Extreme Length and Length Variation in the First Ribosomal Internal Transcribed Spacer of Ladybird Beetles (Coleoptera: Coccinellidae)

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DNA sequences of the first ribosomal internal transcribed spacer (ITS1) were isolated from 10 ladybird beetle species (Coleoptera: Coccinellidae) representing four subfamilies (Coccinellinae, Chilocorinae, Scymninae, and Coccidulinae). The spacers ranged in length from 791 to 2,572 bp, thereby including one of the longest ITS1s and exhibiting one of the most extreme cases of ITS1 size variation in eukaryotes recorded to date. The causes of length variation were therefore analyzed. Almost no putatively homologous sequence similarities were identified for the taxa included. The only exception was for the subfamily Coccinellinae, which yielded sequence similarities in six regions of approximately 550 nucleotide positions, primarily at the 5′ and 3′ ends of ITS1. The majority of differences in ITS1 length between taxa could be attributed to the presence of repetitive elements with comparatively long repeat units. Repetition arose several times independently and was confined to the middle of the spacer which, in contrast to the 5′ and 3′ ends, had not been inferred in previous studies to be subject to functional constraints. These elements were characterized by high rates of evolutionary change, most likely as a result of high substitution rates in combination with inefficient homogenization across repeats. The repeated origin and subsequent divergence of ''long'' repetitive elements should thus be assumed to be an important factor in the evolution of coccinellid ITS1.

Introduction

The first ribosomal transcribed spacer (ITS1) is part of the eukaryotic cistron of ribosomal DNA, located between the genes coding for 18S and 5.8S rRNA. This spacer has become a popular marker for phylogenetic studies of closely related species of animals, plants, and fungi because it shows a comparatively high rate of evolution due to its noncoding structure and can be easily isolated via PCR from almost any taxon, using conserved primers in the adjacent coding regions (e.g., Hillis and Dixon 1991; Bakker, Olsen, and Stam 1995; Buckler and Holtsford 1996; Gouliamova and Hennebert 1998). Interestingly, these studies revealed that the pace of ITS1 sequence evolution does not seem to be uniform either across the spacer region or for different taxonomic groups.

In the majority of metazoans, ITS1 elements generally provide sufficient levels of nucleotide variation for phylogenetic inferences below the species or genus level (e.g., Schlötterer et al. 1994; Bowles, Blair, and McManus 1995; McLain et al. 1995; Miller, Crabtree, and Savage 1996; Blair et al. 1997; Hugall, Stanton, and Moritz 1999; Schilthuizen et al. 1999; Schizas et al. 1999; Harris and Crandall 2000). However, the extents of variation often differ between closely related taxa (e.g., Miller, Crabtree, and Savage 1996; Blair et al. 1997; Harris and Crandall 2000). Moreover, in some groups, this spacer seems to be too conserved for resolving phylogenetic relationships within genera or even subfamilies (e.g., Kuperus and Chapco 1994; Booton et al. 1999). Although the observed differences in the extent of sequence variation between taxonomic groups may have simply resulted from differences in their evolutionary histories (e.g., speciation rates), it is worth noting that they may also reflect different constraints at the molecular level.

ITS1 sequence variation also seems to differ across the spacer region. Throughout the whole subclass Digenea (Platyhelminthes), ITS1 elements have been found to be conserved at the 3′ end, whereas complete spacer sequences could only be aligned with confidence for closely related taxa of the same genus or family (von der Schulenburg, Englisch, and Wägele 1999). Similar findings have also been made for drosophilid dipterans (Insecta) (Schlötterer et al. 1994). In both cases, 3′-end sequence conservation indicated the presence of functional constraints, supported by studies of yeast for which the 3′ end of ITS1 contains recognition sites and conserved secondary structure motifs which have all been found to be essential for rRNA maturation (e.g., Henry et al. 1994; van Nues et al. 1994; Weaver, Sun, and Chang 1997).

In addition, ITS1 evolution seems to be shaped by internal repetition, leading to ITS1 size variation. This repetition includes repetitive elements with comparatively long repeat units, e.g., in trematodes (Platyhelminthes) and dipterans (Paskewitz, Wesson, and Collins 1993; Kane et al. 1996; Tang et al. 1996; van Herwerden, Blair, and Agatsuma 1998, 1999), or, more commonly, ''simple'' repetitive sequence motifs, as in various arthropods and in humans (Gonzalez et al. 1990; Kwon and Ishikawa 1992; Wesson, Porter, and Collins 1992; Wesson et al. 1993; Kuperus and Chapco 1994;

Key words: Coccinellidae, concerted evolution, internal transcribed spacer, repetitive DNA, ribosomal DNA.

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Mol. Biol. Evol. 18(4):648–660. 2001

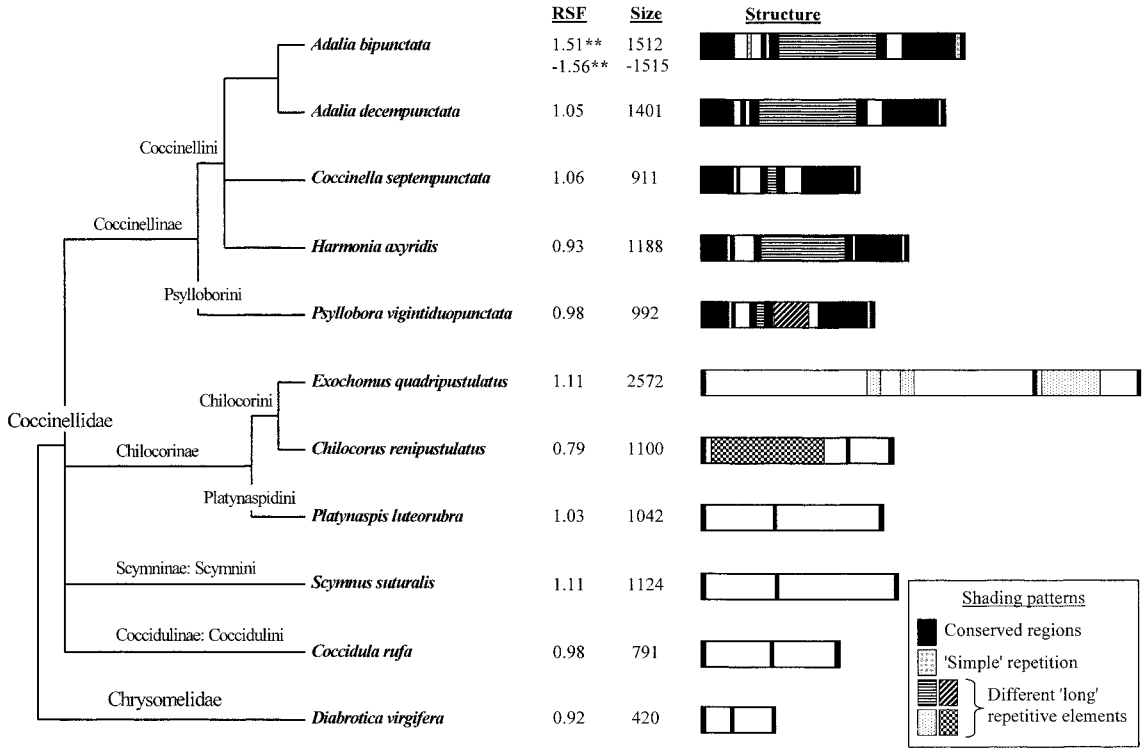


FIG. 1.—Phylogenetic tree of the taxa included in this study and information on the respective ITS1 elements. The phylogeny and classification of the different coccinellid species studied follows Kovář (1996). The tree includes the chrysomelid beetle, *Diabrotica virgifera*, as an outgroup taxon. The relative simplicity factor (RSF), as calculated with the program SIMPLE34 (Hancock and Armstrong 1994), is used as a measure of ''simple'' repetition. Two stars indicate that the RSF value is significantly greater than 1.0 (1% significance level). The size of ITS1 is given in base pairs. For illustration of ITS1 structure, spacers are represented as horizontal bars in proportion to their lengths. Shaded areas denote the presence and approximate position of conserved sequence regions, ''simple'' repeat elements, and ''long'' repetitive elements, as indicated.

Vogler and DeSalle 1994; McLain et al. 1995; Miller, Crabtree, and Savage 1996; Fenton, Malloch, and Moxey 1997; Remigio and Blair 1997; Kumar, Fenton, and Jones 1999; Harris and Crandall 2000). In these cases, evolution of repetitive sequences is subject to molecular processes such as replication slippage, unequal crossing over, and biased gene conversion, which are involved in the generation of length variation and/or lead to concerted evolution (e.g., Dover 1982; Levinson and Gutman 1987; Elder and Turner 1995).

ITS1 evolution may also be affected by the extent of homogenization between repeat units of the ribosomal cistron. Inefficient homogenization between repeats, particularly between those on different chromosomes, may then result in intraindividual ITS1 heteroplasmy, as hypothesized for some insects, crustaceans, and trematodes (Wesson, Porter, and Collins 1992; Vogler and DeSalle 1994; van Herwerden, Blair, and Agatsuma 1998; Harris and Crandall 2000; see also Schlötterer and Tautz 1994).

Consequently, the evolution of these spacers seems to be characterized by a variety of different factors. The respective underlying evolutionary forces and their relationship to each other are as yet poorly understood. Such information should be of value for our general understanding of the evolution of transcribed spacer DNA and is additionally expected to be of importance in employment of ITS1 as a reliable marker in phylogenetic studies.

The discovery of extreme length and length variation of ITS1 in ladybird beetles (Coleoptera: Coccinellidae) gave us an ideal opportunity to extend our current knowledge on the evolutionary dynamics of these spacers. Using a comparative analysis of the complete range of ITS1 size classes from taxa with different degrees of relatedness, we specifically addressed the following questions: What is the origin of extreme length and length variation? Is it associated with previously identified sources of length variation such as ''simple'' repetition or long repeat elements? To what extent is a high degree of length variation reflected by a high rate of sequence evolution? Is there evidence for functional constraints on ITS1 evolution? How do such constraints relate to the factors creating length variation?

Materials and Methods

ITS1 rDNA was analyzed from single individuals of a variety of coccinellid species of different subfamilies and tribes, as illustrated in figure 1. For the two-spot ladybird beetle, *Adalia bipunctata*, we additionally compared DNA sequences from three specimens from different populations: Cambridge, England; Berlin, Germany; and Moscow, Russia. The analysis also included

Table 1
Internal ITSI Sequencing Primers

Code	Sequence (5′→3′)	Taxa[a]
ABFF1	CGCTCGGGTACCTGGAATCG	*Adalia* species
ABRR3	GACGAAAGACAGACGTCTCC	*Adalia* species, *Coccinella septempunctata*
ABRF4	CGAGGTGTTGGAGACGTCTG	*Adalia* species, *C. septempunctata*
ABFR5	ATGTACATCGATATCAACACA	*Adalia* species
ABFF6	AGGTGAAGGCTAGCGTCCACG	*Adalia* species
HA65R	TCATTTCGTGAGCATCTCTAA	*Harmonia axyridis*
HA66F	TAGGTCCATGTTTAGAGATGC	*H. axyridids*
PV450F	CTAGCCGTCTTCCGTGTCGC	*Psyllobora vigintiduopunctata*
PV450R	GCGACACGGAAGACGGCTAG	*P. vigintiduopunctata*
EQFF1	CGTTTCTCTGATACTACCAG	*Exochomus quadripustulatus*
EQFR2	TAGTATCAGAGAAACGTCGA	*E. quadripustulatus*
EQRF3	CGTAAACGGCAATTCTTGTC	*E. quadripustulatus*
EQRR4	GACAAGAATTGCCGTTTACG	*E. quadripustulatus*
EQFF5	TGGTCAATTCACATACATGTC	*E. quadripustulatus*
EQFR6	CAGACATGTATGTGAATTGAC	*E. quadripustulatus*
EQRR7	TCGAGCGAACAATGCGTGGAC	*E. quadripustulatus*
EQRF8	GTCCACGCATTGTTCGCTCGA	*E. quadripustulatus*
CH400F	GGTGTTGAATTGAGCGTGCGC	*Chilocorus renipustulatus*
CH800R	CGACATTGTGATAGCCGATGC	*C. renipustulatus*
PL550R	AAGACGGTGGACGAATCGACA	*Platynaspis luteorubra*
PL680F	GTCCTTTGCGCTGCGGTTCGA	*P. luteorubra*
SS750F	GAACGTACTTAACTGACGTG	*Scymnus suturalis*
SS750R	CACGTCAGTTAAGTACGTTC	*S. suturalis*
CR500F	CGTAACGATCGTTCATTCGA	*Coccidula rufa*
CR500R	CGTTCCGTTCGAATGAACGA	*C. rufa*

[a] Taxa for which primers were used.

the previously published ITS1 sequence of another polyphagous beetle, *Diabrotica virgifera* (Coleoptera: Chrysomelidae; GenBank accession number AF155570; Szalanski et al. 1999). All ITS1 sequences obtained in the course of the present study have been deposited in the EMBL data bank under accession numbers AJ272139–AJ272150.

Total genomic DNA was isolated using a CTAB-based protocol (e.g., Winnepenninckx, Backeljau, and De Wachter 1993; Shahjahan et al. 1995). Whole ladybird specimens were ground in liquid nitrogen, digested overnight at 50°C in 250 μl 2% CTAB buffer (2% w/v CTAB, 0.1 M Tris-HCl [pH 8.0], 0.02 M EDTA, 1.4 M NaCl, 0.5% v/v β-mercaptoethanol, 10 mg/ml proteinase K). DNA was extracted with 2 volumes of chloroform : isoamylalcohol (24:1), precipitated with 2/3 volumes isopropanol, incubated for 1 h at −20°C, and subsequently centrifuged at 13,000 rpm for 30 min. The DNA pellet was washed in 70% ethanol, air-dried, and resuspended in 50–100 μl sterile Millipore H_2O. Ribosomal ITS1 elements were subsequently PCR-isolated from total genomic DNA using the Expand High Fidelity PCR System (Boehringer-Mannheim Ltd.) and the universal invertebrate ITS1 primers BD1 and 4S (e.g., Bowles and McManus 1993; Remigio and Blair 1997). The reaction was controlled with a Progene Thermal Cycler (Techne Ltd.) using the following PCR profile: 2 min at 95°C, followed by 10 cycles of 20 s at 95°C, 30 s at 55°C, and 1–2 min at 72°C, followed by another 20 cycles of 20 s at 95°C, 30 s at 55°C, and 1–2 min at 72°C with an additional 15 s each cycle, and a final extension period of 10 min at 72°C (see instructions to the Expand High Fidelity PCR System, Boehringer-Mannheim Ltd.). Resulting PCR products were purified with Microcon-50 microconcentrators (Amicon Ltd.), and cloned via TA-cloning using the pGEM-T Vector System (Promega Ltd.), chemical transformation of *Escherichia coli* DH5α, and conventional techniques for the identification of recombinants (e.g., Sambrook, Fritsch, and Maniatis 1989). Note that after transformation, cells were recovered for no more than 45 min to prevent duplication of cells prior to plating out on agar plates and thus to ensure a different origin of the resulting colonies. Plasmids containing the insert were purified with the Wizard Minipreps DNA Purification System (Promega Ltd.) and sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Kit, with visualization of results on an ABI Prism 377 DNA Sequencer (Perkin Elmer Ltd.). Sequences were obtained for both strands with the help of pUC/M13 forward and reverse primers and several internal ITS1 primers (table 1). Three independent clones were sequenced for each ladybird specimen. Nucleotide differences between clones never exceeded the level of variation expected from *Taq* polymerase error rates (8.5×10^{-6}; Expand High Fidelity PCR system, Boehringer Mannheim). Majority-rule consensus sequences were therefore generated for each specimen for subsequent analyses.

The program BLAST (Altschul et al. 1990) was employed to identify similarities between the isolated sequences and previously published data. The presence and extent of simple repetition was analyzed following the method of Tautz, Trick, and Dover (1986), using the program SIMPLE34 (Hancock and Armstrong 1994).

Putatively homologous sequence similarities between and within coccinellid ITS1 sequences were analyzed using three approaches: (1) Dotplot analysis was employed to visualize sequence similarities. We used the

program DOTTYPLOT (Gilbert 1990) with a window size of 35 and a stringency of 19, in accordance with previous studies of length-variable sequences (e.g., Hancock and Dover 1988). (2) Sequence similarities were assessed using progressive sequence alignment algorithms. For this purpose, alignments were generated with the program CLUSTAL W (Thompson, Higgins, and Gibson 1994), using a range of gap open and gap extension penalties. (3) Previous studies highlighted that ITS1 elements contain conserved secondary structure motifs, some of which are likely to be of functional importance (cf. von der Schulenburg, Englisch, and Wägele [1999] and references therein). Therefore, further support for putative homologies in primary nucleotide sequence was sought via identification of such conserved secondary structures. For this purpose, secondary structures of complete ITS1 elements were calculated using the program MFOLD (Zuker 1989; see also discussion on relevance of inferred secondary structures as an indication of sequence homologies in Kjer [1995] Hancock and Vogler [1998]). Final alignments were then produced taking into consideration the results obtained in the above analyses.

Phylogenetic analysis was performed on putatively homologous ITS1 sequence regions using maximum likelihood as implemented in the program PAUP*, version 4.0b4a (Swofford 1999). Phylogenetic trees were reconstructed with the heuristic search modus (branch swapping by tree bisection and reconnection) and random addition of sequences based on 10 repetitions. Tree estimation was repeated using a variety of different substitution models. These all yielded the same tree topology. The utility of different substitution models was subsequently assessed with the program MODELTEST (Posada and Crandall 1998), following the strategy outlined by Huelsenbeck and Crandall (1997). The general time-reversible model including rate heterogeneity across sites (Rodríguez et al. 1990; Yang 1996) was identified as the best-fitting model. It was therefore employed for estimation of branch lengths and assessment of the robustness of the inferred topology. The latter was performed via nonparametric bootstrapping (Felsenstein 1985) using the heuristic search modus (branch swapping by tree bisection and reconnection), random addition of sequences with 10 repetitions, and 100 bootstrapped data sets. All alignment illustrations were created with the program GENEDOC, version 2.6.001 (Nicholas and Nicholas 1997), and phylogenetic trees were created with the program TREEVIEW, version 1.5.2 (Page 1996).

Results

General

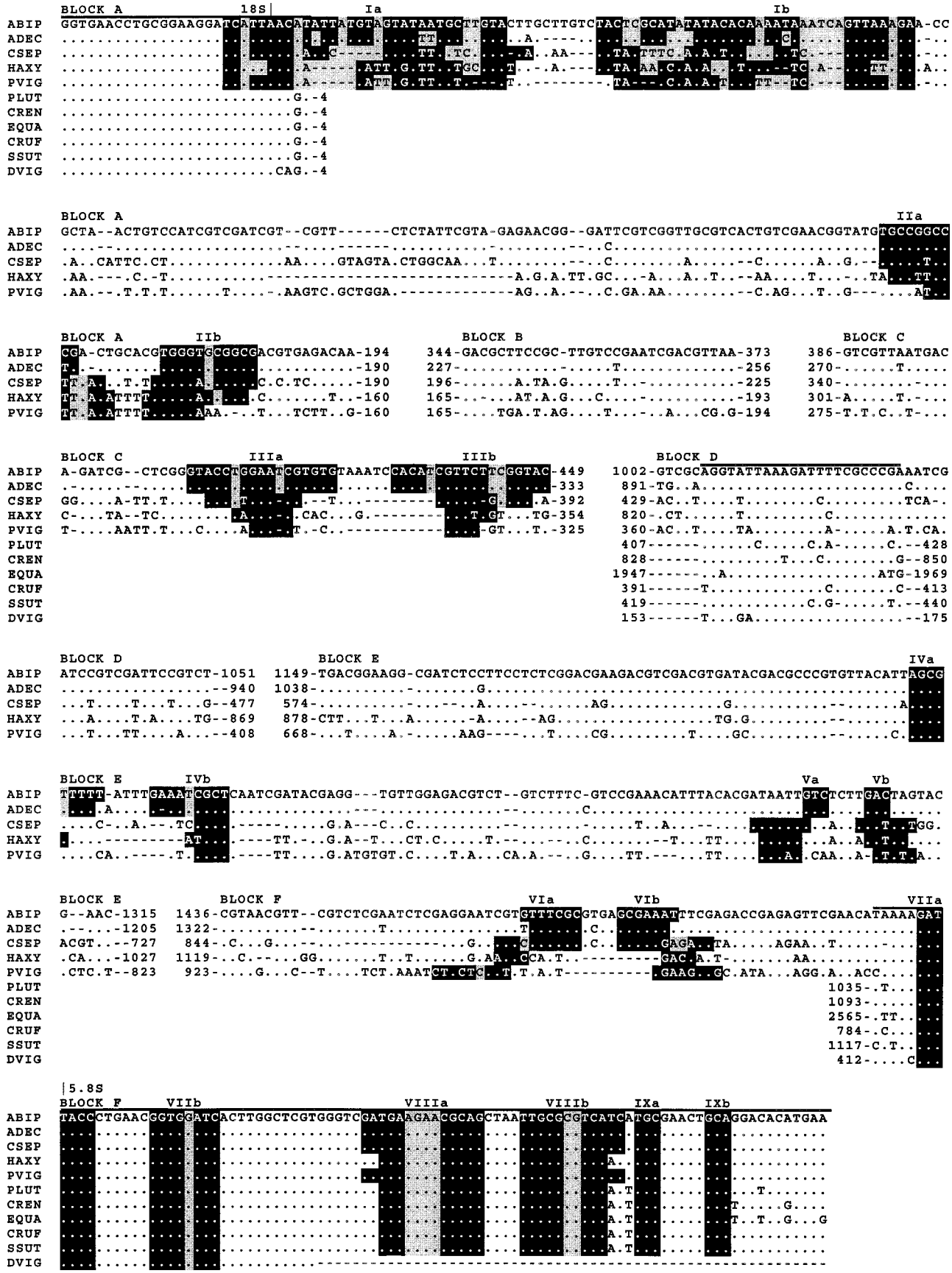
ITS1 elements of ladybird beetles ranged in size between 791 and 2,572 bp (fig. 1). They were all larger than previously studied ITS1s of other polyphagous beetles, e.g., *D. virgifera*, which all showed lengths of $<$550 bp (Szalanski et al. 1999; see also ITS1 sequence from additional chrysomelid beetles that are currently available only from the EMBL/GenBank database). Size variation between coccinellid species, including closely related taxa of the same subfamily or tribe, was extraordinarily high. In contrast, intraspecific variability was low. A PCR assay on multiple specimens of the species considered, collected from different populations across Europe, indicated no major size variation within species (results not shown). This finding was supported by sequence analysis of ITS1 elements from three *A. bipunctata* specimens, which were derived from different populations (Cambridge, Berlin, and Moscow) and showed a maximum size difference of only 3 bp.

There was no unambiguous indication of intraindividual ITS1 sequence variation, as the extent of nucleotide differences between the three clones, sequenced for each specimen, was consistent with the error rate of the *Taq* polymerase used (see *Materials and Methods*). Base compositions of coccinellid ITS1 sequences were highly similar, ranging from 47% A+T in both *Coccidula rufa* and *Scymnus suturalis* to 56% A+T in *Psyllobora vigintiduopunctata* (21%–26% A, 23%–29% G, 25%–31% T, 19%–25% C). However, differences were generally larger between coccinellids and the chrysomelid beetle, *D. virgifera*, which has a base composition of 70% A+T (33% A, 15% G, 37% T, 15% C).

Finally, analysis based on the BLAST algorithm revealed that nucleotide similarities between the isolated sequences and published data are present only in the included part of the 5.8S rRNA gene. Further similarities to any other previously published sequences could not be identified.

DNA Sequence Similarity Between ITS1 Elements

ITS1 sequence regions from different taxa were considered putatively homologous only if they were (1) consistently identified by progressive sequence alignment programs under a range of gap open and extension penalties and (2) indicated by dotplot analysis. Using this strategy, putatively homologous sequence similarities both between all taxa considered and between the different coccinellid species (excluding *D. virgifera*) could be inferred for only three regions (figs. 1 and 2): (1) the 8 bp of the adjacent 18S rRNA gene and 4 bp at the 5′ end of ITS1, (2) about 20 bp in the middle of the spacer (block D in fig. 2), and (3) 87 bp of the adjacent 5.8S rRNA gene and 8 bp at the 3′ end of ITS1. With one exception, further interspecific ITS1 sequence homologies could not be ascertained in any subsets of the data, e.g., closely related taxa such as the members of the subfamily Chilocorinae, or those with similar ITS1 sizes. The only exception was for the subfamily Coccinellinae, which showed a high degree of sequence similarity at about 550 nucleotide positions in six regions within the ITS1 element (figs. 1 and 2): one at the 5′ end ($\sim$190 bp, block A), two at the 3′ end ($\sim$150 bp and $\sim$70 bp for blocks E and F, respectively), and three small dispersed regions of 30–50 bp in the middle of the spacer (blocks B–D). An additional conserved sequence region corresponds to a repetitive element and will be dealt with below. Secondary-structure calculations, based on complete ITS1 and the adjacent partial


BLOCK A
18S
Ia
Ib
IIa
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BLOCK B
BLOCK C
IIIa
IIIb
BLOCK D
BLOCK E
IVa
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BLOCK F
VIa
VIb
VIIa
VIIb
VIIIa
VIIIb
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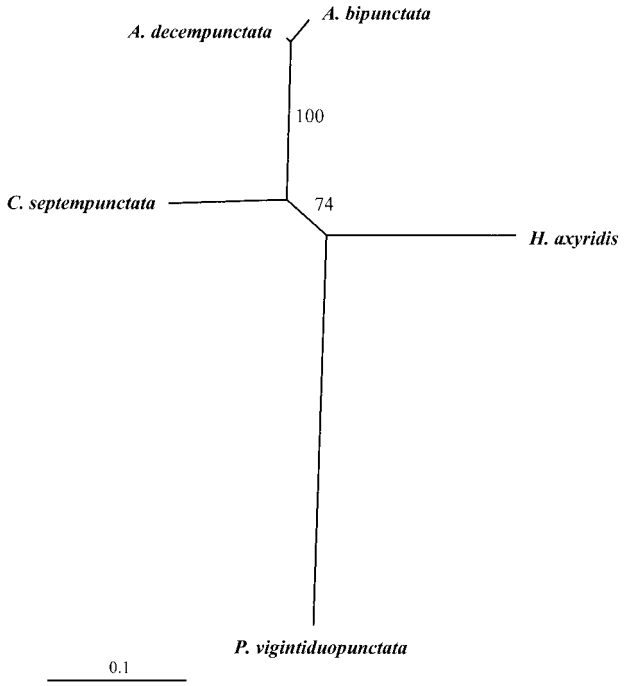

FIG. 3.—Unrooted maximum-likelihood tree topology inferred from the six conserved ITS1 sequence regions of the Coccinellinae. The tree shown was inferred with maximum likelihood as implemented in the program PAUP*, version 4.0b4a (Swofford 1999), using a heuristic search (branch swapping by tree bisection and reconnection), random addition of sequences with 10 repetitions, and the general time-reversible model including substitution rate heterogeneity across sites (four discrete gamma rate categories). The tree had a log likelihood of −2,268.32. Branches are drawn in proportion to the estimated number of substitutions (see bar in bottom left corner). Numbers next to branches refer to the results of bootstrap analysis (100 replicates).

18S and 5.8S rRNA genes, indicated the presence of conserved structural elements in four of these regions (blocks A, C, and E–F), further supporting homology of the observed sequence similarities in these regions.

For the six conserved blocks, the proportion of nucleotide differences ranged from 2.3% to 33.0% (ignoring indels in pairwise comparisons only). The highest degree of divergence was found between *P. vigintiduopunctata* and either *Adalia bipunctata, Adalia decempunctata,* or *Harmonia axyridis* (>30% sequence dissimilarity) and thus always concerned comparisons between taxa of different tribes (Coccinellini, Psyllobori-ni). The two Adalia species produced the highest similarity score (2.3% nucleotide differences). In fact, their ITS1 elements could be aligned without ambiguities for the complete length of the spacer. Moreover, almost complete sequence conservation was observed for the ITS1 of *A. bipunctata* specimens from three different populations which showed no more than three nucleotide changes and two single copy number differences at two ''simple'' repetitive sequence regions (see below). Finally, the conserved sequence regions were shown by phylogenetic tree reconstruction procedures (based on maximum likelihood) to produce unambiguous support for two clades within the Coccinellinae (bootstrap values of >70; fig. 3).

Presence of Internal Repetition

Dotplot analysis indicated the presence of repetitive elements with comparatively long repeat units for six coccinellid species. Both Adalia species bore seven tandemly repeated copies of a ~80-bp-long sequence motif (fig. 4*A*). *Harmonia axyridis* had a repeat element present in eight copies of ~60 bp (fig. 4*A*). *Psyllobora vigintiduopunctata* ITS1 contained three copies of a 26–108-bp-long sequence motif (fig. 4*B*). For *Chilocorus renipustulatus*, three equally long copies of 212 bp were found (fig. 4*C*), and *Exochomus quadripustulatus* contained four tandemly repeated and two dispersed copies, all with sizes of ~80 bp (fig. 4*D*). A high degree of sequence similarity was observed between the repeat elements of *A. bipunctata, A. decempunctata, H. axyridis,* and a single-copy motif within ITS1 of *Coccinella septempunctata* and *P. vigintiduopunctata* (figs. 1, 4, and 5). These repetitive elements and their single-copy counterparts were found at the same relative positions within ITS1. Interspecific sequence similarities could not be identified for any of the other three repetitive elements, which were also present at different relative positions within the spacer (fig. 1).

Sequence divergence between repeats varied both across the repeat unit and between species. *Chilocorus renipustulatus* was the only taxon to produce a highly homogenous distribution of variation across repeats. All other species showed considerable variation. Their respective repeat units nevertheless always contained at least one more conserved sequence region. In certain cases, this finding was also supported by the presence of secondary-structure motifs, found at about the same positions within the repeats in the course of secondary-structure analysis of complete ITS1 elements (fig. 4). Sequence divergence between repeats was computed for the alignments given in figure 4 ignoring gaps in pairwise comparisons only. As the more variable regions could not be aligned with absolute confidence, and as the respective repeat units varied in length between species, the calculated sequence divergence scores were

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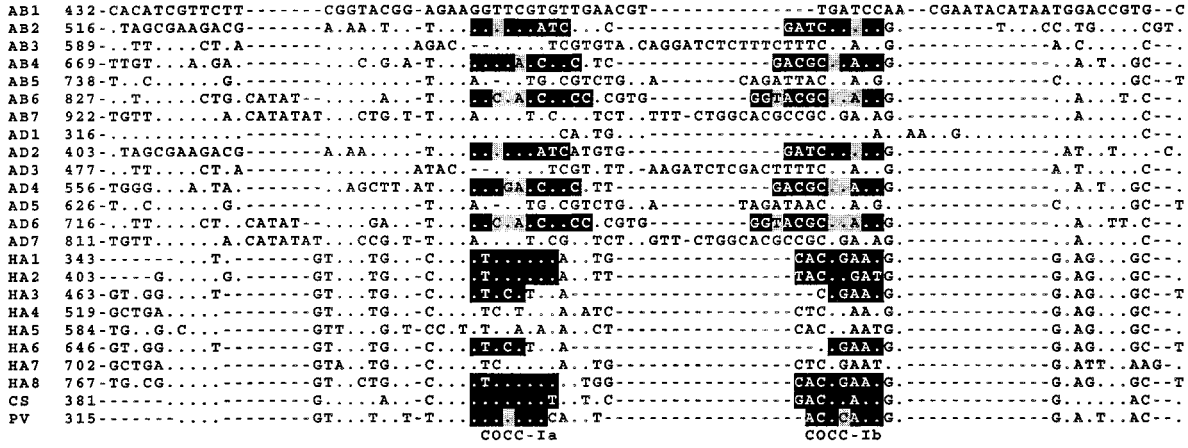
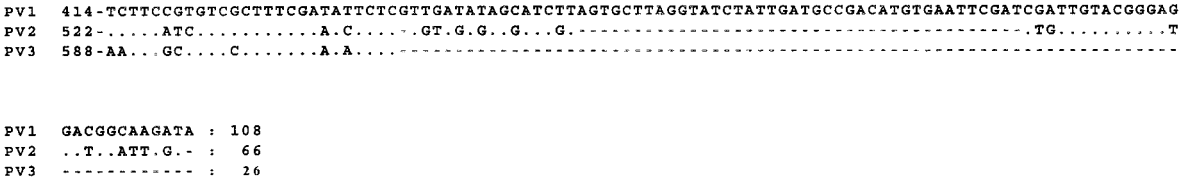
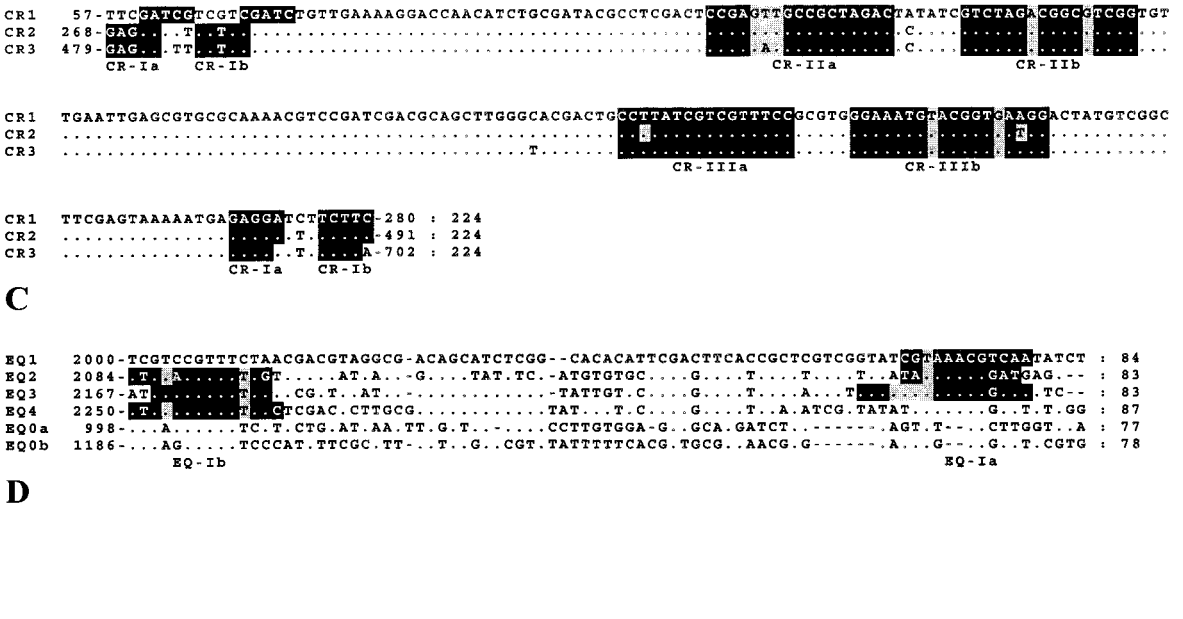
FIG. 2.—Alignment of the six conserved sequence regions of coccinellid ITS1 (blocks A–F). Taxa are indicated with a four-letter code: ABIP = *Adalia bipunctata*; ADEC = *Adalia decempunctata*; CSEP = *Coccinella septempunctata*; HAXY = *Harmonia axyridis*; PVIG = *Psyllobora vigintiduopunctata*; PLUT = *P. luteorubra*; CREN = *Chilocorus renipustulatus*; EQUA = *Exochomus quadripustulatus*; CRUF = *Coccidula rufa*; SSUT = *Scymnus suturalis*; DVIG = *Diabrotica virgifera.* Dots denote identity to the top sequence, and dashes indicate alignment gaps. Numbers at the beginning and end of each block refer to the start and end positions of the conserved regions within each particular ITS1. Roman numerals I–IX denote secondary-structure elements which are found to be conserved between species. Black and gray shadings indicate stem regions (involved in hydrogen bonding) and internal stem loops, respectively. The putative end and beginning of 18S and 5.8S rDNA are indicated by vertical lines, preceded or followed by the respective name of the gene.


COCC-Ia
COCC-Ib

A



B


CR-Ia
CR-Ib
CR-IIa
CR-IIb
CR-IIIa
CR-IIIb

C

EQ-Ib
EQ-Ia

D

	AB2	AB3	AB4	AB5	AB6	AB7	AD1	AD2	AD3	AD4	AD5	AD6	AD7	HA1	HA2	HA3	HA4	HA5	HA6	HA7	HA8	CS	PV
AB1	0.46	0.39	0.43	0.39	0.44	0.42	0.10	0.50	0.38	0.48	0.40	0.44	0.44	0.44	0.42	0.53	0.52	0.54	0.51	0.54	0.45	0.22	0.33
AB2		0.48	0.48	0.62	0.55	0.58	0.48	0.22	0.45	0.50	0.60	0.58	0.56	0.52	0.55	0.56	0.51	0.60	0.57	0.63	0.58	0.38	0.45
AB3			0.49	0.46	0.49	0.57	0.37	0.48	0.13	0.52	0.46	0.49	0.57	0.45	0.46	0.50	0.49	0.59	0.52	0.58	0.51	0.30	0.40
AB4				0.52	0.38	0.34	0.40	0.57	0.49	0.16	0.52	0.39	0.34	0.44	0.44	0.45	0.45	0.46	0.47	0.52	0.44	0.35	0.33
AB5					0.41	0.48	0.35	0.56	0.44	0.55	0.03	0.47	0.45	0.53	0.44	0.47	0.55	0.53	0.49	0.56	0.43	0.31	0.40
AB6						0.45	0.43	0.55	0.45	0.42	0.40	0.08	0.44	0.49	0.51	0.53	0.45	0.56	0.55	0.56	0.51	0.29	0.41
AB7							0.41	0.65	0.52	0.43	0.49	0.45	0.07	0.37	0.40	0.45	0.44	0.51	0.47	0.49	0.38	0.36	0.28
AD1								0.51	0.37	0.45	0.36	0.45	0.41	0.40	0.38	0.51	0.50	0.53	0.49	0.51	0.47	0.28	0.33
AD2									0.48	0.57	0.54	0.53	0.65	0.61	0.63	0.65	0.64	0.70	0.67	0.70	0.63	0.46	0.51
AD3										0.52	0.46	0.45	0.52	0.46	0.48	0.52	0.51	0.60	0.54	0.60	0.55	0.30	0.40
AD4											0.55	0.43	0.42	0.47	0.47	0.47	0.50	0.51	0.49	0.56	0.49	0.42	0.39
AD5												0.47	0.46	0.53	0.46	0.47	0.55	0.53	0.49	0.56	0.43	0.31	0.40
AD6													0.44	0.49	0.54	0.55	0.48	0.61	0.56	0.57	0.53	0.33	0.43
AD7														0.42	0.44	0.47	0.45	0.51	0.49	0.51	0.41	0.36	0.28
HA1															0.14	0.10	0.20	0.27	0.12	0.26	0.12	0.33	0.24
HA2																0.15	0.22	0.21	0.17	0.26	0.19	0.32	0.26
HA3																	0.23	0.30	0.02	0.30	0.15	0.34	0.33
HA4																		0.35	0.25	0.25	0.26	0.32	0.30
HA5																			0.32	0.40	0.29	0.42	0.38
HA6																				0.32	0.17	0.36	0.35
HA7																					0.33	0.42	0.30
HA8																						0.30	0.32
CS																							0.24

A

	EQ2	EQ3	EQ4	EQ0a	EQ0b	CR2	CR3
EQ1	0.41	0.26	0.38	0.56	0.59		
EQ2		0.40	0.45	0.52	0.63		
EQ3			0.31	0.58	0.56		
EQ4				0.59	0.54		
EQ0a					0.57		
CR1						0.03	0.04
CR2							0.02

B

FIG. 5.—Proportion of nucleotide differences between copies of the repetitive elements of coccinellid ITS1. *A,* Comparison between repeats for the Coccinellinae. *B,* Results for *Exochomus quadripustulatus* and *Chilocorus renipustulatus.* Abbreviations of taxa and repeats are as in figure 4. Black and gray shaded areas indicate repeat elements which show nucleotide differences of <20% and <40%, respectively. All comparisons which produced dissimilarity values of <50% are given in bold. Nucleotide differences are calculated for the alignments, given in figure 4*A, C,* and *D,* excluding gaps in pairwise comparisons only.

←

FIG. 4.—DNA sequence alignments of ''long'' repetitive elements within ITS1 of ladybird beetles. *A,* Repetitive element of the Coccinellinae (AB = *Adalia bipunctata*; AD = *Adalia decempunctata*; HA = *Harmonia axyridis*; CS = *Coccinella septempunctata*; PV = *Psyllobora vigintiduopunctata*). *B,* Repetitive element of *P. vigintiduopunctata* (PV). *C, Chilocorus renipustulatus* (CR). *D, Exochumus quadripustulatus* (EQ). The respective copy numbers of the repeats are indicated after species abbreviations. In the case of *E. quadripustulatus,* ''0a'' and ''0b'' refer to the two copies which are found to be dispersed within ITS1. The position of each repeat within ITS1 is given at the beginning of the sequences (also at the end for *C. renipustulatus*), whereas numbers following the colon at the end indicate repeat unit length in base pairs. For *C. renipustulatus,* the sequences shown overlap by 12 bp in order to give a better illustration of conserved secondary-structure motifs at the end/beginning of the repeat unit. The stem regions of secondary-structure motifs, which are found to be conserved for each species across the repeat unit, are indicated by the shaded areas and, below the alignments, by species abbreviations followed by roman numerals. For the Coccinellinae, these are all found at the same position and are therefore denoted ''COCC.'' Black and gray shadings refer to the stems and the internal stem loops, respectively.

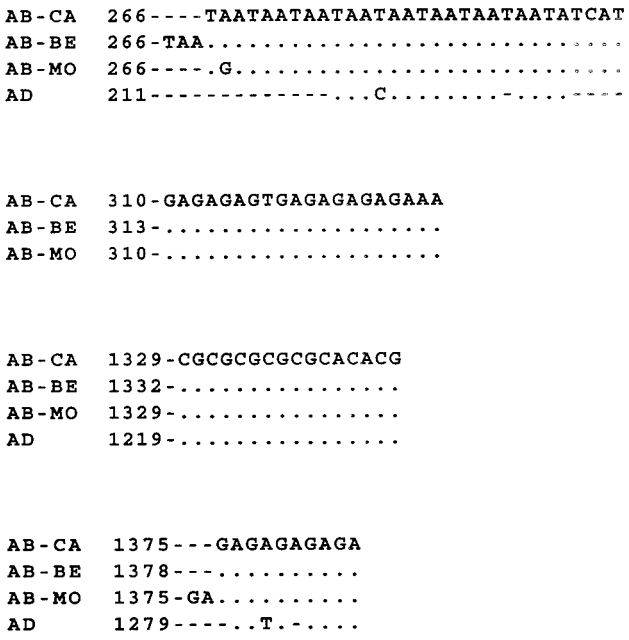


FIG. 6.—Alignment of ''simple'' repeat elements within ITS1 of *Adalia bipunctata* (AB) from three different populations (CA = Cambridge; BE = Berlin; MO = Moscow) and their counterparts in ITS1 of *Adalia decempunctata* (AD). The positions of these elements within ITS1 are given at the beginnings of the sequences.

Table 2
Approximate Increases in ITS1 Size due to Repetitive Elements

TAXON	SIZE INCREASE		ITS1 SIZE WITHOUT REPETITION IN BP
	bp	%	
Adalia bipunctata[a]	493/560	32.5/37.0	1,022/955
Adalia decempunctata . .	497	35.5	904
Harmonia axyridis	422	35.5	766
Psyllobora vigintiduopunctata. . . .	33	3.4	859
Chilocorus renipustulatus	424	38.5	676
Exochumus quadriputulatus[b]	410	15.9	2,162

NOTE.—Size increase, I was calculated excluding one copy of the repeat unit such that $I = s_{average} \times (n - 1)$, where $s_{average}$ is the average length of the repeat unit, and n is the number of repeats. Values for I were then rounded off. Percentage size increase was calculated in relation to the complete length of the spacer (see figs. 1 and 4).

[a] For *A. bipunctata*, values before and after slashes, respectively, indicate ITS1 size increase due to long repetitive elements only, and that due to long repetitive elements plus simple repeats; simple repetitive elements were only considered if they showed at least five copies of the repeat unit. An ITS1 size of 1,515 bp was assumed for calculation of percentage size increase and ITS1 size without repetition (figs. 1, 4*A*, and 6).

[b] For *E. quadripustulatus*, both tandemly repeated and dispersed copies of the repeat unit were considered (figs. 1 and 4*D*).

used only as approximate indicators of the extent of sequence homogenization across repetitive elements. In addition, we decided to exclude *P. vigintiduopunctata* from this part of the analysis. In contrast to other coccinellid species, its repetitive element consists of three copies of the repeat unit with considerable length differences (fig. 4*B*). In this case, sequence similarity values, calculated excluding gaps, did not represent comparable indicators of the degree of homogenization between repeats.

The results thus obtained revealed sequence divergence between repeats to be low for *C. renipustulatus* (<5% nucleotide differences), and, to a lesser extent, for *H. axyridis* (half of the comparisons with <25% and all of them with ≤40% nucleotide differences). For *E. quadripustulatus*, the repeats showed considerable sequence variation (26%–63%). However, variation was consistently lower within the tandem repeats (26%–45%) than between these and the dispersed copies or between the two dispersed copies (52%–63%). The repeats of both *A. bipunctata* and *A. decempunctata* yielded about 34%–65% differences. For these two species, similarity between repeat units was generally higher between than within species, particularly for those copies present in the same order (fig. 5).

Finally, the presence of simple repetition was indicated only for *A. bipunctata.* None of the other species considered produced RSF values significantly greater than 1 (fig. 1). For *A. bipunctata,* four tandemly repeated simple sequence elements with copy numbers of ≥5 were identified: two each at the 5′- and 3′-end regions of the spacer (figs. 1 and 6). The TAA motif of the first of these was, moreover, found by analysis with the program SIMPLE34 to be associated with cryptic simplicity in the adjacent regions. Intraspecific variability among these elements was low, including one nucleotide change and two copy number differences. The latter were, in fact, responsible for intraspecific ITS1 length differences in *A. bipunctata*. Moreover, sequence similarities were present between the two Adalia species for three of these simple repetitive elements, although for two of these, *A. decempunctata* showed a lower copy number.

Consequently, size increases due to repetitive elements (excluding one copy of the respective repeat unit; simple repetitive elements with copy numbers of ≥5 were only considered for *A. bipunctata*) consisted of 13.4%–38.5% of the spacer length. Without such repetition, ITS1 would show lengths of 2,162 in *E. quadripustulatus* and 676–1,022 in the other species (table 2).

Discussion

Coccinellid ITS1 elements are unique regarding both extreme size and size variation. The majority of eukaryotes usually show ITS1 lengths of no more than 800 bp, and almost all of them are shorter than 1,100 bp (e.g., Gonzalez et al. 1990; Pleyte, Duncan, and Phillips 1992; Wesson et al. 1993; Kuperus and Chapco 1994; Schlötterer et al. 1994; Vogler and DeSalle 1994; Bakker, Olsen, and Stam 1995; Bowles, Blair, and McManus 1995; McLain et al. 1995; Buckler and Holtsford 1996; Miller, Crabtree, and Savage 1996; Tang et al. 1996; Fenton, Malloch, and Moxey 1997; Downie et al. 1998; Gouliamova and Hennebert 1998; Kumar, Fenton, and Jones 1999; Schilthuizen et al. 1999; Schizas et al. 1999; van Herwerden, Blair, and Agatsuma 1999;

von der Schulenburg, Englisch, and Wägele 1999; Harris and Crandall 2000). The only two documented exceptions are found in the *Schistosoma japonica* (Platyhelminthes) and the *Anopheles gambiae* (Insecta) species complexes, which produce maximum ITS1 sizes of 1,400 and at least 5,500 bp, respectively (Paskewitz, Wesson, and Collins 1993; van Herwerden, Blair, and Agatsuma 1998). In addition, size variation between closely related taxa is usually less than 400 bp, except for the two above-mentioned cases, which yield length differences of about 900 and at least 3,000 bp.

Sequence analysis on the data from coccinellids indicated a number of factors which characterize ITS1 evolution in this group. In particular, size variation between coccinellid species was associated with a high degree of sequence divergence. For all taxa and almost all subsets of the data, putatively homologous sequence similarities were identified only for three small regions: one in the middle of the spacer and one each at the 3′ and 5′ ends of ITS1. Hence, sequence similarities are present only in restricted areas, suggesting that they are due to functional constraints. This seems likely for the small region in the middle of the spacer, although such a singular conserved fragment has not yet been reported for any other taxon and its functional importance remains to be determined. Functional constraints are known from the 3′ and 5′ end of ITS1 (see below). However, only <10 bp are conserved at both the very end and the beginning of ITS1. Therefore, in these cases, sequence conservation could also have been caused indirectly by selection on the adjacent rRNA genes.

In addition, our analysis included data for more than two taxa from two subfamilies of ladybird beetles, the Coccinellinae and the Chilocorinae. Apparent sequence similarities were observed for almost half of the spacer in the Coccinellinae. These were associated with conserved secondary-structure motifs and were primarily found at the 3′ and 5′ ends. These ITS1 regions have been shown in yeast to contain various elements required for ribosome biogenesis (Henry et al. 1994; van Nues et al. 1994; Weaver, Sun, and Chang 1997). For metazoans, functional importance has been implicated at least for the 3′ end (Schlötterer et al. 1994; von der Schulenburg, Englisch, and Wägele 1999). Moreover, sequence similarities seemed to be correlated with the degree of relatedness of the taxa included (cf. Kovář 1996; see fig. 1), and conserved sequence regions were shown by phylogenetic tree reconstruction to contain a highly informative and consistent phylogenetic signal. These observations, taken together, suggest that sequence similarities in the Coccinellinae are due to identity by descent and are most likely the result of direct functional constraints. In contrast, putatively homologous sequence similarities of such an extent could not be identified for the Chilocorinae. This may reflect a recent origin of the subfamily Coccinellinae, whereas the Chilocorinae represent a phylogenetically older taxon. Interestingly, the latter subfamily includes the longest spacer and also shows a much higher degree of size variation than the Coccinellinae. It is therefore also possible that ITS1 evolutionary rates differ between taxonomic groups due to factors associated with the generation of extreme size variation. However, on the basis of the current knowledge of the phylogeny of ladybird beetles (e.g., Kovář 1996), it is not possible to differentiate between these hypotheses.

Extreme size and size variation seem to be associated with ''long'' repetitive elements. Such a finding is paralleled by previous studies of trematodes and flies. However, in these cases, size variation resulted from copy number differences of homologous repetitive elements (Paskewitz, Wesson, and Collins 1993; Kane et al. 1996; Tang et al. 1996; van Herwerden, Blair, and Agatsuma 1998, 1999), whereas in coccinellids, it is to a large extent determined by the presence or absence of nonhomologous repetition. In addition, coccinellid ITS1 size variation does not seem to be produced by simple repetition, which has previously been recorded for a variety of organisms to generate size increases in different parts of the genome, including ribosomal DNA (e.g., Hancock and Dover 1988; Hancock 1995*a*, 1995*b*). In coccinellids, simple repetition was found to be significant only for *A. bipunctata*, for which it only accounted for minor size differences.

Long repetitive elements, identified for 6 of the 10 coccinellid species studied, were always confined to the middle of the spacer. As functional importance has previously been indicated for the 3′- and 5′-end regions of ITS1, lack of such constraints in the middle of the spacer may have favored the rise of repetition in this region. This is also supported by the fact that repetition has arisen at least four times independently. In particular, three of the six repetitive elements do not show any interspecific similarities, thus implying their independent origins. The remaining elements, all from taxa of the tribe Coccinellini, bear sequence similarities. In this case, the inferred phylogeny supports two equally parsimonious alternatives for their origin: either repetition arose twice independently in the lineages leading to *H. axyridis* and the genus Adalia, or it arose only once at the base of the tribe, followed by its loss in the lineage leading to *C. septempunctata.*

These repetitive elements can occupy up to 40% of the spacer length. Without repetition, five of the six species with repetition (only excluding the exceptional case of *E. quadripustulatus*) would produce a maximum ITS1 length of about 1,000 bp, much more similar in size to those taxa that lack internal repetition. However, extreme size and size variation cannot always be attributed directly to internal repetition. In particular, it is insufficient to explain the size of ITS1 of *E. quadripustulatus*, where absence of repetition would only reduce ITS1 to a length of 2,162 bp. Furthermore, the ITS1s of *Platynaspis luteorubra* and *S. suturalis* are comparatively long ($>1{,}000$ bp) but do not bear internal repeats. Nevertheless, the following characteristics suggest that long repetitive elements have generally played a major role in coccinellid ITS1 evolution and may also represent the original source of size increases in the above cases:

1. DNA sequence evolution in coccinellid ITS1 is generally high, and the rise of long repetition seems to be a frequent event in coccinellid ITS1 evolution (see above).
2. Repetitive elements themselves are subject to high rates of evolutionary change. This is indicated by the observation that copies of the repeat units of a particular repetitive element show considerable sequence variation both within and between taxa. Intriguingly, sequence similarities between copies of the repetitive elements of the two Adalia species are higher between than within species, although the respective ITS1s do not show significant intraindividual or intraspecific variation. This is unusual because the ribosomal cistron itself is tandemly repeated with the different copies being subject to concerted evolution. These observations suggest that there are differences in the factors which determine the extent of homogenization between repeats of the ribosomal cistron and the internal ITS1 repetitive elements. In turn, such differences permit the level of concerted evolution across internal repetitive elements to be reduced even if homogenization across repeats of the ribosomal cistron is perfect.
3. ITS1 of *E. quadripustulatus* contains four tandemly repeated copies, but also two dispersed copies. The latter may have resulted from two transposition events. Alternatively, their presence could indicate that the repetitive element originally extended over a much larger part of ITS1 which was later disrupted by a high substitution rate in combination with imperfect homogenization between repeats. In this case, tandem repetition may have been primarily conserved at the 3′ end region as a consequence of functional constraints in this part of ITS1.

Taken together, these observations suggest that internal repetition has occurred in the past and led to current length variation but is no longer detectable due to the obscuring effects of high substitution rates and inefficient homogenization across repeats. The alternative hypothesis, that ITS1 size increases were the result of insertion events, was not confirmed by the results of the BLAST analysis. There were no sequence similarities indicated between coccinellid ITS1 sequences and those of known insertion elements. An analysis of ITS1s of additional taxa, particularly those which are closely related to *E. quadripustulatus*, *P. luteorubra*, and *S. suturalis*, may help us in the future to understand the origin of ITS1 size increases in these cases if they allow detection either of relics of internal repetition or of insertions.

In conclusion, our results indicate that a variety of factors play a role in the evolution of ITS1 elements. ITS1 data from additional coccinellid species may in the future permit a more detailed assessment of the exact relevance of such factors (see above). Comparative analyses of other taxonomic groups, in each case including taxa with different degrees of relatedness, should also be of great value. In particular, such studies may allow identification of a general pattern in the distribution of conserved and thus putatively functionally important ITS1 regions in relation to those that are highly variable. Similarly, they may aid in establishing whether differences in concerted evolution across repetitive elements and repeats of the ribosomal cistron represent a common feature of rDNA evolution, thus favoring the disruption of internal repetition, which may give rise to the presence of hypervariable regions.

Finally, we note that extreme ITS1 size variation should represent a suitable marker for species diagnostics. This may prove useful in ecological studies which involve analysis of early developmental stages of different coccinellid species, such as eggs and first-instar larvae, which cannot be easily identified using morphological traits.

Acknowledgments

We wish to thank D. Tautz, J. Koene, and two reviewers for comments on the manuscript, and W. Völkl for providing a sample of *P. luteorubra.* J.H.G.v.d.S. was the recipient of a TMR fellowship from the EU, and G.D.D.H. was the recipient of a BBSRC D. Phillips fellowship. The project was funded by a research grant from the Royal Society.

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KEITH CRANDALL, reviewing editor

Accepted December 12, 2000