# Distinguishing Between Laboratory-Reared and Greenhouse- and Field-Collected *Trichoplusia ni* (Lepidoptera: Noctuidae) Using the Amplified Fragment Length Polymorphism Method

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**ABSTRACT** Frequent use of the microbial insecticide, *Bacillus thuringiensis kurstaki* (Berliner) (Bt), in commercial vegetable greenhouses has led to the evolution of resistance in cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), populations. Spatial patterns of Bt resistance suggest that resistant moths disperse from greenhouses selected with Bt to neighboring untreated greenhouses. To quantify dispersal patterns in greenhouse and field populations, molecular markers are desired. We developed a DNA isolation procedure and evaluated the utility of the molecular fingerprinting technique, amplified fragment length polymorphism (AFLP), to analyze the possible population structure of *T. ni* by using laboratory-reared populations. We also assessed the ability of AFLP markers to distinguish between laboratory and wild *T. ni* populations collected from a greenhouse and field in the Fraser Valley of British Columbia, Canada. Due to the complexity of the *T. ni* genome, primer combinations of E+3 and M+4 were required to unambiguously score polymorphic loci. Three of the primer combinations that were examined produced >65 polymorphic bands in laboratory-reared populations, and >90 bands in greenhouse- and field-collected populations. Levels of heterozygosity were higher in wild populations compared with those reared in the laboratory, and AFLP markers reliably distinguished between laboratory and wild populations.

**KEY WORDS** amplified fragment length polymorphism, *Trichoplusia ni*, Bt resistance, population structure, genetic variation

The cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), is an economically important pest in commercial vegetable greenhouses and crucifer field crops in British Columbia, Canada (Janmaat and Myers 2003). The microbial insecticide, *Bacillus thuringiensis kurstaki* (Berliner) (Bt) is frequently used to control *T. ni* in greenhouses. However, the continued use of Bt has been threatened due to the development of resistance (Janmaat and Myers 2003, Franklin and Myers 2008). Surveys of Bt resistance in British Columbia greenhouse populations indicate that resistant moths likely move from greenhouse populations strongly selected for Bt resistance after multiple Bt applications (Dipel and Foray, Valent Biosciences, Libertyville, IL) to untreated greenhouse populations in close proximity (Franklin and Myers 2008). This movement results in elevated levels of Bt resistance in greenhouses that have not been treated with Bt. These patterns of Bt resistance provide indirect evidence for dispersal of resistant moths between greenhouses.

Molecular markers are a potentially valuable tool for quantifying population dispersal. Such markers, with the exception of mitochondrial primer sequences (GenBank, unpublished), have yet to be developed for studying the population structure of *T. ni.* The majority of animal studies have used microsatellite markers, which are highly informative at all loci due to their co-dominant nature (Mariette et al. 2002). The development of microsatellite markers in lepidopteran insects has proven particularly difficult due to a lack of variable loci and a high number of repetitive sequences in flanking regions (Zhang 2004). Therefore, we have chosen to explore the utility of the molecular fingerprinting technique amplified fragment length polymorphism (AFLP) (Vos et al. 1995).

The AFLP technique allows for the amplification of random genomic DNA fragments that are separated based on fragment lengths (Vos et al. 1995). Despite the widespread use of AFLP markers in plant studies, few animal studies and even fewer insect studies have used AFLP techniques (Bensch and Åkesson 2005). AFLP markers are dominant and biallelic and thus suffer the disadvantage of producing poor information content at each locus (Mueller and Wolfenbarger 1999). Sampling a large number of AFLP loci can, however, compensate for the poor information content of each locus (at least 10 times more than a

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codominant marker) (Mariette et al. 2002). This is easily accomplished because each primer combination can usually generate between 50 and 100 restriction fragments (Vos et al. 1995, Behura 2006).

The purposes of this pilot study were to 1) develop a DNA isolation procedure for larval *T. ni* that is suitable for AFLP analysis, 2) screen AFLP primer sets to determine suitable primer combinations for analysis of *T. ni* laboratory populations, and 3) assess the ability of AFLP markers to differentiate laboratory populations from populations collected in a greenhouse and on field crops in British Columbia.

## Materials and Methods

**Insect Material.** Larvae were obtained from six laboratory rearing colonies of *T. ni* that originated from larval collections from vegetable greenhouses in British Columbia in 2001 (GipA, GipB, GlenA, and GlenB), a vegetable greenhouse in 2004 (GH), and a laboratory colony (RC) that had been reared in the laboratory for >15 yr. Wild populations were collected from a tomato greenhouse (49° 05.279′ N, 122° 59.902′ W) on 10 August 2005 and an organic broccoli field (49° 02.450′ N, 123° 03.858′ W) on 8 July 2005 in Delta, British Columbia. The initial screening of AFLP primer combinations was performed using four of the laboratory populations (GipB, GlenA, RC, and GH), whereas three of the laboratory populations (GipA, GlenA, and RC) and the greenhouse and field populations were used to assess the ability of AFLP markers to distinguish between laboratory and wild *T. ni* populations.

Laboratory reared larvae were maintained using methods modified from Ignoffo (1963). Upon emergence, larvae were transferred as groups of 20 to 175-ml polystyrene cups containing artificial wheat germ-based diet and were reared at 26°C under a photoperiod of 16:8 (L:D) h. Greenhouse- and field-collected larvae were reared on artificial wheat germ-based diet for a minimum of 2 d before storage at −80°C to ensure that no plant material was present in their guts.

To avoid misidentification of *T. ni* with the noctuid species, *Autographica californica* (Speyer), which has a similar larval appearance to *T. ni* and is present on field crops and in some greenhouses in British Columbia, *T. ni* larval identification was confirmed by the presence of vestigial prolegs on abdominal segments A3 and A4 and their absence on *A. californica* larvae (Stehr 1987). All larvae, selected at the fifth instar, were killed via decapitation and abdominal segments A9 and A10 were removed. Due to the need for intact DNA molecules, required for AFLP analysis (Reineke et al. 1998), we removed the gut, which contains macromolecule digestive enzymes, by making an incision along the ventral side of the abdomen. All samples were flash frozen in liquid nitrogen and stored at −80°C until analysis.

**DNA Extraction.** DNA was isolated from a total of 24 individuals ($n = 6$ for each GipB, GlenA, RC, and GH) to perform the initial screening of AFLP primer combinations. DNA was extracted from an additional 60 individuals from laboratory populations ($n = 20$ for each GlenA, GipA, and RC) and 12 individuals from both field and greenhouse populations to examine patterns of genetic variation. DNA isolation procedures followed those outlined in Sambrook et al. (1989). The concentration and quality of DNA were determined with a spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Piscataway, NJ), and the quality of the DNA was viewed by using 0.8% agarose gel electrophoresis in 1× Tris-borate-EDTA. DNA was diluted to a final concentration of 100 ng/$\mu$l.

**AFLP Analysis.** AFLP procedures were preformed using the method outlined in Goodwillie et al. (2006). Total genomic DNA (2,000 ng) was digested with EcoRI and MseI (TRU 9 I) (Hoffman-La Roche Limited, Mississauga, ON, Canada), and modified adaptor sequences were ligated to the digest sites with a tailed EcoRI primer site for the preamplification step to improve the clarity of the final gel images for scoring. The tailed primer method was originally designed to create a universal primer based on the M13 phage sequence (LI-COR Inc., Lincoln, NE), which helps with lowering of the cost of infrared primers (Goodwillie et al. 2006) used in the final amplification. The preamplification primers were EcoRI+C and MSEI+A. Amplified fragments were then diluted by 40× and used in the final amplification with the final primer pairs (Table 1); before separation on a LI-COR automated sequencer (LI-COR Inc.).

**Gel Scoring.** Amplified products were denatured and loaded on to a 5.0% polyacrylamide gel, and electrophoresis was performed for 3.5 h on a LI-COR 4200 automated sequencers (LI-COR Inc.). The number of polymorphic bands was scored visually for primer pairs used in the initial screening. Repeatability tests using original tissue samples were performed and 12 of the primer sets were visually inspected to ensure that results were reproducible. Gels from the three primer combinations that were used to examine patterns of genetic variation in laboratory, greenhouse, and field populations were scored using SAGA 2.0 for AFLP bands (LI-COR Inc.) When scoring AFLP fragments, it was assumed that each band corresponded to one locus and comigrating bands shared the same nucleotide sequence. Bands were scored as presence or absence data. Bands that had a strong intensity and were present in >5% of individuals were scored for polymorphism. Faint bands and those present in only a few individuals were excluded from our analyses. Due to the dominant nature of AFLP markers, the presence of a band indicates that the individual is either homozygous (two copies of the allele present) or heterozygous (one copy of the allele present) for that locus and an individual with no band present is considered absent for alleles at that locus (no allele copies present) (Bensch and Åkesson 2005). The absence of a band at a locus may be due to an point mutation, frameshift mutation, or duplication within the restriction site (Kazachkova et al. 2007).

**Data Analysis.** Only loci that were polymorphic (i.e., the band was present in 5–95% of individuals)

**Table 1. AFLP primer combinations and the number of visually scored fragments for the four laboratory *T. ni* populations (GipB, GlenA, RC, and GH) used in the initial screening of AFLP primers**

| EcoRI primer[a] | MseI primer[a] | No. scored fragments |
|---|---|---|
| CACGACGTTGTAAAAC GACGAC TGCGTACCAATTC+CG | GATGAGTCCTGAGT AA+ACCG | 20 |
| CT | AG | |
| | AAG | |
| | ACA | |
| | ACCG | |
| | ACGT | |
| CAA | ACAT | |
| | ACCG | |
| | ACGT | |
| CAC | ACAT | |
| | ACCG | 39 |
| | ACGT | 22 |
| CAG | ACAT | |
| | ACCG | 38 |
| | ACGT | |
| CCA | ACAT | |
| | ACCG | 26 |
| | ACGT | 27 |
| CCC | ACAT | |
| | ACCG | 32 |
| | ACGT | 18 |
| CCG | ACAT | |
| | ACCG | 15 |
| | ACGT | |
| CGA | ACC | |
| | ACAT | 24 |
| | ACCG | 18 |
| | ACGT | 15 |
| | ACTC | |
| | AGCG | |
| | AGCT | 45[b] |
| | ATCC | 24 |
| | ATCG | |
| | ATGA | 30 |
| | ATGG | 51[b] |
| CGC | ACAT | 28 |
| | ACCG | |
| | ACGT | 31 |
| | ACTC | |
| | AGCG | |
| | AGCT | 27 |
| | ATCC | 22 |
| | ATCG | |
| | ATGA | 27 |
| | ATGG | 27 |
| CGG | ACAT | 33[b] |
| | ACCG | 30 |
| | ACGT | 13 |
| | ACTC | |
| | AGCG | |
| | AGCT | 16 |
| | ATCC | 25 |
| | ATCG | |
| | ATGA | 38 |
| | ATGG | 23 |
| CGT | ACCG | |
| | ACGT | |
| CCCC | ACC | |
| | ACG | |
| | AGG | |

Standard primer sequences for EcoRI and MseI primers are listed for the first primer combination (5′-3′). Subsequent primer combinations only differ by their selective nucleotides. The EcoRI primer contains a 5′ tail that is complementary to the M13 sequence (Goodwillie et al. 2006). Primer combinations without scored fragments did not produce bands that could be easily visualized due to either too many or too few polymorphic bands.

[a] Selective nucleotides only at the 3′ prime end of the primer.

[b] Three primer combinations with the highest numbers of polymorphic bands were selected to examine patterns of genetic differentiation among three laboratory populations (GipA, GlenA, and RC) and the greenhouse and field populations.

were used to compare the genetic structure among populations and to perform the cluster analysis. Allele frequencies were estimated in AFLP-SURV 1.0 (Vekemans et al. 2002) with a Bayesian approach assuming Hardy–Weinberg equilibrium and a uniform distribution of allele frequencies to reduce estimation bias from dominant markers (Zhivotovsky 1999). Expected heterozygosities were calculated for each of the three primer combinations and overall in AFLP-SURV 1.0 by using the unbiased estimator of Lynch and Milligan (1994). Wright's pairwise $F_{st}$ values were estimated using AFLP-SURV 1.0 for all population comparisons and 1000 random permutations were performed to test for significant genetic differentiation among populations. Genetic distances were calculated using the minimum distance method for pairwise comparisons of populations (Nei 1972). Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA), and bootstrap support was estimated over 1,000 replicate simulations in the software package TFPGA 1.3 (Miller 1997).

## Results

**Primer Combinations and Levels of Polymorphism Detected.** Sixty selective primer combinations were screened among 24 individuals from four *T. ni* laboratory populations (Table 1). Final amplification with primer combinations of EcoRI +2 selective nucleotides (E+2) and MseI +2 or three selective nucleotides (M+2 or 3) resulted in too many fragments to distinguish on gels. Primer combinations of E+4 and M+3 also resulted in bands that were difficult to visualize. Many primer combinations of E+3 and M+4 and one primer combination of E+2 and M+4 amplified fragments that were easily visualized and were used for the final list of primers chosen for this study. Twenty-nine of these primer pairs produced gels that could be scored (Table 1). The majority of the primer combinations that could be easily distinguished contained the nucleotide sequence CG at the terminal ends of the primers. These primer sets produced between 13 and 51 polymorphic fragments with the majority of bands that could be scored ranging in size from 100 to 550 bp.

From the 29 primer combinations, three primer sets with the most polymorphic bands that could be reliably scored were selected to examine patterns of genetic variation in three *T. ni* laboratory populations (Table 1). In total, 139 fragments were scored among the three primer combinations, with 58, 50 and 31 scoreable loci generated from the following primer sets: E+CGA/M+ATGG, E+CGA/M+AGCT, and E+CGG/M+ACAT, respectively. Of the 139 loci generated 87, 77, and 93 loci were polymorphic from laboratory populations GipA, GlenA, and RC populations, respectively.

**Genetic Variation in Laboratory and Wild Populations.** Field and greenhouse populations had >20% more polymorphic loci and higher heterozygosity than the three laboratory populations surveyed (Fig. 1; Table 2). Mean heterozygosity for laboratory pop-

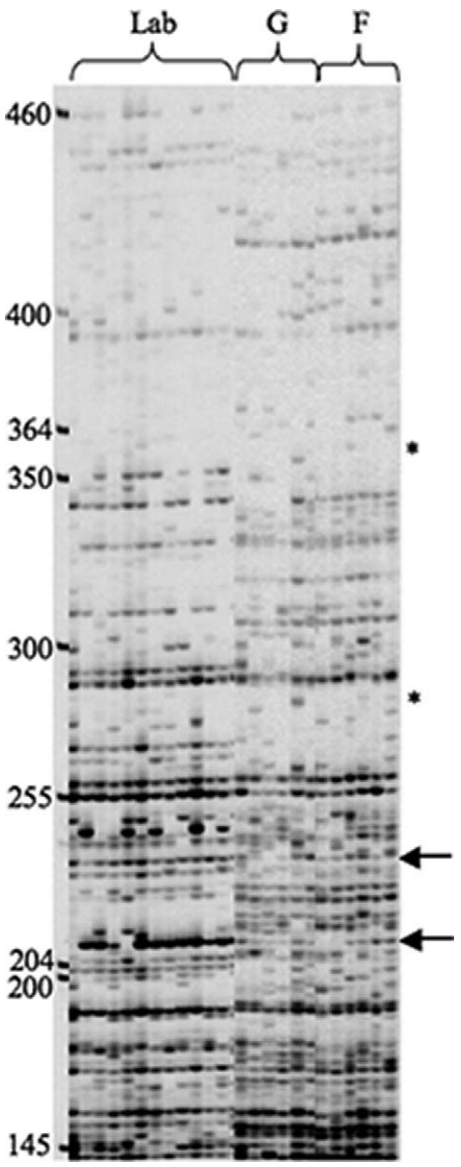


**Fig. 1.** AFLP gel showing fingerprints for 12 laboratory, six greenhouse, and six field *T. ni* individuals by using the primer combination E+CGA and M+AGCT. Size markers are indicated on the left side of the gel. Arrows on the right side of the gel denote two examples of polymorphic loci that were scored for AFLP analysis and asterisks denote two examples of loci that were not be scored because they were faint and could represent potential gel artifacts. Fingerprints for field and greenhouse individuals show a higher level of polymorphism than laboratory reared populations. G and F denote greenhouse and field populations, respectively.

ulations was 0.267, whereas that for greenhouse and field populations was 0.340. Laboratory populations (GipA and GlenA) that had been maintained in the laboratory for 4 yr did not have higher heterozygosity than the laboratory population (RC) that had been reared in the laboratory for >15 yr. These results were consistent with estimates obtained from the analysis of each primer combination separately.

Pairwise $F_{st}$ comparisons indicated that all populations, with the exception of the greenhouse and field populations, were genetically differentiated (Table 3). High levels of differentiation were observed between laboratory and wild-collected greenhouse and field populations ($F_{st}$ = 0.262–0.303, $P < 0.05$), whereas greenhouse and field populations showed no differentiation ($F_{st}$ = 0.0001, $P = 0.11$). The cluster analysis also separated greenhouse and field populations from laboratory populations with a high level of bootstrap support (Fig. 2).

## Discussion

Our results demonstrate that AFLP markers are a valuable molecular tool for the identification of population structure in both laboratory and wild *T. ni* populations. Currently, no other molecular marker has been evaluated to resolve fine scale genetic patterns in *T. ni* populations. AFLP markers are particularly useful for discriminating among populations and identifying population level differentiation due to the large number of polymorphic loci that can be generated genome wide and their high level of reproducibility (Savelkoul et al. 1999). The development of alternative markers, such as codominant microsatellites, would be highly informative, but these markers are expensive to develop (Sunnucks 2000), and their development has proven particularly difficult in lepidopteran species due to a high number of repetitive sequences in flanking regions and multiple copies of microsatellite sequences throughout the genome (Zhang 2004).

Due to the complexity of the *T. ni* genome, primer combinations of E+3 and M+4 were required to produce loci that were easy to score. During the screening process, many of the primer combinations that produced banding patterns that could be reliably scored without too many polymorphic bands included nucleotide combinations of CG. Throughout the genome nucleotide combinations of CG are less common than many other dinucleotides in eukaryotes (Nussinov 1984, Beutler et al. 1989). By selecting primers containing CG combinations, the number of AFLP bands is reduced by over two thirds in insect, fish, and bird species (Bensch and Åkesson 2005). Hence, a possible explanation of the improved resolution of our AFLP gels was the selection of primer combinations containing the CG sequence in either one or both primers.

Differences in AFLP fingerprinting patterns can arise from changes in DNA methylation (Donini et al. 1997), which has been found to change with tissue type and age in many eukaryotic organisms (Regev et al. 1998, Bensch and Åkesson 2005, Ruiz-García et al. 2005). We extracted all DNA from the abdominal tissue of fifth instar *T. ni* to ensure that AFLP fingerprints were not affected by changes in DNA methylation with larval age and tissue type. DNA methylation, however, is absent or rare in many invertebrate species (Regev et al. 1998) and thus may present less of a problem for AFLP analysis in insects. Neverthe-

**Table 2. Summary of genetic diversity for three AFLP primer sets for three laboratory (GipA, GlenA, and RC) and greenhouse and field *T. ni* populations**

| Pop | No. individuals | E+CGA/ M+AGCT | | E+CGG/ M+ACAT | | E+CGA/ M+ATGG | | All loci | |
|---|---|---|---|---|---|---|---|---|---|
| | | $n^a$ | $H^b$ | $N$ | $H$ | $n$ | $H$ | $n$ | $H$ |
| GipA | 20 | 33 | 0.271 | 12 | 0.207 | 31 | 0.261 | 76 | 0.254 |
| GlenA | 20 | 28 | 0.273 | 10 | 0.213 | 30 | 0.280 | 68 | 0.264 |
| RC | 20 | 35 | 0.298 | 11 | 0.213 | 35 | 0.303 | 81 | 0.282 |
| Greenhouse | 12 | 37 | 0.323 | 20 | 0.353 | 40 | 0.341 | 97 | 0.336 |
| Field | 12 | 34 | 0.319 | 21 | 0.373 | 38 | 0.353 | 93 | 0.344 |

[a] *n*, number of polymorphic loci at the 5% level.
[b] *H*, expected heterozygosity under Hardy–Weinberg genotype proportions.

less, we took the precaution of using only fifth-instar larvae.

We observed 20% more polymorphic loci in greenhouse and field populations compared with laboratory populations. The lower heterozygosity in laboratory populations is likely due to the effects of genetic drift that occurred over 4 yr in the laboratory populations GipA and GlenA, and over >15 yr in the RC laboratory population. Our goal in the laboratory is to maintain 200 individuals from each population, but populations have declined to as few as 20–50 individuals when sporadic periods of viral contamination occurred. Furthermore, when moths mate in the laboratory it is probable that the effective population size is smaller than 200 due to the presence of sterile moths, unequal sex ratios, and variation in the number of gametes produced by different individuals (Hedrick 2000). The large reproductive potential of *T. ni* females (up to 1,000 eggs laid per female) (Mitchell and Chalfant 1984) and their ability to mate repeatedly (Landolt 1997) likely allows even small laboratory populations to maintain a moderate level of genetic diversity. In contrast, greenhouse and field populations do not suffer the effects of drift that laboratory populations do, because gene flow among populations and large population sizes likely maintain high levels of genetic diversity.

Pairwise $F_{st}$ values indicated a moderate-to-high level of subdivision among laboratory *T. ni* populations ($F_{st}$ = 0.186–0.253). In agreement with our results, the stored grain pest *Ephestia kuehniella* (Zeller) that is subject to similar containment as laboratory *T. ni* in flour mills reported pairwise $F_{st}$ values ranging from 0.278 to 0.297 (Ryne and Bensch 2008). Furthermore, immobile species, such as *Orchesella cincta* (Collembola) that disperse only a few meters each generation also have $F_{st}$ values in close agreement with those reported for *T. ni* laboratory populations (Timmermans et al. 2005). In contrast, wild-collected greenhouse and field *T. ni* populations showed no differentiation ($F_{st}$ = 0.0001). For other mobile insects, such as the beetle species, *Neochlamisus bebbianae* (Brown) and *Meligethes aeneus* (F.) low-to-moderate levels of differentiation have been observed, depending on the geographic scale in which they are studied (Kazachkova et al. 2007; Egan et al. 2008). $F_{st}$ values for populations of *N. bebbianae* and *M. aeneus* separated by ≈300 km were as low as 0.008. Therefore, it is not surprising that greenhouse and field *T. ni* populations that are highly mobile and separated by <10 km showed no differentiation.

Primer sets that were selected based on the screening of laboratory populations produced too many polymorphic bands for easy evaluation of greenhouse and field populations. Further molecular analysis of greenhouse and field populations would benefit from choosing primer combinations that produced a low number of polymorphic bands during the initial screening of laboratory populations.

Results of our cluster analysis and pairwise $F_{st}$ values indicate that AFLP markers are highly effective for

**Table 3. Pairwise $F_{st}$ values among laboratory (GipA, GlenA, and RC) and greenhouse and field *T. ni* populations**

| Pop | GipA | GlenA | RC | Greenhouse |
|---|---|---|---|---|
| GipA | | | | |
| GlenA | 0.253* | | | |
| RC | 0.186* | 0.212* | | |
| Greenhouse | 0.291* | 0.284* | 0.262* | |
| Field | 0.300* | 0.303* | 0.263* | 0.0001 |

Significance tests are based on the results of 1,000 random permutations.
* Indicates that the populations show significant genetic differentiation at the 5% level.

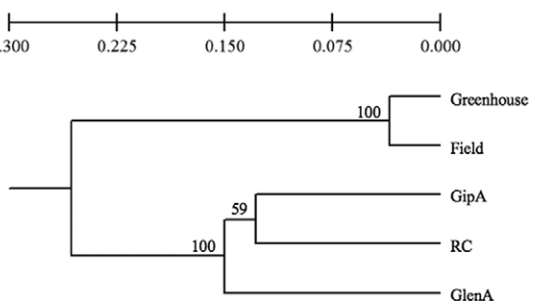


**Fig. 2.** unweighted pair-group method with arithmetic average tree describing the relationship of wild collected greenhouse and field *T. ni* populations and laboratory populations (GipA, GlenA, and RC). The tree was constructed using the minimum distance method (Nei 1972). Bootstrap support (percentage of similar replicates) over 1,000 replicate simulations is indicated above each of the main branches.

distinguishing between laboratory and field and greenhouse populations of *T. ni.* AFLP fragments produced from three primer combinations generated >65 polymorphic loci in laboratory populations and >90 polymorphic loci in greenhouse and field populations. The large number of polymorphic fragments generated through AFLP analysis makes them especially appealing for estimating genetic diversity and studying population structure in wild species where molecular markers are limited (Bensch and Åkesson 2005), as in lepidopteran insects. Our work on the development of DNA isolation procedures and the initial screening of AFLP primers for use in *T. ni* populations provides the foundation for future examination of the population structure of greenhouse and field populations. Furthermore, given the large number of AFLP loci generated genome-wide, it is possible that AFLP markers may assist in the identification of loci that are under selection for Bt resistance.

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