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Research Note

SSR Analysis of *Coffea liberica* var. *liberica* and *Coffea liberica* var. *dewevrei* in the Philippines

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In the Philippines, four coffee varieties (Arabica, Robusta, Liberica and Excelsa) are cultivated for commercial consumption. While Arabica and Robusta varieties are well-known globally, Liberica or Kapeng Barako is being developed as Philippine specialty coffee. Liberica and Excelsa are more similar in morphology compared to Arabica and Robusta since they are varieties of the same species, *Coffea liberica* W. Bull ex Hiern. In this study, 20 SSR markers were amplified for *C. liberica* specimens representing both Liberica and Excelsa varieties in the Philippines. While the SSR markers exhibited high polymorphism, between-site variation was much greater compared to between-variety. The neighbor-joining tree showed specimens from the same site clustering together with moderate to high bootstrap supports. These SSR markers may be used for geographic origin determination of coffee varieties.

Key Words: coffee, Liberica, Excelsa, SSR

Abbreviations: PAUP – phylogenetic analysis using parsimony, PCR – polymerase chain reaction, PIC – polymorphic information content, SSR – simple sequence repeat

INTRODUCTION

Coffea sp. is a valuable agricultural commodity (Lashermes et al. 2008). This genus belongs to the Rubiaceae family and comprises over a hundred species that originated from the African region (Charrier and Berthaud 1985). Among the coffee species being grown for commercial purposes, *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta) are the most cultivated. The Philippines is one of the few countries that cultivate four coffee varieties: Arabica, Robusta, Liberica and Excelsa for commercial purposes (Philippine Coffee Board 2018). The Liberica and Excelsa varieties belong to the species of *Coffea liberica* W. Bull ex Hiern (Davis et al. 2006).

The Liberica variety, *Coffea liberica* var. *liberica*, is locally known as Kapeng Barako. It is being developed as Philippine specialty coffee. This variety is known for its strong woody and bitter taste with an acidic aftertaste and pungent aroma. It has low caffeine content and possesses desirable breeding characteristics in terms of fruit clusters and bean size, which is the largest among the four varieties (N'Diaye et al. 2005). *Coffea liberica* var. *dewevrei*, commonly known as Excelsa or Kapeng Excelsa, has a woody taste and sweet, fruity aroma (Hicks 2002). In terms of morphology, these two are similar but can be differentiated in terms of their leaf shape. The Liberica variety has an elongated leaf shape, while Excelsa variety has a rounder leaf shape (Engelmann et al. 2007).

Since Liberica and Excelsa can only be distinguished through the difference in leaf shape and fruit size, morphological methods cannot be considered as a reliable means for identifying the two varieties. Several molecular techniques such as barcoding (Maurin et al. 2007; Cao et al. 2014), amplified fragment length polymorphism (AFLP) (N'Diaye et al. 2005), and random amplified polymorphic DNA (RAPD) (Bigirimana et al. 2013) are being used for species identification.

Another type of molecular technique uses microsatellites or simple sequence repeats (SSR), which are short, tandem repeats of DNA present in the coding and non-coding portions of the genome (Wang et al. 2009). It has an advantage compared to other molecular markers as it requires only a small amount of DNA for PCR-based screening (Schlotterer 2000). The abundance and highly polymorphic property of SSRs make it a good

marker for plant genetic studies, and identification of cultivars, and evaluating varieties with a narrow genetic base (Vieira et al. 2010; Wang et al. 2009).

The use of SSRs in varietal identification and evaluation of genetic diversity has already been used in the Arabica varieties of coffee (Vieira et al. 2010). In 2012, the genetic diversity of Arabica populations in Nicaragua was found to be low due to its narrow genetic base but significant differentiation was found among the varieties (Geleta et al. 2012). Arabica and Robusta have also been shown to have narrow diversity using SSR markers (Lashermes et al. 1999; Anthony et al. 2001). The diversity of the Robusta gene pool has also been assessed using SSRs (Prakash et al. 2005). In other studies, Arabica DNA fingerprinting using SSR markers has also been developed as a method to discern against the Robusta variety to ensure authenticity of the coffee product sold in the market (Tornincasa et al. 2010).

Aside from fingerprinting and assessment of genetic diversity, SSR markers can also be used for screening of certain traits such as leaf miner resistance in Arabica coffee (Pereira et al. 2011) and for determination of geographical origins such as in the study by Lopez-Gartner et al. (2009) where Arabica varieties grouped together according to source location.

While the literature presents a lot of studies for *Coffea arabica* and *Coffea canephora*, limited data is available about Philippine coffee varieties, especially *Coffea liberica*. In 2016, Santos et al. used SSR markers to analyze Philippine coffee varieties registered with the National Seed Industry Council (NSIC). These markers were able to group together the samples according to species: Arabica, Robusta and Liberica.

This study was conducted to analyze the Liberica and Excelsa varieties of *C. liberica* from different areas in the Philippines using SSR markers. Genetic differentiation between varieties and between different geographic sources was examined.

MATERIALS AND METHODS

Plant Material and DNA Extraction

Around 100 mg of young leaves from two *Coffea liberica* varieties, Liberica (L) and Excelsa (E), were collected from different Philippine coffee farms in Mindanao – Kalamansig in Sultan Kudarat (SK) and Malaybalay in Bukidnon (BK), and in Luzon – the Bureau of Plant Industry in Baguio City, Benguet (BPI) and two areas in Cavite: Amadeo (Am) and the Cavite State University in Indang (CvSU) (Fig. 1). These areas are the main coffee producers in the country. A total of 15 specimens were collected for the Liberica variety and 11 specimens for the Excelsa variety. Genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen, USA) according to manufacturer's instructions. The amount and purity of the extracted DNA were measured using Nanodrop.

SSR Analysis of *Coffea liberica*

For the microsatellite analysis, 20 SSR primers adapted from different coffee studies were used (Table 1). The mixture of the PCR components used were as follows: 3.44 µL Qiagen master mix, 1.2 µL Q buffer, 0.5 µL 25 mM MgCl2, 0.24 µL 10 pM 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 at 72°C for 1 min and final extension at 72°C for 7 min (Teressa et al. 2010). Since the expected band sizes were around 100–150 bp, the amplified PCR products were run in 29:1, 10% polyacrylamide gels for better resolution. A 25 bp ladder (Bioline) and a 100 bp (KAPA) universal ladder were used as molecular weight markers.

The amplified bands were scored by the presence (1) or absence (0) of clear and unambiguous bands. Each of the 20 SSR loci was characterized based on the number of alleles and polymorphic information content (PIC) observed from the specimens analyzed. The PIC was calculated following Seetharam et al. (2009). Genetic relationships were inferred by constructing neighbor-joining trees using PAUP version 4.0b10 for Microsoft Windows 95/ NT (Swofford 2003) and viewed using Tree Explorer 2.12 by Koichiro Tamura 1997–1999. Calculation of pairwise genetic distances was also performed using

Fig. 1. Collection sites for *Coffea Liberica*

Table 1. SSR primers used in this study.

Code	Primer Name	Repeat Motif	Expected Size	Reference
1	ssrR209	GA (16)	161-173 bp	Teressa et al. 2010
2	ssrR268	GA (19)	131-147 bp	Teressa et al. 2010
3	SSR124577	AAG (6)	138-157 bp	Teressa et al. 2010
4	SSR122850	(AGAG)3	132-141 bp	Teressa et al. 2010
5	SSR124195	(AGC)6	83-101 bp	Teressa et al. 2010
6	SSR123557	CTCT (4)	206-270 bp	Teressa et al. 2010
7	ssrCMA008	(CT)14..(TG)10	106-128 bp	Teressa et al. 2010
8	M-24		450-800 bp	Bigirimana et al. 2013
9	Sat235		320-750 bp	Bigirimana et al. 2013
10	Sat172		350-600 bp	Bigirimana et al. 2013
11	Sat227		220-700 bp	Bigirimana et al. 2013
12	Sat229		180-550 bp	Bigirimana et al. 2013
13	Sat254		250-650 bp	Bigirimana et al. 2013
14	ssrCMA059	(CT9)(CA)8	129-165 bp	Teressa et al. 2010
15	ssrCMA198	(TG)9(AG)18	195-236 bp	Teressa et al. 2010
16	SSRCa068	(AGG)7//(GAA)4	236 bp	Missio et al. 2010
17	SSRCa087	(TC)22	143 bp	Missio et al. 2010
18	SSRCa094	(TC)4(TTCT)3// (TTTCCT)3(TTCT)5	195 bp	Missio et al. 2010
19	SSRCa091	(GT)8(GA)10	110 bp	Missio et al. 2010
20	Sat207		82-97 bp	Gichuru et al. 2008

phylogenetic analysis using parsimony (PAUP).

RESULTS AND DISCUSSION

Although the primers were originally developed for Arabica and Robusta, all 20 SSRs were amplified for each specimen of Liberica and Excelsa (Table 2). From the unique bands generated, a total of 242 characters were recorded. The neighbor-joining tree showed close relationships among individuals coming from the same site. Samples from Bukidnon, Sultan Kudarat, Benguet, and Amadeo clustered together with 100%, 89%, 86%, and 50% bootstrap supports, respectively (Fig. 2). Almost all Liberica and Excelsa varieties were differentiated except the ones from BPI Benguet.

The relationships described above can also be summarized based on pairwise distances (Table 3). The distance within sites (0.149) is largely different from the distance between sites (0.275). On the other hand, the distance within varieties (0.238) is only slightly lower than the distance between varieties (0.255).

The SSR markers exhibited high polymorphism for the Liberica and Excelsa varieties. Between-site variation was observed to be greater compared to between-variety variation. It is possible that the pattern observed resulted from low genetic diversity within site. Individuals from one farm may have originated from just a few sets of parents and thus limited the diversification within a site. This low genetic diversity was also reported in populations of Arabica varieties (Geleta et al. 2012; Teressa et al. 2010). On the other hand, variation between the sites may have accumulated due to various ecological factors such as temperature and elevation. Benguet and Bukidnon samples have different phenotypes for the Excelsa variety compared to the ones in Cavite and Sultan Kudarat. One sample from Benguet had a darker leaf color which can be attributed to possible differences in the concentrations of phenolic compounds present in the samples. Genetic differences of Arabica varieties according to source location was also demonstrated by Lopez-Gartner et al. (2009).

The results of this study provide a possible marker for the identification of Liberica and Excelsa varieties and also as a marker in determination of geographic origins of Philippine coffee, which may be used for developing area-specific specialty coffee and add value to the product.

Table 2. Simple sequence repeat (SSR) polymorphism in *Coffea liberica*.

Code	Primer Name	Observed Size	No. of Alleles	Polymorphic Information Content
1	ssrR209	170-1,000 bp	11	0.85
2	ssrR268	150-800 bp	14	0.84
3	SSR124577	125-3,000 bp	13	0.86
4	SSR122850	150-5,000 bp	13	0.87
5	SSR124195	80-260 bp	14	0.86
6	SSR123557	240-5,000 bp	11	0.75
7	ssrCMA008	90-350 bp	12	0.84
8	M-24	160-500 bp	11	0.85
9	Sat235	55-1,600 bp	20	0.90
10	Sat172	150-1,600 bp	12	0.88
11	Sat227	200-800 bp	9	0.86
12	Sat229	125-10,000 bp	6	0.81
13	Sat254	100-1200 bp	14	0.88
14	ssrCMA059	150-800 bp	13	0.83
15	ssrCMA198	160-600 bp	8	0.86
16	SSRCa068	125-1200 bp	13	0.87
17	SSRCa087	125-500	14	0.87
18	SSRCa094	75-1200 bp	12	0.90
19	SSRCa091	85-10,000 bp	10	0.83
20	Sat207	85-10,000 bp	12	0.84

Table 3. Average pairwise distances in *Coffea liberica*.

Comparison	Distance
Average	0.247
Within sites	0.149
Between sites	0.275
Within varieties	0.238
Between varieties	0.255

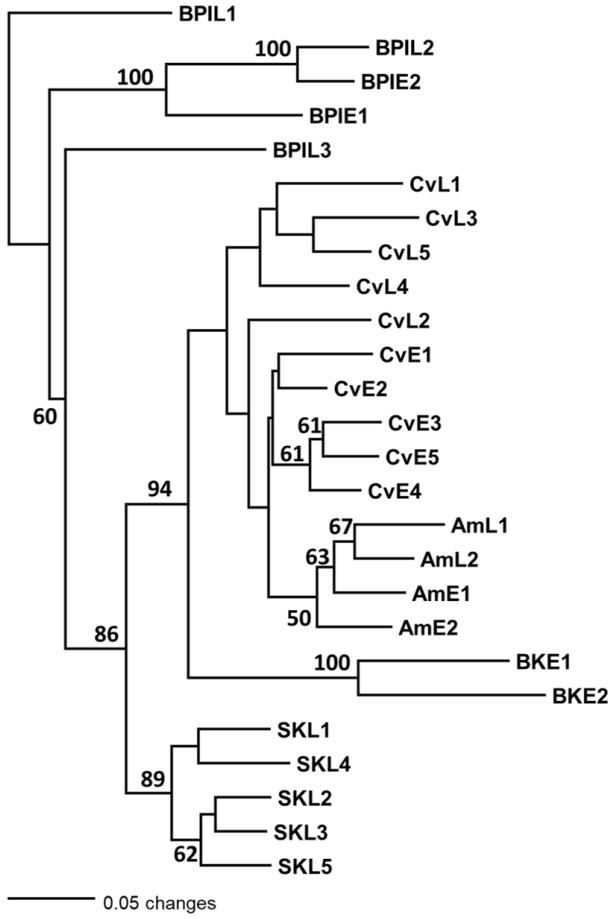

Fig. 2. Neighbor joining tree generated using 20 simple sequence repeat (SSR) markers. Shown are bootstrap support from 1000 replications. The scale bar represents the number of character differences.

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