BioControl (2007) 52:375–383
DOI 10.1007/s10526-006-9034-4

Genetic diversity in *Hirsutella thompsonii* isolates based on random amplified polymorphic DNA analysis

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Received 13 November 2005; accepted in revised form 14 June 2006

Abstract. *Hirsutella thompsonii* Fisher is a host-specific fungal pathogen of the diverse group of arthropods known as the Acari. It particularly affects eriophyid mites, and the identification of its isolates is a difficult task due to its pleomorphic nature. Seven isolates of *H. thompsonii* var. *thompsonii* and *H. thompsonii* var. *synnematosa* collected from various agro-climatic regions of India were subjected to PCR analysis using random decamer primers to differentiate between them. Random amplified polymorphic DNA analysis revealed that all of the isolates from the citrus rust mite, *Phyllocoptruta oleivora* Ashmead (Acari: Eriophyidae), clustered together, with the exception of one, HtCRMB. The only isolate of *H. thompsonii* from *Aceria guerreronis* keifer (Acari: Eriophydae), HtPDBC, clustered separately.

Key words: Citrus, Coconut, Eriophyidae, *Hirsutella thompsonii*, Mites, RAPD analysis

Introduction

Hirsutella thompsonii Fisher is a specific fungal pathogen of the ticks, mites, and their kin that belong to the group of arthropods known as the Acari, and, particularly, of the eriophyid mites inhabiting numerous host plants in sub-tropical and tropical regions. This fungus show a pronounced pleomorphism in in vitro culture (McCoy, 1996). Ultrastructural analysis of the conidiogenous structures of 11 mononematous and synnematous isolates of *H. thompsonii* has revealed three morphologically distinct groups, which have been taxonomically defined as separate varieties, *H. thompsonii* var. *thompsonii*, var. *vinacea* and var. *synnematosa* (Samson et al., 1980). The variety *thompsonii*, isolated from the citrus rust mite, *Phyllocoptruta oleivora*, is

characterized by grayish-green colonies, whereas the variety *vinacea*, isolated from the blueberry bug mite, *Acalitus vaccinii*, is characterized by vinaceous colonies. The strains of the variety *synnematosa*, which have been isolated from the genus *Eriophyes* and related genera, appear to be restricted to the tropics.

Because of the pleomorphic nature of *H. thompsonii*, identification of the isolates is difficult. The results of an isozyme analysis of 17 distinct geographical isolates of *H. thompsonii* suggested that each of the isolates possesses a distinct isozyme pattern (Boucias et al., 1982) which, based on coefficients of similarity data for the isozyme patterns, closely follows the morphological traits on which the identification of the three *H. thompsonii* varieties is based. The results also revealed that the isozyme patterns of the non-synnematous vinaceous varieties of *H. thompsonii* closely resemble those of the non-synnematous grayish-green varieties.

Molecular methods are becoming standard tools for research in fungal taxonomy and systematics (Saghai-Maroof et al., 1984; Bruns et al., 1991; Kumari et al., 1993; Grube et al., 1995; Nicholson and Parry, 1996; Parry and Nicholson, 1996). Mozes-Koch et al. (1995) characterized six isolates of *H. thompsonii*, one isolate each from *H. necatrix* and *H. kirchneri*, on the basis of their random amplified polymorphic DNA (RAPD) profiles. In the present study we discuss the genetic similarity and morphology of *H. thompsonii* isolates of eriophyid mites affecting citrus and palms, collected from different agro-climatic zones of India.

Materials and methods

Fungus isolates

Fruit and leaf samples were collected from various agro-climatic regions of India. The samples were examined for mites infected with *H. thompsonii* under a stereo-binocular microscope and, when present, the fungus was isolated from the mite according to the procedures described by Villalon and Dean (1974) and cultured on potato dextrose agar (PDA) at 24 ± 3°C. Pure cultures of the fungus isolates were obtained after two to three subcultures, with 72 h between each transfer.

Seven isolates of *H. thompsonii* were collected (Table 1).The width of the hyphae and the length of the basal and neck parts of phialide

Table 1. Name and origin of *Hirsutella thompsonii* isolates and their morphological characters following 14 days of culture on PDA

Isolates	Origin of the isolates			Phialide		Hypha width[a] (μm)	Synnemata
	Mite host	Plant host	Location	Length[a,b] (μm)	Length of neck[a](μm)		
HtCRMB	*P. oleivora*	Citrus fruits	Bangalore, Karnataka	9.5–11.5 c	2.5–4.0	3.0–4.0	Absent
HtEMC	Unknown	Unknown	Chandigarh, Panjab	7.0–10.0 d	2.5–4.5	2.5–4.0	Absent
HtPMG	*A. guerreronis*	Palmyra nuts	Govindapuram, Tamil Nadu	8.0–11.5 c	2.5–4.0	2.5–3.5	Present
HtCRMC	*P. oleivora*	Citrus fruits	Chettahalli, Karnataka	10.0–16.0 b	2.5–7.0	2.5–4.0	Present
HtPDBC	*A. guerreronis*	Coconut	Bangalore, Karnataka	8.0–11.5 c	2.5–3.5	2.5–4.0	Absent
HtCRMK1	*P. oleivora*	Citrus leaf	Kolkata, West Bengal	10.0–17.0 a	2.5–7.5	2.5–4.5	Absent
HtCRMK2	*P. oleivora*	Citrus leaf	Kolkata, West Bengal	10.0–16.5 a,b	2.5–7.5	2.5–4.5	Absent

[a]Values are the averages of 30 measurements.
[b]Values followed by the same letter within the column are not significantly different at $p = 0.05$ according to Duncan's multiple range tests.

in PDA medium were measured under the microscope (Table 1). These measurements were compared with the descriptions provided by Samson et al. (1980) for *H. thompsonii* isolates.

DNA isolation

Fungal mycelia grown on potato dextrose broth were harvested and the DNA extracted as described by Porebski et al. (1997) with suitable modifications. Five grams of fungal mycelia finely ground with acetone powder was incubated with the extraction buffer for 1 h at 65°C with intermittent shaking. Following the addition of 6 µl of chloroform:isoamyl alcohol (24:1, v/v), the suspension was mixed well by gentle inversion and then centrifuged at 6000 rpm for 15 min at 4°C. The supernatant was repeatedly washed with chloroform:isoamyl alcohol (24:1, v/v) until a clear supernatant was obtained. A half volume of 5 *M* NaCl was added and mixed thoroughly. About 2.0 ml of the suspension was incubated with one volume of propanol at 4°C overnight to precipitate the DNA. The DNA pellet was recovered by centrifugation at 10,000 rpm for 20 min, then washed with 70% ethanol, air-dried and stored at −20°C until further use.

RAPD analysis

The PCR reactions were carried out in a reaction mixture (final volume: 20 µl) containing 25 ng DNA, 2 µl of 10× buffer, 200 µ*M* of dNTPs (0.8 µl of 5 m*M*), 1 U *Taq* DNA polymerase, 2 m*M* $MgCl_2$ and 5 pmol of each primer. Amplification was achieved in a MJ Research thermocycler (model PTC-100; MJ Research, Waltham, Mass.) programmed for an initial denaturation cycle of 95°C for 5 min, followed by 45 cycles of 94°C for 1 min (denaturation), 35 °C for 1 min (primer annealing), 72°C (primer extension) for 2 min, with a final extension of 10 min at 72°C. The PCR reactions were repeated three times using the same conditions to check the repeatability of the amplification products both within and between reactions. Seven primers from Operon Technologies (Alameda, Calif.) out of the 60 tested produced strong, intense and unambiguous bands; these were selected for analyzing the isolates of *H. thompsonii* (Table 2). The amplified products were resolved by electrophoresis on 1.4% agarose gel containing ethidium bromide (0.5 µg/ml) in 1× TBE buffer (Sambrook et al., 1989) at a constant voltage of 50 V. The gels were

Table 2. List of primers and their products generated through amplification with seven isolates of *H. thompsonii*

Primer	Sequence	Number of bands on gel	Number of polymorphic bands	Number of monomorphic bands	Polymorphism (%)	Total number of bands
OPA-03	5′-AGTCAGCCAC-3′	15	13	2	86.67	52
OPA-04	5′-AATCGGGCTG-3′	13	13	0	100.00	38
OPA-20	5′-GTTGCGATCC-3′	7	7	0	100.00	18
OPB-01	5′-GTTTCGCTCC-3′	16	15	1	93.75	44
OPB-10	5′-CTGCTGGGAC-3′	10	8	2	80.00	41
OPC-06	5-GAACGGACTC-3′	7	7	0	100.00	20
OPC-19	5′-GTTGCCAGCC-3′	9	7	2	77.78	36

subsequently visualized under UV light and documented using a Hero Lab Gel Documentation Unit.

Statistical analysis

Each reproducible band was visually scored as "1" for presence and "0" for absence, and the binary data were used for statistical analysis. Only clear and unambiguous bands were taken into account, and those bands which were faint or diffused were not scored. The band sizes were determined by comparison with the DNA ladder marker (0.5--5 kb). The data were analyzed with STATISTICA package. The dissimilarity matrix was developed using Squared Euclidean Distance (SED) that estimated all pairwise differences in the amplification product (Sokal and Sneath, 1973).

Results and discussion

H. thompsonii isolates exhibited differences with respect to a number of morphological characters (Table 1). Only the HtPMG and HtCRMC isolates of *H. thompsonii* produced synnemata (Table 1). Based on this character two distinct groups could be distinguished morphologically: (1) the non-synnematous greyish-green isolates; (2) the synnematous grayish-green isolates. Accordingly, we were able to classify these isolates into two varieties: *H. thompsonii* var. *thompsonii*, which includes the HtCRMG, HtEMC, HtCRMK1, HtCRMK2 and HtPDBC isolates, and *H. thompsonii* var. *synnematosa*, including the HtCRMB and HtPMG isolates. In general, these findings are similar to those reported by Samson et al. (1980), although this latter group found three morphologically distinct groups among 11 isolates of *H. thompsonii* – a non-synnematous greyish-green strain, a non-synnematous vinaceous strain and synnematous greyish-green strains – which they proposed be placed in three varieties: *H. thompsonii* var. *thompsonii*, var. *vinacea* and var. *synnematosa*, respectively. The general morphological measurements of these isolates are similar to those of the *H. thompsonii* isolates isolated from the citrus rust mite by Fisher (1950).

The selected primers generated a total of 249 bands, with the polymorphic variation ranging from 77.78 to 100% (Table 2). The number of bands produced per primer ranged from 18 to 52, with an average of 5.1 bands per primer for each isolate, and the PCR products ranged in size from 300 up to 2800 bp.

The dendrogram constructed on the basis of the cluster analysis revealed three major groups A, B and C (Figure 1). Cluster A included four isolates of *H. thompsonii*: HtCRMK1, HtCRMK2, HtCRMC and HtEMC. Among these, HtCRMK1 and HtCRMK2 were closely related with 95% similarity. The HtCRMC of this cluster was related to HtCRMK1 and HtCRMK2 with 84 and 87% genetic similarity, respectively. The HtEMC isolate in this cluster formed another subgroup with 72% similarity. Cluster B included two isolates, HtPMG and HtCRMB, that had a genetic similarity of 78% and was separated from cluster A by 32%. The HtPDBC isolate of *H. thompsonii* obtained from *A. guerreronis* formed a separate cluster, C, and showed 63% genetic similarity with other isolates.

The phialide and phialide neck in the HtCRMK1, HtCRMK2 and HtCRMC isolates were significantly longer than those of the other isolates of *H. thompsonii* (Table 1). As the dendrogram shows, these three isolates were grouped in cluster A and collected from *P. oleivora* infesting citrus, while the other isolates were classified in the other two clusters. The two isolates collected on *A. guerreronis* were in separate clusters; however, one isolate collected from Bangalore on citrus rust mite was closer to this group, indicating the possibility of some cross-infectivity between eriophyid hosts in this fungus. Based on morphological characters, the two isolates HtCRMC and HtPMG were identified as belonging to var. *synnematosa*, and the others being placed in var. *thompsonii* (Samson et al. 1980; Aghajanzadeh, 2003). According to this analysis HtCRMC is closely related to HtCRMK1 and HtCRMK2, while HtPMG is related to HtCRMB. These results suggest that the differentiation of the RAPD pattern may not be immediately associated with morphological characteristics (Boucias et al., 1982).

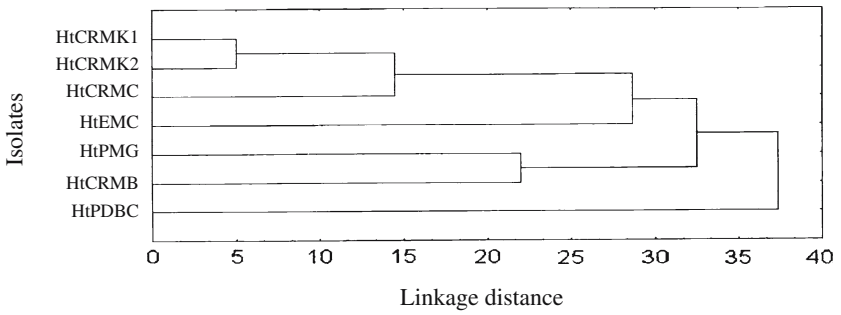

Figure 1. Dendrogram showing the genetic distance between different isolates of *Hirsutella thompsonii* based on RAPD profiles.

Acknowledgements

The authors acknowledge the financial support and the granting of a PhD scholarship by the Agricultural Research, Education and Extension Organization, Ministry of Jihad-e-Agriculture, Islamic Republic of Iran (to SA), and the financial support from the Indian Council for Agricultural Research, Government of India (to BM) through NATP.

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