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An insert in the *Hpy* region of *hscp* in *Heliothis virescens* (Lepidoptera: Noctuidae) reveals a possible *CORE-SINE* of insects

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Abstract A new putative transposon was identified in the tobacco budworm, *Heliothis virescens*. This transposon was characterized as a full length *CORE-SINE* (65 bp of "CORE" core specific nucleotide short interspersed elements) that resembled sequences from three other lepidopterans and humans. In particular, the A-box and B-box regions of this sequence most closely conformed to the signature of *CORE-SINEs* from widely divergent species. This *CORE-SINE* was present as a polymorphism in a hypervariable region of the gene *hscp*, which is the target of pyrethroid insecticides and other xenobiotics in the nerve axon. We described this new putative transposon as *Noct-1* due to its presence in a noctuid moth. This is the first description of a full-length *CORE-SINE* with the A-box, B-box, target site duplication, and candidate core domain from an insect.

Key words A-box, B-box, *CORE-SINE*, *Heliothis virescens*, *Noct-1*, target site duplication

Introduction

The determination of nearly all of the DNA sequences in the human and mouse genomes has revealed an abundance of short interspersed nuclear elements (*SINEs*) and other transposons (Waterston *et al.*, 2002). For example, ~1 500 000 copies of *SINEs* exist in the human genome (Lander *et al*., 2001). In particular, the human *Alu* elements serve as a model for studying the function of *SINEs*, with an estimated 1 283 *Alu* insertion polymorphisms present in the average human (Bennett *et al*., 2004). This frequency, coupled with the observation of epigenetic alterations of phenotype, has led to an increasing awareness of the potential importance of transposons in gene expression that extends beyond the known mutagenic impact (Whitelaw & Martin, 2001). For example, *SILV* (*Silver*) is a pigment gene in an inbred strain of black mice (Theos *et al*., 2005). Interestingly, the characterization of *SILV* in merle, a pattern of coloring in the coat of the domestic dog, revealed a *SINE* insertion at the intron 10/exon 11 boundary (Leigh *et al*., 2006). Another *SINE* (also an insertion of a mutation in *SILV*) is associated with auditory and ophthalmologic abnormalities that are similar to those observed in the human auditory-pigmentation disorder Waardenburg syndrome (Leigh *et al*., 2006). *SINEs*, originally relegated among junk or selfish DNA, could have beneficial functions, such as in stress response (Hagan *et al*., 2003; Smith, 1999). Unlike some other transposons, *SINEs* are found in

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gene-rich regions and are not highly methylated. In the yeast genome, *SINEs* are dramatically juxtaposed with transfer RNA (tRNA) genes and might be related to the nucleolar processing of tRNA genes (Lopez-Giraldez *et al*., 2006; Bertrand *et al*., 1998).

Without intrinsic enzymatic activity such as reverse transcriptase and transposase, *SINEs* are thought to be dependent upon enzymatic activity from other sources, including long interspersed nuclear element (*LINEs*) (Kajikawa & Okada, 2002). For example, *SINEs* can be mobilized by *UnaL2*, a member of the major *LINE* family that encodes the apurine/apyrimidine (AP) endonuclease family in the eel (Kajikawa *et al*., 2005). *SINEs* accumulate by the "Copy and Paste" mechanism in genomes, but there is no known process to remove inserted *SINEs* from the genome (Wang & Kirkness, 2005). Most *SINEs* from mammals, fishes, and plants have been grouped into three superfamilies (*CORE-SINE*, *V-SINE*, and *AmnSINE*) based on conserved regions (Sun *et al*., 2007). The large eukaryotic supra-family of *SINEs* described as *CORE-SINEs* consists of conserved A-boxes and B-boxes resembling the split RNA polymerase III promoter, a core domain, a segment uniquely combined with 3′ *LINE*-like sequence, and a terminal region of simple repeats (Sun *et al*., 2007; Gilbert & Labuda, 1999).

Our previous investigation of insecticide resistance and new eye phenotypes in *Heliothis virescens* led us to examine the role of transposon insertion and analyze DNA inserts in possible regions of the genome (Cho *et al*., 2003). The tobacco budworm, *H. virescens* (F.) (Lepidoptera: Noctuidae), is the world's primary pest of cotton and tobacco. Voltage-gated sodium channels are a common target site for DDT and pyrethroid insecticides (Dong, 2007), and resistance to insecticides is usually conferred by the sodium channel gene (*hscp*). A polymorphic DNA marker, *Hpy*, located in the *IIIS5* of *hscp* lies about 5 kb downstream of *L1029H* (Park *et al*., 1999). Significant association with pyrethroid resistance has been found for *Hpy* (Taylor *et al*., 1996). Herein, we describe a *CORE-SINE* inserted in the *Hpy* marker region of the *hscp* gene in *H. virescens*. This work represents the first characterization and description from an insect of a full-length *SINE* with *CORE-SINE* A-box and B-box signatures.

Materials and methods

Rearing

Both wild-type and several eye mutant strains of *H. virescens* were used in this study. All stages of every strain were maintained under a similar, controlled environment and rearing conditions (Ross & Brown, 1982). All stages were maintained at 27°C with a photoperiod of 16 : 8 (L : D). Adult moths were reared in a single jar containing 10% maltose and honey-water and allowed to oviposit on white cloth that had been placed on the top of a jar. Forty-five to more than 100 newly hatched larvae from each cross were transferred to the plastic jelly cups filled with a modified pinto bean diet (Ross & Brown, 1982).

Crosses and isolation of H. virescens *strains*

The wild-type tobacco budworm has a green body and grey eyes. Yellow-eyed tobacco budworm moths were discovered in a laboratory culture of YEL strain at Clemson University, South Carolina, in October 1998 (Brown *et al*., 2001). Another greenish yellow-eyed strain was supplied by G.T. Payne, West Georgia University. A new mutation, black-eyed moths, were isolated from crosses between yellow-eyed and greenish yellow-eyed moths (called "CP" family) (Cho *et al*., 2003). In pair-mating CP6, five females and one male with black eyes were found among 112 siblings. When two black-eyed hybrid females were mated to their wild-type hybrid brother, we observed two additional new phenotypes for eye pigmentation, which were golden eyes and striped eyes. Thus, the D8, D28, D29, and D63 family is the F6 generation descended from a black-eyed mutant "CP6" family (Cho *et al*., 2003). D families exhibited yellow scales, black eyes, golden eyes, white eyes and wild-type grey eyes. All examined adult moths were members or ancestors of the family D. Adults of the family D63 are moderately resistant to cypermethrin with 11.1% mortality when exposed to 5 μg per vial of cypermethrin for 24 h compared to 89.1% mortality in D8, D28, D29 families (Cho *et al*., 2003); susceptibility test methods have been described previously (Pimprale *et al*., 1997).

DNA isolation and polymerase chain reaction and sequence determination

DNA from black-eyed mutant adults of the D63 family was isolated using a phenol/chloroform technique and amplified in a Rapid Cycler (Idaho Tech, Salt Lake City, UT, US) using primers for the *Hpy* region of *hscp*. The sequences of the primers are Hp4211 (5′ctgatcttcgccatcatggggcgtc3′) and an abbreviated form (5′gttcatgatctgtatcca3′) of CL/Hp4399 (Brown *et al*., 2001; Taylor *et al*., 1996). Known sequences of *IS6*, *IIS6*, and *Hpy* were aligned with the amplified sequences obtained from the pyrethroid-resistant and susceptible

strains of *H. virescens*. Searches employed Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1997) via the internet sites of the National Center for Biotechnology Information and the *Bombyx* Genome Database Working Group. Alignments were performed using the ClustalW (v.1.8) tool of MacVector 7.0 and Lasergene 7.0 computer application (Invitrogen, San Diego, CA, US).

Phylogenetic analysis and secondary structure analysis

The amino acid sequences of various *CORE-SINEs* from mammals and insects were aligned using the Cluster algorithm, ClustalW ver 1.8 (Thompson *et al*., 1994) and the phylogenetic tree was constructed based on alignment by DNASTAR V7.0. Predicted secondary structures were estimated by the M. Zuker RNA mfold programs, available through the server http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1-2.3.cgi.

Results

A new putative transposon in H. virescens

The new putative transposon was a full-length *SINE* and the first transposon identified in *H. virescens*. The transposon was identified from the sequence of the intron of the *Hpy* region, which was amplified from a genomic DNA target (data not shown). Analysis of the sequence revealed a product that was 319 nucleotide bases longer than anticipated based on the published consensus sequence (Taylor *et al*., 1996). A portion of this inserted sequence was 97% identical to a sequence at the beginning of a cosmid clone from which the first three exons of *hscp* had been originally reported (Park *et al*., 1999), as observed in GenBank accession AF072458.1. Examination of the 5′-end sequence and 3′-end sequence of the insert revealed the target-site duplication "AAATTTCTAA" which was <*Hpy* sequences> AAATTTCTAA <*Noct-1*(319bp)> AAATTTCTAA <*Hpy* sequences> (Fig. 1A). This target site sequence was accompanied by several preceding "GAA" repeats at the 3′-end region (Fig. 1A). Among the 26 samples examined in the D63 mutant line, which is resistant to cypermethrin, the frequency of the 319-nucleotide insert allele was 0.23 (Table 1). However, no insert alleles were found in the D8, D28, or D29 eye mutant lines, which are susceptible to cypermethrin (Table 1). We named this new putative transposon *Noct-1* due to its presence in a noctuid moth.

Table 1 Frequencies of 319-nucleotide insertions in lines having mutant eye-color phenotypes in *H. virescens*.

Lines	Domain *Hpy*[§]		Total frequency of the insert allele
	Wild type allele	*319in* allele[¶]	
D63[†]	20/26	6/26	0.23
D8[‡]	26/26	0/26	0
D28[‡]	26/26	0/26	0
D29[‡]	26/26	0/26	0

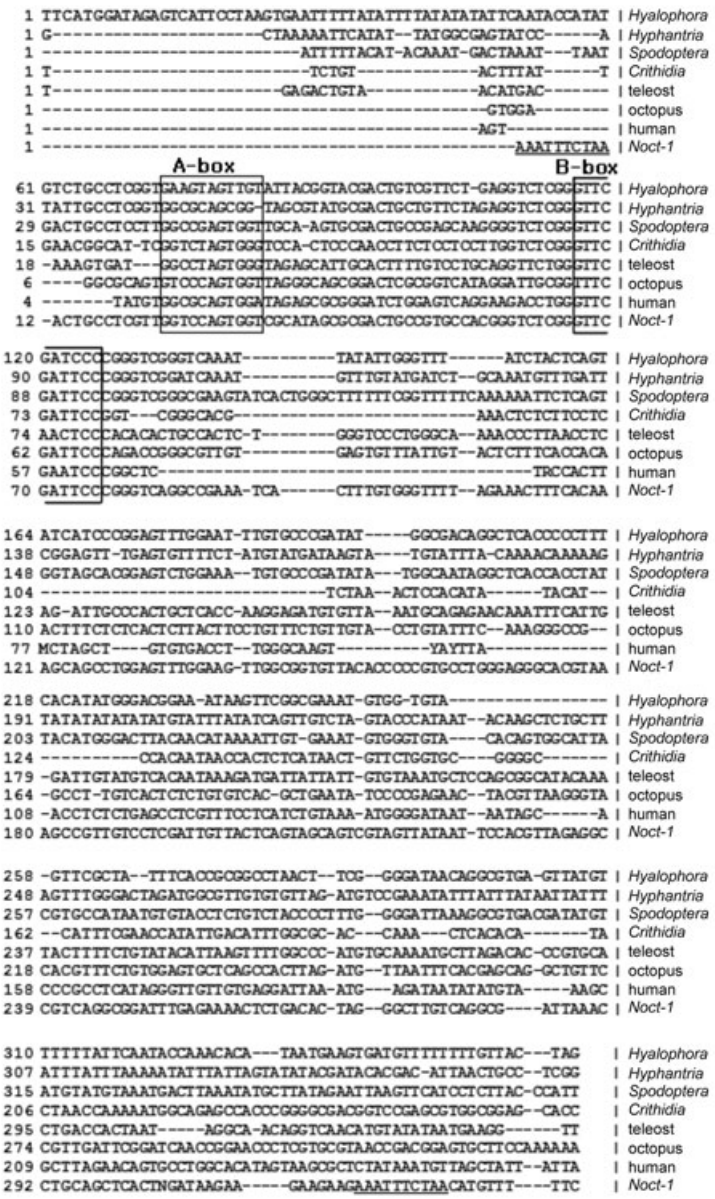
[†]Eye mutant line; most resistant to pyrethroid insecticides described in Cho *et al.* 2003.
[‡]Eye mutant lines; susceptible to pyrethroid insecticides described in Cho *et al.* 2003.
[§]Molecular marker at *IIIS5* (*Hpy* region) in sodium ion channel.
[¶]319 nucleotide insertion in eye mutant lines.

Characterization of Noct-1

Next, we evaluated the homology of *Noct-1* with *Bm1* of *Bombyx mori* (Adams *et al.*, 1986; Mita *et al.*, 2003), *Spodoptera frugiperda, Hyalophora cecropia, Hyphantria cunea* (all lepidopteran species) and with various mammalian *SINEs* (Kawagoe-Takaki *et al*., 2006; Takahashi & Okada, 2002; Takahashi *et al*., 1997). From these results, we confirmed the presence of the A-box and B-box of the *Pol III* promoter (Fig. 1A) (Sun *et al*., 2007; Gilbert & Labuda, 1999). We speculated that *Noct-1* was closer to the *CORE-SINE* superfamily, because it has an A-box and B-box that are highly conserved with *CORE-SINEs* from mammals, fish, and plants. *Noct-1* has 10 bases homologous to the A-box signature of *Ther1*-human (*CORE-SINE*), "GGCGCAGTGG", and 10 bases homologous to the B-box of *Ther1*-human, "GTTCGAATCC" (Zhang *et al*., 2007; Gilbert & Labuda, 1999). This B-box is highly conserved from mammals to insects (Fig. 1A). This finding suggests that a family of *SINEs* exists across a wide variety of animal species.

Next, we carried out a bioinformatics computer software (FASTA) search with only the candidate core domain (~65 bp) with the A-box and B-box of *Noct-1*, and found no similar sequences except *S. frugiperda* (Fig. 1B). Our putative *CORE-SINE* was highly conserved with *S. frugiperda* in the 5′ portion, with the sequence progressively degenerating in the 3′ direction prior to finding a 65-base candidate core domain (assuming a 17-base gap in the core domain from the start of the B-box modeling *CORE-SINEs*) (Fig. 1B). Moving to the 3′ variable region of *Noct-1*, we observed widespread matches near the terminal sequence where there was a "GAA" repeat,

(A)



(B)

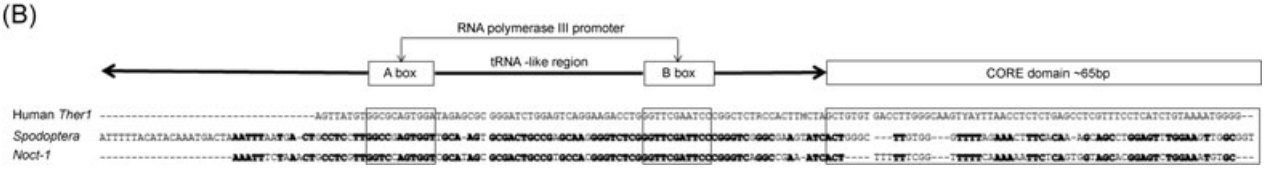


Fig. 1 Comparison of *Noct-1* with putative *CORE-SINEs* of lepidopteran and mammalian sequences. (A) Sequence indicating a short interspersed nuclear elements (*SINEs*) type of transposon, *Noct-1*, in *Heliothis virescens* (AcY452056.1*hscp Hpy* 319*in*) and alignment with similar sequences from *amy2* of *Spodoptera frugiperda* (AF280891.1), *serpin* of *Hyphantria cunea* (AF151527), *attacin* of *Hyalophora cecropia* (X62290.1), human *Ther-1*, and octopus (D32095), teleost (U59855), and trypanosome *Crithidia fasciculate* (M330009.2) *CORE-SINES*. The A-box and B-box are indicated. Target site duplication is underlined prior to the insert and following simple repeats (~GAA) at 3′ terminus of the insert. (B) Schematic structure of *Noct-1* is compared with *Ther1*-human and *S. frugiperda*. RNA polymerase III promoter elements (A-box and B-box), the transfer RNA-like region, and central core (~65 bp) are indicated. Identical nucleotides between *Noct-1* and *S. frugiperda* are in bold.

as expected in *SINEs*, but we found no longer regions of homology in the 3′ region. All described *CORE-SINE* consensus sequences have similar 5′ regions, including core domains, but completely different 3′ regions (Sun *et al*., 2006). Details of the *Noct-1* putative signatures including the A-box, B-box, and candidate core domain are provided in Fig. 1B.

Relationships between Noct-1 *and mammalian* CORE-SINEs

CORE-SINEs are found in mammalian transposon families. The families are defined by the last half of the *LINE*-related segment of the 3′ variable region, including a terminating simple repeat region (Gilbert & Labuda, 2000; Gilbert & Labuda, 1999). One such transposon is *Ther1*-human (Fig. 2). To better understand the phylogenetic relationship between *CORE-SINEs* of mammals and *Noct-1*, we carried out maximum parsimony analyses (e.g., DNASTAR V7.0-generated). We used a tRNA-like region (A-box and B-box) with a putative core domain. In the tree analysis, *Noct-1* clustered with *S. frugiperda*, but not with the other lepidopterans or vertebrate *CORE-SINEs*. Thus, *Noct-1* and *S. frugiperda CORE-SINE* form a separate group of *CORE-SINEs* in insects (Fig. 2).

To confirm these phylogenetic results, we tested whether similar RNA folding patterns could be observed between groups. The possible RNA secondary structures of *SINEs* were constructed using the Mfold RNA free-energy minimization program. We were unable to observe tRNA-like folding patterns from all sequences, because all sequences were not the shorter *SINE* elements composed only of a tRNA-related domain (Fig. 3) (Sun *et al*., 2007). Their RNA secondary structures were composed of three stem-loops, which are present in *Ther1*-human, octopus, and teleost. The three stem-loops can vary due to sequence length. For RNA secondary structures of *Noct-1*, including *S. frugiperda*, *H. cunea*, *H. cecropia*, and *B. mori*, we observed multi-loop and terminal branching with single or two short extended stem loop structures containing several bulges and internal loops (Fig. 3). Although the tested sequences had no significant primary sequence homology and their sequence lengths were different from each other, similar folding patterns were observed (Fig. 3). The *Ther1*-human RNA secondary structure was very similar to the teleost structure and the *Noct-1* RNA secondary structure was similar to the *S. frugiperda* RNA secondary structure, as anticipated (Fig. 3). Sun *et al.* proposed that *SINEs* could have evolved and amplified from a single stem-loop to two, three, and four loops (i.e., a more complex pattern) (Sun *et al*., 2007). If so, the complex folding pattern observed in *Noct-1* indicates that it might be the youngest *CORE-SINE* in *H. virescens* evolution, although we must investigate other *CORE-SINE* sequences in this species (Sun *et al*., 2007).

Discussion

To our knowledge, this is the first description from an insect of a full length *CORE-SINE*. Four sequences from Silkbase (http://morus.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi) were similar to the A-box and B-box region of *Noct-1*, but lacked the "AGTGG" sub-signature of *CORE-SINEs* and did not appear to be full-length *SINEs* (data not shown). However, *Noct-1* contained a clearly defined A-box, B-box, "GAA" repeat, and target site duplication.

Noct-1 was present as a polymorphism in a hypervariable region of the gene *hscp*, which is the target of pyrethroid insecticides and other xenobiotics in the nerve axon. Previously, we reported that *hscp* mutations are associated with insecticide resistance (Lee *et al*., 1999; Park & Brown, 2002). Furthermore, we demonstrated several new visible mutations (eye color) in our strains of this species that contain this transposon (Brown *et al*., 2001; Cho *et al*., 2003). These observations are consistent with the possible mobility of *Noct-1*. The only previous reports of transposons in this species are a partial *hobo* sequence (DeVault & Narang, 1994), a *mariner*-like element (Ren *et al*., 2006), and a partial long terminal

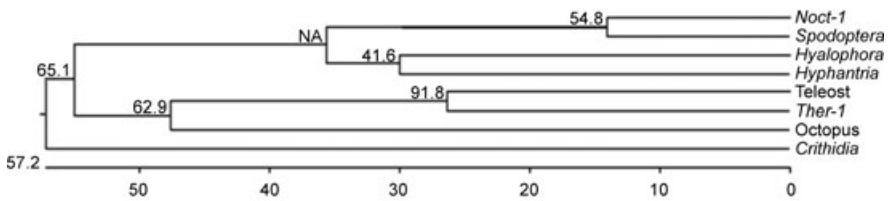


Fig. 2 Relationships of A-box, B-box, and the candidate core domain of putative invertebrate *CORE-SINEs* and vertebrate *CORE-SINEs*. GenBank accession numbers of others are given in Fig. 1. A phylogenetic tree generated by DNASTAR v7.0, Bootstrap trials = 1000. Seed = 111) is used to indicate structural and evolutionary relationships among *CORE- SINEs*.

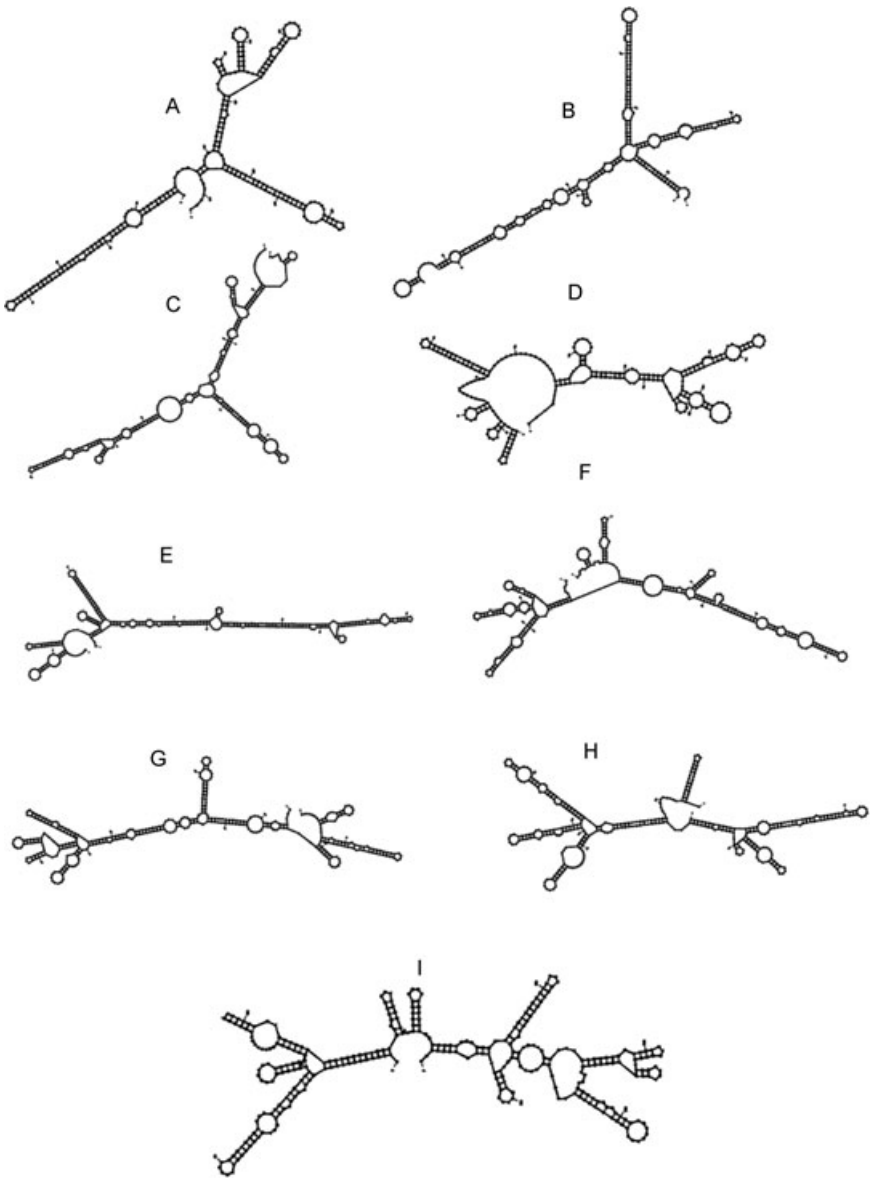


Fig. 3 Possible RNA secondary structures of *CORE-SINE*-related sequences. Predicted secondary structures were performed using Mfold version 3.2 (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1-2.3.cgi) at 37°C for mammal sequences and 25°C for insect sequences. (A) *Ther-1*-human, $\Delta G = -82.20$ kcal/mole; (B) Octopus, $\Delta G = -86.70$ kcal/mole; (C) Teleost, $\Delta G = -74.30$ kcal/mole; (D) Crithidia, $\Delta G = -51.40$ kcal/mole; (E) *H. cunea*, $\Delta G = -124.38$ kcal/mole; (F) *H. cecropia*, $\Delta G = -119.71$ kcal/mole; (G) *S. frugiperda*, $\Delta G = -128.55$ kcal/mole; (H) *Bombyx mori*, $\Delta G = -134.56$ kcal/mole; (I) *Noct-1*, $\Delta G = -130.56$ kcal/mole.

repeat (LTR)-retrotransposon (*Hel-1*) sequence truncating a cadherin-like transcript linked to resistance to *Bacillus thuringensis* toxin (Jurat-Fuentes & Adang, 2006; Gahan *et al*., 2001).

With the recent establishment of *H. virescens* as a model lepidopteran to join the better described silkworm, we look forward to future characterization of this and other *SINEs* among lepidopterans. Information from lepidopterans may help elucidate the phenomenon of transposon control of expression and other newly emerging topics in comparative genomics.

For instance, *SINEs* have been known in regulating the activation of the growth hormone (*GH*) gene in mouse organogenesis as well as in silencing *FWA* gene in *Arabidopsis thaliana* (Kinoshita *et al*., 2007; Lunyak *et al*., 2007). However, there is no information available

about gene activation and silencing associated with *SINEs* in lepidopterans. Thus, the *Noct-1* which is homologous to *SINE* structure and its sequence is very useful in research on the phenomenon of transposon control of gene expression. The phylogenetic relationships in reptiles have been conducted using the *SINE* sequences (Sasaki *et al*., 2004). Thus, our information could be very useful to infer the phylogenetic relationships among lepidopterans as well. In addition, the *SINE* is used as the tool for the introduction of exogenous DNA into genomes. The *SINE*-flanked DNA showed a 4-fold increased integration frequency (Kang *et al*., 2000). The *Noct-1* might also be useful as a tool for production of transgenic lepidopterans.

Acknowledgments

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