African Journal of Biotechnology Vol. 8 (3), pp. 380-390, 4 February, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Genetic variation in *Coffea canephora* L. (Var. Robusta) accessions from the founder gene pool evaluated with ISSR and RAPD

P. Tshilenge[1], K. K. Nkongolo[2]*, M. Mehes[2] and A. Kalonji[1]

[1]University of Kinshasa, Faculty of Agronomy, P.O. 117, Kinshasa XI, Democratic Republic of Congo.
[2]Department of Biology, Laurentian University, Sudbury, Ontario, P3E 2C6, Canada.

Accepted 12 May, 2008

Discovered in Congo in 1898, *Coffea canefora* var. robusta accounts for 25 to 40% of the coffee grown in the world. Most genetic diversity of robusta coffee accessions conserved in *ex situ* collections has been estimated from morphological characteristics. There are limited studies on genetic variability and diversity in *C. robusta*. Inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) markers were used to assess the level of genetic variability among robusta coffee accessions from the founder gene pool in the Democratic Republic of Congo (DRC). The present study clearly established the high variability in the Congolese genepool. RAPD primers detected a higher level of polymorphic loci (95%) than ISSR markers (52%). Each accession could be genotyped using RAPD markers and both markers were efficient in revealing the genetic variability. Jaccard's similarity coefficients generated to determine the genetic distances among accessions, revealed that most of the accessions were genetically distant from each other. The accessions tested represent useful genetic materials for breeding for resistance to tracheomycose and other traits in DRC.

Key words: ISSR, RAPD, genetic variability, *Coffea canephora*, coffee, Congo.

INTRODUCTION

Coffees belong to the Family, *Rubiaceae*. The genus *Coffea* L. comprises over 100 taxa that have been identified so far. Commercial coffee production relies mainly on two species, *Coffea arabica* L. (70%) and *Coffea canephora* Pierre (30%). *Coffea robusta* which is widespread mostly in Africa is generally considered a variety of *C. canephora*. *C. arabica* is a natural allotetraploid (2n = 4X = 44), and is self-fertile (Coste, 1989). Other species are diploids (2n = 22) and generally self-incompatible. *C. canephora* is widely grown in Africa, Indonesia, and to some extent in Northern Brazil. This species accounts for approximately 25 to 40% of the coffee grown in the world. Discovered in 1898 in what is now the Democratic Republic of Congo (DRC), *C. robusta* is the most commonly grown variety of *C. canefora* accounting for up to 95% of *C. canephora* populations worldwide. Unlike *C. arabica,* plants *C. robusta* does not need to grow at high altitude, requires less care to grow because it is hardier, and it tends to be less susceptible to pests and rough handling (Coste, 1989). Its area of distribution is variable and corresponds to hot and humid climatic regions. It is found in low and middle altitudes areas in Africa (Ivory Coast, Democratic Republic of Congo, Cameroon, Uganda, Angola, Ghana, Togo, Madagascar, Tanzania, and Republic of Central Africa), in the far East (India, Indonesia, Philippines, etc), and in Oceania (New Caledonia, etc). Globally, the robusta coffee genepool is conserved in *ex-situ* collection plots in several countries with the Ivory Coast maintaining the largest collection of 700 wild genotypes. In view of the wide geographical distribution of *C. robusta,* characterization and evaluation of its genepool is necessary for effective crop improvement programmes and for better conservation and management of genetic resources (Prakash et al., 2005).

C. robusta is of paramount economic importance in the DRC. It represents almost 90% of all the plantations across the country and is the DRC's biggest cash crop.

*Corresponding author: knkongolo@laurentian.ca.

Most of the *R. coffee* plantations in the DRC derive from progenies of six elite local clones from the Yangambi Research Stations, and one introduced clone from Indonesia, that was previously selected for yield. No specific breeding programs were developed to improve the agronomic traits of genotypes, but selection of genotypes based on yields and morphological traits was carried on by the "Institut National d'Etudes et de Recherches Agronomiques" (INERA). Reoccurrence of some diseases such as tracheomycose (caused by *Gibberella xylariodes*) continues to be a serious threat to coffee production and emphasis is now being directed to disease resistance. For this reason, knowledge of genetic diversity is critical for coffee improvement in the DRC (Fraselle, 1950; and Tshilenge et al., 1998).

Assessment of genetic variability within and among arabica coffee populations using molecular markers such as RAPD, ISSR, ALFP, and SSR has been the subject of several studies (Aga et al., 2005; Agwanda et al., 1997; Anthony et al., 2002; Chaparro et al., 2004; Lashermes et al., 1993; and Masumbuko and Bryngelsson, 2006). Similar analysis in robusta coffee is sketchy, especially for genetic materials from Africa. The main objective of the present study is to evaluate the level of genetic variation among robusta coffee accessions from the DRC using RAPD and ISSR markers.

MATERIALS AND METHODS

Genetic materials

Seeds were collected from accessions randomly chosen and derived from progenies of clones that were intercrossed and grown on experimental plots at Kiyaka Research Station. These original clones represent the main genepool of all the Robusta coffee populations grown in the DRC. The accessions were: KR1/1, KR1/3, KR2/5, KR8/8, KR8/10, KR9/8, KR 10/1, KR 10/8, KR 11/8, KR 12/1, KR 12/4, KR 12/6, KR 14/1, KR 14/10, KR 15/3, KR 5/4, KR 15/6, KR 15/12, KR 16/2, KR 16/5, KR 16/7, KR 17/48, KR 17/55, KR 19/11, KR 19/12, KR 19/18, KR 19/31, KR 20/3, KR 20/50, KR 20/51, KR A/1, and KR B/4. Accessions with the same numerator are progenies of the same half siblings that shared the same maternal parent. The parental trees were initially selected more than 50 years ago based on seed size and yield. Thirty seeds from each accession were cleaned from debris and their seed coat removed prior to storage in liquid nitrogen and DNA extraction. Similarly, embryos were excised from thirty seeds per accession following seed imbibitions for three weeks. The embryos were then frozen in liquid nitrogen and stored at –20°C until DNA extraction.

DNA extraction

To determine the level of genetic variation among accessions, total cellular DNA from 1 g of bulk samples was extracted from whole seeds and excised embryos using the method described by Nkongolo et al. (2005), with some modifications. DNA from 20 individual embryos per accessions was extracted to assess the level of genetic diversity within each accession. The concentration of each bulk sample was determined using the DNA quantitation kit from Bio-Rad and the purity was determined using a Varian Cary 100 UV-VIS spectrophotometer.

Amplification of RAPD and ISSR markers

Ten RAPD primers and ten ISSR primers, synthesized by Invitrogen, were chosen for preliminary amplification of four DNA samples. DNA amplification was performed following the procedure described by Nkongolo et al. (2005). Each PCR reaction was performed in a 25 µl volume containing 5 ng of genomic plant DNA, 10 mM Tris-HCl, pH 8.3 [at 25°C], 50 mM KCl (Applied Biosystems, Foster City, CA), 3.5 mM $MgCl_2$, 200 µM of each dNTP (Applied Biosystems, Foster City, CA), 0.5 µM primer and 0.625 U of *Taq* DNA polymerase (Applied Biosystems, Foster City, Calif). For each primer, a negative control reaction with double distilled water was included. A drop of mineral oil was added to each reaction and the samples were amplified on a DNA thermal cycler (Perkin Elmer, Foster City, CA). The cycles performed were as follows: an initial denaturation at 95°C for 5 min followed by a 2 min incubation at 85°C at which point the polymerase was added; 42 cycles of 90 s at 95°C, 2 min at 55°C, and 60 s at 72°C were performed; a final extension at 72°C for 7 m and a subsequent incubation at 4°C followed. PCR products were loaded onto 1% agarose gels (invitrogen) in 0.5 X Tris-borate-EDTA (TBE) buffer containing ethidium bromide and run at 2.8 V/cm for 90 min. The agarose gels were documented using the Bio-Rad ChemiDoc XRS system and analyzed with the Discovery Series Quantity One 1 D Analysis Software.

ISSR and RAPD analyses

Six SSR and seven RAPD primers that gave consistent profiles across the populations were considered for the analysis (Table 1). Presence and absence of bands were scored as 1 or 0, respectively. Faint bands were not recorded for analysis. Data were analyzed using RAPDistance Program version 1.04 (Armstrong et al., 1994). Jaccard's similarity coefficients were generated to determine the genetic distances among populations. Dendrograms were constructed using the neighbour-joining analysis (Saitou and Nei, 1987). The degree of the relationship between the similarity matrices based on RAPD and ISSR data was calculated using Pearson's correlation coefficient and the significance of the correlation was determined by the Mantel test (Mantel, 1967).

The following parameters were generated using POPGENE 1.31 to describe in detail the level of genetic variation using combined data from amplifications with ISSR and RAPD primers: Nei's gene diversity (h), Shannon's information index (i), the observed number of alleles (Na) and the effective number of alleles (Ne) (Nei, 1973; Yeh et al., 1997).

RESULTS

DNA extraction and amplification

Genomic DNA was isolated from whole coffee seeds and dissected embryos. The quality test revealed that no DNA samples were degraded. DNA concentrations were much lower in samples from whole seeds compared to those from embryos. In fact, average concentrations of DNA from whole seeds and embryos were 76.2 and 500.7 ng/ul, respectively. The amplification of the two sets of samples with RAPD and ISSR primers generated no PCR products from DNA from whole seeds. Interestingly, the RAPD and ISSR primers generated strong and scoreable PCR products with DNA from embryos (Figures 1 and 2).

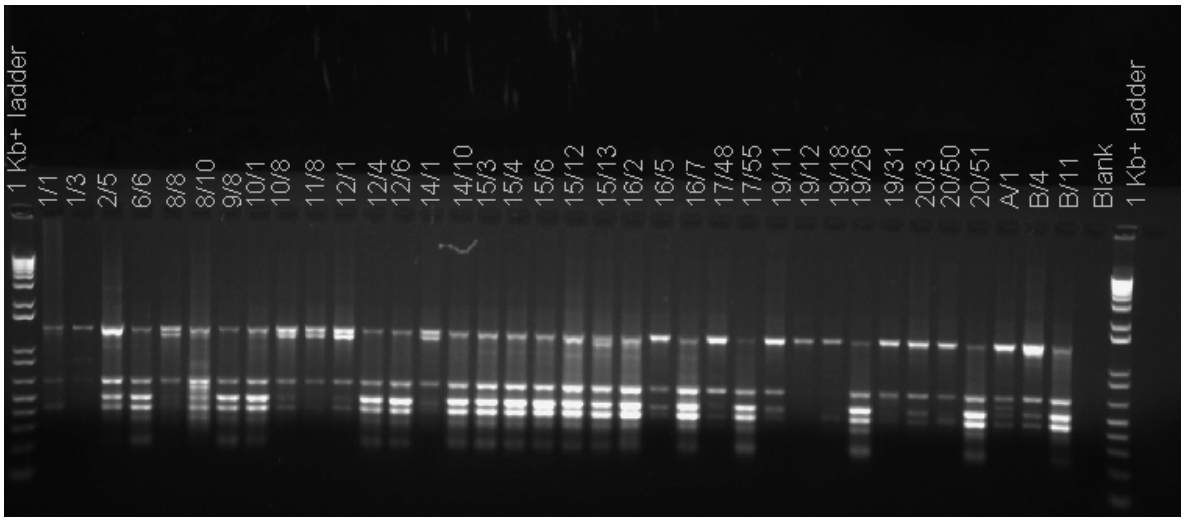

Figure 1. ISSR profiles of genomic DNA from different *coffea canephora (Var. robusta* accessions amplified using primer ISSR 17899 in a 2% agarose gel. Lanes 1 and 39 contain 1-Kb + DNA ladder. Lanes 2 to 37 contain amplified *coffea canephora (Var. robusta)* products from different accessions.

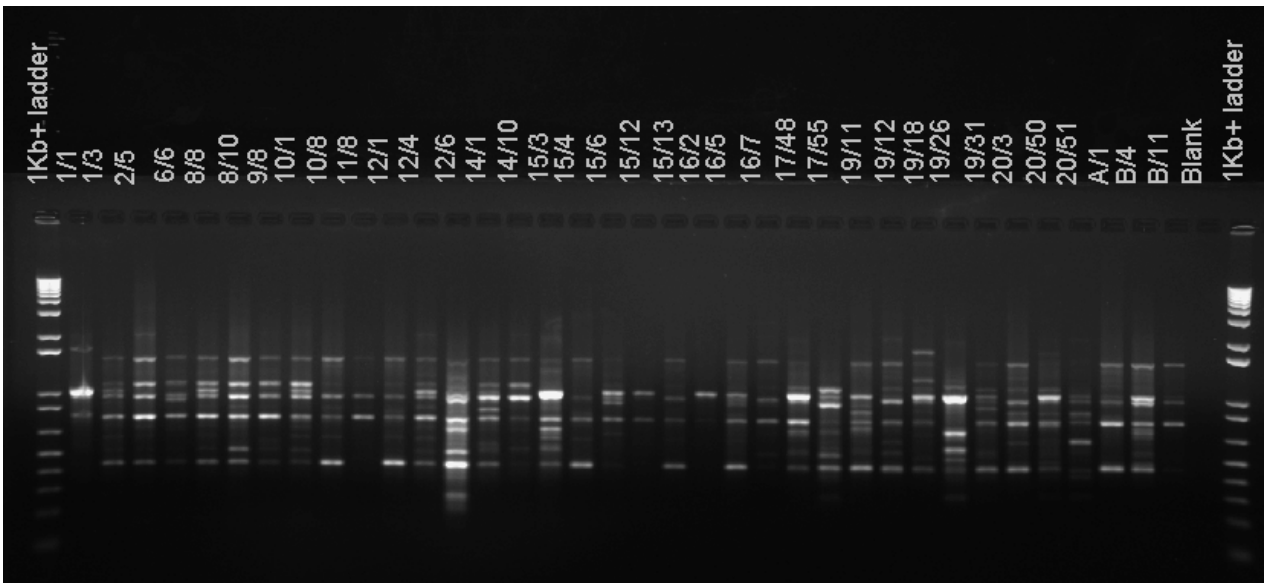

Figure 2. RAPD profiles of genomic DNA from different *coffea canephora (Var. robusta* accessions amplified using primer UBC 186 in a 2% agarose gel. Lanes 1 and 39 contain 1-Kb + DNA ladder. Lanes 2 to 37 contain amplified *coffea canephora (Var. robusta)* products from different accessions.

ISSR analysis

The primer sequence and the size of the amplified fragments are summarized in Table 1. Overall, 52% of the bands generated with the six ISSR primers tested were polymorphic. Primers UBC 825 and 17899A generated intense bands that were highly polymorphic. The genetic relatedness among accession was determined using the Jaccard's similarity coefficients. The scale used for the genetic distance runs from 0 (meaning no genetic difference) to 1 (different for all conditions-criteria). The genetic distance among the coffee accessions varied from 0 to 0.67 (Table 2). About 40% of the genetic distances were equal or greater than 0.40. Accessions KR19/12 and KR19/18 had identical amplification profiles and a genetic distance of 0. Likewise accessions KR10/1 and KR12/6 as well as accessions KR19/31 and KR20/50 revealed identical ISSR amplification profiles. Accessions KR15/12 and KR8/8 were the most distantly related. A dendrogram was constructed from the similarity coefficients using the RAPDistance program v.1.04 (Armstrong et al., 1994). Four main clus-

Table 1. Nucleotide sequences of the RAPD and ISSR primers used to amplify genomic DNA from *Coffea canephora* (Var. robusta) accessions.

RAPD and ISSR Primers	Primer sequences (5' – 3')	Size of the amplified fragments
RAPD		
UBC 186	GTGCGTCGCT	200-2000
Grasse 2	GTGGTCCGCA	150-1200
OPA 4	AATCGGGCTG	0
OPA11	CAATCGCCGT	300-2000
OPH 20	GGGAGACATC	0
Grasse 6	CGTCGCCCAT	300-1200
E 12	TTATCGCCCC	0
ISSR		
Echt 5	AGACAGACGC	250-1000
HB 13	GAGGAGGAGGC	250-800
HB 15	GTGGTGGTGGC	300-1000
UBC 825	ACACACACACACACACT	450-1100
SC ISSR 1	(AG)8RG	250-850
17899A	CACACACACACAAG	250-1400

Table 2. Distance matrix generated from ISSR and RAPD data used in neighbour-joining analysis of Coffea *canephora var. robusta* (RAPDistance version 1.04). ISSR data are in the top diagonal and RAPD data in the lower diagonal.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	**0**	0,23	0,15	0,42	0,39	0,29	0,47	0,15	0,46	0,23	0,33	0,47	0,27	0,53	0,29	0,36	0,36	0,56	0,53	0,09	0,47	0,31	0,21	0,15	0,36	0,36	0,08	0,17	0,08	0,27	0,23	0,21
2		**0**	0,21	0,57	0,33	0,44	0,50	0,21	0,50	0,29	0,38	0,50	0,31	0,56	0,44	0,50	0,50	0,59	0,56	0,31	0,59	0,47	0,38	0,33	0,42	0,42	0,15	0,23	0,15	0,31	0,29	0,27
3			**0**	0,50	0,37	0,38	0,44	0,14	0,43	0,21	0,31	0,44	0,25	0,50	0,38	0,44	0,44	0,44	0,41	0,23	0,44	0,40	0,31	0,27	0,46	0,46	0,08	0,15	0,08	0,25	0,21	0,07
4				**0**	0,56	0,50	0,57	0,38	0,45	0,46	0,63	0,57	0,47	0,64	0,60	0,46	0,46	0,67	0,53	0,50	0,46	0,64	0,53	0,50	0,33	0,33	0,46	0,42	0,46	0,56	0,57	0,43
5					**0**	0,28	0,33	0,28	0,50	0,33	0,22	0,33	0,17	0,39	0,28	0,33	0,33	0,42	0,32	0,44	0,42	0,39	0,22	0,28	0,61	0,61	0,33	0,39	0,33	0,17	0,42	0,32
6						**0**	0,33	0,38	0,63	0,44	0,20	0,33	0,25	0,40	0,14	0,21	0,21	0,44	0,41	0,36	0,21	0,29	0,07	0,14	0,57	0,57	0,33	0,40	0,33	0,13	0,44	0,41
7							**0**	0,44	0,38	0,40	0,14	0,00	0,31	0,08	0,21	0,15	0,15	0,15	0,14	0,43	0,29	0,36	0,27	0,33	0,54	0,54	0,40	0,36	0,40	0,20	0,40	0,47
8								**0**	0,31	0,08	0,31	0,44	0,13	0,50	0,38	0,44	0,44	0,53	0,41	0,23	0,53	0,40	0,31	0,27	0,46	0,46	0,08	0,15	0,08	0,25	0,21	0,07
9									**0**	0,25	0,47	0,38	0,40	0,33	0,53	0,50	0,50	0,38	0,36	0,42	0,60	0,46	0,56	0,53	0,55	0,55	0,38	0,33	0,38	0,50	0,38	0,36
10										**0**	0,27	0,40	0,20	0,47	0,33	0,50	0,50	0,50	0,38	0,17	0,59	0,36	0,38	0,33	0,54	0,54	0,15	0,23	0,15	0,31	0,15	0,14
11											**0**	0,14	0,19	0,21	0,07	0,27	0,27	0,27	0,25	0,29	0,38	0,21	0,13	0,20	0,60	0,60	0,27	0,33	0,27	0,07	0,27	0,35

Table 2. Contd.

12												**0**	0,31	0,08	0,21	0,15	0,15	0,15	0,14	0,43	0,29	0,36	0,27	0,33	0,54	0,54	0,40	0,36	0,40	0,20	0,40	0,47
13													**0**	0,38	0,25	0,31	0,31	0,41	0,29	0,33	0,41	0,27	0,19	0,13	0,53	0,53	0,20	0,27	0,20	0,13	0,31	0,19
14														**0**	0,29	0,23	0,23	0,08	0,21	0,50	0,36	0,31	0,33	0,40	0,62	0,62	0,47	0,43	0,47	0,27	0,47	0,53
15															**0**	0,21	0,21	0,33	0,31	0,23	0,33	0,15	0,07	0,14	0,57	0,57	0,33	0,40	0,33	0,13	0,33	0,41
16																**0**	0,00	0,29	0,27	0,43	0,15	0,36	0,14	0,21	0,42	0,42	0,40	0,36	0,40	0,20	0,50	0,47
17																	**0**	0,29	0,27	0,43	0,15	0,36	0,14	0,21	0,42	0,42	0,40	0,36	0,40	0,20	0,50	0,47
18																		**0**	0,14	0,53	0,29	0,36	0,38	0,44	0,64	0,64	0,50	0,47	0,50	0,31	0,50	0,47
19																			**0**	0,50	0,27	0,44	0,35	0,41	0,60	0,60	0,47	0,44	0,47	0,29	0,47	0,35
20																				**0**	0,53	0,25	0,29	0,23	0,45	0,45	0,17	0,25	0,17	0,33	0,17	0,29
21																					**0**	0,47	0,27	0,33	0,54	0,54	0,50	0,47	0,50	0,31	0,59	0,47
22																						**0**	0,21	0,15	0,62	0,62	0,36	0,43	0,36	0,27	0,36	0,44
23																							**0**	0,07	0,50	0,50	0,27	0,33	0,27	0,07	0,38	0,35
24																								**0**	0,46	0,46	0,21	0,29	0,21	0,13	0,33	0,31
25																									**0**	0,00	0,42	0,36	0,42	0,53	0,54	0,50
26																										**0**	0,42	0,36	0,42	0,53	0,54	0,50
27																											**0**	0,08	0,00	0,20	0,15	0,14
28																												**0**	0,08	0,27	0,23	0,21
29																													**0**	0,20	0,15	0,14
30																														**0**	0,31	0,29
31																															**0**	0,27
32																																**0**

Numbers 1 to 32 represent accessions KR1/1, KR1/3, KR2/5, KR8/8, KR8/10, KR9/8, KR 10/1, KR 10/8, KR 11/8, KR 12/1, KR 12/4, KR 12/6, KR 14/1, KR 14/10, KR 15/3, KR 5/4, KR 15/6, KR 15/12, KR 16/2, KR 16/5, KR 16/7, KR 17/48, KR 17/55, KR 19/11, KR 19/12, KR 19/18, KR 19/31, KR 20/3, KR 20/50, KR 20/51, KR A/1, and KR B/4, respectively.

ters were identified (Figure 3). Accession KR14/1 appeared to be an out group.

RAPD analysis

The level of polymorphism detected with RAPD primers was much higher (95%) compared to ISSR data. Primers UBC 186 and OPA 11 produced 27 and 13 RAPD bands, respectively that were all polymorphic. As with ISSR data, the genetic relatedness among accessions was determined using the Jaccard's similarity coefficients (Table 3). The data revealed that most of the accessions were genetically distant from each other with distance values ranging from 0.17 to 0.89 with more than 75% of genetic distance values ≥ 0.5. Progenies from accessions KR8/8 and KR8/10 were the most genetically closely related followed by progenies from accessions KR 15/4 and KR 12/1. Progenies from accessions related.

A dendrogram KR15/3 and KR1/1 were the most distantly was constructed from the similarity coefficients using the RAPDistance program v.1.04 (Armstrong et al., 1994). No distinctive main clusters were identified. Some accessions from the same family such as KR 8/8 and KR 8/10 clustered together and many from different families were grouped in the same cluster (Figure 4).

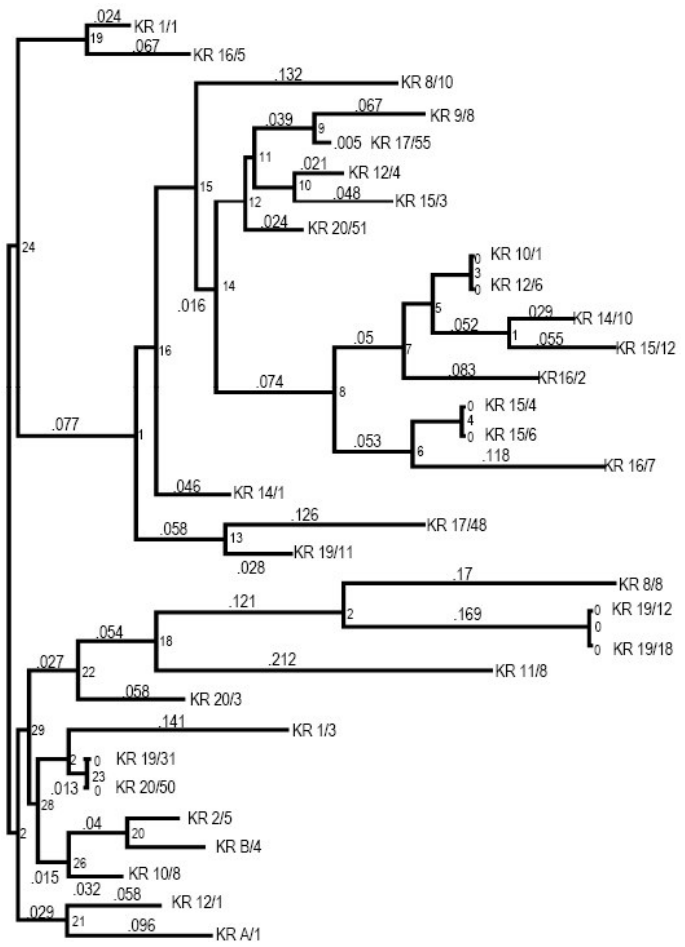

Figure 3. Dendrogram of the genetic relationships among *coffea canephora (Var. robusta* accessions based on the Jaccard similarity matrix using ISSR data. The values above the branches indicate the patristic distances based on the neighbour-joining (NJ) analysis.

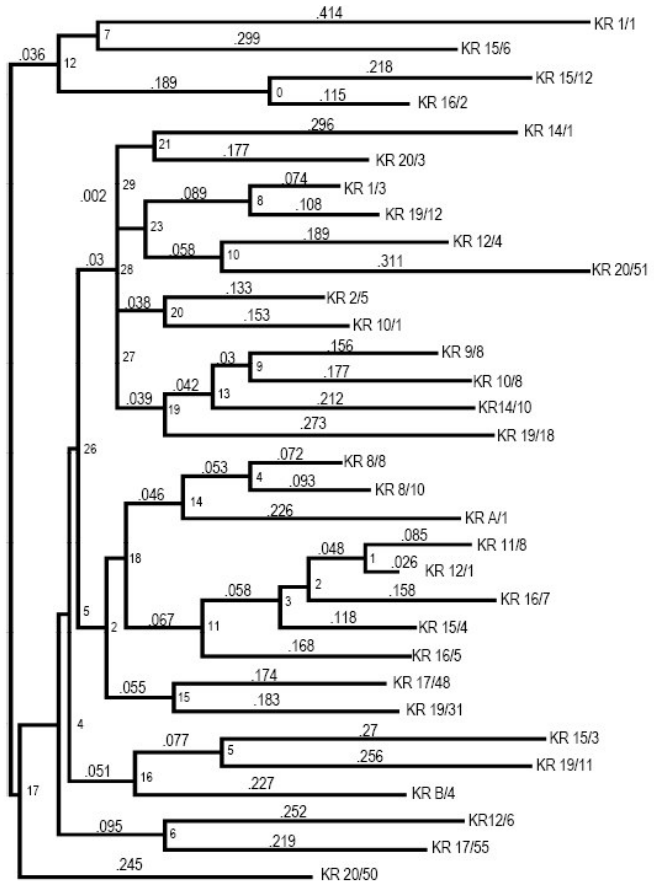

Figure 4. Dendrogram of the genetic relationships among *coffea canephora (Var. robusta* accessions based on the Jaccard similarity matrix using RAPD data. The values above the branches indicate the patristic distances based on the neighbour-joining (NJ) analysis

Interestingly, RAPD primers were able to generate unique fingerprints for each of the accessions analyzed.

ISSR and RAPD combination

Since ISSR and RAPD markers target different regions within a genome, the two data sets were combined. The genetic distance values were as high as those generated with the RAPD system (Table 4). They ranged from 0.24 to 0.79. Over 70% of the genetic distance values were ≥ 0.5. Progenies from accessions KR16/2 and KR15/12 as well as those from KR12/1 and KR 11/8 were the most closely related. Progenies from accessions KR12/6 and KR1/1, KR15/3 and KR11/8, along with progenies from KR15/12 and KR8/8 were the most genetically distant. The dendrogram constructed with the genetic distance values revealed two main clusters including progenies from different families (Figure 5). No pattern was identified.

Detailed analysis of combined ISSR and RAPD data was conducted using POPGENE software. The levels of genetic diversity within accessions were not significantly different for all the parameters studied. As a result, only the mean values are reported. Overall, the level of genetic diversity was high. The mean level of polymorphic loci was 91.5%. The mean Nei's gene diversity (h) value was 0.2076, and the Shannon's index (I) 0.449. The mean observed number of alleles (Na) and effective number of alleles (Ne) were 1.9167 and 1.3383, respectively.

Correlation between ISSRs and RAPDs

The genetic distances matrices obtained through ISSR and RAPD analyses were compared using the Mantel test. The values of the Mantel test showed no significant correlation (r = - 0.082) between individual pair wise genetic distance matrices. These data indicated no good fit of the data obtained by the two markers suggesting that the two systems should be used as complements to each other.

Table 3. Distance matrix generated from RAPD data used in neighbour-joining analysis of *Coffea canephora var. robusta*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	0	0,80	0,83	0,83	0,83	0,78	0,86	0,75	0,78	0,80	0,83	0,88	0,88	0,82	0,89	0,80	0,71	0,83	0,71	0,83	0,78	0,83	0,82	0,83	0,83	0,80	0,85	0,87	0,78	0,92	0,83	0,83
2		0	0,33	0,33	0,46	0,45	0,31	0,40	0,58	0,50	0,46	0,68	0,59	0,54	0,63	0,36	0,64	0,70	0,64	0,46	0,58	0,46	0,54	0,67	0,18	0,50	0,50	0,36	0,45	0,54	0,57	0,46
3			0	0,43	0,43	0,42	0,29	0,50	0,54	0,46	0,53	0,58	0,47	0,50	0,67	0,57	0,69	0,75	0,69	0,53	0,64	0,43	0,50	0,71	0,43	0,46	0,47	0,33	0,42	0,69	0,53	0,43
4				0	0,17	0,42	0,40	0,62	0,54	0,46	0,53	0,65	0,63	0,60	0,67	0,46	0,69	0,75	0,58	0,43	0,42	0,43	0,50	0,63	0,43	0,57	0,36	0,44	0,54	0,69	0,43	0,53
5					0	0,27	0,40	0,50	0,42	0,33	0,53	0,65	0,56	0,50	0,73	0,46	0,79	0,85	0,69	0,53	0,42	0,53	0,60	0,71	0,53	0,57	0,36	0,44	0,64	0,76	0,31	0,63
6						0	0,38	0,33	0,55	0,45	0,42	0,67	0,56	0,36	0,81	0,58	0,73	0,91	0,83	0,64	0,55	0,64	0,62	0,73	0,54	0,45	0,57	0,53	0,67	0,80	0,54	0,64
7							0	0,46	0,50	0,43	0,40	0,47	0,60	0,47	0,64	0,43	0,73	0,69	0,64	0,50	0,50	0,50	0,47	0,74	0,40	0,53	0,53	0,41	0,60	0,65	0,59	0,50
8								0	0,50	0,40	0,36	0,72	0,63	0,45	0,74	0,40	0,70	0,90	0,82	0,62	0,64	0,71	0,79	0,71	0,50	0,55	0,64	0,50	0,75	0,69	0,50	0,62
9									0	0,11	0,64	0,67	0,65	0,50	0,81	0,30	0,73	0,67	0,44	0,42	0,22	0,64	0,80	0,81	0,64	0,69	0,57	0,53	0,77	0,80	0,42	0,64
10										0	0,57	0,61	0,59	0,42	0,76	0,20	0,75	0,70	0,50	0,33	0,30	0,57	0,73	0,75	0,57	0,62	0,50	0,47	0,69	0,73	0,33	0,57
11											0	0,65	0,63	0,50	0,73	0,57	0,58	0,85	0,79	0,63	0,64	0,71	0,69	0,63	0,43	0,57	0,65	0,44	0,64	0,50	0,63	0,63
12												0	0,76	0,70	0,73	0,68	0,78	0,75	0,71	0,50	0,67	0,65	0,47	0,77	0,71	0,75	0,60	0,70	0,74	0,70	0,65	0,58
13													0	0,53	0,72	0,67	0,76	0,88	0,83	0,70	0,72	0,63	0,75	0,70	0,56	0,67	0,65	0,47	0,56	0,75	0,56	0,63
14														0	0,77	0,54	0,77	0,83	0,77	0,60	0,62	0,60	0,75	0,69	0,38	0,54	0,63	0,59	0,71	0,75	0,60	0,60
15															0	0,70	0,85	0,83	0,79	0,67	0,81	0,53	0,71	0,53	0,60	0,76	0,62	0,65	0,75	0,77	0,67	0,60
16																0	0,75	0,70	0,50	0,33	0,30	0,57	0,73	0,75	0,46	0,71	0,60	0,47	0,69	0,64	0,46	0,57
17																	0	0,75	0,67	0,69	0,73	0,79	0,77	0,69	0,69	0,75	0,80	0,67	0,60	0,67	0,79	0,58
18																		0	0,33	0,64	0,67	0,75	0,73	0,85	0,75	0,82	0,77	0,80	0,67	0,73	0,85	0,75
19																			0	0,45	0,44	0,58	0,67	0,79	0,69	0,75	0,62	0,67	0,60	0,77	0,69	0,69
20																				0	0,42	0,43	0,60	0,71	0,53	0,67	0,36	0,53	0,64	0,69	0,53	0,53
21																					0	0,64	0,71	0,81	0,64	0,79	0,67	0,63	0,77	0,80	0,54	0,64
22																						0	0,50	0,63	0,43	0,57	0,36	0,53	0,54	0,76	0,63	0,43
23																							0	0,76	0,60	0,64	0,53	0,67	0,50	0,67	0,76	0,60
24																								0	0,63	0,67	0,65	0,61	0,64	0,76	0,71	0,53
25																									0	0,57	0,56	0,44	0,54	0,50	0,63	0,43
26																										0	0,50	0,56	0,58	0,81	0,67	0,67
27																											0	0,47	0,57	0,78	0,47	0,65
28																												0	0,43	0,59	0,53	0,61
29																													0	0,62	0,73	0,64
30																														0	0,33	0,57
31																															0	0,63
32																																0

Numbers 1 to 32 represent accessions KR1/1, KR1/3, KR2/5, KR8/8, KR8/10, KR9/8, KR 10/1, KR 10/8, KR 11/8, KR 12/1, KR 12/4, KR 12/6, KR 14/1, KR 14/10, KR 15/3, KR 5/4, KR 15/6, KR 15/12, KR 16/2, KR 16/5, KR 16/7, KR 17/48, KR 17/55, KR 19/11, KR 19/12, KR 19/18, KR 19/31, KR 20/3, KR 20/50, KR 20/51, KR A/1, and KR B/4, respectively.

Table 4. Distance matrix generated from combined ISSR and RAPD data used in neighbour-joining analysis of C*offea canephora var. robusta.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	0	0,58	0,57	0,75	0,65	0,58	0,76	0,47	0,72	0,58	0,65	0,79	0,67	0,77	0,72	0,70	0,59	0,74	0,70	0,58	0,70	0,70	0,59	0,57	0,74	0,71	0,57	0,65	0,47	0,64	0,62	0,59
2		0	0,32	0,55	0,44	0,52	0,48	0,35	0,64	0,45	0,48	0,68	0,52	0,64	0,63	0,57	0,65	0,71	0,68	0,45	0,68	0,52	0,54	0,58	0,37	0,55	0,39	0,35	0,35	0,46	0,50	0,42
3			0	0,54	0,45	0,46	0,42	0,36	0,57	0,39	0,48	0,58	0,41	0,58	0,62	0,62	0,64	0,64	0,62	0,46	0,62	0,46	0,48	0,57	0,52	0,55	0,33	0,29	0,29	0,52	0,44	0,29
4				0	0,46	0,55	0,56	0,59	0,60	0,55	0,67	0,70	0,63	0,72	0,73	0,52	0,68	0,79	0,65	0,55	0,52	0,65	0,56	0,65	0,47	0,58	0,48	0,50	0,59	0,70	0,58	0,56
5					0	0,32	0,41	0,42	0,54	0,38	0,41	0,56	0,41	0,50	0,59	0,48	0,61	0,66	0,54	0,55	0,48	0,50	0,41	0,55	0,66	0,68	0,39	0,47	0,54	0,50	0,43	0,52
6						0	0,42	0,43	0,70	0,52	0,35	0,59	0,46	0,45	0,63	0,43	0,52	0,71	0,68	0,58	0,43	0,58	0,42	0,52	0,65	0,62	0,52	0,54	0,57	0,52	0,56	0,59
7							0	0,52	0,52	0,48	0,32	0,33	0,53	0,35	0,55	0,39	0,54	0,48	0,46	0,54	0,46	0,54	0,44	0,63	0,54	0,63	0,54	0,44	0,58	0,48	0,57	0,55
8								0	0,47	0,26	0,39	0,67	0,44	0,57	0,66	0,55	0,64	0,75	0,67	0,50	0,67	0,67	0,58	0,56	0,57	0,60	0,43	0,39	0,48	0,50	0,41	0,39
9									0	0,24	0,64	0,63	0,61	0,50	0,79	0,55	0,71	0,58	0,47	0,50	0,55	0,68	0,74	0,78	0,71	0,75	0,57	0,52	0,68	0,73	0,48	0,58
10										0	0,48	0,59	0,46	0,52	0,67	0,50	0,71	0,65	0,50	0,30	0,57	0,58	0,59	0,63	0,65	0,68	0,39	0,42	0,50	0,58	0,29	0,42
11											0	0,50	0,48	0,42	0,50	0,52	0,48	0,60	0,58	0,54	0,58	0,59	0,50	0,48	0,60	0,68	0,54	0,44	0,52	0,29	0,52	0,55
12												0	0,65	0,54	0,62	0,57	0,59	0,48	0,52	0,54	0,57	0,63	0,45	0,67	0,73	0,76	0,58	0,64	0,67	0,58	0,61	0,59
13													0	0,52	0,61	0,60	0,62	0,71	0,65	0,61	0,65	0,56	0,53	0,52	0,62	0,69	0,52	0,43	0,44	0,52	0,50	0,48
14														0	0,63	0,50	0,59	0,52	0,57	0,64	0,57	0,58	0,64	0,63	0,59	0,68	0,63	0,59	0,68	0,58	0,62	0,64
15															0	0,61	0,68	0,63	0,66	0,58	0,70	0,48	0,55	0,43	0,63	0,78	0,58	0,63	0,66	0,53	0,61	0,59
16																0	0,50	0,57	0,48	0,50	0,22	0,62	0,58	0,62	0,57	0,73	0,62	0,52	0,67	0,50	0,60	0,63
17																	0	0,53	0,50	0,65	0,50	0,69	0,54	0,52	0,67	0,70	0,69	0,60	0,57	0,45	0,73	0,60
18																		0	0,24	0,59	0,50	0,52	0,60	0,69	0,78	0,82	0,64	0,70	0,64	0,58	0,73	0,65
19																			0	0,57	0,40	0,56	0,52	0,67	0,75	0,78	0,62	0,63	0,61	0,56	0,65	0,58
20																				0	0,57	0,46	0,54	0,58	0,59	0,68	0,32	0,48	0,50	0,58	0,43	0,48
21																					0	0,62	0,58	0,67	0,70	0,78	0,67	0,63	0,72	0,62	0,65	0,63
22																						0	0,48	0,52	0,64	0,72	0,46	0,59	0,56	0,62	0,61	0,48
23																							0	0,54	0,65	0,68	0,48	0,60	0,46	0,48	0,67	0,50
24																								0	0,64	0,67	0,52	0,54	0,50	0,52	0,61	0,48
25																									0	0,47	0,58	0,48	0,57	0,58	0,68	0,54
26																										0	0,55	0,57	0,60	0,77	0,71	0,68
27																											0	0,36	0,36	0,57	0,38	0,48
28																												0	0,32	0,48	0,46	0,50
29																													0	0,43	0,54	0,46
30																														0	0,61	0,54
31																															0	0,52
32																																0

Numbers 1 to 32 represent accessions KR1/1, KR1/3, KR2/5, KR8/8, KR8/10, KR9/8, KR 10/1, KR 10/8, KR 11/8, KR 12/1, KR 12/4, KR 12/6, KR 14/1, KR 14/10, KR 15/3, KR 5/4, KR 15/6, KR 15/12, KR 16/2, KR 16/5, KR 16/7, KR 17/48, KR 17/55, KR 19/11, KR 19/12, KR 19/18, KR 19/31, KR 20/3, KR 20/50, KR 20/51, KR A/1, and KR B/4, respectively.

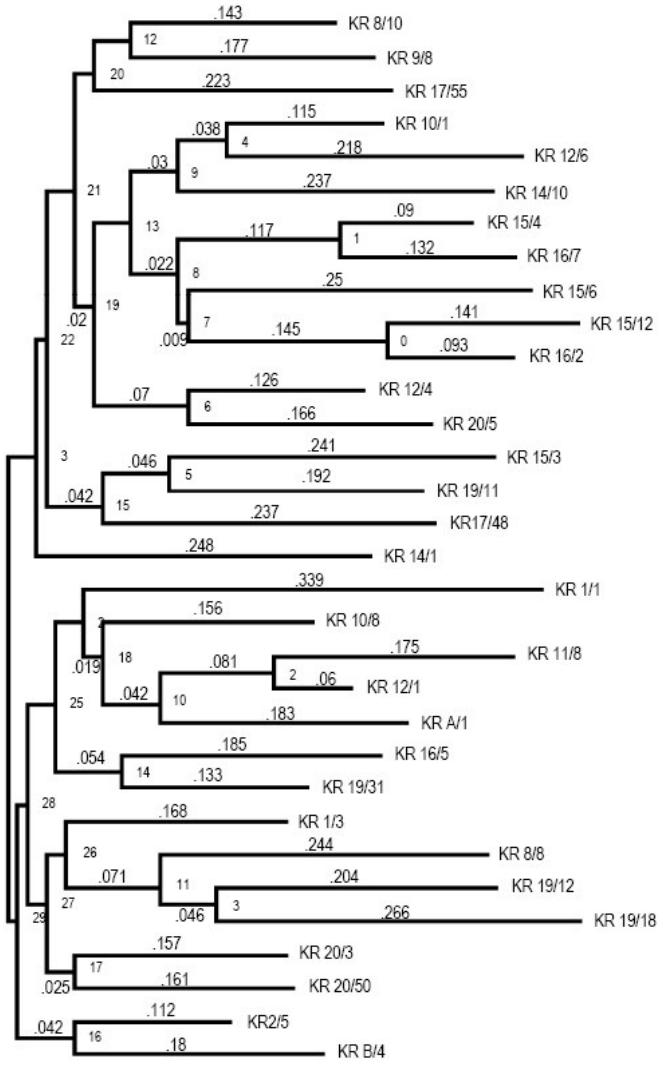

Figure 5. Dendrogram of the genetic relationships among *coffea canephora (Var. robusta* accessions based on the Jaccard similarity matrix using combined ISSR and RAPD data. The values above the branches indicate the patristic distances based on the neighbour-joining (NJ) analysis.

DISCUSSION

Thousands of coffee accessions are conserved in *ex-situ* collections at the seed banks or experimental stations worldwide but the majority are inadequately described for an efficient exploitation in plant breeding. Most of the genetic diversity was estimated from agronomic and morphological characteristics. Morphological and agronomic characters are influenced by the environment and the assessment of diversity based on these parameters are hampered by confusions due to the wide variations for a particular phenotypic trait in a given population specially for a open–pollinated species such as *C. canephora (*Var. *robusta)*. Molecular markers are not influenced by environment; they largely have overcome these limitations (Kuleung et al., 2006). Despite this, studies on variability and diversity in robusta coffee have been relatively few. In the present study we have assessed the genetic variation within the robusta coffee gene pool available in the DRC using ISSR and RAPD markers.

RAPD primers detected a higher level of polymorphic loci (95%) than ISSR markers (52%). Each accession could be genotyped using RAPD markers and both markers were efficient in revealing the genetic variability with the *C. robusta* gene pool in the DRC. The large amount of polymorphic loci detected might be attributed to the allogamous nature of the species, but it also suggested that the original clones that constituted the genetic basis of the populations studied were genetically distant. In fact the progenies tested are the products of a few of generations of inter crossings involving 15 half siblings. The results showed no trend to genetic drift in these populations.

Interestingly, half of the accessions from this collection that was tested for resistance to tracheomycose showed resistance to this disease at different levels (Tshilenge, 2006 unpublished data). This indicates that these accessions can be useful in breeding programs not only for yield but also for resistance to this disease.

Prakash et al. (2005) also analyzed genetic diversity in the Indian Robusta coffee genepool in comparison to a representative of a core collection from the centre of genetic diversity in Africa. They found a high amount of genetic variation in the materials analyzed using SSR and AFLP markers. This is consistent with the high level of variability detected with ISSR markers in DRC accessions. In fact, it has been believed that most of the cultivated robusta coffee types in the world including the Indian genepool were derived from trees grown in DRC in 1895 (Charrier and Berthaud, 1985; Prakash et al., 2005). The accessions analyzed in the present study are progenies of the elite genotypes that were selected at the National Research Institute now called Institut National d'Etudes et de Recherches Agronomiques (INERA) between 1930 and 1960 (Montagnon et al., 1998; Tshilenge et al., 1998)

Aga et al. (2005) reported lower polymorphic loci (25%) in *C. arabica* from Ethiopia using ISSR primers compared to 52% observed in the present study. The level of genetic variability in *C. arabica* from Ethiopia tested by Chaparro et al. (2004) also using RAPD primers was much lower (34.2%) than the 95% observed in *C. robusta* from the DRC. This low level of genetic variability in *C. arabica* reported in many studies could be ascribed in part to its autogamic nature (Chaparro et al., 2004).

Previous studies have shown different level of polymerphisms in different species when RAPD and ISSR were compared. For example, Fang and Roose (1997) reported high level of interspecific variation with RAPD markers than with ISSR in *Citrus spp*. On the other hand, several authors detected high level of polymorphism with the ISSR system than with RAPD in several other plants (Nkongolo et al., 2005; Nagaoka and Ogihara, 1997; Raina et al., 2001; Rus-Kortekaas et al., 1994; Qian et

al., 2001).

In the present study, RAPD markers detected higher level of polymorphism among robusta coffee accessions than did ISSR markers. RAPD analyses showed also greater genetic distances among accessions than did the ISSR data. The discrepancy between the variations revealed by RAPD and ISSR result from different targeted genomic regions. RAPD markers reveal polymorphism in coding and noncoding regions, as well as, repeated or single-copy sequences, covering the entire genome (Williams et al., 1990). Microsatellite loci are dispersed throughout the genome and are hypervariable because of DNA slippage (Echt et al., 1996). Most often ISSR primers detect more polymorphism than RAPD primers because of the high level of variability in microsatellite loci - areas which undergo a different evolutionary process due to selection forces (Qian et al., 2001).

Although in the present study, the RAPD amplifications were reproduced several times under the same conditions, many studies have reported low reproducibility of RAPD profiles compared to ISSR results. This could be the result of a number of factors. First, RAPD primers are composed of 10 arbitrary oligonucleotides. These decamers are used under relaxed amplification conditions which result in multiple priming sites with varying degrees of similarity. Second, it is more difficult to obtain clear and distinct bands between and within species because there are many fragments of similar mobility that originate from non-homologous regions (Sanchez de la Hoz et al., 1996).

In conclusion, RAPD markers appeared to be of higher value than ISSR markers for characterization, analysis and utilization of robusta coffee genetic materials. The two systems are PCR-based and easy to apply to a large screening of breeding materials. For an efficient assessment of the genetic variability they should be used conjointly to target a larger portion of the genome. In the present study, the level of polymorphic loci was very high although the accessions were derived from half siblings originating from limited number of clones. Based on the genetic variability, the resistance to tracheomycose and the seed size, these accessions have great potential in the breeding of robusta coffee for disease resistance and high yield. The accessions can also be distributed to coffee growers for further regeneration of plantations where the genetic superiority of the progenies from this pool has been affected by gene flow from other local accessions.

ACKNOWLEDGEMENTS

We express our appreciation to Paul Michael for technical assistance and to Dr. Victor Clulow for reviewing the manuscript prior to submission. This study was supported by the Canadian International Development Agency through University Partnerships in Cooperation and Development Program involving University of Kinshasa, Laurentian University and Caritas Congo.

REFERENCES

Aga E, Bekele E, Bryngelsson T (2005). Inter-simple sequence repeat (ISSR) variation in forest coffee trees (*Coffea arabica* L.) populations from Ethiopia. Genetica. 124: 213-221.

Agwanda CA, Lashermes P, Trouslot P, Combes M, Charrier A (1997). Identification of RAPD markers for resistance to coffee berry diseases, *Colletotrichum kahawae*, in Arabica coffee. Euphytica 97: 241-248.

Anthony F, Combes MC, Astorga C, Bertrand B, Graziosi G, Lashermes P (2002). The origin of cultivated *Coffea arabica* L. varieties revealed by AFL and SSR markers. Theor. Appl. Genet. 104: 894-900.

Armstrong JS, Gibbs AJ, Peakall R, Weiller G (1994). The RAPDistance Package. 2005, may/20, 30.

Coste R (1989). Caféiers et Cafés. Editions Maisonneuve GP & Larose et Agence de Cooperation Culturelle et Technique, Paris, p. 373.

Chaparro AP, Cristancho MA, Cortina HA, Gaitan AL (2004). Genetic variability of *Coffea arabica* L. accessions from Ethiopia evaluated with RAPDs. Genet. Res. Crop Evol. 51: 291-297.

Charrier A, Berthaud J (1985). Botanical classification of Coffee. In: Clifford M.N. and Wilson K.C. (eds.), Coffee: Botany, Biochemistry and Production of Beans and Beverage, Croom Helm., London, pp. 13-47.

Echt CS, MayMarquardt P, Hseih M, Zahorchak R (1996). Characterization of microsatellite markers in eastern white pine. Genome 39: 1102-1108.

Fang DQ, Roose ML (1997). Identification of closely related citrus cultivars with inter-simple sequence repeat markers. Theor. Appl. Genet. 95: 408-417.

Fraselle J (1950). Obervations preliminaries sur une tracheomycose de *Coffea robusta*. Bull. Agricole du Congo Belge, 41: 361-372.

Kuleung C, Baenziger PS, Kachman SD, Dweikat I (2006). Evaluating the Genetic diversity of triticale with wheat and rye SSR markers. Crop Sci. 46: 1692-1700.

Lashermes P, Cros J, Marmey P, Charrier A (1993). Use of random amplified DNA markers to analyse genetic variability and relationships of *Coffea* species. Genet. Res. Crop Evol. 40: 91-99.

Masumbuko LI, Bryngelsson T (2006). Inter simple sequence repeat (ISSR) analysis of diploid coffee species and cultivated *Coffea Arabica* L. from Tanzania. Genetic Resources and Crop Evolution. Genet. Res. Crop Evol. 53: 357-366.

Mantel M (1967). The detection of disease clustering and a generalized regression approach. Cancer Res. 27: 209-220.

Montagnon C, Leroy T, Eskes EB (1998). Amelioration varietale de Coffea canephora. 2. Les programmes de selection et leurs resultats. Plantation, Recherche, Developpement 5: 89-98.

Nagaoka T, Ogihara YE (1997). Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor. Appl. Genet. 94: 597-602.

Nei M (1973). Analysis of gene diversity in subdivided populations. Proceedings Natl. Acad. Sci. USA 70: 3321-3323.

Nkongolo KK, Michael P, Demers T (2005). Application of ISSR, RAPD, and cytological markers to the certification of *Picea mariana*, *P. glauca*, and *P. engelmannii* trees, and their putative hybrids. Genome 48: 302-311.

Prakash NS, Combes M, Dussert S (2005). Analysis of genetic diversity in Indian robusta coffee genepool (*Coffea canephora*) in comparison with a representative core collection using SSRs and AFLPs. Genet. Res. Crop Evol. 52: 333-343.

Qian W, Ge S, Hong DY (2001). Genetic variation within and among populations of a wild rice *Oryza granulata* from China detected by RAPD and ISSR markers. Theor. Appl. Genet.102: 440-449.

Raina SN, Rani V, Kojima T, Ogihara Y (2001). RAPD and ISSR fingerprints as useful genetic markers for analyis of genetic diversity, varietal identification, and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species. Genome 44: 763-772.

Rus-Kortekaas W, Smulders MJM, Arens P, Vosman B (1994). Direct comparison of levels of genetic variation in tomato detected by a GACA-containing microsatellite probe and by random amplified polymorphic DNA. Genome: 375-381.

Sanchez de la Hoz, MPS, Davila JA, Loarce Y, Ferrer E (1996). Simple

sequence repeat primers used in polymerase chain reaction amplifications to study genetic diversity in barley. Genome 39: 112-117.
Saitou N, Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.
Tshilenge-Djim P, Kalonji-Mbuyi A, Onyembe PML, Mukuna K, Dibwe M, et Oripale M (1998). Caractéristique et évolution spatio-temporelle de la trachéomycose fusarienne du caféier robusta en République Démocratique du Congo (RDC). Rev. Cong. Sci. 14: 132-140.
Tshilenge-Djim P, Munaut F, Kalonji-Mbuyi A, Maraite H (2004). Caractérisation des *Fusarium spp.* associées au dépérissement du caféier Robusta en République Démocratique du Congo. Parasitica 60(3-4): 67-82.
Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.
Yeh F, Yang R, Boyle T (1997). Popgene, version 1.32 edition, Software Microsoft Window-Based Freeware for Population Genetic Analysis. University of Alberta, Edmoton, Canada.