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Differentiation of pear cultivars using PCR with high resolution melting analysis

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Abstract

The aim of this thesis was to investigate if polymerase chain reaction (PCR) coupled with high resolution melting (HRM) analysis can be used for the differentiation of pear cultivars. The differentiation of cultivars is of growing interest due to the increase in food adulteration.

For the differentiation of pear cultivars, DNA was first isolated using commercial DNA extraction kits and then subjected to PCR coupled with HRM targeting microsatellites (SSRs). The differentiation was carried out by pairwise comparing the normalized melting curves.

The first part of the thesis deals with the differentiation of 18 pear cultivars using DNA extracts from leaves. Fifteen primer pairs were applied, including six primer pairs targeting the pear and nine primer pairs targeting the apple genome. With all pear primer pairs and all except one apple primer pair, PCR products were obtained for all cultivars. Some of the apple primer pairs were less suitable for the differentiation of pear cultivars. Differentiation between cultivars of different species (*Pyrus communis* and *Pyrus pyrifolia*) was easier than the differentiation between cultivars of the same species. For the complete differentiation of the 18 pear cultivars, only three primer pairs would have been necessary. One of the 15 primer pairs proved to be the most suitable one, differentiating almost 88 % of the 18 cultivars.

The second part of the thesis focuses on the differentiation of three pear cultivars (Anjou, Cheeky and Williams) using DNA extracts from peel, pulp, and self-produced juices. Due to the low DNA concentration of the DNA extracts of the juices, DNA extraction was optimized using two DNA extraction kits, NucleoSpin Plant II and DNeasy mericon Food Kit. With the modified protocol of DNeasy mericon Food Kit, a higher DNA concentration in the DNA samples was obtained, which were then successfully used for the differentiation with apple and pear primer pairs.

The self-produced juices were heat treated to determine the effect of thermal pasteurisation on the DNA concentration of the DNA extracts and its amplifiability by PCR. The results showed that the heat treatment decreased the DNA concentration in the extracts, leading to increased Ct-values or even no increase in the fluorescence signal within 50 cycles. Different heat treatment conditions had a different impact on the DNA concentration and the Ct-values obtained.

The third part of the thesis compares the derivative and normalized melting curves obtained from leaf, peel, pulp, and juices from the cultivar Williams. The DNA was extracted with two different DNA extraction kits, NucleoSpin Plant II and DNeasy mericon Food Kit, resulting in DNA concentrations from 0.13 to 35.10 ng/μl. In spite of the differences in origin, the procedure of DNA extraction and the DNA concentration in the extracts, very similar normalized melting curves were obtained, which underlines the strength of the genetic fingerprint and the robustness of PCR coupled with HRM.

In summary, PCR-HRM with apple and pear SSR primer pairs was successfully used for the differentiation of pear cultivars of the species *Pyrus communis* and *Pyrus pyrifolia* using DNA samples from leaves, peel, pulp and juices. Pasteurised juices contain low intact DNA and need further investigation for the successful differentiation of cultivars.

Abstract (German)

Das Ziel dieser Masterarbeit war es, zu untersuchen, ob die Polymerase Kettenreaktion (PCR) kombiniert mit der Hochauflösenden Schmelzkurvenanalyse (HRM) für die Unterscheidung von Birnensorten verwendet werden kann. Die Differenzierung von Sorten ist aufgrund zunehmender Lebensmittelverfälschungen von wachsendem Interesse. Für die Differenzierung der Birnensorten wurde zuerst die DNA mittels DNA-Extraktionskits isoliert. Anschließend wurden die DNA-Proben mit Birnen- und Apfel-Primerpaaren, die auf Mikrosatelliten (SSRs) abzielen, mittels PCR und anschließender HRM untersucht.

Der erste Teil der Masterarbeit beschäftigt sich mit der Differenzierung von 18 Birnensorten unter der Verwendung von DNA-Extrakten aus Blättern. 15 Primerpaare (sechs Primerpaare, die auf das Birnengenom abzielen und neun Primerpaare, die auf das Apfelgenom abzielen) wurden getestet. Mit allen Birnen-Primerpaaren und allen Apfel-Primerpaaren, mit Ausnahme von einem, wurden PCR-Produkte für alle Sorten erhalten. Die Unterscheidung zwischen Sorten unterschiedlicher Spezies (*Pyrus communis* und *Pyrus pyrifolia*) war einfacher als die Unterscheidung zwischen Sorten der gleichen Spezies. Für die Differenzierung aller 18 Birnensorten waren nur drei Primerpaare notwendig. Eines der 15 Primerpaare erwies sich dabei als am geeignetsten, weil es die Unterscheidung von fast 88 % der 18 Sorten ermöglichte.

Der zweite Teil der Masterarbeit beschäftigt sich mit der Differenzierung von drei Birnensorten (Anjou, Cheeky und Williams) unter Verwendung von DNA-Proben aus Schale, Fruchtfleisch und selbst hergestellten Säften. Aufgrund der niedrigen DNA-Konzentration in den DNA-Extrakten der Säfte wurde eine Optimierung der DNA-Extraktion mit zwei DNA-Extraktionskits, NucleoSpin Plant II und DNeasy mericon Food Kit, durchgeführt. Mit dem modifizierten Protokoll des DNeasy mericon Food Kits wurde eine höhere DNA-Konzentration in den DNA-Proben erzielt, welche dann erfolgreich für die Differenzierung mit Apfel- und Birnen-Primerpaaren verwendet wurden.

Die selbsthergestellten Säfte wurden hitzebehandelt, um den Effekt der thermischen Pasteurisierung auf die DNA-Konzentration der Extrakte und deren Amplifizierbarkeit mittels PCR zu untersuchen. Die Resultate zeigten, dass eine Hitzebehandlung die DNA-Konzentration in den Extrakten reduzierte, was zu einer Erhöhung der Ct-Werte oder sogar zu keinem Anstieg des Fluoreszenzsignals innerhalb von 50 Zyklen führte. Unterschiedliche Temperatur und Dauer bei der Hitzebehandlung hatten einen unterschiedlichen Einfluss auf die DNA-Konzentration und den Ct-Wert der DNA-Proben.

Der dritte Teil der Masterarbeit vergleicht die ersten negativen Ableitungen der Schmelzkurven und die normalisierten Schmelzkurven, die für Blatt, Schale, Fruchtfleisch und Säfte der Sorte Williams erhalten wurden. Die DNA wurde mit zwei unterschiedlichen DNA-Extraktionskits, NucleoSpin Plant II und DNeasy mericon Food Kit, extrahiert, was zu DNA-Konzentration von 0.13 bis 35.10 ng/µl führte. Trotz der Unterschiede in Herkunft, in der Durchführung der DNA-Extraktion und der DNA-Konzentration in den Extrakten wurden sehr ähnliche normalisierte Schmelzkurven erhalten, was die Stärke des genetischen Fingerabdrucks und die Robustheit von PCR mit HRM verdeutlicht.

Die Arbeit zeigt, dass Birnensorten der Birnenarten *Pyrus communis* und *Pyrus pyrifolia* über DNA-Proben aus Blättern, Schale, Fruchtfleisch und Säften mittels PCR-HRM und SSR-Primerpaaren, die auf das Birnen- oder das Apfelgenom abzielen, unterschieden werden können. Pasteurisierte Säfte enthalten wenig intakte DNA und weitere Forschung für die erfolgreiche Differenzierung der Sorten wird benötigt.

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1. Introduction

1.1 Pears

Pears are sweet fruits, that have been consumed for more than two thousand years. [1] They can be eaten raw or used in a variety of products such as juices, jellies, pies, cakes, and ciders. [1,2] Globally, pears rank at second place in fruit production. Furthermore, the pomace remaining after juice production is high in pectin and can be used to produce vegan gelatine. In 2018, approximately 23.9 million tonnes of pears were harvested. The largest pear producer was China with 20.9 million tonnes followed by Italy, the United States, and Argentina. [2]

Pears are cherished for their taste, nutritional value, and many health benefits due to their components. Additionally, they are easy to grow and to maintain and very robust, able to withstand climatic and stress situations. They can be cultivated in all temperate zones with an elevation ranging from 1700 to 2400 m above sea level. The fruit production starts two to four years after planting and continues for up to 75 years. [2]

1.1.1 Origin and History

Fossils of leaves and fruits of *Pyrus* were found in sediment of the Tertiary period located from Asia Minor through the Caucasus mountains as far as western China. The pears were then dispersed to the east and to the west. [3,4] The first pears cultivated by humans can be traced back to 1000 B.C. in Europe and in East Asia. Homer (850 B.C.) mentioned large orchards of pears in the Odyssey [5] and even described them as 'gifts of the Gods'. [4] Greek philosopher Theophrastus (371 to 287 B.C.) wrote about the propagation of pears from cuttings and seeds and about the difference of wild and cultivated pear species. Cato the Elder (235 to 150 B.C.) describes propagation methods in his book 'De agri cultura' and the roman naturalist Pliny the Elder (23 to 79 A.D.) explains the medicinal values of pears. [4,5]

The major pear production began in Europe in 800 A.D. under Charlemagne who decreed that every garden should cultivate herbs and fruits, including pears. [5] In the eighteenth century, French King Louis XIV promoted the growth of *Pyrus communis*, which increased the selection of new *Pyrus communis* cultivars. In Belgium, more than one thousand new cultivars were cultivated between 1758 and 1900. Abbot Nicholas Hardenpont and Jean-Baptiste Van Mons enhanced the development of new pear cultivars. Especially Van Mons cultivated about 500 different varieties and his work strongly promoted pomology in Europe and America. In the twentieth century, fruit breeding programs were funded to develop new pear cultivars with focus on resistance against pests and diseases, as well as improving their quality, appearance and shelf life. By 2012, around 200 European and 100 Asian pear cultivars were developed and released. [6]

1.1.2 Genomics

Pears belong to the genus *Pyrus* and are part of the Rosaceae family and the Maloideae subfamily. There are 30 different pear species and more than 5000 pear cultivars. [3] According to a theory derived from the 1920s, pears resulted from crossing Prunoideae (basic chromosome number eight) with Spiraeoideae (basic chromosome number nine), both belonging to the Rosaceae family. Typically, the members of the Rosaceae family have the basic chromosome number seven or nine. The crossing between Prunoideae and Spiraeoideae led to the unusual basic chromosome number 17. The majority of pears are diploid, which means that they have two complete sets of chromosomes with a chromosome number of 34 (2×17), however, some of the *Pyrus communis* cultivars and breeds of *Pyrus communis* with *Pyrus bretschneideri* are polyploid. [4] 997 European pear leaves and 166 Asian pear leaves were tested in a study, where approximately 7 % were found polyploid and 88 pear cultivars triploid with a chromosome number of 51 (3×17). Of the 88 pear cultivars, 81 were European cultivars, four interspecific

hybrids and three Asian cultivars. One of the European pear cultivars was tetraploid with a chromosome number of 68 (4x17) and one was aneuploid. [7]

Pears are divided into two main groups, European pears - also known as *Pyrus communis* - and Asian pears. Asian pears are classified into *Pyrus pyrofolia*, *Pyrus bretschneideri*, *Pyrus sinkiangensis* and *Pyrus ussuriensis*. Asian and European pears differ in their geographical origin as well as in their form, texture, components, aroma and flavour. [4, 8] European pears, mostly grown in Europe, South Africa, Oceania, North and South America [4], are softer with a higher starch content [3], but a lower water and sugar content. They also have a stronger aroma than Asian pears. Additionally, European pears are gourd-shaped whereas Asian pears are round [8], similar to an apple, hence the name 'apple pears'. [1] The Asian pears are mostly produced in Asia. [4]

1.1.3 Genetic diversity and differentiation

Genetic diversity refers to the variability between different genotypes of the same species. It is generated either during sexual recombination where homologous chromosomes cross over and new recombinations are developed or due to evolutionary factors such as mutation and migration. Genetic diversity is of great importance for the overall survival of the species. It allows the breeding of plants with special characteristics, including high yields, resistance against pesticides, tolerance against stress and nowadays very important, the breeding of climate resilient cultivars. However, the genetic diversity is decreased due to natural selection of specific traits, inbreeding due to isolation of the species and climate change which alters the environment. Those species, which cannot adapt, are at risk of extinction. [9]

Pears have a high diversity because of their self-incompatibility (SI) system, [10] which rejects pollen from related cultivars, thereby preventing self-fertilization and inbreeding. The pears therefore depend on cross pollination with genetically varied cultivars. SI is divided into heteromorphic SI which prevents inbreeding by producing more morphological flower types and homomorphic SI by genetic and biochemical mechanisms. The Rosaceae family including pears belongs to the homomorphic SI, specifically the gametophytic self-incompatibility (GSI), where the pollen determines the incompatibility. This system is controlled by a S-locus. When the pollen has the same S-locus as the styles, the pollen growth will be blocked. [11]

However, although there are over 5000 pear cultivars, [3] only few are cultivated for production in economic scale. In Europe alone, 80 % of the cultivated and sold pear cultivars consist of e.g. Abbe Fetel, Blanquilla, Conference, Coscia, Doyenne du Comice and Williams. These handful of cultivars dominate the market because they result in sufficient economic outcome. The reduced production of other and even older, traditional pear cultivars may lead to the loss of genetic diversity. [12]

Furthermore, studies found that some *Pyrus* cultivars, especially of the species *Pyrus pyrifolia*, have self-compatible mutants, which allows the self-fertilization/inbreeding. A Japanese study found that these pear seedlings suffered from inbreeding depression which resulted in shorting their height. [13] Inbreeding depression refers to the decreased adaption of the offspring which is often associated with slower growth, reduced germination, height and fertility. [14] Numerous breeding programs around the world were established with the goal to produce new pear cultivars, which can control the ripening time, reduces production costs, and improves the quality and resistance against diseases and climate change. [6, 15] Japanese researchers usually breed by crossing local and closely related pear cultivars (inbreeding). Breeding with foreign cultivars is not performed in order to provide biosecurity when exporting plants from abroad. [15] Inbreeding is generally known to decrease the genetic diversity in the offspring. It will decrease the heterozygosity, viability and even develop inbreeding depression. [14]

Due to the reduced genetic diversity in some modern pear cultivars it is of great importance to preserve especially older, traditional pear cultivars with high genetic diversity. In order to preserve and maintain pear cultivars, a differentiation method is necessary. The differentiation of pear cultivars is challenging because pears are bred either by seed propagation or by interspecific hybridization, which means that two different species within the same genus

can produce an offspring in form of a hybrid. [6] The classification is further complicated since the previous pear names were unknown and replaced with names based on their individual harvest time (spring, summer or autumn), usage and other characteristics. [3, 16]

The first identification and differentiation of pear cultivars was based on their morphological characteristic, but due to the huge similarity between pear cultivars it is a hard skill and was later replaced by the analysis of phenolic compounds, sugar composition, pollen ultrastructure and isozymes. However, the natural components in pears are influenced by for example environmental and storage conditions which made these analyses doubtful. Nowadays, DNA based molecular markers are used to differentiate pear cultivars. [17] Especially, the molecular marker 'simple sequence repeat' (see section 1.3.2) is used to differentiate cultivars and establish genetic fingerprints. [11] The first 'simple sequence repeats' used for pears were actually derived from apples, indicating the strong transferability of 'simple sequence repeat' markers between the two genera. [18] *Malus x domestica*, also known as apple, belongs to the same subfamily (Pomoideae) of the family Rosaceae as pears, has the same chromosome number, similar genome size and secondary metabolites. [19] Studies have confirmed that apple 'simple sequence repeat' markers can be successfully used for distinguishing pear cultivars. [11, 19]

Furthermore, studies found that with DNA based markers, European pear cultivars (*Pyrus communis*) can be easily differentiated from Asian pear cultivars (*Pyrus pyrifolia* and *Pyrus serotina*), whereas the Japanese and Chinese pear cultivars are closely related, which complicates the differentiation. [20]

1.1.4 Components and health benefits

Pears are known for their nutritional value and numerous health benefits. Even in ancient China and Korea, pears were used as a folk remedy. The first traditional Chinese pharmacopoeia 'Shen Nong Ben Cao Jing' published approximately 220 A.D. mentions the effects of pears in relieving thirst, cough, fever, alcoholism and chest tightness. However, it warns that an overconsumption of pears can upset the stomach and the intestines. [8]

Depending on the pear cultivar, the pear consists approximately of 80 % water, 15 % carbohydrates and 2 % fibre plus small amounts of proteins and fats. [8] Most of the carbohydrates consist of sugar, with fructose in the lead, followed by glucose and sucrose. Pears have a glycemic index (GI) of 38 and therefore belong to the low GI food. A low GI value means that the carbohydrates (glucose and insulin) are released slowly into the blood. [2] This supports weight loss and decreases the risk of diabetes. [8] Compared to other fruits, pears have the highest content of insoluble fibre and a high total fibre content. [2] Fibre is prebiotic and supports the activities or growth of microbial species and improves the health of the human body. Additionally, fibre is a good source to treat constipation and intestine inflammation and to prevent obesity. [8] The quality of the fibre of pears is improved by the associated bioactive compounds, such as polyphenols and flavonoids. Since the peel contains most of the fibre, it is recommended not to remove and discard it. [3]

Pears contain nine essential minerals (boron, calcium, copper, iron, magnesium, manganese, potassium, sodium and zinc) where potassium has the highest content followed by magnesium or calcium, depending on the cultivar. The minerals are necessary for both the plant and the human body to function normally as well as for biochemical reactions. Furthermore, they contain a variety of vitamins, including ascorbic acid, vitamin B, choline and all fat-soluble vitamins [3] with the exception of vitamin D. [2]

Pears also have secondary metabolites which play a huge role in the defence against stress and contribute to colour and taste of the fruit. Phenolic compounds, for example, are antioxidative, anticarcinogenic and antimutagenic. The main phenolic compounds in pears are chlorogenic acid, arbutin, and epicatechin. Chlorogenic acid is an antioxidant with chemo preventive properties and might prevent chronic diseases like cancer or cardiovascular diseases. Arbutin has antibiotic properties which enables the plant to defend itself against fire blight [21], a highly destructive bacterial disease of the Rosaceae family. [22] The quantity of phenolic compounds is higher in the peel than in the pulp. [21]

1.2 Fruit juice

Pears are mostly consumed raw or in form of juices. Juice production is a growing market, because juices have a longer shelf life, are more convenient for transport and for consumption (for all age groups and do not require additional utensils) than the fruit itself. Furthermore, fruits with unfavourable sizes, shapes and even blemished portions (excluding spoilage) can still be used. [23]

According to the Codex Alimentarius (General Standard for fruit juices and nectars CXS 247-2005), juices are defined as the liquid extracted from mature and fresh fruits through approved processes. The juice processing has to maintain chemical, nutritional, physical and organoleptic characteristics of the fruits in the juice. [24] The juices can later be filtered, diluted with water, also sugar and acid can be added, depending on the type of juices or their labels. [25]

Fruit juices are categorized based on the fruit, sugar and water content as well as the processing steps. Pure juice 100 % and fresh squeezed juice are juices directly extracted from the fruits with no addition of water or sugar. The fruit content amounts to 100 %. Concentrated fruit juice is when the water content has been removed from the juice to enable better storage, transport and even preservation. The concentrate is diluted with water to its original soluble solids content, also known as Brix level. Fruit nectar has a fruit content of at least 50 % and contains additional water and sweeteners (sugar, honey or syrups), additives and other flavour components. [23]

The guidelines for fruit juices regarding the composition (additives, vitamins, etc.), processing methods (juice production) and labelling are further stated in the European Fruit Juice Directive (Directive 2012/12/EU). [26]

1.2.1 Juice processing

The juice production is divided into many operations. For fruit harvesting, the ripeness, spoilage and contamination of the fruit must be taken into consideration. The fruits are stored under controlled atmosphere, where the temperature, humidity and the amount of oxygen, carbon dioxide and ethylene are modified in order to control the ripening of the fruits and prevent spoiling. The decrease of oxygen and temperature can decrease the spoilage and fermentation, whereas ethylene can accelerate the ripening. The process of fruit harvesting and further handling, such as storage, are described in the good agriculture practise (GAP). [23]

For the juice production process, the fruits are first washed. Clean and chlorinated water is used to ensure the elimination of dirt, pesticides and fungicides. The fruits are then sorted based on the criteria for food safety and quality, such as ripeness, solids level, acidity, the number of defects, the levels of bioburden, mycotoxins and pesticides. The defective fruits (high levels of mycotoxins, pesticides etc.) must be sorted out manually or by sensors. [23, 25]

Stone fruits, oranges, grapefruits and bananas need an additional treatment, where the stones or skins are removed with special extractors. In the following step the fruits are cut or ground in order to increase their surface area, which ensures a higher efficiency and juice yield. [25] The juice extraction and the juice yield can be improved by the addition of macerating enzymes, which hydrolyse fibres, such as cellulose, hemicellulose, and pectin. This process can be shortened by heating the juice to the optimum temperature of the macerating enzymes, 60 °C for about 40 minutes. However, the addition of the enzymes causes the hydrolysis of pectin, with the effect that the pomace cannot be used for vegan gelatine production. Another way to improve juice extraction is by heating the fruits to approximately 70 °C in order to soften the fruit and decrease the viscosity. In the pressing step, the fruits are crushed/pressed and the juice is extracted. Through centrifugation (e.g. decanting centrifugation) and filtration (e.g. membrane filtration, rotary vacuum filter), the pulp is either reduced or completely separated from the juice and the turbidity is reduced. [23]

In the next step, the juice undergoes pasteurisation to inactivate microorganisms, such as yeast, mould, *Lactobacillus*, *Clostridium*, [25] *Escherichia coli* [27] and *Listeria monocytogenes* [28], which endanger the health of the consumers. Furthermore, pasteurisation increases the stability, quality, appearance and the shelf life of the juice by reducing eventual chemical, enzymatic and microbial reactions. [25, 27]

According to the Code of Federal Regulations (CFR) title 21 part 120, the juice production should ensure the food safety by ensuring 5 log reduction (i.e. 10^5) of the juice for the time of the shelf life when stored under normal conditions. 5 log reduction means that the number of pathogens is reduced by a factor of 100,000. [28]

In the classical thermal pasteurisation, the juice has to be heated to a certain temperature for a specific time. [28, 29] Depending on the pH value of the juice, either pasteurisation or sterilisation takes place. Juice with a pH value below 4.5 to 4.6 inhibits the germination of microbial spores and toxin production. [28–30] Therefore, a pasteurisation with temperatures below 100 °C is sufficient. In a juice with a pH value above 4.5 to 4.6, microbial spores can still germinate, therefore the juice is either stored at temperatures below 7 °C (refrigerator) [30] or needs to undergo a sterilisation process at temperatures above 121 °C. [29] The combination of various methods to reduce the extent of one parameter (such as temperature, oxygen, water activity and pH value) is also known as the hurdle principle. [23]

The effect of pasteurisation/sterilisation is determined in form of the processing parameters, the D-, z- and F-values. [31] The D-value is the time required of a certain pasteurisation process at a specific temperature to reduce the pathogens by 90 % (also known as one log reduction). It is described by the first-order kinetics and calculated with formula 1. [30, 31]

$$\log\left(\frac{N}{N_0}\right) = \frac{-t}{D} \quad (1)$$

The z-value is the temperature increase required to achieve the D-value within 1/10 of the time. The F-value is a reference number, which indicates the duration the juice was heated at 250 °F (121 °C). The F-value is used in the food industry to compare different processes and the effectiveness of new pasteurisation methods. [31]

The most common pasteurisation types for fruit juices are the 'low temperature long time' pasteurisation at 63 °C for at least 30 minutes and the 'high temperature short time' pasteurisation at 72 °C for at least 15 seconds. The type of pasteurisation depends on the type of juice and the manufacturer. Higher temperature treatment showed to be more effective to control bacterial growth, but greatly decreases phenolic components compared to lower temperature. [27] As a negative side effect of pasteurisation, heat sensitive compounds such as vitamins, aroma and flavour components are reduced or even eliminated. This leads to a lower quality of the product, [27] however aromatic components can be recovered by distillation. [25]

Finally, the juice is packed into a sterile container. This process can be achieved either by aseptic processing or by hot fill. With aseptic processing, the pasteurised juice is rapidly cooled down and then filled into sterile containers in a sterile environment. This method maintains the quality of the juice, but costs more due to the additional sterilisation steps of room and packaging. With hot fill, the hot, pasteurised juice is filled into the container, thereby sterilising the container and creating a vacuum. The long exposure to high temperature has a negative impact on heat sensitive components. [23]

Apart from the traditional thermal pasteurisation, there are more advanced non-thermal methods, such as membrane filtration, pulsed electric field (PEF), ultraviolet (UV) exposure [27] and high-pressure processing (HPP). [30] Membrane filtration (ultrafiltration and microfiltration) is cold pasteurisation, where large molecules, including microorganisms are separated from small molecules, such as sugars and vitamins. Compared to thermal treatment, the flavours and natural components of the juices remain intact. This method requires less energy, but the membranes must be cleaned and replaced regularly. [27]

PEF inactivates vegetative cells, yeast and microorganisms via pulses at a high electric field intensity. The electrical field damages the cell membrane via irreversible pore formation within microseconds. [27, 32] The degree of

phenolic degradation is less severe than through thermal pasteurisation. However, the effectiveness of PEF is reduced with the complexity of the juices. [27]

UV light is commonly used in the food technology to inactivate microorganisms on surface and on packaging. The UV exposure at the wavelength of 254 nm is used to damage DNA of microorganisms and thus to inhibit their cell growth. [27,33] It causes a lower reduction of total phenolic compounds (29 %) than thermal processing (48 %). Furthermore, no off taste, odour and colour change are developed. [27]

High pressure processing or high hydrostatic pressure (HHP) uses isostatic pressure of 400-600 MPa to inactivate microorganisms. The packaged juices are placed into the HPP treatment chamber, which is then filled with water. The water flow generates a low pressure which increases with time due to the constant addition of water. The high pressure damages the cell membrane and denatures proteins, ultimately leading to microbial cell death. The exclusion of high temperature enables this method to maintain heat sensitive components (e.g. vitamins) and the natural and fresh appearance and flavour. HHP can be combined with heat treatment (above 45 °C) to inactivate spores which increases the juices' shelf life. [30]

1.3 Food adulteration

Food adulteration is defined as the act of intentional substitution of high quality ingredients with lower quality ones, the addition of unauthorized substances, misleading statements, and false labelling, for example regarding origin or cultivars. [34] The goal is to increase the profit through reduction of production costs or the increase of the apparent value of the food product. [35] Food adulteration has existed even in ancient times (Greek and Roman times) but has its rise in the eighteenth and nineteenth centuries, when flour, milk and meat were replaced with inedible substances by the producers. For example, suppliers partly substituted flour with chalk and even coffee and tea with dust to increase the mass of the products and thereby their profits. In 1820, the German scientist Frederick Accum raised awareness of the danger of food adulteration in his book 'A Treatise on Adulteration of Food and Culinary Poisons'. [36]

Although nowadays analytical detection methods are constantly developed and improved, food adulteration has become a global problem, due to the increasing market globalization and the complex food supply chains. Besides, adulteration is often difficult to detect, due to the different types of fraudulent practices, the similar structure of the adulterant to the authentic food, and its potentially low concentrations. [36] According to the US Food and Drug Administration (FDA), food adulteration concerns at least 1 % of the global food production and has a value of 10 to 45 billion US dollar a year. [35] Furthermore, many cases of food adulteration have reduced consumer trust in the food industry and have even caused serious health problems, such as the addition of melamine to milk powder (China's international milk crisis). [36]

1.3.1 Food authenticity

Food authenticity is the correctness of the information on food labels, including details about the composition, origin, production process, and the quality of the product. [37] The development of analytical methods to verify the authenticity of food products is of growing interest due to increasing food adulteration (see section 1.3) and counterfeit food. These methods help to ensure food safety and quality and aim to establish trust between consumers and producers through transparency. [38, 39]

Numerous studies have been carried out to explore and develop more effective methods to detect food adulteration. The most common methods are DNA based methods, e.g. PCR, gel electrophoresis, chromatographic (e.g. HPLC) and spectroscopic methods (e.g. NMR, MS). [39] The choice of method depends on the type of food and the main fraud type, for example substitution of different species/varieties, false geographic origin, or the addition of additives [40]

For species and cultivar identification, especially protein and DNA based methods have been developed. However, protein based methods, such as chromatographic methods or immunological assays, are less commonly used than DNA based methods. Compared to protein, DNA is more stable and present in all organisms and even in most of the tissues. [41] Even in highly processed food, that underwent physical and chemical treatments, sufficient DNA can still be recovered for analysis. Furthermore, DNA is unique for each species/cultivar and remains unaffected by growing conditions or storage environment, unlike proteins. [40] Therefore, DNA based methods, especially methods targeting suitable molecular markers, are mostly used for the identification of species and cultivars in food. [42]

1.3.2 Identification using molecular markers

Molecular markers are DNA sequences that are representative of a genome. They are used in genetic studies, plant biotechnology, and for food traceability to differentiate between individuals, populations, species and/or cultivars. Nowadays, they are especially used in plant biotechnology to identify and to explore the genetic diversity of newly bred plant species and to identify different variants. The molecular marker methods are classified into three types. The first type is based on hybridization (e.g. restriction fragment length polymorphism (RFLP)), the second on PCR (e.g. amplified fragment length polymorphism (AFLP)), and the third on DNA sequencing.

In the RFLP method the DNA is isolated and incubated with restrictive enzymes, which cleave the DNA at their respective cleavage sites. Polymorphisms cause differences in the restriction site and thereby in the length of the DNA fragment. The DNA is then hybridized with a probe, which allows the determination of polymorphisms and thereby the differentiation and even identification of species/cultivar by gel electrophoresis. This method is widely used due to its high reproducibility and specificity. However, RFLP requires high quantity of high quality DNA, toxic and expensive reagents and is furthermore time consuming. Therefore, this method was replaced with other methods, such as the PCR based marker method. [43]

Polymorphisms between different species/cultivars etc. are developed through changes in the DNA sequence, such as point mutation, deletions etc. Common markers are single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs). [43] SNPs are based on polymorphisms in a single nucleotide, due to insertion, substitution or deletion. SSRs also known as microsatellites are short repeatable sequences ranging from 10 to 30 bp [44] of 2 to 6 bp (see figure 1). [40] Microsatellites were first detected in the 1980s and are now the most effective and commonly used PCR based markers. The polymorphism is created during the DNA replication due to DNA polymerase slippage resulting in the different allele length. [45] The variation of the sequence length/repetition influences the amplicon length and the melting temperature, which enables the differentiation of species or cultivars via capillary electrophoresis (see section 3.4) or with HRM (see section 3.3.3). [43]

CAAGGAAATCATCAAAGATTCAAGATTAAAGACGGAGAGAAACAGGCGCCATTATTTGCCTCGCTCTC
TCTCTCTCTCTCTCTCTCTCGAACTTTCTGTCCC**CAACGATCCGAAGCCAACCTTG**

Figure 1 NCBI BLAST: genome sequence of *Pyrus communis* (microsatellite sequence (blue) and binding sites for forward and reverse primer sequences (yellow))

Especially simple sequence repeats have gained huge popularity over the last few years and numerous studies have been carried out to explore their applicability in the identification of food cultivars and the traceability of food production chain. [40] Studies showed, that the analysis of microsatellites by HRM allows to identify and distinguish between cultivars of the same species in non-processed food, e.g. potatoes, corn, tomatoes [43], zucchini, lentils [40] apples and pears [11] and even in processed food e.g. wine, olive oil [40], dried fruits, and juices. [46]

1.3.3 Challenges of food adulteration in fruit juices

Fruit juices are a common target of food adulteration, by diluting them with water, adding sugars (such as fructose, corn syrup), acids and other types of fruit juices, or by replacing cultivars of higher value with those of lower value. [46]

The detection of food adulteration in processed food, including juices, is an important and widely investigated topic. Different methods and techniques have been applied, such as sensory testing (based on colour, odour, and texture) and microscopic examinations, high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), Fourier transform infrared spectrometry (FTIR), and mass spectrometry (MS). [47] Rather new, but commonly used methods are DNA based methods, because DNA can be recovered even from highly processed food. The most used DNA based methods are methods targeting molecular markers, especially SSRs (see section 1.3.2 and 3.3). [46]

However, the occurrence of high amounts of phenolic components and polysaccharides, such as polyphenols and pectin, in food is problematic since these substances can inhibit PCR. During DNA extraction, polysaccharides can precipitate and imitate DNA [48], thereby interfering with enzymes (e.g. DNA polymerase) and inhibiting PCR. [49] Besides, polyphenols can bind to DNA and modify its structure, preventing amplification. [48]

Fruit juices undergo various processing steps (mechanical, chemical, and even enzymatic treatments) [23] and finally pasteurisation, which reduces the amount of phenolic components [27] and thereby PCR inhibitors. Nevertheless, processing and pasteurisation make it more difficult to obtain DNA of high quality and quantity due to DNA degradation. [50] DNA degradation is the process of breaking down DNA into nucleotides, which reduces the amount of DNA template for PCR. Consequently, the number of required reactions to exceed the limit of detection increases. The degree of DNA degradation is influenced by processing and storage conditions, such as temperature, pressure and pH value, as well as the food matrix. [51] These factors can induce deamination, hydrolysis, and oxidation of DNA, lowering its quality, indicated by decreased fragment lengths. [50]

High temperature during pasteurisation or sterilisation can lead to physical damage of DNA. The DNA irreversibly loses its native conformation, and large DNA fragments are sheared into smaller fragments. Naturally occurring smaller DNA fragments, however, are less affected and are therefore still detectable. [50, 51] According to a study, the use of primer pairs targeting shorter DNA fragments (below 100 bp) can improve the amplification of DNA from processed food. [52] Other studies showed that DNA from different food that has undergone high temperature processing apart from pasteurisation and sterilisation, such as baking, frying, autoclaving and roasting, can still be amplified. [50]

A low pH value can cause acid catalysed reactions, however, due to the cell wall DNA is protected from breakdown. However, at extreme pH values ($\text{pH} < 3$) DNA can be depurinated, leading to PCR failure. At high pH values ($\text{pH} 8.5\text{-}9.5$), DNA is stable and no degradation takes place. [50] The pH value of fruit juices depends on the fruit, cultivar, climate, and origin. The pH value of pear juices is typically between 3.3 and 4.5. [53]

2. Aim of this thesis

The motivation behind this thesis was to offer a method to detect food adulteration by differentiating fruit cultivars. Economically motivated food adulteration, also known as food fraud, refers to false labelling, replacement of high quality ingredients with low quality ones, and the addition or removal of certain components. Fruit juices are one of the most common targets of food fraud. There is also growing interest in preserving traditional fruit cultivars, such as old pear and apple cultivars, to maintain genetic diversity. A method that allows the differentiation of fruit cultivars could also be useful for this purpose.

The aim of this thesis was to investigate if DNA can be extracted from pear cultivars using commercial DNA extraction kits. In addition, it was of interest if apple and pear primer pairs targeting microsatellites (SSR) can be used to obtain suitable PCR products for *Pyrus communis* and *Pyrus pyrifolia* that can be subjected to high resolution melting (HRM) analysis. Furthermore, it was to determine if 18 pear cultivars can be differentiated using PCR-HRM with both apple and pear SSR primer pairs. The differentiation of the pear cultivars was performed with DNA extracted from leaves, pulp, peel, and juices. While leaves, peel and pulp were untreated, the juice samples were processed and pasteurised, which typically reduces the amount of intact DNA.

The first aim was to investigate whether 18 pear cultivars can be distinguished by PCR and HRM using apple and pear SSRs. DNA should be extracted from leaves of 15 mostly traditional European cultivars (*Pyrus communis*) and three Asian cultivars (*Pyrus pyrifolia*).

The second aim was to investigate if three pear cultivars (Anjou, Cheeky, and Williams) can be distinguished using DNA extracted from peel, pulp, and self-processed juices. Since fruits and juices generally contain low DNA concentrations due to their high water content and processing steps, different DNA extraction kits should be compared with respect to DNA yield and amplifiability. Furthermore, it was to determine if the three cultivars can be differentiated with PCR coupled with HRM using both apple and pear SSR primer pairs. Since store-bought juices are pasteurised, the influence of heat treatment on DNA concentration and amplifiability was also examined.

The third aim of this thesis was to investigate if DNA extracted from leaf, peel, pulp, and juices of the same pear cultivar (Williams) results in identical melting curves in PCR-HRM analysis. Leaves have a lower water content but a higher content of secondary metabolites than fruits. It was of interest to see how these differences affect the results of HRM analysis.

3. Theoretical background

3.1 DNA extraction

The first DNA extraction was performed in 1869 by Friedrich Miescher. [54] DNA extraction is used to isolate DNA from a sample. The samples are usually fresh or frozen such as hair, blood, leaves, seeds, flowers, viruses and spores. The extraction is based on chemical and physical methods, where the DNA is separated from cell membranes, proteins, lipids, RNA, and other components. [55] There are many methods of DNA extraction based on organic extraction, non-organic or adsorption techniques. Different methods need to be performed depending on the sample analysed. Nowadays, DNA extraction is most commonly performed with kits, which include the solutions and columns required. DNA obtained with DNA extraction kits is usually of high quality and quantity. [54] Before DNA is extracted, sampling and eventually homogenization take place. Homogenization, required for solid and unhomogenized samples, is frequently performed by grinding. [55] The first step of DNA extraction is cell lysis (or cell disruption) where the cell membrane is disrupted. Cell lysis is carried out by chemical (non-ionic detergent), heat, or mechanical disruption. [55, 56] In the next step, DNA is separated from impurities, such as lipids and proteins. [57] The impurities are denatured, precipitated and removed by adding either protease, chloroform-phenol or others. [55]

In this master thesis, two DNA extraction kits were used. For DNA extraction from pear leaves, the NucleoSpin Plant II kit, manufactured by Macherey-Nagel, was used, which is based on the use of silica columns. Chaotropic salts, in form of chaotropic buffer, are added to the samples, which result in denaturation of proteins and the adsorption of DNA onto the silica column. The impurities do not bind onto the surface of the silica column and are washed out. In the last step, DNA is eluted from the column. [58]

For DNA extraction from pear peel, pulp, and juices, the DNeasy mericon Food Kit by Qiagen was used. It is based on the cetyltrimethylammonium bromide (CTAB) method, which is commonly used for extracting DNA from plants. [59] This method was first developed by Doyle in 1990. The samples are added to CTAB (2 %) at an alkaline pH value. The extraction buffer results in the precipitation of DNA and acidic polysaccharides and separates them from impurities. Then DNA is separated from acidic polysaccharides through addition of high salt concentration. [57] Then, DNA is purified and eluted. Depending on the manufacture and the kits, a secondary cleanup may be performed before quantification of the DNA concentration. [59]

3.2 Fluorimetry (Measurement of DNA concentration)

Fluorometric (e.g. Qubit) and spectrophotometric (e.g. NanoDrop) methods are used to quantify the DNA concentration. [60] A precise quantification of DNA is important to obtain consistent and reliable results in the following analyses. [61] Fluorimetry, also called fluorescence spectroscopy, is a simple and fast method to evaluate the concentration of double stranded DNA (dsDNA). It works by exciting the sample with ultraviolet (UV) light and detecting the resulting fluorescence. This method has a high sensitivity and specificity. [60]

For the measurement of fluorescence signals, a light source, such as xenon [62, 63] or mercury lamp [62], emits light to a monochromator. The monochromator selects the excitation wavelength, and the light passes through the sample. [63] For the measurement of the DNA concentration, the wavelength is set at 260 nm, because purine and pyrimidine bases have their absorption peaks at this wavelength. [64] The absorbed light excites electrons in the molecules, and when they return to the singlet ground state, energy in form of radiation is emitted. [62] The light passes through filter and monochromators before being detected. [62, 63]

For quantification, the sample has to contain fluorophores, which are usually compounds with aromatic rings. Common fluorophores for DNA quantification are the intercalating dyes SYBR Green, PicoGreen, and ethidium bromide. [65] These dyes have a positively charged nitrogen atom (see figure 2) and bind to the negatively charged

dsDNA through electrostatic interaction and thereby emit fluorescence. [66] The fluorescence signal is directly proportional to the DNA concentration. [62]

For the quantification of the DNA concentration, calibration standards with known concentrations are needed. The measured fluorescence signals of the standards are plotted against their concentration to obtain a calibration curve, which is then used to calculate the DNA concentration of the DNA sample. [63]

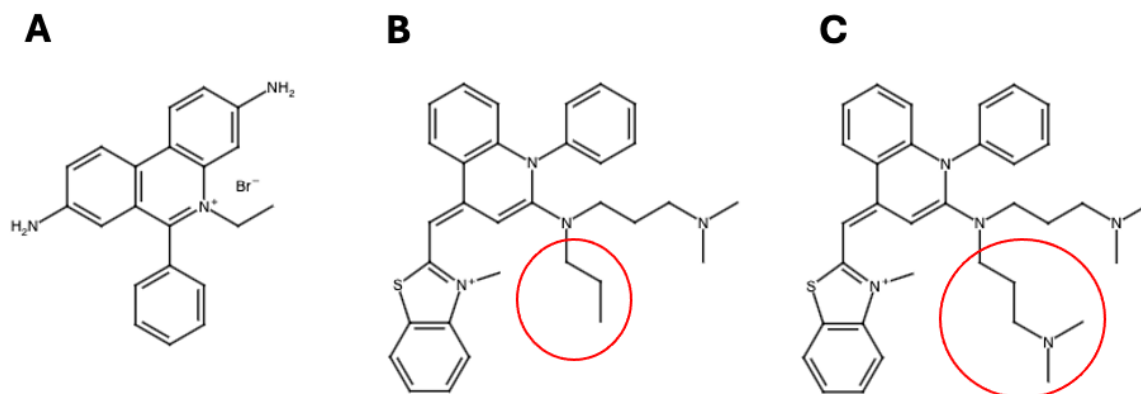


Figure 2 Chemical structure of the intercalating dyes A) ethidium bromide, B) SYBR Green and C) PicoGreen

Ethidium bromide was one of the most popular DNA fluorophores [67]. It intercalates between the DNA base pairs. [66] Its excitation maximum is at 300 nm and its emission maximum is at 605 nm. When ethidium bromide is bound to DNA, the fluorescence signals increase by 20-fold. Compared to other intercalating dyes it is cheap, but it is not used very often anymore because of its toxicity and mutagenicity. [67] Furthermore, studies showed, that it can only be used for DNA samples with high DNA concentrations. [65]

At present, the fluorophores SYBR Green and PicoGreen are the most used for DNA detection due to their selectivity towards DNA, their ability to increase the fluorescence signals up to 1000-fold after binding to DNA [67], and because they also work with low DNA concentrations. [65] Both dyes have their excitation maximum at 480 nm and the emission maximum at 520 nm. [67] They have very similar chemical structures, the only difference is the side chain on the quinolinium ring: SYBR Green has an N-(3-dimethylaminopropyl)-N-propylamino group and PicoGreen an N-bis-(3-dimethylaminopropyl)-amino group (see figure 2; difference marked in red). PicoGreen has one additional dimethylamino group compared to SYBR Green. [68]

PicoGreen shows high stability towards photobleaching [65]. It can also distinguish between intact and degenerated DNA. However, studies found that PicoGreen is sensitive towards contaminations, such as lysis agents (e.g. CTAB), RNA, and proteins. [64] SYBR Green is less expensive than PicoGreen, but it also binds to RNA and ssDNA, making it less selective and less effective compared to PicoGreen. [67]

3.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a simple method to amplify a segment of DNA in vitro. [54] The first PCR was performed by Kary Mullis in 1983. [69] Since then, PCR has not only become a standard method in biological science and medicine [70], but various types of PCR have been developed, for example multiplex PCR, reverse transcriptase PCR, and real time PCR. [54]

PCR is a three-step process. It requires template DNA, primers, deoxynucleotides, and DNA polymerase. [54, 70] The primers are short DNA sequences, that bind to the complementary parts of DNA and define the PCR product (amplicon). DNA polymerase is an enzyme that adds the deoxynucleotides (deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate and deoxythymidine triphosphate) to the primers. These deoxynucleotides are used as building blocks to form the amplicons. [70, 71]

In the first step, the denaturation, double stranded DNA is denatured, which means that it is separated into two single strands (ssDNA). This step is performed at 92 °C to 95 °C. In the second (annealing) step, the primers bind to the complementary parts of the single DNA strands, at 50 °C to 70 °C, depending on the primer pair. In the third (elongation) step, the single DNA strands are extended from the 5' terminus to the 3' terminus with the help of the DNA polymerase at 72 °C. At the end of one PCR cycle, for one dsDNA two dsDNA molecules are obtained (see figure 3). This procedure is repeated for 30 to 50 cycles. [70]

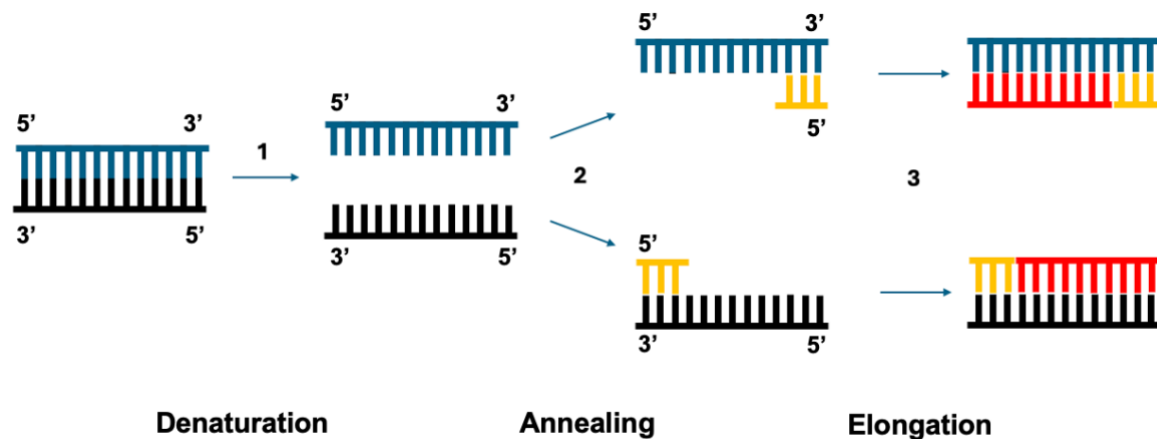


Figure 3 Illustration of a single PCR cycle

PCR is a simple method to rapidly produce numerous copies of a DNA fragment. Due to its high sensitivity, even small amounts of contaminating DNA can lead to the formation of false amplicons. Another limitation of PCR is that the target sequence must be known in order to design primers. This means that unknown DNA sequences cannot be amplified. In addition, primers can anneal to sequences similar to their complementary sequences and form unspecific PCR products. [71]

3.3.1 Primers

Primers, one of the main components used in PCR, are short DNA fragments, also called oligonucleotides. They usually consist of around 18 to 25 bp. [54]

In PCR, the primers must have sequences that are complementary to the DNA template strand. When the dsDNA is dissociated to ssDNA, the primers bind to the matching sequences, which allows the DNA polymerase to initiate DNA synthesis. [70]

For a successful PCR and the production of correct amplicons, primers must be well designed. The primers determine the specificity of the PCR, as well as the amplicon length and its melting temperature. The melting temperature (T_m) is defined as the temperature, at which 50 % of the oligonucleotide molecules (e.g. dsDNA) are dissociated into single strands (e.g. ssDNA; see figure 4, red line). [72]

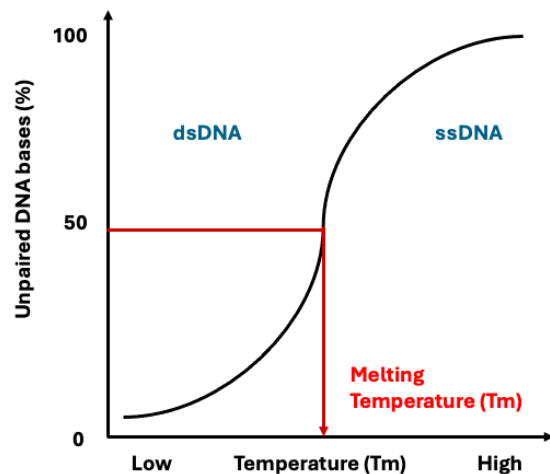


Figure 4 Melting temperature of DNA

The design of the primers accounts for the success of the PCR reaction. Primers are usually designed with the help of bioinformatics (a new research area, combining biological and computational sciences) and then tested with *in silico* tests to determine their suitability. Important criteria for good primers are among others the melting temperature, primer length, and the absence of secondary structures and primer dimers. [73]

The melting temperature of the primer pair should be between 50 °C and 60 °C. Primer sequences containing more guanine (G) and cytosine (C) have a higher melting temperature than those with more adenine (A) and thymine (T) because of the additional hydrogen bond. The G and C content should be approximately 40 to 60 %. [72-74] Primers with a low G/C content can be made longer to increase the T_m above 50 °C. [73] In general, the primer length should be between 18 and 24 bp to ensure specificity of the PCR reaction. Shorter primer sequences (15 bp or less) can cause the formation of unspecific PCR products, whereas very long primers need more time to anneal and can lower the efficiency of the PCR. [75] Furthermore, sequences with repeats of a single base (e.g. four or more Gs in a row) should be avoided. [73] The annealing temperature is the temperature, at which the primers bind to the ssDNA. It is deduced from the melting temperature of the primer. A too low annealing temperature causes the formation of unspecific products or even the lack of amplification due to the insufficient disassociation of dsDNA. [76]

Furthermore, the primers should not form secondary structures (e.g. hair pins) or self-dimers. These structures occur when a primer binds to itself or to another primer instead of to the DNA template [76]. The length of the final amplicon (PCR product) affects the amplification and has to be adjusted depending on the downstream process. [75] For example, for the analysis by HRM, the amplicon should be between 100 to 300 bp long, [74] because longer amplicons show smaller changes in the sequence and in the melting curve, which will complicate the differentiation. [72] Depending on the results of the *in silico* tests (such as probability of the formation of secondary structures and unspecific PCR products) it can be estimated, if the primer pair is suitable. However, the primers still need to be tested *in vitro*, to determine its actual efficiency in the application.

3.3.2 Real time PCR

Real time PCR, also known as quantitative PCR (qPCR), [77] is an innovative and important type of PCR. It enables the monitoring of the PCR progress by measuring fluorescence signals in real time. In basic PCR, a so-called end point detection is performed. End point detection is carried out after completing the PCR run using gel electrophoresis. Real time PCR does not need this additional step and thus, possible cross contaminations, work and time are reduced. Furthermore, real time PCR is more accurate and sensitive than basic PCR, because it measures the fluorescence signals during the exponential phase and not during the plateau phase (section 3.3.4,

figure 7A). The quantification data derived from the exponential phase are more precise and accurate than those from the plateau phase, because in the plateau phase, the reaction slows down because the components are used up. [78]

For real time PCR, a fluorescent reporter is needed. Fluorescent reporters can be divided into two groups, dyes that bind to double stranded DNA and fluorophores bound to oligonucleotides. [78] SYBR Green and EvaGreen belong to the dsDNA intercalating molecules (see figure 5). They detect both specific and non-specific amplicons. SYBR Green replaced the intercalating dye ethidium bromide used before [77], because it is low in costs [78] and has a brighter fluorescence signal. However, SYBR Green was replaced by EvaGreen, because SYBR Green is a mutagen, has high background fluorescence, is instable during storage and has PCR inhibitory effects. EvaGreen has a lower background fluorescence and less inhibitory effects on PCR. Additionally, EvaGreen is not mutagenic and is suitable for HRM analysis. [77]

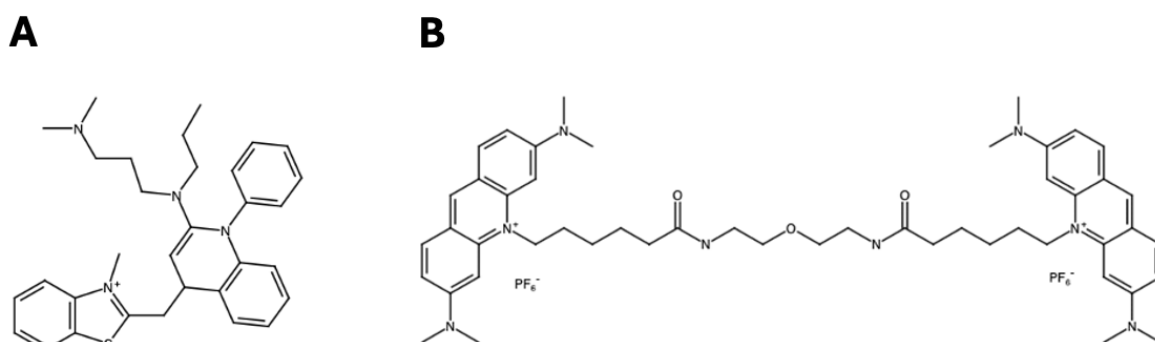


Figure 5 Chemical structure of dsDNA intercalating molecules A) SYBR Green and B) EvaGreen

When the intercalating dyes are not bound to dsDNA, they remain inactive and no or low fluorescence is emitted. When the intercalating dyes are bound to dsDNA, fluorescence intensity increases (see figure 6). The fluorescence signal is proportional to the amount of dsDNA. [78]

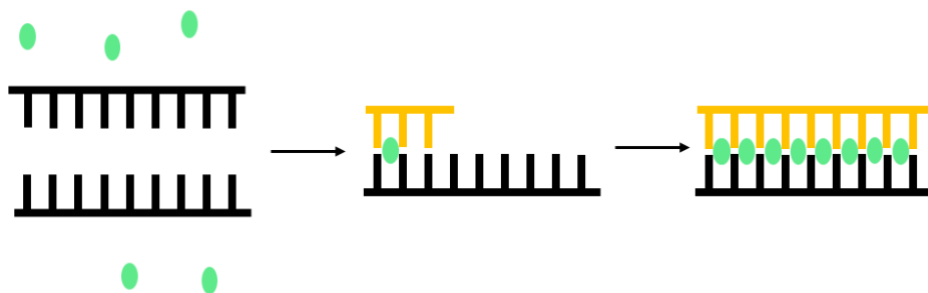


Figure 6 Illustration of the interaction of SYBR Green with DNA

The second class of fluorescent reporter, the fluorophores linked to oligonucleotides, only detect specific amplicons. The most common one is the TaqMan probe. The TaqMan probe is a short DNA sequence, that has a fluorescent reporter dye bound to its 5' and a quencher dye bound to its 3' terminus. The fluorescent reporter and the quencher together form a donor acceptor fluorescence resonance transfer (FRET) pair, which means, that the emitted fluorescence of the reporter is absorbed by the quencher and no fluorescence can be detected, if the reporter and the quencher are in close distance. During the annealing step of the PCR, the TaqMan probe binds to the specific sequence of the DNA from the 3' terminus (downstream from the primer). During the elongation step, the DNA polymerase synthesizes the new DNA strand by extending the primers. When the polymerase reaches the TaqMan probe, it hydrolyses the probe due to its 5' exonuclease activity. This leads to separation of the reporter and the quencher, and the reporter emits a fluorescence signal. With increasing numbers of PCR cycles, more amplicons are produced, leading to an increase in the fluorescence signal. The fluorescence signal is directly proportional to the amount of the amplicons. [78]

3.3.3 High resolution melting (HRM) analysis

Real time PCR is often coupled with high resolution melting (HRM) analysis, which is a fast and low cost method used for the identification of genetic variations between species and/or cultivars. It was invented by the University of Utah in 2003. [74] Since then, HRM has been applied in various clinical diagnostics and in food analysis, such as the identification of different varieties, determination of genetically modified organisms and the detection of food allergens. [72]

After the last PCR cycle is completed, HRM is performed. The amplicons are heated very slowly, e.g. from 65 °C to 95 °C in 0.1 °C steps per 2 seconds. [74] The gradually increasing temperature causes the slow degradation of double DNA strands to single DNA strands. The dissociation of double stranded DNA depends on the amplicon length and the type of base pairs. If the amplicon is longer and/or contains a higher proportion of guanine and cytosine, the dissociation requires a longer time and its melting temperature is increased. With the dissociation of dsDNA, the intercalating dye (e.g. EvaGreen) is released, which causes a decrease in the fluorescence signal. The measured fluorescence signals are plotted against the temperature to obtain the characteristic melting curves, which are used to distinguish between genetic variations based on their shape and/or the melting temperature of the amplicons (see section 3.3.4). [72]

3.3.4 Data evaluation

Data evaluation of PCR coupled with HRM analysis is carried out using specific software. In general, the emitted fluorescence signals are plotted against the number of cycles to obtain the amplification curve or against the temperature to obtain melting and normalized melting curves. [72, 78]

Figure 7A represents a typical amplification curve obtained in real time PCR. The measured fluorescence signal, representing the amount of amplicon, is plotted against the number of PCR cycles. PCR is divided into three phases, the initial, the exponential, and the plateau phase. At the beginning of PCR, the fluorescence signal is too low to exceed the limit of detection, only a baseline is observed. With each PCR cycle, the amount of PCR products doubles, more dye intercalates and thus, the fluorescence signal increases. After the initial phase, the curve reaches the Ct-value, also known as threshold cycle. The threshold cycle is defined as the number of cycles required for the fluorescence signal to exceed the limit of detection. The Ct-value depends on the initial concentration of the DNA template in the sample. If the concentration in the sample is high, fewer PCR cycles are required to reach the limit of detection, resulting in a lower Ct-value. Throughout the initial and the exponential phase, the amplification proceeds exponentially, which means that with each cycle, the amount of PCR products is doubled. Due to the limitation of the reagent (e.g. primers) with increasing cycle number, the amplification efficiency decreases until amplification completely stops and the amplification curve reaches a plateau. [78]

Figure 7B represents the negative derivative of the fluorescence over temperature ($-dF/dT$) plotted against the temperature, also known as derivative reporter ($-Rn'$). It is an HRM derivative plot, used to visualise the melting temperature of PCR products as peaks. At the peak maximum, half of the double DNA strands are dissociated into single strands. The derivative plot provides information regarding the zygosity (homozygous or heterozygous), the melting temperature and possible formation of unspecific products (primer dimers) of the sample. [72]

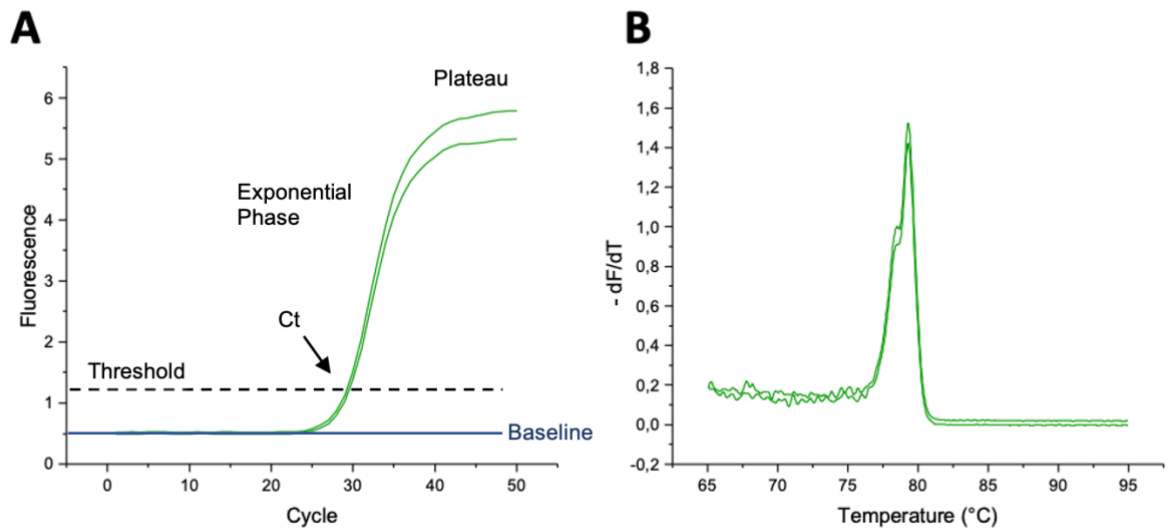


Figure 7 Amplification curve (A) of real time PCR and negative derivative of the fluorescence over temperature ($-dF/dT$) plotted against the temperature (B) of HRM analysis

Zygoty describes the similarity of alleles. 'Homozygous' refers to a specific locus in an individual with identical alleles, whereas 'heterozygous' refers to the occurrence of different alleles. [79] The melting curves of heterozygous samples have complex curve shapes (see figure 8, green curve), which distinguish them clearly from each other and from homozygous samples (see figure 8, blue curve). Homozygous samples lead to similar or identical curve shapes and can only be distinguished by the difference in their melting temperature. [72, 74]

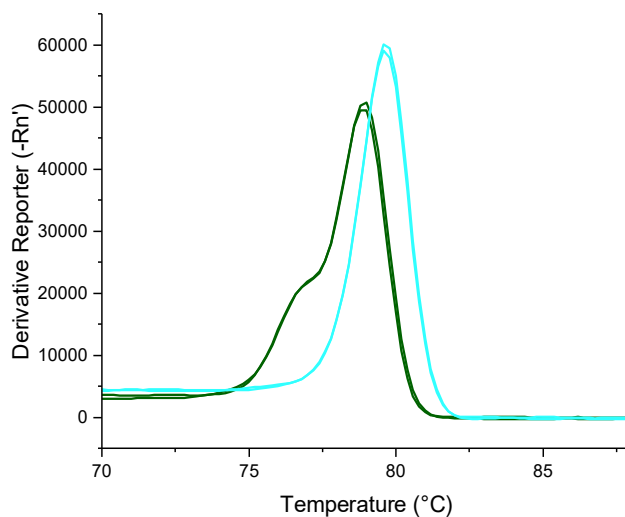


Figure 8 Melting curve of homozygous (blue) and heterozygous (green) samples

For the differentiation of genetic variations between species and/or cultivars, the melting curves are usually normalized [72] by setting a suitable pre- and post-melting region (figure 9 A; blue bar). The pre-melting region is set before the fluorescence signals decrease drastically. The post-melting region is set after the drastic decrease of the fluorescence signal. The fluorescence signals are normalized to relative values of 1 and 0 in order to eliminate differences in background fluorescence signals, thereby improving the ability to detect differences between samples (see figure 9 B). [80] The temperature at the inflection point of the normalized melting curves represents the melting temperature, corresponding to the peak maximum in the derivative plot (see fig. 7B or 8). The differentiation between cultivars is carried out by comparing the normalized melting curves based on their shape and/or the

temperature. Perfectly overlapping curves indicate that the samples have the same amplicon sequence and cannot be differentiated. [72]

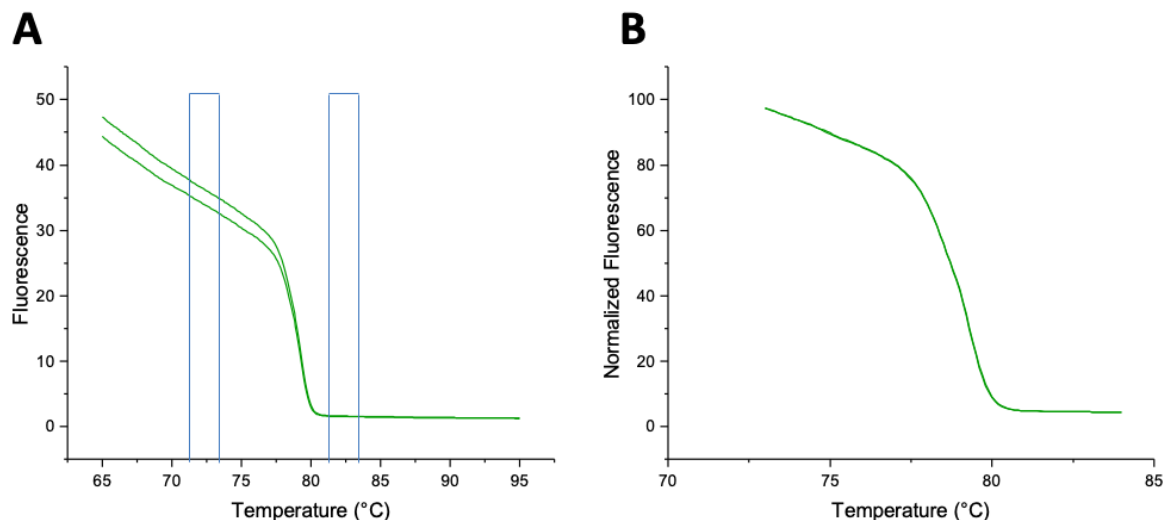


Figure 9 Pre-normalized melting curves with the blue bars indicating the normalization regions (A) and normalized melting curves (B)

3.4 Gel electrophoresis

Before real time PCR was available, PCR products were commonly analysed by gel electrophoresis. After the PCR run, the amplicons were loaded onto a gel [71] (starch, agarose, or acrylamide basis). [81] In addition to the amplicons, a molecular marker containing different DNA fragments with known lengths was applied onto the gel. [71]

When an electrical field is applied, the PCR products and the DNA fragments of the molecular marker move through the gel. Because of the negatively charged phosphate backbone, they move towards the positively-charged anode. This technique separates DNA fragments by size. Smaller fragments migrate faster through the gel, longer ones migrate slowly. The amplicons and the marker are then visualised by staining the gel (e.g. with ethidium bromide) and observing it under UV light. [81]

Figure 10 shows a gel onto which 28 samples were loaded. The first and the last lane represent the molecular marker. The marker consists of 11 DNA fragments with different lengths (25 to 766 bp). The length of amplicons (bp) is determined by comparing the obtained band with those obtained for the marker. Bands at the bottom of the gel usually result from primer dimers (around 50 bp).

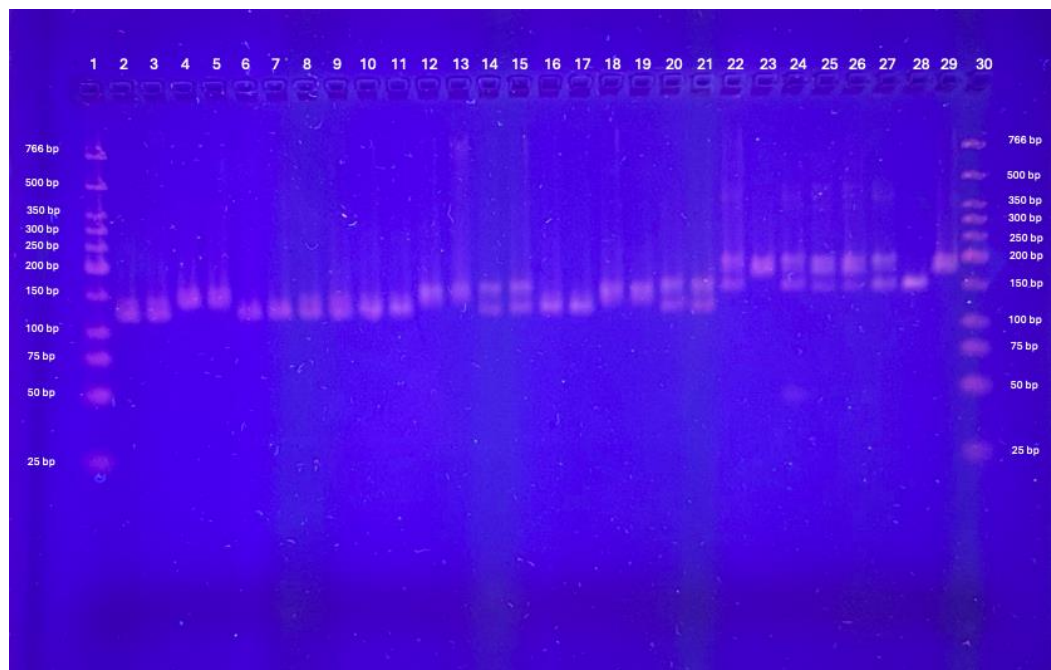


Figure 10 Result of a gel electrophoresis with 28 samples (lane 2 to 29) and the marker (lane 1 and 30)

4 Materials and chemicals

Table 1 List of used instruments

Instruments	Company
691 pH Meter	Metrohm
Analytical Balance Sartorius Talent TE214S	Sartorius
Centrifuge 5424	Eppendorf
Centrifuge Mini Star	VWR International
FastPrep-24 5G	MP
HR1921/20 Juicer	Philips
Galaxy Mini-Centrifuge	VWR International
pH-Indicator Strips	Merck
Pipette Xplorer 0.5-10 µl	Eppendorf
Pipette Xplorer 1-20 µl	Eppendorf
Pipette Xplorer 10-200 µl	Eppendorf
Pipette Xplorer 50-1000 µl	Eppendorf
QuantStudio 5 Thermocycler	Thermo Fisher Scientific
Qubit 4 Fluorometer	Thermo Fisher Scientific
RotorGene Q Thermocycler	Qiagen
Sub-Cell GT Gel Caster	Bio-Rad Laboratories
Sub-Cell GT MP Comb (15 and 30-well)	Bio-Rad Laboratories
Thermomixer C Eppendorf	Eppendorf
Transilluminator UVT-20 M	Herolab
Vortex Classic Advanced Vortex Mixer	VELP Scientifica
Vortexer Vortex-Genie 2	Scientific Industries
Vortex VF2 Mixer	Janke & Kunkel GmbH & Co KG

Table 2 List of used chemicals

Chemicals	Company
Invitrogen Qubit 1x dsDNA Broad Range (BR) Assay Kit	Thermo Fisher Scientific
Invitrogen Qubit 1X dsDNA High Sensitivity (HS) Assay Kit	Thermo Fisher Scientific
2x Type-It HRM PCR Kit	Qiagen
10x TBE Buffer	Invitrogen
Agarose High Resolution	Carl Roth
Chloroform for Analysis	Merck
CoralLoad Concentration 10x	Qiagen
DNA-ExitusPlus IF	PanReac AppliChem ITW
DNeasy Mericon Food Kit	Qiagen
Ethanol Absolute	CHEMSOLUTE
GelRed Nucleic Acid Gel Stain	Biotium
Fumed Hydrochloric Acid	
Low Molecular Weight DNA Ladder	New England BioLabs GmbH
Nuclease Free Water	
NucleoSpin Plant II Kit	Macherey-Nagel
MACERASE Pectinase, Rhizopus sp.	Merck
MeltDoctor HRM Master Mix	Thermo Fisher Scientific
Primer	Sigma-Aldrich
RNase A	Macherey-Nagel
RNase Free Water	Qiagen
ROTI Load DNA-Orange 1 (with Glycerol)	Carl Roth GmbH + Co. KG
Sodium Hydroxide Pellets for Analysis	Merck

Table 3 List of used materials

Materials	Company
Biosphere Filter Tips 0.5 – 20 µl	Sarstedt
Biosphere Filter Tips 2 – 20 µl	Sarstedt
Biosphere Filter Tips 20 – 200 µl	Sarstedt
Biosphere Filter Tips 100 – 1000 µl	Sarstedt
Cell Culture Dish (60 x 15 mm)	Eppendorf
Ceramic Spheres, 6.35 mm (1/4), Bulk	MP Biomedicals
Disposable Hypodermic Needle (0.9 x 40 mm)	B. Braun SE
duoShield PFT Nitril 240 Gloves	SHIELD Scientific
Eppendorf Tubes 0.2 ml	Eppendorf
Eppendorf Tubes 1.5 ml	Eppendorf
Eppendorf Tubes 2 ml	Eppendorf
Eppendorf Tubes 5 ml	Eppendorf
Falcon Tubes 15 ml	Sarstedt
Falcon Tubes 50 ml	Sarstedt
Petri Dish Culture	Sarstedt
PCR Tubes (0.2 ml)	Thermo Fisher Scientific
SC Micro Tube PCR-PT 2 ml	Sarstedt
Sterile Scalpel	B. Braun SE
Strip Tubes and Caps (0.1 ml)	Qiagen
Invitrogen Qubit Assay Tubes	Thermo Fisher Scientific

Table 4 List of used software

Software	Manufacture
High Resolution Melt Software Version 3.1 (2015)	Thermo Fisher Scientific
Microsoft 365 Apps for Enterprise	Microsoft
Origin 2021b	OriginLab Corporation
QuantStudio™ Design & Analysis Software 1.3.1	Thermo Fisher Scientific
Rotor-Gene Q Series Software 2.3.1	Qiagen

Table 5 List of used webservers

Webserver	Website-Link
Chemical Sketch Tool	https://www.rcsb.org/chemical-sketch
NCBI: BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi
OligoAnalyzer	https://www.idtdna.com
Oligo Calc: Oligonucleotide Properties Calculator	http://biotools.nubic.northwestern.edu/OligoCalc.html
RNAfold Webserver	http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi

5 Experimental part

5.1 Samples

5.1.1 Pear leaves

The analysed pear leaf samples were provided from 'Haidegg Experimental Station for Fruit Growing and Viticulture' by Dr. Thomas Rühmer and the research is a cooperation with Assoc. Prof. Dipl.-Ing. Dr. techn. Barbara Siegmund from the Graz University of Technology, Institute of Analytical Chemistry and Food Chemistry. The pear trees in Haidegg were specifically planted for research purposes and for the maintenance of various pear cultivars.

The pear leaves were collected on the 24th of June 2024. Only older pear leaves were picked, because plugging younger leaves would cause damage to the trees. From each pear cultivar, around ten leaves were collected. The collected pear leaves were individually stored in plastic ziplock bags and later placed in a freezer. Table 6 lists the different pear cultivars and their origin.

Table 6 List of the 18 pear leaf samples

Number	Cultivar	Species	Origin
1	Abate Fetel	<i>Pyrus communis</i>	France
2	Celina	<i>Pyrus communis</i>	Sweden
3	Fred	<i>Pyrus communis</i>	Switzerland
4	Xenia	<i>Pyrus communis</i>	Moldavia
5	Cordula	<i>Pyrus communis</i>	Czechia
6	Harovin Sundown	<i>Pyrus communis</i>	Canada
7	Gute Graue	<i>Pyrus communis</i>	France
8	Kärntner Speckbirne	<i>Pyrus communis</i>	Austria
9	Alexander Lukas	<i>Pyrus communis</i>	France
10	Blutbirne	<i>Pyrus communis</i>	Germany
11	Haeng Su	<i>Pyrus pyrifolia</i>	Korea
12	Kimizukawase	<i>Pyrus pyrifolia</i>	Japan
13	Kumoi	<i>Pyrus pyrifolia</i>	Japan
14	Frühe von Trevoux	<i>Pyrus communis</i>	France
15	Conference	<i>Pyrus communis</i>	England
16	Williams	<i>Pyrus communis</i>	England
17	Boscs Flaschenbirne	<i>Pyrus communis</i>	Belgium
18	Cepuna	<i>Pyrus communis</i>	France

5.1.2 Pear juices, peel, and pulp

Three store-bought 100 % direct juices were used for the optimization of the Food Kit protocol by Qiagen, apple juice 1 and pear juice 1 from the manufacturer Rio d'oro and apple juice 2 from the manufacturer Naturland. Optimization was necessary to increase the DNA concentration in the DNA samples.

Self-pressed juices from three different store-bought pear cultivars (Anjou, Cheeky and Williams, see table 7) were produced in-house using a juicer. The juicer allows the production of juices with either low or high fibre content. For each pear cultivar, four kinds of juices were produced, with or without peel, and with high or low fibre content.

Table 7 List of pear samples (juices)

Pear	Cultivar	Origin	Appearance, Texture
Anjou	<i>Pyrus communis</i>	Argentina	Reddish peel, soft
Cheeky	<i>Pyrus communis</i>	South Africa	Greenish-yellow peel, crisp
Williams	<i>Pyrus communis</i>	Argentina	Yellow peel, soft

For the production of the self-pressed juices, the equipment of the juicer was washed twice with water and soap. The equipment was dried and in order to eliminate contaminations, carefully wiped with DNA exitus to destroy existing DNA. Afterwards, the equipment was dried again with kitchen roll to get rid of DNA exitus-residues, which would destroy the DNA of the sample.

The pears were first washed with tap water to remove dirt. Then they were dried and wiped clean with nuclease free water. With sterilised plate and a knife using DNA exitus, the pears were cut into small pieces and the seeds removed. The pears with or without the peel were placed in the juicer, which was set to high or low fibre content, and the juice production started. The juices were collected in 50 ml Falcon tubes and stored in the freezer. Additionally, samples of the raw peel and the pulp were collected and also stored in the freezer. Between producing juices from different pear cultivars, the juicer was cleaned with water, soap and DNA exitus. Furthermore, the gloves were changed when they came in contact with the pears, between the pear cultivars and between the usage of DNA exitus.

The colour of the juices was yellow, but after standing on the counter for a few minutes, the colour turned slightly brownish because of oxidation. With the addition of peel and with a high fibre content the juice became grittier, with lots of small bits of pear pulp and peel.

5.1.3 Heat treatment

For the heat treatment, only juices from Williams pears were selected. An aliquot of the four Williams pear juices (with/without peel; high/low fibre content) was taken and filled into 1.5 ml Eppendorf tubes. The tubes were then incubated at 600 rpm in a thermoshaker at a specific temperature and time shown in table 8. After this procedure, the juices were cooled down quickly in an ice bath.

Table 8 Heat treatment conditions

Pear cultivar	Temperature [°C]	Time [min]
Williams	65	30
Williams	85	3
Williams	85	5
Williams	88	5

For DNA extraction the Food Kit was selected. The procedure was carried out in the same way as for all other pear juices as described in section 5.2.2.

5.2 DNA extraction

5.2.1 NucleoSpin Plant II kit (Plant Kit) by Macherey-Nagel

For DNA extraction from the pear leaves, peel, pulp and the juices, the NucleoSpin Plant II kit by Macherey-Nagel was used. The procedure was carried out by following the protocol of fellow Master student Jakob Scheibelreiter. [82]

Before the DNA extraction started, gloves were put on, the bench surface and the fume hood were cleaned with DNA exitus and dried with paper towels. In order to prevent DNA exitus-residue coming into contact with the samples and destroying DNA, the gloves were changed.

For the homogenization of pear leaves, peel, and pulp, a scalpel, a cell culture dish, a paginated needle and an Eppendorf tube were placed on a fresh paper towel in the fume hood. The mass of the Eppendorf tube was measured and tared with a scale. A small piece of the pear sample was then transferred into the dish tube. For the leaves, the pieces did not include parts of the stem or the veins. The sample was cut into small pieces with the scalpel and secured with the needle. With the needle, the pieces were transferred into the Eppendorf tube.

The mass of the sample should be about 150 mg. However, for two leaf samples (Kimizukawase and Kumoi), only 100 mg were weighed in order to reduce gel formation, which will be described below. The mass of the sample was recorded. Before taking the sample of a different pear cultivar, the paper towel, scalpel, dish tube and the gloves were disposed and replaced with new ones. The needle was kept in the fume hood for later usage.

Two $\frac{1}{4}$ ceramic spheres and 900 μ l PL2 solution were added to each Eppendorf tube. The tubes were closed tightly and placed in the MP instrument. The program 'Saved Programs' and 'Fixed museal tissues' were selected and after correctly closing the instrument, the program was started (see table 9).

Table 9 Homogenization program for the MP instrument

Speed [m/s]	Adapter	Time [s]	Lysing matrix	Quantity [mg]	Cycles	Pause time [s]
6	QuickPrep	40	M	150	3	300

After the end of the program, the samples were removed from the instrument and homogenization efficiency was controlled. The two ceramic spheres were removed from the Eppendorf tubes with the needles dedicated to the individual samples. The needles were slightly bent for an easier removal of the ceramic spheres and in order to reduce spillage of the sample.

For the pear juices, homogenization was not necessary. The Falcon tubes with the juices were taken from the freezer, defrosted and mixed by shaking. The samples were pipetted into the labelled and tared Eppendorf tubes. The mass of the samples should be around 150 mg. 900 μ l PL2 buffer were added to the samples.

The DNA extraction procedure continued for juices, leaves, peel, and pulp samples in the same way. To each sample, 30 μ l RNase A were added, then vortexed to ensure a thorough mixing, spinned down and incubated for 20 minutes at 65 °C with constant shaking at 1400 rpm in a thermoshaker. Afterwards, the samples were removed from the thermoshaker and to each sample, 225 μ l buffer PL3 were added. The solutions were vortexed for a few seconds and incubated on ice for 5 minutes. The samples were centrifuged for 1 minute at 11000 rpm. The supernatants of each sample were placed on an individual NucleoSpin filter with a violet ring for cleaning, which was set on top of a collection tube. The columns were closed and centrifuged for 2 minutes at 11000 rpm. The supernatants were transferred into individual 5 ml Eppendorf tubes. This step was repeated until the entire volume of supernatant per sample had been centrifuged and placed into one 5 ml Eppendorf tube per sample.

In the next step, to each 5 ml Eppendorf tube 1350 µl PC buffer were added and vortexed. In samples where pear leaf samples caused gel formation and did not dissolve even after intense shaking and vortexing, the procedure was continued. Then 675 µl of sample solution were placed on the individual NucleoSpin filter with a green ring for DNA binding, which was set on top of a collection tube. The columns were closed and centrifuged for 1 minute at 11000 rpm. DNA was thereby bound to the filter and the flowthrough containing mostly non-DNA components was discarded. The collection tubes and the ends of the column were tapped on paper towel to empty them from remaining solutions. This step was repeated until all of the solution was centrifuged and the flowthrough discarded. In case, the columns were clogged due to gel formation, centrifugation was repeated. When the columns were completely clogged and the solutions would not flow through after at least five more centrifugation steps, we decided to continue with the next step.

In the next step, the membrane of the column was washed by adding 400 µl PW1 buffer onto the column and centrifuged at 11000 rpm for 1 minute. The flowthrough was discarded. Then 700 µl PW2 were added and centrifuged at 11000 rpm for 1 minute. The flowthrough was discarded and another 200 µl PW2 buffer were added and centrifuged at 11000 rpm for 2 minutes to dry the membrane. Some samples that had resulted in gel formation, needed additional centrifugation steps until the solution passed through the column into the collection tube.

Then the collection tubes were discarded and the columns were placed in Eppendorf tubes. 50 µl PE buffer were aliquoted, incubated at 65 °C and pipetted onto the middle of the column. The columns were incubated at 65 °C for 5 minutes and centrifuged at 11000 rpm for 1 minute in order to elute the DNA. Samples with gel formation on the membrane had a lower yield of eluate because the gel had adsorbed part of the PE buffer. These samples (only from leaves) were slightly yellow and had a lower viscosity, which gave them a sluggish-like consistence.

After DNA extraction, the DNA concentration of the samples was determined (see chapter 5.3). For the samples with a low DNA concentration, a second elution was carried out. The columns were placed in new Eppendorf tubes. 50 µl PE buffer were aliquoted, incubated at 65 °C and pipetted onto the middle of the column. The columns were incubated at 65 °C for 5 minutes and centrifuged at 11000 rpm for 1 minute.

In general, the DNA concentration of the DNA extracts from juices was very low (below 0.01 ng/µl), therefore the following variations of the procedure were performed in order to improve the yield:

- increase the sample mass from 150 mg to 200 mg
- increase the incubation time from 20 minutes to 60 or 90 minutes
- reduce water-content by incubation at 60 °C until sample has a low viscosity
- addition of pectinase (8.14 units) with and without adjusting the pH value [83]

The addition of pectinase with pH adjustment was carried out as follows. 900 µl lysis buffer were added to the homogenous sample and the pH value was adjusted to 4 - 5 with fumed hydrochloric acid. 70 mg pectinase were dissolved in 10 ml nuclease free water. 388 µl of the pectinase (8.14 units) were added to the sample solution and incubated at 37 °C for 30 minutes. Then the pH value was adjusted to approximately 7 using diluted hydroxide solution. Then DNA extraction was performed as described above. Addition of pectinase without the pH adjustment was performed by skipping the treatment with hydrochloric acid and diluted hydroxide solution. [83]

5.2.2 DNeasy mericon Food Kit (Food Kit) by Qiagen

In order to optimize DNA extraction for pear peel, pulp, and juice, a second DNA extraction kit, DNeasy mericon Food Kit by Qiagen, was tested and the protocol slightly adjusted. According to Qiagen, this kit can extract high quality DNA from both raw and highly processed food. It is based on the CTAB extraction method. [84]

Homogenization of the pear peel and pulp was carried out as described in detail in chapter 5.2.1. Approximately 200 mg sample were weighed into a tared Eppendorf tube using scalpel, dish tube, and needle. To each Eppendorf

tube, two ¼ ceramic spheres and 1 ml Food Lysis Buffer were added. To pear peels, an additional 0.5 ml Food Lysis Buffer were added. Then the Eppendorf tubes were closed and placed in the MP instrument. The program 'Saved Programs' and 'Fixed museal tissues' was selected and after correctly closing the instrument, the program was started (see table 9). After the program was finished, the two ceramic spheres were removed from the Eppendorf tubes with the needle.

For juices, the homogenization step was not necessary. 200 mg of the juices were pipetted into tared Eppendorf tubes and mixed with 1 ml Food Lysis Buffer.

The DNA extraction procedure continued for juices, peel, and pulp samples in the same way. To each sample, 2.5 µl Proteinase K solution were added, vortexed and incubated in a thermoshaker for 30 minutes at 60 °C and 1000 rpm. Afterwards, the samples were immediately cooled in an ice bath and centrifuged at 2500 rpm for 5 minutes. 700 µl of the supernatant were transferred into 2 ml microcentrifuge tubes containing 500 µl chloroform. The microcentrifuge tubes were vortexed for approximately 15 seconds and centrifuged at 14000 rpm for 15 minutes. From each sample, 250 µl of the supernatant were added into 2 ml microcentrifuge tubes containing 1 ml Buffer PB and vortexed. 600 µl of the solution were loaded onto the QIAquick spin column, which was set on top of a collection tube. The Eppendorf tubes were centrifuged at 17900 rpm for 1 minute. The flowthrough was discarded and the columns and collection tubes were tapped on a paper towel to empty them from remaining solutions. This step was repeated until all of the solution was centrifuged and the flowthrough discarded.

Then 500 µl Buffer AW2 were loaded onto the columns and centrifuged at 17900 rpm for 1 minute. The flowthrough was discarded and the columns were centrifuged again to dry the membrane. The columns were transferred into 1.5 ml microcentrifuge tubes. For elution, 50 µl Buffer EB were aliquoted, incubated at 65 °C and loaded onto the column. The columns were incubated at 60 °C for 5 minutes and centrifuged at 17900 rpm for 1 minute. If the samples had a low DNA concentration, a second elution was carried out.

As mentioned in section 1.3.3 and as our results showed is the DNA concentration of the DNA extracts from juices very low. Therefore, the protocol of the Qiagen 'DNeasy mericon Food Kit' was modified and various experiments were carried out:

- decrease of the sample mass from 200 mg to 150 mg
- increase Proteinase K solution from 2.5 µl to 5 µl
- removal of water through incubation at 60 °C until sample has a low viscosity
- addition of pectinase (10.86 units; 517 µl of the pectinase solution, see section 5.2.1) with and without adjusting the pH value [83]

5.2.3 DNA concentration measurement by fluorimetry

For measuring the DNA concentration of the DNA extracts, the Qubit 4 fluorimeter with the 1x dsDNA Broad Range Assay Kit was used. The Qubit tubes were placed into 1.5 ml Eppendorf tubes by touching only the caps. This was done to ensure that no dirt or fingerprints were left on the sides of the tubes, which would interfere with the fluorescence signal and thereby yield false results.

The working solution was prepared according to the Qubit 4 fluorimetry calculator. The number of samples and the two standard solutions were entered into the instrument, and the required volume of buffer and of the concentrated assay reagent were calculated. The defined volumes were then pipetted into an Eppendorf tube, vortexed and transferred to the Qubit tubes. 190 µl of the working solution were added for the standards, and 198 µl for the samples. For the standards, 10 µl of the standard solution and for the samples, 2 µl DNA extracts were added to the Eppendorf tubes. The solutions were vortexed for 3 seconds and left to rest for 2 minutes before measuring first the standards then the samples.

The DNA extracts from the pear juices, pulp, and three of the pear leaves (Haeng Su, Kimizukawase and Kumoi) had low DNA concentrations (below 2.5 ng/μl) and therefore, the 1x dsDNA High Sensitivity Assay Kit was used. Because of its high sensitivity, this kit is more suitable for DNA extracts with low DNA concentration. The measurement procedure was the same as described above, using the 1x dsDNA Broad Range Assay Kit. However, to ensure that the fluorescence signal exceed the detection limit, 10 μl DNA extract (instead of 2 μl) and 190 μl working solution (instead of 198 μl) were used. After the measurement, the DNA extracts were stored in the freezer.

5.3 Primer pairs

5.3.1 In silico tests

Primer sequences from five research papers [18, 19, 85-87] were used to identify suitable pear primer pairs. The sequences were tested in silico by using different software tools. These tests were used to determine the melting temperature and the probability of the formation of primer dimers and secondary structures.

First, the melting temperature of the forward and reverse primers were calculated using the software 'Oligo Calc: Oligonucleotide Properties Calculator'. Out of three available types, the 'Salt adjusted' melting temperature was used (see figure 11).

Oligo Calc: Oligonucleotide Properties Calculator

Enter Oligonucleotide Sequence Below
OD calculations are for single-stranded DNA or RNA

Nucleotide base codes

CGT GGA GGA AGA GAA AGA GG

Reverse Complement Strand(5' to 3') is:

CCT CTT TCT CTT CCT CCA CG

5' modification (if any) 3' modification (if any) Select molecule

50 nM Primer 50 mM Salt (Na⁺) 1 Measured Absorbance at 260 nanometers

Calculate **Swap Strands** **BLAST** **mfold**

Physical Constants		Melting Temperature (T _M) Calculations	
Length: 20	Molecular Weight: 6329.2	GC content: 55 %	1 53.8 °C (Basic)
1 ml of a sol'n with an Absorbance of 1 at 260 nm			2 60.5 °C (Salt Adjusted)
is 3.889 microMolar and contains 24.6 micrograms.			3 53.2 °C (Nearest Neighbor)
Thermodynamic Constants Conditions: 1 M NaCl at 25°C at pH 7.			
RlnK 33.404 cal/(°K*mol)		deltaH 163.1 Kcal/mol	
deltaG 25.9 Kcal/mol		deltaS 425.9 cal/(°K*mol)	
Deprecated Hairpin/self dimerization calculations			
5 (Minimum base pairs required for single primer self-dimerization)		Check Self-Complementarity	
4 (Minimum base pairs required for a hairpin)			

Figure 11 Program used for the determination of the melting temperature (Oligo Calc: Oligonucleotide Properties Calculator)

The first criterion for the selection of primer pairs was a melting temperature above 50 °C. A melting temperature below 50 °C would reduce the specificity of the primers and increase the probability of forming unwanted primer dimers and secondary structures.

The second criterion was that the difference in melting temperature between the forward and the reverse primers should not exceed 1 °C. If the temperature difference was greater than 1 °C, a slight adjustment was made. The sequence of the primer with the higher melting temperature was shortened by removing bases at the beginning or the end of the sequence, thereby reducing its melting temperature.

Two software tools were applied to determine the probability of formation of secondary structures and primer dimers. The first was 'RNA fold', which predicts the formation of secondary structures in the primers. The primer sequence was entered into 'Sequence Input' and under 'Energy Parameters' only 'DNA parameters (Matthews model, 2004)'

was selected. The prediction was carried out for both 37 °C and for the 'Salt adjusted' melting temperature minus 5 °C (see figure 12).

Figure 12 In silico test used for the prediction of secondary structures (RNAfold)

Two different minimum free energy (MFE) structures can be seen in figure 13. Figure 13 A shows the MFE structure of a primer sequence at 37 °C and figure 13 B shows the structure at the 'Salt adjusted' melting temperature minus 5 °C. In figure 13 A, the primer forms hair pins, which are considered unwanted secondary structures. At the actual annealing temperature ('Salt adjusted' melting temperature minus 5 °C), the structure changes to a circular shape (figure 13 B). The circular shape indicates that the primer will not form unwanted self-dimers. Therefore, the primer is suitable for the PCR procedure.

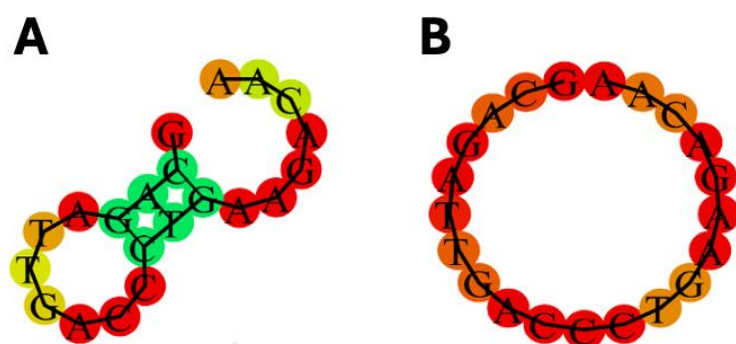


Figure 13 Predicted secondary structures for the reverse primer of MGD154 A) at 37 °C and B) at 54.5 °C (Salt adjusted melting temperature minus 5°C)

The second software was 'Oligo Analyzer', which calculates the delta G value and the number of base pairs required for the formation of self-dimers and hetero-dimers. In the menu either 'Self-Dimer' or 'Hetero-Dimer' was selected and the primer sequence or sequences were entered into 'Sequence' (see figure 14 to 16).

OligoAnalyzer

Figure 14 *In silico* test used for the prediction of dimers (OligoAnalyzer)

Homo-Dimer Analysis

The delta G is calculated by taking into account the longest stretch of complementary bases. These pairs of complementary bases are represented by a solid line. Dotted lines represent additional complementary bases for that dimer structure, but their presence does not impact calculated delta G values. Actual delta G values may vary based on presence of additional complementary bases. The Maximum Delta G value refers to the free energy of the oligo sequence binding to its perfect complement.

Dimer Sequence:
 5'- CGTGGAGGAAGAGAAAGAGG -3'
Maximum Delta G: -37.82 kcal/mol

Delta G: -3.61 kcal/mol **Base Pairs:** 2
 5' CGTGGAGGAAGAGAAAGAGG
 ||
 3' GGAGAAAGAGAGGAGGTGC

Figure 15 Result of the *in silico* test used for the prediction of homo-dimers (OligoAnalyzer)

Hetero-Dimer Analysis

Primary Sequence:

5'- CGT GGA GGA AGA GAA AGA GG -3'

Secondary Sequence:

5'- GCA GAT TGA CCC TGA AGA CAA -3'

CREATE COMPLEMENT CALCULATE

Primary Sequence: 5'- CGTGGAGGAAGAGAAAGAGG -3'
Secondary Sequence: 5'- GCAGATTGACCTGAAGACAA -3'

Maximum Delta G: -38.18 kcal/mol

Delta G: -4.67 kcal/mol **Base Pairs:** 3
 5' CGTGGAGGAAGAGAAAGAGG
 : ||| : :
 3' AACAGAGTCCCAAGTTAGACG

Figure 16 Result of the *in silico* test used for the prediction of hetero-dimers (OligoAnalyzer)

The maximum number of base pairs was set to six, and the minimum delta G value at -10 kcal/mol for the selection of primer pairs.

Primer pairs that did not meet all of the following criteria were considered unsuitable for this research and excluded:

- melting temperature above 50 °C
- temperature difference between forward and reverse primers below 1 °C
- no secondary structures
- no primer dimers (both homo- and hetero-dimers)

Primer pairs that met these criteria were further examined using the 'National Centre for Biotechnology Information: Basic Local Alignment Search Tool' (NCBI BLAST) to verify the correctness of the primer sequences and the presence of the target microsatellite.

The sequence of the forward primer was entered into the search field. Then 'Nucleotide collection (nr/nt)' was selected under 'Database' and '*Pyrus communis* L. (taxid:23211)' was entered as the 'Organism' (see figure 17).

Figure 17 Sequence search on NCBI BLAST

The database then showed all sequences for '*Pyrus communis* L.', that are identical or similar to the entered sequence (see figure 18). The sequence at the top of the list has the highest percentage of identity and the lowest Expect-value (E-value). The E-value is an exponential function of the alignment score and indicates the probability that the observed similarity was by chance. The lower the E-value (below one), the higher the chance that the alignment is statistically significant. [88]

Sequences producing significant alignments

Download

Select columns

Show

100

☒ select all 100 sequences selected

[GenBank](#)

[Graphics](#)

[Distance tree of results](#)

[MSA Viewer](#)

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☒	Pyrus communis genome assembly, chromosome: 4	Pyrus communis	40.1	9138	100%	0.002	100.00%	29965028	OY757071.1
☒	Pyrus communis genome assembly, chromosome: 3	Pyrus communis	38.2	9837	100%	0.008	100.00%	30840920	OY757070.1
☒	PREDICTED: Pyrus communis uncharacterized protein (LOC137737390). mRNA	Pyrus communis	34.2	34.2	85%	0.12	100.00%	1014	XM_068476850.1

Figure 18 Results of NCBI BLAST

The sequence with the lowest E-value was then selected, and the entire sequence of the chromosome was presented via 'Graphics'. The 'search tool' was used to identify the target regions for the forward and the reverse primer (see figure 19; yellow marked sequences). Since this research focuses on the differentiation of cultivars based on the occurrence of SSRs, an SSR identification was carried out. The sequence between the forward and reverse primer binding sites was searched for SSRs (see figure 19, blue marked sequence).

5.4 PCR

Before starting the PCR, the master mix was prepared by calculating the volume of the reaction components according to table 10 and a template was created for the thermocycler. Two different kinds of thermocycler, RotorGene and QuantStudio, were applied in this thesis. The template was created in the thermocyclers' software.

Table 10 Preparation of the Master Mixes

Master Mix for HRM PCR Type-It		Master Mix for HRM PCR Type-It	
Primer concentration	0.2 μ M	Primer concentration	0.4 μ M
Nuclease free water	7.92 μ l	Nuclease free water	7.04 μ l
2x HRM PCR Master Mix	11 μ l	2x HRM PCR Master Mix	11 μ l
Primer 10 μ M forward	0.44 μ l	Primer 10 μ M forward	0.88 μ l
Primer 10 μ M reverse	0.44 μ l	Primer 10 μ M reverse	0.88 μ l
total	19.8 μ l	total	19.8 μ l
Mastermix per well	18 μ l	Mastermix per well	18 μ l
DNA extract	2 μ l	DNA extract	2 μ l

The template outlines the temperature program (see table 11) and the number and order of the samples. The annealing temperature in the program was set according to the melting temperature of the primer pair.

Table 11 Temperature program for the PCR

		Time	Temperature [°C]	Repeats
Hold 1	Denaturation, activation of polymerase	5 minutes	95	
Cycling 1	Denaturation	15 seconds	94	} 50 cycles
	Annealing	30 seconds	50-70	
	Elongation	30 seconds	72	
Hold 2	Final elongation	10 minutes	72	
Hold 3	Denaturation	1 minute	95	
Hold 4	Strand pairing	1 minute	40	
HRM	HRM	-	65-95	0.1°C/step

PCR was prepared in three different laboratories in order to avoid contamination:

- first laboratory: storage of all components used for the PCR (DNA extracts, 2x HRM PCR Master Mix, nuclease free water, primer pair)
- second laboratory: preparation of master mix and addition of nuclease free water for dilution of the DNA extracts
- third laboratory: addition of DNA extracts
- equipment room: execution of the PCR on the thermocycler

Further measures were taken to avoid contaminations, including wearing gloves when touching the equipment in the laboratories. The gloves were changed whenever they came in contact with samples, dirt or DNA exitus. The working bench and fume hood were decontaminated with DNA exitus before and after working on it. It was essential to open tubes containing 2x HRM PCR Master Mix, nuclease free water, or the primer pairs in the second laboratory only. The reaction components were transported in a box that has been decontaminated with DNA exitus. Besides,

laboratory coats were changed between the three laboratories and it was not allowed to enter any of the three laboratories after the contact with amplicons.

In the first laboratory, DNA extracts, primer pairs, 2x HRM PCR Master Mix, and nuclease free water were taken from the freezer and defrosted on the bench. Primer pairs, Master Mix solution and nuclease free water were placed in the decontaminated box with a metal plate on top of a cool bag. Before entering the second laboratory, the lab coat was changed.

In the second laboratory, the master mixes were prepared and the nuclease free water for diluting the DNA extracts was pipetted into the Eppendorf tubes.

The fume hood was cleaned with DNA exitus. Afterwards, the gloves were changed and nuclease free water was placed inside the hood. The required number of Eppendorf tubes (200 µl, 1.5 ml and 2 ml) were shaken out of the packaging bag into the fume hood, without touching them to avoid contamination of the remaining tubes. Nuclease free water was pipetted into a 200 µl tube for the no template control (NTC), which served as negative control. The required amount of nuclease free water was pipetted into the 1.5 ml and 2 ml tubes, used for the dilution of the DNA extracts and the preparation of the master mixes.

DNA extracts from the pear leaves were diluted with nuclease free water to obtain a concentration of 2.5 ng/µl. The extracts with a DNA concentration lower than 2.5 ng/µl were diluted in a ratio of 1:10 with nuclease free water. The dilution of the DNA extracts from the pear leaves was prepared in sufficiently large quantity in order to avoid another dilution process. The diluted extracts were then stored in the freezer and defrosted when needed for PCR. The DNA extracts from the pear peel, pulp, and the four juices were diluted in a ratio of 1:5 with nuclease free water. Long term storage of low concentration DNA leads to poor amplification and inconsistent PCR results (e.g. scattering). Therefore, the dilutions were prepared on the day of the PCR.

The 2x HRM PCR Master Mix was vortexed with Vortex Classic Advanced Vortex Mixer at a speed of 16 three times for approximately five seconds. The required amount of 2x HRM PCR Master Mix was pipetted into the tube containing nuclease free water for the master mix and vortexed at a speed of 16. The 2x HRM PCR Master Mix was then returned to the box. The primer pair was placed inside the fume hood and vortexed at the highest speed (30) for approximately 10 to 15 seconds. The required amount of primer was then pipetted to the master mix. The primer pair was returned to the box and the master mix was vortexed three times at a speed of 16 for five seconds. The metal plate was placed inside the fume hood and the PCR tubes were inserted. 18 µl master mix were pipetted into each PCR tube. The metal plate was placed into the box on top of the cool bag. The fume hood was closed and decontaminated with UV light.

Before entering the first laboratory again, the lab coat was replaced by the third lab coat. In the first laboratory, 2x HRM PCR Master Mix, primer pairs and nuclease free water were returned to the freezer. The defrosted DNA extracts of the samples were vortexed, placed inside the box, and taken to the third laboratory.

In the third laboratory, DNA extracts from the samples were added to the nuclease free water for dilution. The diluted DNA extracts were pipetted to the master mix in the PCR tubes.

The fume hood was opened and cleaned with DNA exitus. The gloves were changed and the DNA extracts and the Eppendorf tubes with the water for the dilutions were placed inside the hood. For the dilution of the DNA extracts, the calculated volume was pipetted into their respective tube and vortexed at highest speed for approximately 15 seconds. Then the metal plate with the PCR tubes was placed inside the hood. The diluted DNA extracts were pipetted according to the template into their corresponding PCR tubes. All DNA extracts were analysed in duplicate. Nuclease free water was pipetted into the PCR tubes for the NTC. As stated before, two different kinds of thermocycler were applied. When using the QuantStudio thermocycler, the PCR tubes were spun down to eliminate air bubbles at the bottom of the PCR tubes. The Quant Studio thermocycler measures the fluorescence signal at the bottom of the tube and air bubbles would distort the results. The RotorGene thermocycler did not

require this additional step because the samples are rotated, thereby eliminating bubbles. The metal plate was returned to the box on top of the cool bag. The fume hood was closed and decontaminated with UV light.

The PCR was carried out in the equipment room. The PCR tubes in the box were placed inside the thermocycler. The prepared template was opened and the program started. After the PCR run was completed, the PCR products (amplicons) were taken from the instrument, put into plastic bags and stored in the freezer.

5.5 Gel electrophoresis

Gel electrophoresis was carried out in a different laboratory than DNA extraction and PCR in order to avoid contamination with amplicons. The working bench was cleaned with 70 % ethanol wearing gloves. Then, approximately 3 g agarose were dissolved and heated in 100 ml 0.5 Tris-Boric acid-EDTA (TBE). When the agarose was completely dissolved, it was poured into the gel chamber containing a gel-comb. While the agarose gel solidified, the 2x LD solution and the 1:4 diluted molecular marker were prepared with nuclease free water, 6x RotiLoad Orange with Glycerin and Low Molecular weight DNA Ladder. Then, 3.5 µl of the 2x LD solution and 3.5 µl of the amplicons were pipetted into 200 µl Eppendorf tubes.

When the gel was hard, the comb was removed and the gel transferred into an electrophoresis chamber, which was filled with 0.5x TBE buffer. The gel marker and the diluted samples were pipetted into the gel slots. Then, the gel chamber was closed, the electrodes were plugged into the power supply and a voltage of 80 V was applied. After approximately two hours, the power supply was turned off and the gel transferred into a plastic box.

A staining solution was added in order to visualise the amplicon bands with UV-light. The staining solution was made by mixing 150 ml distilled water with 45 µl 1000x GelRed Nucleic Acid Gel Stain. The box with the gel was placed in a dark place for 30 minutes for staining. The staining solution was then replaced with distilled water and the gel incubated for another 30 minutes. The water was discarded and the gel was placed on top of the transilluminator in a dark room. To avoid contact with the UV-light, a carton with a hole was positioned over the transilluminator. The UV-light was turned on and photos of the gel were taken through the hole in the carton with a smartphone. Finally, the UV-light was turned off, the transilluminator was cleaned, returned and the gel was discarded. The photo was used for the evaluation (see section 3.4).

6 Results and discussion

6.1 Primer pairs

6.1.1 Pear primer pairs

The sequences of the primer pairs for *Pyrus communis* were taken from five research papers [18, 19, 85-87]. Then, the primer sequences were tested with in silico tests to determine its suitability for this thesis (see section 5.3.1). First, the melting temperature was determined, which should exceed 50 °C. The difference in the melting temperature between forward and reverse primer should not exceed 1 °C. In cases, where the difference was more than 1 °C, the sequence of the primer with the higher melting temperature was shortened, which reduced its melting temperature. Depending on the eliminated bases (adenine, cytosine, guanine or thymine), the temperature would drop drastically or in small steps. Compared to adenine and thymine, the guanine and cytosine pair is held together by a third hydrogen bond. Thus, removing one of these bases results in a more drastic drop in temperature [72]. Primer pairs with a melting temperature below 50 °C and primer pairs where the difference between forward and reverse primer still exceeded 1 °C were excluded.

Overall, 64 primer pairs from the five research papers were tested: 19 [85], 26 [18], 11 [19], 6 [86] and 2 [87]. At the first stage, only 13 primer pairs matched the set criteria. Of the 64 primer pairs, ten had melting temperatures below 50 °C and were automatically excluded. The remaining 41 primer pairs were adjusted to align to the criteria of the temperature difference which should not exceed 1 °C by shortening the sequence of the primer with the higher melting temperature. Finally, 50 primer pairs met the criteria.

These 50 primer pairs were tested using two different in silico tests to determine the probability of the formation of unwanted primer dimers and secondary structures. 16 of the 50 primer pairs formed primer dimers and/or secondary structures and were therefore excluded. The remaining 34 primer pairs were then searched using NCBI to test their applicability to obtain amplicons for *Pyrus communis* and to control if they target microsatellites for the differentiation of cultivars. Primer pairs, where forward or reverse sequences varied from the NCBI, were aligned to sequences in NCBI and the in silico tests were carried out again.

Overall, six primer pairs fulfilled all these criteria and were ordered from Sigma Aldrich and tested via PCR with DNA from Williams pear peel extracted with the Plant Kit. The DNA extract was diluted in a ratio of 1:5 with nuclease free water and analysed in duplicate. The annealing temperature of each primer pair was set at the 'Salt adjusted' temperature minus 1 °C respectively 3 °C and the primer concentration at 0.2 respectively 0.4 µM. For PCR, the QuantStudio thermocycler was used, because it has up to six independent temperature zones, which allows the application of six different annealing temperatures in one PCR run. For the procedure of PCR, see section 5.4.

Table 12 lists the pear primer pairs that were suitable for the analysis and differentiation of pear cultivars according to the in silico tests, NCBI and PCR test results. Furthermore, it lists their sequences (forward/reverse primer and targeted microsatellites) and the optimized PCR parameters (annealing temperature and primer concentration). The PCR parameters were selected based on the PCR results, which includes formation of primer dimers, Ct-value and repeatability of the melting curves. Several primer pairs were modified by shortening their sequences in order to reduce the temperature-difference and by adjusting the sequences according to NCBI. The modified primer pairs are labelled with the letter 'M' at the beginning of their names.

Table 12 Pear primer pairs with their targeted microsatellite sequences (SSR), amplicon lengths (AL) and optimized PCR parameter (annealing temperature and primer concentration)

Name	Primer Sequence 5'→ 3' (Forward and Reverse)	SSR [98]	Temp. [°C]	Conc. [μM]	AL [bp] [98]	Ref.
EMPc08	F.: ATTAATTAGAATACCTAGACACACAC R.: CGGAGGAAGAGCATAGATGG	(CA) ₆	59.1	0.2	160	[85]
MEMPc111t	F.: CCAAACCTTCCAACCCCAACAAT R.: CCGATCAGAAAGAGCTGTGAGT	(GA) ₂₄	59.1	0.4	111	[85]
MEMPc117	F.: GTTCTATCTACCAAGCCACG R.: CGTTTGTGTGTTTTACGTGTTG	(TC) ₁₈	55.4	0.2	119	[85]
MGD127	F.: CTTTCGGATGCCTTTTGCT R.: GCTTTCAGATTCTTCGTCTT	(AG) ₂₁	53.3	0.4	140	[18]
MGD154	F.: CGTGGAGGAAGAGAAAGAGG R.: GCAGATTGACCCTGAAGACAA	(CT) ₂₃	56.4	0.2	185	[18]
NH013a	F.: GGTTTGAAGAGGAATGAGGAG R.: CATTGACTTTAGGGCACATTC	(GA) ₁₁	55.4	0.4	199	[19]

All selected pear primer pairs targeted microsatellites consisting of dinucleotide repeats, but with different repeating bases and lengths. Usually, the primer pairs targeted perfect/uninterrupted microsatellites [44], such as MGD154, as can be seen in figure 20 A. However, the primer pairs MEMPc117 and NH013a targeted imperfect microsatellites, which means that the sequences are interrupted by other bases (see figure 20 B and 20 C). [44]

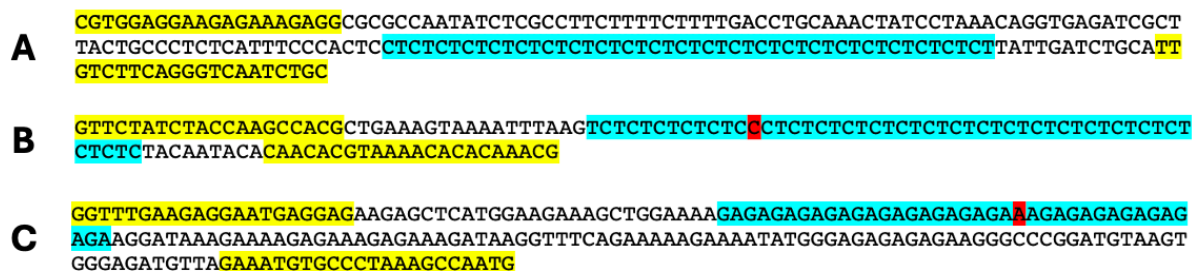


Figure 20 Microsatellite sequence (blue) and target region for forward and reverse primer (yellow) of primer pairs A) MGD154, B) MEMPc117, and C) NH013a. The microsatellite sequences of MEMPc117 and NH013a are interrupted by one base (red).

Although the primer pairs MEMPc117 and NH013a targeted imperfect microsatellites, this did not negatively affect the PCR results (see figure 21 and 23 D). Results did not differ from those obtained with primer pairs targeting perfect microsatellites (figure 22 and 23 A to C). Thus, both were used in this thesis.

For selecting the PCR parameters, the melting curves were analysed with the QuantStudio software.

Figure 21 A shows the melting curves of the PCR products obtained for Williams with primer pair MEMPc117 (imperfect microsatellite) and with a primer concentration of 0.2 μM at 'Salt adjusted' temperature minus 1 $^{\circ}\text{C}$ and minus 3 $^{\circ}\text{C}$. Figure 21 B shows the melting curves obtained with a primer concentration of 0.4 μM .

In figure 21 A, the DNA extracts resulted in the formation of amplicons, whereas the fluorescence signal of the NTC did not increase. In figure 21 B, the melting curves for one of the NTCs (dark blue) had a peak, indicating the development of unwanted primer dimers. The formation of primer dimers leads to inconsistent results, because less reaction components are available for the formation of amplicons. Although the Ct-value obtained with 0.4 μ M primer concentration was slightly lower than that obtained with 0.2 μ M, the primer pair MEMPc117 was used in a concentration of 0.2 μ M (figure 21 A). Figure 21 A indicates that the green melting curves (55.4 $^{\circ}$ C) had a better

repeatability than the blue melting curves (57.4 °C) and since the Ct-value obtained at 55.4 °C was slightly lower (by 1-2) than that obtained at 57.4 °C, the annealing temperature was set at 55.4 °C.

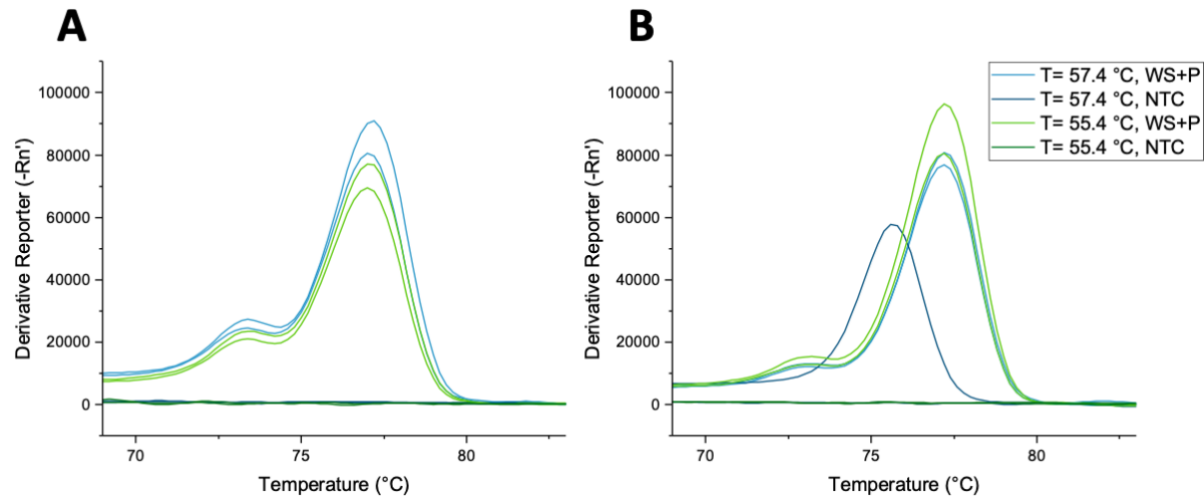


Figure 21 Melting curves of the amplicons obtained for Williams (WS+P, bright blue and bright green) and NTC (dark blue and dark green) with primer pair MEMPc117 with the annealing temperature set at 57.4 °C (blue) and 55.4 °C (green) and primer concentration at A) 0.2 µM and B) 0.4 µM. All extracts were analysed in duplicate.

Figure 22 A shows the melting curves of the amplicons obtained for Williams with primer pair MGD154 (perfect microsatellite) with a primer concentration of 0.2 µM at 'Salt adjusted' temperature minus 1 °C and minus 3 °C. Figure 22 B represents the melting curves obtained with a primer concentration of 0.4 µM. The repeatability was higher at a primer concentration of 0.2 µM (see fig. 22 A) and at 56.5 °C (green curve). Although the Ct-value obtained with a primer concentration of 0.4 µM was lower by 2, it was decided to use a concentration of 0.2 µM, because the higher primer concentration could lead to primer dimer formation.

In subsequent experiments, this primer pair was used at a concentration of 0.2 µM and an annealing temperature of 56.4 °C. This temperature was chosen to match the annealing temperature of the apple primer pair CH02b10, allowing both to be used in a simultaneous PCR run.

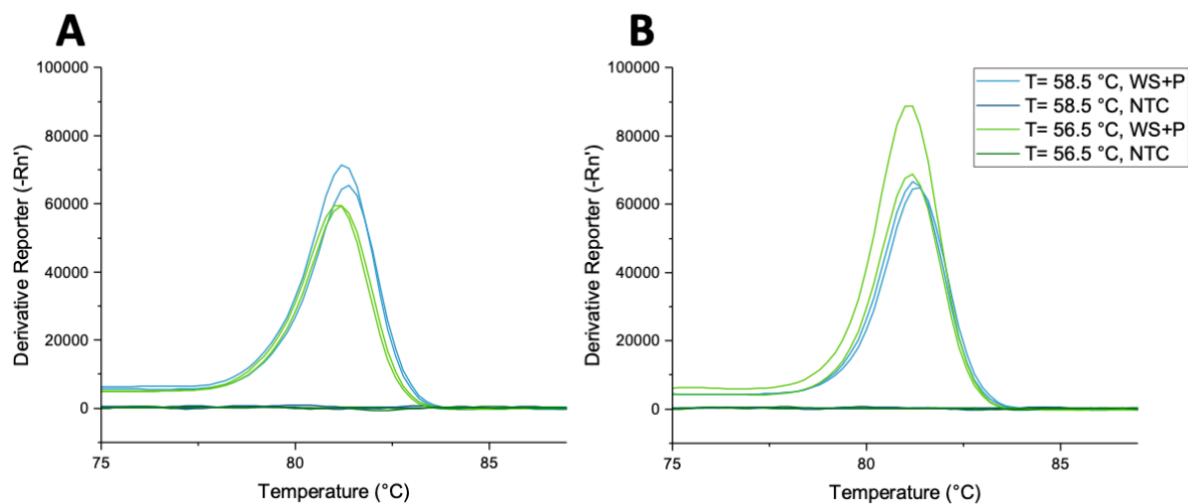


Figure 22 Melting curves of the amplicons obtained for Williams (WS+P, bright blue and bright green) and NTC (dark blue and dark green) with primer pair MGD154 at an annealing temperature of 58.5 °C (blue) and 56.5 °C (green) and primer concentration at A) 0.2 µM and B) 0.4 µM. All extracts were analysed in duplicate.

For the other pear primer pairs, the same procedure was carried out to optimize annealing temperature and primer concentration. Figure 23 illustrates the amplification and melting curves obtained for Williams (WS+P) with the further four pear primer pairs under the optimized PCR parameters (see table 12). Figure 23 B shows that the amplification curves reached a plateau, indicating successful amplification.

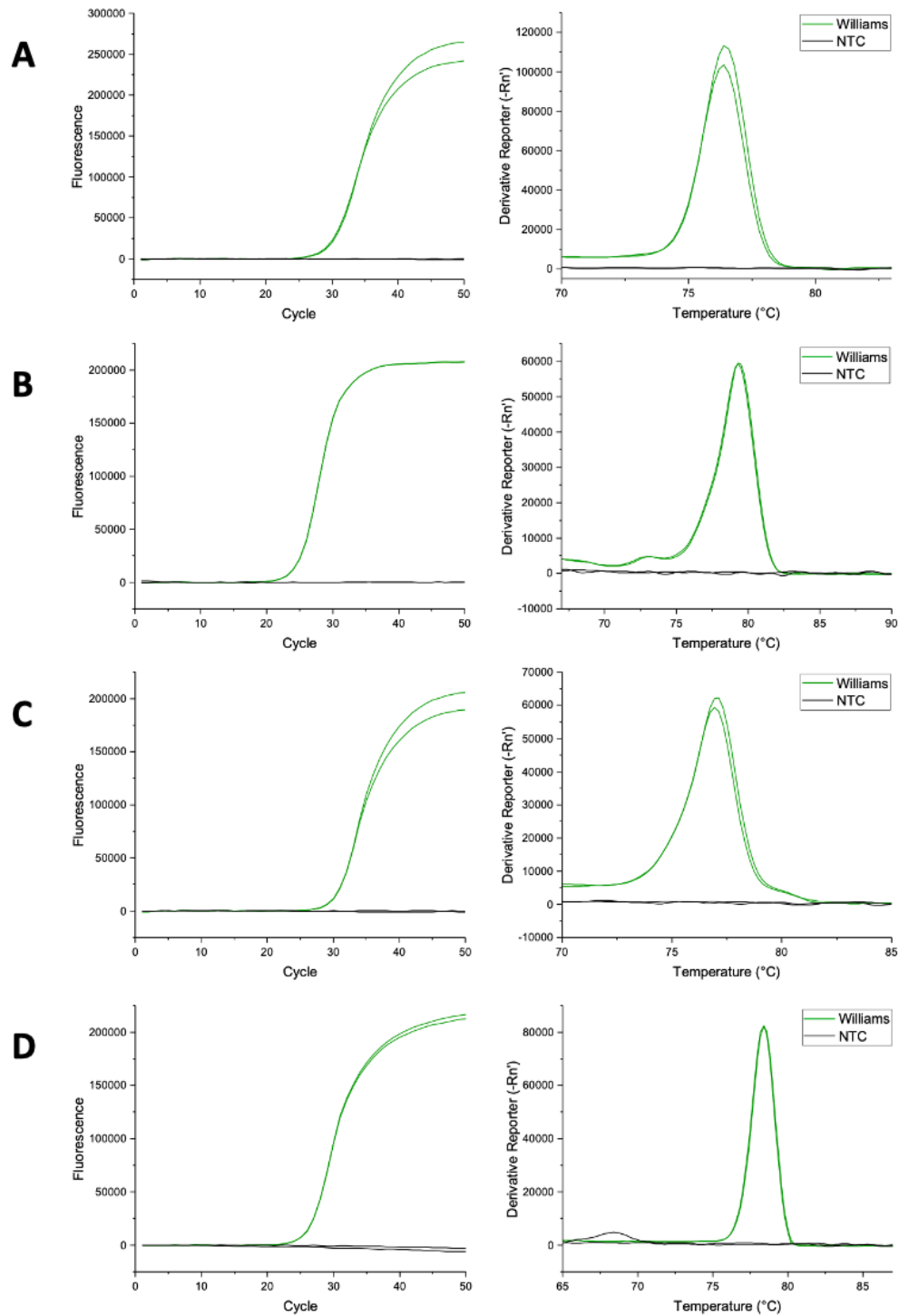


Figure 23 Amplification (left) and melting curves (right) of the amplicons obtained for Williams (green) and NTC (black) with the primer pairs A) EMPc08, B) MEMPc111t, C) MGD127, and D) NH013a. PCR was performed with the optimized PCR parameters (see table 12). All extracts were analysed in duplicate.

6.1.2 Apple primer pairs

In this thesis, 20 apple primer pairs from a former master thesis by Mina Micic [96], used for the differentiation of apple cultivars and obtained from six research papers [89-94] and the HiDRAS database [95], were tested to see if PCR products can be successfully obtained for *Pyrus communis* and if they are suitable for the differentiation of pear cultivars. Apple primer pairs have previously been used for the amplification of *Pyrus communis* DNA samples. [10, 18]

The primer pairs were first tested using NCBI BLAST to determine whether their sequences also appear in the pear genome and if they target microsatellites.

Out of the 20 primer pairs, seven (CH01f12, CH01h01, CH01h10, Ch02b03b, CH02h11a, CH03a09 and CH04c07) [91], targeted microsatellites and their sequences matched those found in NCBI. Four other primer pairs (CH02b10, Ch01f09, Ch03c02 [91] and 01a6 [94]) also targeted microsatellites, but either their forward or reverse sequences differed from the NCBI sequences. The remaining nine primer pairs appeared to be unsuitable based on NCBI. The primer pairs Hi04a02 [95], Hi03d06, Hi02f12 [93], CH01g12 [89], Ch02d11 [91], GD103 [90], 23g4, and 05g8 [94] did not target microsatellites, and their sequences were either absent and/or were partially different from the corresponding NCBI sequences. The forward sequence of primer pair CH02g09 [91] was found twice in the sequence in the *Pyrus communis* genome, which could cause unspecific binding. Since it also did not target a microsatellite, it was considered unsuitable.

Although NCBI results indicated that at least nine of the 20 apple primer pairs were not suitable for the differentiation of pear cultivars, it was decided to test all 20 primer pairs by PCR, since all of them were at hand. The primer pairs were tested with DNA extracted from the pear peel of the cultivars Anjou, Cheeky, and Williams with the Plant Kit. The DNA extracts were diluted 1:5 with nuclease free water to evaluate the probability of differentiating these three cultivars. The primer concentration (0.2 µM) and the annealing temperature were taken from the master's theses of Mina Micic [96] and Michaela Wildbacher. [97] PCR was performed using the QuantStudio thermocycler (see section 5.4). The primer pair CH02b10 was tested with the same DNA extracts diluted 1:15 with nuclease free water and applied in a PCR run with the RotorGene thermocycler.

Based on the PCR results, nine of the 20 apple primer pairs were found to be suitable and subsequently used in this research. Table 13 lists the suitable apple primer pairs, their forward/reverse sequences, the targeted microsatellite sequences and the amplicon lengths.

Table 13 Forward and reverse sequences of apple primer pairs and their targeted microsatellite sequences (SSR), amplicon lengths (AL) and annealing temperatures.

Name	Primer sequence 5' -> 3' (Forward and reverse)	SSR [98]	Temp. [°C]	AL [bp] [98]	Ref.
CH01f12	F.: CTCCTCCAAGCTTCAACCAC R.: GCAAAAACCACAGGCATAAC	(CT) ₁₀	58.5	136	[91]
CH01h01	F.: GAAAGACTTGCAGTGGGAGC R.: GGAGTGGGTTTGAGAAGGTT	(CT) ₁₂	57.5	105	[91]
CH01h10	F.: TGCAAAGATAGGTAGATATATGCCA R.: AGGAGGGATTGTTTGTGCAC	(TC) ₁₆	56.1	105	[91]
CH02b10	F.: CAAGGAAATCATCAAAGATTCAAG R.: CAAGTGGCTTCGGATAGTTG	(CT) ₁₃	56.4	125	[91]
CH02b03b	F.: ATAAGGATACAAAAACCCTACACAG R.: GACATGTTTGGTTGAAAATTG	(CT) ₁₈	56.4	93	[91]
CH02h11a	F.: CGTGGCATGCTTATCATTTG R.: CTGTTTGAACCGCTTCCTTC	(AG) ₈	55.4	110	[91]
CH03a09	F.: GCCAGGTGTGACTCCTTCTC R.: CTGCAGCTGCTGAAACTGG	(CT) ₉	61	119	[91]
CH03c02	F.: TCACTATTTACGGGATCAAGCA R.: GTGCAGAGTCTTTGACAAGGC	(CT) ₁₃	56.4	116	[91]
CH04c07	F.: GGCCTTCCATGTCTCAGAAG R.: CCTCATGCCCTCCACTAACA	(GA) ₁₉	57	112	[91]

Only primer pair Ch03a09 targeted an imperfect microsatellite, which means that the microsatellite sequence is interrupted by other bases (see figure 24, marked red).

GCCAGCTGTGACTCCTTCTCCACCAATACCATTACAACTTCGCAGATGATATTCCAACAGAGAAAAAC**CTCTCTCTCTCGCT**
CTCTCCAAAAAACCATGCCAGTTTCAGCAGCTGCAG

Figure 24 Microsatellite sequence (blue) and target region for forward and reverse primer (yellow) of primer pair CH03a09. The base interrupting the microsatellite sequence is marked red.

The apple primer pairs CH01f12, CH01h01, CH01h10, Ch02b03b, CH02h11a, CH03a09, and CH04c07, which target microsatellites and whose primer sequences matched the NCBI database, successfully produced amplicons for the three pear cultivars in PCR. The melting curves of the duplicate reactions overlapped, indicating high repeatability (see fig. 25 and 26).

Figure 25 shows the melting curves of the amplicons obtained for the pear peel of Anjou, Cheeky, and Williams with primer pair CH1f12. Only the DNA extracts, but not the NTCs resulted in the formation of amplicons. In addition, the melting curves for the three cultivars differed in shape and/or peak maximum, enabling their differentiation. Therefore, primer pair CH01f12 was considered suitable for further analyses.

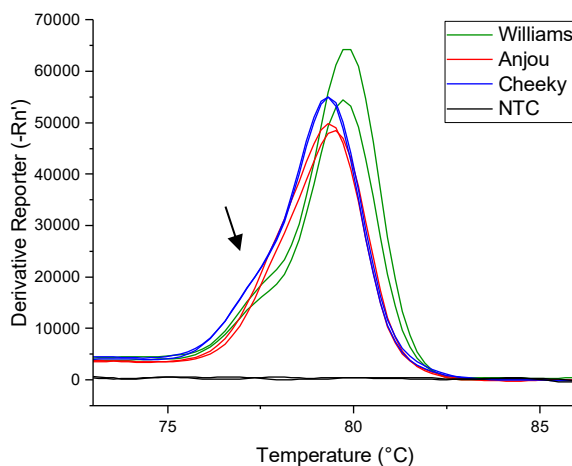


Figure 25 Melting curves of the amplicons obtained for Williams (green), Anjou (red), Cheeky (blue) and NTC (black) using the primer pair CH01f12. All extracts were analysed in duplicate. Black arrow indicates the difference between melting curves for Anjou and Cheeky.

The same procedure was applied to the remaining primer pairs to assess their applicability for this study. The melting curves of the amplicons obtained with primer pairs suitable for the differentiation of cultivars from *Pyrus communis* are shown in figure 26 A to F.

With the primer pairs CH02b03b (fig. 26 C) and CH03a09 (fig. 26 E), all three pear cultivars could be differentiated. In contrast, primer pairs CH01h01 (fig. 26 A), CH01h10 (fig. 26 B) and CH02h11a (fig. 26 D) did not allow clear differentiation of the three cultivars, because the peak maxima were at similar temperatures. Nevertheless, these primer pairs were retained for further analysis, since they target microsatellites and might allow differentiation of other pear cultivars. Primer pair CH04c07 (fig. 26 F) allowed the differentiation of the three cultivars, with peak maxima ranging from 78.1 °C to 78.8 °C. Although DNA from cultivar Anjou was amplified very late (Ct-value 48), resulted in a small peak, this primer pair was still included in subsequent analyses.

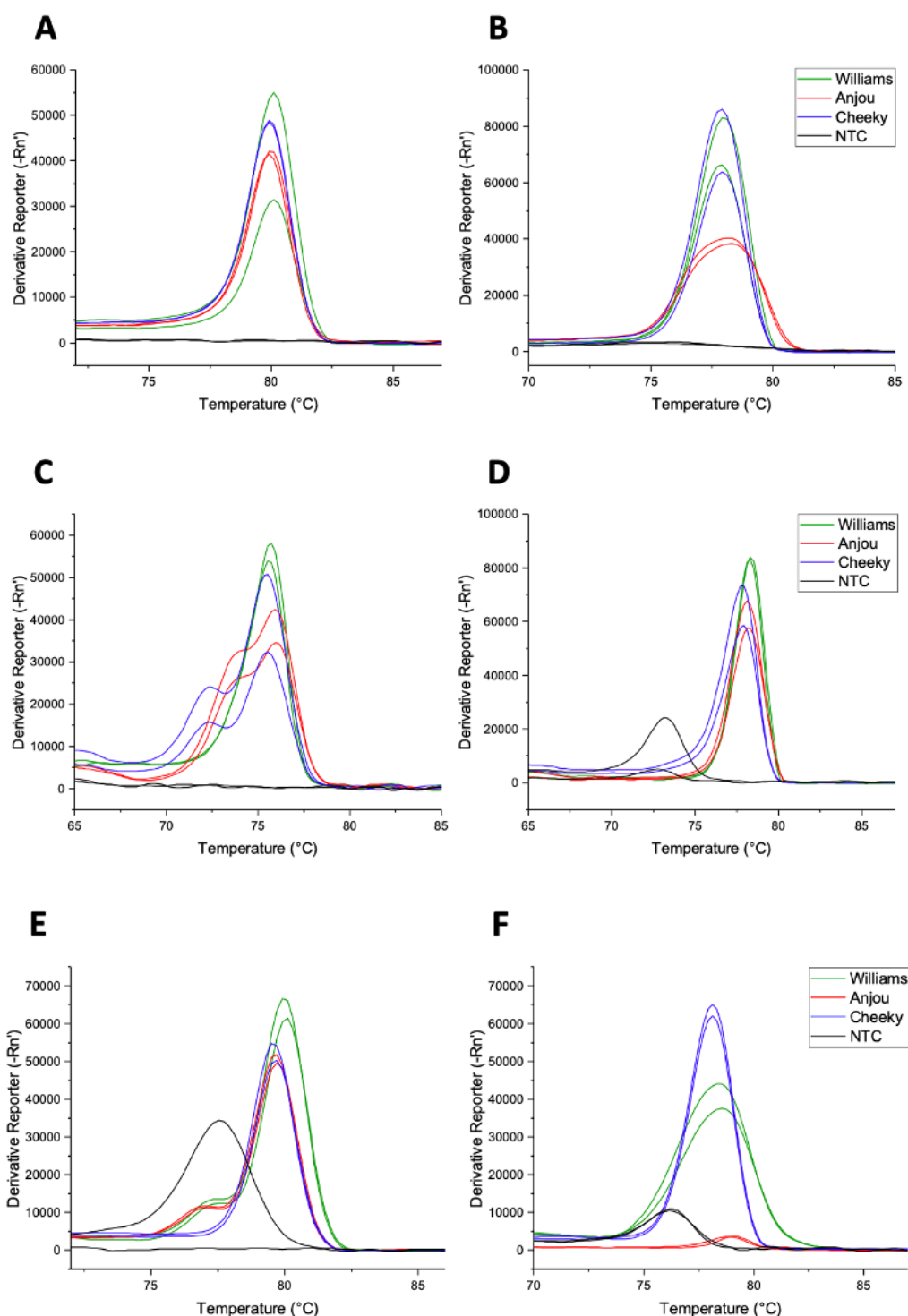


Figure 26 Melting curves of the amplicons obtained for Williams (green), Anjou (red), Cheeky (blue) and NTC (black) with the primer pairs A) CH01h01, B) CH01h10, C) CH02b03b, D) CH02h11a, E) CH03a09 and F) CH04c07. All extracts were analysed in duplicate.

The reverse primer sequences of the primer pairs Ch02b10 and Ch03c02 differed slightly (one to four bases) from the sequences in NCBI. Nevertheless, figure 27 shows that both primer pairs yielded amplicons with distinct melting curves. The melting curves obtained with Ch02b10 (fig. 27 A) differed between the three pear cultivars, enabling clear differentiation. In the case of Ch03c02 (fig. 27 B), the melting curves of Anjou and Cheeky were identical, preventing their differentiation. However, the melting curve obtained for Williams showed a peak maximum at a slightly higher temperature, allowing this cultivar to be distinguished from the other two cultivars.

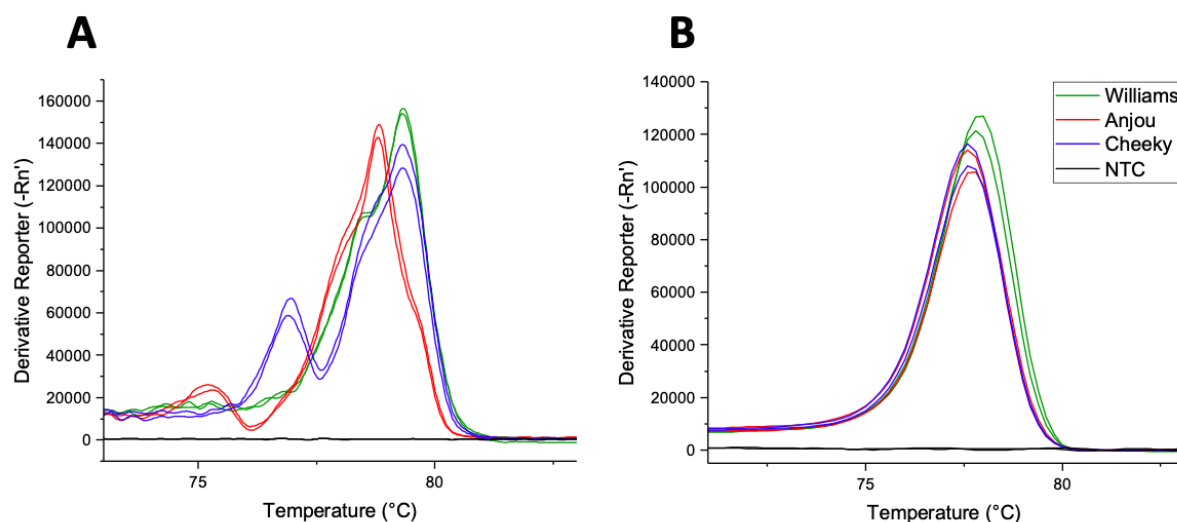


Figure 27 Melting curves of the amplicons obtained for Williams (green), Anjou (red), Cheeky (blue) and NTC (black) with the primer pairs A) Ch02b10 (RotorGene) and B) Ch03c02. All extracts were analysed in duplicate.

The sequences of primer pairs CH01f09 and 01a6 differed slightly from the corresponding sequences in the NCBI database, but both targeted microsatellites. PCR results showed that neither primer pair was suitable for distinguishing pear cultivars, since the melting curves showed poor repeatability between the PCR duplicates (see figure 28).

Figure 28 A presents the melting curves obtained for the three pear cultivars with primer pair CH01f09. The melting curves of the amplicons from Anjou and Cheeky had additional peaks in one of the duplicates, and the melting curves obtained for Williams differed drastically between the duplicates. The Ct-values were between 40 and 42. The melting curves obtained with primer pair 01a6 (figure 28 B) showed poor repeatability, low fluorescence signals, and Ct-values between 38 and 40. Both primer pairs were therefore considered not suitable for distinguishing pear cultivars.

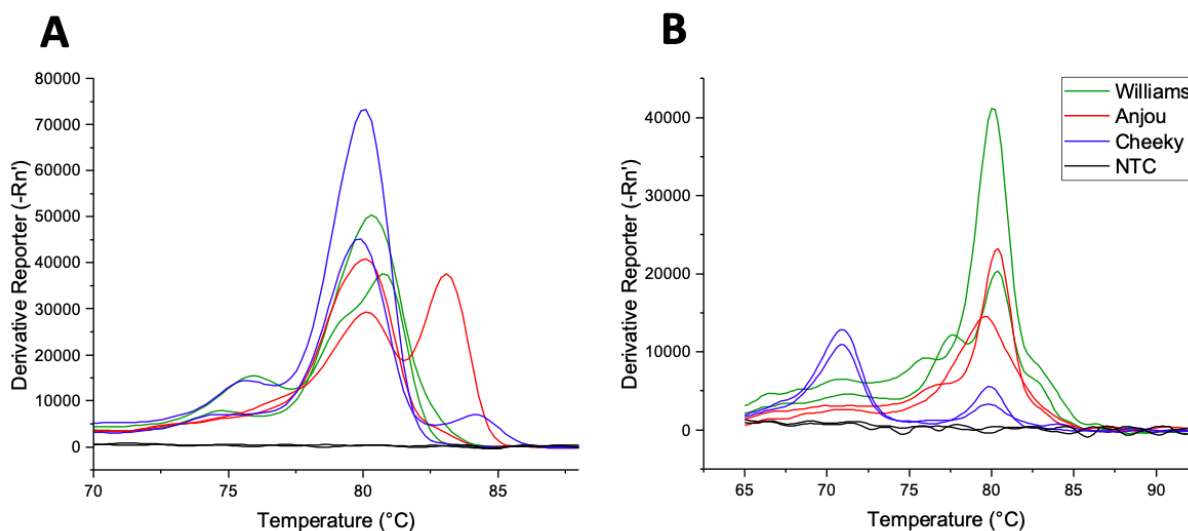


Figure 28 Melting curves of the amplicons obtained for Williams (green), Anjou (red), Cheeky (blue) and NTC (black) with the primer pairs A) Ch01f09 and B) 01a6. All extracts were analysed in duplicate.

Furthermore, the nine primer pairs Hi04a02, Hi03d06, Hi02f12, CH01g12, Ch02d11, GD103, 23g4, and 05g8, which were unsuitable according to NCBI, did either not amplify the respective target regions and/or formed unspecific PCR products and were therefore considered not suitable for distinguishing pear cultivars.

Figure 29 shows the melting curves for Anjou, Cheeky, and Williams obtained with the primer pair Ch01g12. According to Mina Micic, this primer pair was the best one for the differentiation of apple cultivars. [95] However,

the primer pair did not target microsatellite in pears according to NCBI, the forward primer sequence was not complete and the reverse primer sequence could not be found in the sequence of the *Pyrus communis* genome in NCBI. The melting curves obtained with primer pair CH01g12 showed that only primer dimers were produced and not the desired PCR products. Therefore, this primer pair was considered unsuitable for distinguishing pear cultivars.

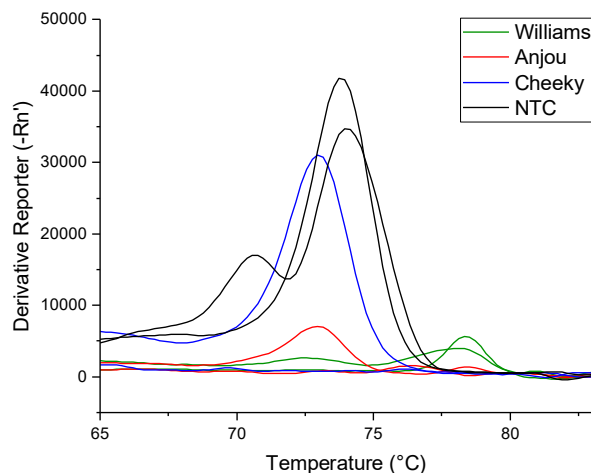


Figure 29 Melting curves of the amplicons obtained for Williams (green), Anjou (red), Cheeky (blue) and NTC (black) using the primer pair Ch01g12. All extracts were analysed in duplicate.

The melting curves obtained with three of the nine unsuitable primer pairs, Ch02d11, GD103, and 05g8, showed that PCR products were successfully formed, since they all had the peak maximum at high melting temperatures (75 to 81 °C) and none of them overlapped with the NTCs (see fig. 30 A to C). The amplicons obtained with primer pair Ch02d11 had their peak maximum at around 77.5 °C to 78.1 °C, primer pair GD103 at 79.3 °C (Cheeky and Anjou) to 80.3 °C (Williams) and primer pair 05g8 at 77.5 °C. The Ct-values obtained with primer pair Ch02d11 were between 26.4 and 28.6, with GD103 between 37.9 and 38.3, and with 05g8 between 31.1 and 32.3. Although none of these three primer pairs targeted microsatellites, most of the melting curves obtained for the three pear cultivars were slightly different. However, none of these three primer pairs were used in further experiments, because nine apple primer pairs had already yielded reliable results and were considered sufficient for the purposes of this thesis.

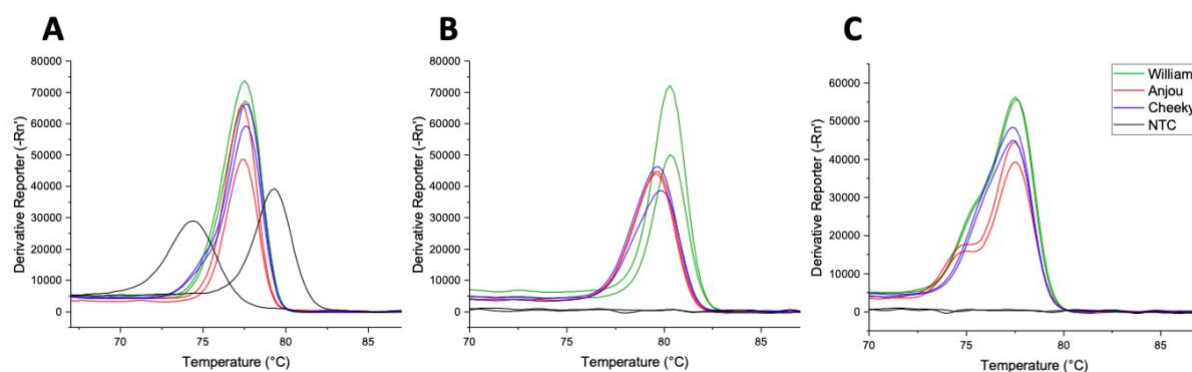


Figure 30 Melting curves of the amplicons obtained for Williams (green), Anjou (red), Cheeky (blue) and NTC (black) using the primer pairs A) Ch02d11, B) GD103 and C) 05g8. All extracts were analysed in duplicate.

6.2 Pear leaves

6.2.1 DNA concentration

DNA extraction from pear leaves was carried out with the NucleoSpin Plant II kit manufactured by Macherey-Nagel (see section 5.2.1). Two extractions were performed to assess repeatability. Table 14 shows the DNA concentrations of the extracts from the first and second extractions. During the second extraction, a second elution step was only performed when the DNA concentration of the first eluate was low (below 35 ng/μl), to ensure that a sufficient volume of DNA extract was available for at least 15 PCR reactions (corresponding to the 15 primer pairs).

Table 14 DNA concentration of DNA extracts from the 18 pear cultivars (from leaves)

Nr.	Pear cultivars	1. Extraction			2. Extraction		
		Mass [g]	Eluate 1 [ng/μl]	Eluate 2 [ng/μl]	Mass [g]	Eluate 1 [ng/μl]	Eluate 2 [ng/μl]
1	Abate Fetel	0.149	63	4.71	0.152	25.2	14.5
2	Celina	0.151	82.4	49.2	0.152	111	-
3	Fred	0.150	85.5	47.6	0.150	69.2	-
4	Xenia	0.151	129	40.9	0.151	35.2	22.6
5	Cordula	0.151	163	72.4	0.151	114	-
6	Harovin Sundown	0.150	127	13.2	0.150	96.3	-
7	Gute Graue	0.150	53.5	25	0.150	17	3.82
8	Kärntner Speckbirne	0.152	11.1	17.5	0.150	10	7.92
9	Alexander Lukas	0.149	33.3	28.7	0.150	9.71	7.9
10	Blutbirne	0.151	13.4	15.2	0.150	8.19	8.83
11	Haeng Su	0.151	5.45	5.29	0.150	1.73	1.51
12	Kimizukawase	0.101	1.39	2.14	0.101	1.72	0.948
13	Kumoi	0.100	2.25	2.42	0.100	1.04	0.458
14	Frühe von Trevoux	0.150	12.2	11.6	0.150	4.62	4.06
15	Conference	0.151	124	18	0.150	147	-
16	Williams	0.150	32.6	25.4	0.150	35.1	-
17	Boscs Flaschenbirne	0.151	16.5	15.1	0.152	5.87	4.69
18	Cepuna	0.151	54	42.6	0.150	34.2	-

The DNA concentration of the extracts from the 18 pear cultivars varied considerably. High concentrations (more than 100 ng/μl) were obtained from Cordula, Harovin Sundown, and Conference in both extractions, and from Xenia in the first extraction. Especially low concentrations (around or below 5 ng/μl) were obtained for Haeng Su, Kimizukawase, and Kumoi in both extractions, and for Frühe von Trevoux and Boscs Flaschenbirne in the second extraction.

During DNA extraction the majority of the pear leaf samples (samples 8–14, 17, 18) formed a gel (see section 5.2.1), which was especially problematic with the Asian pear cultivars. Their DNA extracts had the lowest DNA concentration. The gel plugged the column and prevented the solution from flowing through the membrane. In order to solve this problem for the elution step, the samples were repeatedly centrifuged to dry the membrane as thoroughly as possible before applying the elution buffer. However, the gel absorbed a part of the elution buffer, thereby reducing the final volume of the eluate (to approximately 30 to 40 μl) and consequently lowering the DNA concentration, as can be seen in table 14. Even additional centrifugation steps aimed at drying the gel and recovering the absorbed elution buffer were ineffective.

Most probably, gel formation was caused by secondary metabolites (e.g. phenolic compounds), humic acids (PCR inhibitors), and especially pectin, which coprecipitated with the DNA during the addition of alcohol and even trapped DNA within the gel, resulting in the decreased DNA yield. [83] In a dissertation (University Graz, 2009), difficulties in DNA extraction from pear leaves due to gel formation were associated with the high content of phenolic compounds and polysaccharides. [16] Phenolic compounds and polysaccharides are common in pears (leaves, peel, and pulp) and inhibit molecular reactions such as PCR. [16, 57] Because of their similar chemical structure to DNA, they can interfere with enzymes like DNA polymerases, if they are not completely removed during DNA extraction. The extent of inhibition also depends on their pH value, as acidic polysaccharides show stronger PCR inhibitory effects than basic polysaccharides. [99] In the dissertation, the sample mass was reduced from 17 mg to 15 mg and the incubation time at 65 °C was increased from 10 minutes to 65 minutes in order to decrease the amount of polysaccharides and phenolic compounds, and thereby minimize gel formation. [16]

In order to increase the DNA concentration of the extracts from the Asian pear cultivars, the sample mass was reduced from 150 mg to 100 mg and the DNA extraction carried out as described in section 5.2.1. The DNA concentrations of the extracts from Kimizukawase and Kumoi were slightly increased, whereas it was lower in the extract from Haeng Su compared to performing extraction with 150 mg. Therefore, DNA extraction from Haeng Su was performed with 150 mg sample mass, and from Kimizukawase and Kumoi with 100 mg. It is likely that the three Asian pear cultivars have a higher content of polysaccharides and phenolic compounds than the 15 European pear cultivars. Although some studies have investigated and compared the natural compounds of Asian and European pear cultivars, only a few have explored the leaves. The results of those studies show that the sugar and phenolic content vary among cultivars, regardless of whether they belong to *Pyrus communis* or *Pyrus pyrifolia*. The total content of phenolic compounds was nearly twice as high and the amount of arbutin (one of the main phenolic compounds in pears) three times higher in *Pyrus pyrifolia* than in *Pyrus communis*. In contrast, the amount of chlorogenic acid was higher in *Pyrus communis* than in *Pyrus pyrifolia*. [100]

Studies show that DNA obtained from younger leaves is of higher quality and quantity than DNA from older ones when used for laboratory testing. [16, 57] Furthermore, leaves under stress increase the production of secondary metabolites such as phenolic compounds. [57]

In this study, only older leaves were harvested for the analysis to avoid damaging the pear trees, which had already been harmed by extreme weather conditions and heavy rainfall in 2024. Such environmental stress may have triggered increased production of secondary metabolites in the trees and leaves, thereby exacerbating the problem of gel formation during DNA extraction. The 18 pear cultivars are grown in three different areas at the Haidegg experimental station. Samples 1 to 6, 7 to 14, and 15 to 18 are grown together in separate sections. Sample 7 to 14 (with the exception of sample 7) had the lowest DNA concentration, which may be caused by differences in soil and microclimatic conditions.

Furthermore, it is noticeable, that DNA concentrations were generally higher in the first extracts than in the second. This difference is most likely due to more pronounced gel formation during the second extraction. One possible explanation could be the inclusion of more leaf veins.

For most DNA extracts, the first eluate contained a higher DNA concentration than the second, with the exception of Kärntner Speckbirne, Kimizukawase, and Kumoi in the first extraction, and Blutbirne in both extractions. The reason could be that due to gel formation during the washing step, the membrane was not completely dried. Consequently, residual washing buffer may have been carried over into the first eluate, diluting the DNA and lowering its concentration. In contrast, the second eluate likely contained less impurities, and thus the DNA concentration was slightly higher.

All DNA extracts from pear leaves were slightly yellow, which could indicate low purity, however, the purity of the DNA extracts was not assessed. Besides, the extracts had a lower viscosity than those obtained from pear peel, pulp, and juices.

6.2.2 Performance of PCR-HRM and data evaluation

For the differentiation of the 18 pear cultivars, PCR coupled with HRM was used. The PCR reactions were performed as described in section 5.4 with the QuantStudio thermocycler. The applied primer pairs, primer concentrations and annealing temperatures are listed in table 12 and 13.

In the first PCR series, the 15 primer pairs were applied to DNA extracts from the first DNA extraction round. All extracts were analysed in duplicate. Data evaluation was carried out with the HRM analysis software. First, the amplification curves were analysed with regard to the formation of the desired amplicons and the Ct-values. Then, the melting curves were normalized by setting the normalization ranges. For each primer pair, different normalization ranges were chosen, depending on the melting profiles (see section 3.3.4).

In the second PCR series, the 15 primer pairs were applied to DNA extracts from the second DNA extraction. Additionally, the DNA extracts from the first DNA extraction, for which differences between the PCR duplicates were observed or did not result in the formation of amplicons, were applied in this PCR series. For normalization, the same normalization ranges were used as in the first PCR series. To assess the repeatability, normalized melting curves from the first and the second PCR/extracts were compared by using Origin.

During data evaluation, slight differences were observed between the normalized melting curves obtained in the first and the second PCR run. These differences may have resulted from minor variations in the master mix composition and/or slight fluctuations in the temperature of the thermocycler. In most cases, the shift in the melting and normalized melting curves from 0.01 °C to 1.40 °C, while Ct-values varied between 0.3 to 2.6.

Since the aim of this thesis was to differentiate the 18 pear cultivars based on their normalized melting curves from the first and second DNA extracts, it was of great importance to minimize and eliminate deviations between replicates in order to improve differentiation between the cultivars. Therefore, samples with poor repeatability between the first and second extracts were included in a third PCR run. A third PCR run was not performed when the normalized melting curves exhibited clearly different shapes, making it possible to distinguish the respective cultivars even if their replicates did not perfectly overlap. For example, figure 31 shows the normalized melting curves of the extracts from Kumoi and Cepuna obtained with primer pair NH013a. Compared to the curves obtained for the other cultivars, Kumoi had a slight peak at approximately 74.3 °C (see light blue curves in black box), while Cepuna showed a different curve shape at 79 °C (see purple curves in black box), which clearly differentiate them from the other pear cultivars.

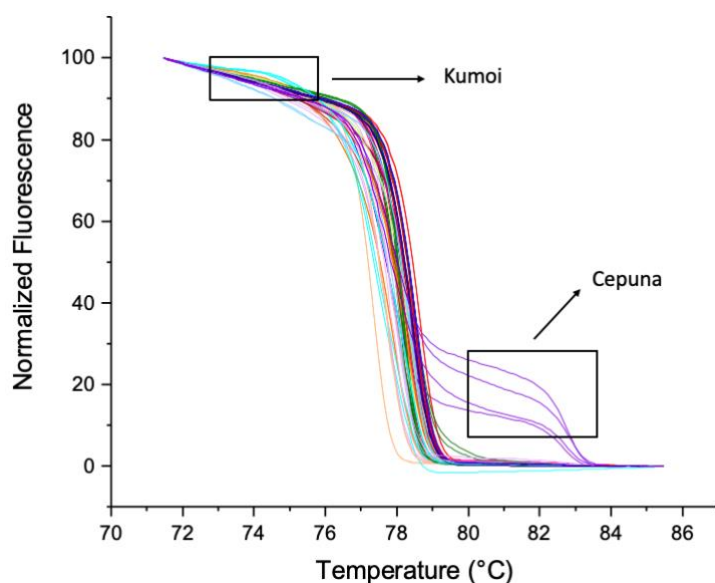


Figure 31 Normalized melting curves of the amplicons of the first and the second extracts from all 18 pear cultivars with primer pair NH013a. All extracts were analysed in duplicate. The black boxes demonstrate different curve shapes of the amplicons from Kumoi and Cepuna.

If the normalized melting curves obtained from the same cultivar showed deviations, the DNA extracts from the first and the second extraction were applied together in the third PCR run. The resulting normalized melting curves were then compared again. This approach helped eliminate inconsistencies arising during PCR performance and proved beneficial, as most normalized melting curves from the same cultivar finally overlapped or at least showed reduced deviation.

Figure 32 shows the normalized melting curves of the amplicons from the two Celina DNA extracts with primer pair CH02b10 in two separate PCR runs (fig. 32 A) and together in a third PCR run (fig. 32 B). The normalized melting curves from the first and the second extraction deviated slightly, whereas they overlapped when both extracts were analysed together in the third PCR run.

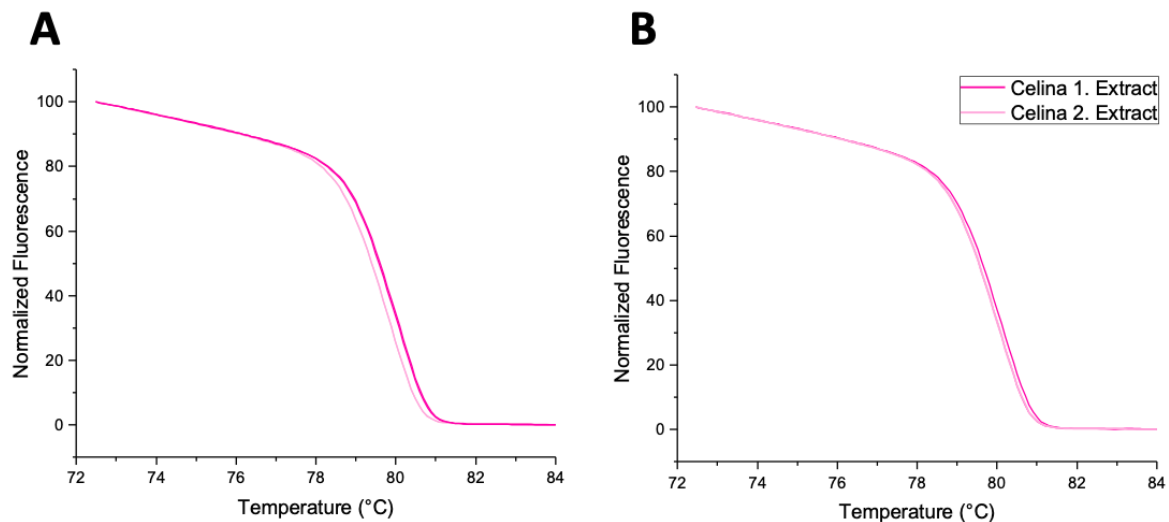


Figure 32 Normalized melting curves of the amplicons from Celina with the first and the second DNA extract applied in A) two separate PCR runs and B) together in the third PCR run with primer pair CH02b10. All extracts were analysed in duplicate.

Figure 33 illustrates the normalized melting curves of the amplicons from Cordula with primer pair MGD154 applied in two separate PCR runs (fig. 33 A) and together in a third PCR run (fig. 33 B). The curves obtained in different PCR runs were slightly shifted, whereas they overlapped completely when the extracts were analysed in the same PCR run.

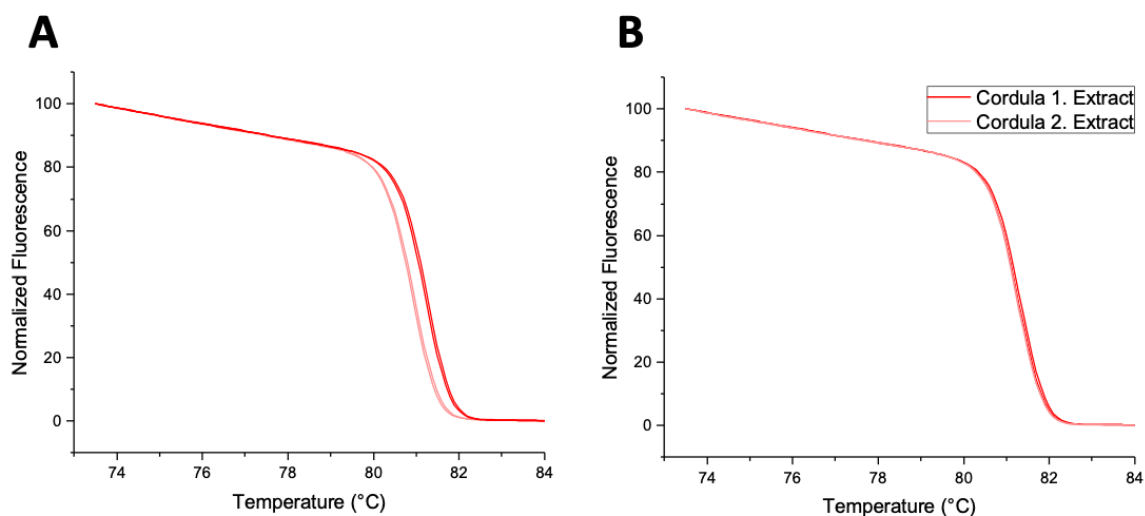


Figure 33 Normalized melting curves of the amplicons from Cordula with the first and the second DNA extract applied in A) two separate PCR runs and B) together in the third PCR run with primer pair MGD154. All extracts were analysed in duplicate.

Some of the normalized melting curves showed higher deviations between the first and second PCR run, and showed a slightly better repeatability in the third (combined) PCR run, but the curves still did not overlap. However,

there are other cases, where the third (combined) PCR did not decrease the deviations, e.g. for Boscs Flaschenbirne with primer pair MEMPc117 (see figure 34).

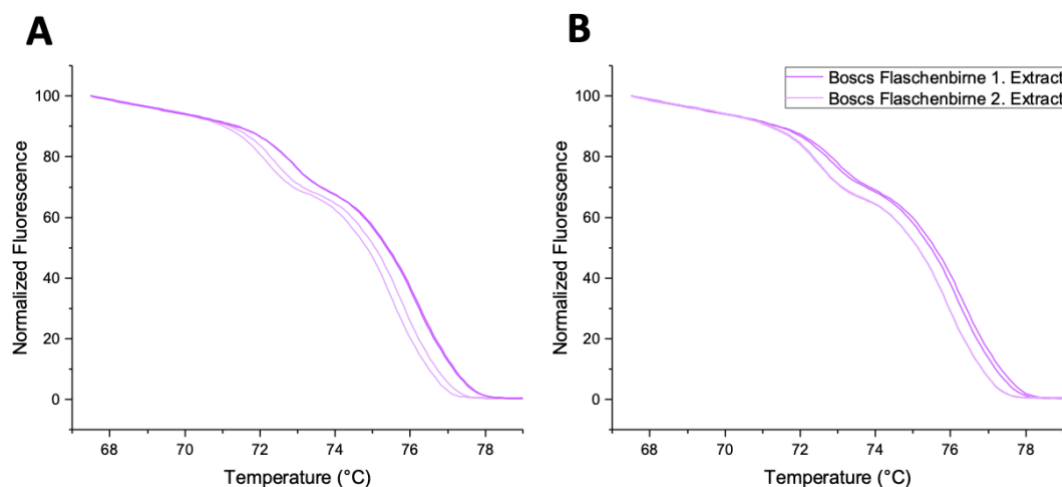


Figure 34 Normalized melting curves of the amplicons from Boscs Flaschenbirne with the first and the second DNA extract applied in A) two separate PCR runs and B) together in the third PCR run with primer pair MEMPc117. All extracts were analysed in duplicate.

Deviating normalized melting curves of the replicates could still be used to distinguish between pear cultivars if they showed a specific curve pattern (see figure 31). If this was not the case, other primer pairs had to be used to differentiate the cultivars. Further experiments, such as a third DNA extraction, using a different extraction protocol, or additional PCRs, were not performed due to time and financial constraints.

During data evaluation, it was noted that the melting curves of Kimizukawase and Kumoi showed clear differences between the first and second PCR runs (or, more precisely, between the first and second DNA extracts). Possible reasons are differences in the PCR setup (e.g. master mix composition) and the varying DNA concentrations of the two extracts. The DNA concentrations were below 2.5 ng/μl, so the extracts were used undiluted in both PCR runs. The first DNA extract had a higher DNA concentration than the second one because gel formation during the second extraction was much stronger. The gel clogged the column and blocked the flow of solutions, including the washing buffers PW1 and PW2. Although additional centrifugation was carried out (see section 5.2.1), the eluate still contained portions of the washing buffers, especially ethanol, which diluted the DNA extract and also acted as a PCR inhibitor. In comparison, gel formation during the first extraction was less pronounced. Despite some clogging, the column could be properly dried before the elution, so less washing buffer remained and the DNA concentration was higher.

The normalized melting curves for Kimizukawase and Kumoi obtained with all 15 primer pairs showed poor repeatability between the replicates, even when both extracts were applied together in a PCR run. The first extract always resulted in a lower Ct-value (about 0.5 to 5) than the second one. The low DNA concentrations of the extracts decreased the amplification efficiency, which led to late amplification and high Ct-values.

All melting curves obtained for the first extract from Kimizukawase were observed at higher temperatures than those of the second extracts. Therefore, the normalized melting curves obtained for the first extract were shifted to higher temperatures (see figure 35 A).

For Kumoi, the temperatures at the peak maximum and the normalized melting curves were not consistent between the first and the second extract, but varied depending on the primer pair. Only for the primer pairs EMPc08, MEMPc111t, MEMPc117, NH013a, CH01h10, CH02b03b, and CH04c07, the maximum peak of the first extract exceeded that of the second. For all other primer pairs, the first extract resulted in a peak maximum at higher temperature.

The fluorescence signals at the peak maxima for Kimizukawase were higher with the first extract than the second extract, except for primer pair CH01h10. For Kumoi, most primer pairs resulted in stronger fluorescence with the

second extract, except for primer pairs MGD127, MGD154, NH013a, CH01h01, CH02b03b, Ch02h11a, Ch03c02, and CH03a09.

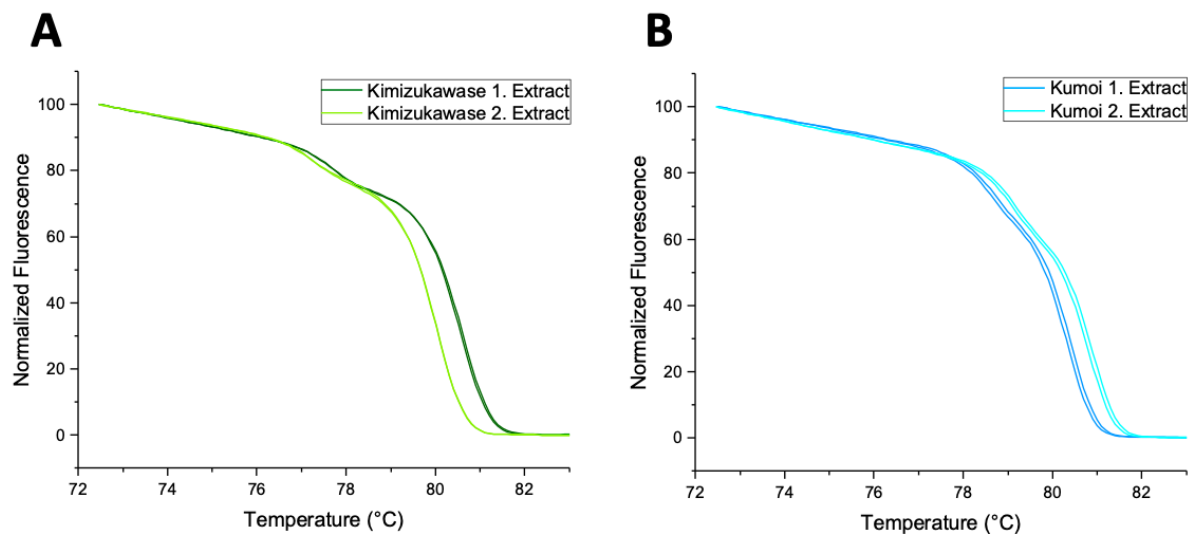


Figure 35 Normalized melting curves of the amplicons from the first (darker colour) and the second (brighter colour) extracts from A) Kimizukawase (green) and B) Kumoi (blue) with primer pair CH03a09. All extracts were analysed in duplicate.

Before the third series of PCR runs was carried out, the first and second DNA extracts from Kimizukawase and Kumoi were diluted in ratios of 1:10 and 1:50 with nuclease free water and applied in a PCR run with primer pair CH03a09 (see figure 36 and 37). The aim was to investigate whether dilution allowed reducing the deviations of the normalized melting curves between replicates and which dilution factor (1:10 or 1:50) would be more suitable.

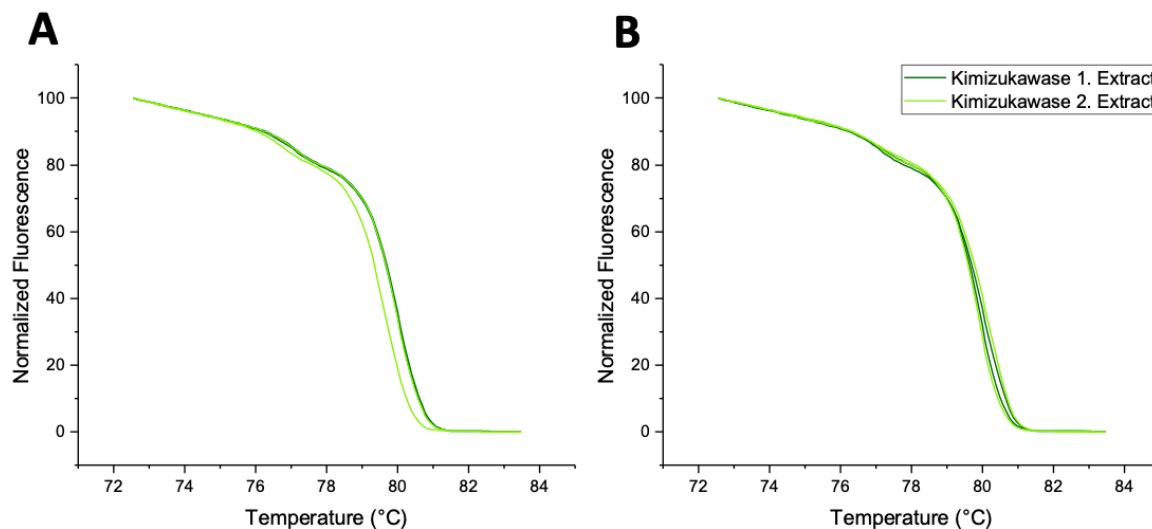


Figure 36 Normalized melting curves of the amplicons from Kimizukawase of the first extract (dark green) and second extract (light green) diluted A) 1:10 and B) 1:50 with primer pair CH03a09. All extracts were analysed in duplicate.

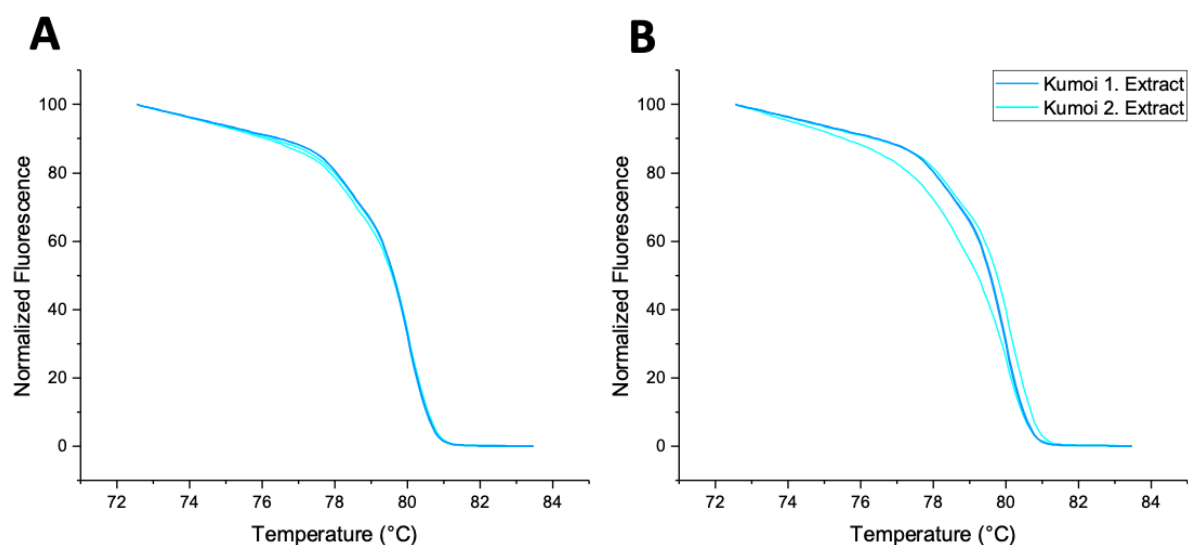


Figure 37 Normalized melting curves of the amplicons from Kumoi of the first extract (dark blue) and second extract (light blue) diluted A) 1:10 and B) 1:50 with primer pair CH03a09. All extracts were analysed in duplicate.

For Kimizukawase, the 1:50 dilution of the DNA extracts gave better results than the 1:10 dilution, because the normalized melting curves for the first and second extract overlapped more closely. In contrast, the curves obtained for the 1:10 dilution deviated considerably (see figure 36). The Ct-values for the extracts diluted 1:10 ranged from 28.4 to 30.0, while those for the 1:50 dilution were between 31.0 and 32.0. For both dilutions, the peak maxima were at 78.8 °C and 80.1 °C, respectively.

For Kumoi, the 1:10 dilution of the DNA extracts showed better repeatability than the 1:50 dilution (see figure 37). The Ct-values for the 1:10 dilution were between 27.4 and 31.5, and for the 1:50 dilution between 30.0 and 34.0. For both dilutions, the peak maxima were at 78.7 °C and 79.9 °C, respectively.

The 1:10 dilution of the extracts from Kimizukawase showed less variation than the 1:50 dilution of Kumoi extracts. Furthermore, the 1:10 dilution resulted in lower Ct-values. To simplify the workflow, it was decided to use a single dilution factor for all DNA extracts from pear leaves with DNA concentration below 2.5 ng/μl. Therefore, a 1:10 dilution was chosen.

6.2.2.1 Evaluation of HRM data obtained with the six pear primer pairs

The results of HRM analysis showed that amplicons were successfully obtained for all 18 pear cultivars with all six pear primer pairs. Typical amplification curves (reaching a plateau phase) were obtained for all cultivars. The Ct-values of the extracts were between 23.5 and 29.9, with some exceptions exceeding 30.

Primer Pair EMPc08

With primer pair EMPc08, the Ct-values obtained in the first PCR run were between 26.0 and 30.6. In the second PCR run, the Ct-values were between 27.5 and 32.2, except for Kimizukawase (33.3), Kumoi (33.5), Frühe von Trevoux (33.7) and Boscs Flaschenbirne (36.9).

In the first PCR, the peak maximum for all 18 cultivars was at lower temperatures than in the second PCR. The fluorescence signal at the peak maximum was higher in the first PCR, except for Fred, Cordula, Blutbirne, Kumoi and Conference. Despite these differences, the repeatability between the first and the second PCR runs (respectively the first and second DNA extracts) was good, because the majority of the normalized melting curves overlapped.

In the third PCR run, those extracts that had shown poor repeatability between the first and second PCR runs were analysed together. This helped to reduce the deviation between the replicates, except for Gute Graue and Boscs

Flaschenbirne. For those two cultivars, differences were observed between the two extracts in the Ct-values (Gute Graue (28.2; 32) and Boscs Flaschenbirne (31.3; 33.8)), and in the intensity of the fluorescence signals at the peak maximum. The signals from the first extracts were higher than those from the second extracts.

The melting curves for Abate Fetel, Xenia, Kärntner Speckbirne, and Blutbirne showed distinct features (see fig. 38), enabling their differentiation from the other pear cultivars. With primer pair EMPc08, Abate Fetel, Xenia, Kärntner Speckbirne, Blutbirne, and Frühe von Trevoux could be distinguished from the other cultivars.

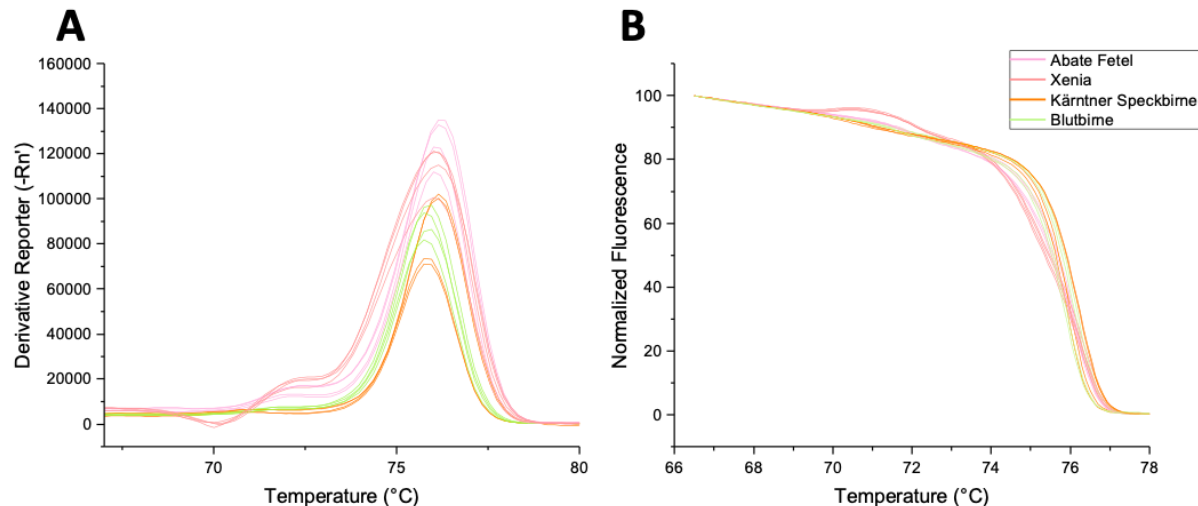


Figure 38 Melting (A) and normalized melting (B) curves of the amplicons of the first and second extracts from certain pear cultivars with primer pair EMPc08. All extracts were analysed in duplicate.

Primer Pair MEMPc111t

With primer pair MEMPc111t, the Ct-values were slightly lower in the first PCR, ranging from 23.2 to 24.3, except for Kärntner Speckbirne (24.8), Kimizukawase (26.0) and Kumoi (25.3). In the second PCR, the Ct-values ranged from 23.2 to 25.2, except for Kimizukawase (27.0) and Kumoi (28.2).

In the first PCR, the peak maximum obtained for all cultivars, except Kumoi and Williams, was at lower temperatures than in the second PCR. The fluorescence signal at the peak maximum was also higher in the first PCR, except for Fred, Cordula, Harovin Sundown, Kumoi, Frühe von Trevoux, Conference, and Williams.

With the third PCR, the normalized melting curves for the replicates overlapped, except for Frühe von Trevoux, which showed the same deviations as when the extracts were applied in two separate PCR runs. Furthermore, the Ct-values of the two extracts from Frühe von Trevoux (24.1; 25.1) still varied.

The melting curves for Cordula, Gute Graue, Conference, and Cepuna had distinct features resulting in complex normalized melting curves (see fig. 39). With primer pair MEMPc111t, Celina and Cepuna could be differentiated from the other pear cultivars.

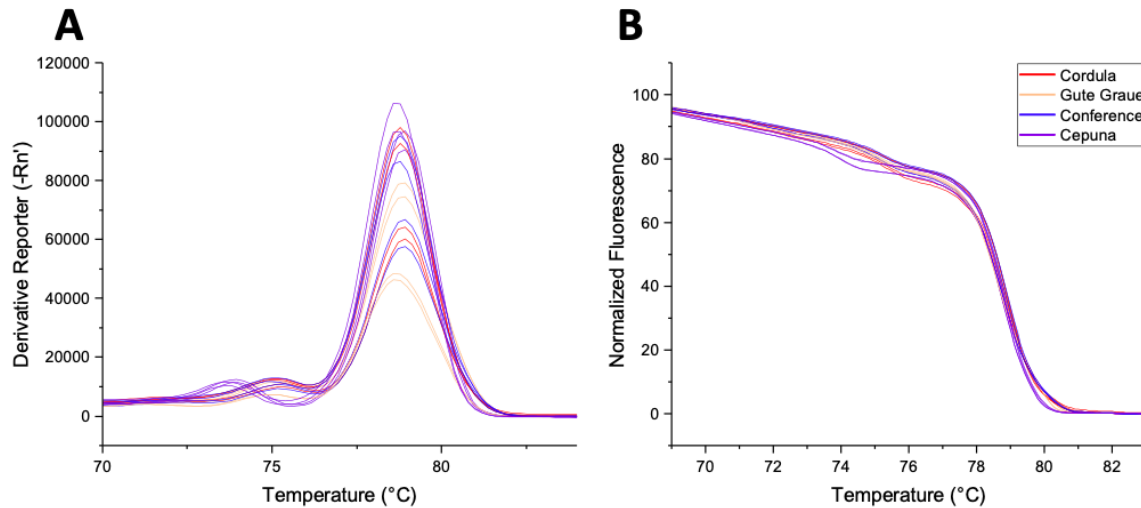


Figure 39 Melting (A) and normalized melting (B) curves of the amplicons of the first and second extracts from certain pear cultivars with primer pair MEMPc111t. All extracts were analysed in duplicate.

Primer Pair MEMPc117

The Ct-values obtained with primer pair MEMPc117 were lower in the first PCR run, ranging from 24.0 to 25.1, except for Kärntner Speckbirne (26.9), Haeng Su (26.0), Kimizukawase (27.1) and Kumoi (26.6), whereas in the second PCR run they ranged from 24.7 to 27.0, except for Haeng Su (29.9), Kimizukawase (30.8) and Kumoi (31.4). The fluorescence signal at the peak maximum was overall higher in the first PCR, except for Celina, Fred, Kumoi and Conference. Regardless, the repeatability between the first and the second PCR/extracts was good.

Although in the third PCR run, differences in the Ct-values between the two DNA extracts from Kärntner Speckbirne (29.1; 27.3), Kumoi (30.1; 34.2), Frühe von Trevoux (25.6; 26.6), and Boscs Flaschenbirne (25; 26.3) were observed and the intensity of the fluorescence signals at the peak maximum obtained for the first extracts were twice as high as for the second extracts, the normalized melting curves for the replicates still overlapped.

The normalized melting curves for Alexander Lukas, Blutbirne, Williams, Boscs Flaschenbirne, and Cepuna had distinct features, corresponding to a complex shape of the normalized melting curve (see fig. 40). With this primer pair, Alexander Lukas, and Cepuna could be differentiated from all other pear cultivars but not from each other; the same applies to Blutbirne, Williams, and Boscs Flaschenbirne. Furthermore, Haeng Su was completely differentiable from the other 17 pear cultivars. Kumoi and Kimizukawase could also be distinguished from all other cultivars, but not from each other.

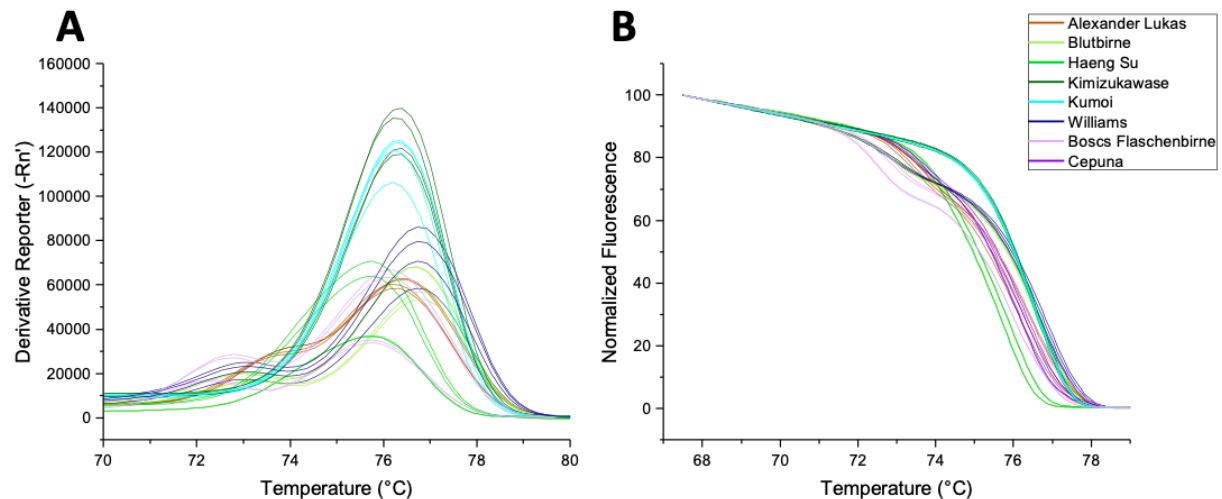


Figure 40 Melting (A) and normalized melting (B) curves of the amplicons of the first and second extracts from certain pear cultivars with primer pair MEMPc117. All extracts were analysed in duplicate.

Primer Pair MGD127

With primer pair MGD127, the Ct-values were in the first PCR run between 26.9 and 29.3, except Celina (34.1). In the second PCR run, the Ct-values were between 27.3 and 30.7, except for Celina (34.8), Haeng Su (33.5) and Kumoi (31.6).

The fluorescence signal at the maximum peak was higher in the first PCR, except for Celina, Fred, Harovin Sundown, and Frühe von Trevoux. The repeatability between the first and the second PCR/extracts was low, because none of the normalized melting curves overlapped. All normalized melting curves obtained in the first PCR were shifted to higher temperatures than those of the second PCR, except for Kumoi which was shifted by about 0.45 °C to lower temperature.

With the third PCR run, the normalized melting curves for the replicates overlapped, except for Gute Graue and Boscs Flaschenbirne. The normalized melting curves for those two cultivars showed the same deviation as when the extracts were analysed in two different PCR runs. Furthermore, the Ct-values for Gute Graue (29.3; 30.5), Kumoi (31.9; 35.7), and Boscs Flaschenbirne (28.2; 29.8) differed strongly between the two extracts and the fluorescence signals at the peak maximum obtained with the first extracts were higher than with the second extracts. The melting curves for Celina, Xenia, Cordula, Gute Graue Kärntner Speckbirne, Haeng Su, Frühe von Trevoux, Williams, and Boscs Flaschenbirne had distinct features, which resulted in the complex normalized melting curves (see fig. 41 B). With primer pair MGD127, Celina, Kärntner Speckbirne, and Haeng Su could be completely differentiated from the other pear cultivars. Frühe von Trevoux could also be differentiated from the other pear cultivars except Boscs Flaschenbirne.

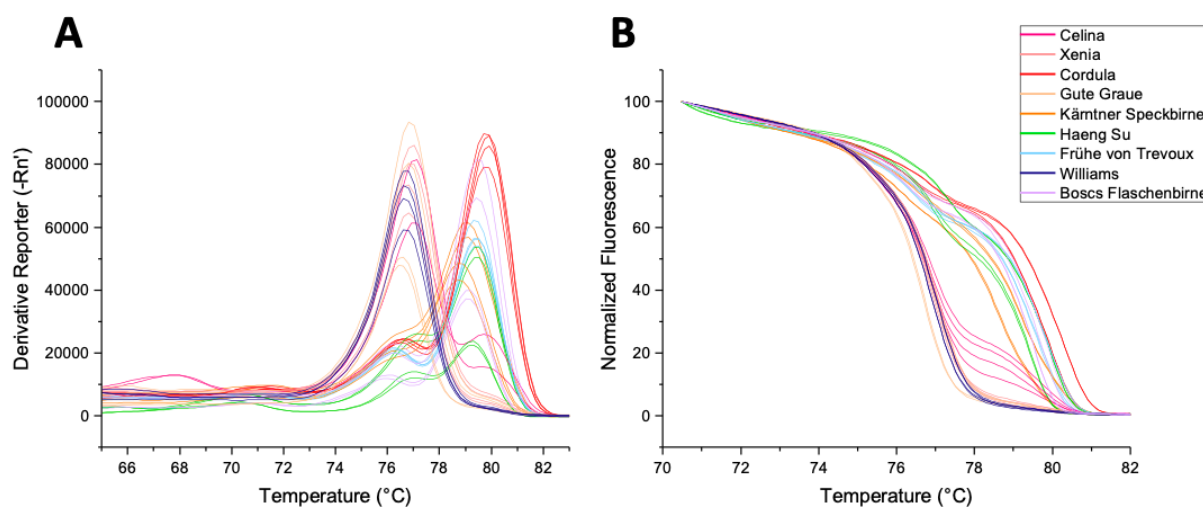


Figure 41 Melting (A) and normalized melting (B) curves of the amplicons of the first and second extracts from certain pear cultivars with primer pair MGD127. All extracts were analysed in duplicate.

Primer Pair MGD154

With primer pair MGD154, the Ct-values were between 23.2 and 25.8 in the first and in the second PCR run, except for Kimizukawase (27.4) and Kumoi (28.2) in the second PCR run.

In the first PCR, the peak maximum was at slightly higher temperatures and the fluorescence signal was also higher than in the second PCR, except for Celina, Fred, Frühe von Trevoux, and Conference. The repeatability of the normalized melting curves between the first and the second PCR/extracts was rather low.

In the third PCR run, the Ct-values still varied between the two extracts from Kärntner Speckbirne (26.5; 24.1) and Kumoi (29.3; 32.3). The melting curves for Kumoi showed the same deviations as when the extracts were analysed in different PCR runs.

The normalized melting curves for Abate Fetel and Frühe von Trevoux had distinct features, which differentiated them from the other cultivars but not from each other (see fig. 42).

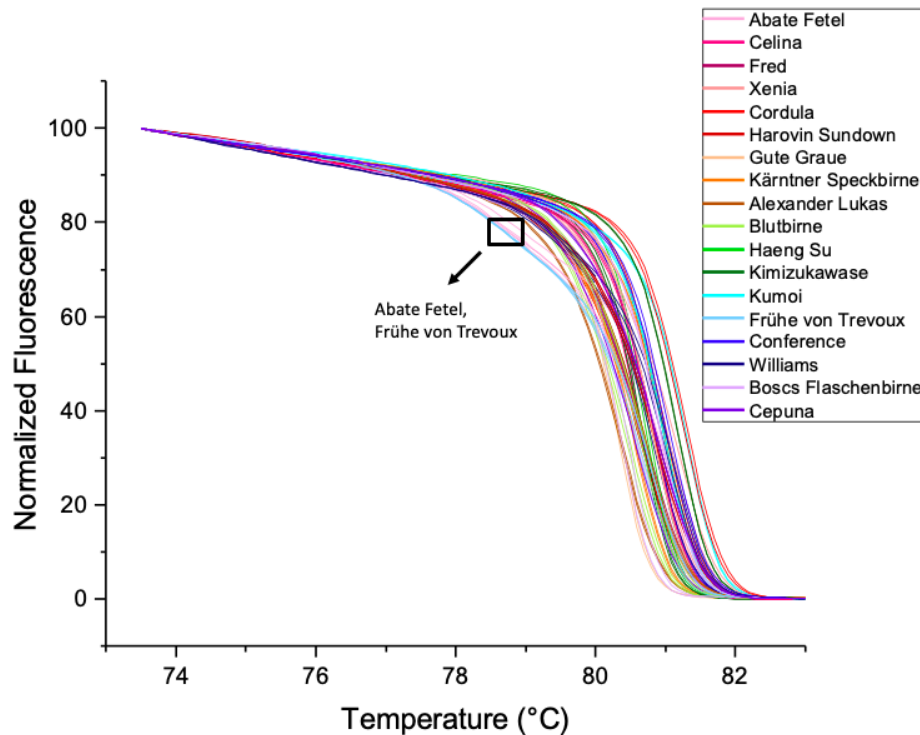


Figure 42 Normalized melting curves of the amplicons of the first and second extracts from the 18 pear cultivars with primer pair MGD127. All extracts were analysed in duplicate.

Primer Pair NH013a

With primer pair NH013a, the Ct-values were between 22.7 and 25.4 in the first PCR run, except for Abate Fetel (26.6), Boscs Flaschenbirne (26.6) and Cepuna (29.9). In the second PCR run, the Ct-values were slightly higher, ranging from 23.3 to 26.5, except for Abate Fetel (28.3), Kimizukawase (27.8), Kumoi (28.6) and Cepuna (31.3). The fluorescence signal at the peak maximum was higher in the first PCR, except for Celina, Fred, and Frühe von Trevoux. The repeatability of the normalized melting curves between the first and the second PCR/extracts was good.

In the third PCR run, differences in the Ct-values between the two extracts from Gute Graue (24.7; 25.9) and Kärntner Speckbirne (26.0; 25.0) were observed. In the third PCR run, the melting curves of both extracts deviated less and most curves even completely overlapped, except for Frühe von Trevoux (see fig. 43). The fluorescence signal at the peak maximum obtained for the first extract was nearly twice as high as that for the second extract. This poor repeatability may be caused by the imperfect microsatellite targeted by the primer pair (see section 6.1.1), resulting in the formation of two different PCR products. However, gel electrophoresis was not carried out to confirm this hypothesis.

With this primer pair, the cultivars Kumoi and Cepuna could be completely differentiated from the other cultivars (see figure 31 in section 6.2.2).

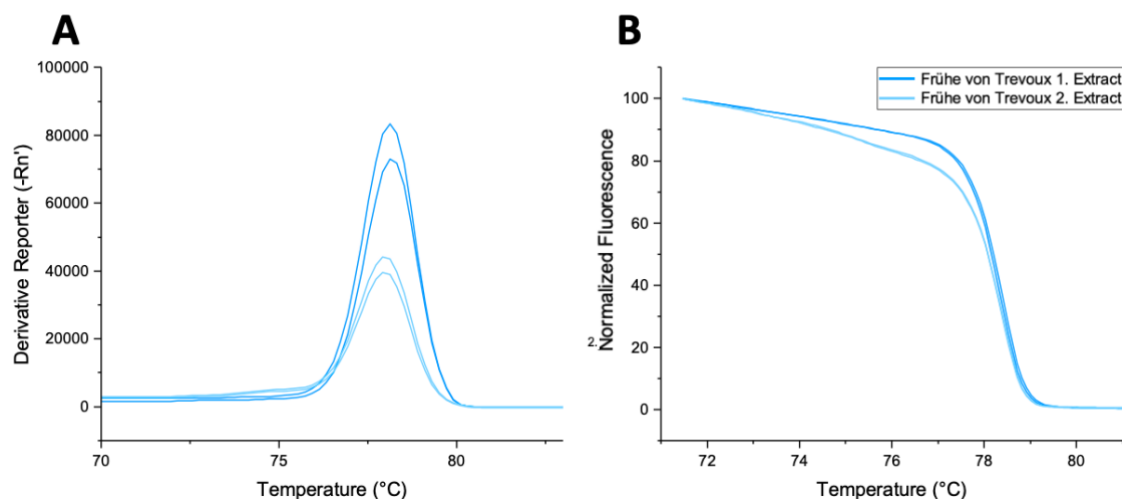


Figure 43 Melting (A) and normalized melting (B) curves of the amplicons of the first and second extracts from Frühe von Trevoux with primer pair NH013a. All extracts were analysed in duplicates.

The HRM results show that PCR products were obtained for all 18 pear cultivars with all six pear primer pairs. In general, the repeatability between the PCR duplicates was good, but the results from the first and second DNA extracts often showed more variation. The normalized melting curves obtained with the primer pairs EMPc08 and MEMPc117 showed good repeatability, since the curves from the replicates overlapped well. However, for several primer pairs, such as MGD127, a few pear cultivars showed more deviating melting curves. It was also noticed that in the first PCR run, the Ct-values were usually slightly lower, and the fluorescence signals and the temperature at the peak maximum were mostly higher than in the second PCR. This could be due to the presence of polysaccharides and secondary metabolites, which can inhibit PCR by inhibiting DNA polymerase [99]. The second extracts probably contained higher amounts of these substances, because of the strong gel formation during DNA extraction (see section 5.2.1 and 6.2.1). This may have resulted in higher Ct-values and lower fluorescence signals.

The DNA extracts from the pear cultivars Gute Graue, Kärntner Speckbirne, Kimizukawase, Kumoi, and Boscs Flaschenbirne showed poor repeatability between the first and second extracts for most primer pairs. In many cases, the deviations could be reduced or even eliminated when both extracts were used together in the same PCR run, although there were some exceptions.

6.2.2.2 Evaluation of HRM data obtained with the nine apple primer pairs

The results of HRM analysis showed, that with the nine apple primer pairs amplicons were successfully obtained for most of the 18 pear cultivars. The Ct-values of the extracts were usually between 23.0 and 30.0 with some exceptions.

Primer Pair CH01f12

With primer pair CH01f12, the Ct-values obtained in the two PCR runs were very similar. In the first PCR run, they were between 24.1 and 26.7, and in the second PCR run between 24.4 and 26.9, except for Haeng Su (29.9) and Kimizukawase (29.1).

In the first PCR run, the peak maximum was at higher temperatures than in the second, except for Harovin Sundown and Kumoi. The fluorescence signals at the peak maximum were also higher in the first PCR, except for Celina, Fred, Cordula, Kumoi, Kimizukawase, Frühe von Trevoux, and Conference. The repeatability between the first and the second PCR/extracts was not good, but repeatability could be increased by analysing the extracts together in the third PCR run, except for Gute Graue and Kärntner Speckbirne (see fig. 44). Differences in the Ct-values between first and second extract were observed for Gute Graue (24.3; 25.8), Kärntner Speckbirne (26.2; 25.5),

Kimizukawase (30.8; 31.5) Kumoi (29; 32.3), and Boscs Flaschenbirne (24.7; 26.4). In general, higher fluorescence signals at the peak maximum were obtained for the first extracts.

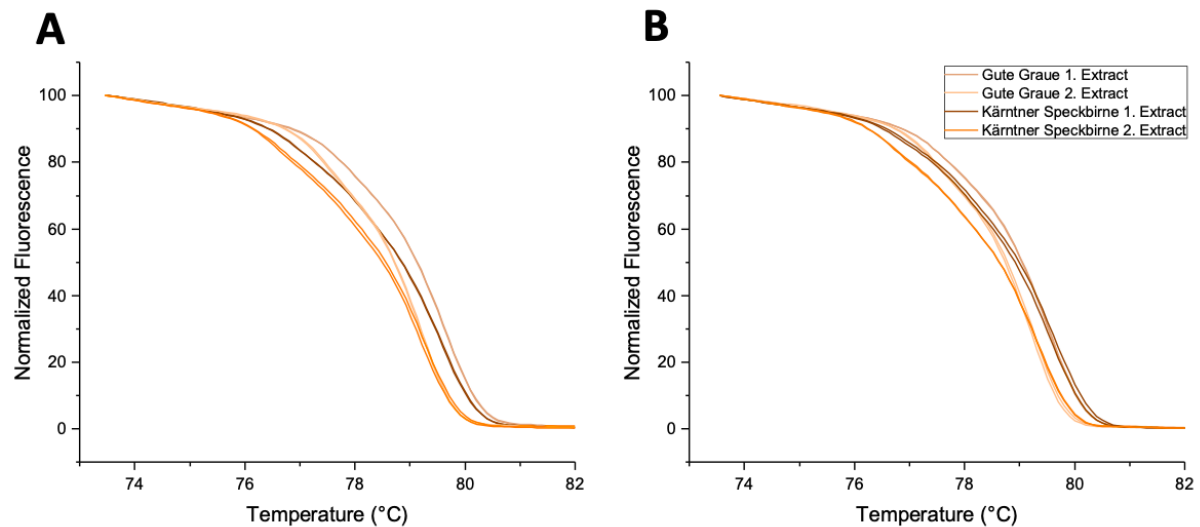


Figure 44 Normalized melting curves of the amplicons of the first and second extracts from Gute Graue and Kärntner Speckbirne with primer pair CH01f12. The extracts were applied A) in two separate PCR runs and B) in the same PCR run. All extracts were analysed in duplicate.

With primer pair Ch01f12, the cultivars Kärntner Speckbirne and Alexander Lukas could be differentiated from the other pear cultivars, except for Gute Graue. This primer pair also enabled differentiation of Kumoi and Cepuna from the other cultivars, except from Kimizukawase (see figure 45).

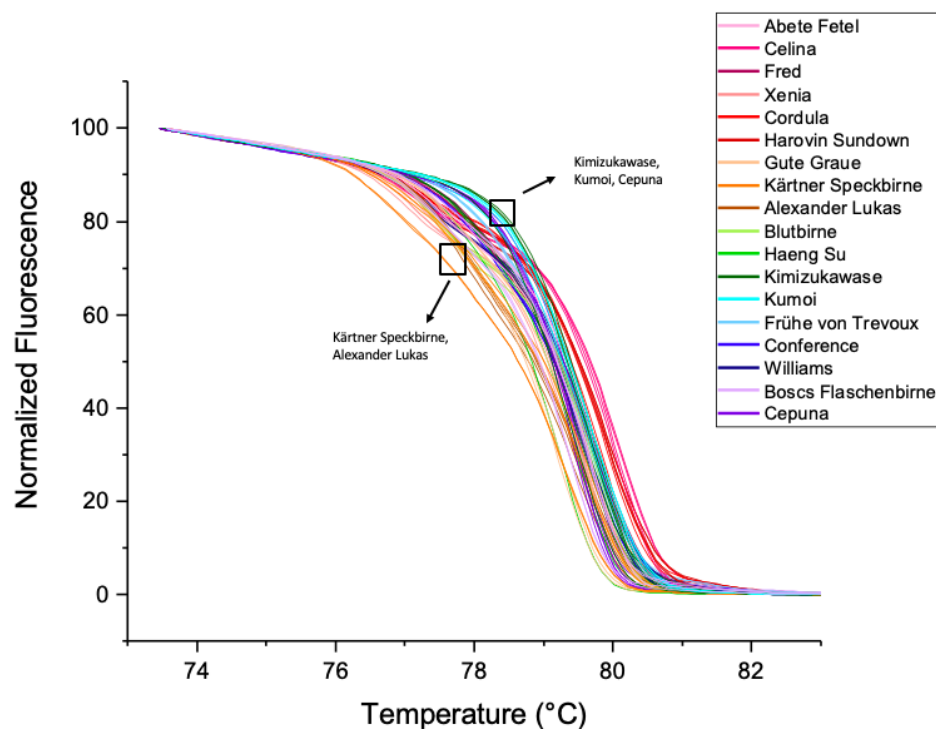


Figure 45 Normalized melting curves of the amplicons of the first and second extracts from the 18 pear cultivars with primer pair Ch01f12. All extracts were analysed in duplicate.

Primer Pair CH01h01

With primer pair Ch01h01, the Ct-values were between 24.1 and 26.2 in the first PCR run, and between 23.5 and 25.9 in the second, except Haeng Su (29.1), and Kumoi (27.5).

In the first PCR, the peak maxima were observed at higher temperatures than in the second PCR run. Furthermore, the fluorescence signal at the peak maximum was higher in the first PCR, except for Harovin Sundown, Kumoi, and Conference.

The repeatability between the first and the second PCR/extracts was not good. In addition, the normalized melting curves for all cultivars except Kimizukawase and Kumoi had the same curve shape and the melting temperatures were also very similar (see figure 46).

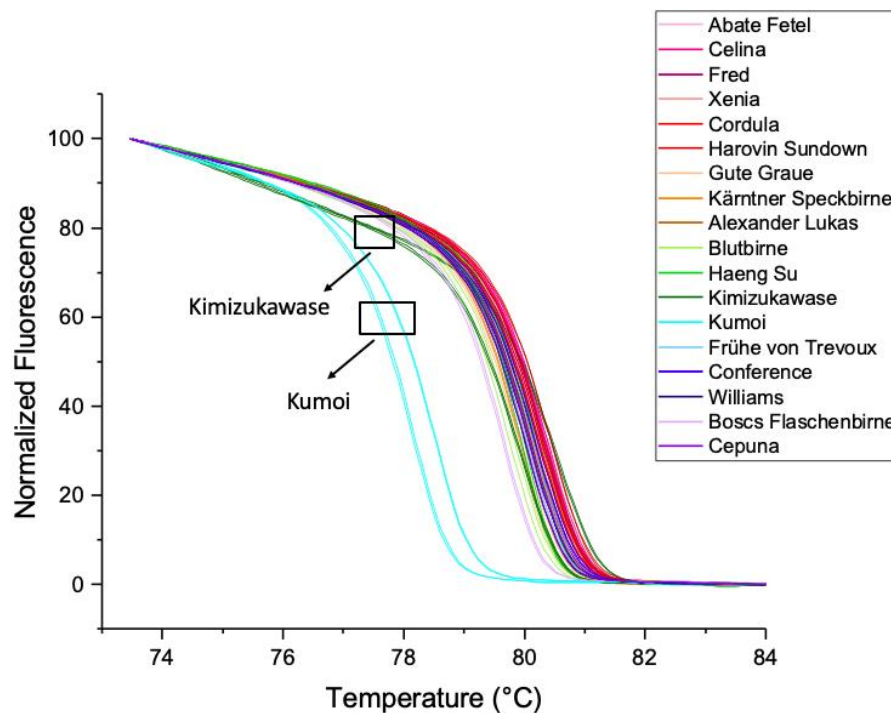


Figure 46 Normalized melting curves of the amplicons of the first and second extracts from the 18 pear cultivars with primer pair Ch01h01. All extracts were analysed in duplicate.

With this primer pair, only Kimizukawase and Kumoi could be distinguished from the other pear cultivars. Due to the limited discriminatory power, this primer pair was not used in further experiments.

Primer Pair CH01h10

With primer pair CH01h10, the Ct-values were very similar between the two PCR runs. In the first PCR, the Ct-values were between 23.3 and 26.4, except for Boscs Flaschenbirne (38.6), and in the second, between 23.2 and 26.8, except for Kumoi (28.1) and Boscs Flaschenbirne (40.5).

In the first PCR, the peak maxima were observed at higher temperatures, except for Blutbirne and Haeng Su, but the fluorescence signal at the peak maxima was higher in the second PCR, except for Abate Fetel, Gute Graue, Kärntner Speckbirne, Haeng Su, and Frühe von Trevoux.

The repeatability between the first and the second PCR/extracts was rather low. The normalized melting curves for the replicates of Boscs Flaschenbirne showed some variations (see fig. 47). But due to their complex shape, Boscs Flaschenbirne could still be clearly distinguished from the other pear cultivars (see fig. 48).

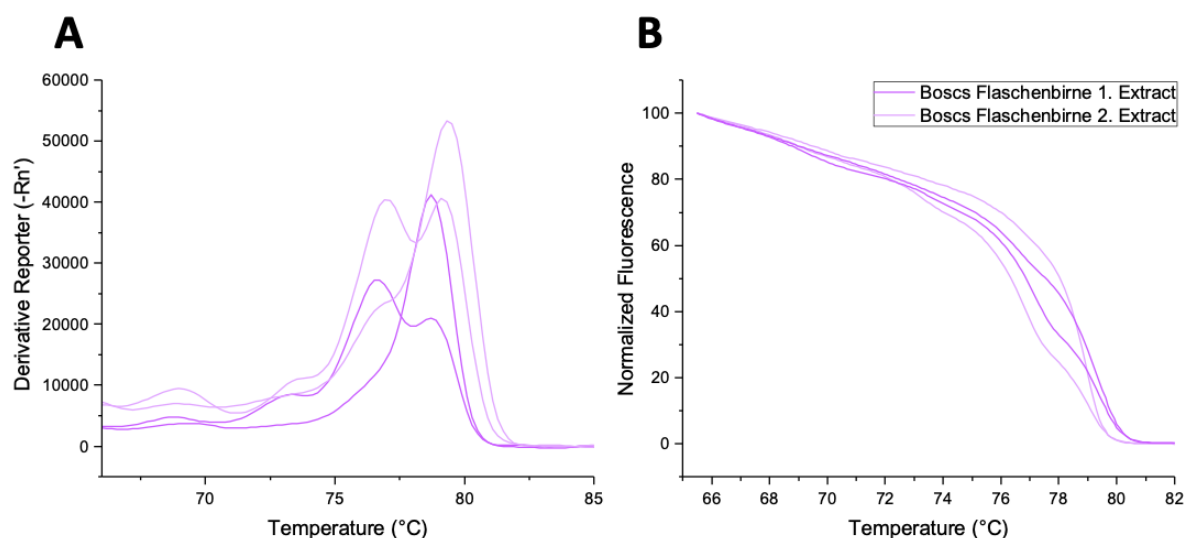


Figure 47 Melting (A) and normalized melting (B) curves of the amplicons of the first and second extracts from Boscs Flaschenbirne with primer pair CH01h10. All extracts were analysed in duplicates.

With this primer pair, the three Asian pears (Haeng Su, Kumoi and Kimizukawase) could be distinguished from all European pears, but not from each other. Cordula and Cepuna could be distinguished from all other pears, except Conference. Amplicons for Abate Fetel to Williams (sample 1–16), except Cordula, Haeng Su, Kimizukawase, Kumoi, and Conference (sample 5, 11, 12, 13, 15) had the same melting temperature and resulted in identical curve shapes and could therefore not be differentiated from each other (see fig. 48).

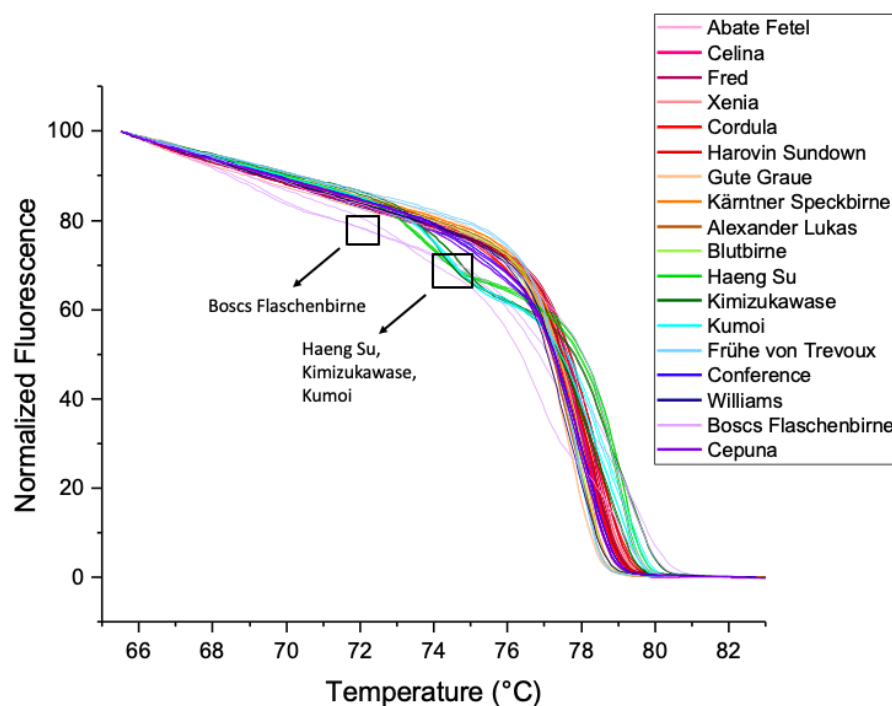


Figure 48 Normalized melting curves of the amplicons of the first and second extracts from the 18 pear cultivars with primer pair Ch01h10. All extracts were analysed in duplicate.

Primer Pair CH02b03b

In general, the Ct-values obtained with primer pair CH02b03b in the first PCR run were lower than those in the second PCR run. The Ct-values were between 27.1 and 28.9 in the first PCR, except for Frühe von Trevoux (29.5)

and Celina (38.9), and between 27.5 and 30.0 in the second, except for Celina (42.0), Haeng Su (36.1), and Boscs Flaschenbirne (31.3).

The fluorescence signals at the peak maximum were higher in the first PCR run than in the second, except for Celina, Fred, Cordula, Harovin Sundown, Conference, and Boscs Flaschenbirne. The temperature at the peak maximum was also higher in the first PCR, except for Fred, Kärntner Speckbirne, Frühe von Trevoux, and Williams. The repeatability between the first and the second PCR/extracts was good, except for the extracts from Fred. Even when both DNA extracts from Fred were analysed together in the third PCR, different Ct-values (39.5; 41.6), melting and normalized melting curves were obtained (see fig. 49). The melting curves obtained for the first DNA extract had two peaks (at 72.5 °C and 76 °C), whereas the second extract only resulted in one peak (at 77.5 °C, see fig. 49 A).

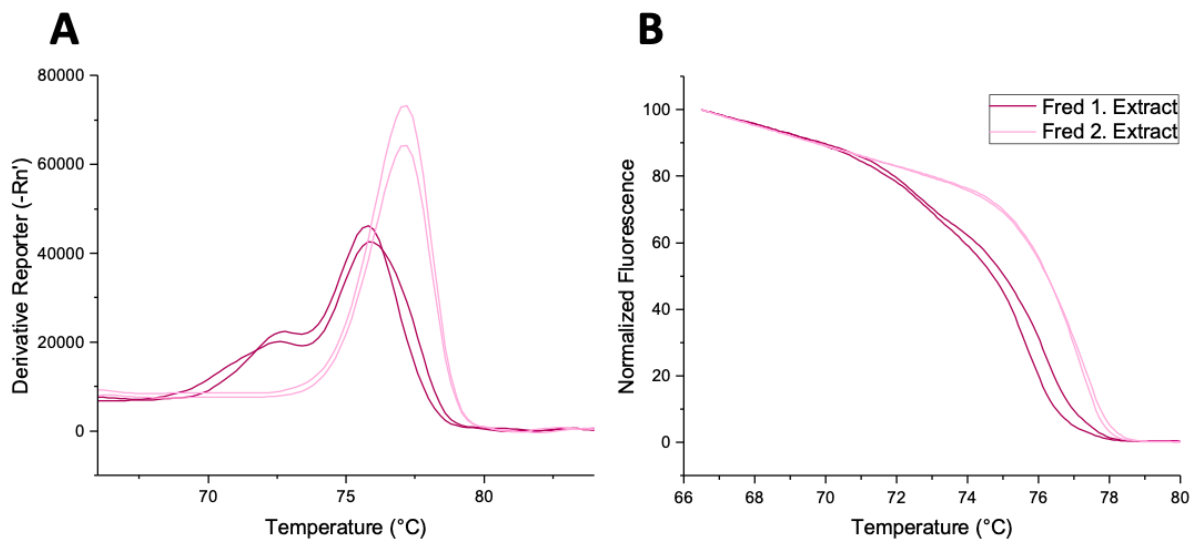


Figure 49 Melting (A) and normalized melting (B) curves of the amplicons of the first and second extracts from Fred with primer pair CH02b03b. All extracts were analysed in duplicates.

A previous study reported that the optimal amplicon length for PCR-HRM is between 100 to 300 bp. [74] According to the NCBI database, the primer pair CH02b03b produced an amplicon of 93 bp [98], which was the shortest amplicon length used in this study. No further investigations (e.g. gel electrophoresis) were performed to identify the reason for the low repeatability.

In the third PCR, the Ct-values still showed strong variation between the replicates from Kumoi (31.9; 36.1) and Frühe von Trevoux (29.9; 31.8), but their normalized melting curves overlapped.

With this primer pair, Haeng Su, Kimizukawase, Williams, and Boscs Flaschenbirne could be clearly differentiated from the other pear cultivars. It also allowed the differentiation of Gute Graue and Alexander Lukas from all other cultivars, but not from each other (see fig. 50). Fred was excluded from the data evaluation due to the inconsistent results.

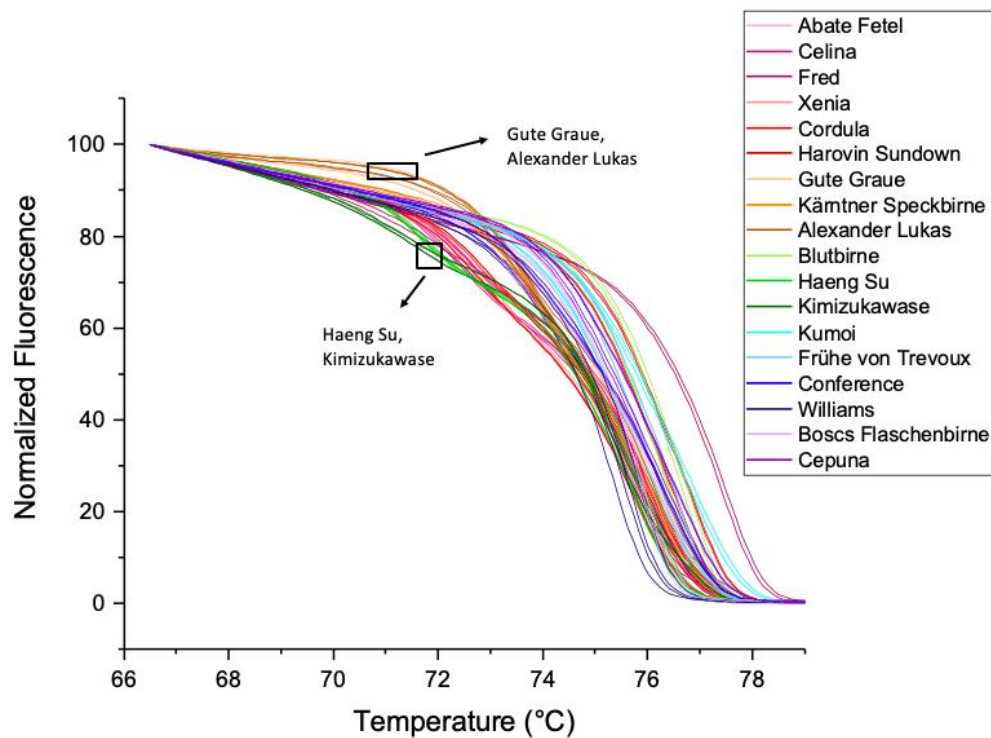


Figure 50 Normalized melting curves of the amplicons of the first and second extracts from the 18 pear cultivars with primer pair Ch02b03b. All extracts were analysed in duplicate.

Primer Pair CH02b10

With primer pair CH02b10, all Ct-values obtained in the first PCR were lower than those obtained in the second PCR, except for Cordula. In the first PCR, the Ct-values were between 26.5 and 29.0, except for Abate Fetel (31.4), Harovin Sundown (31.0), Kärntner Speckbirne (30.5), and Blutbirne (29.8) and in the second PCR, between 26.2 and 30.1, except for Abate Fetel (33.3), Harovin Sundown (31.9), Kärntner Speckbirne (31.6), Blutbirne (31.5), and Boscs Flaschenbirne (31.2).

In the first PCR, the peak maximum was at a higher temperature than in the second PCR, except for Blutbirne, Kumoi, and Williams. Nevertheless, the repeatability of the normalized melting curves between the first and the second PCR/extracts was good. By combined analysis of the first and second extracts in the third PCR run, the repeatability of the melting curves increased, except for Gute Graue and Kärntner Speckbirne. In addition, Ct values for Gute Graue (26.2; 28.1), Kumoi (29.9; 33.9), and Boscs Flaschenbirne (28.2; 31) differed.

Based on the normalized melting curves, the primer pair CH02b10 allowed clear differentiation of Haeng Su, Kimizukawase, and Cepuna from all other pear cultivars (see fig. 51). It could also differentiate Conference from all other pear cultivars except Cordula.

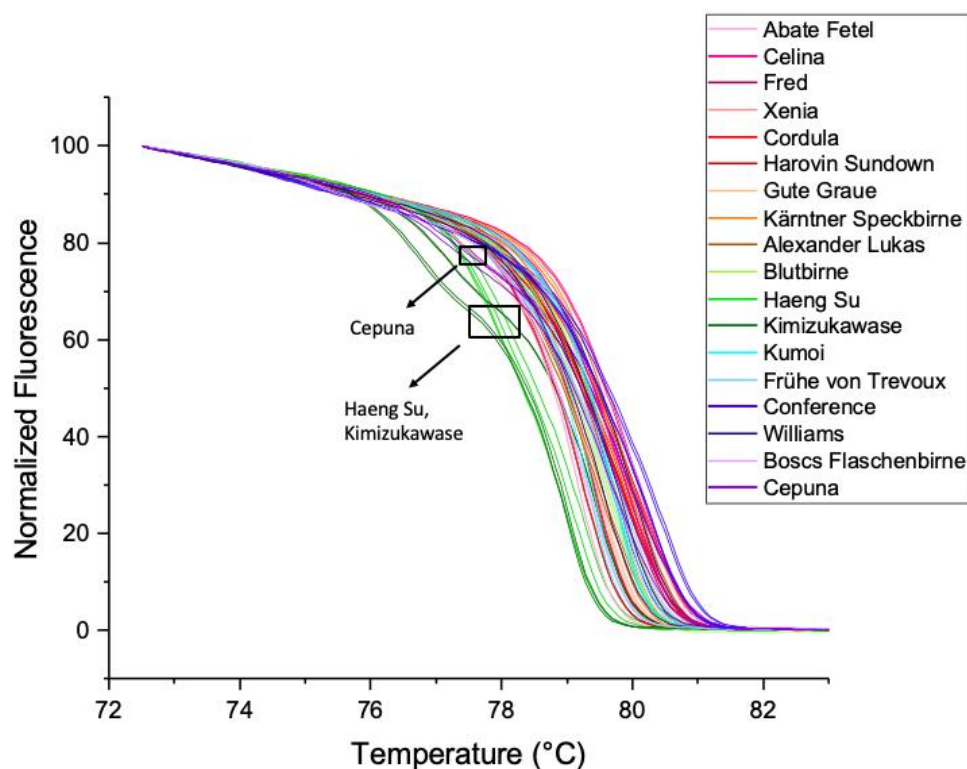


Figure 51 Normalized melting curves of the amplicons of the first and second extracts from the 18 pear cultivars with primer pair Ch02b10. All extracts were analysed in duplicate.

Primer Pair CH02h11a

The Ct-values obtained with primer pair CH02h11a in the first and second PCR ranged from 23.5 to 25.8, except for Haeng Su (28.7), Kimizukawase (26.4), and Kumoi (27.0) in the second PCR.

In the first PCR, the temperature at the peak maximum was higher, except for Kumoi and Williams. The repeatability of the normalized melting curves between the first and second PCR/extracts was quite poor.

However, in the third PCR, the variations between the replicates were reduced, but they remained for Gute Graue and Kärntner Speckbirne. In addition, the Ct-values for both DNA extracts from Gute Graue (23.8; 25.4) and Boscs Flaschenbirne (24; 25.6) differed strongly, and higher fluorescence signals were obtained for the first extracts than for the second extracts.

This primer pair allows the differentiation of Kärntner Speckbirne, Alexander Lukas, and Blutbirne from the other pear cultivars except from each other.

Primer Pair CH03a09

With primer pair CH03a09, the Ct-values in the first PCR were between 23.1 and 25.2, and in the second PCR between 22.8 and 24.7, except for Alexander Lukas (25.4), Kimizukawase (25.9), Kumoi (26.1), and Blutbirne (27.0). The repeatability of the normalized melting curves between the replicates was good. However, the extracts from three pear cultivars (Gute Graue, Kimizukawase and Kumoi) were still tested in a third PCR. It was observed that the Ct-values for Gute Graue (23.5; 24.6), Kimizukawase (25.6; 27.2) and Boscs Flaschenbirne (24.4; 25.3) differed greatly between the two DNA extracts. Due to the differences in the temperature at the peak maximum between the first (80.4 °C) and the second (79.7 °C) extract from Kimizukawase another dilution factor for the extracts from Kimizukawase and Kumoi was tested, as mentioned in section 6.2.2.

With this primer pair Haeng Su, Kimizukawase and Kumoi could be differentiated from all other pear cultivars. The normalized melting curves of the extracts from Abate Fetel, Celina, Frühe von Trevoux, Williams, Boscs Flaschenbirne and Cepuna were very similar and could not be differentiated (see fig. 52).

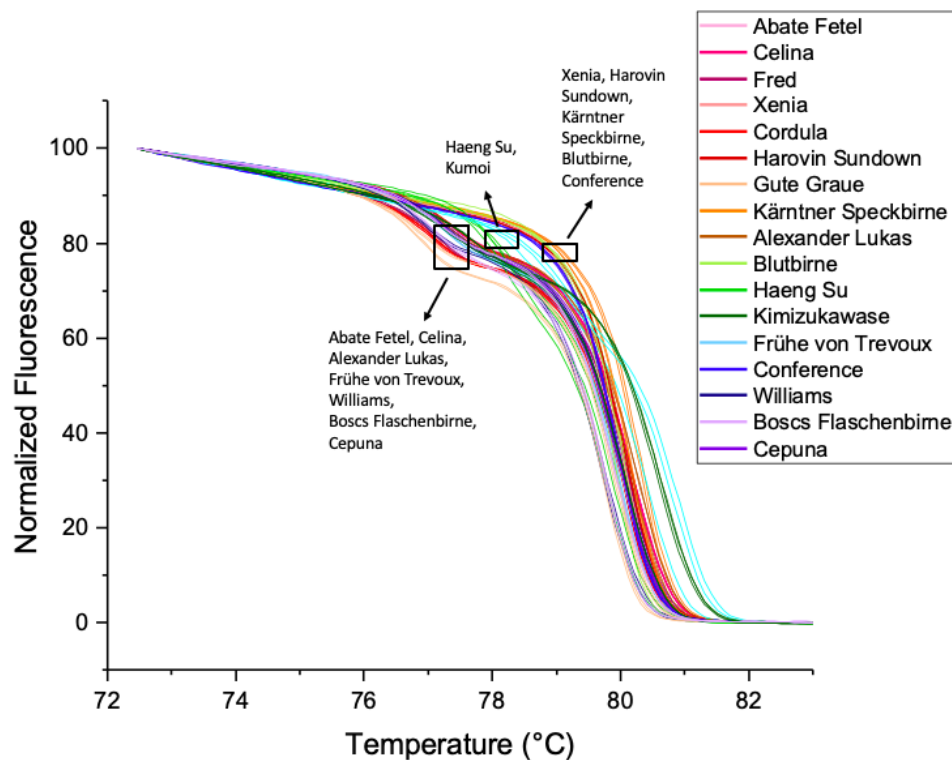


Figure 52 Normalized melting curves of the amplicons of the first and second extracts from the 18 pear cultivars with primer pair Ch03a09. All extracts were analysed in duplicate.

Primer Pair CH03c02

In the first PCR run carried out with primer pair CH03c02, the Ct-values were between 27.1 and 28.9, and in the second PCR between 27.1 and 30.0, except for Boscs Flaschenbirne (30.7).

The fluorescence signal at the peak maximum was higher in the first PCR than in the second, except for Fred, Cordula, Kärntner Speckbirne, and Frühe von Trevoux. In the case of all 18 pear cultivars, in both PCR runs the temperature at the peak maximum was between 77.1 °C and 77.8 °C. The repeatability of the normalized melting curves between the replicates was not good. In addition, for all pear cultivars except Abate Fetel, similar normalized melting curves were obtained (see fig. 53).

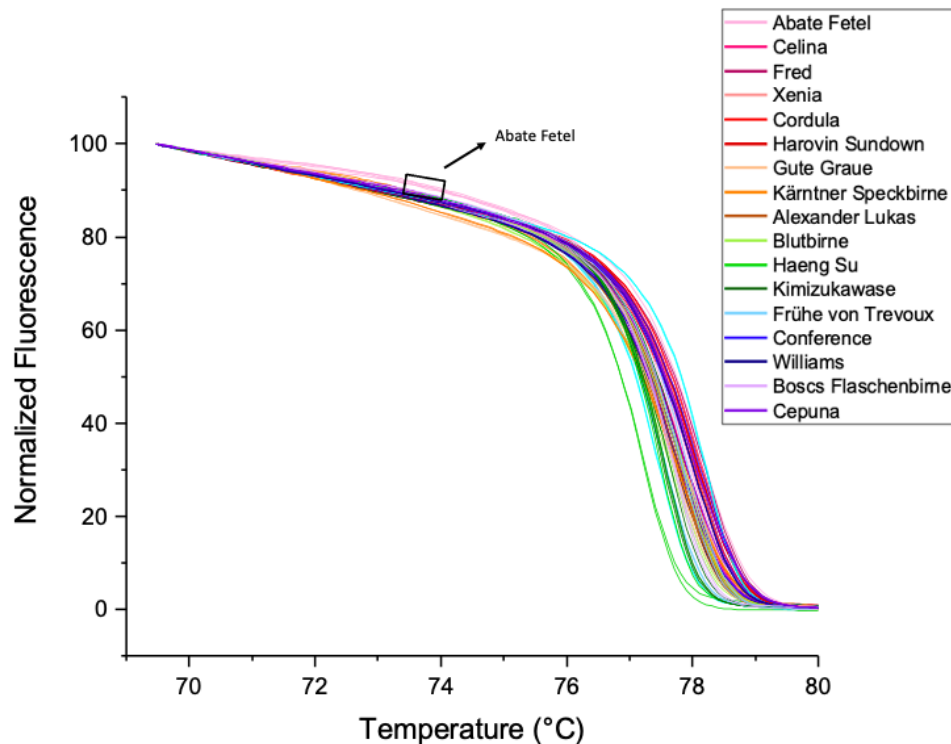


Figure 53 Normalized melting curves of the amplicons of the first and second extracts from the 18 pear cultivars with primer pair Ch03c02. All extracts were analysed in duplicate.

It has to be noted that the sequences of forward and reverse primer differed slightly (four bases) from the NCBI sequences. During the primer tests (see section 6.1.2), this primer pair did not allow the differentiation of the pear cultivars Williams, Anjou, and Cheeky, but was nonetheless applied to the whole set of cultivars. Due to the poor repeatability and the low discriminatory power, it was considered unsuitable for differentiation of the 18 pear cultivars.

Primer Pair CH04c07

Primer pair CH04c07 resulted in quite different Ct-values for the pear cultivars investigated. This holds true for both PCR runs. The Ct-values for Abate Fetel, Xenia, Gute Graue, Kimizukawase, Kumoi, and Boscs Flaschenbirne were between 26.2 and 28.3, whereas those for Fred, Cordula, Harovin Sundown, Frühe von Trevoux, Conference, Williams, and Cepuna ranged from 33.3 to 41.6. The Ct-values for Celina (40.9; 41.4), Blutbirne (47.2; 48.1), and Haeng Su (41.3; 48.1) were even higher. For all 18 pear cultivars, quite complex normalized melting curves were obtained (see fig. 54).

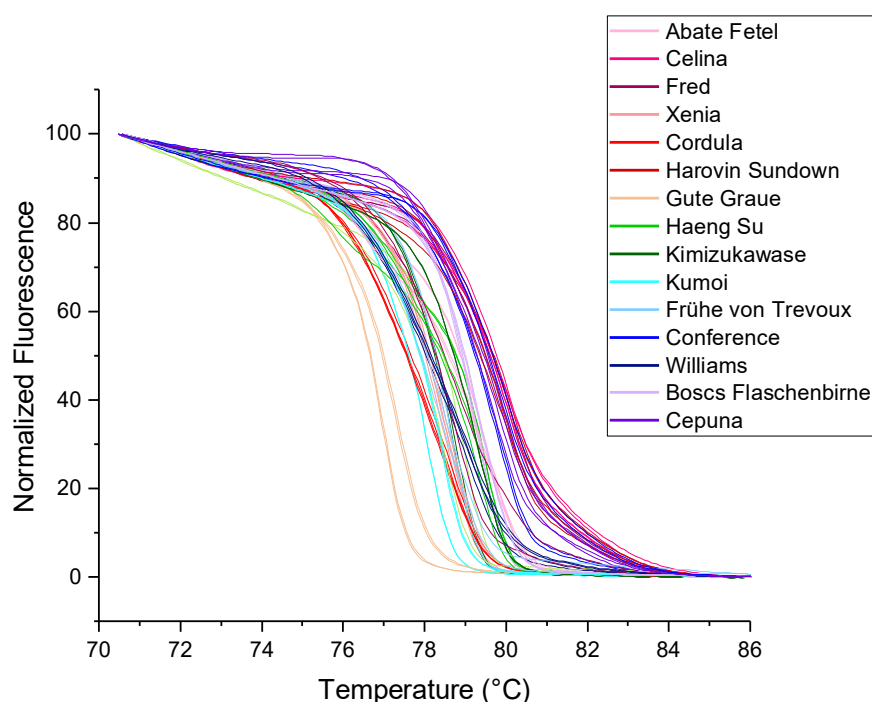


Figure 54 Normalized melting curves of the amplicons of the first and second extracts from the 18 pear cultivars, except for Kärntner Speckbirne, Alexander Lukas, and Blutbirne, with primer pair CH04c07. All extracts were analysed in duplicate.

Due to several limitations, including unsuccessful amplification for Kärntner Speckbirne, Alexander Lukas, and Blutbirne, high Ct-values and low repeatability (see fig. 55), this primer pair was considered unsuitable and was not used further on.

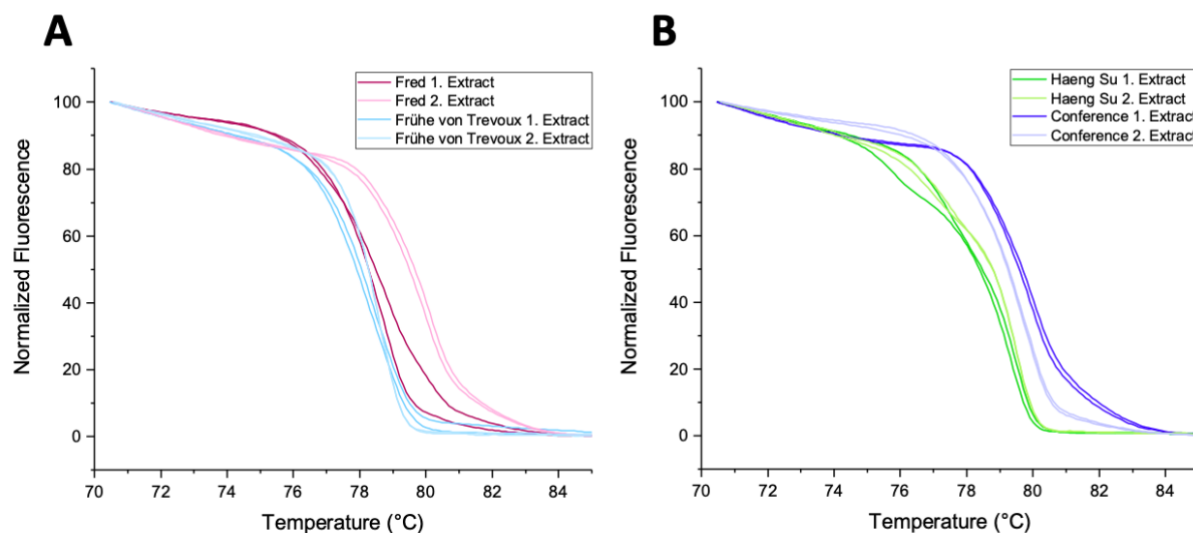


Figure 55 Melting curves of the amplicons obtained with primer pair CH04c04 for the first and the second extract of A) Fred and Frühe von Trevoux and B) Haeng Su and Conference. All extracts were analysed in duplicate.

The results of the HRM analysis indicate that apple primer pairs can be used to obtain PCR products for *Pyrus communis* and *Pyrus pyrifolia*. However, with primer pair CH04c07, DNA from three pear cultivars was not amplified, and therefore, this primer pair was considered less suitable for the analysis of *Pyrus*. With the apple primer pair CH02b03b, different melting curves were obtained for the two extracts from Fred, and the same was observed for the primer pair CH01h10 for Boscs Flaschenbirne. The apple primer pairs CH01h01 and CH03c02 were considered unsuitable for the differentiation of pear cultivars, because they produced very similar normalized melting curves

for most cultivars. In general, the Ct-values obtained with apple primer pairs were slightly higher than those obtained with pear primer pairs, but they were especially high with CH04c07.

Repeatability between PCR duplicates was overall good, but not between the two DNA extracts. Primer pairs CH02b10, CH02b03b, and CH03a09 showed especially good repeatability, since most of the normalized melting curves from the replicates overlapped. However, many normalized melting curves, especially those obtained with primer pair CH02h11a, still showed differences between the two DNA extracts for certain cultivars. The DNA extracts from the pear cultivars Gute Graue, Kärntner Speckbirne, Kimizukawase, Kumoi, and Boscs Flaschenbirne showed poor repeatability between the first and the second extracts with most primer pairs. However, in general, by applying the extracts in the same PCR run, the deviations were greatly reduced.

In addition, it was observed that in most cases, the first PCR resulted in lower Ct-values, higher temperatures at the peak maximum, and higher fluorescence signals for all cultivars than the second PCR.

6.2.3 Differentiation of 18 pear cultivars with 15 primer pairs

Fifteen primer pairs were used to differentiate the 18 pear cultivars, including nine apple and six pear primer pairs (see table 12 and 13). The normalized melting curves of the amplicons from all 18 cultivars were compared based on results from the first and the second DNA extraction (each analysed in PCR duplicates).

Cultivars were considered differentiable when the shape of the normalized melting curve was different and/or when the curve was shifted to a higher or lower temperature (with a temperature difference $> 0.4^{\circ}\text{C}$), leading to a clear differentiation.

Table 15 summarizes the discriminatory power of the PCR assays. It shows the percentage of pear cultivars that could be differentiated with the respective primer pair. Primer pairs CH01h01, CH03c02, and CH04c07 are not included in the list, because they were found to be unsuitable and were therefore not examined further (see section 6.2.2).

Table 15 Percentage of differentiable pear cultivars

Primer pairs	Apple/ pear primer	Differentiable [%]	Non differentiable [%]
MEMPc111t	Pear	87.6	12.4
EMPc08	Pear	86.9	13.1
CH01f12	Apple	85.0	14.5
CH02b10	Apple	84.3	15.7
CH02h11a	Apple	81.7	18.3
CH02b03b	Apple	81.7	18.3
MGD127	Pear	81.7	18.3
CH03a09	Apple	77.8	22.2
NH013a	Pear	74.5	25.5
MEMPc117	Pear	73.9	26.1
MGD154	Pear	70.9	29.1
CH01h10	Apple	62.1	37.9

The pear primer pair MEMPc111t turned out to be the most suitable one, because it showed the highest discriminatory power, allowing differentiation of 87.6 % of the 18 pear cultivars tested. Figure 56 shows the normalized melting curves for the 18 pear cultivars from the first and second extraction, which were used for the differentiation.

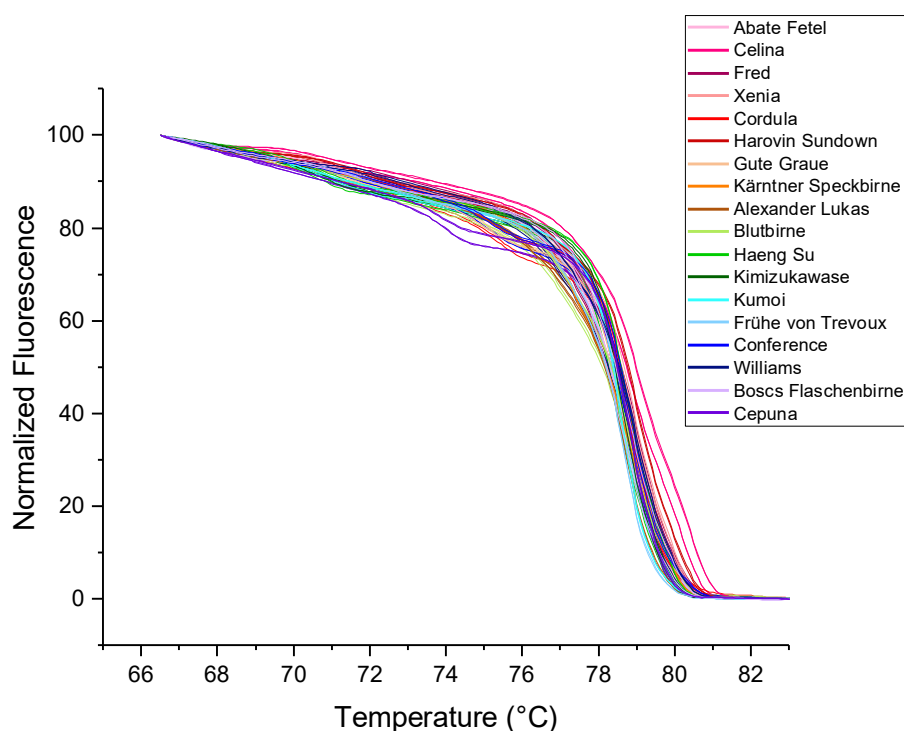


Figure 56 Normalized melting curves of the amplicons of the first and second extracts from the 18 pear cultivars with primer pair MEMPc111t. All extracts were analysed in duplicate.

In the Excel table (figure 57), the 18 pear cultivars are listed both horizontally and vertically. Green markings indicate that the cultivars could be differentiated, while red markings show where they could not be distinguished with primer pair MEMPc111t. With this primer pair, the pear cultivars Celina (sample 2) and Cepuna (sample 18) could be clearly differentiated from all other cultivars. However, MEMPc111t was not able to differentiate all 18 pear cultivars, as indicated by red markings in the table.

		Cultivars																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Cultivars	1																		
	2																		
	3																		
	4																		
	5																		
	6																		
	7																		
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	14																		
	15																		
	16																		
	17																		
	18																		

Figure 57 Differentiation of the 18 pear cultivars using the primer pair MEMPc111t. Green marking indicates that the two cultivars can be differentiated, whereas red markings indicate non differentiable between the cultivars.

The discriminatory tests were carried out with a total of 15 primer pairs. Figure 58 shows that all 18 pear cultivars could be completely differentiated by combining the results from the primer pairs MEMPc111t, CH02h11a, and EMPc08. With MEMPc111t, 87.6 % of the pear cultivars were differentiated. The remaining 12.4 % of cultivars could be distinguished using CH02h11a (9.8 %) and EMPc08 (2.6 %).

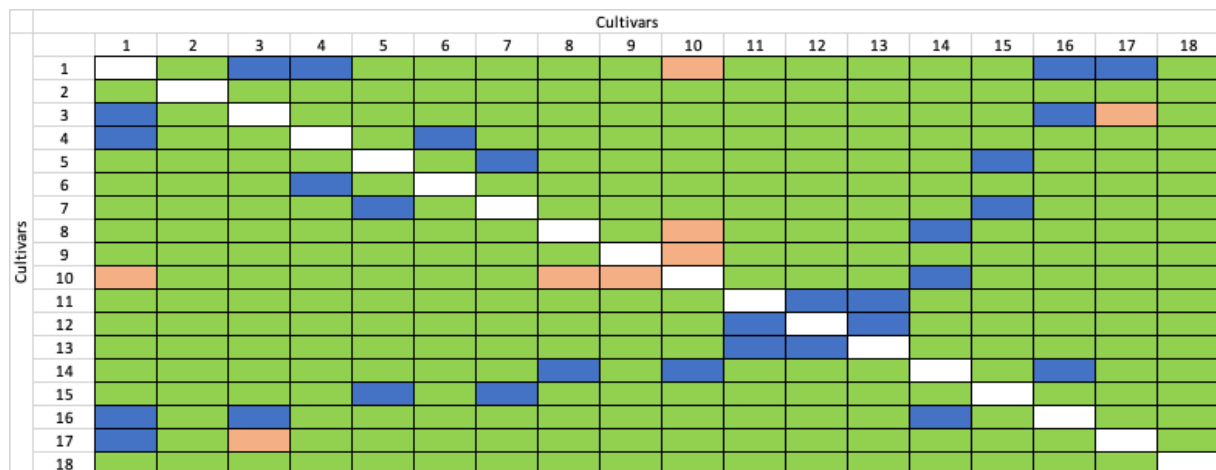


Figure 58 Differentiation of all 18 pear cultivars with the primer pairs MEMPc111t (green), CH02h11a (blue) and EMPc08 (orange). The colours indicate with which primer pair the two cultivars can be differentiated.

6.2.4 Differentiation between pear cultivars of similar origin

It was of great interest to find out, if pear cultivars of similar origin, such as those from the same species or similar crossings, showed similarities in their normalized melting curves.

Asian pears (Haeng Su, Kimizukawase, Kumoi)

Haeng Su, Kimizukawase, and Kumoi are the only Asian pears (*Pyrus pyrifolia*) among the 18 pear cultivars analysed. In this study, the focus was mainly on traditional European pear cultivars, which is why only primer pairs for *Pyrus communis* were chosen during search. Furthermore, the primer sequences were tested with NCBI only for *Pyrus communis*.

In earlier studies, primer pairs targeting microsatellites were developed from *Pyrus pyrifolia* and were also successfully used with *Pyrus communis*. Around 75 % of the microsatellite sequences derived from *Pyrus pyrifolia* are polymorphic in *Pyrus communis* and can therefore be used to distinguish between cultivars of *Pyrus communis*. In recent years, microsatellite primer pairs have also been developed from *Pyrus communis*. [85] However, it is still unclear if primer pairs developed for *Pyrus communis* can be successfully used with *Pyrus pyrifolia*.

The PCR results showed that PCR products were obtained for all three *Pyrus pyrifolia* cultivars with all 15 primer pairs (apple and pear). Furthermore, the three Asian pears could be completely differentiated from the 15 European pears and also from each other using primer pair CH03a09 (see fig. 59 D). With the primer pairs MEMPc111t and CH01h10 (fig. 60 A and 60 C), the three pears could be differentiated from the 15 European pear cultivars. but not from each other.

Primer pairs CH02b03b and CH02b10 were able to differentiate Haeng Su and Kimizukawase, and primer pair CH01h01 could differentiate Kimizukawase and Kumoi from all other pear cultivars (see fig. 59). Primer pairs EMPc08 and MEMPc117 could differentiate Kimizukawase and Kumoi from all other pear cultivars, but not from each other. With primer pairs MEMPc117 and MGD127, Haeng Su, and with primer pair NH013a, Kumoi could be differentiated from all other pear cultivars.

These results show that *Pyrus communis* and *Pyrus pyrifolia* differ genetically, which allows their differentiation. According to the literature, European and Asian pear cultivars differ in their morphological characteristics, molecular markers, and genotypes, which enables differentiation. [20]

However, the differentiation between the Asian pears, especially Kimizukawase and Kumoi, was more difficult. Only the apple primer pairs CH01h01, CH02b10, CH02b03b, and CH03a09 were able to distinguish the three Asian pears (see figure 59 A to D). For primer pair CH01h01, the two extracts were only analysed separately in two

different PCR runs. Therefore, differences in the PCR conditions may have caused deviations between the normalized melting curves of the first and second PCR. A third PCR was not performed due to the similar normalized melting curves between the European pear cultivars and Haeng Su (see section 6.2.2).

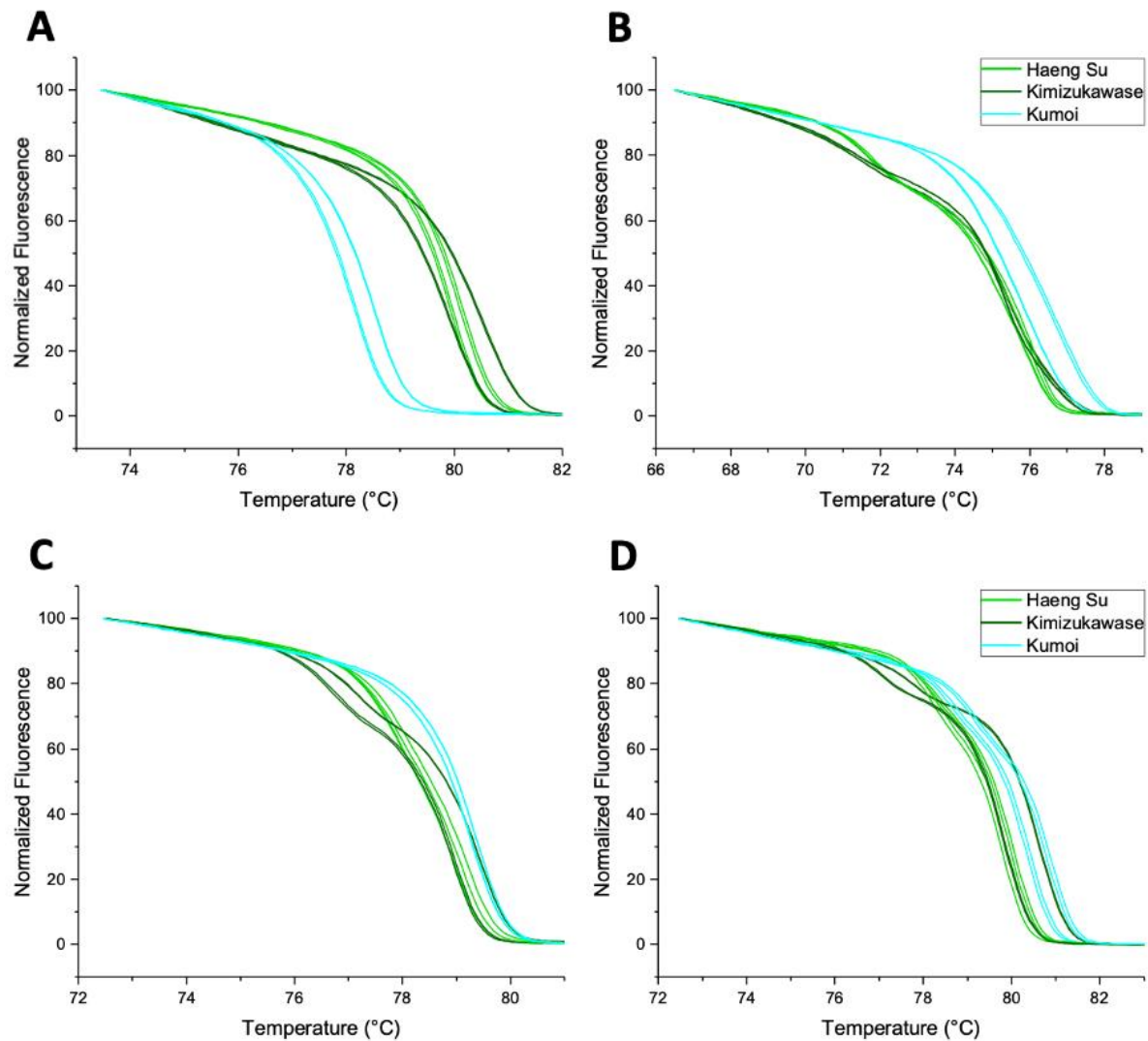


Figure 59 Normalized melting curves of the amplicons of the first and the second extracts from Haeng Su (light green), Kimizukawase (dark green) and Kumoi (light blue) with the primer pairs A) CH01h01, B) CH02b03b, C) CH02b10 and D) CH03a09. All extracts were analysed in duplicate.

Primer pairs MEMPc111t, MGD154, and CH01h10 did not allow differentiation between the three Asian pears (see figure 60 A to C).

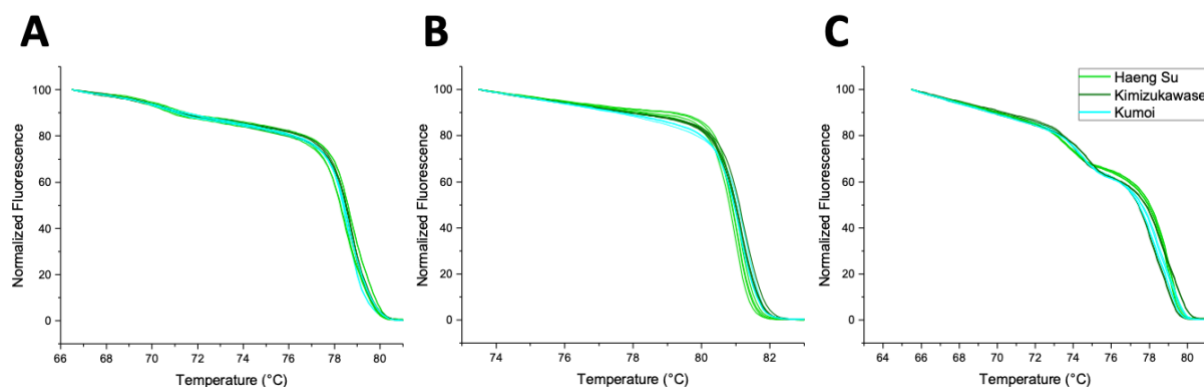


Figure 60 Normalized melting curves of the amplicons of the first and the second extracts from Haeng Su (light green), Kimizukawase (dark green) and Kumoi (light blue) with the primer pairs A) MEMPc111t, B) MGD154 and C) CH01h10. All extracts were analysed in duplicate.

The two Japanese pear cultivars (Kimizukawase and Kumoi) could be differentiated with pear primer pair NH013a and apple primer pairs CH01h01, CH02b03b, CH02b10, CH02h11a, and CH03a09 (see fig. 61 A, 59 A to D and 61 B). With the other nine of the 15 primer pairs, overlapping or similar normalized melting curves were obtained for these two cultivars. Therefore, they were not suitable for differentiating these cultivars.

Haeng Su is a Korean pear cultivar. It could not be differentiated from the Japanese pear cultivars Kimizukawase and Kumoi with primer pairs MEMPc111t, MGD154, CH01h10, CH02h11a, and CH03c02 (see fig. 60 A to D and 61 B and C). With primer pair NH013a, Haeng Su could also not be differentiated from Kimizukawase (see fig. 61 A).

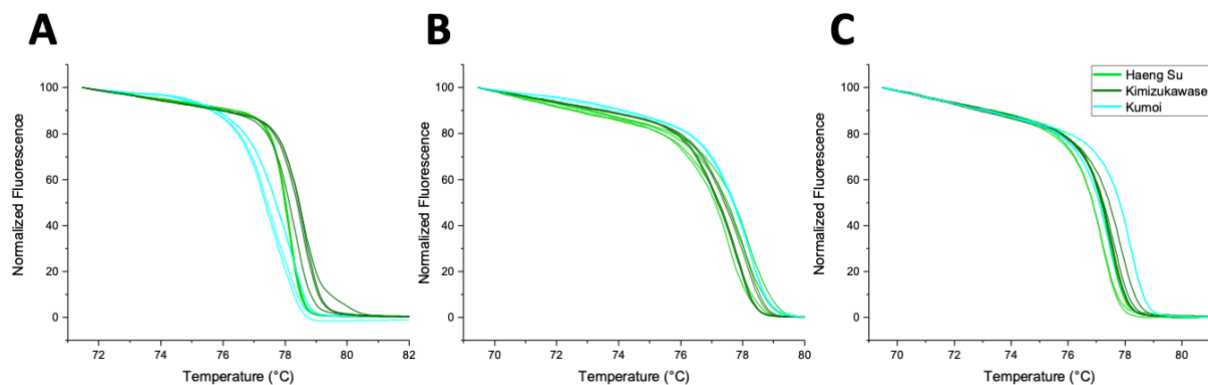


Figure 61 Normalized melting curves of the amplicons of the first and the second extracts from Haeng Su (light green), Kimizukawase (dark green) and Kumoi (light blue) with the primer pairs A) NH013a, B) CH02h11a C) CH03c02. All extracts were analysed in duplicate.

These results showed that the three cultivars of *Pyrus pyrifolia* can be distinguished from the 15 cultivars of *Pyrus communis*. However, the differentiation between the three Asian pear cultivars proved to be more challenging. The two Japanese pear cultivars showed identical normalized melting curves with nine SSR primer pairs, which reflects their close genetic relationship, whereas the Korean pear cultivar showed similar normalized melting curves to the Japanese cultivars with only five to six SSR primer pairs.

Williams and Celina

Celina, also known as Q-Tee, was bred by crossing Williams with Coloree de Juillet. [101] It was of interest if the melting and normalized melting curves for Williams and Celina showed similarities.

The normalized melting curves for the extracts from the two pear cultivars were identical when using the pear primer pairs EMPc08 and NH013a, and the apple primer pairs CH01h10, CH02b10, CH02h11a, CH03c02, and CH03a09 (seven primer pairs in total). All melting curves for Williams and Celina obtained with these primer pairs showed only one peak, which resulted in regularly shaped normalized melting curves (see for example fig. 62).

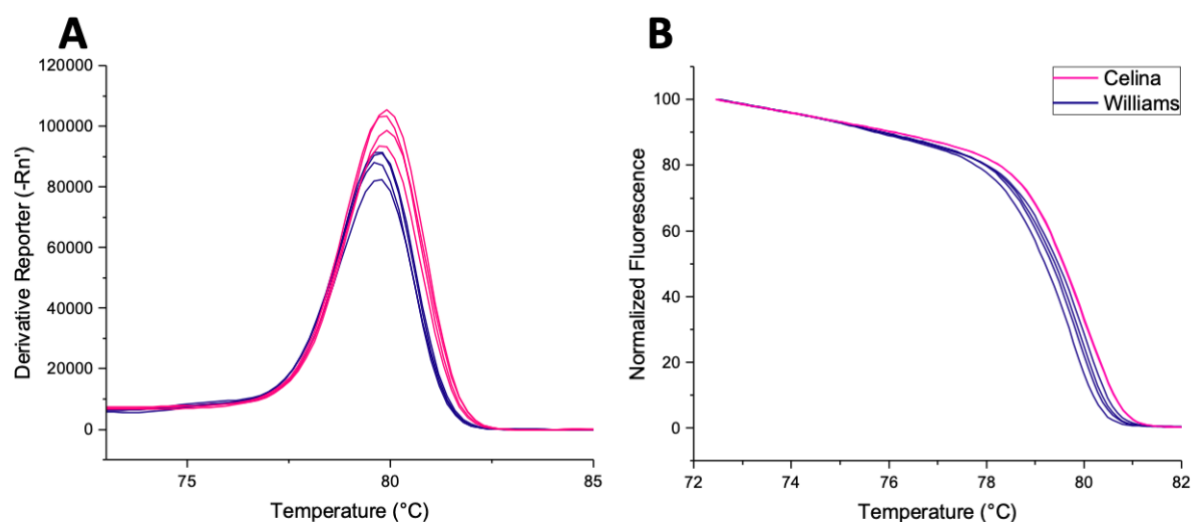


Figure 62 Melting (A) and normalized melting (B) curves of the amplicons of the first and second extracts from Celina (pink) and Williams (dark blue) with the primer pair Ch02b10. All extracts were analysed in duplicate.

The only exception was primer pair CH03a09, with which complex normalized melting curves were obtained for Williams and Celina, but the curves still overlapped (see fig. 63).

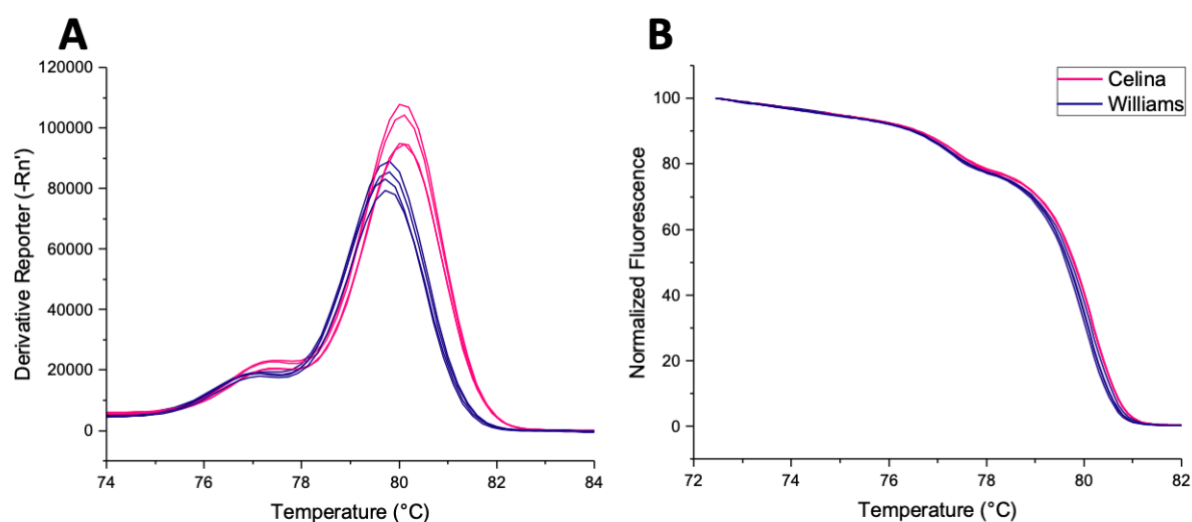


Figure 63 Melting (A) and normalized melting (B) curves of the amplicons of the first and second extracts from Celina (pink) and Williams (dark blue) with the primer pair CH03a09. All extracts were analysed in duplicate.

The two cultivars could be distinguished with pear primer pairs MEMPc111t, MEMPc117, MGD127, and MGD154 and apple primer pairs CH01f12, CH01h01, CH02b03b, and CH04c07. The normalized melting curves for Celina and Williams obtained with primer pairs MEMPc111t, MGD154, CH01f12, and CH01h01 showed similar curve shape, but were still differentiable due to their shift in the melting temperature (see fig. 64).

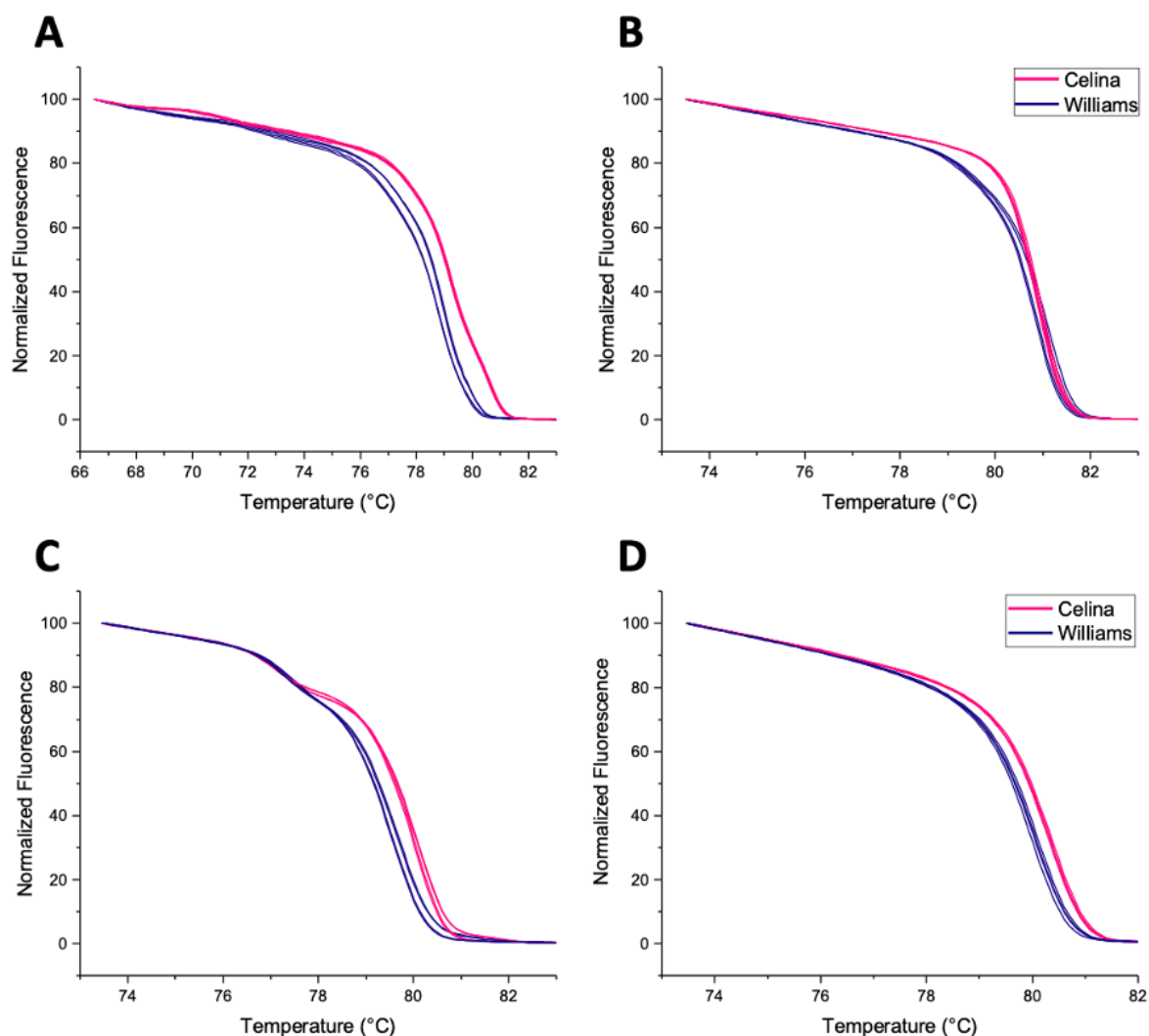


Figure 64 Normalized melting curves of the amplicons of the first and second extracts from Celina (pink) and Williams (dark blue) with the primer pairs A) MEMPC111t, B) MGD154, C) CH01f12 and D) Ch01h01. All extracts were analysed in duplicate.

With primer pairs MGD127, CH02b03b, and CH04c07, the normalized melting curves for Celina had a complex shape, due to the multiple peaks in their melting curves, whereas the normalized melting curves for Williams had a symmetrical shape and only one melt peak (see fig. 65 B to D). With primer pair MEMPC117, the normalized melting curves for Williams were more complex than for Celina (see fig. 65 A).

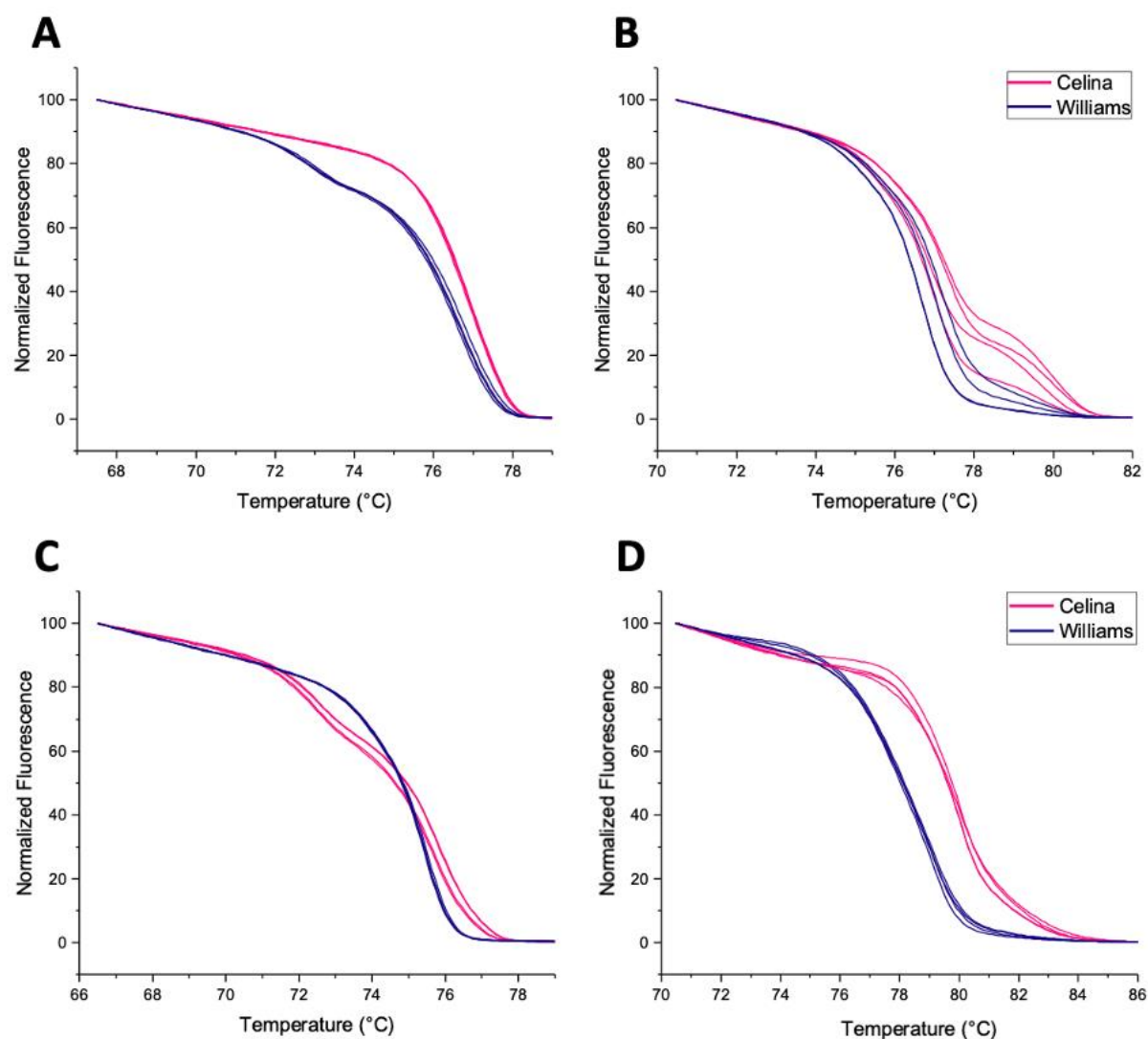


Figure 65 Normalized melting curves of the amplicons of the first and second extracts from Celina (pink) and Williams (dark blue) with the primer pairs A) MEMPC117, B) MGD127, C) CH02b03b and D) Ch04c07. All extracts were analysed in duplicate.

The pear cultivars Williams and Celina could be differentiated with eight primer pairs, where only four primer pairs resulted in more complex melting curves. The close genetic relationship of Williams and Celina is reflected by these results.

Conference, Cordula and Cepuna

Cordula, also known as TE-4179, was bred by crossing Conference with Forellenbirne, and Cepuna by crossing Conference with Doyenné d'Hiver. [101]

With the apple primer pairs CH01h01, CH01h10, CH02h11a, and CH03c02, the three pear cultivars could not be differentiated. However, the apple primer pairs CH01f12, CH02b03b, and CH03a09 allowed their differentiation (see fig. 66).

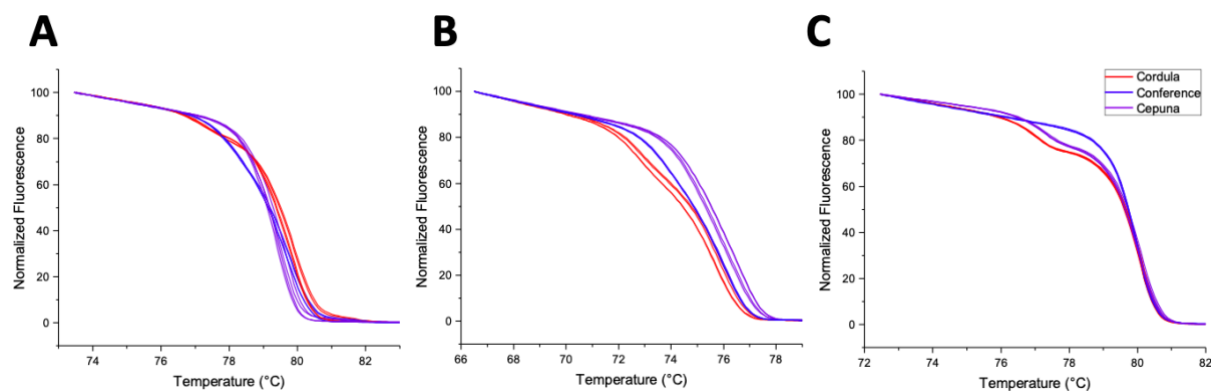


Figure 66 Normalized melting curves of the amplicons of the first and the second extracts from Cordula (red), Conference (blue) and Cepuna (purple) with the primer pairs A) CH01f12 B) CH02b03b and C) CH03a09. All extracts were analysed in duplicate.

The cultivars Cordula and Conference could be differentiated with pear primer pairs EMPc08 and MGD127, and with apple primer pairs CH01f12, CH02b03b, CH03a09, and CH04c07 (see fig. 67).

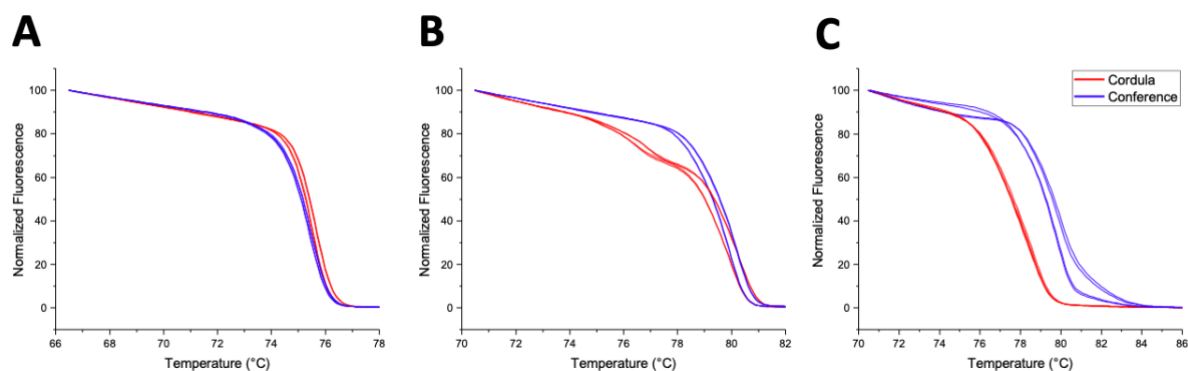


Figure 67 Normalized melting curves of the amplicons of the first and the second extracts from Cordula (red) and Conference (blue) with the primer pairs A) EMPc08 B) MGD127 and C) CH04c07. All extracts were analysed in duplicate.

Cepuna and Conference could be differentiated with the pear primer pairs MEMPc111t, MEMPc117, MGD154, and NH013a, and with the apple primer pairs CH01f12, CH02b10, CH02b03b, and CH03a09 (see fig. 66 and 68).

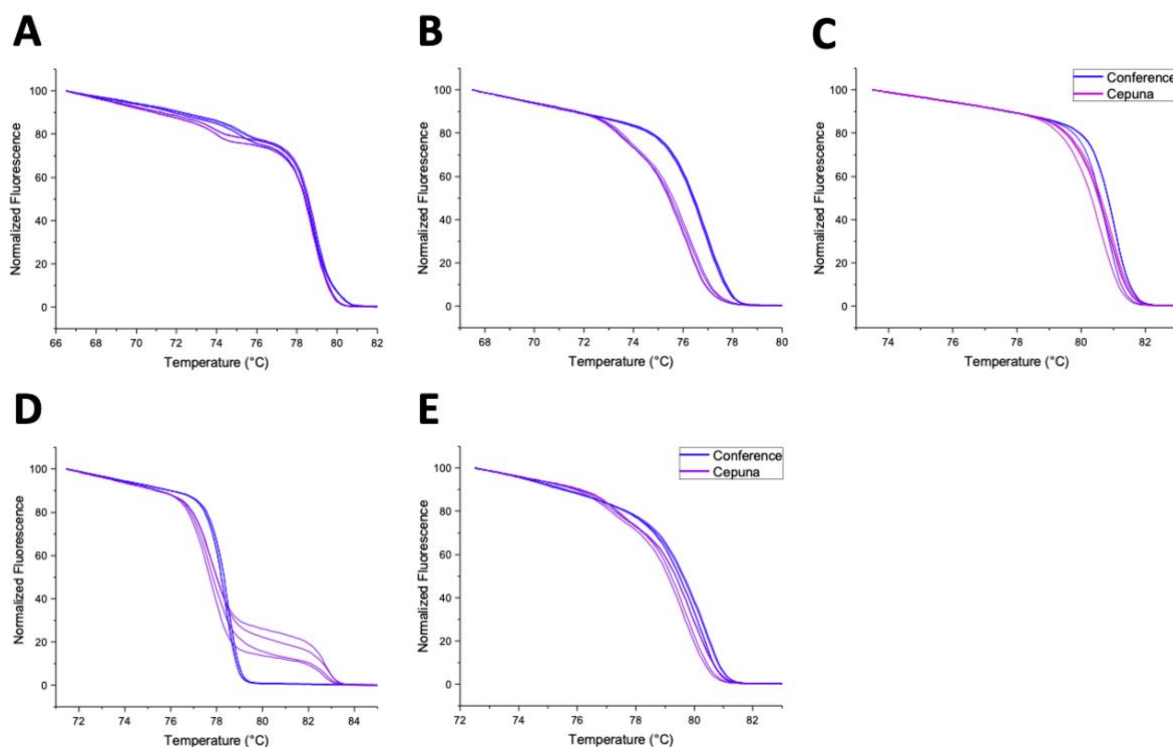


Figure 68 Normalized melting curves of the amplicons of the first and the second extracts from Conference (blue) and Cepuna (purple) with the primer pairs A) MEMPc111t, B) MEMPc117, C) MGD154, D) NH013a and E) CH02b10. All extracts were analysed in duplicate.

Cepuna and Cordula were not differentiable with the primer pairs EMPc08, CH01h01, CH02h11a, and CH03c02. These results show that although both Cepuna and Cordula were bred by crossing with Conference, different normalized melting curves were obtained for the two cultivars with eleven SSR primer pairs, reflecting their genomic differences. For Cordula and Conference, identical normalized melting curves were obtained with eight SSR primer pairs, and for Cepuna and Conference with seven SSR primer pairs, indicating their close genetic relationship.

6.3 Pear peel, pulp, and juices

Another goal of this thesis was to investigate, if it is possible to amplify a segment of DNA from different pear components, including peel, and even heat treated/pasteurised juices, with the aim to differentiate pear cultivars. The first step involved optimization of the protocol of DNA extraction kits to obtain a sufficient amount of intact DNA from these different components.

Four kinds of juices (with or without peel and with low or high fibre-content) were produced in-house from three different pear cultivars. This ensured that the juices contained only the selected cultivars and their natural components, without preservatives or pasteurisation. The pear juices were then heat treated under different pasteurisation conditions to investigate how this affected DNA amplifiability and HRM results.

6.3.1 DNA extraction

DNA was extracted from the peel, pulp, and juices (with or without peel, and with low or high fibre-content) of the pear cultivars Anjou, Cheeky, and Williams using two different kits. The first kit was the Plant Kit by Macherey-Nagel (NucleoSpin Plant II Kit) and the second was the Food Kit by Qiagen (DNeasy mericon Food Kit).

The Plant Kit has previously been used in our working group for successful DNA extraction from apple leaves [96], sunflower seeds [82], and chickpeas [102] in previous master theses dealing with cultivar differentiation by PCR-

HRM. However, one thesis reported unsatisfactory results when extracting DNA from apple juices using this kit, due to the low amount of intact DNA obtained. [97]

This is consistent with publications, which indicate that processed food, including juices, often contain low concentrations of intact DNA due to processing and pasteurisation steps. [34, 50] The low DNA yield is problematic for further analysis. Therefore, it was of interest to optimize the DNA extraction protocol to enhance DNA concentration.

The Food Kit by Qiagen is specially recommended for DNA extraction from processed food and small DNA fragments. [84] In this thesis, both kits were used, compared, and slightly modified (see section 5.2.1 and 5.2.2).

The protocol for the Plant Kit had already been modified by a fellow Master student (see section 5.2.1). [82]

For the optimization of the 'Small Fragment Protocol (200 mg)' of the Food Kit, several experiments were conducted. These included increasing the incubation time from 30 to 90 minutes, doubling the proteinase K volume from 2.5 µl to 5 µl, and reducing the water content of the juices by incubating them at 60 °C and adding pectinase with subsequent pH adjustment [83] (see section 5.2.2). These optimization tests were performed with three commercially available juices (two apple juices and one pear juice), because the in-house produced juices were not yet available at that time. The resulting DNA concentrations under various extraction conditions are listed in table 16.

Table 16 DNA concentration of the DNA extracts from two apple juices and one pear juice obtained with different DNA extraction conditions with Food Kit (n.d. = not defined, because the DNA concentration of the extract was below the limit of detection of the Qubit fluorometer).

DNA extraction conditions	DNA concentration [ng/µl]		
	Apple juice 1	Apple juice 2	Pear juice
Food Kit protocol not modified	n.d.	n.d.	0.0010
Modified protocol (see section 5.2.2)	0.0050	0.0160	0.0570
Modified protocol with 90 min incubation time	n.d.	n.d.	0.0210
Modified protocol with 5 µl proteinase K	0.0050	n.d.	0.0420
Modified protocol with pectinase and pH adjustment	n.d.	n.d.	0.0156
Modified protocol with concentrate (2 ml to 0.5 ml)	0.0055	0.0051	0.0052
Modified protocol with concentrate (200 µl)	0.0050	0.0072	0.0126
Modified protocol with concentrate (2 ml to 0.5 ml) with pectinase and pH adjustments	0.0059	0.0076	0.0057

The results showed that in most cases, the DNA concentrations of pear juice extracts were higher than those from apple juices. This difference could be due to differences in juice components, water content, processing and pasteurisation conditions, and pH values. The pH value was 3.57 for apple juice 1, 3.66 for apple juice 2, and 4.18 for the pear juice. Low pH values can lead to DNA depurination [53] and hydrolysis of the phosphodiester bond, resulting in DNA fragmentation. [48]

Increasing the incubation time and reducing the water content through heat treatment (juice concentration) did not improve DNA yield. The removal of water considerably decreased DNA concentration in the extracts, which might be due to additional DNA degradation caused by heat. The addition of pectinase with pH adjustment, as described in the literature [83], resulted in decreased DNA concentrations. Finally, the modified protocol (see section 5.2.2) was selected, because it led to the highest DNA concentration in the extracts among the tested conditions.

DNA was extracted from peel, pulp, and the four in-house produced juices of the pear cultivars Anjou, Cheeky, and Williams using both the Plant and Food Kit. DNA extraction was performed as described in section 5.2.

The DNA extraction of each sample was performed twice to test the repeatability. The DNA concentration of the extracts obtained in the first and the second extraction with the Plant Kit are listed in table 17, those obtained with the Food Kit in table 18.

Table 17 DNA concentration of the DNA extracts obtained in the first and second extraction of peel (S) and pulp (F) from Williams (W), Anjou (A) and Cheeky (C) with Plant Kit (P)

Cultivar	Abbrev.	Pear component	1. Extraction		2. Extraction	
			Mass [g]	Conc. [ng/μl]	Mass [g]	Conc. [ng/μl]
Williams	PWS	Peel	0.153	17.20	0.154	12.00
	PWF	Pulp	0.144	1.59	0.151	1.59
Anjou	PAS	Peel	0.149	32.20	0.153	13.30
	PAF	Pulp	0.149	0.48	0.152	1.68
Cheeky	PCS	Peel	0.153	17.70	0.154	15.10
	PCF	Pulp	0.148	3.60	0.151	4.19

Table 18 DNA concentration of the DNA extracts obtained in the first and second extraction of peel (S) and pulp (F) from Williams (W), Anjou (A) and Cheeky (C) with Food Kit (F)

Cultivar	Abbrev.	Pear component	1. Extraction		2. Extraction	
			Mass [g]	Conc. [ng/μl]	Mass [g]	Conc. [ng/μl]
Williams	FWS	Peel	0.205	10.20	0.205	5.03
	FWF	Pulp	0.196	4.09	0.198	1.40
Anjou	FAS	Peel	0.201	16.50	0.200	12.40
	FAF	Pulp	0.202	1.58	0.199	1.26
Cheeky	FCS	Peel	0.203	21.60	0.205	12.50
	FCF	Pulp	0.204	6.34	0.197	2.54

Figure 69 shows the mean values of the DNA concentrations of the two DNA extracts obtained with the Plant Kit and the Food Kit from peel and pulp of the pear cultivars Williams, Anjou, and Cheeky.

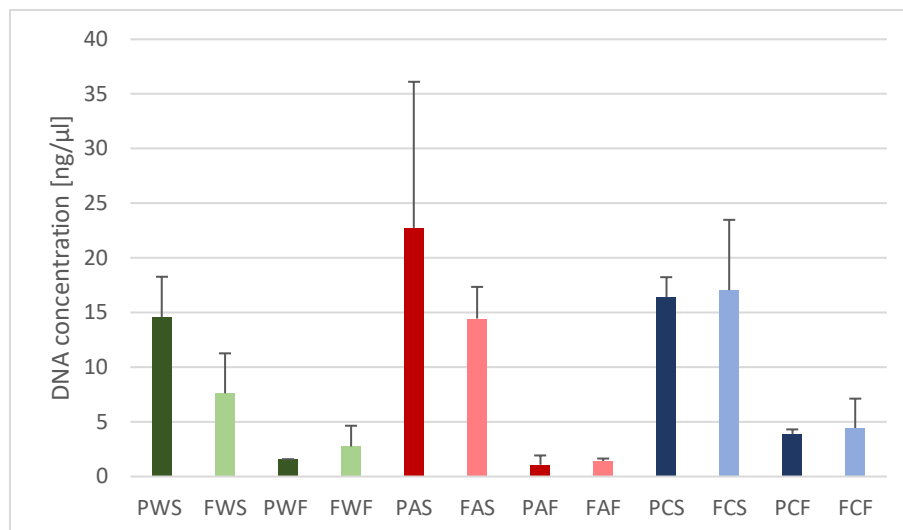


Figure 69 Mean DNA concentration of the two DNA extracts from pear peel (S) and pulp (F) of Williams (W; green), Anjou (A; red) and Cheeky (C; blue) obtained with Plant Kit (P; darker colour) and Food Kit (F; brighter colour). Standard deviations from the two DNA extracts are represented in form of error bars.

Tables 17 and 18 and figure 69 show that the highest DNA concentrations from pear peel were obtained with the Plant Kit, whereas the Food Kit yielded higher DNA concentrations for the pear pulp. The extracts from the peel of Anjou showed the highest DNA concentration. Unlike Williams and Cheeky, the peel of Anjou had red spots. The red colour in the pear peel is a result of accumulation of anthocyanins. Anthocyanins are secondary metabolites with a phenolic structure that are common in plants. They have antioxidative properties and protect the plants against oxidative stress and pests. [103]

Tables 19 and 20 present the DNA concentration of DNA extracts obtained from the four in-house produced juices (without peel WP/ with peel P and high/low fibre-content), using the Plant Kit (table 19) and the Food Kit (table 20).

Table 19 DNA concentration of the two DNA extracts from the four juices from Williams (W), Anjou (A) and Cheeky (C) with Plant Kit (P); WP = without peel, P = with peel

Cultivar	Abbrev.	Peel	Fibre	1. Extraction		2. Extraction	
				Mass [g]	Conc. [ng/μl]	Mass [g]	Conc. [ng/μl]
Williams	PWS1	WP	Low	0.147	0.020	0.151	0.035
	PWS2	WP	High	0.153	0.023	0.150	0.030
	PWS3	P	Low	0.144	0.025	0.151	0.024
	PWS4	P	High	0.154	0.043	0.152	0.036
Anjou	PAS1	WP	Low	0.147	0.024	0.149	0.024
	PAS2	WP	High	0.150	0.027	0.152	0.015
	PAS3	P	Low	0.150	0.033	0.150	0.027
	PAS4	P	High	0.153	0.037	0.149	0.017
Cheeky	PCS1	WP	Low	0.147	0.086	0.149	0.048
	PCS2	WP	High	0.152	0.085	0.151	0.061
	PCS3	P	Low	0.151	0.334	0.152	0.173
	PCS4	P	High	0.150	0.364	0.149	0.254

Table 20 DNA concentration of the DNA extracts from the four juices from Williams (W), Anjou (A) and Cheeky (C) with Food Kit (F); WP = without peel, P = with peel

Cultivar	Abbrev.	Peel	Fibre	1. Extraction		2. Extraction	
				Mass [g]	Conc. [ng/μl]	Mass [g]	Conc. [ng/μl]
Williams	FWS1	WP	Low	0.205	0.132	0.201	0.254
	FWS2	WP	High	0.204	0.284	0.196	0.146
	FWS3	P	Low	0.203	0.222	0.199	0.182
	FWS4	P	High	0.197	0.270	0.204	0.254
Anjou	FAS1	WP	Low	0.204	0.119	0.204	0.197
	FAS2	WP	High	0.205	0.125	0.197	0.108
	FAS3	P	Low	0.204	0.134	0.202	0.079
	FAS4	P	High	0.199	0.108	0.198	0.111
Cheeky	FCS1	WP	Low	0.201	0.454	0.204	0.348
	FCS2	WP	High	0.201	0.378	0.203	0.460
	FCS3	P	Low	0.202	0.850	0.199	1.630
	FCS4	P	High	0.202	1.090	0.203	1.820

With both kits, the DNA concentration of the extracts from Cheeky was up to ten times higher than that of Anjou and Williams. During juice processing it was observed, that Cheeky was crisper and yielded clearer juices containing less grits, whereas Anjou and Williams were much softer, possibly indicating a higher water content.

Furthermore, the pH value of the Cheeky juices (pH 4.47) was higher than that of Anjou (pH 3.97) and Williams (pH 3.84). The pH of fruit juices is influenced by natural compounds (e.g. organic acids), but also by conditions during growth and storage. Studies have shown that food containing a low pH value (< pH 3) causes depurination of DNA [50] and hydrolysis of the phosphodiester bonds, which results in DNA fragmentation [48]. This degradation may result in PCR failure. [50] Thus, the lower pH value of Anjou and Williams juices may have contributed to the reduced DNA integrity and lower DNA concentrations in their extracts.

For a better comparison, the mean values of DNA concentrations from the two DNA extraction kits for each juice type were plotted in diagrams (figure 70 to 72).

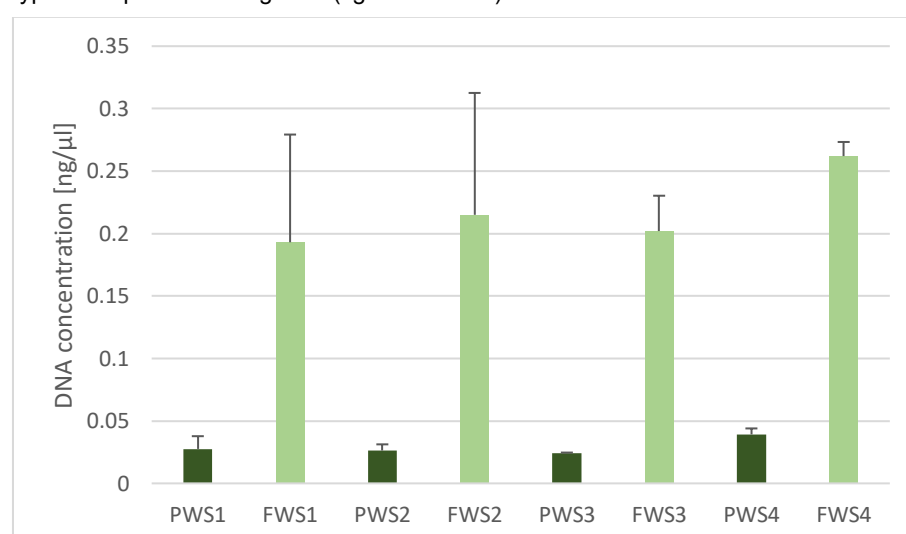


Figure 70 Mean DNA concentration of the two DNA extracts from the Williams juices (WS1-4) obtained from Plant Kit (P; dark green) and Food Kit (F; bright green). Standard deviations from the two DNA extracts are represented by error bars.

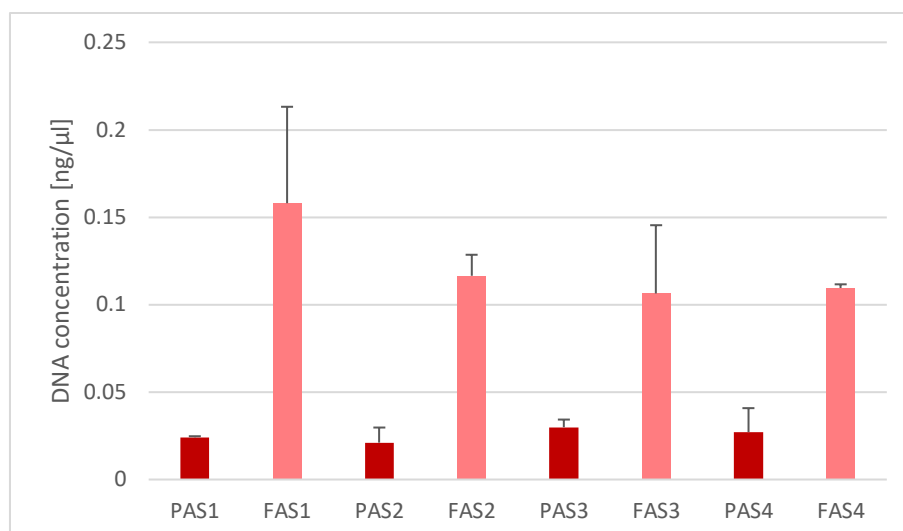


Figure 71 Mean DNA concentration of the two DNA extracts from the Anjou (AS1-4) juices obtained from Plant Kit (P; dark red) and Food Kit (F; bright red). Standard deviations from the two DNA extracts are represented by error bars.

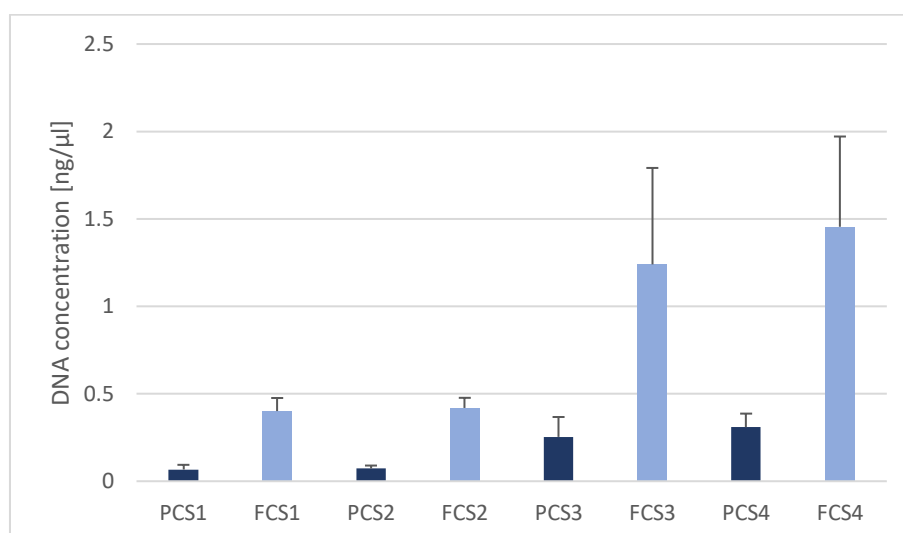


Figure 72 Mean DNA concentration of the two DNA extracts from the Cheeky juices (CS1-4) obtained from Plant Kit (P; dark blue) and Food Kit (F; bright blue). Standard deviations from the two DNA extracts are represented by error bars.

The figures 70 to 72 show that the DNA extracts obtained with the Food Kit had a higher DNA concentration than the DNA extracts obtained with the Plant Kit. Therefore, DNA extracts obtained with the Food Kit were used for analysis with PCR-HRM.

To increase the DNA concentration in the extracts, pectinase was added without pH adjustment during DNA extraction [83] (see section 5.2.2). Table 21 lists the DNA concentration in the extracts from peel, pulp, and the juices with pectinase.

Table 21 DNA concentration of the DNA extracts from peel (FWS+P), pulp (FWF+P) and the four juices (FWS1+P to FWS4+P) from Williams with the addition of pectinase during the DNA extraction with Food Kit (F)

Cultivar	Abbrev.	Pear component	Fibre content	Mass [g]	Concentration [ng/μl]
Williams	FWS+P	Peel	-	0.2021	11.2
	FWF+P	Pulp	-	0.1966	1.9
	FWS1+P	Pulp	Low	0.200	0.171
	FWS2+P	Pulp	High	0.210	0.228
	FWS3+P	Peel, Pulp	Low	0.208	0.222
	FWS4+P	Peel, Pulp	High	0.209	0.34

The addition of pectinase to pear peel seemed to increase the DNA concentration in the extracts (11.2 ng/μl), compared to extracts without pectinase (10.2 ng/μl and 5.03 ng/μl). However, the effect of pectinase on the DNA concentration of DNA extracts from pulp and juices was inconsistent (see table 18, 20 and 21).

To investigate the amplifiability of the extracted DNA, PCR was carried out with the DNA extracts from Williams peel, pulp, and all four juices treated with pectinase. PCR was performed on the RotorGene thermocycler using primer pair CH02b10. Prior to PCR, the extracts were diluted 1:5 with nuclease free water. All extracts were analysed in duplicate. Figure 73 presents the normalized melting curves of the resulting amplicons for Williams peel, pulp, and the juices without (fig. 73 A) and with pectinase (fig. 73 B).

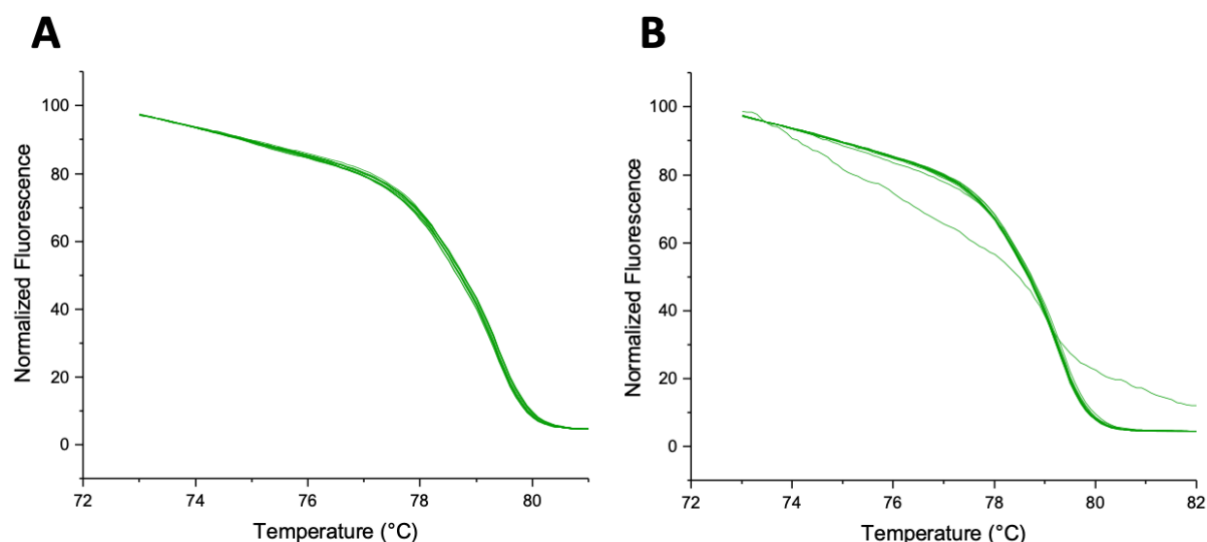


Figure 73 Normalized melting curves of the amplicons obtained with primer pair CH02b10 for Williams peel, pulp and the juices A) without and B) with the addition of pectinase during the DNA extraction. All extracts were analysed in duplicate.

The normalized melting curves of the DNA extracts with and without pectinase were nearly identical. However, in one of the PCR duplicates, DNA from juice 1 with pectinase (FWS1+P) was not amplified. The result was confirmed when PCR was repeated. Therefore, DNA extracts without pectinase were used in further experiments.

6.3.2 Performance of the PCR and dilution factor

PCR coupled with HRM was used to differentiate pear cultivars based on DNA extracted from peel, pulp, and the four juices. All PCRs were performed on the RotorGene thermocycler as described in section 5.4. To determine the optimal dilution factor for PCR amplification, the apple primer pair CH02b10 was used (see table 13).

Sample dilution is a common approach to reduce matrix effects. Matrix effects refer to the influence of co-extracted substances on the analytical result. They can affect the probability of obtaining positive or negative results. Matrix effects can enhance detection, but in most cases, they have a negative impact and inhibit PCR amplification. PCR

inhibitors can be natural sample components or they originate from reagents used during DNA extraction, such as phenol, SDS or EDTA. [104]

To assess the impact of dilution, DNA extracts from Williams peel, pulp, and juices were applied in a PCR run, both undiluted and diluted with nuclease free water in ratios of 1:5 and 1:15.

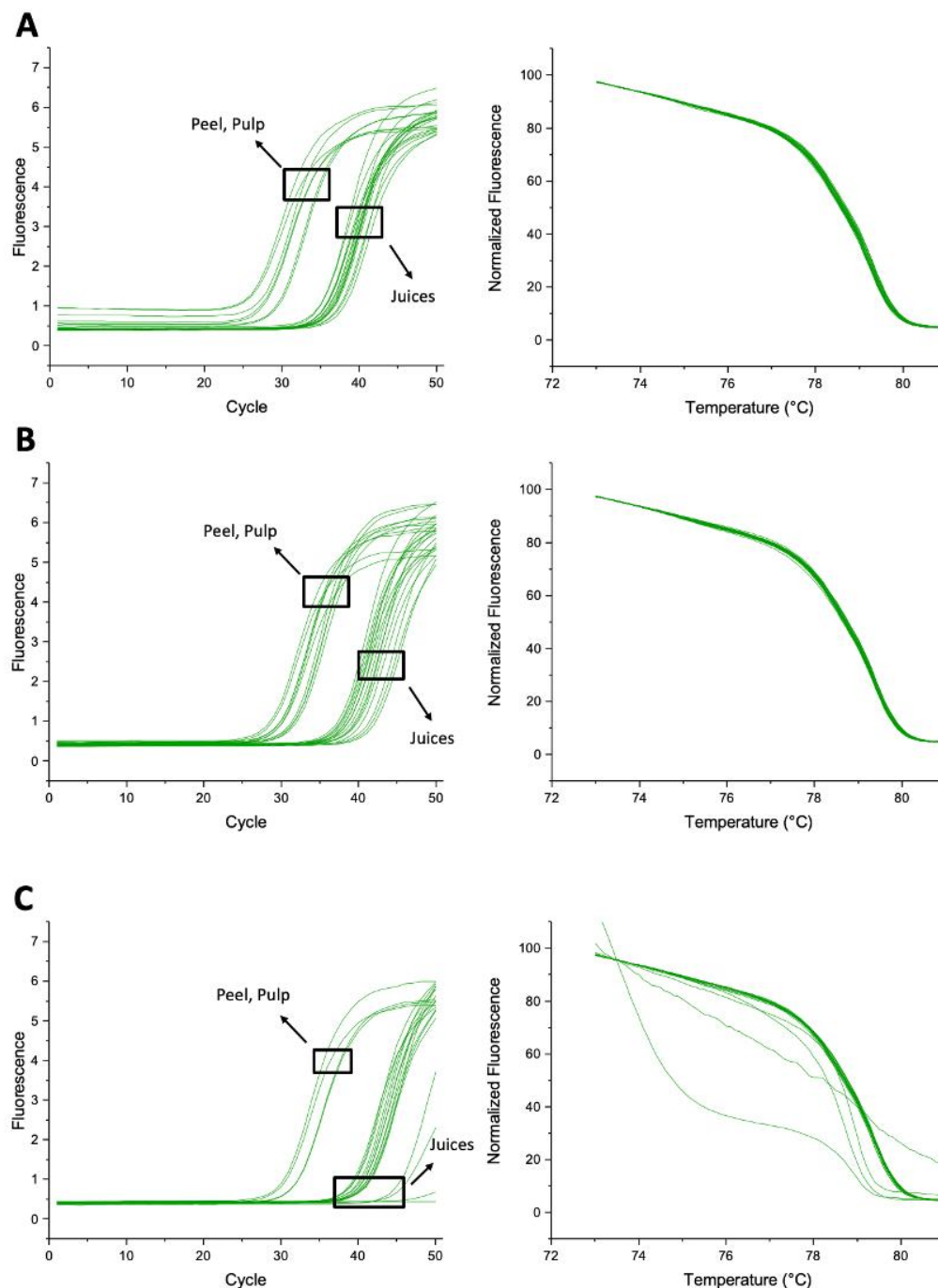


Figure 74 Amplification and normalized melting curves of the amplicons of the two DNA extracts from Williams peel, pulp and juices. The extracts were applied A) undiluted, B) diluted ratio 1:5 and C) diluted ratio 1:15. All extracts were analysed in duplicate.

Figure 74 C shows that a 1:15 dilution resulted in late amplification and considerable signal scattering. In addition, DNA from one of the duplicates of FWS1 and FWS3 was not amplified, indicating poor repeatability and reduced amplifiability. The high dilution factor was therefore considered unsuitable.

The normalized melting curves obtained for the undiluted extracts (fig. 74 A) and those diluted 1:5 (fig. 74 B) showed good repeatability with minimal scattering. The amplification curves for the undiluted extracts showed less variation and lower Ct values than the 1:5 diluted extracts

As a compromise between DNA consumption and PCR performance, a final dilution factor of 1:5 was selected.

6.3.3 Primer pairs for the differentiation of the three pear cultivars

For the differentiation of the pear cultivars Williams, Anjou and Cheeky using PCR coupled with HRM, one apple and one pear primer pair were selected.

The apple primer pair CH02b10 was chosen, because it was the second best primer pair for differentiating apple cultivars. [96] For the selection of a pear primer pair, the six primer pairs EMPc08, MEMPc111t, MEMPc117, MGD127, MGD154, and NH013a (see table 13) were tested. DNA extracts from pulp and juice 3 (with peel and low fibre content) from Williams, Anjou, and Cheeky, obtained with the Food Kit from the first DNA extraction, were used in a PCR run. The DNA extracts were diluted 1:15 with nuclease free water, because this experiment was carried out before the optimal dilution factor was determined (see section 6.3.2). All extracts were analysed in duplicate.

Figures 75 to 80 show the melting and normalized melting curves of the amplicons for Williams, Anjou, and Cheeky obtained with the primer pairs EMPc08, MEMPc111t, MEMPc117, MGD127, MGD154, and NH013a.

With primer pair EMPc08 (fig. 75), Williams could be distinguished from Anjou and Cheeky. However, the DNA from Williams juice (FWS3) was not amplified. The peak maxima of the melting curves for Anjou peel and juices differed in temperature, 75.1 °C for pulp and 75.4 °C to 75.5 °C for juice. With primer pair EMPc08, Anjou and Cheeky could not be differentiated due to the similar melting temperatures of the amplicons.

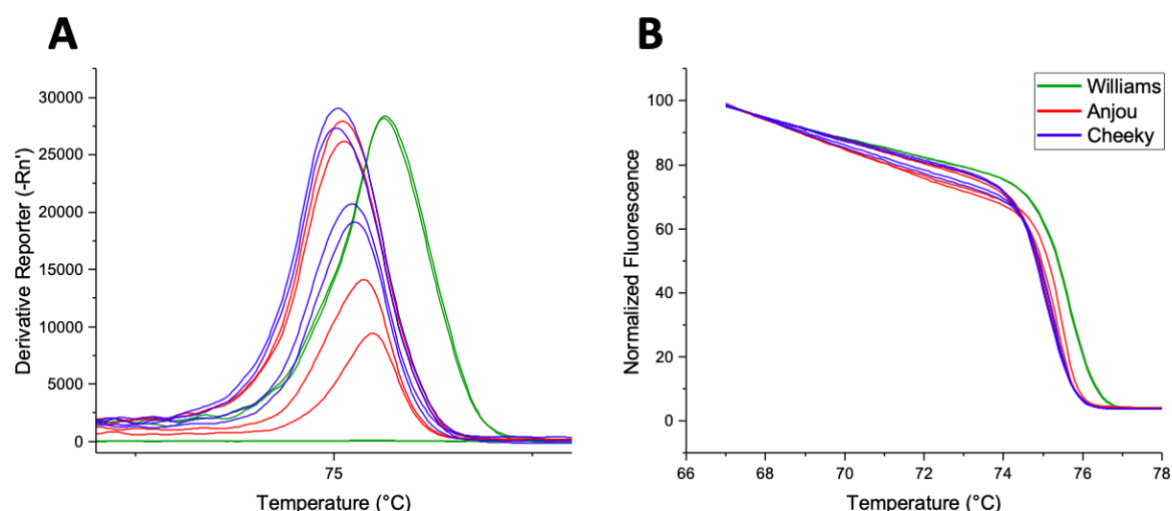


Figure 75 Melting (A) and normalized melting curves (B) of the amplicons for the peel and juice 3 of Williams (green), Anjou (red) and Cheeky (blue) of the first DNA extract obtained with primer pair EMPc08. All extracts were analysed in duplicate. The normalized melting curves for FWS3 are not illustrated in 75 B.

The melting curves for Cheeky, obtained with primer pair MEMPc111t (fig. 76), showed the same curve shape for both pulp and juice extracts. However, for Williams and Anjou, differences were observed between the curves for pulp and juice. For Williams, the melting peak for pulp and one of the PCR duplicates for juice was at 78.3 °C, with a smaller second peak at 79.3 °C, whereas the other juice duplicate showed the main peak at 79.3 °C and a smaller peak at 78.3 °C. A similar result was obtained for Anjou; the main peak for both PCR duplicates from pulp and one from juice was at 78.2 °C, with a smaller peak at 79.4 °C. The second duplicate showed the main peak at 79.4 °C and the smaller peak at 78.2 °C. With this primer pair, the three cultivars could not be clearly differentiated.

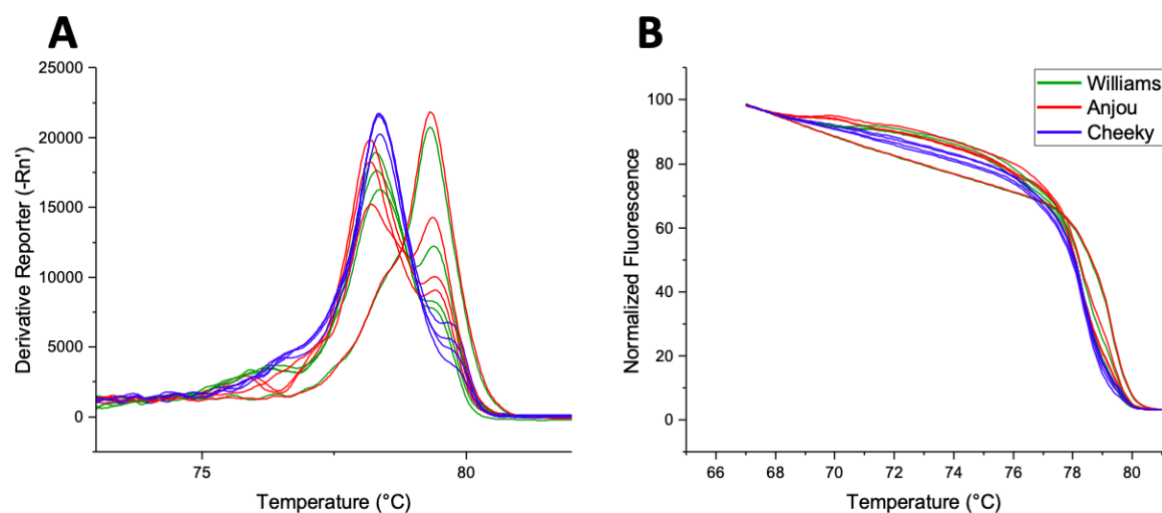


Figure 76 Melting (A) and normalized melting curves (B) of the amplicons for the peel and juice 3 of Williams (green), Anjou (red) and Cheeky (blue) of the first DNA extract obtained with primer pair MEMPc111t. All extracts were analysed in duplicate.

The melting and normalized melting curves for Williams, Anjou, and Cheeky with primer pair MEMPc117 (fig. 77) were very similar. Differentiation between the three cultivars was not possible, due to the deviations in the melting and normalized melting curves for pulp and juice, especially from Anjou and Williams and the similar melting temperature of the amplicons for all three cultivars.

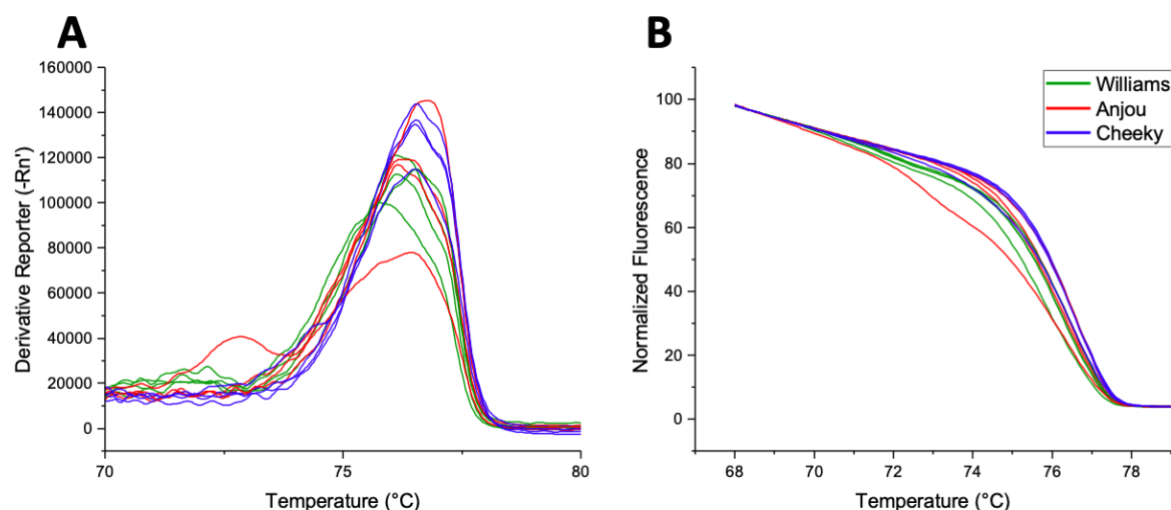


Figure 77 Melting (A) and normalized melting curves (B) of the amplicons for the peel and juice 3 of Williams (green), Anjou (red) and Cheeky (blue) of the first DNA extract obtained with primer pair MEMPc117. All extracts were analysed in duplicate.

Anjou could clearly be differentiated from Williams and Cheeky using primer pair MGD127 (fig. 78). However, the melting curves obtained for one of the PCR duplicates of Anjou juice had its peak maximum at 79.2 °C, instead of 77.2 °C, as observed for both PCR duplicates of pulp and one from juice. These three melting curves had an additional peak at around 71 °C to 72 °C. The melting and normalized melting curves for Williams and Cheeky overlapped completely and could not be differentiated.

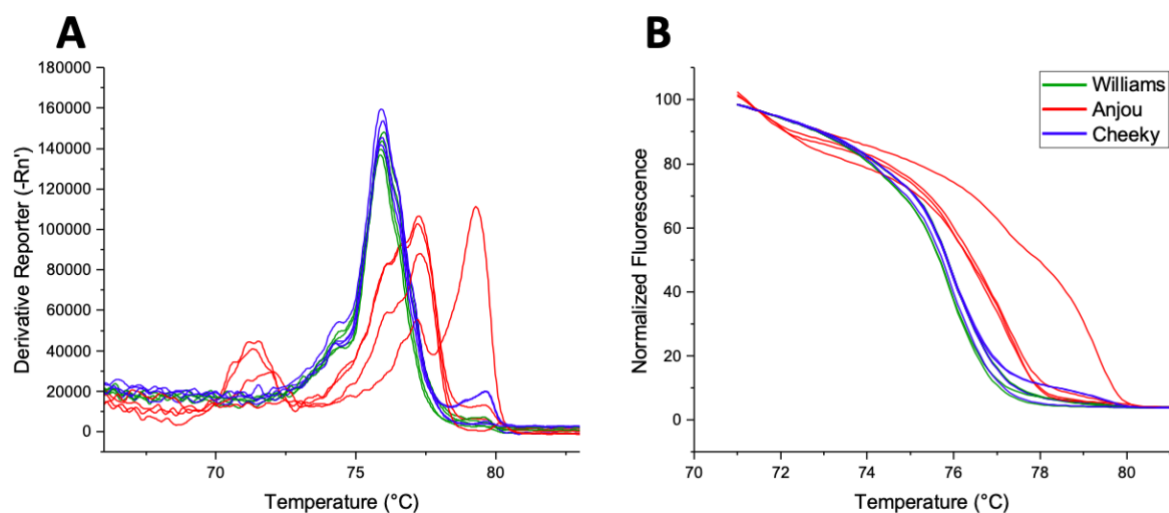


Figure 78 Melting (A) and normalized melting curves (B) of the amplicons for the peel and juice 3 of Williams (green), Anjou (red) and Cheeky (blue) of the first DNA extract obtained with primer pair MGD127. All extracts were analysed in duplicate.

With primer pair MGD154, the three cultivars could be differentiated (fig. 79). However, the normalized melting curve for one of the PCR duplicates from Williams juice overlapped with the curves obtained for Anjou.

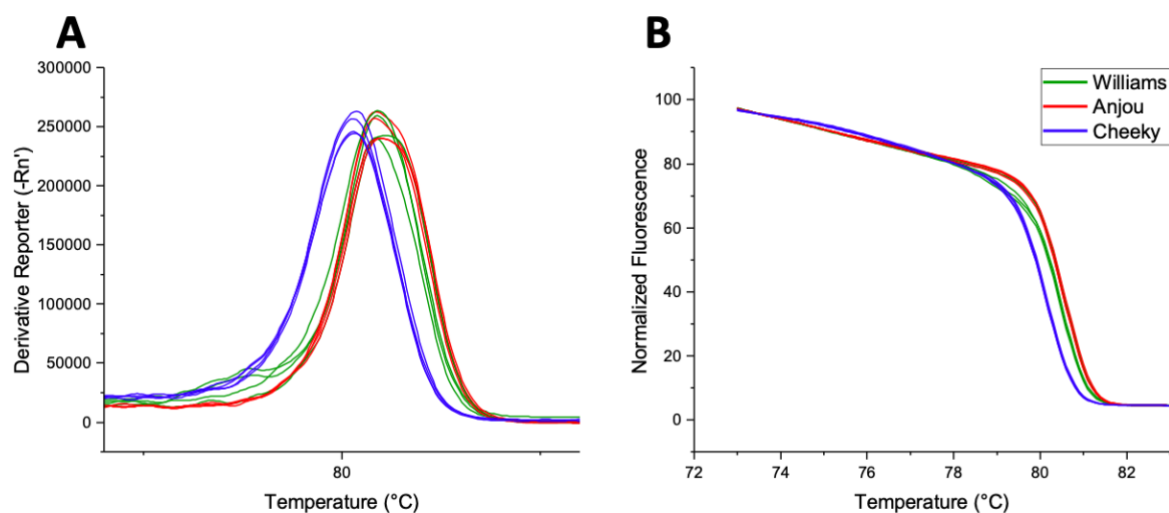


Figure 79 Melting (A) and normalized melting curves (B) of the amplicons for the peel and juice 3 of Williams (green), Anjou (red) and Cheeky (blue) of the first DNA extraction obtained with primer pair MGD154. All extracts were analysed in duplicate.

With primer pair NH013a, Cheeky could be distinguish from Williams and Anjou (fig. 80). However, the melting curves for Anjou juice showed inconsistent results. The melting curves for one of the PCR had the peak maximum at 72.7 °C, whereas for the other duplicate two peaks were obtained, one at 78.2 °C and a higher one at 80.3 °C. The peak maximum obtained for Anjou pulp was at 78.2 °C. The normalized melting curves for Williams overlapped completely with the curve for Anjou pulp.

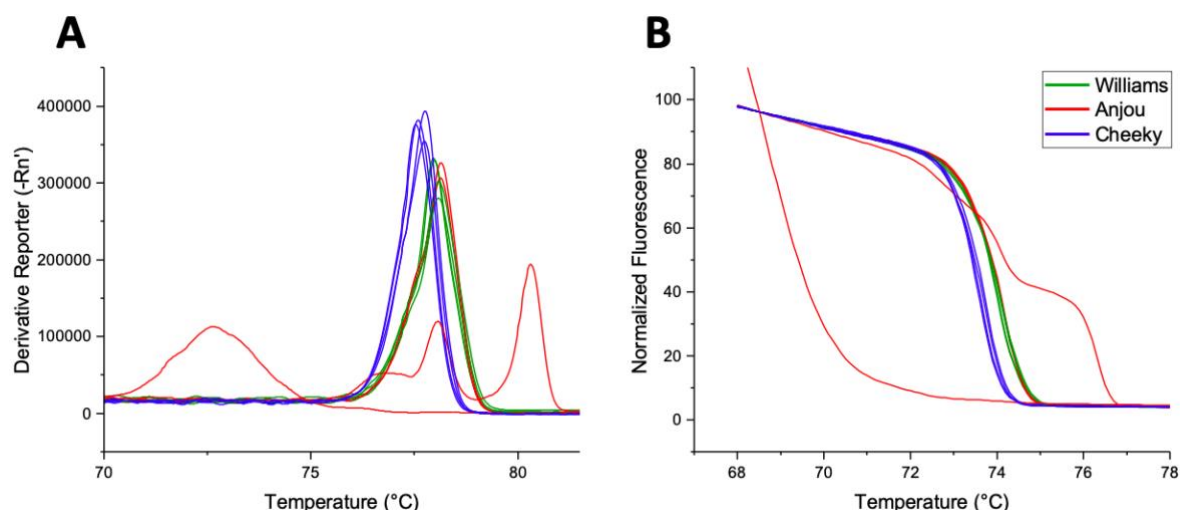


Figure 80 Melting (A) and normalized melting curves (B) of the amplicons for the peel and juice 3 of Williams (green), Anjou (red) and Cheeky (blue) of the first DNA extract obtained with primer pair NH013a. All extracts were analysed in duplicate.

The melting and normalized melting curves of the amplicons obtained for extracts from juices, especially from Anjou and Williams, often proved to be problematic, when using most primer pairs, due to primer dimer formation, poor repeatability and even unsuccessful amplification. It is possible that the poor repeatability and amplification failures were caused by the high dilution factor (1:15). As shown in section 6.3.2, a 1:15 dilution was not suitable, because it led to inconsistent results and poor amplification. However, this experiment was not repeated using the final dilution factor (1:5).

Out of the six pear primer pairs tested, MGD154 was finally chosen for the differentiation of the three pear cultivars.

6.3.4 Differentiation of the three pear cultivars

For the final differentiation of the pear cultivars Williams, Anjou, and Cheeky, DNA extracts from peel, pulp, and the four juices from both the first and second extraction using the Food Kit, were analysed by PCR coupled with HRM, using the apple primer pair CH02b10 and the pear primer pair MGD154. All extracts were diluted 1:5 with nuclease free water and analysed in duplicate. The PCR-HRM was performed with the RotorGene thermocycler.

Data evaluation was based on comparing the normalized melting curves. Cultivars were considered differentiable, when the shape of the normalized melting curve differed and/or when the difference in the melting temperature exceeded 0.4 °C.

Figure 81 shows the normalized melting curves for the three pear cultivars obtained with the apple primer pair CH02b10 (fig. 81 A) and the pear primer pair MGD154 (fig. 81 B). In both graphs, the three pear cultivars have different curve shapes, enabling the differentiation.

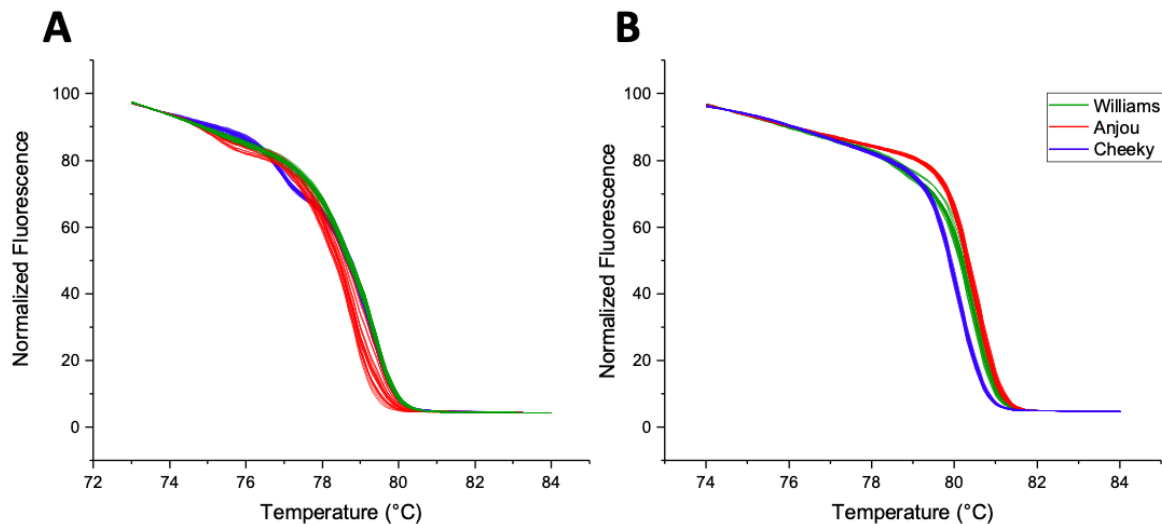


Figure 81 Normalized melting curves of the amplicons for peel, pulp and juices from the pear cultivar Williams (green), Anjou (red) and Cheeky (blue) with the primer pairs A) Ch02b10 and B) MGD154 performed with RotorGene thermocycler.

A PCR run was also performed with the QuantStudio thermocycler to assess the influence of different thermocyclers on the results. Figure 82 shows the normalized melting curves of the amplicons for the three pear cultivars obtained with the same two primer pairs. Both primer pairs, Ch02b10 and MGD154, enabled the differentiation of the three pear cultivars.

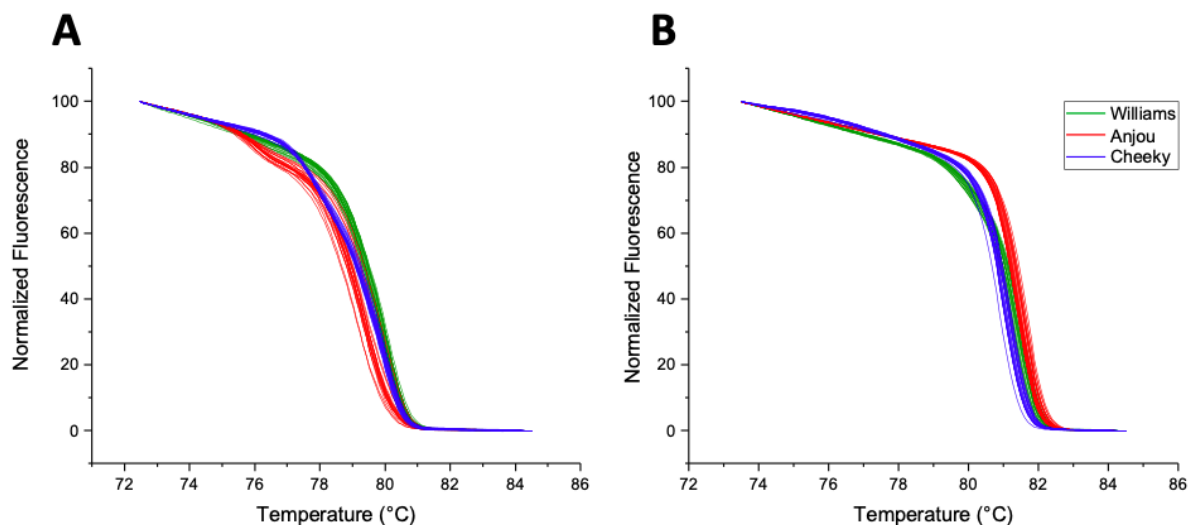


Figure 82 Normalized melting curves of the amplicons for peel, pulp and juices from the pear cultivar Williams (green), Anjou (red) and Cheeky (blue) with the primer pairs A) Ch02b10 and B) MGD154 performed with QuantStudio thermocycler.

The melting curves obtained with RotorGene and QuantStudio were very similar, which confirms the reliability of the results.

This experiment showed that the modified DNA extraction protocol using the Food Kit enabled the extraction of sufficient intact DNA from pear peel, pulp and even juices. The extracts were successfully analysed by PCR coupled with HRM using apple and pear SSR primer pairs, resulting in the clear differentiation of the three pear cultivars.

6.3.5 PCR-HRM with MeltDoctor HRM Master Mix

To differentiate the pear cultivars, PCR coupled with HRM was previously performed with the Qiagen Master Mix (2x Type-It HRM PCR Master Mix). It was of interest to investigate if the MeltDoctor HRM Master Mix from Thermo

Fisher Scientific would also allow the differentiation and to compare its performance with the Qiagen Master Mix, especially, because the MeltDoctor HRM Master Mix and the QuantStudio thermocycler are from the same manufacturer (Thermo Fisher Scientific).

For this experiment, DNA extracts from peel and pulp of all three pear cultivars (Williams, Anjou, and Cheeky) obtained from the second extraction using the Food Kit, were diluted 1:5 with nuclease free water and used in PCR with the apple primer pair CH02b10. The master mix was prepared using the same ratio as for the 2x HRM PCR Master Mix (see table 10). All extracts were analysed in duplicate. This experiment was only performed with the QuantStudio thermocycler, because the RotorGene thermocycler had been sent to the manufacturer for repair.

Table 22 lists the mean Ct-values obtained for the duplicates of the DNA extracts using both the Qiagen and Thermo Fisher Scientific Master Mixes.

Table 22 Mean Ct-values of the extracts for Williams, Anjou and Cheeky peel and pulp with Qiagen and Thermo Fisher Scientific Master Mix.

PCR HRM Master Mix	Williams		Anjou		Cheeky	
	Peel	Pulp	Peel	Pulp	Peel	Pulp
Qiagen	28.43	30.73	25.93	30.00	26.02	28.79
Thermo Fisher Scientific	26.83	29.13	25.91	30.50	25.83	28.09

Table 22 shows that the Ct-values obtained with both Master Mixes were very similar. However, this experiment was only performed once, the repeatability of the results was not assessed.

Figures 83 and 84 show the melting and normalized melting curves of the amplicons from peel and pulp of the three pear cultivars obtained with the Master Mix from Thermo Fisher Scientific (fig. 83) and from Qiagen (fig. 84).

Especially at the beginning of the HRM analysis, the fluorescence signals obtained with the Master Mix from Thermo Fisher Scientific were higher than those obtained with the Master Mix from Qiagen.

Furthermore, the temperature at the peak maximum observed with the Thermo Fisher Scientific Master Mix was lower (around 79 °C) compared to those obtained with the Qiagen Master Mix (around 80 °C). With the Thermo Fisher Scientific Master Mix, the melting curves for Williams had two peaks at 75.5 °C and 78.9 °C, whereas with the Qiagen Master Mix, only one peak was observed at 80 °C. The melting curves for Anjou had two peaks at 75.6 °C and 78 °C to 78.2 °C with the Thermo Fisher Scientific Master Mix, and two peaks at 76 °C and 79.3 °C with the Qiagen Master Mix. The melting curves for Cheeky had the peak maximum at 78.5 °C to 78.7 °C with the Thermo Fisher Scientific Master Mix and at 79.7 °C with the Qiagen Master Mix. With the Qiagen Master Mix, the normalized melting curves for Anjou showed slightly less scattering compared to the Thermo Fisher Scientific Master Mix.

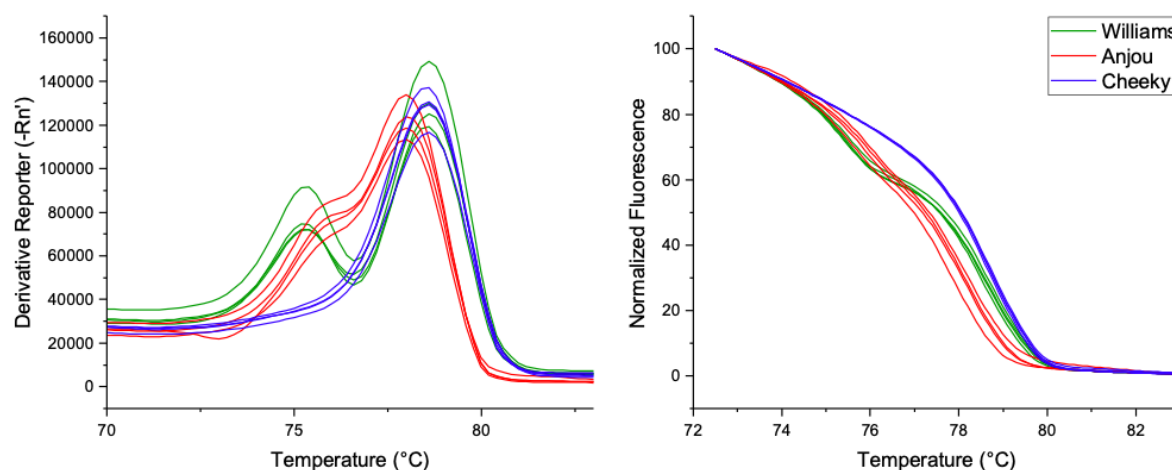


Figure 83 Melting and normalized melting curves of the amplicons for peel and pulp from Williams (green), Anjou (red) and Cheeky (blue) of the second extracts. All extracts were analysed in duplicate. PCR was performed with the MeltDoctor HRM Master Mix from Thermo Fisher Scientific.

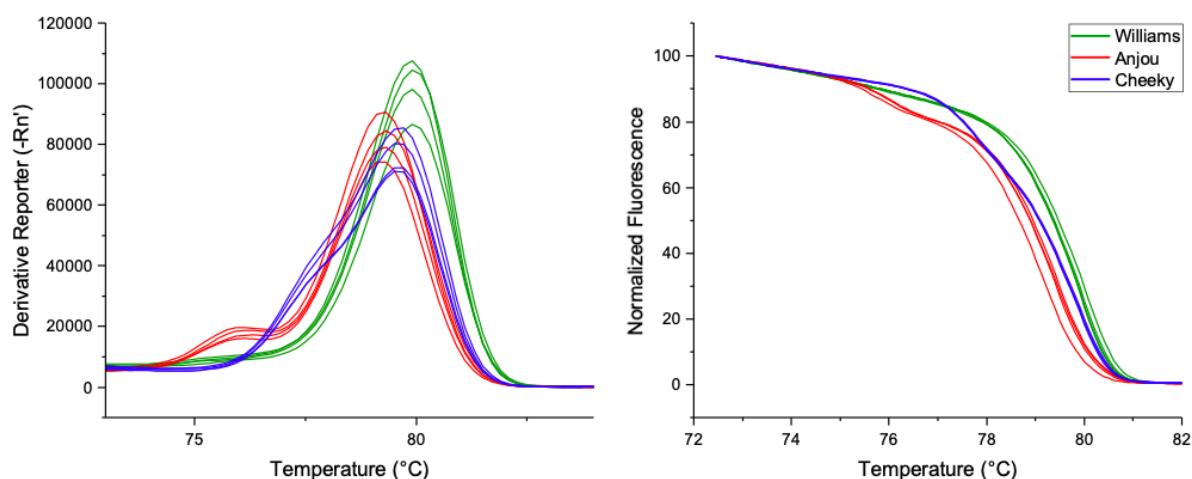


Figure 84 Melting and normalized melting curves of the amplicons for peel and pulp from Williams (green), Anjou (red) and Cheeky (blue) of the second extracts. All extracts were analysed in duplicates. PCR was performed with the 2x Type-It HRM PCR Master Mix from Qiagen.

The experiment demonstrated that using the same DNA extracts and PCR setup, but different Master Mixes, resulted in slightly different HRM results. While the Ct-values were very similar overall, the fluorescence signals were higher when using the Thermo Fisher Scientific Master Mix compared to the Qiagen Master Mix. In spite of these differences, the three pear cultivars could still be clearly distinguished with both Master Mixes.

6.3.6 Heat treatment

The heat treatment was applied to the four pear juices from Williams. The juices were pretreated by incubating them at specific temperatures for defined time periods in a thermoshaker (see table 23) before DNA extraction using the Food Kit. The pasteurisation parameters (temperature and time) were adapted from literature for laboratory use. [27, 105] The incubation period was extended, because the juices were in plastic tubes and therefore not in direct contact with the heating medium, and because the temperature of the juices itself was not monitored. DNA extraction was performed only once. The resulting DNA concentrations are listed in table 23. PCR was performed using the apple primer pair Ch02b10 and the QuantStudio thermocycler, because the RotorGene thermocycler had been sent to the manufacturer for repair. The DNA extracts were diluted 1:5 with nuclease free water and analysed in duplicate. PCR was carried out with the Qiagen Master Mix (2x Type-It HRM PCR Master Mix) and the Thermo Fisher Scientific Master Mix (MeltDoctor HRM Master Mix).

Table 23 lists the conditions of the heat treatment, the DNA concentrations of the extracts and the Ct-values (obtained for two PCR duplicates) of PCR-HRM analysis with Qiagen and Thermo Fisher Scientific Master Mix.

Table 23 DNA concentration of the extracts from Williams juices after heat treatments and the obtained Ct-values (PCR HRM carried out with Qiagen and Thermo Fisher Scientific Master Mix); F= Food Kit, W=Williams, S1= Without peel and low fibre content, S2= Without peel and high fibre content, S3= With peel and low fibre content, S4= With peel and high fibre content

Temperature [°C]	Time [min]	Juice- sample	DNA conc. [ng/μl]	Ct-value			
				Qiagen Master Mix		Thermo Fisher Scientific Master Mix	
65	30	FWS1	0.047	-	41.5	43.1	38.0
		FWS4	0.038	44.3	38.6	36.2	37.0
85	3	FWS1	0.091	38.1	41.0	35.6	36.5
		FWS2	0.056	39.7	40.8	39.1	38.0
		FWS3	0.046	-	40.3	39.1	39.4
		FWS4	0.089	38.6	39.4	35.7	37.0
85	5	FWS1	0.074	40.6	46.0	42.0	42.1
		FWS2	0.022	-	-	-	-
		FWS3	0.031	40.0	40.5	-	42.7
		FWS4	0.038	-	-	37.7	37.8
88	5	FWS1	0.041	-	40.6	48.2	-
		FWS4	0.028	-	-	-	42.3

Table 23 shows that the amplifiability was generally higher and the Ct-values were lower when using the Thermo Fisher Scientific Master Mix, compared to the Qiagen Master Mix, with the exception of FWS3 treated at 85 °C for 5 minutes and FWS1 treated at 88 °C for 5 minutes. As already shown in section 6.3.5, higher fluorescence signals were observed with the Thermo Fisher Scientific Master Mix.

The extracts from FWS2, treated at 85 °C for 5 minutes, had the lowest DNA concentration (0.022 ng/μl) and no increase in fluorescence signals was observed with either Master Mix during PCR. The extracts from FWS4, treated at 88 °C for 5 minutes, had the second lowest DNA concentration (0.028 ng/μl), and only one of the PCR duplicates produced amplicons with the Thermo Fisher Scientific Master Mix.

To compare the heat treatments, table 24 summarizes the mean DNA concentration of the extracts, the Ct-value, and the number of extracts that resulted in successful amplification (including PCR duplicates) for both the Qiagen and Thermo Fisher Scientific Master Mixes.

Only the extracts from Williams peel, pulp, and the heat treated juices were analysed using the MeltDoctor HRM Master Mix. Therefore, no Ct-values, melting curves or normalized melting curves are available for the untreated Williams juices with the MeltDoctor HRM Master Mix. The Ct-value obtained with the MeltDoctor HRM Master Mix was 26.8 for the extract from Williams peel and 29.1 for the extract from Williams pulp.

Table 24 Mean value of DNA concentration of the DNA extracts, Ct-values and number of extracts resulted in successful amplification when analysed via PCR with the Qiagen and Thermo Fisher Scientific Master Mix after different heat treatments

Temp. [°C]	Time [min]	DNA conc. [ng/μl]	Qiagen Master Mix		Thermo Fisher Scientific Master Mix	
			Ct-value	Number of DNA extracts amplified	Ct-value	Number of DNA extracts amplified
-	-	0.209	37.49	8/8 ¹	-	-
65	30	0.043	41.47	3/4 ²	39.58	4/4
85	3	0.071	39.78	7/8 ¹	37.55	8/8
85	5	0.041	41.79	4/8 ¹	40.82	5/8
88	5	0.034	40.63	1/4 ²	45.23	2/4

Note: Mean DNA concentration and Ct-value for not treated juices were taken from table 20 (second DNA extracts from Williams juices)

¹ n=8; four different juices each extract once; four different DNA extracts, each analysed twice in a PCR run (duplicates of the PCR)

² n=4; two different juices each extract once; two different DNA extracts, each analysed twice in a PCR run (duplicates of the PCR)

Figure 85 presents DNA concentrations of DNA extracts from the non-treated and treated pear juices, indicating the influence of different heat treatment conditions.

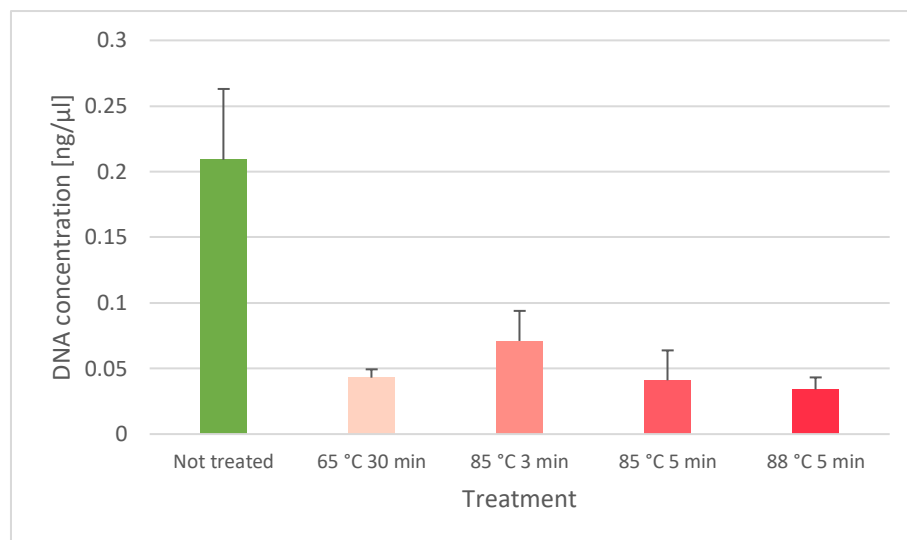


Figure 85 Mean value of DNA concentration of the extracts from the Williams juices without and with heat treatment. Standard deviations from the two to four extracts (see table 23) are represented by error bars.

According to table 24 and figure 85, heat treatment reduced the DNA concentration in the extracts and their amplifiability, while it increased the Ct-value. The extracts from juices treated at 88 °C for 5 minutes had the lowest DNA concentrations, and only a few resulted in successful amplification during PCR. The choice of Master Mix also influenced the Ct-value and amplifiability. It appears that with the Thermo Fisher Scientific Master Mix, lower Ct-values and better amplifiability were achieved, as indicated by the higher number of successful amplifications, compared to the Qiagen Master Mix. However, this experiment was conducted only once, therefore the repeatability of the results cannot be evaluated.

HRM analysis of the heat treated Williams juices with 2x Type-It HRM PCR Master Mix (Qiagen)

Heat treatment at a lower temperature (65 °C) for a longer time period (30 min) reduced the DNA concentration in the extracts more and resulted in higher Ct-values compared to heat treatment at a higher temperature (85 °C) for a shorter time period (3 or 5 min). The extracts from juices treated at 85 °C for 3 minutes had the highest DNA concentrations, lowest Ct-values, and led to positive amplification during PCR, compared to extracts which had

undergone other heat treatments. Extending the treatment duration to 5 minutes at 85 °C decreased the DNA concentration and also the amplifiability. The extracts from juices treated at 88 °C for 5 minutes showed the lowest DNA concentrations and the fewest number of successful PCR amplifications. In most cases, DNA extracted from juices treated at 85 °C and 88 °C for 5 minutes was not amplified.

Figure 86 A shows the amplification curves for Williams peel, pulp, and all juices with and without heat treatment. Only the amplification curves for peel and pulp showed the typical sigmoidal progression, reaching a plateau. The amplification curves for non-treated juices showed an increase in the fluorescence signals at a later PCR cycle than for peel and pulp and did not completely reach the plateau phase. The amplification curves for most treated juices were still in the exponential phase and did not reach the plateau phase. Figure 86 B displays the melting curves of the amplicons. The melting curves for peel, pulp, and non-treated juices showed a small increase in fluorescence before the peak maximum, whereas for the heat treated juices, this additional peak was observed in only one of the PCR duplicates from juices FWS3 and FWS4 treated at 85 °C for 3 minutes.

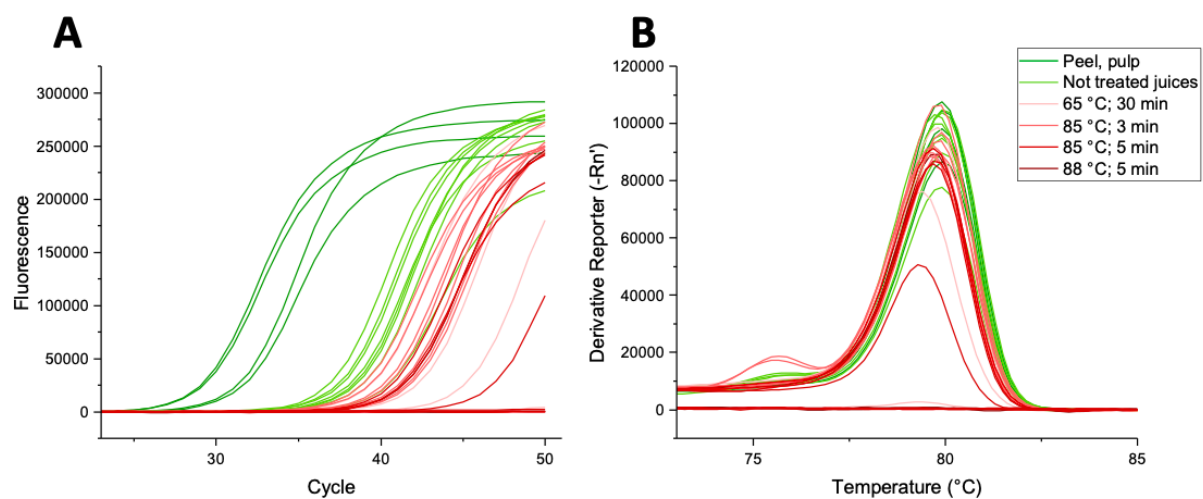


Figure 86 Amplification (A) and melting (B) curves of the amplicons from Williams peel, pulp (dark green), not heat treated juices (light green) and heat treated juices (different colours of red) with CH02b10. All extracts were analysed in duplicate.

Figure 87 shows the normalized melting curves of the amplicons from Williams peel, pulp, and the juices with and without heat treatment. DNA extracts which led to an unsuccessful PCR amplification are not shown. Most of the normalized melting curves for heat treated juices had similar curve shapes as those for peel, pulp, and non-treated juices, except for two curves obtained for juices treated at 85 °C for 5 minutes.

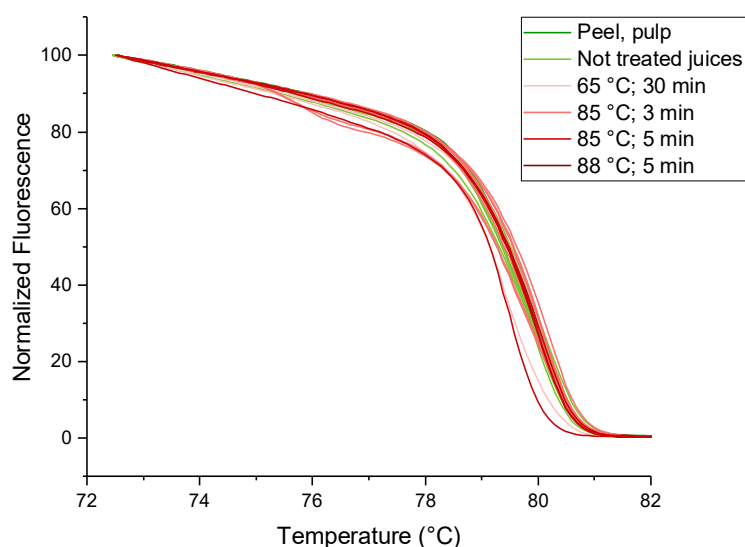


Figure 87 Normalized melting curves of the amplicons from Williams peel, pulp (dark green), not heat treated juices (light green) and heat treated juices (different colours of red) with CH02b10. All extracts were analysed in duplicate. Only the normalized melting curves for the extracts, which led to successful amplification when analysed via PCR are displayed in 87 B.

The figures 88 and 89 show the melting and normalized melting curves of the amplicons obtained for the extracts from FWS1 (figure 88; Williams juice without peel and low fibre-content) and FWS4 (figure 89; Williams juice with peel and high fibre-content) with and without heat treatment. FWS2 and FWS3 were not subjected to heat treatment at 65 °C for 30 minutes and 88 °C for 5 minutes. The curves in figures 88 and 89 show, that not only the type of the heat treatment but also the type of the juices (peel and fibre) had an impact on the HRM results, probably due to the different DNA concentrations in the extracts.

DNA from heat treated FWS1 was not amplified, except for one of the PCR duplicates from FWS1, treated at 65 °C for 30 minutes and 88 °C for 5 minutes. DNA extracts that led to an unsuccessful PCR amplification are not shown in figure 88 B (normalized melting curves). Melting curves for juices treated at 85 °C for 5 minutes had their peak maximum at a slightly lower temperature compared to the others (see fig. 88 A). With the exception of the normalized melting curves for FWS1, treated at 85 °C for 5 minutes, all normalized melting curves for FWS1 overlapped.

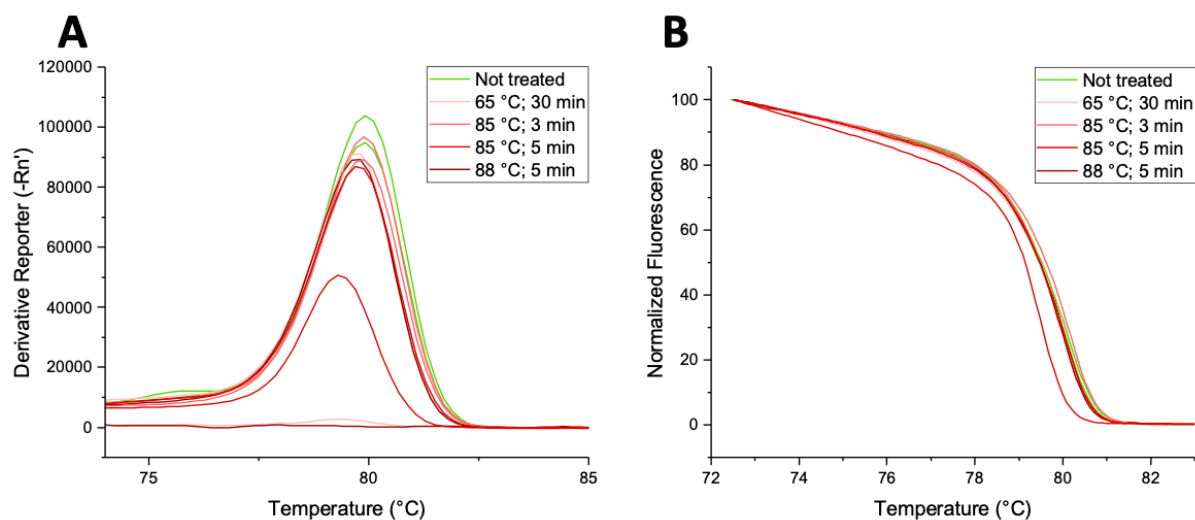


Figure 88 Melting (A) and normalized (B) curves of the amplicons obtained for FWS1 with (different colours of red) and without (green) heat treatments with CH02b10. All extracts were analysed in duplicate. Only the normalized melting curves for the extracts, which led to successful amplification when analysed via PCR are displayed in 88 B.

Only DNA from FWS4, treated at 65 °C for 30 minutes and 85 °C for 3 minutes, was successfully amplified. The fact that the DNA extracts from FWS4 treated at 85 °C and 88 °C for 5 minutes did not lead to a positive PCR amplification may be due to DNA denaturation caused by the heat treatment, which likely reduced the amount of intact DNA. The extracts from FWS4 treated at 88 °C had the lowest DNA concentration (0.028 ng/μl). The extract from FWS4 treated at 65 °C for 30 minutes resulted in the formation of PCR products, whereas the extract from FWS4 treated at 85 °C for 5 minutes did not form PCR products although both extracts had the same DNA concentration (0.038 ng/μl).

Furthermore, the melting curve of one PCR duplicate from FWS4 treated at 85 °C for 3 minutes showed an additional peak at approximately 76 °C, and for one of the duplicates of PCR from FWS4 treated at 65 °C for 30 minutes had its peak maximum at a lower temperature (79.3 °C) than the others (79.8 °C). Figure 89 B displays only the normalized melting curves of FWS4 extracts, with and without heat treatment, that resulted in successful PCR amplification. The additional peak obtained for FWS4 treated at 85 °C for 3 minutes resulted in a more complex normalized melting curve, suggesting differences in DNA integrity.

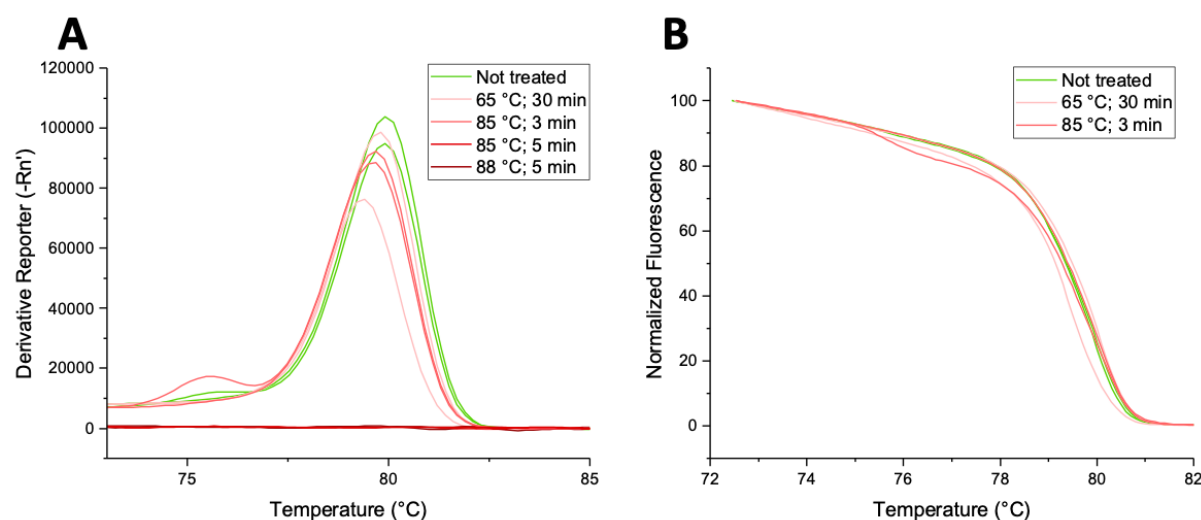


Figure 89 Melting (A) and normalized melting (B) curves of the amplicons obtained for FWS4 with (different colours of red) and without (green) heat treatments with CH02b10. All extracts were analysed in duplicate. Only the normalized melting curves for the extracts, which led to successful amplification when analysed via PCR are displayed in 89 B.

HRM analysis of the heat treated Williams juices with MeltDoctor HRM Master Mix (Thermo Fisher Scientific)

All extracts from juices treated at 65 °C for 30 minutes and 85 °C for 3 minutes led to positive amplification when subjected to PCR. The treatment at 65 °C for 30 minutes had a negative effect, because the Ct-values were even higher than those of extracts treated at 85 °C for 3 minutes. For the juices treated at 85 °C for 5 minutes, four extracts were each analysed in duplicate via PCR. However, of the eight PCRs, only five resulted in a successful amplification. Furthermore, the Ct-values were higher than those obtained for the juices treated at 65 °C for 30 minutes and 85 °C for 3 minutes. Two extracts from juices treated at 88 °C for 5 minutes were each analysed in duplicate via PCR. However, of the four PCRs performed, only two led to positive amplification and the Ct-values were high (45.2).

Figure 90 displays the amplification and melting curves for Williams peel, pulp, and the heat treated juices. Only the amplification curves for peel and pulp had the typical curve progression that reached a plateau. The amplification curves for treated juices showed an increase in fluorescence signals at later cycles and even at the end of the PCR (cycle 50), they were still in the exponential phase and did not reach the plateau phase.

The melting curves of the amplicons obtained with the MeltDoctor HRM Master Mix show that heat treatment decreased the fluorescence signals of the extracts, and the peak at 75.7 °C was either decreased or absent (see

fig. 90 B). Only one of the four melting curves obtained for juices treated at 65 °C for 30 minutes, six of the eight melting curves obtained for juices treated at 85 °C for 3 minutes, two of the eight melting curves obtained for juices treated at 85 °C for 5 minutes and none of the four melting curves obtained for juices treated at 88 °C for 5 minutes showed both peaks.

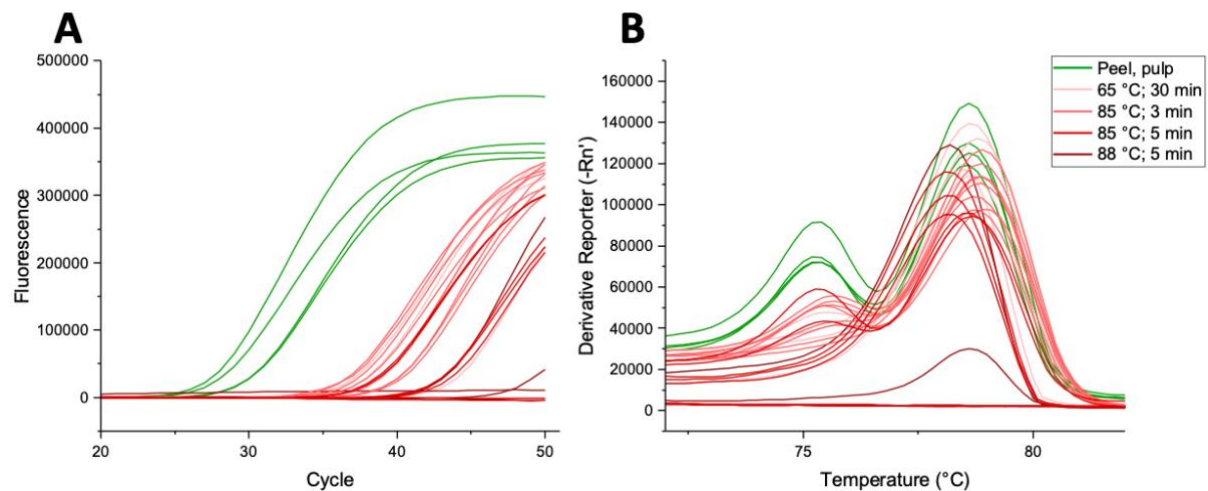


Figure 90 Amplification (A) and melting (B) curves of the amplicons from Williams peel, pulp (dark green) and heat treated juices (different colours of red with CH02b10). All extracts were analysed in duplicate.

Figure 91 displays the normalized melting curves of the extracts from Williams peel, pulp, and all heat treated juices. The normalized melting curves of the DNA extract that led to an unsuccessful PCR amplification are not shown in figure 91. The normalized melting curves for peel and pulp had a knickpoint, whereas most of the treated juices did not show this feature due to the loss of the peak at 75.7 °C (see figure 90). Furthermore, most of the normalized melting curves were shifted to higher temperatures, however, three normalized melting curves for juices treated at 65 °C for 30 minutes, 85 and 88 °C for 5 minutes were shifted to lower temperatures.

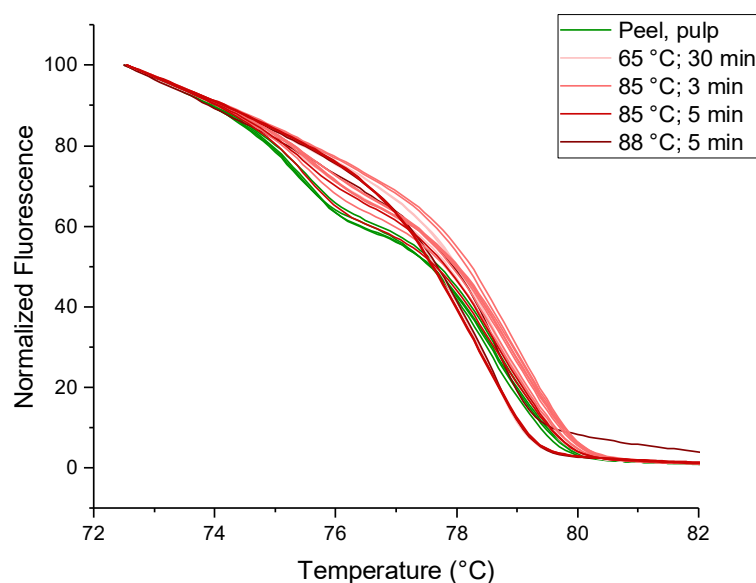


Figure 91 Normalized melting curves of the amplicons from Williams peel, pulp (dark green), not heat treated juices (light green) and heat treated juices (different colours of red) with Ch02b10. All extracts were analysed in duplicate. Only the normalized melting curves for the extracts, which led to successful amplification when analysed via PCR are displayed.

Figures 92 and 93 show the melting and normalized melting curves of the amplicons of the extracts from FWS1 (figure 63; Williams juice without peel and low fibre-content) and FWS4 (figure 64; Williams juice with peel and high

fibre-content), with and without heat treatment. The curves show that the type of the juices also influences the HRM results, probably due to the different DNA concentration in the extracts.

The normalized melting curves of the extracts from FWS1 and FWS4 treated at 85 °C for 3 minutes were similar to those from peel and pulp. However, peaks heights were lower for the treated juices at 85 °C for 3 minutes than those for peel and pulp (see fig. 92 B and 93 B).

The melting curves for FWS1 treated under different conditions (65 °C for 30 minutes, 85 °C for 5 minutes and 88 °C for 5 minutes) did not show the first peak at 75.7 °C. Melting curves for FWS4 treated at 85 °C for 5 minutes and one of the duplicates of PCR from FWS4 treated at 65 °C for 30 minutes showed the peak at 75.7 °C.

DNA from one of the PCR duplicates from FWS1 and FWS4 treated at 88 °C for 5 minutes was amplified and had its peak maximum at a different temperature than that for peel and pulp. Normalized melting curves for most of the heat treated juices did not show the complex curve shape and the repeatability was not good.

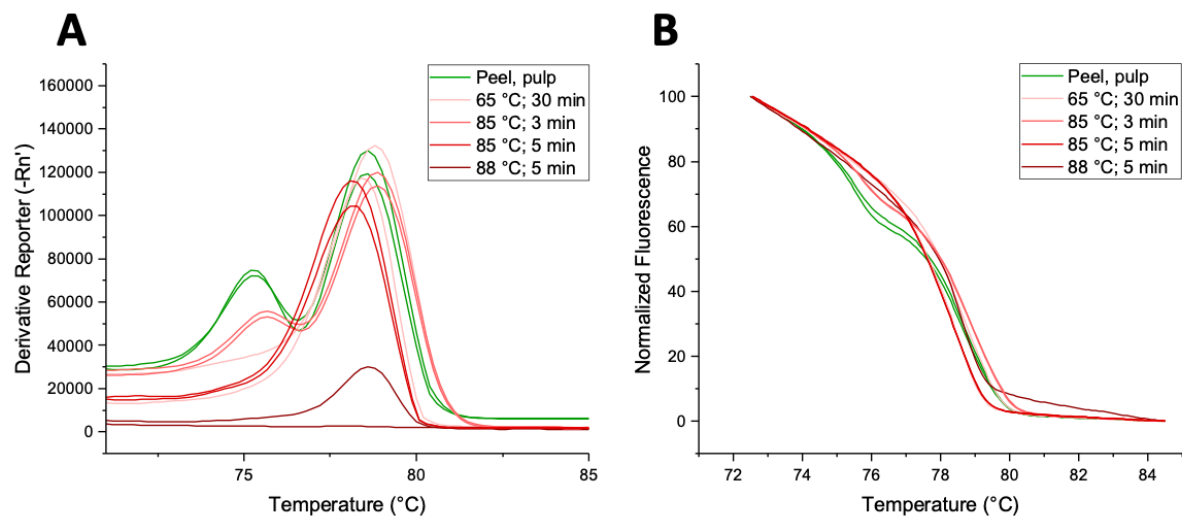


Figure 92 Melting (A) and normalized (B) curves of the amplicons of Williams peel and pulp (green) and FWS1 (different colours of red) with heat treatments with CH02b10. All extracts were analysed in duplicate. Only the normalized melting curves for the extracts, which led to successful amplification when analysed via PCR are displayed in 92 B.

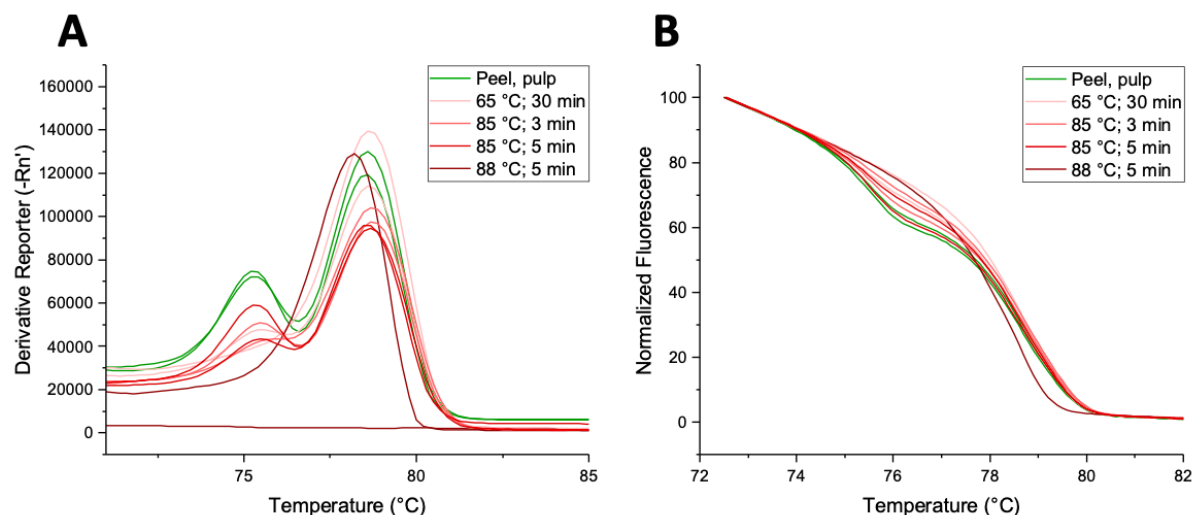


Figure 93 Melting (A) and normalized (B) curves of the amplicons of Williams peel and pulp (green) and FWS4 (different colours of red) with heat treatments with CH02b10. All extracts were analysed in duplicate. Only the normalized melting curves for the extracts, which led to successful amplification when analysed via PCR are displayed in 93 B.

The results of HRM analysis with both Master Mixes (Qiagen and Thermo Fisher Scientific) showed that DNA samples from heat treated juices had higher Ct-values and greatly reduced amplifiability compared to DNA from peel, pulp, and non-heat treated juices. However, the Master Mix from Thermo Fisher Scientific performed better

than that from Qiagen, because it resulted in slightly lower Ct-values and a higher number of successful amplifications. As mentioned before, the fluorescence signals obtained with the Thermo Fisher Scientific Master Mix were generally higher than those with the Qiagen Master Mix.

The amplifiability strongly depends on the DNA concentration, which in turn is influenced by the conditions of the heat treatment and the type of samples. Different heat treatment conditions led to varying DNA concentration. The extracts from juices treated at 85 °C and 88 °C for 5 minutes had the lowest DNA concentrations and consequently resulted in the lowest number of successful amplifications. The amplification curves of DNA samples from heat treated juices were still in the exponential phase and did not reach the plateau phase, even after 50 PCR cycles. This suggests that due to the low DNA concentration, more PCR cycles might be needed to produce sufficient amplicons to exceed the limit of detection and reach the plateau phase. However, the number of PCR cycles was already set at 50, which is higher than the standard range of 25 to 35 cycles. Increasing the PCR cycle number further is not recommended, because it can lead to the formation of unspecific PCR products. Furthermore, in the later stages of PCR, the enzyme activity of the DNA polymerase decreases due to thermal denaturation. [106]

The variations in heat treatment also resulted in a decrease in fluorescence signals, a shift in the peak maximum, and a loss of peaks in the melting curves, resulting in altered normalized melting curves. The normalized melting curves obtained with the Master Mix from Qiagen mostly showed similar curve shapes, making it suitable for cultivar differentiation. However, it is unclear how more complex normalized melting curves, e.g. melting curves with more than one peak, would look like. In contrast, the normalized melting curves obtained with the Master Mix from Thermo Fisher Scientific varied in shape for the DNA extracts from Williams, due to the loss of the first peak at 75.7 °C. This makes this Master Mix less suitable for cultivar differentiation. Based on the normalized melting curves of heat treated juices, differentiation between cultivars is probably not possible with either Master Mix, because even for the pear cultivar Williams different normalized melting curves were obtained. These variations in melting and normalized melting curves were caused by the low DNA concentration in the extracts, which in turn resulted in poor amplification.

As discussed in section 1.3.3, previous studies have shown that DNA from pasteurised food, including juices, can be successfully amplified. [50] Through pasteurisation, the amount of secondary metabolites is reduced [27], which includes PCR inhibitors. However, during processing and pasteurisation, DNA is denatured, resulting in a reduced amount of intact DNA. Nevertheless, original short DNA fragments are more heat stable than longer ones and can still be amplified by PCR. [50, 51] For improving amplification of DNA from processed food, a study suggested the usage of primer pairs targeting shorter amplicons (below 100 bp). [52]

Based on the DNA concentration and the normalized melting curves of the DNA extracts, a more effective DNA extraction method is needed to obtain more intact DNA. Higher DNA concentrations and the use of primer pairs targeting shorter amplicons [52] could improve amplification. A successful amplification, in turn, would improve the repeatability of the normalized melting curves, making them suitable for differentiating various pasteurised juices.

6.4 Williams leaf, peel, pulp, and juices

It was of interest to examine if the extracts from different components (leaf, peel, pulp, and the four juices) of the same pear cultivar resulted in similar melting and normalized melting curves. Only for the pear cultivar Williams were all four components available, therefore this comparison was not possible for other cultivars.

As shown in section 6.3.2, the melting and normalized melting curves for the extracts from peel, pulp, and the four juices were similar. However, section 6.3.2 did not include leaf. The curves for the extracts from some juices (FWS1) were more scattered, probably due to the lower DNA concentration in these extracts. For the comparison, the data from PCRs performed with the Quant Studio thermocycler using primer pairs MGD154 and Ch02b10 were selected and exported to Origin.

Figure 94 illustrates the amplification and melting curves for Williams using primer pair MGD154. The amplification curves of the extracts from leaf, peel, and pulp reached the plateau phase at approximately the same PCR cycle (cycle 35), whereas the amplification curves of the juice extracts reached the plateau only at the end of the PCR run (cycle 47). The Ct-values were 23.7 for leaf, 24.2 for peel, 26.1 for pulp, and 33.7 for the four juices. The fluorescence signals obtained for the extracts from leaf were lower, and the peak maximum was at 80.8 °C, whereas the peak maxima for the other extracts ranged between 81 °C and 81.1 °C.

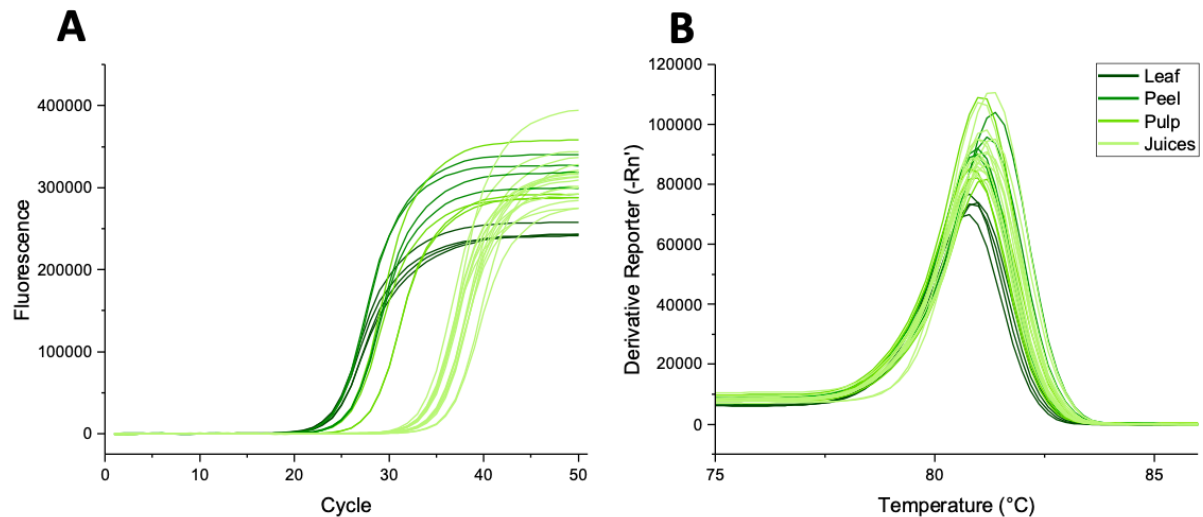


Figure 94 Amplification (A) and melting curves (B) of the amplicons of the first and second extracts from Williams leaf, peel, pulp and juices (different colours of green) with primer pair MGD154. All extracts were analysed in duplicate.

Figure 95 shows the normalized melting curves for the extracts from Williams obtained with primer pair MGD154. The normalized melting curves for the extracts from leaf had the same curve shape as the other extracts but were shifted to a slightly lower temperature.

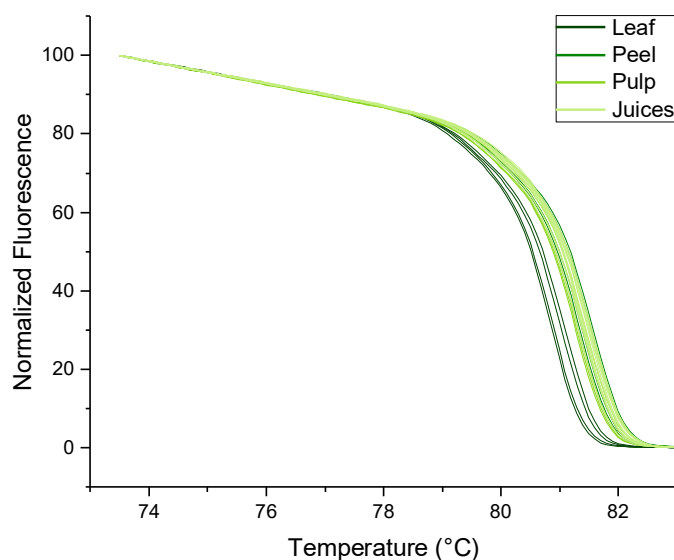


Figure 95 Normalized melting curves of the amplicons of the first and second extracts from Williams leaf, peel, pulp and juices (different colours of green) with primer pair MGD154. All extracts were analysed in duplicate.

Figure 96 displays the amplification and melting curves for Williams with apple primer pair CH02b10. The amplification curves for leaf and peel reached the plateau at approximately the same PCR cycle (cycle 35), whereas the extracts from the juices reached the plateau at the end of the PCR run (cycle 45). The Ct-values were 27.4 for

leaf, 27.9 for peel, 29.6 for pulp, and 37.4 for the four juices. The fluorescence signals obtained for the extracts from leaf were lower and the peak maximum was at 79.7 °C. Peak maxima for the other extracts were between 79.7 °C and 80.1 °C.

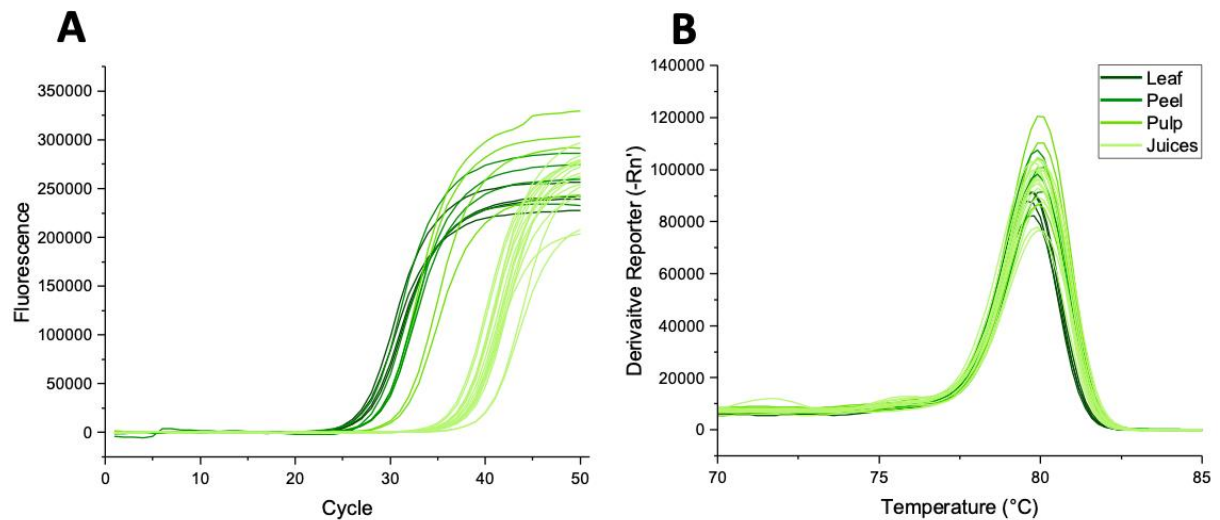


Figure 96 Amplification (A) and melting curves (B) of the amplicons of the first and second extracts Williams leaf, peel, pulp and juices (different colours of green) with primer pair Ch02b10. All extracts were analysed in duplicate.

Figure 97 illustrates the normalized melting curves for Williams with primer pair CH02b10. It shows that the normalized melting curves for the extracts from leaf had the same curve progression as the other extracts but were slightly shifted to a lower temperature.

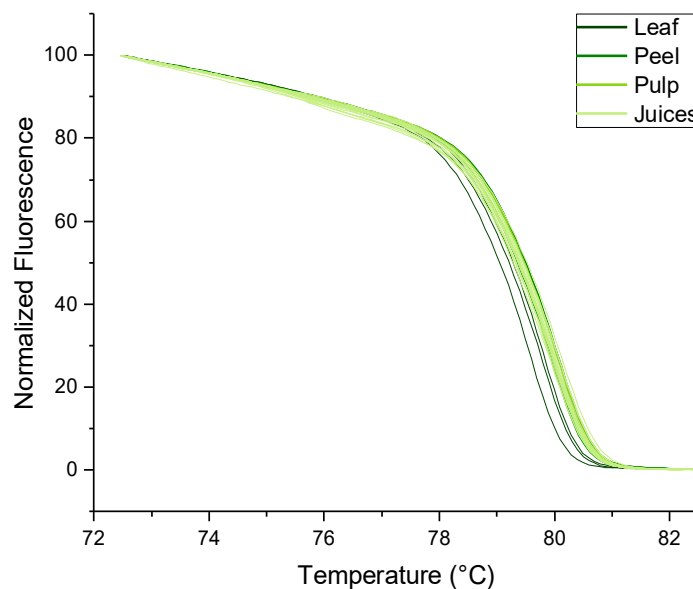


Figure 97 Normalized melting curves of the amplicons of the first and second extracts Williams leaf, peel, pulp and juices (different colours of green) with primer pair Ch02b10. All extracts were analysed in duplicate.

Although the leaf samples (Austria) originated from a different region than the fruits used for peel, pulp, and juices (Argentina), similar normalized melting curves were obtained with both primer pairs. However, the curves did not completely overlap.

The offset of the peak maximum to lower temperatures observed for the extracts from pear leaf could be due to several factors, including the use of different DNA extraction kits (Plant and Food Kit), varying DNA concentrations,

and different dilution factors. For the DNA extraction from leaf, the Plant Kit from Macherey Nagel was used, which is based on silica columns, whereas for peel, pulp, and the juices, the Food Kit by Qiagen was used, which is based on the CTAB method. Originally, the DNA concentration in the extracts from leaf was higher than those from peel, pulp, and juices (see table 14, 18 and 20), but the extracts were diluted to different extents. The DNA concentration of extracts from peel, pulp, and juices ranged from 0.132 ng/μl to 10.2 ng/μl and were diluted 1:5 with nuclease free water. The extracts from leaf were diluted to achieve a final DNA concentration of 2.5 ng/μl. The first DNA extract from Williams leaf had a DNA concentration of 32.6 ng/μl, corresponding to a 1:13 dilution, and the second 35.1 ng/μl, corresponding to a 1:14 dilution.

Another possible reason for the shift is the natural content of leaf-specific compounds, such as polysaccharides and phenolic compounds, which complicate DNA extraction (see section 5.2.1 and 6.2.1) and are known PCR inhibitors. [16, 47] However, polysaccharides and phenols are also naturally present in the fruit [3] and therefore also in juices, although in lower amounts. Juice processing results in a further reduction of secondary metabolites, including phenols. [27] PCR inhibitors are considered negative matrix effects, which are usually reduced by diluting the extracts. [104] In spite of the two or even three times higher dilution of the leaf extracts, their content of PCR inhibitors likely remains higher than in peel, pulp, and juice extracts.

As mentioned before, this experiment was only carried out with cultivar Williams, because leaves and/or fruits from the other cultivars were not available. Therefore, it is currently not known if the normalized melting curves for DNA samples from leaf, peel, pulp, and juices match for other cultivars. Based on the results for Williams, however, such similarity seems possible.

Conclusion

The differentiation of pear cultivars is of great importance to preserve traditional pear cultivars with high genetic diversity and to detect and prevent food adulteration.

In the first part of the thesis, suitable apple and pear SSR primer pairs were identified and tested for their applicability in amplifying DNA from *Pyrus* and for their suitability to differentiate between pear cultivars. From five scientific papers, 64 pear SSR primer pairs were evaluated using in silico tests and NCBI tools. Of these, six pear primer pairs fulfilled the defined criteria and showed good results in PCR combined with HRM analysis. Furthermore, 20 apple SSR primer pairs were tested using NCBI and PCR-HRM, of which nine were found to be suitable.

Subsequently, 18 pear cultivars (DNA from leaves) were analysed using the 15 selected SSR primer pairs. Both apple and pear primers were generally suitable for the differentiation of pear cultivars, however, the pear primer pairs performed slightly better. All six pear primer pairs and eight of the nine apple primer pairs successfully amplified DNA from *Pyrus communis* and *Pyrus pyrifolia*. The apple primer pairs CH01h01 and CH03c02 were considered as unsuitable due to poor repeatability and similar melting curves. With CH04c07, amplification failed for some cultivars. While the differentiation between *Pyrus communis* and *Pyrus pyrifolia* worked quite well, the differentiation of cultivars within the same species was more challenging. Nevertheless, three primer pairs were sufficient to differentiate all 18 pear cultivars. The pear primer pair MEMPc111t was the most suitable one, because it could differentiate almost 88 % of the cultivars.

In the second part, peel, pulp, and self-processed juices from three pear cultivars were analysed to assess if cultivar differentiation is possible using PCR-HRM. Since juices usually contain only low amounts of intact DNA, two DNA extraction kits, (NucleoSpin Plant II (Macherey Nagel) and DNeasy mericon Food Kit (Qiagen)) were compared to optimize the extraction protocol. The Food Kit yielded higher DNA concentrations, allowing successful differentiation of the three pear cultivars from peel, pulp, and juices using the SSR apple primer pair CH02b10 and the pear primer pair MGD154. Results were validated using a second thermocycler and a different Master Mix, confirming the repeatability and robustness of the method.

To simulate commercially available juices, the self-processed juices were heat treated under various conditions. The heat treatment reduced the DNA concentration in the extracts, leading to higher Ct-values and a lower number of successful amplifications. Furthermore, treatment at a lower temperature for a longer time period was more damaging to DNA than a shorter treatment at a higher temperature.

In the third part, the HRM curves for DNA extracts from leaf, peel, pulp, and juices of the cultivar Williams were compared using the primer pairs MGD154 and CH02b10. Although the leaves originated from Austria and the fruits from Argentina, similar normalized melting curves were obtained for all components, underscoring the reliability of genetic fingerprints and the robustness of PCR-HRM.

In summary, the results demonstrate that PCR-HRM using apple and pear SSR primer pairs can be successfully applied for the differentiation of pear cultivars (*Pyrus communis* and *Pyrus pyrifolia*) using DNA from leaves, peel, pulp, and even juices. However, for pasteurised juices, further optimization is recommended, either by improving DNA extraction methods to yield higher DNA concentrations and/or by using primer pairs targeting shorter amplicons [52]. Furthermore, it would be interesting to investigate if leaf, peel, pulp, and juices from the same cultivar consistently yield similar normalized melting curves, thereby confirming the results obtained for Williams.

References

- [1] H. Reiland, J. Slavin, 'Systematic Review of Pears and Health', *Nutrition Today*, vol. 50, no. 6, pp. 301–305, 2015.
- [2] S. Z. Hussain, B. Naseer, T. Qadri, T. Fatima, T. A. Bhat, 'Pear (*Pyrus Communis*)—Morphology, Taxonomy, Composition and Health Benefits', In *Fruits Grown in Highland Regions of the Himalayas*, Cham: Springer, pp. 35–48, 2021.
- [3] X. Li, X. Li, T. Wang, W. Gao, 'Nutritional Composition of Pear Cultivars (*Pyrus* spp.)', In *Nutritional Composition of Fruit Cultivars*, London: Elsevier Science Inc., pp. 573–608, 2016.
- [4] G. J. Silva, T. M. Souza, R. L. Barbieri, A. Costa De Oliveira, 'Origin, Domestication, and Dispersing of Pear (*Pyrus* spp.)', *Advances in Agriculture*, vol. 2014, pp. 1–8, 2014.
- [5] J. E. Jackson, 'Biology of apples and pears', Cambridge: Cambridge University Press, 2003.
- [6] M. Quinet, J. P. Wesel, 'Botany and Taxonomy of Pear' In *The Pear Genome*, Cham: Springer, pp. 1–33, 2019.
- [7] J. Postman, N. Bassil, R. Bell, 'Ploidy of USDA World Pear Germplasm Collection Determined by Flow Cytometry', *Acta Horticulturae*, no. 1094, pp. 75–81, 2015.
- [8] S.-Y. Hong, E. Lansky, S.-S. Kang, M. Yang, 'A review of pears (*Pyrus* spp.), ancient functional food for modern times', *BMC Complementary Medicine and Therapies*, vol. 21, no. 1, pp. 1–14, 2021.
- [9] H.R. Bhandari, A. Nishant Bhanu, K. Srivastava, M.N. Singh, Shreya, A. Hemantaranjan, 'Assessment of Genetic Diversity in Crop Plants - An Overview', *Advances in Plants & Agriculture Research*, vol. 7, no. 3, pp. 279–286, 2017.
- [10] J. Erfani-Moghadam, A. Zarei, 'Assessment of genetic structure among different pear species (*Pyrus* spp.) using apple-derived SSR and evidence of duplications in the pear genome', *Biotechnology & Biotechnological Equipment*, vol. 32, no. 3, pp. 591–601, 2018.
- [11] H. Claessen, W. Keulemans, B. Van de Poel, N. De Storme, 'Finding a Compatible Partner: Self-Incompatibility in European Pear (*Pyrus communis*); Molecular Control, Genetic Determination, and Impact on Fertilization and Fruit Set', *Frontiers in Plant Science*, vol. 10, no. 407, pp. 1–17, 2019.
- [12] F. J. Bielsa, P. Irisarri, P. Errea, A. Pina, 'Genetic Diversity and Structure of Local Pear Cultivars from Mountainous Areas from Aragon (Northeastern Spain)', *Agronomy*, vol. 11, no. 9, pp. 1–17, 2021.
- [13] A. Sato, Y. Sawamura, N. Takada, T. Hirabayashi, 'Relationship between inbreeding coefficients and plant height of 1-year-old seedlings in crosses among Japanese pear (*Pyrus pyrifolia* Nakai) cultivars/selections', *Scientia Horticulturae*, vol. 117, no. 1, pp. 85–88, 2008.
- [14] M. Achrem, E. Stepien, A. Kalinka, 'Epigenetic Changes Occurring in Plant Inbreeding', *International Journal of Molecular Sciences*, vol. 24, no. 5407, pp. 1–17, 2023.
- [15] S. Nishio, N. Takada, T. Saito, T. Yamamoto, H. Iketani, 'Estimation of loss of genetic diversity in modern Japanese cultivars by comparison of diverse genetic resources in Asian pear (*Pyrus* spp.)', *BMC Genetics*, vol. 17, no. 81, pp. 1–12, 2016.
- [16] S. Monschein, 'Genetische Diversität alter Apfel- und Birnensorten (*Malus x domestica* BORKH. und *Pyrus communis* L.) in der Steiermark und Teilen Sloweniens'. Dissertation, University of Graz, Graz, 2009.
- [17] Y. Teng, K. Tanabe, F. Tamura, A. Itai, 'Genetic Relationships of *Pyrus* Species and Cultivars Native to East Asia Revealed by Randomly Amplified Polymorphic DNA Marker', *Journal of the American Society for Horticultural Science*, vol. 127, no. 2, pp. 262–270, 2002.
- [18] M. Hemmat, N. F. Weeden, S. K. Brown, 'Mapping and Evaluation of *Malus x domestica* Microsatellites in Apple and Pear', *Journal of the American Society for Horticultural Science*, vol. 128, no. 4, pp. 515–520, 2003.
- [19] G. Pasqualetto, L. Palmieri, S. Martens, V. G. M. Bus, D. Chagne, C. Wiedow, M. Malnoy, S. Gardiner, 'Molecular characterization of intergeneric hybrids between *Malus* and *Pyrus*', *Horticulture Research*, vol. 10, no. 1, pp. 1–11, 2023.
- [20] S. Kadkhodaei, K. Arzani, A. Yadollahi, G. Karimzadeh, H. Abdollahi, 'Genetic Diversity and Similarity of Asian and European Pears (*Pyrus* Spp.) Revealed by Genome Size and Morphological Traits Prediction', *International Journal of Fruit Science*, vol. 21, no. 1, pp. 619–633, 2021.
- [21] A. Öztürk, L. Demirsoy, H. Demirsoy, A. Asan, O. Gül, 'Phenolic Compounds and Chemical Characteristics of Pears (*Pyrus Communis* L.)', *International Journal of Food Properties*, vol. 18, no. 3, pp. 536–546, 2015.
- [22] Y. Zhao, Y. Tian, L. Wang, G. Geng, W. Zhao, B. Hu, Y. Zhao, 'Fire blight disease, a fast-approaching threat to apple and pear production in China', *Journal of Integrative Agriculture*, vol. 18, no. 4, pp. 815–820, 2019.
- [23] R. P. Bates, J.R. Morris, P. G. Crandall, Food and Agriculture Organization of the United Nations, 'Principles and Practices of Small- and Medium-scale Fruit Juice Processing', Rome, 2001.
- [24] Codex Alimentarius Commission, 'General standard for fruit juices and nectars. No. CXS 247-2005', Rome, 2024.
- [25] D. Mudgil, S. Mudgil, 'Fruits Juice Processing' In '*Beverages: Processing and Technology*', India: Scientific Publisher, pp. 30–50, 2018.
- [26] European Union, 'Directive 2012/12/EU of the European parliament and of the council of 19 April 2012 amending Council Directive 2001/112/EC relating to fruit juices and certain similar products intended for human consumption', 2012.
- [27] Y. Chen, L. J. Yu, H. V. Rupasinghe, 'Effect of thermal and non-thermal pasteurisation on the microbial inactivation and phenolic degradation in fruit juice: a mini-review', *Journal of the Science of Food Agriculture*, vol. 93, no. 5, pp. 981–986, 2013.

- [28] F. Breidt, J. S. Hayes, J. A. Osborne, R. F. McFeeters, 'Determination of 5-Log Pathogen Reduction Times for Heat-Processed, Acidified Vegetable Brines', *Journal of Food Protection*, vol. 68, no. 2, pp. 305–310, 2005.
- [29] M. Glevitzky, I. Bogdan, G. A. Brusturean, D. Silaghi-Perju, 'Use of Pasteurization Units or Equivalent for the Quality Estimation of Fruit Juices Submitted to Different Thermal Treatments', *Chemical Buletin. "POLITEHNICA" University of Timișoara*, vol. 52, no. 66, pp. 18–20, 2007.
- [30] F. V. M. Silva, Evelyn, 'Pasteurization of Food and Beverages by High Pressure Processing (HPP) at Room Temperature: Inactivation of *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella*, and Other Microbial Pathogens', *Applied Sciences*, vol. 13, no. 1193, pp. 1–16, 2023.
- [31] 'Sterilizing Symbols (D, Z, F).', U.S. Food & Drug Administration.
<https://www.fda.gov/inspections-compliance-enforcement-and-criminal-investigations/inspection-guides/sterilizing-symbols-d-z-f>
 [accessed April 06, 2025; 11:23]
- [32] H. W. Woldemariam, S. A. Emire, 'Recent trends in cold pasteurization of fruit juices using pulsed electric fields: A review', *Agricultural and Food Sciences*, vol. 21, no. 1, pp. 1–18, 2020.
- [33] M. Keyser, I. A. Müller, F. P. Cilliers, W. Nel, P. A. Gouws, 'Ultraviolet radiation as a non-thermal treatment for the inactivation of microorganisms in fruit juice', *Innovative Food Science & Emerging Technologies*, vol. 9, no. 3, pp. 348–354, 2008.
- [34] M. E. Dasenaki, N. S. Thomaidis, 'Quality and Authenticity Control of Fruit Juices-A Review', *Molecules*, vol. 24, no. 6, pp. 1–35, 2019.
- [35] 'Economically Motivated Adulteration (Food Fraud).', U.S. Food & Drug Administration.
<https://www.fda.gov/food/compliance-enforcement-food/economically-motivated-adulteration-food-fraud> [accessed April 06, 2025; 11:23]
- [36] M. Kamruzzaman, 'Food Adulteration and Authenticity' In: 'Food Safety: Basic Concepts, Recent Issues, and Future Challenges', Cham: Springer, pp. 127–148, 2016.
- [37] M. Cichna-Markl, I. Mafra, 'Techniques for Food Authentication: Trends and Emerging Approaches', *Foods*, vol. 12, no. 1134, pp. 1–4, 2023.
- [38] G. P. Danezis, A. S. Tsagkaris¹, V. Brusic, C. A. Georgiou, 'Food authentication: state of the art and prospects', *Food Science*, vol. 10, pp. 22–32, 2016.
- [39] I. K. Karabagias, 'Food authentication and adulteration control based on metrics data of foods and chemometrics', *European Food Research and Technology*, vol. 250, pp. 1269–1283, 2024.
- [40] V. Fanelli, I. Mascio, M. M. Miazzi, M. A. Savoia, C. De Giovanni C. Montemurro, 'Molecular Approaches to Agri-Food Traceability and Authentication: An Updated Review', *Foods*, vol. 10, no. 1644, pp. 1–20, 2021.
- [41] I. Mafra, I. M. P. L. V. O. Ferreira, M. B. P. P. Oliveira, 'Food authentication by PCR-based methods', *European Food Research and Technology*, vol. 227, pp. 649–665, 2008.
- [42] A. Lanubilea, L. Stagnatia, A. Maroccoa, M. Busconi, 'DNA-based techniques to check quality and authenticity of food, feed and medicinal products of plant origin: A review', *Trends in Food Science & Technology*, vol. 149, no. 104568, pp. 1–12, 2024.
- [43] B. A. Guler, E. Imamoglu, 'Molecular marker technologies in food plant genetic diversity studies: An overview', *Foods and Raw Materials*, vol. 11, no. 2, pp. 282–292, 2023.
- [44] A. A. Hoshino, J. P. Bravo, P. M. Nobile, K. A. Morelli, 'Microsatellites as Tools for Genetic Diversity Analysis', In *Genetic Diversity in Microorganisms*, InTech, pp. 149–170, 2012.
- [45] M. C. Fischer, C. Rellstab, M. Leuzinger, M. Roumet, F. Gugerli, K. K. Shimizu, R. Holderegger, A. Widmer, 'Estimating genomic diversity and population differentiation – an empirical comparison of microsatellite and SNP variation in *Arabidopsis halleri*', *BMC Genomics*, vol. 18, no. 1, pp. 1–15, 2017.
- [46] D. T. Marinoni, P. Ruffa, V. Pavese, N. R. Giuggioli, 'Apple juice evaluation: Qualitative analysis and microsatellite traceability', *AIMS Agriculture and Food*, vol. 7, no. 4, pp. 819–830, 2022.
- [47] Y. Ren, Y. Ma, Y. Li, Y. Song, W. Zhao, X. Huang, D. Yu, J. Li, Z. Xu, W. Zhao, 'Comparative evaluation of various DNA extraction methods and analysis of DNA degradation levels in commercially marketed Chestnut rose juices and beverages', *BMC Biotechnology*, vol. 25, no. 8, pp. 1–15, 2025.
- [48] L. Fialova, D. Romanovska, I. Marova, 'A Comparative Study of Some Procedures for Isolation of Fruit DNA of Sufficient Quality for PCR-Based Assays', *Molecules*, vol. 25, no. 4317, pp. 1–15, 2020.
- [49] M. H. Rezadoost, M. Kordrostami, H. H. Kumleh, 'An efficient protocol for isolation of inhibitor-free nucleic acids even from recalcitrant plants', *3 Biotech*, vol. 6, no. 61, pp. 1–7, 2016.
- [50] N. Gryson, 'Effect of food processing on plant DNA degradation and PCR-based GMO analysis: A review', *Analytical and Bioanalytical Chemistry*, vol. 396, no. 6, pp. 2003–2022, 2010.
- [51] T. Bauer, W. P. Hammes, N. U. Haase, C. Hertel, 'Effect of food components and processing parameters on DNA degradation in food', *Environmental Biosafety Research*, vol. 3, no. 4, pp. 215–223, 2004.
- [52] L. Zhang, Y. H. Wu, J. Li, W. Li, B. Liu, G. Wu, 'A self-probing primer PCR method for detection of very short DNA fragments', *Analytical Biochemistry*, vol. 514, no. 1, pp. 55–63, 2016.

- [53] W. He, O. Laaksonen, Y. Tian, T. Haikonen, B. Yang, 'Chemical Composition of Juices Made from Cultivars and Breeding Selections of European Pear (*Pyrus communis* L.)', *Journal of Agricultural and Food Chemistry*, vol. 70, no. 16, pp. 5137–5150, 2022.
- [54] N. Gupta, 'DNA extraction and polymerase chain reaction', *Journal of Cytology*, vol. 36, no. 2, p. 116–117, 2019.
- [55] B. B. Kalbande, V. L. Ghonge, S. R. Sutar, S. V. Sable, M. D. Meshram, J. Warade, 'Principals of DNA Extraction', *Agrobios*, vol. 11, no. 51, pp. 6–8, 2012.
- [56] X. Ye, B. Lei, 'The current status and trends of DNA extraction', *BioEssays*, vol. 45, no. 8, pp. 1–11, 2023.
- [57] M. Dairawan, P. J. Shetty, 'The Evolution of DNA Extraction Methods', *American Journal of Biomedical Science and Research*, vol. 8, no. 1, pp. 1–14, 2020.
- [58] C. Katevatis, A. Fan, C. M. Klapperich, 'Low concentration DNA extraction and recovery using a silica solid phase', *PLoS One*, vol. 12, no. 5, pp. 1–11, 2017.
- [59] J. J. Schenk, L. E. Becklund, S. J. Carey, P. P. Fabre, 'What is the "modified" CTAB protocol? Characterizing modifications to the CTAB DNA extraction protocol', *Applications in Plant Sciences*, vol. 11, no. 3, pp. 1–11, 2023.
- [60] B. Bruijns, T. Hoekema, L. Oomens, R. Tiggelaar, H. Gardeniers, 'Performance of Spectrophotometric and Fluorometric DNA Quantification Methods', *Analytica*, vol. 3, no. 3, pp. 371–384, 2022.
- [61] B. Foster, 'A Guide to DNA and RNA Quantification and Quality.', Technology Networks: Genomics Research. <https://www.technologynetworks.com/genomics/articles/a-guide-to-dna-and-rna-quantification-and-quality-392900> [accessed April 06, 2025; 13:24]
- [62] A. Bose, I. Thomas, G. Kavitha, E. Abraham, 'Fluorescence spectroscopy and its applications: A Review', *International Journal of Advances in Pharmaceutical Analysis*, vol. 8, no. 1, pp. 1–8, 2018.
- [63] R. Kotadiya, 'Illuminating the analytical chemistry Profile: A Three-Decade comprehensive review of fluorometric quantitation in pharmaceutical formulations', *Microchemical Journal*, vol. 208, no. 112568, pp. 1–23, 2025.
- [64] X. Li, Y. Wu, L. Zhang, Y. Cao, Y. Li, J. Li, L. Zhu, G. Wu, 'Comparison of three common DNA concentration measurement methods', *Analytical Biochemistry*, vol. 45, pp. 18–24, 2014.
- [65] K. Rengarajan, S. M. Cristol, M. Mehta, J. M. Nickerson, 'Quantifying DNA concentrations using fluorometry: A comparison of fluorophores', *Molecular Vision*, vol. 8, pp. 416–421, 2002.
- [66] A. I. Dragan, R. Pavlovic, J. B. McGivney, J. R. Casas-Finet, E. S. Bishop, R. J. Strouse, M. A. Schenerman, C. D. Geddes, 'SYBR Green I: Fluorescence Properties and Interaction with DNA', *Journal of Fluorescence*, vol. 22, pp. 1189–1199, 2012.
- [67] E. B. A. Kim, 'PicoGreen assay for nucleic acid quantification - Applications, challenges, and solutions', *Analytical Biochemistry*, vol. 692, no. 115577, pp. 1–9, 2024.
- [68] H. Zipper, H. Brunner, J. Bernhagen F. Vitzthum, 'Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications', *Nucleic Acids Research*, vol. 32, no. 12, pp. 1–10, 2004.
- [69] A. Dove, 'PCR: Thirty-five years and counting', *Science*, vol. 360, no. 6389, pp. 670–673, 2018.
- [70] A. S. M. Giasuddin, 'Polymerase Chain Reaction Technique: Fundamental Aspects and Applications in Clinical Diagnostics', *Journal of Islamic Academy of Sciences*, vol. 8, no. 1, pp. 29–32, 1995.
- [71] L. Garibyan, N. Avashia, 'Research Techniques Made Simple: Polymerase Chain Reaction (PCR)', *Journal of Investigative Dermatology*, vol. 133, no. 3, pp. 1–4, 2013.
- [72] B. Druml, M. Cichna-Markl, 'High resolution melting (HRM) analysis of DNA – Its role and potential in food analysis', *Food Chemistry*, vol. 158, pp. 245–254, 2014.
- [73] K. A. Abd-Elsalam, 'Bioinformatic tools and guideline for PCR primer design', *African Journal of Biotechnology*, vol. 2, no. 5, pp. 91–95, 2003.
- [74] N. Kim, J.-S. Kwon, W.-H. Kang, S.-I. Yeom, 'High-Resolution Melting (HRM) Genotyping', In *Plant Genotyping*, New York: Springer, pp. 337–349, 2023.
- [75] C. W. Dieffenbach, T. M. J. Lowe, G. S. Dveksler, 'General Concepts for PCR Primer Design', *Genome Research*, vol. 3, pp. 30–37, 1993.
- [76] 'PCR Primer Design Guidelines.', Premier Biosoft. http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html [accessed April 06, 2025; 13:24]
- [77] Biotium, 'EvaGreen® Dye: The Swiss Army Knife of qPCR', *The Scientist*, 2024.
- [78] I. M. Artika, Y. P. Dewi, I. M. Nainggolan, J. E. Siregar, U. Antonjaya, 'Real-Time Polymerase Chain Reaction: Current Techniques, Applications, and Role in COVID-19 Diagnosis', *Genes*, vol. 13, no. 12, p. 1–22, 2022.
- [79] K. Dirican, 'Zygosity: Implications for Genetics Research and Health Care', *Journal of Genetics and DNA Research*, vol. 7, no. 2, 2023.
- [80] N. M. Sanabria, M. Gulumian, 'The use of HRM shifts in qPCR to investigate a much neglected aspect of interference by intracellular nanoparticles', *PLOS One*, vol. 16, no. 12, pp. 1–22, 2021.
- [81] P. Rabindra and N. Raju, 'Gel-Electrophoresis and Its Applications', In *Gel Electrophoresis - Principles and Basics*, InTech, pp. 15–32, 2012.
- [82] J. Scheibelreiter, 'Sortendifferenzierung von Sonnenblumen und Weinreben mittels hochauflösender Schmelzkurvenanalyse', Master's Thesis, University of Vienna, Vienna, 2024.
- [83] J. Bai, E. Baldwin, H. L. Ling, W. Zhao, I. Kostenyuk, J. Burns, M. Irey, 'Extraction of DNA from Orange Juice, and

- Detection of Bacterium *Candidatus Liberibacter asiaticus* by Real-Time PCR', *Journal of Agriculture and Food Chemistry*, vol. 61, no. 39, pp. 9339–9346, 2013.
- [84] Qiagen, 'DNeasy mericon Food Handbook'.
- [85] F. Fernández-Fernández, N. G. Harvey, C. M. James, 'Isolation and characterization of polymorphic microsatellite markers from European pear (*Pyrus communis* L.)', *Molecular Ecology Notes*, vol. 6, no. 4, pp. 1039–1041, 2006.
- [86] T. Yamamoto, T. Kimura, Y. Sawamura, T. Manabe, K. Kotobuki, T. Hayashi, Y. Ban, N. Matsuta, 'Simple sequence repeats for genetic analysis in pear', *Euphytica*, vol. 124, pp. 129–137, 2022.
- [87] T. Yamamoto, T. Kimura, M. Shoda, Y. Ban, T. Hayashi, N. Matsuta, 'Development of microsatellite markers in the Japanese pear (*Pyrus pyrifolia* Nakai)', *Molecular Ecology Notes*, vol. 2, pp. 14–16, 2002.
- [88] 'BLAST Statistics: The Expect Value.', NIH National Library of Medicine.
https://www.nlm.nih.gov/ncbi/workshops/2023-08_BLAST_evol/e_value.html [accessed April 06, 2025; 14:26]
- [89] C. Guarino, S. Santoro, L. de Simone, O. Lain, C. Cipriani, R. Testolin, 'Genetic diversity in a collection of ancient cultivars of apple (*Malus x domestica* Borkh.) as revealed by SSR-based fingerprinting', *Journal of Horticultural Science & Biotechnology*, vol. 81, no. 1, pp. 39–44, 2006.
- [90] S. C. Hokanson, W. F. Lamboy, A. K. Szewc-McFadden, J. R. McFerson, 'Microsatellite (SSR) variation in a collection of *Malus* (apple) species and hybrids', *Euphytica*, vol. 118, no. 3, pp. 281–294, 2001.
- [91] R. Liebhard, L. Gianfranceschi, B. Koller, C. D. Ryder, R. Tarchini, E. Van De Weg, C. Gessler, 'Development and characterisation of 140 new microsatellites in apple (*Malus x domestica* Borkh.)', *Molecular Breeding*, vol. 10, pp. 217–241, 2002.
- [92] A. Patocchi, F. Fernández-Fernández, K. Evans, D. Gobbin, F. Rezzonico, A. Boudichevskaia, F. Dunemann, M. Stankiewicz-Kosyl, F. Mathis-Jeanneteau, C. E. Durel, L. Gianfranceschi, F. Costa, C. Toller, V. Cova, D. Mott, M. Komjanc, E. Barbaro, L. Kodde, E. Rikkerink, C. Gessler, W. E. van de Weg, 'Development and test of 21 multiplex PCRs composed of SSRs spanning most of the apple genome', *Tree Genetics & Genomes*, vol. 5, pp. 211–223, 2009.
- [93] E. Silfverberg-Dilworth, C. L. Matasci, W. E. Van de Weg, M. P. W. Van Kaauwen, M. Walser, L. P. Kodde, V. Soglio, L. Gianfranceschi, C. E. Durel, F. Costa, T. Yamamoto, B. Koller, C. Gessler, A. Patocchi, 'Microsatellite markers spanning the apple (*Malus x domestica* Borkh.) genome', *Tree Genetics & Genomes*, vol. 2, pp. 202–224, 2006.
- [94] P. Guilford, S. Prakash, J. M. Zhu, E. R. S. Gardiner, H. Bassett, R. Forster, 'Microsatellites in *Malus X domestica* (apple): abundance, polymorphism and cultivar identification', *Theoretical and Applied Genetics*, vol. 94, pp. 249–254, 1997.
- [95] HiDRAS Database.
https://sites.unimi.it/camelot/hidras/HiDRAS-SSRdb/pages/marker_display.php?SelectedSSR=Hi04a02 [accessed July 26 2022 by Mina Micic [95]]
- [96] M. Micic, 'Analyse von Mikrosatelliten mittels hochauflösender Schmelzkurvenanalyse zur Diskriminierung von sterischen Apfelsorten', Master's Thesis, University of Vienna, Vienna, 2021.
- [97] M. Wildbacher, 'Authentizitätsprüfung von Lebensmitteln mittels hochauflösender Schmelzkurvenanalyse', Master's Thesis, University of Vienna, Vienna, 2023.
- [98] BLAST NCBI. <https://blast.ncbi.nlm.nih.gov/Blast.cgi> [accessed June 01, 2025]
- [99] M. H. Rezadoost, M. Kordrostami, H. H. Kumleh, 'An efficient protocol for isolation of inhibitor-free nucleic acids even from recalcitrant plants', *3 Biotech*, vol. 6, no. 61, pp. 1–6, 2016.
- [100] S. H. Yim, S. H. Nam, 'Physiochemical, Nutritional and Functional Characterization of 10 Different Pear Cultivars (*Pyrus* Spp.)', *Journal of Applied Botany and Food Quality*, vol. 89, pp. 73–81, 2016.
- [101] T. Rühmer, 'Prüfung neuer Birnensorten', *Haidegger Perspektiven: Abteilung 10 - Land- und Forstwirtschaft*, 2016.
- [102] I. Niederkofler, 'Differentiation of 24 Chickpea Cultivars by Targeting Microsatellites and Single Nucleotide Polymorphisms using High Resolution Melting Analysis', Master's Thesis, University of Vienna, Vienna, 2024.
- [103] A. Zeb, F. Rahman, 'Phenolic profile, total bioactive contents, and antioxidant activity of pear fruits', *Food Chemistry*, vol. 5, no. 100780, pp. 1163–1170, 2024.
- [104] J. Hedman, M. Lavander, N. Salomonsson, T. Jinnerot, L. Boiso, B. Magnusson, P. Rådström, 'Validation guidelines for PCR workflows in bioterrorism preparedness, food safety and forensics', *Accreditation and Quality Assurance*, vol. 23, pp. 133–144, 2018.
- [105] C. M. G. C. Renard, J. F. Maingonnat, 'Thermal Processing of Fruits and Fruit Juices', In *Thermal Food Processing: New technologies and qualities issues*, Boca Raton: CRC Press Publishing, pp. 413–429, 2012.
- [106] S. M. H. Mubarak, D. A. F. Al-Koofee, O. A. Radhi, J. M. Ismael, Z. F. Al-Zubaidi, 'An Optimization and Common Troubleshooting Solving in Polymerase Chain Reaction Technique', *Systematic Reviews in Pharmacy*, vol. 11, no. 2, pp. 427–436, 2020.

Appendix

List of abbreviations

μM	Micro molar
μl	Micro litre
A	Adenine
Abbrev.	Abbreviation
A.D.	Anno domini
AFLP	Amplified fragment length polymorphism
AL	Amplicon length
B.C.	Before Christ
BLAST	Basic local alignment search tool
bp	Base pair
C	Cytosine
CFR	Code of federal regulations
Conc.	Concentration
Ct	Threshold cycle
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
e.g.	Exempli gratia
EU	European union
F	Fahrenheit
FDA	Food and drug administration
FRET	Fluorescence resonance transfer
FTIR	Fourier transform infrared spectrometry
g	Gramm
G	Guanine
h	hours
GAP	Good agriculture practise
GI	Glycemic index
GIS	Gametophytic self-incompatibility
HHP	High hydrostatic pressure
HPLC	High performance liquid chromatography
HPP	High pressure processing
HRM	High resolution melting
IS	Self-incompatibility
log	Logarithm
MFE	Minimum free energy
mg	Milligram
min	Minute
ml	Millilitre
MPa	Megapascal
MS	Mass spectrometry
NCBI	National centre for biotechnology information
NGS	Next generation sequencing
ng	Nanogram
nm	Nanometre
NMR	Nuclear magnetic resonance spectroscopy
NTC	No template control
PCR	Polymerase chain reaction
PEF	Pulsed electric field
qPCR	Quantitative polymerase chain reaction
RAPD	Random amplified polymorphic deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid

SNP	Single nucleotide polymorphism
ssDNA	Single stranded deoxyribonucleic acid
SSR	Simple sequence repeat
T	Thymine
Temp.	Temperature
TBE	Tris-Borate-EDTA
T _m	Melting temperature
US	United States
UV	Ultraviolet

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