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Multiple alien *Phytophthora* taxa discovered on diseased ornamental plants in Spain

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The plant trade is unwittingly accelerating the worldwide spread of well-known and new or undescribed *Phytophthora* species and creating novel niches for emerging pathogens. The results of a survey carried out from 2001 to 2006 in garden centres and nurseries of the Balearic Islands and eastern Spain combined with the analysis of samples received from ornamental nurseries from northern Spain reflected the extent of this global issue at the local scale. A total of 125 *Phytophthora* isolates were obtained from 37 different host species and 17 putative species identified on morphological features and direct sequencing of the internal transcribed spacer and four mitochondrial and nuclear genes. Five species, *P. ramorum*, *P. hedraiandra*, *P.* 'niederhauserii', *P.* 'kelmania' and *P.* 'taxon Pgchlamydo' were formally unknown to science prior to 2001. In addition, 37 new host/pathogen combinations were first records for Spain, highlighting the risk of non-coevolved organisms from different biogeographic origins coming into contact under managed environments. The problem generated by new or rare taxa of *Phytophthora* found in nurseries for which no prior information on natural habitat and ecology is available for pest risk analysis is discussed.

Keywords: international plant trade, invasive alien species, ornamental plant diseases, pathogenicity, *Phytophthora* taxonomy

Introduction

The genus *Phytophthora* comprises over 70 known species, many of them exhibiting a wide host range. It includes various pathogens of ornamental plants (e.g. Werres *et al*., 2001; de Cock & Lévesque, 2004) and forest trees (e.g. Brasier *et al*., 1993; Jung *et al*., 1999; Rizzo *et al*., 2002; Brasier *et al*., 2004a,b). Despite the significant economic and ecological damages caused by *Phytophthora* species, their centres of origin and natural habitats remain mostly unknown. Of those *Phytophthora* spp. reported from natural ecosystems in Europe, for example *P. quercina*, *P. pseudosyringae* or *P. europaea*, there is little support for them being native to the continent. Indeed, recent population genetic studies indicate that some new *Phytophthora* spp. are probably either introduced, or they may have emerged through hybridization and/or spread via the horticultural trade and forest plantations (Werres *et al*., 2001; Cooke *et al*., 2005; Ivors *et al*., 2006; Brasier, 2007; 2008).

Species that can establish themselves in new environments and out-compete native species are regarded as invasive alien species (IAS), and are recognized as major threats to natural heritage and biodiversity, second only to that of habitat loss (Mooney & Cleland, 2001). Although it is believed that fungal plant pathogens do not quite fit into the common definition of IAS, particularly with regard to invasiveness, there are noteworthy examples of alien fungi and oomycetes within the genus *Phytophthora* causing significant ecological disturbances and biodiversity impoverishment in natural ecosystems (e.g. Weste & Marks, 1987; Anagnostakis, 1988; Brasier *et al*., 1993; Rizzo *et al*., 2002; Desprez-Loustau *et al*., 2007). Because it is estimated that only approximately 10% of the mycoflora have been catalogued (Hawksworth, 2001; Brasier, 2007), plant pathogenic fungi and oomycetes will be part of this unknown diversity, and therefore are likely candidates for causing future invasions.

Among oomycetes recently raised to an IAS status, *P. ramorum* well illustrates the peril posed by pathogens introduced via the ornamental trade. First isolated from *Rhododendron* and *Viburnum* plants in nurseries in the Netherlands and Germany in the mid-1990s (Werres *et al*., 2001), it was initially considered as yet another

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Phytophthora of ornamental plants until its implication as the causal agent of sudden oak death in California (Rizzo *et al*., 2002). In California *P. ramorum* causes a disease at the plant community level, with a million oak trees killed to date (S. Frankel, USDA Forest Service, personal communication) and more than 26 understorey species being infected. In addition, *P. ramorum* exhibits a remarkably wide host range in laboratory inoculations among both ornamentals and wild plants, and probably poses a threat to natural ecosystems in diverse biogeographic areas of the world (Brasier *et al*., 2004a; Denman *et al*., 2005; Moralejo *et al*., 2006b).

The plant trade has largely been recognized as a principal pathway for the introduction of invasive plants (Reichard & White, 2001) and exotic pathogens (Jones & Baker, 2007), but few studies have focused on screening for species diversity within a taxonomic group of microorganisms accompanying the plants being moved. Prior to 2000, little was known about the presence of *Phytophthora* on ornamental plants in Spain. There are more than 278 plant nurseries in the Valencia region, with over 1800 ha dedicated to ornamental plants. About 20% of the plant production is exported to European countries. In contrast, only a few ornamental nurseries are found in the Balearic Islands; most of the plant commodities here are imported from mainland Spain or EU countries and sold through garden centres and small retailers. Therefore, the aims of this work were to investigate the presence of *Phytophthora* on ornamental plants in eastern mainland Spain and the Balearic Islands, to identify and characterize the species involved and their hosts, and to evaluate their pathogenicity.

Materials and methods

Area of study

Valencia and the Balearic Islands are situated in eastern Spain in the western Mediterranean (Fig. 1). The climate of both regions is typically Mediterranean, rainfall mostly occurring between September and April, with mean winter temperatures above 10°C at the coastline and not lower than 6°C in the mountain ranges. Summer months are dry and July mean temperatures are between 24 and 26°C. Both areas belong to the PA1215 ecoregion, which comprises northeastern Spain and southern France Mediterranean forests (World Wildlife Biome Funds http://www.worldwildlife.org/science/data/attributes.cfm). The vegetation is largely maquis and scrub, with Aleppo pine (*Pinus halepensis*) as a main tree species in the lowlands and coastline, and holm oak (*Quercus ilex*) woodlands in the mountain ranges and in the central areas of Mallorca and Menorca in the Balearic Islands.

Phytophthora survey

Phytophthora isolates in this study were obtained from local surveys of garden centres, small retailers, public and botanical gardens and ornamental nurseries of the Balearic Islands and Valencia from 2001 to 2006, and from samples received at the diagnostic laboratory of the Instituto Agroforestal Mediterráneo in Valencia from ornamental nurseries in northern Spain. Nurseries in Valencia were visited when a disease problem was notified. Two nurseries and 13 garden centres were sampled in the Balearic Islands, nine of them in Mallorca, which were visited at least twice during the survey period. In 2002, the quarantine-pathogen *P. ramorum* was detected in Mallorca (Moralejo & Werres, 2002). Until then, the survey was focused on plants showing symptoms commonly associated with root and collar infections caused by *Phytophthora*, such as chlorosis, defoliation, sudden death, shoot dieback or permanent wilting. After its detection, attention was particularly paid to plants showing branch dieback, leaf and stem blight, and leaf necrosis.

Figure 1 Study area of mainland Spain and the Balearic Islands.

Isolation

Isolations were mainly made directly from sections of roots, crowns, stems and leaves of plants showing symptoms and also from potting media of plants with root rots. Soil samples were tested using apple baits or by flooding with distilled water and baiting with young leaves of *Q. ilex* (modified from Jung *et al*., 1999). Diseased tissues, including those of infected baits, were cut into smaller pieces with a sterilized scalpel, washed in tap water, surface-disinfested by dipping in 70% ethanol for 30 s, rinsed in sterilized de-ionized water and blotted dry on filter paper. From the margins of the necrotic lesions, pieces of tissue *c*. 5 × 5 mm were transferred to a *Phytophthora* semi-selective medium P_5ARP (Erwin & Ribeiro, 1996), or on the PARBPH selective culture medium as described by Jeffers & Martin (1986), and incubated at room temperature for 72 h. Pure cultures were obtained by transferring a single hyphal tip from the edge of the colonies onto corn meal agar (CMA, Oxoid LTD or Sigma Chemical Company), potato dextrose agar

(PDA, Biokar Diagnostic) or V8 juice agar (2 g $CaCO_3$, 200 mL V8 juice and 15 g agar in 800 mL distilled water). Stock cultures were maintained on carrot agar (CA; Brasier, 1967) slants covered with paraffin oil, or in sterile soil extract, both stored at 12°C in the dark. Representative specimens of each taxon were deposited at the CBS (Centraalbureau voor Schimmelcultures). The remaining cultures were preserved in the culture collections maintained at the Instituto Mediterráneo de Estudios Avanzados (Esporles, Mallorca) and at the Instituto Agroforestal Mediterráneo (Valencia).

Morphological identification

Phytophthora isolates were identified using colony morphology, growth rate, cardinal growth temperatures, and production, morphology and dimensions of sporangia, oogonia and antheridia (Erwin & Ribeiro, 1996). For morphological examination, isolates were grown on CMA, CA and PDA at 20–24°C in the dark. Radial growth rates (mm day^{-1}) at 20°C were determined on two replicates of each isolate on CA or PDA. Cardinal growth temperatures of some isolates were determined by transferring 5-mm-diameter mycelial plugs onto PDA and incubating at 5–40°C for 7 days in the dark.

The presence of sporangia, hyphal swellings and chlamydospores on all agar media was checked over 2 weeks. To induce the formation of sporangia, three 12-mm-diameter discs were taken from the edge of a 7-day-old colony grown on CA or V8 and placed in a 60-mm-diameter Petri dish previously flooded with 10 mL soil extract (Moralejo *et al.*, 2004). The dishes were kept for 48–72 h at 20°C under a 12-h photoperiod of white light. The shape, papilla formation, and caducity of sporangia were recorded. The presence of hyphal swellings or chlamydospores in soil solution was also examined.

The formation of gametangia on CA and PDA was examined after 10 and 30 days' incubation at 20°C in the dark. Heterothallic species of *Phytophthora* require the presence of opposite mating types known as A1 and A2 for the formation of sexual structures. Heterothallism was investigated for non-self-fertile isolates by pairing them with known A1 and A2 isolates of *P. nicotianae* on CA at 20°C, or with both mating types of *P. cryptogea* on V8 juice agar at 24°C in darkness for 2–6 weeks. Approximately 25 sexual (gametangia) and asexual structures (sporangia and chlamydospores), chosen at random, were measured on CA using a calibrated eyepiece on an Olympus BX-50 compound microscope at ×400 magnification.

DNA extraction, ITS sequencing and molecular phylogeny

Mycelial DNA was extracted either from pure cultures grown in sterile pea broth (Kroon *et al.*, 2004) and checked for quality as previously described (Belbahri *et al.*, 2006a,b), or from pure cultures grown on PDA by scraping the mycelium and mechanically disrupting it by grinding to a fine powder under liquid nitrogen with a mortar and pestle. Total DNA was extracted using the EZNA Plant Miniprep Kit (Omega Bio-tek) following the manufacturer's instructions.

Ribosomal DNA ITS amplifications were carried out using the universal primers ITS4 and ITS6 that target conserved regions in the 18S and 28S rDNA genes (White *et al.*, 1990; Cooke *et al.*, 2000). Isolates showing unique morphological characters, or assigned to a morphospecies with sequence identities below a 99% cutoff in respect to those of ex-type cultures or well-identified species were further investigated by sequencing other nuclear (*EF-1α* and *β-tub*) and mitochondrial (*coxII* and *nadh1*) genes (data not shown). Amplifications for nuclear [translation elongation factor 1 alpha (*EF-1α*) and β-tubulin (*β-tub*)] and mitochondrial [NADH dehydrogenase subunit 1 (*nadh1*)] genes were performed according to Kroon *et al.* (2004). The cytochrome oxidase subunit II gene (*coxII*) was amplified using primers FM75 and FM78 and PCR conditions as described by Martin & Tooley (2003). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH) and DNA sequencing was performed according to Belbahri *et al.* (2006a,b). The sequences obtained for all the isolates studied were registered in GenBank and compared with other *Phytophthora* sequences (Tables 1 and 2). A molecular phylogeny was constructed according to Belbahri *et al.* (2006a,b).

Pathogenicity tests and hosts

New host/pathogen combinations were tested for Koch's postulates over the 6-year period. The inoculation methods varied according to the plant organ of origin. For *Phytophthora* spp. isolated from aerial plant organs, leaves were detached from the original host, bagged, rinsed in sterile distilled water and blotted dry. Sets of four to seven leaves of each host species were placed with the adaxial side down on a metal grid in moist chambers consisting of transparent plastic boxes lined on the bottom with sterile paper towels soaked in water. The abaxial sides of all the leaves except one were inoculated either by placing a single 100-μL drop of a 1×10^4 zoospore suspension at the centre of the unwounded blade or with mycelium plugs after wounding the leaf blade. The remaining leaf was used as a control and inoculated with a drop of sterilized de-ionized water. The boxes were held at 20°C under cool white light from 30-W fluorescent tubes suspended 30 cm above the chambers (12-h photoperiod). Seven days after inoculation, the leaves were scanned and the area of the lesions calculated with Olympus DP12 SOFT version 3·2.

For *Phytophthora* spp. isolated from roots or soil, pathogenicity tests were performed modifying the method described by Babadoost & Islam (2003). The inoculations were performed on potted 6- to 8-month-old plants with at least two isolates of each *Phytophthora* species. Ten plants per isolate were used in each pathogenicity test and 10 plants were used as controls. Inoculum was prepared

Table 1 *Phytophthora* taxa isolated from ornamental plants in this study

Species	Isolate	Host	Disease/environment[a]	Locality	Year of isolation/ season[b]	New disease report[c]	ITS GenBank accession no.
P. cactorum	P1211	*Fragaria* × *ananassa*	fruit rot/nur	Mallorca	2001/w		EF050512
	P1411	*Fragaria* × *ananassa*	fruit rot/nur	Mallorca	2001/w		EF050513
	P1611	*Fragaria* × *ananassa*	fruit rot/nur	Mallorca	2001/w		AY943299
P. cinnamomi	P7223	*Quercus ilex*	soil/seminatural	Mallorca	2003/sp		EF050520
	P7323	*Quercus ilex*	soil/seminatural	Mallorca	2003/sp		EF050521
	P7423	*Quercus ilex*	soil/seminatural	Mallorca	2003/sp		EF050522
P. citricola	P11725	*Quercus ilex*	leaf blight/gc	Mallorca	2005/sp	World	DQ357828
	P7624	*Verbena* hybrid	collar rot/gc	Mallorca	2004/sp	World	EF050523
	P4242	*Ceanothus* sp.	stem blight/gc	Ibiza	2002/au	Spain	AY946259
	P12245	*Viburnum tinus*	leaf blight/gar	Mallorca	2005/au	World	DQ648135
	P15946	*Cistus canariensis*	root and collar rot/bg	Mallorca	2006/au	World	EU244846
	P16246	*Cistus canariensis*	root and collar rot/bg	Mallorca	2006/au		EF153674
	Ps-2	*Rhododendron* sp.	leaf blight	Asturias	2004/sp	Spain	EU194424
	Ps-5	*Rhododendron* sp.	leaf blight	Asturias	2004/sp		EU194425
P. citrophthora	P941	*Citrus sinensis*	fruit rot/nur	Mallorca	2001/au		AY880997
	P2021	*Salvia splendens*	root and collar rot/nur	Mallorca	2001/sp	Spain	AY961611
	P2912	*Arbutus unedo*	twig blight/gc	Mallorca	2002/w	World	AY946249
	P3212	*Arbutus unedo*	twig blight/gc	Mallorca	2002/w		AY961609
	P3742	*Arbutus unedo*	twig blight/gc	Mallorca	2002/au		AY881004
	P4142	*Arbutus unedo*	leaf blight/gc	Mallorca	2002/au		DQ648129
P. cryptogea	PS-73-1	*Pinus* sp.	root rot/nur	Valencia	2006/sp	Spain	EU194426
	PS-73-3	*Pinus* sp.	root rot/nur	Valencia	2006/sp		EU194427
	P7824	*Quercus ilex*	soil/seminatural	Mallorca	2004/au		EF153671
P. drechsleri	PS-43	*Polygala myrtifolia*	root and collar rot/nur	Valencia	2004/sp	World	EU194428
P. hedraiandra	P3642	*Viburnum tinus*	stem canker/gc	Mallorca	2002/sm	Spain	EF050517
	P3842	*Viburnum tinus*	stem canker/gc	Mallorca	2002/sm		AY881005
	P3942	*Viburnum tinus*	stem canker/gc	Mallorca	2002/sm		AY961608
	P11935	*Rhododendron catawbiense*	leaf blight/gc	Girona	2005/sm	Spain[d]	DQ643972
	P12345	*Viburnum tinus*	leaf blight/gc	Girona	2005/w		EF174429
	P12445	*Viburnum tinus*	leaf blight/gc	Girona	2005/w		EF174430
	P12545	*Viburnum tinus*	leaf blight/gc	Girona	2005/w		DQ648145
P. hibernalis	Ps-64	*Rhododendron* sp.	leaf blight/nur	Asturias	2006/sp	Spain[e]	EU194429
	Ps-79	*Rhododendron* sp.	leaf blight/nur	Asturias	2006/sp		EU194430
P. 'kelmania'	P1041	*Gerbera* sp.	collar rot/nur	Mallorca	2001/au	World	AY961613
	P1921	*Coleus* sp.	collar rot/nur	Mallorca	2001/sp	World	AY881000
P. nicotianae	P11613	*Arbutus unedo*	leaf blight/nur	Mallorca	2005/w	World	DQ357827h
	P131	*Cyclamen persicum*	root rot/nur	Mallorca	2001/sm	Spain	EF050510
	P231	*Catharanthus rosea*	collar rot/nur	Mallorca	2001/sm		AY946264
	P331	*Catharanthus rosea*	collar rot/nur	Mallorca	2001/sm		AY880995
	P431	*Salvia officinalis*	collar rot/nur	Mallorca	2001/sm		AY880996
	P531	*Salvia officinalis*	root and collar rot/nur	Mallorca	2001/sm		AY943296
	P631	*Lavandula angustifolia*	root and collar rot/nur	Mallorca	2001/sm		AY943295
	P1141	*Lycopersicon esculentum*	root rot/nur	Mallorca	2001/au		AY880998
	P1311	*Citrus sinensis*	soil/orchard	Mallorca	2001/w		AY880999
	P1511	*Thymus vulgaris*	root rot/nur	Mallorca	2001/w	World	AY943298
	P1821	*Convolvulus* sp.	collar rot/gc	Mallorca	2001/sp	World	AY961612
	P2131	*Catharanthus rosea*	collar rot/nur	Mallorca	2001/sm		EF050514
	P2231	*Capsicum annuum*	collar rot/nur	Mallorca	2001/sm		AY961610
	P2331	*Capsicum annuum*	root and collar rot/nur	Mallorca	2001/sm		AY946263
	P4042	*Ceanothus* sp	stem blight/gc	Ibiza	2002/ au	World	AY946260
	P4342	*Rhododendron* sp.	leaf blight/gc	Mallorca	2002/au		EF050518
	P4442	*Rhododendron* sp.	leaf blight/gc	Mallorca	2002/au		EF050519
	P11615	*Arbutus unedo*	twig blight/gc	Mallorca	2005/w		DQ357827
	P16146	*Cistus canariensis*	root rot/bg	Mallorca	2006/au	World	EU244847
	P16446	*Arbutus unedo*	twig blight/gc	Mallorca	2006/au		EF426559
	P16546	*Arbutus unedo*	twig blight/gc	Mallorca	2006/au		EF426558
	Ps-21	*Rosmarinus officinalis*	root and collar rot/nur	Valencia	2004/sm		EU244849
	Ps-23	*Lavandula angustifolia*	root and collar rot/nur	Valencia	2004/sm		EU194431
	Ps-26	*Rosmarinus officinalis*	root and collar rot/nur	Valencia	2004/sm		EU194432
	Ps-28	*Lavandula angustifolia*	root and collar rot/nur	Valencia	2004/sm		EU194433
	Ps-30	*Rosmarinus officinalis*	root and collar rot/nur	Valencia	2004/sm		EU194434
	Ps-100	*Laurus nobilis*	leaf blight/gc	Barcelona	2006/sm	World	EU194435
P. 'niederhauserii'	P731	*Cistus monspeliensis*	root rot/nur	Mallorca	2001/sm	World	AY943297
	P831	*Cistus salvifolius*	root rot/nur	Mallorca	2001/sm	World	EF050511
	Ps-56	*Hedera* sp.	root and collar rot/nur	Valencia	2005/sp	World	EU244850
	Ps-57	*Hedera* sp.	root and collar rot/nur	Valencia	2005/sp		EU194436
	Ps-101	*Laurus nobilis*	leaf blight/gc	Barcelona	2006/sp	World	EU194437
P. palmivora	PS-47	*Pittosporum* sp.	root and collar rot/nur	Valencia	2004/sp	World	EU194438

Table 1 *Continued*

Species	Isolate	Host	Disease/environment[a]	Locality	Year of isolation/ season[b]	New disease report[c]	ITS GenBank accession no.
P. ramorum	P2512 = (Pram1)	*Rhododendron* sp.	stem blight/gc	Mallorca	2002/w	Spain[f]	EF050515
	P3012	*Rhododendron* sp.	leaf blight/gc	Mallorca	2002/w		EF050516
	P5042	*Viburnum tinus*	leaf blight/gc	Mallorca	2002/au		N/T[i]
	P5142	*Rhododendron* sp.	leaf blight/gc	Mallorca	2002/au		N/T
	P5242	*Rhododendron* sp.	leaf blight/gc	Mallorca	2002/au		N/T
	P5342	*Rhododendron* sp.	leaf blight/gc	Mallorca	2002/au		N/T
	P5442	*Rhododendron* sp.	leaf blight/gc	Mallorca	2002/au		N/T
	P5613	*Camellia japonica*	leaf blight/gc	Mallorca	2003/w		N/T
	P5713	*Camellia japonica*	leaf blight/gc	Mallorca	2003/w		N/T
	P5813	*Rhododendron* sp.	leaf blight/gc	Mallorca	2003/w		N/T
	P5913	*Rhododendron* sp.	leaf blight/gc	Mallorca	2003/w		N/T
	P6213	*Arbutus unedo*	leaf blight/gc	Mallorca	2003/w	World	N/T
	P8014	*Camellia japonica*	leaf blight/gc	Mallorca	2004/w		N/T
	P8114	*Camellia japonica*	leaf blight/gc	Mallorca	2004/w		N/T
	P8214	*Rhododendron* sp.	leaf blight/gc	Mallorca	2004/w		N/T
	P8314	*Rhododendron* sp.	leaf blight/gc	Mallorca	2004/w		N/T
	P9624	*Rhododendron* sp.	leaf blight/gc	Ibiza	2004/sp		N/T
	PR-32	*Rhododendron* sp.	leaf blight/nur	Asturias	2006/w		N/T
	PR-33	*Camellia japonica*	leaf blight/nur	Asturias	2006/w		N/T
P. syringae	P2612	*Arbutus unedo*	twig blight/gc	Mallorca	2002/w	World	AY946262
	P2712	*Arbutus unedo*	twig blight/gc	Mallorca	2002/w		AY946251
	P2812	*Arbutus unedo*	twig blight/gc	Mallorca	2002/w		AY946250
	P3112	*Crataegus laevigata*	twig blight/gc	Mallorca	2002/w	World	AY881002
	P3312	*Arbutus unedo*	twig blight/gc	Mallorca	2002/w		AY881003
	P4542	*Arbutus unedo*	leaf blight/gc	Mallorca	2002/au		DQ648128
	P4642	*Arbutus unedo*	twig blight/gc	Mallorca	2002/au		AY946258
	P4842	*Arbutus unedo*	leaf blight/gc	Mallorca	2002/au		AY946256
	P4942	*Arbutus unedo*	leaf blight/gc	Mallorca	2002/au		AY946255
	P7042	*Arbutus unedo*	leaf blight/gc	Mallorca	2002/au		AY881006
	P7113	*Rhododendron*	leaf blight/gc	Mallorca	2003/w		AY961607
	P7514	*Arbutus unedo*	leaf blight/gc	Mallorca	2004/w		AY946254
	P10113	*Arbutus unedo*	leaf blight/gc	Mallorca	2004/w		DQ648143
	P10213	*Arbutus unedo*	leaf blight/gc	Mallorca	2004/w		DQ648138
	P10313	*Arbutus unedo*	leaf blight/gc	Mallorca	2004/w		N/T
	P10413	*Arbutus unedo*	leaf blight/gc	Mallorca	2004/w		DQ648136
	P10513	*Arbutus unedo*	leaf blight/gc	Mallorca	2004/w		EF050525
	P10613	*Arbutus unedo*	leaf blight/gc	Mallorca	2004/w		AY961606
	P10713	*Arbutus unedo*	leaf blight/gc	Mallorca	2004/w		DQ648142
	P10813	*Arbutus unedo*	leaf blight/gc	Mallorca	2004/w		DQ648130
	P10913	*Arbutus unedo*	leaf blight/gc	Mallorca	2004/w		AY946245
	P11013	*Arbutus unedo*	leaf blight/gc	Mallorca	2004/w		AY881007
	P11113	*Arbutus unedo*	leaf blight/gc	Mallorca	2004/w		AY946246
	P11213	*Arbutus unedo*	leaf blight/gc	Mallorca	2001/w		AY946247
	P11313	*Arbutus unedo*	leaf blight/gc	Mallorca	2001/w		AY946248
	P11413	*Arbutus unedo*	leaf blight/gc	Mallorca	2004/w		DQ648149
	Ps-3	*Rhododendron* sp.	leaf blight/nur	Asturias	2004/sp		EU194439
	Ps-66	*Rhododendron* sp.	leaf blight/nur	Asturias	2006/sp		EU244851
	Ps-65	*Rhododendron* sp.	leaf blight/nur	Asturias	2006/sp		EU194441
P. 'taxon Pgchlamydo'	P16046	*Persea indica*	leaf blight/gc	Mallorca	2006/au	World	EU244848
	Ps-6	*Rhododendorn* sp.	leaf blight/nur	Asturias	2004/sp	Spain	EU194442
	Ps-63 (405)	*Rhododendron* sp.	leaf blight/nur	País Vasco	2006/sp		EU194443
	Ps-67	*Rhododendron* sp.	leaf blight/nur	Asturias	2006/sp		EU194444
	Ps-63	*Rhododendron* sp.	leaf blight/nur	País Vasco	2006/sp		EU244852
P. tentaculata	P2431	*Verbena* sp.	collar rot/nur	Mallorca	2001/sm	World[g]	AY881001
	Ps-33	*Santolina chamaecyparissus*	root and collar rot	Valencia	2004/sp	World[h]	N/T
P. tropicalis	P11515	*Arbutus unedo*	twig blight/gc	Mallorca	2002/w	World	DQ357829
	P4742	*Allamanda* sp.	stem blight/gc	Mallorca	2004/au	World	AY946257

[a] nur, nursery; gc, garden centre; bc, botanical garden.

[b] w, winter; sp, spring; sm, summer; au, autumn.

[c] First reports published during the course of the survey. Information was taken from the Systematic Botany and Mycology Laboratory (SBML) literature database (http://nt.ars-grin.gov/fungaldatabases/literature/Literature.cfm).

[d] Moralejo *et al.* (2007).

[e] Álvarez *et al.* (2007).

[f] Moralejo & Werres (2002).

[g] Moralejo *et al.* (2004).

[h] Álvarez *et al.* (2006).

[i] N/T, not tested.

Table 2 GenBank accession numbers of the sequences of two nuclear and two mitochondrial genes from four putative new species of *Phytophthora*

		Nuclear genes		Mitochondrial genes	
Species	Isolate	*EF 1α*	*β-Tub*	*Nadh1*	*CoxII*
P. 'pseudo-citricola'	P4242	EF050527	EF050526	EF050528	N/T[a]
P. 'niederhauserii'	Ps-57	EU195072	EU195073	N/T	EU195074
	458	EU195075	EU195076	N/T	EU195077
P. 'kelmania'	P1921	EU497938	EU497934	EU497932	EU497936
	P1041	EU497939	EU497935	EU497933	EU497937
P. 'taxon Pgchlamydo'	Ps-63	EU195066	EU195067	N/T	EU195068
	Ps-7	EU195063	EU195064	N/T	EU195065

[a] N/T, not tested.

by growing each isolate in 1 L distilled water containing 200 g oatmeal and 120 mL V8 juice. After inoculation, flasks were incubated in the dark at 20°C for 4 weeks until the mycelium colonized the substratum. The medium colonized by each isolate was buried in the compost mixture around the roots of the test plant at a concentration of 3% (w/v). Control plants were inoculated with similar amounts of a sterile medium. Starting from the following day, the pots, including the controls, were flooded for 2 days and then watered twice a week. Plants were maintained in a glasshouse at 24 ± 5°C. Controls were included in all pathogenicity assays and re-isolations were made from the infected tissue by plating onto PARP medium to confirm Koch's postulates.

Updated information on the host range of *Phytophthora* species was obtained by regularly reviewing new disease reports of ornamental plants caused by *Phytophthora* spp. and checking for new entries in international culture collection databases (i.e. CBS, CABI International, ATCC, DARD) and GenBank accessions (http://www.ncbi.nlm.nih.gov/). Reports of pathogen-host combinations were checked in the Systematic Botany and Mycology Laboratory (SBML) literature database (http://nt.ars-grin.gov/fungaldatabases/literature/Literature.cfm) as well as in the AGRICOLA database (http://agricola.nal.usda.gov/). Information on the centre of origin of the hosts was gathered from published international floras. Each host was assigned to Mediterranean, temperate or tropical biomes.

Results

Phytophthora species on diseased ornamental plants

A total of 125 isolates of *Phytophthora* spp. were obtained from single lesions of 37 plant species showing root and collar rots, stem cankers, and twig and leaf blights, mostly associated with ornamental plants in garden centres, nurseries or, to a lesser extent, public gardens (Table 1). Of these, 94 *Phytophthora* isolates were recovered from ornamental plants in the Balearic Islands. The host list included several growth forms ranging from herb to tree species in 30 distinct genera assigned to 20 families of dicotyledonous angiosperms and coniferous trees of diverse biogeographic origins. Each unique host/pathogen pair was depicted as a simple entity for analysis ($n = 52$) to avoid unbalanced sampling in the analysis by repeated isolation from the same host species. Thus, 53·8% of the *Phytophthora* host species were native to Mediterranean biomes, 28·8% were of tropical or subtropical origin and 17·3% belonged to other temperate climates. Approximately 25% of the 125 *Phytophthora* isolations were made from plants belonging to the Ericaceae and 59% from Mediterranean plants. By contrast, only 35% of plants grown or sold in nurseries and garden centres were estimated to be from a Mediterranean biome.

Phytophthora species diversity

Most of the isolates conformed morphologically to 14 previously known *Phytophthora* species or to species described during the course of the survey. These included common species of ornamental nurseries in Europe such as *P. syringae*, *P. citrophthora*, *P. cactorum*, *P. nicotianae*, *P. cryptogea*, *P. cinnamomi* and *P. citricola*, and less common or rare species such as *P. drechsleri*, *P. palmivora*, *P. tropicalis*, *P. hibernalis* and *P. tentaculata* (Table 1). The morphological identification of the majority of species was confirmed by sequencing the rDNA ITS regions and comparing the sequence alignments in BLAST searches with those in the GenBank database. Thus, 13 isolates could not be assigned to any known or formally described species, having sufficiently distinct ITS DNA sequences and unique morphological traits. Their phylogenetic position within the genus was determined using representative isolates of each species derived from this study, isolates of species included in the phylogenetic study of Cooke *et al.* (2000) and new *Phytophthora* species published subsequently (Fig. 2).

Five taxa identified in this study were formally unknown to science at the onset of the survey in January 2001. These comprised the two recently described species, *P. ramorum* (Werres *et al.*, 2001) and *P. hedraiandra* (de Cock & Lévesque, 2004), and three other putative new species: *P.* 'niederhauserii' and *P.* 'kelmania' (G. Abad, USDA, Beltsville, MD 20705, USA, personal communication) and *P.* 'taxon Pgchlamydo' *sensu* Brasier *et al.* (2003).

Figure 2 Bayesian inference tree using rDNA ITS sequences showing phylogenetic relationships between *Phytophthora* species. Numbers on nodes represent posterior probability/percentage bootstrap support based on Bayesian analysis of the dataset. *Phytophthora* ITS sequences used are indicated by their GenBank accession nos. Scale bar represents 0·1 substitution positions.

Nineteen isolations of *P. ramorum* were made from aerial lesions of *Rhododendron* spp., *Camellia japonica*, *Viburnum tinus* and *Arbutus unedo* from garden centres in Mallorca. All these isolates belonged to the A1 mating type and exhibited colony patterns and growth rates corresponding to the European lineage of *P. ramorum* (S. Werres, personal communication). Ornamental plants infected by *P. ramorum* were detected during winter and early spring. In addition, a progressive decrease in the number of plants infected by *P. ramorum* was noticed from 2003 onwards.

Phytophthora 'niederhauserii' is heterothallic. Based on phylogenetic analyses, it falls in subclade 7b *sensu* Cooke *et al.* (2000), which remarkably only includes host-specific pathogens of crops such as *P. cajani*, *P. vignae*, *P. melonis*, *P. pistaciae* and *P. sojae* (Mirabolfathy *et al.*, 2001). It is closely related and basal to *P. sojae*, probably which has arisen recently as a specific-host pathogen of soyabean (*Glycine max*) from the ancestral species *P.* 'niederhauserii', which is already known to infect five different unrelated hosts (Table 1). Sequence alignment and comparison with two further genes (*β-tub* and *coxII*) indicated that *P.* 'niederhauserii' forms a different taxon, probably with specific status (Fig. 2 and Table 2).

Although morphologically similar to *P. cryptogea*, two isolates, P1041 and P1921, had sequences in their ITS region identical to those of a newly proposed species, *P.* 'kelmania'. Both isolates differed by three base pairs from those of *P. cryptogea*. Five isolates morphologically similar to *P. gonapodyides* were identified as *P.* 'taxon Pgchlamydo'. They readily formed chlamydospores in culture, but did not produce gametangia, either in intraspecific or interspecific matings. The sequences of the *EF-1α*, *β-tub* and *coxII* genes were unique and differed significantly from those of other species (Table 2). A *Phytophthora* isolate morphologically similar to *P. citricola* had a unique ITS sequence only shared with another isolate collected in Australia (DAR34210; GenBank accession no. L41375·1). This *P. citricola*-like isolate differed from its closest relative *P. citricola* in the position of at least four base pairs in the ITS sequence, and in more than seven base pair substitutions in the sequences of the *EF-1α*, *β-tub* and *nadh1* genes (Table 2). Unlike *P. citricola*, it formed unusually small, oogonia with thick-walled oospores. Thus, the *P. citricola*-like taxon was probably a new species or a subpopulation of *P. citricola* that deserves further investigation.

Three other species, *P. tentaculata*, *P. hibernalis* and *P. tropicalis*, previously reported as rare for Europe, were first found in nurseries in Mallorca and mainland Spain. *Phytophthora tropicalis* isolate P11535 from *A. unedo* differed in its (ITS) sequence alignment from other isolates, and needs further research.

New hosts and diseases

Thirty-seven host/pathogen combinations resulted in a number of first records for Spain during the course of this study (Table 1). Of these, 28 were new host/pathogen combinations previously unknown to science. All these first records were tested for Koch's postulates and their pathogenicity confirmed in inoculation assays. All controls, in turn, remained healthy and no *Phytophthora* could be isolated from them. Ornamentals belonging to the Ericaceae received special attention during the survey because of their known susceptibility to *P. ramorum*. As a result, a new infection of *A. unedo* in nurseries, caused by several *Phytophthora* spp. (Moralejo *et al.*, 2008) including *P. ramorum*, was discovered. Seven other *Phytophthora* taxa (*P. citricola*, *P. syringae*, *P. ramorum*, *P. hedraiandra*, *P. nicotianae*, *P. hibernalis* and *P.* 'taxon Pgchlamydo') were found associated with foliage infections of rhododendron.

Phytophthora nicotianae was highly aggressive to irrigated Mediterranean shrubs and scrub plants grown as ornamental plants. The roots and crowns of *Salvia officinalis*, *Rosmarinus officinalis*, *Thymus vulgaris* and *Lavandula angustifolia* were severely infected, often causing high mortality in nurseries. Leaves of *A. unedo* and *Laurus nobilis* and stems of *Convolvulus* sp. and *Ceanothus* sp. were also infected by *P. nicotianae*. Other Mediterranean shrubs likewise introduced as ornamentals, such as *Cistus salvifolius* and *C. monspeliensis*, were fatally affected by *P.* 'niederhauserii' to the extent that a leading producer abandoned production. In contrast, no mortality or decline associated with *Phytophthora* species has been found on Mediterranean scrub plants in natural vegetation in Mallorca.

Discussion

This study presents the results of a 6-year survey of *Phytophthora* species, mainly in ornamental plants of the Balearic Islands and eastern Spain, as an example of their unnoticed introduction through the movement of hosts via the ornamental plant trade. Seventeen *Phytophthora* species were recovered from 37 different hosts, mostly from plant material imported from mainland Spain and other EU member states. For this study, multiple gene sequencing was used to attain reliable identifications of doubtful species or putative new taxa. Accordingly, many of the species found were first reports of host-pathogen combinations for Spain and elsewhere. None of these *Phytophthora* species, however, are considered as native to the Balearic Islands and eastern Spain, on the basis of ongoing surveys in natural ecosystems. Indeed, only *P. nicotianae* and *P. citrophthora* seem to be well established in irrigated soils in citrus orchards and horticultural crops, but have not been detected thus far in natural vegetation (Moralejo *et al.*, 2008). In ecological and biogeographic terms, all these taxa can thus be considered as alien pathogens for these regions.

Several of the *Phytophthora* spp. found in nurseries and garden centres might pose a threat to natural ecosystems in the area of study or in any other region receiving imported plants. For example, there is circumstantial evidence that the soilborne pathogens *P. cinnamomi* and *P. cryptogea* have escaped from nurseries and are currently spreading in *Q. ilex* (holm oak) woodlands, and also infect associated shrubs such as *A. unedo* and *C. monspeliensis* in the lowlands of northern Mallorca. They were isolated in May 2003 from soils associated with holm oak decline and mortality in a private garden in Pollença, north Mallorca. Both pathogens are common on ornamental plants and have recently appeared on *Q. ilex* in forest nurseries in southern Spain (Sánchez *et al*., 2005), and are in addition already established and inflicting damage on holm and cork oaks in huge extensions of the southwest of the Iberian Peninsula.

Although, at present, *P. ramorum* has not been detected outside nurseries in Spain, the presence of the pathogen on various ornamental hosts in garden centres of the Balearic Islands and mainland obviously constitutes a serious threat. The risk posed to Mediterranean vegetation proved high in *in vitro* inoculations (Moralejo *et al*., 2006b), where leaves of over 70% of the plant species found in holm oak woodlands and maquis vegetation were susceptible to some degree. In addition, the inner bark of native *Quercus* trees from Iberia was highly susceptible to *P. ramorum* colonization in wound-inoculation assays (E. Moralejo, unpublished data).

The little-studied newly described species *P. hedraiandra* was isolated in 2002 in Mallorca, 2 years before its formal description by de Cock & Lévesque (2004) from *Viburnum* plants in the Netherlands. *Phytophthora hedraiandra* is rather aggressive to the Mediterranean native species *V. tinus* (Moralejo *et al*., 2007), and shows an increasing host range and a capacity to hybridize with *P. cactorum* (Man in't Veld *et al*., 2007). As occurred with *P. ramorum*, it is spreading worldwide through the ornamental plant trade (Man in't Veld *et al*., 2007; Moralejo *et al*., 2007). In addition, *P. cactorum* can also hybridize with *P. nicotianae* in nurseries (Man in't Veld *et al*., 2007). There is a possibility of genes being transferred between *P. hedraiandra* and *P. nicotianae* mediated by *P. cactorum* intercrosses (Man in't Veld *et al*., 2007), which could confer new virulence factors and environmental traits to the genotypes which acquire them (e.g. tolerance to lower temperatures in *P. nicotianae*).

In this paper circumstantial evidence is provided, at the local scale, of the introduction of alien *Phytophthora* spp. through the international plant trade. Several findings of hitherto rare or new *Phytophthora* spp. in various European countries have emerged following EU mandatory surveys of *P. ramorum*. Beginning with *P. ramorum*, Ivors *et al.* (2006), in a study on population genetics, demonstrated that several isolates with identical AFLP profiles were distributed across the continent. One of these pan-European clonal types (P ram 1 = P25) was isolated in Mallorca (Ivors *et al*., 2006), where there is no production of ornamental rhododendrons. Three other species, *P. tentaculata* (Moralejo *et al*., 2004; Álvarez *et al*., 2006), *P. hedraiandra* (Moralejo *et al*., 2006a) and *P. tropicalis*, are examples of coincident detections in nurseries of southern and northern Europe, and might reflect major trade pathways. This current study shows some interesting parallels with a recent survey carried out in ornamental nurseries in Minnesota, USA, in which *P. hedraiandra* and *P.* 'taxon Pgchlamydo' were also found infecting *Rhododendron* (Schwingle *et al*., 2007). The intercontinental plant trade may well have contributed to converting local *Phytophthora* introductions to a global scale, as for example suggested by the simultaneous findings of *P.* 'niederhauserii', *P. hibernalis* and *P.* 'kelmania' in North America and Spain. Nevertheless, the evidence on trade routes remains circumstantial. The current cosmopolitan distribution of major *Phytophthora* spp., together with the difficulties of elucidating any phylogeographic relationships between *Phytophthora* spp. provides stronger evidence in support of their spread by human activity. It also highlights the lack of knowledge that exists regarding the centre of origin of most *Phytophthora* species.

Such a flow of alien *Phytophthora* spp. may have been facilitated over time by several events. First, plant collectors may have accidentally dispersed infected plant material or infested soil from their natural habitats. Secondly, optimal environmental conditions provided by frequent overhead sprinkling and mild temperatures necessary for stock plants, scion propagation and rooting of stem cuttings may enhance the multiplication of zoosporic pathogens. Once established in commercial nurseries, pathogen spread is also likely to be promoted by the extended practice of spraying ornamentals with preventive/putatively curative fungicides, which favour the development of fungicide resistance or fail to eliminate the pathogen (Brasier, 2005).

Current understanding of the biological properties enabling a *Phytophthora* pathogen to invade a new environment is usually too poor to predict the risk it poses. Indeed, whether some of the *Phytophthora* taxa found in this survey could become established and later inflict economic and/or ecological damage to agriculture or a natural ecosystem is not yet known. This type of scientific gap is one of the 'biological weaknesses' enumerated by Brasier (2005; 2008) which are currently not dealt with in the international plant health system. Thus, recently described species such as *P. hedraiandra*, informally described taxa such as *P.* 'taxon Pgchlamydo', or even genotypes such *P.* 'citricola-like', examples of which were found in this survey, are not yet listed in quarantine schedules, or covered by pest risk analyses. They are therefore subject to continuing spread by the global plant trade (see Brasier, 2007; 2008). This increases the likelihood of new host-pathogen encounters within nurseries, and thus of widening host ranges. The network may broaden when pathogens escape from nurseries to natural vegetation, as exemplified by *P. ramorum*.

The fact is often ignored that exotic microorganisms, apparently harmless in their centres of origin, may alter the population dynamics and structure of invaded plant communities (Mooney & Cleland, 2001; Bohlen, 2006). Currently, the ornamental trade is suspected to be the main vehicle between the often remote original habitat of the pathogen and the newly invaded ecosystem.

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