DETECTION AND IDENTIFICATION OF PHYTOPLASMA DNA IN SYMPTOMATIC MUSHROOMS OF THE GENUS *RAMARIA* (O. GOMPHALES)

by

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Resumen

MARTÍN, M.P., P.P. DANIËLS, E. TORRES & M.T. TELLERÍA (2003). Detección e identificación de fitoplasmas en hongos sintomáticos del género Ramaria (O. Gomphales). *Anales Jard. Bot. Madrid* 60(1): 11-18 (en inglés).

Mediante la reacción en cadena de la polimerasa y el uso de iniciadores específicos para la región 16S rDNA de fitoplasmas, se ha detectado por primera vez en hongos la presencia de estos organismos en basidiomas de *Ramaria* que presentaban un desarrollo anormal. El análisis filogenético de las secuencias por los criterios de parsimonia y de máxima verosimilitud confirman a este fitoplasma como del grupo "Stolbur".

Palabras clave: 16S rDNA, iniciadores específicos, Stolbur, *fungi*.

Abstract

MARTÍN, M.P., P.P. DANIËLS, E. TORRES & M.T. TELLERÍA (2003). Detection and identification of phytoplasma DNA in symptomatic mushrooms of the genus Ramaria (O. Gomphales). *Anales Jard. Bot. Madrid* 60(1): 11-18.

Using polymerase chain reaction assays with specific primers for amplifying phytoplasma 16S rDNA, the presence of phytoplasmas in *Ramaria* basidiomes with abnormal development has been detected for the first time in fungi. Phytoplasmas have not been detected in asymptomatic basidiomes. Sequence analyses based on parsimony and maximum likelihood place the phytoplasma in the Stolbur group.

Key words: 16S rDNA, specific primers, Stolbur, *fungi*.

INTRODUCTION

Phytoplasmas are wall-less bacteria in the class Mollicutes (formerly called mycoplasma-like organisms) associated with diseases of many perennial or herbaceous crops worldwide (LHERMINIER & *al.*, 1999). They have a wide host range and are mostly restricted to phloem tissue. As phytoplasmas are recalcitrant to cultivation, little is known of their biology. They are specially vectored by phloem-feeding insects in which they multiply (BOUDON-PADIEU & *al.*, 1989). During the last decade, DNA-based methods of identification have shown that phytoplasmas form a unique clade within mollicutes (SEEMÜLLER & *al.*, 1994). According to LHERMINIER & *al.* (1999), to control the phytoplasma diseases more information is necessary on host-pathogen relationships and on the replication strategies of phytoplasmas. It has been suggested that herbaceous plants could act as a phytoplasma reservoir when the main host has disappeared.

Most species included in the genus *Ramaria* (O. Gomphales, Basidiomycotina:

JÜLICH, 1981; HAWKSWORTH & *al.*, 1995) are ectomycorrhizal and they have strong fleshy and coralloid basidiomes, branching several times from the base. In general, the branch surface is smooth and basidiome apices are small and delicate, clearly distinct from the branches. Occasionally basidiomes of the ectomycorrhizal species show an abnormal growth: a) the branches become wrinkled; b) the ramification rank decreases with the branches enlarging and fusing together, and c) the apices are indistinct with the terminal branch blunted and inflated (fig. 1). However, under light microscope, no abnormal features were seen in the hyphae or spores.

Comparison of these symptoms in *Ramaria* with those observed in many plants at the Laboratori de Sanitat Agrària in Barcelona (Spain) suggested some kind of disease caused by a virus or phytoplasma infection. Thus, a study was undertaken to verify the presence of these pathogens in the *Ramaria* species with the symptoms described above. Because of their specificity and sensitivity, during the last two years we have been focusing on molecular methods to detect and identify phytoplasmas in plants based on PCR (AHRENS & SEEMÜLLER, 1992). According to LEE & *al.* (1998) and SEEMÜLLER & *al.* (1998) in phytoplasmas the 16S rDNA is a very useful region to separate taxa at the species or genus level.

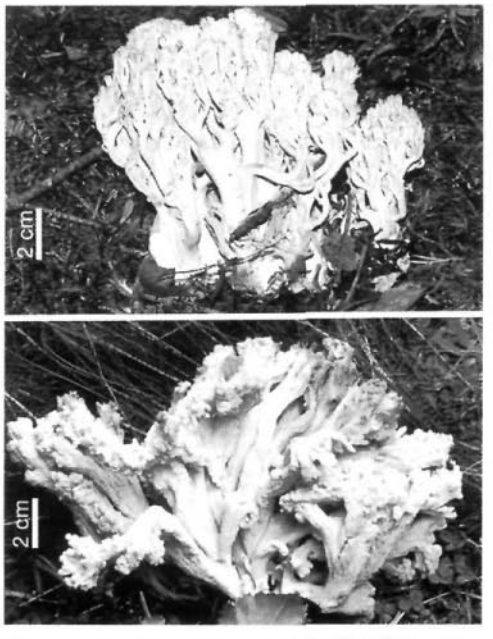

Fig. 1.–Above: *Ramaria pallida* (Schaeff.) Ricken (MA-Fungi 49659) basidiome without phytoplasma symptoms. Below: *Ramaria pallida* (Schaeff.) Ricken (MA-Fungi 49661) basidiome with phytoplasma symptoms.

MATERIAL AND METHODS

DNA from less that 10 mg of healthy and diseased *Ramaria* basidiomes (table 1) was obtained using E.Z.N.A. Plant MiniPrep Kit (Omega-Biotech, Doraville, USA) as described in MARTIN & TORRES (2001).

PCR amplifications. Nested-PCR assays were performed with the primer pair P1/P7 (DENG & HIRUKI, 1991; SMART & *al.*, 1996) followed by the primer pair fU5/rU3 (LORENZ & *al.*, 1995) amplyfing from position 369-386 to position 1251-1231. Amplifications were done using Ready-to-Go PCR Beads (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) as mentioned in MARTÍN & TORRES (2001). From the first PCR, 0.5 µl of amplimer was used as template in nested-PCR. The PCR reactions were performed by using a Techne DNA thermocycler (model Progene). The cycling parameters were 40 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min with a final extension at 72 °C for 10 min. Before initiating the cycling parameters an initial denaturation at 94 °C for 5 min was done. Controls, lacking DNA, were run for each experiment to check for DNA contamination of the reagents.

DNA sequencing. Amplification products were cleaned using the E.Z.N.A. Clean kit (Omega Biotech) and both strands were sequenced separately using primers fU5 and rU3 with an ABI Prism 377 genetic Analyzer and the ABI Prism™ BigDye™ terminator Cycle Sequencing Ready Reaction kit with AmpliTaq®DNA Polymerase (Perkin Elmer Applied Biosystem, Norwalk, Connecticut, USA). Sequence Navigator™ Sequence

TABLE 1

SAMPLES INCLUDED IN THIS STUDY BELONGED TO *RAMARIA FLAVA* (TOURN.: FR.) QUÉL., *R. PALLIDA* (SCHAEFF.) RICKEN AND *R. SCHILDII* R.H. PETERSEN

[indicating collection data (herbarium number, localities and dates), if basidiomes are symptomatic (+) or asymptomatic (–) and the visualization of phytoplasma amplimers (+) after the nested PCR with primers fU5/U3r]

Taxa	MA-Fungi	Localities	Dates	Basidiome	Nested PCR
R. flava	48061	Guadalajara, Spain	20-XI-1998	–	–
R. flava	51163	Valbella, Switzerland	12-IX-2000	+	+ (FITOPL_4)
R. pallida	39878	Segovia, Spain	25-X-1997	+	+ (FITOPL_2)
R. pallida	48066	Huesca, Spain	2-X-1999	–	–
R. pallida	49661	León, Spain	13-IX-1997	+	+ (FITOPL_1)
R. schildii	50726	Lenzerheide, Switzerland	8-IX-1999	+	+ (FITOPL_3)

Comparison software (Perkin Elmer) was used to identify the consensus sequence from the two strands of each ITS region.

To control for possible contamination of extractions, amplifications and sequencing reactions were replicated at the Laboratori de Sanitat Agrària (Barcelona) and at the Real Jardín Botánico (Madrid) following the same protocols as mentioned above, but independent reagents and a thermocycler Perkin Elmer 9700.

Analyses of data. The nucleotide sequence obtained (FITOPL_1) has been logged in the EMBL database and has been compared with 59 sequences retrieved from the EMBL GenBank (table 2). To search for the best alignment Sequence Navigator™ Sequence Comparison for pairwise comparisons and SEQAPP software for multiple sequences were used. Where ambiguities in the alignment occurred, the alignment chosen was the one generating the fewest potentially informative characters. Alignment gaps were marked "-" and unresolved nucleotides or unknown sequences were indicated with "N".

Parsimony analysis was performed using the computer program PAUP 4.0b* (Phylogenetic Program Using Parsimony) of SWOFFORD (1998). *Clostridium innocuum* and *Acholeplasma laidlawii* were used as outgroup. Branch robustness was estimated by bootstrap analysis (FELSENTEIN, 1985) of 10,000 heuristic replicates using the fast stepwise-addition option. In the maximum likelihood analysis (ML) (FELSENSTEIN, 1981) optimality criterion was employed.

RESULTS

No PCR products were visualized with primers P1/P7 from any of the collections analyzed. With fU5/rU3 no amplifications were obtained from asymptomatic collections. The four symptomatic samples give amplifications of 850 bp, in agreement with the expected size, but only from FITOPL_1 was the concentration enough for sequencing. Moreover, in FITOPL_2 and FITOPL_4 an extra band about 800 bp was visualized.

The sequence length obtained is 800 bp and has been logged in the NCBI with the Accession Number AJ430066. The sequences of the 60 taxa were aligned to produce a matrix of 826 characters, of which 203 were parsimony-informative.

Two hundred most parsimonious trees were obtained under heuristic search with a tree length of 723 steps, CI = 0.6321, RI = 0.8845, RC = 0.5591. In the strict consensus tree (fig. 2) the sequence obtained cluster with phytoplasmas from Stolbur group with 99 % bootstrap support. The topology of the ML tree agrees with the consensus tree inferred from heuristic search (fig. 3).

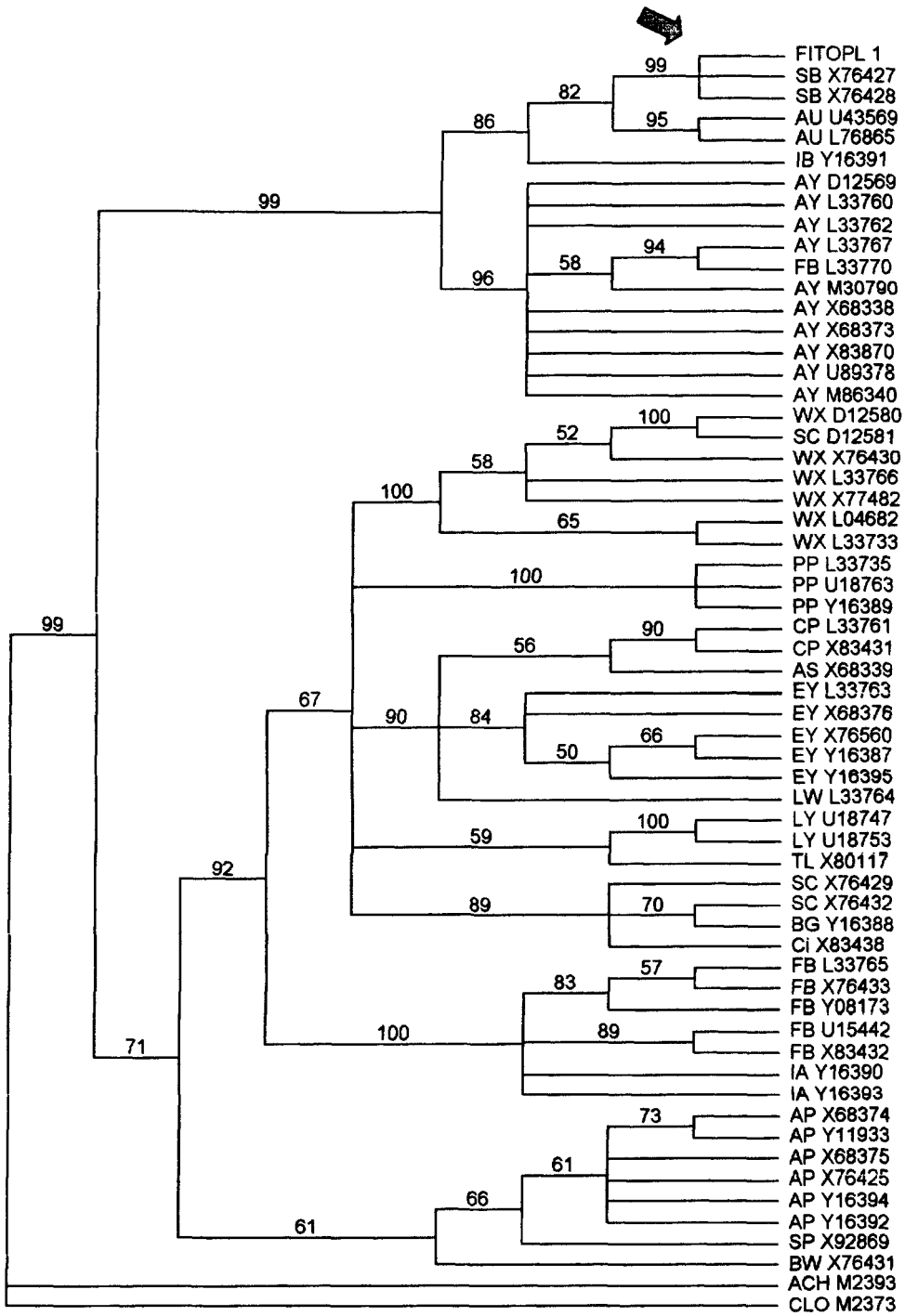

Fig. 2.–Strict consensus tree of 200 MPTs obtained from parsimony analysis under heuristic search. Bootstrap support values are shown at the nodes. Names according to tables 1 and 2.

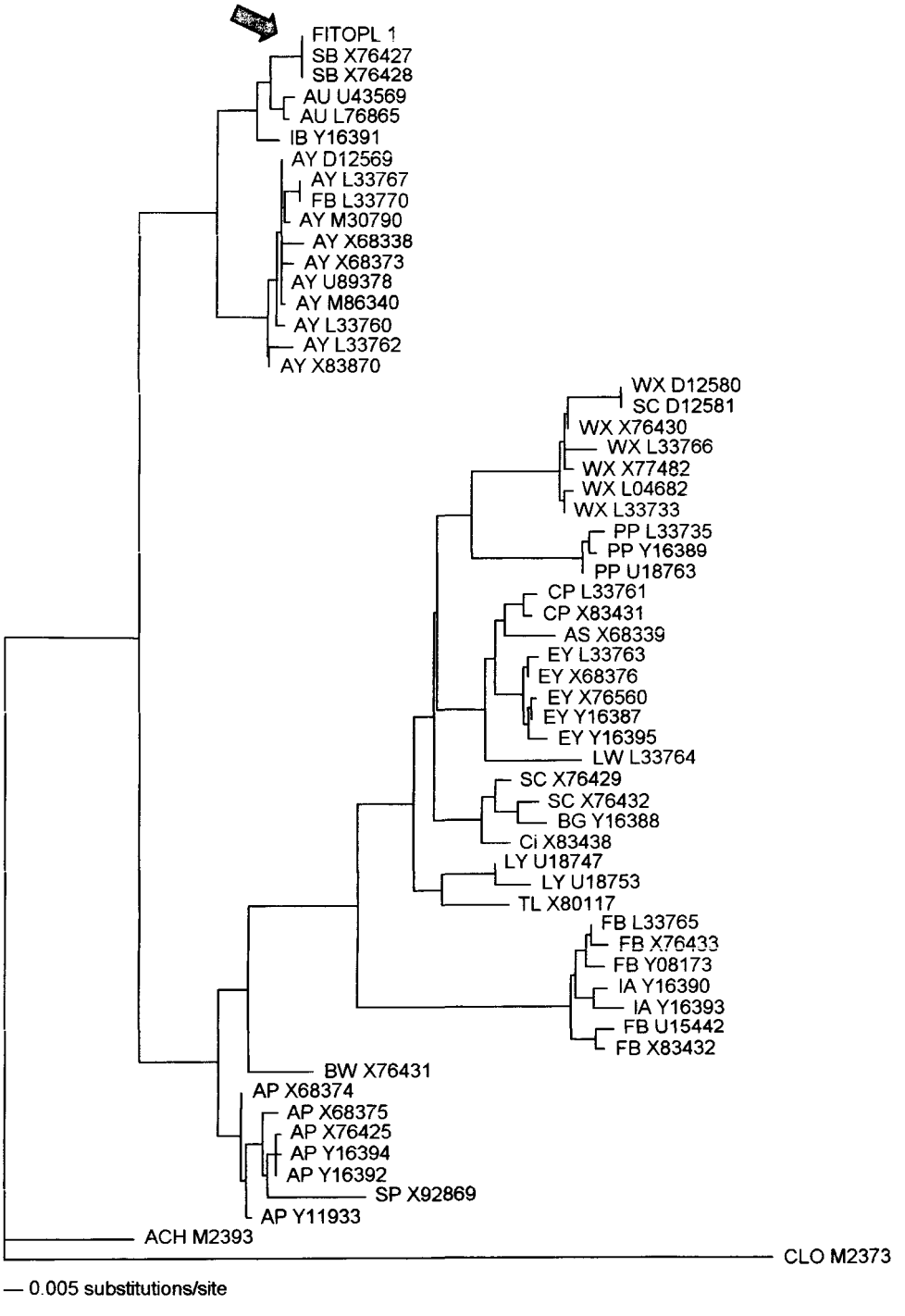

Fig. 3.–The most likely tree found in the maximum likelihood search using a manually optimized alignment. No characters excluded. Names according to tables 1 and 2.

TABLE 2

SEQUENCES RETRIEVED FROM GENBANK INDICATING THE CLASSIFICATION PROPOSED BY SEEMÜLLER & AL. (1998), AS WELL AS THE CODE MENTIONED PREVIOUS TO THE ACCESSION NUMBER (EMBL) INCLUDED IN FIGURES 2 AND 3

Identification	Code	GenBank No.	Identification	Code	Genbank No.
Apple proliferation	AP	X68374	Faba bean phyllody	FB	L33765
Apple proliferation	AP	X68375	Faba bean phyllody	FB	L33770
Apple proliferation	AP	X76425	Faba bean phyllody	FB	U15442
Apple proliferation	AP	Y11933	Faba bean phyllody	FB	X76433
Apple proliferation	AP	Y16392	Faba bean phyllody	FB	X83432
Apple proliferation	AP	Y16394	Faba bean phyllody	FB	Y08173
Ash yellows	AS	X68339	Italian alfalfa witches' broom	IA	Y16390
Aster yellows	AY	D12569	Italian alfalfa witches' broom	IA	Y16393
Aster yellows	AY	L33760	Italian bindweed stolbur	IB	Y16391
Aster yellows	AY	X83870	Loofah witches broom	LW	L33764
Aster yellows	AY	L33762	Phytoplasma australiense	AU	L76865
Aster yellows	AY	L33767	Phytoplasma australiense	AU	U43569
Aster yellows	AY	M30790	Pigeon pea witches' broom	PP	L33735
Aster yellows	AY	M86340	Pigeon pea witches' broom	PP	U18763
Aster yellows	AY	U89378	Pigeon pea witches' broom	PP	Y16389
Aster yellows	AY	X68373	Spartium witches' broom	SP	X92869
Aster yellows	AY	X68338	Stolbur	SB	X76427
Bermuda grass white leaf	BG	Y16388	Stolbur	SB	X76428
Buckthorn witches' broom	BW	X76431	Sugarcane white leaf	SC	D12581
Cirsium phyllody	Ci	X83438	Sugarcane white leaf	SC	X76429
Clover proliferation	CP	L33761	Sugarcane white leaf	SC	X76432
Clover proliferation	CP	X83431	Tanzanian lethal decline of cocconut	TL	X80117
Cocconut lethal yellowing	LY	U18753			
Cocconut lethal yellowing	LY	U18747	Western X-disease	WX	D12580
Elm yellows	EY	L33763	Western X-disease	WX	L04682
Elm yellows	EY	X68376	Western X-disease	WX	L33733
Elm yellows	EY	X76560	Western X-disease	WX	L33766
Elm yellows	EY	Y16387	Western X-disease	WX	X76430
Elm yellows	EY	Y16395	Western X-disease	WX	X77482

DISCUSSION

The highly sensitive nested-PCR combined with the use of specific primers has made possible the detection of phytoplasmas in symptomatic basidiomes of *Ramaria*. The absence of visible products from samples amplified by direct PCR with P1/P7 is probably due to the low concentration of these prokaryotes as described in plant hosts (BERTACCINI & *al.*, 1996).

Plants infected by phytoplasmas exhibit an array of symptoms that suggest profound disturbance in the normal balance of plant hormones or growth regulators. The stolbur phytoplasma is an ubiquitous agent that multiplies in a broad range of cultivated and wild plant species (GARNIER, 2000). The symptoms change according to the host, some affected plants shown variegate branches, as observed in olive-trees (TORRES & MARTÍN, unpublished). These symptoms are the same as detected in *Ramaria* spp. and fit with the molecular analysis, that includes phytoplasmas found in this fungus as belonging to the stolbur group. These phytoplasmas are very frequent in wild plants found in infected grapevines. BATLLE & *al.* (2000) identified stolbur phytoplasmas in *Convolvulus arvensis, Lavandula officinalis, Polygonum con-*

volvulus, Solanum nigrum and *Thymus officinalis*. According to our molecular analysis, stolbur group phytoplasmas are the sister group of the Aster yellows phytoplasma clade.

On the other hand, several taxa of clavarioid fungi have been described as new species, in which the basidiomes show the same features as the symptomatic and positive phytoplama-infected basidiomes included in this study: *Ramaria obtusissima* (Peck) Corner (PECK, 1913; CHRISTAN, 2000), *Ramaria claviramulata* Marr & Stuntz (MARR & STUNTZ, 1973; HUMPERT & *al.*, 2001), *Ramaria rielii* Boud. (BOUDIER, 1897; DANIËLS & TELLERÍA, 2000), *Clavaria strasseri* subsp. *cornucervi* Killermann (KILLERMANN, 1956), *Clavaria anomala* Fr. (FRIES, 1821; SCHAEFFER, 1774), *Clavulina cristata* var. *subrugosa* Corner and *Clavulina rugosa* (Fr.) Schroet. (CORNER, 1950). An accurate molecular analysis should be done in these taxa to know if the basidiomes are infected by phytoplasma. However, only by inoculating phytoplasmas in young basidiomes is it possible to state that the abnormal morphology of the basidiome is clearly related to the presence of the phytoplasma.

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