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Differentiation of Important and Closely Related Cereal Plant Species (Poaceae) in Food by Hybridization to an Oligonucleotide Array

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We report the development of an oligonucleotide microarray for the simultaneous detection of six important cereal food plant species from the Poaceae based on the chloroplast *trnL* intron sequence. We used universal primers to amplify the *trnL* intron from wheat, rye, barley, oat, rice, and maize, followed by a cyclic labeling of oligonucleotides probes and subsequent hybridization to an oligonucleotide microarray. In single taxon analyses, positive signals were produced with a high signal-to-noise ratio. The assay also enabled the analysis of mixed samples. The results obtained for real food samples were in agreement with the ingredient labels, but positive results for grains not declared on the ingredients list were observed in three out of 10 samples, which indicates that the final products and/or the declared ingredients were probably botanically impure or contaminated. The combination of the sensitivity of a universal polymerase chain reaction with the specificity of the labeling reaction allows this protocol to be applied in routine analyses of food samples, as demonstrated by successful analysis of processed composite food products.

KEYWORDS: Species identification; *trnL* intron; oligonucleotide; microarray; ingredient declaration; Poaceae; food analysis; gluten

INTRODUCTION

The need for specific, rapid, and inexpensive methods for differentiation of food species is high and increasing because of the requirements set by the food law (*1*), as a result of consumer awareness and demands, food allergens, introduction of genetically modified organisms (GMOs), and incidents such as the BSE crisis and foot and mouth disease. Immunoassays have been the traditional method of choice for detection and identification of food components, especially sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis, immunoblot, dot-blotting, rocket imunoelectrophoresis, and enzyme immunoassays including enzyme-linked immunosorbent assay (*2*). Many of the protein-based analyses may have limited applicability in processed food due to protein degradation, and DNA-based analytical methods are an alternative for reliable species differentiation. Most of the DNA-based methods apply the polymerase chain reaction (PCR), and methods have been designed for the identification of different animal species (*3*), the detection of genetically modified foods (*4*), and the detection of pathogens in food (*5*). Interestingly, the PCR technology has until recent years had limited applications in relation to other aspects of food composition, although PCR methods specific for different cereals and food crops have now been developed (*6*, *7*).

By combining primer sets for multiple species specific markers, a single PCR can be used to detect several species (*8*−*10*). However, there are practical limitations to these assays (*11*). An alternative approach is to use universal PCR based on a marker present in several target taxa followed by identification analysis of the resulting populations of amplicon products from the reaction (*6*). If more than one sequence is present, as is often the case in a real food sample, DNA microarrays may be used to effectively screen a complex mix of different sequences. Recent developments include various types of DNA arrays, e.g., for differentiation of animal species in food (*12*), fish pathogens (*13*), microbial communities (*14*, *15*), GMOs (*16*), and toxigenic fungi (Ralf Kristensen, personal communication). These assays rely on PCR amplification of homologous sequences from composite populations of target organisms, followed by analysis of the pool of amplification products by array hybridization to specific capture probes. This is achievable when diagnostically informative sequence motifs (hosting the capture probe sequence motif) are flanked by universally conserved regions (sites for PCR amplification). In this format, probes for specific targets are typically deposited on to a glass slide to which PCR products or genomic DNA is hybridized and detected (*12*, *17*). The detection strategy may be broadly grouped into direct and

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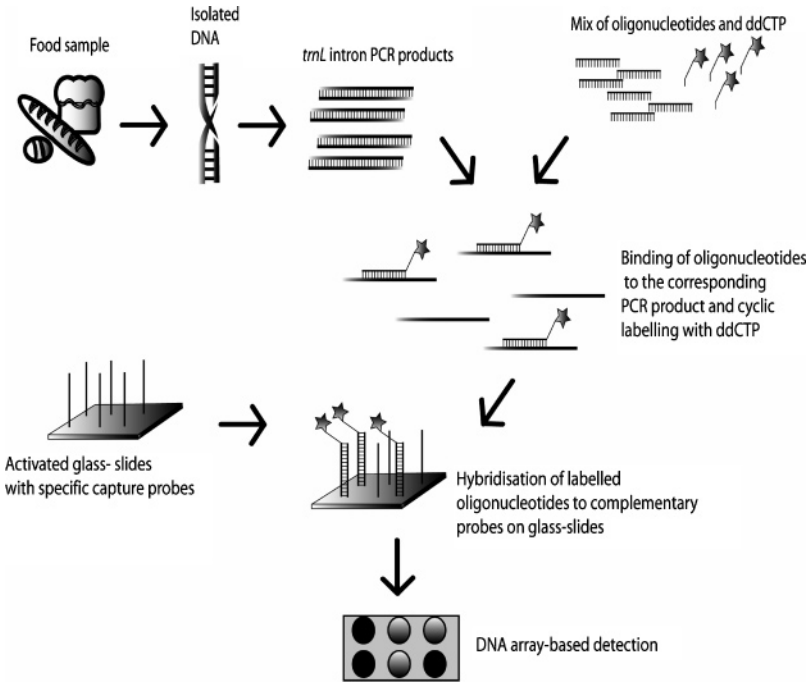

Figure 1. Schematic presentation of the microarray method used in this study. Total DNA is isolated from a food sample and is amplified using the universal *trnL* intron primers. Then, specific oligonucleotides and Fluorescein-12-labeled ddCTP are mixed with the PCR products, and the oligonucleotides are labeled specifically. The labeled oligonucleotides are subsequently hybridized to complementary capture probes on a glass slide, followed by array-based detection.

indirect methods. Indirect strategies usually utilize fluorophore-labeled streptavidin/biotin, which binds to biotinylated targets resulting in an amplification of the signal, while direct methods rely upon labeling of the DNA with fluorophore-coupled cyanine, which can be detected directly (*18*). The principles of the DNA microarray method described here are illustrated (**Figure 1**) (*15*), in which specific oligonucleotides are added to a pool of PCR products and dideoxy CTP (ddCTP) is used in the cyclic labeling. The labeled oligonucleotides (not the PCR products) are hybridized to their respective complement probes on the glass slide. The incorporation efficiency of fluorescently labeled ddCTP is sequence-dependent.

The chloroplast transfer RNA gene $tRNA^{Leu}$ (UAA) intron (*trnL* intron) has proven to be useful in several phylogenetic studies (*19*−*21*), and it has been proposed as a target for detection and identification of specific food crops (*6*). Universal primers have been designed based on sequences of the highly conserved chloroplast tRNA coding genes flanking the non-coding regions of interests (*22*). The primers amplify homologous segments from essentially all higher plant species tested, suggesting that they can be used to study population biology and evolution over a wide taxonomical range. In this study, we used the noncoding *trnL* intron region as a target for the universal PCR reaction, generating a variety of DNA fragments detectable by species specific probes. Species specific probes targeting key food plants were designed based on unique sequence motives (*23*).

In this paper, we demonstrate a plant detection system that couples PCR amplification of the *trnL* intron with an oligonucleotide-based microarray that simultaneously screens for six important cereal food plant species. We also present a crude estimate of the limit of detection based on defined mixed samples. The system may prove valuable to identify whether products contain gluten-rich ingredients, plant material from species that include commercial GM varieties, and to verify ingredient declarations.

MATERIALS AND METHODS

Capture Probe Design. The capture probes were constructed from the criteria that the melting temperature of the probes should preferably be between 50 and 60 °C, and the size of the probes should be between 18 and 35 nucleotides. The probes were constructed so that overlap was minimized, i.e., ideally avoided and never >50%. The specificity of all the probes was tested theoretically by conducting Blastn searches for short, nearly perfectly matching sequences against the EMBL/GenBank sequence database (http://www.ncbi.nlm.nih.gov/BLAST/). All probes carried a T-15 spacer to improve the accessibility on the glass surface of the DNA microarray. All probes were synthesized with C6 amino linker modification to enhance covalent attachment (corning slides) to the activated glass surface. The probes are summarized in **Table 1**. It should be noted that there are cheaper alternatives to using extra spacers, amine modifications, and corning slides, for example, unmodified oligonucleotides can be used and even simple, acid-washed slides may work relatively well as long the as the capture probes are longer than 18 nt.

Cereal Samples. Samples of wheat (*Triticum aestivum*), oat (*Avena sativa*), and barley (*Hordeum vulgare*) were provided by Aksel Bernhoft, National Veterinary Institute, while rice (*Oryza sativa*) was purchased at the local store. Maize (*Zea mays*) was provided by David Zhang, Laboratoire BIOGEVES, France, and rye (*Secale cereale*) was provided by the gene bank at Institute für Pflanzengenetik and Kulturpflanzenforschung (IPK), Germany. Additionally, 10 food samples collected from the local store, The Norwegian Food Safety Authority, and GeMMA (Central Science Laboratory, United Kingdom) (cf. **Table 3**) were included in the study and analyzed according to the protocol. The samples from the Norwegian Food Safety Authority and GeMMA were provided with a detailed ingredient declaration, either as a label on the product or as a product-attached document.

DNA Extraction and Universal Amplification of the *trnL* Intron. Total genomic DNA was extracted from seeds using DNeasy Plant Mini Kits (Qiagen, Hilden, Germany) or a CTAB-based protocol (*24*) modified as described at http://gmo-crl.jrc.it/detectionmethods/NK603−WEB−Protocol%20Validation.pdf. The *trnL* intron was amplified using primer "D" and a modified primer "Cb" missing a G at the 3′-end (*22*). The PCR reaction volume of 50 μL contained 25−75 ng of template DNA, 1 μM each primer, 1 unit of Taq DNA polymerase (AmpliTaq

Table 1. Oligonucleotides Used in the Study

name	sequence 5′–3′[a]	GC (%)[b]	Tm[b]	length
PAsati*b*	ATCCGTGTTTTGAGAGGGGGTT	50	59	22
PErhart	AGAAGGGCTTTCGAAATCTAATACACA	37	60	27
PAndro*a*	CTGTTCTAACGAATCGAAGTAATAA	32	56	25
PAndroa alt.2[c]	GTTCTAACGAATCGAAGTAATAA	30	53	23
PTaest	TAAAAACTCATATCATAATTTTTTTT	12	48	26
PHvulg*a*	TGAGAACTTTTAAAAAGAAAGTGGATTA	25	56	28
PSmont*a*	GGT TTA TAC CTT ATA CAT ATA CAT TTA ATA AAC A	21	58	34
PSmont*b*	GGGGTTTGGTTTATACCTTATACATATA	32	58	28
Hyb.control[d]	AATTTCCCAAGAAAGAAGCCAAGACACCT	43	63	29
Label.control	AAGCGGAAATCTAATTGCCTGGGCA	48	62	25
Label.control template	GAGAATCCTCATCAGTGCCCAGGCAATTAGATTTCCGCTTCCG			

[a] Probes reverse complementary to these oligonucleotides included a 15-T tail at the 5′-end and were spotted onto the glass slides. [b] Calculated at http://www.justbio.com/oligocalc/index.php. [c] PAndro*a* alt.2 was included in the labeling reaction while the capture probe reverse complementary to PAndro*a* was spotted on the glass slide. [d] Fluorescein-12-labeled at the 3′-end.

Gold, Applied Biosystems, Foster City, CA), 0.2 mM dNTP, 2.5 mM $MgCl_2$, and 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl). The PCR program included an initial denaturation step at 95 °C for 10 min followed by 35 cycles of 1 min denaturation at 95 °C, 1 min annealing at 56 °C, and 1 min extension at 72 °C. A final extension for 10 min at 72 °C completed the amplification. The PCR products were visualized on standard agarose gels and treated with 4 U of shrimp alkaline phosphatase (Amersham Biosciences, Little Chalfont, England) and 20 U of exonuclease I (Amersham Biosciences) at 37 °C for 30 min to inactivate the nucleotides and to degrade residual PCR primers. Finally, the shrimp alkaline phosphatase and the exonuclease I were heat inactivated at 95 °C for 10 min. The treated products were then used as templates in the sequence specific labeling.

Sequence Specific Labeling. The cyclic labeling conditions were as follows: 1× Thermosequenace reaction buffer, 2.5 pmol of each species specific oligonucleotide, 25 μM fluorescein-12-ddCTP (Perkin-Elmer, Boston, MA), 3.84 U of Thermosequenace DNA polymerase (Amersham BioSciences), 2×10^{11} copies of labeling control template DNA, and 5 μL of treated PCR product in a total volume of 15 μL. The PCR program included an initial step at 95 °C for 2 min followed by 25 cycles of 30 s of denaturation at 95 °C and 1 min of cyclic labeling at 57 °C.

DNA Array Hybridization. We used Codelink Activated slides (Amersham Bioscience) that were UV cross-linked at 600 mJ. Each glass slide was divided into 8 × 3 individual chambers using a self-made silicone rubber mask. Capture probes (60 μM) reverse complementary to the oligonucleotides used in the labeling reaction were spotted on the glass slides by the Norwegian Microarray Consortium (http://www.mikromatrise.no/). The glass slides were prehybridized in a filtered (0.25 μM) solution of 50% formamide, 5 × SSC, and 0.1% SDS and incubated at 42 °C for 30−60 min. The glass slides were washed twice with distilled H_2O and once with 2-propanol and dried at 700 rpm for 5 min. The hybridization control (0.85 μM) was mixed with 8.3 μL of labeled oligonucleotides. From this mixture, 5 μL was mixed with 50 μL of 6% PEG 8000 and incubated for 5 min at 95 °C. The glass slides were covered with alum-foil, and hybridization was done overnight at 45 °C and 150 rpm. The glass slides were subsequently rinsed at room temperature with 2 × SSC, 0.1 % SDS for 5 min, then 0.1 × SSC and 0.1 % SDS for 10 min, and finally 0.1 × SSC for 1 min. The microarrays were scanned using a GenePix4000B array scanner (Amersham Bioscience). The instrument generates fluorescence images by scanning a laser beam (532 nm) over the sample surface. The data were saved as an inverted, single layer 16-bit TIF file and analyzed with Silverquant analysis v1.0.8 (Beta) (Eppendorf, Germany), where the corrected signal intensities were measured by subtracting the signal from the background and the correction mean. The signal mean is the pixel intensity averaged over the spot; the background is the mean of the local background around the spot, while the correction mean is calculated as the mean of the difference between signal and background of a negative hybridization control on the array. The intensity of the hybridization signal can differ from one spot to another because of variability of the concentration of spotted capture probe and concentration of template DNA across the glass slide and surface conditions. To reduce these variables, we spotted each capture probe in quadruplicate and averaged the intensities for each probe.

SYBR Green I Assay. It is very difficult to determine the exact amount of *trnL* intron present in a sample, and we are not aware of a protocol to achieve this accurately. To estimate the number of *trnL* copies in the validation of the study, we performed real-time PCR analysis using SYBRGreen I. The PCR reaction volume of 25 μL contained 20−40 ng of template DNA, 1 μM each primer (primer Cb and D), 1 unit of Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems), 0.2 mM dATP, dCTP, and dGTP (Applied Biosystems), and 0.4 mM dUTP (Applied Biosystems), 2.5 mM $MgCl_2$, 1× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), and 1:100000 dilution of SYBRGreen I (Sigma Aldrich, St. Louis, MO). The PCR program included an initial decontamination step for 2 min at 50 °C to allow optimal UNG enzymatic activity, followed by 10 min at 95 °C to activate the DNA polymerase, to deactivate the UNG, and to denature the double-stranded template and successively 30 cycles of 15 s denaturation at 95 °C and 1 min of synthesis at 60 °C. Then, the PCR products were heated to 95 °C during 15 s, cooled at 60 °C for 15 s, and then slowly heated back to 95 °C. The PCR reactions were run on an ABI PRISM 7900HT Sequence Detection System device (Applied Biosystems). PCR amplification analysis was performed using the SDS software v2.1 (Applied Biosystems). Melting curve analysis exploits the fact that even a single mismatch between the labeled probe and the amplicon will significantly reduce the melting temperature and a single peak in the Tm curve analysis indicates high specificity of the amplification product. The Ct value may be used to obtain an estimate of the target copy number; a Ct value of ca. 38 equals one target copy number (unpublished data), and a ΔCt of 10 corresponds to a change in copy number by a factor 10^3.

RESULTS

The probes (**Table 1**) were constructed based on the sequence motifs identified by Rønning et al. (*23*). The sequence difference between a specific probe and the DNA sequences of other species available from the EMBL/GenBank sequence database was between one and eight bases. To qualitatively assess the impact of reaction temperature, reactions were carried out at temperatures ranging from 53 to 57 °C. A slight decrease in signal intensity was observed with increased temperatures (data not shown), and we chose 57 °C as the best compromise between specificity and sensitivity. We tested hybridization temperatures between 30 and 50 °C. At 30 °C, some capture probes displayed unspecific binding, in particular the PSmont*a* and PAndro*a* seemed to weakly cross-hybridize with all of the cereal PCR products. When the hybridization temperature was increased to 45 °C, no cross-hybridization was observed for the probes except for the PAndro*a*. At 50 °C, the signal intensity of some of the probes was quite weak (data not shown); hence, 45 °C was selected for subsequent hybridization experiments.

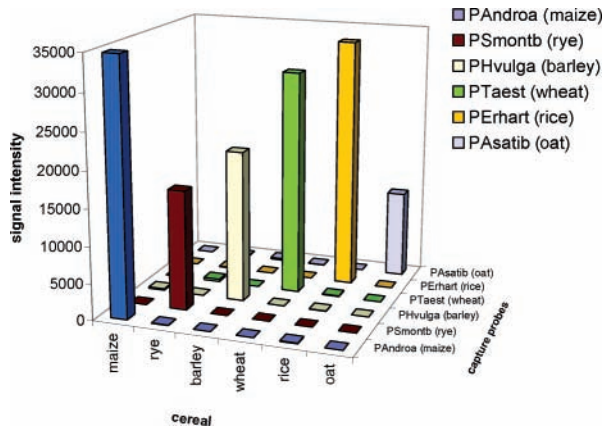

Figure 2. Series of bar graphs that show the average signal intensity for each specific and unspecific capture probe, in which the signal intensity is the mean value from four spots. Each cereal was tested against all of the capture probes.

To avoid the cross-hybridization of PAndro*a*, we increased the stringency by designing a new, shorter oligonucleotide for maize (PAndro alt2), which we included in the labeling reaction instead of the original PAndro*a*. Note that the capture probe spotted on the glass slide was reverse complementary to the original PAndro*a*. The results showed a clear reduction in cross-hybridization with other PCR products without reducing the signal intensity obtained with true PCR products.

When the labeled oligonucleotides were hybridized to the capture probes on the glass slide, distinct signals were detected with the corresponding species specific oligonucleotides (**Figure 2**). Interpretation of the hybridization reactions was straightforward, and it was possible to correctly identify all of the species visually and by calculating the signal intensity using Silverquant. A single nucleotide difference separates PTaest from the corresponding *trnL* motif of rye and barley, but no cross-reactivity was observed. The signal intensities varied among the different capture probes; the probes PAsati*b* and PSmont*a* gave the weakest signals while PErhart and PAndro*a* gave the strongest signals (**Figure 2**).

To obtain a crude estimate of the limit of the detection with the array, mixtures containing approximately 40 ng of template DNA per reaction for each cereal (20 ng of template DNA for maize and rice) were subject to universal PCR, followed by labeling and hybridization. Very little fluorescence signal was found on the spots containing unrelated capture probe and hybridization signals from spots containing related probes exceeded signals from the spots containing unrelated probes and background levels (**Table 2**). Because weak signals were unavoidable at some probe spots, hybridization was considered significant only when the measured hybridization signal determined with Silverquant exceeded 200. With this subjective threshold, we obtained a false negative ratio of approximately 5% (two false negatives out of 36 samples, cf. **Table 2**) and a false positive ratio close to 0% (none observed). A more comprehensive validation study will be needed to establish a more robust threshold value. All of the samples contained high amounts of the *trnL* intron; the rice, maize, wheat, and rye samples contained the most (Ct values ca. 15), while oat and barley contained the least amount (Ct values ca. 17), corresponding to approximately 10^5-10^7 target copy numbers.

The results obtained for the 10 food samples (**Table 3**) were generally in agreement with the declared lists of ingredients. The detection of up to three diverse species in the different food samples was easily achieved with the microarray system. The "GeMMA mixed wheat/soybean flour" and "grain feed sample 80" appeared to contain oat and "Pringles sour cream and onion chips" appeared to contain barley, although these ingredients were not declared on the ingredient label provided on the samples. To verify and eventually quantify the presence of barley in the chips, we performed a barley specific real-time

Table 2. Fluorescence Signals[a] for Hybridization Analysis of PCR Products from Mixed Samples

sample composition	not present	PSmont (rye)	PTeast (wheat)	PErhart (rice)	PAsat (oat)	PHvulg (barley)	PAndro (maize)
wheat, barley, rye, rice, oat	maize	4284 ± 939	9664 ± 625	17059 ± 2368	1741 ± 200	232 ± 46	*123 ± 12*
wheat, barley, rye, rice, maize	oat	665 ± 164	2650 ± 1498	12579 ± 1216	*−1 ± 14*	*78* ± 73	22271 ± 1541
wheat, barley, rye, maize, oat	rice	2360 ± 426	5279 ± 572	*41 ± 19*	1220 ± 122	*154* ± 25	29293 ± 2801
wheat, barley, maize, oat, rice	rye	*26 ± 15*	8818 ± 296	13987 ± 1623	1138 ± 84	375 ± 64	27024 ± 2149
wheat, maize, oat, rice, rye	barley	2364 ± 207	3630 ± 809	11548 ± 1311	2026 ± 1144	*−26 ± 9*	23622 ± 83
maize, oat, rice, rye, barley	wheat	5070 ± 792	*8 ± 9*	17121 ± 3007	2590 ± 376	623 ± 21	31152 ± 2174

[a] Mean values from four spots with standard deviation, in which the measured signal for each spot was determined as described in the Materials and Methods; italic, signal below the threshold of 200.

Table 3. Analysis of DNA Extracts of Food Samples Using Silverquant to Measure Signal Intensities[a]

product	PAndro (maize)	PTeast (wheat)	PSmont (rye)	PHvulg (barley)	PErhart (rice)	PAsat (oat)
oat biscuits with cornflakes	1172 ± 136	4630 ± 228	*53 ± 30*	*73 ± 16*	*42 ± 11*	3230 ± 737
bread	*−145 ± 22*	34658 ± 3139	3999 ± 351	*102 ± 76*	*−39 ± 39*	842 ± 122
Pringles sour cream and onion chips	30521 ± 4819	*122 ± 6*	*185 ± 9*	**261 ± 40**	25234 ± 4496	*27 ± 15*
GeMMA mixed wheat/maize flour	38127 ± 2510	31080 ± 1747	*182 ± 21*	*123 ± 55*	*71 ± 19*	*111 ± 49*
GeMMA mixed wheat flour	*142 ± 72*	32375 ± 5704	*−50 ± 5*	*−41 ± 17*	*−57 ± 54*	**255 ± 56**
grain feed sample 80[b]	*160 ± 72*	6348 ± 2329	*−42 ± 17*	591 ± 110	*−9 ± 91*	**818 ± 88**
grain feed sample 83[b]	*114 ± 29*	2611 ± 346	*−5 ± 25*	10257 ± 611	*54 ± 40*	13646 ± 1268
grain feed sample 84[b]	14650 ± 1350	4473 ± 969	*−39 ± 4*	*145 ± 46*	*33 ± 29*	7576 ± 469
grain feed sample 85[b]	*43 ± 6*	*136 ± 13*	*−44 ± 21*	16784 ± 2978	*−21 ± 23*	3323 ± 156
grain feed sample 86[b]	*170 ± 13*	*92 ± 14*	*48 ± 18*	13437 ± 1265	*123 ± 8*	9858 ± 980

[a] Mean values from four spots with standard deviation, in which the measured signal for each spot was determined as described in the Materials and Methods. [b] The grain feed samples 80–86 were provided from the Norwegian Food Safety Authority. Italic, values below the threshold value; bold, unpredicted positive results.

quantitative PCR analysis based on the single copy gene HvPKAB1 (*25*), and the results indicated the presence of approximately 110 haploid nuclear barley genomes per μL in the DNA extracted from the sample.

DISCUSSION

In single taxon analyses, positive signals were produced and clearly distinguishable from any background. In the analysis of defined samples, we observed false negatives for barley although the real-time PCR demonstrated high amounts of the *trnL* intron (**Table 2**), probably due to competitive effects and differences in the PCR efficiency. We recognize that the PCR technique is subject to amplification efficiency bias, especially in multitemplate reactions (*26*). The bias in multitemplate PCR can be caused by differences in primer binding energy, reannealing of templates, different copy numbers, and low efficiency of template dissociation due to high G−C content (*27*). We observed that in the mixed samples (**Table 2**) maize and rice were overamplified, while barley was underamplified, although the target amount did not seem to differ much according to the real-time PCR.

We selected stringent conditions, with low oligonucleotide concentrations and relatively high labeling and hybridization temperatures. Other stringency reactions could perhaps have minimized cross-reactivity signals further. However, high specificity is achieved at the expense of reduced sensitivity. There are several parameters that influence the rate of heteroduplexes formation during hybridization, including the concentration of both the probe and the target and the sequence composition of the heteroduplexes. The sequence composition is of great concern, and increased duplex stability is observed in G−C rich regions. The size of the capture probe may range from whole PCR products to oligonucleotides consisting of 20 or fewer bases. Another factor with an impact on the heteroduplex formation is the melting temperature. The higher the G−C content, the higher the required hybridization temperatures, but this may be at the cost of excessive signal loss for probes with low G−C content. Letowski et al. (*28*) suggested that the use of different hybridization temperatures may be necessary in the case of probes with dissimilar relative G−C content. We did not determine the impact of G−C content on the specificity and sensitivity of the assay developed in this study. The G−C content of PTaest is 12%, and low signal intensities could therefore be predicted. Contrarily, this probe gave clear and strong signal intensities for all of the samples tested. PAsati*b* on the other hand, with a G−C content of 50%, yielded weak signal intensities. Lane et al. (*29*) demonstrated that the most consistent explanation for probe hybridization failures is target secondary structures. The labeling strategy described in this study may seem unconventional as compared to the labeling strategy used elsewhere (*12*, *13*). An advantage of using the labeling strategy presented here is that any complicating secondary structure that might interfere with longer target hybridization is negated since the slightly lengthened primer is the target that hybridizes to the capture probe on the microarray rather than the PCR product. Alternative chemistries, e.g., using fluorophore-conjugated universal primers, may be cheaper alternatives as the chemistry used in this study may be quite expensive. Longer oligonucleotide probes give better signal intensity than shorter probes, and the potential for target competition among probes of varying sizes on the same microarray could occur during hybridization (*28*).

Several distinct species may share the same sequence identity within the probe region. Although the present assay indicates high specificity of most of the probes, not all potentially cross-reacting species have been tested. For instance, the present assay may not work well to distinguish wheat from members of the genus *Aegilops*, *Heteranthelium piliferum*, and *Taeniatherum caput-medusae*. The latter taxon has been described as both noxious and invasive in California and other parts of the United States (*30*). Some members of the genus *Aegilops* hybridize with wheat and are sometimes crossed with wheat to pass on adaptive features such as cold tolerance and disease resistance. To identify maize, the probe PAndro*a* was used in the method developed. However, this probe is not strictly specific to maize; that is, it will conceivably identify other members of the tribe Andropogoneae. This tribe constitutes mostly weeds but also durra, lemongrass, and members of *Saccharum*; the latter includes medical herbs and sugarcane. Consequently, there is a possible risk of reporting false positives if there are other members of the tribe Andropogoneae present in a food sample. However, this is not considered a major problem in a routine analysis, as we believe the mentioned grasses are rarely used in foods that are normally subject to food analysis or at least their DNA is not present.

In an ideal assay, all truly positive samples must be detected and differentiated. In this assay, the regions where the probes were positioned are in the loop regions of the *trnL* intron and these regions are often associated with hot spots for mutations, both nucleotide substitutions and substitution or insertion−deletion (indel) events (*31*). It is expected that these regions would evolve faster than coding regions and thus provide improved resolution for low-level systematics (*32*). Consequently, a strain that in theory should be identified using this microarray approach may have undergone a substitution or indel event, thus producing a false negative. We do not know the frequency of these events at the population level of the plant species included in this microarray, but a study including 11 specimens of *Poa pratensis* reported several different haplotypes based on *trnL-F* sequences, and the haplotypes varied by both single nucleotide substitutions and indels (*33*). Such chloroplast DNA variability has been observed for other members of the genus *Poa*, including *Poa nemoralis* and *Poa bulbosa* (*34*, *35*).

When analyzing real food samples, we detected undeclared ingredients in three out of 10 samples, which indicates that the final products or their ingredients probably were botanically impure or contaminated. This is quite normal, as botanical impurity and contamination can occur at several stages during growth, harvesting, storage, and transport as well as during milling and further processing. International trade allows some botanical impurity. The risk of contamination is in general higher with increased processing (*36*), and although wheat is the most common contaminant of, e.g., oat products, barley and rye are also frequently observed (*7*, *36*). The level of contamination is generally low in most cases, as was the case for the samples analyzed in the present study.

The largest gain in overall assay sensitivity will be achieved at the level of sample preparation rather than at the level of amplicon detection, and the reason for this is that food and feed samples may produce low template yields and coprecipitated inhibitors may have a considerable impact on the assay sensitivity even before the microarray detection is considered (*29*). This means that to improve the sensitivity of a microarray assay, great care must be taken at the earlier steps in the detection process. The limit of detection is defined as the lowest quantity that can be reliably detected (*4*), but this is a function of sample quality. Grinding, heating, acid treatment, and other processes rapidly degrade the DNA in the food sample, and as

a consequence, many products contain small amounts of low quality DNA. With increased DNA processing, the size of the genomic DNA fragments decreases, and consequently, the amplicon size should be minimized to obtain the highest achievable detectability. The amplicon size of the *trnL* intron using the universal primers ranges between 250 and 1400 bp (*37*), and an amplicon of this relatively large size may very well be degraded in a real food sample. The *trnL* intron is located within the chloroplast genome, which is a high copy number genome relative to the nuclear genome of plants (*38*). Consequently, the probability of detecting the *trnL* intron is higher than for detecting a nuclear marker. A disadvantage of the method is that it does not allow quantification of the targets because of the variability in copy number, together with the influences of the sensitivity and variability between observed fluorescent signals.

Rapid detection and identification of cereals can be achieved by direct detection of characteristic plant specific genes, and real-time PCR has been developed for species specific detection of, e.g., rice (*39*) and maize (*40*). The use of different primer sets for different species is impractical in routine analyses for samples that may contain several cereals, and either a complex PCR with mixtures of primers is needed or a large series of individual PCRs must be run in parallel. Multiplex PCR is a promising tool in diagnostics (*11*), although the presence of more than one primer pair in a multiplex PCR increases the chance of obtaining false amplification products, primarily due to primer−dimer formation. The risk of such primer−dimer formation is reduced when using universal primers combined with microarray technology.

In conclusion, we have developed a simple and accurate DNA method that is able to identify and discriminate between six important and closely related cereal food plant species. This method can identify mixtures of species in real-food samples and is to our knowledge the first report of an oligonucleotide array differentiating between plant species. The multiwell format described here permits higher sample throughput than conventional microarrays where a separate slide is required for each test. The combination of the sensitivity of the universal PCR with the specificity of the labeling reaction provides a powerful tool to identify the origin of food products and to protect consumers against faulty ingredient declaration, e.g., in relation to allergens and GMOs. The experiment demonstrates that this protocol offers sufficient sensitivity to be applicable in routine diagnosis of food samples.

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