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The mitochondrial genomes of *Cambaroides similis* and *Procambarus clarkii* (Decapoda: Astacidea: Cambaridae): the phylogenetic implications for Reptantia

Sanghee Kim, Mi-Hyun Park, Jae-Ho Jung, Dong-Ha Ahn, Tahera Sultana, Sejoo Kim, Joong-Ki Park, Han-Gu Choi & Gi-Sik Min

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We determined the complete mitochondrial (mt) genome sequences of two northern hemisphere freshwater crayfish species, *Cambaroides similis* and *Procambarus clarkii* (Decapoda: Astacidea: Cambaridae). These species have an identical gene order with typical metazoan mt genome compositions. However, their gene arrangement was very distinctive compared with the pan-crustacean ground pattern because of the presence of a long inverted block, which included 19 coding genes and a control region (CR). Because the CR was inverted, their nucleotide frequencies showed a reversed strand-specific bias compared with the other decapods. Based on a comparative analysis of mt genome arrangements between southern and northern hemisphere crayfish and their putative close marine relative (*Homarus americanus*, a true clawed lobster), we postulated that the ancestor of freshwater crayfish had a typical pan-crustacean mtDNA gene order, similar to its marine relatives. Based on this assumption, we traced the most parsimonious gene rearrangement scenario of the northern hemisphere crayfish. In a phylogenetic study on the infraordinal relationships in reptan decapods, the lineage Lineata [Thalassinidea (Brachyura, Anomura)] was well supported, while the infraorder positions of Achelata and Astacidea remained unidentified.

Corresponding authors: *Gi-Sik Min*, *Department of Biological Sciences*, *Inha University*, *Incheon 402-751*, *Korea*. *E-mail: mingisik@inha.ac.kr*; *Han-Gu Choi*, *Division of Life Sciences*, *Korea Polar Research Institute*, *KORDI*, *Incheon 406-840*, *Korea*. *E-mail: hchoi82@kopri.re.kr*
Sanghee Kim, *Division of Life Sciences*, *Korea Polar Research Institute*, *KORDI*, *Incheon 406-840*, *Korea*. *E-mail: sangheekim@kopri.re.kr*
Mi-Hyun Park, *Jae-Ho Jung*, *Dong-Ha Ahn*, *Tahera Sultana and Sejoo Kim*, *Department of Biological Sciences*, *Inha University*, *Incheon 402-751*, *Korea*. *E-mails: bbangmi99@paran.com*, *univeri@hanmail.net*, *dongha@inha.ac.kr*, *tahera328@yahoo.com*, *biosejoo@kordi.re.kr*
Joong-Ki Park, *Graduate Program in Cell Biology and Genetics*, *Department of Parasitology*, *College of Medicine*, *Chungbuk National University*, *Cheongju 361-763*, *Korea*. *E-mail: jkpyou@chungbuk.ac.kr*
Present address for Sejoo Kim, *Deep-sea & Marine Georesources Research Department*, *Korea Ocean Research & Development Institute*, *Gyeonggi-do*, *426-744*, *Korea*. *E-mail: biosejoo@kordi.re.kr*

Introduction

The typical metazoan mitochondrial (mt) genome is a single, circular duplex molecule, about 16 kb in size, containing 37 genes encoding 13 proteins, two ribosomal RNA genes and 22 transfer RNA genes, as well as a major noncoding region or control region (CR) including sites for the initiation of transcription and replication (Anderson *et al.* 1981). The gene organization of mtDNAs along with sequence data can provide useful evolutionary information for comparative genomics and facilitate the reconstruction of phylogenetic relationships (Smith *et al.* 1993; Boore *et al.* 1998; Morrison *et al.* 2002; Lavrov & Lang 2005; Singh 2008).

Most vertebrate mt genomes share a typical gene order, excluding groups such as amphibians and birds (Mindell *et al.* 1998; Mueller & Boore 2005). Gene rearrangements,

however, are much more common in invertebrates (Cameron *et al.* 2007). Many crustaceans such as copepods also have highly rearranged genes (Machida *et al.* 2002; Ki *et al.* 2009b). Meanwhile, decapod genomes are relatively stable and many species have experienced gene arrangements typical of a pan-crustacean (Crustacea + Hexapoda) ground pattern (Liu & Cui 2011); for example, of the 34 decapods from which the complete or near complete mt genomes have been determined, 44% (15 out of 34 species) have identical gene orders with pan-crustaceans. If minor rearrangements are excluded (restricted only to tRNAs), only seven species (21%) have experienced major gene rearrangements (in protein-coding and/or rRNAs genes).

Infraorder Astacidea Latrelle 1802 belongs to the suborder Pleocyemata in Decapoda. It is composed of five superfamilies – one of true clawed lobsters (Nephropoidea), one of reef lobsters (Enoplometopoidea), a number of fossil taxa (Palaeopalaemonoidea) and two of crayfish (Astacoidea and Parastacoidea) – and approximately 780 species have been described so far (De Grave *et al.* 2009). To date, only two complete mt sequences from infraorder Astacidea have been determined: one from an Australian freshwater crayfish species, *Cherax destructor* Clark, 1936 (superfamily Parastacoidea) and another from a true clawed lobster, *Homarus americanus* Edwards, 1837 (Nephropoidea). The mt genome of the American lobster, *H. americanus*, is similar to a typical pan-crustacean ground pattern (Kim *et al.* 2011b), while the southern hemisphere crayfish, *C. destructor*, has a novel mt genome arrangement owing to at least seven gene rearrangement events, namely, five transpositions and two translocations (transposition + inversion) (Miller *et al.* 2004). Recent phylogenetic studies support the monophyly of northern and southern hemisphere freshwater crayfish and their sister group relationship with true clawed lobsters in the Astacidea (Crandall *et al.* 2000; Breinholt *et al.* 2009). The divergence time of crayfish lineages from Nephropoidea was estimated between 239 and 278 millions of years ago (Mya) from the recent molecular clock studies (Porter *et al.* 2005; Breinholt *et al.* 2009). Considering the close relationship between freshwater crayfish and marine clawed lobsters, the large difference between their mt genome structure is somewhat surprising. Thus, it is important to explore more gene rearrangements from additional astacidean mtDNAs, especially from northern hemisphere crayfish, to better understand the evolutionary processes and mechanisms of gene rearrangements in the infraorder Astacidea.

Here, we report the complete mt DNA sequences of two northern hemisphere freshwater crayfish, *Cambaroides similis* (Koelbel, 1892) (originating from Korea) and *Procambarus clarkii* (Girard, 1852) (eastern United States). We postulate the mt genome structure of a freshwater crayfish ancestor based on comparative analysis of the mt genomes of three astacidean superfamilies and hypothesize that the mt gene arrangement process occurred in northern hemisphere crayfish. In addition, we attempted to solve the infraordinal relationships of Reptantia (crawling decapod groups) by phylogenetic analysis using sequence data from mitochondrial protein-coding genes.

Materials and methods

Sampling and molecular techniques

Korean freshwater crayfish (*C. similis*) were collected by hand from a ravine in the Gwanak Mountain in South Korea (37°27′N, 126°57′E). Red swamp crayfish (*P. clarkii*) were purchased from a pet market in Incheon, South Korea.

Total genomic DNA was extracted using a QIAamp tissue kit (Qiagen Inc., CA, USA) and used as a template for polymerase chain reaction (PCR) amplification. The partial sequences of *cox1*, *rrnL* and *nad3* genes were determined using previously published primers for *cox1* (Folmer *et al.* 1994) and *rrnL* (Crandall & Fitzpatrick 1996), and the newly designed *nad3* primers: DecaND3F (5′-AAA AAA ACT ATT AYW GAY CGA GAR AA-3′) and DecaND3R (5-CAC CGT RAT TTC WAC ATC AAA AAT-3′). The PCRs were performed in a total volume of 25 μL containing 10 pg genomic DNA, 2.5 μL 10× AccuPrime™ PCR Buffer II, 10 pmol of each primer and 0.5 units (0.1 μL) of AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen Co., CA, USA) using the following cycle conditions: one cycle of 120 s at 92 °C (initial denaturation), 35 cycles of denaturation-primer annealing-elongation (96 °C for 10 s, 45–55 °C for 30 s, 68 °C for 90 s) and a subsequent 10 min final extension step at 72 °C. The sequences from each gene fragment were then used to design taxon-specific primers (Table S1 Supporting Information) for long PCR amplification using AccuPrime™ *Taq* DNA Polymerase High Fidelity under the following conditions: one cycle of 120 s at 92 °C, 35 cycles of denaturation-primer annealing-elongation (10 s at 96 °C, 20 s at 55 °C and 12 min at 68 °C) and a final extension (10 min at 72 °C). The amplified PCR products were isolated on 1.0% agarose gel containing crystal violet, excised and extracted according to the TOPO XL gel-purifying protocol (Invitrogen Co.). Purified DNA fragments were cloned into *Escherichia coli*-competent cells using the TOPO XL PCR Cloning kit, following the manufacture's protocol. Sequencing reactions of the target fragments were performed in both directions by 'primer walking' using a Big Dye Terminator Cycle-Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and overlapping fragments were assembled to complete the sequence of the entire genome.

Gene annotation and sequence analysis

The location of the 13 protein coding and two rRNA genes was initially identified by DOGMA (Wyman *et al.* 2004) using the default settings and refined through the comparison with the mtDNA genomes of H. americanus (Kim *et al.* 2011b) and C. destructor (Miller *et al.* 2004) by the NCBI's open reading frame (ORF) Finder. We attempted to identify tRNAs using tRNAScan-SE 1.21 (Lowe & Eddy 1997) in the default search mode using mitochondrial/chloroplast DNA as the source and the invertebrate mitochondrial genetic code for tRNA prediction. Remaining tRNA genes were identified by searching for anticodon consensus motif sequences (TxxxR; xxx = anticodon) and manually examining potential secondary structures. The hairpin structure in the CR was predicted using the mfold web server (Zuker 2003) with the default options, except that the folding temperature was set to T = 50 °C. The conserved blocks in the *C. similis* and *P. clarkii* CR were identified by pairwise sequence comparison between two species using ClustalW, which was implemented in BioEdit 7.0.5.3 (Hall 1999) using default settings. Nucleotide frequency, composition, and skewness of protein-coding genes were calculated using DAMBE 5.2.57 (Xia & Xie 2001).

Phylogenetic analysis

To reconstruct a mitochondrial genome phylogeny based on both nucleotide and putative amino acid sequences, we performed maximum likelihood (ML) and Bayesian inference (BI) using PhyML 3.0 (Guindon & Gascuel 2003) and MrBayes 3.12 (Ronquist & Huelsenbeck 2003), respectively.

The nucleotide and amino acid sequences of 13 protein-coding genes from complete mt genomes of 36 decapods and a non-decapod outgroup species, *Euphausia superba* (Euphausiacea: EU583500), were used in the phylogenetic analysis (Table S2 Supporting Information). Nucleotide sequences of all protein-coding genes were translated into amino acid sequences by BioEdit, and then aligned based on their amino acid sequences by ClustalW in BioEdit. For the nucleotide datasets, the amino acid alignment was back-translated into the corresponding nucleotide sequences. All individual gene alignments were concatenated using DAMBE.

Because the mt genomes of the two northern hemisphere crayfish species showed completely reversed strand bias patterns compared with all other decapods (Table 1), the nucleotide dataset was adjusted using the neutral transitions excluded (NTE) method (Hassanin *et al.* 2005): purines were coded by R, while pyrimidines were coded by Y at all third codon positions, first positions of the CTN (*trnL2*) and TTN (*F* and *L1*) codons, and at the first and second positions of ACN (*T*), ATN (*I* and *M*), GCN (*A*), and GTN (*V*) codons. In the amino acid dataset, two northern hemisphere crayfish sequences (*C. similis* and *P. clarkii*) were not included in the phylogenetic analysis to remove false inference caused by the reversed strand bias pattern of their nucleotides.

For the nucleotide dataset, the model GTR + I + G was selected using the Akaike information criterion (AIC) by jModelTest 0.1.1 (Posada 2008) for both ML and BI analyses. The MtRev + I + G was chosen as the best-fit model for the amino acid sequence dataset using ProtTest 2.0 (Abascal *et al.* 2005).

In the ML analysis, the node reliability was estimated by non-parametric bootstrap analysis with 500 random replications. For BI analysis, four Markov Chain Monte Carlo chains were run for 1 000 000 generations and sampled every 1000 generations. Bayesian posterior probability values representing the probability of samples recovering a particular clade were estimated after the initial 200 trees (20%) were discarded as burn-in.

Results and discussion

Basic features of the northern hemisphere crayfish mitochondrial genome

The complete mt genomes of *C. similis* (GenBank accession number, JN991196) and *P. clarkii* (JN991197) were 16 220 and 15 928 bp in length, respectively. The overall A + T content of the mt genome was 71.6% for *C. similis* (38.8% T, 32.8% A, 11.2% C and 17.2% G) and 72.9% for *P. clarkii* (41.1% T, 31.8% A, 9.5% C and 17.7% G), similar to other decapod species (Liu & Cui 2011) but slightly higher than that of *H. americanus* (69.5%) (Kim *et al.* 2011b) and significantly higher than that of *C. destructor* (62.4%) (Miller *et al.* 2004).

Both northern hemisphere crayfish mt genomes contained 13 protein-coding genes, two rRNA genes, 22 tRNAs and a CR that is typical of other metazoans (Boore 1999) (Tables S3 and S4 Supporting Information). The mt gene orders of the two northern hemisphere crayfish were identical, but were significantly different than that of the southern hemisphere crayfish. Their gene arrangement was also very distinctive compared with the pan-crustacean ground pattern (Boore *et al.* 1998) because of the presence of a long inverted block bound by *trnF* and the CR. The inverted block was approximately 10 kb (10 275 bp in *C. similis* and 9973 bp in *P. clarkii*) in length and included 19 genes [six protein-coding genes (*nad1*, *nad4*, *nad4L*, *nad5*, *nad6* and *cytb*), two ribosomal RNA genes (*rrnS* and *rrnL*), and 10 tRNA genes (*trnF*, *H*, *I*, *L1*, *N*, *P*, *Q*, *S1*, *S2*, and *V*) and a CR]. In addition to this inversion, other independent gene rearrangements were observed at four tRNAs (*trnP*, *Q*, *N* and *SI*) (Figs 1 and 4).

Table 1 Genomic characteristics of decapod mtDNAs

	Non-inverted region in northern hemisphere crayfish				Inverted region in northern hemisphere crayfish							
	Atp6* + *Atp8* + *Cox1* + *Cox2* + *Cox3* + *Nad2* + *Nad3				***Cytb* + *Nad6***				***Nad1* + *Nad4* + *Nad4L* + *Nad5***			
Species	Size (bp)	Strand[a]	AC %	GC skew	Size (bp)	Strand[a]	AC %	GC skew	Size (bp)	Strand[a]	AC %	GC skew
Penaeus monodon	5190	+	47.38	−0.12	1653	+	46.34	−0.18	4293	−	40.67	0.15
Marsupenaeus japonicus	5187	+	49.01	−0.17	1647	+	49.00	−0.29	4302	−	38.89	0.27
Litopenaeus vannamei	5187	+	48.75	−0.16	1647	+	46.15	−0.24	4293	−	39.71	0.24
Fenneropenaeus chinensis	5187	+	48.88	−0.17	1647	+	47.78	−0.26	4293	−	38.97	0.25
Farfantepenaeus californiensis	5190	+	48.11	−0.15	1647	+	47.96	−0.30	4290	−	40.46	0.22
Litopenaeus stylirostris	5190	+	48.44	−0.16	1647	+	46.94	−0.25	4293	−	39.38	0.25
Alpheus distinguendus	5175	+	53.99	−0.26	1668	+	55.34	−0.35	4272	−	35.37	0.29
Macrobrachium lanchesteri	5178	+	53.92	−0.25	1647	+	52.34	−0.28	4269	−	33.80	0.30
Macrobrachium nipponense	5175	+	54.59	−0.24	1647	+	55.13	−0.33	4272	−	33.12	0.31
Macrobrachium rosenbergii	5184	+	57.14	−0.28	1644	+	56.50	−0.35	4269	−	32.24	0.31
Halocaridina rubra	5184	+	52.58	−0.26	1635	+	53.89	−0.35	4281	−	34.15	0.36
Exopalaemon carinicauda	5172	+	52.84	−0.26	1647	+	52.21	−0.31	4269	−	34.32	0.32
Pagurus longicarpus	5199	+	48.75	−0.15	1635	+	50.70	−0.27	4269	−	36.34	0.29
Shinkaia crosnieri	5190	+	48.48	−0.24	1641	+	49.18	−0.33	4263	−	36.10	0.41
Portunus trituberculatus	5196	+	47.3	−0.21	1638	+	44.87	−0.23	4311	−	39.71	0.32
Callinectes sapidus	5193	+	49.82	−0.25	1638	+	49.03	−0.30	4293	−	37.97	0.35
Pseudocarcinus gigas	5196	+	48.48	−0.21	1638	+	48.41	−0.28	4317	−	37.71	0.35
Geothelphusa dehaani	5187	+	47.61	−0.24	1632	+	45.89	−0.26	4284	−	37.75	0.40
Charybdis japonica	5187	+	47.83	−0.20	1638	+	48.05	−0.24	4311	−	39.50	0.29
Eriocheir hepuensis	5202	+	47.77	−0.18	1656	+	46.25	−0.22	4296	−	37.98	0.36
Eriocheir japonica	5202	+	47.69	−0.19	1656	+	46.68	−0.24	4296	−	38.10	0.35
Eriocheir sinensis	5202	+	48.09	−0.20	1656	+	46.49	−0.24	4296	−	37.38	0.37
Gandalfus yunohana	5193	+	48.87	−0.24	1638	+	47.74	−0.24	4317	−	37.95	0.36
Scylla olivacea	5196	+	48.05	−0.23	1638	+	48.11	−0.30	4311	−	38.49	0.34
Scylla paramamosain	5196	+	46.73	−0.20	1638	+	46.76	−0.25	4311	−	39.67	0.35
Scylla serrata	5196	+	46.39	−0.20	1638	+	46.03	−0.21	4311	−	39.80	0.35
Scylla tranquebarica	5196	+	45.94	−0.20	1638	+	45.24	−0.24	4314	−	39.48	0.37
Xenograpsus testudinatus	5226	+	48.45	−0.22	1647	+	46.75	−0.25	4281	−	37.80	0.39
Panulirus ornatus	5193	+	50.11	−0.17	1647	+	47.48	−0.20	4308	−	37.14	0.25
Panulirus japonicus	5190	+	47.7	−0.14	1647	+	47.72	−0.23	4308	−	38.46	0.20
Panulirus stimpsoni	5190	+	50.14	−0.19	1647	+	49.43	−0.24	4308	−	36.89	0.27
Upogebia major	5187	+	50.03	−0.21	1641	+	49.06	−0.24	4302	−	36.12	0.31
Homarus americanus	5190	+	47.67	−0.16	1650	+	45.15	−0.19	4299	−	38.15	0.26
Cherax destructor	5193	+	51.72	−0.22	1659	+	52.08	−0.32	4272	−	35.86	0.34
Average	5191	+	49.25	−0.20	1645	+	48.74	−0.27	4294	−	37.49	0.31
Cambaroides similis	5175	+	41.59	0.12	1650	−	44.91	−0.22	4296	+	38.41	0.30
Procambarus clarkii	5175	+	38.34	0.22	1647	−	49.79	−0.31	4302	+	36.03	0.42
Average	5175	+	39.97	0.17	1649	−	47.35	−0.27	4299	+	37.22	0.36

Results of northern hemisphere crayfishes are highlighted in grey.

[a]'+' and '−' in strands indicate heavy and light chains, respectively.

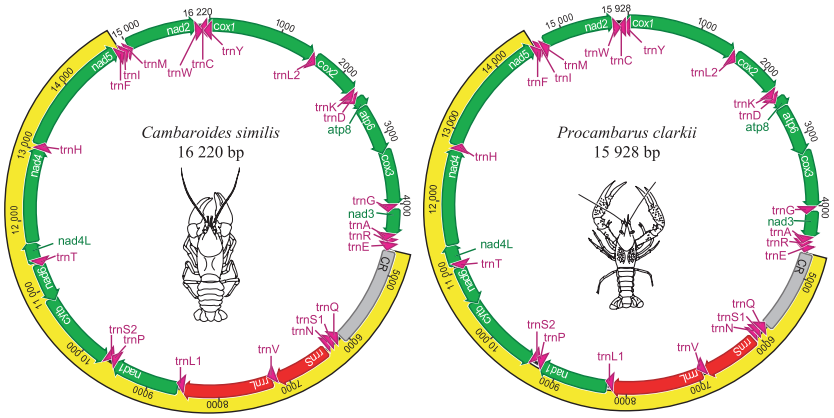

Fig. 1 Mitochondrial genome maps of *Cambaroides similis* and *Procambarus clarkii*. The transcriptional directions are indicated by arrowheads. Yellow arcs on the outside of the gene maps denote the inverted blocks.

The northern hemisphere freshwater crayfish have shorter rRNA genes, but their A + T contents are similar to other decapods. The lengths of *rrnL* in other decapods were 1296–1369 bp, whereas those of *rrnL* in *C. similis* and *P. clarkii* were 1264 bp (AT% = 75.6) and 1262 bp (AT% = 76.5), respectively. Thus, of these decapods, the northern hemisphere crayfishes have the shortest *rrnL* genes. The *rrnS* gene of *C. similis* and *P. clarkii* is 787 (AT% = 75.1) and 786 bp (AT% = 76.5) in length, respectively, whereas those of decapods ranged from 785 to 917 bp. Interestingly, *C. destructor* has the longest *rrnS* (917 bp) among the decapods, while those of northern hemisphere crayfish are the shortest, excluding *Callinectes sapidus* Rathbun, 1896 (785 bp).

Both *C. similis* and *P. clarkii* had the typical 22 tRNA genes, which ranged from 61 to 70 bp in length, and all of them (except *trnS1*) formed a typical cloverleaf secondary structure. The *trnS1* contained no stem in the dihydrouridine (DHU) arm, which is also common in metazoans (Wolstenholme 1992; Yamauchi *et al.* 2003) (Figs S1 and S2 Supporting Information).

Features of northern hemisphere crayfish inversion

Many gene rearrangements in mt genomes, such as gene transpositions and inversions, have been reported in metazoans. This is also a general phenomenon in many crustaceans (Machida *et al.* 2002; Tjensvoll *et al.* 2005; Podsiadlowski & Bartolomaeus 2006; Ki *et al.* 2009b; Ito *et al.* 2010). Typically, transpositions are common in decapods, while inversions are not frequently observed. Inversion events in decapods occur exclusively in tRNAs and have not been reported in other mt gene domains, such as the protein-coding genes, rRNA genes, and the CR. Therefore, this is the first report of decapod inversion occurring both in tRNAs and the other mt regions. To the best of our knowledge, this is the longest inversion block ever observed in metazoan mt genomes.

A long fragment inversion of 8734 bp, including 18 coding genes (six protein-coding, two rRNAs and nine tRNAs genes), was previously reported in the *Priapulus caudatus* Lamarck, 1816 (Priapulida: Priapulidae) mt genome (Webster *et al.* 2007). The *P. caudatus* and two northern hemisphere crayfish shared the same composition of protein-coding genes (*nad1*, *nad4*, *nad4L*, *nad5*, *nad6* and *cytb*) as well as two rRNAs in their inverted fragments. However, the composition of tRNAs were different and the CR was not included in *P. caudatus*. Interestingly, the long fragment inversions from both northern hemisphere crayfish and a priapulid occurred in similar mt regions located between *nad3* and *nad5*, and between the CR and *nad2*.

Change of strand-specific nucleotide frequency bias by inversion

The strand-specific bias in nucleotide frequencies is a common phenomenon in most metazoan mt genomes (Asakawa *et al.* 1991). The heavy (+) strand contains more cytosine (C) and adenine (A), while the light (−) strand consequently contains more guanine (G) and thymine (T). Statistically, this can be reflected by a GC skew (Perna & Kocher 1995). It has been suggested that the strand-specific bias may be associated with the asymmetrical replication of mtDNA that occurs in the CR. Thus, the bias trait can be altered if the CR is inverted (Kilpert & Podsiadlowski 2006). Our result could be an evidence to support this hypothesis because the CRs of northern hemisphere crayfish are included in the inverted fragments. In northern hemisphere crayfish, the average A + C% of (+) genes (*atp6*, *atp8*, *cox1*, *cox2*, *cox3*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, and *nad5*) was 38.7% with a positive GC skew, while those of (−) genes (*cytb* and *nad6*) was 47.4% with a negative GC skew, whose (−) and (+) numbers were completely reversed compared to other decapods (Table 1). This observation further sug-

gests that the strand-specific bias in nucleotide frequencies can be changed by CR inversion.

Structural features of the control region

Cambaroides similis has 13 non-coding regions (1624 bp in length) in the mtDNA, while *P. clarkii* has 14 (1318 bp in length) (Tables S3 and S4 Supporting Information). The whole mt genome length of *C. similis* (16 220 bp) is 292 bp longer than that of *P. clarkii* (15 928 bp). If the length difference between the non-coding regions is excluded, *C. similis* is only 14 bp longer than *P. clarkii*. Therefore, the lengths of the coding genes are very similar between the two species.

Each species has a long, non-coding sequence between the *trnE* and *trnS1* genes, and their lengths are 1455 bp (AT% = 77.7) in *C. similis* and 1188 bp (AT% = 80.9) in *P. clarkii* (Tables S5 and S6 Supporting Information). With the higher A + T ratio, the long lengths and the location in the mt genomes, these major coding sequences are assigned to a putative CR. All other non-coding sequences range between 1 and 76 bp; therefore, they are considered intergenic spacers.

The primary CR DNA sequence exhibits a high level of variation compared with other coding regions in the mtDNA (Arif & Khan 2009). No apparent conserved sequences were observed when our CR sequences were compared to other decapods, including two astacidean species, *H. americanus* and *C. destructor*. However, five highly conserved sequence blocks (blocks A–E) were found between *C. similis* and *P. clarkii*. Furthermore, identical sequence stretches of more than 10 nucleotides were found at five regions (10, 11, 13, 20 and 26 bp in length, respectively) (Fig. 2). The high primary CR sequence conservation between the two species was unexpected considering their distant phylogenetic relationship. Traditionally, *C. similis* and *P. clarkii* have been classified as members of the subfamilies Cambaroidinae and Cambaridinae, respectively, in the family Cambaridae. However, recent molecular studies have shown differences at more than just the family level, and the Asiatic subfamily Cambaroidinae should be separated from Cambaridae and classified as a new family (Ahn *et al.* 2006; Braband *et al.* 2006). The divergence history of these subfamilies is also supported by recent molecular clock data: the two northern hemisphere crayfish subfamilies diverged around 153 Mya, after the divergence of northern and southern hemisphere crayfish, which occurred around 183 Mya (Breinholt *et al.* 2009). Based on these findings, we conclude that the CR sequences in northern hemisphere crayfish are exceptionally conserved, and that the conserved blocks might reflect their functional efficiency, which is important for mtDNA replication and transcription.

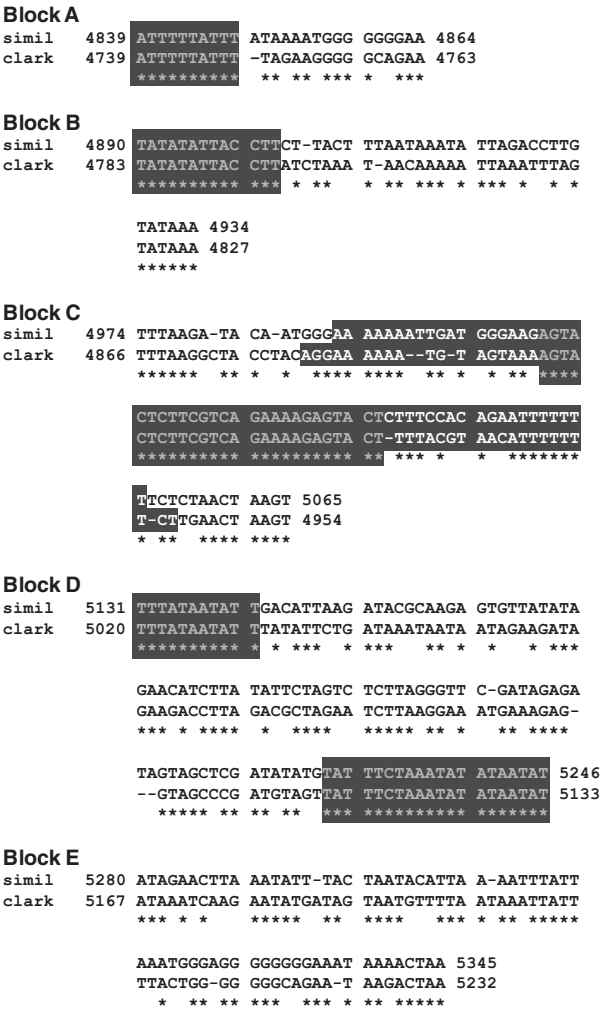

Fig. 2 Conserved sequence blocks in the control regions of *Cambaroides similis* and *Procambarus clarkii*. Identical sequence regions of more than 10 bp between the two species are shaded in grey. Sequences of the hairpin structures are in a box. Asterisks indicate the consensus sequences between the two species.

A putative hairpin structure, which consists of two loops and two stems, was found in the sense sequences of block C from both species (Fig. 3). The identical 26 bp sequence regions between the two species consist of a larger terminal loop (composed of eight nucleotides) and an adjacent upper stem region with nine base pairing. However, the second loop and terminal stem were different between the two species in nucleotide composition and stem length base pairing. The flanking sequences adjacent to the second stem region contain motifs that are conserved across many crustaceans and insects, including the 'TATA' motif in the 5′-flanking region and the 'GACT' motif in the 3′-flanking region (Kilpert & Podsiadlowski 2006). The two

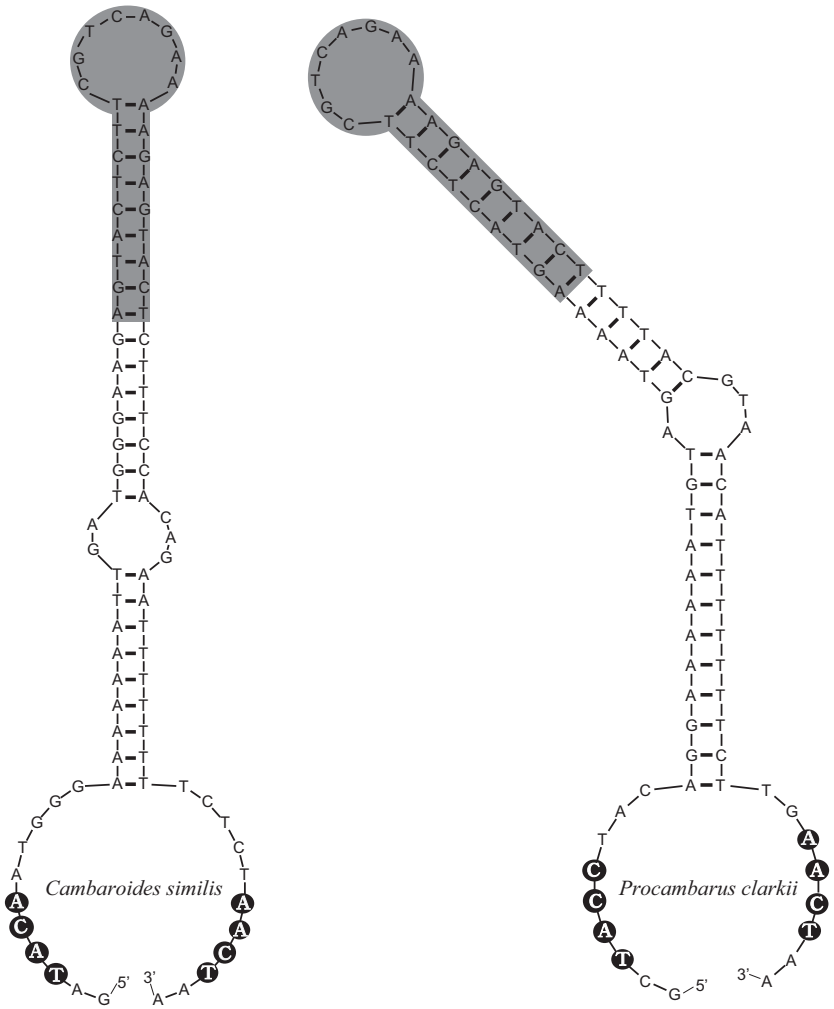

Fig. 3 Hairpin structures in the mitochondrial control regions of *Cambaroides similis* and *Procambarus clarkii*. Conserved motifs are depicted in a black dot with the sequence inside shown in white. The identical sequence regions of both species are shaded in grey.

northern hemisphere crayfish species have identical 'AACT' motifs, which are comparable with the 'TATA' motif at the 5′-flanking region. The 'GACT' motif was different between the two species, and the 'TACA' motif in *C. similis* and 'TACC' in *P. clarkii* were found at the 3′-flanking region (Fig. 3). Also, the flanking motifs of northern hemisphere crayfish were found in the opposite direction relative to most other crustaceans, but they were in the same direction as in *Ligia oceanica* (Linnaeus, 1767) (Crustacea, Isopoda) (Kilpert & Podsiadlowski 2006). This observation provides additional evidence that the CRs of two northern hemisphere crayfish species were inverted as part of a long inverted segment.

Gene order of the mt genome in the freshwater crayfish ancestor

According to a recent study (Kim *et al.* 2011b), *H. americanus* (superfamily Nephropoidea), which may be the closest marine relative to freshwater crayfish (Crandall *et al.* 2000; Ahyong & O'Meally 2004), has a mt genome arrangement identical to pan-crustaceans (Fig. 4B). *Enoplometopus debelius* Holthuis, 1983 (superfamily Enoplometopoidea), another superfamily member of the infraorder Astacidea, also has the same gene order (S. Kim & G.S. Min, unpublished data). There is a long fragment inversion and a small number of tRNA transpositions in the mtDNA of northern hemisphere crayfish. However, their gene order is typical of pan-crustaceans (except for four tRNA transpositions) when inversion is disregarded (Fig. 4). The southern hemisphere freshwater crayfish, *C. destructor*, has an enigmatic gene order with at least seven rearrangements in the complete mt genome (Miller *et al.* 2004) (Fig. 4A). In addition, there is no evidence of a shared plesiomorphic gene rearrangement pattern between southern and northern hemisphere crayfish. Summarizing these results, we inferred that the ancestor of freshwater crayfish had a typical pan-crustacean mtDNA gene order, similar to its marine relatives, and the novel gene orders

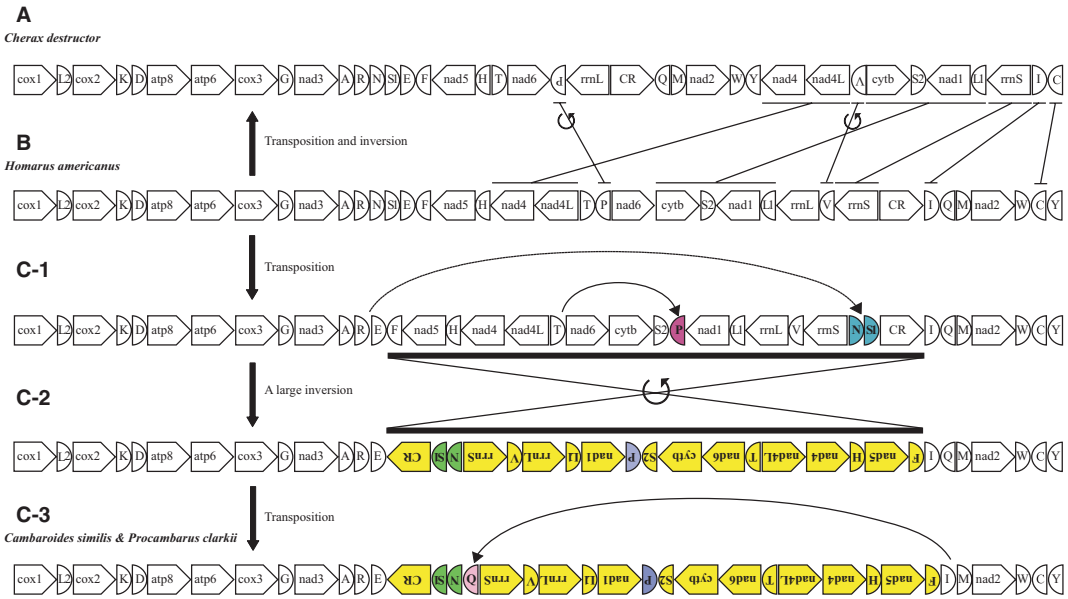

Fig. 4 Comparison of the mitochondrial genome organization of infraorder Astacidea. —A. Gene order of *Cherax destructor* (superfamily Parastacoidea, a southern hemisphere freshwater crayfish). —B. Gene order of *Homarus americanus* (Nephropoidea, a true clawed lobster). The mitochondrial gene order of ancestral pan-crustaceans is well retained in this species. —C. The putative parsimonious gene rearrangement process for *Cambaroides similis* and *Procambarus clarkii* (superfamily Astacoidea, northern hemisphere freshwater crayfishes). —C-1. Transpositions of *trnN/trnS1* cluster (one event) and *trnP* (one event). Because a *trnP* moved within an inverted block, its transposition could be performed by one event regardless of whether it occurred before or after the inversion. —C-2. Inversion occurred in a large fragment bound by *trnF* and CR (one event). —C-3. Transposition of *trnG* (one event). Genes transposed between *H. americanus* and *C. destructor* are underlined and linked by a black line. Inversion is specified by a rotating arrow. Positions of the inverted blocks between *H. americanus* and northern hemisphere freshwater crayfish are shown with thick black lines and specified by the rotating arrow with thin black lines. The transposition route is indicated by an arrowhead line. Genes involved in different rearrangement events are shown in different colours.

of northern and southern hemisphere crayfish were acquired independently after separation.

Parsimonious scenario of northern hemisphere crayfish gene rearrangement

The gene rearrangement pattern of northern hemisphere crayfish is relatively simple, and four events may be sufficient to estimate the most parsimonious gene rearrangement. First, transposition of the *trnN-SI* block occurred before inversion. If this event occurred after inversion, one transposition and one inversion (two events) would be required (Fig. 4C-1). Second, a long fragment inversion occurred (Fig. 4C-2). Third, transposition of *trnQ* occurred. Because *trnQ* is not inverted, transposition of *trnQ* after inversion was most parsimonious, otherwise one more event (inversion) would be required (Fig. 4C-3). Finally, transposition of *trnP* occurred before or after inversion. Because the position of *trnP* (between *trnT* and *nad6*) was originally within the inverted fragment, its transposition/inversion could be completed by one event regardless of its occurrence before or after inversion (Fig. 4C-1).

Mitochondrial molecular phylogeny

Among the major Decapoda lineages, relationships within the Reptantia are the most controversial based on both morphology and molecular data. There is no clear consensus on their phylogeny. To determine decapod phylogeny, especially for the major reptant lineage relationships, we used four more reptant sequences: two from northern hemisphere crayfish species, which were determined in this study, and two published sequences of *Upogebia major* (the first representative of the infraorder Thalassinidea) (Kim *et al.* 2011a) and *H. americanus* (Kim *et al.* 2011b). The latter two sequences were published recently without phylogenetic inference. Therefore, they were not used extensively in the decapod phylogenetic studies.

Phylogenetic trees from the ML and BI inferences using nucleotide and amino acid sequences supported monophyly of all of the major decapod infraorders with high ML bootstrap percentages (BP > 90 in ML) and posterior probability values (BPP = 100 in BI) from both datasets (Fig. 5). The subdivision of Decapoda into two suborders (Dendrobranchiata and Pleocyemata), and a basal position for Caridea within Pleocyemata, are also supported, con-

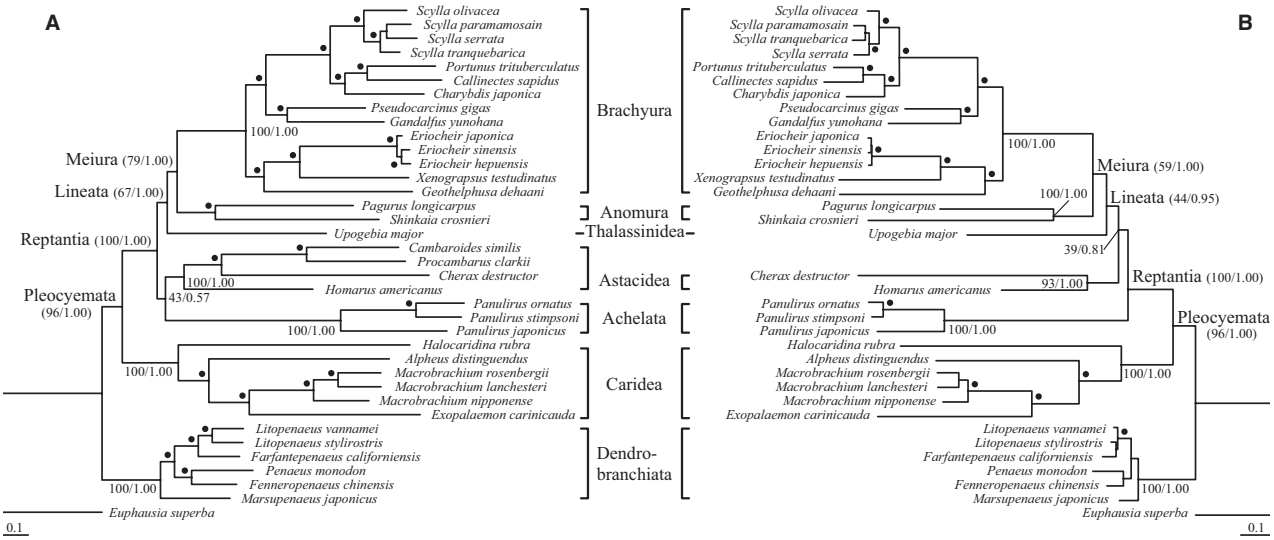

Fig. 5 Phylogenetic analyses of the nucleotide (A) and amino acid sequence datasets (B) derived from the Decapoda using the maximum likelihood analysis and Bayesian inference, respectively. The bootstrap support percentage (BP: left) and Bayesian posterior probability (BPP: right) of each node are denoted directly for infraorders or higher groups. The supporting values within infraorders are denoted by the dots indicating the well-supported values (BP > 90 and simultaneously BPP > 0.90), or otherwise are omitted. Scale bars represent 0.1 mutations per site.

sistent with earlier studies that used morphological and molecular data (Kim & Abele 1990; Scholtz & Richter 1995; Ahyong & O'Meally 2004; Liu & Cui 2011; Qian *et al.* 2011).

Although the monophyly of Reptantia is generally accepted, the interrelationships of infraordinal reptant clades remain unknown (Scholtz & Richter 1995; Ahyong & O'Meally 2004). In our analysis, Thalassinidea clustered with Meiura (Brachyura + Anomura) as a sister group. This relationship was supported by BI analysis (BPP = 1.00 and 0.95 from nucleotide and amino acid datasets, respectively) but not strongly by the ML analysis (BP = 67 and 44). Because Brachyura, Anomura and Thalassinidea share a carapace lineae, a new name, Lineata, was recently proposed to group the three infraorders (Ahyong & O'Meally 2004). The existence of Lineata has been supported by some morphological and molecular studies (Scholtz & Richter 1995; Ahyong & O'Meally 2004; Boisselier-Dubayle *et al.* 2010) but refuted by others (Crandall *et al.* 2000; Porter *et al.* 2005; Tsang *et al.* 2008; Robles *et al.* 2009; Toon *et al.* 2009). The relationship of Achelata and Astacidea with other reptant clades is unclear. In the phylogenetic tree based on the nucleotide dataset in the present study, Achelata grouped with Astacidea (sister to Lineata), but this had weak support (BP = 43, BPP = 0.57: Fig. 5A). In the amino acid dataset (Fig. 5B), Achelata was positioned basally within the Reptantia as sister to Astacidea + Lineata (BP = 39, BPP = 0.81). The sister group relationship with Astacidea and Achelata is consistent with other molecular phylogenetic studies based on molecular data (Tsang *et al.* 2008; Toon *et al.* 2009). However, some previous studies support the basal position of Achelata in Reptantia based on morphology (Scholtz & Richter 1995) and combined analysis of morphology and molecules (Ahyong & O'Meally 2004). An alternative grouping, such as a sister group relationship, between Meiura and Achelata has also been suggested by other studies based on morphology (Dixon *et al.* 2003) and fossils (Schram & Dixon 2004). Many phylogenetic studies have inferred the positions of Astacidea and Achelata in the Reptantia based on the complete mtDNA sequence. Some studies (Miller & Austin 2006; Ki *et al.* 2009a; Boisselier-Dubayle *et al.* 2010; Qian *et al.* 2011) suggest a sister group relationship only, whereas others (Podsiadlowski & Bartolomaeus 2006; Liu & Cui 2010, 2011) support both a sister group relationship of two species and a basal position of Achelata in the Reptantia, based on nucleotide and amino acid datasets.

Appropriate and extensive taxon sampling is one of the most important determinants of accurate phylogenetic estimation. Increased taxon sampling can significantly reduce phylogenetic error (Zwickl & Hillis 2002; Heath *et al.* 2008). Even though more complete mtDNAs from the Reptantia were used in this phylogenetic study, taxon sampling was likely insufficient to obtain reliable infraordinal relationships. For example, although we used three

Achelata mt genome sequences, all of them are in the same genus (*Panulirus ornatus*, *Panulirus japonicus*, and *Panulirus stimpsoni*) of the family Palinuridae. Therefore, mtDNAs from other Achelata families are required to improve the accuracy of the phylogenetic inference of Achelata in Reptantia. Thalassinidea has the same difficulties because only one sequence was available. Therefore, to achieve increased resolution of the infraordinal reptan phylogeny, additional mt genome sequences from phylogenetically distant taxa in Achelata and Thalassinidea, and new sequence information on infraorders of Glypheidea and Polychelida, are required to better establish the decapod phylogeny.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Putative secondary structures for 22 tRNA genes in the mitochondrial genome of *Cambaroides similis*.

Fig. S2. Putative secondary structures for 22 tRNA genes in the mitochondrial genome of *Procambarus clarkii*.

Table S1. Taxon-specific primers used for long PCR amplification of mitochondrial fragments from *Cambaroides similis* and *Procambarus clarkii*.

Table S2. List of species used in the phylogenetic analysis.

Table S3. Mitochondrial genome organization of *Cambaroides similis*.

Table S4. Mitochondrial genome organization of *Procambarus clarkii*.

Table S5. Nucleotide composition of the mitochondrial genome of *Cambaroides similis*.

Table S6. Nucleotide composition of the mitochondrial genome of *Procambarus clarkii*.

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