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## Population structure of the black tiger prawn, *Penaeus monodon*, among western Indian Ocean and western Pacific populations

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**Abstract** We examined the population structure of the black tiger prawn, *Penaeus monodon* Fabricius, 1798, in the Indo-West Pacific by analyzing the geographic distribution of *elongation factor 1-alpha* intron sequences from specimens collected during the winter and spring of 1997. Both the molecular phylogeny of alleles and *F*-statistics indicated very strong differentiation between populations from the western Indian Ocean and western Pacific. This pattern is concordant with other recent studies of marine species in this region, implying that the Indo-Australian Archipelago represents a biogeographic break between populations in the Indo-West Pacific. *F*<sub>ST</sub>-values among populations in the western Indian Ocean also indicate structure within this region, whereas no structure was found among western Pacific populations. Nucleotide diversity was significantly lower in the western Indian Ocean populations than in the western Pacific, implying that the populations have regional differences in demographic history.

### Introduction

Much attention has focused on the Indo-West Pacific as the most diverse assemblage of marine species known (Briggs 1995). The distributions of marine organisms in this region are quite varied. While there are several groups of geminate species pairs or species groups with one species or group in the Indian Ocean and the other in the Pacific Ocean (e.g. many fish species, Woodland 1986; marine water striders, Andersen 1991; corals,

Wallace et al. 1991; butterflyfish, McMillan and Palumbi 1995; and nemerteans, Härlin 1996), many species are distributed throughout much if not all of this region (e.g. at least a dozen prawn species, Dore and Frimodt 1987; the crown-of-thorns starfish *Acanthaster planci*, Benzie 1992; many cone snail species, Röckel et al. 1995; the coconut crab *Birgus latro*, Lavery et al. 1996; the tropical starfish *Linckia laevigata*, Williams and Benzie 1998; and other examples summarized by Briggs 1995). These discordant distributional patterns imply that the processes influencing species distributions in the Indo-West Pacific are not uniform across species.

Information on the population structures of species that span the Indo-West Pacific is minimal, but recent molecular genetic studies have revealed that several species exhibit genetic discontinuities. Based on data from both allozymes and mtDNA restriction sites, a population of the coconut crab, *Birgus latro*, from Christmas Island in the Indian Ocean is genetically distinct from western Pacific populations (Lavery et al. 1995, 1996). Also based on both allozyme and mtDNA restriction site data, populations of the starfish *Linckia laevigata* from South Africa and Thailand in the Indian Ocean are genetically distinct from western Australian and western Pacific populations (Williams and Benzie 1998). Using allozyme data, Lacson and Clark (1995) also found substantial genetic differentiation between Indian and Pacific Ocean populations of four species of damselfish. These studies suggest that population distributions across the Indo-West Pacific can be genetically disjunct while appearing to be continuous.

The black tiger prawn, *Penaeus monodon*, is distributed from southeast Africa to eastern Australia and Japan (Dore and Frimodt 1987) and is cultured commercially in much of its range. The life history of *P. monodon*, comprised of an offshore planktonic larval phase (~2 weeks, Silas et al. 1978), an estuarine post-larval and juvenile phase, and an inshore adult and spawning phase (Dall et al. 1990), may allow moderate gene flow among populations. Both mtDNA and allozyme analyses have indicated though that populations

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from the west coast of Australia are genetically differentiated from populations from the east coast (Benzie et al. 1993). Do these genetic differences reflect a regional discontinuity among populations separated by the Indo-Australian Archipelago?

In order to analyze the genetic diversity of *Penaeus monodon* populations for aquaculture purposes, we collected nuclear sequence data from six populations in the western Indian Ocean and western Pacific Ocean. In the present study, we used these data to test the hypothesis that no significant genetic structure exists among populations from Indian and Pacific Oceans. We tested this hypothesis by examining the geographic distribution of sequences and estimating  $F$ -statistics among six populations from the western Indian and western Pacific Oceans. We analyzed intron sequence data, which can be highly polymorphic within a species due to the apparent neutrality of intron evolution, thus enabling sufficient resolution to determine the population structure of a species (e.g. Palumbi and Baker 1996).

## Materials and methods

Specimens of *Penaeus monodon* Fabricius, 1798 used in this study were collected from shrimp farms in the western Pacific Ocean (Tawau, Sabah, Malaysia; Banjarmasin, Indonesia; Tacloban, Philippines) and western Indian Ocean (west coast of Madagascar; Mauritius; and Bagamayo, Tanzania) in the winter and spring of 1997. Samples were post-larvae, juveniles or adults raised from local, wild-caught broodstock. Whole specimens or pleopods were shipped in 70 to 95% ethanol to our laboratory.

DNA was isolated directly from pleopods or whole post-larvae (around 1 to 5 mg of tissue). The tissues were incubated at 60 °C for a minimum of 2 h in 100 µl of extraction buffer (100 mM EDTA, 25 mM tris pH 7.5, 1% SDS) and 10 µl of 20 mg ml<sup>-1</sup> proteinase K. The homogenates and subsequent aqueous phases were then gently mixed and centrifuged with equal volumes of tris-equilibrated phenol, 1:1 phenol:chloroform, and chloroform. DNA was precipitated in 1.25 M NH<sub>4</sub>OAc and 50% propanol. The pellets were rinsed in 70% ethanol, allowed to air dry, and then re-suspended in 50 µl of H<sub>2</sub>O.

We amplified a region of the *elongation factor 1-alpha* (*efl-a*) gene known to contain an intron in other penaeid shrimps (France et al. 1999). Amplifications were carried out in 25-µl volumes: 1 µl of the extracted DNA (approximately 1 µg of genomic DNA), 0.5 µM of each primer (EF3 s – GACAAGGCCCTCCGCTCTCC and EF4 s – GGGCACTGTTCCAATACCTCC), 0.8 mM dNTPs, 0.30 units of Perkin-Elmer *Taq* DNA polymerase, 1.5 mM MgCl<sub>2</sub>, 10 mM tris (pH 8.3), 50 mM KCl, 0.01% NP-40, and 0.01% TritonX-100. The amplification profile was 30 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s. Amplification products were electrophoresed in a 2% agarose/0.5 × TBE buffer gel in 0.5 × TBE buffer for 20 min. The gels were stained with ethidium bromide and visualized under ultraviolet light.

Successful amplifications were ligated into a t-tailed pBluescript vector (Stratagene, La Jolla, California) following methods of Marchuk et al. (1991). Competent *Escherichia coli* cells were transformed with the ligations, plated out, and allowed to grow overnight as per manufacturer's recommendations. White colonies were screened for vectors containing inserts by amplifications with vector primers (T7 – GTAATACGACTCACTATAGGGC and T3 – AATTAACCCTCACTAAAGGG) in 12.5-µl volumes followed by electrophoresis of amplification products. One to three amplifications which confirmed expected insert size from each individual were purified with the QIAquick PCR purification kit (QIAGEN,

Santa Clarita, California), cycle sequenced with the Perkin-Elmer FS fluorescent sequencing kit (Perkin-Elmer, Foster City, California), and analyzed on an ABI 377 automated sequencer.

Sequences were aligned by eye with the SeqEd program (Applied Biosystems, Foster City, California). Relationships of alleles were analyzed by maximum parsimony with PAUP 3.1.1 (Swofford 1993). Identical sequences were represented only once. Insertions/deletions were coded as single changes such that the difference between sequences with and without an insert was only counted as a single step. A phylogram displaying the relationships among sequences was reconstructed from a consensus of 100 bootstrap analyses.

$F$ -statistics among and within regions were calculated using the  $F_{ST}$ -approach of Hudson et al. (1992) with the Heap Big Analysis program (a Macintosh analysis program written and available from S.R. Palumbi); sequences were resampled 1000 times with a bootstrapping technique which randomized the geographic locations of the sequences to determine significance of the  $F_{ST}$ -values. Pairwise  $F_{ST}$ -values between all populations were also calculated with Arlequin (Schneider et al. 1997) and tested for significance as above. Only a single allele sequence per individual was used in this analysis.

Nucleotide diversity within and among populations was also calculated with the Heap Big Analysis program and calculated as the average Kimura 2-parameter distance (Kimura 1980) between sequences from all individuals of that population. Only a single allele sequence per individual was used in this analysis.

Because results from the analysis of *efl-a* introns in *Penaeus vannamei* suggest the presence of more than one locus (France et al. 1999), we investigated whether this might also be the case for *P. monodon*. Under a 1-locus model, it is expected, assuming 100% heterozygosity and no preferential amplification or ligation of one allele versus another, that when two cloned inserts are sequenced from different colonies containing inserts from the amplification products of an individual, there is a 50% probability of obtaining either identical sequences (i.e. alleles) or different sequences. Under a 2-locus model with the same assumptions, it is expected that there is a 25% probability of obtaining identical sequences and a 75% probability of obtaining different sequences. We compared the observed number of times in which a second sequence was identical to the first sequenced from the same individual. Multiple sequences from an individual were classified as different if they differed by at least four characters or, when they differed by fewer than four characters, if the differences between sequences could not be attributed to polymerase error (i.e. the position was invariant in all other sequences). Using the above expectations under 1- and 2-locus models, a Chi-square test was conducted on the observed values to determine which of these two models more consistently explained the observations. Because of the assumptions, this test is quite conservative such that if heterozygosity is not 100% and there are amplification or ligation biases of particular alleles, the actual expectations for obtaining identical sequences from two random samples are greater than these predictions.

## Results

Amplification product sizes and sequences confirmed the presence of an intron of approximately 200 bp within the *efl-a* gene in *Penaeus monodon*. A total of 112 cloned fragments (Genbank Accession Numbers AF133939 to AF134050) were sequenced from 74 individuals (with either one or two sequences per individual): 18 from Indonesia (from 13 individuals), 17 from Malaysia (from 12 individuals), 19 from the Philippines (from 11 individuals), 18 from Madagascar (from 12 individuals), 29 from Mauritius (from 20 individuals), and 11 from Tanzania (from 6 individuals).

There were 69 different sequences (alleles) among the 112 fragments. These sequences ranged in length from 187 to 213 nucleotides, of which 23 nucleotides were coding sequence; 159 of the nucleotides were invariant. There were 34 phylogenetically informative characters including six insertions/deletions ranging in length from 2 to 14 nucleotides.

In the molecular phylogram, alleles from Indonesia, Malaysia, and the Philippines formed a monophyletic clade distinct from alleles from Madagascar, Mauritius, and Tanzania (Fig. 1).  $F$ -statistics of the sequences further indicated significant structure among the western Pacific and western Indian Ocean populations: the  $F_{ST}$

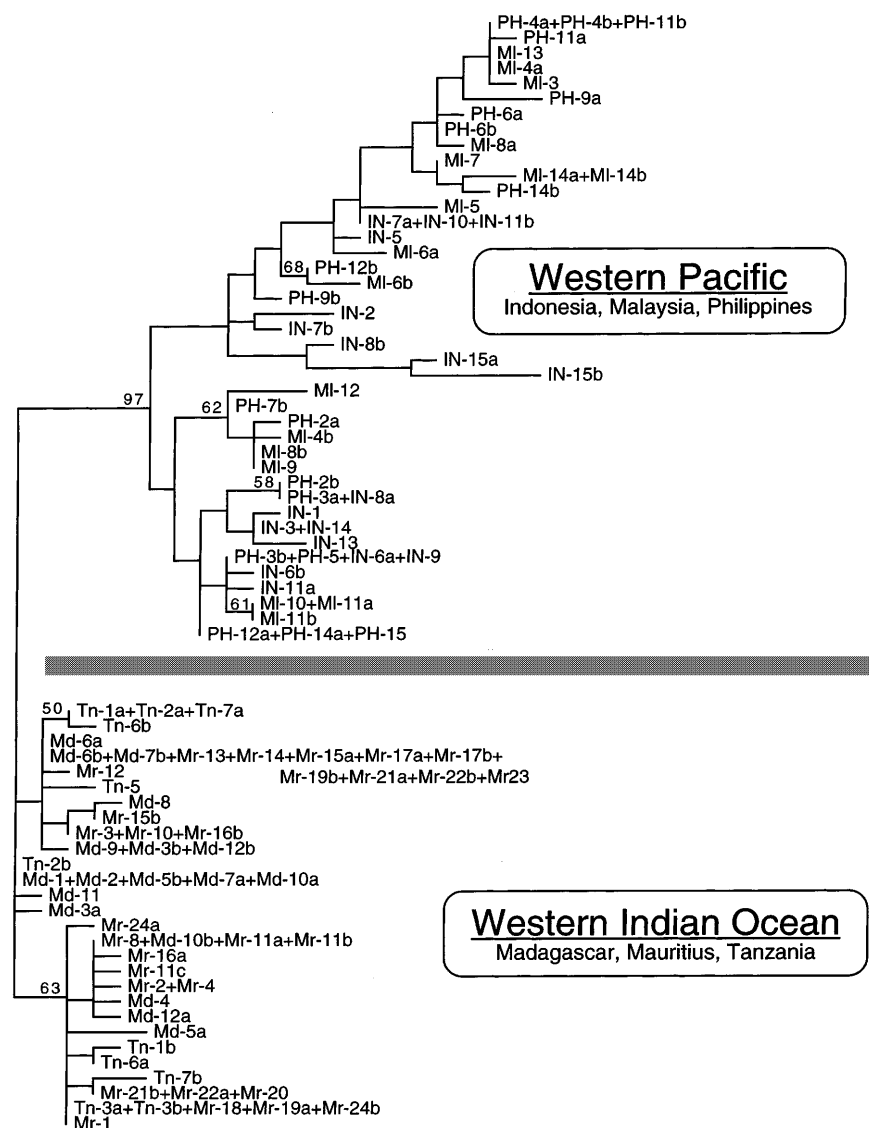
among all populations was 0.398 while the pairwise  $F_{ST}$  of the pooled western Pacific populations versus pooled western Indian Ocean populations was 0.505. Both  $F_{ST}$ -values were significantly larger than  $F_{ST}$ -values from random distributions of sequences among populations and regions (0 out of 1000 bootstraps in tests of both cases had larger  $F_{ST}$ -values). Population pairwise  $F_{ST}$ -values estimated between regions were significantly larger than random distributions of sequences among all pairs from the western Indian and Pacific Oceans (Table 1).

The  $F_{ST}$ -values among the populations in the western Pacific and western Indian Ocean are small and only significantly greater than random distributions of

**Table 1** *Penaeus monodon*. Pairwise  $F_{ST}$ -values among populations from the western Indian Ocean and western Pacific (\*  $p < 0.05$ , \*\*  $p < 0.005$ , \*\*\*  $p < 0.001$ )

Population	Malaysia	Philippines	Madagascar	Mauritius	Tanzania
Indonesia	0.107	0.007	0.499***	0.568***	0.445***
Malaysia	—	0.008	0.493***	0.566**	0.411***
Philippines	—	—	0.494***	0.567***	0.447***
Madagascar	—	—	—	0.065	0.180*
Mauritius	—	—	—	—	0.127

**Fig. 1** *Penaeus monodon*. Molecular phylogram of *efl-a* intron sequences reconstructed with maximum parsimony from a consensus of 100 bootstrap analyses. Tree was rooted to the western Indian Ocean sequences. Tree length = 111 steps; consistency index = 0.559; homoplasy index = 0.444 (IN Indonesia; Md Madagascar; MI Malaysia; Mr Mauritius; PH Philippines; Tn = Tanzania; number following location information used to identify an individual shrimp; lower case letters following individual numbers were used when more than one fragment was sequenced from a single individual; numbers next to nodes indicate bootstrap percentages  $\geq 50\%$ )



sequences within the western Indian Ocean: among western Pacific populations the  $F_{ST}$  was 0.0417 (146 out of 1000 bootstraps had larger  $F_{ST}$ -values), among western Indian Ocean populations the  $F_{ST}$  was 0.109 (37 out of 1000 bootstraps had larger  $F_{ST}$ -values). Nearly identical  $F_{ST}$ -values and significance levels were obtained from the Arlequin AMOVA (analysis of molecular variance) framework. Identical sequences were found in Indonesia and the Philippines (two occurrences), Mauritius and Tanzania (one occurrence), and Madagascar and Mauritius (two occurrences). Five of six of the population pairwise  $F_{ST}$  estimates within the western Indian Ocean and western Pacific are not significantly greater than random distributions of sequences among populations (Table 1). The population pairwise  $F_{ST}$  between the populations from Tanzania and Madagascar was the only significant pairwise  $F_{ST}$  estimated within regions.

Average sequence diversity was significantly (approximately 3.3 times) greater ( $p < 0.001$ ,  $t$ -test) within the western Pacific populations than within western Indian Ocean populations (Table 2). Also, while there were 41 unique sequences identified in western Pacific populations (76% of 54 sequences from 36 individuals), there were only 28 unique sequences identified in western Indian Ocean populations (49% of 57 sequences from 38 individuals).

In general, when two fragments were sequenced from the same individual, they were more likely to be different (Table 3), and differed for a minimum of two characters. The 1-locus model was rejected when counts from all populations were combined.

**Table 2** *Penaeus monodon*. Nucleotide diversity of western Pacific and western Indian Ocean populations

Population	Number of sequences	Nucleotide diversity (%)
Indonesia	13	3.507
Malaysia	12	4.380
Philippines	11	3.835
Western Pacific (mean)	12	3.907 ± 0.44
Western Pacific (pooled)	36	4.077
Madagascar	12	0.967
Mauritius	20	1.041
Tanzania	6	1.536
Western Indian Ocean (mean)	12.7	1.181 ± 0.31
Western Indian Ocean (pooled)	38	1.326
All populations (mean)	12.3	2.544 ± 1.54
All populations (pooled)	74	4.224

## Discussion

### Population structure

Highly significant geographic population structure exists between the western Indian Ocean (WIO) and western Pacific (WP) populations of the black tiger prawn, *Penaeus monodon*. Of the 58 and 54 sequences obtained from each region respectively, no sequences were shared between regions. Sequences from the WIO and WP populations differed by a minimum of seven characters representing either nucleotide substitutions or insertions/deletions. In a molecular phylogram (Fig. 1), sequences from the two regions were distinct. Without inclusion of outgroup sequences the WIO cannot be shown to form a strictly monophyletic clade.

Furthermore, indices of genetic differentiation,  $F_{ST}$ -values, are very large and significantly greater than random distributions of sequences among WIO and WP populations when considering the two regions ( $F_{ST}=0.505$ ) or all six populations ( $F_{ST}=0.398$ ) separately or from population pairwise estimates among WIO and WP populations (Table 1). From these  $F$ -statistics and the geographic distribution of alleles in the molecular phylogram, we can decidedly reject the hypothesis that there is no structure among populations of *Penaeus monodon* in the Indo-West Pacific.

The levels of differentiation between the WIO and WP regions are in accord with observed differences between Indian and Pacific Ocean populations of four species of damselfish (Lacson and Clark 1995), the coconut crab (Lavery et al. 1995, 1996), the tropical starfish *Linckia laevigata* (Williams and Benzie 1998). The concordance of these results and the numerous examples of disjunct species distributions in the Indo-West Pacific (Woodland 1986; Andersen 1991; Wallace et al. 1991; McMillan and Palumbi 1995; Härlin 1996) strongly suggest that species population structure among these regions is shaped by similar vicariant histories (e.g. Avise 1992) and that distinct marine biogeographic provinces exist within the Indo-West Pacific at both inter- and intraspecific levels. An Indo-West Pacific species that is not genetically differentiated across the Indo-Australian Archipelago is the goatfish, *Mulloidichthys vanicolensis*, although populations from the Indian and Pacific Oceans are morphologically differentiated (Stepien et al. 1994). Genetic analyses of species across this range are still few in number.

**Table 3** *Penaeus monodon*. Number of times in which identical or different sequences were obtained when sequencing two fragments from the same individual. Chi-square values are from tests for 1-locus and 2-locus models (see "Materials and methods"; \*  $p < 0.05$ )

Population	Identical sequence	Different sequence	Chi-square 1-locus model	Chi-square 2-locus model
Western Pacific (pooled)	6	12	2.00	0.67
Western Indian Ocean (pooled)	6	14	3.20	0.27
All populations (pooled)	12	26	5.16*	0.88

There does not appear to be significant population structure within the western Pacific populations analyzed – the overall  $F_{ST}$ -value is small and not significantly greater than random distributions of sequences among populations ( $F_{ST}=0.042$ ), none of the pairwise  $F_{ST}$ -values are significantly greater than random (Table 1), and there are instances of shared alleles among populations. However, although sequences within the WIO do not fall independently in geographically defined clades (e.g. sequences from Madagascar do not form a clade distinct from sequences from Tanzania and Mauritius; Fig. 1) and in several cases identical sequences were found in more than one population within the WIO, the  $F_{ST}$ -value among populations within this region ( $F_{ST}=0.109$ ,  $p < 0.05$ ) and the pairwise  $F_{ST}$  between the populations from Madagascar and Tanzania (Table 1) indicate limits to gene flow within the Indian Ocean.

The structure among populations of *Penaeus monodon* within each region implies that while there is significant mixing of individuals occurring within the WP, mixing among populations in the WIO is much more restricted, particularly among populations from Madagascar and Tanzania (Table 1). The geographic distances among populations within each region are similar, so differences in structure among these two regions may be a result of more significant barriers to dispersal in the WIO than in the WP or regional differences in farm management practices.

### Genetic diversity

WP populations are significantly more genetically diverse (approximately 3.3 times) than the WIO populations (Table 2). Within each region, the populations have similar levels of diversity. Based on the similarity of levels of diversity within each local population (Table 2) and the extreme divergence of sequences between regions (e.g. Fig. 1), the populations within each ocean basin likely have shared histories. A similar west-to-east trend of increasing diversity was also found among populations of *Penaeus monodon* from Australia in which a population from the west coast had a lower level of mtDNA diversity than populations from the east coast (Benzie et al. 1993). Differences in diversity are likely due to differences in the demographic histories of populations. For example, the low genetic diversity of the WIO populations may be due to small effective population sizes of WIO populations, recent or historical population bottlenecks in the WIO, or a recent colonization of the WIO from the WP.

None of the other studies of population structures of species occurring in Indian and Pacific Oceans shows this pattern of lower levels of diversity in populations from the Indian Ocean (Stepien et al. 1994; Lacson and Clark 1995; Lavery et al. 1996; Williams and Benzie 1998). In all reported cases, levels of diversity are not significantly different among Indian and Pacific Ocean populations. That *Penaeus monodon* is an exception

implies that although processes affecting species distributions in the Indo-West Pacific have had similar results for several different species, either the dynamics of these actions have varied, *P. monodon* has a unique demographic history which has influenced current levels of diversity, or other biological (e.g. dispersal potential) or ecological (e.g. preferred habitat) factors have influenced the distributions of diversity in this species.

### Number of loci

Evidence from combined populations supports the hypothesis that there is more than one *efl-a* locus within *Penaeus monodon*. This is consistent with data from *P. vannamei* in which *efl-a* appears to be a 2-loci system with up to four alleles per individual (France et al. 1999), which implies that the ancestral *efl-a* locus duplicated prior to the divergence of these two species. Because alleles do not fall into groups that might be recognized as separate loci in *P. monodon*, concerted evolution is likely operating among these loci as suggested for *P. vannamei*. Concerted evolution must be operating fairly quickly, faster than population migration between oceans, for there to be no shared alleles among WP and WIO populations.

Our current molecular phylogenetic analyses assume that the sequences are from a single locus. But failure of this assumption makes tests of geographic differentiation more conservative because it is more likely that alleles would cluster according to which locus they came from as opposed to their geographic source. Also, any within-population homogenization by concerted evolution would only reduce diversity and not bias tests of population structure. Because the sequences may be from two loci, levels of diversity may be over-estimated. However, it is unlikely that the trend of greater diversity in the WP relative to the WIO populations is a consequence of the use of sequence data from two loci instead of one in the analyses.

### Conclusions

Western Indian Ocean and western Pacific populations of the black tiger prawn, *Penaeus monodon*, have separate evolutionary histories, as demonstrated by differences in levels of genetic diversity and degrees of genetic differentiation between the two regions. These results are similar to findings for several other species which showed marked differentiation across the Indo-Australian Archipelago. This in turn implies that many dissimilar marine taxa share similar vicariant histories in the Indo-West Pacific. Results suggest that the *elongation factor 1-alpha* gene occurs in at least two copies in *P. monodon*. If true, phylogenetic distinction among alleles from the two oceans suggests that concerted evolution is operating fairly quickly in this species.

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