inc., Waltham, MA, USA) and stored at -20 °C for
336 further use. Approximately 680 bp were amplified from the 5´ region of the COI gene using the
337 fish-specific primers described by Ivanova et al. [84]: FishF2_t1 TGT AAA ACG ACG GCC
338 AGT CGA CTA ATC ATA AAG ATA TCG GCA C and FishR2_t1 CAG GAA ACA GCT
339 ATG ACA CTT CAG GGT GAC CGA AGA ATC AGA A, respectively. The following primers
340 [18] were used for *Gambusia holbrooki*: FishF2-TCG ACT AAT CAT AAA GAT ATC GGC
341 AC and FishR2-ACT TCA GGG TGA CCG AAG AAT CAG AA. The following primers [85]
342 were used for sisorid catfishes: catF-TCT CAA CCA ACC ATA AAG ACA TTG G and catR-
343 TAT ACT TCT GGG TGC CCA AAG AAT CA.

344 The PCR reactions were performed in a final volume of 25 μL, containing 10–100 ng
345 template DNA, five μmol of each forward and reserve primer, while 12.5 μL of 2× *Taq* Master
346 Mix (Novoprotein, Guangdong, China) and double-distilled water were also used. The thermal
347 conditions consisted of an initial step of 3 min at 94 °C followed by 35 cycles of 0.5 min at 94
348 °C, 45 s at 54 °C, and 1 min 10 s at 72 °C, followed by a final extension of 7 min at 72 °C. The
349 reactions were performed in an Applied Biosystems thermocycler (Veriti™ 96-Well Thermal
350 Cycler, Singapore), and the PCR products were evaluated by electrophoresis using 1% agarose

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351 gel stained with BioRAD (Universal Hood II; Des Plaines, IL, USA). The PCR products were
352 sent to TsingKe Biological Technology Co., Ltd. (Chongqing) for sequencing.
353
354 **Molecular data analysis.** All sequences were manually edited using the SeqMan program
355 (DNAStar software) combined with manual proofreading; all contig sequences started at the first
356 codon position and ended at the third position; no stop codons were also detected. All obtained
357 barcodes were uploaded to the BOLD and GenBank databases, and the details are given in Table
358 S1.
359 The COI barcode sequence of each sample was identified by the scientific name or species
360 using the BLAST and BOLD databases. Specimens were classified by family, genus, and species
361 according to the fish taxonomic systems of Fricke et al. [60], and their status was checked in the
362 IUCN Red List of Threatened Species v. 2020-3. The results of species identification based on
363 the BLAST and BOLD databases are presented in Table S2.
364 We uploaded the entire data set to BOLD under project title 'Freshwater fishes of
365 Uzbekistan'. BOLD version 4 analytical tools were used for the following analyses. The distance
366 summary with the parameter setting the Kalign alignment option [86] and pairwise deletion
367 (ambiguous base/gap handling) was employed to estimate the Kimura 2-parameter (K2P)
368 distances for taxonomic ranks at the species, genus, and family levels. Barcode gap analysis was
369 carried out with the setting of the parameter 'K2P; kalign alignment option; pairwise deletion
370 (ambiguous base/gap handling)' to construct the distribution of intraspecific and interspecific
371 genetic distances [nearest neighbour (NN) analysis]. The BIN discordance report was employed
372 to confirm the exactness of species identification, as well as to check for cases of low levels of
373 genetic differentiation between different species. The Taxon ID tree was used to construct an NJ

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374 tree of the entire 666 sequences with the parameter-setting K2P distance model, the Kalign
375 alignment algorithm [86], and pairwise deletion (ambiguous base/gap handling).

376 To verify intraspecific and interspecific genetic distances, we also used barcode gap
377 analyses in ABGD. ABGD was used with K2P with the transition/transversion ratio (TS/TV) set
378 to 2.0, 10 recursive steps, X (relative gap width) = 1.0; the remaining parameters were set to
379 default values (Pmin = 0.001, Pmax = 0.1, Nb bins = 20).

380 We also used SPECIESIDENTIFIER v1.7.8 to verify species identification success by
381 applying three criteria (BM, BCM, and ASB) to the entire barcode dataset, following Meier et al.
382 [87]. Fish species that had only one sequence (singletons) were automatically assigned as
383 'incorrectly identified' under the BM and BCM criteria, as there were no conspecific barcoding
384 sequences to match.

385 For phylogenetic reconstructions, the datasets were analysed based on the BI methodology
386 using MrBayes 3.2. MrBayes was run with six substitution types (nst = 6), and we considered the
387 gamma-distributed rate variation and the proportion of invariable positions (GTR+G+I) for the
388 *COI* datasets. For BI, we ran four simultaneous Monte Carlo Markov chains for 25,000,000
389 generations, with sampling every 1,000 generations. The chain temperature was set at 0.2. Log-
390 likelihood stability was determined after 10,000 generations, and we excluded the first 1,000
391 trees as burn-in. The remaining trees were used to compute a 50% majority-rule consensus tree.
392 Moreover, to reveal the phylogenetic relationship of some fish species, the NJ tree of the K2P
393 distance was constructed using MEGA7. Phylogenetic trees were visualised and edited using
394 FigTree 1.4.2.

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399

400 **Author contributions**

401 Z.P. initiated the project, acquired funding, and managed project administration. B.S. collected
402 specimens, preformed DNA extraction, analyzed the results and wrote the manuscript. Z.P.
403 reviewed the manuscript. All authors read and approved the final version of the manuscript.

404

405 **Competing interests**

406 The authors declare no competing interests.

407

408 **Data availability**

409 All sequences and associated voucher data are available from BOLD and GenBank.
410 Voucher metadata are available in Supplementary Information.

411

412 **Additional information**

413 Supplementary Information The online version contains supplementary material available at …

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