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FULL LENGTH RESEARCH PAPER

DNA sequence information resolves taxonomic ambiguity of the common mud crab species (Genus *Scylla*) in Indian waters

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Abstract

For several years, mud crabs of genus *Scylla* have been misidentified owing to their high morphological plasticity and the absence of distinct morphological diagnostic characters. The taxonomic confusion of genus *Scylla* de Haan is considered to be a primary constraint to the development of aquaculture. Although genus *Scylla* was revised using morphological and genetic characteristics, taxonomy of *Scylla* species occurring in India is still not clear. In this study, partial sequences of two mitochondrial genes, 16S rRNA and CO1 (Cytochrome C oxidase subunit I) in populations of *Scylla* spp. obtained from eleven locations along the Indian coast were used to differentiate and resolve taxonomical ambiguity of the mud crab species in India. The sequences were compared with previously published sequences of *Scylla* spp. Both trees generated based on 16S rRNA and CO1 indicated that all *S. tranquebarica* morphotypes obtained during this study and *S. tranquebarica* sequences submitted previously from Indian waters reciprocally monophyletic with reference sequence of *S. serrata*. Both sequence data and morphological characters revealed that the species *S. serrata* (Forskal) is the most abundant followed by *S. olivacea*. Further, the 16S rRNA and COI haplotypes of Indian *S. tranquebaric*a obtained in the study significantly differed with the known *S. tranquebarica* by 6.7% and 10.6% respectively whereas it differed with known *S. serrata* by 0.0–0.7% only, a difference that was not statistically significant. From these studies it is clear that ''*S. tranquebarica*'' commonly reported from India should be *S. serrata* (Forskal).

Keywords

16S gene, Cytochrome oxidase 1 gene, mud crabs, phylogeny, *Scylla*, taxonomy

History

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Introduction

Mud crabs of the genus *Scylla*, has been the focus of diversification of coastal aquaculture industry in India and several south-east Asian countries (Paterson & Mann, 2011). They are one of the most widely exploited coastal fishery resources in this region (Jirapunpipat et al., 2009). Identification of *Scylla* species has been contentious because of the morphological plasticity, overlapping morphological and morphometric traits. Mud crabs were originally assigned into three species and one variety: *S. oceanica*, *S. tranquebarica*, *S. serrata*, *S. serrata var. paramamosain* (Estampador, 1949). However, Stephenson & Campbell (1960) questioned this classification as they felt slight morphological changes and difference in color were of little importance to create new species. Thus, they tentatively fused all these forms and suggested one species, *S. serrata*. Nevertheless, subsequent authors provided convincing arguments for the existence of two or three species of *Scylla* in several regions of its distribution (Joel & Raj, 1983; Kathirval & Srinivasagam, 1992). Later, Keenan et al. (1998) revised the taxonomy of genus *Scylla* ''less controversially'' based on the collections made all over the Indo-Pacific region and they recognized four species of *Scylla*: *S. serrata* (former *S. oceanica*), *S. tranquebarica*, *S. olivacea* (former *S. serrata*) and *S paramamosain* (former *S. serrata var. paramamosain*). They used morphological, morphometric and molecular tool, and suggested three most useful morphometic ratios: inner carpus spine to outer carpus spine, frontal median spine height to frontal width and frontal width to internal carapace width. Nevertheless, they reported that no single ratio will discriminate the four species. Although classification of Keenan et al. (1998) is widely accepted, some authors raised doubts about the validity of this revised classification (Ronquillo et al., 1999). Although Keenan et al. (1998) analyzed specimens from almost all over the distributional range of this group, the common species supporting aquaculture and fisheries varies from region to region.

From Indian waters, originally two color morphs (dark green and greenish brown) of *Scylla* were recognized (Joel & Raj, 1983; Kathirvel, 1981; Radhakrishnan & Samuel, 1982). Kathirval & Srinivasagam (1992), later, revised the taxonomy of mud crabs and reported two species of *Scylla*: *S. tranquebarica* and *S. serrat*a for dark green and greenish brown morphs respectively. *Scylla serrata* is the most preferred species for coastal aquaculture

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in India (Kathirvel et al., 2004), however, it has been erroneously reported as *S. tranquebarica* in many scientific literature from India (Jithendran et al., 2010; Mohanty et al., 2006), partly due to the subtle diagnostic characteristics and overlapping morphometric ratios of *S. tranquebarica* and *S. serrata* (Jirapunpipat et al., 2008). Knowledge of Indian mud crab population remains inadequate, despite the importance of this species in both coastal aquaculture and artisanal fisheries of all the coastal states of India. Therefore, there is a clear need to identify the mud crab species in India using molecular tools as morphological diagnostic characteristics of mud crabs are rather weak or specific to life stages or sex. It is crucial to investigate what species is dominant in coastal aquaculture in India, as an incorrect name application can affect the success of aquaculture industry, and it can lead to the farming of a wrong species (Wowor & Ng, 2007), or utilizing the fund to develop a technology for the aquaculture of an economically or biologically unsuitable species. Mitochondrial markers have been widely used to accurately identify, resolve taxonomic ambiguity, forensic identification and describing new species across the tree of life (Hebert et al., 2003). In this paper we have used mitochondrial 16S rRNA and Cytochrome c oxidase subunit I (COI) nucleotide sequences to identify the mud crab species and to compare the present results in relation to those of other studies (Keenan et al., 1998).

Materials and methods

Animals were obtained from 11 different locations along the coast of India (Figure 1). Most specimens used for the study were from artisanal coastal fisheries, although few aquacultured samples were also used. Samples were caught by baited lift net or bottom set gill nets. More than 100 individuals were obtained and for molecular studies 30 representative samples from each location were used. In almost all cases live tissues were examined. For each specimens muscle tissues of the fifth pereopod was dissected and stored in 95% ethanol.

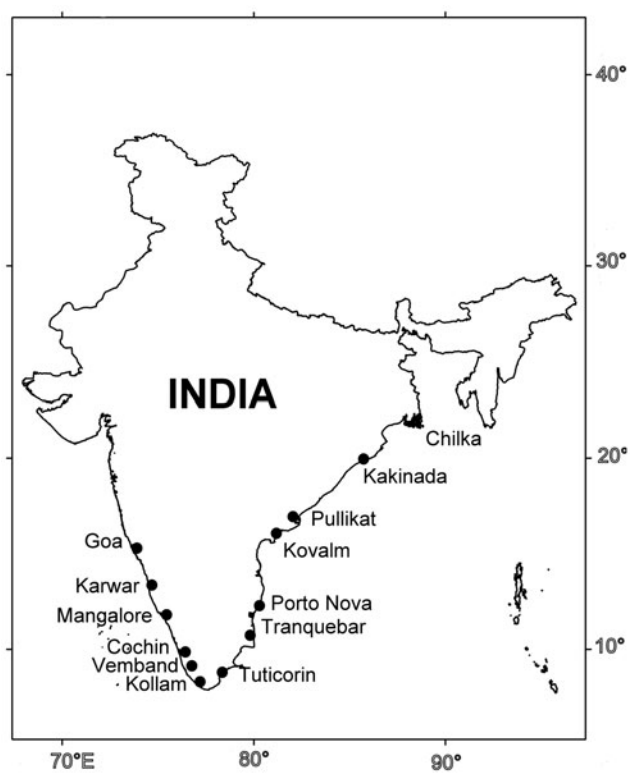

Figure 1. Map of India showing sampling sites of genus *Scylla*.

Classification of specimens

Before proceeding for genetic analysis, specimens were classified on the basis of taxonomically important characters: presence of polygonal marking on the chelipeds and other pereopods, shape and height of the frontal spines, shape of the interspaces of frontal spines, spines on the carpus of chelipeds and convexity of anterolateral spines (Keenan et al., 1998). Most specimens were assigned to two species: *S. serrata* and *S. olivacea*. However, few specimens ambiguous in morphology of *S. tranquebarica* (Keenan et al., 1998) were tentatively assigned as *S. tranquebarica* morphotypes.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from the alcohol preserved muscle tissues using phenol-chloroform extraction protocol of Kocher et al. (1989). Partial 16S rRNA gene was PCR-amplified using the primers 16Sar (5′CGCCTGTTATCAAAACAT3′) and 16Sbr (5′GGTCTGAACTCAGATCACGT3′) described by Iamai et al. (2004) and Somboonna et al. (2010). Polymerase chain reaction (PCR) used 10 ng of template DNA, 0.25 unit of Taq Polymerase, 200 μM of each of four deoxy nucleotide, 200 nM of each of forward and reverse primers and 2.5 μL of 1 X PCR buffer. The cycling profile was: 35 cycles of denaturation (95 °C for 30 s), annealing (47 °C for 40 s) and extension (72 °C for 1 min) with an initial denaturation step of 95 °C for 5 min and final extension step at 72 °C for 10 min. The partial sequence of mitochondrial cytochrome oxidase subunit 1 was gene was amplified and sequenced with primers CO1-f (5′TCCTGCAGGAGGAGGAG A3′) and CO1-d(5′CTGGGTAGTCTGAATAACGT3′) following similar procedure as for the 16S rRNA gene.

PCR products were purified with gel purification kit (Real Biotech Corporation, Banqiao city Taiwan) and sequenced in ABI prism capillary sequencer following manufacture's instruction. The gene sequences of 16S and CO1 of *S. serrata*, *S. olivacea*, *S. tranquebarica* and *Portunus pelagicus* were retrieved from GenBank (Table 2). Additionally, six 16S gene sequences of *S. tranquebarica* deposited in the Genbank from India were also retrieved (Table 2). These sequences were combined with the sequences obtained in the present study and aligned by using ClustalW (Thompson et al., 1994) in MEGA5. Pair-wise evolutionary distance among sequences was determined by the Kimura 2-Parameter methods (Kimura, 1980) using the software program MEGA5 (Tempe, AZ) (Tamura et al., 2011). The statistical significance of the pair wise inter species genetic distance measures between species was evaluated using Students' *t*-test. Neighbor-Joining (NJ) and Maximum Parsimony (MP) trees were constructed using MEGA5, and to verify the robustness of the internal nodes of NJ and MP trees, bootstrap analysis was carried out using 1000 pseudo replication (Felsenstein, 1993).

Results

Thirty new sequences (twenty 16S and ten CO1) of mud crabs were obtained, and these sequences were aligned with publically available mud crab and out group sequences (Tables 1 and 2). No interons or indels were observed in the CO1 gene. After alignment, 16S fragment had 592 nucleotide sites and of which 107 sites were variable and 81 were parsimony informative. Of 549 nucleotide sites of CO1, 122 sites were variable and 85 sites were parsimony informative (Table 3). The AT content of both 16S and CO1 were rich (16S: 68.8%; CO1: 64.7%)

To investigate the relationship of Indian species with other mud crab species, phylogenies were produced using our original data and published sequence data. Both neighbor joining and maximum parsimony trees are identical. Phylogenetic analysis of

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Table 1. Specimens of *Scylla* examined, Genbank accession numbers for genes sequenced and source of materials.

Taxon	Locality	16S	CO1
Scylla serrata 1	Pulicat	JX446640	–
Scylla serrata 2	Tuticorin	JX446641	–
Scylla serrata 3	Kakinada	JX446642	–
Scylla serrata 6	Kollam	KC154084	–
Scylla serrata 7	Cochin	KC154085	–
Scylla serrata 8	Vembanad	KC154086	KC154081
Scylla serrata 9	Karwar	KC154087	KC154082
Scylla serrata 10	Chilka	KC154088	KC154083
Scylla olivacea 1	Porto Nova	JX446635	–
Scylla olivacea 2	Kovalam	JX446636	–
Scylla olivacea 3	Kakinada	JX446638	–
Scylla olivacea 4	Kakinada	JX446638	–
Scylla olivacea 5	Goa	JX446639	–
Scylla olivacea 6	Cochin	KC154070	KC154075
Scylla olivacea 7	Mangalore	KC154071	KC154069
Scylla olivacea 8	Karwar	KC154072	KC154076
Scylla olivacea 9	Karwar	KC154073	KC154077
Scylla olivacea 10	Cochin	KC154074	KC154078
Scylla tranquebarica morphotype 1	Pulicat	JX446643	KC154079
Scylla tranquebarica morphotype 2	Tranquebar	JX446644	KC154080

Table 2. GenBank sequences used in analyses.

Species	Gene region	Locality in GenBank	Accession
Scylla serrata	16S	Taiwan	AF109318
S. olivacea	16S	Taiwan	AF109321
S. tranquebarica	16S	Taiwan	AF109320
S. paramamosain	16S	Taiwan	AF109319
S. tranquebarica	16S	India	KF220544
S. tranquebarica	16S	India	KF220543
S. tranquebarica	16S	India	KF220541
S. tranquebarica	16S	India	KF220539
S. tranquebarica	16S	India	KF220538
S. tranquebarica	16S	India	KF220537
S. serrata	CO1	Red sea	AF097011
S. serrata	CO1	Australia	AF097002
S. olivacea	CO1	Australia	AY373355
S. tranquebarica	CO1	Australia	AY373353
S. paramamosain	CO1	China	AY750930
Outgroup			
Portunus pelagicus	16S	Australia	FJ812329
P. pelagicus	CO1	China	AF082732

Table 3. Summary of sequence characteristics of two mitochondrial gene regions.

Gene region	16S	CO1
Number of samples	30	10
Alignment length	592	549
Base frequency		
A	34.8	26.7
C	12.0	18.6
G	19.2	16.6
T	34.0	38
Transition/transversion	1.83	2.2
No of variable sites	107	122
Parsimony informative sites	81	85
Gamma distribution	0.3466	0.4471

two mitochondrial regions produces trees with similar topology, showing two distinct congeneric clusters supported by high bootstrap value indicating high percentage support for grouping (Figures 2 and 3). In 16S tree all *S. serrata* and *S. tranquebarica* morphotype sequences obtained during this study reciprocally monophyletic with 16S reference sequence of *S. serrata* (Figure 2). Further, six *S. tranquebarica* sequences from India retrieved from Genbank also clustered with *S. serrata* reference sequence. Sequences of *S. olivacea* obtained during this study formed a single cluster grouped with reference *S. olivacea*. Phylogenetic relationship inferred from CO1 sequence data was consistent with those inferred by 16S sequence and showed no difference between *S. serrata* and *S. tranquebarica* morphotypes identified in this study (Figure 3). The average genetic distance between and within species was calculated based on Kimura 2-parameter model. The average distance within *S. serrata* and *S. olivacea* were 0.008639 ± 0.001058 and 0.006837 ± 0.001681, respectively. However, the distance between these two species was more than 10 times (Table 4).

Discussion

The present study demonstrated that mud crabs of India are composed of two species: *S. serrata* and *S. olivacea*, which have long been misidentified as *S. tranquebarica* and *S. serrata* respectively. Further, species identified as *S. tranquebarica* morphotypes in this study and the sequences deposited previously in the GenBank as *S. tranquebarica* from Indian waters were reciprocally monophyletic with *S. serrata* clad. The discrimination of *S. se*rrata and *S. tranquebarica* has often been problematic owing to the overlapping and subtle morphological diagnostic characters (e.g. relative size of frontal teeth and spines on the outer carpus and variation in the polygonal marking on the cheliped and pereopods). Keenan et al. (1998) identified these species using molecular and morphometric tools. Jirapunpipat et al. (2008), however, were unable to identify Thai population of *S. serrata* and *S. tranquebarica* using almost similar morphometric characters used by Keenan et al. (1998). They found overlap of *S. serrata* and *S. tranquebarica* although these two species are clearly distinguishable from *S. olivacea* and *S. paramamosain* using discriminant function analysis. Further, none of the thirty-one 16S sequences analyzed from Thailand matches with reference sequence of *S. tranquebarica* (Somboonna et al., 2010). Regional taxonomic studies carried out in Vietnam (Macintosh et al., 2002), China (Ma et al., 2006, 2012) and South Africa (Davis, 2004), also indicate the absence of *S. tranquebarica* in these regions. Recently, Mandal et al. (2013) also reported the absence of *S. tranquebarica* from Indian waters. The morphotypes assigned for *S. tranquebarica* in this study could be a phenotype extreme of *S. serrata*.

The taxonomy of mud crabs in India was studied by Joel & Raj (1983) and Kathirval & Srinivasagam (1992), and they demonstrated *S. tranquebarica* as a synonym of *S. oceanica*, which is currently synonymized as *S. serrata* by Keenan et al. (1998). Majority of the scientific literature published from India under the name *S. tranquebarica* should be *S. serrata*, and therefore, caution should be taken while interpreting the previously published biological and aquaculture data from Indian sub continent.

As *S. tranquebarica* was originally described from India (type locality: South India; Keenan et al., 1998), it is unlikely to exclude the possibility of existence of *S. tranquebarica* in Indian waters, although none of the sequences obtained during our study matches with reference sequence of *S. tranquebarica*. Further, Keenan et al. (1998) also opined the possibility of existence of this species in the Indian sub continent. The absence of *S. tranquebarica* in the present study could be due to the far less

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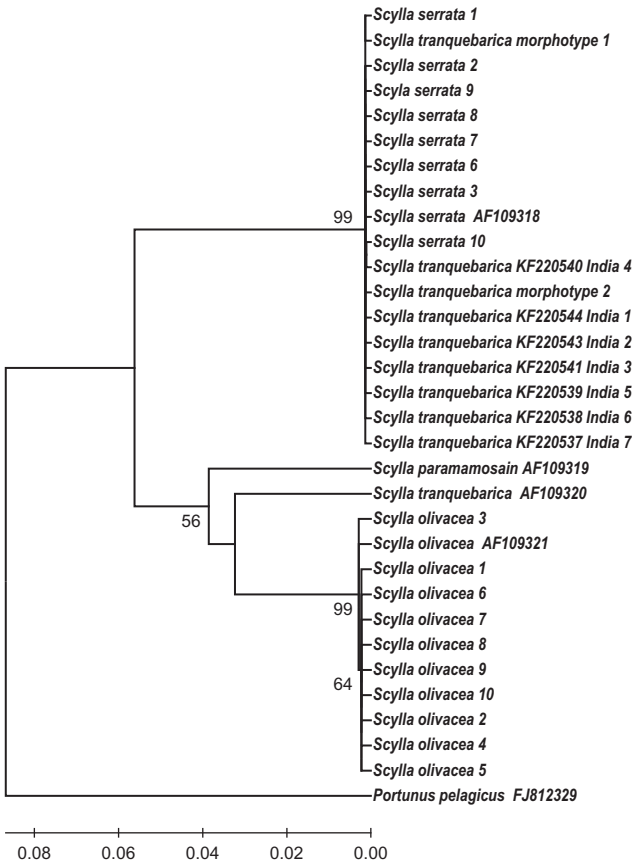

Figure 2. Neighbor Joining (NJ) phylogenetic tree of Indian mud crabs based on the Kimura 2-parameter genetic distance inferred from DNA sequences of mitochondrial gene 16S rRNA. Bootstrap values based on 1000 replications are indicated at the nodes. Only bootstrap values >50% are shown. Refer Table 2 for species designation.

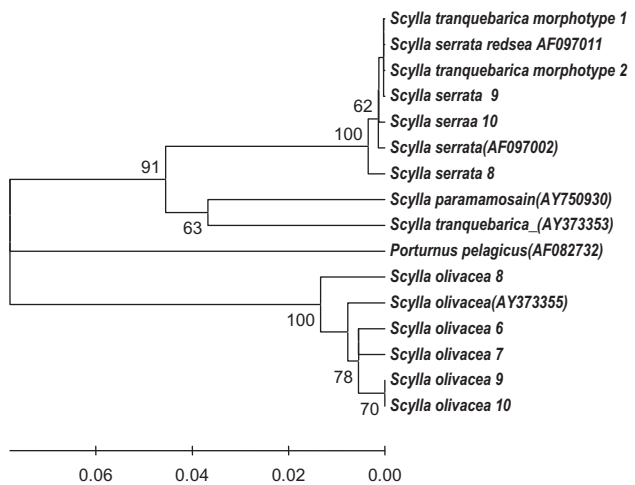

Figure 3. Neighbor Joining (NJ) phylogenetic tree of Indian mud crabs based on the Kimura 2-parameter genetic distance inferred from DNA sequences of mitochondrial gene COI. Bootstrap values based on 1000 replications are indicated at the nodes. Only bootstrap values >50% are shown. Refer Table 2 for species designation.

abundance of this species in Indian waters. Further, this species are found to be relatively less abundant even in its distributional range (Jirapunpipat et al., 2009).

As morphological variations used to discriminate *S. tranquebarica* and *S. serrata* are subtle, it is often difficult to apply morphological data alone with rigor, particularly during the field identification (Jirapunpipat et al., 2009). During the present study, few specimens were identified as *S. tranquebarica* in the field, and subsequent molecular analysis proved them as *S. serrata* with 100% bootstrap support. Therefore, it is suggested that at least in some cases molecular analysis is needed to identify the specimens positively. *S. serrata* is the most preferred species for aquaculture in India (Kathirvel et al., 2004), and many tropical south-east Asian countries, and therefore, it is imperative to select founder population using molecular tools for commercial breeding program.

Table 4. Summary of pair wise distance of 16S and CO1 showing the mean and standard error of K2P distance in different groupings.

	16S	CO1
Within *S. serrata*	0.008639 ± 0.001058	0.003797 ± 0.000824
Within *S. olivacea*	0.006837 ± 0.001681	0.019387 ± 0.002635
S. serrata Vs *S. olivacea*	0.106485 ± 0.004837	0.180726 ± 0.000718

Figure 4. Photograph of *Scylla serrata* (Forskal, 1775), dorsal view of male (carapace width: 96.5 mm).

Although close morphological similarity of *S. tranquebarica* and *S. serrata* have been reported by many workers (Ikhwanuddin et al., 2011; Jirapunpipat et al., 2009), genetic analysis shows that *S. tranquebarica* and *S. olivacea* are more closely related than *S. serrata* (Figure 3). This might indicate that *S. tranquebarica* and *S. olivacea* are the result of very recent speciation. Other studies also found close genetic similarity of *S. tranquebarica* and *S. olivacea* (Fuseya & Watanabe, 1996; Keenan, 1999; Ma et al., 2006). Keenan et al. (1998) delineated *Scylla* as four well supported species with average level of inter specific sequence difference (~12%) more than six times greater than that observed at the intra-specific level (2%). A 3% divergence in the CO1 sequence has been accepted for distinguishing closely related animal species (Hebert et al., 2003). Thus, large genetic difference observed between *S. serrata* and *S. olivacea* in the present study confirm the distinct species level difference.

Diagnosis of the common species of *Scylla* in the Indian waters

Scylla serrata *Forskal*

Carapace colour predominantly greenish to greyish green (Figure 4). Carapace, legs and abdomen (females only) with polygonal patterning. Frontal lobe teeth sharp and equal in size.

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Figure 5. Photograph of *Scylla olivacea* (Herbst, 1796) dorsal view of male (carapace width: 82.9 mm).

Two sharp spines at the outer margin of the carpus of the cheliped. The ratio between frontal lobe width and Internal carapace width is 0.38 ± 0.01.

Scylla olivacea *Herbst*

Carapace colour predominantly red to brown (Figure 5). Carapace rounded with blunt, obtuse frontal teeth, all equal in size with shallow inter space. Short obtuse antero-lateral spines uniform in size and shape. Carpus of chelipeds usually with one obtuse small blunt prominence. Chelipeds legs and abdomen all without obvious polygonal patterning. The ratio between frontal lobe width and internal carapace width is 0.48 ± 0.01.

Conclusion

Identification of mud crabs of genus *Scylla* has been contentious for a long time. In the present paper, the comprehensive DNA sequence analysis of mud crabs of India revealed that species commonly reported as *S. tranquebarica* should be *S. serrata*. Further *S. olivacea* has often been misidentified as *S. serrata* in most scientific literature from India. The present work resolves the taxonomic ambiguity of mud crabs of India.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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