



King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Molecular fingerprinting of *Helicanthus elastica* (Desr.) Danser growing on five different hosts by RAPD



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Received 9 October 2015; revised 24 November 2015; accepted 4 December 2015

Available online 12 December 2015

KEYWORDS

Host-parasite interaction;
Indian mango mistletoe;
Mistletoe biology;
Medicinal plants;
Molecular fingerprint

Abstract Mistletoes are hemiparasitic plants growing on aerial parts of other host trees. Many of the mistletoes are reported to be medicinally important. The hemiparasitic nature of these plants makes their chemical composition dependent on the host on which it grows. They are shown to exhibit morphological dissimilarities also when growing on different hosts. *Helicanthus elastica* (Desr.) Danser (mango mistletoe) is one such less explored medicinal mistletoe found on almost every mango tree in India. Traditionally, the leaves of this plant are used for checking abortion and for removing stones in the kidney and urinary bladder while significant antioxidant and antimicrobial properties are also attributed to this species of mistletoe. The current study was undertaken to evaluate molecular differences in the genomic DNA of the plant while growing on five different host trees using four random markers employing random amplified polymorphic DNA (RAPD) followed by similarity matrix by Jaccard's coefficient and distance matrix by hierarchal clustering analysis. Similarity and distance matrix data employing just 4 random markers, separately and the pooled data as well, revealed significant difference in the genomic DNA of *H. elastica* growing on five different hosts. Pooled data of similarity from all the 4 primers cumulatively showed similarity between 0.256 and 0.311. Distance matrix ranged from of 0.256 to 0.281 on pooling the data

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Peer review under responsibility of King Saud University.



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<http://dx.doi.org/10.1016/j.sjbs.2015.12.002>

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from all the four primers. The result employing a minimum number of primers could conclude that genomic DNA of *H. elastica* differs depending upon the host on which it grows, hence the host must be considered while studying or utilizing this mistletoe for medicinal purposes.

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1. Introduction

Helicanthus elastica (Desr.) Danser (syn. *Loranthus elasticus* Desr. and *Dendrophthoe elasticus* (Desr.) Danser) belonging to family Loranthaceae is a less known underutilized medicinally important species occurring in India. The members of the Loranthaceae family, generally known as mistletoes and mostly distributed in the tropics, are semiparasitic shrubs attached to the hosts by modified root, generally called as haustoria. The leaves of this plant are used for checking abortion and for removing stones in the kidney and urinary bladder (Shanavaskhan et al., 2012). This aerial parasitic plant is grown on a wide range of hosts. Shinde et al. (2007) reported that the chemical composition of a plant species depends upon the genetic identity which gets modified as part of modifications in the physiology of the plant due to the environmental conditions in which the plant grows. To explore the genetic diversity of a plant species, molecular markers independent of environment have been successfully used. Different types of molecular based DNA fingerprinting techniques are in practice for plant materials (Powell et al., 1996). random amplified polymorphic DNA (RAPD) is a polymerase chain reaction (PCR)-based method where arbitrary short primers are used which anneal to complementary DNA sequences. If two such sites are closely situated on the DNA strain, amplification of the interjacent nucleotides are carried out in a PCR reaction (Williams et al., 1990). It is convenient in performance and does not require any information about the DNA sequence to be amplified (Weder, 2002). Ahmed et al., 2006 found RAPD as one of the best tools for detecting species variation among plants.

2. Materials and methods

2.1. Plant material

Tender shoots of *H. elastica* growing on *Croton oblongifolius* Sieber ex Spreng. – Euphorbiaceae (CO), *Mangifera indica* L. – Anacardiaceae (MI), *Samanea saman* (Jacq.) Merr. – Mimosaaceae (SS), *Terminalia chebula* Retz. – Combretaceae (TC) and *Woodfordia fruticosa* (L.) Kurz. – Lythraceae (WF) were collected from the same locality in the Udupi district of Karnataka, India during September 2011. The plant materials were authenticated by Dr. Jacob Thomas, Plant Taxonomist and Curator of the Herbarium of the King Saud University, Riyadh, Saudi Arabia. The specimens were stored separately in a deep freezer at -20°C immediately after collection until isolation of genomic DNA.

2.2. Genomic DNA isolation

The leaves were carefully crushed to a powder using dry ice. 50 mg of this powder was taken in an eppendorf tube. 1 ml of extraction buffer was added and macerated using the tissue

homogenizer. The tubes were incubated at 60°C for 1 h in a dry bath with intermittent mixing and centrifuged at 10,000 rpm for 10 min to separate the unlysed cells. Supernatant was transferred to a fresh eppendorf tube carefully. Equal volumes of Tris saturated phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed well and centrifuged at 10,000 rpm for 10 min. The aqueous layer was pipetted out into the fresh eppendorf tube without taking the interface. Equal volumes of isopropanol and 1/10th volume of 3 M Sodium acetate were added and mixed well and left at room temperature to stand for 5–10 min again, centrifuged at 10,000 rpm for 10–15 min. Then the supernatant was discarded. The pellet was washed with 300 μl of 70% ethanol, air dried and suspended in 30 μl of $1\times$ Tris–ethylenediamine tetraacetic acid (EDTA) buffer.

2.3. Purification of DNA

To the DNA solution 5 μl of RNAase was added and incubated in a water bath at 37°C for 1 h. After 1 h it was removed from the water bath and 1 ml volume of chloroform: isoamyl alcohol (24:1) was added and gently mixed for 10 min. The solution was then centrifuged at 10,000 rpm for 20 min and the aqueous phase was pipetted out. The upper aqueous phase was separated after centrifugation and mixed with 1/10th volume of 3 M sodium acetate (pH 4.8). The DNA was precipitated by adding 2.5 ml of chilled absolute ethanol. The DNA pellet was carefully dried in laminar airflow. The dried DNA was dissolved in minimum amount of TE buffer (pH 8.0). The quantity of total DNA isolated was checked by adding 2 μl $6\times$ orange loading dye (Fermentas, USA) to 2 μl of isolated DNA. Four micro liters of this isolate was loaded in a well of 0.8% w/v agarose gel containing 0.05% of ethidium bromide. Undigested lambda DNA (Fermentas, USA) was used as marker. Agarose gel electrophoresis was carried out for approx. 1 h at 50 V. The gel was visualized under UV light in a gel documentation system (Syngene, UK). The intact double-stranded DNA forming a thick single band of high molecular weight confirmed good quality DNA.

2.4. RAPD analysis

RAPD analysis was performed using 4 randomly and arbitrarily selected 10-base primers (A and B series) obtained from Operon Technologies Inc., Alameda, California. The four random decamer primers used were OPA-02 (TGCCGAGCTG), OPA-13 (CAGCACCCAC), OPA-18 (AGGTGACCGT) and OPB-10 (CTGCTGGGAC). polymerase chain reaction (PCR) was performed based on the protocol of Williams et al. (1990) with some modifications. All 5 DNA samples were diluted to 50 ng/ μl and set for PCR. Amplification reactions were performed with 2.5 μl of $10\times$ PCR buffer with 15 mM MgCl_2 , 0.2 μl of dNTPs mixtures containing 0.2 mM

concentration of each dNTPs, 5 p mole of the primer, 5U of Taq DNA polymerase and 30 ng of genomic DNA. DNA amplification was performed in the thermal cycler (Eppendorf, Hamberg, Germany) programmed for 42 cycles as follows: the first step consisted of holding the sample at 94 °C for 2 min for complete denaturation of template DNA. The second step consisted of 40 cycles and each cycle consisted of three temperature steps *i.e.* 30 s at 94 °C for denaturation of template, one min at 45 °C primer annealing followed by 1 min and 30 s for primer extension. The third step consisted of only one cycle *i.e.* 7 min at 72 °C for complete polymerization followed by holding at 4 °C. After completion of PCR, amplified products were stored at –20 °C till further use.

2.5. Agarose gel electrophoresis (AGE)

The amplified products were separated in 1.5% agarose gel. To 250 ml of Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, 1 mM Na₂EDTA; pH 8.0), 3.75 g of agarose was added and mixed properly using a magnetic stirrer, and was boiled for complete melting of agarose and then cooled to 50 °C. After cooling 12.5 µl of ethidium bromide solution (10 mg/ml) was added, mixed properly and the gel was cast on the gel casting tray. Twenty-six well combs were used for well formation. After complete gelling, the gel was transferred to the gel tank containing 1× TAE buffer and submerged into the buffer. The PCR mixture of 40 µl contained 2× PCR master mix, 1 µl of a single RAPD primer and 19 µl sterile distilled water. Prior to loading the samples, the comb was removed and 2.5 µl of samples were loaded in each well along with a single well loaded with standard 100 bp DNA ladder. The electrophoresis was performed at the constant voltage of 110 V for 1 h and 30 min. After electrophoresis the gel was visualized under UV light and photographed in gel documentation system (Syngene, UK). 38 µl of this was aliquoted into 20 different labeled PCR vials and to this 2 µl of different template DNA was added and the PCR was set.

2.6. Data scoring

The amplicons after PCR were analyzed by electrophoresis on 1.2% (W/V) agarose gels by running in 0.5× TBE buffer. After staining with ethidium bromide the gels were visualized under a UV transilluminator and photographed using Bio-Rad gel documentation system. For comparing the similarity of *H. elastica* growing on different hosts Jaccard's Coefficient (Jaccard, 1912) was employed. Jaccard's Coefficient ($J = a / (a + b + c)$), where a = No. of presence of shared bands in both samples; b = No. of bands present only in sample 2; c = No. of bands present only in Sample 1. Clear and major RAPD-PCR bands were scored as present (1) or absent (0) (Collard and Mackill, 2009). From the similarity matrix calculated using the Jaccard's similarity coefficient, distances between individuals were calculated by clustering analysis (nearest neighbor method) with the help of the StatistiXL program (version 1.7).

3. Results

DNA from tender leaves of *H. elastica* growing on 5 different hosts was extracted using cetyl trimethyl ammonium bromide

(CTAB) method. The DNA in pure form was compared with random primers like OPA-02, OPA-13, OPA-18 and OPB-10 by AGE (Fig. 1(a–d)). The decamer primers were screened generating polymorphic patterns of amplified DNA for the selected plants. The similarity between the species while growing on different hosts was compared by observing shared and specific bands using Jaccard's similarity index (J) (Table 1).

J obtained on comparison of samples with OPA-2 was in the range of 0.280–0.333, the least being between TC (*H. elastica* growing on *T. chebula*) and WF (*H. elastica* growing on *W. fruticosa*) and the highest between CO (*H. elastica* growing on *C. oblongifolius*) and MI (*H. elastica* growing on *M. indica*). On observing the clustering strategy, TC and WF were clustered first at a distance of 0.280; cluster 1 (TC and WF) and MI were clustered together at a distance of 0.296; cluster 2 (TC, WF and MI) was clustered together with CO at a distance of 0.296 and cluster 3 (TC, WF, MI and CO) was clustered with SS at a distance of 0.308. The cophenetic correlation coefficient (between the similarity matrix and the matrix derived from the dendrogram) was found to be 0.559, the degrees of freedom was 8 with a P value of 0.093.

Using OPA-13 the similarity matrix in the range of 0.208–0.333 was obtained, the least being between TC and WF and the highest between WF and MI. TC and WF were clustered first at a distance of 0.208; CO and SS clustered together at a distance of 0.208; cluster 2 (CO and SS) was clustered together with cluster 1 (TC and WF) at a distance of 0.217 and cluster 3 (TC, WF, CO and SS) was clustered with MI at a distance of 0.231. The cophenetic correlation coefficient was found to be 0.508, the degrees of freedom was 8 with a P value of 0.134.

OPA-18 showing a similarity matrix in the range of 0.269–0.310 was obtained, the least being between TC and CO and the highest between MI and WF. TC and WF were clustered first at a distance of 0.250; cluster 1 (TC and WF) and SS clustered together at a distance of 0.269; cluster 2 (TC, WF and SS) was clustered together with CO at a distance of 0.269, and cluster 3 (TC, WF, SS and CO) was clustered with MI at a distance of 0.276. The cophenetic correlation coefficient was found to be 0.644, the degrees of freedom was 8 with a P value of 0.044.

While OPB-10 was employed a similarity matrix was in the range of 0.263–0.318, the least being between SS and WF and the highest between CO and SS. TC and SS were clustered first at a distance of 0.250; cluster 1 (TC and SS) and WF clustered together at distance of 0.263; cluster 2 (TC, SS and WF) was clustered together with CO at a distance of 0.267, and cluster 3 (TC, SS, WF and CO) was clustered with MI at a distance of 0.286. The cophenetic correlation coefficient was found to be 0.460, the degrees of freedom was 8 with a P value of 0.181.

On pooling data obtained from all the four random primers a similarity matrix in the range of 0.256–0.311 was observed, the least similarity was observed between TC and WF and the highest between SS and MI. TC and WF were clustered first at a distance of 0.256; cluster 1 (TC and WF) and SS clustered together at a distance of 0.261; cluster 2 (TC, WF and SS) was clustered together with CO at a distance of 0.280, and cluster 3 (TC, WF, SS and CO) was clustered with MI at a distance of 0.281. The cophenetic correlation coefficient was found to be 0.671, the degree of freedom was 8 with a P value of 0.034.

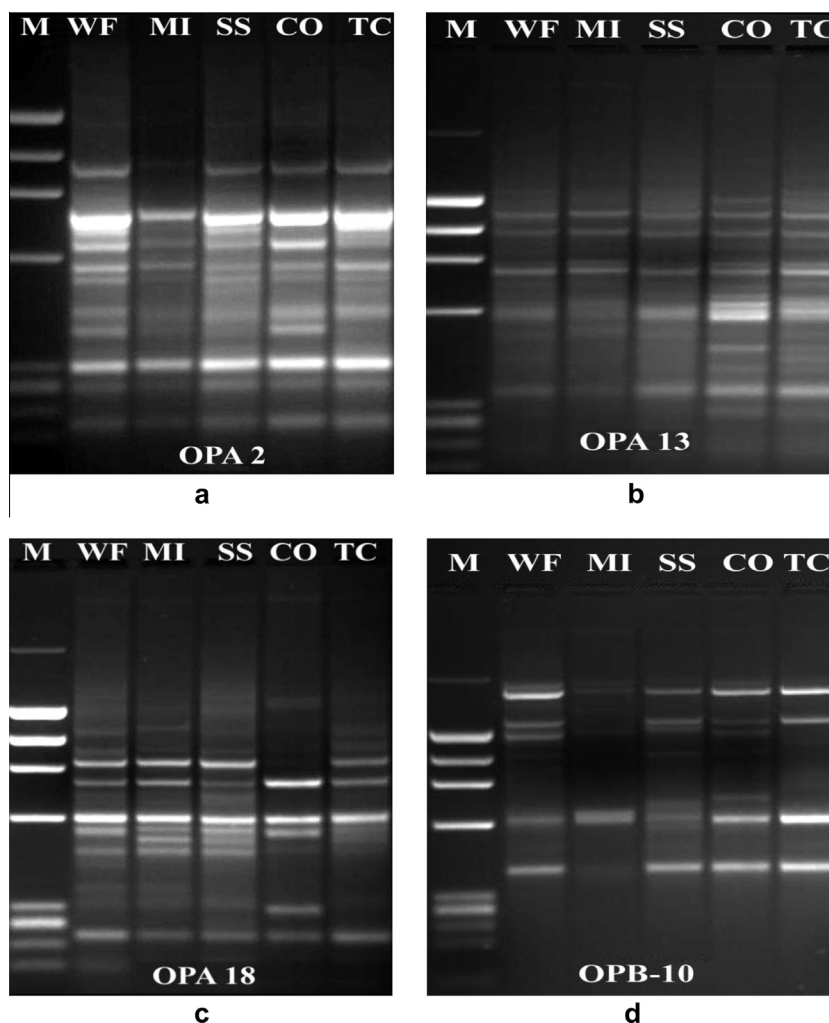


Figure 1 (a–d) Banding pattern of genomic DNA from different host species of *Helicanthus elastica* using four random primers. M, marker; WF, *Helicanthus elastica* growing on *Woodfordia fruticosa*; MI, *Helicanthus elastica* growing on *Mangifera indica*; SS, *Helicanthus elastica* growing on *Samanea saman*; CO, *Helicanthus elastica* growing on *Croton oblongifolius*; TC, *Helicanthus elastica* growing on *Terminalia chebula*.

Table 1 Jaccard's similarity index of *Helicanthus elastica* from five different hosts based on banding patterns obtained from four random primers.

	OPA 2				OPA 13				OPA 18				OPB 10				Pooled data			
	WF	MI	SS	CO	WF	MI	SS	CO	WF	MI	SS	CO	WF	MI	SS	CO	WF	MI	SS	CO
MI	0.296				0.333				0.310				0.294				0.308			
SS	0.310	0.321			0.313	0.313			0.280	0.308			0.263	0.300			0.292	0.311		
CO	0.296	0.333	0.321		0.231	0.231	0.208		0.280	0.280	0.273		0.278	0.316	0.318		0.271	0.290	0.280	
TC	0.280	0.320	0.308	0.320	0.208	0.240	0.217	0.290	0.250	0.276	0.269	0.269	0.286	0.286	0.250	0.267	0.256	0.281	0.261	0.287

CO: *H. elastica* growing on *Croton oblongifolius*; MI: *H. elastica* growing on *Mangifera indica*; SS: *H. elastica* growing on *Samanea saman*; TC: *H. elastica* growing on *Terminalia chebula*; WF: *H. elastica* growing on *Woodfordia fruticosa*.

Dendrogram constructed by the above cluster analysis considering the individual primers and the pooled data from all four primers together showed that DNA of *H. elastica* differs while growing on different hosts (Fig. 2). Four arbitrarily selected 10-bp markers produced reproducible results, based on the number of base pairs common with markers.

4. Discussion

Similarity index with reference to the four decamer primer markers used in the study suggested that *H. elastica* growing on *W. fruticosa*, *M. indica*, *S. saman*, *Croton oblongifolia* and

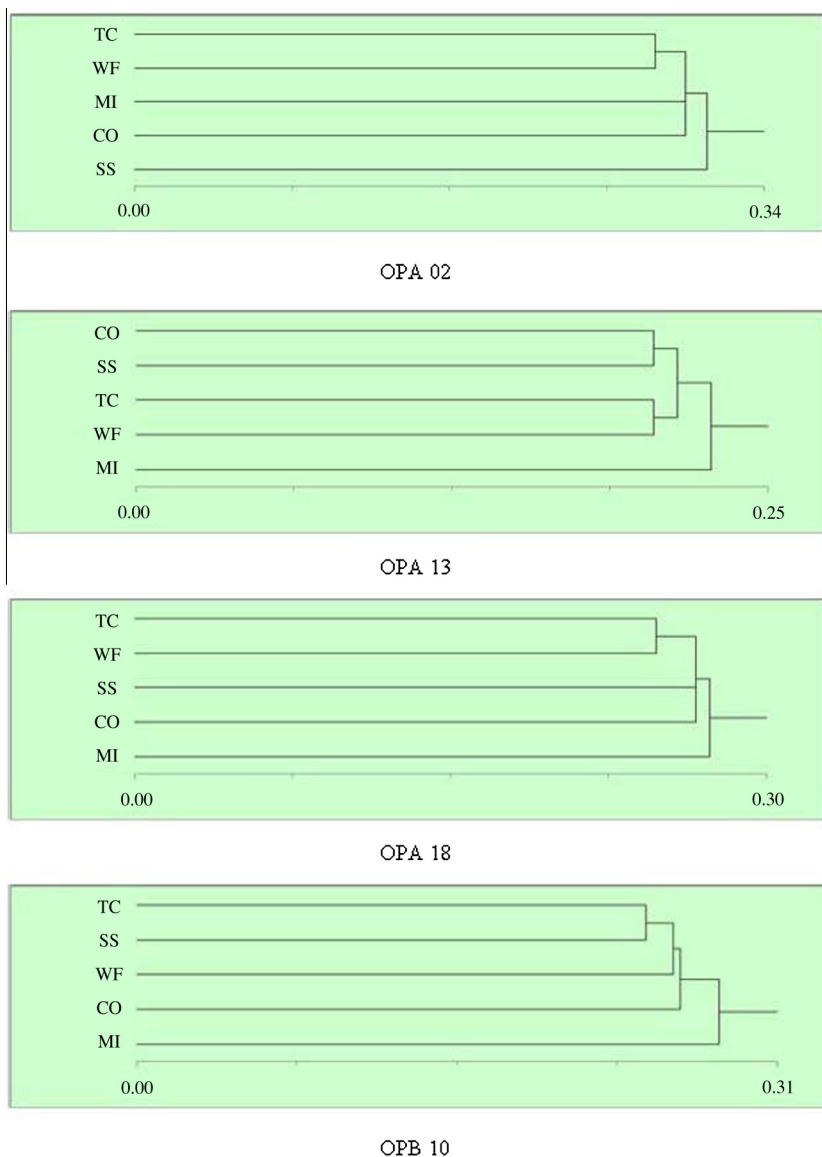


Figure 2 Hierarchical clustering analysis of *Helicanthus elastica* growing on five different hosts using distance matrix obtained by Jaccard's similarity index. CO, *H. elastica* growing on *Croton oblongifolius*; MI, *H. elastica* growing on *Mangifera indica*; SS, *H. elastica* growing on *Samanea saman*; TC, *H. elastica* growing on *Terminalia chebula*; WF, *H. elastica* growing on *Woodfordia fruticosa*.

T. chebula is distinct. Molecular variability among *H. elastica* growing on five different host trees was confirmed by RAPD markers. All the selected primers gave clear and reproducible patterns. The patterns distinguished between the plants and their analysis established an approach to distinguish them based on RAPD markers. The dendrogram clearly differentiated all the five specimens. The study revealed the difference in genetic makeup depending on the host on which the mistletoe grows. The difference in genetic makeup might influence the chemical composition and in turn it might affect the therapeutic property of the mistletoe.

It is reported in studies that the chemical composition of herbal drugs depends upon environmental factors which in turn is due to variation of the genome of plants when growing at different environmental conditions (Echeverrigaray et al., 2001). RAPD fingerprinting has also been reported to be

highly useful in varietal differentiation (Temiesak et al., 1993) and clonal variation (Wang et al., 2009) of plant species. The technique is rapid and less expensive utilizing very less amount of time and money (Arif and Khan, 2009), providing details of genomic difference even below the rank of species and variety (Williams et al., 1990). Mistletoes species, while growing on different hosts show differences in their morphology which may be influenced by the genomic modifications due to the influence of chemicals present in the host plant. *H. elastica* commonly grows on *M. indica* trees though they are reported on plenty of other plants (Sunil Kumar et al., 2015a). The phytochemicals present in the common host *M. indica* are found to occur in *H. elastica* by HPTLC studies (Sunil Kumar et al., 2013) when compounds isolated from *H. elastica* (Sunil Kumar et al., 2015b) were compared to the host extract. This study using a minimum number of primers

could easily differentiate the plant DNA from other hosts. This method can be employed for the development of molecular markers for differentiating mistletoe species in connection with different hosts. The study would also be helpful toward further molecular research on mistletoe biology.

5. Conclusion

DNA from *H. elastica* growing on five different host species was compared by RAPD markers. Differences in the genomic DNA depending on host species was confirmed by selected random markers. This DNA fingerprinting by RAPD will be useful in the identification of the host and comparison of the mistletoe from different hosts.

Acknowledgements

Authors are highly grateful to Vasudha B., Director, Aristogene Biosciences Pvt. Ltd., Bangalore-560044 for helping in RAPD analysis. Fourth and fifth authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University – Saudi Arabia for funding this Research through Research Group No.: RG-1437-002.

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