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RESEARCH PAPERS

Phytophthora ilicis as a leaf and stem pathogen of *Ilex aquifolium* in Mediterranean islands

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Summary. Between 2010 and 2013 several English holly trees showing defoliation, leaf and twig blight were found in natural ecosystems in Sardinia and Corsica. A *Phytophthora* species was consistently isolated from necrotic tissues of leaves and twigs, and bleeding cankers on stems. Isolates obtained were identified as *Phytophthora ilicis*, on the basis of morphological features, colony patterns and growth rates at different temperatures. Identity was confirmed by sequence analyses of the ITS, β-tubulin and *cox*1 gene regions. Two different genotypes within *P. ilicis* were detected. Pathogenicity of *P. ilicis* isolates was verified by inoculating freshly cut twigs of English holly. *Phytophthora ilicis* was originally recorded on English holly in western USA, and to date has been found only in cool temperate regions in northern America and, more recently, in Europe. This is the first report of *P. ilicis* on English holly in the Mediterranean region. Two additional *Phytophthora* species were isolated from other trees and shrubs species growing together with English holly, including *P. bilorbang* from *Alnus glutinosa* leaves and *P. pseudosyringae* from rhizosphere soil samples of *A. glutinosa* and *Castanea sativa*, and from symptomatic leaves of *Hedera helix*.

Key words: holly disease, oomycetes, canker, natural ecosystems, phylogeny.

Introduction

English holly (*Ilex aquifolium* L.) in the *Aquifoliaceae* is an evergreen tree or shrub native to Europe and western Asia (Camarda and Valsecchi, 2008). In Mediterranean regions its distribution is usually restricted to cool and humid mountain ecosystems. In Sardinia (Italy), English holly grows exclusively inland between 600 and 1500 m above sea level (a.s.l.), particularly on Gennargentu Mountain, where it forms ancient woodlots surrounded by grasslands with dispersed shrubs and, locally, almost pure stands. English holly trees are also found associated with *Castanea sativa* Mill. and *Quercus* spp., often as a subordinate shrub/tree layer accompanied by other species such as *Crataegus monogyna* Jacq., *Erica arborea* L., *Hedera helix* L. and *Taxus baccata* L. It is also common along streams together with *Alnus glutinosa* (L.) Gaertn. Similarly, English holly is frequently found in the mountain areas as an understorey species in *Fagus sylvatica* L. and *Quercus* spp. forests in the nearby island of Corsica (France).

In July 2010, severe defoliation and dieback were observed on several English holly trees located along a mountain slope in the Gennargentu area, in Sardinia. Infected trees showed symptoms including twig blight and black spots on leaves, and occasionally cankers/lesions on the main stems and large branches. In October 2013, a similar situation was noticed on English holly trees in natural forests in Corsica. The detection of diseased English holly trees in Mediterranean natural forests was new, and the type of infections observed on the upper parts of affected trees suggested that aerial infection was

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occurring. In both cases, the symptoms detected on English holly trees resembled those described for infections by *Phytophthora ilicis* Buddenh. & Roy A. Young in Oregon and Washington, USA (Buddenhagen and Young, 1957) and in the United Kingdom (Strouts *et al*., 1989). Therefore, the main objective of the study described here was to establish whether *P. ilicis* was involved in the defoliation and dieback of English holly trees in natural Mediterranean forests in Sardinia and Corsica. A second objective was to study and compare the intraspecific variability in terms of morphology, growth rate and phylogeny amongst *P. ilicis* isolates from the two Mediterranean islands and the United Kingdom.

Materials and methods

Sampling and *Phytophthora* isolation

In July 2010, leaves and twigs showing black to dark-brown lesions, as well as necrotic bark tissues from stems, were collected from six English holly trees growing along a stream in Sardinia (site 1, Tonara, 40°02′N, 9°12′E; 1250 m a.s.l.) and from ten old trees (80 cm in diam. at breast height) located on a mountainside (site 2, Desulo, 40°01′N, 9°14′E; 1400 m a.s.l.). Both sites were revisited twice, in November 2010 and April 2011, and disease incidence (% of symptomatic trees) assessed visually on 32 and 60 trees in site 1 and site 2, respectively. Further English holly trees were sampled in October 2013 in Corsica (site 3, Forêt d'Aitone, 42°15′N, 8°50′E; 1120 m a.s.l.). Samples were placed in plastic bags with distilled water, transported in cool boxes to the laboratory and processed within 24 h.

Prior to isolation, samples were washed and surface disinfected with 70% ethanol for 60 s followed by a 60 s rinse in sterile distilled water, and were then left to air dry on a clean paper towel. Small pieces of plant tissue (c. 2 × 2 mm) were aseptically cut from the transition zone between dead and living tissue and plated onto both potato dextrose agar (PDA; Oxoid Ltd) and synthetic Mucor agar (SMA) medium (Elliott *et al*., 1966) supplemented with 50 mL carrot juice and after autoclaving at 121°C for 15 min amended with 0.4 mL of a 2.5% (w:v) aqueous suspension of pimaricin and 3 mL of a 1% (w:v) aqueous solution of rifamycin SV sodium salt (both from Sigma-Aldrich). After 2-3 d, developing colonies were sub-cultured to carrot agar (CA: 15 g agar technical No. 3, Oxoid Ltd; 2.4 g $CaCO_3$; 200 g carrot; 1 L distilled water) (Brasier, 1967). Rhizosphere soil samples were also collected from symptomatic English holly trees and tested for the presence of *Phytophthora* using the baiting method with young leaflets of *Quercus suber* seedlings (Jung *et al*., 1996). Additional isolates sourced from Forest Research - *Phytophthora* culture collection, Farnham, United Kingdom, were used for comparative purposes and are listed in Table 1.

Growth rates and morphological characterization

Colony morphology of *Phytophthora* isolates was assessed visually on CA after 9 d incubation in the dark at 20°C. Temperature-growth relationships were determined on CA at 5, 10, 15, 20, and 25°C (Scanu *et al*., 2014), with three replicates per isolate; the experiment was performed twice.

Sporangia were produced by transferring 5 mm diam. agar plugs of actively growing mycelium on CA to Petri dishes containing non-sterile pond water. Gametangia were examined from 3-week-old colonies grown on CA in darkness at 20°C. Measurements and photographs were made at 200× and 400× magnification and recorded using a digital camera connected to an Olympus BX51 compound microscope and CellD imaging software (Olympus).

DNA extraction, amplification and sequencing

DNA was extracted from mycelium following the CTAB method of Doyle and Doyle (1987). Three gene regions were targeted for PCR and DNA sequencing, including the nuclear ITS and β-tubulin regions, and part of the mitochondrial *cox*1 gene region. ITS regions were amplified using primers ITS-6 (Cooke *et al*., 2000) and ITS-4 (White *et al*., 1990); β-tubulin using primers Btub F1 (Blair *et al*., 2008) and Btub R1 (Kroon *et al*., 2004); *cox*1 using primers FM 84 and FM 83 (Martin and Tooley, 2003). PCR conditions and reaction mixture were those described by Scanu *et al*. (2014). PCR amplification products were purified using the Zymo-SpinTM IC_XL Column extraction kit (ZymoResearch) and sequenced in both directions with a BigDye version 3.1 Ready Reaction Kit on an ABI Prism 3730 capillary sequencer (Life Technologies, Applied Biosystems). DNA sequence chromatograms were viewed and edited using BioEdit v. 5.0.6 software (Hall, 2001) and compared with sequences deposited in GenBank using the BLAST software

Table 1. Locations, hosts, isolation data and GenBank accession numbers for *Phytophthora* isolates used in this study.

Phytophthora sp.	Collection No.[a]	Host, substrate	Location, country, year	Habitat	Collector	GenBank Accession No.[b]		
						ITS	Cox1	β-tubulin
P. ilicis	PH046	*Ilex aquifolium*, twig blight	Sardinia, Italy, 2010	Riparian forest	B. Scanu	KJ458956	KJ458935	KJ458960
	PH061	*I. aquifolium*, twig canker	Sardinia, Italy, 2010	Riparian forest	B. Scanu	n. a.	n. a.	n. a.
	PH136	*I. aquifolium*, leaf blight	Sardinia, Italy, 2011	Natural forest	B. Scanu	KJ458949	KJ458936	KJ458964
	PH137	*I. aquifolium*, stem lesion	Sardinia, Italy, 2011	Natural forest	B. Scanu	KJ458954	KJ458937	KJ458965
	PH181	*I. aquifolium* twig blight	Corsica, France, 2013	Natural forest	B. Scanu	KJ458952	KJ458938	n. a.
	PH182	*I. aquifolium*, leaf blight	Corsica, France, 2013	Natural forest	B. Scanu	KJ458951	KJ458939	n. a.
	8991	*I.* aquifolium, n. a.	n. a.	Large garden	J. Denton	KJ458950	KJ458940	KJ458963
	P1567	*Ilex dipyrena*, branch lesion	Cornwall, UK, 2003	Large garden	S. Denman	KJ458955	KJ458941	KJ458962
	P1900	*I. aquifolium*, leaf blight	Cornwall, UK, 2005	Large garden	n. a.	KJ458953	KJ458942	KJ458961
P. pseudosyringae	PSEU6*	*Quercus robur*, soil	Bavaria, Germany, 1997	Forest	T. Jung	KJ466367	KJ458948	n. a.
	PH040	*Castanea sativa*, soil	Sardinia, Italy, 2010	Natural forest	B. Scanu	KJ466366	n. a.	n. a.
	PH048	*Alnus glutinosa*, soil	Sardinia, Italy, 2010	Riparian forest	B. Scanu	KJ458957	KJ458943	KJ458966
	P2177	*Nothofagus obliqua*, stem lesion	Hampshire, UK, 2011	Plantation	J.F. Webber	*JN542830*	KJ458944	n. a.
	PH157	*Hedera helix*, leaf blight	Sardinia, Italy, 2013	Riparian forest	B. Scanu	KJ458958	KJ458945	n. a.
P. psychrophila	PH097	*Quercus ilex*, soil	Sardinia, Italy, 2011	Natural forest	B. Scanu	KJ458959	KJ458947	n. a.
	PH151	*Q. ilex*, soil	Sardinia, Italy, 2012	Natural forest	B. Scanu	n. a.	KJ458946	n. a.
P. bilorbang	PH164	*A. glutinosa*, leaf blight	Sardinia, Italy, 2013	Riparian forest	B. Scanu	KJ466368	n. a.	n. a.
	PH200	*A. glutinosa*, leaf blight	Sardinia, Italy, 2013	Riparian forest	B. Scanu	n. a.	n. a.	n. a.
	PH201	*A. glutinosa*, leaf blight	Sardinia, Italy, 2013	Riparian forest	B. Scanu	n. a.	n. a.	n. a.

Abbreviations of isolates and culture collections: PH = culture collection of the University of Sassari; P = Forest Research *Phytophthora* culture collection, Farnham, UK. Sequence numbers in italics were retrieved from GenBank, all others were determined in this study; *Ex-type isolate; n. a. = not available.

(http://blast.ncbi.nlm.nih.gov). Sequences obtained in this study were deposited in GenBank and accession numbers are shown in Table 1.

Phylogenetic analysis

Seven ITS sequences of *Phytophthora* species from ITS Clades 3 and 7 (Cooke *et al*., 2000) were downloaded from GenBank, including *P. nemorosa* E.M. Hansen & Reeser (AY332651, HQ643296), *P. pluvialis* Reeser, W. Sutton & E.M. Hansen (KC529657, HN004217), *P. psychrophila* T. Jung & E.M. Hansen (AF449494) and *P. parvispora* Scanu & Denman (GU460376, KC478667), and combined with the sequences derived in this study. DNA sequences were aligned with ClustalX v. 1.83 (Thompson *et al*., 1997). Phylogenetic analyses of sequence data were implemented using PAUP v.4.0b10 (Swofford, 2003) for Maximum-parsimony (MP) analysis and MrBayes v.3.0b4 (Ronquist and Huelsenbeck, 2003) for Bayesian Inference (BI) analysis. The general time-reversible model of evolution (Rodriguez *et al*., 1990), including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories (GTR+Γ+G) was used for BI analysis. Maximum-parsimony analysis was performed using the heuristic search option with 1000 random taxon additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Maxtrees were set to 500, branches of zero length were collapsed, and all multiple equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated from 1000 bootstrap replications (Hillis and Bull, 1993). Bayesian analysis employing a Markov Chain Monte Carlo (MCMC) method were performed. Four MCMC chains were run simultaneously, starting from random trees for 1,000,000 generations. Trees were sampled every 100th generation for a total of 10,000 trees. The first 1,000 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang, 1996) were determined from a majority-rule consensus tree generated with the remaining 9,000 trees.

Pathogenicity tests

Pathogenicity of five *P. ilicis* isolates (PH046, PH136, PH137, P1900 and P1567) from different locations was tested by inoculations of freshly cut twigs of English holly following the method used by Scanu *et al*. (2013). Two isolates of *P. pseudosyringae* T. Jung & Delatour (PH048 and P2177) were also included in the experiment. In April 2011, twigs (30 cm long, 1 cm diam.) were cut from two English holly trees and the cut ends were sealed with Parafilm. A 5 mm diam. hole was punched through the bark to the sapwood surface of each twig using a cork borer. A 5 mm agar plug taken from the margin of 7-d-old *P. ilicis* or *P. pseudosyringae* culture grown on CA was then inserted into the wound and the bark plug replaced. Wounds were wrapped in damp cotton wool and covered with a piece of aluminium foil followed by Parafilm. Inoculated twigs were enclosed in plastic bags and maintained at 20°C for 2 weeks in natural daylight. Six replicates were prepared for each isolate. Six twigs, inoculated with a sterile CA plug, were used as experimental controls. At the end of the experiment, outer bark was removed, then from the area surrounding each inoculation point the visible phloem lesion length was measured. Re-isolation was attempted by transferring ten pieces of diseased tissues taken from the margin of each lesion to CA at 20°C.

Statistical analyses

Morphometric and pathogenicity data were analysed by a one-way analysis of variance (ANOVA) using Tukey's HSD test (Honestly Significant Difference) as a post-hoc test (XLSTAT 2008 software). Differences at $P<0.05$ were considered significant. Analysis of the differences in growth rates between the two *P. ilicis* groups was performed using the Student's t-test ($P<0.01$).

Results

Symptoms

Infections of foliage and twigs of English holly trees (Figure 1) were found in all investigated sites in both Sardinia and Corsica. In the Gennargentu area (Sardinia), symptoms of defoliation and dieback (Figure 1a) were most common on north-facing slopes, particularly alongside pathways and streams. Close inspection of symptomatic trees revealed the presence of twig cankers (Figure 1b) that became ringed with brownish-orange cork tissue

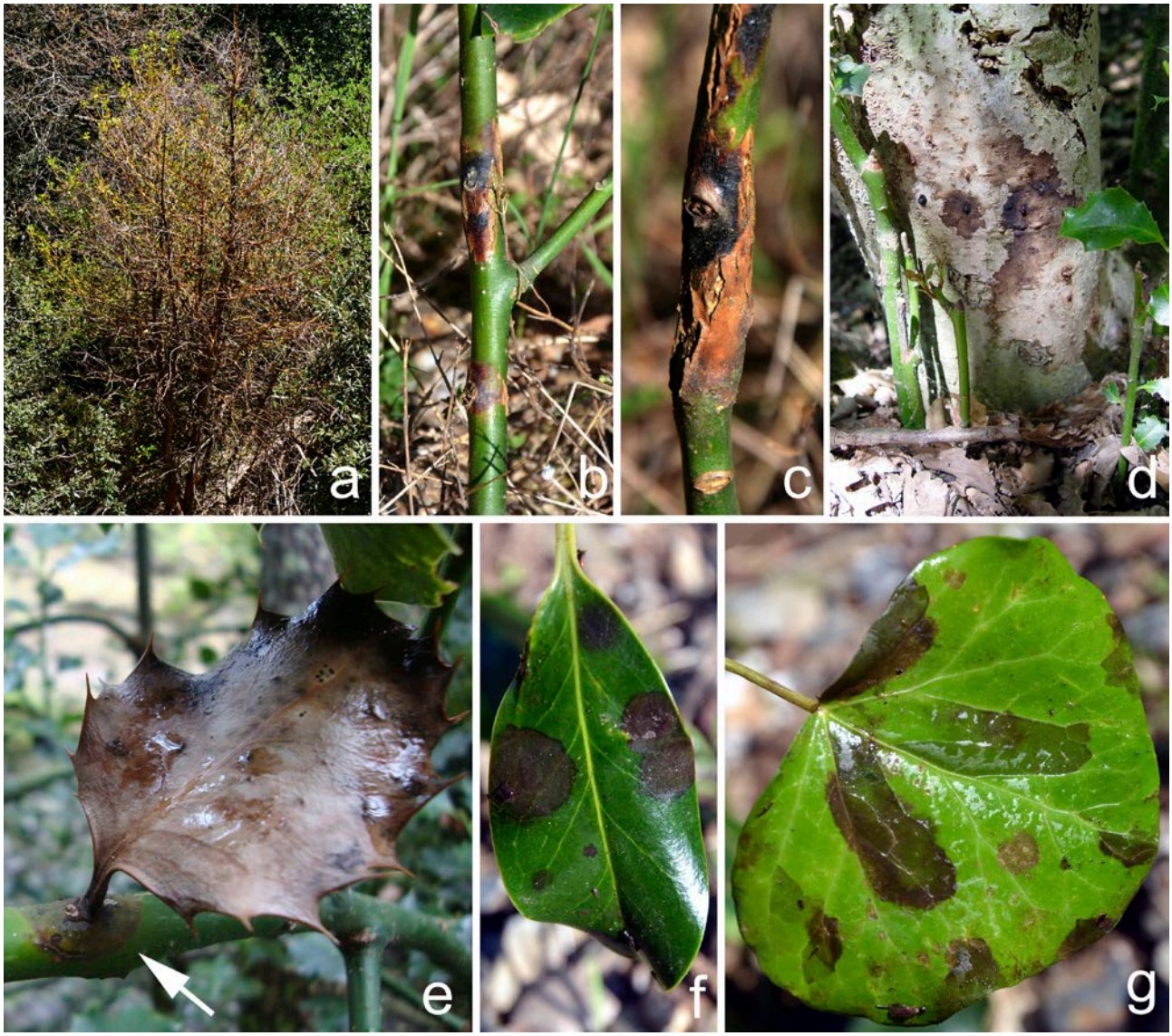

Figure 1. Disease symptoms on *Ilex aquifolium*: mature tree showing complete defoliation (a), twig cankers (b), detail of a twig canker with brownish-orange suberized tissues during the dry season (c), bleeding lesion on the stem (d), fresh lesion around an axillary node (arrow) (e), black rounded spots on the leaf (f). Necrotic lesions on a heavily infected leaf of *Hedera helix* (g).

during the dry season (Figure 1c). Cankers/lesions on the stems and branches were also observed (Figure 1d), often girdling them, and resulting in death of the distal portions. Infection frequently occurred on the axis of petioles, leading to the withering of leaves (Figure 1e). Black spots on the foliage (Figure 1f), generally concentrated in the lower parts of the crown of each tree, were frequently observed. Disease incidence was 62.5% at site 1 and 42.9% at site 2. Seven of the 92 trees assessed were already dead, although the cause could not be attributed to a single factor. Understorey *H. helix* beneath the holly canopy also appeared to be infected, exhibiting heavy foliar and shoot infection as well as defoliation (Figure 1g). Black leaf spots were also detected on young *A. glutinosa* trees.

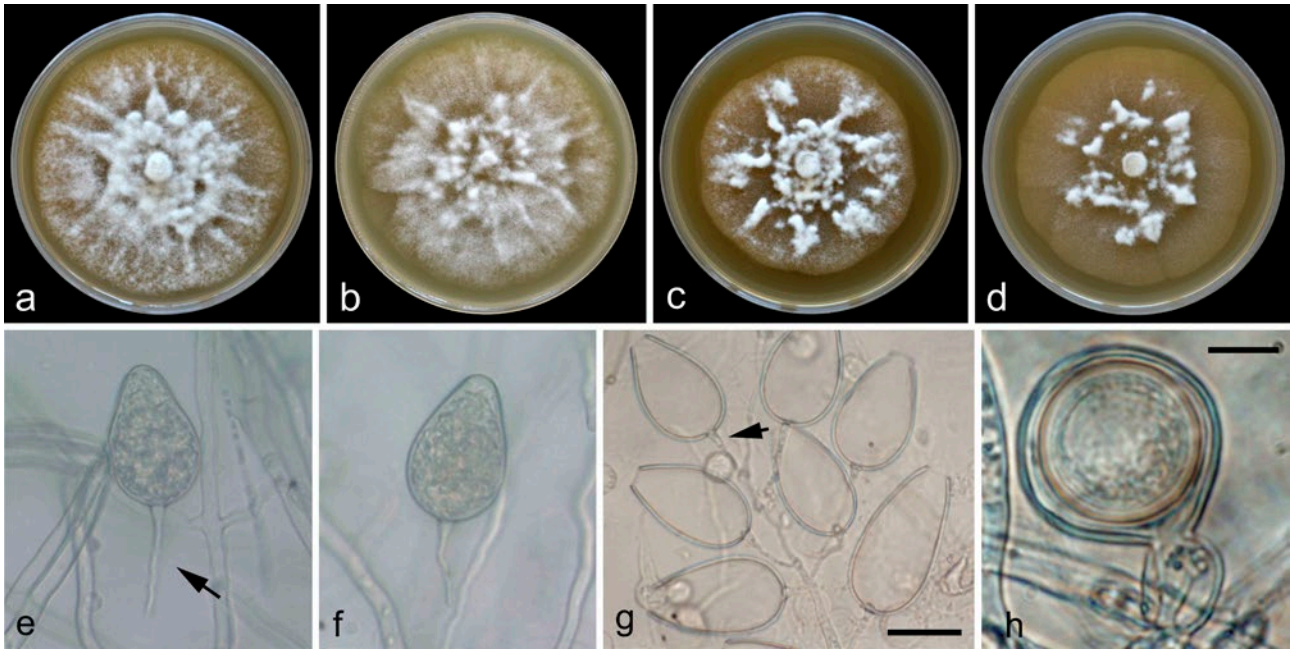

Figure 2. Colony morphologies of *Phytophthora ilicis* isolates after 7 d on carrot agar: PH046 and PH136 from Sardinia (a-b), P1900 and P1567 from the United Kingdom (c-d). *Phytophthora ilicis* structures: non-papillate caducous sporangium with long pedicel (arrow) (e), semi-papillate caducous sporangium (f), sympodial sporangiophores with empty sporangia and short pedicel (arrow) (g), oogonium with amphyginous antheridia (h). Bars = 20 μm for sporangia and 10 μm for oogonium.

Identification of *Phytophthora* isolates

Based on colony morphology in culture and sequencing of the ITS region, the *Phytophthora* species isolated from infected tissues of English holly trees was identified as *P. ilicis*. Isolates obtained from black spots on *A. glutinosa* leaves were identified as *P. bilorbang* Aghighi & T.I. Burgess, whereas those isolates obtained from rhizosphere soil samples of declining *A. glutinosa* and *C. sativa* trees, as well as from symptomatic leaves of *H. helix*, were identified as *P. pseudosyringae* (Table 1).

Growth rate and morphology

Colony growth patterns of *P. ilicis* on CA at 20°C showed low variation amongst isolates from Sardinia and Corsica and were generally stellate with appressed mycelium (Figure 2a, b). The two isolates from the United Kingdom (P1900 and P1567) formed distinctive colony patterns on CA, exhibiting faintly stellate mycelium with fluffy patches and submerged mycelia in the periphery (Figure 2c, d). All *P. ilicis* isolates had identical cardinal temperatures for growth on CA, with a minimum of <5°C and maximum of 24°C; none of the isolates grew at 25°C. *Phytophthora ilicis* isolates from Sardinia and Corsica showed radial growth rates of 5.4 ± 0.2 mm d^{-1} (mean ± SD) at the optimum temperature (20°C). Radial colony growth rates of the two isolates of *P. ilicis* from the United Kingdom at 20°C were significantly less (Student's t-test $P<0.01$) than the overall mean of the isolates from Sardinia and Corsica, which exhibit a mean growth rate of 4.1 ± 0.05 mm d^{-1}. Isolates P1900 and P1567 showed optimum growth at approx. 17°C. Sporangia were produced both on solid agar and in water culture by all *P. ilicis* isolates. Sporangia were ovoid to obpyriform, non-papillate or semi-papillate (Figure 2e, f), borne singly or on sympodial sporangiophores (Figure 2g), each was caducous with a long pedicel ranging from 5 to 26 μm (Figure 2e, f, g). Overall means of sporangial length x breadth of *P. ilicis* isolates from Sardinia and Corsica were 30.9 ± 3.7 × 18.5 ± 2.0 μm, whereas those for the United Kingdom isolates were 34.1 ± 4.4 × 20.0 ± 3.5 μm. Oogonia and oospores averaged 21.2 ± 1.4 μm in diam. for the Sardinian and Corsican isolates and 22.3 ±

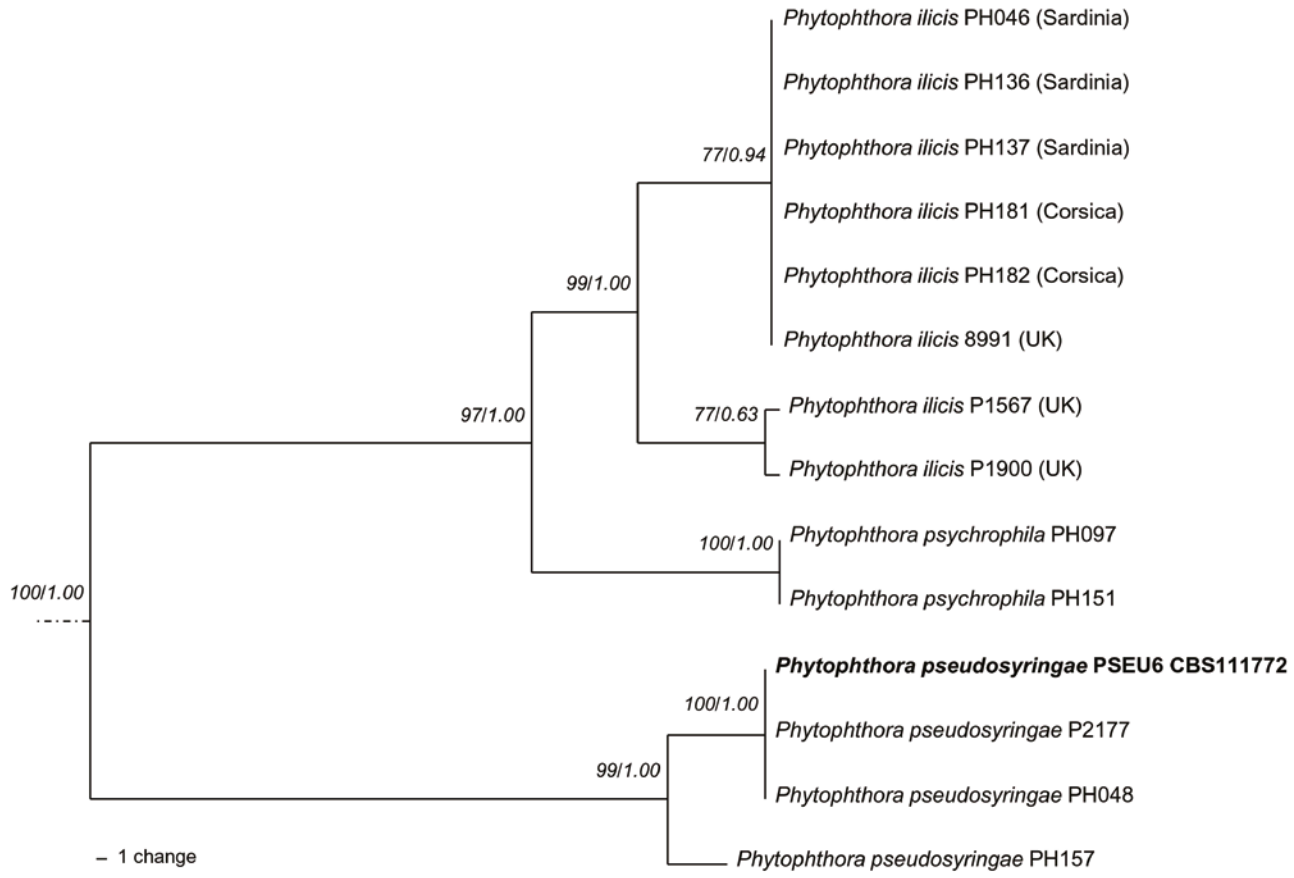

Figure 3. Phylogeny of *Phytophthora* Clade 3 species based on the cytochrome oxidase I gene region. The tree is the most parsimonious, obtained from a heuristic search (CI = 0.928, RI = 0.962). Bootstrap support and posterior probability values are given at the nodes. Ex-type cultures are in bold. The phylogram is rooted to *P. parvispora* (CBS 132771/KC609412 and CBS 132772/ KC609413).

0.7 μm for the United Kingdom isolates. Antheridia were exclusively amphigynous (Figure 2h).

Phylogenetic analyses

The aligned datasets for ITS (20 sequences) and *cox*1 (16 sequences) comprised 832 and 1106 characters, respectively. Based on partition homogeneity tests in PAUP, the ITS and *cox*1 datasets were congruent (P=0.07), but were not combined due to the lack of *cox*1 sequence data for *P. nemorosa* and *P. pluvialis*. Because the β-tubulin sequences were less informative than ITS and *cox*1, they were not used in the phylogenetic analysis. In the phylogenetic tree based on ITS sequences, isolates of *P. ilicis* placed within the ITS Clade 3 (Cooke *et al*., 2000), together with *P. nemorosa*, *P. pluvialis*, *P. pseudosyringae* and *P. psychrophila*. Genetic resolution was well supported at terminal clades and allowed for the differentiation of *P. nemorosa*, *P. pluvialis* and *P. psychrophila* (tree not shown). However, this phylogeny did not recover support both for *P. ilicis* and *P. pseudosyringae* isolates as these two species differed only by 2 bp. The two *P. ilicis* isolates from the United Kingdom (P1900 and P1567) showed a single fixed polymorphism at position 683, having a T rather than a C. The phylogenetic analysis of *cox*1 sequences resolved the two fully supported clades accommodating *P. ilicis*, *P. pseudosyringae* and *P. psychrophila* isolates (Figure 3). As in the ITS phylogeny, the two isolates from the United Kingdom were allocated in a separate clade, but in this case they had greater bootstrap and poste-

Figure 4. Necrotic lesions developed on inoculated twigs of *Ilex aquifolium* after 2 weeks. *Phytophthora ilicis* isolate PH046 (a), PH161 (b), PH136 (c), P1900 (d), P1567 (e) and control (f).

rior probability values (Figure 3). Isolates P1900 and P1567 differed from the other *P. ilicis* isolates at three nucleotide positions. In addition, isolate PH157 of *P. pseudosyringae* showed 25 bp polymorphisms in the *cox*1 gene region compared with all other *P. pseudosyringae* isolates used in this study.

Pathogenicity tests

All five isolates of *P. ilicis* used in pathogenicity tests caused large lesions on freshly cut twigs of English holly after 14 d at 20°C (Figure 4). Lesions averaged 49.4 ± 10.6 mm in length and there were no significant differences in aggressiveness amongst all *P. ilicis* isolates, as determined by lesion size. Necrotic lesions were always longer ($P<0.05$) than those on control twigs (Figure 4). *Phytophthora ilicis* was successfully re-isolated from lesions, thus fulfilling Koch's postulates. *Phytophthora pseudosyringae* isolates also caused larger lesions compared to control twigs ($P<0.05$); however, these were much shorter than those caused by *P. ilicis*.

Discussion

This research began in 2010, as an investigation aiming to establish the causal agent of the severe defoliation and dieback of English holly trees in Sardinia and Corsica. The *Phytophthora* species isolated from infected tissues of leaves and twigs was consistently shown to be *P. ilicis* on the basis of morphological characters, growth/temperature patterns and analysis of the ITS sequences. *Phytophthora ilicis* is believed to have originated in northern America where it was first reported in 1953 (Buddenhagen and Young, 1957), and to have been introduced to the United Kingdom and northern Europe, probably during the last century (Tubby and Webber, 2010).

Until now, its geographic distribution was restricted to cool temperate regions (Buddenhagen and Young, 1957; Strouts *et al*., 1989; Pintos *et al*., 2012). This report is the first to describe detection of *P. ilicis* causing foliar and stem infection on English holly in the Mediterranean region.

The morphological and physiological attributes of *P. ilicis* provide insights into the ecology and survival strategy of this pathogen. Having a lower temperature requirement for growth, *P. ilicis* is well adapted to cool temperate climates. Since both Sardinia and Corsica have typical Mediterranean climates, with long hot and dry summer periods, the occurrence of this pathogen in these areas was unexpected. However, as suggested for other homothallic *Phytophthora* species, such as *P. psychrophila* and *P. quercina* T. Jung (Pérez-Sierra *et al*., 2013), *P. ilicis* is able to survive hot, dry seasons by forming resting spores in infected foliage and the cortex of dead twigs, which resume growth in cool and wet conditions (Buddenhagen and Young, 1957). Furthermore, in the present study when symptomatic foliage and twigs were incubated in water or SMA, caducous sporangia of *P. ilicis* were frequently observed on infected tissues. In addition, the areas where *P. ilicis* was detected in Sardinia and Corsica are characterised by having considerable amounts of precipitation (800–1000 mm) during the mild and wet season from November to April, when the average temperature is 12°C. Based on the high disease incidence found in site 1, the topography of the area is also likely to be an inciting factor: sheltered valleys and densely wooded ravines, heavily populated with English holly trees, provide ideal microclimate for disease spread. These observations suggest that under suitable seasonal conditions, infection and sporangial production by *P. ilicis* on infected tissues of English holly could be frequent in the Mediterranean mountains.

Whether *P. ilicis* was introduced into the Mediterranean natural forests or whether it is endemic to these regions remains unknown. Endemicity has been suggested for the two close relatives *P. pseudosyringae* and *P. psychrophila* (Linzer *et al*., 2009; Pérez-Sierra *et al*., 2013). With *P. ilicis*, all records in northern America came from holly orchards and the pathogen has never been detected in natural environments (Paul Reeser, personal communication). In the United Kingdom, *P. ilicis* was thought to have been accidentally introduced probably via ornamental nursery plants, and since the 1980s it has become widespread on native and ornamental holly trees in parks and gardens (Tubby and Webber, 2010). Likewise, recent detection of the pathogen on English holly trees in Galicia (NW Spain) was again from ornamental gardens (Pintos *et al*., 2012). In contrast, in both Sardinia and Corsica *P. ilicis* was detected in wild environments.

In terms of population structure, the two *P. ilicis* isolates (P1900 and P1567) from the United Kingdom were phylogenetically distinct from the rest of the isolates of the same species used in this study. They also exhibited different colony patterns, lower optimum growth temperature and slower radial growth at 20°C. Such differences could reflect an environmental adaptation or drift resulting from geographic isolation. Further analysis of population structures, including isolates of *P. ilicis* from the North America and Europe should provide more information on the origin and ecology of this pathogen.

In Sardinia, the investigation was also extended to other symptomatic plants, including *A. glutinosa*, *C. sativa* and *H. helix*. Two additional *Phytophthora* species were detected, and subsequently identified as *P. bilorbang* and *P. pseudosyringae*. The recently described species *P. bilorbang* was isolated from necrotic leaves of young *A. glutinosa* trees located along a stream bank. *Phytophthora bilorbang*, previously informally designated as *P*. taxon oaksoil (Brasier *et al*. 2003), was formerly isolated from rhizosphere soil and roots of declining forest trees in Europe (Hansen and Delatour 1999), streams in Oregon (Reeser *et al*., 2011) and more recently from declining European blackberry (*Rubus fruiticosus* L.) in Western Australia (Aghighi *et al*., 2012). In all of these cases, this pathogen was isolated from soil and root samples. Our detection represents the first report of *P. bilorbang* causing foliage infection. However, since infected *A. glutinosa* leaves were found close to the soil, it is most likely that infection occurred when spores were splashed upwards from the stream water. Pathogenicity tests of *P. bilorbang* on *A. glutinosa* are required to establish the role of this pathogen in foliar infections.

The isolation of *P. pseudosyringae* from rhizosphere soil of declining *A. glutinosa* and *C. sativa* trees was not unexpected, as this pathogen has already been recorded from *A. glutinosa* in Germany (Jung *et al*., 2003) and associated with chestnut ink disease in Italy (Scanu *et al*., 2010). One isolate of *P. pseudosyringae* (PH157) was detected from infected leaves of *H.*

helix, and this finding seems quite unusual. Aerial infections are not new for *P. pseudosyringae*, inasmuch as, like other aerial *Phytophthora* spp., it produces partially caducous sporangia that can be aerially or splash dispersed (Jung *et al*., 2003). This pathogen was previously reported causing aerial infections on stems and foliage of forest trees and shrubs in the USA (Wickland *et al*., 2008), foliar blight of *Vaccinium myrtillus* L. and aerial bleeding lesions on stems of *Nothofagus obliqua* (Mirb.) Oerst. in the United Kingdom (Beales *et al*., 2009; Scanu *et al*., 2012). In the phylogenetic analysis based on *cox*1 sequences, isolate PH157 from *H. helix* was grouped in a well-supported terminal clade. This strain may represent a distinct evolutionary lineage that shares a common ancestor with *P. pseudosyringae*. This is consistent with a recent study of Reeser *et al*. (2012), where six unique haplotypes of *P. pseudosyringae* were identified based on *cox* spacer sequence analysis. Likewise, Scanu and Webber (unpublished data) have found different genotypes within a collection of *P. pseudosyringae* isolates from the United Kingdom. Collectively, these results together with the genetic variability of *P. ilicis* found in this study indicate high intraspecific variability within *Phytophthora* major ITS Clade 3. This suggestion, however, should be investigated further.

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