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Partial Mitochondrial Gene Arrangements Support a Close Relationship between Tardigrada and Arthropoda

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Regions (about 3.7–3.8 kb) of the mitochondrial genomes (*rrnL–cox1*) of two tardigrades, a heterotardigrade, *Batillipes pennaki,* and a eutardigrade, *Pseudobiotus spinifer*, were sequenced and characterized. The gene order in *Batillipes* was *rrnL-V-rrnS-Q-I-M-nad2-W-C-Y-cox1,* and in *Pseudobiotus* it was *rrnL-V-rrnS-Q-M-nad2-W-C-Y-cox1*. With the exception of the *trnI* gene, the two tardigrade regions have the same gene content and order. Their gene orders are strikingly similar to that of the chelicerate *Limulus polyphemus* (*rrnL-V-rrnS*-CR-*I-Q-M-nad2-W-C-Y-cox1*), which is considered to be ancestral for arthropods. Although the tardigrades do not have a distinct control region (CR) within this segment, the *trnI* gene in *Pseudobiotus* is located between *rrnL-trnL1* and *trnL2-nad1*, and the *trnI* gene in *Batillipes* is located between *trnQ* and *trnM*. In addition, the 106-bp region between *trnQ* and *trnM* in *Batillipes* not only contains two plausible *trnI* genes with opposite orientations, but also exhibits some CR-like characteristics. The mitochondrial gene arrangements of 183 other protostomes were compared. 60 (52.2%) of the 115 arthropods examined have the *M-nad2-W-C-Y-cox1* arrangement, and 88 (76.5%) the *M-nad2-W* arrangement, as found in the tardigrades. In contrast, no such arrangement was seen in the 70 non-arthropod protostomes studied. These are the first non-sequence molecular data that support the close relationship of tardigrades and arthropods.

Keywords: *Batillipes pennaki*; Gene Arrangement; Mitochondrial Genome; Molecular Phylogeny; Panarthropoda; *Pseudobiotus spinifer*; Tardigrada.

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Introduction

Tardigrades, called "water bears," live in marine, freshwater, or moist terrestrial environments. The first tardigrade species was discovered in 1773, and over 960 species have been described since then (Guidetti and Bertolani, 2005). The phylum Tardigrada is classified into three classes: Heterotardigrada, Mesotardigrada, and Eutardigrada (Ramazzotti and Maucci, 1983). While Heterotardigrada is primarily marine, Eutardigrada is usually terrestrial or freshwater. Mesotardigrada is comprised of only one species, described from a hot spring in Japan by Rahm (1937) and never found again.

The phylogenetic position of the tardigrades in the metazoan animal groups remains unclear. The most recent studies based on morphology and DNA/protein sequences (Brusca and Brusca, 2003; Garey *et al*., 1996; Giribet *et al*., 1996; 2001; Mallatt *et al*., 2004; Neilson, 2001; Regier and Shultz, 2001) consider Tardigrada as a separate phylum belonging to the Panarthropoda, which includes arthropods and their close relatives, the Tardigrada, Onychophora, and Pentastomida. In contrast, a few authors have placed Tardigrada either within the Aschelminthes because of its nematode-like pharynx with stylets (Dewel and Clark, 1973; Kinchin, 1992), or within Arachnoidae *sensu* von Siebold (1854), which includes Tardigrada, Arachnida and Pycnogonida, due to the presence of a multi-articulate cephalothorax and the absence of respiratory organs.

In addition to nucleotide or amino acid sequence data, comparison of mitochondrial gene arrangements could be useful for elucidating controversial metazoan phylogenies (e.g. Boore, 1999; Boore and Brown, 1998; Lim and Hwang, 2006), although examples of dramatic and unexpected mitochondrial gene rearrangements in metazoans have been reported (Shao *et al*., 2004a; 2004b; Sun *et al*.,

2005). One of the most impressive examples of the use of a mitochondrial gene arrangement pattern to examine phylogenetic relationships is the work of Boore *et al*. (1998). They reported that *rrnL*-*L*(*CUN*)-*nad1* and *cox1*-*L*(*UUR*)-*cox2* link Hexapoda to Crustacea, supporting the Pancrustacea hypothesis (cf. Fig. 4).

In order to elucidate the phylogenetic position of the phylum Tardigrada using its mitochondrial gene arrangement pattern, we determined the partial mitochondrial genome sequences (3.7–3.8 kb from *rrnL* to *cox1*) of two tardigrade species, the marine species *Batillipes pennaki* (Heterotardigrada) and the freshwater species *Pseudobiotus spinifer* (Eutardigrada). The sequenced region was annotated, and the gene arrangement patterns were compared with those of the arthropods and other metazoan animals. The sequenced regions are the longest tardigrade mitochondrial genome sequences published thus far, as no complete tardigrade mitochondrial genome sequences have been reported. Our data provide valuable information for defining the tardigrade phylogenetic position.

Materials and Methods

Sample collection A marine heterotardigrade species, *Batillipes pennaki*, and a freshwater eutardigrade species, *Psedobiotus spinifer*, were collected from Gwangyang-City, Jeollanam-do Province and Changnyeong-gun County, Gyeongsangnam-do Province, Korea, respectively.

Extraction of total cellular DNA, PCR amplification, and sequencing Total cellular DNA was extracted using a QIAamp DNA Micro Kit (QIAGEN Co., Germany). The partial mitochondrial genomes (ca. 3.7–3.8 kb from *rrnL* to *cox1*) of the two tardigrade species were amplified using the Expand Long Template PCR System (Roche Biochemicals) with the following primers: 16SB, 5′-CCG GTY TGA ACT CAR ATC A-3′ (Kambhampati and Smith, 1995) and HCO2198, 5′-TAA ACT TCA GGG TGA CCA AAA AAT-3′ (Folmer *et al*., 1994). The cycle setting included a cycle of 2 min at 92°C for the initial denaturation, 15 cycles of 10 s denaturation at 92°C, 30 s annealing at 46°C, and a 4 min elongation step at 68°C. The elongation times during the last 25 cycles were increased by 20 s per cycle. The reaction was terminated following a 7 min final elongation step at 68°C. The PCR conditions used to amplify the two species were exactly the same. The PCR fragments were purified using a PCR Purification Kit (QIAGEN Co., Germany) and cloned in pGEM T-easy vector (Promega, USA). The plasmid DNAs were then extracted with a AtmanBio plasmid purification Kit (Takara Co., Japan) and the inserted DNA was sequenced in both directions with an ABI 3700 model automatic sequencer.

Gene annotation and comparative analysis The DNA sequences were analyzed using GenJockey II v. 1.6 software (Biosoft Inc.). The mitochondrial protein coding genes were identified by a BLAST comparison with other metazoan mitochondrial genomes. We searched for tRNA genes using tRNAscan-SE 1.21 (Lowe and Eddy, 1997) and by eye. The sequence data obtained in the present study are available from GenBank under accession numbers DQ099433 (*B. pennaki*) and DQ099434 (*P. spinifer*).

After annotating the two partial tardigrade mitochondrial genomes, we compared the gene arrangements with those of 185 other protostomes and 646 deuterostomes that have been published thus far (GenBank status on Dec. 20, 2006): 115 arthropods, 16 nematodes, 4 annelids, 29 molluscs, 13 platyhelminthes, 3 brachiopods, 1 echiuran, 1 bryozoan, 1 acanthocephalan and 2 chaetognaths, and 628 chordates, 16 echinoderms, and 2 hemichordates.

Results and Discussion

Partial mitochondrial genomes of two tardigrade species and comparison of the gene arrangements We sequenced and annotated the partial mitochondrial genomes (from *rrnL* to *cox1*) of two tardigrade species, the marine heterotardigrade *Batillipes pennaki* (3,812 bp) and the freshwater eutardigrade *Pseudobiotus spinifer* (3,715 bp). The gene arrangements of *Batillipes* and *Pseudobiotus* were *rrnL*-*V*-*rrnS*-*Q*-*I*-*M*-*nad2*-*W*-*C*-*Y*-*cox1* and *rrnL*-*V*-*rrnS*-*Q*-*M*-*nad2*-*W*-*C*-*Y*-*cox1*, respectively (Fig. 1). The gene arrangement patterns in the sequenced region are identical except for *trnI* (Fig. 2).

The typical arthropod gene arrangement, represented by the pattern of *Limulus polyphemus* (*rrnL*-*V*-*rrnS*-*CR*-*I*-*Q*-*M*-*nad2*-*W*-*C*-*Y*-*cox1*), is very similar to those of the two tardigrades. There were some differences between the tardigrades and *Limulus* (arthropod ground plan) in the position of *trnI* and the presence/absence of the large noncoding region (= control region, CR). Since a CR is not apparent in the sequenced regions of the tardigrades, CR translocations seem to have occurred in both tardigrade species. While *trnI* lies between *trnQ* and *trnM* in *Batillipes*, it is translocated to between *rrnL*-*L*(*CUN*) and *L*(*UUR*)-*nad1* in *Pseudobiotus* (Fig. 1), as reported in another freshwater eutardigrade, *Thulinia* (Boore *et al*., 1998). The Since *trnI* is present in the region examined in *Batillipes*, one of the most primitive marine tardigrades, it is likely that the peculiar *trnI* positioning between *rrnL*-*L*(*CUN*) and *L*(*UUR*)-*nad1* is a eutardigrade lineage-specific characteristic. Positioning of *trnI* between *rrnL*-*L*(*CUN*) and *L*(*UUR*)-*nad1* has not been observed in any other metazoan animals thus far.

Plausible duplication of *trnI* between *trnQ* and *trnM* in *Batillipes* In *Batillipes*, two alternative candidates for *trnI*, *trnI* (1) and *trnI* (2), were identified between the *trnQ* and *trnM* genes with opposite transcriptional orientations, as shown in Fig. 2. Both *trnI* sequences could be folded into

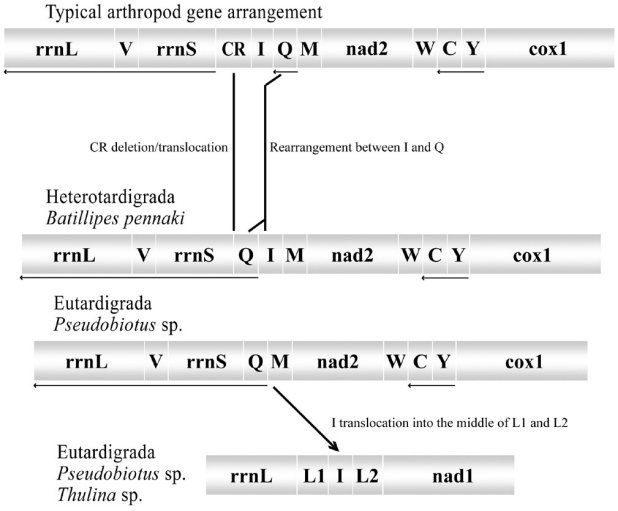

Fig. 1. Comparison of the partial mitochondrial gene arrangements patterns in the tardigrades and arthropods. The two tardigrades, the marine heterotardigrade *Batillipes pennaki* (3,812 bp) and the freshwater eutardigrade *Pseudobiotus spinifer* (3,715 bp), have gene arrangements that are very similar to a typical arthropod gene arrangement pattern (that of *Limulus polyphemus*). All genes are transcribed from left to right except those with an underlined arrow. The asterisk (*) indicates that there are two candidates for *trnI* (I) of opposite orientations in this region in *Batillipes*. Gene abbreviations: large subunit rRNA gene, *rrnL*; small subunit rRNA gene, *rrnS*; control region, CR; NADH dehydrogenase subunit 1 and 2 genes, *nad1* and *nad2*; cytochrome C oxidase subunit I, *cox1*. Transfer RNA genes are indicated by the corresponding one-letter amino acid codes. L1 and L2 indicate *trnL*(*CUN*) and *trnL*(*UUR*), respectively.

plausible $tRNA^{Ile}$ clover-leafs (Fig. 2B). Their stable secondary structures and matching anticodons suggest that a duplication of the *trnI* genes occurred in *Batillipes*.

Apart from instances of *trnS*(*UCA*) and *trnS*(*AGC*) or *trnL*(*CUN*) and *trnL*(*UUR*), which are common in metazoan mitochondrial genomes, only ca. 20 possible cases of tRNA duplication have been reported in metazoan animals (e.g. *Pollicipes polymerus*, *Mytilus edulis, Placopecten magellanicus* and *Schitoma japonicum* etc), and only three different *trnI* duplications have been reported, in *Chrysomia* species (blowflies), thus far (Junqueira *et al*., 2004; Lessinger *et al*., 2004). The complete mitochondrial genome of *C. putoria* was previously reported and was used in this comparative analysis. We did not find any possible additional example of *trnI* duplication in any of the 182 protostomes and 646 deuterostomes examined in this study. This result indicates that *trnI* duplication is very unusual in metazoan mitochondrial genomes.

In *Batillipes*, 50 bp of *trnI* (2), which is 54 bp in length, overlap with *trnI* (1), which is 69 bp in length (Fig. 2A). In contrast, the two *Chrysomia trnI* genes are clearly separated by the CR. Furthermore, duplicated tRNA genes that completely overlap have not been reported thus far (cf. Moritz and Brown, 1997). Therefore, it is likely that either *trnI* (1) or *trnI* (2) in *Batillipes* is a real functional *trnI* gene, although it is impossible to completely exclude the possibility that both are functional. If only one of the two is functional, it appears that the secondary structure predicted for *trnI* (1) (Fig. 2B) is more plausible than that predicted for *trnI* (2) (Fig. 2C) because the former has a relatively stable clover-leaf secondary structure with stable D- and TψC-arms and a more normal length (69 bp). The secondary structure predicted for *trnI* (2) does not have a D-arm, and is shorter (54 bp) than most tRNAs. Assuming only one *trnI* gene in *Batillipes*, the region (106 bp) between *trnQ* and *trnM* in *Batillipes* may be divided into either 9-bp unassigned/69-bp *trnI* (1)/28-bp unassigned regions, or 28-bp unassigned/54-bp *trnI* (2)/24-bp unassigned regions (Fig. 2A).

The CR characteristics of *trnQ* and *trnM* of *Batillipes*
Interestingly this region contains five small repeats and has a very high A + T content (85.7%). We also found some repeat patterns as shown in Fig. 2A. The order of repeats is sr1/sr2/sr2-I/truncated sr1-I (14 bp)/sr1. The large inverted repeats, R1 and R2, can be folded into a large helix having a 33-bp stem and a 4-bp loop (Fig. 2D) that may be related to the regulation of mitochondrial genome replication.

Increased A + T content, tandem repeats and the formation of a large helix by the repeats are typical characteristics of a control region (CR). The extremely high A + T content allows for melting of the sequences with greater ease, which permits replication or transcription enzymes and related machinery to access the region. The repeats and their helix forming ability may also be used to regulate the elongation or termination reactions of transcription and replication. Thus, this region (106 bp including the *trnI* gene) may be a candidate for a CR as well as for a *trnI,* despite its short length. Further sequencing of the remaining parts of the tardigrade mitochondrial genomes and of mitochondrial tRNA transcripts could clarify the numbers and positions of *trnI* and the CR within the mitochondrial genomes.

Comparative analyses of mitochondrial gene arrangements If we do not take the CR and *trnI* gene into consideration, the remaining gene arrangement (*rrnL-V-rrnS-Q-M-nad2-W-C-Y-cox1*) is common to the two tardigrades, and is completely identical to the arthropod ground pattern in terms of both gene order and transcriptional orientations. In view of the wide distribution of *rrnL-V-rrnS* in metazoan animals, we used the gene order in the remaining part in the following comparative analysis (Fig. 3, Tables 1 and 2). We compared the mitochondrial genomes of 115 arthropods (58 hexapods, 31 crustaceans, 4 myriapods, and 22 chelicerates), 2 tardi-

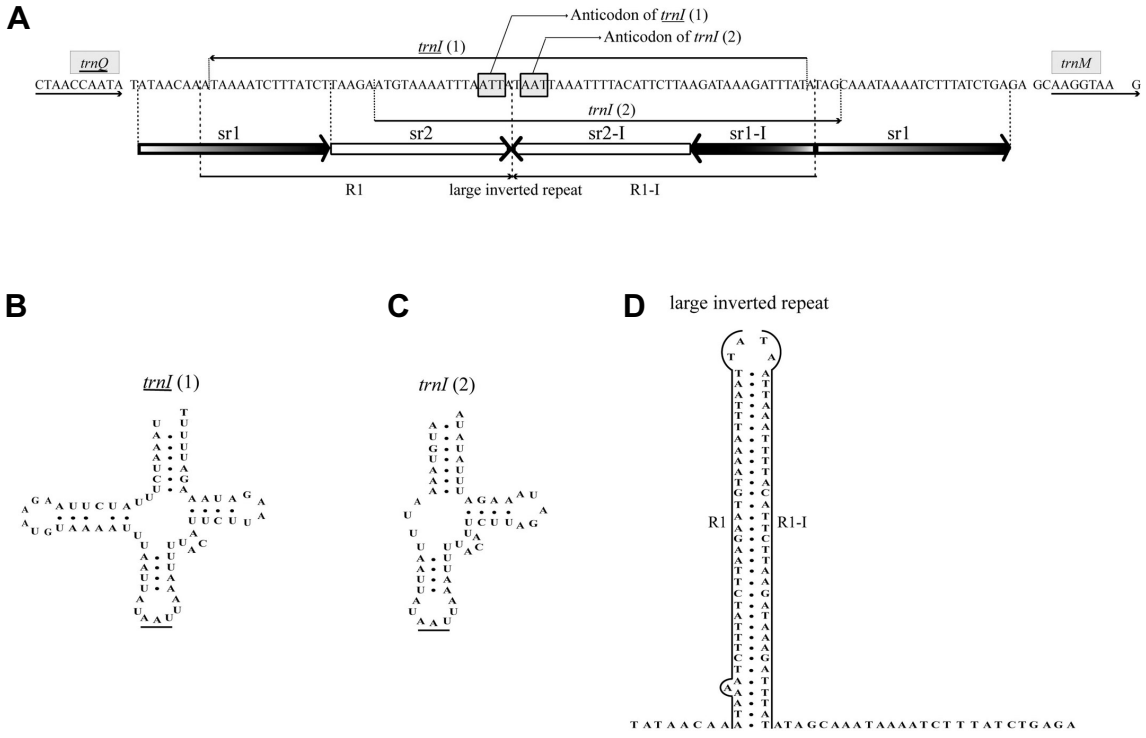

Fig. 2. The primary and potential secondary structures of the 106 bp region between *trnQ* and *trnM* in *Batillipes pennaki*. (A). Primary structure comparison of *trnQ* and *trnM,* showing the two putative *trnI* genes of opposite orientation, and some repeat units. There are two types of repeat units in this region; small repeat type 1, sr1 and inverted sr1 (sr1-I), of 22 bp (black arrows), and small repeat type 2, sr2 or inverted sr2 (sr2-I), of 21 bp (white arrows). The order of the repeats is sr1/sr2/sr2-I/truncated sr1-I (14 bp)/sr1 (B) and (C) are putative $tRNA^{Ile}$ secondary structures. (D) A large helix with a 33-bp stem and a 4-bp loop made up of R1 and R2 that may be involved in the regulation of mitochondrial genome replication.

grades, and 70 other protostomes (16 nematodes, 29 mollusks, 4 annelids, 13 platyhelminths, 3 branchiopods, 1 bryozoan, 1 echiurid, 1 acanthocephalan, and 2 chaetognaths) as shown in Fig. 3. Species names, classification, and GenBank accession numbers are listed in Supplementary Table 1.

Of the 115 arthropods, 55 have the gene arrangement *I-Q-M-nad2-W-C-Y-cox1* (47.9%), which is the most dominant in arthropods (Table 1 and Fig. 3A). Eleven of the others have very similar arrangements, five different types of which were found to have changes in gene positions and transcriptional orientations between *trnI, trnQ* and *trnM* (= *I/Q/M*), or changes in the transcriptional orientations of *trnW, trnC* and *trnY* (= *W-C-Y*). Disregarding the internal variations in *I/Q/M* and *W-C-Y*, we found 66 (57.4%) (*I/Q/M*)-*nad2*-(*W-C-Y*)-*cox1* arrangement patterns (Table 1). Considering that *trnI* is located between *trnQ* and *trnM,* the marine heterotardigrade *Batillipes*, which is considered to be much more primitive than *Pseudobiotus*, seems to possess an arrangement that is more similar to that of the arthropods. Ignoring transcriptional orientations, the *Q-I-M* gene order found in *Batillipes* has been reported in some arthropod species, including two cirripedes (*Tetraclita japonica* and *Pollicipes polymerus*), one hemipteran insect (*Trialeurodes vaporariorum*), and two scorpions (*Mesobuthus gibbosus* and *Centruroides limpidus*), as shown in Fig. 3A.

The differences in *trnI* position between the two tardigrades and between tardigrades and arthropods, the frequent gene rearrangements (especially in the positions of *trnQ* and *trnI*) within the arthropods, and the lack of any such gene arrangement in the four myriapods examined, make it difficult for the gene arrangement to be considered a molecular marker linking the tardigrades and arthropods (Fig. 3A). Therefore, we excluded *trnQ* and *trnI* and conducted a further comparative analysis.

Finding a stable phylogenetic marker joining Tardigrada and Arthropoda based on mitochondrial gene arrangements The mitochondrial gene arrangement *M-nad2-W-C-Y-cox1* found in the two tardigrades was observed in 60 of 115 arthropods (52.2%) (Table 2). The gene arrangement *M-nad2-W-*(*C-Y*)*-cox1*, including variants caused by changes in the transcriptional orientations of *trnC* and/or *trnY*, was found in 69 out of 115 arthropods (60.0%). Such a high level of similarity between the tardigrades and arthropods has never been observed in the mitochondrial genomes of 70 other protostome species,

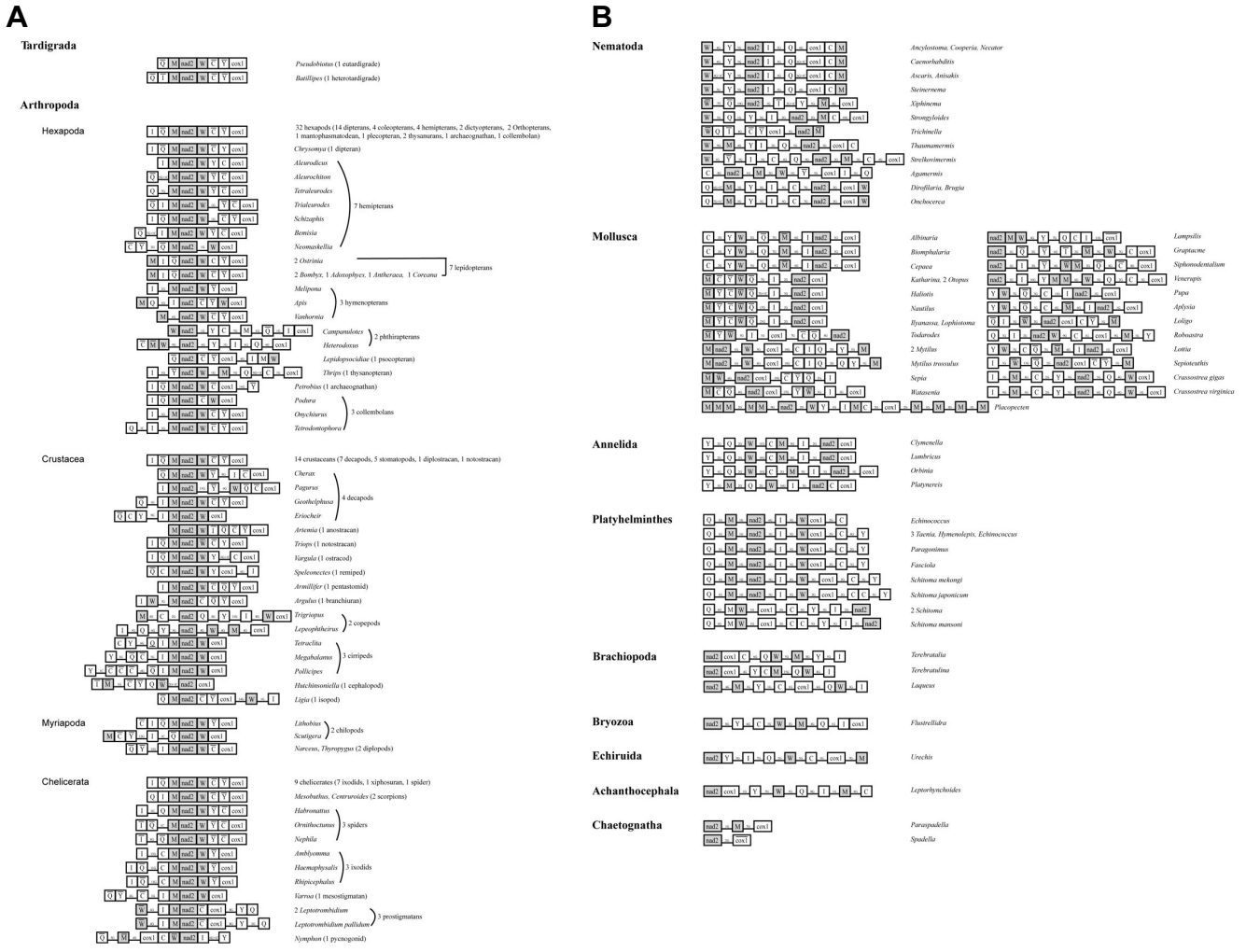

Fig. 3. Comparison of the partial mitochondrial gene arrangements in 185 metazoan animals, including 2 tardigrades, 115 arthropods, 16 nematodes, 4 annelids, 29 molluscs, 13 platyhelminthes, 3 brachiopods, 1 echiuran, 1 bryozoan, 1 acanthocephalan and 2 chaetognaths. Partial mitochondrial gene arrangements of (A) 115 arthropods and 2 tardigrades and (B) 70 other protostomes are shown for comparison. Refer to the Supplementary Table 1 for more detailed information on the taxa employed here. Abbreviations for genes are as noted in Fig. 1. G, gene(s); C, a control region.

nine metazoan phyla (Fig. 3B) or in the genomes of more than 646 deuterostomes (representative examples: Supplementary Fig. 1). Thus, it is likely that the arrangement *M-nad2-W-*<u>*C*</u>*-*<u>*Y*</u>*-cox1* is a marker joining the Tardigrada and Arthropoda phyla.

However, the same arrangement was not found in all four myriapods examined. We therefore excluded <u>*C*</u>*-*<u>*Y*</u>*-cox1* and used only the *M-nad2-W* region for the subsequent comparison in order to search for a much more stable and easily identifiable marker that unambiguously reveals a close relationship between Tardigrada and all four major arthropod groups. Since we found the *M-nad2-W* arrangement not only in the two examined tardigrades, but also in most arthropods (88 of 115 arthropods; 76.5%), this arrangement appears to be a much shorter, but even more convincing, marker joining Tardigrada and Arthropoda (Fig. 3A and Table 2). Three of the four species (75.0%) of myriapods have the same gene arrangement. In addition, it is intriguing that the pattern is strongly conserved in 18 of 22 chelicerates (81.8%), considering that many dramatic mitochondrial gene rearrangements have been reported in chelicerates (Boore, 1999; Qiu *et al*., 2005; Shao *et al*., 2004a; 2004b). It is likely that the gene arrangement pattern, *M-nad2-W-*<u>*C*</u>*-*<u>*Y*</u>*-cox1* (and the much more stable marker, *M-nad2-W*) is the first non-sequence type of molecular element joining Tardigrada and Arthropoda.

The complete mitochondrial genome from a sea spider, *Nymphon gracile* (Pycnogonida, Nymphonidae), was recently sequenced and characterized (Podsiadlowski and Braband, 2006). The arrangement *M-nad2-W* is not found in *Nymphon*, as shown in Fig. 3A. However, our most

Table 1. Frequencies of (*I/Q/M*)-*nad2*-(*W-C-Y*)-*cox1* arrangements in four major arthropod groups.

Gene arrangements	Che[1]	Myr[2]	Cru[3]	Hex[4]	Total
I-Q-M-nad2-W-C-Y-cox1	9/22 (40.9%)	-	14/31 (45.2%)	32/58 (55.2%)	55/115 (47.9%)
I-Q-M-nad2-W-C-Y-cox1	-	-	-	1/58 (1.7%)	1/115 (0.9%)
I-Q-M-nad2-W-C-Y-cox1	-	-	1/31 (3.2%)	-	1/115 (0.9%)
Q-I-M-nad2-W-C-Y-cox1 (similar to that of *Batillipes*)	2/22 (9.1%)	-	-	-	2/115 (1.8%)
M-I-Q-nad2-W-C-Y-cox1	-	-	-	5/58 (8.6%)	5/115 (4.4%)
M-I-Q-nad2-W-C-Y-cox1	-	-	-	2/58 (3.4%)	2/115 (1.7%)
Total (*I/Q/M*)-*nad2*-(*W-C-Y*)-*cox1**	11/22 (50.0%)	0/4 (0.0%)	15/31 (48.4%)	40/58 (69.0%)	66/115 (57.4%)

* (*I/Q/M*) signifies that all the cases in which the gene components within the parentheses directly abut were counted regardless of any changes of position or orientation. (*W-C-Y*) indicates that all cases with the same gene order but different orientations were counted.
[1] Chelicerata; [2] Myriapoda; [3] Crustacea; [4] Hexapoda.

Table 2. Frequencies of mitochondrial gene arrangements joining Tardigrada and Arthropoda in four major arthropod group.

Gene arrangements	Che[1]	Myr[2]	Cru[3]	Hex[4]	Total
M-nad2-W-C-Y-cox1 (tardigrades & a dominant type of arthropods)	11/22 (50.0%)	-	15/31 (48.4%)	34/58 (58.6%)	60/115 (52.2%)
M-nad2-W-C-Y-cox1	-	-	-	1/58 (1.7%)	1/115 (0.9%)
M-nad2-W-Y-C-cox1	3/22 (13.6%)	-	-	3/58 (5.2%)	6/115 (5.2%)
M-nad2-W-Y-C-cox1	-	-	-	1/58 (1.7%)	1/115 (0.9%)
Subtotal *M-nad2-W-(C-Y)*-cox1*	15/22 (68.2%)	0/4 (0.0%)	15/31 (48.4%)	39/58 (67.2%)	69/115 (60.0%)
M-nad2-W	18/22 (81.8%)	3/4 (75.0%)	24/31 (77.4%)	43/58 (74.1%)	88/115 (76.5%)

* (*C-Y*) indicates that all cases with the same gene order but different orientations were included.
[1] Chelicerata; [2] Myriapoda; [3] Crustacea; [4] Hexapoda.

recent data from a more primitive pycnogonid species, *Acelia bituberculata*, indicate that pycnogonids have the same gene arrangement (*M-nad2-W-C-Y-cox1*) as the tardigrades (Park *et al*., 2007). In both *Achelia* and *Nymphon*, *L*(*CUN*) and *L*(*UUR*) are located between *nad1* and *rrnL*, as observed in the myriapods, chelicerates and some other protostomes.

Phylogenetic implications and summary of the present results The phylogenetic implications of the results from this study are illustrated in Fig. 4. The tree shows two discrete protostome lineages: Lophotrochozoa and Ecdysozoa (Aguinaldo *et al*., 1997). Within the Ecdysozoa, Tardigrada and Arthropoda (including Hexapoda, Crustacea, Myriapoda, Chelicerata, and Pycnogonida) are clustered together with a common gene arrangement, *M-nad2-W-C-Y-cox1.* The positioning of *trnI* between *trnL1* and *trnL2* seems to be only characteristic of eutardigrades, although, due to the paucity of tardigrade mitochondrial genome data, it is difficult to completely exclude the possibility that such a pattern appears in heterotardigrades.

Despite its important phylogenetic implications with respect to the joining of Tardigrada and Arthropoda, it is difficult to clearly decide whether Tardigrada is a sister phylum of Arthropoda or a real member of the arthropods.

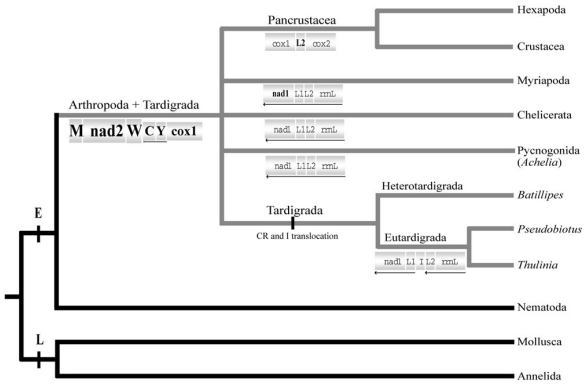

Fig. 4. A new molecular phylogenetic marker inferred from the mitochondrial gene arrangement, *M-nad2-W-C-Y-cox1* (reduced but more stable marker, *M-nad2-W*), joining Tadigrada with Arthropoda. The marker is a plausible ancestral gene arrangement that supports the alliance of Tardigrada and Arthropoda. In the eutardigrades, *Pseudobiotus spinifer* (the present result) and *Thulinia* sp. (Boore *et al*., 1998), *trnI* is located between *nad1-L1* and *L2-rrnL*. The corresponding region in *Batillipes* has not yet been sequenced. Shared-derived gene arrangements of each node/group are presented below the branches. All genes are transcribed from left to right except for those with an underlined arrow. For gene abbreviations, refer to the legend of Fig. 1. E, Ecdysozoa; L, Lophotrochozoa.

By further intensive study of the mitochondrial genomes of a number of tardigrade species, it may be possible to test whether Tardigrada is a sister group of Arthropoda as a separate phylum (supporting Panarthropoda), or a Subphylum (or Class) level taxon of Arthropoda. In addition, it would be interesting to examine whether the remaining panarthropod member, Onychophora, for which no complete mitochondrial genome sequence has been reported, possesses the conserved arrangements commonly observed in Tardigrada and Arthropoda.

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References

Aguinaldo, A. M. A., Turbeville, J. M., Linford, L. S., Rivera, M. C., Garey, J. R., *et al*. (1997) Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature* **387**, 489–493.

Boore, J. L. (1999) Animal mitochondrial genomes. *Nucleic Acids Res*. **27**, 1767–1780.

Boore, J. L. and Brown, W. M. (1998) Big trees from little genomes: mitochondrial gene order as phylogenetic tool. *Curr. Opin. Genet. Dev.* **8**, 668–674.

Boore, J. L., Lavrov, D. V., and Brown, W. M. (1998) Gene translocation links insects and crustaceans. *Nature* **392**, 667–668.

Brusca, R. C. and Brusca, G. J. (2003) Invertebrates 2nd Eds., Sinauer, Associates, Sunderland, Mass.

Dewel, R. A. and Clark, W. H. (1973) Studies on the tardigrades. II. Fine structure of the pharynx of *Milnesium tardigradum* Doyère. Tiss. *Cell* **5**, 161–169.

Folmer, O., Black, M., Hoeh, R., Lutz, R. A., and Vrijekhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol*. **3**, 294–299.

Garey, J. R., Krotec, M., Nelson, D. R., and Brook, J. (1996) Molecular analysis supports a tardigrade-arthropod association. *Inv. Biol.* **115**, 79–88.

Giribet, G., Carranza, S., Baguñá, J., Riutort, M., and Ribera, C. (1996) First molecular evidence for the existence of a Tardigrada + Arthropoda clade. *Mol. Biol. Evol.* **13**, 76–84.

Giribet, G., Edgecombe, G. D., and Wheeler, W. C. (2001) Arthropod phylogeny based on eight molecular loci and morphology. *Nature* **413**, 157–161.

Guidetti, R. and Bertolani, R. (2005) Tardigrade taxonomy: an updated check list of the taxa and a list of characters for their identification. *Zootaxa* **845**, 1–46.

Junqueira, A. C., Lessinger, A. C., Torres, T. T., da Silva., F. R., Vettore, A. L., *et al*. (2004) The mitochondrial genome of the blowfly *Chrysomya chloropyga* (Diptera: Calliphoridae). *Gene* **339**, 7–15.

Kambhampati, S. and Smith, P. T. (1995) PCR primers for the amplification of four insect mitochondrial gene fragments. *Insect Mol. Biol*. **4**, 233–236.

Kinchin, I. M. (1992) What is a tardigrade? *Microscopy (London)* **36**, 628–634.

Lessinger, A. C., Junqueira, A. C., Conte, F. F., and Azeredo-Espin, A. M. (2004) Analysis of a conserved duplicated tRNA gene in the mitochondrial genome of blowflies. *Gene* **15**, 1–6.

Lim, J. T. and Hwang, U. W. (2006) The complete mitochondrial genome of *Pollicipes mitella* (Crustacea, Maxillopoda, Cirripedia): Non-monophylies of Maxillipoda and Crustacea. *Mol. Cells* **22**, 314–322.

Lowe, T. M. and Eddy, S. R. (1997) tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res.* **25**, 955–964.

Mallatt, J. M., Garey, J. R., and Shultz, J. W. (2004) Ecdysozoan phylogeny and Bayesian inference: first use of nearly complete 28S and 18S rRNA gene sequences to classify the arthropods and their kin. *Mol. Phlyogenet. Evol.* **31**, 178–191.

Moritz, C. and Brown, W. M. (1997) Tandem duplications in animal mitochondrial DNAs: variation in incidence and gene content among lizards. *Proc. Natl. Acad. Sci. USA* **84**, 7183–7187.

Nielsen, C. (2001) Animal evolution: interrelationships of the living phyla. Oxford University Press, UK.

Park, S. J., Lee, Y. S., and Hwang, U. W. (2007) The complete mitochondrial genome of the sea spider *Achelia bituberculata* (Pycnogonida, Ammotheidae): arthropod ground pattern of gene arrangement. *BMC Genomics* **8**, 343.

Podsiadlowski, L. and Braband, A. (2006) The complete mitochondrial genome of the sea spider *Nymphon gracile* (Arthropoda: Pycnogonida). *BMC Genomics* **7**, 284.

Qiu, Y., Song, D., Zhou, K., and Sun, H. (2005) The mitochondrial sequences of *Heptathela hangzhouensis* and *Ornithoctonus huwena* reveal unique gene arrangements and atypical tRNAs. *J. Mol. Evol.* **60**, 57–71.

Rahm, G. (1937) Eine neue Tardigraden-Ordnung aus den heissen Quellen von Unzen, Inzel Kyushu, Japan. *Zool. Anz.* **120**, 65–71.

Ramazzotti, G. and Maucci, W. (1983) II Phylum Tardigrada. Terza deizoine rivenduta e corretta. *Me. Ist. Ital. Idrobiol. Dott. Marco Marchi.* **42**, 1–1012.

Regier, J. C. and Shultz, J. W. (2001) Elongation factor-2: A useful gene for arthropod phylogenetics. *Mol. Phlyogenet. Evol.* **20**, 136–148.

Shao, R., Aoki, Y., Mitani, H., Tabuchi, N., Barker, S. C., *et al*. (2004a) The mitochondrial genomes of soft ticks have an arrangement of genes that has remained unchanged for over 400 million years. *Insect Mol. Biol.* **13**, 219–224.

Shao, R., Barker, S. C., Mitani, H., Aoki, Y., and Fukunaga, M. (2004b) Evolution of duplicate control regions in the mitochondrial genomes of metazoan: A case study with Australian *Ixodes* ticks. *Mol. Biol. Evol.* **22**, 620–629.

Sun, H., Zhou, K., and Song, D. (2005) Mitochondrial genome of the Chinese mitten crab *Eriocheir japonica sinensis* (Barachyura: Thoracotremata: Grapsoidea) reveals a novel gene order and two target regions of gene rearrangements. *Gene* **349**, 207–217.

Von Siebold, C. T. E. (1854) In: von Siebold, C.T.E. and Stannius, H (eds), Handbuch der Zootomie. Berlin: Viet.