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Advancing pineapple (*Ananas comosus* (L.) Merr.) cultivar identification and genetic diversity assessment by high resolution melting analysis and SSR markers

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ABSTRACT

Pineapple (*Ananas comosus* var. *comosus*) is one of the most widely cultivated tropical fruits worldwide, with significant economic importance and genetic variability, particularly in Brazil. The identification and certification of cultivars are essential to ensure authenticity, traceability and quality in production. In this context, the present work aimed at developing a new molecular method using high resolution melting (HRM) analysis targeting simple sequence repeats (SSR) for variety and cultivar discrimination. The method allowed to discriminate seven characterised *A. comosus* genotypes into 4 independent clusters with high levels of confidence (≥98.7%): cluster 1 (reference cluster), including cultivars (cv.) Smooth Cayenne, Vitória, Pérola and Turiaçú; clusters 2 with cv. Primavera; cluster 3 with cv. Gigante de Tarauacá; and cluster 4 comprising the *erectifolius* variety. The number of the SSR repeating motif (GA)n is the main polymorphism responsible for discriminating *A. comosus* var. *comosus* from *A. comosus* var. *erectifolius*, while few single-nucleotide polymorphisms distinguished among var. *comosus* cultivars. The applicability of the HRM approach was successfully demonstrated, discriminating uncharacterised genotypes and commercial pineapple samples. Generally, the samples were logically discriminated according to their origins, which was corroborated by sequencing and melt curve profiles, highlighting the robustness of the method. This study represents the first application of HRM for differentiating *A. comosus* genotypes, offering a valuable tool for genetic resource management in pineapple production and food manufacturers.

1. Introduction

Pineapple (*Ananas comosus* var. *comosus*) is a tropical fruit native from South America, specifically, central and southern Brazil, northern Argentina and Paraguay (Hassan et al., 2011). It is ranked among one of the most traded tropical fruits worldwide, being the most economically important species of the family Bromeliaceae and the only member widely cultivated as food (FAO, 2023). It holds notable nutritional significance, as it is rich in fibre, calcium, potassium, copper, vitamins C, B1 and B6 and bromelain, an enzyme complex with potential anti-inflammatory and digestive benefits (Orlandi-Mattos et al., 2019). Moreover, pineapple contains bioactive compounds with antioxidant and antimicrobial properties that have been associated with benefits for metabolic, neurological, and bone health (Ali et al., 2020). Nevertheless, it is important to recognise that biological factors such as ripening stage, climatic conditions and cultivar can modulate the chemical and biochemical properties of the fruit and, consequently, its bioactive properties (Ali et al., 2020; Gondim et al., 2023; Junghans & Souza, 2022).

The pineapple market has been growing due to these appealing aroma compounds and nutritional value, with production and trade experiencing a significant boom in the post–Second World War period. Today, pineapple is one of the most actively traded tropical fruits worldwide, with a global cultivation area exceeding 1 billion hectares in

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2023, being mainly concentrated in Asia, Africa and America. Brazil was the fifth largest producer country, behind Indonesia, Philippines, Costa Rica and China (FAOSTAT, 2025). The economic significance of pineapple extends beyond its direct market value as a fresh fruit or processed product.

In 2003, pineapple taxonomy was revised by D'Eeckenbrugge and Leal (2003) who redefined and simplified the previous classification established by Smith and Downs (1979). Accordingly, it is now simplified in one genus, *Ananas*, with two species, *A. macrodontes* E. Morren (tetraploid, 2n = 100; formerly known as *Pseudananas sagenarius*) and *A. comosus* (L.) Merr. (diploid, 2n = 50). From a botanical perspective, *A. comosus* can be further subdivided into five recognised botanical varieties, *A. comosus* var. *microstachys*, *A. comosus* var. *bracteatus, A. comosus* var. *comosus, A. comosus* var. *erectifolius* and *A. comosus* var. *parguazensis*. From these, *A. comosus* var. *microstachys* and *A. comosus* var. *parguazensis* have limited commercial value, being the latter occasionally used as a genetic resource. *A. comosus* var. *bracteatus* is primarily ornamental and is also occasionally used in breeding, while *A. comosus* var. *erectifolius* is cultivated for fibre production. *A. comosus var. comosus* is the main cultivated variety for food and commercial pineapple production worldwide (Carlier et al., 2010; Chen et al., 2019; Hassan et al., 2011; Lyu et al., 2025), from which more than 100 pineapple cultivars are grown globally. These cultivars can also be classified, from a horticultural and economic perspective, into five main groups based on agronomic and market traits: Cayenne ('Smooth Cayenne'), Queen (e. g. 'Queen', 'Victoria' and 'Mauritius'), Spanish (e.g. 'Red Spanish' and 'Singapore Spanish'), Abacaxi (e.g. 'Pernambuco' and 'Perola') and Maipure (e.g. 'Maipure' and 'Tachirense') (Li et al., 2022; Lyu et al., 2025). The three most economically and traditionally valued are 'Smooth Cayenne', 'Queen' and 'Singapore Spanish', with the first being the most important cultivar due to its high yield potential and favourable characteristics for fresh fruit processing. Consequently, pineapple is a heterozygous species, resulting in considerable diversity in plant and fruit characteristics among cultivars. Each cultivar exhibits distinct traits, including fruit size, sugar content and resistance to diseases. For example, the cv. Smooth Cayenne is globally favoured for its sweetness and low fibre content, while the 'Queen' is smaller and known for its more robust flavour (Junghans & Souza, 2022; Reinhardt et al., 2002, 2018). Despite this natural variability, commercial plantations for example in Brazil are dominated by two cultivars, the international 'Smooth Cayenne', widely cultivated in the country's subtropical regions and the traditional cultivar Pérola, widespread across the national territory (Reinhardt et al., 2002).

At the production level, rural extension and technology transfer to producers are essential to improving growing conditions, as well as increasing productivity and fostering more sustainable and environmentally friendly pineapple production practices. Thus, some genotypes with particular importance to pineapple producers in Brazil and worldwide have received increased research attention (Villalobos-Olivera et al., 2022). Building on this, genetic studies on pineapple have also provided valuable insights into their domestication process and breeding strategies. An example is the development of the Vitória hybrid, which is a cross between the female parent cv. Primavera and male parent cv. Smooth Cayenne, showing how targeted cross-breeding programs is useful to improve agronomic traits such as disease resistance and fruit quality (Ventura et al., 2009).

Despite the global relevance of *Ananas*, having the complete genome been studied, its taxonomy is frequently revised due to the genetic variations and agricultural characteristics associated with the genus (Gondim et al., 2023; Matuszak-Renger et al., 2018; Ogawa et al., 2018). Besides genetic and breeding interests, the pineapple is consumed in various forms, including fresh or processed products such as jams, beverages, candies, dried and canned foods. Additionally, from a consumer perspective, pineapple cultivars differ in fruit weight, shape, size and colour. Therefore, it is imperative that a trustful distinction is made between at least the five main groups in order to align with consumer preferences and market demands (Ali et al., 2022). In this context, studies utilising molecular markers have enhanced the understanding of genetic diversity within *Ananas*. Notably, simple sequence repeat (SSR) markers, also known as microsatellites, have been highly effective in differentiating pineapple germplasm, enabling accurate cultivar identification and comparison (Nashima et al., 2020; Shoda et al., 2012; Wang et al., 2017).

To address the challenges faced by the producers in certifying and authenticating pineapple cultivars, given their morphological similarity, this study aims at exploring the use of SSR markers as a molecular tool to differentiate *A. comosus* varieties and, ideally, cultivars. Molecular markers represent a cost-effective, high-throughput and reliable approach widely applied in plant and fruit authentication, providing robust methods for cultivar identification as they rely on DNA sequences capable of discriminating even closely related taxa (Böhme et al., 2019; Druml & Cichna-Markl, 2014; Grazina et al., 2020). Real-time PCR coupled to high resolution melting (HRM) analysis has emerged as an excellent tool capable of distinguishing related species or cultivar/varieties, owing to its capability to discriminate amplicons differing in one or few nucleotides (Druml & Cichna-Markl, 2014; Grazina et al., 2021). HRM analysis targeting SSR markers has been applied to discriminate varieties common bean (Ganopoulos et al., 2012), lentils (Bosmali et al., 2012), grapevine (Mackay et al., 2008; Pereira et al., 2017), rice (Grazina et al., 2022) and olive oil (Gomes et al., 2018). Nonetheless, to our knowledge, HRM analysis has not been exploited to genetic diversity studies or differentiation of pineapple varieties. Therefore, this study proposed the use of HRM analysis coupled to SSR polymorphisms, as a highly specific approach to differentiate 10 agronomically important pineapple genotypes. This novel strategy aims at improving the certification of pineapple seedlings and reliably distinguish its varieties/cultivars, providing a significant advance for the market.

2. Material and methods

2.1. Plant species and commercial sampling

Seven different cultivars or variants of *A. comosus* (Table 1, Fig. 1) were micropropagated at the Laboratório de Cultura de Tecidos e Células Vegetais do Centro de Pesquisa, Desenvolvimento e Inovação Serrano of the INCAPER, Vitória, Espírito Santo, Brazil. These genotypes were selected for their agronomic importance but also due to their morphological similarities in both plants and fruits. Some morphological traits were examined, such as leaf edges, as well as factors related to resistance to *Fusarium*, a fungus that affects much of the material cultivated and sold in Brazil and worldwide. These aspects are particularly relevant in the adult stage of the plant (Araujo et al., 2012; Marques et al., 2020; Queiroga et al., 2023; Reinhardt et al., 2002; Souza et al., 2017; Ventura et al., 2009).

After sufficient culture growth, the aerial parts of each plant were separated and dried in an oven at 30 °C in the dark for 5 days. The dried tissues were then submerged in liquid nitrogen and ground with mortar and pestle to obtain a fine powder, ensuring mechanical fragmentation of the tissues.

Additionally, thirty-six non-target species (Table S1, supplementary material) were included to evaluate the method specificity. These species represent a variety of plants, including medicinal plants, herbs, spices, cereals, legumes, fruits, and tree nuts. Plant tissues were obtained from commercial shops and botanical gardens, then dried and ground either using a Grindomix GM200 laboratory mill (Retsch, Haan, Germany) or a mortar and pestle.

Finally, to assess the applicability of the method, three uncharacterised isolates from INCAPER were micropropagated and 10 commercial products (e.g. pineapple leaves, fresh fruit and dehydrated) were acquired from commercial markets and specialized shops in Portugal, Spanish and Brazil.

Table 1
Ananas comosus genotypes used in the present study and micropropagated at the Laboratório de Tecidos e Células Vegetais do Centro de Pesquisa, Desenvolvimento e Inovação Serrano of the INCAPER, Vitória, Espírito Santo, Brazil.

Genotype[a]	Cultivar	Horticultural group	Pedigree or origin	Species/ variety	Use	Characteristics				Reference
						Fusarium [b]	Leaf	Ploidy	Fruit	
EC011	Smooth Cayenne	Cayenne	Cultivar originated in French Guiana or French	*A. comosus* var. *comosus*	Processing/ Juice	S	Irregular, thorn at the tip	Diploid	Cylindrical with yellowish pulp colour and ride yellow with green mottling (ripe). 16.6 com in length and 13.6 cm in diameter. Fruit weight with crown 1.773 kg. Acid content 0.7%	Reinhardt et al. (2002); Ventura et al. (2009)
EC013	Primavera	Maipure	Cultivar originated from Brazil	*A. comosus* var. *comosus*	Fresh consumption	R	*Piping*	Diploid	Cylindrical, white pulp, ride yellow (ripe). Fruit weight with crown 1.3 kg. Acid content 0.5%	Queiroga et al. (2023)
EC210	Gigante de Tarauacá	Undefined	Native of Tarauacá region, state of Acre, Brazil	*A. comosus* var. *comosus*	Fresh consumption	ND	*Thorns*	Triploid	White pulp, ride purple (ripe). Fruit weight with crown 4 kg to 15 kg	Marques et al. (2020)
EC088	-	Not belonging to the horticultural cultivar groups	Native to the Amazon region of South America, specifically the Amazon Complex	*A. comosus var. erectifolius*	Not edible, used for fibre production	S	Smooth	Diploid	Not edible, small, cylindrical or subcylindrical, pulp scarce and fibrous, not pleasant flavour	Souza et al. (2017)
EC099	Vitória	Undefined (interspecific hybrid)	Primavera (PRI) x Smooth Cayenne (SC-08)	*A. comosus* var. *comosus*	Fresh consumption	R	*Piping*	Diploid	Cylindrical, whitish pulp, ride bright yellow (ripe), 9.58 cm in length and 12 cm in diameter. Fruit weight with crown 1.427 kg. Acid content 0.8%	Ventura et al. (2009)
EC101	Pérola	Pernambuco	Cultivar originated from Brazil	*A. comosus* var. *comosus*	Fresh consumption	S	*Thorns*	Diploid	Conical, whitish pulp, ride green (ripe), 9.58 cm in length and 11 cm in diameter. Fruit weight with crown 1.473 kg. Acid content 0.5%	Reinhardt et al. (2002); Ventura et al. (2009)
EC197	Turiaçú	Pernambuco	Native from Amazônia Maranhense, State of Maranhão, Brazil	*A. comosus var. comosus*	Fresh consumption	ND	*Thorns*	Diploid	Cylindrical/conical, yellow pulp, ride yellowish (ripe), 20.8 cm in length and 10.4 cm in diameter. Fruit weight with crown 1.620 kg. Acid content 0.4%	Araujo et al. (2012)

[a] The genotype code is retrieved from the Laboratório de Cultura de Tecidos e Células Vegetais do Centro de Pesquisa, Desenvolvimento e Inovação Serrano of the INCAPER, Vitória, Espírito Santo, Brazil, pineapple collection.
[b] ND, not defined; R, resistant; S, susceptible.

2.2. DNA extraction

Genomic DNA was extracted from the plant tissue using the CTAB-PVP-based method as described by Costa et al. (2015), using 200 mg of each sample. Yield and purity of DNA extracts were assessed by UV spectrophotometry using a microplate reader (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany) with a LVis microvolume plate accessory (BMG Labtech, Ortenberg, Germany). The DNA content and purity were evaluated with MARS data analysis software (BMG Labtech, Ortenberg, Germany).

2.3. Target gene selection and oligonucleotide primer design

SSR loci were critically selected from the available literature (Nashima et al., 2020; Wang et al., 2017) for the design of new primers targeting short amplicons (100-200 bp), suitable for HRM analysis (Grazina et al., 2021). Available sequences of selected loci were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/) and six primer pairs were manually designed to encompass the polymorphic regions (Table 2). Primer properties, absence of hairpins and self-hybridization were assessed using OligoCalc properties Calculator (https://oligocalc.eu/). Subsequently, an *in silico* analysis was performed to evaluate the primer specificity using the Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) tools. All primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

Fig. 1. Available photographs of the fruits from the pineapple varieties/cultivars used in the present study. Legend: A, 'Smooth Cayenne'; B, 'Primavera'; C, 'Gigante de Tarauacá'; D, *Ananas comosus* var. *erectifolius*; E, 'Vitória'; F, 'Pérola'; G, 'Turiaçú'. Source: Embrapa (2025); Rodrigues (2013).

Table 2
Key data of primers designed to target different microsatellite loci of *A. comosus* and a conserved eukaryotic region.

Target region	Primer	Sequence (5' → 3')	Amplicon (pb)	Annealing temperature (°C)	Motif	NCBI accession no./ Reference
Chromosome 13	ANC1-F	AGTGTGATTAAAATGGGGTGCCT	160	60	(CT)15	LR828293.1
	ANC1-R	ACATCCTTCTCCTCCGCCAT				
TsuAc189 Chromosome 18	ANC2-F	ACGGATCAAGAAAAAGCTCTAAGA	160	58	(GA)10	LC429608.1
	ANC2-R	TACTACATGGCCCCGCCTAT				
	ANC2-FS	ACCGTAGATCCTTATGACCGGCA	270	62		
	ANC2-RS	AATGGCTTCGCCGGCGCCTA				
TsuAc346 Chromosome 20	ANC3-F	ATCCCACTACACGACTAGCGAA	110	62	(CACACG)5	LC429765.1
	ANC3-R	CTCCTCGTTCGTTACCAAATCGA				
TsuAc230 Chromosome 25	ANC4-F	ATCGAGCGCAAGGGTTTGGTG	163	61	(AG)11	LC429649.1
	ANC4-R	GAATCTTCTCGCTTTTCTCCCTCA				
TsuAc256 Chromosome 22	ANC5-F	GGTGAATATAAAAACCAAACCAGGA	129	58	(GAG)11	LC429675.1
	ANC5-R	TGCTTCTTGTGGGTCTTCTTCT				
TsuAc259 Chromosome 18	ANC6-F	TCGACGCAGTTTTTGCAAGAGA	150	60	(AGA)6	LC429678.1
	ANC6-R	TGGCCTATTAATAGAACCCCCT				
Nuclear 18S rRNA	18SRG-F	CTGCCCTATCAACTTTCGATGGTA	113	65	-	Costa et al. (2013)
	18SRG-R	TTGGATGTGGTAGCCGTTTCTCA				

2.4. Qualitative PCR

Qualitative PCR assays were conducted in a total reaction volume of 25 μL, with 2 μL of DNA extract (10 ng), 1× enzyme buffer (67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20), 3.0 mM of $MgCl_2$, 1 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience GmbH, Ulm, Germany), 200 μM of each dNTP (Grisp, Porto, Portugal) and 200 nM (240 nM for primers 18SRG-F/-R) of each primer (Table 2). PCR amplifications were conducted in a Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA), following the programs: 95 °C for 5 min; 40 cycles (or 33 cycles for primers 18SRG-F/-R) at 95 °C for 30 s, 58-65 °C (Table 2) for 30 s and 72 °C for 30 s; 72 °C for 5 min.

PCR products were confirmed by electrophoresis in 1.5% agarose gels, stained with 1× Gel Red (Biotium, CA, USA) and run in 1× SGTB buffer (GRISP, Porto, Portugal) for 25-30 min at 140 V. The agarose gels were visualised under UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA), and a digital image was recorded using the Image Lab software version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Real-time PCR coupled to HRM analysis

Real-time PCR assays were conducted in a total reaction volume of 20 μL, containing 10 ng of DNA, 1× SsoFast Evagreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 240 nM of each primer (ANC2-F/R). The reactions were performed on a CFX96 Real-time PCR

Detection System (Bio-Rad) under the following conditions: 95 °C for 5 min; 40 cycles at 95 °C for 10 s, 60 °C for 20 s and 72 °C for 30 s with fluorescence signals collected at the end of each cycle. The data were analysed using Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA).

HRM analysis was carried out following amplicon denaturation at 95 °C for 1 min, annealing at 65 °C for 3 min and a melting curve ranging from 65.0 °C to 95.0 °C with 0.2 °C increments every 5 s and with fluorescence signal collected at each step. The Precision Melt Analysis Software 1.2 (Bio-Rad Laboratories, Hercules, CA, USA) was used to analyse the fluorescence data, producing melting curves as a function of temperature, and respective difference curves for easy visual identification of clusters. Real-time PCR assays were repeated in duplicate independent runs using $n = 3$ replicates per assay.

2.6. Sequencing

Sequencing of the selected polymorphic locus was performed with the ANC2-FS/RS primers to confirm the results obtained with the developed method, following the PCR conditions outlined in section 2.4, with small modifications: 40 ng of template DNA, 250 μM of each dNTP and 240 nM of each primer. The amplification program was as follows: 95 °C for 5 min; 40 cycles of amplification at 95 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min; 72 °C for 5 min. The reactions were conducted in a MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were confirmed by electrophoresis and purified using the GRS PCR & Gel Band Purification Kit (GRISP, Porto, Portugal) to eliminate any possible interfering components. The purified products were subjected to Sanger sequencing (Eurofins Genomics, Cologne, Germany). Each target fragment was sequenced twice, with both strands analysed in opposite directions, ensuring high-quality complementary sequences.

3. Results and discussion

3.1. Selection of target marker

SSR markers are widely used in genetic diversity studies because of their high level of polymorphism, including in *A. comosus* genotypes (Nashima et al., 2020; Wang et al., 2017). In the present work, six SSR regions were selected from the literature for the design of new primers to target repetitive motifs in chromosomes 13, 18, 20, 22 and 25 (Table 2). Prior to the specific amplification, genomic DNA extracts were tested with universal eukaryotic primers targeting the 18S rRNA gene, confirming their high amplification capacity (Table 3, Table S1, Supplementary material).

Following the *in silico* assessment of the primers confirming their specificity to *A. comosus*, their amplification capacity across the seven reference pineapple varieties/cultivars was tested with the specific primers. The PCR results show that all primers successfully amplified all the genotypes, producing the expected amplicons (Table 3). However, primers ANC1-F/R, ANC3-F/R, ANC5-F/R and ANC6-F/R appeared to produce cross-annealing or non-specific products (Fig. S1, supplementary results), which makes them unsuitable for HRM analysis. For this reason, the primers ANC2-F/R and ANC4-F/R were selected for further testing.

After assay high-performance optimisation, a 10-fold serial dilution of pineapple DNA (EC011) from 10 ng to 0.1 pg was analysed with primers ANC2-F/R and ANC4-F/R. Both PCR assays proved to be very sensitive, amplifying down to 10 pg and 0.1 ng of pineapple DNA, for primers ANC2-F/R and ANC4-F/R, respectively (Fig. S2, supplementary material). Subsequently, a PCR assay was performed to evaluate primer specificity using 36 non-target species commonly found as food ingredients or closely related to *A. comosus*. Amplification results confirmed that the ANC2-F/R primer pair is highly specific to *A. comosus*, whereas ANC4-F/R exhibit cross-reactivity with almond (Table S1, supplementary material). Taking into consideration the superior sensitivity and specificity, ANC2-F/R primers were selected for further method development by real-time PCR coupled to HRM analysis.

3.2. HRM analysis

Following the selection of the SSR marker with primers ANC2-F/R, an EvaGreen real-time PCR method with HRM analysis was developed for the differentiation of the *A. comosus* varieties/cultivars. Fig. 2 presents the real-time PCR amplification curves (Fig. 2A) and respective melting peaks (Fig. 2B), as well as the HRM data (Fig. 2C and D). The seven pineapple genotypes, using the same initial DNA amount (10 ng), were successfully amplified, showing similar cycle threshold (Ct) values, ranging from 27.19 ± 0.19 to 28.22 ± 0.24 (Fig. 2A–Table 3). These results confirm the amplification of the expected amplicons across all cultivars, in agreement with the qualitative PCR findings. The obtained conventional melting curves revealed closely aligned melt peaks of 83.53 ± 0.07 °C to 83.80 ± 0.01 °C (Fig. 2B–Table 3), suggesting only minute/small differences in the nucleotide composition and/or fragment size among the genotypes. Subsequently, HRM analysis was performed as an approach to differentiate amplicons with small nucleotide variations. This technique monitors the gradual denaturation of double-stranded DNA (dsDNA) amplicons, which is achieved and differentiated from conventional melting curve analysis thanks to the use of high resolution instrumentation, enhanced fluorescent dyes, such as Evagreen, and specific HRM data analysis software (Grazina et al., 2021). After obtaining the melting curve profiles, the HRM analysis software was applied to identify areas of stable pre- and post-melt fluorescence intensity, which enabled normalising the signals to relative values within 0-1.0 (Fig. 2C). This eliminates differences in background fluorescence, while increasing the capacity to detect minute melt profile differences. The resultant difference curve plot allows to visually magnify differences between the melt profiles of distinct clusters (Fig. 2D). HRM data successfully discriminated the *A. comosus* genotypes into 4 independent clusters with high levels of confidence (≥98.7%) (Fig. 2D–Table 3). In detail, cluster 1 (reference cluster) include the 'Smooth Cayenne' (99.6% ± 0.25), 'Vitória' (99.7% ± 0.31), 'Pérola' (99.9% ± 0.06) and 'Turiaçú' (99.8% ± 0.17) cultivars, while clusters 2,

Table 3
Results of qualitative PCR, real-time PCR and HRM analysis targeting a universal eukaryotic region of the 18S rRNA gene (18SRG) and the SSR locus TsuAc189 (ANC2) of characterised *A. comosus* genotypes.

Code	Genotype	Qualitative PCR		Real-time PCR		HRM analysis	
		18SRG	ANC2	Ct ($\bar{X}$ ± SD)	Tm (°C) ($\bar{X}$ ± SD)	Cluster	Level of confidence (%) ($\bar{X}$ ± SD)
EC011	Smooth Cayenne	+	+	27.61 ± 0.25	83.67 ± 0.01	1	99.6 ± 0.25
EC013	Primavera	+	+	27.19 ± 0.19	83.80 ± 0.01	2	99.1 ± 0.15
EC210	Gigante de Tarauacá	+	+	27.63 ± 0.28	83.63 ± 0.03	3	98.8 ± 2.06
EC088	*var. erectifolius*	+	+	28.22 ± 0.24	83.60 ± 0.01	4	99.9 ± 0.10
EC099	Vitória	+	+	27.99 ± 0.19	83.57 ± 0.03	1	99.7 ± 0.31
EC101	Pérola	+	+	27.52 ± 0.21	83.67 ± 0.07	1	99.9 ± 0.06
EC197	Turiaçú	+	+	27.89 ± 0.29	83.60 ± 0.01	1	99.8 ± 0.17

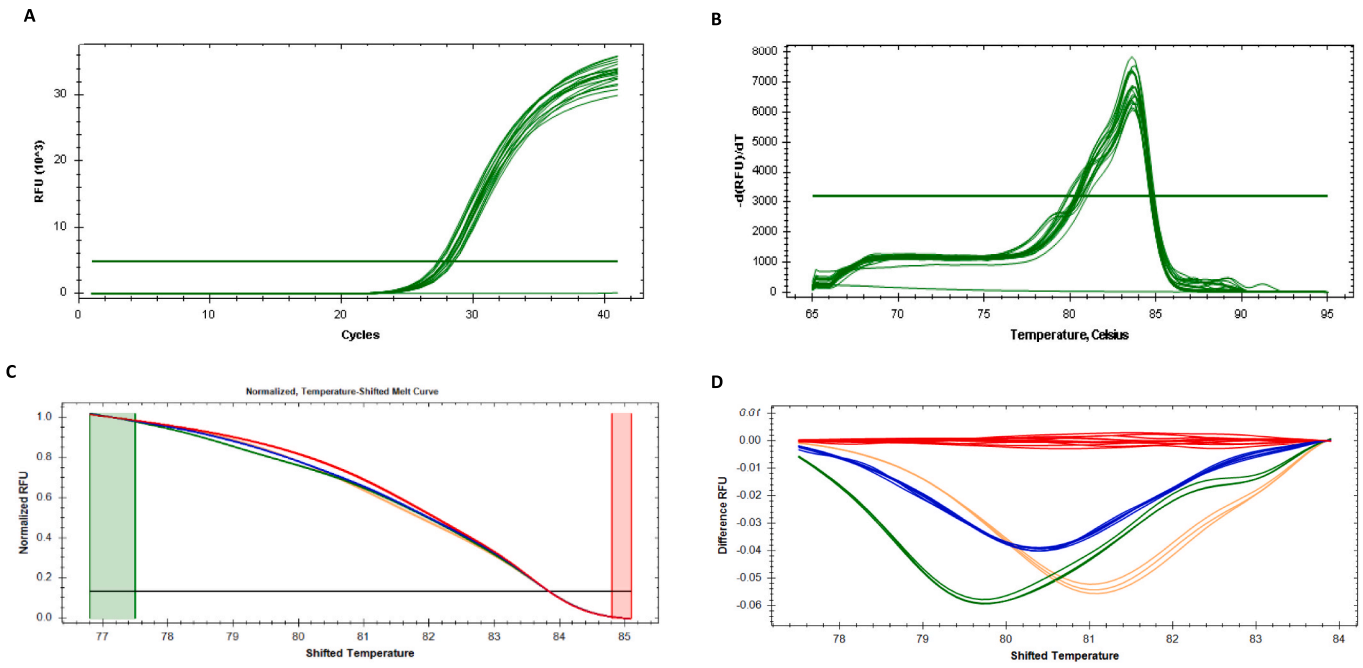

Fig. 2. Representative real-time PCR run and HRM analysis of the seven characterised pineapple genotypes provided by INCAPER. Amplification curves (A), conventional melting curves (B), normalised melting curves (C) and temperature shift difference curves (D). Legend: Cluster 1 (refence cluster, red line): 'Smooth Cayenne' (EC011), 'Vitória' (EC099), 'Pérola' (EC101) and 'Turiaçú' (EC197); Cluster 2 (green line): 'Primavera' (EC013) Cluster 3 (blue line): 'Gigante de Tarauacá' (EC210); Cluster 4 (orange line) *erectifolius* variety (EC088).

3 and 4 were formed with single genotypes, namely cv. Primavera (99.1% ± 0.15), cv. Gigante de Tarauacá (98.8% ± 2.06) and *erectifolius* variety (99.9% ± 0.10), respectively. Generally, the clustering pattern reflects logical genetic relationships within pineapple cultivars/varieties. Cluster 1 grouped commercial cultivars from the Cayenne ('Smooth Cayenne') and Pernambuco ('Pérola' and 'Turiaçú') horticultural groups, along with 'Vitória' (undefined group), a 'Smooth Cayenne' hybrid. Except for the hybrid, cluster 1 cultivars are either susceptible or not yet defined in terms of resistance to *Fusarium*, but they all are diploid. Primavera (Maipure group) formed an independent cluster, being characterised by its resistant to the fungus and generally a sweeter taste, though also diploid. Gigante de Tarauacá cultivar, included in cluster 3, displayed a distinct profile consistent with its triploid status, justifying the lower level of confidence, and unique fruit morphology. Cluster 4 discriminated the only genotype of a distinct variety, *A. comosus* var. *erectifolius*, which is a non-edible wild variety used as ornamental and for fibre production. Its clear genetic divergence from other genotypes is consistent with its distant relationship and further aligned with the discriminatory capacity of the method.

To date, several studies utilising DNA-based methodologies have been conducted to differentiate pineapple genotypes or authenticate pineapple in food products (e.g. beverages). Kato et al. (2005) used amplified fragment length polymorphism (AFLP) markers to evaluate several accessions of *A. comosus* and related species, showing discrete DNA fingerprints among the samples. However, major cultivar groups of pineapple, such as Cayenne, Spanish and Queen, could not be distinguished. Similarly, Shoda et al. (2012) developed SSR-based markers using genomic libraries enriched for GA and CA motifs that could differentiate most pineapple genotypes analysed with values of expected heterozygosity from 0.09 to 0.76. Hidayat et al. (2012) conducted a molecular analysis targeting the internal transcribed spacer (ITS) region in nine Malaysian cultivars. Results classified the cultivars in three groups with high sequence similarity, revealing a high discrimination power of the DNA barcode region that can be applied to Malaysian pineapple cultivars. More recently, Ngorian et al. (2024) analysed several pineapple cultivars/lines with fluorescent-labelled SSR markers, aiming to assess their genetic diversity. Results showed that all SSR markers were able to differentiate the pineapple cultivars/lines, generating numerous polymorphic alleles. Cluster analysis revealed 5 distinct groups, consistent with pedigree and the grouping of cultivars/lines based on morphological characteristics.

Although these methodologies provide valuable contributions and advances for genetic diversity studies and pineapple cultivar discrimination, they are generally complex, time-consuming and, consequently, costly. This work, to our knowledge, represents the first application of HRM analysis to differentiate pineapple genotypes, as a high-throughput, reliable and cost-effective approach. HRM analysis targeting SSR and other nucleotide polymorphisms has already demonstrated its discriminating power when applied to variety differentiation of several plant foods such legumes, olive oil, grapevine, cherry and rice (Bosmali et al., 2012; Ganopoulos, et al., 2011, 2012; Gomes et al., 2018; Grazina et al., 2022; Pereira et al., 2017). Moreover, HRM analysis has proved to be effective in the authentication of species of origin in fruit juices (Faria et al., 2013), berry fruits (Toth et al., 2024) and food traceability applications (Druml & Cichna-Markl, 2014; Grazina et al., 2021; Zhao et al., 2025). However, its applicability to pineapple variety/cultivar discrimination was herein demonstrated for the first time.

3.3. Sequencing

To further correlate the HRM profiles with underlying sequence polymorphisms and validate the obtained results, the targeted SSR locus was amplified and sequenced using the primers ANC2-FS/RS to produce a 270-bp amplicon (Table 2). The sequencing results of the seven genotypes were aligned with the consensus *A. comosus* sequence deposited in the NCBI database with the accession number LC429608.1. and presented in Fig. 3.

The alignment results clearly highlight the genetic distinction of *A. comosus* var. *erectifolius* (EC088) from the cultivars of *A. comosus* var. *comosus* (EC011), thereby supporting its separation into an independent cluster in the HRM analysis. Specifically, eleven nucleotides mismatches were detected, a mismatch in the primer-binding region at position 16,

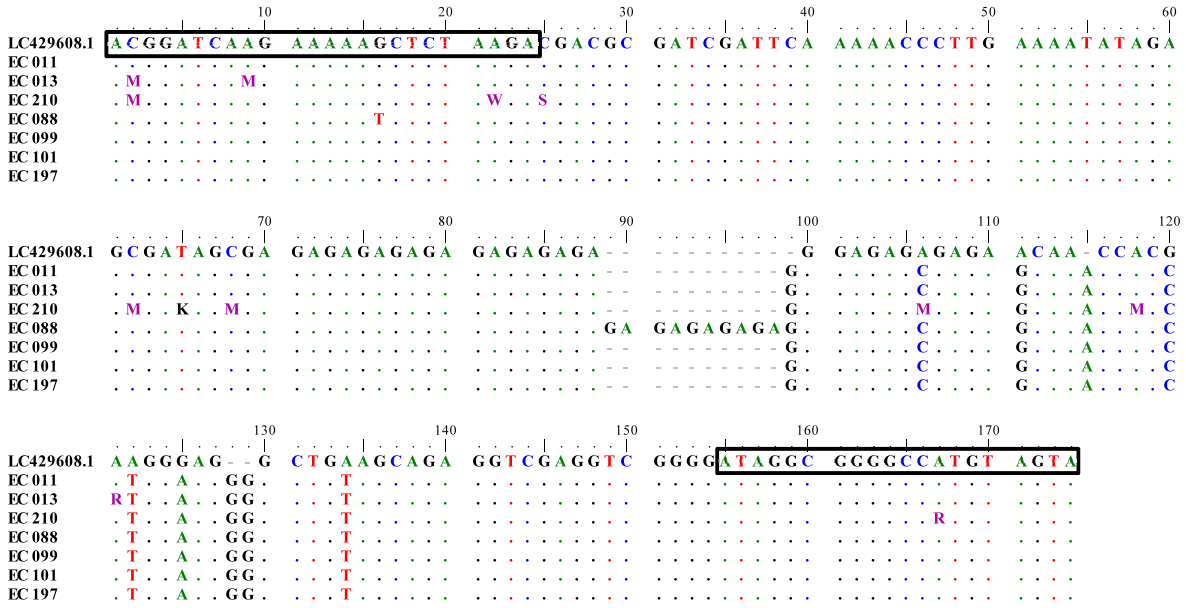

Fig. 3. Alignment of sequence of *A. comosus* from the NCBI database (NCBI accession no. LC429608.1) and the amplicons resulting from the sequencing of the seven characterised *A. comosus* genotypes from INCAPER. Black rectangles identify the region of primer annealing (ANC2-F/R). Legend of undefined bases: K represents G or T; M, A or C; R, A or G; S, C or G; W, A or T.

where G was substituted by T and a length polymorphism spanning positions 71-98, where var. *erectifolius* carries a $(GA)_{15}$ microsatellite, while the other have a $(GA)_{10}$ repeat motif (Fig. 3, Table 4). Cultivars Smooth Cayenne (EC011), Vitória (EC099), Pérola (EC101) and Turiaçú (EC197) displayed identical amplicon sequences, confirming their high genetic proximity and grouping within Cluster 1. 'Gigante de Tarauacá' (EC210) displayed a distinct sequence profile, characterised by nine nucleotide polymorphisms, including substitutions at positions 2 (A/C) and 22 (A/T) of primer forward and at position 167 (A/G) of primer reverse, in addition to position 25 (C/G). The genetic singularity of this cultivar likely reflects its distinct ploidy level (triploid), supporting and validating its separation in cluster 4. Lastly, cv. Primavera (EC013) was discriminated based on three single nucleotide polymorphisms (SNP), two at positions 1 (A/C) and 9 (A/C) of forward primer-binding region and one at position 40 (A/G), justifying the differentiation of this genotype in cluster 3.

Overall, sequencing data clearly justify and corroborate HRM clustering based on the observed SNP and microsatellite variations. In summary, the number of the SSR repeating motif $(GA)_n$ is the main polymorphism responsible for discriminating the *A. comosus* var. *comosus* from *A. comosus* var. *erectifolius*, while few SNP distinguish other cultivars. These results confirm that HRM analysis targeting SSR and SNP is a reliable tool for distinguishing among pineapple genotypes at the level of variety/cultivar, corroborating their phylogenetic relationships.

Table 4
Main polymorphisms identified in the pineapple cultivars/varieties.

Genotypes	TsuAc189 (ANC2) polymorphisms	
	G/T (16)[a]	$(GA)_n$
EC011	G	10
EC013	G	10
EC210	G	10
EC088	T	15
EC099	G	10
EC101	G	10
EC197	G	10

[a] The number in brackets is the position in sequence of Fig. 3.

3.4. Method application to unknown cultivars and commercial samples

Following the development and validation of the HRM method, its application was carried out to assess three uncharacterised germplasm cultivars from INCAPER and ten commercial pineapple samples obtained from markets in Brazil, Portugal and Spain. Accordingly, DNA extracted from pineapple aerial parts, leaves or fruits was amplified by real-time PCR using ANC2-F/R primers, followed by HRM analysis. Fig. 4 shows an example HRM run of the analysed genotypes and commercial pineapple samples, while Table 5 summarises all data. Regarding the uncharacterised genotypes, all three were successfully amplified, yielding similar Ct values ranging from 27.27 ± 0.13 to 27.83 ± 0.22 and closely aligned melt peaks between 83.53 ± 0.07 °C and 83.60 ± 0.01 °C (Table 5). HRM analysis revealed no distinct separation among them. In fact, all were consistently grouped within cluster 1, indicating a close genetic relationship and limited variability at the targeted locus (Fig. 4A–B). This finding was confirmed by sequencing analysis, which revealed almost full alignment among them and with 'Smooth Cayenne' (EC011), as the reference genotype for cluster 1 (Fig. 5). Although these genotypes were originated from pineapple crops in different geographic regions (Brazil and Reunion Island), their crossbreeding history is unknown. Considering this, and given the high commercial value of the cultivar, it is plausible that they belong to the Cayenne group, particularly the Baby type due to its origin, justifying their clustering.

Concerning the commercial pineapple samples, the normalised and respective shift different curves are presented in Fig. 4C–D as an example run, including representative genotypes of clusters 1 and 2 (EC011 and EC013, respectively) because of their commercial relevance. The Ct values of the commercial samples showed some dispersion among them, ranging from 28.04 ± 0.24 to 31.10 ± 0.26, reflecting the variability of leaf or pulp material, while the melt peaks remained closely aligned between 83.27 ± 0.76 °C and 83.73 ± 0.12 °C (Table 5). HRM analysis differentiated the samples into four groups: clusters 1 (AC7) and 2 (AC1 and AC2), including the pineapple samples from Mauritius Island and Brazil, respectively; and two additional clusters, 5 (AC3-AC6, AC9-AC10) and 6 (AC8), which grouped the samples from Costa Rica and Azores, respectively. All commercial products exhibited high confidence levels regarding cluster classification (97.55–99.80%),

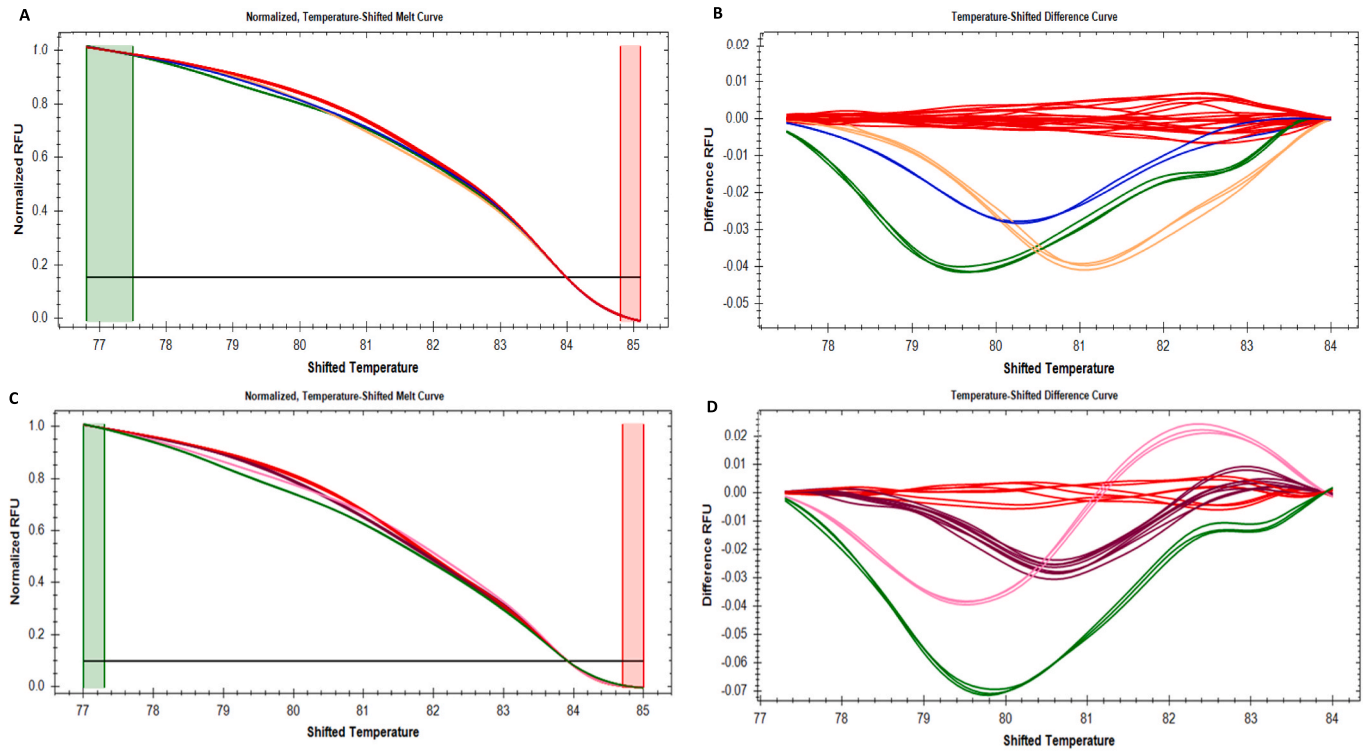

Fig. 4. HRM analysis of the three uncharacterised genotypes from INCAPER and the commercial samples. Representative normalised melting curves (A, C) and temperature shift difference curves (B, D). Legend: Cluster 1, reference cluster, red line; Cluster 2, green line; Cluster 3, blue line; Cluster 4, orange line; Cluster 5, burgundy; Cluster 6, pink line.

Table 5
Results of qualitative PCR, real-time PCR and HRM analysis targeting the universal eukaryotic region of 18S rRNA gene (18SRG) and the SSR locus TsuAc189 (ANC2) of uncharacterised *A. comosus* genotypes and pineapple commercial samples.

Code	Labelled origin	Type of sample	Qualitative PCR		Real-Time PCR		HRM analysis	
			18SRG	ANC2	Ct (mean ± SD)	Tm (°C) ($\overline{X}$ ± SD)	Cluster	Level of confidence (%) (mean ± SD)
Uncharacterised genotypes								
EC211	Incaper/Reunion Island	Baby (Micropropagated tissue)	+	+	27.83 ± 0.22	83.60 ± 0.01	1	99.7 ± 0.36
EC227	Incaper/Brazil	Chico (Micropropagated tissue)	+	+	27.47 ± 0.10	83.60 ± 0.01	1	99.8 ± 0.21
EC229	Incaper/Brazil	Mini (Micropropagated tissue)	+	+	27.27 ± 0.13	83.53 ± 0.07	1	99.5 ± 0.84
Commercial samples								
AC1	Brazil	Pineapple leaf	+	+	30.83 ± 0.41	83.27 ± 0.76	2	97.55 ± 1.34
AC2	Brazil	Pineapple leaf	+	+	31.10 ± 0.26	83.73 ± 0.12	2	98.53 ± 1.16
AC3	Costa Rica	Sweet pineapple leaf	+	+	28.04 ± 0.24	83.37 ± 0.67	5	98.37 ± 1.10
AC4	Costa Rica	Dehydrated pineapple pulp	+	+	30.75 ± 0.38	83.63 ± 0.08	5	99.13 ± 0.99
AC5	Portugal[a]	Fresh pineapple pulp	+	+	29.35 ± 0.35	83.67 ± 0.10	5	99.80 ± 0.10
AC6	Costa Rica	Pineapple leaf	+	+	30.61 ± 0.27	83.47 ± 0.10	5	99.75 ± 0.23
AC7	Mauritius Islands	Baby pineapple leaf	+	+	30.21 ± 0.52	83.60 ± 0.13	1	99.77 ± 0.06
AC8	Azores	Azores pineapple Leaf	+	+	29.35 ± 0.14	83.40 ± 0.01	6	99.65 ± 0.43
AC9	Costa Rica	Sweet Gold pineapple leaf	+	+	28.40 ± 0.30	83.53 ± 0.10	5	99.40 ± 0.50
AC10	Spain[a]	Pineapple leaf	+	+	30.18 ± 0.13	83.40 ± 0.01	5	99.53 ± 0.15

[a] No labelled origin provided, which was replaced by the acquisition country.

validating the robustness of the method even in more processed samples, such as dehydrated pulp (AC4) and fresh pulp (AC5) that have high sugar and polyphenol contents, which can interfere with DNA purification and amplification. The sequencing analysis confirmed that the variations observed in the clusters are associated with few single-nucleotide polymorphisms, consistent with the patterns observed in the reference genotypes (Fig. 5). Sample AC7, originated from Mauritius Islands, was fully aligned with EC011, justifying its inclusion in cluster 1. Samples AC1 and AC2, originated from Brazil, though not fully aligned with EC013, were logically included in cluster 2, representing 'Primavera', a traditional cultivar from Brazil. The potential 5 mismatches identified in sample AC8 justify its inclusion in a single independent cluster 6 with a distinct difference curve profile (Fig. 4D). Although sequencing results do not justify grouping of samples AC3-AC6 and AC9-AC10 in cluster 5, they all display similar difference curve profiles and potentially share the same origin from Costa Rica.

These results demonstrate the ability of HRM to discriminate pineapple varieties and even certain cultivars, as evidenced by the additional clusters observed among commercial samples. This highlights its usefulness for detecting subtle genetic variations, often not captured by

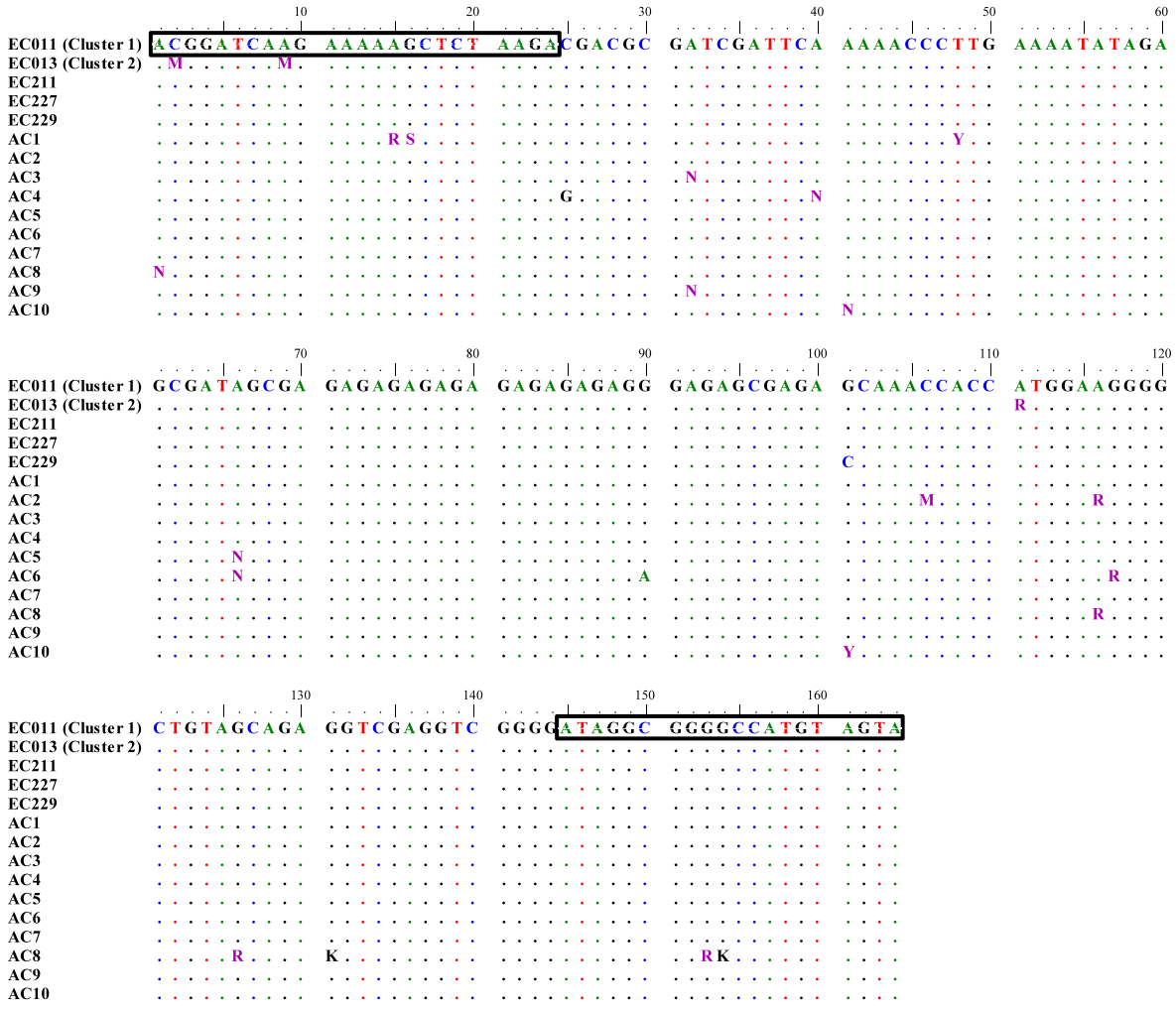

Fig. 5. Alignment of sequences from relevant cluster 1 (EC011) and cluster 2 (EC013) and the amplicons of the three uncharacterised genotypes from INCAPER (EC 211, EC 227 and EC 229) and ten commercial pineapple samples (AC1 to AC10). The black rectangle highlights the region of primer annealing (ANC2-F/ANC2-R). Legend of undefined bases: K represents G or T; M, A or C; R, A or G; S, C or G; W, A or T. N any base or a gap.

morphological methods. Notably, all samples in cluster 5 are originated (AC3, AC4, AC6 and AC9) or most likely (AC5, AC8 and AC10 acquired in Portugal and Spain, with unknown origin) originated from Costa Rica. Considering that Costa Rica is a major producer of the globally dominant MD-2 variety, it is likely that these samples correspond to the MD-2 cultivar. The MD-2 pineapple cultivar, originally developed from hybrid 73-114 by the Pineapple Research Institute in Hawaii, was introduced to Costa Rica for pilot cultivation in 1980 and launched on the global market by Del Monte in 1996. Over the following two decades, MD-2 became the global benchmark in pineapple trade, contributing to increased cultivation areas in Costa Rica and reinforcing the country's leading role in the international market (Paniagua-Molina & Solís-Rivera, 2020).

In contrast, the Azores pineapple is a Portuguese cultivar with Protected Designation of Origin (PDO) that is traditionally associated with the Cayenne group, although with notable differences in acid content and morphological characteristics among others (Table 1). In detail, Portuguese Azorean pineapples are typically smaller, with a concentrated flavour balancing a pronounced sweetness with tangy, unique golden-yellow rough skin, and a vibrant green crown (Rainha et al., 2013). Therefore, the inclusion of the sample originated from Azores in cluster 5, was not expected, considering its Cayenne origin, but justified by the potential mismatches with the cluster 1 representative (EC011) (Fig. 5). However, the Cayenne group category, as for the other groups, is largely defined for commercial and horticultural purposes, meaning it may have limited relevance at the genetic level, explaining this incongruence. Additionally, since these pineapples were collected from commercial markets, the origin or crossbreeding history is unknown.

Similarly, the Baby Mauritius pineapple sample (AC7), originated from Mauritius Island, was grouped with the Smooth Cayenne cluster (cluster 1) rather than with the expected Vitória group (cluster 2). This is in line with the high sequence similarity of the target HRM locus. The limited genetic diversity resulting from the clonal propagation of commercial pineapples could also contribute to this clustering pattern. Samples from Brazil (AC1 and AC2) were grouped in the same cluster 2, corresponding to 'Primavera' of the Maipure group cultivated in Brazil.

In summary, the application of HRM targeting SSR and SNP polymorphisms to commercial products not only validates its utility for authentication of pineapple cultivars/origins, but also exposes gaps in knowledge about the genetic diversity of *A. comosus* at a global scale. These findings support the implementation of this methodology for supply chain traceability, fraud prevention and genetic resource management, aligning with the demands for transparency in international markets.

4. Conclusions

This study successfully demonstrated the development and application of a HRM approach targeting SSR and SNP polymorphisms to differentiate *A. comosus* varieties and cultivars from distinct origins. The

methodology enabled the discrimination of seven characterised *A. comosus* genotypes into 4 independent clusters with high levels of confidence (≥98.7%): cluster 1 (reference cluster) including Smooth Cayenne, Vitória, Pérola and Turiaçú cultivars; cluster 2 having Primavera cultivar; cluster 3 having Gigante de Tarauacá cultivar; and cluster 4 comprising the *erectifolius* variety. Sequencing data corroborated HRM clustering, being the number of the SSR repeating motif (GA) n the main responsible polymorphism for discriminating the *A. comosus* var. *comosus* genotypes from *A. comosus* var. *erectifolius*, while few SNP distinguished other cultivars. The HRM method was successfully applied to discriminate uncharacterised germplasm genotypes and commercial pineapple cultivars. The three uncharacterised genotypes, though having different origins, were all grouped in cluster 1, suggesting their close genetic relationship. The 10 commercial pineapple samples were differentiated into four groups: one in cluster 1 and two in cluster 2, aligned with representatives of 'Smooth Cayenne' and 'Primavera'; one and six samples created additional clusters 6 and 5, including samples from Azores and Costa Rica, respectively. Generally, the samples were logically discriminated according to their origins, which was corroborated by sequencing and melt curve profiles highlighting the robustness of the method. The analysis of commercial samples highlighted the practical applicability of this method for certifying pineapple-derived products, ensuring species authenticity across different international markets. This approach represents a significant advancement for the agricultural sector, offering a rapid, precise and cost-effective tool for cultivar authentication and monitoring genetic diversity in *A. comosus*.

By contributing to the traceability of plant materials, this study strengthens the molecular foundation for future breeding programs and conservation of pineapple genetic variability. The development of a species-specific molecular database opens new paths for genetic research and biotechnological applications, solidifying HRM as an efficient strategy for the molecular characterisation of pineapple cultivars. This work not only advances scientific knowledge, but also supports industry practices, paving the way for improved quality control and genetic resource management in pineapple production.

CRediT authorship contribution statement

Paula Roberta Costalonga Pereira: Writing – original draft, Methodology, Investigation, Formal analysis. **Rita Biltes:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Caterina Villa:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Joana Costa:** Writing – review & editing, Methodology, Investigation. **José Aires Ventura:** Writing – review & editing, Investigation, Conceptualization. **Maria do Carmo Pimentel Batitucci:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Isabel Mafra:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2026.112323.

Data availability

Data will be made available on request.

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