Phylogenetic analysis of wild populations of Exopalaemon carinicauda in three regions of Zhejiang Province based on COI and 16S rRNA genes

Abstract: Phylogenetic analysis is an important technique in molecular systematics, which is often applied to the analysis of evolutionary relationships between organisms. At present, phylogenetic analysis is applied to many branches of biology, such as describing the phylogenetic relationship of different taxa by constructing phylogenetic trees, and analyzing and applying them in terms of their genetic relationship, describing homologous gene relationships, and exploring the historical changes of populations. Exopalaemon carinicauda, belonging to the family Palaemonidae and Exopalaemon, is one of the important economic shrimp species in China. It is mainly distributed in the shallow low-salt waters along the eastern coast of China and the west coast of the Korean Peninsula. It has the characteristics of fast growth, strong adaptability, large individual size and strong reproductive ability. In recent years, as an emerging aquaculture species, the aquaculture industry of white shrimp has developed rapidly, and the coastal area of Zhejiang is one of the important aquaculture production areas of white shrimp. In addition, Zhejiang is located in the central coastal region of China, where the north and south converge, and is a transition zone between the subtropical zone and the warm temperate zone, with a superior geographical location. The natural resources of the spinetail white shrimp along the coast of Zhejiang are very rich. This paper explores the phylogenetic differentiation of wild populations of P. spiniculus in coastal Zhejiang, and provides a theoretical basis for the protection and rational development and utilization of P. spp. resources. The COI and 16S rRNA gene fragments of the mitochondrial DNA (mtDNA) of three populations of S. spine-tailed P. japonica (JX) in northern Zhejiang, Sanmen Sanmen in central Zhejiang (TZ) and Pingyang (WZ) in Wenzhou in southern Zhejiang were amplified and determined, and the phylogenetic differentiation and genetic relationship among S. spinosa populations were studied. The total DNA of the muscle tissue of the spine-tailed white shrimp samples was extracted using the animal tissue genomic DNA extraction kit (Shanghai Sangon Bioengineering Co., Ltd.) from Shanghai Shenggong. After PCR amplification, it was sent to Beijing Qingke Biotechnology Co., Ltd. for sequencing.The obtained sequences were used for further analysis, and the genetic distance between species was calculated based on the Kimura two-parameter model. The phylogenetic phylogenetic tree was constructed by Bayesian Inference (BI) and Maximum Likelihood (ML) method. After comparison, the consistent sequence of COI gene of 529 bp and the consistent sequence of 16S rRNA of 434 bp were obtained, and the combined sequence of 963 bp was obtained. The average contents of T, C, A and G were 34.43%, 20.67%, 27.3% and 17.6%, respectively, the contents of A+T were 61.73%, and the contents of G+C were 38.27%. Among the 21 variable sites, the transition/reversal ratio (R) is 1.74. The average intraspecific genetic distance of the three populations was 0.015. The phylogenetic

This work was supported by the National Natural Science Foundation of China (Grant No. 41276123), the Special Research Fund for the 'Marine Biotechnology and Marine Engineering' Discipline Group of Ningbo University (Grant No. 422004582), and the Key Science and Technology Research Project of Sanmen County Agriculture and Rural Affairs Bureau (Grant No. Sanzhaocai-2021-GK135).

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phylogenetic tree showed that the two groups of JX and WZ were relatively distant, and the TZ and WZ were relatively close. It is speculated that the genetic relationship of S. spiniculus is related to geographical distance, which also indicates that the genetic variation of S. spinosa mainly comes from within the population. The divergence time indicates that the time of the appearance of the spinetail white shrimp along the coast of Zhejiang is about the Cretaceous. The JX population was first differentiated at about 99.39 Ma (95%HDP: 95.03 Ma-130.95 Ma), and then the TZ and WZ populations were differentiated. It is speculated that the differentiation of white shrimp in Zhejiang Province is from north to south. This study clarified the phylogenetic relationship of P. lanceolata along the coast of Zhejiang, and provided a basis for future research on the faunal diversity and resource conservation of this taxon.
Keywords: Exopalaemon carinicauda, COI gene, 16S rRNA gene, Systemic differentiation and development, Kinship.

Molecular systematics is a discipline based on biomacromolecules, exploring biodiversity at the molecular level and delving into the phylogenetic relationships among different organisms [1]. This method traces the genealogical connections between organisms by resolving the relatedness of gene or protein sequences [1]. It has a wide range of applications, including constructing evolutionary trees that demonstrate phylogenetic relationships, assessing relatedness between species, elucidating relationships between homologous genes, and studying the historical dynamics of populations [2]. Given the limitations of single gene sequences in providing comprehensive evolutionary insights, current research trends favor phylogenetic analyses using multi-gene tandem sequences or genome-wide data [3]. For example, Bi et al [4] compared the evolutionary divergence of cod using mitochondrial 16S rRNA, COI, and Cyt b gene fragments, while Xu et al [5] used a combination of multi-gene sequences to explore the origin and evolution of loach fishes.

The ridge-tailed white shrimp (Exopalaemon carinicauda, E. carinicauda) is an important commercial shrimp in China which belongs to the genus Exopalaemon of the family Brachionidae [6]. The species is mainly distributed in shallow, low-salinity waters along the eastern coast of China and the western coast of the Korean Peninsula, with a particular preference for shallow nearshore or estuarine brackish and freshwater habitats [6]. E. carinicauda is well known for its biological characteristics such as rapid growth, adaptability, large size and high fecundity [7]. At present, the national aquaculture area has exceeded 20,000 hectares, with a production of about 50,000 tons [8]. The coastal area of Zhejiang has become the main aquaculture area for this species with its mature shrimp, crab and shellfish mixed culture mode. Zhejiang is located in the middle of China's coast, with both subtropical and warm-temperate transitional climatic characteristics and abundant natural resources [6].

The main purpose of this article is to study the genetic differences and whether they are closer relatives of E. carinicauda in three coastal areas of Zhejiang Province - Jiaxing, Taizhou, and Wenzhou. We used PCR technology to measure the COI and 16S rRNA genes of their mitochondrial DNA, hoping to learn about the gene differentiation of the E. carinicauda in

Zhejiang and even the entire coastal areas of China.

1 Materials and Methods

1.1 Materials

Specimens of E. carinicauda used in this study were collected from three coastal regions of Zhejiang Province, China, during March and April 2021: Haiyan, Jiaxing (Northern Zhejiang); Sanmen, Taizhou (Central Zhejiang); and Pingyang, Wenzhou (Southern Zhejiang). Samples were obtained directly from local fishermen's small-scale nets. Live specimens were transported to the laboratory, where morphological and biological characteristics were measured. Tissue samples were then collected and stored at -80°C for further analysis. Detailed information on the three populations is provided in Table 1.

Table.1 Sampling location and quantity of E. carinicauda

Collection Places	Abbreviation	Quantity	Sampling Part	Latitude and Longitude
Jiaxing City	JX	20	Muscle	120.55° E, 30.50° N
Taizhou City	TZ	20	Muscle	121.22° E, 29.07° N
Wenzhou City	WZ	20	Muscle	120.57° E, 27.66° N

Notes: The sampling sites are all located in Zhejiang Province; Jiaxing population (JX), Taizhou population (TZ), Wenzhou population (WZ).

1.2 Total DNA Extraction

Total DNA was extracted from the muscle tissue of E. carinicauda samples using the Animal Tissue Genomic DNA Extraction Kit (Sangon Biotech, Shanghai, China), following the manufacturer’s instructions. The extracted DNA was stored at -80°C for subsequent use.

1.3 PCR Amplification and Sequencing

The COI gene was amplified using universal primers LCO1490 and HCO2198 [9] (Folmer et al., 1994):

LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3'

HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAAT-3'

The 16S rRNA gene was amplified using universal primers 16Sar and 16Sbr [10] (Macdonald et al., 2005):

16Sar: 5'-CGCCTGTTTATCAAAAACAT-3'

16Sbr: 5'-CCGGTCTGAACTCAGATCACG-3'

The PCR reaction mixture (25.00 μL) consisted of 12.50 μL dNTPs mix, 1.00 μL each of forward and reverse primers, 1.00 μL DNA template, and 9.50 μL DEPC-treated water. The PCR program was as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. The PCR products were detected by 1% agarose gel electrophoresis, photographed and recorded by a gel imager, and sent to Beijing Prime Biotech Co. for sequencing.

1.4 Data Analysis

The sequences were spliced using Vector NTI [11] software and then compared with homologous sequences obtained by BLAST analysis in NCBI using MEGA 5.0 [12] software. In this process, genetic information indexes such as variant sites, base composition parsimony information sites, and conversion/reversal ratios were calculated. At the same time, intra-species genetic distances were calculated based on the Kimura-2-parameter (K-2-P) model [13].

1.5 Phylogenetic Tree Construction

To determine the suitability of the sequences for phylogenetic analysis, the saturation of the sequences was examined by mapping analysis based on transformations and inversions versus Kimura's two-parameter genetic distance [13]. Phylogenetic trees were constructed using Bayesian Inference (BI) in MrBayes software [14] and Maximum Likelihood (ML) in the IQtree program of Phylosuite [15, 16].

1.6 Divergence Time Estimation

In addition to the sequences obtained in this study, DNA sequences of two Macrobrachium species were downloaded from GenBank for estimating the divergence time of E. carinicauda populations in Zhejiang Province (Table 2). The analysis was conducted using BEAST v.2.6.3 software [17]. The GTR+I+G model and Yule tree prior were selected, and a relaxed clock log-normal model was used for the molecular clock. The calibration node was based on the divergence time of the genus Macrobrachium [18]. Markov chain Monte Carlo (MCMC) analysis was run for 10,000,000 generations, with sampling every 1,000 generations. The first 50% of the chains were discarded as burn-in.

Table 2 Selected sample information and Genbank login number

Name	COI	16S
Palaemon gravieri	KT282102.1	KC515045.1
Palaemon serratus	LT717310.1	LT717255.1

2 Results and Analysis

2.1 Sequence Composition Analysis

Following PCR amplification and sequencing, the obtained mtDNA sequences of E. carinicauda, including the cytochrome c oxidase subunit I (COI) gene and the 16S ribosomal RNA (16S rRNA) gene, were rigorously aligned and manually corrected. The final lengths of the sequences were determined to be 529 base pairs (bp) for the COI gene and 434 bp for the 16S rRNA gene. These two sequences were concatenated, resulting in a combined sequence of 963 bp. Within this concatenated sequence, 21 variable sites were identified, indicating potential nucleotide variations. Among these, 17 were parsimony-informative sites, providing valuable information for phylogenetic analysis, and 13 were singleton polymorphic sites, which are significant for studying genetic polymorphism in this species. The transition/transversion ratio (R) was calculated to be 1.74, serving as an important reference for analyzing the types and rates of genetic variation. The average nucleotide compositions of T, C, A, and G were 34.43%, 20.67%, 27.3%, and 17.6%, respectively, with an A+T content of 61.73% and a G+C content of 38.27% (Table 3).

Table 3 The base composition of the joint sequence in the 3 populations of E. carinicauda

Population	T%	C%	A%	G%	A+T%	C+G%
JX	34.4	20.7	27.2	17.7	61.6	38.4
TZ	34.5	20.7	27.3	17.5	61.8	38.2
WZ	34.4	20.6	27.4	17.6	61.8	38.2
Avg	34.43	20.67	27.3	17.6	61.73	38.27

Note: Average (Avg).

A total of 16 haplotypes were identified from 60 individuals (Table 4). Among these, haplotype Hap2 was shared by all three populations (JX, TZ, and WZ). The JX and TZ populations shared one haplotype (Hap5), the JX and WZ populations shared one haplotype (Hap1), and the TZ and WZ populations shared two haplotypes (Hap6 and Hap12). The remaining haplotypes were unique to each population. The JX population exhibited the highest number of haplotypes (8), while the TZ and WZ populations each had 7 haplotypes.

Table 4 Haplotype distribution of joint sequences in the 3 populations

Haplotype16	JX	TZ	WZ	Total
Hap_1	0	1	1	2
Hap_2	4	1	1	6
Hap_3	1	0	0	1
Hap_4	0	2	0	2
Hap_5	1	1	0	2
Hap_6	0	2	1	3
Hap_7	1	0	0	1
Hap_8	1	0	0	1
Hap_9	0	1	0	1
Hap_10	1	0	0	1
Hap_11	0	0	1	1
Hap_12	0	1	2	3
Hap_13	0	0	1	1
Hap_14	0	0	2	2
Hap_15	1	0	0	1
Hap_16	1	0	0	1

2.2 Genetic Distance Analysis

An analysis of molecular variance (AMOVA) was conducted among the populations, treating the three populations as a single group (Table 5). The results revealed an Fst value of 0.2611 ($P < 0.05$), indicating that 26.11% of the total genetic variation was attributed to differences among populations, while 73.89% of the variation occurred within populations. This suggests that, although significant genetic differentiation exists among populations, the majority of genetic variation is still maintained within populations. This pattern may result from factors such as gene flow, mutation, and genetic drift, which are more active within populations. The interpopulation genetic differentiation coefficient of 26.11% indicates a moderate level of genetic differentiation among these populations. Such differentiation may reflect adaptive evolution to different

ecological environments or reduced gene flow due to geographic isolation, reproductive isolation, or other factors.

The average intraspecific genetic distance was 0.0159, a relatively low value indicating a high level of genetic homogeneity within the species. This genetic proximity may contribute to maintaining the species' genetic diversity and adaptive potential. Shrimp from JX and WZ have the highest genetic coefficient of variation, at 0.0183, which suggests that they are genetically quite different from each other, perhaps because of geographic isolation, or historical migration, or some other reason, which restricts the exchange of genes between them anyway. The coefficient of genetic difference between JZ and TZ is 0.0154, which is also quite large, but slightly smaller than that between JZ and WZ. The lowest genetic differentiation coefficient was found between the WZ and TZ (0.014), suggesting that these two groups are genetically the closest, possibly sharing a recent common ancestor or experiencing more frequent gene exchange. In summary, the WZ and TZ exhibited the smallest genetic distance and the closest kinship, whereas the JX and WX showed the largest genetic distance and the most distant kinship.

Table 5 Molecular variation analysis of joint sequence of E. carinicauda (AMOVA)

Source of variation	df	Sum of squares	Variance components	Variance proportion(%)
Among population	2	31.901	0.32374Va	26.11
With population	58	88.272	2.15611Vb	73.89
Total variance	60	120.173	2.47985	

Table 6 Relative genetic distance among the 3 populations in Zhejiang coastal sea

JX	TZ	WZ	
0			JX
0.0154	0		TZ
0.0183	0.014	0	WZ

2.3 Phylogenetic Analysis

The linear relationship between transitions, transversions, and genetic distances was analyzed to assess the saturation of base substitutions in the concatenated sequence. The transition/transversion ratio (R) was calculated to be 1.74. The results showed no evidence of substitution saturation when the Kimura two-parameter (K2P) genetic distance was less than 0.2028. Based on this finding, the concatenated sequence was deemed suitable for constructing a phylogenetic tree.

Phylogenetic trees were constructed using BI and ML methods based on 11 unique haplotypes from the three regional populations of E. carinicauda. The topological consistency between the trees generated by these two methods demonstrated the stability and reliability of the approaches. This consistency typically indicates that the inferred evolutionary relationships are statistically robust. Although the topologies were similar, differences in node confidence values (or support rates) may reflect inherent differences in how the algorithms process the data or certain characteristics of the data itself. In the ML tree, branches with bootstrap support values exceeding 70% were considered well-supported [19], further validating the credibility of the results.

The phylogenetic tree revealed that the TZ and WZ populations clustered together first, followed by the JX. This suggests that the TZ and WZ populations may share more recent gene flow or a common ancestor. In contrast, the JX population exhibited a more distant genetic relationship with the TZ population, indicating that the JX population may have diverged earlier from the common ancestor.

The observation that "genetic relatedness correlates with geographic distance" is a well-known pattern in biogeography, often called Isolation by Distance (IBD). Based on the observed core phenomena it is known that with increasing geographical distance, the genetic exchange between populations slowly becomes less and less, which naturally leads to increasing genetic differences. However, it should be remembered that geographic distance is not the only reason.

In order to comprehensively analyze the evolutionary history and population dynamics of the spiny tailed white shrimp, we plan to expand the sampling range to cover a wider geographical distribution, thereby more accurately reflecting its overall distribution pattern and genetic variation. In addition, we also plan to use genomics technology to obtain more detailed genetic information and systematically study the role of non genetic factors in population differentiation by combining ecological and behavioral data.

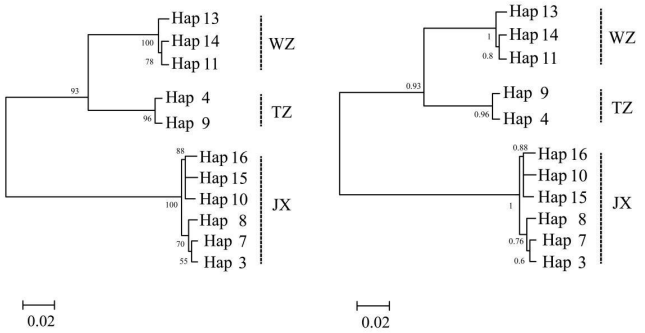

Figure 1 Phylogenetic Tree Built Based on BL Method (Left) and Phylogenetic Tree Built Based on ML Method (Right)

2.4 Divergence Time Analysis

The commonly used method for studying the evolutionary history of species is to analyze cytochrome c oxidase subunit I (COI) and 16S rRNA genes. For the study of E. carinicouda, we combined COI and 16S rRNA gene data and analyzed them using a relaxed molecular clock model. Compared to strict molecular clock models, relaxed models allow for differences in mutation rates between different lineages, which better reflect the complexity of biological evolution and provide more reliable estimates of species divergence time. The research results show that the divergence time of the spintail white shrimp is about 102.27 million years ago. Afterwards, the species underwent further population differentiation, which may have been driven by adaptive evolution in different ecological environments. Considering the different rates of evolution in nature, we purposely chose the “loose molecular clock model” rather than the “one-size-fits-all” strict model. This loose model is like opening a “transmission” for each evolutionary branch, allowing them to have different evolutionary speeds, so that the results are

more reliable. Based on our study of the ridge-tailed white shrimp in three regions, we estimate that they began to diverge around 102 million years ago. Later, in order to adapt to different environments, they further evolved into different types.

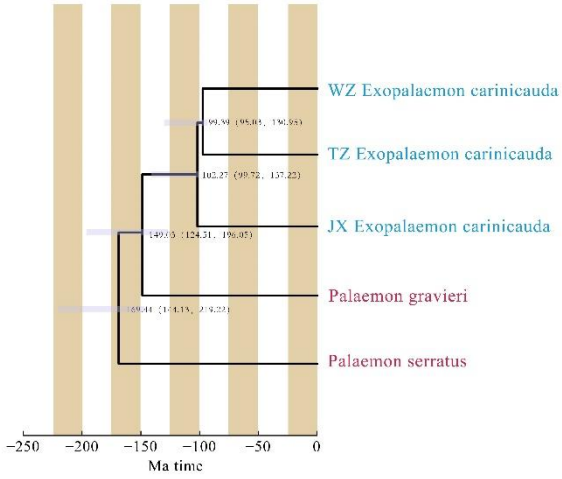

Figure 2 The estimated divergence time of E. carinicauda represents the median value and 95% confidence interval of the node

3 Discussion

E. carinicauda is widely distributed along the coast of China. This study explores the phylogenetic differentiation of E. carinicauda populations along the Zhejiang coast, shedding light on the evolutionary pathways of these populations across the Chinese coastline and the phylogenetic divergence of the genus Exopalaemon in China.

mtDNA, characterized by its small molecular size, simple structure, high copy number, high coding efficiency, lack of tissue specificity, strict maternal inheritance, and rapid evolutionary rate [20], has been extensively utilized in phylogenetics and evolutionary genetics. Sun et al. [21] investigated the differentiation and phylogenesis of three populations of Macrobrachium nipponense using the 16S rRNA gene fragment. Chen et al. [22] analyzed the relationships and differences between Elopichthys bambusa from the Yangtze River and Pearl River regions through multi-gene analysis. Bi et al. [4] studied the phylogenetic relationships of four Gadus species using three mitochondrial gene fragments, 16S rRNA, COI, and Cytb. Zhu et al. [23] analyzed the structure and phylogenesis of the Alpheidae family using the mitochondrial genome. Chen [24] explored the phylogenetic relationships of the Labeoninae subfamily in the Qinling Mountains using multi-gene sequences. Given the high applicability of mtDNA in phylogenetic and evolutionary research, this study utilized the combined sequences of mtDNA COI and 16S rRNA to analyze the systematic evolutionary characteristics of organisms from three regions along the Zhejiang coast.

From the perspective of base composition, the combined gene sequence of E. carinicauda from the three regions in this study showed an A+T content of 61.73% and a G+C content of 38.27%, with A+T base content significantly higher than G+C base content. This result is consistent with the conclusion of Zhao et al. [25], who found a significant bias in the base composition of mitochondrial genes in Decapoda. It also aligns with the conclusion of Asakawa et al. [26], who

reported a significant bias in the base composition of mitochondrial genes in Metazoa. A total of 21 variable sites were detected in the three populations, with a transition/transversion ratio (R) of 1.76, which is consistent with the pattern that amino acid substitutions are mainly transitions, with transitions being more frequent than transversions, demonstrating a high transition bias [27]. The study confirmed that the variations observed in the three E. carinicauda populations fall within the scope of intraspecific variation, with no evidence of intraspecific inbreeding or subspecies differentiation, consistent with the results of Ma et al. [28], who used the COI gene alone.

Analysis of genetic distance revealed that the average intraspecific genetic distance of E. carinicauda in the three regions was 0.0157, indicating a low degree of developmental differentiation. The JX showed a relatively distant kinship with the WZ, which is proportional to the larger geographical distance.

Haplotype analysis of E. carinicauda along the Zhejiang coast based on mitochondrial gene sequences revealed that different geographical populations share a common haplotype. This phenomenon suggests a certain degree of gene flow among different geographical populations within the E. carinicauda population. Furthermore, both private haplotypes and haplotypes shared between geographically distant populations were observed. It is speculated that this may be due to the retention of ancestral haplotype polymorphisms among populations.

ML and BI phylogenetic trees reconstructed from the concatenated mitochondrial gene sequences showed essentially consistent topologies, reflecting similar phylogenetic relationships. The structures of the ML and BI phylogenetic trees based on the concatenated mitochondrial gene sequences indicated a high degree of consistency, reflecting similar phylogenetic relationships. Among the Zhejiang E. carinicauda populations, the JX was the first to diverge, forming a separate clade, while the WZ and TZ formed another clade, suggesting a closer kinship or later divergence time between the WZ and TZ compared to the JX population.

The emergence of E. carinicauda along the Zhejiang coast is estimated to have occurred around the Cretaceous period, which aligns with the findings of Gan [29], Zhu et al. [30], and Yang et al. [31]. The JX was the first to diverge among the E. carinicauda populations along the Zhejiang coast, with the initial divergence occurring approximately 102.27 million years ago. Given that JX is geographically located in the northern part of the Zhejiang coast, it is inferred that the emergence and dispersal of E. carinicauda along the Zhejiang coast proceeded from north to south. Currently, most studies on the phylogenetics and divergence times of E. carinicauda have focused on mitochondrial protein-coding genes. Future studies should incorporate nuclear gene data to more accurately reconstruct phylogenetic trees and estimate divergence times. Additionally, subsequent research should expand the collection range of population materials, increase data from different geographical populations, and explore more comprehensive methods for extracting population material data. Furthermore, it is necessary to integrate morphological characteristics to delve into the origin and dispersal pathways of E. carinicauda in China.

4 Conclusions

The main conclusions of this study are as follows: (1) The combined mitochondrial gene sequence length of E. carinicauda from three regions along the Zhejiang coast was 963 bp. The

average contents of T, C, A, and G in the mitochondrial genome were 34.43%, 20.67%, 27.3%, and 17.6%, respectively, with an A+T content of 61.73%, indicating a significant A+T bias. Phylogenetic analyses using ML and BI methods revealed that the WZ of E. carinicauda exhibited a closer kinship with the TZ. (2) Estimation of the divergence time of E. carinicauda along the coast of Zhejiang showed that the earliest emergence was along the coast of JX, which occurred about 99.39 Ma ago.

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