

## SHORT COMMUNICATION

# Evaluation of roselle based on the internal transcribed spacer sequence of rRNA gene

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## ABSTRACT

Genetic differences in 5-lobed leaf Roselle (*Hibiscus sabdariffa* L.) collected from China, Malaysia, Sudan, and USA were evaluated by the sequence of nuclear internal transcribed spacer 1, 2 regions in rRNA gene (ITS 1, 2 regions). A total of 27 samples were subjected to polymerization chain reaction (PCR) and 729 bp bands were amplified in all samples. Samples were divided into 4 groups based on the single nucleotide polymorphisms at 71, 91, and 249 of the 729 bp band. However, no variations among samples collected from different geographic origins of *H. sabdariffa* are observed.

**Keywords:** *Hibiscus sabdariffa*; ITS 1; 2 regions; Single nucleotide polymorphism (SNP)

## INTRODUCTION

*Hibiscus sabdariffa*, commonly known as Roselle, is produced for tea from the brilliant red sepals or calyces because they have a rich content of vitamins A, C, and several antioxidants (USDA, 2014). The best quality Roselle is produced in the Sudan and China, but Egypt, Jamaica, Mexico, and USA are also important suppliers.

Characterization and identification of genetic difference within the species/varieties is very important for utilization and conservation of Roselle resources. So several methods were performed such as randomly amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR) and inverse sequence-tagged repeat (ISTR) to characterize Roselle (Torres-Moran et al., 2011; Khafaga, 2013; Omalsaad et al., 2014). *Hibiscus sabdariffa* with 5-lobed and a mostly red calyx whereas *H. cannabinus* accessions with 3-lobed leaves and mostly a yellow calyx were separated using RAPD markers, but separation of some accessions in the same species were not successful (Omalsaad et al., 2014). *H. sabdariffa* cultivars collected from different locations and showed three different phenotypes were divided only two group using RAPD and ISSR marker (Khafaga, 2013). *Hibiscus sabdariffa* accessions with 3-lobed

leaves were separated from those with 5-lobed leaves using ISTR markers and it was concluded that accessions collected from China and other areas probably share a common origin among the twelve genotypes grown in western Mexico (Torres-Moran et al., 2011).

From these reports, RAPD, ISSR and ISTR generated useful molecular markers for identification but it was not successful to reveal the genetic difference within closely related *H. sabdariffa* as. Markers generated from the ITS 1, 2 regions of nuclear rRNA gene are considered as useful to study closely related species (Baldwin, 1992). SNPs based on the sequence information of the ITS 1, 2 regions were used in identification of genetic relationships among seedlings of *Kolkwitzia* (Park et al., 2014) and *Ligustrum* (Ma et al., 2014). The objective of this experiment was to understand the genetic relationships of *H. sabdariffa* L. collected from 4 different countries using SNPs of the ITS 1, 2 regions.

## MATERIALS AND METHODS

Roselle (*Hibiscus sabdariffa* L.) seeds were collected from China, Malaysia, USA (purchased from the Seedville), and Sudan. The germinated seeds were grown at greenhouse and transplanted into 5-gallon pots after one year.

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A total of 27 Roselle plants with 5-lobed leaves; 4 from Sudan, 4 from USA, 4 from Malaysia, and 15 from China were randomly selected from 30 plants from each origin. Twenty four weeks after transplanting, young and expanding leaves were collected for extraction of total genomic DNA using Solg<sup>TM</sup> Genomic DNA prep kit (SolGent, Daejeon, Korea). To amplify the ITS 1, 2 regions, PCR was performed with a 18S rRNA gene specific forward primer ITS 1 (5'- TCC GTA GGT GAA CCT GCG G -3') and a 28S rRNA gene specific reverse primer ITS 4 (5'- TCC TCC GCT TAT TGA TAT GC -3'). The PCR reaction mix contained template DNA (20 ng), each primers (10 pmols), 2.5 µl of 10× PCR buffer and 0.15 µl of Pfu DNA Polymerase (SolGent). The PCR amplification condition was: 2 min at 95°C, followed by 35 cycles of 20 s at 95°C, 40 s at 56°C, and 1 min at 72°C. The PCR products were purified with a gel purification kit (SolGent) according to the manufacturer's direction and direct sequenced. Sequences were compared following the BLAST search at NCBI. (<http://blast.ncbi.nlm.nih.gov/>).

## RESULTS AND DISCUSSION

Leaves of all samples collected from 4 countries had palmate 5-lobed leaves with a slight variation in morphology of leaflets (Fig. 1). The shape and color of the calyx showed no significant visual differences among samples collected from each country.

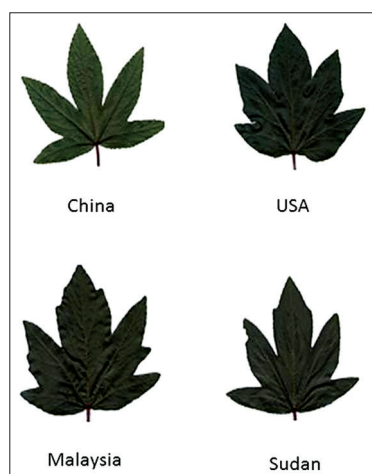
After PCR, 729 bp products were amplified from all samples and 581 bp products were amplified from some samples (Fig. 2). When the 729 bp PCR bands were sequenced, double peaks were observed in 3 SNP positions at 71, 95, 249 (Fig. 3) with ambiguities following International Union of Pure and Applied Chemistry (IUPAC) and nucleotide ambiguity codes revealed 4 different types. The

A type with IUPAC code R in position 249 were observed from 11 samples collected from China and 2 samples from USA (U05, U07), B type with code Y in position 95 in one sample collected from USA (U06), and two samples from Malaysia (M14, M15), C type with code R in position 249 and code Y in position 95 from two samples from China (C05, C11) and one from Malaysia (M16), and D type with code W in position 71 observed in two samples from Sudan (S02, S03) and one from Malaysia (M13).

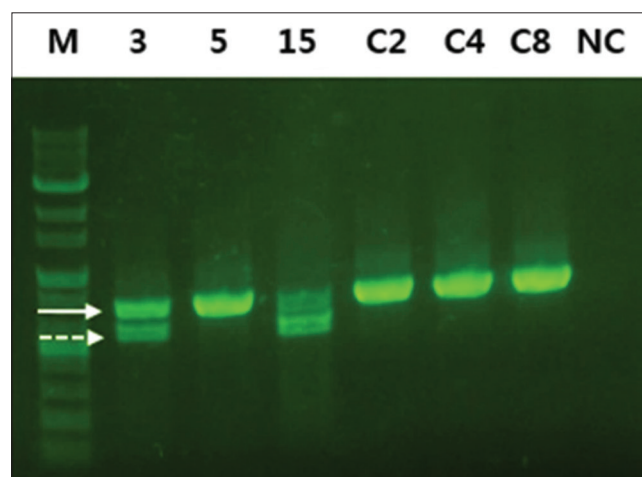
If the nucleotide of ambiguity codes are matched with the code of the remaining samples, for example code W to nucleotide A at position 71, no variances in the nucleotide sequences of ITS 1, 2 regions from two from Sudan and one from China and among all samples are observed. Therefore, there are no variations among all samples collected from different origins based on the ITS 1, 2 regions haplotypes as reported for accessions grown in Mexico (Torres-Moran *et al.*, 2011).

The 729 bp sequences were blasted on NCBI BLAST, and all showed over 99% identity with *Hibiscus sabdariffa* var. *sabdariffa* (FJ527608.1) and *H. sabdariffa* var. *altissima* (JQ609255.1). This suggests that *H. sabdariffa* and *H. cannabinus* cannot be distinguished using SNPs, although they were separated using RAPD markers (Omalsaad *et al.*, 2014). Sequences of ITS 1, 2 regions of one sample each of four types were registered at NCBI GenBank: U05 (A type, KP262031), U06 (B type, KP262033), M16 (C type, KP262032), and S02 (D type, KP262034).

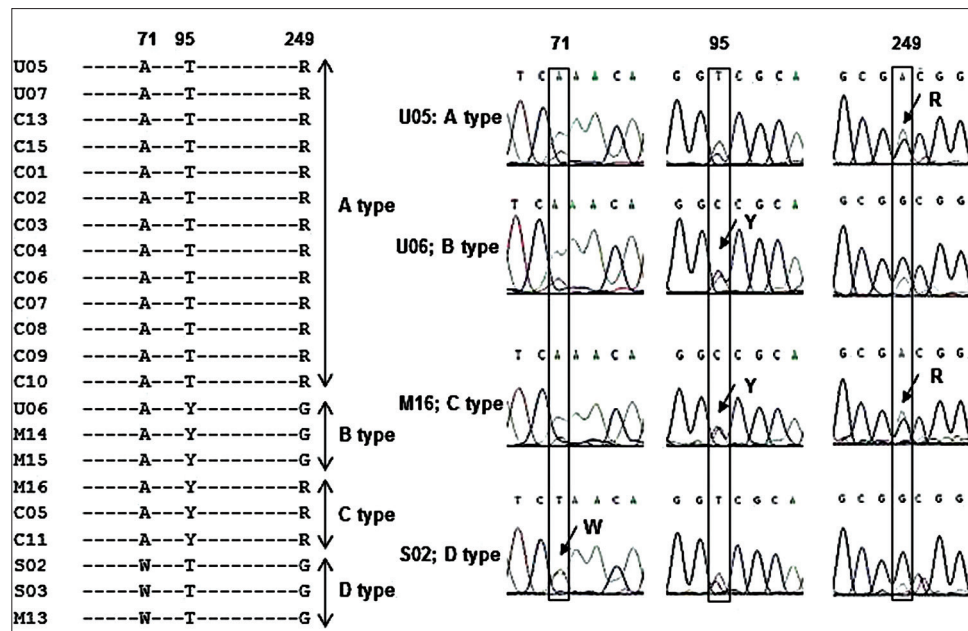
The 581 bp PCR bands were also amplified with 729 bp band from China (C13), Sudan (S03) and Malaysia sample (M15). These 581 bp were isolated and sequenced. The sequences of 581 bp DNA were matched with rRNA ITS 1,2 of *Aureobasidium* species (KF887041, JN031005).



**Fig 1.** General leaf morphology with 5 lobes collected from China, Malaysia, USA and Sudan.



**Fig 2.** PCR analysis using the ITS1, 2 primers in *Hibiscus* leaves showing one large band of 729 bp (solid line) and small band of 581 bp (dashed arrow). M: DNA ladder and NC: negative control.



**Fig 3.** A portion of sequences of ITS nrDNA region and sequence chromatograms of *Hibiscus* samples from China (C01 – C15), Malaysia (M13 – M16), USA (U05 – U07), and Sudan (S02 – S03) are presented. (A) Four different types, A, B, C, and D type with sample numbers and a portion of nucleotide sequence with IUPAC ambiguity codes at the position 71, 95, and 249 are presented. (B) Sequence chromatograms showing two peaks at three sites. Arrows indicated IUPAC ambiguity codes (W: A or T, Y: C or T, R: A or G).

This result suggest that these samples may be contaminated with fungus *Aureobasidium* species which is naturally occurring as epiphyte or endophyte to a wide range of plant species (Andrews et al., 2002).

## CONCLUSION

*Hibiscus sabdariffa* collected from China, Malaysia, Sudan, and USA were evaluated by the ITS 1, 2 regions and they were divided into 4 groups based on the sequence. However, variations among samples collected from different geographic origins were not observed.

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## Author contributions

Y. H. Joung contributed to all aspects of this manuscript. S. H. Park was involved in the DNA research work. M-X. Li and J. K. Suh were involved in the phenotype analysis. F. C. Ginibun and M. S. Roh helped in sample collection and editing of the manuscript.

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