JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Identification of Processed Japanese Green Tea Based on Polymorphisms Generated by STS−RFLP Analysis

SHIV S. KAUNDUN* AND SATORU MATSUMOTO

National Institute of Vegetable and Tea Science, 2769 Kanaya, Kanaya-cho, 425-8501 Shizuoka, Japan

In Japan, tea is generally sold blended, though 90% of the total production is clonal. Due to the increasingly strict consumer need and taste, however, more and more Japanese green teas are being sold under their particular cultivar name. Moreover, tea made from Yabukita, a much appreciated cultivar originally developed in Japan, has recently been produced and imported from a neighboring country. This paper describes a simple and inexpensive methodology capable of identifying fresh and processed Japanese green teas to discourage its fraudulent commercialization. The study was based on 46 main tea cultivars, and polymorphism detected through STS−RFLP analysis of the coding and noncoding DNA regions of three genes, namely phenylalanine ammonia-lyase, chalcone synthase, and dihydroflavonol 4-reductase, for which nucleotide information was available. All 46 tea cultivars analyzed could be easily distinguished using a combination of codominant DNA markers. Yabukita displayed a unique profile when PAL intron was digested with *Dde*I, thus allowing its rapid authentication at low cost.

KEYWORDS: ***Camellia sinensis*; tea; PCR−RFLP; DNA fingerprinting; authentication**

INTRODUCTION

Tea is the most important beverage consumed worldwide after water. Botanically, tea, *Camellia sinensis* (L.) O. Kuntze, can be subdivided into two main varieties: var. *sinensis*, a small-leaved, bushlike plant originating from China, but grown in several countries of Southeast Asia experiencing a cold climate, and var. *assamica*, a large-leaved, tree discovered in the Assam region of India and introduced in several countries enjoying a semitropical climate (*1*, *2*). The a*ssamica* variety contains large amounts of tannin and catechin and is particularly used for black tea, whereas *sinensis* tea accounts for most of the green tea production (*3*).

The difference between these two tea types lies in the processing. In the case of green tea, the enzymes are immediately inactivated through steaming or pan-frying, while freshly plucked leaves are fermented for black tea. A third, intermediate and semifermented type, is also produced, especially in China and Taiwan.

In Japan, the *sinensis* type is widely cultivated and employed mostly in the making of Sencha, a steamed and nonfermented tea. More than 90% of the tea production is clonal with as many as 80 cultivars registered to date. Yabukita, however, a highly productive cultivar, represents 85% of clonal tea, followed by Yutakamidori and Kanayamidori, respectively accounting for 3.1% and 1.4% production only. The other tea cultivars are produced at low tonnage, but are expected to increase soon to provide the public with a larger variety of tastes and flavors.

At present, few teas can be bought according to cultivar, and most of them are labeled with the company brand where they have been processed, but occasionally as to their place of cultivation. Nevertheless, the trend for tea in Japan is likely to follow that of rice and several other fruits and sold under their respective cultivar name to meet stricter consumer needs. Indeed, a recent finding revealed that beneficial effects of tea on human health might sometimes be cultivar dependent. Benefuki, for instance, a hybrid tea between *sinensis* and *assamica* varieties, was found to have strong antiallergic properties due to the presence of high concentrations of methylated forms of epigallocatechin gallate, absent in most other cultivars (*4*). Moreover, the Japanese tea market is now open to competition due to the drastic decrease in custom duty on food products following a World Trade Organization (WTO) agreement, and a first batch of Yabukita produced from a neighboring country was imported recently.

A simple and inexpensive method capable of authenticating tea has, thus, become of prime importance. To this end, we have attempted to fingerprint the 46 most commonly grown tea cultivars in Japan using the Sequence Tagged Site−Restriction Fragment Length Polymorphism (STS−RFLP) methodology. This procedure generates polymorphism by restricting known PCR-amplified DNA sequences, in this case the phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and dihydroflavonol 4-reductase (DFR) genes previously cloned in our laboratory. We have proceeded by fingerprinting fresh plant

* Corresponding author. Present address: Bioscience, Syngenta, Jealott's Hill International Research Centre, Bracknell. Berkshire RG42 6EY, UK. E-mail dkaundun@yahoo.com.

10.1021/jf020821i CCC: $25.00
Published on Web 02/22/2003

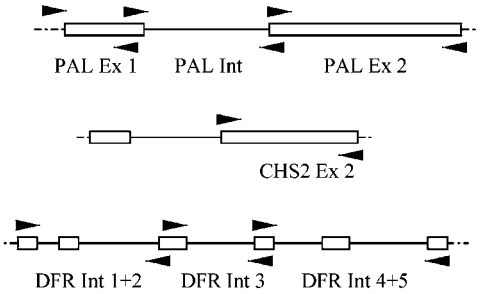

Figure 1. Primer locations on PAL, CHS, and DFR genes used to fingerprint Japanese green tea.

material in the first instance and subsequently tested the applicability of the markers on processed tea.

MATERIALS AND METHODS

Plant Material. Fresh leaves from 46 most commonly grown Japanese tea cultivars (clones) were harvested from the fields of NIVTS, Shizuoka, Japan. These were Asagiri, Asahi, Asatsuyu (P), Benifuji, Benifuki, Benihikari, Benihomare, Fujikaori, Fukumidori, Fushun, Gokou, Hatsumomiji, Horyoku, Izumi, Kanayamidori (P), Karabeni, Komakage, Kuritawase, Kurusawa, Makinoharawase, Meiryoku (P), Natsumidori, Oiwase, Okuhikari, Okumidori (P), Okumusahi, Okuyutaka, Ryohofu (P), Saemidori (P), Samidori, Sayamakaori (P), Sayamamidori, Shunmei (P), Surugawase, Tadanishiki, Takachiho, Tamamidori, Toyoka, Ujimidori, Yabukita (P), Yaeho, Yamakai, Yamanami, Yamatomidori, Yutakamidori (P), and Z1. Ten cultivars (P in brackets), for which "Sencha" steamed for 40 s was available, were also analyzed and the results compared to that obtained with fresh leaves. In addition, different types of processed tea made from Yabukita were analyzed and consisted of "Sencha" steamed for 30, 60, and 120 s, as well as pan-fried tea at 300−350 °C.

DNA Isolation. Total DNA was extracted and measured by a classical CTAB method from 50 mg of fresh or processed tea using the same procedure as described in ref 5.

Primer Design. PCR primers were designed with the Genetix software (Tokyo) and based on cDNA nucleotide sequences of PAL, CHS2, and DFR in tea (cultivar: Yabukita). Putative intron sites for each of the three genes were deduced after alignment with corresponding published sequences from other plant species. A schematic representation of the primer locations is shown in **Figure 1**. All the primers were synthesized by the Grainer Company (Japan).

PCR and Restriction Product Analysis. The polymerase chain reaction was carried out in a 25 μL volume containing 50−100 ng of genomic DNA, 0.5 μM of each primer, 200 μM of each dNTP, 2 mM $MgCl_2$, 50 mM KCl, 500 ng of BSA, 10 mM Tris-HCl, pH 8.3, and 0.5 units of Taq polymerase (Takara, Japan). PCR was performed on a Perkin-Elmer thermocycler model 2400 programmed for an initial denaturation step of 94 °C for 5 min followed by 35 cycles for 30 s at 94 °C, for 30 s at 60 °C and for 1.5 min at 72 °C. A final elongation step for 7 min at 72 °C was included. This basic cycling procedure was used for all DNA fragments except for the longest fragment DFR1+2 for which the extension time was increased to 2 min.

The PCR products were separated on 2% agarose gel and visualized under UV to reveal Amplicon Length Polymorphism (ALP) and STS−RFLP in which case 2 μL of PCR products was digested with 32 different restriction endonucleases, namely *Dde*I, *EcoR*I, *EcoR*V, *Hae*III, *Hap*II, *Hind*III, *Hinf*I, *Hha*I, *Mbo*I, *Nco*I, *Rsa*I, *Alu*I, *BamH*I, *Bgl*II, *Pst*I, *Pvu*II, *Scrf*I, *Spe*I, *Taq*I, *Xho*I, *Nla*III, *EcoT22*I, *Xmn*I, *Cfr13*I, *Hinc*II, *BspH*I, *Dra*I, *Ban*II, *Bgl*I, *Nsp*I, *Sal*I, *and Apa*I, following the manufacturers' recommendations, and separated on either 2% agarose or 3.5% metaphor gel when smaller polymorphic bands were involved. In all cases both the gels and the buffer (1X TBE) contained 0.5 μg/mL ethidium bromide.

Cloning and Sequencing of Amplification Products. To confirm the identity of the PCR bands generated by each of the seven primer pairs, the corresponding amplification products from Yabukita were ligated into pGEM-T easy vector (Promega) and the nucleotide sequences of the inserts determined on an Perkin-Elmer ABI 373 automated sequencer using the dye primer or dye terminator kits (Perkin-Elmer).

Data Analysis. The markers were scored as diploid data, and each allele was assigned an alphabet for a particular primer set and primer set/enzyme combination for amplicon length polymorphism and PCR/RFLP, respectively, and assembled in a table for comparison.

RESULTS

In this study we had recourse to the STS/RFLP methodology to fingerprint and authenticate Japanese green tea. Marker development was based on PAL, CHS2, and DFR, three genes for which nucleotide information was available in the species (*6*, *7*). Seven primer pairs were used to target the three genes as shown on **Figure 1**. Three primer pairs amplified the PAL exon1, the PAL intron, and PAL exon 2. One primer pair was dedicated to the exon 2 of CHS2, while three others targeted the DFR gene. The first amplified fragment of DFR gene consisted of part of exon 1, intron 1, exon 2, intron 2, and part of exon 3 and was denominated as DFR1+2 for simplicity, as it comprised the complete lengths of introns 1 and 2. Likewise the second fragment was named DFR3 and the third fragment DFR4+5. The amplification was first carried out on DNA obtained from fresh leaves of Yabukita and then extended to template DNA obtained from fresh and processed leaves of all other cultivars.

Using template DNA from Yabukita, all the seven sets of primers generated a single band following PCR. The primers targeting the exons amplified a band of expected length as deduced from cDNA sequences, while a fragment greater in length was obtained for those flanking the introns. Sequencing reactions showed that the bands corresponded to the targeted genes with introns varying from 101 bp to 2902 bp for the fourth and second introns of DFR, respectively. The primer sequence information and amplified fragment size for Yabukita are given in **Table 1**.

PCR amplification from the other cultivars also gave a single band for the exons of PAL and CHS2, as well as for DFR3 and DFR4+5 (**Table 2**). Upon restriction with a number of enzymes, polymorphism was observed due to two different allelic forms at these loci as illustrated by CHS2ex2 digested with *BspH*I (**Figure 2a**). One allele contained a restriction site for this enzyme and was fragmented into two while the other did not. The combination of the two different alleles generated three genotypic forms among the cultivars, namely a homozygote for the presence of the polymorphic site, a homozygote for the absence of the site in question, and a heterozygote state with one copy of each of the two alleles. Two different allelic forms and three genotypes were also obtained with CHS2ex2 cut by *Rsa*I. In the case of DFR3 only one enzyme revealed to be polymorphic, whereas for DFR4+5, as many as three enzymes were informative. However, digestion of DFR4+5 with *Xmn*I generated only two genotypes, namely homozygotes for the presence of a polymorphic site for all, but cultivar Karabeni. The same was true with PALex1 digested by *Nla*III in which case Hatsumomiji was individualized as the only one showing a heterozygote state at this locus.

A second scenario occurred when targeting the introns of PAL and DFR1+2 for which one or two bands differing in size could be observed following PCR only. An example of ALP is shown on **Figure 2b** and corresponds to the amplification products obtained with the PAL intron. Two different alleles, denoted A and B, could be observed after PCR generating three genotypes

Table 1. Sequence and Position of STS Primers, PCR Fragment, and Intron Sizes of PAL, CHS2, and DFR Genes[a]

fragment	primer sequence (forward and reverse)	location on cDNA clone	size (bp) in genomic DNA
PAL Ex1	5′-TCCATCAATCTATACACCTACCTG-3′	3–28	497
	5′-CCTTCTTTGGTCCTCCTATGTGA-3′	499–477	
PAL Intron	5′-CACATAGGAGGACCAAAGAAGG-3′	478–499	1818
	5′-GGCAATGTAAGATAGGGGGACT-3′	764–743	Intron: 1531
PALex2	5′-AGTCCCCCTATCTTACATTGCC-3′	743–764	1532
	5′-AACAGATAGGAAGAGGAGCACCATTC-3′	2274–2249	
CHS2ex2	5-AAACCCAAATGTGTGTGCCTAC-3′	308–328	801
	5-AGGATAAACAACACACAAGCGC-3′	1108–1037	
DFR1+2	5′-CGCGGCTATATTGTTCGTGCA-3′	222–242	3262
	5′-GTTGATTGTCGGCTTGATTACC-3′	455–434	Intron1: 126, Intron2: 2902
DFR3	5′-CCAGGAACACCAACAACCCGT –3′	542–561	958
	5′-CCATGCTGCTTTCTCTGCCAA-3′	665–645	Intron3: 834
DFR4+5	5′-AACATTCCCACCAAGCCTAATC-3′	740–761	1365
	5′-ATGAGAACGACACAACTGGCAA-3′	1020–999	Intron4: 101, Intron5 983

[a] Based on cDNA sequences of *Camellia sinensis* (cultivar: Yabukita) of PAL (accession no.: D26596), CHS2 (accession no.: D26594), and DFR (accession no.: AB018685) in GenBank.

Table 2. Origin of Japanese Tea Cultivars and Informative Loci/Restriction Enzyme Used for Fingerprinting[a,b]

cultivar name	origin/descendants	PALex1 /*Hpa*II	PALex1 /*Nla*III	PALint /*Dde*I	PALex2 /*Taq*I	CHS2ex2 /*Rsa*I	CHS2ex2 /*BspH*I	DFR1+2 /*Ban*II	DFR3 /*Hind*III	DFR4+5 /*Xmn*I	DFR4+5 /*Hpa*II	DFR4+5 /*Nla*III
Asagiri	selection	AA	BB	A_1A_2	BB	AB	AB	B_1B_1	AA	BB	AB	BB
Asahi	selection	AA	BB	A_1B	AA	BB	AA	A_1A_1	BB	BB	BB	AA
Asatsuyu (P)	selection	AA	BB	BB	AB	BB	AA	B_1B_1	AA	BB	AA	BB
Benifuji	Benihomare X Ch19	AB	BB	A_1B	AB	BB	BB	B_2C	AB	BB	BB	AB
Benifuki	Benihomare X Cd86	BB	BB	A_1A_1	BB	BB	BB	**CD_1**	BB	BB	BB	BB
Benihikari	Benikaori X Cn1	BB	BB	A_1A_1	BB	AA	BB	**A_2B_1**	AA	BB	AB	BB
Benihomare	unknown	BB	BB	A_1A_1	AB	BB	BB	B_2C	AB	BB	BB	BB
Fujikaori	Yabukita X Inzatsu131	BB	BB	A_1A_2	BB	AB	BB	A_1B_2	AB	BB	BB	AA
Fukumidori	Yabukita X (Yabukita X Sayamamidori)	BB	BB	A_1A_2	AB	AB	AB	A_1B_2	AB	BB	BB	AB
Fushun	Z1 X Kanayamidori	AB	BB	A_1A_2	AB	AB	AB	B_1B_1	AA	BB	AA	BB
Gokou	selection	AB	BB	A_1B	AA	BB	AA	A_1B_1	AB	BB	AB	AB
Hatsumomiji	Aj2 X Nkao5	BB	**AB**	A_1A_1	AB	AB	AB	B_1B_2	AA	BB	AB	BB
Horyoku	Tada hybrids	BB	BB	A_1A_1	AB	AB	BB	B_1B_2	AA	BB	AA	BB
Izumi	Benihomare X unknown	AB	BB	A_1B	BB	AB	BB	**B_1C**	AB	BB	AB	BB
Kanayamidori (P)	Yabukita X S6	AB	BB	A_1A_2	AB	BB	AA	A_1B_1	AB	BB	AB	AB
Karabeni	Kohoku Pref. China	AB	BB	A_1A_2	AB	AB	AB	**B_1E**	AB	**AB**	AB	BB
Komakage	selection	AA	BB	A_1A_1	AA	BB	AB	A_1B_2	AB	BB	BB	AA
Kuritawase	selection	AA	BB	A_1A_2	AA	AB	AB	B_1B_2	AA	BB	AB	AB
Kurusawa	Yabukita X unknown	AB	BB	A_1A_2	BB	BB	AA	**A_1D_2**	BB	BB	BB	AB
Makinoharawase	selection	AA	BB	A_1A_1	AA	BB	AA	B_1B_1	AA	BB	AA	BB
Meiryoku (P)	Yabukita X Z1	AA	BB	A_1A_2	AB	AB	AB	A_1B_1	AB	BB	AB	AB
Natsumidori	selection	AA	BB	A_2B	BB	AB	AB	B_1B_2	AA	BB	AB	AB
Oiwase	Yabukita X Yaeho	BB	BB	A_1A_2	AB	BB	AB	A_1B_2	AB	BB	BB	AA
Okuhikari	Yabukita X G225	AA	BB	A_2B	AB	AB	AB	A_1B_2	AB	BB	BB	AB
Okumidori (P)	Yabukita X Shizuoka-Zaira-16gou	BB	BB	A_1A_2	BB	AA	BB	A_1B_1	AB	BB	AB	AB
Okumusahi	Sayamamidori X Yamatomidori	BB	BB	A_1B	BB	AB	BB	B_1B_2	AA	BB	AB	BB
Okuyutaka	Yutakamidori X NN8	BB	BB	A_2B	AB	BB	AA	**B_1B_1**	AA	BB	AA	BB
Ryohofu (P)	Yabukita X Horyofu	AB	BB	A_1A_2	AB	AB	BB	A_1B_1	AB	BB	AB	AB
Saemidori (P)	Yabukita X Asatsuyu	AB	BB	A_2B	BB	BB	AA	A_1B_1	AB	BB	AB	AB
Samidori	selection	AA	BB	A_1A_1	AA	AB	AB	A_1B_2	AB	BB	BB	AA
Sayamakaori (P)	Yabukita X unknown	BB	BB	A_1A_2	BB	AB	BB	A_1C	BB	BB	BB	AB
Sayamamidori	selection	AB	BB	A_1A_1	AB	AA	BB	A_1B_1	AB	BB	AB	AB
Shunmei (P)	Yutakamidori X NN8	AA	BB	A_1A_1	AB	BB	AA	B_1D_1	AB	BB	AB	BB
Surugawase	Yabukita X unknown	BB	BB	A_1A_2	BB	BB	AB	A_1B_2	AB	BB	BB	AA
Tadanishiki	Tada hybrids	AB	BB	A_1A_1	AB	AA	BB	B_1B_2	AA	BB	AA	BB
Takachiho	selection	AB	BB	A_2B	AB	BB	AB	A_1B_1	AB	BB	AB	AB
Tamamidori	unknown	AA	BB	A_1A_2	AA	BB	AA	A_1B_2	AB	BB	BB	AA
Toyoka	Yabukita X Sayamamidori	AB	BB	A_1A_2	BB	AB	AB	A_1B_1	AB	BB	AB	AB
Ujimidori	selection	AA	BB	BB	AB	AB	AB	A_1B_1	AB	BB	AB	AB
Yabukita (P)	selection	AB	BB	A_2A_2	BB	AB	AB	A_1A_1	BB	BB	BB	AA
Yaeho	selection	AB	BB	A_1A_1	AA	BB	BB	B_1B_2	AA	BB	AB	AB
Yamakai	Yabukita X unknown	AB	BB	A_1A_2	AB	AB	AB	A_1B_2	AB	BB	BB	AA
Yamanami	Kohoku Pref. China	AB	BB	A_1A_2	AB	AA	BB	B_1B_1	AA	BB	AA	BB
Yamatomidori	selection	BB	BB	A_1B	BB	AB	BB	A_1B_2	AB	BB	BB	AB
Yutakamidori (P)	Asatsuyu X unknown	AB	BB	A_1B	AB	BB	AB	**B_1D_2**	AB	BB	AB	BB
Z1	Tamamidori X unknown	AA	BB	A_1A_1	AA	AB	AB	B_1B_2	AA	BB	AB	AB

[a] P in brackets in the first column indicates that processed tea was also analyzed for these cultivars. [b] Unique profiles at a particular locus are represented in bold.

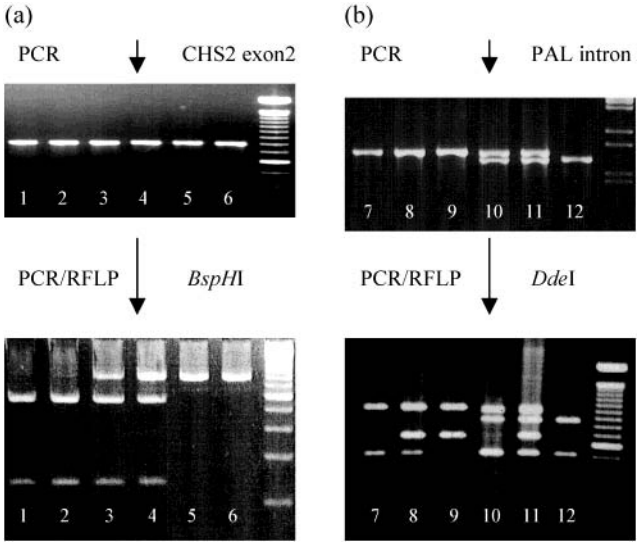

Figure 2. (a) Amplication CHS2 exon2 and corresponding PCR/RFLP profiles obtained with *Bsp*HI among Okumusashi (1: BB), Yamanami (2: BB), Yabukita (3: AB), Oiwase (4: AB), Gokou (5: AA), and Kurusawa (6: AA). (b) Amplification of PAL and corresponding PCR/RFLP profiles with *Dde*I from Shunmei (7: A_1A_1), Kanayamidori (8: A_1A_2), Yabukita (9: A_2A_2), Yutakamidori (10: A_1B), Saemidori (11: A_2B), and Asatsuyu (12: BB).

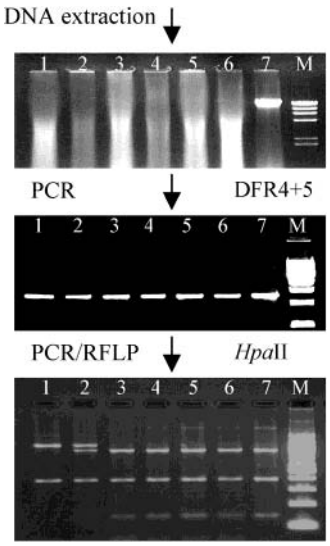

Figure 3. DNA extraction, corresponding PCR amplification of DNA fragment DFR4+5 and PCF/RFLP with *Hpa*II from Asatsuyu steamed for 40 s (1), Saemidori steamed for 40 s (2), and Yakubita (3–7), respectively, steamed for 30 s, 60 s, 120 s, pan-fried, and fresh leaves.

in all. After digestion with a number of restriction enzymes, the allele A could be further divided in two different types due to the presence or absence of a restriction site with *Dde*I. Thus for PAL intron, three different alleles could easily be scored, the combination of which generated six different genotypes in all.

The same was observed with DFR1+2 showing as many as five alleles among the samples denoted A, B, C, D, and E here. Alleles A, B, and D could be further subdivided into two each with *Ban*II, giving a total of eight different alleles. There was, however, a predominance of allele B_1 with a frequency of 0.32 while allele E was present once in a heterozygote state in Karabeni. Considered alone, DFR1+2 locus digested by *Ban*II generated as many as 14 different genotypes including eight unique DNA patterns in the 46 studied cultivars.

The PAL intron and DFR1+2 showed polymorphism with a number of other enzymes, but the presence of two alleles differing in length following PCR, coupled with frequent cuts, especially with enzyme having AT's in their recognition sites, generated complex patterns that were difficult to score. These enzymes were therefore not retained further in order to ensure reliable results.

Extraction from fresh leaves generated DNA of high molecular weight, while that of processed tea yielded a smear in all cases. Nevertheless, no difference in amplification patterns was observed between these two types of plant materials. As shown on **Figure 3**, Asatsuyu and Saemidori steamed for 40 s and Yabukita tea steamed for 30, 60, and 120 s, pan-fried at high temperatures, or obtained from fresh leaves gave the expected DNA fragment using primer pairs tagging the DFR4+5 region. Subsequently upon digestion of DFR4+5 fragment using the *Hpa*II, three different profiles could be observed on an agarose gel. Lane one of **Figure 3** corresponds to the cultivar Asatsuyu for which a restriction site was absent for both of its alleles, but present in Yabukita whatever the processing condition used (lanes 3−7). On the other hand, Saemidori (lane 2) was a heterozygote for the polymorphic site in question. This was expected, as Saemidori is a progeny between Yabukita and Asatsuyu, both parents being a homozygote for opposite characters at this locus.

On the basis of polymorphisms resulting from different point mutations, insertions, and deletions at intron sites, all 46 tea cultivars could be distinguished from each other (**Table 2**). Benihikari and Benifuji, both of which resulted from a cross between an Assam hybrid and a genotype selected from China, were the most distant from each other, differing at as many as eight loci. The two closest cultivars were Kuritawase and Z1, sharing the same genotype at 10 loci and differing at the PAL intron locus only. As shown in **Table 2**, Z1 was a homozygote for allele A_1 for this locus and Kuritawase was a heterozygote with the genotype A_1A_2. The PAL intron locus was also very informative, as it individualized Yabukita (A_2A_2), the leading cultivar in Japan from all other tea samples studied.

DISCUSSION

The precise identification of food products has become prominent in recent years. Retailers and consumers are exigent as to what they are selling and buying, and several protocols using DNA fingerprinting have been proposed to authenticate fish, wine, and ginseng, for example (*8*−*10*). In this study we have developed a similar methodology capable of certifying the main Japanese green teas to discourage the substitution of a particular local tea by another one and to authenticate Yabukita recently imported from a neighboring country.

The procedure developed on fresh plant material and based on three known genes in tea, proved to be easily applicable on different forms of processed green teas. One of the processes that may lead to DNA degradation, and thus PCR failure, is steaming time or pan-frying at high temperatures. Sencha in Japan is normally made by steaming freshly harvested leaves for a period of time of around 1 min. Our study showed that DNA could be effectively recovered and used in PCR from tea heavily steamed for up to 2 min in the process making of Fukamushi-cha, a special type of green tea produced in the Prefecture of Shizuoka. Amplifiable DNA was also obtained with pan-fried or Kamaeri-cha, a very common form of tea made in China and Korea, but also produced in small quantities in Japan. The important factor in PCR, especially targeting short

DNA fragments, is not actually the quality of the template DNA itself, but the absence of inhibitors, such as high levels of polysaccharides and polyphenols in the reaction mixture. Extraction of processed tea with the commonly used CTAB buffer, followed by a chloroform stage, removes most of these two types of products. Also steaming and pan-frying has the purpose of inhibiting oxidative enzymes in the making of green and low astringent tea. This process limits the conversion of polyphenols into highly inhibitive products for PCR, such as theaflavins and thearubigins.

All the tea cultivars could be distinguished based on polymorphism detected in seven DNA fragments digested at 11 polymorphic sites. Identification of the cultivars could also be achieved using a subset of markers, namely PALI/*Hpa*II, PAL intron/*Dde*I, PALex2/*Taq*I, CHS2ex2/*Rsa*I, and DFR4+5 digested by *Hpa*II and *Nla*III. This would avoid using the DFR1+2 fragment which requires more careful allele scoring than the other loci due to relatively small size difference between the alleles. At present the predominant cultivar that needs to be authenticated is undoubtedly Yabukita. This genotype was selected in the early years of the last century and released as cultivar in 1953. Since then it has gained wide acceptance among local farmers and has become a brand tea for Japanese people. To identify Yabukita it is simply needed to target the PAL intron region through PCR and digest the amplification product with *Dde*I, for it is the only cultivar showing a A_2A_2 profile at this locus. To be on a safer side, however, the three easily scored and discriminating loci for Yabukita should be analyzed, that is the PAL intron/*Dde*I, DFR3/*Hind*III, and CHS2ex2/*BspH*I with genotypic frequencies of, respectively, 1/46, 5/46, and 20/46 among the 46 cultivars. In such a case, the chance of encountering a cultivar with a profile identical to Yabukita is only around 1/1000. In practice this probability would be even lower should the tea be imported from other countries, namely Korea and China both producing Yabukita at the present time. Indeed local tea from these countries has been found to differ markedly from that of Japan both from a biochemical point of view and at the DNA level (*11*, *12*). In this very study, the Japanese tea cultivars recently selected from Chinese populations displayed unique or rare profiles; for instance, Karabeni could be individualized with either DFR4+5/*Xmn*I or DFR1+2/*Ban*II. DFR1+2 was also very discriminating for a number of Assam hybrids such as Izumi and Benefuki, while Hatsumomiji showed a unique profile with PALexI digested by *Nla*III.

The uniqueness of Yabukita and its predominance in the Japanese market will make it easy to monitor its fraudulent substitution by another tea and vice versa. For most other genotypes, however, a number of loci have to be investigated to confirm their identity. This is because many tea cultivars have been bred using a few successful cultivars only, namely Yabukita. One such tea is Saemidori, which is a cross between Asatsuyu and Yabukita. This cultivar is well adapted to warm conditions and is an increasingly popular tea in the south of the country. To distinguish Saemidori from the other cultivars, at least three loci should be analyzed, for instance PAL/*Dde*I, CHS2ex2/*BspH*I, and DFR3/*Hind*III.

Though genetically close to each other, complete distinction among cultivars was achieved based on polymorphism detected in PAL, CHS, and DFR only. This could be explained by the fact that these three genes are involved in the phenylpropanoid pathway leading to catechin and tannin synthesis, all playing an important role in tea taste and quality. Indeed, the very existence and cultivation of these main tea cultivars lie in part due to their different organoleptic properties. Asatsuyu for example contains low levels of catechin and is particularly suited for Gyokuro, a mild astringent tea. On the other hand, Hatsumomiji, a hybrid tea between Assam and China genotypes is catechin and tannin rich and is highly praised in the making of Oolong or semifermented tea.

Recently the polyphenol oxidase and caffeine synthase genes, also playing key roles in the quality of tea, have been cloned, and their DNA sequences are freely available on GenBank (*13*). Using the same procedure described here, additional polymorphism could be detected, thus accommodating a more precise identification of a larger number of tea cultivars. The markers detected at these loci will be useful at identifying tailor-made teas that are being bred to produce catechin rich and caffeine poor tea, to serve as a preventive against a number of diseases, and to suit consumers who cannot withstand high concentrations of caffeine, respectively.

In conclusion, the STS−RFLP methodology based on three genes involved in the phenylpropanoid pathway is very effective at identifying the most commonly grown Japanese tea cultivars using both fresh and processed material. This simple, inexpensive, and reproducible procedure has already been successfully applied in the authentication of a first batch of Yabukita tea imported from a neighboring country. It constitutes the first practical application of DNA technology at fingerprinting tea.

ACKNOWLEDGMENT

The authors would like to express their gratitude to Mr. A. Nesumi and Ms. N. Ikeda for providing processed tea used in this study.

LITERATURE CITED

(1) Wight, W. Tea classification revised. *Curr. Sci.* **1962**, *31*, 298−299.

(2) Banerjee, B. Botanical classification of tea. In *Tea: cultivation to consumption*; K. C. Wilson and M. N. Clifford, Eds.; Chapman and Hall: London, 1992; pp 25−51.

(3) Nagata, T.; Sakai, S. Differences in caffeine, flavanols and amino acids contents in leaves of cultivated species of *Camellia. Jpn J. Breed.* **1984**, *34*, 459−467.

(4) Maeda-Yamamoto, M.; Sano, M.; Matsuda, N.; Miyase, T.; Kawamoto, K.; Suzuki, N.; Yoshimura, M.; Tachibana, H.; Hakamata, K. The change of epigallocatechin-3-*O*-(3-*O*-methyl)-gallate content in tea of different varieties, tea seasons of crop and processing method. *Nippon Shokuhin Kakaku Kaishi* **2001**, *48*, 64−68.

(5) Kaundun, S. S.; Zhyvoloup, A.; Park, Y. G. Evaluation of the genetic diversity among Elite tea (*Camellia sinensis* var. *sinensis*) genotypes using RAPD markers. *Euphytica* **2000**, *115*, 7−16.

(6) Matsumoto, S.; Takeuchi, A.; Hayatsu, M.; Kondo, S. Molecular cloning of phenylalanine ammonia-lyase cDNA and classification of varieties and cultivars of tea plants (*Camellia sinensis*) using tea PAL cDNA probe. *Theor. Appl. Genet.* **1994**, *89*, 671−675.

(7) Takeuchi, A.; Matsumoto, S.; Hayatsu, M. Chalcone synthase from *Camellia sinensis*: Isolation of the cDNA and the organ-specific and sugar-responsive expression of the genes. *Plant Cell Physiol.* **1994**, *35*, 1011−1018.

(8) Asensio, L.; Gonzalez, I.; Fernandez, A.; Rodriguez, M. A.; Hernandez, P. E.; Garcia, T.; Martin, R. PCR−SSCP: A simple method for the authentication of grouper (*Epinephelus guaza*), wreck fish (*Polyprion americanus*), and nile perch (*Lates niloticus)* fillets. *J. Agric. Food Chem.* **2001**, *49*, 1720−1723.

(9) Mihalov, J. J.; Marderosian, A. D.; Pierce, J. C. DNA identification of commercial ginseng samples. *J. Agric. Food Chem.* **2000**, *48*, 3744−3752.
(10) Siret, R.; Boursiquot, J. M.; Merle, M. H.; Cabanis, J. C.; This, P. Toward the authentication of varietal wines by the analysis of grape (*Vitis vinifera* L.) residual DNA in must and wine using microsatellite markers. *J. Agric. Food Chem.* **2000**, *48*, 5035−5040.
(11) Choi, S. Studies of the aroma of Korean green tea. *Proceedings of the International Symposium of Tea Science, Shizuoka, Japan*; ISTS, 1991, pp 130−134.
(12) Matsumoto, S.; Kiriwa, Y.; Takeda, Y. Differentiation of Japanese green tea as revealed by RFLP analysis of phenylalanine ammonia-lyase DNA. *Theor. Appl. Genet.* **2002**, 104, 998−1002.
(13) Kato, M.; Mizuno, K.; Crozier, A.; Fujimura, T.; Ashihara, H. Caffeine synthase gene from tea leaves. *Nature* **2000**, *406*, 956−957.

Received for review July 24, 2002. Revised manuscript received December 31, 2002. Accepted December 31, 2002. S.K. was awarded an STA (Japan) fellowship during the 1999−2001 period.
JF020821I