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Short Communication

Identification and taxonomy of some entomopathogenic *Paecilomyces* spp. (Ascomycota) isolates using rDNA-ITS Sequences

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Abstract

A phylogenetic analysis of the 5.8S rDNA and internal transcribed spacer (ITS1 and ITS2) sequences from some entomogenous *Paecilomyces* species supports the polyphyly of the genus and showed the existence of cryptic species. In the Eurotiales, anamorphs *Paecilomyces variotii* and *Paecilomyces leycettanus* were related to the teleomorphs *Talaromyces* and *Thermoascus*. In the Hypocreales, three major ITS subgroups were found, one of which included *Paecilomyces viridis*, *Paecilomyces penicillatus, Paecilomyces carneus* and isolates identified as *Paecilomyces lilacinus* and *Paecilomyces marquandii*. However, the majority of the *P. lilacinus* and *P. marquandii* isolates formed a distinct and distantly related subgroup, while the other major subgroup contained *Paecilomyces farinosus*, *Paecilomyces amoeneroseus, Paecilomyces fumosoroseus* and *Paecilomyces tenuipes*.

Key words: Paecilomyces spp., phylogenetic analysis, rDNA; molecular taxonomy.

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The hyphomycete genus *Paecilomyces* was revised by Samson (1974), who recognized and defined 31 species and divided the genus into two sections. Section *Paecilomyces* contains members which are often thermophilic, the perfect states being placed in the ascomycetous genera *Talaromyces* and *Thermoascus*. Section *Isarioidea* contains mesophiles, including several well-known entomopathogenic or nematophagous species such as *Paecilomyces farinosus*, *Paecilomyces fumosoroseus*, *Paecilomyces amoeneroseus*, *Paecilomyces lilacinus*, *Paecilomyces javanicus* and *Paecilomyces tenuipes*. Classification of these fungi is based on morphological characteristics, which are often highly subjective, with unambiguous identification to the species level often being extremely difficult. Additionally, the morphological differences observed may be the product of simple mutations or media/cultivation effects and thus are not always reliable characters. Definitions of some entomopathogenic or nematophagous species in the genus *Paecilomyces* are subject to doubt when classical identification methods are compared to analysis of genetic markers (Tigano-Milani *et al*., 1995a; Tigano-Milani *et al*., 1995b; Cantone and Vandenberg, 1998). The limitations of traditional identification techniques indicate that alternative methods need to be developed for the identification of these fungi.

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Comparative analysis of ribosomal RNA (rDNA) gene sequence information can be used to clarify natural evolutionary relationships over a wide taxonomic range (Pace *et al.*, 1986). Ribosomal RNA genes (rDNA) typically exist as a tandem repeat that includes coding regions, which are conserved to varying degrees, as well as highly divergent spacer regions. These spacer regions, or internal transcribed spacer sequences (ITS), have been widely used in fungal systematics (Bowman *et al*., 1992; Hibbett, 1992; Driver *et al*., 2000). In the case of the genus *Paecilomyces,* the analysis of sequences of the large and the small subunit rRNA gene has already indicated the polyphyly of the genus (Obornik *et al*., 2001). We therefore decided to use rDNA-ITS sequencing to clarify the taxonomic relationships and identities of some entomopathogenic and nematophagous *Paecilomyces* isolates held in our culture collection.

For our analysis we selected a large number of entomopathogenic Paecilomyces isolates, including several that could not be unambiguously assigned to a known species using morphological characters (Table 1). Monosporic fungal cultures were prepared from liquid nitrogen stored or lyophilized stocks from the Collection of Entomopathogenic Fungi held at Embrapa - Recursos Genéticos e Biotecnologia, Brasília-DF, Brazil or the Insect Pathogen Culture Collection of the Commonwealth Scientific and Industrial Research Organization (CSIRO) Division of Entomology (DE-CSIRO), Canberra Australia).

Table 1 - Origin and conventional classification of isolates, whose ITS1-5.8S-ITS2 DNA was sequenced in this study.

Species designation (classical or as received)	Isolate[a]	Host or substrate	Country	Year	Isogenic group
Nomuraea anemenoides	IMI214110	Soil	Australia	1974	
Nomuraea rileyi	CG129	*Anticarsia gemmatalis*	Brazil (DF)	1988	
P. farinosus	CBS541.81	Spider	Ecuador	1981	
P. farinosus	CG199	*Lymantria dispar*	USA	1986	
P. fumosoroseus	ARSEF1576	*Monophadnus* sp.	Italy	1984	
P. fumosoroseus	CG170	*Pseudococcus* sp.	USA (Florida)	1990	
P. fumosoroseus	CG196	Diptera, Tachnindae	Poland	1983	
P. fumosoroseus	CG204	*Bemisia* sp.	Mexico	1990	
P. fumosoroseus	CG325	*Nilaparvata lugens*	Philippines	1985	
P. fumosoroseus	CG335	Soil	Brazil (MT)	1990	
P. fumosoroseus	CG741	*Lagria villosa*	Brazil (GO)	1982	
P. javanicus	CBS134.22		Ecuador	1922	
P. marquandii	ARSEF3047	Soil	Mali		
P. marquandii	IMI2233	*Meloidogyne* sp.	India		
Paecilomyces carneus	CG525	Soil	Brazil (RS)	1995	
Paecilomyces carneus	IMI058418	Soil	UK		
Paecilomyces leycettanus	IMI178525				
Paecilomyces penicillatus	IMI186962	Mushroom	Belgium		
Paecilomyces sp.	CG177	*Meloidogyne* sp.	Brazil (PA)	1988	
Paecilomyces variotti	CG503	Air	Brazil (SP)	1995	
Paecilomyces variotti	FRR3797	Soil	Brazil		
Paecilomyces viridis	ARSEF2456		France		
Talaromyces leycettanus	FRR3525				
Trichoderma viridis	ARSEF807				
P. lilacinus	CG265	Soil	Brazil (TO)	1991	A
P. lilacinus	CG301	Coleoptera	Brazil (RJ)	1985	A
P. lilacinus	CG348	Soil	Brazil (GO)	1991	A
P. fumosoroseus	CG123	*Spaethiella* sp.	Brazil (AM)	1987	B
P. fumosoroseus	CG197	*Pyrrhalta luteola*	France	1983	B
P. fumosoroseus	CG397	*Bemisia tabaci*	USA (Florida)	1992	B
P. fumosoroseus	CG404	*Bemisia tabaci*	Nepal	1992	B
Paecilomyces sp.	CG499	Coleoptera	Brazil (DF)	1995	B
P. fumosoroseus	CG684	*Bombyx mori*	China	1981	B
P. fumosoroseus	FI1217				B
P. amoeneroseus	CBS738.73	Coleoptera	Ghana	1973	C
P. amoeneroseus	CG162	*Lagria villosa*	Brazil (GO)	1989	C
P. amoeneroseus	CG163	*Lagria villosa*	Brazil (GO)	1989	C
P. fumosoroseus	CG302	*Lagria villosa*	Brazil (GO)	1982	C
P. fumosoroseus	ARSEF1005	*Bombyx mori*	Japan	1998	D
P. fumosoroseus	ARSEF1506	*Pyrrhalta luteola*	France	1983	D
P. fumosoroseus	ARSEF1644	*Musca domestica*	France	1984	D
P. fumosoroseus	ARSEF1645	*Musca domestica*	France		D
P. fumosoroseus	ARSEF2679	*Popillia japonica*	Portugal		D
P. fumosoroseus	ARSEF2745	Soil	Philippines		D
P. fumosoroseus	ARSEF2749	*Plutella xylostella*	Philippines		D
P. fumosoroseus	ARSEF887	Diptera	France		D
P. tenuipes	ARSEF2489	*Spodoptera frugiperda*	Mexico		E
P. tenuipes	ARSEF2490	*Spodoptera frugiperda*	Mexico		E
P. tenuipes	ARSEF2491	*Spodoptera frugiperda*	Mexico		E
P. tenuipes	IMI180610	Lepidoptera	Netherlands		E
P. lilacinus	CBS284.36	Soil	USA	1936	F
P. lilacinus	CG36	*Deois flavopicta*	Brazil (DF)	1992	F
P. lilacinus	CG189	Soil	Brazil (GO)	1991	F
P. lilacinus	CG267	Soil	Brazil (GO)	1991	F
P. lilacinus	CG275	Soil	Brazil (MA)	1991	F
P. lilacinus	CG299	Soil	USA (Florida)	1990	F
P. lilacinus	CG190	Soil	Brazil (RS)	1990	G
P. lilacinus	CG271	Soil	Brazil (GO)	1991	G

[a]Isolate accession numbers from their appropriate culture collection are denoted by the following prefixes: ARSEF, Agricultural Research Service Entomopathogenic Fungus Collection, USDA, Ithaca, NY, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CG, Embrapa Collection, Brasília, Brazil; FI, CSIRO Collection, Canberra, Australia; IMI, International Mycological Institute, Surrey, UK. Isolates with identical ITS1-5.8S-ITS2 gene sequences were assigned to an appropriate isogenic group and are represented in Figure 1 by this group designation. Key to Brazilian states: AM = Amazonas, DF = Distrito Federal, GO = Goiás, MA = Maranhão, MT = Mato Grosso, PA = Pará, RJ = Rio de Janeiro, RS = Rio Grande do Sul, TO = Tocantins.

Genomic DNA was purified from mycelium using the cetyltrimethylammonium bromide (CTAB) extraction method of Rogers and Bendich (1988). A single pair of primers was used to PCR amplify the internal transcribed spacer (ITS) regions ITS1 and ITS2, along with the central 5.8S rDNA, essentially as described by Driver et al. (2000). The primers used were the universal eukaryotic ITS primers, which annealed to the 3' end of the 16/18S rDNA (TW81; 5'GTTCCGTAGGTGAACCTGC) and to the 5' end of the 28S rDNA (AB28; 5'ATATGCTTAAGTTCA GCGGGT) respectively (White *et al.*, 1990). The PCR products were checked using agarose gel electrophoresis and, as expected, produced a product of about 500 bp. Bands were excised and then purified using the Geneclean II kit (Bio 101) and were either cloned using the PCRscript-Amp plasmid vector/cloning kit (Stratagene) prior to sequencing or were sequenced directly using consensus primers. Sequencing of both strands of the PCR products/clones was as described previously (Driver *et al*., 2000).

The ITS1-5.8S-ITS2 gene sequences were aligned with ClustalX v. 1.83.1 (Thompson e*t al*. 1997). Selected sequences available from GenBank were also included in the analysis (Table 1). A Maximum Likelihood with molecular clock tree of divergence data from the ITS alignments was calculated using the DnaMLK program from PHYLIP v. 3.6 (Felsenstein and Churchill, 1996).

The phylogenetic tree, summarizing the relationships between the ITS sequences obtained or used in this study (Figure 1), possesses a major division corresponding to the Ascomycete orders Eurotiales and Hypocreales. These orders respectively, contain members of the two sections of *Paecilomyces* proposed by Samson (1974), where section *Paecilomyces* is found in the Eurotiales (Trichocomaceae) and section *Isarioidea* in the Hypocreales (Clavicipitaceae and Hypocreaceae) (Figure 1). Our ITS analysis data supports the hypothesis that *Paecilomyces* is polyphyletic, which has been previously suggested using rDNA data (Obornik *et al*., 2001) and supports the morphological division of the genus into these two sections. Section *Paecilomyces*, with the type species *Paecilomyces variotii,* contains thermophiles with affinities to *Talaromyces* and *Byssochlamys*, which are related to the class Plectomycetes in the Ascomycota. Section *Isarioidea*, with the type species *P. farinosus*, are mesophiles, mostly entomogenous and with affinities to the order Clavicipitales in the class Pyrenomycetes within the Ascomycota (Mugnier, 1998; Samson, 1974).

The Eurotiales group included a clade containing all Paecilomyces variotii isolates. These strains then join a group comprising *Byssochlamys nivea*, *Thermoascus crustaceus, Paecilomyces leycettanus* and *Talaromyces leycettanus*, which then joins a separate group comprising *Talaromyces purpureus*, *Nomuraea anemenoides* and *Penicillium minioluteum*. The presence of *Talaromyces*

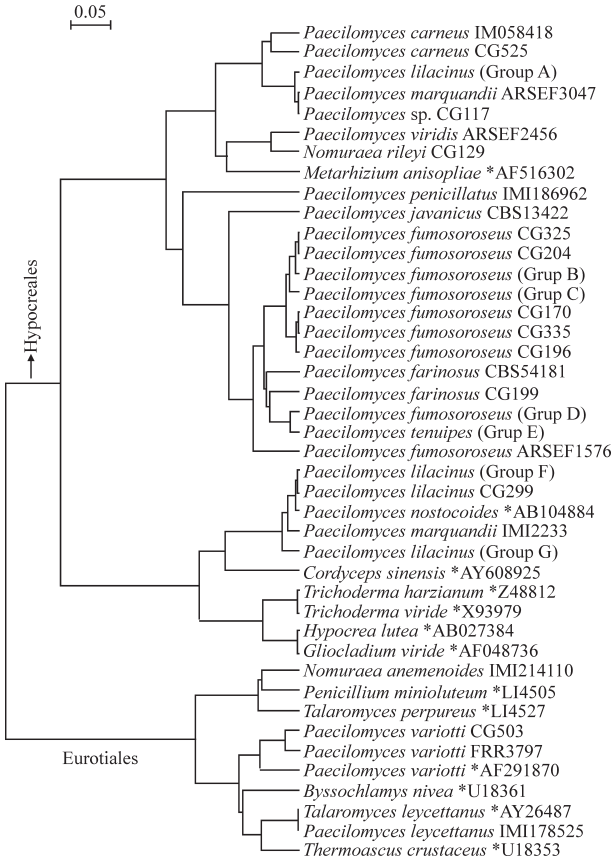

Figure 1 - Phylogenetic tree for *Paecilomyces* species based on internal transcribed spacer (ITS) and 5.8S rDNA sequences using the Maximum Likelihood with molecular clock method. The ascomycetous classes are indicated on the major branches. Sequence divergence is indicated by the scale bar. Samples giving identical ITS1-5.8S-ITS2 gene sequences were assigned to an arbitrary group and are represented in the tree by their group/species designation. Strains marked with an asterisk* are GenBank accessions not sequenced in the study. The tree and its data matrix have been submitted to TREEBASE.

and *Thermoascus* in the same clade as *Paecilomyces leycettanus* and *P. variotii* supports the relationship of these anamorph species from section Paecilomyces to these teleomorph genera. Species of *Talaromyces* were present in both subgroups of the Eurotiales, where some Talaromyces species are more closely related to *Byssochlamys* than others. In fact, these data agree with other studies showing that *Penicillium* and *Talaromyces* are not monophyletic genera (Berbee *et al.*, 1995; LoBuglio *et al*., 1993). The *Talaromyces leycettanus* ITS sequence was a perfect match with *P. leycettanus,* which can therefore be considered the teleomorph for this *Paecilomyces* species. The Hypocreales group possesses a topology that suggests that this group is also not monophyletic. Molecular phylogenetic analyses based on 18S rDNA sequences demonstrated that *P. tenuipes* belongs to the Clavicipitales in the class Pyrenomycetes (Fukatsu *et al*., 1997). Entomogenous species of *Paecilomyces* are also known to be closely related to

other insect-pathogenic fungi, *Beauveria bassiana* and some *Verticillium* species (Oborník *et al*., 2001).

The ITS region was found to be useful in resolving some difficulties within classical Paecilomyces taxonomy and redefined the classification of some of our isolates, where a high level of variability among isolates was previously seen based on analysis of other DNA markers (Tigano-Milani *et al*., 1995a; Tigano-Milani *et al.*, 1995b). Isolates identified morphologically as *P. lilacinus* and *P. marquandii* were located in two, quite distant subgroups. One clade included *P. marquandii* isolate ARSEF3047, which was very close to unidentified isolate CG177, and an isogenic group of three *P. lilacinus* isolates (group A) which may represent a cryptic species closely related to *P. carneus*. This cluster then joined *Paecilomyces viridis* ARSEF2456, which appears to be related to *Nomuraea rileyi* and, to a lesser extent, *Metarhizium anisopliae*. Other isolates originally identified as *P. lilacinus,* including isogenic isolates of Group F (which includes the type strain CBS284.36) and Group G, formed a distinct clade along with *Paecilomyces nostocoides* and *Cordyceps sinensis*. An isolate designated as a 'true' *P. marquandii* strain (IMI2233) was in this group and produced colonies with yellow undersides, a diagnostic trait consistent with the original description of this species (Samson, 1974). This second ITS clade of *P. lilacinus* and *P. marquandii* isolates was in fact more closely related to *Trichoderma*, *Hypocrea* and *Gliocladium* than to the other entomopathogenic *Paecilomyces* species in the Hypocreales.

A large group, containing *P. farinosus* (the type species for the *Isarioidea* section), also contained the other well-known entomogenous species *P. amoeneroseus, P. fumosoroseus* and *P. tenuipes*. A mycoinsecticide of significant commercial interest for the control of whiteflies is produced using *P. fumosoroseus* (Lacey *et al*., 2001) but to exploit the considerable potential of this fungus a reliable and standardized approach to its identification is needed. Our study confirmed that *P. fumosoroseus* lacks a discrete species concept, as has already been suggested based on arbitrarily primed PCR and tRNA fingerprinting (Tigano-Milani *et al*., 1995a) and vegetative compatibility (Cantone and Vandenberg, 1998). A previous phylogenetic analysis of *P. fumosoroseus* using rDNA-ITS sequences showed that there are at least three monophyletic groups within the *P. fumosoroseus* complex and that host selective pressure may be significant in the selection of genotypes (Fargues *et al*., 2002). We found that eight isolates originally classified as *P. fumosoroseus* (represented on the tree by Group D *P. fumosoroseus*) have a similar but distinct ITS sequence to that of the *P. tenuipes* isolates (Group E). These small but consistent differences justify the consideration of these isolates as a different and unknown species, especially considering that the degree of divergence between these isolates and *P. tenuipes* was similar to that obtained between the other *P. fumosoroseus* and *P. amoeneroseus* or *P. farinosus*. In order to obtain more information about their identification these unidentified isolates, which are confused with *P. fumosoroseus,* should be compared to other described *Paecilomyces* species not analyzed in our study. We found six different ITS sequences among the twelve *P. fumosoroseus* isolates closely related to *P. amoeneroseus* (Group C) and an unidentified isolate (CG499) within this cluster had an ITS sequence identical to that of five *P. fumosoroseus* isolates (Group B). Moreover, a misidentified *P. fumosoroseus* isolate (CG302) possessed an identical sequence to the three *P. amoeneroseus* analyzed (Table 1). This group was then joined by *P. javanicus* and *Paecilomyces penicillatus*. These results emphasize the difficulties in using classical morphological traits for taxonomy and the unambiguous identification of these closely related species.

Our results confirm the polyphyly of the genus *Paecilomyces*, already observed by analysis of the large and small-subunit rRNA gene sequences (Obornik *et al*., 2001; Luangsa-ard *et al.*, 2004), where *Paecilomyces* probably represents a form genus only. A major review of the genus *Paecilomyces* is required, including the analysis of a more conserved gene region to clarify the phylogenetic relationships.

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