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Full Length Article

Phylogeny of *Syzygium* Species using Morphological, RAPD and ISSR Markers

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Abstract

The genus *Syzygium* includes more than 700 species globally. Among these, 13 have been described as endemic to Mauritius and one is believed to be extinct. The phylogeny of 6 species of the *Syzygium* genus including *S. commersonii*, *S. coriaceum*, *S. glomeratum*, *S. petrinense*, *S. Samarangense* and *S. venosum* was explored using both morphological characters and molecular techniques. The cluster analysis of the morphological data differentiated *S. commersonii* from the other *Syzygium* species. Ramdom Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) techniques were successfully used to amplify all the 6 *Syzygium* species and polymorphism was observed in all the species. Relationships among the species were further analysed by generating a distance matrix as well as a dendogram using these molecular markers. *S. samarangense* is less closely related to the other *Syzygium* species studied. © 2013 Friends Science Publishers

Keywords: *Syzygium*; RAPD; ISSR; Morphological; Molecular characters

Introduction

Syzygium, commonly known as "Bois de pomme", of family Myrtaceae, has a native range that extends from Africa and Madagascar through southern Asia east to the Pacific. Many members of this genus have a good economic value and they have been used in medicines, food and ornamentals. *Syzygium* has often been considered as a section of the genus *Eugenia* and some species like *S. floribundum* have even been misnamed for a very long time. According to Bosser *et al*. (1990), there are 13 endemic *Syzygium* in Mauritius. They are usually cultivated in the top stratum of the humid forest such as Macchabe, Petrin and Bassin Blanc. The most commonly found *Syzygium* in Mauritius are *S. samarangense* commonly known as "Jamalac" and *S. jambos* commonly known as "Jamrosa". The fruit of Jamalac is consumed raw by many and the flowers are astringent and used in Taiwan to treat fever and halt diarrhoea, while the leaves are used to treat atony whereas the roots are used to treat amenorrhoea (Rouillard and Gueho, 1999). The leaves and stem of *S. glomeratum* which is one of the endemic species, are used in folkloric medicine to treat headaches and coughs (Gurib-Fakim and Brenler, 2004). One of the species that is commonly consumed as a spice is *S. aromaticum*, commonly known as "Giroflier".

Mauritius, which forms part of the Mascarene archipelagos, is an island situated in the Indian Ocean with an area of 2040 km^3. Despite being a small island, Mauritius possesses a rich diversity comprising of both endemic and exotic plant species. However, according to the IUCN red list, 88 plants species are threatened and the main threats include habitat loss, over-exploitation, deforestation alien invasives and climate change. Good conservation strategies need to be set up and a reliable taxonomic classification for the genus *Syzygium* is imperative.

The classical taxonomic classification was based on morphological traits and may be influenced by environmental conditions. DNA-based molecular genetic methods serve as a potential tool in resolving classification problems (Khan *et al.*, 2005; Dogan *et al*., 2010). Different molecular markers have been used to study *Syzygium* species. Shakya *et al*. (2010) made use of both RAPD and the SSR markers in the molecular characterisation of *S. cumini* while AFLP was used in the study of *S. sayeri* (Hillyer *et al*., 2007). In the phylogenetic analysis of another species from the Myrtaceae family, Van der Merwe *et al.* (2005) used RFLP and granted molecular support within the South African species of *Eugenia*. A phylogeny of some *Syzygium* species was obtained from a preliminary chemosystematic study (Lai Fang, 1999).

To date, no molecular study on the local varieties has been conducted in Mauritius. The purpose of the study is primarily to study the phylogeny and understand the biological diversity of this genus with emphasis on the endemic ones found in Mauritius. The identification of taxonomic units and endangered species, whose genetic constitution is distinct from their more abundant relatives, is important in the development of appropriate conservation strategies for *Syzygium* species.

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Materials and Methods

Plant Material

Leaf samples of *Syzygium* species, particularly *S. venosum, S. coriaceum, S. glomeratum, S. commersoni* and *S. petrinense* were collected from Petrin whereas *S. samarangense* was collected in Rose-hill. Young and healthy leaves were collected and kept under moist conditions, in plastic bags containing tissue paper. Those leaves were then brought to the laboratory, cleaned with alcohol to remove contaminants on the lower and upper surfaces and kept in the refrigerator. All experiments were carried out from August 2011 to March 2012 in the Molecular Biology laboratory, Faculty of Agriculture, The University of Mauritius.

Morphological Characterisation

Individual leaf of each species was examined and the morphological characters texture, leaf shape type, midrib colour, leaf colour, venation type, leaf margin, leaf base, leaf apex were noted and entered in a table.

Morphological character state polarization: The characters were polarized using *S. samarangense* as the outgroup and therefore a polarity matrix was obtained (Table 1).

DNA Isolation Protocol and Purification

Genomic DNA was extracted using the protocol of Doyle and Doyle (1987) with minor modifications.

0.075 g fresh leaf tissue was ground in liquid Nitrogen and was then transferred into a tube containing 0.75 mL of preheated (60°C) CTAB buffer followed by addition of 0.2% (v/v) β- mercaptoethanol and 2% (v/v) PVP. The tube was placed in 60°C water bath for 25-30 min with occasional swirling. Afterwards, 2/3 volume chloroform: isoamyl alcohol (24:1) was added and the tubes were inverted several times and then microcentrifuged at 10,000 rpm for 10 min. This step was repeated until no interface was visible and 2/3 volume of ice-cold isopropanol and 0.5 volume of 5M NaCl was added. The tubes were left overnight at 4°C to allow further precipitation of DNA and then the tubes were spun in a microcentrifuge for 30 min at 13,000 rpm. The supernatant was discarded and washed with 70% alcohol. The pellet was air dried under the hood and re-dissolved in 50 µL sterile distilled water. The DNA was purified by incubation with RNAse and a phenol treatment.

PCR Reaction

PCR-RAPD and ISSR reactions were carried out in a volume of 25 µL containing 1X reaction Buffer, 0.2 mM $MgCl_2$, 0.25 mM dNTP, 0.5 µM Primer, 1 Unit Taq DNA Polymerase, 37.5 ng Template DNA and made up to the final volume with nanopure for water using the Applied Biosystems 2720 thermal cycler. However, the thermal profile for RAPD was as follows: 90 s denaturation at 95°C, annealing for 35 cycles for 30 s at 92°C, 1min at 35°C, 3 min at 72°C, extension for 10 min at 72°C and 5 mins at 15°C. 7 operon primers namely OPA 2, OPA 4, OPA 11, OPA 12, OPA 13, OPA 17 and OPA 19 gave clear and polymorphic bands for all the species under study. In case of ISSR, the thermal profile was programmed as: denaturation consisting of 2 cycles of 7 min at 94°C, annealing for 35 cycles for 30 s at 94°C, 1 min at 53°C (depending on the primer used), 2 min at 72°C and a final extension of 10 min at 72°C. 3 ISSR primers namely ISSR 2, ISSR 5 and ISSR 6 gave amplifiable bands. PCR products were separated on 1.5% (w/v) agarose gels using 0.5x TBE Buffer at 90 V and stained with ethidium bromide for visualization under UV light.

Profile Analysis

Each genotype was characterized by its banding pattern using the DNA hyperladder 2 (Bioline) as basepair ladder. The RAPD markers as viewed from the gels after electrophoresis and staining were converted into a matrix of binary data, where the presence of the band corresponded to value 1 and the absence to value 0. The statistical software NTSYS-PC (Rohlf, 2005) and DARwin 5 software (Perrier and Jacquemoud-Collet, 2006) were used to construct a UPGMA dendrogram using hierarchical clustering. Using NTSYS software, a dissimilarity matrix was calculated utilising Jaccard (1908) coefficient. The matrix was converted to a dissimilarity matrix corresponding to the complement (dissimilarity=1−similarity). Cluster analysis based on the dissimilarity matrix, was performed using un-weighted pairgroup method arithmetic averages (UPGMA) (Sneath and Sokal, 1973) of the NTSYS–PC version 2.2 (Rohlf, 2005).

Results and Discussion

Before the advent of molecular markers, genetic variability studies to obtain phylogenetic trees were based upon morphological markers such as leaf shape, colour, size and flower type (Raven *et al*., 2004). Morphological markers correspond to the visually scoring qualitative traits and are influenced by environmental factors, plant biology and the plant developmental stage (Paul *et al*., 2010; Ali *et al.*, 2011).

The *Syzygium* species flower at different times of the year and their characterisation using morphological traits were deemed uninformative and difficult since very limited characteristics, mainly leaf texture, shape, colour, margin, base, apex, colour of midrib and venation type could be used. All species similarly share the "venation type" and the "leaf margin" characteristics (Table 2). In addition, most species share similar morphological characters such as texture and leaf base, therefore these cannot be used to distinguish the species.

Table 1: Polarised state for each character of *Syzygium*

Character Number	Characters	Polarity	
		1	0
1	Texture	Shrub	Tree
2	Type of leaf shape	Obovate/ovate/oval	Elliptic
3	Colour of midrib	Yellow	Green
4	Leaf colour	light green	Green
5	Venation type	Pinnate	Reticulate
6	Leaf margin	Dentate	Entire
7	Leaf base	Cordate	Obtuse
8	Leaf apex	Emmarginate/obtuse	Acuminate

Table 2: Morphological traits of the different *Syzygium* species

Characters	*S.samarangense*	*S. commersonii*	*S. coriaceum*	*S. glomeratum*	*S. petrinense*	*S. venosum*
Texture	Tree	Shrub	Shrub	Shrub	Shrub	Shrub
Type of leaf shape	Elliptical	Elliptical	Obovate	Ovate	Elliptical	Obovate
Midrib colour	Green	Green	Yellow	Green	Yellow	Yellow
Leaf colour	Green	Green	Green	Yellowish green	Green	Green
Venation type	Reticulate	Reticulate	Reticulate	Reticulate	Reticulate	Reticulate
Leaf margin	Entire	Entire	Entire	Entire	Entire	Entire
Leaf base	Obtuse	Cordate	Obtuse	Obtuse	Obtuse	Cordate
Leaf apex	Acuminate	Acuminate	Emmarginate	Obtuse	Obtuse	Obtuse

Table 3: List of primers with most amplifiable bands and their percentage polymorphism

Primer	Nucleotide sequence	Number of markers	Number of polymorphic marker	% polymorphism
RAPD				
OPA 2	TGCCGAGCTG	13	6	41.2
OPA 11	CCATCGCCGT	16	6	37.5
OPA 12	TCGGCGATAG	14	5	35.7
OPA 13	CAGCACCCAC	17	7	41.2
OPA 17	GAAACGGGTG	10	4	40
OPA 19	CCAACGTCGG	13	6	46.2
OPA 4	AATCGGGCTG	12	5	41.7
Total		95	39	
Average		13.6	5.6	41.2
ISSR				
ISSR 5	CTCTCTCTCTCTCTCTG	11	3	27.3
ISSR 2	AGAGAGAGAGAGAGAGC	11	5	45.5
ISSR 6	TCTCTCTCTCTCTCTCTG	11	3	27.3
Total		33	11	
Average		11	3.7	33.3

Fig. 1: Leaves of *S. commersoni*

Fig. 2: Leaf of *S. coriaceum*

Fig. 3: Leaves of *S. glomeratum*

Fig. 4: Leaves of *S. petrinense*

Fig. 5: Leaves of *S. venosum*

Fig. 6: Leaf of *S. samarangense*

Table 4: Distance matrix of *Syzygium* species based on combined RAPD-ISSR marker

	S. commersonii	*S. coriaceum*	*S. glomeratum*	*S. petrinense*	*S. samarangense*
S. coriaceum	0.3279				
S. glomeratum	0.5517	0.4598			
S. petrinense	0.4315	0.2810	0.5518		
S. samarangense	0.8477	0.8477	1.071	0.7175	
S. venosum	0.53565	0.53565	0.8088	0.5044	0.9241

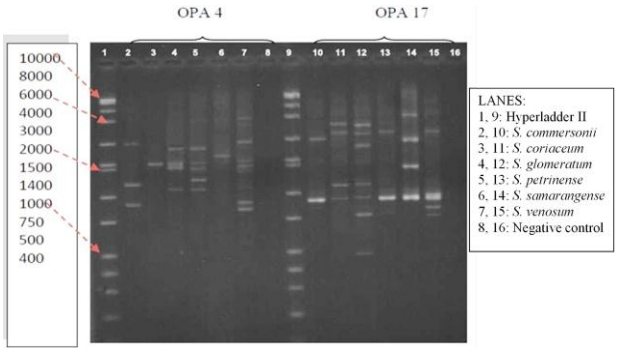

Fig. 7: PCR amplification products using OPA 4 and OPA 17 primers

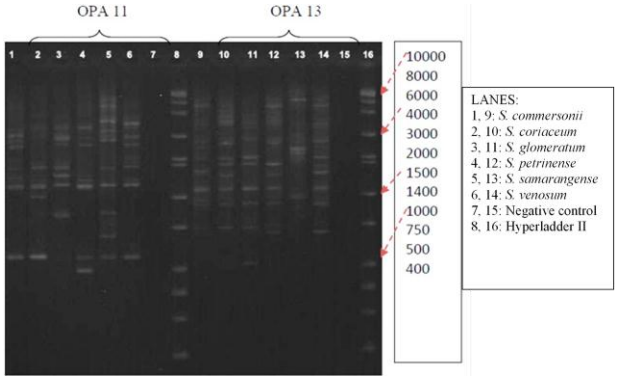

Fig. 8: PCR amplification products using OPA 11 and OPA 13 primers

In the analysis of the morphological data (Fig. 14) *S. commersonii* and *S. samarangense* are sister taxa in one distinct clade, suggesting that they are closely related to each other and less closely related to *S. coriaceum, S. venosum, S. glomeratum* and *S. petrinense*. Although *S. commersonii* and *S. samarangense* share 6 similar characters, the plant texture and the leaf base (Fig. 1 and 6) clearly distinguishes them into two separate clades. The other distinct clade consisted of *S. venosum* and *S. coriaceum* and characters that could differentiate them from other species include leaf base and the leaf apex (Fig. 1-6)

Inclusion of molecular data in taxonomic studies and systematic analyses, is likely to discriminate more among the organisms under study as changes in the genetic constitution from mutations, may not be reflected in the morphological features. Analyses using molecular markers were more informative. In RAPD PCR analysis more polymorphism was observed using primers OPA 2, OPA 4,

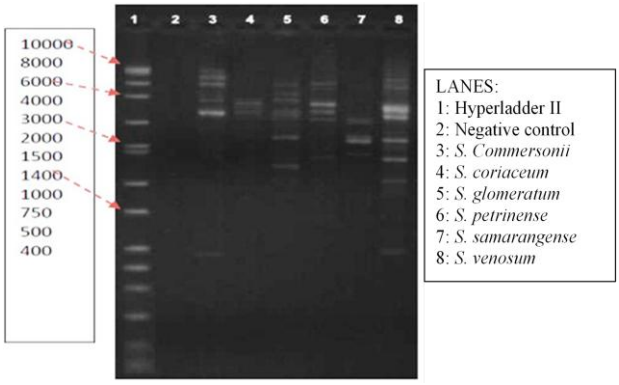

Fig. 9: PCR amplification products using primer OPA 2

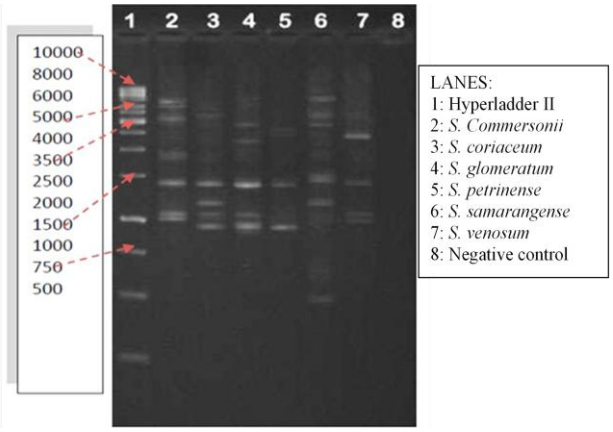

Fig. 10: PCR amplification products using primer OPA 12

OPA 11, OPA 13, OPA 17 and OPA 19 (Table 3; Fig. 7-Fig. 11). From Table 4, it is seen that for the RAPD analysis, an average of 13.6 markers and an average polymorphism of 41.2% per primer was observed as compared to an average of 11 markers and 33.3% polymorphism per ISSR primer. The two markers targeted different parts of the genome (Arif *et al*., 2009). The variation between *S. coriaceum* and *S. petrinense* are the lowest with similarity coefficient of 0.28, whereas *S. glomeratum* and *S. samarangense* have the highest variation. *S. samarangense* is the only *Syzygium* species being studied that was introduced in Mauritius, the others are endemic and this could explain for the high genetic variation.

In the dendogram generated using molecular markers (Fig. 15), *S. samarangense* is clustered separately from the other *Syzygium* spp., confirming that it is genetically

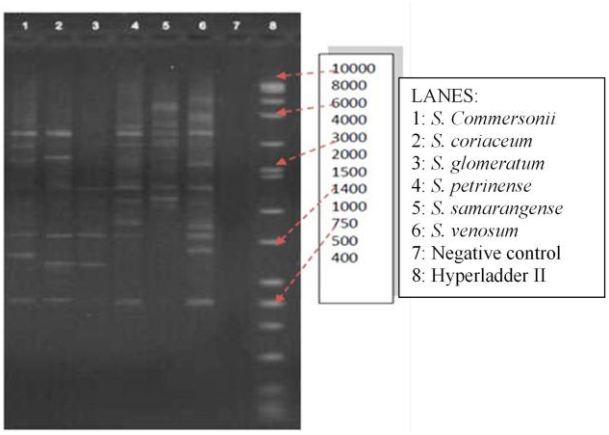

Fig. 11: PCR amplification products using primer OPA 19

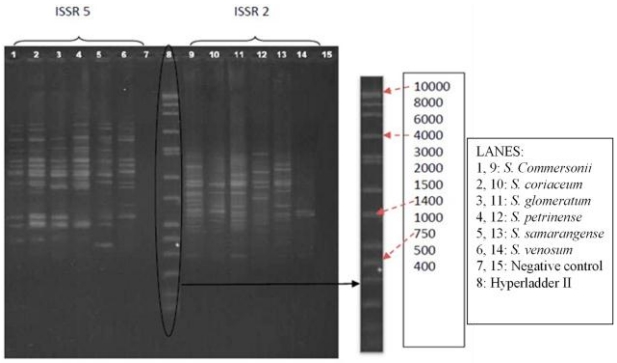

Fig. 12: PCR amplification products using primer ISSR 5 and ISSR 2

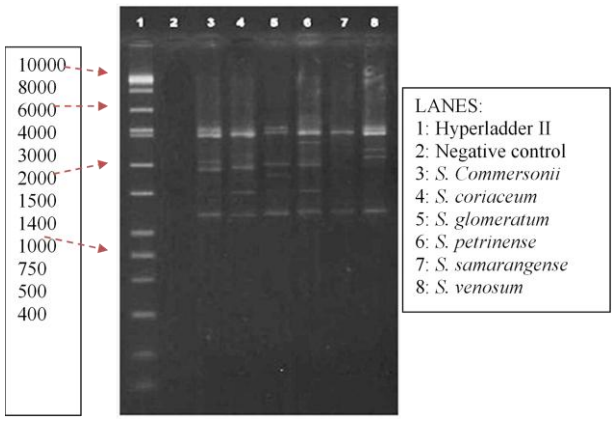

Fig. 13: PCR amplification products using primer ISSR 6

different from other *Syzygium* species in this study. Furthermore, with the exception of *S. samarangense*, primer OPA 19 amplified a 1425 bp marker in every other *Syzygium* species. *S. petrinense* and *S. coriaceum* form a sister taxa inferring that they are closely related to each other and are genetically similar and is further confirmed by the short distance, which exists between them in the similarity matrix (Table 4). The shorter the distance, the

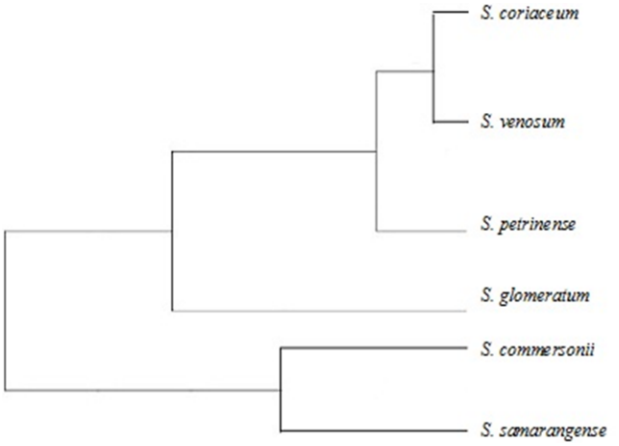

Fig. 14: Dendogram illustrating the phylogenetic relationship among the 6 *Syzygium* using morphological markers

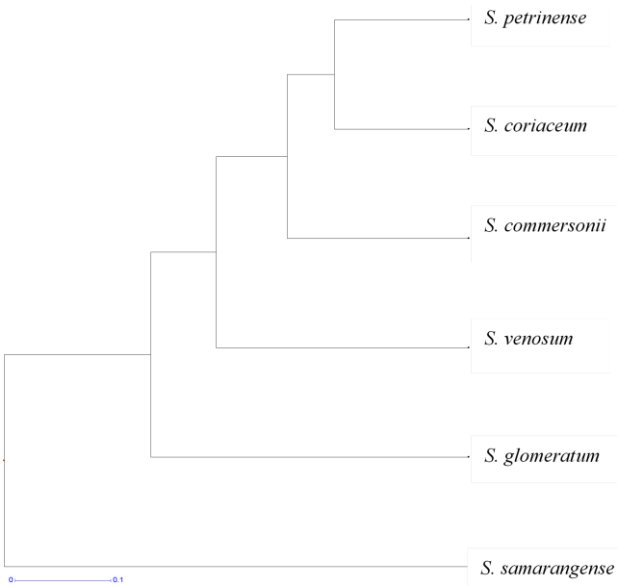

Fig. 15: Combined Dendogram illustrating the phylogenetic relationships among the 6 *Syzygium* spp using both the RAPD and ISSR markers

greater the number of genes shared and the more genetically similar are the species. *S. venosum*, *S. commersonii*, *S. coriaceum* and *S. petrinense* are more closely related to each other than to *S. samarangense*. *S. glomeratum* and *S. venosum* clustered separately from other species in the dendogram (Fig. 15) and can be explained by the common morphological characteristics shared together, which are texture, venation type, leaf margin and leaf apex.

This study is the first of its kind and it has enlightened us on the taxonomy and genetic diversity of the genus *Syzygium*. In this study different phylogenies for *Syzygium* have been obtained using two different marker types; morphological and molecular. Inclusion of other characters/markers such as anatomical, physiological and

ecological features along with phytochemical studies could provide information to construct a better phylogeny. DNA sequencing can reveal important information about the genetic diversity of *Syzygium* and their interrelationship. Baldwin *et al*. (1995) have acknowledged that the Internal Transcribed Spacer (ITS) of the nuclear ribosomal DNA (nrDNA) is a useful source of character for phylogenetic studies and the same can be used for inference of relatedness among the *Syzygium* species. Alternatively several regions in the plastid genome namely rbcL, rpoC1, rpoB, ycf5, psbA-trnH, trnL, atpF-atpH, psbK-psbI can be used as good candidates for plant DNA barcoding and the inference of phylogeny (Taberlet *et al*., 2007; Lahaye *et al*., 2008).

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