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Adulteration Identification of Citrus Juice by Denaturing Gradient Gel Electrophoresis (DGGE)

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ABSTRACT

*trn*L intron and *trn*L-*trn*F non-coding region have become powerful tools to identify plants species in the past few years. In this study, above two regions are used to identify oranges and mandarins via a variable DNA fragment in *trn*L intron. In order to identify the DNA, a primer pair was designed to amplify the variable fragments and the PCR products were analyzed by Denaturing Gradient Gel Electrophoresis (DGGE). The method successfully detected other citrus juices mixed in all of the falsely claimed 100% orange juices. It is suggested that the method is useful to detect the adulteration of 100% pure orange juices.

Key words: *trn*L, DGGE, citrus, chloroplast

INTRODUCTION

Orange juice is the most popular juice worldwide. According to the statistics of the Food and Agriculture Organization (FAO), the export of concentrated citrus juice in 2002 was US$ 6.2 billion and the output of citrus juice increased by 50% from 2000 to 2002. Adulteration of juices is a recurrent problem, which has received extensive media attention. Furthermore, recent health problems related to food industry have also increased consumer concerns[1]. Common methods of adulteration include addition of water, less expensive juices, pulp wash, colorants, and other undeclared additives either alone or in combination to replicate the composition profiles of pure juices[2,3]. Numerous methods of detecting juice adulteration have been developed, such as HPLC[4,5], capillary electrophoresis[1] and pyrolysis mass spectroscopy[6], that analyze the chemical components of juice. Analyzing a single natural juice component is inadequate in obtaining sufficient information to determine juice purity; therefore, multiple component chemical analyses are required to accurately evaluate reliably the differences between adulterated and pure juices. This approach, however, is both time-consuming and expensive[6].

Currently, DNA markers are extensively employed for taxonomy study in many plants[7,8]. These DNA markers provide evidences for plant species identification and are useful for analyzing commercial fruit products. Several DNA markers have been used in citrus analysis, such as Random Amplified Polymorphic DNA (RAPD)[9,10,11], Restriction Fragment Length Polymorphism (RFLP)[12,13], Sequence-Characterized Amplified Regions (SCARs), and chloroplast DNA (cpDNA)[14]. Among these, cpDNA analysis is especially effective in phylogenetic analysis due to its evolutionary conservatism, relative abundance in plant tissue, small size and predominant uniparental inheritance [15]. The most common chloroplast gene utilized to obtain sequence data for cladistic analyses in plants is the large subunit of the ribulose-1,5-bisphosphate carboxylase / oxygenase gene (*rbc*L)[16]. However, when employed alone, *rbc*L is less suitable at lower taxonomic levels than more rapidly evolving genes, introns, and spacers, such as the non-coding region of the chloroplasts DNA *trn*L (leucine) intron and *trn*L-*trn*F (phenylalanine) intergenic spacer[17,18,19].

Denaturing gradient gel electrophoresis (DGGE) separates PCR amplicons of similar length with dissimilar nucleotide compositions on a denaturing gradient gel[20]. The DGGE system can be divided into the perpendicular and parallel DGGE, which are differentiated by the direction of denaturant gradient and electrophoresis. In this study, *trn*L intron and *trn*L-*trn*F intergenic spacer are amplified with a specific primer and the PCR products are employed to run the DGGE. This method is useful and accurate, and can be used in fruit industry.

MATERIALS AND METHODS

I. *Plant Materials and DNA Extraction*

Ten genotypes of *Citrus*, six oranges (*Citrus sinensis* (L.) Osbeck) and four mandarins (*Citrus reticulata* Blanco) are used in this study. More specifically six oranges are Liucheng, Pineapple, Valencia, Hamlin, Parson and Navel Seeding and four mandarins are Ponkan, Tankan, Satsuma Seeding and Murcoot. These plant materials were provided by the National Plant Genetic Resource Center of Taiwan. For sequencing, total DNA was extracted from young leaves according to the method described in Doyle and Doyle[21].

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For DGGE, DNA was extracted from citrus juice according to the method developed by Lipp *et al.*[22].

II. *Amplification and Sequencing of trnL Intron in Chloroplast DNA*

The cpDNA of the 10 genotypes were amplified using one pair of universal primers[23]. The PCR conditions for a total volume of 25 μL were as follows: 200 ng of template DNA; 10× buffer; 6.25 pmoles/each; 0.1 mM dNTP; and 0.5 Unit DynaZyme. The parameters of the amplification reaction were: 1 cycle of 3 min at 96°C; 30 cycles of 1 sec at 96°C, 10 sec at 54°C, 20 sec at 72°C, and an end cycle of 10 min at 72°C. Sequences were generated on an ABI automated sequencer from the Mission Biotech Co. Ltd. (Taiwan) employing the same primers as in amplification. The sequences were aligned with a BioEdit Sequence Alignment Editor.

III. *Oligonucleotide Primers and PCR Condition*

Synthesized primers supplied by the Mission Biotech Co. Ltd. (Taiwan) were diluted with an appropriate volume of water to a final concentration of 100 μmol/L and stored at -20°C until use. The primer pair (B49317 and A50272) designed by Pierre *et al.*[23] was used to amplify the cpDNA

(A)

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Mandarin    CGAAATCGGTAGACGCTACGGACTTAATTGGATTGAGCCTTAGTATGGAA-CTTTCTAAG 59
orange      CGAAATCGGTAGACGCTACGGACTTAATTGGATTGAGCCTTAGATTGGAAACTTACTAAG 60

Mandarin    TGATAACTTTCAAATTCAGAGAAACCCAGGAATTAAAAATGGGTAATCCTGAGCCAAATC 119
orange      TGATAACTTTCAAATTCAGAGAAACCCAGGAATTAAAAATGGGTAATCCTGAGCCAAATC 120

Mandarin    CTCTTCTCTTTTCCAAGAACAAACAGGGGTTCAGAAAGCGAAAAAGGGGGATAGGTGCAG 179
orange      CTCTTCTCTTTTCCAAGAACAAACAGGGGTTCAGAAAGCGAAAAAGGGGGATAGGTGCAG 180

Mandarin    AGACTCAATGGAAGCTGTTCTAACAAATGGAGTTGACTGCCCTTTTTGGTAAAGAAAAAA 239
orange      AGACTCAATGGAAGCTGTTCTAACAAATGGAGTTGACTGCCCTTTTTGGTAAAGAAAAAA 240

Mandarin    GAAAGTAAAATGAATGCTTCTATCGAATATCGAAACTCGATAAAGGATGAAGGATAAGGG 299
orange      GAAAGTCAAATGAATGCTTCTATCGAATATCGAAACTCGATAAAGGATGAAGGATAAGGG 300

Mandarin    TATATAGACTATGGATACGCAGCGAAAAACTAACTCAAAAATCACAACCAAATACGTATT 359
orange      TATATAGACTATGTATACGCAGCGAAAAACTAACTCAAAAATCACAACCAAATACGTATT 360

Mandarin    CCTTTTTATGAAAAAGAAAAAGAAAAGAATTGTTATTGTTATGAATCGATTCTAAGTTGA 419
orange      TCTTTTTATGAAAAAGAAAA------GAATTGGTATTGTTATGAATCGATTCTAAGTTGA 414

Mandarin    AGAAAGAATCGAATATTCCCGGCTCAAATCATTCACTCCACCTCCATGGTCTGATCGATC 479
orange      AGAAAGAATCGAATATTCCCGGCTCAAATCATTCACTCCACCTCCATGGTCTGATCGATC 474

Mandarin    CTTTCTTTTGACTTTTGAAGAACTGATTAATCGGACGAGAATAAAGATAGAGTCCCATTC 539
orange      CTTTCTTTTGACTTTTGAAGAACTGATTAATCGGACGAGAATAAAGATAGAGTCCCATTC 534

Mandarin    TACATGTCAATATCAATACGGGCAACAATGAAATTTAGAGTAAAAGGAAAATCCGTCGAC 599
orange      TACATGTCAATATCAATACGGGCAACAATGAAATTTAGAGTAAAAGGAAAATCCGTCGAC 594

Mandarin    TTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCA 636
orange      TTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCA 631
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(B)

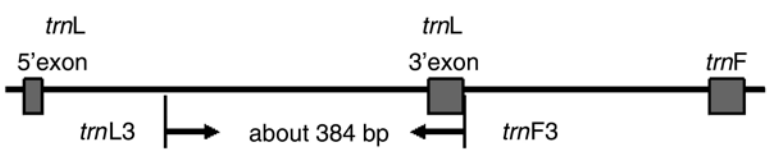

Figure 1. (A) The *trn*L intron sequence of orange and mandarin. The boldface is the primer sequence. (B) Position of *trn*L3/*trn*F3 primer in *trn*L intron.

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Table 1. The universal primers and specific primers used for amplifying the non-coding regions of chloroplast DNA

Primer	Sequence 5'-3'	PCR product	Primer source reference
B49317	CGAAATCGGTAGACGCTACG	About 1070 bp	Pirre *et al.*[23]
A50272	ATTTGAACTGGTGACACGAG		Pirre *et al.*[23]
*trn*L3	GTCAAATGAATGCTTCTATCG	About 384 bp	This study
*trn*F3	AGGGACTTGAACCCTCAC		This study

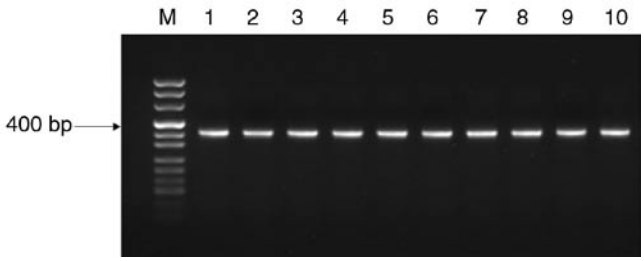

Figure 2. Amplification of the variable *trn*L intron region in six oranges and four mandarins by *trn*L3/*trn*F3 primer. Lane M: 50 bp ladder markers; Lane 1: Liucheng; Lane 2: Pineapple; Lane 3: Valencia; Lane 4: Hamlin; Lane 5: Parson; Lane 6: Navel Seeding; Lane 7: Ponkan; Lane 8: Tankan; Lane 9: Satsuma Seeding; Lane 10: Murcoot.

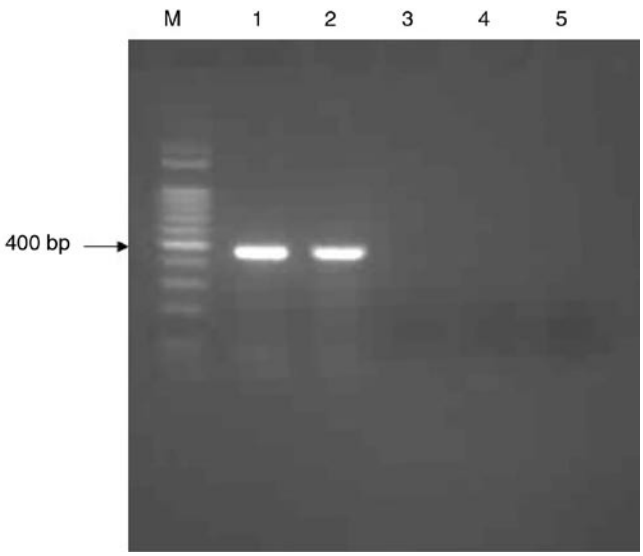

Figure 3. The specificity test of *trn*L3/*trn*F3 primer. Lane M: 100 bp ladder marker; Lane 1: Liucheng (orange); Lane 2: Tankan (mandarin); Lane 3: maize; Lane 4: sugar cane; Lane5: no temple control.

for sequencing. The second primer pair *trn*L3/*trn*F3 was designed and synthesized to amplify the differentiating fragments in oranges and mandarins. The parameters of the amplification reaction were: 1 cycle of 3 min at 96°C; 35 cycles of 1 sec at 96°C, 10 sec at 52°C, 20 sec at 72°C, and an end cycle of 10 min at 72°C. The primer pair *trn*L3/*trn*F3 was designed for DGGE.

IV. *Denaturing Gradient Gel Electrophoresis*

Denaturing gradient gel electrophoresis was performed as described previously[20]. Briefly, 10% polyacrylamide gel (25 mL) with a linear gradient of 15~35% denaturant were poured between glass plates separated by teflon spacers (0.75 mm thick), with a sample well width of 8 mm. Gel was run in DCode™ Universal Mutation Detection System (Bio-Rad, USA) and immersed in an aquarium of running buffer (20 mM sodium acetate, 1 mM EDTA, 40 mM Tris-acetate, pH 7.4) that was maintained at 60°C with a circulating heater. Following electrophoresis for 3 hr at 150 V, the gel was stained with ethidium bromide and photographed.

RESULTS AND DISCUSSION

I. *Primer Design*

The non-coding regions of chloroplast have recently been employed to study the population biology and evolution of plants, and most studies have suggested that these regions could be useful markers for species identification of plants. Araujo *et al.*[24], who utilized three non-coding regions of chloroplast DNA, *trn*T-*trn*L intergenic spacer, *trn*L intron and *trn*L-*trn*F intergenic spacer, to identify the phylogenesis of Citreae, showed that the oranges (*Citrus sinensis*) and mandarins (*Citrus reticulata*) could be divided into two groups. In this study, the chloroplast DNA *trn*L intron and *trn*L-*trn*F intergenic spacers of six orange species and four mandarin species were amplified with the universal primers B49317 and A50272 (Table 1) and the length of the PCR products were roughly 1070 bp (data not shown). These DNA fragments, which were applied for sequencing and were analyzed by GeneDoc, showed a variable DNA fragment between oranges and mandarins (Figures 1A and 1B). Based on the conserved sites flanking the variable regions within *trn*L intron, the primer pair *trn*L3/*trn*F3 (Table 1) was further designed to amplify a 384 bp fragment from all our orange and mandarin samples. It was found that the primer pair could be applied to all samples (Figure 2). To verify the species-specificity of this primer pair, the citrus, maize and sugar cane were tested by PCR and there was no PCR products detected other than citrus (Figure 3). The maize and the sugar cane were used here to confirm that their respective DNA does not interfere with the findings of the study. Experimental results showed that the primer pair *trn*L3/*trn*F3 is specific to citrus, and it can amplify the variable region of *trn*L intron in all citrus samples.

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II. *Condition of Denaturing Gradient Gel Electrophoresis*

The PCR products amplified by the *trn*L3/*trn*F3 primer were used to run the perpendicular DGGE. The goal of running the perpendicular DGGE is to optimize electrophoresis condition. Figure 4 shows the perpendicular DGGE with a denaturing gradient ranging from 0 to 100%. The optimal range for separating the oranges and mandarins was between 15 and 35%. This range was applied to the parallel DGGE for identifying the oranges and mandarins. The DNA fragments of oranges and mandarins were located at different sites on the parallel denaturing gradient gel according to the 6-bp difference between the oranges and mandarins (Figure 5). The orange DNA solution was mixed with mandarin DNA solution at different ratios to run the parallel DGGE (Figure 6). Two banding patterns appeared in the polyacrylamide gel: the heteroduplex molecule type and the homoduplex one[25]. The heteroduplex molecule type of banding can be observed when there is more than one DNA type in the same PCR reaction. A heteroduplex has a mismatch in the DNA double-strand that causes a distortion in its usual conformation, which has a destabilizing effect and causes the DNA to denature at a low denaturant concentration. The migration of heteroduplex bands was slower than that of the corresponding homoduplex bands because the volume of heteroduplex molecules was larger than that of homoduplex molecules. The increase in ratio of mandarin to orange DNA concentrations ratio was accompanied by an increase in heteroduplex molecules. The concentration of heteroduplex molecules peaked when there was 25% orange DNA in the solution (Figure 6). It might be due to the mismatch of forward primer *trn*L3 in the

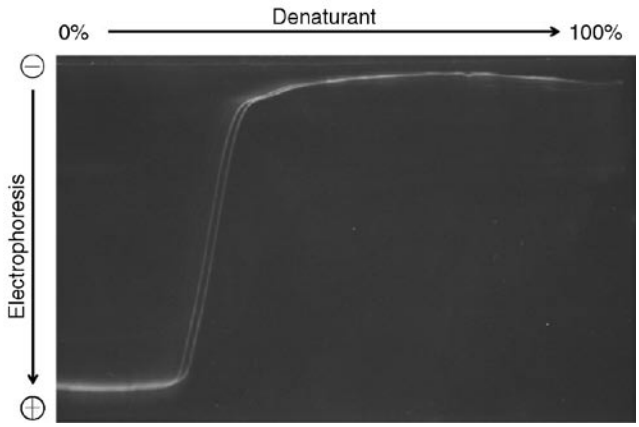

Figure 4. Perpendicular DGGE of orange and mandarin amplified by *trn*L3/*trn*F3 primer. The denaturant concentration of the 10% polyacrylamide gel is between 0 and 100%.

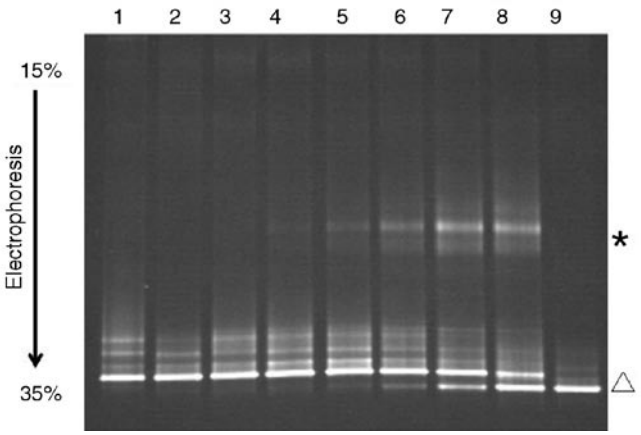

Figure 6. The parallel DGGE of orange DNA solution (O) which was mixed with mandarin DNA solution (M) at different percentage. Lane1: 100% O; Lane 2: 99.5% O + 0.5% M; Lane 3: 95% O + 5% M; Lane 4: 90% O + 10% M; Lane 5: 75% O + 25% M; Lane 6: 50% O + 50% M; Lane 7: 25% O + 75% M; Lane 8: 10% O + 90% M: Lane 9: 100% M. *The heteroduplex molecular. $^{\Delta}$The homoduplex molecular.

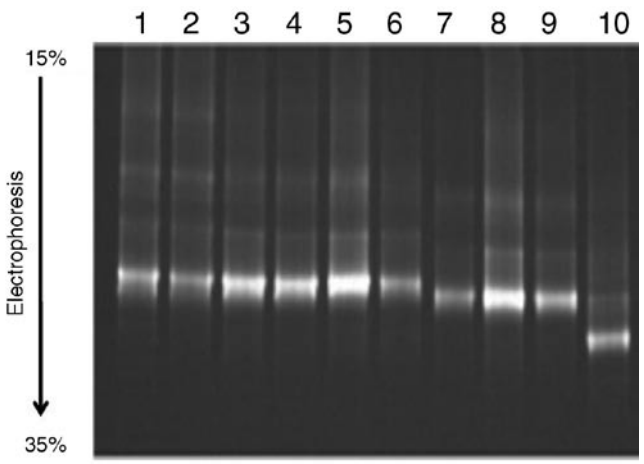

Figure 5. The parallel DGGE of six oranges and four mandarins. Lane 1: Liucheng; Lane 2: Pineapple; Lane 3: Valencia; Lane 4: Hamlin; Lane 5: Parson; Lane 6: Navel Seeding; Lane 7: Ponkan; Lane 8: Murcoot; Lane 9: Satsuma Seeding; Lane 10: Tankan.

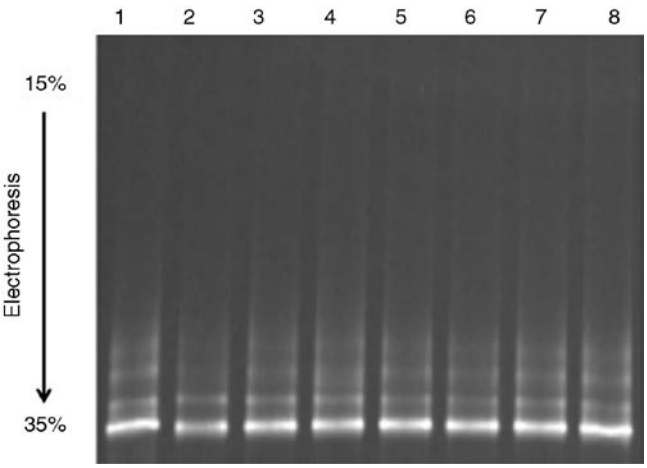

Figure 7. The parallel DGGE of Liucheng DNA solution (L) which was mixed with Navel Seeding DNA solution (N) in different percentage. Lane1: 100% L; Lane 2: 99.5% L + 0.5% N; Lane 3: 95% L + 5% N; Lane 4: 90% L + 10% N; Lane 5: 75% L + 25% N; Lane 6: 50% L + 50% N; Lane 7: 25% L + 75% N; Lane 8: 100% N.

mandarin *trn*L intron sequence, which affects the efficiency of primer. After analysis of the orange and mandarin DNA mixture, the same citrus species were mixed for the feasibility study. Figure 7 shows the parallel DGGE results for the orange DNA solutions mixed with other orange DNA solutions at different percentages. All samples showed only the homoduplex banding pattern. It was found that the heteroduplex molecule type of banding appeared only when the DNA sequences of samples were different.

III. *Detection of Adulteration in Commercial Orange Juice*

Eight commercial orange juice samples which declared to be 100% pure orange juices were employed to run the parallel DGGE for adulteration detection. There was more than one heteroduplex in the commercial samples (Figure 8). Two reasons might account for such finding. First, DNA of other additives present in the 100% orange juice samples was also amplified by the *trn*L3/*trn*F3 primer. Second, the 100% orange juice was mixed with several other citrus juices. However, since the specificity of the *trn*L3/*trn*F3 primer has been confirmed in this study, the identification of more than one heteroduplex in the samples must be attributed solely to the second reason. Commercial orange juices typically have other citrus juices added to increase the flavor and color as well as to promote quality and acceptability of 100% orange juice.

CONCLUSIONS

This study showed that the *trn*L intron of chloroplast DNA is a useful marker for differentiating oranges from mandarins. In previous studies, the primer pair was designed in variable sequences, and the results of these studies were determined by the appearance or absence of banding patterns. By adding the denaturing gradient to gel electrophoresis, the PCR products with the same fragment size and different DNA sequence can be identified in a single electrophoresis. This method is effective for purity detection as well as other processed citrus food products.

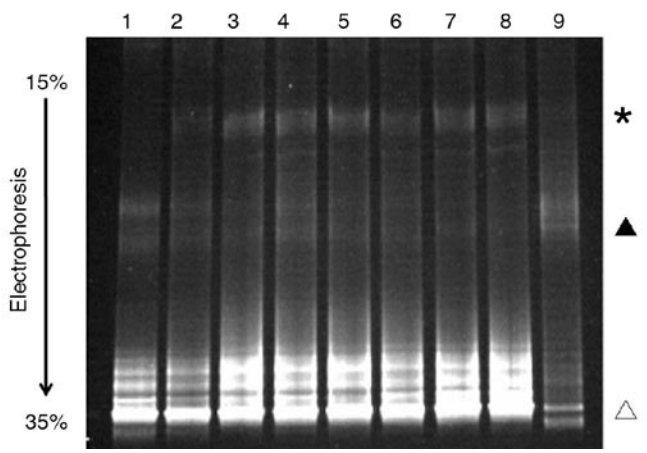

Figure 8. The parallel DGGE of 100% declared commercial citrus juice. Lane 1 to Lane 8: 100% declared commercial orange juice; Lane 9: orange DNA solution mixed with 10% mandarin DNA solution. *The heteroduplex molecular of orange DNA mixed with other unknown citrus DNA. ▲The heteroduplex molecular of orange DNA mixed with mandarin DNA. △The homoduplex molecular of orange or mandarin DNA.

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