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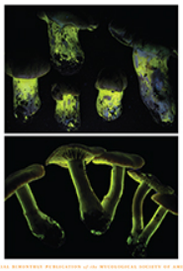

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Botrytis euroamericana, a new species from peony and grape in North America and Europe

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ABSTRACT

A novel species of *Botrytis* isolated from peony in Alaska, USA, and grape in Trento District, Italy, was identified based on morphology, pathogenicity, and sequence data. The grape and peony isolates share sequence homology in the glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), heat shock protein 60 (*HSP60*), DNA-dependent RNA polymerase subunit II (*RPB2*), and necrosis- and ethylene-inducing protein 1 and 2 (*NEP1* and *NEP2*) genes that place them in a distinct group closely related to *B. aclada*, a globally distributed pathogen of onions. Genetic results were corroborated with morphological and pathogenicity trials that included two isolates of *B. cinerea* and two isolates of *B. paeoniae* from peony in Alaska and one isolate of *B. aclada*. The authors observed differences in colony and conidia morphology and ability to cause lesions on different host tissues that suggest that the grape and peony isolates represent a distinct species. Most notably, the grape and peony isolates did not colonize onion bulbs, whereas *B. aclada* readily produced lesions and prolific sporulation on onion tissue. The new species *Botrytis euroamericana* is described herein.

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Paeonia; species recognition; *Vitis*; taxonomy

INTRODUCTION

The genus *Botrytis* includes more than 30 species of cosmopolitan, economically important plant pathogenic fungi, many of which are considered host specific. Together, the putatively host-specific *Botrytis* species along with the omnivorous *B. cinerea* infect more than 1400 monocot and dicot host plant species (Elad et al. 2016). *Botrytis* species have a long history of taxonomic investigation based on morphology; however, infraspecific variation of many *Botrytis* species is incompletely documented (Beever and Weeds 2007). In addition to the classical *Botrytis* species listed by Hennebert (1973), which was the starting point for modern *Botrytis* taxonomy based on multiple-gene genealogies and genetic marker data, other species have been recently described (Fournier et al. 2005; Zhang et al. 2010a, 2010b, 2016; Walker et al. 2011; Li et al. 2012; Leroch et al. 2013; Ferrada et al. 2016; Liu et al. 2016; Saito et al. 2016; Rupp et al. 2017).

Today, the most widely utilized standard in *Botrytis* taxonomy for species delineation involves implementation of the phylogenetic species concept (Taylor et al. 2000) using multigene genealogies with the "housekeeping" genes glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), heat shock protein 60 (*HSP60*), and DNA-dependent RNA polymerase subunit II (*RPB2*) (Staats et al. 2005) and the necrosis- and ethylene-inducing protein genes *NEP1* and *NEP2* (Staats et al. 2007; Walker 2016). The internal transcribed spacer (ITS) region of the nuc rDNA (ITS1-5.8S-ITS2 = ITS) is useful to discriminate *Botrytis* from other fungi in Sclerotiniaceae, but it is not phylogenetically informative within *Botrytis* because of a variability within this region (Beever and Weeds 2007; Walker 2016).

We previously presented information of the morphology, pathogenicity, and phylogenetic relatedness of a single isolate of *Botrytis*, hereafter referred to as "B83," found on withered grapes in Italy and speculated that this isolate represented a new species (Lorenzini and Zapparoli 2014). However, at the time, there were insufficient isolates to fully characterize this species. While conducting surveys on peony (*Paeonia* spp.) in Alaska, USA, we found additional isolates that shared sequence similarity with B83. In this paper, we show that the isolates found in North America also share morphological characteristics and pathogenic traits

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Supplemental data for this article can be accessed on the publisher's Web site.

with isolate B83 from Europe and propose that these isolates represent a new species that occurs on multiple hosts on two continents, described below as *B. euroamericana*.

MATERIALS AND METHODS

Sample collection.—Symptomatic aboveground peony tissues including stems, leaves, and flower buds were collected at commercial peony farms during the 2013 and 2014 growing seasons in Alaska, USA. Twenty-five farms were surveyed in 2013 and 26 farms were surveyed in 2014, with some overlap in farms between years. Farms were located throughout the Fairbanks North Star, Matanuska-Susitna, and Kenai Peninsula boroughs. Tissue from the margin of symptomatic tissue was excised, surface sterilized in a 1% NaOCl solution for 30 s, rinsed twice in sterile water, and plated onto potato dextrose agar (Difco Dehydrated Culture Medium; BD, Franklin Lakes, New Jersey) amended with streptomycin and chloramphenicol (PDA+s/c) at 10 mg L^{-1} each. All isolates were hyphal tipped to ensure only one organism was present in the culture for further analysis. Collection date and location data for isolates used in further analysis in this study are described in TABLE 1.

DNA extraction and PCR.—Hyphal-tipped fungal cultures were transferred onto PDA+s/c overlaid with sterile cellophane membrane (Bio-Rad, Hercules, California) and grown up at room temperature and natural light conditions. Then 10–20 mg of mycelium was scraped off the cellophane into 2.0-mL Eppendorf tubes that contained 3.5-mm glass beads (BioSpec Products, Bartlesville, Oklahoma) to aid in homogenization. Tubes were frozen at −80 C for storage until DNA extraction. Tubes of mycelium were removed from the freezer and homogenized (FastPrep-24; MP Biomedicals, Solon, Ohio) until mycelium resembled a paste. DNA was extracted from homogenized mycelium using the Gentra Puregene Tissue Kit (Qiagen, Redwood City, California) as per the manufacturer's instructions.

Polymerase chain reaction (PCR) was used to amplify the partial sequence of the *G3PDH* gene of selected isolates using primers from Staats et al. (2005), and the resulting product was sequenced using the same primers. Because of the sequence similarity of two isolates (hereafter referred to as AK10 and HA06) to *Botrytis* sp. B83 (Lorenzini and Zapparoli 2014), the partial sequences of the *HSP60, RPB2* (Staats et al. 2005), *NEP1*, and *NEP2* (Staats et al. 2007) genes were subsequently amplified and sequenced. Additional *B. paeoniae* (HA11 and GBG22) and *B. cinerea* (MS05 and HA08) isolates collected from peony in Alaska and an isolate of *B. aclada* (BA5) collected in central Washington (du Toit et al. 2004) (TABLE 1) were also sequenced to confirm their identities for use in morphological and pathogenicity studies. Primer pairs were selected for each isolate according to its genetic clade as described in Staats et al. (2005); however, some of the reactions were performed using primers synthesized without M-13 universal tags (SUPPLEMENTARY TABLE 1). PCR conditions for all reactions were as described in Staats et al. (2005, 2007) and Lorenzini and Zapparoli (2014). All PCR amplifications were carried out in 50-μL reaction that contained 2–27 ng genomic DNA, 1× buffer (Genescript, Nanjing, China), 0.2 μM of each dNTPs (Genescript), 0.1 mg mL^{-1} bovine serum albumin (BSA; New England Biolabs, Ipswich, Massachusetts), 0.2 μM of each primer (Bioneer, Daejeon, Republic of Korea), and 3 U Taq DNA Polymerase (Genescript). PCR products were cleaned up with ExoSAP-IT (Affymetrix, Santa Clara, California) as per the manufacturer's instructions and

Table 1. Collection information for *Botrytis* spp. isolates used in phylogenetic, morphological, and pathogenicity trials in this study.

Species	Isolate code	Host	Collection location	Collection date	Collector
B. euroamericana	AK10	*Paeonia* × *lactiflora*	North Pole, Fairbanks North Star Borough, Alaska, USA	Jul 2013	G.A. Chastagner
	B83	*Vitis vinifera*	Valle dei Laghi, Trento District, Provincia Autonoma di Trento, Italy	Oct 2011	M. Lorenzini and G. Zapparoli
	HA06	*Paeonia* × *lactiflora*	Trapper Creek, Matanuska-Susitna Borough, Alaska, USA	Aug 2014	A.R. Garfinkel and G.A. Chastagner
B. cinerea	HA08	*Paeonia* × *lactiflora*	Trapper Creek, Matanuska-Susitna Borough, Alaska, USA	Aug 2014	A.R. Garfinkel and G.A. Chastagner
	MS05	*Paeonia* × *lactiflora*	Soldotna, Kenai Peninsula Borough, Alaska, USA	Aug 2014	A.R. Garfinkel and G.A. Chastagner
B. paeoniae	HA11	*Paeonia* × *lactiflora*	Trapper Creek, Matanuska-Susitna Borough, Alaska, USA	Aug. 2014	A.R. Garfinkel and G.A. Chastagner
	GBG22	*Paeonia* × *lactiflora*	Fairbanks, Fairbanks North Star Borough, Alaska, USA	Aug. 2014	A.R. Garfinkel and G.A. Chastagner
B. aclada	BA5	Onion seed	Columbia Basin, Washington, USA	2002	L.J. du Toit

sequenced in both directions (Genewiz, South Plainfield, New Jersey) using the same primers used in amplification.

Phylogenetic analysis.—Consensus partial sequences of the *G3PDH, HSP60, RPB2, NEP1*, and *NEP2* genes were developed using Geneious 8.1.4 (Kearse et al. 2012) from forward and reverse sequences for all collected isolates. Gene sequences from this study were aligned with those from type *Botrytis* specimens and outgroups *Monilinia fructigena* and *Sclerotinia sclerotiorum* acquired from GenBank (SUPPLEMENTARY TABLE 2) using Clustal W implemented within MEGA 6.06 (Tamura et al. 2013). The default alignment parameters were used (gap opening penalty at 15, gap extension penalty at 6.66, IUB DNA weight matrix, 0.5 transition weight, and 30% delay divergent cutoff). With the exception of *B. fragariae* sequences that were often shorter than the sequences for the rest of the species used in analysis, sequences were trimmed to equal lengths for all loci.

All sequences were subject to phylogenetic analysis individually and in combined data sets using both neighbor joining (NJ) (maximum composite likelihood method) (Tamura et al. 2004) and maximum likelihood analysis (Kimura 1980; Tamura and Nei 1993) executed in MEGA. Maximum likelihood substitution models were chosen for each individual and combined data set based on the Bayesian information criterion (BIC). The combined data set maximum likelihood tree for the *G3PDH, HSP60*, and *RPB2* genes were made using the Tamura-Nei model with gamma-distributed and invariant sites. The tree for the *NEP1* and *NEP2* combined data set were constructed using the Tamura-Nei model with gamma-distributed substitution rates. Trees from all analyses were inferred with 1000 bootstrap replicates, and all positions with less than 95% site coverage were eliminated from analysis. Alignments and trees for both individual and concatenated data sets were deposited in TreeBASE as study number S21156.

Pathogenicity of* Botrytis *spp.—Isolates B83, AK10, HA06, HA11, GBG22, MS05, HA08, and BA5 were tested for their ability to cause lesions on grape foliage (*Vitis vinifera* 'Interlaken'), yellow onion (*Allium cepa*) bulbs, common bean leaves (*Phaseolous vulgaris* 'Blue Lake Bush'), and peony leaves (*Paeonia* hybrid 'Kansas'). Isolates were transferred onto malt extract agar (MEA) and grown for 5 d in an incubator set at 25 C 12 h light/12 h dark. Then 5-mm plugs were cut from the edge of the colony and placed mycelium-side down onto surface sterilized plant tissues (0.2% NaOCl for 5 min and rinsed twice with sterile water). Tissue either remained unwounded or was wounded with a prick of a sterile minutien pin (Fine Science Tools, Foster City, California) prior to inoculation. A mock inoculation consisting of an MEA plug without fungal tissue was used as a control. Inoculated and mock-inoculated tissues were placed in a clear plastic bin on a wire rack suspended over moistened vermiculite. After placing a lid on the bin, the bin was placed into a clear plastic bag to maintain high humidity and incubated under the same conditions in which the inoculum was produced. Inoculation experiments were performed twice, with five replicates per isolate-wounding combination per trial. Data on lesion development were taken 3–7 d post inoculation (DPI), depending on experiment. The plugs were removed, the diameters of the lesions were measured in two perpendicular directions, and an average was recorded. Isolations for were performed (as described above) to confirm colonization of the plant tissue.

Morphological characterization.—Isolates AK10, HA06, B83, HA08, MS05, GBG22, HA11, and BA5 were used in morphological assays. Colony morphology, sclerotial, and conidial characteristics were assessed on potato dextrose agar (PDA; Oxoid, Basingstoke, UK), malt extract agar (MEA; 20 g L^{-1} malt extract, 20 g L^{-1} glucose, 1 g L^{-1} peptone, 20 g L^{-1} agar), and hay infusion agar (HAY; dried hay composed of ca. 50% *Poa annua* and 50% *Trifolium pratense* with weeds; Atlas 2010). For each isolate, a mycelial agar plug (5 mm diam) was removed from the colony margin of a 5-d-old PDA culture and transferred to Petri dishes (90 mm diam) containing 20 mL PDA, MEA, or HAY. Three replicates were used for each isolate. The plates were incubated at 8, 15, 20, and 25 C for 40 d in the dark. Twenty sclerotia from each strain were randomly measured from plates incubated at 8, 15, and 20 C following the 40-d incubation period. Sclerotia were observed with a stereomicroscope (Leica EZ4D; Leica Microsystems, Wetzlar, Germany) and a light microscope (Leica DM750).

To determine the radial growth rate (RGR; mm d^{-1}), a mycelial plug (5 mm diam) from a PDA culture was placed in the center of Petri dishes (90 mm diam) containing 20 mL PDA, MEA, HAY, or Czapek agar (CzA; K_2HPO_4 10 g L^{-1}, $FeSO_4{\cdot}7H_2O$ 0.01 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.5 g L^{-1}, KCl 0.5 g L^{-1}, $NaNO_3$ 3.0 g L^{-1}, sucrose 30.0 g L^{-1}, agar 15.0 g L^{-1}). The analysis

was performed with three replicate plates for each isolate. The plates were incubated at 20 and 25 C and the colony diameter for each isolate was measured daily for a 7-d period. Experiments were performed twice.

To assess macroconidia and macroconidiophore characteristics, the cultures were incubated for 15 d on PDA or water agar (WA; 2% $w\ v^{-1}$ water agar) at 20 C under 12 h light/12 h dark to promote sporulation. Sporulating structures were mounted on slides, and observations were made with light microscopy (Leica DM750) with camera attached. Length and width of 50 macroconidia (at 1000×) and 10 macroconidiophores (at 400×) from each isolate were measured. The conidial structures of AK10, HA06, and B83 strains were also observed on peony leaves after 9 d of incubation at 20 C 12 h light/12 h dark by scanning electron microscopy (SEM; SEM XL30 ESEM; FEI-Philips, Hillsboro, Oregon) following sample preparation. The samples were treated by critical point dryer (CPD 030; Balzers Instruments, Balzers, Liechtenstein), mounted on metallic specimen stubs and sputter-coated with gold (MED 010; Balzers). Conidiophores and conidia of AK10, HA06, and B83 strains were also observed on HAY by light microscopy and SEM after 10 d at 8 C and 6 d at 20 C on 12 light/12 h dark.

Data analysis.—A three-way analysis of variance (ANOVA) was performed to assess the variables of trial date, wounding, and isolate on lesion development. Where there was no significant trial effect ($P < 0.05$), data from both trials on the same host were pooled and the ANOVA was rerun to only analyze the factors of isolate and wounding. Where a significant trial effect was observed, the trials were assessed separately. All ANOVA calculations were performed using the PROC MIXED procedure in SAS (9.2; SAS Institute, Cary, North Carolina). The physiological data were statistically assessed using a *t*-test procedure. Means for all analyses were separated with Tukey's test multiple range test at $P < 0.05$.

RESULTS

DNA extraction and PCR.—Sequences were obtained for all isolates from this study were deposited in GenBank (SUPPLEMENTARY TABLE 2). Although sequences were trimmed to equal lengths within a locus for phylogenetic analyses (with the exception of *B. fragariae* as described above), edited but untrimmed sequences were submitted to GenBank and ranged from 771 to 1149 base pairs.

Phylogenetic analysis.—Construction of individual gene trees showed consistent branching patterns among the *G3PDH, HSP60*, and *RPB2* genes, and the *NEP1* and *NEP2* genes (SUPPLEMENTARY FIGS. 1–5); thus, concatenated sequences were used to further consider relationships among species. Isolate *B. cinerea* HA08 diverges slightly from *B. cinerea* isolates BcB and MS05, however, probably a result of natural variation in the *NEP1* gene among *B. cinerea* isolates (SUPPLEMENTARY FIG. 4).

Final combined *G3PDH+HSP60+RPB2* data sets included 2781 positions, of which 1991 (71.6%) were identical among all 43 sequences used in the alignment (SUPPLEMENTARY TABLE 2). Combined *NEP1 +NEP2* sequences totaled 1383 base pairs with 844 (61.0%) identical positions among 34 *Botrytis* sequences (SUPPLEMENTARY TABLE 2). Phylogenetic analysis of the combined gene data sets consistently grouped isolates AK10, HA06, and B83 together into a genetically distinct, well-supported clade closely related to *B. aclada* (FIGS. 1 and 2). The phylogenetic analysis also confirmed the identities of the *B. cinerea* and *B. paeoniae* isolates collected from peonies cultivated in Alaska (FIGS. 1 and 2). Neighbor joining trees and maximum likelihood trees from concatenated data displayed identical branching patterns to each other, consistent with those reported in Staats et al. (2005) and Staats et al. (2007); therefore, only maximum likelihood trees are shown (FIGS. 1 and 2).

Pathogenicity of* Botrytis *spp.—A three-way ANOVA did not indicate statistical differences between the two replicated trials conducted to assess lesion development for peony or onion; therefore, results from the two trials were combined for each host species. Statistical differences were detected between the two trials conducted on grape ($P < 0.05$) and bean ($P < 0.001$). Although these results were statistically different, the pattern of lesion development and means separations were similar (data now shown); therefore, results from one trial for grape and bean are presented. An isolate by wounding effect was present for peony ($P < 0.001$) and grape ($P < 0.0001$), but not for onion and bean.

Under our trial conditions, B83, AK10, and HA06 demonstrated the ability to cause lesions greater than 1 mm on all hosts tested except for onion bulbs (TABLE 2). Wounding did not have any significant effect on lesion development for any of the new *Botrytis* species–host species combinations except for B83 on grape leaves where a larger lesion was produced on wounded tissue (TABLE 2). On peony, grape, and bean leaves, B83, AK10, and HA06 caused similar size

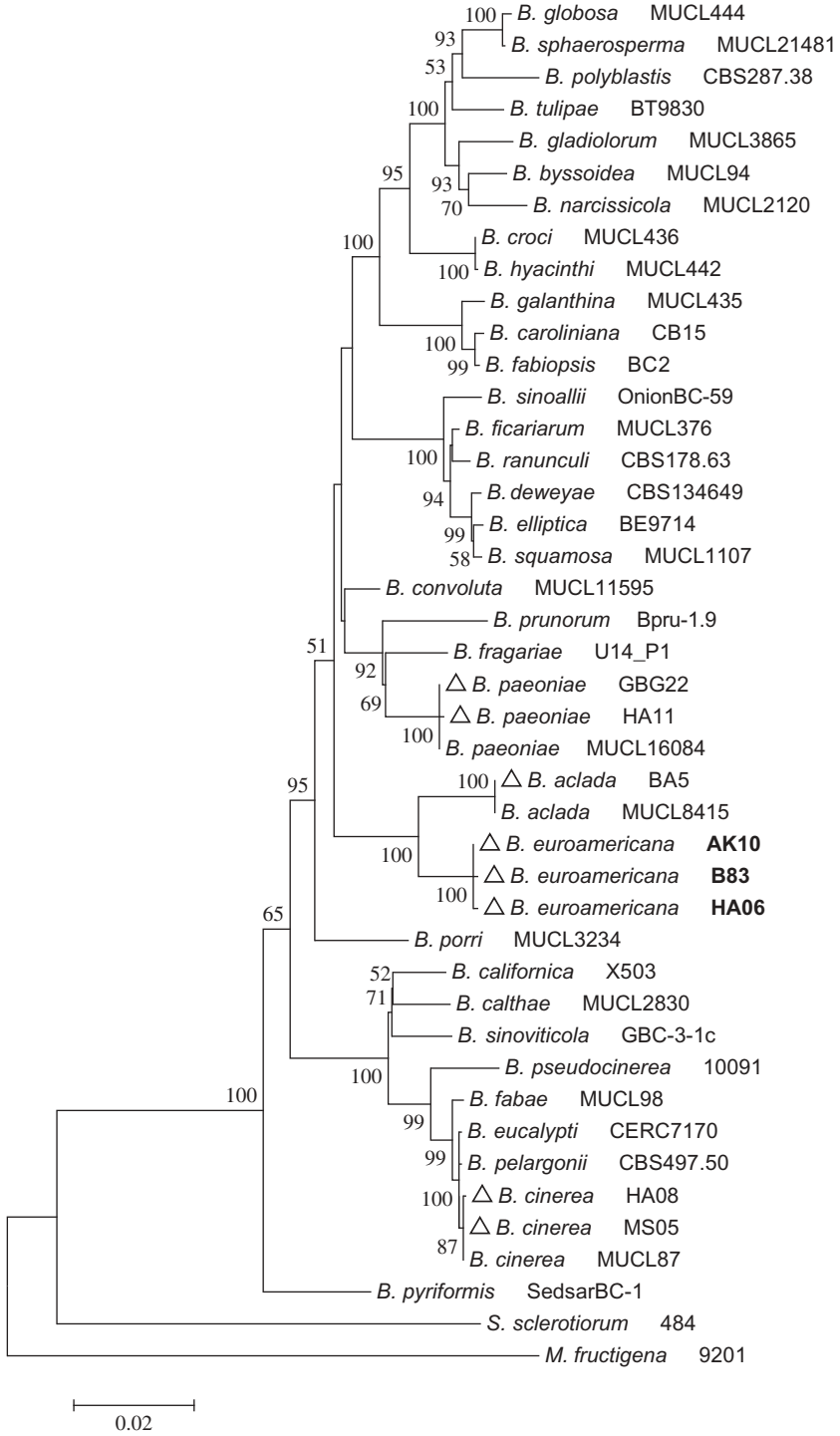

Figure 1. Maximum likelihood tree of combined *G3PDH, HSP60*, and *RPB2* gene sequences (Staats et al. 2005) describing the phylogenetic relationship between isolates used in this study (indicated by Δ) and voucher sequences of named *Botrytis* species obtained from GenBank using *Sclerotinia sclerotiorum* and *Monilinia fructigena* as outgroups. Species names are followed by isolate/strain code. Isolates in boldface type are the proposed new species *B. euroamericana*. A total of 2781 positions were used in analysis. Numerical branch labels represent bootstrap percentages (n = 1000). Branches with <50% bootstrap support are not shown. Branch length is proportional to the numbers of nucleotide substitutions as measured by the scale bar.

lesions as *B. aclada* BA5 (TABLE 2). BA5, however, produced significantly larger lesions than any other isolate on onion bulb tissue (TABLE 2). B83, AK10, and HA06 produced smaller lesions on peony, grape, and bean tissue as compared with *B. cinerea* MS05 in all trials but performed similarly to *B. cinerea* HA08 on peony and grape (TABLE 2). The full results of our pathogenicity trials are reported in TABLE 2.

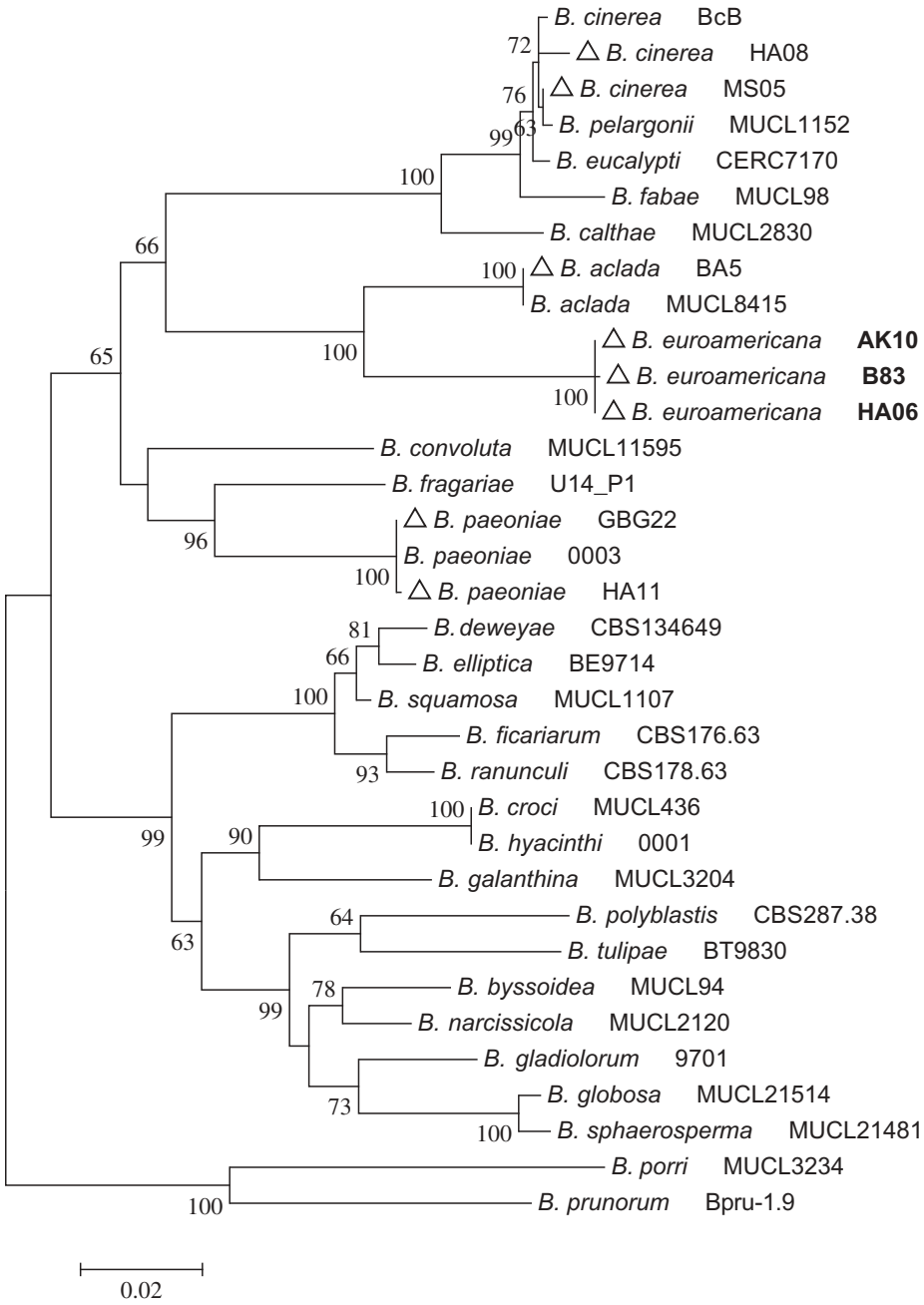

Figure 2. Maximum likelihood tree of combined *NEP1* and *NEP2* gene sequences (Staats et al. 2007) describing the phylogenetic relationship between isolates used in this study (indicated by Δ) and voucher sequences of named *Botrytis* species obtained from GenBank. Species names are followed by isolate/strain code. Isolates in boldface type are the proposed new species *B. euroamericana*. A total of 1383 positions were used in analysis. Numerical branch labels represent bootstrap percentages (n = 1000). Branches with <50% bootstrap support are not shown. Branch length is proportional to the numbers of nucleotide substitutions as measured by the scale bar.

Morphological characterization.—All *Botrytis* spp. isolates grew at 8, 15, 20, and 25 C on PDA. On PDA after 7 d at 20 C, isolates B83, AK10, and HA06, produced whitish-grayish to cream, moderately deep and floccose colonies (FIG. 3A–C), reverse whitish to brownish. *Botrytis cinerea* isolates had grayish to gray and felty colonies, *B. aclada* BA5 developed a brownish and felty colony, and *B. paeoniae* colonies were whitish and moderately wooly. On PDA, MEA, and CzA, isolates displayed differing mycelial growth rates both at 25 and 20 C. Notably, at 25 C, isolates B83, AK10, and HA06 showed significantly higher RGR than *B. paeoniae* and *B. aclada* isolates (SUPPLEMENTARY TABLE 3).

Table 2. Lesion diam on plant tissues inoculated with *Botrytis* spp. on different host plants.[a]

Species	Isolate	Wounding[c]	Lesion diameters (mm)[b]							
			Peony		Onion		Grape		Bean	
B. aclada	BA5	U	2.1	ef	21.8	a	<1	cd	6.3	c
		W	2.2	ef			1.4	cd		
B. cinerea	HA08	U	4.9	def	1.7	b	2.2	cd	32.7	a
		W	10.1	bc			6.8	cd		
	MS05	U	12.1	b	3.1	b	15.2	ab	30.9	a
		W	12.6	b			19.2	a		
B. euroamericana	AK10	U	1.5	f	<1	b	<1	cd	20.0	b
		W	4.6	def			2.8	cd		
	B83	U	2.8	def	—	—	<1	d	8.1	c
		W	6.2	cde			6.9	bc		
	HA06	U	4.8	def	<1	b	4.2	cd	13.1	bc
		W	6.8	cd			4.3	cd		
B. paeoniae	HA11	U	19.8	a	—	—	<1	cd	7.7	cb
		W	21.4	a			1.7	cd		
	GBG22	U	20.3	a	<1	b	3.2	cd	7.8	c
		W	22.0	a			2.7	cd		

[a]Plants were inoculated with mycelial plugs and incubated in high humidity conditions at 25 C (12 h light/12 h dark).
[b]Lesion diameter was measured 7 d post inoculation (DPI) for peony and onion and 3 DPI for grape and bean. No isolate by wounding interaction was observed in either onion or bean; therefore, only simple effects means for isolates are reported. Means followed by the same letter are not significantly different for $P < 0.05$.
[c]"U" indicates tissue remained unwounded, and "W" indicates tissue was wounded prior to inoculation.

Sclerotia production on PDA and/or MEA was observed in all isolates except B83. We previously reported the sclerotial characteristics of B83 after production on PDA and MEA at 4 C after 12 mo (Lorenzini and Zapparoli 2014). On both media at all temperatures, sclerotia of AK10 had a concentric pattern, whereas those of HA06 developed only at 15 C along the edge of the plate (data not shown). AK10 and HA06 isolates produced irregular, subspherical and/or elongate-shaped, concave below, melanized, discrete to arranged sclerotia of variable sizes (FIG. 3H) in concentric rings (FIG. 3G). On PDA after 40 d, AK10 produced an average number of 28 and 94 sclerotia per dish at 8 and 20 C, respectively (TABLE 3), whereas HA06 produced approximately 50 sclerotia per dish at 15 C. This latter strain produced sclerotia that were generally smaller than those of AK10 (data not shown). Sclerotia of AK10 were comparable in size to the *B. aclada* and *B. cinerea* isolates but were bigger than those of the *B. paeoniae* isolates (TABLE 3).

Macroconidiophores typical of *Botrytis* were observed in all isolates, and their length measurements, carried out on WA, varied considerably among and within species (TABLE 3). The density of macroconidiophores produced on PDA varied among B83, AK10, and HA06 isolates, with production quite abundant by B83, rare by AK10, and almost absent by HA06. Wider-diameter conidiophores were formed by the two *B. paeoniae* strains relative to other isolates. All isolates examined, including B83, AK10, and HA06, produced elliptical and oblong macroconidia (FIG. 4B–H). Conidia produced by *B. aclada* were on average smaller than those of the new *Botrytis* species isolates (TABLE 3).

On PDA, microconidia were produced by the new *Botrytis* species (FIG. 4I and K) and *B. paeoniae* isolates (data not shown), whereas microconidia production was not detected by *B. cinerea* and *B. aclada*. A description of the microconidia of the new *Botrytis* species is provided in the TAXONOMY section.

The morphological characterization of B83, AK10, and HA06 strains were also evaluated in HAY medium (FIG. 4; TABLE 4). The colony of B83 had concentric rings of whitish to pale brown mycelium, AK10 developed in uniform way without staining the substratum, which remained translucent, whereas HA06 had whitish colony, dense at the edge (FIG. 3). Conidiophore production by B83 and AK10 on HAY was abundant, whereas for HA06 it was moderate. Conidiophore production by B83 was higher at 20 C than 8 C, whereas for AK10 and HA06 it was greater at 8 C than 20 C. TABLE 4 reports morphology of macroconidiophores and macro- and microconidia formed on HAY by B83, AK10, and HA06 strains. Sclerotia production was not observed on HAY after 40 d.

Macroconidiophores and conidia of AK10 and B83 were also produced on infected leaves of peony at 20 C (FIG. 4B). Macroconidia were not observed on leaves infected by HA06.

TAXONOMY

Botrytis euroamericana.—M. Lorenzini, G. Zapparoli, A.R. Garfinkel & G.A. Chastagner, sp. nov. FIGS. 3 and 4
MycoBank MB821849

Typification: ITALY. PROVINCIA AUTONOMA DI TRENTO: Trento, Valle dei Laghi, +46°07′81″, −10°99′93″. Withered grape (*Vitis vinifera* 'Nosiola'), Oct 2011,

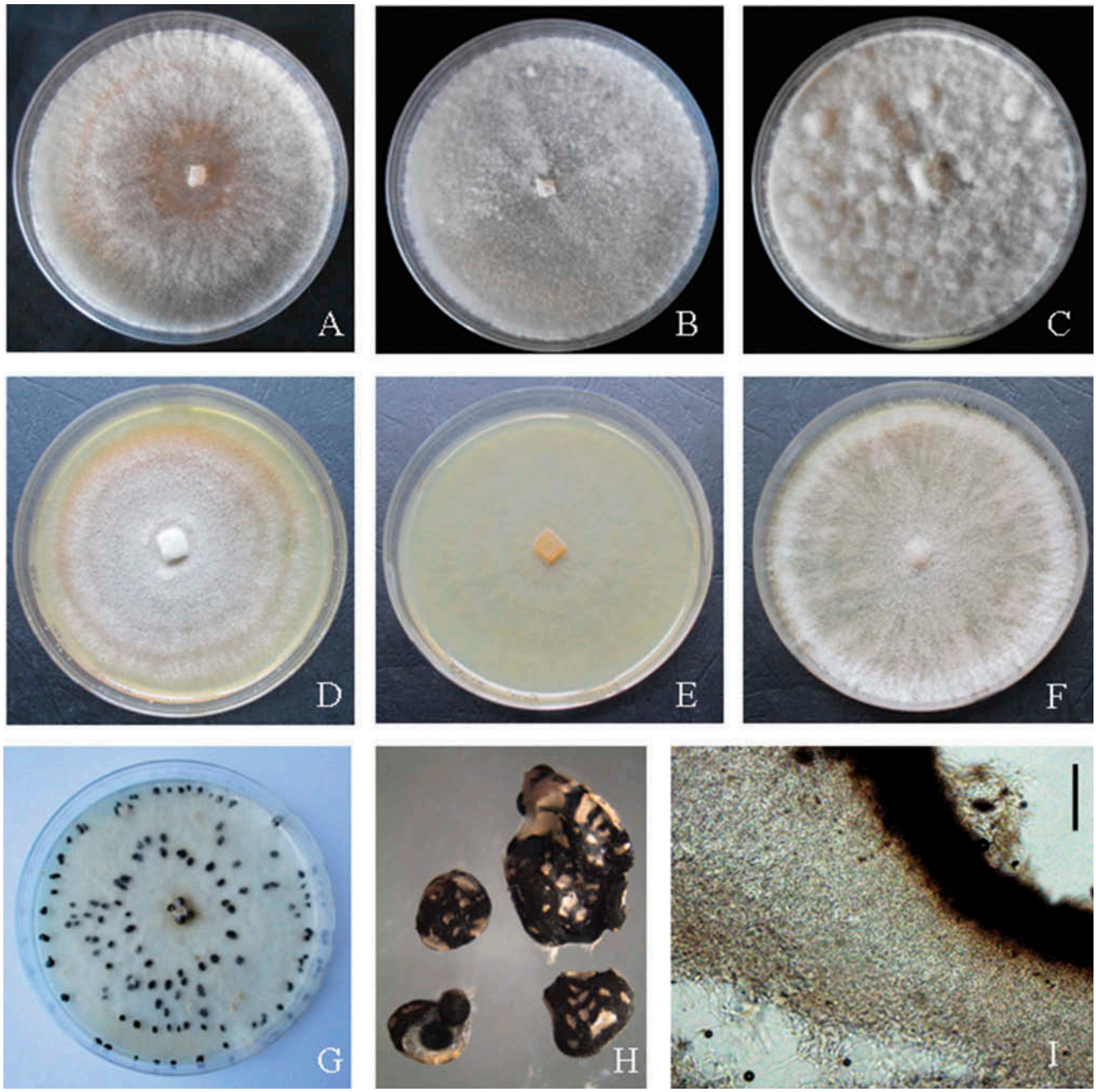

Figure 3. Morphological characteristics of *Botrytis euroamericana*, sp. nov., in culture. A–C. Cultures, respectively, of B83, AK10, and HA06 on PDA in Petri dishes (90 mm diam) incubated for 7 d at 20 C. D–F. Cultures, respectively, of B83, AK10, and HA06 on HAY incubated for 7 d at 20 C. G–H. Single and aggregate black sclerotia produced by AK10 on PDA after 40 d at 20 C. I. Section of an AK10 sclerotium.

M. Lorenzini and G. Zapparoli (**holotype** a living culture at CBS-KNAW: CBS 141699). GenBank accession numbers: ITS = KC191680, *RPB2* = KC191679, *HSP60* = KC191678, *G3PDH* = KC191677, *NEP1* = KC762944, *NEP2* = KC762945.

Etymology: Referring to European and North American continents where the fungus was isolated.

Stroma, a definite sclerotium of the planoconvexoid type, concave below, subspherical and elongate, irregularly shaped, melanized, and covered by cream mycelium spots (FIG. 3H), solitary and aggregated, varying in size from 1–5 × 0.5–3 mm (TABLE 3); in section, typical of the genus, rind cells rounded-polygonal and thick-walled, in 10–30 layers, 4–12.5 × 3–9 µm, a thick cortex, medullary hyphae hyaline, 2.5–5 µm wide, sometimes branched, septate, compactly arranged; in cross-section, showing a loose arrangement of hyphae (FIG. 3I). Production dependant on the isolate, distributed in concentric rings (FIG. 3G).

Macroconidiophores typical of *Botrytis*, arising singly or in groups of two or more from the under surface

Table 3. Morphology of sclerotia, macroconidiophores, and macroconidia formed by *B. euroamericana*, sp. nov., and other *Botrytis* species.

			Sclerotia[a]						
			Size (mm)		No. per dish[b]		Macroconidiophores[c]	Macroconidia[e]	
Species	Strain	CBS number	8 C	20 C	8 C	20 C	Size (µm)[d]	Shape[f]	Size (µm)[g]
B. euroamericana	B83	CBS141699	—	—	—	—	790–1520 × 4.0–14.0	EO, O	7.0–(11.7)–16.0 × 4.0–(5.4)–7.5
	AK10	CBS141559	1.0–5.0 × 0.5–3.0	1.0–3.5 × 1.0–2.5	28 a (± 5)	94 a (± 10)	550–1540 × 6.5–16.5	EO, O	9.5–(13.1)–17.5 × 4.5–(7.5)–10.0
	HA06	CBS141558	—	—	—	—	410–1130 × 5.0–11.5	EO, O	8.5–(11.9)–16.5 × 5.0–(6.2)–8.0
B. aclada	BA5	CBS142339	1.0–3.0 × 1.0–2.0	1.0–6.0 × 1.0–5.0	35 a (± 3)	40 b (± 13)	360–2010 × 7.0–12.0	EO	5.5–(8.4)–12.0 × 3.5–(4.5)–6.5
B. cinerea	MS05	CBS142342	1.5–4.5 × 1.5–3.0	—	61 b (± 5)	—	890–4990 × 5.5–12.0	EO, O	8.0–(11.8)–16.5 × 5.0–(7.8)–12.5
	HA08	CBS142343	3.0–10.0 × 2.0–5.5	—	14 c (± 3)	—	260–1550 × 7.5–13.5	EO, O	5.5–(7.9)–11.0 × 4.5–(6.0)–9.0
B. paeoniae	HA11	CBS142341	0.5–1.0 × 0.5–1.0	0.3–1.0 × 0.3–1.0	345 d (± 71)	493 c (± 52)	720–2090 × 7.5–22.5	EO, O	5.5–(10.3)–17.0 × 4.5–(7.0)–9.5
	GBG22	CBS142340	0.5–1.0 × 0.5–1.0	0.5–1.0 × 0.5–1.0	368 d (± 47)	247 d (± 40)	610–1530 × 7.0–23.5	EO, O	6.5–(12.8)–18.0 × 5.0–(7.5)–11.0

Note. "—" indicates no formation of sclerotia on PDA after 40 d at 8 or 20 C.[a]sclerotia discrete to aggregate produced on PDA after 40 d.
[b]Values (mean ± standard deviation, n = 3) followed by different letters within column are significantly different for $P < 0.05$.
[c]Produced on WA after 15 d at 20 C on 12 h light/12 h dark.
[d]Length × width, minimum–maximum × minimum–maximum.
[e]On PDA at 20 C after 15 d at 20 C on 12 h light/12 h dark.
[f]EO = elliptical/oblong; O = ovoid.
[g]Length × width, minimum–(average)–maximum × minimum–(average)–maximum.

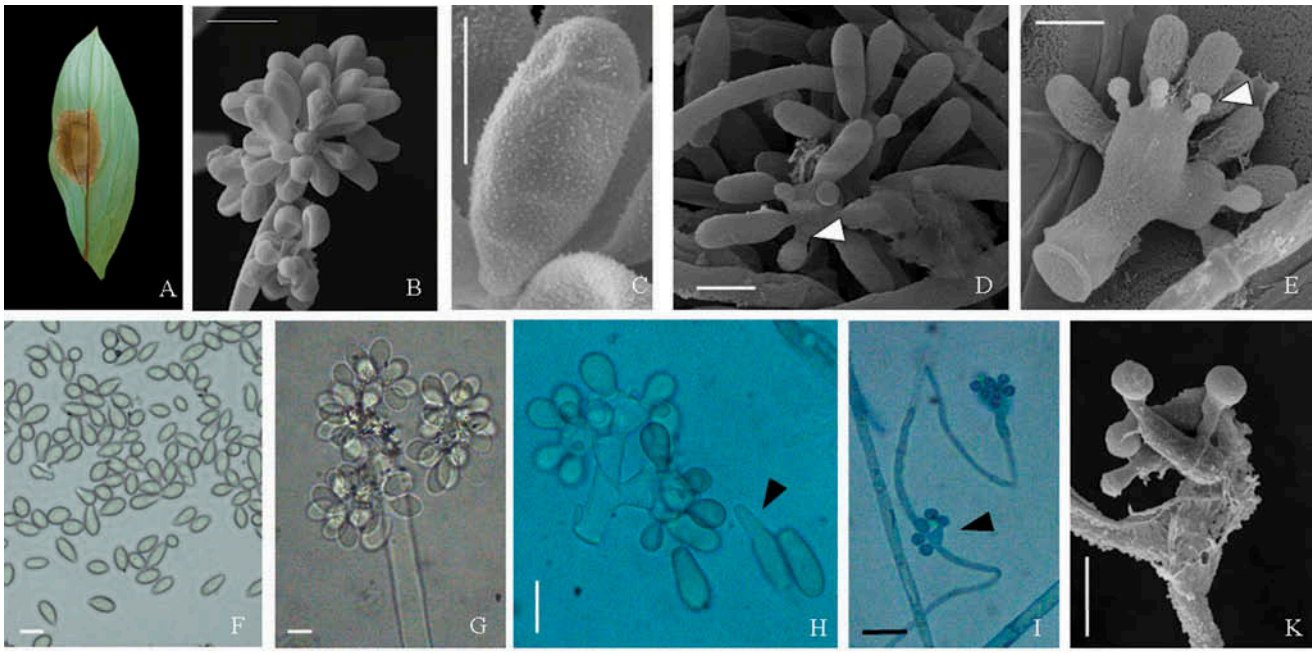

Figure 4. Infected peony leaves and microscopic characteristics of *Botrytis euroamericana*, sp. nov. A. Necrotic lesion on a peony leaflet caused by *B. euroamericana* isolate AK10 after incubation at 25 C for 12 d. B–D. Scanning electronic micrographs of AK10 formed on peony leaves. B. Macroconidiophore. C. Detail of the macroconidium. D. Ampullae showing denticle and rising conidium (arrow). E. Scanning electronic micrograph of B83 ampullae with denticles and rising conidia (arrow) formed on HAY after 6 d at 20 C. F. Dispersed macroconidia formed by AK10 on PDA after 15 d at 20 C. G–K. Light microscopy and scanning electronic micrographs of macro- and microconidia formed by B83 on HAY after 6 d at 20 C. G. Macroconidia arranged in botryose clusters. H. Ampullae carrying macroconidia and a detail of germinated macroconidium indicated by arrow. I. Phialide (arrow) carrying the microconidia. K. Detail of a phialide carrying microconidium. Bars: B, F, G–I = 10 µm; C, D, K = 5 µm; E = µm.

of colony, erect, thick-walled, septate, with prevalently alternate and sometimes whorled branches, brown to subhyaline from the base to apex, 410–1540 × 4–16.5 µm on WA (TABLE 3), 310–2300 × 9–19.5 µm on HAY (FIG. 4; TABLE 4). Macroconidia in botryose clusters, unicellular, hyaline to pale brown in color, ellipsoidal-oblong to ovoid, 7–17.5 × 4–10 µm on PDA (TABLE 3), 7.5–22 × 4.5-9.5 µm on HAY (TABLE 4). On HAY macroconidia are connected by denticles of 0.5–1.5 × 1–2 µm with clavate or subspherical terminal ampullae of 6–21.5 × 3–22 µm formed on dichotomously branching conidiophores. Microconidia unicellular hyaline in color, spherical, 2.0–4.5 µm, borne on phialides, 5.0–8.5 µm (FIG. 4; TABLE 4).

Cultural characters: Colonies on potato dextrose agar growing 15.5–18.5 mm d^{-1} diam (SUPPLEMETARY TABLE 3) aerial mycelium in a concentric to uniform pattern, moderately deep to floccose, sometimes tufted,

Table 4. Morphology of macroconidiophores and macro- and microconidia formed by *B. euroamericana*, sp. nov., on HAY medium at 20 C.

			Ampullae[a]			Macroconidia[a]			Microconidia[a]		
Strain	Macroconidiophores (µm)[a,b]	Branching[a,d]	Shape[d]	No. of conidia	Size (µm)[b]	Denticle (µm)[b]	Shape*	Size (µm)[b]	Shape[d]	Size (µm)[c]	Phialide (µm)[a,c]
B83	470–2300 × 13.0–19.5	A, W	C, Sp	2–9	6.0–20.5 × 6.5–22.0	0.5–1.0 × 1.0–2.0	EO, O	7.5–(13.1)–22.0 × 4.5–(6.2)–8.5	S	2.0–3.0	6.5–8.5
AK10	660–2140 × 13.0–18.0	A, W	C, Sp	3–9	8.0–17.5 × 3.0–13.0	0.5–1.5 × 1.0–2.0	EO, O	7.5–(10.6)–15.5 × 5.0–(7.5)–9.5	S	2.0–3.5	5.0–7.5
HA06[f]	310–900 × 9.0–17.5	A, W	C, Sp	3–9	7.5–21.5 × 7.5–10.0	0.5–1.0 × 1.0–2.0	EO, O	11.0–(15.4)–21.0 × 5.5–(7.0)–9.0	—	—	—

Note. "—" indicates no formation of microconidia at 8 C on 12 h light/12 h dark.
[a]Structures produced at 20 C after 10 d on 12 h light/12 h dark.
[b]Length × width, minimum–(average)–maximum × minimum–(average)–maximum.
[c]Length, minimum–maximum.
[d]A = alternate; C = clavate; EO = elliptical/oblong; O = ovoid; S = spherical; Sp = subspherical; W = whorled.
[f]After 10 d at 8 C on 12 h light/12 h dark.

whitish to pale gray. Sclerotia develop depending of isolate and cultural conditions, subspherical and elongate black sclerotia appearing after few weeks arranged in concentric rings, varying in size (FIG. 3). On HAY, 13–15 mm d^{-1} diam, aerial mycelium in uniform to concentric pattern, low to moderately deep, sometimes not staining the agar, which remains translucent, whitish to pale brown (FIG. 3). Sclerotia not observed.

Habitats: Grapes and peony.

Distribution: Europe (Italy) and North America (United States, Alaska).

Additional cultures examined: UNITED STATES. ALASKA: North Pole, Fairbanks North Star Borough, +64°39′52″, −147°10′40″, on peony (*Paeonia* × *lactiflora*), Jul 2013, *G.A. Chastagner* (a living culture of AK10 CBS-KNAW: CBS141559); Trapper Creek, Matanuska-Susitna Borough, +62°20′54″, −150°39′05″, on peony (*Paeonia* × *lactiflora*), Jun 2015, *A.R. Garfinkel* and *G.A. Chastagner* (a living culture of HA06 CBS-KNAW: CBS141558). GenBank: *G3PDH* = KX266727–KX266728, *HSP60* = KX266733–KX266734, *RPB2* = KX266739–KX266740, *NEP1* = KX266745–KX266746, *NEP2* = KX266751–KX266752.

Notes: B. euroamericana differs from other *Botrytis* species according to the morphological and phylogenetic species concepts and by its pathogenicity and virulence on host plants.

On PDA, the mycelium of *B. euroamericana* differs from *B. cinerea, B. pseudocinerea, B. californica, B. pyriformis*, and *B. aclada*, which have colonies with powdery surfaces because of abundant sporulation. *Botrytis euroamericana* differs in sclerotia production from *B. byssoidea, B. eucalypti, and B. hyacinthi*, which are not sclerotia producers (Chilvers and du Toit 2006; Mirzae et al. 2008; Blomquist and Greene 2011). *Botrytis euroamericana* has macroconidia that are narrower than those of *B. fabae* (10–18.5 µm), *B. fabiopsis* (10–21.5 µm), and *B. hyacinthi* (10–15 µm) (Mirzaei et al. 2008; Zhang et al. 2010a; Blomquest and Green 2011) and shorter than those of *B. sinoallii* (16–25 µm) (Zhang et al. 2010b). Macroconidia of *B. euroamericana* are also smaller than those of *B. squamosa* (12–30 × 8–20 µm) (Chilvers and du Toit 2006). The ellipsoidal shape of *B. euroamericana* macroconidia differs from that of the round *B. globosa* and *B. sphaerosperma* and pyriform *B. pyriformis* (Chilvers and du Toit 2006; Zhang et al. 2016). Moreover, the surface of *B. euroamericana* macroconidia differs from that of the wrinkled *B. fabiopsis* and *B. sinoviticola* and the villiform surface of *B. pyriformis* (Zhang et al. 2010a, 2016; Zhou et al. 2014).

Phylogenetic analysis based on the *G3PDH, HSP60*, and *RPB2* genes (combined) and the *NEP1* and *NEP2* genes (combined) places *B. euroamericana* in a distinct genetic clade closely related to, but separate from, *B. aclada*.

Botrytis euroamericana is less virulent on peony, onion, and grape than *B. cinerea* and less virulent on peony than *B. paeoniae. Botrytis euroamericana* is not pathogenic on onion, whereas *B. aclada* is an aggressive onion pathogen.

DISCUSSION

Morphological, pathogenicity, and sequence data support the recognition of a new species, *B. euroamericana*, for a fungus occurring naturally on peony in Alaska and on grape in Italy. *Botrytis euroamericana* forms a phylogenetic cluster in *Botrytis* Clade 2 (Staats et al. 2005) based on *G3PDH+HSP60+RPB2* (Staats et al. 2005) and *NEP1+NEP2* (Staats et al. 2007) combined gene genealogies, distinct from any currently described *Botrytis* species with 100% bootstrap support, as previously reported by Lorenzini and Zapparoli (2014). Although these isolates are closely related to *B. aclada*, a host-specific pathogen of onion, our morphological data reveal few similarities to *B. aclada* isolate BA5. Not only did the *B. euroamericana* isolates have a larger

conidia size on average than we observed in *B. aclada* BA5, but also the colony morphology and dense sporulation of *B. aclada* BA5 on PDA greatly differed from the relatively sparse to almost absent sporulation of *B. euroamericana*. Macroconidia measured for *B. aclada* BA5 conformed with those reported by previous investigations (Chilvers and du Toit 2006; Mirzaei et al. 2008; Zhang et al. 2008, 2010b). However, the use of conidia size as key to discriminate these two phylogenetic closely related species is unsatisfactory because the ranges overlap. Further analysis of other additional isolates of both species under standardized conditions could be helpful to determine whether conidial size is a reliable character. Size and shape of macroconidia clearly distinguish *B. euroamericana* from other species, as described in the TAXONOMY section.

In this study, we observed that HAY medium was suitable for cultivation of *B. euroamericana* and induced more abundant sporulation than did PDA. The absence of identifiable conidia in leaves in vitro infected by *B. euroamericana* isolate HA06 suggests heterogeneity in sporulation patterns in nature in this new species, as also reported for *B. deweyae* (Grant-Downton et al. 2014). Additional analyses on the sporulation of *B. euroamericana* under different conditions are necessary to understand its importance in the life cycles of natural populations.

Moreover, infrequent or delayed sclerotia production appears to be characteristic of *B. euroamericana* isolates included in this study. Sclerotia were also not formed in cultures following reisolation from host tissue (data not shown). When sclerotia are produced by *B. euroamericana*, they are more irregularly shaped than those produced by *B. aclada* BA5. Data on sclerotia production by *B. euroamericana* strains confirm that this feature greatly depends on culture conditions and can vary among isolates, as already documented for *B. cinerea* (Beever and Weeds 2007; Mirzaei et al. 2008). Some of the most compelling support for species delineation of *B. euroamericana* from *B. aclada* comes from pathogenicity tests on onions, where none of the *B. euroamericana* isolates caused serious lesions on host tissues. Our strain *B. aclada* BA5 rapidly and consistently colonized onion bulb tissue with prolific mycelial growth and sporulation. These results are consistent with the preliminary tests conducted on B83 on onion leaves (Lorenzini and Zapparoli 2014). Even for those isolates of *B. euroamericana* causing small lesions on onion tissue, the fungus was never recovered from the lesion, suggesting that colonization of the host tissue may not have occurred. *Botrytis aclada* was routinely reisolated from inoculated onion tissue in our trials (data not shown).

The pathogenicity trials also served as a means to complete Koch's postulates on host plants from which our strains were originally isolated. These tests indicate that *B. euroamericana* isolates HA06 and AK10, isolated from peony, can be reinoculated onto peony to produce similar symptoms as observed in the original samples. This represents the fourth *Botrytis* species reported to infect peony; *B. cinerea, B. paeoniae*, and *B. pseudocinerea* were all previously reported on peony (Whetzel 1939; Muñoz et al. 2015). Furthermore, despite its being originally isolated from grape berries (Lorenzini and Zapparoli 2014), *B. euroamericana* isolate B83 is able to colonize peony leaves at the same rate as AK10 and HA06 (TABLE 2). Likewise, *B. euroamericana* isolates AK10 and HA06 are able to colonize grape leaves, the original host species of B83. Isolations from diseased tissues confirmed fungal colonization of host tissue (data not shown). Koch's postulates were completed on grape for B83 in a previous study (Lorenzini and Zapparoli 2014). In addition to the first report of *B. euroamericana* as a pathogen of peonies, the completion of Koch's postulates for *B. cinerea* (HA08 and MS05) and *B. paeoniae* (GBG22 and HA11) represent first reports of these pathogens on peonies in Alaska (Cash 1953; Farr and Rossman 2016).

Overall, our *B. euroamericana* isolates appeared to be weakly pathogenic on all tissues we tested, causing significantly less damage than either *B. cinerea* or *B. paeoniae* on peony tissues or *B. cinerea* on bean and grape. The difference in lesion development cannot simply be explained by differences in ability of the isolates to grow at certain temperatures. For example, despite the faster growth rate of *B. euroamericana* as compared with *B. paeoniae* on PDA at 25 C (TABLE 3), *B. euroamericana* did not colonize peony tissue as quickly as *B. paeoniae* at the same temperature, suggesting that it is not as aggressive as the host-specific peony pathogen under these conditions. It is possible that under different environmental conditions or on host tissue of a different age, *B. euroamericana* isolates would be more aggressive. Our initial screening of HA06 on young (not fully expanded) peony leaves showed significantly more necrosis after 7 d in cooler environmental conditions than observed in this trial (data not shown). Furthermore, the extent of necrosis was severe on the original peony foliage from which HA06 was isolated, suggesting that it is capable of causing more than a localized lesion. In some cases with beans, grapes, and peony, *B. euroamericana* displayed the same ability to cause lesions as *B. aclada*, which is a putatively host-specific pathogen of onion. These results are difficult to interpret and could potentially be explained by the observations of Prins (2000)

that some *Botrytis* spp. can cause primary lesions on nonhost tissue.

The full host range of *B. euroamericana* has not yet been determined; however, it is noteworthy to discover the same species on two different host plant species on different continents. Many of the new *Botrytis* species described in the last decade were found on one host crop in a single geographic region (Zhang et al. 2010a, 2010b; Li et al. 2012; Zhou et al. 2014; Ferrada et al. 2016; Saito et al. 2016; Rupp et al. 2017). Furthermore, the two areas where these isolates were found represent regions with diverse environmental conditions and agricultural production histories. The fact that the Alaskan isolates have higher conidia production at lower temperatures than the Italian isolate could be explained by the difference in geographic histories. Nonetheless, this geographical range suggests the ability of *B. euroamericana* to survive and persist in various climates and agro-systems. However, despite our extensive surveys of *Botrytis* on peony across the United States, *B. euroamericana* has only been identified from samples from Alaska (A.R. Garfinkel and G.A. Chastagner, unpublished data). This could be due to sampling bias and the relative prevalence of *B. euroamericana* in each of these locations as compared with other *Botrytis* species. Although in our trials it appears that *B. euroamericana* can also infect *P. vulgaris*, it is unclear if bean is a host of *B. euroamericana* under field conditions. Additional sampling of a more diverse collection of potential hosts is needed to determine the distribution and host range of *B. euroamericana* and, therefore, the economic importance of this pathogen. Given the remote locations and limited agriculture in Alaska where this pathogen has been detected, *B. euroamericana* may be an endemic pathogen on native hosts that has spread onto peonies.

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