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Research Paper

Complete Mitochondrial Genomes of *Carcinoscorpius rotundicauda* and *Tachypleus tridentatus* (Xiphosura, Arthropoda) and Implications for Chelicerate Phylogenetic Studies

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Abstract

Horseshoe crabs (order Xiphosura) are often referred to as an ancient order of marine chelicerates and have been considered as keystone taxa for the understanding of chelicerate evolution. However, the mitochondrial genome of this order is only available from a single species, *Limulus polyphemus*. In the present study, we analyzed the complete mitochondrial genomes from two Asian horseshoe crabs, *Carcinoscorpius rotundicauda* and *Tachypleus tridentatus* to offer novel data for the evolutionary relationship within Xiphosura and their position in the chelicerate phylogeny. The mitochondrial genomes of *C. rotundicauda* (15,033 bp) and *T. tridentatus* (15,006 bp) encode 13 protein-coding genes, two ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes. Overall sequences and genome structure of two Asian species were highly similar to that of *Limulus polyphemus*, though clear differences among three were found in the stem-loop structure of the putative control region. In the phylogenetic analysis with complete mitochondrial genomes of 43 chelicerate species, *C. rotundicauda* and *T. tridentatus* were recovered as a monophyly, while *L. polyphemus* solely formed an independent clade. Xiphosuran species were placed at the basal root of the tree, and major other chelicerate taxa were clustered in a single monophyly, clearly confirming that horseshoe crabs composed an ancestral taxon among chelicerates. By contrast, the phylogenetic tree without the information of Asian horseshoe crabs did not support monophyletic clustering of other chelicerates. In conclusion, our analyses may provide more robust and reliable perspective on the study of evolutionary history for chelicerates than earlier analyses with a single Atlantic species.

Key words: *Carcinoscorpius rotundicauda*; *Tachypleus tridentatus*; Horseshoe crabs; Xiphosura; Mitochondrial genome; Phylogenetics

Introduction

Horseshoe crabs (Xiphosura, Arthorpoda) are marine chelicerates dwelling shallow waters on sandy or muddy bottoms. The Limulidae, only living family of Xiphosura, contains four horseshoe crab species, *Tachypleus tridentatus* Leach, *Tachypleus gigas* Müller, *Carcinoscorpius rotundicauda* Latreille and *Limulus*

polyphemus Linnaeus [1]. The Atlantic horseshoe crab, *L. polyphemus,* occurs along the eastern coast of North America, while the remaining three species are widely distributed across Asia from Southeast to Northeast [1]. The horseshoe crabs have been dramatically decreased in population sizes and many populations have extirpated throughout much of their ranges in the world, probably due to the global degradation of coastal areas.

Despite its name, horseshoe crabs are rather more closely related to arachnids (subphylum Chelicerata) than to crabs (subphylum Crustacea) [1-3]. They are also referred to as living fossil, since little change has been made in their external morphologies over the last 450 million years [1-3]. Phylogenetic relationship among four living horseshoe crabs remains scientifically unresolved yet. Two *Tachypleus* species have been known to compose a monophyletic assemblage on the basis of morphological evaluation [4]. Amino acid sequencing [5] and interspecific crossing experiments [6] showed rather different results suggesting that *T. tridentatus* should be more closely related to *C. rotundicauta* than to *T. gigas*. However, studies based on different mtDNA regions failed to provide a congruent pattern; for example, *T. gigas* and *C. rotundicauda* were clustered as a monophyly in the combined analyses using 16S ribosomal RNA (16S rRNA) and cytochrome oxidase subunit I (*COI*) [7], while *T. gigas* formed the closest relationship with *T. tridentatus* in the combined analysis using 18S rRNA, 28S rRNA and *COI* [8].

Xiphosura has been considered as an ancestral taxon in chelicerates as well as arthropods [9, 10], and the complete mitochondrial genome of *L. polyphemus* has frequently been used to resolve the phylogenetic relationships among chelicerates [3, 11]. However, additional mitochondrial genomic information of horseshoe crab species is critically required to reconstruct the robust evolutionary relationship among chelicerates as well as arthropods, because the phylogenetic relationship between *L. polyphemus* and the remaining Asian species is still vague. Here, the complete mitochondrial genomes of two Asian horseshoe crabs, *C. rotundicauda* and *T. tridentatus* were analyzed to provide novel data for the relationship among species within the order and the placement of horseshoe crabs in the phylogenetic tree of chelicerates.

Materials and methods

Sampling

Specimens of *Carcinoscorpius rotundicauda* was purchased from a pet shop (http://www.hanqua.co.kr, Republic of Korea). The tissue sample of *Tachypleus tridentatus* for analyses was given from Department of Biology, Daegu University (Daegu, Republic of Korea). The remaining tissue specimens were deposited in the Department of Biology, Teacher's College, Kyungpook National University (voucher numbers, HC-13-01: *C. rotundicauda*; HC-13-02: *T. tridentatus*). Total cellular DNA was extracted using the DNeasy Tissue Kit (QIAGEN Co., Germany) according to the manufacturer's protocol.

Amplification and sequencing

The mitochondrial genomes were amplified using long-range PCR [12] with primer sets shown in Table 1 and the Expand Long Template PCR Kit (Roche Co., Germany). The PCR setting was as followed: [92℃ for 2 min], [92℃ for 30 sec, 52℃ for 30 sec, 68℃ for 9 min] × 14, [92℃ for 10 sec, 52℃ for 30 sec, 68℃ for 9 min (+ 20 sec per cycle)] × 24, [68℃ for 5 min]. PCR reactants were loaded on a 1.0 % agarose gel and stained with ethidium bromide to visualize the bands on ultraviolet transilluminator. The PCR products were purified using the PCR Purification Kit (QIAGEN Co., Germany) and sequenced using the primer-walking strategy with the ABI PRISM BigDye terminator system and the ABI3700 model automatic sequencer (Genotech Co., Korea).

Table 1. Primers used in the long-range PCR for the mitochondrial genome analyses of *Carcinoscorpius rotundicauda* and *Tachypleus tridentatus*.

Segment	Primers	Sequence	Citation
a. *Carcinoscorpius rotundicauda*			
*cox*1 - *rrnL*	HCO2498	5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′	34
	16SB	5′-CCG GTY TGA ACT CAR ATC A-3′	35
rrnL - *cox*1	LCO1490	5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′	34
	16SA	5′-CGC CTG TTT AHC AAA AAC AT-3′	35
b. *Tachypleus tridentatus*			
*cox*1 - *rrnL*	HCO2498	5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′	34
	16SB	5′-CCG GTY TGA ACT CAR ATC A-3′	35
rrnL - *cob*	16SA	5′-CGC CTG TTT AHC AAA AAC AT-3′	35
	COB-F1	5'-CGA GTA ATT CAT GCA AAC GGA GC-3'	13
cob - *cox* 1	Tachy-CytbL	5′-GCA GGA ACA GGA TGA ACA GT-3′	This study
	Tachy-CO1H	5′-GCA GGA ACA GGA TGA ACA GT-3′	This study

Sequence analysis

Thirteen protein-coding genes and two ribosomal RNA genes were identified based on sequence similarity under BLAST searches in the NCBI database. The boundary of each gene was determined by alignments with the sequences of the American horseshoe crab, *L. polyphemus* [13] in the Clustal X 2.1 [14]. The position and secondary structure of tRNA genes were determined by using tRNAscan-SE Search Server [15]. Ribosomal RNA (rRNA) genes were identified based on sequence similarity in BLAST searches. Alignments of protein coding genes were translated into amino acid sequences to confirm whether the amplified domains are functional with no frame-shifting or no premature stop codons. The complete mitochondrial genome sequences were deposited in the NCBI GenBank under the accession number JQ178358 (*C. rotundicauda*) and JQ739210 (*T. tridentatus*). The CG-skew values in both coding and non-coding regions (Table 2) were calculated based on CG – skew = (C – G)/(C+G) [11, 16].

Phylogenetic analysis

The mitochondrial genomes of *C. rotundicauta* and *T. tridentatus* were analyzed in the phylogenetic tree using a total of 41 other chelicerate mitochondrial genomes retrieved from NCBI GenBank (Supplementary Table 1). The nucleic acid sequences of 12 protein-coding genes were aligned using Bio-Edit sequence alignment editor (Ibis Biosciences, USA). The atp*8* gene was excluded from the analyses, because the substitution rate was too high to recover the true phylogeny and too short to provide enough numbers of phylogenetically informative characters. Using the annotated gene boundary information, each protein coding gene was excised from the genomic sequence and put into an individual file. The program EMBOSS Transeq [17] was used to translate the nucleotide sequences into amino acid sequences based on the invertebrate mitochondrial genetic code. CLUSTAL X was finally used to align amino acid sequences of 12 genes. Only well-aligned and conserved alignment sites were extracted from each alignment subset using Gblock ver. 0.91b program [18] with the default setting. The extracted conserved blocks of amino acids were subsequently concatenated into a unified, single large alignment set.

The refined alignment was subjected to two different tree-making algorithms: the maximum likelihood (ML) and Bayesian inference (BI) methods. The best fitting model of sequence evolution was tested by ProtTest ver. 1.3 [19] under Akaike information criterion for the amino acid data. The mtREV [20] + I + Γ was consequently selected as the best fitting model and employed for the phylogenetic reconstruction. The ML analysis was carried out using PHYML v2.4.4 [21]. The bootstrap proportions (BP_{ML}; 1000 replicates) of the ML tree were obtained by the fast-ML method using PHYML. The BI analyses implemented in MrBayes v.3.1 [22] with 1,000,000 generations, four MCMC chains (one hot and three cold) and burn-in step of the first 1,000. Node confidence values of the tree were presented with Bayesian posterior probabilities (BPP).

Results

Genome composition

The mitochondrial genomes of *C. rotundicauda* (15,033 bp) and *T. tridentatus* (15,006 bp) included 13 protein coding genes (*nad1 – 6, nad4L, cox1 – 3, cob, atp6 and atp8*), two ribosomal RNA genes (*rrnL* and *rrnS*), 22 tRNA genes and one large non-coding region (putative control region, CR) (Fig. 1; Table 2). In both genomes, nine protein coding genes (*nad*2, *cox*1, *cox*2, *atp*8, *atp*6, *cox*3, *nad*3, *nad*6, and *cob*) were encoded on the heavy strand along with 13 tRNAs (*trn*I, *trn*M, *trn*W, *trn*K, *trn*D, *trn*G, *trn*A, *trn*R, *trn*N, *trn*S2, *trn*E, *trn*T, and *trn*S2), while the remaining four protein coding genes (*nad*5, *nad4*, *nad4L*, *nad1*) with nine tRNAs (*trn*Q, *trn*C, *trn*Y, *trn*F, *trn*H, *trn*P, *trn*L1, *trn*L2, *and trn*V) and two rRNAs (*rrn*L and *rrn*S) were encoded on the light strand (Fig. 1; Table 2). Overall gene arrangement of both mitochondrial genomes was completely identical to that of *L. polyphemus* [13].

The gene components were rather loosely juxtaposed with 51/23 (*C. rotundicauda*) and 40/24 (*T. tridentatus*) of gap/overlapping nucleotides, considering that of *L. polyphemus* (23/19; Table 2) [13]. Although the overall A + T contents of 73.8 % in *C. rotundicauda* and 74.0 % in *T. tridentatus* were relatively higher than that of *L. polyphemus* (67.57 %), those values are within the range (60.2 – 80.4 %) of chelicerates (Supplementary Table 1). The overall pattern of nucleotide skew was highly similar among three mitochondrial genomes including that of *L. polyphemus*, with only an exception found on the putative control region (Table 3).

Protein coding genes

The inferred start/stop codons for protein coding genes of both *C. rotundicauda* and *T. tridentatus* are listed in Table 2. All of the protein coding genes in both mitochondrial genomes were initiated by ATN, with the exceptions in *cox*1, nad1 and *nad5* (Table 2). The open reading frame of *cox1* in both Asian horseshoe crabs started with TTA, while that of *nad1* and *nad5* started with TTG. The canonical stop codon (TAA or TAG) occurs in seven protein coding genes

(*nad1, nad2, cox1, atp6, nad3, nad4 and nad6*; Table 2), while the remaining six (*nad5, nad4L, cob, cox2, cox3* and *atp8*) had incomplete T- or TA- stop codons (Table 2). The codon usage pattern of the 13 protein coding genes is provided in Table 4. A + T rich codons, such as Leu (UUA), Ile (AUU) and Phe (UUU), are frequent in both *C. rotundicauda* and *T. tridentatus* (Table 4).

Transfer and ribosomal RNA genes

A total of 22 tRNA genes ranging from 62 to 73 bp in length were identified in mitochondrial genomes of both *C. rotundicauda* and *T. tridentatus*. The putative secondary structure for each tRNA gene could be predicted based on the sequences (Fig. 2). The sequences, anticodon nucleotides, and secondary structures of tRNA genes from both *C. rotundicauda* and *T. tridentatus* were very similar to those observed in *L. polyphemus* [13]. All of the tRNA genes were capable of forming typical cloverleaf secondary structures (dihydrouridine, DHU), with an exception of trn*S1*; trn*S1* lacked DHU arm in *T. tridentatus* (Fig. 2B) and had a shortened stem (2 bp) in *C. rotundicauda* (Fig. 2A). Two rRNA genes (*rrnS* and *rrnL*) were encoded on the light strand and were separated by a *trn*V in both mitochondrial genomes analyzed. The sizes of *rrnS* and *rrnL* were estimated to be 816 bp (*rrnS*) and 1,301 bp (*rrnL*) for *C. rotundicauda* and 800 bp (*rrnS*) and 1,294 bp for *T. tridentatus*.

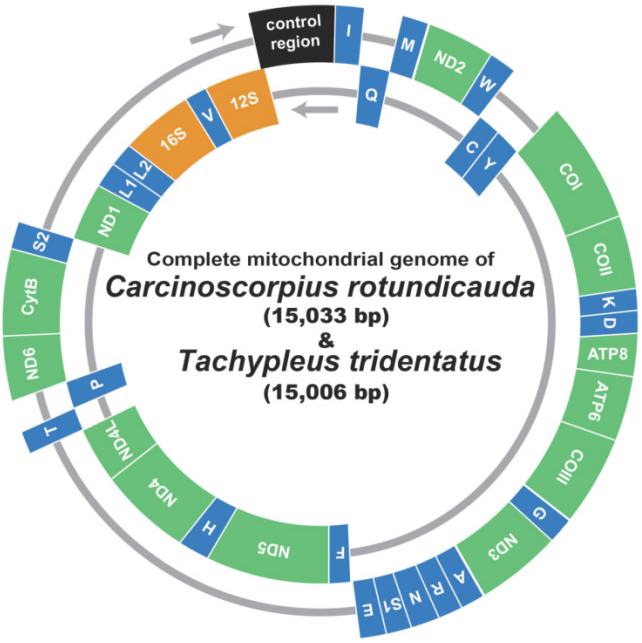


Fig. 1 Gene map of the mitochondrial genome of Asian horseshoe crabs, *Carcinoscorpius rotundicauda* and *Tachypleus tridentatus*. Thirteen protein-coding genes and 2 ribosomal RNA genes are abbreviated as follows: *nad1-6* and *nad4L*, NADH dehydrogenase subunits 1-6 and 4L; *cox 1-3*, cytochrome C oxidase subunits I-III; *cob*, cytochrome b apoenzyme; *atp 6* and *8*, ATPase subunits 6 and 8. The 22 transfer RNA genes are identified by the IUPAC amino acid single-letter codes.

Table 2. The mitochondrial genome profile of two Asian horseshoe crabs, *Carcinoscorpius rotundicauda* and *Tachypleus tridentatus*

Gene	Strand	***Carcinoscorpius rotundicauda*** (15,033 bp)						***Tachypleus tridentatus*** **I** (15,006 bp)					
		position		Size	Codon		Intergenic bp*	position		Size	Codon		Intergenic bp*
		From	to	bp	Start	Stop		From	to	bp	Start	Stop	
*trn*I	H	1	67	67			-2	1	67	67			-2
*trn*Q	L	66	132	67			-2	66	132	67			-2
*trn*M	H	131	199	69			0	131	199	69			0
nad 2	H	200	1219	1020	ATT	TAA	-2	200	1219	1020	ATT	TAA	-2
*trn*W	H	1218	1286	69			-1	1218	1286	69			1
*trn*C	L	1286	1350	65			0	1288	1351	64			0
*trn*Y	L	1351	1415	65			-5	1352	1415	64			-5
cox 1	H	1411	2946	1536	TTA	TAA	3	1411	2946	1536	TTA	TAA	3
cox 2	H	2950	3633	684	ATG	T_ _	0	2950	3633	684	ATG	TAA	1
*trn*K	H	3635	3705	71			0	3635	3705	71			-1
*trn*D	H	3706	3769	64			0	3705	3767	63			0
atp 8	H	3770	3923	154	ATT	T_ _	-5	3768	3923	156	ATT	TAA	-7
atp 6	H	3919	4593	675	ATG	TAA	-1	3917	4591	675	ATG	TAA	-1
cox 3	H	4593	5376	784	ATG	T_ _	0	4591	5374	784	ATG	T_ _	0
*trn*G	H	5377	5442	66			0	5375	5438	64			0
nad 3	H	5443	5787	345	ATA	TAA	6	5439	5783	345	ATT	TAA	6
*trn*A	H	5794	5863	70			0	5790	5857	68			0
*trn*R	H	5864	5925	62			-1	5858	5919	62			-1
*trn*N	H	5925	5994	70			-1	5919	5988	70			-1
*trn*S2	H	5994	6059	66			-1	5988	6054	67			-1
*trn*E	H	6059	6121	63			6	6054	6117	64			11
*trn*F	L	6128	6192	65			0	6129	6192	64			0
nad 5	L	6193	7906	1714	TTG	T_ _	0	6193	7906	1714	TTG	T_ _	0
*trn*H	L	7907	7970	64			1	7907	7970	64			1

Gene	Strand	***Carcinoscorpius rotundicauda*** (15,033 bp)						***Tachypleus tridentatus* I** (15,006 bp)					
		position		Size	Codon		Intergenic	position		Size	Codon		Intergenic
		From	to	bp	Start	Stop	bp*	From	to	bp	Start	Stop	bp*
nad 4	L	7972	9309	1338	ATG	TAG	4	7972	9315	1344	ATT	TAA	1
nad 4L	L	9303	9602	300	ATG	T_ _	2	9317	9602	286	ATG	T_ _	2
*trn*T	H	9605	9668	64			1	9605	9669	65			1
*trn*P	L	9670	9735	66			3	9671	9735	65			3
nad 6	H	9739	10200	462	ATT	TAA	-1	9739	10200	462	ATC	TAA	-1
cob	H	10200	11328	1129	ATG	T_ _	0	10200	11328	1129	ATG	T_ _	0
*trn*S1	H	11329	11396	68			24	11329	11397	69			9
*nad*1	L	11421	12356	936	TTG	TAA	0	11407	12339	933	TTG	TAA	0
*trn*L1	L	12354	12419	66			1	12340	12405	66			1
*trn*L2	L	12421	12487	67			0	12407	12473	67			0
*rrn*L	L	12488	13787	1300			0	12474	13767	1294			0
trnV	L	13788	13857	70			0	13768	13837	70			0
*rrn*S	L	13858	14673	816			0	13838	14637	800			0
CR		14674	15033	360			0	14638	15006	369			0

*Intergenic bp indicates gap nucleotides (positive value) or overlapped nucleotides (negative value) between two adjacent genes

Table 3. AT/CG skews in the mitochondrial protein coding genes (PCG), 2 rRNA genes, CR and the entire mitochondrial genome from three horseshoe crabs, *Carcinoscorpius rotundicauda, Tachypleus tridentatus*, and *Limulus polyphemus*

Gene	AT-skew			CG-skew		
	C. rotundicauda	T. tridentatus	L. polyphemus	C. rotundicauda	T. tridentatus	L. polyphemus
cox1	-0.065	-0.089	0.044	0.153	0.146	0.210
cox2	-0.002	-0.035	0.036	0.305	0.281	0.331
cox3	-0.130	-0.141	-0.073	0.230	0.183	0.333
atp8	-0.108	-0.088	0.097	0.750	0.548	0.721
atp6	-0.107	-0.088	-0.029	0.456	0.398	0.526
nad1	-0.243	-0.200	-0.388	-0.438	-0.414	-0.511
nad2	-0.140	-0.143	-0.052	0.496	0.441	0.570
nad3	-0.183	-0.254	-0.042	0.462	0.341	0.524
nad4	-0.257	-0.251	-0.374	-0.432	-0.393	-0.485
nad4L	-0.162	-0.139	-0.217	-0.697	-0.657	-0.636
nad5	-0.269	-0.250	-0.319	-0.401	-0.348	-0.396
nad6	-0.119	-0.070	-0.006	0.609	0.612	0.629
cob	-0.113	-0.159	-0.081	0.317	0.306	0.427
rrnL	0.044	0.030	0.148	0.456	0.429	0.447
rrnS	0.088	0.104	0.145	0.352	0.333	0.331
CR	-0.026	-0.023	-0.046	-0.111	-0.115	0.077
13 PCG	-0.162	-0.163	-0.160	0.046	0.042	0.088
overall	0.036	0.024	0.111	0.346	0.315	0.399

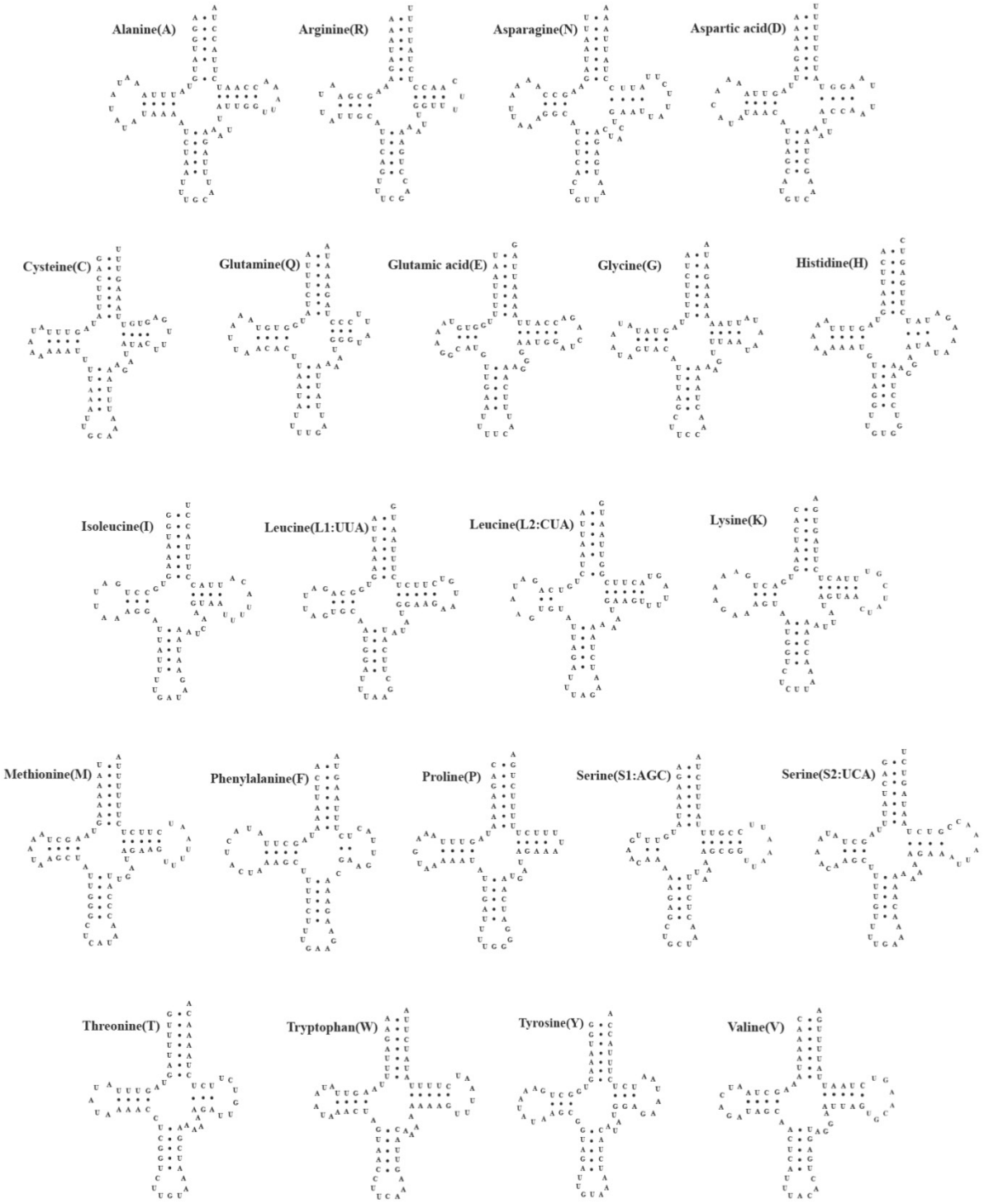
Table 4. Codon usage pattern of the 13 mitochondrial protein-coding genes from three horseshoe crab species, *Carcinoscorpius rotundicauda, Tachypleus tridentatus*, and *Limulus polyphemus*

Amino acids	Codon	No.			Amino acids	Codon	No.			Amino acids	Codon	No.		
		C	T	L			C	T	L			C	T	L
Nonpolar					Phe	UUC	46	66	85	Ser	AGA	75	83	66
Ala	GCA	48	45	55		UUU	333	310	246		AGC	5	**4**	8
	GCC	27	**16**	39	Trp	UGA	101	102	94		AGG	**2**	7	12
	GCG	4	**9**	6		UGG	10	**6**	17		AGU	24	21	22
	GCU	73	84	76							UCA	116	114	98
Ile	AUC	**43**	**42**	107	**Polar**						UCC	49	**31**	65
	AUU	346	335	241	Asn	AAU	126	139	103		UCG	**2**	**3**	6
Leu	CUA	**41**	**42**	94		AAC	35	**20**	44		UCU	106	122	110
	CUC	**17**	**18**	55	Cys	UGC	7	7	9	**Acidic**				
	CUG	**2**	**1**	4		UGU	43	47	40	Asp	GAC	14	**11**	24
	CUU	**85**	**80**	106	Gln	CAA	59	63	52		GAU	48	51	36
	UUA	**348**	**363**	226		CAG	10	**3**	15	Glu	GAA	68	69	64
	UUG	44	**38**	84	Gly	GGA	110	119	109		GAG	14	14	23
Met	AUA	215	229	171		GGC	**8**	11	21	**Basic**				
	AUG	40	23	43		GGG	**32**	**28**	66	Arg	CGA	32	35	34
Pro	CCA	60	64	52		GGU	**67**	59	41		CGC	**3**	**3**	7
	CCC	14	**8**	27	Thr	ACA	78	77	84		CGG	4	**2**	7

Amino acids	Codon	No.			Amino acids	Codon	No.			Amino acids	Codon	No.		
		C	T	L			C	T	L			C	T	L
	CCG	**2**	**2**	4		ACC	29	31	40		CGU	20	20	14
	CCU	60	68	69		ACG	**1**	**0**	2	His	CAC	**19**	**13**	41
Val	GUA	76	62	82		ACU	59	65	53		CAU	56	64	37
	GUC	**6**	**5**	14	Tyr	UAC	26	20	34	Lys	AAA	85	84	66
	GUG	11	19	36		UAU	105	108	87		AAG	16	**8**	19
	GUU	73	86	90										

Bold numbers indicate strong differences (+/-25%) to *Limulus polyphemus*.

(A)



(B)

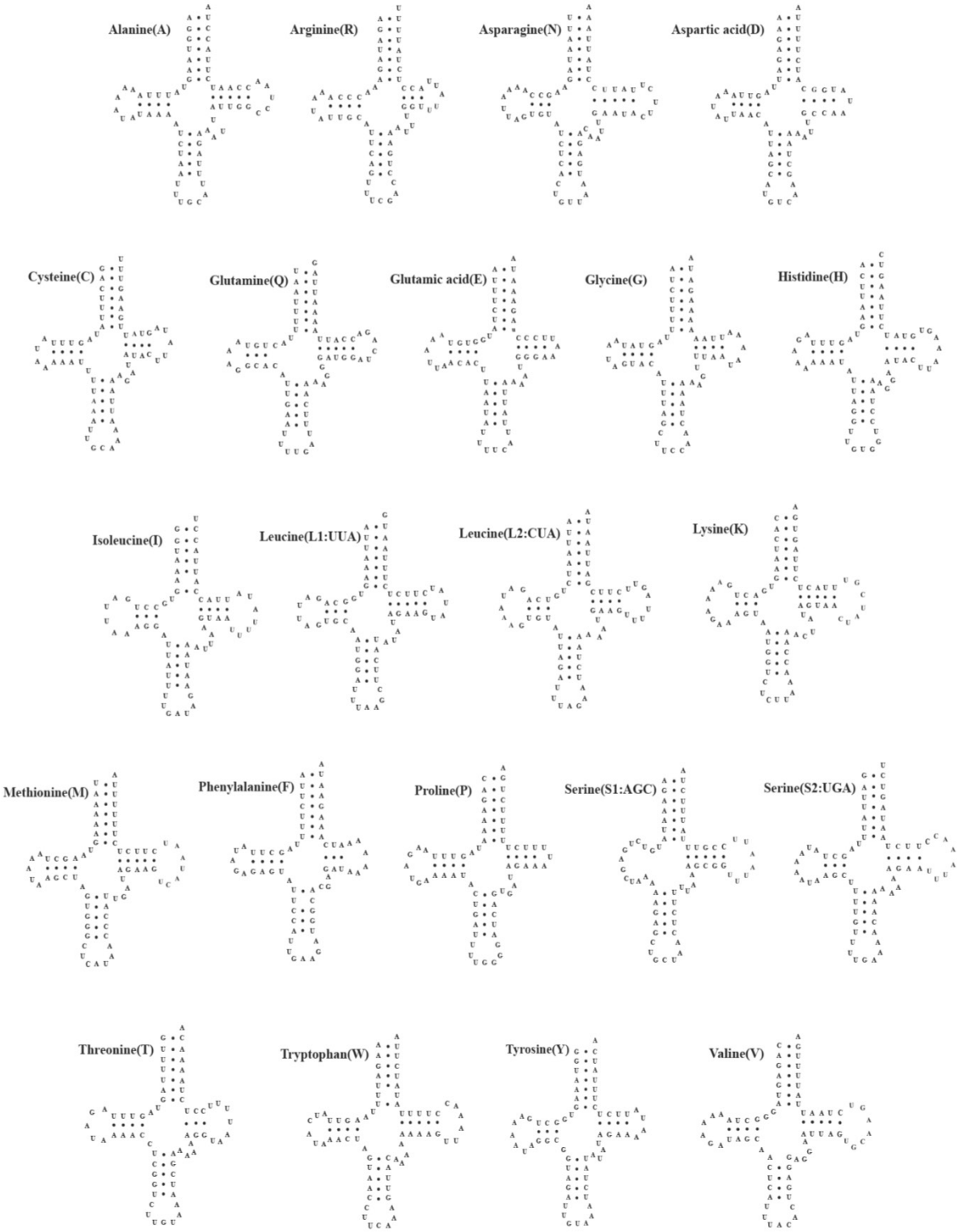


Fig. 2 Putative tRNA secondary structures predicted from the 22 tRNA gene sequences found in the (A) *Carcinoscorpius rotundicauda* and (B) *Tachypleus tridentatus* mitochondrial genome.

Non-coding regions

The major non-coding regions (putative control region, CR) of 360 bp (*C. rotundicauda*) and 369 bp (*T. tridentatus*) were found between *rrn*S and *trn*Q (Table 2). The A + T contents in this region were higher, with 85% and 83.47% in *C. rotundicauda* and *T. tridentatus*, respectively, compared to other regions of the mitochondrial genomes (supplementary Table 1). The sequence of this region is highly conservative among xiphosuran species (Fig. 3A). The stem-loop structure found in *C. rotundicauda* (Fig. 3B) was composed of the 13 bp stem and 11 bp loop. However, the structure of *T. tridentatus* was rather complicated with the 10 bp stem and 13 bp loop followed by the alternative 8 bp stem and 12 bp loop (Fig. 3C). The mitochondrial genome of *L. polyphemus* had such an alternative structure [13], although it does not seem to be stable to build the complete stem-loop structure (Fig. 3D). A small (24 bp) non-coding fragment was found between the *trn*S (UGA) and *nad1* gene in *C. rotundicauda* (Fig. 3E and Table 2. *T. tridentatus* and *L. polyphemus* had just a short (9 bp) and highly conserved non-coding fragment (TTTCTAAA) in this region.

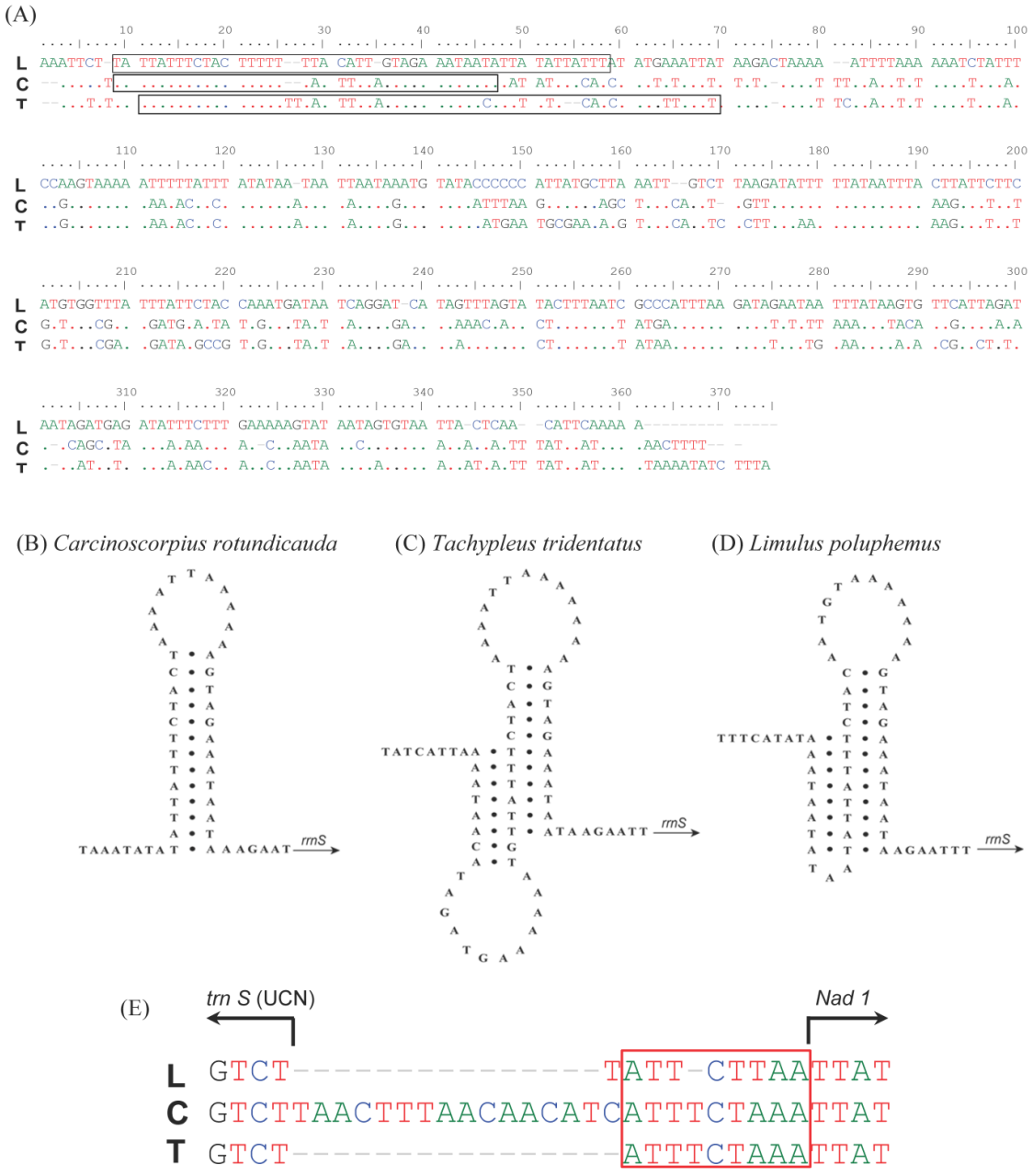


Fig. 3 Comparison in the primary and secondary structures of non-coding regions found in the mitochondrial genomes of *Carcinoscorpius rotundicauda*, *Tachypleus tridentatus* and *Limulus polyphemus*. The CR pair-wise alignment of the three xiphosuran species (A): the dots indicate nts identical to those of the first line in the alignment. Hairpin-like structure (60 bp and 57 bp) from the control region observed in *C. rotundicauda* (B), *T. tridentatus* (C), and *L. polyphemus* (D). An alignment of the gap region between *trn*S1 (UCN) and *nad1* in three xiphosuran species (E): the box indicates a conserved region across three horseshoe crabs. L; *L. polyphemus*, C; *C. rotundicauda*, T; *T. tridentatus*.

Phylogenetic analyses

The phylogenetic trees reconstructed from ML and BI algorithms were completely identical in clustering pattern and overall topology (Fig. 4). *C. rotundicauda* and *T. tridentatus* were recovered as a monophyly (BP_{ML} = 100, BPP = 1.00), whereas *L. polyphemus* solely formed a separate clade from two Asian species (BP_{ML} = 100, BPP = 1.00). Horseshoe crabs were placed as a sister relationship to the Arachnids by strong node-supporting values (Fig. 4A). The tree also supported the monophyletic clusterings of 'Opiliones + Scorpions' (BP_{ML} = 63, BPP = 0.83), 'Uropigy + Amblypygi' (BP_{ML} = 62, BPP = 1.0), Acari (Parasotoformes; BP_{ML} = 62, BPP = 1.00), 'Richinulei + Araneae' (BP_{ML} = 36, BPP = 0.84), Pantopoda (BP_{ML} = 60, BPP = 0.97) and 'Pseudoscorpiones + Acarifomes' (BP_{ML} = 94, BPP = 1.00; Fig. 4A). The monophyletic grouping of 'Opiliones + Scorpions' and 'Uropygi + Amblypygi' are compatible with the result from several previous analyses conducted based on morphological characters [23-25]. When the Asian horseshoe crabs were not considered in the tree, however, the tree was not resolved to support the monophyly of Arachnida, with Solifugae being independently placed at the basal root (Fig. 4B). In addition, such tree did not support the clustering of 'Opiliones + Scorpions' and 'Uropygi + Amblypygi' (Fig. 4B).

Discussion

The present study was designed to provide critical mitochondrial genome information of *C. rotundicauda* and *T. tridentatus* necessary for the concrete inference of phylogenetic relationships among species within Xiphosura and their placement within Chelicerata as well as Arthropoda. The overall architecture of both mitochondrial genomes is highly consistent with those previously reported in the Atlantic horseshoe crab, *L. polyphemus*. Those three mitochondrial genomes share the identical genome content (13 protein coding genes, 2 ribosomal RNA genes, 22 tRNA genes and a single large control region) and overall gene arrangement, as typically shown in many metazoan mitochondrial genomes [13, 26-28]. The mitochondrial genomes of *C. rotundicauda* and *T. tridentatus* are slightly larger than that of *L. polyphemus*, due to more frequent occurrence of gap nucleotides between genes.

In two Asian horseshoe crabs, ten of the protein coding genes (*atp6, atp8, nad2, nad3, nad4. nad6, nad4L, cob, cox2* and *cox3*) have either Met or Ile as the start codon (ATA, ATG or ATT), while the remaining three (*cox1, nad1,* and *nad5*) start with Leu (TTA or TTG). Such start codon pattern was frequently found in a few other chelicerates [25, 26, 29, 30] as well as in several arthropods [31, 32]. Six (*nad5, nad4L, cob, cox2, cox3* and *atp8*) protein coding genes have incomplete stop codon. Such truncated stop codons are commonly observed among many arthropod mitochondrial genomes [30-32] and are expected to be converted into a fully functional TAA via post-translational polyadenylation [29].

The sequences, anticodon nucleotides and secondary structures of tRNA genes are highly consistent with those previously reported in the Atlantic horseshoe crab. Most of the tRNA genes form dihydrouridine (DHU) arm. However, such structure is not found in *trn*S1 of *T. tridentatus*, and incomplete conformation with shortened stem of 2 bp is observed in *C. rotundicauda*. Missing of complete DHU arm in *trn*S1 has been reported among a number of metazoan mitochondrial genomes [29-32].

The A + T rich non-coding regions (putative control regions) were found in both two Asian horseshoe crab mitochondrial genomes. The sequence of this region is highly conservative among xiphosuran species as well as among a variety of arthropods [33]. However, the stem-loop structure is highly variable among three xiphosuran species. An interesting feature of stem-loop structure in *T. tridentatus* is its ability to form an alternative secondary structure that appears to be complete and stable. Although the mitochondrial genome of *L. polyphemus* has an additional 8 nucleotides complementary to the main stem, it does not appear to be stable. It is also surprising that there is a small non-coding fragment (24 bp) between *trn*S and *nad1* in *C. rotundicauda*.

In the phylogenetic analysis among 43 chelicerates, *C. rotundicauda* and *T. tridentatus* were recovered as a monophyly, whereas *L. polyphemus* solely forms a separate clade from two Asian species. Such result supports previous findings showing clear genetic differentiation between *L. polyphemus* and Asian species [7, 8]. Major other chelicerate taxa were clustered in a single monophyletic assemblage, three xiphosuran species being placed at the basal root, suggesting that horseshoe crabs should be considered as an ancestral taxon in arthropods as well as in chelicerates. The phylogenetic tree without the information of Asian horseshoe crabs, by contrast, was not resolved to support monophyletic clustering of chelicerates, since solifuges formed a separate cluster. In conclusion, additional information of Xiphosuran mitochondrial genomes provide more robust and reliable perspective on the evolutionary history of chelicerate as well as arthropods.

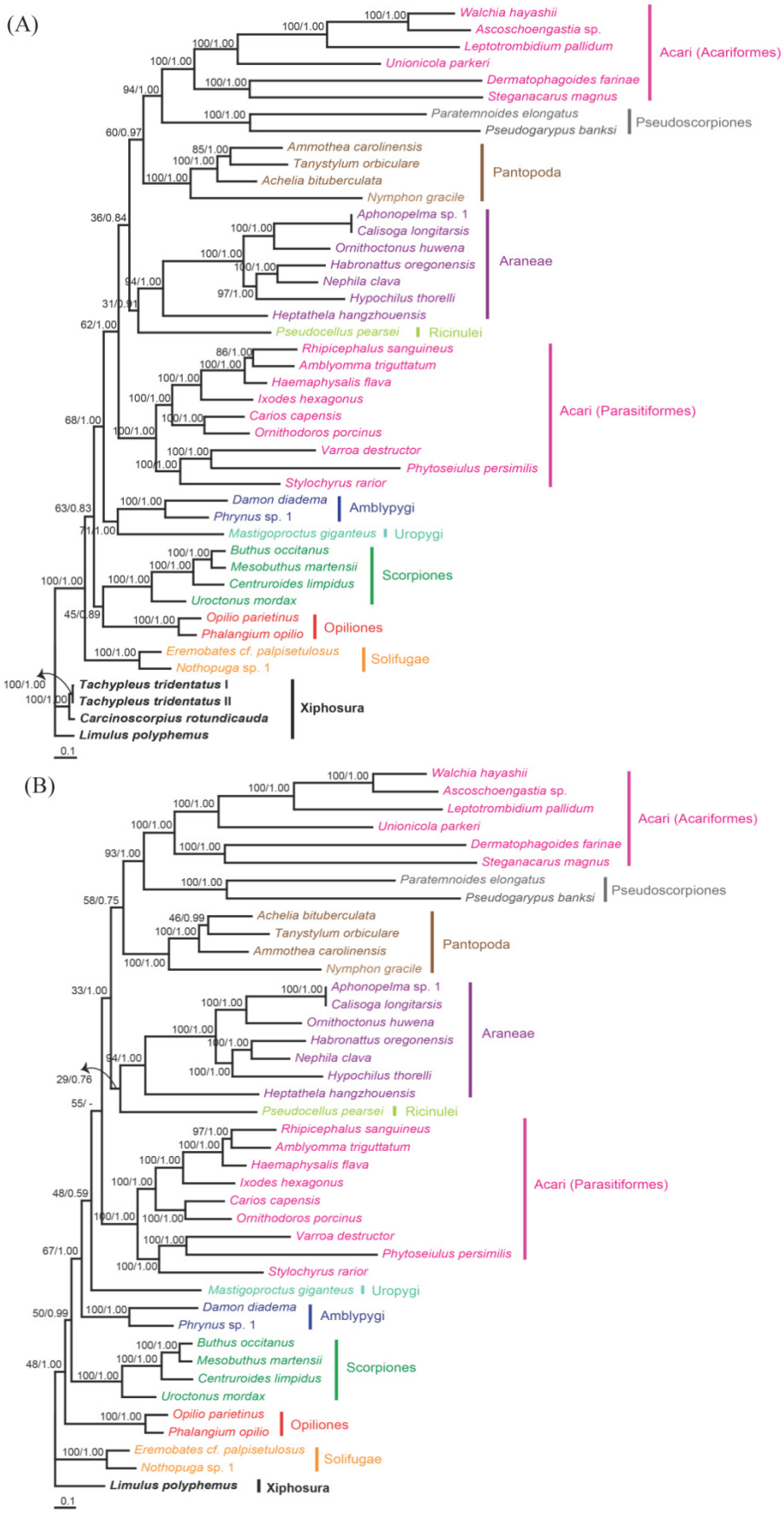


Fig. 4. Maximum likelihood phylogenetic trees of chelicerates based on the amino acid sequences of concatenated 12 mitochondrial protein coding genes. Tree was reconstructed either (A) with three horseshoe species (*C. rotundicauda*, *T. tridentatus* and *L. polyphemus*) or (B) with only *L. polyphemus*. Numbers at the branch indicate the percentages from ML bootstrapping (left) and Bayesian posterior probabilities (right).

Supplementary Material

Supplementary Table 1.
http://www.ijbs.com/v10p0479s1.pdf

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Competing Interests

The authors have declared that no competing interest exists.

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