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Authentication of Coffee by Means of PCR-RFLP Analysis and Lab-on-a-Chip Capillary Electrophoresis

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Coffee is one of the most important world food commodities, commercial trade consisting almost entirely of Arabica and Robusta varieties. The former is considered to be of superior quality and thus attracts a premium price. Methods to differentiate these coffee species could prove to be beneficial for the detection of either deliberate or accidental adulteration. This study describes a molecular genetics approach to differentiate Arabica and Robusta coffee beans. This employs a Polymerase Chain Reaction−Restriction Fragment Length Polymorphism to monitor a single nucleotide polymorphism within the chloroplastic genome. Samples were analyzed with a lab-on-a-chip capillary electrophoresis system. Coffee powder mixtures were analyzed with this technique, displaying a 5% limit of detection. The plastid copy number was found to be relatively constant across a wide range of bean samples, suggesting that this methodology can also be employed for the quantification of any adulteration of Arabica with Robusta beans.

KEYWORDS: Coffee authentication; food forensics; SNP; PCR-RFLP; lab-on-a-chip; chloroplasts

INTRODUCTION

The deliberate misrepresentation of food ingredients on product package labels is a potential problem in the food industry. As a result, food forensics is an emerging discipline that aims to guarantee the authenticity of food products commanding a premium price (*1*). One of these is coffee, which is one of the most important food commodities in world trade. This is mainly due to its global consumption and massive total world production, which amounted to an annual average production of approximately 106 millions of bags for the crop year of 2005/2006 (*2*). Coffee also makes a significant contribution to the economies of many developing countries. The self-pollinating, allotetraploid *Coffea arabica* and the diploid *Coffea canephora* Pierre (*Coffea robusta*) represent the two most commercially significant species. The former is considered to provide superior quality coffee and contributes to >70% of the world's coffee production (*3*, *4*). Thus, Arabica coffees are sold at ∼2−3 times the price of Robustas due to their finer flavor and better quality (*5*). Therefore, there are serious economical reasons to demand that coffee authenticity be guaranteed. Adulteration of coffee can occur at several steps in the field-to-cup production chain, and as a result both green and roasted beans, along with ground coffee, need to be authenticated. Roasting companies require genuine green beans, whereas retailers need to authenticate final products such as roasted intact or ground beans.

Most of the current analytical approaches for the discrimination of Arabica and Robusta coffees arise from the area of analytical/instrumental chemistry. In particular, some indicative analytes such as diterpene-16-*O*-methylcafestol (*6*, *7*), total free amino acids (*8*), trigonelline (*9*), triglycerides (*10*), fatty acids (*11*), sterols (*12*), diterpene kahweol (*5*), and protein profile (*13*, *14*) have been used for the characterization of pure varieties. For the quantitative detection of admixtures, Fourier transform infrared spectroscopy (FT-IR) in combination with principal component analysis (PCA) and classical discriminant analysis (CDA) (*15*) and metal content along with descriptor pattern recognition techniques (*16*) have been used with successful results. Genetic, that is, DNA-based, techniques such as microsatellites (*17*, *18*) and randomly amplified polymorphic DNA (RAPD) (*19*, *20*) analyses have been used only for basic genetic research such as coffee characterization and gene introgression on pure samples. To the best of our knowledge there is no published work on coffee authentication through DNA-based approaches.

In this study, we report the use of a Polymerase Chain Reaction−restriction fragment length polymorphism (PCR-RFLP) and lab-on-a-chip capillary electrophoresis approach to detect contamination of Arabica coffees with Robusta. The choice of target was based on a phylogenetic study, in which a number of single-nucleotide polymorphisms (SNPs) were found among several *Coffea* species, indicating the existence of different chlorotypes between Arabicas and Robustas (*21*). The selected DNA target was the chloroplastic *trn*L(UAA)−

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Table 1. Coffee Samples Used in This Study

no.	coffee sample, origin	species
1	Robusta, Rwanda	*C. canephora*
2	Café de Cuba, Cuba	*C. arabica*
3	Mysore A, India	*C. arabica*
4	Sumatra Lintung	*C. arabica*
5	Jacaranda organic coffee, Brazil	*C. arabica*
6	El Salvador, El Carmen Estate (Icatu)	*C. arabica*
7	India Robusta Mysore	*C. canephora*
8	Maragogype, Nicaragua, Finca el Platanillo	*C. arabica*
9	Mondo Novo, Natural Sertarzinho farm	*C. arabica*
10	Accesion 1s/2, Uganda	*C. canephora*
11	Yellow bourbon, Brazil, Cachoeira farm	*C. arabica*

*trn*F(GAA) intraspacer region, which exhibits three single-base substitutions that lead to different chlorotypes for *C. arabica* and *C. canephora* species (*21*). One of these SNPs resides in a *Psu*I restriction site, resulting in the site being present in Robusta but absent in Arabica. The purpose of this study was to evaluate whether this SNP could be exploited for the qualitative/quantitative detection of Robusta contamination of Arabica using a PCR-RFLP approach.

MATERIALS AND METHODS

Coffee Material. The coffee material used in this study was Arabica and Robusta green beans from several geographical origins (**Table 1**), kindly provided by Mercanta Ltd. "The Coffee Hunters" (London, U.K.). Samples 1 and 2 were used for the admixture of Arabica and Robusta.

DNA Extraction and Quantification. Green beans (20 g) were ground in a milling machine (Glen Creston Ltd., Stanmore, U.K.) using a mesh of 2.0 mm. For the analysis of the admixtures, green coffee bean powders containing 1, 5, 10, 30, and 50% of Robusta in an Arabica background were prepared. DNA was extracted from a 150 mg sample of powder using the GeneSpin DNA extraction kit (GeneScan Analytics GmbH, Freiburg, Germany) according to the manufacturer's instructions. Extracted DNA samples, 5 μL from each DNA, were initially mixed with SYBR Green I (Molecular Probes−Invitrogen Corp., Paisley, U.K.), and after a 1.0% agarose gel electrophoresis, they were analyzed with a Fluoro-S Multi-Imager (Bio-Rad Laboratories, Hertfordshire, U.K.). Uncut lambda DNA (Promega Corp., Southampton, U.K.) in several known concentrations was also used for the quantification of the DNA samples.

PCR-RFLP Methodology. PCR was performed in 5.0 μL of 10× AmpliTaqGold buffer, 5.0 μL of 25 mM $MgCl_2$, 1.0 μL of dNTPs mixture (10 mM each), 1.0 μL of AmpliTaqGold polymerase (Applied Biosystems, Warrington, U.K.), 1.5 μL (10 pmol) of each coffea1 primer (MWG-Biotech GmbH, Ebersberg, Germany) (**Table 2**), and ∼40 ng of total genomic DNA and was made up to 50 μL final volume with nuclease-free water (Sigma-Aldrich, Dorset, U.K.).

PCR conditions were 95 °C for 10 min, 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and 1 cycle at 72 °C for 10 min. The amplification reaction took place in an AB9700 thermocycler (Applied Biosystems, Warrington, U.K.). PCR amplicons were purified directly from the PCR reaction mixture using the QIAquick PCR purification kit (Qiagen, Crawley, U.K.). For the elution of the DNA, 30 μL of molecular grade (Sigma-Aldrich) water was applied into the center of the resin prior to the last centrifugation step. Purified PCR amplicons were quantified using the NanoDrop ND-1000 (NanoDrop Technologies Inc., LabTech, Ringmer, U.K.) according to the manufacturer's procedures.

A 17 μL sample of the purified amplicon was mixed with 2 μL of buffer B (provided with the enzyme) and 1 μL of *Psu*I restriction endonuclease (Fermentas, GmbH, St. Leon-Rot, Germany), vortexed, and incubated at 37 °C for 60 min. Mixtures of Arabica and Robusta amplicons were made to a final volume of 4 μL, and then 1 L of *Psu*I and 4 μL of buffer B were added. The reaction mixture was then made up to a 40 μL final volume with nuclease-free water and treated as above.

DNA Fragment Analysis. The visualization of fragment profiles generated through restriction digest was carried out using a DNA-1000 LabChip with the Agilent 2100 capillary electrophoresis lab-on-a-chip system (Agilent Technologies Ltd., South Queensferry, U.K.). The preparation of both chips and reagents was carried out according to the manufacturer's instructions. The quantification of admixtures was based on the ratio of peak area of Robusta to the total peak area of both species, and the whole analysis was carried out with the 2100 expert software (Agilent Technologies U.K. Ltd.), version B.01.02.SI136.

Samples were also run in a standard 2% agarose gel electrophoresis (Melford Laboratories Ltd., Ipswich, U.K.), stained with ethidium bromide (Invitrogen, Paisley, U.K.) (*22*) and visualized using a UV transilluminator coupled with a GelDoc 2000 imager (Bio-Rad Laboratories, Southampton, U.K.).

Real-Time PCR. A real-time PCR was performed in an ABI Prism 7700 sequence detector (Applied Biosystems, Warrington, U.K.). DNA samples were amplified using both nuclear and chloroplastic specific PCR primers in separate reactions in duplicate. Each reaction contained 12.5 μL of 10× Power SYBR Green master mix (Applied Biosystems, Warrington, U.K.), 0.3 pM of each primer (**Table 2**), and ∼5 ng of total genomic DNA and was run under standard default PCR conditions, which were 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Pooled genomic DNA samples were run in triplicate to generate a standard curve for each PCR primer pair. Data were then analyzed with sequence detector v. 1.7 (Applied Biosystems) software, and the relative concentrations were calculated according to the user's instructions. Statistical analysis between Arabica and Robusta samples was carried out with the *t* test (two-sample assuming equal variances) on Microsoft Excel.

RESULTS AND DISCUSSION

Analysis of the Chloroplastic Target. A 251 bp section of the chloroplastic *trn*L(UAA)−*trn*F(GAA) intraspacer region, containing the *Psu*I restriction endonuclease site, was amplified using coffea1 primers (**Table 2**) and DNA templates extracted from green coffee bean samples as listed in **Table 1**. These primers were designed such that the *Psu*I restriction site was located asymmetrically within the amplicon so that, in the presence of an intact restriction site, digestion with *Psu*I would result in two fragments of 92 and 159 bp, respectively. Amplicons were purified and subjected to digestion with *Psu*I, and the resultant fragments were analyzed by standard agarose gel electrophoresis. The results are shown in **Figure 1**. All Arabica coffees tested gave a single product of ∼251 bp, indicating that, as expected, the *Psu*I restriction site has been disrupted by the SNP. In contrast, the two Robusta samples showed two smaller fragments at around 92 and 159 bp, indicating that, again as predicted, the restriction site is intact.

These results might indicate that this SNP could be used as a diagnostic to differentiate all Arabica and Robusta varieties.

Table 2. PCR Primers Used in This Study

primer	5′–3′ sequence	target	accession no.
Coffea1-F	AATCGATCTGGACGGAAAAGC	*trn*L-*trn*F intragenic region of chloroplast DNA	CAU93387, CCU93393
Coffea1-R	AGCATCCTCATTTTATGAGAAAAGG		
GlydeP-F	GAGAATTGTGGATTCCCCAGT C	*Glycine decarboxylase* P subunit nuclear gene	AF042072, AF043097
GlydeP-R	TCAGCAGGGATTCAAGACGTC		

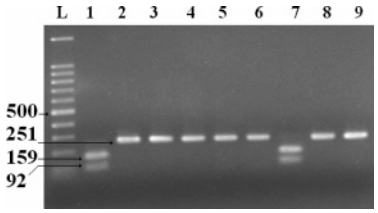

Figure 1. Agarose gel electrophoresis of PCR-RFLP of chloroplastic *trn*L (UAA)–*trn*F (GAA) intraspacer region from several *C. arabica* and *C. canephora* cultivars. Each number corresponds to the cultivar as listed in **Table 1**: Robusta samples (lanes 1 and 7); Arabica samples (lanes 2–6, 8, and 9); (lane L) 100 bp ladder.

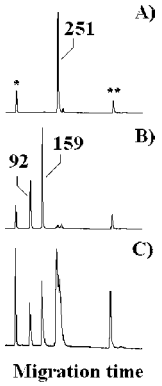

Figure 2. Aligned electropherograms of PCR-RFLP profiles as revealed by the Agilent 2100 Bioanalyzer capillary electrophoresis: (**A**) Arabica; (**B**) Robusta; (**C**) 50:50 powder mixture. Single and double asterisks correspond to 15 and 1500 bp internal markers, respectively, according to the manufacturer's instructions.

However, care should be taken for future applications because *C. canephora* cultivars may have contributed in the past to the production of some commercially important Arabica cultivars (*23*). If the female donor was a *C. canephora* during the last backcrossing, then the chlorotype is likely to differ from that of traditional Arabicas. Thus, further confirmation of the chlorotype on individual Arabica cultivars is necessary before these could be included in any discriminatory analysis.

Arabica and Robusta—samples 2 and 1, respectively—were selected for further analysis (**Table 1**). The PCR-RFLP profiles from these two pure varieties were also analyzed using the capillary electrophoresis lab-on-chip technology (**Figure 2**). In this instance the Arabica generated a single high-intensity band corresponding to a length of 251 bp, whereas the Robusta showed two peaks corresponding to the 92 and 159 bp PCR-RFLP products. A 50:50 mix of Arabica and Robusta amplicons was also subjected to the restriction digest, and the resultant electropherogram showed three clear peaks. These, when analyzed using the 2100 expert software, this mixture gave a ratio of 50:50 for Arabica/Robusta, thus indicating the potential application of this technology to discriminate and quantify coffee admixtures.

Analysis of the Ratio of Plastid DNA Copy Number per Cell. Accurate quantification using a chloroplastic target would require the plastid DNA copy number to be stable between coffee varieties. Thus, the aim of this experiment was to determine the plastid DNA copy number per cell in several commercially important coffee samples. A real-time PCR methodology was set up to compare the relative abundance of a nuclear and plastid target gene. The *glycine decarboxylase* P subunit gene and the *trn*L−*trn*F region were selected as the nuclear and chloroplastic targets, respectively. Gel electrophoresis of the end-point products confirmed that no secondary products or primer dimers were formed (data not shown). Additionally, both PCR primer pairs used displayed amplifications with similar efficiencies between the two targets (data not shown). Therefore, the fluorescence signal accurately reflects the plastid-to-nuclear DNA copy ratio according to

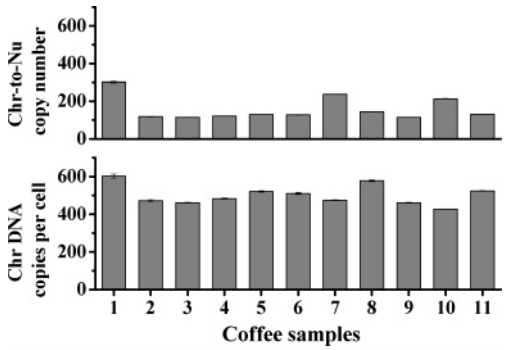

Figure 3. Quantification of the plastid DNA copy numbers as measured through Real-Time PCR: (top) plastid (Chr) to nuclear (Nu) DNA copy number for each coffee sample; (bottom) above numbers converted to plastid DNA copies per cell.

$$\mathrm{PNR} = 2^{(Ct_c - Ct_n)} \quad (1)$$

with PNR denoting the plastid-to-nuclear DNA copy ratio and Ct_c as well as Ct_n denoting the *Ct* values for chloroplast and nuclear targets, respectively. To take into account the difference in ploidy status of Arabica and Robusta samples, plastid DNA copy number per cell was then calculated by multiplying the ratio values by a factor of 2 or 4 depending on whether the DNA sample originated from Robusta (2N) or Arabica (4N) coffee, respectively (**Figure 3**). The results show that the plastid-to-nuclear copy numbers for the three Robusta samples 1, 7, and 10 were all ∼2 times higher than that for the Arabica samples. By converting this to plastid DNA copies per cell, as described above, it was found that both species have the same number of plastid DNA copies per cell ($p = 0.9997$), this being 502 ± 91 and 502 ± 40 copies for Robusta and Arabica, respectively. This value for plastid DNA copy number is at the lower end of the range reported for other plant tissues (*24*, *25*). However, care should still be taken when using chloroplast targets for authentication because whereas copy number was found to be relatively constant between Arabica and Robusta varieties, there was some variability, and this has to be taken into account when accurate quantification of adulteration is required.

Analysis of PCR Amplicon Mixtures. PCR amplicons, generated from either pure Arabica or Robusta DNA, were mixed at several ratios ranging from 50:50 to 99:1 (Arabica/Robusta) and subjected to *Psu*I restriction, and the resultant PCR-RFLP products were analyzed by capillary electrophoresis lab-on-a-chip. The 2100 expert software was used to calculate experimental ratios of Arabica/Robusta using the corrected peak areas of the PCR-RFLP products (**Table 3**). Experimental values plotted against theoretical gave a linear regression ($R^2 = 0.9975$) showing that this approach was efficient for detection down to 1% and as a model system suggested that this approach could be used to authenticate coffee mixtures.

Analysis of Coffee Powder Mixtures. Samples of pure Arabica and Robusta green coffee beans were used to generate a powder. Admixtures of Arabica/ Robusta in the ratios 50:50, 70:30, 90:10, 95:5, and 99:1 were then prepared. DNA was then extracted from these admixtures and subjected to PCR-RFLP analysis. Fragment profiles were initially detected through

Table 3. Theoretical Percentage of Robusta Included in the Model Mixtures versus the Experimental One As Analyzed through the Described PCR-RFLP Approach and Quantification from the Electropherogram As Generated by the 2100 Bioanalyzer

	experimental	
theoretical	amplicon mixtures	powder mixtures
50	50.0 ± 3.7	65.4 ± 0.2
30	30.2 ± 2.1	29.6 ± 2.9
10	13.9 ± 3.5	4.2 ± 0.8
5	NA[a]	1.25 ± 0.0
1	3.7 ± 0.2	ND[b]

[a] Not available. [b] Not detected.

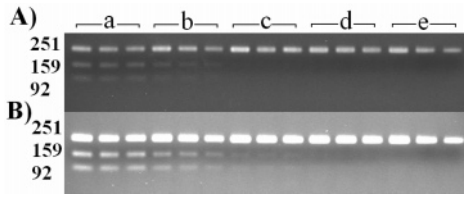

Figure 4. Agarose gel electrophoresis of PCR-RFLP profiles from several ratios of Arabica to Robusta coffee powder mixtures in triplicates: (a) 50:50, (b) 70:30, (c) 90:10, (d) 95:5; (e) 99:1. The gel was exposed in UV light with two different exposure times, leading to nonsaturated (**A**) and saturated (**B**) signal. The former gave a limit of detection of ~30%, whereas the latter allowed us to drop it to ~10%.

standard agarose gel electrophoresis (**Figure 4**). The detection limit for Robusta in this case seemed to be at the 10% inclusion level, but this was only after the gel had been exposed to UV light for a long time. This has resulted in saturation of the signal from the uncut band, making any quantification attempt impossible. The same PCR-RFLP samples were also analyzed using the Agilent 2100 Bioanalyzer. The experimentally determined percentage of Robusta was calculated (**Table 3**) and, when plotted against the theoretical values, gave a linear regression curve ($R^2 = 0.9772$). In this case, the limit of detection for Robusta inclusion dropped to 5%.

In addition to the two cut fragments of 159 and 92 bp and the uncut amplicon of 251 bp, the PCR-RFLP profile also showed peaks with retention times close to that of the uncut amplicon. These were not present when the amplicon originated from one species, leading to the suggestion that they are likely to be heteroduplexes, which are commonly produced during the denaturation and rehybridization steps of a PCR amplification of a template that consists of two different haplotypes (*26*). Similar peaks have been shown to be generated in the analysis of mitochondrial heteroplasmy (*27*). Treatment of the ratio mixture with *CEL*1, an enzyme that cleaves such heteroduplexes, has confirmed this suggestion (data not shown). Therefore, these extra peaks were excluded from the calculations of admixture content.

In the present study, we describe a PCR-RFLP method, which in combination with the Agilent 2100 Bioanalyzer lab-on-a-chip capillary electrophoresis, can be used to quanitatively detect adulteration in green coffee beans. The chloroplastic target used—the *trn*L(UAA)−*trn*F(GAA) intergenic spacer region—was found to be discriminatory for all of the Arabica and Robusta varieties used in this study. However, the ability of this target to discriminate all commercial coffee varieties would need to be further validated using as many cultivars as possible. The use of a chloroplastic target has obvious advantages in terms of copy number, which is likely to make detection more sensitive, compared to nuclear targets. One potential problem with the use of chloroplastic targets is the possibility that copy number may vary. For instance, previous studies using leaf tissue have shown that plastid copy number can vary due to stage of development (*24*), size of the cell (*25*), and senescence or season (*28*). However, there is no information on variability, if any, in plastid copy number in seed (bean) tissues. The results presented here, using a real-time PCR method, would suggest that plastid copy number seems stable in coffee bean tissue and that chloroplastic targets may be suitable for quantitative analysis of coffee admixtures. However, although the beans used in this study originated from a variety of geographical regions, further studies are required to confirm that plastid copy number remains relatively constant across a wider range of varieties and is not influenced significantly by environmental or method of cultivation in the supply chain.

This methodology, as with similar previous study on fish species (*29*, *30*), is very easy to use, and analysis using the LabChip is straightforward and relatively quick—approximately 60 min per 12 samples after the restriction digest step. This would mean that this methodology may be suitable for the routine analysis of coffee samples. A similar approach could be employed for the analysis of roasted beans and even ground coffee providing that DNA suitable for PCR-RFLP can be extracted from these commodities. There is a strong possibility that processing of the bean, either through roasting or other treatments such as decaffeination, will result in the degradation of the DNA. A previous study (*31*) has shown that DNA suitable for PCR analysis can be extracted from beans subjected to various levels of roasting and from commercial coffee powder.

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