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Discovery of a Distinctive Carbamoyl phosphate synthetase in the Air-Breathing Teleost *Heteropneustes Fossilis*: Unveiling Evolutionary Significance

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ABSTRACT

Carbamoyl phosphate synthetase (CPS) is one of the large and ancient gene with tandem duplication, universal distribution, constant function and highly conserved over great phylogenetic distance. The CPS catalyses the formation of carbamoyl phosphate from CO_2, ATP and ammonia or glutamine for pyrimidine biosynthesis, arginine biosynthesis or urea cycle. It has been suggested that all three forms of CPS have evolved from a progenote, kinase gene, which had undergone an ancient duplication. In an attempt to study CPS isoenzyme genes in *H.fossilis* this is first partial unique sequence of CPS gene of 215 base pairs from *H. fossilis*. A comprehensive phylogenetic analysis considering the CPS sequences from representatives of all three domains of classification archea, bacteria and eukarya indicates unique CPSI in air-breathing teleost, *H. fossilis*.

Keywords: Carbamoyl Phosphate Synthetase, Heteropneustes fossilis, evolution, phylogeny, Ureotely

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INTRODUCTION

Common ancestor of all life forms arose between 3500 and 4000 million years ago (Fox et al., 1980). According to recent classification, there are three separate and distinct cell lineages from which all the modern cells have derived: eubacteria, archae bacteria and eukaryotes (Woese & Fox, 1977). As far as evolutionary studies are concerned, only those molecules are helpful which are distributed in all the three lineages and are highly conserved since Carbamoyl phosphate synthatase (CPS) is one of the large and ancient gene with tandem duplication, universal distribution, constant function and highly conserved over great phylogenetic distance. It has been intended to use this as molecular chronometer to study evolution (Schofield, 1993; Anderson, 1994).

CPS catalyses the formation of carbamoyl phosphate from CO_2, ATP and ammonia or glutamine for pyrimidine biosynthesis, arginine biosynthesis or urea cycle. Till now there are three known forms of CPS i.e. CPSI, CPSII and CPSIII. Bacteria generally have a single dimeric CPS II enzyme composed of small subunit (42kDa) and large subunit (120kDa) , which produces CP for both arginine as well as pyrimindine pathway (Meister, 1989). Smaller subunit catalyses the hydrolysis of glutamine (requiring active site cys and his residues) releasing free amino group to large subunit (Miester et al., 1974) whereas large subunit catalyses the formation of carbamoyl phosphate in a complex reaction. Heterodimeric CPS are encoded by genes which are co-transcribed, separately transcribed or even on separate chromosomes (Werner et al.,1985; kwon et al., 1994; Dillon et al., 1995)

It has been suggested that all the three forms of CPS have evolved from a progenote (kinase gene) which had undergone an ancient duplication that has been already proved (Nyunoya & Lusty, 1983). In proteobacteria like *E. coli* and *S. typhimurium* only one CPS is reported. In *E.coli*, small and large subunits are encoded by car A and car B genes (Beckmann et al., 1974). Contrasting to it, a gram positive bacteria *Bacillus subtilis* and *Lactobacillus plantarum* have two different types of CPSII ,each specific to either arginine or pyrimidine biosynthesis (Quinn et al., 1991; Brinjel et al, 2000) where as in protozoans only one CPS is reported till now. Yeast along with all other eukaryotes possess two different CPS for arginine and pyrimidine synthesis (Werner et al., 1985). According to a hypothesis proposed the ancestors of the eukaryotes diverge early from archea (Gouy M & Li W H , 1989; Koonin et al, 2008) and the eukaryotes arose through fusion of an archean and eubacterium which became nucleus and cytoplasm respectively (Lake,1988). Hence, Archean CPS may have significant similarities with mitochondrial CPS forms in eukaryotes.

In eukaryotes, monomeric CPS is found which corresponds to a profused fusion of GAT (Glutamine amidotransferase) and synthetase domains separated by a putative dimerisation

domain corresponding to small and large subunit of *E.coli* with significant internal homology of about 40% between N-terminal and C-terminal domains of synthetase domain (Nyunoya et al., 1985). Moreover, synthetase domain comprises of two homologous halves. The synthesis of CP in higher eukaryotes is catalysed by two separate enzymes: either CPS I or CPS III for urea biosynthesis and CPS II for pyrimidine biosynthes. Encoded by separate nuclear genes, CPSI or CPSIII is transported into the mitochondria (Casey & Anderson, 1982; Mommsen & Walsh,1989; Cao et al., 1991) where as CPS II remains in cytoplasm as a part of CAD complex (with Aspartate transcarbamylase (ATC) and Dihydro-orotase (DHO) (Jones,1980;Evans,1986). The properties of CPSI, CPS III are very similar except that former utilizes ammonia as substrate whereas later is glutamine dependent. CPSI is reported to be present in ureotelic vertebrates like amphibians, birds and mammals. The CPSIII, the most recently discovered CPS, is reported in few invertebrates, ureoosmotic elasmobranchs (Anderson, 1980) , lungfishes (Mommssen & Walsh, 1989,1991; Randall et al.,1989) and in few fresh water teleost like M. salmoides, Oncorynchus mykiss (Anderson 1976; Anderson 1998).

Most interestingly, the functional CPSI along with CPSIII has been reported in the liver of freshwater air-breathing catfish, *H. fossilis* (Saha et al., 1997, Saha & Ratha, 1998). It is apparent that CPS of *H. fossilis* is showing affinity with invertebrates, elasmobranchs, primitive fishes as well as with ureotelic amphibians and mammals suggesting an intermediate evolutionary significance. In addition to it, CPSI is thought to have evolved in the same lineage as fish and invertebrate CPSIII (Mommsen & Walsh, 1989; Campbell & Anderson, 1991; Helbig & Atkinson, 1994). Till now no studies have been carried out to know the conditions at gene level, thus there can be a possibility either these two forms of CPS are products of one gene and become different due to Post-translational modification or there are two different genes coding for two forms independently. Thus, establishing the sequence of isoforms of CPSs in *H. fossilis* is important not only for evolutionary derivation of mammalian CPSI but also in the context of evolution of ureotely in fishes and tetrapods (Griffith, 1991).

In an attempt to study CPS isoenzyme genes in *H.fossilis* so as to elucidate the evolution of CPS, we have given the first sequence of CPS ever reported in this unique fish and a comprehensive phylogenetic analysis considering the CPS sequences from representatives of all three domains of classification archea, bacteria and eukarya.

MATERIALS AND METHOD

Fish

The fish, *H.fossilis*, 40-50g body weight were purchased from commercial sources and acclimatized in the laboratory for 4-6 weeks at about 30ºC with a 12hr: 12hr light and dark period before being used for experiments. No sex differentiation was done while performing this study. Minced goat liver was given as food and the water was changed on alternate days.

Isolation of RNA and synthesis of cDNA

Freshly excised liver and muscle from *H.fossilis* were immediately dropped into liquid nitrogen and then stored at -80ºC.Total RNA from liver tissue was isolated using PureZOL RNA isolation Reagent (BIORAD, USA) from 50mg tissue. Total RNA was stored at -80ºC. Reverse transcription was carried out using iScript Select cDNA synthesis Kit (BIORAD, USA) utilizing MMLV Reverse transcriptase and oligo(dT)20 as primer starting from 1µg of total RNA. The instructions for cDNA synthesis (20µl reaction volume) provided with the kit were followed and the samples were stored at -20ºC until needed.

Amplification of CPS gene fragment

A set of primers corresponding to Synthetase domain of CPS-1 were taken (Anderson et.al, 2007). The *H.fossilis* liver cDNA prepared was used as a template in the PCRs for generating CPS1 specific fragment. Twenty picomoles each of primers-1(5'-GTT CCA TGA AGA GTG TTG GTG AGG TC-3') and primer-2 (5'-CTT AGC TCT TCT TAG TGT CTC TTC-3') with 4µl cDNA in a 50µl standard reaction mixture were used for PCR. The Thermal cycler (Mini MJ, BIORAD, USA) was programmed as follows: 94ºC (denaturation) for 30sec, 60ºC (annealing) for 1min,and 72ºC (extension) for 90sec; this cycle was repeated a total of 35 times.

The β-Actin gene was considered as positive control and primers set with forward primer- TGG CTG GCC GTG ACC TGA CT and reverse primer - GGA AGAGGC AGC AGT GCC CA, specific for β-actin gene were designed and used in the PCR.

Besides this, two more fragments of CPS gene were amplified using different sets of primers one was CPSI specific and the other was CPSIII specific and were submitted to Genbank, accession no. HM031390 and HM031391. Primers used were already established but in different species and were as follows, forward 5'-TAA TGA GGT TGG CCT CAA GC-3'; reverse 5'-GTT CAG TTG TAG GTC TGG AAA-3' for CPSI (Helbing et al.,1994) and forward 5'-GAT CGC AGG CAA TCA AAG CA-3'; reverse 5'-GGA TTT GAG TGG TCA TAG CC-3' for CPSIII (Anderson et al., 1994) but further work is going on establish theses sequence thus these two are not analysed .

PCR resulted in a 350bp product of expected size when analysed by gel electrophoresis on 2% agarose (Lonza,USA) gel in 1X TAE buffer(ethidium bromide staining). This product was isolated from agarose gel using QIAquick gel extraction kit (QIAGEN Inc). The PCR product was sequenced using high throughput sequencing services at TCGA, New Delhi. The

sequence reported in this paper , comprising only CPS gene from *H. fossilis*, has been submitted to Genbank, accession number HQ287216.

DATABASE Sequences

Sequences from following organisms were used with accession number in parentheses.

Escherichia coli (J01597), *Salmonella typhimurium* (U04992), *Pseudomonas stutzeri* (U04993), *Trypanosoma cruzi* (AB005063), *Schistosoma mansoni* (XP_002581898), *Xenopus laevis* (CPSI, NM_001095640), *Opsanus beta* (CPSIII, AF169248), Homo *sapiens* (CPSI, NM_001122633), *Mus musculus* (CPSI, NM_001080809), Rana *catesbiana* (CPSI, U05193), *Rattus norvegicus* (CPSI, NM_017072), *Micropterus selmoides* (CPSIII, AF006491), *Onchorynchus mykiss* (CPSIII,U65893) , *Squalus acanthias* (CPSIII,L31362), *Oreochromis alcalicus grahami* (CPSIII, AF119250), *Danio rerio* (CPSI, NM_001128351), *Gallus gallus* (CPSI, NM_001045841).All sequences were obtained by screening the GenBank and EMBL databases (as on Sept, 2010).

Sequence and Phylogenetic analysis

Multiple alignment of the 26 sequences representing different taxa was carried out using ClustalW. The aligned sequences were analyzed independently with Neighbor joining (NJ), Minimum evolution (ME), maximum parsimony (MP) and UPGMA algorithm in MEGA4.1 (Kumar et al., 2007) to resolve the phylogenetic relationship. The phylogenetic trees were linearized assuming equal evolutionary rates in all lineages (Nei et al., 2004).The clock calibration to convert distance to time was 100 (time/node height). Trees were drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Kumar et al., 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset .There were total of 205 positions in the final dataset.

Bootstrap test was conducted to estimate the robustness of the phylogenetic trees obtained by all the four methods. The bootstrap consensus tree inferred from 500 replicates was used to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using maximum composite likelihood method (Kumar et al, 2004) and are in the units of the number of base substitutions per site. The analysis were conducted in MEGA4 (Kumar et al, 2007). Pairwise sequence divergence data between taxas was also computed using p-distance model.

RESULTS AND DISCUSSION

This is the first partial unique sequence of CPS gene of 215 base pairs from *H. fossilis*. As stated earlier, the primers used in RT-PCR were specific for synthetase domain of CPS which is reported to be relatively conserved during the course of evolution. The nucleotide sequence obtained was also found to be showing significant homology with the synthetase domain of other CPSs from different organisms i.e. Archea to higher vertebrates which proves that the sequences amplified in PCR is of CPS gene however it is difficult to predict the exact class to which it belongs as the percent identity shown with CPSI and III is almost equal and more than vertebrate CPS II although the multiple sequence analysis show that CPS of *H.fossilis* is more closer to primitive CPS gene of lower eukaryotes and invertebrates.

Pair-wise and multiple alignment analysis shows that the nucleotide sequence of *H. fossilis* CPS has more identities with CPSI (of amphibians and mammals) and CPSIII (of teleost fishes) rather than CPSII showing almost 57% ,52% and 50% identities with human, *Rana* and *Xenopus* CPSI, respectively, as well as 54% and 53% identities with *Alcalicus grahami* and trout CPS III, respectively, but only 42 % identity with trout CPS II . Thus, its more likely that the sequence belongs to either CPS I or CPS III rather than CPSII.

Although the partial sequence of *H.fossilis* CPS is showing high level of similarity with human CPS1, amphibians CPSI and CPSIII of alcalicus grahami and trout but a significant level of identity with yeast Arginine biosynthesis-specific CPS, *Sulfobolous solfataricus*, and *Plasmodium falciparum* CPS is also apparent i.e. almost 54% in both the cases. Hence, the possibility of primitiveness of the sequence can also be there.

Table 1: Percentage homology calculated by pairwise alignment of different sequences with *Heteropnuestes Fossilis* CPS partial sequence

Type of organism	Organism	CPS Type	% homology with H. fossilis CPS
Mammals	*Homo Sapiens*	I	56.93
	Homo Sapiens	II	47.19
	Mus musculus	I	49.37
	Mus musculus	II	46.26
	Rattus norvegicus	I	46.61
	Rattus norvegicus	II	48.94
Amphibians	*Xenopus laevis*	I	50.15
	Rana Catesbiana	I	52.32
Aves	*Gallus gallus*	I	52.12
Teleost fishes	*Opsanus beta*	III	47.66
	Onchrynchus mykiss (trout)	III	52.58
	Onchrynchus mykiss (trout)	II	41.99
	Oreochromis alcalicus graham	III	54.30
	Micropterus salmoides	III	46.52
Elasmobranchs	*Squalus acanthias*	III	44.81
Microscopic eukaryote	*Saccharomyces Cerevisae*	Pyr specific	50.91

	Saccharomyces Cerevisae	Arg specific	54.29
Protozoa	*Babesia bovis*		
	Plasmodium falciparum		53.57
Eubacteria (gram +ve)	*Bacillus subtilis*	Pyr specific	46.88
	Bacillus caldolyticus	Pyr specific	45.92
Eubacteria(Gram –ve)	*Esterichia coli*		47.61
	Salmonella typhimurium		43.32
Archaebacteria	*Sulfolobus solfataricus*		48.26
	Pyrococcus furiosus		42.31

Phylogenetic analysis of CPS Genes

Phylogenetic analysis using four methods, Neighbor joining (NJ), Minimum evolution (ME), Maximum parsimony (MP) and UPGMA showed a consistent topology (Fig.1). In some earlier studies, the internal duplication within the synthetase domain of CPS was used for rooting the phylogenetic trees (Lawson et. al, 1996) but later it was contradicted as CPS genes has undergone numerous loss and horizontal gene transfer events which prevents its use for rooting the universal tree of life (Cammarano et. al, 2002)..Hence, unrooted tree was considered for the phylogenetic studies of CPS

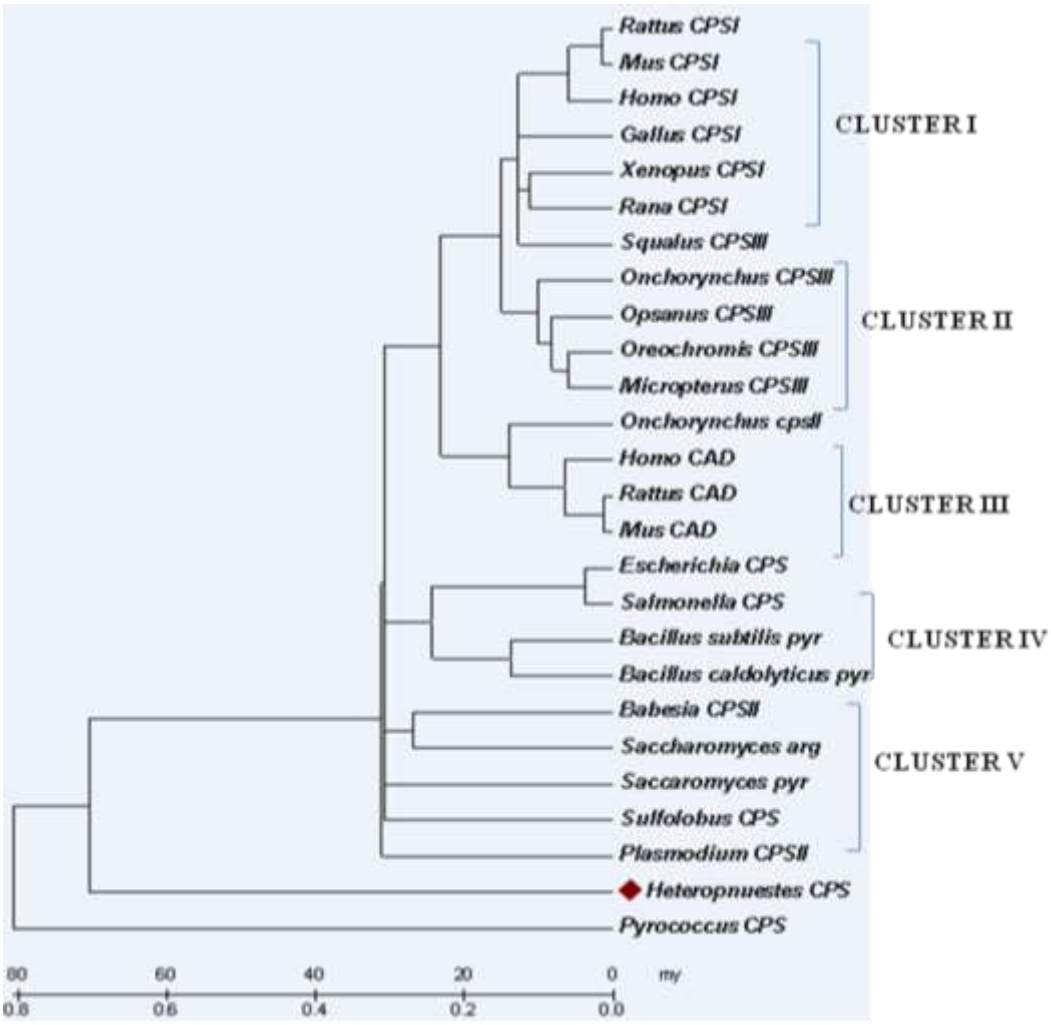

Figure 1: CPS consensus tree of phylogenies with time scale based upon CPS data in different group of organisms.

In consensus tree (figure 1), 26 organism have been distributed in five clusters and 2 outgroups, first cluster consisting of CPS I isoform present in mammals, bird and amphibians. Second cluster comprising of CPSIII isoforms present in elasmobranchs and teleost fishes while third cluster is formed by CPS II isoform present in the form of CAD complex in all vertebrates. CPS isoform present in bacteria including gram positive as well as gram negative bacteria forms the fifth cluster while CPS in *H.fossilis* and *P.furiosus* remains as an outgroup.

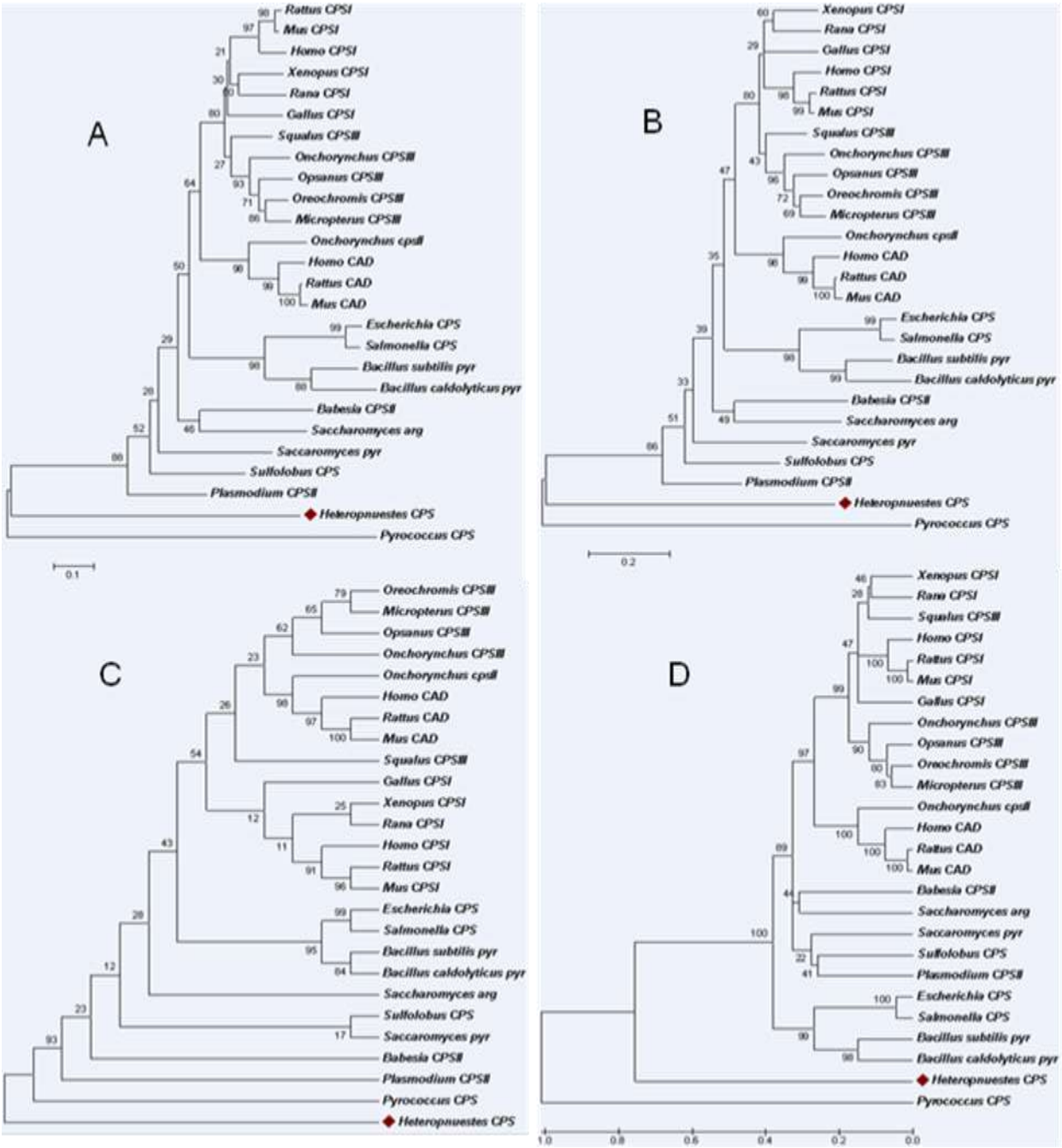

Figure 2: CPS consensus phylogenetic trees based on bootstrap test of phylogeny (500 replicates) (A) Neighbor joining estimate of phylogeny (B) Minimum evolution estimate of phylogeny (C) Maximum parsimony estimate of phylogeny (D) UPGMA estimate of phylogeny.

It is suggested that probably CPSIII has accumulated certain changes and gave rise to amphibious CPSI with the onset of first tetraploidization about 300-450 mya and later it transformed to CPSI in birds and mammals .As it is proposed earlier that *H.fossilis* has evolved during 123-53 My ago along with African clariidae on the basis of 18S DNA studies and is the only survivor of massive K/T extinction (Jansen et al., 2006 ; Agnese and Teugels, 2005) . This study strongly supports the view as *H.fossilis* CPS has not been showing much similarity to CPS III of teleost fishes (modern fishes) but it has been found to have more affinities to the CPS of archae bacteria suggesting that this fish must have been exposed to some of the adverse conditions of warm temperature, low sun light , increased CO_2 , pollution of water bodies apparently leading to drying , at the period of about 65 mya due to K/T crises when there was a mass extinction of marine fauna, which could have led to extinction of all other members of cluster to which *H. fosilis* belongs and urea cycle must have evolved and this unique CPS became functional in this fish to adapt against the drastic conditions at that time only . Hence, it is apparent that *H.fossilis* has monophyletic origin and CPS has been subjected to another evolution during 65mya. However, it requires further confirmation provided by the complete sequence study of CPS in *H.fossilis* as well as Clarias fish which is a close relative to *H.fossilis*.

CONCLUSION:

The phylogenetic analysis of H. fossilis CPS indictes that this fish is the lone survivor of K/T extinction with a monophyletic origin around 65mya. The unique CPS found in this fish became functional under extreme weather conditions and evolved independently. It apparently provided survival benefits to the fish and represents hallmark of the transition from amminotely to ureotely

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