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Pasquale Tripodi *Editor*

# Crop Breeding

## Genetic Improvement Methods

Humana Press

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# Crop Breeding

## Genetic Improvement Methods

Edited by

**Pasquale Tripodi**

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# Preface

The challenges of modern agriculture require increasingly innovative investigation methodologies to be applied to the genetic improvement of crops. Cutting-edge technologies for genome sequencing and plant phenotyping along with computational and bioinformatic tools are speeding the throughput and deepness of the investigation of complex traits. Genome editing approaches are increasingly applied for genetic improvement in many plant species. The successful use of these methodologies requires solid lab-based knowledge in order to prepare the experimental study populations, samples for analysis as well to develop an accurate workflow. The complexity of the methods requires deep specialization and greater interdisciplinarity of skills.

The present volume "Crop Breeding: Genetic Improvement Methods" is a result of a collaboration with leading scientists from main international universities and research institutions working in the crop breeding sector.

Aiming at covering all topics, the present volume describes breeding methods for the development of biparental and multiparental mapping populations, lab protocols for high-throughput isolation of nucleic acids and metabolites, different high-performing genotyping approaches, mapping strategies for QTLs and mutation identifications, computational and bioinformatic pipelines, tissue culture-based and transformation methods for androgenesis, ploidy modification, and RNA interference. The book highlights recent developed genome editing protocols including CRISPR and TALEN methods and methodologies for in-field/in-soil plant phenotyping.

"Crop Breeding: Genetic Improvement Methods," therefore, cover all aspects, being addressed to the broadest audience of students, breeders, and scientists applying current protocols or interested in the knowledge of the described methodologies.

***Pontecagnano, SA, Italy*** ***Pasquale Tripodi***

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# Chapter 5

# High-Resolution Melting Analysis as a Tool for Plant Species Authentication

Liliana Grazina, Joana Costa, Joana S. Amaral, and Isabel Mafra

## Abstract

High-resolution melting (HRM) analysis is a cost-effective, specific, and rapid tool that allows distinguishing genetically related plants and other organisms based on the detection of small nucleotide variations, which are recognized from melting properties of the double-stranded DNA. It has been widely applied in several areas of research and diagnostics, including botanical authentication of several food commodities and herbal products. Generally, it consists of the main steps: (1) in silico sequence analysis and primer design; (2) DNA extraction from plant material; (3) amplification by real-time PCR with an enhanced fluorescent dye targeting a specific DNA barcode or other regions of taxonomic interest (100–200 bp); (4) melting curve analysis; and (5) statistical data analysis using a specific HRM software. This chapter presents an overview of HRM analysis and application, followed by the detailed description of all the required reagents, instruments, and protocols for the successful and easy implementation of a HRM method to differentiate closely related plant species.

**Key words** HRM, Species identification, Authenticity, Botanical origin, Food, Herbal products

## 1 Introduction

Recently, there has been a high number of reports worldwide and a growing evidence regarding the occurrence of fraudulent practices in products of plant origin. These include several foods of high economic importance, such as spices, wine, and olive oil, and different products containing medicinal plants, namely herbal infusions, traditional herbal medicines, and plant food supplements [1–4]. The increasing concern of stakeholders, such as regulatory entities, industries, and consumers, has prompt the development of different methods aiming at the botanical origin authentication. DNA-based methods have undoubtedly proved to be suited for the identification of species, presenting advantages over phenotypic and chemical approaches in terms of specificity and reliability. Advances in molecular biology techniques over the last couple of decades lead to the development of high-resolution melting

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(HRM) analysis, as a simple, fast, and cost-effective tool for plant species authentication.

HRM analysis is a post-PCR approach based on monitoring the gradual denaturation of double-stranded DNA (dsDNA) of amplified fragments, which allows detecting small nucleotide differences. It has emerged from high-resolution real-time PCR instrumentation and new-generation fluorescent dyes. EvaGreen, LCGreen-PLUS, SYTO®9, or ResoLight are examples of enhanced fluorescent DNA-binding dyes that can be used at higher concentrations than the classical SYBR Green I dye, resulting in enhanced fluorescence signals and increased sensitivity, without causing PCR inhibition [5–8]. High-resolution equipment, capable of small temperature increments, high acquisition rate, and high melting accuracy, as well as appropriate software is also required [6–9]. When dsDNA dissociates into single strands (ssDNA), the dye is released, causing a fluorescence decrease that is plotted against temperature, generating a melting curve (Fig. 1a). The melting curve profile and estimated melting temperature depend on the amplicon length, sequence, and GC content. The temperature at which half of the amplicons are single stranded is called melting temperature (Tm). The Tm can be determined from the conventional melting curve analysis, corresponding to the melt peak obtained by plotting the negative derivative of the fluorescence ($F$) over temperature ($T$) ($-\mathrm{d}F/\mathrm{d}T$) versus the temperature [6, 7] (Fig. 1b). Amplicons that substantially differ in length and/or nucleotide composition present distinct melting profiles and, consequently, different Tm, being easily differentiated by simply using standard instrumentation with the SYBR Green dye. However, when amplicons differ in just one or few nucleotides, they may present similar melting curve profiles with small shifts in Tm, disabling their differentiation. In such cases, further data treatment using specific HRM software is required to normalize data. This allows the removal of the fluorescence variance of the pre- and post-melting temperature regions, leaving the curve range between the bars as a new normalized plot that magnifies profile differences (Fig. 1c). To better visualize the differences between individual melting curves, some HRM software applications enable plotting the difference curve data obtained from subtracting the sample melting curves from a pre-defined reference set (Fig. 2).

A key issue in HRM analysis is the selection of the target region. Small-length amplicons (<300 bp) containing sequence variations, such as single-nucleotide polymorphisms (SNP) and small insertions or deletions, among a set of plant species or even cultivars are recommended [7]. DNA barcodes are informative short sequence of nuclear, plastidial, or mitochondrial regions with high potential to serve as taxon identifiers due to their genome low intraspecific and high interspecific variability [10]. The combination of HRM analysis with DNA barcodes, or more precisely mini-barcodes

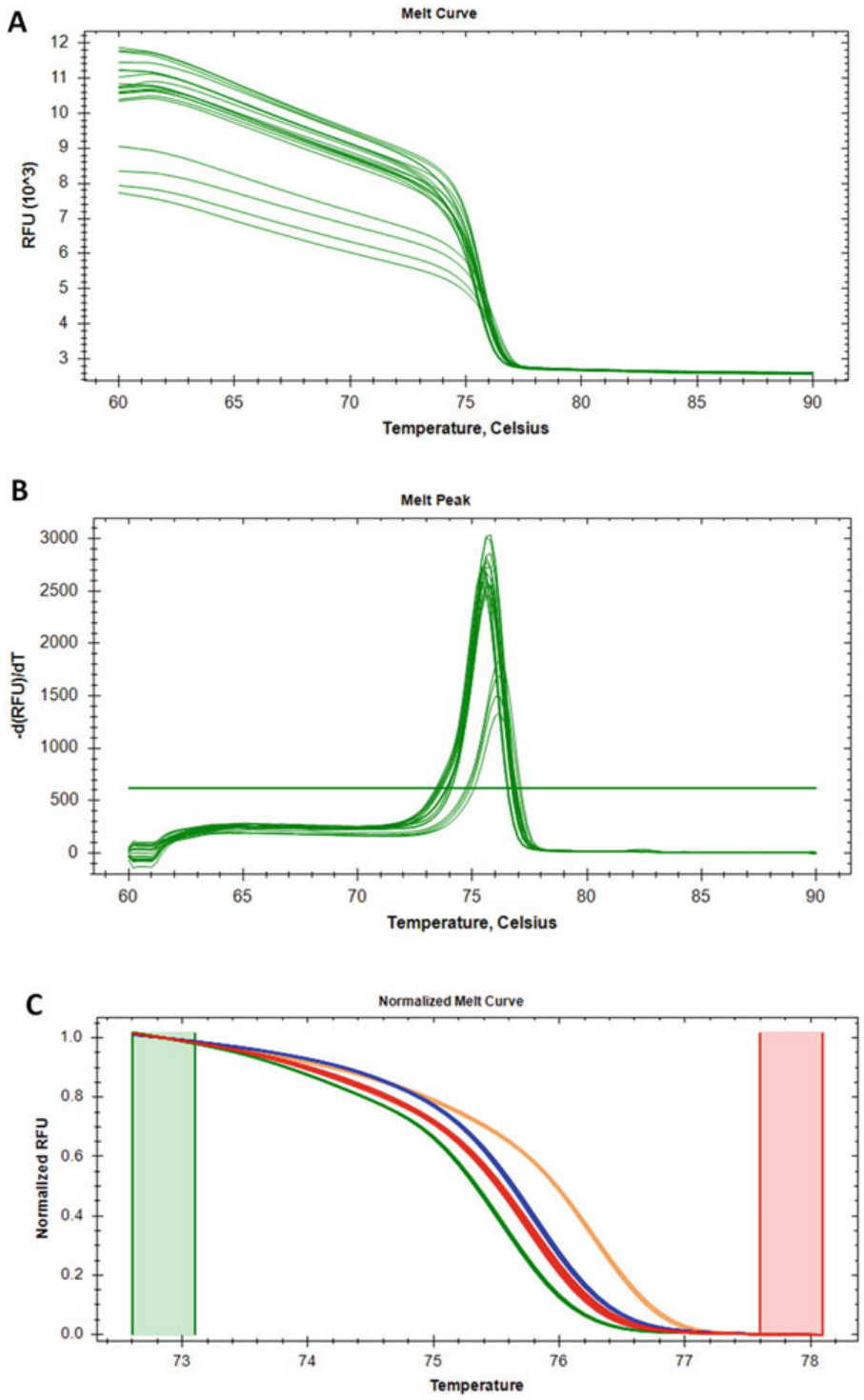


**Fig. 1** HRM analysis applied in the differentiation of *Lavandula stoechas*, *L. pedunculata*, *L. viridis*, and wild *Lavandula.* Raw (**a**) and derivative (**b**) and normalized (**c**) melting curves

(<300 bp), has been designated as Bar-HRM and considered as a powerful tool to differentiate among closely related plant species. In opposition to animal species, mitochondrial regions are not recommended for plants because they present low evolutionary rates and low nucleotide substitution. Therefore, nuclear and

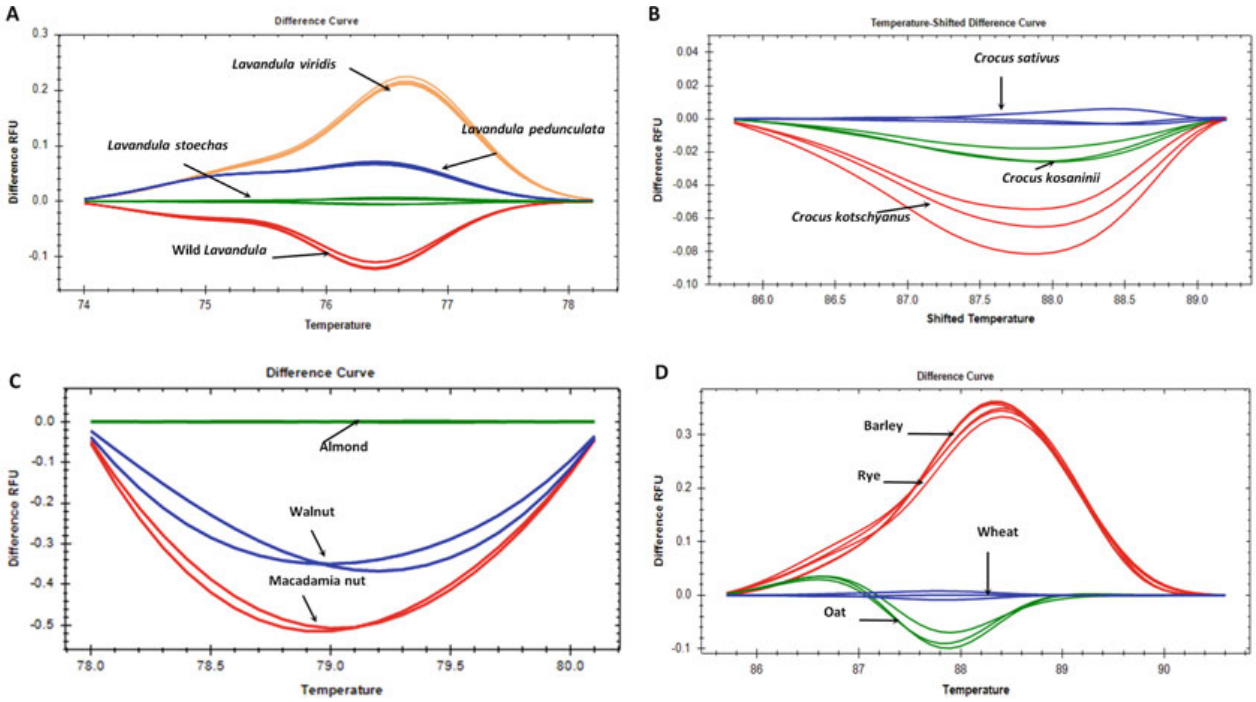


**Fig. 2** Application of HRM analysis (difference melt curves) in the discrimination of plant material at species (**a** and **b**) and genus (**c** and **d**) levels

plastidial regions, such as ribulose-1,5-bisphosphate carboxylase oxygenase (*rbcL*), maturase k (*matK*), intergenic spacer regions (*trnH-psbA*), and internal transcribed spacers 1 and 2 (ITS, ITS2), have been proposed as alternative barcodes for plants. The choice of the best region is often an arduous and challenging task since there is no single locus that works as a universal plant barcode [10–12].

The use of DNA-mini-barcodes coupled with HRM analysis has been successfully applied in the discrimination of plant species in various products, including *Lavandula* spp. to determinate the botanical origin of honey [13] (Fig. 2a), different *Crocus* spp. in commercial saffron spices [14] (Fig. 2b), *Tinospora* spp. to authenticate herbal medicines [15], and *Hypericum* spp. to authenticate herbal infusions [3]. HRM analysis targeting an allergen-encoding gene was successfully applied to discriminate *Prunus dulcis* from other tree nuts [16] (Fig. 2c) and to differentiate wheat (*Triticum* spp.) from other gluten-containing cereals (rye, barley, and oat) in gluten-free foods [17] (Fig. 2d). Overall, HRM analysis is considered a fast and reliable tool to discriminate among closely related plant species, being considered also a cost-effective and high-throughput approach since it does not require any post-PCR analysis or sequencing as in several other DNA-based methods.

## 2 Materials

Prepare all solutions using ultrapure water (deionized water) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Use molecular biology-grade consumables (e.g., tips, reaction tubes, PCR tubes, real-time PCR strips and caps) for DNA extraction and PCR analysis (sterile, DNase and RNase free). The rest of the materials and consumables, which can be bought in non-sterile conditions, should be chemically decontaminated (e.g., DNA-ExitusPlus, AppliChem GmbH, Darmstadt, Germany) or in-house autoclaved (121 °C, 15 min). The use of a PCR workstation, especially when manipulating the DNA extracts and PCR reagents, as well as when performing all tasks associated with the preparation of PCR or real-time PCR mixes, is highly recommended. All waste disposal regulations should be followed when disposing waste materials.

### 2.1 Target Genes and Software

1. Depending on the selected plant species to be differentiated, different coding genes (*rbcL* and *matK*), as well as noncoding spacers (*trnH-psbA*, ITS and ITS2), can be tested to discriminate DNA sequences at species level [18].
2. Table 1 lists software applications that can be used to search for the available DNA sequences, within genes or regions with high sequence homology, but having enough interspecific variability.

### 2.2 Reagents

1. DNA extraction: Nucleospin Plant II (Macherey-Nagel, Düren, Germany) DNA extraction kit (for alternatives *see* **Note 1**).
2. PCR mix: SuperHot *Taq* DNA polymerase (e.g., Genaxxon Bioscience GmbH, Ulm, Germany), chemically inactivated prior to an activation step (normally at 95 °C for several minutes), including respective 10× buffer and 25 mM of $MgCl_2$; PCR-grade water; 10 mM of dNTP mix and primers (forward and reverse) synthesized outsourced (e.g., Eurofins Genomics, Ebersberg, Germany).
3. Agarose gel electrophoresis of PCR products: 1.5% of agarose in 1× SGTB (Grisp, Porto, Portugal) or 2% agarose in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer with 1× GelRed (Biotium Inc., Hayward, CA, USA); DNA marker (e.g., DNA 100 bp marker, Bioron GmbH, Römerberg, Germany); loading buffer (4% (w/v) sucrose, 0.05% (w/v) bromophenol blue, 0.12 M EDTA).
4. Purification of PCR products: GRS PCR and Gel Band Purification kit (Grisp, Porto, Portugal).

**Table 1**
**Examples of algorithms available online for free use listed according to application**

| Software | Description | URL |
|---|---|---|
| Sequence databases | | |
| NCBI database | National Center for Biotechnology Information provides access to biomedical and genomic information | https://www.ncbi.nlm.nih.gov/ |
| Sequence alignment | | |
| BLASTn | Finds regions of similarity between nucleotide sequences to sequence databases and calculates their statistical significance | https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch |
| ClustalW | Multiple Sequence Alignment | https://www.genome.jp/tools-bin/clustalw |
| MEGA | Molecular Evolutionary Genetics Analysis | https://www.megasoftware.net/ |
| Clustal Omega | Multiple Sequence Alignment | https://www.ebi.ac.uk/Tools/msa/clustalo/ |
| BIOEDIT vs7.2 | Biological Sequence Alignment Editor | https://bioedit.software.informer.com/versions/ |
| Primer design | | |
| Primer-Blast | Design primers specific to PCR template | https://www.ncbi.nlm.nih.gov/tools/primer-blast/ |
| Primer3 | Pick primers from a DNA sequence | http://bioinfo.ut.ee/primer3-0.4.0/ |
| GenScript Online PCR Primers Designs Tool | Online tool to design PCR primers | https://www.genscript.com/tools/pcr-primers-designer |
| Eurofins Genomics PCR Primer Design Tool | PCR primer design tool analyzes the entered DNA sequence and chooses the optimum PCR primer pairs | https://www.eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design/ |
| Primer3Plus | Select primer pairs to detect the given template sequence | http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi |
| Primer properties | | |
| OligoCalc | Provide information regarding the physical properties of oligonucleotides, self-complementarity, and hairpin loop formation | http://biotools.nubic.northwestern.edu/OligoCalc.html |
| OligoEvaluator | Provide information about basic physical properties of oligonucleotides, formation of secondary structure, and primer dimer | http://www.oligoevaluator.com/LoginServlet |

(continued)

**Table 1**
**(continued)**

| Software | Description | URL |
|---|---|---|
| Sequencing analysis | | |
| FinchTV | Viewing trace data from Sanger DNA Sequencing (scf or ab1 file formats) | https://digitalworldbiology.com/FinchTV |
| MEGA | Molecular Evolutionary Genetics Analysis | https://www.megasoftware.net/ |
| BIOEDIT vs7.2 | Biological Sequence Alignment Editor | https://bioedit.software.informer.com/versions/ |

5. Mix for real-time PCR with HRM: Use pre-prepared mixes for real-time PCR containing all the reaction components (enzyme, buffer, dNTP, and $Mg^{2+}$) and the new-generation fluorescent DNA-binding dye (e.g., EvaGreen) (e.g., SsoFast EvaGreen Supermix, Bio-Rad Laboratories, Hercules, CA, USA) (other alternatives, *see* **Note 2**); PCR-grade water; 10 mM of dNTP mix; and primers (forward and reverse) synthesized outsourced (e.g., Eurofins Genomics, Ebersberg, Germany).

### 2.3 Equipment

1. Refrigerated centrifuge (e.g., Heraeus Fresco 17, Thermo Scientific, Osterode am Harz, Germany).
2. Thermomixer block (e.g., Thermomixer comfort, Eppendorf AG, Hamburg, Germany).
3. Water bath (0–110 °C).
4. Vortex stirrer.
5. Microplate reader UV/Vis spectrophotometer, with microvolume plate accessory for nucleic acid and protein quantification (Synergy HT with Take 3 plate, BioTek, Winooski, VT, USA) (*see* **Note 3**).
6. Electrophoresis apparatus (electrophoresis tank and power supply).
7. PCR workstation with UV-cleaner-recirculator, UV light, and white lamp (e.g., VWR International GmbH, Darmstadt, Germany).
8. UV light photographic system (e.g., UV light tray Gel Doc™ EZ Imager, Bio-Rad Laboratories, Hercules, CA, USA).
9. Thermal cycler (e.g., MJ Mini personal thermal cycler, Bio-Rad Laboratories, Hercules, CA, USA).
10. High-resolution real-time PCR instrumentation (e.g., CFX96 real-time PCR system, Bio-Rad Laboratories, Hercules, CA, USA) capable of reading one fluorophore (FAM or SYBR

Green) and respective software for real-time PCR data treatment (Bio-Rad CFX manager 3.1, Bio-Rad Laboratories, Hercules, CA, USA), combined with the specific HRM software (Precision Melt Analysis version 1.3, Bio-Rad Laboratories, Hercules, CA, USA) (other alternatives, *see* **Note 4**).

## 3 Methods

### 3.1 In Silico Analysis

1. Select the gene or DNA region for the potential discrimination of the target species and check if there are consensus sequences available at NCBI database. For this purpose, the BLASTn algorithm (Table 1), also at NCBI database, can be used to search for DNA sequences based on their similarity.
2. Download and align the selected sequences using an alignment algorithm (e.g., BIOEDIT vs7.2) (Table 1) (*see* **Note 5**). Within alignment, search for regions of high homology to design primers, but make sure that the amplicons will have some nucleotide mismatches within the entire sequence to allow interspecific variability.
3. Design primers either manually or using primer designing tools, such as Primer-Blast (Table 1). Verify primers' proprieties (physicochemical parameters, absence of hairpins, 3′ complementary, and self-annealing) using specific algorithms (e.g., OligoCalc) (Table 1). Check the complementary of the designed primers toward the target sequences using the software Primer-Blast (Table 1) (*see* **Notes 6–8**).
4. Order primer synthesis in specialized outsourcing facilities (e.g., Eurofins Genomics, Ebersberg, Germany). This step can take 2 or 3 days, depending on the selected production facility.

### 3.2 DNA Extraction

1. To extract DNA from plant material, select an appropriate DNA extraction method, such as Nucleospin Plant II (Macherey-Nagel, Düren, Germany) (*see* **Notes 9** and **10**). Follow kit instructions performing minor alterations, if necessary. The example given below follows the protocol with PL1 buffer.
2. Weigh 20–100 mg of grounded (lyophilized or dried) plant material in a 2.0 mL sterile reaction tube. Add 400 μL of PL1 buffer (preheated at 65 °C) to each tube, make strong vortex, and incubate for 1 h at 65 °C in thermomixer (900 rpm). Make frequent vortex to the samples during the lysis.
3. After incubation, leave tubes at room temperature and add 10 μL of RNase A (10 mg/mL) for 5 min (other conditions can be used, *see* **Notes 11** and **12**).

4. Centrifuge the tubes at 4 °C, 17,000 × *g*, for 10 min. Remove the supernatant carefully to a new tube, transfer it to a Nucleospin filter column, and centrifuge for 2 min at 11,000 × *g* at room temperature.
5. Collect the filtrate to a 1.5 mL sterile reaction tube and add 450 μL of PC buffer (DNA-binding buffer). Mix gently by pipetting and transfer the entire volume to the Nucleospin plant II column. Centrifuge for 1 min, at 11,000 × *g* at room temperature, and discard the flow through (be aware that the column has a maximum volume of 700 μL, so it can only be loaded with 700 μL each time; repeat loading until the entire volume of sample has passed the column).
6. Wash the silica membrane (Nucleospin plant II column) with 400 μL of PW1 and centrifuge for 1 min at 11,000 × *g* (room temperature), discarding the flow through (first washing step).
7. Wash the silica membrane (spin column) with 700 μL and 200 μL of PW2, and centrifuge for 1 and 2 min at 11,000 × *g* (room temperature), respectively (second and third washing steps). After each centrifugation, always discard the flow through. Make sure that the column is dry after the final 2-min centrifugation (residues of ethanol will damage DNA extracts).
8. Place column in a new 1.5 mL sterile reaction tube, add 50 μL of elution buffer (PE) preheated at 65 °C, and incubate for 5 min at 65 °C. Elute through 1-min centrifugation at 11,000 × *g* (room temperature). Repeat last step, in order to obtain 100 μL of DNA extract.

### 3.3 Determination of DNA Yield and Purity

1. Use a microplate UV/Vis spectrophotometer instrument (BioTek Instruments, Inc., Winooski, VT, USA), starting with the calibration of the Take 3 microvolume plate accessory (16 spots) with 4 μL of pure water (e.g., PCR water).
2. Place 4 μL of each DNA extract (in duplicate) on the plate spot and read absorbencies at 260, 280, and 320 nm using the UV/Vis spectrophotometer microplate reader. The yield and purity of each DNA extract will be determined automatically, following the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Winooski, VT, USA).
3. Dilute DNA extracts to a specific concentration (in the case of extracts from plant material, final DNA concentration of 5–10 ng/μL is highly recommended). Store DNA extracts and dilutions at −20 °C until analysis.

### 3.4 Qualitative PCR

1. Prepare the reaction mix by the addition of all the components for a total volume of 25 μL. Each reaction mix must contain PCR-grade water (volume adjusted to the amount of remaining reagents), buffer 10× (2.5 μL), 10 mM of dNTP (2.0 μL), $MgCl_2$ (final concentration of 1.5 up to 3.0 mM), primers (100–500 nM), and 1.0 U of SuperHot Taq DNA polymerase (0.2 μL).
2. Distribute 23 μL of reaction mix by each well or tube and add 2 μL of DNA template (10–20 ng). Positive (DNA from target species) and negative (no-template DNA) controls should be included. Close wells or tubes and place them on the thermal cycler.
3. Define program of temperatures in the thermal cycler. Example of a program: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 65 °C (this temperature must be previously optimized along with the $Mg^{2+}$ concentration for each primer pair) for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min. The number of cycles must also be optimized for each primer pair.
4. To visualize the obtained amplicons, prepare a 1.5% agarose gel stained with GelRed 1× (Biotium Inc., Hayward, CA, USA). Mix 5–20 μL of PCR product with 1–4 μL loading buffer, apply to gel wells, and run electrophoresis using SGTB 1× (Grisp, Porto, Portugal) for 25–30 min at 200 V. For each gel, use a 100 bp DNA marker. If the DNA marker is not pre-stained, add loading buffer.
5. After the electrophoresis, visualize the agarose gel with a UV light tray Gel Doc EZ Imager using GelRed dye protocol. Record a digital image with Image Lab software version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA) and analyze the results.

### 3.5 Real-Time PCR with HRM Analysis

1. When performing a real-time PCR run (e.g., CFX96 Real-Time PCR system) with HRM analysis, set the program of temperatures and design the plate following the steps defined by the software (e.g., Bio-Rad CFX manager 3.1).
2. Open the wizard setup of Bio-Rad CFX manager 3.1, and define the program of temperatures as the protocol. This program must include the real-time PCR amplification, followed by the melt curve. An example of program is presented in Fig. 3 (*see* **Note 13**).
3. Prepare the plate, by selecting the correct fluorophore (SYBR Green) and the plate type (white for white strips/plate, clear for clear strips/plates). Set the number of samples and replicates (3–4 replicates per sample by run are recommended), by defining their place in the plate (example in Fig. 4) (*see* **Note 14**).

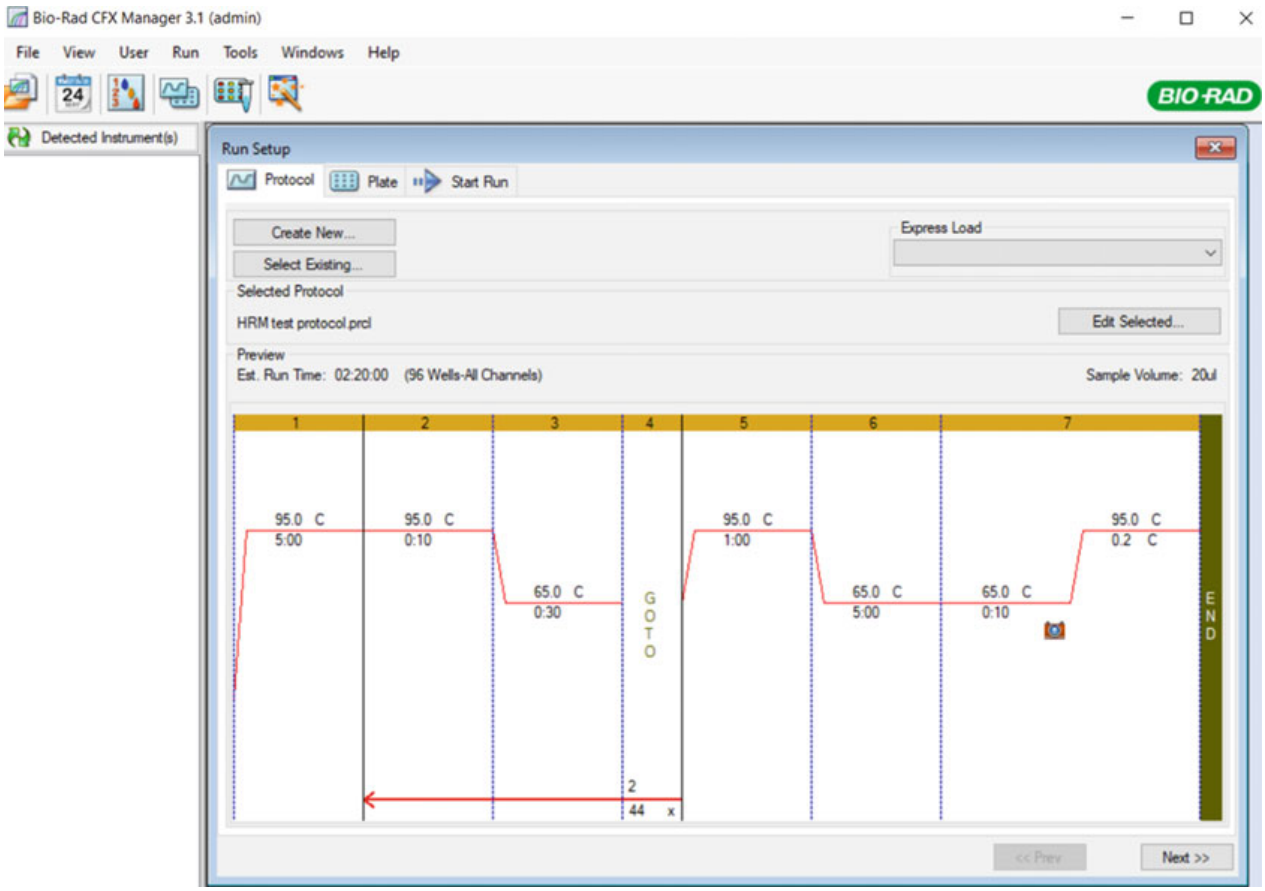


**Fig. 3** Example of a real-time PCR program of temperatures with melt curve protocol adjusted for posterior HRM analysis

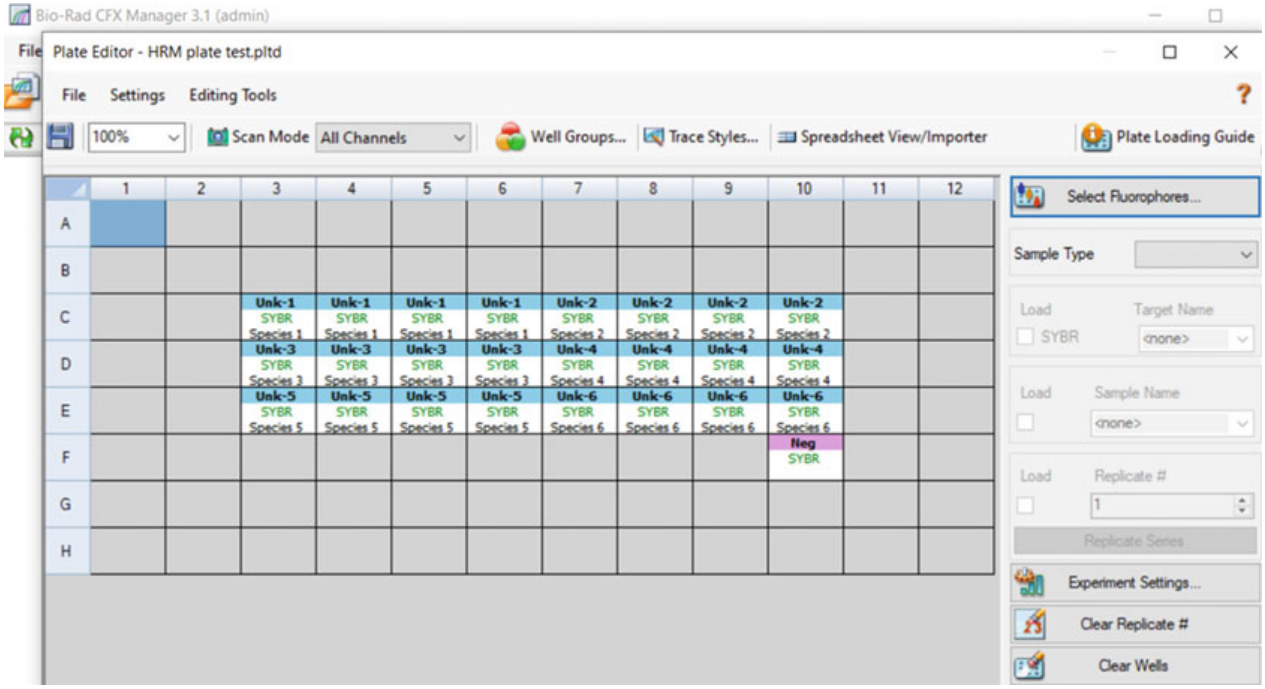


**Fig. 4** Example of a real-time PCR plate

4. Prepare the reaction mix by adding all the components needed for all wells, except the DNA template. Each reaction mix must contain PCR-grade water (volume adjusted to the amount of remaining reagents), 1× of SsoFast EvaGreen Supermix (10 μL), and primers (100–500 nM) for a total of 20 μL reaction volume.
5. Place the necessary number of strips (or plate), distribute 18 μL of reaction mix per each well, and add 2 μL of DNA extract. Include a negative control (no-DNA template). Close wells and use a PCR spinner to ensure that all volume is at the bottom of the wells. Place the reaction strips (or plate) on the thermal cycler and start run.
6. After finishing the real-time PCR run, open the Precision Melt Analysis version 1.3 and create a new melt file by choosing the real-time PCR file recently generated by Bio-Rad CFX manager 3.1. Save the newly generated melt file.
7. Open the melt file and analyze the results (example in Fig. 5). The file is generated using predefined automated parameters, which might be adjusted with respect to the type of analysis that is being processed (*see* **Notes 15–18**).

### 3.6 DNA Sequencing for Method Validation

1. To validate HRM analysis, Sanger sequencing of PCR products of template species (species under study) is recommended (*see* **Notes 19** and **20**).
2. Follow the steps described in this section regarding "Qualitative PCR" to obtain the PCR products from the template species. Use a purification kit (e.g., GRS PCR and Gel Band Purification kit, Grisp, Porto, Portugal) in order to purify the amplified PCR products (removal of components from amplification reaction) according to manufacturers' instructions.
3. Send the purified PCR products for direct sequencing of both strands in opposite directions of each target amplicon in specialized research facilities (Eurofins Genomics, Ebersberg, Germany).
4. Check the quality of electropherograms using FinchTV software (or MEGA software) (Table 1). Only electropherograms with high quality should be analyzed and further aligned (BIOEDIT or MEGA software) (Table 1).
5. Critically analyze HRM results in relation to sequencing data.

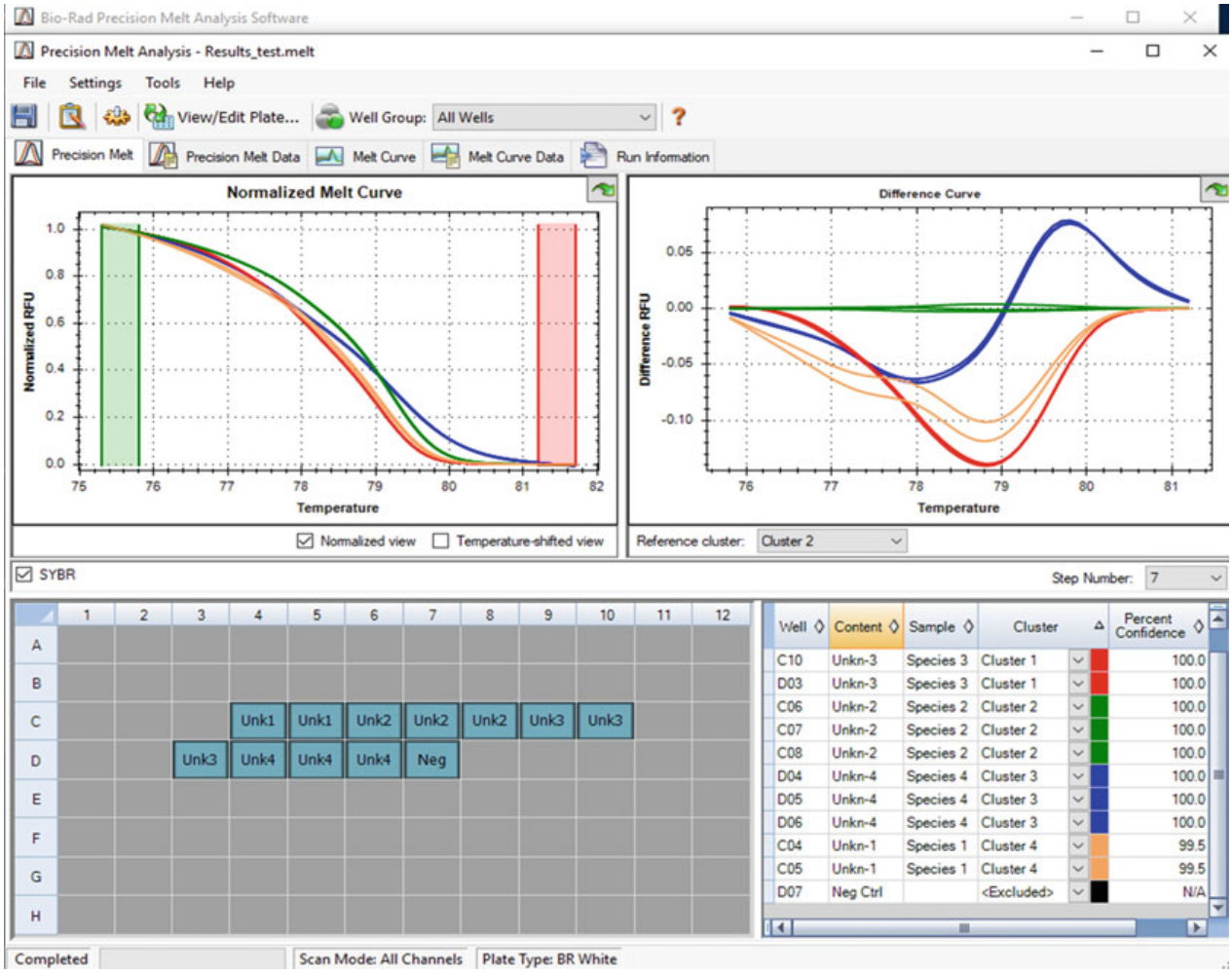


**Fig. 5** Example of a melt file generated with the Precision Melt Analysis version 1.3, showing the normalized melt curve, the difference curve, the plate, and the classification of species by cluster (and respective degree of confidence)

## 4 Notes

1. Several commercial kits specialized in the DNA extraction of plant material can be used as alternative to Nucleospin Plant II (Macherey-Nagel, Düren, Germany) to extract DNA from plant source material, namely DNeasy Plant Mini kit (Qiagen GmbH, Hilden, Germany), E.Z.N.A. plant DNA DS Mini kit (Omega Bio-Tek, Inc., Norcross, GA, USA), and E.Z.N.A. SP Plant DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA). Some of the commercial kits use lysis buffers with cationic (cetyltrimethyl ammonium bromide, CTAB) or anionic (sodium dodecyl sulfate, SDS) detergents, which can be combined with spin columns with silica-based membranes for retrieving high-purity DNA extracts. Nonetheless, most of these commercial kits allow extracting DNA with higher yield than most in-house-developed methods like the CTAB method, thus providing high-yield, -quality, and -purity plant

DNA extracts. Based on bead-beating technology, instead of the common detergents such as CTAB, DNeasy Plant Pro kit (Qiagen GmbH, Hilden, Germany) can also be considered a potential choice to extract high-quality cellular DNA from plant cells, tissues, and seeds. Besides commercial kits, the in-house-developed methods like the CTAB and wizard-based method [19, 20] can also be used.

2. One of the most well-known dyes for HRM analysis is the EvaGreen™, which can be used in a pre-prepared mix as in this protocol (SsoFast EvaGreen Supermix, Bio-Rad Laboratories, Hercules, CA, USA) or acquired separately (Biotium, CA, USA) and added to the in-house-prepared mix. EvaGreen is nonfluorescent, but it becomes highly fluorescent upon "release-on-demand" mechanism of binding to dsDNA. Besides, it is non-mutagenic and noncytotoxic, as it does not cross cell membranes. Other dyes, such as based on LC Green (addition of these dyes increases the melting temperature of DNA by 1–3 °C), Syto 9, and Chromofy (both dyes show high enhancement of fluorescence upon binding to double-stranded nucleic acid sequences), can also be used for successful HRM analysis. Examples of commercially available dyes for HRM application: LightCycler 480 ResoLight High Resolution Melting Dye (Roche Molecular Diagnostics Inc., Pleasanton, CA, USA), LCGreen PLUS (Idaho Technology Inc., Salt Lake City, Utah, USA), MeltDoctor™ HRM Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), and Type-it HRM PCR Kit (Qiagen GmbH, Hilden, Germany).
3. The use of a microvolume UV/Vis spectrophotometer is recommended to allow the direct reading of the extract, using as little extract as possible and avoiding extract dilutions and manipulations. Presently, the most well-known equipment is the nanodrop (e.g., NanoDrop™ 2000, Thermo Fisher Scientific, Delaware, USA), which is capable of doing measurements with just 1–2 μL, but it only allows one reading at a time, while the proposed system with the microvolume plate allows 16 reads simultaneously using similar volumes. To ensure more precise measurements, a volume of 4 μL of each extract is highly recommended.
4. There are other choices for high-resolution real-time PCR instruments (including their respective HRM analysis software): LightCycler® 480 Instrument II with LightCycler® 480 Gene Scanning Software (Roche Molecular Diagnostics Inc., Pleasanton, CA, USA), Rotor-Gene Q HRM System with Rotor-Gene ScreenClust HRM Software (Qiagen GmbH, Hilden, Germany), and 7500 Fast Real-time PCR System with High Resolution Melt (HRM) Software v2.0 (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA).

5. Besides BIOEDIT, there are other algorithms that can be used for DNA sequence alignment, either available online or for free download, namely ClustalW (Multiple Sequence Alignment), MEGA (Molecular Evolutionary Genetics Analysis), and Clustal Omega (Multiple Sequence Alignment) (Table 1).
6. In real-time PCR analysis, especially in HRM analysis, one important parameter to consider is the length of the amplicon. Therefore, to ensure a more accurate HRM analysis, the PCR products should range between 90 and 200 bp, although bigger fragments can also be used.
7. When designing primers manually, consider the following criteria/tips [21–23]: (a) the length of the primers should be 18–24 bp, with 40–60% G/C content (if possible, with the 3′ of a primer ending in C or G to promote binding); (b) avoid more than 1 or 2 G/C pairs at the 3′- and 5′-ends; (c) the Tm of the primers should range between 50 and 60 °C, although primers with Tm closer to 60 °C allow better amplifications; (d) the pair of primers should have closely matched melting temperatures to maximize PCR product yield (difference >5 °C between primers can lead to no amplification); (e) avoid runs of four or more of one base or dinucleotide repeats (for example, ACCCC or ATATATAT); and (f) primer pairs should not have complementary regions.
8. Several online platforms allow designing primers in alternative to the ones presented above. Examples: Primer3, GenScript Online PCR Primer Design Tool, Eurofins Genomics PCR Primer Design Tool, and Primer3Plus. Other software to check primers' properties: OligoEvaluator (Table 1).
9. Extracting DNA from plant material is often a challenge, which means that it is frequently recommended to test different kits or in-house-developed methods for the successful extraction of amplifiable DNA from certain plant species (e.g., seeds, spices, fruits, and leaves).
10. Nucleospin Plant II (Macherey-Nagel, Düren, Germany) allows extracting DNA from plant material using two different and independent protocols. One protocol is based on a lysis buffer containing the cationic detergent CTAB (PL1), while the other protocol uses an anionic (SDS) lysis buffer (PL2), which requires the subsequent precipitation of proteins by adding a potassium acetate solution (PL3). Both methods use a silica-based membrane combined with spin columns to ensure DNA extracts with high yield, quality, and purity.
11. In lysis step, the incubation can be performed for 10 min at 65 °C with previous addition of 10 μL of RNase A (10 mg/mL) (as suggested by the manufacturers), although longer incubation periods are recommended in order to increase DNA yields, which is the case of the example provided above.

12. For plant material, the use of RNase is normally recommended. Its use allows obtaining DNA extracts with more stability and with the adequate purity (1.8–2.0), thus enabling a better performance both by qualitative PCR and real-time PCR. However, care should be taken with its use since it also degrades DNA, reducing drastically the final yield.
13. Before starting the melt curve protocol, it is highly recommended to fully denature the PCR products and allow their correct annealing of the DNA complementary strands (DNA duplexes) by adding two steps, which were referred in the program of temperatures (denaturation of PCR products at 95 °C for 1 min, annealing of DNA duplexes at 65 °C for 5 min).
14. The use of white strips or plates is highly recommended for real-time PCR coupled to HRM analysis because they reduce the background noise and enhance fluorescence.
15. HRM analysis software (e.g., Precision Melt Analysis version 1.3, Bio-Rad Laboratories, Hercules, CA, USA) analyzes the fluorescence signal collected upon each increment of temperature during the melt curve. If the melt curve has big increments of temperature during small intervals of time (e.g., 0.5 °C for 10 s), the HRM software may not have enough data for a correct analysis. In such cases, the software accounts the fact that the generated file by the real-time PCR run with the melt curve does not comply with the recommended melt curve parameters. For optimal high-resolution melt data, the recommended increments of temperature during the melt curve should not exceed 0.2 °C between steps and a hold time minimum of 10 s during the melt curve protocol.
16. The melt file generated by the Precision Melt Analysis Software 1.3 (Bio-Rad Laboratories, Hercules, CA, USA) is analyzed by automated settings. The HRM analysis using the automated settings normally gives the best classification of samples into respective clusters, but depending on the type and number of nucleotide differences among the target sequences, some settings need to be adjusted. The software uses the data from the real-time PCR file and generates melting curves as a function of temperature, followed by the normalized melting curves and respective difference curves for easier visualization of the clusters. The melting curve shape sensitivity establishes the stringency used to categorize melting curves into different clusters. A high percentage value for this parameter allows increasing stringency and presents the results in more heterozygote clusters. The parameter of Tm difference threshold determines the lowest amount of Tm difference among samples. Like the melting curve shape sensitivity parameter, the Tm difference threshold defined to higher levels yields more heterozygote clusters.

17. By adjusting the settings, some nucleotide differences can be highlighted, while others can be neglected, which means that the HRM analysis needs to be controlled. This control is made by the level of confidence (expressed as percent of confidence). Each sample is mapped onto each cluster's probability distribution, based on their similarity to the mean melt curve across each sample in the cluster. The confidence value is an indication of the probability of a sample being included in a cluster; therefore the percentage of confidence should be as close as possible to 100%. Levels above 95% are normally considered as evidence of high confidence levels for the cluster classification.
18. The classification of samples into clusters is highly dependent on the type and number of nucleotide differences. Therefore, it is important to consider the samples/species that are intended to be separated by HRM analysis. When testing many genotypes containing several nucleotide differences, the classification into clusters might not distinguish groups with only one or two nucleotide differences. Even when testing few genotypes, but with few and many nucleotide differences among them, the classification into clusters might not be as evident as expected. For example, when testing three species, one with ten nucleotide differences comparing with two species with only one to two nucleotide mismatches between both, the products might be classified as two clusters instead of the expected three clusters. This can be explained by the high similarity of two species in relation to the third one, which might justify their inclusion in only one cluster in relation to a much distant cluster containing the ten-nucleotide difference species.
19. When developing a new real-time PCR method with HRM analysis, it is highly recommended to validate it by sequencing the PCR products of template species.
20. Before purifying PCR products for sequencing, it is highly recommended to perform an electrophoresis in 1.5% agarose gel (using only 2 μL of the amplified product) following the instructions described in Subheading 3 (Qualitative PCR).

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