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Molecular phylogeny of extant horseshoe crabs (Xiphosura, Limulidae) indicates Paleogene diversification of Asian species

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ABSTRACT

Horseshoe crabs are marine invertebrates well known for their exceptionally low rates of diversification during their entire evolutionary history. Despite the low species diversity in the group, the phylogenetic relationships among the extant species, especially among the three Asian species are still unresolved. Here we apply a new set of molecular genetic data in combination with a wide geographic sampling of the intra-specific diversity to reinvestigate the evolutionary history among the four living limulid xiphosurans. Our analysis of the intraspecific diversity reveals low levels of connectivity among *Carcinoscorpius rotundicauda* lineages, which can be explained by the estuarine-bound ecology of this species. Moreover, a clear genetic break across the Thai–Malay Peninsula suggests the presence of cryptic species in *C. rotundicauda*. The limulid phylogeny finds strong support for a monophyletic genus *Tachypleus* and a diversification of the three Asian species during the Paleogene period, with speciation events well separated in time by several million years. The tree topology suggests that the three Asian species originated in central South East Asia from a marine stem group that inhabited the shallow coastal waters between the Andaman Sea, Vietnam, and Borneo. In this region *C. rotundicauda* probably separated from the Tachypleus stem group by invading estuarine habitats, while *Tachypleus tridentatus* most likely migrated northeast along the Southern coast of China and towards Japan.

1. Introduction

Horseshoe crabs are marine chelicerates closely related to arachnids (Masta et al., 2009; Wheeler and Hayashi, 1998). There are four extant species distributed across the coastal zones of the continental shelfs in North America and South East Asia (Sekiguchi and Shuster, 2009). The American horseshoe crab *Limulus polyphemus* (Linnaeus, 1758) has a distribution along the eastern coast of North America and a genetically and geographically isolated population on the northern east coast of the Yucatán Peninsula in Mexico (Anderson and Shuster, 2003; Faurby et al., 2010; Sekiguchi, 1988). The three remaining species *Tachypleus gigas* (Müller, 1785), *Tachypleus tridentatus* (Leach, 1819), and *Carcinoscorpius rotundicauda* (Latreille, 1802) inhabit the shallow waters in the Indo-Pacific. In this area two species, *Tachypleus gigas* and *C. rotundicauda* have a largely overlapping distribution ranging from the Andaman Sea to the South China Sea, but inhabit different habitats (Sekiguchi, 1988). While *T. gigas* lives in sandy and shallow near-coast habitats, *C. rotundicauda* mostly inhabits estuaries and mangroves. The third Asian species *T. tridentatus* lives in shallow coastal zones of Southern China from Vietnam to Japan (Sekiguchi, 1988).

Horseshoe crabs have puzzled evolutionary biologists for centuries and are often used as prime example for organisms that survived long time periods without any significant changes in their anatomy, earning them the name of 'living fossils' (Eldredge and Stanley, 1984) and 'phylogenetic relicts' (Selander et al., 1970). In the fossil record ancient horseshoe crabs are already known from the Ordovician period (Rudkin et al., 2008; Van Roy et al., 2010), and modern forms which are indistinguishable from recent species appear during Upper Jurassic (Sekiguchi and Sugita, 1980; Briggs et al., 2005). It seems like these forms maintained a static morphology for at least 150 million years (Fisher, 1984). This lack of morphological disparity over time is accompanied by low levels of species diversity throughout the entire evolutionary history of horseshoe crabs (Störmer, 1952, 1955). Even at the peak of their diversification during Carboniferous the group consisted of not more than a few dozen documented species (Anderson and Selden, 1997). Following the Permian–Triassic extinction the diversity of the group was greatly reduced and left behind the only recent group of horseshoe crabs, the Limulidae Zittel, 1885.

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The anatomical similarity among living horseshoe crabs has greatly impaired the elucidation of the phylogenetic relationships among the recent lineages. Although a wide range of non-morphological data has been studied to date (see Avise et al., 1994), the only consensus reached so far is that the Atlantic species, *L. polyphemus*, is a sister taxon to the three Indo-Pacific species. The phylogenetic relationship among the three Indo-Pacific species, however, remains unresolved. Cladistic approaches including fossil evidence resulted in low support for a relationship between *Tachypleus gigas* with *T. tridentatus* (Fisher, 1984; Shuster and Anderson, 2003). In contrast to this an amino acid analysis by Shishikura et al. (1982) suggested *C. rotundicauda* and *T. tridentatus* to be closely related, while *C. rotundicauda* and *T. gigas* were suggested as sister-groups in an analysis of mitochondrial and nuclear genes in the absence of any support measures (Xia, 2000). Other approaches such as interspecific hybridization experiments (Sekiguchi and Sugita, 1980), two-dimensional electrophoresis (Miyazaki et al., 1987) and analyses of two mitochondrial genes (Avise et al., 1994) yielded unresolved genealogies for the Asian species. Such an array of conflicting results has led some authors to the conclusion that the three species constitute a phylogenetically unresolvable trichotomy, resulting from a cladogenetic process in which all three Indo-Pacific species formed within a short geological time (Avise et al., 1994).

In order to solve the controversy about the origin and genealogy of the Asian horseshoe crabs we re-examined the phylogenetic relationships among the recent species with new molecular genetic data. In contrast to previous analyses we maximized the sampling effort by including individuals from geographically distant populations in order to account for the within species diversity and minimize analytical artifacts such as long branch effects.

2. Materials and methods

2.1. Sampling, DNA extraction, amplification, and sequencing

All specimens were collected either by hand or caught in net by local fishermen in their natural coastal environments, and then identified, sexed, photographed, measured, and weighed. 0.2–0.5 ml blood was taken with a sterile syringe and stored in 95% ethanol (Table 1). Total genomic DNA was extracted using the EZNA kit (Omega Bio-Tek) following the instructions of the supplier. Two fragments of nuclear genes from the small and large ribosomal subunit (18S rDNA and 28S rDNA) as well as one fragment of the mitochondrial gene cytochrome *c* oxidase I (COI) were amplified using the primer pairs 18S: 1F-4R (alternatively: 1F-5R); 3F-18Sbi; 18Sa2-9R; 28S: 28SD1F-28Sb (alternatively 28SD1F-28SrD4b); COI: LCO1 1490-HCO1 2198 (Folmer et al., 1994; Giribet et al., 1996; Park and Ó Foighil, 2000; Whiting et al., 1997). Amplification was carried out with PuReTaq PCR beads (Amersham Biosciences) according to the supplier instructions. The PCR-programs were performed in a Thermal Cycler 2720 (Applied Biosystems) (18S: 94 °C/2 min; 35× (94 °C/45 s, 49 °C/45 s, 72 °C/1 min), 72 °C/6 min; 28S: 95 °C/5 min, 35× (95 °C/40 s, 52 °C/40 s, 72 °C/1 min), 72 °C/8 min; COI: 95 °C/5 min, 35× (95 °C/40 s, 45 °C/45 s, 72 °C/1 min), 72 °C/8 min). All PCR products were tested for the presence of amplified products on agarose gels. PCR-amplified samples were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced by Macrogen (Geumcheon-Gu, Seoul, Korea). All chromatograms obtained from the automated sequencer were read and sequence contigs were assembled using Geneious Pro 3.7.0 (Biomatters, Auckland, New Zealand). Sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/) with the accession numbers given in Table 1.

2.2. Alignment and analysis

All sequences were blasted in GenBank, checked for contaminations, and if applicable translated to amino acid sequences. Overlapping sequence fragments were merged into consensus sequences and subsequently aligned and edited using Geneious Pro. Since all alignments appeared reliable, all sequence information was included in the analysis. Phylogenetic analyses were performed with two different methods, maximum likelihood (ML) and Bayesian inference (BI). ML tree estimation was done using RAxML-VI v.7.0.0. (Stamatakis, 2006; Stamatakis et al., 2008). BI

Table 1
Overview over species, individuals, geographic location, and accession numbers used in this study, F – female, M – male.

Species	Code	Region	Locality	GPS data	Sample Date	Gender	Accession no. 18S	Accession no. 28S	Accession no. COI
Acari									
Boophilus sp.	–	–	–	–	–	–	AF018656	–	AY008680
Cyphophtalami									
Siro rubens	–	–	–	–	–	–	AY428818	AY428826	AY428842
Stylocellus sp.	–	–	–	–	–	–	DQ825536	DQ825569	DQ825634
Araneae									
Acanthoscurria sp.	–	–	–	–	–	–	DQ639775	DQ639859	–
Xiphosura									
Limulus polyphemus	LP_W1-1F	New England coast	Woods Hole, MA, USA	41°23′31.36″N 71°0′52.83″W	1/4/2007	F	HQ588742	HQ588729	HQ588751
	LP_F1-1F	Florida East coast	Indian River, FL, USA	27°50′20.10″N 80°28′19.80″W	7/4/2007	F	HQ588740	HQ588731	HQ588753
	LP_M2-1F	Yucatán Peninsula	Río Lagartos, Mexico	21°35′47.19″N 88° 9′52.15″W	22/03/2007	F	HQ588741	HQ588728	HQ588747
Tachypleus tridentatus	TT_K002B1	Eastern China Sea	Shanghai, China	30° 0′37.37″N 122°23′2.87″E	15/10/2006	M	HQ588745	HQ588735	HQ588752
Tachypleus gigas	TG_T1-5F	Andaman Sea	Phuket, Thailand	7°48′58.95″N 98°32′49.66″E	3/12/2006	F	HQ588743	HQ588734	HQ588748
	TG_V1-2	South China Sea	Bac Lieu, Vietnam	9°22′37.30″N 106°13′43.70″E	16/05/2006	F	HQ588744	HQ588736	HQ588749
Carcinoscorpius rotundicauda	CR_T5-3F	Andaman Sea	Phuket, Thailand	7°56′53.74″N 98°29′43.31″E	09/12/2006	F	HQ588738	HQ588732	HQ588754
	CR_T3-2F	Gulf of Thailand	Bang Pu, Thailand	6°54′43.28″N 101°16′32.49″E	05-12-2006	F	HQ588737	HQ588733	HQ588750
	CR_V1-3	South China Sea	Bac Lieu, Vietnam	9°22′37.30″N 106°13′43.70″E	16-05-2006	F	HQ588739	HQ588730	HQ588746

Table 2
Overall molecular diversity within the Limulidae. The number of substitutions and percentages are given for each partition and combined sets within.

Marker	18S	%	28S	%	COI	%	NUC	%	MOL	%
Length (bp)	1768		1090		540		2858		3398	
Indels	No		Yes		No		Yes		Yes	
Variable sites	16	0.9	116	10.6	141	26.1	132	4.6	273	8.0
Parsimony informative sites	15	0.8	104	9.5	120	22.2	119	4.2	239	7.0

Table 3
Intraspecific variation as shown by the pairwise comparison of individuals across the sampled distribution area.

Species	Sample range	Genetic distance		
		18S	28S	COI
C. rotundicauda	Phuket (Thailand) vs. Bac Lieu (Vietnam)	0.0012	0.0038	0.0342
T. gigas	Phuket (Thailand) vs. Bac Lieu (Vietnam)	0.0000	0.0019	0.0074
L. polyphemus	Woods Hole (USA) vs. Río Lagartos river (Mexico)	0.0000	0.0009	0.0149

Table 4
(A) Median of mean and standard deviation parameters from the uncorrelated relaxed clock estimations in the three different BEAST analyses. (B) Divergence estimates for evolutionary radiation of Asian horseshoe crabs through time as inferred from three different trees, using either one or two terminals for *C. rotundicauda*. Values indicate diversification times in Mya with 95% confidence levels in parentheses.

		Four terminals (incl. *C. rotundicauda* Andaman Sea)	Five terminals (incl. *C. rotundicauda* Andaman Sea and Gulf of Thailand)	Five terminals (*C. rotundicauda* Andaman Sea and South China Sea)
A. Partition				
18S	Mean rate	3.4×10^{-5}	3.4×10^{-5}	3.4×10^{-5}
	Stdev	0.23	0.21	0.21
28S	Mean rate	4.6×10^{-4}	4.4×10^{-4}	4.3×10^{-4}
	Stdev	0.18	0.32	0.20
COI-1	Mean rate	4.4×10^{-4}	6.0×10^{-4}	4.8×10^{-4}
	Stdev	0.30	0.41	0.27
COI-2	Mean rate	7.3×10^{-5}	6.7×10^{-5}	6.7×10^{-5}
	Stdev	0.30	0.28	0.28
COI-3	Mean rate	7.6×10^{-3}	7.0×10^{-3}	7.0×10^{-3}
	Stdev	0.45	0.36	0.35
B. Taxon				
Limulidae		135 (114–154)	135 (115–155)	135 (115–155)
Tachypleinae		45 (30–68)	47 (31–67)	47 (32–65)
Tachypleus		24 (14–37)	26 (14–40)	25 (15–37)
Carcinoscorpius		–	7.8 (3.2–15)	8.3 (4.0–14)

analyses were performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Gaps and N's were treated as missing data. The ML analysis consisted of 100 independent runs on the unpartitioned alignment using RAxML under the GTRMIX substitution model (re-estimated all free model parameters) with estimated rearrangement settings, a rate category value of 25 and randomized MP (maximum parsimony) starting trees. This model implies an initial tree inference under the GTRCAT model and thereafter evaluates the final tree topology under the GTRGAMMA model until the likelihood values are stable. Branch support was assessed with 1000 non-parametric bootstrap replicates under the GTRMIX model (random number seed = 12,444) and plotted on the ML tree with the best likelihood value. For the BI analyses, MrModeltest v.2 (Nylander, 2004) was used for search of best fit models for the partitioned datasets; positions 2 and 3 of COI as well as 18S rDNA followed the (GTR + I) model, while position 1 of COI followed the (HKY + I + G) model, and 28S rDNA followed the (GTR + G) model on the Akaike information criterion (AIC). The BI analyses consisted of one to four million generations in two parallel chains executed in three separate runs, and a sampling frequency of 100 generations, while the first 2500 trees were discarded as "burn in". The dataset was tested for incongruence among the genetic markers using the Shimodaira–Hasegawa (SH) test implemented in PAUP* 4.0b10 (Swofford, 2002) with RELL (resampling estimated log-likelihood) and 1000 bootstrap replicates (Goldman et al., 2000; Shimodaira and Hasegawa, 1999) and ML trees derived from those taxa with all three genes sequenced. The partitions were considered to be congruent if the trees derived from one partition were not significantly different from the tree of at least one other partition.

2.3. Divergence time estimates

We dated the tree using BEAST v.1.61. (Drummond and Rambaut, 2007). Since the tree priors assume that each sequence represents a different species, we only included a single specimen from each presumed species in the analyses. The specimens included were *L. polyphemus* from Florida, *T. tridentatus* from the Eastern China Sea, *T. gigas* from the South China Sea, and *C. rotundicauda* from the Andaman Sea. The large genetic gap in *C. rotundicauda* indicated that two cryptic species were present and we therefore conducted additional analyses including *C. rotundicauda* from either the South China Sea or the Gulf of Thailand (Table 4). The data were analysed with five partitions (i.e. COI-1, COI-2, COI-3, 28S, 18S), while jModeltest (Guindon and Gascuel, 2003; Posada, 2008) was employed to define the best substitution models. The models chosen were: GTR + G for 28S and 18S, SYM + G for COI-1, F81 for COI-2, and HKY for COI-3. COI-2 base frequencies were not estimated, but set as equal to empirical values. The priors on all rate parameters were uniform with borders of 0 and 1. The tree was expected to evolve with Yule speciation tree priors and with a lognormal uncorrelated relaxed clock (Drummond et al., 2006). For

all analyses five different chains of 25 million generations were run with sampling every 2500 generations. The first half of all chains were discarded as burnin, while the last halfs were combined and analysed further. Convergence were checked visually as well as by calculating Estimated Sample Size (ESS) of all parameters in Tracer v.1.51 (Rambaut and Drummond, 2007).

The Limulinae–Tachypleinae split is likely to be related to the opening of the Atlantic Ocean about 150–130 Mya (Fisher, 1984). Interestingly, the fossil *Mesolimulus walchi* (Demarest, 1822) a species which is often assumed to represent the stem group of all recent horseshoe crabs, the Limulidae Zittel, 1885 occurred at the same time in Europe (Sekiguchi, 1988; Briggs et al., 2005). For this reason the trees were calibrated with the origin of all recent horseshoe crabs (Limulidae Zittel, 1885) with an age of 140 Mya (normal prior standard deviation 10 Mya).

3. Results

3.1. Molecular genetic diversity

The concatenated alignment contained 3398 basepairs, including the partial 18S (1768 bp), 28S (1090 bp), and COI (540 bp) fragments from nine specimens of all four horseshoe crab species plus four outgroup species (Table 2). Within Limulidae the nuclear genes showed 0.9% (18S) and 10.6% (28S) variable sites while the mitochondrial gene contained 26.1% variable sites. The parsimony informative content of the fragments was 0.8% (18S), 9.5% (28S), and 22.2% (COI). The AT content was relatively balanced in the 18S molecule (49%), but rather low in the 28S fragment (37%) and high in the COI fragment (62%). The intraspecific diversity was very high in *C. rotundicauda*, when compared with *T. gigas* that maintained relatively low levels of genetic diversity across the same region (Table 3).

3.2. Phylogenetic analysis

The SH-test indicated congruence among the data sets, i.e. the trees derived from each partition were not significantly different from those of the other partitions ($P > 0.1$). Therefore the analysis was combined for all nuclear partitions (NUC = 18S + 28S rDNA) as well as for the entire data set (MOL = COI + 18S + 28S rDNA). All analyses yielded an overall stable phylogeny with a topology similar to the combined analysis (Fig. 1). As the only exception, the 18S partition showed a different genealogy. However, it received no statistical support and can be explained by the lack of variation in this marker, i.e. the number of parsimony informative characters within Limulidae is less than 1% (Table 2).

Our analyses consistently showed high support for a sister group relationship between *T. gigas* and *T. tridentatus* and thereby a monophyletic genus *Tachypleus* Leach, 1819 (Fig. 1). Support was also strong for Tachypleinae Pocock, 1902 consisting of a sister-group relationship between *Tachypleus* and *C. rotundicauda*. Like most previous analyses, we found a sister-group relationship between the extant Limulinae (*L. polyphemus*) and Tachypleinae (Asian horseshoe crabs). The phylogeographic relationships among the *L. polyphemus* clades were weakly supported and remain unresolved, while three *C. rotundicauda* clades showed a well-supported subdivision of the geographic locations on each side of the Thai-Malay Peninsula.

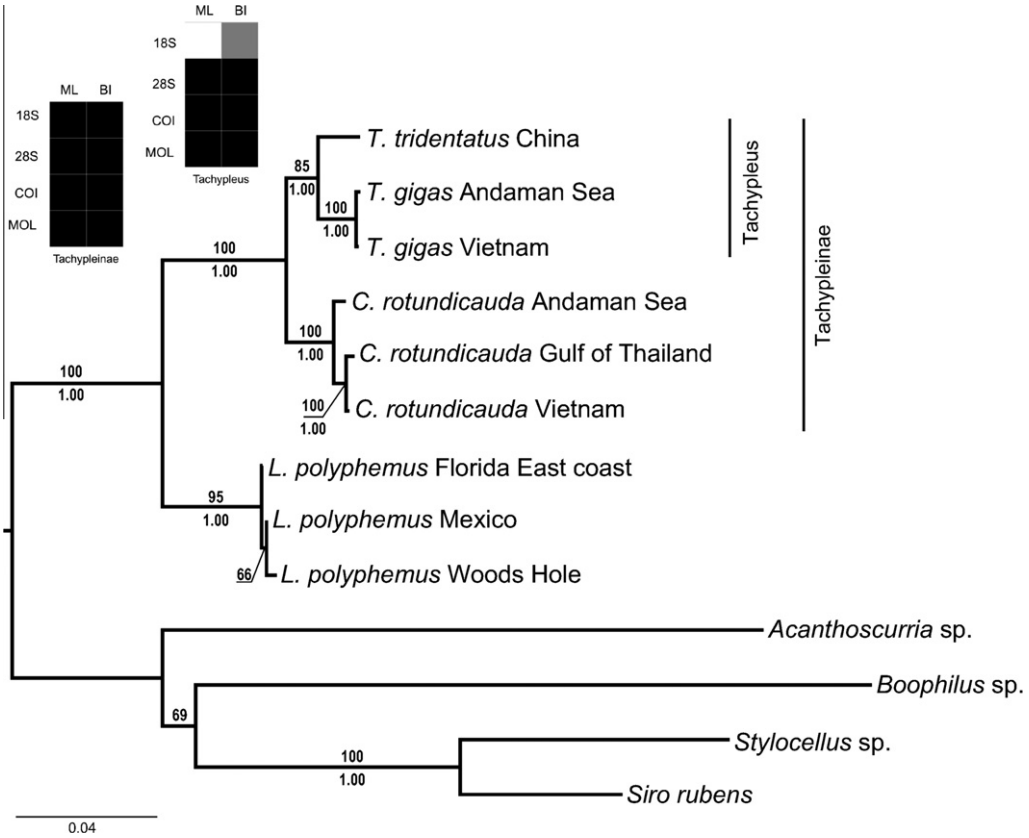

Fig. 1. ML tree from the combined analysis based on all molecular data (MOL) derived from 100 independent runs. Support values are shown above (ML bootstrap) and below (BI posterior probability) the branches. Shaded rectangles summarize all partial and combined analyses. The shading of squares in the rectangles indicates the support from either Bayesian inference (BI) or Maximum likelihood (ML) analysis of partial (18S, 28S, COI) and combined (MOL) datasets. Black squares indicate unambiguous support, grey squares indicate ambiguous support, and white squares indicate absence of support for the monophyly of the two clades, Tachypleinae and *Tachypleus*.

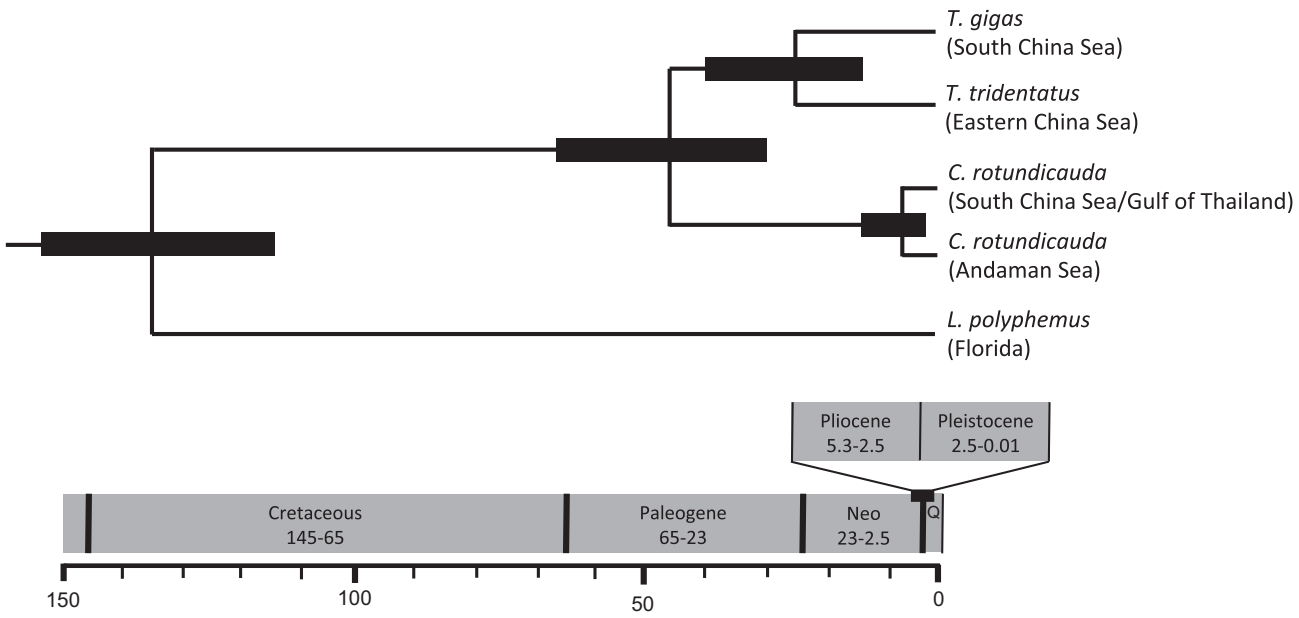

Fig. 2. Chronogram based on the BEAST divergence time analysis showing the estimated evolutionary radiation of Asian horseshoe crabs. Black bars indicate 95% confidence levels. The times scale below the chromatogram features all major geological periods in Mya. Neo, Neogene; Q, Quaternary (2.5–0 Mya).

3.3. Divergence time estimates

All five runs converged to the same parameters for all analyses and the lowest ESS estimate was 190 with most ESS values being above 500. The mean and standard deviation parameters from the three different BEAST analyses are shown in Table 4A. The resulting divergence time for the Asian horseshoe crabs Tachypleinae was dated to app. 45 Mya (30–68), and the origin of the genus *Tachypleus* was dated to app. 25 Mya (15–40), while the internal split of the *C. rotundicauda* across the Thai–Malay Peninsula was dated to app. 8 Mya (3–15), shown in Table 4B and Fig. 2.

4. Discussion

As earlier studies already indicated (Avise et al., 1994), the morphostasis of horseshoe crabs cannot be explained by lack of variation on the molecular genetic level. Among the three genes observed here we find a genetic diversity within and among species, which is similar to that of other marine invertebrates (Havermans et al., 2010; Latiolais et al., 2006; Barber et al., 2002). The genetic diversity within *C. rotundicauda* was larger than in *L. polyphemus* and *T. gigas* which may be explained by the different ecology of this species. As *C. rotundicauda* often inhabits estuarine habitats and mangroves, and less often enters open oceanic water, the gene flow between populations in this species may be much more restricted. A comparative analysis of the population structure in *C. rotundicauda* and *T. gigas* may reveal contrasting patterns of connectivity and possibly even uncover the presence of reproductively isolated lineages within *C. rotundicauda*. Similar substructure across the Thai-Malay Peninsula has been described from other marine coastal species (Alfaro et al., 2004; Crandall et al., 2008). The isolation of different populations on each side of the Thai–Malay Peninsula may have been driven by sea level changes in the past, i.e. lower sea levels may have largely affected the dispersal distance between each side of the peninsula as large parts of the Sunda Shelf were exposed and connected the peninsula to Sumatra and Java. Our results point towards a *C. rotundicauda* split between 3 and 15 Mya, but the inferred divergence rates are relatively low when compared with other marine arthropods (Knowlton and Weight, 1998), e.g. 0.21% pr million years as a lineage mutation rate for *C. rotundicauda* mtDNA. One reason for unusually low divergence rates in horseshoe crabs may be the relatively long generation time in these animals, which usually become sexually mature after 10 years (Shuster, 1958). Such long generation times are expected to produce slower evolutionary rates. Moreover, the divergence rates estimated for *C. rotundicauda* are likely to be biased, as a significant part of the genetic diversity observed in this species may be a part of the variation within the populations on each side of the Thai-Malay Peninsula. If we compare the estimated divergence of *C. rotundicauda* with a fixed rate calculation using the generally accepted mtDNA divergence rate of 1.4% (Knowlton and Weight, 1998), the split would have occurred app. 2.4 Mya. Considering both results it seems likely that the divergence between *C. rotundicauda* on each side of the Thai-Malay Peninsula may have occurred during Plio- or Pleistocene (Fig. 2), which coincides well with the extensive sea-level changes that occurred in the region during this period (Woodruff and Turner, 2009).

Our results agree with previous findings showing American horseshoe crabs as sister taxon to the three Asian species (Fisher, 1984; Avise et al., 1994; Xia, 2000). However, in contrast to all previous analyses of limulid relationships we find strong support for a monophyletic genus *Tachypleus*. Avise et al. (1994) suggested that the lack of anatomical diversity among recent horseshoe crabs together with the apparent problem to resolve the three Asian species was the result of a rather sudden and relatively recent diversification of all recent species. Our analysis however, suggests that the diversification of Asian horseshoe crabs most likely occurred during the Paleogene era (app. 65–23 Mya), with speciation events well separated by several million years (Fig. 2). A possible explanation for the current distribution of recent horseshoe crabs may be that *L. polyphemus* originated in the western Tethys sea and moved westwards with the North American continent during the Atlantic opening. The three Asian species probably originated in the Eastern parts of Tethys sea and survived the eradication of shallow water habitats in central Eurasia following the collision of the continent with the African and Indian plate during the Cenozoic period (Hall, 1998). The tree topology in our analysis suggests that the three Asian species originated in central South East Asia from a marine stem group that inhabited the shallow coastal waters between

the Andaman Sea, Vietnam, and Borneo. In this region *C. rotundicauda* separated from the Tachypleus stem group by invading eustarine habitats, while *T. tridentatus* probably originated by northeast migration along the Southern coast of China and towards Japan. Even though many horseshoe crab fossils suggest that they lived in brackish and freshwater habitats (Moore et al., 2007), our analysis supports the suggestion by Xia (2000) that the common ancestor of modern horseshoe crabs was marine and that *C. rotundicauda's* endurance in freshwater environment is derived.

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