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Intraspecific variation in *Phytophthora citrophthora* from citrus trees in Eastern Corsica

Séverine Cohen[1,*], Valérie Allasia[1], Paul Venard[1], Sylvia Notter[1], Christian Vernière[2,**] and Franck Panabières[1,†]
[1]*INRA, Unité Interaction Plantes-Microorganismes et Santé Végétale, BP 2078, F-06606 Antibes Cedex, France;* [2]*INRA-CIRAD, Station de Recherches Agronomiques de San Giuliano, F-20200, San Nicolao, Corse-France;* [*]*Present address: Equipe Evolution et Génétique des Populations Marines-CNRS UMR 7127; Station Biologique de Roscoff, BP 74, Place Georges Teissier, F-29682 Roscoff Cedex, France;* [**]*Present address: CIRAD-FHLOR, TA50/PS4, F-34398 Montpellier Cedex 5, France;* [†]*Author for correspondence (Fax: +334 93 67 88 88; E-mail: panab@antibes.inra.fr)*

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Abstract

Isolates of *Phytophthora* pathogenic to citrus crops on Eastern Corsica and associated with gummosis were identified by PCR-RFLP of internal transcribed spacers (ITS) sequences and characterized by the random amplified microsatellites (RAMS) technique. A sample of 114 isolates collected from diseased trunks and fruits, and from soil, were overwhelmingly *Phytophthora citrophthora*. Further analysis indicated that the *P. citrophthora* population was not homogeneous in citrus groves. There were two groups, with a few (4%) atypical isolates in two marginal groups. The major groups have been re-examined in the light of mating behaviour, RFLPs of mitochondrial DNA and sequence comparisons of ITS regions of rDNA. They were found distinct with all these criteria and perhaps constitute distinct taxa. The results indicate that important modifications occurred in the population structure of *P. citrophthora* over time in Corsican groves. These changes may have impact on the recent outbreaks of gummosis.

Introduction

Citrus is one of the most important fruit crops in the world with a very wide production area. Among its pathogens, the Oomycete *Phytophthora* represents one of the most serious threats to production. Although 10 species have been reported from diseased trees around the world, three species cause the most serious disease, stem gummosis, as well as root and fruit rot: *Phytophthora citrophthora*, *P. nicotianae* (syn *P. parasitica*) and *P. palmivora* (Erwin and Ribeiro, 1996; Graham and Menge, 2000). They have distinct temporal and climatic requirements, so that their relative distribution and influence vary in the different production areas (Matheron et al., 1997). Hence, *P. nicotianae* and *P. palmivora* are major causes of citrus diseases in the United States, whereas *P. citrophthora* predominates in the Mediterranean Basin and other citrus areas, e.g. China (Ricci et al., 1990; Graham and Menge, 2000; Graham et al., 1998; Zheng and Ward, 1998). Corsica is near the northern limit for citriculture. Citrus trees, mainly citron (*Citrus medica*) were first introduced in antiquity, but as a crop citrus was developed and organized in Eastern Corsica only in the early 1960s. It covers only a moderate area (∼2500 ha), but represents an unique area of commercial production in France.

Citrus varieties have been generally grafted to the rootstock sour orange (*C. aurantium*), which appeared to be resistant to *Phytophthora* following the mid-1800s gummosis epidemics (Laviola et al., 1990). However, sour orange is highly susceptible to other pathogens, such as the citrus tristeza virus (CTV) (Bar-Joseph et al., 1981), nematodes or 'mal secco'

(*Phoma tracheiphila*) (Laviola et al., 1990), and has been replaced in Corsica by close relatives to *Citrus*, such as *Poncirus trifoliata* (trifoliate orange), and hybrids like Troyer and Carrizo citrange (*C. sinensis* × *P. trifoliata*). Scion cultivars are generally susceptible to *Phytophthora* and disease can spread in orchards through rainsplash of infectious structures. Fungicides, such as metalaxyl or fosetyl-Al, control gummosis (Davis, 1982), but require several applications and must be timed correctly (Davino et al., 1990). Hence, effective disease management must integrate the identification of the pathogens with an understanding of their dynamics, to gain maximum benefit at low cost, avoiding fungicide treatments when applications are not needed.

Phytophthora diseases were reported on citrus in Corsica early in the 20th century (Dufrénoy, 1926). A first survey of *Phytophthora* in Corsican groves in the late 1970s yielded predominantly sterile isolates classified as *P. citrophthora* (90%), as well as some strains assigned to *P. nicotianae* (8%) or *P. citricola* Sawada (2%) on the basis of protein profiles (De Vallavieille and Erselius, 1984). However, some isolates of these two species displayed several features common to *P. citrophthora* (De Vallavieille and Erselius, 1984).

The explosion of molecular techniques, mainly the use of internal transcribed spacers (ITS) regions for identifying *Phytophthora* species (White et al., 1990; Crawford et al., 1996; Tooley et al., 1997; Cooke et al., 2000; Förster et al., 2000) permitted the taxonomic status of some taxa to be elucidated and supported recent definitions of new species (Gerlach and Schubert, 2001; Mirabolfathy et al., 2001). It also defined relationships among *Phytophthora* species (Crawford et al., 1996; Cooke et al., 2000), revealed the polyphyletic status of some species (Förster et al., 2000), and allowed the identification of interspecific hybrids (Brasier et al., 1999; Bonants et al., 2000). However, the definition of a *Phytophthora* species cannot rely to the single observation of ITS sequences. Hence, two sympatric species, *P. infestans* and *P. mirabilis*, that are separated by their host specificities and display distinct differences in both nuclear and mitochondrial DNA (mtDNA), possess identical ITS2 sequences (Goodwin et al., 1999), and display two differences in the ITS1 regions (Cooke et al., 2000).

In addition, several studies reported significant variation in *P. citrophthora*, using various independent criteria. Cocoa isolates from Brazil were distinguished from the rest of the species on the basis of mtDNA (Förster et al., 1990), ITS sequences (Lee and Taylor, 1992), and isozyme analyses (Oudemans and Coffey, 1991; Mchau and Coffey, 1994). Furthermore, both sterile and heterothallic (A2) strains have been reported within *P. citrophthora* (Mchau and Coffey, 1994). Lastly, the boundaries between *P. citrophthora* and related species such as *P. citricola* and *P. capsici* are relatively imprecise (Oudemans et al., 1994), so that the distribution of *Phytophthora* species within the pathogenic population of Corsican groves has to be confirmed with additional molecular markers.

The previous *Phytophthora* survey of Corsican groves was completed prior to changes in cultural practices, such as the replacement of sour orange by trifoliate orange, Troyer citrange and Carrizo citrange, and the progressive abandonment of fosetyl-Al applications. More recently, there has been a resurgence of *Phytophthora* in Corsican groves. This may have been due to a change in soil and climatic conditions, a consequence of changing cultural practices, or an adaptation of the *Phytophthora* population to the new rootstocks. Other possibilities are changes in the relative abundance of the various *Phytophthora* species, an introduction of new populations, or interspecific crossings. Thus, a revised evaluation of the diversity of *Phytophthora* species present in diseased orchards was undertaken. The isolate characterization was performed using a combination of morphological and molecular criteria. Random amplified microsatellites (RAMS) were recently developed for assessing genetic variation within fungi (Hantula et al., 1996). It was useful for identifying species-specific patterns among fungi and Oomycetes, including *Phytophthora* (Hantula et al., 1996; 1997; Tooley et al., 2000). In addition, it was used to assess the intraspecific diversity of *P. cactorum* (Hantula et al., 1997; 2000).

In this study, we show that the present population of *Phytophthora* in citrus orchards of Eastern Corsica is primarily composed of strains ascribed to *P. citrophthora*. However, this population consists of four molecular groups, some of which display several features unusual for *P. citrophthora*. We suggest that one group represents a different taxon, and/or that speciation is ongoing in *P. citrophthora*.

Materials and methods

Pathogen strains

Isolates of *Phytophthora* and their origin are listed in Table 1. Reference strains of *P. citrophthora*,

Table 1. Origin, phenotypic, genotypic characteristics of the 16 *Phytophthora* reference strain (listed above the double line) and the 114 field isolates used in this study

Species	Isolate	Location	Date of isolation	Hosts	Origin[x]	Mating type	RAMS-GT[y]	RAMS-CGA pattern	Mitochondrial (mt) DNA pattern	GenBank[z]
P. citrophthora	112	Corsica	—	*C. aurantifolia*	—	ST	GT1	nd	M1	
	144	Corsica	—	*C. clementina*	—	A2	GT2	G2	nd	AY228577
	155	Corsica	1982	Citrus spp	—	A2	GT2	nd	M2	
	163	Morocco	—	*C. sinensis*	—	ST	GT1	G1	M1	AY228564
	244	France	1982	*C. limon*	—	ST	GT1	nd	nd	
	435	Spain	—		—	A2	nd	G2	M2	AY228565
P. nicotianae	44	Corsica	1972	*C. limon*	—	A2	nic	nd	nd	
	113	Corsica	—	*C. reticulata* × *C. paradisi*	—	A2	nic	nd	nd	
	115	Congo	—	*C. reticulata*	—	A1	nic	nd	nd	
	219	Corsica	1977	*C. jambhiri*	—	A1	nic	nd	nd	
P. citricola	61	Italy	—	Citrus spp	—	H	cit	nd	nd	
	277	Italy	—	Citrus spp	—	H	cit	nd	nd	
P. palmivora	178	Venezuela	—	Ficus	—	A1	pal	nd	nd	
	292	Togo	—	Cocoa	—	A2	pal	nd	nd	
	296	Reunion	—	Vanilla	—	A1	pal	nd	nd	
P. syringae	513	Germany	1994	*Aesculus hippocastanum*	—	H	syr	nd	nd	
P. citrophthora	PA10	Corsica	September 1997	*C. clementina*	C1	A2	GT2	G2	M2	
	PA75	Corsica	July 1998	*C. clementina*	C1	ST	GT1	G1	nd	
	PA76	Corsica	July 1998	*C. clementina*	C1	ST	GT1	G1	nd	
	PA133	Corsica	May 2001	*C. clementina* Fruit	C1	ST	GT1	G1	nd	
P. nicotianae	PA9	Corsica	September 1997	*C. clementina*	C1	A1	nic	nd	nd	
P. citrophthora	PA36	Corsica	April 1998	Soil	C2	A2	GT2	G2	M2	AY228570
	PA40	Corsica	April 1998	*C. paradisi*	C2	A2	GT2	G2	M2	
	PA41	Corsica	April 1998	*C. paradisi*	C2	A2	GT2	G2	M2	
	PA46	Corsica	April 1998	*C. volkameriana*	C2	A2	GT2	G2	M2	
	PA47	Corsica	April 1998	*C. volkameriana*	C2	A2	GT2	G2	nd	AY228571
	PA112	Corsica	April 1999	*C. reticulata* Fruit	C2	ST	GT1	G1	nd	
	PA113	Corsica	November 2000	*C. reticulata* Fruit	C2	ST	GT1	G1	nd	
	PA114	Corsica	November 2000	Soil	C2	ST	GT1	G1	nd	
	PA116	Corsica	November 2000	Soil	C2	ST	GT1	G1	nd	
	PA117	Corsica	November 2000	*C. paradisi* Fruit	C2	ST	GT1	G1	nd	
	PA118	Corsica	November 2000	Soil	C2	A2	GT2	G2	nd	
P. nicotianae	PA115	Corsica	November 2000	Soil	C2	A1	nic	nd	nd	
P. citrophthora	PA37	Corsica	April 1998	Soil	C3	ST	GT1	G1	M1	
	PA44	Corsica	April 1998	*C. limon*	C3	A2	GT2	G2	M2	
	PA45	Corsica	April 1998	*C. limon*	C3	A2	GT2	G2	nd	
	PA42	Corsica	April 1998	*C. clementina*	C4	ST	GT1	G1	nd	
	PA43	Corsica	April 1998	*C. clementina*	C4	ST	GT1	G1	M1	
	PA53	Corsica	April 1998	*C. medica Fruit*	C5	ST	GT1	G1	nd	

Table 1. (*Continued*)

Species	Isolate	Location	Date of isolation	Hosts	Origin[x]	Mating type	RAMS-GT[y]	RAMS-CGA pattern	Mitochondrial (mt) DNA pattern	GenBank[z]
	PA62	Corsica	April 1998	*C. medica* Fruit	C5	ST	GT1	G1	nd	
	PA120	Corsica	November 2000	*P. trifoliata*	C6	ST	GT1	G1	nd	
	PA123	Corsica	November 2000	*P. trifoliata*	C6	ST	GT1	G1	nd	
	PA136	Corsica	May 2001	*C. clementina* Fruit	C7	ST	GT1	G1	nd	
	PA111	Corsica	October 2000	*C. limon* Fruit	N1	ST	GT1	G1	nd	
	PA2	Corsica	September 1997	*C. clementina*	Or1	ST	GT1	G1	nd	
	PA3	Corsica	September 1997	*C. clementina*	Or1	ST	GT1	G1	nd	AY228568
	PA4	Corsica	September 1997	*C. clementina*	Or1	ST	GT1	G1	nd	
	PA5	Corsica	September 1997	*C. clementina*	Or1	ST	GT1	G1	nd	
	PA6	Corsica	September 1997	*C. clementina*	Or1	ST	GT1	G1	nd	
	PA7	Corsica	September 1997	*C. clementina*	Or1	ST	GT1	G1	nd	
	PA8	Corsica	September 1997	*C. clementina*	Or1	ST	GT1	G1	nd	
	PA11	Corsica	October 1997	*C. clementina*	Or2	A2	GT2	G2	nd	
	PA12	Corsica	October 1997	*C. clementina*	Or2	A2	GT2	G2	M2	
	PA13	Corsica	October 1997	*C. clementina*	Or2	A2	GT2	G2	nd	
	PA14	Corsica	October 1997	*C. clementina*	Or2	A2	GT2	G2	nd	
	PA15	Corsica	October 1997	*C. sinensis* × *P. trifoliata*	Or3	ST	GT1	G1	nd	
	PA16	Corsica	October 1997	*C. sinensis* × *P. trifoliata*	Or3	ST	GT1	G1	nd	
	PA25	Corsica	October 1997	*C. clementina*	Or3	ST	GT1	G1	nd	
	PA26	Corsica	October 1997	*C. sinensis* × *P. trifoliata*	Or3	ST	GT1	G1	nd	
	PA17	Corsica	October 1997	*C. clementina*	Or4	A2	GT2	G2	nd	
	PA18	Corsica	October 1997	*C. clementina*	Or4	A2	GT2	G2	M2	
	PA19	Corsica	October 1997	*C. clementina*	Or4	ST	GT1	G1	nd	
	PA20	Corsica	October 1997	*C. clementina*	Or4	ST	GT1	G1	nd	
	PA21	Corsica	October 1997	*C. limon*	Or4	ST	GT1	G1	nd	
	PA22	Corsica	October 1997	*C. limon*	Or4	ST	GT1	G1	nd	
	PA23	Corsica	October 1997	*C. limon*	Or4	ST	GT1	G1	nd	
	PA24	Corsica	October 1997	*C. limon*	Or4	ST	GT1	G1	nd	
	PA28	Corsica	November 1997	*C. clementina*	Or5	ST	GT1	G1	nd	
	PA29	Corsica	November 1997	*P. trifoliata*	Or5	ST	GT1	G1	M1	AY228567
	PA30	Corsica	November 1997	*C. clementina*	Or5	ST	GT1	G1	nd	
	PA31	Corsica	November 1997	*C. clementina*	Or6	ST	GT1	G1	nd	
	PA137	Corsica	May 2001	*C. clementina*	Or6	ST	GT1	G1	nd	
	PA138	Corsica	May 2001	*C. clementina*	Or6	A2	GT2	G2	nd	
	PA139	Corsica	May 2001	*C. clementina*	Or6	A2	GT2	G2	nd	
	PA140	Corsica	May 2001	*C. clementina*	Or6	A2	GT2	G2	nd	
	PA32	Corsica	November 1997	*C. clementina*	Or7	ST	GT1	G1	nd	
	PA33	Corsica	November 1997	*C. clementina*	Or7	ST	GT1	G1	nd	

PA34	Corsica	November 1997	*C. clementina*	Or8	A1	GT1	G3	M3	AY228569
PA35	Corsica	November 1997	*C. clementina*	Or8	ST	GT1	G1	nd	
PA93	Corsica	August 1999	*C. clementina*	Or8	ST	GT1	G1	nd	
PA94	Corsica	August 1999	*C. clementina*	Or8	ST	GT1	G1	nd	
PA95	Corsica	August 1999	*C. clementina*	Or8	ST	GT1	G1	nd	
PA96	Corsica	August 1999	*C. clementina*	Or8	ST	GT1	G4	M4	AY228576
PA97	Corsica	August 1999	*C. clementina*	Or8	ST	GT1	G1	nd	
PA98	Corsica	August 1999	*C. clementina*	Or8	ST	GT1	G1	nd	
PA99	Corsica	August 1999	*C. clementina*	Or8	ST	GT1	G1	nd	
PA100	Corsica	Angust 1999	*C. clementina*	Or8	ST	GT1	G1	nd	
PA101	Corsica	August 1999	*C. clementina*	Or8	ST	GT1	01	nd	
PA48	Corsica	April 1998	*C. clementina*	Or9	ST	GT1	G1	nd	
PA49	Corsica	April 1998	*C. clementina*	Or9	ST	GT1	01	nd	
PA50	Corsica	April 1998	*C. clementina*	Or9	ST	GT1	G1	nd	
PA51	Corsica	April 1998	*C. clementina*	Or9	ST	GT1	G1	nd	
PA52	Coisica	April 1998	*C clementina*	Or9	ST	GT1	G1	nd	
PA54	Corsica	April 1998	*C. clementina*	Or10	ST	GT1	G1	M1	
PA55	Corsica	April 1998	*C. clementina*	Or10	ST	GT1	G1	M1	AY228572
PA59	Corsica	April 1998	*C. clementina*	Or10	ST	GT1	G1	nd	
PA60	Corsica	April 1998	*C. clementina*	Or10	ST	GT1	G1	nd	
PA61	Corsica	April 1998	*C. clementina*	Or10	ST	GT1	G1	nd	
PA64	Corsica	April 1998	Soil	Or10	ST	GT1	G1	M1	
PA63	Corsica	April 1998	Soil	Or11	ST	GT1	G1	nd	
PA65	Corsica	April 1998	*C clementina*	Or11	A2	GT2	G2	M2	AY228573
PA66	Corsica	April 1998	*C. clementina*	Or11	A2	GT2	G2	M2	
PA67	Corsica	May 1998	*P. trifoliata*	Or11	ST	GT1	04	M4	AY228574
PA68	Corsica	May 1998	*P. trifoliata*	Or11	ST	GT1	G4	M4	AY228575
PA69	Corsica	June 1998	*C. paradisi* Fruit	Or12	ST	GT1	G1	nd	
PA70	Corsica	June 1998	*C. paradisi* Fruit	Or12	ST	GT1	G1	nd	
PA71	Corsica	July 1998	*C. clementina*	Or13	ST	GT1	G1	nd	
PA72	Corsica	July 1998	*C. clementina*	Or13	ST	GT1	G1	nd	
PA73	Corsica	July 1998	*C. clementina*	Or13	A2	GT2	G2	nd	
PA74	Corsica	July 1998	*C. clementina*	Or13	A2	GT2	G2	M2	
PA77	Corsica	July 1998	*C. clementina*	Or14	ST	GT1	G1	M1	
PA78	Corsica	July 1998	*C. clementina*	Or14	ST	GT1	G1	nd	
PA79	Corsica	July 1998	*C. sinensis* × *P. trifoliata*	Or14	ST	GT1	G1	nd	
PA80	Corsica	July 1998	*C. clementina*	Or15	ST	GT1	G1	nd	
PA81	Corsica	July 1998	*C. clementina*	Or15	ST	GT1	G1	nd	
PA82	Corsica	July 1998	*C. clementina*	Or15	ST	GT1	01	M1	
PA83	Corsica	July 1998	*C. clementina*	Or16	A2	GT2	G2	nd	
PA84	Corsica	July 1998	*C. clementina*	Or17	A2	GT2	G2	M2	
PA85	Corsica	July 1998	*C. clementina*	Or18	A2	GT2	G2	nd	

Table 1. (*Continued*)

Species	Isolate	Location	Date of isolation	Hosts	Origin[x]	Mating type	RAMS-GT[y]	RAMS-CGA pattern	Mitochondrial (mt) DNA pattern	GenBank[z]
	PA86	Corsica	July 1998	*C. clementina*	Or18	A2	GT2	G2	M2	
	PA92	Corsica	July 1998	*C. clementina*	Or18	A2	GT2	02	M2	
P. nicotianae	PA89	Corsica	July 1998	*C. clementina*	Or18	A1	nic	nd	nd	
P. citrophthora	PA87	Corsica	July 1998	*C. paradisi*	Or19	ST	GT1	01	nd	
	PA88	Corsica	July 1998	*C. paradisi*	Or19	ST	GT1	G1	MI	
	PA90	Corsica	July 1999	*C. clementina*	Or20	A2	GT2	G2	M2	
	PA91	Corsica	Jury 1999	*C. clementina*	Or20	A2	GT2	G2	M2	
	PA110	Corsica	October 2000	*C. clementina* Fruit	Or21	ST	GT1	G1	nd	AY228566
	PA142	Corsica	May 2001	*C. clementina*	Or22	ST	GT1	G1	nd	
P. nicotianae	PA141	Corsica	May 2001	*C. clementina*	Or22	A1	nic	nd	nd	
	PA127	Corsica	January 2001	Soil	Or23	A1	nic	nd	nd	

nd: not determined.

[x]: letters correspond to the type of stand (Or = orchards, C = INRA Centre of San Giuliano, N = nursery). Orchards are numbered according to the positions indicated in Figure 1.

[y]: RAMS-GT pattern was defined in the text.

[z]: Accessions for GenBank database.

P. citricola and *P. nicotianae*, *P. palmivora* and *P. syringae* were maintained at INRA, Antibes. These reference isolates were from different geographical origins or isolated from a previous survey in Corsican groves conducted in the late 1970s to early 1980s (De Vallavieille and Erselius, 1984). The isolates constituting the new collection were collected from 23 citrus sites and 2 nurseries in Eastern Corsica from November 1997 to May 2001, where gummosis was reported. They corresponded to locations surveyed two decades earlier (De Vallavieille and Erselius, 1984). Seven additional sites were located at citrus germplasm collection of the INRA Station of San Giuliano, where symptoms were occasionally observed. *Phytophthora* isolates were mainly (96/114) isolated from diseased scions, essentially clones of clementine (*C. clementinia*). However, a few orchards of grapefruit (*C. paradisi*) or lemon (*C. limon*) were also sampled. Nine isolates originated from rootstocks, including Trifoliate orange (*P. trifoliata*), Troyer citrange and Carrizo citrange (*C. sinensis* × *P. trifoliata*). Finally, nine isolates came from soil by baiting with lemon fruit. Eleven were isolated from fruits showing brown rot.

Isolation from diseased material was performed on pea agar (9% blended canned peas, 2% agar) amended with 100 ppm penicillin, 50 ppm pimaricin and 50 ppm polymixin for 2–4 days at 24–25 °C. Hyphal tips of growing colonies were transferred to plates of clarified V8 agar medium. Cultures were maintained on malt agar plates. Mating type was determined by pairing each isolate with known A1 and A2 strains of *P. nicotianae* or *P. citrophthora* on 10% clarified V-8 juice agar plates and checking for oospores from 5 to 8 days until 20 days at 24 °C in the dark. Isolates that failed to produce oospores were considered sterile.

DNA extraction and standard techniques

Mycelia of each isolate were collected after a 6–8-day-period at 24 °C in the dark on defined liquid glucose–asparagine medium (Hall et al., 1969). Total DNA was extracted as described (Panabières and Le Berre, 1999). All manipulations of nucleic acids were performed under standard protocols (Ausubel et al., 1989), unless otherwise indicated. Mitochondrial haplotypes were determined as described previously (Lacourt et al., 1994).

Analysis of ITS sequences

PCR was conducted in 25-µl reaction volumes, containing 100 ng DNA, 0.1 mM dNTP, 0.4 µM ITS4 and ITS6 primers (Table 2, White et al., 1990) and 0.5 unit of *Taq* polymerase (Quantum Appligene), in the buffer supplied by the manufacturer. Cycling parameters were 5 min at 95 °C, followed by 30 cycles of denaturation at 95 °C for 60 s, annealing at 60 °C for 90 s and extension at 71 °C for 90 s, with a final extension at 71 °C for 15 min. Amplification products were precipitated with ethanol, and resuspended in 10 µl H_2O. Aliquots (2 µl) of the amplification products were digested with 10 units of restriction enzymes (New England Biolabs) in a final volume of 10 µl for 2.5 h at 37 °C, and separated on 2% agarose gels in 0.5X TBE. The gels were stained in ethidium bromide and visualized under UV light. Alternatively, amplification products were ligated into pBluescript SK^- vector (Stratagene, La Jolla, USA) and cloned into *E. coli* DH5 α cells under standard protocols (Ausubel et al., 1989). ITS sequences were determined by Genome express SA (Meylan, France). Sequences were edited using Seqpup (version 0.6 for Macintosh), and alignment, tree construction and bootstrap (1000 trials) were performed using CLUSTALW (Thompson et al., 1994).

RAMS analyses

RAMS was carried out after Hantula et al. (1997) with the following modifications: 5–25 ng of DNA were used as templates in a 25-µl reaction volume, containing GT [5′YHY$(GT)_7$G] or CGA [5′DHB$(CGA)_5$] primers at a 0.2 µM final concentration, 0.1 mM of each dNTP and 0.5 units of *Taq* polymerase. Cycling reactions were 5 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 45 s at 50 °C (GT) or 57 °C (CGA) and elongation for 2 min at 71 °C, and a final elongation step for 3 min at 71 °C.

Table 2. Primers used in this study, with their sequence

Name	Sequence
ITS6	5′ GAAGGTGAAGTCGTAACAAGG 3′
ITS4	5′ TCCTCCGCTTATTGATATGC 3′
RAMS GT	5′ YHY(GT)7G
RAMS CGA	5′ DHB(CGA)5

The following designations are used for degenerate sites: B (G, T or C), D (G, A or T), H (A, T or C) and Y (A, C or G).

Amplification products were separated as described above.

Results

Establishment of a Phytophthora *collection*

A total of 114 isolates were obtained from the survey (Figure 1). The sample size varied over seasons, as it represented 33 isolates in Autumn 1997, 49 isolates in Spring and Summer 1998, 13 isolates in Spring 1999, 10 isolates in Autumn 2000, 1 isolate in Winter 2001 and 8 isolates in Spring 2001 (Table 1). The different sites were generally sampled once, although three orchards were surveyed twice, and two locations at the INRA Centre were surveyed three times (Table 1).

Species identification of reference strains

To develop and validate a fast and cheap identification method, we used a set of 16 reference strains in preliminary experiments. They belonged to five *Phytophthora* species pathogenic to citrus.

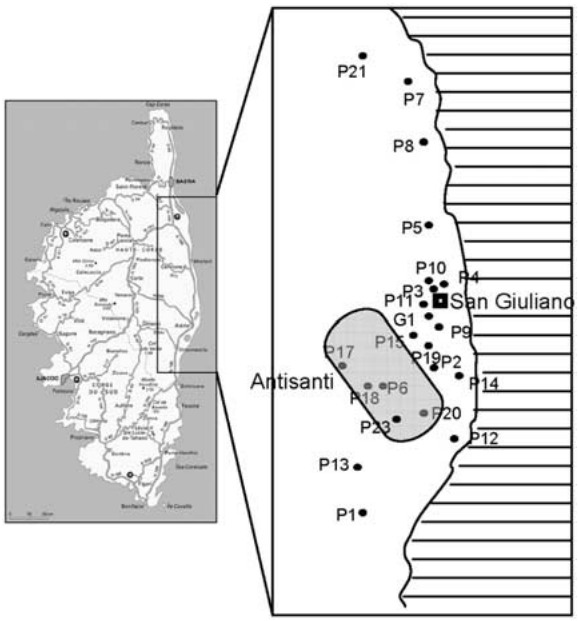

Figure 1. Location of the sampling sites for the *Phytophthora* collection. The black circles represent the private orchards, and the open square represents the INRA Station of San Giuliano. The Antisanti region (see text) is indicated as a grey area.

P. citrophthora, *P. nicotianae* and *P. palmivora* are by far the most common to this crop, but *P. citricola* and *P. syringae* have sometimes been reported on citrus in the Mediterranean Basin, and in Corsica (Kouyeas and Chitzanidis, 1978; De Vallavieille and Erselius, 1984). The identity of these reference strains was confirmed by PCR-RFLP of ITS regions using *Alu*I, and subsequent comparison to restriction pattern data of *Phytophthora* spp. maintained on the CABI web server (www.phytid.org). An additional *Hae*III digestion allowed the separation of *P. citricola* and *P. citrophthora* strains (Figure 2). All isolates used as references were unambiguously assigned to their relative species and could be used for the validation of alternative identification tools.

Following the work on *P. cactorum* (Hantula et al., 1997; Lijla et al., 1998), we tested an anchored-GT primer in RAMS experiments, on the samples previously used in the PCR-RFLP experiments. The resulting patterns generated by the GT primer are shown in Figure 3. The different species yielded distinctive and unambiguous patterns. Isolates of *P. nicotianae* displayed a prominent band of 900 bp (pattern nic), whereas isolates from *P. citricola* exhibited a pattern composed of four major bands of ∼500, 600, 850 and 1500 bp (pattern cit, Figure 3). Isolates of *P. palmivora* displayed a prominent 500 bp band (pattern pal) and the *P. syringae* strain exhibited a major band of 600 bp (pattern syr). Lastly, among the five *P. citrophthora* isolates, two distinct patterns (pattern GT1 of 425, 450 and 550 bp, and pattern GT2 of 425 and 550 bp, respectively) were observed.

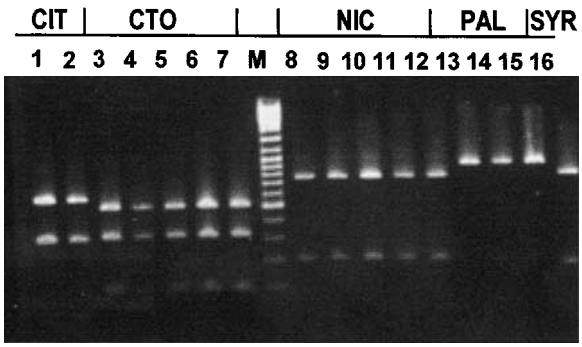

Figure 2. *Hae*III digestion of PCR products amplified with primers ITS4 and ITS6. Lanes 1–2: DNA from *P. citricola* 61 and 277; lanes 3–7: DNA from *P. citrophthora* 112, 114, 155, 163 and 244; M: 123 bp ladder; lanes 8–12: DNA from *P. nicotianae* 44, 113, 115, 219 and 220; lanes 13–15: DNA from *P. palmivora* 292, 296 and 178; lane 16: DNA from *P. syringae* 513.

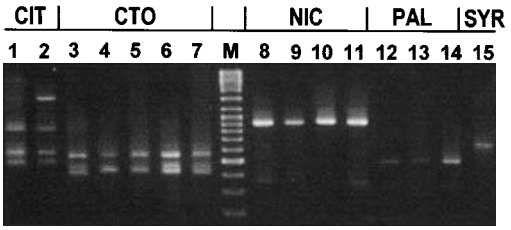

Figure 3. DNA fragments amplified from *Phytophthora* species using RAMS GT primer. Lanes 1–2: DNA from *P. citricola* isolates 61 and 277; lanes 3–7: DNA from *P. citrophthora* isolates 112, 114, 155, 163 and 244; M: 123 bp ladder; lanes 8–11: DNA from *P. nicotianae* 44, 113, 219 and 220; lanes 12–14: DNA from *P. palmivora* 292, 296 and 178; lane 15: DNA from *P. syringae* 513. Amplification products were separated in 2% agarose gels.

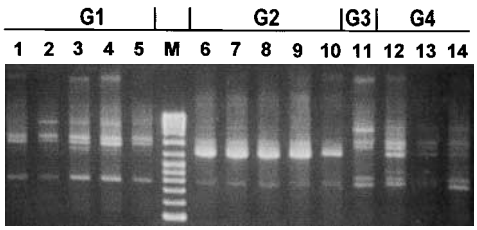

Figure 4. DNA fragments amplified from *P. citrophthora* isolates using RAMS CGA primer. 1: PA3; 2: PA29; 3: PA55; 4: PA110; 5: isolate 163; M: 100 bp ladder; 6: PA36; 7: PA47; 8: PA65; 9: isolate 114; 10: isolate 435; 11: PA34; 12: PA67; 13: PA68; 14: PA96. Amplification products were separated in 2% agarose gels.

Identification of field isolates

Total DNA from 114 field isolates was purified and analyzed with the GT primer. No isolates had patterns corresponding to the *P. citricola, P. palmivora* or *P. syringae* reference isolates. Five strains were identified as *P. nicotianae*. Among the other 109 isolates, 80 and 29 exhibited the GT1 and GT2 patterns respectively, revealed in the reference strains of *P. citrophthora*, and were thus ascribed to this apparently heterogeneous species. This *P. citrophthora* intraspecific variation was further investigated with additional criteria.

Intraspecific diversity within P. citrophthora

The characterization of the Corsican isolates of *P. citrophthora* included an analysis of their mating system. It was determined in several independent experiments, using diverse *P. nicotianae* A1 and A2 testers. More than 72% (79/109) of the isolates failed to produce oospores, even after prolonged incubation, and were therefore considered sterile. In contrast, 29 isolates (26.6% of the population) were identified as A2 after a 5–7-day incubation period. Surprisingly, a single isolate was of the A1 mating type. A few A2 and the A1 *P. citrophthora* isolate were further used as testers in additional matings with isolates previously defined as sterile. No oospores were produced in any situation, confirming their sterility. The five *P. nicotianae* strains were also tested and all produced abundant oospores when paired with A2 testers, and were then defined as A1 strains.

The intraspecific variability of *P. citrophthora* was investigated by RAMS. When the amount of DNA was lowered to 5 ng, an anchored CGA-primer produced four reproducible patterns (Figure 4). Pattern 1, defined by three major bands of 800, 1400, 1500 bp and a fainter 2500 bp band, constituted by far the major group of strains (group G1), in that it represented up to 70% of the sampling (76/109), and encompassed only sterile isolates. All of the 29 A2 isolates exhibited an identical pattern, and therefore constituted the group G2, readily distinguishable with a discrete band of 800 bp and an intense band of 1200 bp. The A1 isolate exhibited a unique, distinct pattern, easily recognized by a doublet of ~750 and 800 bp, a second doublet of 1400 and 1500 bp, and a band of ~2500 bp. This pattern was very similar to that displayed by the isolates of the group G1, and represented the single representative of the molecular group G3. Finally, three sterile isolates displayed a complex pattern defined by two triplets of 800–1000 and 1300–1500 bp, respectively and constituted a fourth molecular group. No significant variation was observed within the pattern of each group (Figure 4).

The diversity was evaluated at the mtDNA level, using a miniprep technique. Thus, total DNA of a subset of 33 strains from the various groups were digested with *Msp*I, and the resulting digests were analyzed after electrophoresis on agarose gels, followed by ethidium bromide staining. This technique had been shown to reveal discrete bands of mtDNA against nuclear background (Panabières et al., 1989; Lacourt et al., 1994). Four closely-related, but distinct patterns were resolved, typical of each molecular subgroup (exemplified in Figure 5).

Lastly, the ITS sequences of 16 isolates from the four groups was determined. ITS1 and ITS2 regions have been shown to resolve at different phylogenetic levels among *Phytophthora* species (Cooke et al., 2000), so separate analyses were performed for each region.

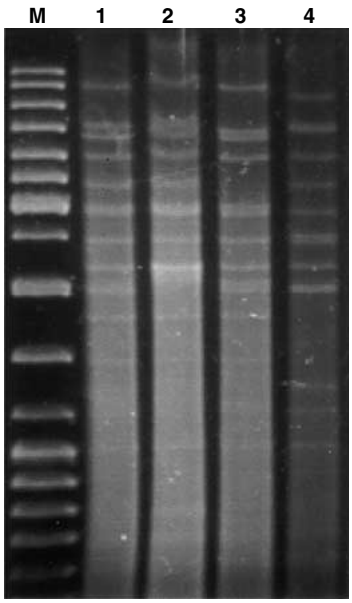

Figure 5. Comparison of the *Msp*I restriction pattern of total DNA from *P. citrophthora* isolates PA55 (1), PA65 (2), PA34 (3) and PA98 (4) representing groups G1–G4, respectively, on 1% agarose gel, after ethidium bromide staining. Discrete bands correspond to mitochondrial DNA. Lane M: 100 bp ladder.

Sequences from reference strains, among which the *P. citrophthora* strain IMI332632 deposited on the CABI web server and isolated from kiwi (accession number AF266785), a *P. citrophthora* strain isolated from citrus in Korea (accession number AF228081), the *P. citrophthora* cocoa isolate 449, whose ITS sequences are known to be divergent from citrus isolates (Lee and Taylor, 1992) were included, as well as ITS sequences of *P. citricola* and *P. capsici* as outgroups.

The sequences of field isolates from the groups G1, G3 and G4 were generally identical to the *P. citrophthora* sequence identified in the citrus strain, except that 3 strains from the G1 group displayed an adenine insertion at position 16, and the G3 isolate displayed a deletion at residue 71. The isolates from the G2 group shared a mutation at position 15 with the *P. citrophthora* kiwi strain, and possessed two additional deletions and two transitions (Figure 6A). The isolate 114, of the group G2, displayed two other T/C transitions at positions 70 and 86.

Alignment of ITS2 sequences revealed that the isolates from the group G2 differed from the *P. citrophthora* strains by at least three diagnostic mutations, among which two are common to *P. citricola* and *P. capsici* (Figure 6B). Lastly, sporadic substitutions were observed in some strains of the G2 group (Figure 6B). The distance-based trees for ITS1 and for ITS2 showed that the group G2 is clearly separate from the other Corsican isolates of *P. citrophthora*, as indicated by the high bootstrap values (77% and 85% for the ITS1 and ITS2 regions, respectively, Figure 7). However, they are also isolated from the cocoa isolate and the outgroup species.

A putative association between the origin of sample and the occurrence of a particular genotype was investigated. No G2 isolate was found in the northern or southern limits of the citrus growing region (Figure 1). The G2/G1 ratio was highly divergent from one location to another. It reached 36% in the INRA Station, decreased to 23.5% in the area surrounding the Station but the G2 isolates constituted more than 68% of the sampling performed in the region of Antisanti, which constitutes an old production area. A single genotype was recovered in 17 out of 23 orchards, and in 4 out of 7 plots surveyed at the INRA Station (Table 1). No obvious association could be observed between a given genotype and the nature of the rootstock and/or variety. As an example, 7 G1 isolates and 4 G2 isolates were collected during the same period in two distinct orchards constituted of clementine grafted on Troyer citrange. Two genotypes (G1 and G2) were found together in 3 orchards (P4, P6 and P13) and 2 plots of the INRA Station (C2 and C3). The ratio G1/G2 within a same location varied from 25% to 75% in the orchards, and from 33% to 83% in the plots at the INRA Station.

Discussion

The present paper reveals that the *Phytophthora* population found in citrus orchards of eastern Corsica is mainly composed of two distinct subgroups within *P. citrophthora* that may represent separate species.

Over a 4-year period, 95.5% of the isolates sampled were assigned to *P. citrophthora*, while less than 4.5% (5/114) of the isolates were *P. nicotianae*, and *P. citricola* was absent. In a previous survey of the same locations, and a similar sample size, *P. citrophthora* was also dominant and constituted 89.7% (78/87) of the isolates, *P. nicotianae* 8% (7/87), and *P. citricola* 2.3% (2/87) of the isolates, respectively (De Vallavieille and Erselius, 1984). These proportions were not significantly different in a chi-square test (not shown). In addition, the identification methods used in the present

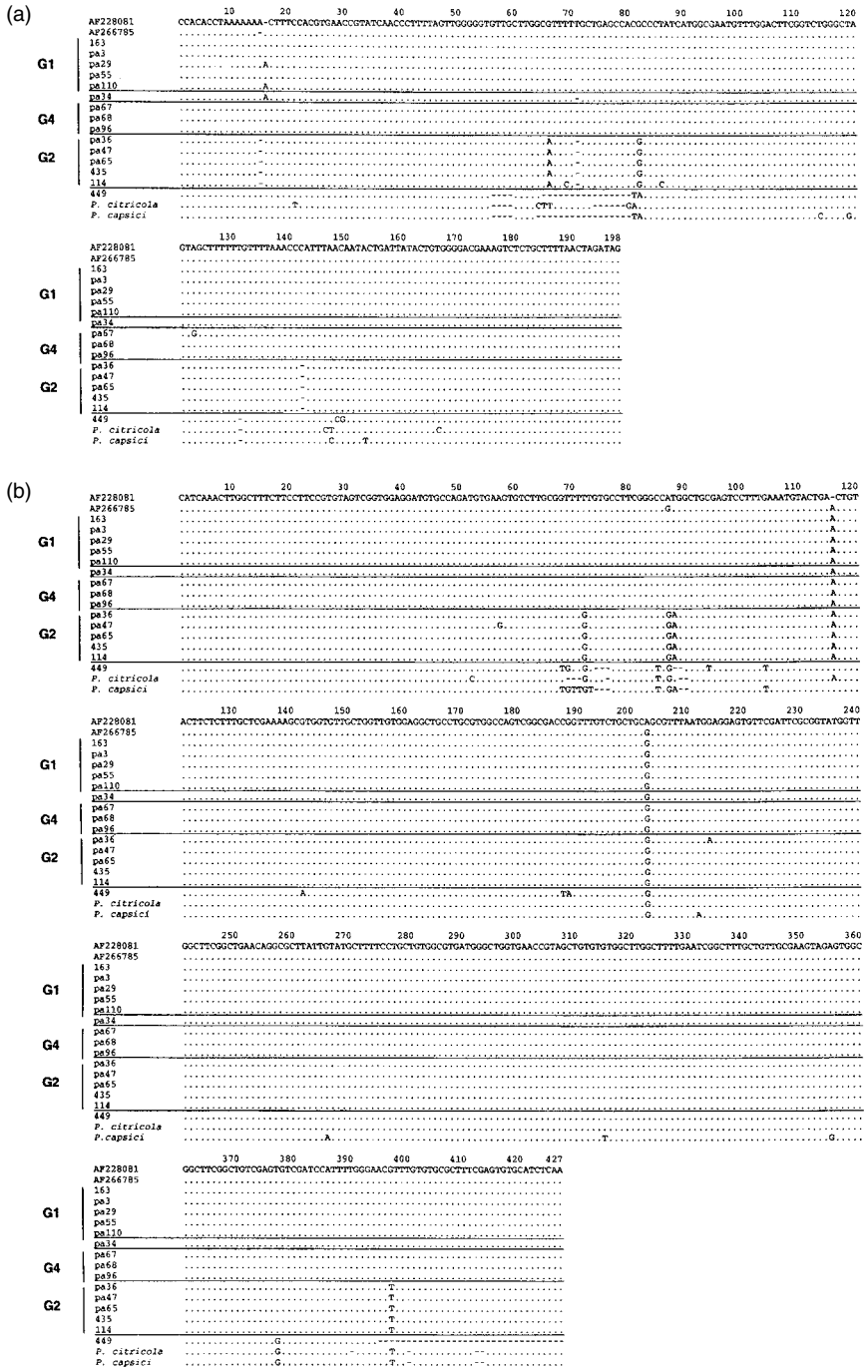

Figure 6. Aligned DNA sequences of ITS regions of *P. citrophthora* isolates collected in citrus groves and some reference strains: *P. citrophthora* from kiwi (accession number AF266785, Cooke et al., 2000), *P. citrophthora* from citrus (accession AF228081), field isolates 163 (sterile), 114 and 435 (A2), *P. citrophthora* from cocoa (isolate 449, Lee and Taylor, 1992), *P. citricola* (accession number L41375, Crawford et al., 1996) and *P. capsici* (accession number AF266787, Cooke et al., 2000). Horizontal bars delineate molecular groups. Dashes (-) indicate introduced gaps. A: alignment of ITS1 region; B: alignment of ITS2 region.

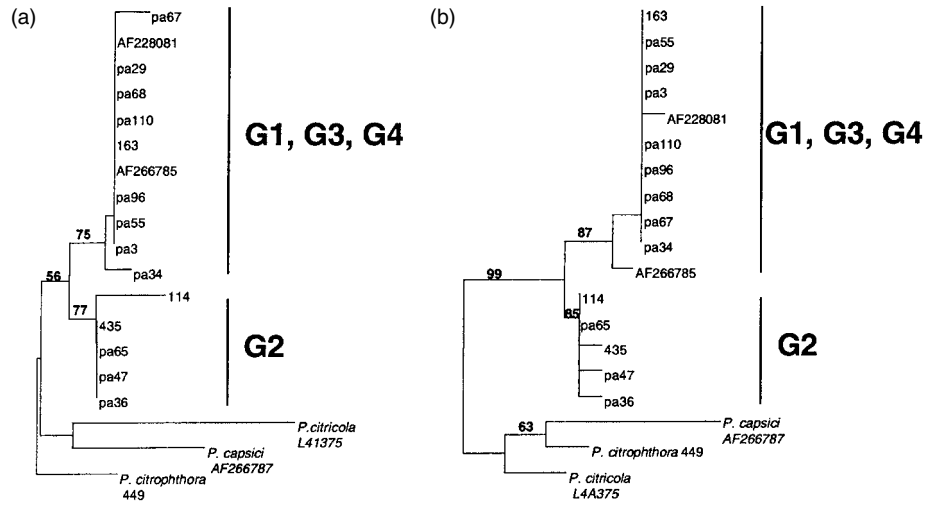

Figure 7. ITS phylogram of Corsican isolates of *P. citrophthora*, relative to reference strains and other *Phytopthora* species. Sequences were clustered with CLUSTALW. A: ITS1 sequences. B: ITS2 sequences. The numbers above the branches indicate percentage bootstrap values from 1000 trials.

study are more robust than in the earlier survey, and the comparison of protein profiles or isozymes of such closely-related species could have lead to misidentifications in the past (De Vallavieille and Erselius, 1984). Hence, 3 out of 7 isolates, identified as *P. nicotianae* on the basis of traditional taxonomical characters, were more akin to *P. citrophthora* when considering crucial features such as the cardinal temperature, the mating type or the protein patterns (De Vallavieille and Erselius, 1984). The proportions of *P. nicotianae* isolates in the 1980s collection and the present one would be highly similar (4.59% and 4.38%, respectively). In a similar context, the *P. citricola* isolates identified in the previous survey more closely resembled *P. citrophthora* than *P. citricola* strains isolated from other hosts (Erselius and de Valavieille, 1984). This similarity was confirmed by comparison of mtDNA profiles (Ricci et al., 1990). Unfortunately, these strains are no longer available for re-examination. We thus conclude that with the exception of changes within *P. citrophthora* strains (see below), the overall proportions of *Phytophthora* species in these citrus orchards has not changed drastically.

The present work indicates that the Corsican *P. citrophthora* population could be composed of two distinct species. Hence, 29 isolates were shown to constitute a homogeneous group with regard to sexual behaviour, RFLP of mtDNA, RAMS-GT, RAMS-CGA and ITS sequences. They differ strikingly from the main group of *P. citrophthora*, designed as group G1 on the basis of molecular data, and on a tendency of the two groups to be reproductively isolated. In addition, the ITS sequence of the group G2 differs from that of the group G1 and *P. citrophthora* sequences published elsewhere (Lee and Taylor, 1992; Cooke et al., 2000; Förster et al., 2000), by three deletions and 6–8 nucleotide substitutions, i.e. 9–11 differences. This is an order of magnitude similar to that delimiting *Phytophthora* species in general (Cooke et al., 2000; Werres et al., 2001). Hence, *P. ramorum* and *P. lateralis* display 11 differences (Werres et al., 2001). Moreover, a number of valid species display less differences. *P. infestans* and *P. mirabilis*, otherwise defined as separate species by various molecular markers (Goodwin et al., 1999), display two substitutions in the ITS1 and have identical ITS2 regions (Cooke et al., 2000). Last, three Group I species, after the Waterhouse classification (Waterhouse, 1963), *P. cactorum*, *P. idaei* and *P. pseudotsugae*, display 4 and 3 nucleotide substitutions, respectively (Cooke et al., 1996). The present results indicate that the *P. citrophthora* isolates of Corsican citrus groves represent at least two distinct taxa. However, they form a distinct branch in phylogenetic trees, and may be considered as members of a *P. citrophthora* complex, unless further analysis of additional characters eventually leads to the erection of the G2 group as a valid species.

Previous analyses had already indicated the existence of separate taxa within *P. citrophthora*, in that cocoa isolates from Brazil can be distinguished from

other isolates on the basis of a lack of hybridization to specific DNA probes (Goodwin et al., 1990), mtDNA patterns (Förster et al., 1990), isozyme profiles (Oudemans and Coffey, 1991; Mchau and Coffey., 1994) and ITS sequences (Lee and Taylor, 1992; Förster et al., 2000). The taxonomic status of this group, named CTR2 (Oudemans and Coffey, 1991; Mchau and Coffey., 1994) is still ambiguous, but it is likely that it constitutes a valid species. In a comparative study performed on 77 *P. citrophthora* isolates, only two A2 strains were found on citrus, among which one was later re-assessed as *P. capsici* (Mchau and Coffey, 1994). In contrast, 11 A2 strains were isolated from various hosts, excluding cocoa isolates. An analysis of ITS sequences of these strains, if still available, and a comparison with those presented in the present work, would indicate whether all these A2 isolates constitute a single species.

Considering that groups 3 and 4 were rarely represented, and related to the group G1 on the basis of RAMS (–GT and –CGA) patterns and ITS sequences, the current structure of *P. citrophthora* can be reduced to two groups, the group G1 (sterile) and the group G2 (A2). A large proportion (26.6%) of this subset was of the A2 mating type. With the allowance for the possible misidentification of three *P. nicotianae* strains of A2 mating type in the previous study (see above), at most 3.7% of A2 isolates were isolated in the 'old' *P. citrophthora* population (De Vallavieille and Erselius, 1984), and at least 96.3% (78/81) of the population were sterile. So the expansion, or emergence, of the A2 group over time, may explain the increased losses due to *Phytophthora* diseases. As mentioned before, most of isolates were collected in diseased orchards. G2 was the only genotype encountered in five orchards, and an INRA plot, where gummosis symptoms were unambiguous. These strains also constitute the main part of the sample collected in different surveys. Several hypotheses may explain the occurrence of A2 isolates in the present population. They were mainly found in two locations. One is the INRA Station that possesses an important germplasm collection, and harbours a wide diversity of rootstocks and varieties. The other corresponds to an old area of citrus production which is rather homogeneous, and primarily consists in clementine grafted on sour orange, or less frequently Troyer citrange. The hypothesis that the A2 strains may have originated from San Giuliano, resulting from an adaptation to the host diversity, can be ruled out, because there is no evidence of preferential abundance of A2 strains in this site. The possibility that this genotype has been introduced recently, maybe during or shortly after the import of new host plants, is also unlikely, because there were no major cultural changes in the old region of production. However, it is possible that the G2 group was present in other native hosts or cultivated crops, and underwent adaptation to citrus plants. In Corsica, citrus orchards have frequently replaced the native bush called 'maquis', which comprises potential hosts for *P. citrophthora*, such as arbutus or rosemary (Erwin and Ribeiro, 1996). In addition, orchards have been surrounded by *Prunus* sp., avocado plantations, and the recently introduced *Feijoa sellowiana* which are all susceptible to *P. citrophthora* (Pane et al., 2001). The two areas where A2 isolates have been found are surrounded by kiwi fruit, which is highly susceptible to *P. citrophthora* (Erwin and Ribeiro, 1996). Last, the ITS sequences of the Corsican A2 isolates share some common nucleotide substitutions with that of the reference strain IMI332632, isolated from kiwi fruit. A survey of *P. citrophthora* in kiwi orchards and other native crops would bring valuable information to test the hypothesis of an adaptation of the G2 isolates from resident plants to citrus.

No RAMS variation is observed within any of the four groups. This observation, with the lack of mtDNA variation and the similarity of mating behaviour within groups suggests that the *P. citrophthora* complex is composed of a limited set of clonal lineages. This is obvious for groups G1 and G4, which were sterile and has to be further investigated for A2 strains. However, in a previous study, the A2 isolates of *P. citrophthora* acted only as inducers of oospore formation in the partner, and did not produce oospores themselves (Mchau and Coffey, 1994). So the potential for sexual reproduction of *P. citrophthora* is in doubt. Nevertheless, further analyses of the genetic diversity within groups is required to assess the role of clonal propagation in their spread.

The results presented here raise several questions. The current *P. citrophthora* population is composed of two distinct taxa, which are frequently separated in space and time, and are reproductively isolated. We now have to evaluate the actual extent of isolation, by an estimation of the potential of gene flow between the two taxa, and by a refined investigation of the molecular diversity of these populations with additional markers. A precise analysis of the relationships between the two *P. citrophthora* taxa, and a comparison with reference strains is needed to define whether the genetic isolation revealed in the present work corresponds to an ongoing speciation event from a common lineage, or

if the G2 group is unrelated to the Corsican population of *P. citrophthora*, and if the sterile isolates collected recently are the same as those present 20 years ago.

The relationship between molecular and pathogenic variation has to be investigated. The degree of correlation between a given pathotype and molecular markers is generally important for fungal populations that reproduce asexually (Asigbetse et al., 1994; Levy et al., 1993), and low in populations that reproduce sexually (Burdon and Roelfs, 1985). Although doubtful, the hypothesis of sexual reproduction among the G2 group cannot be definitively ruled out. So, the potential occurrence of both types of reproduction may have important consequences for the spreading of the disease. It thus remains to evaluate whether the recent outbreaks of citrus diseases are due to a changing population, especially in the ratio of A2 and sterile isolates, to the emergence of new virulences among *Phytophthora* isolates, or to a better fitness of some strains. The evaluation of virulence of the two taxa population towards different rootstocks and varieties is in progress.

Acknowledgements

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