Detection and Discrimination of *Botrytis* Species, Causal Agents of Onion Bulb Rot, Using Quantitative Polymerase Chain Reactions (qPCR) Melting Curve Analysis

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INTRODUCTION

- Onion (*Allium cepa* L.) is an important vegetable crop in the United States, second in farmgate value according to the Agricultural Marketing Resource Center.
- Onion bulbs are susceptible to bulb rots that can cause significant losses.
- *Botrytis aclada*, *B. allii*, and *B. byssoidea* are the primary causal agents of neck rot for onion bulbs in storage.
- Infected onion bulbs typically only develop symptoms after post-harvest curing and some duration of storage.

OBJECTIVES

- Differentiate among species of *Botrytis* associated with bulb rots in storage using melting curve analysis with different DNA primer sets.
- Develop and standardize a molecular detection method for timely management of neck rot by detecting latent bulb infections at harvest.

FUNGAL ISOLATES AND DNA ISOLATION

- Isolates of each of the six *Botrytis* species (*B. aclada*, *B. allii*, *B. byssoidea*, *B. cinerea*, *B. porri*, and *B. squamosa*) were grown on ½ V8 agar medium containing streptomycin sulfate, under fluorescent light for 3 to 7 days, along with isolates of non-target fungi.

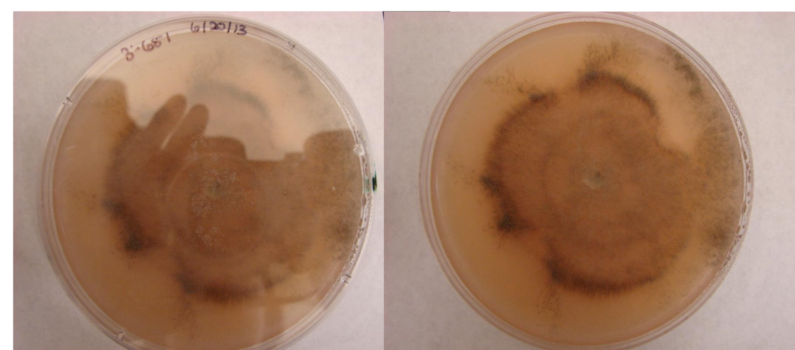

Figure 1. The image on the left shows the plate labeled with the particular isolate on the lid. The image on the right shows how much growth was present prior to isolating *Botrytis* DNA (lid removed).

- DNA was isolated using the UltraClean Soil DNA Extraction kit (MO BIO) following the manufacturer's protocol.

DNA AND PRIMER CONFIRMATION

- Isolated DNA was confirmed via agarose gel electrophoresis.

Figure 2. Gel images confirming DNA isolates were present. A) lane L: 100 bp ladder; lane 1: isolate B347; lane 2: B651; lane 3: BB1; and lane 4: 498 BS. B) lane L: 100 bp ladder; lane 1: isolate BP2; lane 2: B32; lane 3: BA16; and lane 4: B28. See Table 2 for isolate codes.

- Specificity and broad range detection capabilities of the developed primer sets were confirmed using endpoint PCR assays. Primer sets specifically amplified only the *Botrytis* species. No amplification observed with isolates of the genera *Penicillium*, *Aspergillus, or Fusarium.*

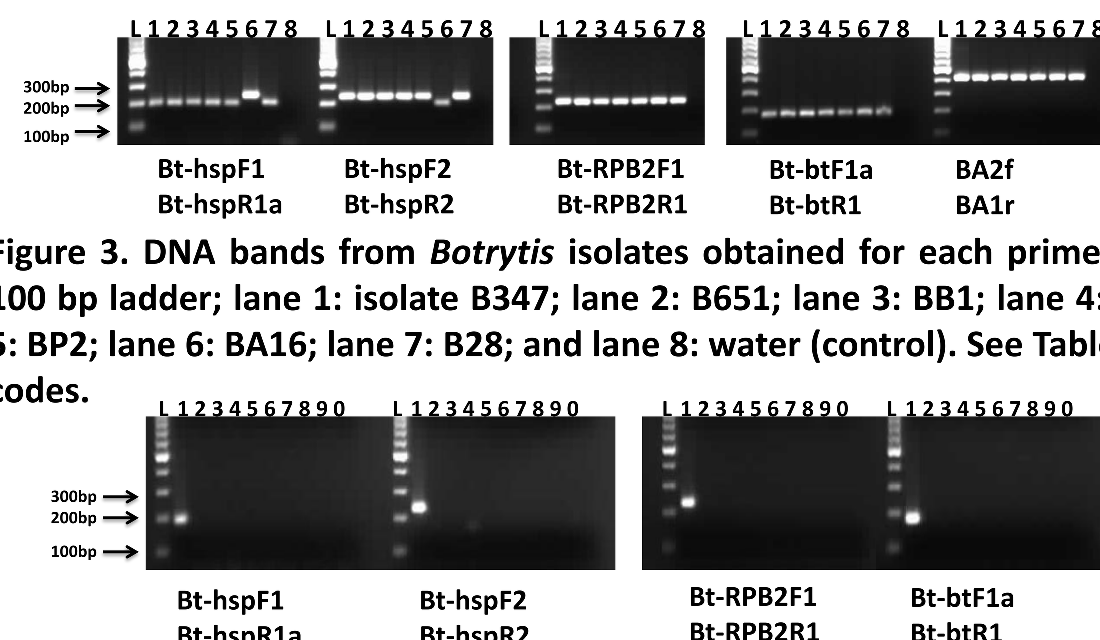

Figure 3. DNA bands from *Botrytis* isolates obtained for each primer set. Lane L: 100 bp ladder; lane 1: isolate B347; lane 2: B651; lane 3: BB1; lane 4: 498 BS; lane 5: BP2; lane 6: BA16; lane 7: B28; and lane 8: water (control). See Table 2 for isolate codes.

Figure 4. Gel images of *Botrytis* primers with DNA template from *Botrytis, Penicillium, Aspergillus, Fusarium,* and *Kluveromyces*. Lane L: 100 bp ladder; lane 1: *B. aclada* (positive control); lane 2: *P. polonicum*; lane 3: *P. brevicompactum*; lane 4: *P. ramulosum*; lane 5: *P. glabrum*; lane 6: *A. niger*; lane 7: *F. oxysporum*; lane 8: *F. proliferatum*; lane 9: *Kluveromyces*; lane 0: water (negative control).

qPCR

- Quantitative PCR reactions run with SYBR Select Master Mix from Applied Biosystems and various primer sets. Reactions were performed in a Bio-Rad MyiQ thermocycler.
- Melting curves and melting points were compared among isolates for different genes.

SYBR GREEN PROPERTIES

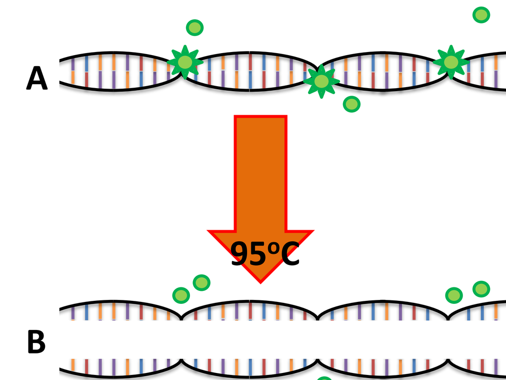

Figure 5. (A) As *Taq* polymerase replicates, SYBR Green binds to any double-stranded DNA at the minor groove. Binding causes SYBR Green to fluoresce, which is quantified. (B) In melting curve analysis, the DNA is heated and, as it denatures, SYBR Green dissociates. The loss of fluorescence is then measured.

TABLE 1: PRIMER SEQUENCES

Gene Region	Primer Name	Nucleotide Sequence (5' → 3')	Reference
Heat Shock Protein	Bt-hspF1	TTGCTGGTGTTGAGACTTTGG	This study
	Bt-hspR1a	GAGGGAAATAGCTCTGGCAAC	This study
RNA Polymerase II, second largest subunit (RPB2)	Bt-RPB2F1	GGAAAGATCGCCAAACCTAGA	This study
	Bt-RPB2R1	ACGAAAACCTTTGTTGCATTG	This study
Beta-tubulin	Bt-btF1a	CTACCTTCTCCGTCGTTCCAT	This study
	Bt-btR1	GATCTCCGTAAGATGGGTTGC	This study
L45-550	BA 2f	GTGGGGGTAGGATGAGATGATG	Nielsen et al. 2002
	BA 1r	TGAGTGCTGGCGGAAACAAA	Nielsen et al. 2002

MELTING CURVES

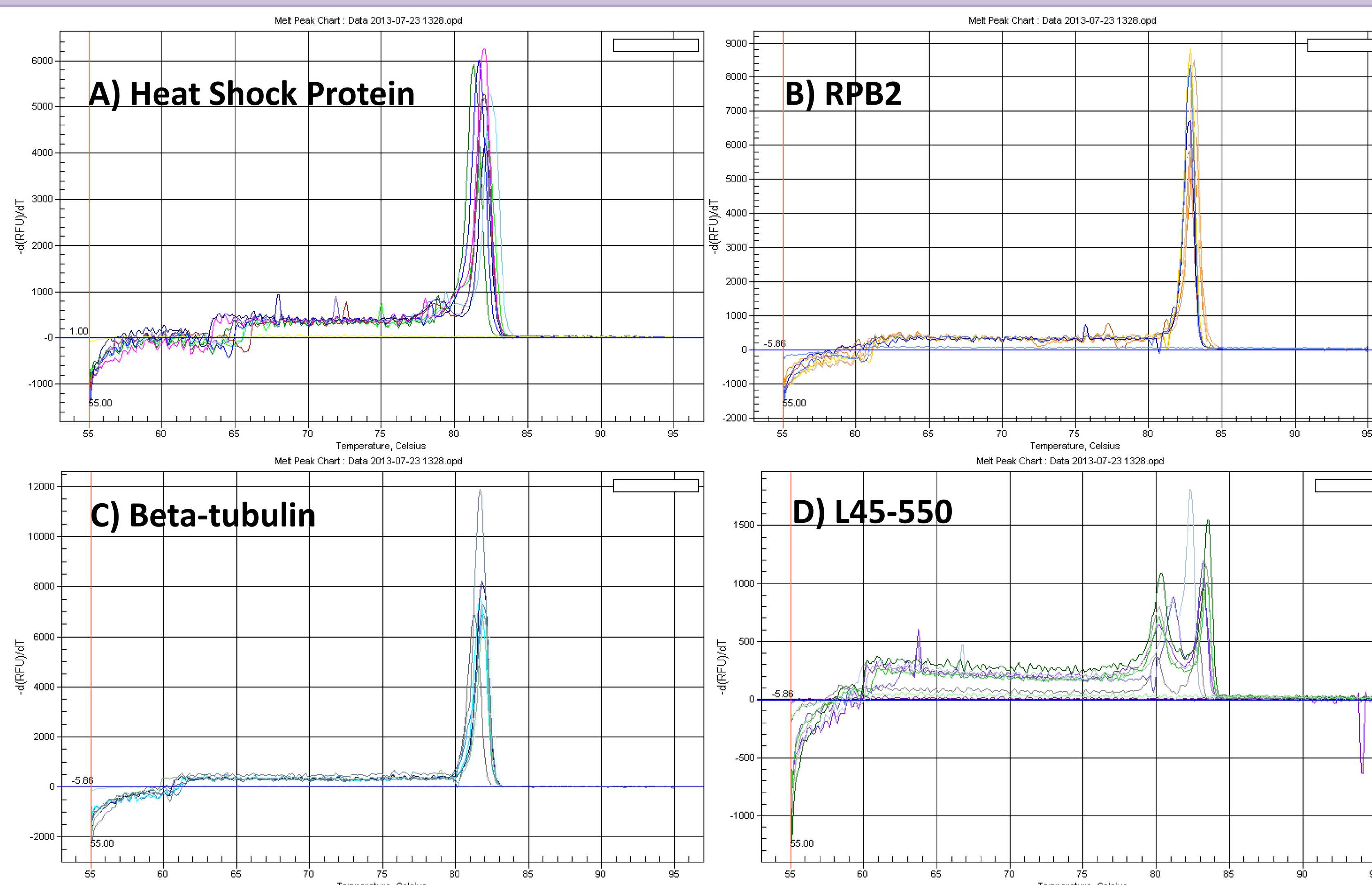

Figure 6. Melting curve generated for each gene using a specific primer set for each of six *Botrytis* species including *B. aclada, B. allii, B. byssoidea, B. cinerea, B. porri,* and *B. squamosa*. (A) The heat shock protein gene was used to design the primer set Bt-hspF1/Bt-hspR1a primer set. (B) The RPB2 gene was used to design the Bt-RPB2F1/Bt-RPB2R1 primer set. (C) The beta-tubulin gene was used to design the Bt-btF1a/Bt-btR1 primer set. (D) The L45-550 DNA region was used to design the BA2f/BA1r primer set (Nielsen et al., 2002). See Table 1 for primer sequences.

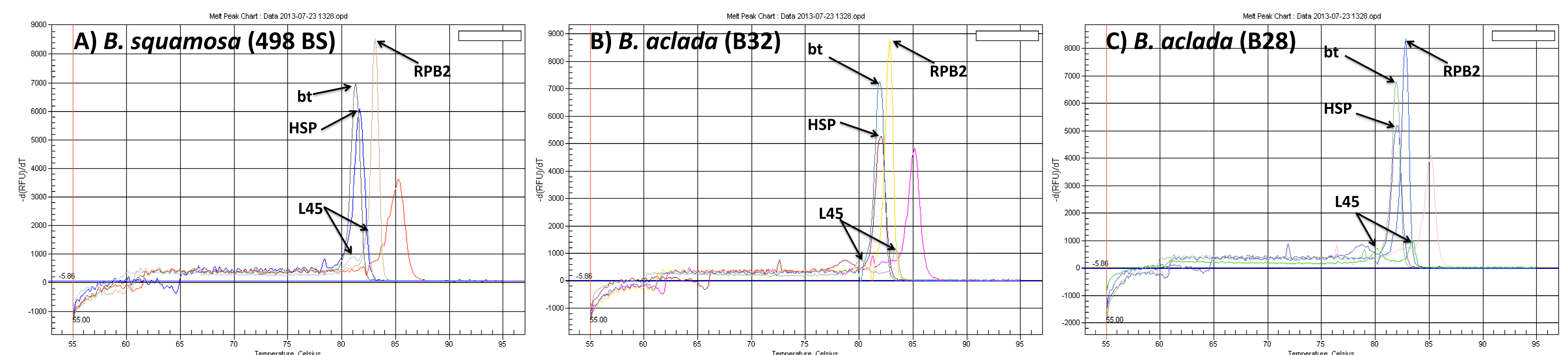

Figure 7. Melting curves for *Botrytis squamosa* or *B. aclada* with each primer set. The melting point values are shown in Table 2. Each curve includes traces for each gene of interest. (B) and (C) show peaks with similar amplitudes and location along the temperature scale.

TABLE 2: MELTING CURVE ANALYSIS SHOWING MELTING POINT(S) FOR ISOLATES OF EACH *BOTRYTIS* SPECIES

Species	Isolate Code	HSP F1/R1a	RPB2F1/R1	btF1a/r1	BA2f/1r
Botrytis aclada	B28	82.00ºC	82.80ºC	82.00ºC	80.20/83.50ºC
Botrytis aclada	B32	82.10ºC	82.80ºC	81.90ºC	80.20/83.40ºC
Botrytis allii	B347	82.20ºC	83.00ºC	81.90ºC	80.20/83.20ºC
Botrytis byssoidea	BB1	82.40ºC	83.00ºC	82.10ºC	81.20/83.20ºC
Botrytis cinerea	B651	81.30ºC	83.00ºC	81.70ºC	--
Botrytis porri	BP2	82.00ºC	82.90ºC	81.80ºC	80.00/82.80ºC
Botrytis squamosa	498 BS	81.70ºC	83.10ºC	81.30ºC	81.20/82.30ºC

CONCLUSIONS AND FUTURE DIRECTIONS

- A combination of primer sets that target different genes distinguished among *Botrytis* species that can infect onion. Two of the species appear to have identical melting points with one primer set, isolates of these two species can be differentiated using additional primer sets.
- Use of multiple primer sets designed from multiple target genes may enhance detection, accuracy, and reproducibility for these onion pathogens.
- Possible applications include detecting latent infections, identifying causal agents during disease outbreaks, sanitation, and onion breeding.
- The protocol of this assay could potentially be applied to the many bacterial pathogens that can cause onion bulb rots.

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