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Using Random Amplified Polymorphic DNA Fingerprinting

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## DISCRIMINATION BETWEEN *HIRSUTELLA LONGICOLLA* VAR. *LONGICOLLA* AND *HIRSUTELLA LONGICOLLA* VAR. *CORNUTA* USING RANDOM AMPLIFIED POLYMORPHIC DNA FINGERPRINTING

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### ABSTRACT

The synnematos, fungal entomopathogen *Hirsutella longicolla* var. *longicolla* is distinguished from a variety *Hirsutella longicolla* var. *cornuta* on the basis of the synnematal morphology. Random Amplified Polymorphic DNA (RAPD) fingerprinting supports the view that these fungi are genetically distinct. Two different banding patterns, one corresponding to isolates of the species and the other to the variety, were generated with two primers and DNA from 19 isolates. Further analysis using nine isolates (four of the species and five of the variety) with three additional primers confirmed the distinction between the species and its variety. This work illustrates the usefulness of the Random Amplified Polymorphic DNA technique in addressing taxonomic questions.

Key Words: fungal entomopathogen, *Hirsutella longicolla* var. *longicolla*, *Hirsutella longicolla* var. *cornuta*, Random Amplified Polymorphic DNA fingerprinting, taxonomy

Fungal species that are pathogenic on plants or animals exhibit variation in many important traits. Variability in morphology can make pathogen identification difficult while physiological variability can affect the evaluation of the potential for damage (virulence) of a particular pathogen. The correct identification of pathogenic species and recognition of physiological strains (pathotypes) is critical information needed to understand the pathogen-host relationship.

Molecular genetic techniques have been used to resolve taxonomic problems and identify strains within pathogenic fungal species (Koch et al., 1991; Kusters-van Someren et al., 1991), to locate genes associated with virulence, and to address questions about the phylogeny and geographical distribution of a pathogen (Bruns et al., 1991). A relatively new molecular technique, Random Amplified Polymorphic DNA (RAPD) fingerprinting (Williams et al., 1990), has been used to characterize strains of the plant pathogen *Leptosphaeria maculans* (Desm.) Ces. & de Not. (Goodwin and Annis, 1991) and *Fusarium solani*

(Mart.) Sacc. f. sp. *cucurbitae* Snyder & Hansen (Crowhurst et al., 1991).

Very few fungal pathogens of insects have been characterized with molecular genetic techniques. Virulence factors in *Beauveria bassiana* (Bals.) Vuill. (Kosir et al., 1991), the relatedness of *B. bassiana* to species of *Tolypocladium* (Rakotonirainy et al., 1991), and the relationship between several *Entomophaga* species (Walsh et al., 1990) have been studied using restriction fragment length polymorphism (RFLP) analysis of genomic or ribosomal DNA.

The genus *Hirsutella* is heterogenous and contains a large number of species pathogenic on insects (see Mains, 1951; Minter and Brady, 1980). The synnematal structure produced by some members of this genus is variable and used only as a secondary characteristic for taxonomic purposes (Mains, 1951). Recently *Hirsutella longicolla* var. *longicolla* Strongman, Eveleigh & Royama was described and a variety *H. longicolla* var. *cornuta* Strongman, Eveleigh & Royama separated on the basis of the synnematal

TABLE I  
*HIRSUTELLA LONGICOLLA* ISOLATES USED FOR RAPD  
 FINGERPRINTING

Isolate No.	Variety	Location <sup>a</sup>	Instar <sup>b</sup>
Hlc1	<i>longicolla</i>	AFES	5
Hlc2	<i>cornuta</i>	AFES	6
Hlc3	<i>cornuta</i>	AFES	6
Hlc4	<i>cornuta</i>	AFES	6
Hlc5	<i>cornuta</i>	AFES	6
Hlc6	<i>cornuta</i>	AFES	6
Hlc7	<i>cornuta</i>	AFES	6
Hlc8	<i>cornuta</i>	AFES	4
Hlc9	<i>longicolla</i>	AFES	3
Hlc10	<i>cornuta</i>	AFES	4
Hlc11	<i>longicolla</i>	AFES	— <sup>c</sup>
H11	<i>longicolla</i>	AFES	5
H12	<i>longicolla</i>	AFES	4
H13	<i>longicolla</i>	AFES	5
H14	<i>longicolla</i>	AFES	6
Hsp1	undetermined	SQ	5
Hsp2	undetermined	SQ	4
Hsp3	undetermined	AFES	—
Hsp4	undetermined	SQ	4

<sup>a</sup> AFES, Acadia Forest Experimental Station, Fredericton, New Brunswick; SQ, St-Quentin, New Brunswick.

<sup>b</sup> All isolates were from spruce budworm except Hsp3 for which the insect species was not determined.

<sup>c</sup> Undetermined instar.

structure (Strongman et al., 1990). The purpose of this work was to confirm, using the RAPD technique, that var. *longicolla* differs genetically from var. *cornuta*.

#### MATERIALS AND METHODS

Isolates of *H. longicolla* var. *longicolla* and *H. longicolla* var. *cornuta* were made from eastern spruce budworm (*Choristoneura fumiferana* (Clem.)) collected over a 3-year period (1986–1988) at two locations, St-Quentin (SQ) in the north and Acadia Forest Experimental Station (AFES) near Fredericton in central New Brunswick, Canada. Isolates were identified from sporulation structures on the budworm cadavers and pure cultures (from hyphal tips) were kept at 4 C on 2% malt extract agar slants. TABLE I shows the identification of the fungal isolates used, the geographic source and the developmental stage of the budworm from which the pathogens were isolated.

For DNA extraction, cultures were prepared by transferring 2.5 ml of an agar stock culture, homogenized in 50 ml of sterile distilled water, into each of three 50 ml volumes of sterile broth composed of K<sub>2</sub>HPO<sub>4</sub> 2 g, peptone 2 g, yeast extract 2 g, malt extract 2 g, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.2 g, NH<sub>4</sub>Cl 3 g and dextrose 20 g in 1 L of distilled water from a Barnstead nanopure II system. Cultures were incubated at 22–25 C on a rotary

shaker (150 rpm) for about 3 wk, and typically yielded 2–3 g (range 1–8 g) of wet mycelium.

DNA was prepared from the entire harvested mycelium as described by Kim et al. (1990) except that the extracts were incubated for 30 min at 37 C in the presence of DNase-free pancreatic RNase A (20 µg per ml; Maniatis et al., 1982) prior to precipitation with ethanol. DNA preparations were dissolved in 2 ml of TE (10 mM Tris, 2 mM EDTA) for use in the amplification reactions.

Amplification conditions were as described in Williams et al. (1990). The 25 µl reaction mixtures contained: 0.5 µl of DNA, 5 µl of a mixture of dATP, dCTP, dGTP and dTTP (0.5 mM of each), 2.5 µl of primer (2 µM), 0.2 µl of *Taq* polymerase (5u/µl) and 2.5 µl of 10× buffer (both from Promega), and deionized water to make 25 µl. A drop of mineral oil was added to each tube and the tubes were briefly centrifuged. An Ericomp Twinblock Thermal Cycler programmed for 45 cycles of 1 min at 94 C, 1 min at 36 C and 2 min at 72 C was used for amplification.

A total of 83 arbitrary sequence oligodeoxyribonucleotide primers were used in this work. Seventy 10-base primers (no. 1–37 and no. 39–71), with five to eight G or C bases, were obtained from Dr. John Carlson, University of British Columbia, Vancouver, Canada. Thirteen nine-base primers (no. 0970-06, -07, -09, -10, -14, -17, -18, -20, -21, -22, -25, -26, and -29) with one to four G or C bases were obtained from Dr. Roland Brosseau, Biotechnology Research Institute, Montreal, Canada. The results presented in Figs. 1–5 were obtained with primers no. 23 (5'-CCCCTTCC-3'), no. 43 (5'-AAAACCGGGC-3'), no. 62 (5'-TTCCCCGTCG-3'), no. 64 (5'-GAGGGCGGGA-3'), and no. 71 (5'-GAGGGCGAGG-3').

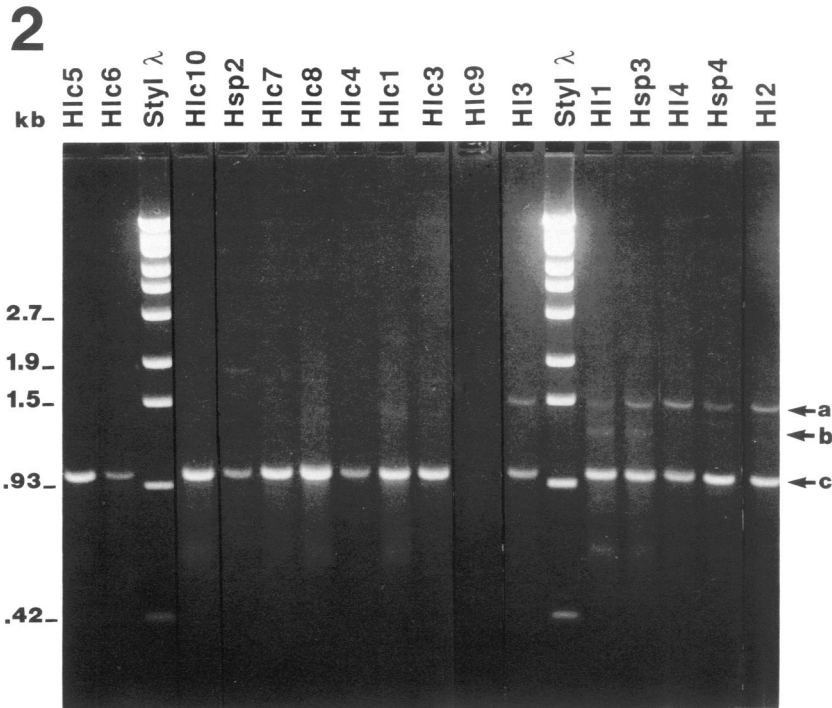
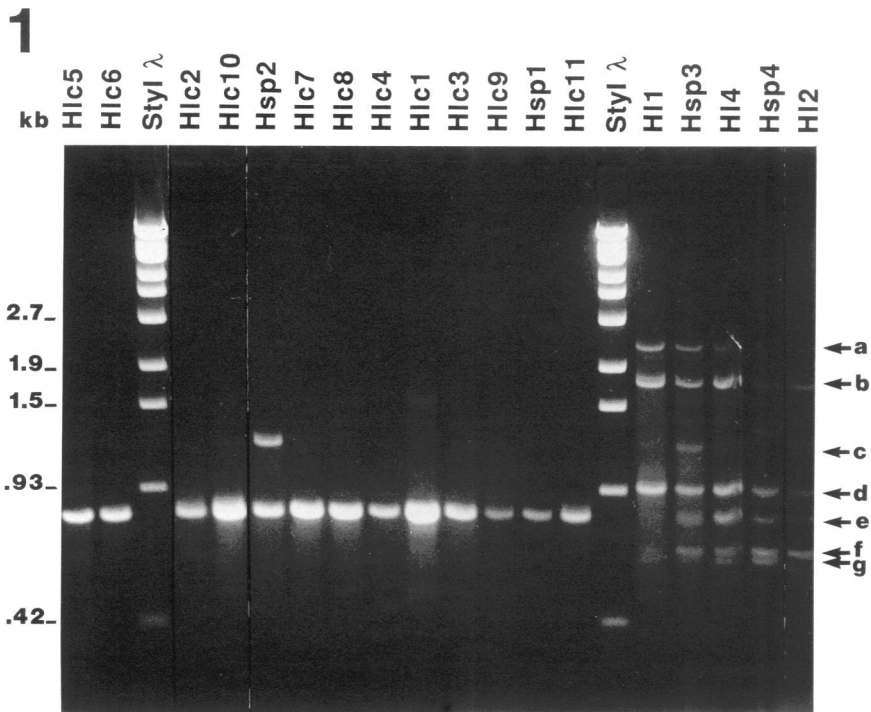
Amplification products were resolved by electrophoresis at 3 V/cm in 1.4% agarose gels submerged in TBE buffer with 0.2 µg of ethidium bromide per ml of buffer (Maniatis et al., 1982).

The patterns of DNA fragments produced by amplification were analyzed by calculating similarity coefficients as described by Gabriel et al. (1988). This coefficient gives double weight to a match of two strong bands, single weight to a match of a strong with a weak band and no weight to a match of two weak bands, and has been recommended for RAPD analysis (Goodwin and Annis, 1991). The legend for Figs. 3–5 indicates which bands were designated as strong or weak for this analysis.

Our study included duplicate DNA preparations from the same batch of fungus and DNA samples from different batch cultures of the same isolate. These checks gave consistent banding patterns, demonstrating the reproducibility of the results.

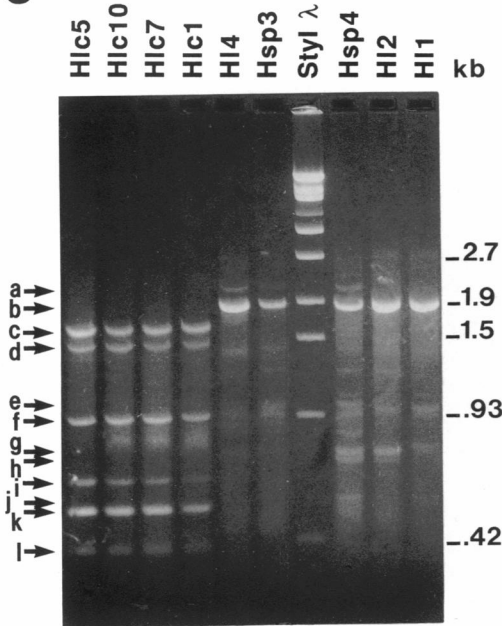
#### RESULTS

We originally screened 83 primers against two DNA samples (Hlc1 and H11) and found that 35 of these produced bands from at least one of the DNAs (data not shown). Two primers, no. 62 and no. 64, were selected for fingerprinting of all the isolates in TABLE I (Figs. 1, 2). Two types of

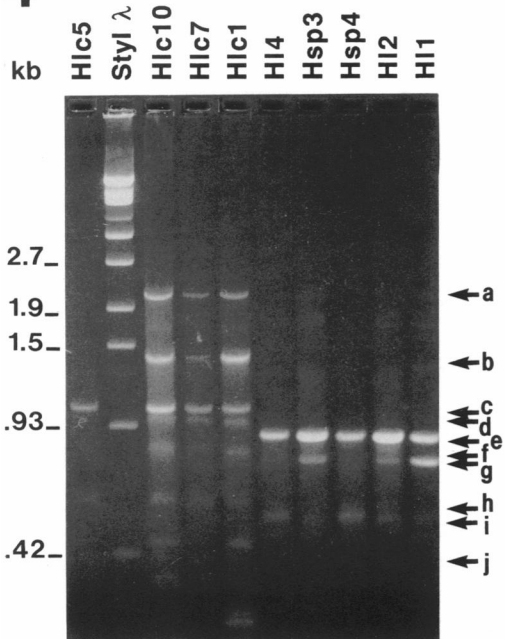


FIGS. 1, 2. Agarose gel electrophoresis of DNA fragments amplified from *Hirsutella* DNAs using arbitrary sequence oligodeoxyribonucleotide primers. 1. *Hirsutella* DNAs with primer no. 62. 2. *Hirsutella* DNAs with primer no. 64. The lanes are labelled according to the isolate names listed in TABLE I. The letters and arrows marked Styl  $\lambda$  are bacteriophage  $\lambda$  DNA digested with the restriction enzyme Styl. The lane marked kb indicates the sizes in kilobase pairs of the  $\lambda$  DNA fragments.

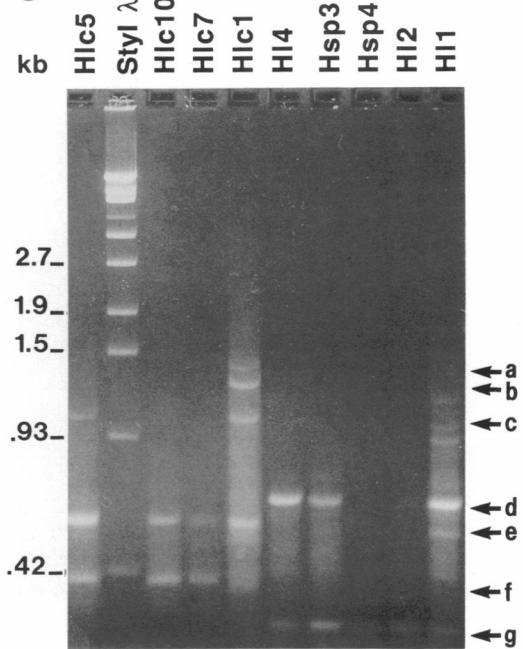
3



4



5



banding patterns were observed with each primer, suggesting the division of the isolates into Group 1 (Hlc1 to Hlc11, Hsp1 and Hsp2 on the left sides of FIGS. 1, 2) and Group 2 (HI1 to HI4, Hsp3 and Hsp4 on the right sides).

FIGS. 3-5. Agarose gel electrophoresis of DNA fragments amplified from *Hirsutella* DNAs using arbitrary sequence oligodeoxyribonucleotide primers. 3. *Hirsutella* DNAs with primer no. 23. 4. *Hirsutella* DNAs with primer no. 43. 5. *Hirsutella* DNAs with primer no. 71. Notations are described in the legend to FIGS. 1 and 2. DNA fragments produced by primer no. 23 (FIG. 3) were used as standards to determine whether other bands would be considered as "strong" or "weak" in the calculation of similarity coefficients (see MATERIALS AND METHODS): If a band was at least as distinct from its background as are band 1 in the Hlc7 lane, band a in the HI4 lane, and band h in the Hsp4 lane, then that band was considered "strong." If a band was less noticeable, then it was "weak."

Four DNA samples from Group 1 (Hlc1, Hlc5, Hlc7 and Hlc10) and five samples from Group 2 (HI1, HI2, HI4, Hsp3 and Hsp4) were fingerprinted with six more primers. Three of these produced data for all nine samples (no. 23, no.

TABLE II

SIMILARITY MATRIX FOR THE RAPD DATA FROM ISOLATES OF *HIRSUTELLA LONGICOLLA* VAR. *LONGICOLLA* AND *H. LONGICOLLA* VAR. *CORNUTA*<sup>a</sup>

	Group 1				Group 2				
	Hlc1	Hlc5	Hlc7	Hlc10	Hl1	Hl2	Hl4	Hsp3	Hsp4
Hlc1		24/29	26/30	31/34	4/26	4/23	5/28	5/31	7/31
Hlc5	0.83		23/25	25/29	4/21	4/18	5/23	5/26	7/26
Hlc7	0.87	0.92		29/30	4/22	4/19	5/24	5/27	7/27
Hlc10	0.91	0.86	0.97		4/26	4/23	5/28	6/31	7/31
Hl1	0.15	0.19	0.18	0.15		14/15	20/20	22/23	19/23
Hl2	0.17	0.22	0.21	0.17	0.93		16/17	19/20	16/20
Hl4	0.18	0.22	0.21	0.18	1.00	0.94		24/25	22/25
Hsp3	0.16	0.19	0.19	0.19	0.96	0.95	0.96		24/28
Hsp4	0.23	0.27	0.26	0.23	0.83	0.80	0.88	0.86	

<sup>a</sup> The fractions in the upper right half of the matrix are the sums of the numbers of strong bands observed for either DNA that match a strong band or a weak band in the other DNA over the sum of the numbers of strong bands observed in each DNA (see legend for Figs. 3–5). The fractions are expressed as coefficients in the lower left half of the matrix.

43 and no. 71; see Figs. 3, 4 and 5, respectively), and the results confirm the distinctiveness of the Group 1 and Group 2 isolates.

Similarity coefficients (Gabriel et al., 1988) were calculated for comparisons of band patterns produced from the nine selected DNAs and the primers no. 23, no. 43, no. 62, no. 64 and no. 71 (TABLE II). The average coefficient for comparisons within Group 1 was 0.89 and for Group 2 it is 0.91. The average coefficient for comparisons between the Groups is 0.20. These results are similar to those of Goodwin and Annis (1991) who distinguished three groups of *Leptosphaeria maculans* isolates by RAPD fingerprinting, obtaining similarity coefficients (calculated as above) of 0.86, 0.71 and 0.76 for intragroup comparisons, and 0.24 for intergroup comparisons.

RAPD analysis showed that three isolates initially identified as var. *longicolla* (Hlc1, 9 and 11) were associated in Group 1 with isolates of var. *cornuta*. Subsequent in vitro sporulation experiments with two of these isolates (Hlc1 and 9) revealed that the initial identifications were incorrect and confirmed the identities determined by RAPD fingerprinting. The original misidentifications were likely a result of using immature synnemata for identification.

Four isolates that could only be identified as *Hirsutella* species, due to the absence of synnemata, were assigned to either var. *cornuta* (Hsp1 and 2) or var. *longicolla* (Hsp3 and 4) on the basis of their Group 1 and Group 2 RAPD banding patterns, respectively. These identifications were not confirmed by in vitro sporulation.

#### DISCUSSION

The separation of *H. longicolla* var. *cornuta* from *H. longicolla* var. *longicolla* is supported by RAPD fingerprinting data. We have shown that, in the case of these taxa, differences in synnematal morphology are useful for separation at the varietal level provided the synnemata are mature. Perhaps synnematal characteristics like the internal hyphal organization and arrangement of the phialides on the synnema as described by Seifert and Okada (1990) would be useful for classification of other *Hirsutella* species.

The isolates used in our study all came from spruce budworm collected at two sites in New Brunswick over 3 years (1986–1988). No clear relationship was detected between this ecological information and Group 1 or Group 2 isolates identified by RAPD analysis. There is some indication that var. *cornuta* is more often associated with the sixth instar and var. *longicolla* with the earlier developmental stages (Strongman, unpubl.). Where the instars are known our RAPD data support this observation; six of 12 isolates from Group 1 (var. *cornuta*) came from sixth instars compared with only one of five from Group 2 (var. *longicolla*). If physiological differences between the sixth instar larvae and the earlier developmental stages are substantial, we postulate that an ancestral *H. longicolla* lineage may have generated the two varieties with var. *cornuta* better adapted for exploiting the sixth instar larvae as a food source.

Can the RAPD technique be used to discriminate among strains within the varieties of *Hir-*

*sutella longicolla*? The primers used in this study show little variability within a group of like DNAs (Figs. 1–5) and most of the variation which does exist may be artifactual due to our failure to detect very faint bands. RAPD fingerprinting with the six primers used in this work may be useful only for discriminating between the varieties but screening with other primers and with more isolates may reveal strain differences. On a broader scope, the technique may also be useful if applied to the multitude of *Hirsutella* species once the range of intraspecific variability is known.

In conclusion, there is a genetic basis for differentiation between *H. longicolla* var. *longicolla* and *H. longicolla* var. *cornuta*. The RAPD technique may prove to be helpful for confirming genetic affinity in taxa grouped by morphological features.

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