ISSN 1996-0700

Asian Journal of Biotechnology

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Asian Journal of Biotechnology 3 (3): 302-309, 2011
ISSN 1996-0700 / DOI: 10.3923/ajbkr.2011.302.309

Molecular Phylogeny of Horseshoe Crab

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ABSTRACT

An effort to assess the utility of 650 bp Cytochrome C oxidase subunit I (DNA barcode) gene in delineating the members horseshoe crabs (Family: xiphosura) with closely related sister taxa was made. A total of 33 sequences were extracted from National Center for Biotechnological Information (NCBI) which include horseshoe crabs (Class: Merostomata), beetles (Class: Insecta), common crabs (Class: Melacostraca) and scorpion (Class: Arachnida) sequences. Constructed phylogram through comprehensive dry lab methodology using advanced software predictive tools showed that beetles are closely related with horseshoe crabs than common crabs (Class: Melacostraca). It was interesting to note that terrestrial Scorpion (Class: Arachnida) were distantly related to horseshoe crabs (Class: Merostomata). Phylogram and observed Genetic Distance (GD) data were also revealed that *Limulus polyphemus* was distantly related to all the other horseshoe crab species. *Tachypleus gigas* was closely related with *Carcinoscorpius rotundicauda* than with *Tachypleus tridentatus*. The observed mean Genetic Distance (GD) value was higher in 3rd codon position in all the selected group of organisms. Among the horseshoe crabs high GC content was observed in *L. polyphemus* (38.32%) and lowest was observed in *T. tridentatus* (32.35%). We conclude that COI sequencing (barcoding) could be used in identifying and delineating evolutionary relatedness with closely related species.

Key words: Cytochrome C oxidase subunit I, genetic distance, codon position, horseshoe crab phylogene, xiphosuran

INTRODUCTION

Mitochondrial DNA (mtDNA) analysis has been employed in the evolutionary study of the animal species for more than 30 years (Brown *et al*., 1979; Avise and Walker, 1999). Its higher mutational rate and lower effective population size than the nuclear DNA make mtDNA a powerful tool to probe for evolutionary studies. This fact provoked a proposal to standardize DNA-based species identification by analyzing a uniform segment of the mitochondrial genome. A library of sequences from taxonomically verified voucher specimens could be built with this approach which could serve as DNA identifiers for species, in short, DNA barcodes (Herbert *et al*., 2003). For animals, 648 bp segment of the mitochondrial gene cytochrome C oxidase I (COI), which can be readily recovered from diverse species with a limited set of primers, was declared as a DNA barcode (Kevin *et al*., 2007). For this DNA barcoding approach to be effective, it must be possible to distinguish between intraspecific and interspecific mtDNA variation. The simplest test is whether the genetic distance within the species is lesser than those between species.

There are four extant species of horseshoe crabs, *Tachypleus tridentatus, Tachypleus gigas, Carcinoscorpius rotundicauda* and *Limulus polyphemus* (Pocock, 1902). The first three species inhabit the Southeast Asian coast and the last species the East Coast of North America.

These are known as living fossils, have maintained their morphology almost unchanged for the past 150 million years. The little morphological differentiation among horseshoe crab lineages has resulted in substantial controversy concerning the phylogenetic relationship among the extant species of horseshoe crabs, especially among the three species in the Indo-Pacific region. Earlier studies suggest that the three species constitute a phylogenetically irresolvable trichotomy (Xia, 2000). For elucidating their phylogenetic relationships, two proteins, coagulogen and hemocyanin, have been investigated (Shishikura *et al*., 1982; Srimal *et al.,* 1985; Sugita, 1986). Miyazaki *et al*. (1989) first investigated tropomyosin which is one of the major structural proteins involved in many types of cells, to elucidate prevailing phylogenetic relationships among horseshoe crabs and his result suggested that *L. polyphemus* is phylogenetically differentiated far from the three Asian species. But the results were not those which were worth studying from the detailed phylogenetic viewpoint, that is, the patterns of the four species were equally different from each other. When the proteins were cleaved with chymotrypsin or trypsin, only smaller differences than those obtained with V8 protease were found (Miyazaki *et al*., 1989).

What remains unresolved is the phylogenetic relationship among the three Indo-Pacific species. *T. gigas* and *T. tridentatus* were grouped together on the basis of morphological traits (Fisher, 1984), but *C. rotundicauda* and *T. tridentatus* appear to be more closely related on the basis of amino acid sequence divergence of a fibrinopeptide like protein (Shishikura *et al*., 1982) and coagulogen (Srimal *et al*., 1985) and on interspecific hybridization studies (Sekiguchi and Sugita, 1980). In a phylogenetic study of the four species of horseshoe crabs employing two-dimensional electrophoresis of skeletal and cardiac muscles, the similarity index is the greatest between *T. gigas* and *C. rotundicauda* for cardiac muscles, but between *T. tridentatus* and *T. gigas* for skeletal muscles (Miyazaki *et al*., 1987). Phylogenetic analyses to find out the genetic relatedness among the four horseshoe crab species based on two partial mitochondrial genes, (16S ribosomal RNA (rRNA) and cytochrome oxidase subunit I (COI)) was also carried out (Xia, 2000). But comprehensive study on genetic relatedness among horseshoe crabs and its related sister taxa is scarce.

As, COI has been proposed as a barcode gene for most of the eukaryotes (Herbert *et al*., 2003). To enhance our understanding on the phylogeny of horseshoe crab and its genetic relatedness with other sister taxa, present study was conducted to find the phylogenetic cues in mitochondrial COI region and to find out evolutionary relationship of horseshoe crabs with closely related taxa.

MATERIALS AND METHODS

Sequence features: A total of 33 barcode sequences belong to horseshoe crab (12), insects including beetle (8), common crab (5) and scorpion (8) were extracted from National Center for Biotechnological Information (NCBI) via FASTA format during March 2010. *Artemia franciscana* (NC_001620.1) was shuffled among the sequences was clearly occupied separate branch in the phylogram as an out-group. Pair wise sequence alignment of nucleotide sequences were performed using ClustalX 2.0.6 (Thomson, 1997). The GC content of all 33 barcodes was estimated by BioEdit sequence alignment editor (Hall *et al*., 1999). MEGA 4.1 Beta3 (Tamura *et al*., 2007) was used to construct phylogenetic trees via Neighborhood joining method using Kimura 2-parameter and to

Table 1: Total number of organisms used to construct the phylogram and their respective accession ID with total sequence length (bp) is given. Percentage of GC content for each organism was calculated using Bioedit sequence alignment editor V.7.0.9.0 (Hall *et al*., 1999)

Organism	Accession ID	Sequence length (bp)	GC content (%)
Limulus polyphemus	EU834780	1677	37.69
Limulus polyphemus	AF218278	552	36.96
Limulus polyphemus	U09392	582	38.32
Limulus polyphemus	U09391	582	38.32
Limulus polyphemus	NC_003057	1536	37.89
Limulus polyphemus	AF216203	1536	37.89
Tachypleus tridentatus	FJ860267	1536	33.07
Tachypleus tridentatus	EF460846	473	32.35
Tachypleus tridentatus	U09387	582	32.65
Tachypleus gigas	U09388	582	33.85
Carcinoscorpius rotundicauda	U09389	582	35.74
Carcinoscorpius rotundicauda	U09390	582	35.91
Centruroides limpidus	NC_006896	1533	39.40
Centruroides limpidus	AY803353	1533	39.40
Centruroides nigrimanus	AY995838	1530	38.43
Centruroides elegans	AY995824	1530	38.76
Centruroides sculpturatus	AY995831	1530	37.25
Centruroides noxius	AY995829	1530	38.24
Centruroides baergi	AY995823	1515	38.81
Centruroides vittatus	AY995835	1530	37.91
Scylla serrata	GU055514	535	32.71
Scylla serrata	GU055513	535	32.71
Portunus pelagicus	AF082732	465	34.41
Portunus pelagicus	FJ812293	1186	37.10
Portunus reticulatus	EF661975	573	35.78
Drosophila quadrisetata	DQ471563	1500	29.60
Drosophila angor	DQ471568	1500	31.93
Drosophila beppui	DQ471581	1500	28.87
Drosophila roehrae	EF570015	1443	31.95
Drosophila beppui	DQ471557	1500	28.87
Paramblopusa borealis	FJ749943	907	29.44
Paramblopusa eoa	FJ749944	907	29.77
Sternhydrus atratus	DQ813703	1265	29.57
Artemia franciscana (Out group)	NC_001620.1	1501	37.69

calculate genetic distance of the given set of sequences in each codon position. Details of sequences used to generate the phylogram are given in Table 1.

RESULTS

Phylogenetic analysis: A phylogenetic tree was constructed to verify the efficiency of coxI gene in delineating closely related species of horseshoe crab and to check its evolutionary relationship with other groups of organisms which were proved to be closest relatives of xiphosurans. The constructed phylogram showed two distinct Clads (A and B). *Artemia franciscana* (NC_001620.1), used as an out group was clearly branched separately, signified the reliability of constructed phylogram. Almost all the internal branches showed high bootstrap value (>90).

Clad A includes horseshoe crabs, insects (beetles) and common crab species and all the scorpion species were distinctly clustered in clad B. As it was expected beetles (Class: Insecta) used in this study was perfectly arranged in clad A showing higher genetic similarity (lesser genetic distance) with horseshoe crab and common crabs were showing higher genetic distance with xiphosurans. Terrestrial scorpion species used in this analysis were clumped together in Clad B indicating their distant genetic relatedness with Horseshoe crab species. The phylogram also showed that *L. polyphemus* was genetically distinct from 3 other species of horseshoe crabs. Among the three Asian horseshoe crab species *T. gigas* showed closer genetic relatedness with *C. rotundicauda* than with *T. tridentatus* hence they occupied same internal nod in the phylogram.

Among the horseshoe crabs *L. plyphemus* had comparatively higher GC content than the other species of horseshoe crabs. Average GC content in *L. polyphemus* was 37.84% followed by *C. rotundicauda* 35.82%. *T. gigas* had higher average GC content (33.85%) than *T. tridentatus* (32.69%) but lower than the other two species.

Mean genetic distance within *L. polyphemus* at 1st, 2nd and 3rd codon position were 0.01, 0.009 and 0.025, respectively. Likewise for *T. tridentatus* it was 0.014, 0 and 0.005 at 1st, 2nd and 3rd codon position, respectively. In case of *C. rotundicauda* the mean genetic distance values were 0.014, 0.014 and 0.05 at 1st, 2nd and 3rd codon position, respectively. Among the Asian horseshoe crab species *T. gigas* showed closer genetic relatedness (lower GD value) with *C. rotundicauda* with the value of 0.043, 0.021 and 0.144 at 1st, 2nd and 3rd codon position, respectively than with *T. tridentatus* with the values of 0.084, 0.028 and 0.242, respectively at 1st, 2nd and 3rd codon position. Calculated genetic distance data showed higher genetic distance in third codon position than its corresponding first and second codon positions (Table 2).

Mean GD value within the four species of horseshoe crabs was 0.095, 0.033 and 0.301 at 1st, 2nd and 3rd codon position respectively. The GD value between Insects (beetle) and horseshoe crabs were 0.201, 0.094 and 0.468 at 1st, 2nd and 3rd codon position respectively proved that horseshoe crabs are genetically more closer to insects followed by common crabs (GD values: 1st = 0.256; 2nd = 0.108 and 3rd = 0.527). Terrestrial scorpion species were showing higher mean GD value with horseshoe crabs (1st = 0.445; 2nd = 0.174 and 3rd = 1.016) clearly demarcated their distant genetic relatedness (Table 3). Figure 1 shows the results.

Table 2: Average genetic distance (GD) between four available species of horseshoe crabs observed in different codon positions

		L. polyphemus				*T. gigas*				*T. tridentatus*				*C. rotundicauda*			
	Codon position	1st+2nd+3rd	1st	2nd	3rd	1st+2nd+3rd	1st	2nd	3rd	1st+2nd+3rd	1st	2nd	3rd	1st+2nd+3rd	1st	2nd	3rd
L. polyphemus	1st+2nd+3rd	0.015				0.198				0.201				0.195			
	1st		0.01				0.143				0.153				0.125		
	2nd			0.009				0.065				0.05				0.047	
	3rd				0.025				0.419				0.462				0.493
T. gigas	1st+2nd+3rd					0				0.111				0.067			
	1st						0				0.084				0.043		
	2nd							0				0.028				0.021	
	3rd								0				0.242				0.144
T. tridentatus	1st+2nd+3rd									0.006				0.12			
	1st										0.014				0.098		
	2nd											0				0.014	
	3rd												0.005				0.281
C. rotundicauda	1st+2nd+3rd													0.260			
	1st														0.014		
	2nd															0.014	
	3rd																0.05

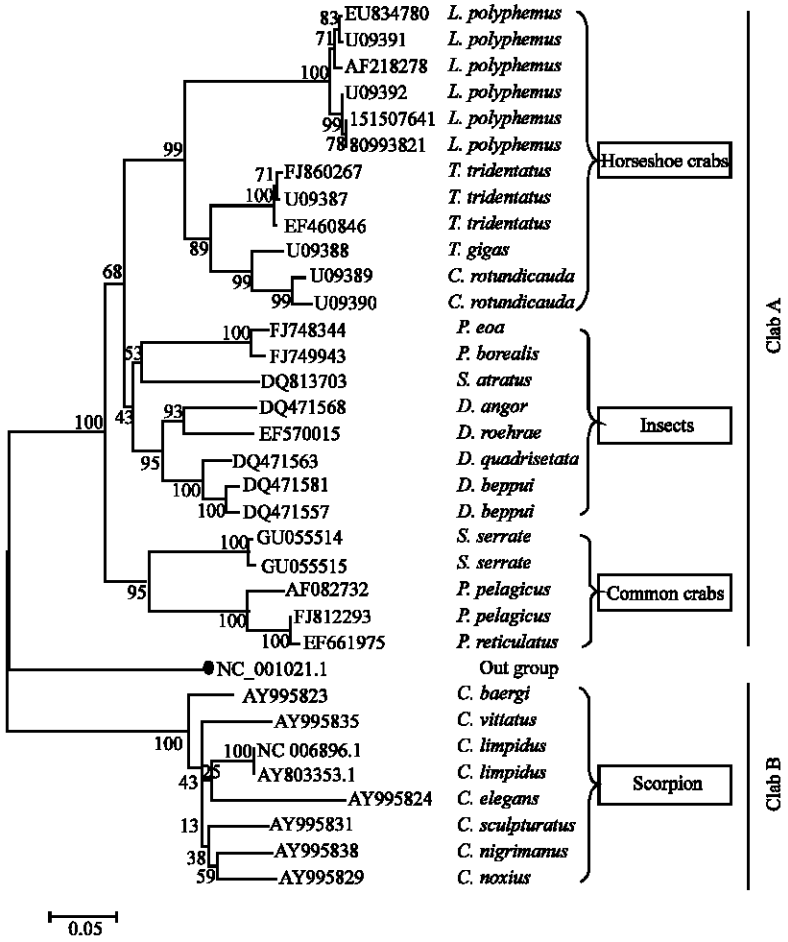

Fig. 1: The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.67404499 is shown in figure. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Codon positions included were 1^{st}+2^{nd}+3^{rd}+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 434 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007)

Table 3: Mean genetic distance (GD) values of different groups of organisms with reference to horseshoe crab at all the possible codon position indicating 3^{rd} codon position shows higher GD value

	1^{st}+2^{nd}+3^{rd} codon position	1^{st} codon position	2^{nd} codon position	3^{rd} codon position
	(Horseshoe crabs)			
Horseshoe crabs	0.13	0.095	0.033	0.301
Insect (beetles)	0.239	0.212	0.094	0.468
Common crabs	0.275	0.256	0.108	0.527
Scorpions	0.45	0.445	0.174	1.016

DISCUSSION

The constructed phylogram clearly indicated that horseshoe crabs are distantly related with scorpions at DNA level. Among the horseshoe crabs *L. polyphemus* showed distant genetic relatedness with other 3 species of Asian horseshoe crabs. This result was also well corresponded with previous finding by Miyazaki *et al.* (1987) where in two dimensional electrophoresis techniques clearly demarcated *L. polyphemus* from the three other species. *T. gigas* showed closer genetic relationship with *C. rotundicauda* than with *T. tridentatus*. This observation was also well coincide with the previous findings (Xia, 2000) where partial mitochondrial DNA sequence analysis revealed grater similarity index between *T. gigas* and *C. rotundicauda* than with *T. tridentatus*.

Insects were showing higher genetic similarity (lesser genetic distance) with xiphosurans (Horseshoe crabs) than the common crabs indicating that horseshoe crab might probably evolved from the ancient aquatic insects. Eurypterids (e.g., sea scorpions) have traditionally been regarded as close relatives of horseshoe Crabs; together forming a group called Merostomata. Subsequent studies placed eurypterids closer to the arachnids (e.g., spiders, terrestrial scorpions, mites and ticks) in a group called Metastomata (Pavlicek *et al.*, 2008). There has also been a prevailing idea that eurypterids are closely related to terrestrial scorpions (Raz *et al.*, 2009). The most recent study of relationships between arachnids and their relatives recognized Eurypterida, Xiphosura and Arachnida as three major groups, but was not able to resolve details between them (Shultz, 2007). Same result was reflected in the present study by separating scorpion species in a clad B indicating that horseshoe crabs have lesser genetic relatedness with terrestrial scorpions (Fig. 1).

Another interesting observation made from the calculated genetic distance data was higher genetic distance was observed in third codon position than its corresponding first and second codon positions. Similar observation was made by Ward *et al.* (2005) while barcoding of fishes from Australian waters. Simmons *et al.* (2006) also observed that greater phylogenetic signal is often found in parsimony-based analyses of third codon positions of protein-coding genes relative to their corresponding first and second codon positions, even for early-derived basal clades (Khan *et al.*, 2010; Siemion and Przemyslaw, 1994). Average genetic distance among the different groups of test organisms used in this study showed higher GD value at 3rd codon position indicating that detailed study on 3rd codon position might reveal possible evolutionary information among the closely related groups of organisms.

CONCLUSION

The COI sequence in the phylogram constructed clearly clustered the selected species in individual group, proving the efficacy of COI gene in delineating the members of evolutionarily cryptic groups of organisms. Constructed phylogram and genetic distance data clearly proved that horseshoe crabs are more genetically related to insects (Class: Insecta) than with common crabs and are distantly related with terrestrial scorpions. But further studies need to be conducted to prove this concept by analyzing total mitochondrial DNA sequence. It is also evident from the present study that the greater phylogenetic signal is often found in third codon position relative to their corresponding first and second codon positions as reported by Ward *et al.* (2005) and Simmons *et al.* (2006).

ACKNOWLEDGMENTS

This research work was funded by IIUM internal endowment fund (Ref No. EDW B100960435). We wish to express our sincere gratitude to International Islamic University Malaysia (IIUM) and Institute of Oceanography and Maritime Study (INOCEM) for providing infrastructural facilities. One of us extent his thanks to Dr. Shahriza, University Malaysia Terengganu (UMT) for his valuable comments on this present work.

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