Integration of Morphology and the Mitochondrial *Cox*1 Gene to Identify Killifishes, *Aplocheilus* Species, in the Attanagalu River, Sri Lanka

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Abstract

Aplocheilus species, often known as killifishes, are popular in the aquarium trade and are used as biological control agents for mosquito larvae. They are endemic to Madagascar, Seychelles, and South-East Asia. *Aplocheilus* species were collected from the Attanagalu River in Sri Lanka, identified using morphological and meristic characters and subjected to truss analysis. The genomic DNA of fresh muscle tissues of 10 fish with different morphological features was amplified by targeting the *Cox*1 gene with FishF1 and FishR1 primers. The amplicons were sequenced and subjected to DNA homology search and phylogenetic analysis. According to morphological identification, *A. dayi* and *A. parvus* were identified, but the truss analysis did not differentiate between the two species. According to a homology search, out of eight sequences, two were best matched with *A. werneri* and the rest with *A. blockii* and phylogenetic analysis formed two separate clusters for two species. We propose that the two individuals showed a closer genetic relationship with *A. werneri* as *A. dayi* and those who showed a closer genetic relationship with *A. blockii* as *A. parvus*. Sequence diversity observed within the proposed two species revealed that intraspecies genetic variation exists in the two populations in the Attanagalu River. The present study suggests the presence of *A. dayi* and *A. parvus* in the Attanagalu River basin, along with the intraspecies genetic variations among individuals within species regarding the mitochondrial *Cox*1 gene.

Keywords: Aplocheilidae, truss analysis, phylogenetic analysis, endangered species

Introduction

Aplocheilus species, commonly known as killifishes, belong to the family Aplocheilidae and are endemic to Madagascar, Seychelles, and South-East Asia (Beck et al., 2017). They are popular aquarium fish due to their striking colour patterns, adaptability to formulated diets and easy keeping in aquaria. *Aplocheilus* species have long been used as biocontrol agents for mosquito larvae in Asia (Manna et al., 2011). According to Froese and Pauly (2022), there are two endemic (Ceylon killifish, *A. dayi* Steindachner (1892) and *Werner's* killifish, *A. werneri* Meinken (1966)) and four native (green panchax, *A. blockii* Arnold (1911); striped panchax *A. lineatus* Valenciennes (1846); blue panchax, *A. panchax* Hamilton (1822) and dwarf panchax, *A. parvus* Sundara Raj (1916)) *Aplocheilus* species in Sri Lanka. However, only three *Aplocheilus* spp., namely *A. dayi*, *A. parvus* and *A. werneri*, have been recorded during the freshwater fish surveys carried out recently in the country (Jayaneththi, 2017; Goonatilake et al., 2020; Surasinghe et al., 2020). The IUCN classifies *A. dayi* and *A. werneri* as critically endangered, whereas *A. parvus* is listed as least concern (Goonatilake et al., 2020).

Aplocheilus werneri has spread only in Sri Lanka's lowland wet zone (Goonatilake et al., 2019) from the Attanagalu to the Gin River basins (Maduranga, 2003). *Aplocheilus parvus* has been reported in moderate saline and freshwaters in the low country wet, intermediate, and dry zones (Pethiyagoda, 1991). No credible information is available on the exact

distribution and abundance of *A. dayi* in the country.

Though the *Aplocheilus* spp. are collected on a large scale from the wild environment for export purposes in the aquarium trade, none of the studies has been conducted to identify them up to species level in Sri Lanka. As such, the exact species of *Aplocheilus* inhabit in Sri Lanka are not known yet. As the traditional use of morphological parameters of fish has several drawbacks, taxonomists use truss analysis in fish identification and classification studies. Truss network measurements include calculating distances between landmarks that form a regular pattern of connected quadrilaterals or cells across the body (Strauss and Bookstein, 1982). It is considered a highly effective method for capturing information about the shape of an organism (Cavalcanti et al., 1999).

DNA barcoding is another technologically advanced, rapid and reliable molecular tool widely used in species identification (Fogelström, 2015; Anupama et al., 2021). A short DNA region (approximately 650 bp) in the mitochondrial genome called cytochrome c oxidase 1 gene (*Cox*1) and its resulting polypeptide (COI) qualifies to serve as a DNA barcode for all animals (Fogelström, 2015). Also, a phylogenetic tree, which can show the evolutionary relationships among the species, can be used to determine the differences among the DNA sequences of different organisms.

Deforestation, urbanisation, gem mining, excessive agrochemical use, water diversions, and the introduction of exotic species have all had a negative impact on the freshwater fish biodiversity in Sri Lanka (Goonatilake, 2007; Goonatilake et al., 2019; Surasinghe et al., 2020). Because *Aplocheilus* species are popular in the aquarium fish export sector and their populations are declining in the natural environment, precise species identification is critical for establishing fish conservation programs in the country. Furthermore, genetic diversity among individuals of the same species is essential for developing conservation management plans. The current study aimed to identify *Aplocheilus* species in the Attanagalu River basin, Sri Lanka, morphologically and molecularly and determine their genetic variation.

Materials and Methods

Ethical approval

This study was approved by the Animal Ethics Committee of University of Kelaniya (No. UOK/ERC/FS/2017/21; 17/12/2017).

Study area and sampling of killifishes

The area selected for the present research study was the basin of the Attanagalu River (Fig. 1). It has an extent of 779 km^2 and extends from Kegalle to the Gampaha districts of Sri Lanka (Fig. 1). Fish were collected from 60 sampling sites at first to third-order streams of the Attanagalu River, which starts from the Dunumala area of the Kegalle district (103 m mean sea level (MSL)) and ends in the Negombo estuary (2 m MSL) of Gampaha District, Sri Lanka. Streams were selected by purposive (judgmental) sampling.

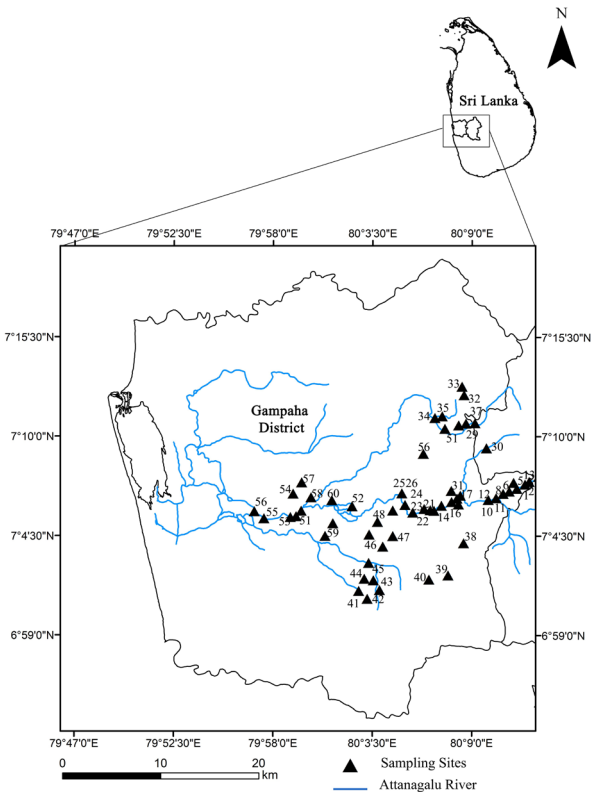


Fig. 1. The selected sampling sites of *Aplocheilus* species at the Attanagalu River basin, which spans through the Kegalle and Gampaha districts of Sri Lanka.

A 3 m stretch of each sampling site was surrounded by a mosquito net with minimum disturbance. *Aplocheilus* species in the enclosed area were collected using a hand net and placed in a glass tank for observation. Fish were identified based on their morphology (Table 1) and photographed at the same collection point (Maduranga, 2003; Goonatilake, 2007). GPS coordinates of each sampling site were recorded by a GPS pointer (eTrex 10, Garmin, USA).

Measurement of meristic characteristics and data analysis

Using the Image J software, a sample of 30 fish phenotypically identified as *A. dayi* and *A. parvus* was used to measure twenty meristic characteristic features (Table 2; Fig. 2).

Measured meristic characteristics were subjected to the first:

Standard measurement = Total length/Standard length

and second:

Truss length of the ith fish ($LT_{(i)}$) = log total length of sample ($LT_{(i)}$[log total mean length ($TL_{(m)}$)/Truss length of the fish ($YL_{(i)}$)])^b

Table 1. Characteristic features used for morphological identification of different *Aplocheilus* spp. in the Attanagalu River, Sri Lanka.

	A. dayi	*A. parvus*	*A. werneri*
Colour on the head	White-colour metallic blotch	White-colour metallic blotch	White-colour metallic blotch
Male	Three black coloured spots as a triangle on the lateral side of the body	Green and red spots are present on single fins	Black vertical stripes extend to the anal fin
Female	Six to seven black coloured vertical stripes at the posterior end of the body and above the anal fin	A black dot at the base of the dorsal fin	A black blotch can be seen in the abdomen due to the merging of the black-coloured stripes
Body length	3–5 cm	2–4 cm	6–9 cm

Table 2. Measurements used to examine morphological variations between *Aplocheilus dayi* and *Aplocheilus parvus* collected from the Attanagalu River, Sri Lanka.

Character	Pin No.*	Character description
1		Total length (TL)
2		Standard length (SL)
3	1–3	Snout to the origin of the dorsal fin
4	1–5	Snout to the origin of the pelvic fin
5	2–3	Posterior point of the eye to the origin of the dorsal fin
6	2–4	Posterior point of the eye to the origin of the pectoral fin
7	2–5	Posterior point of the eye to the origin of the pelvic fin
8	3–5	Origin of the dorsal fin to the origin of the pelvic fin
9	3–4	Origin of the dorsal fin to the origin of the pectoral fin
10	4–5	Origin of the pectoral fin to the origin of the pelvic fin
11	5–6	Origin of the pelvic fin to the posterior end of the dorsal fin
12	3–7	Origin of the dorsal fin to the origin of the anal fin
13	3–6	Origin of the dorsal fin to the posterior end of the dorsal fin
14	5–7	Origin of the pelvic fin to the origin of the anal fin
15	7–6	Origin of the anal fin to the posterior end of the dorsal fin
16	7–9	Origin of the anal fin to the ventral attachment of the caudal fin to the tail
17	6–8	Posterior end of the dorsal fin to the dorsal attachment of the caudal fin to the tail
18	7–8	Origin of the anal fin to the dorsal attachment of the caudal fin to the tail
19	6–9	Posterior end of the dorsal fin to the ventral attachment of the caudal fin to the tail
20	8–9	Dorsal attachment of the caudal fin to the tail to the ventral attachment of the caudal fin to the tail

*Pin numbers: 1 – Snout, 2 – Posterior point of the eye, 3 – Origin of the dorsal fin, 4 – Origin of the pectoral fin, 5 – Origin of the pelvic fin, 6 – Posterior end of the dorsal fin, 7 – Origin of the anal fin, 8 – Dorsal attachment of the caudal fin to the tail, 9 – Ventral attachment of the caudal fin to the tail.

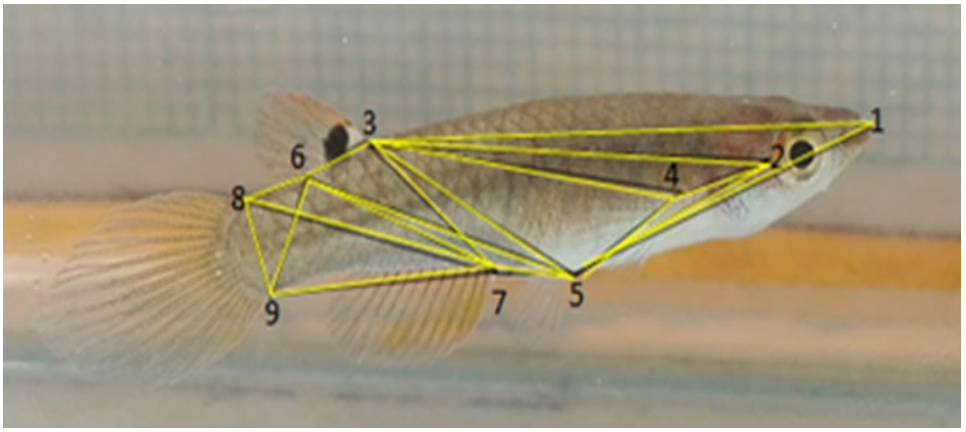


Fig. 2. Nine landmarks used for the truss analysis of *Aplocheilus* spp. 1 – Snout, 2 – Posterior point of the eye, 3 – Origin of the dorsal fin, 4 – Origin of the pectoral fin, 5 – Origin of the pelvic fin, 6 – Posterior end of the dorsal fin, 7 – Origin of the anal fin, 8 – Dorsal attachment of the caudal fin to the tail, 9 – Ventral attachment of the caudal fin to the tail. Redrawn based on Hockaday et al. (2000).

approaches of truss analysis by preparing a principal component analysis (PCA) as described by Doherty and McCarthy (2004) and Fernando and Amarasinghe (2011) using PRIMER 5 software. In the second approach, TL is the total length, $LT_{(i)}$ is the truss length of ith fish, TL_m is the overall mean total length and b is the slope, within areas of the geometric mean regression on the logarithms of LT and TL (Doherty and McCarthy, 2004; Fernando and Amarasinghe, 2011).

DNA extraction and PCR amplification

After the identification based on morphological features, a representation of fish showing different colour patterns sampled from different sampling sites was subjected to genomic DNA extraction. DNA extraction was done with the following method developed and optimised in the present study. Briefly, pieces of muscle tissues (1.0 g) were ground in liquid nitrogen and incubated in 1 mL of pre-warmed CTAB extraction buffer (1.4M NaCl, 20 mM EDTA (pH 8.0), 100 mM Tris (pH 8.0) and 2 % CTAB) at 65 °C. After that, the sample was incubated in 10 µL of Proteinase K (20 $mg.mL^{-1}$) at 65 °C for 1 h and subjected to two times extraction with phenol:chloroform:isoamyl alcohol (25:24:1). Ethanol precipitation was done by adding 3M sodium acetate (0.1 of the volume) and cold 100 % ethanol (1 or 2 volumes). The pellet was washed with 70 % ethanol and the dried DNA pellet was dissolved in 15 µL of TE buffer and treated with RNAse. The extracted DNA's purity and concentration were measured spectrophotometrically (Nanospec, Shimadzu, Japan). A universal primer pair, namely, FishF1/FishR1 (5'TCAACCAACCACAAAGACATTGGCAC3'/5'TAGACTTC TGGGTGGCCAAAGAATCA3') (Ward et al., 2005), was used to amplify the *Cox*1 gene in the mitochondrial DNA (Hubert et al., 2008).

Molecular analysis

Ten PCR products with an expected size of approximately 650 bp (Ward et al., 2005) were DNA sequenced at Asiri Hospital, Sri Lanka and the quality was confirmed by Finch TV chromatogram viewer (Geospiza, Inc.). The sequences were translated into amino acids to check for premature stop codons and to verify that the open reading frame was maintained in the protein-coding loci by using the translate tool in the ExPASY Bioinformatics resource portal (https://www.expasy.org/). The nucleotide sequences were subjected to BLAST searches for highly similar sequences in the GenBank database (https://www.ncbi.nlm.nih.gov/). Additionally, BOLD Identification System (https://www.boldsystems.org/IDS_OpenIdEngine) was used to find out highly identical sequences in 'All Barcode Records' and 'Species Level Barcode Records' databases for species-level identification, having a similarity cutoff of ≥97 % (Hebert et al., 2003).

The sequences generated and deposited in GenBank in the present study and some sequences retrieved from GenBank (Table 3) were aligned in MEGA 7.0 using MUSCLE (Kumar et al., 2016) with default parameters and the evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) with 1000 bootstrap replications. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4409). Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Evolutionary divergence was estimated within and between species using the Kimura 2-parameter model (Kimura, 1980). The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). All positions containing gaps and missing data were eliminated. The analyses were conducted using MEGA7.

Results

Distribution of Aplochielus spp. *in the study area*

A total of 271 individuals of *Aplocheilus* spp. were observed during the study. Based on morphological identification, 181 and 90 individuals were identified as *A. dayi* and *A. parvus*, respectively. The presence of *Aplochielus* spp. in the study area is shown in Figure 3.

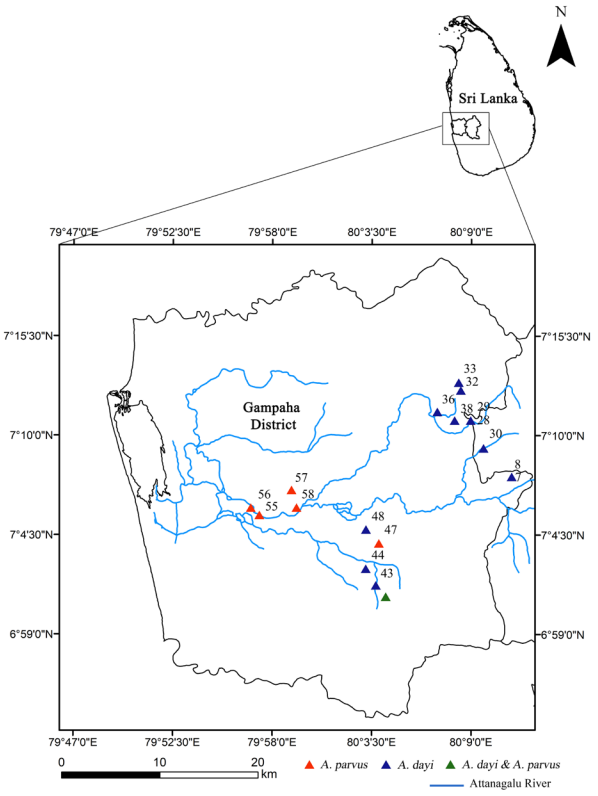


Fig. 3. Map of Sri Lanka depicting Gampaha and Kegalle districts and the locations where *Aplocheilus* spp. were recorded in the Attanagalu River, Sri Lanka.

Of the 60 sampling sites studied, *A. dayi* was present in 13 locations (21.7 %), while *A. parvus* was found only in six sampling sites (10.0 %). Further, *A. blockii*, *A. panchax* and *A. werneri* were not found in any of the sampling sites of the study area. Only one sampling site reported the presence of both *A. dayi* and *A. parvus* (1.7 %). *Aplocheilus dayi* mainly were recorded at comparatively higher altitudes (ranging from 28–74 MSL), while *A. parvus* was more abundant at lower altitudes (14–35 m MSL). The presence of *A. parvus* is clustered more toward the coastal area; in contrast, *A. dayi*'s presence was more towards the inland. As per the observations, most of the time, the two species of *Aplocheilus* were not found in the streams below the paddy fields. However, the *Aplocheilus* spp. were found in the same stream when it is at a higher elevation to the paddy fields.

Identification of Aplocheilus spp. *by morphological features*

Aplocheilus spp. can be identified by the white-colour metallic blotch on their head and characteristic morphological features as described by Maduranga (2003) and Goonatilake (2007). In the wild, the body size of mature fish of *A. dayi* was more significant than that of *A. parvus*. Compared to *A. dayi*, *A. parvus* has shiny scales on the lateral sides of the body, which could differentiate the two species (Goonatilake, 2007). However, in the present study, contrary to the typical morphological features used for identification, several morphological variations were observed in the two species. For example, in *A. parvus*, different colours of the anal fin and in *A. dayi*, different numbers of black-coloured spots along the lateral side of the body were observed (Fig. 4), confirming the possible misidentifications when morphological features are used in species identification.

Use of truss analysis to identify Aplocheilus spp.

Except for seven, all the remaining *Aplocheilus* specimens were clustered together (Fig. 5A) according to the first approach (i.e., Standard measurement = LT/SL) of truss analysis despite being previously phenotypically recognised as two separate species. The second approach resulted in a greater grouping of *A. dayi* for some fish specimens but no different clusters for the two species (Fig. 5B). As a result, the first and second approaches of truss analysis cannot accurately distinguish *A. dayi* from *A. parvus*.

Molecular identification

Out of ten PCR products, eight were successful with the DNA sequencing and the DNA homology search results given by BLAST and BOLD are shown in Table 3. In these searches cutoff of 97 % was used to differentiate different species of *Aplocheilus*. Except for isolate Ap4G, other isolates did not show >97 % sequence identity with any of the sequences available in the public databases (Table 3). However, the nearest best match species were given as *A. blockii* for the isolates Ap1G, Ap1A, Ap2A, Ap2G and Ap3G, and *A. werneri* for the isolates Ap1H and Ap2H. The absence of the *Cox*1 sequences of *A. parvus* and *A. dayi* in nucleotide databases was the limitation in validating these results. On the other hand, isolate Ap4G showed 97.5 % identity with *A. blockii* by BLAST and BOLD search. However, the BOLD analysis failed to find a species level match, but it suggested *A. blockii* as the nearest match from searching in all Barcode Records (Table 3).

Genetic distance among the species of *Aplocheilus* was conducted with the *Cox*1 gene sequences (Table 4). A comprehensive genetic difference was observed within the morphologically identified species in this study, namely *A. parvus* (3.6 %) and *A. dayi* (16.5 %), compared to the other species of the genus *Aplocheilus* from GenBank database. In addition, the genetic difference between *A. parvus* and *A. blockii* (mean difference 4.7 %) and the genetic difference between the species *A. dayi* and *A. werneri* (mean difference 11.1 %) was lower compared to the difference from other species, indicating their close evolutionary relationship.

Only seven sequences (i.e. AP1H, AP2H, AP1A, AP2A, AP3G, AP2G and AP4G) generated from the present study which have more than 500 bp were used for the phylogenetic analysis (Fig. 6). In the maximum-likelihood analysis of DNA sequences, Ap1A, Ap2A, Ap2G, Ap3G and Ap4G formed a distinct clade, and it was well-separated from *A. blockii* isolates from India (Fig. 6). Similarly, DNA sequences Ap1H and Ap2H formed a separate cluster from *A. werneri* isolate (KJ844713), which was earlier reported from Sri Lanka. Both clades were obtained with high (>99 %) bootstrap support values. Both *A. parvus* and *A. dayi*, determined based on morphology, were recovered as a sister group to a clade comprising *A. blockii* and *A. werneri*, respectively, within the analysis of our limited number of *Aplocheilus* samples**.** Intraspecies' genetic difference was observed within the isolates of proposed *A. parvus* and *A. dayi* based on phylogenetic analysis and genetic distance analysis as the isolates Ap3G and Ap1H exhibited more diverged sequences than other isolates in their respective proposed species.

Discussion

Morphological identification revealed the existence of *A. dayi* and *A. parvus* in the Attanagalu River, Sri Lanka, with *A. dayi* being more common than *A. parvus*. *Aplocheilus dayi* was found in greater abundance at higher altitudes and inland locations than at lower coastal locations. To our knowledge, there have been no earlier reports of *A. parvus* in the Attanagalu River basin. The absence of *Aplocheilus* spp. in the downstream areas of paddy fields, which were treated with pesticides or agrochemicals, was observed

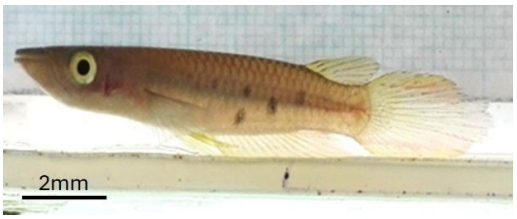
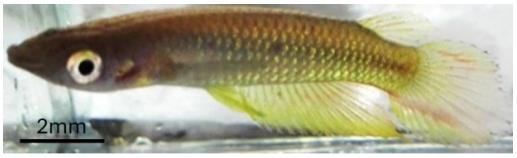
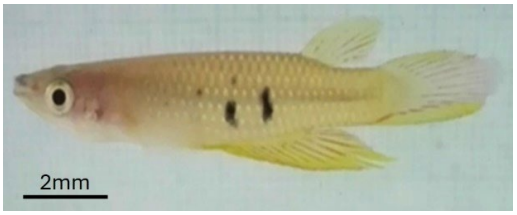
Aplocheilus dayi	*Aplocheilus parvus*
	
Three black-coloured spots on the right lateral side of the body. One is shady	The anal fin is reddish-orange coloured
	
Five shady black-coloured spots on the right lateral side of the body	Fins do not have a particular colour
	
No spots on the left lateral side of the body	The fins are yellow coloured
	
One shady spot on the centre of the left lateral side of the body	The margin of the anal fin is red coloured
	
Four black spots on the left lateral side of the body and the top one is shady	The body is slightly yellowish and the anal and dorsal fins are orange coloured
	
Four differentially sized black-coloured spots on the left lateral side of the body.	Anal and dorsal fins are reddish-orange coloured

Fig. 4. The morphological variations observed in *Aplocheilus dayi* and *Aplocheilus parvus* collected from Attanagalu River, Sri Lanka. Scale bar = 2 mm.

compared to the presence of those fish in the upper stream areas of the paddy fields. Stream water pollution due to agrochemicals could be a possible reason for this observation. The effect of pesticides and fertilisers on declining populations of *A. dayi* has been reported by Goonatilake (2012).

According to the results, morphometric truss analysis was inaccurate in *A. dayi* and *A. parvus* species differentiation. Morphological variations of some individuals may be due to crossbreeding (Aida, 1921) and the impact of climatic parameters that could lead to misidentification. The effect of environmental

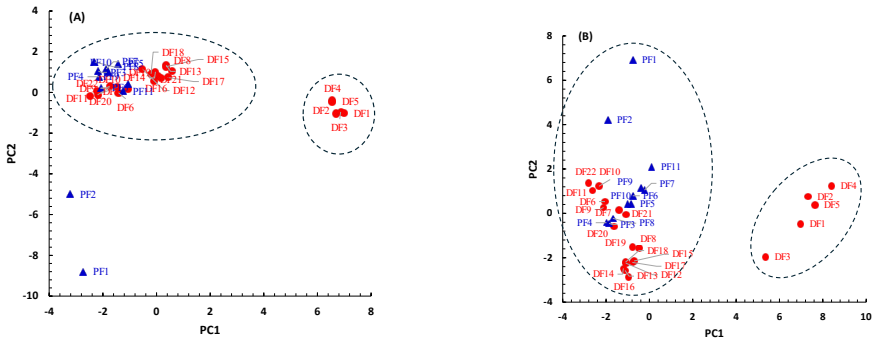


Fig. 5. The principal component analysis for *Aplocheilus dayi* and *Aplocheilus parvus* by the first (A) and the second approach (B) of truss analysis. PF: *A. parvus*; DF: *A. dayi*.

Table 3. Identification of *Aplocheilus* spp. using DNA barcode approaches. Species identification cutoff of 97 % was used for GenBank and BOLD databases.

Sample no./ sample code	GenBank accession number	BLAST search				BOLD search			
		The best homologue species (≥97)	Identity (%)	The best nearest match species	Identity (%)	Species level barcode records	Identity (%)	All barcode records	Identity (%)
1/Ap1G	MT252027	Not found	N/A	*A. blockii* (MK216794)	94	Not found	N/A	*A. blockii*	95
2/Ap1A	MT251879	Not found	N/A	*A. blockii* (KJ844712)	96	Not found	N/A	*A. blockii*	96
3/Ap2A	MT183675	Not found	N/A	*A. blockii* (KJ844712)	96	Not found	N/A	*A. blockii*	96
4/Ap2G	MT246501	Not found	N/A	*A. blockii* (MG813794)	96	Not found	N/A	*A. blockii*	96
5/Ap3G	MT252048	Not found	N/A	*A. blockii* (KJ844712)	92	Not found	N/A	*A. blockii*	91
6/Ap4G	MT252047	*A. blockii* (KJ844712)	97.5	*A. blockii* (KJ844712)	97.5	*A. blockii*	97.5	*A. blockii*	97.5
7/Ap1H	MT250343	Not found	N/A	*A. werneri* (KJ844713)	87	Not found	N/A	*A. werneri*	87
8/Ap2H	MT250196	Not found	N/A	*A. werneri* (KJ844713	96	Not found	N/A	*A. werneri*	96

Table 4. Estimates of evolutionary divergence (%) between the eight sequences used in the present study with the available sequences of species of *Aplocheilus*.

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]
[1] *A. parvus*	**0.0-9.0 (3.6)**							
[2] *A. dayi*	20.3-46.4 (31.2)	**0.0-16.5 (16.5)**						
[3] *A. andamanicus*	26.4-37.1 (29.5)	29.5-41.5 (35.5)	**0.0-0.5 (0.5)**					
[4] *A. armatus*	23.1-32.5 (25.8)	25.4-42.6 (34.4)	13.4-14.1 (13.7)	**0.0-1.0 (0.3)**				
[5] *A. blockii*	2.5-9.3 (4.7)	20.5-40.2 (30.4)	28.5-29.5 (28.9)	24.6-26.0 (25.2)	**0.0-1.0 (0.5)**			
[6] *A. lineatus*	19.9-27.1 (22.2)	23.0-38.0 (30.5)	24.2-24.6 (24.4)	22.0-24.0 (22.5)	21.7-22.6 (22.3)	**0.0-0.2 (0.2)**		
[7] *A. panchax*	23.1-31.9 (25.6)	26.3-40.4 (33.1)	9.9-10.9 (10.4)	7.5-9.1 (7.8)	25.0-26.5 (25.8)	23.3-24.7 (23.8)	**0.0-1.0 (0.7)**	
[8] *A. werneri*	19.5-27.1 (21.4)	4.2-18.1 (11.1)	28.2-28.7 (28.4)	23.2-25.1 (24.6)	19.6-20.6 (20.2)	24.4-25.0 (24.7)	23.1-24.1 (23.5)	N/A

Values in bold are intraspecies distances.
Values in the parentheses are mean genetic distances.
N/A = Not applicable.

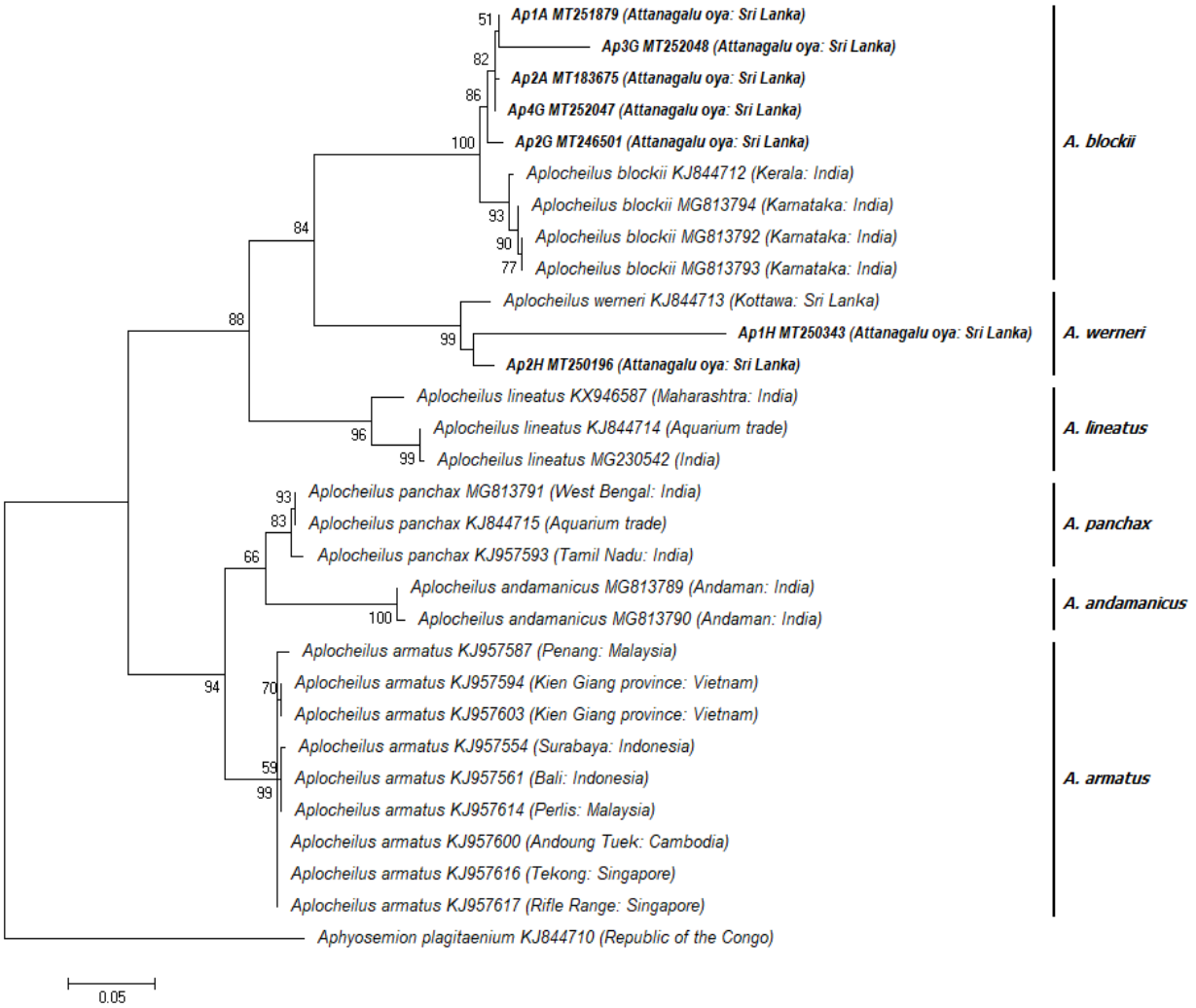


Fig. 6. Molecular phylogenetic analysis by Maximum Likelihood method to show the evolutionary relationship of the amplified region of *Cox*1 of different *Aplocheilus* spp. collected from different locations of the Attanagalu River basin with *Aplocheilus* spp. reported in previous studies. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Aphyosemion plagitaenium* (KJ844710) was used as an outgroup.

factors such as temperature, salinity and dissolved oxygen, water depth and current flow on fish morphology have been well documented (Barlow, 1961; Nishio et al., 2020), and the body shape of fish is not only determined with the genetics of a fish, but it has also been influenced by environment and its habitat (Guill et al., 2003).

In the present study, DNA sequencing of the *Cox*1 gene and subsequent homology search gave the highest per cent identity with the two *Aplocheilus* spp., *A. werneri* and *A. blockii*. However, those specimens in the present study were previously identified using morphological features as *A. dayi* and *A. parvus*. Similarly, when DNA barcoding was used, GenBank gave the best match results with *Pomadasys kaakan* for the morphologically identified *Pomadasys argenteus* (Fogelström, 2015). This result is possible in the homology search due to the non-availability of *A. dayi* and *A. parvus* DNA sequences in the public databases. It was impossible to find DNA sequences of the above two species despite a thorough literature survey and database survey in GenBank, NCBI and MitoFish. The sequence information is available only for *Aplocheilus* species such as *A. werneri*, *A. blockii*, *A. lineatus*, *A. panchax* and *A. andamanicus* (Köhler,1906).

However, the phylogenetic tree shows a clear grouping of the two samples (i.e., Ap1H and Ap2H) from the rest of the five samples (i.e., Ap2G, Ap3G, Ap4G, Ap1A and Ap2A). The two samples, Ap1H and Ap2H, showed a closer relationship to *A. werneri*. However, with a 99 % probability, the two accessions got clustered separately from *A. werneri* (KJ844713) and it strongly supports that Ap1H and Ap2H could represent a different species. *Aplocheilus dayi werneri* is a synonym for *A. dayi* (Froese and Pauly, 2022). This shows the possible genetic closeness between *A. dayi* and *A. werneri*, which is also evident from the findings of the present study (by evolutionary divergence and phylogenetic analysis). Based on the above background, it is reasonable to delineate the MT250343 and MT250196 sequences as *A. dayi*.

The other samples (i.e., Ap1A, Ap2A, Ap2G, Ap3G and Ap4G) used in the study formed a separate cluster showing a closer genetic relationship with the *A. blockii* voucher sample (reference sample). However, it was evident that these samples are a genetically different group to *A. blockii,* creating a well-separated group with a 100 % probability. Due to similarities in the morphological features, it has been reported that *A. parvus* is often misidentified as *A. blockii* (Froese and Pauly, 2022). According to Jayaram (1999), there is a higher possibility of confusing *A. parvus* with *A. blockii*; a few authors have even considered the two synonyms (Talwar and Jhingran, 1991). Phylogenetic analysis of our findings showed the genetic closeness of the five fish samples to *A. blockii*. Therefore, grouping the cluster of these fish samples (i.e., Ap1A, Ap2A, Ap2G, Ap3G and Ap4G) as *A. parvus* is justifiable. However, the non-availability of nucleotide sequences for voucher specimens of *A. dayi* and *A. parvus* in DNA databases is a limitation to delineating the samples studied in the present study, though molecular evidence supports the presence of two species in the study site.

The findings of the present study provided a wealth of information about the population distribution of *Aplocheilus* spp. in the Attanagalu River basin, which will be helpful for taxonomic studies and the conservation of biodiversity and habitats. This is the first molecular-based identification attempted in Sri Lanka for species delineation of *Aplocheilus* spp. Further, the study compares genomic variation in different *Aplocheilus* species concerning the mitochondrial *Cox*1 gene through phylogenetic analysis.

Conclusion

Findings of the present study propose the presence of *A. dayi* and *A. parvus* in the Attanagalu River basin of Sri Lanka, along with the intraspecies genetic variations among individuals within the proposed species with reference to the mitochondrial *Cox*1 gene. According to phylogenetic analysis *A. dayi* and *A. parvus* showed 99 and 100 % variation with the *A. werneri* and *A. blockii*, respectively. Truss analysis, which is used to distinguish morphological variations in fish species, may not be used to identify the two *Aplocheilus* species accurately.

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