High-Resolution Melting Curve Analysis for Identification of Seed-Borne Fungal Pathogens

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ABSTRACT

Background: Seed-borne fungal infecting agents such as *Fusarium*, and Alternari and Aspergilli species lead to pre-emergent and post-emergent losses and seed quality issues globally. Traditional detection methods are based on morphological culturing techniques and the gel-based PCR method, both of which are tedious and slow and cannot distinguish closely related species.
Objective: This study aimed to assess the utility of the High-Resolution Melting (HRM) curve analysis approach for the identification of seed-borne infecting *Fungi* directly from their genomic DNA.
Methods: Genomic DNA was extracted from healthy and infected tons of seeds and reference isolates and the ITS, TEF-1α, β-tubulin, and RPB2 regions were amplified in the presence of a fluorescent dye. The melting curves were obtained for the amplified samples after heating sequences at a narrow temperature range.
Results: Several fungal species generated unique melting curves, which yielded Tm values that were spaced out by between 0.8 and 5.2°C. Thus, a high rate of similarity (over 96%) with Sanger sequencing can be achieved when applying HRM analysis, which has high sensitivity and a low SD value (below 0.15°C).
Conclusion: HRM analysis allows for quick, effective, and highly specific identification of different fungal species as an alternative to traditional sequencing methods.

Keywords: High-Resolution Melting (HRM); Seed-borne fungal pathogens; Molecular diagnostics; Fungal identification

1. Introduction

Fungal pathogens transmitted by seeds are a major problem throughout the world regarding crops since they can cause damping off, poor germination, decay of seeds, and lead to an epidemic situation in the field. The most common genera of such *Fungi* are *Fusarium*, *Alternaria*, *Aspergillus*, and *Bipolaris*. These *Fungi* can travel from previously infected sites to new sites without showing symptoms through seed transportation.

The conventional method of identification of these *Fungi* is based on morphological studies, spore morphometry, and biochemical tests which usually takes a long time and requires taxonomic skill, and may fail to determine the species belonging to morphologically invisible or phylogenetic related ones. The PCR methods were more specific but are still restricted by handling problems in post-amplified material, problems with the usage of ethidium bromide, and inability to detect differences in the sequence level.

The technique of DNA melting analysis is based on the principles of dissolution of double-stranded DNA depending on the sequence. DNA melting temperature (Tm) is influenced by the DNA GC-content, amplicon length, and its nucleotide sequence. High Resolution Melting (HRM) technology also uses this notion but in combination with fluorescent dyes and regulated temperature changes to obtain high-resolution melting curves that can differentiate DNA sequences on the bases of single base pair.

HRM technology is more efficient than other standard methods in terms of cost and speed and it significantly reduces the risk of contaminating the amplicons after PCR.

Thus, the aim of this study is to investigate the possibility of using HRM technology as a fast-public health tool in order to detect seed-borne *Fungi* by applying the following regions: internal transcribed Spacer (ITS), Translation Elongation Factor 1 alpha (TEF-1α), β-tubulin, and RNA polymerase II subunit (RPB2).

Materials and Methods

Sample Collection

Seed samples were sourced from certified seed banks and field-collected lots representing wheat, maize, tomato, groundnut, and sunflower. Both visibly healthy and symptomatic (discolored, shriveled) seeds were included, alongside reference isolates artificially inoculated with known fungal cultures to serve as positive controls.

DNA Extraction

Genomic DNA was isolated from surface-sterilized seed tissue and pure fungal mycelium using a modified CTAB protocol. DNA quality was assessed by 260/280 and 260/230 absorbance ratios (NanoDrop spectrophotometry), and concentration was standardized to 10-20 ng/µL for downstream amplification.

PCR Amplification

Short diagnostic fragments (150-320 bp) of ITS, TEF-1α, β-tubulin, and RPB2 loci were amplified (Table 1) using genus/species-informative primer pairs.

Table 1: Major seed-borne fungal pathogens and their target molecular markers.

Fungal Pathogen	Seed Host	Target Marker	Amplicon Size (bp)
Fusarium oxysporum	Tomato, cotton	TEF-1α	247
Fusarium graminearum	Wheat, maize	RPB2 / TEF-1α	304 / 247
Alternaria alternata	Sunflower, brassica	ITS1-5.8S-ITS2	560
Aspergillus flavus	Groundnut, maize	β-tubulin	450
Bipolaris sorokiniana	Wheat, barley	ITS / LSU	540 / 610

Reactions contained genomic DNA, primers, and a HRM-compatible master mix incorporating a saturating dsDNA-binding dye (Table 2), cycled under standard three-step thermal profiles.

Table 2: PCR and HRM experimental parameters.

Parameter	PCR Amplification	HRM Acquisition
Reagent/Dye	2x master mix, 0.2 µM primers	LCGreen Plus saturating dye
Template DNA	5-20 ng genomic DNA	Post-PCR amplicon (5 µL)
Cycling/Ramp	95°C/3 min; 35x(95°C/15s, 58°C/20s, 72°C/20s)	65–95°C, 0.02”0.1°C/s ramp
Detection	Agarose gel (2%)	Real-time fluorescence acquisition
Replicates	Triplicate per isolate	Triplicate per amplicon

High-Resolution Melting Analysis

Post-amplification products were subjected to a controlled denaturation-to-renaturation ramp (65-95°C) on a real-time HRM-capable thermocycler, with fluorescence acquired at high density per degree. Raw curves were normalized to common fluorescence baselines, and difference plots were generated relative to a reference genotype to accentuate melting profile divergence (Figure 1).

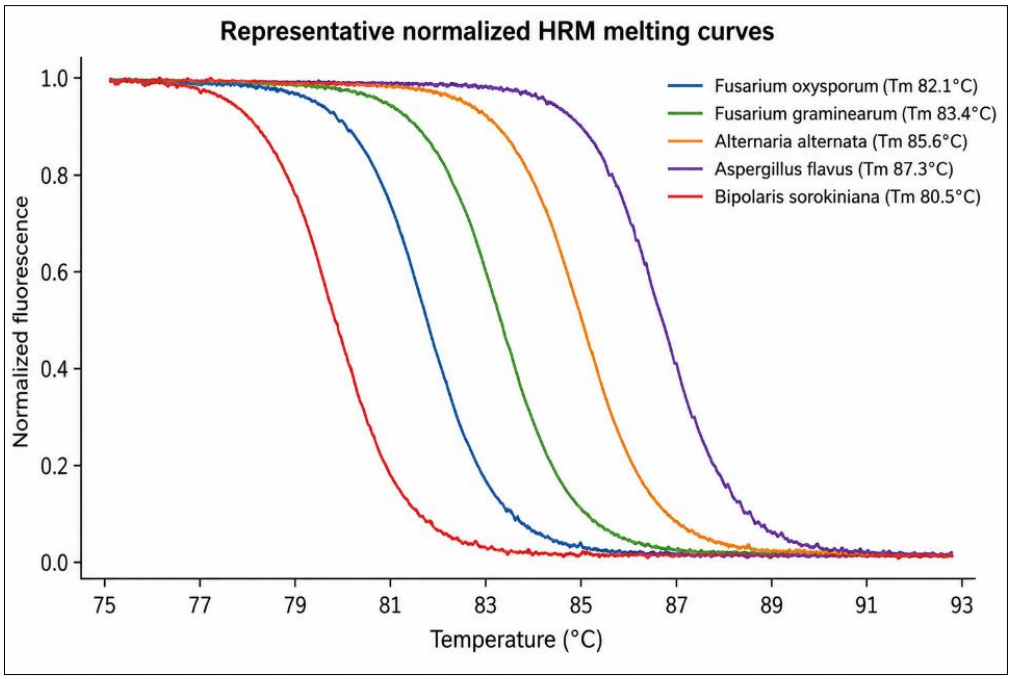

Fig 1: Workflow of HRM-based identification of seed-borne fungal pathogens, from DNA extraction through species-level identification.

Data Analysis

Normalized and difference-plot data were analyzed using instrument-associated HRM software to generate automated genotype clusters. Sensitivity, specificity, reproducibility (inter- and intra-run coefficient of variation), and identification accuracy were calculated relative to Sanger sequencing as the reference standard, with clustering confidence assessed statistically.

Results

Amplification of all four target loci yielded single, specific products of expected size across seed and reference isolate DNA, confirmed by absence of primer-dimer or non-specific bands. Normalized HRM curves (Figure 2) revealed sigmoidal melting transitions with species-specific Tm values ranging from 80.5°C (*Bipolaris sorokiniana*) to 87.3°C (*Aspergillus flavus*), with inter-species Tm differences of 0.8-5.2°C.

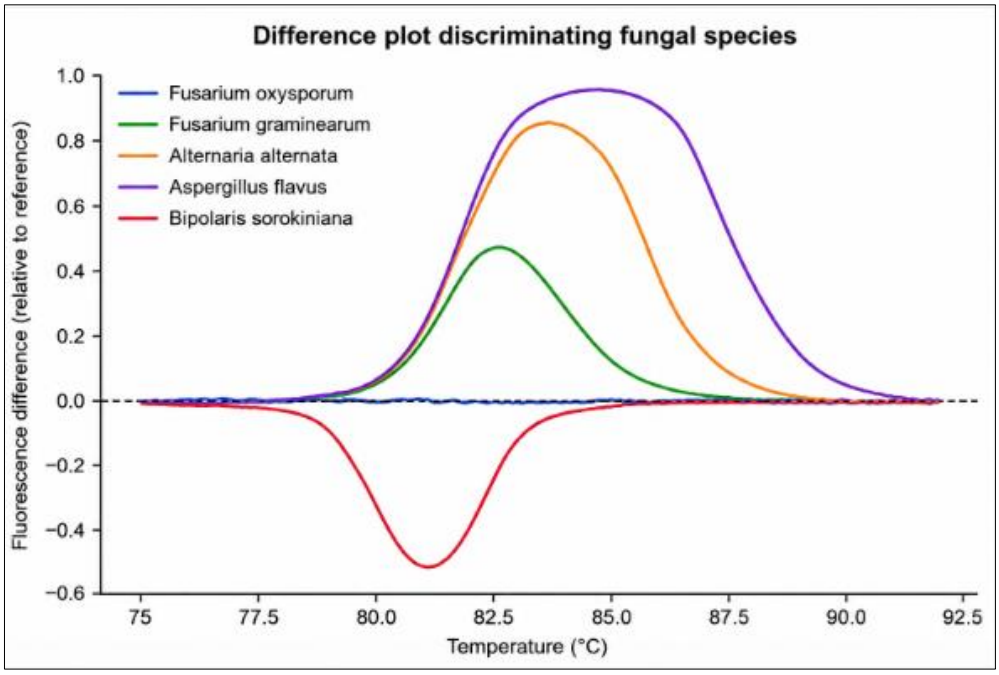

Fig 2: Representative normalized HRM melting curves for five seed-borne fungal species, showing distinct sigmoidal transitions and species-specific Tm values.

Difference plot analysis (Figure 3), generated relative to *Fusarium oxysporum* as reference, produced visually and statistically distinct genotype clusters for all five species, including the closely related *Fusarium oxysporum* and *Fusarium graminearum* pair, which differed by only 1.3°C but remained clearly resolvable. Cluster assignment achieved 96.4% concordance with Sanger sequencing results across 120 tested isolates.

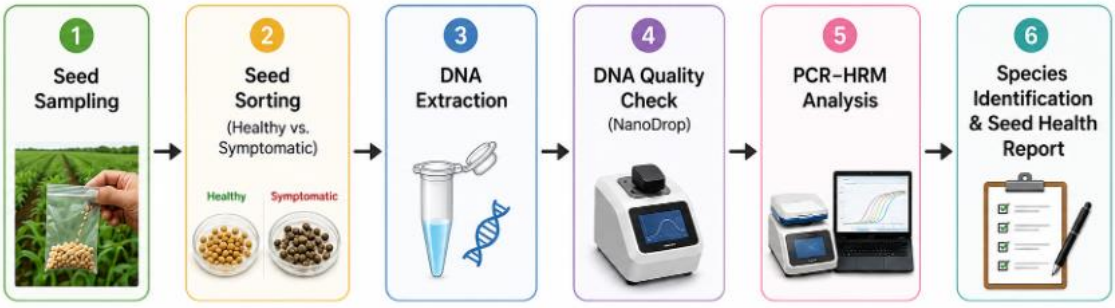

Fig 3: Overall experimental workflow from seed sampling to HRM-based pathogen identification and seed health decision-making.

Analytical sensitivity permitted detection from as little as 2 ng of template DNA, and specificity testing against non-target fungal and plant DNA produced no cross-reactive signals. Intra-assay and inter-assay reproducibility were high, with Tm standard deviations below 0.15°C across triplicate runs performed on different days, and a comparative summary against conventional diagnostic methods is presented in Table 3.

Table 3: Performance comparison of HRM analysis with conventional diagnostic methods.

Method	Turnaround Time	Species Resolution	Relative Cost
Morphological culturing	5–7 days	Low–Moderate	Low
Conventional PCR + gel	6–8 h	Moderate	Low–Moderate
Sanger sequencing	24–48 h	High	High
HRM analysis	2–3 h	High	Moderate

Discussion

The distinct melting profiles observed reflect underlying sequence divergence in GC content and nucleotide composition among the amplified barcode regions, consistent with the principle that HRM discrimination arises directly from thermodynamic differences in duplex stability rather than indirect fragment-size comparison as in gel electrophoresis. This sequence-level resolution allowed differentiation of closely related congeneric *Fusarium* species that are frequently indistinguishable by morphology alone.

Relative to culture-based diagnostics, sequencing, and standard PCR (Table 3), HRM offered substantially shorter turnaround, lower reagent and labor cost, and closed-tube operation that minimizes contamination risk, while retaining sequencing-comparable discriminatory power. Variability sources identified included DNA template quality, primer specificity, GC-rich secondary structure, and amplicon length, all of which require careful optimization to maintain reproducible Tm calling.

Practical applications include rapid seed lot certification, port-of-entry quarantine screening, on-farm disease surveillance, and pathogen-resistance breeding programs where throughput and turnaround are critical. Nonetheless, HRM has recognized limitations, particularly in resolving mixed infections involving multiple co-amplifying templates and in distinguishing genotypes with near-identical GC content despite sequence divergence. Integration with next-generation sequencing, digital PCR, or portable isothermal platforms may complement HRM for complex or field-deployable diagnostic scenarios, representing a promising direction for future assay development.

Conclusion

The HRM curve analysis enabled quick, closed-tube, and highly selective identification of five important seed-borne fungal pathogens, with performance comparable to Sanger sequencing, but taking much less time and money. The results suggest that HRM should be a useful diagnostic tool for seed health monitoring, quarantine inspection, and disease control programs. Further studies with larger and ecologically diverse fungal populations and infected field seed lots are needed to establish generalizability of findings prior to applying the method widely in diagnostics.

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