Gene 520 (2013) 64–72

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

The complete mitochondrial genome of Arctic *Calanus hyperboreus* (Copepoda, Calanoida) reveals characteristic patterns in calanoid mitochondrial genome

Sanghee Kim [a], Byung-Jin Lim [b], Gi-Sik Min [b], Han-Gu Choi [a,*]

[a] Department of Life Sciences, Korea Polar Research Institute, KIOST 12 Gaetbeol-ro, Yeonsu-gu, Incheon 406-840, Republic of Korea
[b] Department of Biological Sciences, Inha University, Incheon 402-751, Republic of Korea

ARTICLE INFO

Article history:
Accepted 20 September 2012
Available online 4 October 2012

Keywords:
Arctic
Calanus hyperboreus
Calanoida
Copepoda
Mitochondria
Genome

ABSTRACT

Copepoda is the most diverse and abundant group of crustaceans, but its phylogenetic relationships are ambiguous. Mitochondrial (mt) genomes are useful for studying evolutionary history, but only six complete Copepoda mt genomes have been made available and these have extremely rearranged genome structures. This study determined the mt genome of *Calanus hyperboreus*, making it the first reported Arctic copepod mt genome and the first complete mt genome of a calanoid copepod. The mt genome of *C. hyperboreus* is 17,910 bp in length and it contains the entire set of 37 mt genes, including 13 protein-coding genes, 2 rRNAs, and 22 tRNAs. It has a very unusual gene structure, including the longest control region reported for a crustacean, a large tRNA gene cluster, and reversed GC skews in 11 out of 13 protein-coding genes (84.6%). Despite the unusual features, comparing this genome to published copepod genomes revealed retained pan-crustacean features, as well as a conserved calanoid-specific pattern. Our data provide a foundation for exploring the calanoid pattern and the mechanisms of mt gene rearrangement in the evolutionary history of the copepod mt genome.

1. Introduction

Calanus hyperboreus Kroyer, 1838 is a dominant Arctic calanoid copepod. It occurs from the surface to the ocean floor in subarctic and Arctic seas, where three *Calanus* species constitute over 70% of the zooplankton biomass: *C. hyperboreus*, *C. glacialis*, and *C. finmarchicus* (Head et al., 2003). It has a 3- to 4-year life cycle and spends the winter in diapause (Head and Harris, 1985; Hirche, 1997). In the Labrador Sea, *C. hyperboreus* contributes a maximum of 54% to the total biomass over the continental shelves, and is most abundant near the deep ocean floor in the Greenland Sea (Head et al., 2003; Hirche et al., 2006). Due to their large biomass, *Calanus* copepods are key components of the food web as food for many ocean animals (Fort et al., 2010; Hopkins and Nilssen, 1991; Jensen et al., 1991; Pendleton et al., 2009). In addition, *Calanus* spp. might function as an indicator of climate change because their spatial distribution is closely associated with variation in the distribution and dynamics of water masses in the Arctic Ocean (Hirche and Kosobokova, 2007; Kwasniewski et al., 2003; Parent et al., 2011).

Because copepods are a highly divergent ancient group, estimated to consist of about 13,000 species in 200 families (but which may represent less than 15% of the actual numbers), the lack of consensus relationships among copepod lineages is not surprising (Humes, 1994; Minxiao et al., 2011; Regier et al., 2005). Mitochondrial (mt) DNA is useful for evolutionary studies and reconstructing the phylogenetic relationships of copepods with their ambiguous morphologies and vast diversity (Braga et al., 1999; Machida et al., 2006; Taniguchi et al., 2004). The order of mt genes is thought to reflect evolutionary history because rearrangement occurs over evolutionary time, resulting in more divergent features in distant phylogenetic groups, but shared gene arrangements among closely related ones. The structure of the mt genome is conserved in most metazoans, while different features have been discovered in crustacean groups, such as gene rearrangements and atypical content (Kilpert and Podsiadlowski, 2006; Kim et al., 2011, 2012a). Specifically, the complete and partial mt genome sequences of copepods available in public databases show extremely diverse gene rearrangements and compositions, even among copepod species (Burton et al., 2007; Jung et al., 2006; Ki et al., 2009; Machida et al., 2002, 2004; Minxiao et al., 2011). Copepod genomes are not well sampled, with only six published complete mt genomes (the cyclopoid copepod *Paracyclopina nana*, harpacticoid copepods *Tigriopus japonicus* and *T. californicus*, and siphonostomatoid copepods *Caligus rogercresseyi*, *Caligus clemensi*, and *Lepeophtheirus salmonis*) and two partial sequences (the calanoid copepods *Eucalanus bungii* and *Neocalanus cristatus*) (Burton et al., 2007; Jung et al., 2006; Ki et al., 2009; Machida et al., 2002, 2004; Minxiao et al., 2011; Tjensvoll et

Abbreviations: *Atp6* and *8*, ATPase subunits 6 and 8; *Nad1–6*, NADH dehydrogenase subunits 1–6; *Cox1–3*, cytochrome *c* oxidase subunits 1–3; *Cytb*, cytochrome *b*; tRNA, transfer RNA; PCG, protein-coding gene; *rrnS* and *rrnL*, small and large subunit ribosomal RNAs; nt, nucleotide; mt, mitochondrial; O_L, origin of replication of the light strand; NCR, non-coding region; CR, control region.

* Corresponding author. Tel.: +82 32 260 6162; fax: +82 32 260 6301.
E-mail address: hchoi82@kopri.re.kr (H.-G. Choi).

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http://dx.doi.org/10.1016/j.gene.2012.09.059

al., 2005; Yasuike et al., 2012). Furthermore, all six of these copepods belong to the superorder Podoplea (Misophrioida, Monstrilloida, Mormonilloida, Siphonostomatoida, Harpacticoida, Gelyelloida, and Cyclopoida) (Huys and Boxshall, 1991), while no published complete mt genomes are available for the superorder Gymnoplea, which includes a single order, Calanoida. Recently, the nearly complete mt genome of *Calanus sinicus* was reported; it includes unique genome features, such as six long non-coding regions (NCR) (>100 bp) and an unusually large genome (≥20 kb). Due to the lack of similarity in the gene order among copepod mt genomes and the limited genome data, it is difficult to use this for phylogenetic analyses, and the conservation of the mt gene order in copepods has been questioned (Ki et al., 2009; Minxiao et al., 2011). A comparative analysis of copepod mt genomes requires more data from copepods, including Calanoida. Despite efforts to obtain additional complete mt genomes from copepods, several researchers have reported problems resulting from the changes in DNA structure disrupting polymerase chain reaction (PCR) amplification, the presence of multiple haplotypes in individuals, and nuclear mt pseudogenes (Machida et al., 2004; Minxiao et al., 2011).

In this study, we determined the mt genome of *C. hyperboreus*, which is the first reported Arctic copepod mt genome and the first complete mt genome of a calanoid copepod. The mt genome is considerably longer than other copepod mt genomes and includes characteristic features. However, comparison to other crustacean mt genomes revealed retained pan-crustacean features and a conserved calanoid-specific pattern.

2. Materials and methods

2.1. Sampling, DNA extraction, PCR amplification, and sequencing

Calanus hyperboreus was collected from the Arctic coast near Dasan Station, Ny-Ålesund, Spitsbergen, Norway (78°55′N, 11°56′E) in 2010 and kept in 95% ethanol until further study. *C. hyperboreus* total genomic DNA was extracted from an individual copepod using a DNeasy tissue kit (QIAGEN, USA) and used as a template for PCR amplification. Partial sequences of the *Cox1*, *Cytb*, and *rrnL* RNA genes were determined using primers newly designed from partial *Cox1* and *rrnL* rRNA sequences of *C. hyperboreus* and a partial *Cytb* sequence alignment of various copepods in the NCBI database (Table 1). PCR of four genes was carried out in 30 μL reaction volumes containing 10× PCR buffer, 2.5 mM dNTP mixture, 10 pmol of each primer, and 2.5 units *Taq* polymerase (TaKaRa, Japan). The amplification consisted of 94 °C for 5 min, 30 cycles of 94 °C for 15 s, 52 °C for 30 s, and 72 °C for 1 min, and a final 3 min at 72 °C. Based on the gene fragment sequences obtained, *C. hyperboreus*-specific primers were designed and used for long PCR amplification (Table 1). Overlapping long PCR fragments of 2585–11,898 bp were amplified using LA *Taq* polymerase (TaKaRa, Japan).

The PCR consisted of 94 °C for 10 min, 40 cycles of 98 °C for 10 s and 68 °C for 10 min, with a final 20 min at 68 °C. The amplified PCR products were purified and cloned into *Escherichia coli* competent cells using a TOPO XL PCR cloning kit (Invitrogen, USA), as recommended by the manufacturer, or sequenced directly with the long PCR primers and a subsequent primer-walking technique (Biomedic, Korea). Overlapping PCR fragments were assembled to complete the sequence of the entire genome. Then many different combinations of primer pairs designed in the primer walking process were used to confirm the overlapping 5′- or 3′-end regions of each contig (Table 1).

2.2. Gene identification and genome analysis

The nucleotide sequences were aligned using the program BioEdit v7.0.1 (Hall, 1999). The locations of the 13 protein-coding genes (PCGs) and two rRNA genes were initially identified using DOGMA (Wyman et al., 2004) with the default settings, and refined through comparison with the mt genome of *C. sinicus* (GU355641) and using the NCBI Open Reading Frame (ORF) Finder. Most tRNA genes were identified using both tRNAscan-SE1.2.1 (Lowe and Eddy, 1997) and ARWEN (Laslett and Canbäck, 2008) in default search mode or with different cutoff values using mt/chloroplast DNA as the source and the invertebrate mt genetic code for tRNA prediction. The remaining tRNA genes were identified by searching for anticodon consensus motif sequences (TxxxR; xxx = anticodon) and examining potential secondary structures manually. The complete *C. hyperboreus* mt genome has been deposited in GenBank under accession number JX678968.

The nucleotide frequency, composition, and GC skew of the PCGs calculated as (G − C)/(G + C) were determined using DAMBE 5.2.57 (Xia and Xie, 2001). The nucleotide sequences of crustacean *ATP8* genes retrieved from NCBI were translated into amino acid sequences with BioEdit, and then aligned based on their amino acid sequences using ClustalW in BioEdit. Repeat sequence patterns in the NCR were checked using the web-based software server Tandem Repeats Finder (http://tandem.bu.edu/trf/trf.html) (Benson, 1999). The hairpin structure in the control region (CR) was predicted using the mfold web server (Zuker, 2003) with the default options.

Table 1
PCR primers used in this work for amplification of the mitochondrial DNA from *Calanus hyperboreus*.

Fragment	Primers	Sequence (5′–3′)	Product
CH COX1	CH CO1F2	TTTTCTCTTCATTTGGCCGGG	*Cox1* gene fragment
	CH CO1R	CGCCCACATCATAGAATGAA	
CH Cytb	Copepod CytbF	AATAYCATTCAGGYTGAATRTG	*Cytb* gene fragment
	Copepod CytbR	CAGATATCTTTYTGRGGGGC	
CH 16S	CH 16SF	AACATGTAAATTAGATTTATAATG	*16S* gene fragment
	CH 16SR	AACATCGAGGTCACAAACACC	
CH#34	CH CytbF	ATGCCCAGGGGATTATTTCT	11,808 bp (*Cytb–Cox3*)
	CH HALF CO3R	CCCATTGAACCAAACAACG	
CH#37	CH#59-33 R3	CCTGTTCTCAGCCATTCCAT	7774 bp
	Calanus HF(+2640)	AGCCGCGTTAGTGTTAAGGT	
CH#59-33	CH 16SF	AACATGTAAATTAGATTTATAATG	2718 bp
	CH CytR	TGGAATGGCTGAGAACAGATT	
CH#4	CH#11-6 R_rev	GGAATGATTGGAACCGGG	4100 bp
	CH#4-60 F1	TCTTTTTTCATCGTTTGATCC	
CH#34-13	CH16SR	AACATCGAGGTCACAAACACC	2587 bp
	CH CO1F2	TTTTCTCTTCATTTGGCCGGG	
CH#48	CH#59-33 R2	TGAAATTTCGGATCCTTGCT	2585 bp
	CH#37 M13R6	AAGGAAGAAACCATGGGGGT	

F, forward primer; R, reverse primer; bp, base pairs.

2.3. Gene order comparison

The orders of mt genes of 11 copepods and one decapod, representative of the pan-crustacean basal pattern, were compared using a linearized representation of each mt genome. The accession numbers of the genomes used for comparison were GU355641 (*C. sinicus*), AB091772 (*E. bungii*), AB091773 (*N. cristatus*), AY959338 (*T. japonicus*), DQ913891 (*T. californicus*), NC007215 (*L. salmonis*), HQ157566 (*Cali. clemensi*), HQ157565 (*Cali. rogercresseyi*), EU877959 (*P. nana*), EU621723 (*Sinergasilus polycolpus*), and NC015607 (*Homarus americanus*).

3. Results and discussion

3.1. Structure of mt genome of C. hyperboreus

The complete mt genome of *C. hyperboreus* (JX678968) was 17,910 bp in length, and contained the typical set of 13 PCGs, 2 rRNA genes, 22 tRNAs, and a long NCR (Fig. 1). Of the 37 genes, 26 (15 tRNA genes, 9 PCGs, and 2 ribosomal RNA genes) were encoded on the + strand and 11 (7 tRNA genes and 4 PCGs) were encoded on the − strand (Table 2). The *C. hyperboreus* genome is considerably longer than those of other copepods such as *P. nana* (15,981 bp), *T. japonicus* (14,628 bp), *T. californicus* (14,578 bp), *L. salmonis* (15,445 bp), *Cali. clemensi* (13,440 bp), and *Cali. rogercresseyi* (13,468 bp), but shorter than that of *C. sinicus* (≥20,460 bp). In the *C. hyperboreus* genome, parts of four mt genes overlapped by a single bp (*trnI*/*Cytb* and *Cox2*/*trnN*) and the intergenic sequences were 1–70 bp in length, except the long NCR (Table 2).

The nucleotide composition of the entire *C. hyperboreus* mt genome was 31.2% A, 35.1% T, 14.2% C, and 19.5% G (Table 3). The A + T contents in both the entire genome and the PCGs were relatively higher in *C. hyperboreus* than most other copepod mt genomes, being 66.3% and 63.8%, respectively, compared to 61.7% and 60.4% in *C. sinicus*, 60.4% and 58.9% in *T. japonicus*, 62.2% and 60.6% in *T. californicus*, and 65.2% and 63.5% in *L. salmonis*. *Cali. rogercresseyi* (66.5% and 66.5%) and *P. nana* (70.8% and 68.3%) had higher values, whereas *Cali. clemensi* had a lower content (65.6%) in the entire genome but a higher content (75.6%) in PCGs.

3.2. Protein-coding genes

Most crustacean mt genomes contain a standard set of 13 PCGs, and missing genes have been reported only in copepods. For example, *Atp8* is absent in the cyclopoid *P. nana* and *Nad4L* is absent in siphonostomatoid copepods in the genus *Caligus* (Ki et al., 2009; Yasuike et al., 2012). The *C. hyperboreus* mt genome contained all 13

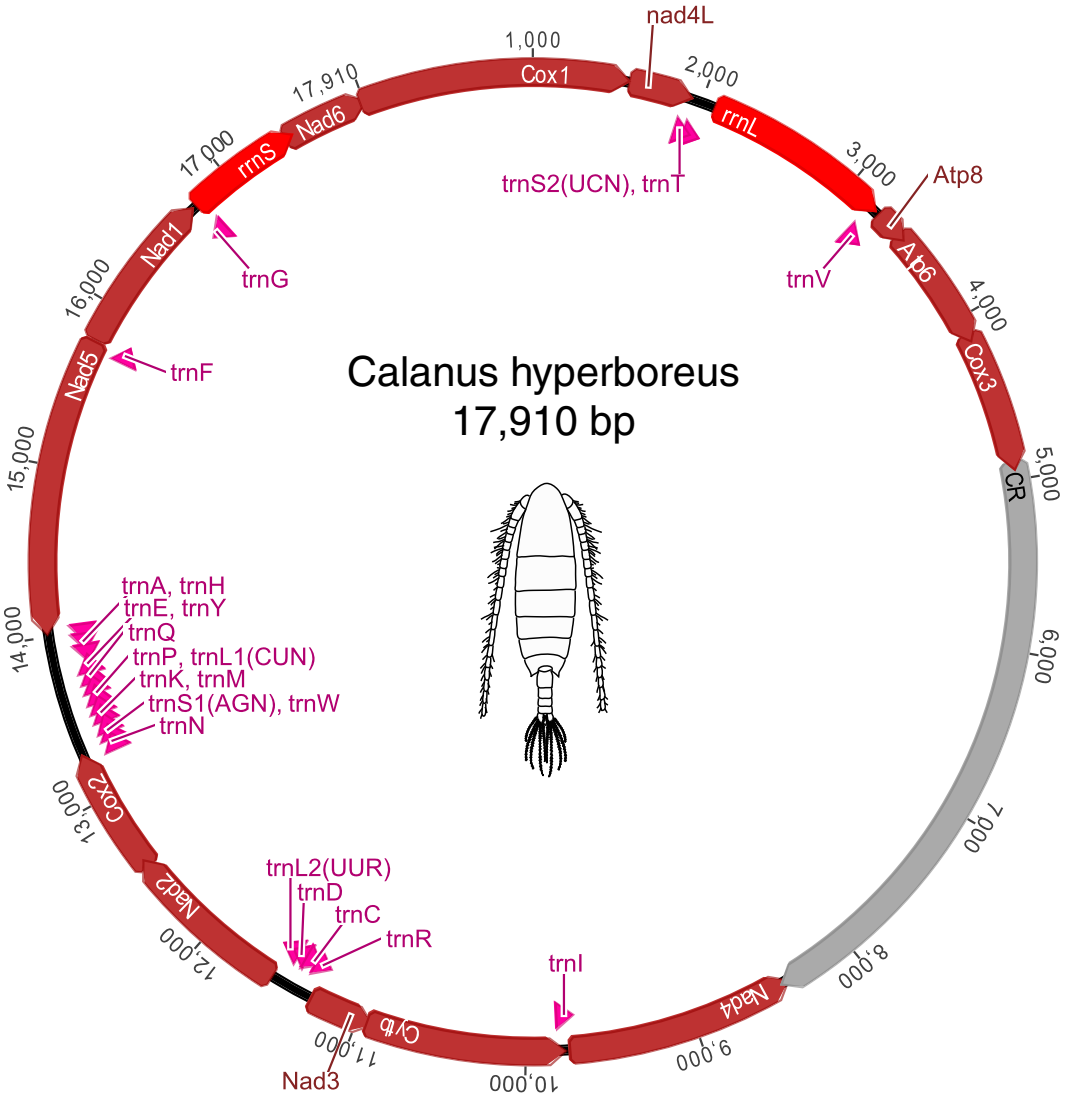

Fig. 1. Mitochondrial genome maps of *Calanus hyperboreus*. The arrows indicate the direction of transcription.

Table 2
Mitochondrial genome organization of *Calanus hyperboreus*.

Gene/region	Position		Size		Codons		Intergenic sequence
	Start	Finish	No. of nt	No. of aa	Initiation	Termination	
Cox1	1	1545	1545	515	ATA	T	41
Nad4L	1588	1920	333	111	ATA	TAA	0
trnS2^{UCN}	1923	1979	57				1
trnT	1981	2043	63				70
rrnL	2114	3189	1076				0
trnV	3190	3252	63				8
Atp8	3261	3416	156	52	ATT	TAA	0
Atp6	3420	4124	705	235	ATG	TAA	2
Cox3	4130	4918	789	263	ATG	TAG	0
CR	4922	8418	3497				0
Nad4	9720	8422	1299	433	ATA	TAA	5
trnI	9790	9726	65				−1
Cytb	10,926	9793	1134	378	ATA	TAA	0
Nad3	11,280	10,930	351	117	ATT	TAA	0
trnR	11,342	11,281	61				11
trnC	11,415	11,354	62				33
trnD	11,512	11,449	64				2
trnL2^{UUR}	11,515	11,578	64				0
Nad2	11,579	12,544	966	322	AAT	TAG	2
Cox2	12,550	13,254	705	235	ATT	TAG	−1
trnN	13,254	13,317	64				3
trnS1^{AGN}	13,321	13,378	58				0
trnW	13,379	13,441	63				1
trnK	13,443	13,502	60				0
trnM	13,503	13,565	63				1
trnP	13,567	13,629	63				0
trnL1^{CUN}	13,630	13,693	64				3
trnQ	13,697	13,765	69				0
trnE	13,766	13,830	65				3
trnY	13,893	13,834	60				7
trnA	13,961	13,901	61				0
trnH	14,025	13,962	64				4
Nad5	15,739	14,033	1707	569	ATA	TAA	3
trnF	15,743	15,803	61				0
Nad1	15,804	16,715	912	304	ATA	TAA	1
trnG	16,783	16,720	64				0
rrnS	17,435	16,784	652				0
Nad6	17,436	17,906	471	157	ATA	TAA	1

Stop codons are not included.
Underlined genes and tRNAs are reverse transcribed.
nt, nucleotide; aa, amino acid.

typical proteins, as shown in Table 2. Of these genes, seven (*Cox1*, *Cytb*, *Nad1*, *Nad4*, *Nad4L*, *Nad5*, and *Nad6*) had ATA as the start codon, whereas the others started with ATG (*Atp6* and *Cox3*) or ATT (*Atp8*, *Cox2* and *Nad3*). In addition, the *Nad2* gene had the rare start codon AAT. Most of the genes ended with a complete TAA (*Atp6*, *Atp8*, *Cytb*, *Nad1*, *Nad2*, *Nad3*, *Nad4*, *Nad4L*, *Nad5* and *Nad6*) or TAG (*Cox*2 and *Cox*3) stop codon, except *Cox1*, which ended with T (Table 2). The total length of the *C. hyperboreus* PCGs was 11,073 bp, which was slightly longer than those of *P. nana* (10,909 bp), *T. japonicus* (10,963 bp), *T. californicus* (10,972 bp), and *L. salmonis* (10,818 bp), but shorter than those of *C. sinicus* (11,136 bp), *Cali. clemensi* (10,635 bp), and *Cali. rogercresseyi* (10,127 bp) (Table 3).

Table 3
Nucleotide composition of the *Calanus hyperboreus* mitochondrial genome.

Nucleotide	Length(bp)	A(%)	C(%)	G(%)	T(%)	A+T(%)	G+C(%)
Entire sequence	17,910	31.10	14.20	19.5	35.1	66.3	33.7
Protein coding sequence	11,073	26.9	17.3	18.9	36.9	63.8	36.2
Codon position[a]							
1st	3691	29.4	15.0	26.2	29.4	58.8	41.2
2nd	3691	16.8	20.4	15.9	46.9	63.7	36.3
3rd	3691	30.2	14.0	19.3	36.6	66.8	33.2
Ribosomal RNA gene sequence	1728	37.5	14.8	8.2	39.5	77	23
Transfer RNA gene sequence	1379	38.9	9.6	13.4	38.1	76.9	23.1
Non-coding region	3698	27.9	13.5	22.0	36.5	64.5	35.5
Major NCR	3497	27.1	14.1	22.5	36.3	63.4	36.6

[a] Stop codons are not included.

The *C. hyperboreus* mt genome included a biased A+T content in the third codon position, as in other decapods (Table 3). The average relative nucleotide frequency in the third position was 30.2% (A), 14.0% (C), 19.2% (G), and 36.6% (T), accounting for a 66.8% A+T content (Table 3). The A+T content was 58.8% and 63.7% at the first and second codon positions, respectively (Table 3). Notably, the second codon position was strongly biased toward T (46.9%) compared to 29.4% at the first codon position (Table 3). The three most frequent codons were UUU (Phe), UUA (Leu), and AUU (Ile), while CGC (Arg) and AGC (Ser) were the least frequent (Supplemental Table 1).

3.3. Ribosomal RNA and tRNA genes

The lengths of *rrnS* and *rrnL* in *C. hyperboreus* were 652 and 1076 bp, respectively. Both rRNA genes were encoded in the same + strand, like other typical arthropods (Table 2). The combined length of the two genes (1728 bp) was shorter than the 1793 bp in *C. sinicus* and 1793 bp in *P. nana*, but longer than the 1614 bp in *T. japonicus*, 1587 bp in *T. californicus*, 1599 bp in *L. salmonis*, 1650 bp in *Cali. clemensi*, and 1651 bp in *Cali. rogercresseyi* (Table 3). The nucleotide composition of the two rRNA genes was strongly biased toward A (37.5%) and T (39.5%), accounting for 77.0% of A+T content, which was higher than in the PCGs (Table 3).

The 22 tRNA genes ranged from 57 (*trnS2*^{AGN}) to 69 (*trnQ*) nucleotides in length and their structures were more or less comparable to those of metazoans, which have the characteristic cloverleaf structure, except *trnS1*^{UCN} and *trnS2*^{AGN} (Supplemental Fig. 1). The lack of the DHU-arm in *trnS* is common in metazoan genomes, which appear to be biased to *trnS2*^{AGN}, but the *C. hyperboreus* mt genome had truncated DHU-arms in both *trnS1*^{UCN} and *trnS2*^{AGN} (Garey and Wolstenholme, 1989). Interestingly, the *C. hyperboreus* mt genome had a cluster of 12 tRNAs (NS_1WKMPL_1QEYAH) located between *Cox2* and *Nad5* (Fig. 1 and Table 2). A similar large tRNA cluster is also found in *C. sinicus* (Minxiao et al., 2011).

3.4. Non-coding regions

Despite its large genome size, *C. hyperboreus* had a relatively compact genome structure. Excluding the longest non-coding sequence, the remaining NCRs were 1–70 bp in size. Like the PCGs, the NCRs were A+T biased with 64.5% for the NCRs overall and 63.4% for the major NCRs and CRs (Table 3). Unlike *C. sinicus*, which has six long NCRs (>100 bp), *C. hyperboreus* had one large NCR, like typical crustaceans. The length of the CR (3497 bp) in *C. hyperboreus* is unusual when compared to 2146 bp in *L. salmonis* (Atlantic form), 1351 bp in *P. nana*, 711 bp in *T. californicus*, 581 bp in *T. japonicus*, and 2959 bp in *C. sinicus* (the largest NCR). The CR in *Cali. clemensi* and *Cali. rogercresseyi* is only 104 and 129 bp, respectively.

Control regions vary greatly in nucleotide composition, length, or location in metazoans. The typical CR is about 1 kb, although some insects have very large CRs, ranging from several hundred bases up to 13 kb (Boyce et al., 1989; Mancini et al., 2008; Taylor et al., 1993). To the best of our knowledge, the largest CR in a complete crustacean mt genome is in *L. salmonis* (Atlantic form, 2146 bp). Therefore, *C. hyperboreus* may have the largest CR not only in copepods, but also in crustaceans. The extended size of the CR may be due to tandem repeats. Three tandem repeat regions were found in the *C. hyperboreus* CR (Fig. 2). One consisted of five copies of 11 nucleotides (ATATTAAATTT) located at positions 251–305 from the 5′-end of the CR. The second was four copies of 12 nucleotides (TTAAATTCATAC) located at positions 310–357. The third was two copies of 399 and 401 bp in length located between positions 925

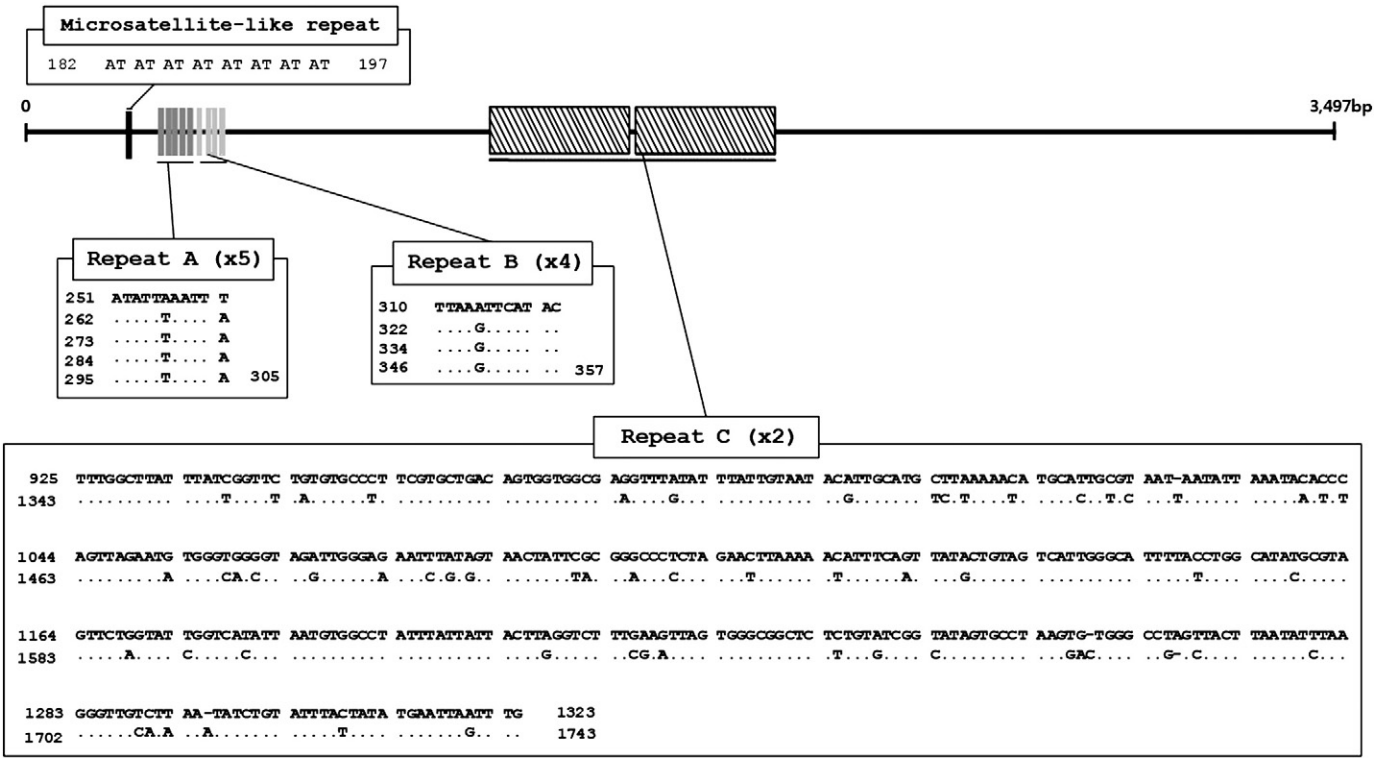

Fig. 2. Structure of the *C. hyperboreus* control region, which contains tandem repeats. Three tandem repeats were found in *C. hyperboreus*. The boxes show the repeated sequences and copy numbers. Dots indicate matching sequences and dashes indicate gaps.

and 1743 and the sequence similarity of the two copies was 85.1%. In copepod mt genomes, apparent tandem repeats with several copies are observed only in *C. hyperboreus*. In addition, a microsatellite-like repeat, $(AT)_8$, was found near the 5′-end of the CR (Fig. 2). Several putative hairpin structures with two loops and two stems, a typical structure of the putative replication origin (O_L) in the arthropod CR, were found in the CR (data not shown). However, conserved flanking motifs such as TATA at the 5′-end and GACT at the 3′-end, which are conserved in the CRs of many crustaceans and insects, were not found adjacent to the second stem regions (Kilpert and Podsiadlowski, 2006).

3.5. Distinct features of the copepod mt genome

3.5.1. Extremely high rate of gene translocation in copepods

The mt genomes of metazoans such as mollusks, brachiopods, and nematodes have highly translocated gene arrangements. Most arthropods, including crustaceans and hexapods, have a conserved mt genome structure referred to as the pan-crustacean basal pattern and 62.2% (46 out of 74 species) of crustaceans have a gene order identical to pan-crustaceans for the major genes, including all 13 PCGs and both rRNAs, if translocations occurring in tRNAs are not counted. The numbers of translocated genes were also very low and they do not exceed eight in all crustacean species, with the exception of copepods (Supplemental Table 2). In contrast, the numbers of translocated genes are extraordinarily high in copepods and all of the copepods shown in Supplemental Table 2 have at least 12 translocations in their major mt genes, and 66.7% of copepods (six out of nine species) have had complete translocation of all of their major mt genes. It is not clear why most copepods have undergone such frequent and unusual translocation events in the evolution of their mt genome. Perhaps this can be explained by their successful adaptation to a wide range of environments, in terms of water type, temperature, and depth, which may have influenced the adaptive evolution of the mt genome. Recent papers have focused on the correlation between the sequence variation in mt-encoded proteins and environmental adaptation to improve metabolic performance (Blier et al., 2001; Fonseca et al., 2008; Rand et al., 2004).

3.5.2. Gene loss in crustaceans has occurred only in copepods

Some metazoans have lost genes from their mt genomes (Adams and Palmer, 2003). However, gene loss has not occurred in crustaceans, except copepods. Therefore, this gene loss is a unique feature of the mt genomes of copepods. The two *Caligus* species lack *Nad4L* and *trnL2*, *L. salmonis* lacks *trnC*, and *P. nana* lacks *Atp8*, and these account for 44.4% (four out of nine species) of the copepod species studied (Fig. 3). These results suggest that the mt genomes of copepods have different evolutionary histories from other crustaceans.

3.5.3. Atp8 deletion or truncation of the Atp8 gene in copepods except Calanoida

Atp8 is the smallest and most rapidly evolving PCG in metazoan mt genomes. Absent or truncated *Atp8* is seen in several metazoan phyla, such as Nematoda, Placozoa, Platyhelminthes, Mollusca, Annelida, and Sipunculida, but not in crustaceans except copepods (Doucet-Beaupre et al., 2010; Dreyer and Steiner, 2004, 2006; He et al., 2005; Hoffmann et al., 1992; Ki et al., 2009; Min and Park, 2009; Serb and Lydeard, 2003; Yasuike et al., 2012; Yatawara et al., 2010). In this study, we found that the *Atp8* gene was conserved in all four calanoids, including the newly determined *C. hyperboreus*, like most crustaceans, whereas *Atp8* was absent (*P. nana*) or truncated in all non-calanoid copepods (Supplemental Fig. 2). About half of the terminal amino acids were deleted in all of the truncated ATP8. It is not clear whether these are the outcome of independent events or a synapomorphic trait shared in several taxa. However, these events will be helpful for tracing the evolutionary process within certain

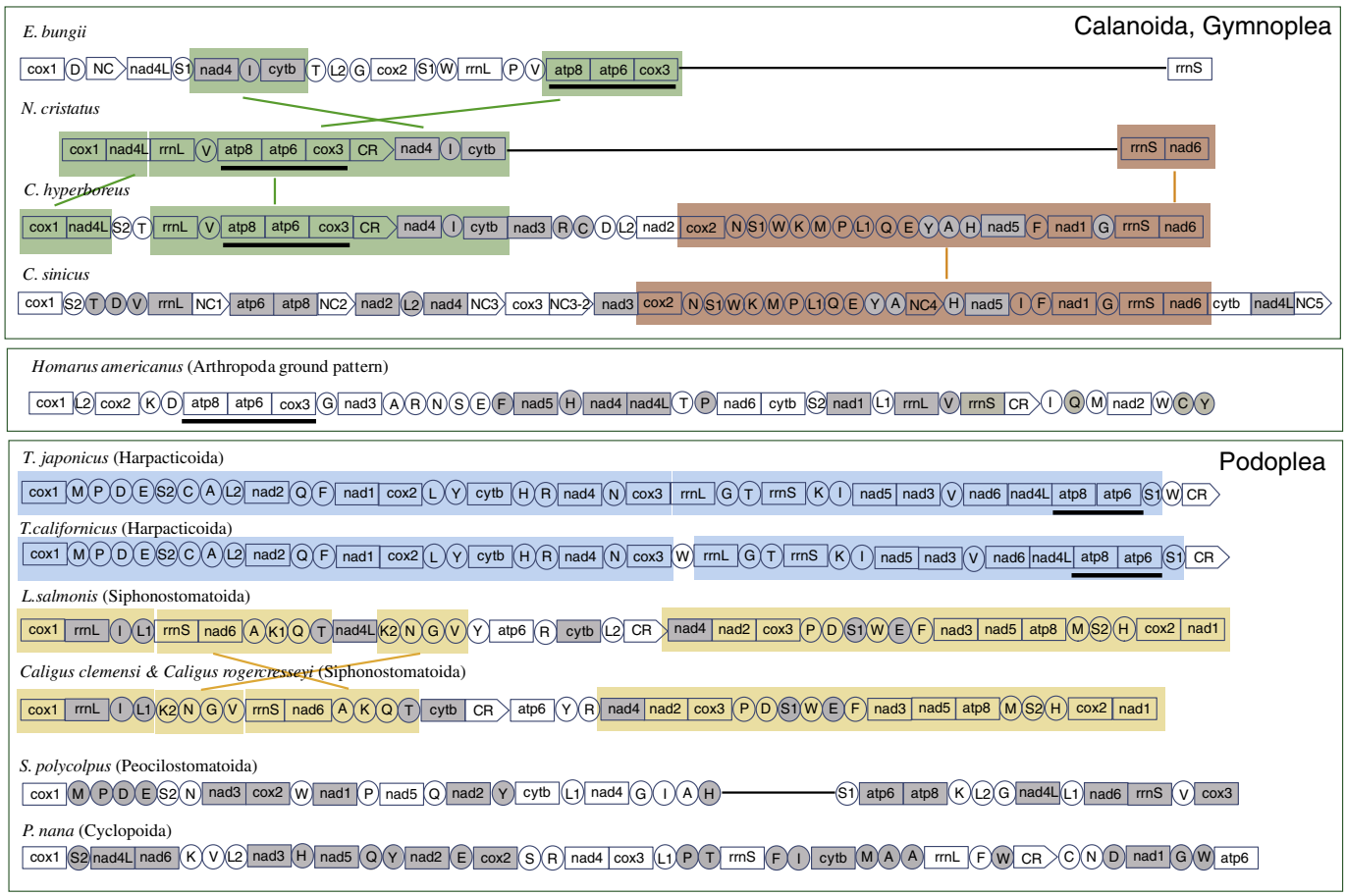

Fig. 3. Comparison of the mitochondrial gene orders among various copepods and *Calanus hyperboreus*. The gene order of *Homarus americanus* (Nephropoidea, true lobster) in the middle represents the pan-crustacean basal pattern. Gene clusters shared between species are represented by boxes with colored overlays. A solid box or circle indicates reversed transcriptional polarity; otherwise the transcription direction is from left to right. The horizontal lines under genes indicate the retained arthropod ground pattern.

taxa, including copepods (Boore and Staton, 2002; Dreyer and Steiner, 2004; Yasuike et al., 2012).

3.5.4. Control region inversion in Copepoda

The GC skew in mt genomes has been considered a useful characteristic in a phylogenetic context (Perna and Kocher, 1995). For example, most crustaceans have a negative GC skew for + strand genes and a positive one for − strand genes (Hassanin, 2006). Many studies have found that the strand-specific bias is associated with asymmetrical replication at the replication origin (O_L) in the CR, and that the GC skew can be reversed (e.g., negative for – strand and positive for + strand genes) if the CR is inverted. This phenomenon has been found in several crustacean taxa, including two decapods (*Cambaroides similis* and *Procambarus clarkii*), two isopods (*Ligia oceanica* and *Eophreatoicus* sp.), an amphipod (*Metacrangonyx longipes*), a branchiopod (*Argulus americanus*), and a cephalocarid (*Hutchinsoniella macracantha*) (Supplemental Table 3) (Braband et al., 2010; Kilpert and Podsiadlowski, 2006; Kim et al., 2012b; Lobry, 1996). In copepods, reversed GC skew is observed in most PCGs in the 11 published mt genomes (Supplemental Table 3). In *C. hyperboreus*, the GC skew was reversed in 11 of 13 PCGs (the exceptions were *Atp6* and *Atp8*), accounting for 84.6% of the reversed GC skew values, whereas 8 genes (61.5%) showed reversed GC skew in *C. sinicus* (Supplemental Table 3). In particular, two Siphonostomatoida (*Cali. clemensi* and *Cali. rogercresseyi*) have a completely reversed GC skew for all PCGs. Most of the PCGs in another Siphonostomatoida (*L. salmonis*) and two Harpacticoida (*T. japonicus* and *T. californicus*) also had reversed GC skews (Supplemental Table 3). These results suggest that the CR is likely inverted in most copepod lineages.

3.6. Features conserved in Calanoida mt genomes

3.6.1. Conservation of pan-crustacean ground pattern in Calanoida

Despite the extremely high rate of gene rearrangement of the mt genome of copepods, our comparative analysis revealed the retained pan-crustacean basal pattern (Fig. 3). The *Cox2–Atp8–Atp6–Cox3* quartet is a highly conserved ancestral gene cluster in most metazoans from Porifera to Chordata (King, 2004). This gene cluster is also conserved in all crustaceans except two anomuran decapods (*Pagurus longicarpus* and *Shinkaia crosnieri*) and all reported copepods. However, the *Atp8–Atp6–Cox3* cluster was conserved in three calanoids (*E. bungii*, *N. cristatus*, and *C. hyperboreus*), but not in *C. sinicus* and all other copepods. It is worth noting that this three-gene cluster is not only the pan-crustacean basal pattern, but also an ancestral gene cluster from unicellular organisms (King, 2004). For *Atp8–Atp6* coupling only, all calanoids, two harpacticoids (*T. japonicus* and *T. californicus*), and *S. polycolpus* have this conserved cluster, while the three siphonostomatoids (*L. salmonis*, *Cali. clemensi*, and *Cali. rogercresseyi*) and a cyclopoid (*P. nana*, *Atp8* is missing) do not. *Atp8–Atp6* is translated from a single mRNA (Berthier et al., 1986). This may be why this *Atp8–Atp6* cluster is conserved in copepods.

3.6.2. A long cluster of tRNAs may be a unique feature of Calanoida

In general, tRNA genes are highly dispersed in the metazoan mt genome and only a few tRNA clusters are conserved, such as ARNSEF located between *Nad3* and *Nad5* (Grisanti et al., 1993; Shen et al., 2009). Strikingly, the mt genome of *C. hyperboreus* had a cluster of 12 tRNAs (NS_1WKMPL_1QEYAH) located between *Cox2* and *Nad5*. This large tRNA cluster is extremely unusual, although it was also found in *C. sinicus* and the arrangement 12 tRNA genes flanked by *Cox2* and *Nad5* was identical after excluding NCR4 between *trnA* and *trnH*. The existence of a large number of tRNA genes in a single cluster has been reported in echinoderms and yeast (e.g., the 13 tRNA cluster in *Asterias forbesi* and 12 tRNA cluster in *Saccharomyces cerevisiae*) (Bonitz et al., 1980; Ghikas et al., 2006; Grisanti et al., 1993; Jacobs et al., 1989). The tRNA cluster is thought to act as an alternative origin of light strand replication, an agent for catalyzing genomic rearrangement in the mt genome, and a hot spot for recombination (Jacobs et al., 1989; Kilpert and Podsiadlowski, 2006; Moritz and Brown, 1987; Seligmann, 2010; Seligmann et al., 2006). In addition, the clustering of tRNA genes appears to be unique to Pezizomycotina and translocations or absences of tRNA may reflect the evolutionary progress of these fungi (Ghikas et al., 2006). Moreover, the absence of some tRNAs has been attributed to functional gain in fungi and yeasts (Kolesnikova et al., 2000; Laforest et al., 1997). Similarly, the long tRNA gene clusters in copepod genomes (12 tRNA genes in two *Calanus* species, 8 in *T. japonicus*, and 6 in *L. salmonis*) can be used as informative criteria to understand the frequent occurrence of rearrangement and consequential evolutionary process in copepods when more data on the mt genome in copepods accumulate.

3.6.3. Comparison of the mt genome structure among closely related Calanoida species

The structure of the mt genome of *C. hyperboreus* is very different from those of other copepods and crustaceans as well as that of another *Calanus* species, *C. sinicus*. Disregarding the 4 NCRs and 2 tRNAs (inverted *trn*G and *trnI*) in *C. sinicus*, the two *Calanus* species share a long gene cluster that includes 4 PCGs, 14 tRNAs and *rrnS* between *Cox2* and *Nad6*, while the other gene regions differ completely between the two species (Fig. 3). The most distinct difference between the two *Calanus* species is the existence of six NCRs (>100 bp) in *C. sinicus*, while *C. hyperboreus* has only a typically long NCR. Because most crustaceans have a single NCR in their mt genomes, multiple NCRs may be an exclusive trait of *C. sinicus*, rather than a common feature of copepods.

The great difference in mt genome structure between the two *Calanus* species is somewhat exceptional because gene order and orientation tend to be identical or very similar among closely related species in Metazoa. This is not exceptional even in the other highly translocated copepods: the two *Caligus* (*Cali. clemensi* and *Cali. rogercresseyi*) are completely identical and the two *Tigriopus* (*T. japonicus* and *T. californicus*) differ only in the position of *trnG*. Furthermore, *L. salmonis* and the two *Caligus* species in the family Caligidae, order Siphonostomatoida, were largely identical (Fig. 3).

Despite being partial sequences, the mt genomes of two calanoid copepods, *E. bungii* and *N. cristatus*, provide considerable information. Interestingly, *C. hyperboreus* and *N. cristatus* were more similar in genome structure than were the two *Calanus* species. *N. cristatus* and *C. hyperboreus*, had largely conserved features: *Atp8*/*Atp6*/*Cox3*, which followed the pan-crustacean basal pattern, and *Nad4*/*trnI*/*Cytb*. In addition, the mt genomes of two calanoid copepods had identical gene orders, except for two tRNA genes: *trnS2* and *trnT* (Machida et al., 2004). From these similarities between two different genera, *Neocalanus* and *Calanus*, we can assume that the mt gene orders among species in family Calanidae are relatively well conserved in the copepod lineages.

We postulate that *C. sinicus* underwent a unique evolutionary process involving its mt genome, which includes multiple NCRs. Alternatively, the greater shared gene arrangement among the other three calanoids excluding *C. sinicus* may be due to adaptation to their habitats. Cameron et al. (2007) and Ki et al. (2009) postulated that there is an interaction between adaptation to a harsh environment and mt genome rearrangement. Note that *N. cristatus*, *E. bungii*, and *C. hyperboreus* are the most abundant copepods in the subarctic Pacific, while *C. sinicus* occurs widely in the East China Sea and coastal waters of Japan (Hulsemann, 1994; Mackas and Tsuda, 1999; Miller et al., 1984; Motoda and Minoda, 1974). To test this hypothesis, adding more mt genomes from calanoid species including *N. cristatus* and *E. bungii* is a prerequisite. It will be helpful to gain an insight into the evolutionary forces responsible for unusual gene rearrangement of *C. sinicus*, as well copepod mitochondrial genome evolution.

3.6.4. Conserved pan-Copepoda pattern in Calanoida

The subclass Copepoda consists of 10 orders. With the exception of the minor order Platycopioida, which includes a single genus, most copepods can be divided into two superorders: Gymnoplea and Podoplea (Huys and Boxshall, 1991). The order Calanoida is the sole member of the superorder Gymnoplea and the remaining orders are clustered in the superorder Podoplea.

As the mt genome of *C. hyperboreus* shares the typical pan-crustacean basal pattern (*Atp8*/*Atp6*/*Cox3*) with two other calanoids and a comparable intact form of the *Atp8* gene, we postulated that it reflects the copepod pattern. This view is supported by phylogenetic analyses based on molecular and morphological data that show the basal status of Calanoida within copepods (Ho, 1994; Huys and Boxshall, 1991; Minxiao et al., 2011). Still, this hypothesis is not conclusive because the mt genomes of other copepods showed no similarity, except those of calanoid copepods, and only two complete mt genomes (*C. hyperboreus* and *C. sinicus*) were used for the genome comparison. Therefore, investigations of additional complete mt genomes from non-Calanidae calanoids and various copepods, such as members of the order Platycopioida, will be useful for clarifying the copepod pattern.

4. Conclusions

We determined the first complete mt genome in the Calanoida, that of *C. hyperboreus*. It contains the typical set of 37 genes (13 PCGs, 2 rRNAs, and 22 tRNA genes) found in metazoans, but has a very unusual gene structure, with the longest crustacean CR reported and a cluster of 12 tRNA genes. In addition, it includes the first tandem repeats found in a copepod CR. Despite the unusual gene structure, our comparative analysis revealed conserved calanoid-specific patterns.

We found two typical pan-crustacean basal patterns from Calanoida while any retained pan-crustacean feature was not found from other copepods except *Atp8*/*Atp6* cluster in harpacticoids. Except *C. sinicus*, other calanoids had an ancestral gene cluster, *Atp8*/*Atp6*/*Cox3* in their mt genome. All calanoids had complete *Atp8* sequences, while those of non-calanoids were absent or partially truncated. These results are consistent with the ancestral position of Calanoida in the previous studies based on morphological and molecular data, supporting the notion that analysis of the mt genome is useful for resolving copepod phylogeny and evolution of the mt genome of copepods.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2012.09.059.

Acknowledgments

This work was supported by the Polar Academic Program (PAP), KOPRI for G.-S. Min, and the Basic Research Program of the KOPRI (PE12030) for S. Kim and H.-G. Choi.

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