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Phylogeny of Trochetia species based on morphological and molecular markers

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Abstract

Trochetia is a genus of six species out of which five are endemic to Mauritius. Its taxonomic classification has been changed from the Sterculiaceae family to the Malvaceae recently. Molecular and morphological characterization was carried out for the five *Trochetia* species as a means to conserve endangered Trochetia species and to understand their genetic diversity. *Hibiscus genevii* and *Dombeya mauritiana* were also included in the study as outgroups to infer the phylogeny of *Trochetia*. A modified protocol was used for DNA extraction using CTAB. Morphological characterization was based on both quantitative and qualitative traits. Random Amplified Polymorphic DNA (RAPD) technique was used for assessing genetic diversity of *Trochetia* species. High levels of polymorphism were noted among the *Trochetia* species using RAPD markers. Both molecular and morphological data were cladistically analyzed using the unweighted pair group method with arithmetic average (UPGMA) based on Jaccard's coefficient. Cluster analysis revealed two different phylogenies of *Trochetia* for the two different markers used. *T. triflora* was found to have more similar features to *D. mauritiana* as compared to its congeneric species, as evident from the dendogram based on morphological characters. Out of the twenty nine morphological characters used, *T. triflora* bears four characters similar to *D. mauritiana* as compared to the other *Trochetia* species. Moreover, both *T. triflora* and *D. mauritiana* are best adapted to similar climatic conditions and have similar geographical distribution, suggesting that their close similarity may possibly be due to convergence. Sterculiaceae family has been reported to be polyphyletic and hence similar characters might have been derived from same or different ancestors for both *T. triflora* and *D. mauritiana*. However, the molecular characters separated the genus *Trochetia* as a different clade from the outgroups. The use of other techniques such as DNA sequencing may elucidate further the phylogenetic relationships of *Trochetia* species.

Keywords: *Trochetia*, morphological data, molecular markers.

**For correspondences and reprints*

INTRODUCTION

Trochetia is a genus of promising ornamental value which consists of six species endemic to the Mascarene Islands (Bosser *et al*., 1987). *Trochetia blackburniana*, *Trochetia boutoniana*, *Trochetia parviflora*, *Trochetia triflora* and *Trochetia uniflora* are endemic to Mauritius, whilst *Trochetia granulata* is endemic to Reunion Island.

Trochetia is a genus of shrubs and small trees with spectacular hermaphroditic flowers. It is also characterized by being one of those limited number of genera consisting of species capable of producing colored nectar (Hansen *et al.,* 2006). The geographical distribution of the *Trochetia* species has spread from more or less dry areas such as the cliffs of Le Morne Brabant and Corps des Gardes to regions of high annual rainfall such as Grand Bassin.

Formerly of the family Sterculiaceae, the genus *Trochetia* has been integrated in the Malvaceae family according to the broad circumscription; the Malvaceae sensu lato (Baum *et al*., 2004; Bayer *et al.,* 2003; Tate *et al*., 2005; Whitlock *et al.*, 2001). However, the issue still remains debatable.

No molecular work has been reported on *Trochetia* species except for DNA extraction and intraspecific variation assessment for *Trochetia boutoniana* (Puchooa and Venkatasamy, 2005). Therefore, this study was initiated to assess the genetic diversity of *Trochetia* species and to infer the phylogeny of different species within the *Trochetia* genus using both morphological and molecular characters. Therefore, this study was undertaken to find out the genetic diversity among Trochetia species and recognize its importance in conserving the endangered *Trochetia* species.

METHODOLOGY

DNA Extraction : A modified Porebski *et al*. (1997) protocol was used for DNA extraction. The final modified 2X CTAB extraction buffer was formulated by using the chemical reagents at a final concentration of 0.1M Tris HCl (Trizma base Hydrochloric acid (pH 8), 0.02M EDTA (Ethylene diamine tetra acetic acid), 1.7M NaCl (Sodium Chloride), 2% CTAB (Hexacetyltrimethyl ammonium bromide), 0.2% β-Mercaptoethanol and 3% PVP (Polyvinylpyrrolidone).

0.5 g of freshly pulverized leaves in liquid nitrogen was poured in tubes containing 5ml of pre-heated extraction buffer at 60°C, and was kept at the same temperature for 30 minutes with intermittent swirling, after which 2/3 volume of chloroform: isoamyl in a ratio of 24:1 (V/V) was added. The tubes were gently rolled until a clear demarcation of separation into two distinct phases could be seen. Thereafter, the tubes were centrifuged at 8000rpm for 10 minutes. The supernatant liquid was collected, to which was added 2/3 volume of 5M NaCl followed by 2 volume of ice-cold isopropanol. The tubes were then kept at -20°C for 30 minutes to allow DNA precipitation. The precipitated DNA was hooked out and washed with 70%

ethanol and dried. The DNA pellet was then dissolved in 200µl of nanopure water. RNAse treatment was carried out by adding 2µl of RNAse to the dissolved DNA and keeping it at 37°c for 1 hour.

RAPD – PCR: RAPD is not only a commonly used molecular technique among plants but also other organisms. Martin *et al.* (1993) and Godwin *et al.* (2001), reported that RAPD has several advantages over other polymorphic DNA-detecting techniques such as RFLP, in terms of quickness, small amount of template DNA required and no need of DNA sequence information. RAPD technique has been appraised for its suitability, and applied in characterization studies such as genotyping of Taro (Godwin *et al*., 2001) and detection of DNA polymorphism and genetic diversity in *Pistacia* (Kafkas *et al.*, 2006). In this study RAPD was used to assess the genetic diversity of the *Trochetia* species.

The reaction mixture for RAPD – PCR consisted of a final molarity of 1X Reaction Buffer, 3mM MgCl2, 0.25mM dNTP, 0.6µM Primer, 1 Unit Taq DNA Polymerase, 50ng Template DNA and made up to the final volume with nanopure water. The final volume per reaction tube was set to 30µl.

The PCR reactions were carried out in a DNA Thermocycler (MJ Research Inc. USA). The cycling conditions of the RAPD-PCR were; 1 cycle of 90 seconds at 95°C for initial DNA denaturation followed by 40 cycles, each consisting of the three following steps; 30 seconds at 92°C for denaturation, 1 minute at 35°C for primer annealing and 3 minutes at 72°C for DNA amplification and thereafter a final time delay cycle of 10 minutes at 72°C and 5 minutes at 15°C. OPC11, OPC17, OPD02, OPI03, OPJ04 were the only five primers that gave suitable amplification for all DNA samples under study.

After the PCR, a 2% agarose gel electrophoresis at 120V was carried out to view the result under ultra violet light after staining with ethidium bromide.

Morphological Characterization : Morphological data recorded for vegetative and reproductive characters were both from qualitative and quantitative traits. Taxonomic features identification and assignment were based on the descriptions by Nath (1987) and Hickey and King (1997). Approximation of quantitative characters was done by complementing personal findings with reference from "Flore des Mascareignes" (Bosser *et al*., 1987).

Morphological character state polarization: The characters were polarized using the two different out groups; *Hibiscus genevii* and *Dombeya mauritiana,* one at a time to form a character matrix for further analysis. All the characters were given equal weight for the construction of the phylogenetic tree. However, flowering characters were not included for the construction of dendogram, owing to the unavailability of flowers for *Dombeya mauritiana*. Firstly, all the morphological data were entered in a table. *Hibiscus genevii* having several primitive characters, based on Hutchinson's phylogenetic system of classification (Nath, 1987), was used as out group for polarization of the characters into a binary state. Those species having

the same character states as *H.genevii* were scored as 1 and those having a derived character state were scored as 0.

Table 1 shows the characters used and their polarized states

S.No.	CHARACTERS	POLARITY	
		1	0
1	Plant texture	Shrub	Tree
2	Approximate plant height	≤5m	>5m
3	Leaf shape	Oval / Cordate	Elliptical
4	Leaf apex	Obtuse	Acuminate/ Acute
5	Incision of leaf blade	Entire	Serrate / Dentate
6	Leaf arrangement	Alternate	-
7	Venation	Reticulate	-
8	Approximate leaf length	≤6cm	>6cm
9	Approximate leaf width	≤4cm	>4cm
10	Approximate petiole length	≤2.5cm	>2.5cm
11	Leaf surface	Glancous / Glabrous	Hairy
12	Approximate stipule length	≤6mm	>6mm
13	Inflorescence	Solitary	Aggregate
14	Approximate pedicel length	>3.5cm	≤3.5cm
15	Bracts	Absent	Present
16	Epicalyx shape	Oblong	Spatulate
17	Approximate epicalyx length	1 – 2 cm	1<x<2
18	Floral symmetry	Actinomorphic	-
19	Sepals – free / fused	Polysepalous	Gamosepalous
20	Approximate sepal length	1 – 2 cm	1<x<2
21	External sepal surface	Glabrous	Hairy
22	Sepal aestivation	Valvate	-
23	Petals colour	Pink / Carmine veined	White
24	Petals – free / fused	Polypetalous	-
25	Approximate petal length	>4cm	<4cm
26	Approximate stamen length	>4cm	<4cm
27	Stamen cohesion	Monoadelphous	-
28	Approximate gynoecium length	>5cm	<5cm
29	Fruit type	Capsule	-

Generating Dendograms : The software NTSYSpc 2.2 and DARwin5 were used to generate dendograms using the morphological and molecular data matrices. Results of both the markers were analyzed separately. The un-weighted pair group

method with arithmetic averages (UPGMA) was used based on Jaccard's coefficients (Jaccard, 1908).

RESULTS

Table 2. Morphological character states - Binary data matrix obtained following polarization of the character matrix with out group

SPECIES	CHARACTER STATES													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
T.blackburniana	1	1	0	0	0	1	1	0	0	1	0	1	1	0
T.boutoniana	1	1	0	0	1	1	1	1	1	1	0	0	1	0
T.parviflora	1	1	0	0	1	1	1	1	1	1	1	1	0	0
T.triflora	0	0	0	0	0	1	1	0	0	0	0	0	0	0
T.uniflora	1	1	0	0	1	1	1	1	1	1	0	1	1	0
H.genevii	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D.mauritiana	0	0	1	0	0	1	1	0	0	0	0	1	0	0

Table 3. Morphological character states - Binary data matrix obtained following polarization of the character matrix with out group

SPECIES	CHARACTER STATES														
	15	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**
T.blackburniana	0	0	1	1	1	1	0	1	1	1	0	0	1	0	1
T.boutoniana	0	0	0	1	0	0	0	1	1	1	1	0	1	0	1
T.parviflora	0	0	0	1	1	0	0	1	0	1	0	0	1	0	1
T.triflora	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1
T.uniflora	0	0	1	1	1	1	0	1	1	1	0	0	1	0	1
H.genevii	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D.mauritiana	0	1	0	1	0	0	0	1	0	1	0	0	1	0	1

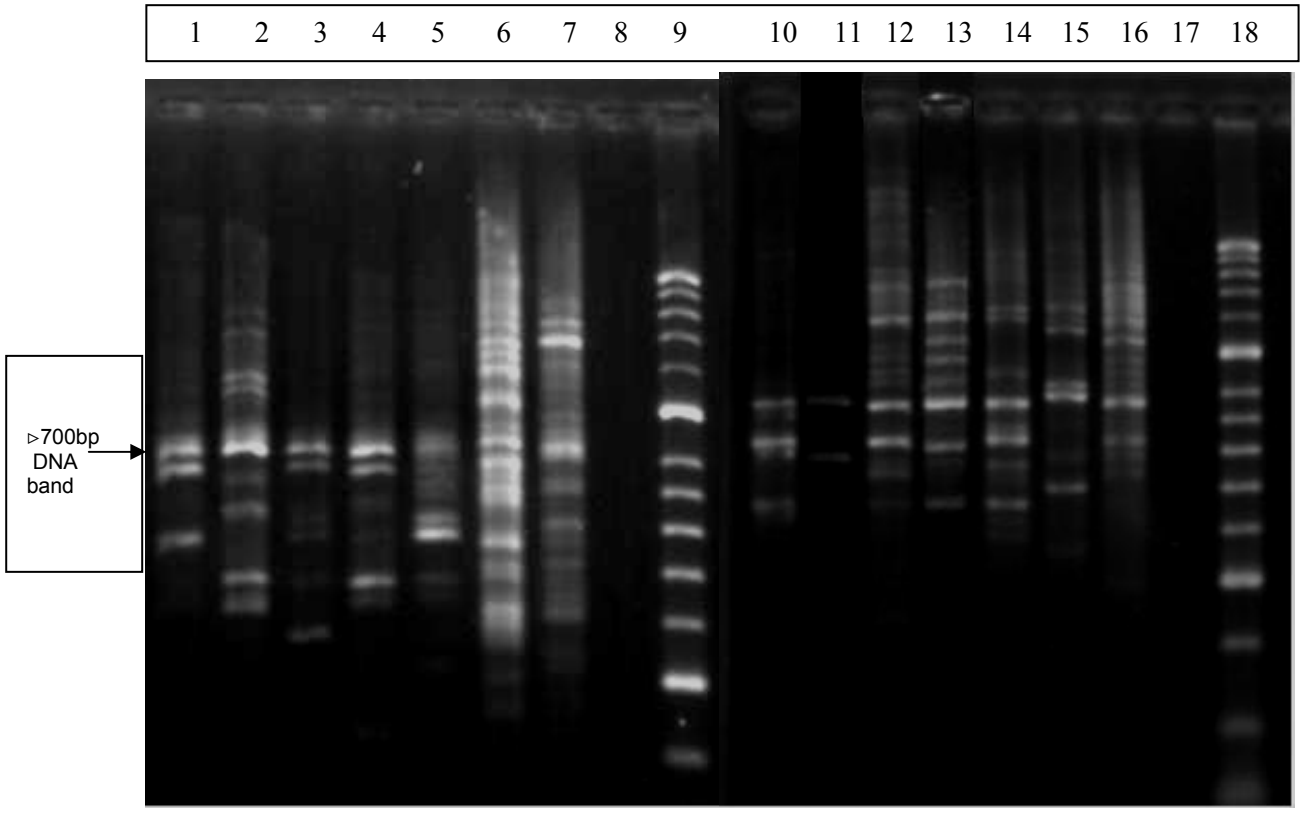

Fig 1. RAPD pattern produced from DNA amplification of the following species using Operon primer OPC-11 and OPI 03 beginning with Lane 1 to 9 consisting of *Trochetia blackburniana, Trochetia boutoniana, Trochetia parviflora, Trochetia triflora, Trochetia uniflora, Hibiscus genevii, Dombeya mauritiana,* Negative control and Hyper Ladder II. Lane 10 to 18 consist of *Trochetia blackburniana, Trochetia boutoniana, Trochetia parviflora, Trochetia triflora, Trochetia uniflora, Hibiscus genevii, Dombeya mauritiana,* Negative control and Hyper Ladder II.

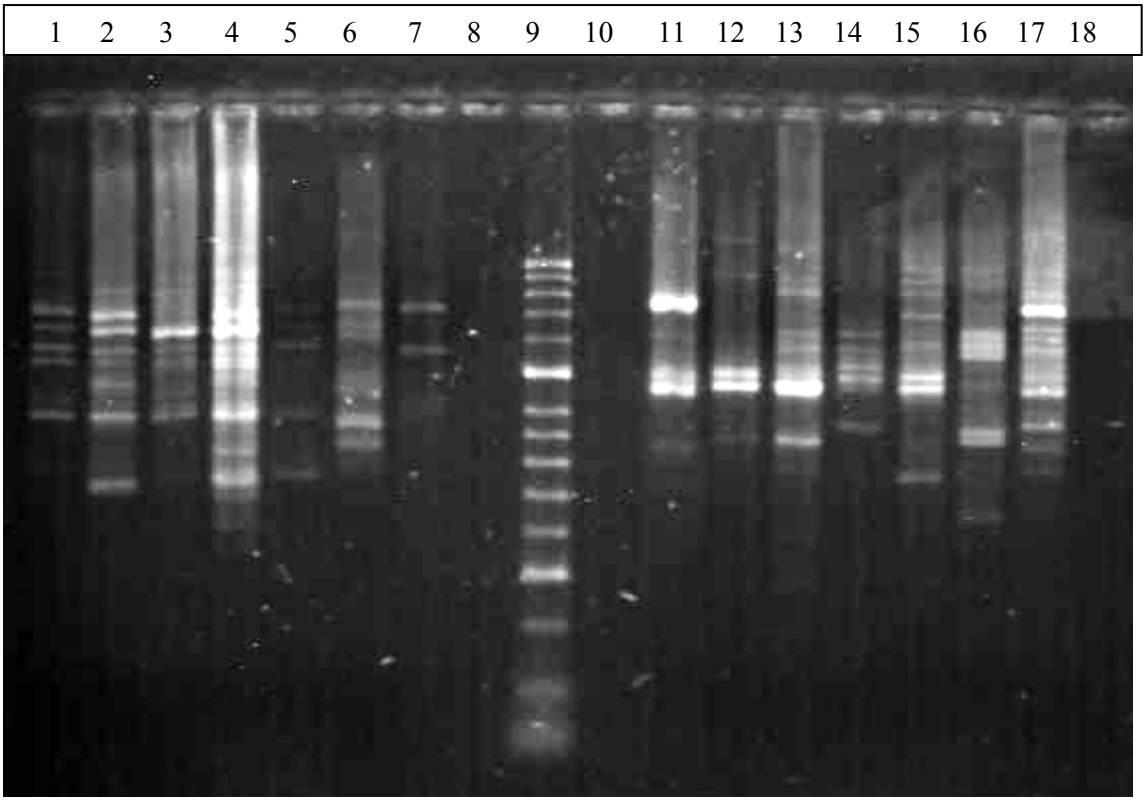

Fig 2. RAPD pattern produced from DNA amplification of the following species using Operon primer OPD 02 and OPJ 04 beginning with Lane 1 to 9 consisting of *Trochetia blackburniana, Trochetia boutoniana, Trochetia parviflora, Trochetia triflora, Trochetia uniflora, Hibiscus genevii, Dombeya mauritiana,* Negative control and Hyper Ladder II. Lane 10 is an empty lane while lane 11 to 18 consist of *Trochetia blackburniana, Trochetia boutoniana, Trochetia parviflora, Trochetia triflora, Trochetia uniflora, Hibiscus genevii, Dombeya mauritiana,* Negative control and Hyper Ladder II.

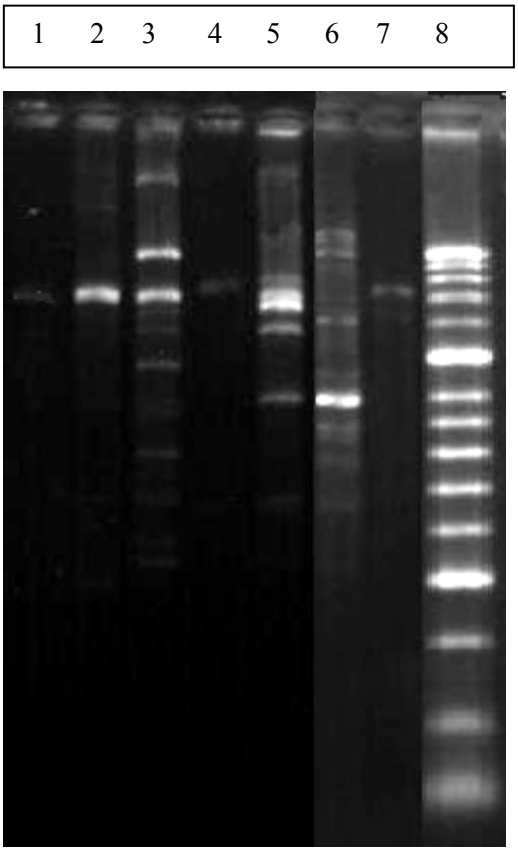

Fig 3. RAPD pattern produced from DNA amplification of the following species using Operon primer OPC 17 beginning with Lane 1 to 9 consisting of *Trochetia blackburniana, Trochetia boutoniana, Trochetia parviflora, Trochetia triflora, Trochetia uniflora, Hibiscus genevii, Dombeya mauritiana* and Hyper Ladder II.

	T.blackburniana	*T.boutoniana*	*T.parviflora*	*T.triflora*	*T.uniflora*	*H.genevii*
T.boutoniana	**0.607143**					
T.parviflora	0.723404	0.735849				
T.triflora	0.714286	0.731707	0.696429			
T.uniflora	0.682927	0.673913	0.633333	0.686275		
H.genevii	0.86	**0.877193**	0.768116	0.833333	0.820896	
D,mauritiana	0.813953	0.791667	0.784615	0.769231	0.672727	0.754098

Table 5. The dissimilarity matrix (also called distance matrix) describes pair-wise distinction between the 6 species studied. Dissimilarity values ranged from 0.6071 to 0.87719. *T.boutoniana* and *H.genevii* were the two species with the most distantly related genotypes as inferred from the dissimilarity matrix in contrast to the two closest varieties *T. boutoniana* and *T.blackburniana* .

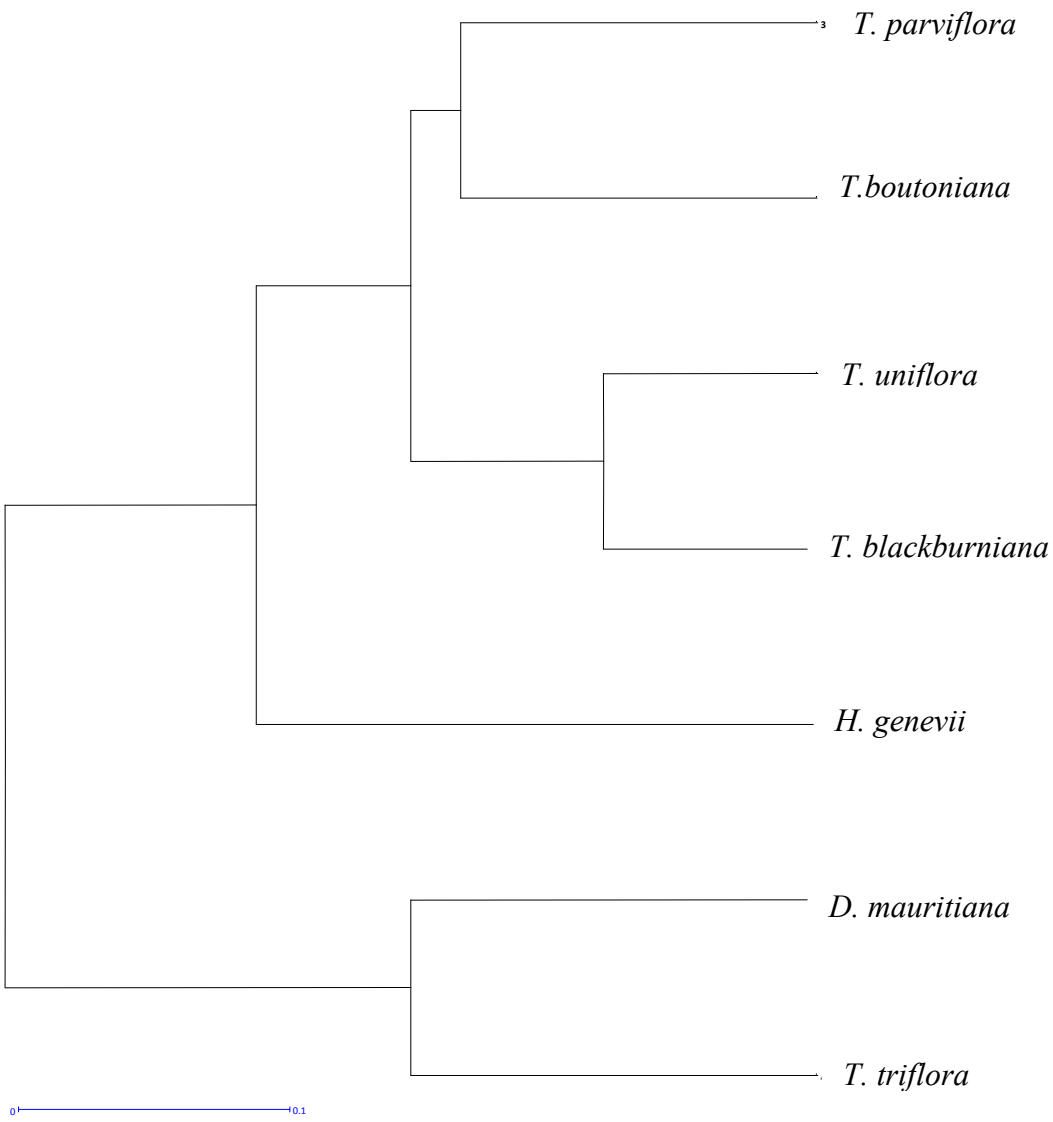

Fig 4. Dendogram based on morphological characters. The *Trochetia* species separated out into 2 distinct clades one with the out group *H.genevii* and the other with *D. mauritiana.*

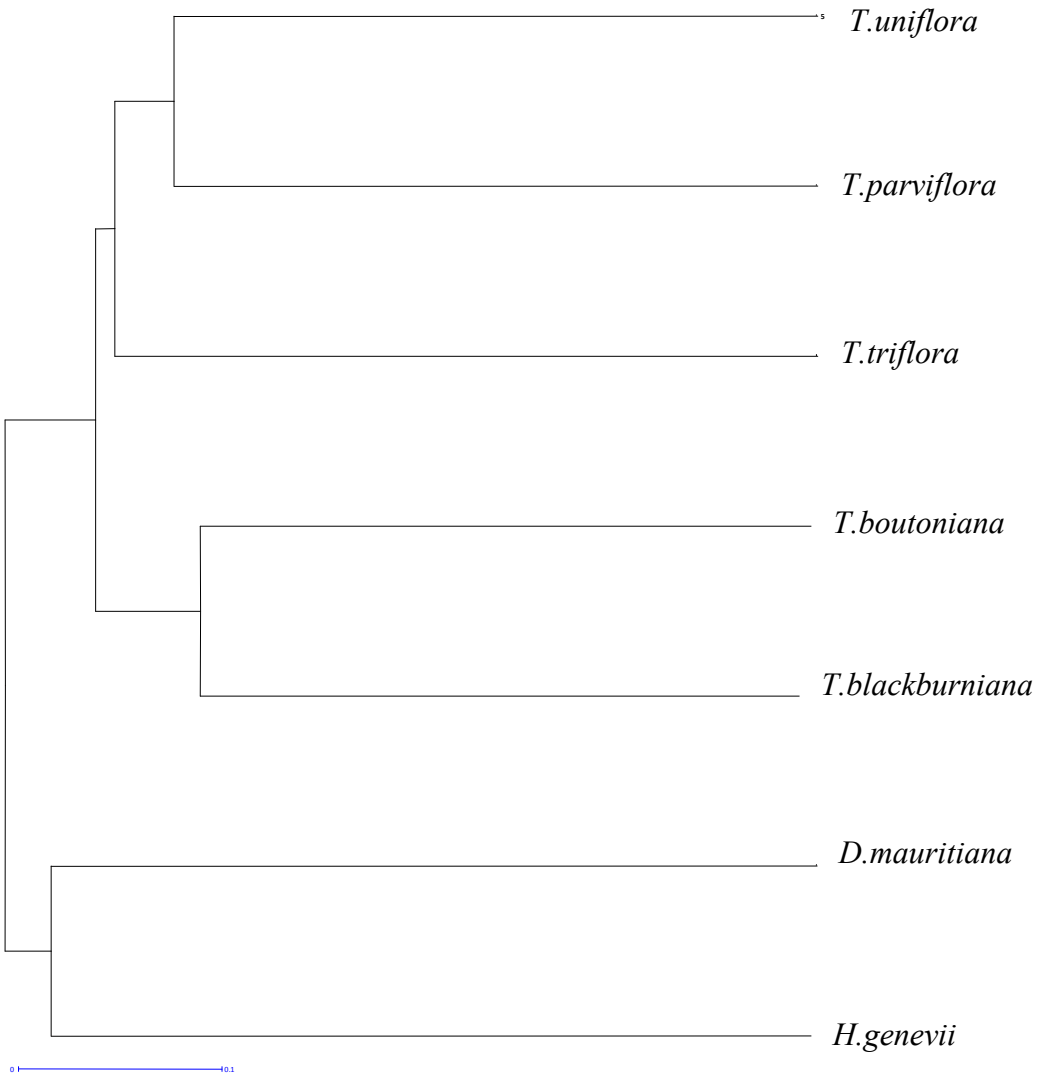

Fig 5 Dendogram based on molecular characters: All the Trochetia species clustered in one clade separate from the two out groups.

DISCUSSION

Each *Trochetia* species possesses a unique combination of morphological features setting it apart from the other species under study. In the analysis of the morphological data, the 5 *Trochetia* species clustered separately into 2 distinct clades (Fig.4). One of them consisted of 4 *Trochetia* species distributed into 2 subclades, one containing *T. parviflora* and *T. boutoniana* and the other *T. uniflora* and *T. blackburniana.* It is interesting to note that four *Trochetia* species clustered separately with *Hibiscus genevii*. The fifth *Trochetia* species - *T. triflora* was cladistically grouped with *Dombeya mauritiana.*

Further analysis of the morphological data showed 44.83% phenotypic resemblance between *T. parviflora* and *T. boutoniana*, and 55.17% for *T. uniflora* and *T. blackburniana*. Despite the fact that *T. parviflora* and *T. boutoniana* have been grouped in a sister clade with 13 polarized similar morphological characters and only one distinctive character - the leaf length separate them into one clade. *T. uniflora* and *T. blackburniana* were held together in a clade with 16 polarized similar characters and have their petal colour as the main phenotypic character for their separation into one clade. Moreover they can be brought closer with a similitude in the quantitative traits such as approximate leaf length, stipule length, pedicel length, sepal length and petal length. However, none of the sister clades do possess any specific polarized morphological characters belonging exclusively to the group.

Four characters; two qualitative and two quantitative were found only in *Trochetia triflora* and *Dombeya mauritiana*. The plant texture and petal colour are the same for *Trochetia triflora* and *Dombeya mauritiana*. Moreover, the petiole length and the plant height were nearly similar for both *Trochetia triflora* and *Dombeya mauritiana*. Both *Trochetia triflora* and *Dombeya mauritiana* are found in places of similar climatic conditions and geographical distribution. *Dombeya mauritiana* was found in the Magenta forest and *Trochetia triflora* was found in Grand-Bassin and Petrin and they are the neighbourhood areas of magenta forest. As Singh (1999), stated, convergence in terms of evolution is brought about by similar climates and habitats and thus bringing about similarity in characters. Homology of characters refers to resemblance due to inheritance from a common ancestor (Singh, 1999). These points may elucidate the much closer resemblance of *Trochetia triflora* with *Dombeya mauritiana* as compared to the other *Trochetia* species. *Trochetia triflora* did not cluster with its congeneric species which may be attributed to the inheritance of characters from different ancestors as the family Sterculiaceae has been reported to be polyphyletic (Wilkie *et al.,* 2006). Polyphyletic taxa are composed of descendents of unrelated ancestors that evolved similar features by convergence and thus bearing analogous characters, which may be the case with the close resemblance in morphological characters of *Trochetia triflora* to *Dombeya mauritiana* being exposed to the same environmental conditions.

High level of polymorphism was recorded in the RAPD-PCR amplifications of the different *Trochetia* species. Amplification using RAPD markers OPC 11, OPD 02,

OPC 17, OPI 03 and OPJ 04 have yielded 98 bands out of which only the 700 bp band obtained from the amplification using the primer OPC 11 was found in all the seven species (Fig.1). Each species has several unique bands suggesting that RAPD can be used as a reliable molecular tool for the identification of *Trochetia* plants in case of mislabeling or uncertainty.

RAPD has proved itself to be a reliable technique for identifying inherent high level of polymorphism among the *Trochetia* species using the molecular markers. Using only five primers, much polymorphism was obtained. Out of 98 bands scored, 76.5% were polymorphic for *Trochetia*, suggesting that RAPD can be used as a molecular marker for the characterization of *Trochetia* and thus infer its genetic diversity. Puchooa and Venkatasamy (2005), noted polymorphism at intraspecific level of *Trochetia boutoniana*, suggesting more polymorphism at interspecific level, among the *Trochetia* species. Mehta (2001) recognized RAPD as being extremely powerful having the ability to separate individuals with intra and interspecific variability.

Analysis of the molecular markers gave a different dendogram (Fig. 5) as the genus *Trochetia* was separated into one clade including all 5 *Trochetia* species and *Hibiscus genevii* and *Dombeya mauritiana* clustered in another clade as distinct out groups. *T. uniflora* and *T. parviflora* were grouped in one clade having a 22.45% similarity banding pattern while *T. boutoniana* and *T. blackburniana* grouped in another clade with 11.22% similarity banding pattern. Remaining *T. triflora* was grouped close to *T. uniflora* and *T. parviflora clade.* Fig. 5.

In contrast, the dendogram of morphological markers (Fig. 4) showed *T. triflora* was separated from the other four *Trochetia* species. Interestingly, *T. triflora* was found to have more similar features to *D.mauritiana* as compared to its congeneric species, as evident from the dendogram based on morphological characters. Further analysis showed that both *T. triflora* and *D. mauritiana* are best adapted to similar climatic conditions and have similar geographical distribution, suggesting that their close similarity may possibly be due to convergence.

The dendograms obtained using molecular characters and that of the morphological characters were showing some differences. In the dendogram based on molecular characters, the genus *Trochetia* is separated in a different clade from the other two genera; *Hibiscus* and *Dombeya*. However, the dendogram based on morphological characters does not support the clustering of taxa as depicted in the dendogram obtained using molecular data. One plausible reason for the difference between the dendograms may be due to the difference in the number of characters used for each of the markers to discriminate among the species. 29 morphological characters were used as compared to 98 bands characterized for the molecular marker. Singh (1999) reported that in numerical studies in plant systematics, at least 60 characters should be used for one marker type.

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 of

an organism. Puchooa and Venkatasamy (2005) reported intraspecific variations in *Trochetia boutoniana*'s genetic constitution, suggesting that though morphological similarities were observed among the individuals of *Trochetia boutoniana* species their genetic constitution may be different. This shows that the systematic studies based on morphological data may be not consistent and also less reliable than molecular analysis.

In this study different phylogenies for *Trochetia* have been obtained using two different marker types; morphological and molecular. But inclusion of other characters/markers such as anatomical, physiological and ecological features along with phyto-chemical studies could provide information to construct a better phylogeny.

Since two different phylogenies have been obtained, DNA sequencing can reveal important information about the genetic diversity of *Trochetia* and their inter-relationship. 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 *Trochetia* species. Moreover, Baldwin (1992) also reported that nrDNA gene family have been subject to pertinent studies and noted that nrDNA gene family undergoes rapid concerted evolution promoting its usefulness for phylogeny reconstruction.

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