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The ectomycorrhizal basidiomycete *Hebeloma circinans* harbors a linear plasmid encoding a DNA- and RNA polymerase

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Bulk DNA isolated from the ectomycorrhizal basidiomycete *Hebeloma circinans* was treated with proteinase K and submitted to agarose gel electrophoresis. In addition to high molecular weight genomic DNA, three minor bands were detected. The band with the highest electrophoretic mobility (2.2 kbp) corresponds to double-stranded RNA. The two other bands, termed pHC1 and pHC2, were shown to be dsDNA molecules of 10.3 and 9.1 kbp, respectively. Treatment of the pHC elements with 3′- and 5′-specific exonucleases revealed a linear structure and proved that the 5′ ends are protected from digestion; for pHC2, linearity was confirmed by restriction mapping. A 3.2 kbp *Hin*dIII fragment of pHC2 was cloned and sequenced; it contains two open reading frames encoding putative viral B type DNA and RNA polymerases. Thus, the fungus harbors a typical linear plasmid, up to now, rarely described for basidiomycetes and hitherto unknown for mycorrhizal species.

Key Words——DNA polymerase; *Hebeloma circinans*; linear plasmid; mycorrhiza; phylogenetic tree; RNA polymerase

While plasmids were formerly considered to be circular molecules, some 20 years ago linear DNA elements were also detected. Originally found in plants, i.e., *Zea mays* (Pring et al., 1977), they are now known as common elements in both pro- and eukaryotes (Meinhardt et al., 1997). In fact, the majority of plasmids in eukaryotes are linear (Esser et al., 1986). In filamentous fungi, linear plasmids are located in the mitochondria and share common structural properties; at both 5′ ends, proteins are covalently attached (terminal proteins; TP) and inverted sequence repeats can be found at the termini (terminal inverted repeats; TIR). In general, linear plasmids of filamentous fungi appear to be uniform in structure. They encode viral-like DNA- and/or RNA-polymerases only, making them independent from their hosts in terms of replication and transcription. Comparative sequence analyses have revealed phylogenetic relations among several linear elements, such as adenoviruses, phages with linear genomes, cytoplasmic killer plasmids of yeasts, and the mitochondrial linear plasmids of filamentous fungi (Griffiths, 1995; Kempken, 1995; Meinhardt et al., 1990; Schründer and Meinhardt, 1995). Linear plasmids have been very rarely described in basidiomycetes and, up to now, for only one element, pEM of *Agaricus bitorquis* (Robison et al., 1991), is sequence data available. The objective of this contribution was to broaden our knowledge about plasmids from basidiomycetes. Thus, we present data on isolation, characterization and partial sequencing of an extrachromosomal element from *Hebeloma circinans.* This fungus is of special interest since it is, according to our knowledge, the first mycorrhizal species in which plasmids were described. *H. circinans* forms an ectomycorrhizal symbiosis with *Picea* spp. and can also be found in association with *Abies pectinata* and *Pinus mugo.*

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Materials and Methods

Strains, plasmids and phages. The bacterial and fungal strains used were as follows: *Escherichia coli* JM107 (Messing et al., 1981) and DH5α (Hanahan, 1983). The strain BR63-10 of *H. circinans* Quélet was kindly provided by Prof. G. Bruchet, Université Lyon 1. It was isolated in 1963 from a sporocarp fragment found under *Picea excelsa* in Samoens (alt. 1,300 m, Haute Savoie, French Alps). The mycelium was

stored at 4°C till 1987 on synthetic culture media with one subculture each year without loosing its plasmids. Bacterial plasmids used were: pUCBM20 and pUCBM21 (Yanisch-Perron et al., 1985; Boehringer Mannheim, Germany). Phages used were M13mp18 (Norrander et al., 1983) and M13BM20/21 (Boehringer Mannheim).

Media and culture conditions. *E. coli* was routinely cultivated on LB-medium (Tryptone 1% (w/v), yeast extract 0.5% and NaCl 1% (w/v), pH 7.2, and grown overnight at 37°C by incubating at 220 rpm on a Model G76 gyrotory water bath shaker (New Brunswick Scientific Co., Inc., Edison, U.S.A.).

The culture medium for *H. circinans* consisted of malt extract 0.8% (w/v), glucose 0.7% (w/v), casitone 0.1% (w/v), KH_2PO_4 0.05% (w/v), $MgSO_4$ 0.05% (w/v), asparagine 0.05% (w/v), biotin 0.4 μg/*l* and thiamine 40 μg/*l*, pH 7.6. The fungus was grown in Petri dishes at 20°C for 3 weeks on this medium solidified with 1% agar. The mycelium grown on a single Petri dish was cut into pieces and used to inoculate a Fernbach flask containing 150 ml of liquid medium. After 10 weeks, this culture was shortly blended and used to inoculate 30 Fernbach flasks with 150 ml medium each.

Isolation of DNA. Plasmid DNA from *E. coli* and single as well as double-stranded DNA from M13 phages were isolated according to Sambrook et al. (1989).

Isolation of fungal nucleic acids. The mycelium was harvested by filtering through cheese cloth and its wet weight determined. The mycelium was ground in a mortar under liquid nitrogen. Powdered mycelium was suspended in 5 ml of 2% SDS in TES buffer (Tris 50.0 mM, EDTA 5.0 mM, NaCl 50.0 mM, pH 8.0) per g and 20 μg proteinase K per ml buffer was added, followed by incubation at 37°C for 4 h and 60°C for 1 h. The slurry was centrifuged at 10,000 rpm at 4°C in a Sorvall SS34 rotor (Dupont, Bad Homburg, Germany) and NaCl was added to the supernatant to a final concentration of 1 M. After mixing, the sample was kept on ice overnight and then again centrifuged for 20 min. To the supernatant, 40% PEG (polyethyleneglycol) 6000 in TE buffer was added and nucleic acids were precipitated for 6 h at 4°C. Centrifugation at 5,000 rpm and 4°C in a SS34 rotor pelleted the sample, which was dissolved in 10 ml TE buffer overnight. Further purification of the nucleic acids was achieved by 3 to 5 phenol-chloroform extractions.

Transformation protocols and DNA analysis. *E. coli* was transformed and transfected according to Sambrook et al. (1989). Agarose gel electrophoresis was performed by conventional methods (Sambrook et al., 1989). Reactions with restriction endonucleases, DNA-ligase and other nucleic acid-modifying enzymes, such as RNase and DNases, were carried out according to the recommendation of the supplier (Boehringer Mannheim). For Southern hybridization (Southern, 1975), the probe was labeled with digoxigenin-dUTP from a DIG DNA Labeling Kit (Boehringer Mannheim) and used at a temperature of 68°C. The detection of DNA/DNA hybrids was performed with a Nucleic Acid Detection Kit (Boehringer Mannheim).

Nucleotide sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) using α-^{35}S-dATP (Amersham Buchler, Braunschweig, Germany), Sequenase (USB, Cleveland, OH, U.S.A.) and the M13 universal sequencing primer (Boehringer Mannheim) or, in the case of the primer walking strategy, with synthesized oligonucleotides. The DNA was either sequenced by creating a set of sequentially overlapping clones (Dale et al., 1985) or by primer walking.

Computer analysis was done using the Husar/GENIUSnet System (Husar: Release 2.1 9/1991) provided by DKFZ Heidelberg, Germany. Phylogenetic calculations were carried out with the TREE program, which is based on the method developed by Feng and Doolittle (1987).

Results and Discussion

Isolation of nucleic acids

Total nucleic acids were isolated from the fungus and submitted to gel electrophoresis; three bands could be visualized in addition to the high molecular weight chromosomal DNA (Fig. 1). When nucleic acid samples were treated with DNase, all bands except the 2.2 kbp band readily disappeared. Treatment with RNase in 2×SSC-buffer (high salt) did not result in the degradation of any of the bands shown in Fig. 1. However, RNase digestion carried out in 0.1×SSC-buffer (low salt) led to the disappearance of the 2.2 kbp band exclusively. DNase sensitivity and RNase resistance confirmed the elements, designated pHC1 and pHC2, to be DNA molecules, whereas the nucleic acid with the electrophoretic mobility of 2.2 kbp evidently consisted of double-stranded RNA. The occurrence of dsRNA representing the genomes of virus-like particles (VLPs) is common and widespread in fungi (Lemke, 1979). pHC bands were detected by gel electrophoresis only after having treated the nucleic acid samples with proteinase K as described in the MATERIALS AND METHODS section. When proteinase K was omitted from the purification procedure, plasmids adhered to the protein fraction and were removed during phenol extraction along with other proteins. In addition, plasmids could not enter the gel without proteinase treatment. Both elements were sensitive to exonuclease III but resistant to λ exonuclease (data not shown), the former being a 3′-spe-

cific nuclease and the latter attacking DNA from the 5′ ends. Thus, both pHC elements were considered to be linear DNA molecules with 5′-attached proteins. Since pHC2 has a higher copy number and a greater electrophoretic mobility than pHC1, it was chosen for further analyses.

Restriction mapping and sequencing

About 50 µg of DNA was isolated from *H. circinans* BR63-10 and submitted to preparative gel electrophoresis. Subsequently, pHC2 DNA was electroeluted and characterized by restriction enzyme digestions, the results of which are summarized in Fig. 2a. The putative linear structure of pHC2 was confirmed by restriction enzyme analysis. The positions of the

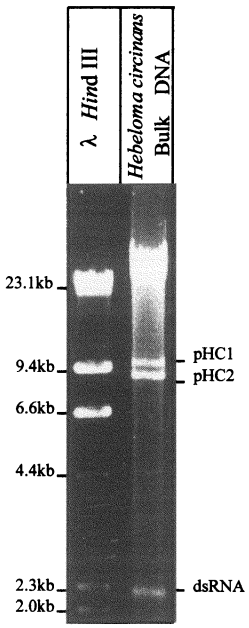

Fig. 1. *H. circinans* BR63-10. Agarose gel electrophoresis of bulk DNA.

The left lane contains λDNA digested with *Hin*dIII as a molecular weight standard. In the right lane, in addition to the high molecular weight chromosomal DNA, three bands of minor molecular weight are visible. The band exhibiting an electrophoretic mobility of 2.2 kbp consists of dsRNA as indicated. Bands designated pHC1 and pHC2 correspond to linear plasmids (for details see text).

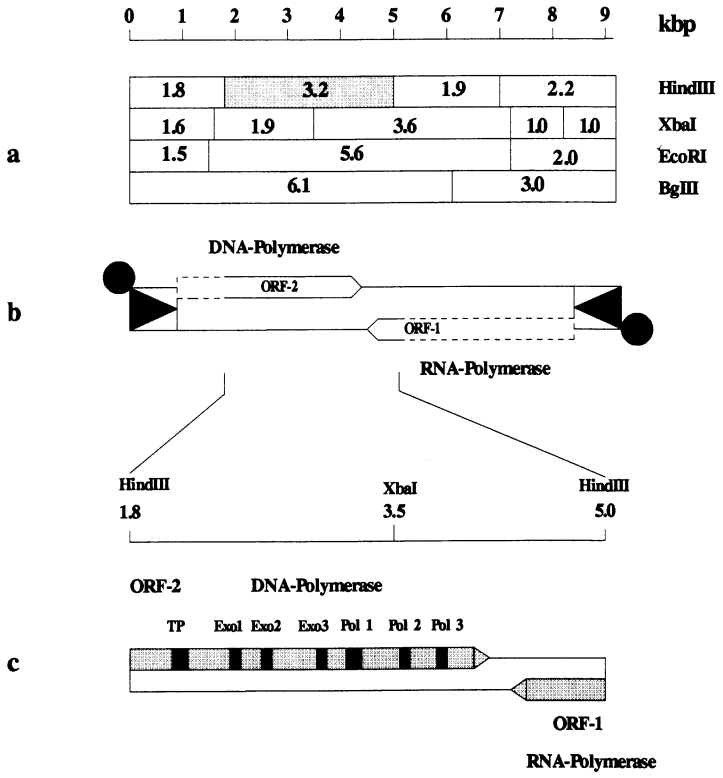

Fig. 2. Schematic representation of linear plasmid pHC2.

a: Restriction enzyme map, the hatched fragment corresponds to the sequenced area of pHC2. b: Organization of pHC2, arrows correspond to ORFs and their directions. Sequenced parts are indicated as solid lines, dotted lines were used to complete ORFs. The sizes of the ORFs were calculated as a mean value from sequence data available for other plasmids (Rohe et al., 1992). Black triangles correspond to presumptive terminal-inverted repeated sequences. Black circles indicate 5′-attached proteins. c: Magnification of the sequenced part of pHC2. The positions of specific functional domains are also indicated. TP, terminal protein; Exo1, 2, 3, exonuclease domains; Pol1, 2, 3, polymerase domains (see also Fig. 3).

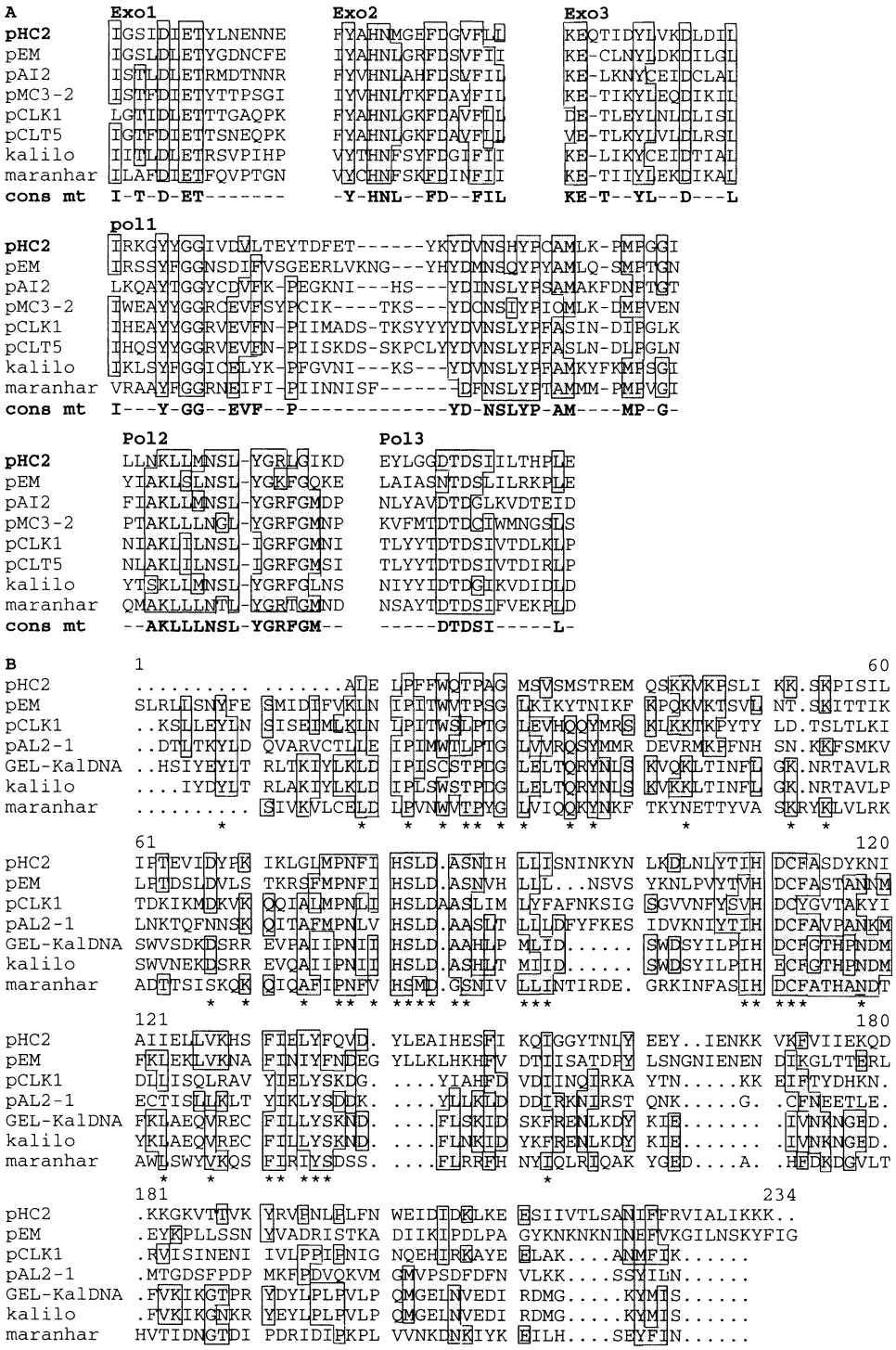

Fig. 3. A: Comparison of conserved domains of the DNA polymerases from filamentous linear mitochondrial plasmids with protein-primed replication.

Exo1, 2, 3 indicate exonuclease domains. Pol1, 2, 3 indicate polymerase domains. Consensus sequences of the mitochondrial linear plasmids from various filamentous fungi are shadowed and conserved amino residues indicated in bold face (cons mt).

B: Aligned polypeptide sequences of the RNA polymerases of linear mitochondrial plasmids of fungi.

Gaps have been introduced to give the best alignment. Identical residues in the five polypeptides are indicated with asterisks.

Source of plasmid sequences (EMBL Heidelberg, Germany): *H. circinans* pHC2 [this work], *Agaricus bitorquis* pEM (Robison et al., 1991), *Ascobolus immersus* pAI2 (Kempken et al., 1989), *Morchella conica* pMC3-2 (Rohe et al., 1991), *Claviceps purpurea* pCLK1 (Oeser and Tudzynski 1989), *Claviceps purpurea* pCLT5 (Oeser et al., 1993), *Neurospora intermedia kalilo* (Chan et al., 1991), *Neurospora crassa maranhar* (Court and Bertrand, 1992), *Podospora anserina* pAL2-1 (Hermanns and Osiewacz, 1992), *Gelasinospora* sp. Gel-kalDNA (Yuewang et al., 1996).

cleavage sites given in the map (Fig. 2a) were determined by single and double digestions.

In our attempts to clone pHC2 DNA in *E. coli*, we used restriction enzymes *Eco*RI, *Xba*I and *Hin*dIII, respectively. However, we were only successful in obtaining the 3.2 kbp *Hin*dIII fragment. Southern hybridization with total fungal nucleic acids, using the cloned fragment as a probe, proved it to be part of pHC2 (data not shown).

Using the internal *Xba*I site of the 3.2 kbp *Hin*dIII fragment, the DNA was subcloned in M13 sequencing vectors as 1.7 and 1.5 kbp fragments, both of which were totally sequenced. The first sequencing reactions involved universal primers, which allowed the identification of about 300 bp from each end of both fragments. Concerning the 1.7 kbp fragment, nucleotide sequences were obtained by primer walking; 9 different primers were used. For sequencing of the 1.5 kbp fragment, a set of sequentially overlapping clones was created (Dale et al., 1985).

The complete nucleotide sequence of the *Hin*dIII fragment was sent to EMBL-Heidelberg and given the accession number HC11504.

Two open reading frames (ORFs) could be detected. With respect to the direction, ORF1 spans nucleotide numbers 3227 to 2600 followed by a stop codon. The deduced amino acid sequence of ORF1 shows striking similarities to RNA polymerases encoded by other linear genomes, especially to linear plasmids from filamentous fungi (Figs. 2b, 3B). Although the N-terminus of the enzyme was not sequenced, the remaining part was sufficient to find homologous counterparts in databases, such as Swiss-Prot or EMBL (Fig. 3B). ORF2 spans nucleotides no. 1 to no. 2575 and is also followed by a TAA stop codon. The deduced amino acid sequence was compared to protein databanks (EMBL, Swissprot). Similarities were found to DNA polymerases, especially to those encoded by linear genomes, such as adenoviruses, phages with linear genomes, and linear plasmids from yeasts and filamentous fungi. Highly conserved regions, which are hatched in Fig. 3A, correspond to functional domains present in viral B-type DNA-polymerases (Bernad et al., 1987). Exo1 to Exo3 are characteristic domains instrumental in the exonuclease activity of the enzyme, whereas Pol1 to Pol3 indicate the polymerase functional domains. TP indicates the presence of a putative terminal protein being encoded as part of the DNA polymerase ORF2 (see also Fig. 2c). Cloning of other parts of pHC2 was

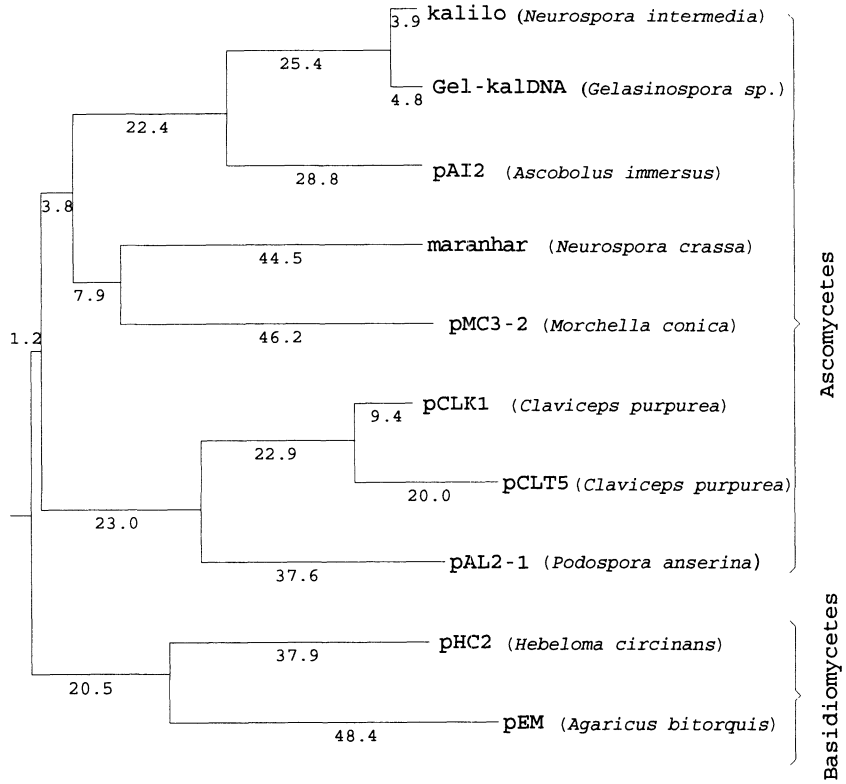

Fig. 4. Cluster dendrogram for DNA polymerases encoded by linear plasmids from filamentous fungi.
Relative evolutionary distances are indicated. The tree was calculated by comparing amino acid sequences spanning Exo1 to Pol3, as indicated in Fig. 3A. The calculation was done by employing the program "Tree" (Feng and Doolittle, 1987) provided by the Husar/Genius System Heidelberg, Germany. The source of sequences is given in the legend of Fig. 3.

hampered by the fact that the copy number of pHC2 decreased constantly when the fungus was grown on artificial culture media. Similar to data obtained for plasmids of the morels (Rohe et al., 1991), pHC2 could only be detected in these continuously propagated mycelium by Southern hybridization using the 3.2 kbp *Hin*dIII fragment as a probe. Taken also into consideration that additional ORFs are not likely to be present (see Fig. 2b), we refrained from cloning other fragments of the plasmid. The high AT content of 73% is consistent with data obtained for many other linear plasmids of fungi, such as 74% for the pEM of *Agaricus bitorquis* (Robison et al., 1991), 67% for the pMC3-2 of *Morchella conica* (Rohe et al., 1991) and 70% for the *kalilo* of *Neurospora intermedia* (Chan et al., 1991).

By comparing our data to other linear plasmids sequenced thus far, it became evident that pHC2 constitutes a typical, mitochondrially associated linear plasmid known to be present in a number of eukaryotes. Based on the obtained sequence data and considering the size of the corresponding ORFs from other linear plasmids of fungi, the structure of pHC2 is schematically presented in Fig. 2b. The position of the functional domains of the DNA polymerase are given in Fig. 2c. Their arrangement corresponds to linear plasmids in general; that is, all linear plasmids so far described show the same succession of the functional domains in their DNA polymerases (Kempken et al., 1992; Rohe et al., 1992).

pHC2 is the second linear plasmid of a basidiomycete for which sequencing data are available. Moreover, it is the first plasmid known from a mycorrhizal fungus. Up to now, all we knew about the molecular biology of the extrachromosomal elements of ectomycorrhizal organisms was by analogy to saprophytic species. The data presented in this contribution prove, however, that symbiotic basidiomycetes may contain very similar plasmids. Moreover, phylogenetic distance calculations based on the sequence data of linear plasmids from filamentous fungi (Rohe et al., 1992) have verified a close relationship of both pEM and pHC2 isolated from basidiomycetes (Fig. 4).

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