

Molecular phylogeny of the *Puntius* (Hamilton, 1822) based on nuclear gene *RAG2*

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Abstract

The tropical Asian cyprinid genus *Puntius* is a major part of the ichthyofauna in Southeast Asia. Systematic status of the genus *Puntius* among Cyprinidae, the most prominent freshwater fish all over the world, remain to be substantiated. The molecular phylogenetic analyses derived from Recombination activating gene sequences (*RAG2*) for 35 representative samples of Malaysian *Puntius* and their allies, indicated paraphyly of the genus *Puntius* among common cyprinid groups of Southeast Asia. At a larger scale, while the monophyly of Cyprinidae and the subfamily Cyprininae were confirmed, the monophyly of the tribe Barbinini, Cyprinini, or *Puntius* (*Systemus*) were not supported.

Keywords: Cyprinidae, *Puntius*, *Systemus*, recombination activating gene 2, phylogeny.

Introduction

Family Cyprinidae with more than 3,268 species and 321 genera is one of the largest freshwater fish families in the world, in which, phylogenetic relationships among Malaysian cyprinids remain largely unresolved (Cunha *et al.*, 2002; Liu and Chen, 2003).

The subfamily Cyprininae is one of the most diverse groups within this family and even within the order Cypriniformes (Nelson, 2006). The subfamily Cyprininae generally split to four main groups; barbines (i.e. *Puntius*, *Barbus* and *Spinibarbus*), cyprinines (i.e. *Cyprinus*, *Carassius*, and *Carassioides*), labeonines (i.e. *Labeo*, *Garra*, and *Osteochilus*), and oreinines (i.e. snow trout) (Yang *et al.*, 2009). The systematic relationships of these groups has been extremely chaotic, as various numbers of groups have been recognized within the subfamily (Chen *et al.*, 2005, 2009; Fang, 2003; Fang *et al.*, 2009, Yang *et al.*, 2009). Amongst different kinds of freshwater fishes of the cyprinid family, the genus *Puntius* Hamilton 1822, commonly known as barb, is a major part of the ichthyofauna in Southeast Asia. The genus,

which contains some 220 nominal species (about 120 of these considered valid), has seen a significant accretion of new species in recent years. Many species are traded internationally as ornamentals (Collins *et al.*, 2012). Carrying the generic name, *Puntius*, was made familiar by Hamilton in 1822, but later researchers have used *Barbonymus*, *Barbus*, *Barbodes*, *Systemus*, *Capoeta*, and *Hypsibarbus*, as generic names and these have not been generally accepted (Mohsin and Ambak, 1983; Rainboth, 1996; Taki, *et al.*, 1977).

Several molecular phylogenetic studies have already used this family to define phylogenetic links or biogeographical inferences (Briolay *et al.*, 1998; Fang, 2003; Fang *et al.*, 2009; He *et al.*, 2008). Among them nuclear genes have been demonstrated to be phylogenetically informative in many studies of phylogenetic relationships (Hansen and Kaattari 1996; Robalo *et al.* 2008). In this study, *RAG2* gene sequences were used to examine the phylogenetic status of *Puntius* and their allies in Peninsular Malaysia and to test the monophyly of the genus *Puntius*, different tribe, subfamily Cyprininae and Southeast Asian

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cyprinids based on nuclear gene (*RAG2*).

Material And Methods

Taxa Sampling

Total 35 samples of Malaysian barb were captured from different parts of Peninsular Malaysia, namely Terengganu, Selangor, Pahang, Perak and Johor (Fig1). Samples of muscle tissues were amplified using the primers (*RAG2*-forward 5'-TTTGGrCArAAGGGCTGGCC-3' and *RAG2*-reverse: 5'-GTrGARrTAGTAGGGCTCCCA-3') that were adapted from Lovejoy and Collette (2001). The DNA from each sample were tested using PCR amplification 20 μ l

reaction volumes of genome DNA containing 2.0 μ l DNA sample, 1.6 μ l MgCl₂, 2 μ l 10x Buffer, 0.4 μ l Tag DNA polymerase, 2.4 μ l dNTPs that contains dATP, dTTP, dGTP and dCTP, 1.0 μ l each primer and 9.6 μ l of double distilled water were amplified. PCR cycling was performed using an additional "touchdown" step, followed by 34 cycles of denaturation for 45 sec at 94 C annealing for 60 sec at temperature specific for the *RAG2* (53 C until 63 C) and 1 min extension at 72 C with a final extension of 72 C for 10 min (Lovejoy and Collette 2001). The sequences generated in this study for *RAG2* gene were deposited in Genbank under accession numbers GU911496 to GU911516 (Table1).

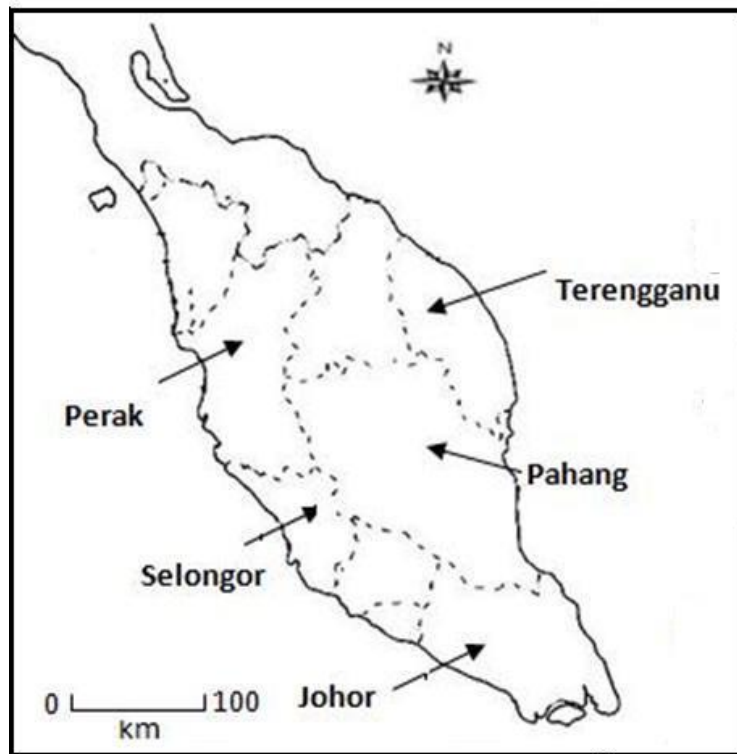


Figure 1. Map showing sampling locations of *Puntius* and their allies in Peninsular Malaysia.

To investigate the phylogenetic position of *Puntius* within the subfamily Cyprininae and their relationships with other cyprinids, we also added eight cyprinids from three genera, 13 barbids from seven genera, and five labeonids from four genera. The phylogenetic tree was rooted with nine species from other cyprinid

subfamilies and three species belong to other Cypriniform families (Table.2).

Phylogenetic Analyses

A total of 74 sequence for *RAG2* (29 nominal genera and 31 species) were aligned with Mafft

Table 1. Taxa examined in this study with Genbank accession numbers.

Cod	Specimen	Accession number (RAG2)	Locality
(JPB4)	<i>Puntius binotatus</i> *	GU911496	Johor
(JPB13)	<i>Puntius binotatus</i> *	GU911497	Johor
(JPB8)	<i>Puntius binotatus</i> *	GU911498	Johor
(JPL1)	<i>Puntius lateristriga</i>	GU911510	Johor
(JPL2)	<i>Puntius lateristriga</i> *	GU911511	Johor
(JPL3)	<i>Puntius lateristriga</i> *	GU911512	Johor
(TPS1)	<i>Barbonymus schwanefeldii</i> *	GU911470	Terengganu
(PPS2)	<i>Barbonymus schwanefeldii</i> *	GU911471	Pahang
(PPS12)	<i>Barbonymus schwanefeldii</i> *	GU911489	Perak
(PPS7)	<i>Barbonymus schwanefeldii</i> *	GU911471	Pahang
(PPBU1)	<i>Puntioplites bulu</i> *	GU911471	Perak
(PPBU2)	<i>Puntioplites bulu</i> *	GU911472	Perak
(BPBU3)	<i>Puntioplites bulu</i> *	GU911475	Selangor
(PPD11)	<i>Hypsibarbus wetmorei</i> *	GU911477	Perak
(PPD14)	<i>Hypsibarbus wetmorei</i> *	GU911478	Perak
(PPD16)	<i>Hypsibarbus wetmorei</i> *	GU911479	Pahang
(PPG06)	<i>Barbonymus gonionotus</i> *	GU911483	Perak
(BPG21)	<i>Barbonymus gonionotus</i> *	GU911484	Selangor
(PPG3)	<i>Barbonymus gonionotus</i> *	U911485	Pahang
(BPG21)	<i>Barbonymus gonionotus</i> *	JN258610	Selangor
(BPT11)	<i>Puntius tetrazona</i> *	GU911491	Selangor
(TPT24)	<i>Puntius tetrazona</i> *	GU911494	Terengganu
(PPT14)	<i>Puntius tetrazona</i> *	GU911495	Terengganu
(PPT14)	<i>Puntius tetrazona</i> *	GU911492	Perak
(TPT15)	<i>Puntius tetrazona</i> *	GU911493	Terengganu
(JPF7)	<i>Puntius johorensis</i> *	GU911508	Johor
(JPF10)	<i>Puntius johorensis</i> *	JN258618	Johor
(JPF4)	<i>Puntius johorensis</i> *	GU911509	Johor
(BPH21)	<i>Puntius hexazona</i> *	GU911501	Selangor
(BPH11)	<i>Puntius hexazona</i> *	GU911502	Selangor
(JPH10)	<i>Puntius hexazona</i> *	GU911503	Johor
(JPE4)	<i>Puntius everetti</i> *	GU911513	Johor
(JPE6)	<i>Puntius everetti</i> *	GU911514	Johor
(JPE2)	<i>Puntius everetti</i> *	GU911515	Johor
(JPE5)	<i>Puntius everetti</i> *	GU911516	Johor

Table 2. Taxa were added from Genbank with accession numbers.

Taxa	Accession number RAG2	Taxa	Accession number RAG2
Order Cypriniformes		Subfamily Cyprininae	
Family Catostomidae		Tribe Cyprinini	
<i>Myxocyprinus asiaticus</i>	DQ367043	<i>Carassius auratus</i>	DQ366941
Family Balitoridae		<i>Cyprinus carpio</i>	DQ366994
<i>Pseudogastromyzon fangi</i>	DQ367042	<i>Cyprinus</i>	DQ366994
<i>Micronemacheilus pulcher</i>	DQ367041	<i>Cyprinus</i>	DQ366939
Family Cobitidae		<i>Cyprinus carpio</i>	AY787041
<i>Paramisgurnus dabryanus</i>	DQ367040	<i>Cyprinus multitaeniata</i>	DQ366939
Family Cyprinidae		<i>Cyprinus carpio</i>	AY787041
Subfamily Leuciscinae		<i>Proocypris</i>	DQ366969
<i>Cyprinella lutrensis</i>	DQ367019	Tribe Barbini	
<i>Cyprinella lutrensis</i>	DQ367019	<i>Barbonymus schwanenfeldii</i>	DQ366961
<i>Ctenopharyngodon idella</i>	EF514220	<i>Barbodes huangchuchieni</i>	DQ366952
<i>Ctenopharyngodon idella</i>	DQ366996	<i>Barbodes vernai</i>	DQ366987
Subfamily		<i>Barbus sp</i>	DQ366990
Hypophthalmichthyina		<i>Barbus</i>	DQ366991
<i>Hypophthalmichthys nobilis</i>	DQ367038	<i>Barbus barbus</i>	DQ366980
<i>Hypophthalmichthys molitrix</i>	DQ367002	<i>Barbus barbus</i>	DQ366990
<i>Hypophthalmichthys molitrix</i>	DQ367001	<i>Barbus sp</i>	DQ366981
Subfamily Gobioninae		<i>Puntius tetrazona</i>	DQ366938
<i>Gobiobotia abbreviata</i>	DQ367033	<i>Puntius semifasciolatus</i>	AY804121
<i>Gobio gobio</i>	DQ367015	<i>Puntius tetrazona</i>	DQ366938
Tribe Labeonini		<i>Spinibarbus hollandi</i>	DQ366973
<i>Lobocheilos melanotaenia</i>	DQ366940	<i>Hampala macrolepidota</i>	DQ366965
<i>Cirrhinus molitorella</i>	DQ366959		
<i>Labeo yunnanensis</i>	DQ366948		
<i>Labeo rohita</i>	JQ346101		
<i>Labeo yunnanensis</i>	DQ366941		

v.6 (Katoet *al.*, 2005). Alignment was based on the inferred amino acid sequence. Maximum likelihood (ML) analysis was performed using PAUP 4.0b10 (Swofford, 2002). Bayesian inferences (BI), using the Markov Chain Monte Carlo method, were performed with MrBayes 3.1.1 (Huelsenbeck and Ronquist 2001) ML models and BI parameters were determined by an Akaike Information Criterion (AIC) as

implemented in Modeltest (Posada and Crandall 1998). The estimated models were used in a subsequent ML heuristic tree search with ten random addition sequences replicates, and TBR branch swapping. To test the robustness of nodes we ran 200 bootstrap replicates under ML with a single random addition sequences per bootstrap replicate. The posterior distributions were summarized as a majority- rule consensus tree

(Aliabadian *et al.* 2007).

The tree structures depicting hypothesized relationships and monophyly of supra specific groups within the Cyprininae (subtribes Barbinini and Cyprinini), as well as hypothesis of monophyly and classifications of *Puntius* and in subsequent generic revisions by Mohsin & Ambak (1983), Rainboth (1996), and Kottelat (1993) was tested against our resulting phylogenetic results using Shimodaira and Hasegawa test (SH test) and RAxML (sequential version). Ten constraint trees were constructed based on different assumed groups using the maximum likelihood searches in RAxML v. 7.0.3 (Stamatakis, 2006; Stamatakis *et al.*, 2008).

The GTR+C+I model was adopted for *RAG2* gene. A total of 100 distinct tests were performed based on each of the ten constraint trees. Shimodaira and Hasegawa tests (SH test) using RAxML were constructed (sequential version) to determine whether the best ML trees resulting from constraint searches are significantly worse than the best ML tree obtained from the original non-constraint search (Shimodaira and Hasegawa, 1999; Stamatakis, 2006; Stamatakis *et al.*, 2008).

Results

Details of selected model for ML and BI analyses are summarized in Table 3.

Phylogenetic analyses of ML and BI revealed that our data formed five major clades that have traditionally been recognized namely, (I) Cyprininae; (II) Leuciscinae; (III) Xenocyprininae (IV) Gobioninae; and (O) outgroups (Fig 2).

The results of *RAG2* data set in Maximum likelihood (ML) and Bayesian inferences (BI) showed monophyly of family Cyprinidae, Cyprininae with high nodal support in all genera. The Bayesian analysis was identical in topology to ML tree. In the BI tree, the nodes were well resolved and most nodes for the recovered tribes were supported thoroughly with significant posterior probabilities (Fig. 2).

Phylogenetic analyses of ML and BI of Cyprininae subfamily in data set, including (*Barbodes*, *Cyprinus*, *Spinibarbus*, *Barbus*, *Hampala*, *Cirrhinus*, *Lobocheiles*, *Labeo*, *Osteochilus*, *Ctenopharyngodon*,

Hypophthalmichthys, *Barbonymus*, *Cyprinella*, *Puntius*, *Procypris*, *Gobiobotia*, *Gobio*) formed five major subclades. The nodes for the recognized subclades are marked with black dot (Fig 2).

These five major subclades (subclades 1–5) were successfully recovered with high bootstrap values (BP) >90% in the ML analysis and high Bayesian posterior probability values (BPP). Subclade 1 (BP=50% and BPP=97%) which is the basal-most group, contains five species of labeonin (*Labeo*, *Lobocheilos*, *Cirrhinus*, *Osteochilus*, *Lobocheiluand*) and *Procypris* of Cyprinini. Subclade 2 (BP=100% and BPP=77%) comprised *Barbus* and *Spinibarbus*. Some taxa of the tribe cyprinini included *Cyprinus* and *Carassius* clustered within Subclade 3 (BP=100% and BPP=99%). Subclade four included some taxa of the tribe barbinini namely, *Barbonymus*, *Barbodes*, *Hypsibarbus*, *Puntioplites* and *Puntius* (BP=100% and BPP=97%). Subclade 5 (BP=100% and BPP=9%) was comprised exclusively of all barbini genera included different species of *Puntius* and *Hampala*.

Cyprinini sensu stricto are found in two clades, clade 3 and clade 4 a relationship that is also strongly supported (BP = 100%; BPP = 100%) (Fig 2). Members of Barbinini are found in all sub-clades except Subclade 1.

SH tests for *RAG2* was identical and revealed that the diagnosis proposed in this study for family cyprinidae and sub family cyprininae. The BI topology was not inferior to the best ML; hence, the monophyly found in our analyses for the best trees was reinforced in SH tests.

The results were reinforced by the SH test showed that only the ML trees resulting from constraint searches obtained enforcing the monophyly for tribe Barbinini and Cyprinini and the genus *Puntius* were significantly worse than the tree obtained from non-constraint search. Comparisons of alternative phylogenetic hypotheses and tree likelihoods compared to the best tree using the SH test with RAxML are outlined in Table 4.

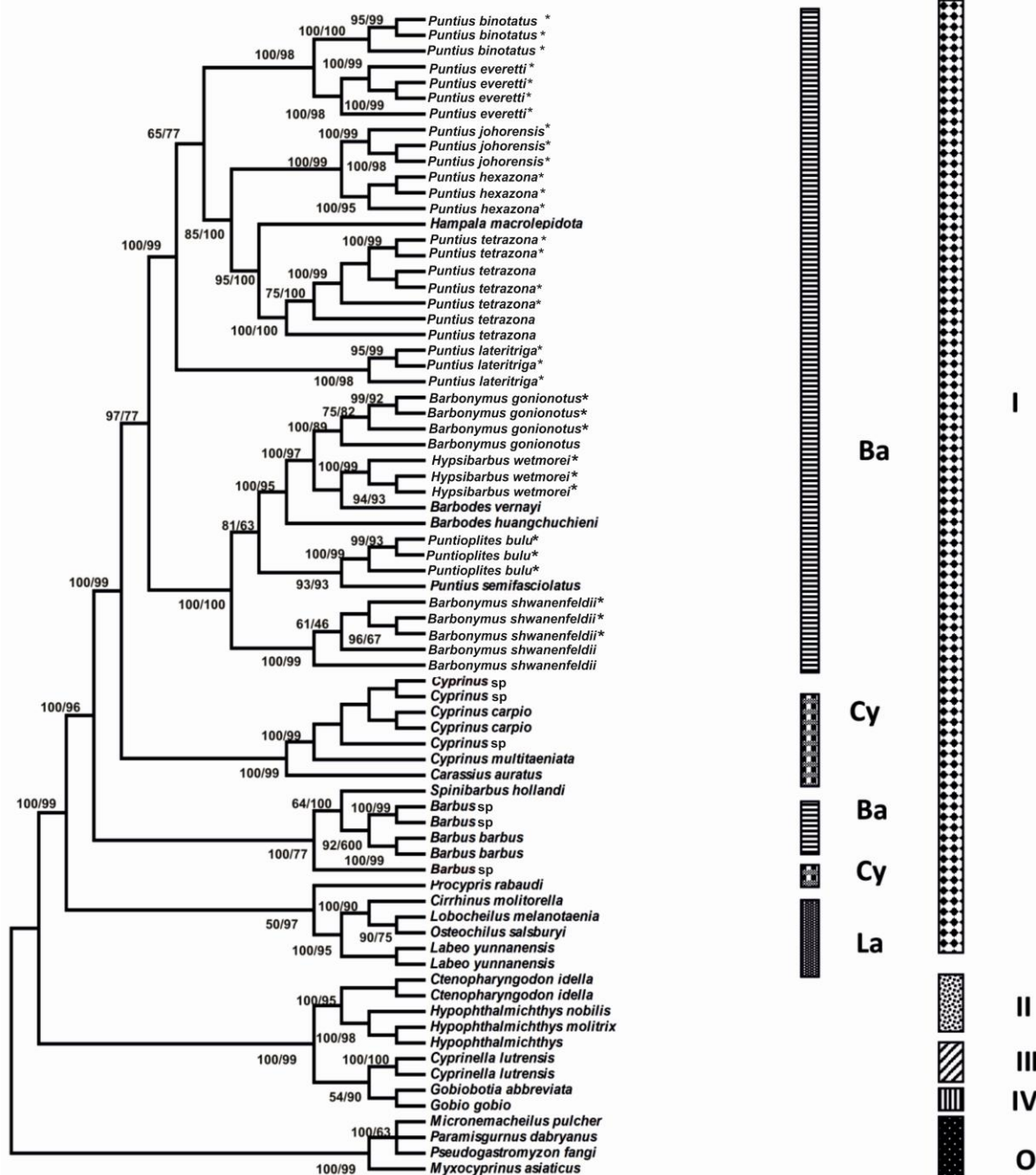


Figure 2. Bayesian consensus tree resulting from analysis of 890 bp of RAG2 among Cyprinidae. The number at each node represents bootstrap values in Bayesian posterior probability (BPP) and the ML analysis (BP) values (all > 0.50/50%). Recognized clades are indicated by Roman numerals on the right side of the figure. (I) Cyprininae; (II) Leuciscinae; (III) Xenocyprinae; (IV) Gobioninae; and (O) outgroups. Nodes for the recognized subclades are marked with black dot. Tribe recognized in Cyprininae: (Ba) Barbinini, (Cy) Cyprinini, (La) Labionini.

Table 3. Characteristic resulting of the appropriate models was selected by Modeltest (Posada and Crandall, 1998).

Characteristics	RAG2
Number of parsimony informative characters	396/890
Number of conserved characters	256/890
Number of variable sites	643/890
The overall transition/transversion bias	$R = 1.872$
Number of most parsimonious trees obtained	250
Consistency index	0.48
Tree length	1342
Retention index	0.76
Model selected by Modeltest	GTR +G+I
Assumed proportion of invariable sites (pinv)	0.0512
Gamma distribution shape parameter (a)	0.8974

Table 4: Comparison of alternative phylogenetic hypotheses using the Shimodaira-Hasegawa test.

Topology tested	best ML tree	D) ln L	SD	Significantly worth
Best tree	-7512.628213			
Monophyly of Cyprinidae	-7512.628207	-0.000006	0.006340	NO
Monophyly of Cyprininae	-7512.628208	-0.000005	0.006441	NO
Monophyly of Barbinini	-7631.005364	-118.37715	20.20483	YES
Monophyly of Cyprinini	-7644.288963	-131.66075	23.50695	YES
Monophyly of <i>Puntius</i> (Kottelat 2000)	-7594.264184	-81.63597	17.84544	YES
Monophyly of <i>Puntius</i> (Mohsin & Ambak 1983)	-7766.651826	-254.02361	30.58707	YES
Monophyly of <i>Systomus</i> (Rainboth 1996)	-7594.264184	-81.63597	17.84544	YES
Monophyly of <i>Hypsibarbus wetmorei</i>	-7512.628209	-0.000003	0.007958	NO
Monophyly of <i>Puntioplites bulu</i>	-7512.628254	-0.000041	0.007715	NO
Monophyly of <i>Barbonymus schwanefeldii</i>	-7512.628209	-0.000003	0.007958	NO
Monophyly of <i>Barbonymus gonionotus</i>	7512.628209-	-0.000003	0.007958	NO

In L: difference in tree likelihood compared to the best tree. NO, not significantly worse than the best topology; YES, significantly worse than the best topology NO, not significantly worse than the best topology, significant, $P < 0.05$.

Discussion

Comparisons and phylogenetic analyses of *RAG2* sequences derived in this study with those from Genbank confirmed the monophyly of Cyprinidae and the subfamily Cyprininae using different methods of analyses (ML, BI), all of which resulted in nodal support for these groups reflecting the robust nature of these clades. But In the present study the monophyly of the tribe Barbinini and Cyprinini were not supported.

The findings were consistent with previous morphological (Cavender&Coburn 1992; Chen *et al.* 1984) and molecular studies (Liu & Chen 2003; Wang *et al.* 2007; He *et al.* 2008; Mayden *et al.* 2009; Yang *et al.* 2009).

The *Puntius* seem to be paraphyletic genus in *RAG2* data set because of unstable position of different species of *Puntius* in phylogenetic tree produced by *RAG2* gene using different data analyses (BI and ML). However, the robustness grouping of the *Puntius* remains to include all individuals of this genus in the phylogenetic tree. In this study the members of the *Hampala* genus placed among different species of *Puntius*. These results are consistent with other findings in molecular phylogenetic analyses by Wang *et al.* (2007).

The type of species of *Puntius* is *Puntius sophore* is described from India. The species *Puntius sophore* (Pool barb) was originally named as *Cyprinus sophore* by Francis Buchanan-Hamilton in 1822, and is also referred to as *Systomus sophore*, and *Barbus sophore* (Hamilton, 1822). *Systomus* having long been treated as a synonym of *Puntius* (Bleeker, 1863; Day, 1878; Banareescu, 1997).

Rainboth(1996)considered *Systomus* to be a valid genus distinct from *Puntius sensu stricto*. Further species were added by subsequent authors (Ng & Tan, 1999; Kottelat & Tan, 2011). In this study following Kottelat (2000, 2001), Kottelat & Widjanarti (2005) and Tan & Kottelat (2009) we retained *Systomus* in the synonymy of *Puntius*, not because we considered *Puntius* to be monophyletic, but because recognizing six species in this study (*Puntius hexazona*, *Puntius binotatus*, *Puntius hexazona*, *Puntius johorensis*, *Puntius everetti*, *Puntius tetrazona* and *Puntius lateristriga*) as *Systomus* out of the large heterogeneous *Puntius* without discussing the

generic position of the remaining species would cause confusion. This confusion is in part because the type species of *Systomus*, *S. immaculatus*, considered a synonym of *S. sarana* since Day (1878), is poorly known.

These taxonomic problems persist because most of the earlier investigations were solely based on classical techniques. A synthesis of classical with recent morphometric and molecular studies may provide a solution to the Southeast Asian Cyprinid taxonomic problem particularly among the *Puntius* complex (Pethiyagoda *et al.* 2012).

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