

Detection and discrimination of cereal and leguminous species in chestnut flour by duplex PCR

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Abstract Chestnut and chestnut-derived products are quite expensive and thus a possible target for fraudulent labeling. To obtain a French Label of Origin, chestnut producers need to certify the purity of their products. Chestnut-derived products are consumed by people who may suffer from celiac disease or are allergic to cereals. For these reasons, we developed a qualitative PCR-based method to detect cereal and leguminous species in chestnut flour. The presence of common wheat and barley was determined by amplifying part of the puroindoline-a gene, the presence of rye by amplifying part of the secalindoline-a gene, the presence of durum wheat, rice, maize and chickpea by amplifying part of lipid transfer protein genes, the presence of oat by amplifying part of a thionin gene, the presence of kidney bean by amplifying part of a late embryogenesis-abundant protein, the presence of soybean by amplifying part of a lectin gene and the presence of fava bean by amplifying part of a nodulin gene. A chestnut thaumatine gene was used as control. PCR conditions were optimized to detect 1% of adulteration. Duplex PCR and specially designed sets of primers that allow amplification of several related species limited the number and cost of analyses required. Interpretation of the results is facilitated by using a decision tree.

Keywords Chestnut · Cereals · DNA · Food labeling · Legumes · PCR

Introduction

In France, about 11 000 tons of chestnut are produced for a total domestic consumption of 20 000 tons. Thus, imports are necessary to satisfy the French demand. Chestnuts are eaten roasted, cooked in water or milk (soups) but also as chestnut purees and flours that are used to make jam, pastry, polenta, special breads and beer. Chestnut flour is produced from chestnuts that are dried for several hours in a flow of hot air, shelled, picked over by hand or mechanically to remove discolored chestnuts and then crushed with a stone grinding mill or hammer. The chestnut flour can be stored for several months at room temperature or several years at 4 °C before use and/or sale. The sale price of chestnut flour is good (5–8 Euros/kg), and because of its high price there is a serious risk of commercial fraud with the addition of low cost cereal flours (wheat, barley, rye, oat or maize) or leguminous species (fava bean, kidney bean, chickpea, soybean, pea or lentil). The risk of accidental contamination is slight because the chestnuts are processed in centers that are quite separate from the sales network. To obtain a substantial benefit, the rate of adulteration needs to be relatively high (more than 10%). International sales regulations allow a low percentage (2–5%) of foreign species provided they are not dangerous for consumers. However, in several parts of France (Cévennes, Ardèche, Corse), chestnut producers wish to have a French Label of Origin (AOC). To achieve this goal, producers and manufacturers must satisfy certain production criteria, confirm the geographic origin and certify the purity of their products.

Chestnut and chestnut-based foods have beneficial nutritional and organoleptic qualities. The biochemical composition of chestnut flour is close to that of many cereals. Starch is the main component and the protein and lipid contents, mineral salts and vitamins (B1, E and C) make chestnut

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flour a very good substitute for people on a gluten-free diet. Indeed, chestnut flour can be consumed by people who suffer from celiac disease, i.e. permanent intolerance to gluten (gliadins and the related prolamins from rye, barley and oats) causing characteristic damage to the small bowel mucosa [1]. For celiac patients it is thus indispensable that the chestnut flour is guaranteed pure. Chestnut flour can also be used instead of wheat or rye flours for people who are allergic to cereals. Food allergy is now recognized as an important food safety issue and Directive 2003/89/EC [2], amending Directive 2000/13/EC [3], ended the 20-year-old “25%” rule and heralds the mandatory inclusion on food labels of the most common food allergen ingredients (of which cereals containing gluten is one of the most important) and their derivatives.

For all these reasons, we propose a polymerase chain reaction (PCR)-based method to detect the presence of the above-mentioned cereals and leguminous species in chestnut flour. To this end, we designed both species-specific primers and primers common to several cereal species. The PCR conditions were optimized to detect 1% of adulteration; the repeatability and the detection limit of the technique were also tested. The interpretation of the results is facilitated using a decision tree.

Materials and methods

Plant materials

The origins and characteristics of the plant materials used are listed in Table 1. The chestnut (*Castanea sativa*) flours we used were specially provided for this study by different producers located in different part of France. The commercial chestnut flours were mixtures of different local varieties all of where were guaranteed free of contamination. Commercial flours were purchased at an organic food store and were guaranteed pure. Seeds from cereal and leguminous species were ground into flour using a ball-grinder (Dangoumau S.A., Clermont-Ferrand, France). For simplex PCRs, the chestnut flours blended with 1 (w/w) of legume or cereal flour were prepared using white chestnut flour from the region of Languedoc. For duplex PCRs, 3% (w/w) of fava bean plus rice, fava bean plus oat, maize plus durum wheat, maize plus common wheat, maize plus rye, chickpea plus barley, chickpea plus common wheat, soybean plus kidney bean, soybean plus lentil or rye plus oat were blended with the chestnut flour from Haute Corse. Before weighing, flours were stored in a control chamber to ensure they all had the same water content (12%).

DNA extraction, quantification and gel analysis

Genomic DNA was extracted from 100 mg of flour using the DNeasy Plant Mini Kit (Qiagen, Hilden., Germany) according to the manufacturer's instructions and modified as follows: we added 600 μ l of AP1 lysis buffer (instead of 400 μ l) containing 400 μ g of RNase A, incubated at 65 °C for 30 min, and then added 195 μ l of buffer AP2. DNA was eluted twice by 50 μ l of AE elution buffer. In each extraction series, a control of extraction was inserted in which the flour was replaced by the same quantity of sterile water. DNA was quantified using the PicoGreen® dsDNA quantitation kit (Molecular Probes, Interchim, Montluçon, France) according to the manufacturer's instructions using λ DNA as a standard. After excitation at 480 nm, the fluorescence emission was measured at 526 nm with a spectrofluorimeter FL2500 (Hitachi, Tokyo, Japan). DNA was analyzed on 0.8% (genomic DNA) or 2% (PCR amplicons) agarose gels in 1 $\times$ TBE buffer [4], stained with ethidium bromide and visualized under UV with the Image Master VDS (Amersham Pharmacia Biotech). The 1 kb + DNA ladder (Invitrogen) was used as a control for size.

Sequences of primers

Sequences of primers listed in Table 2 were designed using the Primer Express Software (Applied Biosystems) or Primer 3 (http://www.genome.wi.mit.edu/genome_software/other/primer3.html). All the primer pairs were designed to obtain amplicons of different sizes and with a T_m close to 58 °C to allow all the PCRs to be run simultaneously.

PCR conditions

Simplex PCRs were performed on 50 μ l aliquots containing 0.2 μ M of each primer, 200 μ M of each dNTP, 1 $\times$ AmpliTaq Gold® DNA polymerase reaction buffer II (Applied Biosystems), 3 mM $MgCl_2$, 0.5 M betaine and 1.25 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems). PCRs were carried out in the GeneAmp® PCR systems 2400 or 9600. For duplex PCRs, primer concentrations were 0.4 μ M for PuroA, PuroAs, LTPa15 and DPH pairs of primers, 0.05 μ M for AveThio, Nod and Lec pairs of primers and 0.2 μ M for all other pairs of primers. Five microliters of DNA extract were put in each PCR that corresponded to between 60 and 100 ng of DNA, depending on the sample. First the AmpliTaq Gold® DNA polymerase was activated at 95 °C for 10 min. This was followed by 35 cycles of 30 s denaturation at 95 °C, 40 s annealing at 58 °C and 45 s elongation at 72 °C. A final cycle with an extension step of 5 min at 72 °C completed the reaction.

Table 1 Characteristics of plant materials

Species	Cultivar or specification	Production area or origin
<i>Castanea sativa</i> (chestnut)	White chestnut ^a	Languedoc
	Commercial flour	Languedoc
	Commercial flour	Rhône-Alpes
	Commercial flour	Corse du Sud
	Commercial flour	Haute Corse
	Commercial flour	Ardèche 2000
	Commercial flour	Ardèche 2002
<i>Hordeum vulgare</i> (barley)	Bomi	INRA
	Clarine	INRA
	Orélie	INRA
<i>Zea mays</i> (maize)	Commercial flour	
	Cathar	Maïsadour
	Bt-176	JRC, Belgium
<i>Triticum aestivum</i> (common wheat)	Scipion	INRA
	Renan	INRA
	Recital	INRA
<i>Triticum durum</i> (durum wheat)	Ardente	INRA
	Neodur	INRA
	Néfer	INRA
<i>Oryza sativa</i> (rice)	Commercial flour	
	Nipponbare	CIRAD
	Ariete	CIRAD
<i>Secale cereale</i> (rye)	Commercial flour	
	Protector	INRA
	Petkus	INRA
<i>Avena sativa</i> (oat)	Evora	GEVES
	Unspecified ^b	
<i>Vicia faba</i> (fava bean)	Commercial flour	
	Unspecified ^b	
<i>Cicer arietinum</i> (chickpea)	Commercial flour	
	Unspecified ^b	
	Unspecified ^b	
<i>Phaseolus vulgaris</i> (kidney bean)	White ^b	
	Red ^b	
	Green ^b	
<i>Glycine max</i> (soybean)	Yellow soybean	
	Roundup Ready	JRC, Belgium
<i>Pisum sativum</i> (pea)	Green ^b	
	Green split peas ^b	
<i>Lens culinaris</i> (lentil)	Green ^b	
	Yellow ^b	

^aGround with a ball-grinder
Dangoumau

^bCommonly available for
cooking

Results and discussion

Quantity and quality of DNA

In order to test the method of DNA extraction, three series of ten extractions were carried out on the white chestnut flour ground in our laboratory and two commercial flours (from Languedoc and Haute Corse). The extraction yield varied (1.15–2.15 μg DNA/100 mg of flour) between flour batches (Table 3). For a given flour, the relative standard deviation ranged from 6.41 to 20.74. The best extraction yields were obtained with the two commercial flours. The

fact the lowest extraction yield was obtained with the flour prepared in our laboratory may be due to a different particle size distribution as millstones are usually used to prepare commercial chestnut flours. As illustrated in Fig. 1, the DNA extracted from four different chestnut flours was of high quality because it was mainly composed of molecules larger than 12 kb.

Specificity of primers

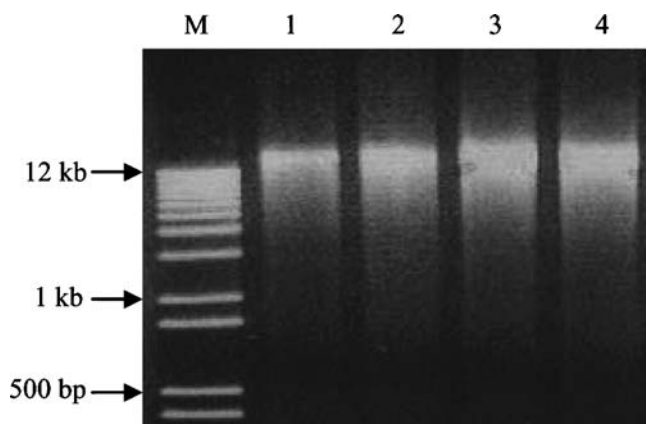
In order to verify their specificity, each pair of primers was tested by PCR on genomic DNA of all the species used

Table 2 Target genes and primer sequences

Species	Target gene	Primer	Sequence (5'→3')	Amplicon (pb)	Accession number or reference
Chestnut	Thaumatococcus	ThaCast1	TGATTGTAGCACCAGGTCAGG	201	AJ242828
		ThaCast2	TTCACGTTGGCTCGACAGC		
Common wheat	Puroindoline-a	PuroAP20F	GAAGTTGTTGGCAGTTACGA	381	X69913
Barley	Hordoindoline-a	PuroAR	TCACCAGTAATAGCCAATAGTG		
Durum wheat	Lipid transfer protein	LTPBd1F	GCGCTCAGGTAATGCTCA	262	X63669
		LTPBd2R	TCCTCGTTGAGATTGTGGAT		
Oat	Thionin	AveThio1	CGAGTTCTGCAACACGGGAT	265	AB072338
		AveThio2	GGCTGCAACTGATGCACG		
Rye	Secalindoline	PuroAP2s	ACACCGAAACACATTGACAC	511	This article
		PuroAM1s	TATGTGACATTTTATTAATTAGCT		
Maize	Lipid transfer protein	LTPA	CATATCTATCCCGCCGTCGT	80	J04176
		LTPD	ATCAAGTAGTGATCATGCGT		
Rice	Lipid transfer protein	LTPa15F	ACGCCGCCAGCATCC	61	X83435
		LTPa15R	ATGGAAGCGCTGATGGTGTAG		
Fava bean	Nodulin	Nod1	CCCACCTTTGATTACCTTGCC	101	AJ250497
		Nod2	AAACCTCCAACAACACGATGAA		
Chickpea	Lipid transfer protein	LTPpc130.1	AGGAACCTTAATTCTGCAGCTGTT	131	AJ002958
		LTPpc130.2	ACCACACATTTGCCAGGGAG		
Kidney bean	Late embryogenesis protein	DPH100L	CATGTGCAGCGAGTGGTACT	100	[8]
		DPH100R	AAGCAAATCCCAAATCCAAA		
Soybean	Lectin	LecF	AACCGGTAGCGTTGCCAG	118	K00821
		lecR	AGCCCATCTGCAAGCCTTT		

Table 3 Quantity of DNA extracted from 100 mg of chestnut flour, standard deviation (SD) and relative standard deviation (RSD) calculated on 10 extractions

Origin	Mean (μg)	SD	RSD
White chestnut	1.153	0.105	9.13
Languedoc	1.717	0.356	20.74
Haute Corse	2.146	0.138	6.41

**Fig. 1** Quality of genomic DNA. DNA extracted from chestnut flours (500 ng) was electrophoresed on 0.8% agarose gel in $1 \times$ TBE buffer. Lane 1 White chestnut flour (Languedoc), lane 2 commercial flour (Languedoc), lane 3 commercial flour (Corse du Sud), lane 4 commercial flour (Haute Corse). Lane M 1 kb⁺ DNA ladder (Invitrogen)

in this study. Representative results of the specificity of primers are presented in Fig. 2. We checked that none of the primers designed to amplify foreign DNA also amplified chestnut DNA. The primer pair ThaCast that amplifies a chestnut thaumatococcus gene was used as a positive PCR control to check that DNA extracted from chestnut flours was free from PCR inhibitors. None of the primers designed to amplify leguminous species amplified cereal species and none of the primers designed to amplify cereal species amplified leguminous species (data not shown). Within cereal species, the primer pair PuroAP20F/AR that amplifies a 381 bp fragment of the common wheat puroindoline-a gene [5] also amplifies the barley hordoindoline-a gene. This is in accordance with previous reports that puroindoline-like genes are present in barley [6]. The primer pair LTPBd1F/2R that amplifies a 262 pb fragment of a durum wheat lipid transfer protein (*Ltp*) gene [7] also amplifies common wheat and rye *Ltp* genes. The primer pair LTPa15F/LTPa15R that amplifies a 61 pb fragment of a rice *Ltp* gene also amplifies an oat *Ltp* gene. The other pairs of primers were maize, rye or oat-specific. None of the legume primers used in this study allow to amplify pea DNA. Within legumes, all primer pairs were species specific except the DHP100L/R pair that amplifies a 100 bp fragment of a kidney bean late embryogenesis protein gene that also amplifies the lentil gene. This cross amplification was verified on three different lentil cultivars.

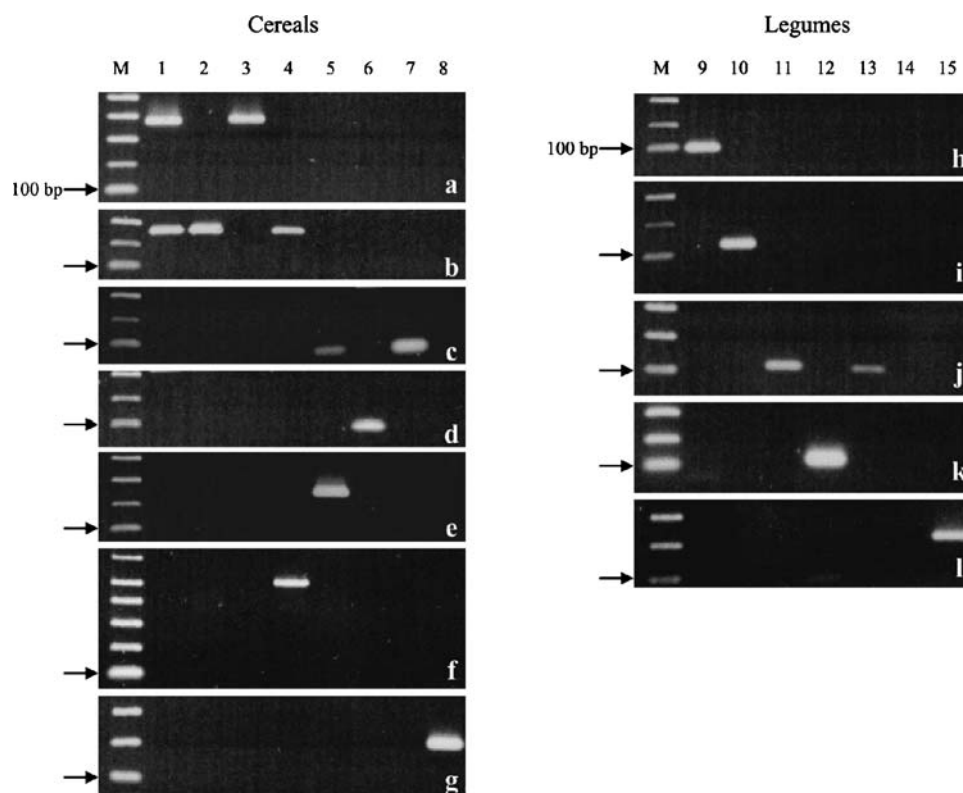


Fig. 2 a–l Specificity of cereal a–g and legume h–l PCR primers. Cereal primers: PCRs were performed with a PuroAP20F/PuroAR primers (381 bp), b LTPBd1F/LTPBd2R (262 bp), c LTPa15F/LTPa15R (61 bp), d LTPA/LTPD (80 bp), e AveThio1/AveThio2 (265 bp), f PuroAP2s/PuroAM1s (511 bp), g ThaCast1/ThaCast2 (201 bp). DNA was extracted from: lane 1 common wheat, lane 2 durum wheat, lane 3 barley, lane 4 rye, lane 5 oat, lane 6 maize, lane 7, rice, lane 8 chestnut.

Legume primers: PCRs were performed with (h) Nod1/Nod2 (101 bp), i LTPpc130.1/LTPpc130.2 (131 bp), j DPH100L/DPH100R (100 bp), k LecF/LecR (118 bp), l ThaCast1/ThaCast2 (201 bp). DNA was extracted from: lane 9 fava bean, lane 10 chickpea, lane 11 kidney bean, lane 12 soybean, lane 13 lentil, lane 14 pea, lane 15 chestnut. Lane M 1 kb⁺ DNA ladder (Invitrogen). Each load corresponds to 15 μ l of PCR reaction

Results that do not corroborate those of Krahulcova et al. [8] who did not amplify lentil DNA with the same primers. This may be due to the different PCR conditions used in these two studies. However, the amplification of lentil DNA with kidney bean DPH primers was weaker than those of kidney bean DNA (Fig. 2j). It should be kept in mind that the lentil genome is six to seven times larger than that of kidney bean [9]. Thus, for the same amount of DNA the number of targets for PCR primers is six to seven times lower for lentil than for kidney bean. The same remark is true when comparing intensity of oat and rice amplicons obtained with LTPa15 primers, indeed the oat genome is 20–25 times larger than that of rice. In addition, oat and lentil DNAs were amplified with primers designed on heterologous species, rice and kidney bean respectively. Comparison of Fig. 2e and c clearly showed that amplification of oat DNA is better with AveThio primers than with LTPa15 primers.

PCR simplex

For this study, DNA was extracted from chestnut flours containing 1% of one adulterating species. All extractions were

carried out in triplicate and a control of extraction was included in each extraction series. All PCRs were performed with the same volume (5 μ l) of DNA extract. As expected, the first PCR carried out with the primer pair ThaCast amplified a 201 bp fragment corresponding to part of a thaumatin-like gene indicating that the DNA was suitable for PCR amplification (data not shown). An aliquot of the control of extraction was then analyzed by PCR with each pair of primers to check that no contamination had occurred during the different steps of DNA extraction (data not shown). Once these controls were completed, the presence of each adulterating species in the chestnut flour was detected at a threshold of 1% (Fig. 3). Using kidney bean DPH primers lentil DNA was not detected in chestnut flours containing 1% of lentil (data not shown). As noticed before (Fig. 2) we observed large variations in PCR efficiencies that may depend on difference in genome size and/or PCR primers.

Repeatability

To test the repeatability of detection, 1% of cereal or legume flours were added to four chestnut flours from different

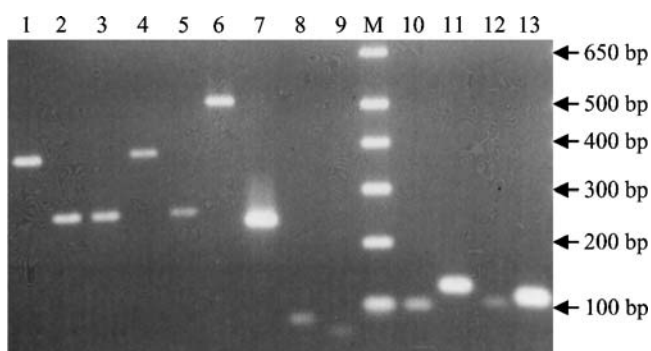


Fig. 3 Detection by simplex PCR of 1% of cereal and leguminous species in chestnut flour (Languedoc). Common wheat, lane 1 PuroAP20F/PuroAR primers (381 bp), lane 2 LTPBd1F/LTPBd2R (262 bp). Durum wheat, lane 3 LTPBd1F/LTPBd2R (262 bp). Barley, lane 4 PuroAP20F/PuroAR (381 bp). Rye, lane 5 LTPBd1F/LTPBd2R (262 bp), lane 6 PuroAP2s/PuroAM1s (511 bp). Oat, lane 7 AveThio1/AveThio2 (265 bp). Maize, lane 8 LTPA/LTPD (80 bp). Rice, lane 9 LTPa15F/LTPa15R (61 bp). Fava bean, lane 10 Nod1/Nod2 (101 bp). Chickpea, lane 11 LTPpc130.1/LTPpc130.2 (131 bp). Kidney bean, lane 12 DPH100L/DPH100R (100 bp). Soybean, lane 13 LecF/LecR (118 bp). Lane M 1 kb⁺ DNA ladder (Invitrogen). Each load corresponds to 15 μ l of PCR reaction

origins or years of production except for oat, kidney bean and soybean (chestnut flour from Haute Corse). Each sample mix was prepared four times, DNA was extracted and simplex PCR performed (Table 4). In all sample mixes and for the four repetitions, an amplicon of the expected size was amplified (data not shown).

Detection limits

The limit of detection was determined on common wheat which is the species which have the largest genome of our study. To this end, serial dilution of DNA extracts were carried out to have 1000, 100, 50, 10 and 1 copies of the puroindoline-a target sequence. The number of copies of the target sequence was calculated on the basis of 33 pg DNA (pg/2C) for the wheat genome [9]. To keep roughly the same amplification conditions as those implemented in simplex or duplex PCR, 45 ng of chestnut DNA were systematically added in each PCR reaction. For 35 PCR cycles the limit of detection was 10 copies and increasing the number of PCR cycles to 38 lowered the detection limit to 1 copy. We used 35 amplification cycles to avoid the detection of the fortuitous presence of traces of common wheat (content lower than 1%) in the chestnut flour. A study aimed at detecting the presence of *Phaseolus vulgaris* in a chestnut puree showed the PCR detection limit to be 1% for this species [8]. However, to be able to compare both results it would have been interesting that these authors specify the number of copies of the kidney bean target sequence with which they worked.

PCR duplex

To reduce the costs of analysis, we used duplex PCR to simultaneously detect two adulterating species. Two amplicons of different size were generated so that they could be distinguished by agarose gel electrophoresis. DNA extractions were carried out in duplicate on mixed flours containing 3% of each of the two adulterating species. To avoid competition between the two PCRs, primer concentrations were optimized. All duplex PCRs allowed detection of the two species added to the chestnut flour (Fig. 4). However, in the duplex PCR soybean plus lentil (lane 12), the 100 bp amplicon corresponding to lentil DNA is quite low compared to the 118 bp amplicon corresponding to soybean DNA. We have already noticed (Fig. 2j) that amplification of lentil DNA with kidney bean DPH primers was weak.

Identification of the adulterating species

Based on primer specificity, the adulterating species in the chestnut flour were identified by referring to a decision tree (Fig. 5). Once all DNA extractions and PCR controls (negative and positive) for each pair of primers have been performed, the decision tree helps identify the adulterating species. If the simplex PCR with chestnut specific primers is positive and all duplex PCRs with primers enabling amplification of cereal or legume DNA are negative, the chestnut sample is free of any of the species we were trying to trace.

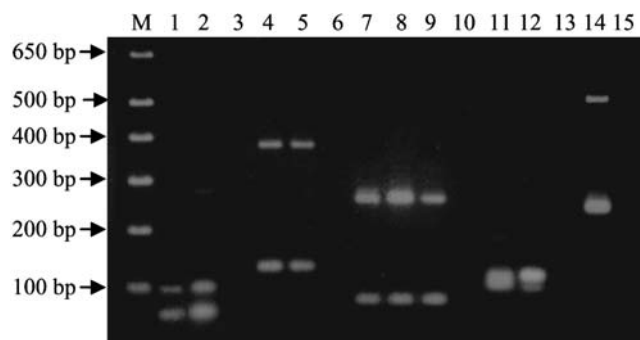
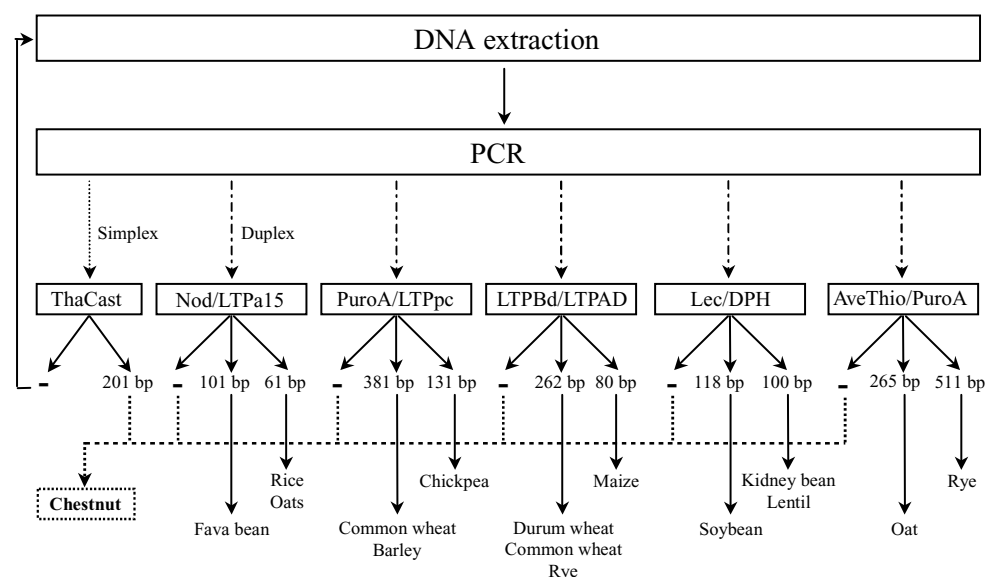


Fig. 4 Detection by duplex PCR of cereal and leguminous species in chestnut flour (Haute Corse) blended with 3% of each adulterating flour. PCRs with Nod1/Nod2 and LTPa15F/LTPa15R pairs of primers (101 and 61 bp): lane 1 fava bean plus rice, lane 2 fava bean plus oat, lane 3 negative control. PCRs with PuroAP20F/PuroAR and LTPpc130.1/LTPpc130.2 (381 and 131 bp): lane 4 chickpea plus common wheat, lane 5 chickpea plus barley, lane 6 negative control. PCRs with LTPBd1F/LTPBd2R and LTPA/LTPD (262 and 80 bp): lane 7 maize plus durum wheat, lane 8 maize plus common wheat, lane 9 maize plus rye, lane 10 negative control. PCRs with LecF/LecR and DPH100L/DPH100R (118 and 100 bp): lane 11 soybean plus kidney bean, lane 12 soybean plus lentil, lane 13 negative control. PCRs with AveThio1/AveThio2 and PuroAP2s/PuroAM1s (265 and 511 bp): lane 14 oat plus rye, lane 15 negative control. Lane M 1 kb⁺ DNA ladder (Invitrogen). Each load corresponds to 15 μ l of PCR reaction

Table 4 Repeatability of simplex PCR detection of adulterating species in chestnut flours at a threshold of 1%

Species	Chestnut origin			
	Corse du Sud	Haute Corse	Ardèche2000	Ardèche 2002
Bean	+	+	+	+
Chickpea	+	+	+	+
Maize	+	+	+	+
Rice	+	+	+	+
Durum wheat	+	+	+	+
Barley	+	+	+	+
Rye	+	+	+	+
Common wheat	+	+	+	+
Oat		+		
Kidney bean		+		
Soybean		+		

Each mixture was carried out four times, the DNA was extracted independently then amplified

Fig. 5 Decision tree to identify the adulterating species in chestnut flours

Although three pairs of primers for cereal detection (LTPa15, PuroA and LTPBd) are not species specific, it is possible to distinguish rice from oats, common wheat from barley, durum wheat from common wheat and rye. Presence of the 61 bp amplicon and absence of the 265 bp amplicon indicate that rice is the adulterating species. Presence of the 381 and 262 bp amplicons indicate that common wheat is the adulterating species. Conversely, presence of the 262 bp amplicon and absence of the 381 and 511 bp amplicons indicate that durum wheat is the adulterating species. Maize, rye and oat can be identified by the presence of one single amplicon. Except kidney bean and lentil that cannot be discriminated, all other leguminous species (fava bean, chickpea and soybean) were easily identified.

Conclusions and future outlook

In reply to a request from chestnut producers to obtain an AOC label for their products we developed a qualitative

PCR-based method to evaluate the purity of their chestnut flours. Different pairs of primers were designed that allow specific amplification of part of the puroindoline-a gene present in common wheat and barley, lipid transfer protein genes present in durum wheat, rice, maize and chickpea, secalindoline gene in rye, thionin gene in oat, bean nodulin gene in fava bean, lectin gene in soybean or late embryogenesis-abundant gene in kidney bean and lentil. Simplex PCR conditions were optimized to detect 1% of adulteration. However, because to obtain a substantial benefit the rate of adulteration needs to be higher (10% or more) PCR duplex were optimized using 3% of adulteration. One simplex PCR with chestnut-specific primers and five duplex PCRs enabled detection of 12 foreign species in chestnut flours thus limiting the cost of analysis. The practical application of the analytical device and the interpretation of the results are facilitated by using a decision tree. This method of detection was developed on chestnut flours and needs to be validated on heat-treated products like chestnut puree and chestnut jam. For people suffering from celiac disease

or who are allergic to cereals, the 1% threshold may be not sufficient. Thus, further advance in analysis would be using real-time quantitative PCR enabling to set a lower threshold for the presence of different breadmaking cereals in chestnut products. The approach would be similar to the one our team developed to quantify common wheat in pasta [10].

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