Structure and molecular evolution of the ribosomal DNA external transcribed spacer in the cockroach genus *Blattella*

Dmitry V. Mukha, Vera Mysina, Valeria Mavropulo, and Coby Schal

Abstract: The ribosomal DNA (rDNA) cluster of insects contains several hundred repeating structural–functional units and, therefore, is a typical example of a multigene family. Eukaryotic ribosomal RNA (rRNA) genes (18S, 5.8S, and 28S like) are arranged in tandemly repeated clusters in the nucleolus organizers, separated by several spacers, namely the non-transcribed spacer, the external transcribed spacer (ETS), and the internal transcribed spacers. The nucleotide sequences of the ETS of the three closely related *Blattella* cockroach species, *Blattella germanica* (Linnaeus, 1767), *Blattella asahinai* (Mizukubo, 1981), and *Blattella lituricollis* (Walker, 1868), were determined and compared. The three species had relatively similar ETS lengths, and sequence differences among them could be explained by two types of rearrangements, namely deletions of subrepeats and nucleotide substitutions. Minor ETS variants in *B. germanica* differed from the major variant in the same way that the major ETS variants of the three *Blattella* species differed from each other. Concerted evolution and the birth-and-death models, which are often invoked to explain the diversity and evolution of the multigene families of rDNA clusters, are discussed in the light of our data. A new model is proposed to explain the evolutionary reorganization of the ETS region: evolution of rDNA by "magnification-and-fixation" is characterized by magnification of minor subrepeats, which become adaptive in a new rapidly changed environment, and subsequent fixation of this variant type as a major component of the multigene family of a new species.

Key words: cockroach, *Blattella*, multigene families, ribosomal DNA, concerted evolution, birth-and-death model, saltational evolution.

Résumé : L'amas d'ADN ribosomique (ADNr) chez les insectes compte plusieurs centaines d'unités fonctionnelles répétées et constitue, par le fait même, un exemple typique d'une famille multigénique. Les gènes (de type 18S, 5,8S et 28S) qui codent pour les ARN ribosomiques (ARNr) sont disposés en tandem au sein des organisateurs nucléolaires et sont séparés par des espaceurs dont l'espaceur non-transcrit, l'espaceur externe transcrit (ETS) et des espaceurs internes transcrits. Les séquences nucléotidiques des ETS chez trois espèces proches de blattes du genre *Blattella* (*Blattella germanica* (Linnaeus, 1767), *Blattella asahinai* (Mizukubo, 1981) et *Blattella lituricollis* (Walker, 1868)) ont été déterminées et comparées. Les trois espèces possédaient des ETS de tailles semblables et les différences de séquences entre elles pouvaient s'expliquer au moyen de deux types de réarrangements, soit des délétions de sous-répétitions ou des substitutions nucléotidiques. Des variants ETS mineurs chez le *B. germanica* différaient du variant majeur de la même manière que les variants majeurs différaient entre eux au sein des trois espèces. À la lumière des résultats obtenus, les auteurs discutent des modèles d'évolution concertée et de naissance-mort, lesquels sont souvent invoqués pour expliquer la diversité et l'évolution des amas multigéniques d'ADNr. Un nouveau modèle est proposé pour expliquer la réorganisation de la région ETS au cours de l'évolution. L'évolution de l'ADNr par « multiplication et fixation » est caractérisée par une multiplication de sous-répétitions mineures, lesquelles deviennent avantageuses dans le contexte d'un environnement récemment modifié, suivie de la fixation de ce type variant en tant que composante majeure au sein de la famille multigénique chez une nouvelle espèce.

Mots-clés : blatte, *Blattella*, familles multigéniques, ADN ribosomique, évolution concertée, modèle de naissance-mort, évolution saltatoire.

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Introduction

The ribosomal DNA (rDNA) cluster of insects contains several hundred repeating structural–functional units and, therefore, is a typical example of a multigene family. The basic organization of the rDNA has been conserved in most eukaryotes. Ribosomal RNA (rRNA) genes (18S , 5.8S , and 28S like) in eukaryotic genomes are arranged in tandemly repeated clusters in the nucleolus organizers of one or more chromosomes, separated by several spacers, namely the NTS (nontranscribed spacer), ETS (external transcribed spacer), and ITS1 and ITS2 (internal transcribed spacers). The NTS separates neighboring repeat units, ETS is located between the promoter and 18S gene, ITS1 is located between the 18S- and 5.8S-like coding regions, and ITS2 lies between the 5.8S- and 28S-like genes (Fig. 1) (Gerbi 1985).

Some multigene families exhibit homogeneity of repeated structural units within a species and greater dissimilarity in representatives of different species (reviewed in Dover 1982; Ohta 1980). Concerted evolution is a common explanation for this characteristic feature of the structural organization of multigene families, and two mechanisms have been proposed to drive this process: (*i*) recombination between repeats, with gene conversion, and (or) (*ii*) unequal crossing over (Arnheim 1983; Brown et al. 1972; Dover and Coen 1981; Eickbush and Eickbush 2007; Li 1997; Nei and Rooney 2005; Ohta 2000; Smith 1976; Zimmer et al. 1980). Owing to unequal recombination exchange, mutant variants of repeated structural units are eliminated, leading to uniformity of members of a multigene family.

A multigene family of a new species, differing from that of the ancestral form, originates at the population level. Experimental data, computer simulation, and mathematical calculations demonstrate that if a multigene family determines a selectively neutral trait, a multigene family of a new type can form because of stochastic processes; i.e., one or several mutant variants may randomly (Ohta 1980), or according to a directional fixation mechanism (Dover 1982) become the major member of a multigene family of a newly formed species. The selective significance of the trait does not alter the nature of formation of the new multigene family but only accelerates this process beacause of selection pressure (Dover 1982; Nevo and Beiles 1988).

The birth-and-death model (Nei et al. 1997) is an alternative explanation of the diversity and evolution of multigene families. According to this model new genes are created by repeated gene duplication, and as some duplicate genes are maintained in the genome for a long time, others degenerate into pseudogenes or get deleted.

We previously compared ~1200-bp 28S rDNA fragments of the three *Blattella* cockroach species (also examined in the current study) and calculated the approximate time of divergence of these species. We also conducted a comparative study to estimate evolutionary variation in the internal transcribed spacers (ITS1 and ITS2) of the three *Blattella* species. These sequences proved to be extremely conserved within species (Mukha et al. 2002). At the same time, important changes occurred during species formation, as shown by comparison of the ITS1 and ITS2 sequences in three *Blattella* species (Mukha et al. 2002).

In this report we describe sequence variability of the rDNA ETS structure within and among the same three *Blattella* species investigated by Mukha et al. (2002): *Blattella germanica* (Linnaeus, 1767), *Blattella asahinai* (Mizukubo, 1981) (sibling species), and *Blattella lituricollis* (Walker, 1868). We discuss the molecular evolution of this region with emphasis on a new mechanism that we propose for evolutionary reorganization within the rDNA multigene family.

Materials and methods

The rDNA ETS structure was determined in 10 *B. germanica* populations, and in 1 population each of *B. asahinai* and *B. lituricollis*. Four *B. germanica* populations from the USA included an insecticide-susceptible standard strain maintained in the laboratory for about 40 years and three recently collected populations from commercial swine farms in North Carolina, USA (see Mukha et al. 2007). Four *B. germanica* populations were collected in Russia (two populations from Moscow, one from Kurgan, and one from Barnaul). Two *B. germanica* populations from Rennes, France, were kindly provided by C. Rivault. *Blattella asahinai* was collected in a soybean field in Weslaco, Texas, USA (Pfannenstiel et al. 2008), and *B. lituricollis* was collected in Kauai, Hawaii, USA, and kindly provided by R. Rice.

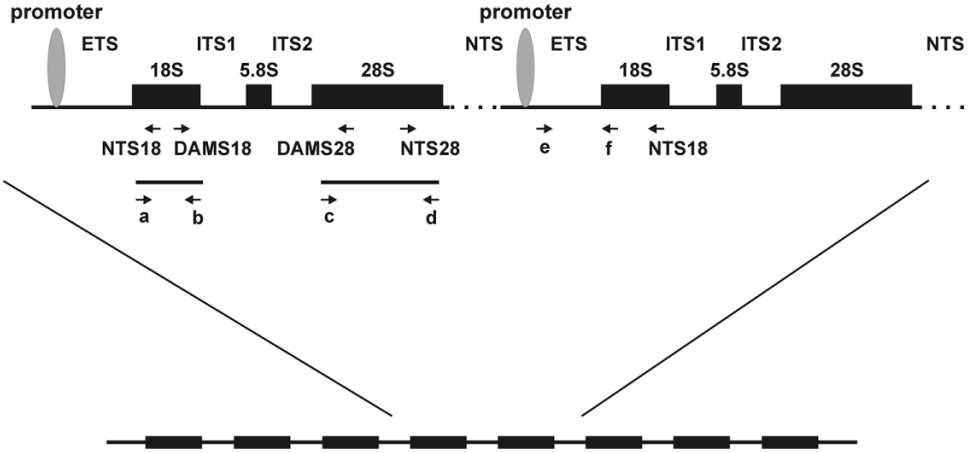
Total cockroach DNA was isolated from whole individuals by homogenization in extraction buffer (Sambrook et al. 1989), followed by phenol–chloroform extraction and ethanol precipitation following standard protocols (Sambrook et al. 1989).

To determine the nucleotide sequence of the entire rDNA repeat unit, two pairs of previously described (Mukha and Sidorenko 1995; Mukha and Sidorenko 1996) "universal" primers (DAMS18/DAMS28, 5′-GTCCCTGCCGTTTGTACACA-3′/5′-CTACTAGATGGTTCGATTAGTC-3′; NTS18/NTS28, 5′-TCCACCAACTAAGAACGGCC-3′/5′-AACTATGACTCTCTTAAGGT-3′) (see Fig. 1) were used to amplify the respective *B. germanica* rDNA fragments, which were cloned and sequenced. Based on these sequences, new species-specific pairs of primers were designed which allowed us to amplify the following gaps: (*i*) between universal primers DAMS18 and NTS18 (*a*/*b*, 5′-GTGGAGCCTGCGGCTTAATTTGAC-3′/5′-CCTTGTTACGACTTTTACTTCC-3′) and (*ii*) between universal primers DAMS28 and NTS28 (*c*/*d*, 5′-GGACCCGAAAGATGGTGAAC-3′/5′-GATCTATCATCTATCCCTGTC-3′) (Fig. 1). The corresponding fragments were also amplified, cloned, and sequenced.

Long PCR amplifications for fragments within the cockroach rDNA repeat unit, located between primers NTS18 and NTS28, were carried out using an enzyme mix containing *Taq* and the thermostable proofreading DNA polymerase *Pwo* (Roche Applied Science, Indianapolis, Indiana) and the PTC-100 Thermal Cycler (MJ Research Inc., Waltham, Massachusetts). Each reaction contained 0.1 μg DNA template, 2.25 mmol/L $MgCl_2$, 2.5 mmol/L of each dNTP, and 0.3 pmol of each primer. The PCR regimen was as follows: initial template denaturation at 94 °C for 3 min; followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 68 °C for 7 min; and a final 10 min elongation step at 68 °C.

For amplification of the other fragments corresponding to

Fig. 1. A schematic representation of the eukaryotic ribosomal genes. Designations: 18S, 5.8S, and 28S, respective genes of ribosomal RNAs; NTS, nontranscribed spacer; ETS, external transcribed spacer; ITS1 and ITS2, internal transcribed spacers; grey oval, marks the RNA polymerase I promoter. Arrows indicate the positions and directions of primer pairs (DANS18/DAMS28, NTS18/NTS28, *a*/*b*, *c*/*d*, *f*/*e*) used for amplification of the respective rDNA fragments.



Blattella rDNA (DAMS18/DAMS28; *a*/*b*; *c*/*d*; *e*/*f*), PCR amplifications were carried out using *Taq* DNA polymerase (Promega, Madison, Wisconsin). Each reaction contained 0.1 μg DNA template, 1.5 mmol/L $MgCl_2$, 1 mmol/L of each dNTP, and 0.2 pmol of each primer. The PCR regimen was as follows: initial template denaturation at 95 °C for 5 min; followed by 30 cycles of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 1 min; and a final 7 min elongation step at 72 °C.

After agarose gel electrophoresis, the amplified fragments were cut from the gel and purified by the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's recommendations. The purified fragments were sequenced directly or first were cloned into the plasmid pGEM-T-Easy Vector (Promega). Sequences were obtained using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (Applied Biosystems Inc., Foster City, California) according to the accompanying protocol and an ABI Prism 310 automated sequencer (Applied Biosystems Inc.).

Two online software programs were used for sequence alignment: BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome) and ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

The following reagents, all from Promega, were used according to the manufacturer's recommendations: SV Total RNA Isolation System (total RNA extraction); DNA 5′ End-Labeling System (primer labeling); Universal Ribo-Clone cDNA Synthesis System (cDNA production).

Results

Structure of the entire *B. germanica* rDNA repeat unit

The assembled repeat unit of *B. germanica* rDNA (see Materials and methods) consisted of 9015 nucleotides (GenBank accession No. AF005243). A segment of the *B. germanica* rDNA repeat unit containing the 3′ end of the 28S gene (blue letters), nontranscribed and external transcribed spacers, and the 5′ end of the 18S gene (green letters) is shown in Fig. 2A.

Because the 28S and 18S rDNA genes are relatively conserved in eukaryotes, the approximate boundaries of the rRNA genes (3′ end of 28S and 5′ end of 18S) could be identified by aligning the *B. germanica* sequence with the corresponding sequences of other insect species represented in the GenBank database using the online BLASTN software (data not shown).

Between the 28S and 18S genes, that is, within the NTS and ETS, we observed several types of subrepeats, indicated in Figs. 2A and 3 by red and dark blue letters, and green, purple, blue, yellow, and grey backgrounds. All the respective subrepeats (of the same background color) were identical, except for the fifth nucleotide within the subrepeats indicated by purple background: the variable nucleotides (T/C) are shown as subscripts (Fig. 2A).

The following conventional approach was applied to determine the boundary between the NTS and ETS, i.e., the approximate location of the promoter. A radioactively labeled primer, located near the 5′ end of the 18S gene (underlined in Figs. 2A, 2B, and 2C) was annealed with the total RNA isolated from the three cockroach species and followed by complementary DNA (cDNA) synthesis catalyzed by reverse transcriptase. The cDNA size was determined by fractionation in 6% polyacrylamide gel under denaturing conditions (Sambrook et al. 1989), using the ϕ-X174 DNA/*Hin*fI markers (Promega) (Fig. 4). Although the transcription initiation site could not be localized by this method to a specific nucleotide, the cDNA in Fig. 4 (lane 1) indicated that in *B. germanica* the transcription start region was ~840 bases upstream of the labeled primer (Fig. 2A).

Thus, the ETS of *B. germanica* comprises three primary domains. The first domain is bounded by the transcription initiation site and a 5′ ETS downstream sequence of ~200 bp. The second domain is a complex series of five subrepeats that differ in length (9–91 bp), structure, and sequence composition. The third domain, adjacent to the 5′ 18S rDNA, comprises ~200 bp of relatively conserved sequence (Figs. 2A and 3).

Fig. 2. Nucleotide sequences containing the rDNA region investigated in the three closely related *Blattella* cockroach species: (A), *B. germanica*; (B), *B. asahinai*; and (C), *B. lituricollis*. Green and blue letters represent the 5′ and 3′ ends of the 18S and 28S rRNA genes, respectively. Subrepeats corresponding to the nontranscribed spacer are indicated by red and dark blue letters. Subrepeats corresponding to the external transcribed spacer are shown with green, purple, blue, yellow, and grey backgrounds. The numbers (1)–(4) follow the respective subrepeats. Bold letters indicate the short sequence that is important in the recombination process (explanation in the text). Subscripts indicate variable nucleotides within the ETS subrepeat denoted by purple background. The location of the primer used for the primer extension reaction is underlined; double underlining shows the position of primers *e* and *f* (see Fig. 1). Deletions (*B. germanica*/*B. asahinai* and *B. germanica*/*B. lituricollis*) in B and C show the difference of ETS subrepeats between the respective species. Superscripts in C indicate variable nucleotides and dashes represent small deletions based on comparison of the *B. germanica* and *B. lituricollis* ETS sequences.

A. ***Blattella germanica***

CTTCGATGTCGGCTCTTCCTATCATTGCGAAGCAGAATTCGCCAAGCGTTGGATTGTTCACCCACCAATAGGGAACG
TGAGCTGGGTTTAGACCGTCGTGAGACAGGTTAGTTTTACCCTACTGATGACAATGTAATTTGGTCGTTGCGACAGT
AATCCTGCTCGGTACGAGAGGAACCGCAGGTTCGGACATTTGGTTCATGCACTCGGTCGAGGGGCCGGTGGTGCGAA
GCTACCATCCGCAGGATTATGCCTGAACGCCTCTAAGGCCGTATCCCGTCTGGTCGATGCAACGATGAGATCGGGAG
TCCCGTGGGTCGAAAGGCTTTAAACAAGGTGATTTTCTTCCTAGGGGGGGACTCTTTCGAGCAGTTAAGGCGGGAAG
CGGAGTGTCGCTGAAATGTTCAACTATCGTAATCAGAAACGGTGGAAGTGGTCTTAGTCCGAGCAGTAGGGCGGACT
GACCATGTTAGGAAGGCTGGGATTGCCGGTTAAGGCGGGAAGCGGAGTGTCGCTGAAATGTTAAACTATCGTAATCA
GAAACGGTGGAAGTGGTCTTAGTCCGAGCAGTAGGGCGGACTGACCATGTTAGGAAGGCTGGGATTGCCGGTTAAGG
CGGGAAGCGGAGTGTCGCTGAAATGATCAACTATCGTAATCAGAAACGGTGGAAGTGGTCTTAGTCCGAGCAGTTGG
GCGGACTGACCATGTTAGGAACGCAGGGATTGCCGGTTAAGGCGGGAAGCGGAGTGTCGCTGAAATGTTCAACTATC
GTAATCAGAAACGGTGGAAGTTGTCTGAGTCCGAGCATTAGGGCGGACTGACCATTTAAGGTCGACCTCATCGGCCG
GTAAATCCTACGCAGATGTCCGGAATGGGTTGTGTTGGAAATTTTCTAAGTCCAGACCTCTTCAAGACATACACGTG
CAGCCGAACCCAAAATCCAGGGGAGTTGAAAAAGTAGCCCCTCGGGAGTTGATGGGTGAACCATGCGGTTTTTGACG
TGATTTAGGGAGGCGACAGATCTACAGACTCGGCAATGTCAAACCATATATGTAGAGGGCAGTCGTCAGGACAGATC
TGTAATGGAAATGTTCTGGCGCTTGGTCGGGTTCGATGCCGGAGGTATGTCTCGTCCTCTCGCCTCCCTTGATGTGC
ATTGACTCGGCCACTCACGGTAGGAATTTTGGTTTCCGGACCACTTTCTGATCCCGTGCACAGCACCTCCGCGGCCA
CTAGGCCCTCCATAGCCTTTGTGTGTCTTATGCGGAGACCCTTTTTATGTCGTATCTAATGATATATACTCTCTTGC
CTCCGGGCGCGAGCTATGTCTCGACTTTCTCATGAGGGTTCTGGTTGCGCCTTTGCGCGCTCCGGGCATTCGTAGAG
ATTGTCAGGTGTGAGAC(1)TGAACCAAGTGTG$_n$GCCTTCGGGCATGAATGAGTTGGGG(1)CGTACGGTTCTAGCTT
GTCTCGGCATATCTGTCCGTATAGGATTGTGTGT(1)GTGTGAGAC(2)GTGGCCTGTGACCGACCCCACAAAGAGT
GTACTC(1)TGTG$_C$GCCTTCGGGCATGAATGAGTTGGGG(2)CAATGCGTGGAAGGTTCGTCCGAGATATGGTCGATC
CCGCTTGAGGCTGCAGAGCCGATGGACGGGGGTAGATCGGGAAAAGAATCCGACA(1)CGTACGGTTCTAGCTTGTC
TCGGCATATCTGTCCGTATAGGATTGTGTGT(2)GTGTGAGAC(3)GTGGCCTGTGACCGACCCCACAAAGAGTGTA
CTC(2)TGTG$_C$GCCTTCGGGCATGAATGAGTTGGGG(3)CAATGCGTGGAAGGTTTGTCCGAGATATGGTCGATCCCG
CTTGAGGCTGCAGAGCCGATGGACGGGGGTAGATCGGGAAAAGAATCCGACA(2)CGTACGGTTCTAGCTTGTCTCG
GCATATCTGTCCGTATAGGATTGTGTGT(3)GTGTGAGAC(4)GTGGCCTGTGACCGACCCCACAAAGAGTGTACTC
(3)TGTGCGCGATCTAGTCTCTCCGCAAGGACCTTTCCCGTCTCGAGATGTATTTCTTGTGTCCGGGGTCTATAGGT
TTTCTTGTCGGCTGTCGGACTTTTTCTGTCGCAGAGTATTAATGACTAGGTTGCCATGCGGGGCTTTTGTACCCGTG
GCGCCTGTTCGGAGCACGTGTAAAACAACGCACGAGTTCCCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAA
AGATTAAGCCATGCATGTCTCAGTGCAAGCTATACTAAAGTGAAACCGCGAATGGCTCATTAAATCAGTTATGGTTC
CTTAGATGAAAGACAGTTAC

B. ***Blattella asahinai***

ATTGTCAGGTGTGAGAC(1)TGAACCAAGTG$_n$GCCTTCGGGCATGAATGAGTTGGGG(1)CAATGCGTGGAAGGTTCG
TCCGAGATACGGTCGATCCCGCTTGAGGCCGCAGAGCCGATGGACGGGGGTAGATCGGGAAATGAATCCGACA(1)C
GTACGGTTCTAGCTTGTCTCGGCATATCTGTCCGTATAGGATTGTGTGT(2)GTGTGAGAC(3)GTGGCCTGTGACC
GACCCCACAAAGAGTGTACTC(2)TGTG$_C$GCCTTCGGGCATGAATGAGTTGGGG(3)CAATGCGTGGAAGGTTCGTCC
GAGATATGGTCGATCCCGCTTGAGGCTGCAGAGCCGATGGACGGGGGTAGATCGGGAAAAGAATCCGACA(2)CGTA
CGGTTCTAGCTTGTCTCGGCATATCTGTCCGTATAGGATTGTGTGT(3)GTGTGAGAC(4)GTGGCCTGTGACCGAC
CCCACAAAGAGTGTACTC(3)TGTGCGCGATCTAGTCTCTCCGCAAGGACCTTTCCCGTCTCGAGATGTATTTCTTG
TGTCCGGGGTCTATAGGTTTTCTTGTCGGCTGTCGGACTTTTTCTGTCGCAGAGTATTAATGACTAGGTTGCCATGC
GGGGCTTTTGTACCCGTGGCGCCTGTTCGGAGCACGTGTAAAACAACGCACGAGTTCCCTGGTTGATCCTGCCAGTA
GTCATATGCTTGTCTCAAAGATTAAGCCATGCGTGTCTCAGTGCAAGCTATACTAAAGTGAAACCGCGAATGGCTCA
TTAAATCAGTTATGGTTCCTTAGATGAAAGACAGTTAC

Deletion (*B. germanica*/*B. asahinai*) – 123 bp

CGTACGGTTCTAGCTTGTCTCGGCATATCTGTCCGTATAGGATTGTGTGT(1)GTGTGAGAC(2)GTGGCCTGTGAC
CGACCCCACAAAGAGTGTACTC(1)TGTGTGCCTTCGGGCATGAATGAGTTGGGG(2)

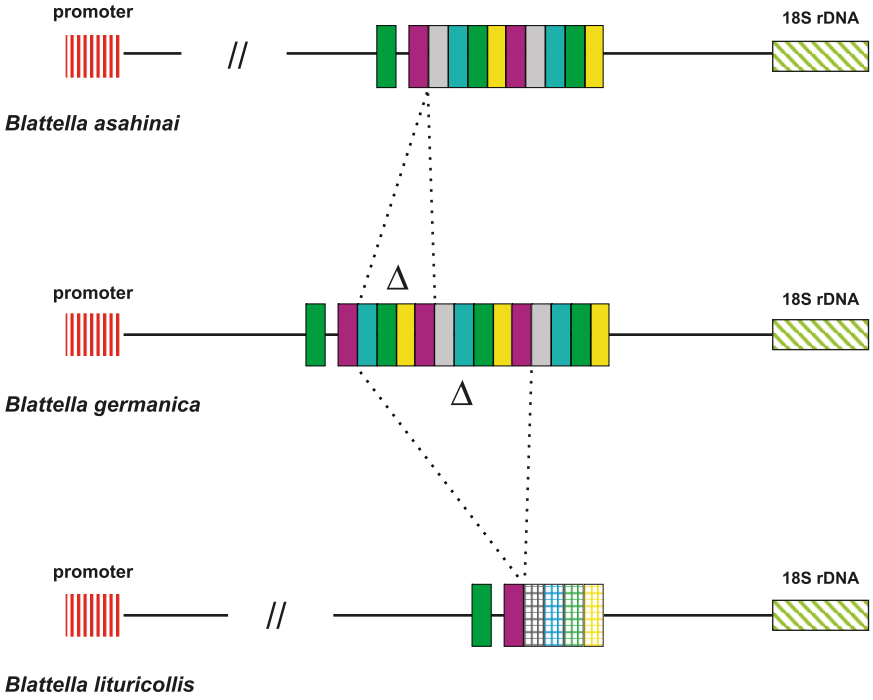
C. ***Blattella lituricollis***

ATTGTCAGGTGTGAGAC(1)TGAACCAAGTG$_n$GCCTTCGGGCAAGAATGAGTTGGGGG(1)CA^{t}TGCGTGGAAGGTT^{t}
GTCCG^{c}TATATGGTCGATCCCGCTTGAGGCTGCAGAGCCG^{c}TGGACGGG--T^{c}GATCGGGAA^{t}A^{t}AATCCGACA(2)C
G^{c}ACGGTTCTAGCTTGTC^{g}C^{atatc}T^{g}TC^{c}GTCC^{c}T^{g}T^{c}GG^{gag}G^{at}TGT(3)GTGT^{ct}GAC(4)^{a}TGGCCTGTGACCG
ACCCCA------GTGTACTC(3)T-TG^{t}GCG-TCTAGTCTCTCCGCAAGGACCTT^{c}CCCG^{a}C^{c}--^{g}GATGTATTTCTT
GT^{t}T--GGGGTCTA--GGTTTTCTTGTCGGCTGTCGGACTT^{g}T^{a}CT--CGCAGAGTAT^{g}AATGACTAGGTTGCCATGC
GGGGCTTT-G^{c}ACCCGTGGCGCCTGTTCGGAGCACGTGTAA^{c}ACAACGCACGAGTTCCCTGGTTGATCCTGCCAGTAG
TCATATGCTTGTCTCAAAGATTAAGCCATGC^{a}TGTCTCAGTGCAAGCTATACTAAAGTGAAACCGCGAATGGCTCATT
AAATCAGTTATGGTTCCTTAGATGAAAGACAGTTAC

Deletion (*B. germanica*/*B. lituricollis*) – 337 bp

CGTACGGTTCTAGCTTGTCTCGGCATATCTGTCCGTATAGGATTGTGTGT(1)GTGTGAGAC(2)GTGGCCTGTGAC
CGACCCCACAAAGAGTGTACTC(1)TGTGCGCCTTCGGGCATGAATGAGTTGGGG(2)CAATGCGTGGAAGGTTCGT
CCGAGATATGGTCGATCCCGCTTGAGGCTGCAGAGCCGATGGACGGGGGTAGATCGGGAAAAGAATCCGACA(1)CG
TACGGTTCTAGCTTGTCTCGGCATATCTGTCCGTATAGGATTGTGTGT(2)GTGTGAGAC(3)GTGGCCTGTGACCG
ACCCCACAAAGAGTGTACTC(2)TGTGCGCCTTCGGGCATGAATGAGTTGGGG(3)

Fig. 3. A schematic representation of the postulated ETS structural differences among the ETS subrepeats of the three *Blattella* species; Δ, deletion of subrepeats relative to *B. germanica* or a similar ancestral sequence; hatched bars denote multiple substitutions within ETS subrepeats.



Organization and interspecific variability of ETS among closely related *Blattella* species

To compare the ETS structures of closely related *Blattella* species, the rDNA fragments of *B. asahinai* and *B. lituricollis*, located between universal primers NTS18 and NTS28 (see Fig. 1), were amplified and cloned. Several randomly selected clones from each species were partially sequenced. Based on comparative analysis of the corresponding rDNA sequences of the three *Blattella* species, we determined that primers *e* (GTGAGACTGAACCAAGTGTG) and *f* (CATATGACTACTGGCAGGATC) (see Fig. 1) are suitable for amplification of the ETS regions of all three species. Figure 5 demonstrates the results of electrophoretic separation of amplification products obtained using these primers. Major bands were then extracted from the gel, purified, and sequenced. The species-specific sequences corresponding to the ETS subrepeats are shown in Figs. 2B and 2C.

Comparative analysis of the ETS nucleotide sequences in *B. germanica* and its sibling species *B. asahinai* revealed a deletion of a 123-bp fragment containing four subrepeats (purple, blue, green, and yellow backgrounds) and two nucleotides (TG) within the first subrepeat indicated by purple background (Figs. 2B and 3). Except for these deletions, the rest of the sequence of *B. asahinai* was identical with that of *B. germanica*.

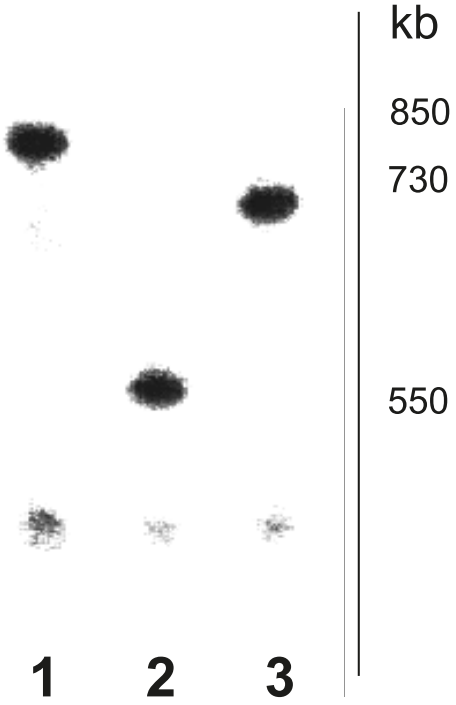
The *B. lituricollis* ETS contained only two intact subrepeats (green and purple backgrounds) but, as with *B. asahinai*, two nucleotides (TG) were deleted within the first subrepeat indicated by purple background (Figs. 2C and 3). Four additional subrepeats (grey, blue, green, and yellow backgrounds) have accumulated numerous nucleotide substitutions (indicated by superscripts) and deletions (indicated by dashes), compared with the *B. germanica* ETS. A 337-bp deletion, relative to the *B. germanica* ETS, contained nine subrepeats (Figs. 2C and 3). The differences in the lengths of the ETS sequences of the three *Blattella* species, represented by deletions relative to the *B. germanica* ETS, were consistent with the ETS lengths of the three species, as measured by the primer extension reaction (Fig. 4).

Analysis of the ETS sequences also suggested that *B. germanica* is significantly more closely related to *B. asahinai* than to *B. lituricollis*, in agreement with our previous inference from phylogenetic analysis of the 28S sequences (Mukha et al. 2002) and the relative positions of these species deduced from morphological data (Roth 1985). These observations indicate that the ETS region may be well suited for resolving phylogenies of closely related cockroach species, especially at a within-genus level.

Intraspecific variability of ETS within *Blattella* species

Intra- and interpopulation heterogeneities in the *B. germanica* ETS structure were determined by comparison of a PCR-amplified rDNA fragment, located between primers *e* and *f* (Fig. 1 and double underlined sequences in Fig. 2). Five individual cockroaches from each of ten *B. germanica* populations from the United States, France, and Russia (four, two, and four populations, respectively) were used in the first part of our studies. Figure 5 (lane 1) shows a typical electrophoretic pattern of the amplifed fragments from one individual cockroach; all 50 individuals exhibited the same pattern. No variation was observed in the length of the main fragment (asterisk in Fig. 5). Moreover, one individual cockroach from each of the 10 *B. germanica* populations was used for the following analysis. The major PCR-amplified

Fig. 4. Approximate mapping of the rDNA promoter location. A radioactively labeled primer, located near the 5′ end of the 18S gene (underlined in Fig. 2), was annealed with the total RNA isolated from the three *Blattella* cockroach species (lane 1, *B. germanica*; lane 2, *B. lituricollis*; and lane 3, *B. asahinai*), followed by complementary DNA (cDNA) synthesis catalyzed by reverse transcriptase and then electrophoresis of the cDNA products in 6% polyacrylamide gel under denaturing conditions. The gel was covered with thin polyethylene film, frozen (–20 °C), and exposed with Kodak BioMax MR film in the dark.

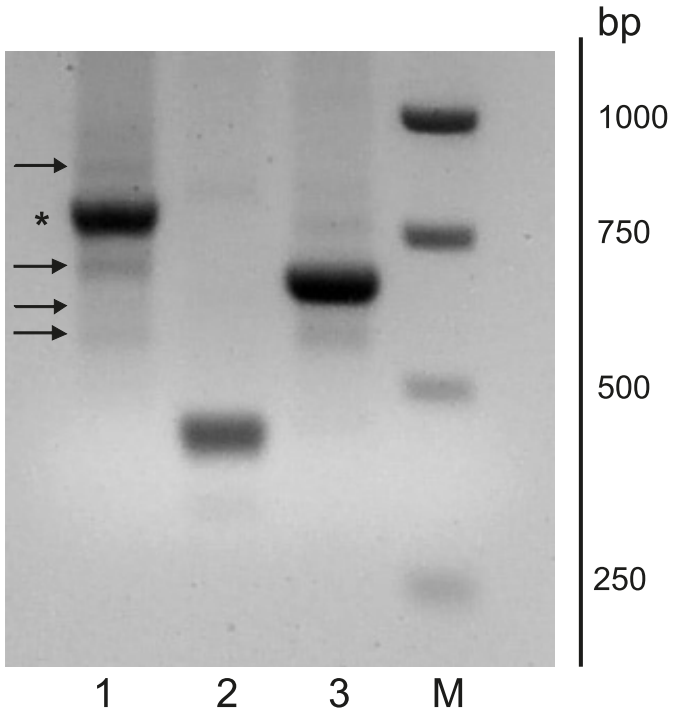


rDNA fragment, located between primers *e* and *f*, was extracted from the gel, purified, and sequenced. To further examine intraspecific variation in the ETS sequence of *B. germanica*, in addition to primers *e* and *f*, we used several additional primers, but oriented to the flanges of the analyzed DNA fragments. No nucleotide variation was detected (data not shown). That is, the ETS of *B. germanica* is a highly conserved, species-specifc region of the genome and the main member of the multigene rDNA family is the same in *B. germanica* individuals that originated in various geographically distinct populations.

More detailed analysis of the PCR-amplified products of the three *Blattella* species total DNA with primers *e* and *f* revealed faint fragments in addition to the main fragment, especially when the agarose gel was overloaded (in Fig. 5, arrows indicate additional *B. germanica* amplified fragments). We used the following approach to analyze structural features of the minor ETS variants of the three *Blattella* species: DNA fragments located above and below the main ETS fragment on a 1% agarose gel were purified from the gel, cloned into pGEM-T Easy Vector (Promega), and sequenced.

The sequences of five *B. germanica* ETS variants are shown in Fig. 6A. A comparison of the nucleotide sequence of the major and minor variants of *B. germanica* showed that the minor variants appeared to be derived from the major variant characteristic of this species by deletion of extended DNA regions consisting of a series of subrepeats. Moreover, the major and minor ETS variants of *B. germanica* appeared to be structurally related in a similar manner as the major ETS variants of the three *Blattella* species were related to each other (Figs. 2 and 6) (see Discussion).

Fig. 5. Agarose gel electrophoresis (0.7%) of the amplified external transcribed spacers of *B. germanica* (lane 1), *B. lituricollis* (lane 2), and *B. asahinai* (lane 3) with primers *e* and *f*. The major *B. germanica* ETS is indicated with an asterisk and minor ETS variants are indicated with arrows (see text). M, marker DNA.



Additionally, two minor ETS fragments of *B. asahinai* and three of *B. lituricollis* were cloned and sequenced. ClustalW2 alignments of the major (typical) and minor variants of the *B. asahinai* and *B. lituricollis* ETS fragments are shown in Figs. 6B and 6C, respectively.

The minor ETS fragments of *B. asahinai* differ from the major ETS in a few single nucleotide substitutions and a long deletion (Fig. 6B). At the same time, all analyzed minor ETS fragments of *B. asahinai* have a specific feature — two nucleotides (TG) within the first subrepeat indicated by purple background (Figs. 2A, 2B, and 6B) — that is characteristic for the major *B. asahinai* ETS fragment (see above). The sequenced *B. lituricollis* minor ETS variants are more similar to the major ETS of this species and differ from the major ETS in a few single nucleotide substitutions and a relatively short insertion (Fig. 6C). Unfortunately, nothing is known about the quantitative relationships between the various minor ETS variants for each of the investigated *Blattella* species, but the ETS variability within each of the *Blattella* species suggests that the minor ETS variants of each species were formed after speciation of the three *Blattella* species.

PCR-amplifcation of subrepeat-containing DNA fragments may potentially be accompanied by the formation of recombinant artificial PCR products as a result of annealing

Fig. 6. Intraspecific variability of ETS within *Blattella* species. (A) Nucleotide sequences of the five minor variants of the *B. germanica* ETS. Superscript letters designate nucleotide substitutions; dashes show deleted nucleotides revealed by comparison of each minor ETS variant with the major ETS. (B, C) ClustalW2 alignments of the major (typical) and minor variants of the *B. asahinai* and *B. lituricollis* ETS, respectively. Double underlined sequences indicate the primers *e* and *f* (Fig. 1) used for amplifcation of the ETS subrepeat region. Green, purple, blue, yellow, and grey backgrounds mark different types of subrepeats (similar to Fig. 2). Green letters mark the 5′ end of the 18S gene.

A. *Blattella germanica*

B. germanica ETS variant #1

GTGAGACTGAACCAAGTGTGTGCCTTCGGGCATGAATGAGTTGGGGCGTACGGTTCTAGCTTGTCTCGGCATATCTG
TCCGTATAGGATTGTGTGTGTGTGAGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCCTTCGGGC
ATGAATGAGTTGGGGCAATGCGTGGAAGGTTCGTCCGAGATATGGTCGATCCCGCTTGAGGCTGCAGAGCCGATGGA
CGGggGTAGaTCGGGAAAAGAATCCGACACGTACGGTTCTAGCTTGTCTCGGCATATCTGTCCGTATAGGATTGTGT
GTGTGTGAGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCGATCTAGTCTCTCCGCAAGGACCTT
TCCCGTCTCGAGATGTATTTCTTGTGTCCGGGGTCTATAGGTTTTCTTGTCGGCTGTCGGACTTTTTCTGTCGCAGA
GTATTAATGACTAGGTTGCCATGCGGGGCTTTTGTACCCGTGGCGCCTGTT^{a}GGAGCACGTGTAAAACAACGCACGAG
TTCCCTGGTTGATCCTGCCAGTAGTCATATG

B. germanica ETS variant #2

GTGAGACTGAACCAAGTGTGTGCCTTCGGGCATGAATGAGTTGGGGCGTACGGTTCTAGCTTGTCTCGGCATATCTG
TCCGTATAGGATTGTGTGTGTGTGAGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCGATCTAGT
CTCTCCGCAAGGACCTTTCCCGTCTCGAGATGTATTTCTTGTGTCCGGGGTCTATAGGTTTTCTTGTCGGCTGTCGG
ACTTTTTCTGTCGCAGAGTATTAATGACTAGGTTGCCATGCGGGGCTTTTGTACCCGTGGCGCCTGTT^{c}GGAGCACGT
GTAAAACAACGCACGAGTTCCCTGGTTGATCCTGCCAGTAGTCATATG

B. germanica ETS variant #3

GTGAGACTGAACCAAGTGTGTGCCTTCGGGCATGAATGAGTT^{t}GGGCAATGCGTGGAAGGTT·GTCCG^{c}GATATGGTC
GATCCCGCTTGAGGCTGCAGAGCCGATGGACGGGGGTAGATCG^{a}GAAAAGAATCCGACACGTACGGTTCTAGCTTGTC
TCG-CATATCTGTCCGTA--GGATTGTGTGTGTG--AGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTG
CG^{t}GATCTAGTCTCTCCGCAAGGACCTTTCCCGTCTCGAGATGTATTTCTTGT--CCGGGGT^{g}TATAGGTTTTCTTGT
CGGCTGTCGGACTTTTTCTGTCGCAGA^{c}TA^{c}TTAATGACTAGGTTGCCATGCGGG^{a}CTTTTG^{c}ACCCGTGGCGCCTGT
TCGGAGCACGTGTAA^{c}ACA^{g}CGCACGAGTTCCCTGG-TTGATCCTGCCAGTAGTCATATG

B. germanica ETS variant #4

GTGAGACTGAACCAAGTGTGTGCCTTCGGGCATGAATGAGTT^{t}GGGCAATGCGTGGAAGGTTCGTCCGAGATATGGTC
GATCCCGCTTGAGGCTGCAGAGCCGATGGACGGGGGTAGATCGGGAAAAGAATCCGACACGTACGGTTCTAGCTTGT
CTCGGCATATCTGTCCGTATAGGATTGTGTGTGTGAGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTG
TGCGCCTTCGGGCATGAATGA^{a}GTTGGGGCAATGCGTGGAAGGTTTGTCCGAGATATGGTCGATCCCGCTTGAGGCTG
CAGAGCCGATGGACGGGGGTAGATCGGGAAAAGAATCCGACACGTACGGTTCTAGCTTGTCTCG-CATATCTGTCCGT
A--GGATTGTGTGT^{c}TG--AGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCGATCTAGTCTCTCC
GCAAGGACCTTTCCCGTCTCGAGATGTATTTCTTGTGTCCGGGGTCTA--GGTTTTCTTGTCGGCTGTCGGACTTTTT
CTGTCGCAGAGTATTAATGACTAGGTTGCCATGCGGGGCTTTTG^{c}ACCCGTGGCGCCTGTTCGGAGCACGTGTAA^{c}AC
AACGCACGAGTTCCCTGGTTGATCCTGCCAGTAGTCATATG

B. germanica ETS variant #5

GTGAGAC(1)TGAACCAAGTGTG$_{T}$GCCTTCGGGCATGAATGAGTTAGGG(1)CGTACGGTTCTAGCTTGTCTCGGCAT
ATCTGTCCGTATAGGATTGTGTGTGTGTGAGAG(2)GTGGCCTGTGACCGACCCCACAAAGAGTGTACTC(1)TGTG$_{C}$
GCCTTCGGGCATGAATGAGTTGGGG(2)CAATGCGTGGAAGGTTCGTCCGAGATATGGTCGATCCCGCTTGAGGCTG
CAGAGCCGATGGACGGGGGTAGATCGGGAAAAGAATCCGACACGTACGGTTCTAGCTTGTCTCGGCATATCTGTCCG
TATAGGATTGTGTGTGTGTGAGAC(1')TGAACCAAGTG$_{T}$GCCTTCGGGCATGAATGAGTTGGGG(1')CAATGCGTG
GAAGGTTCGTCCGCGATATGGTCGATCCCGCTTGAGGCTGCAGAGCCAAGGACGGGGGTAGATCGGGAAAAAGAATC
CGACACGTACGGTTCTAGCTTGTCTCGCATATCTGTCCGTAGGATGGTGTGTGTGAGAC(4)GTGGCCTGTGACCGA
CCCCACAAAGAGTGTACTC(3)TGTGCGCGATTCTAGTCTCTCCGCAAGGACCTTTTCCCGTCTCGAGATGTATTTA
CTTGTCCGGGGTGTAGGTTTTTCTTGTCGGCTGTCGGACTTTTTCTGTCGCAGACTATTAATGACTAGGTTGCCATG
CGGGACTTTTTGCACCCGTGGCGCCTGTTCGGAGCACGTGTAACACAACGCACGAGTTTCCCTGGTTGATCCTGCCA
GTAGTCATATG

of a more extended, partly elongated single-stranded sequence, instead of a primer, during early amplifcation cycles (Bradley and Hillis 1997; Kupriyanova et al. 2004). Therefore, the detection of minor ETS variants in *Blattella* could be explained by PCR errors, and not by the occurrence of recombination in vivo. The differences in the structure of the major ETS variants of closely related *Blattella* species (Figs. 2 and 5), however, cannot be interpreted as the result of artifact amplifcations. Additionally, ETS variants 3 and 4 (Fig. 6) cannot be explained by formation of a recombinant sequence in vitro, because several subrepeats of these ETS variants and the adjacent regions contain multiple nucleotide substitutions. Our observation that the minor fragments 3 and 4 of the *B. germanica* ETS (Fig. 6) are identical with respect to the structure of subrepeats (except for several nucleotide substitutions) to the major ETS variant of *B. lituricollis* and *B. asahinai*, respectively, provides further argument against artificial PCR errors. Finally, the structure of ETS variant 5 (Fig. 6) can be explained only by recombination in vivo.

Fig. 6. (*continued*).

B. ***Blattella asahinai***

```
typical     GTGAGACTGAACCAAGTGTGCCTTCGGGCATGAATGAGTTGGGGCAATGCGTGGAAGGTT 60
minor#1     GTGAGACTGAACCAAGTGTGCCTTCGGGCATGAATGAGTTGGGGCAATGCGTGGAAGGTT 60
minor#2     GTGAGACTGAACCAAGTGTGCCTTCGGGCATGAATGAGTTGGGGCAATGCGTGGAAGGTT 60
            ************************************************************

typical     CGTCCGAGATACGGTCGATCCCGCTTGAGGCCGCAGAGCCGATGGACGGGGGTAGATCGG 120
minor#1     CGTCCGTGATATGGTCGATCCCGCTTGAGGCTGCAGAGCCGATGGACGGGGGTAGATCGG 120
minor#2     CGTCCGTGATA-------------------------------------------------- 71
            ****** ****

typical     GAAATGAATCCGACACGTACGGTTCTAGCTTGTCTCGGCATATCTGTCCGTATAGGATTG 180
minor#1     GAAAAGAATCCGACACGTACGGTTCTAGCTTGTCTCGGCATATCTGTCCGTATAGGATTG 180
minor#2     ------------------------------------------------------------

typical     TGTGTGTGTGAGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCCTTCG 240
minor#1     TGTGTGTGTGAGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCCTTCG 240
minor#2     ------------------------------------------------------------

typical     GGCATGAATGAGTTGGGGCAATGCGTGGAAGGTTCGTCCGAGATATGGTCGATCCCGCTT 300
minor#1     GGCATGAATGAGTTGGGGCAATGCGTGGAAGGTTCGTCCGAGATATGGTCGATCCCGCTT 300
minor#2     ---------------------------------------------TGGTCGATCCCGCTT 86
                                                           ***************

typical     GAGGCTGCAGAGCCGATGGACGGGGGTAGATCGGGAAAAGAATCCGACACGTACGGTTCT 360
minor#1     GAGGCTGCAGAGCCGATGGACGGGGGTAGATCGGGAAAAGAATCCGACACGTACGGTTCT 360
minor#2     GAGGCTGCAGAGCCGATGGACGGGGGTAGACCGGGAAAAGAATCCGACACGTACGGTTCT 146
            ***************************** ****************************

typical     AGCTTGTCTCGGCATATCTGTCCGTATAGGATTGTGTGTGTGTGAGACGTGGCCTGTGAC 420
minor#1     AGCTTGTCTCGGCATATCTGTCCGTATAGGATTGTGTGTGTGTGAGACGTGGCCTGTGAC 420
minor#2     AGCTTGTCTCGGCATATCTGTCCGTATAGGATTGTGTGTGTGAGACGTGGCCTGTGAC 206
            ************************************************************

typical     CGACCCCACAAAGAGTGTACTCTGTGCGCGATCTAGTCTCTCCGCAAGGACCTTTCCCGT 480
minor#1     CGACCCCACAAAGAGTGTACTCTGTGCGCGATCTAGTCTCTCCGCAAGGACCTTTCCCGT 480
minor#2     CGACCCCACAAAGAGTGTACTCTGTGCGCGATCTAGTCTCTCCGCAAGGACCTTTCCCGT 266
            ************************************************************

typical     CTCGAGATGTATTTCTTGTGTCCGGGGTCTATAGGTTTTCTTGTCGGCTGTCGGACTTTT 540
minor#1     CTCGGGATGTATTTCTTGTGTCCGGGGTCTATAGGTTTTCTTGTCGGCTGTCGGACTTTT 540
minor#2     CTCGAGATGTATTTCTTGTGTCCGGGGTCTATAGGTTTTCTTGTCGGCTGTCGGACTTTT 326
            **** ********************************************************

typical     TCTGTCGCAGAGTATTAATGACTAGGTTGCCATGCGGGGCTTTTGTACCCGTGGCGCCTG 600
minor#1     TCTGTCGCAGAGTATTAATGACTAGGTTGCCATGCGGGGCTTTTGTACCCGTGGCGCCTG 600
minor#2     TCTGTCGCAGAGTATTAATGACTAGGTCGCCATGCGGGGCTTTTGTACCCGTGGCGCCTG 386
            ************************** *****************************

typical     TTCGGAGCACGTGTAAAACAACGCACGAGTTCCCTGGTTGATCCTGCCAGTAGTCATATG 660
minor#1     TTCGGAGCACGTGTAAAACAACGCACGAGTTCCCTGGTTGATCCTGCCAGTAGTCATATG 660
minor#2     TTCGGGGCACGTGTAAAACAACGCACGAGTTCCCTGGTTGATCCTGCCAGTAGTCATATG 446
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Discussion

Models for the evolution of multigene families

Extensive theoretical and empirical evidence has accumulated over the last three decades in support of the idea that concerted evolution (meaning that all gene copies in the family are homogenized) is a major driving force in the evolution of multigene families (e.g., Dover 1982; Ohta 1980; Smith 1976). Our results, based on PCR amplification of the rDNA ETS region of *B. germanica* collected from various geographically distant populations, show consistency in the size of the amplified major ETS fragment. This is in agreement with the notion that concerted evolution is the main mechanism maintaining uniformity within members of the *Blattella* rDNA multigene family (Fig. 7A). The nucleolus organizing region, within which the rDNA multigene family is located, is positioned on a single chromosome, the X chromosome in *B. germanica* and one of the autosomes in *B. asahinai* (Ross 1988) (not known for *B. lituricollis*), which would significantly facilitate the process of progressive gene sequence homogenization. On the other hand, our finding that minor members of the multigene rDNA significantly differ from the main member in the same genome shows that the evolutionary history of these families must differ from that predicted by the concerted evolution model.

Because concerted evolution accounts for the lack of genetic variability among rDNA gene copies observed in many animal and plant species, it has been identified as the fundamental mode of rDNA multigene family evolution. However, while the majority of studies indeed support this model, some notable exceptions have been identified in fungi, apicomplexan protists, oak trees, flatworms, mollusks,

Fig. 6. (*concluded*).

C. *Blattella lituricollis*

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typical     GTGAGACTGAACCAAGTGTGCCTTCGGGCAAGAATGAGTTGGGGGCATTGCGTGGAAGGT 60
minor#1     GTGAGACTGAACCAAGTGTGCCTTCGGGCAAGAATGAGTTGGGGGCATTGCGTGGAAGGT 60
minor#2     GTGAGACTGAACCAAGTGTGCCTTCGGGCAAGAATGAGTTGGGGGCATTGCGTGGAAGGT 60
minor#3     GTGAGACTGAACCAAGTGTGCCTTCGGGCAAGAATGAGTTGGGGGCATTGCGTGGAAGGT 60
            ************************************************************

typical     TTGTCCGCTATATGGTCGATCCCGCTTGAGGCTGCAGAGCCGCTGGACGGGTCGATCGGG 120
minor#1     TTGTCCGCTATATGGCCGATCCCGCTTGAGGCTGCAGAGCCGCTGGACGGGTCGATCGGG 120
minor#2     TTGTCCGCTATATGGTCGATCCCGCTTGAGGCTGCAGAGCCGCTGGACGGGTCGATCGGG 120
minor#3     TTGTCCGCTATATGGTCGATCCCGCTTGAGGCTGCAGAGCCGCTGGACGGGTCGATCGGG 120
            *************** ******************************************

typical     AATATAATCCGACACGCACGGTTCTAGCTTGTCGCATATCTGTCCGTCCCTGTCGGGAGG 180
minor#1     AATATAATCCGACACGCACGGTTCTAGCTTGTCGCATATCTGTCCGTCCCTGTCGGGAGG 180
minor#2     AATATAATCCGACACGCACGGTTCTAGCTTGTCGCATATCTGTCCGTCCCTGTCGGGAGG 180
minor#3     TATATAATCCGACACGCACGGTTCTAGCTTGTCGCATATCTGTCCGTCCCTGTCGGGAGG 180
            ************************************************************

typical     ATTGTGTGTCTGACATGGCCTGTGACCGACCCCAGTGTACTCTTGTGCGTCTAGTCTCTC 240
minor#1     ATTGTGTGTCTGACATGGCCTGTGACCGACCCCAGTGTACTCTTGTGCGTCTAGTCTCTC 240
minor#2     ATTGTGTGTCTGACATGGCCTGTGACCGACCCCAGTGTACTCTTGTGCGTCTAGTCTCTC 240
minor#3     ATTGTGTGTCTGACATGGCCTGTGACCGACCCCAGTGTACTCTTGTGCGTCTAGTCTCTC 240
            ************************************************************

typical     CGCAAGGACCTTCC-------------------CCGACCGGATGTATTTCTTGTTTGGGG 281
minor#1     CGCAAGGACCTTCC-------------------CCGACCGGATGTATTTCTTGTTTGGGG 281
minor#2     CGCAAGGACCTTCCTCTCCCGCAAGGACCTTCCCCGACCGGATGTATTTCTTGTTTGGGG 300
minor#3     CGCAAGGACCTTCC-------------------CCGACCGGATGTATTTCTTGTTTGGGG 281
            ************** ************************** ***********************

typical     TCTAGGTTTTCTTGTCGGCTGTCGGACTTGTACTCGCAGAGTATGAATGACTAGGTTGCC 341
minor#1     TCTAGGTTTTCTTGTCGGCTGTCGGACTTGTACTCGCAGAGTATGAATGACTAGGTTGCC 341
minor#2     TCTAGGTTTTCTTGTCGGCTGTCGGACTTGTACTCGCAGAGTATGAATGACTAGGTTGCC 360
minor#3     TCTAGGTTTTCTTGTCGGCTGTCGGACTTGTACTCGCAGAGTATGAATGACTAGGTTGCC 341
            ************************************************************

typical     ATGCGGGGCTTTGCACCCGTGGCGCCTGTTCGGAGCACGTGTAACACAACGCACGAGTTC 401
minor#1     ATGCGGGGCTTTGCACCCGTGGCGCCTGTTCGGAGCACGTGTAACACAACGCACGAGTTC 401
minor#2     ATGCGGGGCTTTGCACCCGTGGCGCCTGTTCGGAGCACGTGTAACACAACGCACGAGTTC 420
minor#3     ATGCGGGGCTTTGCACCCGTGGCGCCTGTTCGGAGCACGTGTAACACAACGCACGTGTTC 401
            ************************************************** **** ****

typical     CCTGGTTGATCCTGCCAGTAGTCATATG 429
minor#1     CCTGGTTGATCCTGCCAGTAGTCATATG 429
minor#2     CCTGGTTGATCCTGCCAGTAGTCATATG 448
minor#3     CCTGGTTGATCCTGCCAGTAGTCATATG 429
            ****************************
```

and fish (Carranza et al. 1999; Carranza et al. 1996; Fujiwara et al. 2009; Gunderson et al. 1987; Rooney 2004; Rooney and Ward 2005; Vierna et al. 2009; Vierna et al. 2010). Other models, such as the birth-and-death model (Nei et al. 1997; Nei and Rooney 2005), for instance, have been proposed to explain the formation of new multigene families that differ from an ancestral species pattern.

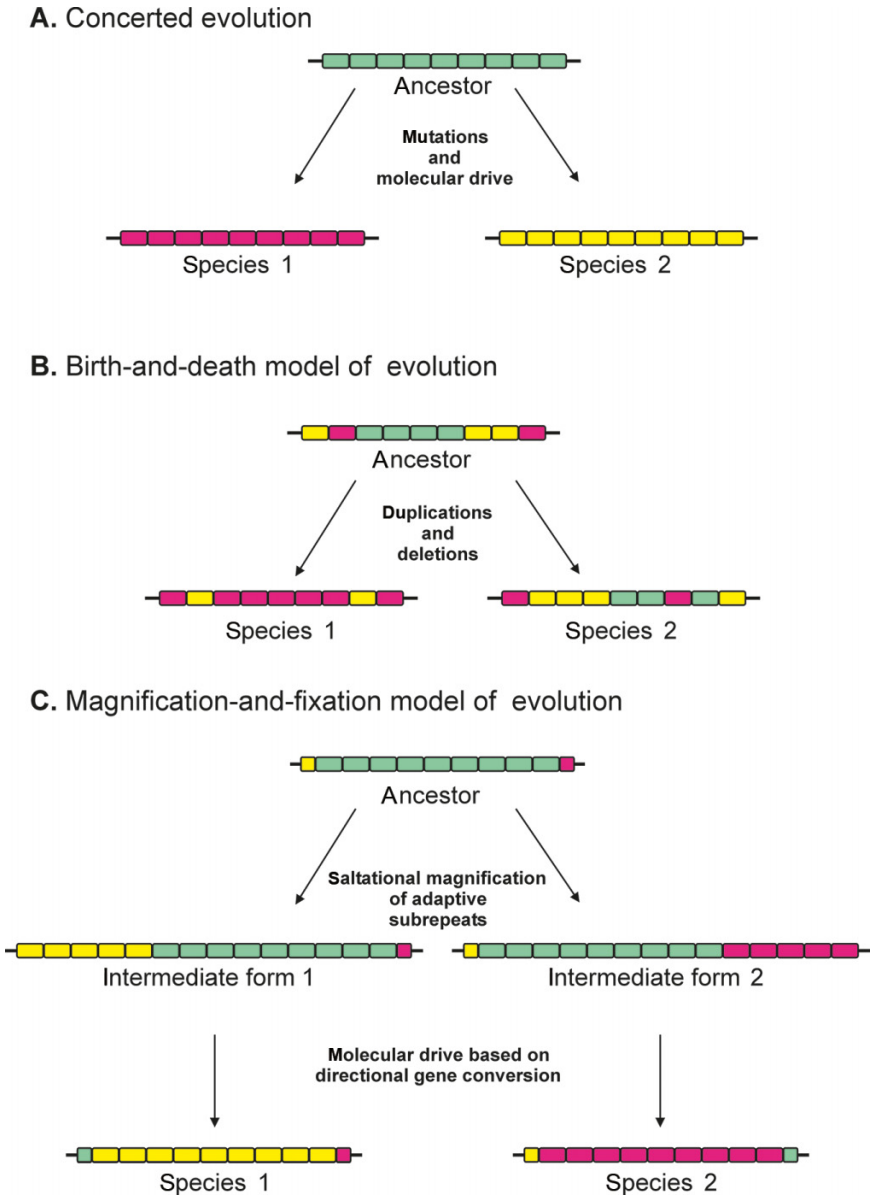
The birth-and-death model, proposed to explain the diversification of immune system genes, is also well supported by patterns of rDNA evolution in some eukaryote species, as well as evolution of other gene families, for example, histone genes (Eirín-López et al. 2009). However, the birth-and-death model fails to explain the evolution of *Blattella* rDNA (Fig. 7B). Our finding that the main member of the multigene rDNA family of *B. germanica* is the same in geographically distinct populations clearly shows that the evolutionary history of these families must differ from that predicted by the birth-and-death model.

A mixed process of concerted evolution, birth-and-death evolution, and selection (Freire et al. 2010; Nei and Rooney 2005) could reconcile the long-term evolution of *Blattella* rDNA. Although our results, in some measure, are consistent with this model of evolution, careful comparisons of the major and minor ETS variants of *Blattella* species allow us to suggest a different model, which, among other features, could also account for the rapid species adaptation to new ecological niches, as would be required for colonizing species such as *B. germanica*.

Relationships among major and minor ETS variants in three *Blattella* species

The structural differences between the major and minor ETS variants of *B. germanica* are similar to the differences observed between the major ETS variants of the three closely related *Blattella* species (Figs. 2 and 6). In all cases, the differences in the ETS structure consists of a deletion of some subrepeats; minor fragment 3 of *B. germanica* is nearly identical with the major ETS variant of *B. lituricollis*, and minor fragment 4 of *B. germanica* is nearly identical with the major ETS variant of *B. asahinai*. The formation of the minor ETS variants 1–4 (Fig. 6) could be explained by homologous recombination between the subrepeats of

Fig. 7. Schematic representation of three models of evolution of multigene families: (A) concerted evolution (after Dover 1982); (B) birth-and-death model of evolution (after Nei et al. 1997); and (C) magnification-and-fixation model of evolution (present study).



the major ETSs; the recombination can occur both at the level of the subrepeat arrays within the ETS, and at the level of the complete rDNA units (genes and spacers), between ETS subrepeats of adjacent rDNA units.

Minor ETS variant 5 of *B. germanica* (Fig. 6) contains, in our view, significant structural information. Comparative analysis of the structure of this fragment and the major ETS variant of *B. germanica* (Fig. 2A), suggests an interesting molecular mechanism responsible for formation of both the minor intraspecific ETS variants and the major ETS of the two closely related species. The sequence of variant 5 (Fig. 6) could be formed only by homologous recombination between subrepeats of the major *B. germanica* ETS (green and purple backgrounds, and the number 1 following each, in Fig. 2A), belonging to one rDNA repeat, and the corresponding subrepeats followed in Fig. 2A by the number 3, belonging to another rDNA repeat. According to this mechanism, subrepeats marked by green and purple backgrounds and the number 1 replace subrepeats marked by green and purple backgrounds and the number 3 of another rDNA repeat; the subrepeat denoted by yellow background followed by the number 2 is deleted. In sequence 5 (Fig. 6), the replaced green and purple subrepeats are followed by the number 1. Interestingly, a characteristic peculiarity of this recombination process is the deletion of the first two nucleotides (TG) of the purple subrepeat that is followed by the number 1 (Fig. 6).

The ETS corresponding to sequence 5 in Fig. 6 is not the main ETS variant within the three closely related *Blattella* species that we analyzed. However, the proposed recombination process involving variant 5 can also form the major ETSs of both *B. asahinai* and *B. lituricollis* from the main ETS variant of *B. germanica* or from a similar ETS of an ancestral species. Indeed, recombination between subrepeats

denoted by the number 1 and green and purple backgrounds (Fig. 2A) of one rDNA repeat unit and the corresponding subrepeats marked by the same background and the number 2 of another rDNA repeat unit would create a sequence corresponding to the main ETS of *B. asahinai*. Similarly, recombination between subrepeats of one rDNA repeat unit denoted by green and purple backgrounds and the number 1 (Fig. 2A) and the corresponding subrepeats of another rDNA unit marked by the same background and the number 3 will result in the sequence corresponding to the main ETS of *B. lituricollis*. Interestingly, within the ETS sequences of both *B. asahinai* and *B. lituricollis*, similar subrepeats (the first purple background) contain the previously noted deletion of the first two nucleotides (TG), providing additional evidence in favor of the recombination mechanism that we suggest for the origin of new ETS variants, preceding the formation of a new rDNA cluster of a new species.

Magnification-and-fixation: a new model for evolution of multigene families

The sequence data for the *Blattella* ETSs are still sparse, and sequences of major and minor ETS variants from other *Blattella* species will provide a more solid foundation for inferring ETS evolutionary history. Nonetheless, based on the current observations, we propose an alternative mechanism for the formation of a multigene family that differs from the ancestral form. It is based on the notion that saltational reorganization of rDNA could occur in response to selection for rapid adaptation to new ecological niches, especially in colonizing species, such as *B. germanica*. We entitle this model "magnification-and-fixation", to convey the two elements of the process (Fig. 7C). In support of this model, we elaborate on the following topics in the following paragraphs: (*i*) support for large scale saltational reorganization of rDNA; (*ii*) involvement of saltational magnification in formation of new multigene families; (*iii*) the functional importance of the ETS repeats and their involvement in regulation of rRNA expression; and (*iv*) features of polymorphic minor rDNA variants that would allow them to escape the homogenization process of concerted evolution and undergo saltational magnification.

Experimental evidence in support of large-scale rDNA reorganization has been obtained in *Drosophila* mutants with large deletions in the ribosomal genes cluster (Henderson and Ritossa 1970). At certain combinations of parental genotypes, heritable restoration of the rDNA repeat copy number was observed in germ-line cells of some first-generation progeny (Henderson and Ritossa 1970). Moreover, this restoration was effected by the magnification of one or several repeats, which lead to an increase in the number of repeated units and restoration of the multigene family characteristic of this species (Endow and Atwood 1988; Komma and Atwood 1994). Similarly, an investigation of the evolution of highly repetitive DNA families in hagfish (Eptatretidae) revealed that the repetitive DNA families arose as an initial magnification of certain ancestral subrepeats that subsequently evolved by saltatory replication and became exposed to a force of concerted evolution (Nabeyama et al. 2000).

In our opinion, experimental evidence for the existence of a genetic mechanism, by which magnification of some members of a multigene family is induced, is extremely important for understanding not only the principles underlying maintenance of the required number of repeats in multigene families, but also trends in formation of multigene families of a new species. Saltational magnification of minor rDNA subrepeats — for example, large-scale genetic reorganization of this genomic region in germ-line cells — could constitute a new mechanism of formation of an rDNA multigene family of a new species.

Recently, based on elegant experiments with yeast, it was shown that the high abundance of rRNA gene copies is important for maintaining genome integrity (Ide et al. 2010). A gene magnification system maintains large cluster(s) of tandemly repeated rDNA copies in the chromosome, with each species having a specific number of copies. Yeast, like other eukaryotes, has many extra untranscribed rDNA copies. When rDNA copy number was experimentally reduced, the low-copy-number yeast strains became sensitive to DNA damage induced by chemical mutagens and radiation. Moreover, this sensitivity was dependent on rDNA transcriptional activity, which interferes with cohesion between rDNA loci of sister chromatids. Ide et al. (2010) found that the extra rDNA copies facilitate condensin association and sister-chromatid cohesion, thereby facilitating recombinational repair. The authors suggest that high concentrations of heavily transcribed genes are toxic to the cells, and therefore magnified genes, such as rDNA, have evolved.

Ribosomes are important in protein synthesis, cellular growth, and organismal development, and because of high rates of transcription of ribosomal genes, rRNA accounts for 80% of the RNA content of growing cells (Moss and Stefanovsky 1995; Paule and Lofquist 1996). The repetitive regions within the NTS and ETS contain enhancers, silencers, and duplications of the core promoter, resulting in regulation of rDNA transcription. It has been assumed that the number of subrepeats within the ETS (i.e., ETS length) affects the rate of rRNA synthesis by affecting the promoter, which is located adjacent to this spacer sequence (Fig. 2A). Interestingly, *B. germanica*, a household pest with high growth and reproductive rates, has a longer ETS region than the other two species, which live outdoors. It would be interesting to know whether selection for certain life history traits (e.g., high growth rate, fast development time) would also select for higher frequency of longer ETS spacers. This hypothesis, and the relationship between ETS length and the rate of rRNA synthesis, will be tested experimentally in our future studies. Importantly, the minor ETS variants in *B. germanica* may represent not only material for further adaptation, evolution of new species, and formation of new variants of rDNA clusters, but also sequences for elucidation of the role and adaptive significance of ETS structure in regulation of rRNA expression.

It is reasonable to postulate that the process of homogenization of repeat sequences proceeds nonuniformly along the multigene family length, and that the flanks of the cluster are less involved in this process than sequences in the core region. In addition, it cannot be ruled out that orphons (rDNA fragments located beyond the cluster of ribosomal genes) may serve as a reservoir of sequences that lead to the evolvement of new functions and impact the evolution of the multigene family (Benevolenskaya et al. 1997; Guimond and Moss 1999; Wei et al. 2003). In these regions,

new structural variants that might have selective advantage under altered environmental conditions may be formed without significantly affecting the phenotype of the organism. The pattern of recombination across rDNA units that we invoke to explain both the major ETS sequences of the three *Blattella* species and the minor variants of *B. germanica* is expected to efficiently spread an adaptive ETS variant across the chromosome.

Selective saltational magnification and subsequent targeted gene conversion are two mechanisms that allow a new structural variant to become the major member of a multigene family or to form a new multigene family (Fig. 7C). At present, nothing is known about the molecular mechanisms by which magnification is induced. Even so, taking into account the above speculations, we hypothesize that two situations could be recognized by an organism and trigger a process that would bring about ribosomal gene magnification: (*i*) the number of adaptive rDNA repeats dramatically decreases and magnification restores the original repeat structure; or (*ii*) under new environmental conditions, major rDNA variants become nonadaptive while one minor variant is adaptive (e.g., green or purple in Fig. 7C) and it, in turn, becomes magnified.

Our magnification-and-fixation model is based on the idea that minor subrepeat types make rapid adaptation to new ecological niches possible, as would be required for colonizing species such as *B. germanica*. Under this model, selective saltational magnification of minor subrepeat types could occur in response to a rapid change in environmental conditions. Saltational magnification is then followed by targeted gene conversion and purifying selection, under which the magnified variant becomes the major member of the multigene family or it forms a new multigene family, while the ancestral major subrepeat type becomes a minor variant (Fig. 7C).

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