Molecular Identification of *Oidium neolycopersici* as the Causal Agent of the Recent Tomato Powdery Mildew Epidemics in North America

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ABSTRACT

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A previous morphological study of *Oidium* anamorphs responsible for the recent tomato (*Lycopersicon esculentum*) powdery mildew outbreaks worldwide suggested that, despite controversial data in the literature, the North American epidemics were caused solely by a newly erected species, *O. neolycopersici*. We report here the first molecular evidence that the North American anamorphs do belong to *O. neolycopersici*. The internal transcribed spacer sequences of the North American anamorphs of this study were identical with those of three Japanese and four European specimens of *O. neolycopersici*. A morphological study confirmed that all the North American *Oidium* anamorphs included in this study produced conidia singly, similar to *O. neolycopersici*. These fungi were readily distinguished from *O. lycopersici*, which produces conidia in chains and is known to infect tomato only in Australia. The phylogenetic analysis showed that *O. neolycopersici* is a distinct powdery mildew species, and it is neither identical nor closely related to any known polyphagous species of the *Erysiphaceae*. Apparently, it was introduced into the United States and Canada only in the 1990s, but its origin is still unknown.

Additional keywords: *Erysiphe* sp.

In the late 1970s, an apparently new powdery mildew disease appeared on tomato (*Lycopersicon esculentum*) in Japan (2) and Australia (52,70). Later, outbreaks of a similar disease were reported from many parts of Europe (9,12,14,19,20,34,38,46–48,64,67) and North America (5–7,31,41,49,57,68). In all these reports, the causal agent of the novel epidemics causing economic damage to tomato has been described as an *Oidium* anamorph and has always been clearly distinguished from *Oidiopsis*, the anamorph stage of *Leveillula taurica*, long known to affect tomato in warmer regions of the world (10). However, the reported data on the conidiogenesis of the novel pathogens were contradictory. Some authors described an *Oidium* anamorph on tomato that produces conidia in chains (2,5,7,14,31,34,46,49,52,68,70), while others reported a pathogen producing conidia singly (6,9,20,38,41,47,48,57,64,67). As the nature of the conidiogenesis is a stable pattern within powdery mildew species (10,11,13,54), the contra-

dictions in the reported data suggested that the tomato powdery mildew epidemics might have been caused by more than one species in different parts of the world or even in the same geographical region. For example, *Oidium* anamorphs with both catenate and non-catenate conidia were reported on tomato in the United States (5,31,41,49,57,68), Canada (6,7), Switzerland (9,14), and Japan (2,32,43).

The conflicting data regarding the conidiogenesis of the tomato powdery mildew pathogens were further complicated by a variation in the pathogenicity of these *Oidium* anamorphs. For example, considerable differences were found in the host range of *Oidium* anamorphs causing epidemics on tomato in England (67), Connecticut (37), the Netherlands (25), and Japan (32). In addition, Lebeda and Mieslerova (39) suggested the existence of three pathotypes based on the differences in the pathogenicity of four European *Oidium* isolates on many accessions of wild *Lycopersicon* spp. Overall, these results suggest a considerable genetic diversity within these poorly delimited pathogens or even the existence of more than one species causing a similar disease on tomato.

Only a few tomato genotypes, such as the breeding line Hirol 3-21 (37) and cv. Grace (33), were reported to be resistant to the novel powdery mildew infections, al-

though Kashimoto et al. (33) have recently showed that cv. Grace is susceptible to a Japanese isolate of the fungus. Most tomato cultivars grown worldwide are susceptible to the disease (25,29,37,40,43). Thus, there is a need to breed cultivars resistant to powdery mildew infections caused by *Oidium* anamorphs (25,29,37,40).

The uncertainties in the identity of the tomato powdery mildew pathogens pose a practical problem for tomato breeders. If two or more species were responsible for the recent outbreaks, this would complicate the breeding strategy, as in the case of certain melon lines that were infected by both *Erysiphe* and *Podosphaera* (syn. *Sphaerotheca*) species (16). Recent studies showed that some *Arabidopsis thaliana* accessions were susceptible to two powdery mildew species, *Golovinomyces cichoracearum* (syn. *E. cichoracearum*) and *E. cruciferarum*, whereas other accessions were resistant to one or both of them (3,65). Differential resistance to more than one powdery mildew species complicates the task of breeding. Thus, there was not only a taxonomical, but also a practical need to determine how many species are responsible for the recent tomato powdery mildew pandemics.

In order to identify the causal agent(s) of the tomato powdery mildew epidemics, Kiss et al. (35) carried out an extensive study of more than 50 tomato powdery mildew specimens, including fungi maintained on living plants and herbarium materials, obtained from all continents where tomatoes are grown. Based on the combined use of classical morphological, scanning electron microscope (SEM), and molecular analyses, these authors concluded that a single *Oidium* anamorph producing single, non-catenate conidia infected tomato in Europe, North and South America, Asia, and Africa. This was described as a new species, *Oidium neolycopersici* Kiss. Surprisingly, a morphologically distinct *Oidium* anamorph, producing conidia in chains, was found on all the tomato materials obtained from Australia. This was neotypified as *O. lycopersici* Cooke & Massee emend. In the molecular part of the study, Kiss et al. (35) determined the internal transcribed spacer (ITS) sequences of the ribosomal DNA (rDNA) in two specimens of *O. neolycopersici*, collected in France and the Netherlands,

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respectively, and found that they were almost identical. The ITS sequence was determined in the Australian chain-forming *O. lycopersici* as well, and a phylogenetic analysis showed that it was not closely related to *O. neolyopersici* (35). Later, Kashimoto et al. (32) showed that the rDNA ITS sequences of three Japanese non-catenate powdery mildew anamorphs were almost identical with those of the two European *O. neolyopersici* specimens sequenced. The other tomato powdery mildew pathogens collected in Europe, Asia, Africa, and the Americas were identified as *O. neolyopersici* based on their morphological characteristics only (29,35,44,67).

In some cases, powdery mildew infections of the same plant species could be caused by more than one species whose anamorphs are sometimes morphologically indistinguishable. If symptoms of the infections and morphology of the pathogens were similar, and the teleomorph stage was not seen, only a detailed microscopic and molecular study could reveal the exact identity of the causal agent of the disease. For example, soybean powdery mildew epidemics in Asia were caused by non-catenate *Oidium* anamorphs of two different species, *Erysiphe glycines* and *E. diffusa* (syn. *Microsphaera diffusa*), which sometimes occurred simultaneously on the same plant (62). However, molecular tools could distinguish them in the absence of the teleomorphs that were produced only sporadically by *E. glycines* and not at all

by *E. diffusa* (62). Similarly, an extensive study showed that powdery mildew epidemics on *Rhododendron* in Europe were caused by at least two species, *E. azaleae* and *E. digitata* (27). It was not until the 1990s that the first ascomata of these two species were observed on *Rhododendron* in Europe, making their identification possible. Until then, only non-catenate *Oidium* anamorphs were found on *Rhododendron*, which could not be distinguished based on their morphological and SEM patterns (27). Other examples of the infection of the same plant species by morphologically similar powdery mildew anamorphs representing different species or even different genera were presented by Cunningham et al. (15) based on the molecular identification of the pathogens involved.

The sexual stage of *O. neolyopersici* is unknown. Attempts to produce ascomata in vitro have failed (35). In light of the case studies presented above, the morphological evidence presented by Kiss et al. (35) might not be sufficient to support claims that all the recent tomato powdery mildew epidemics outside Australia were caused solely by this species. Clearly, more precise methods are needed to reliably identify the causal agent(s) of this disease worldwide. This is particularly important for the North American epidemics, as none of the tomato powdery mildew pathogens collected in the United States and Canada were identified using DNA sequence data. So, it might be possible that they represent

more than one species with morphologically indistinguishable anamorphs.

Phylogenetic studies based on the rDNA ITS region of powdery mildews have resulted in a large data set for molecular identification of various taxa of the *Erysiphaceae* (11,42,45,54,59–62). The analyses of ITS sequences of powdery mildew specimens belonging to the same species showed that they are nearly or completely identical within a given species (23,42,62). This is the case in *O. neolyopersici*, as Kashimoto et al. (32) recently showed that the ITS sequence from six isolates collected in different European countries and Japan is 99 to 100% similar. Thus, we proposed in this work to determine and to analyze the ITS sequences of North American and European tomato powdery mildew pathogens in order to determine whether the recent tomato powdery mildew epidemics in North America were caused solely by *O. neolyopersici*. For comparison, a few specimens of *O. lycopersici*, which produces conidia in chains and whose known distribution is restricted to the southern parts of Australia, were also included in this study.

MATERIALS AND METHODS

Fungal materials and microscopy. A total of 22 tomato samples infected with powdery mildew were obtained from the United States, Canada, Japan, and Europe (Table 1) and were examined as fresh materials or as herbarium specimens using

Table 1. List of the *Oidium neolyopersici* specimens examined in this study using light microscopy and analysis of the rDNA internal transcribed spacer (ITS) sequences^a

Designation	Herbarium accession no. (if available) ^b	Place and year of collection	Collected by	GenBank accession no. of ITS sequence (if determined)	Source of the ITS sequence data
North America					
MD1	BPI 746232	Beltsville, MD, USA, 1999	L. Kiss	AB163915	This paper
MD2	–	Germantown, MD, USA, 1999	L. Kiss	–	
CAN	BPI 746230	Harrow, ON, Canada, 1999	R. Cerkauskas	AB163914	This paper
CA	MUMH775 (BPI 746433)	Oxnard, CA, USA, 1999	R. M. Davis	AB034722	This paper
CT1	BPI 746456	New Haven, CT, USA, 1998	V. L. Smith	–	
CT2	BPI 746457	New Haven, CT, USA, 1998	V. L. Smith	–	
LI	BPI 746228	Riverhead, NY, USA, 1999	M. T. McGrath	–	
GEN	BPI 746231	Geneva, NY, USA, 1999	L. Kiss	–	
ITH	BPI 746229	Ithaca, NY, USA, 1999	D. Kalb	–	
MOR	–	Morristown, NY, USA, 2003	L. Kiss	–	
CAN2	–	Ste-Anne-de-Bellevue, QC, Canada, 2003	L. Kiss	–	
Europe					
Et1	BPI 747013	Aigue Mortes, France, 1989	P. C. Nicot	AF229019	Kiss et al. (35)
NETH	VPRI 20724	Wageningen, Netherlands, 1994	H. van Kesteren	AF229015	Kiss et al. (35)
PV	BPI 746227	Wageningen, Netherlands, 1999	C.-C. Huang	–	
UK1	–	York, UK, 1999	R. T. A. Cook	–	
UK2	VPRI 20381	Wellesbourne, UK, 1994	J. M. Whipps	AB163916	This paper
BULG	VPRI 20360	Bulgaria, 1994	G. Neshev	AB163913	This paper
BP-P5	BPI 746455	Budapest, Hungary, 1998	L. Kiss	–	
SW	BPI 746225	Nyon, Switzerland, 1995	A. Bolay	–	
Japan					
JAP1	MUMH66	Toyama, Japan, 1995	Y. Sato	AB032483	Kashimoto et al. (32)
DNA231	–	Toyama, Japan, 1996	Y. Sato	AB032484	Kashimoto et al. (32)
KTP01	–	Nara, Japan		AB094991	Kashimoto et al. (32)

^a Some specimens were also included in previous studies carried out by Kiss et al. (35) and Kashimoto et al. (32).

^b BPI = U.S. National Fungus Collection, Beltsville, MD; MUMH = Mie University Mycological Herbarium, Japan; VPRI = Plant Disease Herbarium, Institute for Horticultural Development, Victoria, Australia.

light microscopy as described in Kiss et al. (35). Herbarium materials were rehydrated before study using the method developed by Shin and La (56). Three North American and two European tomato powdery mildew specimens producing conidia singly were selected for molecular analyses. For comparative light microscopy studies, three specimens of *O. lycopersici* collected in Victoria, Australia, in 1980 (DAR 35762, DAR 35763, and DAR 35764) were also included in this work.

DNA extraction and amplification of rDNA ITS sequences. Whole-cell DNA was isolated from powdery mildew mycelia using the Chelex method (24,66). The nuclear rDNA region spanning the ITS1, ITS2, and 5.8S rRNA gene was amplified by polymerase chain reaction (PCR). Primers ITS5 (69) and P3 (36) were used for the first amplification, which was performed in a total reaction volume of 50 μ l, including the following reagents: PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatine); 200 μ M of each deoxyribonucleotide triphosphate; 0.4 μ M of each primer with 10 μ l of the template DNA solution; and one unit of *Taq* DNA polymerase (TaKaRa, Tokyo, Japan). The following thermal cycling conditions were performed in a thermal cycler PC-700 (ASTEC, Fukuoka, Japan): an initial denaturing step at 95°C for 2 min; thermocycling for 30 cycles, where each cycle consisted of 30 s at 95°C followed by 30 s at 52°C for annealing, and 30 s at 72°C for extension; and a final extension cycle of 7 min at 72°C. One microliter of the first amplification mixture was used for a second amplification using the nested primer set ITS1 (69) and P3. Components of the reaction mixture and the thermal cycle conditions for the second amplification were the same as for the first one. Each PCR product was subjected to electrophoresis in 1.5% agarose gel in TAE buffer. The DNA product of each amplification was then excised from the ethidium bromide-stained gel and purified using the JETSORB kit (GENOMED, Oeynhausen, Germany) following the manufacturer's protocol.

DNA sequencing. Nucleotide sequences of the PCR products were obtained for both strands using direct sequencing in an Applied Biosystems 373A DNA sequencer. The sequence reactions were conducted using the PRISM Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Four primers, ITS1 (69), P3, T3, and T4 (24), were used for the sequencing in both directions.

Phylogenetic analysis. ITS sequences of five *O. neolycopersici* specimens and of 49 other powdery mildew fungi with non-catenate anamorphs were obtained from DNA databases and were aligned with the newly obtained sequences using the

ClustalX package (63). The ITS sequence of the chain-forming *O. lycopersici* (GenBank accession no. AF229021) was not included in the analysis because it is highly divergent from the sequences of the non-catenate *Oidium* anamorphs (35). The alignment was then refined visually using a word processing program with color-coded nucleotides, and ambiguously aligned sites were removed from the data set for the following analyses. The final alignment was deposited in TreeBASE under the accession number S1201/M2073. Phylogenetic trees were obtained from the data using maximum-likelihood (ML), neighbor-joining (NJ), and maximum parsimony (MP) methods. For ML and NJ analyses, the most appropriate evolutionary model was determined for a given data set using PAUP* 4.0b8 (58) and Modeltest 3.06 (51). A starting tree was obtained with NJ method. With this tree, likelihood scores were calculated for 56 alternative models of evolution by PAUP*. The output file was then imported to Modeltest to compare the models by using Akaike's (4) information criterion. Once a model of evolution was chosen, it was used to construct phylogenetic trees with ML method using Treefinder version March 2003 (G. Jobb, Treefinder. Published online.) and NJ method using PAUP*. For the parsimony analysis, we used the maximum-parsimony (MP) method with a heuristic search using PAUP*. This search was repeated 100 times with different random starting points, using the stepwise addition option to increase the likelihood of finding the most parsimonious tree. Transversions and transitions were treated as equal weight. All sites were treated as unordered, with gaps treated as missing data. The branch-swapping algorithm was TBR, the MULPARS option was in effect, and zero-length branches were collapsed. The strength of the internal branches from the resulting trees was tested by the bootstrap analysis (18) using 1,000 replications in

Fig. 1. A conidiophore of *Oidium neolycopersici* isolate MD1 (BPI 746232) collected in Beltsville, MD, in 1999. Bar = 30 μ m.

MP and NJ analyses and 100 replications in ML analysis.

RESULTS

Morphological studies. All the powdery mildew fungi obtained from North America, Europe, and Japan produced conidia singly (Fig. 1), and their morphological features corresponded to those of *O. neolycopersici*. They were clearly different from the three Australian specimens of *O. lycopersici* included in this study, which produced conidia in chains (Fig. 2). The morphological differences between *O. neolycopersici* and *O. lycopersici* have been discussed previously (35).

Phylogenetic studies. A total of 10 ITS sequences of *O. neolycopersici* were aligned with sequences from species of *Erysiphe* sections *Erysiphe* and *Micropasphaera* obtained from DNA databases. The alignment data matrix consisted of 59 taxa and 614 characters, of which 51 sites were removed because of ambiguous alignment. Of the 563 remaining characters, 181 sites were variable and 123 sites were phylogenetically informative for parsimony analysis. Two isolates of *Erysiphe glycines* were used as outgroup taxa based on Takamatsu et al. (61). Using Modeltest (51), under the Akaike's (4) information criterion, we concluded that a submodel of the GTR model (53), with unequal base frequencies, a gamma-distributed rate heterogeneity model (four rate categories, $G = 0.8657$) (71), and an estimated proportion of invariant sites (0.4668) was the most appropriate model

Fig. 2. A conidiophore of *Oidium lycopersici* collected in South Australia in 1980 and rehydrated in lactic acid from a herbarium specimen (DAR 35763). Bar = 20 μ m.

Of the 10 ITS sequences of *O. neolycopersici* isolates, nine were identical, while the sequence from isolate NETH differed by one base in the ITS2 region. *O. neolycopersici* grouped in a single clade

DISCUSSION

This study has provided the first convincing evidence that the non-catenate *Oidium* anamorphs responsible for the recent tomato powdery mildew epidemics in the United States and Canada are *O. neolycopersici*. This had already been suggested by a morphological examination of some North American tomato powdery

The recent tomato powdery mildew epidemics caused by *Oidium* anamorphs were reported as outbreaks of a previously unknown disease in North America (5–7,31,37,41,49,57,68). Before the 1990s, there was only one record of an *Oidium* anamorph on tomato seedlings (17). This might suggest that *O. neolycopersici* was introduced to the United States and Canada only recently.

The origin of *O. neolycopersici* is unknown, although the center of origin of the host itself, in the Andes, has not been investigated in this respect. Our phylogenetic analysis demonstrated that *O. neolycopersici* is, indeed, a distinct powdery mildew

Fig. 3. A most parsimonious tree based on the nucleotide sequences of the rDNA internal transcribed spacer regions for 59 powdery mildew taxa of *Oidium* subgenus *Pseudoidium*, including 10 isolates from tomato (shown in boldface). This tree has the highest likelihood score among a total of 20 trees consisting of 18 equally parsimonious, one neighbor-joining and one maximum likelihood trees. Bootstrap values of 1,000 replications are shown above nodes. DNA database accession numbers for powdery mildew taxa other than *O. neolycopersici* are also shown on the tree. The consistency index (CI) is 0.5677; retention index (RI) is 0.8002; and rescaled consistency index (RC) is 0.4543.

species, and it is neither identical nor closely related to any known polyphagous species of the *Erysiphaceae* (Fig. 3). European reports, similar to the North American ones, suggest that it has appeared on tomato only recently (29,44,47,67). However, there are records in the older phytopathological and mycological literature of *Oidium* anamorphs infecting tomato in Europe (8,22,28,55), Sri Lanka (50), Peru (1), and Argentina (21), sometimes causing serious epidemics long before the 1990s (21). Unfortunately, to our knowledge, herbarium specimens were not preserved in any of these cases, so the causal agents of the infections reported in these papers cannot be determined. There are a number of herbarium specimens available from Asia, and collected between 1947 and 1987, which were studied by Kiss et al. (35) who concluded that they were all *O. neolyopersici* based on their morphological features. So, powdery mildew infections of tomato caused by non-catenate *Oidium* anamorphs, presumably by *O. neolyopersici*, were recorded, at least in Asia, long before the 1990s.

Although the variability reported in the pathogenicity of some *O. neolyopersici* isolates (39) suggests a considerable genetic diversity, a preliminary report of an amplified fragment length polymorphism (AFLP) analysis revealed only minor genetic differences among five isolates collected from tomato in three different European countries (26). This result, together with the ITS sequence data, and the apparent lack of sexual recombination, suggests that there is little genetic variability in *O. neolyopersici*. Further studies are needed for a more comprehensive genetic characterization of this novel pathogen.

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