Molecular Detection of *Peronospora variabilis* in Quinoa Seed and Phylogeny of the Quinoa Downy Mildew Pathogen in South America and the United States

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ABSTRACT

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Quinoa (*Chenopodium quinoa*) is an important export of the Andean region, and its key disease is quinoa downy mildew, caused by *Peronospora variabilis*. *P. variabilis* oospores can be seedborne and rapid methods to detect seedborne *P. variabilis* have not been developed. In this research, a polymerase chain reaction (PCR)-based detection method was developed to detect seedborne *P. variabilis* and a sequencing-based method was used to validate the PCR-based method. *P. variabilis* was detected in 31 of 33 quinoa seed lots using the PCR-based method and in 32 of 33 quinoa seed lots using the sequencing-based method. Thirty-one of the quinoa seed lots tested in this study were sold for human consumption, with seed originating from six different countries. Internal transcribed spacer (ITS) and cytochrome *c* oxidase subunit 2 (*COX2*) phylogenies were examined to determine whether geographical differences occurred in *P. variabilis* populations originating from Ecuador, Bolivia, and the United States. No geographical differences were observed in the ITS-derived phylogeny but the *COX2* phylogeny indicated that geographical differences existed between U.S. and South American samples. Both ITS and *COX2* phylogenies supported the existence of a *Peronospora* sp., distinct from *P. variabilis*, that causes systemic-like downy mildew symptoms on quinoa in Ecuador. The results of these studies allow for a better understanding of *P. variabilis* populations in South America and identified a new causal agent for quinoa downy mildew. The PCR-based seed detection method allows for the development of *P. variabilis*-free quinoa seed, which may prove important for management of quinoa downy mildew.

Quinoa (*Chenopodium quinoa* Willd.) is the most recent Andean crop to garner global attention as a food crop (4,8,17,25). The majority of quinoa production occurs in the Andean countries of Bolivia, Peru, and Ecuador (35), but production has spread from South America to Europe, North America, and beyond (8). In major quinoa-producing regions, the crop's key disease is quinoa downy mildew (10), caused by *Peronospora variabilis* Gäum (formerly *P. farinosa* f. sp. *chenopodii* Byford) (5), a heterothallic (9) oomycete. Quinoa downy mildew epidemics greatly reduce yield (10), and under experimental conditions, *P. variabilis* reduced seed yields by at least 33% in resistant varieties and up to 99% in susceptible varieties (11). Although quinoa downy mildew is such an important disease of quinoa, there are still several key research areas that are underdeveloped, including molecular detection of *P. variabilis* and the phylogenetic structure of the causal agent of quinoa downy mildew.

Quinoa downy mildew has been reported in countries outside of quinoa's native range, including Canada (34), Denmark (12), India (28), and, most recently, the United States (Pennsylvania in 2011) (33). The original source of inoculum for each *P. variabilis* introduction is unknown, but the pathogen was likely introduced on infected seed. The oospores of *P. variabilis* can be seedborne and remain viable (1), and current oospore detection methods rely on germinating seed under disease conducive conditions (14) or examining seed microscopically (14). If oospores are detectable by these two methods, molecular detection of *P. variabilis* in quinoa seed should also be possible. Molecular methods have been developed to detect *Peronospora* spp. in other seed, including basil (3) and poppy (29). Molecular methods allow for screening more seed for the presence of *P. variabilis* in less time and allow for the standardization of methods to detect the pathogen in seed. These techniques could be further standardized to create certified *P. variabilis*-free quinoa seed.

The genetic diversity of *P. variabilis* has not been properly addressed, but some studies have suggested geographic differences in pathogen populations between European and South American populations based on phylogenies of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) (5) and universally primed polymerase chain reaction (PCR) (13). Both studies indicated that European (Danish) samples and South American (Ecuadorean, Peruvian, and Bolivian) samples formed separate clusters within the same species (5,13). Samples from South America, especially Bolivia, were underrepresented in these studies, and it is possible that the true diversity of the quinoa downy mildew pathogen in South America was not fully explored in previous studies. Also, downy mildew samples from closely related *Chenopodium* weeds have not been examined, likely because previous studies have indicated a limited host range for the quinoa downy mildew pathogen (1,10). No previous studies have used a multigene phylogeny to examine the same population of *P. variabilis* from quinoa; however, multigene analyses have been used extensively to determine the phylogenies of *Peronospora* spp. (20) and other oomycetes (2,27). Phylogenetic studies of *Peronospora* spp. have been conducted using data obtained from ITS (19), large subunit rDNA (21), cyto-

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chrome *c* oxidase subunit 2 (*COX2*) (22,23), NADH dehydrogenase subunit 1 (20), and β-tubulin (20). Due to the obligate nature of *P. variabilis*, it is important to select genetic markers with primers that will only amplify *P. variabilis* DNA, even in the presence of DNA from plant material or other fungi.

The objectives of this research were twofold: to (i) develop a method to rapidly screen quinoa seed for *P. variabilis* and (ii) better understand the phylogenetic relationship of isolates of the quinoa downy mildew pathogen from disease samples collected from endemic and introduced regions.

In this research, we designed specific primers for *P. variabilis* in order to develop a rapid detection method for *P. variabilis* in quinoa seed, including quinoa seed imported for human consumption. This PCR-based method targeted the ITS region and was supported by a sequencing-based method of seed detection of the quinoa downy mildew pathogen. We also examined the phylogenies of two regions, ITS (6) and *COX2* (23), from quinoa downy mildew samples collected in Bolivia, Ecuador, and the United States to determine whether genetic differences in *P. variabilis* existed based on geography.

MATERIALS AND METHODS

Seed, plant, and *P. variabilis* samples. For seed screening studies, 33 seed lots of domestic and imported quinoa seed, most sold for human consumption, along with one seed lot each of *C. pallidicaule* (the Andean crop kañiwa) and *C. album* (lambsquarters), were tested in this study and each seed lot was tested twice. The quinoa seed analyzed in this study originated from six different countries: Bolivia, Ecuador, Peru, India, Canada, and the United States (Table 1).

TABLE 1. Detection of *Peronospora variabilis* in quinoa seed using sequencing and polymerase chain reaction (PCR)-based methods developed in this study

Seed source	Country of origin	Sequencing confirmation[a,b]	PCR amplification[c]
AEP	Bolivia	+/+	–/+
AER	Bolivia	+/+	+/+
AEM	Bolivia	+/+	+/+
WegW	Bolivia	+/+	+/+
WBT	Bolivia	+/+	+/+
WBR	Bolivia	–/+	–/+
WegR	Bolivia	+/+	+/+
TJW	Bolivia	+/+	+/+
TJR	Bolivia	+/+	+/+
TJT	Bolivia	+/+	+/+
TRU	Bolivia	+/+	+/+
WegB	Bolivia	+/+	+/+
EOR	Bolivia	+/+	+/+
EOW	Ecuador	+/+	+/+
AHMW	Bolivia	+/Muc	+/+
ICN	Bolivia	+/+	+/+
COC	Bolivia	+/+	+/+
BLO	Bolivia	+/+	+/+
ECW	Bolivia	+/+	+/+
AHW	Bolivia	+/+	+/+
GIA	Bolivia	+/+	+/+
NPB	Bolivia	+/+	+/+
Chenopodium pallidicaule (KAN)	Bolivia	–/–	–/+
SAH	Peru	+/Asp	+/+
PHW	Peru	+/+	+/+
WGS	India	+/+	+/+
SIS10	United States	–/Asp	–/–
SIS11	United States	+/+	+/+
WMW	United States	+/+	+/+
WMB	United States	–/+	+/+
C. album (CAL)	United States	–/–	–/–
NQG	Canada	+/+	+/–
NQB	Canada	–/+	+/+
NQGO	Canada	+/+	–/–
NQBO	Canada	+/+	+/+

[a] Results are shown for two replicates of each detection method; + indicates *P. variabilis* was detected, – indicates *P. variabilis* was not detected, Asp = sequencing reactions that amplified *Aspergillus* sp., and Muc = sequencing reactions that amplified *Mucor* sp.
[b] Sequencing confirmation of *P. variabilis.*
[c] PCR amplification of *P. variabilis* with primer set PV6.

For phylogenetic analysis, quinoa and *Chenopodium* spp. (*C. album* and *Chenopodium* sp.) leaf samples were collected from mature plants affected by downy mildew (Fig. 1, panels E and F) originating from Bolivia, Ecuador, and the United States (Table 2) during the 2011 to 2013 growing seasons. Samples were dried overnight at 55°C and stored at room temperature prior to DNA extraction. A single lesion was chosen from each leaf for DNA extraction. Sporangia samples collected in Ecuador in 2013 were preserved in 70% ethanol prior to DNA extraction. For Bolivian and U.S. samples, five extractions (leaves taken from different plants) were performed for each field. Total DNA was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands), following manufacturer's instructions.

In 2011, quinoa downy mildew was detected in Centre County and Lancaster County, PA (33). Samples were collected from both field research sites, and *P. variabilis* was maintained on detached quinoa 'Faro' (Siskiyou Seeds, Williams, OR) leaves (14) on water agar. Two lines of *P. variabilis* were maintained: the Rock Springs line (RS) (Centre County) and the Landisville line (LV) (Lancaster County). Mating tests were performed as described by Danielsen et al. (9).

Development of *P. variabilis*-specific primers. *P. variabilis* ITS sequences were obtained from GenBank and from sequences obtained from RS and LV *P. variabilis* lines, following amplification using primer pair DC6/ITS4 (7) (Table 3). Primer set PV6 (*P. variabilis* 6) (Table 3) was developed from the ITS consensus sequence using National Center for Biotechnology Information Primer Blast (37). Optimal PCR conditions for primer set PV6 consisted of the following: initial denaturation step of 94°C for 2 min; followed by 32 cycles of a touchdown PCR (26) with denaturation at 95°C for 30 s, annealing from 66 to 56°C for 45 s, and elongation at 72°C for 90 s; with a final elongation step of 72°C for 5 min. PCR was carried out in 20-µl reactions consisting of GoTaq green master mix (Promega Corporation, Madison, WI), 0.25 µM each primer, and 1 µl of template DNA. All reactions were carried out in an ExpressGene Gradient Cycler (Denville Scientific, Metuchen, NJ).

To assess primer specificity, primer set PV6 was tested to determine whether the primers would amplify DNA from *P. farinosa* f. sp. *spinaciae* on spinach, *Bremia lactucae* on lettuce, *Pythium aphanidermatum* (pure culture), *Phytophthora infestans* on tomato, *Plasmopara obducens* on impatiens, *Peronospora tabacina* on tobacco, *P. manshurica* on soybean, *Plasmopara viticola* on grape, or *Pseudoperonospora cubensis* on cucumber.

To determine primer sensitivity, template DNA from sporangia was amplified using the DC6/ITS4 primer pair. PCR products were run on agarose gels and stained with ethidium bromide, and bands corresponding to *Peronospora variabilis* DNA were excised from the gel and purified using the E.Z.N.A gel extraction kit (Omega Bio-Tek, Norcross, GA) to ensure no carryover of plant DNA. Purified *P. variabilis* DNA was quantified using a NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA) and serial DNA dilutions were amplified using primer pair PV6 to determine primer sensitivity.

Detection of *P. variabilis* in quinoa seed. Seed wash methods for microscopically examining quinoa seed for oospores (14,24) were adapted to screen seed molecularly. Briefly, 5 g (≈1,300 to 1,500 seeds) of quinoa seed was stirred in sterile milliQ water for 30 min at maximum speed. The seed wash suspension was filtered through sterile cheesecloth and centrifuged for 5 min at 4,000 rpm. The obtained pellet was transferred to a tube of Lysing Matrix A

(MP Biomedicals, Solon, OH) and lysed in a FastPrep-24 Instrument (MP Biomedicals) for two cycles of 45 s at speed 4.5. DNA was then extracted using a Qiagen DNeasy Plant Mini Kit. *P. variabilis*-specific primer set PV6 was then used to screen seed for *P. variabilis*. The optimal PCR conditions for this reaction are described above. Amplification was determined by running the PCR products on an agarose gel, followed by ethidium bromide staining.

To validate the specific primer PCR-based method, a sequencing-based detection method was also used to screen quinoa seed. A semi-nested PCR (7) was used to obtain DNA from the extracted seed washes for DNA sequencing. This semi-nested PCR consisted of 25-µl reactions of GoTaq green master mix, 0.2 µM each primer (first reaction: ITS4/DC6 and second reaction: ITS4/ITS6), and 5 µl of each template. PCR reactions were performed according to Cooke et al. (7). PCR products were cleaned with ExoSap IT (Affymetrix/USB, Santa Clara, CA) and submitted for sequencing at the Penn State Genomics Core Facility. Products were sequenced unidirectionally with primer DC6 using an Applied Biosystems 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA).

To validate both molecular detection methods, some seed wash pellets were treated with 5 ml of 1 M KOH for 16 h (14) to visually check for the presence of oospores using a light microscope.

Phylogenetics of the quinoa downy mildew pathogen. Two regions, ITS (7) and *COX2* (22,23), were amplified for phylogenetic study of the causal agent of quinoa downy mildew. For both genes, 25-µl PCR reactions consisted of GoTaq green master

TABLE 2. Samples of quinoa downy mildew used in this study by host, country and region of origin, and year collected

Sample name	Host	Origin, year	Location	GenBank accession numbers[a]	
				ITS	*COX2*
BDM2 A,E	Quinoa	Bolivia, 2011	Cochabamba Department	A:KF269538, E:KF269539	A:KF269649, E:KF269618
BDM3 A,D	Quinoa	Bolivia, 2011	Cochabamba Department	A:KF269540, D:KF269541	A:KF269619, D:KF269645
BDM4 B,D	Quinoa	Bolivia, 2011	Cochabamba Department	B:KF269576, D:KF269542	B:KF269650, D:KF269651
BDM5 A,B	Quinoa	Bolivia, 2011	Cochabamba Department	A:KF269543, B:KF269544	A:KF269620, B:KF269652
BDM6 A	Quinoa	Bolivia, 2011	Cochabamba Department	KF269546	KF269653
BDM8 D,E	Quinoa	Bolivia, 2012	Cochabamba Department	D:KF269547, E:KF269548	D:KF269654, E:KF269621
BDM9 A,B,C,D,E	Quinoa	Bolivia, 2012	Cochabamba Department	A:KF269549, B:KF269550, C:KF269551, D:KF269552, E:KF269553	A:KF269655, B:KF269656, C:KF269646, D:KF269647, E:KF269657
BDM10 A,C,D	Quinoa	Bolivia, 2012	Cochabamba Department	A:KF269554, C:KF269555, D:KF269556	A:KF269658, C:KF269659, D:KF269648
BDM11 B,D,E,	Quinoa	Bolivia, 2012	Chuquisaca Department	B:KF269558, D:KF269559, E:KF269560	B:KF269660, D:KF269622, E:KF269623
BDM12 A,E	Quinoa	Bolivia, 2012	Chuquisaca Department	A:KF269561, E:KF269564	A:KF269624, E:KF269661
BDM13 A,C,D	Quinoa	Bolivia, 2012	Oruro Department	A:KF269565, C:KF269577, D:KF269566	A:KF269674, C:KF269625, D:KF269662
BDM14 A,C,E	Quinoa	Bolivia, 2012	Oruro Department	A:KF269578, C:KF269567, E:KF269568	A:KF269626, C:KF269627, E:KF269628
BDM15 A,E,D	Quinoa	Bolivia, 2012	Chuquisaca Department	A:KF269569, E:KF269571, D:KF269579	A:KF269629, E:KF269630, D:KF269663
BDM16 A,B,C,E	Quinoa	Bolivia, 2012	Chuquisaca Department	A:KF269572, B:KF269573, C:KF269574, E:KF269575	A:KF269664, B:KF269631, C:KF269665, E:KF269666
BDM17 B,C,E	Quinoa	Bolivia, 2012	Chuquisaca Department	B:KF269580, C:KF269581, E:KF269582	B:KF269667, C:KF269668, E:KF269632
BDM18 B,C,D	Quinoa	Bolivia, 2012	Chuquisaca Department	B:KF269583, C:KF269584, D:KF269585	B:KF269669, C:KF269633, D:KF269670
BDM19 A,D,E	Quinoa	Bolivia, 2012	Chuquisaca Department	A:KF269586, D:KF269588, E:KF269589	A:KF269671, D:KF269634, E:KF269635
ECPV4	Quinoa	Ecuador, 2011	Chimborazo Province	KF269605	KF269677
ECPV8	Quinoa	Ecuador, 2011	Chimborazo Province	KF269606	KF269678
ECPV9	Quinoa	Ecuador, 2011	Chimborazo Province	KF269607	KF269679
ECPV10	Quinoa	Ecuador, 2011	Chimborazo Province	KF269608	KF269680
ECPV11	Quinoa	Ecuador, 2011	Chimborazo Province	KF269609	KF269681
ECDM10	Quinoa	Ecuador, 2012	Cotopaxi Province	KF269594	KF269636
ECDM16	*Chenopodium* sp.	Ecuador, 2012	Cotopaxi Province	KF269595	KF269637
ECDM19	*Chenopodium album*	Ecuador, 2012	Tungurahua Province	KF269596	KF269638
ECDM34	Quinoa	Ecuador, 2012	Bolivar Province	KF269597	KF269639
ECDM53	Quinoa	Ecuador, 2012	Chimborazo Province	KF269599	KF269640
ECDM68	*C. album*	Ecuador, 2012	Tungurahua Province	KF269600	KF269641
ECDM69	*Chenopodium* sp.	Ecuador, 2012	Pinchincha Province	KF269601	KF269642
ECDM71	*Chenopodium* sp.	Ecuador, 2012	Pinchincha Province	KF269602	KF269672
ECDM74	Quinoa	Ecuador, 2012	Pinchincha Province	KF269603	KF269643
ECDM75	Quinoa	Ecuador, 2012	Imbabura Province	KF269604	KF269644
EcColta	Quinoa	Ecuador, 2013	Chimborazo Province	KF269610	KF269690
EcColtaSystemic	Quinoa	Ecuador, 2013	Chimborazo Province	KF269617	KF269689
LV	Quinoa	United States, 2011	Lancaster County, PA	KF269590	KF269682
LV1-5	Quinoa	United States, 2011	Lancaster County, PA	1:KF269611, 2:KF269612, 3:KF269613, 4:KF269614, 5:KF269615	1:KF269683, 2:KF269684, 3:KF269685, 4:KF269686, 5:KF269687
RS	Quinoa	United States, 2011	Centre County, PA	KF269591	KF269673
RS2	Quinoa	United States, 2011	Centre County, PA	KF269616	KF269688
SPDM (spinach downy mildew) 1,3	Spinach	United States, 2012	Centre County, PA	1:KF269592, 3:KF269593	1:KF269675, 3:KF269676

[a] GenBank accession numbers are given for multiple samples collected from different quinoa plants in a field. For Bolivian samples, up to five samples were collected (A to E) and, for U.S. samples, up to five were also collected (1 to 5). ITS = internal transcribed spacer and *COX2* = cytochrome *c* oxidase subunit 2.

mix, 0.2 µM each primer, and 5 µl of template DNA. ITS sequences were amplified using primers ITS4/DC6, with PCR conditions described by Cooke et al. (7). Amplification of *COX2* used the peronosporomycete forward/reverse primers (22), with PCR conditions of 96°C for 4 min; 30 cycles of 96°C for 30 s, 50°C for 30 s, and 72°C for 1 min; followed by a final elongation step of 72°C for 4 min. PCR products were visualized on agarose gels following staining with ethidium bromide. PCR products were cleaned with ExoSAP-IT prior to sequencing.

PCR products were sequenced bidirectionally using primers ITS4/DC6 or primers peronosporomycete forward/reverse. Obtained sequences were aligned using MUSCLE (16) in *MEGA*5.10 (32) and alignments were manually checked. Maximum likelihood (ML) trees were generated in *MEGA*5.10 and tested with 1,000 bootstrap replicates. An ML ITS tree was generated using the Hasegawa-Kishino-Yano model with invariant sites, while the ML *COX2* tree was generated using the Tamura 3-parameter model with invariant sites. *Pythium monospermum* was used to root both trees (21).

RESULTS

Primer specificity and sensitivity. Primer set PV6 only amplified DNA from *P. variabilis* (Fig. 2) and consistently produced a 278-bp amplicon. The touchdown PCR protocol was required to avoid amplification of the closely related *Peronospora farinosa* f. sp. *spinaciae*, because only a 3-nucleotide difference existed between both species in the annealing site of the reverse primer. The touchdown PCR also allowed for high primer sensitivity, with a detection limit for *P. variabilis* DNA of 10 fg/µl (Fig. 3). This primer sensitivity is for pure *P. variabilis* DNA and may be reduced in the presence of other DNA such as plant or fungal DNA.

Molecular detection of *P. variabilis* in quinoa seed. Thirty-three seed lots of quinoa were screened using the *P. variabilis*-

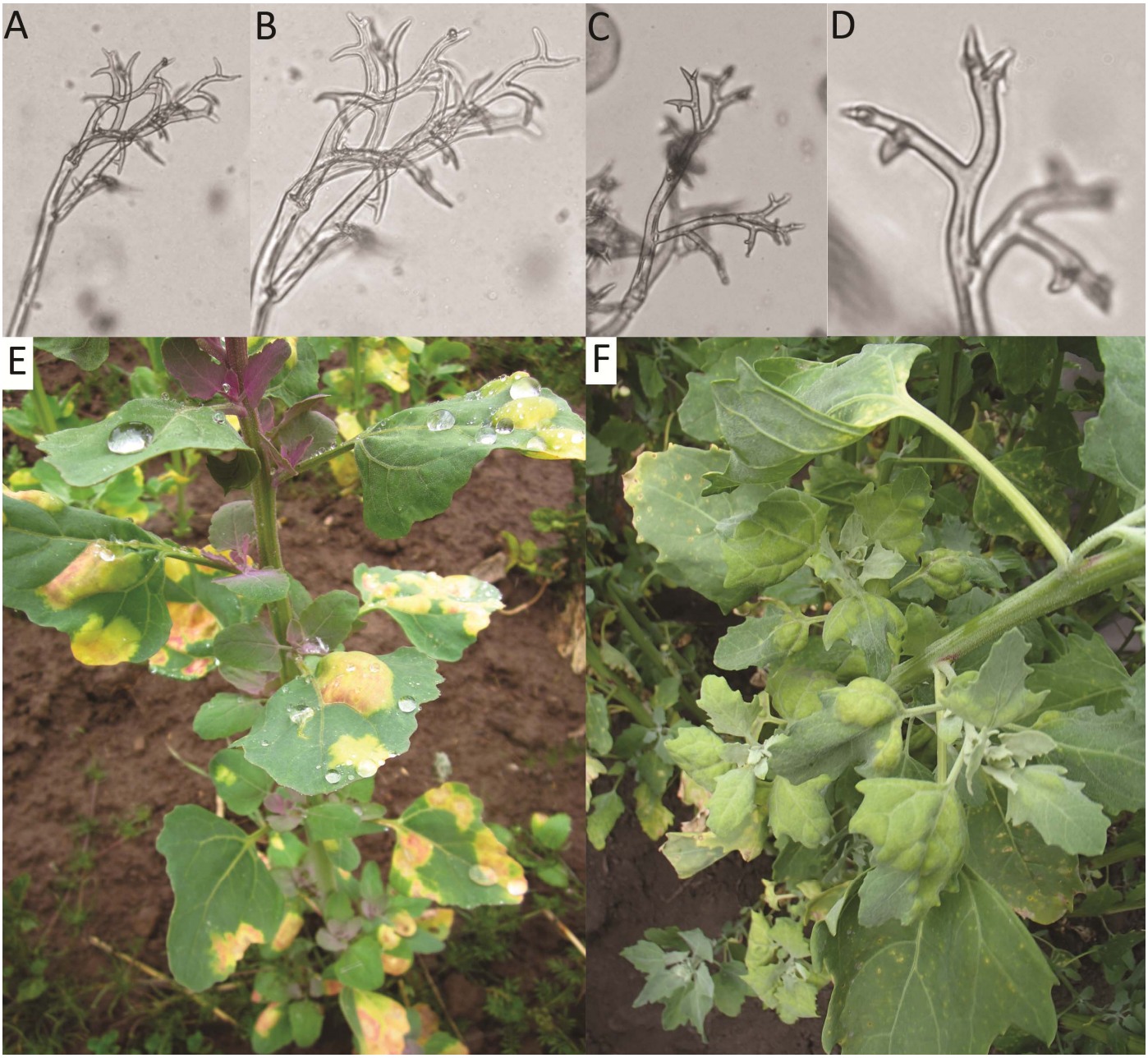

Fig. 1. A and B, Sporangiophore of *Peronospora variabilis*; **C and D,** sporangiophore of a putative *Peronospora* sp. which causes systemic-like downy mildew symptoms; **E,** typical symptoms of quinoa downy mildew, including yellow-pink foliar lesions with sporulation; **F,** atypical symptoms of quinoa downy mildew, including stunting, foliar cupping, chlorosis, and extremely dense sporulation.

specific primer PV6 designed in this study. *P. variabilis* was detected in 31 of 33 seed lots (Table 1; Fig. 4) in at least one of the two replicates. In 28 seed lots, *P. variabilis* was detected in both replicates (Table 1). *P. variabilis* was never detected in seed of *C. album* and was detected once in seed of *C. pallidicaule* (Table 1). Oospores were also visually observed in several seed lots, along with sporangia similar to those of *P. variabilis* (Fig. 5).

A sequencing-based approach was used to validate the PCR-based method and, in this approach, a semi-nested PCR was used (primers ITS4/DC6 for first reaction and ITS4/ITS6 for second reaction) to generate amplicons for sequencing. *P. variabilis* was detected in 32 of 33 samples of quinoa and was never detected in seed of *C. album* or *C. pallidicaule*; therefore, PCR-based detection of *P. variabilis* in *C. pallidicaule* seed was not validated (Table 1). The use of the semi-nested procedure to amplify the ITS region of *Peronospora* spp. (7) led to chimeric amplicons which had the *C. quinoa* ITS region as the first portion of the sequence, followed by the *P. variabilis* ITS region. This chimeric amplicon occurred in all seed lots tested. If *P. variabilis* was not detected by sequencing, only the *C. quinoa* ITS region was present in the sequenced amplicon.

SIS10, from the United States, was the only seed lot in which *P. variabilis* was never detected. However, the sequencing-based detection method amplified *Aspergillus* sp. DNA in this seed lot, and also in the SAH seed lot from Peru. However, *P. variabilis* was never detected with the PCR-based detection method in the SIS10 seed lot. SIS10 and SIS11 were the only seed lots sold for planting; all other seed lots were sold for human consumption.

Phylogeny of the quinoa downy mildew pathogen. High-quality sequences with no polymorphisms were obtained and used to construct phylogenetic trees based on ITS and *COX2* sequences. The ITS phylogenetic tree (Fig. 6) showed little variation among quinoa downy mildew samples regardless of their South or North American origin. Only a single distinct clade (100% bootstrap support) that included all *P. variabilis* from quinoa is shown in this tree. This clade also includes downy

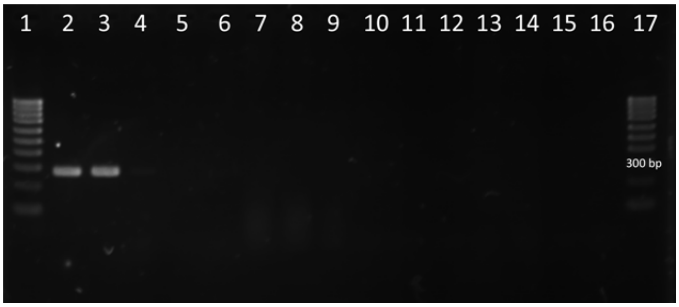

Fig. 2. Specificity of PV6 primers (repeated species are technical replicates). Lane 1, 1-kb ladder; lane 2, *Peronospora variabilis* on quinoa; lane 3, *P. variabilis* on quinoa; lane 4, *P. farinosa* f. sp. *spinaciae* on spinach; lane 5, *P. farinosa* f. sp. *spinaciae* on spinach; lane 6, *P. farinosa* f. sp. *spinaciae* on spinach; lane 7, *P. tabacina* on tobacco; lane 8, *P. manshurica* on soybean; lane 9, *Plasmopara viticola* on grape; lane 10, *P. obducens* on impatiens; lane 11, *Pseudoperonospora cubensis* on cucumber; lane 12, *Bremia lactucae* on lettuce; lane 13, *Phytophthora infestans* on tomato; lane 14, *Pythium aphanidermatum*; lane 15, asymptomatic quinoa tissue; lane 16, negative control; lane 17, 1-kb ladder.

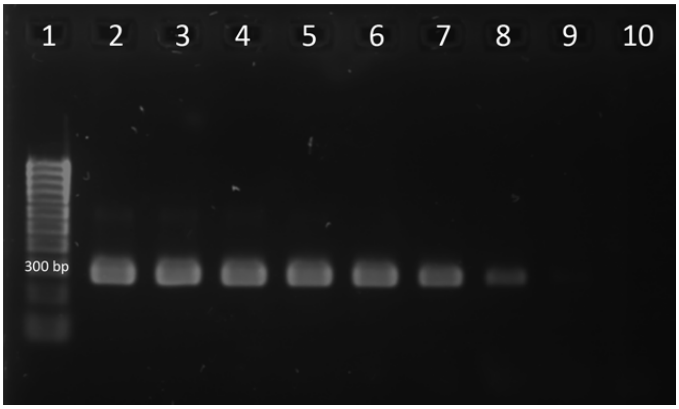

Fig. 3. Sensitivity of primer set PV6 for detection of *Peronospora variabilis*. Lane 2, 10 ng/µl; lane 3, 1 ng/µl; lane 4, 100 pg/µl; lane 5, 10 pg/µl; lane 6, 1 pg/µl; lane 7, 100 fg/µl; lane 8, 10 fg/µl; lane 9, 1 fg/µl; lane 10, 100 ag/µl.

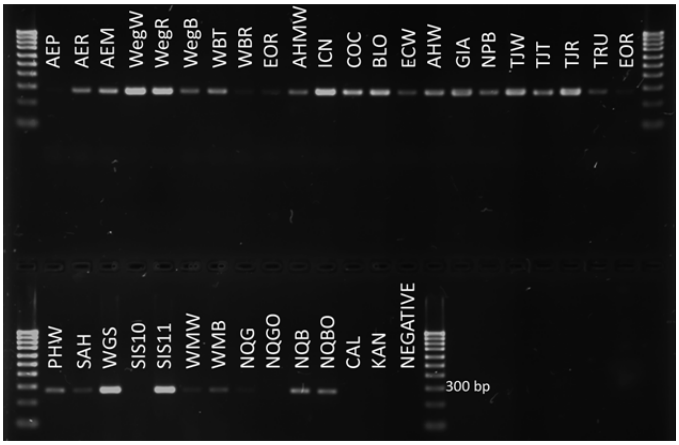

Fig. 4. Electrophoresis gel showing results of polymerase chain reaction-based detection of *Peronospora variabilis* in quinoa seed washes. Product size is approximately 278 bp. Codes for the sources of the seed washes are found in Table 1.

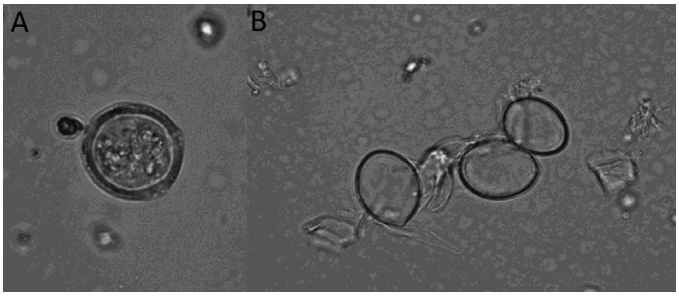

Fig. 5. A, Oospore and **B,** sporangia, likely of *Peronospora variabilis*, from quinoa seed wash.

TABLE 3. Primers used in this study

Primer name	Sequence (5′ to 3′)	Source
PV6F	GTTGCTGGTTGTGAAGGCTG	This study
PV6R	ATGCTACGCAACCGAAGTCA	This study
DC6	GAGGGACTTTTGGGTAATCA	7
ITS4	TCCTCCGCTTATTGATATGC	36
ITS6	GAAGGTGAAGTCGTAACAAGG	36
Peronosporomycete forward	GGCAAATGGGTTTTCAAGATCC	22
Peronosporomycete reverse	CCATGATTAATACCACAAATTTCACTAC	22

mildew that was collected from *Chenopodium* weed species in Ecuador (from *C. album* [unfilled boxes]: ECDM19 and -68 and from *Chenopodium* sp. [black boxes]: ECDM16, -69, and -71), which may indicate that weedy *Chenopodium* spp. can be a reservoir of the pathogen. The only samples that were not included in the quinoa downy mildew *P. variabilis* clade were samples ECPV9, ECPV10, and ECColtaSystemic, collected in Ecuador from quinoa that did not show the characteristic symptoms of typical downy mildew but of a systemic-like infection. These samples were grouped in a separate clade (100% bootstrap support) more closely related to *P. boni-herici* and *P. farinosa* f. sp. *spinaciae*, and are a separate species from *P. variabilis*.

For the ML tree generated by *COX2* sequences (Fig. 6), there was a major clade that included all samples of quinoa downy mildew; however, there was a clear separation of the U.S. lineage from the South American samples (97% bootstrap support). RS, a *P. variabilis* line collected in Pennsylvania, is phylogenetically placed within the South American lineage, whereas LV, the other *P. variabilis* line collected in Pennsylvania, is closest to the U.S. lineage. As per the ITS phylogeny, samples ECPV9, ECPV10, and ECColtaSystemic from Ecuador were placed in a separate clade, with 100% bootstrap support. The clear separation from the main quinoa downy mildew pathogen in both phylogenies indicates that samples ECPV9, ECPV10, and ECColtaSystemic

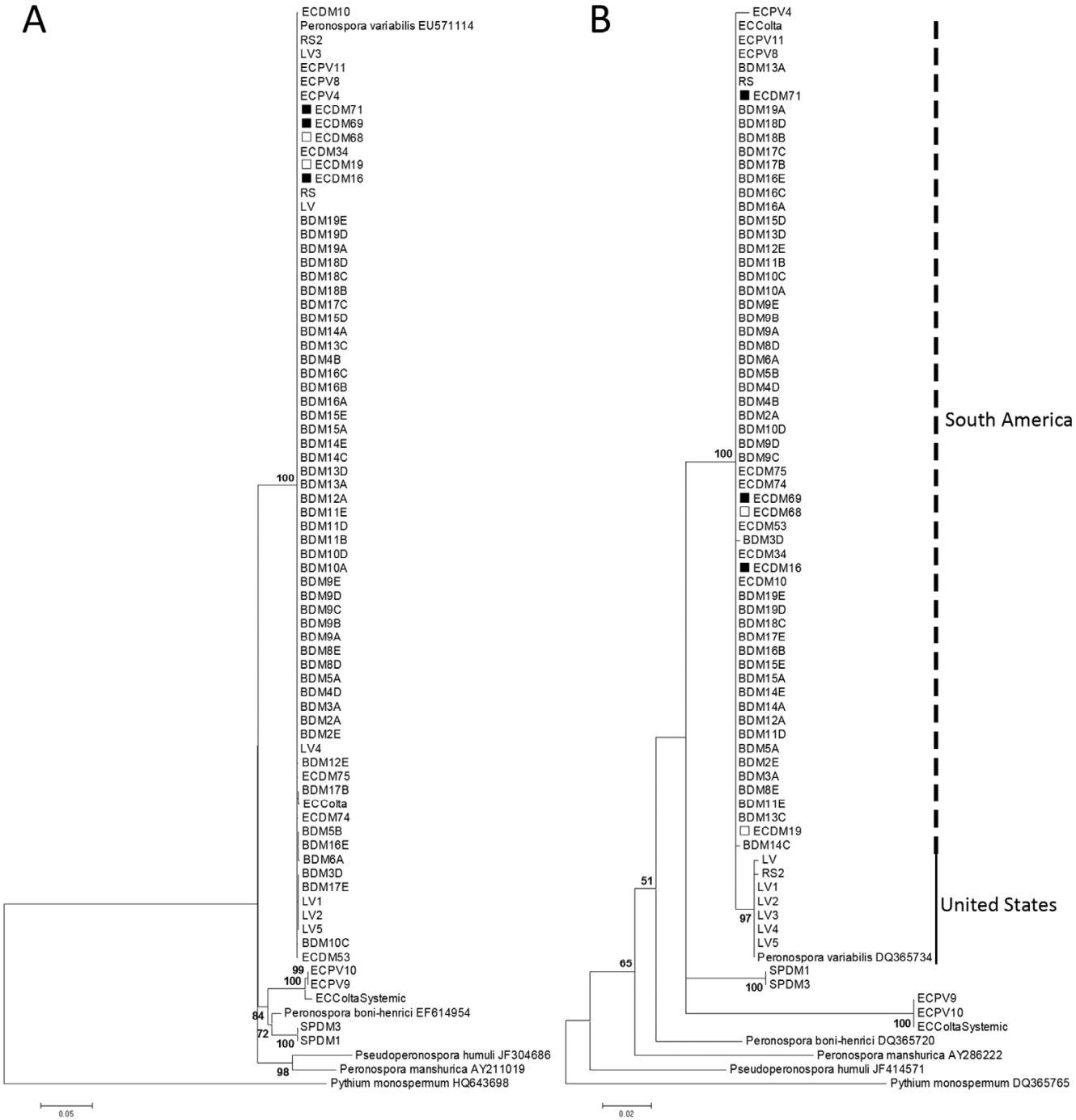

Fig. 6. A, Maximum likelihood (ML) tree of internal transcribed spacer sequences of the downy mildew pathogen, *Peronospora variabilis*, on *Chenopodium quinoa*. **B,** ML tree of cytochrome *c* oxidase subunit 2 sequences of the downy mildew pathogen on *C. quinoa*. For both trees, sequences from either *C. album* (□) or *Chenopodium* sp. (■) collected in Ecuador are specially marked. BDM samples are from Bolivia, ECDM and ECPV are samples collected in Ecuador, while LV and RS are U.S. samples. SPDM sequences are from the spinach downy mildew pathogen, *P. farinosa* f. sp. *spinaciae*. Taxa with GenBank accession numbers were taken from other studies of *Peronospora* phylogeny for comparison.

are a separate species from *P. variabilis*. For both ITS and *COX2* phylogenies, no within-field or within-country differences were observed for *P. variabilis* populations.

DISCUSSION

In this research, we developed a PCR-based molecular method for the identification of *P. variabilis*, the causal agent of quinoa downy mildew. This methodology allows for the rapid, high-throughput screening of quinoa seed for the presence of *P. variabilis*. This methodology is the first step in creating certified *P. variabilis*-free quinoa seed. Use of the seed wash allows for screening of many more seed than other methodologies that require direct grinding and a DNA extraction method. The seed wash method was utilized to reduce the numerous inhibitory compounds, such as phenolic compounds in seed, which could interfere with DNA amplification. Generally, sequencing supported the findings of the PCR-based detection method, except for two seed lots (SAH and SIS10), where sequencing repeatedly amplified nontarget fungal DNA, which may have hindered detection of *P. variabilis*. Only one seed lot (NQGO) tested negative in the PCR-based assay but positive in the sequencing-based assay (Table 1), whereas seed from *C. pallidicaule* tested positive once in the PCR-based assay but *P. variabilis* was never detected in the sequencing-based method.

P. variabilis was detected in nearly every seed lot of quinoa that was screened. All but two quinoa seed lots (SIS10 and SIS11) were sold for direct human consumption, which means the seed underwent extensive post-harvest processing to remove saponins in the quinoa seed coat (15). Saponins are removed either by washing or abrasion (15), and this intensive processing has been shown to reduce fungal contamination of seed (31). However, the effects of the desaponification process on *P. variabilis* contamination are unknown. Danielsen et al. (14) visually detected oospores in the pericarp of quinoa seed in 15% of lots observed. We detected *P. variabilis* in 94% of seed lots tested. The seed wash procedure screens 1,300 to 1,500 seeds simultaneously versus visually screening 100 seed coats at a time. However, our test does not indicate the viability or cell type of *P. variabilis* that is detected. Viability of *P. variabilis* needs to be determined by growing seedlings under disease-conducive conditions, and these assays are time consuming. In some seed washes, we visually observed oospores and, in one seed lot, we also observed sporangia (Fig. 5). Visual inspection of seed washes was time consuming and oospores were difficult to detect due to the similarity in size, shape, and color to *Chenopodium* pollen and other seed debris (data not shown).

Based on ITS and *COX2* phylogenies of this study's quinoa downy mildew samples, we can infer more about the pathogen population in South America and the United States. First, based on the *COX2* phylogeny, there is support for geographic diversity in the pathogen. Samples collected in the United States form a clade (with strong bootstrap support, 97%) distinct from South American samples. U.S. and South American samples form a separate clade from samples with systemic-like downy mildew symptoms (Fig. 1).

Based on ITS and *COX2* phylogenies, *Peronospora* spp. infecting weedy *Chenopodium* spp. in Ecuador appear to be genetically identical to *P. variabilis* that infects quinoa. *Chenopodium* weeds may serve as reservoirs of *P. variabilis* between quinoa production seasons. In Pennsylvania, there was never any indication of *P. variabilis* infections of *C. album* under field conditions. Genetic markers with finer resolution than ITS and *COX2* are needed to detect differences in virulence groups (30) and mating types of *P. variabilis* (9), and there may still be differences in *P. variabilis* populations from quinoa and weedy *Chenopodium* spp. These genetic markers could help quinoa breeders rapidly characterize local *P. variabilis* populations.

This study was initiated to determine how quinoa downy mildew was introduced to Pennsylvania in 2011 (33). It is likely that *P. variabilis* was introduced into Pennsylvania on infected quinoa seed. There are several lines of evidence to support this. First, downy mildew-like symptoms were never observed on lambsquarters adjacent to heavily sporulating quinoa plants. Other authors have noted downy mildew-like symptoms on *Chenopodium* weeds growing next to infected quinoa (12,34). *C. album*, grown from locally collected seed, never developed symptoms of downy mildew during pathogenicity assays under controlled conditions (data not shown). It appears that there were at least two separate introductions of the pathogen to Pennsylvania. *P. variabilis* line LV is genetically similar to the U.S. lineage determined by the *COX2* phylogeny, whereas *P. variabilis* line RS was more genetically similar to the South American *COX2* lineage. It should be noted that another sample (RS2) of *P. variabilis* collected at Rock Springs was most similar to the U.S. *COX2* lineage. Oospores of *P. variabilis* were observed in leaves collected at Rock Springs which, because *P. variabilis* is heterothallic (9), supports that two separate introductions of the pathogen occurred at this single Pennsylvania field site. Further, the LV and RS lines formed oospores in mating experiments (14) and represent two mating types from two geographically distinct sites in Pennsylvania. Therefore, there were likely at least two introductions of *P. variabilis* to Pennsylvania, one from seed from South America and one from *P. variabilis* of the North American lineage, possibly from seed from North America.

Genetically isolated populations of quinoa may harbor genetically distinct populations of the causal agent of quinoa downy mildew. Quinoa plants with unique symptoms were observed in Colta Canton, Chimborazo Province, Ecuador, a region of indigenous quinoa cultivation isolated from other quinoa-producing regions, in 2011 (ECPV9 and ECPV10) and 2013 (ECColtaSystemic). These plants exhibited stunting, leaf cupping and distortion, foliar chlorosis, and extremely dense sporulation on the undersides of leaves. The symptoms on these plants were distinct from typical symptoms of quinoa downy mildew (Fig. 1) and conidiophores collected from these plants are morphologically different from *P. variabilis* (Fig. 1). These atypical symptoms were thought to be due to systemic infections originating from oospores that infected seed or seedlings (1,18) but our findings suggest these samples were infected with a *Peronospora* sp. that is genetically distinct from *P. variabilis*. It should be noted that quinoa leaf samples exhibiting typical downy mildew samples collected in the same fields in 2011 (ECPV4, -8, and -11) and 2013 (ECColta) were genetically similar to other South American *P. variabilis* samples. As is indicated by our phylogenetic analyses, the samples collected in Colta Canton may represent an undescribed species of *Peronospora* that also causes a downy mildew on quinoa. Comparisons of ITS sequences from this undescribed *Peronospora* sp. to known *Peronospora* spp. in GenBank have only 96% maximum identity (*P. pulveracea* FJ384778 and *P. corydalis* AF528562) and 94 to 95% maximum identity with *P. farinosa* from *C. album* (AF528556 and AF528557). The ecology of this species of *Peronospora* is unknown at this time but deserves further research to determine its role in quinoa downy mildew epidemics, especially because this *Peronospora* sp. was demonstrated to occur in the same field as *P. variabilis*.

Demand for quinoa will not decrease and sustainable disease management strategies must be developed to ensure continued quinoa production in Andean countries. The methodology developed in this research will allow for rapid screening of quinoa seed to detect *P. variabilis*. Exclusion of pathogens from production fields is a key step in integrated management of any disease, and this research allows for the development of *P. variabilis*-free quinoa seed lots. Our findings also support previous research that indicated geographic differences in *P. variabilis* populations

from quinoa (5,13). Further, a new species of *Peronospora* was found in Ecuador that causes a systemic-like downy mildew infection in quinoa.

This study highlights the importance and ubiquity of seedborne *P. variabilis* in quinoa. Seedborne *P. variabilis* likely plays important roles in the spread of *P. variabilis* to new quinoa-producing regions of the world and the maintenance of *P. variabilis* in endemic regions.

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LITERATURE CITED

1. Alandia, S., Otazu, V., and Salas, B. 1979. Enfermedades. Pages 137-148 in: La quinua y la kañiwa: cultivos andinos. M. Tapia, ed. Bib. Orton IICA/CATIE, Bogata, Columbia.
2. Azevedo, M. I., Botton, S. A., Pereira, D. I. B., Robe, L. J., Jesus, F. P. K., Mahl, C. D., Costa, M. M., Alves, S. H., and Santurio, J. M. 2012. Phylogenetic relationships of Brazilian isolates of *Pythium insidiosum* based on ITS rDNA and cytochrome oxidase II gene sequences. Vet. Microbiol. 159:141-148.
3. Belbahri, L., Calmin, G., Pawlowski, J., and Lefort, F. 2005. Phylogenetic analysis and real time PCR detection of a presumably undescribed *Peronospora* species on sweet basil and sage. Mycol. Res. 109:1276-1287.
4. Bhargava, A. 2006. *Chenopodium quinoa*—an Indian perspective. Ind. Crops Prod. 23:73-87.
5. Choi, Y. J., Danielsen, S., Lubeck, M., Hong, S. B., Delhey, R., and Shin, H. D. 2010. Morphological and molecular characterization of the causal agent of downy mildew on quinoa (*Chenopodium quinoa*). Mycopathologia 169:403-412.
6. Choi, Y. J., Denchev, C. M., and Shin, H. D. 2008. Morphological and molecular analyses support existence of host-specific *Peronospora* species infecting *Chenopodium*. Mycopathologia 165:155-164.
7. Cooke, D. E. L., Drenth, A., Duncan, J. M., Wagels, G., and Brasier, C. M. 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. Fungal Genet. Biol. 30:17-32.
8. Cusack, D. F. 1984. Quinua: grain of the Incas. Ecologist 14:21-31.
9. Danielsen, S. 2001. Heterothallism in *Peronospora farinosa* f. sp. *chenopodii*, the causal agent of downy mildew of quinoa (*Chenopodium quinoa*). J. Basic Microbiol. 41:305-308.
10. Danielsen, S., Bonifacio, A., and Ames, T. 2003. Diseases of quinoa (*Chenopodium quinoa*). Food Rev. Int. 19:43-59.
11. Danielsen, S., Jacobsen, S. E., Echegaray, J., and Ames, T. 1999. Impact of downy mildew on the yield of quinoa. CIP Program Rep. 1999-2000:397-401.
12. Danielsen, S., Jacobsen, S. E., and Hockenhull, J. 2002. First report of downy mildew of quinoa caused by *Peronospora farinosa* f. sp. *chenopodii* in Denmark. Plant Dis. 86:1175.
13. Danielsen, S., and Lubeck, M. 2010. Universally primed-PCR indicates geographical variation of *Peronospora farinosa* ex. *Chenopodium quinoa*. J. Basic Microbiol. 50:104-109.
14. Danielsen, S., Mercado, V. H., Ames, T., and Munk, L. 2004. Seed transmission of downy mildew (*Peronospora farinosa* f. sp. *chenopodii*) in quinoa and effect of relative humidity on seedling infection. Seed Sci. Technol. 32:91-98.
15. Dominguez, S. S. 2003. Quinoa-postharvest and commercialization. Food Rev. Int. 19:191-201.
16. Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792-1797.
17. El Hafid, R., Aitelmaalem, H., Driedger, D., Bandara, M., and Stevenson, J. 2005. Quinoa…The Next Cinderella Crop for Alberta? Alberta Agriculture, Food and Rural Development Ag Entrepreneurship, Edmonton, Alberta, Canada.
18. Frinking, H. D., and Linders, E. G. A. 1986. A comparison of two pathosystems: Downy mildew on *Spinacia oleracea* and on *Chenopodium album*. Neth. J. Plant Pathol. 92:97-106.
19. Göker, M., Garcia-Blazquez, G., Voglmayr, H., Telleria, M. T., and Martin, M. P. 2009. Molecular taxonomy of phytopathogenic fungi: A case study in *Peronospora*. PLoS One 4:e6319.
20. Göker, M., Voglmayra, H., Riethmuller, A., and Oberwinkler, F. 2007. How do obligate parasites evolve? A multi-gene phylogenetic analysis of downy mildews. Fungal Genet. Biol. 44:105-122.
21. Göker, M., Voglmayr, H., Riethmuller, A., Weiss, M., and Oberwinkler, F. 2003. Taxonomic aspects of Peronosporaceae inferred from Bayesian molecular phylogenetics. Can. J. Bot. 81:672-683.
22. Hudspeth, D. S. S., Nadler, S. A., and Hudspeth, M. E. S. 2000. A COX2 molecular phylogeny of the Peronosporomycetes. Mycologia 92:674-684.
23. Hudspeth, D. S. S., Stenger, D., and Hudspeth, M. E. S. 2003. A cox2 phylogenetic hypothesis for the downy mildews and white rusts. Fungal Divers. 13:47-57.
24. Inaba, T., Takahashi, K., and Morinaka, T. 1983. Seed transmission of spinach downy mildew. Plant Dis. 67:1139-1141.
25. Jacobsen, S. E. 1998. Developmental stability of quinoa under European conditions. Ind. Crops Prod. 7:169-174.
26. Korbie, D. J., and Mattick, J. S. 2008. Touchdown PCR for increased specificity and sensitivity in PCR amplification. Nat. Protocols 3:1452-1456.
27. Kroon, L. P. N. M., Bakker, F. T., van den Bosch, G. B. M., Bonants, P. J. M., and Flier, W. G. 2004. Phylogenetic analysis of *Phytophthora* species based on mitochondial and nuclear DNA sequences. Fungal Genet. Biol. 41:766-782.
28. Kumar, A., Bhargava, A., Shukla, S., Singh, H. B., and Ohri, D. 2006. Screening of exotic *Chenopodium quinoa* accessions for downy mildew resistance under mid-eastern conditions of India. Crop Prot. 25:879-889.
29. Landa, B. B., Montes-Borrego, M., Munoz-Lesma, F. J., and Jimenez-Diaz, R. M. 2007. Phylogenetic analysis of downy mildew pathogens of opium poppy and PCR-based in plant and seed detection of *Peronospora arborescens*. Phytopathology 97:1380-1390.
30. Ochoa, J., Frinking, H. D., and Jacobs, T. 1999. Postulation of virulence groups and resistance factors in the quinoa downy mildew pathosystem using material from Ecuador. Plant Pathol. 48:425-430.
31. Pappier, U., Fernandez Pinto, V., Larumbe, G., and Vaamonde, G. 2008. Effect of processing for saponin removal on fungal contamination of quinoa seeds (*Chenopodium quinoa* Willd.). Int. J. Food Microbiol. 125:153-157.
32. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony method. Mol. Biol. Evol. 28:2731-2739.
33. Testen, A. L., McKemy, J. M., and Backman, P. A. 2012. First report of quinoa downy mildew caused by *Peronospora variabilis* in the United States. Plant Dis. 96:146.
34. Tewari, J. P., and Boyetchko, S. M. 1990. Occurrence of *Peronospora farinosa* f. sp. *chenopodii* on quinoa in Canada. Can. Plant Dis. Surv. 70:127-128.
35. UNFAO. 2011. FAOSTAT. Food and Agriculture Organization of the United Nations, FAOSTAT database. http://faostat.fao.org
36. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.
37. Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinf. 13:134.