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Molecular Identification and Phylogenetic Relationships of Threadfin Breams (Family: *Nemipteridae*) Using mtDNA Marker

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Abstract

Cytochrome c oxidase-1 gene sequences of mitochondrial genome were analyzed for species identification and phylogenetic relationship among the commercially important *Nemipterus* species. Sequence analysis of COI gene clearly indicated that all the nine fish species fell into distinct clads, which are genetically distant from each other and exhibited identical phylogenetic reservation. All the COI gene sequences provide sufficient phylogenetic information and evolutionary relationship to distinguish the nine *Nemipterus* species unambiguously. As per the neighbour-joining (NJ) and maximum likelihood (ML) trees, all the nine species are genetically distant from each other and exhibited identical phylogenetic reservation. Based on the NJ and ML phylogenetic trees *N. mesoprion, N. zysron, N. hexodon, N. nematophorus, N. virgatus* and *N. bipunctatus* were closely related with high bootstrap value (97). The overall mean Kimura two parameter (K2P) distances between the nine species was 0.109. The intra species K2P distance was high in *N. japonicus* (0.069) followed by *N. peronii* (0.050) and *N. mesoprion* (0.002). This study proves the use of mtDNA COI gene sequence based approach is an alternative tool for identifying fish species at a faster pace.

Keywords: cytochrome oxydase-1, intraspecific, *Nemipterus*, phylogeny; taxonomy

Abbreviation used: mtDNA: mitochondrial DNA, CO1: Cytochrome Oxydase-1, NJ: Neighbour-joining, ML: Maximum Likelihood, K2P: Kimura Two Parameter, FAO: Food and Agricultural Organization, dNTP: Deoxyribonucleotide triphosphate, mM: Milli Molar, TAE: Tis Acetate EDTA

Introduction

The threadfin breams, also called pink perch constitute an important demersal finfish resource in the Indian EEZ. These fishes are abundant beyond 50 m but show higher concentration at 100-200 m depth as revealed by the exploratory surveys and experimental fishing (Murty *et al*., 2001). Threadfin breams are one of the most dominant components among the demersal fisheries of India being exploited by commercial trawlers and available all the entire year (Russell, 1990). The classification of nemipterid fishes into different taxa is much confusing as, they are the members of one of the most confusing families (Russell, 1990). Threadfin bream's catches are usually mixed in India with representation of three or more species of genus *Nemipterus* (Murty *et al*., 2001). They are rarely reported as separate species because of problems in proper identification (Pawar *et al*., 2011). Morphometric characters and colour patterns are the most useful taxonomic tools which helps in segregation of fish species (Russell, 1990). But taxonomic ambiguity exists for juvenile and pre-adult fishes and it may lead to another synonym to a fish species. DNA-based approaches for taxon diagnosis exploiting DNA sequence diversity among species can be used to identify fishes and resolve taxonomic ambiguity including the discovery of new/cryptic species (Hebert *et al*., 2003). As there is no paternal contribution of mtDNA and no known recombination between mitochondrial genomes, the mtDNA genes are selected for species identification (Thangaraj and Lipton, 2011). Earlier studies have demonstrated that the COI gene is appropriate and accepted as a universal barcode for discriminating between closely related species across diverse animal phyla and this has been used for marine and freshwater fishes (Hebert *et al*., 2003; Hubert *et al*., 2008; Lakra *et al*., 2011; Ward *et al*., 2005).

Considering the importance of molecular identification and as there are no attempts to study genetic relation-

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ship between various *Nemipterus* species, in spite of its economic importance and significant contribution in marine fishery, the present study was achieved. In this study, the genetic difference between four commonly available threadfin bream species in Bay of Bengal and other *Nemipterus* species were assessed using mitochondrial gene sequence.

Materials and methods

Sample collection

Forty specimens from four species were collected from the Pondicherry coastal waters (11°46' and 12°03' N and 79°36' and 79°53' E). Immediately after the collection specimens were kept in the iceboxes for further studies. All the fishes were identified up to the species level using the FAO Fish Identification Sheets (Thomson, 1984) and further confirmation were carried out at Zoological Survey of India, Southern Regional Centre, Chennai. The voucher specimens are maintained in the Department of Zoology, Kanchi Mamunivar Centre for Post-Graduate Studies, Pondicherry. Approximately 100 mg of white muscle tissue and fin-clips from two to five individuals of each species were preserved in 95% ethanol and stored at 4°C until used.

DNA isolation

The DNA was isolated by standard Proteinase-K/Phenol-Chloroform-ethanol method (Sambrook *et al*., 1989) and the concentration of isolated DNA was estimated using a UV spectrophotometer. The DNA was diluted in TAE buffer to a final concentration of 100 ng/µL.

Amplification and sequencing

The COI gene was amplified in a 50 µL volume with 5 µL of 10X Taq polymerase buffer, 2 µL of $MgCl_2$ (50 mM), 0.25 µL of each dNTP (0.05 mM), 0.5 µL of each primer (0.01 mM), 0.6 U of Taq polymerase and 5 µl of genomic DNA. The primers used for the amplification of the COI gene were FishF1-5'TCAACCAACCACAAAGACATTGGCAC3' and FishR1-5'TAGACTTC TGGGTGGCCAAAGAATCA3' (Ward *et al*., 2005). The thermal regime consisted of an initial step of 2 min at 95°C followed by 35 cycles of 40 s at 94°C, 40 s at 54°C and 1 min 10 s at 72°C followed in turn by final extension of 10 min at 72°C.

The PCR products were visualized on 1.5% agarose gels, and the most intense products were selected for sequencing. The cleaned up PCR product was sequenced by a sequencing facility (Bioserv Biotechnologies Pvt Ltd, Hyderabad, India).

Sequence analysis

The CO1 gene partial sequences of the four *Nemipterus* species were unambiguously edited using BioEdit sequence alignment editor14 and aligned using CLUSTAL-W in BioEdit, and checked manually. Identical sequences were assigned in the same haplotype identity and only a single example of each species used in the phylogenetic divergences assuming that identical haplotypes shared the

Tab. 1. Threadfin bream fish species and their COI sequence Genbank accession numbers

Sl. No	Scientific name	Common name	Accession number
1	*Nemipterus zysron*	Slender threadfin bream	JN992287.1
2	*Nemipterus nematophorus*	Doublewhip threadfin bream	JN992286.1
3	*Nemipterus bipunctatus*	Delagoa threadfin bream	HQ423413.1
4	*Nemipterus hexodon*	Ornate threadfin bream	EF609414.1
5	*Nemipterus furcosus*	Fork-tailed threadfin bream	EF609413.1
6	*Nemipterus japonicus* 1	Japanese threadfin bream	FJ347947.1
7	*Nemipterus japonicus* 2	Japanese threadfin bream	EF609555.1
8	*Nemipterus japonicus* 3	Japanese threadfin bream	EF609553.1
9	*Nemipterus japonicus* 4	Japanese threadfin bream	HQ149889.1
10	*Nemipterus japonicus* 5	Japanese threadfin bream	JN992288.1
11	*Nemipterus mesoprion* 1	Mauvelip threadfin bream	EF609561.1
12	*Nemipterus mesoprion* 2	Mauvelip threadfin bream	EF609559.1
13	*Nemipterus mesoprion* 3	Mauvelip threadfin bream	EF609557.1
14	*Nemipterus mesoprion* 4	Mauvelip threadfin bream	EF609560.1
15	*Nemipterus virgatus* 1	Golden threadfin bream	FJ237835.1
16	*Nemipterus virgatus* 2	Golden threadfin bream	FJ237835.1
17	*Nemipterus virgatus* 3	Golden threadfin bream	FJ237837.1
18	*Nemipterus virgatus* 4	Golden threadfin bream	FJ237839.1
19	*Nemipterus peronii* 1	Notchedfin threadfin bream	EF609415.1
20	*Nemipterus peronii* 2	Notchedfin threadfin bream	HQ149890.1
21	*Nemipterus peronii* 3	Notchedfin threadfin bream	HQ149891.1
22	*Lates calcarifer* (Outgroup)	Seabass	EU189376.1

same evolutionary origin. Haplotype definitions have been submitted to the NCBI GenBank (Acc. No. HQ423413, JN992286, JN992287, JN992288). To support the present data, selective CO1 sequences in other five *Nemipterus* species and one outgroup (*Lates calcarifer*) were retrieved from Genbank (Tab. 1). Nucleotide diversity, genetic variation and nucleotide composition and pairwise evolutionary distance among haplotypes was determined by the Kimura 2-Parameter method (Kimura, 1980) using the software program MEGA 3.1 (Molecular Evolutionary Genetics Analysis) (Kumar *et al*., 2004). The neighbour-joining (NJ) and maximum likelihood (ML) trees were constructed using MEGA 3.1 and to verify the robustness of the internal nodes of these trees, bootstrap analysis was carried out using 1000 pseudoreplications.

Results and discussion

A total of 21 sequences were analysed from nine *Nemipterus* species in this study. Simplicity and un-ambiguity were observed among all the sequences and no introns, deletions or stop codons were observed any of the sequences. The sequence analysis revealed the average nucleotide frequencies as A = 23.4 ± 0.75%, T = 31.6 ± 1.17%, G = 18.0 ± 0.60, C = 26.9 ± 0.76 (Tab. 2). The average number of nucleotide difference (K) = 67.51 and nucleotide diversity (Pi) = 0.1342. Tajma's statistics (D) =-0.1109 and it was not significantly (p<0.01) different among sequences. Kimura 2 Parameter (K2P) genetic distance in threadfin bream fish species is given in Tab. 3. The overall K2P distance between the nine species was 0.109. The K2P genetic distance was high (0.150) between *N. perinii* and *N. hexodon*. Very low K2P distance (0.009) was exhibited between *N. mesoprion* and *N. zysron*.

The intra-species K2P distance (Fig. 1) was high in *N. japonicus* (0.069) followed by *N. peronii* (0.050) and *N. mesoprion* (0.002). The codon based genetic distance in nine *Nemipterus* species is depicted in Tab. 4 (below diagonal). Based on the CO1 sequence data, the codon based genetic distance was high (0.372) between *N. hexodon* and *N. nematophorus*. The minimum level (0.007) of codon based genetic distance was displayed between *N. mesopri-*

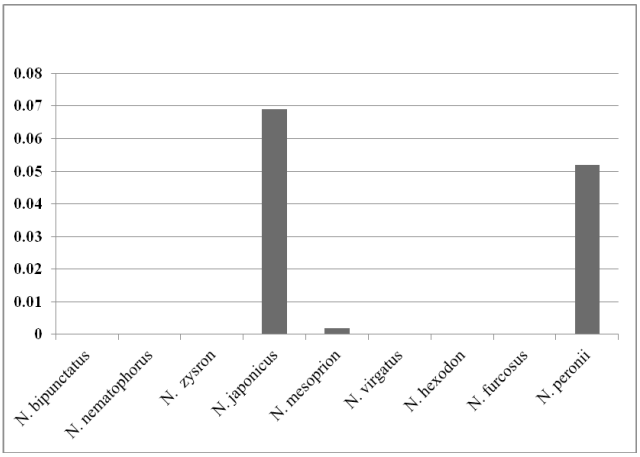

Fig. 1. Intra-species Kimura 2 Parameter (K2P) genetic distance in threadfin bream fish species

Tab. 2. Nucleotide base composition of threadfin bream fish species

Species	A	T	G	C	GC	GC 1	GC2	GC3
N. zysron	23.6	32.2	17.1	27.2	44.3	36.1	54.1	42.4
N. nematophorus	25.0	33.2	16.3	25.5	41.8	30.7	53.2	41.5
N. bipunctatus	23.7	32.3	18.6	25.4	44.0	33.8	54.9	43.4
N. hexodon	24.3	30.5	18.2	27.0	45.2	38.4	54.6	42.7
N. furcosus	23.4	29.8	17.9	29.0	46.9	42.0	56.0	42.7
N. japonicus	23.1	32.4	17.8	26.8	44.7	35.4	56.4	42.7
N. mesoprion	22.7	32.7	18.4	26.2	44.6	36.2	55.1	42.7
N. virgatus	23.6	31.1	18.1	27.1	45.2	39.5	54.4	42.8
N. peronii	24.1	30.0	18.3	27.6	46.3	39.2	56.3	42.9
Mean	23.4 ± 0.75	31.6 ± 1.17	18.0 ± 0.60	26.9 ± 0.79	44.7 ±1.45	36.8 ± 3.38	55.0 ± 1.07	42.64 ± 0.50

Tab. 3. Kimura 2 Parameter (K2P) genetic distance in threadfin bream fish species

Species	*N. zysron*	*N. nematophorus*	*N. bipunctatus*	*N. hexodon*	*N. furcosus*	*N. japonicus*	*N. mesoprion*	*N. virgatus*	*N. peronii*
N. zysron	****								
N. nematophorus	0.075	****							
N. bipunctatus	0.099	0.095	****						
N. hexodon	0.078	0.100	0.098	****					
N. furcosus	0.133	0.140	0.122	0.142	****				
N. japonicus	0.133	0.133	0.141	0.138	0.122	****			
N. mesoprion	0.009	0.076	0.093	0.069	0.134	0.130	****		
N. virgatus	0.086	0.079	0.090	0.095	0.114	0.123	0.084	****	
N. peronii	0.145	0.144	0.141	0.150	0.064	0.120	0.140	0.134	****

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Tab. 4. Codon based distance (below diagonal) and disparity index (above diagonal) in threadfin bream fish species

Species	*N. zysron*	*N. nematophorus*	*N. bipunctatus*	*N. hexodon*	*N. furcosus*	*N. japonicus*	*N. mesoprion*	*N. virgatus*	*N. peronii*
N. zysron	****	0.161	0.107	0.085	0.000	0.000	0.000	0.000	0.011
N. nematophorus	0.256	****	0.000	0.249	0.099	0.000	0.172	0.121	0.020
N. bipunctatus	0.231	0.030	****	0.171	0.087	0.000	0.121	0.082	0.000
N. hexodon	0.185	0.372	0.292	****	0.000	0.050	0.158	0.000	0.000
N. furcosus	0.093	0.262	0.233	0.030	****	0.000	0.000	0.000	0.005
N. japonicus	0.075	0.060	0.060	0.206	0.111	****	0.000	0.000	0.000
N. mesoprion	0.007	0.268	0.238	0.247	0.145	0.087	****	0.000	0.024
N. virgatus	0.044	0.221	0.195	0.062	0.010	0.070	0.081	****	0.000
N. peronii	0.126	0.166	0.102	0.066	0.056	0.076	0.159	0.053	****

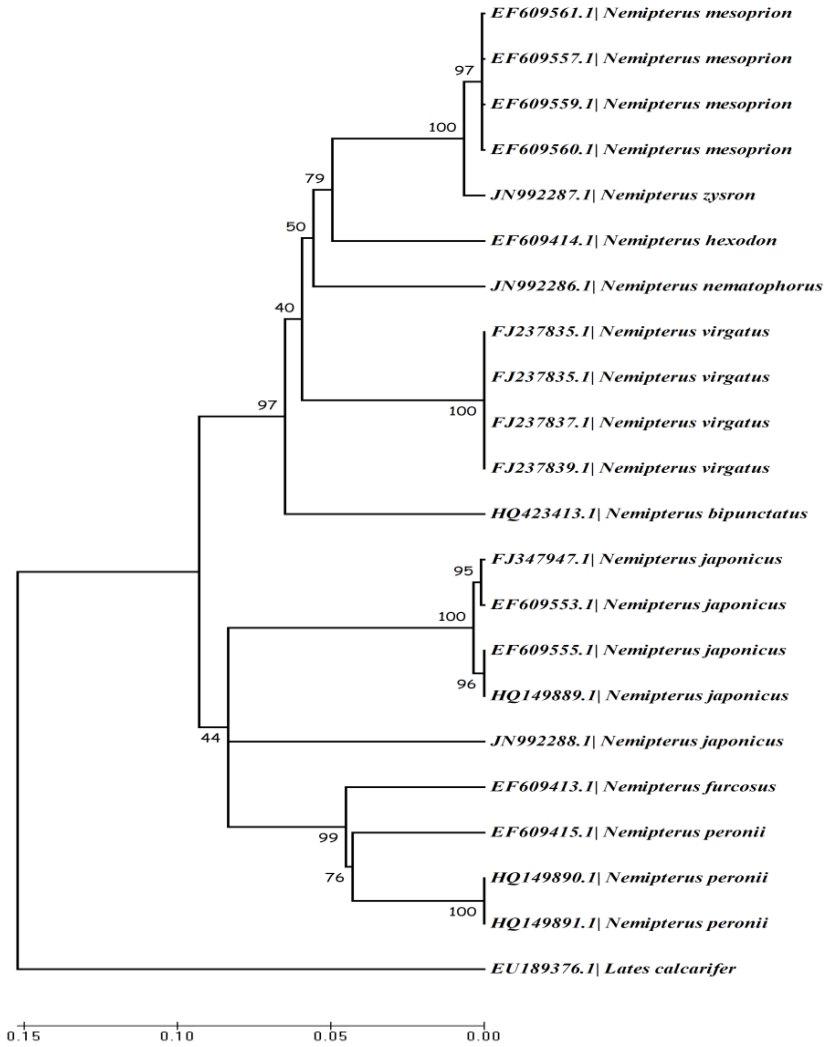

Fig. 2. Neighbour Joining (NJ) phylogenetic tree of threadfin bream fish species from COI gene sequences

on and *N. zysron*. The disparity index in nine *Nemipterus* species is displayed in Tab. 4 (above diagonal). The maximum disparity index (0.249) was observed between *N. nematophorus* and *N. hexodon*.

All the twenty two sequences were subjected in the phylogenetic analysis. The neighbour joining tree (NJ) and maximum likelihood tree (ML) of K2P distance were created to provide a graphical representation of the patterning of divergence of nine *Nemipterus* species (Fig. 2 and 3). As per NJ tree two distinct clads as two sub-trees within the same genus were recognized with high bootstrap value. Among the two sub-trees the larger one has an independent assemblage of *N. mesoprion*, *N. zysron, N. hexodon, N. nematophorus, N. virgatus* and *N. bipunctatus*

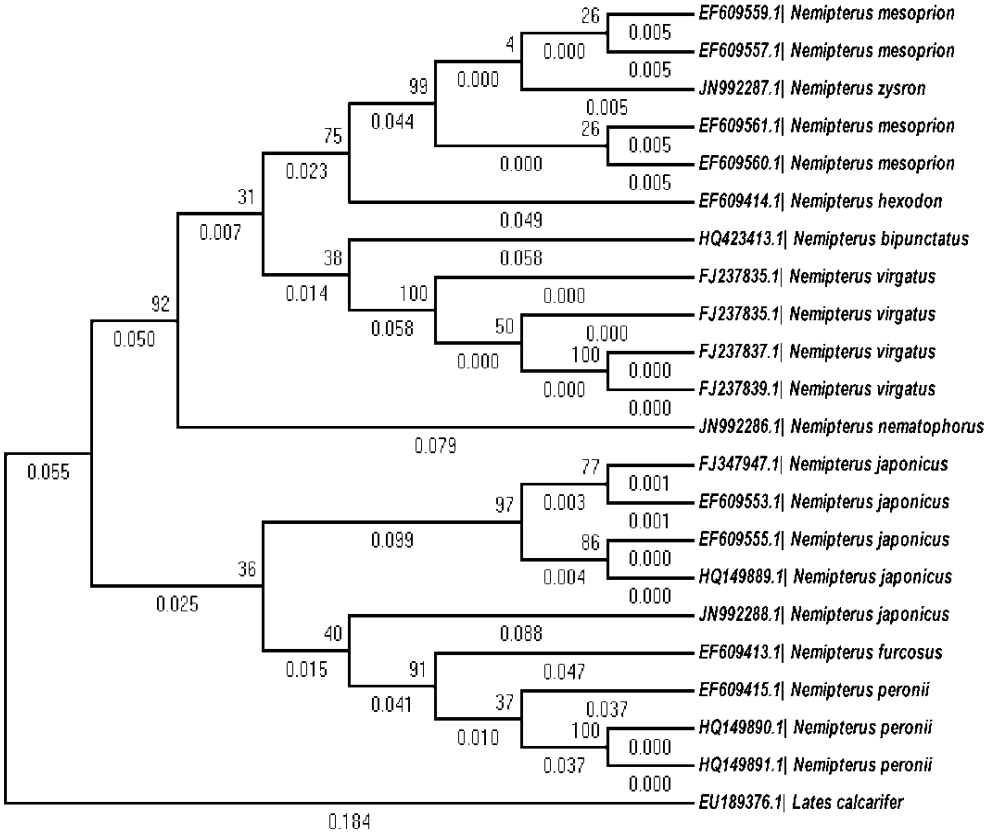

Fig. 3. Maximum likelihood (ML) phylogenetic tree of threadfin bream fish species from COI gene sequences

with 97% bootstrap value. Another clad representing all the other three species such as *N. japonicus, N. furcosus* and *N. peronii* with only 44% bootstrap value. The outgroup, *Lates calcarifer* was highly divergent and deviated in to a separate clad forming the root to the phylogenetic tree. The ML tree also showed the same type of divergence and formed two sub-trees having the bootstrap value of 92% and 36% respectively.

Species identification and phylogenetic relationship based on traditional methods and molecular methods are mostly concordant (Ward *et al*., 2005). In this study nine *Nemipterus* species were found genetically distinct from each other based on CO1 gene sequence which demonstrates simplicity and unambiguity. Morphologically very similar species like *N. mesoprion, N. zysron* and *N. virgatus* form a sister clad. Whereas, *N. japonicus, N. furcosus* and *N. peronii* form an independent sister clad in both NJ and ML trees of CO1 gene sequence. Estimates of genetic divergence from CO1 gene were sufficient to differentiate individuals of different threadfin bream species. In this study the level of intra-species variation was low which may be due to low number of haplotype identified in the sample with limited numbers collected for this study. Similarly, Lakra *et al*. (2011) reported very low intra-specific genetic divergence for scombroid fished and Ward *et al*. (2005) in many marine teleost species. Peris *et al*. (2009) also reported very low within species genetic distance for Indian carangid fishes.

Ward *et al*. (2005) reported an overall higher GC content in fishes based on complete MtDNA genome ranging from 38.4-43.2% and in CO1 alone it was 42.2-47.1%, which reflects the 3rd base variation. Peris *et al*. (2009) also reported considerable variation was exhibited in carangids in the 3rd base position. In this study it has been observed the mean GC content was 36.8 (GC1)-42.6 (GC3) among the nine *Nemipterus* species. The range of K2P intra-specific genetic distance in the present study was 0.000-0.069, which was slightly higher when compared with the previous studies of Indian carangids, 0.000-0.015 (Peris *et al*., 2009), Australian fishes (Ward *et al*., 2005), North American Birds (Hebert *et al*., 2004), Moths (Hebert *et al*., 2003), and Primates (Hajibabaei *et al*., 2006). The mean nucleotide diversity (Pi) among all the species was estimated as 0.1342. In earlier studies it was estimated as 0.2029 in Indian marine fishes (Lakra *et al*., 2011). It has been shown that lineage diversity more quickly within species than between species (Pons *et al*., 2006).

An effective DNA-based identification system requires the satisfaction of three important conditions: it must be possible to recover the target DNA from all the species; the sequence information must be easily analysed; and the information content of the target sequence must be suf-

ficient to enable species-level identification (Peris *et al*., 2009). All these three requirements were met in the present study, as all fish species examined could be recovered and targeted CO1 fragment aligned and analysed easily. Specimens of all species formed distinctive cluster and congruent with conventional morphological taxonomy. As per Ward *et al*. (2005), the CO1 analysis seeks only to delineate species boundaries yet there is some clear phylogenetic signal in CO1 sequence data, which is evident by the clustering of most of congeneric and confamilial species. The absolute clustering of conspecifics in the present study indicates the diagnostic ability of CO1 to correctly identify species.

Conclusions

The study has successfully proved the utility of COI divergences in identifying many of the *Nemipterid* fishes. The present analysis was not meant to be exhaustive, but to highlight the most important feature of COI based studies, that is the diagnostic ability of COI sequences in distinguishing closely related species and the intra-specific distances are lower than the inter-specific distances which was proved beyond doubt. The slight difference in the resolution factor in the present study and previous studies on Indian, Australian fishes and other groups of animals may be due to the low sample size. Further studies involving all the threadfin breams in the world and also by increasing the sample size in future studies will clarify the issue.

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