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# Cross-species investigation of *Helicoverpa armigera* microsatellites as potential markers for other related species in the *Helicoverpa* - *Heliothis* complex

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## Abstract

Primers previously designed to amplify microsatellite DNA markers in the Old World bollworm, *Helicoverpa armigera*, larvae were tested in three closely related species: the corn earworm, *Helicoverpa zea*, tobacco budworm, *Heliothis virescens*, and *Heliothis subflexa*. Of the fourteen loci surveyed, only four loci (HaB60, HaC14, HaC87, HarSSR1) consistently demonstrated scorable single-copy microsatellite bands. Of these four, length polymorphism was identified only in the HaB60 marker (160 bp, 140 bp) of the *H. virescens* and *H. subflexa* sampled laboratory populations. Partial DNA sequences of all the identified single-copy microsatellites are presented as well as alignments to their respective *H. armigera* microsatellite.

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Keywords: Lepidoptera, *Helicoverpa armigera*, *Helicoverpa zea*, *Heliothis virescens*, *Heliothis subflexa*, microsatellites  
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## Introduction

The lengthy process and expertise required to isolate and identify potential microsatellite markers often precludes the use of this valuable technique in studies to determine genetic variation in natural populations. If microsatellite markers identified and developed from one biological source could be applied to other similar species, the usefulness of these genetic markers could be broadened. Fortunately, a number of microsatellites markers have been developed to study the population genetic variation in the Old World bollworm, *Helicoverpa armigera*, a serious insect pest of several agriculturally important grain and fiber crops (Tan et al., 2001; Ji et al., 2003; Scott et al., 2004). We therefore undertook a survey of some of the available lepidopteran species that are used routinely in our laboratory, namely *Helicoverpa zea*, *Heliothis virescens*, and *Heliothis subflexa*, to determine if previously designed microsatellite markers for *H. armigera* from several published sources could be applied to these closely related lepidopteran species. Selection of these species for study was also contingent on their importance as field crop pests. The host range of *H. zea*, the corn earworm, includes over 100 plants with the most significant crops being corn, cotton and tomato. Occasional hosts include bean, broccoli, cabbage, chrysanthemum, eggplant, head cabbage, green bean, lettuce, okra, pea, pepper, soybean, strawberry and watermelon. The tobacco budworm, *H. virescens*, is also principally a field crop pest, attacking such crops as alfalfa, clover, cotton, flax, soybean, corn, and tobacco. However, it sometimes feeds on such vegetables as cabbage, cantaloupe, lettuce, pea, pepper, pigeon pea, squash, and tomato, especially when cotton or other favored crops are not abundant. *H. virescens* is a common pest of geranium and other flower crops such as ageratum, bird of paradise, chrysanthemum, and gardenia, to name a few. In contrast, *H. subflexa* is of minor agricultural importance feeding on a few plant species such as *Solanum nigrum* and *Physalis* spp, but serves as a unique laboratory subject in studies to determine and compare host range infectivity and genetic resistance to baculoviruses.

## Materials and Methods

Based on a previously published protocol (McIntosh et al., 1996), genomic DNA was extracted from 2<sup>nd</sup> or early 3<sup>rd</sup> instar *H. zea* and *H. virescens* larvae obtained from the North Carolina

State University- Entomology Insectary, Raleigh, North Carolina, whereas 2<sup>nd</sup> or 3<sup>rd</sup> instar *H. subflexa* larvae were obtained in-house at the USDA, ARS, Biological Control of Insects Research Laboratory, Columbia, Missouri. Sample sizes are shown in Tables 1 and 2. Fourteen published primer sets designed to amplify the following microsatellite loci of *H. armigera* were employed in this study: (1) (HaB60) -- (CTG)<sub>2</sub> (TTG)<sub>3</sub> (CTG)<sub>5</sub> (TTG)<sub>2</sub>, (2) (HaC14) -- (ATTT)<sub>5</sub>, (3) (HaD47) -- (CA)<sub>5</sub> (TCA)<sub>4</sub>, (4) (HaC87) - (TC)<sub>5</sub> (Scott et al., 2004); (5) Ham2 -- (TTTTGA)<sub>9</sub>, (6) Ham3 -- (TAAA)<sub>2</sub> (TAAAT)<sub>4</sub>, (7) Ham4 -- (TCTG)<sub>6</sub> TCTT (TCTG)<sub>6</sub>, (8) Ham5 -- (T)<sub>n</sub> (G)<sub>n</sub>, (9) Ham6 -- (GAT)<sub>2</sub> TT (GAT)<sub>2</sub> TT.....(AATA)<sub>5</sub> (Tan et al., 2001); (10) HarSSR1 - (TGC)<sub>2</sub>GAT (TGY)<sub>4</sub>GAT(TGY)<sub>35</sub>(TGA)<sub>2</sub> AGC(TGY)<sub>8</sub> (11) HarSSR2 - (ATG)<sub>7</sub>, (12) HarSSR3 - (TCA)<sub>6</sub>, (13) HarSSR4 - (GYT)<sub>25</sub>, and (14) HarSSR5 - [T(T)AA]<sub>6</sub> (Ji et al., 2003). DNA microsatellite amplification was conducted under the following two polymerase chain reaction conditions using a Hybaid OmniGene thermal cycler (Midwest Scientific, www.midsci.com) in 25 l of puReTaq Ready-To-Go™ PCR bead reaction mixture (Amersham Biosciences, www.apbiotech.com), including 100-200 ng of genomic DNA template. First, after initial denaturing at 94° C for 5 min, the reaction mixture underwent 35 cycles at 94° C for 1 min, variable annealing temperature (see Tables 1 and 2) for 30 sec, 72° C for 40 sec, and a final extension at 72° C for 5 min (Tan et al., 2001). Second, after initial denaturing at 94° C for 1 min, the reaction mixture underwent 35 cycles at 94° C for 1 min, 50° C for 1 min, 73° C for 1 min, and a final extension at 72° C for 5 min (Scott et al., 2004). These two previously published PCR conditions with their respective primers were used to establish a comparative baseline for the three lepidopteran species examined in this study. However, if the expected fragment size(s) was not detected under the original PCR conditions for a particular microsatellite, empirical studies with various annealing temperatures were conducted in an attempt to resolve these problematic microsatellite markers (Table 1). A 10 l aliquot of each amplified sample was run on a 2.5% Metaphor™ agarose gel (10 mM Sodium hydroxide-Boric acid buffer, pH 8.5) for ca. 1 h at a constant 120 v using a Bio-Rad Wide Mini-Sub Cell-gel system.

Results

Initially, annealing temperatures previously published for the various microsatellites detected in *H. armigera* were employed in this study with resulting mixed success. Therefore, as indicated in Table 1, several annealing temperatures were tested for each locus in all three species in an attempt to determine the optimal running conditions for successful microsatellite amplification. Table 2 shows the microsatellite loci that failed to show distinct single-copy bands under the various PCR amplification conditions tested. Of the fourteen loci surveyed, only four loci (HaC14, HaB60, HaC87, and HarSSR1) consistently demonstrated scorable single-copy microsatellite bands that might lead to the potential detection of population polymorphism in subsequent studies (Fig.1). The phrase “potential detection” must be emphasized since the samples tested were limited to only laboratory reared insects. Of the four loci that consistently demonstrated scorable single-copy microsatellite bands, length polymorphism was identified only in the HaB60 marker (160 bp and 140 bp). The remaining microsatellites investigated showed multiple banding patterns, which have typically been observed in a number of lepidopteran species during the process of microsatellite clone development, and further indicate the repetitive nature of the flanking regions of microsatellites throughout the genome of Lepidoptera (Zhang, 2004). The HaC14 270 bp band (Fig. 2) detected among all three species, and first thought to be a microsatellite repeat variation, appears to be actually caused by a duplication of the downstream

primer sequence used to amplify the microsatellite (sequence data not shown).

To obtain a more accurate picture of the nucleotide base composition of some of the detected single-copy microsatellites that showed either the expected allele size or a variant, direct DNA sequencing of PCR products was performed at the University of Missouri DNA Core Facility, Columbia, Missouri using an Applied Biosystems (www.appliedbiosystems.com) 3730 DNA Analyzer. Because of the known potential for amplification errors during the PCR reaction due the inherent nature of the *Taq* polymerase, 2-3 replicate samples of each locus were sequenced from individual insects and a single consensus sequence was generated employing VisCoSe (Spitzer et al., 2004). Partial sequence alignments of five alleles from four microsatellites are indicated in Figure 3. In addition to the generated sequence alignments, the T-coffee program also provides an index of Consistency of the Overall Residue Evaluation (CORE), an objective measure that identifies which regions of the compared sequences are correctly aligned by averaging the scores of each of the aligned pairs involving a base within a column (Notredame et al., 2000). A CORE value > = 3 would indicate a properly aligned base position and is considered the best compromise between a level of sensitivity and specificity required for proper base alignment. All of the aligned portions of the expected microsatellites showed reasonably high CORE scores for their individual alleles (70% for HaC14; 70%, 51% for HaB60 (160 bp, 140 bp, respectively); 91% for HaC87 (118bp); and 71% for

**Table 1.** Microsatellite markers previously published for *Helicoverpa armigera* found to successfully amplify similar microsatellite loci in three other related lepidopteran species.

| Species             | Locus   | Annealing temp (Co) - DNA band fragment profile               | Number of replicates | Sample size (n) | Reference for PCR running conditions              |
|---------------------|---------|---------------------------------------------------------------|----------------------|-----------------|---------------------------------------------------|
| <i>H. zea</i>       | HaB60   | 50° - multiple bands > 200 bp marker                          | 1                    | 7               | Tan et al., 2001(modified)*                       |
|                     |         | 55° - single 160 bp band                                      | 3                    | 18              |                                                   |
|                     |         | 60° - multiple bands > 200 bp marker and a single 160 bp band | 2                    | 12              |                                                   |
| <i>H. zea</i>       | HaC87   | 50° - single-copy band 118 bp band                            | 2                    | 14              | Scott et al., 2004;<br>Tan et al., 2001(modified) |
|                     |         | 55° - multiple bands                                          | 1                    | 3               |                                                   |
|                     |         | 60° - single-copy 118 bp band                                 | 2                    | 12              |                                                   |
| <i>H. zea</i>       | HaC14   | 50° - single-copy 160 bp band                                 | 1                    | 12              | Scott et al., 2004<br>Tan et al., 2001(modified)  |
|                     |         | 55° - single-copy 160 bp band                                 | 1                    | 3               |                                                   |
| <i>H. zea</i>       | HarSSR1 | 50° - single-copy 240 bp band                                 | 1                    | 2               | Scott et al., 2004                                |
|                     |         | 58° - single-copy 240 bp band                                 | 2                    | 5               |                                                   |
|                     |         | 50° - multiple bands                                          | 2                    | 12              |                                                   |
| <i>H. virescens</i> | HaC14   | 55° - single-copy 160 bp                                      | 2                    | 15              | Scott et al., 2004<br>Scott et al., 2004          |
|                     |         | 50° - multiple bands                                          | 2                    | 4               |                                                   |
| <i>H. virescens</i> | HaB60   | 55° - 160 bp and 140 bp single-copy bands                     | 3                    | 11              | Tan et al., 2001(modified)                        |
|                     |         | 50° - 240 bp single-copy band                                 | 1                    | 5               |                                                   |
| <i>H. virescens</i> | HarSSR1 | 58° - multiple bands                                          | 2                    | 4               | Scott et al., 2004                                |
|                     |         | 60° - multiple bands                                          | 1                    | 2               |                                                   |
| <i>H. subflexa</i>  | HaB60   | 50° - multiple bands                                          | 2                    | 4               | Scott et al., 2004                                |
|                     |         | 55° - 160 bp and 140 bpsingle-copy bands                      | 3                    | 14              |                                                   |
| <i>H. subflexa</i>  | HaC14   | 50° - 160 bp single-copy band                                 | 3                    | 14              | Scott et al., 2004                                |
|                     |         | 55° - multiple bands                                          | 1                    | 2               |                                                   |
| <i>H. subflexa</i>  | HaSSR1  | 58° - 240 bp single-copy band                                 | 2                    | 4               | Scott et al., 2004                                |

**Table 2.** Microsatellite markers previously published for *Helicoverpa armigera* found to unsuccessfully amplify similar microsatellite loci in three other related lepidopteran species.

| Species             | Locus   | Annealing temperature (Co) - DNA band fragment profile                                   | Number of replicates | Sample size | PCR running conditions                         |
|---------------------|---------|------------------------------------------------------------------------------------------|----------------------|-------------|------------------------------------------------|
| <i>H. zea</i>       | HaD47   | 500 - single-copy 240 bp band                                                            | 1                    | 2           | Scott et al., 2004; Tan et al. 2001 (modified) |
|                     |         | 550 - multiple bands                                                                     | 3                    | 18          |                                                |
|                     |         | 530 - multiple bands                                                                     | 1                    | 6           |                                                |
| <i>H. zea</i>       | Ham3    | 600 - multiple bands > 200 bp marker                                                     | 1                    | 6           | Tan et al., 2001; Tan et al. 2001 (modified)   |
|                     |         | 660 - multiple bands > 200 bp marker                                                     | 2                    | 12          |                                                |
| <i>H. zea</i>       | HarSSR2 | 520 - fb                                                                                 | 1                    | 2           | Scott et al., 2004; Tan et al. 2001 (modified) |
|                     |         | 600 - nb                                                                                 | 1                    | 4           |                                                |
|                     |         | 590 - nb                                                                                 | 1                    | 2           | Scott et al., 2004; Tan et al. 2001 (modified) |
| <i>H. zea</i>       | HarSSR3 | 600 - multiple bands                                                                     | 1                    | 4           |                                                |
|                     |         | 550 - multiple bands > 200 bp marker                                                     | 1                    | 3           |                                                |
| <i>H. zea</i>       | HarSSR4 | 60 - single-copy 240                                                                     | 1                    | 2           | Scott et al., 2004; Tan et al. 2001 (modified) |
|                     |         | 660 - multiple bands > 240 bp band                                                       | 1                    | 3           |                                                |
| <i>H. zea</i>       | HarSSR5 | 660 - multiple bands                                                                     | 1                    | 4           | Tan et al., 2001                               |
| <i>H. zea</i>       | Ham5    | 520 - multiple bands                                                                     | 1                    | 2           | Tan et al., 2001                               |
|                     |         | 500 - multiple bands                                                                     | 1                    | 2           | Scott et al., 2004; Tan et al. 2001 (modified) |
| <i>H. virescens</i> | HaC87   | 550 - multiple bands                                                                     | 1                    | 4           |                                                |
|                     |         | 600 - nb                                                                                 | 1                    | 4           |                                                |
|                     |         | 520 - fb                                                                                 | 1                    | 2           | Tan et al. 2001(modified); Scott et al., 2004  |
| <i>H. virescens</i> | HarSSR2 | 660 - multiple bands                                                                     | 2                    | 7           |                                                |
|                     |         | 590 - multiple bands                                                                     | 1                    | 2           | Scott et al., 2004                             |
| <i>H. virescens</i> | HarSSR3 | 600 - multiple bands                                                                     | 1                    | 3           |                                                |
|                     |         | 500 - multiple bands                                                                     | 2                    | 7           |                                                |
|                     |         | 600 - multiple bands                                                                     | 1                    | 2           | Scott et al., 2004                             |
| <i>H. virescens</i> | HarSSR4 | 530 - multiple bands                                                                     | 1                    | 2           |                                                |
| <i>H. virescens</i> | Ham3    | 550 - multiple bands                                                                     | 1                    | 4           | Tan et al., 2001                               |
| <i>H. virescens</i> | Ham5    | 520 - multiple bands                                                                     | 1                    | 2           | Tan et al., 2001                               |
|                     |         | 550 - nb                                                                                 | 1                    | 4           | Tan et al., 2001                               |
| <i>H. virescens</i> | Ham6    | 660 - nb                                                                                 | 1                    | 3           |                                                |
|                     |         | 500 - multiple bands                                                                     | 1                    | 2           | Scott et al., 2004                             |
| <i>H. subflexa</i>  | HaC87   | 550 - multiple bands > 200 bp marker and strongly stained bands at 55, 70, 75 bp markers | 2                    | 18          |                                                |
|                     |         | 500 - multiple band                                                                      | 1                    | 2           | Scott et al., 2004                             |
| <i>H. subflexa</i>  | HaD47   | 550 – suspected single-copy band at 140 bp                                               | 2                    | 10          |                                                |
| <i>H. subflexa</i>  | HaD47   | 500 - multiple bands                                                                     | 1                    | 2           | Scott et al., 2004                             |
|                     |         | 530 - multiple bands >180 bp marker band                                                 | 2                    | 16          | Tan et al., 2001; Tan et al. 2001 (modified)   |
| <i>H. subflexa</i>  | Ham3    | 590 - multiple bands                                                                     | 1                    | 4           |                                                |
| <i>H. subflexa</i>  | Ham6    | 530 - multiple bands                                                                     | 1                    | 4           | Tan et al., 2001                               |
| <i>H. subflexa</i>  | HaSSR2  | 520 - nb                                                                                 | 1                    | 2           | Tan et al., 2001                               |
| <i>H. subflexa</i>  | HaSSR3  | 590 - fb                                                                                 | 1                    | 2           | Tan et al., 2001                               |
| <i>H. subflexa</i>  | HaSSR4  | 600 - fb                                                                                 | 1                    | 2           | Tan et al., 2001                               |
| <i>H. subflexa</i>  | HaSSR5  | 540 - fb                                                                                 | 1                    | 2           | Tan et al., 2001                               |

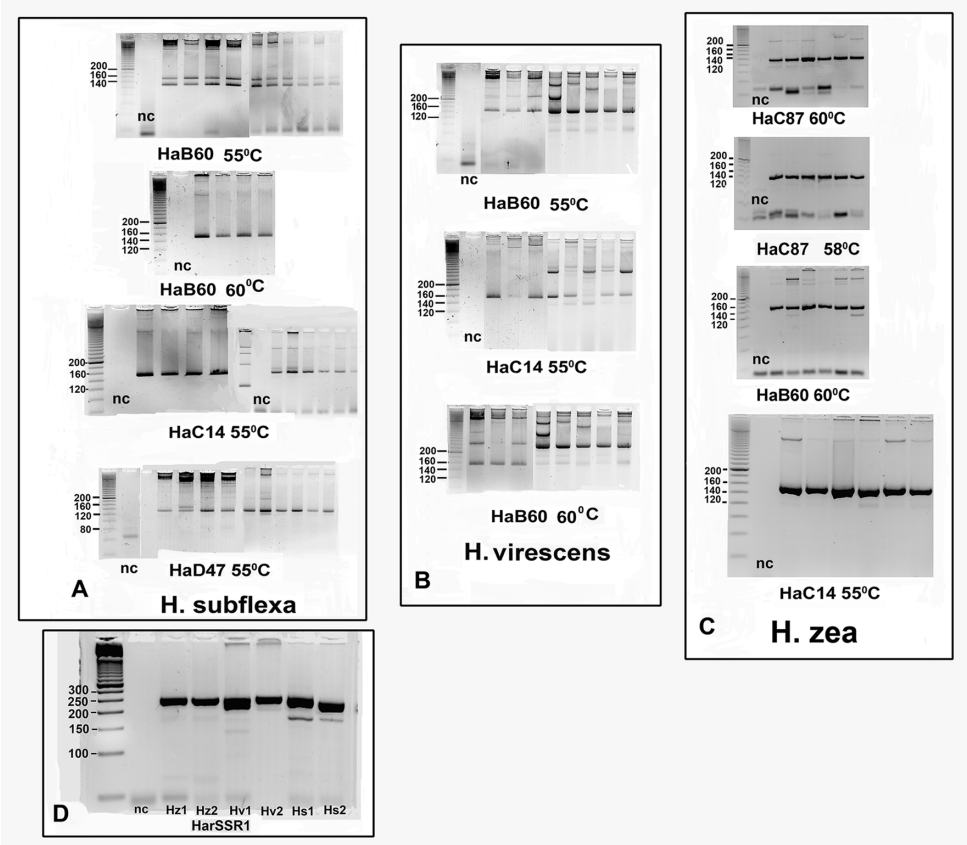
nb= no bands detected; fb = faint bands  
Repetitive sequences are defined as repeated genomic regions containing microsatellite motifs and their flanking regions.

HarSSR1 (240bp), indicating at least for the most part a good portion of the base positions were properly aligned. Based on the aligned regions generated by the T-coffee program ([www.ch.embnet.org/software/TCoffee.html](http://www.ch.embnet.org/software/TCoffee.html)), the identity of the nucleotide sites of the partially sequenced microsatellites relative to *H. armigera* was found to be 78% for all three species at the HaC14 160 allele, 83% for all three species at the HaB60 160 allele, 41% for *H. virescens* and *H. zea* at the HaB60 140 allele, and 84% for *H. zea* and *H. virescens* at the HaC87 118 allele, and 76% for *H. zea* and *H. virescens* at the HarSSR1 240 allele. As indicated in Fig. 3 (A-D) only the downstream primer used in PCR amplification for each locus appeared in the sequence along with the microsatellite marker. However, the upstream primer that would typically be included as part of the 5'-end of the microsatellite marker was not sequenced during the automatic analysis.

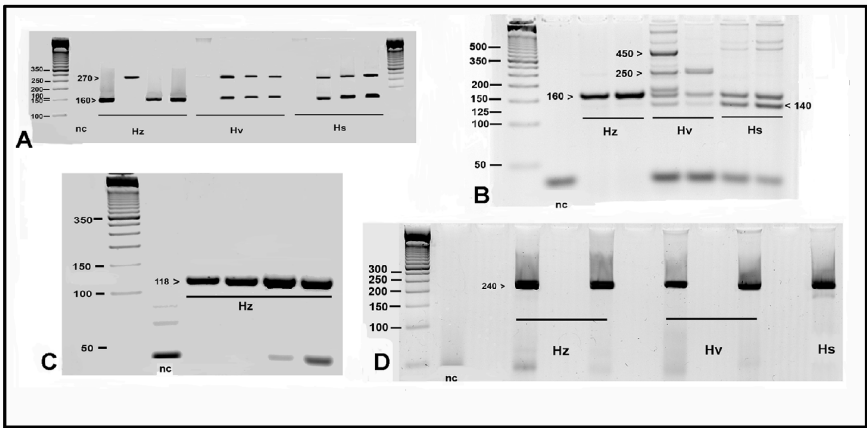
Several reports have shown that comparing allele sizes can result in inaccurate allele size differences for microsatellites (Estoup et al., 1995; Haberl and Tautz, 1999). One can approach this potential problem of size homoplasy by either employing single-strand conformation polymorphism analysis (SSCP) or sequence analysis of the DNA fragments. However, Liepelt et al. (2001) has shown that even sequenced, aligned microsatellites can show differences in repeat numbers occurring among clones and samples from the same individual. Their solution was to split the analyzed complex locus into two new loci. Nevertheless, we chose sequence analysis to determine if our unknown fragments contained not only the microsatellite but also to obtain an overall view of the alignment patterns of the fragments relative to the *H. armigera* markers.

Overall, the alignments of the four microsatellite loci detected in the three species, but with the

**Figure 1.** An assortment of PCR amplifications depicting several potential microsatellite primer pairs. (A) PCR amplification of three single-copy microsatellites from 10 individual *Heliothis subflexa* larvae; (B) PCR amplification of two single-copy microsatellites from eight *Heliothis virescens* larvae; (C) three single-copy microsatellites detected in *H. zea*, the more closely related of the three species to *Helicoverpa armiger* a. Base pair markers are indicated on the left of each gel. The size of specific bands that were sequenced is indicated for each of the microsatellite loci. nc = negative control.



**Figure 2.** Successful identification of PCR amplified single-copy microsatellites from sampled individuals of the three species. (A) HaC14; (B) HaB60; (C) HaC87; and (D) HarSSR1. Hz = *Helicoverpa zea*; Hv = *Heliothis virescens*; Hs = *Heliothis subflexa*. Base pair markers are indicated on the left of each gel. nc = negative control. The size of specific bands that were sequenced is also indicated for each of the microsatellite loci.



**Figure 3.** Partial sequences of the four simple sequence loci. All sequences were aligned employing the T-Coffee multiple sequence alignment package. Microsatellite alleles are shown for (A) HaC14, (B,C) HaB60, (D) HaC87 and (E) HaRSSR1. Bold letters indicate the location of the simple sequence repeat and the box-shaded regions indicate identities. A CORE index for each base position is indicated in the outlined box below each alignment. The primer sequences flanking the loci are shown in lowercase letters.

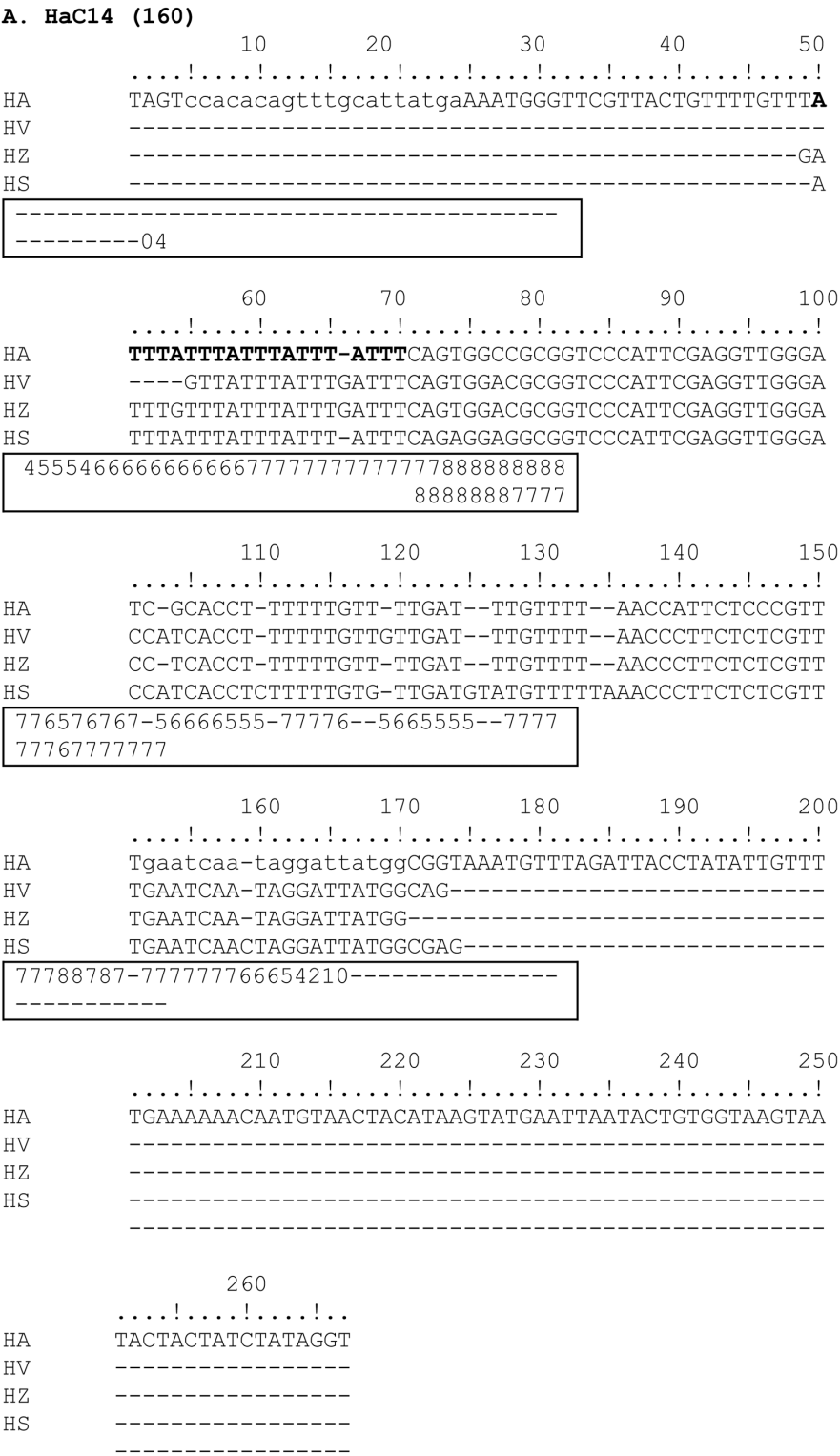


Figure 3 (B).

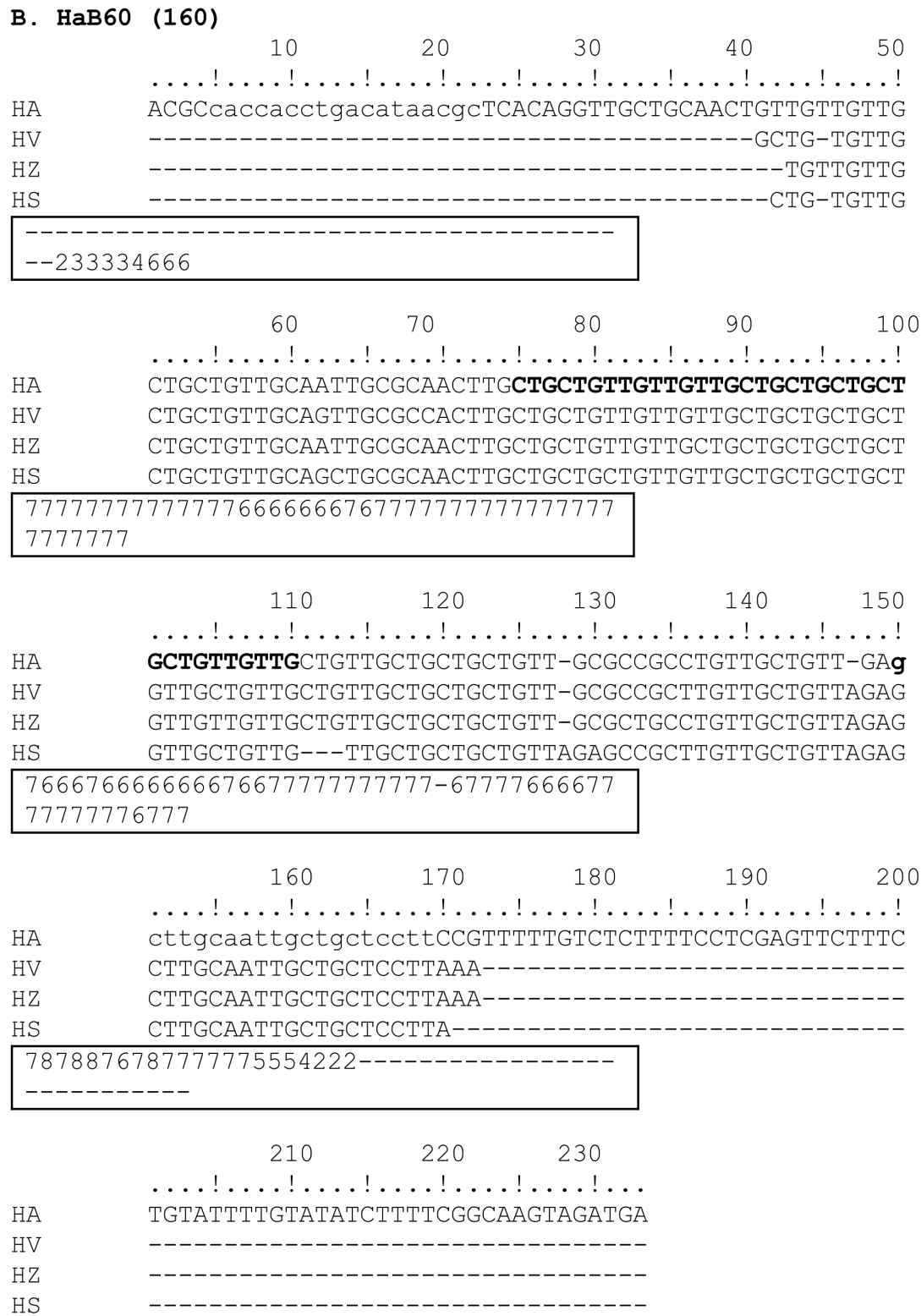




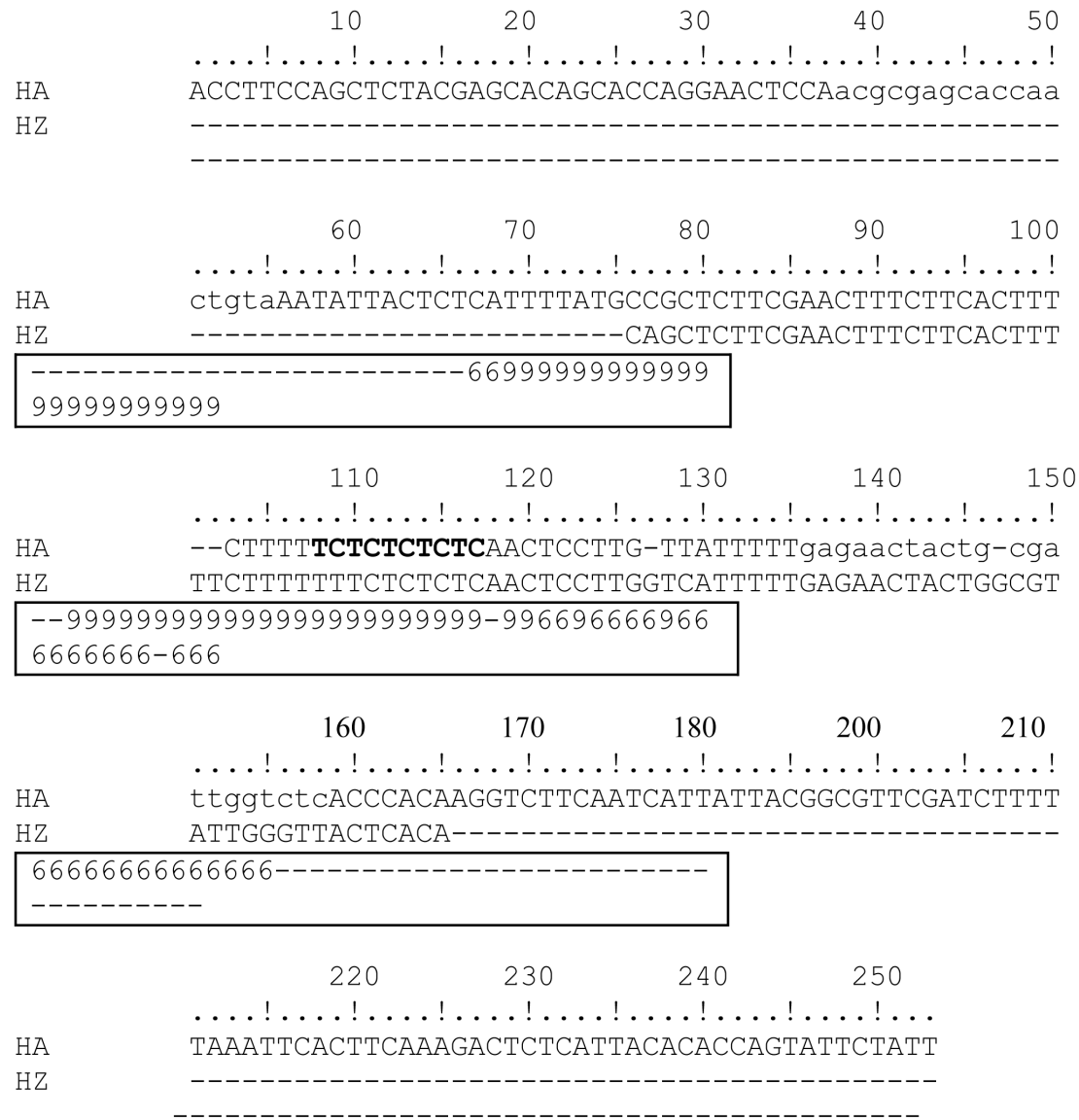
Figure 3 (C).

C. HaB60 (140)

|    |                                                          |                                 |            |                      |        |
|----|----------------------------------------------------------|---------------------------------|------------|----------------------|--------|
|    | 10                                                       | 20                              | 30         | 40                   | 50     |
|    | .....!                                                   | .....!                          | .....!     | .....!               | .....! |
| HA | ACGCcaccac                                               | ctg-acataacgc                   | TCACAGGTTG | CTGCTGCAA-CTGTTGTTGT |        |
| HV | A---CATC-TCGGTACTTGG-----                                |                                 |            | GAGCATCTAGCCCACGGGGA |        |
| HS | -----A-TCGGTACTTGG-----                                  |                                 |            | AGCATCTAGCCAACGGGGA  |        |
|    | 1---1112-23337444443-----                                |                                 |            | 144444444            |        |
|    | 73333333334                                              |                                 |            |                      |        |
|    | 60                                                       | 70                              | 80         | 90                   | 100    |
|    | .....!                                                   | .....!                          | .....!     | .....!               | .....! |
| HA | TGCTGCTGTTGCAATTGCGCAACTTG                               | <b>CTGCTGTTGTTGTTGCTGCTGCTG</b> |            |                      |        |
| HV | GAC-GCTATTACCATGGC-CTTCAT-CT-CTGA-----                   |                                 |            |                      |        |
| HS | GAC-GCTATTACCATGGC-CTTCAT-CT-CTGA-----                   |                                 |            |                      |        |
|    | 444-4555544445444-344444-44-4443-----                    |                                 |            |                      |        |
|    | 444-4555544445444-344444-44-4443-----                    |                                 |            |                      |        |
|    | -----                                                    |                                 |            |                      |        |
|    | 110                                                      | 120                             | 130        | 140                  | 150    |
|    | .....!                                                   | .....!                          | .....!     | .....!               | .....! |
| HA | <b>CTGCTGTTGTTG</b> CTGTTGCTGCTGCTGTTGCGCCGCTGTTGCTGTTGA | g                               |            |                      |        |
| HV | ---CAACAGGAACTA-----                                     |                                 |            | GATT-----GTTGC-      |        |
| HS | ---CAA-AGGAACTA-----                                     |                                 |            | GATT-----GTTGCG      |        |
|    | ---222234444443-----                                     |                                 |            | 2333-----545442      |        |
|    | ---222234444443-----                                     |                                 |            | 2333-                |        |
|    | -----545442                                              |                                 |            |                      |        |
|    | 160                                                      | 170                             | 180        | 190                  | 200    |
|    | .....!                                                   | .....!                          | .....!     | .....!               | .....! |
| HA | cttgcaattgctgctCCTTCCGTTTTGTCTCTTTTCCTCGAGTTCTTTC        |                                 |            |                      |        |
| HV | -TTGCAATTGCTGCTCCTTAA-----                               |                                 |            |                      |        |
| HS | -TTGCAATTGCTGCTCCTTAA-----                               |                                 |            |                      |        |
|    | -66666667777777654322-----                               |                                 |            |                      |        |
|    | -66666667777777654322-----                               |                                 |            |                      |        |
|    | -----                                                    |                                 |            |                      |        |
|    | 210                                                      | 220                             | 230        |                      |        |
|    | .....!                                                   | .....!                          | .....!     |                      |        |
| HA | TGTATTTTGTATATCTTTTCGGCAAGTAGATGA                        |                                 |            |                      |        |
| HV | -----                                                    |                                 |            |                      |        |
| HS | -----                                                    |                                 |            |                      |        |

Figure 3 (D).

D. HaC87 (118)



E. HarSSR1 (240)

```

      10      20      30      40      50
      ....!....!....!....!....!....!....!....!....!
HZ -----
HV -----
HA AAACAAGGACATAGGTTAACAAAGTTATTTACATCAGTAGTTTGTGTGG

      60      70      80      90     100
      ....!....!....!....!....!....!....!....!....!
HZ -----
HV -----
HA GACTCCTGAGTTCCCATTA CTGTT aggtgattgtggctcagTTTTTGAA

      110     120     130     140     150
      ....!....!....!....!....!....!....!....!....!
HZ -----TGTGTGAGTTGCTGCGAATTGCTGTTGCTGTGATTG
HV -----CTG----TGTGATGGTTGCTGTGAATTGCTGTTGTTGTGAA--
HA TTTGATTCTGCTGTTGAGATGGTTGCTGCGA-TTGCTGTTGCTGTGATTG

-----222----344443356666655545666666
    66777766488



      160     170     180     190     200
      ....!....!....!....!....!....!....!....!....!
HZ TTGTTGCTGCTGCTGCTGCTGTTGTTGTTGTTGCTGCTGCTGTTGTTGTT
HV TTGCTGCTGCTGCTGTTGTTGTTGTTGCTGCTGCTGCTGCTGCTGTTGTT
HA TTGTTGCTGCT---GCTGCTGTTGTTGTTGTTGCTGCTGCTGTTGTTGCT

57777777777666766666666666666666667777
    76666666667

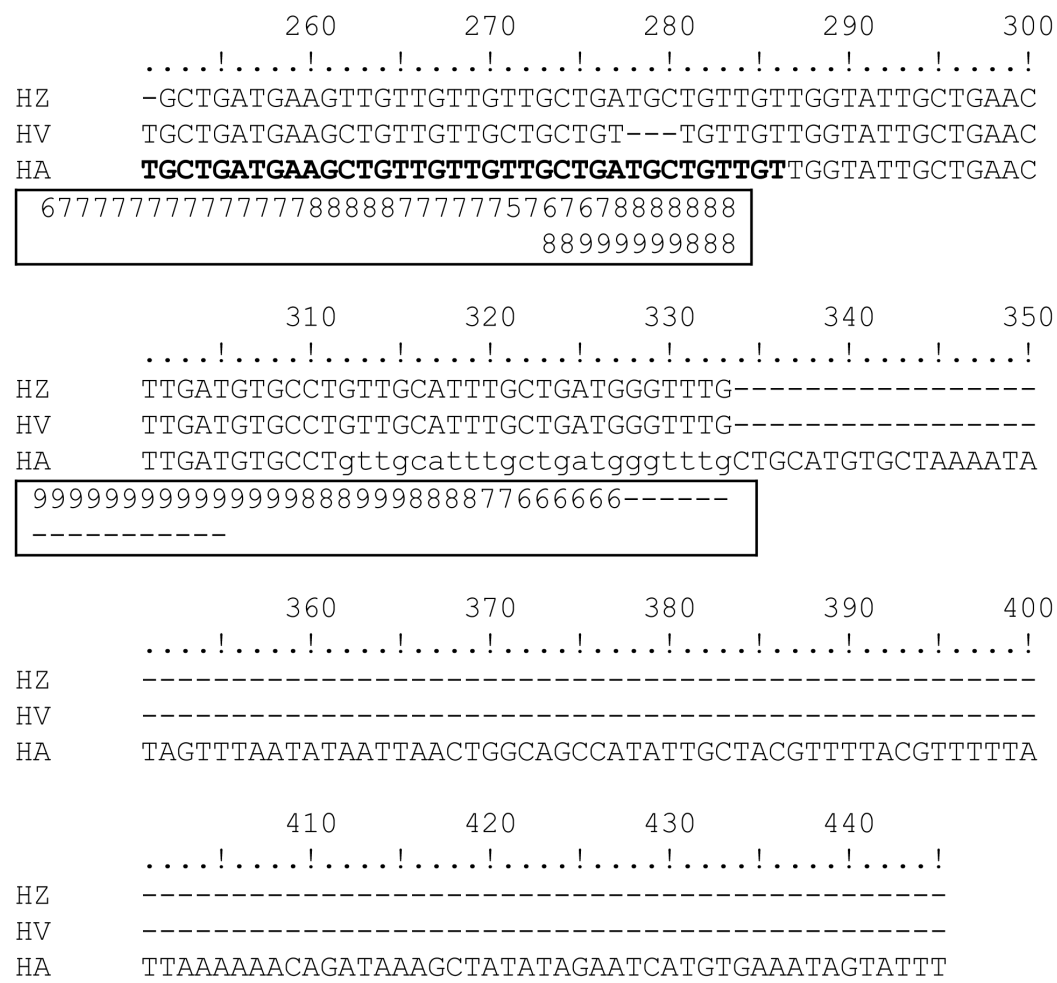


      210     220     230     240     250
      ....!....!....!....!....!....!....!....!....!
HZ GCTGCTGCTGC---TGTTGTTGCTGCTGCTGTTGCT-----
HV GTTGTTGTTGCT---TGTTGTTGCTGCTGCTGTTGCTGTTGTTGTTGCTGC
HA GCTGCTGCTGCTGTTGTTGTTGCTGCTGCTGCTGCTGTTGCTGCTGCTGC

77777777777---77777766666666666666665555
    5555555666


```

Figure 3 (E, con't).



occurrence of some inversions at HaB60, a substitution in HaC87, and deletions in HaC14 and HarSSR1, showed a high number of identical nucleotide sites with the *H. armigera* repetitive motifs (Table 3). The length polymorphism detected in *H. virescens* and *H. subflexa* at the HaB60 locus revealed a large deletion of the repetitive array in the 140 bp allele of both species. However, with the complete sequence of one primer and a partial of the other 5'-end primer contained in the sequence read, it was still deemed to be a factual allele (Fig.3C).

The occurrence of null alleles in microsatellites is known to be an impediment to their successful application as markers in population genetic studies (Pemberton et al., 1995; Schlötterer and Pemberton, 1998; Liewlaksaneeyanawin et al., 2002), and have been implicated as a possible

cause for the low levels of heterozygosity found in Lepidoptera (Meglec et al., 2004). Since only samples collected from laboratory populations were employed in this study, we probably restricted ourselves from determining some level of polymorphism, if any, in the loci studied from the three species, though the number of polymorphic microsatellites to date has been found to be typically low in Lepidoptera (Ji and Zhang, 2004). Given the inherent variability of the microsatellite flanking regions in Lepidoptera, further work, in particular controlled mating studies, will be needed to elucidate the frequency of null alleles in these species.

The specific repetitive nature of the microsatellite flanking regions found in Lepidoptera demonstrates the difficulty of isolating similar microsatellites from closely related species.

**Table 3.** Comparison between four *H. armigera* microsatellites and the repetitive sequences identified in three related lepidopteran species.

| Locus               | Species             | Microsatellite sequence                                                                                     |
|---------------------|---------------------|-------------------------------------------------------------------------------------------------------------|
| HaC14 (160 bp)      | <i>H. armigera</i>  | ATTT ATTT ATTT ATTT ATTT                                                                                    |
|                     | <i>H. zea</i>       | ATTT GTTT TAIT ATTT                                                                                         |
|                     | <i>H. virescens</i> | GTT ATTT ATTT                                                                                               |
|                     | <i>H. subflexa</i>  | ATTT ATTT ATTT ATTT ATTT                                                                                    |
|                     | <i>H. armigera</i>  | CTG CTG TTG TTG TTG (CTG) <sub>5</sub> (TTG) <sub>2</sub>                                                   |
| HaB60 (160 bp)      | <i>H. zea</i>       | CTG CTG TTG TTG TTG (CTG) <sub>4</sub> TTG CTG TTG                                                          |
|                     | <i>H. virescens</i> | CTG CTG TTG TTG TTG (CTG) <sub>4</sub> TTG CTG TTG                                                          |
|                     | <i>H. subflexa</i>  | CTG CTG CTG TTG TTG (CTG) <sub>4</sub> TTG CTG TTG                                                          |
|                     | <i>H. armigera</i>  | CTG CTG TTG TTG TTG (CTG) <sub>5</sub> (TTG) <sub>2</sub>                                                   |
| HaB60 (140 bp)      | <i>H. armigera</i>  | CTG CTG TTG TTG TTG (CTG) <sub>5</sub> (TTG) <sub>2</sub>                                                   |
|                     | <i>H. zea</i>       | -                                                                                                           |
|                     | <i>H. virescens</i> | (CT-) (CTG) (A-)(--)- <sub>6</sub> (CAA)(CAG)(GAA)                                                          |
|                     | <i>H. subflexa</i>  | (CT-) (CTG) (A-)(--)- <sub>6</sub> (CAA) (-AG)(GAA)                                                         |
|                     | <i>H. armigera</i>  | (TC) <sub>5</sub>                                                                                           |
| HAC87 (118 bp)      | <i>H. zea</i>       | TT (TC) <sub>4</sub>                                                                                        |
|                     | <i>H. virescens</i> | -                                                                                                           |
|                     | <i>H. subflexa</i>  | -                                                                                                           |
|                     | <i>H. armigera</i>  | (TGC) <sub>2</sub> GAT (TGY) <sub>4</sub> GAT (TGY) <sub>35</sub> (TGA) <sub>2</sub> AGC (TGY) <sub>8</sub> |
|                     | <i>H. zea</i>       | (TGC) <sub>2</sub> GAAT (TGY) <sub>4</sub> GAT (TGY) <sub>30</sub> AGT (TGY) <sub>8</sub>                   |
| HarSSR1<br>(240 bp) | <i>H. virescens</i> | TGC TGT GAAT (TGY) <sub>4</sub> GAAT (TGY) <sub>31</sub> AGC (TGY) <sub>7</sub>                             |
|                     | <i>H. subflexa</i>  | -                                                                                                           |

|                     |                                                                                                                        |
|---------------------|------------------------------------------------------------------------------------------------------------------------|
| (TGY) <sub>4</sub>  | TGC TGT TGC TGT                                                                                                        |
| (TGY) <sub>30</sub> | TGT TGT TGC TGC TGC TGC TGC TGT TGT TGT TGT TGC TGC TGC TGT TGC TGC TGA<br>TGC TGT TGT TGC TGC TGC TGT TGC TGC TGA TGA |
| (TGY) <sub>8</sub>  | TGT TGT TGT TGC TGA TGC TGT TGT                                                                                        |
| (TGY) <sub>4</sub>  | TGC TGT TGT TGT                                                                                                        |
| (TGY) <sub>31</sub> | TGT TGT TGT TGT TGC TGC TGC TGC TGC TGT TGT TGT TGT TGT TGC TGC TGC TGT<br>TGC TGT TGT TGT TGC TGC TGC TGA TGA         |
| (TGY) <sub>7</sub>  | TGT TGT TGC TGC TGT TGT TGT                                                                                            |
| (-)                 | not detected                                                                                                           |

However, some of the data presented here extends the utility of previously developed microsatellites of one species to closely related members, and has the potential to be used as population genetic markers in other related lepidopteran species.

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