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The mycoparasitic fungus *Clonostachys pityrodes*: phylogenetic analysis as a tool for molecular identification

Abstract

Biological control is a promising and sustainable strategy to reduce damage caused by agricultural pests and the use of chemical fungicides. Fungal strains of the genus *Clonostachys* are studied as biocontrol agent of fungi and nematodes. However, the presence of this fungus in the soils of Misiones remains unexplored. Traditional fungal identification is generally carried out by morphological characterization in Petri dishes, and by observing their reproductive structures under the microscope. In general, with this methodology it is possible to identify to the genus level, however determining up to the species level is usually very complicated in some genera and many times ambiguities are achieved. In this context, molecular data emerges as an important tool to complement morphological information and thus achieve a correct fungal identification. The objective of this work was to molecularly identify with ITS markers a strain of the mycoparasitic fungus *Clonostachys* HEP30. The nucleic acids were isolated for molecular corroboration. From the extracted genetic material, the ITS1-5,8S-ITS2 region was amplified and sequenced. Once the region of interest was obtained, the information obtained was compared with that existing in the databases, using the Blast (Basic Local Alignment Search Tool) of the NCBI (National Center for Biotechnology Information) and the fungal barcoding database and then phylogenetic analysis was done. The molecular identification and phylogenetic analysis allowed us to classify the fungal isolate *Clonotachys* HEP 30 with high percentage of identity as a member of *Clonostachys pityrodes* species.

Keywords: identification, mycoparasitic, ITS, fungi, biocontrol

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Introduction

Biological control is a promising and sustainable strategy to reduce damage caused by agricultural pests and the use of chemical fungicides. Fungal mycoparasites include many living microorganisms that can act against another fungus to obtain different nutrients from the host to live.[1] Species from the *Clonostachys* genus (Ascomycota, Bionectriaceae) are common soil inhabitants and plant decomposers, and also some species are referred as endophytes commonly found in tropical and subtropical regions.[2,3] The *Trichoderma* genus is the best studied biocontrol agent (direct and indirect) of many microbial phytopathogens (fungi and bacteria).[3–8] However, nowadays some members of the *Clonostachys* genus are also referred as unspecific destructive mycoparasites, including against some microorganisms referred as important plant pathogens.[3,4,9,10] Traditional fungal identification is generally carried out by morphological characterization in Petri dishes, and by observing their reproductive structures under the microscope. In general, with this methodology it is possible to identify to the genus level, however determining up to the species level is usually very complicated in some genera and many times ambiguities are achieved. Molecular identification often is recommended as a valuable tool since many fungal isolates could not be differentiated easily and adequately by traditional cultural and morphological methods.[3,11,12] So, the objective of this work was to molecularly identify a strain of the mycoparasitic fungus *Clonostachys* HEP30 with ITS markers.

Material and methods

Biological material

The *Clonostachys* HEP 30 strain was isolate in soil samples from non-anthropic environments from the Capital department of Misiones province, Argentina. The *Clonostachys* HEP 30 strain was stored at 4°C under the accession number LBM247 in the Biotechnological Fungal Strain Culture Collection of the Misiones Biotechnology Institute.

Molecular identification

For DNA extraction and amplification the mycelia of the *Clonostachys* HEP 30 strain was grown in the dark in flask with malt extract broth with a concentration of 1.27% (w/v) (ME - Britania SA) at 28±1°C for 5 days. The extraction of genomic DNA was performed with standard protocols.[11–13] The obtained DNA was resuspended in 30 µL of sterile distilled free of DNAse water (Biopak®). Also, the obtained DNA was further examined by electrophoresis agarose gels at standard concentration of in 1% (w/v - InBio) and stained with a solution of Gel Red (Biotium, 10,000 X). A portion of the ITS region (ITS1-5.8S-ITS2) of approximately 550 bp was amplified by the polymerase chain reaction (PCR). This PCR amplifications were done in a standard 20µL reaction mixture composed of 1X PCR Buffer, 200 µM of dNTP mix, 2.5mM $MgCl_2$, 10 pmol of each of the amplification primers described below, 0.5 u of Taq polymerase (InBio), and approximately 1µg of genomic DNA.[11–13] The universal fungal primers used were the ITS1 F- (5'-TCCgTAggTgAACCTgCgg-3') and ITS4 R- (5'-TCCTCCgCTTATTgATATgC-3') (White et al., 1990).14 A stadard amplification protocol was used; it included an initial denaturation at 94°C for 4 min, followed by 35 PCR amplification iterations of 94°C for 40 s, 53°C for 40 s and 72ºC for 40 s. Also a final PCR-extension step of 72°C for 10 min was included.[11] The amplified fragment was further examined by electrophoresis agarose gels at a concentration of 2% (w/v - InBio) and stained with a solution of Gel Red (Biotium, 10,000X). Both strands of the amplicón (PCR product) were further sequenced by Macrogen Korea for the phylogenetic studies.[12]

Phylogenetic analysis

The ITS1-5.8S-ITS2 nucleotide sequence of *Clonostachys* HEP 30 isolate was compared against different nucleotide sequences deposited in the GenBank and Fungal barcoding databases for the molecular species identification. Twenty-two ITS sequences were retrieved from the GenBank and Fungal barcoding databases. All these sequences represented species within the *Clonostachys* genus (Table 1). Nucleotide sequences retrieved in this study consisted of about 600 bp corresponding to the complete ITS and partial 18S and 28S regions.[15] A sequence of *Trametes versicolor* (NR_154494.1) was used as an outgroup to root the *Clonostachys* phylogenetic tree. All DNA sequences selected were aligned using the Clustal W program.16 The analyses used were based on a distance-based method (Neighbor joining, NJ) and the MEGA 6.0 package[17] was used for the analyses. Also, for supporting the specific clades represented in the tree obtained bootstrap analyses of 1,000 replicates were carried out. The nucleotide divergences were estimated using Kimura's two-parameter method.

Table 1 Genbank accession numbers retrieved from databases

Species	Strain number	ITS rDNA accession number
Clonostachys chlorina	CBS 287.90	MH862212.1
Clonostachys intermedia	CBS 508.82	AF210682.1
Clonostachys intermedia	CBS 508.82	NR_137652.1
Clonostachys miodochialis	CBS 997.69	AF210674.1
Clonostachys miodochialis	CBS 997.69	NR_137649.1
Clonostachys miodochialis	CBS 997.69	MH859506.1
Clonostachys divergens	CBS 967.73b	AF210677.1
Clonostachys divergens	CBS 967.73b	NR_137532.1
Clonostachys grammicospora	CBS 209.93	MH862392.1
Clonostachys grammicospora	CBS 209.93	NR_137650.1
Clonostachys chlorina	CBS 287.90	NR_137651.1
Clonostachys candelabrum	CBS 504.67	MH859044.1
Clonostachys candelabrum	61_NO.ST78.TLOM1	KY977560.1
Clonostachys candelabrum	ccy1	KY315566.1
Clonostachys capitata	CBS 218.93	AF358240.1
Clonostachys capitata	CBS 218.93	MH862394.1
Clonostachys rosea	1087	HM052819.1
Clonostachys rosea	CBS 154.27	NR_165993.1
Clonostachys pityrodes	-	JQ234965.1
Clonostachys pityrodes	CBS 126394	MH864280.1
Clonostachys pityrodes	BR69	MN637804.1
Clonostachys pityrodes	C40376SNA1CC1089	JQ411387.1

Results and discussion

For molecular identification the genomic DNA of *Clonostachys* HEP 30 strain was extracted and amplified using a pair of primers, ITS 1 and ITS 4. The ITS sequence obtained had 563 bp after sequencing and contig construction. The ITS1-5.8S-ITS2 nucleotide sequence obtained of *Clonostachys* HEP 30 strain was deposited in GenBank-NCBI database under the accession number MH048667. The comparison and molecular analysis of *Clonostachys* HEP 30 sequence with the selected sequences in both genetic databases allowed us to state this sequence as belonging to *Clonostachys pityrodes* species. Our sequence obtained high percentages of identity of 99% with the MN637804 accession number from NCBI database, and with a sequence belonging to *Bionectria pityrodes* species (anamorph of *C. pityrodes*) with the CBS126394 accession number from Fungal barcoding database. The ITS1-5.8S-ITS2 phylogenetic trees obtained by the NJ method revealed that our fungal isolate under study belongs to e monophyletic clade of *C. pityrodes* species (92% bootstrap) (Figure 1). Also, our phylogenetic analyses revealed close positioning of *C. pityrodes* with *Clonostachys candelabrum* in a closely related group, separated of the others species of *Clonostachys* genus.

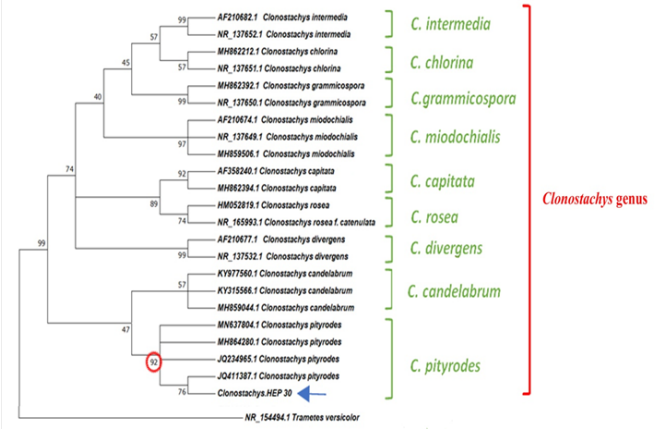

Figure 1 ITS1-5.8S-ITS2 phylogenetic trees obtained by the NJ method of *Clonostachys* HEP 30.

The identification of species belonging to this genus is notoriously difficult by traditional morphological methods. Nowadays the molecular identification methods are commonly referred to do not replace the morphological traditional identification of fungi; conversely, they complement the morphological methods.[12] It is proposed that the effective and safe application of any biocontrol agents (liquid or solid) depend on environmental conditions and target species, but also in an accurate and reliable identification of the biocontrol agents in time and space.[3]

Conclusion

The molecular identification and phylogenetic analysis allowed us to classify the fungal isolate *Clonotachys* HEP 30 with high percentage of identity as a member of *Clonostachys pityrodes* species.

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None.

Conflicts of interest

The authors declare that there is no conflict of interest.

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