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Polymerase chain reaction and chemometrics detected several *Pinus* species including *Pinus armandii* involved in pine nut syndrome

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ABSTRACT

A dramatic peak in reported cases of pine mouth or pine nut syndrome (PNS) was observed in Europe and in the United States of America in 2008–2012. The PNS symptoms involve a constant bitter and/or metallic taste that appear 1–2 days after ingestion and disappear within 5–14 days. The chemical compound responsible for the symptoms is unknown, but symptoms are related to ingestion of pine nuts from the species *Pinus armandii*. *P. armandii* used industrially for non-food purposes has entered the food chain through mislabeling. Consequently, species determination of pine nuts has gained focus in governmental control of food authenticity. In this study, a PCR primer design targeted conserved DNA sequences that span an area of variation between *P. armandii* and other relevant species. Principal component analysis (PCA) of high-resolution melting curves from PCR amplicons was used to cluster pine species from reference material, and to determine the species of unknown samples. The PCA successfully clustered 2 subspecies/varieties of *P. armandii*, *Pinus bungeana*, *Pinus massoniana*, *Pinus pinea*, and *Pinus wallichiana*. *Pinus koraiensis*/*Pinus pumila* and *Pinus sibirica*/*Pinus cembra* had identical PCR amplicons, respectively, and formed 2 distinct clusters. 12 pine nuts from 4 unknown samples were analyzed. 10 pine nuts clustered together with *P. armandii* and *P. koraiensis*/*P. pumila*. 2 pine nuts were not part of clusters, but probabilities suggested *P. armandii*, and *P. sibirica*/*P. cembra*. These determined species were comparable to external results obtained elsewhere.

1. Introduction

Pine nut syndrome (PNS), first described in the scientific literature as taste disturbances in 2001 (Mostin, 2001), was followed by an absent scientific focus for several years. It was not until a dramatic peak in reported cases of PNS between 2008 and 2012, that it became more broadly known to the scientific community. In France, 3000 cases were reported in 2009 (Flesch et al., 2011) and, in the United States of America 501 cases were reported from 2008 to 2012 (Kwegyir-Afful et al., 2013). It is obvious that the vast majority of cases were unreported due to a lack of linkage between pine nut ingestion and the symptom involved. In general, the PNS symptoms appear 1–2 days after ingestion and disappear within 5–14 days; involving a constant bitter and/or metallic taste intensified upon ingestion of certain foods (Hampton, Scully, Gandhi, & Raber-Durlacher, 2013; Kwegyir-Afful et al., 2013; Munk, 2010). During PNS symptoms, exclusion of certain foods may feel necessary for some. No spontaneous relapse or other side effects were shown within a year (Ballin, 2012). The agent responsible for the symptoms is unknown, but symptoms are related to ingestion of pine nuts from the species *Pinus armandii* (Destaillats, Cruz-Hernandez, Giuffrida, & Dionisi, 2010; Destaillats et al., 2011; Kobler et al., 2011; Mikkelsen, Jessen, & Ballin, 2014; Zonneveld, 2011). *P. armandii*, used for industrial non-food purposes, have entered the food chain because of mislabeling. In addition, fraudulent substitution or admixtures might also occur as pine nuts from different species are differently prized. The problems with *P. armandii* and PNS have showcased the need for authentication of pine nut species, especially within the closely related pine nut species from the genus *Pinus*. *P. armandii* is one out of more than 100 different species from the genus *Pinus* that exist worldwide. Not all pine nuts are regarded edible, and only 29 pine species produce edible nuts according to the Food and Agriculture Organization (FAO) http://www.fao.org/docrep/x0453E/X0453e12.htm. The genus *Pinus* encompasses two subgenera (Gernandt, López, García, & Aaron, 2005). The subgenus *Strobus* includes species, such as *P. armandii*, *Pinus bungeana*, *Pinus cembra*, *Pinus koraiensis*,

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Pinus pumila, *Pinus sibirica*, and *Pinus wallichiana*. The subgenus *Pinus* includes *Pinus densata*, *Pinus massoniana*, *Pinus pinea*, *Pinus tabuliformis*, and *Pinus yunnanensis*. Sections and subsections further divide each subgenus. Pine nuts from several of the above-mentioned species are visually interchangeable, addressing the need for non-morphological authentication methods.

Genome size (Zonneveld, 2011), polymerase chain reaction (PCR) (Handy, Timme, Jacob, & Deeds, 2013), and DNA sequencing (Handy et al., 2011) are published molecular biology techniques, whereas, nuclear magnetic resonance (Kobler et al., 2011) and gas chromatographic analysis of fatty acids (Destaillats et al., 2010, 2011) coupled with principal component analysis (PCA) (Mikkelsen et al., 2014) are published chemical techniques in pine species determination. So far, no specific chemical compound is identified in *P. armandii*, and species determination through chemical techniques is, therefore, primarily performed through fingerprinting (Kobler et al., 2011) and profiling methods (Mikkelsen et al., 2014). Consequently, pine nuts subjected to these methods should belong to a single species to avoid ambiguous results because of mixed species. It is, therefore, important to morphologically separate the pine nuts prior to analysis or perform the analysis on single pine nuts. Fortunately, DNA based methods offer an advantage, as different species have different DNA. Handy et al. (2013) developed a rapid streamlined PCR assay based on locked nucleic acid that determined *P. armandii* in mixtures of different species and in single pine nuts. Nevertheless, PCR monitoring systems that can detect several pine species are interesting for authentication purposes and homogeneity of pine nut batches. High-resolution melting (HRM) curve analysis of PCR amplicons has shown potential in single nucleotide differences (Royo, Muniz, & Hueros, 2011), and its applicability has been demonstrated in detection of allergens in food (Costa, Mafra, & Oliveira, 2012). In this study, we have tried to design PCR primers that amplify several pine species, producing amplicons of different size and sequence. These amplicon differences combined with HRM curve analysis and PCA were explored for a rapid discrimination of several pine nut species.

2. Materials and methods

2.1. Pine nut reference material

Reference material from common commercial pine nuts and from pine nuts in close relation to *P. armandii* was gathered and named RM1 – RM12. Reference materials from the subgenus *Strobus* included 3 x *P. armandii* (RM1 – RM3), *P. bungeana* (RM4), *P. cembra* (RM5), 2 x *P. koraiensis* (RM6, RM7), *P. pumila* (RM8), *P. sibirica* (RM9), and *P. wallichiana* (RM10). Reference material from the subgenus *Pinus* included *P. massoniana* (RM11) and *P. pinea* (RM12). Pine species, reference material number, geographical origin, year of harvest, and company name are presented in Table 1. Three randomly chosen pine nuts from each reference material and their HRM curves examined species uniformity.

2.2. Pine nut and cross-reactivity samples

Four samples (S1 – S4) were chosen for investigation. Samples S1 – S3 were collected as a part of national regulatory control and originated from batches involved in PNS consumer complaints in 2010 (S1) and 2011 (S2, S3). To include a proposed *P. armandii* species, sample S4 was bought as *P. armandii* from a Chinese web shop. Pine nut samples might be heterogeneous in respect to species, and three pine nuts from each sample were, therefore, randomly chosen, and labeled $S1_{a,b,c}$, $S2_{a,b,c}$, etc. Each of the 12 pine nuts from samples S1 – S4 were individually analyzed with the presented method and compared to results from a chemical species determination method performed earlier on the same reference material and samples (Mikkelsen et al., 2014).

To demonstrate in vitro specificity, PCR analyses of common foods were included as a cross-reactivity assay. The negative controls for the cross-reactivity assay included almond, cashew nut, hazelnut, peanut, walnut, and soybean; all officially sampled for routine purposes in other control areas.

2.3. PCR primer design and DNA alignment

To ensure a low detection limit, primers were targeted towards mitochondrial DNA (Ballin, Vogensen, & Karlsson, 2009). In relation to species, primers targeted *P. armandii* and other morphologically similar pine nuts from the subgenus *Strobus*, including *P. bungeana*, *P. cembra*, *P. koraiensis*, *P. pumila*, *P. sibirica*, and *P. wallichiana*. Their different DNA sequences were obtained from the NCBI webpage (http://www.ncbi.nlm.nih.gov/) and aligned with the ClustalW software (http://www.genome.jp/tools/clustalw/) to find suitable sequences for primer design. Preferably, sequences should have conserved regions for primer design, and in addition spanning species-specific variations enabling species determination of several other species. An attempt to design primers with the same specifications in respect to length, melting temperature (T_m), and the combined content of guanine (G) and cytosine (C) was done. In addition, amplicon size from the different species should be as short as possible to optimize separation of the PCR amplicon T_{ms} (Reed, Kent, & Wittwer, 2007).

The ClustalW alignment of different mitochondrial sequences from various pine species showed an area of variations suitable for primer design in the NADH subunit 5 (nad5) region. This sequence area enabled us to design primers that span a sequence unique for *P. armandii* and with several species-specific variations that provides the possibility to determine several other species. Primers named Pin4 and Pin5 were equal in length, T_m, and GC content, Table 2. The Eurofins MWG Operon (Ebersberg, Germany) provided the primers.

The alignment of the sequence spanning the primers Pin4 and Pin5 from the subgenus *Strobus*, including *P. armandii* (DQ983609.1, AB455857.1), *P. bungeana* (EU369320.1), and *P. wallichiana* (AB455864.1) showed size and/or sequence differences between the species. The alignment of *P. koraiensis* (AB675846.1)/*P. pumila* (AB455868.1) and *P. cembra* (AB455866.1)/*P. sibirica* (AB455869.1) showed no sequence differences, respectively, Table 3.

Pine nuts from the subgenus *Pinus* also contain edible species available on the commercial marked. It would therefore be interesting to include these in the species determination. An alignment of the sequences spanning the primers Pin4 and Pin5 was performed on the species *P. densata* (HM467728.1), *P. massoniana* (FJ906702.1), *P. pinea* (KM233704), *P. tabuliformis* (EU369315.1), and *P. yunnanensis* (EU369318.1). Compared to the species from the subgenus *Strobus*, *P. densata*, *P. massoniana, P. tabuliformis*, and *P. yunnanensis* from the subgenus *Pinus* had a conserved 46-nucleotide insertion. With the exception of *P. pinea*, species from the subgenus *Pinus* were found to have a nucleotide mismatch in the sequence corresponding to primer Pin4 (Supplementary data). The *P. pinea* sequence (Ballin, 2014) had no primer Pin4 mismatch but lacked a 60-nucleotide region compared to the other species from the subgenus *Pinus*. Instead, a *P. pinea* insertion of 107 nucleotides was present, see Supplementary data. The *P. pinea* difference compared to the *P. densata*, *P. massoniana*, *P. tabuliformis*, and *P. yunnanensis* was in agreement with the phylogenetic relationship. *P. densata*, *P. massoniana*, *P. tabuliformis*, and *P. yunnanensis* belong to the subsection *Pinus*, which is distinct from the subsection *Pinaster* encompassing *P. pinea* (Frankis, 1993; Wang, Tsumura,

Table 1
Species of reference material, reference number, geographical origin, year of harvest, and company name.

Pine species, from the genus of *Pinus*	Reference material	Geographical origin	Year of harvest	Company name
Subgenus of *Strobus*				
P. armandii	RM1	China	2010	International Nut & Dried Fruit, Spain
P. armandii	RM2	United Kingdom	2011	Sandeman Seeds, UK
P. armandii	RM3	China	[a]	F.W. Schumacher, USA
P. bungeana	RM4	China	2010	OMC Seeds, Lithuania
P. cembra	RM5	Germany	2010/2011 (mixed)	OMC Seeds, Lithuania
P. koraiensis	RM6	Denmark	2011	University of Copenhagen, Denmark
P. koraiensis	RM7	China	2010	International Nut & dried Fruit, Spain
P. pumila	RM8	Russia	2010	Prime Seeds, Denmark
P. sibirica	RM9	China	2010	International Nut & Dried Fruit, Spain
P. wallichiana	RM10	Kashmir	2010	Prime Seeds, Denmark
Subgenus of *Pinus*				
P. massoniana	RM11	China	2009	Prime Seeds, Denmark
P. pinea	RM12	[b]	[b]	Supermarket, Denmark

[a] Purchased in 2013 (unknown year of harvest).
[b] Obtained from a local supermarket with no information on geographical origin and year of harvest.

Table 2
Primer specifications.

Primer name	Primer sequence in the NADH dehydrogenase subunit 5	Number of base pairs	Melting temp. (T_m)[a]	GC content	Size of amplicon
Pin4	ACCCTTCTCACTCTTTGAGG	20	57.3 °C	50%	85–278
Pin5	ACCGTATGTCCGAACAGGAT	20	57.3 °C	50%	85–278

[a] T_m [°C] = 69.3 + [41(n_G + n_C)/s − (650/s)], n_G = number of guanines, n_C = number of cytosines, s = number of all nucleotides per sequence.

Table 3
Alignment of relevant DNA sequences from the subgenus of *Strobus* spanning the primer pair Pin4 and Pin5.

Accession number and species	DNA sequence from an intron in the NADH dehydrogenase subunit 5 spanning the area between primers Pin4 and Pin5	Amplicon size
DQ983609.1 *P. armandii*	ACCCTTCTCACTCTTTGAGGG–AAGAAATTCTAGT–AAAACCCTATAGAGGGGGAAGGGGGGGGGGGGATCCTGTTCGGACATACGGT	86
AB455857.1 *P. armandii*	ACCCTTCTCACTCTTTGAGGGGAAGAAATTCTAGTAAAAACCCTATAGAGGGGGAAGGGGGGGGGGG–ATCCTGTTCGGACATACGGT	87
EU369320.1 *P. bungeana*	ACCCTTCTCACTCTTTGAGGGGAAGAAATTTTAGTCAAAACCCTATAGAGGGGGAAGGGGGGGGGG––ATCCTGTTCGGACATACGGT	86
AB455866.1 *P. cembra*	ACCCTTCTCACTCTTTGAGGGGAAGAAATTCTAGTAAAAACCCTATAGAGGGGGAAGGGGGGGGG–––ATCCTGTTCGGACATACGGT	85
AB675846.1 *P. koraiensis*	ACCCTTCTCACTCTTTGAGGGGAAGAAATTATAGTAAAAACCCTATAGAGGGGGAAGGGGGGGGGG––ATCCTGTTCGGACATACGGT	86
AB455868.1 *P. pumila*	ACCCTTCTCACTCTTTGAGGGGAAGAAATTATAGTAAAAACCCTATAGAGGGGGAAGGGGGGGGGG––ATCCTGTTCGGACATACGGT	86
AB455869.1 *P. sibirica*	ACCCTTCTCACTCTTTGAGGGGAAGAAATTCTAGTAAAAACCCTATAGAGGGGGAAGGGGGGGGG–––ATCCTGTTCGGACATACGGT	85
AB455864.1 *P. wallichiana*	ACCCTTCTCACTCTTTGAGGGGAATAAATTATAGTAAAAACCCTATAGAGGGGGAAGGGGGGGGG–––ATCCTGTTCGGACATACGGT	85

Yoshimaru, Nagasaka, & Szmidt, 1999). The limited number of available sequences from *P. densata*, *P. massoniana*, *P. tabuliformis*, and *P. yunnanensis* questioned the sequence validity, and a PCR investigated if Pin4 and Pin5 could amplify these species despite the theoretical Pin4 mismatch. As shown later, *P. massoniana* was amplified and, therefore, included as reference material in this study.

2.4. DNA extraction

A semi-automated KingFisher robot and the Wizard® Magnetic DNA Purification System for Food (Promega, USA) extracted DNA from three individual pine nuts from reference materials and samples, according to a standard protocol. Negative controls were homogenized and extracted in triplicate. In brief, one deshelled pine nut or 0.1 g of negative control was placed in a 2 ml micro-centrifuge tube. 500 μl of Lysis Buffer A (Promega, USA) and 5 μl of RNAse A (4 mg/ml) (Qiagen Inc., Germany) were added and vortexed for 10 s 250 μl of Lysis Buffer B (Promega, USA) was added and vortexed. After 10 min of incubation at room temperature (22–25 °C), 750 μl of Precipitation Solution (Promega, USA) was added and vortexed. After 10 min of centrifugation at 15,870 × g, 1000 μl of the supernatant was transferred to a new 2 ml micro-centrifuge tube. 50 μl of well dispersed magnetic beads, Magnesil®, (Promega, USA) was added to the supernatant and inverted. A volume of isopropanol equivalent to 0.8 volume of the transferred supernatant was added and inverted before 5 min of incubation at room temperature. Racks were placed in the KingFisher robot as follows. Well 1: 1.8 ml of sample with the magnetic beads. Well 2: 250 μl of Lysis Buffer B. Well 3: 1000 μl of ethanol 70%. Well 4: 1000 μl of ethanol 70%. Well 5: 300 μl of PCR grade water. Start the KingFisher protocol (Software version: BindIt Software 3.1 for KingFisher Instruments). Recover the liquid and transfer it to a new tube.

2.5. DNA quantification

As described earlier (Ballin, Vogensen, & Karlsson, 2012), DNA quantification was performed with the PicoGreen dsDNA Quantitation Kit (Molecular Probes Inc, The Netherlands), a TBS-380 Mini-Fluorometer, and a Minicell adaptor (Turner BioSystems, Sunnyvale, CA).

2.6. PCR amplification and high-resolution melting curve analysis

Each reaction mix consisted of 10 μl of a 2 X Type-it HRM PCR Kit with HotStarTaq Plus DNA Polymerase, EvaGreen Dye (QIAGEN, Hilden, Germany), and 750 nM of each primer in 0.1 ml tubes (Corbett Research Inc., Australia). Except for the non-template control (NTC), all tubes contained 20 ng of template DNA in TE-buffer. DNase and RNase free water (Sigma–Aldrich Inc.) were added to reach a final volume of 20 μl. A Rotor-Gene Q 5-plex (QIAGEN, Hilden, Germany.) carried out the PCR. PCR temperature conditions were set as follows: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, and 60 °C for 30 s.

The Rotorgene Q Series Software 2.0.2 (build 4) recorded data from the HRM channel in the Rotor-Gene machine. The HRM channel operated at an excitation at 460 ± 20 nm and an emission at 510 ± 5 nm. All C_T values were obtained from threshold values of 0.008 fluorescence units. High-resolution melting curves were obtained from 0.1 increments every 2 s from 70 to 85 °C. Peak temperatures from the HRM curves were obtained from threshold values of 0.008 fluorescence units. DNA from reference material and samples were analyzed in triplicates; i.e. three individual pine nut DNA extractions from each reference material and sample.

2.7. Principal component analysis of high-resolution melting data

The Rotor-Gene Screen-Clust HRM software version 1.10.1.2 (QIAGEN, Hilden, Germany) evaluated the HRM curves. The software performed a normalization of the HRM data. A subsequent subtracting of all the differentiated curves by the composite median of all curves generated the residual plot used for the PCA. The supervised mode of the software grouped the unknowns into known groups. Probabilities and typicalities provided statistical information that indicates the likelihood that an unknown belongs to a known cluster (Reja et al., 2010). According to the Screen-Clust HRM software, probabilities below 0.7 and typicalities below 0.05 should be treated with caution.

2.8. DNA sequencing

The primers Pin4 and Pin5 together with DNA from *P armandii* reference materials (RM1 – RM3) were send to Eurofins Genomics GmbH (Germany) for sequencing. In accordance with the Eurofins sample submission guide, 15 μl of genomic DNA with a concentration of 25 ng/μl, and 25 μl of each primer with a concentration of 10 pmol/μl were shipped in Safe-lock tubes.

3. Results and discussion

3.1. In vitro specificity

A PCR cross-reactivity assay with negative controls, and *P. armandii* and *P. koraiensis* as positive controls investigated the in vitro specificity. Amplification of positive controls at Ct 19–26, and the lack of amplification of negative controls (cut of Ct 40) supported the in vitro specificity towards pine nuts.

3.2. High-resolution melting curve analysis of reference material

Three individual pine nuts from each reference material (RM1 – RM12) were analyzed. Fig. 1 presents the HRM curves and the normal melting curves. Reference material (RM1 – RM10) from the subgenus *Strobus* showed T_{ms} between 76.43 and 77.89 °C, whereas *P. massoniana* (RM11) and *P. pinea* (RM12) from the subgenus *Pinus* showed T_{ms} at 79.65 and 82.32 °C, respectively, with a satisfactory standard deviation between 0.00 and 0.13, Table 4. The PCR melting curve of *P. massoniana* (RM11) was unexpected because of the nucleotide mismatch in the sequence (FJ906702.01) corresponding to the Pin4 primer. A search for other *P. massoniana* sequences revealed two additional entries (FJ906701.01, EU369319.1), but with the same nucleotide mismatch as FJ906702.01. One explanation could be that sequences from subspecies/varieties of *P. massoniana* were not yet published. As expected from the DNA insertions described in Section 2.3, *P. massoniana* (RM11) and *P. pinea* (RM12) showed higher T_{ms} compared to the species from the subgenus *Strobus*.

As expected from a lack of DNA sequence differences between *P. koraiensis* (RM6, RM7)/*P. pumila* (RM8), and *P. sibirica* (RM9)/*P. cembra* (RM5), no species determination could be extracted from their HRM curves, Fig. 1A. DNA from the three reference materials of *P. armandii* (RM1 – RM3) showed two different HRM curves suggesting different *P. armandii* subspecies/varieties. Amplified *P. armandii* DNA from RM1 shows a typical melting curve whereas RM2 and RM3 showed melting curves with a shoulder, Fig. 1B. These results indicate a mixture of amplicons. To investigate this further, the *P. armandii* amplicons from RM1 – RM3 were sequenced.

3.3. DNA sequencing

In agreement with the melting curve profiles in Fig. 1B, the amplicon from RM1 showed a single sequence, whereas RM2 and RM3 showed mixed sequences (data not shown). The mixed sequences are most likely not a contamination issue since DNA was extracted from single seeds. Possible explanations could be heteroplasmy, horizontal gene transfer, or other phenomena that causes sequence variations in the same gene.

3.4. Principal component analysis and species determination of pine nuts

All three HRM curves (Fig. 1A) from each reference material (RM1 – RM12) were used to build the PCA model. The PCA discriminated between *P. armandii* (RM1), *P. armandii* (RM2 – RM3), *P. bungeana* (RM4), *P. massoniana* (RM11), *P. pinea* (RM12), *P. wallichiana* (RM10), *P. koraiensis* (RM6, RM7)/*P. pumila* (RM8), and *P. sibirica* (RM9)/*P. cembra* (RM5), Fig. 2. As expected, the similar melting curves in *P. koraiensis* (RM6, RM7)/*P. pumila* (RM8), and *P. sibirica* (RM9)/*P. cembra* (RM5), shown in Fig. 1, disabled PCA clusters of the individual species and incapacitated their species discrimination. Fortunately, discrimination between *P. koraiensis*/*P. pumila,* and *P. sibirica*/*P. cembra* was of less importance compared to the identification of *P. armandii* and *P. massoniana* that were not intended for human consumption according to FAO (http://www.fao.org/docrep/x0453e/x0453e12.htm). Reference materials were placed in the above species clusters with satisfactory probabilities ranging from 0.83 to 1.00, with the exception of on pine nut from *P. armandii* (RM3). This pine nut was placed on the boundaries between the *P. armandii* (RM2, RM3) and the *P. sibirica* (RM9)/*P. cembra* (RM5) clusters with probabilities of 0.40 and 0.60, respectively.

The 3 pine nuts from the unknown samples, $S1_{a,b,c}$, clustered together with *P. armandii* (RM2, RM3). The 6 pine nuts from the unknown samples, $S2_{a,b,c}$ and $S3_{a,b,c}$, clustered together with *P. koraiensis* (RM6, RM7)/*P. pumila* (RM8). The pine nut $S4_b$ clustered together with the *P. armandii* (RM2, RM3) and the pine nuts $S4_{a,c}$ were placed outside, but close to the *P. armandii* (RM2, RM3) cluster, Fig. 2. To verify the PCA clustering of the samples $S1_{a,b,c}$ – $S4_{a,b,c}$, results were compared to a species determination through fatty acid profiling (Mikkelsen et al., 2014) (data not shown) performed prior to the present work. Note that species determination

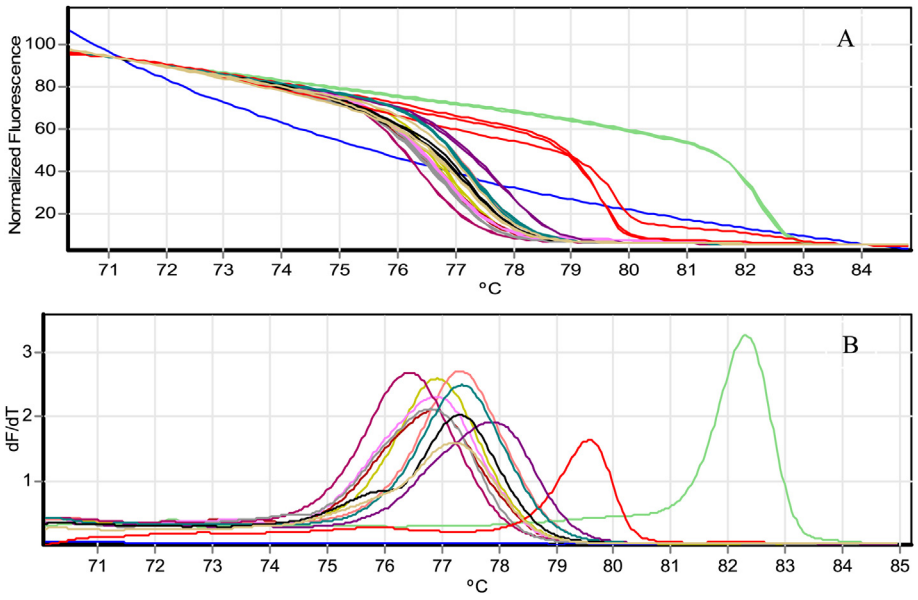

Fig. 1. Panel A shows normalized high-resolution melting curves from PCR amplicons. Panel B shows normal melting curves; only one replicate is shown for clarity. Color codes: NTC,*P. armandii* (RM1),*P. armandii* (RM2),*P. armandii* (RM3),*P. bungeana* (RM4),*P. cembra* (RM5),*P. koraiensis* (RM6),*P. koraiensis* (RM7),*P. pumila* (RM8),*P. sibirica* (RM9),*P. wallichiana* (RM10),*P. massoniana* (RM11),*P. pinea* (RM12). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

through fatty acid profiling was performed on a mixture of several pine nuts from each sample, in contrast to the presented PCA performed on individual pine nuts from each sample. This contrast impeded a comparison between the individual pine nuts $S1_{a,b,c}$, $S2_{a,b,c}$, etc. As shown in Table 5, the fatty acid profile determined the same species in samples S1 – S3. Results were less clear for sample S4. The fatty acid profiling determined sample S4 to be *P. armandii*, but the pine nuts $S4_{a,c}$ were placed outside the *P. armandii* (RM2, RM3) cluster in the PCA model. The $S4_c$ had a probability of 0.99 of belonging to the *P. sibirica* (RM9)/*P. cembra* (RM5) cluster, and the pine nut $S4_a$ had a probability of 0.99 of belonging to the *P. armandii* (RM2, RM3) cluster, even though it was placed outside the cluster. Another interpretation could be that the pine nuts $S4_{a,c}$ belong to a *P. armandii* subspecies/variety with a different PCR amplicon sequence, but a fatty acid profile that resembles the *P. armandii* profile. As a measure of how well a sample falls within its classified cluster, typicalities were calculated in the Screen-Clust software

Table 4
High-resolution melting temperatures from PCR amplicons and their standard deviation (n = 3).

Color code	Pine species	Mean T_m, (°C)	Standard deviation (°C)
	Non template control	–	–
Subgenus of *Strobus*			
	P. armandii (RM1)	76.98	0.07
	P. armandii (RM2)	77.31	0.02
	P. armandii (RM3)	77.31	0.08
	P. bungeana (RM4)	77.89	0.01
	P. cembra (RM5)	77.33	0.03
	P. koraiensis (RM6)	76.88	0.03
	P. koraiensis (RM7)	76.90	0.00
	P. pumila (RM8)	76.86	0.05
	P. sibirica (RM9)	77.34	0.02
	P. wallichiana (RM10)	76.43	0.00
Subgenus of *Pinus*			
	P. massoniana (RM11)	79.65	0.13
	P. pinea (RM12)	82.32	0.03

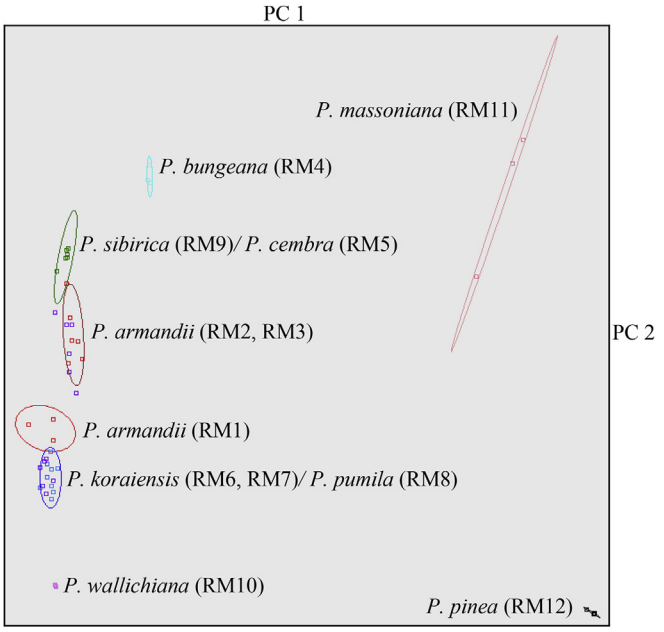

Fig. 2. Principal component (PC) analysis of high-resolution melting curves. Circles and squares of the same color represent clusters of species.□Represent the individual pine nut samples, $S1_{a,b,c}$, $S2_{a,b,c}$, $S3_{a,b,c}$, and $S4_{a,b,c}$.$S1_{a,b,c}$ and $S4_b$ were grouped in the *P. armandii* (RM2, RM3) cluster. $S2_{a,b,c}$ and $S3_{a,b,c}$ were grouped in the *P. koraiensis* (RM6, RM7)/*P. pumila* (RM8) cluster. $S4_{a,c}$ were placed outside the *P. armandii* (RM2, RM3) cluster. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5
Results from the analyzed samples and a comparison with results obtained elsewhere.

Sample[a]	PCA clustering results from samples	Source	Results from external analysis
$S1_{a,b,c}$	*P. armandii* (RM2, RM3) cluster	Governmental control	*P. armandii*[b]
$S2_{a,b,c}$	*P. koraiensis* (RM6, RM7)/*P. pumila* (RM8) cluster	Governmental control	*P. koraiensis*[b]
$S3_{a,b,c}$	*P. koraiensis* (RM6, RM7)/*P. pumila* (RM8) cluster	Governmental control	*P. koraiensis*[b]
$S4_{a,b,c}$	*P. armandii* (RM2, RM3), $S4_b$; outside cluster, $S4_{a,c}$	A Chinese web shop, labeled *P. armandii*	*P. armandii*[b]

[a] Three pine nuts from each sample were individually analyzed.
[b] Species determination results obtained from a fatty acid profile (Mikkelsen et al. 2014).

(Reja et al., 2010). The typicality was 0.11 for the pine nut $S4_a$ and 0.07 for $S4_c$. All other pine nuts from samples had typicalities ranging from 0.17 to 0.92.

4. Conclusion and perspectives

The presented HRM curves of PCR amplicons in combination with PCA discriminated several species and subspecies/varieties in a single PCR analysis. This strategy can easily discriminate more species from each other compared to traditional multiplex real time PCR. In addition, multiplex real time PCR requires careful and often difficult primer and probe design. Yet, another alternative method in species determination is DNA sequencing, which provides a high degree of confidence. One notable difference between DNA sequencing and the presented method is the information that can be extracted from a negative result. In the presented method, a sample result outside a cluster means that the sample belongs to a species excluded from the species present in the model. In contrast, a DNA sequencing result that does not match any DNA sequence from the DNA library is not as informative since the result does not easily exclude any specific species. The impact of this difference increases, as the PCA model gets more robust with additional species.

The present PCA model based on the HRM analysis showed that different *P. armandii* reference material formed distinct clusters. A DNA sequencing analysis of the PCR amplicons confirmed the differences between the *P. armandii* reference materials. Genomic differences between *P. armandii* subspecies/varieties are not well characterized in the literature, and we, therefore, suggest that future research will include the analysis of a wide selection of *P. armandii* subspecies/varieties to better understand the genomic intraspecies diversity.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2015.12.036.

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