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RESEARCH ARTICLE

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A new family of satellite DNA sequences as a major component of centromeric heterochromatin in owls (Strigiformes)

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Abstract We isolated a new family of satellite DNA sequences from *Hae* III- and *Eco* RI-digested genomic DNA of the Blakiston's fish owl (*Ketupa blakistoni*). The repetitive sequences were organized in tandem arrays of the 174 bp element, and localized to the centromeric regions of all macrochromosomes, including the Z and W chromosomes, and microchromosomes. This hybridization pattern was consistent with the distribution of C-band-positive centromeric heterochromatin, and the satellite DNA sequences occupied 10% of the total genome as a major component of centromeric heterochromatin. The sequences were homogenized between macro- and microchromosomes in this species, and therefore intraspecific divergence of the nucleotide sequences was low. The 174 bp element cross-hybridized to the genomic DNA of six other Strigidae species, but not to that of the Tytonidae, suggesting that the satellite DNA sequences are conserved in the same family but fairly divergent between the different families in the Strigiformes. Secondly, the centromeric satellite DNAs were cloned from eight Strigidae species, and the nucleotide sequences of 41 monomer fragments were compared within and between species. Molecular phylogenetic relationships of the nucleotide sequences were highly correlated with both the taxonomy based on morphological traits and the phylogenetic tree constructed by DNA-DNA hybridization. These results suggest that the satellite DNA sequence has evolved by concerted evolution in the Strigidae and that it is a good taxonomic and phylogenetic marker to examine genetic diversity between Strigiformes species.

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Introduction

According to de Boer (1984), the Strigiformes comprises 12 species of two genera in the Tytonidae and 134 species of 27 genera in the Strigidae; the karyotypes have been described in two Tytonidae and 23 Strigidae species. The karyotypes of several owl species have been additionally reported by Schmutz and Moker (1991) and Rebholz et al. (1993). The diploid chromosome numbers of the owls range from 66 to 86, and in the Strigidae the karyotypes are generally composed of 6–10 pairs of macrochromosomes, including Z and W chromosomes, and a large number of microchromosomes. The karyotypes of Strigidae species are also characterized by large heterochromatin blocks in the centromeric regions of chromosomes. The C-banded karyotypes have been reported in 12 species of seven genera in the Strigidae, in which large C-positive bands are distributed on all macrochromosomes and microchromosomes except for several species with C-negative bands on Z chromosomes (Rebholz et al.1993; Sasaki et al.1994). In contrast, the karyotypes of Tytonidae species are quite different from those of Strigidae species. The size difference of chromosomes is sequential in two species of the Tytonidae, *Phodilus badius* and *Tyto alba*, and their macrochromosomes and microchromosomes are not distinguished clearly. In addition, the distribution of C-banded heterochromatin is not uniform in the Tytonidae, suggesting that frequent chromosome rearrangements occurred between the Strigidae and Tytonidae.

Only a few centromeric repetitive sequences have been described in avian orders and families, and the distributions of the centromeric repeats can be classified into three patterns: (1) on several pairs of macrochromosomes;

(2) on all or most chromosomes; and (3) only on microchromosomes. Repetitive sequences distributed on several pairs of macrochromosomes were isolated from the Falconiformes, Gruiformes and Columbiformes (Longmire et al.1988; Chen et al.1989; Solovei et al.1996). Centromeric repetitive sequences located on all or most chromosomes were cloned in the Psittaciformes and Passeriformes (Madsen et al.1992; Saifitdinova et al.2001), and microchromosome-specific repetitive sequences were isolated from the Galliformes and Struthioniformes (Matzke et al.1990,1992; Tanaka et al.2000; Yamada et al. 2002a, 2002b). On the other hand, no centromeric repetitive sequences have been reported in the Strigiformes.

To expand the understanding of evolution of centromeric repetitive sequences, we isolated a new family of satellite DNA sequences from the Blakiston's fish owl (*Ketupa blakistoni*), and analyzed their nucleotide sequences, chromosomal distribution, organization in the genome and sequence conservation in the Strigiformes. In addition, we isolated satellite DNA sequences from seven other Strigidae species, and examined the intra- and interspecific divergence of the nucleotide sequences. We discuss the molecular evolution of the satellite DNA and its use in taxonomic and phylogenetic analyses in the Strigiformes.

Materials and methods

Specimens, cell culture and chromosome preparation

Nine Strigidae species, *Bubo bubo* (eagle owl), *Bubo virginianus* (great horned owl), *K. blakistoni* (Blakiston's fish owl), *Ninox scutulata* (brown hawk owl), *Nyctea scandiaca* (snowy owl), *Otus bakkamoena* (collared scops owl), *Otus scops* (scops owl), *Pulsatrix perspicillata* (spectacled owl), *Strix uralensis hondoensis* (Ural owl), and one Tytonidae species, *T. alba* (barn owl), were used for the present study. The skin tissues were collected by biopsy and used for fibroblast cell culture. The fibroblast cells were cultured in 199 medium supplemented with 15% fetal bovine serum at 39°C in 5% CO_2, and used for DNA extraction and chromosome preparation.

C-banding

The C-banding patterns were examined in the above-mentioned ten Strigiformes species. Chromosome C-banding was performed with the BSG (Barium hydroxide/Saline/Giemsa) method (Sumner 1972).

Molecular cloning of repetitive sequences

Genomic DNA of *K. blakistoni* was extracted from whole blood cells by the standard techniques, and in other species the cultured fibroblast cells were used for DNA extraction. Genomic DNA of *K. blakistoni* was digested with 18 restriction endnucleases, *Apa* I, *Bam* HI, *Bgl* I, *Bgl* II, *Eco* RI, *Eco* RV, *Hae* III, *Hin* dIII, *Hin* fI, *Nsi* I, *Pvu* II, *Rsa* I, *Sac* I, *Sau* 3AI, *Sma* I, *Taq* I, *Xba* I and *Xho* I, size fractionated by electrophoresis in a 3% agarose gel, and stained with ethidium bromide. The prominent DNA bands of repetitive sequences were isolated from the gel. The DNA fragments were eluted using Suprec-01 (Takara), and cloned into pBluescript II SK (-) (Stratagene). The cloned fragments were sequenced using a Thermo Sequenase pre-mixed cycle sequencing kit (Amersham) with Texas Red-labeled T3 and T7 primers, and an SQ-5500 DNA sequencer (Hitachi).

Fluorescence in situ hybridization (FISH)

Chromosome preparation and FISH were performed as described previously (Matsuda and Chapman1995; Suzuki et al. 1999b). The DNA fragments were labeled with biotinylated 16-dUTP using a nick translation kit (Roche Diagnostics) and ethanol-precipitated with sonicated salmon sperm DNA and *Escherichia coli* tRNA. After hybridization, the slides were incubated with fluorescein isothiocyanate-avidin (Roche Diagnostics) and stained with propidium iodide. The FISH images were captured with the 550CW-QFISH application program of Leica Microsystems Imaging Solutions (Cambridge, UK) using a cooled CCD camera (Micro-MAX 782Y, Princeton Instruments) mounted on a Leica DMRA microscope.

Southern blotting and slot-blot hybridization

For Southern blot hybridization genomic DNA extracted from *K. blakistoni* was digested with four restriction endonucleases: *Eco* RI, *Hae* III, *Pvu* II and *Rsa* I. Digested DNAs were fractionated by electrophoresis in a 3% agarose gel, and DNA fragments were transferred onto Hybond N+ nylon membrane (Amersham). For slot-blot hybridization genomic DNAs from eight Strigiformes species, *K. blakistoni* , *B. bubo* , *Ny. scandiaca* , *S. u. hondoensis, P. perspicillata* , *Ni. scutulata* , *O. bakkamoena* and *T. alba*, were blotted on the nylon membrane using Bio-Dot SF blotting equipment (Bio-Rad). The repeated element cloned from *K. blakistoni* was labeled with [α-^{32}P]dCTP using Multiprime DNA Labeling System (Amersham), and hybridized to the membranes. Hybridization was carried out overnight at 42°C in 50% formamide, 5×SSPE, 5×Denhardt's, 0.5% SDS and 20 μg/ml denatured salmon sperm DNA. The membrane was washed at 42°C in 2×SSC and 1×SSC, and exposed to BioMax MS Autoradiography Film (Kodak).

Estimation of copy numbers of the repeated elements

The copy numbers of the repeated elements were estimated by slot-blot hybridization. RNase-treated whole genomic DNAs and polymerase chain reaction (PCR) products of the repeated elements were denatured with NaOH, and transferred onto Hybond N^+ nylon membrane using Bio-Dot SF blotting equipment. DNA probes of the repeated elements were labeled with digoxigenin (Dig) using PCR DIG Labeling Mix (Roche Diagnostics), and hybridized to the membranes at 42°C in DIG Easy Hyb solution (Roche Diagnostics). The luminescent signals were detected with Anti-Digoxigenin-AP Fab fragments and CDP-Star (Roche Diagnostics). The intensity of luminescent signals was measured by Scanning Imager and analyzed using Image Quant software (Molecular Dynamics).

Results

Karyotype of *K. blakistoni*

Karyotype analysis of *K. blakistoni* with Giemsa-stained metaphase spreads revealed that the chromosome number was 2 n =82. There were three pairs of large acrocentric chromosomes, the 4th largest was the metacentric Z chromosome, two pairs of medium-sized chromosomes — submetacentric (5th) and acrocentric (6th), 4 pairs of

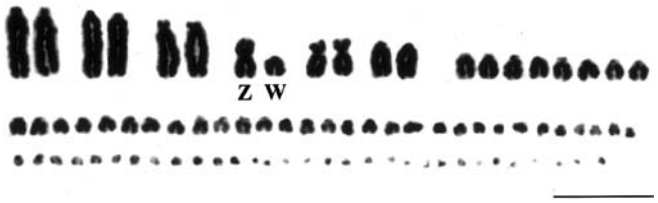

Fig. 1 Giemsa-stained karyotype of *Ketupa blakistoni*. *Bar* represents 10 μm

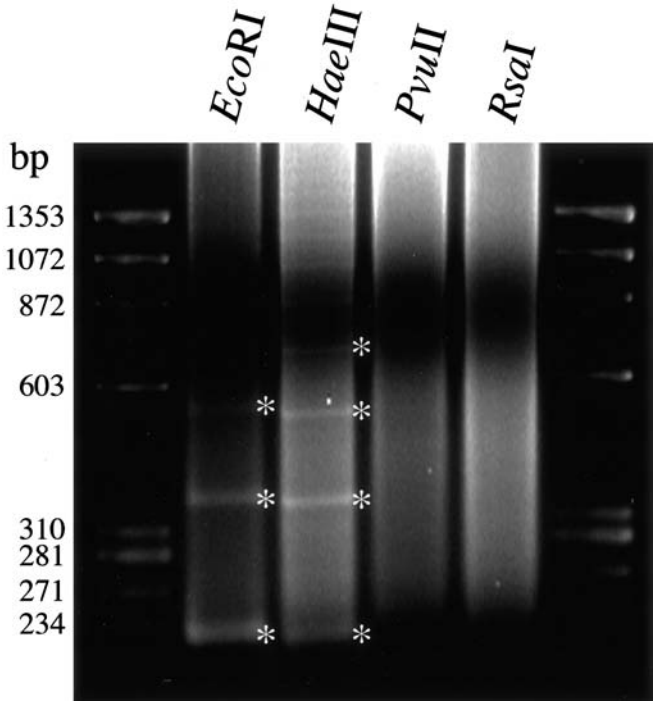

Fig. 2 Ethidium bromide-stained gel of *K. blakistoni* genomic DNA digested with the restriction endonucleases *Eco* RI, *Hae* III, *Pvu* II and *Rsa* I. *Asterisks* indicate the prominent DNA bands resulting from *Eco* RI and *Hae* III digestion that were used for molecular cloning of the repetitive sequences

small acrocentric chromosomes (the 7–10th) and 62 microchromosomes of mostly acrocentric nature (Fig. 1). The W was a small acrocentric chromosome.

Molecular cloning and nucleotide sequences of the repeated elements

Genomic DNA of *K. blakistoni* digested with *Eco* RI and *Hae* III revealed prominent DNA bands of repetitive sequences arranged in a ladder pattern by agarose gel electrophoresis (Fig. 2). DNA fragments were isolated from the DNA bands, and cloned into the plasmid. Sixteen, 13 and 15 clones were isolated from the approximately 180, 360 and 540 bp bands resulting from *Eco* RI digestion, and 23, 12, 7 and 13 clones from the 180, 360, 540 and 720 bp bands from *Hae* III digestion, respectively. A new family of repetitive sequences was contained in the

a

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                          10        20        30        40        50        60
1U37   (AB103253) CCAAGAGTTC ACATTGTTT- CGCAGAATCG CGTTTCTGCA GAGGACACAA ACGCTTGTTT
1U42   (AB103254) .....T.... .....T...- .........A .......... .......... ..........
1U48   (AB103255) .....T.... .....C...- .......... ......C... .......... .........C
1U51   (AB103256) .....T.... .........G G......... ......C.A. .......... ..........
1U54   (AB103257) .....T.... .....T...- .......... ....A..... .......... ....C..A.C
4U4-1  (AB103258) AG....T... .....CG..- .......... ......C... ...C...... .........C
4U4-2             A....T.... ......C..- .......... ......C... ...C...... .........C
4U4-3             AG...T.... ......C..- .......... ......C... ...C...... .........C
4U11-1 (AB103259) A....T.... ......C..- .......... ......C... ...C......T.G..T..C
4U11-2            A....T.... ......C..- .......... ......C... ...C.....T.....C
4U11-3            A....T.... ......C..- .......... ......C... ...C.....T.....C
4U33-1 (AB103260) AG...T.... ......C..- .......... ......C... ...C...... .........C
4U33-2            A....T.... ......G..- .........C .....-C... ...C...... .........C
4U33-3            A....T.... ......G..- .......... .......... ...C...... .........C
4U37-1 (AB103261) .....T.... .....T...- .........A .......... .......... ..........
4U37-2            A....T.... .........G G......... ..A...C... .......... ..........
4U37-3            A....T.... .....T...G G......... ......C..G .....T.... ..........
4U37-4            A....T.... .........G G...T...A. A.A...C... .......... ...T......
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                          70        80        90        100       110       120       130
1U37   AGGACAAAAG AAAACACTGA GCCCCACATT CACTGTTGCC CTGGAGAGCT TGCAGAGCAC TGGGGAAAGG
1U42   .......... .......... .......... .......... .....C.... .......... ..........
1U48   .......... ...G.C.A.. ....A..... .......... .......... .....A.... ..........
1U51   .T........ .......... .......... .......... .......... .......... ..........
1U54   ...G....T. ...G.C.A.. .......... .......... .........C .......... ..........
4U4-1  ...G.G.... ...G...A.. .......... ..G....... ...C.C.... .......... ..CA......
4U4-2  ...G.G.... ...G...A.. .......... ..G....... ...C.C.... .......... ..CA......
4U4-3  ...G.G.... ...G...A.. .......... ..G....... ...C.C.... .......... ..CA......
4U11-1 .CTGT.G..T .......A.. .......... .....C.... .......... .......... ..........
4U11-2 .......... .......A.. .......... .......... .......... .......... ..........
4U11-3 .......... .......A.. .......... .......... .......... .......... ..........
4U33-1 ...G.G.... ...G...A.. .......... ..G....... ...C.C.... .......... ..CA......
4U33-2 ...G.G.... ...G...A.. .......... ..C....... ...C.C.... .......... ..CA......
4U33-3 ...G.G.... ...G...A.. .......... ..G....... ...C.C.... .......... ..CA......
4U37-1 .......... .......... .......... .......... .......... .......... ..........
4U37-2 .T........ .......... .......... .......... .......... .......... ..........
4U37-3 .......... .......... .......... .......... .......... .......... ..........
4U37-4 .T........ .......... .......... .......... .......... .......... ..........
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                          140       150       160       170
1U37   CAGGCAGAGA ATTCCCTGCT AGCACCTTCT CTGTGCCTTG GAAGG
1U42   .........T .......... ....A..... T.......G. .....
1U48   .......... T......... .......... ......A.G. .....
1U51   .......... .......... .......... .......G. .....
1U54   .......... .......... .....C.... ......A.G. .....
4U4-1  .......... T......... .......... .......G. ....C
4U4-2  .......... T......... .......... .......G. ....C
4U4-3  .......... T......... .......... .......G. ....C
4U11-1 ..A....... .......... ....G..... .......G. .....
4U11-2 ..A....... .......... ....G..... .......G. ....C.
4U11-3 ..A....... .......... ....G..... .......G. .....
4U33-1 .......... T......... .......... .......G. ....C
4U33-2 .......... T......... .......... .......G. ....C
4U33-3 .......... T......... .......... .......G. ....C
4U37-1 .......... .......... .......... .......G. .....
4U37-2 .......... .......... .......... .......G. .....
4U37-3 .......... .......... .......... .......G. .....
4U37-4 .......... .......... .......... .......G. .....
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Fig. 3a, b Nucleotide sequences of the repeated elements isolated from *Hae* III- and *Eco* RI-digested genomic DNA of *K. blakistoni*. The nucleotide sequences of five monomeric fragments, three tetrameric fragments including three internal monomer units and one tetrameric fragment including four monomer units cloned from the *Hae* III-digested DNA, and 13 monomeric fragments and 9 trimeric fragment clones from the *Eco* RI-digested DNA are demonstrated in **a** and **b**, respectively. *Hyphens* indicate gaps in the nucleotide sequences. *Underlines* in **a** and **b** indicate the internal restriction sites of *Eco* RI and *Hae* III, respectively

13 monomeric (Accession numbers AB103240–AB103252), 12 dimeric (AB103228–AB103239) and 9 trimeric (AB103219–AB103227) fragments from *Eco* RI digestion, and 5 monomeric (AB103253–AB103257), 4

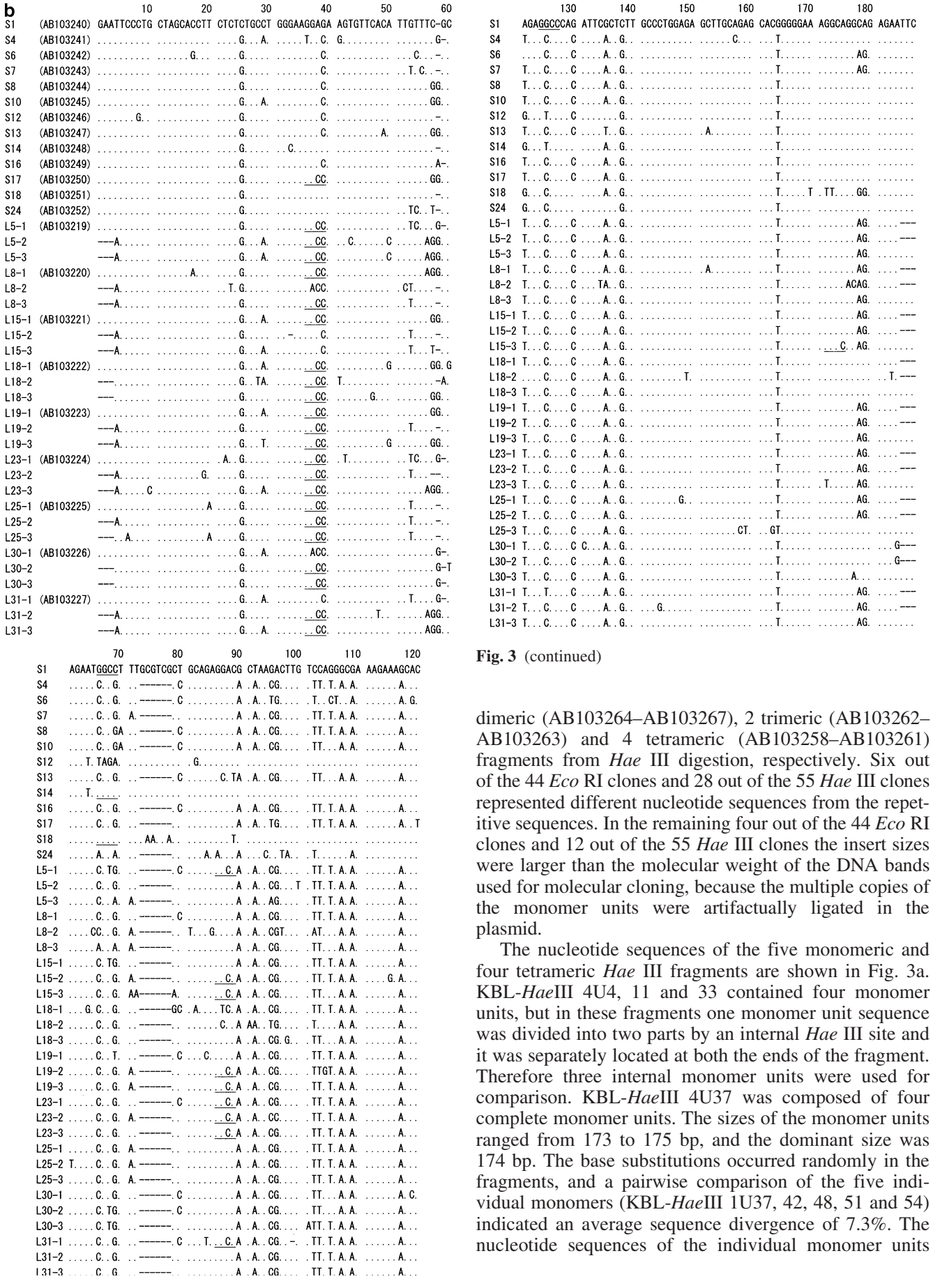


Fig. 3 (continued)

dimeric (AB103264–AB103267), 2 trimeric (AB103262–AB103263) and 4 tetrameric (AB103258–AB103261) fragments from *Hae* III digestion, respectively. Six out of the 44 *Eco* RI clones and 28 out of the 55 *Hae* III clones represented different nucleotide sequences from the repetitive sequences. In the remaining four out of the 44 *Eco* RI clones and 12 out of the 55 *Hae* III clones the insert sizes were larger than the molecular weight of the DNA bands used for molecular cloning, because the multiple copies of the monomer units were artifactually ligated in the plasmid.

The nucleotide sequences of the five monomeric and four tetrameric *Hae* III fragments are shown in Fig. 3a. KBL-*Hae*III 4U4, 11 and 33 contained four monomer units, but in these fragments one monomer unit sequence was divided into two parts by an internal *Hae* III site and it was separately located at both the ends of the fragment. Therefore three internal monomer units were used for comparison. KBL-*Hae*III 4U37 was composed of four complete monomer units. The sizes of the monomer units ranged from 173 to 175 bp, and the dominant size was 174 bp. The base substitutions occurred randomly in the fragments, and a pairwise comparison of the five individual monomers (KBL-*Hae*III 1U37, 42, 48, 51 and 54) indicated an average sequence divergence of 7.3%. The nucleotide sequences of the individual monomer units

differed by averages of 1.1%, 3.8% and 2.1% in KBL-*Hae*III 4U4, 11 and 33 fragments, respectively, and 4.3% in KBL-*Hae*III 4U37. One base addition was observed at position 20 in KBL-*Hae*III 1U51 and in three monomer units of KBL-*Hae*III 4U37. The G+C content of the 18 monomer units was 52.4% on average, ranging from 49.1 to 54.6% (not significantly AT-rich). *Eco* RI sites were contained in more than half of the units, and therefore repeated elements of the same family as the *Hae* III family were also isolated from *Eco* RI-digested genomic DNA (Fig. 3b).

There were no internal repeats in the repeated elements, and a search of the DDBJ sequence database failed to find any significant homology to known nucleotide sequences.

Figure 3b shows the nucleotide sequences of the 13 monomeric fragments and nine trimeric fragments isolated from *Eco* RI-digested genomic DNA. The dominant size of the monomeric *Eco* RI fragment was 180 bp with a 6 base *Eco* RI restriction site. There was an addition of six bases at position 73–78 in *Eco* RI-S1, S12, S14 and S18 fragments. The average sequence divergence between the 13 monomeric fragments was 11.0% by pairwise comparison, ranging from 1.1 to 17.6%. The sequences were diverged 8.1% on average between the monomer units within the trimeric fragments, and the divergence ranged from 3.4 to 13.5%. The average G+C content of the repeated elements was 51.0% on average.

Chromosomal distribution

One of each size of the *Eco* RI and *Hae* III fragments was subjected to FISH analysis. All three *Eco* RI fragments, KBL-*Eco*RI S1, M17 (AB103228) and L5, and four *Hae* III fragments, KBL-*Hae*III 1U37, M2 (AB103264), L6 (AB103262) and 4U4, demonstrated the same hybridization patterns with signals located in the large centromeric heterochromatin regions of all macrochromosomes, including the Z and W chromosomes, and microchromosomes (Fig. 4a). The chromosomal distribution was completely consistent with the centromeric C-positive bands, indicating that the repetitive sequence is a major component of centromeric heterochromatin in *K. blakistoni* chromosomes (Fig. 4b). Consistency of the hybridization patterns with the centromeric C-banded heterochromatin was also seen in two other species, *P. perspicillata* and *Ny. scandiaca* (Fig. 4c–f). No fluorescence signals were obtained for the six *Eco* RI and 28 *Hae* III fragments that contained sequences different from the repeated sequences. The karyotypes of *P. perspicillata* and *B. bubo* were different from those of other Strigiformes species. They had pairs of the largest metacentric- and submetacentric no. 1 chromosomes in *P. perspicillata* (Fig. 4d) and *B. bubo* (data not shown), respectively, in contrast to the largest acrocentric chromosomes in other species (Belterman and de Boer1984; Sasaki et al.1984; Rebholz et al.1993; present study).

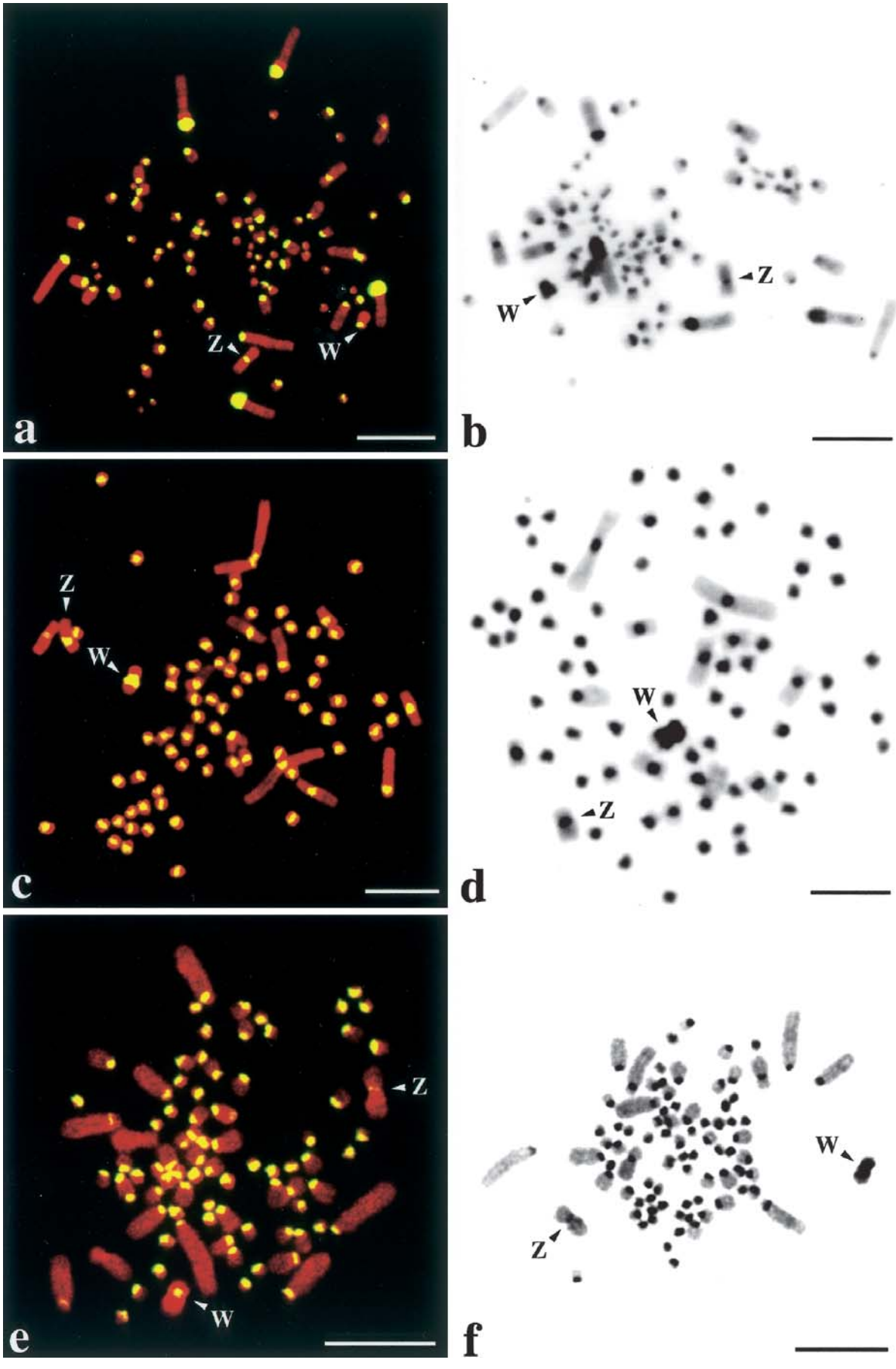
Organization in the genome

To study the organization of the repetitive sequences in the genome, total genomic DNA of *K. blakistoni* digested separately with four restriction endonucleases was subject to Southern blot hybridization with the 174 bp element of *K. blakistoni* (KBL-*Hae*III 1U48) as a probe. The hybridization resulted in polymeric ladder signals of tandem repeats with 174 bp monomer length (Fig. 5), indicating that the repetitive sequence can be categorized as satellite DNA. In *Eco* RI and *Hae* III digests the monomer unit was present in the greatest abundance, with progressively decreasing copy numbers of each higher order. In contrast, *Pvu* II and *Rsa* I digests produced higher intensity of hybridization bands with increasing size of the multimers. These results indicate that the *Pvu* II and *Rsa* I cleavage sites are rare in the tandem arrays of the 174 bp monomer units.

The relative amount of the satellite DNA sequences in the genome was estimated by slot-blot hybridization in *K. blakistoni, P. perspicillata* and *Ny. scandiaca*. The intensity of the signals was compared between genomic DNAs and PCR products (Fig. 6). The satellite DNA sequences represented 10.0%, 14.5% and 9.6% of the total genomes of *K. blakistoni, P. perspicillata* and *Ny. scandiaca,* respectively. The abundance of copy numbers in *P. perspicillata* corresponded to the large sizes of C-band-positive heterochromatin (Fig. 4c, d).

Sequence divergence between the repeated elements

The sequence homologies of the repeated elements were examined for the monomeric and polymeric fragments in the *K. blakistoni* genome. The monomeric fragments cloned randomly were probably derived from different chromosomes in *K. blakistoni* whose karyotype is composed of 41 pairs of chromosomes. Thus, comparison between the monomeric fragments and between the multiple units within the polymeric fragment is very likely indicative of the between-chromosomes and within-chromosome comparisons, respectively. The molecular phylogenetic comparison of the nucleotide sequences was made with the *Hae* III fragments shown in Fig. 3a. The molecular phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei1987) using the computer software MEGA2 (http://www.megasoftware.net/). The monomer units of the same polymeric fragments were clustered in the same group except that one unit of KBL-*Hae*III 4U33 (4U33-1) was contained in the cluster of KBL-*Hae*III 4U4 fragments (Fig. 7). These results suggest that the homology of the nucleotide sequences was higher within the same chromosomes than between the different chromosomes. The base substitution was 7.3% and 11.0% on average between the five monomeric *Hae* III fragments and between the 13 monomeric *Eco* RI fragments, in comparison with 1.1–4.3% (2.8% on the average) and 3.4–13.5% (8.1% on the average) sequence divergence between the monomer units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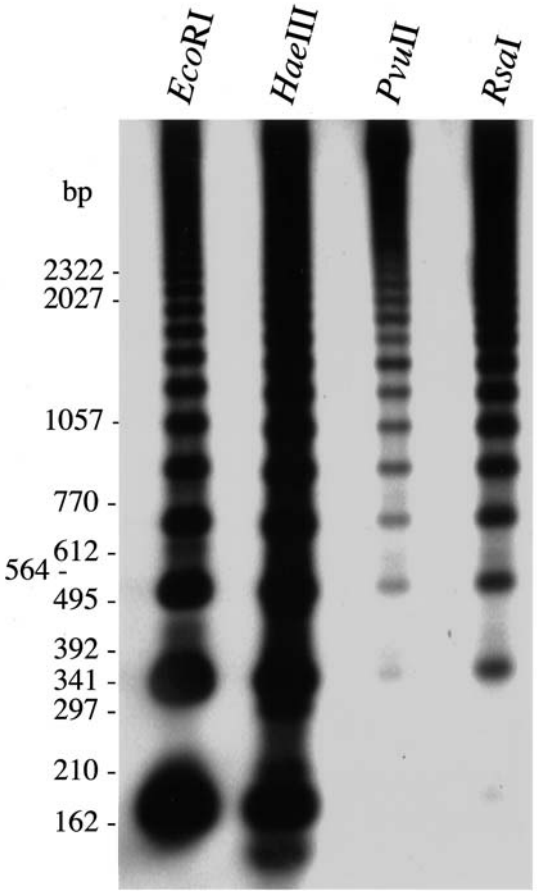

Fig. 5 Autoradiographs of Southern blot hybridization of *K. blakistoni* genomic DNA probed with the 174 bp element (KBL-*Hae*III 1U48). The genomic DNA was digested with the restriction enzymes, *Eco* RI, *Hae* III, *Pvu* II and *Rsa* I. Each lane contains 10 μg genomic DNA. A mixture of λDNA digested with *Hin* dIII and ϕX174 phage DNA digested with *Hin* cII is used as molecular weight marker

within the polymeric *Hae*III and *Eco*RI fragments, respectively. No chromosome compartmentalization with high sequence divergence was observed because all the chromosomes must recombine interchromosomally at the centromere.

Fig. 4 Comparison of fluorescence in situ hybridization patterns of the repeated elements (**a**, **c**, **e**) with C-banded patterns (**b**, **d**, **f**) in metaphase spreads of three Strigidae species: *K. blakistoni* (**a**, **b**), *Pulsatrix perspicillata* (**c**, **d**) and *Nyctea scandiaca* (**e**, **f**). The 174 bp element of *K. blakistoni* (KBL-*Hae*III 1U48) was used as a biotinylated probe. The fluorescence signals and large C-band-positive regions are located in the centromeric regions of all macrochromosomes, including Z and W chromosomes, and microchromosomes in *K. blakistoni* (**a**, **b**), *P. perspicillata* (**c**, **d**), and *Ny. scandiaca* (**e**, **f**). *Bars* represent 10 μm. *Arrowheads* indicate the Z and W chromosomes

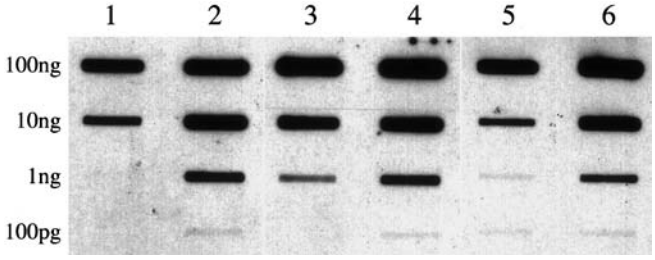

Fig. 6 Estimation of copy numbers of the satellite DNA by slot-blot hybridization in three Strigidae species, *K. blakistoni* (KBL), *P. perspicillata* (PPE) and *Ny. scandiaca* (NYS). *Lane 1* genomic DNA of *K. blakistoni*; *lane 2* KBL-*Hae*III1U48 fragment (Accession no. AB103255); *lane 3* genomic DNA of *P. perspicillata*; *lane 4* PPE2 (AB103283) fragment; *lane 5* genomic DNA of *Ny. scandiaca*; *lane 6* NYS4 (AB103273) fragment. The hybridization probes were KBL-*Hae*III1U48, PPE2 and NYS4 fragments in *lanes 1* and *2*, *lanes 3* and *4*, and *lanes 5* and *6*, respectively. DNA samples of the three fragments were amplified by the polymerase chain reaction using their plasmid clones, and blotted on the membranes

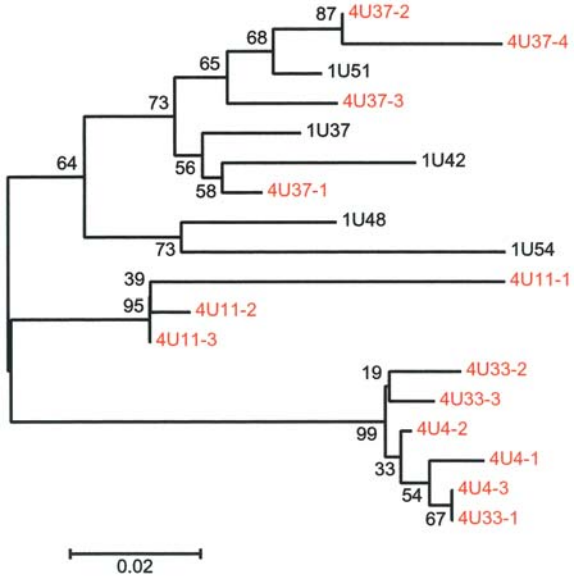

Fig. 7 The phylogenetic relationship of the nucleotide sequences of the five monomeric fragments (KBL-*Hae*III 1U37, 42, 48, 51, 54), three tetrameric fragments containing three internal monomer units (KBL-*Hae*III 4U4, 11, 33) and a tetrameric fragment containing four monomer units (KBL-*Hae*III 4U37) shown in Fig. 3a. The molecular phylogenetic tree was constructed by the neighbor-joining method

Sequence homology in Strigiformes species

Figure 8 demonstrates the slot-blot hybridization patterns resulting from probing with the 174 bp *Hae* III element (KBL-*Hae*III 1U48) of *K. blakistoni* in genomic DNAs of seven Strigidae species, *K. blakistoni* (KBL), *B. bubo* (BBU), *Ny. scandiaca* (NYS), *S. u. hondoensis* (SUH), *P. perspicillata* (PPE), *Ni. scutulata* (NIS), and *O. bakkamoena* (OBA), and one Tytonidae species, *T. alba* (TAL). The probes solidly cross-hybridized to the ge-

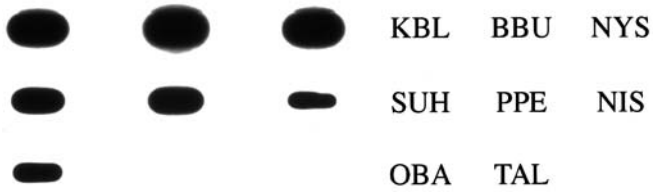

Fig. 8 Autoradiographs of slot-blot hybridization of genomic DNAs of seven Strigidae and one Tytonidae species probed with the 174 bp element of *K. blakistoni* (KBL-*Hae*III 1U48). KBL *K. blakistoni*; BBU *Bubo bubo*; NYS *Ny. scandiaca*; SUH *Strix uralensis hondoensis*; PPE *P. perspicillata*; NIS *Ninox scutulata*; OBA *Otus bakkamoena*; TAL *Tyto alba.* Each slot contains 500 ng DNA

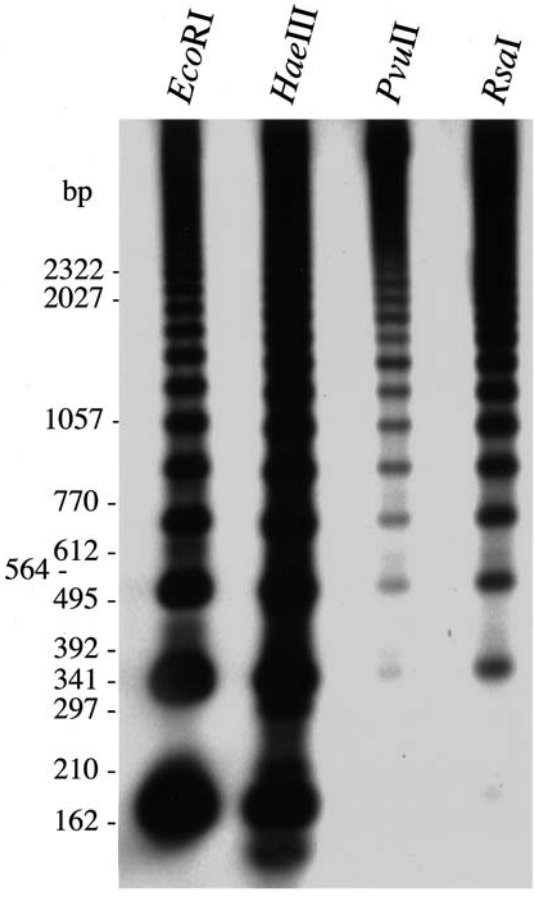

Fig. 9 Nucleotide sequences of the 41 monomeric fragments of the repetitive sequences cloned from *Hae* III-digested genomic DNA of *B. bubo* (BBU), *Bubo virginianus* (BVI), *K. blakistoni* (KBL), *Ny. scandiaca* (NYS), *Otus scops* (OSC) and *P. perspicillata* (PPE), and from *Eco* RI-digested genomic DNA of *S. u. hondoensis* (SUH) and the *Hin* fI-digested genomic DNA of *Ni. scutulata* (NIS)

nomic DNAs of seven Strigidae species, but did not yield any hybridization signals in *T. alba*. The intensity of hybridization was weaker in SUH, PPE, NIS and OBA compared with KBL, BBU and NYS, although the same amount of genomic DNA was blotted on the membranes. The sizes of centromeric C-band-positive heterochroma-

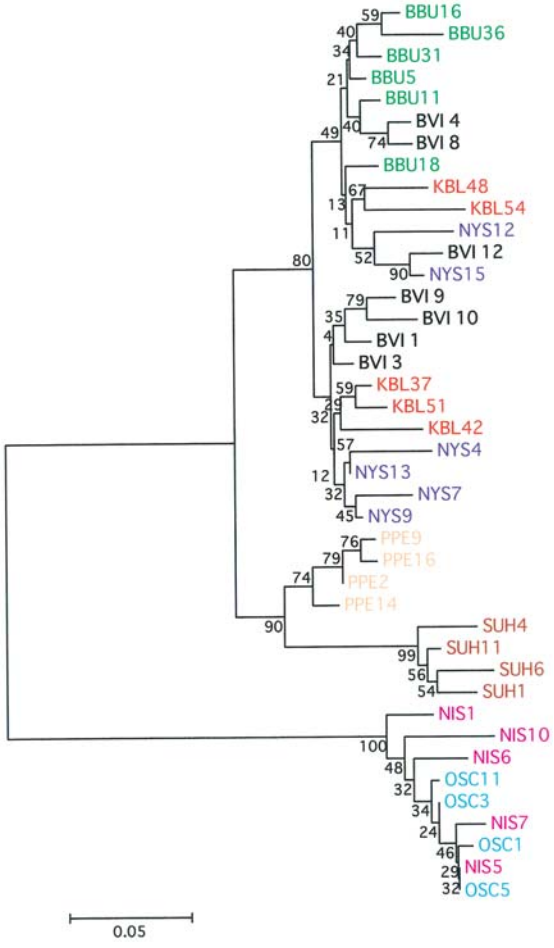

Fig. 10 Molecular phylogenetic relationship of the nucleotide sequences of the 41 fragments that were randomly cloned from eight Strigidae species shown in Fig. 9. The molecular phylogenetic tree was constructed by the neighbor-joining method. BBU *B. bubo*; BVI *B. virginianus*; KBL *K. blakistoni*; NYS *Ny. scandiaca*; PPE *P. perspicillata*; SUH *S. u. hondoensis*; NIS *Ni. Scutulata*; OSC *O. scops*

tin were not so different between the two groups (Fig. 4, the data of the other four genera are not shown). The satellite DNA accounts for 14.5% of the genome in PPE, which is higher than in KBL (10%) and NYS (9.6%), but the intensity of the hybridization signals was much weaker in PPE than KBL and NYS. The difference in hybridization intensity appears to be due to differences in nucleotide sequence homology, not to differences in copy numbers of sequences between the two groups.

Phylogenetic relationship of the nucleotide sequences in other Strigidae species

A total of 41 monomeric fragments of the satellite DNA sequences were isolated from eight Strigidae species, *B. bubo* (BBU), *B. virginianus* (BVI), *K. blakistoni* (KBL), *Ni. scutulata* (NIS), *Ny. scandiaca* (NYS), *O. scops* (OSC), *P. perspicillata* (PPE) and *S. u. hondoensis* (SUH),

and their nucleotide sequences were determined. Six BBU, 7 BVI, 5 KBL, 6 NYS, 4 OSC, 4 PPE fragments were isolated from genomic DNA digested with *Hae* III, and 4 SUH fragments and 5 NIS fragments were isolated from *Eco* RI- and *Hin* fI-digested genomic DNA, respectively. The nucleotide sequences were comparatively aligned for maximum matching of base pairs (Fig. 9). There were three highly variable sequence regions among the species, where nucleotides were partially added or deleted in several species. Nucleotide additions were located at position 23–27 in PPE, at position 41–50 in NIS, OSC and PPE (or deletions in BBU, BVI, KBL, NYS and SUH) and at position 157–162 in SUH.

The molecular phylogenetic relationship of the 41 monomeric fragments was compared at the intra- and interspecific levels (Fig. 10). We conducted a phylogenetic construction including all the gaps of the nucleotide sequences. This alignment was informative, and the phylogenetic inference method revealed that the genetic distances between the repeated elements were smaller within the same species than those between different species. In addition, the phylogenetic analysis clearly showed the existence of two separate clades of monomer variants supported by 100% of bootstrap value, one leading to *Ninox* and *Otus*, and the other leading to *Bubo, Ketupa, Nyctea, Pulsatrix*, and *Strix.* In the latter group the nucleotide sequences were divided into three subgroups; a group of four species of three genera, *Bubo, Ketupa* and *Nyctea,* a *Pulsatrix* group and a *Strix* group.

Discussion

A novel family of repetitive sequences was isolated from both *Hae* III- and *Eco* RI-digested genomic DNA of *K. blakistoni*. Southern hybridization and chromosome in situ hybridization revealed that the 174 bp elements are arranged into large tandem arrays and localized to centromeric C-band-positive regions of all chromosomes. The sequence represented 10% of the total genome. These results imply that the repeated element is a major component of centromeric heterochromatin of chromosomes in *K. blakistoni* and categorized as satellite DNA (Singer1982). The average G+C content was 52.4% and 50.1% in the *Hae* III and *Eco* RI fragments, respectively, and sequence homology search by BLAST and FASTA in DDBJ revealed no related DNA sequences. The evolutionary features of the satellite DNA sequences, as rapidly evolving sequences, make them useful markers for both taxonomic and phylogenetic studies. Species-specific satellite DNA is an excellent diagnostic marker for identifying species, and the sequence divergence discloses relationships between closely related species that share the same repeated family. Thus, the satellite DNA family cloned in this study, which is conserved in a broad group of owl species, is useful for revealing evolutionary relationships in the Strigiformes. Slot-blot hybridization indicated that the eight Strigiformes species used in this study can be classified into three groups by the intensity of cross-hybridization signals with the repeated element of *K. blakistoni*: (1) three genera, *Ketupa, Bubo* and *Nyctea*; (2) four genera, *Strix, Pulsatrix, Ninox* and *Otus*; and (3) one genus*, Tyto,* in which the repeated element of the same family is absent*. Tyto alba* belongs to the Tytonidae family, which is different from the Strigidae family, and this classification of the eight genera is consistent with that from traditional taxonomy based on morphological traits (Sibley and Monroe1990) and classification by DNA-DNA hybridization (Sibley and Ahlquist1990). The molecular phylogeny of the 41 monomeric fragments isolated from the eight Strigidae species also supports a concerted mode of evolution for this satellite DNA family and the previous classification of the Strigidae (Sibley and Ahlquist1990). The Strigidae species are grouped into two separate clades; *Otus* and *Ninox* are distant from the group of *Ketupa, Bubo, Nyctea, Pulsatrix* and *Strix.* These results suggest that the satellite DNA is a good taxonomic and phylogenetic marker in the Strigiformes.

In the Galliformes, 41–42 bp tandemly repeated sequences, which are distributed primarily on microchromosomes, have been isolated from four species of the Phasianidae: chicken (*Gallus gallus*; CNM repeat; Matzke et al.1990), turkey (*Meleagris gallopavo*; TM repeat; Matzke et al.1992), Japanese quail (*Coturnix japonica*; Tanaka et al.2000), and the blue-breasted quail (*Coturnix chinensis*; Yamada et al. 2002b). Microchromosome-specific repetitive sequences have also been found in two Struthioniformes species, the lesser rhea (*Pterocnemia pennata*) and the greater rhea (*Rhea americana*) (Yamada et al. 2002a). The homogenization of centromeric heterochromatin between macro- and microchromosomes is not found in these orders, which are classified at the basal position of the avian phylogenetic tree (Sibley and Ahlquist1990; Sibley and Monroe1990). Microchromosomes differ from macrochromosomes not only in physical size but also in structure and function. Chicken microchromosomes contain a high density of CpG islands (McQueen et al.1996), high acetylation of histone H4 (McQueen et al.1998), elevated gene density (Suzuki et al. 1999a; Smith et al.2000) and low frequency of microsatellite DNA sequences (Primmer et al.1997). These structural differences between macro- and microchromosomes might be due to the limitation of homogenization between the two types of chromosomes. In contrast to Galliformes and Struthioniformes, in the Strigidae the satellite DNA is distributed in the centromeric regions of both macro- and microchromosomes as large heterochromatin blocks. The avian karyotypes are highly conserved among different orders excepting the family Accipitridae, and the karyotypes conserved through the majority of species are characterized by the morphology of the large three pairs: submetacentric no. 1 and no. 2, and subtelocentric or acrocentric no. 3 (Takagi and Sasaki1974; Belterman and de Boer1984). The ancestral karyotype of the Strigidae species has been deduced from comparison with other orders and between many Strigidae species (Rebholz et al.1993). It consists of

acrocentric no. 1, and acrocentric no. 2 and no. 3 of comparable size, which are not much larger than other macrochromosomes. This result suggests that the karyotype of the Strigidae is one of the variant types of the primitive avian karyotype. It is probable that the difference in the chromosomal distribution of the satellite DNA sequences is caused by the difference in karyotypes between the Strigidae species and the Phasianidae and Struthioniformes species.

The molecular phylogeny based on DNA-DNA hybridization indicates that the modern Strigiformes diverged late from other Neognathous birds (Sibley and Ahlquist1990; Sibley and Monroe1990). Recently we investigated chromosome homologies among more than ten species of Galliformes by comparative chromosome painting with paints for chicken chromosomes 1–9 and Z. We concluded that chicken retains the ancestral karyotype of the Galliformes, which is quite similar to the karyotype of emu (Shetty et al.1999), and confirmed also that few chromosome rearrangements have occurred between macro- and microchromosomes in the Galliformes (Shibusawa et al. 2004). We applied comparative painting to *K. blakistoni* chromosomes with probes for chicken chromosomes 1–9 and Z. One interchromosomal rearrangement of macrochromosomes and two rearrangements of macro- and microchromosomes were found between the two species (Nishida-Umehara et al. unpublished data). Chromosome rearrangements have frequently occurred between macro- and microchromosomes in the Strigiformes compared with the other two orders, suggesting that the chromosome rearrangements might be related to the homogenization of the satellite DNA in centromeric heterochromatin of macro- and microchromosomes. The mechanism of homogenization and concerted evolution of the centromeric repetitive sequences between macro- and microchromosomes will be unclear until the cytogenetic organization in microchromosomes and the arrangement of macro- and microchromosomes in the nuclei have been defined in the Strigidae.

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