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Source: Journal of Insect Science, 5(47) : 1-13

Published By: Entomological Society of America

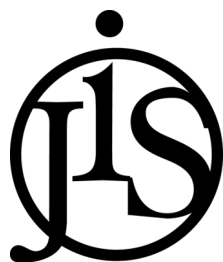
URL: [https://doi.org/10.1673/1536-2442\(2005\)5\[1:CIOHAM\]2.0.CO;2](https://doi.org/10.1673/1536-2442(2005)5[1:CIOHAM]2.0.CO;2)

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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.

Cite this paper as: Grasela JJ and McIntosh AH. 2005. Cross-species investigation of *Helicoverpa armigera* microsatellites as potential markers for other related species in the *Helicoverpa* - *Heliothis* complex. 13pp. *Journal of Insect Science* 5:47, available online: insectscience.org/5.47

Keywords: Lepidoptera, *Helicoverpa armigera*, *Helicoverpa zea*, *Heliothis virescens*, *Heliothis subflexa*, microsatellites
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Received 16 July 2005 | Accepted 20 September 2005 | Published 31 December 2005
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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 2nd or early 3rd instar *H. zea* and *H. virescens* larvae obtained from the North Carolina

State University- Entomology Insectary, Raleigh, North Carolina, whereas 2nd or 3rd 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)₂ (TTG)₃ (CTG)₅ (TTG)₂, (2) (HaC14) -- (ATTT)₅, (3) (HaD47) -- (CA)₅ (TCA)₄, (4) (HaC87) - (TC)₅ (Scott et al., 2004); (5) Ham2 -- (TTTTGA)₉, (6) Ham3 -- (TAAA)₂ (TAAAT)₄, (7) Ham4 -- (TCTG)₆ TCTT (TCTG)₆, (8) Ham5 -- (T)_n (G)_n, (9) Ham6 -- (GAT)₂ TT (GAT)₂ TT.....(AATA)₅ (Tan et al., 2001); (10) HarSSR1 - (TGC)₂GAT (TGY)₄GAT(TGY)₃₅(TGA)₂ AGC(TGY)₈ (11) HarSSR2 - (ATG)₇, (12) HarSSR3 - (TCA)₆, (13) HarSSR4 - (GYT)₂₅, and (14) HarSSR5 - [T(T)AA]₆ (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 to 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; 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 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 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 bp single-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	
<i>H. zea</i>	HarSSR3	600 - multiple bands	1	4	Scott et al., 2004; Tan et al. 2001 (modified)
		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
<i>H. virescens</i>	HaC87	500 - multiple bands	1	2	Scott et al., 2004; Tan et al. 2001 (modified)
		550 - multiple bands	1	4	
		600 - nb	1	4	
<i>H. virescens</i>	HarSSR2	520 - fb	1	2	Tan et al. 2001(modified); Scott et al., 2004
		660 - multiple bands	2	7	
<i>H. virescens</i>	HarSSR3	590 - multiple bands	1	2	Scott et al., 2004
		600 - multiple bands	1	3	
		500 - multiple bands	2	7	
<i>H. virescens</i>	HarSSR4	600 - multiple bands	1	2	Scott et al., 2004
		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
<i>H. virescens</i>	Ham6	550 - nb	1	4	Tan et al., 2001
<i>H. virescens</i>	Ham6	660 - nb	1	3	Tan et al., 2001
<i>H. subflexa</i>	HaC87	500 - multiple bands	1	2	Scott et al., 2004
		550 - multiple bands > 200 bp marker and strongly stained bands at 55, 70, 75 bp markers	2	18	
<i>H. subflexa</i>	HaD47	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>	Ham3	500 - multiple bands	1	2	Scott et al., 2004
<i>H. subflexa</i>	Ham3	530 - multiple bands > 180 bp marker band	2	16	Tan et al., 2001; Tan et al. 2001 (modified)
<i>H. subflexa</i>	Ham6	590 - multiple bands	1	4	Tan et al., 2001
<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), 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.

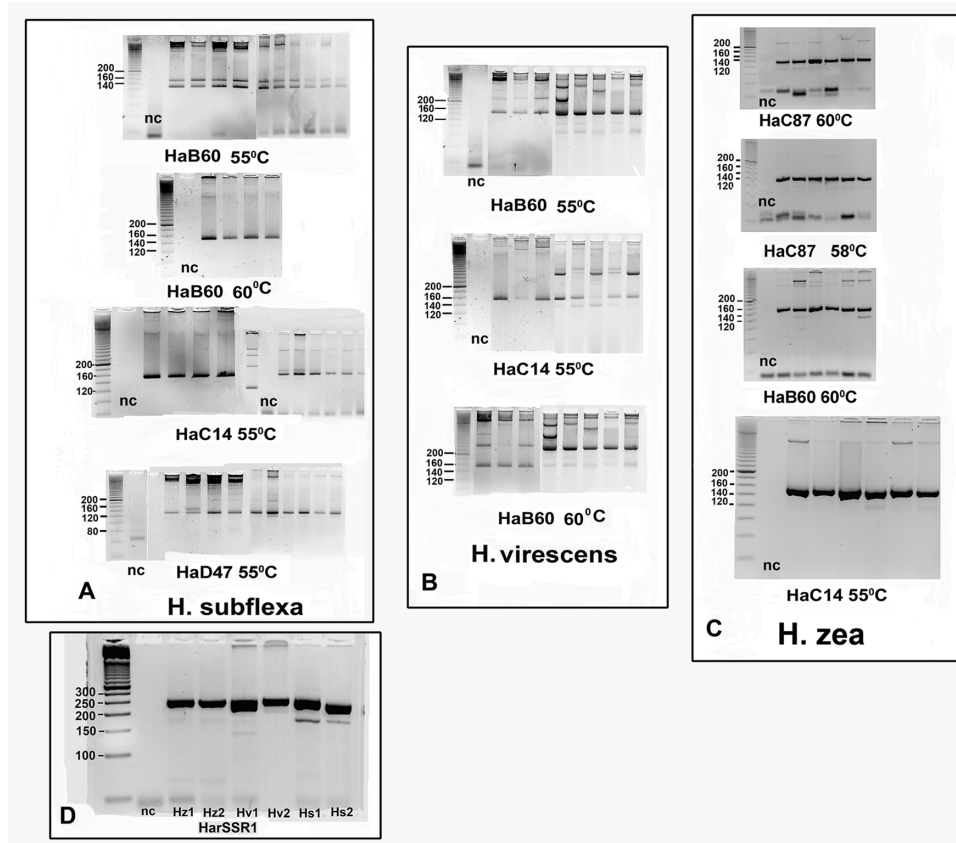


Figure 2. Successful identification of PCR amplified single-copy microsatellites from sampled individuals of the three species. (A) HaC14; (B) HaB60; (C) HaC87; and (D) HarSSR1. Hz = *Helicoverpa zea*; Hv = *Heliothis virescens*; Hs = *Heliothis subflexa*. Base pair markers are indicated on the left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

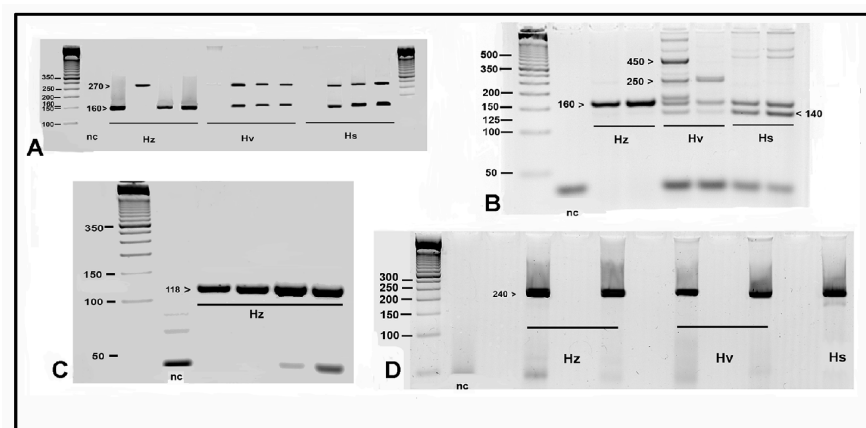


Figure 3 (B).

B. HaB60 (160)

```

          10      20      30      40      50
    ....!....!....!....!....!....!....!....!....!....!....!
HA   ACGCcaccacctgacataacgcTCACAGGTTGCTGCAACTGTTGTTGTTG
HV   -----GCTG-TGTTG
HZ   -----TGTTGTTG
HS   -----CTG-TGTTG
    
```

```

-----
--233334666
    
```

```

          60      70      80      90      100
    ....!....!....!....!....!....!....!....!....!....!....!
HA   CTGCTGTTGCAATTGCGCAACTTGCTGCTGTTGTTGTTGCTGCTGCTGCT
HV   CTGCTGTTGAGTTGCGCCACTTGCTGCTGTTGTTGTTGCTGCTGCTGCT
HZ   CTGCTGTTGCAATTGCGCAACTTGCTGCTGTTGTTGCTGCTGCTGCTGCT
HS   CTGCTGTTGAGCTGCGCAACTTGCTGCTGCTGTTGTTGCTGCTGCTGCT
    
```

```

777777777777766666667677777777777777777
7777777
    
```

```

          110     120     130     140     150
    ....!....!....!....!....!....!....!....!....!....!....!
HA   GCTGTTGTTGCTGTTGCTGCTGCTGTT-GCGCCGCCTGTTGCTGTT-GAGg
HV   GTTGTGTTGCTGTTGCTGCTGCTGTT-GCGCCGCTTGTGCTGTTAGAG
HZ   GTTGTGTTGCTGTTGCTGCTGCTGTT-GCGCTGCCTGTTGCTGTTAGAG
HS   GTTGTGTTG---TTGCTGCTGCTGTTAGAGCCGCTTGTGCTGTTAGAG
    
```

```

766676666666676677777777777-67777666677
77777776777
    
```

```

          160     170     180     190     200
    ....!....!....!....!....!....!....!....!....!....!....!
HA   cttgcaattgctgctccttCCGTTTTTGTCTCTTTTCCTCGAGTTCTTTC
HV   CTTGCAATTGCTGCTCCTTAAA-----
HZ   CTTGCAATTGCTGCTCCTTAAA-----
HS   CTTGCAATTGCTGCTCCTTA-----
    
```

```

7878876787777775554222-----
-----
    
```

```

          210     220     230
    ....!....!....!....!....!....!....!....!....
HA   TGTATTTTGTATATCTTTTCGGCAAGTAGATGA
HV   -----
HZ   -----
HS   -----
    
```


Figure 3 (D).

D. HaC87 (118)

```

          10          20          30          40          50
      ....!.....!.....!.....!.....!.....!.....!.....!.....!.....!
HA      ACCTTCCAGCTCTACGAGCACAGCACCAGGAACTCCAacgcgagcaccaa
HZ      -----
-----

          60          70          80          90          100
      ....!.....!.....!.....!.....!.....!.....!.....!.....!.....!
HA      ctgtaAATATTACTCTCATTTTATGCCGCTCTTCGAACCTTCTTCACTTT
HZ      -----CAGCTCTTCGAACCTTCTTCACTTT
-----66999999999999
999999999999

          110         120         130         140         150
      ....!.....!.....!.....!.....!.....!.....!.....!.....!.....!
HA      --CTTTTTCTCTCTCTCAACTCCTTG-TTATTTTTgagaactactg-cga
HZ      TTCTTTTTTTCTCTCTCAACTCCTTGGTCATTTTTGAGAACTACTGGCGT
-----999999999999999999999999-9966966666966
66666666-666

          160         170         180         200         210
      ....!.....!.....!.....!.....!.....!.....!.....!.....!.....!
HA      ttggtctcACCCACAAGGTCTTCAATCATTATTACGGCGTTCGATCTTTT
HZ      ATTGGGTTACTCACA-----
-----6666666666666666-----
-----

          220         230         240         250
      ....!.....!.....!.....!.....!.....!.....!.....!.....!.....!
HA      TAAATTCACCTTCAAAGACTCTCATTACACACCAGTATTCTATT
HZ      -----
-----

```


Figure 3 (E, con't).

```

                260          270          280          290          300
                .....!.....!.....!.....!.....!.....!.....!.....!.....!.....!.....!
HZ      -GCTGATGAAGTTGTTGTTGTTGCTGATGCTGTTGTTGGTATTGCTGAAC
HV      TGCTGATGAAGCTGTTGTTGCTGCTGT---TGTTGTTGGTATTGCTGAAC
HA      TGCTGATGAAGCTGTTGTTGTTGCTGATGCTGTTGTTGGTATTGCTGAAC
        6777777777777777888887777775767678888888
        88999999888
                310          320          330          340          350
                .....!.....!.....!.....!.....!.....!.....!.....!.....!.....!.....!
HZ      TTGATGTGCCTGTTGCATTTGCTGATGGGTTTG-----
HV      TTGATGTGCCTGTTGCATTTGCTGATGGGTTTG-----
HA      TTGATGTGCCTgttgcatttgctgatgggtttgCTGCATGTGCTAAAATA
        999999999999999888999888877666666-----
        -----
                360          370          380          390          400
                .....!.....!.....!.....!.....!.....!.....!.....!.....!.....!.....!
HZ      -----
HV      -----
HA      TAGTTTAATATAATTAAGTGGCAGCCATATTGCTACGTTTTACGTTTTTA
                410          420          430          440
                .....!.....!.....!.....!.....!.....!.....!.....!.....!.....!.....!
HZ      -----
HV      -----
HA      TTAAAAAACAGATAAAGCTATATAGAATCATGTGAAATAGTATTT

```

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.

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