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DNA fingerprinting of *Paecilomyces* strains of potential use for the biological control of pests

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Summary

Telomeric fingerprinting was found to be highly differentiating for *Paecilomyces fumosoroseus* and *Paecilomyces lilacinus* isolates in comparison to intron splice site PCR and is therefore a good method for quality control of future products based on these fungi. Although the telomeric restriction length polymorphisms correctly divided the isolates into their appropriate species, further correlation with host range or geographical origin of the isolates was not found. In this respect, intron splice site PCR was more informative taxonomically. The chromosome numbers inferred from telomeric fingerprints were seven chromosomes for *P. lilacinus* and between six and nine chromosomes for *P. fumosoroseus*.

Introduction

Paecilomyces fumosoroseus and *Paecilomyces lilacinus* (Deuteromycotina: Hyphomycetes) are fungi currently receiving attention for their potential in biological control programmes. *P. fumosoroseus* isolates are commonly entomogenous, are found infecting many different orders of insects in all stages of the life-cycle, are geographically widespread and can also be isolated from soil samples (Samson 1974). This fungus is being developed for use against a range of agricultural pests, including the whitefly, *Bemisia tabaci* (Osborne & Landa 1992; Lacey *et al.* 1993), where biocontrol may offer an effective alternative for control of insects becoming highly resistant to conventional pesticides. *P. lilacinus* is frequently isolated from soil, particularly in samples originating from warmer regions (Domsch *et al.* 1980). Some strains have been shown to be mycoparasitic, colonizing fungal sclerotia (Gupta *et al.* 1993). There have also been reports of invasive mycoses caused by opportunistic *P. lilacinus* in immunocompromised patients (Orth *et al.* 1996). Of particular interest, however, are the entomogenous (Rombach *et al.* 1986) and nematogenous (Carneiro 1992; Holland *et al.* 1999; Gunasekera *et al.* 2000) strains of *P. lilacinus*.

An analysis of the efficacy of biopesticides requires the accurate monitoring of the released strain in the field and the reliable differentiation of that strain from those possibly endemic to the application site or target. The initial identification and differentiation of fungal strains is often difficult, where a combination of morphological, biochemical and virulence tests, and access to type-strains are required for reliable identification (Samson 1974). Molecular markers are attractive alternatives for fungal identification and fingerprinting, where the often simple and standard techniques are now generally available. Arbitrarily primed PCR (Williams *et al.* 1990) was previously used to analyse strains of *P. fumosoroseus* (Tigano-Milani *et al.* 1995a; Cantone & Vandenberg 1998) and *P. lilacinus* (Tigano-Milani *et al.* 1995b; Gunasekera *et al.* 2000). Another technique, which has successfully differentiated commercial yeast strains, is PCR using consensus intron splice site primers (de Barros Lopes *et al.* 1996). Such analyses can reveal genetic diversity among strains and furthermore can demonstrate the extent of geographical or host distribution of related strains.

Although simple and economical for the screening of large numbers of isolates, PCR-based methods may not be sensitive or reproducible enough to differentiate closely related strains, particularly where quality control or verification of released strains is required. In this respect, detection of restriction fragment length polymorphisms (RFLPs) using multi-copy genes as probes is an alternative technique that has been applied in several fungi as a distinguishing marker. Telomeric DNA probes were successfully used to fingerprint strains of *Beauveria bassiana* (Viaud *et al.* 1996), *Botrytis cinerea* (Levis *et al.* 1997) and *Metarhizium anisopliae* var. *acridum* (Inglis *et al.* 1999). We therefore evaluated the

potential of telomeric fingerprinting to differentiate some *P. fumosoroseus* and *P. lilacinus* strains in comparison with a PCR-based technique using fungal intron splice site primers (de Barros Lopes *et al.* 1996).

Materials and methods

Fungal strains

P. fumosoroseus and *P. lilacinus* isolates (Table 1) were obtained as liquid nitrogen-stored cultures from the EMBRAPA Recursos Genéticos e Biotecnologia collection of entomopathogenic fungi. The strains selected for analysis conformed to the typical descriptions of *P. fumosoroseus* and *P. lilacinus* , using classical morphological characteristics (Samson 1974). Cultures for preparation of conidia were plated on potato dextrose agar (Difco) and incubated at 28 °C for 7–10 days.

DNA fingerprinting methods

Isolates for extraction of genomic DNA for restriction enzyme digests and PCR, were grown for 48 h in 200 ml *Aspergillus* complete medium (Pontecorvo *et al.* 1953) in 500 ml Erlenmeyer flasks at 28 °C with shaking at 200 rev/min. Mycelium was then harvested by filtration, washed with sterile distilled water and ground to a powder in liquid nitrogen. Genomic DNA was then extracted using a CTAB extraction method (Rogers & Bendich 1988), modified by the addition of a phenol extraction step to remove residual nuclease activity. Genomic DNA (1 μg) from each isolate was digested with *Eco*RI for 3 h in the appropriate buffer. Following electrophoresis, DNA fragments were alkaline (0.4 M NaOH) blotted on to a positively charged nylon hybridization membrane (Hybond XL, Amersham). Similar blots were prepared with DNA digested with the restriction enzymes *Bam*HI and *Hin*dIII. Telomeric DNA was synthesized by PCR using a pair of self-complimentary telomeric primers ($[TTAGGG]_4$, and $[CCCTAA]_4$) (Ijdo *et al.* 1991) and was labelled with $[\alpha-^{32}P]$dCTP using a random primer labelling kit (Rediprime II, Amersham). Hybridizations, high stringency washes and signal detection were carried out using standard techniques.

PCR-based fingerprinting using fungal intron splice site primers: EI1, CTGGCTTGGTGTATGT; EI2, CTGGCTTGCTACATAC; LA1, GCGACGGTGTACTAAC; LA2, CGTGCAGGTGTTAGTA, was carried out essentially as described previously (de Barros Lopes *et al.* 1996), using 20 ng fungal DNA, prepared as above, per reaction. PCR products were resolved by electrophoresis on 1.5% w/v agarose gels and bands detected by ethidium bromide staining.

Data analysis

Bands hybridizing with the telomeric probe were scored (1 = present; 0 = absent) as binary characters and data combined from the three different restriction enzymes utilized. Data was then analysed using the NT-SYS-pc V1.8 package (Rohlf 1993). Similarity matrices were calculated using the Jaccard coefficient (Sneath & Sokal 1973), which considers only the joint presence of a character as an indication of similarity. Clustering of the similarity data was done using the unweighted pair-group method, arithmetic mean (UPGMA; Sneath & Sokal 1973) and a summary dendrogram of similarity data produced. Amplified PCR products from gel photographs were similarly scored.

Table 1. Origin and classification of *Paecilomyces* isolates.

Isolate[a]	Host or substrate	Location	Year
P. fumosoroseus			
CG35	Soil	Brasília-DF, Brazil	1991
CG202	*Musca domestica*	France	1985
CG203	*Bemisia tabaci*	Florida, USA	1990
CG205	*Bemisia tabaci*	Florida, USA	1992
CG404	*Bemisia tabaci*	Kathmandu/Nepal	1992
CG436	*Bemisia tabaci*	India	1992
CG456	*Pieris brassicae*	France	1978
CG686	*Bemisia* sp.	Mexico	1990
CG688	*Bemisia tabaci*	Texas, USA	1992
CG736	*Bombyx mori*	Japan	1998
CG738	*Plutella xylostella*	Philippines	1989
P. lilacinus			
CG189	Soil	GO, Brazil	1991
CG262	Soil	TO, Brazil	1991
CG362	Soil	GO, Brazil	1991
CG638 (=CBS284.36)	Soil	USA	1936

[a] CG = CENARGEN/EMBRAPA Entomopathogenic Fungus Collection, Brasília, Brazil.

Results and discussion

Telomeric fingerprinting was found to be highly differentiating for the strains of *P. fumosoroseus* and *P. lilacinus* tested in this study, producing unique profiles for all strains with each of the three restriction enzymes used (Figure 1). The three different restriction enzymes yielded a total of 167 binary characters derived from unambiguously readable bands. An UPGMA dendrogram summarizing the results of cluster analysis of the data (Figure 2) showed that *P. fumosoroseus* and *P. lilacinus* isolates formed distinct clades with very low similarity to each other. However, the similarity between individuals in these clades was also extremely low, approaching that of the inter-species branch. No host or geographical relationships could be discerned in the dendrogram, where *P. fumosoroseus* strains isolated from soil, the whitefly, *Bemisia tabaci* or from other insects failed to form coherent groups. The apparent hypervariability of the subtelomeric DNA in these strains could indicate high genetic instability in these loci. For this reason, temporal studies are required to determine short and medium-term stability of the fin-

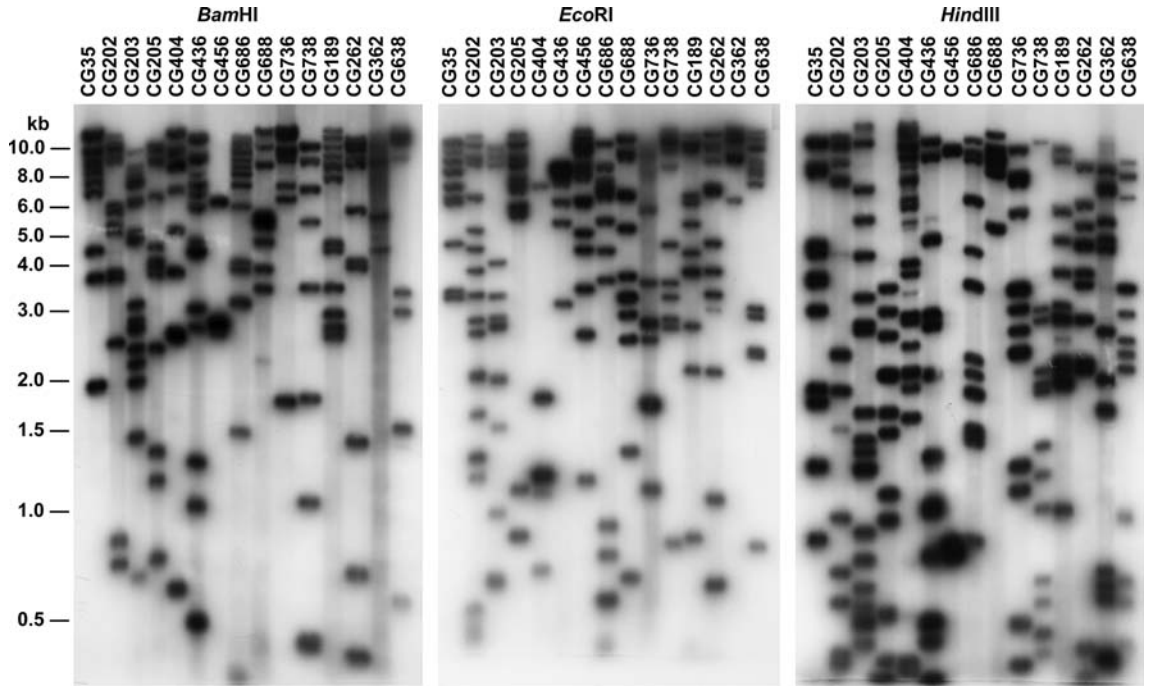

Figure 1. Telomeric fingerprint autoradiograms of *P. fumosoroseus* and *P. lilacinus* isolates. Molecular weight scale was estimated using the 1 kb DNA ladder (Amersham).

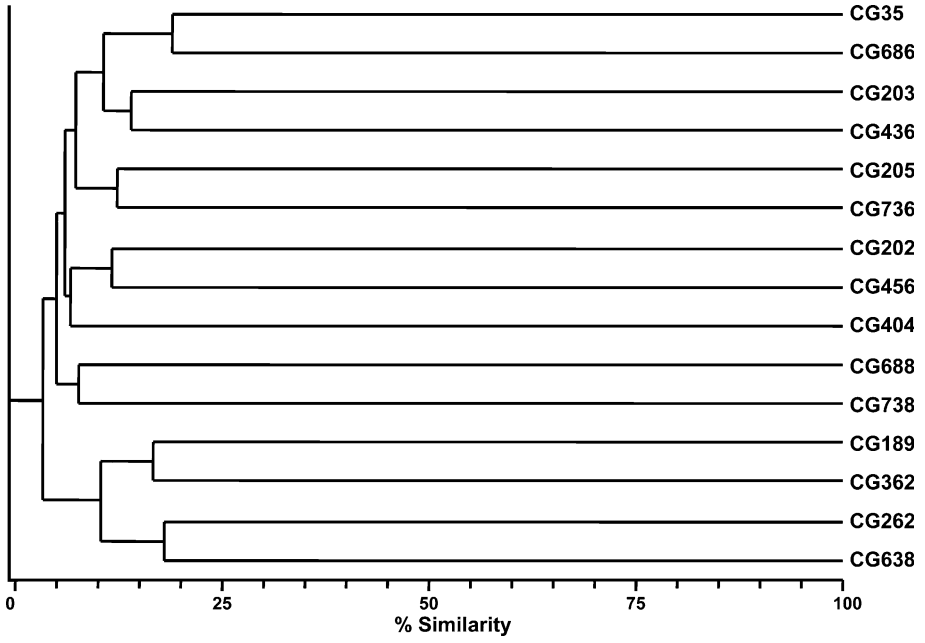

Figure 2. Dendrogram of combined telomeric fingerprinting data. A similarity matrix was calclulated, based on the Jaccard coefficient, and the tree drawn using the unweighted pair group arithmetic mean method (UPGMA).

gerprints obtained. In this respect, it was interesting to note that among the four *P. lilacinus* strains analysed, the telomeric fingerprint of one strain isolated in 1936 in the USA (CG638) was closer to one of three Brazilian strains isolated in 1991 (CG262) than were the three Brazilian strains to each other.

In all four *P. lilacinus* isolates examined, a chromosome number of seven may be inferred by the number of bands resolved in the telomeric fingerprints (Table 2). In contrast, the inferred chromosome number among the *P. fumosoroseus* isolates varied between six and nine chromosomes. These chromosome number estimates and variation within species are consistent with previously published data on entomopathogenic Deuteromycetes. Here, pulsed field electrophoretic karyotyping showed that the DNA of a single strain of *P. fumosoroseus* could be resolved into six chromosome-sized bands (Shimizu *et al.* 1991) and in *Metarhizium anisopliae*, an

Table 2. Number of chromosomes in *Paecilomyces* isolates inferred by the number of bands which hybridized with the telomeric probe.

Isolate	Number of bands per restriction enzyme			Inferred Chromosomes
	*Bam*HI	*Eco*RI	*Hin*dIII	
CG35	11	14	13	7
CG202	14	17	14	9
CG203	18	12	18	9
CG205	13	12	11	7
CG404	12	6	18	9
CG436	17	9	13	9
CG456	4	15	2	8
CG686	15	16	12	8
CG688	11	12	6	6
CG736	9	13	13	7
CG738	9	13	13	7
CG189	14	14	14	7
CG262	11	14	12	7
CG362	10	6	14	7
CG638	10	14	14	7

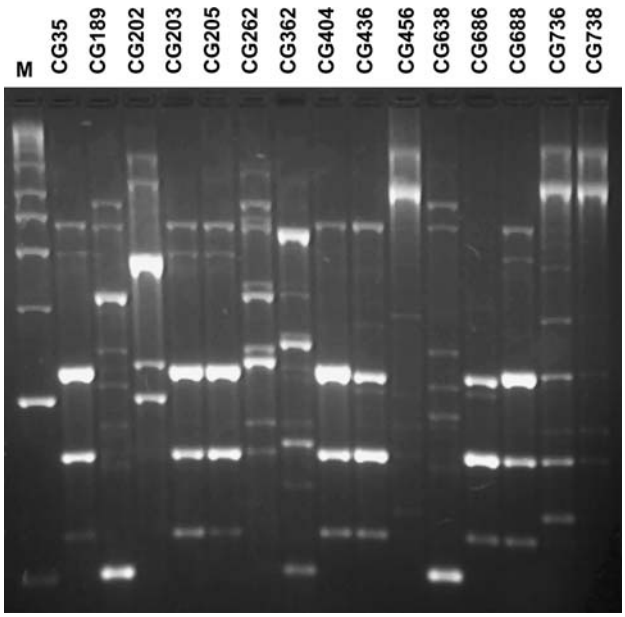

Figure 3. Example of intron splice site PCR, using primer EI2, of *P. fumosoroseus* and *P. lilacinus* isolates. Molecular weight marker (M) was the 1 kb DNA ladder (Amersham).

electrophoretic karyotype of between seven and eight chromosomes was reported for several strains (Valadares-Inglis & Peberdy 1998). Electrophoretic karyotype estimates appear to be generally consistent with chromosome numbers inferred from telomeric fingerprints in these fungi, where in *Metarhizium flavoviride* between five and seven chromosomes were inferred among a collection of eight isolates (Inglis *et al.* 1999). Gain or loss of dispensable chromosomes by anastomosis and non-reciprocal crosses with other compatible strains in the field could be an explanation for variations in telomere banding pattern and inferred chromosome number, apart from that resulting from possible limitations of electrophoretic resolution (both standard agarose gel electrophoresis of restriction fragments and pulsed field electrophoresis). At least 20 vegetative compatibility groups have been detected in *P. fumosoroseus* isolates, indicating that chromosome exchange among field strains is possible (Cantone & Vandenberg 1998). Interestingly, digestion with certain restriction enzymes produced an anomalously low number of bands in fingerprints in certain strains, such as CG456, where a particular band could appear with greater intensity than usual. This effect could be the result of duplication of a telomere-associated sequence to different telomeres.

Intron splice site PCR of the *Paecilomyces* isolates was found to be less differentiating than telomeric fingerprinting and required the combination of the results from the four different primers to distinguish all strains. PCR with primers EI1, EI2 or LA1 gave rise to multiple products with a wide molecular weight range (Figure 3). In contrast, primer LA2 resulted in poor amplification of *Paecilomyces* DNA and reproducibly failed to produce a PCR product with *P. fumosoroseus* strain CG738 DNA. A large variation in PCR band intensity was found, where some products were clearly preferentially amplified in some strains and bands of the same apparent molecular weight varied in intensity between certain samples. Additionally, a significant proportion of the higher molecular weight bands could not be reliably scored under the electrophoretic conditions used. The variability in band intensities obtained is a potential disadvantage of the technique, which is probably related to exhaustion of PCR reagents in individual reactions. This could clearly lead to variation in profiles obtained, which are already dependent on multiple technical factors, including different sources and quality of PCR reagents, use of different PCR machines with varying thermal properties and variation in the quality of template DNA, which is difficult to standardize. Nevertheless, the four different intron splice site primers yielded a total of 65 binary characters.

Cluster analysis of the PCR data (Figure 4) showed that, as in the case of telomeric RFLPs, the strains analysed grouped in their respective species. In this case, however, all the *P. fumosoroseus* strains isolated from *Bemisia* spp. formed a distinct, apparently monophyletic group. This group then formed a clade with the *P. fumosoroseus* strains CG736 and CG738, which although isolated from different hosts, namely *Bombxy mori* and *Plutella xylostella*, both originated in Asia. The remaining *P. fumosoroseus*, both originating in France, then completed the *P. fumosoroseus* group. This data is consistent with a proposed co-evolution of host specialized *P. fumosoroseus* genotypes on *Bemisia* whiteflies originating in India (Brown 1994), which have then recently spread along with their host to Europe, Africa and the Americas (Horowitz 1986). The molecular evidence for this theory has been provided elsewhere by restriction analysis of amplified rDNA-ITS, where clustering of *P. fumosoroseus* isolates from *Bemisia* was

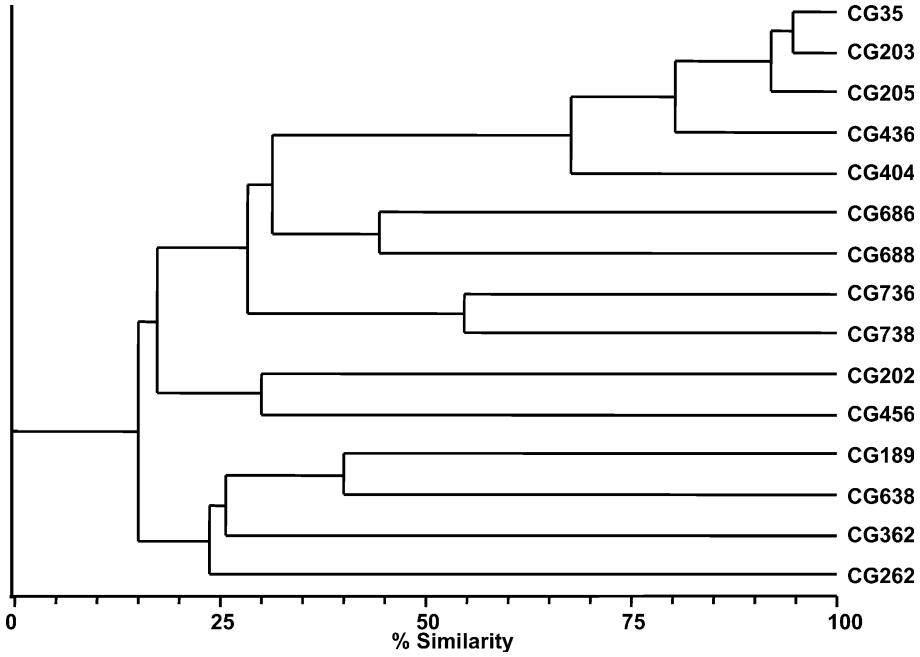

Figure 4. UPGMA dendrogram of combined intron splice site PCR data.

evident, regardless of geographical origin (Fargues *et al*. 2002).

Conclusions

Our results indicate that telomere fingerprinting is highly suitable for strain definition and quality control of potential biocontrol agents based on *Paecilomyces* strains. However, the technique is probably not suitable for large-scale monitoring of numerous environmental isolates, where cost and time are important considerations. Here, our results show that intron splice site PCR could be suitable for this type of monitoring, where even a single primer could be sufficient for screening large numbers of isolates recovered from field release trials. The taxonomic information provided by the two tested techniques differed notably, where our data indicated that telomeric fingerprinting is probably not suitable or reliable for defining taxonomic relationships above the strain level in *P. fumosoroseus* and *P. lilacinus*. Nor is the technique suitable for an assessment of the overall taxonomic similarity between two related individuals. In this case, our results suggest that multi-locus PCR-based techniques using primers binding throughout the genome can provide more significant information in these fungi.

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