Phylogenetic Analysis of Downy Mildew Pathogens of Opium Poppy and PCR-Based In Planta and Seed Detection of *Peronospora arborescens*

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ABSTRACT

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Severe downy mildew diseases of opium poppy (*Papaver somniferum*) can be caused by *Peronospora arborescens* and *P. cristata*, but differentiating between the two pathogens is difficult because they share morphological features and a similar host range. In Spain, where severe epidemics of downy mildew of opium poppy have occurred recently, the pathogen was identified as *P. arborescens* on the basis of morphological traits. In this current study, sequence homology and phylogenetic analyses of the internal transcribed spacer regions (ITS) of the ribosomal DNA (rDNA) were carried out with DNA from *P. arborescens* and *P. cristata* from diverse geographic origins, which suggested that only *P. arborescens* occurs in cultivated *Papaver somniferum* in Spain. Moreover, analyses of the rDNA ITS region from 27 samples of downy-mildew-affected tissues from all opium-poppy-growing regions in Spain showed that genetic diversity exists within *P. arborescens* populations in Spain and that these are phylogenetically distinct from *P. cristata*. *P. cristata* instead shares a more recent, common ancestor with a range of *Peronospora* species that includes those found on host plants that are not members of the Papaveraceae. Species-specific primers and a PCR assay protocol were developed that differentiated *P. arborescens* and *P. cristata* and proved useful for the detection of *P. arborescens* in symptomatic and asymptomatic opium poppy plant parts. Use of these primers demonstrated that *P. arborescens* can be transmitted in seeds and that commercial seed stocks collected from crops with high incidence of the disease were frequently infected. Field experiments conducted in microplots free from *P. arborescens* using seed stocks harvested from infected capsules further demonstrated that transmission from seedborne *P. arborescens* to opium poppy plants can occur. Therefore, the specific-PCR detection protocol developed in this study can be of use for epidemiological studies and diagnosing the pathogen in commercial seed stocks; thus facilitating the sanitary control of the disease and avoidance of the pathogen distribution in seeds.

Additional keywords: genetic diversity, *Papaver* spp., *Peronospora cristata*, seedborne transmission.

The exact characterization and understanding of the genetic relationships among taxonomically related plant pathogenic species are key elements for the design of disease management strategies. Opium poppy (*Papaver somniferum* L.), the only source of the alkaloid drugs (morphine, codeine, and thebaine) for the pharmaceutical industry, can be severely affected worldwide by downy mildew diseases caused by *Peronospora arborescens* and *P. cristata* (11,36,37,45). These two *Peronospora* species were first differentiated on the basis of the average conidium dimensions (33). However, conidium dimensions of the two species were later found to overlap, making it difficult to differentiate between them solely on the basis of morphological characters (36). A more accurate differentiation of the two species was accomplished on the basis of sequence homology and phylogenetic analyses of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) (36).

In addition to overlapping morphological characters, *P. arborescens* and *P. cristata* also exhibit an overlap in their host range. *P. arborescens* has been reported to infect *Argemone mexicana* (29), as well as several *Meconopsis* spp., including *M. betonicifolia*, *M. cambrica*, *M. latifolia*, *M. napaulensis*, *M. polyanthemos*, and *M. simplicifolia* (2,16,18,33), and *Papaver* spp., including *Papaver alpinum*, *Papaver argemone*, *Papaver caucasicum*, *Papaver dubium*, *Papaver hybridum*, *Papaver lecoqii*, *Papaver litwinowii*, *Papaver nudicaule*, *Papaver orientale*, *Papaver pavonimumm*, *Papaver rhoeas*, *Papaver Setigerum*, and *Papaver somniferum* (2,4,6,15,16,18,19,23,24,34). Similarly, *P. cristata* was reported to infect *M. betonicifolia* (31), *M. cambrica* (33), *Papaver argemone*, *Papaver hybridum*, and *Papaver rhoeas* (11,21,23,33), and recently, *Papaver somniferum* (36). Interestingly, *P. cristata* has only been reported on host species that are also recorded hosts of *P. arborescens.*

In Spain, opium poppy is grown annually on approximately 7,500 ha, primarily in the southern (Andalucia) and central (Castilla-La Mancha and Castilla-León) regions of the country. This acreage accounts for at least 5% of the legally cultivated opium poppy worldwide, making Spain the fifth largest European producer of poppy seeds and straw (28,30,32). During the last few years, yields of opium poppy crops in Spain have decreased as a consequence of losses from several diseases, including poppy fire caused by *Pleospora papaveracea* (anamorph = *Dendryphion penicillatum*) and others of unknown etiology (30). In the spring of 2004, severe epidemics of downy-mildew-affected commercial opium poppy crops in different growing regions across the country. This time period was characterized by exceptionally wet and mild weather, particularly in southern Spain; the mean monthly rainfall was higher (46 to 182 mm) and the temperature was lower (10 to 17°C) than the means of 31 to 55 mm and 12 to 19°C, respectively, recorded between 1971 and 2000 (26). At that time,

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the pathogen was identified as *P. arborescens* based on morphological features (28). Although this was the first report of this pathogen in Spain, opium poppy growers had previously observed symptoms of downy mildew (irregularly shaped, chlorotic lesion on upper poppy leaves and brown, angular necrotic spots on the lowermost ones) (F. J. Muñoz-Ledesma, *personal communication*). Therefore, this downy mildew disease may have occurred in Spain with low prevalence and incidence since opium poppy was first established as a crop in the drier areas of Andalucía in the early 1970s. If this is the same disease, then it has now spread and increased in incidence and severity as production of the crop has expanded to new cooler and more humid irrigated areas in central Spain for improving harvest yields (30). This geographical extension of the downy mildew disease to areas where opium poppy had not been cropped before, together with the patchy aggregation of affected plants often observed (15,36; M. Montes-Borrego, B. B. Landa, F. J. Muñoz-Ledesma, and R. M. Jiménez-Díaz, *unpublished data*), suggested that primary inoculum for the disease may have been seedborne and/or distributed from infected wild poppy plants. Additionally, the risk of *P. cristata* infection resulting from indigenous inoculum should be considered since *P. arborescens* and *P. cristata* have been reported in Europe and several of their common hosts are present in Spain (i.e., *M. cambrica*, *Papaver argemone*, *Papaver hybridum*, and *Papaver rhoeas*) (8). However, the role that *P. cristata* may play in epidemics of opium poppy downy mildew in Spain still remains to be investigated.

The specific objectives of this research were to: (i) confirm the taxonomic identity of the main opium poppy downy mildew pathogen in Spain; (ii) determine the phylogenetic relationships that exists between and within *P. arborescens* and *P. cristata* populations and with related genera within the Peronosporales on the basis of analysis of ITS rDNA sequences; (iii) develop a polymerase chain reaction (PCR) assay for the specific identification and detection of *P. arborescens*; and (iv) determine if *P. arborescens* can be seedborne in opium poppy seeds.

MATERIALS AND METHODS

Sample collections. Diseased and healthy opium poppy plants were sampled in the spring (April–June) of 2004–2006 from commercial crops in all growing areas of the country, including the provinces of Córdoba, Málaga, and Sevilla in Andalucía, southern Spain and Albacete and Toledo in the Castilla-La Mancha Region of central Spain (Table 1). Samples included roots and symptomatic leaves, stems, and capsules (showing pathogen sporulation, irregularly shaped chlorotic lesions, and brown, angular necrotic spots) from diseased plants, as well as asymptomatic tissues from infected and healthy plants. DNA extracted from samples of *M. cambrica* (United Kingdom) and *Papaver somniferum* (Australia) infected by *P. cristata*, *Allium cepa* infected by *Peronospora destructor*, *Lactuca sativa* infected by *Bremia lactucae*, *Helianthus annuus* infected by *Plasmopara halstedii*, and from other fungi isolated from opium poppy tissues (*Beauveria bassiana*, *Fusarium* spp., and *Pleospora papaveracea*) were included in the study (Table 1).

DNA extraction and quantification. Total genomic DNA was extracted from the oomycetes mycelia and sporangia scrapped from sporulating leaves, as well as from symptomatic and asymptomatic plant tissues with the Fast DNA kit (Qbiogene, Madrid, Spain). This latter system was selected as the most suitable for DNA extraction in the study because of the: (i) high number of samples that could be processed per operator; (ii) consistency in the amount of total DNA extracted; (iii) quality and stability of the extracted DNA; and (iv) total DNA extracted can be used directly for PCR assays.

The obligate biotrophy of *P. arborescens* makes it difficult to obtain DNA of PCR quality of the oomycete that is also free from contaminant DNAs (mainly bacterial and plant DNA). For this reason, pathogen DNA was extracted from sporangiophores bearing sporangia that were carefully removed from naturally or artificially induced sporulating leaves. These structures were collected with a sterile needle and placed in 1.5-ml microcentrifuge tubes containing 1,000 µl of sterile distilled water and a drop of Tween 20 (Sigma-Aldrich, Madrid, Spain). The suspension was vortexed for a few seconds, filtered through a 0.8-µM Millipore filter, and the filter was washed three times with ultrapure, sterile water (40). Sporangia and sporangiophores retained on the filter were resuspended in 500 µl of sterile water and centrifuged at 12,000 × *g* for 2 min and the pellet was finally resuspended in 200 µl of ultrapure, sterile water. For plant material, fresh tissues were cut into small pieces with a sterile blade and used for DNA extraction. Approximately 100 to 200 mg of plant tissue or sporangiophores and sporangia suspensions were placed in a 1.5-ml Fast DNA tube containing lysing matrix A, 800 µl of CLS-VF solution, and 200 µl of protein precipitation solution (PPS) for plant material, and 1,000 µl CLS-Y solution for suspensions of pathogen structures. Cells were mechanically disrupted in a Fast Prep System Bio 101 (Qbiogene) by reciprocal shaking of the samples for 30 s at a 5.5 speed, twice. Samples were incubated on ice for 2 min between successive homogenizations. Then, the supernatant was collected by centrifugation (10 min at 12000 × *g*) and processed with the Fast DNA kit according to the manufacturer's instructions.

For seed assays, approximately 400 to 500 opium poppy seeds (average weight = 130 to 150 mg) were placed in a 1.5-ml Fast DNA tube containing lysing matrix A, 800 µl of buffer CLS-VF and 200 µl of PPS, and allowed to soften for 1 h before processing as described above. Thereafter, the DNA pellet was resuspended in ultrapure, sterile water, quantified with the Quant-iT DNA Assay Kit Broad Range fluorometric assay (Molecular Probes Inc., Leiden, the Netherlands) and a Tecan Safire fluorospectrometer (Tecan Spain, Barcelona, Spain) according to the manufacturer's instructions, diluted with ultrapure, sterile water, and used for PCR assays.

PCR protocols using universal primers. The ITS1-5.8S-ITS2 regions of the rDNA of different species of Peronosporaceae, fungi, and plants used in the study (Table 1) were amplified in PCR assays using universal primers (44) as well as primer DC6 (7) specific for species in the orders Pythiales and Peronosporales of the Oomycota.

ITS5/ITS4 proved the most effective universal primers in a reaction mix (final volume of 50 µl) of 5 µl of 10× reaction buffer (166 mM $[NH_4]_2SO_4$, 670 mM Tris-HCl [pH 8.0, 25°C], Tween 20), 1 µM of each primer, 200 µM of each dNTP, 2 units of *EcoTaq* DNA polymerase (EcoGen, Madrid, Spain), 1.5 mM $MgCl_2$, and 1 µl of template DNA (5 to 20 ng of DNA). Amplifications were performed in Perkin-Elmer 9600 (Perkin-Elmer, Norwalk, CT) and PTC 100 (MJ Research Inc., Watertown, MA) thermocyclers. The cycling program included an initial denaturation step of 4 min at 95°C, followed by 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 56°C, 2 min extension at 72°C, and a final 10 min extension step at 72°C followed by a 4°C soak. The DC6/ITS4 primer pair was used for the selective amplification of the complete ITS region of rDNA together with a portion of the 18S rDNA. PCR reactions were conducted in 50-µl volumes as described before, except that 0.6 µM of each primer and 60 µM of each dNTP were used. PCR conditions were initial denaturation of 5 min at 95°C, followed by 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 62°C, 2 min extension at 72°C, and a final extension of 10 min at 72°C followed by a 4°C soak.

All reactions were repeated at least twice and always included a positive control (*P. arborescens* DNA obtained from sporangiophores with sporangia scraped from sporulating leaves) and negative controls (*Papaver somniferum* DNA or no DNA). Amplification products were separated by electrophoresis in 1.5% agarose

gels in 1× TAE buffer for 60 to 120 min at 80V, stained with ethidium bromide, and visualized under UV light. The GeneRuler DNA ladder mix (Fermentas, St Leon-Rot, Germany) was used for electrophoresis.

Sequencing of amplified products and phylogenetic analysis. PCR products amplified with primer pairs ITS5/ITS4, DC6/ITS4, or the primer pair pdm3/pdm4 (36; see below) were purified with a gel extraction kit (Geneclean turbo, Qbiogene, Illkirch, France), quantified with the Quant-iT DNA Assay Kit Broad Range fluorometric assay as described before, and used for direct DNA sequencing or for cloning. Purified PCR products were ligated into pGEM-T easy vector system II using *Escherichia coli* strain JM109 for transformation according to the manufacturer's procedure (Promega, Madison, WI). Transformed clones were cultured and the plasmid DNA extracted from them (two clones per PCR amplicon) with the Wizard Plus SV Minipreps DNA purification system (Promega) was used for sequencing. PCR amplicons and cloned inserts were sequenced in both directions with a terminator cycle sequencing ready reaction kit (BigDye; Perkin-Elmer Applied Biosystems, Madrid, Spain) using DC6, ITS4, and ITS5 primers (PCR amplicons) or universal primers (M13-20fw/M13rev) (cloned inserts), according to the manufacturer's instructions. The resulting products were purified and run on a DNA multicapilar sequencer (ABI Prism 3100 genetic analyzer; Perkin-Elmer Applied Biosystems) at the University of Córdoba sequencing facilities. All ITS1-5.8S-ITS2 sequences obtained from *P. arborescens* collections in the study were deposited in GenBank (Table 1). In addition, ITS1-5.8S-ITS2 sequences from *Papaver somniferum*, *Fusarium* spp., and *Pleospora papaveracea* isolated from opium poppy, *P. destructor* infecting onion, and *P. cristata* infecting *M. cambrica* were obtained (Table 1).

The ITS sequences of *P. arborescens* (27 sequences), *P. destructor* (1 sequence), and *P. cristata* (2 sequences) obtained in this study were aligned with 41 published DNA sequences of Oomycete species belonging to the Peronosporaceae (Table 2), including the genera *Hyaloperonospora*, *Perofascia*, *Peronospora*, and *Pseudoperonospora*. The *Peronospora* and *Pseudoperonospora* spp. were selected because they: (i) are closely related to *P. arborescens* (36,43); (ii) have their hosts within the Ranunculales (Papaveraceae and Fumariaceae) (17); or (iii) have been recorded in Spain (24).

TABLE 1. List of *Peronospora* spp. and specimens for which PCR amplicons were sequenced in the study and fungi and oomycetes used to determine specificity of the species-specific primers

Pathogen species	Host species, sampled tissue	Geographic origin	Collection code	Year	GenBank accession no.
Peronospora spp.					
P. arborescens	*Papaver somniferum*, mycelium	"Casa Arriba Los Llanos", Albacete, Spain	P13ACALL	2005	DQ885367
	Papaver somniferum, leaves	"Casilla San José", Écija , Sevilla, Spain	P2ESJ	2004	AY695805
			P5ESJ	2005	AY695807
	Papaver somniferum, mycelium	"Casilla San José", Écija, Sevilla, Spain	P38ESJ	2004	DQ886487
			P6ESJ	2005	DQ885363
	Papaver somniferum, leaves	"San Rafael", Écija, Sevilla, Spain	P1ESR	2004	AY695804
			P4ESR	2004	AY695806
	Papaver somniferum, mycelium	"San Rafael", Écija, Sevilla, Spain	P37ESR	2005	DQ885364
	Papaver somniferum, leaves	"Ruidero", Écija, Sevilla, Spain	M2ERUI	2004	DQ885362
	Papaver somniferum, mycelium	Viso alto", Écija, Sevilla, Spain	P8EVA	2005	DQ885365
	Papaver somniferum, leaves		P7EVA	2005	DQ885364
	Papaver somniferum, leaves	"La Estrella", Écija, Sevilla, Spain	P9ELE	2005	DQ885366
	Papaver somniferum, mycelium	"Monteluna", Antequera, Málaga, Spain	P29MAM	2006	DQ885378
			P30MAM	2006	DQ885379
	Papaver somniferum, mycelium	"Valsequillo", Antequera, Málaga, Spain	P27MAV	2006	DQ885376
	Papaver somniferum, leaves	"Valsequillo", Antequera, Málaga, Spain	P28MAV	2006	DQ885377
	Papaver somniferum, mycelium	"El Pontón", Antequera, Málaga, Spain	P31MEP	2006	DQ885380
	Papaver somniferum, mycelium	"Cortijo del Río", Antequera, Málaga, Spain	P32MACR	2006	DQ885381
			P33MACR	2006	DQ885382
			P34MACR	2006	DQ885383
	Papaver somniferum, leaves	"Hormigos", Malpica de Tajo, Toledo, Spain	TO	2004	AY702098
			M1THO	2005	DQ886488
	Papaver somniferum, mycelium	"Valdemerino", Malpica de Tajo, Toledo, Spain	P16TVMT	2005	DQ885368
			P17TVMT	2005	DQ885369
			P19TVMT	2005	DQ885371
	Papaver somniferum, mycelium	"El Torrejón", Malpica de Tajo, Toledo, Spain	P18TTMT	2005	DQ885370
			P20TTMT	2005	DQ885372
	Papaver somniferum, stem	"El Torrejón", Malpica de Tajo, Toledo, Spain	P21TTMT	2005	DQ885373
P. cristata[a]	*Meconopsis cambrica*, leaves	England, UK	P22MC	2005	DQ885374
			P24MC	2005	DQ885375
	Papaver somniferum, leaves	Tasmania, Australia (F. S. Hay)		2005	Np[b]
	Papaver somniferum, leaves	Tasmania, Australia (P. J. Cotterill)		2005	Np
P. destructor[a]	*Allium cepa*, leaves	Norway	P46N	2005	DQ885385
Other fungi and Oomycetes					
Fusarium sp.	*Papaver somniferum*, leaves	Écija, Sevilla, Spain	FVM	2004	DQ885387
Fusarium sp.	*Papaver somniferum*, leaves	Écija, Sevilla, Spain	FVS3	2004	DQ885388
Beauveria bassiana	*Papaver* somniferum, leaves	Carmona, Sevilla, Spain	EaBb 04/01	2004	DQ364698
Pleospora papaveracea	*Papaver* somniferum, seeds	Carmona, Sevilla, Spain	D2	2004	DQ885386
Bremia lactucae[a]	*Lactuca sativa*	Córdoba, Spain		2006	Np
Plasmopara halstedii[a]	*Helianthus annuus*	Córdoba, Spain		2004	Np
------	*Papaver somniferum* var. nigrum	ALCALIBER, S.A., Carmona, Sevilla, Spain		2004	DQ364699

[a] Plant samples infected by *P. cristata, P. destructor, Plasmopara halstedii*, and *B. lactucae* were provided by D. E. L. Cooke (Scottish Crop Research Institute, Invergowrie, Dundee, Scotland, UK), F. S. Hay (Tasmanian Institute of Agricultural Research, University of Tasmania, Burnie, Australia), P. J. Cotterill (GlaxoSmithKline, Latrobe, Tasmania, Australia), B. Nordskog (Plante forsk, The Norwegian Crop Research Institute, Norway), L. Molinero (IAS-CSIC, Córdoba, Spain), and J.A. Navas-Cortés (IAS-CSIC, Córdoba, Spain).

[b] Np = not performed.

Sequences were initially aligned with the ClustalX 1.83 software (41) with default options. Then, the Bionumerics 4.5 software (Applied Maths, Kortrijk, Belgium) was used to generate phylogenetic trees with the neighbor-joining (NJ) and maximum-parsimony (MP) methods and UPGMA cluster analysis. The phylograms were bootstrapped 1,000 times to assess the degree of support for the phylogenetic branching indicated by the optimal trees. Trees were rooted with *Phytophthora infestans* AF266779 ITS1-5.8S-ITS2 gene sequence as an outgroup (43).

Design of *P. arborescens* specific primers. A subset of nonredundant ITS1-5.8S-ITS2 sequences of *P. arborescens* and *P. cristata* selected from GenBank, as well as sequences obtained in this study, were aligned using the ClustalX 1.83 software and utilized for designing *P. arborescens*-specific PCR primers (Table 3). Primers were designed with Primer3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and Beacon Designer (http://www.premierbiosoft.com/netprimer/netprimer.html) software to ensure that at least one of the primers contain less than 85% homology and includes one nucleotide insertion as compared with *P. cristata* ITS1-5.8S-ITS2 sequences and ensure lack of homology with ITS1-5.8S-ITS2 sequences of *Papaver somniferum* or fungi commonly isolated from this plant (see below). An in silico test of primer specificity was conducted by running the primer sequences against the nonredundant GenBank dataset with parameters set for the identification of short, nearly exact matches.

Specific PCR assays. Reaction conditions such as annealing temperature and $MgCl_2$ and primer concentrations were adjusted experimentally to optimize the amplification with each primer pair (Table 3). Optimized PCR reactions were (final volume of

TABLE 2. List of published GenBank accession numbers and associated host species of Peronosporales taxa used in the current study

Species	Host species	Geographic origin	GenBank accession nos. (reference)
Perofascia lepidii	*Lepidium virginicum*	Korea	AY211013 (9)
Perofasica lepidii	*Capsella bursa-pastoris*	Romania	AF465760 (12)
Peronospora spp.			
P. alpicola	*Ranunculus aconitifolius*	Germany	AY198271 (43)
P. aparines	*Galium aparine*	Austria	AY198300 (43)
P. arborescens	*Papaver rhoeas*	Austria	AY198292 (43)
P. arborescens	*Papaver rhoeas*	Romania	AY465761 (12)
P. bulbocapni	*Corydalis cava*	Austria	AY198272 (43)
P. conglomerata	*Geranium molle*	Austria	AY919304 (5)
P. conglomerata	*Geranium molle*	Austria	AY198246 (43)
P. cristata	*Meconopsis cambrica*	England, UK	AY374984 (D.E.L. Cooke and N.A. Williams, *unpublished data*)
P. cristata	*Papaver somniferum*	Tasmania, Australia	AY225472-AY225482, AY225484 (36)
P. chenopodi	*Chenopodium album*	Austria	AY198285 (43)
P. corydalis	*Corydalis ocotensis*	Korea	AY211015 (9)
P. corydalis	*Corydalis speciosa*	Korea	AY211016 (9)
P. destructor	*Allium cepa*	Tasmania, Australia	AY225469 (36)
P. dicentrae	*Dicentra canadensis*	Tennessee, USA	AY198273 (43)
P. farinose	*Chenopodium album*	Romania	AF465762 (12)
P. farinose	*Chenopodium album*	Korea	AY211017 (9)
P. farinose	*Chenopodium serotinum*	Korea	AY211018 (9)
P. manshurica	*Glycine soja*	Korea	AY211019 (9)
P. manshurica	*Glycine max*	Unknown	AB021711 (M. Saito, *unpublished data*)
P. ranunculi	*Ranunculus acris*	Austria	AY198267 (43)
P. sparsa	*Rosa* sp.	England, U.K.	AF266783 (13)
P. sparsa	*Rosa* sp.	Tasmania, Australia	AY225470 (36)
P. tabacina	*Nicotiana alata*	Austria	AY198289 (43)
P. viciae	*Pisum sativa*	Tasmania, Australia	AY225471 (37)
P. viciae	*Vicia angustifolia*	Austria	AY198230 (43)
Hyaloperonospora spp.			
H. parasitica	*Thlaspi arvense*	Romania	AF465759 (12)
H. parasitica	*Capsella bursa-pastoris*	Austria	AY198254 (43)
Pseudoperonospora spp.			
Pseudoperonospora cubensis	*Cucumis sativa*	Austria	AY198306 (43)
Pseudoperonospora humuli	*Humulus lupulus*	Unknown	AF448225 (J. Patzak, *unpublished data*)
Pseudoperonospora humuli	*Humulus lupulus*	Austria	AY198304, AY198305 (43)
Pseudoperonospora urticae	*Urtica dioica*	Austria	AY198307 (43)

TABLE 3. PCR primers designed in this study, their sequences, origins, and specificities

Primer name[a]	Sense	Primer set name	Detects	Source	Sequence (5′-3′)	Melting temperature (°C)	Amplicon size (bp)
P3Pa1fw[b]	Forward	P1	*Peronospora arborescens*	ITS2	GCTATGGCGATAATGGAGGA	60.0	200
P3Pa1rv[b]	Reverse		Peronosporales	ITS2	CAAATTTCCCAAATGGGTTG	60.0	
P3Pa2fw	Forward	P2	*Peronospora* spp.	ITS1	TGATCTCGGTCGGAGCTAGT	60.0	545
P3Pa2rv[b]	Reverse		*Peronospora* spp.	ITS2	TCACCAGTTATACCGCCACA	60.0	
OMPac1fw	Forward	P3	Oomycetes	ITS1	CCACACCTAAAAACTTTCC	52.3	594
OMPa1rv[b]	Reverse		*P. arborescens*	ITS2	AACACTCCTCCATTATCG	50.5	
OMPac7fw	Forward	P6	Oomycetes	5.8S	GAACGCATATTGCACTTCC	56.7	456
OMPa7rv[b]	Reverse		*P. arborescens*	ITS2	CGCACAACACAAATTTCC	55.9	

[a] The use of the sequences described for a diagnostic test for *P. arborescens* is covered by a Spanish patent application (number assignation P200603319) owned by University of Córdoba and ALCALIBER S.A.

[b] Primers include a nucleotide that is a deletion in the ITS1-5.8S-ITS2 sequence of *P. cristata.*

25 µl) 2.5 µl of 10× reaction buffer, 0.75 µM of each primer, 50 µM of each dNTP, 0.75 unit of *EcoTaq* DNA polymerase (EcoGen), 1 mM $MgCl_2$, and 1 µl of the template DNA. The cycling program consisted of an initial denaturation step of 3 min at 95°C, followed by 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 61°C, 1 min at 72°C, and a final extension step of 5 min at 72°C. Gel electrophoresis was performed as described for universal primers.

Specificity of the designed primer pairs was tested by including the following controls in PCR assays: (i) *P. arborescens* DNA extracted from sporangiophores and sporangia scraped from sporulating opium poppy leaves; (ii) total DNA extracted directly from opium poppy leaves infected with *P. arborescens*; (iii) DNA extracted from healthy *Papaver somniferum*; (iv) total DNA extracted from tissues of *Papaver somniferum* and *M. cambrica* infected with *P. cristata*; (v) DNA extracted from fungi isolated from opium poppy tissues (i.e., *B. bassiana*, *Fusarium* spp., and *Pleospora papaveracea*); (vi) DNA extracted from other oomycetes within the Peronosporaceae (i.e., *B. lactucae*, *P. destructor*, and *Plasmopara halstedii*); and (vii) no DNA as a negative control.

Primers pdm3 and pdm4, reported as specific for the identification and detection of *P. cristata* (36), were also used as described to test for their specificity against populations of *P. arborescens.*

Sensitivity of the diagnostic PCR was assessed by determining the minimum amount of *P. arborescens* DNA that could be detected in PCR reactions. For this purpose, *P. arborescens* DNA (10 ng/µl) was diluted (1:2, 1:10, 1:20, $1:10^2$, $1:10^3$, $1:10^4$, and $1:10^5$) in ultrapure, sterile water, as well as in *Papaver somniferum* DNA extracted from healthy opium poppy leaves (10 ng/µl) or seeds (20 ng/µl) to account for any possible influence that the host DNA might have on the amplification of pathogen DNA. Special care was taken to get accurate pathogen and host DNA concentrations. For this objective, both the stock DNA and its dilutions were quantified fluorimetrically in triplicate in two independent plates using the Quant-iT DNA Assay kit as described above. The host DNA concentrations used were selected as representative of DNA amounts yielded with the Fast DNA protocol described above from 100 mg of leaf tissue and 100 seeds of opium poppy, respectively. All PCR reactions were repeated at least three times by independent operators using the conditions referred to as above and always included *Papaver somniferum* DNA or no DNA as negative controls.

Species-specific PCR assays using samples from opium poppy crops. During the spring of 2005 and 2006, plants were sampled from a commercial opium poppy field with a high incidence of the downy mildew disease in Écija, Seville Province of Andalucía. Samples were representative of severely through mildly affected tissues as well as asymptomatic tissue. Some affected plants showed dwarfing and rosetting, with the uppermost leaves showing light chlorosis, suggesting systemic infection of the plants. All plant tissues were surface sterilized (1% NaOCl for 3 min) prior to DNA extraction. Total DNA was extracted from complete stem samples, the epidermal and cortex tissues of stems, capsules, and leaves affected with necrosis, light chlorosis, or no symptoms. PCR reactions were conducted using three selected specific primer sets (P2, P3, and P6; Table 3).

Detection and transmission of *P. arborescens* in opium poppy seeds. Artificially infested and naturally infected or infested seed samples were used to test the reliability of the developed assay for detecting *P. arborescens* DNA in or on seed. For artificial infestation, healthy seeds (i.e., that gave no amplification for *P. arborescens* in previous assays using the species-specific PCR protocol) were surface sterilized with 1% NaOCl for 3 min, rinsed with distilled, sterile water, dipped in 1 ml of sporangium suspensions adjusted to get 1×10^4, 5×10^3, 2.5×10^3, 2.5×10^2, and 25 sporangia per 100 seeds, and vortexed. The treated seeds were allowed to dry at room temperature in a Vacufuge TM Concentrator 5301 system (Eppendorf Ibérica S.L., Madrid, Spain). Total DNA was extracted from the artificially infested seeds as described above.

Stocks of naturally infected or infested seeds were obtained from capsules of diseased plants sampled from opium poppy fields with high incidence of downy mildew. These capsules showed a range of disease symptoms, from nonsymptomatic but having light sporulation on the capsule peduncle to the entire capsule showing severe necrosis. Seeds were obtained from capsules through a hole at the base of a capsule made by aseptically removing its peduncle at the site of junction and emptying the seeds into a sterile eppendorf tube. Four samples of 500 seeds each were processed for DNA extraction as described above. PCR assays using DNA extracted from either artificially infested seeds or naturally infected or infested seeds sampled from infected capsules were conducted with the three specific primer sets as described before.

To further investigate whether seedborne *P. arborescens* can give rise to infected opium poppy plants, we conducted an experiment in nonirrigated field microplots that had never been cropped with *Papaver somniferum* (sandy loam soil, pH 8.5, 1.4% organic matter) at the Alameda del Obispo Research Station near Córdoba (37.5°N, 4.8°W, altitude 110 m). The microplots (1.25×1.25 m^2, 50-cm depth) were sown with seed stocks numbers 471, SR, 432, and 431 of the commercial opium poppy cv. Nigrum on 3 January 2005. Seeds of these seed stocks were provided by ALCALIBER S.A. (Carmona, Sevilla, Spain), the only enterprise officially authorized for opium poppy cultivation in Spain, and originated from fields severely affected by downy mildew. Seeds of stock number 451, which had been washed with 1% NaOCl for 3 min and further treated with metalaxyl (Apron XL; Syngenta Agro, Madrid, Spain), were used as a control. Each microplot consisted of four furrows, 0.2 m apart and 0.2 m from the closest microplot edge barrier (a 25 cm tall, 0.5 cm width fiber cement sheet). A microplot was hand sown by carefully spreading 100 seeds per furrow. There were four replicated microplots per seed stock in a completely randomized design. Weeds (especially *Papaver* spp.) that developed inside or outside of the microplots were removed by hand as soon as they developed. Daily mean temperature and rainfall were recorded in a weather station located at the experimental site. After emergence, plants were observed at weekly intervals for development of symptoms of opium poppy downy mildew.

RESULTS

PCR assays using universal primers. Amplification of DNA extracted from *P. arborescens* sporangiophores and sporangia scraped from downy-mildew-affected opium poppy with primers ITS4 and ITS5 yielded fragments of approximately 900 bp. However, amplification of total DNA from symptomatic leaves yielded the 900-bp band and an approximately 800-bp product similar in size to that amplified using DNA extracted from healthy *Papaver somniferum* in a previous study (32).

Amplification of DNA extracted from *P. arborescens* sporangiophores and sporangia with primers DC6 and ITS4 yielded a single PCR amplicon of approximately 1,200 bp. There was no amplification when those two primers were used with DNA extracted from tissues of healthy opium poppy plants.

PCR assays using *P. cristata*-specific primers. Amplification of total DNA extracted from samples of *M. cambrica* and opium poppy infected with *P. cristata* with primer pair pdm3/pdm4 (reportedly specific for *P. cristata*; 36) (Table 1) yielded a single band of approximately 389 bp. Interestingly, when primers pdm3/pdm4 were used for PCR assays of more than 200 samples of diseased opium poppy from Spain, 15% of samples amplified DNA from *P. arborescens* (see below).

Sequencing of amplified products and phylogenetic analysis. Sequence homology and phylogenetic analysis of the ITS1-5.8S-ITS2 regions of rDNA amplified from samples of the downy

mildew pathogen confirmed that *P. arborescens* was the sole causal agent of the disease affecting opium poppy in Spain (Fig. 1). Sequencing and phylogenetic analysis confirmed that all pathogen samples that had previously yielded a 389-bp amplicon with primers pdm3/pdm4 were *P. arborescens* rather than *P. cristata*. The 27 collections of the downy mildew pathogen recovered from *Papaver somniferum* in Spain showed high sequence homology (99.8 to 100%) and were differentiated into four ITS sequence types on the basis of sequence differences in a total number of eight nucleotide positions in the ITS1, 5.8S, and ITS2 regions. These differences were not related to geographical origin (region, province, or field) of isolates (Table 1). ITS sequence homology between collections of *P. arborescens* from opium poppy in Spain and *P. arborescens* AF465761 from Romania (12) and AY198292 from Austria (43) ranged from 99.3 to 99.6%. Sequence homology between *P. arborescens* from Spain and *P. cristata* AY225472–AY225482 (36) from Tasmania and *P. cristata* AY374984 from Europe (D. E. L. Cooke and N. A. Williams, *unpublished data*) ranged from 80.9 to 93.7% and 93.6 to 94%, respectively.

The topology of the phylogenetic trees produced with the NJ (Fig. 1) and MP method were almost identical (data not shown). *P. arborescens* from *Papaver rhoeas* in Romania (AF465761) and Austria (AY198292) grouped with all poppy downy mildew collections from Spain, with bootstrapping values of 100% both for NJ and MP analyses. The two methods of analysis indicated that *P. arborescens* from *Papaver somniferum* and *Papaver rhoeas* formed a single, highly supported clade, phylogenetically distinct to the clade comprising *P. cristata.* Interestingly, *P. cristata* from *Papaver somniferum* and *M. cambrica* is more closely related to other *Peronospora* spp. that have their host in the Ranunculales (*P. corydalis* and *P. cristata* [Papaveraceae], *P. alpicola* and *P.*

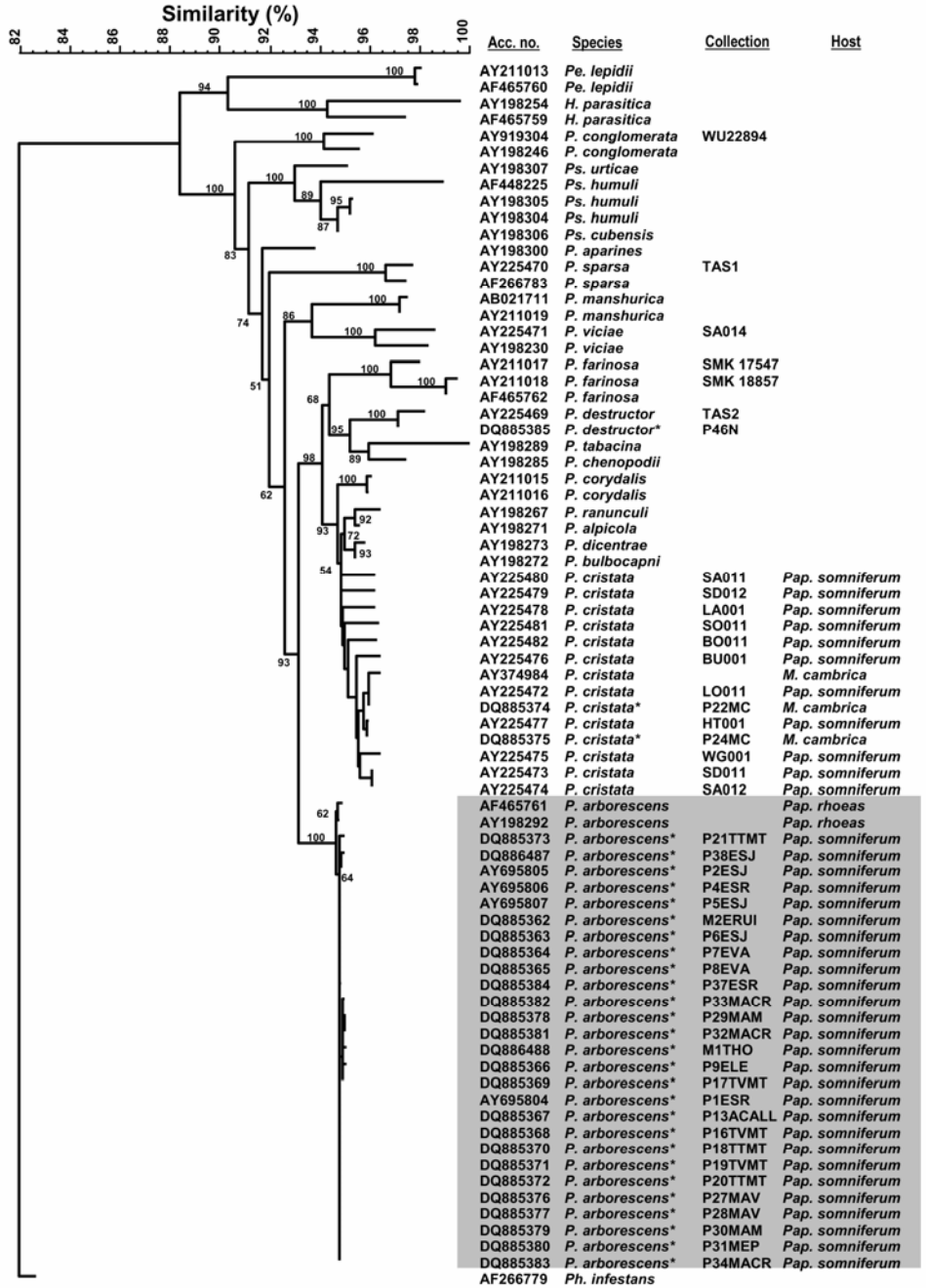

Fig. 1. Phylogenetic tree inferred from neighbor-joining analysis of the complete ITS region (ITS1, 5.8S rDNA, and ITS2) indicating relationships between downy mildew species. Bootstrap supports of more than 50% are given for appropriate main order clades. Asterisks (*) show taxa whose data were obtained in this study. H. = *Hyaloperonospora*, Pe. = *Perofascia*, P. = *Peronospora*, Ps. = *Pseudoperonospora*, and Ph. = *Phytophthora*.

ranunculi [Ranunculaceae], and *P. bulbocapni* and *P. dicentrae* [Fumariaceae]) (Fig. 1).

Design of *P. arborescens*-specific primers. Four primer pairs were designed with the criteria described above, namely P3Pa1fw/P3Pa1rv, P3Pa2fw/P3Pa12rv, OMPac1fw/OMPa1rv, and OMPac7fw/OMPac7rv that were designated P1, P2, P3, and P6 primer sets, respectively. The primer sequences (Table 3) were compared with the NCBI nonredundant nucleotide database (May 7, 2006) using BLAST 2.2.14 (3). Sets P1, P2, P3, and P6 were predicted to be specific for *P. arborescens.*

Specificity of the newly developed *P. arborescens*-specific primers. Primer sets P1, P2, P3, and P6 reproducibly amplified DNA fragments of 200, 545, 594, and 456 bp, respectively, in PCR assays with DNA extracted from all collections of *P. arborescens* sporangiophores and sporangia (Fig. 2) as well as from infected *Papaver somniferum* tissue and samples of *Papaver somniferum* that previously had yielded the 389-bp band (potentially specific for *P. cristata*) with the pdm3/pdm4 primer pair (Fig. 3). No cross amplifications were observed when the tested primer sets were used with DNA extracted from plant samples infected with *P. cristata* either from the United Kingdom or Tasmania or DNA from healthy plants (Fig. 3). Likewise, no cross amplification occurred with DNA of other fungi and Oomycetes tested (data not shown). Use of primer set P1 also yielded a DNA band larger than 200 bp, but of much lower intensity, in addition to the predicted 200-bp amplicon (Fig. 2). Attempts were made to increase the specificity of this primer set with no success; consequently, P1 primer set was disregarded for further studies.

PCR quality of all DNA samples, including samples containing *P. cristata* DNA, was confirmed with the universal ITS5/ITS4 or DC6/ITS4 primer pairs.

Sensitivity of *P. arborescens*-specific primers. Adding DNA from opium poppy in the PCR reaction mix did not influence sensitivity of the specific PCR assays (Table 4). Use of reaction mixes containing 10 ng of opium poppy DNA extracted from healthy leaves resulted in a PCR detection limit of 10, 1 to 10, and 1 to 10 pg of *P. arborescens* DNA for primer sets P2, P3, and P6, respectively (Table 4). Interestingly, the detection limit for each of the primer sets was increased when DNA from surface-sterilized, healthy seeds rather than from leaves was used for dilutions. This occurred even though the background plant DNA concentration was double for seed DNA and both sets of assays were performed with the same stock solutions of *P. arborescens* DNA. Therefore, using reaction mixes containing 20 ng of opium poppy DNA extracted from seeds allowed a detection limit of PCR assays of 1, 1 to 0.1, and 1 pg (Fig. 4) of *P. arborescens* DNA for primer sets P2, P3, and P6, respectively. Adding DNA from poppy seeds or leaves to PCR reaction mixes using primer pair DC6/ITS4 resulted in a detection limit of 100 pg of *P. arborescens* DNA, irrespective of the source of plant DNA (Table 4). Use of the detection method by different operators did not influence reproducibility and consistency of results.

Species-specific PCR assays with samples from opium poppy crops. PCR assays using P2, P3, and P6 primer sets and DNA extracted from leaf, stems, and capsules of diseased opium poppy plants, as well as from roots of plants with dwarfing and resetting symptoms, resulted in nearly 100% positive detection of *P. arborescens* in the infected tissues (Fig. 5). There were no amplification products in PCR assays with DNA extracted from leaves and capsules of apparently healthy opium poppy plants. When DNA was extracted from infected plants showing very light chlorosis or no symptoms, detection of the pathogen on leaves and epidermal stem tissues was more efficient using primer set P3 compared with use of primer sets P2 and P6 (Fig. 5).

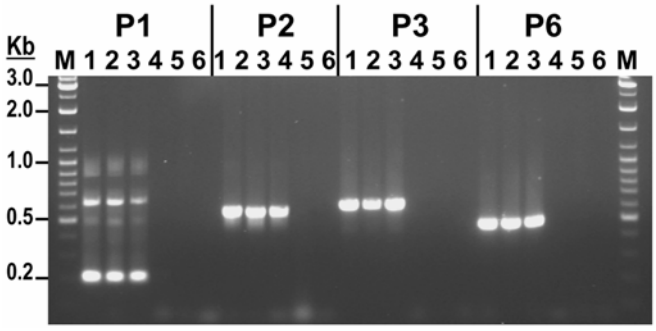

Fig. 2. Specificity of *Peronospora arborescens*-specific primers P3Pa1fw/P3Pa1rv (P1), P3Pa2fw/P3Pa12rv (P2), OMPac1fw/OMPa1rv (P3), and OMPac7fw/OMPac7rv (P6). M, GeneRuler DNA ladder mix (Fermentas, St Leon-Rot, Germany); lane 1, *P. arborescens* sporangiophore and sporangia from sporulating opium poppy leaves (positive control); lane 2, *P. arborescens*-infected leaves of *Papaver somniferum*; lane 3, *P. arborescens*-infected stem of *Papaver somniferum*; lane 4, healthy leaves of *Papaver somniferum*; lane 5, healthy stem of *Papaver somniferum*; and lane 6, no DNA template.

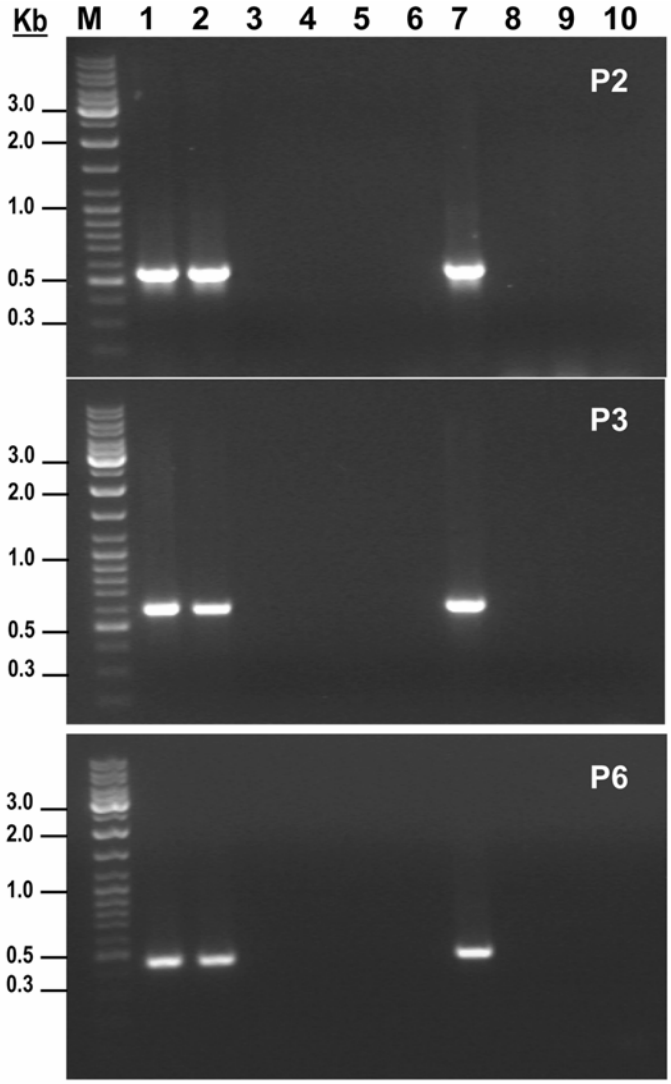

Fig. 3. Specificity of *Peronospora arborescens*-specific primers P3Pa2fw/P3Pa12rv (P2), OMPac1fw/OMPa1rv (P3), and OMPac7fw/OMPac7rv (P6). M, GeneRuler DNA ladder mix (Fermentas, St Leon-Rot, Germany); lane 1, *P. arborescens* sporangiophores and sporangia from sporulating opium poppy leaves (positive control); lane 2, *P. arborescens*-infected leaves of *Papaver somniferum*; lanes 3–5, *P. cristata*-infected leaves of *Papaver somniferum* from Australia; lane 6, *P. cristata*-infected leaves of *Meconopis cambrica* from the United Kingdom; lane 7, *P. arborescens*-infected leaves of *Papaver somniferum* that also yielded amplification with primers pdm3/pdm4 (described as specific for *P. cristata,* 37); lane 8, healthy leaves of *Papaver somniferum*; lane 9, healthy, surface-sterilized seeds of *Papaver somniferum*; and lane 10, no DNA template.

Detection and transmission of *P. arborescens* in opium poppy seeds. PCR assays with primer sets P2, P3, or P6 were equally effective in the detection of *P. arborescens* in artificially infested seeds and allowed detection of up to 25 sporangia per 100 seeds with any of the primers (data not shown). Similarly, each of the three primer sets was effective in the detection of seedborne *P. arborescens* in seed stocks collected from infected capsules harvested from affected plants (Fig. 5). Conversely, *P. arborescens* was not detected on seed stocks collected from asymptomatic capsules (data not shown).

Seed stocks collected from fields severely affected by downy mildew and used for field experiments resulted in positive amplification for *P. arborescens* in 75% of samples using the species-specific primers. Seedlings in the field microplots emerged 30 to 40 days after sowing. Typical symptoms of downy mildew (i.e., chlorotic foliar lesions on lower leaves that later became brown, angular spots) appeared on the opium poppy plants in microplots approximately 40 to 60 days after seedling emergence. All symptomatic leaves showed sporulation of the pathogen. In general, there was a low incidence of downy mildew for all seed stocks tested. No disease developed in microplots sown to control seeds. The disease occurred in 25 to 100% of microplots sown to an infected seed stock, with 25 to 50% of rows of plants within a microplot showing affected plants and only a few plants within these rows showing disease symptoms. Usually, affected plants in a microplot were aggregated (three to six plants showing symptoms clustered in a patch). Unusual warm and dry weather conditions occurred during the year of experiment, which caused symptomatic plants to become necrotic and die very early in the season (7 to 14 days after pathogen sporulation). These conditions were characterized by monthly average mean maximum and minimum temperatures from March through May that were 1.8 to 5.8°C higher than those occurring in regular years. Monthly average rainfall and mean relative humidity from January through May were 7 to 64 mm and 2 to 17% lower than normal, respectively (26). The unusually warm and dry weather probably accounted for the low incidence of diseased plants and reduced pathogen spread within a microplot. The presence of *P. arborescens* on symptomatic plants was confirmed by the species-specific PCR assay (data not shown).

DISCUSSION

Phylogenetic analysis of the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) demonstrated that *P. arborescens* is the downy mildew pathogen of *Papaver somniferum* in Spain. We also documented that there is a low degree of genetic diversity within *P. arborescens* populations occurring in Spain. *P. arborescens* has been reported in different countries throughout the world, including Afghanistan, Algeria, Argentina, Australia, Austria, Azerbaijan, Belgium, Bulgaria, Canada, China, Egypt, Finland, Germany, Greece, India, Iran, Italy, Japan, Korea, Libya, Pakistan, Poland, Romania, South Africa, Sweden, Thailand, Turkey, United Kingdom, United States, and Uzbekistan. Comparatively, the geographic range of *P. cristata* appears to be more reduced, since this species has been reported only in Australia, Bulgaria, England, and Ireland (18,19). In the Iberian Peninsula, *P. arborescens* has been reported to be infecting *Papaver dubium*, *Papaver hybridum*, and *Papaver rhoeas* in Spain, and infecting wild *Papaver somniferum* and *Papaver rhoeas* in Portugal (24). Since *P. arborescens* and *P. cristata* over-

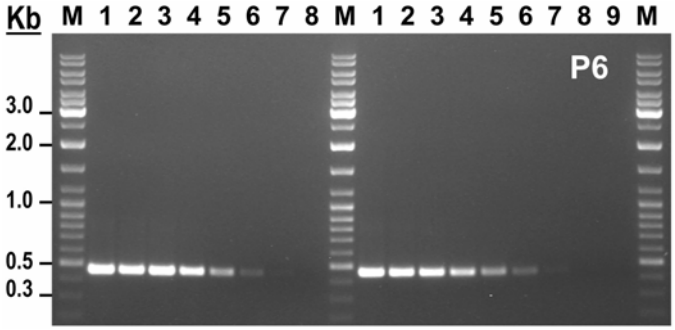

Fig. 4. Sensitivity of *Peronospora arborescens*-specific primers OMPac7fw/OMPac7rv (P6) with *P. arborescens* DNA (10 ng/µl) diluted in water (H_2O Series) or in DNA of *Papaver somniferum* (20 ng/µl) extracted from seeds. M, GeneRuler DNA ladder mix (Fermentas, St Leon-Rot, Germany); lanes 1–7, DNA dilution series 1:2 (lane 1), 1:10 (lane 2), 1:20 (lane 3), $1:10^2$ (lane 4), $1:10^3$ (lane 5), $1:10^4$ (lane 6), and $1:10^5$ (lane 7); lane 8, no DNA template; and lane 9, healthy, surface-sterilized seeds of *Papaver somniferum*.

TABLE 4. Sensitivity of *Peronospora arborescens*-specific primers designed in this study and Oomycete universal primers with purified DNA of *P. arborescens* or mixed with host DNA

Primer pair	Dilution Series[a]	Background DNA (ng/ul)[a]	DNA of *Peronospora arborescens* (ng/µl)[a] 5	1	0.5	0.1	0.01	0.001	0.0001	Negative control	H_2O control
P3Pa2fw/P3Pa12rv (P2)	I	0	+/+/+[b]	+/+/+	+/+/+	+/+/+	–/+/+	–/–/–	–/–/–		–/–/–
		10	+/+/+	+/+/+	+/+/+	+/+/+	±/+/+	–/–/–	–/–/–	–/–/–	–/–/–
	II	0	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	–/+/±	–/–/–		–/–/–
		20	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	–/+/±	–/–/–	–/–/–	–/–/–
OMPac1fw/OMPa1rv (P3)	I	0	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	–/±/±	–/±/±		–/–/–
		10	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	–/±/+	–/±/±	–/–/–	–/–/–
	II	0	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/–	±/±/–		–/–/–
		20	+/+/+	+/+/+	+/+/+	+/+/+	+/+/–	±/±/+	±/±/–	–/–/–	–/–/–
OMPac7fw/OMPac7rv (P6)	I	0	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	±/±/–	–/–/–		–/–/–
		10	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/±/+	–/–/–	–/–/–	–/–/–
	II[c]	0	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/±/+	–/–/–		–/–/–
		20	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/±/–	–/–/–	–/–/–	–/–/–
DC6/ITS4[d]	I	0	+/+	+/+	+/+	+/	±/+	–/–	–/–		–/–
		10	+/+	+/+	+/+	+/	–/–	–/–	–/–	–/–	–/–
	II	0	+/+	+/+	+/+	+/	+/±	–/–	–/–		–/–
		20	+/+	+/+	+/+	+/	–/–	–/–	–/–	–/–	–/–

[a] *P. arborescens* DNA (10 ng/µl) was serially diluted to obtain 5 to 0.0001 ng of pathogen per microliter of PCR reaction in distilled water (0 ng background DNA) or in *Papaver somniferum* DNA extracted from leaves (10 ng/µl background DNA; dilution series I) or seeds (20 ng/µl background DNA; dilution series II) to investigate any possible influence of host DNA on amplification of pathogen DNA.

[b] Results presented were obtained by three independent operators with same dilution series. + = positive amplification; ± = positive weak amplification; and – = no amplification.

[c] Representative results from this dilution series experiment are shown in Figure 4.

[d] This primer pair combination was assessed only in two independent PCR reactions.

lap in morphological characters (33,36) and host ranges, and most of the reports referred to above were based on symptoms on the hosts and morphology of the pathogen only, it is possible that some of the descriptions attributed to one of the two species in a country might in fact correspond to the other. In the same way, the possibility that *P. cristata* might be present in Spain on hosts where it has been reported from other countries, such as *Meconopsis cambrica*, wild poppies *Papaver argemone*, *Papaver hybridum*, and *Papaver rhoeas*, wild *Papaver somniferum*, or other *Papaver* spp. (8), should not be ignored since our study focused on cultivated *Papaver somniferum* only. In fact, this was the scenario that occurred in Australia where downy mildew of *Papaver nudicaule*, *Papaver hybridum*, and *Papaver dubium* in different states of the country and downy mildew of commercial opium poppy in Tasmania were initially attributed to *P. arborescens* (15,37). However, a detailed phylogenetic analysis of the pathogen population demonstrated that *P. cristata* rather that *P. arborescens* was the causal agent of *Papaver somniferum* downy mildew in Tasmania (36). Since the Tasmanian poppy industry was founded from England (36), it would be noteworthy to determine whether *P. cristata* and/or *P. arborescens* are the causal agents of downy mildew of cultivated *Papaver somniferum* in the United Kingdom. Future studies are worth conducting to determine if *P. cristata* and *P. arborescens* differ in virulence on *Papaver somniferum*, host specialization on *Papaver* spp. or *Meconopsis* spp., and worldwide geographic distribution.

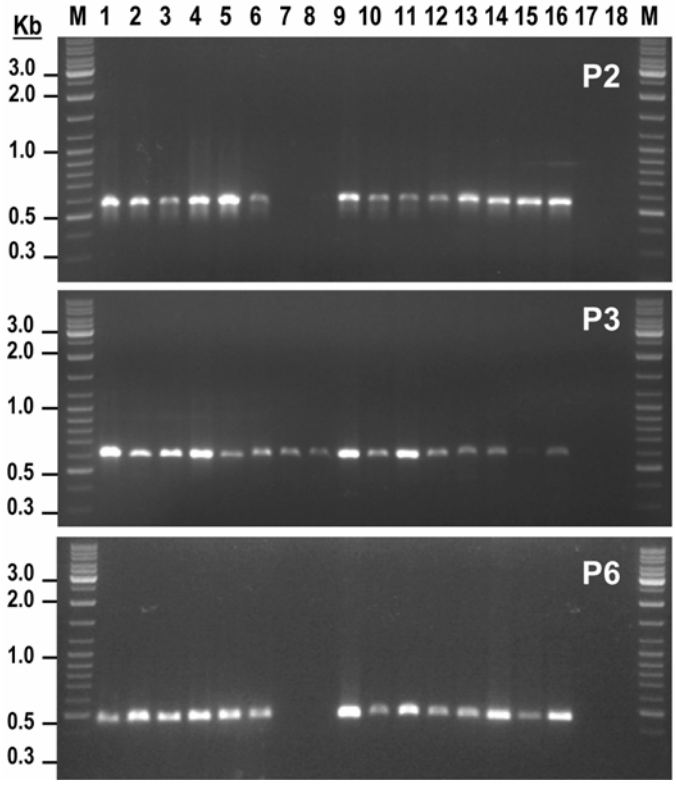

Fig. 5. Detection of *Peronospora arborescens* in naturally infected opium poppy tissues with specific primers P3Pa2fw/P3Pa12rv (P2), OMPac1fw/OMPa1rv (P3), and OMPac7fw/OMPac7rv (P6). M, GeneRuler DNA ladder mix (Fermentas, St Leon-Rot, Germany); lanes 1–2, *P. arborescens* sporangiophores and sporangia from sporulating opium poppy leaves (positive control); lanes 3–7, *P. arborescens*-infected leaves with different severity of symptoms (necrosis, pathogen sporulation, chlorosis, light chlorosis, and no symptoms, lanes 3 to 7, respectively); lanes 8–9, surface-sterilized, symptomatic stem (epidermal and cortex tissue, lanes 8 and 9, respectively); lane 10, peduncles of infected capsule; lanes 11–12, surface-sterilized root tissue from plants showing dwarfing and rosseting; lane 13–14, seeds from symptomatic capsules; lanes 15–16, capsule tissue (with light symptoms or pathogen sporulation, lanes 15 and 16, respectively); lane 17 healthy *Papaver somniferum* seeds; and lane 18, no DNA template.

The phylogenetic analysis also revealed that *P. cristata* is more distantly related to a clade that comprises *P. arborescens* infecting *Papaver somniferum* and *Papaver rhoeas* than to other *Peronospora* spp., whose hosts are outside the Papaveraceae. Interestingly, all *Peronospora* spp. infecting species in the order Ranunculales grouped together in the same clade (with the exception of *P. arborescens*). This clade was more closely related to a clade containing *Peronospora* spp. infecting species within the Caryophyllales than to all *P. arborescens* collections, which may indicate host jumping between distantly related host families (43). Similar results were obtained by Scott et al. (36) for *P. arborescens* and *P. cristata* with a data set for phylogenetic analysis smaller than that used in the current study.

In addition to use for phylogentic studies, ITS rDNA sequences have been proved useful in the development of species-specific PCR protocols for in planta detection and identification of plant pathogenic oomycetes (1,7,9,36,38). We employed a similar method to develop *P. arborescens*-specific primers and developed a PCR-based protocol that was employed to answer several lingering questions in this pathosystem. First, we were able to efficiently differentiate *P. arborescens* from *P. cristata* and demonstrate that only *P. arborescens* occurs in cultivated *Papaver somniferum* in Spain. Second, the assay was used to detect *P. arborescens* in symptomatic and asymptomatic opium poppy plant parts and, third, we documented that *P. arborescens* can be seedborne in seeds from infected opium poppy plants. Because of the obligate biotrophy of Peronosporales, availability of molecular protocols for their in planta and seed detection and identification is particularly important for the implementation of health certification schemes. Such a diagnostic technology should be both sensitive enough to detect small amounts of pathogen before evident symptom expression on the host, as well as specific to avoid cross-reaction problems with other related pathogens. The species-specific PCR protocol developed in this study satisfies those desirable characteristics because it shows: (i) considerable flexibility, since it can be applied to different plant samples including capsules, leaves, roots, seeds, and stems; (ii) high sensitivity, since the pathogen can be detected in symptomless plant tissues and seed stocks where the pathogen may be present at very low concentrations, i.e., as little as 0.1 to 10 pg of *P. arborescens* DNA (depending upon the primer set) against a background of 10 to 20 ng of opium poppy DNA; and more important (iii) high specificity, since no cross amplification occurred with other closely related pathogens, especially with *P. cristata*-infected opium poppy tissues.

In similar studies, it was usually necessary to develop a nested-PCR protocol to increase flexibility and sensitivity in the amplification of pathogen DNA from infected plant samples (38) or concentrations of template pathogen DNA similar to that amplified with the single-PCR protocol developed in our work (7,38). Also, in contrast to other studies (e.g., 38) the presence of plant DNA did not reduce the sensitivity of the PCR protocol. Interestingly, the detection limit of the single round-PCR assay that we developed is rather similar to that reported for quantitative real-time PCR assays developed for quantifying *Plasmopara viticola* on leaves of *Vitis vinifera* (42), different *Phytophthora* spp. on decaying leaves (35), and *Alternaria brassicae* in cruciferous seeds (22). In those latter studies, the standard deviations of replicates increased greatly at a concentration of pathogen DNA <1 pg or when host DNA was present in the reaction mix, thus reducing the accuracy of the quantitative technique.

A final advantage of the three primer sets designed in this study concerns their high specificity for *P. arborescens*. In our study, we aimed for an identification and detection protocol that would avoid cross amplification of *P. cristata* DNA because we found that the *P. cristata*-specific primers designed by Scott et al. (36), pdm3/pdm4, yielded cross reaction when used for PCR assays of some *P. arborescens* samples from Spain. These authors had to

base the specificity of the pdm3/pdm4 primer pair only on sequence comparison and BLAST searches on GenBank database, since apparently they could not test plant samples infected with *P. arborescens*. In our study, the comparison of pdm3/pdm4 sequences with all the *P. arborescens* sequences that we used identified only one or two single nucleotide difference for both primers, which may account for the cross amplification of *P. arborescens* DNA. While designing our primer sets, we ensured specificity by including one nucleotide that was a deletion in the ITS sequence of *P. cristata* and/or 100% homology in its sequence with *P. arborescens* in at least one of the primers of each set. This theoretical specificity was experimentally demonstrated with DNA extracted from samples of *P. cristata*-infected leaves from Australia and the United Kingdom.

Although the *P. arborescens*-specific primer sets and PCR protocol developed in this study was not aimed at quantitative assays, we believe that it may be of great use in opium poppy production to avoid the introduction of *P. arborescens*-contaminated seeds in areas free from the pathogen and when introducing seeds of germplasm for plant breeding purposes. Also, this protocol can be of use for epidemiological studies of the disease, helping to demonstrate the occurrence of asymptomatic, systemic infections, or to choose appropriate timing of fungicide treatments for chemical control of the disease before symptom expression.

Downy mildew of opium poppy is one of the most destructive diseases of this crop worldwide (27,36,37). Consequently, efforts should be made to avoid dispersal of the pathogen to opium-poppy-growing areas free from *P. arborescens*. In Spain, the rapid spread of the opium poppy downy mildew to areas where opium poppy had not been cropped before, together with the progressive increase in the incidence and severity of the disease during the last few years, suggested seedborne transmission of the pathogen. Results from this study confirm the potential of this hypothesis. Thus, by using the species-specific PCR assay developed, we demonstrated that *P. arborescens* was seedborne on commercial seed stocks, and further, that seedborne inoculum can give rise to infected plants under field conditions. To our knowledge, this is the first demonstration that *P. arborescens* can be seed transmitted. Further research is needed to determine location of the pathogen in seed tissues as well as factors that determine efficacy of transmission. Thin- and thick-walled mycelia and/or oospores of different *Peronospora* spp. (i.e., *P. ducomati*, *P. effusa*, *P. farinosa*, *P. manshurica*, and *P. viciae*) have been detected on the seed surface, as well as in infected seed coat (pericarp and spermoderm layer), persistent calyx, and pods of their respective hosts (25,39,46). In our study, seedborne transmission of *P. arborescens* occurred with rather low frequency, perhaps because of the unusual warm and dry weather conditions that took place during the year of the field experiment. The day/night temperatures and relative humidity that occurred at the site during the field experiment have been reported unfavorable for sporulation, germination of sporangia, appresorium formation, and infection for other *Peronospora* spp. (14,20). Nevertheless, the efficacy of seed transmission might also be low in nature, as shown for *P. lactucae*, with a level of infection that reached 0.3 to 2.9% in commercial seed stocks of *Lactuca sativa* (25). In contrast, sporangia of *Peronospora* spp. from infected sporulated leaves can be readily dispersed by wind and rain splashing, and consequently, infected leaves could be an important source of inoculum (14,20). In our field study, we are confident that the possibility of external aerial inoculum giving rise to plant infection did not occur, since the field microplots of the study were: (i) located 60 to 120 km apart from the closest cultivated opium poppy fields; (ii) surrounded by fields within the research station that were kept free of weeds (including *Papaver* spp.) by conventional tillage or herbicide treatment; and (iii) that the disease did not show up in the control plots planted close to plots planted with infected seeds.

An additional important finding of this study was the detection of putative systemic infection of opium poppy by *P. arborescens*, since the pathogen DNA was amplified from roots of plants showing dwarfing and rosetting in the field with no other evident symptom of downy mildew. The systemic infection of these plants may have taken place from infected seeds or by soilborne oospores of the pathogen during seed germination and/or seedling growth. Systemic infection of buckwheat and soybean seedlings from infected seeds and oospores has been demonstrated for *P. ducomati* and *P. manshurica*, respectively (39,46). Systemic infections of opium poppy by *P. arborescens* may be of significance in the epidemiology of downy mildew if, as reported for sunflower downy mildew caused by *Plasmopara halstedii* (10), asymptomatic systemic infections give rise to viable, infected seeds.

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