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Cloning of Molluscan Telomere DNA with (TTAGGG)n Repeat and its Chromosomal Location in the Freshwater Snail *Biwamelania habei*

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ABSTRACT—In the present study, we have cloned and determined the nucleotide sequence of telomeric DNA of a freshwater gastropod *Biwamelania habei*. The sequence was comprised of (TTAGGG)n tandem repeat, which is identical with the sequence of vertebrate telomere. The characteristic sequences, together with the chromosomal localization revealed by FISH analysis and the Bal 31 exonuclease sensitivity of the hybridization signal, support the sequences to be located at chromosomal ends. This is the first report for identification of molluscan telomere DNA and is also the first report of invertebrate telomere with (TTAGGG)n sequence. Although the telomere sequence of *B. habei* is different from that of any other invertebrates reported to date, the sequence universally occurs among various eukaryotes including some metazoans. It is thus evident that *B. habei* retains ancestral sequence for invertebrate telomeres. Interstitial telomeric signals were observed in some chromosomes. Some of such interstitial telomeric sites coincide with the presumed fusion point of Robertsonian rearrangement.

INTRODUCTION

Telomeres are structures that form the termini of linear eukaryotic chromosomes and are responsible for complete replication, meiotic pairing, and stability of chromosomes (Zakian, 1989; Blackburn, 1991). In most eukaryotic organisms, telomeres consist of tandem repeats of GC-rich sequences.

Telomere sequence shows a high degree of evolutionary conservation. The human telomere sequence (TTAGGG)n is present in all vertebrates from *Mus musculus* (Kipling *et al*., 1995), cattle (Tsao *et al*., 1998) to several fishes (Garrido-Ramos *et al*., 1998; Perez *et al*., 1999). In addition, this sequence also occurs in lower and distantly related eukaryotes, such as trypanosomes (Van der Ploeg *et al*., 1984), several slime molds (Forney *et al*., 1987) and fungi (Javerzat *et al*., 1993). The ubiquitous nature of the sequence shows the (TTAGGG)n to be the universal telomeric consensus sequence in eukaryotic genomes.

On the other hand, there is considerable diversity among the different telomeric DNAs. For example, in some invertebrates, telomeric repeat sequence is TTAGG for Bombyx (Okazaki *et al*., 1993), TTAGGC for *Caenorhabditis* and *Ascaris* (Muller *et al*., 1991) and TTGCA for *Parascaris* (Teschke *et al*., 1991). In *Drosophila*, DNA at the end of chromosomes is composed of a transposable element, instead of simple tandem repeat sequence (Biessmann *et al*., 1990; Saiga and Edstrom, 1985; Traverse and Pardue, 1989; Young *et al*., 1983). It is notable that TTAGGG universal telomere sequence has never been found in invertebrate animals. In addition, many lower eukaryotes, such as *Tetrahymena*, *Paramecium, Dictyostelium, Saccharomyces,* have species-specific telomeric sequences, which deviated from the universal TTAGGG (review by Zakian, 1995).

Mollusca is one of the largest phylum of invertebrate animals. Although many cytological studies on various molluscan taxa have been carried out, the telomeric structure is poorly understood. There are only a few reports for FISH (Fluorescence *in situ* hybridization) and southern hybridization analyses on molluscan chromosomes using the human telomeric probe (Guo and Allen, 1997; Okazaki *et al*., 1993). Molluscan telomere sequence has never been determined yet. Identification of precise telomere sequence from some molluscs should provide invaluable information for understanding the diversity and evolution of invertebrate telomere. Further systematic studies on various eukaryotic groups are necessary

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to obtain an overview of the diversity of the telomeric sequence.

In the present study, we have cloned and determined telomere sequence from a freshwater gastropod *Biwamelania habei*. We also confirmed the chromosomal localization of the telomere sequence obtained. This is the first report for identification of molluscan telomeric DNA.

MATERIALS AND METHODS

Southern hybridization

B. habei was collected from Lake Biwa, Shiga Prefecture, Japan. In the following experiment, the standard protocols for the DNA analyses were carried out as described by Sambrook *et al.* (1989). Total genomic DNA was extracted from hepatopancreas of adult snails and purified with the CTAB method as described by Shahjahan (1995).

Total genomic DNA of *B. habei* was digested with the restriction enzyme, *Eco* RI, *Hind* III and *Pst* I (Takara). Digested DNA fragments were separated on 0.7% agarose gel and transferred onto nylon membrane Hybond N (Amersham). Southern analysis was performed using digoxigenin (Dig) DNA Labeling and Detection Kit (Roche Diagnostics). As the third nucleotide of the telomeric repeat sequence is occasionally variable among some taxa, (TTNGGG)n sequence was employed as a probe for the primary screening. Highly repetitive probe sequence was amplified by PCR as described by Ijdo *et al.* (1991) and was labeled with Dig by random primed DNA synthesis. Hybridization was performed in a low-stringent condition: 0.02 μg/ml probe DNA, 50% formamide, 50 mM $NaPO_4$ (ph 7.0), 5% sodium dodecyl sulfate (SDS), 0.1% lauloylsarcosine and 2% skim milk at 37°C overnight. Post-hybridization washes were performed twice in 2×SSC, 0.1% SDS at room temperature for 5 min, followed by twice washes in 0.1×SSC, 0.1% SDS at 68°C for 15 min. The washed membrane were immunodetected with anti-digoxigenin-AP (alkaline phosphatase) and visualized with NBT (nitro blue tetrazolium salt) / X-phosphate (5-bromo-4-chloro-3-indolylphosphate).

Cloning and sequencing

Total genomic DNA was digested with *Eco* RI and separated on 0.7% agarose gel. After the electrophoresis, DNA fraction larger than 20 kbp in size was excised from the gel and purified by the prep-A-Gene DNA purification systems (BIO RAD). These procedure was intend to increase the efficiency of cloning, as we confirmed by the southern analysis that the putative telomeric DNA was not digested by *Eco* RI at all (see below). Then, the extracted DNA was sonicated to decrease the size into 0.1 to 1 kbp, blunted with DNA Blunting Kit (TAKARA), and ligated into the *Sma* I site of the vector pUC 118. The ligation mixture was used to transform competent cells of DH5 α strain of *Escherichia coli*. The positive clones were screened by colony hybridization with Dig-labeled (TTNGGG)n probe under the conditions described above.

Nucleotide sequence of the positive clones were determined by an ABI 377 automated sequencer (Perkin-Elmer) using ABI Prism Big Dye Terminator Cycle sequencing Ready Reaction Kit (Applied Biosystem).

Bal 31 exonuclease analysis

Total DNA of *B. habei* (5 μg/ml) was digested with Bal 31 nuclease in 20 mM Tris-HCl (pH 8.0), 600 mM NaCl, 12 mM $MgCl_2$, 1 mM EDTA for 15, 30, 60, 180 min using 2.5 U enzyme/5 μg DNA at 30°C. As a positive control for Bal 31 activity, 0.05 μg/μl of Lambda phage DNA digested with *Hind* III were also added. Then the aliquots were ethanol precipitated, digested with *Hind* III and separated by electrophoresis on 0.7% agarose gel. DNAs were transferred onto nylon membrane. Southern analysis was performed with Dig-labeled (TTAGGG)n, an insert sequence of clone pBT-1 obtained from *B. habei* (see below), under the conditions described above.

Fluorescence *in situ* hybridization

Chromosome spreads of spermatogonia were obtained from adult male testes of 11 individuals of *B. habei* by air-drying method. Chromosomal preparations were stored at –20°C until use for hybridization. Slides were denatured in 70% deionized formamide and 2(SSC for 2 min at 70°C and were dehydrated through an ice-cold ethanol series. As we have identified the telomere sequence of *B. habei* to be identical with that of human telomere (see below), we used biotin-labeled human telomere probe supplied by Oncor, Inc.. Prior to incubation, DNA probes were denatured at 95°C for 10 min. 10 μl of the hybridization solution (0.5 μg/ml biotin-labeled probe DNA; 50% formamide; 2×SSC, pH 7.0) was put on a slide, and was incubated in a moisture chamber at 37°C over-night. Then slides were washed in 50% formamide and 2×SSC for 15 min at 37°C, followed by 2×SSC, 1(SSC (for 10 min at room temperature each) and then rinsed in 4×SSC.

The hybridization signals were detected by anti-rabbit serum conjugated to fluorescein isothiocyanate (FITC) after incubation with anti-biotin rabbit serum. Slides were observed under a fluorescence microscope after mounting in a fluorescence antifade solution including DNA counterstain (propidium iodide). At least 10 cells per individual were evaluated to detect hybridization signals.

RESULTS

Detection of telomeric repeat sequence in *B. habei*

As shown in Fig. 1, the total genomic DNA of *B. habei* contains some sequences hybridizing with the (TTNGGG)n probe. Positive hybridization signals were detected as a smear band larger than 20 kbp even after digestion with *Eco* RI, *Hind* III or *Pst* I. The strong hybridization signal, together with the undigested nature of the signal, suggest that the (TTNGGG)n-like sequences exist in high copy number of repetitive manner within *B. habei* genome.

Cloning of telomere sequence

After screening of the genomic library constructed as above, we obtained three positive clones, pBT-1, -2 and -3. We also performed southern analysis to *B. habei* genome using Dig-labeled these positive clones as a probe, and obtained same hybridization signal as shown in Fig. 1 (data not shown). Although we tried to determine the sequences of these clones for both DNA strands, sequencing reaction was successful

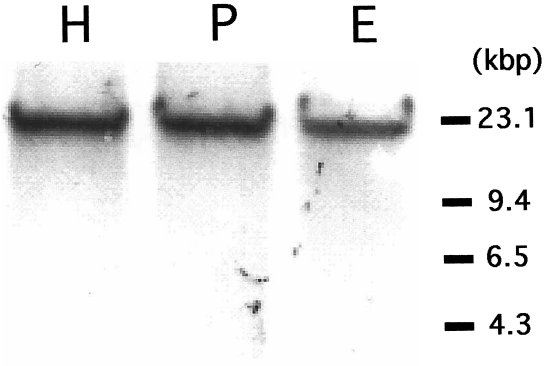

Fig. 1. Southern hybridization of *B. habei* DNA with (TTNGGG)n probe. Total DNA was digested with *Eco* RI (E), *Hind* III (H) and *Pst* I (P), respectively, separated on 0.7% agarose gel, and hybridized with Dig-labeled (TTNGGG)n probe.

a pBT-1 (**TTAGGG**)$_{90}$

b pBT-2 (**TTAGGG**)$_4$TAGGG(**TTAGGG**)$_5$TTAGAG(**TTAGGG**)$_6$(TTTAGGG)$_2$(**TT**
AGGG)$_4$TTTAGGG (**TTAGGG**)$_3$TTTAGGGTTAGAG (**TTAGGG**)$_4$TTAG
GGGG(**TTAGGG**)$_5$TTAGGTTTAGGG(**TTAGGG**)$_{16}$TTAGG(**TTAGGG**)T
TAGG(**TTAGGG**)$_5$TTAGGTTTAGGG(**TTAGGG**)$_5$TTAGGTTTAGGG(**TT**
AGGG)(TTAGAGGG)$_2$(**TTAGGG**)$_4$TTAGG(**TTAGGG**)$_2$

c pBT-3 (**TTAGGG**)$_2$TTAGGTTTAGGG(**TTAGGG**)$_4$TTTAGGG(**TTAGGG**)$_2$TTAG
GTTTAGGG(**TTAGGG**)TTAGAGGG(**TTAGGG**)$_4$TTAGG(**TTAGGG**)$_5$TTA
GTTATG(**TTAGGG**)$_2$TTAGAGG(**TTAGGG**)$_5$TTAGAGG(**TTAGGG**)$_6$TTA
GAGGG(**TTAGGG**)$_4$TTAGAGGG(**TTAGGG**)TTAGG(**TTAGGG**)$_6$TTAGA
GGG(**TTAGGG**)TTAGAGGG(**TTAGGG**)$_6$TTAG(**TTAGGG**)$_5$TTAG(**TTAG**
GG)$_3$TAAGGG(**TTAGGG**)$_4$TTAGG(**TTAGGG**)$_4$TTAGG(**TTAGGG**)$_3$TTAG
GTTTAGGG(**TTAGGG**)$_3$TAGGG(**TTAGGG**)$_2$TTTAGGG(**TTAGGG**)$_4$

Fig. 2. Partial or complete insert sequences of three positive clones, pBT-1 (**a**), pBT-2 (**b**) and pBT-3 (**c**). TTAGGG, the dominant repeat unit, is indicated by bold letters.

only in a certain strand, probably due to the tandemly repeated GC-rich sequence. In the clone pBT-2, sequencing was completed and the insert was demonstrated to have 534 nt. However, in other two clones, the sequence reaction could not be extended to the terminal. The insert lengths of pBT-1 and -3 were roughly estimated by electrophoresis as 700 and 800 kbp, respectively.

Complete or partial insert sequences of the clones are shown in Fig. 2. Clone pBT-1 contains at least 90 copies of completely conserved tandem array of the hexanucleotides TTAGGG, the universal telomere consensus sequence.

pBT-2 and -3 also contain repetitive sequences composed of TTAGGG and its related sequences (Fig. 2b, c and Table 1). In a total of 187 repeat sequences, we found 10 variants of TTAGGG. The frequency of these variants was low and most of the repeated sequences were composed of complete TTAGGG (Table 1). The variants displayed dispersed distribution and did not show local enrichment within the insert DNA (Fig. 2b, c).

All the variant units observed in pBT-2 and -3 are similar to TTAGGG and are presumed to be derived from the consensus sequence. For example, TAGGG, TTAGG and TTAG can be arisen from TTAGGG by deletion of T, G and GG, respectively. TTTAGGG, TTAGGGGG, TTAGAGG and TTAGAGGG are apparently derived from the consensus sequence by insertion of single or di-nucletides. TTAGAG and TAAGGG are variants with single nucleotide substitution. In the last variant, TTATG, both deletion and substitution should be involved in.

Table 1. Frequencies of variant repeat unit in pBT-2 and pBT-3.

	clones		
repeat unit	pBT-2	pBT-3	total
TTAGGG	65	77	142
TTAGG	7	7	14
TTTAGGG	7	5	12
TTAGAGGG	2	5	7
TTAG		3	3
TAGGG	1	1	2
TTAGAGG		2	2
TTAGAG	2		2
TTAGGGGG	1		1
TAAGGG		1	1
TTATG		1	1

Bal 31 exonuclease analysis

To determine whether the (TTAGGG)n repeats are located directly at the end of *B. habei* chromosomes, total DNA of high molecular weight was treated with Bal 31 exonuclease, which digests linear DNA from the both termini, for increasing time of exposure. As shown in Fig. 3b, hybridization signals detected by (TTAGGG)n disappeared after 180 min digestion. Judging from the digestion pattern of Lambda DNA added as control, Bal 31 exonuclease activity can be roughly estimated as c.a. 2 kbp/60 min under the experimental condition (4.3 kbp fragment disappeared at 60 min in Fig. 3a). Thus, (TTAGGG)n sequences should be located within 6 kbp from its chromosomal end in *B. habei*.

Chromosomal localization of (TTAGGG)n sequence

We examined chromosomal localization of the (TTAGGG)n sequence in *B. habei* by FISH. Fig. 4a and b show the results of FISH analysis obtained from different individuals to metaphase and prophase chromosomes, respectively. Fluorescence signals were observed at most of the chromosome ends, though the size and intensity of the signals varied among the termini. Doublet signals were detected at the telomeric ends at the metaphase chromosomes (Fig. 4a). In addition to such chromosomal termini, hybridization signals were also detected within some chromosomal arms (Fig. 4a, b). Such interstitial telomeric signals (ITS)s were

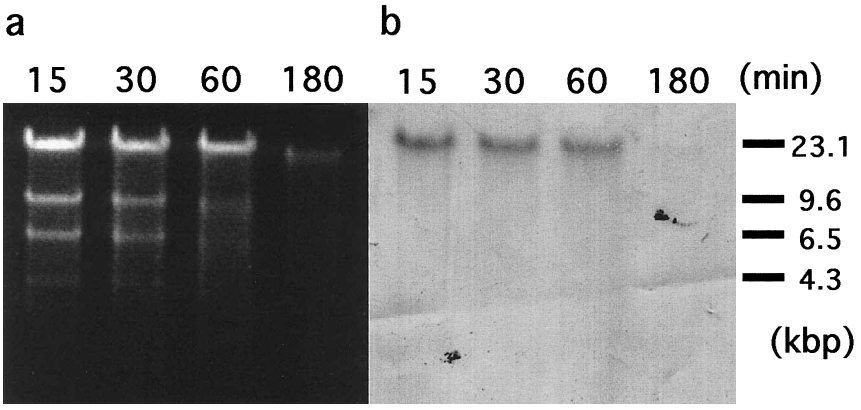

Fig. 3. Bal 31 exonuclease sensitivity of (TTAGGG)n sequence in *B. habei*. Total genomic DNA of *B. habei* was digested with Bal 31 for 15, 30, 60 and 180 min. Lambda DNA digested with *Hin*d III was also added as positive control for Bal 31 activity in the reaction. After treatment of Bal 31, the DNA was further digested with *Hin*d III, separated with 0.7% agarose gel, transferred to nylon membrane and hybridized with Dig-labeled (TTAGGG)n probe. **a:** Electrophoresis of the gel visualized by Ethidium bromide-staining. In this electrophoretic condition, genomic DNA of *B. habei* cannot be separated from 23.1 kbp fragment of Lambda DNA. **b:** Southern hybridization of Bal 31 treated DNA with Dig-labeled (TTAGGG)n probe. The hybridization signals show Bal 31 sensitivity of *B. habei* DNA, because the probe does not hybridize with Lambda DNA.

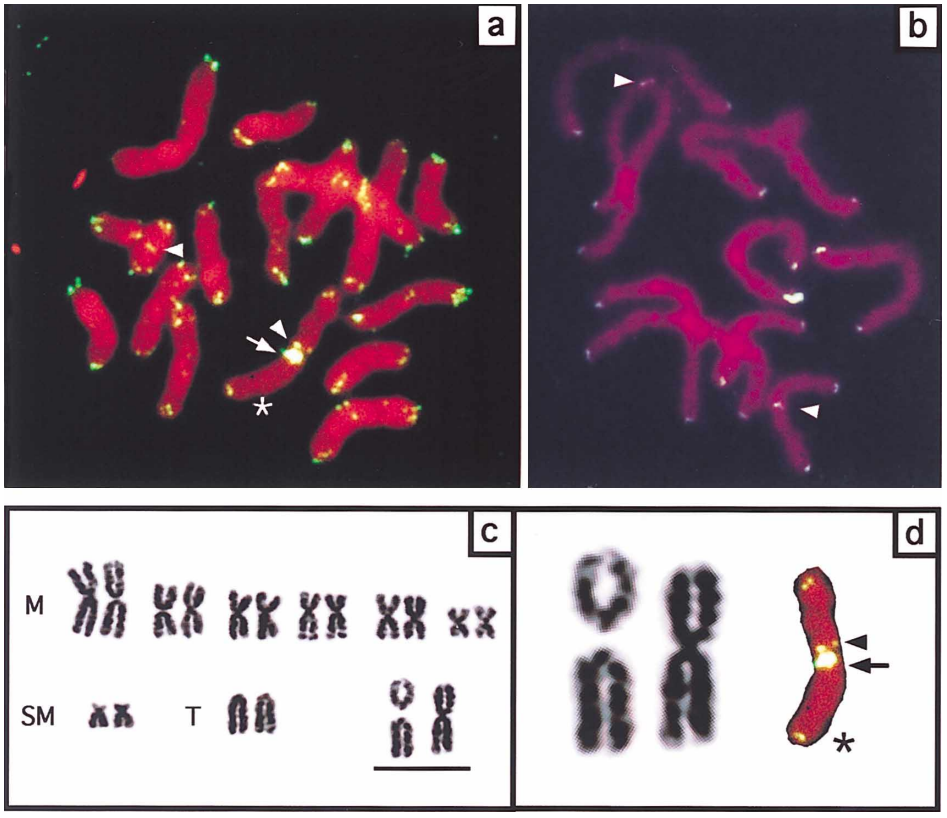

Fig. 4. *In situ* hybridization of chromosomes of *B. habei* with biotin-labeled (TTAGGG)n probe. Hybridization signals were detected by FITC fluorescence (yellow), while the chromosomes were counterstained with propidium iodide (red). Metaphase (**a**) and prophase (**b**) chromosomes obtained from different individuals are shown. Arrowheads and arrow indicate ITSs (see text). Asterisk shows a large chromosome involved in Robertsonian rearrangement. **c**: Karyotype of an individual of *B. habei* shown in Fig. 4a. Chromosomes were stained with Giemsa. Underlined chromosomes are involved in Robertsonian rearrangement. M, SM and T represent metacentric, submetacentric and telocentric chromosomes, respectively. **d**: Proposed mechanism for an ITS. A large metacentric chromosome produced by fusion of two telocentric chromosomes (shown by asterisk in Fig. 4a) has a large ITS at the presumed fusion site (shown by arrow). Near the large ITS, there is also a small ITS (shown by arrowhead).

observed in some chromosomes. In a remarkable case shown in Fig. 4a, a large and intense ITS was observed in a large chromosome. Interestingly, this individual is heterozygous for a pair of chromosomes presumed to be involved in Robertsonian rearrangement (Fig. 4c). The strong ITS, locating at the centromeric position of the large metacentric chromosome, coincides with the presumed fusion point (Fig. 4d).

We also performed FISH analysis on some congeneric species, *B. niponica* and *B. reticulata*, and confirmed that the probe can be hybridized at the chromosomal ends in those species, as well as *B. habei* (data not shown).

DISCUSSION

Molluscan telomere

In the present study, we have obtained three clones containing (TTAGGG)n sequence from a freshwater snail *B. habei*. This hexanucleotide tandem repeat has been known as the universal consensus sequence of eukaryotic telomere. Chromosomal localization revealed by FISH analysis and Bal 31 exonuclease sensitivity of the hybridizing signal also support the sequence to be telomeric DNA of *B. habei*. The present paper is the first report for the isolation of molluscan telomeric DNA.

Although telomeric repeat sequence is completely conserved in vertebrates, invertebrate telomere is highly variable. Telomeric repeat sequences are TTAGG in *Bombyx* (Okazaki *et al*., 1993), TTAGGC in *Caenorhabditis* and *Ascaris* (Muller *et al*., 1991) and TTGCA in *Parascaris* (Teschke *et al*., 1991). In *Drosophila*, DNA at the end of chromosomes is composed of a transposable element, instead of simple tandem repeat sequence (Biessmann *et al*., 1990; Saiga and Edstrom, 1985; Traverse and Pardue, 1989; Young *et al*., 1983). Most of the invertebrates examined to date have different telomeric sequences of their own. *B. habei* is the first case of invertebrate telomere comprised of TTAGGG. The telomere repeat TTAGGG occurs in wide variety of organisms, not only in mollusca and vertebrates, but also in far distantly related lower eukaryotes such as *Tetrahymena*, *Paramecium*, *Dictyostelium*, *Saccharomyces* and so on (review by Zakian, 1995). It is thus evident that *B. habei* retains an ancestral sequence for invertebrate telomere DNA. Other types of unique telomeric sequence occur in some nematodes and insects must have derived from TTAGGG independently in each invertebrate lineage. Our finding provides important data for determining the polarity of evolutionary trend for invertebrate telomere sequence. Examination of more invertebrate taxa would shed light on the detailed process for diversification of telomere sequence in invertebrates.

In the present study, we detected many variant telomeric repeat units to be occurred in the clones, pBT-2 and pBT-3. Such a sequence variation has also been reported in telomeric sequences from human and mouse genome (Wells *et al*., 1990; Ijdo *et al*., 1991; Yen *et al*., 1997). Interestingly, such variant repeats are demonstrated to be located in the internal telomeric sequences, while the telomeric (TTAGGG)n array in the exact chromosomal termini is very homogeneous in sequence (Moyzis *et al*., 1988; Kipling *et al*., 1995). One of the possible mechanism of which sequence variation occurs exclusively in internal telomeric sequence is mutation accumulation. Although telomeric sequences at exact chromosome ends are regenerated by telomerase, internal telomeric sequence is no longer subject to such turnover (Kipling *et al*., 1995). Taken together with the frequent chromosomal rearrangements and presence of many ITSs in *B. habei*, it is possible that the two clones with many variants are derived from such internal telomeric regions.

Interstitial telomeric signals

ITSs were detected in some metaphase and prophase chromosomes of *B. habei* (Fig. 4a, b). Such ITSs have been reported in many vertebrates (Meyne *et al*., 1990). Two possible mechanisms have been proposed to explain the generation of these interstitial telomeric sites: fusion of ancestral chromosomes at telomeric sites or amplification of endogenous, short (TTAGGG)n tandem repeats within the chromosome arms.

In a certain case of *B. habei* shown in Fig. 4d, the exact location of an ITS coincides with the presumed fusion site of two telocentric chromosomes (Robertsonian fusion). The interstitial telomeric site at least in this case, can be regarded as a remnant of past telomeric sites of ancestral chromosomes.

One of the remarkable features of *Biwamelania* is unusually high level of chromosomal variability. In *B. habei*, chromosome number ranged from 2n=17 to 21, and almost all individuals show different karyotype of its own (Burch, 1968; Nomoto, unpublished). It is thus difficult to distinguish between Robertsonian fusion and fission event only by the standard Giemsa staining method. Presence of ITS provides a convincing evidence for chromosomal fusion by detecting ancestral telomeric sites in rearranged chromosomes.

It is notable that the ITS located at the presumed fusion site is extremely large and intense. Such a large signal cannot be explained by a fusion alone, because the observed signal is much larger than those of telomeric sites of unfused chromosomes. The interstitial telomeric sequences must be amplified by unequal crossing-over and/or tandem duplication after the chromosomal fusion. The observation that interstitial telomeric sequence is a hot spot for recombination (Ashley and Ward, 1993) may favor for such phenomena. In addition to the large ITS, we also detected a small ITS near the presumed fusion site (Fig. 4d; indicated by arrowhead). Such an additional signal suggests multiple fusion involving a minute chromosome or occurrence of a small inversion event near the interstitial telomeric junction after the chromosomal fusion. In any case, ITS uncovers detailed mechanism of chromosomal rearrangement, which is undetectable in the standard Giemsa staining and would provide invaluable information for understanding the complex pattern of chromosomal evolution in *Biwamelania*.

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