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Molecular analysis of the major *Phytophthora* species on cocoa

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The internally transcribed spacer (ITS) regions of the ribosomal RNA (rRNA) gene cluster of 161 isolates of *Phytophthora* species involved in pod rot, stem canker and leaf blight of cocoa were analysed to determine inter- and intraspecific variation in this disease complex. The species *P. palmivora*, *P. megakarya*, *P. capsici*, *P. citrophthora* and *P. nicotianae* could all be clearly distinguished by PCR amplification of the ITS region followed by restriction analysis with *Hae*III, *Hinf*I, *Pvu*II and *Alu*I. This method provided a relatively rapid identification procedure for these species, and was able to distinguish isolates that had previously been misidentified by morphological methods. Sequence analysis showed that the four main cocoa-associated species formed two distinct groups, one comprising *P. capsici* and *P. citrophthora*, and the other *P. palmivora* and *P. megakarya*. Detailed sequence analysis and comparison with published literature suggested that *P. capsici* isolates from cocoa may be closely related to *P. tropicalis*, a species recently described from *Cyclamen* and *Dianthus*.

Keywords: interspecific variation, rDNA-ITS region, RFLPs

Introduction

Phytophthora black pod is the most serious disease constraint to cocoa production worldwide, especially in West and Central Africa, where some 65% of the world's cocoa is largely cultivated by subsistence farmers (Assoumou, 1997; ICCO, 1998). Four main species of *Phytophthora* are known to cause black pod disease, and these vary in both their aggressiveness and the level of crop loss caused. *Phytophthora megakarya* is the most aggressive and can cause between 60 and 100% crop loss (Djiekpor *et al*., 1981; Dakwa, 1988). In contrast, *P. palmivora* is less aggressive and can cause crop losses of 4·9–19% (Blencowe & Wharton, 1961; Dakwa, 1984); this species is more aggressive than *P. capsici* (Lawrence *et al*., 1982). *Phytophthora citrophthora* is more aggressive than *P. palmivora* or *P. capsici*, and requires less time for zoospore germination and penetration on unwounded, detached pods (Campêlo *et al*., 1982). Single reports of other *Phytophthora* species causing black pod include *P. botryosa* (Chee & Wastie, 1970), *P. heveae* (Lozano & Romero, 1984), *P. katsurae* (Liyanage & Wheeler, 1989) and *P. megasperma* (Zentmyer, 1988), although these are not considered major problems for cocoa production.

Phytophthora species have historically been delimited by their morphology, cytology and biochemistry (Sansome *et al*., 1975; Brasier & Griffin, 1979; Kaosiri & Zentmyer, 1980; Hardham *et al*., 1986; Appiah *et al*., 2003). There are, however, difficulties in using phenotypic taxonomic characters, in that some characters overlap between species, and significant variation can, and does, occur among isolates of the same species (Brasier & Griffin, 1979; Erwin & Ribeiro, 1996; Appiah, 2001; Appiah *et al*., 2003). Molecular approaches can provide reliable methods for pathogen identification and disease diagnosis, and the technology available has sufficient sensitivity to enable detection of variation between organisms at the level of a single base change (Schlick *et al*., 1994). Various DNA-based techniques have been used to evaluate inter- and intraspecific variation in *Phytophthora* species, including nuclear and mitochondrial DNA RFLPs (Förster *et al*., 1987, 1989; Oudemans & Coffey, 1991; Lee *et al*., 1993; Ersek *et al*., 1994), and analysis of sequences in the

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ribosomal RNA (rRNA) gene cluster (Cooke & Duncan, 1997; Förster *et al*., 2000). Analysis of the internal transcribed spacer (ITS) region of the nuclear rRNA repeat unit has been used to determine interspecific variation (Cooke *et al*., 1996; Crawford *et al*., 1996; Cooke & Duncan, 1997), and sequence differences have been used to distinguish between some *Phytophthora* species (Lee & Taylor, 1992; Ristaino *et al*., 1998). ITS sequence data have also been used to study the phylogenetic relationships of many *Phytophthora* species (Lee & Taylor, 1992; Brasier *et al*., 1999) and have clarified some of the taxonomic relationships within the genus (Cooke *et al*., 2000; Förster *et al*., 2000). However, most of these studies have involved noncocoa *Phytophthora* species, and in cases where some of the species occurring on cocoa were studied, only a small number of isolates were included. Selection of these isolates has been based on a number of different criteria, and so the results may not be entirely representative of the cocoa *Phytophthora* species. For example, Lee & Taylor (1992) included only seven isolates from cocoa (belonging to four species) out of 27 isolates sequenced, and Cooke *et al*. (2000) included 17 isolates belonging to two species found on cocoa in a large sequence analysis involving 234 isolates of 50 species of *Phytophthora* and other Oomycetes. Similarly, Förster *et al*. (2000) included single isolates of *P. capsici* and *P. citrophthora* from cocoa in a study where 57 isolates of *Phytophthora* were sequenced.

Rapid identification and diagnosis of the major *Phytophthora* species (*P. palmivora*, *P. megakarya*, *P. capsici* and *P. citrophthora*) that cause pod rot of cocoa worldwide remains a problem. This paper presents a detailed analysis of the ITS regions of 161 isolates mainly received as *P. palmivora* and *P. megakarya*, but also including isolates from the less frequently reported species *P. capsici* and *P. citrophthora*, predominantly from cocoa in West and Central Africa. The analysis was undertaken in order to characterize the variability within and between the main species of *Phytophthora* causing pod rot and other diseases of cocoa, and to determine possible phylogenetic relationships between them.

Methods

Collection of isolates

One hundred and forty isolates of *Phytophthora* spp. were collected from cocoa-growing countries worldwide. Twenty-one additional isolates from plants other than cocoa were also obtained for comparison (Table 1). All identifications were confirmed by morphological characterization and mating type designation as reported by Appiah (2001) and Appiah *et al*. (2003).

Growth of cultures and DNA extraction

Cultures were grown in a shaking incubator at 27°C for 3–5 days in 250 mL conical flasks containing 60 mL V8 broth (20% V8 juice in distilled water). Mycelium was harvested by vacuum filtration, washed with sterile distilled water (SDW) and freeze-dried overnight. DNA was extracted from ground, freeze-dried mycelium following a modification of the method of Raeder & Broda (1985). The modification involved the introduction of two incubation steps: the first after addition of the extraction buffer (0·2 M Tris-HCl pH 8·5, 0·25 M NaCl, 25 mM EDTA, 0·5% SDS) where the homogenate was incubated at 60°C for 20 min; the second incubation was under the same conditions after the phenol-chloroform-isoamyl alcohol (25 : 24 : 1) extraction was completed. The extracted DNA was redissolved in $T_{10}E_1$ buffer (10 mM Tris; 1 mM EDTA, pH 8) and stored at –20°C.

PCR amplification and restriction of the ITS region

Polymerase chain reaction (PCR) amplification of the ITS region of the template DNA was performed using the primers ITS1 and ITS4 as described by White *et al*. (1990). PCR was undertaken in 50 μL volumes consisting of 33·25 μL HPLC-grade water, 5 μL 10× PCR buffer, 4 μL dNTPs (at 5 mM each), 1·5 μL 25 mM $MgCl_2$, 2 μL (20 pmol) each of ITS1 and ITS4 primers, 0·25 μL (0·5 U) T*th* polymerase and 2 μL template DNA. Cycling conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 45 s and 72°C for 2 min. After the cycling reactions there was a final step of 72°C for 6 min. Eleven restriction endonucleases (*Alu*I, *Ava*II, *Cfo*I, *Hae*III, *Hha*I, *Hin*fI, *Msp*I, *Pst*I, *Pvu*I, *Pvu*II and *Xba*I) were screened to determine restriction sites with two isolates of each species. The restriction enzymes that produced clear and potentially diagnostic patterns were selected for analysis of all of the *Phytophthora* isolates. Approximate sizes of the digested products were determined by comparison with a standard 100 bp molecular marker ladder (Promega Corporation, Madison, WI, USA) after 3% NuSieve agarose gel electrophoresis in TBE buffer (Tris-base 10·8 g L^{-1}, boric acid 5·5 g L^{-1}, EDTA 0·93 g L^{-1}, pH 8) and staining in ethidium bromide (0·5 μg mL^{-1}).

Cloning and sequencing of ITS-PCR products

The ITS regions of the rDNA of 18 selected isolates (see Table 3) were cloned using pGEM-T Easy Vector System II kits (Promega). The transformed cultures were screened on duplicate LB/ampicillin/IPTG/X-Gal plates overnight at 37°C. Five white colonies were picked randomly from each isolate and cultured in 2 mL LB/ampicillin medium overnight. The selected recombinant plasmids were checked for the presence of the inserted DNA fragment by PCR by boiling 10 μL of the bacterial culture in 40 μL SDW at 95°C for 10 min, removing the cell debris by centrifugation, and setting up a standard PCR with the ITS primers using 5 μL of the supernatant. PCR cycling conditions were the same as above. The resulting PCR products were visualized after gel electrophoresis and staining in ethidium bromide.

One millilitre of each recombinant plasmid culture was purified using the Wizard Plus Minipreps DNA Purification System kit (Promega). The purified plasmid DNA was

Table 1 Details of isolates used in study

Study no.	Collection/ other number	Country (region/location)	Year of isolation	Host	RFLP group
Phytophthora capsici					
1	IMI 325899	Brazil	1988	*Theobroma cacao*	PCAP1
2	IMI 325900	Brazil	1988	*T. cacao*	PCAP1
3	IMI 304412[a]	Cote d'Ivoire (Abidjan)	1988	*T. cacao*	PCAP1
4	IND 44	India (Kerala, Kanara)	1999	*T. cacao*	PCAP3
5	IND 42	India (Kerala, Rani)	1999	*T. cacao*	PCAP3
6	IMI 357546	Mexico	1993	*T. cacao*	PCAP2
7	IMI 230564	Nigeria	1989	*Capsicum* sp.	PCAP2
8	PPaE9[b]/IMI 386149	Taiwan	1993	*Hedera helix*	PCAP2
P. citrophthora					
9	93 P61/IMI 386150	Indonesia (North Sulawesi)	1993	*T. cacao*	PCIT
P. megakarya					
10	CAM 187	Cameroon	–	*T. cacao*	PM1
11	NS 270	Cameroon (Fako, Bagimgili)	–	*T. cacao*	PM1
12	NS 264/IMI 386156	Cameroon (Fako, Idewa)	–	*T. cacao*	PM1
13	NS 268/IMI 386157	Cameroon (Fako, Sange)	–	*T. cacao*	PM1
14	NS 260/IMI 386154	Cameroon (Manyu, Ayoke)	–	*T. cacao*	PM1
15	NS 128/IMI386153	Cameroon (Mefou, Nomays)	–	*T. cacao*	PM1
16	CAM 184/IMI 386159	Cameroon (Meme)	–	*T. cacao*	PM1
17	CAM 309/IMI 386160	Cameroon (Meme)	–	*T. cacao*	PM1
18	NS 275/IMI 386161	Cameroon (Meme)	–	*T. cacao*	PM1
19	NS 308/IMI 386162	Cameroon (Meme)	1995	*T. cacao*	PM1
20	NS 229/IMI 386155	Cameroon (Ndé, Sangwa)	–	*T. cacao*	PM1
21	IMI 337098	Equatorial Guinea	1990	*T. cacao*	PM2
22	EG 63/IMI 386164	Equatorial Guinea (Rio Muni)	–	*T. cacao*	PM1
23	EG 1B/IMI 386163	Equatorial Guinea (Bioko)	–	*T. cacao*	PM1
24	G4·112·1/IMI 386165	Gabon (Oyem-Est)	1982	*T. cacao*	PM1
25	BBPSI-4	Ghana	1999	*T. cacao*	PM1
26	GHA 12/IMI 386217	Ghana	1990	*T. cacao*	PM1
27	IMI 337104	Ghana	1990	*T. cacao*	PM1
28	ASH-A 25	Ghana (ASH, A'buokrom)	1998	*T. cacao*	PM1
29	ASH-AK 20	Ghana (ASH, A'buokrom)	1998	*T. cacao*	PM1
30	AK/IMI 386172	Ghana (ASH, Akomadan)	1998	*T. cacao*	PM1
31	AK 1/IMI 386173	Ghana (ASH, Akomadan)	1998	*T. cacao*	PM1
32	AK 2/IMI 386174	Ghana (ASH, Akomadan)	1998	*T. cacao*	PM1
33	AKA 22	Ghana (ASH, Akomadan)	1998	*T. cacao*	PM1
34	AKA 23	Ghana (ASH, Akomadan)	1998	*T. cacao*	PM1
35	AKA 24	Ghana (ASH, Akomadan)	1998	*T. cacao*	PM1
36	AKA 25	Ghana (ASH, Akomadan)	1998	*T. cacao*	PM1
37	AKP 2 /IMI 386179	Ghana (ASH, Akomadan)	1998	*T. cacao*	PM1
38	CS-AKO/IMI 386180	Ghana (ASH, Akomadan)	1998	*T. cacao*	PM1
39	ASH-AB 11	Ghana (ASH, Bibiani)	1998	*T. cacao*	PM1
40	ASH-AM 16	Ghana (ASH, Mpasaaso)	1998	*T. cacao*	PM1
41	ASH-M 29	Ghana (ASH, Mpasaaso)	1998	*T. cacao*	PM1
42	ASH-M 44	Ghana (ASH, Mpasaaso)	1998	*T. cacao*	PM1
43	ASH-M 49	Ghana (ASH, Mpasaaso)	1998	*T. cacao*	PM1
44	ASH-A 14	Ghana (ASH, Nkawie)	1998	*T. cacao*	PM1
45	DYT-NYI/IMI 386187	Ghana (ASH, Nyinahin)	1997	*T. cacao*	PM1
46	ASH-O 48	Ghana (ASH, Otaakrom)	1998	*T. cacao*	PM1
47	PK 1/IMI 386189	Ghana (ASH, Pokukrom)	1998	*T. cacao*	PM1
48	PK 2/IMI 386190	Ghana (ASH, Pokukrom)	1998	*T. cacao*	PM1
49	PKA 1/IMI 386191	Ghana (ASH, Pokukrom)	1998	*T. cacao*	PM1
50	PKA 2 /IMI 386192	Ghana (ASH, Pokukrom)	1998	*T. cacao*	PM1
51	ASH-DA 39	Ghana (ASH, Tepa)	1998	*T. cacao*	PM3
52	ASH-T 10	Ghana (ASH, Tepa)	1998	*T. cacao*	PM1
53	ASH-AW 37	Ghana (ASH, Wansanbire)	1998	*T. cacao*	PM1
54	ASH-W 21	Ghana (ASH, Wansanbire)	1998	*T. cacao*	PM1
55	ASH-W24	Ghana (ASH, Wansanbire)	1998	*T. cacao*	PM1
56	BBA 13	Ghana (B/A, Bechem)	1998	*T. cacao*	PM1

Table 1 *Continued*

Study no.	Collection/ other number	Country (region/location)	Year of isolation	Host	RFLP group
57	CSD-BEC-1/IMI 386199	Ghana (B/A, Bechem)	1997	*T. cacao*	PM1
58	YKK-BEC/IMI 386200	Ghana (B/A, Bechem)	1997	*T. cacao*	PM1
59	KA-NNON/IMI 386201	Ghana (B/A, Nnonkokrom)	1997	*T. cacao*	PM1
60	NYS-NNON/IMI 386202	Ghana (B/A, Nnonkokrom)	1997	*T. cacao*	PM1
61	GHVR 37	Ghana (V/R)	1994	*T. cacao*	PM1
62	MAG-AVA/IMI 386204	Ghana (V/R, Avatime)	1997	*T. cacao*	PM1
63	ADZA-KLA/IMI 386205	Ghana (V/R, Klave)	1997	*T. cacao*	PM1
64	VR 10/IMI 386206	Ghana (VR, Kpeve)	1997	*T. cacao*	PM1
65	VR 11	Ghana (VR, Kpeve)	1997	*T. cacao*	PM1
66	AGM-ESS/IMI 386208	Ghana (W/R, Essam)	1997	*T. cacao*	PM1
67	GHWR 12	Ghana (W/R, Essam)	1998	*T. cacao*	PM1
68	MAN-ESS/IMI 386209	Ghana (W/R, Essam)	1997	*T. cacao*	PM1
69	KBM-JBO/IMI 386210	Ghana (W/R, Juabeso)	1997	*T. cacao*	PM1
70	NGR 16	Nigeria (Ondo)	1995	*T. cacao*	PM1
71	TG 3	Togo (Kloto, Kouma)	1991	*T. cacao*	PM1
72	TG 6	Togo (Kloto, Kouma)	1991	*T. cacao*	PM1
P. nicotianae					
73	IMI 359462	Malaysia (Ranau)	1988	*Bougainvillea* sp.	PN
P. palmivora					
74	IMI 325923	Costa Rica	1988	*T. cacao*	PP1
75	CR 196	Costa Rica (La Lola)	1988	*T. cacao*	PP1
76	CR 197	Costa Rica (Turrialba)	1988	*Eucalyptus* sp.	PP1
77	MMP1	Ghana	1999	*Persea americana*	PP1
78	AKC	Ghana (ASH, Akomadan)	1998	*T. cacao*	PP1
79	IMI 324466	Ghana (ASH, Akomadan)	1988	*T. cacao*	PP1
80	JA-NYI/IMI 386223	Ghana (ASH, Nyinahin)	1997	*T. cacao*	PP1
81	FF-OFF/IMI 386224	Ghana (ASH, Offinso)	1997	*T. cacao*	PP1
82	JANASH 02/IMI 386225	Ghana (ASH, Offinso)	1997	*T. cacao*	PP1
83	NBA-OFF/IMI 386226	Ghana (ASH, Offinso)	1997	*T. cacao*	PP1
84	OWOFF6/IMI 386227	Ghana (ASH, Offinso)	1997	*T. cacao*	PP1
85	CSD-BEC-2/IMI 386228	Ghana (B/A, Bechem)	1997	*T. cacao*	PP1
86	KM-GSO/IMI 386229	Ghana (B/A, Goaso)	1997	*T. cacao*	PP1
87	BA 31/IMI 386230	Ghana (B/A, Mim)	1997	*T. cacao*	PP1
88	KER 01/IMI 386231	Ghana (E/R, Kukurantumi)	1998	*T. cacao*	PP1
89	OPRI 29/IMI 386232	Ghana (E/R, Kusi)	1998	*T. cacao*	PP1
90	OPRI 33/IMI 386233	Ghana (E/R, Kusi)	1998	*T. cacao*	PP1
91	OPRI 35/IMI 386234	Ghana (E/R, Kusi)	1998	*T. cacao*	PP1
92	GHER 1/IMI 386235	Ghana (E/R, Tafo)	1997	*T. cacao*	PP1
93	PPSR 2/IMI 386236	Ghana (E/R, Tafo)	1997	*T. cacao*	PP1
94	TP 1/IMI 386258	Ghana (E/R, Tafo)	1997	*T. cacao*	PP1
95	TP 2/IMI 386259	Ghana (E/R, Tafo)	1997	*T. cacao*	PP1
96	TP 3/IMI 386260	Ghana (E/R, Tafo)	1997	*T. cacao*	PP1
97	TP 4/IMI 386261	Ghana (E/R, Tafo)	1997	*T. cacao*	PP1
98	TP 5/IMI 386262	Ghana (E/R, Tafo)	1997	*T. cacao*	PP1
99	VR 3	Ghana (VR, Kpedze)	1998	*T. cacao*	PP1
100	VR 13	Ghana (VR, Kpeve)	1998	*T. cacao*	PP1
101	GHWR 7/IMI 386265	Ghana (W/R, Adukromyede)	1998	*T. cacao*	PP1
102	GHWR 34/IMI 386266	Ghana (W/R, Akontombra)	1998	*T. cacao*	PP1
103	GHWR 85/IMI 386267	Ghana (W/R, Akontombra)	1998	*T. cacao*	PP1
104	W46/IMI 386268	Ghana (W/R, Asankragwa)	1998	*T. cacao*	PP1
105	GHWR 38/IMI 386269	Ghana (W/R, Asawinso)	1998	*T. cacao*	PP1
106	GHWR 89/IMI 386270	Ghana (W/R, Asawinso)	1998	*T. cacao*	PP1
107	GHWR 48/IMI 386271	Ghana (W/R, Ayinabirim)	1998	*T. cacao*	PP1
108	GHWR 77/IMI 386272	Ghana (W/R, Buako)	1998	*T. cacao*	PP1
109	W10/IMI 386291	Ghana (W/R, Buako)	1998	*T. cacao*	PP1
110	W11/IMI 386292	Ghana (W/R, Buako)	1998	*T. cacao*	PP1
111	GHWR 3/IMI 386274	Ghana (W/R, Essam)	1998	*T. cacao*	PP1
112	GHWR 31/IMI 386289	Ghana (W/R, Juabeso)	1998	*T. cacao*	PP1
113	GHWR 58/IMI 386290	Ghana (W/R, Juabeso)	1998	*T. cacao*	PP1

Table 1 *Continued*

Study no.	Collection/ other number	Country (region/location)	Year of isolation	Host	RFLP group
114	W52/IMI 386293	Ghana (W/R, Juabeso)	1998	*T. cacao*	PP1
115	GHWR 33/IMI 386276	Ghana (W/R, Juabeso)	1998	*T. cacao*	PP1
116	W63/IMI 386275	Ghana (W/R, Juabeso)	1998	*T. cacao*	PP1
117	GHWR 36/IMI 386277	Ghana (W/R, Kukumso Dade)	1998	*T. cacao*	PP1
118	W64/IMI 386294	Ghana (W/R, Kukumso)	1998	*T. cacao*	PP1
119	GHWR 19/IMI 386282	Ghana (W/R, Nyamebekyere)	1998	*T. cacao*	PP1
120	GHWR 32/IMI 386283	Ghana (W/R, S/Bekwai)	1998	*T. cacao*	PP1
121	GHWR 71/IMI 386284	Ghana (W/R, S/Bekwai)	1998	*T. cacao*	PP1
122	GHWR 76/IMI 386285	Ghana (W/R, S/Bekwai)	1998	*T. cacao*	PP1
123	GHWR 90/IMI 386286	Ghana (W/R, S/Bekwai)	1998	*T. cacao*	PP1
124	W1/IMI 386287	Ghana (W/R, S/Bekwai)	1998	*T. cacao*	PP1
125	W40/IMI 386288	Ghana (W/R, W/Akropong)	1998	*T. cacao*	PP1
126	IMI 325922	Hawaii	1988	*Carica papaya*	PP1
127	92 P14	Indonesia	1992	*T. cacao*	PP1
128	94 P42/IMI 386297	Indonesia (East Java)	1994	*T. cacao*	PP1
129	94 P43/IMI 386298	Indonesia (East Java)	1994	*T. cacao*	PP1
130	94 P44/IMI 386299	Indonesia (East Java)	1994	*T. cacao*	PP1
131	94 P54/IMI 386300	Indonesia (East Java)	1994	*T. cacao*	PP1
132	94 P55/IMI 386301	Indonesia (East Java)	1994	*T. cacao*	PP1
133	94 P56/IMI 386302	Indonesia (East Java)	1994	*T. cacao*	PP1
134	94 P57	Indonesia (East Java)	1994	*T. cacao*	PP1
135	94 P05/IMI 386304	Indonesia (Jambi)	1994	*T. cacao*	PP1
136	94 P15/IMI 386305	Indonesia (Jambi)	1994	*T. cacao*	PP1
137	94 P18	Indonesia (Jambi)	1994	*T. cacao*	PP1
138	90P26/IMI 386307	Indonesia (North Sulawesi)	1990	*T. cacao*	PP1
139	94 P37/IMI 386308	Indonesia (North Sulawesi)	1994	*T. cacao*	PP1
140	IMI 359456	Malaysia (Tawau)	1988	*T. cacao*	PP1
141	IMI 359457	Malaysia (Tawau)	1988	*T. cacao*	PP1
142	IMI 182591	Nigeria	1974	*T. cacao*	PP1
143	IMI 344485	Papua New Guinea (Keravat)	1991	*T. cacao*, bark	PP1
144	BNP/IMI 386309	Taiwan	1996	*Areca catechu*	PP1
145	PPaC55	Taiwan	1988	*Citrus* sp.	PP2
146	PPaC59/IMI 386311	Taiwan	1989	*Citrus* sp.	PP1
147	PPaCa3	Taiwan	1992	*Cattleya* sp.	PP1
148	PPaCa5/IMI 386313	Taiwan	1993	*Cattleya* sp.	PP1
149	PPaCu2	Taiwan	1995	*Hedychum* sp.	PP1
150	PPaCy3/IMI 386315	Taiwan	1992	*Cymbidium oiwakensis*	PP1
151	PPaDn1	Taiwan	1990	*Dendrobium* sp.	PP1
152	PPaE1/IMI 386317	Taiwan	1990	*Hedera helix*	PP1
153	PPaM2	Taiwan	1997	*Mangifera indica*	PP1
154	PPaO9	Taiwan	1996	*Oncidium* sp.	PP1
155	PPaP33/IMI 386321	Taiwan	1989	*Carica papaya*, root	PP1
156	PPaP72/IMI 386322	Taiwan	1997	*Carica papaya*, fruit	PP1
157	PPaPa2/IMI 386323	Taiwan	1995	*Raphiopedillum* sp.	PP1
158	PPaWa2	Taiwan	1990	*Syzyguim samaragenes*	PP1
159	TRIN 02/IMI 386325	Tobago (Les Coteaux)	1988	*T. cacao*	PP1
160	6604	Togo (Kloto, Palime)	1987	*T. cacao*	PP1
161	TRIN 01IMI 386327	Trinidad (St Augustine)	1988	*T. cacao*	PP1

[a]Isolate previously identified as *P. katsurae* but found to be *P. capsici*.
[b]Isolates received from source as *P. palmivora* but found to be otherwise.

eluted in 100 μL nuclease-free water, and the final DNA concentration was determined by UV spectrophotometry from a 500 μL (1 : 100 dilution) volume of each sample.

The DNA samples were sequenced on a LI-COR Automated DNA Sequencer 4200 IR2 (LI-COR Inc., Lincoln, NE, USA) using LI-COR SequiTherm EXCEL II DNA Sequencing Kits-LC and a single-labelled primer protocol. The cycle sequencing protocol employed a 17 μL premix of 7·2 μL 3·5× SequiTherm EXCEL II sequencing buffer, 1 μL each of IRD700 or IRD800 labelled M13 universal and M13 reverse primers, 1 μL SequiTherm EXCEL II DNA Polymerase (5 U μL^{-1}), 250 fmoles of template DNA and 5·8 μL deionized water. Two microlitres each of SequiTherm EXCEL II-LC Termination mix A, C, G or T and 4 μL of

premix were added to each reaction tube. Cycling conditions were 95°C for 5 min, followed by 30 cycles of 95°C at 30 s, 50°C at 15 s and 70°C at 1 min. On completion of PCR 3 μL of stop/loading buffer was added to each reaction.

One microlitre of each reaction was loaded onto a precast sequencing gel composed of 7·5 mL Rapid Gel XL Solution 40% (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, UK), 21 g urea, 5 mL 10× TBE Long Run buffer, 28 mL distilled water, 500 μL DMSO, 350 μL 10% fresh ammonium persulphate and 75 μL TEMED. The electrophoresis conditions were 1500 V, 35 mA and 40 W at 50°C. Gels were prerun for 15–30 min before loading samples. Sequence data were collected using the BASEIMAGE IR[2] software (LI-COR).

Sequence analysis

SEQUENCHER 2·0 software (Gene Codes Corporation, Michigan, WI, USA) was used to remove vector contamination, correct miscalled bases, and generate contigs from the forward and reverse sequences of individual clones. The contigs from clones belonging to the same species were aligned to generate consensus sequences representing the various species. A BLAST search (Altschul *et al*., 1997) was performed to compare the consensus sequences generated with those deposited in GenBank (www.ncbi.nlm.nih.gov). The consensus sequences for each species were aligned with each other through CLUSTALW (www.ebi.ac.uk/clustalw/) to determine variable regions or base sequences unique to each species or isolate. Puzzle coefficients (transition/transversion ratio and coefficient of variation) were calculated using TREE PUZZLE 5·0. Distance matrices between all pairs of sequence from the multiple alignments were calculated and trees were generated by neighbour joining (NJ). Bootstrap analyses of 500 interactions were performed by the Consense routine in the phylogenetic inference package (PHYLIP) (www.hgmp.mrc.ac.uk). The dendrogram was viewed by importing the calculated values into TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

Results

ITS-PCR amplification and restriction analysis

Generally, PCR amplification of the ITS region of the *Phytophthora* isolates gave products of approximately 900 base pairs in length, with small variations in a few isolates. Four of the 11 restriction enzymes tested (*Hae*III, *Hin*fI, *Pvu*II and *Alu*I) gave fragment patterns that distinguished between *P. megakarya*, *P. palmivora* and *P. capsici*, and these four enzymes were used to digest all PCR products. The diagnostic restriction patterns produced with the four restriction enzymes for the three major species (*P. palmivora*, *P. megakarya* and *P. capsici*) are shown in Fig. 1, and full details are given in Table 2.

Sixty isolates identified as *P. megakarya* gave consistent patterns, and two (21 and 51) differed in their *Hin*fI and *Alu*I patterns, respectively (Table 2). Eighty-eight isolates identified as *P. palmivora* gave consistent patterns, and one isolate received as *P. palmivora* (145) produced different fragment patterns with *Hae*III and *Hin*fI, but the same pattern as the other isolates belonging to the species with *Pvu*II and *Alu*I restriction enzymes (Table 2).

Within *P. capsici*, ITS-PCR product size differences of approximately 20–40 bp were observed from isolates from the same, as well as different, geographic origins. Eight isolates were considered to belong to *P. capsici*. These were the six isolates received as *P. capsici*, one isolate from cocoa received as *P. katsurae*, and one isolate from *Hedera helix* received as *P. palmivora* (Appiah *et al*., 2003). These isolates produced one of three different sets of patterns with the four restriction enzymes (Tables 1 and 2). In eight of the 12 individual patterns, the sum of the restriction fragments was greater than the original size of the PCR products. For instance, *Alu*I produced three RFLP groups of *P. capsici* with those designated PCAP2 and PCAP3 isolates (from India and Taiwan, and Mexico and Nigeria, respectively) having fragments with a greater total than the approximate 900 bp PCR product (Table 2), and a similar situation was seen with *P. megakarya* 51, and with *Hin*fI for *P. megakarya* isolate 21. PCAP1 isolates from Brazil/Côte d'Ivoire differed from the others in producing an additional *Hin*fI fragment of 170 bp.

DNA sequencing and phylogenetic analyses

The sequences of the entire ITSI/5·8s/ITSII regions, together with short termini from the large and small subunit genes, were obtained from the 18 isolates selected (Table 3). The BLAST searches confirmed identification of the sequences from *P. megakarya*, *P. palmivora* and *P. capsici* isolates. The two isolates received as single representatives of *P. citrophthora* and *P. nicotiana* were

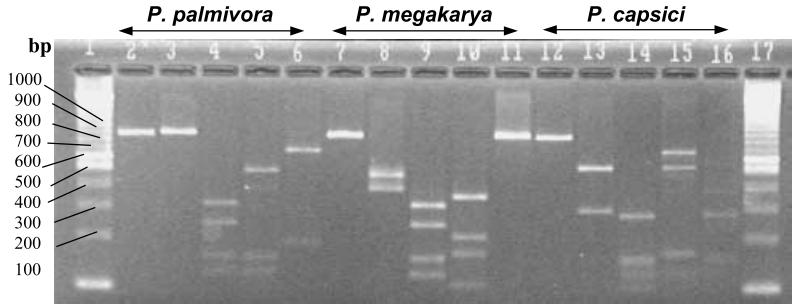

Figure 1 Diagnostic restriction patterns of ITS-PCR products for the three main *Phytophthora* species on cocoa. Lanes 2–6: *P. palmivora* undigested PCR product; PCR product digested with *Hae*III, *Hin*fI, *Alu*I and *Pvu*II; lanes 7–11: *P. megakarya*, samples as *P. palmivora*; lanes 12–16: *P. capsici* samples as *P. palmivora*; lanes 1 and 17: 100 bp molecular markers.

Table 2 Restriction profiles of RFLP groups within *Phytophthora* species on cocoa

RFLP group[a]	No. of isolates	Approximate ITS-PCR product size (bp)	ITS-RFLP sizes (bp) obtained with selected restriction endonuclease enzymes			
			*Hae*III	*Hinf*I	*Pvu*II	*Alu*I
PCAP1	3	≈900	570, 330	290, 170, 150, 130, 110	900	720, 180
PCAP2	3	≈860	550, 310	290, 150, 130, 110	860	720, 550, 180
PCAP3	2	≈900	550, 310	290, 150, 130, 110	900	700, 550, 180, 120
PCIT	1	≈900	490, 330, 80	320, 200, 130, 110	900	550, 190, 160
PM1	60	≈900	490, 410	340, 260, 170, 130	900	370, 220, 180, 120
PM isolate 21 (IMI 337098)	1	≈900	490, 410	340, 290, 260, 170, 130	900	370, 220, 180, 120
PM isolate 51 (ASH-DA39)	1	≈900	490, 410	340, 260, 170, 130	900	370, 350, 220, 180, 120
PN	1	≈900	750, 400, 150	340, 260, 170, 130	900	720, 220, 180, 120
PP1	88	≈900	900	340, 260, 170, 130	700, 200	520, 170, 150
PP isolate 145 (PpaC55)	1	≈900	320, 250, 180, 120	900	700, 200	520, 170, 150

[a]PCAP, *P. capsici*; PCIT, *P. citrophthora*; PM, *P. megakarya*; PN, *P. nicotiana*; PP, *P. palmivora.*

Table 3 Sizes of rDNA ITS regions and flanking sequences in five species of *Phytophthora* from cocoa

Isolate	Study number	GenBank Accession number	Origin	Sections of rDNA sequenced and their sizes (bp)					
				18S-SSU	ITS I	5·8S	ITS II	28.S-LSU	Total
P. capsici									
IMI 325900	2	AF467083	Brazil	11	173	153	426	39	802
IMI 304412	3	AF467084	Côte d'Ivoire	11	173	153	428	39	804
IND 44	4	AF467085	India	11	173	153	427	39	803
P. citrophthora									
93P61	9	AF467086	Indonesia	11	195	153	435	39	833
P. nicotianae									
IMI 325462	73	AF467087	Malaysia	11	229	153	422	39	854
P. palmivora									
IMI 325923	74	AF467088	Costa Rica	11	215	154	432	40	852
94P43	129	AF467089	Indonesia	11	213	153	420	39	836
GHWR 48	107	AF467090	Ghana	11	214	153	421	39	838
VR 13	100	AF467091	Ghana	11	220	156	423	40	850
TP 1	94	AF467092	Ghana	11	215	157	419	39	841
PPaP33	155	AF467093	Taiwan	11	213	153	420	39	836
PPaC55	145	AF467094	Taiwan	11	214	153	420	39	837
P. megakarya									
ASH-A25	28	AF467095	Ghana	11	213	153	417	39	833
ASH-DA39	51	AF467096	Ghana	11	213	153	417	39	833
MAG-AVA	62	AF467097	Ghana	11	213	153	421	39	837
NS 264	12	AF467098	Cameroon	11	213	153	418	39	834
CAM 184	16	AF467099	Cameroon	11	214	153	417	39	834
TG 3	71	AF467100	Togo	11	213	154	417	39	834

also correctly identified by BLAST searches, whereas the isolate received as *P. katsurae* matched to *P. capsici*, in agreement with the RFLP results. All sequences were deposited in GenBank with accession numbers AF467083 to AF4670100 (Table 3). The average sizes of the ITS I/5·8s/ITS II regions were 753 bp for *P. capsici*, 783 bp for *P. citrophthora*, 804 bp for *P. nicotianae*, 791 bp for *P. palmivora* and 784 bp for *P. megakarya*. The termini of the 18S and 28S subunits were highly conserved within all the *Phytophthora* species, and the little sequence variation within the 5·8S subunit was limited to point mutations and a few indels (insertions/deletions; 1–2 bases), predominantly among the *P. palmivora* isolates. There was significant variation in terms of deletions, insertions and substitutions between species within the ITS regions, especially within ITS I, although several highly conserved

Table 4 Conserved regions within the ITS I and II regions of *Phytophthora* species

Position[a]	Length[b]	Remarks
ITS I region		
12–24	13	Single (C) substitution in *P. nicotianae*
26–47	22	Single (T) substitution in *P. nicotianae*
73–77	5	No species variation present in this segment
80–84	5	No species variation present in this segment
87–92	6	No species variation present in this segment
94–103	10	No species variation present in this segment
108–112	5	No species variation present in this segment
120–132	13	No species variation, a single (C) insertion in one *P. palmivora* isolate
140–185	46	Single (A) substitution in *P. nicotianae*; single substitution and insertions in some *P. palmivora* isolates
ITS II region		
339–355	17	Single (T) substitution in *P. nicotianae*
358–364	7	Single (T) substitution in one *P. palmivora* isolates
366–386	21	Single substitutions in *P. citrophthora* (C) and *P. palmivora* (T)
389–411	23	Single substitution in *P. nicotianae* (T) and *P. megakarya* (G)
419–424	6	No species variation present in this segment
434–442	9	Single (C) substitution in *P. megakarya*
447–478	32	Single substitutions in *P. nicotianae* (2As) and in *P. palmivora* (C)
494–506	13	Single (A) substitution in *P. nicotianae*
521–526	6	No species variation present in this segment
529–545	17	Single (T) substitution in *P. palmivora* and *P. megakarya*
549–595	47	Single substitutions in *P. nicotianae* (2As and T); in *P. megakarya* (A) and in some *P. palmivora* isolates
599–604	6	Single (C) substitution in *P. megakarya*
614–620	7	Single (A) substitution in *P. palmivora*
635–643	9	Single (A) substitution in *P. megakarya*
657–671	15	No species variation present in this segment
679–683	5	No species variation present in this segment
695–714	20	Single substitutions (T) in *P. citrophthora*
717–728	12	No species variation present in this segment
755–763	9	No species variation present in this segment

[a]Nucleotide positions were determined using *P. capsici* isolate IMI 325900 sequence as reference (Fig. 4).
[b]Number of nucleotides in the conserved region counted along the reference sequence.

regions were present in both ITS I and ITS II sequences. The conserved regions varied considerably in length, ranging from 3 to 73 nucleotides (Table 4). Generally the 5′- and 3′-termini of both ITS I and ITS II sequences were highly conserved in all the *Phytophthora* species, with variation mostly limited to a few (one to three) base changes.

The neighbour-joining tree grouped the species on three distinct branches (Fig. 2). *Phytophthora palmivora* isolates formed a single group, with the *P. megakarya* isolates forming a sister group. These groups were separated by approximately five base changes per 100 bases. The three isolates of *P. capsici* together with the single *P. citrophthora* isolate formed a distinct group, and this was placed separately from the *P. palmivora*/*P. megakarya* grouping.

All ITS sequences for *P. capsici* available in the EMBL database were downloaded, together with sequences for the recently described *P. tropicalis* (Gerlach & Schubert, 2001). These were aligned with the sequences for *P. capsici* obtained here. Due to differences in sequence lengths two different alignments were undertaken, one for ITS I sequences only (which included most sequences), and one for the full ITS I/5·8/ITS II region (results not shown). In both of these alignments the cocoa isolates formed a group with *P. tropicalis*. The ITS I alignment included an additional sequence from *P. capsici* from cocoa used in the study of Förster *et al*. (2000), and this sequence also grouped closer to *P. tropicalis*.

Discussion

This study has shown that RFLP analysis of the ITS region can be used effectively to rapidly distinguish between the four main species of *Phytophthora* involved in black pod disease of cocoa.

The *Alu*I restriction fragments obtained with isolates of *P. capsici* and *P. megakarya* 51, and the fragments obtained with *Hin*fI for *P. megakarya* 21, gave total sizes that were in excess of the apparent size of the original PCR products. This could be attributed to a number of causes, including experimental errors such as contamination, or low enzyme activity due to unoptimized conditions. However, low enzyme activity is unlikely in these cases, as all the isolates were subjected to the same treatment and the complex patterns were largely restricted to the

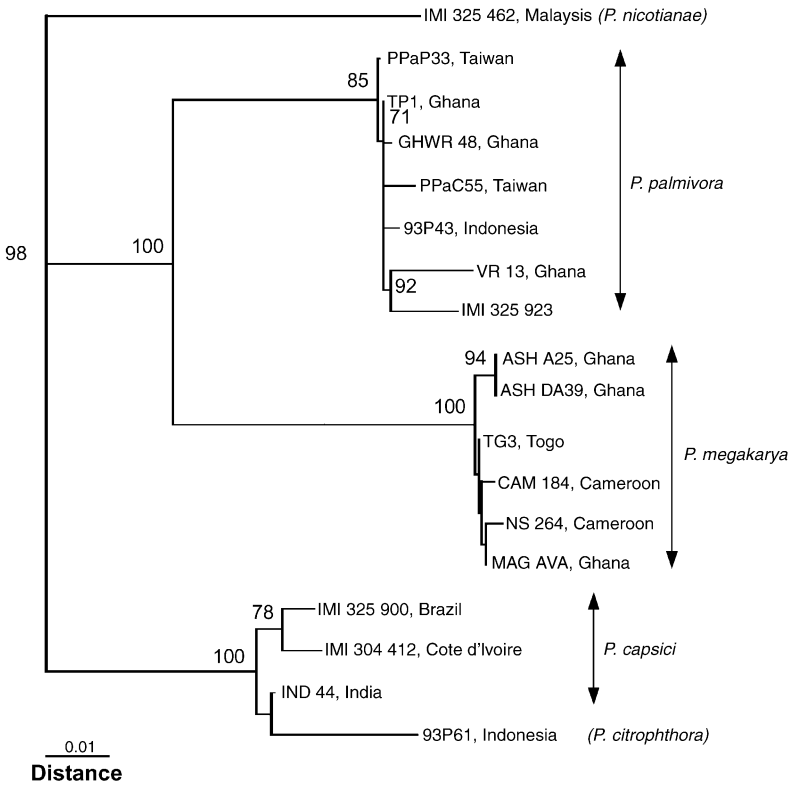

Figure 2 Relationships of *Phytophthora* species on cocoa based on neighbour-joining analysis of rDNA-ITS region sequences. Numbers adjacent to branch points are bootstrap values from 500 interactions.

eight *P. capsici* isolates. Contamination is also unlikely, as repeated digestions with freshly extracted DNA samples produced the same results and similar complex patterns were not seen with other enzymes. Similar restriction profiles after digestion with *Alu*I have been observed independently for *P. capsici* (D. Brayford, CABI, personal communication). A possible explanation for the anomalous patterns is the presence of multiple forms of the rDNA-ITS gene cluster in single isolates. This is not uncommon in fungi, and has been reported in several species including *Beauvaria brongniartii*, the arbuscular mycorrhizal Glomales, *Sclerotium rolfsii* and *Ascochyta* spp. (Neuvéglise *et al*., 1994; Harlton *et al*., 1995; Sanders *et al*., 1995; Fatehi & Bridge, 1998). Harlton *et al*. (1995) and Fatehi & Bridge (1998) suggested that the different ITS regions might be associated with different nuclei, as the genera they examined were multinucleate. The genus *Phytophthora* is diploid and multinucleate, and so this may provide an explanation. In many such reported cases the addition of the restriction fragments gave a total that was a multiple of the single PCR product, although this was not the case for the isolates reported here. In some instances the sum of the restriction fragments was less than the size of the complete PCR product. This is due to the production of small fragments that are not visible on the gels, and also to the production of more than one restriction fragment of the same size, that appear as a single band. Both these events are present in these data, and an example is *P. capsici* RFLP group 1, where theoretical digestion with *Hin*fI based on the sequence data gives two fragments of approximately 130 bp (130 and 133 bp) and one fragment of 8 bp. This illustrates that simple band patterns cannot be considered to be completely representative of sequences, and that RFLP data should be supported by sequence data before detailed interpretations are made.

The ITS analysis indicated the presence of subgroups within the *P. capsici* clade, determined by the presence or absence of the additional fragments seen in the *Alu*I ITS digestions. Although the exact nature of the additional fragments found within the ITS region of the *P. capsici* isolates has not been determined, several earlier reports have suggested *P. capsici* to be highly diverse. These include the separation of *P. capsici* into two or three forms based on isoenzyme criteria (Oudemans & Coffey, 1991; Mchau & Coffey, 1994); a high degree of mtDNA diversity (Förster *et al*., 1989); and, more recently, the detection of two groups based on ITS I sequence analysis (Förster *et al*., 2000).

Recently, Aragaki & Uchida (2001) used morphological distinctions to describe a new species, *P. tropicalis*, that included isolates previously assigned to '*P. palmivora* MF4' on cocoa. BLAST searching of the *P. capsici* sequences from isolates from cocoa obtained here, against other *Phytophthora* sequences held at EMBL, showed that the consensus *P. capsici* sequence was a closer match to two sequences described as from *P. tropicalis* (from *Cyclamen*) than to sequences labelled as *P. capsici* (from *Capsicum*). However, the differences between the most similar sequences were very marginal (98·73 compared with 98·38% similarity), so the correct designation of these isolates remains unclear. The only differences between the consensus ITS1 sequence obtained here and that obtained

by Förster *et al*. (2000) from an isolate from cocoa (AF242788) are two unresolved bases. This sequence, in turn, differs by only two bases from the sequence obtained from *P. capsici* from *Spondias purpurea* (AF242785) by Förster *et al*. in the same study. The alignment of the complete ITS I/5·8/ITS II regions also grouped the cocoa isolates obtained here with *P. tropicalis*; unfortunately only the ITS I sequences are available from the isolates reported from Förster *et al*., so comparisons of the entire ITS I/5·8S/ITS II region cannot be used to provide a more complete comparison. On the basis of the high similarity values it is possible that *P. tropicalis* is synonymous with *P. capsici*, but further complete sequences will be required to confirm this.

The high degree of similarity in the ITS I region suggests that the different species are closely related. For instance, the ITS I regions from *P. capsici* and *P. citrophthora* differed by only 21 bases. This size difference was the result of a single base indel and a single continuous indel of 20 bases in *P. capsici* near the beginning of the ITS I region (Fig. 2). This size difference may have arisen from a single or several mutational events which resulted in a deletion of nucleotides in *P. capsici* or an insertion in *P. citrophthora*. Deletion is the most likely explanation, as the 20 bases absent in *P. capsici*, plus another 12 continuous bases, are present at the comparable point in the other species sequenced here (*P. nicotianae*, *P. palmivora* and *P. megakarya*), and in other species where sequence data have been deposited (e.g. Cooke *et al*., 2000). Alternative hypotheses include an insertion event, as found in the other species, or an introgression, which occurred through hybridization (Goodwin, 1997). A close evolutionary relationship is also apparent between *P. palmivora* and *P. megakarya*, where the length of the ITS I regions differ by only two nucleotides, and the base sequence differences were limited to one to three base substitutions. The sequence analysis showed that the ITS I region was more variable than the ITS II region between species, and this may indicate different evolutionary rates for the two regions.

RFLPs of PCR products can clearly be used to identify species of *Phytophthora* on cocoa, and although gel visualization of the fragments may not reflect all the sequence information, very minor sequence variations can be identified within species. The use of a PCR-ITS-RFLP approach will help in the monitoring of infections and the spread of the different *Phytophthora* species causing black pod diseases.

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