Virulence Structure Within Populations of *Pseudoperonospora cubensis* in the United States

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ABSTRACT

Cucurbit downy mildew (CDM), caused by the obligate oomycete *Pseudoperonospora cubensis*, has resurged around the world during the past three decades. A new pathotype or genetic recombinant of *P. cubensis* have been suggested as possible reasons for the resurgence of CDM in the United States in 2004. In total, 22 isolates collected between 2004 and 2014, mainly in the eastern United States, were tested for their compatibility with a set of 15 cucurbit host types. The virulence structure within these isolates was evaluated on a set of 12 differential genotypes from eight genera. All isolates were highly compatible with the susceptible cultivar of *Cucumis sativus*, whereas the least compatibility was observed with *Luffa cylindrica* and *Momordica charantia*. Based on the compatibility with the differential host set, five pathotypes (1, 3, 4, 5, and 6) were identified among the 22 isolates examined. Pathotypes 1 and 3 had not been previously described in the United States and isolates of these two new pathotypes were also compatible with ‘Poinsett 76’, a cultivar of *C. sativus* known to be resistant to CDM prior to 2004. Virulence within the pathogen population was

expressed based on virulence factors, virulence phenotypes, and virulence complexity. The number of virulence factors ranged from two to eight, indicating a complex virulence structure, with 77% of the isolates having five to eight virulence factors. Thirteen virulence phenotypes were identified; the mean number of virulence factors per isolate and mean number of virulence factors per virulence phenotype was 5.05 and 5.23, respectively, indicating that complex isolates and phenotypes contributed equally to the complex virulence structure of *P. cubensis*. Gleason and Shannon indices of diversity were 3.88 and 2.32, respectively, indicating a diverse virulence structure of *P. cubensis* within the United States population. The diverse virulence and high virulence complexity within the pathogen population indicate that host resistance alone in available cucurbit cultivars will not be effective to control CDM. An integrated approach involving a combination of fungicide application and introduction of cultivars with new resistance genes will be required for effective management of CDM.

Cucurbitaceae is a fairly large family and includes more than 118 genera and 825 species, with members distributed widely throughout the tropics and warm temperate regions of the world (Lebeda et al. 2007). In the United States, cucurbits are an important group of vegetable crops and account for approximately 10% of the total value of vegetables produced nationally (Anonymous 2016). The most widely grown cucurbits include cucumber, cantaloupe, pumpkin, squash, and watermelon. In 2014, cucurbits were produced on approximately 390,000 acres concentrated mainly in the eastern half of the United States and in Arizona, California, and Texas and were valued at \$1.5 billion (Anonymous 2016). Florida, Georgia, and North Carolina are among the major producers of cucumber, squash, and melon, accounting for more than 32% of the total value of the crop produced in the United States (Anonymous 2016).

Cucurbit downy mildew (CDM), caused by the obligate oomycete *Pseudoperonospora cubensis*, is one of the most economically important limiting factors in cucurbit production worldwide (Lebeda and Cohen 2011; Ojiambo et al. 2015). This is particularly the case because the resurgence of CDM in many parts of the world during the last decade has resulted in severe epidemics and crop losses (Cohen et al. 2015; Holmes et al. 2015). In the United States, the disease was only of minor concern on cucumber prior to 2004 and required very little application of fungicides due to resistant cultivars deployed in 1960s using the resistance source Plant Introduction (PI) 197087 (Barnes and Epps 1954). However, this long-standing resistance that was effective for approximately 40 years was overcome, resulting in a resurgence of the disease in the United States in 2004 (Holmes et al.

2006). Resurgence of CDM was also reported in Israel in 2002 (Cohen et al. 2003) and in Europe in 1985 and 2009 (Lebeda et al. 2011). In the United States, resurgence of CDM resulted in severe epidemics in the eastern part of the country in 2004 and 2005, resulting in significant crop losses (Holmes et al. 2006). In the United States, CDM now occurs annually, posing a significant threat to cucurbit production (Holmes et al. 2015).

Resurgence of CDM has dramatically affected production of cucurbits and deployment of disease management options worldwide (Holmes et al. 2015). Thus, considerable efforts have been initiated to determine and understand possible causes for the resurgence of CDM in Europe (Kitner et al. 2015; Lebeda et al. 2013), Israel (Cohen et al. 2015), and the United States (Holmes et al. 2015; Ojiambo et al. 2015) to devise effective disease management strategies. Anthropogenic introduction of new pathotypes of *P. cubensis*, possibly from Asia, has been suggested as a plausible explanation for the resurgence of CDM around the world (Runge et al. 2011). In *P. cubensis*, pathotypes are physiological forms of the pathogen that differ in host specificity on the level of genus, species, or subspecies of various cucurbits (Lebeda and Widrechner 2003). As in other oomycetes, the interaction between *P. cubensis* and cucurbits is discontinuous and displays a compatible or incompatible reaction pattern. This compatible or incompatible reaction with a set of differential host species is the basis for classifying pathotypes of *P. cubensis*. Thomas et al. (1987) first identified pathotypes of *P. cubensis* based on a differential set composed of *Cucumis sativus*, *C. melo* var. *reticulatus*, *C. melo* var. *conomon*, *C. melo* var. *acidullus*, *Citrullus lanatus* and *Cucurbita pepo*. Isolates capable of infecting *Cucumis sativus* and *C. melo* var. *reticulatus* were identified as pathotype 1, while those capable of infecting *C. melo* var. *conomon* in addition to the former hosts were considered to be pathotype 2. Isolates capable of infecting all the *Cucumis* spp. were considered to be pathotype 3.

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All isolates capable of infecting *Citrullus lanatus* in addition to the *Cucumis* spp. were identified as pathotype 4 and all those that were capable of infecting *Cucurbita* spp. in addition to *Cucumis* spp. and *C. lanatus* were considered to be pathotype 5. Isolates from Japan belonged to pathotype 1, 2, or 3, whereas those from the United States belonged to pathotype 4 or 5 (Thomas et al. 1987). The host differential set proposed by Thomas et al. (1987) to designate pathotypes was composed of 6 differentials from three genera and it was later expanded to 12 genotypically well-defined differentials from six genera that also include *Benincasa*, *Luffa*, and *Lagenaria* (Lebeda and Widrechner 2003). Recently, the latter host differential set has been expanded to 15 differentials to include hosts from two additional genera, *Momordica* and *Trichosanthes* (Cohen et al. 2015).

New pathotypes and shifts in virulence within the population of *P. cubensis* have been associated with the resurgence of CDM around the world. For example, Lebeda et al. (2013) reported new pathotypes and an increase in the host range of *P. cubensis* following the resurgence of CDM in Czech Republic in 2009. A new pathotype (pathotype 6) that did not infect watermelon but caused severe disease outbreaks on pumpkin and summer squash appeared in Israel in 2002 (Cohen et al. 2003). Similarly, pathotype 5 that was aggressive on squash appeared in Italy in 2002 (Cappelli et al. 2003). Some surveys (Cohen et al. 2015) have indicated the presence of pathotypes 7, 8, 9, and 10 in China, Vietnam, Russia, and India, respectively. Systematic studies on the virulence structure within the population of *P. cubensis* are still lacking in the United States. Earlier work by Thomas et al. (1987), which included only two isolates, indicated the presence of only pathotypes 4 and 5 in the United States. Colucci (2008) later conducted a more comprehensive study on the host range of *P. cubensis* following the 2004 resurgence of CDM and concluded that the pathogen population was very diverse after disease resurgence in the United States. However, no pathotypes were identified, nor was the level of virulence diversity quantified in that study. In addition, a link between changes in virulence of *P. cubensis* and the resurgence of CDM in the United States has not been established.

Knowledge of prevailing pathotypes is essential for deployment of host resistance for effective disease management. In the United States, Cespedes-Sanchez et al. (2015) recently reported no differences in the dominant pathotypes of *P. cubensis* in Michigan between 2010 and 2011. However, no specific pathotypes of *P. cubensis* were identified in that study to determine whether they were different from those reported earlier by Thomas et al. (1987). Documentation of prevailing pathotypes is a useful reference for the study by

Thomas et al. (1987) when determining whether pathotypes other than pathotypes 4 and 5 are present in the United States. In a recent study (Thomas et al. 2017) based on a limited sample size, isolates characterized as pathotypes 1 and 3 were all of the A1 mating type, whereas those characterized as pathotypes 4 and 5 were of the A2 mating type. It would be interesting to determine whether this association between pathotype and mating type holds using a robust set of isolates. Given that host range studies are laborious and time consuming, genotypic markers associated with mating types (Lebeda et al. 2014) could be used to rapidly determine and narrow down potential pathotypes and, hence, the host range of *P. cubensis* within a given field. To further elucidate the pathotype and mating type structure of *P. cubensis*, this study focused on the following specific objectives: (i) establish the current host range of *P. cubensis* collected from various cucurbit hosts, (ii) determine the prevailing pathotypes of *P. cubensis* within the pathogen population, (iii) establish virulence and virulence diversity within the *P. cubensis* population, and (iv) characterize the prevalence and distribution of the mating types of *P. cubensis* within the prevailing pathotypes of *P. cubensis* in the United States.

MATERIALS AND METHODS

Isolate collection and maintenance. In total, 22 isolates of *P. cubensis* generated from single lesions were used in this study. The isolates were obtained from different cucurbit host types that included cucumber, cantaloupe, watermelon, pumpkin, butternut squash, and acorn squash, primarily from the eastern United States (Table 1). All isolates were collected from 2005 to 2013 except Pc1982, which was originally isolated from cantaloupe leaves collected in South Carolina in 1982. Isolate Pc1982 was previously stored at -80°C and maintained by periodic reinoculation on detached cantaloupe leaves under controlled conditions.

Single-lesion isolates were obtained from field samples of infected leaves of cucurbits collected by cooperators in the CDM ipmPIPE disease monitoring network (Ojiambo et al. 2011) and shipped to the Plant Pathology Laboratory at North Carolina State University in Raleigh. Leaves of infected samples were carefully examined and isolates from leaves with discrete lesions were subsequently maintained on their respective hosts in the laboratory as single-lesion isolates. Leaf samples without discrete single lesions were further processed to generate single-lesion isolates using host types from which isolates were originally obtained.

TABLE 1. Source and description of *Pseudoperonospora cubensis* isolates used to determine the current host range and pathotype structure of pathogen population in the eastern United States

Isolate	Host of origin	Host species	Year	State	County
08F1	Acorn squash	<i>Cucurbita pepo</i>	2008	Georgia	Tift
2013F2	Acorn squash	<i>C. pepo</i>	2013	South Carolina	Charleston
2013F11	Acorn squash	<i>C. pepo</i>	2013	New York	Suffolk
D3	Butternut squash	<i>C. moschata</i>	2012	South Carolina	Charleston
2013D2	Butternut squash	<i>C. moschata</i>	2013	Florida	Hillsborough
2013D4	Butternut squash	<i>C. moschata</i>	2013	North Carolina	Johnston
2013D6	Butternut squash	<i>C. moschata</i>	2013	Alabama	Escambia
Pc1982	Cantaloupe	<i>Cucumis melo</i>	1982	South Carolina	Charleston
2013B17	Cantaloupe	<i>C. melo</i>	2013	New York	Ontario
05A1	Cucumber	<i>C. sativus</i>	2005	Michigan	Oceana
08A1	Cucumber	<i>C. sativus</i>	2008	California	Salinas
A11	Cucumber	<i>C. sativus</i>	2012	North Carolina	Johnston
2013A3	Cucumber	<i>C. sativus</i>	2013	Georgia	Worth
2013A10	Cucumber	<i>C. sativus</i>	2013	South Carolina	Charleston
2013A15	Cucumber	<i>C. sativus</i>	2013	Alabama	Macon
2013A18	Cucumber	<i>C. sativus</i>	2013	New Jersey	Salem
2013A19	Cucumber	<i>C. sativus</i>	2013	Ohio	Sandusky
2013A20	Cucumber	<i>C. sativus</i>	2013	Ohio	Medina
2013C3	Giant pumpkin	<i>Cucurbita maxima</i>	2013	North Carolina	Johnston
08C1	Pumpkin	<i>C. maxima</i>	2008	New York	Suffolk
08E1	Watermelon	<i>Citrullus lanatus</i>	2008	Texas	Bryan
2013E1	Watermelon	<i>C. lanatus</i>	2013	Florida	Collier

Sporangia from such lesions were washed off infected leaves using a Preval sprayer (Complete Unit 267; Precision Valve Corporation, Yonkers, NY) and the suspension was used to inoculate whole plants at a concentration of approximately 1×10^4 sporangia/ml. Inoculated plants were bagged with polythene bags and incubated in a growth chamber at 18°C with no light for 24 h. After 24 h, plants were unbagged and incubated at 21 and 18°C (day and night, respectively) for disease development, with a photoperiod of 12 h. Plants were examined daily for symptoms and, when discrete single lesions were visible, plants were bagged again and kept under dark conditions for 24 h to stimulate sporangia production. An individual lesion was subsequently cut from a leaf and collected sporangia were used to inoculate the original host to multiply sporangia of each isolate. To propagate isolates, first or second leaves were placed on sterile, moist paper towels in clear acrylic boxes. Abaxial sides of the leaves were then inoculated with approximately 2×10^4 sporangia/ml and incubated in a growth chamber at 21 and 19°C (day and night, respectively) and a photoperiod of 12 h. All isolates were maintained in the laboratory by propagating them on their respective hosts using the detached-leaf assay until they were ready for use in subsequent experiments.

Host range and pathotypes. Isolates collected in this study were used to determine the host range of *P. cubensis* based on compatibility reaction with cultivated and wild cucurbit hosts belonging to eight genera: *Cucumis* (*Cucumis sativus*, *C. melo* var. *reticulatus*, and *C. melo* var. *conomon*), *Citrullus* (*Citrullus lanatus*), *Cucurbita* (*Cucurbita moschata*, *C. pepo*, and *C. maxima*), *Luffa* (*Luffa cylindrica*), *Lagenaria* (*Lagenaria siceraria*), *Trichosanthes* (*Trichosanthes cucumerina*), *Benincasa* (*Benincasa hispida*), and *Momordica* (*Momordica charantia*) (Table 2). The differential *Cucumis melo* var. *acidulus* used by Thomas et al. (1987) was not included in this study due to lack of seed. To promote germination, seed of *C. melo* var. *reticulatus*, *C. melo* var. *conomon*, *Citrullus lanatus*, *Luffa cylindrica*, and *Lagenaria siceraria* were pregerminated on moist filter papers in glass Petri plates in an incubator at 30°C for 5 to 6 days. Seed of *T. cucumerina* and *B. hispida* were first soaked in warm water for 24 h, whereas seed of *M. charantia* were soaked in water at room temperature for 24 h prior to the pregermination treatment described above. For *T. cucumerina*, tips of the seed area where the radicle emerges were also slightly broken off to facilitate emergence of radicles prior to the pregermination treatment. Seeds of *Cucumis sativus* and all pregerminated seeds were then planted in small plastic cups and placed in the greenhouse at a cycle of 26 and 22°C (day and night, respectively) where all plants were maintained.

Compatibility of isolates with differential hosts was determined using a detached-leaf assay. First and second primary leaves from 4 to 5-week-old plants were placed with the adaxial side on moist paper towels in clear acrylic boxes and inoculated as described

above with 5×10^3 sporangia/ml. A negative control with water-only inoculation was included in the assay. Inoculated leaves were then incubated in a growth chamber at 21 and 18°C under a cycle of 12 h each of light and darkness and monitored for symptoms. Disease severity (DS) (percent leaf area infected) and sporulation were assessed at 5 and 7 days after inoculation, respectively. To assess sporulation, two leaf discs measuring 9 mm in diameter were cut from each leaf and vortexed for 20 min in microcentrifuge tubes containing 500 µl of 50% ethanol to dislodge the sporangia (Cohen et al. 2003). Sporangial counts were then determined using a hemocytometer and expressed as sporangia per square centimeter of leaf tissue. Acrylic boxes with inoculated leaves were arranged in the growth chamber in a completely randomized design and the assay was repeated at least once.

Compatibility of *P. cubensis* with cucurbit hosts has been assessed previously based only on sporulation intensity (Cohen et al. 2003; Lebeda et al. 2012) but also using both sporulation intensity and DS (Salati et al. 2010). Here, both sporulation and DS were used to provide a comprehensive assessment of compatibility reaction as follows: (i) high compatibility = sporulation rate of approximately 5×10^3 sporangia/cm² of leaf tissue and DS $\geq 50\%$, (ii) moderate compatibility = sporulation rate of approximately 3×10^3 sporangia/cm² and $20\% \leq DS < 50\%$, (iii) low compatibility = sporulation rate of approximately 2×10^3 sporangia/cm² leaf tissue and $5\% < DS < 20\%$, (iv) very low compatibility = sparse sporulation and $1\% < DS \leq 5\%$, and (v) incompatible = no evidence of sporulation or disease symptoms. To classify isolates into specific pathotypes, compatibility reactions were grouped into two categories as follows: (i) compatible (+) = highly compatible reaction as described above and (ii) incompatible (–) = moderate, low compatibility, very low compatibility, or an incompatible reaction as described above. Isolates were subsequently classified into pathotypes based on their patterns of compatible and incompatible reaction with the differential host set, composed of eight genera (Cohen et al. 2003, 2015; Lebeda and Gadasová 2002; Thomas et al. 1987).

Mating type determination. The mating type of *P. cubensis* isolates was determined as described by Cohen and Rubin (2012) by pairing of sporangia of an unknown isolate with sporangia of a tester strain (Thomas et al. 2017) with a known mating type. Here, equal proportions (1:1, vol/vol) of sporangial suspension (2×10^4 spores/ml) of the tester and unknown isolate were mixed together and the resultant suspension was used to inoculate detached first true leaves of cantaloupe ('Ananas') or cucumber ('Straight Eight') plants. Cantaloupe and cucumber have been reported as favorable hosts for oospore production (Cohen and Rubin 2012). Plants were grown and maintained in the greenhouse as described above and repeated seeding was conducted to ensure a steady supply of the first true leaves that were used in the assay. The adaxial side of the detached leaves was placed on moist paper towels in clear acrylic boxes,

TABLE 2. Description and source of host types used to determine the reaction of a range of cucurbit host species to *Pseudoperonospora cubensis* under controlled inoculation assays in the United States

Host type	Acronym	Common name	Cultivar	Seed supplier
<i>Benincasa hispida</i>	BH	Wax gourd	Lai Jud	Kitazawa Seed
<i>Citrullus lanatus</i>	CL	Watermelon	Mickylee	Hollar Seed
<i>Cucumis sativus</i>	CS1	Cucumber	Straight Eight	Wyatt-Quarles
<i>C. sativus</i>	CS2	Cucumber	Poinsett 76	Clifton Seed
<i>C. melo</i> var. <i>reticulatus</i>	CMr	Cantaloupe	Ananas	Willhite Seed
<i>C. melo</i> var. <i>conomon</i>	CMc	Pickling melon	Green Striped	Kitazawa Seed
<i>Cucurbita pepo</i> subsp. <i>ovifera</i>	CP1	Summer squash	Dixie	Seminis
<i>C. pepo</i> subsp. <i>ovifera</i>	CP2	Acorn squash	Table Queen	Wyatt-Quarles
<i>C. pepo</i> subsp. <i>pepo</i>	CP3	Pumpkin	Jack-O'-Lantern	Burpee
<i>C. moschata</i>	CMo	Butternut squash	Waltham	Wyatt-Quarles
<i>C. maxima</i>	CMA	Giant pumpkin	Big Max	Wyatt-Quarles
<i>Lagenaria siceraria</i>	LS	Bottle gourd	Nam Tao Yao	Kitazawa Seed
<i>Luffa cylindrica</i>	LC	Sponge gourd	Short	Kitazawa Seed
<i>Momordica charantia</i>	MC	Bitter gourd	Futo Spindle	Kitazawa Seed
<i>Trichosanthes cucumerina</i>	TC	Snake gourd	Buag Ngu	Kitazawa Seed

while the abaxial side of the leaf was spot inoculated on at least 20 different spots with 10 µl of the sporangial mixture. Inoculated leaves were then incubated in a growth chamber at 21°C at 50 to 60% relative humidity under a cycle of 12 h each of light and darkness. At 7 to 10 days postinoculation, 11-mm-diameter leaf discs were cut from infected leaves and clarified for 24 h in ethyl alcohol-acetic acid solution (3:1, vol/vol). Clarified leaf discs were washed three times in deionized water and then examined under a compound microscope (×100 magnification) for the presence of oospores. Unknown isolates were designated as A1 or A2 if oospores were produced in coinoculations with an A2 or A1 tester strain, respectively.

Data analysis. Sporulation intensity and DS data from experimental runs were first tested for homogeneity of variance using GLIMMIX procedure in SAS (version 9.4; SAS Institute, Cary, NC). Homogeneity tests were not significant for both sporulation ($P = 0.1045$) and DS ($P = 0.4072$) and data from experimental runs were combined for subsequent analysis. The effect of isolate and host on sporulation intensity and DS was analyzed using PROC GLIMMIX to determine the degree and extent of heterogeneity in the compatibility of *P. cubensis* with cucurbit hosts.

Virulence was quantified based on (i) number of compatible reactions for an isolate (i.e., virulence factor [v-factor]) and (ii) number of unique compatibility and incompatibility patterns for all isolates (i.e., virulence phenotype [v-phenotype]). In the first set of analysis, virulence complexity of the pathogen population was estimated as described by Andrivon (1994) based on (i) the mean number of v-factors per isolate (C_i) and (ii) the mean number of v-factors per v-phenotype (C_p) as:

$$C_i = \sum_j (p_j \cdot v_j) \quad (1)$$

and

$$C_p = \sum_j (v_j) / N_p \quad (2)$$

in which p_j is the frequency of the j th v-phenotype in the population, with j ranging from 1 to N_p ; v_j is the number of v-factors in the v-phenotype j in the population; and N_p is the number of v-phenotypes identified in the population. In the second set of analyses, diversity of v-phenotypes (Lebeda 1982) was estimated using both Gleason (H_G) and Shannon (H_S) indices, because the former accounts only for richness whereas the latter accounts for both evenness and richness. The indices were calculated as follows:

$$H_G = [(N_p - 1) / \ln N_p] \quad (3)$$

$$H_S = -\sum_j (P_j \cdot \ln P_j) \quad (4)$$

where N_i is number of isolates tested, and p_j and N_p are as defined above. To allow for direct comparison of these diversity estimates with those reported in other studies, relative indices for the Gleason (H_{GR}) and Shannon (H_{SR}) were also calculated as follows:

$$H_{GR} = (N_p - 1) / (N_i - 1) \quad (5)$$

and

$$H_{SR} = H_S / \ln N_i \quad (6)$$

The association of pathotype with mating type among isolates collected in this study was examined using contingency tables based on the frequency of isolates within a pathotype and the number of isolates with the corresponding mating type. Differences in the distribution of A1 and A2 mating type isolates within each pathotype were examined based on Fisher's exact test using PROC FREQ in SAS.

RESULTS

Sporulation intensity and DS. Inoculation of the differential host set with *P. cubensis* isolates collected in this study resulted in

different levels of sporulation intensity ranging from no sporulation to 9.54×10^4 sporangia/cm² of leaf tissue (Table 3). Sporulation intensity was significantly affected by isolate ($F = 10.37$, $P < 0.0001$), differential host ($F = 25.39$, $P < 0.0001$), and the interaction between isolate and differential host ($F = 3.17$, $P < 0.0001$) (Table 4). Sporulation intensity across all differential hosts was significantly ($P < 0.0001$) higher for isolate 2013D6, with a sporulation intensity of 2.09×10^4 sporangia/cm², than for all other isolates tested, except Pc1982 and 2013D2 that had an intensity of 1.66×10^4 and 1.85×10^4 sporangia/cm², respectively. Similarly, sporulation intensity across all isolates was significantly ($P < 0.0001$) higher on CMA that had 2.13×10^4 sporangia/cm² than on all other differential hosts, except CMO and CS1, that had 1.82×10^4 and 2.11×10^4 sporangia/cm², respectively. Inoculation of CMO with isolate 2013D6 resulted in the highest level of sporulation observed among all isolate-differential host combinations. Other isolate-differential combinations that resulted in high levels of sporulation included 2013D2 × CP3 and 2013D6 × CMA, with 6.66×10^4 and 5.81×10^4 sporangia/cm², respectively. DS also differed significantly among isolates ($F = 7.22$, $P < 0.0001$), differential hosts ($F = 30.65$, $P < 0.0001$), and the interaction between isolates and differential hosts ($F = 2.57$, $P < 0.0001$) (Table 4).

Compatibility reactions and pathotypes. A wide range of compatibility reactions ranging from no compatibility to highly compatible were observed when cucurbit host types were inoculated with isolates collected in this study (Table 5). For example, all isolates were highly compatible with CS1, CMr, and CMA, except 2013A20, 2013F11, and 2013D2 that had a very low compatibility with CMr and 2013A10 that had a very low compatibility with CMA. Similarly, all isolates had either an incompatible or a very low compatible reaction with LC, TC, and MC, except 2013A20 and Pc1982 that were highly compatible with TC (Table 5). Approximately 50% of the isolates had a highly compatible reaction with CP1, CP2, CP3, CMO, and CS2, while the remaining isolates had either an incompatible or a very low compatible reaction with these host types.

A wide range of variation in compatibility was evident when sporulation intensity data were grouped into compatible (+) or incompatible (−) reactions to establish pathotypes present in the study (Fig. 1). For example, the differentials CS1, CMA, CMr, and CMC were compatible with >60% of the isolates evaluated in this study. The remaining host differentials had a compatible reaction with 18 to 40% of the isolates evaluated, except host differentials LC, MC, and TC that had a compatible reaction with <10% of the test isolates (Fig. 1). Based on the pattern of compatibility and incompatibility reactions with the differential host set, the isolates were classified into a total of five pathotypes (Table 6). Pathotypes 1 and 3 were the most frequent, constituting 25 to 30% of the isolates evaluated, whereas pathotype 4 was the least frequent, with <10% of the isolates tested, and included Pc1982 (Fig. 2A). None of the isolates collected in this study were classified as pathotype 2.

v-Factors, phenotypes, complexity, and diversity. The number of v-factors per isolate ranged from two to eight, indicating a complex virulence, with an average of 5.05 v-factors present across all isolates examined (Table 6). The most commonly occurring v-factor was v-5, which was present in 41% of all the isolates tested (Table 6). Isolate Pc1982, collected in 1982, had seven v-factors. In total, 13 v-phenotypes were described in this study, with v-phenotypes P2, P3, and P9 being the most frequent and most of the remaining v-phenotypes, including P6, represented by the historical isolate Pc1982, having only a single isolate (Table 6). Most v-phenotypes differed from one another by one or two v-factors.

Virulence complexity as estimated by the mean number of v-factors per isolate (C_i) and mean number of v-factors per v-phenotype (C_p) was 5.05 and 5.23, respectively, for the entire pathogen population studied. Values of C_i and C_p were very similar indicating that approximately 50% of the v-phenotypes in the population were the complex v-phenotypes. Values for H_G and H_S for the population were 3.88

and 2.32, respectively. Relative indices for the two measures of diversity, H_{GR} and H_{SR} , were 0.57 and 0.75, respectively. Given that H_{GR} was slightly higher than 0.5, richness had a large contribution (compared with evenness) to the overall diversity of the *P. cubensis* population in this study.

Association of pathotype with mating type. The distribution of isolates with either A1 or A2 mating type among the five pathotypes differed significantly ($\chi^2 = 20.0$, $P < 0.0001$) for the isolates evaluated in the study (Fig. 2B). For example, pathotypes 1 and 3 were exclusively composed of isolates belonging to the A1 mating type. In contrast, pathotypes 4, 5, and 6 were exclusively composed of isolates of the A2 mating type (Fig. 2B).

DISCUSSION

The virulence structure of *P. cubensis* was examined in this study to determine the level and variation in the compatibility of the pathogen with cucurbit hosts, identify existing pathotypes and determine the diversity of virulence within the pathogen populations in the eastern United States. A high degree of variability in compatibility was

observed between the differential host set and isolates collected from various hosts across the United States. Further, we identified a total of five pathotypes, with three pathotypes being reported for the first time in the United States. Thirteen v-phenotypes were identified, with some phenotypes and pathotypes being more frequent than others and estimates of virulence diversity and complexity being high across the entire pathogen population. This study provides the first comprehensive assessment and documentation of virulence characteristics of *P. cubensis* and serves as a useful foundation to examine the potential role of changes in its virulence structure in the 2004 resurgence of CDM in the United States.

The interaction of *P. cubensis* with cucurbits is very specific and results in clear patterns of compatibility or incompatibility (Thomas et al. 1987) that provide a framework to describe the population and virulence structure of the pathogen. Although *P. cubensis* is known to have a wide host range and to exhibit a high degree of pathogenic variation (Colucci 2008; Lebeda 1992; Lebeda and Cohen 2011; Lebeda et al. 2012), systematic studies on this aspect of the pathogen in the United States were still lacking. In this study, differences in compatibility of *P. cubensis* with cucurbit host types

TABLE 3. Sporulation of *Pseudoperonospora cubensis* on detached leaves of cucurbit host types following inoculation with isolates collected from different cucurbits in the United States^a

Isolate	Sporulation ($\times 10^3$) on inoculated cucurbit host type ^b														
	CS1	CS2	CMr	CMc	CP1	CP2	CP3	CMo	CMA	CL	BH	LC	LS	TC	MC
05A1	16.5 (2.2)	16.6 (16.2)	5.2 (4.8)	7.3 (3.9)	0.0 (0.0)	0.2 (0.2)	0.5 (0.5)	2.9 (2.9)	18.6 (13.6)	1.3 (1.3)	0.0 (0.0)	0.0 (0.0)	19.9 (6.8)	5.0 (5.0)	0.0 (0.0)
08A1	14.5 (1.7)	13.7 (7.2)	6.0 (3.2)	3.1 (0.1)	0.6 (0.6)	0.0 (0.0)	0.3 (0.3)	0.0 (0.0)	4.1 (1.6)	0.0 (0.0)	1.2 (1.2)	0.0 (0.0)	13.1 (4.7)	0.5 (0.1)	0.0 (0.0)
2013A18	25.0 (19.0)	10.9 (3.7)	9.2 (1.1)	13.2 (0.7)	0.0 (0.0)	0.0 (0.0)	0.2 (0.2)	8.1 (6.2)	6.7 (2.0)	0.5 (0.2)	0.0 (0.0)	4.2 (3.3)	10.6 (2.5)	0.0 (0.0)	0.0 (0.0)
2013A19	12.3 (1.7)	7.2 (5.6)	5.5 (0.1)	6.9 (4.7)	5.5 (2.3)	0.0 (0.0)	2.8 (2.8)	0.0 (0.0)	8.8 (0.9)	1.1 (1.1)	2.5 (2.1)	3.6 (2.3)	6.7 (5.1)	3.1 (1.2)	0.3 (0.3)
2013A10	7.8 (1.9)	1.4 (0.5)	15.9 (11.6)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	2.9 (2.9)	0.0 (0.0)	1.7 (1.1)	0.0 (0.0)	1.2 (1.2)	1.7 (1.7)	0.0 (0.0)	3.6 (0.2)	0.0 (0.0)
2013A3	24.7 (15.9)	3.3 (0.8)	7.1 (7.1)	7.0 (3.9)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	11.5 (9.7)	9.2 (3.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.6 (0.3)	0.0 (0.0)	0.0 (0.0)
A11	10.9 (4.4)	13.3 (1.4)	11.5 (2.2)	7.9 (1.4)	1.6 (1.6)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	21.7 (1.7)	0.4 (0.4)	7.0 (2.0)	0.0 (0.0)	24.3 (11.1)	3.1 (3.1)	0.6 (0.6)
2013A15	48.0 (28.4)	9.2 (7.0)	4.8 (3.6)	5.6 (2.5)	5.1 (5.1)	0.0 (0.0)	0.6 (0.3)	0.0 (0.0)	12.9 (2.9)	0.3 (0.0)	4.4 (2.8)	0.3 (0.0)	6.9 (5.0)	0.0 (0.0)	0.6 (0.6)
2013A20	20.4 (1.7)	9.1 (0.6)	2.5 (0.9)	1.6 (0.0)	0.0 (0.0)	0.3 (0.3)	10.5 (8.3)	17.8 (17.8)	29.4 (16.9)	3.3 (2.0)	6.6 (2.2)	0.3 (0.3)	5.3 (4.4)	6.1 (5.4)	0.5 (0.5)
2013B17	48.3 (33.9)	11.5 (7.5)	6.1 (3.9)	3.0 (2.6)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	14.3 (5.6)	0.0 (0.0)	4.9 (0.9)	0.6 (0.6)	3.2 (3.2)	0.3 (0.3)	0.3 (0.3)
2013C3	28.1 (9.4)	14.2 (0.5)	8.4 (3.4)	3.7 (2.2)	2.5 (1.9)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	7.5 (7.2)	0.0 (0.0)	0.8 (0.8)	0.0 (0.0)	1.7 (1.1)	2.8 (2.8)	0.9 (0.3)
08C1	46.7 (15.4)	4.5 (1.1)	6.7 (0.1)	9.0 (9.0)	1.7 (1.7)	0.6 (0.6)	4.4 (3.7)	0.4 (0.4)	12.9 (4.8)	0.3 (0.3)	5.0 (2.8)	4.2 (2.6)	4.8 (6.4)	5.6 (5.6)	1.6 (0.3)
2013E1	7.3 (4.5)	0.0 (0.0)	23.0 (15.2)	5.1 (2.0)	1.7 (1.7)	1.1 (0.5)	9.7 (5.4)	3.0 (3.0)	17.7 (1.4)	8.4 (2.8)	0.6 (-)	3.7 (0.9)	3.2 (1.1)	2.8 (2.5)	0.0 (0.0)
08E1	7.8 (5.05)	0.6 (0.6)	3.6 (2.0)	4.1 (0.6)	15.5 (9.5)	5.0 (2.5)	6.5 (1.5)	1.2 (0.9)	6.1 (2.3)	17.1 (3.7)	14.1 (11.2)	0.0 (0.0)	9.6 (4.3)	1.9 (0.6)	0.0 (0.0)
08F1	6.4 (6.4)	0.0 (0.0)	6.4 (3.9)	4.3 (1.2)	26.8 (5.3)	11.6 (2.3)	25.7 (18.9)	25.4 (2.6)	26.3 (15.7)	2.7 (1.1)	0.9 (0.3)	0.0 (0.0)	1.7 (0.5)	4.5 (0.5)	1.7 (0.2)
D3	32.9 (14.1)	16.4 (16.4)	5.6 (3.1)	4.2 (0.8)	23.6 (12.5)	46.2 (26.6)	12.8 (3.1)	39.5 (2.0)	27.6 (6.7)	4.1 (0.3)	2.6 (2.6)	0.3 (0.0)	1.2 (0.6)	2.0 (0.2)	0.0 (0.0)
2013D4	5.4 (4.5)	0.0 (0.0)	10.1 (1.7)	3.9 (1.4)	34.8 (11.4)	19.3 (11.2)	24.8 (4.2)	31.7 (1.1)	33.7 (13.1)	4.1 (3.1)	4.8 (4.8)	3.4 (2.5)	2.5 (2.5)	3.7 (1.9)	2.0 (1.1)
2013D6	26.6 (21.4)	0.3 (0.3)	16.1 (6.0)	25.0 (15.0)	34.2 (4.6)	16.9 (4.4)	30.3 (6.0)	95.4 (32.4)	58.1 (7.5)	3.6 (2.0)	0.0 (0.0)	0.3 (0.3)	4.0 (2.7)	1.4 (1.4)	0.8 (0.8)
2013F2	9.5 (3.3)	1.1 (1.1)	3.9 (2.0)	7.8 (2.8)	23.7 (8.1)	32.6 (3.9)	27.9 (9.8)	51.3 (15.8)	33.4 (0.9)	9.2 (7.3)	9.5 (2.6)	1.2 (0.3)	1.2 (0.6)	2.2 (0.9)	0.3 (0.0)
2013F11	10.1 (3.9)	0.0 (0.0)	0.2 (0.1)	0.3 (0.3)	35.5 (12.7)	41.9 (24.7)	47.9 (2.9)	53.1 (5.6)	27.5 (1.9)	0.3 (0.0)	1.9 (1.9)	0.9 (0.9)	4.2 (2.3)	3.4 (1.2)	0.9 (0.3)
2013D2	9.2 (7.3)	0.0 (0.0)	0.9 (0.6)	12.2 (6.2)	24.6 (1.8)	28.7 (6.6)	66.6 (12.9)	56.2 (25.0)	55.9 (4.1)	0.8 (0.5)	3.7 (2.2)	0.1 (0.1)	14.1 (5.9)	2.3 (0.5)	1.7 (0.5)
Pc1982	45.6 (2.5)	0.2 (0.2)	43.4 (6.6)	51.6 (7.5)	0.0 (0.0)	1.9 (0.0)	14.1 (5.0)	3.1 (1.2)	36.4 (7.3)	16.1 (5.5)	0.0 (0.0)	1.2 (1.2)	18.9 (4.8)	12.6 (0.5)	3.9 (0.2)

^a Sporulation values are number of sporangia produced per square centimeter of leaf tissue and are means of two to four replications, except for 2013E1 on BH, that was not replicated. Italicized values shown in parenthesis are standard errors of the mean for sporulation.

^b Cucurbit host types are defined based on acronyms described in Table 2.

were observed and all isolates evaluated were compatible with the susceptible cultivar of *C. sativus*, as reported in previous studies (Cohen et al. 2003; Lebeda and Gadasová 2002; Thomas et al. 1987). However, unlike the susceptible cultivar of *C. sativus*, approximately 50% of the isolates evaluated had either a very low compatibility or were incompatible with *C. sativus* 'Poinsett 76'. Poinsett 76 is a resistant cultivar developed from PI 197087 in the 1950s. It contains the *dm-1* gene that had been effective until the resurgence of CDM in 2004 (Criswell et al. 2010; Cohen et al. 2015). *Cucurbita maxima* had the second highest number of compatible reactions, where all isolates except 2013A10 resulted in a compatible reaction. In the study by Thomas et al. (1987), isolates of *P. cubensis* that were incompatible with other *Cucurbita* spp. were also incompatible or lowly compatible with *C. maxima*. The observation by Thomas et al. (1987) is in contrast to results from the present study and studies conducted in the Czech Republic (Lebeda and Gadasová 2002) and Malaysia (Salati et al. 2010), where most of the isolates that were incompatible with other *Cucurbita* spp. were highly compatible with *C. maxima*. Differences in the pathogenic profile of isolates and host genetic makeup may explain the high compatibility with *C. maxima* observed in the present study, unlike in the study by Thomas et al. (1987). Similar to other studies conducted in Israel (Lebeda and Cohen 2011) and the Czech Republic (Lebeda and Widrechner 2003; Lebeda et al. 2013), *Luffa cylindrica* and *M. charantia* exhibited the lowest level of

compatibility with our test isolates. However, *L. cylindrica* and other *Luffa* spp. have been reported to be highly compatible with *P. cubensis* isolates in China (Cohen et al. 2015) and India (Jamadar and Desai 1999). Thus, the *P. cubensis* populations in the United States or Czech Republic may be composed of a pathotype that is distinctively different from that in the pathogen populations in India or China.

As is the case for many other oomycete plant pathogens, description of pathotypes provides a useful means to characterize and, thereby, understand the virulence structure of *P. cubensis* within a given population (McDonald and Linde 2002). Following the 2004 resurgence of CDM in the United States, Colucci (2008) observed a broader virulence pattern of *P. cubensis* than previously reported by Thomas et al. (1987) but did not describe the pathotypes or their distribution. In the present study, five pathotypes were described from a total of 22 isolates evaluated. Three of these pathotypes—pathotype 1, which includes 1(A); pathotype 3, which includes 3(B) and 3(C); and pathotype 6, which includes 6(A)—had previously not been reported in the United States. Pathotype 6 was reported for the first time following the resurgence of CDM in Israel in 2002 (Cohen et al. 2003). Pathotypes 1 and 3, which were previously reported to be present only in East Asia and Israel (Cohen et al. 2003; Thomas et al. 1987), were also the most predominant in the present study. Pathotype 4 and 5 isolates were previously present in the United States, with all *Cucurbita* spp. being compatible with pathotype 5 but not with pathotype 4 isolates (Thomas et al. 1987). However, although pathotype 4 isolates in the present study were not compatible with *C. pepo* subsp. *ovifera*, they were compatible with *C. pepo* subsp. *pepo*. This suggests that a specific interaction between *P. cubensis* and *Cucurbita* spp. exists at the host subspecies level that may need to be considered in selecting host types to include in a differential set when determining pathotypes of *P. cubensis*. This partly supports an earlier hypothesis by Lebeda et al. (2006) on the existence of *P. cubensis* races on various genera and species of cucurbits and the importance of their specification. This assumption was recently confirmed by detailed studies on virulence variability of *P. cubensis* on *Cucumis melo* (Lebeda et al. 2016b) and *Cucurbita* spp. (Lebeda et al. 2016a).

TABLE 4. Summary of analysis of sporulation intensity and disease severity on inoculated host differentials to determine compatibility of *Pseudoperonospora cubensis* with cucurbit host types

Source	ndf ^a	ddf ^a	Sporulation		Disease severity	
			<i>F</i>	Pr > <i>F</i>	<i>F</i>	Pr > <i>F</i>
Isolate	21	325	10.37	0.0001	7.22	0.0001
Differential	14	325	25.39	0.0001	30.65	0.0001
Isolate × differential	280	325	3.17	0.0001	2.57	0.0001

^a Abbreviations: ndf = numerator degrees of freedom and ddf = denominator degrees of freedom.

TABLE 5. Compatibility of *Pseudoperonospora cubensis* with a range of cucurbit host types under controlled conditions following inoculation with isolates collected from different cucurbits across the United States

Isolate	Cucurbit host type ^a														
	CS1	CS2	CMr	CMc	CP1	CP2	CP3	CMo	CMa	CL	BH	LC	LS	TC	MC
05A1	+++	+++	+++	+++	—	±	—	±	+++	±	—	—	+++	±	—
08A1	+++	+++	+++	++	—	—	—	—	+++	—	±	—	+++	—	—
2013A18	+++	+++	+++	+++	—	—	±	±	+++	±	—	±	+++	—	—
2013A19	+++	+++	+++	+++	—	—	±	—	+++	±	—	±	+++	—	—
2013A10	+++	±	+++	—	—	—	±	—	±	—	±	±	±	—	—
2013A3	+++	±	+++	+	—	—	—	±	+++	—	—	—	±	—	—
A11	+++	+++	+++	+++	—	—	—	—	+++	—	+++	—	+++	+	—
2013A15	+++	+++	+++	+++	+++	—	±	±	+++	—	++	—	+++	—	—
2013A20	+++	+++	+	±	—	±	±	++	+++	++	+++	—	+++	+++	±
2013B17	+++	+++	+++	++	±	—	—	±	+++	—	+	—	++	—	—
2013C3	+++	+++	+++	++	+	—	—	—	+++	—	—	—	+	+	±
08C1	+++	+++	+++	++	±	—	+	—	+++	—	++	±	++	±	±
2013E1	+++	±	+++	+++	±	±	+++	±	+++	+++	—	±	+	+	±
08E1	+++	±	++	±	++	++	+++	±	+++	+++	+++	—	+++	±	—
08F1	+++	—	+++	+	+++	+++	+++	+++	+++	+	±	—	—	++	—
D3	+++	+	+++	+++	+++	+++	+++	+++	+++	++	±	—	±	±	±
2013D4	+++	—	+++	+++	+++	+++	+++	+++	+++	+++	++	±	+	+	±
2013D6	+++	—	+++	+++	+++	+++	+++	+++	+++	++	—	±	++	±	—
2013F2	+++	±	+++	+++	+++	+++	+++	+++	+++	+++	+++	±	±	±	—
2013F11	+++	—	±	±	+++	+++	+++	+++	+++	±	±	±	++	++	±
2013D2	+++	—	+	+++	+++	+++	+++	+++	+++	±	±	—	+++	+	±
Pc1982	+++	—	+++	+++	—	±	+++	±	+++	+++	—	±	+++	+++	+

^a Cucurbit host types are defined based on acronyms described in Table 2. Compatibility reactions are as follows: +++ = high compatibility, with a sporulation rate of approximately 5×10^3 sporangia/cm² of leaf tissue and disease severity (DS) $\geq 50\%$; ++ = moderate compatibility, with a sporulation rate of approximately 3×10^3 sporangia/cm² leaf tissue and $20\% \leq DS < 50\%$; + = low compatibility, with a sporulation rate of approximately 2×10^3 sporangia/cm² leaf tissue and $5\% < DS < 20\%$; ± = very low compatibility, with sparse sporulation; and — = incompatible, with no evidence of sporulation or disease symptoms.

A distinct pattern in the distribution of pathotypes across the United States was evident in this study. Most of the isolates collected from the northern states (Michigan, New Jersey, New York, and Ohio) were either pathotype 1 or pathotype 3, whereas isolates collected from the southern states (Alabama, Florida, Georgia, North Carolina, South Carolina, and Texas) were diverse and belonged to all five pathotypes reported in this study. The diversity of pathotypes in southern states could be due to the proximity of these states to the overwintering sources in southern Florida and along the edge of the Gulf of Mexico, where a diverse range of cucurbits are cultivated year round. Overwintering hosts and seasonally cultivated cucurbit species exert different degrees of

selection pressure that result in differences in prevailing pathotypes in specific geographic areas (Thomas et al. 1987). A high genetic diversity has also been detected in *P. cubensis* populations in the southern states of Georgia and North Carolina (Quesada-Ocampo et al. 2012). A closer examination of the compatibility reactions shows that isolates belonging to pathotypes 1 and 3 were compatible with *C. sativus* Poinsett 76, whereas isolates belonging to all other pathotypes exhibited either an incompatible or a very lowly compatible reaction with Poinsett 76. Given that Poinsett 76 was a highly resistant cultivar (Criswell et al. 2010) and pathotypes 1 and 3 had not been reported in the United States prior to 2004, the breakdown of resistance in Poinsett 76 and other resistant cucumber cultivars that resulted in the resurgence of CDM in the United States may have been due to the introduction of an aggressive strain of pathotype 1 or 3.

Pathotypes of *P. cubensis* in the United States have not been characterized following the initial study by Thomas et al. (1987). Thus, the host differential set developed by Thomas et al. (1987) serves a useful baseline for establishing whether new pathotypes are present in the current population of *P. cubensis* in the United States. Unavailability of some differentials (e.g., *C. melo* var. *acidulous*) used earlier (Thomas et al. 1987) led to some isolates not grouping perfectly within pathotypes described by Thomas et al. (1987). For example, isolate 2013A3 was compatible with *C. sativus* and *C. melo* var. *reticulatus* and would be designated as pathotype 1 based on Thomas et al. (1987). However, 2013A3 was compatible with *C. maxima*, unlike in the study by Thomas et al. (1987), where pathotype 1 isolates were only lowly incompatible with *C. maxima*. The high sporulation cutoff value used by Thomas et al. (1987) may partly explain differences observed between the two studies. Compatibility of *C. maxima* with several *P. cubensis* isolates based on the sporulation cutoff used in the present study has also been reported elsewhere (Lebeda and Gadasová 2002; Lebeda and Widrechner 2003; Lebeda et al. 2013). Thus, rather than assign 2013A3 a new pathotype, that isolate was designated as pathotype

Fig. 1. Variation in the susceptibility of cucurbit host types used as differentials to establish the virulence structure of *Pseudoperonospora cubensis* in the United States. Each bar represents the proportion of isolates with a compatible reaction relative to the total number of isolates examined.

TABLE 6. Pathotype designation, virulence characteristics, and mating type of *Pseudoperonospora cubensis* isolates collected from different cucurbits across the United States

Isolate	Host differential ^a												PP ^b	Vf ^c	Vp ^d	MT ^e
	CS1	CMr	CMc	CL	CP2	CMa	CMo	LS	LC	MC	BH	TC				
2013A10	+	+	—	—	—	—	—	—	—	—	—	—	1	2	P1	A1
2013A3	+	+	—	—	—	+	—	—	—	—	—	—	1 (A)	3	P2	A1
2013B17	+	+	—	—	—	+	—	—	—	—	—	—	1 (A)	3	P2	A1
2013C3	+	+	—	—	—	+	—	—	—	—	—	—	1 (A)	3	P2	A1
08C1	+	+	—	—	—	+	—	—	—	—	—	—	1 (A)	3	P2	A1
05A1	+	+	+	—	—	+	—	+	—	—	—	—	3 (A)	5	P3	A1
08A1	+	+	+	—	—	+	—	+	—	—	—	—	3 (A)	5	P3	A1
2013A18	+	+	+	—	—	+	—	+	—	—	—	—	3 (A)	5	P3	A1
2013A19	+	+	+	—	—	+	—	+	—	—	—	—	3 (A)	5	P3	A1
2013A15	+	+	+	—	—	+	—	+	—	—	—	—	3 (A)	5	P3	A1
A11	+	+	+	—	—	+	—	+	—	—	+	—	3 (B)	6	P4	A1
2013A20	+	—	—	—	—	+	—	+	—	—	+	+	3 (C)	5	P5	A1
Pc1982	+	+	+	+	—	+	—	+	—	—	—	+	4	7	P6	A2
2013E1	+	+	+	+	—	+	—	—	—	—	—	—	4	5	P7	A2
08E1	+	—	—	+	—	+	—	+	—	—	+	—	4 (A)	5	P8	A2
D3	+	+	+	+	+	+	+	—	—	—	—	—	5	7	P9	A2
2013D4	+	+	+	+	+	+	+	—	—	—	—	—	5	7	P9	A2
2013D6	+	+	+	+	+	+	+	—	—	—	—	—	5	7	P9	A2
2013F2	+	+	+	+	+	+	+	—	—	—	+	—	5 (A)	8	P10	A2
2013D2	+	—	+	—	+	+	+	+	—	—	—	—	6	6	P11	A2
08F1	+	+	—	—	+	+	+	—	—	—	—	—	6 (A)	5	P12	A2
2013F11	+	—	—	—	+	+	+	—	—	—	—	—	6 (B)	4	P13	A2

^a Cucurbit host differentials are defined based on acronyms described in Table 2.

^b Proposed pathotype designation is based on the pattern of compatibility reaction between an isolate and the host differential set, where + = highly compatible reaction and — = moderate, low, and very low compatibility or incompatible reaction based on the compatibility scheme originally developed by Thomas et al. (1987) and later expanded by Lebeda and Widrechner (2003) and Cohen et al. (2015). Letters in parenthesis refer to a variant of a pathotype that largely conforms to the Thomas et al. (1987) model but differs in one or two compatibility reactions.

^c Virulence factor of an isolate based on the number of compatible reactions with the host differential set.

^d Virulence phenotype of an isolate based on a unique pattern of compatible or incompatible (i.e., + or —) reactions with the differentials.

^e Mating type.

1A (i.e., a variant of pathotype 1 that is compatible with *C. maxima*). This approach was adopted to assign pathotypes to isolates that did not perfectly fit the Thomas et al. (1987) model. Studies conducted on *P. cubensis* populations in the Czech Republic (Lebeda and Gadasová 2002) and Malaysia (Salati et al. 2010) have also reported a lack of perfect fit with the pathotype model proposed by Thomas et al. (1987). Based on the differential set used in the present study, Lebeda and Gadasová (2002) observed that only 1 of their 22 isolates tested corresponded to the pathotype model of Thomas et al. (1987). This was attributed to the limited taxonomic range of the differential set used by Thomas et al. (1987), making it unable to capture the extent of virulence variability within *P. cubensis* (Lebeda and Widrechner 2003). Nonetheless, the pathotypes described in the present study serve as a useful working framework for subsequent studies on the structure and variability of virulence of *P. cubensis* in the United States.

The level of virulence of all isolates examined in this study was moderate, with a mean v-factor of 5.1 across the entire population. Although virulence of *P. cubensis* isolates in the United States has previously been examined (Colucci 2008), the level of virulence was not quantified in that study and, thus, we are unable to compare our findings with those reported by Colucci (2008). Additionally, the level of virulence observed in the present study is comparatively lower than that reported for *P. cubensis* in the Czech Republic, where the pathogen population has been reported to have a high level of virulence, with a mean v-factor of 9.0 (Lebeda et al. 2013). In the present study, v-phenotypes, complexity, and diversity were

analyzed to determine differences in the *P. cubensis* population in the United States. Thirteen v-phenotypes were identified and it is likely that v-phenotypes P6, P7, P8, P9, and P10 were present in the United States prior to 2004 because they were identified as pathotypes 4 and 5 described earlier by Thomas et al. (1987). All the other v-phenotypes were not associated with previously described pathotypes and could be considered new v-phenotypes. In addition, v-phenotypes P1 and P2 (pathotype 1) and P3, P4, and P5 (pathotype 3) were compatible with Poinsett 76 and they may be associated with the resurgence of CDM in 2004. Diversity estimates indicated that virulence diversity within the *P. cubensis* population in the United States is high. Although virulence diversity estimates for *P. cubensis* have not been reported prior to this study, the normalized estimate for Shannon index of diversity reported here is comparable to that of *Phytophthora infestans* ($H_{SR} = 0.72$) infecting potato and tomato in the United States (Andrison 1994). This high virulence diversity is consistent with the high virulence variability observed in this study. High virulence variation of *Pseudoperonospora cubensis* has also been reported in the Czech Republic (Lebeda et al. 2013). Although specific estimates of virulence diversity were not presented in the study by Lebeda et al. (2013), 67 pathotypes were determined from a total of 389 isolates of *P. cubensis*. Further, the mean virulence complexity ($C_i = 5.05$ and $C_p = 5.23$) observed in the present study was high, with v-factors per isolate and v-factors per v-phenotype equally contributing to virulence complexity of *P. cubensis*. The high virulence variability observed in this study verifies the genetic flexibility of *P. cubensis* and its ability to evolve and adapt more rapidly to fungicides and changes in cucurbit hosts (Quesada-Ocampo et al. 2012).

The pathotypes of isolates evaluated in this study were significantly associated with the mating type of the corresponding isolates. Specifically, all pathotype 1 and 3 isolates were of the A1 mating type, whereas pathotype 4, 5, and 6 isolates were of the A2 mating type. Molecular studies conducted on *P. cubensis* in the Czech Republic showed that isolates sampled before the shift in virulence in 2009 exhibited the genotype of the subspecies of clade II, whereas isolates sampled after the shift in virulence exhibited a genotype of the subspecies of clade I (Kitner et al. 2015). It was suggested that genotypes within clade II, probably indigenous to East Asia, were of the A1 mating type, whereas genotypes in clade I were the A2 mating type of *P. cubensis* (Lebeda et al. 2014). Isolates belonging to pathotypes 1 and 3 were also of the A1 mating type and had not been previously reported in the United States. Given that pathotype 1 and 3 isolates were indigenous to East Asia, it is highly possible that they were introduced from East Asia to the United States via Europe (Runge et al. 2011) through anthropogenic activities. In addition, because only isolates of pathotypes 1 and 3 were compatible with Poinsett 76, which was resistant to CDM until 2004, the findings in Kitner et al. (2015) provide additional support that pathotype 1 and 3 isolates may have been responsible for the resurgence of CDM in the United States in 2004. New pathotypes of *P. cubensis* that are aggressive and cause severe epidemics on *Cucurbita* spp. and *Citrullus lanatus* have also been reported since 2009 in the Czech Republic (Kitner et al. 2015; Lebeda et al. 2012, 2014). These new pathotypes have been linked with an increase in virulence within the pathogen population in the Czech Republic, and most of the isolates belonging to these new pathotypes also contain signatures of sexual recombination (Kitner et al. 2015). Although genetic signatures of pathotype 1 and 3 isolates (A1 isolates) reported in this study were not examined, it is also possible that a recombinant F1 heterozygote of the A1 mating type that was fit and able to survive and rapidly spread across the eastern states may have been responsible for the resurgence of CDM in the United States.

The high pathogenic variability observed in this study indicates that *P. cubensis* has the genetic plasticity to evolve rapidly. Recently, the pathogen has also been shown to have the ability to reproduce sexually in the United States (Thomas et al. 2017). The latter characteristic is expected to facilitate the rapid evolution (McDonald and Linde 2002) of *P. cubensis*, leading to a high risk for

Fig. 2. Frequency distribution of pathotypes of *Pseudoperonospora cubensis* based on isolates collected from various cucurbit host types in the United States. **A**, Frequency distribution of all *P. cubensis* isolates within each pathotype and **B**, frequency distribution of isolates of the A1 and A2 mating type within each pathotype of *P. cubensis*.

developing resistance to available fungicides and less durability of resistance in new cultivars that may be deployed for controlling CDM. Indeed, reduced efficacy for specific fungicides used to control CDM in the United States has been reported (Adams and Quesada-Ocampo 2014; Ojiambo et al. 2010). Thus, it is unlikely that resistance alone in cucurbit cultivars will be effective to control CDM. An integrated approach involving a combination of timely application of fungicides, rotation of fungicides with different chemistries, and introduction of cultivars with new resistance genes will be required for effective disease management.

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