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RESEARCH ARTICLE

INTERNAL TRANSCRIBED SPACER (ITS) SEQUENCE ANALYSIS OF NUCLEAR RIBOSOMAL DNA (nrDNA) IN *AVERRHOA* L

***Soumya, S. L. and Bindu R. Nair**

Department of Botany, University of Kerala Kariavattom, Thiruvananthapuram, Kerala, India

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ABSTRACT

The present study focuses on the insights of intraspecific and interspecific variability to address the questions related to diversity among the collections of *Averrhoa* (*Averrhoa bilimbi* and *A. carambola*). Internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) from fifteen collections, belonging to six districts of Kerala state were analyzed. Nucleotide sequences obtained were deposited in the database and showed high percentage of similarity with the already deposited sequences in the database. Multiple sequence alignments entailed 561 conserved sites, 54 variable characters and 33 parsimony informative sites out of 615 characters aligned. Based on Maximum composite likelihood analysis, the estimated transition/transversion bias (R) was 0.53. The estimated mean nucleotide diversity of ITS1-5.8S-ITS2 within subpopulations was 0.005, mean diversity in entire population was 0.035 and mean inter population diversity was 0.030. Phylogenetic and molecular evolutionary analyses revealed that the *Averrhoa* collections used in this study could be classified distinctly into two groups, those belonging to *A. bilimbi* and the rest to *A. carambola*. A higher sequence similarity could be noticed among the sour and sweet *A. carambola* collections, while three *A. bilimbi* collections, could be discriminated from the rest.

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INTRODUCTION

Internal Transcribed Spacer (ITS) sequences are located in eukaryotic rDNA genes between the 18S and 5.8S rDNA coding regions (ITS1) and between the 5.8S and 23S rDNA coding regions (ITS2). Studies of restriction site variation in the ribosomal DNA (rDNA) in populations of plants and animals have shown that while coding regions are conserved, the spacer regions are variable (Gerbi, 1985). These spacer sequences have a high evolution rate and are present in all known nuclear rDNA genes of eukaryotes (Jorgensen *et al*. 1987; White *et al*. 1990). Several studies successfully revealed the usefulness of ITS sequences in studying phylogenetic and genomic relationships of plants at lower taxonomic levels (Feliner *et al*., 2004; Volkov *et al*., 1999). White *et al*. (1990) described a set of primers that were useful for amplifying ITS sequences from most plant and fungal phyla. This obviated the necessity for primer design or prior sequence knowledge, meaning that ITS sequence data could be more readily obtained than perhaps any other nuclear marker. One of the more remarkable properties of rDNA genes is that the individual copies may appear to evolve more or less in unison. That is, instead of each gene copy acquiring unique sequence variation due to the evolutionary accumulation of mutations, all repeat copies within an array (or genome) may jointly share the same set of mutations. This uniformity arises from one or more processes of inter-genic sequence homogenization that collectively are referred as concerted evolution (Elder and Turner 1995). The ITS polymorphism might occur at the genus, species or individual levels, making it a useful source of molecular character for phylogenetic studies in many angiosperms (Nwakanma *et al*., 2003). ITS sequences are considered most useful for phylogenetic analysis among related species and/or among populations within a species (Doyle and Beachy, 1985). This makes the ITS region an interesting subject for evolutionary/phylogenetic investigations (Nwakanma *et al*., 2003; Gulbitti-Onarici *et al*., 2009) as well as biogeographic investigations (Dubouzet and Shinoda 1999; Sharma *et al*., 2002). The genus *Averrhoa* is represented by two species, *A. bilimbi* L. (Bilimbi) and *A. carambola* L. (Carambola). There is no knowledge on interspecific and intraspecific polymorphism of ITS region in the genus *Averrhoa*. Hence, the objective of the present study is to determine the genetic relationship as well as intra-individual polymorphism of ITS1–5.8S–ITS2 region between and among nine collections of *A. bilimbi* and six collections of *A. carambola* (sour and sweet) collected from different regions of Kerala state and also to evaluate the utility of ITS markers for geneticstudies. Since, knowledge of germplasm diversity is vital for plant conservation and improvement, it is imperative to determine the genetic diversity in *Averrhoa* germplasm.

****Corresponding author: Soumya S.L.,***
Department of Botany, University of Kerala Kariavattom, Thiruvananthapuram, Kerala, India.

Table 1. Details of collections of *Averrhoa* from various localities

S. No.	Sample	Id. No.	District	Locality	Latitude	Longitude
1				Trikaripur	12° 8' 30"N	75° 10' 19"E
2		Ab1	Kasaragod	Koliyoor	12° 44' 24"N	74° 57' 10"E
3				Kudlu	10° 32' 44"N	76° 17' 8"E
4				Thamarassery	11° 24' 54"N	75° 56' 25"E
5		Ab2	Kozhikkode	Ramanattukara	12° 44' 24"N	74° 57' 10"E
6				Beypore	11° 10' 44" N	75° 48' 14" E
7				Irumbuzhi	11° 4' 42"N	76° 6' 05"E
8		Ab3		Thenhipalam	11° 8' 1"N	75° 53' 38"E
9			Malappuram	Chaliyar	11° 0' 5"N	76° 0' 17"E
10				Nilambur	11° 16' 17"N	76° 13' 26"E
11				Thevara	9° 56' 24"N	76° 18' 5"E
12		Ab4		Ramamangalam	9° 56' 7"N	76° 29' 17"E
13			Ernakulam	Thrikkakkara	10° 1' 57"N	76° 19' 54"E
14				Odakkali	10° 5' 42"N	76° 33' 0.9"E
15				Guruvayur	10° 35' 41"	76° 2' 18"E
16	*A.bilimbi*			Vellanikkara	10° 32' 42"N	76° 16' 26"E
17		Ab5	Thrissur	Chavakkad	10° 34' 59"N	76° 1' 7"E
18				Kaipamangalom	10° 32' 44"N	76° 17' 8"E
19				Ollur	10° 28' 51"N	76° 14' 33"E
20				Ponkunnam	9° 33' 53"N	76° 45' 20"E
21		Ab6	Kottayam	Vazhoor	10° 32' 44"N	76° 17' 8"E
22				Ettumanoor	9° 40' 7" N	76° 33' 30"E
23				Maniyar	9° 19' 48"N	76° 52' 29"E
24		Ab7	Pathanamthitta	Elanthoor	10° 32' 44"N	76° 17' 8"E
25				Konni	9° 13' 28"N	76° 50' 51"E
26				Puthoor	9° 2' 32"N	76° 42' 48"E
27				Anchalummoodu	10° 32' 44"N	76° 17' 8"E
28		Ab8	Kollam	Paravoor	10° 8' 25"N	76° 13' 49"E
29				Sasthamkotta	9° 2' 36"N	76° 37' 31"E
30				Kalyapuram	10° 32' 44"N	76° 17' 8"E
31				Kariavattom	8° 34' 8"N	76° 53' 11"E
32				Poojappura	8° 31' 41"N	76° 55' 44"E
33				Thonnakkal	8° 38' 15"N	76° 50' 46"E
34				Kanjiramkulam	8° 21' 35"N	77° 3' 12"E
35		Ab9	Thiruvananthapuram	Chirayinkeezhu	8° 39' 53"N	76° 47' 8"E
36				Konchiravila	8° 31' 41"N	76° 55' 44"E
37				Attingal	8° 40' 48"N	76° 49' 48"E
38				Vellayani	8° 26' 3"N	76° 59' 30"E
39				Kilimanoor	8° 46' 8"N	76° 53' 0.72"E
40				Varkala	8° 46' 16"N	76° 43'18"E
41				Koliyoor	12° 44' 24"N	74° 57' 10"E
42		Ac1	Kasaragod	Kudlu	10° 32' 44"N	76° 17' 8"E
43				Kumbala	12° 23' 35"N	75° 8' 2"E
44				Thenhipalam	11° 8' 1"N	75° 53' 38"E
45		Ac2	Malappuram	Nilambur	11° 16' 17"N	76° 13' 26"E
46	*A.carambola*			Tavanur	10° 51' 8"N	75° 58' 57"E
47	(Sour)			Koodal	9° 8' 22"N	76° 51' 6"E
48		Ac3	Pathanamthitta	Kaviyoor	9° 24' 7"N	76° 37' 36"E
49				Konni	9° 13' 28" N	76° 50' 51"E
50				Chavakkad	10° 34' 59"N	76° 1' 7"E
51		Ac4		Vellanikkara	10° 32' 42"N	76° 16' 26"E
52			Thrissur	Guruvayoor	10° 35' 41"N	76° 2' 18"E
53				Peramangalam	10° 28' 51"N	76° 14' 33"E
54				Thonnakkal	8° 38' 15"N	76° 50' 46"E
55		Ac5	Thiruvananthapuram	Nedumangad	8° 36' 8"N	77° 0' 11"E
56	*A.carambola*			Attingal	8° 40' 48"N	76° 49' 48"E
57	(Sweet)			Kallambalam	8° 45' 52"N	76° 47' 18"E
58				Tripunithura	9° 56' 58"N	76° 20' 40"E
59		Ac6	Ernakulam	Ramamangalam	9° 56' 7"N	76° 29' 17"E
60				Elamkulam	10° 54' 33"N	76° 13' 49"E

MATERIALS AND METHODS

Plant materials

The preliminary screening was carried out in *Averrhoa bilimbi* (40 trees) collected from different localities of nine districts (collections) and *A. carambola* (20 trees) collected from six districts in Kerala state (Table 1). Since the variations between collections were less, the trees collected from each district were considered to represent one collection in the subsequent analysis thereby reducing the effective sample size as fifteen collections for both species taken together.

Fresh, young, disease free leaves were used for DNA extraction. The leaves of all samples were surface sterilized and stored frozen at -80°C until use.

Genomic DNA extraction

Total genomic DNA was extracted from 100 mg tissue of each collection based on the method of Doyle & Doyle (1990). The quality of the DNA samples was determined by observing the ratio of absorbance at A_{260}:A_{280} using Biospectrophotometry (Biophotometer Plus, SI No 6132 ZG 4026 Eppendorf, Germany). Presence of DNA was confirmed by subjecting

samples to 0.8% agarose gel electrophoresis and staining with ethidium bromide. Staining intensities of the total DNA were compared visually with a 500 bp molecular weight DNA marker. After quantification, DNA was diluted to a final concentration of 50 ng μl^{-1}. All the required chemicals were obtained from Genei (Bangaluru, India).

Primer Selection

The internal transcribed spacers (ITS 1 and ITS 2) and the 5.8S coding region were amplified by forward primer (ITS 5: 5'-GGAAGTAAAAGTCGTAACAAGG-3') and reverse primer (ITS 4: 5'-TCCTCCGCTTATTGATATGC-3'). The primers were chosen based on ITS sequences published by White *et al*. (1990). Different primer concentrations (0.1µM, 0.3µM, 0.5µM) were also screened.

Polymerase Chain Reaction (PCR) mixture

Polymerase chain reactions were carried out in a total volume of 25µl reaction cocktail consisting of one unit of (0.25 µl) Taq DNA Polymerase, 200mM each of (2 µl) dNTP mixture, 2 µl of oligonucleotide primers, 2.5µl of 1 X Thermos table PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$and 50 mM KCl, pH 8.3) and 50 ng of isolated genomic DNA and final volume was adjusted to 25 µl with sterile double distilled water. All the chemicals except primers were obtained from Genei (Bangalore, India). From this, 22.5 µl was taken to each PCR tube and finally the template DNA was added for the PCR. A negative control containing all components except genomic DNA was included in each set of reactions to ensure that no self-amplification or DNA contamination occurred. PCR conditions, including the concentration of template DNA, primer, dNTP and Taq DNA polymerase were optimized to generate high intensity and sharp bands with a clear background.

PCR conditions of genomic DNA

The thermal cycling profile employed for ITS analysis includes: 1 cycle of 94^0C for 5 minutes, 35 cycles of 94^0C for 30 seconds, 50^0C for 1 minute, and 72^0C for 2 minutes followed by a final extension of 72^0C for 10 minutes.

Gel Electrophoresis and Documentation

Amplified products were subjected to electrophoresis on different concentrations of Agarose gel with 1X Tris Acetate Ethylene Diamine tetra acetic acid (1X TAE Buffer), stained with Ethidium Bromide and visualized with the help of UV trans-illuminator equipped with Gel Documentation System (Alpha Innotech Corporation, USA). Electrophoresis was performed in a horizontal Gel Electrophoresis unit (Scie Plas, UK) and the gels were documented and analyzed in detail by the Gel Documentation System. The molecular size of the amplified fragments was estimated using the100 bp DNA ladder (Genei, Bangalore).

ITS Sequence analysis

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA).The PCR mix consisted of 10-20ng of PCR product, 3.2pM (either Forward or Reverse) of primers, 0.28µl of sequencing Mix, 1.86µl of 5X reaction buffer and made up to 10µl with sterile distilled water. The sequencing PCR temperature profile consisted of a first cycle at 96^oC for 2 minutes followed by 30 cycles at 96^oC for 30 seconds, 50^oC for 40 seconds and 60^oC for 4 minutes for all the primers.

Post Sequencing PCR Clean up

About 10µl of milli Q and 2 µl of 125mM EDTA per reaction was mixed to prepare master mix I. From this master mix about 12µl was added to each reaction containing 10µl of reaction contents and properly mixed. Master mix II of 2µl 3M sodium acetate (pH 4.6) and 50µl of ethanol per reaction was prepared. Then about 52µl of master mix II was added to each reaction, mixed well and incubated at room temperature for 30 minutes. Then centrifuged at 14,000 rpm for 30 minutes and decanted the supernatant and 100µl of 70% ethanol was added. Again centrifuged at 14,000 rpm for 20 minutes, decanted the supernatant and repeated the 70% ethanol wash. The supernatant decanted and the pellet was air dried. The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al*., 2010). ITS sequences obtained were checked for homology using the NCBI BLAST program (http:/www.ncbi.nlm.nih.gov/ BLAST/). Nucleotide sequence alignments were performed using Clustal X version 1.81 (Thompson *et al*., 1997). Sequence alignments were manually adjusted by using BioEdit (Hall, 1999). Gaps were treated as missing data in phylogenetic analyses. Maximum Likelihood Estimate of Transition/Transversion Bias was estimated under the Kimura 2-parameter model (Kimura, 1980). Evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis, MEGA5 (Tamura *et al.*, 2011). The estimate of nucleotide diversity (π) of ITS1-5.8S-ITS2 region of *A. bilimbi* and *A. carambola* collections were also calculated using MEGA5. Phylogenetic trees were constructed by using MEGA software version 5.1 (Tamura *et al*., 2007) using Maximum Likelihood and UPGMA method (Sneath and Sokal, 1973; Tamura *et al*., 2007) and phylogenetic relationships were analysed. The estimate of the pattern of nucleotide substitution was computed by using Maximum Composite Likelihood (MCL) method.

RESULTS

The concentration of ITS primers for the analysis of *Averrhoa* collections was standardized as 0.5µM.The suitable annealing temperature for amplification was 50^0C. The concentration of agarose gel which provided the better resolution of ITS products was standardized as 1%. Fifteen collections of *Averrhoa* samples were tested for the variations in rDNA ITS region. There was no length variation in the PCR product obtained from all the collections studied. The amplified products from all the samples had very similar molecular size *i.e*., 132 bp (Figure 1). All the rDNA ITS sequences obtained for each *Averrhoa* collections studied presently were submitted directly to GenBank through Sequin software. The sequence can be located by collection numbers - AbITSSequin.sqn AbITS-1 KR905594 to AbITSSequin.sqnAbITS-9 KR905602 and AbITSSequin.sqnACITS-1 KR905603 to AbITSSequin. sqnACITS-6 KR905608.The GenBank collection numbers are represented in Table 2.

Table 2. Averrhoa samples sequenced with collection number

Sl.No.	Sample	Sample code	Collection number	
1		Ab1.	AbITSSequin.sqn AbITS-1	KR905594
2		Ab2.	AbITSSequin.sqn AbITS-2	KR905595
3		Ab3.	AbITSSequin.sqn AbITS-3	KR905596
4		Ab4.	AbITSSequin.sqn AbITS-4	KR905597
5		Ab5.	AbITSSequin.sqn AbITS-5	KR905598
6	*Averrhoa bilimbi*	Ab6.	AbITSSequin.sqn AbITS-6	KR905599
7		Ab7.	AbITSSequin.sqn AbITS-7	KR905600
8		Ab8.	AbITSSequin.sqn AbITS-8	KR905601
9		Ab9.	AbITSSequin.sqn AbITS-9	KR905602
10		Ac1	AbITSSequin.sqn ACITS-1	KR905603
11		Ac2.	AbITSSequin.sqn ACITS-2	KR905604
12	*Averrhoa carambola*	Ac3.	AbITSSequin.sqn ACITS-3	KR905605
13		Ac4.	AbITSSequin.sqn ACITS-4	KR905606
14		Ac5.	AbITSSequin.sqn ACITS-5	KR905607
15		Ac6.	AbITSSequin.sqn ACITS-6	KR905608

Table 3. Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	T	C	G
A	-	*4.82*	*7.33*	18.99
T	*4.36*	-	13.09	*7.91*
C	*4.36*	8.61	-	*7.91*
G	10.48	*4.82*	*7.33*	-

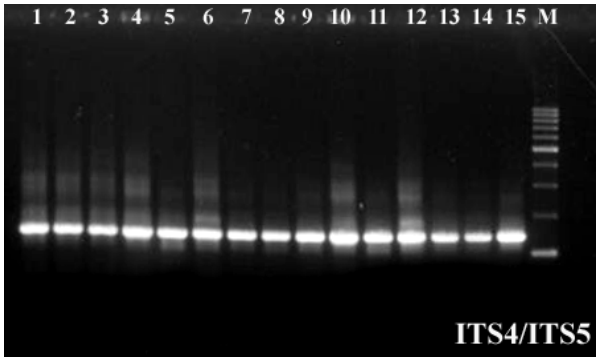

Figure 1. Amplification profile of *Averrhoa* collections based on ITS primers

Averrhoa bilimbi ITS sequences deposited in the GenBank Database showed high similarity with the *Averrhoa* sequences already deposited in the NCBI database [*A. bilimbi* (~99%) and *A. carambola* (~95%)]. Likewise, the presently deposited *A. carambola* ITS sequences also showed high similarity with the *Averrhoa* sequences stored at the NCBI database [*A. carambola* sequences (~100%) and *A. bilimbi* sequences (~95%)]. Multiple sequence alignment of ITS sequences obtained from 15 collections of *Averrhoa* consisted of a total of 615 characters. Of these 561 characters were conserved, 54 characters were variable and 33 characters were parsimony informative or potentially informative sites. Maximum composite likelihood (MCL) estimate of the pattern of nucleotide substitution are provided in table3. MCL analysis revealed that the estimated transition/transversion bias (R) was found to be 0.53. The estimate of nucleotide diversity of ITS1-5.8S-ITS2 fragments displayed that mean diversity (π) within subpopulations of both species of *Averrhoa* (*A. bilimbi* and *A. carambola* collections) was 0.005, mean diversity in entire population was 0.035, mean inter population diversity was 0.030 and the coefficient of differentiation was 0.865. Alignment of sequences further revealed the presence of both insertions/deletions (indels) and substitutions (transitions and transversions) in both ITS1 and ITS2 regions and is represented in Figure 2.

Phylogenetic and molecular evolutionary analyses were conducted on ITS sequences using MEGA version 5 and UPGMA and Maximum likelihood (ML) tree were constructed (Figure 3 and 4). Based on the ITS sequences, the 15 collections of *Averrhoa* under study were differentiated into two main clusters, those belonging to *A. bilimbi* and the rest to *A. carambola*. A higher sequence similarity could be noticed among the *A. carambola* collections as thesour and sweet collections of *A. carambola* grouped together to form a single group. However, the *A. bilimbi* cluster formed two groups, three of the *A. bilimbi* collections (Ab2, Ab3 and Ab4), could be discriminated from the rest. Similar discrimination of these three collections of *A. bilimbi* was also noticed in the other molecular marker studies (Soumya *et al*., 2017).

DISCUSSION

Ribosomal ITS sequences are generally used for phylogenetic reconstruction because they are ubiquitous and exist in high copy number. Ribosomal DNA repeats often undergo rapid concerted evolution within and among arrays (Alvarez and Wendel, 2003). A number of studies have shown that ITS polymorphism within individuals is relatively common (Zhang and Ge, 2007).

Figure 2. Complete alignment matrix of *Averrhoa* collections based on rDNA ITS sequences

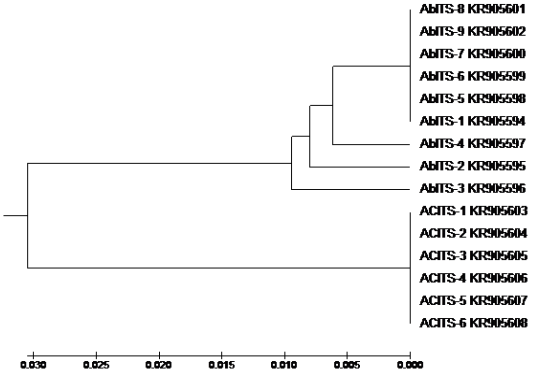

Figure 3. UPGMA dendrogram based on rDNA ITS sequence

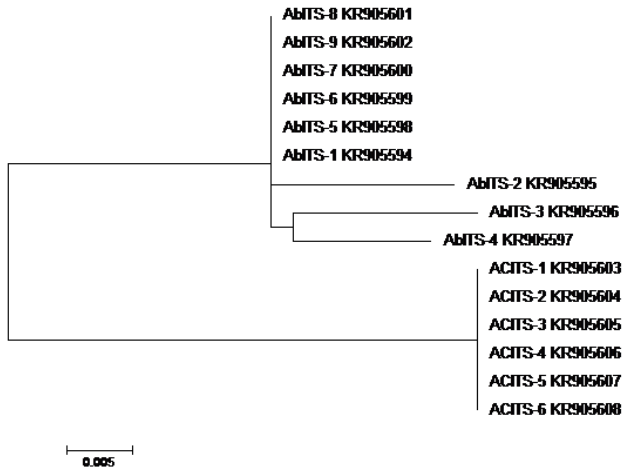

Figure 4. Maximum Likelihood dendrogram based on rDNA ITS sequence

Knowledge of genetic diversity within a population and among populations is important for conservation management, especially in identifying genetically unique structural units, within a species and determining the populations that need protection (Zhuravlev *et al*., 2010). In the present study, rDNA ITS amplicons on agarose gel electrophoresis revealed that all the collections of both species of *Averrhoa* had similar molecular size (132 bp). This would indicate that during evolution and selection there has been no selection for or against ITS length variation (Souframanien *et al*., 2003). The sequence data showed that total variable sites present in the ITS region of *Averrhoa* collections were8.78%. The dendrogram obtained from ITS sequence data showed that these variations were noticed only in *A. bilimbi* collections. However, there were no such variations in sour and sweet collections of *A. carambola*. The percentage of variation observed in *Averrhoa* collections was slightly higher than values reported in wild barley 7.4% and 6.9% in wheat collections by Sharma *et al*. (2002). It was explained that these variable sites could be produced by the substitutions which arise due to point mutations at specific sites, or indels that presumably result from slippage replication (Stephan, 1989). In the current study, it was observed that out of a total of 615 characters, 561 were conserved, 54 variable and 33 sites parsimony informative. These parsimony informative sites are suggested to give information about the evolutionary relationship among taxa. ITS sequence analysis therefore appears to be a useful tool for evaluating genetic diversity in *Averrhoa* at the species level.

It has been suggested that the spacer sequences may accumulate mutations in the form of base substitution, duplication, deletion and insertion (Scoles *et al*., 1987) and chromosomal rearrangements in genomes (Hautea *et al*., 1993). While studying the ITS sequences in cowpea lineages in the Sahara regions, Pasquet (2000) observed a low variation within and between collections of cowpea, and explained that it may be due to the extreme isolation of collections. Similarly low level of ITS sequence variation was observed in the present study. Homology of the nucleotide sequence of 5.8S rDNA genes of closely related species and the presence of varying degree of non-homology in the ITS1 and ITS2 region of the unrelated species makes the ITS region especially useful for quantifying relatedness among species (Chatterton *et al*., 1992). The evolutionary history was constructed using UPGMA and Maximum likelihood method and was inferred in units of the number of base substitutions per site. Similar to other molecular studies (RAPD and SRAP) conducted previously, 15 collections of *Averrhoa* could be grouped into two main groups (*A. bilimbi* and *A. carambola*). From the analysis, it was clear that there was no ITS sequence variation in sour and sweet collections of *A. carambola*. However, three collections of *A. bilimbi* could be clearly delineated from the rest.

Conclusion

This is the first in-depth report of genetic characterization in *Averrhoa* using ITS sequences. A higher sequence similarity could be noticed among the *A. carambola* collections, while three *A. bilimbi*collections, could be discriminated from the rest. ITS sequence analysis therefore appears to be a useful tool for evaluating genetic diversity in *Averrhoa* at the species level. The study could be extended further to collections from other geographical regions in order to derive a better picture for interspecific and intraspecific genetic variations.

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