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The *Cryphonectria parasitica* plasmid pUG1 contains a large ORF with motifs characteristic of family B DNA polymerases

E. Gobbi*, A. Carpanelli, G. Firrao and R. Locci

Dipartimento di Biologia applicata alla Difesa delle Piante, Università di Udine, Via Scienze 208, Udine I-33100, Italy

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ABSTRACT

The isolation and characterization of the circular mitochondrial plasmid pUG1 from the ascomycete *Cryphonectria parasitica* is described. The entire sequence (4182 bp) was obtained and high similarities to DNA-dependent DNA polymerases were revealed. Strikingly common features with the DNA polymerases encoded by the *Neurospora intermedia* plasmids Fiji and LaBelle, such as matches to the conserved motifs A and B and the presence of TTD instead of DTD in motif C, were found, suggesting the existence of a distinct group of members of the B DNA family polymerases. These strong similarities between the plasmids might suggest a common origin of the *C.parasitica* and the *Neurospora* plasmids.

INTRODUCTION

Plasmids are very commonly found in filamentous fungi where they generally have a mitochondrial localization (1). Some plasmids represent defective forms of mitochondrial DNA (mtDNA), while others lack any homology to the mtDNA.

The majority of the plasmids so far identified, belonging to the second type, are linear but their functions are still unclear with a few exceptions such as the kalilo and maranhar DNA of *Neurospora* spp. and pAL2-1 of *Podospora anserina* (2).

Circular plasmids have been reported to occur in fungi, and to date they include only the Mauriceville, Fiji, LaBelle, Java, Mb1, VS, Harbin 2 plasmids in *Neurospora* spp. (3), the plasmids of *Absidia glauca* (4) and of *Pythium* spp. (5). The circular plasmids are not associated with a particular phenotypic trait with the exception of a plasmid of *A.glauca* which encodes for a surface protein present only in mating type positive strains harbouring the plasmid (4).

In the plant pathogenic fungus *Cryphonectria parasitica* a circular plasmid, pUG1, has been found in virulent strains exhibiting a senescent phenotype of the colony reported as heteroauxesis (6). Morphological alterations of plasmid-containing virulent strains of *C.parasitica* appeared during prolonged vegetative propagation as abnormalities of the colony development. The phenomenon also includes cessation of growth of the colony with limitations of the morphological development and possibly consequences on the dynamics of the development of the pathogen in the environment (7).

Cryphonectria parasitica has a great economic and ecological relevance to forestry as the causal agent of Chestnut Blight, the severe disease that destroyed hectars of chestnut stands mostly in USA and Europe (8,9). Transmissible hypovirulence, i.e. a dramatic decrease in pathogenicity, is associated with an extra-chromosomal viral ds-RNA (10) and characterizes hypovirulent strains of the fungus, making biological control treatments possible and efficient in Europe (9,11).

The *Neurospora* plasmids are some of the best studied and characterized plasmids, nonetheless their origin, functions and the mechanisms of their maintenance are still not well understood. A reverse transcriptase activity has been proposed to explain the formation and replication of the Mauriceville homology group plasmids (12), while the LaBelle and Fiji plasmids were found to encode a DNA-dependent DNA polymerase (13) like other linear plasmids such as kalilo from *Neurospora intermedia* (14), maranhar from *Neurospora crassa* (15), pEM from *Agaricus bitorquis* (16), pClK1 from *Claviceps purpurea* (17), pAI2 from *Ascobolus immersus* (18) and S1 from maize (19).

In the present study, the first reported isolation, the characterization and complete sequence of pUG1 are given. The plasmid shows a high degree of similarity with two *N.intermedia* plasmids, Fiji (13) and LaBelle (20), and might encode for a DNA polymerase. The plasmid mitochondrial location is confirmed.

MATERIAL AND METHODS

The sequence reported in this paper has been deposited in the EMBL database (accession number Y12637).

Strains and plasmids

Three isolates of *C.parasitica* harbouring plasmids, Cp5, Cp9 and Cp13, were isolated from evolutive cankers on chestnut trees (21), Cp5pl$^-$ is a derivative of strain Cp5, which lost its plasmid spontaneously during vegetative propagation. Strain Ep155 (ATCC 38755) is a laboratory standard plasmid-free strain provided by N.K.Van Alfen (Texas A&M University, Texas, USA). Ccp29 and Ccp28 were donated by G.J.Boland (University of Guelph, Ontario, Canada) and are dsRNA-free hypovirulent strains.

The plasmids pECUG1a and pECUG1b contained respectively the 2.4 and 1.7 kb *Kpn*I fragments of the pUG1 plasmid inserted into the *Kpn*I site of pUC19. pECUG1a was cleaved by *Eco*RI and subcloned in two plasmids, pECUG182 and pECUG322, used in sequencing.

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* To whom correspondence should be addressed. Tel: +39 432 558503; Fax: +39 432 558501; Email: micol@pldef.uniud.it

Fungal DNA isolation

The isolation of mtDNA and nuclear DNA (nDNA) by CsCl–bisbenzimide gradient, was performed according to Gobbi *et al*. (22). The DNAs were digested by *Kpn*I for 2–8 h. MtDNA was treated with Ribonuclease A from bovine pancreas (Sigma, St Louis, MO, USA) at 37°C for 20 min. The digested nDNA and mtDNA were electophoresed in 0.7% agarose gel in TBE buffer with EtBr (0.5 µg/ml) at 50 V/m for 16–18 h at 4°C.

For rapid extraction of total DNA, fungi were grown on PDAmb agar plates covered by a membrane (Bio-rad Laboratories #165-0963, Hercules, CA, USA) for 7 days at 25°C in the dark.

The DNA was extracted from the mycelia as described in Lecellier and Silar (23).

Cloning and hybridization procedures

The procedures used in plasmid cloning, preparation and transformations were performed according to Ausubel *et al*. (24).

Plasmids pECUG1a and pECUG1b DNAs were labelled by random priming according to the manufacturer (Boehringer Mannheim Biochemicals, Mannheim, GE) with 7dUTP digoxigenin (BMB). The visualization of membranes was performed following the manufacturer's instructions (BMB). After hybridization, the membranes were stripped and rehybridized with different probes.

Isolation of mitochondria

Fresh mycelium was obtained from 8 day-old biomass grown in 250 ml complete medium (25) inoculated with 5 × 10^5 conidia and incubated at 25°C with agitation (100 r.p.m.). The mycelium was ground, with glass beads, on ice in a mortar containing cold extraction buffer (10 mM TES-NaOH pH 7.5, 1 mM EDTA pH 8, 0.33 M saccharose) as described (26). The homogenate was centrifuged at 1100 *g* for 10 min to remove cell debris and the supernatant was centrifuged at 15 000 *g* for 20 min to give a pellet enriched in mitochondria. The pellet was suspended in extraction buffer and purified by a discontinuous sucrose gradient. Mitochondria were recovered from the 1.6–1.2 M interface and washed with extraction buffer prior to the DNAse digestion at 37°C for 1 h. Mitochondrial DNA was isolated by proteinase K treatment (100 µg/ml) in the presence of sarkosyl (10%), followed by phenol extractions and ethanol precipitation.

DNA sequencing

Both strands were sequenced using the Thermosequenase flourescent labelled primer cycle sequencing kit (Amersham International plc, Buckinghamshire, UK) and the automated sequencing system A.L.F. DNA Sequencer (Pharmacia Biotech Europe, Brussels, Belgium). M13 universal and reverse primers were used to sequence inwards and the internal regions of clones pECUG1b and pECUG182 were sequenced by primer walking. Each region was sequenced at least twice. The analyses of the sequence data were performed using the sequence analysis software package from the Genetics Computer Group of the University of Winsconsin (27) and by the BLAST (28) programs for database searches.

RESULTS

Detection of plDNA

Low molecular weight plasmid-like DNA bands were detected during a screening of mtDNA of *C.parasitica* strains isolated from nature. All these bands disappeared when treated with DNAse but were resistant to the RNAse treatment (data not shown). In the *Kpn*I-digested mtDNA of strain Cp9, two bands, which fluoresced more intensely than the larger fragments derived from the mtDNA digestion (Fig. 1A, lane 1), were supposed to be of plasmid origin and cloned. When used as probes in Southern analysis, the two resulting recombinant plasmids pECUG1a and pECUG1b can only detect the two corresponding bands of 2.4 and 1.7 kb in the *Kpn*I-digested mtDNA (Fig. 1B and C, lanes 1). In Figure 1, the additional fainter hybridization signal, corresponding to a DNA fragment 4.1 kb in size and not belonging to the standard pattern of *Kpn*I-digested mtDNA, was an incomplete cleavage product, as proved by longer digestion (data not shown).

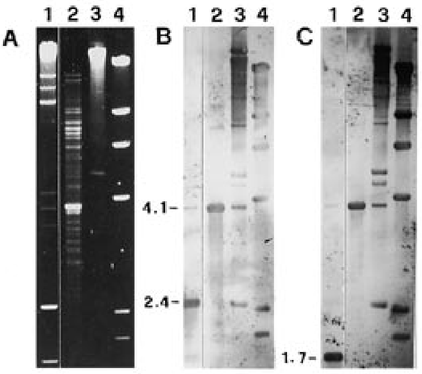


Figure 1. Southern hybridization analysis of strain Cp9 of *C.parasitica* harbouring the plasmid. The mtDNA was separated by CsCl–bisbenzimide gradient. (**A**) Ethidium bromide-stained agarose gel. Blotted DNA from (A) was hybridized using pECUG1a (**B**) and pECUG1b (**C**) as a probe. Lanes 1, *Kpn*I-digested mtDNA; lanes 2, *Eco*RI-digested mtDNA; lanes 3, undigested mtDNA; lanes 4, λDNA digested by *Hin*dIII as a marker.

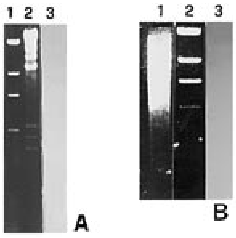


Figure 2. Absence of the plasmid in strain Ep155 of *C.parasitica.* The nDNA and the mtDNA were separated by CsCl–bisbenzimide gradient, digested and blotted. (**A**) Lane 1, λDNA digested by *Hin*dIII as a marker; lane 2, *Kpn*I-digested mtDNA; lane 3, blotted DNA from lane 2 was hybridized with the probe pECUG1a. (**B**) Lane 1, *Kpn*I-digested nDNA; lane 2, λDNA digested by *Hin*dIII as a marker; lane 3, blotted DNA from lane 1 was hybridized with the probe pECUG1a.

Figure 1 also shows undigested mtDNA of strain Cp9 (lanes 3). Up to nine bands may be visualized by EtBr staining and Southern analysis; these bands resolved into a single 4.1 kb DNA fragment when the DNA was cleaved by *Eco*RI (Fig. 1 lanes 2). These DNA bands, which migrate independently from the mtDNA molecule, share homology with both pECUG1a and pECUG1b and are therefore reported as being a plasmid present in Cp9 as a multimeric molecule. The 4.1 kb fragment is considered to

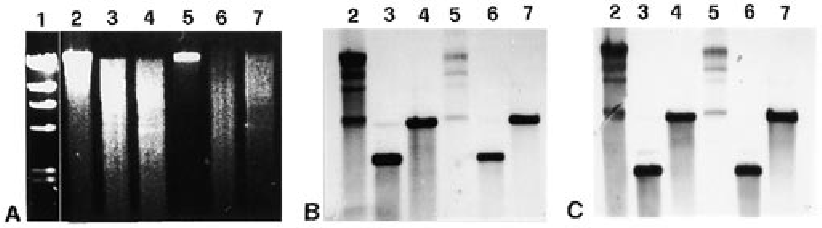


Figure 3. Distribution of plasmids homologous with pUG1 in strains of *C.parasitica*. The DNAs of strains Cp5 and Cp13 were extracted from the PDAmb agar plates, electrophoresed and blotted. (**A**) Ethidium bromide-stained agarose gel of total DNAs. Blotted DNA from (A) was hybridized using pECUG1a (**B**) and pECUG1b (**C**). Lane 1, λDNA digested by *Hin*dIII as a marker; lanes 2, undigested total DNA of Cp5; lanes 3, *Kpn*I-digested total DNA of Cp5; lanes 4, *Eco*RI-digested total DNA of Cp5; lanes 5, undigested total DNA of Cp13; lanes 6, *Kpn*I-digested total DNA of Cp13; lanes 7, *Eco*RI-digested total DNA of Cp13.

represent the basic unit and the monomer is called pUG1. According to Griffiths (1), the many bands present in the undigested sample are assumed to be different degrees of relaxation of concatamers of the basic unit. No plasmid bands were detected in Ep155 nDNA and mtDNA (Fig. 2) or in Ccp29 and Ccp28 DNAs (data not shown).

Homology to other DNAs

The sequence homology of the plasmid with nDNA and mtDNA was evaluated by hybridization studies.

The plasmid showed no homology with the nDNA or with the mtDNA of plasmid-containing and plasmid-free strains (Figs 1, 2 and 3), the probes pECUG1a and pECUG1b hybridized to their homologous targets, 2.4 and 1.7 kb respectively, when present but not to other genomic DNAs. Moreover, when the two strains Cp5 and its isogenic Cp5pl$^-$ were examined, signals appeared only in strain Cp5 (Fig. 4, lanes 3 and 4) corresponding to the plasmid fragments, this result confirms the absence of homology between pUG1 and mtDNA. In addition, the mtDNA patterns of these two strains were indistinguishable, as shown in detail in the *Eco*RI and *Kpn*I digestions, suggesting that no gross mutation has taken place as a consequence of the plasmid existence. Southern analysis (Fig. 3) showed a significantly high homology of pUG1 with the plasmids of similar sizes present in the strains Cp5 and Cp13; these plasmids also show an identical undigested and *Eco*RI- or *Kpn*I-digested pattern, apparently very similar to that of pUG1.

Localization of pUG1

The plasmid pUG1 has always been detected only in the mitochondrial fraction from CsCl–bisbenzimide gradient. The conclusive proof of the mitochondrial localization of pUG1 came from the isolation of the plasmid after the purified mitochondria of Cp9 were treated with DNAse. The digested DNA extracted from DNAse treated mitochondria contained the plasmid (Fig. 5).

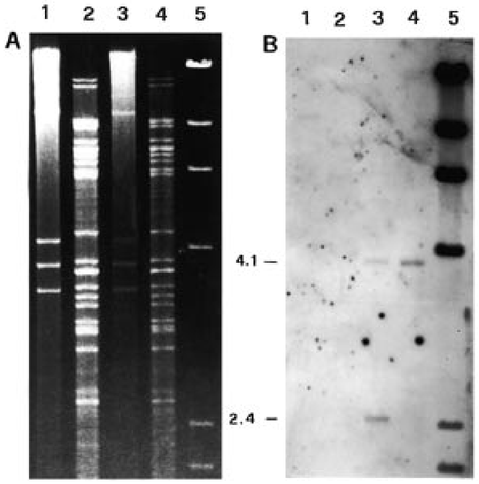


Figure 4. Southern hybridization analysis of strains Cp5 and Cp5pl$^-$ of *C.parasitica*. The mtDNA was separated by CsCl–bisbenzimide gradient, digested and blotted. (**A**) Ethidium bromide-stained agarose gel of mtDNAs. (**B**) Blotted DNA hybridized with the probe pECUG1a. Lanes 1, *Kpn*I-digested mtDNA of Cp5pl$^-$; lanes 2, *Eco*RI-digested mtDNA of Cp5pl$^-$; lanes 3, *Kpn*I-digested mtDNA of Cp5; lanes 4, *Eco*RI-digested mtDNA of Cp5; lanes 5, λDNA digested by *Hin*dIII as a marker.

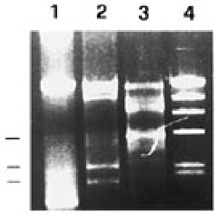


Figure 5. Evidence of the localization in the mitochondria of the plasmid pUG1 of *C.parasitica*. Ethidium bromide-stained agarose gel of DNA extracted from the DNAse-treated mitochondria of strain Cp9. Lane 1, undigested DNA; lane 2, *Kpn*I-digested mtDNA; lane 3, *Eco*RI-digested mtDNA; lane 4, λDNA digested by *Hin*dIII as a marker.

The structure of plasmid pUG1

The plasmid pUG1 is 4182 bp long and is a circular molecule as indicated by electrophoresis analysis. In Figure 1, the undigested mtDNA sample contains many bands in addition to the high molecular weight mtDNA band. The fastest migrating band hybridizes with both pECUG1a and pECUG1b. The apparent size of this band, if considered linear, would be 2.4 kb (Fig. 3B and C lanes 3), while, if compared to a supercoiled molecular weight marker, the resulting size of 4.0 kb (data not shown) would be consistent with the hybridization results, suggesting the supercoiled conformation of pUG1.

The plasmid sequence was determined from the two *Kpn*I clones pECUG1a and pECUG1b. To confirm that pECUG1a and pECUG1b actually represent the entire pUG1, we isolated PCR fragments obtained by using primer pairs derived from the ends of both pECUG1a and pECUG1b and total DNA as template. The sequence data of these PCR fragments are consistent with the

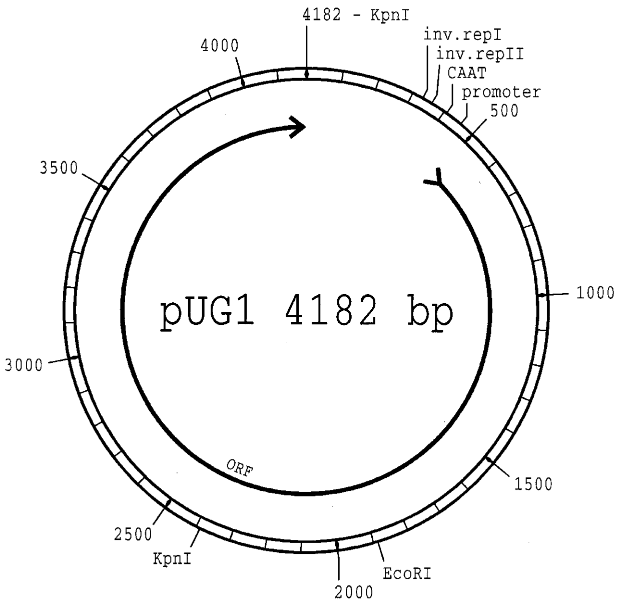


Figure 6. Physical map of the plasmid pUG1 isolated from strain Cp9 of *C.parasitica*. The long ORF, encoding a putative DNA polymerase, is indicated by the inner arrow. On the outer ring the putative CAAT box and promoter region, and the two longest inverted repeats, at position 331–364 and 359–408 bp respectively, are reported.

notion that the fragments are oriented as shown in the physical map in Figure 6 (data not shown).

The entire sequence of pUG1 was determined and the A+T content was estimated to be ~68.6%.

To determine the potential coding regions, the nucleotide sequence of pUG1 was translated in all possible reading frames using the genetic code typical for mitochondrial genes of filamentous fungi (TGA coding for Trp) (29). The codon usage of pUG1 was found to be similar to that of the mtDNA of *P.anserina*, and respectively slightly less similar to the codon usage of the Fiji plasmid, the mt ds-RNA of *C.parasitica* (30) and the LaBelle plasmid (data not shown). One long open reading frame (ORF) of 3645 bp was detected, which may encode a polypeptide of 1214 amino acids and its position is indicated on the map. Comparison of the ORF to the proteins in the databases revealed extensive regions of similarity with two proteins produced by the mitochondrial plasmids Fiji (PIR accession no. A47462) and LaBelle (PIR accession no. S03772) from *N.intermedia* (Fig. 7). These proteins have been proved to encode similar DNA dependent DNA polymerases (13).

The level of similarity of pUG1 ORF product to these proteins, is 56 and 57% for Fiji and LaBelle respectively. This similarity suggests a relatedness between them and also implies that pUG1 codes for a putative DNA polymerase.

By scanning the putative pUG1 polymerase sequence, some good matches to motifs A and B of the family B DNA polymerase (31) were found. Like Fiji and LaBelle polymerases, the pUG1 putative polymerase lacks a strict match to motif C of this family, it contains the sequence TTD instead of the sequence DTD, but the region surrounding the core of the motif contains several matches to the other polymerases of the B DNA family. The pUG1 putative DNA polymerase matches five of the 14 diagnostic amino acids for protein primed DNA polymerase motifs and only one of the 20 diagnostic amino acids for nucleic acid primed DNA polymerase motifs (32).

The plasmids pUG1, Fiji (GenBank accession no. L08781) and LaBelle (EMBL accession no. X13912) also share some other similarities such as being circular mitochondrial plasmids of similar length (4070 bp for LaBelle, 4182 bp for pUG1 and 5268 bp for Fiji), where a short non-coding region exists in addition to the long ORF. Moreover they have a G+C content that is approximately equal (35% for LaBelle, 31.4% for pUG1 and 29% for Fiji) and an interesting degree of identity in the region corresponding to their long ORFs (49% to the LaBelle plasmid and 50% to the Fiji plasmid).

The plasmid pUG1 has a 537 bp non-coding region which revealed no significant similarities to any other sequences in the databases. A putative promoter region, TTATAATCCTA, with two mismatches to the *Neurospora* consensus promoter TTAG-$^{A}/_{T}$RR$^{G}/_{T}$$^{G}/_{C}N^{A}/_{T}$ (33), might occur at position 457–467 bp. Also there is an upstream TCAATCT box at position 407 bp. In addition, in this region there are many repetitive elements and also inverted repeats, capable of forming potential stem–loop structures. The significance of these features is not known. In many organisms such structures have been implicated in transcription termination, RNA processing and mtDNA replication (20).

DISCUSSION

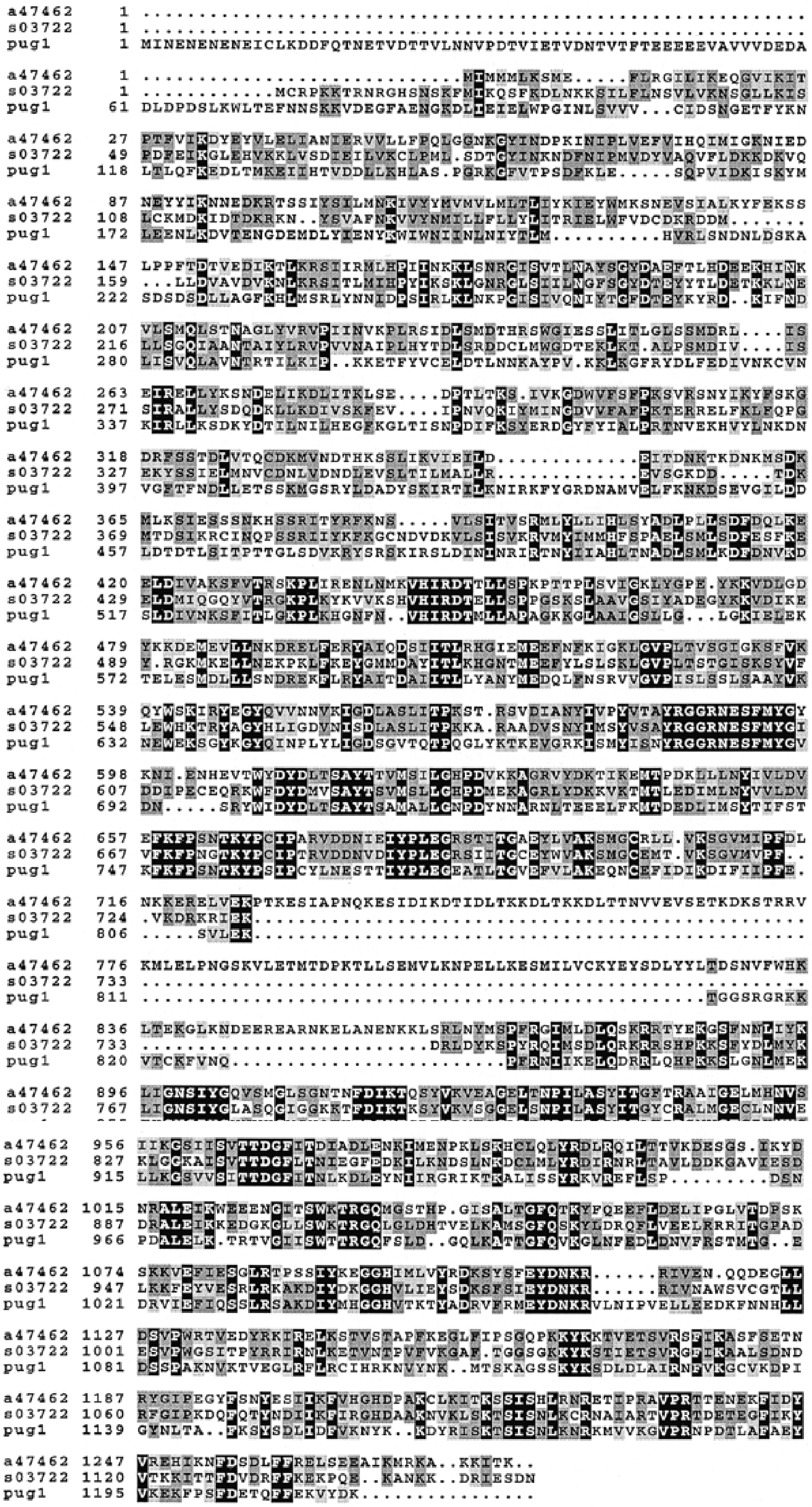
A plasmid-like DNA was reported in strains of *C.parasitica* in 1990 (34,35). It was described as being associated with a degenerative phenomenon called heteroauxesis. More recently Bell *et al.* (36), during the construction of the physical map of mtDNA of *C.parasitica*, found a plasmid of a similar size to pUG1.

The aims of this study were to characterize and localize the extrachromosomal plasmid, pUG1, and to assess the extent of sequence similarity with other DNAs to gain more insight into its biological role.

We report here the characterization and complete sequence of the plasmid pUG1 which exists as a mixture of multimeric molecules in mitochondria.

The mitochondrial plasmid pUG1 appears to constitute a distinctive genetic element: it shows virtually no homology with the standard mtDNA in DNA–DNA hybridizations, it achieves a high copy number without suppressive behaviour towards wild type mtDNA, and it also shows no homology with the nDNA. Since the detection of pUG1 from strain Cp9, all preparations of mtDNA of *C.parasitica*, from cultures isolated directly from natural populations, were assayed for the presence of plasmid DNA. The strains Cp5 and Cp13 harbour a plasmid very similar to pUG1 as confirmed by the hybridization experiments. Using pUG1 as a probe, plasmids were detected from 41% of the strains which belong to a population from a very restricted geographic area (E.G., unpublished), hence, confirming the notion that the plasmid-containing strains can be the rule rather than the exception in fungal populations (1).

Figure 7. Comparison of amino acid sequences from the putative pUG1 ORF protein and the ORF proteins of the Fiji and LaBelle plasmids. The residues that are identical in two polypeptides are boxed in dark grey. Light grey boxes indicate residues that are members of the same amino acid family. Black boxes indicate exact matches in all polypeptides.


a47462 1 ....
s03722 1 ....
pug1 1 MINENENENEICLKDDFQTNETVDTTVLNNVPDTVIETVDNTVTFTEEEEEVAVVVDEDA
a47462 1 ....MIMMMLKSME.....FLRGILIKEQGVIKIT
s03722 1 ............MCRPKKTRNRGHSNSKFMIKQSFKDLNKKSILFLNSVLVKNSGLLKIS
pug1 61 DLDPDSLKWLTEFNNSKKVDEGFAENGKDLLEIELWPGINLSVVV...CIDSNGETFYKN
a47462 27 PTFVIKDYEYVLELIANIERVVLLFPQLGGNKGYINDPKINIPLVEFVIHQIMIGKNIED
s03722 49 PDFEIKGLEHVKKLVSDIEILVKCLPML.SDTGYINKNDFNIPMVDYVAQVFLDKKDKVQ
pug1 118 LTLQFKEDLTMKEIIHTVDDLLKHLAS.PGRKGFVTPSDFKLE.....SQPVIDKISKYM
a47462 87 NEYYIKNNEDKRTSSIYSILMNKIVYYMVMVLMLTLIYKIEYWMKSNEVSIALKYFEKSS
s03722 108 LCKMDKIDTDKRKN..YSVAFNKVVYNMILLFLLYLITRIELWFVDCDKRDDM.......
pug1 172 LEENLKDVTENGDEMDLYIENYKWIWNIINLNIYTLM..........HVRLSNDNLDSKA
a47462 147 LPPFTDTVEDIKTLKRSIIRMLHPIINKKLSNRGISVTLNAYSGYDAEFTLHDEEKHINK
s03722 159 ...LLDVAVDVKNLKRSITLMIHPYIKSKLGNRGLSIILNGFSGYDTEYYTLDETKKLNE
pug1 222 SDSDSDLLAGFKHLMSRLYNNIDPSIRLKLNKPGISIVQNIYTGFDTEYKYRD..KIFND
a47462 207 VLSMQLSTNAGLYVRVPIINVKPLRSIDLSMDTHRSWGIESSLITLGLSSMDRL....IS
s03722 216 LLSGQIAANTAIYLRVPVVNAIPLHYTDLSRDDCLMWGDTEKLKT.ALPSMDIV....IS
pug1 280 LISVQLAVNTRTILKIP..KKETFYVCELDTLNNKAYPV.KKLKGFRYDLFEDIVNKCVN
a47462 263 EIRELLYKSNDELIKDLITKLSE....DPTLTKS.IVKGDWVFSFPKSVRSNYIKYFSKG
s03722 271 SIRALLYSDQDKLLKDIVSKFEV....IPNVQKIYMINGDVVFAFPKTERRELFKLFQPG
pug1 337 KIRLLKSDKYDTILNILHEGFKGLTISNPDIFKSYERDGYFYIALPRTNVEKHVYLNKDN
a47462 318 DRFSSTDLVTQCDKMVNDTHKSSLIKVIEILD.............EITDNKTKDNKMSDK
s03722 327 EKYSSIELMNVCDNLVDNDLEVSLTILMALLR.............EVSGKDD.....TDK
pug1 397 VGFTFNDLLETSSKMGSRYLDADYSKIRTILKNIRKFYGRDNAMVELFKNKDSEVGILDD
a47462 365 MLKSIESSSNKHSSRITYRFKNS.....VLSITVSRMLYLLIHLSYADLPLLSDFDQLKE
s03722 369 MTDSIKRCINQPSSRIIYKFKGCNDVDKVLSISVKRVMYIMMHFSPAELSMLSDFESFKE
pug1 457 LDTDTLSITPTTGLSDVKRYSRSKIRSLDININRIRTNYIIAHLTNADLSMLKDFDNVKD
a47462 420 ELDIVAKSFVTRSKPLIRENLNMKVHIRDTTLLSPKPTTPLSVIGKLYGPE.YKKVDLGD
s03722 429 ELDMIQGQYVTRGKPLKYKVVKSHVHIRDTELLSPPGSKSLAAVGSIYADEGYKKVDIKE
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pug1 692 DN.....SRYWIDYDLTSAYTSAMALLGNPDYNNARNLTEEELFKMTDEDLIMSYTIFST
a47462 657 EFKFPSNTKYPCIPARVDDNIEIYPLEGRSTITGAEYLVAKSMGCRLL.VKSGVMIPFDL
s03722 667 VFKFPNGTKYPCIPTRVDDNVDIYPLEGRSIITGCEYWVAKSMGCEMT.VKSGVMVPF..
pug1 747 KFKFPSNTKYPSIPCYLNESTTIYPLEGEATLTGVEFVLAKEQNCEFIDIKDIFIIPFE.
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s03722 724 .VKDRKRIEK..
pug1 806 .....SVLEK..
a47462 776 KMLELPNGSKVLETMTDPKTLLSEMVLKNPELLKESMILVCKYEYSDLYYLTDSNVFWHK
s03722 733 ....
pug1 811 ....TGGSRGRKK
a47462 836 LTEKGLKNDEEREARNKELANENKKLSRLNYMSPFRGIMLDLQSKRRTYEKGSFNNLIYK
s03722 733 ..........................DRLDYKSPYRQIMSDLQRKRRSHPKKSFYDLMYK
pug1 820 VTCKFVNQ.........................PFRNIIKELQDRRLQHPKKSLGNLMEK
a47462 896 LIGNSIYGQVSMGLSGNTNFDIKTQSYVKVEAGELTNPILASYITGFTRAAIGELMHNVS
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s03722 827 KLGGKAISVTTDGFLTNIEGFEDKILKNDSLNKDCLMLYRDIRNRLTAVLDDKGAVIESD
pug1 915 LLKGSVVSITTDGFITNLKDLEYNIIRGRIKTKALISSYRKVREFLSP.........DSN
a47462 1015 NRALEIKWEEENGITSWKTRGQMGSTHP.GISALTGFQTKYFQEEFLDELIPGLVTDPSK
s03722 887 DRALEIKKEDGKGLLSWKTRGQLGLDHTVELKAMSGFQSKYLDRQFLVEELRRRITGPAD
pug1 966 PDALELK.TRTVGIISWTTRGQFSLD..GQLKATTGFQVKGLNFEDLDNVFRSTMTG..E
a47462 1074 SKKVEFIESGLRTPSSIYKEGGHIMLVYRDKSYSFEYDNKR......RIVEN.QQDEGLL
s03722 947 LKKFEYVESRLRKAKDIYDKGGHVLIEYSDKSFSIEYDNKR......RIVNAWSVCGTLL
pug1 1021 DRVIEFIQSSLRSAKDIYMHGGHVTKTYADRVFRMEYDNKRVLNIPVELLEEDKFNNHLL
a47462 1127 DSVPWRTVEDYRKIRELKSTVSTAPFKEGLFIPSGQPKKYKKTVETSVRSFIKASFSETN
s03722 1001 ESVPWGSITPYRRIRNLKETVNTPVFVKGAF.TGGSGKKYKSTIETSVRGFIKAALSDND
pug1 1081 DSSPAKNVKTVEGLRFLRCIHRKNVYNK..MTSKAGSSKYKSDLDLAIRNFVKGCVKDPI
a47462 1187 RYGIPEGYFSNYESIIKFVHGHDPAKCLKITKSSISHLRNRETIPRAVPRTTENEKFIDY
s03722 1060 RFGIPKDQFQTYNDIIKFIRGHDAAKNVKLSKTSISNLKCRNAIARTVPRTDETEGFIKY
pug1 1139 GYNLTA..FKSYSDLIDFVKNYK..KDYRISKTSISNLKNRKMVVKGVPRNPDTLAFAEY
a47462 1247 VREHIKNFDSDLFFRELSEEAIKMRKA..KKITK..
s03722 1120 VTKKITTFDVDRFFKEKPQE..KANKK..DRIESDN
pug1 1195 VKEKFPSFDETQFFEKVYDK................

The impact of pUG1 on the host phenotype is still unknown. It should not be associated with reduction or variation in virulence as all the plasmid-containing and virus-free strains, except one, were virulent on chestnut trees (E.G., unpublished). As far as the senescent phenotype is concerned, no definitive association has been determined between heteroauxesis and the presence of pUG1. There is no correlation between a specific mitochondrial RFLP complex and the existence of mitochondrial plasmids. No plasmid was detected in the Canadian strains which are hypovirulent with a defective mitochondrial function.

The possible existence of a genetic mobile element was postulated to explain the strikingly high level of RFLP shown by the mtDNA of the strains of *C.parasitica* (34,36). However there is no evidence yet to confirm such a role for pUG1 because of the absence of integration of the plasmid sequence into the mtDNA, as shown by hybridization. Therefore the plasmid biological implications have not yet been elucidated.

Based on sequence similarity between the mitochondrial plasmid ORF of *C.parasitica* and the two of *Neurospora*, it seems likely that the putative DNA polymerase coded by pUG1 represents a particular group B DNA polymerase. The characteristics of this group of enzymes are that they contain TTD instead of the highly conserved DTD in the motif C region and that they are closely related to the linear plasmid polymerases because of their protein primed initiation. pUG1 as a new member of this group enforces the suggestion that TTD is a possible alternative motif C as noted in Fiji and LaBelle polymerases (13).

The origin of pUG1 is unknown, neither nDNA nor mtDNA share any homology with the plasmid DNA which apparently is not derived from the nDNA because of the distinct codon usage of pUG1 from that of nDNA of *C.parasitica* (data not shown). Although the genetic code of the mtDNA of *C.parasitica* has not been formally demonstrated, two reported mitochondrial elements, pUG1 and a mt ds-RNA (30) of *C.parasitica*, which use the genetic code of mitochondrial DNA of molds (37), might provide some hints.

To our knowledge only one case of natural intergeneric distribution of plasmids in fungi has been reported, the kalilo plasmid of *Neurospora* and *Gelasinospora* spp. (38); recently one case of artificial transfer of a mitochondrial plasmid, pAI2 from *A.immersus* to *P.anserina* has been described (39). This accumulating evidence for horizontal gene transfer contributes to the hypothesis of a common origin of pUG1 and the Fiji and LaBelle plasmids, considering the strong similarities of the nucleotidic and amino acid sequences and the fact that *Cryphonectria* and *Neurospora* are possibly taxonomically close genera.

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