Universally Primed Polymerase Chain Reaction Alleles and Internal Transcribed Spacer Restriction Fragment Length Polymorphisms Distinguish Two Subgroups in *Botrytis aclada* Distinct from *B. byssoides*

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ABSTRACT

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Fifty-one isolates representing the four *Botrytis* spp. associated with onion neck rot were clustered by unweighted pair group method with arithmetic mean based on universal-primed polymerase chain reaction (UP-PCR) fingerprints. Bootstrap analysis of the consensus phenogram clearly demonstrated five strong clusters among the four *Botrytis* spp.: *B. cinerea* (C), *B. squamosa* (S), *B. byssoides* (B), and *B. aclada* (AI and AII). Subdivision of the 30 *B. aclada* isolates, AI (14) and AII (16), from Europe,

Egypt, North America, and Japan was further supported by restriction analysis of the internal transcribed spacer of the ribosomal genes and spore size measurements. Gene diversities (*H*) among AI and AII isolates were very low (0.007 and 0.043, respectively). A likelihood ratio chi-square test (G^2) of Nei's coefficient of genetic differentiation (G_{ST}) showed that both *B. aclada* subgroups, AI and AII, were significantly different from *B. byssoides* ($P < 0.001$), and that *B. aclada* subgroups AI and AII were significantly different from each other ($P < 0.001$). No UP-PCR alleles were shared by AI and *B. byssoides* isolates, whereas 10 and 12 alleles were shared by AI:AII and AII:*B. byssoides*, respectively. The hypothesis that AII may be a hybrid between AI and *B. byssoides* is discussed.

Additional keywords: *Botryotinia*, DNA fingerprint, hybrid species.

Botrytis is a fungal form genus including 22 species (6) that causes disease on a wide range of crops and ornamentals. Onions (*Allium cepa* L.) are attacked by at least seven *Botrytis* spp. (5), and Walker (27) distinguished three that cause neck rots in onions: *B. aclada* Fres. (syn. *B. allii* Munn), causal agent of neck rot (name originally proposed by Walker [27] was gray mold neck rot); *B. byssoides* Walker (teleomorph *Botryotinia allii* [Sawada] Yamamoto), causal agent of mycelial neck rot (27); and *Botrytis squamosa* Walker (teleomorph *Botryotinia squamosa* Vien.-Bourg), causal agent of small sclerotial neck rot of white onion cultivars (27). *B. squamosa* is better known as the causal agent of onion leaf blight. *B. cinerea* Pers. Fr. (teleomorph *Botryotinia fuckeliana* [de Bary] Whetz.) causes leaf spots and brown staining of onion bulbs (7) and is occasionally isolated from diseased onion bulbs. Of these onion pathogens, *B. aclada* is most commonly associated with onion neck rot disease, where storage losses in excess of 50% have been observed (13-15).

B. aclada and *B. byssoides* cause similar symptoms and their spore sizes overlap (5). This has led several researchers to postulate that *B. byssoides* and *B. aclada* are conspecific (7,9). However, Owen et al. (22) studied the variability of *B. aclada* and *B. byssoides* and concluded that the two species are valid. Morphological characteristics of the conidiophore and cytological measurements of the average number of nuclei in conidia have been used to separate *B. aclada* and *B. byssoides* (17,26). However, no consensus has yet been reached on the taxonomic status of *B. byssoides* and *B. aclada*.

B. aclada consists of two subgroups based on spore measurements (5), and chromosome number in the nuclei (26). Shirane et al. (26) found that cells in the mitotic phase of seven *Botrytis* spp. (including *B. aclada*, *B. byssoides*, *B. cinerea*, and *B. squamosa*) all contained 16 chromosomes. However, one subgroup of the *B. aclada* isolates contained 32 chromosomes per nucleus and produced spores that were bigger than the *B. aclada* isolates with 16 chromosomes. Shirane et al. (26) speculate that the 32 chromosome subgroup is an autotetraploid of the 16 chromosome subgroup.

We are working with development of molecular markers for detection of neck rot organisms (21), and to accomplish this it is essential that (i) inter- and intraspecific variability of *Botrytis* spp. pathogenic to onions, and (ii) the taxonomic status of *B. byssoides* and *B. aclada* be addressed. We have obtained isolates from Europe, Japan, North America, and North Africa and calculated their genetic variability by universal primed polymerase chain reaction (UP-PCR), a variant of random amplified polymorphic DNA (29) with greater reproducibility due to use of longer primers and higher annealing temperature (2,3,18). Objectives of this work were to study the molecular and morphological variability of *Botrytis* spp. associated with neck rot of onions, with particular focus on *B. aclada*.

MATERIALS AND METHODS

Fungal material and spore measurements. *Botrytis* isolates were obtained from fungal culture collections or were collected in different onion fields in Denmark (Table 1). Fungal isolates were grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit) and, for long-term preservation, were stored in a 15% glycerol suspension at -80°C . For production of conidia, isolates were grown on onion leaf extract agar (OEA; 15 g of dried onion

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leaves and 16 g of agar per 1 liter of dH₂O) for 14 days at 21°C under black light. Length and width of 100 conidia from each isolate of *B. aclada* were measured at ×500 magnification under Nomarski optics. Morphological verification of isolates of *B. byssoides* and *B. squamosa* was difficult because these isolates did not sporulate on either PDA or OEA.

DNA extraction. For DNA extraction, fungi were grown for 5 to 7 days at room temperature in the dark on PDA covered with a dry sterilized (120°C for 12 h) disc of cellophane. Mycelia were scraped from the cellophane and ground in liquid nitrogen with a mortar and pestle. Powdered mycelium (30 mg) was transferred to an Eppendorf tube, and DNA was extracted according to the

TABLE 1. *Botrytis* spp. isolates used in this study

Isolate no.	Original no.	Origin	Isolated from	Spore size (µm) ^a	UP-PCR group ^b	ITS <i>Sph1</i> ^c
<i>B. aclada</i>						
BA1	BA1 ^d	Denmark	Bulb of <i>Allium cepa</i>	9.0 × 5.0	AI	3
BA2	BA2 ^d	Denmark	Bulb of <i>Allium cepa</i>	9.0 × 4.8	AI	3
BA3	MUCL 8 ^e	Belgium	Bulb of <i>Allium cepa</i>	8.9 × 4.6	AI	3
BA4	MUCL 403	Netherlands	Bulb of <i>Allium cepa</i>	9.9 × 5.3	AII	2
BA5	MUCL 99	U.S.	Bulb of <i>Allium cepa</i>	10.4 × 6.5	AII	2
BA6	MUCL 961	Belgium	Bulb of <i>Allium cepa</i>	10.3 × 5.9	AII	2
BA7	MUCL 1150	Norway	Bulb of <i>Allium cepa</i>	10.1 × 5.7	AII	2
BA8	MUCL 3037	Canada	Bulb of <i>Allium cepa</i>	9.1 × 4.8	AI	3
BA9	MUCL 3106	U.S.	Bulb of <i>Allium cepa</i>	9.2 × 5.2	AI	3
BA10	MUCL 3109	Japan	Leaf of <i>Allium cepa</i>	10.6 × 5.8	AII	2
BA11	MUCL 3614	Egypt	Bulb of <i>Allium cepa</i>	14.1 × 6.3	AII	2
BA12	MUCL 494	Denmark	Bulb of <i>Allium ascalonicum</i>	9.7 × 5.8	AII	2
BA13	MUCL 8415	Germany	Bulb of <i>Allium cepa</i>	ns	AI	3
BA14	MUCL 9114	Netherlands	Leaf of <i>Allium cepa</i>	ns	AII	2
BA16	MUCL 18868	Scotland	Bulb of <i>Allium cepa</i>	13.0 × 5.9	AII	2
BA17	MUCL 18967	England	Leaf of <i>Allium ascalonicum</i>	12.1 × 5.9	AI	3
BA18	B4065 ^f	UK	Bulb of <i>Allium cepa</i>	9.2 × 5.2	AI	3
BA19	B4090 ^f	UK	Bulb of <i>Allium cepa</i>	10.4 × 5.6	AI	3
BA20	61-2 ^g	U.S.	<i>Allium cepa</i>	10.2 × 5.9	AII	2
BA21	3-DK99 ^d	Denmark	Bulb of <i>Allium cepa</i>	9.8 × 5.5	AI	3
BA22	8-DK99 ^d	Denmark	Bulb of <i>Allium cepa</i>	9.6 × 5.2	AI	3
BA23	13-DK99 ^d	Denmark	Bulb of <i>Allium cepa</i>	10.1 × 6.2	AII	2
BA24	MUCL 1105	U.S.	— ^h	12.2 × 7.3	AII	2
BA25	MUCL 3528	—	—	11.4 × 5.6	AII	2
BA26	MUCL 3537	—	—	10.7 × 6.0	AII	2
BA27	MUCL 3556	UK	Bulb of <i>Allium cepa</i>	11.9 × 5.9	AII	2
BA28	MUCL 3810	Belgium	Bulb of <i>Allium cepa</i>	8.4 × 5.5	AI	3
BA29	MUCL 20329	Belgium	<i>Allium schoenoprasum</i>	9.2 × 4.8	AI	3
BA30	CBS 260.71 ⁱ	Denmark	Bulb of <i>Allium cepa</i>	9.2 × 4.9	AI	3
<i>B. byssoides</i>						
BB1 ^j	MUCL 94	U.S.	Bulb of <i>Allium cepa</i>	ns	B	2
BB2	MUCL 283	U.S.	Bulb of <i>Allium cepa</i>	ns	AII	2
BB3	MUCL 3108	Japan	Leaf of <i>Allium cepa</i>	ns	S	2
BB4	MUCL 3844	Netherlands	<i>Allium cepa</i>	ns	S	2
BB5	MUCL 9113	Netherlands	<i>Allium cepa</i>	ns	B	2
BB6	B3730 ^f	UK	<i>Allium porrum</i>	11.8 × 7.0	B	2
BB7	MUCL 30498	—	—	ns	S	2
<i>B. cinerea</i>						
BC1	BC1 ^d	Denmark	Leaf of <i>Allium cepa</i>	11.4 × 7.7	C	2
BC3	MUCL 3107	U.S.	Leaf of <i>Allium cepa</i>	10.4 × 7.9	C	2
BC4	MUCL 3647	Kenya	<i>Coffea arabica</i>	ns	C	2
BC5	MUCL 9115	Netherlands	Leaf of <i>Allium cepa</i>	ns	C	2
BC6	MUCL 10117	Israel	Bulb of <i>Allium cepa</i>	ns	C	2
BC7	MUCL 21596	Netherlands	Leaf of <i>Allium cepa</i>	ns	C	2
BC8	61-34 ^g	U.S.	—	ns	C	2
<i>B. squamosa</i>						
BS2	MUCL 3821	Belgium	Leaf of <i>Allium cepa</i>	ns	S	2
BS3	MUCL 3848	Netherlands	<i>Allium cepa</i>	ns	C	2
BS6	MUCL 28710	U.S.	Leaf of <i>Allium cepa</i>	ns	S	2
BS7	B3808 ^f	UK	<i>Allium cepa</i>	ns	S	2
BS8	B3834 ^f	UK	<i>Allium cepa</i>	ns	S	2
BS9	MD-16 ^g	U.S.	—	ns	S	2
BS10	64A ^g	U.S.	—	ns	S	2
BS11 ^j	MUCL 1107	U.S.	Bulb of <i>Allium cepa</i>	ns	S	2

^a Average length × width of 100 conidia; and ns = nonsporulating in culture.

^b Groups of *Botrytis* isolates based on unweighted pair group method with arithmetic mean cluster analysis of universal-primed polymerase chain reaction (UP-PCR) DNA markers (Fig. 2).

^c Number of fragments after *Sph1* digestion of internal transcribed spacer (ITS) ITS1 to ITS4 amplification product (Fig. 1).

^d Provided by the Danish Institute of Agricultural Sciences, Research Center, Denmark.

^e Provided by the Belgium Coordinated Collection of Microorganisms, Louvain-la-Neuve.

^f Provided by S. R. Kenny, HRI, Wellesbourne, Warwick, UK (8).

^g Provided by J. W. Lorbeer, Cornell University, Ithaca, NY.

^h Missing information.

ⁱ Provided by Centraalbureau voor Schimmelcultures, the Netherlands.

^j Culture of type specimen.

method of Möller et al. (16). DNA was dissolved in 50 µl of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C until use. DNA concentration, estimated on agarose gels, ranged from 0.1 to 1 mg/µl. DNA samples were diluted 10-fold for use in internal transcribed spacer (ITS) and UP-PCR analysis.

ITS restriction analysis. PCR amplification of the ITS rDNA region (Fig. 1) was performed in a final reaction volume of 20 µl. The following, with the final concentration indicated, were added: 2.5 mM MgCl₂, 0.25 mM each deoxyribonucleotide triphosphates (dNTP), dATP, dGTP, dCTP, and dTTP, 1 unit of *Taq* DNA polymerase (Promega, Madison, WI), 50 mM KCl, 10 mM Tris-HCl at pH 9.0, 0.1% Triton X-100, 0.5 pmol of each primer ITS1 and primer ITS4 (Table 2), and 10 to 100 ng of fungal DNA were used. A programmable controller (PTCTM-100; MJ Research Inc., Watertown, MA) was used with the following program: 94°C (3 min), 1 cycle, 92°C (30 s), 53°C (30 s), 72°C (30 s), 29 cycles, 72°C (3 min), 1 cycle. PCR product (3 µl) was separated on 1.5% agarose (Sigma Chemical, St. Louis) gels in 1× Tris-acetate-EDTA (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide, visualized under UV light, and inspected for presence of more than one DNA band. Ten microliters of PCR product was precipitated by adding 1 µl of 3 M NaOAc (pH 4.8) and 25 µl of 96% EtOH, and placing the mixture on ice for 10 min. Precipitation mix was centrifuged for 20 min (14,000 rpm, 4°C). The pellet was washed with 500 µl of 70% EtOH and resuspended in 20 µl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA (1 µl) was restriction digested with *Sph*I (New England Biolabs Inc., Beverly, MA) according to the manufacturer's recommendation and, after digestion, separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

UP-PCR. In a preliminary experiment, eight primers were screened for use in the UP-PCR analysis and four primers were selected for the present study (Table 2). The DNA used for ITS amplification was also used for UP-PCR. The following, with the final concentration indicated, were added: 2.5 mM MgCl₂, 0.35 mM each dNTP, dATP, dGTP, dCTP, and dTTP, 1.2 units of DNA polymerase (DyNAzyme II; Finnzymes, Espoo, Fin), 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.5 pmol of primer (Table 2), and 10 to 100 ng of fungal DNA were used. A programmable controller was used with the following program: 94°C (3 min), 1 cycle, 92°C (50 s), 52°C (1 min, 10 s), 69°C (1 min), 29 cycles, 69°C (3 min), 1 cycle. PCR reactions were performed in a total volume of 20 µl. The PCR products were separated on 1.5% agarose gels (as described previously). Presence and absence of DNA fragments were scored as 1 or 0, respectively (Table 3). UP-PCR amplification was performed at least twice to confirm pattern of DNA fragments.

Data analysis. Variation among spore measurements for *B. aclada* subgroups AI and AII was evaluated by Hotelling's *T*² test (two-sample multivariate analysis of variance [Proc MANOVA], version 6.12, SAS Institute, Cary, NC) where conidia width and length are included in the same analysis. The two groups were treated as fixed variables (AI and AII), based on the UP-PCR data.

Fig. 1. Schematic diagram of the nuclear ribosomal DNA gene cluster consisting of the small subunit (SSU), 5.8S, the large subunit (LSU), and the two internal transcribed spacers (ITS1 and ITS2) based on White et al. (28). *Sph*I^b restriction site found in the ITS1 to ITS4 amplification product for all *Botrytis* spp. (Table 1). *Sph*I^a restriction site unique for *B. aclada* subgroup AI. Primer sequences are shown in Table 2.

A distance-matrix ($1 - S_j$) of pairwise comparisons of the UP-PCR markers was constructed from Jaccard similarity coefficients (S_j), where $S_j = \sum_{mn} (1,1) / [\sum_{mn} (0,1) + \sum_{mn} (1,0) + \sum_{mn} (1,1)]$; and 1 indicates presence of a band, 0 indicates absence, and m and n indicate the two isolates in comparison. All negative matches (0,0) are excluded from the coefficient. A cluster analysis of all isolates was performed using the unweighted pair group method with arithmetic mean (UPGMA) algorithm in the program NEIGHBOR from the PHYLIP program package (J. Felsenstein, version 3.5c, Phylogenetic inference package, Department of Genetics, University of Washington, Seattle). In the UPGMA analysis, PHYLIP generates a tree file, containing information of the ordering of isolates and branch lengths. Using this tree file, a phenogram was visualized using the program TREEVIEW (23). There was made no corrections or change of ordering in this tree. Statistical support for the branches in the phenogram was tested by bootstrap analysis (4). One thousand random reassembled (with replacement) data sets were made from the original data set using the program DNAsim (N. Holst, unpublished data). These 1,000 data sets were used for generating 1,000 trees using the UPGMA algorithm in the program NEIGHBOR, and a consensus tree was generated with the program CONSENCE in the PHYLIP program package. Gene diversity (H) (19) was calculated for each UP-PCR cluster, where $H = \sum (1 - \sum x_k^2) / n$, and x is the allele frequency of the k th UP-PCR allele, and n is the number of UP-PCR loci. Two alleles per UP-PCR locus were considered a null allele and a positive allele, represented by the absence or the presence of a band, respectively. Nei's coefficient of genetic differentiation (G_{ST}) (19) was calculated for all pair-wise combination of the two subgroups of *B. aclada* (AI and AII) and *B. byssoides* (B), where $G_{ST} = (H_T - H_S) / H_T$. The total gene diversity (H_T) over all loci was calculated as $H_T = \sum (1 - \sum x_k^2) / n$, where x is the allele frequency of the k th UP-PCR allele in the total population, and n is the number of UP-PCR loci. The subpopulation gene diversity (H_S) over all loci was calculated as $H_S = \sum (1 - \sum x_{ik}^2) / mn$, where x is the allele frequency of the k th UP-PCR allele in the i th subpopulation, m is the number of subgroups, and n is the number of UP-PCR

TABLE 2. Nucleotide sequences of primers in this study

Primer ^a	Nucleotide sequence 5'→3'	Reference
ITS1	TCCGTAGGTGAACCTGCGG	28
ITS4	TCCTCCGCTTATTGATATGC	28
L21	GGATCCGAGGGTGGCGGTCT	18
AS4	TGTGGGCGCTCGACAC	10
AS15inv	CATTGCTGGCGAATCGG	11
3-2	TAAGGGCGGTGCCAGT	3

^a ITS = internal transcribed spacer.

TABLE 3. Universally primed polymerase chain reaction (UP-PCR) genotypes of group AI, AII, and B

Group ^a	UP-PCR genotype ^b	N ^c
AI-1	100101110000110001101010101011	16
AI-2	1001011100001100011010101011011	15
AII-1	111111111001101111011111101111	4
AII-2	111111111101101111111111101111	3
AII-3	111111111101101111011111101111	4
AII-4	101101111101101111011111101111	6
AII-5	01111111101011001110111111101111	7
AII-6	111111111101001111111111101111	4
B-1	01111001101100111011011101100101	12

^a AI-1 (BA1, BA2, BA3, BA8, BA9, BA13, BA17, BA19, BA21, BA22, BA28, BA29, and BA30); AI-2 (BA18); AII-1 (BA4); AII-2 (BA5, BA7, BA11, BA14, and BA23); AII-3 (BA6, BA10, BA12, BA16, BA24, BA26, and BA27); AII-4 (BA20); AII-5 (BA25); AII-6 (BB2); and B (BB1, BB5, and BB6).

^b Multilocus genotypes: 1 and 0 represent positive and null alleles, respectively, at 33 UP-PCR loci.

^c Number of null alleles.

loci. A likelihood ratio chi-square test (G^2) was performed with the null hypothesis of no difference in UP-PCR allele frequencies in the two subgroups tested (1,24).

RESULTS

UP-PCR and cluster analysis. The four selected primers generated 82 reproducible bands from the 51 *Botrytis* isolates analyzed, collected as members of the four *Botrytis* spp. Each primer generated between 5 to 10 strong, reproducible bands for each of the five *Botrytis* groups. The isolates were clustered by UPGMA based on the matrix of Jaccard similarities and the consensus phenogram (Fig. 2) of the UP-PCR data, shows five strong clusters among the four *Botrytis* spp.: B (*B. byssoides*), C (*B. cinerea*), S (*B. squamosa*), and AI and AII for the two observed subgroups of the *B. aclada* isolates. The bootstrap values calculated for statistical support of the UP-PCR groups, AI, AII, B, C, and S, were all higher than 94%, giving strong support for the individual groups. Support for the B:AI:AII branch and the C:S branch was also high (93%), indicating a close relationship

between *B. aclada* and *B. byssoides*. The branch connecting AI and AII was supported by a bootstrap value of 54%, which gives no support for the two *B. aclada* subgroups clustering separately from *B. byssoides*. This means that the three groups, AI, AII, and B, were very strongly supported, but the relationship between them remains unresolved by this analysis.

Subdivision of *B. aclada*. As discussed previously, the UPGMA cluster analysis showed a clear subdivision of the *B. aclada* isolates into two subgroups (Fig. 2), and this subdivision was supported by restriction analysis of the ITS rDNA region (Table 1). *SphI* restriction analysis of the ITS rDNA region (Fig. 1) showed that all AII isolates contained two fragments (band size of approximately 220 and 320 bp, Table 1) and all group AI isolates contained three fragments (band size of approximately 120, 200, and 220 bp). The MANOVA showed the spore sizes (length $\pm$ SE $\times$ width $\pm$ SE) of the two subgroups of *B. aclada*, AI ($9.5 \pm 0.3 \times 5.1 \pm 0.1$) and AII ($11.0 \pm 0.3 \times 6.0 \pm 0.1$), to be significantly different ($F_{2,24} = 13.27$; $P < 0.0001$). Some overlap in spore size between the two groups was observed (Table 1). Most isolates of *B. aclada* (except for BA13 and BA14 which did not

Fig. 2. Unweighted pair group method with arithmetic mean cluster analysis of Jaccard similarities among universally primed polymerase chain reaction markers scored for 51 isolates of *Botrytis cinerea* (C), *B. squamosa* (S), *B. byssoides* (B), and two subgroups of *B. aclada* (AI and AII). Number at major branches indicates percent occurrence of the cluster to the right of the branch in 1,000 bootstrap trees generated from 82 bands sampled with replacement. Bar indicates genetic distance. Only branches occurring in 50% or more of the bootstrap trees (i.e., majority-rule consensus tree) are labeled.

sporulate) easily produced abundant conidia on OEA with characteristic flat and sparse production of mycelium, leaving no doubt about identity.

Genetic variability within UP-PCR groups. All eight C isolates and nearly all 10 S isolates (except BS11 and BS6 which were identical) had unique UP-PCR genotypes (data not shown), and the gene diversities (*H*) among the isolates were 0.143 and 0.102, respectively. For the *B. aclada* and *B. byssoidea* isolates, 33 positive UP-PCR alleles were identified (Table 3). Of 30 *B. aclada* isolates, obtained from all over the world, 14 were AI types. These isolates were almost identical, and only one isolate (BA18) differed from the others, by a single UP-PCR band. The gene diversity among the AI isolates was 0.007, and the similarity coefficient between the two AI genotypes was 0.94 (Table 4). Subgroup AII showed more variation, and six UP-PCR genotypes were observed among the 16 isolates in the present study. However, similarity coefficients between the AII genotypes ranged from 0.83 to 1.00 (Table 4), and the gene diversity among isolates was 0.043, indicating low genetic variation in this subgroup as well. The three *B. byssoidea* isolates designated to group B were identical (Table 3).

Genetic differentiation of intraspecies groups. The UPGMA analysis showed that AI, AII, and B were clustered strongly, but it also showed that differentiation of the AI:AII cluster from the B cluster was not supported. This indicates a very close relationship between *B. aclada* and *B. byssoidea*. In all pair-wise comparisons there was a highly significant genetic differentiation between the subgroups (Table 5). AI:AII genotypes and AII:B genotypes have almost identical similarity coefficients, respectively, between 0.52 to 0.61 and 0.50 to 0.61. However, AI and B genotypes have similarity coefficients between 0.22 to 0.23 (Table 4), and this is comparable to the similarity coefficient between C:S genotypes of 0.22 to 0.28 (data not shown). Seven UP-PCR alleles were present in all AI, AII, and B isolates; 10 alleles were exclusively associated with AI:AII and 12 with AII:B, but no UP-PCR alleles were exclusive to AI:B (Table 3). These data indicate that AII is genetically intermediate between AI and B.

Isolate identity. Identity of some of the isolates from the culture collections was not supported by our UP-PCR analysis. We have, nevertheless, chosen to keep our original designations of the isolates (i.e., as the species received from culture collections) because we do not have morphological evidence for the identity of the *B. byssoidea* and *B. squamosa* isolates. Of the seven *B. bys-*

soidea isolates obtained from culture collections, only two isolates (BB5 and BB6) grouped strongly with a culture of the *B. byssoidea* type specimen (BB1) (Table 1). Isolates BB2, BB3, BB4, and BB7 were, based on UP-PCR fingerprint analysis, grouped with *B. aclada* subgroup AII (BB2) or *B. squamosa* (BB3, BB4, and BB7). The history of isolate BB7 shows that MUCL received it as *B. squamosa*, but that it was reassigned to *B. byssoidea*. Our analysis indicates that the reassignment was probably incorrect and that BB7 is a *B. squamosa* isolate. Isolate BS3 was received as a *B. squamosa*, but the UP-PCR DNA fingerprint showed a closer relation to the *B. cinerea* group (Table 1). Similar to *B. byssoidea*, *B. squamosa* seldom sporulate on artificial media, and lack of sporulation makes it very difficult to make a correct diagnosis of the isolate by traditional methods.

DISCUSSION

The UPGMA and bootstrap analysis of UP-PCR fingerprints of the *Botrytis* isolates gave strong support for the *B. cinerea* group (C), the *B. squamosa* group (S), and the *B. byssoidea* group (B). *B. aclada* isolates clustered in two genetically distinct subgroups, AI and AII. Spore size measurements and ITS restriction analysis supported this subdivision of *B. aclada* isolates. Previous work has shown that *B. aclada* consisted of two subgroups (5,26), but this is the first investigation, to our knowledge, that clearly demonstrates a substantial genetic difference between the two *B. aclada* subgroups. Differentiation between the two subgroups of *B. aclada* cannot be made solely on the basis of spore size, however restriction fragment length polymorphism analysis of the ITS rDNA region with *SphI* can differentiate the two subgroups of *B. aclada*. The ITS rDNA region had been sequenced for a few isolates of *B. aclada*, *B. byssoidea*, *B. squamosa*, and *B. cinerea* in a previous study (21). This sequence information revealed that restriction digest with the enzyme *SphI* was able to cut *B. aclada* (AII), *B. byssoidea*, *B. squamosa*, and *B. cinerea* once, whereas *B. aclada* (AI) would be cut twice. This was true for all isolates investigated. Shirane et al. (26) have shown that cells of the large-spored subgroup of *B. aclada* contain 32 chromosomes, whereas cells of the small-spored subgroup contain 16 chromosomes. In the present work, the large-spored *B. aclada* subgroup is called AII and the small-spored subgroup is called AI. Shirane et al. (26) suggested that the 32 chromosomal *B. aclada* subgroup is an auto-diploid of the 16 chromosomal *B. aclada* subgroup. If this hy-

TABLE 4. Jaccard similarity coefficients between universally primed polymerase chain reaction (UP-PCR) genotypes

UP-PCR genotype	AI-2	AII-1	AII-2	AII-3	AII-4	AII-5	AII-6	B
AI-1	0.94	0.61	0.57	0.59	0.63	0.59	0.53	0.23
AI-2	...	0.59	0.55	0.57	0.61	0.57	0.52	0.22
AII-1	...	...	0.93	0.97	0.90	0.86	0.90	0.53
AII-2	...	...	...	0.97	0.90	0.87	0.97	0.59
AII-3	...	...	...	...	0.93	0.90	0.93	0.56
AII-4	...	...	...	...	...	0.93	0.87	0.50
AII-5	...	...	...	...	...	...	0.83	0.57
AII-6	...	...	...	...	...	...	...	0.61

TABLE 5. Likelihood ratio chi-square test (*G*²) of Nei's coefficient of genetic differentiation (*G*_{ST}) of pair wise comparisons of *Botrytis aclada* and *B. byssoidea* subgroups AI, AII, and B

UP-PCR groups ^a	<i>H</i> _S ^b	<i>H</i> _T ^c	<i>G</i> _{ST} ^d	df	<i>G</i> ^{2e}	<i>P</i> ^f
AI-AII	0.023	0.198	0.884	33	473	<0.001
AI-B	0.003	0.366	0.992	33	381	<0.001
AII-B	0.024	0.222	0.892	33	209	<0.001

^a The 33 universally primed polymerase chain reaction (UP-PCR) loci used for calculation shown in Table 3.

^b Genetic diversity within subpopulations.

^c Total genetic diversity over all subpopulations.

^d Nei's genetic differentiation between subpopulations.

^e Likelihood ratio chi-square test. Null hypothesis of no difference in allele frequencies among subpopulations over the 33 UP-PCR loci.

^f *P* = probability for *G*².

pothesis were correct then it would be expected that the genetic differentiation between the two subgroups would be small because of identity among the copies of the genome in the 32 chromosomal subgroup. Our data do not support this conclusion, because analysis of genetic differentiation of the subgroups AI and AII shows that they are very different; *B. aclada* subgroups AI and AII could be two separate species.

The *B. byssoidea* isolates, although identical, were supported by a bootstrap value of only 95%. This is a consequence of the bootstrap method, where random data sets are generated with replacement resulting in new data sets in which some characters are represented more than once and other characters are excluded. In approximately 5% of these data sets, characters separating *B. byssoidea* from other isolates were excluded.

Many researchers have speculated that *B. aclada* and *B. byssoidea* are conspecific, mainly because they have overlapping spore size and because they cause similar symptoms of disease (onion neck rot). Our analysis of genetic differentiation between *B. byssoidea* isolates (B) and *B. aclada* (AI and AII) isolates clearly shows that the three subgroups are very different, and most likely are not conspecific. The pair-wise similarity coefficients between AI, AII, and B isolates show that AII is equally similar to both AI and B, but AI and B are as different from each other as *B. squamosa* isolates (S) are from *B. cinerea* isolates (C). This might indicate that AII is a hybrid species between AI and B. This hypothesis fits with the results of Shirane et al. (26), because AII could have become polyploid (32 chromosomes) by fusion of AI (16 chromosomes) and B (16 chromosomes). Another indication is that AII isolates consisted exclusively of UP-PCR bands that were present in either AI or B. Similar DNA band patterns have recently been reported in analysis of plant and oomycete hybrids using PCR-mediated DNA markers (12,25). Resolution of the hybridization hypothesis is inferred from sequences of specific genes, in which ambiguous sites in the AII sequences were shown at positions in the sequence where AI and B have different nucleotides (20). Although we found no evidence for pathogenic specialization between the two *B. aclada* groups (data not shown), screening of new germ plasm and evaluation of new management strategies would be strengthened by mixing a diverse sample of the pathogens from both *B. aclada* groups and *B. byssoidea*.

Great variation was observed in *B. cinerea* isolates and *B. squamosa* isolates. All *B. cinerea* isolates and nearly all *B. squamosa* isolates had unique genotypes. Both species reproduce sexually in nature, and thus have the potential for generating high levels of genetic variation within populations, as observed. No perfect state has been identified for *B. aclada*, and the almost complete lack of variation at DNA level of *B. aclada* subgroup AI can be taken as strong support for clonality of this subgroup. The variation in *B. aclada* subgroup AII was somewhat greater; six UP-PCR genotypes were identified among 16 isolates. The samples of AI and AII isolates originated from many different regions in Europe and Northern America and were collected over nearly a century (data not shown). This supports both the separation of the two subgroups and the relative invariability among isolates in each of the *B. aclada* subgroups. *B. byssoidea* was only represented by three isolates (including a culture of the type specimen), and thus our finding of no variation within this group must be taken cautiously. The perfect state of *B. byssoidea*, *Botryotinia allii*, has only been observed in Japan, where it causes leaf blight, whereas *B. byssoidea* causes disease to onion bulbs (5). The type specimen of *B. byssoidea* was collected in the United States in 1925 and the two other authentic *B. byssoidea* isolates in this work were collected in the United Kingdom in 1984. It may be that *Botryotinia allii* mainly reproduces asexually in Europe and in the United States, or it could be that *B. byssoidea* and *Botryotinia allii* represent two separate species. Resolution of this question is beyond the scope of the present study.

B. byssoidea is traditionally identified on artificial media by sparse or absent sporulation, and by production of white fluffy mycelium and lack of sclerotial production (5). Five of six *B. byssoidea* isolates obtained from culture collections were incorrectly identified, according to our analysis. This illustrates the inadequacy of traditional morphological methods when nonsporulating isolates are to be identified. More certain diagnoses of nonsporulating *Botrytis* isolates can be obtained with DNA fingerprinting methods.

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