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One-Step PCR Amplification of Complete Arthropod Mitochondrial Genomes

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A new PCR primer set which enables one-step amplification of complete arthropod mitochondrial genomes was designed from two conserved 16S rDNA regions for the long PCR technique. For this purpose, partial 16S rDNAs amplified with universal primers 16SA and 16SB were newly sequenced from six representative arthropods: *Armadillidium vulgare* and *Macrobrachium nipponense* (Crustacea), *Anopheles sinensis* (Insecta), *Lithobius forficatus* and *Megaphyllum* sp. (Myriapoda), and *Limulus polyphemus* (Chelicerata). The genomic locations of two new primers, HPK16Saa and HPK16Sbb, correspond to positions 13314–13345 and 12951–12984, respectively, in the *Drosophila yakuba* mitochondrial genome. The usefulness of the primer set was experimentally examined and confirmed with five of the representative arthropods, except for *A. vulgare*, which has a linearized mitochondrial genome. With this set, therefore, we could easily and rapidly amplify complete mitochondrial genomes with small amounts of arthropod DNA. Although the primers suggested here were examined only with arthropod groups, a possibility of successful application to other invertebrates is very high, since the high degree of sequence conservation is shown on the primer sites in other invertebrates. Thus, this primer set can serve various research fields, such as molecular evolution, population genetics, and molecular phylogenetics based on DNA sequences, RFLP, and gene rearrangement of mitochondrial genomes in arthropods and other invertebrates.

Key Words: **universal primer; long PCR; Arthropoda; mitochondrial genome.**

INTRODUCTION

The mitochondrial genome of multicellular animals consists of a closed circular DNA molecule except for some cnidarians and an isopod, *Armadillidium vulgare*, in which it consists of one or two linear molecules (Warrior and Gall, 1985; Bridge *et al.*, 1992; Raimond *et al.*, 1999). Its usual size ranges from 14 to 17 kb (Wolstenholme, 1992). The mitochondrial genome has been considered useful for resolving a number of phylogenetic problems in both low and high categorical levels (Baldwin *et al.*, 1998; Ballard *et al.*, 1992). While its primary sequence except for the 12S rDNA region has been used mainly in lower categorical levels, its gene rearrangement pattern and 12S rDNA sequence have been used in higher categorical levels (Hwang and Kim, 1999).

The four major arthropod groups are Chelicerata (scorpions and horseshoe crabs), Crustacea (crabs and brine shrimp), Myriapoda (centipedes and millipedes), and Insecta (flies and beetles). Phylogenetic relationships among them remain contentious. According to recent publications (Boore *et al.*, 1995, 1998), gene arrangements of the mitochondrial genome are highly conserved within the phylum Arthropoda, although a dramatic exception in the hard ticks has been reported (Black and Roehrdanz, 1998). Thus, when rearrangements occur, they are considered powerful markers for inferring deep evolutionary history.

Characterization of the complete mitochondrial genome, however, has been established mainly in vertebrates and rarely in invertebrates. One of the reasons for this may be that many invertebrates are relatively small in size (e.g., Protozoa, Tardigrada, Gastrotricha, Arthropoda, etc.), making it difficult to extract sufficient purified mtDNA for subsequent processing. To solve this problem, numerous universal PCR primers for partly amplifying certain regions of the mitochondrial genome have been designed and published (Kocher *et al.*, 1989; Simon *et al.*, 1994; Sorenson *et al.*, 1999).

Recently, as the long PCR technique was being developed, primers for PCR amplification of the entire mitochondrial genome in one or two pieces were designed and reported (Roehrdanz, 1995; Nelson *et al.*, 1996). Nevertheless, neither primers nor experimental

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results for the one-step PCR amplification of the complete mitochondrial genome have been reported for invertebrates.

Here, we present a primer set that can be easily used to amplify entire arthropod mitochondrial genomes with one-step PCR.

MATERIALS AND METHODS

Total Cellular DNA Extraction

Total cellular DNA with high molecular weight for the subsequent long PCR was isolated from a representative of each of the four major arthropod groups. For DNA isolation, ethanol-preserved samples or frozen samples stored in a −70°C deep freezer were ground to powder in liquid nitrogen and digested with proteinase K (15 mg/ml) in lysis buffer (100 mM Tris–Cl, pH 8.0, 160 mM sucrose, 80 mM EDTA, and 0.5% SDS) for 3 h at 65°C. Purification was done via ethanol/chloroform extraction. During the process, the centrifuge speed did not exceed 960 *g* to prevent the extracted total cellular DNA from physically fragmenting into small sizes. This is a critical step to successfully amplify the complete mitochondrial genome, because DNA fragments should be larger than 50 kb to get larger than 10 kb product of long PCR.

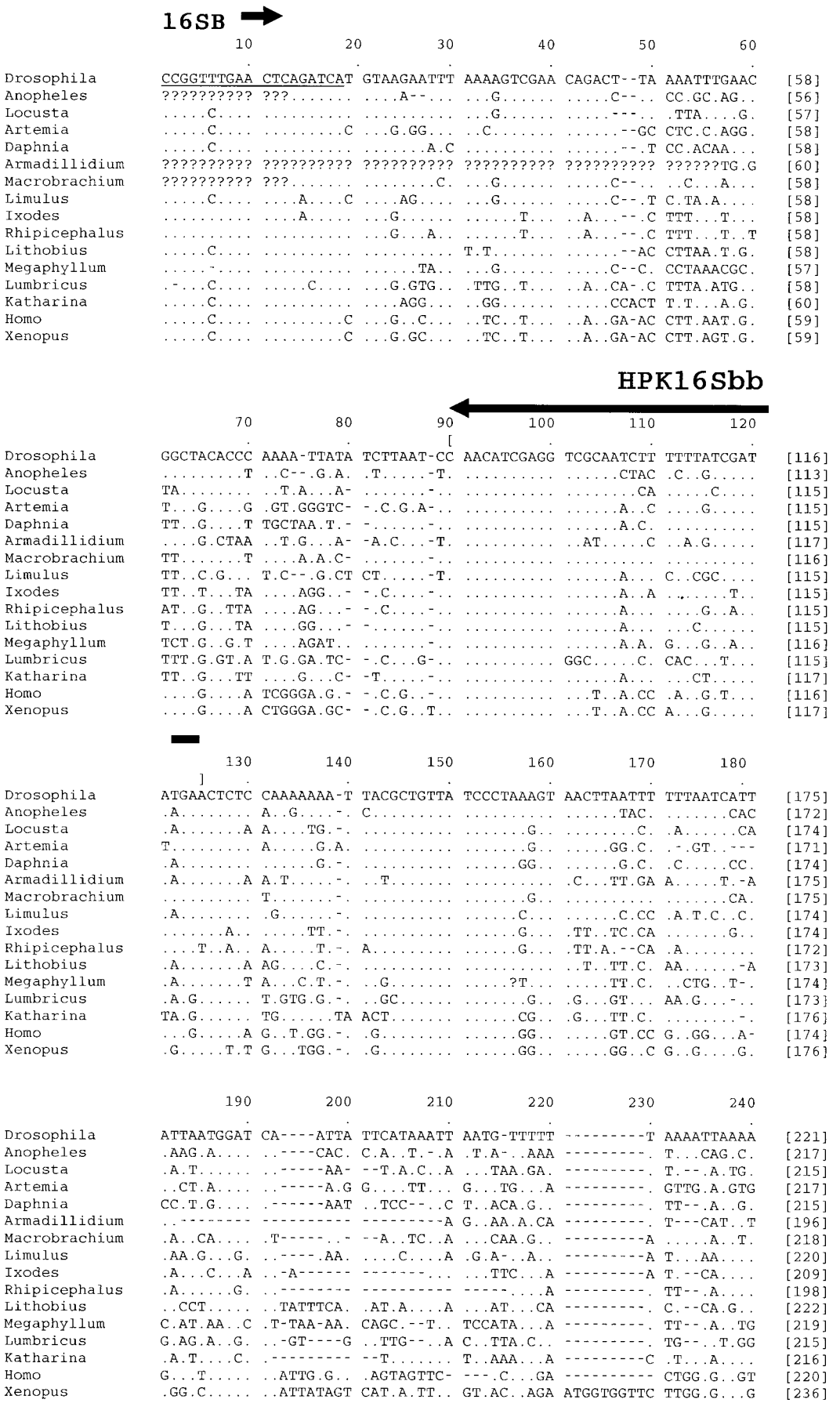
Primer Design

Prior to the performance of the long PCR, the partial portions of the large subunit ribosomal RNA genes (16S rDNA) from the prepared total cellular DNAs of *Armadillidium vulgare* (Accession No. AF373610), *Macrobrachium nipponense* (AF373611), *Anopheles sinensis* (AF373609), *Lithobius forficatus* (AF373608), *Megaphyllum* sp. (AF373607), and *Limulus polyphemus* (AF373606) were amplified with universal primers 16SA (20 mer) 5′-CGC CTG TTT ATC AAA AAC AT-3′ (Simon *et al.*, 1994) and 16SB (18 mer) 5′-CCG GTT GAA CTC AGA TCA-3′ (Kambhampati and Smith, 1995) shown in Fig. 1. The amplification products were cloned into pGEM T-easy Vector (Promega Co.) and sequenced with a Big-Dye Terminator sequencing kit (Perkin–Elmer Co.) and an ABI 310 automated sequencer (Perkin–Elmer Co.) with M13 forward and reverse commercial vector primers. The nucleotide sequences of partial 16S rDNAs were aligned with the Clustal X multiple alignment program (Thompson *et al.*, 1997) with previously published sequences of two insects (*Drosophila melanogaster*, U37541; *Locusta migratoria*, X80245), two chelicerates (*Ixodes hexagonus*, AF081828; *Rhipicephalus sanguineus*, AF081829), two crustaceans (*Artemia franciscana*, X69067; *Daphnia pulex*, G4927669), one mollusk (*Katharia tunicata*, G557273), one annelid (*Lumbricus terrestris*, G984290), and two chordates (*Homo sapiens sapiens*, X93334; *Xenopus laevis*, M10217). Four possible primers for subsequent long PCR were designed from the most conserved regions based on the 16SA-B mutiple sequence alignment (Fig. 1). Using the Oligo ver. 4.0 program (National Biosciences Inc.), we confirmed that the primers do not form a significantly strong helix or duplex within each primer or between primers. The long PCR primers designed were synthesized and then purified through HPLC by Operon Inc.

One-Step PCR Amplification of Complete Mitochondrial Genomes in Arthropods

Long PCR for amplification of complete arthropod mitochondrial genomes was carried out by use of four combinations of four designed primers and the Expand Long Template PCR System (Boehringer Mannheim Co.). For long PCR, two types of reaction mixtures were prepared according to the manufacturer's manual: mixture 1 (25 μl) contained 1 μg of total cellular DNA, 0.3 μM each primer, and 0.5 μM each dNTP; mixture 2 (25 μl) contained 5 μl of 10× buffer system 3 (20 mM Tris–HCl, pH 7.5, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% Tween 20, 0.5% Nonidet-P40, 50% glycerol), 1 mM $MgCl_2$, and 2.5 units of enzyme mix (*Taq* + *Pwo*) supplied by the manufacturer. The two separate reaction mixtures were placed together in a 0.2-ml thin-walled tube (Sarstedt Co.) and mixed well. Mineral oil (30 μl) was overlayed on top of the final reaction mixture (50 μl) to prevent evaporation of the reaction mixture. A Perkin-Elmer GenAmp 9700 Thermal cycler was used and the reaction conditions were 1 cycle (2 min at 92°C), 10 cycles (10 s at 92°C, 30 s at 65°C, and 13 min at 68°C), 20 cycles (10 s at 92°C, 30 s at 65°C, and 13 min at 68°C + cycle elongation of 20 s for each cycle), and 1 cycle (a prolonged elongation for 7 min at 68°C). PCR products were loaded onto 0.7% TAE agarose gel with appropriate DNA size marker and bands were observed on a UV transilluminator.

FIG. 1. Sequence alignment of partial 16S rDNAs from three insects, four crustaceans, three chelicerates, two myriapods, one annelid, one mollusk, and two vertebrates. The positions and orientations of universal primers 16SA and 16SB, which were used to amplify partial 16S rDNA regions, are shown at both ends of the alignment (underlined sequences). The orientations and positions of two one-step PCR primers are also indicated. These were employed for full-length amplification of the arthropod mitochondrial genomes in the present study. Drosophila, *Drosophila melanogaster;* Anopheles, *Anopheles sinensis;* Locusta, *Locusta migratoria;* Artemia, *Artemia franciscana;* Daphnia, *Daphnia pulex;* Armadillidium, *Armadillidium vulgare;* Macrobranchium, *Macrobrachium nipponense;* Limulus, *Limulus polyphemus;* Ixodes, *Ixodes hexagonus;* Rhipicephalus, *Rhipicephalus sanguineus;* Lithobius, *Lithobius forficatus;* Megaphyllum, *Megaphyllum* sp.; Lumbricus, *Lumbricus terrestris;* Katharina, *Katharia tunicata;* Homo, *Homo sapiens sapiens;* Xenopus, *Xenopus laevis.* ".", Base identical to *Drosophila* sequence located in the first line of each alignment set; "-", alignment gap; "?" unknown sequence.


16SB
HPK16Sbb

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                250        260        270        280        290        300
                 .          .          .          .          .          .
Drosophila      GTTT------ ---------- ---------- TTTAAATTTT AAT-ATCACC CCAATAAAA-   [253]
Anopheles       ...AC----- ---------- ---------- ACATT..A.C T.C-...T.. ....CG...T   [251]
Locusta         AGAG------ ---------- -------TTT AA.T.T.C.. C..-G..... ....C....-   [250]
Artemia         T..A------ ---------- ---------- ..CTGT.C.. .CC-G..G.. ....CCG..-   [249]
Daphnia         AAG.GG---- ---------- T------ACT ...T..C... CT--G..G.. ....C....-   [252]
Armadillidium   ...------- ---------- --------AT ...------- ---------- -------..-   [206]
Macrobrachium   AAAG------ ---------- -------TTA A......... ...-.C.... ...G.....-   [253]
Limulus         .A.A------ ---------- ---------- AG.TTC...C TC.-.C.G.. ...G.....-   [252]
Ixodes          .A.------- ---------- -------ATA .A.TCT.... T.--...... ....CC...-   [242]
Rhipicephalus   AG.------- ---------- -------TTT AAAT.T.... CC--GC.G.. ...GCT...-   [231]
Lithobius       ...------- ---------- --------TT .C.T.T.C.A C.--..TG.. ....CT...-   [254]
Megaphyllum     T.G.TT---- ---------- T------ATT AA.T.--CA. TT--GC.G.. ...GCC...-   [254]
Lumbricus       .A.G------ ---------- -------ATG GG...T.CCC TG--G..G.. ....CCG..-   [249]
Katharina       .AAG------ ---------- -------CTT .A.TTG..C. TT.-G..G.. ....CT...A   [252]
Homo            C..AGCATGT ACTGCTC--- --GGAGGTTG GG.TCTGC.C CGAGG..G.. ....CCG..A   [275]
Xenopus         .G.GGTAGGC CCTATTCTTC AAGGAGGATT ...T.T.C.C CG.GG..G.. ....CCG..A   [296]

                310        320        330        340        350        360
                 .          .          .          .          .          .
Drosophila      ---------- TATTTTAATT TATTAAAATT AAATTAATCT T---TATAAT TAAAATAAA-   [299]
Anopheles       A---TAAGCC ..A.AA.GC. AC..TTT.C. C..-.-T.-- -----G...G CC....T..-   [298]
Locusta         ---------- ---CA...AA AT.A.C.T.A .......A.. A---...... A.....T..-   [293]
Artemia         ---------- ---CA.TGC. .TACTCTG.C T.....CCTA C---CGC..G AC...AG..-   [292]
Daphnia         ---------- ---...C..C AT--.-.T.. TTG...TAAA A---AG...C CT...ACTT-   [292]
Armadillidium   ---------- ---C...TGG ----.G...A .-....CCAC C----CC.G. A...TATTT-   [243]
Macrobrachium   ---------- ---...T..C ..AA.T.TAA .TT....AA. .---CT.T.. A.TTTAT..-   [296]
Limulus         ---------C .C.C...GCC .T.CCC..CC G..--...TA -----....A A....CC..-   [295]
Ixodes          ---------- ---..AG... .C--...TAA TTC.C.TCA- ------C... CT..------   [274]
Rhipicephalus   ---------- ---A..TT.. ..--.T.G.A GTTAC.TAT- ------...A .....--TT-   [266]
Lithobius       ---------- ---CG....A ----.....A .T..A.T.TC A----T...A .....AT.T-   [292]
Megaphyllum     ---------- ---C.A..C. A.--.CG..A .T.CA.TAA. A---ATA..C GT...AT.T-   [295]
Lumbricus       ---------- ---...T.G. -----..... TTT.C.TAT- ------.T.. .T...-----   [279]
Katharina       A---TGGA-- --.A.A.... .TG.TTTTC. ...AG.T.T. A---.T..TA C.T..A--T-   [299]
Homo            TTTTTAATGC AGG..-TGG. AG..T.GGAC CTG.GGG.T. ----GT..GG ..CTG.TTG-   [329]
Xenopus         ACTTTAGGTC AG...CTGC. .G..T..T.G TT...TCCT. ATGGGT..GG .TC..CTTGG   [356]

                370        380        390        400        410        420
                 .          .          .          .          .          .
Drosophila      TA--A----A ATATAAAGAT TTATAGGGTC TTCTCGTCTT TTAAATTAAT TTTAGCTTTT   [353]
Anopheles       AC--T----T ........T. .......... ..A.....CC ACT..A.... ..G...C...   [352]
Locusta         .T--TAATA. ..G.....C. C......... ........CC AAG..A..-. ..A...C...   [350]
Artemia         .------TA. ..G..... C......... ..A...C.C. CC..G.AC.. ..A.......   [346]
Daphnia         ----TAATG. -A......C. CC.A...... .GA.....CC CC...CGC.. ..A.......   [347]
Armadillidium   -------AT. -.....G.T. .--..... ..A..... .C--TAATT. A.A..AAC..   [291]
Macrobrachium   .T--TA-TA. .A..... C......... ........C. ....T..... ..........   [353]
Limulus         A.-------- .AG.....C. .A..... ........CC ...GTAA... ..C.......   [347]
Ixodes          ---------- ---....A.. .C..... ....T...CC .A.TT.A... ...T....C.   [321]
Rhipicephalus   ---------- ----.T.A.. .C..... ....T...C. .A.T..... AA.T.T..C.   [313]
Lithobius       -------AC. -CG....AC. .A..... .......... ...TTA.T.. A.A..TA.C.   [344]
Megaphyllum     C.--TAATAT TA..C..CT. .A.A..... ....T...CC ...T.A.C.. ...T.TC...   [353]
Lumbricus       ---------- ---.T...C. C......... ....T...C. .CG.CGCT.. C.A.CAC.C.   [326]
Katharina       ---------- T--.....C. CG..... ..T..... ..G.GA.T.. ..A.....C.   [347]
Homo            C.--TTAAT. .AT.....C. CC..... .......... GCTGTG.T.. GCCC..C.C.   [387]
Xenopus         C.GTTCTTT. G.T.....C. CC......-. .......TC. A.GGT.CT.. CCCC..C.C.   [415]

                430        440        450        460        470        480
                 .          .          .          .          .          .
Drosophila      TGACTAAAAA ATAA--AATT CTATTTTAAA TTTAAATGAA ACAGTTAATA TTTCGTCCAA   [411]
Anopheles       .C...C.... G.T.--.... .A.A..ACGT AAAGG.---G ....C.C..C CC........   [407]
Locusta         .A...T.... G.T.AATTC. AA.AA.ATT. ..A.G.---G .....A...T .C........   [407]
Artemia         .C...T...G ..T.--.C.. .A....A.TC GAAG.G---- ....ACC..C .C.....A..   [400]
Daphnia         .C...T.... ..G.--.... .A.CAAAT.T ....G.---G ....C.TT.T .C.....TC.   [402]
Armadillidium   .TTTAT-TCT TAG.TT.... TA.CAAA.-. AAAT..---T .A.....T.C ...T...GGG   [346]
Macrobrachium   .A........ ....AGTTC. A.T.AAATTT .AA..---. .....AT... .C..A.T...   [410]
Limulus         .C...... ....--.... .A.AA.CT.T ..CC..---G ....A.TT.T ..A.......   [402]
Ixodes          .C..A..... ..T.--.... .AT...ATT. A.....---G .A..-.T..T AA....TA..   [375]
Rhipicephalus   GC..A..TT. .A..--.... TC.A...TT. AC.TG.---. .A.A-GTT.T ....TGAA.T   [367]
Lithobius       .C..CT.TCT TA..--.... .ACC.A.CT. AAAT..---G .....-.CAT CCC.A..A..   [398]
Megaphyllum     .A..A..... GA..--.... .A...A.T.. A..T.---G ....-.GCCT CCGT...A..   [407]
Lumbricus       .CGG.T.G.G GGT.--TT.. TA.GCGGCTT A.AG.G---. CAGT-ATT.. ......TT.T   [380]
Katharina       .C...T.... ..T.--.T.. T.....A.TG .AAT.G--.G .....CTTGC CCA....A.G   [403]
Homo            .C..GGGC.G G.C.---... TC.C.GGTT. AAAGT.A..G ....C.G.AC CC....GG.G   [444]
Xenopus         GC..GG--.G G.C.---G.. TA..GGATTG GA.GC.G..G ......G.AC C.....GG--   [468]
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FIG. 1—*Continued*

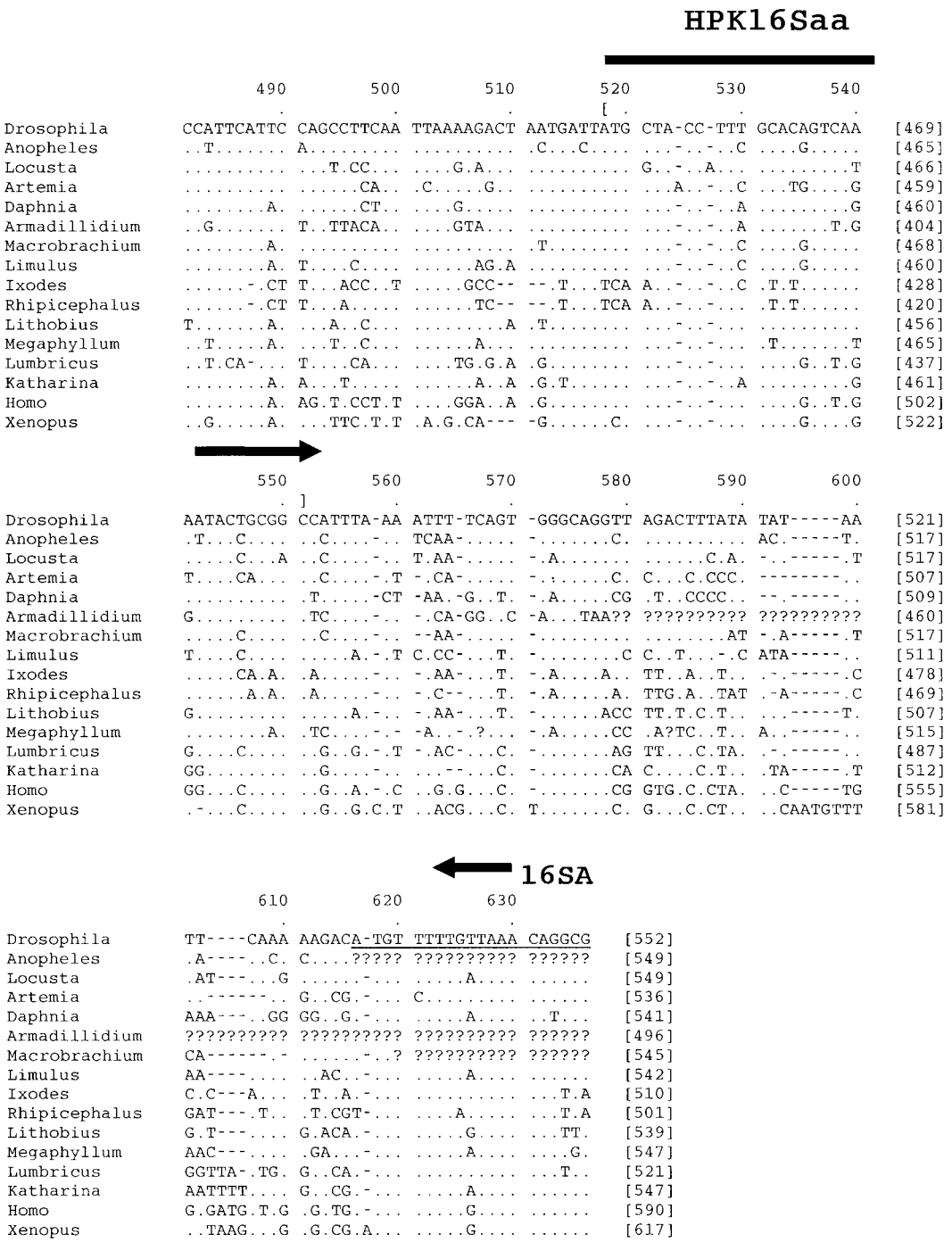

FIG. 1—*Continued*

RESULTS

Universal Primers for One-Step Long PCR

In the present study, nucleotide sequences of 16SA-B regions from *A. sinensis* (Insecta), *A. vulgare* and *M. nipponense* (Crustacea), *L. forficatus* and *Megaphyllum* sp. (Myriapoda), and *L. polyphemus* (Chelicerata) were newly determined prior to the designing of long PCR primers. Four primers were designed from the most conserved regions in the sequence alignment of 16SA-B regions constructed with the newly sequenced data and those of other arthropods retrieved from the EMBL database (Fig. 1). Of the four combinations of the primers, the best combination of primers was fi-

HPK16Saa

```
                          10         20         30
                          .          .          .
(32mer)       5′- ATGCTA-CC-TTTGCACRGTCAAGATACYGCGGC - 3′

Drosophila          ......-..-.......A.....A....T.....
Anopheles           ......-..-..C....G.....AT...C.....
Locusta             ...G..-..A.......A....TA....C...A.
Artemia             ......A..-..C...TG....GT....CA....
Daphnia             ......-..-..A....A....GA....T.....
Armadillidium       ......-..-..A....A..T.G.....T.....
Macrobrachium       ......-..-..C....G.....A....C.....
Limulus             ......-..-..C....G.....T....C.....
Ixodes              TCAA..-..-..C.T.TA.....A....CA.A..
Rhipicephalus       TCAA..-..-....T.TA.....A....TA.A..
Lithobius           ......-..-.......A..........T.....
Megaphyllum         ......-..-....T..A....TA....T..A..
Lumbricus           ......-..-.......G..T.G.....C.....
Katharina           ......-..-..A....A....G.G...T.....
Homo                ......-..-.......G..T.G.G...C.....
Xenopus             .C....-..-.......G....GA-...C.....
```

HPK16Sbb

```
                          10         20         30
                          .          .          .
(34mer)       5′ - CTTATCGAYAAAAAAGWTTGCGACCTCGATGTTG - 3′

Drosophila          .A......T.......A.................
Anopheles           .A......C..G.GTAG.................
Locusta             .......GT....TG.A.................
Artemia             .AA.....C....G..T.................
Daphnia             ........T.....G.T.................
Armadillidium       ........C.T..G..A..AT.............
Macrobrachium       .A......T.......A.................
Limulus             .......GCG..G...T.................
Ixodes              .A...A..T....T..T.................
Rhipicephalus       .A...T..C.......T.................
Lithobius           ........TG......T.................
Megaphyllum         .....T..C...C.T.T.................
Lumbricus           ......A.T.GTG.G.A...GCC...........
Katharina           ..A.....AG......T.................
Homo                .A....A.C..T.GG.T..A..............
Xenopus             .C......C...TGG.T..A..............
```

FIG. 2. Sequences of two one-step long PCR primers, HPK16Saa and HPK16Sbb, and sequence alignments of the two primer sites of three insects, four crustaceans, three chelicerates, two myriapods, one annelid, one mollusk, and two vertebrates. Drosophila, *Drosophila melanogaster;* Anopheles, *Anopheles sinensis;* Locusta, *Locusta migratoria;* Artemia, *Artemia franciscana;* Daphnia, *Daphnia pulex;* Armadillidium, *Armadillidium vulgare;* Macrobrachium, *Macrobrachium nipponense;* Limulus, *Limulus polyphemus;* Ixodes, *Ixodes hexagonus;* Rhipicephalus, *Rhipicephalus sanguineus;* Lithobius, *Lithobius forficatus;* Megaphyllum, *Megaphyllum* sp.; Lumbricus, *Lumbricus terrestris;* Katharina, *Katharia tunicata;* Homo, *Homo sapiens sapiens;* Xenopus, *Xenopus laevis.* ".", Base identical to primer sequence located in the upper region of each alignment set; "-", alignment gap.

nally selected through long PCR experiment: HPK16Saa (32 mer) 5′-ATG CTA CCT TTG CAC RGT CAA GAT ACY GCG GC-3′ and HPK16Sbb (34 mer) 5′-CTT ATC GAY AAA AAA GWT TGC GAC CTC GAT GTT G-3′ (Fig. 2). The locations of HPK16aa and HPK16bb correspond to positions 13314–13345 and 12951–12984, respectively, of the *Drosophila yakuba* mitochondrial genome.

Usefulness of One-Step Long PCR Primers in Arthropods

To examine the usefulness of the selected primers in arthropods, long PCRs were repetitively performed with total cellular DNAs extracted from representatives of the four major arthropod groups: a chelicerate (*L. polyphemus*), an insect (*A. sinensis*), two myriapods (*L. forficatus*, *Megaphyllum* sp.), and a crustacean (*M. nipponense*). The result showed that the primer set can be used to successfully amplify complete mitochondrial genomes from all five arthropods examined as shown in Fig. 3. The obtained PCR products are about 15.5 kb in length.

To confirm that the long PCR products were amplified from mtDNA, all the PCR products were eluted from the gel, and then both ends of them were directly sequenced with the primers HPK16Saa and HPK16Sbb, which had been used in the long PCR. DNA sequencing was carried out by a Big-Dye Terminator sequencing kit and an ABI 310 automated sequencer (Perkin–Elmer Co.). The sequences were the same as the 16S rDNA sequences known through the experiment to design long PCR primers (data not shown). It indicates that PCR products of ca. 15.5 kb in length were amplified from mtDNA.

Considering the sequence alignment of the two primer sites (Fig. 2) and the result of the PCR experiment (Fig. 3), the primer set seems to be very powerful over a wide range of arthropods. Thus, with this set, we can easily and rapidly amplify complete mitochondrial genomes with small amounts of arthropod genomic DNA.

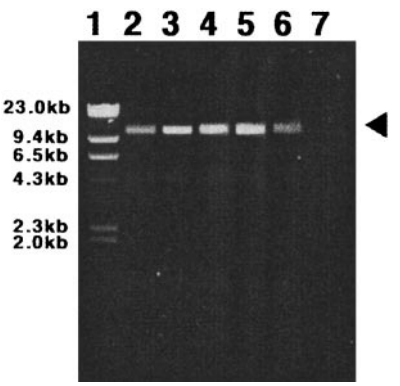

FIG. 3. One-step PCR amplification with HPK16Saa and HPK16Sbb of complete mitochondrial genomes from five arthropods representing the major arthropod groups. PCR products were loaded onto 0.7% TAE agarose gel. Lane 1, λ DNA/*Hin*dIII size marker (Promega Co.); lane 2, *Anopheles sinensis* (Insecta); lane 3, *Macrobrachium nipponense* (Crustacea); lane 4, *Limulus polyphemus* (Chelicerata); lane 5, *Lithobius forficatus* (Myriapoda, Chilopoda); lane 6, *Megaphyllum* sp. (Myriapoda, Diplopoda); lane 7, negative control performed without any cellular DNA. The arrowhead indicates long PCR products of full-length mitochondrial genomes, which are ca. 15.5 kb in length.

DISCUSSION

Prior to the designing of one-step PCR primers, nucleotide sequences of the 16SA-B regions should be determined from selected arthropods, *A. sinensis* (Insecta), *A. vulgare* and *M. nipponense* (Crustacea), *L. forficatus* and *Megaphyllum* sp. (Myriapoda), and *L. polyphemus* (Chelicerata). Because no 16SA-B sequences have been published in myriapods so far, we sequenced those regions from a chilopod (*L. forficatus*) and a diplopod (*Megaphyllum* sp.) representing the two major groups of myriapods. In chelicerates, although complete mitochondrial genomes from two hard ticks were characterized, their sequence evolution and gene arrangement showed peculiar patterns compared to those of the other arthropods examined (Black and Roehrdanz, 1998). Therefore, 16SA-B sequence of *L. polyphemus*, known as having a relatively slow evolutionary rate and as being one of the most representative chelicerates, was also determined and used. Compared to myriapods and chelicerates, relatively more sequences from insects and crustaceans have been published (Crease, 1999; Flook *et al.*, 1995; García-Machaco *et al.*, 1999). We added 16SA-B sequence of *A. vulgare* (Raimond *et al.*, 1999) because its mitochondrial genome is linearized and has evolved peculiarly as shown in some cnidarians (Warrior and Gall, 1985; Bridge *et al.*, 1992). The finding of a linearized mitochondrial genome in arthropods is surprising because all the arthropods known so far have circular mitochondrial genomes. In addition to this, it was recently revealed that primary and secondary structures of its nuclear SSU rDNA were extremely expanded and unique, especially in V4 and V7 (Choe *et al.*, 1999). Thus, if the primer sites designed are conserved in *A. vulgare* which has a highly deviated molecular evolutionary pattern, it is likely to become strong evidence supporting the proposal that the primers designed from those sites could function over a wide range of arthropods. Thereby, *A. vulgare* among crustaceans was chosen for this study. However, due to the linearized shape of its mitochondrial genome, it is impossible to conduct long PCR. It was employed only for sequence comparison of long PCR primers.

Development of one-step PCR primers to amplify full-length mitochondrial genomes has significant implications for various studies performed with microscopic invertebrates. Due to the difficulties of isolating sufficient purified mtDNA from minute invertebrates, complete mitochondrial genomes have not been explored as fully in invertebrates as in vertebrates. The one-step primer set enables us to easily and rapidly obtain sufficient mtDNA through one-step PCR. A separate step extracting purified native mtDNA, typically requiring a large amount of samples, is no longer necessary. Although the primer set presented here was examined only in major arthropod groups, it is likely that the set will successfully work for other invertebrates, considering the relatively high degree of sequence conservation in two primer sites of various other invertebrates (Fig. 2). Thus, this primer set can serve various research fields such as molecular evolution, molecular phylogenetics, and population genetics based on mitochondrial genomes not only in arthropods but even in other invertebrates. In particular, it will shed light on phylogenetic relationships among major arthropod groups based on gene order of the entire mitochondrial genome.

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