Genetics and Molecular Biology, 29, 4, 715-721 (2006)
Copyright by the Brazilian Society of Genetics. Printed in Brazil
www.sbg.org.br

Research Article

Genetic diversity of *Hirsutella thompsonii* isolates from Thailand based on AFLP analysis and partial β-*tubulin* gene sequences

Myrian S. Tigano[1], Byron Adams[2], Saowanit Maimala[3] and Drion Boucias[4]

[1]*Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica, Final, Brasília, DF, Brazil.*
[2]*Department of Microbiology & Molecular Biology, Brigham Young University, Provo, UT, USA.*
[3]*Department of Entomology, Kasetsart University, Bangkok, Thailand.*
[4]*Department of Entomology and Nematology, University of Florida, Gainesville, FL, USA.*

Abstract

Amplified fragment length polymorphism (AFLP) was used to investigate polymorphism among 43 *Hirsutella thompsonii* isolates (33 from Thailand) obtained from various mite species. The outgroups were an unidentified *Hirsutella* isolate along with *Hirsutella nodulosa* and *Hirsutella kirchneri*. Phylogenetic analyses of the AFLP data showed significant variation among isolates and the existence of three *H. thompsonii* clades. We also investigated the isolates using PCR with specific primers for the *Hirsutella* exotoxin gene *Hirsutelin A* (*HtA*) and 18 of these isolates were used for sequencing of the partial β-*tubulin* gene. Phylogenetic analyses of β-*tubulin* sequences showed two distinct *H. thompsonii* clades, one of which included AFLP clades I and II. For both markers grouping of the *H. thompsonii* isolates was not related to either host mite species or geographical origin, although for the *HtA* gene one clade contained only isolates with no detectable *HtA* band. These results confirm the high intraspecific polymorphism of *H. thompsonii*, and maximum likelihood analysis showed no monophyletic group within this species. To refine the taxonomy of this genus other studies should be undertaken using additional molecular markers and several other *Hirsutella* isolates.

Key words: entomopathogenic fungus, mites, molecular markers.

Received: October 18, 2005; Accepted: February 14, 2006.

Introduction

The genus *Hirsutella* (Hypocreales) contains a variety of fungal pathogens isolated from numerous mite, nematode, and insect hosts (McCoy, 1996; Jaffee, 2000; Chandler *et al.*, 2000; Van der Geest *et al.*, 2000). For example, the *Hirsutella* species *H. nodulosus, H. citriformis* and *H. gigantean*, infect lepidopterans, hemipteran and dipterans producing structures composed of a compact group of erect conidiophores (synnemata) while *H. rhossiliensis* and *H. minnesotensis* infect plant-parasitic nematodes. The most wide-studied species from this genus is the mite-specific pathogen *Hirsutella thompsonii* (McCoy, 1981), a mononematous species that has been separated into three morphologically distinct groups consisting of the tropical variety *synnematosa*, the subtropical variety *vinacea* and the temperate variety *thompsonii*. This fungus has a worldwide distribution on different eriophyoid and tetranychid mite hosts and during hot, humid weather can cause spectacular natural epizootics among mite populations (*e.g.* citrus rust mites, blueberry, coconut and tomato mites, etc.) and is considered to be a major natural enemy of various mite pests (Chandler *et al*., 2000).

Isolates of *H. thompsonii* have displayed phenotypic plasticity *in vitro* and certain isolates produce multiple conidial states (McCoy, 1996). Isoenzyme analyses of 15 geographically diverse *H. thompsonii* isolates has shown a high degree of intra-specific variability even among morphologically similar *Hirsutella* isolates (Boucias *et al.*, 1982). Maimala *et al.* (2002) reported that the major hosts of *H. thompsonii* are eriophyoid mites displaying specific host plant preferences and that many *H. thompsonii* isolated from a range of eriophyiids varied in their ability to produce exotoxins such as Hirsutellin A (HtA). More recently, (Maimala, 2004) reported extensive plasticity in the growth characteristics of *H. thompsonii* isolated from a range of eriophyiids and one may speculate that the variation exhibited by *H. thompsonii* strains may have co-evolved with the specific mite-plant interactions.

Send correspondence to Myrian S. Tigano. Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica, Final Av. W/5 Norte, 02372, 70770-900 Brasília, DF, Brazil. E-mail: myrian@cenargen.embrapa.br.

A major objective of the work described in this paper was to determine if the variation observed in *H. thompsonii* is linked to phenotypic plasticity or genotypic variation. Amplified fragment length polymorphism (AFLP) were used to examine *H. thompsonii* isolates exhibiting a spectrum of phenotypic differences and the results were complimented by an analysis of *HtA* and β-*tubulin* gene data.

Material and Methods

Fungal strains

In this study we used 43 *Hirsutella thompsonii* Fisher isolates collected from various mite species, 33 isolates having been collected from various mite species inhabiting different plants in various regions of Thailand (Table 1, Maimala *et al*., 2002). The outgroups consisted of *Hirsutella* isolate 624 (unidentified to species level), *Hirsutella nodulosa* and *Hirsutella kirchneri*, from the United States Agricultural Research Service (USDA-ARS) Entomopathogenic Fungal Collection.

DNA extraction

Isolates were inoculated into 250 mL flasks containing 40 mL of malt extract broth (glucose, 1%; malt extract 0.3%; peptone 0.5%; yeast extract, 0.3%) and incubated at 25 °C at 250 revs min^{-1} for 4 days. Mycelial samples were collected on Whatman number 1 filter-paper using vacuum filtration, frozen at -70 °C, lyophilized and stored at -20 °C. About 25 mg of the lyophilized mycelia of each sample were frozen in liquid nitrogen, crushed in a mortar and the total genomic DNA extracted using a modification of the cetyltrimethylammonium bromide (CTAB) DNA extraction protocol of Boucias *et al.* (2000a).

AFLP analysis

For each sample, 0.3-2.0 μg of genomic DNA was digested with *EcoRI* and ligated to *EcoRI* adaptors in a single overnight reaction at 37 °C (Suazo and Hall, 1999). The digestion-ligation reactions were diluted with TE buffer to a final volume of 200 μL and stored at -20 °C. A series of six 19-mer primers (synthesized by Gemini Biotech Houston, TX) were used, consisting of the *EcoRI* adapter sequence GACTGCGTACCAATTC plus three 3' selective nucleotides (AGG, AGT, GGC, ACC, CGA and GGG). The amplification reactions were conducted in a volume of 25 μL containing the anchor-annealed *EcoRI* fragments, primer and *Taq* DNA polymerase using the conditions established by Suazo and Hall (1999) and the PCR products separated by electrophoreses on 1.5% (w/v) agarose-synergel (0.7% (w/v) agarose and 0.4% (w/v) synergel; Diversified Biotech, Boston, MA), stained with ethidium bromide and photographed under UV light.

Analysis of the β-*tubulin* and *Hirsutelin A* genes

Partial β-*tubulin* sequences were PCR amplified using primers designed from consensus regions of selected fungal, algal and protozoan β-*tubulin* genes, the primers being βtubF 5' TGGGCYAARGGYCACTACACYGA 3' and βtubR 5' TCAGTGAACTCCATCTCRTCCAT 3' (Tartar *et al*., 2002). PCR reactions were performed in a final volume of 25 μL containing 1 μL of genomic DNA, 0.4 μM of primers, 200 μM of dNTPs, 1x *Taq* recommended buffer, and 1 unit of *Taq* DNA polymerase (Fisher Scientific, Pittsburg, PA). The cycling program was 1 min at 94 °C and then 30 cycles (1 min denaturation at 94 °C followed by 1 min annealing at 55 °C and 2 min extension at 68 °C) with a final 6 min elongation step at 68 °C. The PCR products were purified using a QIAquick PCR purification kit (QIAGEN Inc., Santa Clara, CA) and then sent to the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida for sequencing.

The *HtA* gene was PCR amplified from the different DNA samples using the primers DGB35 (5'-CATATGAA AGCCTTTACTGCCATTCTC-3') and DGB14 (3'-ATCT CAGAACCAACGAGCCTAGG-5') (Maimala *et al*., 2002). The PCR conditions were the same as for the β-*tubulin* gene amplification, except that the annealing temperature used for these primers was 60 °C. The PCR products were separated by electrophoreses on 1% (w/v) agarose gel and visualized using ethidium bromide staining.

Phylogenetic analyses

Bands from AFLP analysis were scored as present or absent using digitized photographs of the gels. Experiments were repeated at least once, and only DNA fragments consistently present were recorded and considered to be binary characters. DNA fingerprints of each isolate were converted into a binary matrix. The data were assumed unordered with no *a priori* weighting.

The partial β-*tubulin* gene sequences were initially aligned with GenBank data retrieved using the Clustal X program version 1.83 (Thompson *et al*., 1997) and the alignments refined visually using the MacClade program version 4.02 (Sinauer Associates, Inc., Sunderland, MA, USA). Three homologous GenBank β-*tubulin* gene sequences from the fungi *Neurospora crassa* (M13630) *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (U14138) and *Cordyceps bassiana* (AY366063) were included in the alignment. Gene genealogies were reconstructed using the distance-based, neighbor joining (NJ; Saitou and Nei, 1987), and the discrete character-based, maximum parsimony (MP) and maximum likelihood (ML), algorithms included in the PAUP* program version 4.0b10 (Swofford, 2002). Maximum parsimony analysis was performed using a heuristic search including 1,000 bootstrap replicates of random addition sequences and the

Table 1 - Data on the *Hirsutella* isolates examined in this study. The *HtA* gene was detected using the PCR reaction (- no band; + weak band; ++,normal band) and the AFLP and β-*tubulin* clades (I, II, III and IV) were constructed by maximum parsimony tree analysis of AFLP and partial β-*tubulin* gene sequence data (NA = not analyzed and an asterisk (*) represents the isolates standed separated from the clades).

Hirsutella species and isolate number	Host mite	Isolation location	Year isolated	*HtA* gene	AFLP clade	β-*tubulin* clade
H. thompsonii isolates						
13	*Phyllocoptruta oleivorus*	Yala, Thailand	1991	-	I	I
18 II	*P. oleivorus*	Nan, Thailand	1991	+	*	II
21	*P. oleivorus*	Phrae, Thailand	1991	-	II	II
1707	*P. oleivorus*	Chanthaburi, Thailand	1999	-	II	NA
H4	*P. oleivorus*	Pathumthani, Thailand	1992	-	II	NA
HT72	*P. oleivorus*	USA	1972	++	I	NA
HTF74	*P. oleivorus*	USA	1974	++	I	NA
HTF75	*P. oleivorus*	USA	1975	++	I	I
HTF87	*P. oleivorus*	USA	1987	++	I	NA
HTFPB	*P. oleivorus*	USA	Unknown	++	I	NA
HTDowAl	*P. oleivorus*	USA	Unknown	++	I	NA
HTDowPh	*P. oleivorus*	USA	Unknown	++	I	NA
JAB04	*P. oleivorus*	Brazil	Unknown	++	I	NA
NC	*Acalitus vaccinii*	USA	Unknown	++	I	NA
884	*Phyllocoptruta musae*	Chai Nat, Thailand	1998	++	I	NA
887	*Phyllocoptruta malligai*	Chai Nat, Thailand	1998	++	I	NA
1289	*P. malligai*	Trat, Thailand	1999	++	II	NA
1962	*Phyllocoptruta* sp.	Nakhon Pathom, Thailand	2000	++	II	NA
2089	*Phyllocoptruta* sp.	Phitsanulok, Thailand	2000	++	I	NA
898	*Aculops caricae*	Chai Nat, Thailand	1998	++	I	NA
966	*A. caricae*	Chon Buri, Thailand	1998	++	I	NA
1730	*A. caricae*	Chanthaburi, Thailand	1999	-	II	II
1764	*A. caricae*	Chanthaburi, Thailand	1999	++	II	NA
885	*Aculops cratevi*	Chai Nat, Thailand	1998	++	II	NA
2121	*Aculops grabrati*	Phitsanulok, Thailand	1900	++	I	I
3B	*Vilaia cythereae*	Nonthaburi, Thailand	1990	++	II	NA
1840	*Vilaia pamithus*	Chiang Mai, Thailand	1999	++	II	II
225	*Vilaia strebli*	Nakhon Pathom, Thailand	1996	++	II	NA
1690	*V. strebli*	Rayong, Thailand	1999	++	II	II
915	*V. strebli*	Chai Nat, Thailand	1998	-	III	III
2077	*V. strebli*	Uttrradit, Thailand	2000	-	III	III
968	*Vilaia morindae*	Chon Buri, Thailand	1998	-	III	III
1722	*Vilaia thaianae*	Chanthaburi, Thailand	1999	-	III	III
2057	*Vilaia sandorici*	Phitsanulok, Thailand	2000	-	*	III
2110	*V. sandorici*	Phitsanulok, Thailand	2000	-	*	III
873	*Vasates spondiasi*	Chai Nat, Thailand	1998	++	I	I
1401	*V. spondiasi*	Nakhon Pathom, Thailand	1999	++	I	NA
1614	*V. spondiasi*	Chachoengsao, Thailand	1999	++	II	NA
Pink	*Aculus pelekassi*	USA	Unknown	++	I	NA
2109	*Aculus menoni*	Phitsanulok, Thailand	2000	-	II	II
1305	*Aculus* sp.	Trat, Thailand	1999	++	II	NA
1813	*Aculus* sp.	Chiang Mai, Thailand	1999	-	I	NA
1773	*Aculus* sp.	Nakhon Pathom, Thailand	1999	-	III	III
Outgroups						
H. kirchneri ARSEF5550	*Lolium perenne*	United Kingdom	1981	-	NA	IV
H. nodulosa ARSEF5473	*Dioryctria zimmermani*	USA	1995	-	NA	IV
Hirsutella isolate 624	*Vilaia elaeocarpi*	Chanthaburi, Thailand	1997	-	NA	IV

tree-bisection-reconnection branch swapping algorithm with the steepest descend option not in effect. The NJ tree was obtained using the mean-character difference for the AFLP dataset and the Log Determinant distance measure for the β-*tubulin* sequences, each using 1,000 bootstrap replicates. The ML analysis was applied only to the β-*tubulin* sequences dataset. The GTR+G model favored by ModelTest program version 3.06 (Posada and Crandall, 1998) was used for the ML searches which were conducted heuristically with 100 bootstrap replicates.

Results

The *HtA gene* PCR amplification with *HtA* gene-specific primers confirmed the results previously obtained by Maimala *et al*. (2002). A consistent ~600 bp band (data not shown) was produced by 67% of the *H. thompsonii* isolates (HtA+), 17% of which produced weak bands (Table 1). The *Hirsutella* species other than *H. thompsonii* showed no detectable *HtA* bands.

The AFLP analysis of the 43 *H. thompsonii* isolates and *Hirsutella* isolate 624 (Table 1) with the five different primers produced identical AFLP profiles for isolates HTF75 and HTF87 and also for isolates 1707 and H4, so in these cases only the profiles of isolates HTF75 and 1707 were used in the final analyses. The amplification of the anchor-ligated *EcoRI* fragments with all the primers used produced an array of scoreable bands with abundant polymorphisms that were resolved on ethidium bromide-stained agarose synergels (Figure 1). The number of polymorphic AFLP bands selected varied from 25 to 39 per primer and their sizes ranged from ~250 to 3,000 bp. A total of 183 fragments were scored as AFLP markers. No single fragment was found to be amplified in all of the 42 isolates analyzed (*i.e.*, no fragment was monomorphic) and 161 were parsimony-informative characters. The topologies of the NJ and MP trees were identical, so only the parsimony tree is shown in Figure 2. Three clades (I, II and III) were identified, with the *Hirsutella* isolate 624 and three *H. thompsonii* isolates (18 II, 2057 and 2110) being outliers which remained independent from the clades. In both MP and NJ analyses clades I and III were well defined with bootstrap support (bs) of 86%-100%, while clade II was poorly supported and included two groups with bs of 79-84%. Each of these groups contained subgroups that were supported by bootstrap values from NJ and MP analysis. Interestingly, the AFLP clades were not related to original host or geographical location (Table 1, Figure 2). For example, seven *H. thompsonii* isolates obtained from the mite *Phyllocoptruta oleivorus* were included among the nine isolates from the USA (Table 1) which all grouped in clade 1, while three isolates from the same host species were placed in clade II. Furthermore, the well-supported Clade III included isolates from different regions from Thailand and from various hosts (Table 1, Figure 2). In several instances, clade designation seemed to be correlated with the expression of the *HtA* gene (Table 1). For example, all of the members in clade III did not produce the *HtA* PCR product (HtA$^-$) whereas clade I contained mainly HtA$^+$ isolates (17 out of 19) while clade II contained a mix of HtA$^+$ and HtA$^-$ isolates (Table 1, Figure 2).

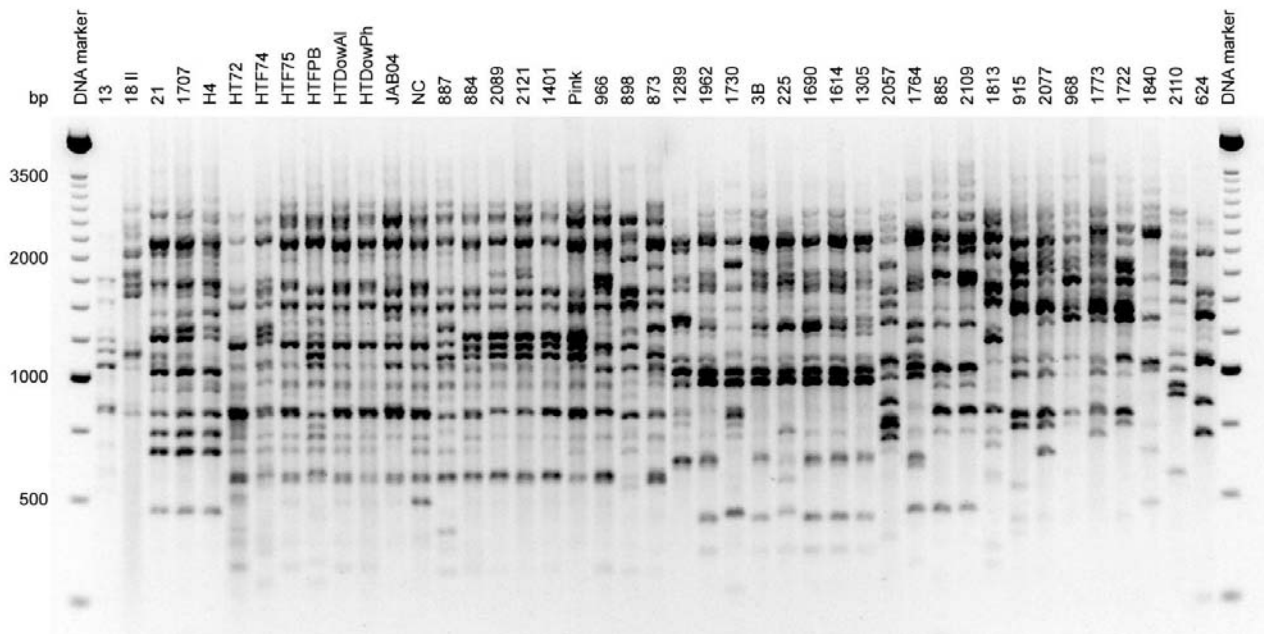

Figure 1 - Example of ethidium bromide-stained AFLP reaction products electrophoresed on 1.5% agarose-synergel. The first and last lanes represent the 250 bp DNA Ladder (Invitrogen).

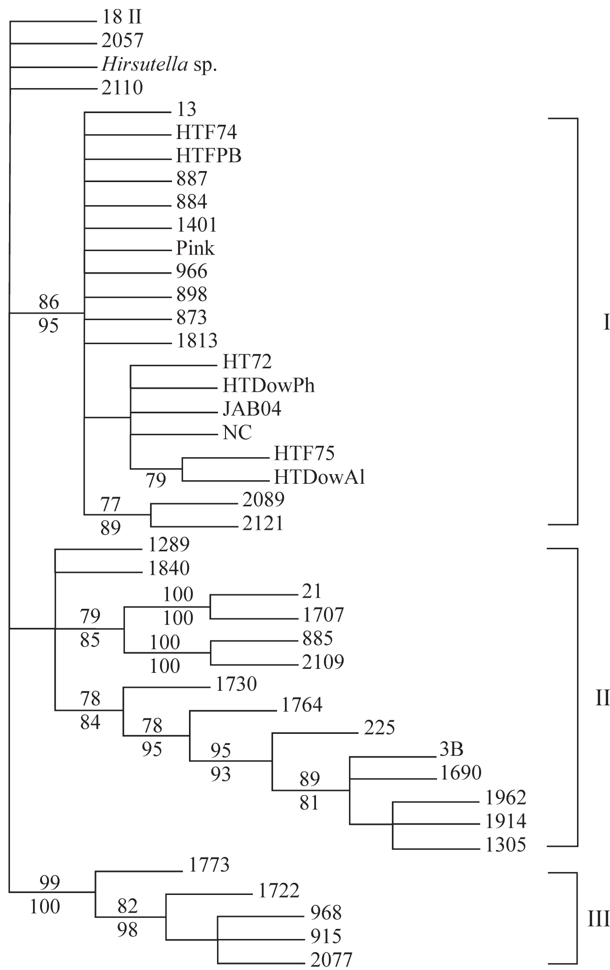

Figure 2 - Maximum parsimony (MP) tree based on AFLP characters. This tree also represents the topology obtained with the neighbor joining (NJ) analysis. Numbers next to the branches are bootstrap percentage values, based on 1,000 replicates and obtained with MP (above) and NJ (below) analyses. The outgroup is *Hirsutella* isolate 624.

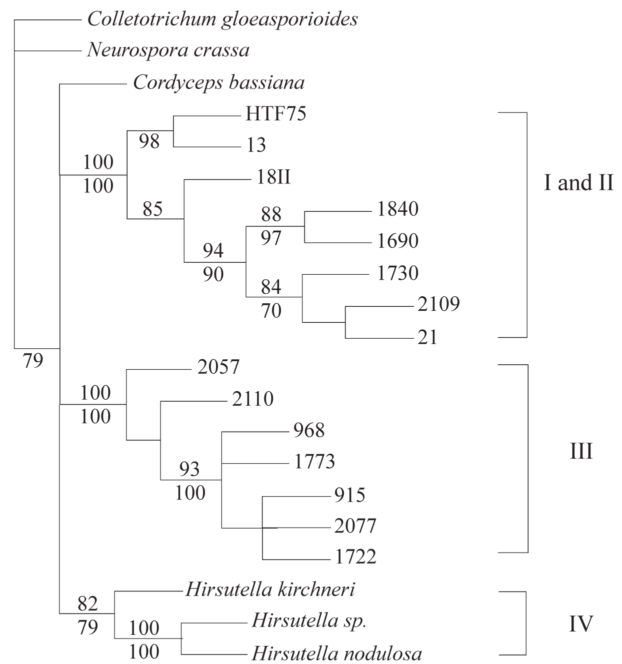

Figure 3 - Maximum parsimony (MP) tree based on β-*tubulin* sequences. This tree also represents the topology obtained with the neighbor joining (NJ) analysis. Numbers next to the branches are bootstrap percentage values, based on 1,000 replicates and obtained with MP (above) and NJ (below) analyses. The outgroups are *Neurospora crassa* and *Colletotrichum gloeosporioide*.

The β-*tubulin* sequences analyses showed that the βtubF and βtubR primers amplified a segment of the β-*tubulin* gene that includes 'intron F' from the *Neurospora crassa* β-*tubulin* gene sequence (GenBank M13630). The partial β-*tubulin* sequences obtained from the 20 *Hirsutella* isolates analyzed consisted of 750 (isolate 2109) to 805 (isolate 1730) bp. Identical sequences were obtained for isolates HTF75, 873 and 2121 so only the HTF75 sequence was included in subsequent analyses. The 57 bp related to the intron was not included in the alignment of the sequences. The final alignment contained 681 positions, of which 186 were variable and 124 were parsimony-informative characters. The sequences have been deposited in GenBank. Phylogenetic trees generated by both distance and parsimony methods had similar topologies, and are represented by the maximum parsimony tree (MP) shown in Figure 3 which shows four clades (I to IV). Some of the isolates that were in clade I and II in the trees obtained with the AFLP dataset were shown to be in a strongly supported group (100% bs for both NJ and MP analyses) that included the well-supported clade I (98% bs) and clade II (85% bs). The maximum likelihood tree (ML) showed a slightly different topology, showing also clades I and II as a unique clade, but a close relationship (77% bs) between clade III and clade IV (Figure 4).

Discussion

Our research showed that, in general, *H. thompsonii* isolates obtained from various mite species showed high polymorphism for the molecular markers studied. Amplified fragment length polymorphism (AFLP) has proved to be a powerful DNA marker technique to evaluate polymorphism among isolates of *H. thompsonii* as well for other fungi (Boucias *et al.*, 2000a, b). Based on AFLP analyses, the different *H. thompsonii* were grouped into three clades uncorrelated with either location or mite species. Different strains isolated from the same mite species were often found in two or more of the delineated clades (Table 1). Similarly, no correlation was found between phenotypic data (Maimala *et al.*, 2002; Maimala, 2004) and clade designation, with, for example, isolates 873 (clade I) and 1690 (clade II) showing both polyblastic conidia and synnemata whereas other isolates (13, 1614, 1707, 1722, 1730, 1764 and 1813), distributed in all three clades, produce polyblastic conidia without synnemata (Maimala *et al.*, 2002). Comparisons of other phenotypic data, such as plant host,

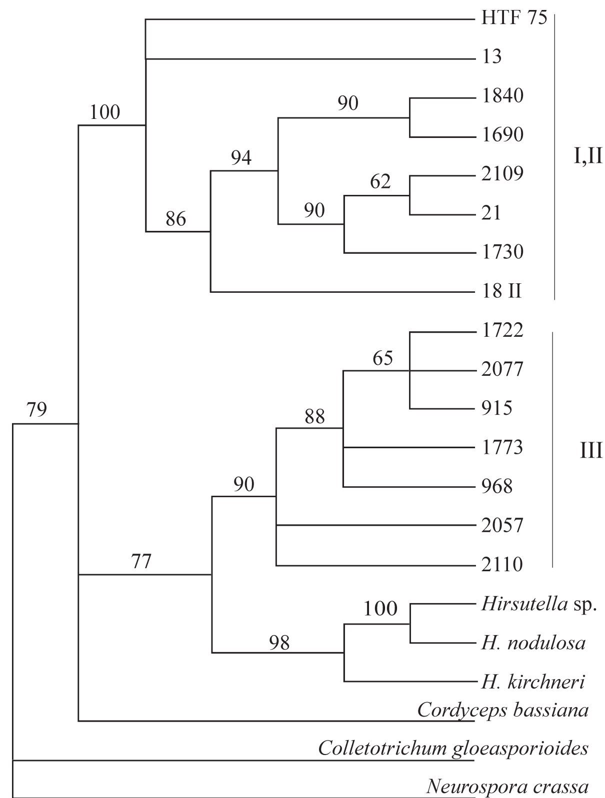

Figure 4 - Maximum likelihood tree based on β-*tubulin* sequences. Numbers next to the branches are bootstrap percentage values, based on 1,000 replicates. The outgroups are *Neurospora crassa* and *Colletotrichum gloeosporioide*.

fungal growth rate, colony color and morphology, and phialide size (Maimala, 2004) failed to correlate any morphological phenotype(s) to the genotypic data resulting from our AFLP analysis. However, clades I and III showed a relationship with the presence of the *HtA* gene, with Clade I members (except for one isolate) being HtA+ and all clade III members being HtA-.

Because of the high AFLP marker polymorphism observed among the *H. thompsonii* isolates studied, we conducted partial sequencing of the β-*tubulin* gene on a select group of isolates. Analyses of sequence data is a widely used approach in the study of species delimitation and the analysis of DNA sequence information within the β-*tubulin* region has provided robust phylogenetic information for fungi (Jong *et al*., 2001; ODonnell *et al*., 1998), including the entomopathogenic fungi of the genus *Paecilomyces* and *Nomuraea* (Han *et al*., 2002, Luangsa-Ard *et al*., 2005). Based on β-*tubulin* analysis, we have observed that *Hirsutella* grouped with a branch that included *C. bassiana*, suggesting that this genus possesses affinities to the Hypocreales. Stensrud *et al.* (2005) reported that *Cordyceps* species are commonly the teleomorphic stage of anamorph genera of many entomopathogenic fungi. The connection of *Hirsutella* and *Cordyceps* was first observed for the ant pathogen *Cordyceps unilateralis* whose anamorph is *Hirsutella formiacarum* (Petch, 1924) and several other *Cordyceps* species have been reported to be the teleomorphs of various *Hirsutella* species (Kendrick and Carmichael, 1973; Samson *et al.*, 1988).

Our β-*tubulin* gene analysis allowed the detection of intraspecies variability. In both β-tubulin trees (MP and ML), clades I and II from AFLP data were placed in a unique well-supported clade, and clade III included two isolates which were in separate clades in the AFLP analyses. We also found that β-*tubulin* clade IV included *Hirsutella* isolate 624, *H. nodulosa* and *H. kirchneri* (the non-*thompsonii* isolates), indicating that *Hirsutella* isolate 624 obtained in Thailand was closely related to the mite pathogen *H. nodulosa*.

The ML tree indicates that *H. thompsonii* is not monophyletic because clade III isolates were more closely related to *H. nodulosa* and *H. kirchneri* than to the rest of the *H. thompsonii* isolates studied. Clade III isolates were all HtA^- and were isolated from Thailand, albeit from different locations and hosts. The fact that *H. thompsonii* isolates are difficult to identify is due to the pleomorphic character of this fungus and the high polymorphism detected among different isolates in regard to several biological characteristics and molecular markers (Boucias *et al*., 1982, Mozes-Koch *et al*., 1995, Van der Geest *et al*., 2000). Our results have confirmed that the taxonomy of *Hirsutella* is complex (Samson *et al*., 1988) and that genomic characterization, through the analysis of other DNA sequences, should be developed in order to refine the taxonomy of *H. thompsonii*.

Acknowledgments

This research was supported in part by funds provided to M. Tigano by the Brazilian agencies Embrapa and Capes. The authors thank R. McTiernan and D. Purcell for technical support, R. Humber (USDA) for providing isolates and for a critical review of the manuscript and A. Jeyaprakash for a critical review of the manuscript.

References

Boucias D, Stokes C, Suazo A and Funderburk J (2000a) AFLP analysis of the entomopathogen *Nomuraea rileyi*. Mycologia 92:638-648.

Boucias D, Tigano MS, Sosa-Gomez DR, Glare TR and Inglis PW (2000b) Genotypic properties of the entomopathogenic fungus *Nomuraea rileyi*. Biological Control 19:124-138.

Boucias D, McCoy CW and Joslyn DJ (1982) Isozyme differentiation among 17 geographical isolates of *Hirsutella thompsonii.* Journal of Invertebrate Pathology 39:329-337.

Chandler D, Davidson G, Pell JK, Ball BV, Shaw K and Sunderland KD (2000) Fungal biocontrol of acari. Biocontrol Science and Technology 10:357-384.

Han Q, Inglis GD and Hausner G (2002) Phylogenetic relationships among strains of the entomopathogenic fungus,

Nomuraea rileyi, as revealed by partial β-tubulin sequences and inter-simple sequence repeat (ISSR) analysis. Letters in Applied Microbiology 34:376-383.

Jaffe BA (2000) Augmentation of soil with the nematophagous fungi *Hirsutella rhossiliensi* and *Arthrobotrys haptyla*. Phytopathology 90:498-504.

Jong SN, Lévesque CA, Verkley GJM, Abeln ECA, Rahe JE and Braun PG (2001) Phylogenetic relationships among *Neofabraea* species causing tree cankers and bull's-eye rot of apple based on DNA sequencing of ITS nuclear rDNA, mitochondrial rDNA, and the β-tubulin gene. Mycological Research 105:658-669.

Kendrick WB and Carmichael JW (1973) Hyphomycetes. In: Ainsworth GC, Sparrow FK and Sussman AS (eds) The Fungi: An Advanced Treatise. Academic Press, New York, pp 323-509.

Luangsa-Ard JJ, Hywel-Jones NL, Manoch L and Samson RA (2005) On the relationship of *Paecilomyces* sect. *Isarioidea* species. Mycological Research 109:581-589.

Maimala S (2004) Screening strains of *Hirsutella thompsonii* (FISHER) for mass production by solid state fermentation technology. PhD Thesis, Kasetsart University, Bangkok.

Maimala S, Tartar A, Boucias D and Chandrapatya A (2002) Detection of the toxin Hirsutellin A from *Hirsutella thompsonii*. Journal of Invertebrate Pathology 80:112-126.

McCoy CW (1996) Pathogens of eriophyoid mites. In: Lindquist EE, Sabelis MW and Bruin J (eds) Eriophyoid Mites – Their Biology, Natural Enemies and Control. Elsevier Science B.V., Amsterdam, pp 481-490.

McCoy CW (1981) Fungi: Pest control by *Hirsutella thompsonii*. In: Burges HD (ed) Microbial Control of Insects, Mites and Plant Diseases. Academic Press, New York, pp 499- 512.

Mozes-Koch R, Edelbaum O, Liveneh O, Sztejnberg A, Uziel A, Gerson U and Sela I (1995) Identification of *Hirsutella* species, isolated within a species and heterokaryons by random amplified polymorphic DNA (RAPD). Z Pflanzenkrankh Pflanzensch 102:284-290.

O'Donnell K, Cigelnik E and Nirenberg HI (1998) Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90:465-493.

Petch T (1924) Studies in entomogenous fungi. IV. Some Ceylon *Cordyceps*. Transactions of the British Mycological Society 10:28-45.

Posada D and Crandall KA (1998) MODELTEST: Testing the model of DNA substitution. Bioinformatics 14:817-818.

Saitou N and Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Samson RA, Evans HC and Latgé JP (1988) Atlas of Entomopathogenic Fungi. Springer Verlag, Berlin, 187 pp.

Stensrud O, Hywel-Jones NL and Schumacher T (2005) Towards a phylogenetic classification of *Cordyceps*: ITS rDNA sequence data confirm divergent lineages and paraphyly. Mycological Research 109:41-56.

Suazo A and Hall HG (1999) Modification of the AFLP protocol applied to honey bee (*Apis mellifera* L.) DNA. Bio-Techniques 26:704-709.

Swofford DL (2002) PAUP*: Phylogenetic Analysis Using Parsimony (*And Other Methods). Sinauer Associates, Inc., Sunderland, 140 pp.

Tartar A, Boucias DG, Adams BJ and Becnel JJ (2002) Phylogenetic identifies the invertebrate pathogen *Helicosporidium* sp. as a green alga (Chlorophyta). International Journal of Systematic and Evolutionary Microbiology 52:273-279.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG (1997) The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24:4876-4882.

Van der Geest LPS, Elliot SL, Breeuwer JAJ and Beerling EAM (2000) Diseases of mites. Experimental and Applied Acarology 24:497-560.

Associate Editor: Sérgio Olavo Pinto da Costa