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Simple Sequence Repeat Analysis of Selected NSIC-registered Coffee Varieties in the Philippines

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ABSTRACT

Coffee (*Coffea* sp.) is an important commercial crop worldwide. Three species of coffee are used as beverage, namely *Coffea arabica, C. canephora*, and *C. liberica*. *Coffea arabica* L. is the most cultivated among the three coffee species due to its taste quality, rich aroma, and low caffeine content. Despite its inferior taste and aroma, *C. canephora* Pierre ex A. Froehner, which has the highest caffeine content, is the second most widely cultivated because of its resistance to coffee diseases. On the other hand, *C. liberica* W.Bull ex Hierncomes is characterized by its very strong taste and flavor. The Philippines used to be a leading exporter of coffee until coffee rust destroyed the farms in Batangas, home of the famous *Kapeng Barako*. The country has been attempting to revive the coffee industry by focusing on the production of specialty coffee with registered varieties on the National Seed Industry Council (NSIC). Correct identification and isolation of pure coffee beans are the main factors that determine coffee's market value. Local farms usually misidentify and mix coffee beans of different varieties, leading to the depreciation of their value. This study used simple sequence repeat (SSR) markers to evaluate and distinguish Philippine NSIC-registered coffee species and varieties. The

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neighbor-joining tree generated using PAUP showed high bootstrap support, separating *C. arabica, C. canephora*, and *C. liberica* from each other. Among the twenty primer pairs used, seven were able to distinguish *C. arabica*, nine for *C. liberica*, and one for *C. canephora*.

Keywords: Coffea, NSIC-registered varieties, SSR

INTRODUCTION

Coffee is an economically important crop in the global market. It belongs to the caffeine-containing subgenus *Coffea* from the family Rubiaceae, which comprises over a hundred species originating from the African region (Charrier and Berthaud 1985). Among the coffee species used for commercial consumption, *C. arabica* L. is the most cultivated, accounting for 70% of the global coffee production. It is the only allotetraploid in the genus and is self-pollinating. This species also has the highest market value (Tornincasa et al. 2010) because of its low caffeine content, excellent taste, and aroma (Vidal et al. 2010; Vieira et al. 2010). The species *C. canephora* Pierre ex A. Froehner is second to *C. arabica* in terms of production, contributing the remaining 30% of global coffee production. It has certain advantages in terms of production due its high-yielding properties and tolerance to diseases. However, its taste, which is characterized as woody bitter and of high caffeine content, is inferior to *C. arabica* (Reyes 2010).

Most studies report that only *C. arabica* and *C. canephora* are cultivated for commercial consumption. The Philippines is one of the few countries that commercially produce, in addition to *C. arabica* and *C. canephora*, varieties of the species *C. liberica* W. Bull ex Hierncomes. The Liberica variety, *C. liberica* var. Liberica, was an economically important commodity during the 1930s. Locally known as the *Kapeng Barako*, it is distinguished for its strong, woody, and bitter taste, acidic aftertaste, and pungent aroma. Apart from its strong taste, this variety also possesses desirable reproductive characteristics in terms of fruit clusters, bean size (the largest among the four varieties), and low caffeine content (N'Diaye et al. 2005). *Coffea liberica* var. Dewevrei, commonly known as Excelsa coffee, has a woody taste, and sweet, fruity aroma (Reyes 2010).

The identity and purity of the coffee produce determine its market value. Owing to the economic importance of coffee, it is of interest to assess its genetic diversity

and to come up with markers that will identify and distinguish species, as well as varieties within a species. Since morphological methods are sometimes not reliable in differentiating coffee species and varieties, molecular techniques are being used and developed to address this concern. The CBOL (Consortium for the Barcoding of Life) Plant Working Group has recommended two universal plant barcodes for species identification, namely the *matK* and *rbcL* genes (Janzen 2009). These two genes have been used in verifying the identities of the coffee species in the farms located in Cavite, Philippines. The said genes were able to distinguish among the species *C. arabica*, *C. canephora*, and *C. liberica*. However, the varieties *C. liberica* var. Liberica and *C. liberica* var. Dewevrei were not successfully differentiated and clustered together in a single clade (Cao et al. 2014). The *matK* and *rbcL* markers could discriminate between species but not varieties within species.

Microsatellite or simple sequence repeat (SSR) markers are short, tandem repeats present in the coding and non-coding portions of the genome (Wang et al. 2009). SSRs require only a small amount of DNA for polymerase chain reaction (PCR)-based screening and can reveal multiple alleles at a single locus. Automated allele detection and sizing are also readily available (Schlotterer et al. 2000). The abundance and highly polymorphic property of SSRs make it a good marker for plant genetic studies, identification of cultivars, and evaluation of varieties with a narrow genetic base (Vieira et al. 2010; Wang et al. 2009).

SSRs have been used in varietal identification and the evaluation of genetic diversity in *C. arabica* varieties (Vieira et al. 2010). In 2012, low genetic diversity was observed in the *C. arabica* populations in the Nicaraguan regions due to their narrow genetic base, but significant differentiation was found among the varieties (Geleta et al. 2012). Both *C. arabica* and *C. canephora* have also been shown to have narrow diversity using SSR markers (Anthony et al. 2001; Anthony et al. 2002; Lashermes et al. 1999). In other studies, *C. arabica* DNA fingerprinting using SSR markers has also been developed as a method to test against *C. canephora*, in order to ensure the authenticity of the coffee products sold in the market (Tornincasa et al. 2010). SSRs have also been used to evaluate leaf miner resistance in Arabica coffee (Pereira et al. 2011). The diversity of the *C. canephora* gene pool was also assessed using SSRs (Prakash et al. 2005).

Since coffee variety misidentification and coffee bean sample impurity are major factors that affect the income of small-scale farmers, this study aims to identify potential molecular markers with different SSR primers for variety identification

using NSIC-registered varieties as standards. The NSIC under the Department of Agriculture, Bureau of Plant Industry was established in 1992 under Republic Act 7308. This office functions to approve and register crop varieties. Currently, there are 22 registered coffee varieties across the country (NSIC 2012).

MATERIALS AND METHODS

Plant Material and DNA Extraction

NSIC-registered coffee samples were collected from Benguet, Cavite, and Bukidnon (Table 1). Two plants from each available variety were collected. Around 100 mg of young leaves were obtained from each plant for DNA extraction. Genomic DNA was extracted using Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Automated quantification of the amount and purity of the extracted DNA was performed using Nanodrop. On average, about 100 ng per µL of DNA was extracted for each specimen.

Polymerase Chain Reaction and Electrophoresis

Twenty SSR primers reported in published literature (Table 2) were used for amplification in each specimen. The concentration of the PCR components for a 14 µL reaction were as follows: 3.44 µL Qiagen master mix, 1.2 µL Q buffer, 0.5 µL 25 mM MgCl2, 0.24 µL 10 µM primers, 7.38 µL DNAse/RNase-free water, and 1.0 µL 20 ng DNA.

The following PCR conditions were used: initial denaturation at 94°C for 10 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension

Table 1. NSIC varieties used in this study

Species Identity	Variety	Source
Coffea arabica	Red Bourbon	Bureau of Plant Industry, Baguio City, Benguet
Coffea arabica	Yellow Bourbon	Bureau of Plant Industry, Baguio City, Benguet
Coffea arabica	Caturra	Bureau of Plant Industry, Baguio City, Benguet
Coffea canephora	Ivory Coast 2	Cavite State University, Indang, Cavite
Coffea canephora	Ivory Coast 7	Cavite State University, Indang, Cavite
Coffea canephora	Ivory Coast 8	Cavite State University, Indang, Cavite
Coffea canephora	S247	Cavite State University, Indang, Cavite
Coffea liberica	BS1 (for registry)	Cavite State University, Indang, Cavite
Coffea canephora	FRT23	Nestle Philippines, Inc., Malaybalay, Bukidnon
Coffea canephora	FRT 65	Nestle Philippines, Inc., Malaybalay, Bukidnon

at 72ºC for 1 min; and final extension at 72ºC for 7 min (Teressa et al. 2010). The PCR products were run in 2% agarose gels for confirmation. For better resolution of the bands, the PCR products were run in 10% native polyacrylamide gels. Both 100 bp (KAPA) and 25 bp (Bioline) DNA ladders were used as molecular weight markers.

Table 2. Primer sequences used for SSR analysis

Primer Name	Sequence	Repeats	Reference
ssrR209 F	5'CGGGGGTAAAAAGATTGTAA3'	GA (16)	Teressa et al. 2010
ssrR209 R	5'TTGGTGGGAGGGGAGTA3'		
ssrR268 F	5'GTATCCCACAATGAAATCAC3'	GA (19)	Teressa et al. 2010
ssrR268 R	5'AGTAGAATTTTCAACATATAAG3'		
SSR124577 F	5'GATGGCTTTTCTCCGTTATCC3'	AAG (6)	Teressa et al. 2010
SSR124577 R	5'GGATTCGACTGCTGGATGAT3'		
SSR122850 F	5'TCCAGTTTGATCAGCAACCA3'	(AGAG)3	Teressa et al. 2010
SSR122850 R	5'CCATCTTGGGGATAGAGCAA3'		
SSR124195 F	5'ATCCCCATCAGAAGACCTCA3'	(AGC)6	Teressa et al. 2010
SSR124195 R	5'CCTCCACCGCCTGTTTATTA3'		
SSR123557 F	5'ATCTCCTCGTTCTTCCCCAT3'	CTCT (4)	Teressa et al. 2010
SSR123557 R	5'GCTTGTAGCAGGCAGGAAAC3'		
ssrCMA008 F	5'CATTCTGGTCCTGATGCTCT3'	(CT)14..(TG)10	Teressa et al. 2010
ssrCMA008 R	5'TCATTCACTTATTAACGTCCATC3'		
M-24 F	5'GGCTCGAGATATCTGTTTAG3'	Not specified	Bigirimana et al. 2013
M-24 R	5'TTTAATGGGCATAGGGTCC3'		
Sat235 F	5'TCGTTCTGTCATTAAATCGTCAA3'	Not specified	Bigirimana et al. 2013
Sat235 R	5'GCAAAATCATGAAAATAGTTGGTG3'		
Sat172 F	5'ACGCAGGTGGTAGAAGAATG3'	Not specified	Bigirimana et al. 2013
Sat172 R	5'TCAAAGCAGTAGTAGCGGATG3'		
Sat227 F	5'TGCTTGGTATCCTCACATTCA3'	Not specified	Bigirimana et al. 2013
Sat227 R	5'ATCCAATGGAGTGTGTTGCT3'		
Sat229 F	5'TTCTAAGTTGTTAAACGAGACGCTTA3'	Not specified	Bigirimana et al. 2013
Sat229 R	5'TTCCTCCATGCCCATATTG3'		
Sat254 F	5'ATGTTCTTCGCTTCGCTAAC3'	Not specified	Bigirimana et al. 2013
Sat254 R	5'AAGTGTGGGAGTGTCTGCAT3'		
ssrCMA059 F	5'GATGGACAGGAGTTGATGGT3'	(CT9)(CA)8	Teressa et al. 2010
ssrCMA059 R	5'TTTTAACACTCATTTTGCCAAT3'		
ssrCMA198 F	5'AGCAACTCCAGTCCTCAGGT3'	(TG)9(AG)18	Teressa et al. 2010
ssrCMA198 R	5'TGGAAGCCCGCATATAGTTT3'		
SSRCa068 F	5'ATGTTGTTGGAGGCATTTTC3'	(AGG)7//(GAA)4	Missio et al. 2011
SSRCa068 R	5'AGGAGCAGTTGTTGTTTTCC3'		
SSRCa087 F	5'TCACTCTCGCAGACACACTAC3'	(TC)22	Missio et al. 2011
SSRCa087 R	5'GCAGAGATGATCACAAGTCC3'		
SSRCa094 F	5'GTGTCCTAGGGAAGGGTAAG3'	(TC)4(TTCT)3// (TTTCCT)3 (TTCT)5	Missio et al. 2011
SSRCa094 R	5'GAGTGCTAGGAGAGGGAGAG3'		
SSRCa091 F	5'CGTCTCGTATCACGCTCTC3'	(GT)8(GA)10	Missio et al. 2011
SSRCa091 R	5'TGTTCCTCGTTCCTCTCTCT3'		
Sat207 F	5'AAGCCGTTTCAAGCC3'		Pereira et al. 2011
Sat207 R	5'CAATCTCTTTCCGATGCTCT3'		

Data Analysis

The PCR products were evaluated by scoring the presence (1) or absence (0) of clear and unambiguous bands. A neighbor-joining tree with 1,000 bootstrap replicates was constructed using PAUP version 4.0b10 for Microsoft Windows 95/ NT and viewed using TreeExplorer 2.12 by Koichiro Tamura 1997-1999. Pairwise genetic distances were also calculated using PAUP.

RESULTS AND DISCUSSION

A total of 236 unique bands were identified from the 20 SSR markers. Based on the neighbor-joining tree generated, the *C. arabica, C. canephora, and C. liberica* species were differentiated into separate clades (Figure 1). Of the 20 SSR markers, seven primer pairs distinguished *C. arabica*, nine for *C. liberica,* and one for *C. canephora* (Table 3). This shows that the SSR markers can be used in delineating species despite

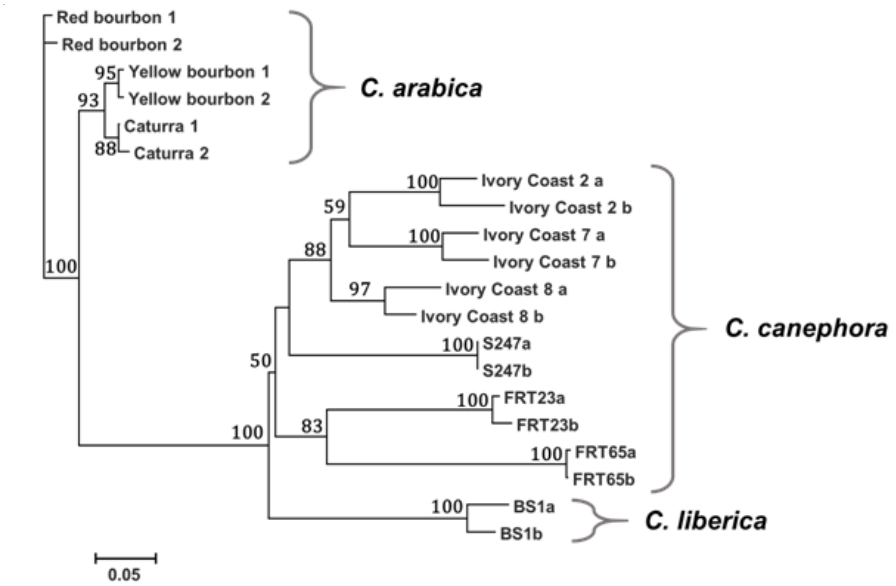

Figure 1. Neighbor-joining tree of 20 NSIC-registered coffee varieties generated from banding profiles from 20 microsatellite markers. Branch lengths are drawn to scale and represent uncorrected p-distances. Bootstrap supports of 1000 replicates are shown.

Table 3. Species distinguished by each primer pair

Primer pair	Diagnosable species
ssrR209	-
ssrR268	-
SSR124577	*C. arabica*
SSR122850	*C. liberica*
SSR124195	*C. arabica*
SSR123557	*C. arabica*
ssrCMA008	*C. arabica, C. liberica, C. canephora*
M-24	*C. liberica*
Sat235	-
Sat172	-
Sat227	*C. liberica*
Sat229	*C. liberica*
Sat254	*C. liberica*
ssrCMA059	*C. arabica, C. liberica*
ssrCMA198	*C. arabica*
SSRCa068	*C. arabica*
SSRCa087	-
SSRCa094	-
SSRCa091	*C. liberica*
Sat207	*C. liberica*

Table 4. Generic distances in coffee species and varieties. n, number of pairwise comparison; p, uncorrected distance

Pairwise comparisons	p
Average between species (n=108)	**0.382**
Average between varieties of the same species (n=72)	0.285
Average between varieties of *C. arabica* (n=12)	0.060
Average between varieties of *C. canephora* (n=72)	0.330
Average within varieties (n=10)	**0.034**
Red bourbon (n=1)	0.017
Yellow bourbon (n=1)	0.008
Caturra (n=1)	0.009
Yellow Bourbon and Caturra combined (n=4)	0.024
Ivory Coast2 (n=1)	0.083
Ivory Coast7 (n=1)	0.068
Ivory Coast8 (n=1)	0.072
S247 (n=1)	0.000
FRT23 (n=1)	0.021
FRT65 (n=1)	0.004
BS1 (n=1)	0.057

very high polymorphisms. In particular, the ssrCMA008 primer pair was able to differentiate the three species. Teressa et al. (2010) used this primer pair to compare varieties of *C. arabica*. This is the first study that demonstrates its utility for species diagnosis. A 100% bootstrap support was observed for *C. arabica* and *C. liberica* species, whereas the support for *C. canephora* was only at 50%. The low bootstrap support for *C. canephora* is likely due to the large genetic distance between the Ivory Coast and S247 varieties from Cavite, and the FRT varieties from Bukidnon. The average genetic distance among varieties of *C. canephora* (p = 0.330) was comparable to the distances among species (p = 0.382; Table 4).

The SSR markers were also able to differentiate among the varieties. Bootstrap supports of 100% were observed for the Red bourbon, Ivory Coast 2, Ivory Coast 7, S247, FRT23, FRT65, and BS1 varieties. A bootstrap support of 97% was observed for Ivory Coast 8. Bootstrap supports of 95% and 88% were observed for Yellow Bourbon and Caturra varieties, respectively.

Among the *C. arabica* varieties, the red bourbon variety can be distinguished from the others using the SSR124577 (Figure 2) primer pair. The allele number for this primer pair was higher in this study (n=8) compared to that of Teressa et al. (2010), indicating higher diversity among the *C. arabica* varieties in the Philippines. The Red bourbon variety was shown to be distinct: a 150-bp band from SSR124577, and 150-bp and 350-bp bands from SAT229 primer pairs can distinguish the Red Bourbon from the other *C. arabica* varieties. The Yellow bourbon and Caturra varieties clustered together with 93% bootstrap support. Although the bootstrap support for each of these clades is moderately high, the values obtained were lower compared to the support for the clades of the other varieties (Figure 1). The average pairwise

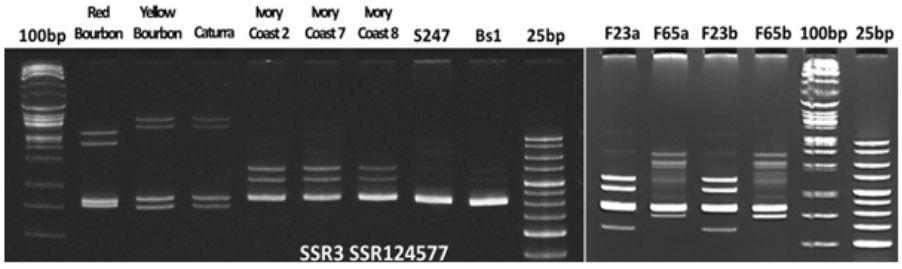

Figure 2. Banding patterns observed for the different Coffea varieties using the SSR124577 marker.

genetic distance within these two varieties combined is small (p = 0.024) and is even lower than the average genetic distance within single varieties (p = 0.033; Table 4). These two varieties were distinguished by the SSRCa087 primer pair. Apart from this, they share the same banding profile based on the other markers. Moreover, the 140-bp, 1,000-bp, and 1,200-bp bands from SSR124577 (Figure 2) were found to be unique to Yellow bourbon and Caturra. These varieties are commonly considered to be identical, but were registered as distinct varieties (Prof. Valentino Macanes, pers. comm.). Results in this study show partial support for this claim, but the current dataset is insufficient to generate conclusions, considering that the two varieties did form distinct clades. The Yellow bourbon and Caturra varieties were observed to have leaves that are similar in shape and size, but Caturra had shorter internodes. According to the NSIC registry (NSIC 2012), they also differ in berry color, but this was not observed in this study because there were no berries during the time of sampling.

The SAT235 is linked to disease resistance against coffee berry disease (Gichimu et al. 2014; Gichuru et al. 2008). It is not clear from these papers, however, what fragment size correspond to the marker for the disease. Based on the NSIC registry (NSIC 2012), only IC8 has a record of moderate resistance against coffee berry disease. The bands exhibited by IC8 for the SAT235 primer pair is shared by other varieties of *C. canephora*, except for the FRT varieties from Bukidnon. No entries are available for other varieties. Among the *C. canephora* varieties, the FRT varieties developed by Nestle Philippines, Inc. in Bukidnon highly diverged from the Ivory Coast and S247 varieties from Cavite—a phenomenon manifesting even in terms of morphology. FRT varieties take a much longer time to flower and fruit, but produce greater yield and more berries per leaf node.

These results show the potential of SSR markers for use in varietal identification of coffee. The findings also indicate the possible application of SSR markers in other existing cultivars available in the country. Proper identification is important to ensure homogeneity and increase marketability. Moreover, the application of SSRs could later be extended for marker-assisted selection of important traits, such as disease resistance, aroma, and yield. Marker-assisted selection would provide a bottom-up evolutionary approach in genetic improvement, which is more acceptable to society compared to the top-down approach of genetic engineering.

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