11688 J. Agric. Food Chem. 2010, 58, 11688–11694
DOI:10.1021/jf102414q

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Efficiency of DNA Typing Methods for Detection of Smoked Paprika "Pimenton de la Vera" Adulteration Used in the Elaboration of Dry-Cured Iberian Pork Sausages

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The purpose of this work was to develop a PCR method for the identification of smoked paprika "Pimentón de la Vera" adulteration with paprika elaborated from varieties of pepper foreign to the la Vera region, in central western Spain. Three autochthonous varieties of pepper, Jaranda, Jariza, and Bola, and the varieties Papri Queen, Papri King, Sonora, PS9794, and Papri Ace, foreign to the La Vera region, were used in the study. Analyses of the ITS and 5.8S rDNA, RAPD-PCR, SSR, and ISSR were tested. RAPD-PCR, SSR, and ISSR analyses allowed differentiation among the varieties of paprika analyzed. There was no difference in the sequence of ITS1−5.8S rDNA−ITS2. In addition, with the RAPD-PCR primers S13 and S22, two molecular markers were obtained of 641 and 704 bp, respectively, which allowed all of the smoked paprika varieties to be differentiated from paprikas elaborated with the five foreign varieties. These two molecular markers were investigated as a basis for detecting the adulteration of smoked paprika with paprika elaborated from foreign varieties of pepper.

KEYWORDS: Smoked paprika; adulteration; RAPD; SSR; ISSR; ITS1−5.8S rDNA−ITS2

INTRODUCTION

Smoked paprika is an important ingredient for the final sensory characteristics of the Iberian pork sausage "chorizo", a traditional fermented cured sausage of major relevance in the meat industry of Spain. This spice, recognized as Protected Designation of Origin "Pimentón de la Vera", gives a typical color, flavor, and taste to sausages (*1*). The use of high-quality paprika in chorizo imparts a greater redness to the product, an increase in shelf life because of improved preservation and color stability (*2*, *3*), and a greater guarantee of hygienic safety for consumers (*4*).

In elaborating the "Vera" paprika, oak logs are used as a heat source in the traditional and laborious drying process. For this process only the Jaranda, Jariza (*Capsicum annuum* subsp. *longum*), and Bola (*C. annuum* subsp. *cerasiforme*) pepper varieties are suitable, as their thin pericarp is appropriate for this slow drying process. Smoking contributes to a more highly valued paprika pepper aroma than that obtained with systems of sun-drying or by forced air. Adulteration of la Vera paprika with sun-dried imported paprika of lower quality has been a troubling issue for many years in the paprika and meat industries. To manufacture these sun-dried peppers, pepper varieties are used that give a greater yield but are not suitable for smoking because of their thick pericarp. The use of imported or mixed paprikas means reduced quality of the Iberian pork sausage chorizo. In addition, these foreign paprikas can be an important source of microbial contamination due to poor sanitary conditions during growing, harvesting, processing, and storage (*5*−*8*). Few studies have been able to determine the fraudulent admixture of peppers. Determination of the color components has been the most widely used technique for the characterization of peppers subjected to different types of drying systems (*9*). In previous work our group has studied protein profiles for the determination of mixtures of smoked peppers with sun-dried peppers (*10*, *11*).

Techniques based on nucleic acids are quick, economic, and safe methods for the characterization of plants. Specifically, DNA markers are widely established for food authenticity purposes involving detection of plant material (*12*−*14*). Today, molecular markers such as random amplified polymorphic DNAs (RAPD), simple sequence repeats (SSRs), and inter-simple sequence repeat (ISSR) have been developed for the genetic mapping of *Capsicum* species (*15*−*17*). RAPD-PCR markers have been effective in routine variety identification and assessment of hybrid pepper seed purity (*18*−*24*). The ISSR markers have been shown to be useful in genetic fingerprinting of varieties of pepper (*C. annuum*) (*25*). SSRs or microsatellite markers have been developed for varietal identification of the genus *Capsicum* (*26*−*29*). In this sense, PCR techniques could be an appropriate method to detect mixed peppers quickly and safely in the processing of chorizo sausage and used in addition to the determination of mixtures using protein profiles. The aim in this work was to develop a PCR method for the rapid and accurate screening for adulteration in the smoked paprikas used in making chorizo sausage.

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 Published on Web 10/19/2010

Table 1. Primers, Range of Molecular Weights, Total and Polymorphic Bands, and Adulteration-Specific Bands (Characterized by Molecular Mass in Base Pairs)[a] Amplified with RAPD Primers

					adulterant paprika varieties				
primer	primer sequence (5′−3′)	range of mol wt (bp) of bands	total bands	polymorphic bands	Sonora	PS9794	Papri Ace	Papri Queen	Papri King
S1	GTGTGCCCCA	1050 to 225	10	3	–[b]	–	–	–	–
S2	ACGCGCCCT	1545 to 234	13	0	–	–	–	–	–
S3	GTAACGCC	1406 to 430	7	3	–	–	–	430	430
S4	ATGTAACGCC	1452 to 432	6	2	1452	–	1452	–	1452
S5	AGCAGCGTGG	1409 to 194	15	5	–	581	–	581	–
S6	AGTCAGCCAC	989 to 280	10	1	–	–	–	–	–
S7	CTGAAGCGCA	976 to 329	9	2	–	–	–	–	–
S8	AAGACCGGGA	2002 to 287	14	8	571	2002, 1692, 1537, 1149, 287	2002, 1692, 1537, 1149, 571, 287	2002, 1692, 1537, 1149, 287	2002, 1692, 1537, 1149, 571, 287
S9	CTACGGCTTC	1262 to 290	11	5	334, 290	334, 290	–	407, 334, 290	407, 334, 290
S10	GGTGGCCAAG	1383 to 142	13	1	–	–	–	–	–
S11	CACAGACCTG	1651 to 176	11	0	–	–	–	–	–
S12	CCGAGGGGTT	1848 to 240	13	0	–	–	–	–	–
S13	TTGCGGCTGA	912 to 276	13	10	**641**	**641**	**641**	**641**	**641**
S14	GGAAAGCGTC	1595 to 183	13	4	–	–	–	399	399
S15	GTTTCGCTCC	1646 to 172	13	5	–	–	1494, 1371, 1267	–	–
S16	TGCGCCCTTC	1330 to 250	9	0	–	–	–	–	–
S17	TGCTCTGCCC	1577 to 299	12	0	–	–	–	–	–
S18	ACGCGCCCT	1156 to 168	13	3	599	599	–	–	–
S19	CTGCTGGGAC	1908 to 282	18	13	1630	–	–	1630	–
S20	ACGGCGTATG	1968 to 211	19	5	1768, 1639	–	–	1969, 1768, 1639, 522	1969, 1768, 1639, 522
S21	CCGAATTCCC	1544 to 279	10	1	–	–	–	–	–
S22	GGGAATTCGG	1439 to 376	8	2	**704**	**704**	**704**	**704**	**704**
S23	GGGATATCGG	1817 to 274	16	5	–	–	–	–	–
S24	TTACGGTGGG	1818 to 254	10	0	–	–	–	–	–
S25	CTCCCAGGGT	1583 to 169	11	2	–	1060	–	–	1060
L1	GAGGGTGGCGGTTCT	1273 to 289	15	6	–	–	625	625	–
L2	GGAAGTAAAAGTCGTAACAAGG	1496 to 191	12	0	–	–	–	–	–
L3	GCACGGACCATATTGTATGCCTG	1000 to 229	6	0	–	–	–	–	–
L4	AGAGTTTGATCCTGGCTCAG	2721 to 172	17	9	–	–	684	2721, 2329, 1578, 950	684
L5	TTTCACGAACAACGCGACAA	1005 to 329	11	6	694	694	–	–	694
L6	CCGTGCCAGGACTGGGTCTT	1267 to 572	8	6	1267, 1046, 814, 580	814, 580	580	580	814
L7	ATCTTCTCCGACTGGTTCGG	914 to 184	15	10	366, 315	–	366	–	366
L8	AGAGTTTGATCATGGCTCAG	1151 to 315	11	2	–	–	–	–	–
L9	ATAAAGTGAAGAGATTCGTACC	735	1	0	–	–	–	–	–
L10	GTATTCCTGAATGGAATACAC	1191 to 280	16	9	1104, 738, 689	738, 422	422	738, 724, 422	1104, 738, 422
total		**2721 to 142**	**422**	**131**	**20**	**17**	**17**	**27**	**26**

[a] The amplicons listed in the table were found in the RAPD profiles generated by the adulterant varieties Sonora, PS9794, Papri Ace, Papri Queen, or Papri King (*Capsicum annuum*) and absent in the RAPD profiles obtained from the smoked varieties Jaranda, Jariza, and Bola (*C. annuum*) used as controls. [b] Contaminant-specific bands not detected.

MATERIALS AND METHODS

Plant Material. Samples of paprika elaborated with eight varieties of pepper were studied. Three pure batches of smoked paprika of appropriate varieties for smoke-drying, Jaranda, Jariza (*C. annuum* subsp. *longum* L.), and Bola (*C. annuum* subsp. *cerasiforme* L.), were used. These three autochthonous varieties of pepper were smoke-dried for 15 days. They were separated into batches and taken to the processing plant to be milled into paprika. Samples were collected from five different processing lines. A total of 15 samples of each batch (three from each processing line) were collected.

Also, five pure pepper batches of each of the sun-dried foreign varieties Papri Queen, Sonora, Papri King, PS9794, and Papri Ace (*C. annuum* L.) were used. In the laboratory, these peppers were ground with a stone mill (provided by Protected Designation of Origin "Pimentón de la Vera") to obtain different samples of paprika.

All of the samples (50 g approximately) were put into plastic bags and kept under dry conditions prior to assay in the laboratory within 1−2 days.

With respect to the analysis of artificially adulterated samples, six adulteration levels (1, 5, 10, 20, 40, and 60%) were tested. These adulteration levels were prepared for each of the three varieties of smoked paprika with each of the five nonsmoked varieties. Each adulteration level sample was analyzed in triplicate.

DNA Isolation. The paprika samples were triturated in a mincer followed by grinding in a mortar. DNA was isolated according to the method including CTAB as detergent in the extraction buffer used by Porebski et al. (*30*). The samples were incubated at 65 °C for 30 min with 10 mL of extraction buffer (100 mM Tris-HCl (pH 8), 20 mM EDTA, 3 M NaCl, 2% (w/v) cetyltrimethylammonium bromide (CTAB), and 0.2% (v/v) of β-mercaptoethanol). The concentration and purity of extracted DNA were measured spectrophotometrically and brought to a final value of 10 ng/μL (Biophotometer, Eppendorf AG, Hamburg, Germany).

Sequence Analysis of the Partial ITS1−5.8S rDNA−Complete ITS2 Region of the Paprika Varieties. The amplification of the 5.8S rDNA−ITS region was carried out under the following conditions: each

Table 2. Primers, Range of Molecular Weights, Total and Polymorphic Bands, and Adulteration-Specific Bands (Characterized by Molecular Mass in Base Pairs)[a] Amplified with SSR Primers

					adulterant paprika varieties				
primer	ref	range of mol wt (bp) bands	total bands	polymorphic bands	Sonora	PS9794	Papri Ace	Papri Queen	Papri King
$(AG)_5$	*29*	1078 to 108	11	2	**457**	**457**	**457**	**457**	**457**
$(CT)_6$	*29*	716 to 69	13	10	—[b]	415, 92	—	—	415, 92
$(CCA)_8$	*29*	1406 to 92	11	1	902	902	902	—	902
cmaflp24	*32*	935 to 200	11	4	—	—	—	—	—
cmaflp18	*33*	1610 to 194	22	8	—	924, 812	—	695	812
cmaflp110	*32*	718 to 117	8	1	—	—	—	—	—
cmaflp11	*32*	902 to 139	8	0	—	—	—	—	—
total		**1610 to 69**	**84**	**26**	**2**	**6**	**2**	**2**	**5**

[a] The amplicons listed in the table were found in the SSR profiles generated by the adulterant varieties Sonora, PS9794, Papri Ace, Papri Queen, or Papri King (*Capsicum annuum*) and absent in the RAPD profiles obtained from the smoked varieties Jaranda, Jariza, and Bola (*C. annuum*) used as controls. [b] Contaminant-specific bands not detected.

30 μL reaction mixture contained 10 ng of template DNA, 10 mM Tris-HCl, pH 9.0, 2.8 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 200 ng of each primer, and 1 U of DNA polymerase (Biotools, Madrid, Spain). The reaction tubes were overlaid with 20 μL of mineral oil. Reaction without template DNA was used as the negative control in all amplifications. The primers ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ (*31*) were used. Amplification was performed in a MyCycler thermal cycler (Bio-Rad, Hercules, CA), using an initial denaturation of 5 min at 94 °C followed by 40 cycles consisting of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C and a final step of 5 min at 72 °C. Amplification products were separated by electrophoresis in 1% (w/w) agarose gels and detected by staining with ethidium bromide (0.5 μg/mL). A DNA molecular size marker VI of 2.1−0.15 kbp (Roche Farma, Indianapolis, IN) was used to determine the size of the PCR products. Electrophoretic patterns were compared using Image Analysis software (Genetools, SynGene, Cambridge, U.K.).

Sequence analysis of the partial ITS1−5.8S rDNA−complete ITS2 region purified PCR products was done in sense and antisense directions at the Sequence Centre, SECUGEN S.L. (Madrid, Spain), with primers ITS1 and ITS4. Sequences were edited with Chromas version 1.43 (Griffith University, Brisbane, Queensland, Australia) and compared with the EMBL and GenBank database using the BLAST algorithm. The identities of the PCR products were determined on the highest score basis. For sequence alignment, the Clustal W algorithm of MegAlign (Lasergene 7, GATC Biotech, Konstanz, Germany) was applied, and the percentages of identity and divergence were obtained.

RAPD-PCR Analysis of the DNA. The amplification of the RAPD-PCR was carried out under the following conditions: Each 30 μL reaction mixture contained 10 ng of template DNA, 10 mM Tris-HCl, pH 9.0, 3 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 200 ng of primer, and 1 U uf DNA polymerase (Biotools). Also, each reaction tube was overlaid with 20 μL of mineral oil. Reaction without template DNA was used as the negative control in all amplifications. A total of 35 RAPD-PCR primers were used, of which 25 were 10-mer (S1−S25) and 10 were 15 or more base primers (L1−L10) (**Table 1**). Amplification was performed in a MyCycler thermal cycler (Bio-Rad) with the following cycling conditions: one cycle of 94 °C for 5 min, 35 cycles of 94 °C for 1 min, hybridization temperature of 40 or 50 °C for 1 min and 72 °C for 1 min, and a final extension of 72 °C for 5 min. The hybridization step differed according to the length of the primers: for the 10-mer 40 °C was used and for the rest, 50 °C. The amplification products were separated by electrophoresis in 2% (w/w) agarose gels and detected by staining with ethidium bromide (0.5 μg/mL). A DNA molecular size marker VI of 2.1−0.15 kbp (Roche Farma) was used to determine the size of the PCR products. Electrophoretic patterns were compared using Image Analysis software (Genetools, SynGene).

SSR Analysis of the Paprika Varieties. Seven SSR primers (**Table 2**) (*29*, *32*, *33*) were used to amplify the DNA of each paprika. PCR reactions were performed in a final volume of 30 μL of reaction mixture containing 10 ng of template DNA, 10 mM Tris-HCl, pH 9.0, 2.0 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.2 μM of each primer, and 1 U of DNA polymerase (Biotools). Each reaction tube was overlaid with 20 μL of mineral oil. Reaction without template DNA was used as the negative control in all amplifications. PCR was performed in a MyCycler thermal cycler (Bio-Rad), programmed for a hot start of 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at the average of the melting temperatures (T_m) of two flanking primers, and 1 min at 72 °C, plus a final extension of 5 min at 72 °C. All amplification products were separated by electrophoresis in 2% (w/w) agarose gels and detected by staining with ethidium bromide (0.5 μg/mL). A DNA molecular size marker VI of 2.1−0.15 kbp (Roche Farma) was used to determine the size of the PCR products. Electrophoretic patterns were compared using Image Analysis software (Genetools, SynGene).

ISSR-PCR Analysis of Paprika Varieties. A total of 10 ISSR-PCR primers (**Table 3**) (*25*) were used. ISSR-PCR amplifications were set up in a 20 μL reaction mixture containing 10 ng of template DNA, 10 mM Tris-HCl, pH 9.0, 2.0 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.8 μM primer, and 1 U of DNA polymerase (Biotools). Each reaction tube was overlaid with 20 μL of mineral oil. Reaction without template DNA was used as the negative control in all amplifications. Amplification was performed in a MyCycler thermal cycler (Bio-Rad), with a program of initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, and a final extension of 72 °C for 5 min. The products were electrophoresed on 2% (w/w) agarose gels and detected by staining with ethidium bromide (0.5 μg/mL). A DNA molecular size marker VI of 2.1−0.15 kbp (Roche Farma) was used to determine the size of the PCR products. Electrophoretic patterns were compared using Image Analysis software (Genetools, SynGene).

Sequencing of Specific Bands. To verify that the adulteration markers selected were identical in the five nonsmoked varieties, the PCR bands selected for the identification of nonsmoked paprikas were gel-purified before cloning into a pCR2.1-TOPO vector (Invitrogen, Paisley, U.K.). After confirmation that transformation of *Escherichia coli* 2H5α (Invitrogen) yielded recombinant clones containing the PCR markers, the cloned fragments were sequenced at the Sequence Centre, SECUGEN S.L., Madrid, Spain. Sequences were edited with Chromas version 1.43 (Griffith University) and compared with the EMBL and GenBank database using the BLAST algorithm. Comparison was done by aligning the sequences using the Clustal W algorithm of the program MegAlign (Lasergene, GATC Biotech).

RESULTS AND DISCUSSION

Sequence Analysis of the Partial ITS1−5.8S rDNA−Complete ITS2 Region of the Paprika Varieties. Amplification of the partial ITS1−5.8S rDNA−complete ITS2 region resulted, for all paprika varieties, in a single fragment with a molecular size of approximately 730 bp. The sequences of the eight paprika varieties showed an identical fragment of 729 bp. The homology sequences in the BLAST databases showed a high homology with *Capsicum eximuim* AY665841.1 (96% identity), *Capsicum baccatum* AF244708.1 (95% identity), and *Capsicum lycianthoides* DQ314158.1 (91% identity), as the partial ITS1−5.8S rDNA−complete ITS2 region sequence of *C. annuum* was not available

Table 3. Primers, Range of Molecular Weights, Total and Polymorphic Bands, and Adulteration-Specific Bands (Characterized by Molecular Mass in Base Pairs)[a] Amplified with ISSR Primers

				adulterant paprika varieties				
primer	range of mol wt (bp) bands	total bands	polymorphic bands	Sonora	PS9794	Papri Ace	Papri Queen	Papri King
TGT9	770 to 192	11	0	—[b]	—	—	—	—
GCC4	1537 to 257	11	0	—	—	—	—	—
CAG4	980 to 227	13	3	—	—	—	—	—
GA8C	1343 to 145	14	3	—	—	—	—	—
AAT4	1163 to 224	8	1	—	—	—	—	—
2ATT	1619 to 410	7	0	—	—	—	—	—
TG7T	821 to 202	9	3	—	281	—	—	281
CA7	852 to 179	21	7	256	256	—	256	256
T(GA)8	733 to 157	8	1	—	—	—	—	—
C(GA)7	733 to 157	8	0	—	—	—	—	—
total	**1619 to 145**	**110**	**18**	**1**	**2**	**0**	**1**	**0**

[a] The amplicons listed in the table were found in the ISSR profiles generated by the adulterant varieties Sonora, PS9794, Papri Ace, Papri Queen, or Papri King (*Capsicum annuum*) and absent in the RAPD profiles obtained from the smoked varieties Jaranda, Jariza, and Bola (*Capsicum annuum*) used as controls. [b] Contaminant-specific bands not detected.

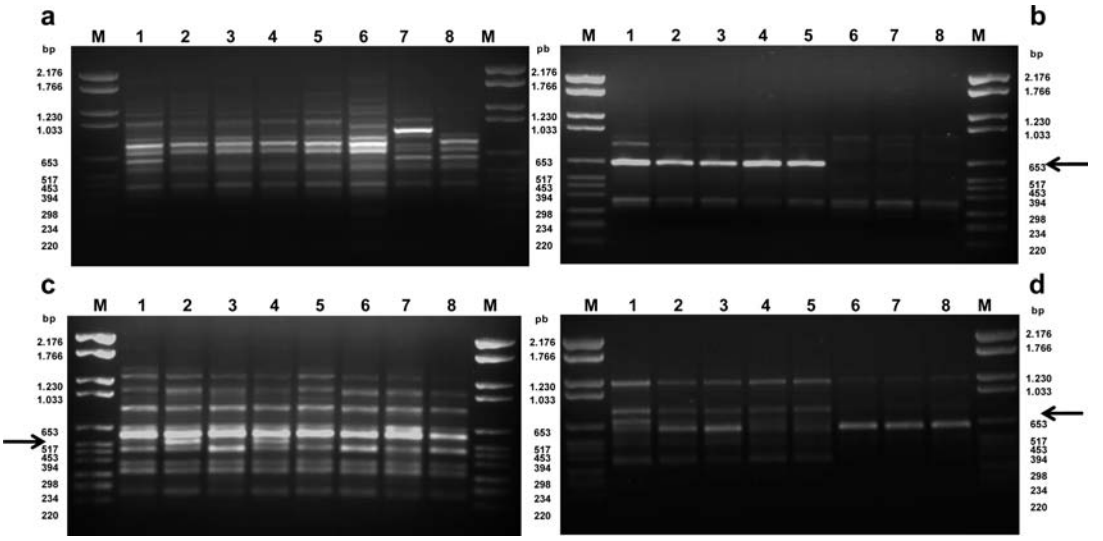

Figure 1. RAPD amplification patterns generated by primers S5 (**a**), S13 (**b**), S19 (**c**), and S22 (**d**): lanes 1−5, nonsmoked varieties Papri King, Papri Queen, Papri Ace, PS9794, and Sonora, respectively; lanes 6−8 smoked varieties Jariza, Bola, and Jaranda, respectively; lane M, DNA molecular size marker VI of 2.1−0.15 kbp (Roche Farma). Black arrows indicate the position of the specific markers for nonsmoked varieties of (**b**) 641 bp (lanes 1−5), (**c**) 581 bp (lanes 2 and 4), and (**d**) 704 bp (lanes 1−5).

in the database. These sequences were submitted to GenBank and are currently published under the accession no. GU944973. Although the analysis of internal transcribed spacers (ITS) has been adequate for inter- and intraspecific identification in different plants (*34*−*36*), this technique was not suitable for differentiation of the varieties of paprika pepper studied, because they presented no polymorphism.

Analysis of Paprika Varieties by RAPD-PCR. Analysis of 35 RAPD-PCR primers produced a total of 422 distinct bands of PCR among the 8 varieties of pepper studied, which is an average of 12 bands per primer. The bands ranged from approximately 142 to 2721 bp (**Table 1**). The S20 primer amplified 19 different bands, whereas the L10 primer amplified one band. Paran et al. (*22*) obtained 7.5 different bands per primer, and Ilbi (*19*) obtained 17 bands per primer, using RAPD primers to characterize pepper varieties (*C. annuum*).

A total of 131 polymorphic bands were obtained, which is an average of 3.74 polymorphic bands per primer. These values are in agreement with Rodriguez et al. (*24*), who reported an average of 3.12 polymorphic bands per primer, and greater than those obtained by Paran et al. (*22*), with 1−3 polymorphic bands per primer. The largest numbers of polymorphic bands were obtained with primers S19 (**Figure 1a**), S13 (**Figure 1b**), and L7 with 13, 10, and 10 different bands, respectively (**Table 1**).

This survey permitted us to characterize pepper varieties by finding molecular markers that differentiate between the three smoked varieties used to elaborate Iberian chorizo pork sausage and the five varieties not allowed under the regulations of "Pimenton de la Vera" Protected Designation of Origin. In particular, 43 molecular markers obtained with 19 RAPD primers allowed us to differentiate at least one of the nonsmoked paprika varieties (**Table 1**). With 54.3% of the primers, at least one marker was obtained that identifies nonsmoked varieties. The varieties Papri Queen and Papri King had 26 different molecular markers, whereas Sonora, Papri Ace, and PS9795 had 20, 17, and 17 different molecular markers, respectively (**Table 1**). The S8 primer amplified 6 molecular markers of 2002, 1692, 1537, 1149, 571, and 287 bp, although none of them were in the 5 nonsmoked paprika varieties. The S5 primer amplified one molecular marker of 581 bp in Papri Queen and PS9794 (**Figure 1c**). The molecular markers of 641 and 704 bp amplified with S13 and S22 primers, respectively (**Figure 1b**,**d**) were in the 5 nonsmoked paprika varieties, but not in

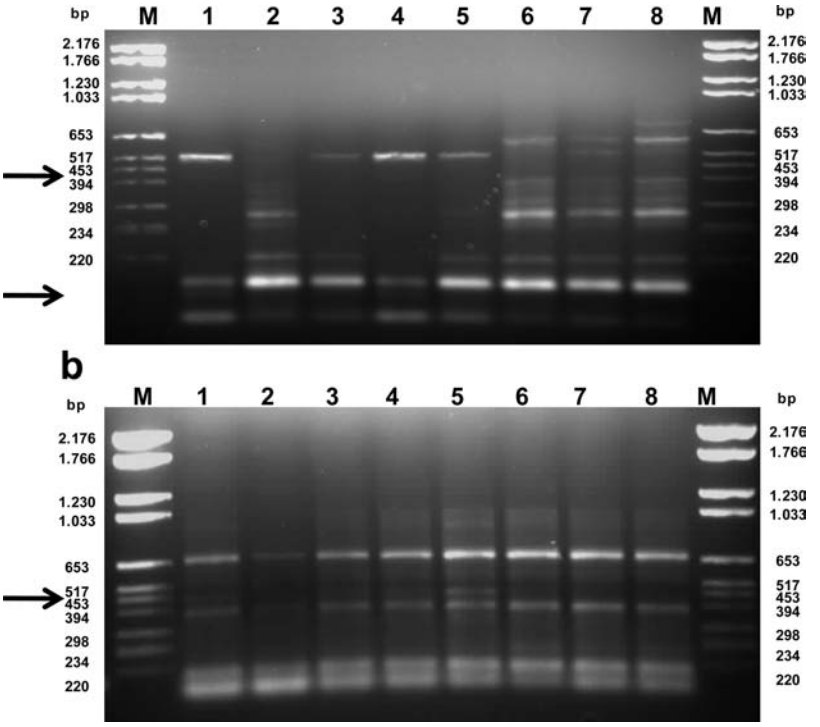

Figure 2. SSR amplification patterns generated by primers $(CT)_6$ (**a**) and $(AG)_5$ (**b**): lanes 1−5 nonsmoked varieties Papri King, Papri Queen, Papri Ace, PS9794, and Sonora, respectively; lanes 6−8, smoked varieties Jariza, Bola, and Jaranda, respectively; lane M, DNA molecular size marker VI of 2.1−0.15 kbp (Roche Farma). Black arrows indicate the position of the specific markers for nonsmoked varieties of (**a**) 415 and 92 bp (lanes 1 and 4) and (**b**) 457 bp (lanes 1−5).

the 3 smoked varieties. In addition, the sequencing of these specific markers of the 5 paprika varieties used to adulterate the smoked paprika showed bands of 641 and 704 bp to be the same in the 5 varieties. The degree of nonpolymorphism shown by these diagnostic markers is able to detect the adulteration of smoked paprika by admixtures of foreign varieties of paprika.

Analysis of Varieties of Paprika by SSR. Eighty-four distinct PCR bands were amplified by SSR markers (**Table 2**) with the 8 varieties of pepper studied, an average of 12 bands per primer. This number of bands per primer is greater than that reported by Sanwen et al. (*29*) and Ince et al. (*26*). The bands ranged from 69 to 1602 bp. The primer cmaflp18 amplified 22 bands, and cmaflp11 and cmaflp110 each amplified 8 bands. A total of 26 polymorphic bands were obtained (30.95%), an average of 3.71 polymorphic bands per primer. The $(CT)_6$ primer amplified 10 polymorphic bands (**Table 2**; **Figure 2a**), whereas no polymorphic bands were obtained with the cmaflp11 primer. Our study allowed us to identify varieties of nonsmoked paprika because 7 of 26 polymorphic bands were specific molecular markers of the nonsmoked varieties. PS9794 had 6 different molecular markers, Papri King had 5 molecular markers, and Sonora, Papri Ace, and Papri Queen had 2 markers (**Table 2**). The cmaflp18 primer amplified 3 molecular markers of 924 (PS9794 paprika variety), 812 (PS9794 and Papri King), and 695 bp (Papri Queen). The $(CT)_6$ primer amplified two bands of 415 and 92 bp, which were characteristic of the PS9794 and Papri King varieties (**Figure 2a**). Finally, molecular markers of 457 bp amplified with the $(AG)_5$ primer were in the 5 nonsmoked paprika varieties and were not scored in the smoked varieties Jaranda, Jariza, or Bola (**Figure 2b**).

The sequencing of the specific marker of the 5 paprika varieties used to adulterate the smoked paprika showed that the bands of 457 bp are the same in the 5 varieties. In this sense, this diagnostic marker will be used to detect the adulteration of smoked paprika by admixture of foreign varieties of paprika.

Analysis of Varieties of Paprika by ISSR. A total of 110 distinct bands of PCR were amplified by ISSR primers with the 8 varieties of pepper studied, corresponding to an average of 11 bands per primer. The bands ranged from 145 to 1619 bp (**Table 3**). The greatest number of different bands was obtained with the CA7 primer, whereas 7 bands were amplified by 2ATT. Eighteen of 110 bands were polymorphic (16.4%), corresponding to 1.8 polymorphic bands per primer. These ISSR primers had been used successfully by Kumar et al. (*25*) for the differentiation of closely related chilli seed varieties (*C. annuum*), obtaining far more amplification products and polymorphism. Only two specific bands of the nonsmoked paprika varieties were obtained by ISSR primers. The 281 bp band, amplified using the TG7T primer, was scored in the PS9794 and Papri King varieties, and the 256 bp band obtained with the CA7 primer was scored in all nonsmoked varieties except Papri Ace (**Table 3**; **Figure 3**).

Detection of Adulteration in Smoked Paprika. The paprika variety used to adulterate smoked paprika may change from case to case. Therefore, it would be desirable to find specific molecular markers present in all varieties of nonsmoked paprika and absent from varieties of smoked paprika. The primers were selected on the basis of this criterion. In particular, they were S13, S22 (RAPD markers) (**Figure 1**), and $(AG)_5$ (SSR marker) (**Figure 2**). These three primers amplified specific bands for nonsmoked paprika pepper varieties of 641, 704, and 457 bp, respectively. The effect of smoked paprika adulteration with a mixture of paprika obtained from Jariza and Papri Queen varieties on the intensity of the 641 and 704 bp bands using primers S13 and S22, respectively, is illustrated in **Figure 4**. The intensity of the 641 bp band increases as the percentage of adulteration is greater (60%) and disappears at the lowest level of adulteration (1%) (**Figure 4a**). This was repeated in the analyses of the mixtures made with other varieties (data not shown). In the cases corresponding to primer S22, the intensity of the 704 bp band also varied with the level of

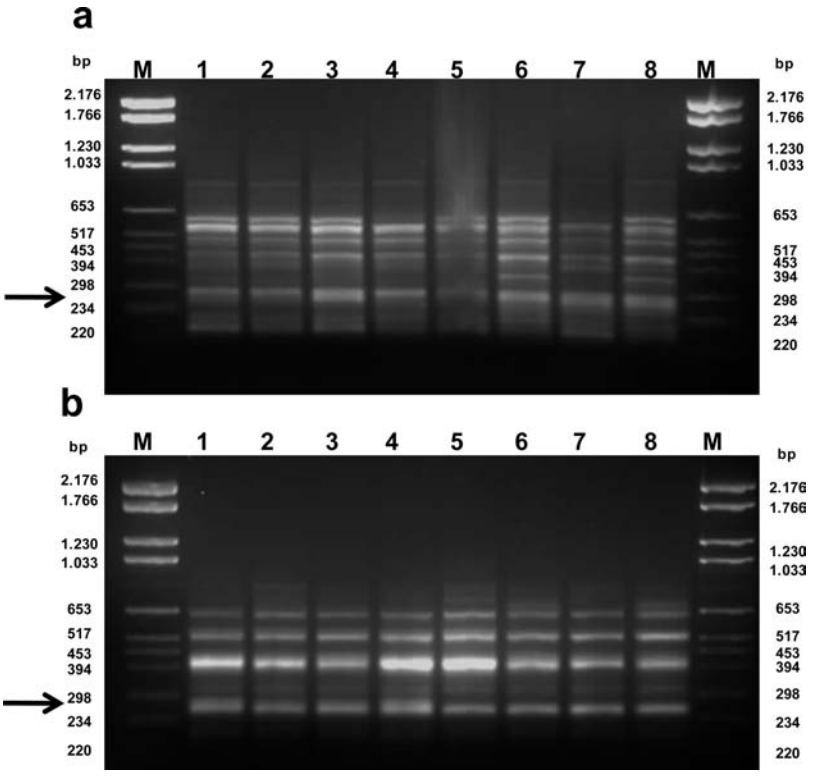

Figure 3. ISSR amplification patterns generated by primers CA7 (**a**) and TG7T (**b**): lanes 1−5, nonsmoked varieties Papri King, Papri Queen, Papri Ace, PS9794, and Sonora, respectively; lanes 6−8, smoked varieties Jariza, Bola, and Jaranda, respectively; lane M, DNA molecular size marker VI of 2.1−0.15 kbp (Roche Farma). Black arrows indicate the position of the specific markers for nonsmoked varieties of)**a**) 256 bp (lanes 1, 2, 4, and 5) and (**b**) 281 bp (lanes 1 and 4).

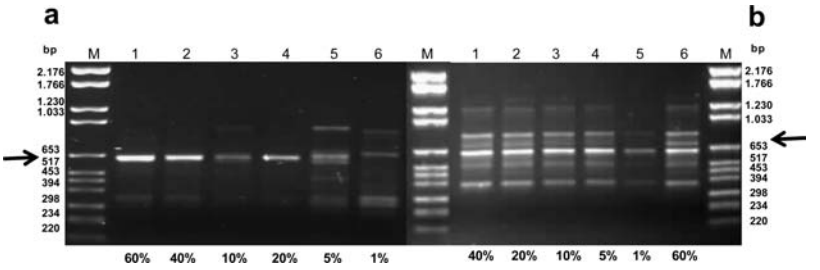

Figure 4. RAPD amplification patterns generated by primers S13 (**a**) and S22 (**b**) of the mixtures with Papri Queen and Jariza varieties in six different percentages (shown at the bottom of the figure, lanes 1−6): lane M, DNA molecular size marker VI of 2.1−0.15 kbp (Roche Farma). Black arrows indicate the position of the specific markers for adulteration detection of (**a**) 641 bp and (**b**) 704 bp.

adulteration, with the 704 bp marker disappearing at 1% of admixture (**Figure 4b**).

With respect to the intensity of the 457 bp specific band, there was a decrease of the intensity from the higher mixtures to a minimum for the 20% of mixture (data not shown). The differences found with this specific band permitted only the detection of adulteration of PDO-protected smoked paprika with paprika from the Papri Queen and Sonora varieties. This may be because the band amplified with the primer $(AG)_5$ showed low intensity in the five foreign varieties tested (**Figure 2b**). In this sense the 457 bp specific band was inappropriate for the purpose of the present study.

Classical molecular biology techniques have been reported to be very good tools to detect food adulteration related to extraneous plants in commercial samples of oregano (*37*) and for the identification of meat and poultry species (*38*, *39*) and fish (*40*). The detection of about 5−10% of foreign paprika represents a very good limit of detection for the Iberian chorizo pork sausage industry. This could justify the use of a method with the markers of 641 and 704 bp for routine quality control at the beginning of the processing of Iberian pork chorizo to reliably screen for the adulteration of smoked paprika with foreign varieties.

In conclusion, our study has demonstrated that RAPD analysis with the S13 and S22 primers is a useful tool for differentiating all of the smoked paprika from all of the foreign paprika varieties studied. The specific molecular bands of 641 and 704 bp were found to be the best markers for determining the adulteration of smoked paprika with the Papri Queen, Papri King, Papri Ace, PS9794, and Sonora varieties. Consequently, RAPD analysis could be a useful method as routine quality control to detect the adulteration of the raw material of smoked paprika used in the elaboration of the Iberian chorizo pork sausage.

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Received for review June 22, 2010. Revised manuscript received September 16, 2010. Accepted September 20, 2010. This work was supported by Grant 2PR01B012 from the Consejería de Educación y Tecnología (Junta de Extremadura) and FEDER funds. A.H. was the recipient of a grant from the Regional Government of the Junta de Extremadura (Consejería de Educación, Ciencia y Tecnología).