

**Genetic variability in the natural populations of *Carinotetraodon travancoricus* revealed using mitochondrial COI sequences**

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**Abstract**

*Carinotetraodon travancoricus* or Malabar puffer fish is an endemic species described from rivers originating from the Western Ghats in S. India. This species is captured extensively as an aquarium fish and is having substantial demand in global markets. However, being prone to overfishing and impacts of anthropogenic alterations in its habitats, IUCN has categorized it as a threatened/vulnerable species. Since, knowledge on variability of wild populations could help in their conservation and management, morphometric and genotypic analyses were carried out in natural populations of *C. travancoricus* inhabiting two geographically separated rivers Pamba and Chalakkudy. Mean values of eleven length parameters measured in 456 males and 439 females inhabiting these rivers revealed significant difference (ANOVA,  $F = 10.2$   $p < 0.001$ ) between sexes and between females inhabiting two rivers. Principal component analysis revealed two factors in males and three factors in females, explained variance of 83.62 and 89.94% in respective sexes. Results of both PCA and discriminant function analysis indicated perceptibly high degree of separation between individuals inhabiting the two rivers. A total of twenty-five COI sequences were generated from *C. travancoricus* collected from rivers Pamba ( $n=14$ ) and Chalakkudy ( $n=11$ ). Sequence alignment revealed considerable base substitutions between samples from both rivers, indicating possibility of population differences. AMOVA analysis also provided significant  $F_{st}$  value (0.622;  $P$ -value 0.00) in support of population difference between individuals of both rivers. Interpopulation genetic distance reached upto 2.50%, high enough to confirm genetic diversity among individuals, revealing perceptible population events within this species. The present results indicated high degree of population difference between *C. travancoricus* inhabiting geographically separated rivers Pamba and Chalakkudy as evidenced from both morphometric and genotypic analyses.

**Keywords.** *Carinotetraodon travancoricus*, Morphometry, COI, Population difference

## 1. Introduction

Tetraodontidae (Order: Tetraodontiformes) represents one of the most speciose family of pufferfishes with 19 genera and 189 species (Yamanoue et al., 2011). Most of them occur in tropical regions, inshores and estuarine waters while a minor group (~30 species) invaded freshwater for their entire lifespan. Freshwater pufferfishes are reported from tropical regions of southeast Asia, central Africa and South America (Yamanoue et al., 2011; Lakra, Goswami, & Singh, 2013). In the southeast Asia (viz. southwest India, Indochina and the Sunda Islands), approximately 21 species of freshwater pufferfishes within five genera are accounted. Genus *Carinotetraodon*, comprising of six valid species of dwarf pufferfishes, is one among them. Its congeners correspond to *C. travancoricus* and *C. imitator* (restricted to peninsular India), *C. borneensis*, *C. salivator* and *C. irrubesco* (inhabiting Sundaland) and *C. lorteti* (reported from Thailand, Cambodia and Vietnam (Indochina)) (Yamanoue et al., 2011).

*C. travancoricus* (Hora & Nair, 1941) or Malabar puffer fish is considered as an endemic species inhabiting in freshwaters and estuaries of Western Ghats in India, specifically Kerala and Karnataka (Dahanukar, Raut, & Bhat, 2004; Talwar, 1991; Jayaram 1999; Devi, Indra, & Raghunathan, 2000). It was first described from Pamba River in Kerala by Hora & Nair (1941). Presently, occurrence of this fish is reported from 13 rivers of Kerala including Pamba, Chalakkudy, Vamanapuram, Bharathapuzha, Periyar, Kabani and Muvattupuzha. Its presence is also reported in Vembanad Lake and kole wetlands of Trichur (Easa & Basha 1995; Prasad, Sabu, & Prathibhakumari, 2012; Anupama & Harikrishnan 2015). *C. travancoricus* is captured extensively from Periyar and Achenkovil rivers and Vembanad Lake for ornamental purpose (Easa & Shaji, 2003). It is also having substantial demand in global aquarium markets, with respect to other ornamental fishes of India (Jayalal & Ramachandran, 2013; Anupama & Harikrishnan, 2015). However, considerable demand of this species in international markets resulted in threats like over harvesting and anthropogenic alterations of its natural habitats. Presently, it is considered as one of the threatened/vulnerable fish species of India by IUCN (Dahanukar 2013; Anupama & Harikrishnan, 2015; Ansar et al., 2017). Altogether, stock management of *C. travancoricus* inhabiting the natural waters is important since it is a commercially important aquarium fish enlisted under threatened category.

Management of wild populations depends on knowledge regarding the variations existing within locally reproductive populations (Carvalho, 1993). Identifying populations through morphometric and meristic approaches has long been adopted towards achieving this objective

(Almeida et al., 2008; Siddik et al., 2016). Conservation and management of wild populations could be performed by understanding their genetic variability (Mukhopadhyay & Bhattacharjee, 2014). Valdez-Pineda, Morán-Angulo, Voltolina, & Castillo-Vargasmachuca (2014) studied the population structure and reproductive aspects of the local stocks of puffer fish, *Sphoeroides annulatus* (Jenyns, 1842) (Osteichthyes: Tetraodontidae), in Mexico using morphometric characters. It is known that ecological and genetic factors influence development and maturation of individual fish species leading to distinguishable stock characteristics which can be perceived through morphometric analyses (Cadrin, 2000). Investigating genetic stocks of endemic species and characterizing genetic variability within individuals of populations may delineate their fitness, through which they adapt and survive in changing environmental conditions and stress. This may contribute to conservation and sustainability of wild stocks (Mukhopadhyay & Bhattacharjee, 2014). Determination of genetic variability and population structure could be performed using molecular markers (Mukherjee & Mandal, 2009; An et al., 2012; Lakra, Goswami, & Singh, 2013; Moghim et al., 2013; Deepak & Harikrishnan, 2016; Huang, Xu, & He, 2019; Zhong, Li, Kong, & Yu, 2017). Studies using nucleotide sequences representing typical barcode gene regions provided a new dimension in molecular taxonomy (Hebert, Cywinska, Ball, & Dewaard, 2003; Deepak & Harikrishnan, 2016; Jose et al., 2019). *Mitochondrial gene cytochrome c oxidase I (mtCOI)* is considered as a major molecular marker for bio-identification of fishes as its sequence divergence differentiates even closely allied species (Abbas, Megahed, Hemeda, & ElNahas, 2018; Dhar & Ghosh, 2014). Nucleotide sequences of a single species could be accurately identified when they form monophyletic clade within a phylogenetic tree with intraspecific divergence below a threshold value (Srivathsan & Meier 2012). This is an efficient method applicable to fisheries management (Shen, Guan, Wang, & Gan, 2016).

Hitherto, a study by Lakra, Goswami, and Singh (2013) regarding the genetic relatedness of Indian pufferfishes inhabiting fresh and marine waters remains as a major molecular level investigation on pufferfishes. There are sparse reports regarding the genetic assessment of *C. travancoricus* populations inhabiting its natural abode. Since *C. travancoricus* is an endemic species of Kerala, molecular genetic studies over their natural populations could provide sufficient knowledge beneficial for stock conservation, management and trade. This study attempts both morphological and molecular analyses on natural populations of *C. travancoricus* for evaluating its genetic variability as a prerequisite for stock identification and conservation.

## 2. Materials and methods

### Sample collection

A total of 476 and 419 samples of *C. travancoricus* were collected from two rivers Pamba (9° 10' to 9° 40' N latitude; 76° 15' to 77° 20' E longitude) and Chalakkudy (10° 05' to 10° 35' N latitude; 76° 15' to 76° 55' E longitude). Specimens were transported to the laboratory under chilled condition and subjected to morphological identification following the taxonomic keys provided by Hora & Nair (1941) and Britz & Kottelat (1999). Sexes were identified as described by Anupama et al. (2019) and the samples were subjected for detailed morphometric measurements after wiped with tissue paper for cleaning and draining. Eleven linear morphometric measurements such as total length (TL), standard length (SL), eye diameter (ED), pre orbital (PRO), post orbital (POO), body depth, base of pectoral fin (PFL), base of anal fin (AFL), base of dorsal fin (DFL), pre dorsal fin length (PRDF), post dorsal fin length (PODF) , were recorded using vernier calipers to nearest millimeter and body weight (BDW) was measured in an electronic balance of 0.001g. The size dependant variations in linear morphometric measurements were normalized following Elliott et al. (1995) as

$$\text{Madj} = M(Ls/L0)^b$$

where, Madj is size dependent normalized measurement, M is original measurement, Ls is mean of standard lengths, L0 is standard length and b is regression coefficient of log M on log L0. Significant variations in linear morphometric parameters recorded in river wise population data were delineated using analysis of variance (ANOVA), principal component analysis (PCA), linear discriminant functions (DFA) and cluster analysis (CA) using statistical softwares MS Excel, 2013; PAST v.3.26 and SPSS v.16.

### Genotyping

For molecular analysis, samples were randomly selected at a 1:10 ratio from morphologically congruent individuals of Pamba (n=140) and Chalakkudy (n=110) rivers (modified Jose et al., 2019). Abdominal tissues of collected specimens were used to extract genomic DNA following spin column method proposed in DNeasy Blood and Tissue Kit (Qiagen). Concentration and purity of DNA extracts were estimated using UV spectrophotometer (Nanodrop 2000) at a wavelength range of 260/280 nm. Optical density (OD) of DNA extracts were measured and good quality samples (showing OD between 1.7 and 2.0) were selected for thermal amplification. PCR amplification was performed using a 50µl reaction solution prepared using Sigma Aldrich ReadyMix™ Taq PCR

Reaction Mix with MgCl<sub>2</sub>. Partial mitochondrial COI gene sequences from genomic DNA was amplified using primer pair Fish F1: 5'-TCAACCAACCACAAAGACATTGGCAC-3' and Fish R1: 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3') (Ward et al., 2005). Amplification was performed in a Corbett gradient thermal cycler following the temperature regime according to Lakra, Goswami, & Singh (2013). Thermal regime consisted of an initial denaturation at 95 ° C for 2 min followed by 35 cycles of denaturation at 94° C for 40 s, 54 ° C (annealing temperature) for 40s, extension at 72 ° C for 1:10 min and a final extension at 72 ° C for 10 min. PCR amplicons were visualized on agarose gel (1.2%) and samples exhibiting intense bands were selected, purified and outsourced for sequencing.

### **Nucleotide sequence analyses**

COI sequences were manually edited using BioEdit 7.0.9 (Hall 1999). Primary screening of nucleotide sequences was done by searching National Center for Biotechnology Information (NCBI) database with Basic Local Alignment Search Tool (BLASTn). Clustal X was used for aligning and compiling these sequences (Thompson et al., 1997). Genetic diversity indices of aligned sequences were analyzed using DnaSP 5.10 (Librado & Rozas, 2009) and Arlequin 3.1 (Excoffier, Laval, & Schneider, 2005). Population structure (based on Median joining network) using identified haplotypes were generated using PopART (Population Analysis with Reticulate Trees) (Bandelt, Forster, & Röhl, 1999; Leigh & Bryant, 2015). Phylogram based on Neighbour Joining and Minimum Evolution analyses with 1,000 bootstraps (differentiating haplotypes and populations) was also generated. Pairwise sequence distance data between haplotypes based on Kimura 2 Parameter model was calculated using MEGA 5 (Tamura et al., 2011; Deepak & Harikrishnan, 2016).

## Results

The details of morphometric measurements recorded in male and female *C. travancoricus* inhabiting rivers Pamba and Chalakkudy are given in Table 1 and 2 respectively.

	Males						Females						Pooled population		
	No.	Min.	Max.	Mean	±	SD	No.	Min.	Max.	Mean	±	SD	Mean	±	SD
TL	236	19.08	24.71	20.71	±	0.89	240	17.11	41.69	21.07	±	1.52	20.89	±	1.26
SL	236	8.00	24.00	17.14	±	3.29	240	7.00	26.00	16.75	±	3.49	16.95	±	3.39
PRO	236	1.59	3.57	2.57	±	0.47	240	1.38	7.33	2.30	±	0.52	2.44	±	0.51
ED	236	1.14	2.75	2.00	±	0.19	240	0.83	5.66	2.10	±	0.43	2.05	±	0.33
POO	236	4.28	7.79	6.62	±	0.68	240	2.79	12.98	4.40	±	0.83	5.50	±	1.35
Depth	236	6.67	16.12	10.62	±	1.18	240	5.67	14.29	7.37	±	1.10	8.98	±	1.98
PFL	236	1.08	2.19	1.99	±	0.13	240	1.24	6.28	1.85	±	0.52	1.92	±	0.39
AFL	236	1.10	2.73	1.50	±	0.35	240	1.19	3.62	1.92	±	0.36	1.71	±	0.41
DFL	236	1.10	2.73	1.50	±	0.35	240	1.18	3.53	1.93	±	0.36	1.71	±	0.41
PRDF	236	9.81	18.22	13.40	±	1.13	240	1.98	25.12	12.16	±	1.73	12.77	±	1.59
PODF	236	4.37	8.04	7.16	±	0.60	240	5.35	12.02	6.03	±	0.58	6.59	±	0.82
BDW	236	0.90	0.79	0.32	±	0.15	240	0.90	0.89	0.38	±	0.15	0.37	±	0.15

**Table 1.** Descriptive data on various morphometric measurements recorded in male, female and pooled populations of *C. travancoricus* inhabiting river Pamba

	Males						Females						Pooled population		
	No.	Min.	Max.	Mean	±	SD	No.	Min.	Max.	Mean	±	SD	Mean	±	SD
TL	220	16.71	44.78	20.38	±	2.17	199	16.48	40.70	20.16	±	2.51	20.28	±	2.34
SL	220	5.00	26.00	16.13	±	3.07	199	6.00	23.00	15.83	±	2.89	15.99	±	2.99
PRO	220	1.29	5.53	2.17	±	0.47	199	1.22	5.91	2.15	±	0.56	2.16	±	0.51
ED	220	1.19	5.19	2.09	±	0.51	199	1.23	5.47	2.00	±	0.44	2.05	±	0.48
POO	220	2.54	10.36	4.29	±	0.82	199	2.44	10.65	4.15	±	0.94	4.22	±	0.88
Depth	220	4.51	18.23	7.28	±	1.55	199	3.95	23.14	7.30	±	2.44	7.29	±	2.02
PFL	220	1.22	10.11	2.17	±	0.71	199	1.14	5.53	2.00	±	0.46	2.09	±	0.61
AFL	220	0.72	5.55	1.75	±	0.59	199	0.99	4.27	1.49	±	0.45	1.63	±	0.55
DFL	220	0.72	5.55	1.75	±	0.59	199	0.99	4.27	1.49	±	0.45	1.63	±	0.55
PRDF	220	11.06	25.75	13.37	±	1.26	199	11.01	27.49	13.19	±	1.67	13.29	±	1.47
PODF	220	2.94	9.50	5.45	±	0.82	199	3.86	9.89	5.98	±	0.97	5.70	±	0.93
BDW	220	0.10	0.66	0.30	±	0.12	199	0.11	0.85	0.30	±	0.14	0.30	±	0.14

**Table 2.** Descriptive data on various morphometric measurements recorded in male, female and pooled populations of *C. travancoricus* inhabiting river Chalakkudy

Mean values of eleven length parameters measured in males and females inhabiting these rivers revealed significant difference (ANOVA,  $F = 10.2$   $p < 0.001$ ) between sexes and between females inhabiting two rivers. Univariate analysis of variance of these parameters showed significant differences in all measurements except AFL and DFL (Table 3).

	Wilks lambda	F value	p
TL	0.974	7.6	$p < 0.050$
SL	0.978	19.7	$p < 0.001$
ED	0.991	8.0	$p > 0.050$
PRO	0.987	11.7	$p < 0.005$
POO	0.830	182.8	$p < 0.001$
Depth	0.935	62.1	$p < 0.001$
PFL	0.936	60.9	$p < 0.001$
AFL	1.000	0.3	$p > 0.050$
DFL	1.000	0.1	$p > 0.050$
PRDF	0.889	111.9	$p < 0.001$
PODF	0.871	132.3	$p < 0.001$

**Table 3.** Results of ANOVA conducted on morphometric measurements recorded from two river populations (sexes pooled) of *C. travancoricus*

All length measurements except AFL and DFL were further used for principal component analysis, discriminant function analysis and cluster analysis. In principal component analysis, out of eleven morphometric measurements used for study, two factors in males and three factors in females had eigen values  $> 1$  which explained variance of 83.62 and 89.94% in respective sexes (Table 4).

	Eigen values		Percentage of variance	
	Males	Females	Males	Females
PC1	8.041	9.446	60.15	69.51
PC2	3.137	1.604	23.47	11.81
PC3		1.172		8.62

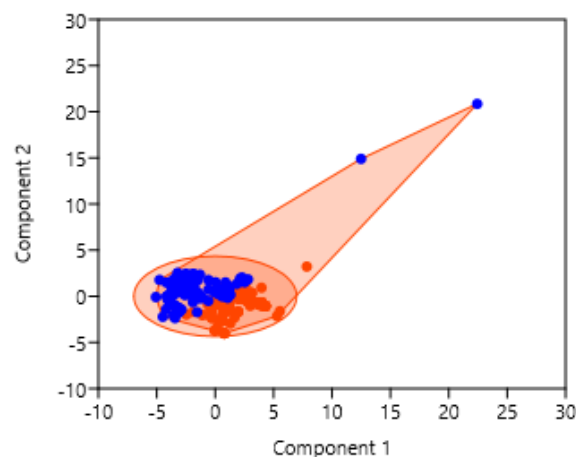
**Table 4.** Details of eigen values and percentage of variance recorded in principal component analysis of two river populations of *C. travancoricus*

In males, PC1 explained 60.15% variance and had significant loadings in factors BD, POO, TL and PODF while in PC2, significant loadings were recorded in TL and PRDF (Table 5). In

females, PC1 explained 69.51% variance with significant loadings in TL, BD and PRDF where as PC2 and PC3 explained 11.80 and 8.62% of variances with significant loadings in BD and TL respectively (Table 5).

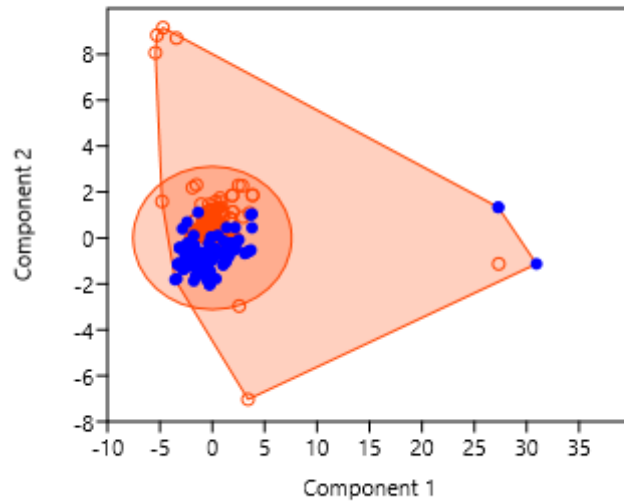
Morphometric measurements	PC1		PC2		PC3	
	Males	Females	Males	Females	Males	Females
TL	0.374	0.639	0.680	0.257	-	0.488
SL	0.131	0.007	0.155	0.211	-	0.147
PRO	0.118	0.132	0.014	0.048	-	0.162
ED	0.031	0.099	0.075	0.037	-	0.099
POO	0.407	0.230	0.239	0.085	-	0.261
BD	0.717	0.509	0.282	0.340	-	-0.778
PFL	0.051	0.102	0.189	-0.044	-	0.087
AFL	0.015	0.075	0.148	0.131	-	0.099
DFL	0.015	0.074	0.148	0.134	-	0.096
PRDF	0.235	0.458	0.490	-0.851	-	-0.063
PODF	0.301	0.145	0.228	0.039	-	-0.019

**Table 5.** Details of factor loadings in principal component analysis in two river populations of *C. travancoricus*



**Fig 1.** Scatter plot of scores in PC1 (60.15%) and PC2 (23.47%) of morphometric measurements recorded from male *C. travancoricus* inhabiting two river populations





**Fig 2.** Scatter plot of scores in PC1 (69.51%) and PC2 (11.81%) of morphometric measurements recorded from female *C. travancoricus* inhabiting two river populations

The scatterplots of scores in PC1 and PC2 in respect of both male (Fig.1) and female (Fig. 2) of *C. travancoricus* indicated two groups with low overlapping. Out of nine length measurements used, six measurements revealed significant difference in discriminant analyses of males and females of two rivers. Further, one function was found highly significant in each sex (Table 6).

Group	Functions	Wilk's Lambda	chi square	df	p
Male	1	0.037	1488.63	6	0.000
Female	1	0.562	250.05	7	0.000

**Table 6.** Results of Wilk’s test conducted in morphometric measurements in males and females of *C. travancoricus* inhabiting river Pamba and river Chalakkudy

The results of DFA showed highly successful classification rate in both male and females in two rivers. 100% of males in river Pamba and 98.6% of males in river Chalakkudy were correctly classified and cross validated (Table 7).

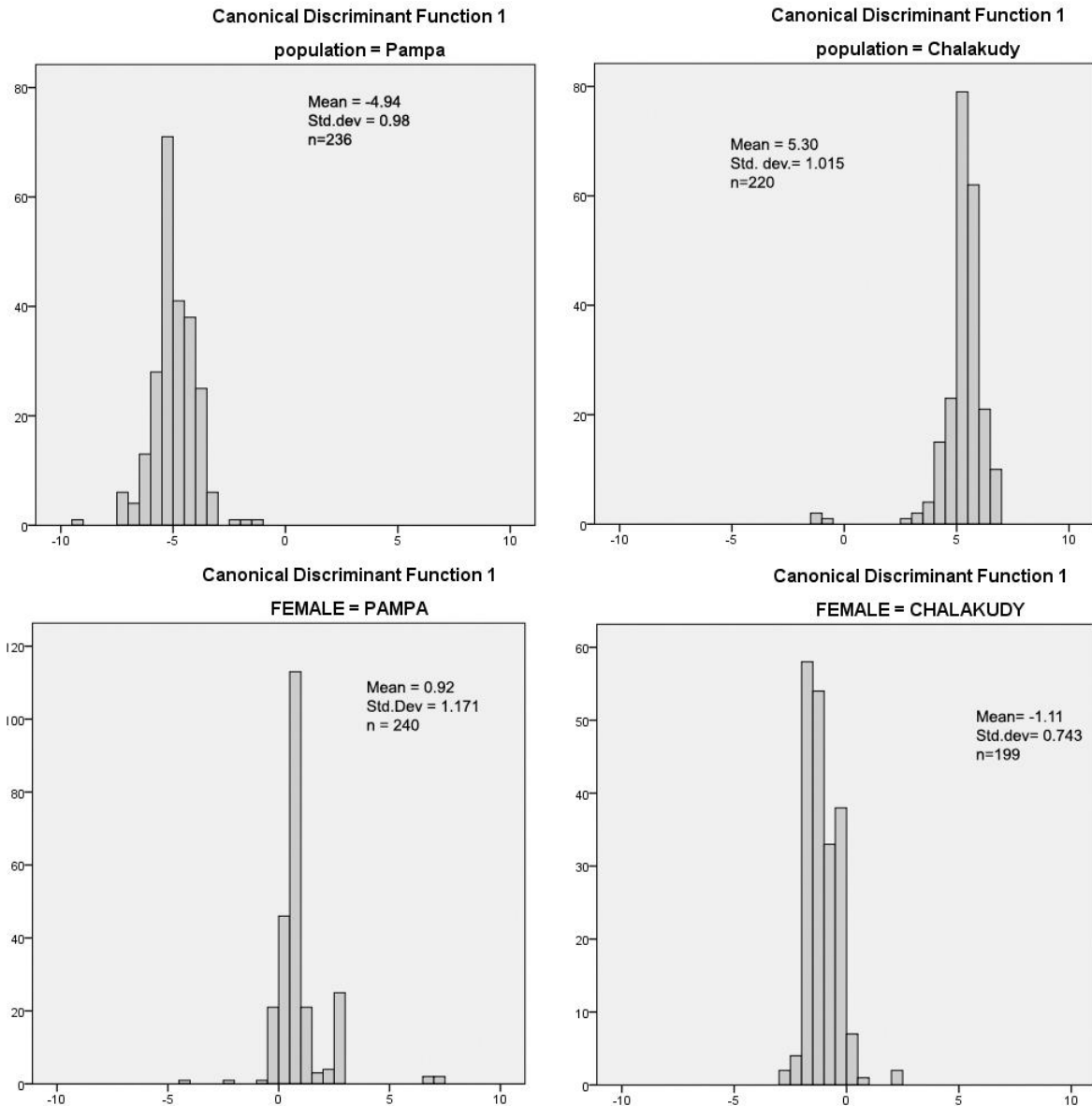
		Predicted group membership			
		Males			
		Poplations	Pamba	Chalakkudy	Total
Original	Count	Pamba	236	0	236
		Chalakkudy	3	217	220
	%	Pamba	100.0	0.0	100.0
		Chalakkudy	1.4	98.6	100.0
Cross validated	Count	Pamba	236	0	236
		Chalakkudy	3	217	220
	%	Pamba	100.0	0.0	100.0
		Chalakkudy	1.4	98.6	100.0

**Table 7.** Details of specimens classified in each group and cross validation in respect of morphometric characters of male *C. travancoricus*

In females, more than 90% of females were correctly classified and cross validated in river Pamba (91.3%) and river Chalakkudy (91%) (Table 8).

		Predicted group membership			
		Females			
		Poplations	Pamba	Chalakkudy	Total
Original	Count	Pamba	220	20	240
		Chalakkudy	16	183	199
	%	Pamba	91.7	8.3	100.0
		Chalakkudy	8.0	92.0	100.0
Cross validated	Count	Pamba	219	21	240
		Chalakkudy	18	181	199
	%	Pamba	91.3	8.7	100.0
		Chalakkudy	9.0	91.0	100.0
Cross validation is done only for those cases in the analysis.					

**Table 8.** Details of specimens classified in each group and cross validation in respect of morphometric characters of female *C. travancoricus*



**Fig 3.** Coordinate plots of two river populations of *C. travancoricus* in discriminant function analysis

It could also be noted that coordinate plots of males and females inhabiting two rivers showed perceptibly high degree of separation (Fig 3).

A total of twenty-five COI sequences were generated for *C. travancoricus* samples collected from Pamba (n=14) and Chalakkudy (n=11) rivers using the mentioned primer pair and thermal regime. Homology and species identity of these nucleotide sequences were evaluated using BLASTN annotations of National Centre for Biotechnology Information (NCBI) (Deepak & Harikrishnan, 2016). Since all these sequences were having more than 97% identity score towards

their homologous sequences in public database, all of them were selected for molecular analyses (Shen, Guan, Wang, & Gan, 2016). COI sequences were 581 to 605 bp in length. All these sequences were submitted in NCBI database (Accession numbers: KF245927- KF245933; KY033371- KY033388).

Among the 25 aligned sequences, major base substitution pattern was exhibited at ten base pair locations (87, 102, 195, 255, 261, 359, 460, 465, 501 and 552) revealing possibilities of population difference in *C. travancoricus* individuals. Based on these substitutions, COI sequences of individuals inhabiting Chalakkudy river (with accession numbers KY033377- KY033384; KY033386- KY033388: hereafter designated as population1) and Pamba river (KF245927- KF245933; KY033371- KY033376; KY033385: hereafter designated as population 2) could be differentiated into two populations. Genetic diversity indices (detailed in Table 9) analyzed also confirmed population level divergence among these groups.

Eleven COI sequences representing *C. travancoricus* inhabiting Chalakkudy river were accounted within the population 1. These sequences with 603 base pairs (excluding sites with gaps / missing data) contained 601 monomorphic and two polymorphic sites constituting three haplotypes. Haplotype 1 included four individuals viz. KY033384, KY033386, KY033388 and KY033383). Haplotype 2 was represented by five individuals (KY033387, KY033377; KY033379- KY033381). Within the 3<sup>rd</sup> haplotype, two individuals (KY033378 and KY033382) were present.

Population 2 constituted fourteen individuals from Pamba river and their sequence lengths ranged from 581 (for a single sequence with accession number KF245933) to 605 base pairs. Total number of sites excluding gaps or missing data was 579 base pairs. 570 monomorphic and nine polymorphic sites were present which accounted for five haplotypes (4<sup>th</sup>, 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> after considering the previous haplotypes in population 1). Haplotype four and five were represented by KY033385 and KY033372 respectively while haplotype six contained three inhabitants (KY033373; KY033375- KY033376). Two individuals (KY033371, KY033374) formed the seventh haplotype and eighth haplotype contained seven individuals (KF245927- KF245933). Diversity indices ('Hd' and ' $\pi$ ') and base frequencies also supported the presence of two different populations of *C. travancoricus* in each river (refer Table 9).

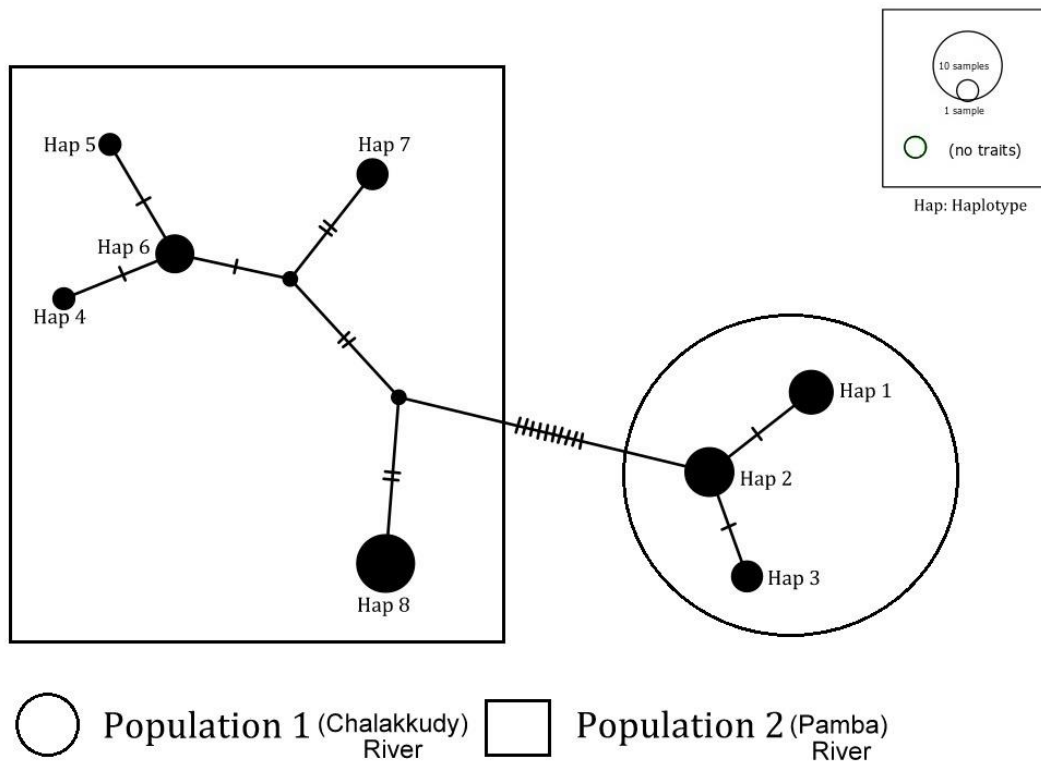
Taxon	<i>Carinotetraodon travancoricus</i>	
	Population 1	Population 2
<b>Number of Sequences</b>	11	14
<b>Alignment length</b>	603	605
<b>Number of monomorphic sites</b>	601	570
<b>Number of polymorphic sites (S)</b>	2	35
<b>Number of haplotypes (h)</b>	3	5
<b>Haplotype diversity (Hd)</b>	0.691 +/- 0.086	0.725 +/- 0.104
<b>Nucleotide diversity (<math>\pi</math>)</b>	0.00139 +/- 0.0012	0.0289 +/- 0.0153
<b>Base frequency (%)</b>		
<b>A</b>	23.88	24.25
<b>C</b>	27.66	28.34
<b>G</b>	17.25	16.80
<b>T</b>	31.21	30.61

**Table 9.** Details of genetic parameters inferred from *COI* sequences of *C. travancoricus* populations inhabiting Pamba and Chalakkudy rivers

Analysis of molecular variance (AMOVA) calculated for obtaining ‘Fixation index (Fst)’ with respect to the population difference revealed higher fixation index (0.622 ( $p \leq 0.00$ )), confirming the presence of population difference between population 1 and population 2 of *C. travancoricus*.

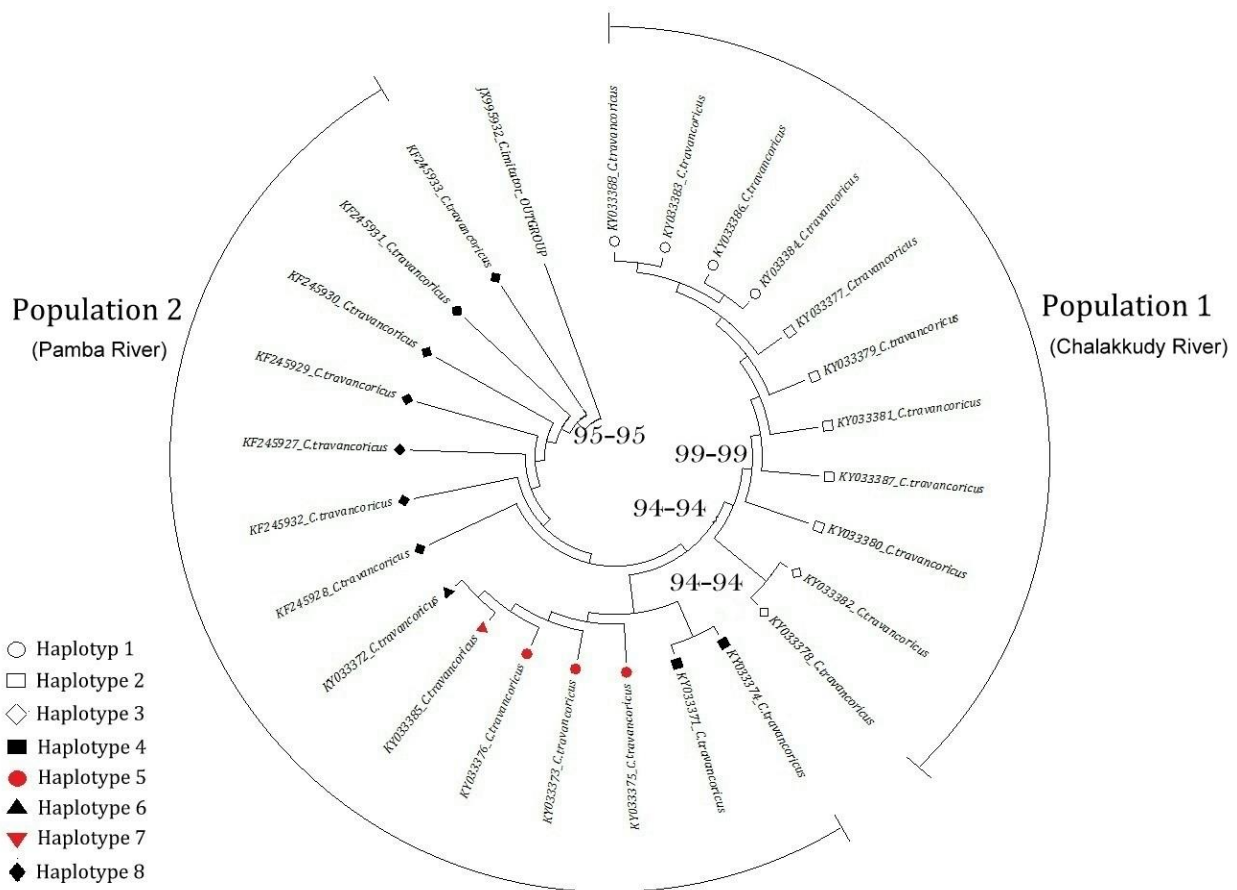
#### ***Haplotype network, Phylogram and genetic distance***

The relationship between haplotypes of *C. travancoricus* inhabiting Pamba and Chalakkudy rivers were identified through haplotype network based on Median joining analysis (Fig. 4).



**Fig 4.** Haplotype network based on Median joining analysis

Phylogram based (Fig. 4) on Neighbour Joining and Minimum Evolution analysis justified previous findings regarding the presence of two different populations of *C. travancoricus* in Pamba and Chalakkudy rivers. Cladistic array exhibited eight haplotypes within two populations with two independent lineages.



**Fig 5.** Phylogram based on Neighbour Joining and Minimum Evolution analyses

Genetic distance between Population 1 and 2 were also calculated using pairwise distance (supplementary file) that reached up to 2.50%, confirming the presence of population difference among the haplotypes. Genetic distance was low (upto 0.30%) within population 1 and moderate in population 2 (upto 1%).

## Discussion

Ornamental fishes, most popular pets of the world are considered as ‘living jewels’ (Anna Mercy, Gopalakrishnan, Kapoor, & Lakra, 2007; Haridas et al., 2019). Keeping aquarium fishes has been traditionally practiced for centuries and has been regarded as the second largest hobby practised worldwide (Jayalal & Ramachandran, 2013; Anna Mercy, Gopalakrishnan, Kapoor, & Lakra, 2007; Haridas et al., 2019). Globally, ornamental fish trade has become a multibillion industry and its retail turnover is estimated to worth 6-8 billion US \$ (Jayalal & Ramachandran, 2012; Haridas et al., 2019) with exports around 337 million US \$ (FAO, 2008). Developing countries are major exporters of ornamental fishes in which Asian countries account for nearly 60% of the total export. Tropical

fishes are mainly considered for ornamental fish trade and hence, their culture under confined aquatic systems has grown as a major commercial business (Anna Mercy, Gopalakrishnan, Kapoor, & Lakra, 2007; Haridas et al., 2019). Thus, 'Aquariculture' or the ornamental fish culture formed an important commercial component of aquaculture (Haridas et al., 2019).

The Western Ghats is a major biodiversity hotspot in India, known for its rich diversity of endemic freshwater fish fauna in rivers originating from this region (Anna Mercy, Gopalakrishnan, Kapoor, & Lakra, 2007). However, India was way behind in ornamental fish trade and the export was worth only 1.17 million US\$ during 2009-2010 (Jayalal & Ramachandran, 2012). Low diversity of non-unique fish species offered to aquarium fish trade has mainly been attributed to this (Kawada 2007). Generally, more species varieties available in large quantities are demanded by the ornamental fish trade (Sane 2007). Later, the export potential of the country was enhanced with introduction of *C. travancoricus*, *Scarlet badis* and Drape fin which acquired higher acceptance in global markets (Kawada 2007; Jayalal & Ramachandran 2012). Presently, India plays a major role in ornamental fish export by providing more than 150 commercially important ornamental fishes.

*C. travancoricus* or dwarf pufferfish is the smallest pufferfish reported from 13 rivers of Kerala including Pamba (Hora & Nair, 1941; Anupama & Harikrishnan, 2015) and Chalakkudy (Ajithkumar, Remadevi, Thomas, & Biju, 1999; Biju, Thomas, Ajithkumar, & George, 1999; Raghavan, Prasad, Ali, & Pereira, 2008; Beevi & Ramachandran, 2009). Dahanukar (2013) categorized this species under vulnerable category since the fish was found to prone to anthropogenic interventions and habitat modifications. Decline of *C. travancoricus* populations (30-40% within a period of ten years), lack of conservation action plans towards its stocks and necessity for investigating its population status were also reported.

The results of present study indicated distinct population differentiation in *C. travancoricus* inhabiting two Western Ghats rivers of Kerala, river Pamba and river Chalakkudy. Analyses of morphometric characteristics demonstrated significant between two populations and also between sexes. Since size dependent variations were removed through allometric normalization, the significant differences in phenotypic characters reflected population differences (Vatandoust et al., 2015). Discriminant function analysis has been regarded a successful tool in distinguishing stock differences in one species (Karakousis et al., 1991). Perceptibly high degree of classification into groups in discriminant function analysis in the present study indicated highly heterogenous nature of phenotypic characteristics in *C. travancoricus* which could distinctly distinguished the two river populations. The results of discriminant function analysis were also confirmed by principal



component analyses which indicated differentiation of morphometric parameters such as body depth, post orbital length, post dorsal fin length, total length and pre dorsal fin. Identification of populations within a species is a fundamental problem in fisheries management and this could be attained through identification of stock(s) (Carvalho & Hauser, 1995). Therefore, fish stocks are considered as the fundamental biological units in fisheries management (Reiss, Hoarau, Dickey-Collas, & Wolff, 2009).

Phenotypic plasticity leading to heterogeneity in morphometric parameters of populations has been attributed to changes in environmental conditions existing in geographically separated habitats (Paugy and Levequw, 1999; Poulet et al., 2004; Turan et al., 2006; Ferrito et al., 2007). The population segregation in *C. travancoricus* observed in the present study could well be attributed to differential environmental conditions existing between geographically separated rivers Pamba and Chalakkudy. Further, these rivers have been prone to many human interventions leading to severe ecological alterations (David and Jennerjahn, 2013; David et al., 2016). Anthropogenic activities like deforestation, construction of dams, reclamation of water areas, pollution, over fishing, exotic fish introduction etc. have largely been attributed to hampering natural habitats and life of many riverine ichthyofauna in many rivers originating from the Western Ghats including river Pamba (Renjithkumar et al., 2011) and river Chalakkudy (Raghavan, Prasad, Ali, & Pereira, 2008).

Phenotypic variations in populations have significant bearing on genetic variability also. Interactions of environmental and genetic factors are attributed to cause perceptible variations in morphological characteristics (Poulet et al., 2004). However, possibilities of variations within the morphological characters of an organism based on their developmental stages and presence of cryptic species (species that are morphologically similar and genetically different) could cause severe confusions (Binpeng et al., 2018). Considering these complications regarding traditional morphology-based approach, need for implementation of modern technological aids like DNA barcoding is important for species identification and population analysis (Hebert, Cywinska, Ball, & Dewaard, 2003; Mohammed-Geba 2015; Deepak & Harikrishnan, 2016).

Yamanoue et al., (2011) investigated the evolutionary history of freshwater tetraodontids using whole mitogenome sequences from tetraodontids. Lakra, Goswami, & Singh (2013) studied the genetic relatedness and phylogenetics of five Indian pufferfishes using mitochondrial markers COI and 16S rRNA. However, a molecular approach towards the populations of pufferfishes has not yet been performed. Hence, this study was carried out using COI gene to identify the populations of

*C. travancoricus* inhabiting natural waters of Kerala viz. individuals inhabiting Pamba and Chalakkudy rivers.

Mohammed-Geba (2015) performed a bioinformatic simulation using COI gene sequences of *Epinephelus akaara*, an endangered Serranid fish species in South Korean waters, for analysing its population structure. The author was able to identify presence of different haplotypes indicating the possibilities of a recent population expansion. Abbas, Megahed, Hemeda, & ElNahas (2018) performed DNA barcoding and investigated the genetic population structure (using mitochondrial COI gene) of two congeners of genus *Diplodus*, *D. sargus* and *D. vulgaris* (Family Sparidae) inhabiting Egyptian Mediterranean Sea. The generated results supported no significant population subdivision for these congeners of genus *Diplodus*, suggesting the presence of same genetic material in all populations. Tonay et al. (2020) assessed the population connectivity, evolutionary history, and conservation status of short-beaked common dolphin using mitochondrial DNA D-loop fragments. Genetic diversity and population structure of a cyprinid fish (*Ancherythroculter nigrocauda*) was performed using mitochondrial cytochrome b gene (cyt b) and 13 microsatellite (SSR) loci (Zhai et al., 2019). A study based on RAPD-PCR method by Mukhopadhyay & Bhattacharjee (2014) also reported lack of genetic diversity in threatened freshwater ornamental fish, *Badis badis* (dwarf chameleon fish), inhabiting Terai, the western sub-Himalayan region of West Bengal.

This study has revealed population level genetic divergence within the natural stocks of *C. travancoricus* inhabiting Pamba and Chalakkudy rivers. Presence of high number of haplotypes and their relationships could be accounted for adaptations of this species according to its changing environments as proposed by Ciftci & Okumu (2003) and Mukhopadhyay & Bhattacharjee (2014). Anupama & Harikrishnan (2015) reported the beneficial role of *Cabomba* plants on adaptation and survival of *C. travancoricus* in aquarium tanks. Low lying fallow areas associated with river Pamba are known to support *Cabomba* extensively (Alexander, Nair, & Shaji, 2010), their impact on establishing *C. travancoricus* populations need to be further investigated. The abundance of aquatic plants could cause population increase in many fishes like Sunfish, *Lepomis* spp. and *Pomoxis* spp. (Ware & Gasaway, 1977; Shireman, Colle, & Durant, 1981).

Genetic diversity within *C. travancoricus* has brought up the possibilities of broodstock development. Higher market acceptance of pufferfishes including *C. travancoricus* in international markets could promote sincere attempts towards the optimization of their culture. Genetic variations in species and populations enable them to adapt to their changing environments. Therefore, molecular marker assisted assessment of natural populations provides scientific data for protection of

weaker populations and long-term management of fisheries resources. Considering all these facts, aquaculture could be implemented for *C. travancoricus* so that broodstock management, captive breeding and supply of fishes could be practised by reducing the over exploitation of the local stocks of this species.

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### **Data sharing**

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

### **Disclosure statement**

The authors declare responsibility for the entire contents of this paper and have no conflicts of interest.

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