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Phylogenetic relationship of *Phytophthora cryptogea* Pethybr. & Laff and *P. drechsleri* Tucker

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ABSTRACT

The phylogeny and taxonomy of *Phytophthora cryptogea* and *Phytophthora drechsleri* has long been a matter of controversy. To re-evaluate this, a worldwide collection of 117 isolates assigned to either *P. cryptogea*, *P. drechsleri* or their sister taxon, *Phytophthora erythroseptica* were assessed for morphological, physiological (pathological, cultural, temperature relations, mating) and molecular traits. Multiple gene phylogenetic analysis was performed on DNA sequences of nuclear (internal transcribed spacers (ITS), ß-tubulin, translation elongation factor 1α, elicitin) and mitochondrial (cytochrome c oxidase subunit I) genes. Congruence was observed between the different phylogenetic data sets and established that *P. drechsleri* and *P. cryptogea* are distinct species. Isolates of *P. drechsleri* form a monophyletic grouping with low levels of intraspecific diversity whereas *P. cryptogea* is more variable. Three distinct phylogenetic groups were noted within *P. cryptogea* with an intermediate group providing strong evidence for introgression of previously isolated lineages. This evidence suggests that *P. cryptogea* is an operational taxonomic unit and should remain a single species. Of all the morphological and physiological traits only growth rate at higher temperatures reliably discriminated isolates of *P. drechsleri* and *P. cryptogea*. As a homothallic taxon, *P. erythroseptica*, considered the cause of potato pink rot, is clearly different in mating behaviour from the other two species. Pathogenicity, however, was not a reliable characteristic as all isolates of the three species formed pink rot in potato tubers. The phylogenetic evidence suggests *P. erythroseptica* has evolved from *P. cryptogea* more recently than the split from the most recent common ancestor of all three species. However, more data and more isolates of authentic *P. erythroseptica* are needed to fully evaluate the taxonomic position of this species.

Introduction

The discrimination of *Phytophthora cryptogea* Pethybridge & Lafferty (1919) and *Phytophthora drechsleri* Tucker (1931) is an ongoing controversial issue in *Phytophthora* taxonomy (Erwin *et al.* 1983; Mills *et al.* 1991; Erwin & Riberio 1996; Cooke *et al.* 2000). In Tucker's original comparison of *P. cryptogea*, *P. drechsleri* and *Phytophthora erythroseptica* Pethybridge (1913), he attested to all three species being alike, but since isolates of *P. drechsleri* were able to grow well at 35 °C, he indicated that

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they could be separated using temperature relations (Tucker 1931). Although temperature was originally used as a criterion to separate *P. cryptogea* from *P. drechsleri*, Waterhouse (1963) used maximal sporangial length as the primary distinguishing feature in her key with maximum growth temperature being of secondary importance. She added that *P. drechsleri* could also be distinguished by its narrower hyphal diameter, larger oospores, more elongated sporangia (larger, with a tapered base) and occasional homothallic behaviour. These additional distinctions further complicated identification procedures. Moreover, the high-temperature criterion did not always correlate with the other identifying features (Klisiewicz & Beard 1976; Banihashemi & Ghaisi 1993) and as a result, some isolates were described as intermediate between both species (Flowers *et al.* 1973; Shepherd & Pratt 1973; Klisiewicz 1977; Stanghellini & Kronland 1982). This led some investigators to cast doubt upon the validity of temperature response as the main distinguishing feature (Shepherd & Pratt 1973; Klisiewicz 1977), while others simply rejected *P. drechsleri* as an acceptable species (Bumbieris 1974; Gerrettson-Cornell 1979). Some maintained that *P. drechsleri* should be kept as an acceptable species until more conclusive data were obtained (Kannaiyan *et al.* 1980; Kröber 1981). The high degree of morphological and physiological variability encountered did not allow Ho & Jong (1986) to discriminate the two species in their study. They did, however, consider the possibility of *P. drechsleri* being a variant of *P. cryptogea* that accumulated minor changes in morphological traits alongside its adaptation to higher temperatures and infection of hosts of warmer areas.

Mills *et al.* (1991) combined results of isozyme and mtDNA analysis to identify at least seven distinct molecular subgroups represented by the 123 isolates described as *P. cryptogea* and *P. drechsleri* in their study. They highlighted the fact that a wide range of genetically different isolates had been described as *P. cryptogea* or *P. drechsleri* over the years, described the groups and estimated relatedness but did not consider taxonomic revision.

With the benefit of molecular sequence data we can now see that several species are morphologically similar to *P. drechsleri* and *P. cryptogea* which has lead to them being used as 'catch all' names for superficially similar taxa that grow or fail to grow at or above 35 °C, respectively. For example, Pal *et al.* (1970) initially reported *P. drechsleri* var. *cajani* as the cause of the stem rot disease of pigeon pea (*Cajanus cajan* (L.) Millsp.) but this was later described a *Phytophthora cajani* (Amin *et al.* 1978). Kannaiyan *et al.* (1980) then re-examined several isolates and renamed it *P. drechsleri* f. sp. *cajani* on the basis of morphological similarity to *P. drechsleri*. Isozyme and mtDNA RFLP analysis however identified these isolates as a group (G) distinct from the typical *P. drechsleri* isolates in group 'A' (Mills *et al.* 1991). This result was supported by phylogenetic analysis of ITS rDNA sequences that showed *P. cajani* as a distinct and distantly related species to *P. drechsleri* (Cooke *et al.* 2000). Similarly, isolates from various hosts in North America, designated by Mills *et al.* (1991) as *P. cryptogea*/*P. drechsleri* group J and K have been reclassified into *Phytophthora gonapodyides* (Brasier *et al.* 1993) and *Phytophthora* taxon Pgchlamydo (Brasier *et al.* 2003), respectively.

P. erythroseptica was first described in Ireland by Pethybridge (1913) as the causal agent of pink rot of potato tubers. Tucker (1931) in his very first comparison of *P. cryptogea*, *P. drechsleri* and *P. erythroseptica*, based on a single isolate of each species, indicated that all three taxa were morphologically similar; discriminated only on the basis of temperature relations and oospore diameter. *P. erythroseptica* was discriminated on the basis of yellowish appearance of oogonia, homothallism, larger oospores, and an inability to grow at 35 °C. Isozyme analysis of *P. erythroseptica* revealed that it is a uniform and distinct taxon termed group 'Per' (Mills *et al.* 1991). On the basis of the internal transcribed spacer regions (ITS) sequences of genomic rDNA, Cooke *et al.* (2000) showed that this species was consistently differentiated as a distinct taxon more closely related to *P. cryptogea* than to *P. drechsleri*. In contrast, a phylogenetic analysis based on only the ITS1 region of rDNA questioned the validity of retaining *P. erythroseptica* as separate taxonomic entity (Förster *et al.* 2000). More recently, Mirabolfathy *et al.* (2001) studied two non-papillate species of *Phytophthora* as the causal agents of pistachio gummosis in Iran. Their previous descriptions as *P. drechsleri* and *Phytophthora megasperma* was re-examined by RFLPs and sequence comparison of ITS regions of rDNA. The isolates from pistachio described as *P. drechsleri* had ITS sequences identical to *Phytophthora melonis*, *Phytophthora sinensis*, and isolates described as *P. drechsleri* from cucurbits in Iran (five isolates). They concluded that these taxa should be considered conspecific and all subsumed within *P. melonis*.

Overall, the literature is littered with such conflicts in taxonomy and phylogenetic position of these taxa and a comprehensive investigation by means of new taxonomic tools, of isolates from different parts of the world and various hosts, is clearly necessary. The objectives of this study were therefore to re-assess the status of these taxa using molecular methods. A preliminary ITS-based screen of as many isolates described as *P. drechsleri*, *P. cryptogea*, and *P. erythroseptica* as possible was followed by more detailed phylogenetic analysis based on a range of nuclear and mitochondrial genes. Finally, such data were interpreted in light of a re-examination of their mating systems and morphological and physiological characteristics.

Material and methods

Preliminary isolate identification

To define the scope of the study we pre-screened 117 isolates according to their internal transcribed spacer (ITS) sequence (see below) to define the principal groups. From this, misidentified isolates were excluded and those within the *Phytophthora drechsleri*, *Phytophthora cryptogea*, and *Phytophthora erythroseptica* groups were analysed further for their morphological and physiological characteristics, mating systems and more detailed molecular analysis with additional nuclear and mitochondrial genes. Q3

Organisms and cultural conditions

Details of the 47 *Phytophthora* isolates examined in this study are listed in Table 1. The isolates were sourced from the culture collection of the authors or in case of some Iranian isolates

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Table 1 – Species and origins of their isolates studied and their GenBank sequence accession numbers. Q5

Species	Phylogenetic group[m]	Isolate code		Mating type	Host	Location	Year isolated	GenBank accession no.[a]				
		Local[n]	International					ITS	*TUB*	*ELO*	*COX*	*PEX1*
P. drechsleri[j]		SCRP222[o]		A2	*Solanum tuberosum*	Wales	?	AY659435	AY659481	AY659528	AY659575	AY659622
P. drechsleri (T)	'A'	SCRP232[b]	ATCC46724, CBS292.35, P1087A	A2	*Beta vulgaris* var. *altissima*	USA	1935	AY659442	AY659488	AY659535	AY659582	AY659629
P. drechsleri	'A'	SCRP236[b]	IMI040500, P3901	S	*Solanum tuberosum*	Argentina	1949	AY659444	AY659490	AY659537	AY659584	AY659631
P. drechsleri[k]		SCRP239		S	*Oryza sativa*	USA	1990	AY659446	AY659492	AY659539	AY659586	AY659633
P. drechsleri		SUAh4		A1	*Beta vulgaris*	Iran	2002	AY659452	AY659498	AY659545	AY659592	AY659639
P. drechsleri		SUAk2		A1	*Beta vulgaris*	Iran	2002	AY659453	AY659499	AY659546	AY659593	AY659640
P. drechsleri[j]		SUC5		A2	?	USA	1992	AY659456	AY659502	AY659549	AY659596	AY659643
P. drechsleri[j]		SUC18		A1	*Beta vulgaris*	Iran	1992	AY659457	AY659503	AY659550	AY659597	AY659644
P. drechsleri[j]		SUC20		A1	*Helianthus annuus*	Iran	1993	AY659458	AY659504	AY659551	AY659598	AY659645
P. drechsleri		SUKv3		A2	*Beta vulgaris*	Iran	2002	AY659459	AY659505	AY659552	AY659599	AY659646
P. drechsleri		SUSa1		A1	*Beta vulgaris*	Iran	2002	AY659461	AY659507	AY659554	AY659601	AY659648
P. drechsleri		SUSa2		A1	*Beta vulgaris*	Iran	2002	AY659462	AY659508	AY659555	AY659602	AY659649
P. drechsleri		SUSd3		A1	*Beta vulgaris*	Iran	2002	AY659463	AY659509	AY659556	AY659603	AY659650
P. drechsleri		SUSr1		A1	*Beta vulgaris*	Iran	2002	AY659464	AY659510	AY659557	AY659604	AY659651
P. cryptogea	I 'B'	SCRP205	IMI34684, P1693T	A1	*Solanum tuberosum*	Northern Ireland	?	AY659423	AY659469	AY659516	AY659563	AY659610
P. cryptogea	I	SCRP206		A1	?	England	?	AY659424	AY659470	AY659517	AY659564	AY659611
P. cryptogea	I 'B'	SCRP207	IMI045168, P1739	A1	*Lycopersicon esculentum*	New Zealand	1951	AY659425	AY659471	AY659518	AY659565	AY659612
P. cryptogea	I	SCRP212[c]		S	*Lycopersicum esculentum*	France	1987	AY659428	AY659474	AY659521	AY659568	AY659615
P. cryptogea	I	SCRP214[c]		A1	*Gerbera jamesonii*	France	1973	AY659430	AY659476	AY659523	AY659570	AY659617
P. cryptogea	I	SCRP219[c]		A2	*Lycopersicum esculentum*	France	1983	AY659432	AY659478	AY659525	AY659572	AY659619
P. cryptogea	I	SCRP225[d]		A1	*Ozothamnus* sp.	England	1995	AY659437	AY659483	AY659530	AY659577	AY659624
P. cryptogea	I	SCRP226[e]	IMI 382781	A1	*Pinus laricio*	?	?	AY659438	AY659484	AY659531	AY659578	AY659625
P. cryptogea[l]	I	SCRP229		A1	*Rubus idaeus*	England	1987	AY659440	AY659486	AY659533	AY659580	AY659627
P. cryptogea[l]	I	SCRP230	IMI 323058	S	*Rubus idaeus*	England	1988	AY659441	AY659487	AY659534	AY659581	AY659628
P. cryptogea	I	SUC4		A1	?	USA	1992	AY659455	AY659501	AY659548	AY659595	AY659642
P. cryptogea f. sp. *begoniae*	II 'D'	SCRP201[b]	IMI260685, CBS468.81, P3265	S	*Begonia eliator*	Germany	1981	AY659421	AY659467	AY659514	AY659561	AY659608
P. cryptogea	II	SCRP204	IMI379121 (3134)	S	*Abies nobilis*	Ireland	?	AY659422	AY659468	AY659515	AY659562	AY659609
P. cryptogea	II 'E'	SCRP210[b]	P3198	A2	*Abies nobilis*	USA	?	AY659427	AY659473	AY659520	AY659567	AY659614
P. cryptogea	II	SCRP213[c]		S	*Gerbera jamesonii*	France	1972	AY659429	AY659475	AY659522	AY659569	AY659616
P. cryptogea	II	SCRP217[c]		A2	*Solanum melongena*	Spain	?	AY659431	AY659477	AY659524	AY659571	AY659618
P. cryptogea	II	SCRP221[f]		S	*Rubus idaeus*	Australia	?	AY659434	AY659480	AY659527	AY659574	AY659621
P. cryptogea	II	SCRP223[d]		S	*Choisya* sp.	England	1995	AY659436	AY659482	AY659529	AY659576	AY659623
P. cryptogea[l]	II 'div'	SCRP228	IMI303922, P3355	A2	*Rubus idaeus*	Ireland	1985	AY659439	AY659485	AY659532	AY659579	AY659626
P. cryptogea[l]	II 'E'	SCRP235	IMI129907, P3494	S	Soil	Australia	?	AY659443	AY659489	AY659536	AY659583	AY659630
P. cryptogea	II	SUC2		A1	*Solanum melongena*	Iran	1992	AY659454	AY659500	AY659547	AY659594	AY659641
P. cryptogea	II	SUKv15		A1	*Beta vulgaris*	Iran	2002	AY659460	AY659506	AY659553	AY659600	AY659647

(*continued on next page*)

Table 1 (*continued*)

Species	Phylogenetic group[m]	Isolate code		Mating type	Host	Location	Year isolated	GenBank accession no.[a]				
		Local[n]	International					ITS	*TUB*	*ELO*	COX	*PEX1*
P. cryptogea	II	SUSt1		S	*Beta vulgaris*	Iran	2002	AY659465	AY659511	AY659558	AY659605	AY659652
P. cryptogea	II	SUSt3		A1	*Beta vulgaris*	Iran	2002	AY659466	AY659512	AY659559	AY659606	AY659653
P. cryptogea	III 'C'	SCRP209[b]	P1811	S	*Juglans hindsii*	USA	?	AY659426	AY659472	AY659519	AY659566	AY659613
P. cryptogea	III	SCRP220[c]		S	*Rosmarinus officinalis*	France	1989	AY659433	AY659479	AY659526	AY659573	AY659620
P. cryptogea	III	SCRP731[g]		S	*Rosmarinus officinalis*	Italy	2003	AY659450	AY659496	AY659543	AY659590	AY659637
P. cryptogea	III	SCRP732[g]		S	*Rosmarinus officinalis*	Italy	2003	AY659451	AY659497	AY659544	AY659591	AY659638
P. erythroseptica	'Per'	SCRP238	ATCC36302, P1699	H	*Solanum tuberosum*	USA	1997	AY659445	AY659491	AY659538	AY659585	AY659632
P. erythroseptica		SCRP240[h]		H	*Solanum tuberosum*	Netherlands	?	AY659447	AY659493	AY659540	AY659587	AY659634
P. erythroseptica		SCRP241[h]		H	*Solanum tuberosum*	Netherlands	?	AY659448	AY659494	AY659541	AY659588	AY659635
P. erythroseptica		SCRP242[i]		H	*Solanum tuberosum*	Australia	?	AY659449	AY659495	AY659542	AY659589	AY659636
P. lateralis (T)		SCRP390	IMI040503, CBS168.42	–	*Chamaecyparis lawsoniana*	USA	1942	AF266804	AY659513	AY659560	AY659607	AY659654

(T) = Type isolate, ? = unknown.
a H = homothallic, ITS = Internal transcribed spacers, S = sterile, *PEX1* = elicitin.
b Clive Brasier, Forest Research, UK.
c Franck Panabieres, INRA France.
d D. Whitehead, RHS Wisley, UK.
e CABI Bioscience, Egham, UK.
f G. McGregor, AgVictoria, Australia.
g Santina Cacciola, University of Catania, Italy.
h Wilbert Flier, PRI, Wageningen.
i Eileen Scott, University of Adelaide, Australia.
j Formerly identified as *P. cryptogea*.
k Formerly identified as *P. erythroseptica*.
l Formerly identified as *P. drechsleri*.
m Molecular groupings identified in this study and other codes to matches (where known) to the groupings of isolates studied by Mills *et al.* (1991) indicated by a letter or descriptor (Per – *Phytophthora erythroseptica*; div – diverse isolates).
n Source of culture to SCRI; SCRI culture unless stated.
o David Shaw, University of Wales.

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directly isolated from the host tissue on PARPH media (CMA, amended with 10 μg ml^{-1} pimaricin, 200 μg ml^{-1} ampicillin, 10 μg ml^{-1} rifampicin, 25 μg ml^{-1} PCNB, and 50 μg l^{-1} hymexazol) (Jeffers & Martin 1986). Isolates were stored on cornmeal agar (CMA; Sigma, Poole, UK) slopes at 15 °C. Routine stock cultures for research studies were grown on French bean agar (FBA; ground French beans 30 g l^{-1}, agar 15 g l^{-1}) at 20 °C.

Colony morphology and growth rate

The isolates were grown at 20 °C on CMA (Sigma, Poole, UK), clear V8-juice agar (CV8-100 ml V8 juice (Campbell's, New Jersey, USA), 900 ml distilled water, and 15 g agar), malt extract agar (MEA, Sigma, Poole, UK), potato-dextrose agar (PDA, Sigma, Poole, UK), and hemp seed agar (HSA; extract of 60 g ground hemp seed, 900 ml distilled water, and 15 g agar). Petri dishes (9 cm diam.) containing 20 ml of the test media were inoculated with 5 mm diam. discs cut from the edge of a 5–10-d-old culture. The discs were placed upside down in the centre of each plate, and the plates were incubated in the dark. Colony morphology was noted after 8 d and growth rate measurements made after the onset of growth along two lines intersecting at right angles at the centre of the inoculum. Growth rate (mm d^{-1}) was recorded on all media. For temperature–growth relationships, CMA plates were inoculated using three replicate plates per isolate and incubated at 5, 10, 15, 20, 25, 30, 35, 37 and 40 °C. Growth rate was recorded 5 d after the onset of linear growth. The test was repeated for the key range of 30–37 °C.

Sporangia

One disc (10 mm diam.), cut from the growing edge of a 7-d-old culture grown on CV8 at 20 °C in the dark, was placed in a 9 cm Petri dish and flooded, just over its surface, with non-sterile soil extract (100 g soil flooded with 1 l distilled water for 24 h at room temperature and then filtered). After incubation at 20 °C in the dark for 48–72 h, dimensions and characteristic features of 50 fully mature sporangia, chosen at random, were determined at ×400 magnification for each isolate.

Breeding system and morphology of oogonia, oospores and antheridia

Oospores were produced in dual culture with either A1 (IMI268688) or A2 (IMI207770) mating type isolates of *Phytophthora nicotianae* on HSA (amended with 30 mg β-sitosterol l^{-1}) plates using 0.2 μm polycarbonate membrane to prevent gametangia of the different species from mixing. For isolates which did not produce oospores the test was repeated using A1 (02B-05) and A2 (02-B10) mating types of *Phytophthora infestans* on amended HSA plates. For each isolate, 50 oogonia, oospores and antheridia, chosen at random, were measured from 4–6-week-old cultures grown at 20 °C in the dark on amended HSA. Measurements were made at ×400 magnification.

Pathogenicity

All isolates were evaluated for their ability to cause pink-rot symptoms on potato tubers. Potato tubers (*Solanum tuberosum* var. Alpha and *S. tuberosum* var. Pentland Javelin) were washed and steeped in 0.5 % W/V of sodium hypochlorite for five minutes before rinsing with sterile water. Once dry, a 7 mm diameter plug was removed from the tuber and a 5 mm mycelial disc (grown on CMA) was inserted to the hole and the potato plug returned to its original position. The cut was sealed with Nescofilm (Bando Chemical Ind. Ltd., Kobe, Japan) to avoid desiccation. The potatoes were incubated in the dark for 5 d at 20 °C, cut open and exposing to the air for 30 min before observations of the symptoms were recorded. (Mostowfizadeh-Ghalamfarsa *et al.* 2006).

Isolates of *Phytophthora drechsleri* (SCRP232), *Phytophthora cryptogea* (SCRP207), and *Phytophthora erythroseptica* (SCRP242) were evaluated about their ability to cause disease in a range of different plant species (*Cucumis sativus* (cucumber), *Cucurbita pepo* conv. *giromontina* (courgette), *Cucurbita maxima* (pumpkins), *Beta vulgaris* (sugar beet), *Solanum lycopersicum* (tomato), *Helianthus annuus* (sunflower), *Carthamus tinctorius* (safflower), *Pisum sativum* (pea) and *Onobrychis viciifolia* (sainfoin)). A 1 l conical flask of Vermiculite (500 ml) was amended with 300 ml of strained French bean extract and autoclaved twice in 24 h intervals. Each flask was inoculated with 8–10 agar blocks of 7-d-old culture and incubated at 25 °C for 3 weeks in the dark. Fifty ml of this inoculum were used to inoculate each of the pots that contained 4–7-d-old seedlings of the above plant species. The pots were flooded for 24 h and grown in the growth chamber at 25 °C. Plants were observed over a 4-week period.

DNA extraction

Isolates were grown in 20 ml still culture of pea broth (boiled extract of 125 g frozen green peas in 1000 ml distilled water at pH 6.2) at 20 °C. After vacuum filtration, the mycelium was freeze-dried for extended storage at −20 °C. DNA was extracted from mycelium using a Puregene DNA extraction kit, Flowgen (Lichfield, England).

DNA amplification

DNA of the internal transcribed spacer regions (ITS) were amplified using the universal primers ITS6 and ITS4 (Cooke *et al.* 2000; White *et al.* 1990). ITS6 is a version of ITS5 (White *et al.* 1990) modified by comparison against 18S sequences of *Phytophthora* to improve the amplification of DNA from oomycetes (Cooke & Duncan 1997). Fragments of the translation elongation factor 1 α (*ELO*) gene and the ß-tubulin (*TUB*) gene were amplified using, ELONGF1 and ELONGR1, TUBUF2 and TUBUR1 (Kroon *et al.* 2004) primers, respectively. No introns were present in these regions. The region containing the mitochondrial cytochrome c oxidase subunit I (*COX*) gene fragment was amplified using COXF4N and COXR4N (Kroon *et al.* 2004) primers. For elicitin (*ELI*) gene primer selection, the *Phytophthora infestans* EST sequence of accession BE776632 was used (Torto *et al.* 2003). Amplification with PEX1F (5′ GATGAACTTYCGYGCTCTG 3′) and PEX1R (5′ GCGTACGAGTASACGTTGAG 3′) yielded a fragment of 329 bp, with no introns present.

Amplifications were performed in a Primus 96 plus thermocycler (MWG-BIOTEC, Ebersberg, Germany). The PCR mixture contained: 10–20 ng of template DNA, 1 μM of each primer, 100 μM of dNTPs, 0.4 U *Taq* DNA polymerase (Promega,

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Southampton, England), 1.5 mM of $MgCl_2$, 2.5 µl of 10× PCR buffer, 100 mM BSA, in a reaction volume of 25 µl. For mtDNA gene amplification, the $MgCl_2$ concentration was raised to 3.5 mM. All PCRs consisted of one cycle of 94 °C (95 °C for ITS) for 2 min; 35 cycles (30 for ITS) of 94 °C (95 °C for ITS) for 20 s, the locus-specific annealing temperature for 25 s, 72 °C for 50 s; and a final cycle of 72 °C for 10 min. Annealing temperatures were 55, 60, 60, 52 and 57 °C for ITS, *TUB*, *ELO*, *COX* and *ELI* loci, respectively. Successful amplification was confirmed by gel electrophoresis (1 h at 70 V) on 1.0 % agarose gels (BIOLINE, London, UK) in 1× TBE buffer. Gels were stained using ethidium bromide and DNA fragments were visualised under UV light.

Sequencing of amplified product

The amplification products of all isolates were purified through Wizard Prep columns (Promega, Southampton, England) to remove excess primers and nucleotides. PCR products were sequenced in forward and reverse orientation using the primers used for amplification and a dye terminator cycle sequencing kit (BigDye sequencing kit, Applied Biosystems, Warrington, UK) on an ABI377-96 automated sequencer (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions.

Phylogenetic analysis

A multiple gene genealogy approach as well as single gene comparisons were applied in the study of the phylogenetic relationships. A preliminary alignment of sequences was made using ClustalX (Thompson *et al.* 1997) with subsequent visual adjustment. The alignments of each of the four regions and a concatenated single alignment of all regions were analysed by both distance-based and maximum likelihood methods in PHYLIP (Felsenstein 1993). The transition/transversion parameter was estimated using the PUZZLE program (Strimmer & von Haeseler 1996). This parameter was used in the PHYLIP DNAML (Felsenstein & Churchill 1996) and DNADIST (Felsenstein 1993) program. The robustness of the DNAML tree was tested using 500 bootstrap trials. The trees were drawn using Treeview (Page 1996). All isolates were sequenced as part of this study with the exception of the following GenBank accession number: *Phytophthora lateralis* (AF266804).

Results

Preliminary isolate identification

Of 117 isolates that were pre-screened by ITS analysis, 62 were confirmed as *Phytophthora drechsleri*, *Phytophthora cryptogea* or *Phytophthora erythroseptica* and 58 were misidentified and subsequently identified as *Phytophthora gonapodyides*, *Phytophthora inundata*, *Phytophthora melonis*, *Phytophthora pistaciae* or *Phytophthora parsiana* (Supplementary table). Based on these preliminary analyses, 46 isolates of *P. drechsleri*, *P. cryptogea* or *P. erythroseptica* were selected from the global collection to represent the full range of genetic diversity of these taxa (Table 1). An isolate of another ITS-clade 8 species, *Phytophthora lateralis*, was selected as an outgroup.

For these 47 isolates, fragments of three additional nuclear genes and one mitochondrial gene were sequenced, including *TUB*, *ELO*, a putative *ELI*, and *COX*.

The combined nuclear and mitochondrial DNA data set comprised 3888 characters for 47 taxa which contained 198 (5.09 %) potentially phylogenetic informative sites with a final expected transition/transversion ratio of 1.22. Maximum likelihood and neighbour-joining analysis of the combined nuclear and mitochondrial DNA set revealed five different lineages among isolates: *P. drechsleri*, *P. cryptogea* Group I (GI), *P. cryptogea* Group II (GII), *P. cryptogea* Group III (GIII) and *P. erythroseptica* (Fig 3f).

Neighbour-joining as well as maximum likelihood (data not shown) analysis of the five individual loci showed gene–gene concordance in the five observed lineages with only a few exceptions (Fig 3, TreeBASE accession 23241). The positions of isolates SCRP201, SCRP213, SCRP214, and SCRP228 in the phylogenetic trees were atypical and varied according to the sequenced region (see below).

The *P. drechsleri* clade was resolved as monophyletic in the five individual neighbour-joining gene trees with bootstrap support ranging from 90 to 100 % (with the exception of the *ELO* gene tree) (Fig 3). These isolates, which includes the type isolate of *P. drechsleri*, consistently grouped in a clade distinct from all other isolates which we consider as *P. drechsleri sensu stricto*.

The *P. cryptogea* dominated clade was a monophyletic group with bootstrap support ranging from 59 to 96 % in different gene trees (Fig 3). The 32 isolates comprised a clade of three separate *P. cryptogea* lineages and a *P. erythroseptica* clade. The combined gene neighbour-joining tree indicated that *P. cryptogea* GII is ancestral to the other two lineages but this was not consistent in all individual trees. In each case, however, *P. cryptogea* GII and *P. cryptogea* GIII isolates were more closely related to each other than to the other group (Fig 3). In each case the *P. erythroseptica* clade was rooted amongst the GI *P. cryptogea* isolates. Double peaks in sequencing electropherograms indicated heterozygosity and these were reflected as ambiguity codes in the multiple alignments (data not shown). Four *P. cryptogea* isolates (SCRP214, SCRP201, SCRP213 and SCRP228) had a mean of 6.25, 8.25 and 3.5 heterozygous sites per gene for the *TUB*, *ELO* and *ELI* regions, respectively, compared to 0.5, 2.5 and 1.1 amongst the remaining 24 *P. cryptogea* isolates This increase in heterozygosity was reflected in a phylogenetic position of these four isolates intermediate between the *P. cryptogea* GI and GII clades (marked with asterisks in Fig 3). With one exception, the *P. erythroseptica* isolates formed a closely related cluster that grouped amongst the *P. cryptogea* clades. The exception was isolate SCRP238 which grouped with either other isolates of *P. erythroseptica* or with *P. cryptogea* GI or GIII (Fig 3).

Temperature relations

The mean growth rate of *Phytophthora drechsleri*, *Phytophthora cryptogea* and *Phytophthora erythroseptica* differed markedly (Fig 2). However, within each taxon the range was large (Table 3). There were some significant differences in growth

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rate between the molecular subgroups of *P. cryptogea* (Table 3). In general, isolates identified as *P. drechsleri* had an optimum temperature of 30 °C and grew well (more than 3.5 mm d^{-1}) at 35 °C, whereas *P. cryptogea* did not. Exceptions were the isolates SCRP209, SCRP217 and SCRP220 which could grow 3.3, 3 and 2.8 mm d^{-1} at 35 °C, respectively, but even in this case all *P. cryptogea* isolates had an optimum temperature of 25 °C. None of the isolates identified as *P. erythroseptica* could grow at 35 °C. A notable exception was the *P. drechsleri* isolate SCRP239 that showed a markedly reduced growth rate over the whole temperature range (see below).

Colony growth pattern

Most isolates produced a uniform to irregular colony pattern on almost all of the media. The patterns were more distinct on PDA (Supplementary Fig 1) but, overall, the colony patterns could not be used to clearly distinguish the groups of isolates.

Sporangium morphology

Sporangia of *Phytophthora drechsleri*, *Phytophthora cryptogea* and *Phytophthora erythroseptica* were non-papillate and ranged in shape from obpyriform, ellipsoid to ovoid; with or without a tapered base. Morphological plasticity was, however, evident, with one isolate producing sporangia with both tapered and non-tapered bases under the same environmental conditions. The range of sporangial shapes for isolates of the three species is shown (Fig 1). All isolates produced proliferating sporangia and some had sympodial sporangiophores. In general, the sporangia of *P. drechsleri* isolates were more elongated than *P. cryptogea* with a higher length:breadth ratio, but this trait could not be used to reliably discriminate between the species (Table 2).

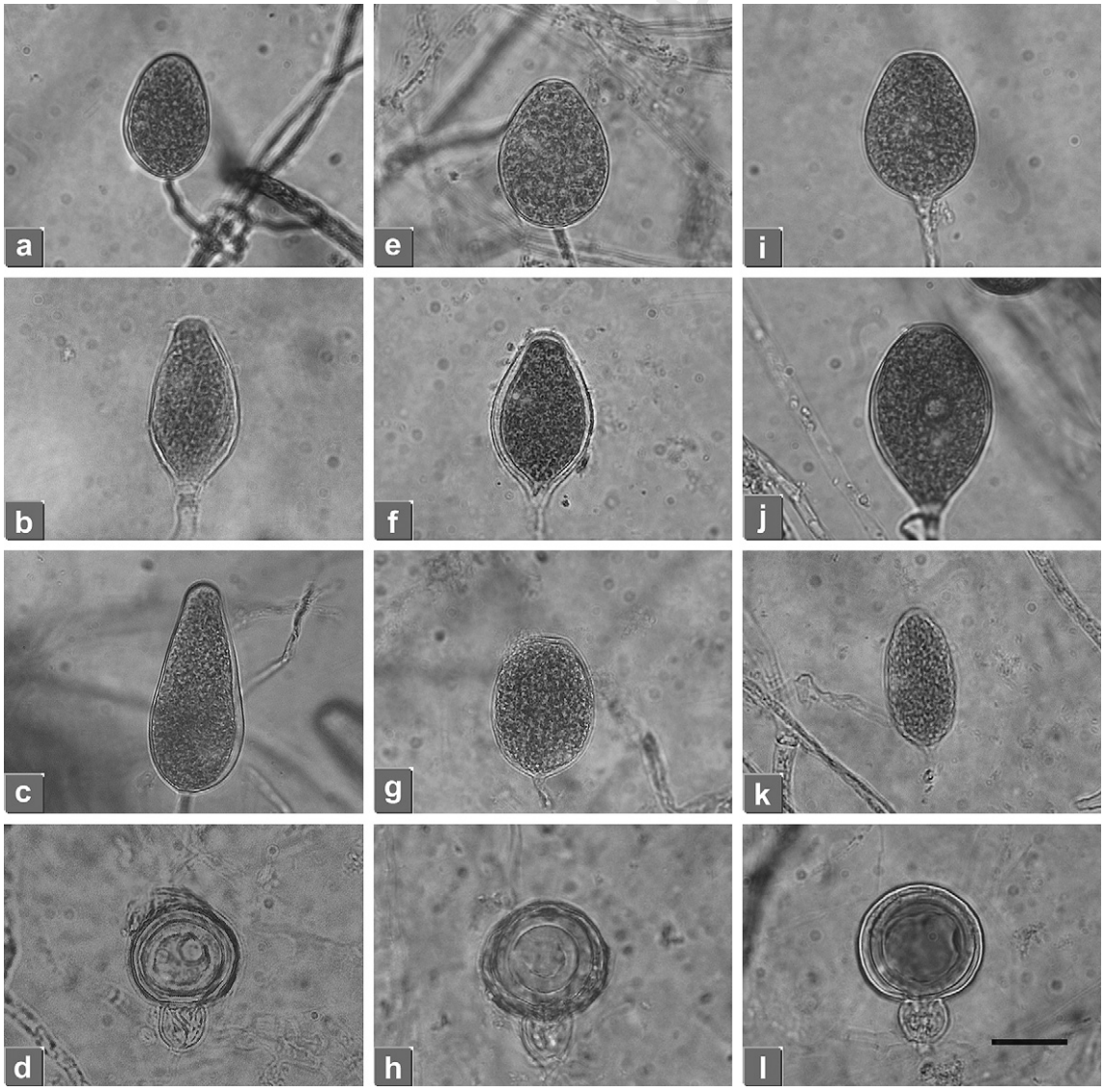

Fig 1 – Range of sporangial morphology (a–c), (e–f), and (i–k) and characteristics of the oospores and antheridia (d, h and l) of *Phytophthora drechsleri* [(a) SCRP232, (b) SCRP222, (c) SCRP222, (d) SUKv15], *P. cryptogea* [(e) SCRP731, (f) SUC1, (g) SuC2, (h) SCRP219] and *P. erythroseptica* [(i–l) SCRP242]. Bar = 20 μm.

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Table 2 – Comparison of morphological characters of *Phytophthora drechsleri*, *P. cryptogea*, and *P. erythroseptica*.

Character	*P. drechsleri*	*P. cryptogea*				*P. erythroseptica*
		(All groups)	GI	GII	GIII	
Sporangia						
Papilla	–	–	–	–	–	–
Average length (μm)	35.5 ± 12.7[a]	35.7 ± 9.45	34.5 ± 9.17	36.4 ± 9.69	38 ± 9.98	31 ± 7.82
Range[b] length (μm)	17.3–87.5	17.3–75	17.3–75	19.2–74.9	19.2–71	17.3–53.8
Average breadth (μm)	21.1 ± 6.2	22.8 ± 5.35	19.2 ± 5.59	23.9 ± 5.04	24 ± 4.79	21 ± 5.99
Range breadth (μm)	12.5–55	11.5–49.9	11.5–44.2	11.5–49.9	15.4–40.3	11.5–36.5
Isolate averages						
Isolate length (μm)	21.3–57.7	27.1–46.4	27.1–45.5	27.3–46.4	31.2–42.1	26–39.9
Isolate breadth (μm)	13.9–34.6	16.7–29.6	16.7–29.6	19.3–29.6	19.8–25.5	17.5–27.5
Length:breadth ratio	1.7:1	1.6:1	1.6:1	1.5:1	1.6:1	1.5:1
Isolate averages	1.4:1–2.3:1	1.3:1–1.9:1	1.4:1–1.9:1	1.3:1–1.8:1	1.5:1–1.7:1	1.4–1.5
Shape(s)	El,Op,Ov	El,Op,Ov	El,Op	El,Op	El,Op,Ov	El,Op,Ov
Distorted shapes	–	–	–	–	–	–
Tapered base	+	+	+	+	+	+
Caducity	–	–	–	–	–	–
Proliferation	+	+	+	+	+	+
Sympodial	(+)	(+)	(+)	(+)	(+)	(+)
Average pore diam. (μm)	5.8 ± 1.57	6.8 ± 1.8	6.4 ± 1.75	7.1 ± 1.86	6.7 ± 9.7	6.2 ± 2.01
Isolate averages (μm)	2.5–9.5	1.9–15.2	1.9–15.2	3.8–12.5	5.7–9.5	1.9–15.2
Homothallism	–	–	–	–	S	+
Oogonia						
Average diam. (μm)	29.9 ± 6.18	32.3 ± 6.34	33.4 ± 5.85	35.4 ± 5.51	S	37.2 ± 3.64
Range (μm)	17.3–46.1	15–49.9	17.5–48	15–49.9		25–44.2
Isolate averages (μm)	22.4–37.4	26.4–43.9	26.4–42.4	30.1–43.9		36.1–38.9
Tapered base	+	+	+	+		+
Oospores						
Average diam. (μm)	26.7 ± 5.25	28.5 ± 6.12	27.9 ± 5.75	33 ± 5.87	S	30.6 ± 3.49
Range (μm)	15.4–42.5	13.4–49.9	13.4–46.1	15–49.9		17.3–36.5
Isolate averages (μm)	20.9–33.8	23.1–43.9	23.1–38.3	29.6–43.9		29.6–32.1
Plerotic	+	(+)	(+)	+		–
Aplerotic	(+)	(+)	(+)	(+)		+
Oospore wall						
Average diam. (μm)	3.6 ± 0.93	3.9 ± 0.86	3.8 ± 0.83	4.1 ± 0.95		3 ± 0
Isolate averages (μm)	2.5–5	2.4–5	3–5	2.4–5		3
Antheridia	Am	Am	Am	Am	S	Am
Average diam. (μm)	12.8 ± 2.9	13.8 ± 2.7	14.3 ± 1.96	13.2 ± 3.26		14.9 ± 1.9
Range (μm)	7.7–21.1	5–19.2	11.5–19.2	5–19.2		9.6–19.2
Isolate averages (μm)	9.1–15.6	8–17.3	13.1–17.3	8–17.3		14.3–15.4
Hyphae						
Average width (μm)	5.5 ± 0.91	5.4 ± 0.6	5.4 ± 0.5	5.3 ± 0.48	5.5 ± 0.58	6 ± 0.82
Isolate averages (μm)	5–7.5	5–7.5	5–6	5–6	5–6	5–7
Hyphal swellings						
In water	(+)	(+)	(+)	(+)	(+)	–
On agar	–	(+)	(+)	(+)	(+)	–
Colony morphology on						
CMA	Uni	Uni	Uni	Uni	Uni	Cor
CV8	Uni	Uni, Cry, Irg	Uni, Cry, Irg	Uni	Uni	Uni
MEA	Uni	Uni, Irg, Cry, Ros	Uni, Irg, Cry, Ros	Uni, Irg, Ros, Cry	Uni, Irg	Uni, Ros, Irg
HSA	Uni	Uni, Irg	Uni, Irg	Uni	Uni	Uni
PDA	Ros	Ros, Cry, Uni, Irg	Ros, Uni, Irg	Ros, Cry, Uni, Irg	Ros, Uni	Ros
Average radial growth rate at 20 °C (mm d^{-1}) on						
CMA	6 ± 2.77	4.6 ± 1.93	3.2 ± 1.35	5.5 ± 1.98	5.2 ± 0.71	3 ± 0.35
CV8	6.1 ± 1.67	6.8 ± 1.17	6.5 ± 1.17	6.8 ± 1.04	7.4 ± 1.61	6.4 ± 0.97
MEA	4.4 ± 1.52	4.7 ± 1.01	4.6 ± 0.99	4.6 ± 1.09	5.3 ± 0.77	4.3 ± 0.38
HSA	6.2 ± 2.16	7.2 ± 1.32	6.4 ± 1.32	7.5 ± 1.22	7.9 ± 0.85	6.5 ± 0.5
PDA	4.2 ± 1.33	4.7 ± 1.17	4.9 ± 1.35	4.5 ± 1.15	4.8 ± 0.9	4.7 ± 0.57

+ = Feature occurring frequently. (+) = Feature occurring infrequently. – = Feature not observed. Am = Amphigynous. Cor = Coralloid. Cry = Chrysanthemum. El = Ellipsoid sporangia. Irg = Irregular. Op = Obpyriform sporangia. Ov = Ovoid sporangia. Ros = Rose shaped. S = Sterile. Uni = Uniform.

a Figures are mean ± standard deviation of all isolates from a particular group.

b Minimum–maximum of isolates.

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Mating behaviour and morphology of sex organs

The majority of isolates of *Phytophthora drechsleri* and *Phytophthora cryptogea* were heterothallic and produced amphigynous terminal antheridia in response to the opposite mating type and generating oogonia with a mean diameter of 29.9 and 32.3 respectively. A single isolate (SCRP232) also produced intercalary antheridia. The isolates of *P. cryptogea* GIII, however, failed to produce oospores when crossed with *P. cryptogea*, *P. drechsleri* or other species and were thus considered sterile. On average, the dimensions of all the measured features of the sex organs were marginally larger in *P. cryptogea* isolates than those of *P. drechsleri* (Table 2). However, the range of sizes between species, isolates and amongst organs formed by a single isolate was sufficiently large to make them taxonomically useless (Table 2). All *Phytophthora erythroseptica* isolates were homothallic producing oogonia, antheridia and oospores in single culture (Table 2). Again, on average these structures were slightly larger than those of *P. cryptogea* and *P. drechsleri* but the range of sizes prevented their effective use in discriminating the species.

Pathogenicity

After 5 d incubation in potato tubers all isolates (except *Phytophthora drechsleri* isolate SCRP239) produced the characteristic pink-rot symptoms described by Pethybridge (1913). A distinct pink colour change was observed when the infected potato was sliced open and exposed to the air for several minutes, while the non-inoculated control maintained its original colour. The inoculated isolates were re-isolated from each of the diseased potatoes and their identity was verified.

Isolate SCRP207 (*Phytophthora cryptogea*) caused damping-off on one-week-old seedlings of sugar beet and pea and suppressed normal growth of sunflower, safflower and tomato (Table 4). Isolate SCRP232 (*P. drechsleri*) caused damping-off on one-week-old seedlings of pea and suppressed normal growth of sugar beet. No symptoms were observed on the cucumber, courgette or pumpkin seedlings. Isolate SCRP242 (*Phytophthora erythroseptica*) caused no disease symptoms on any of the plants tested. The inoculated *Phytophthora* species were re-isolated from diseased plants and their identity verified by ITS sequencing. No *Phytophthora* species were recovered from the roots or crowns of the healthy plants.

Discussion

Phytophthora drechsleri and *Phytophthora cryptogea* could not be discriminated consistently on the basis of morphology yet the sequence data provides strong support for their status as distinct taxa. Low levels of intraspecific variation were found in *P. drechsleri* compared to *P. cryptogea*, within which the molecular signatures of three subgroups were demonstrated. Phylogenetic analysis of the DNA sequences of the five regions provided evidence of introgression between the *P. cryptogea* groups. The cluster of four *Phytophthora erythroseptica* isolates consistently branched from within one *P. cryptogea* subgroup suggesting this homothallic species is derived from the heterothallic *P. cryptogea*.

Resolution of a long-standing debate over the status of *P. drechsleri* is provided in this study. With the benefit of objective DNA-based methods we were able to pre-screen the collection and, after confirming the identity of fourteen isolates as *P. drechsleri*, we studied them further. This is in stark contrast to previous studies (*e.g.* Ho & Jong 1986, 1991) in which detailed observations were made on isolates grouped by, what we now know to be, subjective and sometimes unhelpful morphological criteria. Without the benefit of a molecular identification, much previous analysis was confounded by misidentified isolates and, inevitably, the conclusions were flawed. For example, we were able to use the accession numbers to trace 11 of the 14 isolates that Ho & Jong (1986) considered to be *P. drechsleri* and note that only one of them is now considered a true representative of this taxon. Observations in the current study confirmed the difficulty in discriminating *P. drechsleri* from *P. cryptogea* using morphological criteria alone; in fact no single discriminatory morphological character was identified. The Waterhouse (1963) key for example, suggested the presence of elongated sporangia with tapered bases is a feature of *P. drechsleri* yet we observed such structures in *P. drechsleri*, *P. cryptogea* and *P. erythroseptica*. Growth rate at higher temperatures proved a more consistent feature with isolates of *P. drechsleri* (with the exception of SCRP239 discussed below) having an optimum temperature for growth of 30 °C and continuing to grow at a mean of 6 mm d^{-1} at 35 °C compared to a mean of 2 mm d^{-1} or less in *P. cryptogea* and *P. erythroseptica* (Table 3; Fig 2). In the study of Mills *et al.* (1991), all isolates of group 'A' also showed such high growth rates at 35 °C.

The phylogenetic data in this study provided clear support for *P. drechsleri* as a distinct and monophyletic taxon. In each of the five single gene phylogenies, all 14 isolates formed a distinct monophyletic clade strongly supported by the bootstrap analyses (Fig 3). A degree of substructure was noted within this clade; for example in the case of the tree based on the nuclear *TUB* gene (Fig 3b) the clustering reflects the geographical origin of the isolates with American isolates (SCRP232, SCRP236 and SUC5) basal to those of European (SCRP222) or Asian origin. This pattern is clearer in the analysis based on the mitochondrial *COX* gene (Fig 3e). Such minor intraspecific sequence variation is perhaps unsurprising in a species of broad host range and global origin and may, for example, reflect past geographic isolation of sub-populations. The *ELO* and *COX* sequences of isolate SCRP239 are atypical amongst *P. drechsleri* isolates. It also grew slowly at all temperatures, had an optimum temperature of 25 °C and poor pathogenicity on potato. However, apart from the production of intercalary antheridia, the morphology of SCRP239 did not differ from other isolates. This isolate, unusual in that it was isolated from rice, was originally identified as *P. erythroseptica* but grouped within *P. drechsleri* by Gunnell & Webster (1988). Some of the properties reported by Gunnell & Webster (1988) differ from this publication perhaps as a consequence of its long period in culture. The *P. drechsleri* isolate tested in this study did not cause any disease on the various cucurbit species included in our preliminary screen (Table 4). Previous reports of cucurbit disease are almost certainly associated with misidentified isolates of what we now know to be the unrelated *Phytophthora melonis* (Cooke *et al.* 2000). The results of Mills *et al.* (1991) support this as all their isolates reported as

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Table 3 – Radial growth rate of *Phytophthora* isolates on CMA at different temperatures (growth rate at optimum temperature shown in bold).

	Average radial growth rate (mm d^{-1}) at °C								
	5	10	15	20	25	30	35	37	40
P. drechsleri[a] (14)[b]	0.4	2.3	4.1	6.4	8	**10.9**	6.8	3.2	0.2
Isolate average	0.0–1	0.7–2.9	2.1–6.2	2.3–8.7	3.8–11.7	4.5–15.5	3.7–10.1	0.8–4	0.0–1.2
P. cryptogea (28)	0.2	2	3.5	4.6	**5.9**	4.6	0.8	0.2	0
Isolate average	0.0–2.1	0.9–5.1	1.6–6.5	1.1–8.9	2.8–11	2.8–10.3	0.0–3.3	0.0–1.5	0
P. cryptogea (G I) (11)	0	1.3	2.7	3.2	**4.6**	3.7	0.3	0	0
Isolate average	0.0–0.4	0.9–3.2	1.6–4.1	1.1–6.5	2.8–6.9	2.9–4.4	0.0–1.8	0	0
P. cryptogea (G II) (13)	0.4	2.2	3.9	5.5	**7**	5.4	0.8	0.1	0
Isolate average	0.4–2.1	1–3.6	1.7–6.5	2.9–8.9	4.7–11	3–10.3	0.0–3	0.0–0.9	0
P. cryptogea (G III) (4)	0.4	3.4	4.5	5.2	**6.2**	4.5	2.3	0.7	0
Isolate average	0.0–0.8	1.2–5.1	2.7–5.9	4.1–5.7	5.3–7.5	2.8–6.3	1.1–3.3	0.1–1.5	0
P. erythroseptica (4)	0	1.1	2.4	3	**3.6**	2.3	0	0	0
Isolate average	0	0–1.6	1.4–3	2.6–3.3	2.1–4.3	1.8–2.8	0	0	0

a Isolate SCRP239 excluded.
b Number of isolates tested.

P. drechsleri which caused cucumber crown-rot were clustered in their group 'F' which also included all *P. melonis* isolates.

As described above for *P. drechsleri*, our pre-screen of isolates on the basis of ITS sequence allowed a detailed analysis of the traits of 28 isolates we considered as *P. cryptogea*. No single morphological character discriminated *P. cryptogea* from *P. drechsleri* but its optimal temperature for growth of 25 °C was distinct from the higher optimum of 30 °C in *P. drechsleri* (Table 3; Fig 1). There were only minor differences in pathogenicity between the two species (Table 4). The sequence data was, however, definitive, indicating *P. cryptogea* shares a recent common ancestor with, but is clearly distinct from, *P. drechsleri*. The DNA sequencing and subsequent phylogenetic analysis provided no evidence of any recent introgression between the 14 *P. drechsleri* and 28 *P. cryptogea* isolates sampled in this study. In contrast to *P. drechsleri*, the phylogenetic analysis of the five sequenced genes resolved distinct sub-populations within *P. cryptogea*. Three groups (termed GI, GII and GIII) were consistently demonstrated (Fig 3). Careful cross-referencing with isolates common to other studies (Mills *et al.* 1991; Förster *et al.* 2000) confirmed that our groups corresponded to those of 'B' (our GI), 'E', 'D' and 'diverse' (our GII) and 'C' (our GIII) defined on the basis of isozymes and mtDNA RFLPs by Mills *et al.* (1991). It is clear that these subgroups share a recent common ancestor but an evolutionary divergence has occurred. Possible drivers of such divergence are host specificity and/or geographic origin. Marked differences in pathogenicity of *P. cryptogea* isolates on *Gerbera jamesonii* and *Begonia-Elatior*-Hybrids led Kröber (1981) for example, to define isolates specific to *Begonia* as *P. cryptogea* f. sp. *begoniae*. These isolates were defined as group 'D' by Mills *et al.* (1991) and fall within our GII. However, there is little other support for isolation either by host range or geographic origin amongst the isolates examined in this or other studies (Mills *et al.* 1991; Erwin & Riberio 1996) with isolates of each group being recovered from a wide range of host plants on different continents. *P. cryptogea* infects a very wide range of plant species being widely reported in horticulture, forestry and natural ecosystems on a global scale since early in the 20th century (Erwin & Riberio 1996). It is thus probable that any biogeographical boundaries have been blurred by widespread distribution of the pathogen in international trade of infected plants (Brasier 2008).

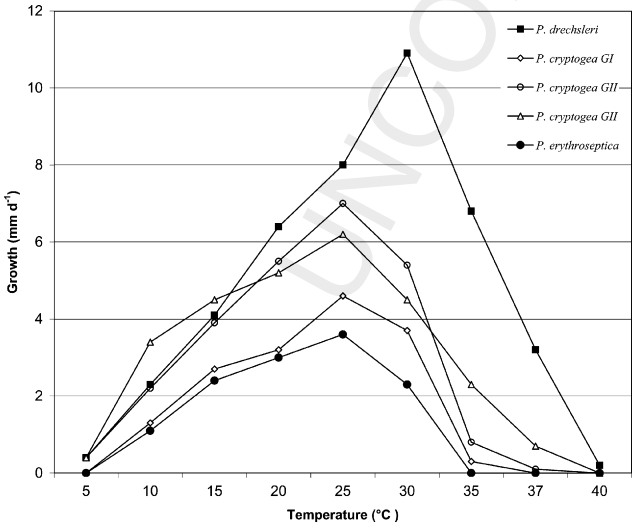

Fig 2 – Average radial growth rate of different *Phytophthora* isolates on CMA at 5–40 °C.

The subgroups of *P. cryptogea* defined here are based on the combined gene tree (Fig 3f) which indicates a basal position of GII and GIII that are more closely related to each other and ancestral to the more distantly related GI. Examination of the individual gene trees provides more detail on the relationships and possible origins of these subgroups. In four of the five trees, the four isolates of GIII form a distinct sister group to GII. Less diversity was noted in the *PEX* gene sequence and

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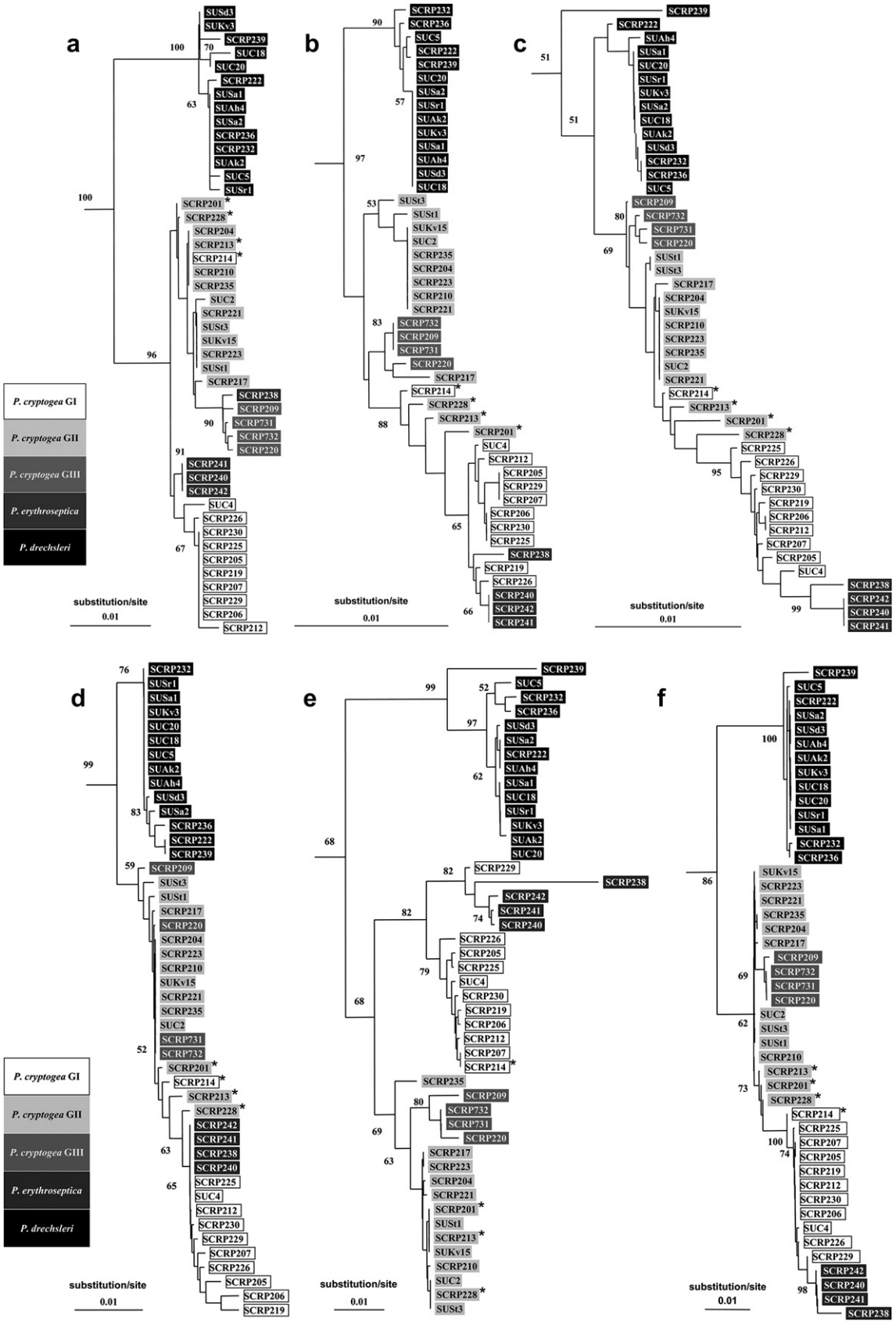

Fig 3 – Phylogenetic relationship of *Phytophthora drechsleri*, *P. cryptogea* groups and *P. erythroseptica* based on neighbour-joining method. The numbers at the branch points indicate the percentages of bootstrap values ≥50 %. (a) ITS1, 5.8S subunit, and ITS2 regions of the genomic ribosomal RNA tandem gene repeat; (b) *TUB* gene; (c) *ELO* gene; (d) *ELI* gene; (e) *COX* gene; (f) combined genes (ITS1, 5.8S subunit, and ITS2 regions of rDNA; *TUB*; *ELO*; *ELI*; and *COX*).* = GI/GII introgressants.

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Table 4 – Pathogenicity of *Phytophthora drechsleri*, *P. cryptogea*, and *P. erythroseptica* isolates on different plant species.

Host	*P. drechsleri* (SCRP232)	*P. cryptogea* (SCRP207)	*P. erythroseptica* (SCRP242)
Cucumis sativus (cucumber) var. Venlo pickling	–	–	–
Cucurbita pepo conv. giromontina (courgette) var. All green bush	–	–	–
Cucurbita maxima (pumpkin) var. Mammoth	–	–	–
Beta vulgaris (sugar beet) var. Duke	(+)	+	–
Solanum lycopersicum (tomato) var. Moneymaker	–	(+)	–
Helianthus annuus (sunflower) var. Little Dorrit	–	(+)	–
Carthamus tinctorius (safflower) var. Grenade mixture	–	(+)	–
Pisum sativum (pea) var. Onward	+	+	–
Onobrychis viciifolia (sainfoin)	+	+	–
Solanum tuberosum (potato)[a]	+	+	+

+ = Damping-off. (+) = Stunted growth. – = No symptoms observed.
a All isolates in this study tested for potato pink rot.

the GIII isolates, which, in this case, are placed amongst those of GII in a loose basal clade. The single isolate of this group that could be cross-referenced is from *Juglans hindsii* and in Mills *et al.* (1991) represents group 'C'. The other three isolates in our study were from *Rosmarinus officinalis* in European nurseries but the common host is likely coincidental, given the broad range of hosts of the eleven other group 'C' isolates identified by Mills *et al.* (1991) from the USA, Australia and Papua New Guinea. As stated, our GII corresponds to Mills *et al.* (1991) groups 'D' and 'E' and the close relationship of 'C', 'D' and 'E' isolates was also noted in their analysis. Isolates of this group also closely match the ITS sequence of an isolate informally described as *Phytophthora* sp. *'kelmania'* (AY117032) (see discussion below).

The majority of isolates of our *P. cryptogea* GI form a distinct group with minor sequence differences reflected in subclusters in each tree. An exception is that based on the ITS region (Fig 3a) which, in general, displays less diversity within the groups and likely reflects within-group sequence homogenisation *via* concerted evolution or 'molecular drive' (Dover 1982). Our GI corresponds to group 'B' from Mills *et al.* (1991) who also observed such within-group variation and noted the type isolate of *P. cryptogea* was a member of group 'B'.

The phylogenetic placements of a group of four *P. cryptogea* isolates, in particular, provide very strong evidence for introgression, or gene flow, between lineages GI and GII. Isolates SCRP201, SCRP213, SCRP214, and SCRP228 group in intermediate positions in the three trees based on single-copy nuclear genes (*TUB*, *ELO*, *PEX*). Examination of the electropherograms of these genes indicated a higher incidence of 'double' peaks consistent with heterozygosity at a rate higher than amongst the other isolates. These isolates were placed within their GII (SCRP201, SCRP213, and SCRP228) or GI (SCRP214) clades in the case of the uniparentally inherited COX mtDNA data. Furthermore, the GI isolate (SCRP214) grouped within the GII isolate sister clade in the ITS tree which is consistent with recombination between GII and GI ITS types followed by a directional concerted evolution (Wendel *et al.* 1995) fixing the ITS sequence to the GII form. Such processes have been observed previously in *Phytophthora* hybridisation (Brasier *et al.* 1999). The data is consistent with an introgression between these groups but it is unclear whether this reflects a recent or ancient genetic exchange. However, ITS polymorphism and intermediate position of SCRP201, SCRP213, SCRP214, and SCRP228 in all gene trees except *COX* suggest a more recent origin. These four isolates were collected between 1972 and 1985 on species of *Gerbera*, *Rubus* and *Begonia* in Europe. The *Begonia* isolate is that examined by Kröber (1981) and described as group 'D' by Mills *et al.* (1991). Interestingly, the *Rubus* isolate was also examined by Mills *et al.* (1991), but its isozyme data did not allow it to be grouped in 'B', 'C', 'D' or 'E' so it was lumped within a miscellaneous 'diverse' assemblage. It is not clear how such introgressants were derived; examination of their ploidy and mating behaviour and attempts to reconstruct such forms would reveal more about their nature and origins. Both A1 and A2 mating types occur amongst GI and GII *P. cryptogea* isolates examined with no clear relationship between molecular lineage and mating type. Conventional mating is thus plausible but other mechanisms are available (Brasier 1992). Although no barriers for mating across molecular types of *P. cryptogea* are apparent, comprehensive reciprocal mating studies are needed to examine this more in detail. The *P. cryptogea* isolates, SCRP210, SCRP213 and SCRP214 were from a comprehensive INRA collection from the European horticulture industry in the 1970s to 1980s. An examination of 37 INRA isolates by ITS RFLP analysis also supports the introgression described above. Three digest patterns with the *Msp*I enzyme were observed (data not shown) with 11 and 12 isolates corresponding to GI and GII, respectively and a third group of 14 isolates having an ITS digest pattern indicative of a polymorphic ITS region and matching those of the introgressant isolates SCRP213 and SCRP214. This indicates that these three forms of *P. cryptogea* were commonly found in the European horticulture trade 30–40 y ago.

The extent of molecular diversity observed in *P. cryptogea* in this study is not without precedent in other *Phytophthora* taxa. However, in contrast to the closely related assemblage of taxa such as *Phytophthora megasperma* and *Phytophthora gonapodyides* in ITS-clade 6 (Brasier *et al.* 1993, 2003) where intraspecific sequence polymorphism was related to obvious changes in colony morphology and mating behaviour, there is no clear evidence of such differences amongst *P. cryptogea* groups. The sterility of all four isolates of our GIII *P. cryptogea* isolates is likely coincidental as the corresponding group 'C' of Mills *et al.* (1991) comprised A1, A2 and sterile isolates.

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These data are consistent with the generation of novel stable and fit pathogenic forms of *P. cryptogea* from previously isolated and, presumably, allopatric populations which is significant in two respects. Firstly, it suggests that *P. cryptogea* is an operational taxonomic unit and should remain a single species. And secondly, it further highlights the risks posed by international plant trade (Brasier 2008) in transporting isolates capable of generating stable and fit new forms of *P. cryptogea*.

For a long time the homothallic nature of *P. erythroseptica* has led to it being considered as a distinct monophyletic species. However, in the combined and individual gene trees (Fig 3) our data indicate that the *P. erythroseptica* isolates are derived from *P. cryptogea*. With the exception of the ITS tree, all four isolates of *P. erythroseptica* group closely within the *P. cryptogea* GI isolates clade. All isolates are from potato but isolate SCRP238 from the USA differs in DNA sequence from the others in four of the five sequenced regions. In the case of the ITS analysis, SCRP238 is most closely related to *P. cryptogea* GIII isolates, it groups amongst the GI isolates in the *TUB* tree and a *P. cryptogea* isolate (SCRP229) groups with *P. erythroseptica* in the case of the mtDNA *COX* gene analysis (Fig 3). Collectively this provides evidence of introgression from *P. cryptogea* and is consistent with the hypothesis that *P. erythroseptica* is a secondarily derived homothallic form of *P. cryptogea*. Such a phenomenon has been reported in the case of *P. drechsleri* (Mortimer *et al.* 1977) and other studies support the derivation of homothallic taxa from heterothallic ones (*e.g.* Cooke *et al.* 2000). Clearly the ability to cause a pink rot of potato tubers is not a trait specific to *P. erythroseptica* as, with the exception of a single isolate, all isolates of all three species examined in this study caused such symptoms in our laboratory assay. Potato pink-rot symptoms were also caused by isolates of *P. cryptogea* from Kiwi fruit in Chile (Latorre *et al.* 1995). Further studies of isolates from field-infected tubers would be valuable to ascertain the pathogenicity of these three species under natural conditions. The single isolate of *P. erythroseptica* tested (SCRP242) was not pathogenic on other plant species tested which also distinguished it from *P. cryptogea* and *P. drechsleri* (Table 4). The evidence presented here suggests that *P. erythroseptica* and *P. cryptogea* are conspecific. However, more data on a wider selection of *P. erythroseptica* isolates should be examined prior to any formal taxonomic change.

Consideration of *P. drechsleri*, *P. cryptogea* and *P. erythroseptica* in a wider selection of *Phytophthora* species (Fig 4) supports their position in clade 8a and is consistent with other studies (Cooke *et al.* 2000; Kroon *et al.* 2004; Blair *et al.* 2008). Noteworthy in the Blair *et al.* (2008) publication is the position of the undescribed taxon *P.* sp. *'kelmania'* as basal to *P. cryptogea* and the presence of *Phytophthora richardiae* in clade 8a. The sequence data from the undescribed species *P.* sp. *'kelmania'* (Abad *et al.* 2006; Blair *et al.* 2008; Moralejo *et al.* 2009) places it amongst the *P. cryptogea* GII or GIII isolates described in this study. Closely related isolates from *Gerbera* sp. and *Colea* sp. reported to match *P.* sp. *'kelmania'* were also described as ''morphologically similar to *P. cryptogea*'' (Moralejo *et al.* 2009). It is thus highly likely that this taxon is conspecific with *P. cryptogea*. The case of *P. richardiae* reported by Blair *et al.* (2008) in clade 8a also needs resolution. There are few isolates described as *P. richardiae* available in international

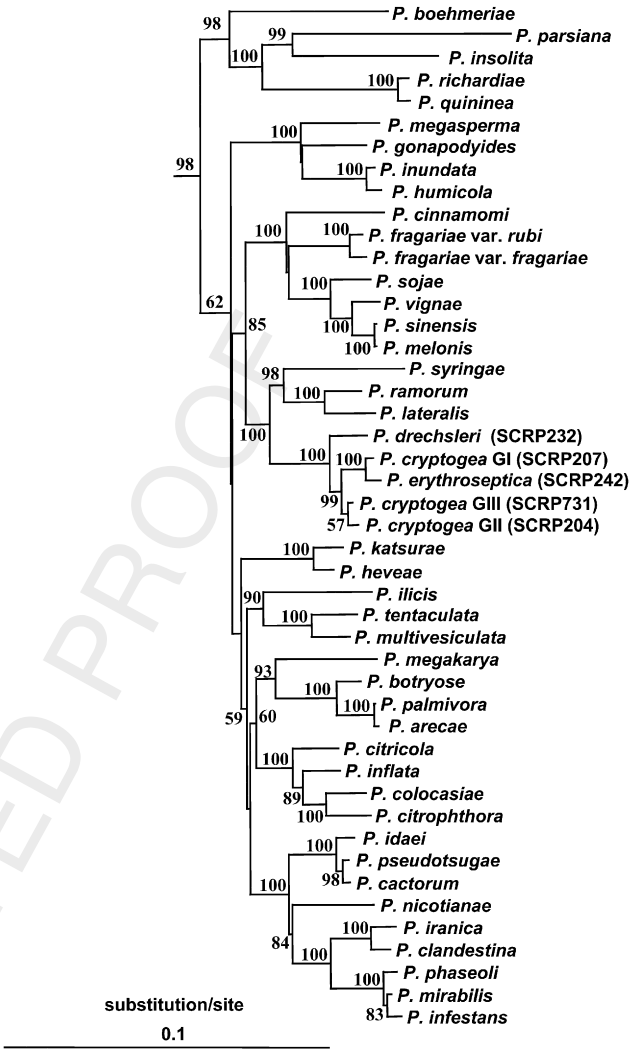

Fig 4 – Phylogram of a neighbour-joining analysis of the combined gene matrix of *Phytophthora drechsleri*, *P. cryptogea* groups and *P. erythroseptica* together with 41 *Phytophthora* species. The numbers within parentheses indicate the isolates numbers. The combined sequence matrix contained the ITS1, 5.8S subunit, and ITS2 regions of the rDNA, *TUB*, *ELO* and *COX* genes. The numbers at the branch points indicate the percentages of bootstrap values ≥50 %.

culture collections. The Buisman isolate deposited with CBS in 1930 (CBS 240.30) and an isolate reported to be from *Zantedeschia* in the Netherlands in 1927 (IMI 340618) have been examined by Kroon *et al.* (2004) and Cooke *et al.* (2000), respectively. In both cases, *P. richardiae* was found to be most closely related to *Phytophthora macrochlamydospora* in ITS-clade 9. In the United States, the CBS accession 240.30 is recorded as ATCC46734 (corresponding to ATCC60353) and its ITS sequence submitted to GenBank as FJ801949 also groups in clade 9. However, the sequences of five isolates published as *P. richardiae* in Blair *et al.* (2008) and recorded as *P. richardiae* in the *Phytophthora* database (www.phytophthora-db.org) all group within clade 8 alongside the GI isolates of *P. cryptogea* from this study. Four of the five are from calla lily in Japan isolated in the late 1980s and the fifth is purportedly

CBS 240.30, ATCC46734. The balance of the evidence suggests that the taxon originally isolated from calla lily by Buisman was *P. richardiae* (clade 9) and subsequently *P. cryptogea* has also been reported from calla lily and incorrectly named *P. richardiae* on the basis of its plant host.

This detailed study of the phylogenetic relationships amongst worldwide collections of *P. cryptogea* and *P. drechsleri* has resolved several issues. It is clear that misidentification of cultures has confused the taxonomy of this group and this has impacted our understanding of the pathogenicity and origins of these taxa of pathogens that remain significant plant health threats, particularly in the plant nursery industries. In this study we have confirmed that *P. drechsleri* is genetically, but not morphologically, distinct from *P. cryptogea* with growth at higher temperatures remaining a helpful means of discrimination. *P. cryptogea* itself comprises at least three molecularly distinct but, again, morphologically identical groups. Our evidence indicates a more recent introgression of the genomes of two of these groups and such processes are likely to be ongoing and widespread with increasing movement of these pathogens internationally. Broad host range and widely distributed heterothallic species such as *P. cryptogea* and *P. drechsleri* have greater opportunities for genetic exchange among and within sub-populations and this may explain the molecular diversity we observed. Inevitably the focus, to date, has been on isolates of lineages recognised for the problems they cause on the horticultural plants that act as their hosts and 'vectors'. It will be interesting to examine additional isolates of these clade 8 taxa from natural ecosystems to understand more about their centre of diversity, ecological role, distribution and potential future threat to plant industries worldwide.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.funbio.2010.02.001.

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