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rRNA Sequence Comparison of *Beauveria bassiana*, *Tolypocladium cylindrosporum*, and *Tolypocladium extinguens*

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Five strains of *Tolypocladium cylindrosporum*, one strain of *Tolypocladium extinguens*, and nine strains of *Beauveria bassiana* were analyzed using a rapid rRNA sequencing technique. The sequences of two highly variable domains (D1 and D2) located at the 5′ end of the 28S-like rRNA molecule were determined. The phylogenetic tree computed from the absolute number of nucleotide differences shows the separation between the genus *Beauveria* and the genus *Tolypocladium* and points out that *T. cylindrosporum* and *T. extinguens* probably do not belong to the same genus.

INTRODUCTION

The morphoontogenic criteria generally used for the determination of the genus and species of filamentous Fungi sometimes raise controversy. Thus, the genus *Tolypocladium* (Gams, 1971) was recently integrated with the genus *Beauveria* (Von Arx, 1986) based on the morphological similarities.

In the Hyphomycete genus *Tolypocladium*, two entomopathogenic species are known: *Tolypocladium cylindrosporum* and *Tolypocladium extinguens*, which are distinguished by the morphological structure (Samson and Soares, 1984) as well as by their isoenzymatic profiles (Soares et al., 1985).

The phyletic relationship which exists between the group *Tolypocladium* and the group *Beauveria* can be studied by the analysis of the sequences of the 28S rRNA subunit. In fact, ribosomal RNAs (rRNAs) provide a powerful taxonomic indicator since they are highly conserved in living organisms. The 5S rRNA, too short, has been much more rapidly evolving domains (D1 and D2) (Clark et al., 1984).

A comparative analysis of the sequences of these regions (D1 and D2) has been undertaken for several isolates of *T. cylindrosporum*, *T. extinguens*, and *B. bassiana* in order to get an idea of the phylogenetic relationship among these species.

MATERIALS AND METHODS

Fungal Strains

All strains used in this study originated from the fungal collection of the biological Control Research Station of La Minière, INRA (Table 1).

Growth Conditions and Preparation of 28S rRNA

Fungi were grown at 25°C on a semisynthetic (CM) agar or liquid medium containing the following: 0.4 g of KH_2PO_4; 1.4 g of $NA_2HPO_4, 2H_2O$; 0.6 g of $MgSO_4, 7H_20$; 1 g of KCl; 0.7 g of NH_4NO_3; 10 g of glucose, 5 g of yeast extract; and 20 g of agar in 1000 ml of distilled water.

TABLE 1
LIST OF SEQUENCED STRAINS

Species	Isolate	Host species	Geographical origin	Date
Beauveria bassiana	Bb 28	*Leptinotarsa decemlineata*	France	1971
	Bb 70	*Lasiocampidae*	Madagascar	1973
	Bb 73	?	USRR	1974
	Bb 103	*Gargaphia* sp.	Colombia	1975
	Bb 147	*Ostrinia nubilalis*	France	1978
	Bb 169	*Sitona discoideus*	France	1981
	Bb 182	*Sitona discoideus*	France	1982
	Bb 216	*Sitona discoideus*	Morocco	1983
	Bb 377	*Ostrinia nubilalis*	France	1986
Tolypocladium cylindrosporum	TC 1	*Aedes sierrensis*	United States	1971
	TC 7	*Aedes australis*	New Zealand	?
	TC 8	Fern	England	1965
	TC 9	Soil	Holland	?
	TC 10	Soil	Czechoslovakia	1967
Tolypocladium extinguens	TC 16	*Arachnocampa luminosa*	New Zealand	1977
Fusarium oxysporum	FOM 15	Melon (Fungal Collection Cryptogamie, Univ. Paris XI)		

washed with sterile water, lyophilized, and stored at −20°C. Mycelium (120 mg) of each lyophilized strain was powdered in liquid nitrogen and soaked in 1 ml of an extraction buffer: 150 mM NaCl; 50 mM Tris, *p*H 7.4; 5 mM EDTA, and 5% sodium dodecyl sulfate.

Nucleic acids were extracted and purified twice with 1 ml of a solution of phenol:chloroform (1:1). Samples were centrifuged for 15 min at 10,000*g* during each extraction. A series of precipitation with LiCl (4 and 2 M) and ethanol permitted the samples to be washed, eliminating double-stand DNA.

rRNA Sequencing

The dideoxynucleotide chain termination method was used, modified for the use of reverse transcriptase (Qu et al., 1983). RNA synthesis was initiated using standard synthetic oligonucleotide primers (P1, P3) 5′ end labeled with γ P32 ATP and polynu-

which has also been sequenced using a third primer (P2).

Nucleotide fragments were separated by gel electrophoresis (6 and 8%) polyacrylamide in Tris–borate buffer. Gels were dryed before autoradiography and revealed after 3 days of exposure in Amersham film.

Analysis of Data

For each domain, the sequences were aligned manually and each one was compared with the others. Dendrograms were constructed by using the Fitch program of Felsenstein's Phylip package. This method translates directly punctual differences observed in phyletic distances measured in terms of the proportions of different nucleotides (Fitch and Malgoliash, 1967).

One approach by the parsimonious method was also studied using the DNA PENNY program from the same package. Parsimony selects the trees that require the minimum of events. One strain of *Fusarium*

in three distinct groups. The first comprises all the strains of *T. cylindrosporum*, the second comprises the strains of *B. bassiana*, and the third consists of one strain of *T. extinguens*. The sequences of the strains which comprise each group are strictly identical, regardless of the geographical origin and the host species. The three groups of sequences are sufficiently homologous to enable them to be aligned (Fig. 1).

The region we selected, about 500 nucleotides long, should provide enough data and should reduce the risk of spurious fluctuations in the statistics of nucleotide substitutions. The substitution of nucleotides constitutes the type of variation most often observed. As already observed in other organisms, the differences are particularly concentrated in the domain D2. The nature and the position of the majority of the substitutions between *T. extinguens* and *B. bassiana* are also found between *T. cylin-*

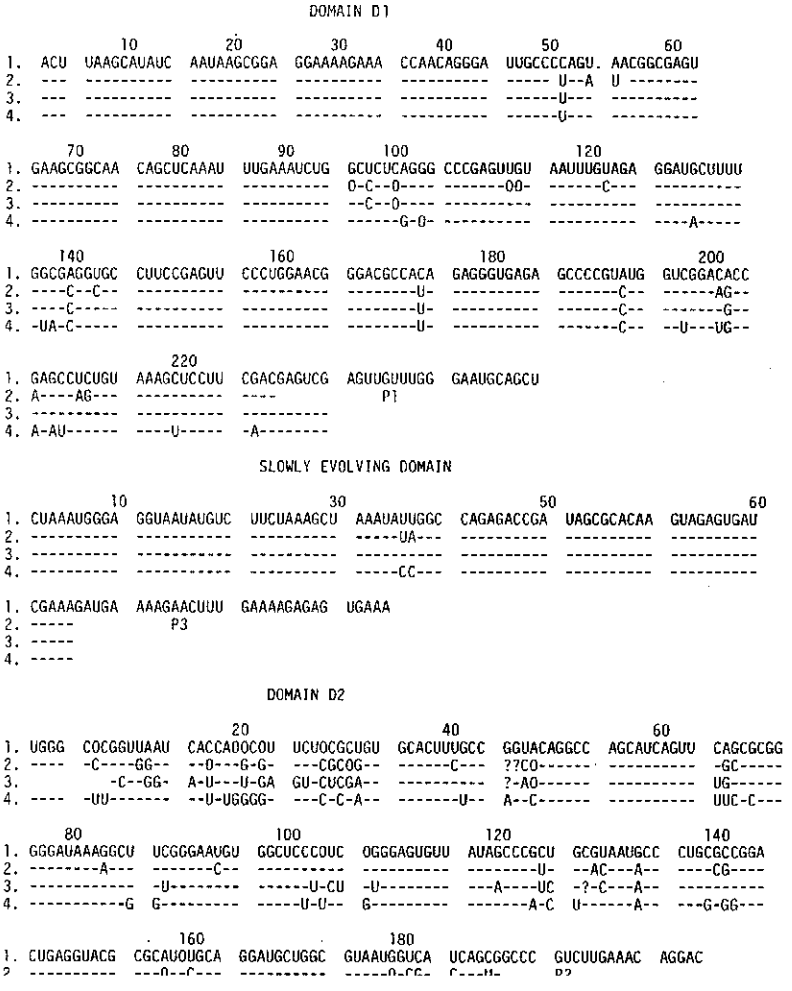

TABLE 2
DISTANCE MATRIX AMONG THE THREE CLASSES OF IDENTICAL SEQUENCES, PLUS ONE REFERENCE SPECIES: *Fusarium oxysporum*

	B. bassiana (Bb)	*T. cylindrosporum* (Tc)	*T. extinguens* (Te)	*F. oxysporum* (Fo)
Bb	—	51	39	47
Tc	0.315	—	47	65
Te	0.234	0.287	—	57
Fo	0.291	0.418	0.359	—

Note. Distances are expressed in absolute value of differing bases (up per matrix) within the D1 + D2 regions and in lower matrix the corresponding knuc value.

drosporum and *B. bassiana*. Within D1, containing 227 nucleotides, 18 sites of substitutions or deletions were denoted and 48 within the 190 sequenced nucleotides of D2 and finally only two substitutions were observed in the evolutionarily conserved region.

A representative comparison by a distance matrix is shown in Table 2. Only the two domains D1 and D2 have been taken into account. It appears that variation of D1 and D2 between *T. cylindrosporum* and *B. bassiana* is greater than that between *B. bassiana* and *T. extinguens*. The differences between *B. bassiana* and *T. cylindrosporum* is as important as that between *B. bassiana* and *F. oxysporum*.

The dendrograms (Fig. 2) produced by the two selected programs give a better understanding of the phyletic relationship among the four groups of sequences. The two approaches give the same topology. The dendrogram shown in Figure 2 was derived using the Fitch program of the Phylip package. Branch lengths were obtained with crude distance values without modification.

T. extinguens is relatively closer to *B. bassiana* than to *T. cylindrosporum*. Conversely, *T. cylindrosporum* is very different from *T. extinguens* and from *B. bassiana*. *B. bassiana* is as distant from *T. cylindrosporum* as from *F. oxysporum*. However, the latter is taken as an outgroup species. It is evident that the three groups are really very distinct.

DISCUSSION

T. cylindrosporum has been characterized by Von Arx (1986) as the synonym *B. cylindrospora* on the structural similarities of conidiogenous elements and the appearance of the colonies. Several authors have questioned the validity of this arrangement (Mugnai et al., 1989; Samson et al., 1988).

The two genera *Tolypocladium* and *Beauveria*, despite a similarity in certain morphological structures, have important

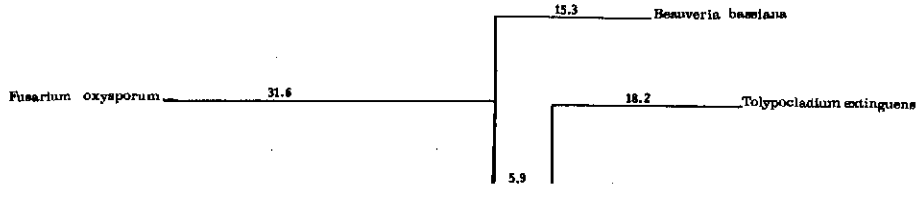

TABLE 3
COMPARISON OF DIFFERENT MORPHOLOGICAL CHARACTERS OF THE THREE GROUPS

Conidiogenous cells production	*Beauveria bassiana* Sympodial	*Tolypocladium cylindrosporum* Basipetal	*Tolypocladium extinguens* Basipetal
Structure of Conidiophores	Not in sporodochia, without stipe and swollen vesicles	Verticillate branches bearing lateral or terminal whorls of phialides	Bearing several regularly verticillates branches which terminate in whorls on phialides
Phialides	Absent	With swollen cylindrical base	Small with swollen base and a thin neck
Conidia	Different shape, one celled, globose	Cylindrical with rounded ends, often slightly bent	Subglobose to ellipsoidal
Conidiogenous cells	Smooth-walled, flask shaped with a swollen basal part terminating in a zig-zag rachis		

ontogenic differences, notably due to the fact that *Beauveria* has sympodial conidiogenis while that of *Tolypocladium* is basipetal (Table 3). Additionally, the molecular approach by the analysis of rRNA sequences enhances the differences between the genus *Tolypocladium* and the genus *Beauveria*. Moreover, the rRNA sequences show that the differences between *T. extinguens* and *T. cylindrosporum* are almost as great as the differences between the genus *Tolypocladium* and the genus *Beauveria* (Table 2; There are 47 variations between *T. extinguens* and *F. oxysporum* and 51 between *B. bassiana* and *T. cylindrosporum*). Since the rRNA of *T. cylindrosporum*, *T. extinguens*, and *B. bassiana* are as different between each other as they are from Fusarium (ex. 51 variations between *T. cylindrosporum* and *B. bassiana* and 47 between *B. bassiana* and *F. oxysporum*), we suggest on the one hand that the genus *Beauveria* and the genus *Tolypocladium* are not related and on the other hand that *T. cylindrosporum* and *T. extinguens* probably used for the determination of *T. cylindrosporum* and *T. extinguens* does not have phylogenetic significance. Moreover, *T. extinguens* is weakly virulent against mosquitoes species while strains of *T. cylindrosporum* are highly virulent.

In conclusion, the rRNA polymorphisms, the isozymes variability, and the morphoontogenic characters have complementary data from where we conclude the separation between the genus *Beauveria* and the genus *Tolypocladium*.

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