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USE OF DNA BARCODING IN THE INVESTIGATION OF DISPUTABLE MERISTIC FEATURES FOR *HYPSELOBARBUS JERDONI* (DAY, 1870) FINGERLINGS

Shamima Nasren[1&2], Nagappa Basavaraja[1], Malathi Shekar[3], Md. Abdullah Al-Mamun[1 & 2], Sanjay Singh Rathore[1], Purandara Ballyaya Abhiman[1] and Kevin Dsouza Ronald[4]

[1]Department of Aquaculture, College of Fisheries, Mangaluru-575 002, India.
[2]Fisheries Faculty, Sylhet Agricultural University, Bangladesh.
[3]DBT Bioinformatics Centre, Department of Aquatic Animal Health Management, COF, Mangaluru, India.
[4]Department of Applied Zoology, Mangalore University, Mangaluru, India.
e-mail : snasren-fbg@sau.ac.bd

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ABSTRACT : *Hypselobarbus jerdoni* is an important medium-sized barb, indigenous to central Western Ghats, with an aquaculture potential and of conservational value. The aim of this study was to use mithochondrial DNA (mtDNA) COI and 16SrRNA genes as a supportive tool to precisely identify the two sister species, *H. jerdoni* and *H. pulchellus*, which show a lot of variation in meristic characters such as the number of lateral line scales. DNA barcoding included Cytochrome oxidase subunit I (COI) and 16SrRNA genes were done for representatives from different groups (sixty-two number of fishes) of Lateral line scales (Lls) counts. The results showed that there was no difference among the specimens based on DNA barcoding (COI and 16SrRNA genes), but there was a difference among them based on the number of llsc. This study reveals the use of llsc for the identification of these two sister species is questionable.

Key words : *Hypselobarbus jerdoni*, *H. pulchellus*, barbs, COI, 16SrRNA, meristic characters, llsc.

INTRODUCTION

Approximately 30,000 species of fishes are recorded in the world and around 35% of them inhabit freshwater (Nelson, 2006). The Cyprinidae is the largest family of freshwater fishes and it includes about 367 genera and 3006 species (Nelson *et al*, 2016). The generic name of some species has been modified several times (Zheng *et al*, 2016) notably small and medium-sized barbs of Cyprinidae. Identification of several species still remains obscure within the *Hypselobarbus* genus, due to lack of proper records from earlier descriptions (Knight *et al*, 2013a). Among them, *H. pulchellus* (Day, 1870), *H. jerdoni* (Day, 1870) and *H. dobsoni* (Day, 1876) have caught the attention of several researchers owing to their taxonomic ambiguity (Hora and Misra, 1942; Jayaram, 1991; Talwar and Jhingran, 1991; Jayaram, 1999; Daniels, 2002). In particular, distinction between *H. pulchellus* and *H. jerdoni* is externally difficult as they possess similar morphological characters (Devi and Ali, 2011). These two species are similar and are often confused with each other. Still, it is the distinctive keys that cause doubt in determining the proper identification between these two species. Ichthyologists demonstrated that lateral line scale count is an important feature because their number is specific for each species (Levin, 2010) (Table 1). Hence, there is a need to combine llsc with molecular techniques to verify the position of *H. jerdoni* and *H. pulchellus* (Knight *et al*, 2013a; Knight *et al*, 2016; Shamima *et al*, 2019a; Shamima *et al*, 2019b).

Proper identification of species is mandatory in fisheries research, management and conservation. But there are limitations to perform it accurately at eggs, larvae, juvenile and even in adult stages (Mohanty *et al*, 2015). The mtDNA markers like COI and 16S RNA are extensively used for identifying fish species where morphological features are inadequate. DNA barcoding is the short, standardized portion of the DNA sequence, which enables ichthyologists to identify up to species level. DNA barcoding has emerged as a mighty tool to identify species accurately (Herbert *et al*, 2003). It is reported that DNA barcoding is efficient for species identification and used in many species like cultivable carps (Mohanty *et al*, 2015), whiting (Vinod *et al*, 2013), asian sea bass

Corresponding author: Shamima Nasren

(Ward *et al*, 2008), sciaenids (Lakra *et al*, 2009), skates cichlids (Shirak *et al*, 2009), and Amazonian fishes (Ardura *et al*, 2010)

The Jerdon's carp, locally known as Cha-meen, *H. jerdoni*, is categorized as Least Concern (IUCN, 2019) but the populations are declining (M. Arunachalam pers. comm., 2010). This species is overexploited for food and aquarium trade. The Pulchellus locally known as Haragi or Hullu gende, *H. pulchellus*, is categorized as Critically Endangered / possibly Extinct (IUCN, 2019). Both of them are morphologically similar, particularly in juvenile stage, when they exhibit a very little variation in morphological characteristics between them. Hence, the present study was aimed to assess the reliability of lateral line scale counts vis-a-vis mtDNA genes for identification of *H. jerdoni* and *H. pulchellus*.

MATERIALS AND METHODS

Specimen collection and morphological identification

Sixty-two live fish specimens were collected from the Netravathi River of Dakshina Kannada (South Canara) district (12°51'31"N 75°07'54"E to 12°55'40"N 75°21'55"E) during February-May, 2018 using drag net and photographed with a Canon 1300D digital camera. The photographs were used for meristic counts, i.e. llsc and were named the voucher as College of Fisheries, Mangluru (COFM 16058 to COFM 16065) (Fig. 1). The primary identification of fishes was carried out based on llsc and body coloration (Day, 1870 and Day, 1878).

Table 1 : Lateral line scales count (llsc) for *H. jerdoni* and *H. pulchellus*.

Lateral line scales (Lls) count		Reference
H. jerdoni	*H. pulchellus*	
27-28	30-32	23
-	30-32	24
Synonym of *H. pulchellus*	Synonym of *H. jerdoni*	6
29-30 + 1	29-30 + 1	4
-	32-34+1-2	25
27-29	30-32	26
-	30-32	27

DNA extraction and molecular identification

DNA was sampled from individual fish via non-destructive finclip samples (1 cm^2) and preserved in 95% ethanol at -20°C until use. Total genomic DNA was extracted using the standard phenol-chloroform method (Sambrook *et al*, 1989). A 707 bp fragment of COI was amplified using universal primers, Fish F1-5' CAACCAACCACAAAGACATTGGCAC-3'and

FishR2-5'ACTTCAGGGTGACCGAAGAATCAGAA-3' gene (Ward *et al*, 2005). Similarly, a 621 bp fragment of 16SrRNA was amplified using the universal primer pair 16SF (5'ACGCCTGTTTATCAAAAACAT-3') and 16SR (5'CCGGTCTGAACTCAGATCACGT-3') gene (Palumbi,1996).A 20 µl PCR mixture contained 2.0 µl of DNA template (50 ng/ml), 2.5 µl 10x assay PCR buffer with $MgCl_2$, 2 µl of each primer (10 pm/ml), 1.6 µl dNTP mix (2.5mM each) and 0.20µl of Taq DNA polymerase. The reactions were performed on a C1000 Touch™ Thermal Cycler (BIO-RAD) for initial denaturation 3 minutes 95°C and 34 cycles under the conditions: (30 s at 95°C, 30 s at 54.5°C and 36 s at 72°C) for 16 S and (30 s at 95°C, 30 s at 55°C and 50 s at 72°C) for COI, with final extension at 72°C for 5 min. The PCR products were visualized on 1.5% agarose gels (Fig. 2). Then PCR products were purified using UltraClean® PCR Clean-Up kit and sent for sequencing in AgriGenome Lab Pvt Ltd, Kochi, Kerala. The sequences were analyzed using bioinformatics tools, Multialin (Corpet, 1988) used for alignment and Blastn (Zhang *et al*, 2000) was used for comparing the sequences. Then the sequences were submitted in NCBI through Banklt following the author's instructions.

Table 2 : Body weight, body length, body height, lateral line scale count (llsc) and colouration of eight voucher specimens.

Number of the fishes in each group	Morphospecies (based on llsc and colouration)	Representative Voucher name	Weight (g)	Length (mm)	Body height (mm)	Length: height	Coloration	
							Dorsal tip	Caudal tip (Black or grey)
Ten	*H. pulchellus*(30-30)*	COFM 16058	18	110	27.1	4.06	Black	Absent
Eight	*H. pulchellus* (29-30)*	COFM 16059	17	110	28.8	3.82	Black	Both black tipped
Five	*H. pulchellus*(30-29) *	COFM 16065	19	115	27	4.26	Black	Upper caudal black
Nine	*H. jerdoni*(28-28) *	COFM 16060	15	110	25	4.4	Black	Absent
Seven	*H. jerdoni*(29-29) *	COFM 16061	20.5	125	30	4.17	Black	Upper caudal black
Thirteen	*H. jerdoni*(29-28) *	COFM 16062	13	95	22.2	4.28	Black	Upper caudal black
Six	*H. jerdoni*(27-28)*	COFM 16063	20.5	120	22	5.45	Black	Upper caudal grey
Four	*H. jerdoni*(28-29)*	COFM 16064	26	120	37	3.24	Black	Upper caudal black

*The numbers in parenthesis represent llsc on the left and right side of the fish, respectively.

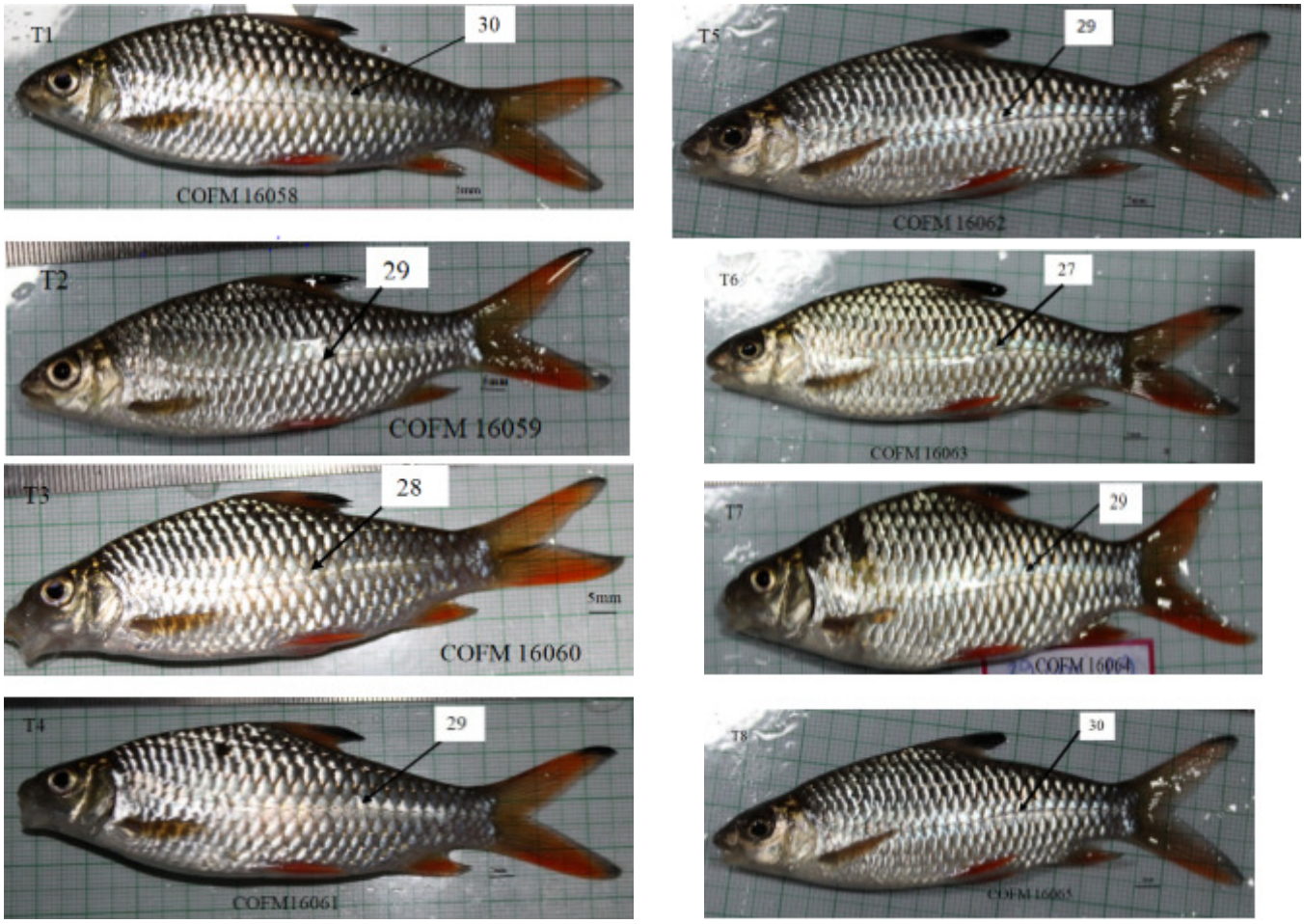

Fig. 1 : Voucher specimen COFM 16058 to COFM 16065 (T1 to T8). Number indicates Llsc.

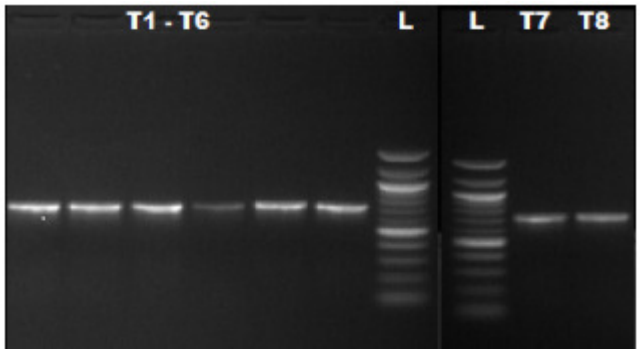

Fig. 2 : Photograph of agarose gel containing PCR products of COI region of T1-T8 specimens, L:marker.

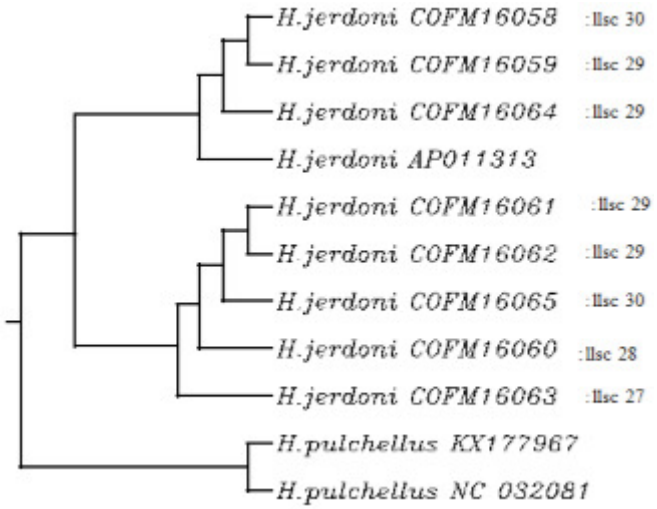

Fig. 3 : Rooted phylogenetic tree (UPGMA) of eight voucher specimens, KX177967, NC032081 and AP011313 sequences based on COI.

RESULTS AND DISCUSSION

A total of sixty voucher specimens were studied and were found to be advanced fingerlings (Basavaraja, 2007) length ranged between 95 and 125mm (av. 98.75 mm) and lls count varied between 27 and 30. The local aquarium fish collectors revealed that *H. jerdoni* often mixed with congeneric species and hence was difficult to identify in their early life stages, since at this stage these species phenotypically look similar (Ali *et al*, 2013). *H. pulchellus* was first described in by Day (1870) from South Canara (probably Mangalore), Karnataka and adults having silver or bronze coloured band running across the length of the body, had the lateral line scale row and one scale row above it (Collected by F. Day, (BMNH 889.2.1.4328) (Dry skin) (Knight *et al*, 2013a). Interestingly, since then, no pre-adult specimen of this

species with the band has been found from the type locality. So it is thought that the fish with lls count 30 or more might be *H. pulchellus* as shown in Table 1.

Meristic counts and morphological identification

The eight specimens studied were 13 - 20.5 g (av. 16.5 g) in weight and 22 - 37 mm (av. 27.39mm) in body depth. The height of the body to the total length was 3.82 to 5.45. On the other hand, earlier report showed 4.0 to 4.5 for *H. pulchellus* and 4.0 for *H. jerdoni*[23], indicating overlapping of body depth for both the species. Excepting COFM 16063, all the fish had bright silvery body colour, dorsal fin tip black in colour and dorsal finrays count 12, with caudal fin having orange shade and black tip (Fig. 1 and Table 2). The anal fin of all fishes had light orange colour, with the exception of COFM 16062 voucher specimen, which had anal fin reached somewhat beyond the root of the caudal fin. The lateral line was complete, concave for all the specimens, barring COFM 16062, which had lateral line complete and straight (Fig. 1 and Table 2). COFM 16060 and COFM 16061 had 28 and 29 llsc, respectively, on both left and right side of lateral line; rest of the six vouchers had different lls count on both side, e.g. COFM 16058 had 29 llsc on left side and 30 llsc on the opposite side. The features were overlapping for all specimens with respect to lls count (Knight *et al*, 2013a; Day, 1870; Day, 1878; Arunachalam *et al*, 2016; Basavaraja, 2014). As a result, two types of morphospecies were selected: a) *H. pulchellus* (COFM 16058, COFM 16059 and COFM 16065) and b) *H. jerdoni* (COFM 16060 to COFM 16064) (Table 2).

DNA barcoding and molecular identification

In this study, the partial 16SrRNA sequences of seven specimens (COFM 16058 to COFM 16064) were 621 bp nucleotides. In NCBI's BLAST (Basic Local Alignment Search Tool) (Zhang *et al*, 2000) the per cent nucleotide identity (PNI) is used to clearly identify related isolates in GenBank for comparison. The identity percentage was 98.39 - 98.71%, with AP011313.1:1998-2618 *H. jerdoni* mitochondrial DNA, complete genome, on 27 March 2019 (Miya, 2016). Furthermore, the nucleotide variation in seven amplified regions of 16SrRNA (GenBank accession numbers MK675503.1 to MK675509.1) was found in thirteen positions, e.g. 41, 43, 69, 73, 74, 218, 287, 290, 306, 342, 395,611 and 620. Our results indicated higher levels of nucleotide variation in the amplified region of COI (19 positions), compared to that of 16SrRNA amplicons (Table 3).

Nucleotides variation profiles were random for all

Table 3 : Nucleotide variation in position numbers in COI sequences of eight voucher specimens.

Morphospecies with voucher name	The position numbers where nucleotides are different																COI GenBank accession no.
	35	36	37	42	53	60	84	225	234	258	261	336	378	486	498	543	561	663	666	
H. pulchellus, COFM 16058	T	G	T	C	C	A	T	G	A	T	A	G	C	T	A	C	A	T	G	MK736826
H. pulchellus, COFM 16059	A	T	G	C	C	A	T	G	A	T	A	G	C	T	A	C	A	T	G	MK736827
H.pulchellus, COFM 16065	T	T	G	C	C	A	C	A	G	C	G	A	T	C	G	T	G	C	A	MK736825
H. jerdoni, COFM 16060	A	T	G	C	C	A	T	G	A	T	A	G	C	T	A	C	A	T	G	MK736822
H. jerdoni, COFM 16061	T	T	G	T	A	C	C	A	G	C	G	A	T	C	G	T	G	C	A	MK736823
H. jerdoni, COFM 16062	T	T	G	T	C	A	C	A	G	C	G	A	T	C	G	T	G	C	A	MK736828
H. jerdoni, COFM 16063	T	T	G	T	C	A	C	A	G	C	A	G	T	C	G	T	G	C	A	MK736829
H.jerdoni, COFM 16064	T	T	G	C	C	A	T	G	A	T	A	G	C	T	A	C	A	T	G	MK736824

the amplified COI amplicons. Morphospecies (a) *H. pulchellus* (COFM 16058) had three nucleotides (35, 36 and 37) different from *H. pulchellus* (COFM 16059). *H. pulchellus* (COFM 16058) had four nucleotides (35, 42, 53 and 60) similar with *H. pulchellus* (COFM 16065). Moreover, the morphospecies (b) *H. jerdoni* (COFM 16061 to COFM 16063) had the variations in nucleotides positions at 53, 60 and 336. But the DNA barcoding confirms that (a) and (b) morphospecies were *H. jerdoni*, the identity percentage was 97.59 - 99.29%, with AP011313.1:1998-2618, *H. jerdoni* mitochondrial DNA, complete genome, on 27-March-2019 (Miya, 2016). The phylogenetic tree (Larkin *et al*, 2007) based on COI showed two main clades (Fig. 3). Also, *H. pulchellus* (KX177967) (Sahoo *et al,* 2016) and NC032081 (Sahoo *et al,* 2017) sequences from NCBI formed distinct clades. Both the morphospecies (a) and (b) are included in one clade, but two sub-types were found (Figure 3). COFM 16058 (llsc 30), COFM 16059 (llsc 29) and COFM 16064 (llsc 29) formed one clade, while COFM 16061 (llsc 29), COFM 16062 (llsc 29), COFM 16065 (llsc 30), COFM 16060 (llsc 28) and COFM 16063 (llsc 27) formed another clade (Fig. 3).

CONCLUSION

Our results reveal that though all the specimens studied were phenotypically different, but they were genetically same. In clade, our specimens are showing separate than *H. pulchellus* clade. Published work indicates that fish with lateral line scales 30 or more are *H. pulchellus* (Knight *et al*, 2013a; Day, 1870; Day, 1878; Arunachalam *et al*, 2016; Basavaraja, 2014). Our study confirms that all the specimens with lateral line scale count 27 to 30 were *H. jerdoni*. The mtDNA markers (COI and 16SrRNA) prove that all the specimens collected from the Netravathi River were *H. jerdoni* or two strains of *H. jerdoni.* Thereafter, the fingerlings which having 30 Llsc were thought to be *H. pulchellus* is resolved by our findings.

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