Chromosomal localization of the 18S-28S and 5S rRNA genes and (TTAGGG)n sequences of butterfly lizards (Leiolepis belliana belliana and Leiolepis boehmei, Agamidae, Squamata)

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Abstract
Chromosomal mapping of the butterfly lizards Leiolepis belliana belliana and L. boehmei was done using the 18S-28S and 5S rRNA genes and telomeric (TTAGGG)n sequences. The karyotype of L. b. belliana was 2n = 36, whereas that of L. boehmei was 2n = 34. The 18S-28S rRNA genes were located at the secondary constriction of the long arm of chromosome 1, while the 5S rRNA genes were found in the pericentromeric region of chromosome 6 in both species. Hybridization signals for the (TTAGGG)n sequence were observed at the telomeric ends of all chromosomes, as well as interstitially at the same position as the 18S-28S rRNA genes in L. boehmei. This finding suggests that in L. boehmei telomere-to-telomere fusion probably occurred between chromosome 1 and a microchromosome where the 18S-28S rRNA genes were located or, alternatively, at the secondary constriction of chromosome 1. The absence of telomeric sequence signals in chromosome 1 of L. b. belliana suggested that its chromosomes may have only a few copies of the (TTAGGG)n sequence or that there may have been a gradual loss of the repeat sequences during chromosomal evolution.

Key words: chromosomal mapping, FISH, interstitial telomeric site, Leiolepidinae, ribosomal RNA gene, telomeric sequence.
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Squamate reptiles, the most diverse reptilian order, have traditionally been classified into three suborders: Amphisbaenia (worm lizards), Serpentes (snakes) and Lacertilia (lizards). Lizards can be further classified into six infraorders (Iguania, Gekkota, Scincomorpha, Diploglossa, Dibamia and Platynota) (Uetz, 2011). The butterfly lizards (Leiolepis, Agamidae, Iguania) are burrowers inhabiting Southeast Asia. These lizards show a wide variety of karyotypes and sexual systems. In Thailand, there are three species of Leiolepis (L. belliana, L. reevesii rubritaeniata and L. boehmei) that are barely distinguished from other congeneric species by their typical scale and skin coloration (Peters, 1971). Leiolepis belliana occurs as two subspecies, Leiolepis belliana belliana and L. belliana ocellata. Leiolepis b. belliana occurs throughout the Thailand, while L. b. ocellata is found in the upper northern region and L. reevesii rubritaeniata occurs only in the northeast. All of these species are bisexual. Putative unisexualy has been reported for the diploid L. boehmei, for which only females have been detected in the provinces of Songkhla and Nakhon Si Thammarat in southern Thailand (Darevsky and Kupriyanova, 1993; V. Aranyavlai, 2003, PhD thesis, Chur-
Ribosomal RNA genes are tandemly arrayed repeats that are believed to have evolved in a concerted manner. Since all copies of ribosomal RNA genes are homogenous within individuals and species, they represent an important source of information for karyological characterization and evolutionary relationships (Srikulnath, 2010). Telomeric (TTAGGG)n sequences are commonly found at telomeres (Meyne et al., 1990) but are also observed at non-telomeric sites known as interstitial sites (ITSs) (Ventura et al., 2006; Srikulnath et al., 2009a). ITSs may form large blocks of telomeric sequences known as heterochromatic ITSs (het-ITSs) that are located mainly in centromeric or pericentromeric regions (Ruiz-Herrera et al., 2008). ITSs may be the remnants of chromosomal rearrangements that provided the information involved in karyotypic evolution (Meyne et al., 1990). The 18S-28S rRNA genes are generally located on a pair of microchromosomes or chromosome 2q in Iguania (Porter et al., 1991).

In our previous study, we mapped the 18S-28S and 5S rRNA genes and telomeric (TTAGGG)n sequences of L. reevesii rubritaeniata chromosomes (Srikulnath et al., 2009a). The 18S-28S gene was located at the secondary constriction of the long arm of chromosome 1, and the 5S rRNA gene at the pericentromeric region of chromosome 6. Hybridization signals for (TTAGGG)n sequences were observed at the telomeric ends of all chromosomes and interstitially at the same position as the 18S-28S rRNA genes, suggesting that in L. reevesii rubritaeniata telomere-to-telomere fusion probably occurred between chromosome 1 and a microchromosome where the 18S-28S rRNA genes were located (Srikulnath et al., 2009a).

In the Leiolepidinae, a cytogenetic map has been reported for only one species (L. reevesii rubritaeniata) (Srikulnath et al., 2009a,b). In the present study, we used fluorescent *in situ* hybridization (FISH) to map the 18S-28S and 5S rRNA genes and telomeric (TTAGGG)n sequences in L. b. belliana and L. boehmei as representative species of *Leiolepis* in Thailand.

Live specimens of L. b. belliana and L. boehmei were collected from Chonburi (13°4'0" N and 101°0'0" E) and Songkla (7°12'36" N and 100°33'36" E) provinces, respectively. Their sexes were determined morphologically and confirmed by internal genital anatomy. All experimental procedures with the lizards conformed to the guidelines established by the Animal Care Committee at Hokkaido University (Japan). Although L. b. ocellata used to be found in Thailand it was not available for this study. Mitotic chromosomal preparations of L. boehmei were obtained from fibroblast cultures of heart, lung and mesentery, as described by Srikulnath et al. (2009a), whereas mitotic and meiotic chromosomes of L. b. belliana were prepared from testes, according to Imai et al. (1981).

Chromosomal locations of the 18S-28S rRNA genes, 5S rRNA genes and telomeric (TTAGGG)n sequences were determined by FISH, as previously described (Matsuda and Chapman, 1995; Srikulnath et al., 2009a). Dual-color FISH was done to compare the chromosomal locations of telomeric (TTAGGG)n sequences with those of the 18S-28S rRNA genes, whereas single FISH was used to investigate the chromosomal location of the 5S rRNA genes.

The chromosomal numbers of L. b. belliana and L. boehmei were 2n = 36 (12 macrochromosomes and 24 microchromosomes) and 2n = 34 (12 macrochromosomes and 22 microchromosomes), respectively (data not shown). These results were identical to those previously reported (V. Aranyavali, 2003, PhD thesis, Chulalongkorn University, Bangkok).

Chromosomal mapping of the 18S-28S and 5S rRNA genes is important for karyological characterization of a species and for establishing phylogenetic relationships since these genes occur in multiple copies that facilitate detection (Pendás et al., 1994; Srikulnath, 2010). In Iguania, the 18S-28S rRNA genes are generally located on a pair of microchromosomes or chromosome 2q (Porter et al., 1991), whereas the nucleolar organizer region is located on chromosome 6 of *Tropidurus* species, the karyotypes of which are similar to *Leiolepis* (Kasahara et al., 1987). In contrast, FISH signals for the 18S-28S rRNA genes in L. b. belliana and L. boehmei were restricted to the secondary constriction in the subtelomeric region of the long arm of chromosome 1 (Figure 1A,C,D,F,G,I). These features were comparable to those of L. reevesii rubritaeniata (Srikulnath et al., 2009a).

In previous work, we showed that the 5S rRNA genes in L. reevesii rubritaeniata were located in the pericentromeric region of the long arm of chromosome 6 (Srikulnath et al., 2009a). The same localization was also observed in L. b. belliana and L. boehmei (Figure 2). These findings indicate that the position of major and minor ribosomal RNA genes may be the same among species of *Leiolepis*. Cytogenetic studies of more leiolepideine species, especially *Uromastyx* sp. which is classified in the same subfamily, are required to confirm this suggestion. Such studies would shed light on the chromosomal locations of the 18S-28S and 5S rRNA genes in the conserved karyotypes of Leiolepideinae and Iguania.

The distribution of telomeric sequences provides preliminary information on the processes involved in karyotypic evolution (Meyne et al., 1990; Srikulnath, 2010). In the present study, the (TTAGG)Gn sequences were localized to the telomeric ends of all chromosomes of L. b. belliana and L. boehmei (Figure 1B,C,E,F,H,J). The hybridization signals were weak on macrochromosomes, whereas high intensity signals were observed on almost all...
microchromosomes, which suggests site-specific amplification of the (TTAGGG)$_n$ sequences on these chromosomes. These findings were similar to those of *L. reevesii rubritaeniata* in our previous study (Srikulnath et al., 2009a). However, microchromosome-specific amplification of telomeric repeats has not been reported in other squamate reptiles (Pellegrino et al., 1999). Comparable hybridization patterns have also been observed in birds, including several species of Galliformes, Anseriformes and Passeriformes (Nanda et al., 2002).

Figure 1 - Chromosomal localization of the 18S-28S rRNA genes and (TTAGGG)$_n$ sequences in *L. b. belliana* mitotic metaphase (A-C) and meiotic (D-F) chromosome, and *L. boehmei* mitotic metaphase chromosome (G-I). Hybridization patterns of the 18S-28S rRNA genes (red) (A,D,G) and (TTAGGG)$_n$ sequences (green) (B,E,H) in DAPI-stained chromosomes and their co-hybridization pattern (C,F,I). Arrows indicate FISH signals of the 18S-28S rRNA genes (A,C,D,F,G,I), and interstitial telomeric sites (ITSs) (H,I). Scale bars = 10 µm.

Interstitial telomeric sites (ITSs) appear to be relics of chromosomal rearrangements such as fusions or inversions (Go et al., 2000). Hybridization signals for interstitial telomeric sites were found at the secondary constriction in the subtelomeric region of the long arm of chromosome 1 in *L. reevesii rubritaeniata*, where the (TTAGGG)$_n$ sequences co-localized with the 18S-28S rRNA genes (Srikulnath et al., 2009a). This same arrangement was also found in *L. boehmei*. In contrast, 18S-28S rRNA genes are generally located on a pair of microchromosomes or chromosome 2q.
in Iguania (Porter et al., 1991). These results suggest the possible occurrence of telomere-to-telomere fusion between a microchromosome with the 18S-28S rRNA genes and the distal end of chromosome 1 in the lineage of L. reevesii rubritaeniata and L. boehmei.

Comparison of the chromosomal maps for the butterfly lizard (L. reevesii rubritaeniata) and Japanese four-striped rat snake (Elaphe quadrivirgata) also indicated that co-localization of the 18S-28S rRNA genes and ITSs in the subtelomeric region of LRE1q may be the result of a small paracentric inversion (Srikulnath et al., 2009b). This inversion may have occurred between the proximal region of the DYNCHI (dynein, cytoplasmic 1, heavy chain 1) gene and the distal region on LRE1q after the telomere-to-telomere fusion of the ancestral LRE1q and a microchromosome with the 18S-28S rRNA genes, which generally persist in Iguania (Srikulnath et al., 2009b).

An alternative explanation could be that since telomeres cap the end of chromosomes to protect them from deteriorating and fusing with neighboring chromosomes then chromosomal reorganization would lead to telomere exclusion and an absence of ITS at the fusion site. In contrast, the blockade of ITS may produce a fragile site leading to chromosomal breakage (Bolzán and Bianchi, 2006). Hence, ITTs may represent possible fission points at which new telomeres can be formed by pre-existing telomeric repeats (Ruiz-Herrera et al., 2008). This conclusion suggests that telomere formation may have occurred at the secondary constriction of chromosome 1 in the lineage of L. reevesii rubritaeniata and L. boehmei. Comparison of the karyotypes of L. reevesii rubritaeniata and L. boehmei indicated that the macrochromosomal features were identical and conserved throughout the suborder Iguania (Olmo and Signorino, 2005).

Thus, the evidence of comparative mapping and the location of 18S-28S rRNA genes and ITS might also verify the process of their transposition to different chromosomes in Leiolepis and Iguania. However, no ITS was found in chromosome 1 of L. b. belliana. In equids, ITS signals have been observed on chromosome 1 of Equus quagga burchelli, but are absent from the chromosomes of other equids. The absence of ITSs on chromosome 1 of L. b. belliana could be the result of a low number of copies of (TTAGGG)n, making it impossible to detect the sequence, or may represent a gradual loss of the repeat sequences during chromosomal evolution (Santini et al., 2002). Further molecular cytogenetic characterizations of other Leiolepis and Uromastyx species are required to clarify the possible location of (TTAGGG)n sequences in Leiolepidinae and Iguania.

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