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Mitochondrial plasmids of the pCp family are spread worldwide in *Cryphonectria parasitica* populations

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A worldwide collection of strains of *Cryphonectria parasitica* was examined to draw a precise picture of the incidence and diversity of mitochondrial plasmids related to the plasmid pUG1. Amplification by specific PCR of 199 strains showed the presence of pUG1-like plasmids in 22% of the populations examined. The entire plasmid molecules were amplified by multiplex PCR and the products showed different RFLP patterns. The variability was mostly in a non-coding region of the molecule that has been sequenced in some representative strains, enabling the molecular evolution of the molecule to be elucidated. The data show that mitochondrial plasmids of *C. parasitica* comprise an almost homogeneous family (designated pCp) that can be divided into two clusters based on the presence/absence respectively of a 60 nucleotide region in North American and European plasmids.

INTRODUCTION

Plasmids are common extra-chromosomal DNA molecules that can reproduce independently from chromosomal DNA in both pro- and eukaryotes. Plasmids have been discovered more recently in the latter, but the number of reports concerning different eukaryotic organisms, with the exception of animals, is now considerable (Griffiths 1995). Although the origin and persistence of plasmids is still not understood, their presence can be considered the rule, particularly in filamentous fungi where they are generally localized in mitochondria (*mt*) and are of linear type (Nargang 1985). Most circular *mt*plasmids are derived from *mt*DNA and have sequence homology with the host nucleic acids and should be considered plasmid-like molecules. However, a minority, the 'true plasmids', have their own independent sequence (Griffiths 1995). This second group includes the plasmids LaBelle, Fiji, Varkud, Mauriceville, Java, Mb1, VS and Harbin 2 of *Neurospora* spp., and the *Cryphonectria parasitica* mitochondrial plasmid, pUG1, which has a high degree of similarity to the Fiji and LaBelle plasmids of *N. intermedia*. These three *mt*plasmids all encode a particular family B DNA polymerase which is characterized by a specific signature, TTD instead of DTD, in the motif C typical of this family. Moreover, they are closely related to the linear plasmid polymerases (Li & Nargang 1993, Gobbi *et al.* 1997). The existence of these unique features in their coded enzymes suggests that pUG1 and the plasmids Fiji and LaBelle of *Neurospora intermedia* constitute a new subgroup of circular *mt*plasmids, and that they may share a common origin.

Cryphonectria parasitica is the pathogenic agent of chestnut blight, a disease that almost completely destroyed the chestnut population of the USA and Europe (Anagnostakis 1987, Heiniger & Rigling 1994). Some strains of the pathogen, termed 'hypovirulent', are infected by a cytoplasmic mycovirus of the genus *Hypovirus* (Hillman *et al.* 1995). The viral double-stranded (ds) RNA produces an attenuation of fungal virulence and its spread in the chestnut stands by means of the hypovirulent strains can be used as a tool for biological control of the disease (Grente & Berthelay-Sauret 1978).

While hypovirulence has been successfully employed to heal chestnut trees in Europe (Bisiach, De Martino & Intropido 1991, Heininger & Rigling 1994), it did not produce similar results in North American forest ecosystems (Anagnostakis *et al.* 1998). A different way of reducing the virulence of this pathogen would be of great interest and importance.

Plasmids of parasitic fungi are generally considered to be correlated with the pathogenicity of their host (Griffiths 1995). In *C. parasitica*, plasmid pUG1 was initially found in strains showing a senescent phenotype called heteroauxesis (Gobbi *et al.* 1985, Firrao & Gobbi 1989, Gobbi, Firrao & Locci 1989). Since then, it has been hypothesized that the plasmid might reduce the fitness of the strain and consequently lower pathogenicity in chestnut trees (Gobbi & Locci 1990). A second

C. parasitica plasmid, named pCRY, with 99.8% nucleotide sequence identity to pUG1, has recently been reported (Monteiro-Vitorello *et al.* 2000). The two plasmids appear to be closely related and pCRY has been proven to affect the virulence of at least one strain of *C. parasitica* when tested on apples or chestnut stems. Moreover mitochondrial hypovirulence has been proven to exist (Baidyaroy *et al.* 2000).

Among filamentous fungi, only a few fungal species, mostly *Neurospora* spp., have been well-investigated for *mt*plasmids, usually in connection with the senescence of the mycelium. The Fiji and LaBelle plasmids are commonly present in *Neurospora* spp. strains and are very well characterized. In contrast, little is known about *C. parasitica* mitochondrial plasmids. Since these plasmids may have a role as the agents of a new kind of hypovirulence, we undertook an extensive survey on a worldwide collection of 199 strains from natural populations of *C. parasitica*. Our aim was to draw a precise picture of the incidence and diversity of pUG1-like *mt*plasmids and to determine possible evolutionary groupings. Amplification experiments were performed to detect the presence of pUG1-like plasmids, to amplify the entire plasmid molecule, if present, and to sequence the most diverse regions of the molecule for phylogenetic studies. The relationships of the plasmid to a specific genome (*mt*DNA or viral dsRNA), to specific hosts, to the virulence of the host and to the geographic origin of the strains were also investigated. Since plasmids are useful markers in the study of fungi at the population level, pUG1 or similar plasmids could help to clarify aspects of the biology of *C. parasitica*, such as the modality of its worldwide diffusion and the structure of its populations (Milgroom 1995).

We describe here a family of mitochondrial plasmids of *C. parasitica* named pCp. A huge number of plasmids belonging to this family have been found around the world. Since among the Western plasmids no significant diversity has been found, we suggest that the European and the North American populations have a common ancestor.

MATERIALS AND METHODS

Fungal isolates

The strains of *Cryphonectria parasitica* used in this study are listed in Table 1. They were isolated from *Castanea* spp. and *Quercus* spp. tissues. All isolates were grown on Potato Dextrose Agar (PDA; Difco Laboratories, Detroit, MI) and are preserved in the National Culture Bank, Dipartimento di Biologia applicata alla Difesa delle Piante, Udine.

DNA extraction

Extraction of total DNA was carried out as described in Lecellier & Silar (1994) from 0.2 g of mycelium grown on PDA agar plates covered by BIO-rad membrane

Table 1. Subpopulations of *Cryphonectria parasitica* used in the present study (*n* = number of isolates).

Origin	Host	*n*
Europe		
Italy		
Friuli Venezia Giulia	*Castanea sativa*	10
Trentino	*C. sativa*	2
Lombardia[a]	*C. sativa*	9
Piemonte[a]	*C. sativa*	4
Liguria[a]	*C. sativa*	1
Toscana[a]	*C. sativa*	3
Basilicata 1	*C. sativa*	3
Basilicata 2	*Quercus pubescens*	3
Puglia 1	*Q. pubescens*	2
Puglia 2	*C. sativa*	2
Calabria 1	*C. sativa*	5
Calabria 2	*Q. pubescens*	2
Campania 1	*Q. frainetto*	1
Campania 2	*C. sativa*	5
Sardegna[a]	*C. sativa*	2
Italy*[b]	*C. sativa*	1
Italy*[c]	*C. sativa*	1
	Subtotal	56
France		
Ardeche	*C. sativa*	2
Lozere	*C. sativa*	4
Dordogne	*C. sativa*	3
Var	*C. sativa*	1
Pyrenees atlantiques	*C. sativa*	1
Corse	*C. sativa*	4
	Subtotal	15
Switzerland		
Bregaglia	*C. sativa*	4
Novaggio	*C. sativa*	3
Laboratory cross[d]	*C. sativa*	2
Copera	*C. sativa*	2
Claro	*C. sativa*	4
Choex	*C. sativa*	4
	Subtotal	19
Greece		
Mt. Pelion	*C. sativa*	4
Messologi	*C. sativa*	3
Ioannina	*C. sativa*	2
Chalkidiki	*C. sativa*	2
Arkadia	*C. sativa*	1
	Subtotal	12
Albania		
Tropoja	*Castanea sativa*	2
Albania*	*Castanea sativa*	1
	Subtotal	3
	Europe subtotal	105
North America		
Canada		
Ontario	*C. dentata*	1
USA		
Texas	*C. pumila*	1
California	*C. dentata*	5
Connecticut	*C. dentata*	2
Maryland	*C. dentata*	10
	America subtotal	19
Asia		
Japan		
Yasato[e]	*Castanea* sp.	2
Yachiyo[e]	*Castanea* sp.	7
Sowa[e]	*Castanea* sp.	2
Sanwa[e]	*Castanea* sp.	4
	Subtotal	15

Table 1. (*cont.*)

Origin	Host	*n*
China		
Suzhow	*Castanea* sp.	2
Schuchen[e]	*Castanea* sp.	2
Nanjing[e]	*Castanea* sp.	1
Guandong[e]	*Castanea* sp.	1
Guixi[e]	*Castanea* sp.	3
Jianyang[e]	*Castanea* sp.	1
Xiuming A[e]	*Castanea* sp.	3
Xiuming B[e]	*Castanea* sp.	3
Qian xi	*Castanea* sp.	3
Huai rou	*Castanea* sp.	3
Zhen an	*Castanea* sp.	3
Tong cheng	*Castanea* sp.	3
Yi chang	*Castanea* sp.	3
Dong nan	*Castanea* sp.	1
Yang shuo	*Castanea* sp.	2
Yu ping	*Castanea* sp.	1
	Subtotal	31
Korea		
Korea*	*Castanea* spp.	29
	Subtotal	29
	Asia subtotal	75
	Total	199

[a] Subpopulations reported in Cortesi, Milgroom & Bisiach (1996).
[b] Strain corresponding to ATCC 52574, obtained by the conversion of Ep67 with the French hypovirulent Ep 113.
[c] Strain corresponding to the ATCC 38753, isolated by L. Mittempergher in Italy.
[d] Strain obtained by the laboratory conversion of a virulent isolate.
[e] Subpopulations reported in Milgroom *et al.* (1996).
* Place of isolation unknown.

(BIO-rad Laboratories, Hercules, CA) for up to 5 d at 25 °C in the dark. DNA samples were resuspended in 50 µl of water.

Primers design, PCR and electrophoresis

Primers were designed depending upon the sequence of pUG1 (EMBL accession no. Y12637); their sequences and localization are reported in Fig. 1 and Table 2. Four PCR experiments were performed: detection PCR, control PCR, multiplex PCR and preparative PCR for sequencing. Each PCR reaction contained 10 × buffer (Boehringer Mannheim Biochemicals, Mannheim), 150 ng of each primer, 200 µM dNTPs and 2 U *Taq* Polymerase in a standard reaction of 50 µl. All reactions were performed in a 9600 thermocycler (Perkin Elmer, NJ).

Control PCR was performed with the NS7/NS8 primer pair (White *et al.* 1990) for partial amplification of the nuclear 18 S rDNA. The reaction was cycled 40 times with 95 ° (30 s), 60 ° (60 s) and 68 ° (165 s) as parameters. For the detection of pUG1-like plasmids, PCR amplification was performed with primers PSR and PSF for 35 cycles of 95 ° (30 s), 60 ° (75 s), and 72 ° (90 s). Multiplex PCR was conducted using three pairs of primers, PM1F and PM1R, PM2F and PM2R, PM3F and PSR, with a touchdown program, 28 cycles of 95 ° (30 s), 64 ° $-\Delta t = 0.5$ ° (60 s) and 72 ° (90 s) followed by seven cycles of 95 ° (30 s), 50 ° (60 s) and 72 ° (90 s). Fragments for sequencing were produced from a PCR performed with primers PUGF and PUGR for 35 cycles of 95 ° (30 s), 56 ° (75 s) and 72 ° (90 s).

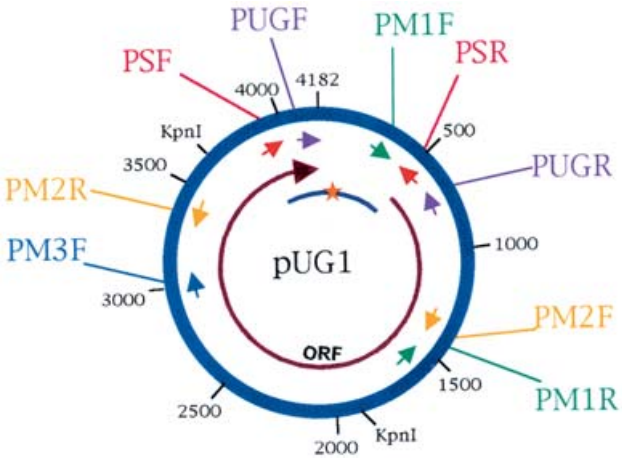

Fig. 1. Physical map of the plasmid pUG1 of *Cryphonectria parasitica*. Positions of the primers mentioned in the text are indicated. The open reading frame (ORF) (semi-circular arrow), the polymorphic region (semi-circle), and the 60 bp insertion of the pCRY-like plasmids (star) are also shown.

All PCR experiments were performed at least twice from independent DNA extractions. Amplified DNA was routinely electrophoresed on 1 % (w/v) agarose gels at 100 V for 1 h in Tris-acetate buffer, stained with EtBr (0.5 µg ml^{-1}) and photographed under UV light.

Analysis of RFLPs

DNA samples amplified with multiplex PCR were digested with *Rsa*I, *Eco*RI, *Kpn*I and *Hin*fI as indicated by the manufacturer (Boehringer Mannheim Biochemicals). Profiles were resolved on 1 % agarose gels run at 100 V for 40 min and stained with EtBr (0.5 µg ml^{-1}).

Sequencing and phylogenetic analysis

After PCR, amplified DNAs were purified by the PCR purification kit protocol (Qiagen, Germany) and sequenced by standard methods. Sequences were aligned manually and analysed with the PAUP program version 3.1.1 (Swofford 1993). The strength of the tree topology was assessed by the bootstrap method.

RESULTS

Occurrence of pUG1-like plasmids

All DNA samples were amplified by the control primers NS7 and NS8 (data not shown). Forty-four of 199 (22.1 %) fungal isolates tested consistently gave amplification products with specific primers PSF and PSR (Table 3). Only a single band was detected of approximately 800 bp, the size expected from pUG1. Five isolates (2.5 %) did not give consistent results, and the remaining 150 (75.4 %) failed to amplify despite giving positive control reactions.

Table 2. Primers used for the amplifications of the plasmid DNAs of *Cryphonectria parasitica*.

Primer	Sequence 5′–3′	Position[a]	PCR type
NS7	GAGGCAATAACAGGTCTGTGATGC	n.r.[b]	Control
NS8	TCCGCAGGTTCACCTACGGA	n.r.[b]	Control
PSF	GACCTGGATTTAGCTATTAGAAA	3894–3916	Detection
PSR	GCCCCACTTCCACTTAAACTAAA	485–507	Detection
PM1F	CGAAAAATAACGGGTGTGGGATA	340–362	Multiplex
PM1R	TAAGTTTCTTAACAGGGTAGGC	1470–1491	Multiplex
PM2F	TTCAGTTGGCCGTTAATACAAG	1381–1402	Multiplex
PM2R	TTCTATAACTTGATATTAACGCTTT	3372–3396	Multiplex
PM3F	CAAGATCGACGACTACAACAC	3042–3062	Multiplex
PUGF	ATACATTGGCATTCGCTG	4093–4110	Sequence
PUGR	TCAGCATCTTCATCAACAAC	696–715	Sequence

[a] Primer position as shown in Fig. 1.
[b] Position reported in White *et al*. (1990).
n.r., not relevant.

There was no correlation between the presence of a pUG1-type plasmid and a specific genotype of mitochondrial DNA of the host strain (Table 4). The presence of viral dsRNA did not influence the presence of the plasmids as pUG1-like plasmids were found both in virus-affected and virus-free isolates with different degrees of virulence, nor were plasmids associated with a specific plant host.

Global distribution of the plasmids

Amplifiable pUG1-like plasmids were common in the Western populations of *Cryphonectria parasitica* where 32.3% (40/124) of the isolates harboured such plasmids (Table 3). The frequencies were higher in the French and Swiss populations, with 12/15 (80%) and 10/19 (52.7%) strains respectively hosting a plasmid. Plasmids were detected in 16/56 (28.6%) of the Italian strains, while no amplification was obtained from any strain from Greece or Albania. In contrast, the frequency of the presence of plasmids in the North American population was only 10.5% (2/19) and only 4% (3/75) in the eastern Asian populations.

Plasmid restriction site polymorphisms

The entire DNA of the plasmids was amplified by multiplex PCR in all the strains harbouring pUG1-like plasmids, with the exception of the Asiatic ones, and three fragments were obtained (Fig. 2). These measured 1991, 1647 and 1151 bp in size as expected from pUG1, were designated m1, m2 and m3 respectively and covered, with overlaps, the entire circular molecule.

Restriction enzymes *Rsa*I and *Hin*fI were chosen for the RFLP analysis. *Rsa*I digestion of the m1 fragment produced three restriction patterns, while digestion of the fragments m2 and m3 gave two patterns. When *Hin*fI was used, 3 RFLPs were detected in fragment m1, none in fragment m2 and three in fragment m3 (data not shown). The most polymorphic region of the plasmid was detected, corresponding to the non-coding region of pUG1 between 3800 and 500 bp.

Table 3. Percentage of isolates in each of the *Cryphonectria parasitica* subpopulations which contained pCp plasmids DNAs amplified by PCR primed with PSF and PSR. Subpopulations not listed did not contain any isolates carrying amplifiable plasmid DNA.

Origin	% isolates with plasmids
Italy ($n=56$)	
Friuli Venezia Giulia	70
Trentino	50
Lombardia	22.2
Piemonte	25
Basilicata 1	33.4
Basilicata 2	66.6
Calabria 1	20
Calabria 2	50
Italian isolates %	28.6
France ($n=15$)	
Lozere	100
Dordogne	100
Var	100
Pyrenees atlantiques	100
Corse	75
French isolates %	80.0
Switzerland ($n=19$)	
Bregaglia	100
Novaggio	66.6
Laboratory cross	50
Claro	75
Swiss isolates %	52.6
USA ($n=19$)	
California	20
Connecticut	100
USA isolates %	10.5
Japan ($n=15$)	
Sanwa	25
Japanese isolates %	6.6
China ($n=16$)	
Guixi	66.6
Xiuming A	33.3
Chinese subpopulations %	18.7
Total ($n=155$)	
Overall percentage	28.4

The multiplex primers did not produce any amplification under any of the experimental conditions tested when used with the Asiatic strains.

Table 4. Description of the *Cryphonectria parasitica* isolates that harbour pCp plasmids.

Origin	Strain	Host	mtDNA[a]	dsRNA[b]	Virulence[c]
Italy					
Friuli Venezia Giulia					
Udine 1	CP42	*Castanea sativa*	D		VIR[1]
Pordenone	CP6	*C. sativa*		−[1]	VIR[1]
Pordenone	CP2b	*C. sativa*			HYPO[1]
Udine 2	CP13	*C. sativa*	B		VIR[1]
Udine 2	CP9	*C. sativa*	E		VIR[1]
Udine 2	CP16	*C. sativa*	B		VIR[1]
Udine 3	CP17	*C. sativa*			VIR[1]
Trentino					
Trento	CP34	*C. sativa*			HYPO[1]
Lombardia					
Bergamo	EN31	*C. sativa*			
Bergamo	EN70	*C. sativa*			
Piemonte					
Verbania	Vo14	*C. sativa*			
Basilicata 1					
Potenza 1	77	*C. sativa*			
Basilicata 2					
Potenza 2	F1	*Quercus pubescens*			
Potenza 2	F5	*Q. pubescens*			
Calabria 1					
Catanzaro	287	*C. sativa*			
Calabria 2					
Catanzaro	F30	*Q. pubescens*			
Italy*	Ep802	*C. sativa*		+[1]	HYPO[1]
Italy*	Ep67	*C. sativa*		−[1]	VIR[1]
Switzerland					
Bregaglia	M1392	*C. sativa*			HYPO[2]
Bregaglia	M1483	*C. sativa*			VIR[2]
Bregaglia	M1487	*C. sativa*			VIR[2]
Bondo	M1390	*C. sativa*			HYPO[2]
Novaggio	M1665	*C. sativa*			VIR[2]
Novaggio	M1672	*C. sativa*			HYPO[2]
Switzerland*	TIX	*C. sativa*		+[2]	HYPO[2]
Claro	TI17-3.X	*C. sativa*			VIR[2]
Claro	TI21-1.0	*C. sativa*			HYPO[2]
France					
Lozere 1	48.4D	*C. sativa*			
Lozere 2	48.5I	*C. sativa*		+[3]	HYPO[3]
Lozere 3	48.3H	*C. sativa*			
Lozere 4	48.2A	*C. sativa*			
Dordogne 1	24.B1	*C. sativa*		−[3]	VIR[3]
Dordogne 2	MSD14	*C. sativa*		−[3]	VIR[3]
Dordogne 3	MSD3	*C. sativa*		−[3]	VIR[3]
Var	2022	*C. sativa*		+[3]	HYPO[3]
Pyrenees	2106	*C. sativa*			
Corse 1	SamA2	*C. sativa*		−[3]	VIR[3]
Corse 2	BocB8	*C. sativa*		+[3]	HYPO[3]
Corse 2	BocA3	*C. sativa*			
America					
California	1-3CA	*C. dentata*			
Connecticut	Ep44	*C. dentata*	A	−	VIR
Japan					
Sanwa	JA104	*Castanea* sp.		−[4]	
China					
Guixi	09 370	*Castanea* sp.		−[4]	
Guixi	09 383	*Castanea* sp.		−[4]	
Xiuming A	09 509	*Castanea* sp.		−[4]	

[a] Mitochondrial haplotypes as reported in Gobbi & Locci (1990).

[b] Presence (+) or absence (−) of dsRNA as reported by [1]Gobbi (unpubl.), [2]D. Rigling (pers. comm.), [3]C. Robin (pers. comm.) and [4]Peever *et al*. (1998). Blank spaces indicate data not available.

[c] Normal (VIR) or lower virulence (HYPO) of the isolates of *C. parasitica* determined on chestnut trees or *in vitro* and reported by [1]Gobbi & Locci (1990), [2]D. Rigling (pers. comm.), [3]C. Robin (pers. comm.). Blank spaces indicate data not available.

* Place of isolation unknown.

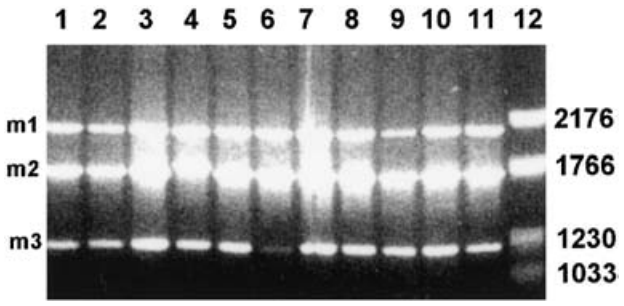

Fig. 2. EtBr agarose gel of DNAs from strains of *Cryphonectria parasitica* containing pCp plasmids, amplified by multiplex PCR with the primers PM1F, PM1R, PM2F, PM2R, PM3F and PSR.

Lane 1, strain BocA3 (F); *lane 2*, strain 48.4 (F); *lane 3*, strain M1483 (CH); *lane 4*, strain Ep44 (USA); *lane 5*, strain F1 (I); *lane 6*, strain En31 (I); *lane 7*, strain Cp34 (I); *lane 8*, strain Cp2b (I); *lane 9*, strain Cp9 (I); *lane 10*, strain Cp13 (I); *lane 11*, strain Cp6 (I); *lane 13*, marker VI (BMB). The sizes of fragments m1, m2 and m3 are 1991, 1647 and 1151 bp respectively, those of the markers (bp) are indicated.

Plasmid sequences and diversity

In order to evaluate the degree of sequence similarity among the different plasmids, the polymorphic region from 16 representative strains was amplified with the primers PUGF and PUGR (Fig. 3). The Asiatic strains produced a single band of 1200 bp in size, larger than that expected of 800 bp. Repeated attempts to sequence these amplification products failed as the PCR products were heterogeneous, and difficulties in their cloning were encountered, and so they were no longer investigated in this study. Seven sequences were aligned with those of pUG1 and pCRY (GenBank® accession no. AF031368), a pUG1-like plasmid detected in one American strain (sequence alignment available upon request from the first author).

When the sequences were analysed with PAUP to infer phylogenetic relationships, all the plasmids were very closely related with a low level of divergence. A maximum parsimony analysis resulted in the tree shown in Fig. 4. The separation of the American plasmid cluster from the European one was supported by a 100% bootstrap value. The short distance between the two groups indicates that the sequences are very stable and presumably of relatively recent divergence. Thus the pCp family of mitochondrial plasmids of *Cryphonectria parasitica* includes the pUG1 and the pCRY groups.

DISCUSSION

Previously, two smaller surveys of *Cryphonectria parasitica* provided only a sporadic estimate of the diffusion of mitochondrial plasmids similar to pUG1 (Gobbi *et al.* 1997, Monteiro-Vitorello *et al.* 2000). The present study offers a more detailed, global picture of the distribution of these plasmids. The data show that they comprise an almost homogeneous group, where diversity is not highly significant and is due mostly to base pair substitutions. The family of plasmids (designated pCp) can be divided into two groups, the European with pUG1-like plasmids and the American with pCRY-like plasmids, based respectively on the absence/presence of a 60 bp repeat.

The PCR for the detection of plasmids was specific and sensitive enough to allow the umambiguous amplification of plasmids in *C. parasitica* even starting from a small amount of mycelium. While most of the strains gave the same results in all the PCR experiments, a minority had inconsistent data probably due to the plasmid instability or to a variable concentration of DNA that was sometimes too low to be detectable with the technique employed. The incidence of *C. parasitica* strains that have a pCp plasmid is surprisingly high and is comparable only to the relative incidence of the plasmid Fiji in *N. intermedia* as reported by Arganoza *et al.* (1994). pCp plasmids are dispersed abundantly both in Europe and in North America, while in Asia the estimate of their distribution is still uncertain.

Among the oriental strains, the multiplex PCR always failed while the detection and preparative PCR were possible albeit the sizes of the preparative amplified fragments were larger than expected. Although only one band was visible on the gels after their amplifications, the PCR products were heterogeneous so their direct sequencing was not feasible. The putative Asiatic plasmids are apparently characterized by a sequence different from pUG1, although they could be distantly related, and they require a more detailed characterization. Their cloning and sequencing is in progress in our laboratory, and will be discussed elsewhere.

In Europe, the frequency of the plasmids is very variable and depends on the geographic location. A large heterogeneity was noted in the subpopulations, possibly due to founder effects. The high frequency of pCp plasmids in Italy, France and Switzerland is probably due to the fact that the plasmid was already present in the fungal host when first introduced into these regions. Each sample size was not vast but the tested strains were homogeneously distributed, and therefore the estimates of the plasmid frequency are believed to be accurate.

Conversely, in Greece no plasmids were found, although the disease is reported to exist there since 1964 (Xenopulos 1985). It could be speculated that the Greek fungal population is clonal as it was recently introduced, there are few vc-groups, no sexual and very reduced asexual reproduction. This situation is presumably due to a single introduction of a plasmid-free strain of *C. parasitica*, or the absence of plasmids could derive from the local climate, which is unfavourable for its maintenance. It could not depend on the background genomes of the fungal host as it has been demonstrated that this has no influence.

In North America, 10.5% of the analysed populations contained plasmids. It should be noted that the American isolates analysed in this study originated mostly from one site, while the sample size from the other locations was very small, so this result should be

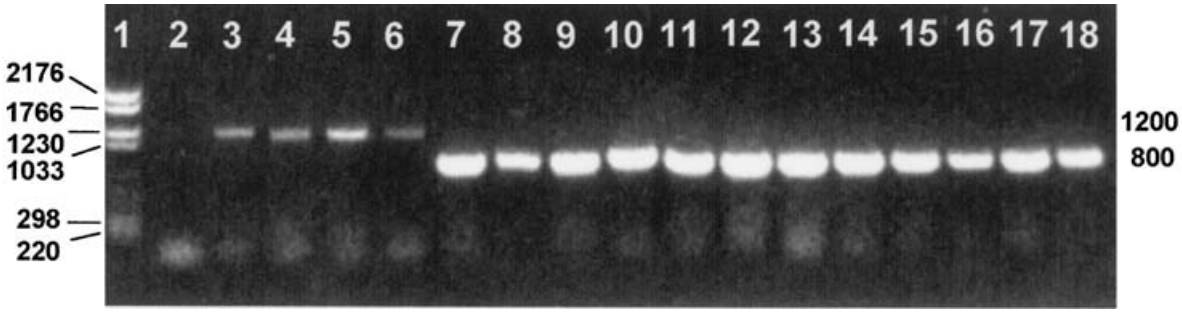

Fig. 3. Amplification of total DNA from strains of *Cryphonectria parasitica* by PCR with the primers PUGF and PUGR; EtBr-stained agarose gel.

Lane 1, marker VI (BMB); *lane 2*, negative control; *lane 3*, strain JA104 (J); *lane 4*, strain 09370 (C); *lane 5*, strain 09383 (C); *lane 6*, strain 09509 (C); *lane 7*, strain BocA3 (F); *lane 8*, strain 48.4 (F); *lane 9*, strain M1483 (CH); *lane 10*, strain Ep44 (USA); *lane 11*, strain F1 (I); *lane 12*, strain En31 (I); *lane 13*, strain Cp34 (I); *lane 14*, strain Cp2b (I); *lane 15*, strain Cp9 (I); *lane 16*, strain Cp13 (I); *lane 17*, strain Cp6 (I); *lane 18*, strain Cp42 (I). The sizes of the standard molecular weights markers (bp) and of the PCR products (bp) are indicated.

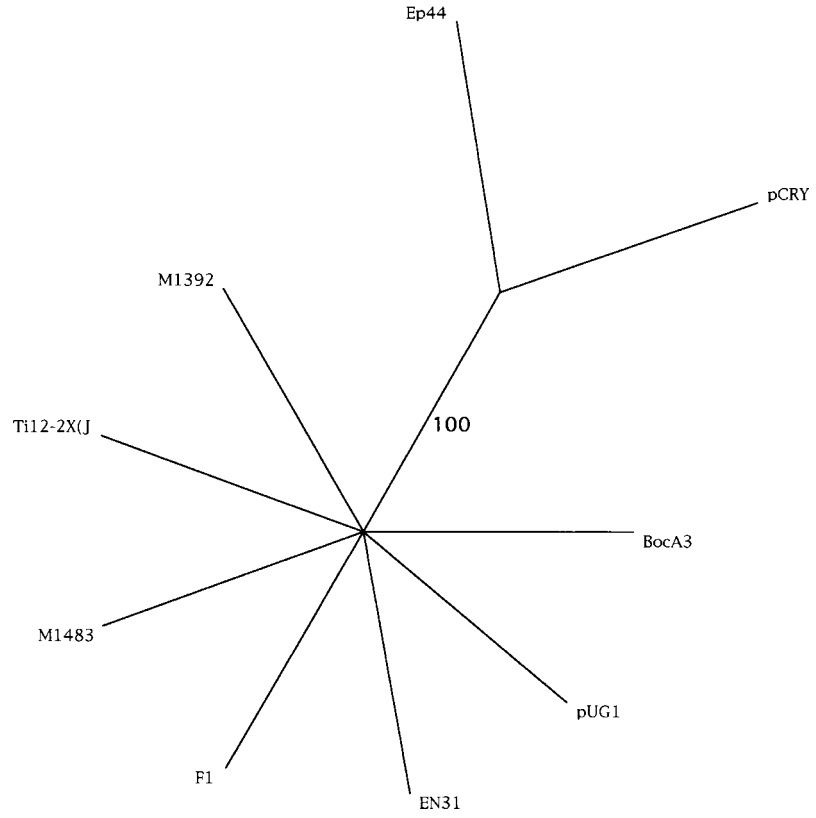

Fig. 4. Unrooted tree generated with portions (pUG1 positions from 4111 to 701) of the sequences of 9 mt pCp plasmids of *Cryphonectria parasitica*. Plasmids: pCRY (USA); Ep44 (USA); BocA3 (F); pUG1 (I); En31 (I); F1 (I); M1483 (CH); M1392 (CH); Ti 12-2X (CH).

considered with caution even though it agrees with a previously reported lower occurrence of pCp plasmids in the American strains (Monteiro-Vitorello *et al.* 2000). The finding of only one plasmid out of the five Californian isolates is very interesting; the fungi were sampled in the only commercial chestnut orchard of the state infected with chestnut blight. The collection site is outside the natural range of American chestnut trees and therefore both the trees and the fungi are separated from the wider natural populations; moreover the outbreak of *C. parasitica* in this plot is recent and results in a clonal population of fungi (Gobbi & van Alfen, unpubl.). Their low diversity is probably due to a founder effect and genetic drift. In such a homogeneous and restricted group of isolates it is surprising that the pCp plasmid could not be detected in all the strains as expected from the rate of incidence shown in other populations and from its reported capability to spread among fungi (Baidyaroy *et al.* 2000).

The sequences of the European and North American plasmids are very similar and strongly conserved. This could be due to selective pressure but it is intriguing how

diverse the evolutionary rates of *mt*DNA and *n*DNA are compared to plasmid DNA. High frequencies of RFLPs of mtDNA have been reported (Gobbi & Locci 1990, Liu *et al*. 1996), as well as a moderate to high degree of genetic differentiation among *C. parasitica* subpopulations depending on the geographical localization considered (Peever, Liu & Milgroom 1997). However, the pCp plasmids, with their very stable sequences, indicate an evolution not matching that of their fungal host, or the extrachromosomal hypovirus (Chung, Bedker & Hillman 1994).

Data on pCp plasmid phylogeny could shed light on the dynamic interaction of plasmid and fungus. Nothing is known of the origin of the pCp plasmids; the simplest hypothesis is that the plasmids were present in the fungal ancestor of both European and North American populations of *C. parasitica* and that they were spread worldwide with their fungal hosts. In spite of finding the European populations of dsRNA in Asia, suggesting the direct introduction of the Japanese populations into Europe, it still remains an open question whether *C. parasitica* was introduced into Europe from North America or directly from Japan (Milgroom *et al*. 1996, Peever *et al*. 1998). The characterization of the diversity of Asiatic plasmids could be helpful in clarifying this point. Moreover the definition of the distribution of the pCp family plasmids across species of this fungus and even across closely related genera would establish if an event of horizontal transfer between *Neurospora* and *Cryphonectria* through intermediate related genera had occurred. Conceivably, the Asiatic population of pCp plasmids, if it exists, is the ancestor of the Western ones, and would constitute the link between the different genera.

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