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Resolving the identity of *Adhatoda beddomei* C.B. Clarke using morphological and molecular (RAPD) techniques

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Abstract

Adhatoda is one of the most widely used medicinal plants of the present world. Two species of *Adhatoda* – namely *Adhatoda vasica* and *Adhatoda beddomei* have been used for curing respiratory ailments. Both are known to inhabit India but, *A. beddomei* is reported to be a critically endangered, endemic plant of Kerala (south India). Over the years the dormant controversy regarding authentic identity of *A. beddomei* has resurfaced. Hence, the present study was carried out to help resolve this controversy by amalgamation of robust tools -morphological and molecular characterization to classify *A. vasica* and suspected *A. beddomei* as two separate individuals. Morphological studies indicated *A. beddomei* as being very different from *A. vasica* and molecular studies revealed the latter and former to be placed far apart based on Squared Euclidean Distance.

Key words: Medicinal plant, critically endangered, endemic, morphological characterization, molecular characterization, squared euclidean distance, *Adhatoda beddomei, A. gingiana, A. vasica*

Introduction

A. vasica and *A. beddomei* are members of family Acanthaceae and are well known in indigenous systems of medicine for their beneficial effects (Anonymous, 1985). *A. vasica* is globally distributed species to a moderately sufficient extent from India to sub-continent of Malaysia while *A. beddomei* is a species endemic to the state of Kerala in India (Anonymous, 1985). *A. vasica* has been described as an evergreen perennial attaining height up to 6m and has been naturalized in India as a hedge plant (Anonymous, 1985). This species is generally identified by the presence of long and broad (30x5cm) leaves and long inflorescence stalks bearing white flowers with purple streaks on the lower lip (Anonymous, 1985). On the contrary, *A. beddomei* has been described as a medium sized, 1-2m tall shrub (Anonymous, 1985) with narrow leaves and bearing white flowers without the purple streaks. Both species are largely propagated by vegetative means (though incidence of seed propagation in *A. vasica* has been reported) and are known to contain the same active principle- vasicine and vasicinone. *A. vasica* is used medicinally in bronchial ailments while *A. beddomei* is used in haemorrhages, haemoptysis and mennorrhagea (Anonymous, 1985). For sometime now, a controversy has arisen and the identity and very existence of *A. beddomei* is being questioned. Kerala physicians, however, recognize two varieties of 'vasa', locally known as *Atalotakam* & *Cittaatalotakam*, which is known only under cultivation. The latter is so called because of its smaller stature, small leaves and flowers. Consequently, most of the recent authors on Ayurvedic *Materia Medica* have considered them as two different species and have equated the former with *A. zeylanica* (*A. vasica*) and the latter with *A. beddomei*, respectively (Shivarajan and Indira, 1994). Sebastine and Ramamurthy (1964) for the first time reported *A. gingiana* as a new species from South India. There is no doubt about the taxonomic identity as far as *A. vasica* is concerned. But doubts about the identity of *A. beddomei* (*Cittaatalotakam*) still remain owing to the fact that it has been known to be rare and endangered species for sometime (Henry *et al.*, 1978; Sunitha, 2005). After an exhaustive floristic survey of the southern Western Ghats, a team of scientists from NBPGR, Thrissur, could not locate this plant in the area (Amalraj *et al.*, 1991). *A. vasica* being a widely distributed species is considered somewhat variable; therefore, the belief has settled on *Cittaatalotakam* being a variant form of *A. vasica* itself (Shivarajan and Indira, 1994). No research work is reported on the variantion of these plant types using molecular resolution. The need of the hour was therefore, to help resolve this seemingly perennial problem. Hence, this study was carried out at the Indian Institute of Horticultural Research (IIHR), to help solve the dispute over the identity of this species.

Materials and methods

Plant material: Cuttings of *A. beddomei* were collected from Kerala, Karnataka and Tamil Nadu states of Southern India. *A. vasica* was collected from Gandhi Krishi Vignan Kendra (GKVK), Bangalore, Karnataka and subsequently brought to IIHR, Bangalore for establishment (Table 1).

Establishment: The collected accessions of *A. vasica* and *A. beddomei* were initially established in earthen pots containing soil and FYM in the ratio 1:1 for 2 months. Subsequently they were transferred to 3 x 1.8m plots, FYM and neem cake were applied in the ratio 2:1 and they were successfully established in IIHR Field Gene Bank (FGB). Morphological characterization was carried out for 25 qualitative and 20 quantitative traits for *A. vasica* and *A. beddomei* accessions after four years of establishment in Field Gene Bank (FGB). Simultaneously, molecular characterization was also performed using leaves, excised from the second and third node of field established plants,

Table 1. Source of plant material

Species	Collection source	Collection site	Part collected
A. vasica	Cultivated, GKVK, Bangalore	GKVK	Cuttings
A. beddomei-1	Kerala	Potted plant at IIHR	Cuttings
A. beddomei-2	Unknown	GKVK nursery	Cuttings
A. beddomei-3	Cultivated, Thrissur, Kerala	Homestead Courtyard	Cuttings
A. gingiana	Cultivated, FRLHT, Bangalore	Gingi hills, TN	Cuttings

as material for DNA extraction using CTAB method (Ravishankar *et al.*, 2000) and followed by RAPD analysis (Williams *et al.*, 1990).

DNA extraction: Two grams of leaves excised from the second and third node of FGB established plants were cut, washed with double distilled water, blotted dry with blotting paper and finally chopped to small pieces using sterile pair of scissors. The trimmed leaf pieces were crushed to fine powder in liquid nitrogen. 100 mg PVPP (Poly vinyl polypyrrolidone) was added and mixed before suspending the mixture in 10ml of pre-heated extraction buffer containing 10% β-mercaptoethanol. After incubation for 1 hour at 60°C (with periodic gentle mixing every 15 min), the mixture was extracted with 10ml chloroform:isoamylalcohol (24:1 v/v) and centrifuged at 4000g for 15 min. The supernatant was carefully pipetted into fresh, sterile autoclaved centrifuge tube and incubated overnight at -20°C after adding 2.5ml of 5M NaCl followed by 10ml cold ethanol and gently mixing the contents. After subsequent centrifugation at 5000g for 10 minutes, the supernatant was discarded and the pellet was washed with 2 ml of 76% ethanol thrice. The pellet (genomic DNA) was dried at 37°C for 30 minutes, re-suspended in 1 ml TE buffer and incubated at 37°C after adding 10µg/ml ribonuclease. DNA concentrations were calculated by measuring the absorbance at 260nm using UV spectrophotometer. For RAPD analysis 20ng of genomic DNA was used.

RAPD Analysis: 20ng of extracted genomic DNA was used for RAPD analysis prior to which the concentration of $MgCl_2$, which produced polymorphic bands with ten random primers, was standardized. The concentration of $MgCl_2$ standardized was 2.5mM for *Adhatoda* species. The ten random primers (Operon Technologies kits, Califonia, USA) used for PCR amplification were OPE-7, OPE-15, OPE-20, OPI-1, OPI-6, OPI-7, OPI-9, OPS-3, OPS-13, OPS-19. The total reaction volume (25µl) for PCR consisted of 2.5µl RE buffer, 2.5µl primer, 2.5µl dNTP (1mM), 3.0U/µl of Taq-polymerase, 2.2mM $MgCl_2$, 50ng/µl genomic DNA,. The PCR conditions were:

1. 93°C for 3 min, **2.** 92°C for 2 sec, **3.** 35°C for 2 sec, **4.** 72°C for 1 min, **5.** Step 2. to 4. repeated 42 times, **6**. 72°C for 8 minutes. The reaction ended with an indefinite hold at 4°C.

The PCR products were separated by electrophoresis on 1.5% agarose gel containing 0.5µg/ml-ethidium bromide. The RAPD bands were visualized under UV light and photographed. The bands were subsequently scored for each primer as '0' (if bands were absent) and '1' (if bands were present in the corresponding position).

Data analysis: The binary data obtained was analyzed using cluster analysis program to form a dendrogram based on Ward's method in based on Squared Euclidean Distances.

Results and discussion

Morphological characterization: Significant variations were observed within the four accessions (Table 2a). The three *A. beddomei* accessions showed similar growth patterns while *A. vasica* showed significant difference in morphological characters.

Among the quantitative traits, there was a tendency among the three *A. beddomei* accessions to be *at par* (Table 2a), while *A. vasica* showed distinct dissimilarity. When plant height was compared, *A. beddomei*-1 (49.2) and *A. beddomei*-2 (58.6) were *at par* and *A. beddomei*-3 was *at par* with *A. beddomei*-2 (64.4), indicating similar growth in the *A. beddomei* accessions. *A. vasica* showed significant difference and measured 212.8cm. Similar trend was observed with leaf width and inflorescence length where *A. vasica* showed significantly different values of 6.66 and 16.7, respectively. In case of leaf length, *A. beddomei*-3 and *A.-beddomei*-1 were *at par* and *A. beddomei*-2 was *at par* with *A. beddomei*-1 while *A. vasica* showed significantly longer leaves (25.48). Similar trend was seen with petiole length. With respect to peduncle length, bract length, bracteole length, calyx length, corolla length, corolla tube length, corolla upper lip length and corolla lower lip length, the 3 *A. beddomei* accessions were at par and the observations did not vary significantly while *A. vasica* showed significantly different values (Table 2a).

All the 25 qualitative traits observed showed similarity in *A. beddomei* accessions. In *A. vasica*, 19 traits were

Table 2a. Morphological characterization of *A. beddomei* accessions and *A. vasica* (quantitative trait)

.	Traits	*A. beddomei*-1	*A. beddomei*-2	*A. beddomei*-3	*A. vasica*
1	Plant height	49.20 ^{a}	58.60 ^{ab}	64.40 ^{b}	212.80 ^{c}
2	Leaf size- length	8.70 ^{ab}	9.94 ^{b}	8.14 ^{a}	25.48 ^{c}
3	Leaf size- width	2.70 ^{a}	2.72 ^{a}	2.80 ^{a}	6.66 ^{b}
4	Petiole length	0.62 ^{ab}	0.92 ^{b}	0.48 ^{a}	4.06 ^{c}
5	Inflorescence length	4.52 ^{a}	5.04 ^{a}	4.64 ^{a}	16.70 ^{b}
6	Peduncle length	2.30 ^{a}	2.50 ^{a}	2.28 ^{a}	7.86 ^{b}
7	Bract length	1.32 ^{a}	1.26 ^{a}	1.28 ^{a}	2.64 ^{b}
8	Bract width	0.52 ^{a}	0.52 ^{a}	0.52 ^{a}	1.46 ^{b}
9	Bractiole length	1.00 ^{a}	1.00 ^{a}	1.00 ^{a}	1.88 ^{b}
10	Calyx length	0.78 ^{a}	0.78 ^{a}	0.80 ^{a}	1.00 ^{b}
11	Corolla length	1.66 ^{a}	1.66 ^{a}	1.64 ^{a}	3.60 ^{b}
12	Corolla lip size- upper	1.70 ^{a}	1.70 ^{a}	1.70 ^{a}	2.96 ^{b}
13	Corolla lip size- lower	1.60 ^{a}	1.60 ^{a}	1.60 ^{a}	2.86 ^{b}
14	Numbre of nerves on bract	4-5	4-5	4-5	5-6
15	Numbre of nerves on bractiole	3	3	3	3-4
16	Numbre of nerves on calyx	3	3	3	3
17	Locules in cross section	2	2	2	2
18	Numbre of ovules	2	2	2	2
19	Fruit length	No fruit set	No fruit set	No fruit set	2.32
20	Seed size (l / b)	No seed set	No seed set	No seed set	0.8 / 0.78 (*)

Mean values super scribed by the same letters are not significantly different (*P*=0.05)

Table 2b. Morphological characterization of *A. beddomei* accessions and *A.vasica* (qualitative trait)

	Character	*A.beddomei*-1	*A.beddomei*-2	*A.beddomei*-3	*A. vasica*
1	Growth habit	Medium sized shrub	Medium sized shrub	Medium sized shrub	Dense shrub (*)
2	Branching type	Opposite ascending	Opposite ascending	Opposite ascending	Opposite ascending
3	Stem shape	Terate	Terate	Terate	Terate
4	Stem texture	Glabrous	Glabrous	Glabrous	Glabrous
5	Stem bark colour	Yellowish green	Yellowish green	Yellowish green	Yellowish green
6	Leaf shape	Oblong-lanceolate	Oblong-lanceolate	Oblong-lanceolate	Elliptic-lanceolate (*)
7	Leaf tip	Acuminate	Acuminate	Acuminate	Acuminate
8	Leaf pubescence	MPWY	MPWY	MPWY	MPWY
9	Mature leaf texture	Glabrous	Glabrous	Glabrous	Glabrous
10	Leaf margin	Entire	Entire	Entire	Entire
11	Leaf colour	DGPG	DGPG	DGPG	DGPG
12	No. of main nerves	8-10 pairs	8-10 pairs	8-10 pairs	13-15 pairs (*)
13	Venation	Reticulate between nerves	Reticulate between nerves	Reticulate between nerves	Reticulate between nerves
14	Inflorescence type	Axillary spike	Axillary spike	Axillary spike	Axillary spike
15	Peduncle size	Shorter than leaves	Shorter than leaves	Shorter than leaves	Shorter than leaves
16	Bract type	Elliptic, subacute, glabrous	Elliptic, subacute, glabrous	Elliptic, subacute, glabrous	Elliptic, subacute, glabrous
17	Calyx texture	Slightly pubescent	Slightly pubescent	Slightly pubescent	Slightly pubescent
18	Calyx type	Oblong-lancoelate	Oblong-lancoelate	Oblong-lancoelate	Oblong-lancoelate
19	Corolla colour	White without purple streaks	White without purple streaks	White without purple streaks	White with purple streaks (*)
20	Corolla pubescence	Pubescent on outer surface	Pubescent on outer surface	Pubescent on outer surface	Pubescent on outer surface
21	Corolla type	Bi-lipped	Bi-lipped	Bi-lipped	Bi-lipped
22	Anther type	2-celled, basifixed	2-celled, basifixed	2-celled, basifixed	2-celled, basifixed
23	Ovary pubescence	Pubescent on outer surface	Pubescent on outer surface	Pubescent on outer surface	Pubescent on outer surface
24	Seed shape	No seed set	No seed set	No seed set	Orbicular-oblong and flattened(*)
25	Seed texture	No seed set	No seed set	No seed set	Glabrous (*)

MPWY= minutely pubescent when young, DGPG=darkgreen above, palegreen below
The 6 quantitative traits which showed difference have been indicated by an asterix (*)

similar to that of *A. beddomei* while six differed. In *A. beddomei*, there was no incidence of fruit-set inspite of considerable inflorescence development. Apparently, many inflorescence stalks were observed to turn yellow and fall off before flowering. On the contrary, in *A. vasica* profuse flowering (Dhore and Tidke, 2005) was observed and a minimum of 1 fruit was produced per inflorescence stalk.

Molecular characterization: Good quality DNA was obtained from all the four accessions based on the A_{260}/ A_{280} ratio obtained. Yield was similar in all the lines *i.e.* a range of 0.67 to 2.33 µg/mg of DNA was obtained (Table 3).

Table 3. DNA concentrations of *Adhatoda* accessions by measuring the absorbance at 260 and 280nm

Sl No.	Species	A_{260}	A_{280}	A_{260}/ A_{280}	Amount of DNA (µg/mg)
1	*Adhatoda vasica*	0.073	0.042	1.755	0.73
2	*Adhatoda beddomei*-1	0.080	0.045	1.787	0.80
3	*Adhatoda beddomei*-2	0.080	0.046	1.739	0.80
4	*Adhatoda beddomei*-3	0.067	0.040	1.687	0.67
5	*Adhatoda gingiana*	0.233	0.168	1.389	2.33

Banding pattern of PCR products revealed greater monomorphism between *A. beddomei*-1, *A. beddomei*-2 and *A. beddomei*-3 accessions (suspected *A. beddomei* accessions). *A. vasica* and *A. gingiana* produced greater polymorphism in comparison with *A. beddomei* accessions indicating the prevailing difference between the species. There was distinct similarity between the *A. beddomei* accessions while the pattern of *A. vasica* was different. *A. gingiana* banding pattern was most distinct (Fig 1). DNA-based techniques have been widely used for authentication of plant species of medicinal importance (Kalpana *et al.*, 2004). This technique is also useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phytochemically indistinguishable.

Dendrogram constructed using squared euclidean distances further showed *A. beddomei* accessions to be different from *A. vasica*. However, it also revealed *A. vasica* and *A. gingiana* to be linked as they formed a cluster and the *A. beddomei* accessions grouped in another cluster (Fig 2) indicating their similarity at molecular level. *A. beddomei* accessions and *A. vasica* were placed far apart indicating that *A. beddomei* accessions and *A. vasica* were not the same. This outcome is of great importance because primarily, *A. beddomei* was considered as a form of *A. vasica* (Shivarajan and Indira, 1994). The morphological and molecular analysis obtained from the present study supports *A. beddomei* as a separate species, quite different from *A. vasica* and *A. gingiana*.

Conclusively, the above study is a decisive indicator for accepting *A. beddomei* as a species separate from *A. vasica* based on the significantly different qualitative and quantitative morphological data. This difference was further substantiated by RAPD analysis, at the molecular level. Molecular analysis indicates *A. gingiana* as a probable variant or form of *A. vasica* due to the cluster formed by these two species were separated by same linkage distance. Morphological and molecular findings support *A. beddomei* as a separate individual different from *A. vasica* and *A. gingiana*. Thereby, this study helps and clarifies the prevailing confusion wherein *A. beddomei* was considered to be a variety

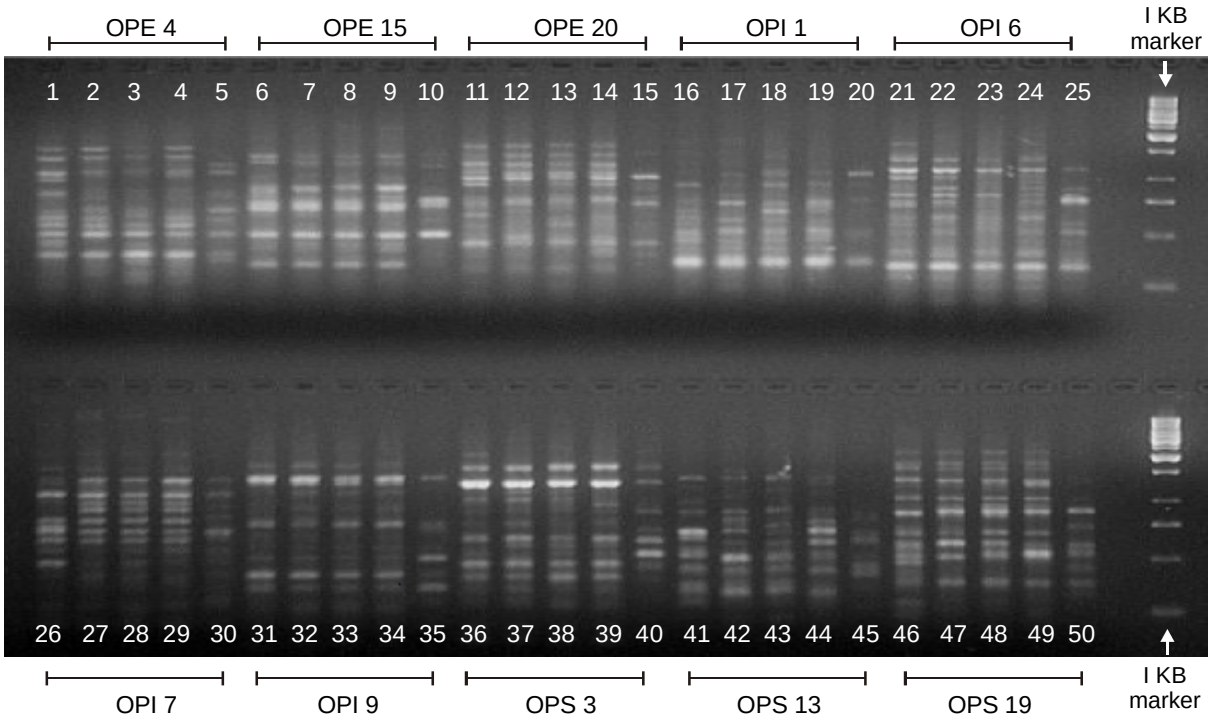

Fig. 1. Banding pattern of *Adhatoda* accessions using 10 Operon primers

Note: Lanes 1, 6, 11, 16, 21, 26, 31, 36, 41 & 46 are of *A. vasica* with 10 different Operon primers; Lanes 2, 7, 12, 17, 22, 27, 32, 37, 42 & 47 are of *A. beddomei*-2 with 10 different Operon primers; Lanes 3, 8, 13, 18, 23, 28, 33, 38, 43 & 48 are of *A. beddomei*-3 with 10 different Operon primers; Lanes 4, 9, 14, 19, 24, 29, 34, 39, 44 & 49 are of *A. beddomei*-3 with 10 different Operon primers; Lanes 5, 10, 15, 20, 25, 30, 35, 40, 45 & 50 are of *A. gingiana* with 10 different Operon primers.

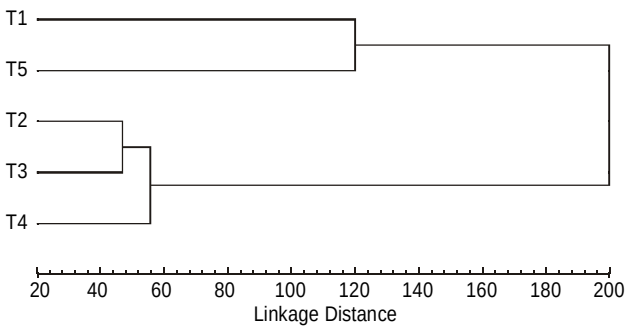

Fig. 2. Dendrogram showing linkage distance among different accessions of *Adhatoda* spp. T1- *A. vasica*, T2-*A. beddomei*-1, T3-*A. beddomei*-2, T4-*A. beddomei*-3, T5- *A. gingiana,* respectively

of *A. vasica*. The study has also indicated the presence of variations within *A. beddomei* accessions, which could be attributed to region specific variability. Moreover, *A. beddomei* could still be considered critically endangered or on the verge of extinction, due to their absence in the wild (in their endemic regions). However, owing to their important medicinal property they have been cultivated in various pockets of Kerala indicating possibility of sustainable harvest.

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References

Amalraj, V.A., K.C. Velayudhan and Z. Abraham, 1991. Threatened medicinal plants of western ghats *In:* Proc. Symp. on Rare, Endangered and Endemic Plants of Western Ghats (30-31, Aug. 1991), Thiruvananthapuram.

Anonymous, 1985. *The Wealth of India*. CSIR Publication. New Delhi. Vol. I: A.

Dhore, M.M. and J.M. Tidke, 2005. Pollination ecology of *Adhatoda zeylanica* (Acanthaceae). *Med. Arom. Plant Abstr.,* 27(3): 270.

Henry, A.N., K. Vivekanandan and N.C. Nair, 1978. Rare and threatened flowering plants of South India. *J. Bombay Nat. Hist. Soc.,* 75: 684-697.

Kalpana, J., C. Preeti, D. Warude and P. Bhushan, 2004. Molecular markers in herbal drug technology. *Curr. Sci.,* 87(2): 25.

Ravishankar, K.V., A. Lalitha and M.R. Dinesh, 2000. Assessment of genetic diversity relatedness among mango cultivars of India using RAPD markers. *Jour. Hort. Sci. Biotech.,* 75(2): 198-20.

Sebastine, K.M and K. Ramamurthy, 1964. A New Species of *Justicia* from South India. *Bull. Bot. Surv. India.,*Vol. 6. No.1: Pp 99-100.

Shivarajan, V.V. and B. Indira, 1994. *Ayurvedic Drugs and Their Plant Sources.* Oxford publication. pp. 502-03.

Sunitha Bhaskaran. 2005. *Ex situ* conservation strategies for threatened medicinal plant species- *Acorus calamus* Linn. and *Adhatoda beddomei* C.B.Clarke. Ph.D. Thesis. Department of Applied Botany. Kuvempu University. Shimoga. Karnataka.

Williams, J.G.K., A.R. Kubelik, K. Livark, A. Rafalski and S.V. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.,*18: 6531-6535.