Phytopathologia Mediterranea (2013) 52, 1, 46–65

RESEARCH PAPER

Characterisation of *Phytophthora inundata* according to host range, morphological variation and multigene molecular phylogeny

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Summary. *Phytophthora inundata* is an Oomycetous plant pathogen that has been isolated from a range of different plant species. Although formally described in 2003, relatively little further characterisation of this taxon has been reported. This study presents an approach to phylogenetic discrimination of *P. inundata* based on concordance of multiple gene genealogies in relation to the host range and morphological traits of isolates from Iran (*Beta vulgaris*, *Pistacia vera*, *Solanum nigrum* and river water), United Kingdom (*Salix matsudana*), Spain (*Olea* sp.) and the United States of America (unknown host). Isolates were identified by rDNA internal transcribed spacer (ITS) sequence analysis, followed by assessment of pathogenicity, morphological, physiological (cultural, temperature relations and mating) and molecular traits. Multiple gene genealogy analyses were performed on regions of nuclear (ITS, β-tubulin, translation elongation factor 1α) and mitochondrial (cytochrome c oxidase subunit I) gene sequences of this and other major *Phytophthora* species. Congruence was observed in different phylogenetic data sets. Maximum likelihood phylogenetic reconstruction based on the different genes indicated that, although there is some variation between morphological and mating behaviour of *P. inundata* isolates, this taxon is a distinct and homogeneous species which always appeared as a sister taxon to *P. humicola*. *Phytophthora inundata* is shown to be a uniform species, with isolates from wide-ranging geographic origins having similar host ranges, molecular, and morphological characteristics.

Key words: Multiple gene genealogy, intraspecific variation.

Introduction

Phytophthora inundata Brasier, Sanch. Hern. & S.A. Kirk is a plant-pathogenic member of Oomycota that was first isolated from roots of *Aesculus hippocastanum* (horse chestnut) in Buckinghamshire, United Kingdom and roots of *Salix matsudana* in Kent, United Kingdom in 1970 and 1972, respectively (Brasier *et al.*, 2003b). The isolates had non-papillate sporangia, large oogonia, and were heterothallic with A1 and A2 compatibility types. The isolates were sexually sterile, but behaved as 'silent' A1 or A2 types, unable to produce their own gametangia but able to induce gametangial formation in the opposite sexual compatibility type of another species. Although showing a superficial resemblance to *P. gonapodyides*, the isolates were much faster growing at their optimum temperature, had a distinctive colony type, and exhibited an unusually high upper temperature limit for growth of 37°C. This *Phytophthora* was considered to be a new taxon, and was informally designated as 'O-group' (*i.e.* other group; Brasier *et al.*, 1993). This taxon has since been isolated from a variety of riparian habitats in Europe (Sánchez-Hernández *et al.*, 2001; Brasier *et al.*, 2003b), infected sugarbeet roots, *Solanum nigrum*, and pistachio in Iran (Mostowfizadeh-Ghalamfarsa *et al.*, 2006b), soil around carrot and parsley crops in Australia (Cunnington *et al.*, 2006), diseased alfalfa roots in USA (Ho *et al.*, 2006), infected roots and crown tissues of citrus trees

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ISSN (print): 0031-9465
ISSN (online): 1593-2095
www.fupress.com/pm

in Chile (Vial et al. 2006), dead *Xanthorrhoea preissii* in the south-west of Western Australia (Stukely *et al.*, 2007), stem pith and rhizosphere soil of *Nicotiana tabacum* in Virginia (Parkunan *et al.*, 2010), and decaying plants of *Zostera marina* in brackish water in the Netherlands (Man in't Veld *et al.*, 2011).

Molecular sequencing showed that *Phytophthora* sp. O-group had a distinct internal transcribed spacer (ITS) profile. It was a member of the major *Phytophthora* ITS clade 6 (Cooke *et al*., 2000) and subclade I of ITS clade 6 (Brasier *et al*., 2003a) together with *P. humicola*, *Phytophthora* taxon Walnut and *Phytophthora* sp. Apple-cherry. Brasier *et al.* (2003b) formally named *Phytophthora* sp. O-group as *P. inundata* sp. nov. They described *P. inundata* as a 'difficult' taxon to diagnose and recommended a test of growth rate at 28–30ºC to determine the upper temperature limit for growth and the analysis of the ITS sequence for accurate identification.

Kroon *et al.* (2004) demonstrated the utility of multiple-gene genealogies for investigating species boundaries among morphologically cryptic *Phytophthora* species, although they did not include some controversial *Phytophthora* species such as *P. melonis* and *P. inundata* in their study. Consideration of one isolate of *P. inundata* from the United Kingdom in a wider selection of *Phytophthora* based on multiple-gene genealogies confirmed its position in clade 6 (Blair *et al.*, 2008).

Studies on *P. inundata* to date have included only a few isolates associated with a limited number of plant species. The present study widens the investigation to a comparison of a diverse collection of *P. inundata* isolates associated with different plant species, and considers host range and pathogenicity data, molecular and morphological traits. The study also reports the phylogenetic discrimination and intra-specific diversity of *P. inundata* based on multiple gene genealogies.

Materials and methods

Organisms and cultural conditions

Details of the *P. inundata* isolates examined in this study are listed in Table 1. The isolates were sourced from the culture collections of the authors, or in the case of the Iranian isolates, directly isolated from the host tissue on PARP-H medium (Table 2) (Jeffers and Martin, 1986). For long-term storage, isolates were cultured on cornmeal agar (CMA, Table 2) slopes at 15°C. Routine stock cultures for research were grown on CMA at 20°C.

Colony morphology and growth rate

The isolates were grown at 20°C on CMA, CV8, PDA, HSA, MEA, and CA (Table 2). Petri dishes (9 cm diam.) containing 20 mL of the respective test media were inoculated with 5 mm diam. discs each cut from the edge of a 5-10 d-old culture. A disc was placed upside down in the centre of each plate, and the plates were incubated in the dark. Colony morphology was noted after 8 d. Growth rate measurements were 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 after 5 d. For temperature-growth relationships, CMA plates were inoculated using three replicate plates per isolate and incubated at 5, 10, 15, 20, 25, 30, 35 and 37°C. Growth rate was recorded 5 d after the onset of linear growth. Tests were repeated twice for the range of 30–37°C.

Morphology of sporangia and hyphal swellings

One disc (10 mm diam.), cut from the growing edge of a 7 d-old-culture grown on either CMA, PDA, HSA, CA, FBA, WA or PBA (Table 2) 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 suspended in 1 L distilled water for 24 h at room temperature and then filtered). Isolates were also grown individually on 9 cm Petri dishes containing either CMA, PDA, HSA, CA or FBA. As well, five sterile hemp seeds were put on a 7 d-old culture grown on CMA at 20°C in the dark. After 12 h hemp seeds were placed in a 9 cm Petri dish and individually flooded with non-sterile soil extract, Petri solution (Waterhouse and Blackwell, 1954) and Schmitthenner solution (Schmitthenner, 1970). Finally, Petri dishes were placed under two conditions: at 25°C in continuous fluorescent light or at 20°C in the dark for 24–72 h. Dimensions and characteristic features of 50 fully mature sporangia, chosen at random, were determined at ×400 magnification for each isolate.

Chlamydospore production

Isolates were grown individually in 9 cm Petri dishes containing CMA, PDA, HSA, CA, FBA, WA,

Table 1. Isolates of *Phytophthora inundata* examined in this study and their accession numbers for the GenBank database.

Isolates [a]	Host (matrix)	Year of isolation	Source of isolate	Accession [b]			
				ITS	*TUB*	*ELO*	*COX*
SCRP644	*Salix matsudana*	1972	UK	EF210200	EF210202	EF210204	EF210206
SCRP645	*Olea* sp.	1996	Spain	EF210201	EF210203	EF210205	EF210207
SUC21	*Pistacia vera*	1993	Iran, Ker.	AY659712	AY659722	AY659727	AY659732
SUC22	-	1999	USA	AY659713	-	-	-
SUC23	-	1999	USA	AY659714	AY659723	AY659728	AY659733
SUD47	water	1994	Iran, Far.	AY659715	-	-	-
SUKv1	*Beta vulgaris*	2002	Iran, Far.	AY659716	-	-	-
SUMp1	*Beta vulgaris*	2002	Iran, Far.	AY659717	AY659724	AY659729	AY659734
SUMp8	*Beta vulgaris*	2002	Iran, Far.	AY659718	-	-	-
SUSt6	*Solanum nigrum*	2002	Iran, Far.	AY659719	AY659725	AY659730	AY659735
SUSt7	*Solanum nigrum*	2002	Iran, Far.	AY659720	-	-	-
SUGK4	*Beta vulgaris*	2007	Iran, Far.	-	-	-	-

[a] SCRP644 = P246b (IMI389751) and SCRP645 = P894 (IMI390121) (Brasier *et al.*, 2003b);
[b] *COX*, cytochrome c oxidase subunit I; *ELO*, translation elongation factor 1α; Far., Fars province of Iran; ITS, Internal transcribed spacers; Ker., Kerman province of Iran; *TUB*, β-tubulin.

Table 2. Recipes of media used in this study.

Media [a]	Recipes
Carrot agar (CA)	Carrot extract 250 g L^{-1}; agar 15 g L^{-1}
Clear V8-juice agar (CV8)	V8 juice (Campbell's, UK) 100 ml L^{-1}; agar 15 g L^{-1}
Cornmeal agar (CMA)	Ground corn extract [b] 40 g L^{-1}; agar 15 g L^{-1}
French been agar (FBA)	Ground French been 30 g L^{-1}; agar 15 g L^{-1}
Hemp seed agar (HSA)	Ground hemp seed extract 60 g L^{-1}; agar 15 g L^{-1}
Malt extract agar (MEA)	Malt extract 25 g L^{-1}; agar 15 g L^{-1}
PARP-H	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 mg L^{-1} hymexazol
Pea broth agar (PBA)	Pea extract 125 g L^{-1}; agar 15 g L^{-1}
Potato dextrose agar (PDA)	Potato extract 300 g L^{-1}; dextrose 20 g L^{-1}; agar 15 g L^{-1}
Water agar (1%)	Agar 10 g L^{-1}

[a] Media such as carrot broth (CB), cornmeal broth (CM), French been broth (FB), hemp seed broth (HS), pea broth (PB), and potato dextrose broth (PD) prepared with the same recepies as above, without agar.
[b] Extracts were made by boiling each material in distilled water for one hour and filtering through cheese cloth.

or PBA, and 5 mL capacity bottles containing CM, PD, HS, CB, FB, or PB (Table 2), sterile distilled water and sterile soil extract. All media were supplemented with 30 mg L^{-1} β-sitosterol and 1 g L^{-1} $CaCO_3$. Petri dishes and bottles were placed in the dark at 25°C and 4°C. Chlamydospore production was examined every 15 d for 8 months. Isolates were also grown individually in 500 mL capacity flasks containing CB supplemented with 30 mg L^{-1} β-sitosterol and 1 g L^{-1} $CaCO_3$. Flasks were placed in the dark at 25°C. After 24 h flasks were shaken vigorously and placed horizontally. After 6 d, 100 mL sterile distilled water was added to every flask and flasks were placed vertically in the dark at 25°C. Chlamydospore production was examined every 15 d for 3 months. Two isolates of *Phytophthora cinnamomi* and *Phytophthora nicotianae* were used as positive controls.

Breeding system and morphology of oogonia, oospores and antheridia

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 HSA (amended with 30 mg L^{-1} β-sitosterol) plates using a 0.2 μm polycarbonate membrane to prevent gametangia of the different species or isolates from mixing. All isolates were paired with themselves, and with other isolates of *P. inundata* and A1 (IMI2668688) or A2 (IMI207770) mating types of *P. nicotianae* on amended HSA plates. Oospores produced on the *P. inundata* isolate side of each membrane were selected for measurements at ×400 magnification.

Pathogenicity

Host range

Inoculum of isolates was produced on mixtures of vermiculite and hemp seed extract (Banihashemi and Fatehi, 1989). Ten species of perennial plants and 20 species of annual plants were screened for their susceptibility to *P. inundata* (Table 3). Perennial plants were propagated as cuttings in plastic pots (one stem per pot) containing steamed soil mix (soil:sand, 1:1 v/v), and were grown on a greenhouse bench at 18 to 30°C. One to 2 year-old perennial plants were inoculated by spreading 50 mL of inoculum under the soil surface around the plant in each pot. Control plants received 50 mL of vermiculite and hempseed juice mixture. To assess the host range in annuals, seeds of the plants were sown in 10 cm diam. plastic pots (one seed per pot) containing steamed soil mix (soil:sand, 1:1 v/v) and were grown on a greenhouse bench at 18 to 30°C. One or 2 week-old seedlings were inoculated by adding 10 mL of inoculum over the soil surface around the plant in each pot. Control plants received 10 mL of a vermiculite and hempseed juice mixture. Mature (6 month-old) sugarbeet plants were also examined by adding 50 mL of inoculum under the soil surface around them. Roots were wounded by a sterile scalpel before inoculation, and unwounded control roots were also examined. After inoculation, plants were irrigated to keep the soil moist for 24 h, then the plants were watered daily and evaluated for wilt, defoliation and development of lesions on the stems every day for 8 weeks (for herbs) and 18 months (for perennial plants). The experiment was performed using a randomized complete block design with three replications.

Pathogenicity test on detached tree twigs

Five isolates from different plants and geographical areas were selected for pathogenicity tests. The 1 year-old detached twigs (*ca* 9–12 mm diam.) of 21 tree species (Table 4) were detached from leaves and cut to about 30 cm length and surface-disinfected using 96% ethanol. The cut ends of every detached twig and any other sites of damage were covered with melted paraffin. A *ca.* 7 mm diam. plug was removed from the bark in each detached twig and a 5 mm disc, cut from the edge of a 5–10 d-old culture, was placed under the bark and the cut was sealed with Nescofilm (Bando Chemical Ind. Ltd., Kobe, Japan) to avoid desiccation. Control plants received a CMA disc only. The detached twigs were placed in a bowl containing 150 mL sterile distilled water and kept at 25°C. The progress of the isolates in the detached twigs was examined by culturing the infected tissue on Petri dishes containing PARP-H medium. The experiment was performed in active (summer) and dormant (winter) growth stages and repeated six times.

Pathogenicity test on fruits, tubers and roots

The fruits of eight plant species (Table 5), tubers of potato and roots of carrot and sugarbeet were washed and disinfected with 96% ethanol. A 7 mm diam. plug was removed from each fruit, tuber or root. A 5 mm disc cut from a CMA culture was inserted in the gap and the plug was sealed with Nescofilm. Control plant organs each received a CMA disc. The

Table 3. Susceptibility of roots of different plant species to *Phytophthora inundata* isolates.

Family	Common name	Scientific name	Re-isolation					PCR detection
			SUGK4	SUMp1	SUC21	SUC23	SCRP 644	
Anacardiaceae	Pistachio	*Pistacia vera*	-	-	-	-	-	Negative
Amaranthaceae	Amaranth*	*Amaranthus* sp.	-	-	-	-	-	Negative
Moraceae	Fig	*Ficus carica*	-	-	-	-	-	Negative
Oleaceae	Olive	*Olea europaea*	-	-	-	-	-	Negative
Rosaceae	Sweet almond	*Prunus amygdalus* var. *dulcis*	+	+	+	+	+	Positive
	Apple	*Malus domestica*	-	-	-	-	-	Negative
	Peach	*Persica vulgaris*	-	-	-	-	-	Negative
	Cherry plum	*Prunus caspica*	-	-	-	-	-	Negative
Rutaceae	Lemon	*Citrus medica* var. *limonium*	-	-	-	-	-	Negative
Salicaceae	Weeping willow	*Salix babylonica*	-	-	-	-	-	Negative
Vitaceae	Grape	*Vitis sylvestris*	-	-	-	-	-	Negative
Asteraceae	Sun flower	*Helianthus annuus*	-	-	-	-	-	Negative
Chenopodiaceae	Sugarbeet	*Beta vulgaris*	-	-	-	-	-	Negative
	Sugarbeet**	*Beta vulgaris*	+	+	+	+	+	Positive
	Garden spinach	*Spinacia oleracea*	-	-	-	-	-	Negative
	Wild spinach*	*Chenopodium album*	-	-	-	-	-	Negative
Cucurbitaceae	Water melon	*Citrullus vulgaris*	-	-	-	-	-	Negative
	Melon	*Cucumis melo*	-	-	-	-	-	Negative
	Pumpkin	*Cucurbita pepo*	-	-	-	-	-	Negative
	Cucumber	*Cucumis sativus*	-	-	-	-	-	Negative
Fabaceae	Chick pea	*Cicer arietinum*	-	-	-	-	-	Negative
	Lentil	*Lens esculenta*	-	-	-	-	-	Negative
	French bean	*Phaseolus vulgaris*	-	-	-	-	-	Negative
	Vetch	*Vicia sativa*	-	-	-	-	-	Negative
Gramineae	Corn	*Zea mays*	-	-	-	-	-	Negative
	Bermuda grass*	*Cynodon dactylon*	-	-	-	-	-	Negative
Malvaceae	Gumbo	*Abelmoschus esculentus*	-	-	-	-	-	Negative
Solanaceae	Tomato	*Lycopersicon esculentum*	-	-	-	-	-	Negative
	Egg plant	*Solanum melongena*	-	-	-	-	-	Negative
	Thorn apple*	*Datura stramonium*	-	-	-	-	-	Negative
Umbelliferae	Parsley	*Petroselinum hortense*	-	-	-	-	-	Negative

*, Weed; **, Wounded.

Table 4. Susceptibility of detached twigs of different tree species to *Phytophthora inundata* isolates tested in active (Act.) or dormant host growth stage (Dor.).

Family	Common name	Scientific name	Re-isolation									
			SUGK4		SUMp1		SUC21		SUC23		SCRP644	
			Act.	Dor.	Act.	Dor.	Act.	Dor.	Act.	Dor.	Act.	Dor.
Aceraceae	Plane maple	*Acer platanoides*	-	-	-	-	-	-	-	-	-	-
Anacardiaceae	Pistachio	*Pistacia vera*	-	-	-	-	-	-	-	-	-	-
Caesalpiniaceae	Judas tree	*Cercis siliquastrum*	-	+	-	+	-	+	-	+	-	+
Juglandaceae	Walnut tree	*Juglans regia*	-	+	-	+	-	+	-	+	-	+
Magnoliaceae	Big laurel	*Magnolia grandiflora*	-	+	-	+	-	+	-	+	-	+
Moraceae	Fig	*Ficus carica*	-	-	-	-	-	-	-	-	-	-
	Red mulberry	*Morus rubra*	-	-	-	-	-	-	-	-	-	-
Oleaceae	Flowering ash	*Fraxinus ornus*	-	-	-	-	-	-	-	-	-	-
	Olive	*Olea europaea*	-	-	-	-	-	-	-	-	-	-
Papilionaceae	Locust tree	*Robina pseudacacia*	-	-	-	-	-	-	-	-	-	-
Platanaceae	Plane tree	*Platanus orientalis*	-	-	-	-	-	-	-	-	-	-
Punicaceae	Pomegranate	*Punica granatum*	-	-	-	-	-	-	-	-	-	-
Rosaceae	Sweet almond	*Prunus amygdalus* var. *dulcis*	-	-	-	-	-	-	-	-	-	-
	Plum	*Prunus domestica*	-	+	-	+	-	+	-	+	-	+
	Apple	*Malus domestica*	-	-	-	-	-	-	-	-	-	-
	Peach	*Persica vulgaris*	-	-	-	-	-	-	-	-	-	-
	Dog rose	*Rosa canina*	-	+	-	+	-	+	-	+	-	+
Rutaceae	Bitter orange	*Citrus bigaradia*	-	-	-	-	-	-	-	-	-	-
	Sweet lime	*Citrus limon*	-	-	-	-	-	-	-	-	-	-
Salicaceae	Weeping willow	*Salix babylonica*	-	-	-	-	-	-	-	-	-	-
Hippocastanaceae	Horse chestnut	*Aesculus hippocastanum*	-	-	-	-	-	-	-	-	-	-
Ulmaceae	Common elm	*Ulmus carpinifolia*	-	-	-	-	-	-	-	-	-	-

fruits, tubers and roots were placed in paper pockets and kept at 25°C. Three to 20 d after inoculation, the pieces of infected tissue of the fruits, tubers or roots were transferred on PARP-H medium to determine the progress of the isolates. The experiment was repeated six times with different fruits, tubers or roots.

Pink rot symptoms on potato tubers

As part of the procedure to discriminate *P. inundata* from *P. cryptogea*, *P. drechsleri* and *P. erythroseptica*, all isolates were evaluated for their ability to cause pink rot symptoms on potato tubers. Tubers were washed and steeped in 10% w/v sodium hypochlorite for 15 min, rinsed with sterile water and dried. A 7 mm diam. plug was removed from each tuber and a 5 mm diam. mycelial disc cut from a CMA culture was inserted before re-sealing with the original potato plug. The cut was sealed with Nescofilm to avoid desiccation and the potatoes were incu-

Table 5. Susceptibility of fruits, tubers and roots of different plant species to *Phytophthora inundata* isolates.

Family	Common name	Scientific name	Infected plant part	Re-isolation					PCR detection
				SUGK4	SUMp1	SUC21	SUC23	SCRP 644	
Actinidiaceae	Kiwifruit	*Actinidia arguta*	Fruit	+	+	+	+	+	Positive
Chenopodiaceae	Sugarbeet	*Beta vulgaris*	Root	+	+	+	+	+	Positive
Cucurbitaceae	Cucumber	*Cucumis sativus*	Fruit	-	-	-	-	-	Negative
	Pumpkin	*Cucurbita pepo*	Fruit	-	-	-	-	-	Negative
Rosaceae	Apple	*Malus domestica*	Fruit	+	+	+	+	+	Positive
Rutaceae	Lemon	*Citrus medica* var. *limonium*	Fruit	+	+	+	+	+	Positive
	Mandarin	*Citrus nobilis*	Fruit	+	+	+	+	+	Positive
Solanaceae	Tomato	*Lycopersicon esculentum*	Fruit	+	+	+	+	+	Positive
	Egg Plant	*Solanum melongena*	Fruit	+	+	+	+	+	Positive
	Potato	*Solanum tuberosum*	Tuber	-	-	-	-	-	Negative
Umbelliferae	Carrot	*Daucus carota*	Root	-	-	-	-	-	Negative

bated in the dark for 5 d at 20°C. The tubers were cut open and exposed to the air for 30 min before observations were recorded. An isolate of *P. erythroseptica* (SCRP242) also used as a positive control.

In all cases symptomatic tissues were assayed for the presence of *P. inundata* using PCR with specific primers (Mostowfizadeh-Ghalamfarsa and Safaie-Farahani, 2009).

DNA extraction

Isolates were each grown in 20 mL still culture of pea broth at 20ºC. After vacuum filtration, the mycelium was freeze-dried for extended storage at -20°C. DNA was extracted from the mycelium of each isolate using a Puregene DNA extraction kit (Flowgen, Lichfield, UK).

DNA amplification

DNA of the internal transcribed spacer regions (ITS) was amplified using the universal primers ITS6 and ITS4 (White *et al*., 1990; Cooke *et al*., 2000) (Table 7). 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 Oomycota (Cooke and Duncan, 1997). Fragments of the translation elongation factor 1 alpha gene (*ELO*) and the β-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 (Table 7). The region containing the mitochondrial cytochrome c oxidase subunit 1 (*COX*) gene fragment was amplified using COXF4N and COXR4N primers (Kroon *et al*., 2004; Table 7).

Amplifications were performed in a Primus 96 plus thermocycler (MWG-BIOTEC, Germany). The PCR mixture contained: 10 to 20 ng of template DNA, 1 μM of each primer, 100 μM of dNTPs, 0.4 U *Taq* DNA polymerase (Promega, Madison, WI, USA), 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. Successful amplification was confirmed by gel electrophoresis (1 h at 70 V) on 1.0% agarose gels in 1× TBE buffer. Gels were stained using ethidium bromide and DNA fragments were visualised under UV light. The PCR conditions for all regions are summarised in Table 8.

Table 6. Variation in morphological, physiological and colony characteristics of *Phytophthora inundata* isolates examined in this study.

Character		Isolate [a]										
		SUP644	SUGK4	SUC21	SUC22	SUC23	SUD47	SUKv1	SUMp1	SUMp8	SUSt6	SUSt7
Colony characteristics on												
CMA												
	Pattern	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni
	Growth rate[b]	N.P	N.P	6.4	5.8	5.8	5.3	7.1	6.7	6.6	7.1	6.9
CV8												
	Pattern	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni
	Growth rate	N.P	N.P	4.9	4.7	4.6	4.6	5	4.6	4.3	4.3	4.3
MEA												
	Pattern	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni
	Growth rate	N.P	N.P	6.1	5.5	5.5	3.3	5.6	5.3	5.5	5.7	5.9
HSA												
	Pattern	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni	Uni
	Growth rate	N.P	N.P	5.1	5.3	4.5	5	7.7	7	6.7	7	7
PDA												
	Pattern	Ros	Ros	Ros	Ros	Ros	Ros	Ros	Ros	Ros	Ros	Ros
	Growth rate	N.P	N.P	5.1	4.3	5.1	4.8	5.7	5.5	5.6	4.6	4.8
Average growth rate (mm d^{-1}) on CMA[c]												
5°		0.6	1.4	0.7	1.1	0.8	1.4	1.2	1.5	1.9	1.5	1.1
10°		2.2	3.2	3.2	3.4	3.2	3.1	3.7	3.7	3.2	3.7	3.5
15°		3.5	4.9	4.4	4.2	4.1	4.5	5.3	5.3	4.3	5.1	5.4
20°		3.9	6.7	6.8	6.1	4.8	5.4	6.2	6.2	5.6	6.3	6.1
25°		5	6.3	7.3	6.5	6.4	6.4	6.4	6.4	6.3	6.4	6.4
30°		**5.6**	**7.7**	**7.6**	**7.3**	**7.7**	**6.9**	**7.8**	**7.8**	**7.1**	**7.2**	**6.8**
35°		2.4	1.7	2.3	2.3	0.7	0.8	0.6	0.6	0.1	0.6	0.1
37°		0	0	0	0	0	0	0	0	0	0	0

(*Continued*)

Table 6. *Continues.*

Character	Isolate [a]										
	SUP644	SUGK4	SUC21	SUC22	SUC23	SUD47	SUKv1	SUMp1	SUMp8	SUSt6	SUSt7
Hyphae											
Average width (μm)	5	6	6	5	6	5	5	5	5	5	5
Hyphal swellings											
Production on solid media	–	–	–	–	–	–	–	–	–	–	–
Production in liquid media	+	+	+	+	+	+	+	+	+	+	+
Average length (μm)	41.20±6.12	43.10±6.3	40.58±5.3	37.71±5.5	43.63±5.7	40.28±5.3	39.90±8.7	42.35±5.2	40.54±5.5	36.18±5.8	39.35±4.5
Average width (μm)	36.52±5.21	37.80±5.45	35.19±5.1	36.11±4.6	40.81±6.2	37.12±4.2	35.50±3.8	37.71±4.6	39.72±5.8	30.81±5.3	37.25±4.6
Chlamydospores	–	–	–	–	–	–	–	–	–	–	–
Sporangia											
Papilla	–	–	–	–	–	–	–	–	–	–	–
Average length (μm)	68.8±8.3	77.5±3.1	71.2±4.4	73.25±4.3	75.58±4.9	73.88±4.5	72.42±5.1	72.59±6.1	75.45±4.5	72.25±4.1	74.32±10.3
Range length (μm)	59–77	65–78.5	70–80	70–82	67–85	61–81	71–82	62–87	64–77	65–81	60–95
Average width (μm)	50.5±8.7	57.9±3.7	58.3±6.32	58.9±4.1	61.33±3.6	59.35±4.2	60.02±6.1	58.55±6.7	54.61±4.7	58.92±4.3	54.42±8.8
Range width (μm)	45–61	50–67.5	50–70	49–71	55–70	44–68	51–72	45–70	50–66	51–72	42–72
Length: width ratio	1.36	1.23	1.21	1.24	1.36	1.24	1.36	1.23	1.38	1.22	1.36
Shape(s)	Op	Op	Op	Op,El	Op	Op,El	Op	Op	Op	Op,El	Op
Tapered base	+	+	+	+	+	+	+	–	–	+	–
Caducity	–	–	–	–	–	–	–	–	–	–	–
Proliferation	P/N	P/N	P/N	P/N	P/N	P/N	P/N	P/N	P/N	P/N	P/N
Average pore diam. (μm)	10	10	10	11	10	11	11	11	11	10	10
Production on solid media	–	–	–	–	–	–	–	–	–	–	–
Production in liquid media	–	–	–	–	–	–	–	–	–	–	–
Production on hemp seeds	+	+	+	+	+	+	+	+	+	+	+
Mating behaviour	A2	A1	A1	A1	A1	A1	S	A1	A1	S	A1

(*Continued*)

Table 6. *Continues.*

Character	Isolate [a]										
	SUP644	**SUGK4**	**SUC21**	**SUC22**	**SUC23**	**SUD47**	**SUKv1**	**SUMp1**	**SUMp8**	**SUSt6**	**SUSt7**
Oogonia											
Average diam. (μm)	34.81±5.1	32.18±5.7	33.28±5.1	35.21±2.6	35.90±2.9	34.58±3.2	–	34.08±4.4	36.5±2.5	–	35±3.3
Range (μm)	24–42	23–40	23–40	25–43	25–41	26–44	–	26–41	24–41	–	10–40
Tapered base	–	–	–	–	–	–	–	–	–	–	–
Oospores											
Average diam. (μm)	33.28±5.1	30.08±2.5	30±4.1	31.84±2.6	32.27±2.9	31.12±3.1	–	30±4.4	32.4±2.4	–	31.63±3.1
Range (μm)			19–38	19–35	23–33	23–38	–	21–33	23–36	–	21–40
Plerotic	–	–	–	–	–	–	–	–	–	–	–
Aplerotic	+	+	+	+	+	+	–	+	+	–	+
Wall average thickness (μm)	4	4	3	4	4	3	–	4	4	–	4
Antheridia											
Average diam. (μm)	18.9±1.1	15.5±0.6	15.9±1.4	16.8±1.2	16.36±1.4	15.25±1.3	–	15.16±0.5	16.66±1.1	–	16.27±1.4
Shape(s)	Sph, Cyl	Sph, Cyl	Sph, Rec	Sph, Cyl	Sph	Sph, Cyl	–	Sph	Sph	–	Sph
Inception	Amph	Amph	Amph	Amph	Amph	Amph	–	Amph	Amph	–	Amph

[a] Morphological data of SCRP645 are available in Brasier *et al.* (2003b) as P894.
[b] Average radial growth rate at 20°C (mm d^{-1}).
[c] Growth rate at optimum temperature shown in bold. +, Feature occurring frequently; –, Feature not observed; A1, A1 mating type; Amph, Amphigynous; Cyl, Cylindrical; El, Ellipsoidal sporangia; H, Homothallic; N, Nesting proliferation; Op, Obpyriform sporangia; P, Proliferating isolate; Rec, Rectangular; Ros, Rose–shaped; S, Sterile; Sph, Spherical; Uni, Uniform; N.P., Not performed.

Table 7. The primers used in this study and their characters.

Target DNA	Primer	Orientation	Primer sequence	Accession number[a]	Primer location[b]	Size[c] (bp)	Authors
Internal transcribed spacers 1, 2 and 5.8S gene of rDNA[a]	ITS6	Forward	5′ GAA GGT GAA GTC GTA ACA AGG 3′			860	Cooke *et al.*, 2000
	ITS4	Reverse	5′ TCC TCC GCT TAT TGA TAT GC 3′				White *et al.*, 1990
β-tubulin	TUBUF2	Forward	5′ CGG TAA CAA CTG GGC CAA GG 3′	U22050	570-589	989	Kroon *et al.*, 2004
	TUBUR1	Reverse	5′ CCT GGT ACT GCT GGT ACT CAG 3′	1538-1558			
Translation elongation factor 1α	ELONGF1	Forward	5′ TCA CGA TCG ACA TTG CCC TG 3′	AJ249839	180-199	972	Kroon *et al.*, 2004
	ELONGR1	Reverse	5′ ACG GCT CGA GGA TGA CCA TG 3′		1132-1151		
Cytochrome c oxidase subunit I	COXF4N	Forward	5′ GTA TTT CTT CTT TAT TAG GTG C 3′	U17009	9126-9147	972	Kroon *et al.*, 2004
	COXR4N	Reverse	5′ CGT GAA CTA ATG TTA CAT ATA C 3′	10076-10097			

[a] Reference to the GenBank accession containing the DNA sequence, on which the primer is based.
[b] Reference to the location of the primer within the original DNA sequence.
[c] Average amplicon length.

Table 8. PCR conditions for genes studied.

Gene[a]	Initial denaturation	Number of cycles	Denaturation	Annealing	Extension	Final Extension
ITS	95(120)[b]	30	95(20)	55(25)	72(50)	72(600)
TUB	94(120)	35	94(20)	60(25)	72(50)	72(600)
ELO	94(120)	35	94(20)	60(25)	72(50)	72(600)
COX	94(120)	35	94(20)	52(25)	72(50)	72(600)

[a] ITS, internal transcribed spacers 1, 2 and 5.8S gene of rDNA; *TUB*, β-tubulin; *ELO*, translation elongation factor 1α; *COX*, cytochrome c oxidase subunit I.
[b] Temperature '°C' (time 'sec').

Sequencing of amplified products

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

Phylogenetic analysis

A multiple gene genealogy approach as well as single-gene comparisons were applied for studying the phylogenetic relationships of *P. inundata* and other *Phytophthora* spp. *Phytophthora inundata* sequences generated in this study were compared to those of other taxa obtained from GenBank (Table 9). 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 both by distance-based and maximum likelihood methods in PHYLIP (Felsenstein, 1993). The transition/transversion parameter was estimated using the PUZZLE program (Strimmer and von Haeseler, 1996). This parameter was used in the PHYLIP DNAML (Felsenstein and Churchill, 1996) and DNADIST (Felsenstein, 1993) programs. The robustness of the DNAML tree was tested using 500 bootstrap trials. The trees were drawn using Treeview (Page, 1996). The GenBank accession numbers of *Phytophthora* species used for phylogenetic studies are shown in Tables 1 and 9.

Results

Sequence data and phylogenetic analyses

All isolates were confirmed as *P. inundata* in comparison with reference *P. inundata* ITS sequences (Brasier *et al*., 2003b) (Table 1). For four isolates representing the range of different matrices, as well as two isolates from the original description of the species (Brasier *et al*., 2003b), fragments of two additional nuclear genes and one mitochondrial gene were sequenced, including β-tubulin (*TUB*), translation elongation factor 1α (*ELO*), and cytochrome c oxidase subunit 1 (*COX*) (Table 1).

The rRNA gene ITS sequences of nine putative *P. inundata* isolates and the two reference isolates were 100% identical and they had an average of 99.3 and 94.5% identity with close matches, *P. humicola* and *P.* taxon Walnut respectively, over a *ca*. 816 bp sequence. The sequences of the *TUB*, *ELO* and *COX* genes also showed very low levels of intraspecific variation with 7, 6, and 4 base changes, respectively, amongst the six isolates. This corresponded to 99.0–99.8 % identity within *P. inundata* and an average of 98.2 and 94.9% identity with their closest matches *P. humicola* and *P.* taxon Walnut, respectively, over an alignment length of *ca.* 3560 bp. Phylogenetic trees based both on distance-based and maximum likelihood analysis of the four individual loci showed gene to gene concordance in all of the four trees (data not shown). The *P. inundata* clade was resolved as monophyletic in the four individual gene trees as well as in the combined tree (Figure 1) and consistently appeared as a sister group of *P. humicola*.

Growth rate and colony morphology

A comparison of the average radial growth rate of *P. inundata* isolates at different temperatures showed that the optimum temperature was 30°C (Table 6). All isolates produced uniform colony patterns on almost all media except PDA, on which colonies were rose-shaped in all cases (Table 6 and Figure 2).

Sporangia, hyphal swellings and chlamydospores

No sporangia were produced on sterile solid or liquid media. However, many sporangia were produced from infected hemp seeds or agar discs of isolates grown on FBA or PBA and flooded with non-sterile soil extract**.** Sporangia were non-papillate, non-caducous and ranged in shape from obpyriform to ellipsoid (Figure 3); with an average length of 73.5±10.3 μm, an average width of 57.6±10.3 μm, and an average length/width ratio of 1.3±1.1 : 1; with or without tapered bases. All isolates produced internal or external proliferation in their sporangia and some displayed sympodial sporangiophores. The average pore diameter was 10.6 μm (Table 6). Sporangia could be in groups of two to four. Spherical hyphal swellings were produced in liquid media, but not on solid media (Table 6). No chlamydospores were seen in liquid or on solid media using the dif-

Table 9. Accession numbers for the GenBank database of isolates of *Phytophthora* spp. used for phylogenetic analysis.

Species	ITS[a]	*TUB*[b]	*ELO*[c]	COX[d]
P. arecae	AF266781	AY564049	AY564105	AY564164
P. boehmeriae	DQ297406[e]	AY564050	AY564106	AY564165
P. botryosa	AF266784	AY564051	AY564107	AY564166
P. cactorum	AF266772	AY564052	AY564108	AY564167
P. cinnamomi	AF266764	AY564054	AY564110	AY564169
P. citricola	AF266784	AY564055	AY564111	AY564170
P. citrophthora	AF266785	AY564056	AY564112	AY564171
P. clandestina	AJ131989	AY564057	AY564113	AY564172
P. colocasiae	AF266786	AY564058	AY564114	AY564173
P. cryptogea	AF266796	AY564059	AY564115	AY564174
P. drechsleri	AF266798	AY564060	AY564116	AY564175
P. erythroseptica	AF266797	AY564061	AY564117	AY564176
P. fragariae	AF266762	AY564062	AY564118	AY564177
P. rubi	AF266761	AY564064	AY564120	AY564179
P. gonapodyides	AF266793	AY564066	AY564122	AY564181
P. heveae	AF266770	AY564067	AY564123	AY564182
P. humicola	AF266792	AY564069	AY564125	AY564184
P. idaei	AF266773	AY564070	AY564126	AY564185
P. ilicis	AJ131990	AY564071	AY564127	AY564186
P. infestans	AF266779	AY564035	AY564093	AY564150
P. inflata	AF266789	AY564072	AY564128	AY564187
P. insolita	AF271222	AY564073	AY564129	AY564188
P. iranica	AJ131987	AY564074	AY564130	AY564189
P. katsurae	AF266771	AY564075	AY564131	AY564190
P. lateralis	AF266804	AY564076	AY564132	AY564191
P. megakarya	AF266782	AY564078	AY564134	AY564193
P. megasperma	AF266794	AY564079	AY564135	AY564194
P. mirabilis	AF266777	AY564038	AY564095	AY564153
P. multivesiculata	AF266790	AY564080	AY564136	AY564195
P. nicotianae	AF266776	AY564081	AY564137	AY564196
P. palmivora	AF266780	AY564082	AY564138	AY564197
P. phaseoli	AF266778	AY564044	AY564101	AY564159
P. pseudotsugae	AF266774	AY564084	AY564140	AY564199
P. quininea	DQ275189[f]	AY564085	AY564141	AY564200
P. ramorum	AY54049[g]	AY564092	AY564149	AY564208

(*Continued*)

Table 9. *Continues.*

Species	ITS[a]	*TUB*[b]	*ELO*[c]	COX[d]
P. richardiae	AF271221	AY564086	AY564142	AY564201
P. sinensis	AF266768	AY564087	AY564143	AY564202
P. sojae	AF266769	AY564047	AY564104	AY564162
P. syringae	AF266803	AY564088	AY564144	AY564203
P. taxon Walnut[h]	AY659711	AY659721	AY659726	AY659731
P. tentaculata	AF266775	AY564089	AY564145	AY564203
P. vignae	AF266766	AY564090	AY564146	AY564205
Pythium aphanidermatum	AF271227	AY564048	EF426555[i]	AY564163

[a] ITS, internal transcribed spacers 1, 2 and 5.8S gene of rDNA (Cooke *et al.*, 2000). [b] *TUB,* β-tubulin (Kroon *et al.*, 2004). [c] *ELO,* translation elongation factor 1α (Kroon *et al.*, 2004). [d] *COX,* cytochrome c oxidase subunit I (Kroon *et al.*, 2004). [e] Dick *et al.*, 2006. [f] Belbahri *et al.* 2006. [g] Giltrap *et al.*, 2004. [h] Isolate SCRP233 (USA, *Juglans hindsii*, 1988) genes sequenced for this study. [i] *Pythium vexans* (Belbahri *et al.*, personal communication).

ferent methods, whereas the two control isolates of *P. cinnamomi* and *P. nicotianae* produced abundant chlamydospores in every case.

Breeding system and morphology of oogonia, oospores and antheridia

Isolates mainly were of the A1 heterothallic mating type, whereas one isolate from United Kingdom was A2 and some others were sterile and could not produce or induce any sexual organs (Table 6). Isolates produced oospores when paired with their opposite mating type isolates of *P. inundata* or *P. nicotianae*, but pairing *P. inundata* isolates produced significantly more oospores in all cases. Isolates produced globose oogonia with rounded bases. Oospores were aplerotic with average diameter of 32.3±3.3 µm. Isolates produced only amphigynous terminal antheridia with average diameter of 16.2±1.5 µm (Table 6, Figure 3).

Pathogenicity

Four months after inoculation of the perennial plants, only almond trees showed decline symptoms in the shoots in combination with rotting symptoms on the roots with all isolates tested. The pathogen was re-isolated from infected roots. No symptoms of disease were observed on any other perennial plants or seedlings of annual plants (Table 3). However, re-isolation from soil in pots showed that the isolates were alive and active. All isolates were pathogenic on wounded roots of mature sugarbeet plants causing rot lesions on the roots, whereas unwounded controls did not show any symptoms. All isolates caused spreading lesions in the detached twigs of five tested tree species in their dormant growth stage, but none could progress on twigs sampled during their active growth stage (Table 4). In addition, all of the isolates caused spreading lesions on some species of fruits (Table 5). None of the isolates produced any symptoms in potato tubers. In no case did control plants, detached twigs, fruits and tubers show any disease symptoms.

Discussion

Several new species have recently been described within *Phytophthora* ITS clade 6 (Hansen *et al.,* 2009; Jung *et al.*, 2011; Man in't Veld *et al.*, 2011), but *P. inundata* remains a distinct taxon. Maximum likelihood phylogenetic reconstruction of the ITS region of rDNA confirmed that all *P. inundata* isolates are distinct and uniform (data not shown). The *P. inundata* isolates grouped within the major ITS clade 6 (Cooke *et al.*, 2000) and, as confirmed in a recent study of *Phytophthora* species from ITS clade 6 subclade I (Jung *et al.*, 2011), their closest relatives in the clade was the homothallic *P. humicola*. Some GenBank accessions incorrectly named as *P. inundata* from a sister clade

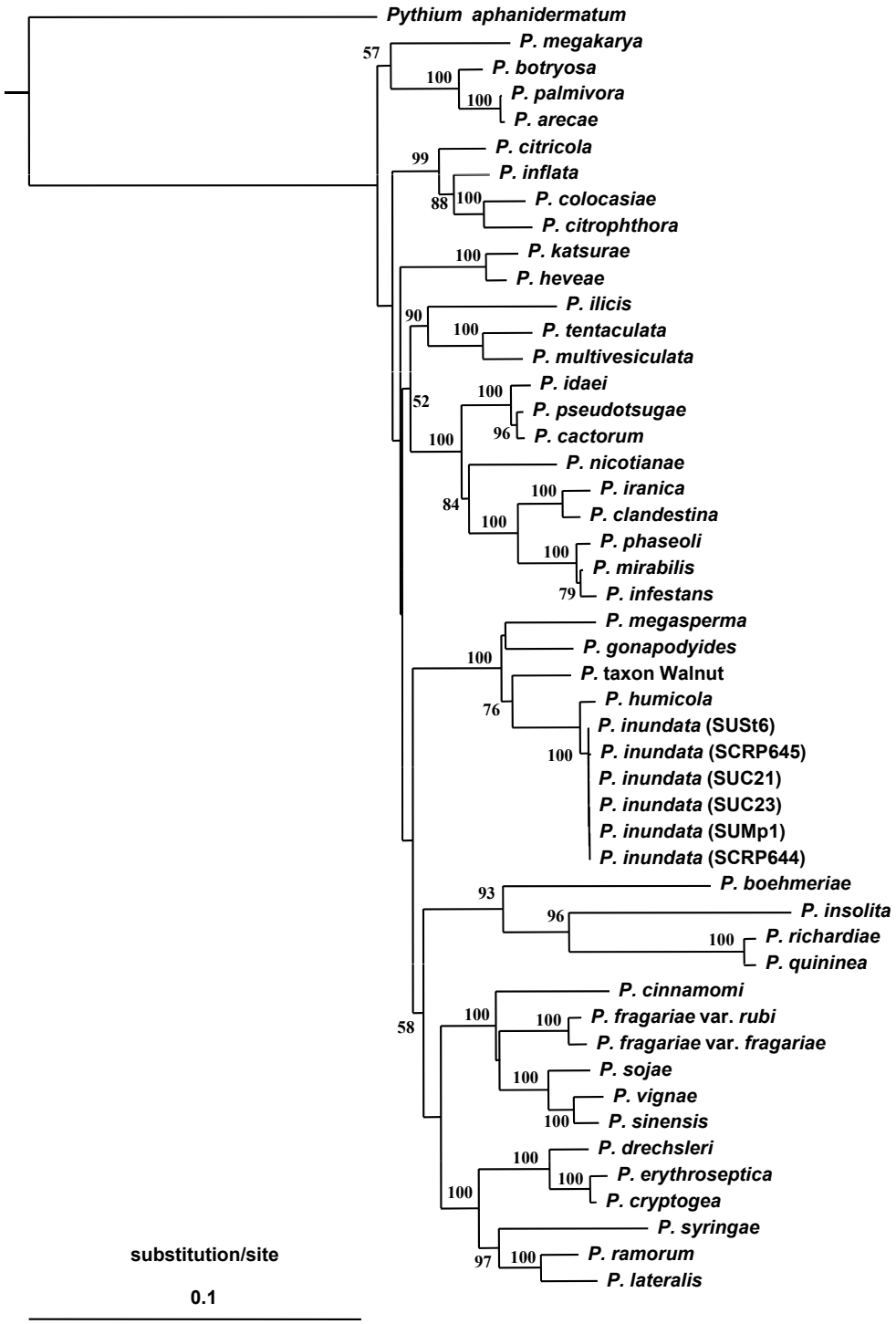

Figure 1. Combined genes phylogram of 42 *Phytophthora* taxa and six *Phytophthora inundata* isolates with different origins chosen as representatives of *P. inundata* isolates studied. The numbers within parentheses are those of the isolates. The phylogram was constructed after maximum likelihood analysis of the combined ITS1, 5.8S subunit, and ITS2 regions of the rDNA (Cooke *et al.*, 2000), β-tubulin, translation elongation factor 1α and cytochrome c oxidase subunit I (Kroon *et al.*, 2003) genes. The bootstrap values are indicated at the branch points (only values of 50% or more shown).

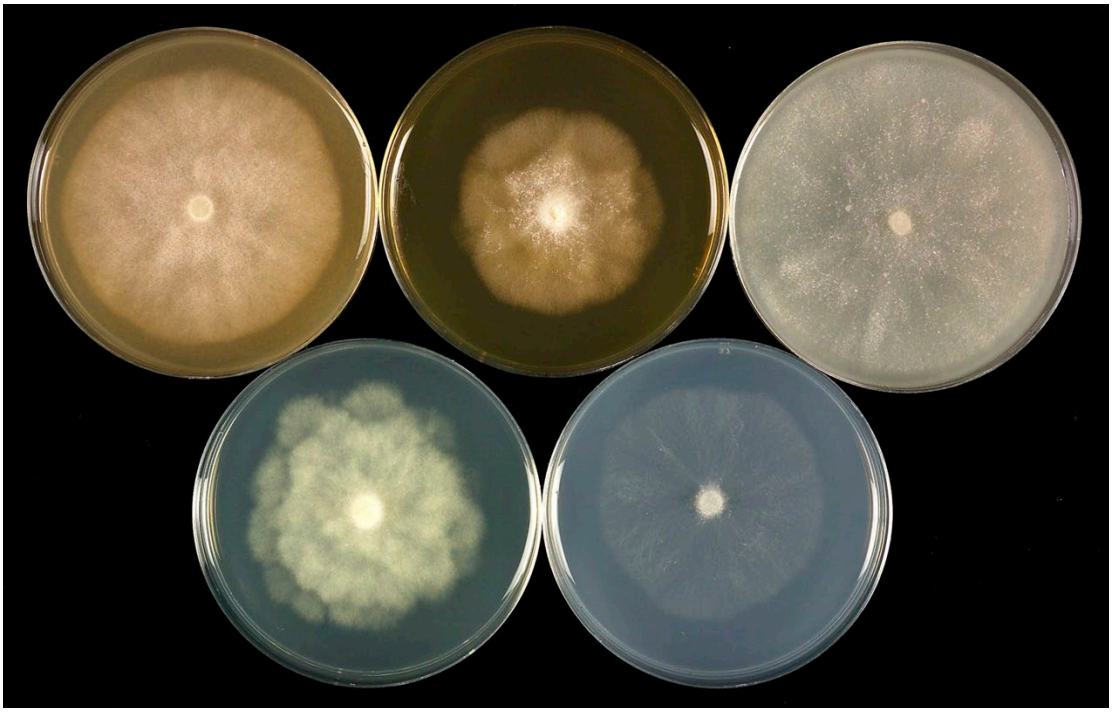

Figure 2. Colony morphology of *Phytophthora inundata*, isolate SUSt6 after 8 d at 20ºC; from left to right clarified V8 juice agar, malt extract agar, hemp seed agar, potato dextrose agar and cornmeal agar.

to *P. inundata* alongside an as yet undescribed, *Phytophthora* sp. (G. Abad, personal communication). Subclade II included the described morphospecies *P. gonapodyides*, *P. megasperma s. str.*, *P. thermophila, P. litoralis, P. gregata, P. gibbosa* and some other unnamed taxa (Brasier *et al.*, 2003a). The ITS regions of approximately 40 other *P. inundata* isolates available in GenBank were almost identical to those examined in this study, indicating that this is a widespread and uniform taxon (Table 1).

Maximum likelihood analysis of other genes for a subset of isolates as well as the combined gene tree (Figure 1) confirmed the homogeneity of *P. inundata*. Variation in the *TUB, ELO* and *COX* regions for the *P. inundata* isolates was limited and all phylogenies were congruent (data not shown). This taxon always appeared as a sister taxon to *P. humicola*. However, other studies have indicated a greater intraspecific diversity in *COX* sequences than ITS (Jung and Burgess, 2009; Jung *et al.*, 2011). Although Kroon *et al.* (2004) did not include this newly described species in their study, the present study confirms the location of this taxon within clade 6 of *Phytophthora sensu* Kroon *et al.* (2004).

Phytophthora inundata isolates produced rose-shaped colonies on PDA and CA, and uniform colonies on other media, supporting Brasier *et al.*'s (2003b) contention that the colony pattern on CA (irregular, stellate to broadly lobed) is a discriminative feature.

The isolates showed optimal growth at 30°C. The upper temperature limit for the growth of all isolates was 35°C (Table 6). Brasier *et al.* (2003b) reported a high optimum temperature for growth of 28–30°C and a high upper temperature limit for growth of 35–37°C.

None of the isolates produced sporangia on solid media, consistent with the finding of Waterhouse (1963), who reported that *Phytophthora* species with non-papillate sporangia do not produce sporangia on solid media. The best method to produce sporangia was to use hemp seeds and non-sterile soil extract. Light was not necessary for sporangium production. The average length/width ratio of *P. inundata* isolates was low (1.2:1), so that the sporangia were subglobose. This species has large sporangial

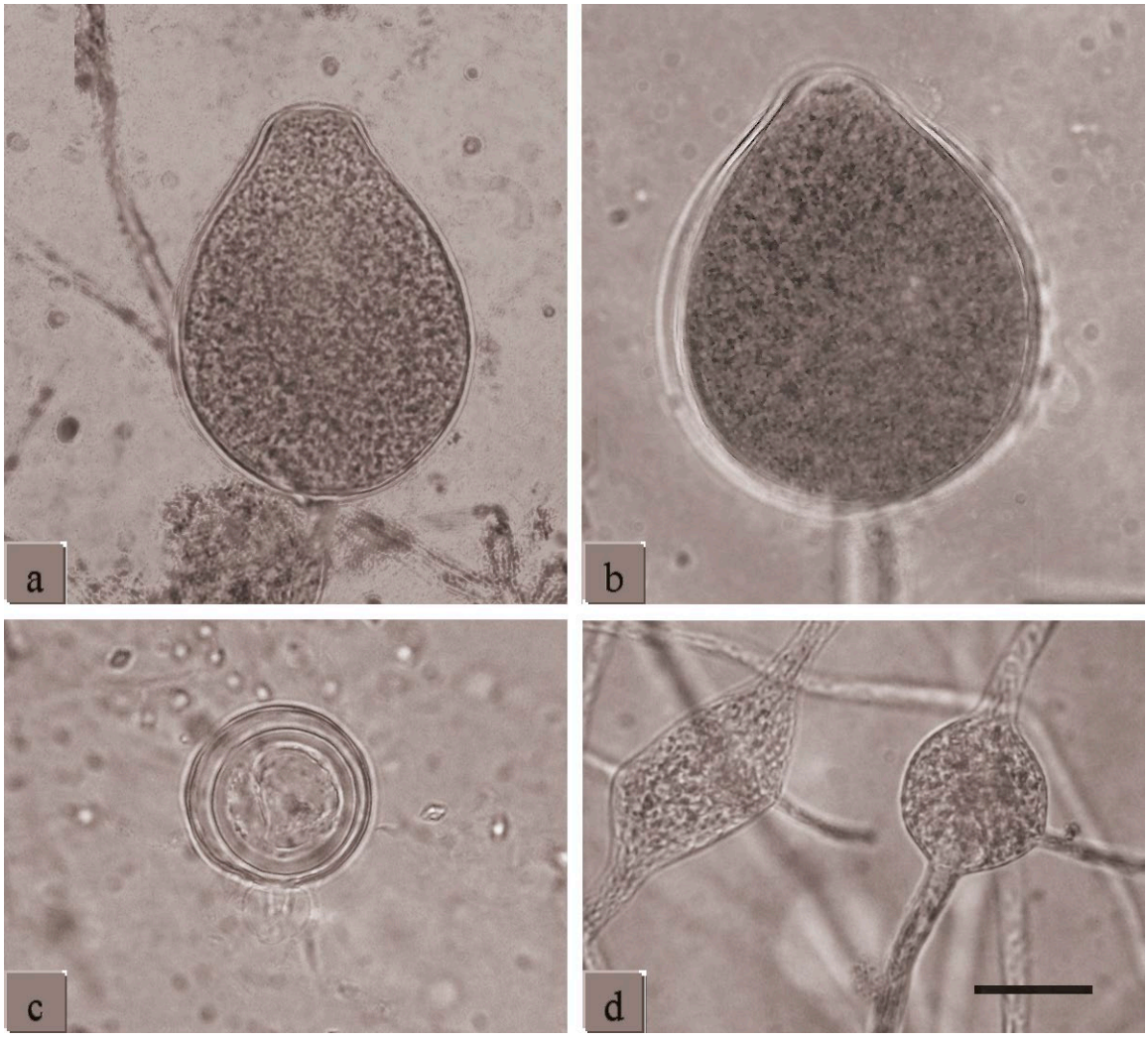

Figure 3. Sporangial morphology (a–b), characteristics of the oospores and antheridia (c) and hyphal swellings (d) of *Phytophthora inundata* (a = SUD47, b, c = SUC23 and d = SUSt6). Bar = 20 μm.

pores (average size 10.6 μm). Tightly sympodial sporangiophores, as well as internal proliferation, were observed in all isolates. No chlamydospores were produced from any of the applied methods, whereas two isolates of *P. cinnamomi* and *P. nicotianae* produced many chlamydospores using different methods. These results confirm the observation by Brasier *et al.* (2003b), so *P. inundata* is a species that does not produce chlamydospores, at least in artificial media. There is no other report about chlamydospore formation, but this should be examined in host tissues.

Brasier *et al.* (2003b) showed that *P. inundata* isolates are either normally heterothallic as A1 or A2 mating types or partially heterothallic, mating only with some A1s or A2s of their own species. This also explained that while some isolates appeared to be sterile, they behaved as 'silent' A1 or A2 sexual compatibility types, inducing oogonial formation in A1s or A2s of other heterothallic species. This indicates partial breakdown of the sexual mechanism in the species. Cunnington *et al*. (2006) and Parkunan *et al.* (2010) also reported that the *P. inundata* isolates in their studies were sterile. Similarly, in the present study, 18% of isolates were sterile and others were either A1 or A2. None of the isolates behaved as 'silent' A1 or A2 (Table 6). Oospores of our isolates (average

diam. 31.8±3.8 μm) were slightly smaller than those reported by Brasier *et al.* (2003b) for the type isolate (average diam. 35.7 μm).

The study of Sánchez-Hernández *et al*. (2001) indicated that *P. inundata* isolates were highly aggressive on roots of olive trees. In the present study, however, we examined olive trees for 18 months after inoculation and could not reproduce this result. We did not observe symptoms on olive shoots or roots, but re-isolation from soil suggested that the isolates had remained active in this experimental system. The difference in findings may relate to the differences in environmental conditions, cultivars or age of the trees tested. In our tests *P. inundata* isolates were not pathogenic on lemon trees, which is consistent with the observations of Vial *et al*. (2006). Parkunan *et al.* (2010) isolated *P. inundata* from *N. tabacum* plants, but in their pathogenicity tests these isolates were not pathogenic on *N. tabacum*. In the present study, none of the isolates resulted in any disease symptoms on seedlings of the 30 annual plants tested (Table 3), whereas sweet almond trees were susceptible to *P. inundata* isolates. Brasier *et al.* (2003b) reported the isolation of *P. inundata* from diseased roots of *Salix* sp., particularly after flooding events. However Koch's postulates have not been completed on these hosts. In our tests the *P. inundata* isolates were not pathogenic on *Salix* sp. under the conditions tested.

Phytophthora inundata was not pathogenic in tests on detached tree twigs when tested in an active growth stage, but detached twigs of five different tree species were susceptible to *P. inundata* when tested during dormancy (Table 4). This may be a result of lower activity of resistance mechanisms in trees in winter. Consistent with our results, Vial *et al.* (2006) reported that *P. inundata* was not pathogenic on some detached citrus twigs, and twigs of various trees were more susceptible to *P. parsiana* in dormant than in active growth stages (Hajebrahimi and Banihashemi, 2011). In another study, however, detached twigs of apple were more susceptible to *P. cactorum* in an active growth stage than during dormancy (Jeffers and Aldwinckle, 1986). Our results showed that eight of the different fruits, tubers or roots tested were susceptible to *P. inundata* (Table 5). Fruits of sweet lime developed small lesions after inoculation as also observed by Vial *et al.* (2006). *Phytophthora inundata* isolates did not induce pink rot or any other signs of disease in potato tubers, This could be a reliable trait for discrimination of *P. inundata* from other morphologically similar species such as *P. cryptogea* and *P. drechsleri*, which readily produce these symptoms (Mostowfizadeh-Ghalamfarsa *et al.*, 2006a). Cucumber fruits were also resistant to *P. inundata* isolates, whereas they are highly susceptible to some other morphologically similar species such as *P. melonis* (Erwin and Ribeiro, 1996), suggesting that this trait could also be employed to discriminate *P. inundata*.

In our studies, *P. inundata* isolates were not pathogenic on any of the weed species tested; therefore these weeds are not likely to be effective for disseminating this pathogen. However, our previous studies showed that roots of amaranth were colonized by *P. inundata* without any obvious symptoms (Safaie Farahani and Mostowfizadeh-Ghalamfarsa, 2010), so it is possible that weeds allow survival of the pathogen. We isolated *P. inundata* from infected roots of sugarbeet several times. Our tests showed that *P. inundata* is not pathogenic to unwounded sugarbeet plants and roots. However, all isolates infected wounded roots. Therefore, in many unproven reports of root rot in various plants, wounded tissue could have been involved. The pathogen may be an opportunistic invader of plants in which the natural defenses have been compromised by wounding.

On several occasions, *P. inundata* has been isolated from roots of trees after flooding (Brasier *et al.*, 2003b) or following exceptionally heavy rain (Sánchez-Hernández *et al.*, 2001). On other occasions, *P. inundata* has been isolated from river water or from pond debris (*e.g.* Brasier *et al.*, 2003b), suggesting that *P. inundata* is an inhabitant of, and adapted to, riparian ecosystems. The origins of our isolates, however, indicate that the species is not restricted to riparian ecosystems and can be found even in semiarid ecosystems (Table 1). Therefore, more studies of the aetiology, origin and genetic diversity of this species are required to fully elucidate habitat diversity.

This study has extended knowledge of this little-studied species by broadening the range of known habitats, examining the molecular diversity of a wider selection of isolates with additional sequenced regions and examining its morphology and pathology. The isolates examined are homogeneous in pathogenicity, molecular, and morphological traits and there are no differences among isolates originating from various plants. The homogeneity may be a result of recent derivation of *P. inundata* from other

Phytophthora species and an insufficient time period to generate and express variation. The observed uniformity could also be due to the distribution of a single clone from an as yet unknown centre of origin, and/or a reflection of a non-functional breeding system restricting the pathogen to clonal reproduction.

Acknowledgments

Dr. David A. Ratkowsky is specially thanked for his valuable comments on the paper. This study was funded by the Iran National Science Foundation (award number 86015.03). Dr David Cooke was funded by the Scottish Government, and cultures used in this study are held under licence number PH/20/2011.

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Accepted for publication: July 30, 2012