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RESEARCH ARTICLE

Complete mitochondrial genomes of two cockroaches, *Blattella germanica* and *Periplaneta americana*, and the phylogenetic position of termites

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Abstract The mitochondrial genomes are one of the most information-rich markers in phylogenetics. The relationships within superorder Dictyoptera have been debated in the literature. However, the closely related termites (Isoptera) are retained as unranked taxon within the order Blattaria (cockroaches). In this work, we sequenced the complete mitogenomes of two cockroaches, reconstructed the molecular phylogeny and attempted to infer the phylogenetic position of termites in Blattaria more reliably. The complete mtDNA nucleotide sequences of the peridomestic American cockroach (*Periplaneta americana* L.) and the domestic German cockroach (*Blattella germanica* L.) are 15,025 and 15,584 bp in size, respectively. The genome shares the gene order and orientation with previously known Blattaria mitogenomes. Most tRNAs could be folded into the typical cloverleaf secondary structure, but the tRNA-Ser (AGN) of *P. americana* appears to be missing the dihydrouridine arm. Using nucleotide and amino acid sequences as phylogenetic markers, we proposed that termites should be treated as a superfamily (Termitoidea) of cockroaches. We suggested that Polyphagoidea was the sister group of Termitoidea in Blattaria and supported that the suborder Caelifera is more closely related to the Phasmatodea than to the suborder Ensifera of Orthoptera.

Keywords Mitogenome · Phylogenetic analysis · Cockroach · Blattaria · Dictyoptera · Isoptera

Introduction

The growing interest in the mitochondrial genomes (mitogenomes) has triggered a rapid increase in the number of published complete mitogenome sequences (Curole and Kocher 1999). The number of mitogenome sequences determined from vertebrates is larger than that from insects, despite the fact that insects constitute the most species-rich class among animals with almost a million of taxa described to date. Until now, more than 8,634 complete metazoan mitogenomes have been reported and among them 337 are from insects (http://www.ncbi.nlm.nih.gov).

The Orthopteroidea include Blattaria (cockroaches), Isoptera (termites), Mantodea (mantids), Orthoptera (grasshoppers and crickets), Phasmatodea (stick and leaf insects) and Mantophasmatodea (gladiators). Phylogenetic relationships among these insects are still quite controversial (Plazzi et al. 2011). Termites, cockroaches and mantids form a well-established lineage, the Dictyoptera, uniquely defined by having a perforation in the tentorium (the internal skeletal part of the head) and enclosing their eggs within a specialized case (ootheca). Currently, cockroaches are classified into three superfamilies: Blattoidea,

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Blaberoidea, and Polyphagoidea. Within the Dictyoptera, there is agreement that both termites and mantids are monophyletic groups (Inward et al. 2007). Thus, phylogenetic analyses among cockroach families, as well as the superorder Dictyoptera, have been a focus of several studies during the past decade (Inward et al. 2007; Klass and Meier 2006; Lo et al. 2003; Lo et al. 2007; Lo et al. 2000; Terry and Whiting 2005; Zhang et al. 2010). According to their results, the support for ranking termites is inconsistent. Among them, Inward et al. assuredly did a comprehensive molecular phylogenetic study, however, only using five genes to infer phylogenetic relationships within the Dictyoptera. Zhang et al. (2010) explored it based on the mitogenome data for the first time. However, Isoptera is still retained as an unranked taxon within Blattaria.

The cockroaches, among the most reviled of all insects, have long been recognized as potential mechanical vectors of human intestinal parasites and animal pathogens, as well as sources of human allergens (Yoon et al. 2009). Cockroaches often carry microorganisms that are important in nosocomial infections, and their medical importance in the spread of bacteria cannot be ruled out (Fakoorziba et al. 2010). Unfortunately, although there are approximately 4,000 species of cockroaches (Velez et al. 2006), there are only two mitogenomes available in Blattaria, *Periplaneta fuliginosa* and *Eupolyphaga sinensis* (Yamauchi et al. 2004; Zhang et al. 2010).

The peridomestic American cockroach (*Periplaneta americana* L.) and the domestic German cockroach (*Blattella germanica* L.), are ubiquitous pest species that are obligate commensally with humans, with heavy infestations associated strictly with human habitations, farms, food stores, waste areas and other anthropogenic habitats (Mukha et al. 2007). In most places they are the most prominent and important of the public health insects which occur with poor standards of hygiene (Fakoorziba et al. 2010; Gore and Schal 2005; Lee et al. 2003; Ritchie et al. 2009). They are resistant to all major groups of insecticides and have also been regarded as model for studying insect physiology (Irles et al. 2009; Lee et al. 2000; Xu et al. 2009; Yoon et al. 2009). Some partial mtDNA sequences have been published in GenBank (http://www.ncbi.nlm.nih.gov/GeneBank), but their complete mitogenome sequences have not yet been reported.

With more than 14,900 bp of nucleotide data and 37 genes, mitogenomes are one of the most information-rich markers in phylogenetics (Fenn et al. 2008). Compared to single or multi-gene analyses, mitogenomes could provide better resolution for deep phylogenetic relationships (Secq et al. 2006). The study of the evolution of mitogenomes instead of the evolution of mitogenes may become a new instrument for understanding of the mechanisms of the biological speciation and lineage divergence (Ravin et al. 2010). Besides its use as a phylogenetic marker, the mtDNA genome represents a "full" small genome, in which several structural genomic features can be systematically and quite easily investigated. These features allow the description of evolutionary trends in phylogenetically distant organisms (Flegontov et al. 2011; Gissi et al. 2008; Pantou et al. 2006).

The objective of this study was to determine, annotate and describe the mitogenomes of *B. germanica* and *P. americana*; to target Orthopteroidea insects phylogeny on using mitogenome data; and to infer the clear phylogenetic position of Isoptera. The newly determined *B. germanica* mitogenome is the first complete sequence for the superfamily Blaberoidea and for the family Blattellidae (synonym Ectobiidae). The *P. americana* mitogenome is the second complete sequence for the superfamily Blattoidea. The sequences given in the present study may not only provide useful information to phylogenetic researches of Blattaria and other insects, but also for developing mitogenome genetic markers for species identification of cockroaches. In addition, they may facilitate research on phylogenetics of the Dictyoptera.

Materials and methods

Samples and DNA extraction

The cockroaches used in this study were collected on public land, which was not a protected area or a national park, and their collections and the study were approved by the government. Specimens of *B. germanica* and *P. americana* were sampled from the offspring of cockroaches collected from Danyang in Jiangsu Province, People's Republic of China, and maintained as a lab culture. Total genomic DNA was extracted from the muscle of the fresh specimen's femurs by the standard proteinase K and phenol/chloroform extraction method, then stored at −20°C and used as a template for subsequence PCR reactions.

Primer design and PCR amplification

Some partial sequences were amplified and sequenced at first using general primers based on Simon et al. (1994). These fragments of the mitogenomes were amplified by long PCR using Takara LA Taq^{TM} (Takara Bio, Otsu, Shiga, Japan): an initial denaturation for 2 min at 94°C, followed by 15 cycles of denaturation 30 s at 94°C, annealing 30 s at 50–58°C (depending on primer combinations), elongation 60–300 s (depending on putative length of the fragments) at 68°C; then followed by 15 cycle of denaturation 30 s at 94°C, annealing 30 s at 50–58°C,

Table 1 Sequencing primers used in the analysis of *B. germanica* (Bg) and *P. americana* (Pa)

Primer name	Upstream primers sequences (5′–3′)	Downstream primers sequences (5′–3′)	Anneal temperature (°C)
Bg 1	TM-J-159: GCTAAATAAAGCTAATGGGTTCAT	C1-N-2314: ACAGTAAATATATGATGAGCTCA	52
Bg 2	C1-J-2124: CAACATTTATTTTGATTCTTTGG	TK-N-3764: GTTTAAGAGACCACCACTTG	50
Bg 3	C2-J-3555: GCAGACGCTACTCCAGGTCGTCTT	N4-N-8554: CATATACTTTAATAATTGCTCATGG	62
Bg 4	N4-J-8374: GAAGGGGGTGCTGCTATATTAC	CB-N-11240: ATTACTCCTCCTAATTTATTAGGAAT	59.5
Bg 5	CB-J-10849: CAATGAGTATGAGGAGGATTTGCTGT	SR-N-14603: TGTGCCAGCAGTCGCGGTTATACA	56
Bg 6	SR-J-14420: AGGGTATCTAATCCTAGTTT	N2-N-321: TCTAATCCTATTCACGCCCCT	59
Pa 1	TM-J-166: GCTAAWTAAAGCTAATRGGTTCAT	C1-N-2657: GTTAAACCTGTAAATAGAGGGTATC	52
Pa 2	C1-J-2164: CAACACTTATTCTGATTCTTTGGTC	TL-N-3033: TCCATTGCACTTATCTGCCAT	50
Pa 3	C1-J-2784: ACGATACTCCGATTATCCCGATGCT	C3-N-5425: TGGTGGTTTGATGAAAAATGTAGTG	55
Pa 4	C3-J-5153: TACAGCTATTTTATTAGCATCAGGAG	N4-N-8657: GCTTATTCCTCKGTWGCTCATATAGG	55.5
Pa 5	N4-J-8426: GAAGGTGGAGCGGCTATATTAC	CB-N-11419: GGTCGWGCWCCAATTCATGT	56
Pa 6	CB-J-10819: TGAGGACAAATATCATTTTGAGG	LR-N-13065: GGACGAGAAGACCCTATAGAGT	59.3
Pa 7	LR-J-12754: CCGGTCTGAACTCAGATCATGT	SR-N-14362: TAGAGGAATCTGTCCCTTAATCG	55.5
Pa 8	SR-J-14121: GAGCCAAAATCACTATTACAATC	N2-N-419: GGAACAGTAGAGTTGATGATGCT	60
Pa 9	SR-J-14121: GAGCCAAAATCACTATTACAATCTAC	AT-N-15500: CCATTAAATGTTGGTAACTCGAT	53

elongation 60–300 s + 6 s/cycle at 68°C and a final extension period of 68°C for 10 min. PCR products were tested by electrophoresis on an agarose gel (1%). Subsequently, based on information from these sequence fragments, new pairs of primers were designed of which each segment overlapped the adjacent sequence by 160–390 bp. The larger mtDNA fragments were also amplified by long PCR using Takara LA Taq. Primers are shown in Table 1.

Purification, sequencing and sequence assembly

When a single band was observed in the PCR products, it was purified using a V-gen PCR Clean-up Purification Kit. If more than one band was present, the appropriately sized PCR product was cut from the gel and extracted using a Biospin Gel Extraction Kit. All fragments were sequenced in both directions, and large PCR products were sequenced with a primer walking strategy. Sequences were checked and assembled by the program seqman (DNASTAR, 2001), BioEdit and Chromas 2.22, then checked manually to obtain the complete mitogenome sequences of *B. germanica* and *P. americana*. The coverage for each mitogenome was above 2×.

Data analysis

Genes encoding proteins, ribosomal RNAs (rRNA) and transfer RNAs (tRNA) were identified according to their amino acid translation or secondary structure features, respectively. Individual gene sequences were compared with the available homologous sequences of *P. fuliginosa* and *E. sinensis* in GenBank. The 22 *tRNA* genes of each were identified using software *tRNA* Scan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE) and their cloverleaf secondary structures and anticodon sequences were identified using DNASIS (Ver.2.5, Hitachi Software Engineering).

The reconstruction of phylogenetic trees

In order to discuss the phylogenetic relationships within Orthopteroidea, we used concatenated nucleotide/amino acid sequences of 13 protein-coding genes and whole mtDNA sequences (Coucheron et al. 2011; Jung et al. 2010; Kim et al. 2006; Minegishi et al. 2005) of 29 relevant species whose complete mitogenome sequences were available in GenBank as the markers to reconstruct phylogenetic trees. The sequences of one Plecoptera specie (*Pteronarcys princeps*) and three Ephemeroptera species (*Parafronurus youi*, *Ephemera orientalis* and *Siphlonurus immanis*) were used as outgroup. The mitogenome sequences used in this text were downloaded from GenBank (Table 2).

The nucleotide and amino acid sequences were aligned by Muscle in MEGA 5.05 (Piłsyk and Paszewski 2009; Tamura et al. 2011) with manual refinements. One alignment was based on the complete mtDNA sequences, except for the highly variable extended termination-associated sequence (ETAS) domain within major noncoding region, creating a sequence of 15,965 base pairs (bp). The second

Table 2 The mitochondrial genome sequences used to reconstruct phylogenetic trees

Order	Species	Accession number
Dictyoptera/Blattaria	*Blattella germanica*	EU854321
	Periplaneta americana	GU947663
	Periplaneta fuliginosa	AB126004
	Eupolyphaga sinensis	FJ830540
Dictyoptera/Isoptera	*Reticulitermes flavipes*	EF206314
	Reticulitermes santonensis	EF206315
	Reticulitermes hageni	EF206320
	Reticulitermes virginicus	EF206318
	Coptotermes formosanus	AB626145
Dictyoptera/Mantodea Mantophasmatodea Phasmatodea	*Tamolanica tamolana*	DQ241797
	Sclerophasma paresisensis	DQ241798
	Phraortes illepidus	AB477460
	Ramulus irregulariterdentatus	AB477463
	Entoria okinawaensis	AB477459
	Megacrania alpheus adan	AB477471
	Heteropteryx dilatata	AB477468
	Micadina phluctainoides	AB477466
Orthoptera/Caelifera	*Locusta migratoria tibetensis*	HM219224
	Thrinchus schrenkii	GU181288
	Gomphocerus sibiricus tibetanus	HM131804
	Atractomorpha sinensis	EU263919
	Acrida cinerea	GU344100
	Physemacris variolosa	GU945504
Orthoptera/Ensifera	*Elimaea cheni*	GU323362
	Elimaea cheni	EU137664
	Gryllotalpa orientalis	AY660929
	Anabrus simplex	EF373911
	Ruspolia dubia	EF583824
	Gampsocleis gratiosa	EU527333
Plecoptera Ephemeroptera	*Pteronarcys princeps*	AY687866
	Parafronurus youi	EU349015
	Ephemera orientalis	EU591678
	Siphlonurus immanis	FJ606783

alignment was built from the complete set of codons (except stop codons) creating a concatenated sequence of 11,025 bp (3,675 amino acid positions) corresponding to the 13 protein genes (*ATP6*, *ATP8*, *COI*, *COII*, *COIII*, *Cytb*, *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5* and *ND6*) (Coucheron et al. 2011). In the second alignment, the amino acid sequences of each protein were aligned respectively before concatenation. The nucleotide sequence alignments were made based on amino acid sequence alignments.

Phylogenetic analyses based on the nucleotide and amino acid datasets were performed using maximum likelihood (ML) and Bayesian (BI) methods. The best fitting substitution model for the ML and BI nucleotide analyses, as judged by the Akaike information criterion (AIC), was determined by ModelTest 3.7 (Posada and Crandall 1998) and MrMODELTEST 2.3 (Nylander 2004). The best-fit model GTR (general time reversible) + I (invariant) + G (gamma) for the nucleotide dataset was used for the ML analyses. For BI nucleotide analyses, we assessed the best fit model of evolution for each gene, which resulted in four partitions with the following models: (1) 13 protein genes, 2 rRNA genes = GTR + I + G; (2) tRNA-Arg, tRNA-Cys, tRNA-Lys, tRNA-Ser(AGN), tRNA-Ser(UCN), tRNA-Thr, tRNA-Tyr, tRNA-Val = GTR + G; (3) tRNA-Ala, tRNA-Asn, tRNA-Gln, tRNA-Glu, tRNA-Gly, tRNA-His, tRNA-Ile, tRNA-Leu(CUN), tRNA-Met, tRNA-Phe, tRNA-Pro = HKY + G; tRNA-

Asp, tRNA-Leu(UUR), tRNA-Trp = HKY + I + G. For the amino acid dataset, model selection was performed with ProtTest v. 2.4 (Abascal et al. 2005) and under the AIC, MtArt (Model of Amino Acid Replacement for Arthropoda) + I + G (Abascal et al. 2007) was the best-fit model. As MtArt model is not implemented in the current version of phylogenetic software we used, we chose the best scoring alternative model of MtRev (the general reversible Markov model for mtDNA-encoded proteins) + I + G for the amino acids in phylogenetic analyses.

The BI analyses were performed by MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003) and ML analyses by RAxML 7.0.4 (Stamatakis 2006), with gaps treated as missing data in both. Confidence values for the maximum-likelihood tree were obtained by bootstrapping (1,000 replicates). In BI analyses, two independent sets of Markov chains were run, each with one cold and three heated chains for 1×10^6 generations, and every 100 generations were sampled. Convergence was inferred when a standard deviation of split frequencies <0.01 was achieved or the likelihood of the cold chain stopped increasing and began to randomly fluctuate. The burn-in parameter was estimated by plotting –ln L against the generation number using TRACER v1.4.1 (Rambaut and Drummond 2007), and the retained trees were used to estimate the consensus tree and the BI posterior probabilities. Three replicates of these BI runs were conducted, retrieving the same topology.

Results

Genome organization and gene arrangement

The organizations of two mitogenomes are shown in Fig. 1 were drawn by the software OGDraw (Lohse et al. 2007). The cockroach mitogenomes are circular molecules, 15,025 bp (*B. germanica*) and 15,584 bp (*P. americana*) in size and have been deposited in GenBank (GenBank accession numbers EU854321 and GU947663). The two sets of 37 genes found in a typical insect mitogenome were present: 13 protein-coding genes (*ATP6, ATP8, COI-III, ND1-6, ND4L, Cytb*), 2 ribosomal RNA genes (*srRNA* and *lrRNA*), 22 transfer RNA genes and a putative major noncoding region (Table 3). The gene order and orientation is identical to that of *Drosophila melanogaster* (Clary et al. 1982). The structure of mtDNA is conserved in divergent insect orders and even some crustaceans (Crease 1999; Nardi et al. 2003), and has been inferred to be ancestral for insects (Boore et al. 1998).

We calculated the base composition of the mtDNA in both species using DNASTAR, and the results are also listed with the other two species of Blattaria (Table 4). It corresponds well to the AT bias generally observed in insect mitogenomes, which ranges from 69.5 to 84.9% (Crozier and Crozier 1993; Dotson and Beard 2001). The coding of the genome is very compact and some of the genes overlap each other in the mitogenomes. In *B. germanica*, this occurs

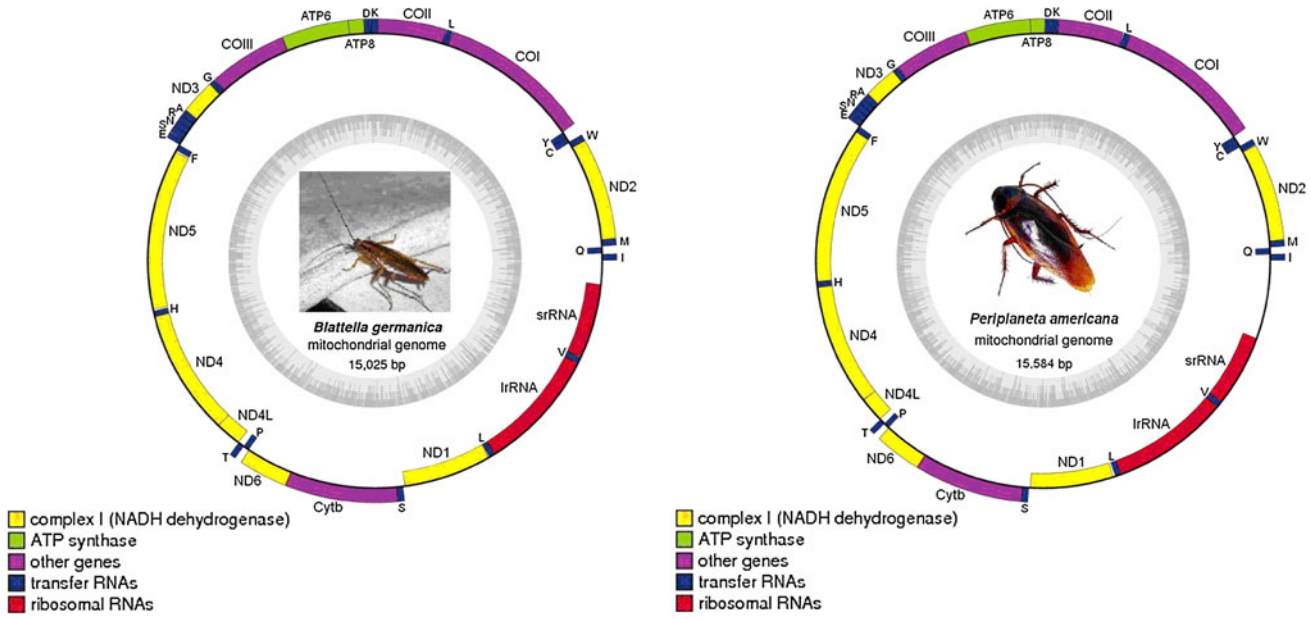

Fig. 1 Circular map of the mitogenomes of *B. germanica* and *P. americana*

Table 3 Annotation of the mitochondrial genomes of *B. germanica* (Bg) and *P. americana* (Pa)

Feature	Strand	Position		Initiation codon/stop codon		Anticodon
		Bg	Pa	Bg	Pa	
trnI	J	1–67	1–69			GAT
trnQ	N	75–139	67–135			TTG
trnM	J	149–216	156–221			CAT
ND2	J	218–1243	222–1247	ATG/TAA	ATG/TAA	
trnW	J	1,242–1,310	1,247–1,317			TCA
trnC	N	1,302–1,368	1,310–1,378			GCA
trnY	N	1,368–1,433	1,403–1,472			GTA
COI	J	1,437–2,972	1,477–3,012	TTG/TAG	TTG/TAA	
trnL(UUR)	J	2,975–3,039	3,027–3,096			TAA
COII	J	3,042–3,734	3,100–3,784	ATG/AGA	ATG/T–	
trnK	J	3,728–3,798	3,785–3,855			CTT
trnD	J	3,800–3,866	3,856–3,919			GTC
ATP8	J	3,867–4,025	3,920–4,078	ATA/TAA	ATT/TAA	
ATP6	J	4,019–4,699	4,072–4,752	ATG/TAA	ATG/TAA	
COIII	J	4,699–5,487	4,752–5,540	ATG/TAA	ATG/TAA	
trnG	J	5,490–5,554	5,545–5,608			TCC
ND3	J	5,555–5,908	5,609–5,962	ATA/TAG	ATT/TAG	
trnA	J	5,907–5,971	5,961–6,025			TGC
trnR	J	5,971–6,033	6,025–6,089			TCG
trnN	J	6,035–6,099	6,089–6,154			GTT
trnS(AGN)	J	6,100–6,168	6,155–6,221			GCT
trnE	J	6,172–6,234	6,223–6,293			TTC
trnF	N	6,235–6,302	6,292–6,359			GAA
ND5	N	6,318–8,033	6,360–8,090	ATT/TAA	ATT/TAA	
trnH	N	8,049–8,112	8,091–8,159			GTG
ND4	N	8,113–9,452	8,164–9,501	ATG/TA-	ATG/TAA	
ND4L	N	9,446–9,727	9,495–9,779	ATG/TAA	ATG/TAA	
trnT	J	9,730–9,794	9,782–9,845			TGT
trnP	N	9,795–9,860	9,846–9,911			TGG
ND6	J	9,863–10,363	9,914–10,414	ATA/TAA	ATT/TAA	
Cytb	J	10,363–11,496	10,414–11,547	ATG/TAA	ATG/TAA	
trnS(UCN)	J	11,496–11,564	11,548–11,618			TGA
ND1	N	11,584–12,525	11,636–12,583	ATA/TAG	ATG/TAA	
trnL(CUN)	N	12,532–12,598	12,587–12,652			TAG
lrRNA	N	12,599–13,906	12,653–13,944			
trnV	N	13,907–13,977	13,945–14,015			TAC
srRNA	N	13,979–14,772	14,016–14,820			
A + T-rich		14,773–15,025	14,821–15,584			

Strand J the ORF/gene is located in the plus strand, *Strand N* the minus strand

Table 4 Base composition of the known complete mitochondrial genome in Blattaria

Species	%T	%C	%A	%G	%G + C	%A + T	Total (bp)
B. germanica	35.4	15	39.2	10.4	25.4	74.6	15,025
E. sinensis	31.6	17.5	40.4	10.5	28	72	15,553
P. americana	32.1	15.6	42	10.3	25.9	74.1	15,584
P. fuliginosa	33	14.5	42.1	10.3	24.8	75.1	14,996

Table 5 Estimates of relative synonymous codon usage (RSCU) for each codon of *B. germanica* (Bg) and *P. americana* (Pa)

Amino	Codon	RSCU		Amino	Codon	RSCU		Amino	Codon	RSCU		Amino	Codon	RSCU	
		Bg	Pa			Bg	Pa			Bg	Pa			Bg	Pa
Phe	UUU	1.76	1.68	Ser	UCU	1.99	1.94	Tyr	UAU	1.77	1.72	Cys	UGU	1.82	1.73
	UUC	0.24	0.32		UCC	0.50	0.36		UAC	0.23	0.28		UGC	0.18	0.27
Leu	UUA	4.60	3.65		UCA	2.33	2.41	TER	UAA	–	1.85	Trp	UGA	1.84	1.79
	UUG	0.34	0.81		UCG	0.15	0.21		UAG	–	0.15		UGG	0.16	0.21
	CUU	0.61	0.39	Pro	CCU	2.18	1.58	His	CAU	1.54	1.36	Arg	CGU	0.52	1.31
	CUC	0.04	0.07		CCC	0.32	0.23		CAC	0.46	0.64		CGC	0.14	0.14
	CUA	0.38	1.05		CCA	1.41	2.1	Gln	CAA	1.78	1.81		CGA	1.64	2.28
	CUG	0.04	0.03		CCG	0.09	0.09		CAG	0.22	0.19		CGG	0.42	0.28
Ile	AUU	1.83	1.71	Thr	ACU	1.62	1.3	Asn	AAU	1.68	1.67	Ser	AGU	0.88	1.02
	AUC	0.17	0.29		ACC	0.36	0.45		AAC	0.32	0.33		AGC	0.15	0.19
Met	AUA	1.80	1.76		ACA	1.96	2.16	Lys	AAA	1.48	1.61	Arg	AGA	3.23	1.63
	AUG	0.20	0.24		ACG	0.07	0.08		AAG	0.52	0.39		AGG	0.05	0.24
Val	GUU	1.66	1.69	Ala	GCU	2.01	1.59	Asp	GAU	1.76	1.8	Gly	GGU	1.69	1.93
	GUC	0.19	0.12		GCC	0.35	0.44		GAC	0.24	0.2		GGC	0.07	0.23
	GUA	1.97	2.06		GCA	1.59	1.89	Glu	GAA	1.82	1.75		GGA	1.86	1.5
	GUG	0.17	0.12		GCG	0.04	0.09		GAG	0.18	0.25		GGG	0.38	0.34

11 times, spanning 1–9 bp, and is a total of 39 bp in length. The mtDNA genome of *P. americana* also contains 11 overlapping regions, spanning 1–8 bp, for a total of 34 bp. Besides the noncoding regions, there are 17 non-coding regions, spanning 1–19 bp, totaling 91 bp and 12 non-coding regions, spanning 1–24 bp, totaling 95 bp in mtDNA genomes of *B. germanica* and *P. americana*, respectively. Some overlapping gene segments are in identical positions in Blattaria mitogenomes, e.g. the region between tRNA-Trp (trnW) and tRNA-Cye (trnC), which is usual in other Orthoptera species.

The each mitogenome contains 22 *tRNA* genes, which are interspersed in the genome and range from 63 to 71 bp in length, and they were identified in the same relative genomic positions as observed in *P. fuliginosa* (Yamauchi et al. 2004) and *E. sinensis* (Zhang et al. 2010). The predicted secondary structure for the 22 *tRNA* genes of *B. germanica* and *P. americana* can be seen in Figure S1 and S2 (supporting information). Putative secondary structures of tRNAs indicate that most of them have typical cloverleaf secondary structures and their anticodons are similar to those found in other metazoan animals. However, the tRNA-Ser (AGN) of *P. americana* could not form a stable stem loop structure in the dihydrouridine (Oliveira et al. 2008) arm as shown in many other insect tRNA-Ser (Kim et al. 2005).

Protein-coding regions

The protein-coding genes typically use ATN as the preferred initiation codon. ATG is the most used initiation codon (15 genes, in total combining results from both species), then ATT (5 genes), ATA (4 genes), and TTG (2 genes, *COI* of two cockroaches) (Table 3). The initiation codon of the cytochrome oxidase subunit I (*COI*) gene has been extensively discussed in several arthropod species including insects (Caterino and Sperling 1999). Tetranucleotides (ATAA, TTAA, and ATTA) and a hexanucleotide (ATTTAA) were postulated as the initiation codon of the *COI* gene (Hong et al. 2009; Zhou et al. 2007). However, the ATN initiation codon, postulated tetranucleotides or hexanucleotide of the both *COI* genes have not been detected here. To weigh the existence or none of the initiation codon, we postulated that the TTG was the initiation codon, similar to some genes of Nematoda species (Jex et al. 2008; Okimoto et al. 1990).

The protein-coding genes use TAA and TAG as termination codons, except the *COII* gene, AGA (*B. germanica*) and T (*P. americana*). The *ND4* and *COII* genes have incomplete termination codons TA (*B. germanica*) and T (*P. americana*), respectively. The incomplete termination codon is commonly found in metazoan mitogenomes, and the reasonable interpretation is that mRNA polyadenylation makes complete TAA stop codon (Anderson et al. 1981). The observed A + T content strongly influences the use of degenerate synonymous sites in protein coding genes, with relaxed pressure probably responsible for the most frequent use of NNA and NNU codons (Table 5).

The noncoding region

The only major non-coding region in insect mitogenome is heavily biased towards A + T nucleotides and seems to

Fig. 2 The noncoding region sequence of the mitochondrial genomes. *Framed and underlined* segments indicate A-/T-homooligomers and TA repeats, respectively. The *bold and dot segments* indicate the 42 bp conserved sequences in *Periplaneta*

B. germanica

GAAGCCAATAATCGAAAGATCACTTCAACTATTAAGTAAACTGTTATTTATACTTAA
AAAAAAAAATAGAGTACAAATTCCTCCCCAAATCCAATAATTCAACCTCTTCCTTCAACC
TTCTTAAATTTTCAACCTTTTAACCTTTTTCGAAGCCAATATTTTTACTCGTTACGATCCAC
TATAAATAAAAATACAGTAATTTAAGATGTCAATCTTATCAAACTTTTTTTTTATAAAATA
AAATAGAAAAAAA

P. americana

ATCAAATAAATACTGAAACAAAAATCGGAACTAAATAAAAAAATAAAGACTAAATAAAATC
AAAAATAAAAAAAGTAAAACCCGAATTTCTCCATACATTTCAACCAACTCACAACGTAT
TTTTAACACAACTACTAACATCAACTCTTTCAACTCTTAAGAAAACATTTAAAACCACGGA
CCCAAAAAAAAAAAACTGAAACATTAACCTTAACCTATAATAAAATTCTCTTTTCCTATAAT
AGTAACAGTATAACTACAGATCTCATTTTTTTTTTAATTATTAAATGAGATCTGTAGTTATACT
GTTACTATTATTTATACACTAAAGGTTTAATGTCACTGTTAACAATAATTGTATCCCGTCAATT
ATTTATTTTATATATACATATATATATATAAGGACCTATTTTGAATAATTAATTATTGATAAATAG
ATTTAACAAGAATATATTTTTTATATAAATAATATATATTCTAATAATTTAGATTTATATTAATAT
AAATCATATATATATATATACCTCTATTTTTCAACAATAATGCTGTATATTCATAGATAATCTGT
ATTATATATAAATTAATTATTATAATATAAATAAACCTATTTTTTAGTAAAATAGGTCATTTTAT
TTAAATAATTAATTTATATATATATATATAATCTTATATTAATAAAAAATCGAGTTACCAACATTTA
ATGGAATACTTATTATAATCCATAAATATAGAACCAATAAAGAAAATGTTATCTTAAAATAAC
AAA

P. fuliginosa

ATTAGACAATATATTTAAACTAGGTATGGGTCAACATTAAAAAATGTTATAAAATAACAAAAT
AATATAAATAAAAAAGTAAAACCCGAATTTCTCCATACATTTTAACCAACTTATCAACCA
AGTACAAATTTCTACTACTTCACAACTTCAAACAACACTTAAATTAATTATTTAAACTAGGT
ATGGGTCAACATTAAAAAATGTT

evolve under a strong directional mutation pressure (Zhang and Hewitt 1997). The noncoding region varies greatly in insects, from 73 bp in *Heterodoxus macropus* to 4,601 bp in *Drosophila melanogaster* (Garesse 1988; Shao et al. 2001). The noncoding regions of the two determined cockroach mitogenomes are located between small rRNA and $tRNA^{Ile}$. This region of *B. germanica* is 253 bp long with 75.1% A + T content, longer than that of *P. fuliginosa* (208 bp), but shorter than average when compared with other insects. Short repeating sequences except Poly A and Poly T could not be found, in the whole noncoding region of *B. germanica* (Fig. 2).

The noncoding region of *P. americana* is 764 bp long with 81.6% A + T content, shorter than 857 bp of *E. sinensis* (Zhang et al. 2010). Noncoding region of *P. americana* has three TA repeats (Fig. 2). Compared with the other three cockroaches, this region has one 42 bp conserved sequence in *Periplaneta* (Fig. 2).

Phylogenetic analyses

The phylogenetic trees are shown in Fig. 3 (BI) and Figure S3 (ML).

Discussion

The relationships among Blattellidae (*B. germanica*), Blattidae (*P. fuliginosa* and *P. americana*) and Polyphagidae (*E. sinensis*) have been argued for many years.

According to analyses of morphological and anatomical data (Grandcolas 1996; Roth 2003), Blattidae is the sister group of all other cockroaches and the (monophyletic) Polyphagidae is the sister group of Blattellidae. The relationship of the three families based on mitochondrial *COII* gene sequences, was [Polyphagidae, (Blattidae, Blattellidae)] (Maekawa and Matsumoto 2000). Analyses of sequence data of CAPA peptides have recovered the relationships [Blattellidae, (Blattidae, Polyphagidae)] (Roth et al. 2009). The phylogenetic results based on nucleotide sequences (Fig. 3a, b; Figure S3.A, S3.B) support the [Polyphagidae, (Blattidae, Blattellidae)] relationship, while the results based on amino acid sequences suggest [Blattidae, (Blattellidae, Polyphagidae)] (Fig. 3c) or the [Polyphagidae, (Blattidae, Blattellidae)] (Figure S3.C).

DeSalle et al. (1992) reckoned that Mantodea and Isoptera were sister groups in Dictyoptera. But, in recent years, based on morphological features such as ootheca and life history, or via methods of molecular systematics, more and more researchers have supported the hypothesis [(Blattaria, Isoptera), Mantodea] within Dictyoptera, and even suggested that termites are social cockroaches and no longer merit classification as a separate order (Isoptera) distinct from the cockroaches (Blattodea) (Inward et al. 2007; Klass and Meier 2006; Nalepa and Lenz 2000; Zhang et al. 2010). In this study, Isoptera (monophyletic) and Blattaria (paraphyletic) form a large clade, which together with its sister group Mantodea forms a monophyletic group. Some studies have indicated that the termites should be sister to Cryptocercidae and belong to the superfamily Blattoidea, which

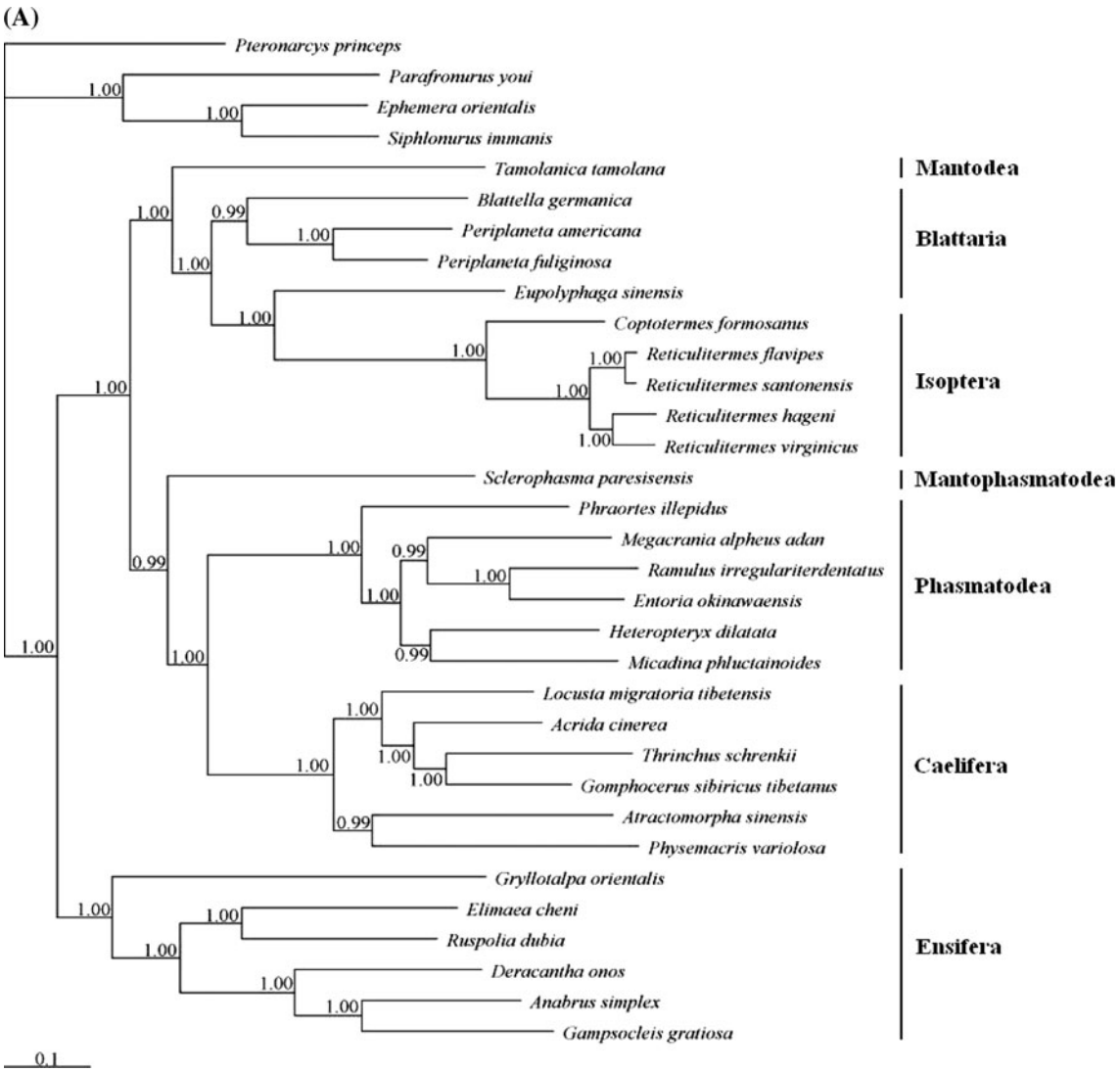

Fig. 3 Bayesian phylogenetic trees based on **a** whole mtDNA sequences, **b** concatenated nucleotide sequences and **c** concatenated amino acid sequences

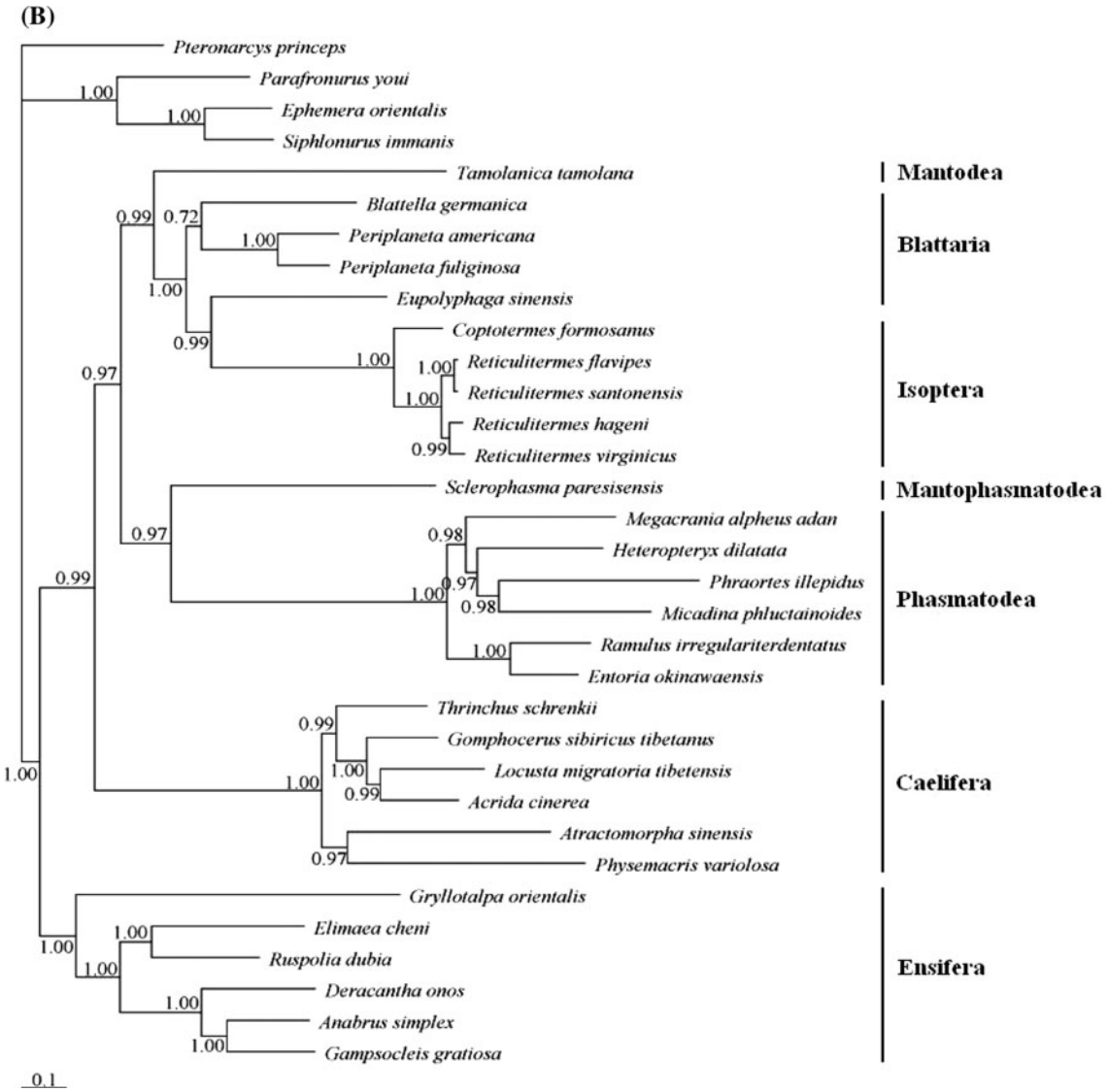

Fig. 3 continued

was sister group of Blaberoidea (DjernÆS et al. 2011; Inward et al. 2007). The Termitoidea was then regarded as the synonym of Blattoidea, which included epifamilies Blattoidae, Termitoidae and Cryptocercoidae (http://blattodea.speciesfile.org/Database.aspx). In Figure S3.C, the termites (monophyletic) are sister groups of Blattoidea with low confidence value (34 of 100). But in Fig. 3, S3.A and S3.B, the termites (monophyletic) are sister groups of Polyphagoidea (*E. sinensis*) with high support values, and this cluster is sister to the (Blattoidea, Blaberoidea) (Fig. 3a, b; Figure S3.A, S3.B) or the Blaberoidea (Fig. 3c). So we suggested that the termites should be treated as independent Termitoidea and the possible [(Termitoidea, Polyphagoidea), (Blattoidea, Blaberoidea)] relationship in Blattaria. This view will be tested by using the new mitogenome data of Cryptocercidae species.

A sister group relationship between Phasmatodea and Mantophasmatodea receives strong support (Fig. 3; Figure S3). In BI analyses based on nucleotide or amino acid sequences of 13 genes (Fig. 3b, c) and ML analysis based on the amino acid sequences (Figure S3.C), the clade (Phasmatodea and Mantophasmatodea) is more closely related to Dictyoptera (Blattaria, Isoptera and Mantodea), than to others in Orthopteroidea. However, based on whole mtDNA sequences (Fig. 3a, BI; Figure S3.A, ML) and ML analysis of nucleotide sequences of 13 genes, the (Phasmatodea and Mantophasmatodea) is the sister group of Caelifera (Orthoptera). So the evolutionary position of Dictyoptera in orthopteroid insects is still uncertain but our results support that the suborder Caelifera is more closely related to the Phasmatodea than to the suborder Ensifera (Hennig 1981; Lind 1994).

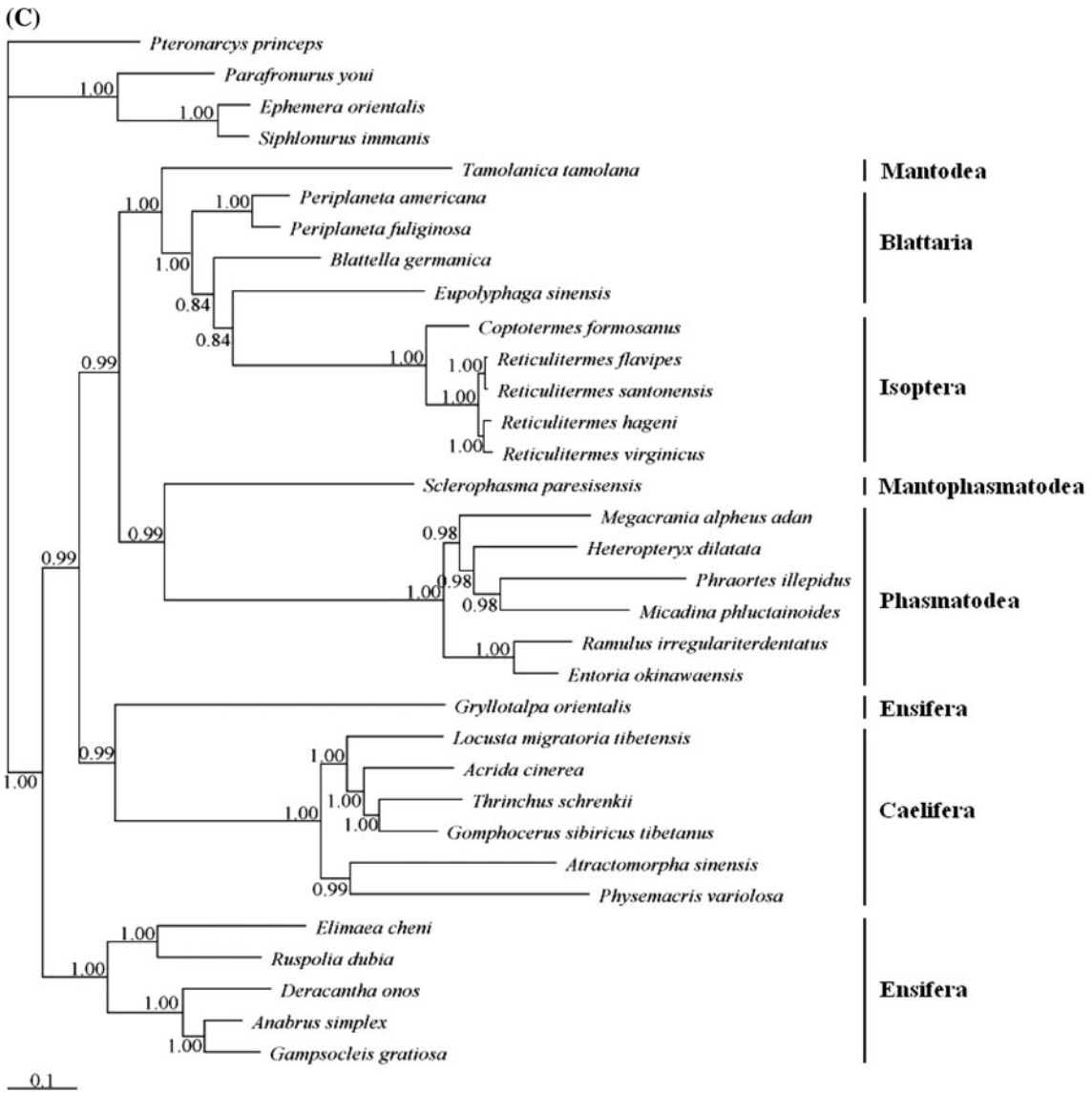

Fig. 3 continued

In protein trees (Fig. 3c, BI; Figure S3.C, ML), the *Gryllotalpa orientalis* (Ensifera) is more closely related to Caelifera than to other Ensifera, which might be due to an artifact of the phylogenetic reconstruction or the alternative MtRev + I + G model used for the amino acid dataset (not the best-fit MtArt + I + G model). Also, there is a view that DNA might be more useful than amino acid sequences for closely related species: the synonymous substitutions are invisible at the amino acid level (Goldman and Yang 1994). The amino acid sequences of mitochondrial genes might be unsuitable for classification within the Orthoptera.

In summary, this paper confirmed the monophyly of Dictyoptera and Phasmatodea, and supported Mantophasmatodea as sister group of Phasmatodea. The obtained results provide evidence that whole mtDNA sequences and concatenated nucleotide sequences of 13 protein genes were suitable markers to unravel the ancient splits leading to the orthopteroid orders. This work represents one important step towards a more stable phylogeny of orthopteroid insects. Further work and additional complete mitogenomes are needed to better address the placement of branches within the Orthopteroidea tree.

Conclusion

The complete mitogenomes of *B. germanica* and *P. americana* were sequenced. Both species, together with other cockroach species, share the same mitochondrial genome organization. Like other invertebrates, they are rich in A + T throughout the entire mitogenomes.

Based on the analysis results of this text and Zhang et al. (2010), we supported the view that "death of an order Isoptera" (Inward et al. 2007), and followed the suggestion that "a higher family level rank such as superfamily or epifamily" (Lo et al. 2007). Therefore, we propose that termites should be treated as a superfamily (Termitoidea) of cockroaches. Furthermore, we expect that more morphological and molecular data would be used to unravel the relationships within Dictyoptera.

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