

**PHYLOGENETIC RELATIONSHIPS OF FIFTEEN SPECIES OF
CYPRINIDS (TELEOSTEI) OF SRI LANKA, BASED ON BIOCHEMI-
CAL AND MORPHOLOGICAL CHARACTERISTICS.**

A. B. Kotalawala

*Department of Zoology, University of
Sri Jayewardanapura, Nugegoda.*

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Abstract

Phylogenetic relationships for fifteen species of Sri Lankan *Cyprinids* were revealed by the cladistic analysis of separate and combined biochemical and morphological characters. Different data sets yielded different cladograms. The consensuses of cladograms based on biochemical data conflict with the existing classification of subfamilies and include few Cyprinine species with Rasboranine species in one clad. The consensuses of cladograms based on morphology favour the existing classification of subfamilies and include Cyprinine species and Rasboranine species in separate clads. Despite these differences both cladograms favour the close relationship of some species. Both cladograms indicate that, *P. nigrofasciatus*, *P. Cumingi* and *P. bimaculatus* have a common origin and are closely related. Both data sets and the combined data favour that the four rasboranine species, *Rasbora daniconius*, *Rasbora vaterifloris*, *Amblypharyngodon melettinus* and *Danio malabaricus* have a common origin and are closely related.

Key Words: Phylogenetic relationships, biochemical characters, morphological characters, cladistic analysis.

1. Introduction

In the past, morphological approach is the main technique used in the systematics of animal groups. But during the last two decades molecular investigation of systematic problems have progressed from uncommon curiosities to a standard means of elucidating phylogenetic history. The allozyme analysis, which is based on the differences of protein molecules is the most common molecular technique used in the phylogenetic studies of animal groups. This technique was used to show the phylogenetic relationships among several reptile groups in different countries by different authors ('Nilson et. al. 1994, Caddie et. al. 1990, Queiroz and Lawson 1994') Allozyme analysis was used to clarify the colour polymorphism, geographic variations and intra and interspecific variations of amphibia (Karin and Gutman 1986, Shaffer et. al. 1991, Titus et. al. 1989). Phylogenetic patterns of the avian genus *Ammodramus* was studied by Zink and Avise in 1990 by using both mitochondrial DNA and allozyme variation. Allozyme

technique was used to determine the genetic variations, population subdivisions and post larvae identifications of species of prawns (Mulley and Latter 1981, Mathaeis et.al. 1981, Lavery and Staples 1990, Saini 1987, Mathaeis et.al. 1983, Benzie & Silva 1984). This technique was used in the gene duplications and systematics of fish by Shaklee et.al. in 1990.

Some phylogenetic relationships of Sri Lankan Cyprinids based on morphology and osteology was studied by Kotalawala and Jinadasa (Kotalawala and Jinadasa 1992, Kotalawala 1992, Kotalawala 1994). However these Cyprinids were not studied biochemically. The aim of the present study is to investigate the phylogenetic relationships based on biochemical characters and to compare it with the phylogenetic relationships based on morphological characters.

2. Materials and Methods

Fifteen Cyprinids were subjected to electrophoretic and morphological studies. For electrophoretic studies these fishes were collected from Wak-oya, one of the main feeding canals of the Kelani River, brought to the laboratory alive and kept in fish tanks until the beginning of the experiment. Just before the loading of the gel, these fishes were killed, skeletal muscle tissues (0.75 gms) were obtained into disposable plastic centrifuge tubes and homogenized in distilled water. The extracts were centrifuged for 10 minutes at 10000 rpm and the supernatant directly used for the loading of the gel.

Standard starch gels were prepared and horizontal gel electrophoresis was carried out as described by Hillis and Moritz (1990). Ten enzymes were tested and scored for interspecific variations (Table I). Allozyme characters were qualitatively coded into discrete states for the analysis in the two computer programs PAUP (Phylogenetic Analysis Using Parsimony version 3.0 Swofford 1991) and MacClade (Analysis of Phylogeny and Characters Evolution, version 3.0, Maddison and Maddison, 1992). For allozymes, the locus was considered the character, with alleles as the character states. Most of the characters were coded into two states while few characters were coded into multiple states. The character matrix for allozymes were shown in table II. All the characters were coded as unordered. The heuristic search in PAUP was employed to obtain the most parsimonious cladograms. 50% majority rule and strict consensus of these trees were searched. A bootstrap with 500 replicates was conducted to estimate the best fit cladogram.

Table I Enzymes tested for the interspecific variation of different species of Cyprinds.

Enzyme	Commision number	Buffer system	Locus
Malic enzyme (ME)	1.1.1.40	AM	ME1, ME2, ME3, ME4
Isocitric dehydrogenase (DH)	1.1.1.42	AM	IDH1, IDH2
Alcohol dehydrogenase (ADH)	1.1.1.1	AM	ADH1, ADH2
Phosphoglucose isomerase (PGI)	5.8.1.9	Ridgway	PGI, PGI2
Superoxide dimutase (SOD)	1.15.1.1	Ridgway	SOD1, SOD2
Malate dehydrogenase (MDH)	1.1.1.37	AM	MDH1, MDH2
Lactate dehydrogenase (LDH)	1.1.1.27	Ridgway	LDH1, LDH2, LDH3, LDH4
Esterase (EST)	3.1.1.1	Ridgway	EST1, EST2
Phosphoglucomutase (PGM)	5.4.2.2	Ridgway	PGM1, PGM2
Aspartate aminotranseferase (AAT)	2.6.1.1	Ridgway	AAT1, AAT2

Table II Allozyme characters coded into discrete states for the analysis of two computer programs PAUP and MacClaid, *P. do* = *P. dorsalis*, *P. fil* = *P. filamentosus*, *P. ni* = *P. nigrofasciatus*, *P. vit* = *P. P. vittatus*, *P. tit* = *P. titteya*, *P. bi* = *P. bimaculatus*, *P. ch* = *P. chola*, *P. am* = *P. amphibius*, *P. sa* = *P. sarana*, *R. da* = *Rasbora daniconius*, *R. va* = *R. vaterifloris*, *D. ma* = *Danio malebaricus*, *Eso* = *Esomus denrica*, *Amb* = *Amblypharingodon melettinus*.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		<i>P. do</i>	<i>P. fil</i>	<i>P. ni</i>	<i>P. cu</i>	<i>P. vit</i>	<i>P. tit</i>	<i>P. bi</i>	<i>P. ch</i>	<i>P. am</i>	<i>P. sa</i>	<i>R. da</i>	<i>R. va</i>	<i>D. ma</i>	<i>Eso</i>	<i>Amb</i>
1	ME1	A&B	B&C	B	C	A	B	?	B	E	B	A	A	-	-	-
2	ME2	-	-	A	A	-	A	?	-	C	-	A&B	A&B	A&B	B	A&B
3	ME3	-	-	B	B	B	-	?	-	-	-	-	-	-	A&B	B
4	ME4	-	-	A	-	B	B	?	B	B	-	A&B	A&B	-	A&B	-
5	PGI1	B	A&C	-	A&B	-	-	?	B	E	A&B	-	D	D	-	-
6	PGI2	-	A&C	A	A	A	A&B	?	-	-	-	A&B	A&C	A&C	-	-
7	AAT1	A&C	-	B&C	B&C	C	C	?	?	?	?	A&B	A&B	C	C&D	B&D
8	AAT2	A&B	-	A&C	A&C	-	-	?	?	?	?	-	C	C	-	-
9	EST1	-	-	B	B	A&B	B	A&B	?	?	?	-	B	-	-	-
10	EST2	A&B	A&B	-	-	-	-	?	?	?	?	B	-	B	-	B
11	LDH1	A&B	B	-	-	-	B&C	-	A&B	?	?	B&C	B	B	?	-
12	LDH2	A	B	B&C	B&C	B	B	B	A	?	?	D	D	D	?	-
13	LDH3	A&C	A	B	B	A&B	A	A&B	?	?	?	D&E	-	E	?	E&F
14	LDH4	A	A	-	-	A&B	A	A&B	?	?	?	C	-	-	?	A
15	PGM1	-	B	-	-	-	-	?	?	?	?	A	-	A	?	-
16	PGM2	A	-	A	B	A&B	B	B	?	?	?	C	C	-	?	B
17	MDH1	-	A	-	-	A&B	A	-	-	-	A	-	-	-	?	-
18	MDH2	A&B	A&B	C&D	C&D	C	C&D	A&C	B	B	A	-	-	E	?	B
19	IDH1	-	A	-	-	-	C	B	A	B	A	D	D	D	?	D
20	IDH2	A	A	A&B	-	-	C	B	A	A	A	-	-	-	?	-

The morphological data obtained from the previous studies (Kotalawala & Jinadasa 1994, Kotalawala 1994) were coded into discrete two state or multistate characters to analyse in the two computer programs, PAUP and MacClaid (Table III). The strict and 50% majority rule consences and bootstrap consences of morphological analysis were compared with those of biochemical data. A third computer analysis was carried out for combined biochemical and morphological data (Table IV). Strict, 50% majority rule and bootstrap consences of combined data was compared with those of morphological and biochemical data.

Table III The morphological characters of fifteen Cyprinids coded into character states, LL = Nature of lateral line, C = complete 1 = Incomplete, N.D. Spi = Nature of dorsal spine A = smooth B = serrate, Tr = Number of scales over and below the lateral line A = 3.5/3.5 B = 4.5/3.5, C = 5.5/3.5, Gill = number of gill rakers A = 4-10, B = 10-15, C = >15, Vert = Number of vertebrae, A = 25-30, B = 30-35 C = 35-40, Pect R = Number of pectoral Fin rays, A = 10-15, B = 15-20, D. Spi = Number of dorsal fin spines, A = 0-2, B = 3, C = 4 D. ray = Number of dorsal fin rays, A = <8, B = 8-10, C = 10-15, A. ray = Number of anal fin rays A = 5, B = 5-10, C = >10, Barb = Number of barbels, A = 0, B = 2, C = 4, Pty = Number of dorsal fin Pterygiophores, A = 1-10, B = >10, Pred = Predorsal bones, A = 1-5, B = 5-10 *P. bi* = *P. bimaculatus*, *P. cho* = *P. chola*, *P. cum* = *P. cumingi*, *P. fil* = *P. filamentosus*, *P. nig* = *P. nigrofasciatus*, *P. sar* = *P. sarana*, *P. tit* = *P. titteta*, *P. vit* = *P. vittatus*, *D. mal* = *Danio malabaricus*, *R. vat* = *Rasbora vaterifloris*, *R. dan* = *R. daniconius*, *E. dan* = *Esomus danrica*, *Ambly* = *Amblypharyngodon melettinus*, *P. do* = *P. dorsalis*, *P. amp* = *Puntius amphibius*.

	1	2	3	4	5	6	7	8	9	10	11
	LL	N.D. Spi	Tr	Gill	Vert	Pect R	D. Spi	D. ray	A. ray	Barb	Pty
1	<i>P. amp</i>	C	A	B	C	C	A	C	B	A	B
2	<i>P. bin</i>	C	A	A	B	B	A	B	A	A	B
3	<i>P. cho</i>	C	A	C	B	B	B	C	B	A	B
4	<i>P. cum</i>	I	B	A	A	A	A	B	B	A	A
5	<i>P. dos</i>	C	A	B	C	B	B	C	B	A	B
6	<i>P. fil</i>	C	A	B	A	B	A	C	B	A	B
7	<i>P. nig</i>	C	B	B	A	A	A	B	C	A	A
8	<i>P. sar</i>	C	B	B	A	B	B	C	B	A	C
9	<i>P. tit</i>	I	A	A	A	A	A	B	A	A	B
10	<i>P. vit</i>	I	A	A	B	A	A	A	B	A	A
11	<i>D. mal</i>	-	-	E	A	B	A	A	C	C	-
12	<i>R. vat</i>	-	-	B	B	B	A	A	B	B	-
13	<i>R. dan</i>	-	-	B	B	B	A	A	B	B	-
14	<i>E. dan</i>	-	-	F	C	B	A	A	B	B	-
15	<i>Ambly</i>	-	-	E	B	B	A	A	B	B	-

Table IV Combine molecular and morphological character matrix of Cyprinids *P. fil* = *Puntius filamentosus*, *P. nig*= *P. nigrofasciatus*, *P. do* = *P. dorsalis*, *P. cu*= *P. cuningi*, *P. vit*=*P. vittatus*, *P. tit*=*P. titteya*, *P. bi* =*P. bimaculatus*, *P. ch*= *P. chola*, *P. am*= *P. amphibius*, *P. sa*= *P. sarana*, *R. da* = *Rasbora daniconius* , *R.va*= *R. vaterifloris*, *D.ma*=*Danio malabaricus*, *E.da*= *Esomus danrica*, *Amb*= *Amblypharyngodon melettinus*, Gill= number of gill rakers, A= 10, B= 10-15, C=> 15, Vert= number of vertebrae, A= 25=30, B=30-35, C=35-40, Pect=number of pectoral fin rays, A=10-15, B=15-20, D. spi= number of dorsal fin spines, A=0, B=3, C=4, D. ray= Number of dorsal fin rays A=4-8, B=8-10, C=10-15, Array= num

		8BIM Applic														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		P.do	P.fil	P.nig	P.cu	P.vit	P.tit	P.bi	P.ch	P.am	P.sa	R.da	R.va	D.ma	E.da	Amb
1	ME1	A&B	B&C	B	C	A	B	?	B	E	B	A	A	-	-	-
2	ME2	-	-	A	A	-	A	?	-	C	-	A&B	A&B	A&B	B	A&B
3	ME3	-	-	B	B	B	-	?	-	-	-	-	-	-	A&B	B
4	ME4	-	-	A	-	B	B	?	B	B	-	A&B	A&B	-	A&B	-
5	PG11	B	A&C	-	A&B	-	-	?	B	E	A&B	-	D	D	-	-
6	PG12	-	A&C	A	A	A	A&B	?	-	-	-	A&B	A&C	A&C	-	-
7	AAT1	A&C	-	B&C	B&C	C	C	?	?	?	?	A&B	A&B	C	C&D	B&D
8	AAT2	A&B	-	A&C	A&C	-	-	?	?	?	?	-	C	C	-	-
9	EST1	-	-	B	B	A&B	B	A&B	?	?	?	-	B	-	-	-
10	EST2	A&B	A&B	-	-	-	-	?	?	?	?	B	-	B	-	B
11	LDH1	A&B	B	-	-	-	B&C	-	A&B	?	?	B&C	B	B	?	-
12	LDH2	A	B	B&C	B&C	B	B	B	A	?	?	D	D	D	?	-
13	LDH3	A&C	A	E	B	A&B	A	A&B	?	?	?	D&E	-	E	?	E&F
14	LDH4	A	A	-	-	A&B	A	A&B	?	?	?	C	-	-	?	A
15	PGH1	-	B	-	-	-	-	-	?	?	?	A	-	A	?	-
16	PGH2	A	-	A	E	A&B	B	B	?	?	?	C	C	-	?	B
17	MDH1	-	A	-	-	A&B	A	-	-	-	A	-	-	-	?	-
18	MDH2	A&B	A&B	C&D	C&D	C	C&D	A&C	B	B	A	-	-	E	?	B
19	IDH1	-	A	-	-	-	C	B	A	B	A	D	D	D	?	D
20	IDH2	A	A	A&B	-	-	C	B	A	A	A	-	-	-	?	-
21	GILL	C	A	A	A	B	A	B	B	C	A	B	B	A	C	B
22	VERT	B	B	A	A	A	A	B	B	C	B	B	B	B	B	B
23	PECT	B	A	A	A	A	A	A	B	A	B	A	A	A	A	A
24	D.SPI	C	C	B	B	A	B	B	C	C	C	A	A	A	A	A
25	D.RAY	B	B	B	B	B	A	A	B	B	B	B	B	C	B	B
26	A.RAY	A	A	A	A	A	A	A	A	A	A	B	B	C	B	B
27	BARB	B	B	A	A	A	E	B	B	B	C	-	-	-	-	-
28	PTY	A	A	A	A	A	A	A	A	B	A	A	A	B	A	A
29	PRED	A	A	A	A	A	A	A	A	B	A	B	A	B	B	B

3. Results

Analysis of biochemical data produced 100 equally parsimonious cladograms, each with a consistency index of 0.976 and a length of 83 steps. The strict, 50% majority rule and bootstrap consensus with 500 replicates is shown in fig. 1. 50% majority rule and bootstrap consensus show a good separation of clusters. It confirmed that the four species *Rasbora daniconius*, *Rasbora vaterifloris*, *Amblypharyngodon melettinus* and *Danio Malabaricus* have a common origin and are closely related species. In relation to the other species three types of consensus give different phylogenetic relationships.

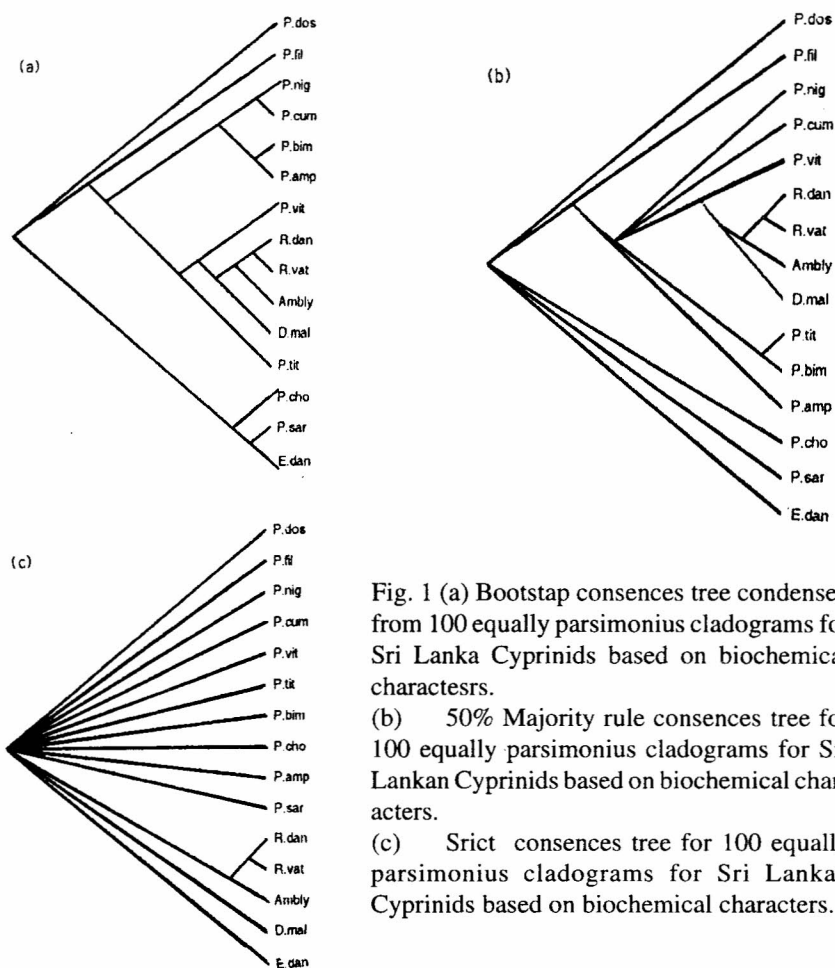
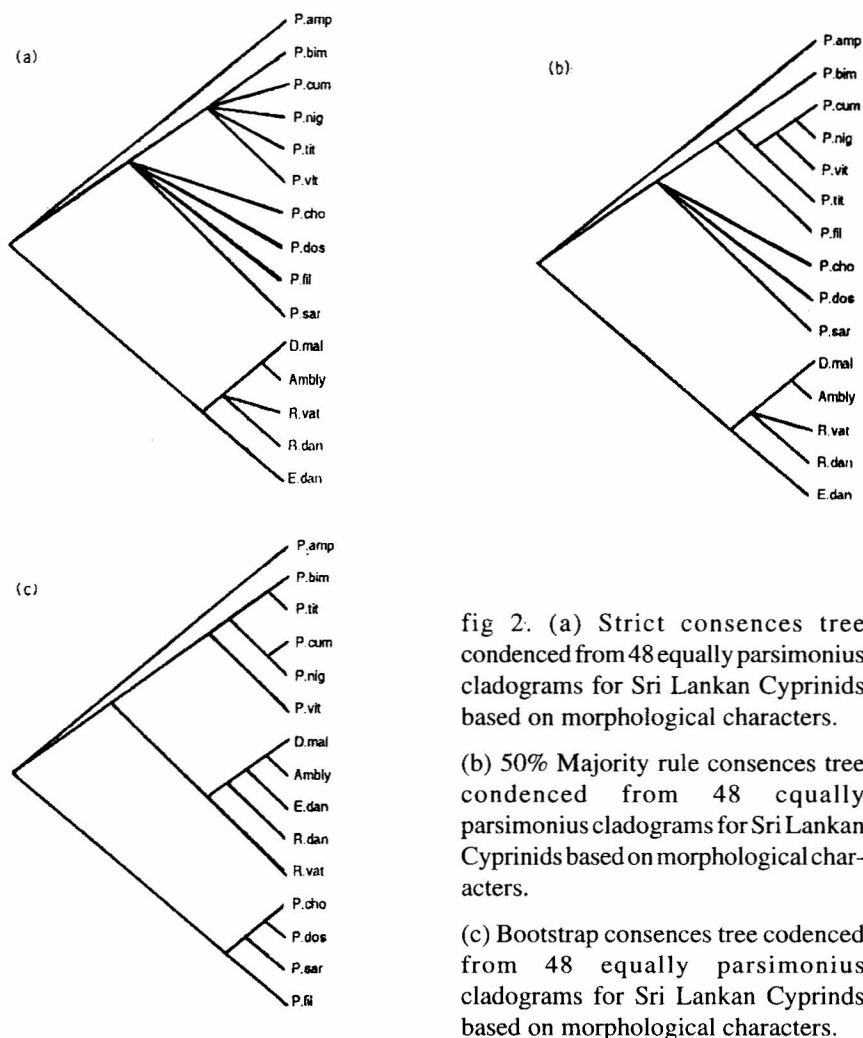


Fig. 1 (a) Bootstrap consensus tree condensed from 100 equally parsimonious cladograms for Sri Lanka Cyprinids based on biochemical characters.

(b) 50% Majority rule consensus tree for 100 equally parsimonious cladograms for Sri Lankan Cyprinids based on biochemical characters.

(c) Strict consensus tree for 100 equally parsimonious cladograms for Sri Lankan Cyprinids based on biochemical characters.

Analysis of morphological data produced 48 equally parsimonious cladograms, each with a consistency index of 0.55 and with a length of 40 steps. Strict, 50% majority rule and bootstrap consensus with 500 replicates are shown in fig 2. It shows that all three types of consensus trees are very much similar to each other. Based on these consensus trees, all Cyprinine species were separated from Rasboranine species. The five smaller *Puntius* species, *Puntius nigrofasciatus*, *P. cuningi*, *P. titteya*, *P. vittatus* and *P. bimaculatus* have a common origin and are closely related. The other four *Puntius* species *Puntius dorsalis*, *P. chola*, *P. sarana* and *P. filamentosus* also have a common origin and are closely related. *P. amphibius* is distantly related to all the other species. Five rasboranine species, *Rasbora daniconius*, *R. vaterfioris*, *Danio malabaricus*, *Amblypharingodon melettinus* and *Esomus danrica* have a common origin and are closely related to each other.



Analysis of combined biochemical and morphological data produces 54 equally parsimonious cladograms each with a consistency index of 0.897 and a length of 107 steps. The three types of consensus trees which shown in fig. 3. are similar to each other. It shows that five Rasboranine species *Rasbora daniconius*, *R. vaterifioris*, *Amblypharingodon melettinus*, *Danio malabaricus* and *Esomus denrica* clustered together and are closely related. The five smaller *Puntius* species *P. nigrofasciatus*, *P. cumingi*, *P. titteya*, *P. vittatus* and *P. bimaculatus* have a common origin and are closely related to each other, The relationship of all the other species vary in different consensus trees. Based on combined data *P. amphibius* has a common origin with Rasboranine species.

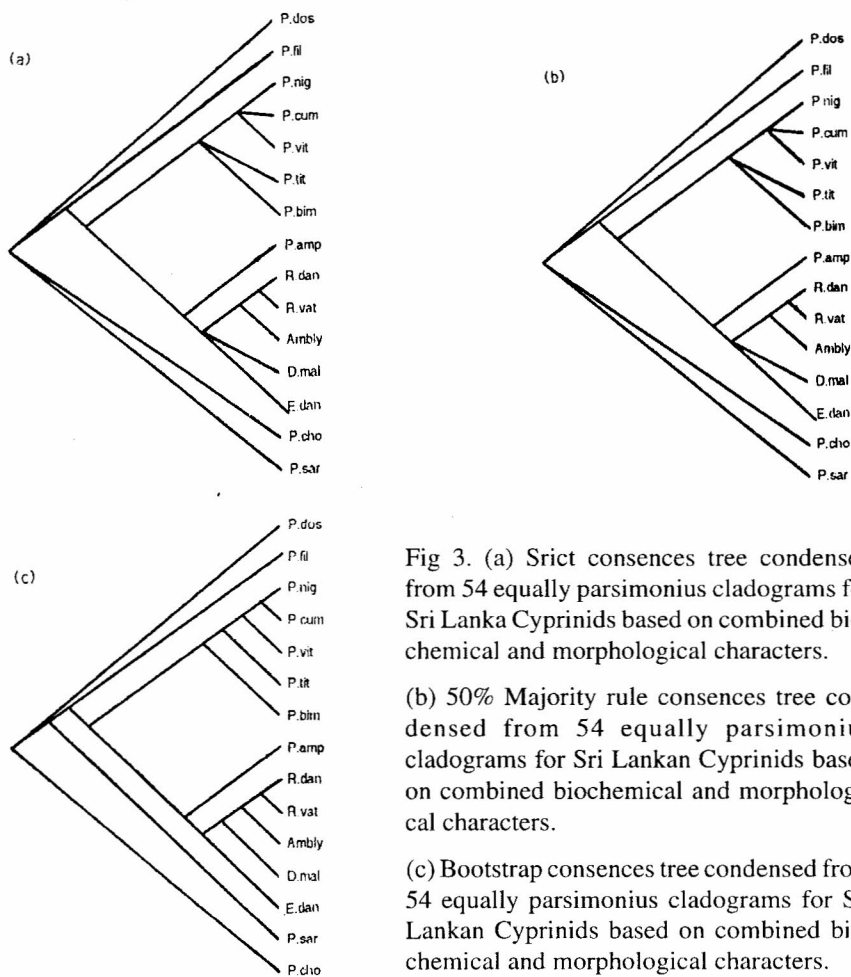


Fig 3. (a) Strict consensus tree condensed from 54 equally parsimonious cladograms for Sri Lanka Cyprinids based on combined biochemical and morphological characters.

(b) 50% Majority rule consensus tree condensed from 54 equally parsimonious cladograms for Sri Lankan Cyprinids based on combined biochemical and morphological characters.

(c) Bootstrap consensus tree condensed from 54 equally parsimonious cladograms for Sri Lankan Cyprinids based on combined biochemical and morphological characters.

4. Discussion

A primary objective of phylogenetic studies is to reconstruct the evolutionary history of a group of organisms. Because of the organisms under study have a single history, systematic studies of any set of genetically determined characters should be congruent with other such studies based on different set of characters in the same organisms. Congruence between studies is a strong evidence that the underlying historical pattern has been discovered; conflict may indicate that theoretical or procedural problems in one or both analysis or it may indicate that additional data are needed to resolve the phylogenetic relationships in question (Hillis 1987).

Both molecular and morphological characters used in this study are genetically determined. But the phylogenetic relationships obtained in the analysis of two data sets are not totally congruent. The molecular data point at an early separation of *P. dorsalis* from *P. filamentosus*. This type of separation is not clearly demonstrated by the cladogram based on morphology. Strict and bootstrap consensus of morphological data shows that *P. dorsalis* and *P. filamentosus* are closely related species and have a common origin with *P. chola* and *P. sarana*. Based on the molecular data, the two small *Puntius* species, *P. titteya* and *P. vittatus* are clustered together with four Rasboranine species, *Rasbora daniconius*, *R. vaterifloris*, *Amblypharyngodon melettinus* and *Danio malabaricus*. But, based on morphological data, all *Puntius* species were separated from Rasboranine species at an early stage. Molecular data point at an early separation of *P. dorsalis* from *P. Chola*. However, bootstrap consensus of morphological data indicate that two species *P. chola* and *P. dorsalis* are closely related and clustered together with *P. sarana* and *P. filamentosus*.

Although phylogenetic relationships based on molecular data are not totally congruent with those of morphological data, there are similarities of phylogenetic relationships of the two data sets. Analysis of both biochemical and morphological data indicate that the three cyprinine species, *Puntius nigrofasciatus*, *P. cumingi* and *P. bimaculatus* have a common origin and are closely related species. In the same way the four rasboranine species *Rasbora daniconius*, *R. vaterifloris*, *Amblypharyngodon melettinus* and *Danio malabaricus* have a common origin and are closely related. The consensus trees based on combined data is similar to consensus trees of both molecular and morphological data sets. This is an indication of the effect of one data set on the other. The addition of morphological data to the molecular data enhance the separation of clusters.

Two different data sets related to the same group of animals can make the drawing of conclusions of phylogenetic relationships more complex. (Shaffer et.al.1991). This is proven by the present study. The phylogenetic relationships based on morphology favour the existing classification of cyprinids into the two sub families sub family Rasborinae and sub family Cyprininae. But the phylogenetic relationships based on molecular data and combined data conflict with the existing classification of these cyprinids.

The morphological characters are direct and observable while the molecular characters are indirect and their appearance depend on number of complex chemical reactions. Character matrix of molecular data depend on the interpretation of the band pattern by the observer. Therefore there are more chances for errors to occur during the making of data matrix of molecular data than in the making of data matrix of morphological data. In the combined data analysis, the addition of morphological data into molecular data increase the number of characters and this can make operational problems in computer programs. These drawbacks and the conflict of phylogenetic relationships of molecular data with the existing classification of sub families, lead to assume that the phylogenetic relationships based on morphology is more favourable than the phylogenetic relationships based on molecular data and combined data.

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